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Sensory mother cells are selected from among mitotically quiescent cluster of cells in the wing disc of *Drosophila*

KAZUYA USUI* and KEN-ICHI KIMURA

Laboratory of Biology, Hokkaido University of Education, Iwamizawa Campus, Iwamizawa 068 Japan

*Author for correspondence

Summary

The large sensory bristles on the head and thorax of *Drosophila* are arranged in a precise pattern. Each bristle is formed by the progeny of a sensory mother cell (SMC) which is generated in the imaginal wing disc. The proneural genes *ac* and *sc* play an important role in the correct positioning of these SMCs by conferring to reproducibly located clusters of cells the competence to become SMCs. Indirect evidence suggested that the mitotic state of a cell could also play an important role in SMC positioning. In order to analyze the relation between the formation of SMC and the mitotic activity in the developing wing disc, we used BrdU immunolabeling to monitor the mitotic activity in an enhancer-trap transformant line, A101, where the SMCs can be visualized. Our results indicate that SMCs arise from clusters of mitotically quiescent cells (MQC), and that the cell that becomes a SMC was itself arrested at the G_2 stage of the cell cycle. The emergence of MQCs follows a precise temporal and spatial pattern which is not affected by the absence of the *ac* and/or *sc* genes. We propose that the reproducible emergence of MQCs could be an important factor in the formation of SMCs.

Key words: sensory mother cell (SMC), pattern formation, mitotically quiescent cluster of cells (MQC), mitotic activity, cell differentiation, neurogenesis, *Drosophila.*

Introduction

The pattern of sense organs is often remarkably constant in arthropods. How such a precise and reproducible pattern is generated has been extensively studied in the case of the mechanosensory bristles on the notum of *Drosophila*. Three types of innervated bristles are present on the notum of the adult: large bristles (macrochaetes), small bristles (microchaetes) and trichoid sensilla. The macrochaetes and trichoid sensilla form in invariant numbers and at invariant locations (summarized in Bryant, 1975, 1978; Hartenstein and Posakony, 1989). Each macrochaete is generated by the progeny of a sensory mother cell (SMC), which is derived from the epithelial cells of the imaginal wing disc. The SMC undergoes two differential divisions (Lawrence, 1966; Bate, 1978; Hartenstein and Posakony, 1989) to produce four cells that differentiate as a neuron, a sheath cell (thecogen), a socket-producing cell (tormogen) and a shaft-producing cell (trichogen), forming together a sensory bristle.

The formation of a sense organ is a progressive process, involving several classes of genes (for review, see Ghysen and Dambly-Chaudière, 1989, Jan and Jan, 1990). Genetic evidence has led to the conclusion that the expression of the genes *achaete* (*ac*) and *scute* (*sc*) plays an essential role in the decision of epithelial cells to become SMCs (Garcia-Bellido and Santamaria, 1978; reviewed by Ghysen and Dambly-Chaudière, 1989). Furthermore, since the expression of *ac* and *sc* is spatially restricted to the locations where SMCs will form later (Romani et al., 1989; Skeath and Carroll, 1991; Cubas et al., 1991), it has been suggested that the expression of *ac* and *sc* defines the regions of the imaginal disc where the SMCs can develop. Recently, however, Rodriguez et al. (1990) have reported that the ubiquitous expression of *sc* in an ac^- and sc^- background leads to the development of macrochaetes at the normal positions and not elsewhere. They concluded that the capability to develop sensory bristles is temporally and spatially regulated independently of the restricted expression of *ac* and *sc*.

In a developing tissue, the mitotic cell state can be important for processes such as commitment, determination and differentiation. The mitotic activity of imaginal disc cells has been extensively studied in *Drosophila* (Dale and Bownes, 1980; Dunne, 1981; James and Bryant, 1981; Adler, 1981; Adler and MacQueen, 1981; Fain and Stevens, 1982; Graves and Schubiger, 1982; Adler and MacQueen 1984; O'Brochta and Bryant, 1985; Schubiger and Palka, 1987). During the larval life, the number of cells in the wing disc increases exponentially until the beginning of metamorphosis (Fain and Stevens, 1982; Adler and MacQueen, 1984). Two regional differences in mitotic activity have been described in the wing region of the wing disc: a

zone of non-proliferating cells (ZNC) at the presumptive wing margin (O'Brochta and Bryant, 1985) and a midline stripe (M-stripe) of mitotically quiescent cells which intersects the center of the ZNC (Schubiger and Palka, 1987). In this report, we show that the mitotic activity in the notum region is regulated spatially and temporally and that SMCs are singled out from among mitotically quiescent clusters of cells.

Materials and Methods

Fly stocks

Flies were reared on cornmeal-yeast medium at 25°C under constant illumination. Males and females from the wild-type strain Canton-Special were used for the analysis of normal development. The transformant line A101 was used to identify the SMCs. This line contains a P-lArB construct which carries a fusion of part of the P element transposase with the bacterial *lacZ* gene under the control of the weak P-transposase promoter (Bellen et al., 1989, Wilson et al., 1989). The resultant β-galactosidase-transposase fusion protein is specifically produced in SMCs and in their progeny. The product is located in the nucleus due to the existence of nuclear targeting sequences in the amino-terminal region of the P transposase (Mlodzik et al., l990). *lacZ* is expressed very early in SMC determination (Huang et al., 1991). $sc^{10.1}$, a double mutant for *ac* and *sc*, is balanced over $FM7^c$, y^{31d} sc^8 w^a sn^{X2} *v B*. $sc^{10.1}$/Y larvae have yellow Malpighian tubules and can be distinguished from $FM7^c$/Y larvae with colorless tubules. $sc^{10.1}$/Y adult flies have neither macrochaetes nor microchaetes on the notum. $In(1)ac^3$ homozygous flies lack the aDC, pDC and pAP macrochaetes and many microchaetes.

Staging

The spatial and temporal pattern of SMC emergence has been described in detail by Huang et al. (1991) using the transformant line A101. Based on this description we deduced the age of the A101 discs from the SMC pattern. The age of larvae at the time of BrdU injection was determined by recording the time until puparium formation (PF) and given in hours before puparium formation (BPF).

X-gal staining

X-gal staining of wing discs was performed using the method described by Hiromi et al. (1985) with slight modifications. Discs were dissected from larvae and fixed for 15 minutes in 0.1 M sodium cacodylate buffer, pH 7.2, containing 2% glutaraldehyde and 0.02% Triton X-100. After fixation, the discs were washed in PBS, and stained for β-galactosidase activity by incubation in 0.08% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 5 mM potassium ferricyanide, and 5 mM potassium ferrocyanide in sodium citrate/phosphate buffer (pH 8.0) for 4-24 hours at 25 or 37°C. After several washes in PBS, preparations were dehydrated, cleared in methyl benzoate and mounted in Bioleit (Oken Co.).

Application of BrdU in vitro (incubation)

In order to monitor the replication of the wing disc cells of the third instar larvae, the thymidine analogue 5-bromo-2′-deoxyuridine (BrdU) was used. BrdU (Sigma) was applied by the incubation of discs in a 50-500 mg/ml solution of BrdU in Schneider's medium for 30 minutes, as described by Hartenstein and Posakony (1989).

Application of BrdU in vivo (injection)

BrdU was applied by injection into living larvae at various stages, using a micropipette (approximately 0.03 ml of a 5 mg/ml solution of BrdU in PBS), and the larvae were allowed to grow until 24-30 hours after puparium formation (APF) at 25°C. The pupae were then dissected in PBS. BrdU becomes unavailable or inactivated within less than 4 hours at 25°C after injection (Hartenstein and Posakony, 1989; Schubiger and Palka, 1987, Wolff and Ready, 1991). Therefore, the pattern of BrdU-containing nuclei at 24-30 hours APF reflects the pattern of mitotic activity in the wing disc about the time when BrdU was injected. We focus on three bristles: the pDC, aDC, and pPA. The stained samples were classified into three classes according to the distribution of labeled nuclei around the sensory organs as follows: in the first class, a sample with no labeled cells around the sensory organ was scored as 1.0; in the second class, a sample with a few labeled cells around the sensory organ was scored as 0.5; and in the third class, a sample with many labeled cells around the sensory organ was scored as 0. Ten, eight and fourteen heminota injected at 12-18, 6-12, 0-6 hours BPF, respectively, were examined.

Immunohistochemistry

The preparations were fixed for 15 minutes in modified Carnoy's fixative (100% ethanol: glacial acetic acid, 3:1), rehydrated, washed in PBS containing 0.3% Triton X-100 (PBS-TX) and hydrolyzed in 2 N HCl for 30 minutes. After several washes in PBS-TX the preparations were incubated in 10% fetal calf serum in PBS-TX for 1 hour. A mouse monoclonal antibody against BrdU (Amersham) was added to the above solution at a dilution of 1:20, and the preparations were incubated for 2 hours at room temperature. In the notum preparation, the patterns of BrdU incorporation and the progeny of SMCs were simultaneously visualized by anti-BrdU antibody and monoclonal antibody, mAb 22C10 (kindly provided by Dr S. Benzer), which specifically binds to neurons and their accessory cells (Zipursky et al., 1984; Canal and Ferrus, 1986; Hartenstein and Posakony, 1989). mAb 22C10 was added simultaneously with anti-BrdU antibody for double labeling at a dilution of 1:20. After several washes in PBS-TX, the preparations were incubated for 2 hours in HRP-conjugated rabbit anti-mouse IgG antibody (Amersham) diluted 1:20 in PBS-TX. After several washes in PBS-TX and then PBS, the preparations were stained for HRP activity by incubation for 15 minutes in 0.05% diaminobenzidine (DAB, Sigma) in PBS containing 0.04% NH_4Cl, 0.2% β-D-glucose, and 0.1% glucose oxidase. The reaction was interrupted by diluting the substrate with PBS. After dehydration and clearing with methylbenzoate, the preparations were mounted, observed and photographed under Nomarski optics.

Double staining method for BrdU and X-gal

We modified the condition of fixation for double staining with BrdU and X-gal because glutaraldehyde fixation removes BrdU immunostaining and Carnoy's fixative probably kills the activity of β-galactosidase. After incubation in the BrdU solution, discs were fixed in 0.04% glutaraldehyde in 0.1 M cacodylate buffer containing 0.02% Triton X-100 for 15 minutes. After several washes in PBS, the preparations were stained according to the X-gal staining method. Preparations were rinsed and post-fixed with modified Carnoy's fixative for 15 minutes. After several washes in PBS, the preparations were digested with 0.2% trypsin 2000 E/g (Merck) for 30 minutes at 37°C, rinsed with PBS-TX and treated with 2 N HCl for 30 minutes. The following treatments were done according to BrdU immunostaining.

Results

General description

The sensory organs of the notum and wing blade are arranged in a reproducible pattern (Fig. 1B,C). Eleven large mechanosensory bristles and two trichoid sensilla arise on each heminotum at fixed positions. Each bristle is formed by the progeny of a single sensory mother cell (SMC) which appears in the developing imaginal disc. The wing disc consists of three layers of cells: the squamous cells of the peripodial membrane, the columnar epithelial cells which will form the adult epidermis, and the adepithelial cells (myoblasts) in the notum region, which will form the flight muscles. SMCs are singled out from the columnar epithelial cells. Fig. 1A shows the SMCs in the right wing disc from a late third instar larva of the enhancer-trap line A101, where the bacterial *lacZ* gene is expressed only in the SMCs. Since *lacZ* expression in A101 line is one of the earliest markers for SMCs, it is possible to follow the appearance of SMCs and to show that they form according to a fixed temporal and spatial pattern (Huang et al., 1991).

Pattern of mitotic activity of wing disc cells

The number of cells in the wing disc increases exponentially during larval life, with a doubling time of 7.5 to 8 hours during the first and early second instar (Madhavan and Schneiderman, 1977), and 8.5 hours in average during larval life (Garcia-Bellido and Merriam, 1971; Haynie, 1975). The discs of early third instar larva are very small and flat; as they grow in size they become folded.

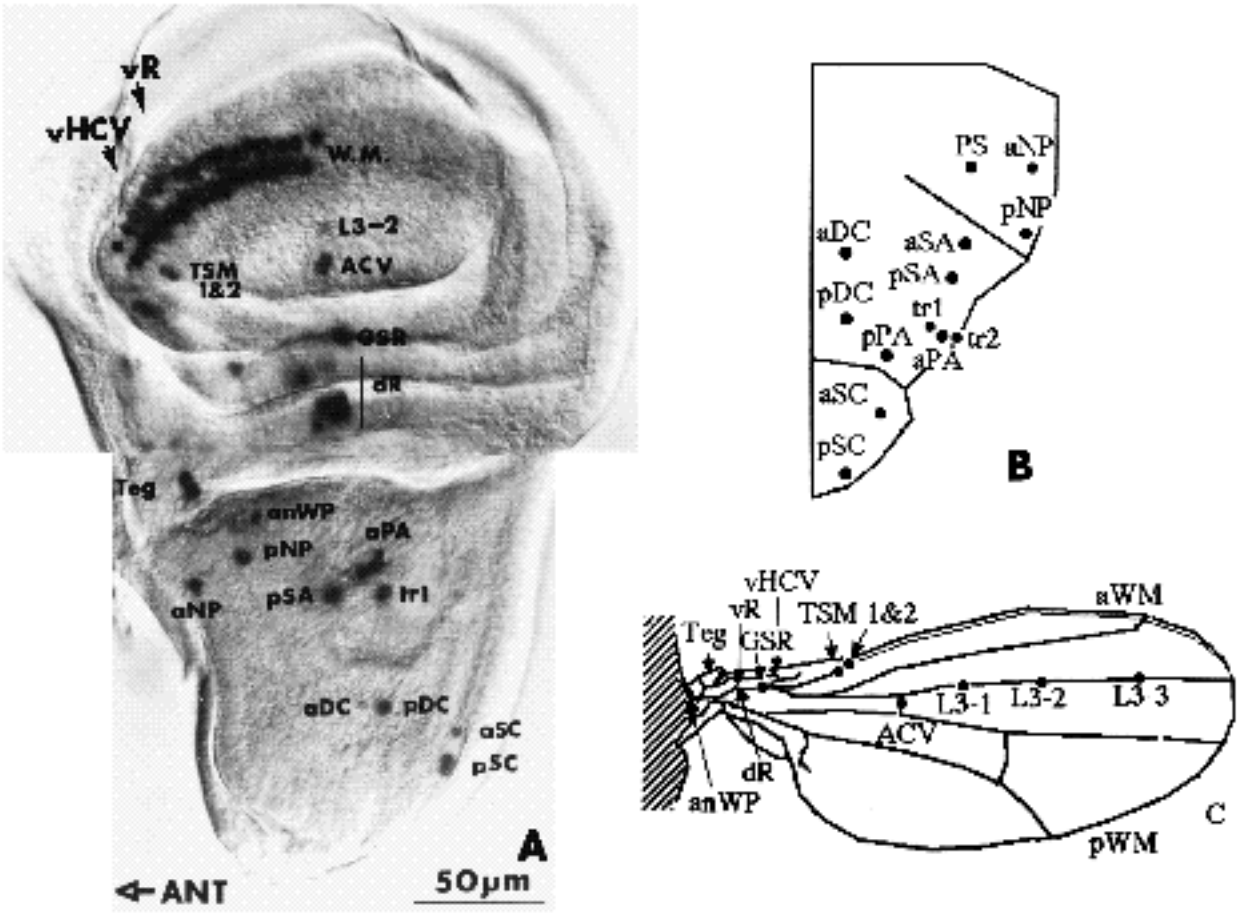
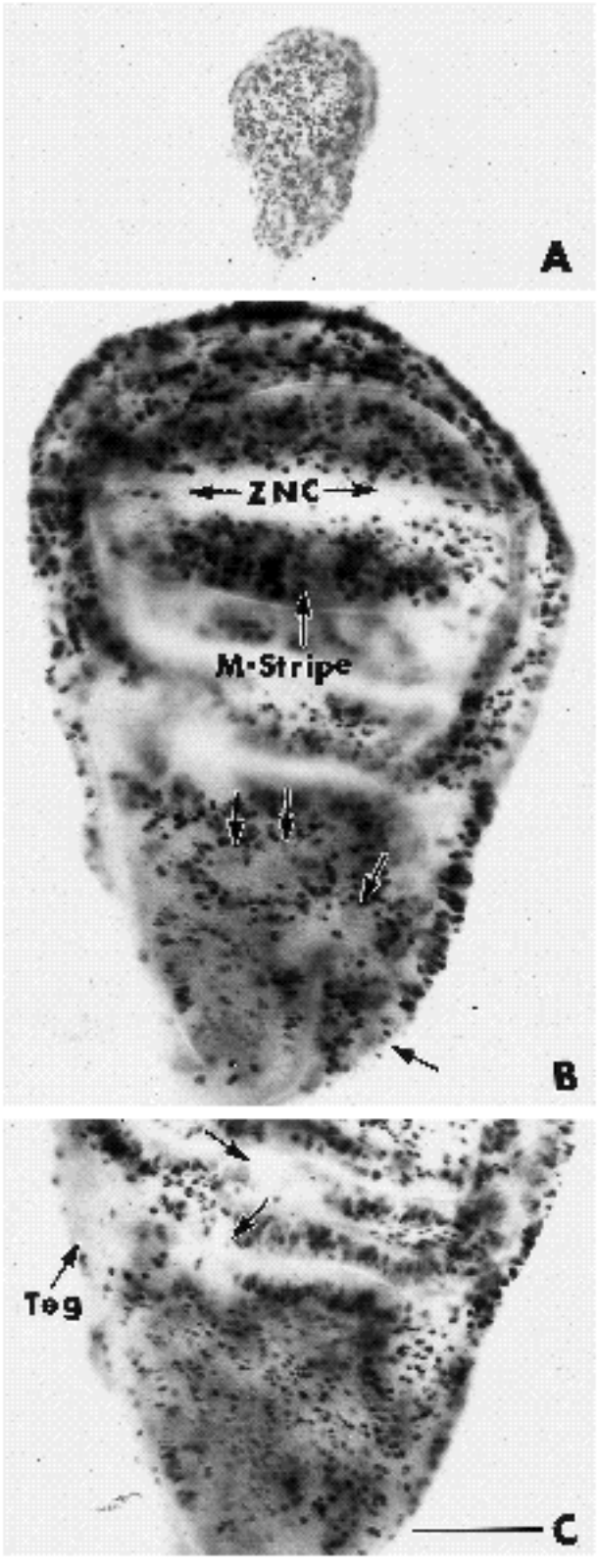


Fig. 1. (A) SMCs on the right wing disc of a wandering third larva from the transformant line A101. SMCs and their progeny express β-galactosidase and can therefore be labeled with X-gal in this line. Site nomenclature and SMC assignment are according to Huang et al. (1991) and Cubas et al. (1991). TSM 1 & 2, twin campaniform sensilla 1 and 2; L3-2, campaniform sensillum of the third wing vein; ACV, campaniform sensillum of the anterior crossvein; GSR, giant sensillum of the radius; dR, sensilla of the dorsal radius; Teg, sensilla and bristles of the tegula; anWP, companiform sensilla of anterior wing process; W.M., chemosensory bristles of the anterior wing margin; vHCV, sensillum of the ventral humeral cross vein (out of focus, arrowhead); vR, sensilla of the ventral radius (out of focus, arrowhead). aNP, anterior notopleural bristle; pNP, posterior notopleural bristle; aDC, anterior dorsocentral bristle; pDC, posterior dorsocentral bristle; pSA, posterior supraalar bristle; aPA, anterior postalar bristle; aSC, anterior scutellar bristle; pSC, posterior scutellar bristle; trl; trichoid sensillum 1. Anterior is to the left. (B) Schematic representation of the distribution of eleven macrochaetes and two trichoid sensilla on the adult right heminotum. PS, presutural bristle; aSA, anterior supraalar bristle; pPA, posterior postalar bristle; tr2, trichoid sensillum 2. Other symbols are explained in the legend of Fig. 1A. (C) Schematic representation of the distribution of adult wing sensory organs. L3-1, -2, -3, campaniform sensilla of the third wing vein; aWM, chemosensory bristles of anterior wing margin; pWM, non-innervated bristles of posterior wing margin. Anterior is to the top.

We examined the pattern of mitotic activity in the developing wing disc, by following the pattern of incorporation of BrdU in S-phase nuclei. For this, we incubated the imaginal discs in a BrdU solution for 30 minutes, and processed them for immunodetection of incorporated BrdU immediately after the incubation. Only nuclei of cells that were in S phase will be labeled in these conditions.



In early third instar larvae, the nuclei of the wing disc cells are strongly labeled with BrdU, reflecting a high mitotic activity (Fig. 2A). The spatial distribution of the labeled cell is random. In the late third instar larva, however, the distribution of BrdU-labeled cells becomes non-uniform (Fig. 2B,C). The most obvious feature is a band (several cells in width) of non-labeled cells, running in the anterior - posterior direction in the wing region (Fig. 2B). This band was called the ZNC (zone of non-proliferating cells) by O'Brochta and Bryant (1985) and coincides with the prospective wing margin (Fig. 1C). Perpendicular to this band, another band of non-labeled cells was observed (Fig. 2B). This band was named midline stripe (M-stripe) by Schubiger and Palka (1987) and corresponds to the prospective vein L3 (Fig. 1C). Another region of the prospective wing where cells are reproducibly unlabeled is the presumptive tegula region (Fig. 2C), which will make the most proximal part of the wing blade (Fig. 1C). Smaller clusters of non-labeled cells are also observed at other locations in the wing region (Fig. 2C, arrows).

In the notum region, labeled nuclei were observed both among epithelial and adepithelial cells (Fig. 2B,C). Strongly labeled nuclei are randomly distributed in the adepithelial layer (Fig. 2C). In the epithelial layer, however, the spatial distribution of labelled nuclei is not uniform (Fig. 2B). Clusters of non-labeled cells were reproducibly detected in most discs of late third instar larvae (Fig. 2 B and see later), in particular in the central region of the notum where the SMCs of the aDC, pDC, pSA, trl and aPA will appear (Fig. 1A).

Regional relationship between sensory mother cells (SMCs) and mitotically quiescent cluster of cells (MQCs)

Double staining with BrdU and X-gal in late wing discs of the enhancer-trap line A101 reveals that SMCs form within the clusters of non-labeled cells (Fig. 3A,B,C and D). The two rows of SMCs of the chemosensory bristles of the anterior wing margin (Fig. 1C) are located in the anterior half of the ZNC (Fig. 3A). The SMCs of the campaniform sensilla L3-2, ACV, and GSR form in the dorsal half of the M-stripe. Other SMCs such as vR and vHCV in the wing region (Fig. 3C), or unidentified SMCs in the tegula (Fig. 3B, Bryant, 1975) were also surrounded by clusters of mitotically quiescent cells (MQCs). The same correlation is observed in the notum region. The SMCs of the pSA,

Fig. 2. Patterns of BrdU-labeled cells in the wing disc. (A) Wing disc from an early third instar larva. Random distribution is observed in the wing disc at this stage. (B) Wing disc from a late third instar larva. The zone of non-proliferating cells (ZNC) is obvious in the wing pouch. The midline stripe (M-stripe) of non-labeled cells is visible perpendicular to the ZNC. In addition to the ZNC and M-stripe, cell clusters where BrdU has not been incorporated were observed in the tegula and notum regions (arrows). (C) Notum region of the same disc at a different focal plane. The adepithelial cells (myoblasts) are also labeled randomly. Bar: 50 μm. Anterior is to the left.

aPA, trl, pDC, aNP (Fig. 3B), aSC, and pSC (Fig. 3D) are located within clusters of cells that have not incorporated BrdU. The SMCs are not always located at the center of the cluster. For example, the SMC of the pDC is located at an eccentric position in the cluster (Fig. 3B).

When wing discs from the A101 line were stained simultaneously with X-gal and BrdU, we sometimes observed that the two sister cells resulting from the first division of the SMC were doubly labeled with X-gal and BrdU (not shown). However, we never observed a SMC which was doubly labeled with X-gal and BrdU. This indicates that the SMC may be arrested not in G_1 phase but in G_2 phase.

Temporal relationship between the emergence of SMCs and the presence of MQCs

We examined the temporal relationship between SMCs and MQCs to determine which appears earlier, the SMC or its surrounding MQC. Fig. 3F shows a doubly labeled wing disc from a middle third instar larva, the stage of which is judged to be about 24 hours BPF from the description by Huang et al. (1991) because the SMCs of dR, pSC, L3-2 and GSR have already appeared. Although the resolution of the analysis for this stage is low because the disc is small and few SMCs are available as landmarks to estimate the position where other SMCs will appear later, MQCs are observed at or near the prospective positions of SMCs that will emerge soon after, specifically, the aPA, pNP, aNP, and tegula SMCs (Huang et al., 1991). In the wing region, the ZNC appears earlier than the SMCs of the wing margin. A ZNC void of any SMC is often observed in mid/late third instar larva (about 12 hours BPF) (Fig. 3E).

The same situation occurs in late third instar larvae. In the disc shown in Fig. 3B, the SMCs of the aPA, pDC, aNP, pSA and tr1 are surrounded by MQCs (Fig. 3B). Judging from the description of the temporal pattern of SMC appearance by Huang et al. (1991), the stage of this disc is about 6 hours BPF. In the same disc, MQCs are also observed at the positions where the SMCs of the aDC and PS will appear several hours later. Thus, MQCs appear earlier than the corresponding SMC.

Sequential appearance of MQCs

In order to reconstruct the dynamics of appearance and disappearance of MQCs, we performed the BrdU injection experiment. BrdU was injected into third instar larvae at various stages, and the larvae were subsequently allowed to grow until 24-30 hours APF. The pattern of BrdU-containing nuclei at a pupal stage reflects the pattern of mitotic activity in the wing disc at the time when BrdU was injected (Fig. 4A). We focus on the temporal pattern of MQC of three bristles: the pDC, aDC, and pPA (Fig. 4B,C). Fig. 4D shows the relation between the frequency of a MQC and the developmental stage when BrdU was injected.

In the pPA region, we observed the formation of the MQC. When BrdU was injected at 12-18 hours BPF or at 6-12 hours BPF, no MQC could be detected around the prospective pPA site (Fig. 4D). However, when BrdU was injected at 0-6 hours BPF, a MQC surrounded the pPA site (Fig. 4B). The SMC of the pPA is first observed at 1 hour APF (Huang et al., 1991). This means that the MQC appears a few hours before the formation of the SMC.

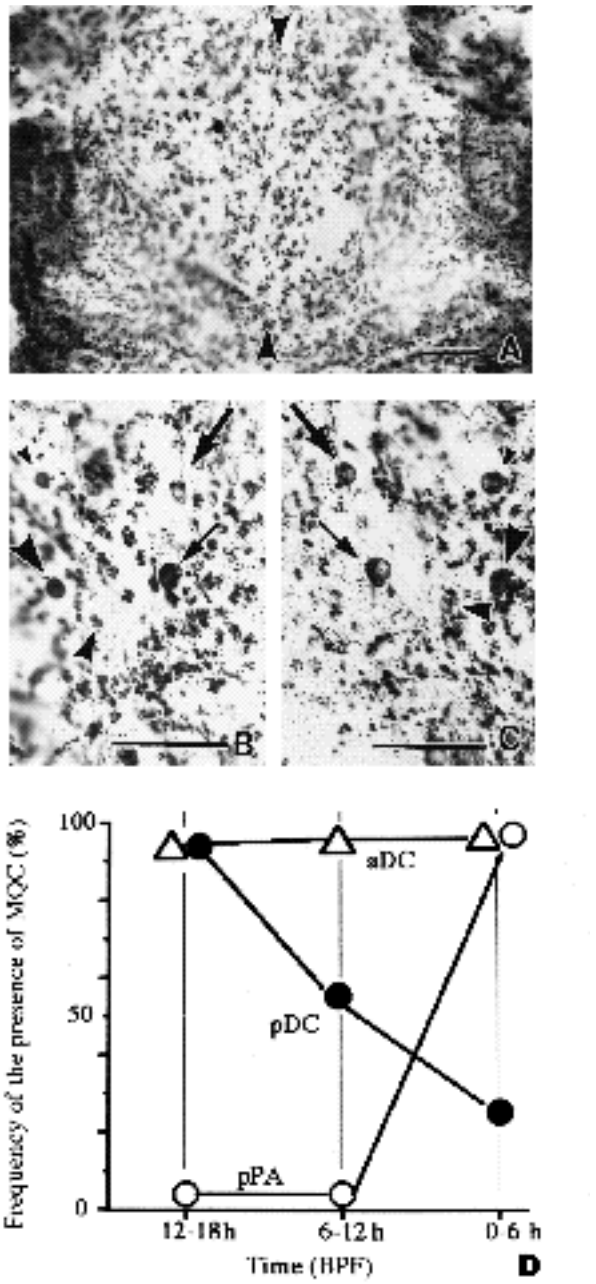


Fig. 4. (A) Non-random distribution of BrdU-labeled cells in the notum of a 24 hours APF pupa. BrdU was injected in third instar larvae, and the grown-up pupae were dissected at 24 hours APF. Arrowheads, dorsal midline. Bar, 100 μm. (B, C) The central region of a pupal heminotum (24-30 hours APF) double-labeled with BrdU and mAb 22C10. Large arrow, aDC; small arrow, pDC; small arrowhead, pSA; middle arrowhead, pPA; large arrowhead, aPA. Anterior is to the top. Bar, 100 μm. In B, BrdU was injected at 0-6 hours BPF. Left heminotum. The aDC and pPA are within a MQC, while the pDC is surrounded by labeled cells. (C) BrdU was injected 12-18 hours BPF. Right heminotum. The aDC and pDC are within a MQC, while the pPA is surrounded by labeled cells. (D) Frequency distribution of the MQC corresponding to three bristles (pDC, pPA, and aDC) at different developmental stages, suggestive of a sequential appearance of the MQCs during the third larval instar.

In the aDC region, a MQC was seen in most of the pupae that were examined. The SMC of the aDC is singled out later than 12 hours BPF and remains undivided until 1 hour APF (Huang et al., 1991). Thus in the case of the aDC the MQC is formed prior to the SMC, and is maintained around the SMC for several hours (Fig. 4B, C).

In the case of the pDC region, we could observe the disappearance of a MQC. When BrdU was injected at 12-18 hours BPF, at a time when the SMC of the pDC is already formed (Huang et al., 1991), we observed the presence of a MQC surrounding this SMC. However, in about 75% of the larvae that have been injected with BrdU at 0-6 hours BPF, the cells surrounding the SMC are labeled. This shows that the cells that were mitotically arrested at the time when the SMC was formed resume mitotic activity at later times (Fig. 4B). The SMC of the pDC remains undivided at least until 6 hours BPF and thereafter divides to produce two sister cells by 0 hours BPF (Huang et al., 1991). The disappearance of the MQC seems therefore to occur around the time when the SMC undergoes its first cell division.

MQC in ac *and* sc *mutants*

It has been suggested that *ac/sc* protein may be involved in the cell cycle of SMC (Cubas et al., 1991). We examined the effect of the *ac* and/or *sc* mutations on the pattern of MQCs to examine whether or not the formation of MQCs is regulated by the *ac* or *sc* gene. The adult notum of ac^3 mutant flies lacks the *ac*-dependent pSA, aDC and pDC macrochaetes due to the lack both of *ac* and *sc* expressions in the corresponding proneural cluster (Skeath and Carroll, 1991), since expression of both of these genes is controlled by the *ac* regulatory regions which directly activate *ac* transcription and indirectly cross-activate *sc* expression by means of the *ac* protein (Martinez and Modolell, 1991; Skeath and Carroll, 1991). The SMCs of these bristles were not found in the ac^3; A101 wing disc (data not shown). If the expression of *ac* were responsible for the formation of MQCs, the MQCs corresponding to *ac*-dependent sites should be lost in the mutant disc. However, MQCs were present at the position of the aDC and pDC (Fig. 5B, small arrow and small arrowhead, respectively) and, more generally, no significant differences from the wild type (Fig. 5A) were found in the BrdU incorporation pattern.

The $sc^{10.1}$ mutant is doubly mutant for *ac* and *sc*; it synthesizes no *ac* mRNA and a truncated *sc* protein (Campuzano et al., 1985; Villares and Cabrera, 1987). Adult $sc^{10.1}$ mutant flies completely lack macrochaetes and microchaetes on the notum as well as all chemosensory bristles on the anterior wing margin (Garcia-Bellido, 1979, Cubas et al., 1991). No SMCs are found in $sc^{10.1}$; A101 mutant discs (Cubas et al., 1991, our unpublished data). No apparent changes of MQCs, M-stripe and ZNC pattern were recognized in $sc^{10.1}$ mutant disc (Fig. 5C, D). In the region of the aDC and pDC, both of which depend on the *ac* gene, a typical MQC pattern was observed (small arrow and small arrowhead, respectively, in Fig. 5C). Other MQCs were also found, for example, in the regions of the pSA, which depends both on the *ac* and *sc* genes, and in that of the aPA, which depends on the *sc* gene (large arrow and large arrowhead, respectively, in Fig. 5C).

Discussion

The regulation of mitotic activity in imaginal discs may play a role not only in the control of the growth, but also in the developmental processes such as commitment, differentiation, compartmentalization and regeneration (Dale and Bownes, 1980; Adler, 1981; Graves and Schubiger, 1982; Adler and MacQueen, 1984). The spatial distribution of replicating cells was thought to be uniform in *Drosophila* (James and Bryant, 1981; Adler and MacQueen, 1984) until O'Brochta and Bryant (1985) described a regional departure from uniform mitotic activity, the zone of non-proliferating cells (ZNC) that corresponds to the presumptive wing margin in the wing disc of late third instar larva. Schubiger and Palka (1987) described an additional region of mitotic quiescence, the midline stripe (M-stripe), which intersects the ZNC perpendicularly. This M-stripe coincides with the third vein. Here we report that the spatial distribution of mitotic activity is not uniform in late wing discs, either in the wing region or in the notum region. Mitotically quiescent clusters of cells (MQCs) are found around the SMCs and around the positions where SMCs will appear later, suggesting that the emergence of MQCs may be correlated to the acquisition of a proneural fate by these cells.

Sensory organs are generated through multiple steps operated by many classes of genes (Ghysen and Dambly-Chaudière, 1989; Jan and Jan, 1990). First, a prepattern is formed according to positional cues given by prepattern genes. Clusters of ectodermal cells respond to the pre-existing positional cues, and acquire a proneural state due to the expression of proneural genes such as *ac* and *sc*. A SMC is then singled out from the proneural cluster by cell-cell interactions and inhibits its neighbours from adopting the same fate (Wigglesworth, 1940; review by Simpson, 1990). This step is mediated by the neurogenic genes (review by Campos-Ortega and Jan, 1991). The type of sensory organ is then selected by neuronal type selector genes, and cell lineage genes will then control the divisions of the SMC and the differentiation of the sister cells formed by the ensuing cell divisions, thereby forming the different components of the sensory organ.

It was thought that the spatially restricted expression of *ac* and *sc* was one of determinants of where SMCs will arise (Romani et al., 1989). However, it has been reported recently that the ubiquitous expression of *sc* obtained by heat-shocking a hsp-*sc* chimeric gene in a mutant where the endogenous *ac* and *sc* genes are mutated, results in the formation of some macrochaetes at positions roughly appropriate for wild-type macrochaetes, indicating that within tissues there are local heterogeneities which make some sites more sensitive than others to the ubiquitous expression of *sc* (responsiveness). This result implies that a mechanism must exist that regulates spatially and temporally the responsiveness to the *sc* product (Rodriguetz et al., 1990). We think that one of the most likely candidates for this local responsiveness is the mitotically quiescent state of the cells that constitute the MQCs. In the wild-type fly, *sc* is expressed only in the anterior half of the ZNC, and SMCs appear only along the anterior margin of the wing (Fig. 1A). However, if the *sc* gene is overexpressed as in the mutant (Hw^{49C}), extra SMCs form in the posterior region of the

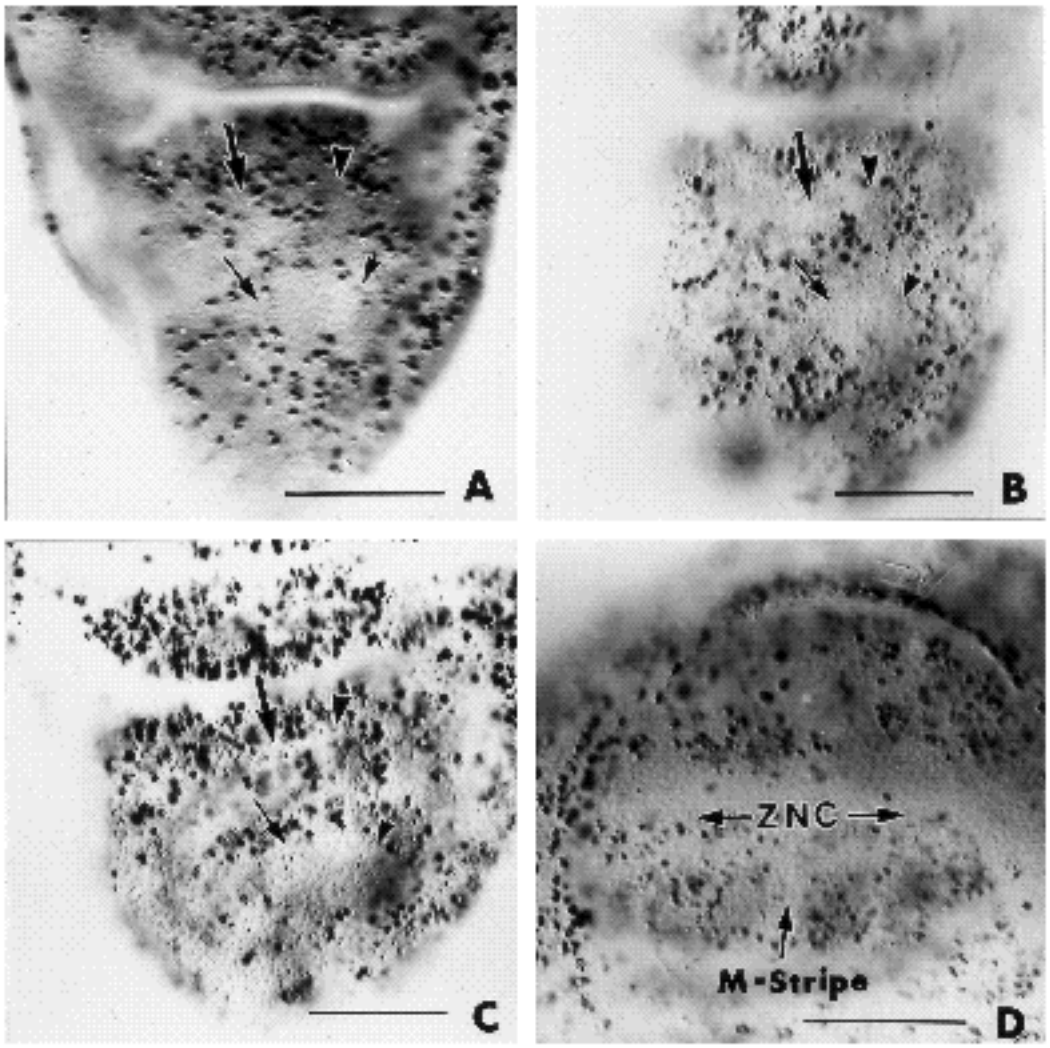


Fig. 5. The formation of a normal pattern of MQCs does not require the *ac* and *sc* genes. BrdU labeling patterns of the wing disc from wild-type (A) and mutant (B-D) late third instar larvae. (A) Notum region of a wild-type wing disc. MQCs corresponding to the aDC (small arrow), pDC (small arrowhead), pSA (large arrow), and aPA (large arrowhead) are observed in a reproducible and typical pattern. (B) Notum region of an ac^3 mutant. No significant difference from the wild type is detectable in the BrdU incorporation pattern. MQCs are present at the position of the aDC (small arrow) and pDC (small arrowhead) which are removed in this mutant. (C, D) Wing discs of a $sc^{10.1}$ disc (doubly mutant for *ac* and for *sc*). (C) The MQC pattern on the notum does not appear significantly different from the wild type. (D) The ZNC and M-stripe of the wing region are also observed in this mutant. Bar, 50 μm.

wing margin (our unpublished data), resulting in the differentiation of extra bristles along the posterior margin of the adult wing (Balcells et al., 1988). This implies that the posterior region of the ZNC is responsive to the expression of the *sc* gene. Thus we propose that the formation of a SMC requires the acquisition of responsiveness, due to a depression of mitotic activity, in addition to the acquisition of ability to become SMC (competence), due to the expression of the proneural genes *ac* or *sc* (Goriely et al., 1991).

Competence and responsiveness are both spatially and temporally regulated. The MQCs appear prior to the formation of the SMC, in a temporal and spatial pattern that is very similar to that of *ac* and *sc* expression. This raises the question of whether the MQCs appear as a result of *ac* and *sc* expression. Both *ac* and *sc* genes encode a basic helix-loop-helix protein (Villares and Cabrera, 1987), which is characteristic of a family of transcriptional regulators, including the mammalian MyoD1 which promotes myogenesis (Davis et al., 1987). MyoD1 inhibits cell proliferation when introduced in cultured cells, independently of myogenic differentiation (Sorrentino et al., 1990). The similarity in structure and function between *ac*, *sc* and MyoD1 raises the possibility that the products of *ac* and *sc* also alter the mitotic behavior of the epithelial cells. However, this is not the case because the appearance of MQCs is not affected in ac^3 (ac^-) and $sc^{10.1}$ (ac^- and sc^-) mutants. Therefore, the local appearance of MQCs is unlikely to be regulated by the local expression of the *ac* and *sc* genes. However, we cannot eliminate a possibility that the disappearance of MQC after the SMC appearance is involved in the *ac/sc* expression. For example, once *ac/sc* are repressed in the MQC, their absence may enable the cells in the MQC

to start dividing again. Cubas et al. (1991) have suggested that the disappearance of *ac/sc* expression from the SMC is a prerequisite for its division.

If the appearance of MQCs does not depend on the expression of *ac* and *sc*, how is it regulated? It is believed that the embryo and the imaginal discs are subdivided into smaller domains by the combined or combinatorial action of 'prepattern genes' (reviewed by Ingham, 1988, Wilkins and Gubb, 1991). The spatially and temporally restricted appearance of the MQCs may be regulated by the same combinations of prepattern genes that also control the expression of *ac* and *sc*. The gene *wingless* (*wg*), a segment polarity gene that shows a spatially restricted pattern of expression both in the embryo (Baker, 1988a) and in the wing disc (Baker, 1988b), may be one of the candidates for the MQC regulatory genes. In the wing disc, *wg* is expressed in the ZNC, the prospective tegula and part of the notum region (Baker, 1988b; Peifer et al., 1991). In viable *wg* mutants, the ZNC is reduced or absent (R.G. Phillips, personal communication). The expression of *wg*, however, does not cover all the areas where MQCs are observed, and therefore other genes are probably involved as well.

The importance of the mitotic state of the cell in developmental processes has already been documented in other cases. For example, when undifferentiated myoblasts begin to differentiate, cells are withdrawn from the cell cycle and arrested in the G_1 phase (Linkhart et al., 1980). In the case of the fly sense organs, the importance of the mitotic state for SMC singling out has been already suggested by some groups (Hartenstein and Posakony, 1989; Huang et al., 1991; Cubas et al., 1991). Our results give us indirect evidence about the stage of the cell cycle at which the cells of the MQCs are blocked. We observed that the two daughter cells of a SMC may display both BrdU and X-gal labeling, but no doubly labeled SMC was ever observed. This indicates that the SMC is derived from a MQC cell which will not incorporate BrdU before undergoing mitosis and, therefore, that this cell was arrested in G_2. After the cell division has taken place, the two daughter cells will enter S phase for the next cell division and, therefore, may be doubly labeled with X-gal and BrdU. Thus we conclude that commitment to become a SMC only occurs in cells that are arrested in the G_2 phase of the cell cycle.

The idea that the SMC is derived from MQC can explain the early dispensability of the genes *ac* and *sc* for the formation of bristles. When mitotic recombination is induced before the appearance of the SMC (48 hours BPF, Garcia-Bellido and Merriam, 1971; 10-25 hours before the time when SMCs become detectable by their increased accumulation of *ac* and *sc* protein, Cubas et al., 1991), cells that have become genotypically ac^- and sc^- can nevertheless become bristles. On the other hand, it has been shown that the expressions of *ac* and *sc* genes start before the appearance of SMCs and continue until the first division of the SMC (Cubas et al., 1991; Skeath and Carroll, 1991), suggesting that their expressions are necessary not only for making the cell competent to become SMC, but also for the maintenance of SMC state (Cubas et al., 1991). If the cells that will become SMC later are indeed arrested mitotically, these paradoxical results are readily explained (Fig. 6). If mitotic recombination is induced in one of these cells, the cell can nevertheless express *ac* and *sc* long after the recombination has been induced, since it is only after the next division that one of the daughter cells will effectively become homozygous for the mutation. Therefore, the cell can attain the identity of a SMC. As *ac* and *sc* products are not present, and, therefore, presumably not required, after the first division of the SMC (Cubas et al., 1991; Skeath and Carroll, 1991), the ac^- sc^- progeny of the SMC, which will be formed as a result of the recombination event, can form a bristle in spite of the absence of functional *ac* and *sc* genes.

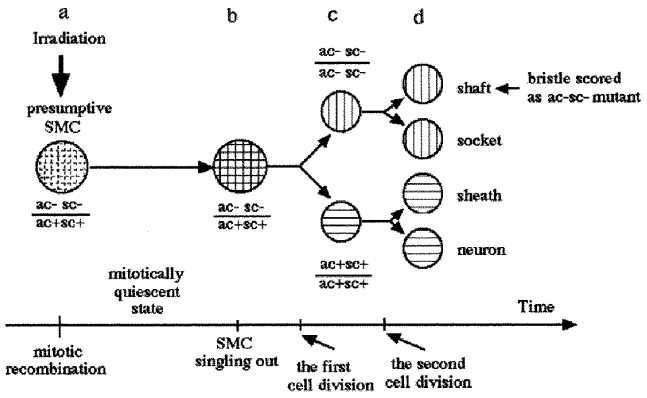


Fig. 6. Hypothetical explanation for the early dispensability of *ac/sc* genes for bristle formation in mitotic recombination experiments. (a) Mitotic recombination in a cell (a presumptive SMC) (ac^-sc^-/ac^+sc^+) is induced by irradiation. If the cell is arrested mitotically, the genotype of the cell remains ac^+sc^+/ac^-sc^- for a long time until its first division. (b) The cell can be singled out as a SMC, because the cell still remains genotypically ac^+sc^+/ac^-sc^-. (c) After the first division of the SMC, one of the daughter cells becomes genotypically ac^-sc^-/ac^-sc^-. (d) After the second division, the cell of ac^-sc^- genotype cell becomes two type of cells, a shaft cell and a socket cell. They can be scored as an ac^-sc^- mutant bristle.

Finally, we would like to summarize our view of the commitment to SMC fate in relation to the mitotic activity (Fig. 7). The epidermal cells of the wing disc are proliferating exponentially during most of the larval life. No regional differences of mitotic activity can be seen in discs at the early third instar stage (Fig. 7A). Reproducibly located clusters of cells are then withdrawn from the cell cycle and arrested, probably in the G_2 phase (Fig. 7B); the other cells continue to proliferate actively. The arrested cells form the MQCs and respond to the expression of proneural genes by acquiring a proneural potential. One cell is singled out among the cells of the MQC and becomes SMC (Fig. 7C). The SMC resumes its mitotic activity, enters the M phase without synthesizing DNA, and produces two daughter cells (Fig. 7D). The other cells of the MQC may then be released from mitotic arrest and resume their mitotic activity (Fig. 4D, pDC), or remain arrested in G_2 (Fig. 4D, aDC and pPA) until early metamorphosis (Graves and Schubiger, 1982). As the expression of *ac* and *sc* is eventually turned off in the proneural clusters, these cells will lose their proneural potential even though they remain arrested in G_2, and eventually develop as epidermal cells (Fig. 7E).

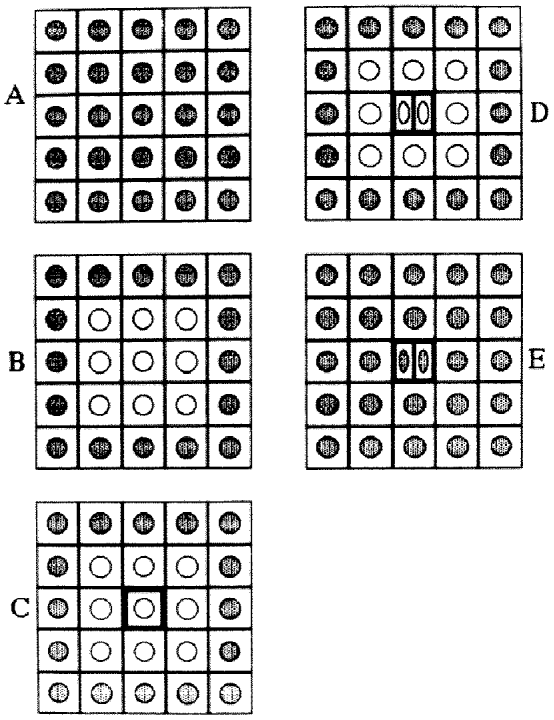


Fig. 7. Schematic representation of the summary of the first steps in the development of a sense organ in relation to the mitotic activity. Each square represents a cell. Filled circle, mitotically activate cell; open circle, mitotically arrested cell; bold box, sensory mother cell and its progeny. See text for explanations.

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References

Adler, P. N. (1981). Growth during pattern regulation in imaginal discs. *Dev. Biol.* **87**, 356-373.

Adler, P. N. and MacQeen, M. (1981). Partial coupling of the cell cycles of neighboring imaginal disc cells. *Exp. Cell Res.* **133**, 452-456.

Adler, P. N. and MacQueen, M. (1984). Cell proliferation and DNA replication in the imaginal wing disc of *Drosophila melanogaster*. *Dev. Biol.* **103**, 28-37.

Baker, N. E. (1988a). Localization of transcripts from the *wingless* gene in whole *Drosophila* embryos. *Development* **103**, 289-298.

Baker, N. E. (1988b). Transcription of the segment-polarity gene *wingless* in the imaginal discs of *Drosophila*, and the phenotype of a pupal-lethal *wg* mutation. *Development* **102**, 489-497.

Balcells, L., Modolell, J. and Ruiz-Gomez, M. (1988). A unitary basis for different *Hairy-wing* mutations of *Drosophila melanogaster*.*EMBO J.* **7**, 3899-3906.

Bate, C. M. (1978). Development of sensory systems in arthropods. In *Handbook of Sensory Physiology*, vol.9 (ed. M. Jacobson), pp. 1-53. Berlin, New York: Springer Verlag.

Bellen, H. J., O'Kane, C. J., Wilson, C., Grossniklaus, U., Pearson, R. K. and Gehring, W. J. (1989). P-element-mediated enhancer detection: a versatile method to study development in *Drosophila*. *Genes Dev.* **3**, 1288-1300.

Bryant, P. J. (1975). Pattern formation in the imaginal wing disc of *Drosophilamelanogaster*: fate map, regeneration and duplication. *J. Exp. Zool.* **193**, 49-78.

Bryant, P. J. (1978). Pattern formation in imaginal discs. In *The Genetics and Biology of Drosophila*, vol. 2c (eds. M. Ashuberner and T. R. F. Wright), pp. 230-325. London and New York: Academic Press.

Campos-Ortega, J. A. and Jan, Y. N. (1991). Genetic and molecular bases of neurogenesis in *Drosophila melanogaster*. *Ann. Rev. Neurosci*. **14**, 399-420.

Campuzano, S., Carramolino, L., Cabrera, C. V., Ruiz-Gomez, M., Villares, R., Boronat, A. and Modolell, J. (1985). Molecular genetics of the achaete-scute gene complex of *D. melanogaster*.*Cell* **40**, 327-338.

Canal, I. and Ferrus, A. (1986). The pattern of early neuronal differentiation in *Drosophila melanogaster*. *J. Neurogen.* **3**, 297-319.

Cubas, P., de Celis, J.-F., Campuzano, S. and Modolell, J. (1991). Proneural clusters of *achaete-scute* expression and the generation of sensory organs in the *Drosophila* imaginal wing disc. *Genes Dev.* **5**, 996-1008.

Dale, L. and Bownes, M. (1980). Is regulation in *Drosophila* the result of epimorphic regulation? *Wilhelm Roux's Arch.Dev. Biol.* **189**, 91-96.

Davis, R. L., Weintraub, H. and Lassar, A. B. (1987). Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **51**, 987-1000.

Dunne, J. F. (1981). Growth dynamics in the regeneration of a fragment of the wing imaginal disc of *Drosophila melanogaster*. *Dev. Biol.* **87**, 379-382.

Fain, M. J. and Stevens, B. (1982). Alterations in the cell cycle of *Drosophila* imaginal disc cells precede metamorphosis. *Dev. Biol.* **92**, 247-258.

Garcia-Bellido, A. (1979). Genetic analysis of the *achaete-scute* system of *Drosophilamelanogaster*. *Genetics* **91**, 491-520.

Garcia-Bellido, A. and Merriam, J. R. (1971). Parameters of the wing imaginal disc development of *Drosophila melanogaster*. *Dev. Biol.* **24**, 61-87.

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

Ghysen, A. and Dambly-Chaudière, C. (1989). Genesis of the *Drosophila* peripheral nervous system. *Trends Genet.* **5**, 251-255.

Goriely, A., Dumont, N., Dambly-Chaudière, C. and Ghysen, A. (1991). The determination of sense organs in *Drosophila*: effect of the neurogenic mutations in the embryo. *Development* **113**, 1395-1404.

Graves, B.J. and Schubiger, G. (1982). Cell cycle change during growth and differentiation of imaginal leg discs in *Drosophila melanogaster*. *Dev. Biol.* **93**, 104-110.

Hartenstein, V. and Posakony, J. W. (1989). Development of adult sensilla on the wing and notum of *Drosophila melanogaster*. *Development* **107**, 389-405.

Haynie, J. (1975). Intercalary regeneration and pattern formation in imaginal discs of *Drosophila melanogaster*. Ph. D. Thesis, Univ. Calif., Irvine.

Hiromi, Y., Kuroiwa, A. and Gehring, W. (1985). Control elements of the *Drosophila* segmentation gene *fushi tarazu*.*Cell* **43**, 603-613.

Huang, F., Dambly-Chaudière, C. and Ghysen, A. (1991). The emergence of sense organs in the wing disc of *Drosophila*.*Development* **111**, 1087-1095.

Ingham, P. W. (1988). The molecular genetics of embryonic pattern formation in *Drosophila*.*Nature* **335**, 25-34.

James, A. A. and Bryant, P. J. (1981). Mutations causing pattern deficiencies and duplications in the imaginal wing disk of *Drosophila melanogaster*.*Dev. Biol.* **85**, 39-54.

Jan, Y. N. and Jan, L. Y. (1990). Genes required for specifying cell fates in *Drosophila* embryonic sensory nervous system. *Trends Genet.* **13**, 493-498.

Lawrence, P. A. (1966). Development and determination of hairs and bristles in the milkweed bug. *Oncopeltus fasciatus* (Lygaeidae, Hemiptera). *J. Cell. Sci.* **1**, 475-498.

Linkhart, T. A., Clegg, C. H. and Hauschka, S. D. (1980). Control of mouse myoblast commitment to terminal differentiation by mitogens. *J. Supramol. Struct.* **14**, 483-498.

Madhavan, M. M. and Schneiderman, H. A. (1977). Histological analysis of the dynamics of growth of imaginal discs and histoblast nests during the larval development of *Drosophila melanogaster*. *Wilhelm Roux's Arch. Dev. Biol.* **183**, 269-305.

Martinez, C. and Modolell, J. (1991). Cross-regulatory interactions

between the proneural *achaete* and *scute* genes of *Drosophila*. *Science* **251**, 1485-1487.

Mlodzik, M., Baker, N. E. and Rubin, G. M. (1990). Isolation and expression of *scabrous*, a gene regulating neurogenesis in *Drosophila*. *Genes Dev*. **4**, 1848-1861.

O'Brochta, D. A. and Bryant, P. J. (1985). A zone of non-proliferating cells at a lineage restriction boundary in *Drosophila*. *Nature* **313**, 138-141.

Peifer, M., Rauskolb, C., Williams, M., Riggleman, B. and Wieschaus, E. (1991). Segment polarity gene *armadillo* interacts with *wingless* signaling pathway in both embryonic and adult pattern formation. *Development* **111**, 1029-1043.

Rodriguez, I., Hernandez, R., Modolell, J. and Ruiz-Gomez, M. (1990). Competence to develop sensory organs is temporally and spatially regulated in *Drosophila* epidermal primordia. *EMBO J*. **9**, 3583-3592.

Romani, S., Campuzano, S., Macagno, E. R. and Modolell, J. (1989). Expression of *achaete* and *scute* genes in *Drosophila* imaginal discs and their function in sensory organ development. *Genes Dev*. **3**, 997-1007.

Schubiger, M. and Palka, J. (1987). Changing spatial patterns of DNA replication in developing wing of *Drosophila*. *Dev. Biol.* **123**, 145-153.

Simpson, P. (1990). Lateral inhibition and the development of the sensory bristles of the adult peripheral nervous system of *Drosophila*. *Development* **109**, 509-519.

Skeath, J. B. and Carroll, S. B. (1991). Regulation of *achaete-scute* gene expression and sensory organ pattern formation in the *Drosophila* wing. *Genes Dev*. **5**, 984-995.

Sorrentino, V., Pepperkok, R., Davis, R. L., Ansorge, W. and Philipson, L. (1990). Cell proliferation inhibited by *MyoD1* independently of myogenic differentiation. *Nature* **345**, 813-815.

Villares, R. and Cabrera, C. V. (1987). The *achaete-scute* gene complex of *D. melanogaster*: Conserved domains in a subset of genes required for neurogenesis and their homology to *myc*. *Cell* **50**, 415-427.

Wigglesworth, V. B. (1940). Local and general factors in the development of "pattern" in *Rhodnius prolixus*. *J. Exp. Biol.* **17**,180-200.

Wilkins, A.S., and Gubb, D. (1991). Pattern formation in the embryo and imaginal discs of *Drosophila* - What Are the Links. *Dev. Biol.* **145**, 1-12.

Wilson, C., Pearson, R. K., Bellen, H. J., O'Kane, C. J., Grossniklaus, U. and Gehring, W. J. (1989). P-element-mediated enhancer detection: an efficient method for isolating and characterizing developmentally regulated genes in *Drosophila*. *Genes Dev*. **3**, 1301-1313.

Wolff, T. and Ready, D. F. (1991). The beginning of pattern formation in the *Drosophila* compound eye: the morphogenetic furrow and the second mitotic wave. *Development*, **113**, 841-850.

Zipursky, S. L., Venkatesh, T., Teplow, D. B. and Benzer, S. (1984). Neuronal development in the *Drosophila* retina: Monoclonal antibodies as molecular probes. *Cell* **36**, 15-26.

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Fig. 3 Colour

Fig. 3. Regional and temporal relationships between sensory mother cells (SMC) and mitotically quiescent cluster of cells (MQC). Discs were stained with BrdU immunohistochemistry (brown) and X-gal reaction product (deep blue). (A) Wing region of the right wing disc from a late third instar larva. SMCs of the wing chemosensory bristles are aligned in two rows in the anterior half of the ZNC. SMCs of L3-2, ACV, and GSR are located in a line in the dorsal half of the M-stripe. (B) Notum region of a right wing disc from a late third instar larva (about 6 hours BPF). SMCs of the tegula (Teg) and several notum macrochaetes (aNP, pSA, aPA, trl, and pDC) are located within the clusters of BrdU non-labeled cells (the SMC of the pNP is out of focus). Other SMCs such as PS and aDC have not appeared yet. MQCs are observed around the sites where the SMCs of the PS and aDC bristles will appear later (arrows). (C) SMCs of the vR and vHCV sensilla, in the wing region, also form within MQCs (Same preparation as in A). (D) Scutellum region of the notum of a right wing disc (older than B). SMCs of the aSC and pSC are located in the cluster of BrdU non-labeled cells. The SMC of the pSC bristle has already undergone its first division. (E) Wing region of the wing disc from a mid/late third instar larva (about 12 hours BPF). The ZNC has appeared but the SMCs of the chemosensory bristles of the anterior wing margin have not appeared yet. (F) A wing disc from the mid third instar larva (about 24 hours BPF). Only the SMCs of the dR sensilla and pSC bristle (faintly labeled, arrowhead), L3-2 and GSR have appeared (L3-2 and GSR are out of focus). MQCs are observed at the positions where the SMCs of the tegula (Teg), aNP, pNP, and aPA will appear later (arrows). Bar, 50 μm. Anterior is to the right.