Pattern formation in the nematode epidermis: determination of the arrangement of peripheral sense organs in the *C. elegans* male tail

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Summary

The developmental process that determines the arrangement of ray sensilla in the *Caenorhabditis elegans* male tail has been studied. It is shown that the adult arrangement of rays is determined by the placement of ray cells at specific sites in the epidermis of the last larval (L4) stage. Placement of ray cells at specific epidermal sites results from the generation of neurons and support cells in the epidermis near to their final positions, and the subsequent refinement of these positions by an active mechanism involving specific cellular associations. Positions of ray cells and adjacent epidermal cells have been studied during ray development by means of indirect immunofluorescence staining with an antibody to a cell junctional antigen. Mutations are described in six genes that alter the adult arrangement of the rays, frequently resulting in fusion of rays. Changes in the adult pattern of rays in mutants appear to result from prior changes in the epidermal positions of ray cells, and for two mutants it is suggested that this may be due to the inappropriate clustering of processes from neurons and support cells of adjacent rays. Development of the wild-type arrangement of rays appears to require the specification of molecular differences between the rays that affect the specificity of their cellular associations.

Key words: morphogenesis, pattern formation, peripheral sense organs.

Introduction

The epidermis of the nematode *C. elegans* contains simple peripheral sense organs, known as sensilla. These sensilla, numbering some 30 in the hermaphrodite and 53 in the male, are located at reproducible positions in the head, along the body, or in the tail, and mediate the animal's interaction with its environment *via* various sensory modalities (Ward *et al.* 1975; Sulston *et al.* 1980; Chalfie and White, 1988). Most of the sensilla are present at hatching, and additional sensilla are added during postembryonic development, particularly those present in the male tail required for copulatory behavior. As a model for understanding how animal morphology is determined by genes, we are carrying out a genetic analysis of the postembryonic development of one class of male-specific sensilla known as rays.

Rays, used by the male to sense contact with the hermaphrodite prior to copulation, are present in the posterior region within the fan, a male-specific copulatory organ. Each of the nine bilateral pairs of rays lies at a reproducible position in the fan, thereby giving rise to a male tail morphology that is constant within a species. Closely related species have variant patterns of

rays, attesting to the genetic specification of the overall pattern (for example, some species have ten instead of nine pairs, or a different anteroposterior distribution) (Cobb, 1920; Chitwood and Chitwood, 1974; Andrássy, 1983).

Although the rays are required for copulation, they are not required for strain viability, as the other sex in *C. elegans* is a self-fertile hermaphrodite. This property has facilitated the isolation of mutations in several genes that affect ray development (Hodgkin, 1983; Waring and Kenyon, 1990; Baird and Emmons, 1990). Mutations previously described either prevent the generation of the specialized cells of the rays (Hodgkin, 1983; Kenyon, 1986; Shen and Hodgkin, 1988; Chisholm and Hodgkin, 1989; Waring and Kenyon, 1990), or affect morphogenesis of individual rays (Hodgkin, 1983; Baird and Emmons, 1990). In this paper, we describe mutations in 6 genes that alter the positions of the rays in the epidermis. These genes define components of an active mechanism that determines the arrangement of the rays in the fan.

It is expected that morphology rests both on the generation of specialized cells at correct positions, and on the morphogenetic properties of individual cells. The latter include cell migration, contact guidance, cell

adhesion, cell shape and asymmetric differentiation. Organogenesis and tissue architecture are particularly dependent on the ordered formation of homotypic and heterotypic cell contacts, adhesions, and junctions between cells (Bard, 1990). Both the generation of the cells in the male tail and their differentiation, arrangement, movements and contacts, have been described, making this a favorable system for genetic studies of the morphogenetic mechanism. Sulston and Horvitz (1977) and Sulston et al. (1980) reported the postembryonic cell lineages that follow from the division of a small number of blast cells, giving rise to the male-specific structures of the tail, including the rays. Sulston et al. (1980) determined the positions and characteristics of cells in the male tail by reconstructing the tail from electron micrographs of serial thin sections, and also described the morphogenetic movements that give rise to the fan and rays.

We have extended this work by determining the dynamically changing positions and shapes of cells in the epidermis during the developmental process. We have found that the adult arrangement of the rays is dependent upon the positioning of ray cells at reproducible sites in the epidermis of the preceding larval stage. The epidermal positions of ray cells depend both upon the initial positions where cells are born, and upon their selective cellular affinities which refine these initial positions. Formation of each ray at a distinct site appears to be guided by the expression of a unique pattern of affinities for epidermal cells and ray cells. Mutations in the 6 identified genes either directly block the activity of molecules participating in cell contacts, or affect specification of differences between the rays that ultimately determine the pattern of affinities of each.

Materials and methods

Isolation and mapping of mutations

Mutant and wild-type strains of C. elegans were maintained at 20° as described by Brenner (1974). Unless otherwise indicated, all strains contained him-5(e1490) (Hodgkin et al. 1979). This mutation increases the frequency of males in a selfing population to 30 % due to an increase in the rate of X chromosome loss due to meiotic nondisjunction (C.. elegans males are XO, hermaphrodites are XX).

Mutations were generated in the N2 strain background by treatment with ethylmethanesulfonate (EMS) as described by Brenner (1974). Mutageneses were conducted in strains carrying either him-5(e1490) or tra-1(e1488). The tra-1(e1488) mutation results in self-fertile intersexes with hermaphrodite gonads and male tails (Hodgkin, 1987). Mutations causing abnormal male tail morphology were identified by screening clonally propagated populations (derived from mutagenized P_o hermaphrodites) at a magnification of 400× using differential interference contrast (Nomarski) optics (Sulston and Horvitz, 1977). In him-5 screens, F_2 or F_3 males were scored, and mutations were fixed by picking sibling hermaphrodites. In tra-1 screens, homozygous F_2 mutants were picked and propagated directly.

Newly identified mutations were outcrossed at least twice to eliminate extraneous mutations and mapped following the procedures of Brenner (1974). Two- and three-factor cross data are available from the Caenorhabditis Genetics Center. Some two-factor crosses with X-linked mutations were conducted in a trans configuration, as the ability to score hemizygous X chromosomes in males obviated the need to construct cis-doubles (Baird and Emmons, 1990). Such crosses were scored from him-5/+F_1 hermaphrodites because the rate of X-linked recombination is reduced in him-5 homozygous hermaphrodites (Hodgkin et al. 1979).

The mutations bx23, bx24, bx28, bx41, bx53 and bx61 were isolated in screens for Mab (male abnormal) mutations causing abnormal adult male tail morphology, as described above. These six mutations, and a putative allele of sma-2 that was subsequently lost, were isolated by screening 2448 mutagenized haploid genomes. The average frequency of null mutations at a single locus produced by the mutagenesis protocol followed is 1/2000 haploid genomes (Brenner, 1974).

The mab-18 allele bx79 was found to be present in a strain maintained by E. Hedgecock carrying the mutation lin-20(e1796). A. Chisholm has shown that e1796 is a hypomorphic allele of vab-3 (Brenner, 1974) (personal communication). We found that the strain harboring the e1796 mutation has a male tail phenotype identical to mab-18(bx23), and fails to complement bx23 for this defect. Strong alleles of vab-3 do not have a male tail defect, and mab-18(bx23) does not have the morphological and lineage defects found in vab-3 mutants. Therefore, the strain of Hedgecock appears to be a double mutant, to which we assign the genotype mab-18(bx79)vab-3(e1796). mab-18 and vab-3 map to a 3 map unit interval on X, and the two mutations have not been separated.

The mutation mab-21(sy155) was separated from a strain kindly sent to us by H. Chamberlin and P. Sternberg.

A number of mutations causing abnormal body morphology in C. elegans were previously surveyed by us for defects in the male tail (Baird and Emmons, 1990). Of 37 bli, dpy, lon, rol, sma, sqt, and vab mutants examined, only sma-2 and sma-3 mutants had defects causing fusion of rays. All of the available alleles of these two genes were examined (see Tables 1 and 2).

Linkage of the autosomal mutation bx28 remains unknown, because this mutation failed to show linkage to markers in the centers of the each of the 5 autosomes.

Determination of cell lineages

Cell lineages were determined as described by Sulston and Horvitz (1977). Three mab-18 sides examined had wild-type cell lineages, confirming that the thin, displaced ray that usually fuses with ray 4 was derived from V6.ppppa, the precursor to ray 6 in wild type. Lineage analysis of 6 mab-21 sides revealed that an ectopic ray was derived from T.apapa in each case.

Indirect immunofluorescence

Animals were either freeze fractured and fixed with methanol and acetone (Kenyon, 1986; Priess and Hirsh, 1986) or fixed with 1 % paraformaldehyde and permeabilized by reduction and oxidation of the cuticle (Ruvkun and Giusto, 1989; Finney and Ruvkun, 1990). Permeabilized animals were incubated for up to five hours at 37° with a 1:50 dilution of primary monoclonal antibody MH27 (generously provided by R. Waterston), and then for 5–12 h at 37° with a 1:50 dilution of secondary antibody (rhodamine isothiocyanate conjugated goat anti-mouse, Boehringer Mannheim Biochemicals, Indianapolis, IN). Antibody-stained animals were mounted in a solution containing 10 mM Tris pH 7.5, 30 mM NaCl, 80 % glycerol, and 2 % N-propylgallate, and observed and photo-

graphed with a Zeiss Axioplan microscope equipped for epifluorescence.

Results

Rays have a reproducible arrangement in the adult male tail

The fan and rays are the most prominent external specializations of the *C. elegans* male tail, which is modified for copulation (Fig. 1A). The structure and postembryonic somatic development of the male have been described by Sulston and Horvitz (1977) and by Sulston *et al.* (1980). The acellular fan consists of lateral extensions of the outer layer of adult cuticle, and is used by the male to clasp the hermaphrodite during a search for the vulva. The eighteen rays are peripheral sensory organs extending outward from the body within the fan, and usually opening through a hole in the cuticle. The rays are used by the male to sense contact of its tail with another nematode, and are essential for successful copulation (Hodgkin, 1983; J. Sulston, personal communication; unpublished observations).

Each ray comprises processes of three cells contained within an epidermal sheath (Sulston *et al.* 1980). Two of the cells are neurons, and the third is an epidermal support cell, known as the ray structural cell, which forms the opening containing the dendritic endings of the neurons (Fig. 1B). The cell bodies of all three cells are in lumbar ganglia located anterior of the anus, and
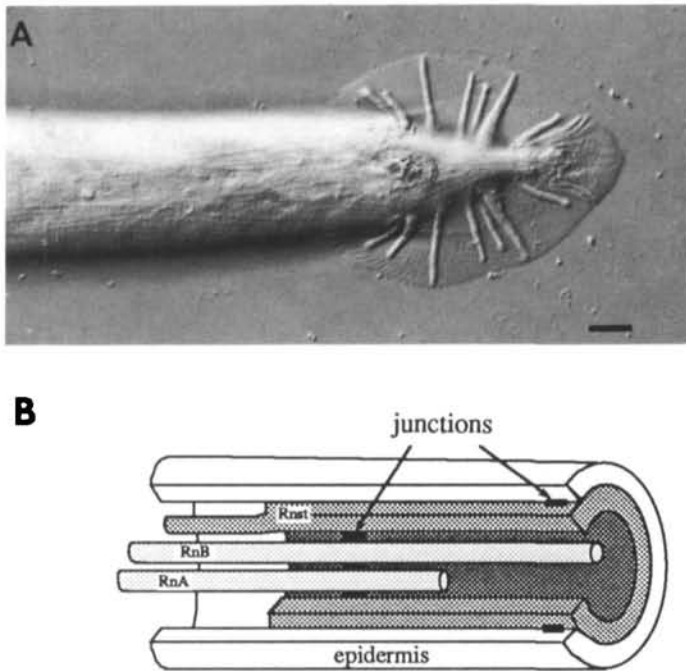
Fig. 1. (A) Ventral view of adult male tail, showing fan and rays (Nomarski optics). The nine rays are numbered in order from anterior to posterior. Scale=10 microns.
(B) Diagram of a ray tip (Sulston *et al.* 1980). The terminal one third of a ray is depicted; the ray tip and the exterior of the animal are to the right. RnA, A type neuron; RnB, B type neuron; Rnst, structural cell.

the neurons have axonal processes extending into the preanal ganglion. The connectivity of these axons has not been determined. The neurons are of two types, designated A and B, distinguishable by the ultrastructure of their dendrites and cell bodies. Each of the nine rays contains one neuron of each type, and with the exception of ray 6, the rays have similar ultrastructure. Ray 6 differs from the other rays in having a thicker, tapering profile, a B-type neuron of distinct morphology, and in not opening to the exterior.

Individual rays have reproducible positions in the fan, resulting in an overall arrangement that is a constant and distinguishing property of nematode species (Cobb, 1920; Chitwood and Chitwood, 1974; Andrássy, 1983). The *C. elegans* male tail is characterized by having rays 1 and 2 anterior of the cloaca, ray 3 at the cloaca, and rays 4 through 6 and 7 through 9 in two postcloacal clusters (Nigon and Dougherty, 1949). Furthermore, the rays of *C. elegans* open reproducibly on one or the other surface of the fan, or at the fan margin: rays 1, 5 and 7 open on the dorsal surface, rays 2, 4 and 8 open on the ventral surface, and rays 3 and 9 extend to and open at the margin (Sulston and Horvitz, 1977). Thus each ray tip has a precise anteroposterior and dorsoventral position on the surface of the animal.

The arrangement of rays in the fan is determined by the arrangement of ray cells in the epidermis of the L4 larva

The fan, the rays and the distinctive shape of the posterior body region arise from a morphogenetic rearrangement that occurs at the end of the last, or L4, larval stage (Fig. 2). Until the last molt, a larval male has a simple tapered tail like that of a hermaphrodite. At the end of the L4 stage, cells lying in the posterior region retract and move anteriorly and dorsally (Sulston *et al.* 1980). Consequently, the tail becomes shorter and thinner, and the outer layer of adult cuticle, previously laid down underneath the L4 cuticle, dissociates from the body laterally and folds to produce the fan.

Prior to this morphogenetic episode, papillae, which are the precursors of ray tips, appear in the lateral epidermis (Fig. 2B). The formation of papillae corresponds to the attachment of rays to the fan cuticle. Later, as the fan extends from the body the papillae elongate and the rays are formed (Fig. 2C,D).

Papillae form at reproducible sites on the L4 surface, and these sites determine the positions where the corresponding rays will open on the surface of the fan. Papillae are arrayed along the anteroposterior axis in the order of the adult rays. Papillae of rays destined to open on the dorsal surface of the fan are located more dorsally, papillae of rays destined to open on the ventral surface are located more ventrally, and papillae of rays destined to open at the edge of the fan are located at an intermediate position on a line that demarcates where the fold of the fan will form.

The positions where papillae form appear to result from the targeting of the processes of the ray structural cells to specific sites in the epidermis. In the adult, the
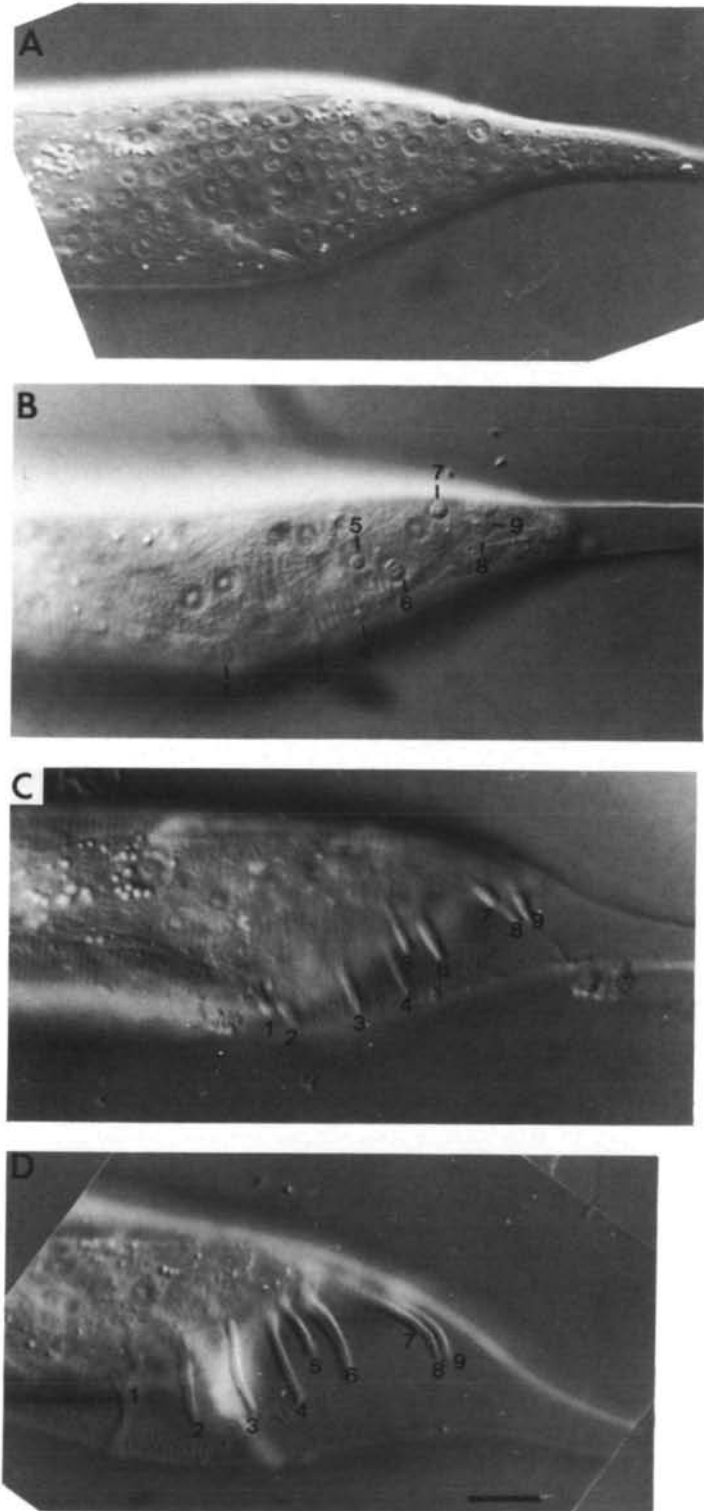
Fig. 2. Morphogenesis of the fan and rays during the L4 larval stage. (A) Nuclei of epidermal cells and ray cells are clustered in the posterior region. (36 h after hatching.) (B) Beginning of retraction. Ray papillae are visible; the papilla of ray 2 is below the plane of focus. (40 h after hatching.) (C) Mid-retraction; fan and rays forming. The tips of rays 1, 5, and 7 can be seen to lie in the dorsal surface of the fan. (43 h after hatching.) (D) Retraction complete. A fully formed male tail is present inside the L4 cuticle. (45 h after hatching.) Scale = 10 microns.

structural cell process surrounds the ray opening and is joined to the epidermal sheath and to the A and B neurons by belt junctions (Fig. 1B). This role is essential for ray formation: if the structural cell is ablated with a laser microbeam, no ray forms (Sulston and Horvitz, 1977). We infer that it is the structural cell that is largely responsible for attachment of the ray to the cuticle and formation of the papilla. It follows that the formation of papillae at specific sites is dependent upon mechanisms that act to position the processes of the structural cells.

Mutations defining six genes alter the arrangement of rays and papillae

Mutations in six genes have been identified that disrupt the pattern of rays, causing displacements and fusions (Fig. 3). The defects in these mutants are described in Table 1, and their expressivity is given in Table 2. Mutations in four of these 'fused-ray genes', *mab-18*, *mab-20*, *mab-21*, and an unmapped *mab* gene defined by *bx28*, primarily affect ray development, although defects elsewhere in the seam are seen occassionally. Mutations in the other two genes, *sma-2* and *sma-3*, cause small body size in addition to fused rays (Brenner, 1974). The ray defects in *sma-2* and *sma-3* males apparently are not a result of small body size *per se*, as mutations in other *sma* and *dpy* genes that cause similar or more severe alterations in body morphology do not affect the ray pattern.

Mutations in *mab-18*, *mab-21*, *sma-2* and *sma-3* also result in morphological transformations of rays. In wild-type males, most rays have a slender cylindrical morphology, whereas ray 6 is thicker and more conical (Fig. 1A). In *mab-18* and *mab-21*, ray 6 is transformed into a slender cylindrical ray. This defect can be observed in the minority of sides in which rays 4 and 6 are not fused (Fig. 3A). Mutations in *sma-2* and *sma-3* cause an opposite transformation affecting rays 5 and 7, which frequently adopt a thicker conical morphology, typical of ray 6 in wild-type (Fig. 3E,F).

In *mab-21* mutants there is a tenth ray lying between rays 6 and 7. This ray usually fuses with ray 5 or ray 7. In 6 cases examined, the tenth ray arose from the anterior sister of R7, T.apapa, which divided following a normal ray sublineage. This was the only consistent lineage abnormality observed in fused ray mutants.

Multiple alleles in two of six fused-ray genes have been isolated in our mutant hunts, and altogether we have multiple alleles of 5 out of the 6 genes identified. Therefore, it is likely that few, if any, genes remain to be identified that are easily mutable to give a fused-ray phenotype in a viable and otherwise nearly normal male. Additional genes are expected to be involved in determining ray assembly and pattern formation; however, these genes might also act elsewhere in the nervous system or in other tissues, and hence mutations in these genes would be pleiotropic.

The frequency of fused ray mutations in our screens was $7/2448 = 2.9 \times 10^{-3}$ per mutagenized haploid genome. Assuming there are six genes that can mutate to give a viable, nearly normal male with fused rays, the
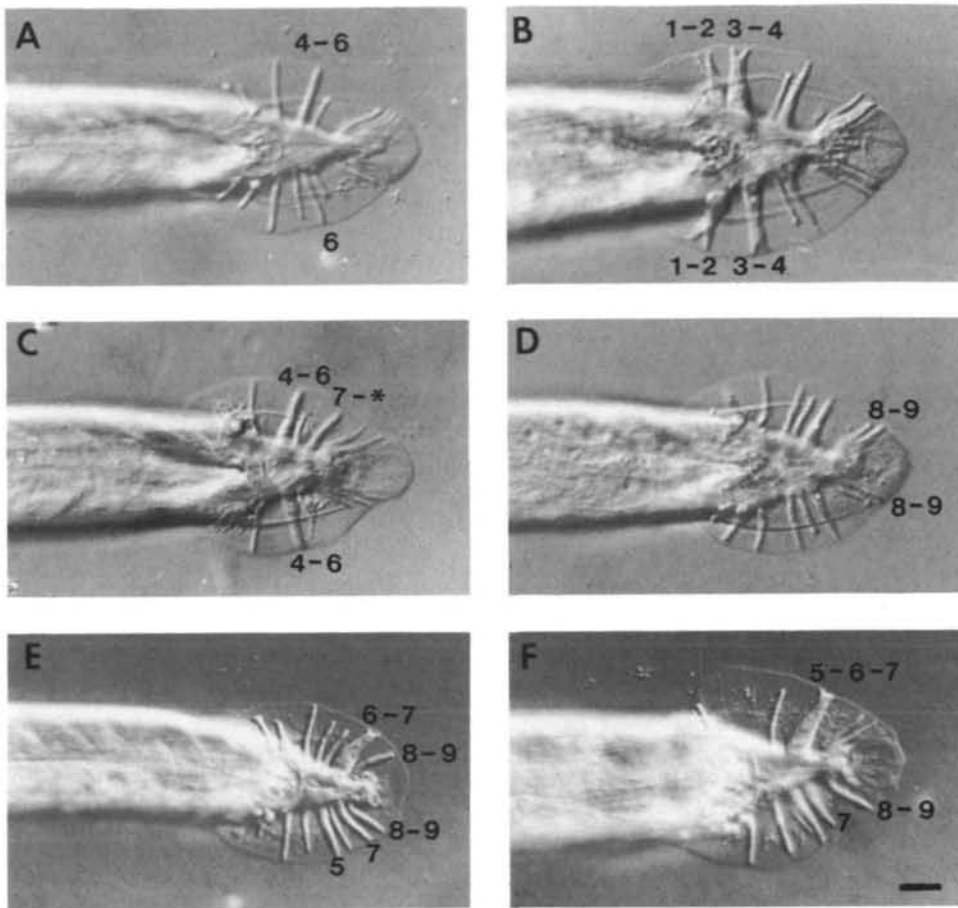
Fig. 3. Mutations causing fused rays, ventral view of male tail. Fused rays and single rays in abnormal positions or with abnormal morphology are labelled. (A) *mab-18(bx23)*; (B) *mab-20(bx24)*; (C) *mab-21(bx53)*. Rays 1 and 2 on both sides are out of the plane of focus and obscured by material in the fan. (D) *mab-(bx28)*; (E) *sma-2(e502)*; (F) *sma-3(e491)*. Ray 1 on the left and rays 1 and 2 on the right are out of the plane of focus. On the left ray 3 is absent, which occurs in wild type at a frequency of about 10 %. Scale=10 microns.

Table 1. *Summary of fused-ray mutant phenotypes*

Gene	Alleles	Map position	Mab phenotype
mab-18	bx23	+4.8, X	Anterior displacement and morphological transformation* (thick to thin) of ray 6.
	bx79		Frequent fusion of rays 4 and 6.
mab-20	bx24	−12, I	Displacements and fusions of rays 1–4, rays 8 and 9, and to a lesser extent, ray 6.
	bx61		Typically (*bx24*) rays 8 and 9 fuse, as do various combinations of rays 1–4. Ray 6 only fuses in combinations including ray 4. *bx61* is a weak allele that primarily results in fusions of rays 3 and 4.
mab-21	bx53	−8.5, III	Anterior displacement and morphological transformation (thick to thin) of ray 6.
	bx41		Frequent fusion of rays 4 and 6. Generation of a tenth ray from the anterior sister
	syl55		of the ray 7 precursor cell (T.apapa), which usually fuses with ray 7 or ray 5.
mab-	bx28	?	Frequent fusion of rays 8 and 9.
sma-2	e502	−0.1, III	Displacement and morphological transformations (thin to thick) of rays 5 and 7.
	e172		Frequent fusions of ray 6 and 7 and of rays 4 and 5. Occasional fusions of rays 5 and 6.
	e297		*e1491* is a weak allele that has less severe effects on body morphology and ray pattern.
	e1491		
sma-3	e491	−0.9, III	Displacement and morphological transformations (thin to thick) of rays 5 and 7.
	e637		Frequent fusions of ray 6 and 7 and of rays 4 and 5.
	e958		

*In wild-type males, ray 6 is thicker and more highly tapered than the other rays.

frequency per gene was 0.48×10^{-3}. Brenner determined an average forward mutation rate of 0.5×10^{-3} per gene under the conditions of mutagenesis used here. Therefore the mutations described here do not appear to be rare alleles, and are most likely to be hypomorphic or null alleles. Based on this inference, we conclude that fused ray genes play a role in determining ray pattern during wild-type development. However, the conclusion that these mutations are loss-of-function and possibly null alleles is tentative, as it rests at this stage only on the frequency at which mutations were isolated and the assumption about the number of genes of this type. Further direct genetic evidence is required to confirm this conclusion. All

Table 2. *Frequency of ray fusions in mutants**

Gene	Alleles scored	Ray 1	Ray 2	Ray 3	Ray 4	Ray 5	Ray 6	Ray 7	Ray 8	Ray 9	N
Wild-type									12	12	129
mab-18	bx23				95	95			13	13	134
	bx79				82	82			ns	ns	73
mab-20	bx24	90	95	95	87	1	44	4	79	79	77
	bx61			16	16				18	18	173
	bx61/bx24	8	10	33	31				21	21	48
mab-21†	bx53			1	96	19	98	11	ns	ns	80
	bx41				84	48	97	24	21	21	90
	bx53/bx41				96	12	96	12	ns	ns	25
mab-	bx28								95	95	202
sma-2	e502				15	18	62	62	14	14	61
sma-3	e491			5	28	28	34	37	9	9	67

*The body of the table gives the frequency in percent at which each ray is fused with another ray (N=number of sides scored). Fusion frequencies of less than 1% are not listed; ns=ray not scored. †The ectopic ray generated by T.apapa fused at a frequency of 75%.

fused ray mutations are recessive and, with two exceptions, multiple alleles in single genes have similar phenotype and expressivity. The exceptional genes are *mab-20*, which has one strong allele and one weak allele, and *sma-2*, which has three strong alleles and one weak allele. The weaker alleles of these genes differ from the stronger alleles in expressivity. The hetero-allelic combination of *mab-20(bx24/bx61)* has an intermediate phenotype (Table 2).

Ray development in mutant animals

Comparisons of papillae and ray patterns in fused-ray mutant males confirm the conclusion that the position of papilla formation determines the position of the adult ray in the fan. Most papillae in mutant males are present at their normal positions. However, some papillae are displaced, and these displacements of papillae correspond to the displacements and fusions of rays. In every case examined, displacement of an adult ray was preceded by the expected displacement of the corresponding papilla on the surface of the L4. Fused rays arose when multiple papillae formed close together at a single site. This might be because the epidermis is unable to sheathe such rays individually, or because the processes of the rays adhered to each other (Fig. 4).

Except for displacements of papillae, ray development in mutant animals is essentially normal. For mutations in all the genes except *mab-21*, nine papillae of normal morphology and nearly normal arrangement appear at the appropriate time. In *mab-21* animals, there are ten papillae; the tenth papilla, which is derived from T.apapa, is usually located between the papillae of rays 5 and 7 and gives rise to a tenth ray that frequently fuses with ray 5 or 7. As distinct papillae form from each ray cell group present, fused rays do not arise from fusions of ray structural cells or from the failure of some structural cells to produce a papilla and the adoption of their neurons by other rays.

Light microscopic observations of fused rays and electron microscopic observations of thin sections through fused rays support the conclusion that fused rays consist of processes of two or more rays in a single epidermal sheath. In adult males, ray tips visualized by

Nomarski optics have a characteristic ring and dot structure that probably corresponds to the external opening of the rays (Sulston and Horvitz, 1977). Fused rays always have two or more apparently normal ray tips, indicating the presence of multiple channels, each presumably formed by a distinct structural cell. In electron micrographs of thin sections through fused rays, multiple sets of ray cell processes are apparent, each set consisting of two dendrites surrounded by one structural cell process (2/2 observed, data not shown). These sets of ray processes are surrounded by a single sheath of epidermis.

Development of cells in the posterior epidermis

In order to understand how ray structural cells are localized to distinct sites in the epidermis, as well as to ascertain why these sites are altered in mutants, we analyzed the arrangement and geometry of cells in the surface of the male tail during the developmental events of the late L3 and L4 larval periods. The arrangement of the cells was determined by indirect immunofluorescence staining with the monoclonal antibody MH27. *C. elegans* epidermal cells are joined together by belt junctions, which form a closed ring around each cell just below the apical surface (White, 1988). The belt junction is associated with microfilaments (Priess and Hirsh, 1986), and probably corresponds to the adherens junction described in a number of vertebrate cells and tissues (Geiger *et al.* 1985). A component of this belt junction is the presumed target of MH27 antibody, and immunofluorescence staining with this antibody outlines the apical surface of each cell in the epidermis (Priess and Hirsh, 1986).

The origins of the cells in the male tail have been described by Sulston and Horvitz (1977) and by Sulston *et al.* (1980). Dorsally and ventrosublaterally, the tail epidermis consists of a large syncytial cell, known as hyp7, that also covers the dorsal and ventral surfaces of the body anterior of the tail (White, 1988). Laterally, the epidermis consists of a single row, or seam, of postembryonic blast cells, which synthesize new cuticle before each molt (Singh and Sulston, 1978; Edwards and Wood, 1983). Seam cells generally divide in a stem

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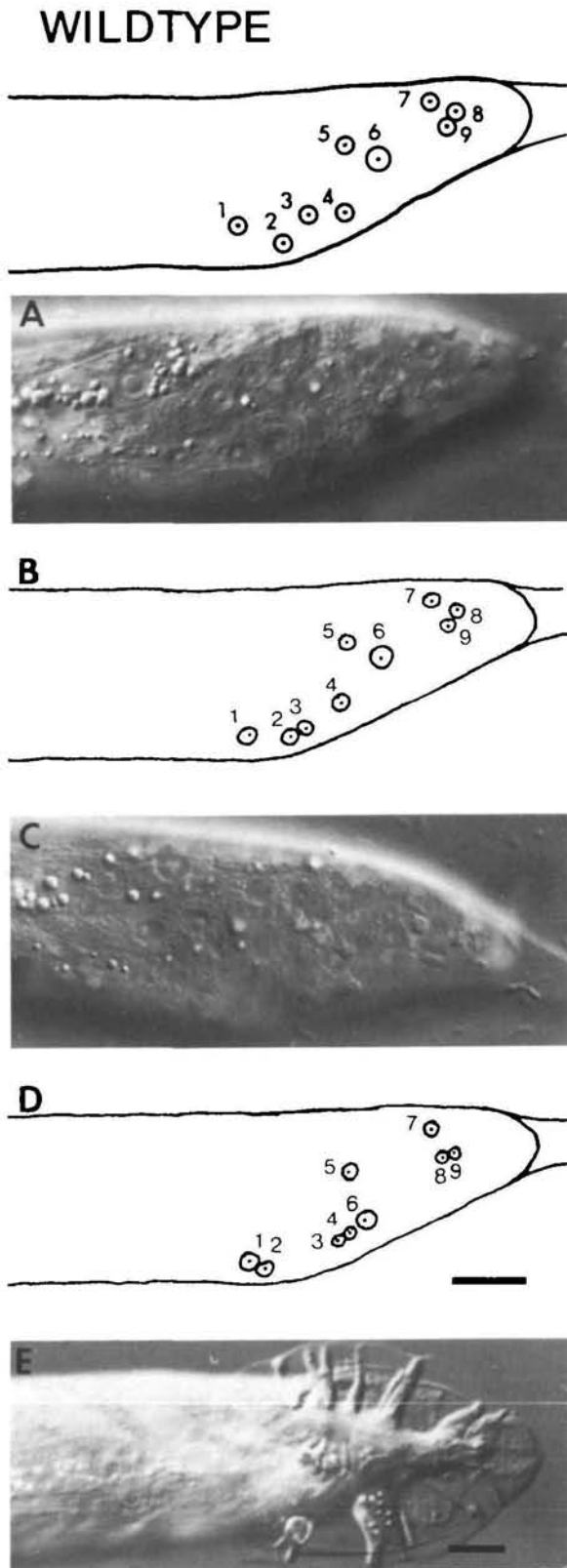
Fig. 4. Formation of fused rays from closely adjacent papillae. Nomarski photomicrographs and drawings of the left-hand side (A and B) and right-hand side (C and D) of a single L4 larva of *mab-20(bx24)*, compared to the adult form (E) of the same animal, and to wildtype (top). Scales=10 microns.
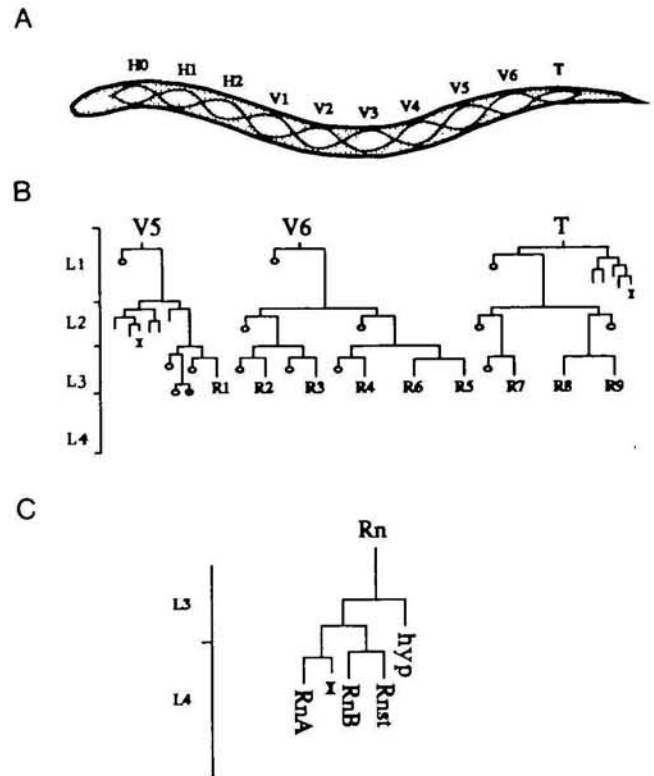
Fig. 5. Cell lineages of seam cells. (A) The location of seam blast cells in the lateral epidermis of an L1 larva after hatching. (B) The cell lineages of V5, V6, and T seam cells. Open circles, cells that fuse with the hypodermal syncytium; closed circle, cell that remains in the seam; (X) programmed cell death; R1–R9, ray precursor cells for rays 1–9. Each ray precursor cell generates a ray sublineage. (C) The ray sublineage. RnA, A type neuron; RnB, B type neuron; Rnst, structural cell; hyp, hypodermal cell.

cell pattern generating anterior daughters that fuse to hyp7 and posterior daughters that remain in the seam and divide further (Fig. 5). Following the final stem cell divisions and seam cell-hpy7 fusions in L4, the remaining seam cells fuse with each other to form a bilateral pair of syncytia extending along the length of the body.

In males, the three posterior-most pairs of seam cells, V5, V6 and T, follow a modified lineage and generate nine bilateral pairs of ray precursor cells (Rn, $n=1$–9). Ray precursor cells divide during late L3 and early L4 following a stereotypical pattern known as the ray sublineage, which generates the three cells of a ray, one cell that undergoes programmed cell death, and one epidermal cell, the Rn.p cell (Fig. 5C). The cells of the ray sublineage are generated in the epidermis, joined by belt junctions (Fig. 6A).

Patterns of Rn.p cells during ray development

Antibody staining revealed that during the L4, ray structural cells are localized to specific sites in the epidermis in a multistep process that apparently depends on the birth positions of cells, and on
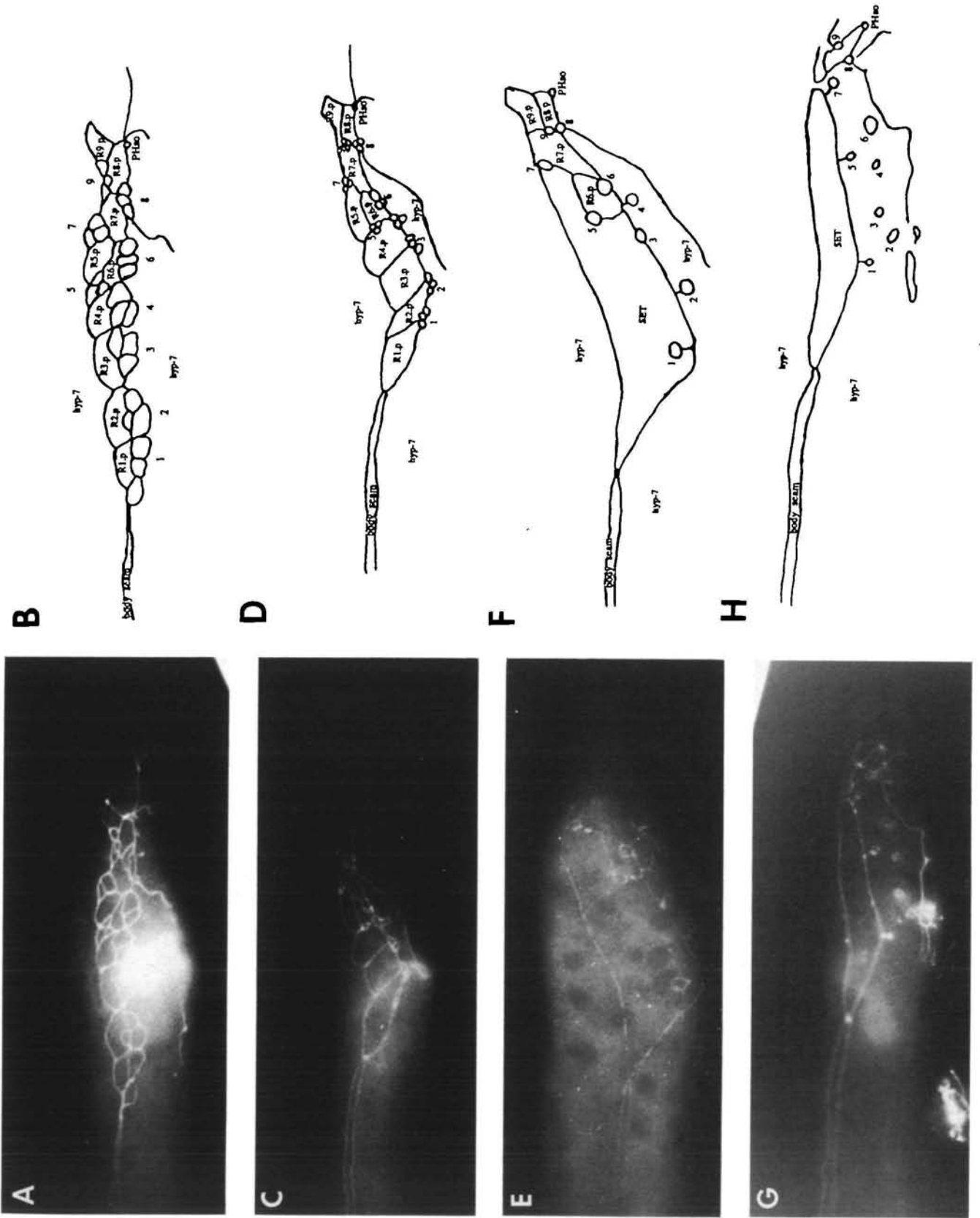
Fig. 6. Development of the posterior lateral epidermis in the wild-type L4 male. Fluorescence photomicrographs of MH27 antibody-stained animals (left side), and drawings (right side) of the posterior region of males at progressively later times during the L4 larval period. Representative animals are shown in the photomicrographs; the drawings are composites, following the photomicrographs, but also utilizing information from additional animals to give a more complete or representative depiction. For example, the boundary between R1.p and R2.p is shown in D, whereas these cells have fused in C. R1.p, R2.p, etc., the posterior daughters of the ray precursor cells; 1, 2, etc., the ray cell groups; hyp7, the syncytial hypodermal cell that covers most of the body; SET, tail seam; PHso, phasmid socket cell. Scale=10 microns.

interactions between epidermal cells, between ray cells, and between epidermal cells and ray cell processes. A rough anteroposterior pattern of ray structural cells, first established by cell birth positions, is progressively refined as the ray cells and epidermal cells differentiate, until the final pattern is achieved.

During L4, the Rn.p cells undergo a series of shape changes and cell fusions, resulting in a reproducible arrangement of cell boundaries. Shortly after their birth, left and right R9.p cells extend across the dorsal surface of the tail and contact each other (Fig. 6A and B). The anterior Rn.p cells enlarge during early L4 so that they eventually cover most of the lateral surface of the tail (Fig. 6C and D). During mid-L4, R1.p–R5.p fuse together to form a syncytium called the tail seam (SET) (Fig. 6D and F). SET fusion is progressive: R1.p and R2.p fuse first, followed by R3.p, R4.p, and R5.p in anteroposterior order. During late L4, the SET shrinks dorsally and becomes a thin extension of the body seam (Fig. 6G and H). Concurrent with SET retraction, R6.p–R9.p fuse with hyp7. The positions, shapes and fusions of these cells and of the SET are distinctive and reproducible throughout tail development.

Because of their lineal relationships to Rn.p cells, cells of the rays are generated at particular positions in the epidermal cell pattern. These positions establish the overall anteroposterior arrangement of the rays, as well as some dorsoventral skewing of the positions of rays 5 through 9. There are no long range migrations of cells or significant relative movements, and hence the general features of the cellular arrangement are established by the cell lineage.

Refinement of the positions of ray structural cells

During the L4, the ray neurons develop from undifferentiated precursor cells in the epidermis into mature neurons. Likewise, the ray structural cells differentiate into mature neuronal accessory cells. At the surface, as viewed by MH27 antibody staining, differentiation of ray neurons and structural cells progresses through the following stages (Fig. 6): first the neuronal and struc-

tural cell bodies migrate subepidermally, leaving only narrow processes extending to the surface; next two of the three processes of each ray, presumably those of the neurons, disappear, whereas one, presumably that of the structural cell, remains at the surface; and finally, the structural cell process becomes fixed to the appropriate site in the surface.

As differentiation of ray cells proceeds, there is a progressive refinement of the positions of the ray cell processes in the epidermis. This refinement appears to be guided by three types of cellular interactions: clustering of ray cell processes, localization of clustered processes to sites associated with junctions of multiple epidermal cells, and engulfment of ray structural cells by neighboring epidermal cells.

First, neuronal and structural cell processes form ray-specific clusters, as the first step in ray assembly. Immediately after birth, cells of adjacent rays touch one another (Fig. 6A and B). However, after the subepidermal migration of cell bodies, processes belonging to individual rays no longer contact processes belonging to adjacent rays, but adhere specifically to one another, forming a cluster of three ray processes for each ray (Fig. 6C and D). This ray-specific clustering appears to involve selective cellular affinities between the cells belonging to individual rays, as specificity of clustering is lost in mutations, as discussed below.

Second, clusters of ray processes are localized to specific sites in the epidermis, each site being associated with, and perhaps defined by, the junction of three or more epidermal cells (Fig. 6C and D). The targeting of ray-process clusters to these sites is reproducible and might be mediated by the expression of ray-specific affinities for multiple epidermal cells. By these interactions with epidermal cells, the anteroposterior positions of rays 1 through 4, and both the anteroposterior and dorsoventral positions of rays 5 through 9 could be determined by the geometry of Rn.p cells and hyp7. As an alternative explanation for the localization of ray processes, the positions of both the ray cells and epidermal cells could be determined by external cues, located in the basal lamina, for example.

Third, following the disappearance of neuronal processes from the surface, and the fusion of R1.p–R5.p to form the SET, three ray structural cell processes are selectively engulfed by neighboring epidermal cells (Fig. 6E and F). Ray cell groups 1 through 4 are at first arrayed in a line between R1.p–R4.p and ventral hyp7. However, following SET fusion, structural cell processes of rays 1, 2, and 4 are displaced dorsally or ventrally and move either into the SET or into hyp7. The engulfing hypodermal cell does not fuse around the ray process, which consequently appears in the antibody-stained preparations as a stem-loop figure projecting into the SET or hyp7 domain. The process of ray 3 remains at the SET:hyp7 boundary. After engulfment is complete, all of the ray structural cells are at positions where papillae appear as observed by Nomarski microscopy.

After their final positions are attained, structural cells remain fixed despite further changes of epidermal cell

positions (Fig. 6G and H). Towards the end of L4, the ventral boundary of the SET moves dorsally until the SET reaches its narrow adult configuration. As it does so, ray structural cell processes maintain their positions and become progressively engulfed by hyp7. At first they remain connected to the retreating SET by a line of staining, but later this disappears as hyp7 fuses with itself around each ray process, leaving isolated circles of staining for each ray. Likewise, structural cell positions are unaffected by fusions of R6.p–R9.p with hyp7. Eventually, when morphogenesis of the fan and rays begins, all the rays will be ensheathed by hyp7.

Mutations in mab-18 and mab-20 prevent separate clustering of ray processes

Does the correct positioning of ray structural cells among the posterior epidermal cells come about by an active or a passive process? Certainly the final step of engulfment would appear to be an active and selective mechanism acting differently on different rays. Localization of processes to sites defined by the junction of three or more epidermal cells, on the other hand, might be a passive process of movement to a position of minimum energy. From evidence gained by examining the cellular configuration in two fused ray mutants, mab-18 and mab-20, we infer that this is not the case. Selective clustering of ray processes and the formation of contacts between ray cell processes and specific epidermal cells appear to be active processes, the first of these being disrupted in the mutants.

MH27 staining revealed that the initial placement of Rn.p cells and ray cell groups was not affected (not shown). Except as discussed below, subsequent differentiation of the epidermal cells was also generally unaffected, so that a typical pattern of epidermal cell boundaries was formed. The first defect to appear was at the stage where processes of single rays cluster separately in the epidermis (Fig. 7). In mab-18, rays 4 and 6 formed a single cluster, and in mab-20, clusters containing processes from rays 1 through 4 formed.

Remarkably, despite their displacement into abnormal clusters, ray processes appeared to maintain the same set of epidermal cell contacts as in wild type. This required distortion of the shapes of R7.p, in the case of mab-18, and of R1.p, R2.p, and R3.p in the case of mab-20. In the mutants, each of these cells was stretched such that contacts with the appropriate clusters of ray cell processes were maintained. This may be indicative of an active mechanism working to maintain ray cell–epidermal cell contacts.

mab-20 is also required for the selective engulfment of ray structural cells. In mab-20 males, structural cells of rays 1, 2 and 4 remained at the Rn.p–hyp7 boundary following SET fusion (3/3 sides scored). In mab-18 males the structural cells of rays 1 and 2 were engulfed normally, and the structural cell of ray 4, which does not form a stem-loop figure due to its association with the structural cell of ray 6, was localized ventral of the R6.p–hyp7 boundary (4/4 sides scored).
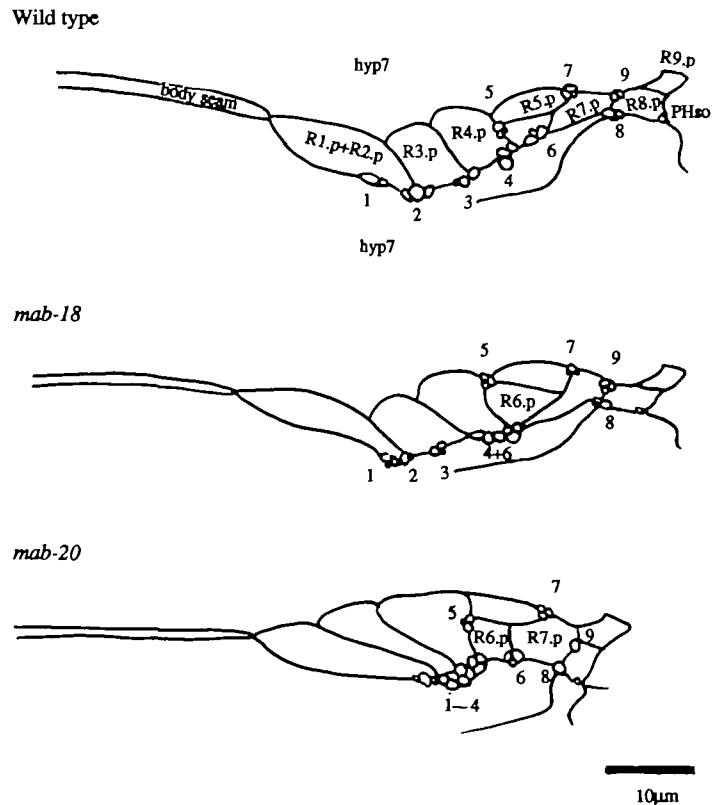

Fig. 7. Posterior lateral epidermis in mutant L4 males. Drawings of the posterior lateral epidermis of representative mab-18 and mab-20 L4 males stained with MH27. Symbols as in Fig. 6. Scale=10 microns.

Discussion

Epidermal cell boundaries appear to guide ray pattern formation and delineate the dorsal and ventral surfaces of the fan

We have described cellular events leading to a reproducible pattern of ray sensilla in the C. elegans adult male tail, and mutations defining six genes that appear to play a role in this process. These results establish the male tail as a system in which several important issues in developmental neurobiology may be addressed. Further genetic and molecular studies of the genes described here should lead to insights into such general processes as specification of neuronal non-equivalence, cellular recognition and interaction in assembly of sense organs, and pattern formation in organogenesis.

It is shown that the arrangement of rays in the adult fan results from the targeting of ray structural cell processes to specific locations in the L4 lateral epidermis. During L4, a reproducible pattern of Rn.p cells is formed between the dorsal and ventral domains of the large syncytial epidermal cell hyp7. Structural cell processes are targeted to specific sites within this pattern in a dynamic, multistep process. A rough approximation to the final anteroposterior pattern is established by the cell lineage, which generates cells near to their final positions. This pattern is refined by

the localization of structural cell processes at sites associated with specific epidermal cell junctions. This mechanism establishes the anteroposterior positions of all the rays, as well as the dorsoventral postions of rays 3 and 5-9. Finally, the dorsoventral pattern of rays 1, 2 and 4 is established through the engulfment of their structural cells by either hyp7 or the SET.

The targeting of structural cell processes to specific epidermal sites may come about as a result of selective interactions between ray cells and the cells of the surrounding epidermis. The specification of these sites may involve the recognition of particular Rn.p cells by individual structural cells, and the engulfment of structural cells may involve selective affinities for either hyp7 or the SET. By this model, fused ray genes might encode *cellular recognition or adhesion molecules* required for bringing certain pairs of cells together. An alternative model is that refinements in the ray structural cell pattern may reflect the movement of structural cell processes to target sites defined by extracellular cues, for example in the extracellular matrix.

The correspondence between ray cell position on the L4 surface and position of ray opening on the fan surface implies that there is a correspondence between the dorsal and ventral surfaces of the fan and distinct domains of epidermal cells. Thus, a dorsal fan domain, consisting of R1.p–R7.p and R9.p, and a ventral fan domain, consisting of hyp7 and R8.p, can be defined. The position where the fan joins the body dorsally corresponds to the dorsal boundary of the SET, and the fan folds along a line following the ventral boundary of the SET, R7.p and R9.p.

The existence of two distinct fan domains may be relevant to the manner in which the fan forms. The fan extends during a period of general tail retraction in late L4 (Sulston and Horvitz, 1977; Sulston *et al.* 1980). As the fan extends, it appears to be inflated and its dorsal and ventral surfaces are not in contact. Later, the fan deflates and these surfaces anneal to each other. Fan annealing always results in the fan folding along a line corresponding to the boundary of the dorsal and ventral fan domains, as defined above. This precise folding may result from simple geometrical constraints on the cuticle at the time of annealing. Alternatively, cuticle synthesized by the dorsal and ventral fan domains may be biochemically distinct, and the dorsal and ventral fan surfaces may selectively anneal to each other. Link *et al.* (1988) have provided evidence for a biochemical difference between cuticle of the fan and of the body.

Ray identities and the mechanism of ray pattern formation

We suggest that an essential early step in determining the pattern of rays is the specification of distinct and intrinsic ray identities, and that some fused-ray genes act in the process that specifies these identities. Established ray identities would guide the subsequent expression of a unique spectrum of cellular properties during development of each ray. This hypothesis accounts for the observation that at a number of points

during development of the ray pattern, the rays behave differently from each other, in spite of existing in the same general cellular milieu. In separately clustering, and in contacting specific epidermal cells, the rays appear to express distinct cellular affinities, which we infer are due to the expression of cell adhesion molecules with different properties. Similarly, in the process of engulfment by the SET and hyp7, rays 1, 2, 3, and 4 behave differently, and in some cases oppositely, in spite of being in contact with the same two epidermal domains. The ray-specific defects caused by mutations in fused-ray genes show that the genetic requirements for development of the individual rays are also not equivalent.

Further ray differences appear in the adult. The A neurons of rays 5, 7 and 9 express dopamine, whereas the A neurons of the other rays do not (Sulston *et al.* 1980). Ray 6 has a distinct morphology, which preliminary analysis of electron micrographs suggests is due to an enlarged structural cell (data not shown). Finally, the B neuron of ray 6 has a morphology distinct from the B neurons of the other rays (Sulston *et al.* 1980). Hence, variants are produced of each of the three ray cell types.

Like the ray cells, the epidermal cells generated by the ray sublineages are also not equivalent. R1.p to R5.p fuse together, whereas R6.p to R9.p fuse at a later time with hyp7. The bilateral pair of R9.p cells reach across the dorsal surface and make contact. Further distinctions among epidermal cells must be postulated in order to account for the unique epidermal cell–ray cell contacts observed during ray development. Possibly differences between the rays and between the epidermal cells are established simultaneously at the level of the ray precursor cells, Rn, which subsequently generate ray sublineages with subtly different properties. An alternative hypothesis is that a cascade of instructive cellular interactions, involving cells at various stages, guides the development of individual cellular differences.

Mutations in *mab-18*, *mab-21*, *sma-2* and *sma-3* affect several ray properties, and hence these genes are candidates for regulatory genes governing establishment of ray identities or expression of ray properties. In addition to causing fusions between rays, mutations in these genes affect ray morphology. In *mab-18* and *mab-21*, ray 6 loses it distinctive tapering shape, whereas in *sma-2* and *sma-3*, rays 5 and 7 appear to take on this shape. In further studies of additional ray properties, such as neurotransmitter usage and B neuron ultrastructure, it will be possible to determine whether neurons as well as structural cells are affected by these mutations. A regulatory role for *mab-21* is strongly suggested by the effect of mutations in this gene on the epidermal cell T.apapa, which is transformed into a ray precursor cell.

Non-equivalence among ray cells and epidermal cells

Sublineages occur in the *C. elegans* cell lineage in the generation of multiple copies of sets of cells (Sulston, 1988). Variants of a sublineage arise to adapt the

cellular products to specific positional requirements. For example, a 12-fold repeated sublineage which generates a set of 5 motor neurons gives rise to a VC neuron only in the vicinity of the vulva, where the neuron is required, and a programmed cell death elsewhere (Sulston, 1976). Likewise, the rays in the male tail differ in morphology and neurotransmitter usage, although the functional significance of such differences is not yet known. We have shown that further distinctions appear to affect neurons, structural cells, or epidermal cells of all the ray sublineages. These distinctions are necessary for generation of the wild-type arrangement of the ray dendrites in the epidermis.

Lewis and Wolpert (1976) have pointed out that non-equivalent behavior of cells of a single cell type is typical of multicellular organisms and is essential to their development and morphogenesis. Non-equivalence is particularly pronounced in the nervous system, where positional cues can dictate a very large number of neurons with distinct properties (Udin and Fawcett, 1988). It is likely that ray differences are established by interactions between cells, a possibility that can be explored by means of cell ablation experiments with a laser microbeam. Hence this simple system may serve as a model for studying how positional information leads to the generation of non-equivalent cells of a single cell type.

References

Andrássy, I. (1983). A Taxonomic Review of the Suborder Rhabditina (Nematoda:Secernentea). Paris: Orstom.

Baird, S. E. and Emmons, S. W. (1990). Properties of a class of genes required for ray morphogenesis in Caenorhabditis elegans. Genetics 126, 335-344.

Bard, J. (1990). Morphogenesis. The Cellular and Molecular Processes of Developmental Anatomy. Cambridge University Press, Cambridge.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics 77, 71-94.

Chalfie, M. and White, J. (1988). The nervous system. In The Nematode Caenorhabditis elegans (ed. W. Wood) Cold Spring Harbor Laboratory. pp. 337-391.

Chisholm, A. and Hodgkin, J. (1989). The mab-9 gene controls the fate of B, the major male-specific blast cell in the tail region of Caenorhabditis elegans. Genes Dev. 3, 1413-1423.

Chitwood, B. G. and Chitwood, M. B. (1974). Introduction to Nematology. Baltimore: University Park Press.

Cobb, N. A. (1920). One Hundred New Nemas (Type Species of 100 new genera). Waverly Press, Baltimore.

Edwards, M. K. and Wood, W. B. (1983). Location of specific messenger RNAs in C. elegans by cytological hybridization. Devl Biol. 97, 375-390.

Finney, M. and Ruvkun, G. (1990). The unc-86 gene product couples cell lineage and cell identity in C. elegans. Cell 63, 895-905.

Geiger, B., Volk, T. and Volberg, T. (1985). Molecular heterogeneity of adherens junctions. J. Cell Biol. 101, 1523-1531.

Hodgkin, J. (1983). Male phenotypes and mating efficiency in Caenorhabditis elegans. Genetics 103, 43-64.

Hodgkin, J. (1987). Sex determination and dosage compensation in Caenorhabditis elegans. Ann. Rev. Genet. 21, 133-154.

Hodgkin, J., Horvitz, H. R. and Brenner, S. (1979). Nondisjunction mutants of the nematode Caenorhabditis elegans. Genetics 91, 67-94.

Kenyon, C. (1986). A gene involved in the development of the posterior body region of C. elegans. 46, 477-487.

Lewis, J. H. and Wolpert, L. (1976). The principle of non-equivalence in development. J. theor. Biol. 62, 479-490.

Link, C. D., Ehrenfels, C. W. and Wood, W. B. (1988). Mutant expression of male copulatory bursa surface markers in Caenorhabditis elegans. Development 103, 485-495.

Nigon, V. and Dougherty, E. C. (1949). Reproductive patterns and attempts at reciprocal crossing of Rhabditis elegans Maupas 1900, and Rhabditis briggsae Dougherty and Nigon. J. exp. Zool. 112, 485-503.

Priess, J. R. and Hirsh, D. I. (1986). Caenorhabditis elegans morphogenesis: The role of the cytoskeleton in elongation of the embryo. Devl Biol. 117, 156-173.

Ruvkun, G. and Giusto, J. (1989). The Caenorhabditis elegans heterochronic gene lin-14 encodes a nuclear protein that forms a temporal developmental switch. Nature 338, 313-319.

Shen, M. M. and Hodgkin, J. (1988). mab-3, a gene required for sex-specific yolk protein expression and a male-specific lineage in C. elegans. Cell 54, 1019-1031.

Singh, R. and Sulston, J. E. (1978). Some observations on moulting in Caenorhabditis elegans. Nematologica 24, 63-71.

Sulston, J. E. (1976). Post-embryonic development in the ventral cord of Caenorhabditis elegans. Philos. Trans. R. Soc. Lond. B Biol. Sci. 275, 287-297.

Sulston, J. E. (1988). Cell lineage. In The Nematode Caenorhabditis elegans (ed. W. B. Wood) Cold Spring Harbor Laboratory. pp. 123-155.

Sulston, J. E., Albertson, D. G. and Thomson, J. N. (1980). The Caenorhabditis elegans male: Postembryonic development of nongonadal structures. Devl Biol. 78, 542-576.

Sulston, J. E. and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode Caenorhabditis elegans. Devl Biol. 56, 111-156.

Udin, S. B. and Fawcett, J. W. (1988). Formation of topographic maps. Ann. Rev. Neurosci. 11, 289-327.

Ward, S., Thomson, N., White, J. G. and Brenner, S. (1975). Electron microscopical reconstruction of the anterior sensory anatomy of the nematode Caenorhabditis elegans. J. comp. Neurol. 160, 313-337.

Waring, D. A. and Kenyon, C. (1990). Selective silencing of cell communication influences anteroposterior pattern formation in C. elegans. Cell 60, 123-131.

White, J. (1988). The anatomy. In The Nematode Caenorhabditis elegans (ed. W. Wood) Cold Spring Harbor Laboratory, pp. 81-122.