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Properties of a Class of Genes Required for Ray Morphogenesis in *Caenorhabditis elegans*

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ABSTRACT

We have identified eight mutations that define at least five terminal differentiation genes (*ram* genes) whose products are required during the extension of the male-specific ray sensilla in *Caenorhabditis elegans*. *ram* gene mutations result in morphological abnormalities in the sensory rays but do not appear to interfere with ray functions. A similar ray morphology phenotype was observed in males harboring mutations in three previously defined genes, *dpy-11*, *dpy-18* and *sqt-1*, that also affect body shape. One of these genes, *sqt-1*, is known to encode a collagen. Mutations in different *ram* genes failed to complement, from which we infer that their gene products functionally interact. For one *ram* gene, failure to complement was shown to result from haploinsufficiency. Intergenic noncomplementation did not extend to the body morphology genes. The temperature-sensitive periods of both *ram* and body morphology mutations corresponded to the period of development in which ray extension occurs. We propose that *ram* gene products act together in a critical interaction between the rays and the cuticle required for wild-type ray morphology.

THE final form of adult animals results from the combined actions of developmental control genes, or regulatory genes, and terminal differentiation genes. The products of regulatory genes determine the generation of specific cells in a lineally or spatially defined manner, and ultimately govern the expression of other genes. The products of terminal differentiation genes are directly responsible for the differentiated characteristics of the cell and organism. Numerous genetic investigations have resulted in the identification of control genes affecting many kinds of developmental events (*e.g.*, see INGHAM 1988; STERNBERG and HORVITZ 1984; BELOTE *et al.* 1985; HODGKIN 1987a). Further understanding of the developmental process will require the identification of the terminal differentiation genes upon which these control genes act, and elucidation of how the products of terminal differentiation genes dictate the form and function of the organism.

We have chosen to focus on the tail structures of the *Caenorhabditis elegans* male as a model to study the action of both regulatory genes and differentiation genes. The tails of *C. elegans* males are highly specialized for copulation. This specialization entails the execution of male-specific postembryonic cell lineages, the differentiation of male-specific structures, and the morphogenesis of adult body shape (SULSTON and HORVITZ 1977; SULSTON, ALBERTSON and THOMSON 1980). Genetic studies of male tail development are facilitated by the ability to propagate male-infertile mutant strains through the self-fertilizing, hermaphroditic sex. Furthermore, male copulatory structures are generally not required for male viability. Genes required for male-tail development have previously been identified and studied by HODGKIN (1983). Mutations in three genes, *mab-3*, *mab-5* and *mab-9*, affect male-specific cell lineages and thus have the properties of regulatory genes (SHEN and HODGKIN 1988; KENYON 1986; COSTA *et al.* 1988; CHISHOLM and HODGKIN 1989). The downstream targets of these genes are not known.

In this paper we describe mutations in five new genes that have the properties of male-specific terminal differentiation genes, and in three known genes that result in a similar male-specific phenotype. Mutations in these genes affect the morphology of the nine bilateral pairs of male-specific sensory rays. Their gene products are required at the end of development, during tail morphogenesis and after the completion of the male-specific lineages, and thus are likely to function in the implementation, rather than the regulation, of ray development. Based on their genetic properties, we conclude that these genes function in the interaction of the cells of the ray with the surrounding extracellular cuticle.

MATERIALS AND METHODS

***Caenorhabditis elegans* strains and maintenance:** Mutant and wild-type strains of *C. elegans* were maintained as described by BRENNER (1974). Unless otherwise noted, strains that contained nonconditional mutations were grown at 20°, and strains that contained temperature-sensitive (ts) mutations were grown at 25°. The following mutations and chromosomal aberrations were used for genetic mapping:

LG I: *dpy-5(e61)*; *unc-29(e1072)*.
LG II: *dpy-10(e128)*; *unc-4(e120)*; *bli-2(e768)*.
LG III: *dpy-17(e164)*
LG IV: *dpy-13(e184)*; *unc-17(e113)*; *unc-22(e66)*; *unc-24(e138)*; *mDp1*; *mDf9*; *nT1a*.
LG V: *unc-42(e270)*; *nT1b*.
LG X: *dpy-6(e14)*; *unc-3(e151)*; *unc-18(e81)*.

Unless otherwise indicated, all strains used in this study contained *him-5(e1490)*. This mutation increases the frequency of males in a selfing population to 30% because of an increase in the rate of *X* chromosome loss due to meiotic nondisjunction (HODGKIN, HORVITZ and BRENNER 1979). Other mutations that were scored for morphological defects or used for complementation tests are listed in the RESULTS. All mutations and genes have been named according to the conventions of HORVITZ *et al.* (1982).

Identification and mapping of mutations: Mutations were generated by treatment with ethylmethanesulfonate (EMS) as described by BRENNER (1974). This treatment results in a forward mutation rate of 5×10^{-4} mutations per locus per haploid genome in *C. elegans*. Mutageneses were conducted in strains carrying either *him-5(e1490)* or *tra-1(e1488)*. This *tra-1* mutation generates self-fertile intersexes with hermaphrodite gonads and male tail structures (HODGKIN 1987b) (see also KENYON 1986). All mutations were identified by screening clonally propagated populations (derived from mutagenized P_0 hermaphrodites) at a magnification of 400× using differential interference contrast (Nomarski) optics (SULSTON and HORVITZ 1977). In *him-5* strains, F_2 or F_3 males were scored for sensory ray defects, and mutations were fixed by picking sibling hermaphrodites. In the *tra-1* screens, homozygous F_2 mutants were picked directly. A total of 2448 haploid genomes were screened. All mutations described in this paper are independent isolates.

All newly identified mutations were outcrossed at least twice to eliminate extraneous mutations. Concurrently, various marked autosomes were introduced and linkage was determined by repulsion of the mutant phenotype from these markers. Ram phenotypes, in these and all other crosses, were scored at 400×. Linkage to the *X* chromosome was determined by scoring the F_1 males from crosses of mutant hermaphrodites to wild-type males. (*C. elegans* males are *XO*, and therefore *X*-linked mutations will be expressed in F_1 males.)

Map positions were determined from two- and three-factor crosses as described by BRENNER (1974) with the following exceptions. Two-factor crosses with *X*-linked mutations were conducted in a *trans* configuration. This approach was taken as the ability to score hemizygous *X* chromosomes in *XO* males obviated the need to construct *cis* doubles. These crosses were scored from *him-5*/+ F_1 hermaphrodites because the rate of *X*-linked recombination is reduced in *him-5/him-5* hermaphrodites (HODGKIN, HORVITZ and BRENNER 1979). Male progeny were generated by crossing *him-5* males to the F_1 hermaphrodites. To determine the map positions of temperature-sensitive mutations, progeny laid by hermaphrodites at 20° were shifted to 25° after 1 day of development.

Construction of doubly and triply mutant strains:
ram-1(bx34) I; ram-4(bx25ts) IV: Hermaphrodite progeny of a *ram-1*/+; *ram-4/unc-17 dpy-13* hermaphrodite were propagated clonally at 16°. A population in which all of the males were Ram (*i.e.*, in which *ram-1* was fixed) and that segregated no Unc Dpy animals (*i.e.*, in which *ram-4* was fixed) was retained as tentative *ram-1*; *ram-4* double. This genotype was confirmed by mating males from this population to triply marked (*dpy-5* I; *bli-2* II; unc-22) hermaphrodites and scoring (at 25°) for Unc Ram and Dpy Ram males in the F_2 generation.

ram-1(bx34) I; ram-2(bx39ts) II; ram-4(bx25ts) IV: ram-1; ram-4 males were mated to *dpy-5*; *ram-2*; *unc-22* hermaphrodites. NonUnc nonDpy F_2 hermaphrodites were clonally propagated and F_3 Unc Dpy males were scored for Ram defects. F_3 hermaphrodites were clonally propagated from a plates in which 100% of the F_3 Unc Dpy males were Ram (*i.e.*, in which the F_2 hermaphrodite was *ram-1/dpy-5*; *ram-2/ram-2*; *ram-4/unc-22*). A population that did not segregate Unc or Dpy animals was retained as a *ram-1*; *ram-2*; *ram-4* strain. This genotype was confirmed by crossing triply *ram* males to triply marked hermaphrodites (see above) and by scoring for Unc Dpy Ram, Unc Bli Ram, and Dpy Ram F_2 males. (*dpy-5* is epistatic to *bli-2*, and therefore Dpy Bli Ram males could not be scored for directly.)

Temperature-sensitive periods: L4 males, grown at the permissive (16°) and restrictive (25°) temperatures, were staged by microscopic observation at either 12× or 400×, and then shifted to 25° or 16°, respectively. At most time points, between 10 and 30 animals were temperature shifted, although in one case, as few as four animals were temperature shifted. Temperature shifted animals were allowed to mature overnight, and ray morphology was scored at 400×. Criteria used for staging males were: (1) general tail morphology (mid L4, 38 ± 2 hr); (2) retraction of hypodermis from tail spike (40 hr); (3) appearance of papillae (41 hr); (4) degree of ray extension (43 hr, 44 hr); and (5) molting (45 hr). The time points defined for these stages were based on published descriptions of male tail development (SULSTON, ALBERTSON and THOMSON 1980) and on our unpublished observations. These stages are normalized to hours posthatching at 20° (HIRSH, OPPENHEIM and KLASS 1976).

RESULTS

A common ray morphology phenotype is conferred by mutations in eight genes: The nine bilateral pairs of sensory rays are male-specific tail sensilla that are essential for copulation in *C. elegans* (SULSTON and HORVITZ 1977; SULSTON, ALBERTSON and THOMSON 1980). They each comprise the dendritic endings of two neurons and the process of one structural cell. These three processes are contained within a tube of hypodermis, which, in turn, is surrounded by a tube of inner-layer cuticle. Wild-type rays project radially from the tail, have a smooth, slightly tapered morphology, and are embedded in the fan, which is composed of outer-layer cuticle (Figure 1A).

We identified 13 mutations that resulted in a similar lumpy, amorphous ray morphology by screening mutagenized populations and by surveying existing mutant strains (Figure 1, B–L). We will refer to the ray phenotype in these strains as the "*Ray morphology*," or Ram, phenotype. Mutations that conferred a Ram phenotype were of two types: those that affected only ray morphology, and those that also affected body shape, resulting in short, fat, or dumpy (Dpy) animals. Eight of 13 mutations (*bx25ts*, *bx29*, *bx30*, *bx32*, *bx34*, *bx39ts*, *bx48* and *bx56ts*) affected only ray morphology (Figure 1, B–I). These mutations defined five *ray morphology*, or *ram*, loci on four linkage groups (Fig-

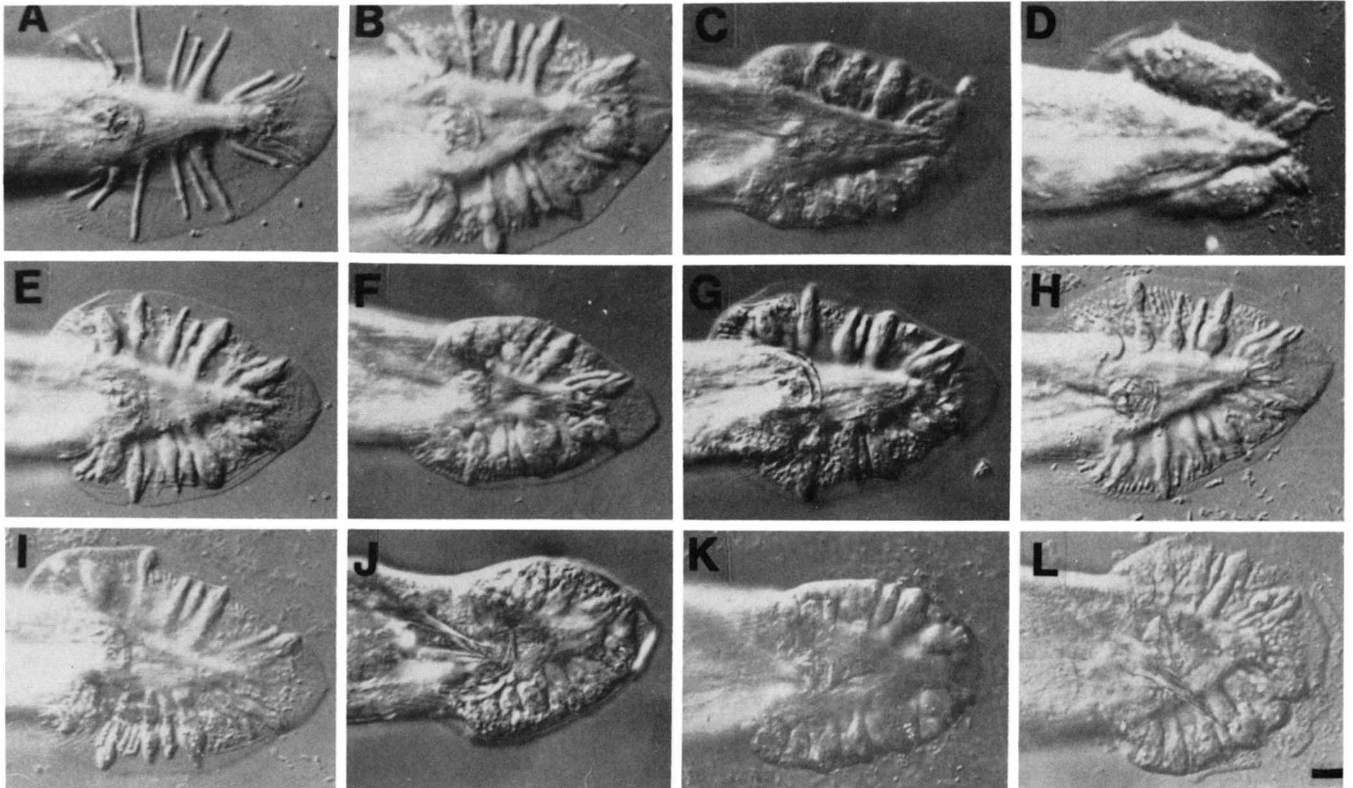

FIGURE 1.—Ventral views of wild-type and mutant adult male tails. A) wild type; B) *ram-1(bx34)I*; C) *ram-2(bx39ts)II*; D) *ram-3(bx32)II*; E) *ram-4(e25ts)IV*; F) *ram-?(bx48)IV*; G) *ram-?(bx56ts)*; H) *ram-5(bx30)X*; I) *ram-?(bx29)X*; J) *dpy-11(e224)V*; K) *dpy-18(bx26)III*; L) *sqt-1(e1350)II*. All strains shown contain *him-5(e1490)V*. This mutation does not affect the anatomy of the adult male. Temperature-sensitive mutations were grown at 25°. Scale bar = 10 μm.

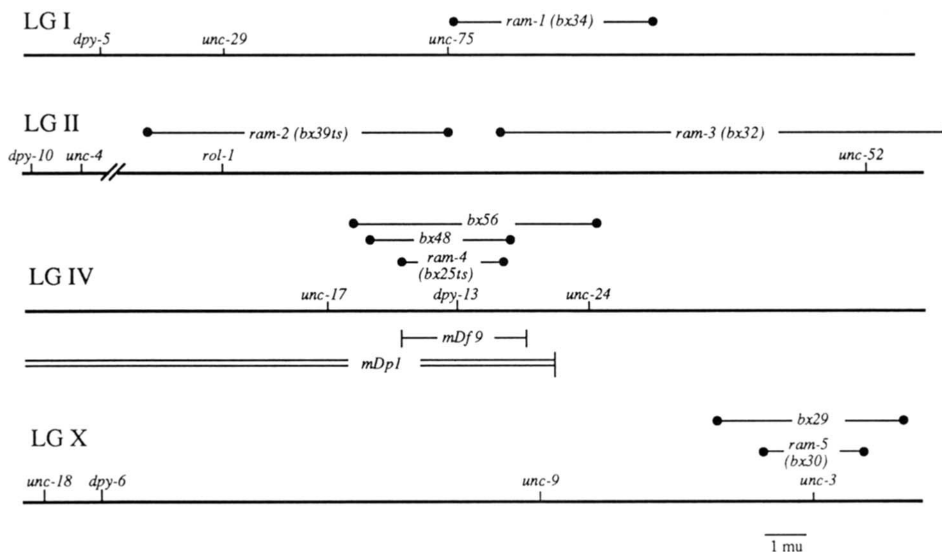

FIGURE 2.—Map positions of the *ram* genes. Portions of LGs I, II, IV and X are shown. Map positions of markers used in two- and three-factor crosses are indicated, as well as the map positions of other markers not used but provided for reference. The regions covered by the chromosomal deficiency, *mDf9*, and the chromosomal duplication, *mDp1*, are shown below the LG IV map. The *ram* gene map positions indicate 95% confidence intervals obtained from two-factor crosses (Table 2). The map positions of the markers were taken from the 1989 *Caenorhabditis elegans* genetic map (M. L. EDGLEY and D. L. RIDDLE, personal communication).

ure 2). Three of the *ram* mutations were temperature-sensitive, being completely wild type at 16°. All *ram* mutations were semidominant, having variably swollen ray tips as heterozygotes (see Figure 3). The penetrance of the semidominant trait was incomplete and its expressivity was low, *i.e.*, not all of the rays in affected animals had swollen tips. Five of 13 mutations (*e224*, *bx26*, *e364*, *e1096* and *e1350*) affected both ray and body morphology (Figure 1, J-L). These mutations were in three previously identified genes, *dpy-11*, *dpy-18* and *sqt-1* (BRENNER 1974; COX *et al.* 1980). Ram defects did not significantly interfere with ray functions, as most Ram males mated efficiently. None of the mutations resulting in Ram defects affected the hermaphrodite vulva, a structure that is affected by mutations in some classes of genes that are required

TABLE 1

***ram* gene mapping: three-factor data**

Gene	Allele	LG	Markers	Recombinant strains
ram-1	*bx34*	I	*dpy-5 unc-29*	DpyRam – 2
				Unc – 1
ram-2	*bx39ts*	II	*dpy-10 unc-4*	DpyRam – 6
				Unc – 9
ram-3	*bx32*	II	*dpy-10 unc-4*	DpyRam – 3
				Unc – 4
ram-4	*bx25ts*	IV	*unc-17 dpy-13*	UncRam – 5
				Dpy – 5
			dpy-13 unc-24	UncRam – 4
				Dpy – 2
	bx48	IV	*unc-17 dpy-13*	UncRam – 2
	bx56ts	IV	*unc-17 dpy-13*	UncRam – 3
				Dpy – 3
ram-5	*bx30*	X	*unc-18 dpy-6*	UncRam – 5
				Dpy – 3

TABLE 2

***ram* gene mapping: two-factor data**

Gene	Allele	LG	Marker	F_2 phenotypes W	R	M	RM	Percent recombination
ram-1	*bx34*	I	*dpy-5*	407	27	34	91	11.6 ± 2.7
ram-2	*bx39ts*	II	*dpy-10*	160	9	9	32	9.0 ± 3.9
ram-3	*bx32*	II	*dpy-10*	163	22			20.0 ± 5.8
ram-4	*bx25ts*	IV	*unc-17*	403	8	10	148	3.2 ± 1.4
	bx48	IV	*unc-17*	249	3	6	62	2.9 ± 1.8
	bx56ts	IV	*unc-17*	101	2	3	29	3.8 ± 3.2
ram-5	*bx30*	X	*unc-3*	1	149	207	1	0.55 ± 0.77
	bx29	X	*unc-3*	3	159	166	1	1.2 ± 1.2

W = wild-type, R = Ram, M = Marker (Unc or Dpy), RM = RamMarker. Recombination on LG X measured for hermaphrodite oogenesis (P_0).

for normal male tail development (HODGKIN 1983).

ram genes were defined solely on the basis of genetic map data (Tables 1 and 2) because all *ram* gene mutations failed to complement, including those at distinct loci (see below). From these data, five *ram* loci can be defined. Multiple *ram* mutations were present at two of these loci. Since in these instances we could not distinguish between allelic mutations and mutations in closely linked genes, only one such mutation was assigned to a *ram* gene, whereas the others were left unassigned. A single *ram* mutation, *bx34*, was located on LG I and defined the gene *ram-1*. Two *ram* mutations, *bx39ts* and *bx32*, were located on LG II. The map positions determined for *bx39ts* and *bx32* were nonoverlapping, and therefore they were assigned to two different genes, *ram-2* and *ram-3*, respectively. This assignment was confirmed by measuring the recombination frequency in the *bx39ts* to *bx32* interval. For this purpose, *trans* doubly heterozygous males (*bx39ts +/+ bx32*) were mated to *unc-4* hermaphrodites and F_1 (*i.e.*, nonUnc) males were scored for Ram defects. As a Ram phenotype was expressed by doubly heterozygous males, but not by singly heterozygous males, it was assumed that F_1 males that expressed a Ram phenotype harbored a recombinant, *bx39ts bx32* chromosome (*i.e.*, were *bx39ts bx32/unc-4*). Three Ram males were observed among 77 F_1 males scored. Thus, the recombination frequency between *bx39ts* and *bx32* was 3/77, or 0.08, which was consistent with their respective distances from *dpy-10* derived from two-factor data (Table 2). Three *ram* mutations were located within overlapping regions of LG IV and two were located within overlapping regions on LG X (Figure 2). Close linkage of the three LG IV mutations (*bx25ts*, *bx48* and *bx56ts*) was confirmed by scoring directly for recombination in the interval separating *bx25ts* and *bx48*, and in the interval separating *bx25ts* and *bx56ts*. This was accomplished by scoring for nonRam male progeny of *bx25ts/bx48* and *bx25ts/bx56ts* hermaphrodites, respectively. As these mutations fail to complement, nonRam males would have to harbor a recombinant wild-type chromosome IV. No nonRam male progeny were observed in either case (0/152 and 0/160, respectively). These results indicate close linkage (< 0.15 m.u.), but they are not inconsistent with intergenic distances in this region of the *C. elegans* genome. Therefore, *bx25ts* was assigned to *ram-4*, whereas *bx48* and *bx56ts* were left unassigned. Similarly, on LG X, *bx30* was assigned to *ram-5*, whereas *bx29* was left unassigned. These mutations were not mapped with respect to each other.

Mutations in three additional genes affected both ray and body morphology. The Ram phenotype conferred by these mutations was identical to that observed in *ram* gene mutants. One such mutation, *bx26*, was recovered in a screen for mutations affecting ray development. In addition to the Ram phenotype, *bx26* animals were Dpy. We were not able to separate the Ram and Dpy phenotypes. By genetic map data and complementation tests, *bx26* was shown to be an allele of the previously identified gene, *dpy-18*. Other alleles of *dpy-18* have been isolated on the basis of Dpy body morphology (BRENNER 1974). Males homozygous for two such *dpy-18* alleles, *e364* and *e1096*, were examined and found to have a Ram phenotype. *bx26* failed to complement both the Ram and Dpy phenotypes of these mutations. The Ram phenotype of all three *dpy-18* mutations was not expressed at 16°. The mutant body morphology was not temperature-sensitive.

The results with *dpy-18* prompted us to survey other mutations that affected body morphology for defects in ray morphology. In all, at least 42 mutations in 38 different genes were scored (Table 3). In addition to *dpy-18*, we identified two other genes, *dpy-11* and *sqt-1*, that could be mutated to give a Ram defect (Figure 1, J and L, respectively). A single allele of *dpy-11*, *e224*, was scored. This mutation expressed a temper-

TABLE 3

Ray sensilla phenotypes of mutations affecting gross body morphology

Ray phenotype	Gene (allele(s))
Ram	*dpy-11(e224)*; *dpy-18(e364am, e1096, bx26)*; *sqt-1(e1350)*[a]
nonRam	*bli-2 (e768)*; *bli-3 (e767)*; *bli-4 (e937)*; *bli-5 (e518)*; *dpy-1 (e1)*; *dpy-2 (e8)*; *dpy-3 (e27, e182)*; *dpy-4 (e1166)*; *dpy-5 (e61)*; *dpy-6 (e14)*; *dpy-7 (e88)*; *dpy-8 (e130)*; *dpy-9 (e12)*; *dpy-10 (e128)*; *dpy-13 (e184)*; *dpy-14 (e188)*; *dpy-19 (e1259ts)*; *dpy-20 (e1282ts)*; *dpy-24 (s71)*; *dpy-25 (e817)*; *lon-1 (e185)*; *lon-2 (e678)*; *rol-1 (e91)*; *rol-4 (sc8)*; *sma-1 (e60)*; *sma-2 (e502)*[b]; *sma-3 (e491)*[b]; *sma-4 (e729)*[b]; *sma-5 (n678)*; *sma-6 (e1482)*; *sma-8 (e2111)*; *sqt-1 (sc1, sc100, sc103)*[a]; *sqt-3 (sc63)*; *vab-2 (e96)*; vab-10(e698)

[a] *sqt-1* was the only gene identified with alleles in both Ram and nonRam classes.

[b] *sma-2* and *sma-3* males had fused rays. *sma-4* males were missing rays and had posterior alae. These phenotypes were distinct from the Ram phenotype.

ature-sensitive Ram defect, being nonRam at 16° and Ram at 25°. Four alleles of *sqt-1* were scored. Two, *e1350* and *sc1*, were antimorphic alleles that confer a recessive Dpy phenotype and a dominant roller (Rol) phenotype, and two, *sc100* and *sc103*, were putative null alleles that express no visible phenotype (KUSCH and EDGAR 1986). A non-ts Ram defect was observed in *e1350* males but not in males homozygous for the other *sqt-1* alleles. No Ram defects were observed in *sqt-1*/+ males. None of the other body morphology mutations examined expressed a Ram defect. In particular, no mutation in any body morphology gene whose map position overlapped with that of a *ram* gene expressed a Ram phenotype. Furthermore, the body morphology phenotypes of all such mutations (*dpy-13*, *dpy-24*, *rol-1* and *vab-10*) were complemented by the corresponding *ram* gene mutations (data not shown). Hence, the *ram* mutations described above define a class of genes distinct from previously identified body morphology genes.

***ram* gene products functionally interact but are not functionally redundant:** An unusual property of the *ram* genes was that mutations in different *ram* genes failed to complement (Figure 3). This was shown by scoring males doubly heterozygous for all pairwise combinations of autosomal *ram* gene mutations (Table 4). [*ram-5(bx30)* and *ram-(bx29)* were not included in these tests as *C. elegans* males are XO, and thus hemizygous for X-linked genes.] All tests were carried out at 25°, as some *ram* gene mutations were temperature-sensitive. Noncomplementation did not extend to mutations in *dpy-11*, *dpy-18* or *sqt-1*. The Ram phenotype of mutations in these body morphology genes was not expressed in doubly heterozygous combinations with mutations in the autosomal *ram* genes, or with mutations in the other body morphology genes (Table 4).

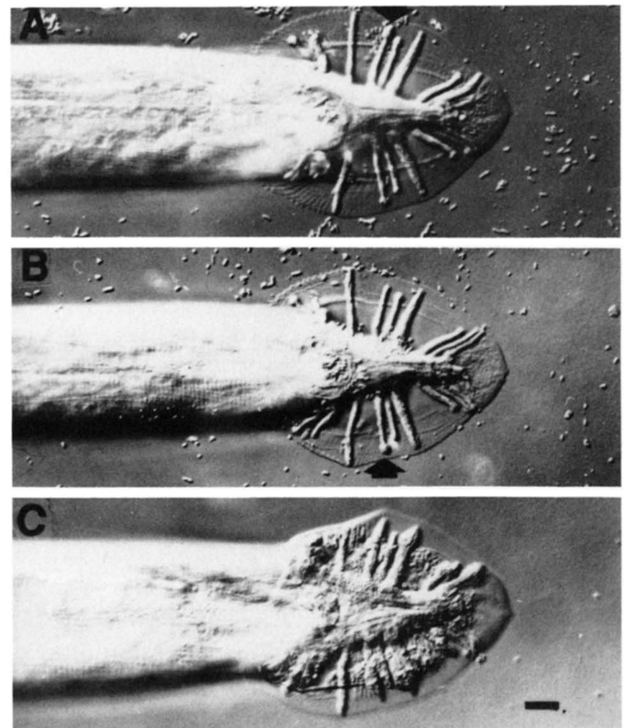

FIGURE 3.—Noncomplemetation between *ram* gene mutations. Ventral views of A) *dpy-5(e61) ram-1(bx34)*/+; B) *unc-17(e113) ram-4(bx26ts)*/+; and, C) *ram-1(bx34)*/+; *unc-17(e113) ram-4(bx25ts)*/+ males. Arrowheads indicate examples of swollen ray tips in A and B. All animals grown at 25°. Scale bar = 10 μm.

Intergenic noncomplementation, or "dominant enhancement," of recessive mutations is an indication of functional interactions between the products of the genes involved (BOTAS, DEL PRADO and GARCÍA-BELLIDO 1982; KUSCH and EDGAR 1986; TRICOIRE 1988; DAMBLY-CHAUDIERE *et al.* 1988; HOMYK and EMERSON 1988). Thus, *ram* gene products appear to participate in a common developmental function that is distinct from the functions provided by *dpy-11*, *dpy-18* and *sqt-1*. Dominance can result from either gain-of-function or loss-of-function mutations (MULLER 1932). Examples of each type of dominance have been described in studies of dominant enhancement (KUSCH and EDGAR 1986; DAMBLY-CHAUDIERE *et al.* 1988). With gain-of-function mutations, dominant enhancement can result from inappropriate (*e.g.*, elevated) expression of essentially normal gene products (*e.g.*, HOMYK and EMERSON 1988), or from the expression of aberrant gene products that interfere with the functions of wild-type gene products (*e.g.*, KUSCH and EDGAR 1986). With loss-of-function mutations, dominant enhancement must result from a combined haploinsufficiency of function (*e.g.*, DAMBLY-CHAUDIERE *et al.* 1988).

The dominant enhancement of *ram-4* resulted from haploinsufficiency. This conclusion was based on stud-

TABLE 4

Complementation patterns among Ram mutations

	ram-1 (*bx34*)	*ram-2* (*bx39ts*)	*ram-3* (*bx32*)	*ram-4* (*bx25ts*)	*dpy-11* (*e224*)	*dpy-18* (*e364*)	*sqt-1* (*e1350*)
Wt	Tip[a]	Tip	Tip	Tip	Wt	Wt	Wt
ram-1 (*bx34*)		Ram	Ram	Ram	Wt	Wt	Wt
ram-2 (*bx39ts*)			Ram	Ram	Wt	Wt	Wt
ram-3 (*bx32*)				Ram	Wt	Wt	Wt
ram-4 (*bx25ts*)[b]					Wt	Wt	Wt
dpy-11 (*e224*)						Wt	Wt
dpy-18 (*e364am*)							Wt

[a] All crosses were scored at 25°. Tip = semidominant *ram* phenotype; swollen ray tips, incomplete penetrance Ram = lumpy, amorphous rays, Wt = wild-type ray morphology.

[b] The *ram* mutations *bx48* and *bx56ts* failed to complement all other *ram* gene mutations, they were not tested with *dpy-11*, *dpy-18* or *sqt-1*. These mutations may be allelic to *ram-4* (*bx25ts*).

TABLE 5

Complementation of *ram* mutations with *mDf9*

Cross[a]	F_1 males[b]		Percent Ram
	Ram	nonRam	
Wt × *mDf9*/+	0	50	0
ram-1 (*bx34*) × *mDf9*/+	11	13	46
ram-2 (*bx39ts*) × *mDf9*/+	11	23	32
ram-3 (*bx32*) × *mDf9*/+	14	20	41
ram-4 (*bx25ts*) × *mDf9*/+	16	27	37

[a] All strains except the *mDf9*/+ strain contained *him-5* (*e1490*).

[b] All crosses were scored at 25°. The semidominant, swollen-ray-tip phenotype, was scored as nonRam.

ies with a deficiency of *ram-4* (Table 5). The properties of this deficiency, *mDf9*, were identical to the properties of *ram-4*(*bx25ts*). A single copy of *mDf9* in a wild-type background produced a semidominant swollen ray tip phenotype as was observed in *ram-4*(*bx25ts*)/+ males. A single copy of *mDf9* in combination with a single mutant copy of any other autosomal *ram* gene resulted in a Ram phenotype. Thus, *ram-4* is a haploinsufficient gene, and the haploinsufficiency is greatly enhanced by heterozygous mutations in other *ram* genes. These results were obtained by mating *ram* males to balanced hermaphrodites that contained *mDf9* (*mDf9*/*nT1*(*IV*); *nT1*/+(*V*), and by scoring F_1 males for Ram defects. In the *ram-4*(*bx25ts*) × *mDf9*/+ cross, 37% of the F_1 males had a Ram phenotype. This was lower than the expected frequency of 50%, probably because of a reduction in viability or a retardation in growth of deficiency heterozygotes. Comparable frequencies of Ram males were observed in crosses with the other *ram* gene mutations, consistent with the failure of *mDf9* to complement these mutations.

We also concluded from these results that *ram-4*(*bx25ts*) was a null mutation. The complementation pattern of *bx25ts* with other *ram* gene mutations was the same as that of *mDf9*. Furthermore, the Ram phenotype of *bx25ts*/*mDf9* males was no more severe than the Ram phenotype of *bx25ts* homozygotes.

Since *ram-4*(*bx25ts*) was a typical *ram* mutation, it was possible that many or all of the other *ram* mutations were null mutations as well, and that all *ram* genes were haploinsufficient singly (swollen ray tips), and in combination (Ram). Such combined haploinsufficiency could have been because all *ram* genes made equivalent products. That this was not the case was shown by studying the effect of an additional, wild-type copy of the *ram-4* gene in males homozygous for various *ram* gene mutations. A single wild-type copy of *ram-4* on the free duplication *mDp1* (Figure 2) complemented two chromosomal copies of *ram-4*(*bx25ts*) but not two copies of mutant alleles in other *ram* genes (Table 6). *mDp1* was tested for complementation with *ram-4*(*bx25ts*) by constructing an *unc-17 ram-4*; *him-5*; *mDp1* strain (*unc-17* is covered by *mDp1*). We scored both Unc (animals that had lost *mDp1*) and nonUnc (animals harboring *mDp1*) males (≥50 of each) for Ram defects. All nonUnc males had wild-type rays whereas all Unc males expressed a Ram phenotype. This result was consistent with the presence of a wild-type copy of *ram-4* on *mDp1*, and with the conclusion that *bx25ts* was a null mutation. To test other autosomal *ram* gene mutations for complementation with *mDp1*, *unc-17 dpy-13*; *mDp1* males were mated to *ram*; *unc-17 dpy-13* hermaphrodites, and nonUnc nonDpy F_2 self progeny males (*i.e.*, F_2 males harboring *mDp1*) were scored for Ram defects. Approximately 25% of such F_2 males had a Ram phenotype, as expected if *mDp1* failed to complement the *ram* gene mutation. Complementation of *mDp1* with *ram-5* was tested by mating *unc-17 dpy-13*; *mDp1* males to *ram-5* (nonHim) hermaphrodites. One hundred percent of the F_1 males (*i.e.*, *ram-5*/O cross progeny) expressed a Ram phenotype. Many of these males should have contained *mDp1*. Thus, *mDp1* does not complement homozygous or hemizygous mutations in *ram* genes other than *ram-4*. We conclude that although the products of the *ram* genes functionally interact, the product of the *ram-4* gene is not redundant with those of the other *ram* genes.

The effects of *ram* gene mutations were not additive: If the *ram* genes acted in a concerted manner during ray development, the elimination of a single *ram* gene should have had the same effect as the elimination of multiple *ram* genes. This prediction was shown to be correct as the Ram phenotype of doubly (*ram-1 I*; *ram-4 IV*) and triply (*ram-1 I*; *ram-2 II*; *ram-4 IV*) mutant *ram* strains was indistinguishable from the Ram phenotype of the corresponding singly mutant strains (Figure 4). Furthermore, both of these strains were able to mate reasonably well, indicating that ray functions were not significantly affected. There was only one indication that either multiply mutant strain was more severe than singly mutant

TABLE 6

Complementation of *ram* mutations with *mDp1*

Cross[a]	F_2 males[b]		Percent Ram
	Ram	nonRam	
mDp1 × *ram-1 (bx34)*	12	32	27
mDp1 × *ram-2 (bx39ts)*	8	21	28
mDp1 × *ram-3 (bx32)*	4	16	20
mDp1 × *ram-5 (bx30)*[c]	50	0	100

[a] *mDp1* = *unc-17 dpy-13; him-5; mDp1*. The ram strains (except *ram-5*) were *ram; unc-17 dpy-13; him-5*.

[b] All crosses were scored at 25°. Only nonUnc nonDpy males were scored. The semidominant, swollen-ray-tip phenotype, was scored as nonRam.

[c] F_1, *ram-5 (bx30)*/O, males scored.

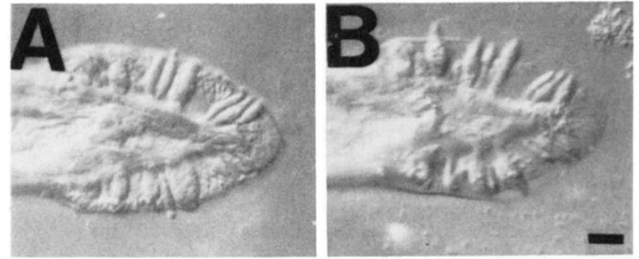

FIGURE 4.—The effects of *ram* gene dosage on male tail morphology. Shown are ventral views of tails of adult males that were grown at 25°: A) *ram-1(bx34)I; ram-4(bx25ts)IV; him-5(e1490)V*; B) *ram-1(bx34)I; ram-2(bx39ts))II; ram-4(bx25ts)IV; him-5(e1490)V*. Scale bar = 10 μm.

strains. In the triply mutant strain, a low percentage (<1%) of males had a "bent head" phenotype. This was a permanent ventral bend in the head region that caused such males to move in small irregular circles. Bent head males tended to be slightly small, but their Ram phenotype was no more severe than that of their siblings without bent heads. No bent head hermaphrodites were observed.

The *ram* and *dpy* gene products are required during ray extension: Temperature-sensitive periods of temperature-sensitive *ram* and *dpy* mutations were determined to define the developmental stage in which their gene products acted. An initial indication of the period of gene activity was obtained by observing ray morphogenesis in mutant animals. Such rays were defective at their first appearance, indicating that *ram* and body morphology genes acted at, or before, this stage. Based on this observation our temperature shift experiments focused on the period of ray extension.

The most thorough studies were of *ram-2(bx39ts)* and *dpy-18(e364)*. Their temperature-sensitive periods were coincident with tail retraction (Figure 5). This was determined by shifting *ram-2(bx39ts)* and *dpy-18(e364)* males between permissive (16°) and restrictive (25°) temperatures at various stages of development. These experiments focused on the period from mid to late L4, following the completion of the ray precursor cell lineages [early L3 (SULSTON and HORVITZ 1977)]. Interestingly, one downshifted *ram-2* male was observed in which the sensory rays had wild-type morphology at their bases but were Ram at their tips. This animal had been downshifted following the formation of papillae, the first visible sign of ray attachment to the cuticle, but prior to the beginning of ray extension. From this result, it appeared that the *ram-2* gene product acted at the bases of sensory rays as they were extended. Further attempts to obtain males with partially abnormal sensory rays were unsuccessful.

A rough determination of temperature-sensitive periods was made for *ram-4(bx25ts)* or *dpy-11(e224)* by shifting mutant males between restrictive and permissive temperatures at, or prior to, mid-L4. For both mutations, males upshifted prior to mid-L4 expressed Ram phenotypes and males downshifted prior to mid-L4 had wild-type sensory rays. These results were consistent with the temperature-sensitive periods determined for *ram-2* and *dpy-18*.

DISCUSSION

We have identified eight mutations that affect sensory ray morphology in *C. elegans* males. These mutations define at least five genes that have been designated *ray morphology*, or *ram* genes. Mutations in *ram* genes have no apparent affect other than ram morphology defects. We have also identified five mutations in three previously known body morphology genes that result in a similar ray defect. Our results indicate that the products of these two types of genes act in a common process, but provide distinct functions. These genes are terminal differentiation genes by the criterion that they act at the end of development, after the cell lineage is complete and during final morphogenesis of the fan and rays. We suggest that they encode structural components of the ray-cuticle ensemble. Alternatively, they might be required for the assembly, or even the expression, of structural components. The genes affecting body morphology are very likely to be components of the cuticle, and *sqt-1* is known to encode a collagen (KRAMER *et al.* 1988). *ram* gene function is essential only in males, as *ram* gene mutations have no evident effect on hermaphrodites.

At least one *ram* mutation, *ram-4(bx25ts)*, is a loss-of-function, and most likely a null mutation. This conclusion is based on the observation that the properties of *ram-4(bx25ts)* are identical to those of a deficiency of *ram-4*, *mDf9*. Furthermore, the Ram phenotype of *ram-4(bx25ts)/mDf9* males is no more severe than that of *ram-4(bx25ts)* homozygotes. Two other *ram* mutations are located near to, or within, *ram-4*. One, *bx56ts*, is temperature sensitive, whereas the other, *bx48*, is not. The presence of a non-tem-

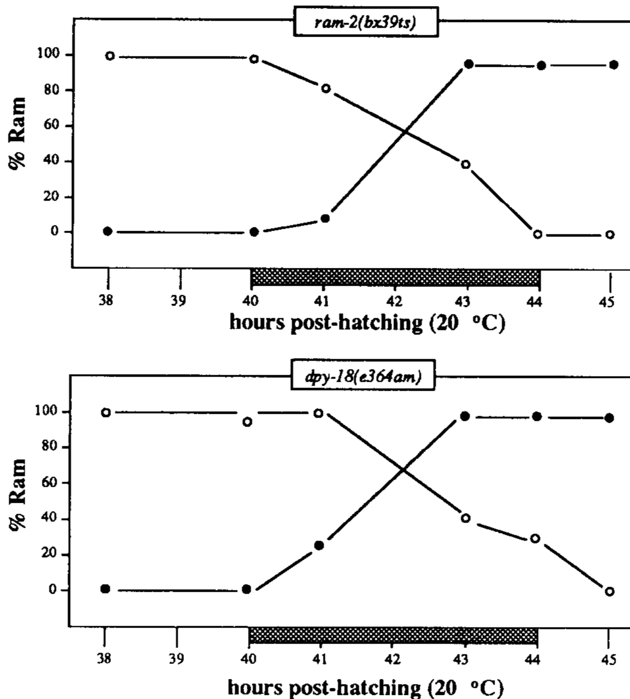

FIGURE 5.—Ray morphology temperature-sensitive periods of A) *ram-2(bx39ts)II*; and B) *dpy-18(e364)III*. Males were shifted, at various stages of L4, between permissive (16°) and restrictive (25°) temperatures. The open circles denote animals that were upshifted and the closed circles denote animals that were down shifted. The percent of males expressing a Ram phenotype were plotted against the stage at which they were temperature shifted (normalized to 20°). The stippled box on the time axis indicates the period of tail retraction (SULSTON, ALBERTSON and THOMSON 1980).

perature-sensitive mutation in *ram-4* might suggest that *bx25ts* is not a null mutation. However, it has been shown that the phenotypes of null mutations in some genes are less severe than the phenotypes of non-null mutations (GREENWALD and HORVITZ 1980; LANDEL *et al.* 1984; PARK and HORVITZ 1986; KUSCH and EDGAR 1986). Furthermore, it has not been established that *bx48* is in *ram-4*, and not in another, closely linked, *ram* gene.

ram-4(bx25ts) appeared to be a typical *ram* gene mutation. Its Ram phenotype, at 25°, was identical to the Ram phenotype expressed by other *ram* gene mutations, it failed to complement mutations in other *ram* genes, and it was semidominant. Therefore, it was possible that the other *ram* mutations were loss-of-function, and possibly null, mutations as well. This conclusion was consistent with the frequency at which *ram* gene mutations were isolated. Loss-of-function mutations are the most frequent class of mutations (HERMAN 1988). Assuming that *ram* gene mutations were loss-of-function mutations, and based on their frequency of isolation (8 mutations/2448 haploid genomes screened) and on the known forward mutation rate of our EMS treatment (5×10^{-4} mutations/locus/haploid genome) (BRENNER 1974), we estimated that the *C. elegans* genome contained six to seven *ram* genes. This estimate was consistent with our identification of at least five *ram* genes. Gain-of-function mutations are isolated at much lower frequencies (PARK and HORVITZ 1986). Had we assumed that *ram* gene mutations were gain-of-function mutations, our estimate would have been 10- to 100-fold higher. We have isolated multiple mutations at two *ram* loci, although, at present, we cannot discriminate between allelic mutations and mutations in closely linked genes. Based on these results, we believe that few, if any, *ram* genes remain to be identified.

By several criteria, *ram* gene products act in a common process during ray development. First, all *ram* mutants shared a common male-specific phenotype. Second, the Ram phenotype of doubly and triply mutant *ram* males was no more severe than the Ram phenotype of the corresponding singly mutant *ram* males. This result would not be expected if *ram* gene products acted independently during ray development. Finally, *ram* gene mutations displayed an unusual pattern of intergenic noncomplementation. Intergenic noncomplementation, or dominant enhancement, has generally been interpreted as an indication of functional interactions between the products of the genes involved (BOTAS, DEL PRADO and GARCÍA-BELLIDO 1982; BELOTE *et al.* 1985; KUSCH and EDGAR 1986; TRICOIRE 1988; DAMBLY-CHAUDIERE *et al.* 1988; HOMYK and EMERSON 1988).

Studies of dominant enhancement include examples of interactions between the products of regulatory genes (BOTAS, DEL PRADO and GARCÍA-BELLIDO 1982; BELOTE *et al.* 1985; TRICOIRE 1988; DAMBLY-CHAUDIERE *et al.* 1988), and of interactions between the products of terminal differentiation genes (KUSCH and EDGAR 1986; HOMYK and EMERSON 1988). We favor the latter model here, because *ram* gene products act at the end of development, following the terminal differentiation of the ray cell groups, and because it appears that the site of *ram* gene product action is the adult cuticle (see below), an unlikely site-of-action for regulatory gene products. It is possible that *ram* gene products physically interact. Physical interaction is the most likely basis of noncomplementation among cuticle defective alleles of *sqt-1*, *sqt-2*, *sqt-3* and *rol-8* in *C. elegans* (KUSCH and EDGAR 1988) and among flight defective alleles of *Mhc*, *fliA*, *up*, *hdp* and *rsd* in *Drosophila melanogaster* (HOMYCK and EMERSON 1988). However, these interactions differ from *ram* gene product interactions in that they are allele-specific and probably do not represent the phenotype of doubly heterozygous combinations of null mutations. The cuticle of *C. elegans* and the flight muscles of *D. melanogaster* are composed of, or contain, stable macromolecular complexes. The assembly of these complexes may be insensitive to doubly heterozygous combinations of null mutations. *ram* gene products, on

the other hand, may assemble into unstable complexes that are in equilibrium with their unassembled components. In males doubly heterozygous for null mutations in any two *ram* genes, such an equilibrium could be shifted so that not enough functional "*ram* complexes" are present to establish proper ray morphology during development. This type of equilibrium shift could also be the basis of dominant enhancement observed between the *da* and *AS-C* regulatory genes in Drosophila. Deletions of *da* and *AS-C* are haploinsufficient in combination (DAMBLY-CHAUDIERE *et al.* 1988) and it has been proposed that the products of these genes may physically interact via a shared helix-loop-helix domain (GHYSEN and DAMBLY-CHAUDIERE 1989).

The concerted activity of *ram* gene products is dependent upon multiple components. This was demonstrated by showing that a wild-type duplication of *ram-4*, which complemented *ram-4(bx25ts)*, did not complement mutations in any other *ram* genes. Therefore, assuming that most, or all, *ram* mutations were loss-of-function mutations, the product of *ram-4* was not functionally equivalent to the products of the other *ram* genes. A similar conclusion may be drawn for additional *ram* genes provided our mutations in these genes were null. The Ram phenotype of *ram-3(bx32)* was more severe than that of any other *ram* gene mutation, and mutations in two *ram* genes were temperature sensitive, whereas mutations in the others were not. Such differences could not result from null mutations in functionally equivalent genes. Thus, the wild-type product of *ram-3* may provide some residual *ram* function in the absence of other *ram* gene products, and the wild-type products of *ram-2* and *ram-4* may be entirely dispensable at low temperature.

We conclude from the Ram phenotype of mutations in *dpy-11*, *dpy-18* and *sqt-1*, that ray morphology is defined, in part, by the cuticle. Body morphology in *C. elegans* is determined by the external cuticle, and most genes that can be mutated to affect body morphology are thought to encode components of the cuticle (COX *et al.* 1980; OUAZANA, GARRONE and GODET 1985; KUSCH and EDGAR 1986; KRAMER *et al.* 1988; VON MENDE *et al.* 1988). In particular, *sqt-1* is known to encode a collagen (KRAMER *et al.* 1988). Collagens are the primary components of the cuticle (COX, KUSCH and EDGAR 1981). Therefore, the Ram phenotype expressed by mutations in *dpy-11*, *dpy-18* and *sqt-1* probably resulted from defects in cuticle structure. We suggest further that it is the inner layer of adult cuticle that is critical for ray morphology. The adult cuticle of *C. elegans* is composed of two layers (WHITE 1988). During ray extension, the surfaces of the inner and outer layers become disassociated laterally in the tail region (SULSTON, ALBERTSON and THOMSON 1980). The outer layer loops out to form the fan and, during tail retraction, is in contact with the extending rays at their tips, but not along their lengths. On the other hand, the inner layer of adult cuticle remains in contact with the body and surrounds the ray sensillum along its length as it extends (Figure 6). In mutant males, a Ram phenotype is apparent in incompletely extended rays. As the outer layer of adult cuticle is not in contact with the rays during this period, the Ram defect must involve the inner layer of cuticle, the cells of the rays, or both.

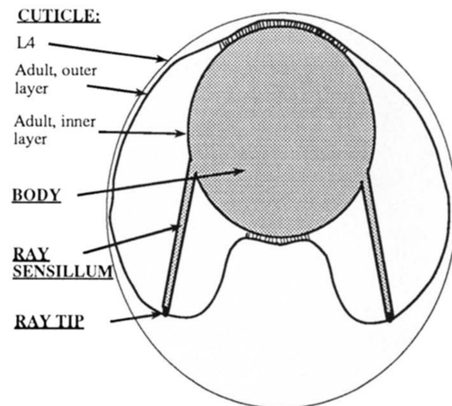

FIGURE 6.—Hypothetical transverse section through an L4 male showing the spatial relationships of the inner and outer layers of adult cuticle to the extending ray sensilla. Based on electron micrographs of N. THOMSON.

These arguments apply to the actions of the *ram* gene products as well as the body morphology gene products. The *ram* gene products appeared to be required for the same critical interactions as the body morphology gene products, since mutations in both had similar or identical phenotypes. Furthermore, *ram* and body morphology genes had the same times of action as defined by temperature shift experiments with temperature-sensitive alleles. However, *ram* gene mutations had several properties that were distinct from those of body morphology gene mutations, indicating that the activities of these two classes of genes were functionally distinct. We suggest that *ram* gene products either interact in a concerted manner with, or comprise a specialized part of, the inner layer cuticle. They may reside in the inner layer cuticle or in the apical surface of the underlying hypodermis.

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