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Sensory mother cell division is specifically affected in a *Cyclin-A* mutant of *Drosophila melanogaster*

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Cyclin proteins are one of the important components of the mechanism regulating mitosis in eukaryotic cells. We isolated a *Drosophila Cyclin-A* mutant in which the progenitor cells of the peripheral nervous system (the sensory mother cells) do not divide properly, causing the loss and other abnormalities of mechanosensory organs in the adult fly. Sequence analysis of the mutant genome reveals that a P element is inserted into the first intron of the *Cyclin-A* gene. A 13 kb wild-type genomic DNA containing the *Cyclin-A* transcription units rescued the mutant phenotype when introduced into the mutant fly. The regulation of cell type specific expression of the *Cyclin-A* gene is discussed.

Key words: Cyclin A/*Drosophila melanogaster*/sensory organ

Introduction

Cyclins are highly conserved proteins in eukaryotes. At the G_2-M transition of the cell cycle, cyclins work as a partner of *cdc2* kinase, a catalytic subunit of mitosis promoting factor (Evans *et al.*, 1983; Swenson *et al.*, 1986; Standart *et al.*, 1987).

In *Drosophila*, A- and B-type cyclins have been found (Whitfield *et al.*, 1989; Lehner and O'Farrell, 1989, 1990). Both types of cyclin proteins are coexpressed in epidermal cells of the early embryo (Lehner and O'Farrell, 1989, 1990) and in giant neuroblasts of the larval brain (Whitfield *et al.*, 1990). While cyclin B degrades at the metaphase-anaphase transition of the cell cycle, cyclin A does so at metaphase, suggesting their different roles in regulating cell cycle progression (Whitfield *et al.*, 1990; Lehner and O'Farrell, 1990). The functional requirement of cyclins in cells of developing organisms is amenable to mutation analysis. To date, only A-type cyclin mutants have been identified and characterized. The *l(3)183* and noe114 mutants harboring a null mutation in the *Cyclin-A* gene (*CycA*) cause embryonic lethality. In mutant embryos, the cessation of epidermal cell division is observed at cell cycle 16, probably because at that stage maternally stored cyclin A is exhausted (Lehner and O'Farrell, 1989). However, the contribution of cyclin A to the division of other types of cells has not been characterized.

During the ontogeny of multicellular organisms, cell proliferation in various tissues is strictly regulated to give rise to an organized morphology. Most of the adult cuticle of *Drosophila* develops from the imaginal discs, which are composed of monolayered epithelial cells. These cells proliferate at a constant rate during larval development (Postlethwait, 1978). Toward the end of larval and early pupal stages, the sensory mother cells (SMCs) are singled out from the population of disc cells, while the remaining cells are fated to the epidermis. Then, each SMC undergoes two differential divisions giving rise to a neurone and three support cells, together they compose a mechanosensory organ, for instance, a large bristle of the adult fly (Bate, 1978; Huang *et al.*, 1991).

We have isolated a *Cyclin-A* mutant, in which the division of SMCs in imaginal discs is specifically affected, resulting in bristle abnormalities. This mutation is due to the insertion of a P element into the first intron of the gene, suggesting that the regulation of cell type specific expression of *Cyclin-A* may depend on this intron.

Results

Phenotype of the hari mutant

We isolated a third chromosome recessive mutant, initially named *hari*, which lacks bristles on the dorsal side of the head and thorax (Figure 1b). In homozygous *hari* flies, several types of bristle defects are observed: loss of both shaft and socket, loss of either shaft or socket, bipolar shafts without socket, and shafts extending under the epidermis. The frequency of these defects in the large bristles on the head and thorax varies according to the position of the bristle (Figure 1c). The bristles on the dorsal side of the notum, the dorsocentral (DC) and scutellar (SC) bristles, are most frequently affected. In addition to these bristles, the microchaetae on the notum, the central row of bristles of the triple row at the wing margin, and the bristles on the abdominal tergites are slightly affected, while the bristles on the legs are normal.

Except for these bristle defects, mutant flies have normal morphology. For example, the distance between the pPA bristles and the number of epidermal cells between them are virtually unaffected (Table I). The overall morphology of the compound eyes of the mutant flies, including the number of ommatidia, also appears normal under the scanning electron microscope.

hari flies are fertile enough for propagating in homozygous condition. Eighty-one per cent of the mutant embryos hatch normally, while in most of the unhatched embryos neither cellularization nor segmentation is observed. In contrast to the adult sensory organs, the peripheral nervous system of the *hari* homozygous embryos develops normally (Dr C.Dambly-Chaudiere, personal communication).

Cloning of the hari gene

The *hari* mutation was induced by a P element insertion. To clone the *hari* gene, the genomic DNA fragments flanking the insertion site were recovered by the plasmid rescue

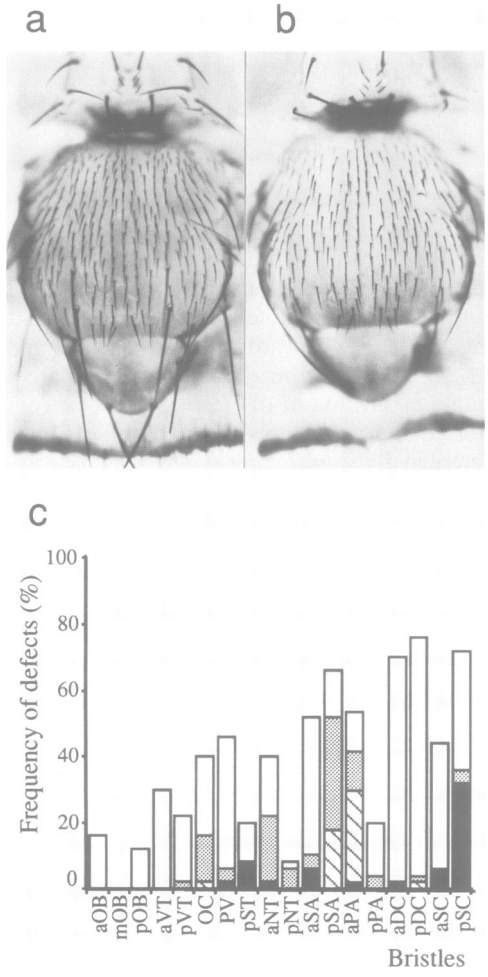

Fig. 1. Bristle phenotype of the *hari* mutant. Dorsal view of a wild-type fly (**a**) and of a fly homozygous for the *hari* mutation (**b**). (**c**) Expressivity of defects in 18 bristles located on the head (aOB-PV) and thorax (pST-pSC) of the *hari* mutant. For abbreviation of bristle nomenclature, see Bryant (1978). Types of defects are as follows; open bars, loss of both shaft and socket; shaded bars, loss of either shaft or socket; hatched bars, bipolar shaft without socket; solid bars, shaft extending under the epidermis. n = 50 for each bristle.

Table I. The distance and number of epidermal cells between pPA bristles

	pPA−pPA	
	Distance (μm)[a]	Number of trichomes[a]
Wild-type	454.9 ± 3.9	214 ± 3
hari	446.9 ± 2.9	225 ± 4

[a]Mean ± SE n = 10.

method. In the developmental Northern blot analysis using these fragments as probes, fragments from both sides of the insertion site detected the transcripts showing the same expression pattern, which suggested that the P element was inserted in an intron of the *hari* gene. cDNAs were obtained from an embryonic cDNA library of the *Canton-S* wild-type strain. Wild-type genomic DNA clones were also isolated. The sequences of the cDNA and of the corresponding genomic DNA (M.Takahisa *et al.*, in preparation) suggest that *hari* is a *Cyclin-A* (*CycA*) mutation. The *CycA* gene of

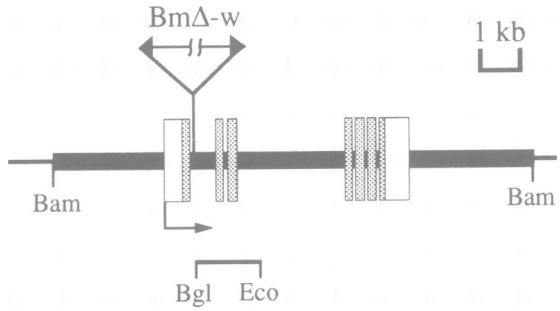

Fig. 2. Schematic representation of the *Cyclin-A* gene of *Drosophila melanogaster*. Seven boxes represent exons and arrow indicates the direction of transcription. The shaded area shows a putative protein coding region (Lehner and O'Farrell, 1989). In the *hari* mutant, the BmΔ-w transposon (Pirrotta *et al.*, 1985) is inserted in the first intron of the *Cyclin-A* gene. The bold line represents the wild-type genomic DNA fragment (*Bam*HI−*Bam*HI fragment) used to construct p[DCA13]. The *Bgl*II−*Eco*RI fragment used to screen genomic and cDNA clones is also shown.

Drosophila has seven exons and in the *hari* mutant the P element is inserted into the first intron (Figure 2).

Rescuing of hari with the CycA$^+$ gene

To test whether *hari* is caused by the *CycA* gene modification, the wild-type *CycA* gene (*CycA*$^+$) was introduced into flies by P element-mediated transformation. A 13 kb genomic DNA fragment containing all exons flanked by 2.7 kb of upstream and 3.0 kb of downstream sequences was cloned into the pUChsneo vector (p[DCA13], Figure 2). Following the injection of *Canton-S* embryos with p[DCA13], neomycin selection was performed to establish transformant fly lines. Among six independent lines established, five harbored the transgene on the second chromosome. This chromosome was introduced into the *hari* mutant by genetic crossing (see Materials and methods). As shown in Table II, the *CycA*$^+$ transgene of the A25-1 line could almost completely rescue the *hari* phenotype of the mutant flies. The remaining four lines showed the same level of rescuing as that of the A25-1 line (data not shown). This result verifies that *hari* is a *CycA* mutation and that the *cis*-regulatory sequence(s) included in the 13 kb fragment are sufficient for the expression of the *CycA* gene in SMCs (see below).

Genetic complementation

That the *hari* mutant is affected in the *CycA* gene was also confirmed by genetic complementation. For this test we used the *l(3)183* mutant, reported as a null mutation of *CycA* (Lehner and O'Farrell, 1989), and the *Df(3R)vin-3* that lacks this chromosomal region (Hoogwerf *et al.*, 1988). As shown in Figure 3, both flies heterozygous for *hari* and *l(3)183* and hemizygous for *hari* and *Df(3R)vin-3* showed a *hari* phenotype, demonstrating that *hari* is a *Cyclin-A* mutation. Moreover, complementation tests show that *hari* is not a hypomorphic mutation, because the phenotypes of the *hari*/*l(3)183* heterozygote and *hari*/*Df(3R)vin-3* hemizygote are not stronger than that of *hari* homozygous flies (Figure 3). Thus, *hari* seems to be a null mutation that only affects the function for bristle formation of the *CycA* gene.

Sensory mother cell division in the hari mutant

Bristles are formed by the progeny of SMCs, each of which divides twice to produce the four cells that will form a bristle

Table II. Rescue of *hari* phenotype by a *CycA* transgene

Genotype	No. of adults examined	Adults with abnormal bristles (%)	Abnormal bristles[a] (%)
+/+; *hari/hari*	192	192 (100)	771 (50.2)
p[DCA13]/+; *hari/hari*	254	31 (12.2)	35 (1.7)
p[DCA13]/p[DCA13]; *hari/hari*	115	5 (4.3)	5 (0.5)

[a]Eight bristles, pairs of two DC and two SC bristles, were counted in each fly.

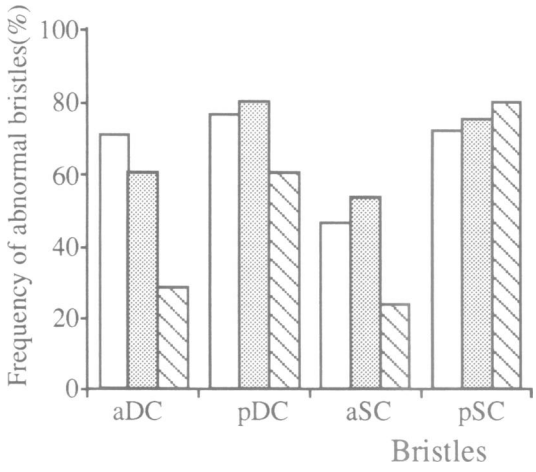

Fig. 3. Genetic complementation of mutants. The four bristles most severely affected in the *hari* mutant were counted in *hari* homozygous flies (open bars), *hari/Df(3R)vin3* hemizygotes (shaded bars) and *hari/l(3)183* heterozygotes (hatched bars). Values of the four types of defects (cf. legend of Figure 1) are combined. $n = 50$ for each bristle.

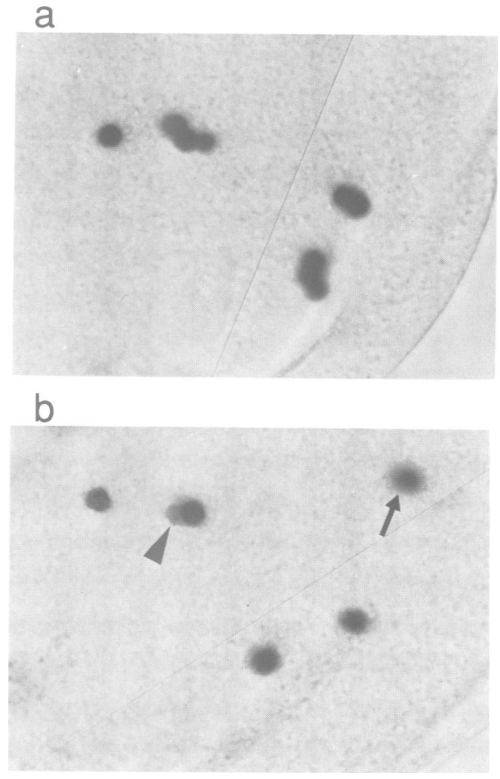

Fig. 4. SMC divisions in the wing imaginal discs of the *hari* B52/+ + control fly (**a**) and the *hari* B52/*hari* + fly (**b**). Imaginal discs were dissected from white pupae at 3 h after puparium formation, and stained for β-galactosidase activity. Presumptive notum part of the disc is shown in both panels, oriented anterior to the left and proximal downwards. In (a), the four cell clusters are the progenies of SMCs, for aDC, pDC, pSC (lowermost) and aSC bristle, from left to right. These SMCs independently divide in a developmentally regulated fashion (Huang *et al.*, 1991). In (b) no cell division is observed in any of these SMCs. A weak label associated with the pDC mother cell (arrowhead) may represent a 'multiple precursors of SMC', which sometimes appears transiently before the determination of a single SMC (Huang *et al.*, 1991). The appearance of a pPA mother cell (arrow) indicates that this imaginal disc is slightly older than that in (a).

organ. Considering that the *CycA* gene is affected in the *hari* mutant, we examined the bristle SMCs in *hari* wing discs to elucidate whether the bristle defects are associated with defects in the SMC divisions. In order to mark the SMCs and their progeny, we utilized the enhancer-trap line B52. The *lacZ* reporter gene in this line is specifically expressed in SMCs (Dr A.Ghysen, personal communication). A fly strain homozygous for *hari* and heterozygous for B52 was constructed and its wing imaginal discs were stained for β-galactosidase activity. In control discs 3 h after puparium formation, three of the four SMCs corresponding to the two dorsocentral and two scutellar bristles have already divided to generate two to three daughter cells (Figure 4a). As shown in Figure 4b, these four SMCs appear at the appropriate position in *hari* discs. However, none of them had divided 3 h after puparium formation. These four SMCs correspond to the most severely affected bristles in the *hari* mutant (cf. Figure 1c). Although these SMCs had divided in some *hari* discs, the number of progeny cells was smaller than those in the control discs. The SMC divisions were also affected in other regions. The degree of abnormality in the SMC division seemed parallel with that of the bristle in the corresponding region.

Discussion

The above results allow us to conclude that in imaginal discs cyclin A is essential for SMC division. *hari* proved to be a *Cyclin-A* mutation; therefore, we rename it $CycA^{har}$. Although the $CycA^{har}$ mutation interferes with SMC divisions in the wing disc, epidermal cell proliferation is not affected (Table I). Furthermore, the P element insertion appears to have little or no effect on the overall transcription of the *CycA* gene, since Northern analyses of the RNA extracted from the whole body at various developmental stages show that the expression pattern of *CycA* in the mutant is essentially the same as in the wild-type (data not shown). Therefore, we postulate that the transcription of *CycA* is specifically regulated in the SMC, and that the *cis*-regulatory element(s) for SMC specific expression reside(s) in its first intron and/or near it. In the $CycA^{har}$ mutant, the P element

insertion would interfere with the function of this regulatory element, so that SMC division is altered, thus causing the bristle abnormalities. Of course, at present we cannot exclude other possibilities like that the P element insertion interferes with the splicing of the cyclin A pre-mRNA specifically in the SMC. Anyway, the interpretation that the *hari* mutation affects the SMC specific regulation of *CycA* expression is strongly supported by our observation that $CycA^{har}$ behaves not as a hypomorphic mutation, but rather as a null mutation for SMC specific function (Figure 3).

In *Drosophila*, sensory organ formation is a multistep developmental process in which the proneural genes play a significant role in the determination of SMC (Romani *et al.*, 1989; Ghysen and Dambly-Chaudiere, 1989; Cubas *et al.*, 1991). It is known that all of the proneural genes identified to date encode DNA binding proteins, bHLH proteins (Alonso and Cabrera, 1988; Caudy *et al.*, 1988; Gonzalez *et al.*, 1989; Rushlow *et al.*, 1989; Eliis *et al.*, 1990; Garrell and Modolell, 1990; Garrell and Campuzano, 1991). They are believed to regulate the expression of the downstream genes that are essential for SMC's differentiation. Recently, the *hunchback* gene expression was shown to be controlled by the proneural gene(s) in the central nervous system (Cabrera and Alonso, 1991). However, none of such target genes have yet been identified for SMCs. Since the first intron of the *CycA* gene contains five copies of the CANNTG motif (M.Takahisa *et al.*, in preparation), which is known to be the consensus binding site for bHLH proteins (Murre *et al.*, 1989; Blackwell and Weintraub, 1990; Blackwell *et al.*, 1990), it may be that this intron is a direct target for the proneural genes. This would be consistent with a crucial role of this intron in the division pattern of SMCs. Tissue specific enhancer elements in introns are known to be present in *engrailed* (Kassis, 1990), *Toropomyosin-1* (Schultz *et al.*, 1991) and *β_3-tubulin* (Gasch *et al.*, 1989) genes of *Drosophila*. We are now introducing modified *CycA* genes into the fly to test this possibility.

Materials and methods

Fly strains

The *hari* mutant was induced by the jumpstart method using Δ2-3 transposase source and mw474 strain (provided by Dr V.Pirrotta), which contains a BmΔ-w transposon on the X chromosome (Pirrotta *et al.*, 1985). Outcrossing *hari* mutant five times to a *w* strain did not change the mutant phenotype. Reversion tests with Δ2-3 transposase source gave 37% reversion rate of the *hari* mutant.

Df(3L)vin3/TM3 was obtained from Mid-America Drosophila stock center. *l(3)183/TM3* was provided by Dr D.Roberts.

To mark SMCs in the *hari* wing disc, the *hari* B52 chromosome was constructed by recombination between *hari* and *ru h th st cu* B52 chromosomes (provided by Dr A.Ghysen). To avoid some suppressive effect of B52 on the *hari* phenotype, males homozygous for *hari* B52 were crossed to *hari* homozygous females and F1 progenies were used to strain SMCs.

All of the flies were raised on a standard yeast−corn meal−sugar−agar food at 25°C.

SEM observation of the fly

To count the number of epidermal cells on the notum, we used the pPA bristles as landmarks because they are the only bristles that frequently remained in the dorsal-most part of the mutant notum. After the usual fixation and critical point drying, the flies were observed and photographed under a Hitachi S-800 scanning electron microscope. After enlargement on the screen at ×580 magnification, the stripe of 10 mm wide was marked off between the two pPA bristles. All of the epidermal cells (trichomes) in this stripe were counted in the mutant and in *Canton-S* wild-type female flies.

Staining of wing disc SMCs

In the imaginal discs of the enhancer trap line B52, β-galactosidase activity is solely detected in SMCs, as it occurs in the A37 (Ghysen and O'Kane, 1989) and A101 lines (Huang *et al.*, 1991). Wing imaginal discs were dissected in PBS from pupae at 3 h after puparium formation. The discs were fixed with 0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3) for 15 min at room temperature and washed in PBS containing 0.3% Triton X-100 for 10 min. After two additional washings in PBS, discs were incubated in the prewarmed staining solution {0.2% X-gal, 0.3% Triton X-100, 150 mM NaCl, 1 mM $MgCl_2$, 3.1 mM $K_4[Fe^{II}(CN)_6]$, 3.1 mM $K_3[Fe^{III}(CN)_6]$, in 10 mM phosphate buffer [pH 7.2]}. After overnight incubation at 37°C, discs were washed and mounted in PBS to observe under the microscope.

Cloning

The genomic DNA fragments flanking the insertion site of BmΔ-w transposon were cloned by the plasmid rescue method (Steller and Pirrotta, 1985). λDashII genomic library (provided by Dr Y.Sano) and embryonic λZapII cDNA library (Caudy *et al.*, 1988; provided by Dr T.Uemura) of *Canton-S* wild-type strain were screened with random primer labeled *BglII−EcoRI* fragment of the rescued genomic DNA (Figure 2).

Transformation of flies

A 13 kb *BamHI* fragment of a *Cyclin-A* genomic clone was introduced into the *BamHI* site of pUChsneo vector to yield the p[DCA13]. In this construct, the *Cyclin-A* gene is transcriptionally oriented in the same direction as the heat shock promoter in the vector. p[DCA13] was injected into *Canton-S* embryos with phsπ helper plasmid. For a detailed procedure of injection, selection of transformed flies, and identification of the integrated chromosome in the transformant, see Ueda *et al.*, 1987.

To examine the activity of the transgene in rescuing the 'hari' phenotype P[DCA13]/SM1; *Pr Dr/TM3* males were crossed to *Sp/SM1*; $CycA^{har}/CycA^{har}$ females. Among the F1 progenies, p[DCA13]*/SM1*; $CycA^{har}$*/TM3* males and females were selected and crossed to each other. F2 progenies homozygous for $CycA^{har}$ were divided into two groups according to whether they had one or two copies of the transgene, and examined for the bristle phenotype. To count many flies, only four pairs of bristles, two DCs and two SCs, those showing the severest phenotype in the mutant, were examined. Four types of bristle defects (see text) were combined into one category, 'abnormal bristles'.

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