The role of yan in mediating the choice between cell division and differentiation

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SUMMARY

An allele of the yan locus was isolated as an enhancer of the Ellipse mutation of the Drosophila epidermal growth factor receptor (Egfr) gene. This yan allele is an embryonic lethal and also fails to complement the lethality of anterior open (aop) mutations. Phenotypic and complementation analysis revealed that aop is allelic to yan and genetically the lethal alleles act as null mutations for the yan gene. Analysis of the lethal alleles in the embryo and in mitotic clones showed that loss of yan function causes cells to overproliferate in the dorsal neuroectoderm of the embryo and in the developing eye disc. Our studies suggest that the role of yan is defined by the developmental context of the cells in which it functions. An important role of this gene is in allowing a cell to choose between cell division and differentiation. The relationship of the Egfr and Notch pathways to this developmental role of yan is discussed.

Key words: Drosophila melanogaster, eye, embryonic development, neuronal determination, cell cycle, yan, aop

INTRODUCTION

Intercellular signalling mechanisms are a primary means of cell fate determination in developmental systems. In the Drosophila eye, receptor tyrosine kinase (RTK) mediated cell-cell interactions have been shown to be important for the determination of neuronal fate. The mechanism for the development of the R7 photoreceptor cell has been elucidated by extensive genetic and molecular analysis (reviewed by Zipursky and Rubin, 1994). In this system, the activation of the Sevenless RTK on the precursor of the R7 cell initiates the Ras cascade, leading to the determination of the R7 precursor as a photoreceptor neuron. The Ras signal is transmitted into the nucleus by mitogen-activated protein kinase (MAPK), which phosphorylates nuclear factors. Two ETS domain-containing nuclear factors have been shown to be direct targets of MAPK in this system (reviewed by Dickson, 1995). The first is Pointed, a transcription factor that is activated by MAPK through phosphorylation. The second protein, Yan, is a negative regulator that binds to DNA at the same consensus sites as Pointed, but functions as an inhibitor of transcription. Phosphorylation of Yan by MAPK inactivates its function as a repressor, and allows the activation of genes by Pointed. In this model, a signal through Sevenless is tightly regulated, resulting in the inactivation of a negative component of the pathway and the activation of a positive one. Consistent with this mode of signal transduction, genetic experiments show that loss of Pointed causes a loss of R7 cells (O’Neill et al., 1994; Brunner et al., 1994) while a decrease in Yan function results in an increased number of cells developing as R7 neurons (Lai and Rubin, 1992; O’Neill et al., 1994). In recent studies, activated versions of Yan were created by mutagenizing each of the eight MAPK phosphorylation sites (Rebay and Rubin, 1995). By transforming Drosophila S2 cells with these constructs, it was shown that, in response to a Ras signal, Yan is transported out of the nucleus and rapidly degraded. Using the activated constructs, it was further shown that Yan is a general inhibitor of differentiation in many cell types (Rebay and Rubin, 1995). Despite the interest in Yan as a negative regulator of development, there has been no published analysis of null alleles of yan. The Yan protein shows abundant expression in the embryo suggesting that Yan normally functions in cells other than the R7 precursor. In this paper, we have analyzed the null mutant phenotype of yan and show that at two different stages of development, the yan gene participates in the decision-making process that allows a cell to choose between differentiation and division pathways.

The cells predestined to give rise to the adult Drosophila eye are set aside in the embryo as an eye disc which grows through the first three instars of larval development. In the third instar disc, a wave of morphogenesis commences at a front called the morphogenetic furrow (Ready et al., 1976). This furrow initiates at the posterior edge of the disc and moves anteriorly (reviewed by Heberlein and Moses, 1995). Cells anterior to the...
furrow are undifferentiated and divide asynchronously. However, within five cell diameters anterior to the furrow, all the cells are forced into a state of G1 arrest (Thomas et al., 1994). At the posterior edge of the morphogenetic front, each cell faces a binary choice of either joining a synchronous band of cells undergoing division, or joining a preclustering that will eventually differentiate into photoreceptor neurons (Wolff and Ready, 1991a). In this paper, we demonstrate the involvement of the yan gene product in this process.

Many genes are involved in the patterning of neurons in the eye disc. Interactions of yan with two of these genes, the Drosophila EGF receptor gene (called DER or Egfr) and the neurogenic gene Notch were critical for the functional analysis presented in this paper. Previous studies have shown that Egfr (Baker and Rubin, 1992; Xu and Rubin, 1993; Tio et al., 1994; Freeman, 1994) and Notch (Fortini et al., 1993; Cagan and Ready, 1989) play prominent roles in eye development. In the Egfr gain-of-function mutation, Ellipse (Egfr(E)), photoreceptor clusters develop normally, but they are spaced further apart than in wild type. Many of the intervening uncommitted cells incorporate BrdU but eventually apoptose (Baker and Rubin, 1992). Xu and Rubin (1993), have shown that mosaic clones of Egfr loss-of-function mutations proliferate poorly and fail to express neuronal markers. Genetic analysis of spitz, a ligand for Egfr (Schweitzer et al., 1995), has suggested that Egfr may have an early role in the initial formation of the cluster, as well as a later function in proper patterning of the R-cells (Tio et al., 1994; Freeman, 1994). The lethal alleles of yan described in this paper interact prominently with mutations in Egfr suggesting a role for yan in multiple pathways initiated by RTKs.

The Notch gene product functions in many different cell-cell interactions in the developing eye, allowing cells to choose between a determined and an undetermined state. Ectopic activation of Notch during cluster formation causes cells to remain undifferentiated (Fortini et al., 1993), while loss of Notch function causes excessive development of photoreceptor neurons (Cagan and Ready, 1989). In this paper we show that yan mutations interact with Notch and examine the possible roles of Notch in relation to the function of Yan as a member of RTK pathways.

MATERIALS AND METHODS

Scanning electron microscopy

Adult flies were serially incubated for 12 hours in each of the following solutions: 25%, 50%, 75%, 100% and 100% EtOH; then in 25%, 50%, 75%, 100% and 100% hexamethyldisilazane (Sigma) in EtOH. After the final incubation, the hexamethyldisilazane was poured off and the flies were allowed to desiccate under vacuum for several days. They were mounted in colloidal silver paste (Ted Pella), dried for a day and sputter coated with gold/platinum. Adult eyes were analyzed on an ISI DS-130 scanning electron microscope at 10 kV.

Embryo preparations

Cuticle preparations of embryos were made following Wieschaus and Nusslein-Volhard (1986). To stain for BrdU incorporation, staged and dechorionated embryos were permeabilized with octane (Sigma) for 3 minutes and then spread on 1 mg/ml BrdU in Grace medium. Embryos were allowed to develop at 25°C for 30 minutes, then collected and prepared according to the standard protocol (Ashburner, 1989). For anti-FasII antibody staining, the protocol described by Grenningloh et al. (1991) was followed.

Recombination mapping of e2d

Standard recombination mapping was employed to map e2d between the el and dp markers and to D62L, dp79b on 2L. To determine if e2d was separable from yan by recombination, e2d/Cyo flies were crossed to yan(pok-x8)P[yan(pok-x8)] homozygotes. The e2d yan(pok-x8) escaper females were then mated back to e2d/Cyo males. The progeny were then screened for recombinants of either the e2d yan+ or e2d yan(pok-x8) genotype. No such recombinants were isolated upon screening 2,488 flies. Thus e2d was found to be inseparable from yan(pok-x8) to less than 0.04 mu.

Generation of mosaic patches

Mosaic clones were generated by 1200 R X-ray irradiation of 12-36 hour old collections of hatching larvae. The w+ gene, which gives rise to a red pigment in the eye, was used as a marker. In wild-type controls, white patches in an otherwise red eye were generated in adult eyes at a frequency of 8% (n=500). Using an identical scheme, homozygous clones of e2d, aop-lp and aop-ls were identified with the same frequency as in the wild-type controls, as visible scars within the otherwise highly ordered retinal pattern of the adult eye.

To mark yan+/yan- patches in the developing eye imaginal discs, yan/Cyo males were crossed to w~/w+; P[Arm-lacZ.w+]/28A/P[Arm-lacZ.w+] females carrying P-element insertions containing the armadillo promoter fused to the lacZ gene (Vincent et al., 1994). This construct drives lacZ expression autonomously in every cell of the eye disc. Both wild-type and yan+/yan- clones were generated as above and were identified by staining for lacZ expression. Double staining with anti-Elav antibody or for BrdU incorporation showed that yan+/yan- patches, lacking lacZ expression, did not express Elav, but did stain extensively for BrdU. In corresponding wild-type controls, patches lacking lacZ expression showed a normal staining pattern of Elav expression. The frequency of mosaic patches generated in the eye disc was similar to the frequency seen in adults.

Antibody and BrdU protocols for eye discs

BrdU staining was done essentially as described by Truman and Bate (1988) using an ABC kit with Horseradish Peroxidase (HRP) conjugates as the secondary antibody (Vector Labs) and anti-BrdU monoclonal antibody (Becton Dickenson). For BrdU/mAb22C10 double staining, discs were first taken through the entire BrdU protocol and then, after the HRP color reaction, were stained with mAb22C10 as the secondary antibody (Vector Labs) and anti-BrdU monoclonal antibody (Becton Dickinson). For BrdU/Elav double staining, discs were then mated back to the BrdU protocol, substituting the Carnoy’s fix with a 40 minute fix in PLP followed by three, 10 minute washes in PBS. After the first HRP color reaction for the BrdU staining, which included 0.4 mg/ml Nickel chloride, the discs were washed 4x 10 minutes in PBS, incubated 10 minutes in PBS with 0.4% NP-40 (PBNS) and 10% goat serum, incubated overnight in anti-Elav antibody, in PBNS with 10% goat serum, washed 6x 20 minutes in PBNS and incubated overnight in anti-SP-Biont antibody (Jackson labs) which was diluted 1:400, and preabsorbed for 1 hour against fixed tissue. The discs were then washed 6x 20 minutes in PBST, incubated overnight in the avidin/biotin complex solution at 4°C, washed 6x 20 minutes in PBS, and then taken through the color reaction for 10 minutes without any nickel chloride to distinguish the precipitates of the two different staining reactions.

RESULTS

The e2d mutation interacts with Egfr

In a screen to identify modifiers of the activated EGF receptor...
mutant Ellipse (EgrfE), we isolated a mutation called e2d as an enhancer. EgrfE/+ flies have a weak rough eye phenotype (Fig. 1B) in which the regular array of facets (ommatidia) seen in the wild-type compound eye (Fig. 1A) is disrupted. This rough appearance is significantly enhanced when one copy of the e2d mutation is introduced (Fig. 1C). When made homozygous, the e2d mutation was found to be an embryonic lethal. The embryonic phenotype was analyzed by comparing cuticular preparations of mutant embryos (Fig. 1G) with wild type (Fig. 1F). The most obvious external phenotype in the mutant is a severe defect in the cephalopharyngeal skeleton and a large hole in the dorsoanterior region of the cuticle.

Since e2d interacts with EgrfE, we determined if the embryonic phenotype of e2d would interact with the loss-of-function mutant allele, faint little ball, flb1F26 (called EgrfE here for simplicity). Chromosomes containing both EgrfE and e2d were constructed by recombination and embryos that were homozygous for both mutations were generated. As shown in Fig. 1H, ventral denticle bands are entirely absent in EgrfE/EgrfE embryos, whereas in the double mutant combination (Fig. 1I), this phenotype is partially suppressed, and some denticle bands can be seen. Thus the e2d mutation interacts with both gain- and loss-of-function mutations in EgrfE, enhancing EgrfE and partially suppressing EgrfE, respectively.

e2d, aop and yan are allelic

Standard recombination mapping methods were employed to map e2d to the 22D region of the arm of the second chromosome. The lethality of e2d maps to the deficiency Df(2L)dp79b, which uncovers the anterior open (aop) (Nusslein-Volhard et al., 1984) and yan mutations. The previously described aop mutations were characterized by a severe head defect with an open anterior region of the cuticle (Lindsley and Zimm, 1992; Nusslein-Volhard et al., 1984). This phenotype is remarkably similar to that seen for e2d (Fig. 1G). Hence, complementation crosses were initiated between the aop alleles aop-1P, aop-1Is and the e2d mutant. No aop/e2d flies were ever recovered in these crosses (Table 1B). Furthermore, both aop alleles were found to enhance the rough eye phenotype of EgrfE in a manner similar to that seen for e2d (Fig. 1D,E). Thus the map position, phenotype and complementation tests suggest that the e2d and aop alleles represent mutations in the same essential gene.

The map position of aop corresponds closely to that of yan and recombination analysis showed us that e2d was inseparable from yan (see Materials and Methods for details). To determine if aop and e2d were allelic to yan, we initiated a complementation analysis. Of the three previously described alleles of yan used in this study, yan1 is the weakest, giving rise to an almost wild-type external appearance of the eye. The yanpok-x8 (Tei et al., 1992) and yanl (Lai and Rubin, 1992) alleles are essentially equivalent and are stronger than yan1, but both are hypomorphic since their phenotypes are enhanced over a deficiency for the region (Fig. 2C; Table 1A). In complementation tests (Table 1), e2d, aop-1P and aop-1Is were found to be allelic to yan. Besides being recessive lethals on their own, these mutations were semilethal over yanpok-x8, giving rise to escapers with small rough eyes (Table 1; Fig. 2D-F). These escapers have eyes that are smaller and rougher than that seen for yanpok-x8/yanpok-x8 flies (compare Fig. 2B with D-F). In tangential sections of yanpok-x8/yanpok-x8 eyes, ectopic R7 cells are seen, but the overall organization of the ommatidial array is normal (Fig. 2H). In contrast, yanpok-x8/e2d flies have a more severe phenotype (Fig. 2I) that includes fusion of ommatidia, loss of the regular pigment cell array and a more extensive ectopic conversion into R7 cells in every ommatidium. In all phenotypes and complementation tests described thus far, the three alleles aop-1P, aop-1Is and e2d give results that are similar to those obtained with Df(2L)dp79b. Genetically, these alleles behave as nulls for the yan locus. The similarity between these three alleles extends through the developmental and functional analyses that will be presented below.

Three different names, aop (Nusslein-Volhard et al., 1984; Lindsley and Zimm, 1994), pok (Tei et al., 1992) and yan (Lai and Rubin, 1992) have been used to describe the same gene. Since most of the recent publications have used the name yan, we have followed the same nomenclature in this paper, and have used aop, pok and e2d as allele names.

Effect of lethal alleles of yan on R7 development

The availability of sensitized genetic backgrounds makes it feasible to study the effect of loss of one copy of a gene on the development of the R7 cell (reviewed in Daga and Banerjee, 1994). We employed the sensitized genetic background, seyE4/Y;Starr3C2/+ (Rogge et al., 1991) to analyze the effect on R7 development of alleles of yan that have been described before, as well as the newly characterized alleles. In seyE4/Y;Starr3C2/+ flies, R7 cells develop in only 16% of the ommatidia (Table 2). Loss of one copy of a positive regulator of the sevenless pathway, such as boss or ras-1, causes no R7

### Table 1(A). e2d fails to complement yan

<table>
<thead>
<tr>
<th>Allele</th>
<th>Df(2L)dp79b</th>
<th>e2d</th>
<th>I</th>
<th>pok-x8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Df(2L)dp79b</td>
<td>Lethal (0/500)</td>
<td>Lethal (0/352)</td>
<td>Semi-lethal, escapers w/very rough eye (12/240)</td>
<td>Semi-lethal, escapers w/very rough eye (19/501)</td>
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<tr>
<td>e2d</td>
<td>Lethal (0/500)</td>
<td>Semi-lethal, escapers w/very rough eye (1/104)</td>
<td>Semi-lethal, escapers w/very rough eye (29/206)</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Semi-lethal, escapers w/mild rough eye (8/71)</td>
<td></td>
<td>Viable, mild rough eyes</td>
<td></td>
</tr>
<tr>
<td>pok-x8</td>
<td></td>
<td></td>
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</table>

### Table 1(B). aop fails to complement yan

<table>
<thead>
<tr>
<th>Allele</th>
<th>aop-1P</th>
<th>aop-1Is</th>
<th>e2d</th>
</tr>
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<tbody>
<tr>
<td>pok-x8</td>
<td>Semi-lethal escapers w/very rough eyes (6/138)</td>
<td>Semi-lethal escapers w/very rough eyes (16/104)</td>
<td>Semi-lethal escapers w/very rough eyes (29/206)</td>
</tr>
<tr>
<td>e2d</td>
<td>Lethal (0/367)</td>
<td>Lethal (0/245)</td>
<td>Lethal (0/500)</td>
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</table>

The numbers shown represent the number of flies of the indicated genotype that were recovered in the cross as well as the total number of flies scored. The Mendelian ratio expected for non-lethal genotypic combinations would be 1/4.
cells to develop in this genetic background (Bonfini et al., 1992), whereas the loss of a negative regulator such as GAP1 causes an increased number of R7 cells to develop (Rogge et al., 1992). When one copy of yan is removed from a sevE4/Y;SosJC2/+ fly, the number of R7 cells that develop significantly increases (Table 2). Quantitatively, the effect is similar for all alleles of yan, including e2d, aop and the deficiency for the region. Thus, in this genetic background, the lethal mutations in yan affect the development of R7 cells with the same severity as the hypomorphic mutations in the yan gene. While many of the earlier arguments regarding the role of yan in R7 development were based on the development of ectopic R7 cells, or on biochemical analyses in cell lines, this assay further establishes a role for yan in the specification of the fate of the cell that develops into R7 from the true R7 precursor.

**Early function of yan during eye development**

To determine the null phenotype of yan in the eye, yan<sup>e2d</sup>/yan<sup>e2d</sup> clones were generated in eyes of flies that were otherwise yan<sup>e2d</sup>+/ (see Materials and Methods for details). Loss of yan function causes a scar in the adult eye that shows no organized, faceted structure. When the borders of the clones were analyzed, no mosaic ommatidia containing yan<sup>e2d</sup>/yan<sup>e2d</sup> cells could be found (Fig. 3). 20 independent scars were analyzed and 125 ommatidia were scored along the boundaries of the scars. Many of these ommatidia lacked the complete complement of R-cells. However all R-cells that developed
contained at least one wild-type copy of yan. This suggests that the function of the yan gene product is autonomously required for the development of a cell as a photoreceptor neuron. This is consistent with the autonomous function of yan, seen in ectopic R7 development, using partial loss-of-function alleles (Lai and Rubin, 1992). This result also demonstrates that complete loss of yan during development does not give rise to an adult retina with extra photoreceptor neurons.

Table 2. Effects of yan alleles in sensitized genetic background for R7 development

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ommatidia containing R7 cells (%)</th>
<th>Total ommatidia counted</th>
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<tr>
<td>sevE4/Y; SosJC2/yan+</td>
<td>16</td>
<td>567</td>
</tr>
<tr>
<td>sevE4/Y; SosJC2/yan-</td>
<td>66</td>
<td>560</td>
</tr>
<tr>
<td>sevE4/Y; SosJC2/yanpok-x8</td>
<td>80</td>
<td>605</td>
</tr>
<tr>
<td>sevE4/Y; SosJC2/yan1</td>
<td>77</td>
<td>563</td>
</tr>
<tr>
<td>sevE4/Y; SosJC2/yanpok-1P</td>
<td>77</td>
<td>577</td>
</tr>
<tr>
<td>sevE4/Y; SosJC2/yanpok-1IS</td>
<td>64</td>
<td>616</td>
</tr>
<tr>
<td>sevE4/Y; SosJC2/yanpok-x2d</td>
<td>81</td>
<td>629</td>
</tr>
<tr>
<td>sevE4/Y; SosJC2/Df(2L)dp79b</td>
<td>66</td>
<td>540</td>
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Individual ommatidia were scored for the presence of R7 by using the optical technique of pseudopupil (Franceschini and Kirshfeld, 1971). The deficiency, Df(2L)dp79b deletes bands 22A2-3 to 22D5-E1 and includes the yan locus. The numbers for different yan alleles are significantly increased over wild type, but are not significantly different from one another.

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Mutant phenotypes seen in adult patches are often terminal effects that result from a cascade of events during development. Thus, cells could develop as neurons and die later, or not develop into neurons at all, in both cases giving rise to a scar in the adult eye clone. To determine the phenotype resulting from loss of yan function during development, we analyzed eye discs that contained yan pok-x8 patches. In initial experiments, we marked the wild-type chromosome with lacZ driven in all cells by the armadillo promoter. This allowed us to visualize the region made homozygous for the mutant chromosome (see Materials and Methods for details). On double staining, we confirmed that the cells that fail to stain with lacZ were the ones that show the phenotypes described below.

In wild-type eye discs, the anti-Elav antibody stains a
incorporate BrdU. Some cells in the clones that were posterior wild-type cells are normally arrested in G1 and would not staining pattern extended anteriorly through the domain where The clones often straddled the morphogenetic furrow and the labeled with BrdU were readily visible in the mutant tissue. rounds of cell division. As a result, large numbers of cells mutant for identified by BrdU staining and two examples of labelled cells (arrowheads in Fig. 4E,F). Over 25 patches were cell divisions can be visualized as a tight band of BrdU- wild-type part of the mosaic eye disc, a synchronous wave of clones as evidenced by their ability to incorporate BrdU. In the tissue led us to analyze the cell division profile in the mutant that is mutant for cell divisions, suggesting that yan function is autonomously required in these cells so that they may develop as neurons. Tissue showing a wild-type staining pattern could be seen extending around the edges of the clone, clusters containing less than the usual component of cells can be identified (Fig. 4A), once again suggesting that yan function is autonomously required in these cells so that they may develop as neurons. The scarring of the eye shown in this mutant clone is representative of the phenotype that was seen in twenty different mosaic animals. No yan- photoreceptors, lacking pigment granules (arrowheads), were seen along the edge of the mutant clone.

Fig. 3. Tangential section through a yan2d/yan2d clone in the adult eye. Bar, 1 µm. The region close to the clone is highlighted for clarity. The scarring of the eye shown in this mutant clone is a regular array of neurons commencing at the morphogenetic furrow and extending posteriorly. Over 50 yan2d/yan2d mutant clones were readily identified by their failure to express this neuronal marker. Wild-type flies that were similarly irradiated never gave rise to these patches (see Materials and Methods). In mosaic animals, the clones range in size from including only a few clusters to virtually half the disc. Along the edges of the clone, clusters containing less than the usual component of cells can be identified (Fig. 4A), once again suggesting that yan function is autonomously required in these cells so that they may develop as neurons. Tissue showing a wild-type staining pattern could be seen extending around the patch when the latter straddled the furrow (Fig. 4B) often enclosing the patch within the differentiating area and restarting a furrow posterior to it (Fig. 4C). Larger patches were always associated with extra folds or clefts (Fig. 4D) indicating the appearance of an abnormal number of cells in the region that is mutant for yan.

The observation that larger patches include extra folds of tissue led us to analyze the cell division profile in the mutant clones as evidenced by their ability to incorporate BrdU. In the wild-type part of the mosaic eye disc, a synchronous wave of cell divisions can be visualized as a tight band of BrdU-labelled cells (arrowheads in Fig. 4E,F). Over 25 patches were identified by BrdU staining and two examples of yan2d/yan2d clones are shown (Fig. 4E,F). The patches of cells that are mutant for yan were found to re-enter the cell cycle for further rounds of cell division. As a result, large numbers of cells labeled with BrdU were readily visible in the mutant tissue. The clones often straddled the morphogenetic furrow and the staining pattern extended anteriorly through the domain where wild-type cells are normally arrested in G1 and would not incorporate BrdU. Some cells in the clones that were posterior to the furrow also continued to divide. This too is never seen in wild-type flies. Clones homozygous for aop-Ip or aop-IIs have the same BrdU and anti-Elav patterns as yan2d (Fig. 4G,H).

To verify that the same cells that incorporate BrdU in the clones are the ones that fail to stain with neuronal markers, discs with mutant patches were double stained with mAb22C10 as well as for BrdU incorporation. Two examples (Fig. 4I-L) show the complementarity of the two staining patterns. The cells in the patch of tissue that lacks the yan gene product fail to differentiate as neurons and instead re-enter the cell cycle. These results suggest that complete loss of yan function interferes with the proper interpretation of signals at the furrow that allow a cell to choose between cell division and cluster formation.

The embryonic phenotype of yan

As mentioned previously, loss of yan function leads to embryonic lethality, probably due to the massive head defects seen in cuticle preparations. The underlying cause of these defects is an overproliferation and loss of normal differentiation of the dorsal head ectoderm, a region that expresses yan at a high level (Lai and Rubin, 1992). The dorsal head ectoderm has a complex fate, giving rise to the visual system (larval eye, eye imaginal disc, optic lobe), medial parts of the brain, as well as part of the epidermis that covers the head of the embryo (Fig. 5A).

In wild-type development, the lateral region of the head gives rise to typical neuroblasts, which delaminate and eventually constitute most of the brain (Younossi-Hartenstein et al., 1995). Cells that remain at the surface become part of the epidermis. Differentiation of the dorsal neurectoderm begins later, during mid-embryogenesis, after neuroblasts forming the brain have delaminated and initiated proliferation. Cells at a dorsoposterior position invaginate to form the optic lobe primordium (Green et al., 1993). A small cluster of cells adjacent to the region of optic lobe invagination remain in the head ectoderm and differentiate into the larval eye (Bolwig’s organ). More anteriorly, cells of the dorsal neurectoderm move inside the embryo in several clusters and become incorporated into the brain hemispheres. During late embryogenesis, the cells that have remained at the surface fold inside as the process of head involution begins, giving rise to the dorsal pouch, as well as the eye imaginal disc.

In yan mutants, normal differentiation of the dorsal head ectoderm is blocked. This phenotype is exhibited by all the lethal alleles (Fig. 5C-D). Anteriorly, the neural precursors fail to separate from the epidermis and cells remain in a disorganized multilayered epithelium. These cells continue to proliferate throughout late embryogenesis (Fig. 5F). Head involution fails and, hence, the dorsal pouch and eye imaginal discs do not form. The Bolwig’s organ is either strongly reduced or absent altogether. FasII staining suggests that the optic lobe primordium does form, although the normal infolding is disrupted (Fig. 5H).

Another head structure that shows severe abnormalities in all yan lethal alleles is the stomatogastric nervous system (SNS). The SNS consists of a series of ganglia that flank the esophagus (Hartenstein et al., 1994). In wild-type embryo, these cells develop from three primordia invaginating from the roof of the stomodeum. In yan mutants, a higher number of SNS precursors appear, and it seems that these cells undergo an additional round of mitosis, leading to a final number of
Fig. 4. Phenotype of \textit{yan\textsuperscript{-}/yan\textsuperscript{-}} clones in the developing eye disc. For all discs, posterior is to the left. (A-D) Development of neurons marked with anti-Elav antibody in mosaic discs. Each panel shows an independently generated \textit{yan\textsuperscript{e2d}/yan\textsuperscript{e2d}} clone. The regions homozygous for the mutation do not stain with this neuronal marker. The morphogenetic furrow is marked in A with an arrow. Ommatidia with less than the normal component of R cells (arrowheads in A and C) and folds of tissue developing within the patch (open arrow in D) can be seen. (E,F) Two examples of \textit{yan\textsuperscript{e2d}/yan\textsuperscript{e2d}} patches in the eye stained for incorporation of BrdU. A thin band of BrdU incorporation (arrow), immediately posterior to the furrow, is seen in the wild-type region of the mosaic disc. The mutant patches (arrowheads) show ectopic incorporation of BrdU. (G,H) Double staining with anti-Elav antibody (brown) and BrdU incorporation (black). (G) An example of a \textit{yan\textsuperscript{aop-}}\textit{b/yan\textsuperscript{aop-}}\textit{b} mutant clone that extends over the lower half of the disc is shown. Due to the extensive folding of tissue associated with large clones of this nature, only a small fraction of the BrdU staining is visible at this one plane of focus. (H) An example of a \textit{yan\textsuperscript{aop-}}\textit{b/yan\textsuperscript{aop-}}\textit{b} clone. (I-L) Double staining of discs containing \textit{yan\textsuperscript{e2d}/yan\textsuperscript{e2d}} clones with the neuronal marker mAb22C10 (I and K, fluorescent secondary antibody) and for BrdU incorporation (J, L, biotin coupled secondary antibody). mAb 22C10 is a cytoplasmic neuronal marker that is easier to visualize than anti-Elav in discs double stained for BrdU incorporation. Two double stained discs are shown; the first in I and J and the second in K and L. The mutant areas of the tissue (arrowheads) show increased incorporation of BrdU, and whenever posterior to the furrow, are the precise regions that do not stain with the neuronal marker. Bars: (A-C), 7.8 \textmu m; (D-F and I-L), 50 \textmu m; (G-H), 29 \textmu m.
SNS neurons that is increased over wild type by a factor of 3-4. Despite their strongly increased number, SNS precursors seemed to differentiate into neurons, as evidenced by the markers 22C10 and FasII (Fig. 5C,D,H).

**yan interacts with Notch**

Two different classes of interactions, instructive and permissive, are important for a cell to receive signals. Examples of instructive interactions include those initiated by RTKs such as Sevenless and Egfr, while permissive interactions are represented by members of the *Notch* pathway. A cell is not competent to receive an instructive signal, such as the one initiated by Sevenless, if the permissive *Notch* pathway is disrupted (Cagan and Ready, 1989). The role of *yan* in the Sevenless pathway has been widely described (Lai and Rubin, 1992; Brunner et al., 1994; Lai and Rubin, 1992; R. Rogge and others
Rebay and Rubin, 1995), and its interactions with Egfr were demonstrated in this study. In addition, we found that Notch interacts with yan mutations. Since the strongest alleles of yan are lethal and yan has no dominant component to its phenotypes, we used weaker alleles of yan in this study. When yanP or yanpok-x8 are combined with a duplication of the Notch locus, Dp(1;2)w51b7 (called Dp(N+) for simplicity), the eye phenotype is synergistically enhanced giving a very rough eye (Fig. 6). Essentially, in combination with extra copies of Notch, the weak hypomorphic yanP/yanP combination now resembles the stronger allelic combination, yanpok-x8/yanpok-x8. The fraction of ommatidia with ectopic R7 cells is increased from 19.8% (n=444) in yanP/yanP to 91.4% (n=478) in yanP, Dp(N+)/yanP, Dp(N+), similar to that seen in yanpok-x8/yanpok-x8. Similarly, the phenotype of yanpok-x8/yanpok-x8 is also enhanced in combination with Dp(N+)/Dp(N+) to a level resembling that of yanpok-x8/Df(2L)dp79b (compare Fig. 6G with K and Fig. 6K with 2C). The yanpok-x8/yanpok-x8 genotype in this combination resembles that of yanpok-x8/yanpok-x8/Df(2L)dp79b, a phenotype that is further enhanced over yanpok-x8/yanpok-x8 and resembles that of yanpok-x8/yanpok-x8/Df(2L)dp79b.

DISCUSSION

Loss of yan function causes embryonic lethality

The role of yan in the regulation of the sevenless pathway has been well established. It seems clear that MAPK negatively regulates yan by phosphorylating it on one or more critical sites (Rebay and Rubin, 1995). This causes yan to be transported out of the nucleus and degraded, allowing R7-specific transcription to take place in the cell that has received the signal. This is also true of the development of other cell types. The loss-of-function alleles of yan used in the previous studies have
been hypomorphic, thus separating out the function in R7 development from all other functions of yan. In this paper, we have shown that a more severe loss of yan function results in embryonic lethality. This was also the unpublished observation of Rebay and Rubin (1995). We have further analyzed the yan locus genetically to establish that lethal yan alleles are in fact allelic to the previously characterized aop mutations. Moreover, phenotypic analysis has shown that yan<sup>aop</sup>-<i>lr</i>, yan<sup>2d</sup> and yan<sup>aop</sup>-<i>dis</i> behave in a manner similar to a deficiency for the region, suggesting strongly that they result from complete loss of function in the embryo and in the eye.

**Context dependence of yan function**

The differences in phenotypes between the lethal alleles and the previously described hypomorphic alleles appear in clones of mutant tissue generated in the eyes of mosaic animals. Given the role of yan as a negative regulator of photoreceptor development in the sevenless pathway, it was surprising to find that yan<sup>−</sup>/yan<sup>−</sup> cells fail to differentiate as neurons and instead enter into S-phase to reinitiate the cell division cycle. It is possible to reconcile the obvious dichotomy between the development of extra R cells in hypomorphic alleles, with the observation that R cells fail to differentiate in the null alleles, by recognizing that the interpretation of a tyrosine kinase-derived signal is dependent entirely on the predisposition of the cell by which it is received. Thus the Ras-derived signal in the R7 precursor is interpreted as a differentiation signal, whereas for the cells at or anterior to the furrow such a signal could be mitogenic and cause a cell to enter the cell cycle. In this model, the function of yan is context dependent, either mediating the transition between an undifferentiated and a differentiated cell fate (for R7), or a choice between differentiation and cell division (at the furrow). We propose that the Yan protein not only represses the genes that are needed to differentiate as R7 but also, at the furrow, the genes that are responsible for entering the cell cycle.

The Egfr gene product is a likely candidate for initiating a signal that regulates cell division at the morphogenetic furrow. Baker and Rubin (1992) have previously shown that in the Egfr gain-of-function mutation, Egfr<sup>E</sup>, one can find increased incorporation of BrdU in the cells posterior to the morphogenetic furrow. Furthermore, Egfr<sup>E</sup> clones are smaller in size than their wild-type twin spots. This is not due to cell death, but underproliferation of cells within the clones (Xu and Rubin, 1993). The simplest interpretation of our results is that the patches of yan<sup>−</sup>/yan<sup>−</sup> cells in the eye disc represent an extreme example of the Egfr<sup>E</sup> phenotype and mimic gross activation of the Egfr signal. The strong genetic interaction observed between Egfr and yan is consistent with this model. Thus it seems likely that, in wild-type development, an Egfr signal at the furrow leads to inactivation of Yan, releasing cells from G1 arrest and causing them to enter a second round of division. In the absence of Yan, entry into cell cycle would become signal independent. In hypomorphic alleles, this initial function is intact, probably due to residual activity of the protein. In null allele patches, the earlier phenotype obscures the later function in R7 development. However, the R7 developmental role of Yan can also be extracted from the null allele phenotypes by placing these alleles either in sensitized genetic backgrounds, or in heteroallelic combinations with the weaker alleles.

The fact that the R7 precursor interprets the down-regulation of Yan in the context of differentiation and not cell division is not surprising. Many examples are known where two different Ras signals could cause very different effects within the same cell. For example, PC12 cells divide in response to EGF and differentiate into neurons in response to NGF even though the same Ras pathway is involved, albeit with a different profile of MAPK activation (Traverse et al., 1992; Yan et al., 1991). Also, two cells with distinct developmental programs often interpret identical Ras signals quite differently. In the eye disc, it has been demonstrated previously, that the specific fate that a cell will adopt in response to the triggering effect of the Ras signal depends upon downstream transcription factors that are prepatterned into the cell (Basler et al., 1990). Our results indicate that the predisposition of cells at or anterior to the furrow is such that yan has a role in regulating the cell division of these cells. In this context, it is interesting to note that a link between Ras pathways and cell division has also been suggested from genetic studies by Thomas et al. (1994), who have isolated Ras alleles as modifiers of roughex, a regulator of cell division at the morphogenetic furrow.

**Role of Notch in yan signalling**

In addition to interacting with two different RTK pathways, we found that yan mutants also interact with Notch. Specifically, loss of Notch partially suppresses yan and extra Notch enhances its mutant phenotype. These genetic interactions suggest one of the following two scenarios: either yan acts downstream of Notch and the Notch pathway has elements in common with RTK pathways; or that the Notch pathway allows the proper interpretation of RTK-derived signals. Genetic analysis cannot distinguish between these two possibilities. The precedence for either direct or indirect involvement of Notch in RTK pathways linked to photoreceptor development is quite strong. For example, in a Notch<sup>−</sup> background, an R7 cell does not develop, even when the Sevenless signalling system is intact (Cagan and Ready, 1989).

**Loss of function versus activated alleles of yan**

The observed phenotypes of the yan null mutations in the eye...
can be easily reconciled with the observed results for the phenotype of activated yan (Rebay and Rubin, 1995). When this version of yan is expressed ubiquitously in the eye disc, the cells apoptose at the morphogenetic furrow. This is a phenotype opposite to that observed by us for the null alleles. A cell expressing excessive amounts of activated versions of yan will essentially be insulated from all tyrosine kinase-derived signals, those triggering it towards division, as well as those that initiate differentiation. Such a cell has no fate to follow and it consequently chooses to apoptose. It is known that cells in the eye disc that fail to join the cluster as neuronal or nonneuronal cells are ultimately fated to apoptose (Wolff and Ready, 1991b). It is likely that a cell expressing activated yan follows that same fate at an earlier stage. The terminal phenotype of adult patches resulting from loss of yan function is a scar lacking differentiated cells, presumably due to the later apoptosis of the cells dividing inappropriately within the yan+/yan- patch. While the terminal phenotype may be similar, the developmental phenotype of the activated mutation in yan and that of null alleles of yan are quite the opposite of each other.

**Similarity of yan function in the eye and the embryo**

When comparing the function of yan in adult eye development and embryonic development, several strong parallels become apparent. In the embryo, Yan does not participate in all developmental pathways that involve Egfr. For example, yan mutants do not appear to have any phenotypes that would suggest a role in the determination of the dorsoventral axis of the embryo. Instead, Yan function is required in a relatively small ectodermal territory of the head, which includes the anlagen of the larval and adult eye, as well as other parts of the nervous system. Here, as in the eye disc, yan functions to suppress proliferation. Thus, the most dramatic effect of loss of yan function is the failure of the dorsal head ectoderm to split up into neural and epidermal precursors. Instead, cells in yan mutant embryos remain an irregular surface layer which continue to proliferate, although there is at least some degree of differentiation of these cells as neurons. Furthermore, as proposed for the eye disc, yan function in the embryo may also be mediated by the EGF signalling pathway. Thus, loss of function of yan is able to ameliorate somewhat the cuticle phenotype resulting from Egfrf1 mutations. The head defects of Egfrf1 mutant embryos have not been thoroughly investigated; however, our results indicate (V. H., unpublished) wide-spread cell death in this mutant. It is likely that reduced proliferation levels contribute to these defects. Thus, in Egfrf1/Egfrf1 embryos, the brain hemispheres are reduced in size and exposed to the surface due to the early death of head epidermal precursors and the SNS is entirely absent. These phenotypes are the opposite of the overproliferation seen for these tissues in lethal yan mutations.

In summary, the wild-type function of yan is dependent upon the developmental context of a given cell. In general, the Yan protein seems to be involved in keeping cells quiescent until they receive either differentiation or cell-division signals. The response of any given cell to mutations in the yan gene will depend upon many different factors. Not all cells receiving RTK signals phenotypically respond to a loss of yan since the Yan expression pattern is tightly controlled. For example, once cells are committed to a differentiated fate in the eye disc, the expression of Yan is immediately terminated (Lai and Rubin, 1992). Cells that do normally express Yan will respond to a loss of yan differentially, depending upon which downstream effectors they express. Pointed is unlikely to be the only activator that Yan functions against since the expression patterns of Yan and Pointed are overlapping, but not identical (Lai and Rubin, 1992; Klaes et al., 1994). Finally, the response to loss of yan may depend upon which incoming RTK signal a particular cell is receiving. In this context, it is interesting to note that ELK-1, the mammalian counterpart of yan, has been proposed to function by integrating different MAPK signal pathways, balancing the cell’s response to disparate extracellular signals (Whitmarsh et al., 1995).

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