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Identifying targets of the *rough* homeobox gene of *Drosophila*: evidence that *rhomboid* functions in eye development

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Summary

In order to identify potential target genes of the *rough* homeodomain protein, which is known to specify some aspects of the R2/R5 photoreceptor subtype in the *Drosophila* eye, we have carried out a search for enhancer trap lines whose expression is *rough*-dependent. We crossed 101 enhancer traps that are expressed in the developing eye into a *rough* mutant background, and have identified seven lines that have altered expression patterns. One of these putative *rough* target genes is *rhomboid*, a gene known to be required for dorsoventral patterning and development of some of the nervous system in the embryo. We have examined the role of *rhomboid* in eye development and find that, while mutant clones have only a subtle phenotype, ectopic expression of the gene causes the non-neuronal mystery cells to be transformed into photoreceptors. We propose that *rhomboid* is a part of a partially redundant network of genes that specify photoreceptor cell fate.

Key words: *Drosophila*, homeobox, *rough*, *rhomboid*, enhancer trap, retina, photoreceptor.

Introduction

Genes that contain the homeobox sequence (McGinnis et al., 1984; Scott and Weiner, 1984) control a range of cell fates in all eukaryotes examined. They appear to act as transcriptional regulators that are responsible for establishing a pattern of gene expression appropriate for a given cell type or developmental region (for recent reviews see Hayashi and Scott, 1990; Affolter et al., 1990; Andrew and Scott, 1992). In order to understand more fully how homeobox genes so profoundly affect the fate of a cell during development, it will be necessary to identify the main components of the pathway downstream of these genes. The search for downstream genes has been carried out by a variety of strategies. These include analysing the effect of homeobox gene mutations on the expression of candidate target genes (for example, see Hafen et al., 1984; Struhl and White, 1985; Bienz and Tremml, 1988; Winslow et al., 1989; Reuter et al., 1990) and screening for genetic modifiers of homeobox gene mutations (Kennison and Russell, 1987; Kennison and Tamkun, 1988). The approach that we have taken in searching for genes downstream of *rough* relies on enhancer traps, and combines some of the advantages of both these previous strategies. A similar approach was recently used to identify potential targets of the *Antenna-pedia* gene (Wagner-Bernholz et al., 1991).

The approximately 750 individual ommatidia that form the compound eye differentiate in a monolayer epithelium called the eye imaginal disc. Each ommatidium has eight photoreceptors, and the differentiation of these photoreceptors occurs in a precise sequence: first R8 is determined, then R2 and R5, R3 and R4, R1 and R6, and finally R7. It is believed that this stereotypical development is regulated by a series of inductive signals: as each new cell or pair of cells differentiates, it induces its neighbours to adopt a specific fate (for reviews see Tomlinson, 1988; Ready, 1989). The product of the *rough* gene is known to be necessary for the specification of R2 and R5 identity in the developing imaginal disc (Tomlinson et al., 1988; Basler et al., 1990; Kimmel et al., 1990). In *rough* mutants, the presumptive R2/R5 cells lose some aspects of their normal identity, and appear to be partially transformed into other photoreceptor subtypes (Heberlein et al., 1991; Van Vactor et al., 1991).

In an effort to understand how the activity of *rough* in the presumptive cells R2 and R5 leads to their acquiring that specific fate, we have compared the expression of 101 enhancer trap lines in wild-type and *rough*$^{-}$ flies. Enhancer trap lines result from the chromosomal insertion of an *E. coli lacZ* gene next to a transcriptional enhancer, thereby generating a pattern of β-galactosidase expression that reflects the pattern of expression of the gene at the site of insertion (O'Kane and Gehring, 1987; see Freeman, 1991, for recent review). We chose to examine enhancer trap lines

that had been previously shown to be expressed early in the developing photoreceptors, and sceened for lines with *rough*-dependent expression in the presumptive R2 and R5 cells. This approach has allowed us to identify several loci that appear to be downstream of *rough* in R2 and R5. It is important to stress that these experiments alone cannot distinguish between genes that are directly transcriptionally controlled by *rough*, and those that are more indirectly dependent on *rough* function for their expression.

One of the enhancer traps that we have thus identified as being downstream of *rough* is in the previously identified gene, *rhomboid*. *rhomboid* is known to have a function in several stages of embryogenesis, including the formation of some ventral structures and parts of the nervous system (Mayer and Nüsslein-Volhard, 1988; Bier et al., 1990). Until now there has been no evidence for *rhomboid* functioning in eye development. We have investigated the role of *rhomboid* in photoreceptor differentiation, and our results suggest that it is part of the process that causes cells to adopt a photoreceptor fate, although this function appears to be largely redundant.

Materials and methods

Fly strains and genetics

The enhancer trap lines that were crossed into a *rough*$^{-}$ background were isolated in a screen for genes expressed posterior to the morphogenetic furrow (M. F., U. Gaul, J. S. Heilig, L. S. Higgins, and G. M. R., unpublished data). We followed the procedure described by Mlodzik and Hiromi (1991), but used the plwb element, which contains the *white* gene as a dominant marker (kindly provided by U. Grossniklaus and W. Gehring). The enhancer trap lines used to examine the identity of the extra photoreceptors were also isolated in this screen. Enhancer trap lines were crossed into a ro^{x63} background by standard techniques; ro^{x63} is a null allele of *rough* (Heberlein et al., 1991). Care was taken to generate fly stocks which were homozygous for individual enhancer trap lines and ro^{x63}, thus eliminating the two-fold differences in the level of staining that are observed between heterozygous and homozygous individuals. Enhancer traps that were homozygous lethal were screened as heterozygotes.

rhomboid alleles used in this study include rho^{7M43} (Mayer and Nüsslein-Volhard, 1988), kindly provided by C. Nüsslein-Volhard; rho^{del1} (Bier et al., 1990), kindly provided by E. Bier; and the three excision alleles described in the text and shown in Fig. 2A, $rho^{P\Delta5}$, $rho^{P\Delta16}$ and $rho^{P\Delta38}$. These excision alleles were made by crossing X81 flies to flies carrying a stable source of P transposase (Robertson et al., 1988), and selecting individuals that had lost the P element, as detected by loss of the $white^{+}$ marker. Lethal lines thus generated were tested for complementation with rho^{7M43}, and all new *rhomboid* alleles were analysed by genomic DNA blotting (see legend to Fig. 2).

Mitotic clones were induced as described by Tomlinson et al. (1988). The dominant marker that we used to detect clones of *rhomboid* tissue in the adult eye was the P[w]33 element at cytogenetic position 70C (our unpublished data). Adult *Drosophila* heads were fixed and sectioned as described by Freeman et al. (1992).

Molecular techniques

All DNA manipulation and analysis were done according to the protocols of Sambrook et al. (1989). The *sevenless-rhomboid* ectopic expression construct was made in several steps. First, a *rhomboid* fragment was amplified using a two-step PCR protocol (see Higuchi, 1989, for a description of this procedure). The primers were designed so that a novel *Xba*I site was added 16 nucleotides 5′ of the initiating AUG codon, and the *rhomboid* fragment extended as far as the *Hin*dIII site 3′ of the transcription unit, as shown in Fig. 2A. The *rhomboid* coding region was sequenced in order to ensure that no PCR errors were incorporated into the ectopic expression constructs. This *Xba*I to *Hin*dIII *rhomboid* cassette was then cloned downstream of a fragment containing the *sevenless* promoter (−967 bp to +89 bp, Bowtell et al., 1988), giving a transcriptional fusion. This promoter/transcript fragment was then cloned into a plasmid that contained *sevenless* enhancer sequences (+90 bp to +9.3 kb; see Bowtell et al., 1989) inserted into pDM30, a P-element transformation vector (Mismer and Rubin, 1987). We also made a variant of this construct, in which most of the 3′ untranslated region of the *rhomboid* gene had been deleted. We found no difference between transformants with the two constructs, and have treated them as equivalent when producing flies with multiple sev-rho copies.

The heat shock-*rhomboid* construct was made by cloning the *Xba*I to *Hin*dIII *rhomboid* cassette described above downstream of a 306 nucleotide fragment of the *hsp70* gene (corresponding to coordinates −189 to −495 in Fig. 4 of Ingolia et al., 1980), which includes the heat-shock element and the TATA box. This HS-rho fusion was then cloned into the transformation vector pw8 (Klemenz et al., 1987). P-element transformation (Spradling and Rubin, 1982) with both sev-rho and HS-rho was done by standard procedures. Several transformants were isolated for each construct; flies with multiple copies of sev-rho were made by recombining different insertions onto the same chromosome, and by crossing insertions on different chromosomes into the same fly.

Activity and immunostaining of imaginal discs and embryos

The expression of enhancer traps in imaginal discs and embryos was assayed by an X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) activity stain for β-galactosidase (Bellen et al., 1989; Wagner-Bernholz et al., 1991). Eye imaginal discs were immunostained as described previously (Freeman et al., 1992), except for the disc shown in Fig. 3D which was fixed in 4% paraformaldehyde in the presence of 1% NP-40; all subsequent stages were done by standard methods. Embyros were fixed and immunostained as described by Patel et al. (1987). The anti-neuroglian is a monoclonal antibody (Hortsch et al., 1990), and the supernatant was used at a dilution of 1:5. mAbro1 is a monoclonal antibody raised against the *rough* protein (Kimmel et al., 1990), and was used at a dilution of 1:10,000. Anti-β-galactosidase was bought from Promega, and was used at 1:500; Biorad horseradish peroxidase-coupled goat anti-mouse IgG was used at 1:300.

Embryonic cuticle preparations

Cuticle preparations were done according to the protocol of Wieschaus and Nüsslein-Volhard (1986). Embryos were removed from their vitelline membranes prior to fixation and were mounted in 1:1 Hoyer's mountant:lactic acid.

Producing an antibody against rhomboid

Using standard PCR techniques, a 458 bp DNA fragment encoding the N-terminal 153 amino acids of the *rhomboid* protein was generated and ligated into the pATH11 trpE fusion protein vector (Koerner et al., 1991). The resulting plasmid produced a trpE-rhomboid fusion protein of approximately $60\times10^{3}M_{r}$ which was used as an immunogen for monoclonal antibody production by standard methods (Harlow and Lane, 1988). Two anti-rhomboid monoclonal antibodies were isolated and called mAbrho1 and mAbrho2.

Results

Using enhancer traps to search for genes downstream of rough

We have looked for genes that might lie downstream of *rough* by examining the effect of its absence on the expression pattern of a collection of enhancer traps. The *rough* gene encodes a homeodomain protein that controls some of those aspects of R2 and R5 development that provide them with an identity different from the other six photoreceptor cells. *Rough* protein is expressed in cells R2, R5, R3 and R4, but the *rough* gene is only required in cells R2 and R5 for normal eye development (Tomlinson et al., 1988; Kimmel et al., 1990). When comparing the expression of the enhancer traps in wild-type and *rough*$^-$ larvae, we screened for the most obvious differences: for example, greatly reduced expression in R2 and R5. More subtle reductions or enhancements of expression may have been missed.

As listed in Table 1, only seven of the 101 enhancer traps tested have a significantly altered expression pattern in the absence of *rough* function. Of these, four (A135, AE33, P7, X81) are lines that are normally expressed in R2 and R5, and are therefore candidates for *rough*-dependent genes that might play a part in the specification of the R2/R5 subtype. The other three lines (A65, O8, S1) show alterations in cells other than R2 and R5, and these are most easily explained as reflecting aspects of the *rough* phenotype. Interestingly, three of the lines that might be downstream of *rough* in cells R2 and R5 (AE33, P7, X81) are normally expressed predominantly in photoreceptor precursors R8, R2 and R5: expression is first seen in cell R8 near the morphogenetic furrow, and cells R2 and R5 begin to express the marker a few rows more posteriorly - at about the position where they start to express neural antigens. All three of these enhancer traps have reduced β-galactosidase expression in R2 and R5 in the *rough* mutant eye disc. The best examples of this are AE33 and X81, whose expression patterns are shown in Fig. 1. In total, 3 of the 8 enhancer trap lines that are expressed in cells R8, R2 and R5, are affected by loss of *rough.*

Effects in cells other than R2 and R5

The effects of removing wild-type *rough* function in cells other than R2 and R5 are likely to reflect indirect consequences of the loss of *rough*, due to the abnormal development of *rough*$^-$ discs. Recently, it has been demonstrated (Heberlein et al., 1991; Van Vactor et al., 1991) that additional cells expressing R8-specific markers differentiate in many of the ommatidia of an eye disc missing *rough* function, and this effect can be seen with some of the enhancer traps that we have examined. For example, in *rough*$^-$ discs

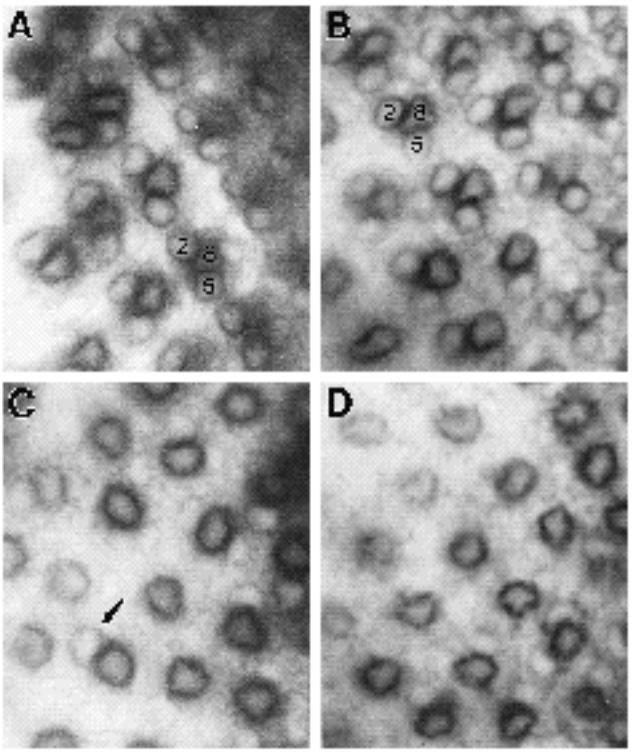

Fig. 1. The effect of *rough* on enhancer trap expression. Groups of ommatidia from third instar eye imaginal discs are shown, stained for the expression of the β-galactosidase reporter gene by X-gal activity stain. (A and C) The enhancer trap line X81 is dependent on *rough* function in cells R2 and R5: compare the expression of the reporter gene in wild-type (A) and *rough*$^-$ (C) discs. Instead of the three cell groups (R8, R2, R5) stained in wild-type discs, only one, or occasionally two cells, per cluster are stained in the *rough*$^-$ disc. The arrow in C points to an example of a pair of R8-like cells staining (see text for explanation). Although this phenomenon is seen in 30-40% of ommatidia, it is less frequent towards the dorsal and ventral edges of the disc than closer to the equator. The panels show regions away from the equator - hence the apparent rarity of the R8 pairs. Staining in the presumptive cells R2 and R5 is abolished. The X81 enhancer trap also directs β-galactosidase expression in photoreceptors in the pupal retina. As in the larval disc, R2, R5 and R8 stain most strongly in 40 hour X81 pupal retinas stained with X-gal (data not shown). (B and D) The enhancer trap line AE33 is also normally expressed in cells R8, R2 and R5 (B), and in a *rough*$^-$ disc shows the same effect as X81: staining is abolished in cells R2 and R5, but remains in R8 (D).

Table 1. *Seven enhancer trap lines whose expression pattern is altered in the absence of* rough *function*

Enhancer trap #	Chromosome position	*rough*$^+$ expression pattern	*rough*$^-$ expression pattern
A65	32B1-2	R8 strong; R1-R7 weak	25% of ommatidia have 2 strongly staining cells.
A135	40A1-4	R1-R8; Fades at disc posterior	Reduced expression and fades sooner.
AE33	51C5-6	R8, R2 and R5	R2 and R5 expression reduced.
O8	32F1-2	R3, R4, R7 and cone cells	R3 and R4 expression altered
P7	90D4-5	R8, R2, R5 strong; R1, R3, R4, R6, R7 weak	R2 and R5 expression reduced.
S1	42F1-2	R3, R4 strong; R1, R2, R5-R8 weak	Early R3 and R4 expression missing?
X81	62A1-2	R8, R2, R5 strong; R1, R3, R4, R6, R7 weak	R2 and R5 expression is reduced or absent.

expressing the R8/R2/R5-specific enhancer trap lines AE33, P7, and X81, it is quite clear that the β-galactosidase expression in the R2 and R5 precursor cells is reduced. In these lines a single R8 cell is seen near the furrow, but further posterior in the disc, approximately 40% of the ommatidial clusters have two cell nuclei which stain strongly (Fig. 1C). Furthermore, the line A65 normally expresses β-galactosidase most strongly in the R8 precursor cell and more weakly in the other cells, while in a *rough*⁻ disc many doublets of cell nuclei staining with the intensity of the R8 precursor cell are observed.

All of the enhancer traps that express β-galactosidase in the R3 and R4 photoreceptor reflect the developmental defects that occur in the R3 and R4 photoreceptors in a *rough*⁻ eye disc. However, the O8 and S1 enhancer traps show additional defects with respect to the development of the R3 and R4 photoreceptors. Thus in S1, the early β-galactosidase expression in R3 and R4 appears to be completely absent in a *rough*⁻ background. In a *rough*⁻ disc, the O8 enhancer trap directs expression of β-galactosidase in pairs of nuclei near the morphogenetic furrow which resemble R3 and R4 precursors; further posterior only one of these nuclei remains apical, and the other appears to sink basally. These basal nuclei have an odd cylindrical morphology which is never observed for wild-type β-galactosidase-expressing nuclei, and they may represent cells that are undergoing cell death. On rare occasions, O8 is also expressed in more than two cells per ommatidium in a *rough*⁻ disc.

The X81 enhancer trap line is an insertion into the rhomboid *locus*

The X81 enhancer trap insertion was localised by in situ hybridisation to cytological position 62A1,2; this is the same location as the previously characterised gene, *rhomboid*, which functions in embryonic development (Bier et al., 1990). We isolated genomic DNA flanking the X81 insertion point, and mapped the P element insertion to between 100 and 150 nucleotides 5′ of the predicted transcriptional start site of *rhomboid* (see Fig. 2A). Flies homozygous for the X81 insertion are wild-type.

The embryonic pattern of β-galactosidase expression in the enhancer trap line X81 (Fig. 2B-2D) is similar in almost all detail to the *rhomboid* RNA expression described by Bier et al. (1990). The only difference is that we have seen no evidence of a longitudinal dorsal stripe of expression in the blastoderm. Outside the embryo, a major difference in the expression pattern of X81 and the rho^{lac1} enhancer trap studied by Bier et al. (1990) is that X81 is expressed in the eye imaginal disc, as described above, while rho^{lac1} is not. (Another enhancer trap in *rhomboid* is also expressed in the eye disc; U. Gaul and G. M. R., unpublished observations.) A possible reason for this difference is that, although the X81 and rho^{lac1} elements are only about 100 nucleotides apart, they are in different transcriptional orientations. The two enhancer traps might therefore detect significantly different components of the complete *rhomboid* expression pattern.

Loss of function rhomboid *mutations*

Upon mobilisation, P-elements frequently excise imprecisely, causing the formation of small deletions of genomic DNA around their insertion point (Daniels et al., 1985). We took advantage of this phenomenon to generate new *rhomboid* mutations. We screened 897 lines of flies from which the *white* gene marker carried by the P-element had been lost, and from these we isolated 40 lethal lines, all of which failed to complement the rho^{7M43} mutation (Mayer and Nüsslein-Volhard, 1988). These 40 new *rhomboid* alleles were then screened by genomic DNA blotting to identify deletions that extended as far as the translational initiation site. Two such excision lines were found, $rho^{P\Delta 5}$ and $rho^{P\Delta 38}$; a third line, $rho^{P\Delta 16}$, is also very likely to remove the translational start site (see legend to Fig. 2). Since these alleles are deletions that extend from at or near the point of insertion of the X81 enhancer trap element, through the whole 5′ end of the *rhomboid* transcript and into the coding region of the gene, they almost certainly represent complete loss-of-function mutations of *rhomboid*. Previously existing *rhomboid* mutations have not been sufficiently characterised to know whether they result in a complete loss of function. Embryonic cuticle preparations of each of these new *rhomboid* mutations look identical, and they appear not to be significantly different from those described for the rho^{7M43} mutation (Mayer and Nüsslein-Volhard, 1988; see Fig. 2E and 2F).

rhomboid appears to act downstream of *rough* in the developing eye; however, we have been unable to detect any evidence of a genetic interaction between mutations in these two genes. In particular, a decrease in the gene dosage of *rhomboid* has no apparent effect on the phenotype of a weak *rough* allele (data not shown).

The distribution of the rhomboid *protein*

We have raised monoclonal antibodies against a fusion protein containing the N-terminal 153 amino acids of the *rhomboid* protein and used these to investigate the expression of the *rhomboid* protein (Fig. 3). Although the staining that we observe is rather weak, the *rhomboid* protein expression that we observed in embryos (data not shown) corresponds closely to *rhomboid* RNA expression, as described by Bier et al. (1990).

The sequence of the *rhomboid* protein indicates that it has several membrane spanning domains, and this leads to the prediction that it is a membrane-associated protein (Bier et al., 1990). Our data show that the *rhomboid* protein in embryos appears to be predominantly localised in vesicles (Fig. 3A). The nature of these vesicles is not known, but they resemble those in which the *Drosophila sevenless*, *boss*, *scabrous* and *wingless* proteins are found (Tomlinson et al., 1987; Krämer et al., 1991; Baker et al., 1990; Van den Heuvel et al., 1989). Sevenless is a transmembrane receptor protein, and boss is its membrane-associated ligand; these vesicles in the R7 precursor cell have been shown to contain internalized ligand/receptor complexes (Krämer et al., 1991). Scabrous and wingless are both secreted proteins.

Rhomboid protein is also detected in the third instar eye imaginal disc, where it is limited to the developing ommatidia (Fig. 3B). This confirms that the X81 enhancer trap expression does reflect real expression of *rhomboid* in the

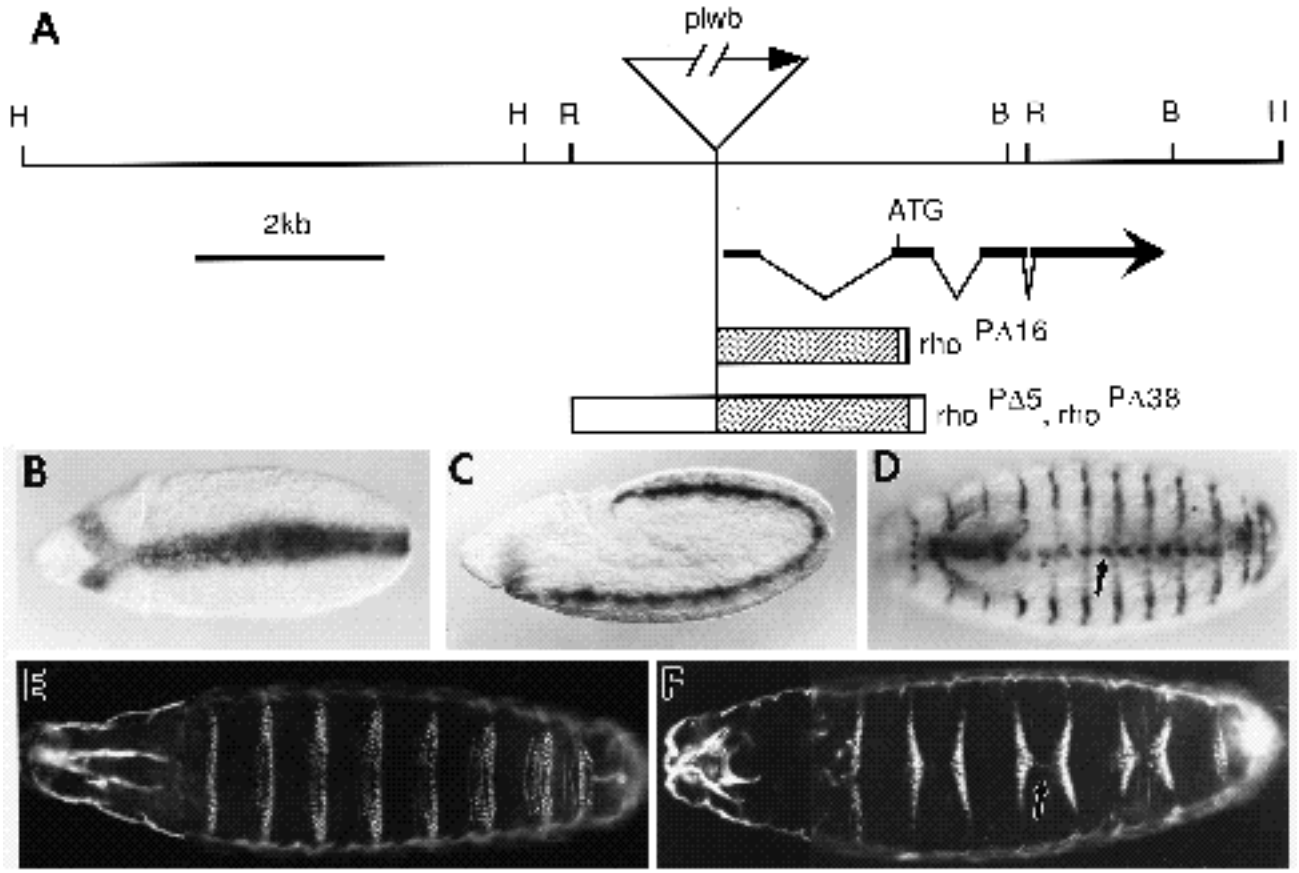

Fig. 2. (A) Map of the region around *rhomboid*. The X81 enhancer trap element is inserted between 100 and 150 nucleotides 5′ of the presumed transcriptional start site of the *rhomboid* gene (Bier et al., 1990), and they are in the same transcriptional orientation. Sequencing of the genomic DNA encompassing the gene indicated that there is one more small intron in the coding sequence than has been previously reported. This intron is between nucleotides 1114 and 1115 in the coordinates used by Bier et al. (1990), and is approximately 140 nucleotides long; it contains an *Eco*RI site. The extent of small deletions caused by the imprecise excision of the enhancer trap element is shown: the hatched bars represent sequences that are missing in the mutations indicated, and the open bars indicate the uncertainty in mapping the ends of the deletions. $rho^{P\Delta 16}$ leaves the left end of the element intact, and deletes to between an *Xmn*I site 17 nucleotides 5′ of the presumed initiating ATG codon, and an *Sph*I site 88 nucleotides 3′ of the ATG; it is therefore likely, but not certain, that $rho^{P\Delta 16}$ deletes the beginning of the protein. $rho^{P\Delta 5}$ and $rho^{P\Delta 38}$ both delete the whole P element, and may delete DNA as far as the *Eco*RI site to the left of the insert; they both delete as far to the right as between the *Sph*I site 88 nucleotides 3′ of the ATG, and an *Eco*RV site 267 nucleotides 3′ of the ATG: both these mutations therefore remove the N terminus of the protein, as well as the entire 5′ end of the transcript. (B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI.). (B-D) X-gal staining to indicate the expression pattern of the X81 enhancer trap in the embryo (anterior to the left). (B - ventral view). In the gastrulating embryo, the expression is limited to a few rows of cells on either side of the ventral furrow; soon after the stage shown here, the expression narrows to a single row of cells at the ventral midline. There is also expression in a broad band in the head region. (C - lateral view) At the germband extended stage, the mesectoderm is stained. (D - ventral view) In a germband retracted embryo staining is seen in ventral epidermal stripes in each segment, and in a subset of cells in the midline of the nervous system (an example is indicated by the arrow). Not seen in this view are the dorsal stripes, which are similar to the ventral ones. (E) An embryonic cuticle preparation of a wild-type embryo, and (F) of a $rho^{P\Delta 5}$ homozygous embryo (anterior to the left). Note the characteristic fusions of the denticle belts in the *rhomboid* embryo (indicated by an arrow), as well as the abnormal head skeleton.

developing eye disc. Under normal fixation conditions, the staining is punctate, and is reminiscent of the vesicle staining in the embryo, making it difficult to distinguish precisely in which cells the *rhomboid* protein is expressed (Fig. 3C). However, it appears to be limited to only a subset of the developing cells and, in discs fixed in the presence of the detergent NP-40 (see Materials and Methods), this subset can be identified as cells 8, 2 and 5 (Fig. 3D). This observation is consistent with the expression of the X81 enhancer trap, which is transcribed in predominantly cells 8, 2 and 5.

Mitotic clones of rhomboid *in the adult eye*

In order to investigate the role of *rhomboid* in eye development, we have characterised the phenotype of *rhomboid* mutations in the eye. Since all *rhomboid* mutations are lethal, this can only be done by generating mitotic clones of homozygous $rhomboid^{-}$ tissue in an otherwise heterozygous fly. An eye color marker was used to allow the identification of the homozygous mutant clones in the eye.

We have made mitotic clones with both of our null *rhomboid* mutations ($rho^{P\Delta 5}$ and $rho^{P\Delta 38}$), and with two existing mutations, rho^{7M43} and rho^{del1}, and find them all to have

an identical, subtle phenotype in the adult eye. There is a very slight disruption of the ommatidial array associated with the clones (Fig. 4). This defect most often occurs at the boundary between wild-type and mutant tissue (as shown in Fig. 4). The significance of this boundary effect is not clear, nor is the underlying cause of the phenotype. It is worth stressing that, while this phenotype is very subtle and only affects a small number of ommatidia, it is highly reproducible and is likely to represent the true loss-of-function phenotype of *rhomboid*: four different mutations, induced in different genetic backgrounds, show the same effect, and two of those mutations are almost certainly complete protein nulls.

Ectopic expression of rhomboid

In order to examine further what role *rhomboid* might have in ommatidial determination, we tested the effect of altering its expression pattern in the developing eye. We chose two different ectopic expression procedures (Fig. 5A). In the first experiment we linked the *rhomboid* coding region to the enhancer and promoter of the *sevenless* gene and introduced them into flies by P-element transformation. These control sequences have been previously shown to confer the *sevenless* expression pattern on heterologous genes (Basler et al., 1989; Bowtell et al., 1989; Kimmel et al., 1990). *sevenless* is expressed at least transiently in all the cells of the developing ommatidium except for R8, R2 and R5, so transformants that are wild type for their own *rhomboid* gene, and which contain a *sevenless-rhomboid* (sev-rho) fusion gene, will express *rhomboid* in all ommatidial cells including the cone cells and mystery cells (Fig. 5B). The second ectopic expression construct that we made was to put the *rhomboid* coding sequences under the control of the *hsp70* promoter. Upon heat shock, transformants carrying this construct will express *rhomboid* in all cells, including the uncommitted cells surrounding each ommatidium.

Ectopic expression under the sevenless *promoter*

sev-rho transformants have *rough* eyes. The degree of roughness appears to be quite sensitive to the dose of the *rhomboid* gene since different transformant lines show varying amounts of disorder, and all transformants show increasing roughness with increasing copy number of the sev-rho construct. In general two copies are enough to show clear roughness, and when six copies are combined in the same fly there are severe disruptions in the pigment lattice and lens structures. Sections through transformant eyes show that the primary defect in sev-rho eyes is the presence of one, two and occasionally three extra photoreceptor cells (compare Fig. 5C and 5D). These extra cells have the characteristic morphology of the outer photoreceptors R1-R6. Often the addition of these cells to the ommatidium does not greatly disrupt the overall architecture, and in these cases the additional cells can be seen to be in the vicinity of cells R3 and R4. The penetrance of this extra cell phenotype depends on the dose of the sev-rho construct: Fig. 5D shows a section through an eye containing two copies, in which about 30% of the ommatidia have extra cells; up to four copies produces a similar phenotype, although the proportion of ommatidia with extra cells increases to about 70%; when the copy number is over four, other defects are frequent. These include missing pigment cells leading to fusions between adjacent ommatidia, loss

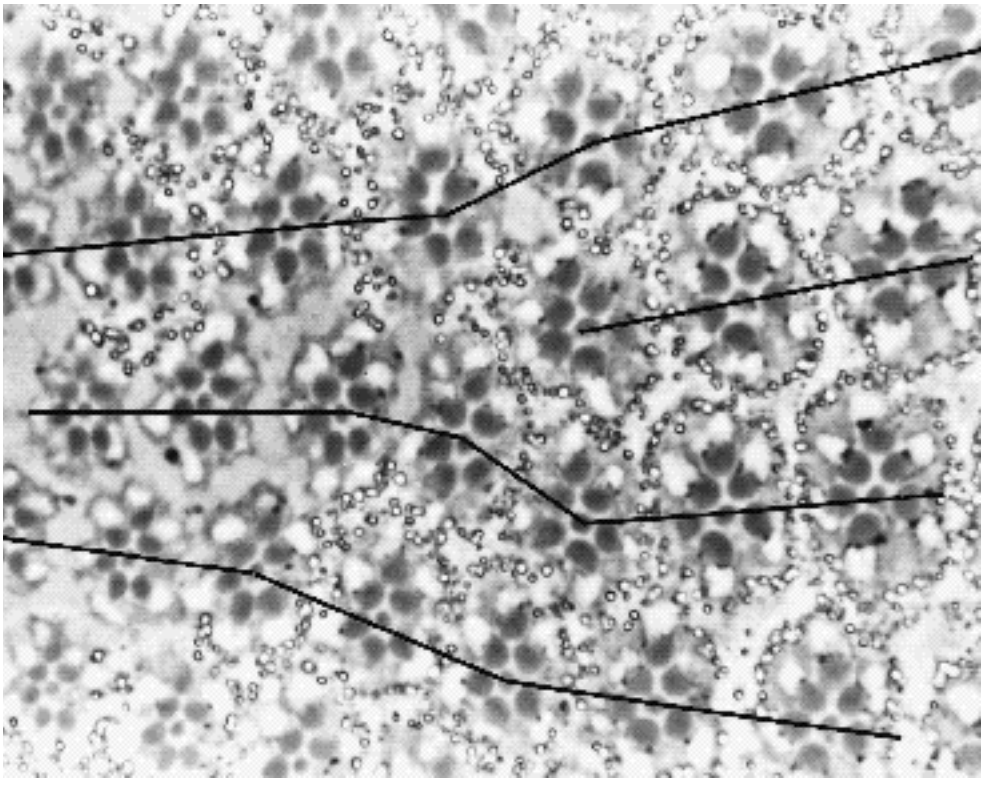

Fig. 4. The phenotype of *rhomboid* mutations in the eye. Mitotic clones of several different *rhomboid* mutations were sectioned, and all showed the same subtle but reproducible phenotype. Typically, a small number of ommatidia were missing, leading to a slight local roughening in the clone. Often a whole row of ommatidia is missing in the clone, and this is shown in this 2 micron section: the edge of a clone is pictured, with the mutant, *white*$^{-}$, tissue to the left of the panel, and the wild-type, *white*$^{+}$, tissue towards the right. At the clone boundary a row of ommatidia from the wild-type tissue terminates. We have tried to indicate this phenomenon more clearly by drawing a line through each ommatidial row.

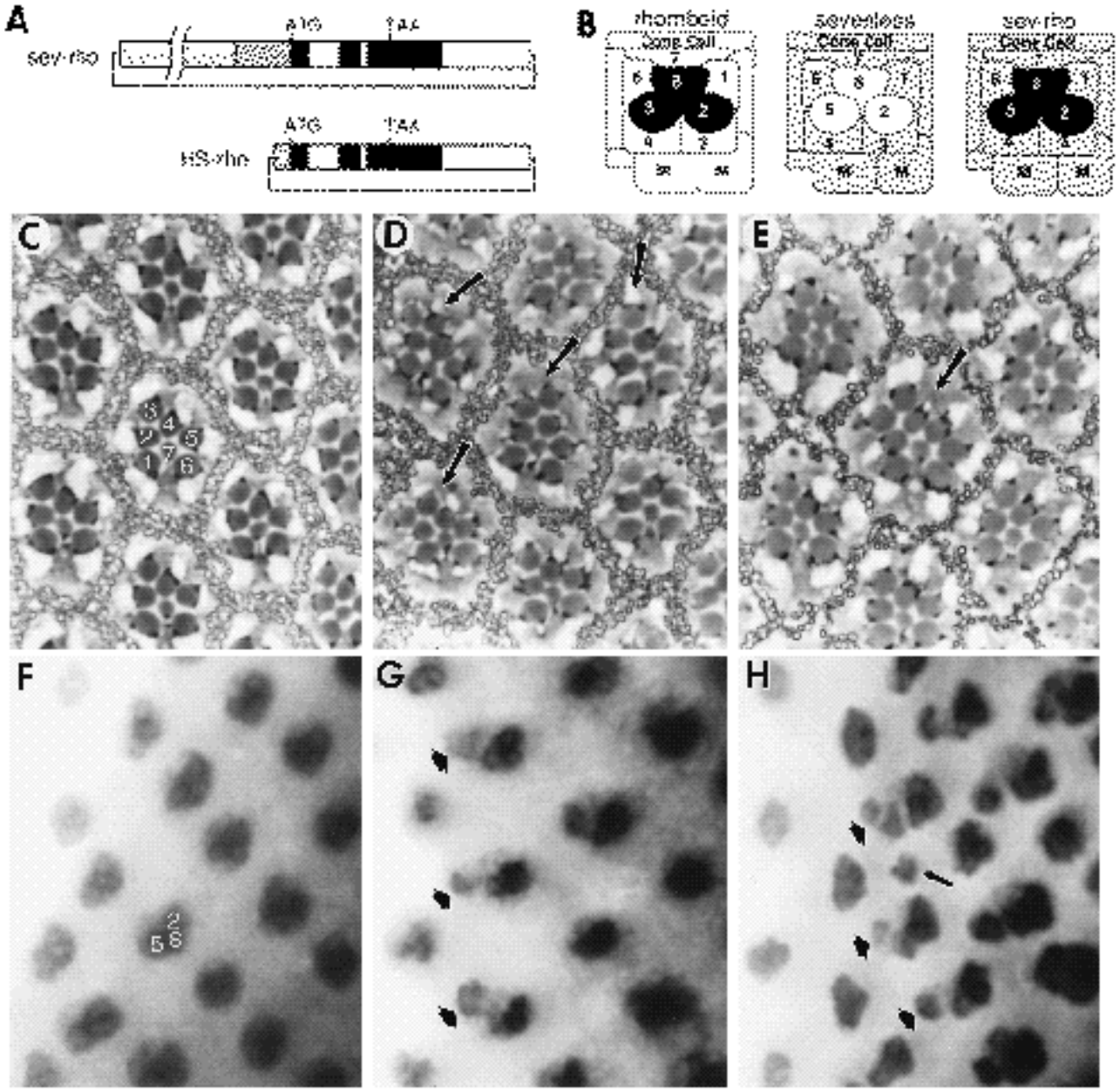

Fig. 5. Ectopic expression of rhomboid. (A) The two misexpression constructs. The sev-rho construct includes a *sevenless* enhancer fragment (speckled), and a *sevenless* promoter fragment (hatched), linked to the *rhomboid* transcription unit from which the first, non-coding, exon and the first intron have been removed (see Materials and Methods). The HS-rho construct has a fragment containing the sequences necessary to confer heatshock inducibility upon a heterologous gene (see Materials and Methods) linked to the same *rhomboid* fragment as the sev-rho construct. (B) The sev-rho misexpression experiment. *rhomboid* appears to be predominantly expressed in cells R8, R2 and R5; *sevenless* is expressed in all the other cells of the developing ommatidia (Tomlinson et al., 1987); in sev-rho flies, which are wild type for their own copy of the *rhomboid* gene, the gene is expressed in all the cells. These diagrams represent all the cells that are associated with the ommatidia throughout larval development, rather than any particular stage of their differentiation. (C) A 2 micron tangential section through a wild-type adult eye. Note that in this plane of section seven photoreceptors can be seen in each ommatidium; the photoreceptors are easily identified by their rhabdomeres, the dark organelles used for light trapping. The identity of each photoreceptor can be determined by its position in the asymmetric trapezoid, and these identities are indicated. (D) A similar section through a fly carrying two copies of the sev-rho construct: note that several of the ommatidia have an extra outer photoreceptor (examples of such ommatidia are indicated with arrows). (E) A section through a HS-rho eye, which was heatshocked as a third instar larva. One of the ommatidia (indicated by an arrow) has an extra outer photoreceptor that is indistinguishable from those in sev-rho eyes. (F-H) Third instar eye imaginal discs stained with an antibody against neuroglian, which is a ubiquitous neural antigen expressed early in neuronal differentiation (Hortsch et al., 1990); in each case the morphogenetic furrow is to the left. (F) A wild-type disc. Cells R8, R2, and R5 are indicated. Cells R3 and R4, adjacent to R2 and R5, can be seen to stain weakly at this stage in some of the clusters. (G) A sev-rho disc from a larva with two copies of the sev-rho construct. Cells in the position of the mystery cells that are expressing neuroglian are indicated with arrowheads. (H) A disc from a larva with six copies of the sev-rho construct. In these discs, a greater proportion of ommatidia have extra cells differentiating neuronally, and more overall disruption of the disc is apparent. The broad arrowheads indicate some examples of neuronally differentiating cells in the mystery cell position; the arrow indicates an extra cell, between clusters, that is undergoing neural differentiation.

of regularity of spacing and orientation, and occasional extra photoreceptors with inner photoreceptor morphology; the lens defects apparent in eyes from flies with six copies of sev-rho suggest that there are also disruptions in cone cell development when the ectopic dose of *rhomboid* becomes very high.

In order to understand what the initial defects were in the development of eyes of sev-rho flies, we examined the earliest stages of their development in the eye imaginal disc. During the third larval instar, a dorso-ventral indentation, known as the morphogenetic furrow, sweeps anteriorly across this monolayer epithelium. As cells emerge from the posterior of the furrow, they begin to show morphological signs of differentiating, and they begin to express neural antigens. Cells are recruited into the developing ommatidia in a precise sequence, and every differentiating cell can be recognised as it develops (Tomlinson, 1988; Ready, 1989). In discs containing two copies of the sev-rho construct, extra cells undergoing neural differentiation can be seen in many of the clusters. This is most easily observed at the stage when three cells (R8, R2 and R5), and then, soon after, five cells (R8, R2, R5, R3 and R4), are normally expressing neural antigens. The additional one or two cells are in the position of the mystery cells in wild-type discs, adjacent to cells R3 and R4 (Fig. 5G). The mystery cells are so called since their ultimate fate is unclear. In wild-type discs they join the precluster of the first five photoreceptor precursors, but they never express neural antigens, and eventually they leave the cluster and appear to rejoin the pool of uncommitted cells (Tomlinson et al., 1987). In sev-rho flies, some of the mystery cells never leave the cluster, and differentiate as morphologically normal outer photoreceptor cells. The proportion of ommatidia with these transformed mystery cells correlates well with that seen in sections of adult eyes. With two copies of sev-rho, the transformation of the mystery cells is the only defect that we can detect in sev-rho discs. With higher copy numbers, other defects become apparent including abnormal spacing and orientation, and the occasional additional photoreceptor that is not in the position of a mystery cell (Fig. 5H), which may represent a different cell adopting an inappropriate photoreceptor fate.

Ectopic expression under the hsp70 *promoter*

Flies carrying the HS-rho construct are wild type at 25°C. However, upon a series of heat shocks during the third

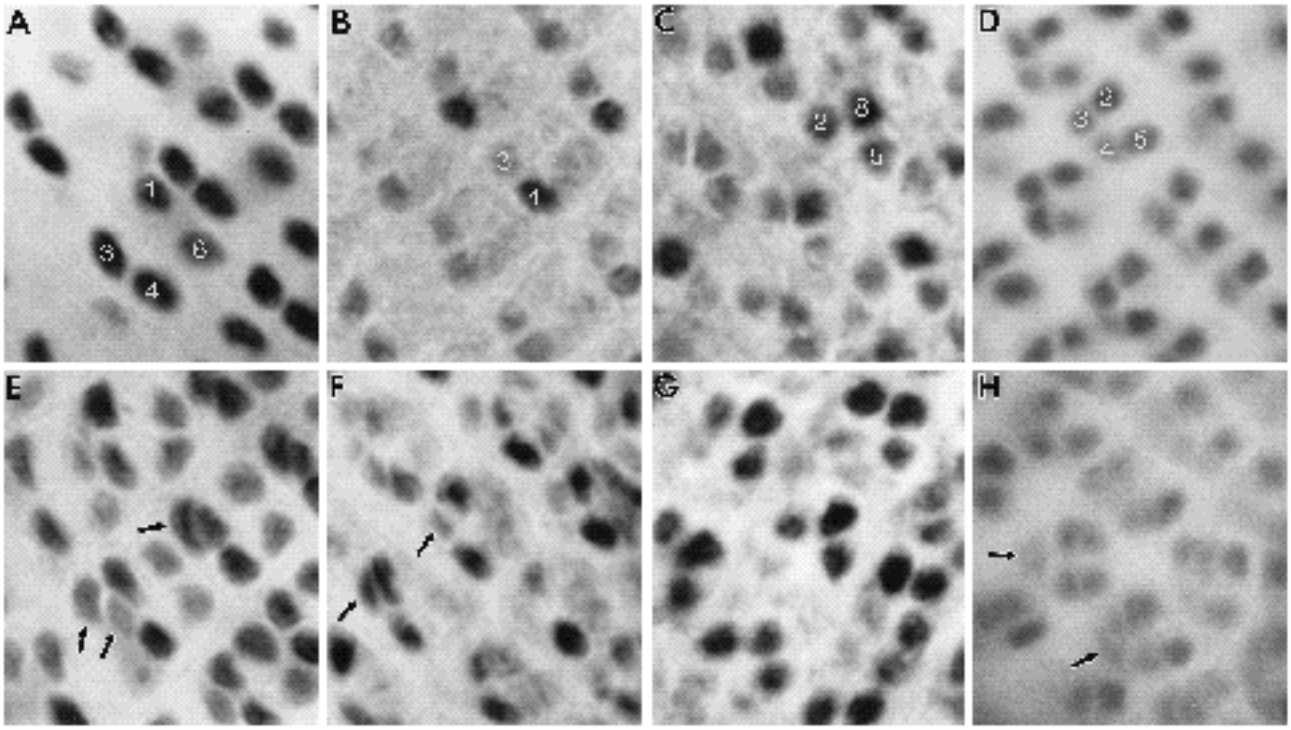

Fig. 6. The identity of the extra photoreceptors in sev-rho imaginal discs. A to C are wild-type discs carrying enhancer trap insertions specific for particular cells; E to G are discs with three copies of the sev-rho construct carrying the same enhancer trap lines. All these discs were immunostained for β-galactosidase, and the morphogenetic furrow is to the left. A and E show discs carrying an enhancer trap in the *seven-up* gene (Mlodzik et al., 1990). This marker is expressed only in cells R3, R4, R1 and R6, as is shown in A; E in sev-rho discs, many ommatidia have additional staining cells adjacent to cells R3 and R4 (see arrows). B and F show discs carrying an enhancer trap called O32 (our unpublished data). This is expressed predominantly in cells R3, R4 and R7, but in the region near the furrow, where this field is from, only cells R3 and R4 are stained (B); (F) in sev-rho discs additional cells adjacent to R3 and R4 are seen to express O32 in many ommatidia (example indicated by an arrow). C and G show discs carrying the AE33 enhancer trap (described in this work), which is predominantly expressed in cells R8, R2, and R5 (C); sev-rho discs do not have additional AE33-expressing cells (G). D and H are wild-type and sev-rho discs, respectively, stained with mAbro1, an antibody against the rough protein. D shows the normal expression pattern in cells R2, R5, R3 and R4. H shows the staining in sev-rho discs: many ommatidia are found to have extra rough-expressing cells, adjacent to cells R3 and R4, in the mystery cell position (indicated by arrows).

larval instar, when photoreceptor determination is occurring, a stripe of slight roughness is induced in the adult eye. Sections through this rough portion of the eye indicate an ommatidial phenotype that is indistinguishable from that of sev-rho flies: one or two extra outer photoreceptors are found in the vicinity of R3 and R4 (Fig. 5E). We presume that these are also transformed mystery cells. In order to see this effect, quite severe heat shock regimes are required, for example four 30 minute pulses over a period of 6.5 hours to larvae carrying two copies of the HS-rho construct. Even then, the penetrance of this extra cell phenotype is low: typically two or three abnormal ommatidia in an eye. We do not know why the HS-rho constructs appear less effective at producing the transformed mystery cell phenotype than the sev-rho constructs, but it may be that the level per cell of ectopic *rhomboid* expression is quite low in HS-rho discs. Interestingly, even though *rhomboid* is ectopically expressed in all cells, including all the uncommitted cells in the disc, we see no evidence for any cells other than mystery cells being transformed into photoreceptors.

The identity of the transformed mystery cells

We have used cell-type-specific markers to try and determine the identity of the transformed mystery cells in sev-rho flies. These markers are enhancer trap lines that have previously been shown to express the *E. coli lacZ* gene in various subsets of the developing photoreceptors (M. F., U. Gaul, J. S. Heilig, L. S. Higgins, G. M. R., unpublished; Mlodzik et al., 1990). By crossing these enhancer traps into flies carrying sev-rho, we were able to examine which markers are expressed by the extra cells, and therefore to determine their identity. The transformed mystery cells express an enhancer trap in the *seven-up* gene, which is specific to R3, R4, R1 and R6 (Fig. 6E) and one that is specific to cells R3, R4 and R7 (Fig. 6F); they do not express an enhancer trap specific to cells R8, R2 and R5 (Fig. 6G). Furthermore, using an antibody against the *rough* protein, we find that the transformed cells express *rough*. Normally, only four cells per ommatidium (R2, R5, R3, R4) express *rough* protein posterior to the morphogenetic furrow (Kimmel et al., 1990). However, in sev-rho discs, we often see five or six cells per ommatidium staining, and the additional cells are in the mystery cell position (Fig. 6H). On the basis of these results, it appears as if the transformed mystery cells have an identity closest to cells R3 and R4.

Discussion

The identification of homeobox target genes should elucidate some of the mechanisms by which a cell chooses between alternative fates. The approach that we have used to identify genes downstream of the *rough* gene is to cross enhancer traps, which were previously known to be expressed in the developing ommatidium, into a *rough* mutant background and thus to look for genes whose expression was *rough*-dependent. *rough* has previously been shown to be required only in cells R2 and R5, and it has a critical role in the specification of their fate (Tomlinson et al., 1988; Basler et al., 1990; Kimmel et al., 1990; Heberlein et al., 1991). Our strategy made no assumptions about what genes might be regulated by *rough*, and allowed us to examine directly whether the transcription of a gene was *rough*-dependent. Our search has identified several candidates, and we have characterised further the role of one of these, *rhomboid*, during eye development. We have shown that *rhomboid* is expressed in the developing ommatidia, where its expression in cells R2 and R5 is *rough*-dependent; *rhomboid* mutations subtly affect normal eye development, and its ectopic expression causes a profound fate change in the mystery cells, which are transformed into photoreceptors.

Cell identities and rough *target genes*

It is important to note that cells R2 and R5 do not differentiate normally in *rough* mutants. Nevertheless, in most cases the two cells that would become R2 and R5 in a wild-type disc can still be identified by their position in the ommatidial precluster, and they still differentiate as neurons (Tomlinson et al., 1988). In *rough* mutants these two presumptive R2/R5 cells lose at least part of their normal identity, and instead appear to acquire a variety of abnormal fates. For example, Heberlein et al. (1991) have shown that many of these cells take on some characteristics of R3, R4, R1 and R6 cells in that they express and become dependent on the product of the *seven-up* gene, which is normally required and expressed in only cells R3, R4, R1 and R6. In other cases, one of the presumptive R2/R5 cells acquires an R8-like fate. Van Vactor et al. (1991) observed ectopic boss expression in many of the presumptive R2/R5 cells, suggesting a more frequent transformation toward R8. One interpretation of these results is that in the absence of *rough* the presumptive R2/R5 cells are receptive to inappropriate cues, and their fate may then be decided stochastically or by small variations in local environment. This view is consistent with the variability in the composition of individual ommatidia seen in the eyes of *rough* adults. It is also possible that the presumptive R2/R5 cells adopt a hybrid cell fate, simultaneously expressing cell-type-specific markers that are never co-expressed in the same cell in wild type.

Additional evidence that *rough* controls some part of the R2/R5 fate comes from the results of its ectopic expression under the *sevenless* promoter: this causes the presumptive R7 cell to be transformed into an outer photoreceptor (Basler et al., 1990; Kimmel et al., 1990), although the specific subtype of this transformed cell is unclear. Our data suggest that *rough* does not control all aspects of the R2/R5 identity. In our screen, 5 out of 8 lines, which are normally expressed in the three cells R8, R2 and R5, were unaffected by the loss of *rough* function.

In total only 4 out of 101 lines that we examined appeared to detect genes that were *rough* targets in cells R2 and R5. We have found that approximately 5% of all enhancer trap lines are expressed at moderate to high levels in the developing ommatidia (M. F., U. Gaul, J. S. Heilig, L. S. Higgins, G. M. R., unpublished). Taken together, these numbers suggest that the total number of genes dependent on *rough* for R2 and R5 specification is not large. Previous analysis of the *rough* phenotype indicates that, minimally, *rough* must regulate the genes necessary for providing the inductive signal to specify the photoreceptor

identity of cells R3 and R4, and for specifying some part of the outer photoreceptor subtype identity.

In the simplest view, it would be expected that those genes that were required for the R2/R5 subtype, and which were downstream of *rough*, would be expressed in only cells R2 and R5: although *rough* is expressed in cells R2, R5, R3 and R4, it is not required in R3 and R4 (Tomlinson et al., 1988), and furthermore, those genes that specify the R2/R5 identity seem unlikely to be expressed in R3/R4. However, in the enhancer trap screen on which this study was based, no lines that were expressed only in R2 and R5 were isolated. Instead, most of those lines that do appear to be downstream of *rough* in R2 and R5 are expressed in the triplet of cells R8, R2 and R5. This could indicate a relatively close developmental relationship between R8 and R2/R5, a conclusion previously suggested by a characterisation of the *Star* gene in eye development (Heberlein and Rubin, 1991): *Star* is required in cells R8, R2 and R5 for normal ommatidial differentiation. Genes identified by the enhancer trap lines that are expressed in R8, R2 and R5 must be regulated by factors other than *rough* in R8, since *rough* is not significantly expressed in that cell.

The role of rhomboid *in eye development*

The X81 enhancer trap, whose expression is dependent on *rough*, is an insertion at the *rhomboid* locus. This was the first indication that *rhomboid*, known to be necessary for embryonic development (Mayer and Nüsslein-Volhard, 1988; Bier et al., 1990), was also likely to have a role in eye development. We have confirmed that the enhancer trap expression pattern does reflect real expression of *rhomboid* in the eye imaginal disc. Although our antibody does not stain strongly enough to characterise unambiguously the details of the protein's expression - either because the amount of *rhomboid* protein is low, or because the antibody is of low affinity - it is quite clear that *rhomboid* is expressed in the developing photoreceptors, beginning early in their differentiation. It is likely that the expression is limited to cells R8, R2 and R5, as detected by the enhancer trap.

When misexpressed under the *sevenless* promoter/enhancer, *rhomboid* profoundly alters the fate of the mystery cells, causing them to become outer photoreceptors, with characteristics of cells R3 and R4. This result suggests that *rhomboid* may function in the process by which at least some cells acquire a photoreceptor fate. When ectopically expressed under the control of the heat shock promoter, and therefore expressed in all cells, it is still only the mystery cells that inappropriately adopt a photoreceptor fate. Even in wild-type discs, the mystery cells undergo some of the early stages of photoreceptor differentiation, although they ultimately appear to rejoin the pool of uncommitted cells. They undergo similar morphological changes to the photoreceptors of the early five-cell precluster; they express *sevenless*; and they are particularly susceptible to acquiring a photoreceptor fate in a variety of mutants (Tomlinson et al., 1987; Mlodzik et al., 1990; Fischer-Vize et al., 1992; Freeman et al., 1992). Thus the mystery cells seem to be developmentally quite close to photoreceptors in the precluster and ectopic expression of *rhomboid* is sufficient to alter their fate.

Although there is a dramatic effect of ectopically expressing *rhomboid*, loss of *rhomboid* function has a relatively mild effect in the eye. The only visible phenotype in mutant clones is a slight disruption of the ommatidial array. The easiest way to reconcile this observation with the ectopic expression results is to suggest that *rhomboid* is part of a largely redundant network of gene products: because of this redundancy, the removal of *rhomboid* only has minor effects.

There are two alternative explanations for our ectopic expression results: in one *rhomboid* acts autonomously, and its expression in the mystery cell under the *sevenless* promoter causes the cell's transformation into a photoreceptor; in the other view, *rhomboid* acts non-autonomously, in which case its ectopic expression in cells R3 and R4 induces the adjacent mystery cells to adopt a photoreceptor fate. It is tempting to speculate that, since *rhomboid* appears to be downstream of *rough* in cells R2 and R5, it may be part of the inductive signal presented to R3 and R4, and this would favour the non-autonomous model of *rhomboid* action. In this view, *rhomboid* is part of a complex network of overlapping signals that are used to specify a photoreceptor fate in the developing eye. In sev-rho flies, cells R3 and R4 ectopically express *rhomboid*, thus presenting an inappropriate inductive signal to their neighbours, the mystery cells, causing the transformation of the latter into R3/R4 like cells. Since *rhomboid* appears to be only part of a mechanism that causes photoreceptor determination, its ectopic expression only affects the mystery cells, which may already express many of the gene products necessary for this developmental pathway, and therefore only need a slight 'push' to adopt that fate. This model provides a testable hypothesis: the question of whether or not *rhomboid* acts autonomously can be addressed by mosaic analysis. Unfortunately, the sev-rho transformation does not produce a penetrant-enough phenotype to allow a simple analysis in the adult eye, so this experiment will have to be done by analysing clones in the developing eye disc - which will be technically challenging.

Functional redundancy of rhomboid

rhomboid encodes a protein whose function appears to be partially redundant in the eye. While it may not be possible to select for fully-redundant functions in evolution, largely overlapping functions may be quite common. This could account for the observation that a large majority of mutations in genes adjacent to enhancer traps expressed in the developing eye, have no obvious effect on eye development (our unpublished observations). The view that a majority of genes will produce clear phenotypes that illuminate their normal function may be heavily biased by the fact that most genes characterised to date have been initially identified by having a clear mutant phenotype. As more genes are isolated by reverse-genetic techniques, which do not rely on a phenotype, a more accurate picture of the extent of redundancy of developmental mechanisms should emerge. It is notable that in those cases where small regions of the genome have been extensively analysed for transcription units and genes with detectable phenotypes, there appears to be a two- to three-fold excess of transcripts over phenotypes (for example see Hall et al., 1983). There

are some attractive theoretical features of a system with such overlapping functions. These include robustness, since a small perturbation is less likely to affect a process deleteriously; and the ability to adapt relatively easily, there being less selection pressure against a change in any single gene in such a redundant network.

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Fig. 3. The expression of rhomboid protein in the embryo and the eye imaginal disc. Immunohistochemical staining with the monoclonal antibody mAbrho1 is shown. (A) A view of two lateral stripes of *rhomboid* expression in a germband retracted embryo; note that *rhomboid* protein is largely restricted to vesicles (arrows). (B) *rhomboid* protein is expressed in the third instar eye imaginal disc, posterior to the morphogenetic furrow (anterior to the left, arrow marks position of furrow). C and D are higher magnification views of a portion of an eye disc. (C) Under normal fixation conditions, *rhomboid* staining in the eye disc is punctate, making it difficult to determine the identity of individual cells. (D) Using a fix that includes the detergent NP-40 (see Materials and Methods), the *rhomboid* protein is apparently somewhat solubilised, allowing a clearer identification of the cells in which it is expressed; an example of the characteristic triplet of cells R8, R2 and R5 is indicated.