mirror encodes a novel PBX-class homeoprotein that functions in the definition of the dorsal–ventral border in the *Drosophila* eye

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The *Drosophila* eye is composed of dorsal and ventral mirror-image fields of opposite chiral forms of ommatidia. The boundary between these fields is known as the equator. We describe a novel gene, *mirror* (*mrr*), which is expressed in the dorsal half of the eye and plays a key role in forming the equator. Ectopic equators can be generated by juxtaposing *mrr* expressing and nonexpressing cells, and the path of the normal equator can be altered by changing the domain of *mrr* expression. These observations suggest that *mrr* is a key component in defining the dorsal–ventral boundary of tissue polarity in the eye. In addition, loss of *mrr* function leads to embryonic lethality and segmental defects, and its expression pattern suggests that it may also act to define segmental borders. Mirror is a member of the class of homeoproteins defined by the human proto-oncogene PBX1. *mrr* is similar to the Iroquois genes *ara* and *caup* and is located adjacent to them in this recently described homeotic cluster.

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The spatial control of differentiation is a central issue in the study of the development of multicellular organisms. A primary step in pattern formation is the division of homogeneous cells into distinct fields. The border between these fields can then act as signaling centers for further patterning and growth (Peifer and Bejsovec 1992; Blair 1995). An example of such a border is found in the *Drosophila* eye. The eye is composed of dorsal and ventral fields of photoreceptor clusters called ommatidia. Ommatidia in the dorsal half of the eye are the mirror image of those in the ventral region (see Fig. 1B, below; Deitrich 1909; Ready et al. 1976; Tomlinson 1985, 1988; Tomlinson and Ready 1987). The boundary where the dorsal and ventral fields meet is known as the equator. The equator bisects the eye from anterior to posterior, rarely deviating by more than one ommatidial width as it crosses the eye. How the equator is determined is not well understood, but clonal analysis has indicated that cell lineage is not an important factor in forming the equator. Therefore, it has been proposed that cell–cell interactions are critical for establishing the dorsal/ventral (D/V) boundary within the eye (Ready et al. 1976).

The eye develops from a monolayer epithelium called the eye imaginal disc (for review, see Wolff and Ready

1993). During the third instar larval period preclusters at the posterior of the eye disc begin to form, and their differentiation induces the formation of new preclusters anterior to them. This leads to a wave of differentiation that sweeps across the disc. This wave is marked by an indentation known as the morphogenetic furrow, which separates the undifferentiated and differentiating regions of the disc. Initially, preclusters emerging from the morphogenetic furrow appear identical. As they mature, the preclusters in the dorsal and ventral regions of the eye rotate in opposite directions (see Fig. 1A, below). Asymmetries are incorporated into the preclusters as they rotate, resulting in ommatidia of opposing polarity and chirality in the adult eye. Recent work has demonstrated that the degree of ommatidial rotation is controlled by a number of genes including *nemo* and *roulette* (Choi and Benzer 1994) and has suggested that tissue polarity genes such as *spinylegs*, *prickly-spinylegs* (Gubb 1993; Choi et al. 1996), *frizzled* (Zheng et al. 1995), and *dishevelled* (Theisen et al. 1994) are necessary for regulating D/V polarity in the eye.

Several experiments suggest that the equator might be source of D/V patterning information. Analysis of patterning defects in clones of *frizzled*[−] cells suggests that *frizzled* is required to interpret and relay a signal emitted by the equator (Zheng et al. 1995). Consistent with this proposal is the observation that a number of genes ap-

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pear to be expressed either solely at the equator or in a gradient from the equator (Sun et al. 1995; Villano and Katz 1995; Brodsky and Steller 1996). Furthermore, morphological analysis has indicated that ommatidial differentiation in each row proceeds from the equator outwards (Wolff and Ready 1991) and has been used to suggest that more mature ommatidia provide D/V polarity information to their less differentiated neighbors (Gubb 1993).

We describe here a novel homeodomain-containing protein, Mirror (Mrr), that plays an important role in defining the equator. *mrr* is expressed in the dorsal half of the eye, with expression terminating at the equator. Using loss-of-function *mrr* alleles and ectopic expression of *mrr*, we have analyzed the role of *mrr* during eye development. Our data suggest that the juxtaposition of

mrr expressing and nonexpressing cells plays an important role in defining the equator. In addition, analysis of the *mrr* expression pattern and the *mrr* loss-of-function phenotype in embryos suggests that *mrr* also acts in the definition of the segmental border.

Results

Identification of a gene expressed in the dorsal half of the eye

mrr was first detected using a P element (*mrr*^{P1}) that carries *white* and *lacZ* and is inserted into the 69D region of the third chromosome. Strong *white* gene expression was restricted to cells dorsal to the equator (Fig. 1C,D). This expression pattern suggested that *mrr*^{P1}

Figure 1. Identification of a gene expressed in the dorsal half of the eye. (A) Development of polarity and chirality in an eye imaginal disc. For this and all subsequent pictures, dorsal is up, anterior is to the left. Undifferentiated cells are sequentially recruited into the precluster. The acquisition of neuronal identity is indicated by the acquisition of color; R2/5 (purple), R3/4 (green), R8 (yellow), R7 (red). Preclusters begin to rotate as they differentiate, rotating in opposite directions above and below the equator. The final arrangement shown is not achieved until pupal stages. (B) Diagram of a wild-type equator. Above the equator ommatidia are organized such that the apex of the trapezoid formed by the ommatidium points up, whereas in the ventral half of the eye the mirror image form exists. Each black circle represents a rhabdomere projected by a photoreceptor into the middle of the ommatidium. (C) *mrr*^{P1} flies express the *white* gene (which results in red pigmentation) in the dorsal half of the eye. Expression of *white* does not cross the equator but terminates at variable positions in the dorsal ommatidia by the equator. Position of the equator is indicated by the sawtooth red line. (D) Section of a *mrr*^{P1} eye. Note the restriction of *white* (seen as yellow pigment granules in sections) to the dorsal half of the eye. (E) β -Galactosidase staining of a *mrr*^{P1} eye imaginal disc. Staining is restricted to the dorsal half of the eye disc. Staining decreases after the furrow has passed. Note the patch of staining in the antennal portion of the disc. The white arrow indicates the position of the morphogenetic furrow; the black arrowhead indicates the presumed position of the equator. (F) In situ hybridization of *mrr* riboprobe to an eye imaginal disc. Transcript is restricted to the dorsal anterior half of the eye disc, with expression ceasing at the morphogenetic furrow. Arrows as in E. (G) β -Galactosidase staining of a *mrr*^{P1} embryo. Staining reflects a segmentally repeated expression pattern of this enhancer trap. (H) In situ hybridization of *mrr* riboprobe to a wild-type stage 11 embryo. Segmentally repeated staining resembles staining of embryos carrying *mrr*^{P1}.

might be inserted close to a gene, that we call *mrr*, which is involved in determining dorsal identity or in forming the D/V boundary. To determine if *mrr* is expressed at the time of the establishment of D/V polarity, we examined developing eye discs. β -Galactosidase activity was restricted to the dorsal cells both in front of the morphogenetic furrow and in the first few rows behind the furrow (Fig. 1E). Analysis of β -galactosidase staining in *mrr*^{P1} embryos (Fig. 1G) suggests that *mrr* might also play a role in embryonic development.

mrr encodes a homeodomain protein

To identify *mrr*, the P element was used to obtain genomic DNA adjacent to the insertion site. A chromosomal walk was conducted in both directions from the *mrr*^{P1} insertion point, and a portion of the walk was then used

to probe an eye imaginal disc cDNA library (Fig. 2A). Nine overlapping cDNAs were isolated that define a 23-kb transcription unit with five exons. Hybridization of these cDNAs to eye imaginal discs demonstrated that this gene is expressed in the dorsal half of the eye disc in a pattern similar to that of β -galactosidase expression in *mrr*^{P1} eye discs (Fig. 1E,F). Hybridization of these cDNAs to embryos also revealed expression that was very similar to β -galactosidase activity in *mrr*^{P1} embryos (Fig. 1G,H). We conclude that this transcription unit is *mrr*.

The 3.5-kb *mrr* transcript contains a single long open reading frame (ORF) of 1.9 kb. Conceptual translation of the *mrr* cDNA (Fig. 2B) reveals that the *mrr* protein (Mrr) is a putative transcriptional activator containing both a homeodomain and an acidic transactivation domain (for review, see Triezenberg 1995; Burglin 1994). The Mrr homeodomain contains 3 additional amino acids be-

Figure 2. Molecular characterization of *mrr*. (A) Genomic structure of the *mrr* locus. A restriction map is shown. (X) *Xho*; (R) *Eco*RI; triangles indicate the positions of P elements determined by genomic sequencing. White boxes indicate the UTR of the *mrr* cDNA; black boxes indicate coding regions. Positions of exons were determined through a combination of Southern hybridization and genomic sequencing of the intron-exon boundaries. 5' and 3' ends shown are those of the cDNA clones. Overlapping lines beneath the map indicate phage and P1 clones used to analyze the genomic region. The hatched region of the restriction map indicates the region excised in the embryonic lethal excision allele *mrr*^{e48}, as determined by genomic Southern analysis of the mutant chromosome. (B) Amino acid sequence of the Mrr protein deduced from the cDNA sequence. Coding begins with the first methionine of the ORF; however, the second methionine (downward arrows) conforms more closely to the Kozack consensus sequence. Bold characters indicate regions conserved with Ara and Caup. The underlined region indicates the position of the homeodomain. Shaded letters indicate acidic regions, which could act as transcriptional activators. (C) Homeodomain homology. Amino acids identical to Mrr are silhouetted in black; conservative changes are shaded. (D) Notch interaction domain homology. (E) Novel homology region conserved between Mrr and Ara and Caup.

tween helix 1 and helix 2 that are not present in most homeodomains. This places it in a class of homeodomain proteins defined by human PBX1 and its *Drosophila* homolog Extradenticle (see Fig. 2C) (Kamps et al. 1990; Nourse et al. 1990; Flegel et al. 1993; Rauskolb et al. 1993). These proteins are notable for forming heterodimers with other homeodomain proteins and increasing their partners' target specificity and affinity (Chan et al. 1994; van Dijk and Murre 1994, 1995; Chang et al. 1995; Knoepfler and Kamps 1995; Lu et al. 1995; Mann 1995; Phelan et al. 1995).

Mrr is related most closely to the two homeodomain proteins of the Iroquois complex, Araucan (*Ara*) and Capolican (*Caup*), although the Iroquois proteins are related more closely to each other than to *Mrr* (Gomez-Skarmeta et al. 1996). The homology to these proteins is particularly striking in three regions (Fig. 2B). The major region of homology lies within the homeodomain and the 15 amino acids immediately after the homeodomain. Over the homeodomain, 57/60 amino acids are identical, with three conservative changes (Fig. 2C). In the amino-terminal region there is a highly conserved Notch interaction domain, which has been proposed to be involved in protein-protein interactions (Gomez-Skarmeta et al. 1996) (Fig. 2D). In addition, a highly conserved novel region close to the carboxyl terminus is also conserved among all these family members (Fig. 2E). The similarity of *mrr* to *ara* and *caup* and its coincident location at 69D (within 50 kb of *caup*; Gomez-Skarmeta et al. 1996) suggests that *mrr* is a new member of the Iroquois complex.

Patterning defects in *mrr* alleles

To analyze the function of *mrr* we sought to obtain mutant *mrr* alleles. Because the *mrr*^{P1} insertion site is within 300 bp of the beginning of the *mrr* transcriptional unit, the insertion of the P element might have disrupted *mrr* expression. We therefore began our phenotypic analysis by examining homozygous *mrr*^{P1} eyes to determine whether there were defects in patterning. *mrr*^{P1} eyes are smooth, with no apparent disruptions in the packing of the ommatidial facets. However, closer analysis revealed subtle patterning defects. In ~20% of *mrr*^{P1} eyes (9/50), at least one ommatidium close to the equator is misspecified, having ventral chirality and polarity but residing in the dorsal field, or vice versa. Such misspecified ommatidia are not found in wild-type eyes (0/50; see also Franceschini and Kirchfeld 1971; Ready et al. 1976). In addition, *mrr*^{P1} eyes had defects in the path of the equator across the eye. In wild-type eyes, the equator moves up and down one ommatidial width as it traverses the eye and rarely if ever moves more than two ommatidial widths at one time. In contrast, ~10% of *mrr*^{P1} eyes displayed an irregular equator. These irregularities were either three consecutive steps in a single direction or a single step of three ommatidial widths (data not shown). These defects in *mrr*^{P1} flies suggested that *mrr* might have a role in defining the equator.

The weak and variable defects in *mrr*^{P1} eyes and the lack of a *mrr*^{P1} embryonic phenotype despite strong ex-

pression of *mrr* in the embryo (Fig. 1H) suggested that the *mrr*^{P1} might retain substantial function. We therefore sought stronger *mrr* alleles. We took advantage of the fact that transposase-catalyzed excision of P elements is often imprecise and can result in the deletion of flanking sequences. Among 95 transposase-induced excision events, we isolated eight excision alleles that fail to complement each other and have either embryonic or early larval lethal phenotypes. Consistent with these alleles being defective for *mrr* function, in situ hybridization of embryos homozygous for *mrr*^{e48}, a strong allele that contains a 1-kb deletion in the promoter region, had only very low levels of *mrr* transcript. We also obtained an independent insertion into the *mrr* locus, *mrr*^{P2} (Brodsky and Steller 1996). *mrr*^{P2} is an insertion 234 bp upstream of the 5' end of the *mrr* cDNA and results in early larval lethality that cannot be complemented by *mrr*^{e48}. This recessive lethal phenotype could be reverted at high frequency (45/58 excision events) upon excision of the P element, indicating that the P-element insertion near *mrr* was the cause of the lethal phenotype. Taken together, these results indicate that the defects in each of these alleles are the result of reduced *mrr* expression.

Loss of *mrr* expression can define a new equator

Because strong *mrr* alleles have an embryonic lethal phenotype, we studied *mrr* function in the eye by examining clones of cells homozygous for loss-of-function *mrr* mutations. Clones of homozygous mutant cells were generated by mitotic recombination and lacked the *white* gene and therefore pigment granules. Loss of *mrr* function had different effects in different parts of the eye. In the ventral half of the eye, there were no effects, consistent with our observation that *mrr* expression is restricted to the dorsal half of the eye. Dorsal clones also had no significant disturbances in ommatidial polarity or chirality within the clone. These results suggested that *mrr* is not required for the implementation of dorsal identity (see Fig. 3B,C).

In contrast to the lack of defects within *mrr*-minus clones, we noted a dramatic alteration in patterning at the border of certain clones. Ectopic equators formed at the equatorial borders of dorsal anterior clones where *mrr*-minus tissue abutted *mrr*-expressing tissue (see Fig. 3B). The wild-type ommatidia just outside of the clone adopted ventral polarity and chirality. Ventral patterning extended for one to two ommatidial widths, then the tissue resumed normal dorsal patterning. The formation of ectopic equators where *mrr*-expressing and -nonexpressing cells meet suggests that the juxtaposition of *mrr*-expressing and -nonexpressing cells serves to define the normal equator (Fig. 3A).

Loss of *mrr* changes the path of the normal equator

If the juxtaposition of *mrr*-expressing and -nonexpressing cells defines the normal equator, then loss of *mrr* function in dorsal cells along the equator should affect its

Figure 3. A border of *mirror* expression occurs at normal and at ectopic equators. Diagrams show approximate placement of sections: Red indicates *mirror* expression; white indicates lack of *mirror* expression. Aberrant or ectopic equators are indicated by a yellow line; a black line indicates a normal equator. Diagram of ommatidial polarity is adjacent. Black arrows indicate normal polarity; red arrows indicate abnormally oriented ommatidia. In sections *B* and *C* the loss-of-function clone is marked by the loss of yellow pigment granules. (*A*) Section from a *mirror*^{P1} heterozygote eye, illustrating a border of *mirror* expression at a wild-type equator. (*B*) Ectopic equator generated at the ventral border of an anterior *mirror*-minus clone. All clones in the dorsal anterior region of the eye extended into head cuticle; therefore, analysis of the polar face of the clones was not possible. (*C*) Equatorial clones demonstrating *mirror* expression guiding the path of the equator as it enters a *mirror*-minus clone. (*D*) Ectopic *mirror* expressed at the ventral margin of the eye can produce ectopic equators. The yellow line indicates the region of the ectopic equator. An eye imaginal disc from a UAS-*lacZ*; 30A Gal4 fly, stained for β -galactosidase activity to show the expression of the Gal4 line.

path. We therefore examined *mirror*-minus clones that crossed the normal equator. Where the equator approaches a patch of *mirror*-minus tissue, the path of the equator is diverted to follow the new border of *mirror* expression (Fig. 3C). This diversion is consistent with the proposal that the border of *mirror* expression is important for defining the position of the equator. However, after

taking several dorsal steps paralleling the border of *mirror*-expressing and -nonexpressing cells, the equator resumes its posterior to anterior path across the eye rather than continuing to follow the edge of the clone. This result suggests that the pattern of *mirror* expression is not the sole determinant of equator position and that there may be spatial restrictions on the ability of *mirror* to define the equator.

Ectopic expression of mirror alters ommatidial patterning

To further test the hypothesis that the pattern of *mirror* expression defines the equator, we analyzed the effects of ectopic expression of *mirror*. Our first approach was to place *mirror* under the control of a heat shock promoter and observe the effects of ubiquitous expression during third instar larval development. Heat shock-driven *mirror* disrupts normal development and results in flies with rough eyes (data not shown). Sections of these eyes revealed numerous defects, including randomized polarity and chirality of ommatidia. The pleiotropic nature of these defects are difficult to interpret but are consistent with a requirement for spatially restricted *mirror* expression. To test whether a border of *mirror* expression is important for its function, we used the Gal4 system (Brand and Perrimon 1993) to express *mirror* at the ventral margin of the eye. Sections of these eyes revealed occasional ommatidia (in three of five eyes examined) that had dorsal polarity and chirality close to the ventral margin of the eye, near where ectopic *mirror* was expressed (Fig. 3D). No abnormal ommatidia were observed in regions of the ventral field away from where *mirror* was ectopically expressed. These data suggest that ectopic expression of *mirror* can result in the perception of an equatorial border by nearby ommatidia and can lead them to orient around this new border.

mirror expression during development

Loss of *mirror* function results in embryonic lethality, which indicates that *mirror* function is important during early development. We therefore examined *mirror* expression during embryogenesis by in situ hybridization. *mirror* expression was detected first at the cellular blastoderm stage as an anterior ventral patch at the site of the presumptive anterior midgut invagination. Shortly thereafter, *mirror* is expressed at the dorsal folds prior to their formation (Fig. 4A). *mirror* continues to be expressed in these tissues (Fig. 4B,C). *mirror* is expressed in a segmental pattern by the beginning of stage 10 (Fig. 4D) and then later in delaminating neuroblasts (Broadus et al. 1995). *mirror* is also expressed at this time in more dorsal groups of cells. As the embryo undergoes germ-band retraction, *mirror* expression is retained in the nerve cord and appears transiently in the proventriculus as it undergoes folding (Fig. 4E).

mirror is also expressed in a complex pattern in larvae and adults. This pattern includes portions of the wing, haltere, and genital imaginal discs, as well as the dorsal anterior follicle cells in the ovary (data not shown). A

Figure 4. In situ hybridization with riboprobes to staged embryos reveals dynamic expression of *mrr* during embryogenesis. Embryos are oriented so that dorsal is *up* and anterior is to the *left*. Embryos were staged according to Campos-Ortega and Hartenstein (1985). (A) Expression at dorsal folds and anterior midgut invagination; (B) early gastrulation maintains pattern at dorsal folds and anterior midgut invagination; (C) early germ-band extension maintains expression at dorsal folds and anterior midgut invagination; (D) *mrr* is expressed in a segmentally repeated pattern in a germ-band extended, early stage 11 embryo; (E) stage 13 embryos show strong staining in the proventriculus and weak staining in anterior portion of the ventral nerve cord; (F) costaining with antibodies to *engrailed* (brown) and in situ *mrr* probes (blue) demonstrate that *mrr* is expressed at the anterior border of the segment.

role for *mrr* function in the development of these tissues is reflected by defects in these structures in *mrr*^{P1} flies. These defects include held-out wings, missing thoracic bristles, crumpled halteres, and the production of eggs lacking dorsal appendages and anterior chorion.

mrr function in embryonic segmentation

The expression pattern of *mrr* suggests that its function may be important for many steps in development. We were particularly interested in the expression of *mrr* in each segment. To determine where in the segment *mrr* is expressed, we costained wild-type embryos with antibodies to *Engrailed*, which defines the posterior border of each segment, and with *mrr* riboprobes. This experiment revealed that *mrr* expression is adjacent and posterior to *Engrailed* in every segment (Fig. 4F). This placed *mrr* expression at the anterior border of each segment.

To determine whether *mrr* expression in the segment is important for patterning, we examined the cuticles of *mrr* loss-of-function embryos. *mrr* mutant cuticles were smaller than wild type and had sparse denticles (Fig. 5). Anterior denticles were often missing from each segment, and denticle rows 2 and 3 often were fused together. These defects in *mrr* mutant embryos confirm that *mrr* has a role in patterning within segments. Interestingly, a small percentage of embryos also had fusions of adjacent segments (Fig. 5E), suggesting that *mrr* may also play a role in defining the border between segments.

Discussion

The *Drosophila* eye is divided precisely into dorsal and ventral mirror-image fields, which are separated by a

sharp boundary known as the equator. We have identified a novel homeobox-containing gene, *mrr*, which functions to define the equator. Three lines of evidence suggest that a border of *mrr* expression defines the equa-

Figure 5. Loss of *mrr* results in segmental defects. (A) Wild-type cuticle segments A5; (B) *mrr* mutant cuticle segment A5; (C) wild-type cuticle segment A6; (D) *mrr* mutant cuticle segment A6; (E) rare fusion event in *mrr* mutant embryo. Abdominal segment A4 and A5 have fused partially. Embryos were collected for 3 hr and aged for 28 hr, dechorionated, devitellinized, and mounted in Hoyer's/lactic acid.

tor. First, the expression of *mrr* is consistent with a role in defining the equator. *mrr* is expressed in the dorsal half of the eye days before D/V asymmetries become apparent, and expression ceases once these asymmetries are established. Furthermore, *mrr* expression terminates at the midpoint of the eye, producing a natural border of *mrr* expression where the equator will form. Second, our mosaic analysis has revealed that changes in *mrr* expression can create ectopic equators or alter the path of the normal equator. For example, *mrr* loss-of-function clones situated in dorsal regions of the eye can produce an ectopic equator along the side of the clone that faces the normal equator. In addition, when the normal equator enters a region of *mrr* loss of function, the path of the equator is diverted toward the new border of *mrr* expression. Third, ectopic expression studies are consistent with the hypothesis that a border of *mrr* expression defines the equator. Ectopic expression of *mrr* in a small region at the ventral margin of the eye results in the adoption of dorsal polarity and chirality of some nearby ommatidia. This suggests that an ectopic equator is being defined in this region. In contrast, ubiquitous expression of *mrr* led to rough and disordered eyes, which suggests that spatial restriction of *mrr* expression is important for the ability of *mrr* to define a border. Together, these data suggest a model in which the juxtapositioning of *mrr*-expressing and -nonexpressing cells defines a border.

One important aspect of the induction of ectopic borders in *mrr* clones is that only the edge of the clone that is parallel to the normal equator produces an ectopic equator. One interpretation of this observation is that the polarizing signal is transmitted from an equator toward the dorsal and ventral poles. Consistent with this idea is the observation that clones of the tissue polarity mutant, *frizzled*, behave as though a signal is being transmitted from the equator (Zheng et al. 1995).

Although our results suggest that juxtaposing *mrr*-expressing and -nonexpressing cells can define a border, it is not always sufficient. Only clones in the anterior region form ectopic equators. All clones in the dorsal anterior-most region of the eye developed ectopic equators at their ventral face. One explanation for this restriction may be that *mrr* is not the sole determinant of the equator. In this regard, it is interesting to note that *ara* and *caup* are also expressed in dorsal regions of the eye (J. Gomez-Skarmeta and J. Modellel, pers. comm.). In addition, there is evidence for the differential expression of other genes along the D/V axis of the eye (Sun et al. 1995; Brodsky and Steller 1996). Such genes might contribute to equator formation. Alternatively, as our alleles are not entirely lacking *mrr* transcript, the possibility remains that clones without any *mrr* function could induce equators throughout the dorsal half of the eye.

How is the equator formed?

We propose that dorsal identity is determined through a gradient of a dorsal morphogen, which activates *mrr* transcription in a graded fashion. If *mrr* is then able to

regulate its own transcription (a characteristic of many transcription factors), this could result in a sharp border of *mrr* expression. Interactions between the *mrr*-expressing and -nonexpressing cells could then lead to the production of signals that pattern the eye. Three observations are consistent with this model. First, a potential *Mrr*-binding site (based on the defined DNA binding site of Ara; Gomez-Skarmeta et al. 1996) is present in the *mrr* upstream region (H. McNeill, unpubl.). Second, *mrr* expression includes both a gradient that is strongest in the most dorsal regions and a sharp termination at the equator (see Fig. 1E,F). Third, there are several enhancer trap lines that are expressed at the equator, which provide candidates for control by *Mrr*.

Previous studies have been divided as to how D/V polarity and the equatorial boundary are established (Chanut and Heberlein 1995; Ma and Moses 1995; Strutt and Mlodzik 1995; Wehrli and Tomlinson 1995). Some groups suggest that D/V polarity responds to global coordinates, whereas others propose that D/V polarity results solely from progression of the morphogenetic furrow. The restriction of *mrr* expression to dorsal cells during the second larval instar clearly indicates that D/V differences in positional identity exist prior to the furrow.

The mechanism of D/V boundary determination in the wing may provide a model for understanding equator formation in the eye. Dorsal identity in the wing is controlled by homeodomain protein Apterous, which is expressed solely by dorsal cells (Bourgouin et al. 1992; Cohen et al. 1992; Williams et al. 1993). The juxtaposition of Apterous-expressing and -nonexpressing cells defines the D/V border in part by directing dorsal expression of the secreted protein Fringe (Diaz and Cohen 1993; Blair et al. 1994; Irvine and Wieschaus 1994; Kim et al. 1995). Fringe then directs patterning at the D/V border in part by controlling the Notch pathway (Kim et al. 1995, 1996). Because Apterous is not expressed in the eye, it is interesting to speculate that *mrr* may control D/V border formation in the eye by controlling some of these same genes. However, there are some differences in the actions of *apterous* and *mrr*. Although the equator can cross a clone of *mrr* cells, the D/V border of the wing cannot cross through an *apterous* clone. Another difference is that cells within dorsal *apterous* clones have ventral identity while ommatidia within dorsal *mrr* clones always retain dorsal morphology. However, the lack of independent markers for D/V identity in the eye prevents us from ruling out the possibility that the ommatidia within the dorsal *mrr* clones are actually ventral with respect to the ectopic equator.

Mrr in the embryo

Our study of *mrr* function in the eye has led us to propose that *mrr* acts to define the equatorial border. Our *in situ* analysis of *mrr* expression demonstrated that *mrr* is also expressed at another well-studied boundary, the segmental border. In this case, *mrr* is expressed at the anterior side of the border. The time of onset of *mrr* expres-

sion in the segment is after the establishment of parasegmental signaling, but prior to and during the determination of the segmental border and the refinement of Engrailed and Wingless expression. As the border between Engrailed- and Wingless-expressing cells determines the parasegmental boundary, so might the juxtapositioning of *mrr*-expressing and -nonexpressing cells be important for defining the segmental border. Consistent with this hypothesis is our observation that *mrr* mutant embryos have segmental patterning defects. Our data in the D/V boundary in the eye and on the segmental border in the embryo suggest that *mrr* may function in both of these systems to define borders.

Materials and methods

Isolation and analysis of *mrr* mutations

The *mrr*^{P1} insertion was obtained during a screen for imprecise excision of a P element in the *Src64* locus, and was identified by the dorsal specific expression of the *white* gene. No *Src64* interactions are observed. The P-element insertion site was localized to 69D on polytene chromosomes by in situ hybridization using biotin-labeled probes specific for P-element sequences and *mrr* cDNA probes.

mrr^{P1} homozygous flies have numerous subtle defects. Flies have abnormal scutellar bristles, drooping halteres, dichae wings, and are missing alulae. Homozygous mothers lay eggs lacking dorsal appendages and often have an open anterior chorion. Hatch rate analysis indicates that <30% of eggs laid by homozygous mothers hatch. Reciprocal crosses to *w*¹¹¹⁸ stocks demonstrated that this is a maternal effect. Pseudopupul (Franceschini and Kirschfeld 1971) analysis of eyes indicated occasional subtle defects in the orientation of ommatidia and the regularity of the equator.

To obtain stronger *mrr* alleles the *mrr*^{P1} insertion was mobilized using the $\Delta 2-3$ transposase source (Robertson 1988) and excisions were identified by loss of *white* expression.

Immunohistochemistry and in situ analysis

Digoxigenin-labeled *mrr* riboprobes were made using the Boehringer Mannheim Genius kit, as described. In situ hybridizations utilized antisense-strand riboprobes and were performed according to standard procedures (Tautz and Pfeifle 1989). Cuticle preparations in the eggshell were obtained by collecting the progeny of *mrr*^{e48}/*w*¹¹¹⁸ for 3 hr, aging embryos for 28 hr, devitellinizing, dechorionating, and mounting in Hoyers/lactic acid (Wieschaus and Nüsslein-Volhard 1986). Cuticle preparations were viewed after 24 hr at 65°C on a slide warmer.

Analysis of *mrr*^{P1} β -galactosidase activity

Imaginal discs were dissected from third instar *mrr*^{P1} homozygous larvae in phosphate-buffered saline (PBS), fixed in 1% glutaraldehyde for 5 min at 22°C, and rinsed three times in PBS. Discs were immersed in X-gal staining solution for 1–3 hr at 22°C. β -Galactosidase staining of *mrr*^{P1} and *mrr*^{P2} flies demonstrated that *mrr* is expressed in wing imaginal discs in dorsal and peripheral regions, in ovaries in the anterior dorsal follicle cells, in genital disc in a lateral stripe, in the larval CNS, and intestinal tract. We did not observe staining in leg discs.

Analysis of 30A *Gal4*: UAS *mrr* flies

30A *Gal4*:UAS (upstream activating sequence) *mrr* flies were obtained from a cross between 30A *Gal4*/Cyo flies and UAS *mrr*/TM3 flies. The resulting flies were raised at 18°C for 2 weeks and shifted to 23°C until eclosion. Flies carrying *mrr* expressed under the 30A *Gal4* control were identified by lack of TM3 and Cyo markers. Eyes from five flies were fixed with glutaraldehyde and osmium tetroxide as described (Tomlinson and Ready 1987) and analyzed in 1 μ m sections under a light microscope.

Clonal analysis

Mitotic clones were generated by irradiating the progeny of males (homozygous for a *white*⁺ P element at 70°C) crossed to *w*⁻; *mrr*^{e48}/TM3 females. Embryos were collected for 24 hr, aged for 24 hr, and irradiated with 1000 rads. This generated *mrr* mutant clones marked by the loss of *white*. The positions of clones were marked prior to embedding, and standard histological methods were used for sections of adult eyes (Tomlinson and Ready 1987).

Molecular biology

Genomic DNA flanking *mrr*^{P1} (a 1.5-kb *Xho*I rescue fragment) was isolated by plasmid rescue (Pirrotta 1986) and used as a probe to screen a genomic library cloned into the λ FixII vector (Stratagene, Inc.). A 4.5-kb genomic fragment was then used to probe an eye imaginal disc cDNA library in λ gt10 (constructed by Dr. A. Cowman, Walter and Eliza Hall Institute, Melbourne, Australia). *Eco*RI fragments of *mrr* cDNA were cloned into Bluescript (Stratagene, Inc.) and used for sequencing and production of riboprobes. Molecular analysis of *mrr*^{P1} imprecise excisions was done using genomic Southern analysis. Additional genomic DNA was obtained by screening P1 cosmid libraries with a *mrr* cDNA. P1C was subcloned as a *Sac*-*Cla* fragment from P1446. Manipulations of DNA and RNA were done according to the protocols of Sambrook et al. (1989). Expression constructs containing *mrr* cDNA were made by fusing the two fragments of *mrr* cDNA obtained from *Eco*RI and *Hind*III subcloning of the λ gt10 cDNA library to a common *Hind*III site. The construct containing the 5'-untranslated region (UTR), the entire coding region of *mrr* and 0.7 kb of 3' UTR were cloned into the *Kpn*I site of pHXK and subcloned into the *Not*I site of hscasPer. The same *Kpn*I fragment was cloned into the *Kpn*I site of the *Gal*UAS vector pUAST (Brand and Perrimon 1993). Constructs were injected into *w*¹¹¹⁸ flies using standard methods (Rubin and Spradling 1982).

Sequencing

All DNA sequences were determined using the dideoxy chain termination method with a Sequenase kit (U.S. Biochemical). Templates for the determination of the 3505-bp *mrr* cDNA sequences were generated by sonication of plasmid DNA and insertion of the sonicated fragments into the vector M13mp10. The entire sequence of *mrr* was determined on both strands. Compressions were eliminated using a combination of deaza-nucleotide and dITP nucleotides and analysis of templates on formamide-containing gels. The genomic structure of *mrr* was determined through a combination of exonuclease-deletion of the 5.5-kb plasmid rescue fragment of *mrr*^{P2} and primer-based sequencing of the remainder of *mrr* using oligonucleotide primers on genomic subclone P1C.

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Note added in proof

The sequence data described in this paper have been submitted to GenBank under accession no. U95021.

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