Pointed regulates an eye-specific transcriptional enhancer in the *Drosophila hedgehog* gene, which is required for the movement of the morphogenetic furrow

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Summary

Drosophila **development depends on stable boundaries between cellular territories, such as the embryonic parasegment boundaries and the compartment boundaries in the imaginal discs. Patterning in the compound eye is fundamentally different: the boundary is not stable, but moves (the morphogenetic furrow). Paradoxically, Hedgehog signaling is essential to both: Hedgehog is expressed in the posterior compartments in the embryo and in imaginal discs, and posterior to the morphogenetic furrow in the eye. Therefore, uniquely in the eye, cells receiving a Hedgehog signal will eventually produce the same protein. We report that the mechanism that underlies this difference is the special regulation of *hedgehog* (*hh*) transcription through the dual regulation of an eye specific enhancer. We show that this enhancer requires the Egfr/Ras pathway transcription factor Pointed. Recently, others have shown that this same enhancer also requires the eye determining transcription factor Sine oculis (So). We discuss these data in terms of a model for a combinatorial code of furrow movement.**

Key words: *Hedgehog*, Pointed, *Drosophila*, Eye, Morphogenetic furrow, Transcription, Enhancer

Introduction

The *Drosophila* compound eye is comprised of about 750 similar facets or ommatidia, each containing eight photoreceptor neurons and 12 other cells (Wolff and Ready, 1993). Patterning in the presumptive eye epithelium begins in the third larval instar when a wave of cell-type specification and patterning passes across the eye, from posterior to anterior: the morphogenetic furrow (Ready et al., 1976). A key event in the furrow is the specification of a spaced array of single cells via Notch-mediated lateral inhibition, which become the ommatidial founder cells, later to differentiate as the first photoreceptor neuron, the R8 (Cagan and Ready, 1989; Baker et al., 1996). The furrow lays down a new column of R8/founder cells roughly every 2 hours (Ready et al., 1976; Basler and Hafen, 1989).

Posterior to the furrow, after the founder cell is specified, the rest of the ommatidial cells are recruited by successive rounds of Ras pathway induction, beginning with the other seven photoreceptors (Ready et al., 1976; Tomlinson, 1985; Tomlinson, 1988; Wolff and Ready, 1993; Voas and Rebay, 2004). In all cases, this Ras signal involves MAPK phosphorylation and the activation of the Ets domain transcription factors *pointed* and *anterior open* (also known as Yan) (O'Neill et al., 1994; Dickson, 1995; Rubin et al., 1997). An early effect in these receiving cells is the upregulation of Pointed, and later the cells differentiate (Tomlinson and Ready, 1987). In contrast to the other photoreceptors, the R8 does not require the Egfr/Ras pathway or Pointed for its initial specification or early neural differentiation (but does require Egfr signaling later for its maintenance) (Kumar et al., 1998; Baonza et al., 2001; Yang and Baker, 2001).

Progression of the morphogenetic furrow depends on *hedgehog* signaling: if *hedgehog* function is removed by means of a conditional mutation, mosaic clones or by an eye specific allele, the furrow arrests (Heberlein et al., 1993; Ma et al., 1993). If, anterior to the furrow, *hedgehog* is ectopically and locally expressed, or if the negative *hedgehog* pathway elements *patched* or *Pka* are locally removed, an ectopic furrow propagates away from the triggering site in all directions (Heberlein et al., 1995; Li et al., 1995; Ma and Moses, 1995; Strutt et al., 1995). From in situ hybridization and the expression of two lacZ enhancer-trap lines, *hedgehog* is thought to be expressed in all the developing photoreceptor cells posterior to the furrow (Lee et al., 1992; Heberlein et al., 1993; Ma et al., 1993). Thus, *hedgehog*, expressed posterior to the furrow is necessary and sufficient for furrow progression.

Cells anterior to the furrow respond to the Hedgehog signal via the receptor Smoothened (Smo) and other downstream elements, including the transcription factor Cubitus interruptus (Ci) (Strutt and Mlodzik, 1996; Strutt and Mlodzik, 1997; Fu

and Baker, 2003; Lum and Beachy, 2004). Cells in and anterior to the furrow respond to the *hedgehog* signal and activate the expression of several target genes, including *hairy*, *decapentaplegic*, *patched* and *atonal* (Heberlein et al., 1993; Ma et al., 1993; Ma and Moses, 1995; Baker and Yu, 1997; Shyamala and Bhat, 2002). In addition to furrow progression, *hedgehog* signaling from outside the eye field is required for furrow initiation (from the posterior disc margin) (Domínguez and Hafen, 1997; Borod and Heberlein, 1998; Chen et al., 1999; Curtiss and Mlodzik, 2000; Pappu et al., 2003). On the margins of the eye disc Wingless signals antagonize *hedgehog* and limit the rate of furrow progression (Ma and Moses, 1995; Treisman and Rubin, 1995).

Decapentaplegic expressed in the furrow was first thought to act as a second signal to relay the *hedgehog* signal forward (Blackman et al., 1991; Heberlein and Moses, 1995). However, *decapentaplegic* pathway loss of function does not arrest the furrow and the ectopic expression of *decapentaplegic* anterior to the furrow does not produce an ectopic furrow that propagates away from this source, but rather begins some distance away at the eye disc margin (Burke and Basler, 1996; Wiersdorff et al., 1996; Chanut and Heberlein, 1997b; Pignoni and Zipursky, 1997; Fu and Baker, 2003). Thus, unlike *hedgehog*, *decapentaplegic* is neither necessary nor sufficient for furrow progression at the center of the disc, but may be downstream of and redundant to *hedgehog* (Greenwood and Struhl, 1999). *decapentaplegic* is also expressed at the eye disc margin and may be more directly involved in furrow initiation and progression there, together with members of the retinal determination gene complex, such as *dachshund* (Mardon et al., 1994; Wiersdorff et al., 1996; Chanut and Heberlein, 1997b; Chanut and Heberlein, 1997a; Pignoni and Zipursky, 1997).

Thus, in the developing eye Hedgehog signaling drives a moving wave: the morphogenetic furrow. Cells that once received a Hedgehog signal on the anterior side will later express Hedgehog on the posterior side; essentially a cyclic and progressive phenomenon. By contrast, in the embryonic cuticle and imaginal discs, Hedgehog, Decapentaplegic and Wingless also interact, producing stable (not moving) boundaries, to form segments and/or lines of clonal restriction (Morata and Lawrence, 1977; García-Bellido et al., 1979; Lawrence, 1981; Akam, 1987; Ingham and Martinez-Arias, 1992; Kornberg and Tabata, 1993; DiNardo et al., 1994; Perrimon, 1994; Hidalgo, 1996). Unlike the eye, in these places a cell that receives Hedgehog does not later send it and there is no progressive cyclical process (Burke and Basler, 1997).

We suggest that there may be a special mode of *hedgehog* regulation so that in the eye (and nowhere else) a cell that receives Hedgehog will later express it (after a delay). A mechanism for this may be a dually regulated, eye-specific transcriptional enhancer for the *hedgehog* gene. This enhancer would require two simultaneous inputs to become active: one that is eye specific and another that is downstream of *hedgehog* (directly or indirectly). To test this possibility, we took advantage of two alleles of *hedgehog* that stop the furrow, but that have little or no phenotypic effect outside the eye. Both of these mutations delete DNA from the same 1.9 kb region of the first intron. We therefore reasoned that the regulatory elements responsible for a unique mode of *hedgehog* expression in the eye may reside in this region.

Here, we show that a 1.9 kb region of the first intron of *hedgehog* is necessary and sufficient to direct reporter *lacZ* expression posterior to the furrow in the developing eye, but that it is inactive (almost) everywhere else. We find that a minimal sequence of 203 bp confers this regulation and contains three consensus Ets domain transcription factor binding sites. We show that this enhancer drives expression in all the ommatidial cells except the R8: the pattern of Egfr/Ras pathway signaling and Pointed activation. We show that the three Ets sites in the minimal fragment are bound by Pointed in vitro, and that the function of this enhancer genetically requires *pointed* in vivo. Pointed is a major effector of photoreceptor differentiation and is therefore downstream of the furrow and indirectly downstream of *hedgehog* function. This fragment also contains one of the two So sites recently shown to be required by others (Pauli et al., 2005). We propose that So and Pointed confer eye-specific dual regulation on *hedgehog* and may explain why the furrow moves, and is not a stationary compartment boundary.

Materials and methods

Drosophila stocks and mosaic analysis

hh^{+} stocks

Canton-S, w^{1118} and w^{1118}; *P{(w, ry)D}3* gl^{3} *e*.

hedgehog mutant stocks

w^{1118}; hh^{bar3}, w^{1118}; hh^{fse}, w^{1118}; hh^{8}/TM6 *Tb Hu* and w^{1118}; hh^{AC}/TM3 *Sb*.

dpp reporter

dpp:lacZ cn; ry^{506} (BS3.0 from Blackman et al., 1991). For, P-element transformation, ry^{506} were injected.

Genotype for the *bar3:lacZ pointed* null clones

y w ey:FLP; *bar3:lacZ*; *P{neoFRT}82B* $pnt^{delta88}$/*P{neoFRT}82B P{Ubi-GFP(S65T)nls}3R*.

SEM and facet counts

Scanning electron microscopy (SEM) was as described previously (Tio and Moses, 1997). Statistical analyses of ommatidium numbers by calculating the mean (μ), standard deviation (s.d.) and 95% confidence interval range (95%CI). For each genotype, one right eye each from three females was counted. hh^{fse}/+: μ=736.0, s.d.=19.61, 95%CI 698-774; hh^{bar3}/+: μ=725.33, s.d.=5.31, 95%CI 715-736; hh^{8}/+: μ=717.67, s.d.=5.79, 95%CI 706-729; wild type: μ=710.0, s.d.=8.98, 95%CI 692-728; hh^{AC}/+: μ=640.67, s.d.=39.38, 95%CI 563-718; hh^{fse}/hh^{8}: μ=511.67, s.d.=9.74, 95%CI 493-531, hh^{fse}/hh^{fse}: μ=498.33, s.d.=18.73, 95%CI 462-535; hh^{bar3}/hh^{8}: μ=340.67, s.d.=15.8, 95%CI 310-372; hh^{fse}/hh^{bar3}: μ=241.0, s.d.=2.94, 95%CI 235-247; hh^{bar3}/hh^{bar3}: μ=232.0, s.d.=23.76, 95%CI 185-279; hh^{fse}/hh^{AC}: μ=159.67, s.d.=8.58, 95%CI 143-176; hh^{bar3}/hh^{AC}: μ=133.67, s.d.=5.44, 95%CI 123-144.

Microscopy, antibodies and immunohistochemistry

Embryos and eye discs were as described previously (Kumar and Moses, 2001). Eye discs were mounted in Vectashield (Vector Labs, H-1000), imaged by confocal microscopy (Zeiss LSM510) or by DAB staining with Ni/Co, then DPX (Zeiss). Primary antibodies used were rabbit anti-Hedgehog (1:625, a gift from I. Guererro), mouse anti-Boss for R8 (1:1000, a gift from S. L. Zipursky) (Cagan et al., 1992), mouse anti-β-gal (1:1000, Promega 23783), rabbit anti-β-gal (1:1,000 Cortex BioChem CA2196), rat monoclonal anti-Elav 7E8A10 (1:1000 from DSHB) (Bier et al., 1988), guinea pig anti-Senseless, (1:1000, a gift from G. Mardon) (Nolo et al., 2000), mouse anti-Futsch (1:100,

also known as 22C10, a gift from S. L. Zipursky) (Fujita et al., 1982). Secondary antibodies (Jackson ImmunoResearch): goat anti-mouse HRP (1:40, 115-035-003), goat anti-mouse minX FITC (1:200, 115-095-166), goat anti-rabbit HRP (1:100,111-035-003), goat anti-guinea pig FITC (1:200, 106-095-003), goat anti-rat Cy5 (1:200, 112-175-003) and goat anti-rabbit TRITC (1:250, 111-025-003).

Plasmids

Deletions in hh^{bar3} (6053-7938) and hh^{fse} (6456-7469) were determined by PCR and sequencing from *P{(w, ry)D}3 gl*^{3} *e* (bases numbered from the site of P30) (Lee et al., 1992). Four putative Ets protein binding sites [5′-(C/G)(A/C/G)GGA(A/T)(A/G)-3′ (Xu et al., 2000)] were found at 6296, 6308, 6330 and 7034. The sequences of transgene constructs, all inserted into *Not*I site of pDM30hslacZ (Bowtell et al., 1989), were amplified by PCR from Canton-S DNA, with engineered *Not*I sites, to yield pDM30(bar3)hslacZ, pDM30(fse)hslacZ, pDM30(bar3L)hslacZ, pDM30(bar3R)hslacZ, pDM30(bar3L1)hslacZ and pDM30(bar3L2)hslacZ (Fig. 1A). For Ets site deletion construct, pDM30(bar3L2deltaETS)hslacZ, oligonucleotides with substitutions in the three Ets sites (see Fig. 6) were assembled into 203 bp Bar3L2deltaEts fragment (with engineered *Not*I sites). For pDM30(6xETS)hslacZ, an oligo with six tandem consensus Ets binding sites was synthesized. All constructs were confirmed by DNA sequencing. Positions of constructs was: pDM30(bar3)hslacZ, 6053-7938; pDM30(fse)hslacZ, 6456-7468; pDM30(bar3L)hslacZ, 6053-6455; pDM30(bar3R)hslacZ, 7469-7938; pDM30(bar3L1)hslacZ, 6053-6252; pDM30(bar3L2)hslacZ and pDM30(bar3L2deltaETS)hslacZ, 6253-6455. The oligonucleotide sequences used, with the Not1 sites italicized and mutagenic base changes underlined, were as follows. For pDM30(bar3)hslacZ, 6053AForward (AAAAAA*GCGGCCGC*-AGGGTGGGAAAAAGGCCCGC) and 7938AReverse (AAAAA-GCGGCCGCGGATCCGCGACACGAAGATCCTTTTC); for pDM30(fse)hslacZ, 6456Forward (AAGAAAAAA*GCGGCCGC*-*TCTAGA*AGCTTATATATAAAAAAAGGGGGTGACTCCCC) and 7469Reverse (AAGGAAAAAA*GCGGCCGC*GAATTCTGCGCT-GGACGCGCAATGAAC); for pDM30(bar3L)hslacZ, 6053BForward (CCGCGGCCGCAGGGTGGGAAAAAGGCCCG) and 6456Reverse (CCGCGGCCGCACATATATGTATGTATATATGC-AGC); for pDM30(bar3R)hslacZ, 7469Forward (CCGCGGCCGCTCGATTCG-AATTCGAGCTCAATGCA) and 7938BReverse (CCGCGGCG-CCTTGCGACACGAAGATCCTTTTCTTC); for pDM30(bar3L1)-hslacZ, 6053BForward (above) and 6253Reverse (CCGCGGCCG-CCCCACCTAAACGATTCACACACACA); for pDM30(bar3L2)-hslacZ, 6253Forward (CCGCGGCCGCTGACGTGATTTCTTCAG-AGTTTCAACTCG) and 6456Reverse (above); for pDM30(bar3L2deltaETS)hslacZ, 6258MutagenicForward (TGATTT-CTTCAGAGTTTCAACTCGTATTTTTTCGACTATCACGTGTGT-CGCTGCGCAAGTTGTAAGTTTT), 6363MutagenicReverse (CC-TTTGATTCACGGCACTGATTGAGATCGCAGAGCATGCGAAA-ACTTACAACTTGCGCAGCGACA), 6294Reverse (AGTCG-AAAAAATACGAGTTGAAACTCTGA), 6337Forward (TGCGAT-CTCAATCAGTGCCGTGAATC), 6053BForward (above), 6253Forward (above) and 6456Reverse (above); for pDM30(6xETS)hslacZ, 6XForward (GGCCCAGGAAGCCAGG-AAGTCAGGAAGCCAGGAAGTCAGGAAGCCAGGA-AGT) and 6XReverse (GGCCACTTCCTGGCTTCCTGACTTCCTGGCTTCC-TGACTTCCTGGCTTCCTG).

Transformation

Embryo injections were as described previously (Rubin and Spradling, 1982) with constructs, at 1:1 ratio (500 μg/ml each) with S129A enhanced P-transposase plasmid (Beall et al., 2002). Numbers of independent transgenic lines for each construct were as follows: pDM30(bar3)hslacZ, 13; pDM30(fse)hslacZ, 7; pDM30(bar3L)hslacZ, 5; pDM30(bar3R)hslacZ, 4; pDM30(bar3L1)hslacZ, 2; pDM30(bar3L2)hslacZ, 6; pDM30(bar3L2deltaETS)hslacZ, 5; pDM30(6xETS)hslacZ, 3. Some lines have additional, ectopic patterns that we attribute to enhancer trap effects, e.g. 2/13 of the pDM30(bar3)hslacZ lines. In all cases, we studied multiple independent lines.

EMSA (gel shift)

GST-PntP2 protein, expressed in *E. coli*, purified as described by (Kauffmann et al., 1996). The DNA probe was from positions 6234 to 6347, and was end labeled (gamma-^{32}P-ATP, T4 Polynucleotide Kinase, New England Biolabs). The 203 bp wild-type and delta-Ets competitor DNAs were synthesized by PCR from pDM30(bar3L2)hslacZ, pDM30(bar3L2deltaETS)hslacZ. The oligo competitors were the unlabelled annealed sequences given below. The protocol was as described previously (Buratowski and Chodosh, 2002), modified as follows: the binding reactions were in 18 μl total volume in 'binding buffer' (Xu et al., 2000) comprising 13 mM HEPES (pH 7.9), 40 mM KCl, 44 mM NaCl, 4.4 mM Tris (pH 7.5), 0.7 mM EDTA, 0.3 mM DTT and 9% glycerol. Poly dI-dC (1 μg), 5 μg of BSA and double-stranded labeled probe (6 fmol, 8000 CPM) were added to each reaction with unlabeled DNA competitors, as indicated. GST-PntP2 (200 fmol) was added last. Incubations were for 30 minutes at room temperature. Samples analyzed by 4% non-denaturing, poly-acrylamide gel and dried for autoradiography. The oligonucleotide used for wild type was TTTTTCGACTATATCCTGT-GTCGCTTCCTCAGTTTAAGTTTTCGCTTCCTCTGCGATCCAA. The oligonucleotide used the Ets sites mutant was TTTTT-CGAC-TATCACGTGTGTCGCTGCGCAAGTTGTAAGTTTTCGCAGCT-CTGCGATCTCAA.

Results

eye-specific *hedgehog* mutations: lesions and allelic series

There are two known eye-specific *hedgehog* (*hh*) mutations: hh^{bar3} (also known as hh^{1}) and $hh^{furrow\ stops\ early}$ (or hh^{fse}). Both are associated with deletions in the first intron (Fig. 1A) (Lee et al., 1992; Ma et al., 1996). hh^{bar3} is a homozygous viable allele with a strong recessive eye phenotype resulting from arrest of the morphogenetic furrow (Ives, 1950; Mohler, 1988; Heberlein et al., 1993). hh^{fse} is a gamma-induced viable allele with a weaker eye phenotype (Ma et al., 1996). We used PCR and direct sequencing to determine the precise end-points of the deletions (see Materials and methods). The hh^{bar3} deletion is 1885 bp and the hh^{fse} deletion lies within the span of hh^{bar3}, but is shorter (1013 bp, Fig. 1A). Both hh^{bar3} and hh^{fse} are viable and can be maintained as homozygous stocks, although they are not as vigorous as wild type. This is probably not due to second-site recessive lethal mutations, as we derived lines that are isogenic for the X and major autosomes and they are no more vigorous. We examined the cuticles and nervous system (by anti-Elav and anti-Futsch stains) of the isogenic hh^{bar3} and hh^{fse} embryos, and found no detectable phenotypes (data not shown).

To determine if either of these two eye-specific alleles are null for *hedgehog* function in the eye, we derived all viable pair-wise combinations of these alleles, wild-type and two zygotic lethal alleles (hh^{AC} and hh^{8}). hh^{AC} is a single gene deletion that removes both the start sites for transcription and translation (Fig. 1A) (Lee et al., 1992). hh^{8} (also known as hh^{13C}) is a chain-terminating mutation in the coding sequence (Fig. 1A) (Lee et al., 1994). Both alleles are zygotic lethal with strong cuticle phenotypes. hh^{AC} is thought to be a null because of the strength of its phenotype and the nature of its lesion (Lee et al., 1994). On phenotypic grounds and comparison with

other alleles, five groups have also reported hh^8 to be functionally amorphic (Mohler, 1988; Lee et al., 1992; Heemskerk and DiNardo, 1994; Hooper, 1994; Park et al., 1996).

We find that these alleles form a series for adult eye phenotype (Fig. 1B-I). We quantified this by counting eye facets in adult females (Fig. 1J) and find that hh^{fse}, hh^{bar3} and hh^8 heterozygotes are not significantly different from wild type. However, hh^{AC} is slightly dominant, with an eye that is about 10% smaller than wild type (although this difference is not statistically significant, see 95% confidence limits in the Materials and methods, and in Fig. 1J).

By facet number, hh^{bar3} is a strong, eye-specific hypomorph. It is fully recessive in trans to wild type, has a severely reduced eye when homozygous (68% smaller than hh^{bar3}/hh^+) and in trans to the null hh^{AC} it is smaller still (82% smaller than hh^{bar3}/hh^+). This suggests that hh^{bar3} is not an amorph for eye size by Muller's test: the phenotype becomes stronger in trans to the null (Muller, 1932). hh^{fse} is similar to but weaker than hh^{bar3}: the hh^{fse} homozygous eye is only 32% smaller than hh^{fse}/hh^+ and in trans the null (hh^{AC}), it is further reduced to 78%. Thus, by both measures (phenotype as a homozygote and in trans to a null), hh^{bar3} is a strong hypomorphic allele and hh^{fse} is a weaker hypomorph. From the 95% confidence limits, all these results are statistically significant.

Another way to view these eye specific mutations is by the number of columns of ommatidia produced, a more direct measure of how far the furrow progresses in the larval disc. Wild-type eyes have about 28 to 30 columns, hh^{fse} homozygotes have about 20, hh^{bar3} homozygotes have about 10 and the strongest mutant genotype we have studied (hh^{bar3}/hh^{AC}) has about six. The furrow may stop early in hh^{fse}, but it stops much earlier in hh^{bar3}.

More interesting are the anomalous phenotypes of both eye-specific alleles when placed in trans to the second purported null (hh^8): hh^{fse}/hh^8 has significantly larger eyes (512 mean facets, or 30% fewer than hh^{fse}/hh^+) than hh^{fse}/hh^{AC} (160 mean facets or 78% fewer than hh^{fse}/hh^+), and hh^{bar3}/hh^8 has significantly larger eyes (341 mean facets, or 53% fewer than hh^{bar3}/hh^+) than hh^{bar3}/hh^{AC} (134 mean facets or 82% fewer than hh^{bar3}/hh^+). Thus, although hh^8 may be functionally amorphic for non-eye phenotypes it appears to be much weaker than hh^{AC} in this assay. This may be due to transvection (Lewis, 1954; Duncan, 2002). It could be that hh^{AC} deletes cis-regulatory elements (in addition to the coding sequence), which are also deleted from hh^{bar3} and hh^{fse}, while hh^8 leaves these putative regulatory elements intact. Thus, hh^8 may supply some degree of essential regulation in trans when heterozygous to hh^{bar3} or hh^{fse}, both of which have their coding sequences intact.

Two mechanisms suggest themselves for the apparent eye-specificity of hh^{bar3} and hh^{fse}.

(1) It is possible that the requirement for *hedgehog* function is higher in the eye than in the

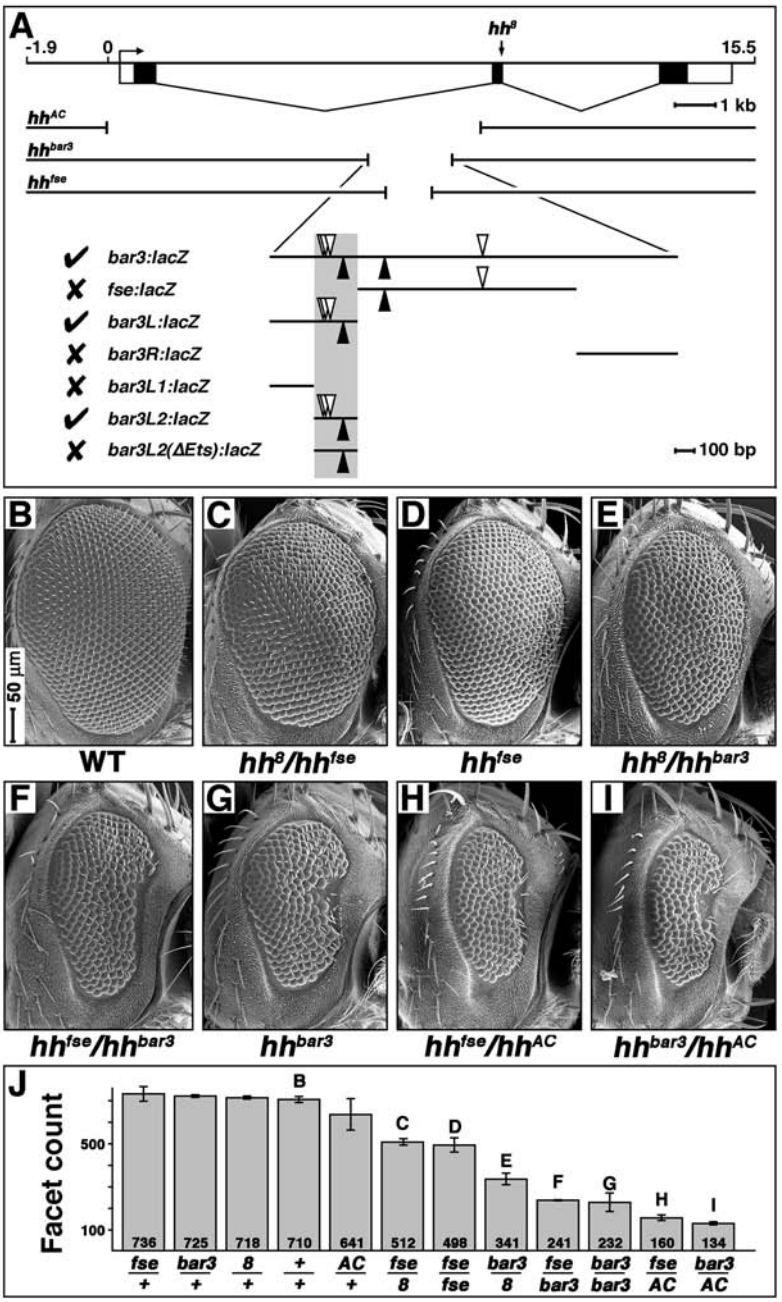

Fig. 1. *hedgehog* mutations form an allelic series. (A) The *hedgehog* locus and the transgenic reporter constructs used. The lesions of four *hedgehog* alleles is indicated (see text). Seven transgenic constructs are drawn to a larger scale (indicated). The checks and crosses indicate the constructs that express or do not express *lacZ* reporter posterior to the morphogenetic furrow. The gray box indicates the minimal region required for eye-specific expression. The white triangles indicate Pointed-binding sites (this paper) and the black triangles indicate Sine oculis (So)-binding sites (Pauli et al., 2005). (B-I) Adult eyes, anterior rightwards and dorsal upwards. Genotypes are indicated below the panels. (J) Histogram of female facet counts for different *hedgehog* genotypes as indicated. Bars show mean facet counts; number of individuals counted indicated in bar. Error bars show the 95% confidence limits. Letters above some bars refer to panels of same genotype. Scale bar: 50 μm in B for B-I.

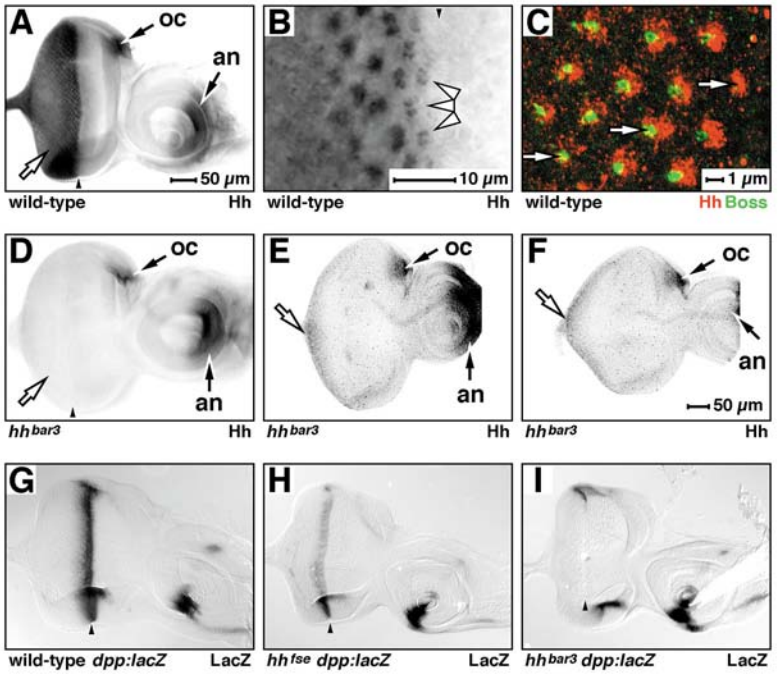

Fig. 2. hh^{bar3} phenotypes in the developing eye. Eye-antennal imaginal discs, shown anterior rightwards and dorsal upwards. Black arrowheads indicate the morphogenetic furrow. Genotypes are indicated below each panel. (A-F) Hedgehog antigen (red in C and black in the other panels). Boss is in green in C. (G-I) *lacZ* driven by the BS3.0 *dpp* enhancer (Blackman et al., 1991). an, antennal disc; oc, ocellar domain. In hh^{bar3}, Hedgehog antigen is undetectable in the presumptive eye field (compare at white arrows in A and D) but is normal in the ocellar region and in the antennal disc (black arrows in A,D). Hedgehog is first expressed in only two cells per ommatidium (white arrowheads in B) and that these are not Boss-expressing R8 cells (white arrows in C). A low level of anti-Hedgehog stain is seen at the posterior margin near the time of furrow initiation (white arrows in E,F). *dpp:lacZ* expression is reduced in the furrow in hh^{fse} and eliminated from the central furrow in hh^{bar3}. Scale bar in A: 50 μm for A,D,G-I; 10 μm in B; 1 μm in C; in F, 50 μm for E,F.

rest of the animal, so that weak mutations which reduce the quantity of *hedgehog* function uniformly, may affect only the eye. If so, then hh^{bar3} may simply have less function than hh^{fse}.

(2) It is possible that hh^{bar3} and hh^{fse} specifically affect *hedgehog* expression or function in the eye. If so, then hh^{bar3} may stop expressing *hedgehog* earlier than hh^{fse}.

These two possibilities need not be exclusive. Probably, hh^{bar3} and hh^{fse} affect a transcriptional enhancer and not the protein itself or the gene promoter, because neither lesion directly affects the coding sequence. In sequencing 23 cDNAs from eye-imaginal discs, we found no alternative first exon or start site in the region of the two mutations (Ma et al., 1996).

Hedgehog protein expression and function in the eye are affected by hh^{bar3} and hh^{fse}

We used an antibody to detect Hedgehog antigen in the developing third larval eye-antennal imaginal disc (Fig. 2). We find that Hedgehog antigen is detectable in three territories: posterior to the morphogenetic furrow (white arrow in Fig. 2A), and outside the eye field in the presumptive ocellar domain ('oc' in Fig. 2A) and the anterior compartment of the antenna ('an' in Fig. 2A). The expression in the ocellar domain is consistent with reported *hedgehog* function in the head vertex (Royet and Finkelstein, 1996; Royet and Finkelstein, 1997; Amin et al., 1999). The expression in the anterior compartment of the antennal disc appears anomalous, as *hedgehog* functions in the posterior compartments of most imaginal discs, and may be explained by the inversion of the antenna (Struhl, 1981).

In the region of the morphogenetic furrow, Hedgehog antigen first appears in two cells (white arrowheads in Fig. 2B). These cells appear to be the R2 and R5, and later antigen is also expressed in R3 and R4. The central cell of the precluster (R8) appears not to express Hedgehog antigen. Similar differences have been observed between the R8 and the other cells, such as later expression of Elav, and independence of the Egfr/Ras pathway (Kumar et al., 1998). To confirm this, we doubly stained eye discs for Hedgehog and the R8-specific antigen Boss (Fig. 2C). We first observe Boss antigen slightly later than Hedgehog. In the early clusters there is a gap in the Hedgehog stain (resembling a keyhole), and in the next few columns this gap is filled by Boss (white arrows in Fig. 2C). Soon after this, Hedgehog expression fades. Thus, we suggest that Hedgehog is not expressed in the R8: at least we cannot detect it to the limits of our resolution. It may be that the furrow is driven forwards by expression from all cells except the R8.

In late third instar, hh^{bar3} homozygous eye discs no Hedgehog antigen is detectable posterior to the morphogenetic furrow, while it remains unaffected in the ocellar region and in the antenna (Fig. 2D). We have also stained other hh^{bar3} homozygous imaginal discs (wings and legs) and find the Hedgehog antigen pattern is not detectably different from wild type (data not shown). Thus, hh^{bar3} does specifically affect Hedgehog expression in the eye, which suggests that it is indeed an eye-specific regulatory allele. This argues strongly that the eye specific phenotype of hh^{bar3} is not only due to a higher requirement for Hedgehog in the eye. We also stained hh^{fse} eye discs and find a reduced but detectable level of Hedgehog antigen in the eye (data not shown).

We deliberately overstained discs and find that the weaker and earlier domain of Hedgehog expression in the posterior margin, previously described by others in wild type, is not abolished in hh^{bar3} (see white arrows in Fig. 2E,F) (Domínguez and Hafen, 1997; Borod and Heberlein, 1998). After furrow initiation in hh^{bar3} homozygous eye discs, we detect no Hedgehog antigen in the first 12 ommatidial columns of photoreceptor cells. Thus, we suggest that in hh^{bar3} homozygotes the furrow initiates normally under the influence of marginal Hedgehog, and moves for the first 12 columns or so under this or other influences. Then the furrow fails as it has not begun to express Hedgehog locally.

We also tested the function of *hedgehog* signaling in the

developing eye through the activity of a target gene. The BS3.0 element from the *decapentaplegic* gene (*dpp:lacZ*) drives strong reporter expression in the furrow, in the eye disc margins and in the compartment boundaries of other discs (Fig. 2G) (Blackman et al., 1991). We find that hh^{fse} greatly reduces, but does not eliminate, *dpp:lacZ* expression in the furrow but not the compartment boundaries of other discs (see furrow and antenna in Fig. 2H). However, as previously reported, hh^{bar3} has a stronger effect (Fig. 2I) (Heberlein et al., 1993), as does the conditional allele hh^{ts2} (Ma et al., 1993).

Taken together, these data suggest that hh^{bar3} is a null for Hedgehog protein expression in the eye field, while hh^{fse} is a weaker allele: we can detect no Hedgehog expression or function (to drive *dpp:lacZ*) in hh^{bar3}.

How then do hh^{bar3} homozygotes manage to make 12 columns of ommatidia before the furrow arrests, and how can hh^{bar3} be enhanced when placed in trans to the null? It is possible that there is a very low level of Hedgehog antigen present in hh^{bar3}. However, we have grossly over stained hh^{bar3} discs and have never seen any. Alternatively, it could have been that Hedgehog antigen is expressed in hh^{bar3} for the first 12 columns and then ceases to do so. However, when we stained younger eye discs we saw no such early expression. Thus, we propose that the furrow may be induced and produce the initial columns of ommatidia through a signal from elsewhere. This is likely to involve Decapentaplegic expressed on the posterior margin, perhaps with a contribution from Hedgehog (less affected by hh^{bar3}) and this is why the facet count in hh^{bar3}/hh^{AC} is less than that in hh^{bar3} homozygotes. We suggest that later, as the furrow moves away from the margin, it becomes an automobile organizer, supplying on its own inducer (Hedgehog) as it moves, and at this point the hh^{bar3} homozygous furrow arrests, having failed to make any Hedgehog.

The hh^{bar3} deletion removes an eye-specific transcriptional enhancer

We placed the DNA contained within the hh^{bar3} deletion before a truncated *hsp70* promoter driving *lacZ* and derived transgenic flies (‘*bar3:lacZ*’, see Fig. 1A). We recovered 13 lines and all show strong reporter expression posterior to the furrow, but not in the ocellar domain and the antenna (Fig. 3A). This is strikingly reciprocal to the effect on Hedgehog antigen expression of hh^{bar3} itself (compare Fig. 2C with Fig. 3A), and demonstrates that this DNA contains elements that are sufficient for this eye-specific regulation. We also stained other imaginal discs and whole embryos (0-24 hour collection) and found only one other consistent expression pattern: three regions of the embryonic gut, of unknown significance (data not shown).

We did a similar experiment using the hh^{fse} deleted DNA (Fig. 1A). We recovered seven lines and only two had barely detectable *lacZ* expression, and this is in the disc margin (not posterior to the furrow, Fig. 3B). Thus, the hh^{fse} deletion contains elements that contribute to the eye-specific expression pattern, but are not sufficient for it. To further delimit this *hedgehog* gene eye specific enhancer we constructed a series of smaller fragment transgenes (Fig. 1A, Fig. 3C-F). We find that the minimal sufficient element to function as the *hedgehog* eye enhancer is a 203 bp fragment (*bar3L2:lacZ*), which lies to the left of the hh^{fse} deletion and within the hh^{bar3} deletion

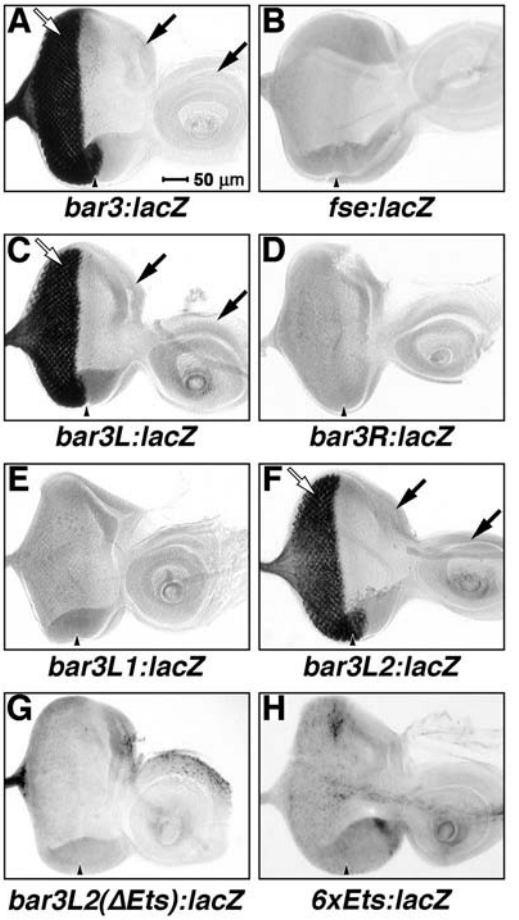

Fig. 3. β-Gal expression from *hedgehog* enhancer constructs. Third instar eye-antennal imaginal discs, stained for β-gal antigen, and shown anterior towards the right and dorsal upwards. Arrowheads mark the morphogenetic furrow. Transgenic *lacZ* lines are named as shown in Fig. 1A and indicated below the panels, see text. Constructs containing the three adjacent Pointed-binding sites have robust β-gal expression posterior to the morphogenetic furrow (white arrows in A,C,F) but not in the antennal disc or the ocellar region (black arrows in A,C,F). The 203 bp fragment *bar3L2* is sufficient to drive expression of *lacZ* reporter in the eye (F). Mutation for all three Pointed-binding sites eliminates this reporter expression (G). Six tandem Pointed-binding sites are not sufficient to drive expression of β-gal (H). Scale bar: 50 μm.

(Fig. 3F, bases 6253 to 6455 using previous numbering) (Lee et al., 1992). Very recently another group has shown that the hh^{bar3} deletion has this eye specific function (Pauli et al., 2005). For other reasons (see below), they deleted a longer left region of a very similar fragment and showed that it is required, although they have not shown that it is sufficient (Pauli et al., 2005). Their data are entirely consistent with ours.

The *hedgehog* eye enhancer drives expression in all cells expect the R8

To better define which cells express the *hedgehog* eye enhancer we stained eye imaginal discs for β-gal and other cell specific markers (Fig. 4). We find that β-gal antigen is detected first in R2 and R5, then the other photoreceptor cells and later the accessory cone cells (not shown). By staining simultaneously

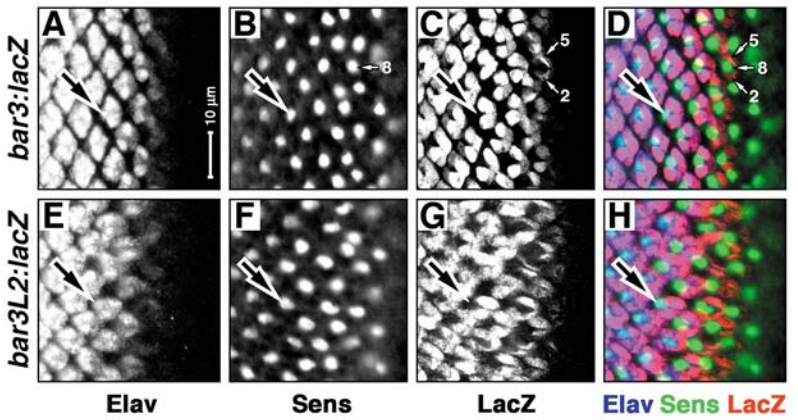

Fig. 4. The *hedgehog* eye enhancer drives expression in all photoreceptors except for the R8 photoreceptor. Third instar eye imaginal discs, anterior rightwards. The fields shown are just posterior to the furrow. A-D and E-H are the same fields in the same eye discs. Genotypes are shown on the left; antigens are shown below; colors as indicated. Elav marks all photoreceptor nuclei (A,D,E,H;, Senseless (Sens) marks only the R8 photoreceptor nuclei (B,D,F,H); *lacZ* reporter (C,D,G,H). Black arrows indicate a Sens positive R8 nucleus in a single ommatidium. White arrows (B-D) mark R8, 2 and 5 as indicated. Both the 1.9 kb full-length *bar3:lacZ* (A-D) and the minimal 203-bp *bar3L2:lacZ* (E-H) have indistinguishable *lacZ* expression patterns. There is no LacZ expression in the R8 photoreceptor. Scale bar: 10 μm.

for the R8-specific protein Senseless (Frankfort et al., 2001) we find that the β-gal reporter is not detectable in the R8, at least in the third instar larva (arrows in Fig. 4). This pattern is driven by both the full *bar3:lacZ* element and the 203 bp minimal fragment (*bar3L2:lacZ*).

The β-gal reporter expression pattern driven by the *hedgehog* eye enhancer (all the photoreceptors except the R8) is the same as the genetic requirement for *Egfr* signaling (Kumar et al., 1998), and of the expression of the *pointed* enhancer trap pnt^{1277} (data not shown) (Brunner et al., 1994). This suggests that the Egfr pathway Ets-domain transcription factor Pointed may be a direct regulator of the *hedgehog* eye enhancer.

The *hedgehog* eye enhancer is activated by the Egfr pathway transcription factor Pointed

If Pointed directly regulates the *hedgehog* eye enhancer, then we might expect to find Pointed-binding sites there. We used a published Ets domain binding site consensus (Xu et al., 2000), and found four such sites (white arrowheads in Fig. 1A), three of which lie in the minimal 203 bp element, and one of which is outside it but in the hh^{fse} deletion. To test the cis-acting requirement for these sites in vivo, we modified the minimal 203 bp *hedgehog* eye enhancer by introducing mutations into the three Ets sites [*bar3L2(deltaEts):lacZ*, see Materials and methods]. We derived transgenic flies and found that these do not express the *lacZ* reporter in the developing eye (compare Fig. 3G with 3A,C,F). Thus, these are the only three Ets sites in the 203 bp fragment that are required for function in vivo.

To test the function of Pointed-binding sites alone in vivo, we derived a hexamer concatenated construct and tested it in flies (*6xEts:lacZ*, see Materials and methods). This construct does drive *lacZ* expression in the embryonic nervous system, as described for the expression of *pointed* itself (data not shown) (Klämbt, 1993). However, it does not drive expression in the developing eye (Fig. 3H). Thus, the cis-acting Ets binding sites appear to be necessary, but not sufficient for the activity of the *hedgehog* eye enhancer.

To test if *pointed* is necessary in trans for the *hedgehog* eye enhancer, we derived retinal mosaic clones for the null allele $pnt^{delta88}$ (Scholz et al., 1993) and stained for a neural marker and for *bar3:lacZ* expression (Fig. 5). Interestingly, as previously reported (Baonza et al., 2002; Yang and Baker, 2003), we find that *pointed* is not required for the neural differentiation or maintenance of the R8 cells (black arrows in Fig. 5B,D), suggesting that the late requirement for Egfr in R8 maintenance (Kumar et al., 1998) does not require the Pointed transcription factor. These clones do show, also as previously reported (Baonza et al., 2002; Yang and Baker, 2003), that *pointed* function is absolutely required for the differentiation of the other photoreceptor cells. We find that *bar3:lacZ* expression is undetectable in the clones (Fig. 5B,D). This is consistent with direct regulation of the *hedgehog* eye enhancer by Pointed.

We tested these sites for Pointed protein binding in vitro by EMSA (gel-shift) assays (see Materials and methods). We found that Pointed protein can bind to and shift an oligonucleotide probe containing the three putative sites (Fig. 6, track 2). The 203 bp minimal *hedgehog* eye enhancer can compete effectively with the labeled probe for Pointed binding (Fig. 6, tracks 3-7). The same 203 bp fragment with the three

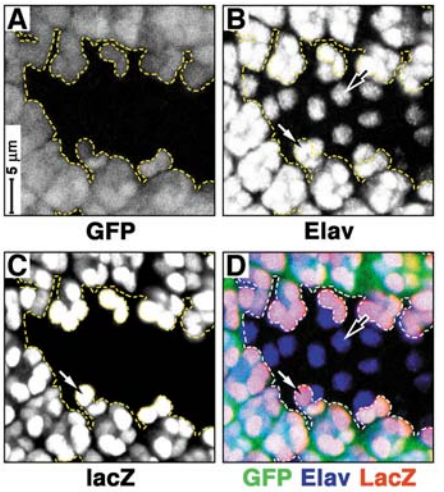

Fig. 5. *pointed* regulates the expression of *hedgehog* from an eye specific enhancer. (A-D) Third instar *bar3:lacZ* eye imaginal disc. The field shown is several columns posterior to the morphogenetic furrow, anterior towards the right. The disc contains a mosaic clone that is homozygous for the null mutation $pnt^{delta88}$. (A,D) GFP negatively marks the outlined clone. (B,D) Elav marks all the photoreceptors. (C,D) *lacZ* reporter. *lacZ* reporter expression is absent from within the *pointed* clone (a wild-type example outside the edge of the clone is indicated with a white arrow). The single R8 cells are Elav positive in the clone (black arrow). Scale bar: 5 μm.

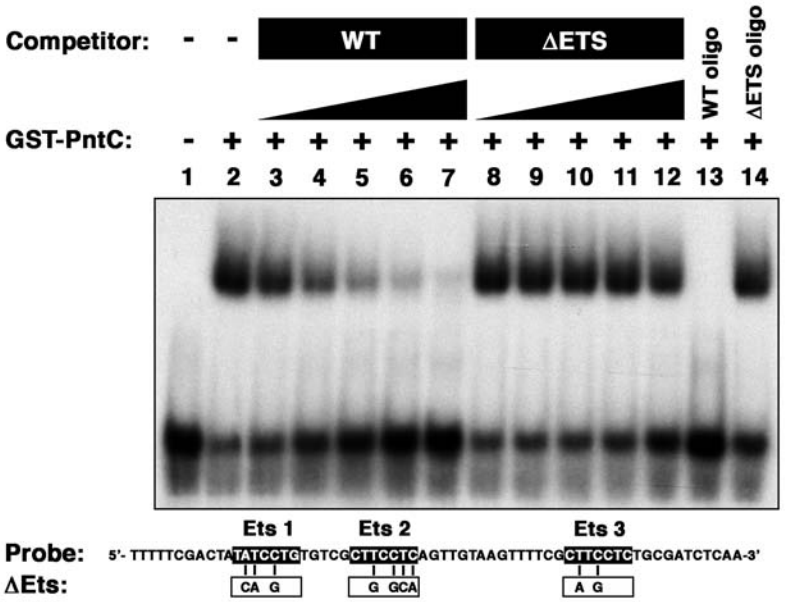

Fig. 6. Pointed P2 protein binds specifically to the *hedgehog* eye specific enhancer. Electrophoretic mobility shift assay (EMSA) gel for GST-Pointed binding. Competitor DNA is indicated at the top, with the rising level indicated by ramps. The presence or absence of GST-Pointed is indicated by + or –. Lane numbers are indicated. Lane 1, probe alone; lane 2, no competitor; lanes 3-7, 203 bp *Bar3L2* competitor, ranging from 15 to 240 times molar excess; lanes 8-12, 203 bp *Bar3L2(deltaETS)* competitor, ranging from 15 to 240 times molar excess; lane 13, cold probe as competitor and lane 14 Ets site mutated cold probe as competitor. The probe sequence is shown below gel with the three Ets consensus sites highlighted. The nine mutations made in *Bar3L2(deltaETS)* are shown in white boxes.

Pointed sites mutated does not compete at all [as shown in Fig. 6, tracks 8-12, and below the gel, the same site mutations as used in *bar3L2(deltaEts):lacZ*]. Thus, the 203 bp fragment does contain Pointed-binding sites and the three Ets-binding sites we identified in silico are the only three that function as Pointed-binding sites in vitro.

Discussion

We have characterized the expression of Hedgehog at the protein level in the developing eye and confirm the previously published RNA level expression data: Hedgehog is expressed posterior to the furrow in the developing eye (Lee et al., 1992; Heberlein et al., 1993; Ma et al., 1993). We now report that Hedgehog is expressed in other photoreceptor cells, but not the R8. We have characterized two eye-specific alleles of *hedgehog* and show that they delete elements that are specifically necessary for expression in the developing eye, posterior to the morphogenetic furrow. This *hedgehog* eye enhancer drives expression in all of the developing ommatidial cells except the R8. We reduced this element to a 203 bp minimal fragment that is sufficient for reporter expression. We have shown that the *hedgehog* eye enhancer is regulated by *pointed* in vivo and bound by Pointed in vitro. As Egfr/Ras-driven Pointed activates reporters in all the cells except the R8, we suggest that the *hedgehog* expression in the developing eye is driven by this enhancer and that Hedgehog is expressed in the developing ommatidial cells excepting the R8.

We propose that hh^{bar3} is indeed null for *hedgehog* expression in the developing eye, consistent with the loss of detectable antigen. This appears to contradict our facet count data, which show that hh^{bar3} is not null for eye size. We suggest that *hedgehog* functions elsewhere (probably in the eye disc margin), expressed at some lower level, and acts redundantly with Decapentaplegic to drive the early phases of furrow progression. This is consistent with data from others for an early role for *hedgehog* in the eye margin for furrow initiation (Domínguez and Hafen, 1997; Borod and Heberlein, 1998), and with a proposed redundancy between *hedgehog* and *dpp* in the furrow (Greenwood and Struhl, 1999). The enhancement of the hh^{bar3} phenotype when it is placed in trans to a null (hh^{AC}) suggests that hh^{bar3} may reduce, but not eliminate this early function.

Several examples of eye-specific transcriptional enhancers have been characterized. A number of these are in genes that act early in retinal determination (*eyes absent*, *dachshund* and *sine oculis*), and are not directly involved in the morphogenetic furrow (Bui et al., 2000; Punzo et al., 2002; Pauli et al., 2005). Some enhancers that function in and posterior to the morphogenetic furrow have also been studied. One example is the *atonal* gene, which has been shown to have two regulatory enhancers with specific and different activities in the furrow (Sun et al., 1998). Interestingly the *atonal* enhancers produce almost the reciprocal expression pattern of the *hedgehog* eye enhancer we describe here: *hedgehog* is expressed in all cells except the R8 and *atonal* expression is in only the R8, posterior to the furrow. Furthermore, *atonal* mutations can affect *hedgehog* signaling, although this may be indirect (White and Jarman, 2000), and indeed, *hedgehog* is also known to regulate *atonal* (Dominguez, 1999). Other enhancers that act posterior to the furrow have been characterized in the *rough*, *sevenless* and *prospero* genes, but none of these appears to show the particular type of regulation which we describe here (Basler et al., 1989; Bowtell et al., 1989; Heberlein and Rubin, 1990; Bowtell et al., 1991; Xu et al., 2000).

Very recently, others have also reported that a similar DNA fragment from the hh^{bar3} region confers post-furrow, eye-specific expression on a *lacZ* reporter (Pauli et al., 2005). The authors characterize the consensus binding site for another transcription factor: the retinal determination protein Sine oculis (So). They find two So-binding sites in the hh^{bar3} region (black arrowheads in Fig. 1A), and, as we did for the Pointed sites, they show that these are necessary for the normal function of the *hedgehog* eye enhancer. They show that a So site tetramer is sufficient to drive reporter expression in the entire presumptive eye field in the third instar disc, but that one is not. One of their two So sites lies within our 203 bp minimal element.

Taken together, our data and theirs suggest that Pointed and So activation at the minimal element are each necessary, but that neither is sufficient for the specific activation of the *hedgehog* eye enhancer posterior to the furrow. We propose that they act

together to confer this dual regulation. This is consistent with the model we discussed previously (see Introduction): that special dual regulation of *hedgehog* is the mechanism which makes the morphogenetic furrow move, unlike the stable compartment boundaries. We suggest that this dual regulation depends on one 'selector' signal that is eye specific (So), to differentiate the furrow from boundaries in other organs. The second component must act to close a loop such that cells which receives the furrow inducing signal will later send it after a delay, to make the boundary move forward. This 'signal' component is Pointed, acting downstream of Egfr/Ras signaling in the assembling ommatidia. This may be a case of 'selector' and 'signal' transcriptional integration (Affolter and Mann, 2001; Mann and Carroll, 2002). Indeed, *pointed* itself has been shown to integrate 'selector' factors in muscle development (Halfon et al., 2000). We propose that by this dual regulatory mechanism, a system that first evolved to divide the bauplan into metameric parasegments has been co-opted to drive a moving wave of differentiation in the developing eye.

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