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The Pax-homeobox gene *eyegone* is involved in the subdivision of the thorax of *Drosophila*

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SUMMARY

The *eyegone (eyg)* gene is known to be involved in the development of the eye structures of *Drosophila*. We show that *eyg* and its related gene, *twin of eyegone (toe)*, are also expressed in part of the anterior compartment of the adult mesothorax (notum). We report experiments concerning the role of these genes in the notum. In the absence of *eyg* function the anterior-central region does not develop, whereas ectopic activity of either *eyg* or *toe* induces the formation of the anterior-central pattern in the posterior or lateral region of the notum. These results demonstrate that *eyg* and *toe* play a role in the genetic subdivision of the notum, although the experiments indicate that *eyg* exerts the principal function. However, by itself the Eyg product cannot induce the formation of notum patterns; its thoracic function requires co-expression with the Iroquois (Iro) genes. We show that the restriction of *eyg* activity to the anterior-central region of the wing disc is achieved by the antagonistic regulatory activities of the Iro and *pnr* genes, which promote *eyg* expression, and those of the Hh and Dpp pathways, which act as repressors. We argue that *eyg* is a subordinate gene of the Iro genes, and that *pnr* mediates their thoracic patterning function. The activity of *eyg* gives rise to a new notum subdivision that acts upon the pre-extant one generated by the Iro genes and *pnr*. As a result the notum becomes subdivided into four distinct genetic domains.

Key words: *eyg*, *toe*, Thorax, Pax genes, *Drosophila*

INTRODUCTION

In *Drosophila*, much is known about genetic subdivisions of the body. An early and fundamental one is the segregation of anterior (A) and posterior (P) compartments along the anteroposterior (AP) axis of the body, which establishes the parasegmental trunk (reviewed by Lawrence and Morata, 1994; Lawrence and Struhl, 1996; Mann and Morata, 2000). The various Hox genes become active in specific positions, thus generating the morphological diversity along this axis.

However, within each segment, the morphological diversity does not depend on the Hox genes but on other developmental genes that are expressed in specific regions within segments. For example, *engrailed (en*) determines the difference between A and P compartments (Morata and Lawrence, 1975), *apterous (ap*) is involved in distinguishing the dorsal and ventral regions of the wing (Diaz-Benjumea and Cohen, 1993), and *pannier (pnr)* discriminates the dorsal-medial and dorsal-lateral regions of the thorax and abdomen (Calleja et al., 2000; Herranz and Morata, 2001).

The second thoracic segment develops in the dorsal region of the wing and the corresponding part of the thorax, known as mesothorax. These structures derive from the wing imaginal disc, which is subdivided from the beginning into A and P compartments (Lawrence and Morata, 1977). This subdivision affects both the wing and the thoracic region. In the mesothorax, the A compartment forms the greater part, the notum, whereas the P compartment is a small featureless region known as the postnotum.

The notum is made up of approximately 15,000 cells that develop a complex and highly stereotyped bristle pattern in which no lineage restriction has been found (Calleja et al., 2000). Thus, its genetic/morphological diversity has to be generated by non-lineage subdivisions. The notum has been shown to be subdivided into two major regions defined by the activities of the *pnr* and Iroquois (Iro) genes (Calleja et al., 2000; Gomez-Skarmeta et al., 1996). The Iro genes are expressed originally in all the notum cells and specify the development of the entire notum (Diez del Corral et al., 1999). Later, *pnr* restricts Iro gene activity to the lateral region and also specifies dorsal-medial development (Calleja et al., 2000).

However, *pnr* and the Iro genes are expressed in comparable domains in head, thoracic and abdominal segments, suggesting that they encode general properties such as dorsal-medial or dorsal-lateral, which apply to all segments. That is, they do not determine the development of one specific segment, but are probably involved in a general combinatorial mechanism together with other general factors, such as the Hox genes or *engrailed* (reviewed by Mann and Morata, 2000). It follows that there should exist other genes, regulated by the combinations of the above selector genes, which would be responsible for the morphological diversity within the different segments.

Using the ‘yellow’ method (Calleja et al., 1996), we have

isolated a number of Gal4 lines conferring expression in various parts of the thorax. One of them (EM461) yielded Gal4 activity in most of the scutum, the part of the notum from the anterior border to the suture with the scutellum (Fig. 1). No activity was observed in the abdomen. We report a functional study of a gene whose expression directs Gal4 activity in the EM461 line. The gene was discovered to be *eyegone (eyg)*, which encodes a homeodomain Pax protein (Jun and Desplan, 1996). *eyg* is one of the elements of the genetic network activated during eye development (Hazelett et al., 1998; Hunt, 1970), and it is also involved in the development of the salivary gland duct (Jones et al., 1998). Our results demonstrate that *eyg*, and its related and adjacent gene *twin of eyegone (toe)*, play a role in the genetic subdivision of the thorax. The *eyg*/*toe* function is necessary for scutum formation, and its ectopic activity in the scutellum transforms this into a scutum-like tissue. *eyg* expression in the scutum is regulated by a positive input of the Iro genes and *pnr*, and by the repressor activity of the Hh and Dpp signalling pathways.

MATERIALS AND METHODS

Molecular localisation of EM461

By using plasmid rescue, we cloned and sequenced the flanking genomic sequence 3′ of the pGalW element (Brand and Perrimon, 1993). The insert is located at 157,094 bp within the scaffold AE 003541 (FlyBase), 102 bp upstream of the eyg transcription start. toe is located 30 kb downstream of eyg (Flybase).

Drosophila strains

The following *Drosophila* strains were used in this work to generate loss of function clones: *Df(3L)iro* (Leyns et al., 1996); eyg^{20MD1}, $Gal4\text{-}eyg^{gv5}$ (generous gift of Maria Dominguez); *FRT80* iro^{DfM1}*/TM6* (Gomez-Skarmeta et al., 1996); *FRT42*ptc^{16}*/CyO*, *FRT40A*$Atkv^{a12}$*/Cyo* (Nellen et al., 1996); *FRT40A* Mad^{B1}*/Cyo* (Wiersdorff et al., 1996); *FRT40A*smo^{3}*/Cyo* (Strutt and Mlodzik, 1997); *FRT2A* eyg^{SA2}*/TM6B*; and *FRT2A mwh/TM3 Sb*. The FLP/FRT technique (Xu and Rubin, 1993) was used to generate loss of function clones. Larvae of the appropriate genotype were heat shocked for 1 hour at 37°C, at different larval stages. The clones were visualised in discs by either loss of GFP or of β-galactosidase expression.

For gain-of-function experiments, the following Gal4 lines were used: ywf^{36a} *hsFLP122*; *abx FRT* f^{+} *FRT Gal4-UAS-lacZ/Cyo* (de Celis and Bray, 1997); *ywflp122; act-FRT* y^{+} *FRT Gal4 UAS-GFP/SM5 Tb* (Ito et al., 1997); *ap-Gal4* (Rincon-Limas et al., 2000); *455-Gal4* (Martin-Blanco, 1998); *ush-Gal4* (Calleja et al., 2002); *248-Gal4* (Sanchez et al., 1997); *pnr-Gal4* (Heitzler et al., 1996); and *638-Gal4* (M. Calleja and G.M., unpublished). The gain-of-function clones were generated by recombination at the FRT sequences. The gain of *Gal4* and *lacZ* activity can be detected by β-Gal staining. In the adult cuticle, the clones can be scored because they are mutant for f^{36a} and contain y^{+} activity. The *UAS-ara* (Diez del Corral et al., 1999) and *UAS-*tkv^{QD} (Hoodless et al., 1996) lines used have been described previously. *UAS-eyg* and *UAS-toe* lines were generated by cloning the whole ORF into the pUAST vector, using the following cloning sites: *Not*I/*Xho*I for *eyg*, and *Eco*RI/*Xho*I for *toe*. The constructs were injected into *yw* embryos and stable lines were selected by rescue of the *white* phenotype. *eyg* cDNA was kindly provided by C. Desplan, and the *toe* cDNA by the BDGP. For the apoptosis experiments we used an UAS-P35 line (Hay et al., 1994).

The *lacZ* reporter lines used were: *en-LacZ* (Simcox et al., 1991), *neu-LacZ* (Flybase) and *esg-lacZ* (Whiteley et al., 1992).

P-element mutagenesis

Males homozygous for the pGal4 insertion (EM461) were crossed to females carrying the hop transposase. Excisions of the pGal4 transposon were selected by the loss of the w^{+} eye in the F1 progeny. Individual revertants were crossed to TM3/TM6 flies and balanced. PCR analysis was carried out with individual stocks with strong phenotypes over *Df(3L)iro*. We used one primer located 2.5 kb downstream of the P-element insertion site and two primers located upstream of the insertion site, one 3 kb upstream and the other 11.5 kb upstream.

Downstream primer, 5′-CCGGTGGACTATGGCGCGAACGG-ACGCG-3′;

Upstream 1, 5′-CGGCGTGGCCACCTTGGGCTTTGAGCC-3′; and

Upstream 2, 5′-CGGCGAGGGGAGTGGGGCCTGATGGG-3′.

Generation of a polyclonal anti-Eyg antibody

The complete *eyg* ORF was cloned in the pQE vectors (Qiagen) and the recombinant protein purified by following the QIA express protocols. The protein was injected into guinea pigs and the serum obtained was used as a polyclonal antibody.

Inmunostaining of embryos and discs

Discs were dissected in PBS and fixed in 4% paraformaldehyde for 20 minutes at room temperature. Discs were subsequently washed in PBS, blocked in blocking buffer (PBS, 0.3% Triton, 1% BSA), and incubated overnight with the primary antibody: anti-β-Gal (1:2000; rabbit), anti-Eyg (1:200; guinea pig) or anti-Ara (1:200; rat) diluted in blocking buffer at 4°C. Washes were performed in blocking buffer, and the appropriate fluorescent secondary antibody was added for 1 hour at room temperature. Following further washes in blocking buffer, the discs were mounted in Vectashield. Anti-Ara antibody was kindly by S. Campuzano, anti-β-Gal (rabbit) and anti-caspase 3 were purchased from Cappel and from Cell Signalling, respectively. Images were taken in a laser MicroRadiance microscope (Bio-Rad) and subsequently processed using Adobe Photoshop.

In situ hybridisation and antibody/in situ hybridisation-double labelling were performed as described (Azpiazu and Frasch, 1993), and embryos were mounted in Permount (Fisher Scientific). Digoxigenin-labelled RNA probes were synthesised as described (Tautz and Pfeifle, 1989). *eyg*-specific antisense RNA probe was synthesised from a 570 bp *Sac*I/*Kpn*I fragment of the cDNA provided by C. Desplan. The *toe*-specific antisense RNA probe was synthesised from a plasmid provided by the BDGP and consists of an 475 bp *Ear*I/*Xho*I fragment.

Preparation of larval and adult cuticles

Adult flies were prepared by the standard methods for microscopic inspection. Soft parts were digested with 10% KOH, washed with alcohol and mounted in Euparal. Embryos were collected overnight and aged an additional 12 hours. First instar larvae were dechorionated in commercial bleach for 3 minutes and the vitelline membrane removed using heptano-methanol 1:1. Then, after washing with methanol and 0.1% Triton X-100, larvae were mounted in Hoyer's lactic acid (1:1) and allowed to clear at 65°C for at least 24 hours.

RESULTS

Cloning of the EM461 Gal4 line

The EM461 Gal4 line directs expression in most of the scutum, antennae and the genitalia. *EM461>UAS-y* adults show no y^{+} rescue either in the legs or in the abdomen. The insertion was located in the third chromosome and is homozygous viable with no visible phenotype.

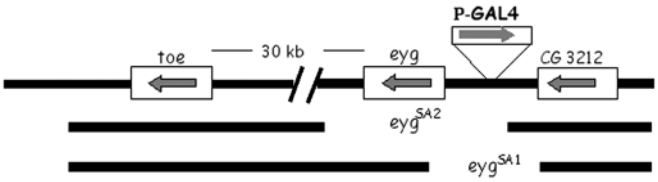

Fig. 1. Molecular map of the *eyg/toe* genomic region. The three transcription units located in the region (*eyg*, *toe* and CG3212) are shown as boxes, and their transcriptional orientation is indicated by arrows. The location of the *EM461Gal4* insert with respect to the genes is also indicated. As shown, neither the eyg^{SA1} nor the eyg^{SA2} deletions eliminate *toe*, which is located 30 kb downstream from *eyg*.

By using plasmid rescue, we cloned sequences at the insertion point of the P-element (see Materials and Methods), and found that it was inserted 102 bp 5′ of the transcription start site of *eyg* (FlyBase). The expression pattern found in *EM461>UAS-lacZ* embryos was similar to that of *eyg* (see below). Also, the imaginal expressions of EM461 and of *eyg* were largely coincident.

The analysis of the genomic region flanking the insertion point revealed the presence of another transcription unit, *toe*, and one predicted gene (CG32102; Fig. 1). *toe* is located 35.7 kb upstream from the P-element insertion, and CG32102 1.1 kb downstream from the insertion point. No cDNA has been reported for the latter gene. By contrast, *toe* has been previously cloned and shows high sequence identity with *eyg* (FlyBase). Their sequence conservation and location indicates that *eyg* and *toe* derive from a duplication of a primordial *eyg*-like gene, and raises the possibility that they may have redundant functions.

The expression domains of *eyg* and *toe*

We have analysed the expression of *eyg* and *toe* during embryonic and imaginal disc development using the *UAS-lacZ* construct, *eyg* and *toe* specific RNA probes, and an anti-Eyg polyclonal antibody generated in our laboratory. The distribution of the *eyg* and *toe* transcripts appears to be very similar during embryogenesis, and also in the wing imaginal disc, although the expression levels of *toe* are consistently lower than those of *eyg*. The β-Gal distribution in *eyg-Gal4/UAS-lacZ* embryos and imaginal discs matches that observed for the *eyg* and *toe* transcripts, and that observed with the anti-Eyg antibody. Thus, we shall use the anti-Eyg antibody as indicative of the expression of both genes.

Our results on the embryonic expression of *eyg/toe* are in agreement with those already reported for *eyg* using in situ hybridisation (Jones et al., 1998) (see also Fig. 3), and will not be considered further. We checked the possibility that *eyg* might be expressed in the embryonic primordia of dorsal discs by double-label experiments with *escargot (esg)* (Whiteley et al., 1992) and found that none of the cells expressing *eyg* show *esg* activity (data not shown). This result suggests that at the beginning of the wing disc development *eyg* is not yet active.

eyg/*toe* is expressed in the eye, wing and haltere discs. The expression and function of *eyg* in the eye disc has been reported (Hazelett et al., 1998); expression in the wing disc is shown in Fig. 2. The first sign of activity is observed at the beginning of the third instar (Fig. 2B), and, by that stage, the Eyg product already is restricted to a part of the thoracic region of the disc. In mature discs there are, in addition to the major domain in the thorax, two small expression domains in the hinge (Fig. 2B,D) and the pleura.

We have delimited the *eyg*/*toe* domain in the thorax by double labelling wing discs with anti-Eyg antibody and probes from genes also expressed in the thorax: the Iro genes, *pnr* and *en*. The expression domain does not overlap with that of *en* (Fig. 2B), indicating it is only active in the anterior compartment, but it overlaps partially with the *pnr* and Iro gene domains in the anterior notum (Fig. 2D,E). The *eyg*/*toe* domain does not extend to the whole of the lateral region of the notum, as indicated by the X-Gal staining of *eyg-Gal4/UAS-lacZ* flies (Fig. 2A), where some of the macrochaetes are not labelled, and also by double labelling with *neu-lacZ* (Fig. 2C), which marks all the macrochaetes; several of them are outside the eyg/toe domain.

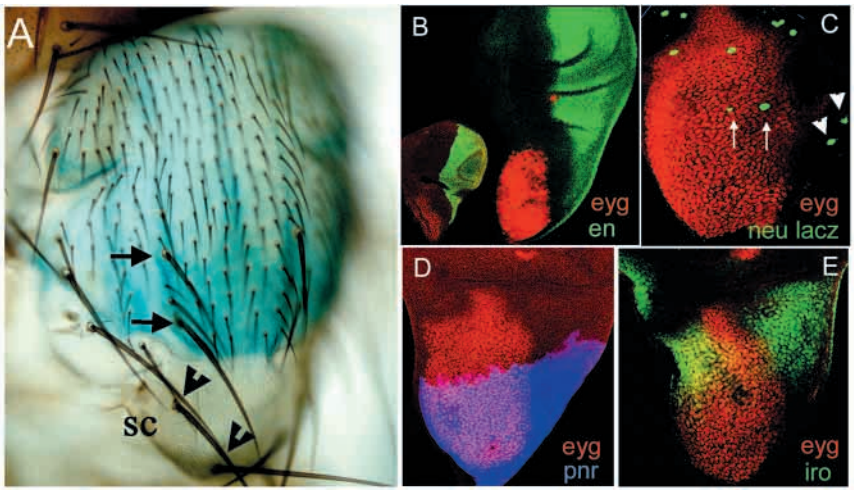

Fig. 2. Expression domain of *eyg* in the adult mesothorax and the wing imaginal disc. (A) Adult mesothorax of a fly of genotype *eyg-Gal4 > UAS-lacZ* stained with X-Gal (blue). The *eyg* domain occupies most of the notum, except the scutellum (sc) and the lateral region, which includes some macrochaetes. The dorsocentral bristles are marked with arrows and the scutellar bristles with arrowheads. (B) Early (left) and mature (right) wild-type third instar wing discs stained for *en* (green) and *eyg* (red). The eyg expression domain occupies the greater part of the anterior compartment, but does not extend to the posterior compartment. Note the dot of expression in the wing hinge. (C) The thoracic region of a wild-type disc stained for *neuralizer* (*neu*; green), which marks the precursors of the macrochaetes, and *eyg* (red). The precursors of two dorsocentral bristles (arrows), as well as some of the pre-sutural ones, are inside the *eyg* domain. The scutellar bristles (arrowheads) and the more lateral ones are outside the *eyg* domain. (D) Double staining for *pnr* (blue) and *eyg* (red), showing the overlap of their expression domains. (E) Thoracic region of a wild-type wing disc doubly stained for Iro genes (green) and *eyg*, showing that the expression domains of the genes overlap in part.

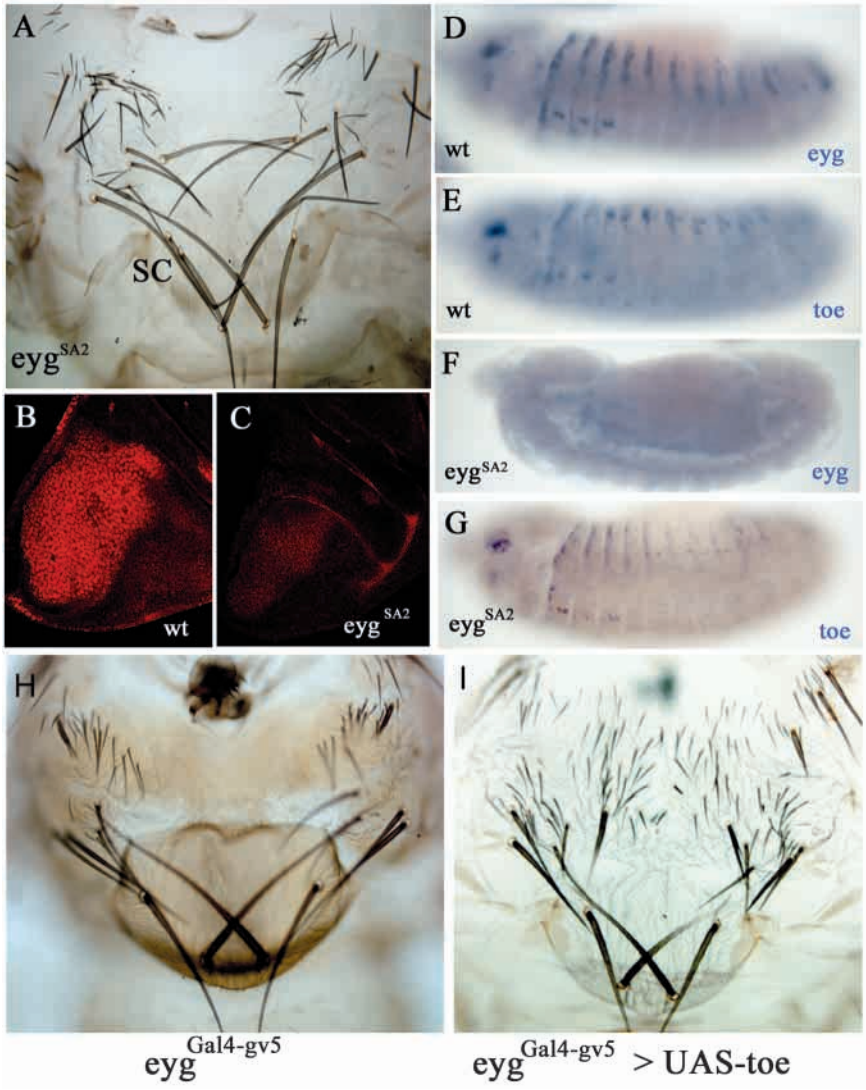

Fig. 3. Phenotype of *eyg* mutants. (A) Adult thorax of an $eyg^{SA2}/Df(3L)$ *iro* fly. The scutellum (sc) is present, as well as some of the lateral macrochaetes, but most or all the *eyg* domain is missing. Note the very low number of microchaetes, which compose most of the *eyg* domain (compare with Fig. 1A,B). (B,C) Anti-Eyg antibody of the thoracic region of a wild-type disc (B) and a eyg^{SA2}/eyg^{20MD1}-mutant disc (C). Low levels of anti-Eyg staining can still be observed in the mutant disc, suggesting that the antibody also recognises the Toe protein. (D) *eyg* expression at late embryogenesis of a wild-type embryo, as revealed by in situ hybridisation with a specific eyg probe. (E) In situ hybridisation using a specific *toe* probe reveals a similar expression pattern. (F) In eyg^{SA2}-mutant embryos there is no detectable *eyg* transcription. (G) In eyg^{SA2}-mutant embryos *toe* transcription appears normal. (H,I) Adult thorax of a fly of genotype $eyg^{Gal4\text{-}gv5}/Df(3L)iro$ (H), showing a phenotype very similar to that of the fly in A. The high levels of *toe* activity in $eyg^{Gal4\text{-}gv5}/Df(3L)iro > UAS\text{-}toe$ flies (I) give rise to a partial rescue of the mutant phenotype. Note the appearance of dorsocentral bristles and of numerous microchaetes.

The general conclusion from these experiments is that in the wing disc *eyg/toe* expression is activated approximately at the onset of the third larval period. Its expression is restricted from the beginning to a subgroup of thoracic cells that will form the anterior central portion of the mesothorax.

Loss- and gain-of-function studies of *eyg* and *toe*

Phenotype of *eyg* mutations in the notum

We have generated a large number of revertants of the *eyg*-Gal4 line that lose the w^+ gene. Of these, the eyg^{SA1} and eyg^{SA2} revertants fail to complement and their trans combination shows a clear notum phenotype, lacking most of the bristles. They also fail to complement eyg^{20MD1}, a P-*lacZ* insertion located 3′ of the *eyg* gene. The eyg^{SA2} and eyg^{20MD1} alleles show a stronger phenotype in their trans combinations, and over *Df(3L)iro*, a deletion of *eyg*, *toe* and several other genes (see Materials and Methods). eyg^{SA2} is a 9 kb deletion including the entire *eyg* transcription unit, but not that of *toe*. eyg^{SA1} is a 3.8 kb deletion that removes part of the *eyg* transcription unit. Most of the phenotypic analysis has been carried out using the eyg^{SA2} allele and the *Df(3L)iro*. There are no individual mutants for the *toe* gene.

The phenotype of $eyg^{SA2}/Df(3L)iro$ is shown in Fig. 3A. The notum is much reduced owing to the lack of practically the entire *eyg*/*toe* domain. The majority of the microchaetes of the central region and the dorsocentral bristles are missing, but the scutellar and lateral bristles are present. The zones not affected by the mutations are those that in wild-type flies do not possess *eyg/toe* activity.

As most of the normal eyg domain is lacking in $eyg^{SA2}/Df(3L)iro$ flies, there was the possibility that the loss of *eyg* function may cause apoptosis in cells normally expressing *eyg*. It has been reported that *eyg* mutants produce apoptosis in the eye cells anterior to the morphogenetic furrow (Jang et al., 2003). However, in our experiments, we do not find apoptosis in the mutant wing discs and there is no detectable caspase activity in the notum region (using an anti-caspase antibody; data not shown). Moreover, we carried out an experiment designed to assay the contribution of apoptosis to the *eyg*-mutant phenotype. The mutation eyg^{gv5} is a Gal4 insertion at *eyg* with a strong phenotype (Fig. 3H), which can be used to drive the activity of the baculovirus gene *P35*, a general inhibitor of apoptosis (Hay et al., 1994). Flies of the genotype $eyg^{gv5}/Df(3L)iro$; *UAS-P35* show the same phenotype as their siblings $eyg^{gv5}/Df(3L)iro$, indicating that apoptosis is not a major factor contributing to the *eyg* syndrome in the notum.

We have also induced *eyg*-mutant clones cells in the notum. As expected, these clones behaved normally outside the eyg domain, but those inside the domain show loss of bristles and abnormal patterning, which is often associated with alterations in the normal polarity of surrounding bristles and trichomes. In the disc they adopt a round shape, suggesting that they tend

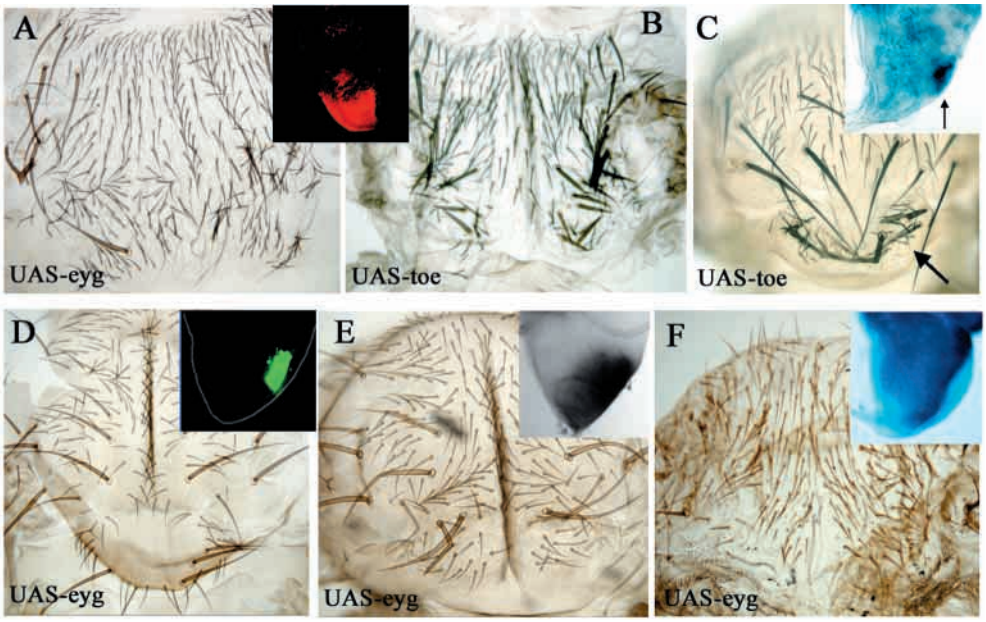

Fig. 4. Phenotypic consequences of ectopic *eyg* and *toe* expression. The expression of the different Gal4 lines driving *UAS-LacZ* or *UAS-GFP* is shown in the insets. (A) Notum of a fly of genotype *248-Gal4 > UAS-eyg*. The scutellum is transformed toward scutum as indicated by the change of morphology and the presence of microchaetes in the posterior notum. Note the opposite polarity of the bristles in the transformed and untransformed territories. (B) Transformation observed in the notum of *248-Gal4 > UAS-toe* flies, resembling that portrayed in A. (C) Notum of a *638-Gal4 > UAS-toe* fly. The ectopic expression of *toe* is limited to the scutellum region (arrow in inset), which differentiates microchaetes (arrow). (D) Similar transformation found in *455-Gal4 > UAS-eyg*. The driver directs expression to the scutellum region (inset). (E) The broader expression (inset) of *eyg* in the posterior notum region produces more extensive transformation. (F) In *ap-Gal4 > UAS-eyg* flies the Eyg product is uniformly expressed in all the notum cells. It results in an expansion of the normal anterior-central region of the notum, and the disappearance of the scutellum and the lateral region.

to sort out from surrounding *eyg*-expressing cells. These results indicate that the activity of *eyg* is connected with the acquisition of specific cell affinity properties.

The interpretation of the phenotype of *eyg* mutations may be complicated by the possibility that although the eyg^{SA2} is a complete loss-of-*eyg* transcription, there could be some rescue by *toe*, which is expressed in the same domain. However, we note that, in *eyg*-mutant flies, most or all the *eyg* domain is lost (Fig. 3A,H), suggesting that if there is rescue by *toe* it has to be weak. The ability of the Toe product to rescue the *eyg*-mutant phenotype was tested in an experiment in which we used the eyg^{gv5} Gal4 line to drive the Toe product. As shown in Fig. 3H,I, flies of the genotype eyg^{gv5} > *UAS-toe* show partial rescue of the eyg^{gv5} phenotype in the notum. This experiment indicates that the Toe product has a developmental potential similar to that of Eyg (see also below and Fig. 4). However, *toe* transcript levels appear normal in eyg^{SA2} mutants (Fig. 3G), and still are not able to rescue the strong phenotype of eyg^{SA2}, suggesting that the normal *toe* levels are not sufficient to substitute for the loss of *eyg* activity.

We have used *eyg* and *toe* specific RNA probes to examine the transcriptional activity of the two genes in *eyg* mutants. In eyg^{SA2} embryos there is no *eyg* transcription (Fig. 3E,), whereas that of *toe* appears normal (Fig. 3F). In wing discs of the same genotype we detect, using the anti-Eyg antibody, a small but consistent amount of cross-reacting product (Fig. 3C). As this suggested that the anti-Eyg antibody recognises both the Eyg and Toe products, we carried out an experiment expressing *toe* under the control of an *Ubx-Gal4* driver line. In *Ubx-Gal4 > UAS-toe* embryos, *eyg* transcription is not altered, but anti-Eyg antibody staining depicts the ectopic activity of the *toe* gene (data not shown).

Altogether, the preceding results indicate that normal *eyg* function is required for the appearance of the anterior central part of the notum. There is little, if any, rescue of the *eyg*-mutant phenotype by *toe*, in spite of the fact that it is expressed in the same domain. The results also demonstrate that *toe* is not regulated by *eyg*, as the loss of *eyg* activity does not affect *toe* expression.

Misexpression of *eyg* and *toe*

To study the effects of ectopic *eyg* and *toe* expression by the Brand and Perrimon method (Brand and Perrimon, 1993), we generated *UAS-eyg* and *UAS-toe* transformants, which then were crossed to various Gal4 lines. We also used the flip-out method (Chou and Perrimon, 1992) to induce marked clones of *eyg*-expressing cells.

The effect of misexpressing *eyg* and *toe* in the notum was analysed using the 248-Gal4, *pnr*-Gal4, 638-Gal4, *ap*-Gal4, 455-Gal4 and *ush*-Gal4 driver lines. The combinations of the Gal4 lines with *UAS-eyg*, and with *UAS-toe*, give essentially the same results (Fig. 4A-C), indicating that the two gene products have a similar, or the same, developmental capacity.

In the combinations with 248-Gal4, 455-Gal4, 638-Gal4, *ush*-Gal4 and *pnr*-Gal4 the scutellum is modified and develops a scutum-like pattern, as indicated by shape alterations and the appearance of microchaetes (Fig. 4A-E). We also observe that there is an abrupt change in polarity of the bristles and trichomae close to the border of the transformation, suggesting that *eyg* and *toe* might have a role in defining polarity of the epidermal elements.

In contrast to the preceding combinations, in *ap-Gal4/UAS-eyg* flies, the scutellum is absent but there is no indication of scutellum to scutum transformation (Fig. 4F). As *ap* is expressed in all notal cells after the second larval period (Diaz-Benjumea and Cohen, 1993; Rincon-Limas et al., 2000) and precedes *eyg* expression, we believe that inducing the activity of *eyg* early in notum development programmes the whole notum to develop as the *eyg*/*toe* domain, thus preventing the formation of scutellum (see Discussion).

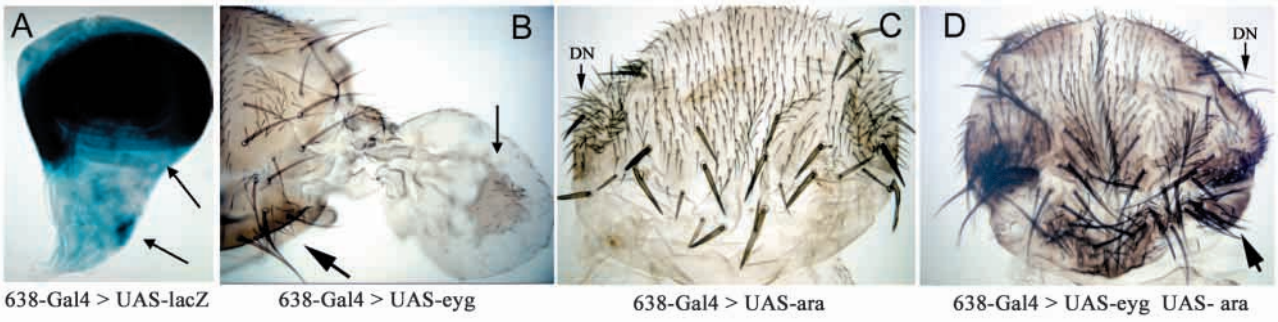

Fig. 5. Ectopic *eyg* expression. (A) The 638-Gal4 line drives expression in the posterior thorax and the wing pouch (arrows). (B) In *638-Gal4 > UAS-eyg* there is a clear transformation of the scutellum towards scutum (thick arrow), but there is little transformation in the wing, except in the centre (thin arrow), where there is some transformation towards notum. (C) Thorax of a fly of genotype *638-Gal4 > UAS-ara*. The wing is replaced by a duplication of the notum (DN). (D) Thorax of a fly of genotype *638-Gal4 > UAS-eyg UAS-ara*. The wing is replaced by a notum duplication (DN), but the scutellar regions of the normal and the duplicated notum are transformed towards scutum (thick arrow). Note the presence of microchaetes, which are indicative of the transformation.

The transformations observed in clones of *eyg*-expressing cells (data not shown) fit well with those described with the Gal4 lines. In the normal *eyg* territory these clones develop normally, indicating that increased levels of *eyg* activity do not appear to have a major effect. In the scutellum and lateral region they tend to sort out from surrounding tissue and also exhibit a change of fate; this is especially clear in the scutellum, where they develop scutum-like tissue.

These results suggest that *eyg* and *toe* are involved in the determination of the specific development of the anterior central region of the notum. This function is also reflected by the acquisition of specific cell affinities, which may have a role in the partitioning of the thorax (see Discussion). *eyg*-expressing clones in the disc are of normal shape in the *eyg* domain but have a round shape in the scutellum region, again indicating that *eyg* affects cell affinities.

eyg requires Iro gene activity to specify notum patterns

One significant result of the misexpression experiments is that whereas ectopic *eyg* or *toe* expression in the thorax induces scutum-like tissue, their activity in the wing does not produce a comparable transformation. The expression of *eyg*, or of *toe*, alone produces defects in growth and differentiation in the wing, but there is no clear indication of transformation towards notum (Fig. 5B). This behaviour is clearly illustrated by the *638-Gal4* line which drives expression in the posterior notum as well as in the wing pouch (Fig. 5A). In *638-Gal4 > UAS-eyg* flies the scutellum, but not the wing, is transformed towards scutum. Only in a small region in the centre of the wing is there is some tissue resembling notum (Fig. 5B).

This suggested that *eyg* may require a developmental co-factor normally present in the thorax but not in the wing. As the obvious candidates were the Iro genes, which we show to be activators of *eyg* (see below), we have compared the effect of *eyg* in the wing in either the presence of the absence of the *ara* gene. Of the three genes of the Iro-complex, *araucan (ara)*, *caupolican (cau*), and *mirror* (*mirr*), the first two are expressed in the same domain of the wing disc and appear to have the same developmental role (Gomez-Skarmeta et al., 1996).

Flies of genotype *638-Gal4 > UAS-ara* exhibit normal notum development and in addition the wing also becomes transformed towards notum (Fig. 5C). Thus the Ara product has the capacity to induce notum development, as expected from previous studies (Diez del Corral et al., 1999). In the favourable cases, it is possible to discriminate the presence of scutellum structures in the ectopic notum (Fig. 5C). In *638-Gal4 > UAS-ara UAS-eyg* flies, *ara* and *eyg* are co-expressed in the same cells of the wing. In these flies, the ectopic notum produced in place of the wing displays, additionally, a scutellum to scutum transformation (Fig. 5D). This result strongly suggests that *ara* (and probably *cau*) is providing the necessary 'thoracic' context for the normal function of *eyg*/*toe*. We believe that the notum-like tissue observed in the centre of the wing of *638-Gal4 > UAS-eyg* flies (Fig. 5B) is caused by the Iro gene activity normally present in that region (Gomez-Skarmeta et al., 1996).

Altogether the results obtained about the developmental consequences of eliminating or misexpressing *eyg* and *toe* indicate that these genes have a role in the subdivision of the notum into an anterior-central and a posterior-lateral region. To achieve this function they require the contribution of the Iro genes.

Regulation of *eyg* expression in the notum

The *eyg*/*toe* expression domain in the thorax covers only part of the notum; as illustrated in Fig. 2, *eyg/toe*-expressing cells in the mature disc are restricted to the anterior and central region of the disc. The misexpression experiments show that this spatial restriction is important for the normal patterning of the thorax. Thus, the factors controlling *eyg/toe* expression are significant components of the pattern formation process of the thorax.

The Iro genes and *pnr* upregulate *eyg* expression

We first checked the regulatory role of the Iro genes, for although in mature discs *eyg* and the Iro genes are not co-extensive (Fig. 2D), there is evidence that in early disc development Iro gene expression covers much or all the mesothorax (Diez del Corral et al., 1999). Thus the eyg domain would be a subset of the initial Iro gene domain, which suggests that the Iro genes might function as early activators of *eyg*. Moreover, cells lacking Iro gene activity are not able to differentiate thoracic structures (Diez del Corral et al., 1999), suggesting that they also lack *eyg* activity.

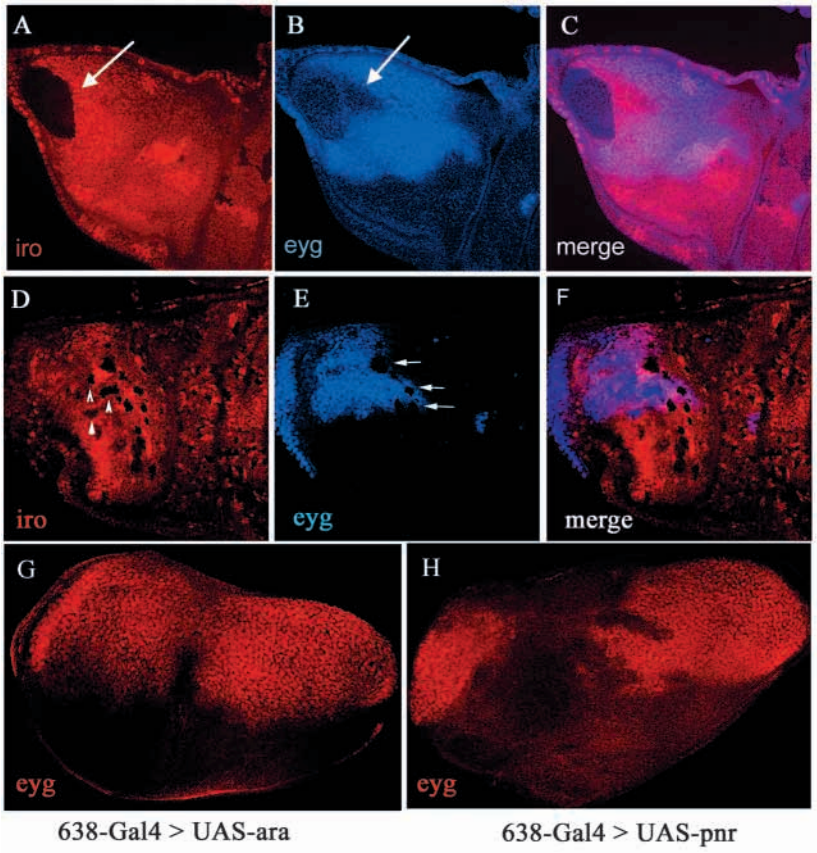

Fig. 6. Regulation of *eyg* by the Iro and *pnr* genes. (A-C) Large Iro gene-mutant clone (black; arrows) induced at first instar. The cells of the clone show no *eyg* expression, and the effect extends to wild-type cells close to the clone. (D-F) Thoracic region of a wing disc containing clones deficient for Iro gene activity (black). The medial region is to the left and the lateral is to the right. The clones located towards the lateral region (arrows) lose *eyg* expression (blue), whereas those located more medially (arrowheads) do not. (G) Wing disc of *638-Gal4 > UAS- ara* showing that the ectopic *ara* expression induces *eyg* activity (red). (H) *638-Gal4 > UAS- pnr* wing disc also showing activation of *eyg* by *pnr*.

We find that, in absence of Iro gene activity, *eyg* expression is lost: clones of Iro gene mutant cells induced in the first instar abolish *eyg* expression even in regions in which the Iro genes are not active in mature discs (Fig. 6A-C). An intriguing feature of these clones is that their effect on *eyg* extends to the zone outside the clone. Similar non-autonomous effect of the Iro genes have been reported (Diez del Corral et al., 1999).

However, in the third larval period the eyg and Iro gene domains are not co-extensive (Fig. 2). Clones of cells lacking Iro gene function induced at this stage lack *eyg* activity, but only if they localise in the Iro gene domain; Iro$^-$ clones in the *pnr* domain retain *eyg* activity (Fig. 6D-F). This indicates that there should be other factor(s) responsible for the maintenance of *eyg* expression in the part of the domain where the Iro genes are not active at this stage. The obvious candidate is *pnr*, which in late disc development is expressed in a notum domain complementary to that of the Iro genes (Calleja et al., 2000). We have induced *pnr*-mutant clones in third instar discs (72-96 hours of development) and examined the effect on *eyg* expression. These clones are small, and because they have differential cell adhesion properties (Calleja et al., 2000), tend to be round in shape and sort out from surrounding cells. In these clones, *eyg* expression is not modified, probably because the loss of *pnr* function results in gain of Iro gene activity (Calleja et al., 2000), which upregulates *eyg*. Nevertheless, ectopic *pnr* expression induces ectopic activation of *eyg* (Fig. 6I), just like the Iro genes do (Fig. 6H). This effect is not mediated by concomitant Iro gene expression as *pnr* does not activate (but rather repress) the Iro genes (Calleja et al., 2000). These results strongly suggest that both the Iro genes and *pnr* act as positive regulators of *eyg*.

The hh and Dpp pathways repress *eyg* activity

Another important aspect of *eyg* regulation is how its expression is inhibited in the scutellum and lateral notum. These regions are close to the AP compartment border, suggesting that the Hh and Dpp pathways might have a repressing role. There is evidence (Mullor et al., 1997) that the scutellum and the zone close to the AP border is patterned by Hh. This region is also expected to contain high levels of Dpp.

The role of the Hh pathway was tested by examining *eyg* expression in clones of cells that either are defective in [by eliminating the activity of the *smoothened* (*smo*) transducer] or express high levels of *hh* activity (by removing *ptc* activity or misexpressing *hh*). As illustrated in Fig. 7A-C, *smo*$^-$ clones located in the proximity of the AP border gain *eyg* activity. This gain of *eyg* activity is also reflected in the cuticular differentiation of these clones, which form scutum-like structures (Fig. 7D). On the contrary, *ptc*$^-$ clones localised in the *eyg* domain show loss of *eyg* expression (Fig. 7E-G). Similarly, ectopic expression of *hh* in the *eyg* domain results in loss of *eyg* expression (data not shown).

The regulatory role of Dpp was assessed by inducing clones of cells mutant for *Mothers against Dpp (Mad)*, a gene necessary for the transduction of the Dpp signal (Newfeld et al., 1996). The results are shown in Fig. 8A-F: the behaviour of the mutant clones regarding *eyg* expression depends on their position in the disc. In those clones located inside the *eyg* domain, *eyg* expression remains normal, although in some cases there is a noticeable increase of expression (Fig. 8D-F). *Mad*$^-$ clones outside but close to the *eyg* domain exhibit gain of expression (Fig. 8A-F), but in those located further away *eyg* is not de-repressed. This effect of position can be observed in the larger *Mad*$^-$ clones, like the one depicted in Fig. 8A-C; the zone of the clone close to the border of the domain expresses *eyg* ectopically whereas the zone opposite does not. We believe the reason for this differential behaviour of the clones is due to the various levels of Hh signalling in this region (see Discussion). We have found several *Mad*$^-$ clones in the posterior compartment, as judged by their position, one of which is shown in Fig. 8D-

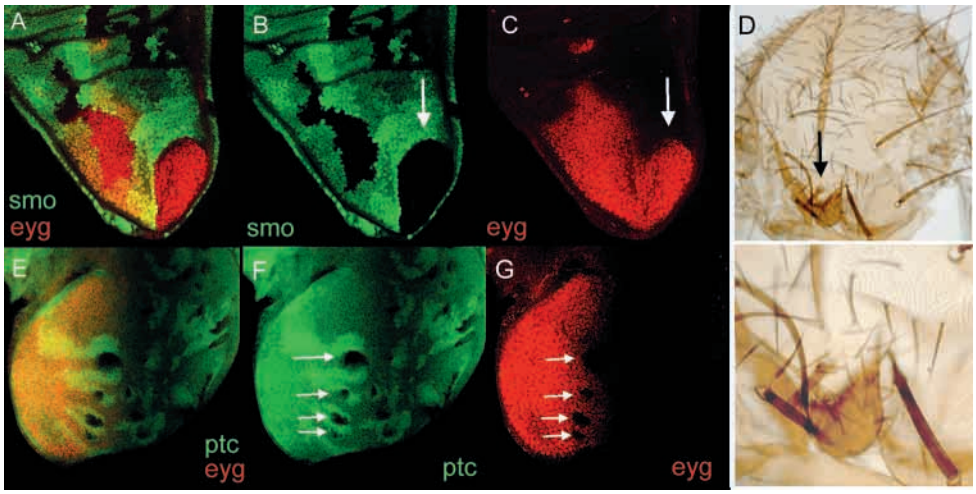

Fig. 7. Negative regulation of *eyg* by Hh signalling. (A-C) Clone of cells mutant for *smo* (arrow; marked black in B) showing gain of *eyg* activity (red). (D) A *smo* clone marked with *yellow* (arrow) in the adult cuticle. An amplification is shown in the lower panel. The clone differentiates notum structures resembling the scutum, as indicated by the microchaetes. (E-G) Clones mutant for *ptc* (arrows) exhibit loss of *eyg* expression.

F. None of these clones show gain of *eyg* expression. Because *hh* does not play a role in the posterior compartment (Basler and Struhl, 1994), the failure of *Mad*$^{-}$ clones to de-repress *eyg* expression suggests that the latter is regulated by a different mechanism in the posterior compartment. The repressor role of Dpp upon *eyg* expression is further supported by the results obtained from forcing high levels of Dpp activity in the *eyg* domain: in cells mutant for a constitutive form of the Dpp receptor, *thick veins* (Nellen et al., 1996), *eyg* activity is abolished (Fig. 8G-I).

In conclusion, the Iro and *pnr* genes appear to act as activators of *eyg*, whereas the Hh and Dpp pathways repress *eyg* activity. The combination of these two antagonistic activities yield the normal *eyg* expression pattern.

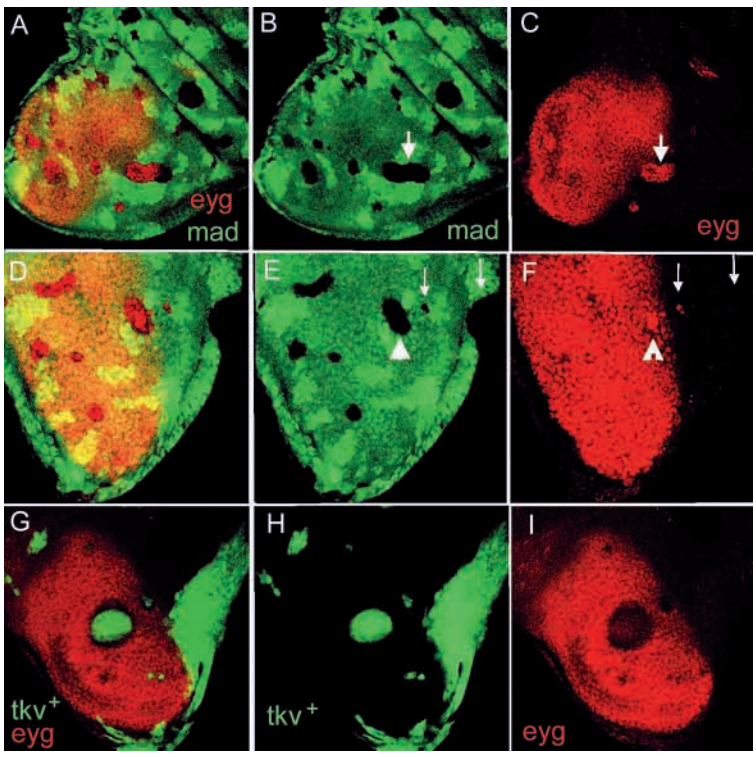

Fig. 8. Negative regulation of *eyg* by Dpp signalling. (A-C) Derepression of *eyg* in *Mad*-mutant clones (black). Note that in the larger clone (arrow), *eyg* (red) is activated only in the cells closer to the eyg domain. (D-F) Two *Mad* clones (arrows), a small one close to the eyg domain, showing ectopic *eyg* activation, and a larger one in the posterior compartment, in which *eyg* is not activated. Note the increase of *eyg* expression in the *mad* clone close to the border (arrowhead). (G-I) Several clones mutant for a constitutive form of the Tkv receptor, showing repression of *eyg*.

DISCUSSION

The homeobox gene *eyg* is known to play a role in eye development (Hazelett et al., 1998; Hunt, 1970; Jang et al., 2003), where it acts in combination with other eye forming genes. We describe a new, hitherto unknown, function of *eyg* in the development of the thorax. We also show that *eyg* and its twin gene, *toe*, perform similar and redundant roles, although *eyg* exerts the principal role. Given the similarity in expression and function of the two genes, we will refer to *eyg/toe* as a single functional unit.

As are the other segmental structures of *Drosophila*, the mesothorax is subdivided into anterior (notum) and posterior (postnotum) compartments by the activity of the *en* gene. Subsequently, these compartments are further subdivided (Calleja et al., 2000) into medial and lateral regions by the activities of the *pnr* and Iro genes. These regions are not defined by restricted lineages, but are kept developmentally segregated by the differential affinities of the *pnr*- and Iro gene-expressing cells (reviewed by Mann and Morata, 2000; Calleja et al., 2000). We now find that the notum, but not the postnotum, is further subdivided by the activity of *eyg/toe*. Its role in this subdivision is clearly demonstrated both by the experiments in which its function is eliminated and when it is ectopically expressed. The lack of *eyg* results in flies in which the central anterior region, corresponding to the *eyg*/*toe* notum domain, is missing (Fig. 3A). Conversely, ectopic expression of either *eyg* or *toe* in the scutellum transforms it towards scutum (Fig. 4). The conclusion is

that *eyg/toe* is involved in a new step in the subdivision of notum, which is superimposed over the previous one established by the Iro genes and *pnr*. The result is the appearance of four distinct regions, according to the active or inactive state of *eyg/toe* in the *pnr* and the Iro gene domains. The specific activation of *eyg/toe* in the notum, the anterior mesothoracic compartment, but not in the postnotum, the posterior mesothoracic compartment, appears to be an important factor in the generation of diversity in the thorax.

A significant functional feature of *eyg/toe* is that it is unable to induce notum structures by itself, but requires co-expression of its activator the Iro gene (Fig. 5), and probably *pnr*. For example, whereas ectopic *eyg/toe* activity induces scutum-like structures in the scutellum (which is also part of the notum and which expresses *pnr*), it fails to do so in most of the wing. Interestingly, it only induces notal structures in the middle of the wing (Fig. 5B), precisely the place where there is Iro gene activity in normal development (Gomez-Skarmeta et al., 1996). This mode of action is unlike that of selector or selector-like genes, such as the Hox genes, *en*, *Dll*, *pnr* or the Iro genes (reviewed by Mann and Morata, 2000), which are able to induce, out of context, the formation of the patterns they specify. This indicates that *eyg/toe* is not of the same rank, but that it is developmentally downstream of the Iro genes and *pnr*, and appears to mediate their 'thoracic' function. The restriction of *eyg/toe* activity to the thorax, unlike the Iro genes and *pnr*, which are also expressed in the abdomen, is fully consistent with this role. We note that *eyg/toe* is also expressed in a similar domain in the metathorax, suggesting that it may perform a parallel role in this segment.

The elaboration of the *eyg/toe* expression domain in the notum

The *eyg*/*toe* expression domain occupies the larger part of the notum, extending from the anterior border to the suture between the scutum and the scutellum. This domain coincides with the region affected in the *eyg* mutations, and is consistent with the gain-of-function experiments. Thus the *eyg*/*toe* expression domain corresponds to the zone where *eyg/toe* function is required. As it is only a part of the notum, a question of interest is to find out how *eyg/toe* expression is restricted to this zone. This restriction is necessary for the appearance of distinct anterior-central and posterior-lateral subdomains, for if *eyg/toe* are expressed uniformly, as *in ap-Gal4 > UAS-eyg* flies (Fig. 4F), the entire notum develops as the anterior-central domain.

Our experiments indicate that the localised expression of *eyg/toe* is achieved by the activity of two antagonistic factors: the promoting activity induced by the Iro and *pnr* genes, and the repressing activities exerted by the Hh and the Dpp pathways. The latter are probably mediated by Hh and Dpp target genes that are yet to be identified.

Both Iro genes and *pnr* act as activators of *eyg/toe* expression. In Iro gene-mutant clones *eyg* is abolished, and ectopic Iro gene activity results in ectopic *eyg* expression (Fig. 6). Although *pnr*$^-$ clones do not lose *eyg* activity, the probable explanation is that they show Iro gene activity (Calleja et al., 2000), which upregulates *eyg*. However, ectopic *pnr* expression induces *eyg* activity (Fig. 6). Because the Iro gene and *pnr* expression domains cover the entire notum, in the absence of any other regulation they would induce *eyg* activity in the whole structure.

The elimination of *eyg* activity from the scutellum and lateral notum is caused by the Hh and Dpp pathways. Because the AP compartment border is displaced posteriorly in the notum, these two pathways are active at high levels in the posterior region of the mesothorax. Assuming that the two signals behave as in the wing (Lecuit et al., 1996; Nellen et al., 1996), Hh activity will be higher in the region close to the AP border, whereas the effect of Dpp will extend further anteriorly. Thus the repressive role of Hh will be greater in the proximity of the AP border and that of Dpp will be greater in more anterior positions. This is precisely what our results indicate. In the region close to the AP border the high Hh levels alone are sufficient to repress *eyg* (Fig. 8C). However. in more anterior positions, close to the border of the *eyg* domain, Hh levels are lower and, although Hh is still necessary, it is not sufficient to repress *eyg*. Here there is an additional requirement for Dpp activity (Fig. 8A-F).

Thus, the part of the notum that does not express *eyg* can be subdivided into two distinct zones according to the mode of *eyg* regulation: a region close to the AP border that requires only Hh, and a more anterior region that requires both Hh and Dpp. In the posterior compartment, the repression of *eyg* has to be achieved by a different mechanism because neither the inactivation of the Hh pathway in *smo*$^-$ clones, nor the inactivation of the Dpp pathway (Fig. 8D-F), induces ectopic *eyg* activity. A probable possibility is that *en* itself may act as repressor.

The result of the antagonistic activities of the Iro genes and *pnr* on the one hand, and of the Hh and Dpp signalling pathways on the other, subdivides the notum into an *eyg*/*toe* expressing domain and a non-expressing domain. The localised expression of *eyg/toe* contributes to the morphological diversity of the thorax, as it distinguishes between an anterior-central region and a posterior-lateral one. It provides another example of a genetic subdivision of the body that is not based on lineage. It also provides an example of a patterning gene acting downstream of the combinatorial code of selector and selector-like genes. Its mode of action supports a model in which the genetic specification of complex patterns, such as the notum, is achieved by a stepwise process involving the activation of a cascade of regulatory genes.

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