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1(2)gl gene regulates late expression of segment polarity genes in *Drosophila*

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Abstract

To analyse the possible roles of *Drosophila* tumour suppressor genes, *1(2)gl* and *1(2)gd*, in differentiation programmes of imaginal cells, we investigated their interactions with two segment polarity genes, viz., *cubitus interruptus Dominant* (*ci-D*) and *engrailed* (*en*), by examining their patterns of expression in tumourous imaginal discs of $1(2)gl^4$ or $1(2)gd^1$ homozygous larvae. While the $1(2)gd^1$ mutation did not have much effect, the areas of expression of *ci-D* and *en* in the tumourous discs of *1(2)gl* homozygous larvae were significantly increased and the anterior-posterior compartment boundary was no longer identifiable. To examine if the loss of *en* expression compartment boundary in *1(2)gl* tumourous discs was due to overproliferation of the posterior compartment cells or due to a deregulated expression of *en* in the anterior compartment cells, $1(2)gl^4$ homozygous cell clones were generated in $1(2)gl^4$ $en^{lacZ}/+$ + background. A distinct X-gal staining in *1(2)gl* homozygous clones in the anterior compartment in wing imaginal discs or in adult wings confirmed deregulated ectopic expression of *en* in *1(2)gl* mutant anterior compartment cells. We suggest that *1(2)gl* is involved in regulating post embryonic expression of segment polarity genes.

Keywords: *1(2)gl*; *Drosophila* tumour suppressor genes; *engrailed*; *cubitus interruptus Dominant*; Segment polarity genes; Flip recombination

1. Introduction

A number of gene loci have been identified in *Drosophila* as tumour suppressors as recessive mutations at these loci result in malignant or benign tumours in different tissues (Gateff and Mechler, 1989; Woods and Bryant, 1989; Jursnich et al., 1990). Recessive mutations of the *1(2)gl* gene lead to malignant transformation of neuroblasts of the presumptive adult optic centres in the larval brain and of the imaginal disc cells resulting in hypertrophy of brain and excessive growth of imaginal discs which coalesce into poorly organized large tumourous masses (Gateff and Schneiderman, 1969; 1974). Recessive lethal mutations of *1(2)gd* gene, on the other hand, produce hyperplastic overgrowth of imaginal discs (Bryant and Schubiger, 1971). Molecular and developmental analyses clearly indicate that the *1(2)gl* and *1(2)gd* tumour suppressor genes play important roles in the regulation of specific cell fates and patterns of differentiation rather than just controlling the rate of cell proliferation (Bryant, 1987; Gateff and Mechler, 1989; Szabad et al., 1991; Strand et al., 1994).

In order to understand how the *1(2)gl* and *1(2)gd* genes affect cell fates and differentiation, we examined the expression of two segment polarity genes, viz., *cubitus interruptus Dominant* (*ci-D*) and *engrailed* (*en*) in *1(2)gl* and *1(2)gd* mutant individuals. These two segment polarity genes are expressed in a complementary fashion in anterior and posterior lineage compartments, respectively, and have critical roles in defining and/or maintaining the anterior and posterior identity of cells (DiNardo et al., 1985; Kornberg et al., 1985; Brower, 1986; Eaton and Kornberg, 1990; Orenic et al., 1990).

Our results showed that while the anterior-posterior compartment boundary was unaltered in hyperplastic imaginal discs of *1(2)gd* individuals, anterior-posterior compartment boundaries appeared to break down in the

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tumourous imaginal discs of *l(2)gl* mutant individuals. The disruption of anterior-posterior compartment boundary and increase in the area of expression of *en* in tumourous discs of *l(2)gl* mutant individuals was found to be due to its deregulated ectopic expression in the anterior compartment cells. We suggest that the *l(2)gl* gene is involved in regulating expression of *en* and other compartment specific genes like *ci-D* during post-embryonic stages of development. In addition, our results also confirmed the cell autonomous function of *l(2)gl* gene and its possible roles in regulating adult epidermal differentiation programmes.

2. Results

2.1. *en* expression is normal in *l(2)gl* and *l(2)gd* mutant larvae prior to onset of tumourigenesis

The expression of *en* in *l(2)gl* and *l(2)gd* mutants was examined through X-gal staining in embryos and in imaginal discs from different stages of larval development in the progeny derived from crosses between $l(2)gl^4$ en^{lacZ}/*SM5* and $l(2)gl^4$ *or*/*SM5* or between $l(2)gd^1$ en^{lacZ}/*SM5* and $l(2)gd^1$ *a px or*/*SM5*. All of the >2000 embryos examined for X-gal staining in each case showed the typical wild type *en* stripped pattern. Since about 50% of the stained (en^{lacZ} carrying) embryos were expected to be homozygous for the $l(2)gl^4$ or $l(2)gd^1$ mutant allele, a typical *en* expression in all embryos suggested that the absence of *l(2)gl* or *l(2)gd* function in embryos did not affect the *en* expression. Similarly, all imaginal discs from second, early and mid third instar larvae hetero- or homozygous for *l(2)gl* or *l(2)gd* (about 100 wing imaginal discs from each stage in both cases) showed the characteristic en^{lacZ} X-gal staining pattern (Fig. 1A). Although several of these discs would have been homozygous for *l(2)gl* or for *l(2)gd*, the fact that none of the discs displayed any aberrant X-gal staining pattern showed that the *en* expression was not affected in the *l(2)gl* or *l(2)gd* homozygous individuals till mid third instar stage, i.e. before the onset of tumourigenesis.

2.2. *en* expression is disrupted in *l(2)gl* but not in *l(2)gd* tumours in imaginal discs

The tumourous imaginal discs of *l(2)gd* homozygous late third instar larvae (identified by their prolonged larval life and bloated appearance) did not show a significant alteration in *en* expression during their extended larval life (Fig. 1B). However, imaginal discs from *l(2)gl* homozygous larvae exhibited a progressive disruption in *en* expression pattern during their extended larval life with increasing areas of discs showing X-gal staining. The anterior-posterior compartment boundary was completely lost (Fig. 1D and E). In situ hybridization with *en* probe to transcripts in intact *l(2)gl* tumourous discs also confirmed the disruption of compartmental boundary and expression of *en* in almost the entire area of imaginal discs (not shown).

2.3. Pattern of *ci-D* expression is disrupted in *l(2)gl* tumours

The *ci-D* promoter driven expression of *lacZ* in imaginal discs from early and mid third instar *l(2)gl* larvae, prior to the onset of tumourigenesis, was restricted, as in wild type larvae, to the anterior compartment, i.e., the area of the discs where *en* was not expressed (Fig. 1C). The tumourous imaginal discs in *l(2)gl* homozygous late third instar larvae, however, exhibited progressive increase in area of *ci-D* expression during their extended larval life leading to a complete disruption of the anterior-posterior compartment boundary (Fig. 1F and G).

2.4. *l(2)gl* transcripts are present in the entire area of wild type wing imaginal discs

In situ hybridization with the *l(2)gl* probe to transcripts in wing imaginal discs of wild type late third instar larvae revealed presence of *l(2)gl* transcripts in whole of the disc (Fig. 2A). Imaginal discs from *l(2)gl* deficient larvae were taken as control and, as expected, they did not show any hybridization (Fig. 2B).

2.5. *l(2)gl* mutant somatic clones in anterior compartment show expression of *en*

As described in Materials and methods, we used the yeast '*FLP-FRT*' site-specific recombination system to obtain mutant *l(2)gl* clones in $l(2)gl^+$ and en^{lacZ} heterozygous background. If the *l(2)gl*-deficient cell clone in the anterior compartment expressed *en* ectopically, one could detect *en* promoter-driven β-galactosidase activity in such a clone. Since the boundary of anterior-posterior compartment is most distinctly demarcated in wing imaginal discs or in adult wings, we examined the en^{lacZ} staining in *l(2)gl* mutant clones in these tissues only. After heat shock to first instar larvae, only a few distinct *l(2)gl* deficient clones in wing imaginal discs of late third instar larvae were identifiable; however, such clones were more frequent in adults. Out of about 350 wing discs of *w hsFLP1*; $l(2)gl^4$ *pFRT40A* en^{lacZ}/ + *pFRT40A*+ or *w hsFLP1*; + *pFRT40A* +/*SM5* genotypes examined, 4 discs showed small 'tumourous' structure in the anterior compartment; all of these showed a distinct clonal blue staining for β-galactosidase activity (see Fig. 3A and B).

Out of 375 *l(2)gl* deficient clones obtained in different body parts in the 720 *w hsFLP1*; $l(2)gl^4$ *pFRT40A* en^{lacZ}/+ *pFRT40A* + adult flies, 73 individuals showed *l(2)gl* mutant clones on antennae, 68 showed clones on eyes, 53 on both eyes and antennae, 47 on thorax, 59 on wings, 29 on both wings and thorax, 21 on eyes, antennae and wings, 18 on legs and 7 on genitalia. In most of these cases, the mutant clones were

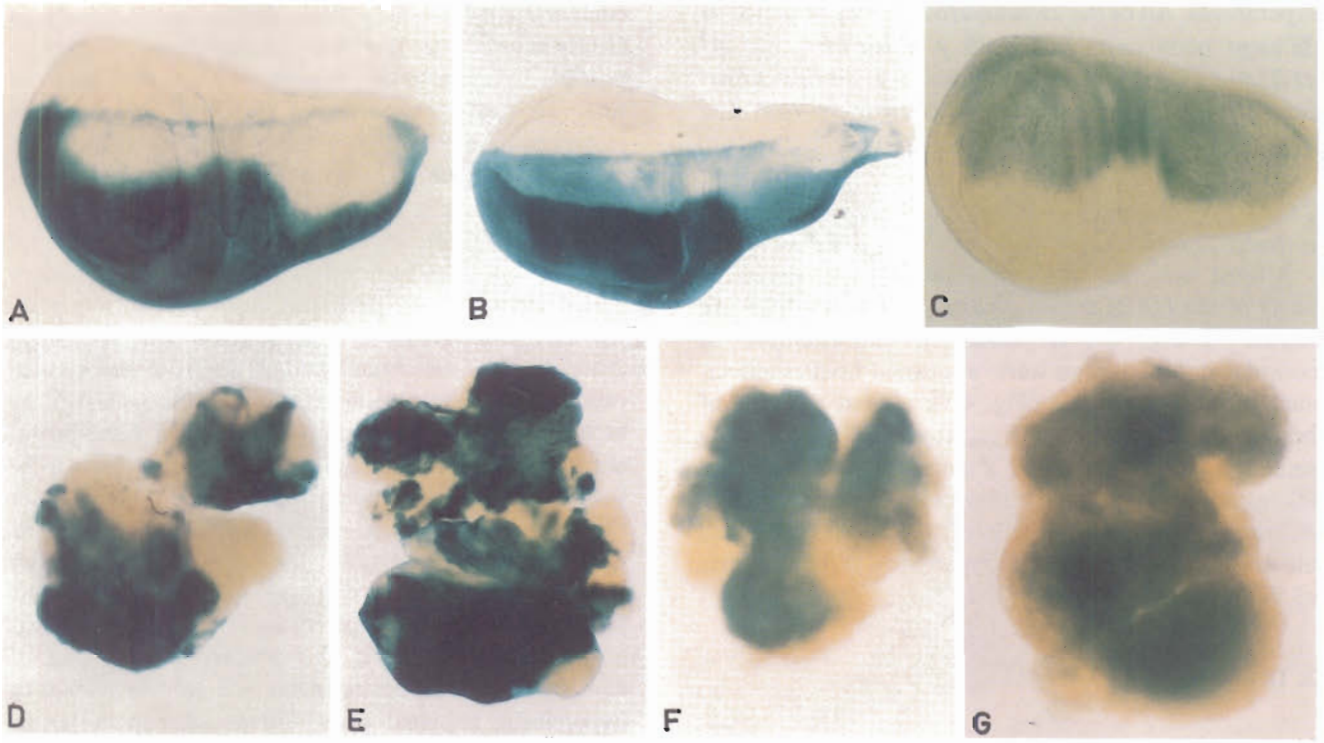


Fig. 1. Expression of *en* (A, B, D and E) or *ci-D* (C, F and G) in wing imaginal discs from third instar larvae of $l(2)gl^4\ en^{lacZ}/l(2)gl^4$ + (A, D and E), $l(2)gd^1\ en^{lacZ}/l(2)gd^1$ + (B) or $l(2)gl^4/l(2)gl^4;\ ci\text{-}D^{plac}/+$ (C, F and G) genotypes as revealed by X-gal staining. Note the normal pattern of *en* and *ci-D* expression in imaginal disc from 7-days-old $l(2)gl^4\ en^{lacZ}/l(2)gl^4$ + (A) or $l(2)gl^4/l(2)gl^4;\ ci\text{-}D^{plac}/+$ (C) larvae prior to onset of tumourigenesis, an unaltered anterior-posterior compartment boundary in imaginal disc from 9-days-old $l(2)gd^1\ en^{lacZ}/l(2)gd^1$ + larva as revealed by en^{lacZ} expression (B) and a progressively disrupted pattern of *en* (D and E) or *ci-D* (F and G) expression in imaginal discs from 9- (D and F) and 12- (E and G) days old $l(2)gl^4\ en^{lacZ}/l(2)gl^4$ + larvae.

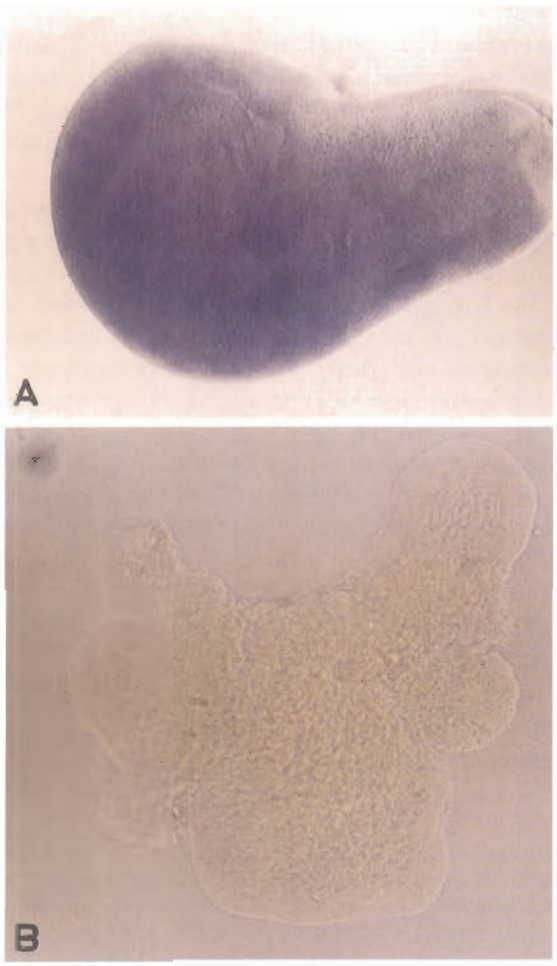


Fig. 2. In situ hybridization of DIG-labelled *l(2)gl* probe to transcripts in wing imaginal disc from wild type late third instar larva (A) and from *l(2)gl* homozygous larva (B). While in imaginal disc from wild type larva, the hybridization is seen all over; no hybridization is detected in the imaginal discs from $l(2)gl^4$ mutant larva.

identifiable due to formation of small tumourous structures with disrupted cuticular patterns. Thirty two clones on the anterior compartment of wings were examined for β-galactosidase staining to monitor *en* expression; as revealed by X-gal staining, *en* was expressed in 29 of these 32 anterior compartment *l(2)gl* clones (Fig. 3C and D).

To confirm that the above somatic clones were indeed *l(2)gl* homozygous, *yellow* cuticular marker was also used in conjunction with *l(2)gl* in a separate set of

experiments. All of the 25 tumourous clones obtained in different body parts in the 19 *y w hsFLP1; 1(2)gl*^{4} *pFRT40A en*^{lacZ}/+ *y*$^{+}$*25F pFRT40A* + adult flies showed *yellow* cuticular phenotype confirming that the tumourous clones were indeed *1(2)gl*$^{-}$.

2.6. *1(2)gl mutant somatic clones show defects in cuticular differentiation*

Altered cuticular differentiation was observed in many of the *1(2)gl* deficient clones: some examples are shown in Fig. 4A–D. Common abnormalities in cuticular differentiation were: abnormal bristles associated with clones in eye (Fig. 4A), altered patterns of bristles in thorax and wings, abnormalities in wing veination (Fig. 4B and D), abnormal folding of wing blade, formation of supernumerary appendages (Fig. 4C) and bifurcation of palpus. Melanotic dead cell masses were also frequently observed in different parts of the body.

3. Discussion

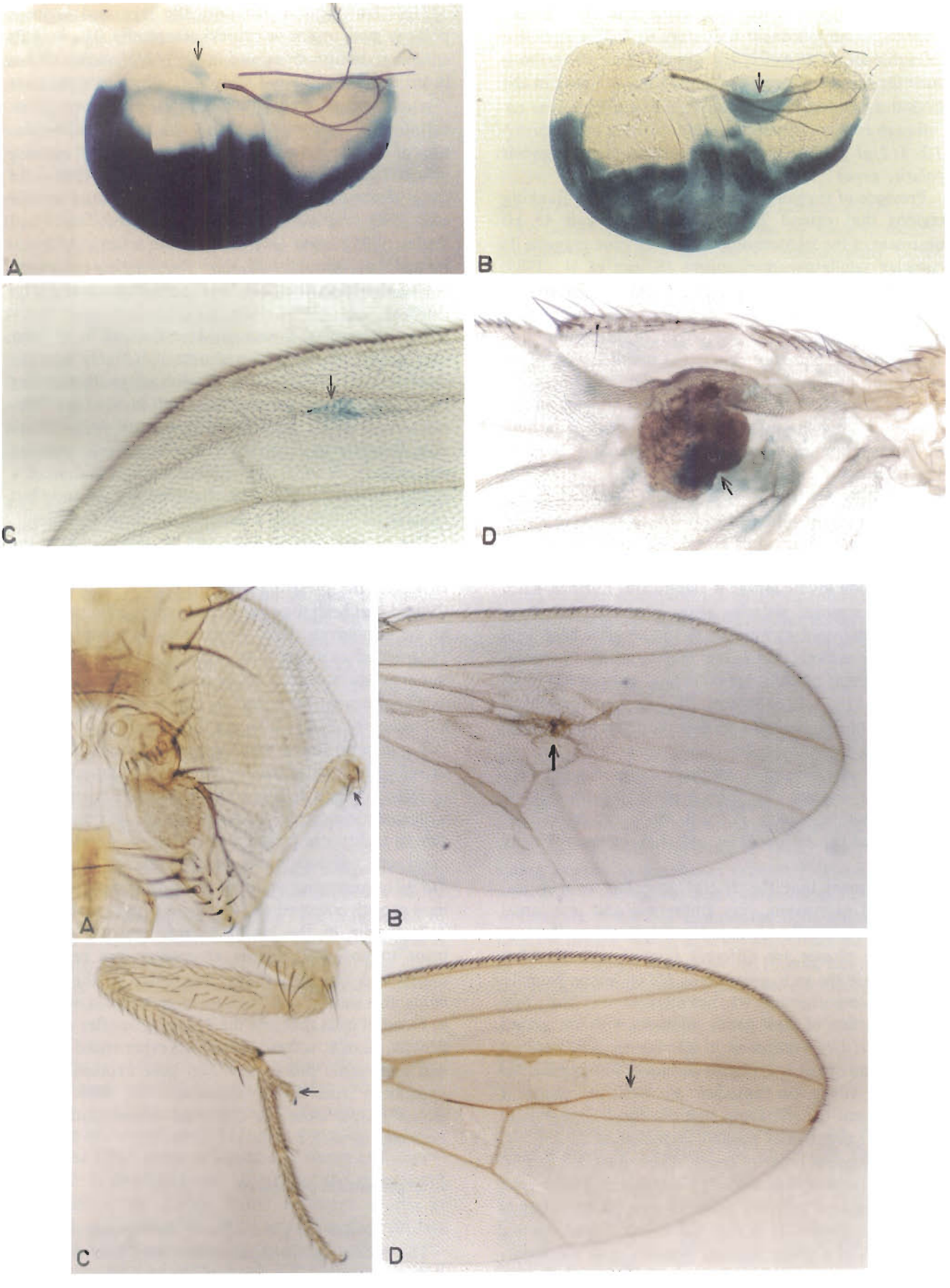
3.1. *Lack of 1(2)gl gene function leads to disruption of anterior-posterior compartment boundary in tumourous imaginal discs*

Segmentation of *Drosophila* begins in the early gastrula and from inception, each segment comprises of an anterior and a posterior developmental compartment whose cells do not intermingle (Garcia-Bellido et al., 1973; Kornberg and Tabata, 1993). The anterior-posterior lineage compartments can be defined by regions of differential gene activity established during embryonic development. The *en* gene is expressed in the posterior lineage compartment and it plays a critical role in defining and maintaining the posterior identity of these cells and controlling their proliferation (Kornberg et al., 1985; DiNardo et al., 1985; Brower, 1986; Hidalgo, 1994). Likewise, *ci-D* is expressed in the anterior lineage compartment (Eaton and Kornberg, 1990; Orenic et al., 1990). The *en* and *ci-D* genes are expressed in complementary, non-overlapping regions within each segment. However, beginning with the late third instar stage, *en* and *ci-D* expressions in developing wing disc overlap in a narrow region just anterior to the lineage compartment boundary (Blair, 1992). Our observations clearly showed that there was no disruption of anterior-posterior compartment boundary until the onset of tumourigenesis in third instar stages of *1(2)gl* larvae. However, with the onset of tumourigenesis, there was a progressive disruption of anterior-posterior compartment boundaries in tumourous imaginal discs of *1(2)gl* homozygous individuals during the extended larval period. This disruption of the compartment boundary would be due to overproliferation of cells of one compartment and their invasion into the other compartment (Siha, P., pers. commun.) and/or due to a deregulated expression of these compartment-specific genes in the other compartment in tumourous discs. Over-proliferation of one compartment cells does not appear to be solely responsible for the disrupted pattern of the *en* or the *ci-D* expressing cells, since if one particular compartment cells were proliferating more than the other, either the *en* or the *ci-D* expressing region should have actually become smaller than the other in *1(2)gl* discs. This was not seen. Furthermore, the absence of function of another tumour suppressor gene, *1(2)gd*, which leads to hyperplastic imaginal discs, did not disrupt anterior-posterior compartment boundary since in these discs the *en* expression remained restricted to the posterior compartment. The tumourous larval brain ganglia of *1(2)gl* larvae (data not presented) too did not show any disruption in the *en* expression pattern. Thus the disruption of anterior-posterior compartment boundary in *1(2)gl* discs is not simply related to the over-proliferation phenotype.

The distinct ectopic expression of *en* in *1(2)gl* somatic clones in the anterior compartment in imaginal discs and in adult wings clearly showed that in *1(2)gl*$^{-}$ cells, the compartment-specific expression of *en* was deregulated and even anterior compartment cells began to express *en* ectopically. Since these clones were induced in a wild type background and did not show any trail of *en*-expressing cells connecting the clones to posterior compartment, it is unlikely that *1(2)gl* mutant cells of posterior compartment invaded the anterior compartment and were, therefore, expressing *en*. Even if these clones were due to invasion of posterior compartment cells, the *1(2)gl*$^{-}$ function must be responsible for this since in wild type discs such invasions are not per-

Fig. 3. Ectopic expression of *en* detected by X-gal staining in *1(2)gl*^{4} *pFRT40A en*^{lacZ}/*1(2)gl*^{4} *pFRT40A* + clones (arrows) in the anterior compartment of wing imaginal discs from late third instar *1(2)gl*^{4} *pFRT40A en*^{lacZ}/+ *pFRT40A* + larvae (A and B) and in wings from freshly emerged *1(2)gl*^{4} *pFRT40A en*^{lacZ}/+ *FRT40A* + adults (C and D).

Fig. 4. Examples of *1(2)gl* mutant somatic clones showing defects in cuticular differentiation (A–D): Note the *1(2)gl* homozygous clone (arrow) on the eye of *y w hsFLP1; 1(2)gl*^{4} *pFRT40A enlacZ*/+ *y*$^{+}$*25F pFRT40A* + individuals (A), the lightly pigmented yellow marked bristles on the clone are difficult to see in photograph; a *1(2)gl* homozygous clone (arrow) on adult wing with abnormal wing veination (B); a supernumerary appendage (arrow) with well differentiated distal regions developing from the first tarsal segment of first leg (C); bifurcation of IV longitudinal vein (arrow) without an apparent overgrowth (D).


A
B
C
D
A
B
C
D

mitted. Since, like *en*, the *ci-D* expression also did not respect the compartment boundary in *1(2)gl* discs, we infer that an absence of *1(2)gl* gene product was responsible for the ectopic expression of the *ci-D* gene in the posterior compartment cells.

3.2. *1(2)gl gene is a later-acting regulator of segment polarity genes*

Presence of transcriptionally silent extensive flanking regions that extend 20 kb downstream and 45 kb upstream of the transcription unit of *en* gene suggests its complex regulatory mechanisms (Kuner et al., 1985; Drees et al., 1987; Soeller et al., 1988). Earlier genetic and molecular analyses have defined four temporally overlapping phases of *en* regulation: (a) initiation of *en* expression by pair-rule genes, (b) maintenance of *en* expression by *wingless* protein secreted from the adjacent anterior cells, (c) *en* autoregulation and, subsequently, (d) regulation of *en* in an independent fashion by later acting *en* regulators (Busturia and Morata, 1988; Dura and Ingham, 1988; Heemskerk and DiNardo, 1994; Noordermeer et al., 1994; Siegfried et al., 1994). At present, little information is available about the regulation of *en* after embryonic development. In this context, the present demonstration of *1(2)gl* affects *en* expression in late larvae is significant. Further, since the other segment polarity genes like *ci-D* also show altered pattern of expression in the absence of *1(2)gl* function, it is likely that *1(2)gl* may be a general regulator of segment polarity genes. Strand et al. (1994) suggested that the *1(2)gl* protein, p127, is a component of cytoskeleton which helps to maintain the apical-basal polarity of the imaginal disc cells; it is likely that the loss of apical-basal polarity in differentiating imaginal discs due to the absence of *1(2)gl* protein disrupts cell-cell communication which in turn leads to ectopic or deregulated expression of *en* and *ci-D* segment polarity genes.

It is known that the *1(2)gl* gene has two critical periods of expression, viz., embryonic and late larval and that its transcriptional and translational products at these two phases are different (Gateff and Mechler, 1989). Since the *en* expression in *1(2)gl* larvae prior to onset of tumourigenesis was not affected, it is likely that the interaction of these genes coincides with the second phase of *1(2)gl* expression in late larvae. The uniform distribution of the *1(2)gl* gene product in imaginal discs and the effect on anterior as well as posterior compartment-specific genes rule out the *1(2)gl* gene to be a direct negative or positive regulator of the *en*, *ci-D* or other segment polarity genes. There must be other interacting factor/s that mediate the effect of *1(2)gl* gene on the segment polarity genes in late larval imaginal discs. One possibility is that the compartment-specific inhibitor/s of a given segment polarity gene work only in the presence of *1(2)gl* gene product so that in *1(2)gl*$^-$ background, the normally repressed segment polarity gene begins to express ectopically. The various cuticular abnormalities seen in the *1(2)gl* mutant clones in various parts of adult body also point to a significant role of the 'tumour-suppressor' gene in various differentiation programmes. Possibly the cascade of regulation of differentiation by *1(2)gl* is via segment polarity genes. This finds support in the observed duplicated distal structures in leg (see Fig. 6C), the kind usually seen when *wingless* is ectopically expressed (Struhl and Basler, 1993); other results in our laboratory (Mukherjee and Roy, unpublished) show that *wingless* expression is also altered in imaginal discs in the absence of *1(2)gl* function.

The absence of β-galactosidase staining in at least some cells in anterior compartment of *1(2)gl* tumourous discs or in the *1(2)gl* clones in adult wings showed that the *en* gene did not express by default in all of the anterior compartment cells in the absence of *1(2)gl* function. Whether the anterior compartment cells that did not express *en* in *1(2)gl* mutant background were a specific subset could not be identified in this study but remains an interesting possibility for further studies.

The *en* gene product is known to repress *ci-D* in the posterior compartments (Eaton and Kornberg, 1990). However, the present results with *lacZ* reporter gene showed that both the *en* and *ci-D* expressed in most cells of the tumourous discs. Apparently, in absence of the *1(2)gl* product in late third instar larval discs, this negative regulation of *ci-D* by *en* does not operate.

In agreement with earlier results showing cellular autonomy of *1(2)gl* function (see Gateff and Mechler, 1989), we also found that the neighboring normal cells in *1(2)gl* heterozygotes did not prevent tumourigenesis of *1(2)gl* mutant cells in somatic clones. Earlier studies with somatic mosaics suggested that *1(2)gl* gene expression in embryonic period was critical for prevention of malignant tumourigenesis since in animals mosaic for *1(2)gl* homozygous and heterozygous genotype, malignant growth occurred only in clones of cells that lost the *1(2)gl* gene in the preblastoderm syncitial embryos prior to any *1(2)gl* gene expression (see review by Gateff and Mechler, 1989). The results of the present study also showed that malignant growth did not occur in clone of cells that lost the *1(2)gl* gene after embryogenesis; all cells in these individuals experienced the normal embryonic phase of *1(2)gl* gene expression. The apparently tumourous appearance of these *1(2)gl* clones results from the disrupted differentiation programmes of such cells.

4. Experimental procedures

4.1. *Fly strains and generation of recombined stocks*

The following fly stocks were used (for details of mutations not specifically referred to in the following,

see Lindsley and Zimm, 1992): *1(2)gl^4 or/SM5*; *1(2)gd^1 a px or/SM5*; *en^{lacZ}/CyO* (Hama et al., 1990); *ci-D^{plac}* (Eaton and Kornberg, 1990); *w hsFLP1*; *pw$^+$30C pFRT40A* and *y w hsFLP1*; *y$^+$25F pFRT40A* (Xu and Rubin, 1993). All flies and larvae were reared in uncrowded conditions on standard yeast supplemented food at 22 ± 1°C.

The following stocks were generated from the above through appropriate crossings: (i) *1(2)gl^4 en^{lacZ}/SM5;* (ii) *1(2)gd^1 en^{lacZ}/SM5*; (iii) *1(2)gl^4/SM5*; *ci-D^{plac}*; (iv) *1(2)gl^4 pFRT40A en^{lacZ}/SM5*; (v) *y w hsFLP1*; *1(2)gl^4 pFRT40A en^{lacZ}/SM5*.

To obtain individuals hetero- or homozygous for *1(2)gl* and heterozygous for *en^{lacZ}*, the *1(2)gl^4 en^{lacZ}/SM5* flies were crossed to *1(2)gl^4 or/SM5* flies. Likewise, to obtain individuals hetero- or homozygous for *1(2)gd* and heterozygous for *en^{lacZ}*, the *1(2)gd^1 en^{lacZ}/SM5* flies were crossed to *1(2)gd^1 a px or/SM5* flies. Persisting larvae with a bloated appearance were identified as *1(2)gl^4* or *1(2)gd^1* mutants, respectively.

4.2. X-gal staining of embryos, imaginal discs and adult wings

X-gal staining of embryos was carried out essentially as described by Bellen et al. (1989). For X-gal staining of larval imaginal discs and brain ganglia, the tissues were dissected in modified Poels' salt solution (Lakhotia and Mukherjee, 1980), fixed in 2.5% gluteraldehyde in 50 mM sodium phosphate buffer (pH 8.0) for 5 min, washed three times for 5 min each in 50 mM sodium phosphate buffer (pH 8.0) and finally incubated in X-gal solution (0.3% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 2 mM $K_4[Fe(CN)_6]$, 2 mM $K_3[Fe(CN)_6]$, 50 mM Na-phosphate buffer, pH 8.0) for 4 h at 37°C. After washing in the 50 mM sodium phosphate buffer, they were mounted in 80% glycerol in PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.4).

For X-gal staining of adult wings, the wings of freshly eclosed flies were fixed in 25% gluteraldehyde in PBS for 20–30 s, immediately washed with PBS, transferred to the X-gal solution as above and incubated overnight at 37°C. After washing in the phosphate buffer and dehydration in ethanol, the wings were mounted in 80% glycerol.

4.3. In situ hybridization to RNA in intact imaginal discs

Digoxigenin labelled *1(2)gl* DNA probe was used to hybridize in situ to transcripts in intact imaginal discs of wild type or homozygous *1(2)gl* mutant larvae. Since small sized DNA probe is reported (Kramer and Zipursky, 1992) to facilitate tissue penetrance and subsequent hybridization, the 5.3-kb *Eco*RI fragment of *1(2)gl* cDNA clone (Jacob et al., 1987) was digested with *Alu*I and *Rsa*I to generate smaller fragments. These fragments were labelled with dig-dUTP (Boehringer and Mannheim) by random priming. The probe was diluted to 40 ng/100 μl hybridization buffer (50% deionized formamide, 5× SSC, 0.1% Tween-20, 200 μg/ml yeast tRNA and 100 μg/ml sonicated salmon sperm DNA) and heat denatured before use. The procedure for in situ hybridization and colorimetric detection was as described by Kramer and Zipursky (1992).

4.4. Generation of 1(2)gl deficient somatic clones

To obtain mutant *1(2)gl* clones by somatic recombination in *1(2)gl$^+$* and *en^{lacZ}* heterozygous background, we used the yeast *'FLP-FRT'* site-specific recombination system (Golic, 1991; Golic and Lindquist, 1989; Harrison and Perrimon, 1993; Xu and Rubin, 1993). Since the *1(2)gl* gene maps in the 21A region of salivary gland polytene chromosomes, a stock carrying *FRT* insertion on left arm of chromosome 2 (i.e. 40A) was used and a *1(2)gl^4 pFRT40A en^{lacZ}* recombinant chromosome was generated by appropriate genetic crosses. To generate *1(2)gl* deficient clones in *1(2)gl* heterozygous individuals, virgin *w hsFLP1*; *pw$^+$30C pFRT40A* or *y w hsFLP1*; *y$^+$25F pFRT40A* females were mated to *1(2)gl^4 pFRT40A en^{lacZ}/SM5* or to *y w hsFLP1*; *1(2)gl^4 pFRT40A en^{lacZ}/SM5* males, respectively. The progeny first instar larvae were subjected to heat shock for 1 h at 37°C, following which they were returned to 22°C to continue further development. Heat shock to *w hsFLP1*; *+ pFRT40A +/1(2)gl^4 pFRT40A en^{lacZ}* or to *y w hsFLP1*; *+ y$^+$25F pFRT40A +/1(2)gl^4 pFRT40A en^{lacZ}* first instar larvae would induce homologous mitotic recombination specifically at 40A on the left arm of the second chromosome so that any resulting *1(2)gl*-deficient cell clones would always carry the *en^{lacZ}* gene in heterozygous condition. Wing imaginal discs from late third instar larvae or wings of adult flies were examined for *1(2)gl* mutant clones and processed for X-gal staining to detect the β-galactosidase activity under the *en* promoter. The adult flies were also examined for other cuticular defects with or without the *y* marker. The desired body parts of these flies, i.e. thorax, wings, legs, etc were dissected, softened in 10% potassium hydroxide for 10 min, dehydrated in alcohol and mounted in Euparal for microscopic examination.

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