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Drosophila tissue polarity requires the cell-autonomous activity of the *fuzzy* gene, which encodes a novel transmembrane protein

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SUMMARY

The tissue polarity gene *fuzzy* (*fy*) has two roles in the development of *Drosophila* wing hairs. One is to specify the correct orientation of the hair by limiting the site of prehair initiation to the distal vertex of the wing cell. The other is to control wing cell hair number by maintaining the integrity of the cytoskeletal components that direct hair development. The requirement for *fy* in these processes is temperature dependent, as the amorphic *fy* phenotype is cold sensitive. Analysis of mosaic wings has shown that the *fy* gene product functions cell autonomously. We have cloned the *fy* transcript, which encodes a novel four-pass transmembrane protein that shares significant homology with proteins encoded by vertebrate cDNAs. The fourth putative transmembrane domain does not appear to play a significant role in tissue polarity as it is deleted in a weak *fy* hypomorph. Expression of the *fy* transcript is developmentally regulated and peaks sharply at the time of wing cell pre-hair initiation.

Key words: *Drosophila*, *fuzzy*, tissue polarity, cold-sensitivity

INTRODUCTION

The regular orientation of pattern elements on an epithelium is a common feature of animal morphology. One striking example is the organisation into parallel arrays of the bristles and hairs on the cuticle of adult insects. To produce such regular arrays, the cells of the cuticular epithelium must be polarised within the plane of the epithelium and this planar polarity must be aligned with respect to the body axes. Mutations in the *Drosophila* tissue polarity genes result in novel patterns of bristle and hair orientation (Gubb and Garcia-Bellido, 1982; Adler, 1992; Gubb, 1993). These patterns indicate that epithelial cells in tissue polarity mutants still acquire a planar polarity, although the bristle and hair alignments differ from the wild-type pattern. The study of tissue polarity genes should, therefore, provide some understanding of the mechanism by which polarised epithelial cells are aligned with the body axes. In this report, we present the phenotypic and molecular characterisation of the tissue polarity gene *fuzzy* (*fy*).

All tissue polarity mutations affect the development of wing hairs. In the wild-type wing, each cell secretes a single distally pointing hair, the initiation of which is preceded by the accumulation of F-actin at the distal vertex of the hexagonal pupal wing cell. In the pupal wing of tissue polarity mutants, the subcellular distribution of F-actin is altered and, as a direct consequence, the final orientation of cell hairs is changed (Wong and Adler, 1993). On the basis of differences in development of their wing hairs, the tissue polarity genes have been placed in three classes. In the pupal wing of group I mutants (*frizzled* (*fz*), *dishevelled* (*dsh*) and *prickle-spiny legs* (*pk-sple*)), the F-actin bundle is frequently displaced towards the apical centre of the cell and a single hair with aberrant polarity results. In group II mutants (*inturned* (*in*) and *fy*) up to three actin bundles form at the cell periphery, so that many cells secrete extra hairs as well as displaying mutant hair polarity. Group III consists of a single gene, *multiple wing hairs* (*mwh*). The pupal wing cells of *mwh* show a similar F-actin distribution to the group II mutants, but have additional late-forming actin bundles that produce small secondary hairs. The extra hairs formed in *fy* and *in* mutants suggest a role for these genes in maintaining the integrity of cytoskeletal components required for wing hair development, as well as in hair polarity. A similar function has been proposed for RacI, a member of the rho family of small GTPases, as the expression of a dominant negative form (RacN17) in the wing phenocopies the multiple hair phenotype of the group II genes (Eaton et al., 1995, 1996).

The wing hair phenotypes of combinations of the tissue polarity mutations have suggested epistatic relationships between the three classes of genes (Wong and Adler, 1993). These interpretations are based upon the multiple hair phenotypes. The possible epistatic relationships between these genes based on the polarity phenotypes are less clear cut, although both *mwh* and *in* are close to being epistatic to *fy* and *pk* (Gubb and Garcia-Bellido, 1982; Wong and Adler, 1993; Coulson, 1994). Flies mutant for *fy* or *in*, in combination with the group I mutations, have a wing hair phenotype that more closely resembles the *fy* or *in* single mutant. In contrast, the double mutant combination of *fy* or *in* with *mwh* more closely resembles *mwh*. These observations have led to the proposal of a model in which *fy* and *in* function down-

stream of the group I genes and upstream of *mwh* in a regulatory pathway that controls the site of hair initiation (Wong and Adler, 1993).

The *fz* gene encodes a seven-pass transmembrane protein that functions both non-cell autonomously, to transmit tissue polarity information, and cell autonomously, to direct the site prehair initiation (Vinson et al., 1989; Krasnow and Adler, 1994). The cell-autonomous function of Fz is proposed to act through the product of the *dsh* gene (Krasnow et al., 1995), which encodes an evolutionary conserved protein that is also part of the *wingless* (*wg*) signaling pathway (Theisen et al., 1994; Klingensmith et al., 1994; Noordermeer et al., 1994). Both Fz and an homologous *Drosophila* protein, Dfz2, have been shown to mediate *wg* signaling in a cell culture assay (Bhanot et al., 1996). On the basis of its in vivo expression pattern Fz2 has been proposed to be a receptor for *wg*, although an analogous Wnt ligand for Fz has not been identified. Therefore, the *wg* and tissue polarity signaling pathways act through Fz-like receptors and converge on *dsh*. Although the downstream effectors of *wg* signaling in *Drosophila* have been well characterised (see, for example, Klingensmith and Nusse, 1994), little is known about the genes proposed to act downstream of *fz* and *dsh* in the tissue polarity pathway. To date, only the *in* gene has been molecularly characterised and found to encode a novel protein containing two putative membrane-spanning domains (Park et al., 1996). Here we show that *fy*, a second downstream component of this pathway, also encodes a novel transmembrane protein.

MATERIALS AND METHODS

Phenotypic analysis

Flies were cultured at 25°C on yeasted cornmeal agar unless otherwise stated. Second chromosomes used for mapping and for phenotypic analysis were *fy^2 bw*, *fy^3 cn bw sp*, *T(Y;2) fy^4 cn bw sp*, *cn bw sp*, *fy^5 b pr*, *fy^6 b pr cn*, *Df(2L)N22-14* and *Df(2L)N22-5*. Wings for phenotypic analysis were dissected from flies that had been stored at −70°C or in a 1:1 solution of 70% ethanol:glycerol at room temperature and were mounted in aquamount (BDH Ltd) with isopropanol. A representative hair polarity phenotype for each genotype was drawn after examining at least six mounted wings. Mitotic clones of *fy^2* were generated by crossing *fy^2 bw* males to *f^{36a}*; *f$^+$30B M(2)z/CyO* virgin females and irradiating F_1 larvae (dose 1000 R; 300 R/minute, 100 kV, 15 mA, 2 mm Al filter) at between 72 and 96 hours after egg laying (AEL). Wings from irradiated *f^{36a}*; *f$^+$30B M(2)z/fy^2 bw* males were mounted as described above and *f^{36a}*; *fy^2 M$^+$* clones identified by the *f* wing hair phenotype.

Nucleotide sequence analysis of cDNA and genomic clones

*Eco*RI restriction fragments from the genomic phage clone W5-p9 (Neumann-Silberberg and Schuepbach, 1993) that flank the site of the *fy^3* deletion were used to screen 5×10^5 clones of an Oregon R imaginal disc plasmid cDNA library by the protocols described in Brown and Kafatos (1988). The longest *fy* cDNA recovered (*fcJ*) was subcloned into pBluescript SK+ (Stratagene Ltd.) and deletion constructs from 5′ to 3′ of the sense strand made using exonuclease III. Single-stranded templates were produced by using the R408 helper phage (Stratagene Ltd) and were sequenced from the M13-20 primer following the Sequenase protocol (US Biochemical Corp.). Sequence data from these clones were assembled into a contig using the GCG (Genetics Computer Group, Inc.) GELASSEMBLE program. The *fcJ* cDNA was subcloned into pBluescript KS+ and the antisense strand sequenced from oligonucleotide primers complementary to the sense strand by the same protocol. The same primers were used to characterise subclones of a second *fy* cDNA (*FcH*) and the 3.2 kb *Bam*HI fragment from phage W5-p9 containing the corresponding region of genomic DNA. The best open reading frame within the composite cDNA sequence was identified by the Positional Base Preference method in the Standard Staden Programs for Nucleotide Interpretation. The encoded polypeptide was compared to protein database entries using the Blastp and Fasta programs and to DNA databases using the tBlastn program (Altschul et al., 1990; Pearson and Lipman, 1988). Prediction of helical transmembrane domains was performed by the PHDhtm program of the PredictProtein server (Rost et al., 1995) and refined by alignment with the peptide sequence encoded by a homologous *D. virilis* locus (S. C., unpublished results).

Rescue of *fy^2* phenotype

The 3.2 kb *Bam*HI restriction fragment spanning the *fy* transcription unit (see Fig. 4) was subcloned from phage W5-p9 into the pWhite Rabbit transformation vector (Dunin-Borkowski and Brown, 1995). A solution of 1 μg/μl pWhiteRabbit*fy*B3.2 and 0.25μg/μl pHsπΔ2-3 DNA in Spradling buffer was microinjected into *y w* embryos following standard protocols (Spradling, 1986). G_0 flies were crossed back to *y w* and *w$^+$* G_1 progeny crossed to a *w*; *Sco*/*In(2LR)O*, *Cy*; *In(3LR)TM2*, *Ubx^{130}*/*In(3LR)TM6B*, *Hu* stock to balance the inserts. To test for rescue of the *fy^2* phenotype, *w*; *In(2LR)O Cy*/+; fyB1/*In(3LR)TM2 Ubx^{130}*males were crossed to *y w*; *fy^2* homozygous females and the *fy^2*/*In(2LR)O Cy*; *fyB1*/+ progeny crossed to each other. Wings from *w*; *Cy$^+$* and *w$^+$*; *Cy$^+$* progeny were mounted and hair polarity patterns compared.

Characterisation of *fy* mutant alleles

The *fy^3* deletion was characterised by amplifying a DNA fragment spanning the deleted *Eco*RI restriction site from *fy^3* homozygote and progenitor genomic DNA using the oligonucleotide primers 5′-GCTACACGGACTGTCTGCTG-3′ and 5′-GCACTCATGGCATGTGCATG-3′ following the PCR protocol described in Collier et al. (1992). PCR products were purified using the Prep-a-Gene kit (Bio-Rad), heat-denatured and sequenced from the same primers following the standard Sequenase protocols (US Biochemical Corp.). To identify the *fy^2* and *fy^5* mutations, the 3.1 kb *Bam*HI/*Xho*I fragment spanning the *fy* locus (see Fig. 4) was amplified from *fy^2* homozygote genomic DNA using the flanking oligonucleotide primers 5′-CTGACCACATACTTCTTGATTC-3′ and 5′-CACACATCTTACAGTTGGATC-3′. The PCR product was digested with *Bam*HI and *Xho*I, cloned into pBluescript KS+ and characterised by single-stranded sequencing from *fy*-specific oligonucleotide primers as described above. Clones from three independent PCR reactions were sequenced to eliminate the possibility that base changes had arisen from nucleotide misincorporation by *Taq* polymerase. The *fy^2* mutation also introduces a novel *Bgl*II restriction site into the *fy* coding region, the presence of which has been confirmed by comparative Southern blot analysis.

Northern and in situ analyses of *fy* expression

For northern analysis, developmentally staged wild-type (Canton-S) animals were homogenised in a guanidinium isothiocyanate solution and total RNA isolated according to the method of Chomczynski and Sacchi (1987). Poly(A)+ RNA was prepared from the total RNA using oligo(dT)-cellulose by the method of Aviv and Leder (1972). Approximately 0.5 μg of poly(A)+ RNA from each developmental stage was separated on an agarose-formaldehyde gel, blotted to a nylon membrane and hybridised with radiolabelled *FcJ* cDNA and Rp49 probes following standard procedures. For whole-mount in situ hybridisation, sense and antisense digoxigenin-labelled RNA probes were prepared using the *FcH fy* cDNA as a template. Hybridisation

of the riboprobes to pupal wings was performed as described in Sturtevant et al. (1993) but with hybridisation and washing steps increased to 55°C.

RESULTS

The *fy* gene is not easily mutated

The original *fy* allele (fy^1) has been lost. A mutant with a similar phenotype was recovered in an EMS screen and designated fy^2 due to its' similar map position (Grell, 1969), although a test of allelism was not possible. An X-ray mutagenesis screen has been undertaken in this laboratory to create new *fy* alleles. Of 35,000 chromosomes screened, however, only two (fy^3 and fy^4) showed a *fy* phenotype in combination with the fy^2 allele (Clare Henchcliffe, unpublished results). Polytene chromosome analysis has shown that the fy^3 chromosome is cytologically normal and the fy^4 mutation is associated with a T(Y:2) translocation that breaks in the interval 29B4-C2. We have recently acquired two EMS-induced *fy* alleles fy^5 (previously fy^{JN11}) and fy^6 (previously fy^{JN12}) isolated in Paul Adler's laboratory (University of Virginia, USA).

The fy^2 and fy^3 alleles behave as amorphs as they do not give appreciably stronger phenotypes in combination with a deletion of the region (*Df(2L)N22-14*) than when homozygous. The fy^5 and fy^6 alleles are weak hypomorphs displaying only mild deviations from wild-type wing hair polarity and a low incidence of wing cells displaying extra hairs. Flies of the genotypes fy^2/fy^5 and fy^2/fy^6 have significantly stronger phenotypes than fy^5/*Df(2L)N22-14* and fy^6/*Df(2L)N22-14*, respectively, implying that the fy^2 allele displays some antimorphic character in combination with hypomorphic *fy* alleles. Since stocks homozygous for the fy^2 or fy^3 alleles can be maintained, the *fy* gene appears not to be required for viability or fertility. In contrast, the fy^4 translocation is lethal in combination with a deletion of the region (*Df(2L)N22-14*), although this lethality maps to an adjacent locus (Glynnis Johnson and S. C., unpublished results).

The amorphic *fy* phenotype is cold sensitive

Mutant *fy* flies display an altered pattern of bristle and hair orientation. The microchaetae and macrochaetae of the notum and abdomen are turned towards the midline, rather than pointing posteriorly, and the bristles of the anterior wing margin stands more erect than wild type (Grell, 1969). The cuticular hairs, which usually point distally on the appendages and posteriorly on the rest of the body, are arranged into novel patterns of polarity. The wing hair patterns of *fy* mutant flies of the same genotype that have been cultured at the same temperature are almost identical. Diagrammatic representations of the hair polarity pattern on the dorsal wing surface of fy^3/*Df(2L)N22-14* mutant flies cultured at 18°C and 29°C and also the wild-type polarity of *cn bw sp* progenitor flies are shown in Fig. 1. The hair polarity pattern on the ventral surface of the mutant wing is similar to the dorsal surface at the margins, but shows a more pronounced deviation from wild type towards the interior of the wing blade.

Flies carrying either the fy^2 or the fy^3 allele display a sig-

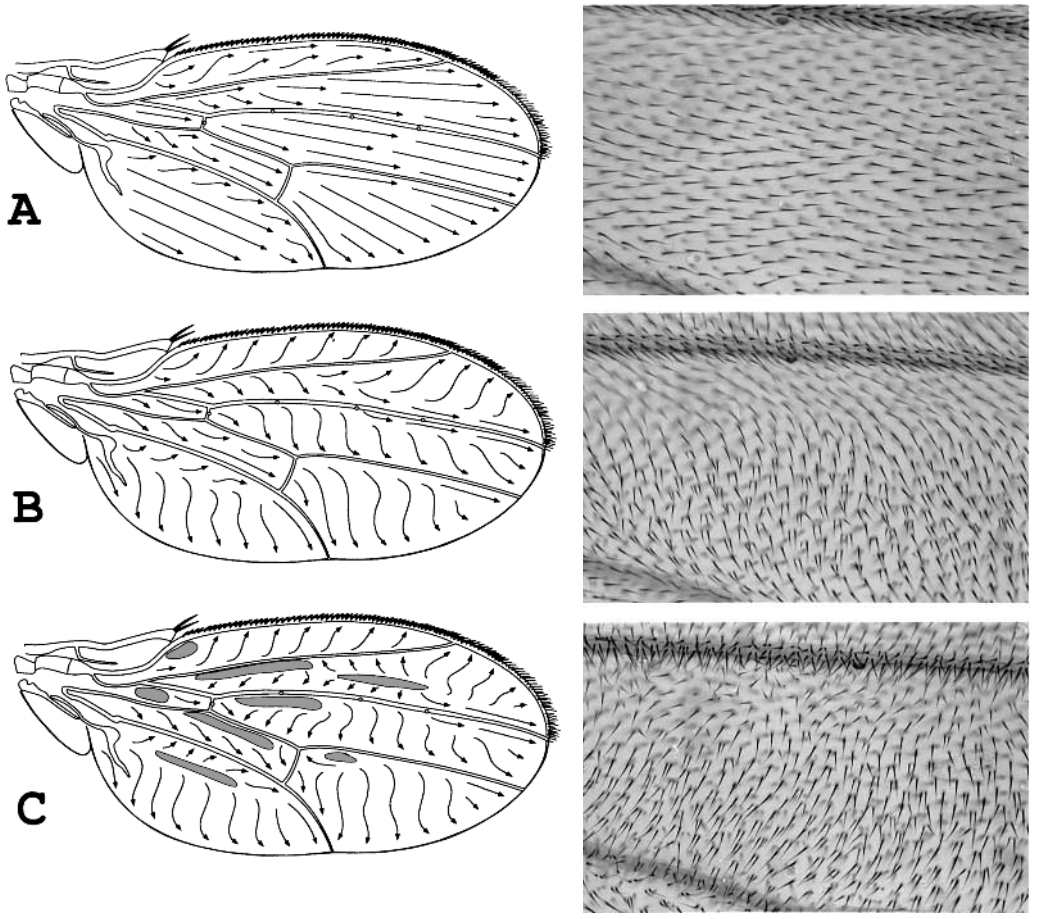

Fig. 1. Diagrammatic representation of hair polarity patterns on the dorsal wing surface of *fy* mutant and progenitor flies. Vectors delineate the direction of hair polarity for each region of the wing blade. Shaded areas represent regions for which a polarity could not be assigned either because of variation between individual wings, or because all wings show close to random orientation. Each diagram represents an average phenotype from six wings of flies raised contemporaneously. The erect triple row of the anterior wing margin associated with the *fy* phenotype is not represented. Next to each diagram is a photograph of the C region of a wing of the same genotype immediately anterior to the posterior cross vein. Phenotypes represented are; (A) *cn bw sp* (fy^3 progenitor) homozygous flies cultured at 18°C, (B) fy^3/*Df(2L)N22-14* flies cultured at 29°C and (C) fy^3/*Df(2L)N22-14* flies cultured at 18°C.

nificantly stronger phenotype when cultured at 18°C than at 29°C (Fig. 1B,C). The phenotype at 25°C is intermediate but much closer to that at 29°C. The stronger phenotype is characterised by an increase in the overall deviation from wild-type hair polarity and the proportion of the wing for which a specific polarity cannot be assigned. These changes are more evident towards the interior of the wing blade than at the margins (Fig. 1B,C). As both the fy^2 and fy^3 alleles are amorphs, this cold-sensitivity appears to derive from a temperature-dependent requirement for the *fy* gene product, rather than from the products of these alleles being inherently cold sensitive.

A proportion of *fy* wing cells secrete up to four hairs instead of the single hair produced by wild-type cells. This phenotype is not uniform across the wing blade. Cells close to the wing margin still secrete a single hair, with the exception of cells of the first two rows next to the posterior margin, between the alula and the distal tip of the L4 vein, which frequently produce two (Fig. 2A). There is also a region of wild-type hair number a few cell diameters either side of the distal region of the wing veins, particularly L3 and L5 on the dorsal surface (Fig. 2B). This possibly correlates with the fact that L3 and L5 are dorsal veins, although a reciprocal phenotype with respect to the ventral veins is less apparent on the ventral wing surface. In general, cell hair number increases with the deviation of hair polarity from wild type (e.g. Fig. 1B,C). An exception to this rule is the behavior of cells with reversed (distal-to-proximal) polarity, which usually retain wild-type hair number (Fig. 2C).

Previous studies have found that *fy* flies do not have the ectopic inverted joints in the tarsi of the leg that are associated with some tissue polarity mutants (Held et al., 1986; Coulson, 1994). However, double mutant combinations of the fy^2 allele with either *pk* or *mwh* show ectopic tarsal joints at a low frequency, which are never observed in *pk* or *mwh* mutants themselves (Coulson, 1994). These results suggest that *fy* has a redundant role in tarsal joint development.

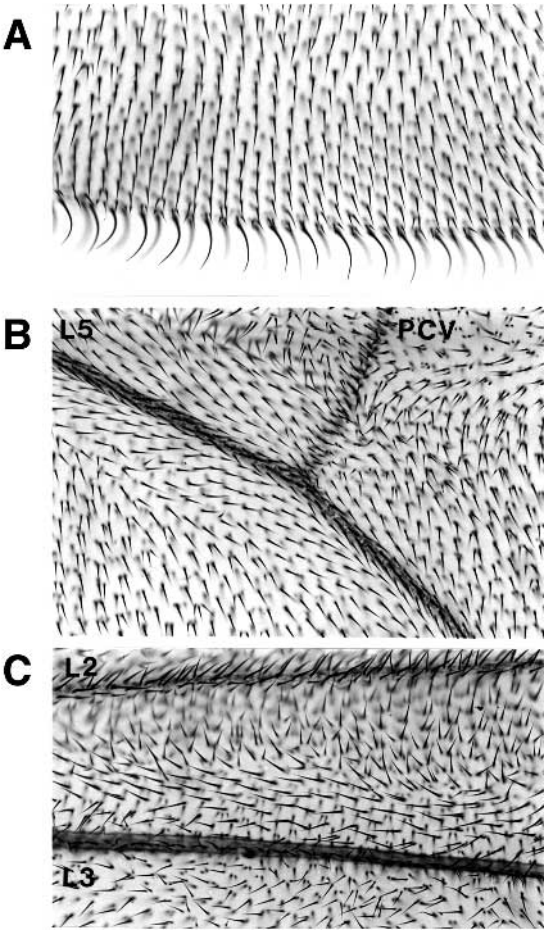

Fig. 2. Examples of the heterogeneity of the *fy* wing cell hair number phenotype from wings of $fy^3/Df(2L)N22\text{-}14$ flies cultured at 18°C. The proximal to distal axis of each wing is from left to right. (a) E region of the dorsal wing surface close to the posterior margin. Most cells retain wild-type hair number but the cells of the row immediately adjacent to the margin secrete two hairs. (B) Region surrounding the junction of the L5 and the posterior cross vein (PCV) on the dorsal surface. Most cells within four rows on both sides of the L5 vein retain wild-type cell hair number and close to wild-type hair polarity. (C) B region of the ventral wing surface. A proportion of cells anterior to the L3 vein display reversed (distal to proximal) hair polarity. The majority of these retain wild-type hair number.

Clones of fy^2 tissue mimic polarity in homozygous fy^2 wings

Clones of fy^2 mutant tissue marked with $forked^{36a}$ (f^{36a}) were induced in a f^+30B background by X-ray-induced mitotic recombination. 20 clones displaying the f^{36a} wing hair phenotype ranging from 20 cells to approximately one third of the dorsal wing surface were examined for changes in hair orientation. In each case the changes in planar polarity exhibited by cells within these clones resembles that shown by cells at an equivalent position within a fy^2 homozygous wing (Fig. 3). The exceptions were the cells towards the borders of the clones that adopt a polarity that is intermediate between mutant and wild type. It was evident that fy^2 clones do not cause domineering non-cell autonomy of the degree shown by clones of the *fz* gene (Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987), as hair polarity and hair number of fy^+ cells surrounding the clones appeared normal. However, the rescue of the f^{36a} wing hair phenotype by f^+30B construct is not complete, which made it difficult to be confident about the precise boundaries of some clones. Therefore it was not possible to rule out a limited degree of non-cell autonomy.

The *fy* locus maps close to *gurken*

The fy^2 allele has a genetic map location of 2-24.1 (Grell, 1969) and had previously been localised to the interval 29C2-29D1.2 using deficiencies of this region recovered by Wustmann et al. (1989; Clare Henchcliffe, personal communication). The cytology of the fy^4 translocation (29B4-C2) places the gene towards the distal end of this interval. We have refined this mapping by showing that *fy* is uncovered by $Df(2L)N22\text{-}14$ (29C1.2;30C8.9), but not by $Df(2L)N22\text{-}5$ (29C3.5;30C8.9). This places the *fy* locus between the distal endpoints of these two deficiencies (29C1.2;29C3.4), the same interval to which the female-sterile mutation *gurken* (*grk*) had previously been mapped. A 48 kb phage walk, initiated from the distal end of YAC clone DY51 and spanning this region, had been undertaken to clone *grk* (Neumann-Silberberg and Schuepbach, 1993) and was used to assist with the identification of the *fy* transcript. The *fy* locus was subsequently localised to the distal

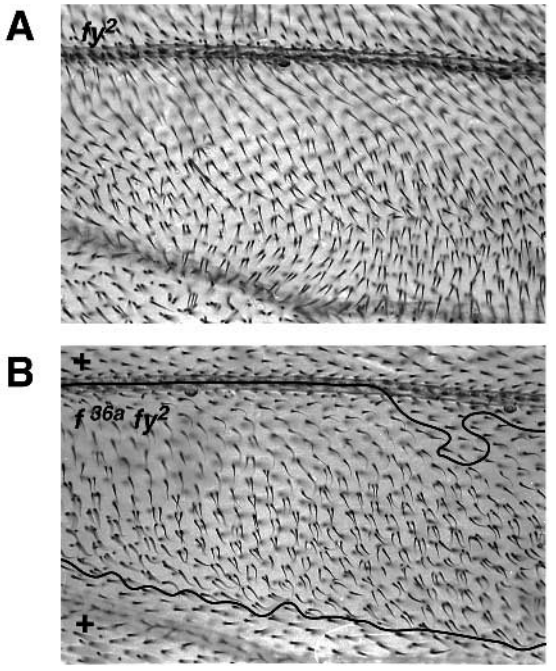

Fig. 3. Hair polarity of fy^2 clone compared to the equivalent region of a fy^2 homozygous wing. The proximal to distal axis of both wings is from left to right. (B) f^{36a} fy^2 clone from a fly cultured at 25°C. The approximate borders of the clone are marked. (A) Equivalent region of the wing of a fy^2 homozygous fly cultured at 25°C. Hair polarity within the clone mimics that of the mutant wing with the exception of cells near to the clone boundary which display a polarity intermediate between the mutant and the wild-type orientation.

end of the *grk* walk by the identification of a 70 bp deletion on the fy^3 chromosome (see below).

The *fy* transcript encodes a novel transmembrane protein

We used the genomic *Eco*RI restriction fragments from the *grk* phage walk that flank the site of the fy^3 deletion to screen approximately 5×10^5 plasmid clones from an imaginal disc cDNA library (Brown and Kafatos, 1988). Three cDNA clones spanning the deletion were isolated (*fcH*, *fcI* and *fcJ*) and also clones representing the transcripts lying immediately proximal and distal (Fig. 4). The two shorter clones *fcH* (1.6 kb) and *fcI* (1.1 kb) are polyadenylated at almost identical positions. The longer clone *fcJ* (1.9 kb) is shorter at the 3′ end and has no poly(A) tail but has 344 bp of additional 5′ sequence. The nucleotide sequences of the *fcH* and *fcJ* clones are identical in their overlapping region and the 1880 bp composite cDNA sequence is indicated in Fig. 5. The site of polyadenylation of the shorter cDNAs indicates that the 5′ to 3′ orientation of the transcript is from proximal to distal on the chromosome (Fig. 4).

The longest open reading frame (ORF) within the composite cDNA sequence is 416 amino acids in length (Fig. 5). The putative start codon is not embedded within a good *Drosophila* consensus sequence (Cavener and Ray, 1991), but alignment with the ORF encoded by a homologous *Drosophila virilis* locus suggests that it is indeed the start site for translation (S. C., unpublished results). The encoded polypeptide is predicted by the PHDhtm neural network system to have four membrane-spanning helices (Rost et al., 1995, see Fig. 5), but otherwise contains no recognisable functional motifs. The primary sequence of the *fy* gene product has been compared to protein databases using BLAST (Altschul et al., 1990) and FASTA (Pearson and Lipman, 1988) homology searches, but no significant matches were found. However, the conceptual translation of a human-expressed sequence tag (EST, I.M.A.G.E. clone 45228) from an infant brain cDNA library gives a peptide with 32% identity to a 119 amino acid portion of the *fy* sequence between the first (TM1) and the third (TM3) putative membrane-spanning regions (Fig. 6). A mouse embryonic cDNA (Life Tech clone 555546) encodes a peptide with similar degree of homology. We conclude that the *fy* gene product is a novel transmembrane protein that contains at least one domain that is conserved in vertebrate proteins.

The 1.9 kb *fy* transcript maps entirely within a 2.3 kb *Bam*HI/*Hin*dIII genomic restriction fragment. The alignment of the nucleotide sequence of this fragment with that of the composite cDNA has identified two small introns, of 58 and 59 bp, that lie towards the 5′ and 3′ ends of the open reading frame, respectively. The nucleotide sequence of the *Bam*HI/*Hin*dIII fragment is presented in Fig. 5.

A small genomic region completely rescues the fy^2 phenotype

The 3.2 kb genomic *Bam*HI fragment that spans the *fy* tran-

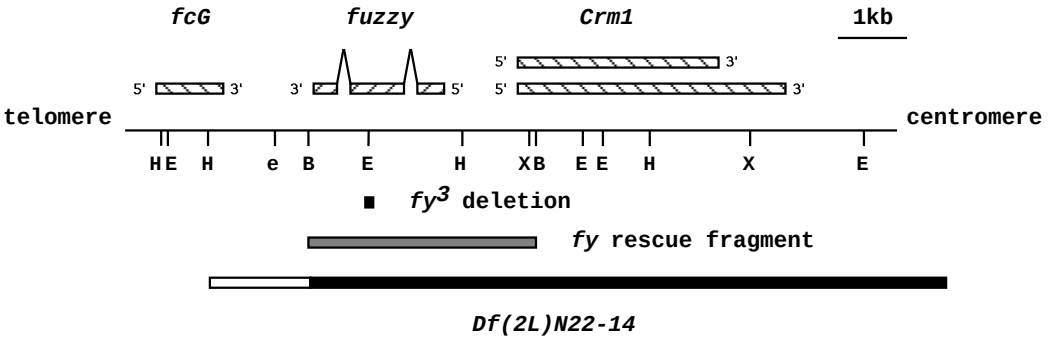

Fig. 4. Molecular map of the *fy* locus at 29C1,2. Transcripts are indicated by hatched boxes. The size of the *fy* introns is exaggerated for clarity. The intron/exon structure of the flanking genes is not shown. The *Crm1* transcripts encode a peptide with high homology to the Chromosomal Region Maintenance protein of yeast (Adachi and Yanagida,, 1989; Toda et al., 1992, Collier et al. unpublished data). *fcG* is an uncharacterised cDNA clone from an imaginal disc library. The 3.2 kb *Bam*HI fragment sufficient to rescue the fy^2 phenotype is indicated by the lightly shaded box. The regions deleted on the fy^3 chromosome and by *Df(2L)N22-14* are represented by black boxes. The unshaded portion of *Df(2L)N22-14* box indicates the degree of uncertainty concerning the distal breakpoint of the deletion. Restriction sites are B; *Bam*HI, E; *Eco*RI, H; *Hin*dIII and X; *Xho*I. The lower case 'e' is a polymorphic *Eco*RI site present on *cn bw sp* and derivative chromosomes.

script (Fig. 4) was cloned into the wRabbit transformation vector and introduced into flies by P-element-mediated transformation. A single copy of the construct on the third chromosome (*fy*B1) has proved sufficient to rescue completely the *fy*^{2} homozygous mutant phenotype. Individual rescued flies, most noticeably those cultured at 18°C, can display occasional wing cells with doubled hairs, but this is not consistent from animal to animal. In addition to the *fy* transcription unit, the construct contains just 50 bp downstream of the site of polyadenylation and the small region (around 600bp) of untranscribed sequence between the 5′ ends of the *fy* and *Crm1* loci. The complete rescue of the *fy*^{2} phenotype by a small genomic fragment may mean that all elements necessary for the wild-type expression of the *fy* transcript are contained within this region. However, as the *fz*, *dsh* and *in* phenotypes can be rescued by uniform expression driven by the *hsp70* promotor (Krasnow and Adler, 1994; Klingensmith et al., 1994; Park et al., 1996), there are precedents for tissue polarity gene function not being dependent upon wild type expression.

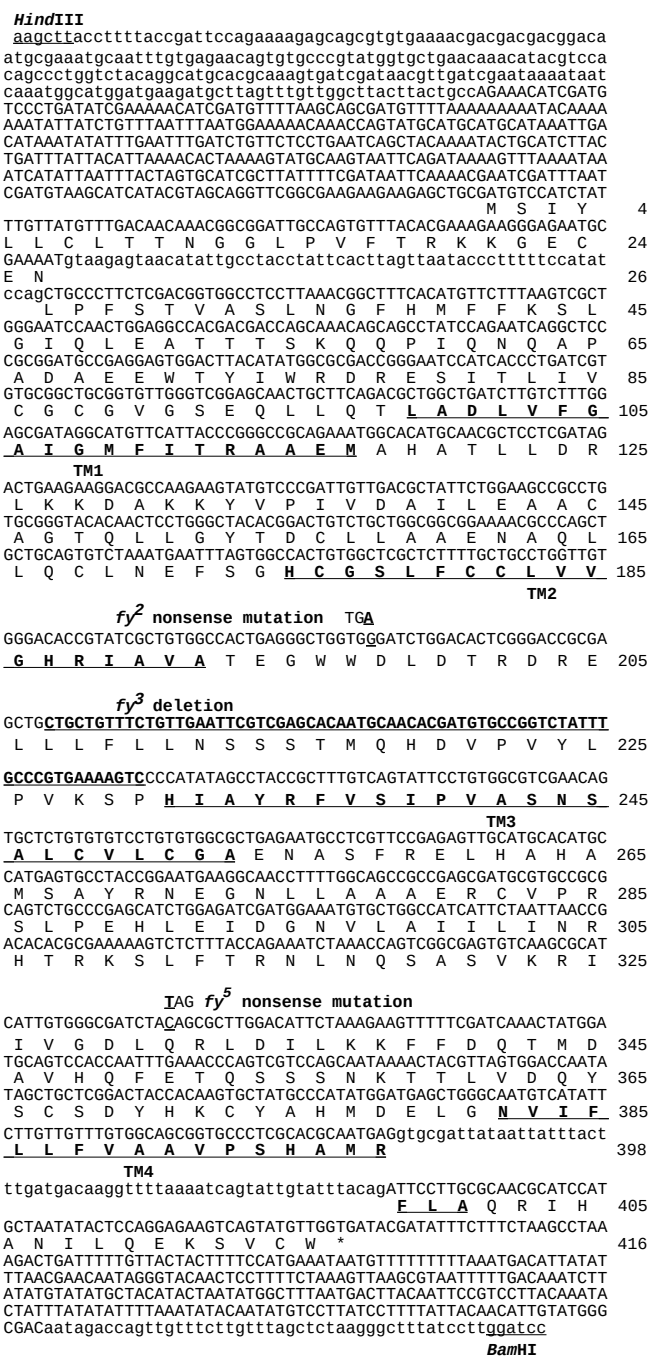

Fig. 5. Nucleotide sequence of the 2.3 kb *Hin*dIII/*Bam*HI genomic fragment that spans the *fy* locus (see Fig. 4). Sequence present in cDNA clones is shown in upper case. The G to A transition introducing the *fy*^{2} nonsense mutation, the 70 bp deleted on the *fy*^{3} chromosome and the C to T transition introducing the *fy*^{5} nonsense mutation are shown in bold type. A one-letter translation of the *fy* open reading frame is shown below the nucleotide sequence, numbers refer to amino acid positions within the coding sequence. Amino acids highlighted with bold type and underlined are within the putative membrane-spanning regions TM1 to TM4. GenBank accession no. for the cDNA sequence is AF022891.

Both strong and weak *fy* alleles encode truncated proteins

Phenotypically the *fy*^{2} and *fy*^{3} alleles behave as amorphs as they are not appreciably stronger in combination with a deficiency of the region than when homozygous. The *fy*^{5} allele is an extremely weak hypomorph that displays almost wild-type wing hair polarity and number. Molecularly the *fy*^{3} chromosome lacks an *Eco*RI site present in its progenitor strain (*cn bw sp*) and *Bam*HI and *Hin*dIII restriction fragments spanning this site are slightly reduced in size. Direct sequencing of a PCR product spanning the missing *Eco*RI site amplified from *fy*^{3} homozygote DNA, has identified a deletion of 70 bp in comparison with *cn bw sp*. The deletion is entirely within the ORF of the cDNAs (Fig. 5) and is predicted to result in a protein product in which the 210 C-terminal amino acids are replaced with just two residues, a proline and an isoleucine. The *fy*^{2} and *fy*^{5} lesions were identified by sequencing clones of PCR products spanning the *fy* coding region that had been amplified from homozygote genomic DNA. All *fy*^{2} clones contained a G to A transition within the ORF that introduces a nonsense mutation (Fig. 5) and is predicted to result in a protein lacking the 220 C-terminal amino acids. Consequently the *fy*^{2} and *fy*^{3} alleles encode similar truncated proteins that lack the two putative C-terminal membrane-spanning domains and interrupt the region of homology shared with products encoded by human and mouse ESTs (Fig. 6). The *fy*^{5} allele has a C to T transition at codon 331 that introduces a nonsense mutation resulting in a protein lacking the fourth putative membrane domain. The very weak phenotype of the *fy*^{5} allele suggests that this part of the protein, which is not conserved in the human I.M.A.G.E. clone 45228 (S. C., unpublished results), is not required for the greater part of the *fy* gene's role in tissue polarity specification.

Expression of the *fy* transcript is developmentally regulated

Hybridisation of the longest *fy* cDNA (*fcJ*) to a northern blot of poly(A)+ RNA isolated from wild-type (Canton-S) animals at a series of developmental stages is shown in Fig. 7. An mRNA species of just under 1.9 kb was identified consistent with the length of the composite cDNA sequence presented in Fig. 5. A low level of expression of this transcript is detectable at all stages of development, with a relatively strong peak of expression in the 2-day-old pupa that coincides with the period of prehair initiation. Significant expression is also detectable

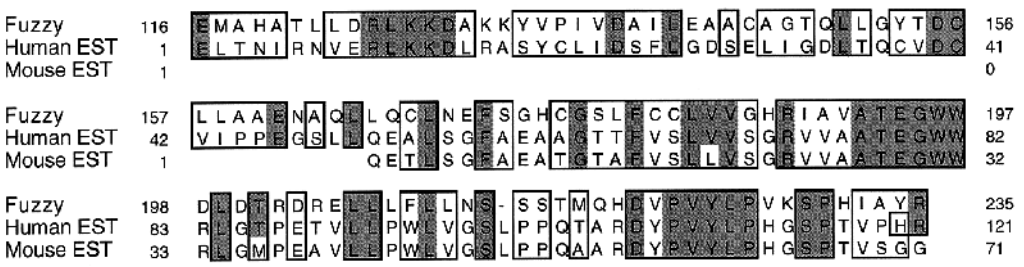

Fig. 6. Alignment by homology of the primary peptide sequences encoded by the *fy* gene and by Expressed Sequence Tags from human (EST, I.M.A.G.E. clone 45228) and mouse (Life Tech clone 555546) using the ClustalW program. Identical residues are shaded and homologous residues boxed. Our preliminary nucleotide sequence analysis of clone 45228 has identified an error in the sequence of the database entry that results in a reading frame shift and that has been corrected to create this alignment.

in the embryo and female, but not male, adult flies suggesting that the embryonic expression has a maternally derived component. The maternal and embryonic transcripts appear slightly shorter than the pupal transcript (Fig. 7). As no evidence has been found for alternatively spliced *fy* transcripts, this size difference may reflect differences in polyadenylation.

We have hybridised pupal wings at the time of highest *fy* expression with sense and antisense riboprobes. The pattern shown by the antisense strand is shown in Fig. 7B, the sense strand did not produce significant hybridisation. At this stage the *fy* transcript is present in all wing cells consistent with the *fy* gene having a cell-autonomous phenotype that affects all regions of the wing blade. The transcript is relatively abundant adjacent to the wing margin and in bands of cells flanking each of the wing veins with the exception of L2. This pattern may simply reflect cell densities in the pupal wing at this stage. The lack of staining surrounding L2, however, is reminiscent of the BrdU-staining patterns at 15-20 hours after pupation (Schuebiger and Palka, 1987; Milan et al., 1996), raising the possibility that *fy* expression is related to the exit of pupal wing cells from their last cell division.

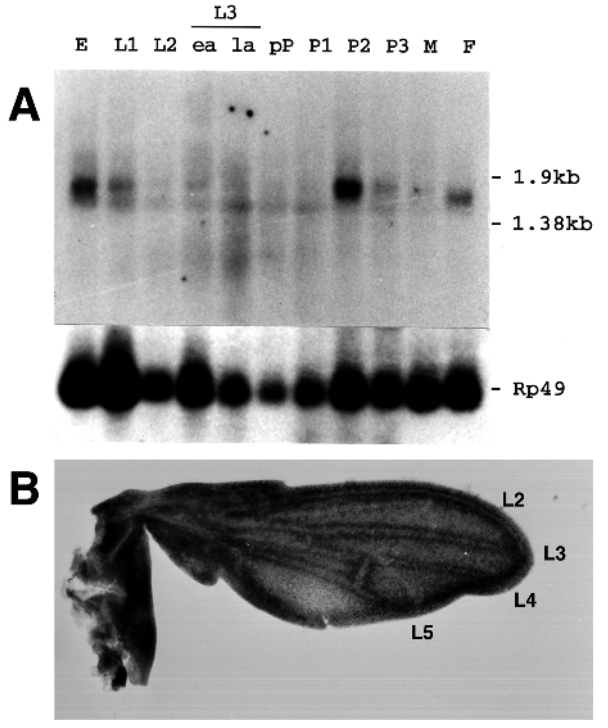

Fig. 7. (A) Hybridisation of a radiolabeled *fy* cDNA (*fcJ*) probe to a northern blot of poly(A)$^+$ RNA from *Drosophila* developmental stages. Stages are; E; embryo, L1; first larval instar, L2; second larval instar, L3; third larval instar (ea; early, la; late), pP; prepupal, P1; first day pupa, P2; second day pupa, P3; third day pupa; M; adult male, F; adult female. Sizes refer to migration of RNA marker bands. Hybridisation of an Rp49 probe to the same blot is shown below as an indication of the relative amounts of RNA loaded. (B) Hybridisation of a digoxigenin-labeled *fy* antisense riboprobe to a pupal wing 31 hours after pupal formation. The distal ends of the L2, L3, L4 and L5 veins are indicated.

DISCUSSION

The *fy* wing hair polarity phenotype

Mutations in the *fy* gene result in new patterns of wing hair polarity (see Fig. 1B,C). As a rule, hairs in the anterior third of the *fy* mutant wing point more anteriorly than wild type and those in the remainder of the wing point more posteriorly than normal. Similar patterns are produced by the other tissue polarity mutations (with the exception of the *pk* single mutant where the pattern is almost reversed) and also by the double mutant combinations of tissue polarity genes (Gubb and Garcia-Bellido, 1982; Wong and Adler, 1993; Coulson, 1994). It seems, therefore, that there is an underlying pattern that results from reduced tissue polarity gene activity. The existence of such a 'default' pattern would imply that mutant cells still express a planar polarity that depends upon their position within the wing blade, even without information from the tissue polarity gene pathway. It is not clear, however, what information the mutant cells are responding to. Tissue polarity mutant hair patterns do not appear to be influenced by the anterior-posterior compartment boundary of the wing and the domineering non-cell-autonomy associated with *fz* clones can cross the compartment boundary (Gubb and Garcia-Bellido, 1982). These observations suggest that the orientation of a wing cell's planar polarity is independent of its lineage.

It is clear that the *fy* phenotype is influenced by the presence of wing veins. Veins often correspond to discontinuities in the polarity pattern (see Fig. 1B,C) and mutations that remove wing veins also remove the associated discontinuities in the *fy* wing hair pattern (S. C., unpublished observations). Cells surrounding the wing veins often show close to wild-type polarity (see Fig. 2B), implying that veins can direct polarity in the absence of *fy* gene function. However, wing vein differentiation is not required to align cell polarity in the wing as the

veinlet (*ve*) *vein* (*vn*) double mutant combination, which eliminates all veins but L1, displays normal hair polarity.

The *fy* wing hair number phenotype

One property of the *fy* wing phenotype that is shared by *in* and *mwh*, is a high incidence of cells producing two or more hairs. As a rule, the further a mutant cell's planar polarity is from wild type, the more likely it is to produce multiple hairs (see for instance Fig. 1B). There are, however, two pieces of evidence from the *fy* mutant wing that suggest that mutant cell hair number is separable from hair polarity. First, the cells that are most strongly mutant for polarity, i.e. those producing hairs pointing from distal to proximal, usually retain wild-type hair number (Fig. 2C). Second, although the cells close to the posterior wing margin show a comparable hair polarity, only those in the two rows immediately adjacent to the margin display an abnormal hair number (Fig. 2A). Indeed, these are the cells that are most likely to show multiple hairs on fy^5 mutant wings that retain close to wild-type hair polarity. In addition, some combinations of *fy* alleles display an incidence of split, rather than doubled, wing hairs. Since wing hairs have been proposed to elongate from the tip (Eaton et al., 1996), the formation of split hairs suggests that *fy* has a role in maintaining the integrity of the hair as well as controlling its initiation. Pupal wings treated with microtubule antagonists phenocopy the multiple hair phenotype of *fy* wing cells (Chris Turner and Paul Adler, personal communication), suggesting that *fy* is required for the integrity of the microtubule arrays that direct prehair development. The cold-sensitivity of amorphic *fy* alleles is consistent with a role in stabilising microtubules as microtubules are inherently cold sensitive. A similar mechanism has been previously proposed to explain the cold-sensitivity of *in* alleles (Adler et al., 1994). Flies mutant for the *fritz* (*frtz*) gene have a similar phenotype to *fy* and *in* (Gubb, 1993). Strong *frtz* alleles are also cold sensitive (S. C., unpublished observations) supporting a general role for the group II genes in a temperature-sensitive developmental process.

fy specifies tissue polarity cell autonomously

The wing hair polarity within fy^2 clones mimics that at the equivalent position on the wing of a fy^2 homozygous mutant fly, implying that the planar polarity of a fy^- cell is expressed cell autonomously and is dependent upon its position within the wing blade. The exceptions to this observation are the cells at the borders of fy^2 clones which display a polarity between mutant and wild type (see Fig. 2B). This could be due to partial rescue of the mutant cell polarity phenotype by wild-type tissue. Alternatively, there may be an mechanism, independent of *fy* function, by which hair polarity is aligned with neighbouring cells (Adler et al., 1987). Such an activity would explain why adjacent hairs on wings mutant for *fy* wing are orientated to form smooth, rather than irregular, curves (see Fig. 1B,C). The fy^2 clones examined did not affect the polarity or hair number shown by adjacent wild-type cells over a significant distance. Therefore, whilst the *fy* gene is required for cells to interpret polarity information correctly, it is probably not needed for the transmission of such information between cells. This is consistent with the proposed role of the *fy* gene downstream of *fz* and *dsh* in a cell-autonomous pathway that specifies prehair initiation (Krasnow et al., 1995). The *in* gene is proposed to function at the same step in this pathway and has a similar cell-autonomous phenotype to *fy* (Gubb and Garcia-Bellido, 1982; Park et al., 1996). The finding that both *fy* and *in* encode putative transmembrane proteins suggests their gene products may be co-localised within the cell.

fy expression coincides with polarity determination

There is an abrupt peak of *fy* expression in the 2-day-old pupa. At this time, cell division in the wing is complete (Schuebiger and Palka, 1987; Milan et al., 1996) and the formation of wing cell prehairs, which is characterised by the localisation of F-actin to the distal vertex of the cell (Wong and Adler, 1993). The pattern of *fy* expression in the pupal wing at this time (Fig. 7B) resembles the pattern of incorporation of BrdU in the final cell divisions of the wing (Schuebiger and Palka, 1987; Milan et al., 1996). As the patterns of last cell divisions in the wing do not appear to be as strictly regulated as they are, for example, in the *Drosophila* eye, a reference point for gene activation would be the time at which a cell becomes mitotically quiescent. The transient expression of *fy* after cell division prior to cell differentiation suggests that the *fy* gene product is expressed specifically to interpret polarity information. This raises the possibility that one of the components of the putative tissue polarity signal transduction pathway will be a transcription factor that promotes *fy* expression. None of the tissue polarity genes characterised so far encodes a transcription factor, but it has been reported that the *fz* protein is required for normal expression of the *nemo* gene and in the eye (Zheng et al., 1995). A simple model of *fz* activating *fy* transcription seems unlikely, however, as data on epistatic interactions have suggested that the *fz* gene regulates the activity of *fy* and *in* (Wong and Adler, 1993). It is possible, therefore, that *fy* expression is activated by a mechanism that promotes cell differentiation at the time the cell has completed division and that the *fy* gene product is regulated by *fz* through protein-protein interactions rather than transcriptional control.

The embryonic expression of the *fy* transcript is intriguing, as *fy* mutants have no known embryonic phenotype. However, the tissue polarity genes *fz* and *in* are also expressed in the embryo and have no associated phenotype (Adler et al., 1990; Park et al., 1996). This suggests either that the putative tissue polarity signaling pathway is redundant during embryogenesis, or that it plays a role in establishing planar polarity in the embryo that is reflected in a less conspicuous way than the orientation of bristles and hairs on the adult cuticle.

Two roles for the *fuzzy* gene in wing hair development

In summary, the *fy* gene encodes a novel four-pass transmembrane protein that plays two roles in the development of hairs on the *Drosophila* wing. The first is to specify the correct orientation of the hair by restricting its initiation to the distal vertex of the cell. This activity is proposed to be directed by polarity information received by the Fz receptor, possibly through Wnt signaling, and transmitted by the Dsh protein. The second role of *fy* is to permit the development of just a single cell hair by maintaining the integrity of the F-actin and microtubule arrays that are required for hair development, a process that may also require the activity of the small GTPase Rac1.

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