

# The *Drosophila* tissue polarity gene *inturned* acts cell autonomously and encodes a novel protein

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## SUMMARY

Mutations in the *inturned* (*in*) gene result in abnormal wing hair polarity and in many wing cells forming two or more hairs instead of the normal single hair. We have generated genetic mosaics in a number of different experiments and find that the *in* gene is required in all regions of the wing and that it functions in a cell autonomous fashion. We report the molecular cloning of the *in* gene, the molecular mapping of *in* mutations and the isolation and sequencing of an *in* cDNA clone. The *in* gene encodes a novel protein

whose sequence suggests it will be membrane bound. The ability of an *in* cDNA, the expression of which is driven by the basal activity of the *hsp70* promoter to rescue an *in* mutation suggests that patterned expression of *in* is unlikely to play a role in the function of this gene.

Key words: *Drosophila*, tissue polarity, *inturned*, wing hair development

## INTRODUCTION

Polarized structures that decorate the adult cuticle of insects such as *Drosophila* typically display a common orientation (Adler, 1992; Gubb, 1993). For example, on appendages sensory bristles and hairs point distally. Mutations in tissue polarity genes result in bristles and hairs pointing in abnormal directions (Gubb and Garcia Bellido, 1982; Wong and Adler, 1993). On the wing essentially all cells produce a single distally pointing cuticular hair derived in pupal wing cells from a cellular extension called the prehair. The prehair contains abundant actin filaments and microtubules (Mitchell et al., 1983; Fristrom et al., 1993; Wong and Adler, 1993), and is typically assembled at the distal most vertex of the pupal wing cells (Wong and Adler, 1993). Genetic studies place the tissue polarity mutations into 3 phenotypic/epistasis groups (Wong and Adler, 1993). Mutations in each group alter prehair initiation in distinctive ways. For example, in *inturned* (*in*) mutants, prehair initiation is not restricted to the vicinity of the distal vertex. This results in a majority of cells forming more than one prehair initiation center and adult hair (Wong and Adler, 1993). The formation of prehairsts at alternative locations is correlated with hairs having non-distal polarity. We previously suggested a model where the *frizzled* (*fz*)-like genes function upstream of the *in*-like genes, which are in turn upstream of *multiple wing hair* (*mwh*) (Wong and Adler, 1993). These genes are suggested to function as part of an intercellular signaling and intracellular signal transduction system that regulates the subcellular location for prehair initiation (Wong and Adler, 1993).

The temperature sensitive period of *in<sup>ts</sup>* is short and ends a couple of hours prior to the first visible sign of prehair morphogenesis (Adler et al., 1994). This supports the hypothesis that *in* functions in the regulation of prehair initiation, but that it has no role in the actual outgrowth of the prehair. Previous studies suggested that *in* functioned cell autonomously in regulating hair number and polarity (Gubb and Garcia-Bellido, 1982), but this conclusion was based on unmarked clones where the mutant clone boundaries could not be unambiguously determined. We have therefore reexamined the question of cell autonomy for *in*. We first repeated and confirmed the results of Gubb and Garcia-Bellido (1982). To determine if *in* cells could be rescued by a neighboring wild-type cell we generated twin spots of cells homozygous for either *in* or a cell autonomous hair morphology mutant. We found cells with an *in* phenotype (i.e. two hairs) juxtaposed to cells that displayed the morphological marker and hence were genetically wild type for *in*. This shows that the presence of a wild-type neighbor does not rescue a genetically *in* cell. We also generated marked *in* clones and found that these clones typically have no effect on the behavior of neighboring wild-type cells (as clones of *frizzled* and *prickle* do; Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987). Rare examples of a possible effect on neighboring wild-type cells were seen. Our conclusion is that *in* is cell autonomous in action.

We have used a YAC based walking technique to molecularly clone the *in* gene. The DNA sequences that encode *in* were identified by mapping the location of mutations on genomic Southern blots, via northern analysis of mutations that alter the abundance of the *in* mRNA, via the sequencing of

point mutations and via transformation rescue. The almost complete rescue obtained from a fusion gene where the expression of the *in* open reading frame is driven by an *hsp70* promoter indicates that patterned transcription does not play an important role in *in* function. The sequence of the *in* cDNA indicates that it encodes a novel protein with a number of potential transmembrane domains.

## MATERIALS AND METHODS

### Mutant strains

Most of the *in* alleles used were isolated in this lab and described in detail previously (Adler et al., 1994a). Most of our *in* alleles were isolated in two mutant screens. The I series of alleles (e.g. *in<sup>IH56</sup>* was isolated in an *ri* mutant background. The G series of alleles (e.g. *in<sup>GN4a</sup>*) was isolated in a different genetic background that was wild-type for *ri* (the original third chromosome on which these were induced carried a *P[w<sup>+</sup>]* transposon. The *in<sup>CAH3a</sup>* allele was isolated from the progeny of F<sub>1</sub> flies obtained by crossing the Oregon R (M) and Harwich (P) wild-type stocks. The cell autonomously acting hair morphology mutant *starburst* (*strb*) was isolated in a large FLP/FRT based mutant screen carried out in our lab, and will be described elsewhere. The stocks carrying the *hs-FLP* gene, the FRT80 stock and the N-myc cell marker were constructed by Xu and Rubin (1993) and obtained from the Drosophila Stock Center at Indiana University. Stocks carrying the various balancer chromosomes and other marker genes were obtained from the Drosophila Stock Centers at Indiana University and Bowling Green State University.

### Mitotic clone analysis

Mitotic clones were generated either by the use of the FLP/FRT system (Golic and Lindquist, 1989; Xu and Rubin 1993), or via  $\gamma$ -ray irradiation (1000 R). In most experiments, 2- to 4-day old larvae were treated to induce mitotic crossing over.

### Preparation of mini genomic library of 76F3-77C2 region

Genomic DNA from the DY317 containing yeast strain (Garza et al., 1989) was isolated and a library derived from it was made in  $\lambda$  FIXII (Stratagene). A total of 3,000 plaques from this library was screened with two probes. One consisted of the YAC DNA band (about 220 kb) cut out from a FIGE (field inversion gel electrophoresis) gel. Since this DNA was inevitably contaminated with the yeast chromosome 2, which is similar to the YAC DY317 in size, we also used as a negative control a probe made from a yeast chromosome 2 band. DNA was isolated from each gel using GeneClean resin (Bio 301), labeled by oligolabelling (Oligolabelling Kit, Pharmacia) and used for screening the genomic library. Forty nine putative *Drosophila* clones were picked which were hybridized to the DY317/chromosome 2 probe, but not to chromosome 2 probe. The  $\lambda$  clones were placed in one new plate in a grid pattern and transferred to a nitrocellulose filter (Pieretti et al., 1991).

### Genomic walking

In the YAC vector, the 5' sequence of the P element is juxtaposed to one end of the *Drosophila* DNA insert and the 3' sequence to the other end (Garza et al., 1989). We identified three 5' P end clones and four 3' P end clones from the library. The inserts of these end-specific clones were used for in situ hybridization to salivary gland polytene chromosomes. The 5' P end probe hybridized to 76F and the 3' P end probe to 77C confirming that DY317 contained the *in* locus. We initiated genomic walking from the 3' P end. The four 3' P end clones isolated earlier were digested with restriction enzymes that allowed the insert DNA fragments to be distinguished from  $\lambda$  vector arms. The best clone was selected, and insert DNA of this clone was used to probe the phage grid filter to identify overlapping phage. This process was repeated ten times and the results are shown in Fig. 3. More details can be found in Park (1993).

### cDNA library screening

The screening of cDNA libraries was performed essentially as described by Maniatis et al. (1989). A  $\lambda$ gt11 *Drosophila* pupal cDNA library, purchased from Clontech, and an early embryo (4-8 hrs) cDNA library (Brown and Kafatos, 1988) were screened. Clones for neighboring genes were isolated from the pupal library, but no *in* clones were recovered from more than 500,000 plaques screened. We obtained six identical *in* cDNA clones from the early embryonic library after screening 3,000,000 colonies.

### Sequencing of *in* mutants

Ten 300-600 bp PCR products were generated from *in* mutant DNA that covered the *in* genomic DNA sequences and these were analyzed for mutations on an MDE gel (AT Biochem). PCR products showing double bands were directly sequenced (Exon-flu DNA Sequencing Kit, Stratagene).

### Construction of transformation plasmids

A *SalI-EcoRI* fragment containing the whole *in* open reading frame was cut from the original pNB40 clone (Brown and Kafatos, 1988) and subcloned to pBluscriptII (Stratagene) resulting in pBInW. A *ClaI-XbaI* fragment from pBInW was subcloned to pBHS (Park et al., 1994) resulting in pBHSInW. Finally a *KpnI-BamHI* from pBHSInW was subcloned to pW8 (Klemenz et al., 1987) resulting in the *hs-in* injection construct, pW8InW.

Site-directed mutagenesis was performed according to Ho et al. (1989) to generate an RGE mutant *hs-in* construct, pW8InM. In the first round of PCR, primer sets used were: (a) 5'-AGT CGA CAT TCA GAG CTG-3'; (b) 5'-CAA GCG GGG AGA GTG GTT TAG-3'; (c) 5'-CTA AAC CAC TCT CCC CGC TTG-3'; (d) 5'-GTT CTC CAC CTT GCA CAC-3'. The second PCR was performed using primers (a) and (d). The PCR product was digested with *StuI* and *AvrII* and subcloned to pBInW.

A *XbaI-BamHI* fragment, about 12 kb long, from the genomic clone 37 was subcloned to Casper (Pirrota, 1988) to generate a genomic *in* injection construct, CasperIn. Embryos were injected with transformation constructs by standard techniques (Spradling and Rubin, 1982).

## RESULTS

### *inturned* acts cell autonomously

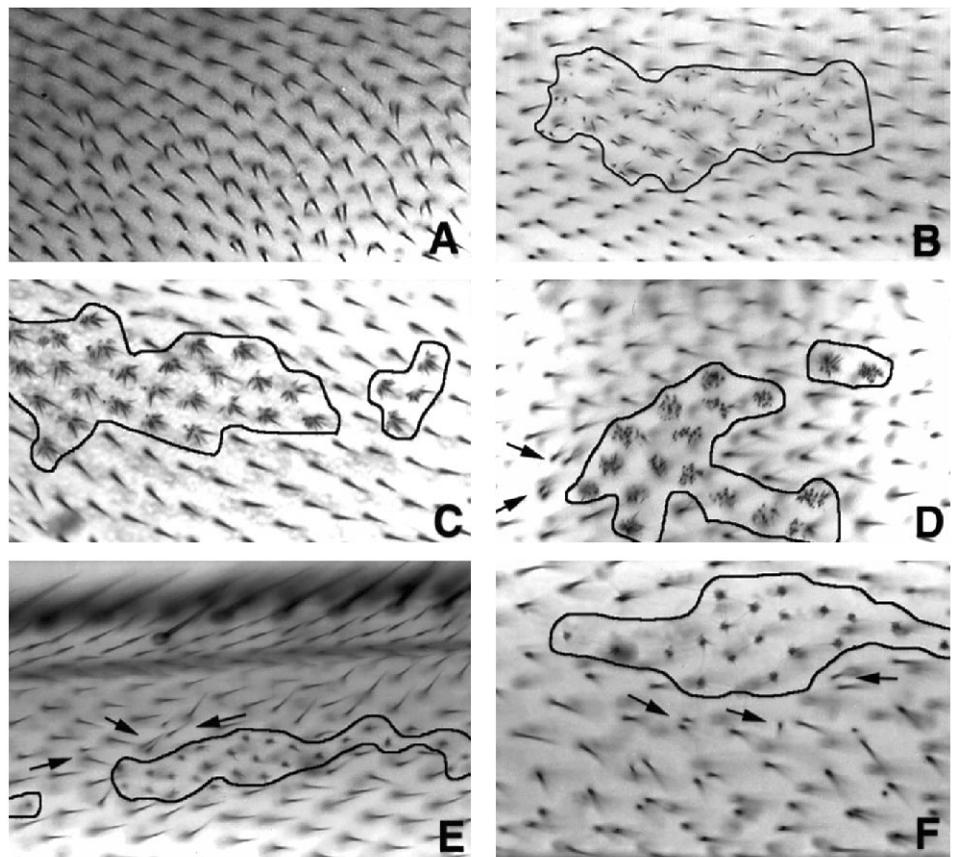
Previous experiments by Gubb and Garcia-Bellido (1982) showed that it was possible to see an *in* phenotype in mosaic wings carrying *in* clones. In these experiments the clones were not marked in any other way, so that it was not possible to determine if *in* acted in a truly cell autonomous fashion. As a first experiment we also made unmarked clones. We examined three *in* alleles (*in<sup>IH56</sup>* (68 clones), *in<sup>IH52</sup>* (22 clones) and *in<sup>I</sup>* (17 clones)) and for all of them we found clones in all regions of the wing where cells displayed an *in* phenotype. That is, we saw cells that formed more than one hair, and hairs with an abnormal polarity (Fig. 1A). That we could find cells in all regions of the wing that displayed an *in* phenotype suggests that *in* function is required in all regions of the wing.

We next made *mwh in* clones to determine if the presence of neighboring *in* cells resulted in wild-type cells being induced to produce an *in* phenotype. Since *mwh* is epistatic to *in* with respect to the multiple hair phenotype (Gubb and Garcia-Bellido, 1982; Wong and Adler, 1993), *mwh* acts cell autonomously, and all *mwh* cells produce a mutant phenotype, we could define the boundary of a clone by the *mwh* phenotype and ask if any neighboring cells displayed an *in*-like phenotype (i.e. altered polarity and many cells forming two hairs). We

found and scored 67 *mwh in* clones in all regions of the wing and found no evidence of clones affecting the morphogenesis of the neighboring wild-type cells (Fig. 1B). That is, we saw no evidence of single hair cells with abnormal polarity. An occasional cell was seen that produced only two hairs instead of the three or more that *mwh* cells typically produce. However, in *mwh* (and *mwh in*) wings we have found that about 5% of cells produce only two hairs (P. N. Adler, unpublished data), thus the occasional two hair cells in the *mwh in* clones are expected, and there is no reason to believe that they represent genetically wild-type cells that are induced to display an *in*-like phenotype. As a second experiment to address this question we made *trc in* clones. Cells mutant for *trc* (Ferrus, 1976), like those mutant for *mwh*, produce extra hairs. The phenotype of *trc* is more severe, however, with *trc* cells producing an average of almost 6 hairs per cell (with some cells producing 10 or more hairs; P. N. Adler, unpublished data) versus about 4 hairs per cell for *mwh* (Wong and Adler, 1993). A major difference between the *mwh in* clones versus the *trc in* clones is that in these later clones the cells display a much more severe phenotype than cells singly mutant for either gene alone. Thus, *trc in* mutant cells produce on average more than 14 hairs, with a range of from 5-25 hairs per cell (Fig. 1C,D). We examined 90 *trc in* clones and in 82 of them there was no hint of the mutant cells affecting the differentiation of the neighboring wild-type cells (Fig. 1C). In 8 clones however, we saw one or two cells at the periphery of the clone that produced two hairs and resembled a typical *in* cell (Fig. 1D). Given the extreme phenotype of the *trc in* cells, the cells that produce two hairs are well out of the phenotypic distribution of the *trc in* cells and hence unlikely to represent a phenotypic tail as might be the case in the experiment that used *mwh* as a cell marker. These rare cells could be evidence for a cell non-autonomous action of *in* over a short distance, although it is not the majority result and other explanations are possible.

The observation that the vast majority of wild-type cells appeared to differentiate normally when bordering *mwh in* or *trc in* clones would indicate that *in* does not act cell non-autonomously in a domineering manner as do *fz* and *pk* (Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987). The experiments reported above, could not however, determine if neighboring wild-type cells could rescue neighboring *in* cells. The results from the unmarked *in* clones indicated that such rescue could not be complete, but it could be

partial (e.g. only cells directly touching a wild-type cell would be rescued). To test this possibility we have generated twin spots between *in* and a second cell autonomously acting mutation that produces hairs with a grossly abnormal morphology. In this experiment we used a newly discovered hair morphology gene *starburst* (*strb*). Mutations in this gene results in thin, short, and often split hairs (J. Charlton, J. Liu and P. N. Adler, unpublished data). *w hsf1p; in FRT80/strb FRT80* larvae were heat shocked to induce FLP catalyzed crossing over. This should generate twin spots of neighboring clones that are homozygous for either *in* or *strb*. In this experiment we can be confident that any *strb* cells seen are genetically wild type for *in*. We frequently found cells that produced an *in* phenotype (defined as two or three hairs for this experiment) that were touching one or more *strb* cells (Fig. 1EF). For example, in one experiment we identified 22 twin spots (defined as the presence of phenotypically *strb* and *in* cells within 3 cells of each other), and in 18 of these at least one (and typically several) *in*-like hair cell was juxtaposed to one or more *strb* cells. In those twin spots where we did not see the juxta-



**Fig. 1.** Light micrographs of *in* clones. In all micrographs distal is to the left and proximal to the right. (A) An unmarked *in*<sup>IH56</sup> clone. Note the many double-hair cells and the abnormal polarity. (B) An *mwh in*<sup>IH56</sup> clone. The outline of the clone as determined by those cells showing the *mwh* multiple hair cell phenotype is drawn. Note the normal morphology and polarity of the neighboring wild-type cells. C and D show *trc in*<sup>IH56</sup> clones. The outline of the clone is drawn in. Note the extreme multiple hair cell phenotype of the *trc in*<sup>IH56</sup> cells. Many of the smallest hairs produced by these cells are not obvious in the photographs. These clones had a tendency to become separated as is shown in both of these micrographs. The *trc in*<sup>IH56</sup> hairs in D have a more upright orientation than those in C resulting in their different appearance in the micrograph. Arrows point to two *in*-like cells at the periphery of the *trc in*<sup>IH56</sup> clone. E and F show twin spots of *in*<sup>IH56</sup> and *strb* clones. The boundary of the *strb* clones are outlined. Arrows point to double hair cells juxtaposed to *strb* cells.

position of phenotypically *in* and *strb* cells there appeared to be only a very short (2-4 cells) boundary where the two clones touched. Hence the lack of phenotypically touching *in* and *strb* cells in these twin spots may be due to chance and the lack of complete cellular penetrance of *in* mutations (i.e. not all *in* cells form more than one hair). We conclude that the presence of a direct wild-type neighbor does not result in the rescue of an *in* cell.

### Molecular cloning of *in*

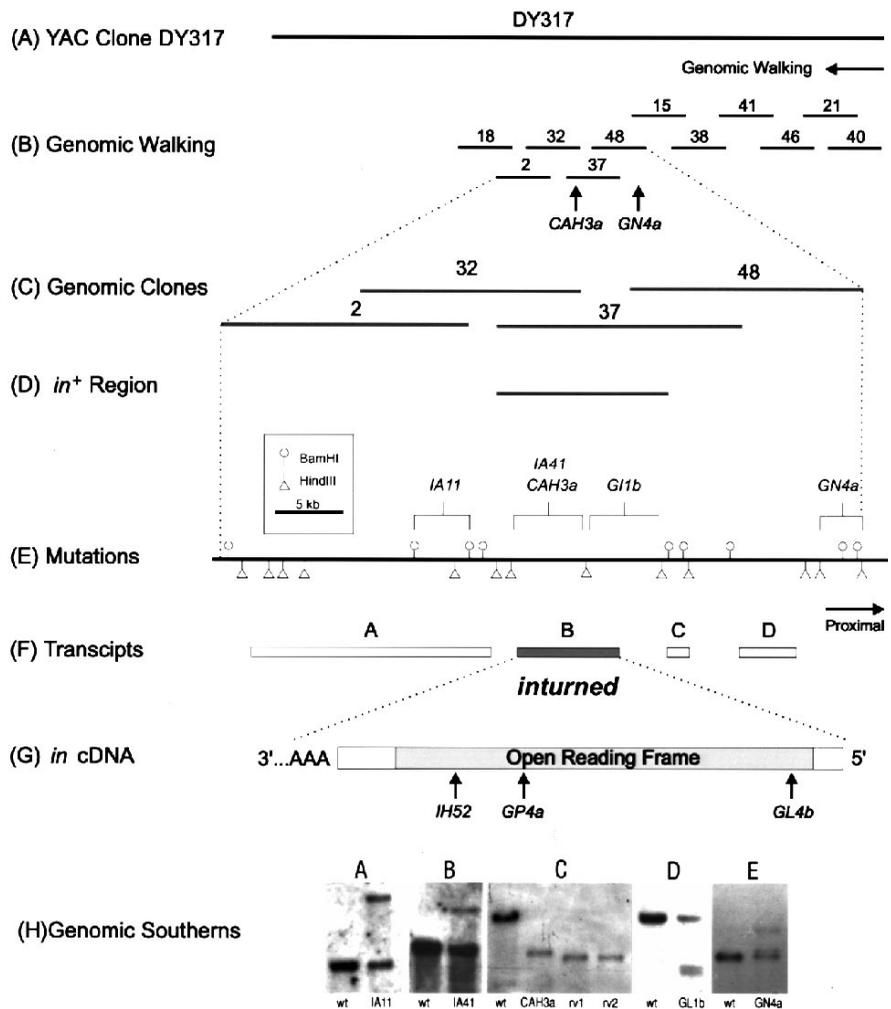
In a previous study, we found that *in* was located in the 77B2-77C1 region (Adler et al., 1994). To analyze this region and clone the *in* gene, we utilized a YAC clone, DY317, which spans 76F3-77C2 (Garza et al., 1989). We isolated a  $\lambda$  phage library of 49 clones derived from DY317 (see Materials and Methods), and carried out a walk in this mini-library (Fig. 2B). Inserts from phage in the walk were used to probe genomic Southern blots to identify RFLPs associated with a number of *in* mutations where we had expectations that a gross change in DNA structure had occurred (Fig. 2H). We were able to map the locations of five *in* mutations. Four of these are strong *in* alleles that appear to completely inactivate the *in* gene, while one is a weak allele. All four of the strong alleles were mapped to an approximately 15 kb region in the walk (Fig. 2EH). The *in*<sup>GN4a</sup> mutation is associated with a heterochromatic inversion and displays a weak and variable phenotype. It was localized to a fragment that mapped more than 10 kb proximal to any other mutation (Fig. 2EH). We suggest that this mutation inactivates *in* via position effect variegation (Henikoff, 1990) and that this breakpoint is outside of the *in* gene. The clustering of the five *in* mutations to the region of DNA diagrammed in Fig. 2 gave us confidence that the *in* gene was localized to this region.

### Transcription of the *in* region

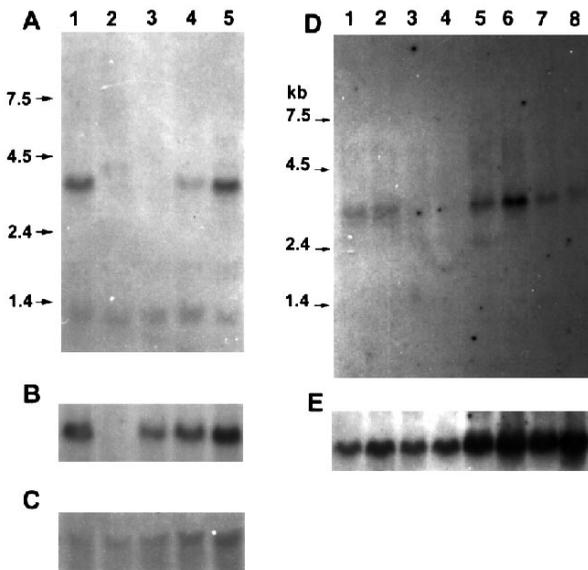
Four non-overlapping transcripts were identified in this approximately 50 kb region by northern analysis (data not shown, see Fig. 2F). We concentrated on the three transcripts that flanked the region where the 4 strong *in* alleles mapped.

We isolated cDNAs derived from transcripts A, B and C by screening embryonic and pupal cDNA libraries. The location of the DNA sequences that encoded these cDNAs was determined via

hybridization to blots of cloned, restricted DNA from the region. As a first test of which if any of these cDNAs were related to the *in* gene we made probes from each of these



**Fig. 2.** On line A the YAC clone DY317 is represented. Line B shows the steps in the genomic walk taken in the minilibary we constructed from the DY137 DNA. Line C shows an enlargement of the *in* region bacteriophage. Line D shows the extent of the genomic DNA that showed complete rescue of *in* mutants. Line E shows the restriction map of the *in* region and the location of five *in* mutations as determined from genomic Southern analysis. Line F shows the location of genomic sequences that encoded mRNAs detected on northern blots. Line G shows the orientation and location of mutations in the *in* cDNA clone. H shows examples of the genomic Southern analysis. Within H, A shows an RFLP associated with *in*<sup>IA11</sup> revealed by probing a genomic Southern blot of *Bam*HI restricted *in*<sup>+</sup>/*TM3* and *in*<sup>IA11</sup>/*TM3* DNA with a 4.4 kb *Bam*HI fragment from bacteriophage 32. B shows the RFLP associated with *in*<sup>IA41</sup>, revealed by probing a genomic Southern blot of *Hind*III/*Not*I restricted *in*<sup>+</sup>/*TM3* and *in*<sup>IA41</sup>/*TM3* DNA with a 5.1 kb *Hind*III/*Not*I fragment from bacteriophage 32. C shows the RFLPs associated with *in*<sup>CAH3a</sup> revealed by probing a genomic Southern blot of *Hind*III/*Not*I restricted *Oregon R*, *in*<sup>CAH3a</sup>, *in*<sup>CAH3aRVB2a</sup>, and *in*<sup>CAH3aRVC1a</sup> DNA (these two latter strains are revertants of *in*<sup>CAH3a</sup>) with a 5.1 kb *Hind*III/*Not*I fragment from bacteriophage 32. D shows the RFLP associated with *in*<sup>GL3b</sup> revealed by probing a genomic Southern blot of *Hind*III/*Not*I restricted *in*<sup>+</sup>/*TM3* and *in*<sup>GL3b</sup>/*TM3* DNA with a 3 kb *Hind*III/*Not*I fragment from bacteriophage 48. E shows the RFLP associated with *in*<sup>GN4a</sup> revealed by probing a genomic Southern blot of *Hind*III restricted *in*<sup>+</sup>/*TM3* and *in*<sup>GN4a</sup>/*TM3* DNA with a 3.2 kb *Hind*III fragment from bacteriophage 15. For A,B,D and E the control DNA is from the parental chromosome. The parental chromosome for *in*<sup>CAH3a</sup> is uncertain (it is derived from the Harwich strain which is polymorphic), but *in*<sup>CAH3a</sup> is the parental chromosome for the two revertants. The RFLP between *Oregon R* and the other three DNAs in C is not likely to reflect a functionally significant difference.



**Fig. 3.** A, B, and C show the results of probing a northern blot of pupal RNA from a series of *in* mutants. Poly(A)<sup>+</sup> RNA of from 1-2 day pupae was isolated, and about 5  $\mu$ g was fractionated per lane on a 1% agarose gel containing formaldehyde. The RNA was transferred to Nytran filter paper (S&S) and hybridized with random priming labeled probes. The position of molecular mass markers is shown. Lane 1 contains Oregon R RNA, lane 2 *in*<sup>IA11</sup>, lane 3 *in*<sup>IA41</sup>, lane 4 *in*<sup>GLAb</sup>, and lane 5 *in*<sup>GP4a</sup>. The blot in A was probed with cDNA B (*in*), that in B was probed with cDNA A and that in C with cDNA C. In A, in wild type, there is a 3.5 kb RNA and a faint band at around 1 kb (this was not seen in all experiments). This 3.5 kb RNA is altered in size or abundance in four of the *in* mutants. D and E show a developmental northern showing that the *in* gene is widely expressed. Approximately 50  $\mu$ g of RNA was isolated from a variety of stages, fractionated by gel electrophoresis and blotted to Nytran. The blot in D was probed with the 5.1 kb *Hind*III/*Not*I fragment from bacteriophage 32. E is the same blot reprobred with *hsp83* as a loading control. Lane 1, 0- to 12-hour embryo RNA; lane 2, 12- to 24-hour embryo RNA; lane 3, 2nd instar larval RNA; lane 4, third instar larval RNA; lane 5, 0- to 1-day pupal RNA; lane 6, 1- to 2-day pupal RNA; lane 7, 3-5 day pupal RNA; lane 8, adult RNA.

cDNA clones and used them to probe a northern blot of RNA from various *in* mutants (Fig. 3). A 3.5 kb band was detected with the cDNA B (Fig. 3A). This band was greatly reduced in two *in* inversions, *in*<sup>IA11</sup> (a hint of a larger hybridizing band is seen in this mutant) and *in*<sup>IA41</sup> (lane 2 and 3, respectively), and moderately reduced in one cytogenetically normal  $\gamma$ -ray-induced mutant, *in*<sup>GLAb</sup> (lane 4). This suggested that the 3.5 kb band was derived from the *in* gene. The presence of an apparently normal 3.5 kb band in one *in* mutant, *in*<sup>GP4a</sup> (lane 5), implied that this  $\gamma$ -ray-induced mutation might be due to a single base change or a small deletion or insertion. We later found that this mutation was associated with a deletion of one net nucleotide (see below). The cDNA A hybridized to a 3.7 kb band (Fig. 3B), which was present in normal amounts in all *in* mutants except *in*<sup>IA11</sup>. This was consistent with the mutation mapping data (Fig. 3) and suggested that *in*<sup>IA11</sup> affects expression of both transcript A and B. A 2.2 kb band detected with the cDNA C was present in normal amounts in all tested *in* mutants (Fig. 3C). The northern analysis of the *in* mutants suggested that the transcript B was the product of the *in* gene.

A developmental northern blot was probed with sequences from cDNA B. It showed that this transcript was present at all developmental stages, and was most abundant in embryos and in pupae 24-48 hours after white prepupae formation (Fig. 3D).

### The *inturned* cDNA encodes a putative membrane protein

The cDNA derived from transcript B (hereafter this will be referred to as the *in* cDNA) was sequenced (Fig. 4). The longest open reading frame encoded a 869 amino acid long polypeptide. A stop codon was present upstream of the putative Met start site in this reading frame. A consensus poly(A) addition site (AATAAA) was found 35 bases upstream of the oligo A run at the 3' end of the cDNA clone, consistent with this oligo A run being derived from the poly(A) tail of the mRNA. Based on the sequence data, we designed primers to amplify genomic DNA. We found no differences in the sizes of bands amplified from genomic or cDNA templates suggesting that the *in* gene does not have any introns (at least within the coding region). We next amplified genomic DNA from several of the *in* mutants. By direct sequencing of the PCR products, sequence changes associated with two cytogenetically normal  $\gamma$ -ray-induced and one EMS-induced mutation were found in the open reading frame of the *in* cDNA (see Fig. 4). In *in*<sup>GLAb</sup>, a deletion of seven nucleotides was identified early in the open reading frame (nucleotides 187-193). This deletion would cause a frame shift and a premature termination of translation. Thus, only a small fragment of the presumed *in* protein could be produced. This mutation also appeared to decrease the level of *in* mRNA, perhaps by affecting the stability of the mRNA (Fig. 3). In *in*<sup>GP1a</sup>, two nucleotides (nucleotide 2080 and 2081) were replaced by one nucleotide resulting in a frame shift, which should also result in a truncated protein. In the EMS-induced mutation *in*<sup>IH52</sup> a C to T transition at nucleotide 2407 was found, which results in a stop codon. This should result in a truncated protein that lacks the carboxy-terminal 107 amino acids. Since this allele is a phenotypic null allele (Adler et al., 1994) the carboxy-terminal region is likely to be essential for *in* function. These data confirmed that the presumptive *in* cDNA was indeed the *in* cDNA.

The predicted *In* protein is a novel protein. Fasta (Pearson and Lipman, 1988), BlastP (Altschul et al., 1990), and Smith-Waterman (Smith and Waterman, 1981) searches revealed no sequences in the databases with a dramatic similarity to *in*. The BlastP search reported one sequence with a statistically significant similarity ( $P=0.016$ ) to *in*. This sequence is for a component of the mitochondrial ribonuclease P from *Saccharomyces cerevisiae* (PIR S30802) (Dang and Martin, 1993) (Fig. 5). There are six segments of sequence similarity. Over the 207 amino acids in these segments the two proteins are 29.5% identical and 47.8% similar.

The *in* protein was found to contain a number of common protein sequence motifs for posttranslational modification (e.g. N-linked glycosylation and both Ser/Thr and Tyr phosphorylation sites). The significance of these remains to be determined. One particularly interesting motif was the presence of an RGD sequence. This sequence serves as an integrin binding site in many proteins (D'Souza et al., 1991). A mutagenesis experiment (see below) showed that this was likely not to be the case for *in*. Hydrophobicity analyses suggested that the *In* protein could contain at least one transmembrane domain (Fig. 6). Programs based on several different algorithms predicted Domain 1 (TM1

– amino acids 344–364) to be a transmembrane domain (e.g. Kyte and Doolittle, 1982; Klein et al., 1985; Rao and Argos, 1986, and Eissenberg et al., 1984), and this prediction was not sensitive to altering parameters such as the size of the window scanned. A second domain 2 (TM2 – amino acid 469–489) was predicted to be a transmembrane domain by a subset of these programs (Rao and Argos, 1986; Eissenberg et al., 1984).

**Rescue of *in* phenotypes**

As a further test that we had correctly identified the *in* gene and transcript, we carried out transformation rescue. We subcloned a 12 kb fragment from bacteriophage 37 into the pW8 P element transformation vector. This fragment contained

the entire coding region found in the *in* cDNA clone. Transformants carrying this vector were obtained and one copy of this transgene was found to provide complete rescue of an *in* null genotype (Fig. 7B). This data confirmed that the A and C transcripts were not essential for *in* function, as the sequences encoding them are not present in the rescuing construct.

We next constructed a gene where the putative *in* open reading frame was placed behind the *hsp70* promoter and obtained germ line transformants that carried this transgene. We found that one copy of this transgene could provide nearly complete rescue of a null *in* genotype by simply raising the flies at 25°C (Fig. 7C). No evidence of any polarity abnormalities were seen on the wings of such flies and the number

COGCAGTCGACATTGAGAGCTGAGCTGAGCGGTATCAACCGGGGAGATAGTTGAGTG TGTAACCGGAAATAGTAGGCCACTAATAACTATCCACCCAGTTAACTATCCCGGTAACT	60 120	GAGCCCACTCACCCATCGGAGTTCCTCATCTAGCGCAGTGCCTGTACTACGATGGC E P L T T H R E F F I Y G S A L Y Y D G	1560 1620
ATGGCAAAATCGCGGCGAGCCTGCGGAGGCGCTTCTCCAGTTCGAAGTCTCGCTG H R K S P A S L P S E A F S S S N S S L	180 240	TTCTGGTGGCAGCGCTGTCACCGGAAGTGAAGGATTCCTCGCG F L V A S L L P P E V R V S V E G F L R	500 560
AGCAACTCCTACAGCGGATGGCAGCGACTGGCCAACTGGGAGGAGTACGTGTCCACAGAC S N S Y S D G S D L A N W E E Y V S T D	240 300	TGCGGGGAATCTTCAGTGTGCTCGGAGTGTCCCGGAATCAAAGTTCGCGAGATGTAC C R G I F E L L G A A P G I K V R E M Y	1680 1740
GGCAGTCTCTTACATGAGTATGTGCGAATGTGAGAAGACTGGCGTCTCGGACAGCGG G S L F Y M E Y V R C E K T G V S A E R	300 360	GTGTGGGAGGATAGTGTGCCAGTGCACAGGACGATYTTCTTACCATTCTGCACC V W E E I V L P S A T G R Y F L T I C T	1740 1800
CGGTGGAGTTCATGCGTGTGCTGCGCTGGGAAAGTGTGCGTGTCCGACGCGG R V E F M R R S L R L G K K S S R R Q P	360 420	AAGAACCCTGTAGTCTGCGGTGATCTTGAAGATCTTCGATGCTCCAGACATGGCACCG K N H L S L A V I L K I P D A P D M A P	1800 1860
CACAGCAATAATCAGAATCAGAGTCAGAACCAACAGTCCACCGCCGAGCAGGTG H S N N Q M Q S Q N Q N Q S H G P S R L	420 480	GATGGGTGGTGGTCCCTCTTTTACATCGAGGAGATTCAAGAGACGCTGGACCAC D A V V G P S L F Y I E E I Q E T L D H	1860 1920
GATAGCCAGCGGAGCGCAAGAGTTCGCGTCTCACAGTCAAGAAGGCTGCCCGGAT D S S Q T E P Q E F R F C F K T A A P D	480 540	CTAGTACAGTGGCGATTGAATCCCTGGCCATGTTCTGGTCCGTTTCCAATAAGAGACCC L V Q C G I E S L A M F W S V S N K R P	1920 1980
CCCAAGAACTGGATCTGGTGTACAGCGCGGATGCGCTTCGCTTTCGAGCAGCATCC P K K L D L V I T A A D R F R F G R R S	540 600	GAGTGTGGAGCGCACTGCCAGGAGTAGAGATCAGGAGAAGGACCGCAACCGA E V L D A T A S E S R D Q E K E P N N R	1980 2040
AGCGCGTGGAGTCCATACTGGGCTTCGCGTCTGCGCTTTCGAGCAACGAGAATGC T A V E S I L G F R V L P F P D Q P E C	600 660	CTGGAGTCTCTCAACGAGCAAGTAAACGCTGCTGAGTCTTCGTTGGAGGAGGAGCC L E S F L K Q K L T V L S P S V E E A	2040 2100
CTGATGTCGATGGGTTGTTCAAGATCTGAGTGGCGTGCAGCAGCGCATCAAGCGGGA L M V D G F V H D L S A L Q H G I K R G	660 720	CAACTGTGCTGCTCCCTGGGTTGCTTCCATACATAGTCTGACTCCCGATGAGGAOAG Q L C S S L G G S S I H S L T P S E D E	2100 2160
GATTGGTTAGATCCGTAATGGCATAGAGGTGTACGCGAGCAATGTGGATGAGTTGCTC D W F R S L N G I E V Y A S N V D E L L	720 780	TCCTGTGCGAGGATGACCCCATCCATGGTACTGCGGAGGACTCGGACAGCGGTTCC S C R R R L T P I H G T A E D S D S G S	2160 2220
CAGCAGTTCGTTGAGCCACTCAGGTGTGCTAGGCTTCCAGTCTGCGGTGCGGCCAGT Q Q F V E P T Q V C L G F Q S C G A A S	780 840	GACTGGGAGAATTCGCGTACAGCATCCACTTCACTATGAGTGAATCTCGGGCCGAG D W E N F A V Q H P L H Y G L N L G A E	2220 2280
GCAGTTCGCGCCAGGATCCGGGTATAATCAAATGTCCGCGAGGAGCAGGTGTCAAG A V S P T D P G Y N Q N V R E E Q V C	840 900	AGCCACGTCAGAGCAAAATGACAGATCCATGTGGAAGGAGATCAACAACGTTGGTCCG S H S Q S M T E S M W K E I N N V P	2280 2340
GTGGAGAATTTCCCATGTTCCAGGAGAAGTTGAGCAGATGTTGATAACGGAGGGGAAC V E N F P M F T E K F E Q M L I T E G N	900 960	GTCAAGATATCGCTGGCTGGAAGAATTCGTTCTGCTACTAGTCTACATTGACATAGCC V K I S A G W K N S V L H Y V Y I D I A	2340 2400
TTGCGTATCCCGTAGTTCGTTGAGCATAAAGTCCGCTTTCGCTATGCTGCTACTCCCA F G I P G S S V D I R M P F A M L L L P	960 1020	AATGGTCTTGTGTTGGCCATTTACCGAGCTCCGAGAATCCAACTACCTCTCCGAG N G S L F A P F T D S S E N S N Y L S E	2400 2460
COGGAGTGTATCAGCATGAACAGCAACAGGATCCCTCTACTYFTTCGGAATCCCC P E C Y Q H E Q Q D S L Y Y F P E I P	1020 1080	ATTCGTAGGCGGTGCACATAATCCACCGCTGCTGCGAGAATCCAAAGCACTATYCRGAG I R Q A C H I I H A V G L Q K S K H Y R	2460 2520
GACAACCTCCTATTCAAGGCCGTTGGAGTTCCTCACCTGCACGCGTACTCAGTGAA D N F L F K A R G S F L T L H A V L S E	1080 1140	CATCTATCGGAATCCTCGAGGACAGGAGCACTCGAACCGGCGACAGTAGCCACACAGT H L S E S S R A Q T N S N G H S S H T V	2520 2580
CTGCACACCAGCGCTGAGTTCAGGCTGCTGGTGGATCAGGTGCAATACCACTGCAAC L H T Q P L S S R L L V D Q V Q Y H V N	1140 1200	TCGCTGATCAAGGAGCAGGCGATGATCCTGGAGGTGAGCAGCAACCAAGTCCGAGCGG S L I K E R G M I L E V S T Q P K S D G	2580 2640
TACCGCAACTGAATGGGTTCTGATCTCTTCGCTACCGCGGAGGACTCTGCTGCGC Y R Q L N G F L V L F A Y A G G L C C A	1200 1260	CAATCCAACTCTCGACGAGCAGTCTGCTTCTGGTGGTGGTCTCTTTCAGTCCGCA Q S Q S T S S R F V V G R L F Q S P	2640 2700
GGGAGTGCAGCCTGCGATCGATGAGTAAATCGGATACATAGCCTTCTCTGCGCGGG A E C S L R S D E L I G Y I R F S L P G	1260 1320	GCCAAGGAGTGTAGCTGTGACCGGTGCGATGTTCCCGAGAATAGTCCGAATGGCC A K E V Y V C H R S D V P Q N I V E M A	2700 2760
TTGGTCTGGAGGCTTCAATCAGGAGGAGAGCGCGGAATAGTTCGAGACTGAAGCTG L V L E S F N Q E G E P G N S S R L K L	1320 1380	TTCCGGTGTCTCTTCTCAATGGGATGACATCTTGGAAAGGATACTACCACTTAGATA F R L S F F S M G	2760 2820
TTCTTGCAGACTTTTTCGAAATCCAAAGAACCCGGCTCGTAGCCAGATGCCATGGGCAT F L R H F C E I Q R T R L V A R C H G H	1380 1440	CTGGCTATGCGTAGAATCTTGCTATAATATAAACACAAATATTTAAATGTCGTTTAA CTAAACAAATTTATATAGGCATTCAAACAATTTTTAAATATATTTAAGAATG	2820 2880
ATTGTTTCGAGGAGTCTCGGCGCAATCCGCGAGCCTGCCACTGCCAAGGAAGCGCAC I R F E E L L G Q S R S L P L P K E A H	1440 1500	CATAATTAATCATTTTCAGCAATTCATTAAGCAAGCATTTGATTTAGGGGAAGTCAAT TTGTATTTTCAAAATTCGAAAGCGCAGCTTGCCAAAACCTTAAGAAATGAAATA	2880 2940
GTGCGATCTTTGATGCTGAGTGAATGGAGGCGATGACTGCAACTGGAACGAC V P I F D A L S E M E A M D Y R N W N D	1500 1560	TGTACATTTCTCTGAAACCGCAGCGCAAAATATGATGATGAGGATTTTCAAGAGGA ACACAAAAA	2940 3000
	1560 1620		3000 3060

**Fig. 4.** The sequence of the *in* cDNA clone is shown. The locations and nature of the changes associated with three *in* mutations are shown. The two potential transmembrane domains are underlined and a putative poly(A) addition sequence is in bold. Also in bold are potential N linked glycosylation sites and the RGD sequence.

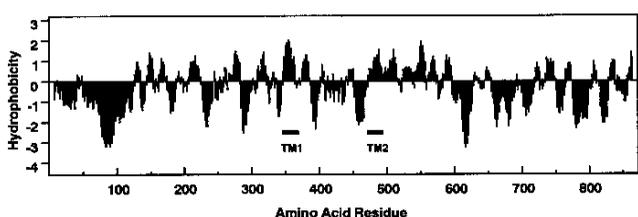
<i>in</i>	74	KSSRRQPHSNQNGQNSQNGSHGPSRLDSOTEPQEFRFSSQFK	116		
		K + + NN N +Q++ S GP+ +Q +P F F+		12/42	identity
RPM2	103	KQOOSTHYVNNNNHRRHYSTGPTLPTNOYDPLNFSNRNFQ	144	21/42	positive
<i>in</i>	221	AVSPTDPGYNQNVREEQVCVNFPMTEKFEQML	255		
		++ + P Q E + K EN P++ E E L		10/33	identity
RPM2	147	SLKTSQPSVQQPQNEYSLLKDNENAPVWKEDEPCL	181	19/33	positive
<i>in</i>	309	GSFLTLHAVLSELHTQPLSSRLLYDQVQYHVNRYQLNGFLVLF	351		
		G F ++ + + + S + Q++ +N L G LF		10/35	identity
RPM2	935	GEFKKYESLSKRFNDKISESSKIDIQLEYLKNKDLKGAFTLF	977	16/35	positive
<i>in</i>	464	TTHREFFIYGSAIYYDGF	481		
		T H+ +Y AL+ D F		10/43	identity
RPM2	986	TPHKTMDLYTFALFLDSF	1003	19/43	positive
<i>in</i>	614	EKEPNRRLSFLKQKLTVLSPVVEEAQLCSSL	646		
		+KE N++ + L + +LSPS+E + + L		7/18	identity
RPM2	1058	KKEELNGMLNLLYDSIRLLSPSIEIDKSKKEKL	1090	10/18	positive
<i>in</i>	743	SLFAPFTDSSESNYLSEIROACHIIVHVLQKSKHY	778		
		S PFT S +N + I H VLQ Y		12/36	identity
RPM2	1122	SFLNPFPTPSMLFNNLITETIYINEHASSLVLQGLIY	1157	14/36	positive
		All 6 segments		61/207	identity
				99/207	positive

**Fig. 5.** The alignment of *in* and the mitochondrial RNase P component is shown. The alignment is shown for the six regions of similarity that contributed to the BLASTP score. The accession number for the *inturned* cDNA sequence is U37134.

of multiple hair cells fell by about four orders of magnitude from the estimated more than 10,000 in an *in* wing (estimated from the fraction of multiple hairs cells in defined regions and the total number of hairs on a wing; Garcia-Bellido and Merriam, 1971) to an average of about 1 double hair cell per wing (we found individual wings with 0, 1 or 2 double hair cells per wing). This suggests that the *in* cDNA is sufficient for all *in* function for tissue polarity on the wing.

Heat-shock induced overexpression of *in* at various stages of development did not produce any gain-of-function phenotypes (data not shown) as has been seen for *fz* (Krasnow and Adler, 1994). The In protein seems not to be a limiting factor in the signaling pathway for tissue polarity formation.

When we examined the sequence of the predicted In protein we noticed that it contained an RGD (Arginine-Glycine-Aspartate) motif which is found in many integrin binding proteins. To test whether this sequence is functionally important for *in* function, we made a mutant construct which encoded RGE (Arginine-Glycine-Glutamate) instead of RGD. In other systems such a mutational change dramatically reduces integrin binding (Plow et al., 1985). The mutant transgene rescued the *in* phenotype and indeed was indistinguishable from the wild-type *hs-in* construct, implying that the In protein is not an integrin binding protein (Fig. 7D).



**Fig. 6.** Shown is a plot of the hydrophobicity analysis of the In protein by the method of Kyte and Doolittle (1982). The location of the two potential transmembrane domains predicted by several programs is noted (TM1 and TM2).

## DISCUSSION

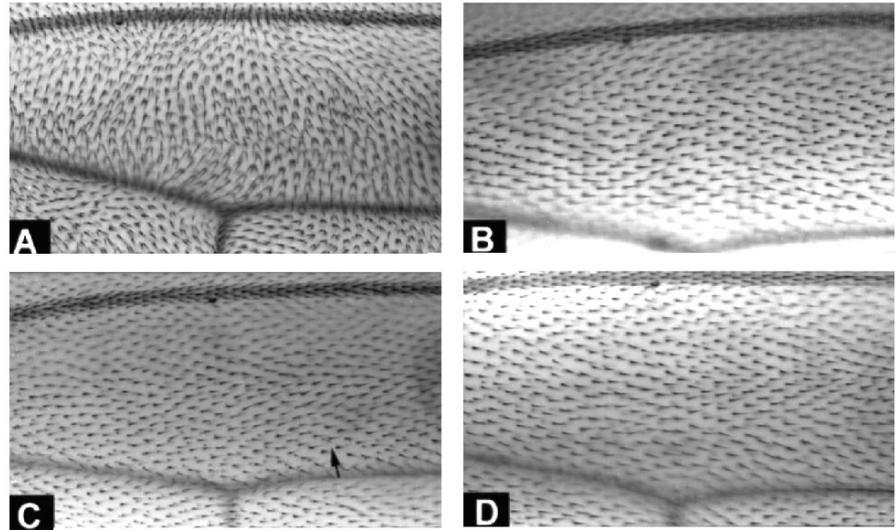
### The *inturned* gene encodes a novel protein

Previous studies had found that the *frizzled* and *disheveled* tissue polarity genes encoded novel proteins (Vinson et al., 1989; Klingensmith et al., 1994; Theisen et al., 1994). Our molecular analysis of the *inturned* gene has shown that it also encodes a novel protein. These data provide a further indication that the tissue polarity system in *Drosophila* is likely to involve a novel intercellular signaling/signal transduction mechanism. Only one protein was picked out of the databases as having a sequence that was statistically significantly related to the sequence of the In protein. This sequence encodes a component of the mitochondrial RNase P from yeast. The function of this protein component is not clear but it may have RNA binding activity (Dang and Martin, 1993). Given the relatively low level of similarity between the sequences of the two proteins it is not clear if the similarity is biologically significant. One way to think about the function of the *in* gene in tissue polarity is that it is essential for localizing (and hence activating) an inhibitor of prehair initiation to the periphery of the pupal wing cells (Adler et al., 1994). One way to localize an activity to a specific subcellular region is to localize the mRNA (Ding and Lipshitz, 1993), as is seen for maternally derived determinants in the *Drosophila* early embryo (Wang and Lehmann, 1991; Ephrussi et al., 1991; Kim-Ha et al., 1991; Berleth et al., 1988). Thus, it is plausible that the In protein might have an RNA binding activity and through this activity localize factors that regulate prehair initiation. Previous genetic experiments suggested a model where the activity of the *fz*-like genes functions to inactivate the product of the *in*-like genes at the distal vertices of the pupal wing cells (Wong and Adler 1993; Krasnow and Adler, 1994). The suggestion from the sequence, that the In protein will be a membrane protein, as the Fz protein is (Park et al., 1994), raises the possibility that this putative inactivation could involve a direct interaction between complexes of membrane bound proteins.

### Transcriptional regulation does not appear to play an important role in *in* function

The impressive rescue of an *in* mutation by a chimeric gene where the *in* cDNA is under the control of the *hsp70* promoter provided compelling evidence that we had correctly identified the *in* gene, and that this cDNA is sufficient for *in* function. If other forms of the *in* transcript exist they cannot encode any factors with unique activities. In these experiments the flies were simply cultured at 25°C. Given what is known about the activity of the *hsp70* promoter it is likely that the *hs-in* transgene is expressed at a relatively uniform low level in all cells in the pupal wing. Hence it is quite unlikely that patterned transcriptional regulation (temporal, tissue specific, or position specific) plays a key role in the functioning of the *in* gene. It remains quite possible that translational or post-translational gene regulation mechanisms might be important for *in* function, and that these are retained in our transgenic lines. It is worth noting that rescue by a chimeric gene driven by the *hsp70* promoter is also seen for the two other tissue polarity genes tested in this way (i.e. *fz*; Krasnow and Adler, 1994 and *dsh*; Klingensmith et al., 1994). This suggests that patterned gene regulation may be relatively unimportant in the functioning of this system as a whole.

Mutations in *in* do not result in any known phenotype in



**Fig. 7.** Rescue of *in* mutations by transgenes. A shows an *in*<sup>H56</sup>/*Df in* wing. B shows an *P[w<sup>+</sup> in<sup>genomic</sup>]; in*<sup>H56</sup>/*Df in* wing. C shows a *P[w<sup>+</sup> hsp70-in]* cDNA; *in*<sup>H56</sup>/*Df in* wing. The fly from which this wing was obtained was grown at 25°C with no heat shock. The arrow points to a rare double hair cell found on this wing. D shows the rescue obtained from an *hsp70-in* (*RGE*) cDNA.

embryos or larvae. Hence it was not expected that the *in* gene would be expressed in these developmental stages, as the developmental northern analysis indicates it is. It is possible that *in* mutants have a subtle phenotype that we have missed or that it causes a phenotype that is only seen under conditions that are not replicated in the lab. Alternatively, it is possible that the *in* gene has an important function in these developmental stages, but that it is redundant. Only further analysis will be able to determine if any of these hypotheses are correct. Once again there is an interesting parallel with *fz*, as this gene is also expressed in embryos and larvae while no obvious phenotype is seen in these developmental stages in mutants (Adler et al., 1987, 1990).

### *in* functions cell autonomously

Previous work by Gubb and Garcia-Bellido (1982) suggested that *in* functioned cell autonomously, as cells that displayed an *in* phenotype were seen when mitotic clones were induced by irradiation of *in*<sup>+</sup> larvae. We have extended their observations in several ways. The tissue polarity genes are unusual in that several of the genes (*fz* and *pk*; Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987) display domineering cell non-autonomy. That is, the presence of mutant cells results in neighboring genetically wild-type cells showing the mutant phenotype. We did two experiments to test if this could be the case for *in*. In them we used the adult cuticular hair markers *mwh* and *trc* to identify the clone of *in* cells in the adult wing. Only in the experiment where we marked the *in* cells with *trc* did we obtain any clear evidence for cell non-autonomy, and in this experiment it was a rare result. The *in*-like differentiation of one or two cells at the periphery of about 10% of the *trc in* clones might represent a weak cell non-autonomous function of *in*, however this need not be the case. While *trc* typically acts in a cell autonomous fashion (Ferrus, 1976; Vinson and Adler, 1987) the mutant phenotype of the *trc in* cells was much more severe than is seen in cells mutant only for *trc*. It is possible that the presence of such unusually aberrant cells is the cause of the cell non-autonomy, and that this is a synthetic phenotype derived from the extreme phenotype that results from the interaction of *in* and *trc*.

We also carried out experiments to determine if the presence of a neighboring wild-type cell could rescue a genetically *in*

cell. In this experiment we generated twin spots for *in* and a second mutation that causes grossly abnormal hair morphology. We obtained unambiguous evidence for cells which both displayed the two hair cell *in* phenotype and had direct wild-type neighbors. On the organismal level, null *in* mutations display complete penetrance and strong and relatively consistent expressivity. However, the *in* phenotype does not display complete penetrance on a cellular level. Some wing cells produce only a single hair (e.g. in experiments reported previously we found that almost 30% of the cell in a central region of the wing form a single hair; Adler et al., 1994) and the degree of cellular penetrance varies across the wing, further complicating the situation (e.g. it is generally more severe in central versus peripheral regions and on the ventral as opposed to dorsal surface; P. N. Adler, unpublished data). In addition, some regions of mutant wings do not show dramatically altered polarity (Gubb and Garcia-Bellido, 1982; Adler et al., 1994). This lack of complete cellular penetrance provides a limitation on the interpretation of some of our mosaic experiments. Our data show that genetically *in* cells can display an *in* phenotype when juxtaposed to wild-type cells. However, it is possible that the presence of direct wild-type neighbors does alter the probability that an *in* cells will produce two or more hairs (e.g. it could decrease the probability from 70% to 40%). Since cells that displayed an *in* phenotype and were juxtaposed to wild-type cells were common, a dramatic decrease in the probability of a cell displaying an *in* phenotype is unlikely.

### Interaction of *in* and *trc*

The observation that the phenotype of *mwh in* cells closely resembles that of *mwh* (Wong and Adler, 1993), while *trc in* cells display a much more severe phenotype than either *trc* or *in* alone suggests that *mwh* and *trc* cause their multiple hair cell phenotypes in different ways and/or affect different genetic pathways. In previous experiments we found that in both *in* and *mwh* cells prehair initiation is not limited to the distal vertex, but rather is seen at a variety of locations along the cell periphery (Wong and Adler, 1993). This spread in conditions permissive for prehair initiation leads to the formation multiple prehair initiation centers, which is at least partly responsible for the multiple hair phenotypes seen in the adult wing (*mwh*

appears to have two effects; Wong and Adler, 1993). Based on these phenotypes and on the epistasis of *mwh* we suggested previously that *mwh* is downstream of *in* in a pathway that restricts prehair initiation to the distal vertices of the pupal wing cells (Wong and Adler, 1993). We have not examined the behavior of *trc* cells in pupal wings, but based on the interaction of *trc* and *in* it would not be surprising if *trc* affected a process other than localizing prehair initiation. For example, *trc* could cause the growing prehair to fall apart, generating multiple hairs. If this was the case, we would predict the interaction seen between *in* and *trc* as all of the extra prehairsts initiated due to *in* would split into several hairs due to the *trc* mutation. Further studies on *trc* and its interactions with *in* and other tissue polarity genes will be required to explain the interaction of *in* and *trc*.

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