Molecular Analysis of EMS-Induced *frizzled* Mutations in *Drosophila melanogaster*

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ABSTRACT

The *frizzled* (*fz*) gene of Drosophila is essential for the development of normal tissue polarity in the adult cuticle of Drosophila. In *fz* mutants the parallel array of hairs and bristles that decorate the cuticle is disrupted. Previous studies have shown that *fz* encodes a membrane protein with seven putative transmembrane domains, and that it has a complex role in the development of tissue polarity, as there exist both cell-autonomous and cell nonautonomous alleles. We have now examined a larger number of alleles and found that 15 of 19 alleles display cell nonautonomy. We have examined these and other alleles by Western blot analysis and found that most *fz* mutations result in altered amounts of Fz protein, and many also result in a Fz protein that migrates aberrantly in SDS-PAGE. We have sequenced a subset of these alleles. Cell nonautonomous *fz* alleles were found to be associated with mutations that altered amino acids in all regions of the Fz protein. Notably, the four cell-autonomous mutations were all in a proline residue located in the presumptive first cytoplasmic loop of the protein. We have also cloned and sequenced the *fz* gene from *D. virilis*. Conceptual translation of the *D. virilis* open reading frame indicates that the Fz protein is unusually well conserved. Indeed, in the putative cytoplasmic domains the Fz proteins of the two species are identical.

THE adult cuticle of Drosophila contains many polarized structures such as sensory bristles and hairs, which are typically arranged in a parallel array (ADLER 1992; GUBB 1993). On appendages they usually point distally and on the body posteriorly. The function of the tissue polarity genes is essential for the development of hairs and bristles that display normal polarity (GUBB and GARCIA-BELLIDO 1982; ADLER 1992; GUBB 1993).

frizzled (*fz*) is one of the well studied tissue polarity genes (ADLER *et al.* 1987, 1990, 1994; VINSON and ADLER 1987; VINSON *et al.* 1989; KRASNOW and ADLER 1994; PARK *et al.* 1994a,b). Mutations in *fz* result in alterations in bristle and hair polarity in all body regions and in the formation of a rough eye due to an abnormal arrangement of ommatidia (ZHENG *et al.* 1995; L. MARSH, personal communication cited in ADLER *et al.* 1990). Previous analysis of *fz* alleles in genetic mosaics indicated that *fz* had two mutably separate functions (VINSON and ADLER 1987). Three *fz* alleles (including a null allele) displayed domineering directional cell nonautonomy in clones in all regions of the wing; that is, wild-type cells located distal (but not proximal) to a mutant *fz* clone produced hairs with abnormal polarity. This suggests that *fz* is required for the distal transmission of an intercellular polarity signal. Two *fz* alleles, however, were cell-autonomous in mitotic clones, suggesting that *fz* is required for the interpretation of the polarity signal. Our conclusion from these results was that *fz* is required for both signaling and receiving or interpreting a signal. One intriguing observation was that the two cell-autonomous alleles were unusual "tissue specific" *fz* alleles in that unlike other *fz* alleles they did not cause a rough eye (ADLER *et al.* 1987). In addition, the pattern of wing hair polarity disruption they produced was unusual (ADLER *et al.* 1987). To assess whether or not this correlation between cell autonomy and tissue specific phenotype was significant, we analyzed an additional 14 *fz* alleles. Two additional cell-autonomous alleles were found that did not show the tissue specific phenotype of the original cell-autonomous alleles.

Previous studies showed that *fz* encodes a single protein that functions in the development of tissue polarity in the epidermis (ADLER *et al.* 1990; KRASNOW and ADLER 1994). Sequence analysis, cell fractionation and immunostaining argue that this protein is an integral membrane protein that contains an odd number (likely seven) of transmembrane domains (VINSON *et al.* 1989; PARK *et al.* 1994a). We report here a molecular analysis of a collection of EMS-induced *fz* alleles. We first characterized by Western blot analysis the protein produced by 23 *fz* alleles and found a range of molecular phenotypes including altered amounts and/or migration rates of Fz protein. We next determined the molecular sequence changes associated with a subset of these EMS-induced mutations. We found cell nonautonomous *fz* missense mutations in putative extracellular, transmembrane and cytoplasmic domains. In contrast, the four cell-autonomous alleles were all due to changes in a

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single amino acid: a proline located in the putative first cytoplasmic loop.

As a complement to the mutational studies noted above, we also cloned and sequenced the *fz* gene of *D. virilis*, a species that diverged from *D. melanogaster* as much as 60 million years ago (BEVERLY *et al.* 1984). We found that the overall gene structure was conserved, as the two *fz* genes each contained five homologous exons. Conceptual translation of the *D. virilis* Fz protein showed that the protein sequence was 92% identical to the *D. melanogaster* sequence. Remarkably, not a single substitution was found in the putative cytoplasmic domains. As a test of the functional conservation of the *D. virilis fz* gene, we constructed a transgene that expressed the *D. virilis fz* gene behind an *hsp70* promoter in *D. melanogaster*. This transgene showed rescue activity that mimicked that of an equivalent *D. melanogaster fz* transgene, indicating that the few amino acid differences seen were not of functional importance.

MATERIALS AND METHODS

Drosophila strains and mutant isolation: Fly cultures were maintained at 25°. Descriptions of genetic markers and balancer chromosomes used are located in LINDSLEY and ZIMM (1992).

The EMS-induced *fz* mutations were isolated in two large F_1 screens carried out in this lab, one of which was described previously (ADLER *et al.* 1987). These mutations were induced in a lethal free third chromosome marked with the recessive visible mutations *th* and *st*. The second screen was carried out in a similar manner but employed a chromosome marked with the recessive visible marker *ri*. In our original paper characterizing *fz* mutations we characterized alleles as being strong, moderate or weak (ADLER *et al.* 1987). In that paper we discussed the possibility that some of this variation might be due to modifier mutations that accumulate in stocks that carry *fz* mutations. In that paper we concluded that the described differences were not due to modifiers. With further experience with these mutations we now feel that we underestimated the effects of modifier mutations, and that some of the alleles previously described as moderate should be classified as strong. Some corrections are listed in Table 1.

RNA isolation: Total RNA was prepared from *D. melanogaster* mutants flies heterozygous for the allele in question and $Df(3L)fz^{D21}$. Approximately 0.35 g of each mutant stock was used to isolate RNA by the procedure of CHOMCZYNSKI and SACCHI (1987) (for details see JONES 1995). Poly A+ RNA was isolated via oligo dT cellulose chromatography as described by MANIATIS *et al.* (1989). RNA was isolated from *D. virilis* in a similar way.

cDNA preparation: First strand cDNA was synthesized using the cDNA PCR cycle kit from InVitrogen. 1.0 micrograms of polyA(+) RNA was used in each reaction. The protocol provided by the kit was followed, using the random primers provided.

PCR amplification of *D. melanogaster* cDNA: Detailed description of the PCR amplification is presented in JONES (1995). Reagents used to amplify mutant cDNAs were from Perkin Elmer Cetus PCR Amplification Kit. The first round of PCR amplifications was performed with sense and antisense primers 72R1 and 73H3 (see Table 1 for primers). Second round amplification of each mutant allele was done in two separate reactions, using an aliquot of the first round amplification reaction and primers 72R1 and 130 in one reaction mix and primers 131 and 73H3 in the other reaction mix. The amplified products were gel purified before their use in sequencing.

Sequencing of mutant genes: Sequencing reactions were done using Invitrogen's PCR Sequencing kit, following the protocol provided in the kit. Amplified mutant cDNA (described above) was used in each sequencing reaction. Reactions were performed using a Perkin Elmer PCR machine. Some early experiments were done by amplifying genomic DNA and sequencing this directly with Sequenase after asymmetric amplification to generate single-stranded templates. All identified mutations were confirmed by reamplification and sequence analysis of mutant cDNAs. Sequencing primers were based on the *fz* cDNA sequence obtained previously (VINSON and ADLER 1987) and are listed in Table 1.

Cloning of the *D. virilis fz* gene: A *D. virilis* genomic library (constructed by R. BLACKMAN cited in THUMMEL 1993) was screened with ^{32}P-labeled *D. melanogaster fz*-specific genomic probes for exons one, two, three-four, and five. Hybridizations were done at 42° in 50% formamide, 5× Denhardt's, 5× SSPE, 0.1% SDS and 0.1 mg/ml salmon sperm DNA. Washing was done at 50° in 2× SSC. A total of 28 clones were identified as containing *fz*-specific sequences that fell into three groups. Southern blot analysis of *Sal*I digests from isolated phage DNA was used to identify restriction fragments that contained *D. virilis fz* sequences. Exon one was isolated in a 15-kb fragment, exons two, three, and four were isolated together in a 15-kb fragment, and exon five in a 7.5-kb fragment. These fragments were subcloned and analyzed further.

Sequencing of *D. virilis* genomic DNA: A set of processive deletions were made and sequenced in both directions using the dideoxy method (SANGER *et al.* 1977). The sequence was confirmed via sequencing *D. virilis* cDNA using sequencing primers derived from the genomic sequence. The *D. virilis* cDNA was made and sequenced in the same way as is described for the mutant *D. melanogaster* cDNAs. The accession number for the *D. virilis* cDNA sequence is L43163, and for the exon 1–5 sequences they are L43340, L43341, L43342, L43343, and L43344, respectively.

Construction of an *hsp70 D. virilis* transgene: A complete *D. virlis fz* cDNA was subcloned behind the *hsp70* promoter and in front of the *SV40* polyA addition sequence in the pBHS vector (PARK *et al.* 1994). A fragment consisting of the cDNA and regulatory sequences was then transferred to the pW8 vector (KLEMENZ *et al.* 1987) for transformation into Drosophila germ line (SPRADLING and RUBIN 1982).

Western analysis: Western blot analysis of the Fz protein was done as described previously (PARK *et al.* 1994). Since the Fz protein aggregates when boiled in SDS sample buffer (PARK 1993), in our experiments we either did not heat the sample at all or heated it at 85° for 10 min. After the initial probing with the anti-Fz monoclonal antibody 1C11, the blots were washed and reprobed with an anti-actin monoclonal antibody (Amersham) to control for loading. The 1C11 monoclonal antibody is directed against an epitope from the amino terminal domain of the Fz protein (PARK *et al.* 1994). It is possible that for one or more mutations the reduced signal on immunoblots is due to the mutation disrupting the epitope. As is discussed later among the mutations sequenced mutations in this domain of the protein were infrequent.

Mitotic clone analysis: We used two separate approaches in studying *fz* mutations in genetic mosaics. For some *fz* alleles the cell marker *trc* was recombined onto *fz th st* chromosomes. In these experiments mitotic recombination was induced by irradiating larvae 3–4 days after egg laying with 1000R of gamma rays. Adult *fz trc*/+ fly wings were dehydrated in ethanol and then mounted on slides in Euparol. The FLP/FRT

TABLE 1

Oligonucleotide primers

Primer no.	Start	Sense/antisense	Oligomer sequence
8	1020	A	CAA CTG GAG GTC ATC ACT GC
12	1312	S	GGA TTC GTG TGC CCC GT
72RI	678	S	CTG ATC GAA TTC TTT GAC AGC GAT ATC G
73H3	2534	A	CAT CGG AAG CTT CTA AGT AAC TTT CGT TAG C
130	1847	A	TGC GAC TTG TTC TCA ATC GCC TCC TG
131	1617	S	GAC TCT GTG TCG TGC CGC GAA CCA T
157	1202	A	CCG ATG ATG TGG TAT TCT CC
168	2208	A	ATA TCC CTG TGC CAT TGG ATC ATC C
169	834	A	TGA TGT GGC AGT CCA TCT AGT TCG G
171	1636	A	TTT GAC GGG CGG CGG AAA TGG TTC G
176	964	S	GAG GTC CAT CAG TTT GCT CCG CTC G
178	1836	S	GAA CAA GTC GCA CTT ATT CCA CCT GG
189	2109	S	GCC TGA TGT TGC GAA TTG G
194	2398	S	TTC GTG GAG AGG TTG CAG GG
195	1203	S	CGG AGA ATA CCA CAT CAT CGG

system was used for other alleles. In these experiments *fz* alleles were placed onto a third chromosome that carried an FRT sequence at 80 and the cell marker *mwh*. Mitotic recombination to produce mosaic clones was done by crossing *mwh fz FRT80/TM3* and *w hsflp; FRT80* females. One-day egg lays were subjected to a 1-hr heat shock at 38° on day 3–4 after egg lay. Adult fly wings from *w hsflp; mwh fz FRT80/FRT80* flies were removed and examined as described above.

Analysis of *fz* mutant phenotype and rescue: We assessed the phenotype of a *fz* null genotype and the ability of the *hs-fz*^{wt} transgene to rescue this phenotype as described previously (KRASNOW and ADLER 1994). Briefly, we estimated the fraction of the dorsal surface of the wing showing altered hair polarity, and we counted the number of multiple hair cells in the C region on the dorsal surface of the wing as measures of *fz* function.

RESULTS

Most *fz* alleles act cell nonautonomously: We analyzed 14 additional *fz* alleles in genetic mosaics. We found 12 of these alleles acted cell nonautonomously and two acted cell-autonomously (Table 2). When combined with our previous data (VINSON and ADLER 1987), we now have a total of 15 cell-nonautonomous and four cell-autonomous alleles. As shown in Figure 1, in the cell nonautonomously acting mutations (Figure 1, A and B) hairs produced by wild-type cells located distal to marked *fz* clones had altered polarity. Often the hairs appeared to point in toward the clone. In the case of the cell-autonomously acting *fz* mutations (Figure 1, C and D), the polarity of the hairs produced by the surrounding wild-type cells was normal.

Western blot analysis of mutant Fz proteins: We first analyzed wing disc tissue from 23 *fz* alleles (Table 2, Figure 2). Four of these alleles either disrupt the chromosome within the region that encodes the *fz* transcript (*fz*^{K21}, *fz*^{C21}, and *fz*^{3}) or delete an entire exon (*fz*^{KD4a}) (ADLER *et al.* 1990). As expected none of these genotypes produced any detectable protein. In our initial experiments we also detected no Fz protein produced by three additional mutations (*fz*^{P21}, *fz*^{R52}, and *fz*^{EAB2a}). As is discussed later when *fz*^{R52} was studied in more depth, we were able to detect a very small amount of Fz protein. Seven mutations (*fz*^{1}, *fz*^{HB52}, *fz*^{HB41}, *fz*^{HD21}, *fz*^{GL31}, *fz*^{HC52}, and *fz*^{CI9}) resulted in less (<50% normal) Fz protein than wild type being detected on the Western blots. We could, however, routinely detect the Fz protein encoded by these mutant genes (Figure 2). Ten mutations (*fz*^{F31}, *fz*^{H51}, *fz*^{N21}, *fz*^{R53}, *fz*^{J22}, *fz*^{R54}, *fz*^{R51}, *fz*^{HE11}, *fz*^{J22} and *fz*^{J12}) resulted in normal or only moderately reduced amounts of Fz protein (less than a twofold difference from wild type).

In addition to changes in the amount of Fz protein produced, we also saw differences in the size of the Fz protein in a number of mutations. Fz protein from seven mutants (*fz*^{HC52}, *fz*^{HB41}, *fz*^{HB52}, *fz*^{R54}, *fz*^{HD21}, *fz*^{GL31}, and *fz*^{CI9}) was shifted to a common slightly larger apparent molecular weight when assayed by SDS-PAGE. One allele, *fz*^{H51}, resulted in a protein that migrated faster than the wild-type protein (Figure 2A4). We estimated the size of this mutant protein to be ~48 kd.

Sequence analysis of *fz* mutant alleles: Previous sequence analysis demonstrated that the *fz* mRNA that functions in the epidermis (KRASNOW and ADLER 1994) encodes a protein with 582 amino acids (VINSON and ADLER 1989). We determined the sequence changes associated with 14 EMS-induced *fz* alleles. One EMS-induced allele (*fz*^{P21}) was found to be associated with an insertion into the first exon that shifted the reading frame (JONES 1995).

Cell nonautonomous *fz* alleles due to missense mutations map throughout the fz gene: Several EMS-induced *fz* mutations that act cell nonautonomously were sequenced and found to be associated with single base pair changes. Only one of these (*fz*^{HE11}) mapped within the putative large extracellular N-terminal domain (Figure

TABLE 2

Autonomy and Western blot analysis of fz alleles

Mutant allele	Strength of allele	No. of clones examined	Percent showing nonautonomy	Technique	Amount of Fz protein[b]	Apparent size of Fz protein[c]
122	S[a]	18	83	γ-ray	Normal[d]	Normal
GL31	S	5	100	γ-ray	Low[e]	Larger
EAB2a	S	15	93	γ-ray	None	—
C19	S	12	92	γ-ray	Low	Larger
P21	S	8	100	γ-ray	None[f]	—
H51	S	9	100	γ-ray	Normal	Smaller
R52	S	10	100	γ-ray	Very low[g]	Smaller
HD21	M	13	100	γ-ray	Low	Larger
I12	S	18	0	FLP/FRT	Normal	Normal
HE11	S	11	100	FLP/FRT	Normal	Larger
HC52	S	11	100	FLP/FRT	Low	Larger
HB41	S	16	100	FLP/FRT	Low	Larger
J22	S	12	0	FLP/FRT	Normal	Normal
R53	W	16	81	FLP/FRT	Normal	Normal

[a] S, strong; M, moderate; W, weak.

[b] The amount of Fz protein was assayed by Western blot analysis as described in MATERIALS AND METHODS.

[c] A reduced rate of migration during electrophoresis was interpreted as indicating an increased molecular weight, and conversly an increased rate of migration was interpreted as indicating a reduced molecular weight.

[d] The amount of protein appeared to be within twofold of normal.

[e] The protein could be routinely detected but the immunosignal was less than half of that seen in a wild-type fly.

[f] No protein was detected.

[g] The protein could not routinely be detected, and when it was, the signal was decreased about fivefold or more compared to wild type.

3). This mutation was associated with a codon change of TGT to TAT. Two mutations were due to missense mutations in putative transmembrane domains. These were fz^{HC52} (GGG to AGG) and the previously described cold-sensitive allele fz^{HD21} (ADLER *et al.* 1994).

fz^{HB41} and fz^{HB52} were both found to be due to valine 454 being replaced by a phenylalanine (GTT to TTT). This amino acid is predicted to be in the third cytoplasmic loop of the Fz protein (Figure 3). Since both of these mutations were isolated from the same group

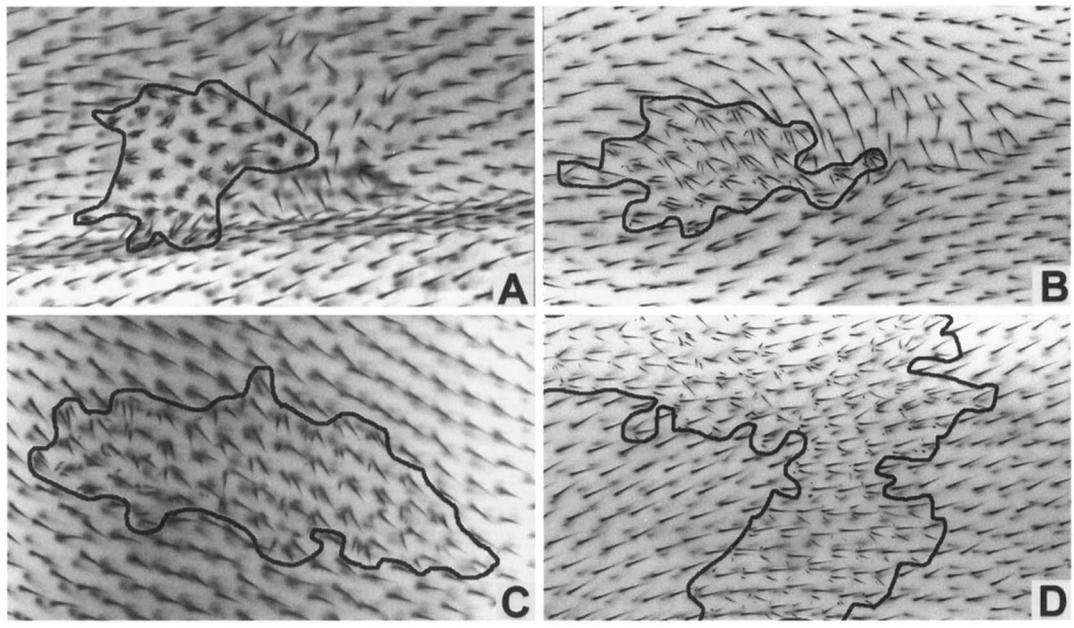

FIGURE 1.—Examples of marked fz mitotic clones. (A) fz^{R52} *trc* clone that displays the domineering directional cell nonautonomy typical of most *fz* alleles. (B) fz^{R53} *mwh* that also displays the cell nonautonomy. (C and D) fz^{J22} *mwh* and fz^{I12} *mwh* clones that are cell autonomous. The boundary of the clones are outlined. All wings are oriented with proximal being on the left and distal on the right.

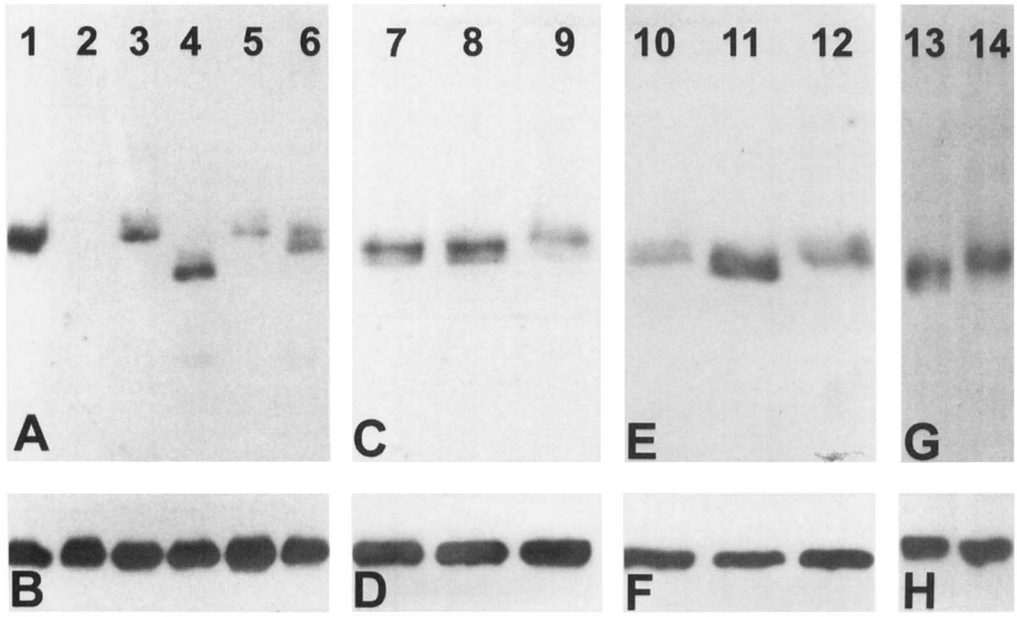

FIGURE 2.—Western blot analysis of the Fz protein. The upper panels (A, C, E and G) were probed with the 1C11 anti-Fz monoclonal antibody (PARK *et al.* 1994a). The lower panels (B, D, F and H) are derived by reprobing the blot with an anti-actin monoclonal antibody to act as a loading control. Four different experiments are shown. Lane 1, Oregon-R; lane 2, fz^{EAB2a}; lane 3, fz^{GL31}; lane 4, fz^{H51}; lane 5, fz^{CI9}; lane 6, fz^{J22}; lane 7, fz^{N21} R; lane 8, Oregon R; lane 9, fz^{HE11}; lane 10, fz^{HC52}; lane 11, Oregon R; lane 12, fz^{HB41}; lane 13, *D. melanogaster;* lane 14, *D. virilis.*

of mutagenized parental flies, we suspect that they are not independent.

The fz^{H51} mutation is a nonsense mutation: The fz^{H51} mutation was found to be due to a change of tryptophan 500 (TGG) to a stop codon (TGA) (Figure 3). The Fz*H51* protein migrated more rapidly on SDS-PAGE as expected for a truncated protein (Figure 2). This allele is a phenotypic null that behaves cell nonautonomously in mitotic clones.

Complexity within the R5 series of alleles: In our original screen we recovered four mutations from the same bottle of parental flies (R5 bottle). In this screen we did not remove the male flies, and the eggs from which the R5 series of mutations were isolated were laid almost 2 weeks after the mutagenesis. Thus, it is possible that these mutations are not independent. Three of the mutations produced a strong phenotype (fz^{R51}, fz^{R52} and fz^{R54}), while one (fz^{R53}) produced a very weak phenotype. The fz^{R52} allele was found to contain the same nonsense mutation as fz^{H51} (Figure 3). This mutant gene results in much less protein being produced than fz^{H51} (Table 2), hence we suspect that there must be a second hit in nontranslated sequences (which we did not sequence) to account for the lowered amount of mutant protein. In our RT-PCR experiments we needed an additional round of amplification to amplify the fz^{R52} cDNA for sequencing. This suggests that the lowered protein levels are due to lowered mRNA levels. The protein produced by fz^{R52} migrated the same on SDS-PAGE as the protein produced by fz^{H51}. Since there is no phenotypic difference between fz^{H51} and fz^{R52}, the truncated protein likely lacks any activity.

The two remaining strong alleles from the R5 series (fz^{R51} and fz^{R54}) both produced moderate amounts of a protein that displayed a slightly slower than normal migration rate. We sequenced fz^{R54} and found that this gene contained two mutations. The first mutation results in a substitution of methionine 469 for arginine (ATG to AGG), and the second results in the substitution of trp-500 by an arginine (TGG to CGG) (Figure 3). We note that trp-500 is the location of the nonsense mutation in fz^{R52}.

The final allele from the R5 series is the weak hypomorphic mutation fz^{R53}. Sequencing showed that this mutation was associated with a substitution of alanine-374 by a glycine residue (GCG to ACG). This residue is located in the presumptive second cytoplasmic loop of the protein (Figure 3).

Cell-autonomous *fz* alleles share missense mutations at proline residue 278: The two cell-autonomous alleles (fz^{F31} and fz^{N21}) (VINSON and ADLER 1987) that are unusual in not sharing the rough eye phenotype exhibited by other *fz* alleles (ADLER *et al.* 1987) were both found to cause the substitution of pro 278 by ser (CCG to TCG). These identical mutations were isolated independently. The two additional cell-autonomous alleles (fz^{J12} and fz^{J22}) were found to cause the substitution of pro 278 by leu (CCG to CTG). Unlike fz^{F31} and fz^{N21}, the mutant phenotypes of fz^{J12} and fz^{J22} are typical of strong *fz* alleles. fz^{J12} and fz^{J22} were isolated independently. Proline 278 is predicted to be in the first cytoplasmic loop of Fz (Figure 3).

Molecular cloning and sequencing of the *D. virilis fz* gene: The *D. melanogaster fz* gene is composed of five

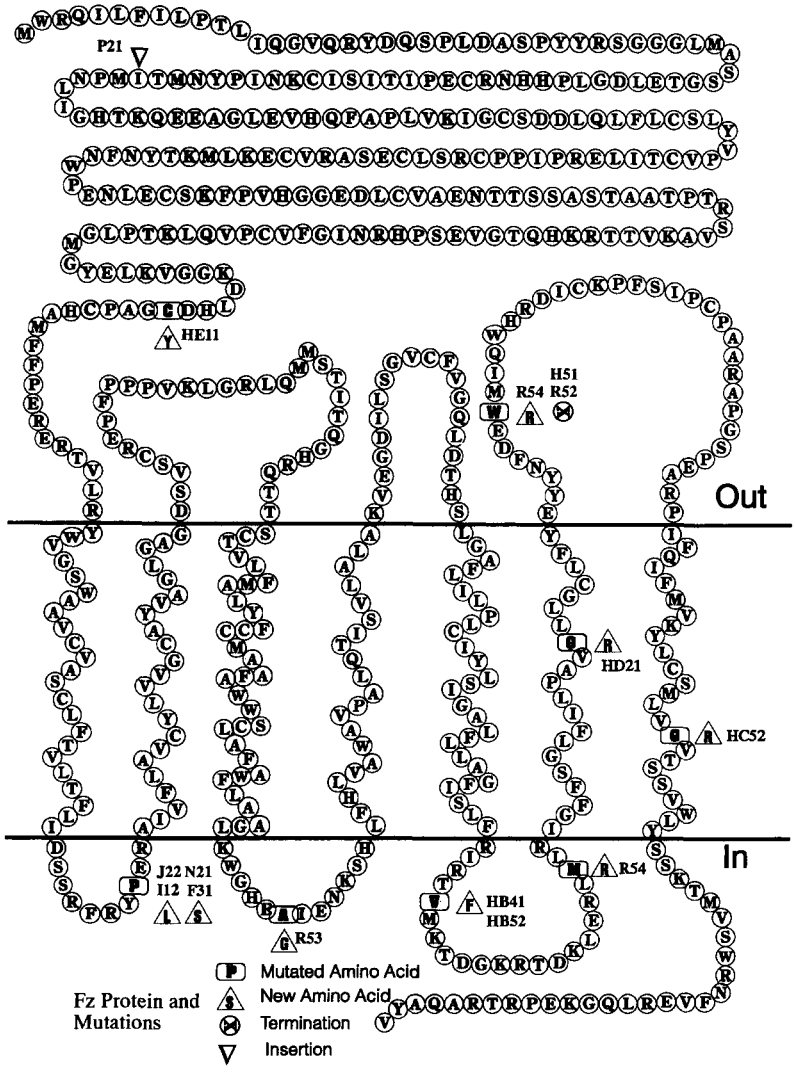

FIGURE 3.—A diagrammatic representation of the Fz protein and the changes associated with *fz* mutations.

exons spread out over more than 90 kb of genomic DNA (ADLER *et al.* 1991). The first and fourth introns are ~25 and 60 kb, respectively, while the second and third introns are quite small. Our cloning and analysis of the *D. virilis fz* exons indicates that this same general gene structure has been conserved. We found the 5′-most exon of the *D. virilis fz* gene in one group of recombinant bacteriophage, the middle three exons of the *D. virilis fz* in a second group of recombinant bacteriophage and the 3′-most exon in a third discrete group of bacteriophage. We did not attempt a chromosomal walk to determine the length of the first and fourth introns in *D. virilis,* but our results are sufficient to say that the general structure of the *fz* gene is conserved in the two Drosophila species.

We sequenced the exonic sequences from our cloned genomic DNA and then confirmed this sequence via sequencing amplified cDNA obtained by RT-PCR. The locations of the exon-intron junctions were identical to those found in *D. melanogaster.* Assuming that the *D. virilis* mRNA is translated using the 5′-most AUG as a start codon, the conceptually translated *D. virilis* Fz protein is 583 amino acids, two residues longer than its *D. melanogaster* homologue (Figure 4). The two proteins are predicted to be 91.6% identical over their entire lengths. Over much of the proteins the identity is actually substantially higher, as the first 46 amino acids are only ~33% (15/46) identical. Discounting this region, we find the two sequences are 96% identical. The degree of conservation is high in all other regions. It is highest in the presumptive cytoplasmic loops and COOH terminal domains that are identical in the two species. The putative transmembrane domains are 97% identical, being somewhat more conserved than the presumptive extracellular domains. Consistent with this very high degree of sequence conservation an anti-*fz* monoclonal antibody raised against the *D. melanogaster* Fz protein recognized the *D. virilis* Fz protein. This protein migrated slightly slower than the *D. melanogaster* homologue (Figure 2).

The *D. virilis fz* gene is functionally conserved: We constructed and obtained germ line transformants for a gene where the *D. virilis fz* cDNA was placed behind the *hsp70* promoter. We tested the ability of this

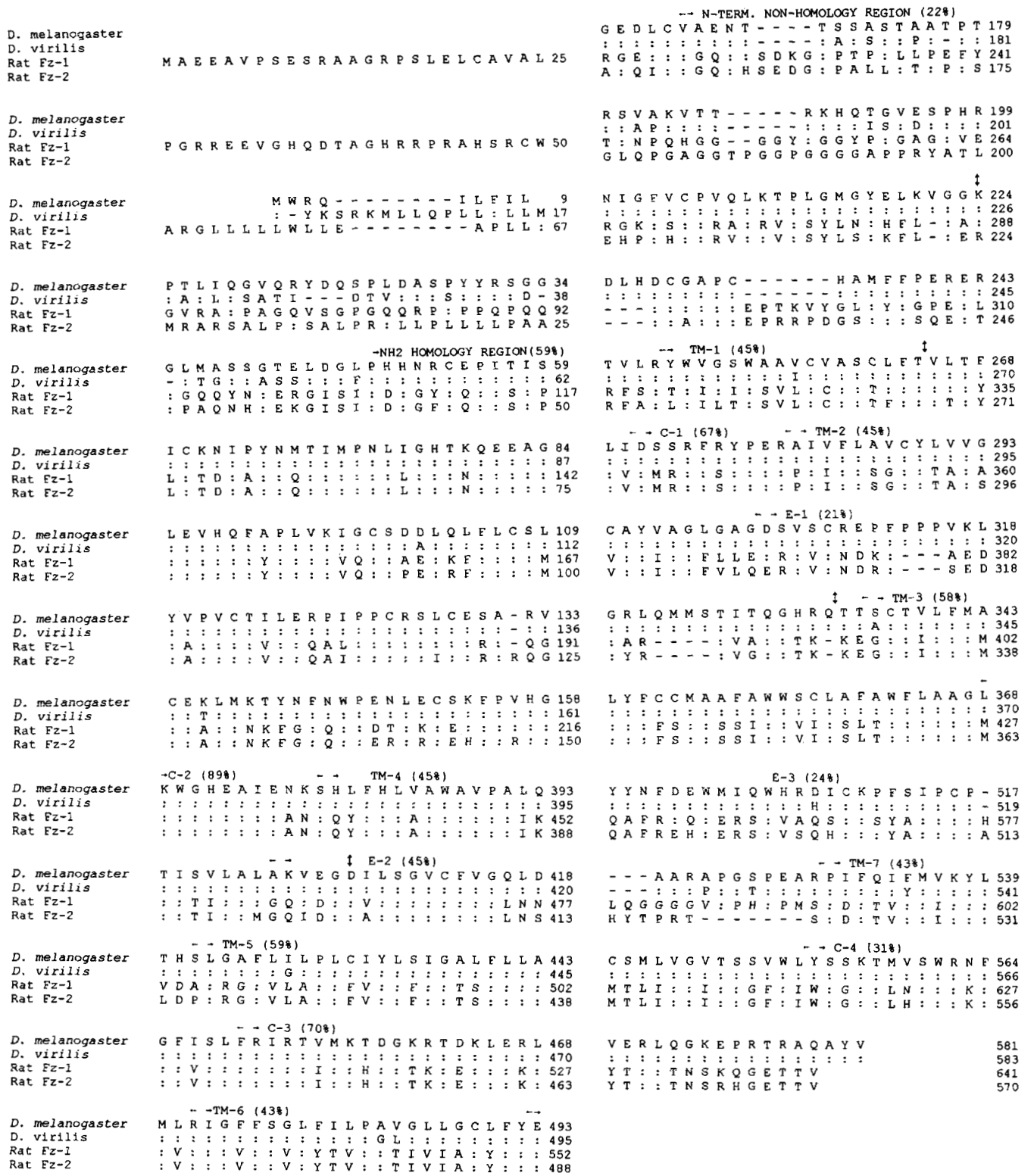

FIGURE 4.—A comparison of the Fz proteins from flies and rat. A, identity to the *D. melanogaster* sequence. Arrows above the sequence indicate putative protein domains. TM, transmembrane domain; C, cytoplasmic loop (or tail); E, extracellular loop. The percentage identity for the four sequences in each putative domain is given in parentheses. The vertical double headed arrows mark the location of the exon-intron junctions in the Drosophila genes.

transgene to provide *fz* rescue activity in the same way we did for the *D. melanogaster hsfz* transgene (KRASNOW and ADLER 1994). We crossed the transgene into a *fz* null genetic background and scored the wings of flies reared at 29° for the fraction of the wing showing abnormal polarity and for the number of multiple hair cells

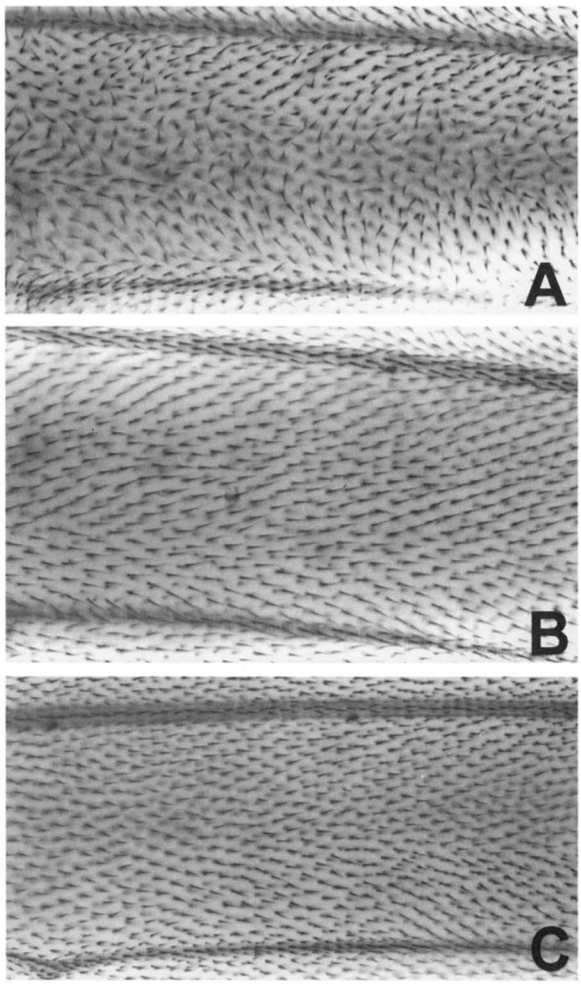

FIGURE 5.—Rescue of a null *fz* genotype by the *hs-fz*^{vir} gene. (A) Region on the ventral surface of the wing from a fz^{K21}/fz^{D21} (inversion/deletion) fly. This represents the null genotype. (B) The same region of a *hs-fz*^{vir}; fz^{K21}/fz^{D21} fly. (C) The same region of an Oregon-R fly.

produced in the dorsal C cell of the wing. As was seen for the comparable constructs with the *D. melanogaster fz* cDNA, we obtained substantial rescue using both criteria. In a *fz* null mutant ~85% of the wing shows abnormal polarity (KRASNOW and ADLER 1994). The *hsfz*^{vir} transgene reduced this to ~5% of the wing surface (Figure 5, Table 3). This is comparable to what is seen for the *hsfz*^{mel} transgene (KRASNOW and ADLER 1994). The number of multiple hair cells was reduced about sevenfold from 35 to 5.5 per C cell (Table 3).

The overexpression of the *D. melanogaster fz* gene produces a gain of function tissue polarity phenotype (KRASNOW and ADLER 1994). The heat shock induction of the *D. virilis fz* transgene induced a similar gain of function phenotype, further demonstrating the functional conservation of the *D. virilis fz* gene.

DISCUSSION

Structure and function in the Fz protein: Many of the *fz* mutations studied caused at least some decrease

TABLE 3

Rescue of fz by the hs-fz^{vir} transgene

Genotype	Fraction of wing with abnormal polarity	No. of multiple hair cells
fz^{K21}/fz^{D21}	86.4 ± 5.1	34.5 ± 4.9
hs-fz^{vir}; fz^{K21}/fz^{D21}	5.3 ± 1.7	5.5 ± 2.0

Four wings were scored for each genotype/phenotype. Values are average ± SE. All differences are significant ($P < 0.05$, Whitney-Mann nonparametric test).

in the amount of Fz protein present in cells as assayed by Western blotting. In principal, some *fz* mutations might decrease *fz* activity by decreasing the amount of Fz protein and not by producing a Fz protein that has less (or no) activity. In previous experiments we found that the expression of a *hsfz* transgene at 29° provided almost complete rescue of a null mutant genotype for the endogenous *fz* gene (KRASNOW and ADLER 1994). Under these conditions we found ~20% of the normal levels of Fz protein was present in the pupal wing cells. This genotype (*hsfz;* fz^{-}) produced a weaker mutant phenotype than any of our *fz* alleles. Thus, for any mutation to cause of loss of *fz* function via decreasing the amount of Fz protein as opposed to decreasing the activity of the Fz protein, we would expect that substantially <20% of the normal amount of Fz protein would need to be present in the mutant. Since many of our *fz* mutations do not result in substantially <20% of normal Fz protein levels, we argue that at least these *fz* mutations must be producing a protein of decreased activity.

The wild-type Fz protein is localized to the apical plasma membrane and to the subapical regions of the lateral plasma membranes (PARK *et al.* 1994). One way that *fz* mutations could result in a decrease in Fz protein activity would be to cause the protein to become mislocalized. Given the likely seven transmembrane domains of Fz, it seems unlikely that any missense mutation would result in the protein not being inserted into a membrane, however, it seems plausible that a missense mutation might result in the protein being localized to alternative membrane locations. Unfortunately, our antibody reagents are only marginally able to detect the endogenous Fz protein *in situ,* hence we are unable to unambiguously detect the mutant proteins that are typically less abundant to determine if any resulted in an abnormal subcellular localization.

Genetic mosaic studies have shown that the *fz* gene is required both for signaling and for cells to respond to the polarity signal (VINSON and ADLER 1987). An extensive cDNA clone analysis provided evidence for two potential Fz proteins (ADLER *et al.* 1991), however, the ability of a transgene that expressed only one of these (FzI) to provide complete rescue of *fz* mutations argued that only one of these two proteins functioned

in tissue polarity in the epidermis (KRASNOW and ADLER 1994). The lack of consequence of expression of the second putative *fz* protein also argued that only the FzI protein functioned in the epidermis. Our finding here that a phenotypic null mutation (*i.e.*, fz^{H51}) maps to the fifth exon, which is specific to the *fzI* transcript, provides further independent evidence for a single *fz* protein functioning in tissue polarity in the epidermis.

The difference between the cell-autonomous and cell-nonautonomous *fz* alleles is likely to be qualitative and not quantitative in nature. Among the cell-nonautonomous alleles are both null alleles (*e.g.*, fz^{H51}) and our weakest hypomorphic allele (fz^{R53}), and among the cell- autonomous alleles are both strong (*e.g.*, fz^{J22}) and moderate alleles (*e.g.*, fz^{N21}).

Several possible models can explain how a single Fz protein can provide both of the tissue polarity functions. We have previously suggested that the Fz protein functions as a tissue polarity receptor, and that receptor binding ligand leads to the activation of two signal transduction pathways (Figure 5 in PARK *et al.* 1994) (this model will be referred to as the receptor model). One of these would lead to the relaying of the signal (*e.g.*, the release of additional ligand) and the other would lead to the cell assembling the microvillus-like prehair at the distal vertex (WONG and ADLER 1993) (*i.e.*, the cell-autonomous function of *fz*). In many ways this model suggests that the Fz protein functions in a way that is analogous to the cAMP receptor in Dictyostelium (DEVREOTES 1989). In an alternative model the cell-autonomous and cell-nonautonomous functions of the Fz protein might be quite independent. For example, the Fz protein could function directly as a ligand (signaling function) and also function independently in some way (perhaps as a receptor) to provide for the cell-autonomous function) (receptor/ligand model). While both of these models remain possible, we feel the data reported in this paper favor the former. If the Fz protein served directly as a ligand, it seems likely that it would be the extracellular domains of the protein that would be recognized by it's receptor. This possibility is not supported by the distribution of cell nonautonomously acting missense mutations. The large amino terminal domain of the Fz protein contains most of the amino acid residues that are likely extracellular (PARK *et al.* 1994). On a per amino acid basis this region contained few missense mutations (1 mutation/247 amino acids), an observation that would not be predicted by the Fz receptor/ligand model. Furthermore, no missense mutations were found to be localized in the 89 amino acids present in the three putative extracellular loops, regions that also might be recognized by a receptor. (In this discussion we ignore the fz^{R54} allele that is associated with changes in both the third extracellular loop and in the third cytoplasmic loop, since we do not know the relative importance of these two changes for inactivating *fz*.) In our experiments we found a number of mutations with a cell-nonautonomous phenotype mapped to the presumptive cytoplasmic (two mutations/72 amino acids) and transmembrane domains (two mutations/173 amino acids). It is possible that these mutations act cell nonautonomously via indirectly disrupting the structure of the extracellular domains of the protein so that they were no longer recognized by a receptor, however, it would be surprising if such mutations were more common on a per amino acid basis than mutations that directly affected the extracellular domains. These data are, however, easily accommodated by the receptor model for *fz* function. Mutations in transmembrane and cytoplasmic domains of the protein would be expected to be cell nonautonomously acting if they disrupted the activation of the intracellular signal transduction pathway that results in the production of additional signal.

Any model for *fz* function must be able to accommodate the existence of the four cell- autonomously acting *fz* alleles. In the receptor model, we might expect that the activated Fz receptor interacts with two different sets of signal transduction proteins and does so using different parts of its cytoplasmic domains. This model would predict that cell-nonautonomously acting and cell-autonomously acting mutations would map to different regions within the cytoplasmic domains. The receptor/ligand model does not make any similar prediction. Our finding that all four of the cell-autonomous alleles map to a single proline residue in the putative first cytoplasmic loop of the protein while cell-nonautonomously acting mutations are found in other cytoplasmic loops is consistent with the expectations of the receptor model for *fz* function, although the number of relevant mutations is admittedly small.

The ligand-binding domains of a number of G-coupled receptors lies in transmembrane domains (DIXON *et al.* 1988; WESS *et al.* 1993), as does the rhodopsin chromophore (NAKAYAMA and KHORANA 1990). Our finding that two *fz* mutations are in putative transmembrane domains is consistent with the possibility that the Fz protein functions as a receptor with a transmembrane ligand-binding pocket. It is possible, however, that these mutations disrupt the function of the Fz protein via indirectly affecting the structure of other regions of the Fz protein or via interfering with the ability of the protein to allosterically communicate ligand binding extracellularly to cytoplasmic domains.

Why do many mutant Fz proteins migrate anomalously on SDS-PAGE?: Seven of the *fz* mutations analyzed by Western blot analysis revealed a Fz protein that ran slightly more slowly than normal. Four of these were sequenced and found to be associated with missense mutations that do not change the molecular weight of the protein enough to expect to be able to detect a change on SDS-PAGE. For example, the fz^{HB41} mutation is due to a substitution of a phenylalanine for a valine, which would change the expected molecular weight of

the Fz protein from 62,341 Da to 62,389 Da. Two possible explanations are that these mutant proteins have been covalently modified or that they are not completely denatured. The Fz protein is known to be glycosylated (PARK *et al.* 1994), but no other covalent modifications are known. Given the general similarity of the Fz protein to G-coupled receptors, we considered that the Fz protein might be phosphorylated as a means of regulating its activity. We were unable, however, to find any evidence for phosphorylation of Fz (*e.g.*, treatment by phosphatase altering the migration of Fz) (J. LIU, W. J. PARK and P. N. ADLER, unpublished results). At the current time we cannot distinguish between the two suggested possible explanations for the slowed migration of many mutant Fz proteins.

The *fz* gene is very highly conserved: The *fz* genes of *D. melanogaster* and *D. virilis* are unusually well conserved. Over their entire length they are 92% identical, and if we disregard the amino terminal 46 amino acids the two proteins are 96% identical. This level of conservation is similar to that seen for the seven transmembrane domain protein rhodopsin (NEUFELD *et al.* 1992) and is substantially greater than was seen for the Boss 7 transmembrane protein (HART *et al.* 1993). The identity of the putative cytoplasmic domains of the two *fz* proteins suggest that this part of the protein is under particularly stringent selection. This might be expected if these domains interacted with a number of cytoplasmic signal transduction proteins.

Two *fz* homologues have been cloned and sequenced from the rat (CHAN *et al.* 1992). These proteins are 46 and 43% identical to that of the *D. melanogaster* Fz protein and 45 and 43% identical to the *D. virilis* Fz protein (Figure 4). The highest degree of conservation is seen in the three presumptive cytoplasmic loops of the protein that are 70, 89 and 70% identical for the four proteins. The large putative extracellular domain of *fz* is interesting in that it shows one large region that is strongly conserved (59% identity) (amino terminal homology region). This region is flanked by regions that show low conservation. Included in the conserved amino acids are proline 278, the site of the four cell-autonomous mutations. Also conserved are cys 229, ala 374 and gly 545, three sites of cell-nonautonomous mutations in *D. melanogaster*. Further, both of the sites mutated in fz^{R54} (met-469 and trp-500) are also conserved in the rat. Indeed, only two of the sites where we detected missense mutations are not conserved in the *fz* genes of flies and rat. Thus, for sites that we know to be important for *fz* function in *D. melanogaster*, there is a higher degree of conservation between fly and rat than over the protein as a whole. Ignoring the amino terminal 46 amino acids, there are 20 amino acid differences between the *D. melanogaster* and the *D. virilis* sequences. These are sites where at least some amino acid variation is compatible with *fz* function in flies. Of these 20 sites 16 have amino acids in the rat that are not found in either of the fly proteins. Thus, among sites where we have evidence that amino acid variation is compatible with function, the rat genes are conserved at a lower than average extent. These data support the argument for functional homology of the fly and rat *fz* genes.

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