Functional Domains of Fused, a Serine-Threonine Kinase Required for Signaling in Drosophila

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ABSTRACT

fused (*fu*) is a segment-polarity gene encoding a putative serine-threonine kinase. In a wild-type context, all *fu* mutations display the same set of phenotypes. Nevertheless, mutations of the *Suppressor of fused* [*Su(fu)*] gene define three classes of alleles (*fu0, fuI, fuII*). Here, we report the molecular analysis of known *fu* mutations and the generation of new alleles by *in vitro* mutagenesis. We show that the Fused (Fu) protein functions *in vivo* as a kinase. The N-terminal kinase and the extreme C-terminal domains are necessary for Fu^+ activity while a central region appears to be dispensable. We observe a striking correlation between the molecular lesions of *fu* mutations and the phenotype displayed in their interaction with *Su(fu)*. Indeed, *fuI* alleles which are suppressed by *Su(fu)* mutations are defined by in-frame alterations of the N-terminal catalytic domain whereas the C-terminal domain is missing or altered in all *fuII* alleles. An unregulated FuII protein, which can be limited to the 80 N-terminal amino acids of the kinase domain, would be responsible for the neomorphic costal-2 phenotype displayed by the *fuII-Su(fu)* interaction. We propose that the Fu C-terminal domain can differentially regulate the Fu catalytic domain according to cell position in the parasegment.

THE *fused* (*fu*) gene is one of 16 segment-polarity genes identified so far. These genes are involved in cell-cell signaling events that allow cells to recognize their position within the embryonic segment and thus acquire their intrasegmental identities along the antero-posterior axis (NÜSSLEIN-VOLHARD and WIESCHAUS 1980; MARTINEZ-ARIAS *et al.* 1988; INGHAM 1991; PEIFER and BEJSOVEC 1992). They encode varied types of products involved in at least two major signal transduction pathways: the *wingless* (*wg*) and the *hedgehog* (*hh*) pathways. The Wingless (Wg) and Hedgehog (Hh) secreted proteins serve at least two separate roles in specifying embryonic cell fates (for review, see HOOPER and SCOTT 1992; DINARDO *et al.* 1994; KLINGENSMITH and NUSSE 1994; PERRIMON 1994). Nevertheless, the different steps of these pathways are not precisely known, and the hierarchical relationships between these genes are not yet clearly understood. Two of these genes, the *fu* gene (PRÉAT *et al.* 1990; THÉROND *et al.* 1993) and the *zeste-white3* (*zw3*) gene (BOUROUIS *et al.* 1990; SIEGFRIED *et al.* 1990) encode putative serine-threonine kinases suggesting that post-translational modifications play a key role in intrasegmental pattern formation. From our work (LIMBOURG-BOUCHON *et al.* 1991; PRÉAT *et al.* 1993) and that of others (INGHAM 1993), it has been assumed that the Fused (Fu) kinase could play a central role in the *hh* signaling pathway that is necessary for the transcription of the *wg* gene (FORBES *et al.* 1993).

In previous papers, the pleiotropic phenotype of *fu* mutants was described; it includes the maternally determined embryonic segment-polarity phenotype that consists of the loss of the posterior part of each segment and mirror-image duplication of the remaining anterior part (NÜSSLEIN-VOLHARD and WIESCHAUS 1980; MARTINEZ-ARIAS 1985; PERRIMON and MAHOWALD 1987), and several zygotic defects such as LV3-LV4 wing vein fusion (FAUSTO-STERLING 1971, 1978) and ovarian tumorigenesis (KING *et al.* 1957). More than 40 *fu* mutants have been studied that all display these embryonic and adult phenotypes (BUSSON *et al.* 1988). These mutants behave as a single complementation group. Intragenic complementation has not been observed, suggesting that the same function is required in the different developmental processes where the Fu product is involved.

A complete suppressor of the embryonic and adult fused mutant phenotypes, *Suppressor of fused* [*Su(fu)*], has been isolated and characterized (PRÉAT 1992; PHAM *et al.* 1995). Amorphic *Su(fu)* mutations lead to viable flies with no detectable mutant phenotype. They are able to suppress all the embryonic and adult phenotypes of *fu* mutants in a semidominant manner. However, a systematic study of the genetic interactions between the amorphic $Su(fu)^{LP}$ mutation and numerous

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fu alleles has revealed an unexpected complexity of the *fused* gene and permitted us to define three classes of *fu* alleles (PRÉAT *et al.* 1993). Class 0 (*fu0*) and class I (*fuI*) *fu* mutants are completely suppressed for all their mutant phenotypes by $Su(fu)^{1.P}$; for example, *fuI*; $Su(fu)^{1.P}$ flies are apparently wild-type. Class II (*fuII*) *fu* mutants, although suppressed for their fused phenotype, display a new maternal and zygotic phenotype in interaction with $Su(fu)^{1.P}$, very similar to that presented by the mutants of another segment-polarity gene, *costal-2* (*cos-2*): embryos lack the median anterior part of each segment with mirror-image duplication of the remaining most anterior structures and adults display duplications of the wing and leg anterior compartments (GRAU and SIMPSON 1987, PRÉAT *et al.* 1993). In addition, class I mutants are dominant over class II in *fuI/fuII;Su(fu)* flies whereas class 0 mutants are recessive over class II in *fu0/fuII;Su(fu)* flies.

The 805 amino acid Fused protein is composed of at least two regions: a 268 amino acid (aa) N-terminal domain sharing ~30% identity with the catalytic domain of serine-threonine kinases and a C-terminal part that has no significant similarity with any known protein and may represent the regulatory domain of the kinase (THÉROND *et al.* 1993). Molecular characterization of two class I and one class II *fu* mutations suggests that only class II alleles possess a mutant C-terminal domain (PRÉAT *et al.* 1993). All class 0 alleles identified have large deletions encompassing the whole *fu* gene and some neighboring genes. From the genetic and molecular data of the *fu-Su(fu)* interaction, it has been proposed that the Fu, Suppressor of fused [Su(fu)] and Costal-2 (Cos-2) products interact with each other. These interactions may be regulated in the different parts of the segment by post-translational modifications implicating modifiers as yet to be identified (PRÉAT *et al.* 1993).

To gain further insights into the molecular basis of *fused* genetic complexity, we have identified the molecular lesions of nine other *fu* alleles (eight known *fu* alleles at the endogenous *fu* locus and one new transgenic *fu* allele obtained in a *P* element excision mutagenesis) and generated 11 new alleles by *in vitro* mutagenesis. We present evidence that kinase activity is necessary for Fu function, and we show that the Fu protein can be divided into three domains with each playing a different role. Two domains are required for Fu activity, the kinase and C-terminal domains, separated by an apparently dispensable region. We observe a striking correlation between the nature of the molecular lesion and the phenotypic class as revealed by the *fu-Su(fu)* interaction. Class I alleles, which give wild-type flies in interaction with *Su(fu)* mutations, bear alterations in the kinase domain but keep the C-terminal domain intact whereas class II alleles, which display a costal-2 like neomorphic phenotype in interaction with *Su(fu)* mutations, have this C-terminal domain altered or absent. Furthermore, we present evidence for the first 80 amino acids of the kinase domain in generating this neomorphic cos-2 like phenotype. Gene dosage experiments show that the C-terminal domain alone is able to reverse this phenotype in a non competitive manner. We discuss a possible double role of this C-terminal domain in differentially regulating the Fu catalytic activity according to the cell position in the parasegment.

MATERIALS AND METHODS

Stocks: The origins and phenotypes of endogenous *fused* mutations analyzed in this work have been described in BUSSON *et al.* (1988) and PRÉAT *et al.* (1993). $Su(fu)^{1.P}$ is an EMS-induced mutation previously described that behaves as an amorphic mutation and gives viable flies with a karmoisin phenotype (PRÉAT 1992). This allele corresponds to a 1.5-kb deficiency affecting the *Su(fu)* and *karmoisin* transcription units (PHAM *et al.* 1995). Endogenous *fu* mutations have been previously classified into three classes on the basis of their interaction with $Su(fu)^{1.P}$: the fu^{1}, fu^{62}, fu^{mH63} and fu^{JB3} alleles belong to class I, the fu^{A}, fu^{M1}, fu^{MC2}, fu^{C10}, fu^{G3}, fu^{RX15}, fu^{W3}, fu^{RX16} and fu^{RX2} alleles to class II, and the $Df(1)fu^{Z4}$ allele to class 0 (PRÉAT *et al.* 1993 and Table 2). New *fu* mutations induced on a fu^{+} transgene were maintained as *w*; $P(w^{+}fu^{x})/P(w^{+}fu^{x})$ or + strains (Tables 1 and 2). Stocks were kept on a yeast/maize/agar medium (GANS *et al.* 1975). Crosses were made at 20-22° unless otherwise specified.

Generation of new *fu* alleles by *P*-element excision mutagenesis: Two different $P(w^{+}fu^{+})$ insertions, localized on the second chromosome, were used in this mutagenesis, the FU-S 5a insertion carrying the FU-S insert and the FU-L 29 insertion carrying the FU-L insert (THÉROND *et al.* 1993). The FU-S (a 5.025-kb *Bgl*II-*Kpn*I genomic DNA fragment) and FU-L (a 7.4 kb *Sph*I-*Kpn*I genomic DNA fragment) inserts, containing, respectively, 817 and 3117 bp DNA upstream of the transcription start site, include all the *fused* transcription unit and are presented in Figures 1 and 2. $wfu^{A}/wfu^{A};P(w^{+}fu^{+})/CyO;+/+$ females were crossed with $w^{+}fu^{+}/Y;CyO/Sp;\ P(\Delta 2\text{-}3\ ry^{+})Sb/TM6$ males carrying the $P(\Delta 2\text{-}3\ ry^{+})$ insertion as a source of transposase. Transposition and perfect and imperfect excision events occur in the germ line of $wfu^{A}/Y;P(w^{+}fu^{+})/CyO;P(\Delta 2\text{-}3\ ry^{+})/+$ F_1 males (DANIELS *et al.* 1985). These F_1 males were crossed with $FM3/wfu^{A}$ females. Imperfect excision events specifically altering the *fused* gene were selected by screening $wfu^{A};P(w^{+}fu^{x})/+$ F_2 progeny showing a $w^{+}fu^{-}$ phenotype. These $w^{+}fu^{-}$ flies were individually taken and subsequent crosses performed to verify their fu^{-} phenotype and test the class of the new fu^{x} allele (see Table 1). To recover independent events, the dysgenic cross was repeated 100–120 times in separate vials and only one $w^{+}fu^{-}$ F_2 individual per vial was kept for subsequent crosses. Twenty independent mutant lines were obtained from the FU-S transgene and 21 independent mutant lines from the FU-L transgene.

Molecular analysis of *fu* alleles induced by *P*-element excision: The mutant strains obtained from the P[FU-S] insertion were analyzed by Southern blot analysis using *Kpn*I and *Sal*I digestions and the 5.025-kb *Bgl*II-*Kpn*I fragment as a probe. The mutant strains obtained from the P[FU-L] insertion were analyzed using *Bam*HI, *Bgl*II and *Eco*RI digestions and the 7.4-kb *Sph*I-*Kpn*I fragment as a probe. The fu^{XS37} mutant strain was also analyzed using *Pvu*II, *Pst*I and *Kpn*I digestions and the 1.4-kb *Bam*HI-*Sal*I fragment as a probe (Figures 1 and 2). The genomic DNA of the wfu^{+}; $P(w^{+}fu^{XS37})$ strain was amplified in two different polymerase chain reaction (PCR) experiments (SAIKI *et al.* 1985), the products of the amplification

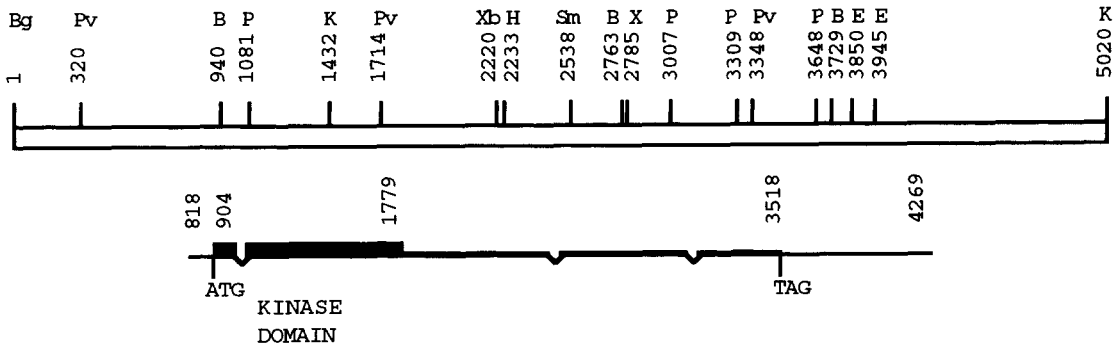


FIGURE 1.—Molecular structure of the FU-S genomic region. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; Sm, *Sma*I; X, *Xho*I; and Xb, *Xba*I. Coordinate 1 corresponds to the *Bgl*II site, 817 bp upstream the *fu* transcription start. The *fused* transcription unit (solid line) extends from coordinate 818 to coordinate 4269, the coding sequence (black bar) from coordinate 904 to coordinate 3518 and the kinase domain (plain black box) from coordinate 904 to coordinate 1779. Intron positions are respectively, 1015–1086 for the first intron, 2457–2516 for the second intron and 3092–3159 for the third intron (THÉROND *et al.* 1993).

were cloned and independent clones were sequenced (Figure 3B and Table 2).

Generation of new *fu* alleles by deletion mutagenesis: The FU-S genomic DNA fragment was cloned into the pBluescript vector digested by *Bam*HI and *Kpn*I, giving the pB-FU-S recombinant vector. The restriction map of the FU-S insert is given in Figure 1. The *Pst5, Pst9* and *Pst11* alleles were obtained by partial *Pst*I digestion of the pB-FU-S vector; the vector-containing portions were gel purified and allowed to recircularize and the resulting deleted vectors, pB-Pst5, pB-Pst9 and pB-Pst11, selected by subsequent restriction map analysis. The *HS* allele was obtained by complete digestion with *Hind*III and *Sma*I of the pB-FU-S vector, filling in the 3′ *Hind*III ends with Klenow enzyme and recircularization. The *BS* allele was obtained by complete digestion with *Sma*I and partial digestion with *Bam*HI, filling in the *Bam*HI site and recircularization. The *HP* allele was obtained by complete digestion of the *Hind*III site (genomic position 2233) and digestion of one of the four *Pst*I sites (genomic position 3007) of the pB-FU-S vector, gel purification of the vector containing fragment and treatment with Klenow enzyme; it corresponds to a filling in of the 3′ *Hind*III ends and a degradation of the 5′ protruding *Pst*I ends and recircularization. In all cases, the boundaries of the expected deletions were verified by sequencing. The genomic alterations of these deleted alleles and their expected consequences at the protein level are given in Table 2 and Figure 4. For each deleted allele, the mutagenized FU-S fragment was isolated from the pBluescript vector by complete digestion with *Xba*I (polylinker site, note that the internal genomic site, coordinate 2220, does not cut) and partial digestion with *Kpn*I and introduced into the pW6 transformation vector (KLEMENZ *et al.* 1987) opened with the same enzymes.

Generation of new *fu* alleles by site-directed mutagenesis: From the pB-FU-S vector carrying the *Bgl*II-*Kpn*I insert (coordinates 1–5025), the *Bam*HI/*Bgl*II-*Sal*I fragment (coordinates 1–2281) was isolated and subcloned into pEMBL18 vector. From this intermediate recombinant vector, two smaller fragments, the *Bam*HI-*Kpn*I (coordinates 940–1432) and the *Sac*I-*Kpn*I (coordinates 1–1432, *Sac*I is in the polylinker site) fragments were subcloned into M13mp19 and M13mp18 respectively. Oligonucleotide-directed *in vitro* mutagenesis was carried out by using the Bio-Rad Muta-gene M13 *in vitro* mutagenesis kit. The *K33R, K33E, ST1* and *ST3* alleles were obtained from the M13mp19 recombinant vector and the *G13V* allele from the M13mp18 recombinant one. The molecular alterations of these *in vitro* induced alleles and their consequences at the protein level are given in Table 2 and Figure 4. The new *fu* alleles were obtained with the following oligonucleotides: the *G13V* allele with 5′-CCCGAAGGAGACTTGCCCCACC-3′, the *K33E* allele with 5′-GGAGATCACTTCGATGGCCACCAC-3′, the *K33R* allele with 5′-GGAGATCACCCTGATGGCCACCAC-3′, the *ST1* allele with 5′-CTTGGAGATCACTCGATGGCCAC-3′ and the *ST3* allele with 5′-GCGGATGCTTCACCGGGCCTG-3′ oligonucleotide. Each mutagenized fragment was purified from the M13 vectors and exchanged with the corresponding wild-type fragment carried in the intermediate pEMBL18 recombinant vector. The *Bam*HI/*Bgl*II-*Sal*I fragments bearing each mutation were then isolated and exchanged with the corresponding wild-type fragment carried by the pB-FU-S vector. The FU-S fragments bearing each mutation were finally introduced into the pW6 transformation vector, using two different procedures. For the *ST1* and *ST3* mutations, the mutagenized FU-S fragments were isolated from the pBluescript vectors by complete digestion with *Xba*I (vector site) and partial digestion with *Kpn*I and introduced into the pW6 vector digested with the same enzymes. For the *G13V, K33E* and *K33R* mutations, the pB-FU-S vectors bearing the mutations were digested with *Xba*I and *Xho*I (coordinate 2785) and with *Xho*I and *Kpn*I; the desired fragments were gel purified and used for a triple ligation with the pW6 vector digested with *Xba*I and *Kpn*I. At each step of these cloning procedures, the presence of the expected mutations was verified by sequencing the mutated region.

Germ line transformation: The pW6-FU-S vectors bearing the *in vitro* induced new *fu* mutations were injected into the w^{1118} host line, with the pUC(*hs*-Δ2-3) vector as a helper, under standard conditions (SPRADLING and RUBIN 1982). For each mutation, several independent transformant lines were established, carrying the $P(w^{+}fu^{+})$ transposon either on the second or the third chromosome. In all these constructs, the expression of the *fu* gene is under a minimal *fused* promoter (THÉROND *et al.* 1993).

Molecular analysis of endogenous *fu* mutations: The endogenous *fu* mutations were localized by Southern analysis using acrylamide gels as described by PRÉAT (1990). For each mutant allele, the genomic region bearing the mutation was amplified by PCR using appropriate primers (data not shown). DNA isolated from about one-half of an adult fly provided sufficient template for a typical PCR amplification. PCR buffer contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3) and 2 mM $MgCl_2$, Taq polymerase was from Perkin Elmer Cetus. Amplification conditions were as follows: 1 cycle, 2 min 96°, 45 sec hybridization t°, 60 sec 74°/35 cycles, 30 sec 92°,

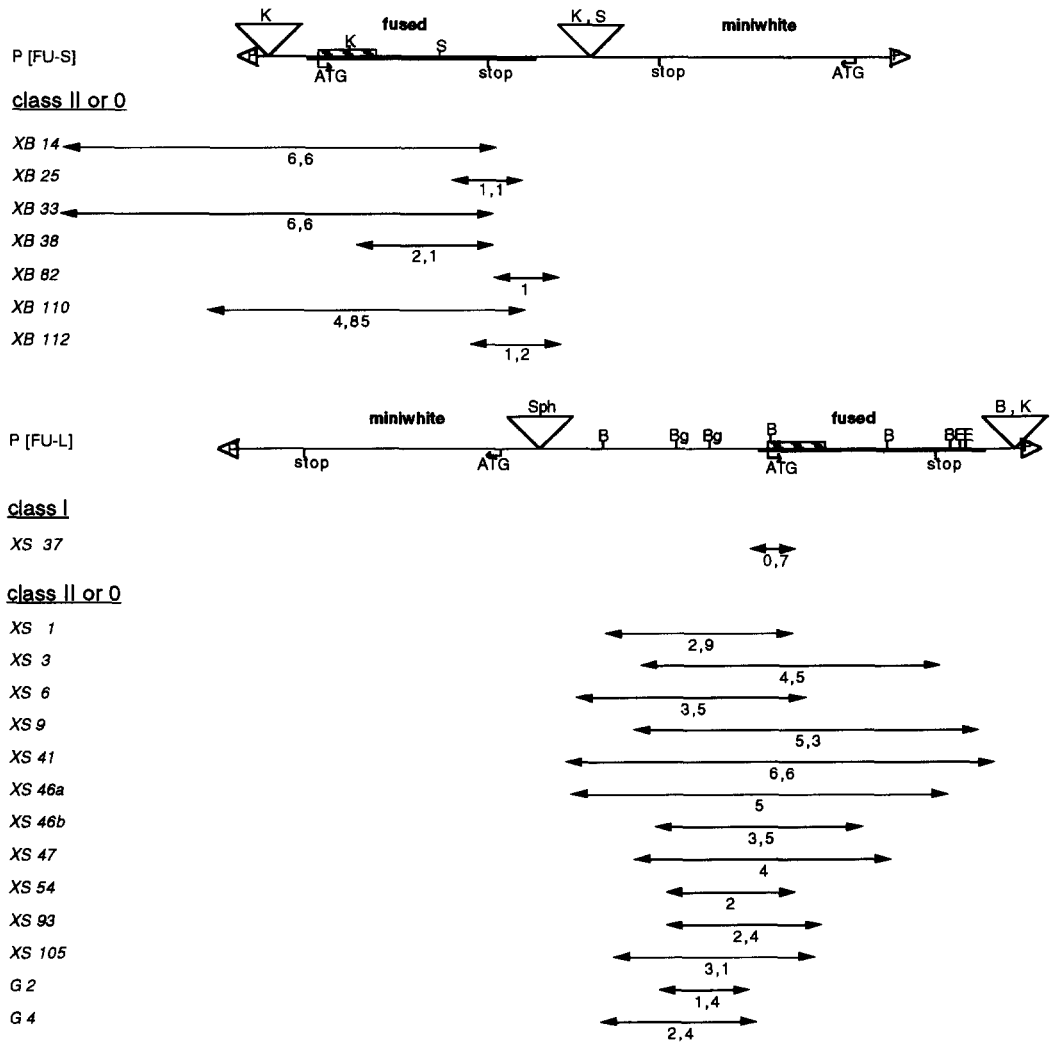


FIGURE 2.—Molecular analysis of the *fu* mutations induced on a transgenic fu^+ gene. B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; K, *Kpn*I; S, *Sal*I; Sph, *Sph*I. P[FU-S] and P[FU-L] are two different Pw^+fu^+ insertions on chromosome 2; they contain the entire fu^+ transcription unit and 817 and 3117 bp, respectively, upstream of the fu^+ transcriptional start and 756 bp downstream the fu^+ transcriptional end; P[FU-L] also contains a part of the neighboring C4 transciption unit (THÉROND *et al.* 1993). ▽, the polylinker sites present in the pW6w^+ and pW8w^+ transformation vectors used to obtain the P[FU-S] and P[FU-L] transgenic insertions. The fu^+ transcription unit is represented by a thick line and the kinase domain by a hatched box. The fu^+ promoter region is internal with respect to the ends of the P[FU-L] insertion while it is external in the P[FU-S] insertion. Southern blot analysis (see MATERIALS AND METHODS) was performed on seven strains obtained from the P[FU-S] insertion (XB strains) and on 14 strains obtained from the P[FU-L] insertion (XS and G strains). Deletions are indicated by lines with arrowheads at both extremities. The class of the new *fu* alleles was defined by the test described in Table 1.

45 sec hybridation $t°$, 60 sec 74°/10 min 74°. For fu^{62}, fu^{mH63}, fu^{JB3}, fu^{M1}, fu^{RX15} and fu^{RX2} alleles, the PCR products were cloned into the pBluescript vector and the fragments sequenced. In all cases, except fu^{mH63}, two independent clones were sequenced; the fu^{mH63} alteration identified was also detected by a *Hae*III site polymorphism at genomic position 1413. For fu^{MC2}, fu^{C10}, fu^{G3}, fu^{W3} and fu^{RX16}, the PCR products were isolated and directly sequenced using the Pharmacia sequencing kit with T7 polymerase. For fu^{MC2}, fu^{W3} and fu^{RX16}, the products of two different PCR amplification were sequenced. In addition to the expected mutations, we observed natural polymorphism as a result of strain background differences, the same variations being observed in different mutants arising from the same reference strain (data not shown). The molecular alterations of endogenous *fu* mutations are given in Table 2 and Figure 3A. The fu^{62}, fu^{mH63} and fu^{M1} allele sequences were previously given in PRÉAT *et al.* (1993). The *fused* genomic regions of both the fu^{RX2rev} revertant strain and the fu^{RX2} original mutant strain were amplified by PCR as three overlapping fragments, extending respectively from nucleotides 801 to 2054, 1695 to 3033 and 2673 to 3796. These fragments were cloned into the pBluescript vector and sequenced. For each fragment, two PCR experiments were performed and several clones sequenced on both strands. The genomic region was sequenced from coordinate 801, upstream the transcriptional start site (818), to coordinate 3700, covering the genomic region translated in the fu^{RX2} mutant (new stop at coordinate 3631, see Table 2 and Figure 3A). Numerous variations due to natural polymorphism were recognized in both fu^{RX2rev} and fu^{RX2} genomic DNAs. Only one substitution was observed at position 3043, leading to the replacement of a G in the fu^{RX2} sequence by a T in the fu^{RX2rev} sequence and thus to the replacement of a valine by a phenylalanine at position 670.

RT-PCR analysis of fu^{JB3} transcripts: fu^{JB3} and fu^{+} females were collected and frozen at −70°. Total RNA extractions were performed by the hot phenol method (PALMITER 1974). DNase I treatment was performed during 30 min at 37° in 100 mM KCl, 1.5 mM MgCl2, 50 mM Tris-HCl pH 8.3 with 40 units of DNase I (Pharmacia) plus the RNA template and distilled water to 25 μl. To inactivate the enzyme, the reaction mixture was then treated at 75° during 15 min. Reverse transcription and amplification reactions were assembled in the same tube by adding 50 mM of each dNTP, 12.5 pmol of each primer, distilled water to 50 μl and 2 drops of paraffin oil. Denaturation and annealing were performed in the PCR apparatus for 3 min at 94° and followed by a programed step at 55°. AMV RTase (2.5 units; Pharmacia) was then added and the tube left for 20 min at 55°. After addition of 2.5 units of Taq polymerase (Perkin Elmer Cetus), the amplification reaction was performed by 30 cycles of 94°, 55°, and 72°, 1 min each step. The primers used (coordinates 861–884 and 1339–1358, respectively) are located on both sides of the intron boundaries, the normal intron extending from coordinate 1015 to coordinate 1086, the putative alternative intron extending from coordinate 988 to coordinate 1086. The amplified fragments were cloned and sequenced.

Western blot and immunoprecipitation analysis: The embryos (age 0–12 hr) were harvested, dechorionated, and resuspended in a buffer containing 50 mM Hepes pH 7.6, 50 mM KCl, 1 mM EGTA, 1 mM EDTA, and 0.05% NP40 supplemented with a mix of protease inhibitors (10 μM benzamidine HCl, 1 μg/ml phenanthroline, 100 μM Pefablock, and 10 μg/ml each of aprotinin, leupeptin, pepstatin A). The embryos were immediately homogenized at 4° by several passes of a Teflon dounce homogenizer. Insoluble material was sedimented and supernatant was collected. After measuring protein concentration by O.D. each sample was incubated at 100° for 5 min in gel loading buffer. Protein extracts were separated on SDS, 6% acrylamide gels and blotted onto nitrocellulose (Schleïcher and Schuell) using a semidry electrotransfer blotter (E and K Scientific Products). Protein levels were examined by staining the filters with Ponceau S solution. The following procedures were done at room temperature. The membranes were blocked for 1 hr with PBTM (5% low-fat dry milk in phosphate-buffered saline, 0.1% Tween 20) and then incubated with Fused polyclonal antibody (P. THÉROND and M. BISHOP, unpublished data) for 2 hr. The membranes were washed three times with PBTM and incubated with HRP coupled donkey anti-rabbit IgG for 30 min (Amersham) at a 1/10,000 dilution. After washing three times with PBTM, the blots were developed using the ECL system (Amersham) and Fuji X-ray film. For immunoprecipitation, 2 mg of protein lysates were precleared by incubation with Protein A-Sepharose beads (Sigma) for 1 hr at 4°. After Protein A-beads were removed by centrifugation, the cleared lysates were incubated with 2.5 μg of purified rabbit anti-Fu antiserum for 1 hr at 4°. The immunocomplexes were precipitated with 50 μl of protein A-Sepharose beads for 1 hr at 4°, washed three times with lysis buffer. Samples were then heated 5 min at 100° in SDS loading buffer. Samples were analyzed on 6% polyacylamide gel as described above.

Germ line clone analysis of transgenic *fu* alleles: Germ line mitotic recombinants were selected using the ovo^{D1} dominant sterile mutation (BUSSON *et al.* 1983; PERRIMON and GANS 1983). For the *ST3 K33R, Pst9* and *Pst11* alleles, *FM6/ywsnfDf(1)fu*^{Z4}*;P(w*$^{+}$*fu*^{x}*)/P(w*$^{+}$*fu*^{x}*);Su(fu)*^{LP}*/Su(fu)*^{LP} virgin females were crossed to $ovo^{D1}v/Y;+/+;Su(fu)^{LP}/Su(fu)^{LP}$ males, for the *ST1* allele, *FM6/ywsnfDf(1)fu*^{Z4}*;+/+;Su(fu)LP/Su(fu)*^{LP} virgin females were crossed to $ovo^{D1}v/Y;Xasta/P(w^{+}fu^{x});Su(fu)^{LP}/Su(fu)^{LP}$ males and these females were transferred daily to new bottles. First instar larvae were irradiated with 1000 Roentgen of X-rays. Upon emergence, *ywsnfDf(1)fu*^{Z4}*/ovo*^{D1}*v;P(w*$^{+}$*fu*^{x}*)/+;Su(fu)*^{LP}*/Su(fu)*^{LP} F_1 females were allowed to mate with $FM6/Y;+/+;Su(fu)^{LP}/Su(fu)^{LP}$ males. Only those F_1 females with a germ line ovo^{+} clone laid eggs and were analyzed individually. Embryos were studied for their hatching ability and their cuticular phenotype. For the *ST3* and *Pst9* alleles, the frequency of F_1 females carrying a germ line clone was of 1%. For the *K33R* allele, no clone was obtained and for the *Pst11* and *ST1* alleles, the only clones giving fertilized eggs corresponded to recombination between $Df(1)fu^{Z4}$ and ovo^{D1}.

RESULTS

Class determination of the transgenic *fu* alleles: Endogenous *fu* mutations have been previously classified into three classes on the basis of both maternal and zygotic phenotypes displayed by the *fu-Su(fu)* interaction (PRÉAT *et al.* 1993). Class I *fu* mutants are totally suppressed by the amorphic $Su(fu)^{LP}$ mutation for both their embryonic and adult phenotypes. Class II *fu* mutants, although suppressed, display an additional phenotype in interaction with $Su(fu)^{LP}$, very similar to that presented by *cos-2* mutations for both embryonic and adult phenotypes. Class 0 *fu* mutants like class I are also totally suppressed by $Su(fu)^{LP}$ but the two class 0 mutants identified so far [$Df(1)fu^{Z4}$ and $Df(1)fu^{P1}$] are large deficiencies including other genes than *fused* and display an additional larval lethality; thus their suppressed phenotype could only be seen on the embryonic phenotype of $Df(1)fu/Df(1)fu;Su(fu)^{LP}/Su(fu)^{LP}$ germinal clone progeny (PRÉAT *et al.* 1993). In a $Su(fu)^{-}$ background, class I alleles are dominant over class II, which are dominant over class 0: this dominance/recessivity relationship between *fu* alleles is observed both for embryonic and adult traits.

Transgenic *fu* mutations were obtained in two ways: by selecting imperfect excisions from a fu^{+} gene carried by a $P(w^{+}fu^{+})$ insertion located on chromosome 2 and by creating *in vitro fu* mutations that were reintroduced in the flies by *P*-mediated transformation. They were tested for the fu^{+} or fu^{-} phenotype of $wfu/Y;P(w^{+}fu^{x})/+$ flies. Their class was determined by testing their ability to rescue the zygotic lethality due to the *fuII-Su(fu)* interaction, in $wfuII/Y;P(w^{+}fu^{x})/+;Su(fu)^{LP}/Su(fu)^{+}$ flies (see Table 1). By analogy with the dominance/recessivity relationship of endogenous *fu* alleles, we supposed that a transgenic class I allele would be dominant over an *in situ* class II allele whereas a transgenic class II or 0 allele would be recessive over an *in situ* class II allele. We expected that a new transgenic *fu* allele would be of class I if able to rescue the lethality of $fuII/Y;Su(fu)^{LP}/+$ flies, whereas it would be of class 0 or class II if not able to correct the *fuII;Su(fu)* lethal interaction. This test alone does not permit us to distinguish class II from class 0 alleles; due to the zygotic lethality of endogenous class 0 alleles identified so far, we are not able to test the phenotype of $wfu0/Y;P(w^{+}fu^{x})/+;Su(fu)^{LP}/Su(fu)^{+}$ flies that would have been expected

TABLE 1

Determination of the class of the transgenic *fu* mutations

Allele	Phenotype[a] of the following genotypes[b]: *fu I or II* / *Y* — *fu*^{x} / + — *Su(fu)*$^+$ / *Su(fu)*$^+$	*fu II* / *Y* — *fu*^{x} / + — *Su(fu)*$^-$ / *Su(fu)*$^+$	Deduced class
G13V *K33E* *K33R* *XS37*	[fu$^-$]	[fu$^{+/-}$]	class I
BS	[pupal lethal] [strong fu wing phenotype]	[fu$^{+/-}$]	class I
ST1 *ST3* *HP* *Pst9* *Pst11* *Pst5*	[fu$^-$]	[cos-2-like]	class 0 or class II
HS	[fu$^+$]	[fu$^+$]	class +

[a] Adult wing phenotype and zygotic viability. In a *Su(fu)*$^+$/*Su(fu)*$^+$ background, [fu$^+$], wild-type phenotype (see Figure 6B); [fu$^-$], fu phenotype, adult viable with LV3-LV4 wing vein fusion; [pupal lethal and strong fu wing phenotype], flies die as pharate adults with LV2-LV3 wing vein fusion and loss of LV4 vein (see Figure 6J). In a *Su(fu)*$^-$/*Su(fu)*$^+$ background, [fu$^+$], wild-type phenotype; [fu$^{+/-}$], partially suppressed fu phenotype, adults fully viable with LV3-LV4 wing veins only partially fused; [cos-2-like], flies die as pharate adults with mirror-image duplication of the wing anterior compartment.

[b] The dominance/recessivity relationship between endogenous *fu* alleles in *Su(fu)*$^-$ context is as follows: *fu0* or *fuI; Su(fu)*$^-$, [fu$^+$] (see Figure 6C); *fuII; Su(fu)*$^-$, [cos-2-like] (see Figure 6K); *fuI/fuII; Su(fu)*$^-$, [fu$^+$]; *fu0/fuI; Su(fu)*$^-$, [fu$^+$]; *fu0/fuII; Su(fu)*$^-$, [cos-2-like] (PRÉAT *et al.* 1993).

to present a suppressed phenotype if the transgenic *fu*^{x} allele was of class 0 and a cos-2 phenotype if the transgenic *fu*^{x} allele was of class II. To recognize these two classes, we examined the embryonic phenotype of eggs laid by *wfu0/wfu0;P(w*$^+$*fu*^{x}*)/+;Su(fu)*^{LP}*/Su(fu)*^{LP} germinal clones.

Different classes of *fu* alleles can be obtained from a transgenic *fu*$^+$ gene: Among the 31 viable *fu* alleles so far studied, seven belong to class I and 24 to class II (PRÉAT *et al.* 1993). In a diepoxybutane mutagenesis performed with a wild-type Oregon R strain (OreR-6), we recovered 12 class II and one class I alleles, while in an X-ray mutagenesis performed on a *car* strain, we recovered seven class II alleles. The six other class I alleles were obtained from different laboratories and were therefore generated from different reference strains.

The neomorphic cos-2 phenotype could be associated with a cryptic mutation near the *fu* gene. To rule out the involvement of this mutation, we tried to recover different types of *fu* alleles from a transgenic insertion containing only the *fu*$^+$ gene and none of the neighboring units (THÉROND *et al.* 1993). For this purpose, we used the ability of *P* elements to be imprecisely excised, leading to internal deletions of sequences contained in the transposon (DANIELS *et al.* 1985). Two different transgenic lines containing a *P(w*$^+$*fu*$^+$*)* insertion on the second chromosome were used to select partial excisions affecting only the *fu*$^+$ gene (MATERIALS AND METHODS). Several lines with a w$^+$fu$^-$ phenotype were obtained and tested both for the type of *fu* allele recovered (see Table 1) and the extent of the corresponding deletions (Figure 2).

Among 41 transgenic lines studied, only one class I allele, *fu*^{XS37}, was recovered, the other 40 being of class 0 or II. To characterize the deletions induced by the imperfect excisions of the *P(w*$^+$*fu*$^+$*)* element, a Southern blot analysis was performed on the genomic DNA of the *wfu*$^+$*; P(w*$^+$*fu*^{x}*)* strains studied. Figure 2 gives a summary of the results obtained for 21 transgenic strains. The deletions extend from 700 bp to 6.6 kb. Several alleles retain some *fu* sequence, other deletions remove the entire *fused* gene and, for some of them, adjacent genomic sequences. The new transgenic class I allele, *fu*^{XS37}, corresponds to the smallest deletion (700 bp); it lacks the *Bam*HI and *Kpn*I sites located in the kinase domain but keeps the *Pvu*II site at the end of this domain (Figure 1). As the *Bam*HI site is located 35 bp downstream of the first ATG translation initiation codon, a more precise analysis of this allele was undertaken to define the exact limits of this deletion. The analysis of *fu*^{XS37} and its interpretation with respect to the class of the allele are presented in Table 2 and Figure 3B.

TABLE 2

Molecular alterations associated with *fused* mutations

Allele[a]	Mutagen	Genomic position	Nucleotide alteration[b]	Amino acid alteration[c]
Endogenous *fu* mutations				
Class I				
62	γ-rays	1389–1397	Δ CGCGAAACT	Δ ALA139 to LEU141
mH63	EMS	1414	G to A	ALA147 to THR
JB3	DEB	1002–1041	Δ AGTG . . . to AAAG	See text
Class II				
M1	DEB	1216–1243	Δ GAGT . . . to CGCT	ΔGLU81 to THR805 + 15aa[d]
MC2	DEB	1450–1503	Δ TCGA . . . to GCCA + 1G	ΔSER159 to THR805 + 59aa[d]
C10	DEB	2153–2185	Δ TGCG . . . to ATAG + GGCG	ΔLEU393 to THR805 +17aa[d]
G3	DEB	219–2210	Δ CAAC . . . to CGCG	ΔTHR407 to THR805 + 3aa[d]
RX15	X-rays	2398–2399	CA to TTCTCAGTT	ΔGLN475 to THR805 + 7aa[d]
W3	DEB	2872–2897	Δ CTCT . . . to CCCC	ΔLEU613 to THR805 +11aa[d]
RX16	X-rays	2951–2962	Δ TCGAGGAGCTGG + 1C	ΔVAL639 to THR805 +84aa[d]
RX2	X-rays	3347–3362	Δ TCAGCTGCTGCGCCTG	ΔGLN749 to THR805 +89aa[d]
Transgenic *fu* mutations				
Class I				
G13V	M13	941–942	GGA to GTC	GLY13 to VAL
K33E	M13	1000	AAA to GAA	LYS33 to GLU
K33R	M13	1001–1002	AAA to AGG	LYS33 to ARG
BS	DEL	945–2540	Δ CTTG . . . to TCCC	Δ PHE15 to PRO502 see text
XS37	PM	879–1533	Δ ATCC . . . to ACTG + CTGTGATA	See text
Class II or 0				
ST1	M13	1000–1002	AAAGTGA to GA.GTGA	LYS33 to GLU and STOP
ST3	M13	1147	CGGCTGA to CGG.TGA	STOP after ARG57
HP	DEL	2238–3011	Δ TAGT . . . to TGCA	Δ SER422 to GLN659
Pst9	DEL	3012–3313	Δ GCAG . . . to TGCA	Δ GLN660 to GLN736
Pst11	DEL	3012–3652	Δ GCAG . . . to TGCA	ΔGLN660 to THR805 +64aa[d]
Pst5	DEL	3314–3652	Δ GAGC . . . to TGCA	ΔSER738 to THR805 +64aa[d]
Class +				
HS	DEL	2238–2540	Δ TAGT . . . to TCCC	Δ SER422 to PRO502

[a] The origin and class of *fu* alleles induced at the *fused* locus have been described in BUSSON *et al.* (1988) and PRÉAT *et al.* (1993). The generation (MATERIALS AND METHODS) and class determination (RESULTS) of transgenic *fu* alleles is described in this work.

[b] Δ CGCGAAACT, deleted nucleotides; Δ AGTG . . . to AAAG, the internal limits of the deleted fragment are given. GGA to GTC, the modified codons are given and the mutated nucleotides are underlined.

[c] Δ ALA139 to LEU141, deleted amino acids; the internal limits of the deleted fragment are given. ALA147 to THR, nature of the amino acid substitution.

[d] The additional residues come from another reading frame of the translated or untranslated regions of the wild-type sequence (see Figures 3 and 4).

From our characterization of the fu^{XS37} allele, we have demonstrated that a mutation within *fu* alone is able to confer the class I phenotype characterized by the dominance of class I over class II allele in *Su(fu)*$^-$ background (see Table 1). It is therefore unlikely that the class II phenotype itself results from the presence of a neomorphic mutation outside the *fu* gene. Among 41 alleles induced in this experiment, only one belongs to class I. This can be explained by the fact that deletions resulting from imprecise excisions are quite large. Most of them delete the whole coding sequence and/or the promoter region, probably leading to class 0 alleles, expected to be totally devoid of Fu product. By analogy with the molecular structure of endogenous alleles (see below), the few small deletions affecting the distal part of the *fu* coding sequence could be class II alleles.

This experiment demonstrates that different classes of *fu* alleles can be obtained from a fu^+ transgene, and we conclude that the type I or type II interaction is only dependent on the *fu* allele itself. It is thus possible to

create *in vitro fu* mutations and classify their phenotypic differences.

Sequence analysis of *fu* alleles: Sequence analysis of three *fu* alleles (two class I: fu^{62} and fu^{mH63} and one class II: fu^{M1}) presented previously (PRÉAT *et al.* 1993) suggested a structure-function relationship between the part of the protein affected and the class of the *fu* allele. To further examine this question, we extended this study to eight other *fu* alleles, one of class I (fu^{JB3}) and seven of class II (fu^{MC2}, fu^{C10}, fu^{G3}, fu^{RX15}, fu^{W3}, fu^{RX16} and fu^{RX2}); the mutations were localized by precise Southern analysis as previously described (PRÉAT 1990) and, for each mutant, the mutated region was amplified by PCR and sequenced (see MATERIALS AND METHODS). The results obtained are presented in Table 2 and Figure 3A. The consequences of the mutations at the protein level were deduced from the DNA sequence analysis and are given on Figure 4.

The class I allele, fu^{62}, corresponds to a 9-bp deletion (PRÉAT *et al.* 1993). The three deleted amino acids (Ala139-Lys140-Leu141) lie adjacent to the DFG (Asp143-Phe144-Gly145) triplet. This DFG triplet represents the most highly conserved motif in the kinase catalytic domain and is implicated in ATP binding (HANKS *et al.* 1988); it is flanked on either side by two hydrophobic or near neutral residues; the nonpolar Leu141 residue, located at two positions upstream of the DFG triplet and deleted in fu^{62} is highly conserved (HANKS *et al.* 1988). The class I allele, fu^{mI163}, is a single base substitution and results in a change of the nonpolar Ala147 to a polar Thr (PRÉAT *et al.* 1993); this amino acid, located at two positions downstream of the DFG triplet is also a relatively conserved residue. For these two alleles, the catalytic kinase domain is present but altered at positions otherwise conserved in serine-threonine kinases.

The nature of the product encoded by the newly sequenced class I allele, fu^{JB3}, was more hypothetical. This allele bears a 40-bp deletion that spans the 3′ part of the first exon and the 5′ part of the first intron and thus removes the 5′ GT donor site of splicing (Figure 3A). If left unspliced, the mutant allele would encode a 55 aa protein beginning with the first 32 normal aa followed by 23 aa translated from intron sequences and would lack the carboxy-terminal domain. To detect if upstream sites could be used as splicing donor sites, fu^{JB3} mRNAs were amplified using RT-PCR (see MATERIALS AND METHODS) and sequenced (data not shown). Three types of mRNAs were detected, two minor species corresponded respectively to nonspliced forms and to the use of an alternative 5′ GT splicing site at position 951–952, the major species corresponded to the use of an alternative 5′ GT splicing site at position 988–989. The **AAGGT**GGT sequence (from position 985 to position 992) fits well with the $^{C}/_{A}$**AGGT**$^{A}/_{G}{}^{A}/_{G}$T consensus sequence for intron 5′ donor sites (Mount *et al.* 1992) and is very similar to the normal *fu* splicing site (**AAGGTGAG**). When this alternative splicing site is used, the normal reading frame is conserved, the resulting protein lacks the nine VVAIKVISK amino acids (aa 29 to aa 37) of the catalytic kinase domain but keeps the rest of the protein intact, in particular the carboxy-terminal part. Residues Ala31 and Lys33 deleted in this sequence are highly conserved, present in all kinases, the invariant lysine being directly involved in the phospho-transfer reaction (KAMPS and SHEFTON 1986).

The eight class II alleles sequenced have alterations that are located either in the kinase domain (fu^{M1}, fu^{MC2}), or in the second domain, the middle part for fu^{C10}, fu^{G3}, fu^{RX15} or the terminal part for fu^{W3}, fu^{RX16}, fu^{RX2}, leaving intact the kinase domain (Table 2 and Figure 3A). Nevertheless, in all cases, the mutations create a frameshift in the open reading frame and at least a part of the carboxy-terminal domain is altered (Figure 4). The two extreme situations are represented by fu^{M1} and fu^{RX2}. The fu^{M1} allele corresponds to a 28-bp deletion; it leads to a putative 95 aa long miniprotein that retains only the 80 first aa of the kinase domain; the 188 remaining aa of the catalytic domain and the entire carboxy-terminal part of the protein are absent. The fu^{RX2} allele bears a 16-bp deletion that affects the C-terminal part of the coding sequence; the resulting putative protein lacks the 57 last aa of the normal protein that are replaced by 89 new ones. Thus, the common features of these eight class II *fu* alleles is the presence of the amino-terminal part of the kinase domain (including the 80 first aa of the protein) and the absence or alteration of the extreme carboxy-terminal part of the second domain (including the 57 last aa of the protein) (see Figure 4).

The overall number of class I alleles is small compared with that of class II alleles (seven class I and 24 class II over 31 viable *fu* alleles). However, the sequence analysis of class I and class II *fu* alleles is in accordance with our previous interpretation: class I *fu* alleles have the kinase domain altered but the second domain intact, whereas class II *fu* alleles have this second domain altered.

The class I allele, fu^{XS37}, obtained in the P excision experiment was also sequenced (Figure 3B). This allele bears an 8-bp insertion and a 655-bp deletion extending from coordinates 879 to 1533, which removes the translational start codon (coordinate 904). However, this allele keeps some function because it is dominant over a class II allele in a *Su(fu)*$^{-}$ background (Table 1). The transcriptional start (coordinate 818) is still present and another ATG codon must be used as an alternative translational start. Indeed, Western blot analysis on immunoprecipitated protein extracts (MATERIALS AND METHODS) reveals a Fu^{XS37} protein of ~80 kD molecular weight (Figure 5). Downstream of the deletion, several ATG codons are found in the same frame as the normal Fu protein. The first one (coordinate 1555) is located at 90 nucleotides from the transcriptional start, while, in

A)

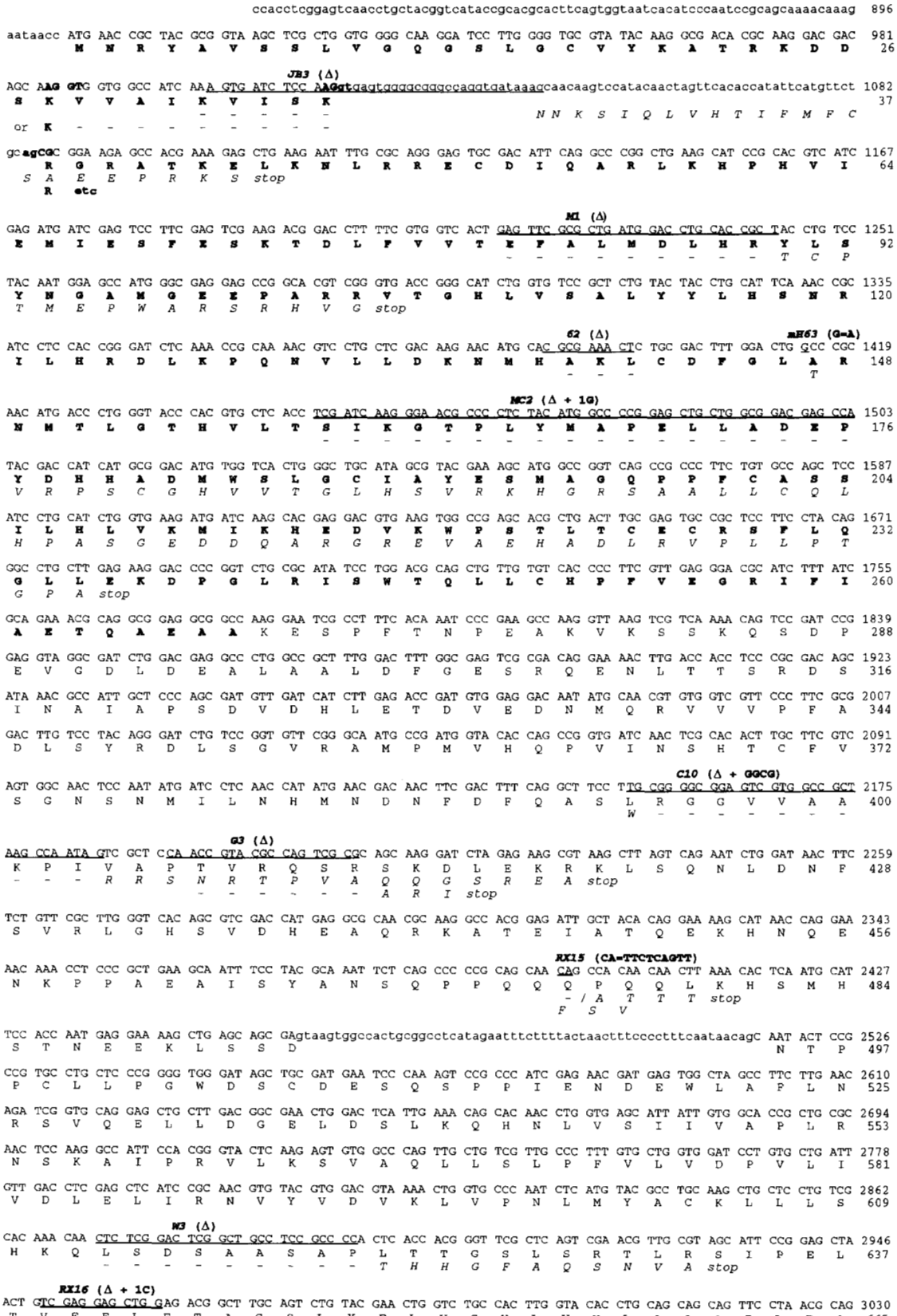

JB3 (Δ)
M1 (Δ)
62 (Δ)
mH63 (G→A)
MC2 (Δ + 1G)
C10 (Δ + GGCG)
G3 (Δ)
RX15 (CA→TTCTCAGTT)
N3 (Δ)
RX16 (Δ + 1C)

FIGURE 3.

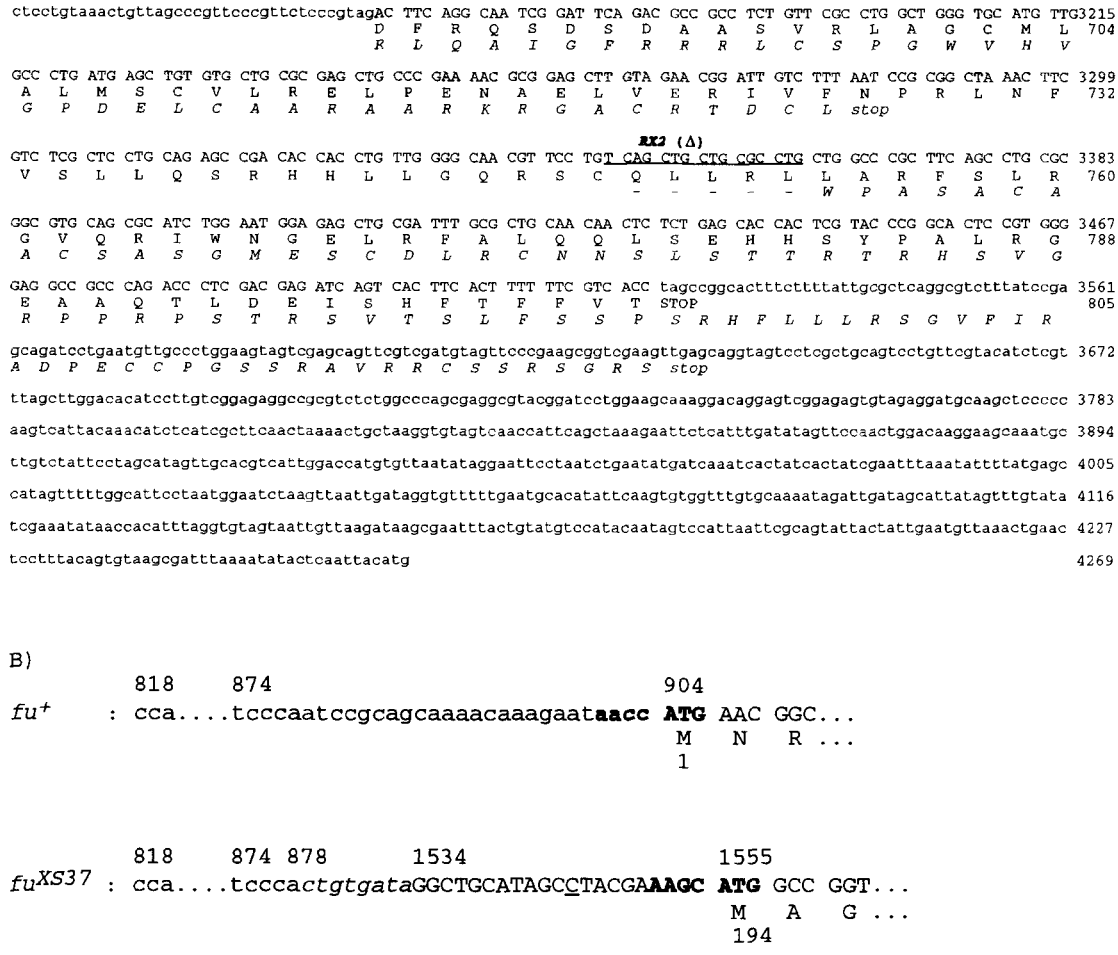
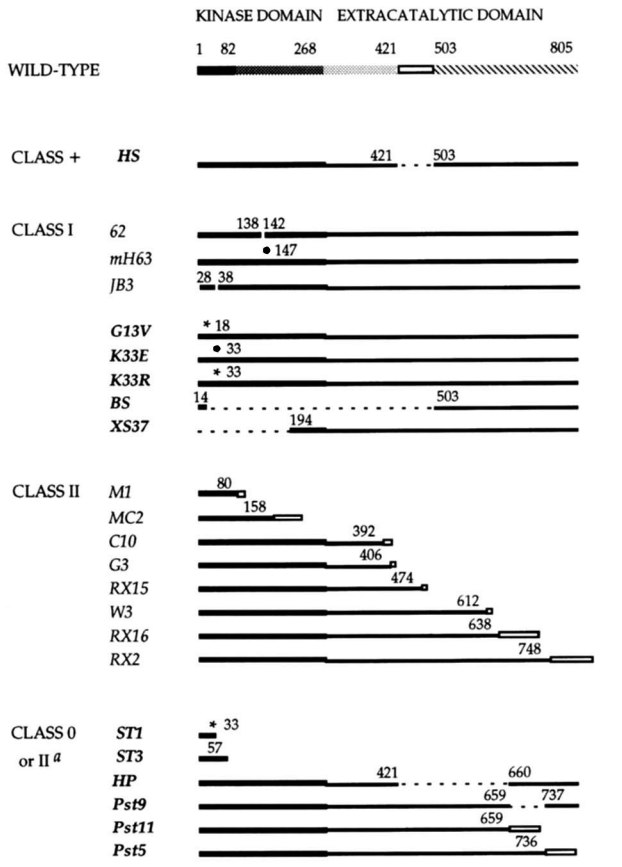


FIGURE 3.—*Continued* Genomic sequence alterations of *fu* alleles and their consequences at the protein level (see also Table 2 and Figure 5). (A) Endogenous *fu* alleles. The wild-type *fused* genomic sequence is given between coordinates 818 (putative transcription start) and 4269 (end of the transcript) (THÉROND *et al.* 1993). The coding sequence is given in capital letters and extra-coding sequences in lower-case letters. For each mutant allele, the nucleotides altered are underlined. *JB3*, *M1*, *62*, *G3*, *W3* and *RX2* alleles correspond to deletions (Δ), *MC2*, *C10* and *RX16* to deletions with 1 or more remaining nucleotides (Δ + x), *mH63* to a single substitution (G = A) and *RX15* to the substitution of two nucleotides by nine others (CA = TTCTCAGTT). The wild-type amino acid sequence, with the kinase domain in bold letters, is presented in the single letter code underneath the coding sequence. The modified parts of the expected mutant proteins are written in italics under the wild-type protein sequence. Two possibilities are given for the *JB3* allele, whether or not alternative splicing takes place. The normal AGgt and putative AGGT 5′ splicing consensus are given in bold letters (see text). For the *RX15* allele, F, S, and V represent the three additional amino acids encoded by the nine nucleotide insertion. (B) P-excision induced fu^{XS37} transgenic allele. The fu^{XS37} allele corresponds to a 655 bp deletion (between coordinates 879 and 1533), associated with an 8-bp insertion (CTGTGATA). The deletion does not affect the transcriptional start (coordinate 818). The normal translational start codon (ATG, coordinates 904–906), removed by the deletion, the alternative ATG translational start (coordinates 1555–1557) and the four nucleotides preceding the ATG are in bold. The eight added nucleotides in the fu^{XS37} sequence are in italics. The C, which replaces a G at position 1545, in the normal sequence is underlined. The expected fu^{XS37} protein sequence would begin at amino acid 194 and then extend normally until amino acid 805 (see text).

the wild-type gene, the ATG is at 86 nucleotides from it. This putative alternative ATG is preceded by a AAGC sequence, very close the AACC *fu* sequence preceding the normal ATG, these two sequences fit well the $^{C}/_{A}$ A $^{C}/_{A}$ $^{C}/_{A}$ Drosophila translation initiation consensus (CAVENER 1987). If this alternative ATG is effectively used, the resulting mutant protein would have the observed size; starting from aa 194, it would contain the last 75 aa of the kinase domain and an entirely normal carboxy-terminal domain (Figure 4). The structure of this class I protein would fit with our previous hypothesis. This interpretation is further strengthened by the fact that the *BS in vitro* allele is also a class I allele (see below).

Mutations in conserved residues of the putative kinase domain lead to a fused mutant phenotype: We have previously shown that the N-terminal domain of the putative Fu protein shares up to 32% identity with the catalytic domain of serine-threonine kinases (PRÉAT *et al.* 1990) and that the 15 invariant or nearly invariant residues characteristic of all kinases are present (HANKS *et al.* 1988). Nevertheless, it has not been demonstrated so far that the Fu protein actually functions *in vivo* as



[a] The test used does not permit us to distinguish class 0 from class II *fu* alleles (see Table 1 and text for the interpretation of these mutants).

FIGURE 4.—Structure of the Fused protein according to the different classes of *fu* alleles (see also Table 2). The different phenotypic classes are defined by genetic tests. Endogenous *fu* alleles are in plain letters, transgenic alleles in bold letters. The different domains of the wild-type Fused protein are as follows: the kinase domain (1–268) is in dark, the plain black box corresponding to the small lobe (1–82), the stippled black box to the large lobe of the kinase (83–268); in the second part (269–805), the diagonally hatched box (503–805) represents the C-terminal domain, the white box (422–502) the dispensable part and the stippled grey box (269–421) a part whose exact function is as yet not known. For *fu* mutants, the kinase domain is represented by a thick line and the second domain by a thinner line, the deleted parts are represented by a dotted line and the out of frame parts by open rectangles; single amino-acid substitutions are marked by an asterisk. Numbers indicate residues still present in deletion mutants or the affected residue in point mutants.

a kinase. In fact, sequence analysis of *fu* mutants suggests that the two domains of the protein are equally required for its activity since mutations restricted to either of these domains lead to the same fu mutant phenotype. On the other hand, at least one case has been reported where the kinase-like domain of a protein (the transmembrane ANP receptor) has no catalytic function and plays only a regulatory role on the second intracellular domain which has guanylate cyclase activity (CHINKERS and GARBERS 1989).

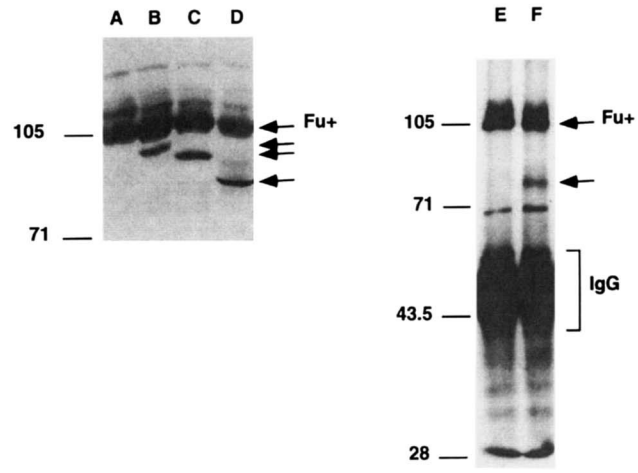


FIGURE 5.—Fused truncated proteins in transgenic embryos. Wild-type embryos (0–12 hr) have a Fused protein (Fu+) at an apparent molecular weight of 105 kD (lanes A and E). In $fu^+;P(w^+fu^{Pst11})$, $fu^+;P(w^+fu^{Pst9})$, $fu^+;P(w^+fu^{HS})$ and $fu^+;P(w^+fu^{XS37})/CyO$ embryos (lanes B–D and F, respectively), an additional band is present at 80–90 kD (arrows). Positions of molecular weight markers are indicated in kilodaltons. Embryonic protein extracts in A–D have been analyzed by direct Western blot analysis whereas Fu proteins were immunoprecipitated before Western analysis in E and F (see MATERIALS AND METHODS).

In known kinases, the two first glycines of the Gly-X-Gly-X-X-Gly consensus found at the N terminus of the catalytic kinase domain (subdomain I of HANKS *et al.* 1988) are highly conserved residues, implicated in ATP binding (WIERENGA and HOL 1983; BOSSEMEYER 1994). Downstream of this consensus, 14–23 residues from the last conserved glycine (subdomain II of HANKS *et al.* 1988), lies an invariant lysine, involved in the phospho-transfer reaction (KAMPS and SHEFTON 1986). Many cases have been reported where substitution of this conserved lysine by alternate amino-acids, including arginine, resulted in loss of protein kinase activity (HANKS *et al.* 1988; CELENZA and CARLSON 1989; MELNICK *et al.* 1993).

We used oligonucleotide-directed mutagenesis to replace either the second conserved glycine of the Gly-X-Gly-X-X-Gly consensus of the Fu protein (Gly13) by a valine or the conserved lysine residue (Lys33) by a glutamic acid or an arginine residue (see MATERIALS AND METHODS and Table 2). The glycine to valine and lysine to arginine substitutions are relatively conservative whereas the lysine to glutamic acid change is nonconservative. The mutated *fu* genes bearing these mutations (respectively, *G13V, K33E* and *K33R*) were introduced in the flies by *P*-mediated transformation and several transgenic strains were obtained for each construction (see MATERIALS AND METHODS). In all three cases, the transgenic $wfu/Y;P(w^+fu^x)/+$ flies obtained displayed a fu^- phenotype (Table 1), showing that all three mutations led to the loss of Fu activity. This fu^- phenotype was particularly strong in the case of the

TABLE 3

Dosage effects between *fu* alleles in various *Su(fu)* backgrounds

	fu and *Su(fu)* genotypes[a]							
	fu^1/fu^X			fu^A/fu^X			$fu^A/fu^1;fu^X$	
Transgenic *fu* allele	$\frac{Su^+}{Su^+}$	$\frac{Su^-}{Su^+}$	$\frac{Su^-}{Su^-}$	$\frac{Su^+}{Su^+}$	$\frac{Su^-}{Su^+}$	$\frac{Su^-}{Su^-}$	$\frac{Su^-}{Su^+}$	$\frac{Su^-}{Su^-}$
Class I								
G13V	fu^W	$fu^{+/-}$	fu^+	fu^M	fu^+	fu^+	$fu^{+/-}$	fu^+
K33R	fu^M	fu^W	fu^+	fu^S	fu^W	fu^+	fu^W	fu^+
BS	fu^S	fu^W	fu^+	fu^{Ext}	fu^W	fu^+	fu^S	fu^+
No. of I and II doses		4 [I]/0 [II]			2 [I]/2 [II]		3 [I]/1 [II]	
Class II								
Pst5	fu^W	$fu^{+/-}$	fu^+	fu^M	cos-2	ND	$fu^{+/-}$	fu^+
Pst9	fu^W	$fu^{+/-}$	fu^+	fu^M	cos-2	ND	$fu^{+/-}$	fu^+
Pst11	fu^W	$fu^{+/-}$	fu^+	fu^M	cos-2	ND	$fu^{+/-}$	fu^+
HP	fu^W	$fu^{+/-}$	fu^+	fu^M	cos-2	ND	$fu^{+/-}$	fu^+
No. of I and II doses		2 [I]/2 [II]			0 [I]/4 [II]		1 [I]/3 [II]	

Phenotypes are written in plain letters and are illustrated in Figure 6. fu^+, wild type; $fu^{+/-}$, fu partially suppressed; fu^W, fu weak; fu^M, fu medium; fu^S, fu strong; fu^{Ex}, fu extreme; cos-2, late pupal lethality with cos-2 like phenotype; ND, not determined.

[a] fu^1 is a class I allele with a fu^W phenotype, fu^A is a class II allele with a fu^M phenotype. fu^X is the transgenic allele tested; *G13V, K33R* and *BS* are class I transgenic *fu* alleles, *Pst5, Pst9, Pst11* and *HP* are expected to be class II transgenic *fu* alleles.

K33R fu transgene when associated with the class II fu^A endogenous allele, giving near complete fusion or disappearance of the LV4 vein (Table 3 and Figure 6H). These results provide strong evidence that Fu is functioning *in vivo* as a kinase. As these three alleles behave as class I alleles, dominant over class II in a $Su(fu)^-$ background (Table 1), we are confident they actually encode a product. These class I constructs affected in the kinase domain but retaining the C-terminal domain intact support our model.

***In vitro* mutations reveal three domains in the Fused protein:** The endogenous *fu* mutations sequenced so far showed that the extreme C-terminal part of the protein is required for Fu activity but did not allow to check the function of more central parts of the large second domain of the Fused protein (from aa 269 to aa 805). To characterize this second domain further, we created *in vitro* mutations (see MATERIALS AND METHODS) corresponding to a series of in frame deletions encompassing different parts of this domain, *BS, HS, HP, Pst9 in vitro* mutations (Table 2 and Figure 4), as well as terminal deletions mimicking the effects of some of the endogenous mutations, *Pst11* and *Pst5* to be compared with *RX16* and *RX2* endogenous mutations and *ST1* and *ST3* to be compared with *M1* endogenous one (Table 2 and Figure 4). The transgenic flies bearing these *in vitro* mutations were tested for their fu^- or fu^+ phenotype as well as for their class in a $Su(fu)^-$ background (Table 1).

Unexpectedly, the *HS* mutation corresponding to the internal 81 amino acid long deletion from aa 422 to aa 502, located approximately in the middle of the protein, behaves as a fu^+ allele both in $wfu/Y;P(w^+fu^{HS})/+$ and in $wfuII/Y;P(w^+fu^{HS})/+;Su(fu)^-/Su(fu)^+$ combinations (Table 1 and Figure 6B). This result demonstrates that this region of the Fu protein is dispensable.

All other *in vitro* mutations affecting the second domain displayed the same behavior: they give a fu^- phenotype in a $wfu/Y;P(w^+fu^x)/+$ combination and do not rescue the lethality of the $fuII\text{-}Su(fu)^-$ interaction in a $wfuII/Y;P(w^+fu^x)/+;Su(fu)^-/Su(fu)^+$ combination (Table 1). This result shows that they belong either to class II or to class 0. By analogy with class II endogenous alleles, we expected *HP, Pst9, Pst11* and *Pst5* to belong to class II but no prediction could be made for *ST1* and *ST3*, compared to the class II endogenous fu^{M1} allele and to the class 0 $Df(1)fu^{Z4}$ deletion. Western blot analysis performed on *Pst9* and *Pst11* protein extracts showed the presence of a truncated protein of the expected size (Figure 5) while the same analysis performed on *ST1* and *ST3* products did not permit us to detect any Fu product (data not shown). In a germ line clone analysis (see MATERIALS AND METHODS), we obtained three females with $Df(1)fu^{Z4}/Df(1)fu^{Z4};P(w^+fu^{ST3})/+;Su(fu)^{LP}/Su(fu)^{LP}$ germinal clones and 10 females with $Df(1)fu^{Z4}/Df(1)fu^{Z4};P(w^+fu^{Pst9})/+;Su(fu)^{LP}/Su(fu)^{LP}$ germinal clones. The former laid wild-type suppressed eggs able to hatch as expected from a $fu0;fu0;Su(fu)^-$ germ line. The latter laid eggs unable to hatch as expected from a $fu0;fuII;Su(fu)^-$ germ line; these eggs were not segmented but displayed a duplicated tuft characteristic of a cos-2 phenotype. Taken together, these results are in agreement with the assignment of *ST1* and *ST3* alleles to class 0 and that of *Pst9* and *Pst11* alleles to class II.

All *in vitro fu* mutations leading to alterations in the kinase domain but not affecting the extracatalytic do-

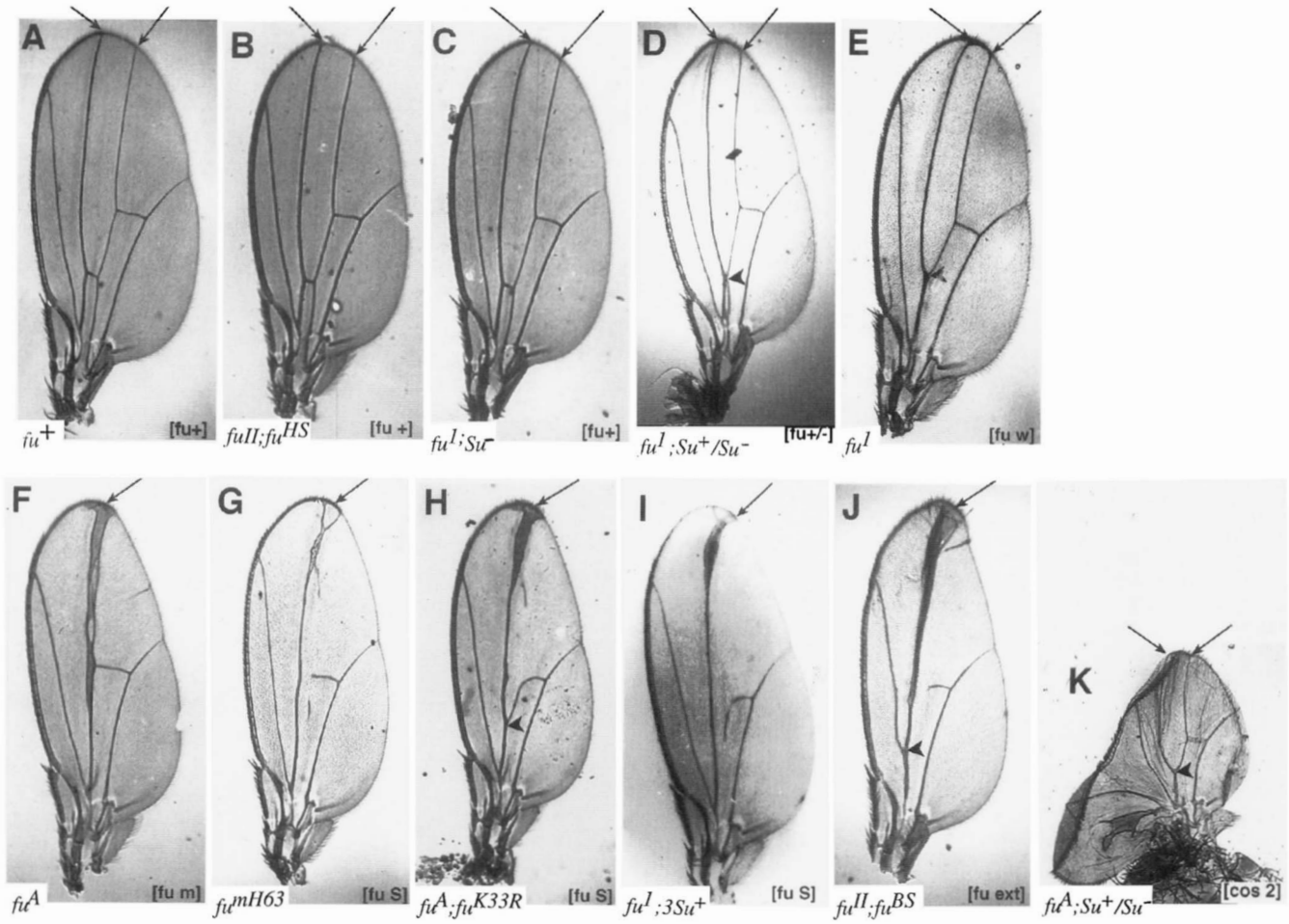


FIGURE 6.—Wing phenotypes of different *fu* and *fu;Su(fu)* genotypes. The phenotypes are presented in a series corresponding to increasing strength of the fu mutant phenotype; [fu^+], wild-type phenotype; [$fu^{+/-}$], partially suppressed fu phenotype; [fu^W], fu weak; [fu^M], fu medium; [fu^S], fu strong; [fu^{Ext}], fu extreme phenotypes; [cos-2], cos-2-like phenotype. (A and B) fu^+ phenotype of (A) wild-type strain. (B) $fuII;fu^{HS}$ genotype. The transgenic fu^{HS} allele confers a perfect fu^+ phenotype to all endogenous *fu* alleles tested. (C–E and I) Phenotypes of the class I fu^1 allele in, (C) $Su(fu)^-/Su(fu)^-$, (D) $Su(fu)^-/Su(fu)^+$, (E) $Su(fu)^+/Su(fu)^+$, (I) three maternal and three zygotic $Su(fu)^+$ doses, genotypes, showing the suppressor effect of decreasing doses and the enhancer effect of increasing doses of the $Su(fu)^+$ allele on the fu phenotype. In (E), note the extension of the bristle carrying double row, between LV3 and LV4 veins at the wing margin (arrows). (F and K) Phenotypes of the class II fu^A allele in, (F) $Su(fu)^+/Su(fu)^+$ and (K) $Su(fu)^-/Su(fu)^+$ genotypes. Note the partially suppressed fu phenotype (arrows and arrowhead) and the cos-2 phenotype displayed in the $Su(fu)^-/Su(fu)^+$ background. (G, H and J) Strong and extreme fu phenotypes displayed by (G) class I fu^{mH63}, (H) $fu^A;fu^{K33R}$ and (J) $fuII;fu^{BS}$ genotypes. Note the nearly total (G and H) or total (J) proximal loss of the LV4 vein. Note also the proximal fusion of LV2 and LV3 veins displayed by the $fu^{II};fu^{BS}$ genotype. The distal end of LV3 and LV4 wing veins at the wing margin is marked by arrows, double arrows (A–E and K), LV3 and LV4 separated, single arrow (F–J), LV3 and LV4 fused. The proximal fusion of LV3-LV4 veins (D, E and H), partial (D) or total (E and H), and of LV2-LV3 veins (J) is indicated by arrowhead. Scale ×20.

main (*G13V, K33E, K33R*) behave as class I *fu* alleles (see above). The *BS* mutation is a large internal deletion that removes 488 residues, from amino acid 15 to 502, and keeps only the 14 first aa of the kinase domain and the last 303 aa of the second domain. The $wfu/Y;P(w^+fu^{BS})/+$ genotype leads to a fu^- phenotype (Figure 6J) while the $wfuII/Y;P(w^+fu^{BS})/+;Su(fu)^-/Su(fu)^+$ genotype is rescued with a partially suppressed fu^+ phenotype (Table 1). Thus *BS* behaves as a class I *fu* allele.

These results lead to several conclusions concerning the role of the different parts of the Fu protein (Figure 4). First, both the integrity of the kinase domain and that of the C-terminal part, extending from at least aa 503 to aa 805, are necessary for wild-type Fu function. Second, the central part of the protein, from aa 422 to aa 502, appears to be dispensable; the exact size of this dispensable region remains to be determined. Third, the presence of the C-terminal end of the protein, from aa 503 to aa 805, is sufficient for producing a class I *fu* allele phenotype. Fourth, the 80 N-terminal amino acids of Fu appear involved in the production of the class II phenotype, in the absence of the C-terminal domain.

Dosage relationship between class I and class II *fu*

alleles in *Su(fu)*$^+$ and *Su(fu)*$^-$ backgrounds: All endogenous *fu* alleles studied so far display the same set of maternal and zygotic traits and behave as a unique complementation group. The strength of the fused phenotype depends on the allele studied and a rather good correlation was observed between the strength of the wing and the embryonic phenotype (BUSSON *et al.* 1988). The fu wing phenotype can be classified into three groups depending on the extent of the apparent LV3 and LV4 wing vein fusion: a fu^{weak} (fu^W) type (*fu^1* allele phenotype), a fu^{medium} (fu^M) type (*fu^A* allele phenotype) and a fu^{strong} (fu^S) type displayed by pupal lethal alleles as *fu^{mH63}* (Figure 6, E–G) without obvious correlation with the class of the allele (*fu^1* and *fu^{mH63}* are class I alleles, *fu^A* is a class II allele). Like the embryonic phenotype in which pattern elements are deleted and replaced by a duplication of remaining ones, this apparent wing vein fusion has been interpreted as a disappearance of the LV4 vein from the posterior compartment and a duplication of the LV3 vein from the anterior one (FAUSTO-STERLING 1978). It must be noticed that the anterior double row composed of bristles, which normally stops at the LV3 level on the wing margin, extends between LV3 and LV4 veins in *fu* mutants (Figure 6, compare A and E). Thus the wing domain affected in *fu* mutants seems to extend on both sides of the antero-posterior boundary.

The *Suppressor of fused* gene was shown to affect the fused phenotype in a dose-dependent manner. Increasing the number of *Su(fu)*$^+$ maternal and zygotic doses was shown to enhance the fused phenotype of both class I and class II *fu* alleles, leading to strong wing phenotypes (Figure 6I) and with sufficient doses (for example, three maternal and three to five zygotic doses), to a total lack of viability (PHAM *et al.* 1995). On the other hand, decreasing the number of *Su(fu)*$^+$ doses was shown to rescue the fused phenotype of class I alleles, also in a dose-dependant manner, one *Su(fu)*$^+$ dose leading to a partial rescue (fu$^{+/-}$ wing phenotype, Figure 6D), 0 dose to a total rescue (fu$^+$ wing phenotype, Figure 6C). Decreasing the number of *Su(fu)*$^+$ doses, although correcting the wing phenotype of class II alleles (Figure 6K), led to zygotic lethality: one and 0 *Su(fu)*$^+$ dose leads to late (with cos-2 phenotype, Figure 6K) and early pupal lethality, respectively. In these *Su(fu)*$^-$/*Su(fu)*$^+$ and *Su(fu)*$^-$/*Su(fu)*$^-$ backgrounds, class I are dominant over class II as they are able to totally rescue the *fuII;Su(fu)*$^-$ lethality.

To study the possible dosage effect of *fused*, we constructed flies containing different doses of class I and class II *fu* alleles and tested their zygotic phenotype in various *Su(fu)*$^+$ or *Su(fu)*$^-$ backgrounds. Results are presented in Table 3 and Figure 6.

In a *Su(fu)*$^+$/*Su(fu)*$^+$ background, all transgenic alleles, except *HS*, gave a fused mutant phenotype when tested against endogenous *fu^1* (class I) and *fu^A* (class II) alleles. Two of these transgenic alleles, *K33R* and *BS*, which belong to class I, enhanced both *fu^1* and *fu^A* mutant phenotypes, giving a very strong phenotype when combined with the *fu^A* allele: *fu^A;K33R* flies have mutant wings with a nearly total loss of LV4 vein (fu^S phenotype, Table 3 and Figure 6H) whereas *fu^A;BS* flies are late pupal lethal and display an extreme mutant wing phenotype with total loss of LV4 vein and a fusion of the proximal parts of LV2 and LV3 veins (fu^{Ext} phenotype, Table 3 and Figure 6J); this last characteristic corresponds to an extension of the mutant phenotype in the anterior compartment and was never observed for a *fu* mutant. This enhancement of the fused phenotype is only observed with those two class I transgenic alleles; it must be noted that *BS* is an unusual allele, completely lacking the kinase domain and only containing the C-terminal part of the second domain.

In *Su(fu)*$^-$/*Su(fu)*$^+$ and *Su(fu)*$^-$/*Su(fu)*$^-$ backgrounds, no dosage effect could be observed between class I and class II alleles, one dose of class I allele being sufficient to rescue the lethality of up to three class II doses (Table 3). In these contexts, the *BS* allele behaves as a normal class I allele. We have shown that the distinction between class I and class II alleles only depends on the integrity of the C-terminal domain. Thus, in a *Su(fu)*$^-$ background, one dose of intact C-terminal domain is able to reverse the deleterious effect of up to three doses of Fu protein with an altered C-terminal.

One revertant of the class II *fu^{RX2}* allele, named *fu^{RX2rev}*, was fortuitously obtained while maintaining the *FM3/fu^{RX2} car* strain and a homozygous *fu^{RX2rev}* strain was established. This revertant recovered both a zygotic and maternal fu$^+$ phenotype as *fu^{RX2rev}* flies bear perfectly wild-type wings and *fu^{RX2rev}* females are fully fertile. Nevertheless it has the class II sensitivity to *Su(fu)* as the *fu^{RX2rev};Su(fu)*$^-$/*Su(fu)*$^+$ flies still present a lack of viability and a cos2-like phenotype, although this *Su(fu)* sensitivity is somewhat weaker than that of the *fu^{RX2}* allele. This revertant was combined with different *fu* alleles in various *Su(fu)* backgrounds. The results are presented in Table 4. In a *Su(fu)*$^+$/*Su(fu)*$^+$ background, some genetic combinations reveal that the *fu^{RX2rev}* revertant is not equivalent to a wild-type *fu*$^+$ allele as a fu$^-$ phenotype reappears when a transgenic class I allele is added. This effect of class I alleles on the *fu^{RX2rev}* phenotype (Table 4) looks very like their effect on the enhancement of the *fu^1* and *fu^A* phenotypes (Table 3). It is also reminiscent of the effect of increasing doses of the *Su(fu)*$^+$ allele on the fu phenotype (PHAM *et al.* 1995 and Figure 6I).

In a *Su(fu)*$^+$/*Su(fu)*$^-$ background, the cos2-like phenotype of the *fu^{RX2rev}* revertant can be rescued if one dose of a class I allele is added, showing that the revertant presents the same recessive behavior as other class II alleles. Genetic and molecular analysis was undertaken to identify the nature of the reversion event. Recombination analysis shows that the reversion event is located at or very near the *fused* locus (data not

TABLE 4

***fu*^{RX2rev} revertant phenotypes in various genetic backgrounds**

rev, 0, I and II *fu* allele dosage	*fu* genotype[a]	Phenotype[b] *Su+/Su+* genotype	Phenotype[b] *Su−/Su+* genotype
rev/rev	*RX2rev/RX2rev*	[fu$^+$]	[cos-2]
rev/I	*RX2rev/fu^1*	[fu$^+$]	[fu$^+$]
rev/II	*RX2rev/fu^A*	[fu$^+$]	[cos-2]
rev/0	*RX2rev/fu^{Z4}*	[fu$^+$]	[cos-2]
rev/0/I	*RX2rev/fu^{Z4}; BS/+*	[fu$^+$]	[fu$^+$]
rev/I/I	*RX2rev/fu^1; BS/+*	[fu^M]	[fu^W]
	RX2rev/fu^1; K33R/+	[fu^W]	ND
	RX2rev/fu^1; G13V/+	[fu$^{+/-}$]	ND
rev/I/II	*RX2rev/fu^1; HP/+*	[fu$^+$]	ND
	RX2rev/fu^1; Pst9/+	[fu$^+$]	ND
	RX2rev/fu^1; Pst5/+	[fu$^+$]	ND
rev/II/I	*RX2rev/fu^{RX2}; BS/+*	[fu$^+$]	ND
	RX2rev/fu^{M1}; BS/+	[fu$^+$]	ND

[a] *fu^1* and *fu^{Z4}* are, respectively, class I and class 0 endogenous *fu* allele; *fu^A*, *fu^{RX2}* and *fu^{M1}* are class II endogenous *fu* alleles. *BS*, *K33R* and *G13V* are class I transgenic *fu* alleles, *HP*, *Pst9* and *Pst5* are expected class II transgenic *fu* alleles.

[b] The phenotypic symbols are the same as in Table 3 and Figure 6. ND, not determined.

shown) and the entire sequencing of the *fu^{RX2rev}* revertant and *fu^{RX2}* original mutant alleles was done (MATERIALS AND METHODS). The only difference between both alleles lies in the *fu* C-terminal domain where a G to T substitution leads to the replacement of a valine by a phenyl alanine at position 670, in a 16 amino-acid stretch totally conserved between *Drosophila melanogaster* and *D. virilis* Fu proteins (BLANCHET-TOURNIER *et al.* 1995). To be confirmed, this result would need the construction of the corresponding transgenic allele, bearing the substitution event associated with the original *fu^{RX2}* deletion. If it is, it would permit us to distinguish two subdomains in the C-terminal domain, one implicated in the kinase activity (that affected by the reversion event) and one involved in the generation of the ectopic cos-2 phenotype (that affected by the deletion).

DISCUSSION

The Fused protein has two recognizable regions: a typical kinase domain and an extracatalytic domain showing no significant homology with any known protein (PRÉAT *et al.* 1990; THÉROND *et al.* 1993). In this study, we performed genetic and molecular analysis on both endogenous and *in vitro* induced *fu* mutant alleles to further characterize the function of Fused and to assess the role of the different domains.

The Fused protein functions *in vivo* as a kinase and two separate domains are required for this activity: Sequencing of endogenous *fu* mutant alleles and genetic analysis of transgenic flies bearing *fu* mutations generated *in vitro* reveal that two domains are required for Fused activity. The first is the catalytic domain itself located at the N-terminal part of the protein and extending from amino acid 1 to amino acid 268. The second domain is located toward the C terminus of the protein, from amino acid 503 to the last amino acid (aa 805). Between these two domains is a region apparently dispensable for Fu activity that extends at least over 81 residues, from aa 422 to aa 502. Indeed, these residues are deleted in the protein encoded by the *HS* allele that confers a perfectly wild-type phenotype. It is noteworthy that this part of the Fu protein belongs to an hydrophilic region extending from amino acid 410 to amino acid 530 (THÉROND *et al.* 1993). This central region may have no specific function but could serve as a hinge between the N-terminal catalytic and C-terminal domains. Sequence comparison of the *fu* genes from *D. melanogaster* and *D. virilis* (two species whose estimated divergence time is 60 million yr) reveals a high degree of conservation both in the kinase region (88% identity from aa 1 to aa 268) and in the C-terminal region (73% identity from aa 494 to aa 805), emphasizing their functional importance. The central part of the protein is less conserved, with 57% identity (aa 422 to aa 502) (BLANCHET-TOURNIER *et al.* 1995).

We have shown that *in vitro* mutations affecting any of the two totally invariant residues implicated in the activity of all kinases (the glycine G13 of the GXG XXG motif involved in ATP binding and the lysine K33 involved in phospho-transfer) lead to a fused mutant phenotype when reintroduced into *fu$^-$* flies. We are confident that these transgenes are actually expressed

as they belong to class I *fu* alleles dominant over class II ones in a *Su(fu)*$^-$ background. Thus, this result provides a strong indication that Fused effectively functions *in vivo* as a kinase.

The C-terminal part of the extracatalytic domain is absolutely required for Fused activity but its precise role remains to be determined. It could activate the kinase domain either directly or by binding regulatory proteins that would modulate Fused activity. Modeling of the Fused structure, based upon crystallographic studies of the mouse cAMP-dependant kinase (PKA), reveals the presence of a putative amphipathic helix in the C-terminal part of the protein, from aa 756 to aa 771 (P. ALZARI and M. VÉRON, personal communication). This helical structure, which is recognized in many other kinases, is supposed to anchor in a hydrophobic pocket lying on the core opposite to the active site and is suspected to be important for the stability of the protein and (or) as a docking site for different effectors (KNIGHTON *et al.* 1991a; VÉRON *et al.* 1993, 1994). The C-terminal domain could also be involved in the correct subcellular localization of the protein, allowing the targeting of Fused toward its substrate(s). Indeed, several recent studies have emphazised the role of the regulatory subunits of different protein kinases and phosphatases for their differential subcellular localization (SCOTT and CARR 1992; HUBBARD and COHEN 1993; VAN AELST *et al.* 1993; VOJTEK *et al.* 1993; ZHANG *et al.* 1993).

The lack of Su(fu) activity reveals new properties of the different Fu domains: The Fu kinase is known to be required for the transduction of the Hedgehog signal, which leads to the transcription of *wg* in the most posterior cells of the embryonic parasegment (LIMBOURG-BOUCHON *et al.* 1991; FORBES *et al.* 1993). However, we have previously shown that the Fu activity is dispensable when the *Su(fu)* gene is inactivated, as *Su(fu)*$^-$ mutations completely suppress the phenotype of *fu* deficiencies (class 0 alleles). This observation suggests that *fu* and *Su(fu)* have antagonistic roles in the pathway. Furthermore, the *Su(fu)*$^-$ background allowed us to reveal some unexpected properties of *fu* alleles, previously classified as class I and class II alleles (PRÉAT *et al.* 1993).

In a *Su(fu)*$^-$ background, class II *fu* alleles display an embryonic and adult cos-2 phenotype. In the embryo, it is characterized by an extension of the *wg* transcription domain, which could result from the inactivation of the Cos-2 product itself. Molecular analysis demonstrates that all class II alleles have alterations in the extracatalytic domain. Among these alleles, fu^{M1} encodes the shortest FuII product, which contains the first 80 amino acids of the normal Fu protein (fu^{ST1} and fu^{ST3} *in vitro* alleles, which are likely to encode smaller products behave as class 0 ones). This short Fu^{M1} protein is sufficient to induce the cos-2 phenotype in the absence of Su(fu). Interestingly, this part of Fu corresponds precisely to the so-called small lobe of the kinase catalytic domain (KNIGHTON *et al.* 1991a), primary involved in the binding of the ATP nucleotide (KNIGHTON *et al.* 1991b; TAYLOR *et al.* 1992). In addition, a role in binding regulatory proteins has also been recognized for this small lobe in other kinases (HATAKEYAMA *et al.* 1991; DE BONDT *et al.* 1993; WELCH and WANG 1993). We propose that this structure is able to bind and thus sequester or inactivate the Cos-2 product.

Class I *fu* alleles as class 0 ones are completely suppressed by *Su(fu)*$^-$ mutations. All *fuI* alleles studied so far bear alterations in the kinase domain suggesting that one possible function of Fu could be to phosphorylate Su(fu) to inactivate it. The products encoded by most *fuI* alleles (and by fu^+) contain the small lobe, but do not induce the cos-2 phenotype in a *Su(fu)* mutant background. In addition, any class I allele is able to fully rescue the cos-2 phenotype presented by any *fuII;Su(fu)*$^-$ genotype. We thus propose that the C-terminal domain extending from aa 503 to aa 805, which is present in all FuI proteins and totally or partially altered in all FuII ones, is able to counterbalance the effect exerted by the small lobe of the kinase. This regulation can be perfomed through intra- but also intermolecular interactions, as *fuI* alleles suppress the cos-2 phenotype in *fuI/fuII;Su(fu)*$^-$ transheterozygotes. This dominance, observed even when one *fuI* allele is confronted with three doses of *fuII*, is unlikely to result from a direct equimolecular interaction between the kinase and the C-terminal domain. It probably corresponds to an indirect interaction via one or several other product(s) and/or to the formation of complexes involving several Fu molecules.

The Fu extracatalytic C-terminal domain could differentially regulate the Fu catalytic activity according to the cell position in the parasegment: Our results reveal complex relationships between the Fu, Su(fu) and Cos-2 products. In a previous paper (PRÉAT *et al.* 1993), we reported that *cos-2* mutations behave as *Su(fu)*$^-$ mutations toward class I and class II *fu* alleles. In *cos-2;fuI* double mutants, the fu phenotype is suppressed, and these flies display the phenotype of the *cos-2* allele, whereas in *cos-2;fuII* double mutants, the fu phenotype is also suppressed, but the cos-2 phenotype is strongly enhanced. These effects are observed both for the maternally determined embryonic phenotypes and for the zygotic phenotypes. In addition, *Su(fu)* mutations enhance the cos-2 mutant phenotype. These observations show that Cos-2 and Su(fu) act in the same way, antagonistically to Fu.

We propose that in posterior parasegmental cells, the Fused kinase would be activated in response to the Hh signal. This process requires the integrity of the C-terminal part of the protein and must occur via intramolecular interactions, as pairs of mutant alleles altered in the catalytic and the extracatalytic domains cannot complement. Fused kinase activity would inhibit both Cos-2 and Su(fu) activities (via phosphorylation of these pro-

teins?), allowing *wg* transcription. In this hypothesis, the fu$^-$ phenotype would result from ectopic Cos-2 activity in posterior parasegmental cells. Expression of Cos-2 in these cells would require the Su(fu) activity, accounting for the suppression of the fu mutant phenotype by *Su(fu)* mutations.

On the contrary, in cells that do not receive the Hh signal, the Fu C-terminal domain would prevent the catalytic domain from binding to Cos-2, thus allowing Cos-2 activation and *wg* repression. This regulation could be achieved via intermolecular interactions, as previously discussed. The above hypothesis accounts for the cos-2 phenotype observed when both Su(fu) and the C-terminal domain are absent. It accounts also for the observation that an extra-dose of the C-terminal domain enhances the fu phenotype of all *fu* mutant alleles (including those with altered kinase activity) and reveals the fu phenotype of the fu^{RX2rev} allele. In fact, it predicts that an extra C-terminal domain could increase the release of the Cos-2 product from the catalytic domain in posterior parasegmental cells, providing more active Cos-2 and thus increasing *wg* repression.

Taken together, these different results support the hypothesis that the C-terminal domain of the Fu protein plays two alternative roles: activation of the Fu kinase in cells receiving the Hh signal and inhibition of this activity in other cells. Whether these two functions depend upon the same part of the C-terminal domain remains to be determined. This question will be approached by screening for second site mutations (such as fu^{RX2rev}) that separate these two functions. However, differential activation of Fu clearly involves unidentified transregulators. Such factors could be identified by a genetic screen for new *fu* suppressors or by screening for proteins that interact with Fu and Su(fu) using the yeast two hybrid system (FIELDS and SONG 1989).

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