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

Pascal Thérond,1 Georges Alves, Bernadette Limbourg-Bouchon,2 Hervé Tricoire,3 Elizabeth Guillemet,2 Jeanine Brissard-Zahraoui, Claudie Lamour-Isnard and Denise Busson

Institut Jacques Monod, C.N.R.S., Université Paris VII, 75251 Paris Cedex 05, France

Manuscript received April 7, 1995
Accepted for publication January 3, 1996

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, *fu* 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 anterior-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 Nuss 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-white 3* (*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)* mutation and numerous

Corresponding author: Denise Busson, Laboratoire de Génétique du Développement et Evolution, Institut Jacques Monod, 2 Place Jussieu, 75251 Paris Cedex 05, France. E-mail: dbusson@ccr.jussieu.fr

1 Present address: G. W. Hooper Foundation, HSW 1542, University of California, San Francisco, CA 94143.
2 Present address: Centre de Génétique Moléculaire, C.N.R.S., F-91198 Gif-sur-Yvette Cedex, France.
3 Present address: Institut de Physique Nucléaire, Université Paris XI, BP No. 1, 91406 Orsay Cedex, France.
fu alleles has revealed an unexpected complexity of the fused gene (PREAT et al. 1993). Class 0 (fu0) and class I (fuI) fu mutants are completely suppressed for all their mutant phenotypes by Su(fu)12, for example, fuI, Su(fu)12 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)12, 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, PREAT 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 fuI/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 (THEROND 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 (PREAT 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 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 (PREAT 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 BUSON et al. (1988) and PREAT et al. (1993). Su(fu)12 is an EMS-induced mutation previously described that behaves as an amorphic mutation and gives viable flies with a karmoisin phenotype (PREAT 1992). This allele corresponds to a 1.5-kb deficiency affecting the Su(fu) and karmoisin transcription units (PATH et al. 1995). Endogenous fu mutations have been previously classified into three classes on the basis of their interaction with Su(fu)12: the fu1, fu2, fu6 and fu21 alleles belong to class I, the fu12, fu13, fu4 and fu19 alleles to class II and the fu22 allele to class 0 (PREAT et al. 1993 and Table 2). New fu mutations induced on a fu+ transgene were maintained as w; P(w+fu+)/ P(w+fu+) 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°C 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 FUS-5a insertion carrying the FU-S insert and the FU-L29 insertion carrying the FU-L insert (THEROND et al. 1993). The FUS (a 5.025-kb BglII-KpnI genomic DNA fragment) and FU-L (a 7.4-kb SplII-KpnI genomic DNA fragment) inserts, containing, respectively, 817 and 3117 bp DNA upstream of the transcription start site, include all the fused transcribed unit and are presented in Figures 1 and 2. wfu+/wfu+/ P(w+fu+)/ GyO+/+ females were crossed with w+fu+/ Y; GyO/+ P(Δ2-3 ry') Sb/TM6 males carrying the P(Δ2-3 ry') insertion as a source of transposase. Transposition and perfect and imperfect excision events occur in the germ line of wfu+/ Y; P(w+fu+)/ GyO/P(Δ2-3 ry')/+ F1 males (DANIERS et al. 1985). These F1 males were crossed with F3 H4 wfu females. Imperfect excision events specifically altering the fused gene were selected by screening wfu+/ P(w+fu+)/ F2 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 allele (see Table 1). To recover independent events, the dysgenic cross was repeated 100-120 times in separate vials and only one w+fu− F2 individual per vial was kept for subsequent crosses. Twenty independent mutant lines were obtained from the FUS 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 FUS insertion were analyzed by Southern blot analysis using KpnI and Sall digestions and the 5.025-kb BglII-KpnI fragment as a probe. The mutant strains obtained from the FU-L insertion were analyzed using BamHI, BglII and EcoRI digestions and the 7.4-kb SplII-KpnI fragment as a probe. The fu22 mutant strain was also analyzed using PstI, Pml and KpnI digestions and the 1.4-kb BamHI-Sall fragment as a probe (Figures 1 and 2). The genomic DNA of the wfu+: P(w+fu+2) strain was amplified in two different polymerase chain reaction (PCR) experiments (SAKI et al. 1985), the products of the amplification
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 BamHI and KpnI, giving the pB-FU-S recombinant vector. The restriction map of the FU-S insert is given in Figure 1. The PstI, PsfI and PstII alleles were obtained by partial PstI digestion of the pB-FU-S vector; the vector-containing portions were gel purified and allowed to reccircularize and the resulting deleted vectors, pB-PstS, pB-PsfS and pB-PstII, selected by subsequent restriction map analysis. The HS allele was obtained by complete digestion with HindIII and Smal of the pB-FU-S vector, filling in the 3' HindIII ends with Klenow enzyme and recircularization. The BS allele was obtained by complete digestion with Smal and partial digestion with BamHI, filling in the BamHI site and recircularization. The HP allele was obtained by complete digestion of the HindIII site (genomic position 2235) and digestion of one of the four PstI 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' HindIII ends and a degradation of the 5' protruding PstI 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 XbaI (vector site) and partial digestion with KpnI and introduced into the pW6 transformation vector (KLEEMAN 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 BglII-KpnI insert (coordinates 1-5025), the BamHI/BglII-SalI fragment (coordinates 1-2281) was isolated and subcloned into pEMBL18 vector. From this intermediate recombinant vector, two smaller fragments, the BamHI-KpnI (coordinates 940-1432) and the Sad-KpnI (coordinates 1-1432, Sad 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 Mutagene M15 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 G13Val allele with 5'-CCCGAAGGAGACCTTGGCCCACC3'; the K33E allele with 5'-GGAGATCATTCGATGGCCACCAC3'; the K33R allele with 5'-GGAGATCATCGATGGCCACCAC3'; the ST1 allele with 5'-CTTGGATTGCATCGATGGCCACCAC3' and the ST3 allele with 5'-GGCGATGCTTCACCGGCCGCTG3' 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 BamHI/BglII-SalI 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 XbaI (vector site) and partial digestion with KpnI 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 XbaI and Xhol (coordinate 2785) and with Xhol and KpnI; the desired fragments were gel purified and used for a triple ligation with the pW6 vector digested with XbaI and KpnI. 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 w118 host line, with the pUC(hs-A2-3) 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 (THEROND et al. 1993).

Molecular analysis of endogenous fu mutations: The endogenous fu mutations were localized by Southern analysis using acrylamide gels as described by PETERS (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-HC1 (pH 8.3) and 2 mM MgCl2, Taq polymerase was from Perkin Elmer Cetus. Amplification conditions were as follows: 1 cycle, 2 min 96°, 45 sec hybridization °, 60 sec 74°/35 cycles, 30 sec 92°,
FIGURE 2.—Molecular analysis of the fu mutations induced on a transgenic fu' gene. B, BamHI; Bg, BglII; E, EcoRI; K, KpnI; S, SstI; Sph, SphI. 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 transcription unit (THEROND et al. 1993). V, 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 hybridization time, 60 sec 74°/10 min 74°. For fu62, fuMC10, fuMT, fuMK and fuRS alleles, the PCR products were cloned into the pBluescript vector and the fragments sequenced. In all cases, except fuMK, two independent clones were sequenced; the fuMK alteration identified was also detected by a HadII site polymorphism at genomic position 1413. For fuMC10, fuMK, fuMT and fuRS, the PCR products were isolated and directly sequenced using the Pharmacia sequencing kit with T7 polymerase. For fuMC2, fuMT and fuRS, 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 fu62, fuMC10 and fuMT allele sequences were previously given in PréAT et al. (1993). The fused genomic regions of both the fuRS revertant strain and the fuRS 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 fuRS mutant (new stop at coordinate 3631, see Table 2 and Figure 3A). Numerous variations due to natural polymorphism were recognized in both fuRS and fuRS genomic DNAs. Only one substitution was observed at position 3043, leading to the replacement of a G in the fuRS sequence by a T in the fuRS sequence and thus to the replacement of a valine by a phenylalanine at position 670.
RT-PCR analysis of fu

transcripts: fu

and fu

females were collected and frozen at −70°C. Total RNA extractions were performed by the hot phenol method (PALMITER 1974). DNase I treatment was performed during 30 min at 37°C 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°C 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°C and followed by a programed step at 55°C. AMV RTase (2.5 units; Pharmacia) was then added and the tube left for 20 min at 55°C. After addition of 2.5 units of Taq polymerase (Perkin Elmer Cetus), the amplification reaction was performed by 30 cycles of 94°C, 55°C, and 72°C, 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 30 mM Hepes pH 7.6, 50 mM KCl, 1 mM EGTA, 1 mM EDTA, and 0.85% NP40 supplemented with a mix of protease inhibitors (10 μM benzamidine, 1 μg/ml phenanthroline, 100 μM Pefablock, and 10 μg/ml each of aproitin, leupeptin, pepstatin A). The embryos were immediately homogenized at 4°C 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°C for 5 min in gel loading buffer. Protein extracts were separated on SDS, 6% acrylamide gels and blotted onto nitrocellulose (Schleicher 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 amplified fragments were cloned and sequenced.

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 (PRAET et al. 1993). Class I fu mutants are totally suppressed by the amorphic Su(fu)0P mutation for both their embryonic and adult phenotypes. Class II fu mutants, although suppressed, display an additional phenotype in interaction with Su(fu)0P, 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)0P but the two class 0 mutants identified so far [Df(l)fup1 and Df(l)fup2] 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(l)fup1/Df(l)fup1; Su(fu)0P/Su(fu)0P germinal clone progeny (PRAET et al. 1993). In a Su(fu)0 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+)/ + flies. Their class was determined by testing their ability to rescue the zygotic lethality due to the full Su(fu) interaction, in wfuU/Y;P(w’ fu+)/ +; Su(fu)0P/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 full U/Y; Su(fu)0P/+; + flies, whereas it would be of class 0 or class II if not able to correct the full U/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+)/ +; Su(fu)0P/Su(fu)+ flies that would have been expected
we recovered 12 class alleles were therefore generated from different reference strains. An X-ray mutagenesis performed on a wild-type Oregon R strain (OreR-6), an allele was of class I alleles were obtained from different laboratories and far studied, seven belong to class I and 24 to class II. Among the 31 viable transgenic lines containing a P[w+fu+] gene: several lines with a w+fu- phenotype were obtained and tested both for the type of partial excisions affecting only the fu gene and, for some of them, adjacent genomic sequences. The new transgenic class I allele, fuX37, corresponds to the smallest deletion (700 bp); it lacks the BamHI and KpnI sites located in the kinase domain but keeps the PvuII site at the end of this domain (Figure 1). As the BamHI 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 fuX37 and its interpretation with respect to the class of the allele are presented in Table 2 and Figure 3B.

Table 1: Determination of the class of the transgenic fu mutations

<table>
<thead>
<tr>
<th>Allele</th>
<th>fu I or II fu O Su(fu) Y + Su(fu) Y</th>
<th>fu II fu O Su(fu) Y + Su(fu) Y</th>
<th>Deduced class</th>
</tr>
</thead>
<tbody>
<tr>
<td>G13V</td>
<td>[fu -]</td>
<td>[fu*<del>/</del>]</td>
<td>class I</td>
</tr>
<tr>
<td>K33E</td>
<td>[fu -]</td>
<td>[fu*<del>/</del>]</td>
<td>class I</td>
</tr>
<tr>
<td>K33R</td>
<td>[pupal lethal]</td>
<td>[fu*<del>/</del>]</td>
<td>class I</td>
</tr>
<tr>
<td>BS</td>
<td>[strong fu wing phenotype]</td>
<td>[fu*<del>/</del>]</td>
<td>class I</td>
</tr>
<tr>
<td>ST1</td>
<td>[fu -]</td>
<td>[cos-2-like]</td>
<td>class 0 or class II</td>
</tr>
<tr>
<td>ST3</td>
<td>[fu -]</td>
<td>[cos-2-like]</td>
<td>class 0 or class II</td>
</tr>
<tr>
<td>HP</td>
<td>[fu -]</td>
<td>[cos-2-like]</td>
<td>class 0 or class II</td>
</tr>
<tr>
<td>Pst9</td>
<td>[fu -]</td>
<td>[cos-2-like]</td>
<td>class 0 or class II</td>
</tr>
<tr>
<td>Pst11</td>
<td>[fu -]</td>
<td>[cos-2-like]</td>
<td>class 0 or class II</td>
</tr>
<tr>
<td>Hs</td>
<td>[fu*]</td>
<td>[fu*]</td>
<td>class +</td>
</tr>
</tbody>
</table>

*4 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 6I). 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.

*5 The dominance/recessivity relationship between endogenous fu alleles in Su(fu)- context is as follows: fu0 or fu1; Su(fu)+, [fu1] (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).

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 (Théron 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, fuX37, 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') 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 fusel gene and, for some of them, adjacent genomic sequences. The new transgenic class I allele, fuX37, corresponds to the smallest deletion (700 bp); it lacks the BamHI and KpnI sites located in the kinase domain but keeps the PvuII site at the end of this domain (Figure 1). As the BamHI 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 fuX37 and its interpretation with respect to the class of the allele are presented in Table 2 and Figure 3B.
### Molecular alterations associated with fused mutations

<table>
<thead>
<tr>
<th>Allele</th>
<th>Mutagen</th>
<th>Genomic position</th>
<th>Nucleotide alteration</th>
<th>Amino acid alteration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous fu mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class I</td>
<td>62</td>
<td>γ-rays</td>
<td>1389–1397</td>
<td>Δ CGCGAAACT</td>
</tr>
<tr>
<td></td>
<td>mH63</td>
<td>EMS</td>
<td>1414</td>
<td>G to A</td>
</tr>
<tr>
<td></td>
<td>jB3</td>
<td>DEB</td>
<td>1002–1041</td>
<td>Δ AGTG... to AAAG</td>
</tr>
<tr>
<td>Class II</td>
<td>M1</td>
<td>DEB</td>
<td>1216–1243</td>
<td>Δ GAGT... to CGCT</td>
</tr>
<tr>
<td></td>
<td>MC2</td>
<td>DEB</td>
<td>1450–1503</td>
<td>Δ TCGA... to GCCA + 1G</td>
</tr>
<tr>
<td></td>
<td>C10</td>
<td>DEB</td>
<td>2153–2185</td>
<td>Δ TGGC... to ATAG + GCCG</td>
</tr>
<tr>
<td></td>
<td>G3</td>
<td>DEB</td>
<td>219–2210</td>
<td>Δ GAAC... to GCCG</td>
</tr>
<tr>
<td></td>
<td>RX15</td>
<td>X-rays</td>
<td>2598–2999</td>
<td>CA to TTTCGAST</td>
</tr>
<tr>
<td></td>
<td>W3</td>
<td>DEB</td>
<td>2872–2897</td>
<td>Δ CTCT... to CCCC</td>
</tr>
<tr>
<td></td>
<td>RX16</td>
<td>X-rays</td>
<td>2951–2962</td>
<td>Δ TGGAGGAGCTG + 1C</td>
</tr>
<tr>
<td></td>
<td>RX2</td>
<td>X-rays</td>
<td>3347–3362</td>
<td>Δ TCGACTGCTGCGCTG</td>
</tr>
<tr>
<td>Transgenic fu mutations</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class I</td>
<td>G13V</td>
<td>M13</td>
<td>941–942</td>
<td>GGA to GTC</td>
</tr>
<tr>
<td></td>
<td>K33E</td>
<td>M13</td>
<td>1000</td>
<td>AAA to GAA</td>
</tr>
<tr>
<td></td>
<td>K33R</td>
<td>M13</td>
<td>1001–1002</td>
<td>AAA to AGG</td>
</tr>
<tr>
<td></td>
<td>BS</td>
<td>DEL</td>
<td>945–2540</td>
<td>Δ CTGC... to TGCC</td>
</tr>
<tr>
<td></td>
<td>XS37</td>
<td>PM</td>
<td>879–1533</td>
<td>Δ ATCC... to ACTG + GTGTGATA</td>
</tr>
<tr>
<td>Class II or 0</td>
<td>ST1</td>
<td>M13</td>
<td>1000–1002</td>
<td>AAAGTTG to GA.GTGA</td>
</tr>
<tr>
<td></td>
<td>ST3</td>
<td>M13</td>
<td>1147</td>
<td>GGGTTG to GGG.TGA</td>
</tr>
<tr>
<td></td>
<td>HP</td>
<td>DEL</td>
<td>2238–3011</td>
<td>Δ TACG... to TGCA</td>
</tr>
<tr>
<td></td>
<td>Ps19</td>
<td>DEL</td>
<td>3012–3313</td>
<td>Δ GCCG... to TGCA</td>
</tr>
<tr>
<td></td>
<td>Ps11</td>
<td>DEL</td>
<td>3012–3652</td>
<td>Δ GCCG... to TGCA</td>
</tr>
<tr>
<td></td>
<td>Ps5</td>
<td>DEL</td>
<td>3314–3652</td>
<td>Δ GCCG... to TGCA</td>
</tr>
<tr>
<td>Class + HS</td>
<td>DEL</td>
<td>2238–2540</td>
<td>Δ TAGT... to TGCC</td>
<td>SER422 to PRO502</td>
</tr>
</tbody>
</table>

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

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.

ALAL39 to LEU141, deleted amino acids; the internal limits of the deleted fragment are given. ALA147 to THR, nature of the amino acid substitution.

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 fuX37 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 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<sup>62</sup> and fu<sup>116</sup> and one class II: fu<sup>Mt</sup>) presented previously (Praet 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<sup>B2</sup>) and seven of class II (fu<sup>W2</sup>, fu<sup>10</sup>, fu<sup>Y3</sup>, fu<sup>Y155</sup>, fu<sup>W3</sup>, fu<sup>Y160</sup> and fu<sup>Y22</sup>); the mutations were localized by precise Southern analysis as previously described (Praet 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<sup>62</sup>, corresponds to a 9-bp deletion (Praet 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 kinase domain 5' part of the first intron and the 5' part of the first intron and is implicated in ATP binding (Hanks et al. 1988). The class I allele, fu<sup>116</sup>, is a single base substitution and results in a change of the nonpolar Ala147 to a polar Thr (Praet 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 serinethreonine kinases.

The nature of the product encoded by the newly sequenced class I allele, fu<sup>B2</sup>, 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<sup>B2</sup> 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 AAGGTGTTG sequence (from position 985 to position 992) fits well with the C<sup>A</sup>/AGGT<sup>C</sup>/C<sup>C</sup>/T consensus sequence for intron 5' donor sites (Mount et al. 1992) and is very similar to the normal fu splicing site (AAGGTGTTG). 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<sup>M</sup>, fu<sup>Nc2</sup>), or in the second domain, the middle part for fu<sup>Y10</sup>, fu<sup>Y3</sup>, fu<sup>R155</sup> or the terminal part for fu<sup>W3</sup>, fu<sup>R160</sup>, fu<sup>Y22</sup>, 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<sup>M</sup> and fu<sup>NX2</sup>. The fu<sup>M</sup> 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<sup>NX2</sup> 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 absence 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<sup>NST7</sup>, 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<sup>NST7</sup> 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
The wild-type amino acid sequence, with the kinase domain in bold letters, is presented in the single letter code underneath. Putative AGGT 5' splicing consensus are given in bold letters (see text). For the sequence. Two possibilities are given for the JB3 allele, whether or not alternative splicing takes place. The normal AGGT and the coding sequence. The modified parts of the expected mutant proteins are written in italics under the wild-type protein. 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. This putative alternative ATG is preceded by a AAGC sequence, very close to the AACC sequence are in italics. The C, which replaces a G at the putative AGGT and putative AGGT S' splicing consensus are given in bold letters (see text). For the RX3 allele, F, S, and V represent the three additional amino acids encoded by the nine nucleotide insertion. (B) P-excision induced Drosophila transgenic allele. The fuX37 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 expected fuX37 protein sequence would begin at amino acid 194 and then extend normally until amino acid 805 (see text).

**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) (Therond 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. fu+ corresponds to deletions (A), fuX37 to deletions with 1 or more remaining nucleotides (A). fuXY37 to a single substitution (G->A). fuM15 to substitutions (A<->T). fuRX16 to deletions with 1 or more remaining nucleotides (A<->T). fuM20 to a single substitution (G->A). fuRX15 to the substitution of two nucleotides by nine others (CA = TTAGCTAG). 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 S' splicing consensus are given in bold letters (see text). For the RX3 allele, F, S, and V represent the three additional amino acids encoded by the nine nucleotide insertion. (B) P-excision induced fuX37 transgenic allele. The fuX37 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 expected fuX37 protein sequence would begin at amino acid 194 and then extend normally until amino acid 805 (see text).
The different domains of the wild-type Fused protein are as follows: the kinase domain (1–268) is in dark, the plain 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 G-terminal domain, the white box (422–503) 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 by an asterisk. Numbers indicate residues still present in deletion mutants or the affected residue in point mutants.

![Figure 4](image)

**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.

**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(wu fuXex), fu+;P(wu fuex), fu+;P(wu fuXex) and fu+;P(wu fuXex)/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-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-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, GI3V, 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(wu fuXex+)/+ 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
K33R fu transgene when associated with the class II fuO 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 new product. This 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 502). 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, GS, HS, HP, Pst9 in *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 RX:6 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 and are illustrated in Figure 5. 

<table>
<thead>
<tr>
<th>Class I fu allele</th>
<th>fu±; fu−</th>
<th>fuO; fuO</th>
<th>fuO; fuO</th>
</tr>
</thead>
<tbody>
<tr>
<td>fuO; fuO</td>
<td>fuO</td>
<td>fuO</td>
<td>fuO</td>
</tr>
<tr>
<td>fuO; fuO</td>
<td>fuO</td>
<td>fuO</td>
<td>fuO</td>
</tr>
<tr>
<td>fuO; fuO</td>
<td>fuO</td>
<td>fuO</td>
<td>fuO</td>
</tr>
</tbody>
</table>

Phenotypes are written in plain letters and are illustrated in Figure 6. fu+ wild type; fu−, fu partially suppressed; fuO, fu weak; fuO, fu medium; fuO, fu strong; fuO, fu extreme; cos-2, late pupal lethality with cos-2 like phenotype; ND, not determined.

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− fuO)/+ and in *wfuII/Y;P(w− fuO)/+; Su(fu)−/Su(fu)+ combination and do not affect the extracatalytic 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 502). 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, GS, HS, HP, Pst9 in *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 RX:6 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− fuO)/+ and in *wfuII/Y;P(w− fuO)/+; Su(fu)−/Su(fu)+ combination (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− fuO)/+ combination and do not rescue the lethality of the *fuO/Su(fu)−* interaction in a *wfuII/Y;P(w− fuO)/+;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 STI and ST3 compared to the class II endogenous fuO allele and to the class 0 *Df(1)fuO* 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)fuO*/*Su(fu)−/Su(fu)+* and *Su(fu)−/Su(fu)+* germline clones and 10 females with *Df(1)fuO*/*Df(1)fuO*/*Su(fu)−/Su(fu)+* germline clones. The former laid wild-type suppressed eggs able to hatch as expected from a fuO;fuO;Su(fu)− germ line. The latter laid eggs unable to hatch as expected from a fuO;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.
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/) genotype leads to a fu phenotype (Figure 6J) while the wfu/Y;P(w 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 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 fu phenotype, in the absence of the C-terminal domain.

Dosage relationship between class I and class II fu

Figure 6.—Wing phenotypes of different fu and 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⁺⁺], fu weak; [fu⁻⁺⁺⁺], fu medium; [fu⁻⁻⁻⁻], fu strong; [fu⁻⁻⁻⁻⁻⁻], fu extreme phenotype; [cos⁻⁻⁻⁻⁻⁻], cos-2-like phenotype. (A and B) fu⁻⁻⁻⁻ phenotype of (A) wild-type strain. (B) [fu⁻⁻⁻⁻] genotype. The transgenic [fu⁻⁻⁻⁻] allele confers a perfect fu⁻⁻⁻ phenotype to all endogenous fu alleles tested. (C–E and I) Phenotypes of the class I [fu⁻⁻⁻] 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⁺⁺], (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⁻⁻⁻⁻⁻⁻], (H) [fu⁺⁺⁺⁺⁺] and (J) [fu⁺⁺⁺⁺⁺] 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⁻⁻⁻⁻⁻⁻] 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.
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 fuweak (fuW) type (fuI allele phenotype), a fumedium (fuM) type (fuI allele phenotype) and a fustrong (fuI) type displayed by pupal lethal alleles as fumh63 (Figure 6, E–G) without obvious correlation with the class of the allele (fuI and fumh63 are class I alleles, fuI is a class II allele). Like the embryonic phenotype in which pattern elements are deleted and affected in fu mutants seems to extend on both sides of the anterior-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). The fu wing phenotype can be classified into three groups depending on the extent of the apparent LV3 and LV4 wing 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 anterior-posterior boundary.

In a Su(fu)+/Su(fu)+ background, the cos2-like 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 fuI allele, named fuI92, was fortuitously obtained while maintaining the FM3/fuI2 car strain and a homozygous fuI92 strain was established. This revertant recovered both a zygotic and maternal fuI allele phenotype as fuI92 flies bear perfectly wild-type wings and fuI92 females are fully fertile. Nevertheless it has the class II sensitivity to Su(fu) as the fuI92/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 fuI 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 fuI92 revertant is not equivalent to a wild-type fuI allele as a fuI allele reappears when a transgenic class I allele is added. This effect of class I alleles on the fuI92 phenotype (Table 4) looks very like their effect on the enhancement of the fuI and fuI alleles (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 6J).

In a Su(fu)+/Su(fu)+ background, the cos2-like phenotype of the fuI92 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

fuRX2w revertant phenotypes in various genetic backgrounds

<table>
<thead>
<tr>
<th>rev, 0, I and II fu allele dosage</th>
<th>fu genotype(^a)</th>
<th>Su+/Su+ genotype</th>
<th>Su−/Su+ genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>rev/rev</td>
<td>RX2rev/RX2rev</td>
<td>[fu(^+)]</td>
<td>[cos-2]</td>
</tr>
<tr>
<td>rev/I</td>
<td>RX2rev/fu(^i)</td>
<td>[fu(^+)]</td>
<td>[fu(^+)]</td>
</tr>
<tr>
<td>rev/II</td>
<td>RX2rev/fu(^A)</td>
<td>[fu(^+)]</td>
<td>[cos-2]</td>
</tr>
<tr>
<td>rev/0</td>
<td>RX2rev/fu(^{24})</td>
<td>[fu(^+)]</td>
<td>[cos-2]</td>
</tr>
<tr>
<td>rev/0/I</td>
<td>RX2rev/fu(^{24}); BS/+</td>
<td>[fu(^+)]</td>
<td>[fu(^+)]</td>
</tr>
<tr>
<td>rev/I/I</td>
<td>RX2rev/fu(^i); BS/+</td>
<td>[fu(^A)]</td>
<td>[fu(^N)]</td>
</tr>
<tr>
<td></td>
<td>RX2rev/fu(^{A}); K33R/+</td>
<td>[fu(^+)]</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>RX2rev/fu(^{A}); G13V/+</td>
<td>[fu(^−)]</td>
<td>ND</td>
</tr>
<tr>
<td>rev/I/II</td>
<td>RX2rev/fu(^i); HP/+</td>
<td>[fu(^+)]</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>RX2rev/fu(^{i}); Pst9/+</td>
<td>[fu(^+)]</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>RX2rev/fu(^{i}); Pst5/+</td>
<td>[fu(^+)]</td>
<td>ND</td>
</tr>
<tr>
<td>rev/II/II</td>
<td>RX2rev/fu(^{K2}); BS/+</td>
<td>[fu(^+)]</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>RX2rev/fu(^{M1}); BS/+</td>
<td>[fu(^+)]</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) fu\(^i\) and fu\(^{24}\) are, respectively, class I and class 0 endogenous fu allele; fu\(^A\), fu\(^{RX2}\) and fu\(^{M2}\) 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.

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

**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; Théron 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 vitro 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 a hydrophilic region extending from amino acid 410 to amino acid 530 (Théond 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-tourrier 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 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-dependent 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. VERON, 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; VERON 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 emphasized 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, fuM1 encodes the shortest FuII product, which contains the first 80 amino acids of the normal Fu protein (fuN1 and fuT1 in vitro alleles, which are likely to encode smaller products behave as class 0 ones). This short FuM1 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 BONDY 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 1 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 performed through intra- but also intermolecular interactions, as fuI alleles suppress the cos-2 phenotype in fuI/fuI;Su(fu)+ transheterozygotes. This dominance, observed even when one fu 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 signal, the Fu C-terminal domain would prevent the fu phenotype from ectopic 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 fuX2 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 fuX2) 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).

We thank J. Bache and P. Vandurka for excellent technical assistance. We are grateful to M. F. Blanchet-Tournier, A. Goldsborough and A. Plessis for critical reading of the manuscript and helpful discussions. We thank F. Chalvet and F. BiHL, students of the Molecular and Cellular Genetic DEA of Paris VI and Paris XI Universities, for their contribution to this work. This work was supported by grants No. 6770 from Association de la Recherche contre le Cancer, No. 910103 from Institut National pour la Sante et la Recherche Medicale and No. 92.C.0797 from Ministére de la Recherche et de l’Education.

LITERATURE CITED


Knighton, D. R., J. Zhang, I. F. T. Fick, V. A. Ashford, N. Xuong et al., 1991a Crystal structure of the catalytic subunit of cyclic

Functional Domains of Fused 1197


Communicating editor: T. Schupbach