Development 120, 569-578 (1994)
Printed in Great Britain © The Company of Biologists Limited 1994

The sevenless signalling cassette mediates *Drosophila* EGF receptor function during epidermal development

Fernando J. Diaz-Benjumea[2] and Ernst Hafen[1]

[1]Zoologisches Institut, Universität Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland
[2]EMBL, Meyerhoferstrasse 1, Postfach 1022.40, 69012 Heidelberg, Deutschland

SUMMARY

In *Drosophila*, Drk, an SH2 adaptor protein, Sos, a putative activator of Ras1, Ras1, raf and rolled/MAP kinase have been shown to be required for signalling from the sevenless and the torso receptor tyrosine kinase. From these studies, it was unclear whether these components act in a single linear pathway as suggested by the genetic analysis or whether different components serve to integrate different signals. We have analyzed the effects of removing each of these components during the development of the adult epidermal structures by generating clones of homozygous mutant cells in a heterozygous background. Mutations in each of these signalling components produce a very similar set of phenotypes. These phenotypes resemble those caused by loss-of-function mutations in the *Drosophila* EGF receptor homolog (DER). It appears that these components form a signalling cassette, which mediates all aspects of DER signalling but that is not required for other signalling processes during epidermal development.

Key words: *Drosophila*, *sevenless*, torso, EGF, DER, receptor, signal transduction, Drk, Sos, Ras1, raf, MAP kinase, wing development, vein formation

INTRODUCTION

Cellular interactions play an important role during growth and morphogenesis of multicellular organisms. Genetic and biochemical studies in different organisms have shown that a family of cell surface receptors with intrinsic tyrosine kinase activity mediates a wide variety of different responses to extracellular signals. The c-kit receptor tyrosine kinase that is encoded by the *White* locus of the mouse is required for the migration of germ cells and the differentiation of melanocytes (Chabot et al., 1988; Geissler et al., 1988). In *Caenorhabditis elegans*, the differentiation of the vulval precursor cells depends on the activation of the let-23 receptor tyrosine kinase (Aroian et al., 1990). In *Drosophila*, the torso receptor tyrosine kinase is required for the specification of terminal structures in the embryo (Klingler et al., 1988; Casanova and Struhl, 1989; Sprenger et al., 1989) and the sevenless receptor tyrosine kinase controls the specification of the R7 photoreceptor cell fate in the developing eye (Banerjee et al., 1987; Hafen et al., 1987; Basler and Hafen, 1988; Bowtell et al., 1988). In contrast to the single mutant phenotypes associated with *torso* and *sevenless*, mutations in the gene encoding the *Drosophila* homolog of the EGF receptor (DER), produce pleiotropic phenotypes: viable *DER* alleles prevent dorsoventral patterning of the follicle cells during oogenesis (Schüpbach, 1987). Strong loss-of-function alleles cause embryonic lethality (Price et al., 1989; Schejter and Shilo, 1989; Raz and Shilo, 1993). During postembryonic development, DER function is required for the differentiation of wing veins, the control of cell size and the development of photoreceptor cells (Diaz-Benjumea and Garcia-Bellido, 1990a; Clifford and Schüpbach, 1992; Xu and Rubin, 1993). The gain-of-function allele *Ellipse* (*Elp*) causes the formation of a reduced number of ommatidial units in the eye and additional veins in the wing (Baker and Rubin, 1989, 1992).

In spite of the plethora of developmental decisions controlled by receptor tyrosine kinases, genetic as well as biochemical characterization of components involved in signal transmission suggests that the different receptors utilize a common set of signal transduction components. The response of rat PC12 cells to treatment by phorbol esters or NGF is mediated by Ras and results in the activation of the c-Raf serine/threonine kinase and the mitogen-activated kinase (MAPK or ERK1) (Howe et al., 1992; Thomas et al., 1992; Wood et al., 1992). In *C. elegans*, the Sem-5 SH2 adaptor protein, let-60 Ras and lin-45 raf are required for signalling from the let-23 receptor during vulval development (Beitel et al., 1990; Han and Sternberg, 1990; Clark et al., 1992; Han et al., 1993). In *Drosophila*, genetic screens for dominant modifiers of either hypomorphic or gain-of-function *sevenless* mutations have indicated that Drk, an SH3-SH2-SH3 adaptor protein, Sos, a putative guanine-nucleotide releasing factor, and Ras1 are essential components of the sevenless signalling cascade (Rogge et al., 1991; Simon et al., 1991, 1993; Olivier et al., 1993). Mutations in *Drk*, *Sos* and *Ras1* also act as dominant modifiers of gain-of-function mutations of *torso* and *DER* (Simon et al., 1991; Doyle and Bishop, 1993). Mutations in *raf* block signalling from torso and sevenless (Ambrosio et al., 1989; Dickson et al., 1992). Recently, it has been shown that MAP kinase, which is encoded by the *rolled* gene, plays

an essential role in the torso and the sevenless signalling pathway downstream of raf (Brunner et al., 1994; Biggs et al., unpublished). Constitutively activated versions Ras1 and raf have been constructed and shown to be sufficient to induce differentiation of terminal structures or R7 photoreceptor cells when expressed in the embryo or in the developing eye, respectively (Dickson et al., 1992; Fortini et al., 1992; Lu et al., 1993). Similarly, a gain-of-function mutation in *rolled* (rl^{Sem}) results in the activation of the torso, sevenless and DER pathways (Brunner et al., 1994). This suggested that the activation of these components is not only necessary but also sufficient to transduce the signal mediated by sevenless, torso and DER.

Since most of the signal transduction components in the sevenless and the torso pathway have been identified as dominant modifiers in individual genetically sensitized pathways, it is not known in how many different signalling processes these components are involved. Animals homozygous for loss-of-function mutations in any of these genes die during embryonic or larval development. We decided to analyze the phenotype of loss-of-function mutations in *Drk*, *Sos*, *Ras1*, *raf* and *rolled* in clones of homozygous mutant cells in the adult epidermal structures. The phenotypes observed for loss-of-function mutations in *Drk*, *Sos*, *Ras1*, *raf*, and *rolled/MAP kinase* should reflect the sum of the phenotypes observed for the different receptors that signal via these components. If we observed phenotypes not accounted for by mutations in the known receptor tyrosine kinases, it is likely that there are additional receptors that utilize these signalling components. Furthermore, it is also possible that the phenotypes of the individual signal transduction components differ from each other. This may suggest that some of the components receive input from multiple signalling pathways whereas others do not.

The results presented here indicate that the phenotypes of cell clones mutant for either *Drk*, *Sos*, *Ras1*, *raf* or *rolled* are identical in all aspects to the phenotypes described for cell clones mutant for *DER*. This suggests that also during development of the adult epidermis Drk, Sos, Ras, raf and rolled/MAP kinase act in a linear pathway mediating the different aspects of differentiation controlled by DER. The absence of additional phenotypes for any one of these components indicates that they are not essential for other signalling processes that occur during epidermal development.

MATERIALS AND METHODS

The clonal analysis was carried out using the strongest available loss-of-function mutations at each locus. Drk^{R1} is a single amino acid substitution in the SH2 domain, which abolishes the ability of Drk to bind the activated receptor (Olivier et al., 1993). Sos^{X122} behaves genetically as a null mutation and Sos^{dm7} is a partial loss-of-function mutation (Rogge et al., 1991). $Ras1^{e2F}$ results in a single amino acid substitution (Simon et al., 1991). It is likely that Drk^{R1} and $Ras1^{e2F}$ are not complete loss-of-function mutations. The raf^{EA75} allele is a strong loss-of-function mutation but has not been characterized molecularly (Melnick et al., 1993). The hypomorphic raf^{C110} allele was also analyzed in clones (data not shown). It exhibits a very weak variable phenotype similar to that seen for Sos^{dm7}. Three different *rolled* (*rl*) alleles were used: $Df(2R)rl^{10a}$ is a deficiency for *rl*; rl^{la8} and rl^{EMS698} are strong loss-of-function mutations (Hilliker, 1976). The gain-of-function allele rl^{Sem} results in a single amino acid substitution in the catalytic domain of MAP kinase and activates the sevenless, torso and DER pathway (Brunner et al., 1994).

Two strategies were followed to mark the clones genetically (Fig. 2). In the case of *raf* and *rl*, the clones were marked in *cis* with the mutations f^{36a} and *pwn*, respectively. In the case of *Drk*, *Sos* and *Ras1*, the flies were f^{36a} and the clones were marked by the loss of a P(f^+) insertion in the homologous chromosome. As shown in the Fig. 2, the cell markers used in the clonal analysis of *Drk* and *raf* are located between the mutant and the centromere. This permits an unambiguous identification of the mutant cell clones. In the cases of *Sos*, *Ras1* and *rl* only a subset of the marked clones correspond to events of mitotic recombination proximal to the mutation analyzed. The number, frequency and size of clones in the wing and the tergites are summarized in Table 1. The number of clones analyzed in legs were 9 for *Drk*, 16 (7 with phenotype) for *Sos*, 31 (13 with phenotype) for *Ras1* and 12 for *raf*. *Minute* alleles were used to obtain large clones, which facilitated the analysis of the phenotypes. Mutant clones that are $Minute^+$ have an improved viability compared to $Minute^-$ cells. The developmental parameters of the *Minute* alleles used in this study can be found in Ferrus, 1974.

Mitotic recombination was induced by X-rays from a Philips H.T. generator operating at 100 kV, 15 mA, 2 mm filter, at a dose of 1000 rad ($=10^{-2}$ J. kg^{-1}). The age of irradiation was dated in hours after egg laying (A.E.L.). Different parts of the fly were dissected in a mixture of ethanol/glycerol (7/1), fat was removed with KOH (10%) and after dehydration, wings, nota, legs and abdomens were mounted separately in a mixture of lactic acid/ethanol (6/5) and sealed with nail polish. The clones were examined under the compound microscope. Clones were drawn using a digitizing tablet and plotted as shown in Fig. 3.

RESULTS

We performed a clonal analysis in the adult epidermis of all known lethal mutations that affect the sevenless signal transduction cascade. The phenotypes of mutant clones in the wing were analyzed in greater detail since a similar analysis carried out for *DER* indicated that DER function is required for a number of different decision during wing development (Diaz-Benjumea and Garcia-Bellido, 1990a). The *Drosophila* wing is made of a dorsal and a ventral layer of epidermal cells. Each cell differentiates a small hair or trichome. The only visible

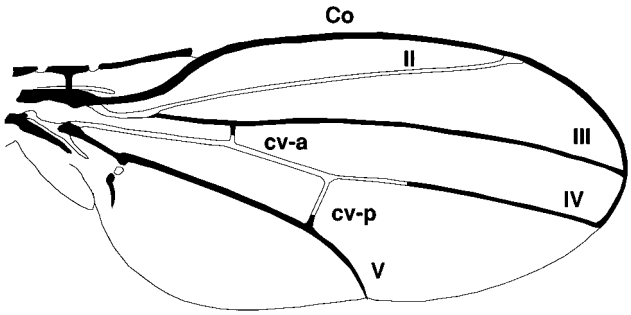


Fig. 1. Pattern of veins in the *Drosophila* wing. Camera-lucida drawing of a wild-type wing showing the dorsal and the ventral character of the different veins. Filled-in veins are dorsal and empty veins are ventral. The longitudinal veins are labelled with roman numerals. Abbreviations: Co, costal vein; cv-a, anterior cross vein; cv-p, posterior cross-veins.

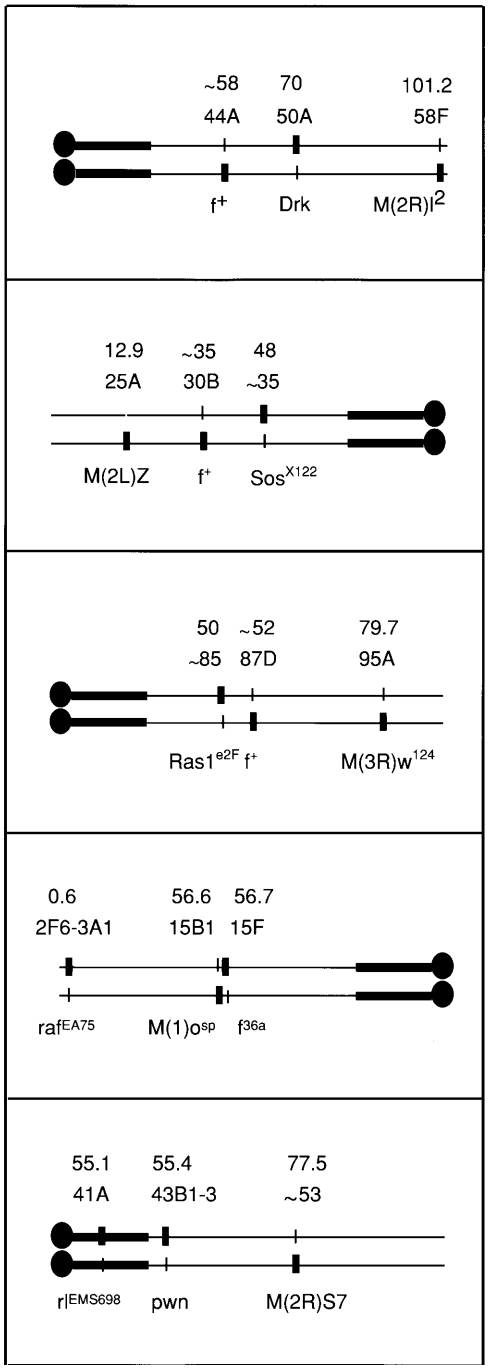


Fig. 2. Chromosomes used in the different clonal analysis experiments. Meiotic (top line) and cytological (bottom line) location of the mutations used in the different clonal analyses. Genotypes involving a P(*forked*$^+$) insertion carry on the X chromosome the f^{36a} allele. This allele permits the analysis of clones in bristles as well as in trichomes. (~) estimated location.

structures on the wing blade are veins and sensory organs. The veins are longitudinal ridges running along the proximodistal axis of the wing on either surface (Fig. 1).

The clones of cells homozygous for either *Drk*, *Sos*, *Ras1*, *raf* or *rl* exhibit a set of phenotypes that is very similar to the phenotypes observed in the clonal analysis of DER. The phenotypes can be grouped into six classes (see Fig. 7): the six classes are listed in the order of the phenotypes observed with an allelic series of *DER* mutations (Diaz-Benjumea and Garcia-Bellido, 1990a). The lack-of-vein phenotype (class 1) is observed with weak viable alleles of *DER* whereas a phenotype in the abdominal tergites (class 6) is only observed with strong loss-of-function mutations. Since the same set of six phenotypes was observed in clones mutant for any of the signalling components tested, we will describe each of these phenotypic classes separately.

Class 1: absence of veins

Clones of cells mutant for *DER* or any of the signalling components prevent the formation of veins anywhere on the wing surface. A clone of homozygous *Drk* cells is shown in Fig. 4A. The dorsal and the ventral component of the vein L4 is removed by the clone. This phenotype is strictly cell autonomous; mutant cells are unable to differentiate veins. Clones in either surface do not affect the differentiation of veins on the other surface suggesting that the proposed inductive mechanism for the differentiation of ventral veins is not affected (Garcia-Bellido 1977). Weak viable alleles of *DER* and *rolled* also result in the disruption of the vein L4 suggesting that already a small reduction in the function of these components prevents the differentiation of veins (Fig 6A). The formation of veins is also the only process affected in clones homozygous for the weak Sos^{dm7} allele. Conversely, gain-of-function mutations in *DER* and *rl* result in the formation of extra veins (Fig. 6C).

Class 2: non-autonomous differentiation of ectopic veins

Narrow clones adjacent to one vein can produce small pieces of extra veins surrounding the clone independently of whether the wild-type vein is removed by the clone. A clone of cells mutant for Sos^{X122} marked with *f* is shown in Fig. 4B. No vein is formed within the clone but adjacent to the mutant cells, wild-type (f^+) cells differentiate veins (arrowheads). A similar non-autonomous behaviour for the *DER* mutations has been observed in the developing eye. Clones of *DER* mutant cells in the eye imaginal disc prevent the neuronal differentiation of these cells but cause the formation of ommatidia with additional photoreceptor cells adjacent to the clone (Xu and Rubin, 1993).

Class 3: reduced cell size and class 4: extra bristles

Cells homozygous for mutations in *DER* or any of the signalling components tested produce a higher cell density suggesting that the cells are smaller. As a result of the change in cell density, the wing is folded towards the surface bearing the clone and the dorsal and ventral components of the veins are uncoupled. That is most evident in vein L4, where the vein ridge is dorsal in the distal part of the wing and ventral in the proximal part. An example of a ventral Sos^{X122} clone producing a fold (arrowheads) in the overlaying dorsal side is shown in Fig. 4C. The difference in cell density is most evident in the Ras^{e2F} clone shown in Fig. 4D. Flies homozygous or hemizygous for the viable rl^1 allele also possess bent down wings (Morgan et al., 1925). The reduced function of rolled/MAP kinase in rl^1 mutants appears to affect cell size stronger on the ventral than on the dorsal side.

Table 1. Number, frequency and size of the clones obtained in the wings and the abdominal tergites in the different experiments

			Wing		Abdomen	
Expt.	Genotype	Age (h.AEL)	Number of Clones (*)	Cells per Clone (†)	Number of clones (*)	Clones per Abdomen
A	f^{36a}; Drk / f^{+44A} M(2R)l^2	63+/–12	38 (38)	1.050	33 (1)	0.26
B	f^{36a}; Sos^{X122} / M(2L)Z f^{+30B}	63+/–12	80 (61)	2.180 (420)	24 (0)	0.34
C	f^{36a}; Sos^{dm7} / M(2L)Z f^{+30B}	63+/–12	24 (4)	1.850 (2.030)	16 (0)	0,41
D	f^{36a}; $Ras1^{e2F}$ / f^{+87D} M(3R)w^{124}	63+/–12	52 (52)	790	24 (8)	0,15
E	Raf^{EA75} f^{36a} / M(1)o^{sp}	42+/–12	76 (76)	410	29 (0)	0.83
F	f^{36a}; rl^{10a} / f^{+44A} M(2R)l^2	63+/–12	27 (0)	—	—	—
G	rl^{EMS698} pwn / M(2R)S7	63+/–12	57 (15)	2.370 (330)	130 (2)	0,53
H	f^{36a}; Sem / f^{+44A} M(2R)l^2	63+/–12	34 (0)	1.900	19 (0)	0,50

*Number of clones that show a phenotype (in parenthesis). Note that in the wing this is 100% when the cell marker used is more proximal on the chromosome than the mutation analysed in each case (see Fig. 2). and <100% when the cell marker is distal to the mutation. This suggests that the penetrance of the phenotype is 100% and the clones without a phenotype correspond to the mitotic recombination events between the cell marker and the mutation analysed in each case.
†Average number of cells per clone in clones that show a phenotype (in parenthesis). Note the reduction in the size of the clones mutant for the strong *Sos*, *raf* and *rolled* alleles used in the experiments B, E, and G in comparison with the size of wild-type clones (around 2,000 cells) induced at the same time.

A higher cell density is also observed in clones in the notum, the legs and the head. Examples are shown in Fig. 5A for *Ras1* and in Fig. 5B for *Sos*. In addition to the higher cell density of epidermal cells, the number of bristles is also increased in the clone compared to the regions of wild-type tissue surrounding the clone (arrowheads in Fig. 5A and Diaz-Benjumea and Garcia-Bellido, 1990a).

Class 5: cell viability

Strong loss-of-function alleles are cell lethal in clones in imaginal discs. However, using the Minute$^+$ technique, it was possible to recover very small clones. These clones are very narrow, only two or three cells wide and are recovered at low frequency. This observation suggests that the absence of mutant clones under normal circumstances is caused by the competition with faster growing heterozygous cells. In the case of *rolled/MAP kinase* for which complete loss-of-function mutations are available, it was not possible to detect any clones suggesting that the complete removal of MAP kinase function is lethal for the cell. Similarly, with strong loss-of-function mutations in DER, it is also only possible to recover very small clones.

Due to the strongly reduced viability of *rolled* clones, it was not possible to cover the entire wing surface (Fig. 2). To further establish the similarity between DER and rolled phenotypes, we used as a complementary approach an analysis of genetic interactions between *rolled* lack- and gain-of-function alleles and other mutations affecting the wing vein pattern. The expectation was that both mutations, *rolled* and *DER*, show interactions with the same wing vein mutants. As shown in Fig. 6, genetic combinations of either lack-of-function or gain-of-function alleles of *rolled* and *DER* show a strong enhancement of the wing phenotype. The results of this analysis are summarized in Table 2. Essentially the same mutations that showed an interaction with *DER* (Diaz-Benjumea and Garcia-Bellido, 1990a), also interact with *rolled*. The results from the clonal analysis and the genetic interactions indicate that DER and rolled/MAP kinase function in the same pathway.

Class 6: abdominal tergites

Strong loss-of-function alleles of *DER* and *rolled* produce small narrow clones in the abdominal tergites. In the Ras^{e2F} analysis, we have found a similar phenotype but with a low penetrance. An example of a Ras^{e2F} clone is shown in Fig. 5C. Interestingly, wild-type bristles from the region around the clone shift their position and come to lie inside the clone. Consequently, the mutant clones contain both mutant and wild-type bristles, whereas the surrounding region contains fewer bristles. This last phenotype is not observed with the alleles of *Drk*, *Sos* and *raf* tested. It is possible that in these mutations there is sufficient residual activity left for the normal development of the epidermis in the tergites.

DISCUSSION

The clonal analysis of the signalling components Drk, Sos, Ras1, raf and rolled/MAP kinase during the development of the adult epidermis presented here indicates that mutations in each of these components produce the same set of six phenotypes as mutations in *DER*. Furthermore, for each of the genes for which multiple alleles were tested, the six phenotypes can be ordered in the same phenotypic series. This indicates that these components, initially identified by their role in mediating signalling in the developing eye, also mediate every aspects of *DER* function during the development of epidermal structures. In addition to the lethal mutations analyzed here, mutations in *Gap1* and *sina* have been shown to be involved in the sevenless signalling pathway (Carthew and Rubin, 1990; Gaul et al., 1992). The existing alleles of *Gap1* and *sina* are viable in homozygous condition and do not display phenotypes characteristic of hypomorphic mutations in *DER* or *rolled*. The gene products of *Gap1* and *sina* may therefore act specifically in sevenless pathway.

DER signalling controls two different aspects of differentiation in epidermal cells

In the wing, cells homozygous for loss-of-function mutations in *DER* or for any of the downstream signalling components are smaller and are unable to differentiate veins. It is possible that the two phenotypes are linked, if smaller cells are unable to differentiate vein structures. There are, however, a number

of other mutations that cause a reduction in cell size without preventing the formation of veins. For example the mutation *l(3)Me10* produces small cells similar to those produced in *DER* clones but it does not affect the formation of veins (Ferrus and Garcia-Bellido, 1976). Likewise there are many mutations that affect the differentiation of veins without affecting cell size. The mutation *veinlet*, a viable allele of *rhomboid*, removes the distal portions of veins without altering the cell size (Diaz-Benjumea and García-Bellido, 1990b; Sturtevant et al., 1993). Furthermore *veinlet*$^{-}$ clones on the dorsal side affect in a non-autonomous manner the differentiation of ventral veins (Garcia-Bellido, 1977). This is not the case for the mutations analyzed here. None of the clones on one side of the wing affect the formation of veins on the other side (Fig. 3). It is likely then that cell size and vein differentiation are two independent characteristics acquired by cells in the wing and that DER activity, mediated via the same signalling cascade, controls these two different aspects of differentiation in each cell.

The sevenless signalling cassette is exclusively required for signalling from DER during epidermal development

All phenotypes resulting from the reduction of the function of either Drk, Sos, Ras1, raf or rolled/MAP kinase resemble those caused by loss-of-function mutations of *DER*. This suggests that none of these components is involved in other signalling processes that can be revealed by clonal analysis during the development of the adult epidermis. The process of lateral inhibition mediated by the products of *N* and *Dl* plays a role not only in the differentiation of bristles but also in the differentiation of wing veins. In contrast to the phenotypes detected in the clonal analysis for mutations in the TK pathway, clones of cells mutant for loss-of-function alleles of *Dl* and *N* produce thicker veins. In the case of *Dl*, the proposed ligand, this phenotype is non-autonomous and a row of cells surrounding the clone also differentiates as vein tissue (Garcia-Bellido and de Celis, 1992). In the mutations analyzed here, the non-autonomous phenotype consists of the differentiation of a normal-sized vein adjacent to the mutant clone. The differentiation of the wing margin is dependent on wingless function (Phillips and Whittle, 1993). Although many of the mutant clones tested crossed the wing margin its differentiation was not affected

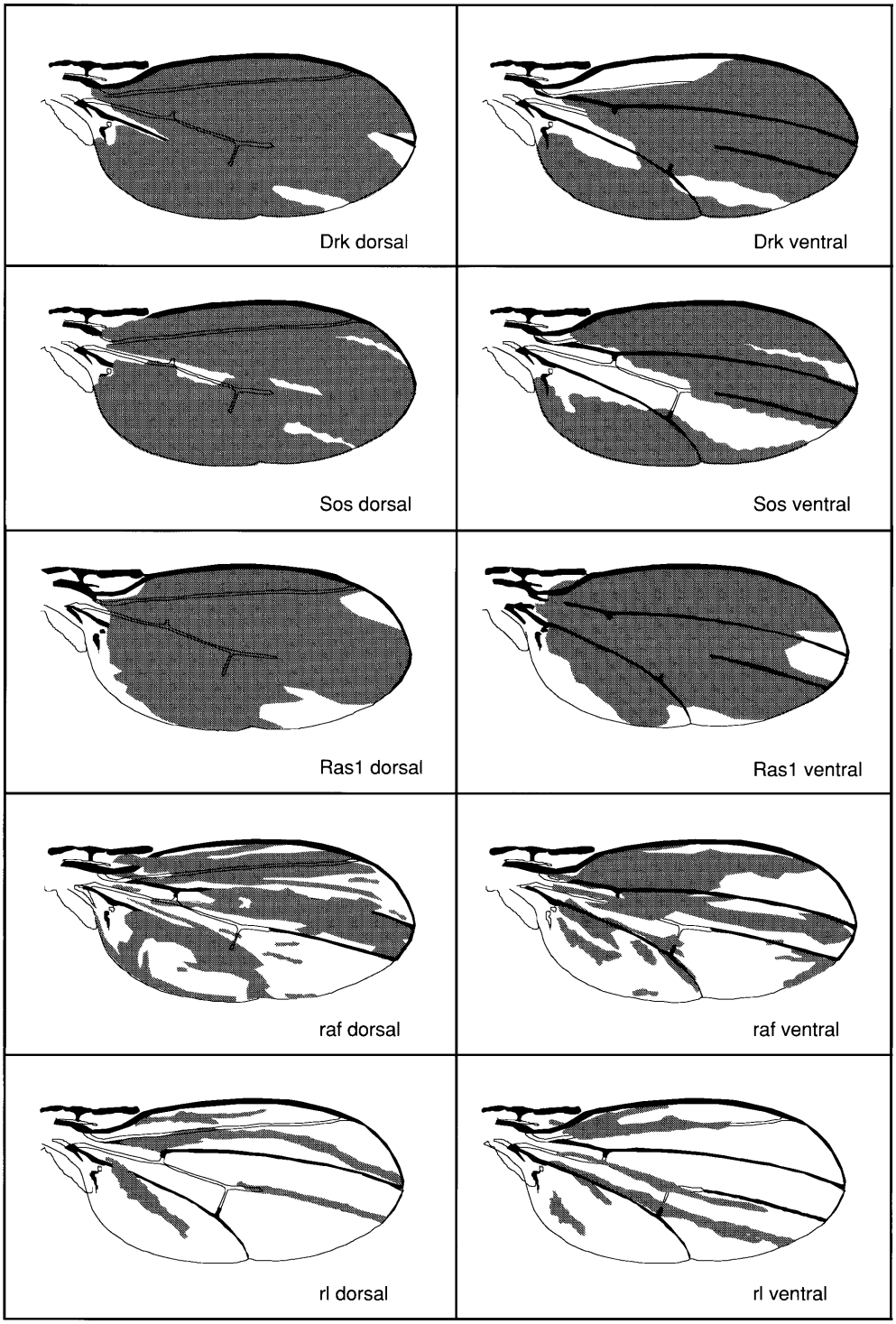
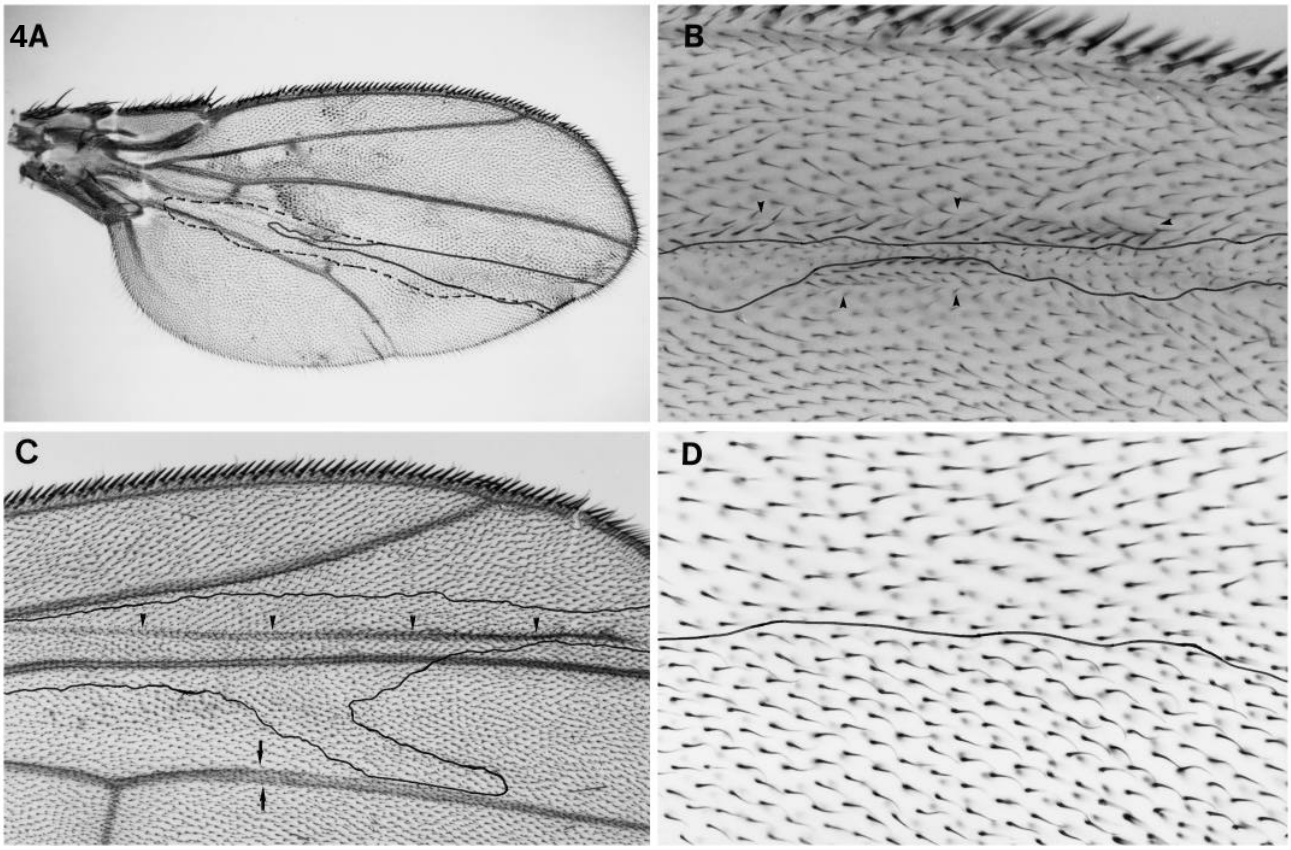


Fig. 3. Regions of the wing covered in the different clonal analyses. Shaded areas represent areas that were covered by mutant clones. Veins whose differentiation is prevented in the different clones are not drawn. In each case analyzed, the mutant clone removed the veins in the surface of the wing where the clone was located but did not affect the vein on the opposite surface.


4A
B
C
D

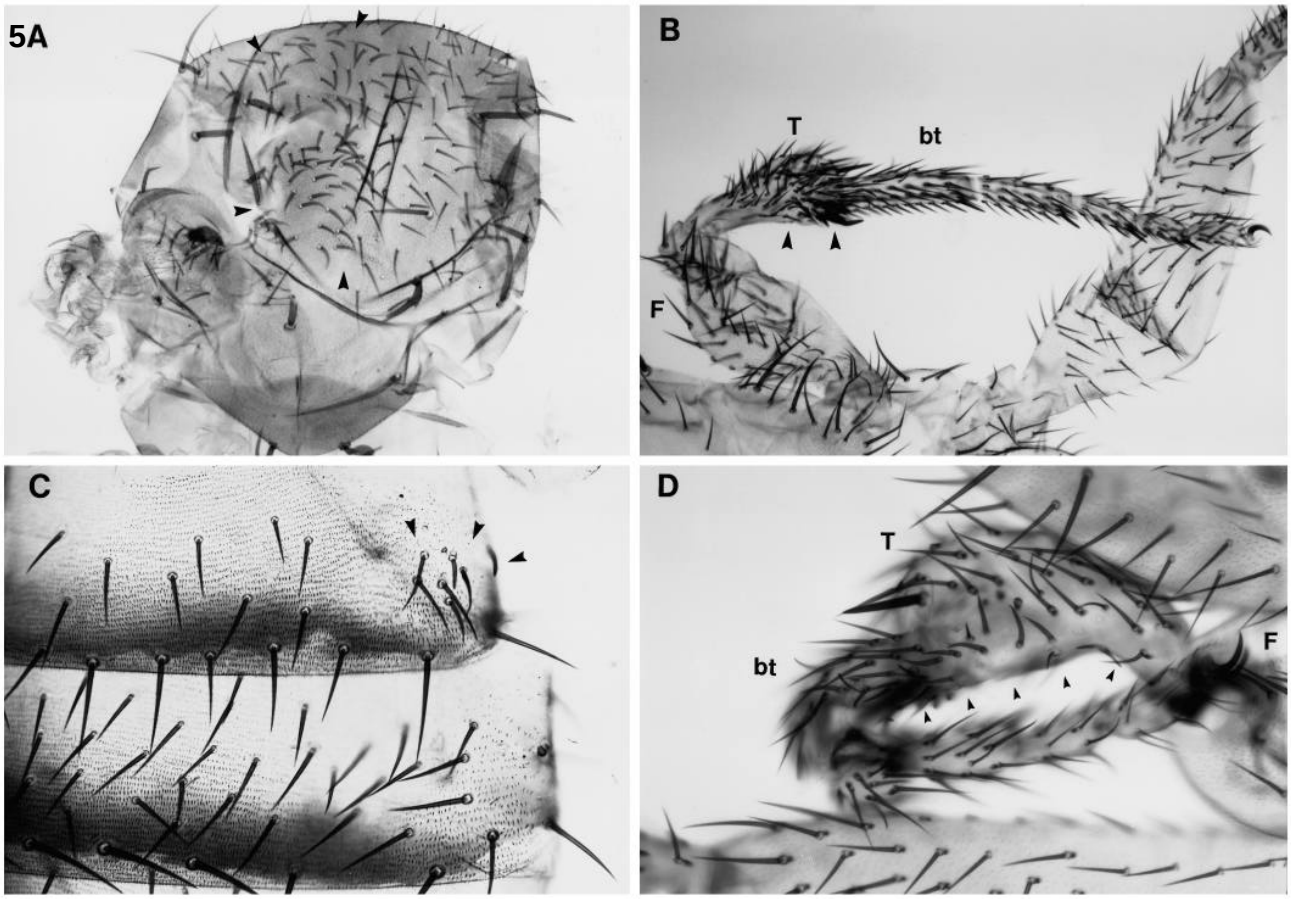

5A
B
T
bt
F
C
D
T
bt
F

Fig. 4. Clonal phenotypes in the wing. (A) *f Drk* (Minute$^+$) clone in the wing. The clone extends as a stripe of cells in both the dorsal (solid line) and ventral surfaces (dashed line) of the posterior compartment. Both the dorsal and the ventral component of the vein L4 is removed. (B) Ectopic veins produced by a *f Sos^{X122}* clone. The *f* trichomes occupy a narrow stripe which splits the presumptive vein region and induces the differentiation of two veins (arrowheads), which have the same width as the wild-type veins. Note that both veins are formed by wild-type cells adjacent to the clone. (C) *f Sos^{X122}* (Minute$^+$) clone in the ventral surface of the wing (outline) produces a fold in the dorsal surface (arrowhead). As a result of this clone, the wing is folded ventrally. Note that the dorsal and ventral components of the vein L4 (arrows), which overlap in the wild-type wing, are shifted as a result of the change in cell density in the ventral surface. The viable allele *rolled^1* shows a similar phenotype. (D) High magnification of a *Ras1^{e2F} f* clone. The clone covers the bottom part of the figure as a transverse stripe. The black line outlines the boundary between the clone and the wild-type tissue. Note the higher density of *f* trichomes compared with the wild-type trichomes.

Fig. 5. Clonal phenotypes in the notum, leg and tergites. (A) *Ras1^{e2F} f* clone in the left hemi-notum. Note the higher density of *f* bristles (arrowheads) as compared with the wild-type bristles in the right half of the notum. (B) Low magnification of a *f Sos^{X122}* (Minute$^+$) clone in the tibia (arrow). Note the reduction in size. (C) *Ras1^{e2F} f* clone in an abdominal tergite. Note the higher density of *f* and wild-type bristles (arrowheads) within the clone and the region devoid of bristles surrounding the clone. (D) High magnification of a *f Sos^{X122}* (Minute$^+$) clone (arrowhead) covering part of the femur (F), the tibia (T) and the basitarsus (bt) of the second leg. Note the deformation and the reduction in size of the segments affected by the clone in comparison with the normal tarsal segments.

(Figs 3, 4C). Clones of *dpp* cells prevent the formation of wing veins and affect the development of the imaginal disc cells in a non-autonomous fashion when the mutant cells occupy a region anterior to the AP compartment boundary (Posakony et al., 1990). It is unlikely that the lack of vein phenotype and the reduced viability observed for mutations analyzed here are caused by blocking the dpp signalling pathway. *dpp* mutant clones do not produce extra veins outside the clone as it is observed for all mutations analyzed here. Furthermore, the allelic series of *dpp* mutations and hypomorphic mutations in *DER* and *rolled* exhibit a different set of phenotypes (Segal and Gelbart, 1985; Clifford and Schüpbach, 1989). It appears therefore that none of the signalling components that are common to different receptor tyrosine kinases are required in the as yet unknown signalling pathways controlled by wingless and dpp.

It has been proposed that the different steps in the signal transduction cascade serve to integrate different intracellular signals. Studies in vertebrate cell culture using dominant negative forms of p21-ras suggest that Ras functions not only downstream of receptor tyrosine kinases but also downstream of src-related kinases (Kremer et al., 1991) as well as protein kinase C (Mulcahy et al., 1985). Furthermore, MAP kinase can be activated by a Ras-dependent and a Ras-independent pathway. In vertebrates, the dual specificity kinase Mek (MAPK or ERK kinase) activates MAP kinase by phosphorylation on both tyrosine and threonine. Mek can be activated by raf or by Mek kinase. Mek kinase, similar to its yeast homolog STE11, mediates signals from heterotrimeric G protein coupled receptors (Crews and Erikson, 1993). Therefore Mek, like Ras, appears to act as an integration point of signals from

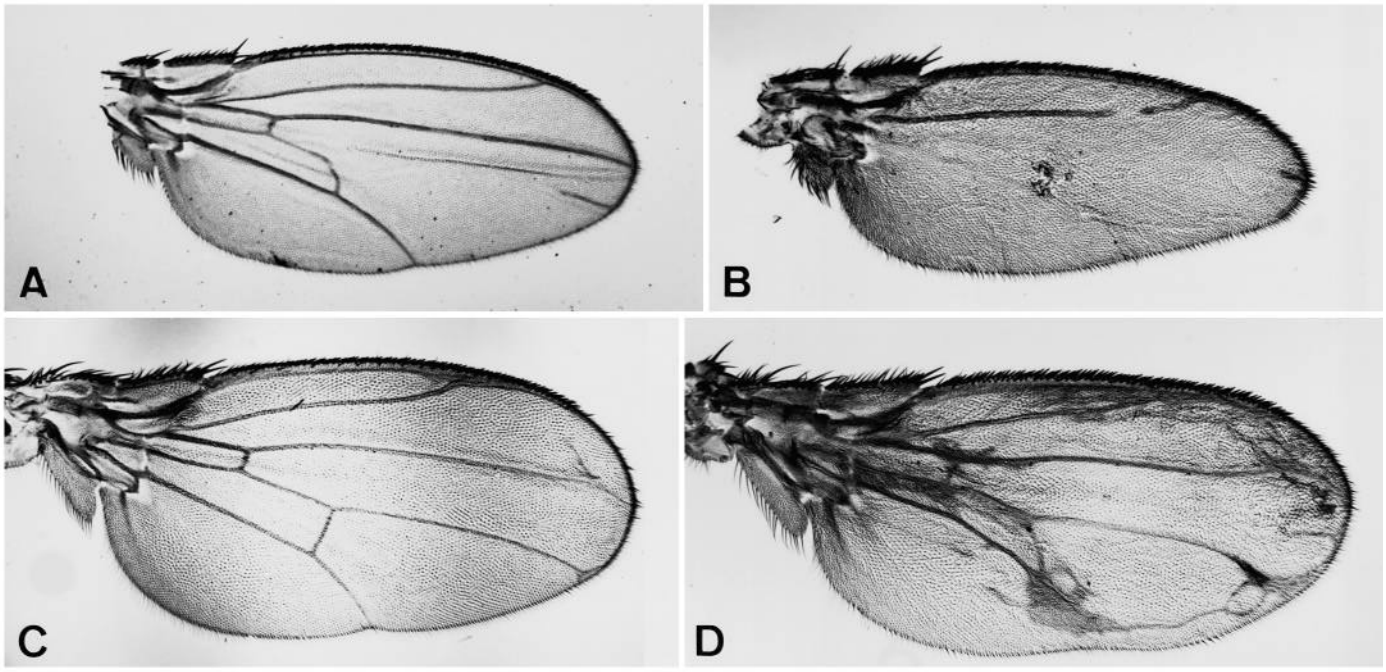


Fig. 6. Genetic interaction between *rolled* and alleles of *DER*. (A) Wings of flies with the following genotypes are shown: *rl^1/rl^{10a}* (A), *rl^1 DER^{top1}/rl^{10a} DER^{top4A}* (B), *Elp^{B1}/+* (C), and *Elp^{B1}/rl^{Sem}* (D). Wings of flies hemizygous for *rl^1* are slightly reduced in size and exhibit a disruption of vein L4 (A). The phenotype of *top^1* hemizygous flies is very similar (not shown). The lack of veins phenotype and size reduction is strongly enhanced in the *rl top* double mutant (B). Similarly, the formation of additional veins in the gain-of-function mutation *Elp^{B1}* (C) and *rl^{Sem}* (not shown) is strongly enhanced in the *Elp^{B1}/rl^{Sem}* double mutant (D). All the pictures were made at the same magnification.

Table 2. Genetic interactions between *rolled* alleles and other mutations affecting the wing vein morphogenesis

Allele	Locus	rl^1/rl^{10a}	*Sem* / +
top^1/top^{4A}	(2-100)	●	
Elp^{B1} (*Elipse*)	(2-100)	○	●*
ras-2†	(1-)	○	
ast (*asteroid*)	(2-3.8)	●	
ve (*veinlet*)	(3-0.2)	●	
vn (*vein*)	(3-16.2)	●	
ci (*cubitus interruptus*)	(4-0)	○	
Vno /+ (*Vein off*)	(3-Rear.)	●	
ri (*radius incompletus*)	(3-47.0)	+	
tt (*tilt*)	(3-40.0)	●	
vvl^{M3}/*In sep* (*ventral veinless*)	(3-Rear.)	●	
net (*net*)	(2-0.0)	○	●*
px (*plexus*)	(2-100.5)	○	●*
Dl^{M1}/+ (*Delta*)	(3-66.2)	+	●
N^{55E11}/+ (*Notch*)	(1-3.0)	●	
Ax /+ (*Abruptex*)	(1-3.0)	●	

The results of these interactions with *DER* alleles have been described previously (Diaz-Benjumea and Garcia-Bellido, 1990a). ●, strong enhancement; ○, suppression; +, additive; *, studied in heterozygous condition; †, *Drosophila* activated *ras-2* expressed under the control of the actin promoter (Bishop III and Corces, 1988). The phenotypes of viable combinations of different *rl* alleles can be ordered according their strength as follows: Phenotypic series of different viable allelic combinations of *rl*: $rl^{10a}/rl^1 \geq rl^{EMS64}/rl^1 > rl^{la8}/rl^1 > rl^{EMS698}/rl^1 > rl^1/rl^1$.

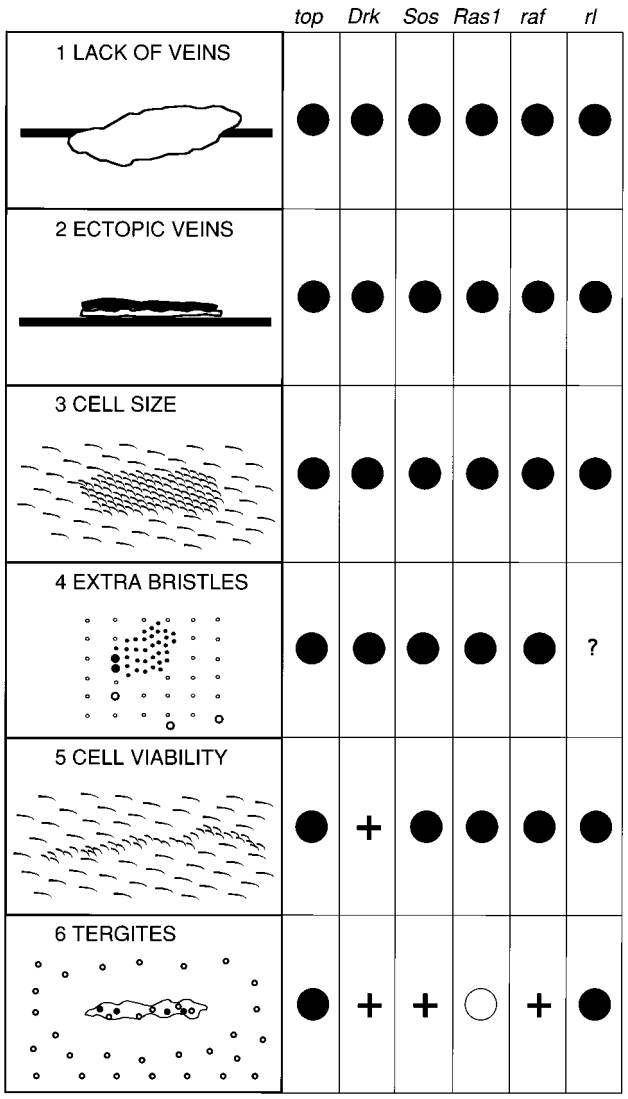


Fig. 7. Summary of the classes of phenotypes observed. The phenotypes are ordered according to the strength of the allele required to produce it. Lack of veins (1) is seen with very weak hypomorphic alleles whereas the recruitment of wild-type bristles into the clone in tergites is only observed with strong loss-of-function alleles. Filled dots indicate that the phenotype has been observed. Open circles indicate that the phenotype was not seen in every clone analyzed. (+) The phenotype has not been observed with the alleles tested. (?) The results were ambiguous.

different signalling pathways. In light of this high degree of integration of different signals observed in vertebrate cell culture systems, it is surprising that in our studies the mutant phenotypes for the different signalling components are so similar. The fact that mutations in *Drk*, coding for an SH2 adaptor protein that is likely to be involved primarily in signalling from receptor tyrosine kinases and mutations in *Ras1* and *rolled/MAP kinase* produce similar phenotypes suggests that during epidermal development, Ras1 and MAP kinase are not responding to a larger variety of signals than Drk. The results presented here are consistent with the finding that also during the development of the compound eye clones of *Drk* and *Ras1* produce identical phenotypes (Simon et al., 1991). Therefore during the development of neither the compound eye nor the adult epidermis is there functional evidence that this signalling cascade may serve to integrate different intracellular signals.

Different cellular responses to a common signalling cassette

The components analyzed here have been shown to mediate signalling from sevenless, torso and DER in the eye (Simon et al., 1991; Doyle and Bishop, 1993; Olivier et al., 1993). Our results indicate that even within the same cell two aspects of differentiation, cell size and vein formation, are controlled by the same receptor signalling system. In rat PC12 cells, stimulation with EGF induces cell proliferation whereas stimulation with NGF induces neural differentiation. These different responses are mediated by a very similar signalling cascade including Ras, raf and MAP kinase (Chao, 1992). It is possible that the strength of the signal or its duration triggers the qualitatively different responses. Consistent with this hypothesis is the finding that the activation profile of MAP kinase upon EGF and NGF stimulation differs significantly. Whereas MAP kinase activity increases within 2 minutes of stimulation with either growth factor, the activity decreases very rapidly upon EGF stimulation but is sustained for 60-90 minutes after NGF stimulation (Traverse et al., 1992). In the wing, weak alleles of *DER* and *Sos* affect only the differentiation of veins but do not reduce cell size. This suggests that these two cellular responses are also dependent on different levels of DER signalling.

It is clear, however, that simple quantitative differences in the amount of signalling are not sufficient to explain the variety of phenotypes observed in mutations of the different receptor tyrosine kinases. The gain-of-function mutation in *rolled*, rl^{Sem}, mimics the gain-of-function phenotypes of *torso* in the

embryo, *sevenless* in the eye and *DER* in the wing (Brunner et al., 1994). This suggests that the different interpretation of MAP kinase activity is likely to occur downstream of MAP kinase. In vertebrates, MAP kinase has been shown to activate transcription factors such as Elk-1, a component of the serum response factor complex (Marais et al., 1993). In the developing eye, the *sina* gene encodes a nuclear protein required for R7 development that is specifically expressed in the cells competent to become R7 cells (Carthew and Rubin, 1990). Although this factor may not be a direct target of the sevenless signalling cascade, it appears to be essential for the correct interpretation of the signal in these cells. Genetic screens to identify components that act downstream of *rolled/MAP kinase* should help to identify mutations with phenotypes corresponding to only a subset of those observed for mutations used in the present study and thereby help to identify genes involved in the tissue- and stage-specific interpretation of a common signal.

We would like to thank M. Simon for providing *Ras1* and *Sos* alleles, N. Perrimon for raf^{EA75}, A. Hilliker for the *rolled* alleles, V. Corces for the P(*forked*$^{+}$) insertions and A. Garcia-Bellido for the stocks of P(f^{+}) insertions with *Minutes*. We thank K. Basler, S. Cohen, B. Dickson, M. Dominguez and M. Mlodzik for comments on the manuscript. F. D-B. was supported by an EMBO short-term fellowship. The work was supported by a grant of the Swiss National Science Foundation to E. H.

REFERENCES

Ambrosio, L., Mahowald, A. P. and Perrimon, N. (1989). Requirement of the *Drosophila raf* homologue for *torso* function. *Nature* **342**, 288-291.

Aroian, R. V., Koga, M., Mendel, J. E., Ohshima, Y. and Sternberg, P. W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**, 693-699.

Baker, N. E. and Rubin, G. M. (1989). Effect on eye development of dominant mutations in *Drosophila* homologue of the EGF receptor. *Nature* **340**, 150-153.

Baker, N. E. and Rubin, G. M. (1992). *Ellipse* mutations in the *Drosophila* homologue of the EGF receptor affect pattern formation, cell division, and cell death in eye imaginal discs. *Dev. Biol.* **150**, 381-396.

Banerjee, U., Renfranz, P. J., Pollock, J. A. and Benzer, S. (1987). Molecular characterization and expression of *sevenless*, a gene involved in neuronal pattern formation in the Drosophila eye. *Cell* **49**, 281-291.

Basler, K. and Hafen, E. (1988). Control of photoreceptor cell fate by the sevenless protein requires a functional tyrosine kinase domain. *Cell* **54**, 299-312.

Beitel, G. J., Clark, S. G. and Horvitz, R. (1990). *Caenorhabditis elegans ras* gene *let-60* acts as a switch in the pathway of vulval induction. *Nature* **348**, 503-509.

Bishop III, J. G. and Corces, V. (1988). Expression of an activated *ras* gene causes developmental abnormalities in transgenic *Drosophila melanogaster*. *Genes Dev.* **2**, 567-577.

Bowtell, D., Simon, M. A. and Rubin, G. M. (1988). Nucleotide sequence and structure of the *sevenless* gene of *Drosophila melanogaster*. *Genes Dev.* **2**, 620-634.

Brunner, D., Oellers, N., Szabad, J., Biggs III, W. H., Zipursky, S. L. and Hafen, E. (1994). A gain of function mutation in Drosophila MAP kinase activates multiple receptor tyrosine kinase signalling pathways. *Cell* in press.

Carthew, R. W. and Rubin, G. M. (1990). *seven in absentia*, a gene required for specification of R7 cell fate in the Drosophila eye. *Cell* **63**, 561-577.

Casanova, J. and Struhl, G. (1989). Localized surface activity of *torso*, a receptor tyrosine kinase, specifies terminal body pattern in *Drosophila*. *Genes Dev.* **3**, 2025-2038.

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P. and Bernstein, A. (1988). The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse *W* locus. *Nature* **335**, 88-89.

Chao, M. V. (1992). Growth factor signaling: Where is the specificity? *Cell* **68**, 995-997.

Clark, S. G., Stern, M. J. and Horvitz, H. R. (1992). *C. elegans* cell-signalling gene *sem-5* encodes a protein with SH2 and SH3 domains. *Nature* **356**, 340-344.

Clifford, R. and Schüpbach, T. (1992). The torpedo (DER) receptor tyrosine kinase is required at multiple times during *Drosophila* embryogenesis. *Development* **115**, 853-872.

Clifford, R. J. and Schüpbach, T. (1989). Coordinately and differentially mutable activities of *torpedo*, the *Drosophila melanogaster* homolog of the vertebrate EGF receptor gene. *Genetics* **123**, 771-787.

Crews, C. M. and Erikson, R. L. (1993). Extracellular signals and reversible protein phosphorylation: What to Mek of it all. *Cell* **74**, 215-217.

Diaz-Benjumea, F. J. and Garcia-Bellido, A. (1990a). Behaviour of cells mutant for an EGF receptor homologue of *Drosophila* in genetic mosaics. *Proc. R. Soc. Lond. B* **242**, 36-44.

Diaz-Benjumea, F. J. and García-Bellido, A. (1990b). Genetic analysis of the wing vein pattern of *Drosophila*. *Roux's Arch. Dev. Biol.* **198**, 336-354.

Dickson, B., Sprenger, F., Morrison, D. and Hafen, E. (1992). Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. *Nature* **360**, 600-603.

Doyle, H. J. and Bishop, J. M. (1993). Torso, a receptor tyrosine kinase required for embryonic pattern formation, shares substrates with the Sevenless and EGF-R pathways in *Drosophila*. *Genes Dev.* **7**, 633-646.

Ferrus, A. (1974). Parameters of mitotic recombination of *Minute* mutants of *Drosophila melanogaster*. *Genetics* **79**, 589-599.

Ferrus, A. and Garcia-Bellido, A. (1976). Morphogenetic mutants detected in mitotic recombination clones. *Nature* **260**, 425-426.

Fortini, M. E., Simon, M. A. and Rubin, G. M. (1992). Signalling by the *sevenless* protein tyrosine kinase is mimicked by Ras1 activation. *Nature* **355**, 559-561.

Garcia-Bellido, A. (1977). Inductive mechanisms in the process of wing vein formation in *Drosophila*. *Roux's Arch. Dev. Biol.* **182**, 93-106.

Garcia-Bellido, A. and de Celis, J. F. (1992). Developmental genetics of the venation pattern of Drosophila. *Annu Rev Genet* **26**, 277-304.

Gaul, U., Mardon, G. and Rubin, G. M. (1992). A putative Ras GTPase activating protein acts as a negative regulator of signaling by the sevenless receptor tyrosine kinase. *Cell* **68**, 1007-1019.

Geissler, E. N., Ryan, M. A. and Housman, D. E. (1988). The dominant *white spotting* (*W*) locus of the mouse encodes the *c-kit* proto-oncogene. *Cell* **55**, 185-192.

Hafen, E., Basler, K., Edstroem, J. E. and Rubin, G. M. (1987). *sevenless*, a cell-specific homeotic gene of *Drosophila*, encodes a putative transmembrane receptor with a tyrosine kinase domain. *Science* **236**, 55-63.

Han, M., Golden, A., Han, Y. and Sternberg, P. W. (1993). *C. elegans lin-45 raf* gene participates in *let-60 ras*-stimulated vulval differentiation. *Nature* **363**, 133-140.

Han, M. and Sternberg, P. W. (1990). *let-60*, a gene that specifies cell fates during C. elegans vulval induction, encodes a *ras* protein. *Cell* **63**, 921-931.

Hilliker, A. J. (1976). Genetic analysis of the centromeric heterochromatin of chromosome 2 of *Drosophila melanogaster*: deficiency mapping of EMS-induced lethal complementation groups. *Genetics* **83**, 765-782.

Howe, L. R., Leevers, S. J., Gómez, N., Nakielny, S., Cohen, P. and Marshall, C. J. (1992). Activation of the MAP kinase pathway by the protein kinase raf. *Cell* **71**, 335-342.

Klingler, M., Erdélyi, M., Szabad, J. and Nüsslein-Volhard, C. (1988). Function of *torso* in determining the terminal anlagen of the *Drosophila* embryo. *Nature* **335**, 275-277.

Kremer, N. E., D, A. G., Thomas, S. M., DeMarco, M., Brugge, J. S. and Halegoua, S. (1991). Signal transduction by nerve growth factor and fibroblast growth factor in PC12 cells requires a sequence of src and ras actions. *J. Cell Biol.* **115**, 809-819.

Lu, X., Chou, T. B., Williams, N. G., Roberts, T. and Perrimon, N. (1993). Control of cell fate determination by *p21ras/Ras1*, an essential component of *torso* signaling in *Drosophila*. *Genes Dev.* **7**, 621-632.

Marais, R., Wynne, J. and Treisman, R. (1993). The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. *Cell* **73**, 381-393.

Melnick, M. B., Perkins, L. A., Lee, M., Ambrosio, L. and Perrimon, N. (1993). Developmental and molecular characterization of mutations in the *Drosophila-raf* serine/threonine protein kinase. *Development* **118**, 127-138.

Morgan, T. H., Bridges, C. B. and Sturtevant, A. H. (1925). The genetics of Drosophila. *Biobliog Genet* **2**, 233.

Mulcahy, L. S., Smith, M. R. and Stacey, D. W. (1985). Requirement for *ras*

proto-oncogene function during serum-stimulated growth of NIH3T3 cells. *Nature* **313**, 241-243.

Olivier, J. P., Raabe, T., Henkemeyer, M., Dickson, B., Mbamalu, G., Margolis, B., Schlessinger, J., Hafen, E. and Pawson, T. (1993). A Drosophila SH2-SH3 adaptor protein implicated in coupling the Sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* **73**, 179-191.

Phillips, R. G. and Whittle, J. R. S. (1993). *wingless* expression mediates determination of peripheral nervous system elements in late stages of *Drosophila* wing disc development. *Development* **118**, 427-438.

Posakony, L. G., Raftery, L. A. and Gelbart, W. M. (1990). Wing formation in Drosophila melanogaster requires decapentaplegic gene function along the anterior-posterior compartment boundary. *Mech. Dev.* **33**, 69-82.

Price, J. V., Clifford, R. J. and Schüpbach, T. (1989). The maternal ventralizing locus *torpedo* is allelic to *faint little ball*, an embryonic lethal, and encodes the Drosophila EGF receptor homolog. *Cell* **56**, 1085-1092.

Raz, E. and Shilo, B.-Z. (1993). Establishment of ventral cell fates in the Drosophila embryonic extoderm requries the EGF receptor homolog (DER). *Genes Dev*. in press.

Rogge, R. D., Karlovich, C. A. and Banerjee, U. (1991). Genetic dissection of a neurodevelopmental pathway: *Son of sevenless* functions downstream of the *sevenless* and EGF receptor tyrosine kinases. *Cell* **64**, 39-48.

Schejter, E. D. and Shilo, B. Z. (1989). The Drosophila EGF receptor homolog (DER) gene is allelic to *faint little ball*, a locus essential for embryonic development. *Cell* **56**, 1093-1104.

Schüpbach, T. (1987). Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in Drosophila melanogaster. *Cell* **49**, 699-707.

Segal, D. and Gelbart, W. M. (1985). *shortvein*, a new component of the *decapentaplegic* gene complex in *Drosophila melanogaster*. *Genetics* **109**, 119-143.

Simon, M. A., Bowtell, D., Dodson, G. S., Laverty, T. R. and Rubin, G. M. (1991). Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. *Cell* **67**, 701-716.

Simon, M. A., Dodson, G. S. and Rubin, G. M. (1993). An SH3-SH2-SH3 protein is required for p21Ras1 activation and binds to sevenless and Sos proteins in vitro. *Cell* **73**, 169-177.

Sprenger, F., Stevens, L. M. and Nüsslein-Volhard, C. (1989). The *Drosophila* gene *torso* encodes a putative receptor tyrosine kinase. *Nature* **338**, 478-483.

Sturtevant, M. A., Roark, M. and Bier, E. (1993). The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Dev.* **7**, 961-973.

Thomas, S. M., DeMarco, M., D'Arcangelo, G., Halegoua, S. and Brugge, J. S. (1992). Ras is essential for nerve growth factor- and phorbol ester-induced tyrosine phosphorylation of MAP kinases. *Cell* **68**, 1031-1040.

Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992). Sustained activation of the mitogen-activated protein (MAP) kinase cascade may be required for differentiation of PC12 cells. Comparison of the effects of nerve growth factor and epidermal growth factor. *Biochem. J.* **288**, 351-355.

Wood, K. W., Sarnecki, C., Roberts, T. M. and Blenis, J. (1992). *ras* mediates nerve growth factor receptor modulation of three signal-transducing protein kinases: MAP kinase, Raf-1, and RSK. *Cell* **68**, 1041-1050.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.

(Accepted 3 December 1993)