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Ras1 and a Putative Guanine Nucleotide Exchange Factor Perform Crucial Steps in Signaling by the Sevenless Protein Tyrosine Kinase

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Summary

We have conducted a genetic screen for mutations that decrease the effectiveness of signaling by a protein tyrosine kinase, the product of the Drosophila melanogaster *sevenless* gene. These mutations define seven genes whose wild-type products may be required for signaling by *sevenless*. Four of the seven genes also appear to be essential for signaling by a second protein tyrosine kinase, the product of the *Ellipse* gene. The putative products of two of these seven genes have been identified. One encodes a *ras* protein. The other locus encodes a protein that is homologous to the S. cerevisiae *CDC25* protein, an activator of guanine nucleotide exchange by *ras* proteins. These results suggest that the stimulation of *ras* protein activity is a key element in the signaling by *sevenless* and *Ellipse* and that this stimulation may be achieved by activating the exchange of GTP for bound GDP by the *ras* protein.

Introduction

Protein tyrosine kinases (PTKs) are important regulatory proteins that control many aspects of cellular growth, differentiation, and metabolism (Cantley et al., 1991). Many polypeptide hormones are known to regulate the metabolism, differentiation, and growth of target cells by binding transmembrane receptors that possess intracellular PTK domains (Cross and Dexter, 1991). In addition, mutations that alter the normal regulation and activity of either receptor PTKs or the closely related cytoplasmic PTKs can generate oncogenes that are capable of altering cellular growth control (Bishop, 1991; Hunter, 1991). Understanding how the PTKs influence cellular activities could provide important insights into how these cellular processes are regulated and coordinated.

Both the ligand-bound receptor PTKs and the oncogenic PTKs show elevated PTK activity relative to their unbound or unmutated counterparts. This increase in PTK activity is presumed to be a key element in initiating the signal transduction pathway, because mutations that destroy PTK activity almost invariably abolish function (for a partial exception, see Henkemeyer et al., 1990). The primary biochemical consequence of the increased PTK activity is generally phosphorylation of the PTK itself, but a number of other cellular proteins are also phosphorylated on tyrosine residues to varying extents. A number of proteins whose content of phosphotyrosine increases after a PTK is activated have been characterized. Among these molecules are several proteins that are known or thought to be elements of signaling pathways and are thus potential mediators of PTK signaling (reviewed by Cantley et al., 1991). These include phospholipase C-γ, the ras GTPase activating protein (rasGAP), a subunit of a PtdIns 3-kinase, and the c-*raf1* serine/threonine kinase. A second consequence of PTK activation is the recruitment of several of these same cellular proteins into complexes with the activated PTK. The precise composition of these complexes is not clear, but in several cases the binding appears to be mediated by SH2 domains of the target proteins binding to the phosphotyrosine moieties of the activated PTK. Complex formation may act as a mechanism for localizing signal transducing molecules to their site of action.

These studies have identified a number of proteins that may play important roles in signal transduction by PTKs. However, the functional significance of these potential components of PTK signaling pathways has been difficult to determine because there has been no easy way to ask whether these proteins are an essential part of the signaling pathway. Studies of mutations that affect development in flies and nematodes have provided in vivo evidence for the participation of two proteins in PTK signaling. The Drosophila *l(1)polehole* locus, which encodes a c-*raf1* homolog, is required for the action of the PTK encoded by the *torso* gene (Ambrosio et al., 1989; Sprenger et al., 1989; Casanova and Struhl, 1989). Studies of a Caenorhabditis elegans vulval development likewise indicate that a *ras* protein, the product of the *let-60* gene, is essential for the action of the PTK encoded by the *let-23* gene (Han and Sternberg, 1990; Beitel et al., 1990; Aroian et al., 1990).

We have attempted to study the mechanism of PTK signaling by first systematically identifying genes whose products may be essential for normal strength signaling by a particular PTK, the product of the Drosophila melanogaster *sevenless* (*sev*) gene, and then characterizing the products of these candidate loci. Our hope is that these experiments will both provide in vivo evidence for the involvement of suspected elements of PTK pathways and perhaps identify new signaling proteins.

The *sev* gene encodes a transmembrane PTK (Sevenless) that is most related to the vertebrate c-*ros* protein (Hafen et al., 1987; Basler and Hafen, 1988; Bowtell et al., 1988). The sole known function of Sevenless is for the development of the Drosophila compound eye. The eye is composed of approximately 800 repeating units called ommatidia. Each ommatidium consists of a precise array of eight photoreceptors and 12 accessory cells. In the absence of functional Sevenless, each ommatidium lacks a particular photoreceptor, the R7 cell. Studies of the development of flies mutant for *sev* show that the cell that would

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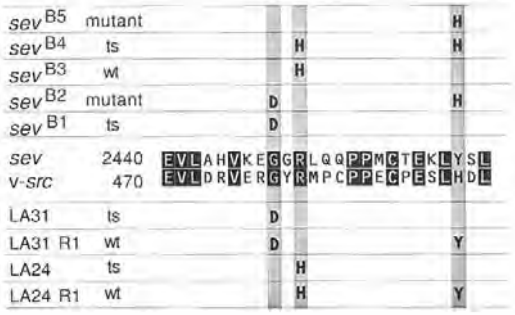

Figure 1. The Construction of Temperature-Sensitive *sev* Alleles

Site–directed mutagenesis was used to generate five new *sev* alleles, which were then placed into sev^{d2} flies by P element–mediated germline transformation. These mutations were based on the sequences of two temperature-sensitive alleles of v-*src*, LA24 and LA31, and two revertants, LA24 R1 and LA31 R1 (Fincham and Wyke, 1986). The central portion of the figure shows a comparison of the amino acid sequences of portions of the Sevenless protein and $pp60^{v\text{-}src}$. The phenotype of the v-*src* alleles and the changes in the $pp60^{v\text{-}src}$ amino acid sequence are shown below. The mutations that were induced in *sev* and their phenotypes are indicated above the comparison. The phenotypes of flies carrying the mutant *sev* alleles were examined by the reduced corneal pseudopupil method at 18°C and 29°C.

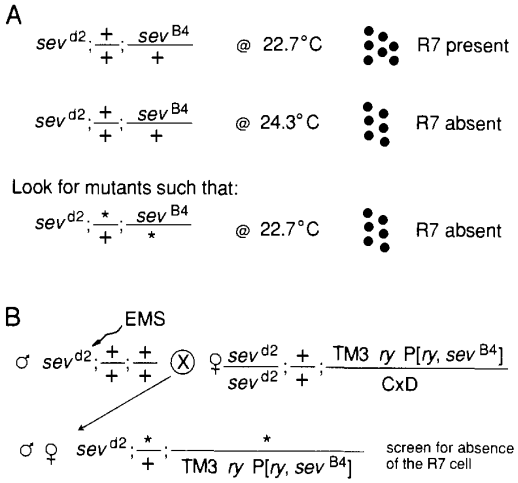

Figure 2. The Scheme for Isolating *E(sev)* Mutations

(A) is a summary of the phenotypes of the sev^{B4} allele at different temperatures. The black circles are a depiction of the observed reduced corneal pseudopupil when the flies are reared at the indicated temperature. The reduced corneal pseudopupil represents an optical projection of the positions of the rhabdomeres, the light-sensing organelles of the photoreceptors, from a group of neighboring ommatidia. The R7 rhabdomere is represented by the central circle of the phenotypically wild-type image observed when sev^{B4} flies are reared at 22.7°C. Each asterisk represents a mutagen-treated chromosome. (B) is a more detailed description of the screen for *E(sev)* mutations. Mutagenized sev^{d2} male flies were mated with sev^{d2}; *CxD*, *D/TM3*, *Sb*, *ry*, P[*ry*, sev^{B4}] females at 22.7°C. The male and female *Sb* progeny (genotypes sev^{d2}/Y: */+; */*TM3*, *Sb*, *ry*, P[*ry*, sev^{B4}] or sev^{d2}/sev^{d2}; */+; */*TM3*, *Sb*, *ry*, P[*ry*, sev^{B4}]) were scored for the absence of R7 photoreceptors. At this temperature, approximately 2% of the female *Sb* flies and less than 1% of the male *Sb* flies display a reduced corneal pseudopupil lacking an R7 image in the absence of mutagenesis. Flies with reduced corneal pseudopupils lacking the R7 image were backcrossed to sev^{d2}; *CxD*, *D/TM3*, *Sb*, *ry*, P[*ry*, sev^{B4}] flies in order to determine which flies carried *E(sev)* mutations. The mutant progeny of this cross were then used to balance the *E(sev)* mutation. The mutagen-treated sev^{d2} flies were isogenic for both the second and third chromosomes.

normally become the R7 photoreceptor cell instead develops as a nonneuronal lens-secreting cone cell (Tomlinson and Ready, 1986, 1987). Analysis of ommatidia that are mosaic for the *sev* mutation has demonstrated that the requirement for Sevenless function is limited to the R7 cell itself. Thus, Sevenless is proposed to act as a receptor for a signal that determines whether the presumptive R7 cell becomes a photoreceptor or a nonneuronal cell.

In this report we describe a genetic screen for mutations that decrease the effectiveness of signaling by Sevenless. This screen has allowed us to identify seven genes, *Enhancers of sevenless*, that when mutated can make the phenotype of a temperature-sensitive *sev* allele more severe. We have molecularly characterized two of the loci. One locus, *Ras1* (formerly *Dras1*; Neuman-Silberberg et al., 1984; Brock, 1987), encodes a *ras* protein. The other locus, *Son of sevenless* (*Sos*), has been previously identified on the basis of a dominant allele that enhances signaling by a mutant *sev* protein (Rogge et al., 1991). We show that *Sos* encodes a protein that is homologous to the *CDC25* protein, an activator of guanine nucleotide exchange by *RAS* proteins in budding yeast (Broek et al., 1987; Robinson et al., 1987; Jones et al., 1991). These results imply that an increase of *ras* protein activity is a key element in signaling by the *sev* PTK and further suggest that this activation may be achieved by stimulating GDP–GTP exchange of the *Ras1* protein.

Results

Enhancers of sevenless Define Seven Loci

The simplest way to isolate mutations that might affect signaling by the *sev* PTK would be to isolate new recessive mutations that result in the absence of the R7 cell. This approach has led to the discovery of two genes: *bride of sevenless*, which appears to encode the ligand for Sevenless (Reinke and Zipursky, 1988; Hart et al., 1990; Krämer et al., 1991), and *seven in absentia*, which encodes a nuclear protein and is therefore unlikely to be directly involved in the early steps of Sevenless-mediated signaling (Carthew and Rubin, 1990). We chose a different strategy, because we suspected that much of the pathway downstream of the *sev* PTK would be shared with other PTKs. While Sevenless function is entirely dispensible for the viability of the fly, at least one other PTK is essential (Schejter and Shilo, 1989; Price et al., 1989). Furthermore, if other PTKs have crucial roles in the development of the eye, disruption of their signaling pathways might result in such disorganized eyes that the presence of the R7 cell would be impossible to score. Mutations that inactivate the common PTK signaling components would therefore be missed in a genetic screen for recessive mutations that cause the absence of the R7 cell. We therefore devised a

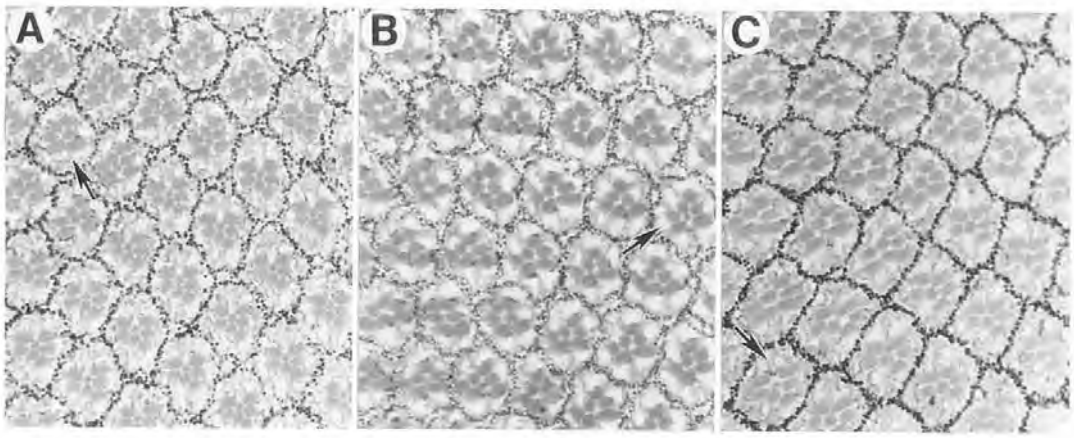

Figure 3. The Phenotype of *E(sev)* Mutations

Each panel is a photomicrograph of a sectioned eye. The flies were reared at 22.7°C. The anterior of each eye is to the right. (A) shows the phenotype of an eye from a female fly containing the sev^{B4} allele in the absence of *E(sev)* mutations. The genotype is sev^{d2}; +/*TM3*, *Sb*, *ry*, P[*ry*, sev^{B4}]. The dark circular structures in each ommatidial cluster are the rhabdomeres, the light-sensing organelles, of the photoreceptors. The rhabdomere of the R7 cell is in the center of each cluster and is surrounded by the rhabdomeres of photoreceptors R1–R6. The rhabdomere of R8 lies proximally in the retina and is not visible in these sections. The R7 cell rhabdomere is present in all but one ommatidial cluster. The arrow points to the exception. (B) and (C) show the phenotype of female flies carrying the $E(sev)3C^{e1B}$ or $E(sev)2A^{e4G}$ alleles, respectively. The genotypes are (B) sev^{d2}; $E(sev)3C^{e1B}$/*TM3*, *Sb*, *ry*, P[*ry*, sev^{B4}] and (C) sev^{d2}; $E(sev)2A^{e4G}$/+; +/*TM3*, *Sb*, *ry*, P[*ry*, sev^{B4}]. In each fly, nearly all of the ommatidial clusters are lacking an R7 rhabdomere and thus an R7 photoreceptor. One exceptional ommatidium with an R7 cell is seen in each panel and is indicated with an arrow. In each panel, the most posterior row of ommatidia is approximately three rows from the posterior edge of the eye. We chose this region because this is the area of the eye that we assay by the reduced corneal pseudopupil method. There is an apparent difference in the quantity of Sevenless activity that is required in different regions of the eye. When Sevenless activity is limited, such as when flies carrying the sev^{B4} allele are reared at 22.7°C, ommatidia in the anterior region of the eye generally lack an R7 cell, while more posterior clusters are still phenotypically wild-type.

more sensitive genetic screen that allows the identification of mutations that reduce rather than abolish the activity of downstream signaling proteins.

Systematic studies of the effects of chromosomal deletions on fly development have indicated that there are very few loci that have observable haploid phenotypes (Lindsley et al., 1972). Other studies have shown that, in general, the level of expression of a locus is proportional to the gene copy number (Muller et al., 1931). Given these considerations, our strategy was to create conditions in which signaling through the Sevenless pathway is so barely adequate that even 2-fold reductions in the gene activity of downstream elements of the pathway might disrupt signaling. Under these sensitized conditions, a recessive loss-of-function mutation in a gene encoding a component of the signaling pathway might yield a dominant R7-minus phenotype even though it only inactivates one copy of the gene. We refer to such mutations as *Enhancers of sevenless*.

An essential requirement for conducting such a genetic screen for *Enhancers of sevenless* is the ability to precisely control the strength of signaling by Sevenless. Since we could not predict the exact level of signaling that would be ideal for this genetic screen, we constructed two temperature-sensitive alleles of *sev* that allow fine control of signaling strength. The temperature-sensitive alleles were produced by site-directed mutagenesis of the *sev* gene based on the sequence of two temperature-sensitive v-*src* alleles that change amino acid residues conserved between v–*src* and *sev* (Fincham and Wyke, 1986). Five *sev* mutations were constructed and placed in flies using P element–mediated transformation (Figure 1). Two alleles, sev^{B1} and sev^{B4}, are temperature-sensitive.

The screen for *Enhancers of sevenless* was conducted as outlined in Figure 2 using the sev^{B4} allele. The presence of the R7 cell was scored by the reduced corneal pseudopupil method, which allows the rapid scoring of the *sev* phenotype in live flies (Franceschini and Kirschfeld, 1971). At 22.7°C, the reduced corneal pseudopupils of flies carrying the sev^{B4} allele are wild type, while at 24.3°C, the reduced corneal pseudopupil is clearly lacking the R7 cell image. We screened for ethylmethylsulfonate-induced mutations that result in a reduced corneal pseudopupil lacking the R7 cell image at 22.7°C.

Approximately 30,000 progeny of mutagenized flies were screened for the absence of R7 cells. Twenty *Enhancer of sevenless* (*E(sev)*) mutations were isolated and localized to individual chromosomes. None of these mutations as heterozygotes can prevent the development of the R7 cell of a fly that contains a sev^{+} gene (data not shown). Two examples of enhancement of the temperature-sensitive sev^{B4} phenotype by *E(sev)* mutations are shown in Figure 3. Eyes from *E(sev)*/+ flies generally contain less than 10% wild-type ommatidia, while eyes from flies that do not carry an *E(sev)* mutation generally contain greater than 90% wild-type ommatidia. However, the extent of enhancement is variable and dependent on the exact temperature at which the flies are reared.

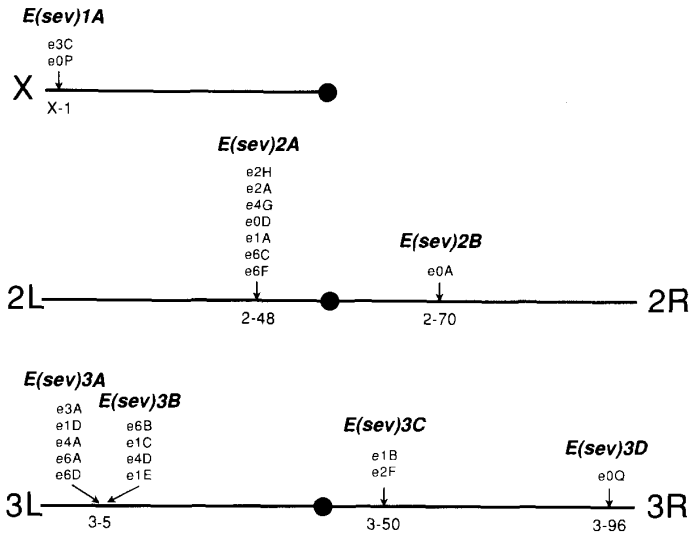

Figure 4. Genetic Mapping of *E(sev)* Mutations

The *E(sev)* mutations were mapped and tested for possible allelism as described in Experimental Procedures. The approximate recombination map positions (±5 centimorgans) of each locus are shown below the arrows. The map positions of *E(sev)3A* and *E(sev)3B* are only accurate to within 10 centimorgans due to their distance from the nearest marker. The *E(sev)3A*, *E(sev)3B*, and *E(sev)3C* mutations define three essential genes. Animals carrying two mutations of the same groups rarely or never survive to adulthood. The *E(sev)2A* mutations also define an essential gene; however, several of the alleles retain some activity. Animals carrying two strong alleles of the locus, such as *E(sev)2A*^{e4G} and *E(sev)2A*^{e2H}, die before pupation, while animals that carry two weak alleles, such as *E(sev)2A*^{e0D} and *E(sev)2A*^{e1A} frequently eclose as small adults with extremely rough and disordered eyes. The placement of the two X-linked *E(sev)* mutations, *E(sev)1A*^{e3C} and *E(sev)1A*^{e0P}, into a single group is provisional. The *E(sev)1A*^{e3C} mutation is viable in hemizygous males and has a weak effect on the phenotype of the *sev*^{B4} allele, while the *E(sev)1A*^{e0P} mutation is tightly linked to a recessive lethal mutation and has a strong effect on the phenotype of *sev*^{B4} allele. We have chosen to display these mutations as allelic because the two mutations map to the same region and are both capable of suppressing the phenotype of the *Elp* mutation. This interpretation of the data suggests that the *E(sev)1A*^{e3C} allele retains some residual activity. However, the possibility that these two mutations affect two separate genes that each modify signaling by the *sev*^{B4} and *Elp* PTKs cannot be excluded.

The *E(sev)* mutations were then mapped by recombination to approximate chromosomal positions. To test whether mutations that map to the same region are allelic, we crossed flies carrying nearby *E(sev)* mutations to each other. Assuming that these genes are essential for viability, then an animal carrying two *E(sev)* mutations that inactivate the same gene should be inviable. The results of the mapping and complementation experiments are shown in Figure 4 (see also Table 1). The 20 *E(sev)* mutations define seven loci, which can be mutated to disrupt signaling by the *sev*^{B4} protein. Multiple mutant alleles of *E(sev)2A*, *E(sev)3A*, *E(sev)3B*, and *E(sev)3C*, have been isolated and are recessive-lethal mutations, indicating that these loci encode essential functions. A fifth locus, *E(sev)1A*, may also have been identified by two mutations, but one allele is viable (see Figure 4 for a discussion of this locus). Two loci, *E(sev)2B* and *E(sev)3D*, have each been identified only once. Although the chromosomes carrying each of these unique mutations carry a recessive-lethal mutation, more alleles will be required to determine whether the recessive-lethal phenotypes are due to the *E(sev)* mutations. The distribution of the *E(sev)* mutations among only seven loci suggests that we have identified most of the loci that can be easily mutated to enhance the phenotype of the *sev*^{B4} allele.

Four of the *E(sev)* Loci Appear to Be Required for Photoreceptor Development

We next wished to examine the consequences of mutating both copies of the *E(sev)* loci for the development of the R7

Table 1. Summary of Properties of *Enhancer of sevenless* Loci

Locus	Number of Mutant Alleles	Required for Photoreceptor Development	Suppressor of Ellipse	Acts in R7	Potential Biochemical Activity
E(sev)1A	2[a]	Yes	Yes	Yes	
E(sev)2A/Sos	7	Yes	Yes	Yes[d]	Putative guanine nucleotide exchange factor
E(sev)2B	1	Yes	Yes		
E(sev)3A	5	?[b]	No		
E(sev)3B	4	?[b]	No		
E(sev)3C/Ras1	2	Yes	Yes	Yes	GTPase
E(sev)3D	1	No[c]	No		

[a] See Figure 4 for a discussion of this locus.
[b] No clones were observed. These results are consistent with a requirement for cell growth or viability.
[c] Occasional ommatidia with extra photoreceptors are observed.
[d] Rogge et al. (1991) have shown that a dominant allele of this locus acts in the R7 cell.

cell and other photoreceptors. Since the *E(sev)* mutations are, with one exception, present on recessive-lethal chromosomes, we generated clones of cells that were homozygous for the *E(sev)* mutations. For the loci that are represented by multiple alleles, at least two alleles were tested. The analysis of these clones allowed us to divide the *E(sev)* loci into three classes (Figure 5 and Table 1). Mutations at four loci appear to greatly reduce the ability of a cell to develop as either an R7 cell or as any other photoreceptor. This class includes *E(sev)1A*, *E(sev)2A*, *E(sev)2B*, and *E(sev)3C*. Despite the fact that these mutations were identified owing to their ability to interfere with R7 cell development, our results suggest that they must also play a role in some process that is common to the development of all photoreceptors. Homozygous clones of two of the loci, *E(sev)3A* and *E(sev)3B*, were never or rarely seen. This result is consistent with the conclusion that these loci are essential either for cell growth or viability. One locus, *E(sev)3D*, appears to be dispensable for both photoreceptor and R7 cell development. The homozygous E(sev)3D ommatidia are generally wild type, though occasionally an ommatidium has an extra photoreceptor. However, this locus is represented by a single mutant allele, which may retain some activity.

We examined embryos homozygous for mutations of either the *E(sev)2A* or the *E(sev)3C* loci to try to determine which steps of development require the function of these loci. In each case the mutant embyos hatch at frequencies equivalent to wild-type controls but die before the onset of pupal development. This may imply that the products of these loci do not have important roles during embryonic development. However, an equally likely possibility is that there are maternal contributions of these products that are sufficient for embryonic development. Analysis of embryos derived from homozygous mutant germline clones will be required to address this issue.

E(sev) Loci Function in the Developing R7 Cell

If the *E(sev)* loci do encode proteins that participate in the transduction of a signal from the activated *sev* PTK into the cell, then the proteins should function within the R7 cell itself rather than in the cell that transmits the signal. We have tested this prediction for two of the *E(sev)* loci, *E(sev)1A* and *E(sev)3C*, by generating marked clones of wild-type cells in a genetic background that contains the temperature-sensitive sev^{84} allele and is heterozygous for the *E(sev)* mutation. The ommatidia that are outside of the clone lack the R7 cell because of the enhancement of the sev^{84} phenotype by the *E(sev)* mutation, while the ommatidia that are entirely within the clone are genetically wild-type for the *E(sev)* locus and thus are phenotypically wild-type. Since there are no absolute cell lineage relationships between cells within an ommatidium, the ommatidia that are at the boundary of the marked clone will contain cells that are heterozygous for the *E(sev)* mutation and cells that are wild-type for the *E(sev)* locus. If the *E(sev)* locus exerts its effect in the developing R7 cell, then we would expect R7 cells developing in a mosaic ommatidium to be wild-type for the *E(sev)* locus. The genotype of the other photoreceptors should be irrelevant.

The results of such an analysis of mosaic ommatidia are shown in Figure 6 (see also Table 1). For the $E(sev)1A^{e0P}$ mutation, 40 of 41 R7 cells from phenotypically wild-type ommatidia were wild-type for the *E(sev)1A* locus. Similar results (34 of 35) were obtained for the $E(sev)3C^{e1B}$ mutation. The two exceptions are most likely due to the presence of a small fraction of R7 cells even in the presence of the *E(sev)* mutations (see Figure 2). These results are entirely consistent with the conclusion that the *E(sev)1A* and *E(sev)3C* mutations act in the developing R7 cell to attenuate signaling by the *sev* PTK. The tendency of the R1 and R6 cells to be related by lineage to R7 and the incomplete penetrance of the *E(sev)* phenotypes makes it difficult to absolutely eliminate the possibility that these mutations also act in the R1 and R6 cells to block the formation of the R7 cell. However, the data clearly rule out the possibility that the two *E(sev)* mutations act by decreasing the strength of signaling by the R8 cell from which the *bride of sevenless*–mediated signal originates (Reinke and Zipursky, 1988; Krämer et al., 1991).

Mutations of Four *E(sev)* Loci Attenuate Signaling by Another PTK

An underlying assumption of our study was that different PTKs use at least some common signaling pathways. We asked whether the *E(sev)* loci might encode such elements of a common signaling pathway by testing whether the *E(sev)* mutations could attenuate signaling by a PTK other than Sevenless.

The PTK that we chose to test was the *Ellipse* (*Elp*) allele of the *faint little ball* locus (also known as *torpedo* and *DER*). The *faint little ball* gene encodes a PTK receptor that is most homologous to the vertebrate EGF receptor and the *neu* proteins (Livneh et al., 1985; Schejter and Shilo, 1989; Price et al., 1989). Recessive loss-of-function mutations of *faint little ball* cause embryonic death. The mutation *Elp* is a dominant allele of the locus, which causes moderate roughness of the eye when heterozygous and severe eye roughness and reduced eye size when homozygous (Baker and Rubin, 1989). The basis for these phenotypes seems to be impaired formation of ommatidial clusters. The eyes of *Elp*/+ flies have normal ommatidia, but fewer of them than wild-type eyes. The *Elp* allele appears to encode an overly active protein that is so slightly activated that the activity provided by the normal wild-type copy of the gene is still a phenotypically significant fraction of the total activity; while *Elp*/+ flies have rough eyes, *Elp*/− flies have wild-type eyes. The sensitivity of the mutant eye to this slight change in gene activity suggested that if the *E(sev)* mutations did compromise signaling by the *Elp* PTK, the effect would be seen as a suppression of the *Elp* phenotype.

Flies carrying each *E(sev)* mutation were crossed to flies carrying the *Elp* mutation in order to generate flies that were heterozygous for both mutations. These flies were examined for the roughness of their eyes. The results are illustrated in Figure 7 (see also Table 1). Mutations in the *E(sev)3A*, *E(sev)3B*, and *E(sev)3D* loci had no effect on the *Elp* phenotype. Mutations in the remaining four loci were capable of suppressing the *Elp* phenotype. Three loci,

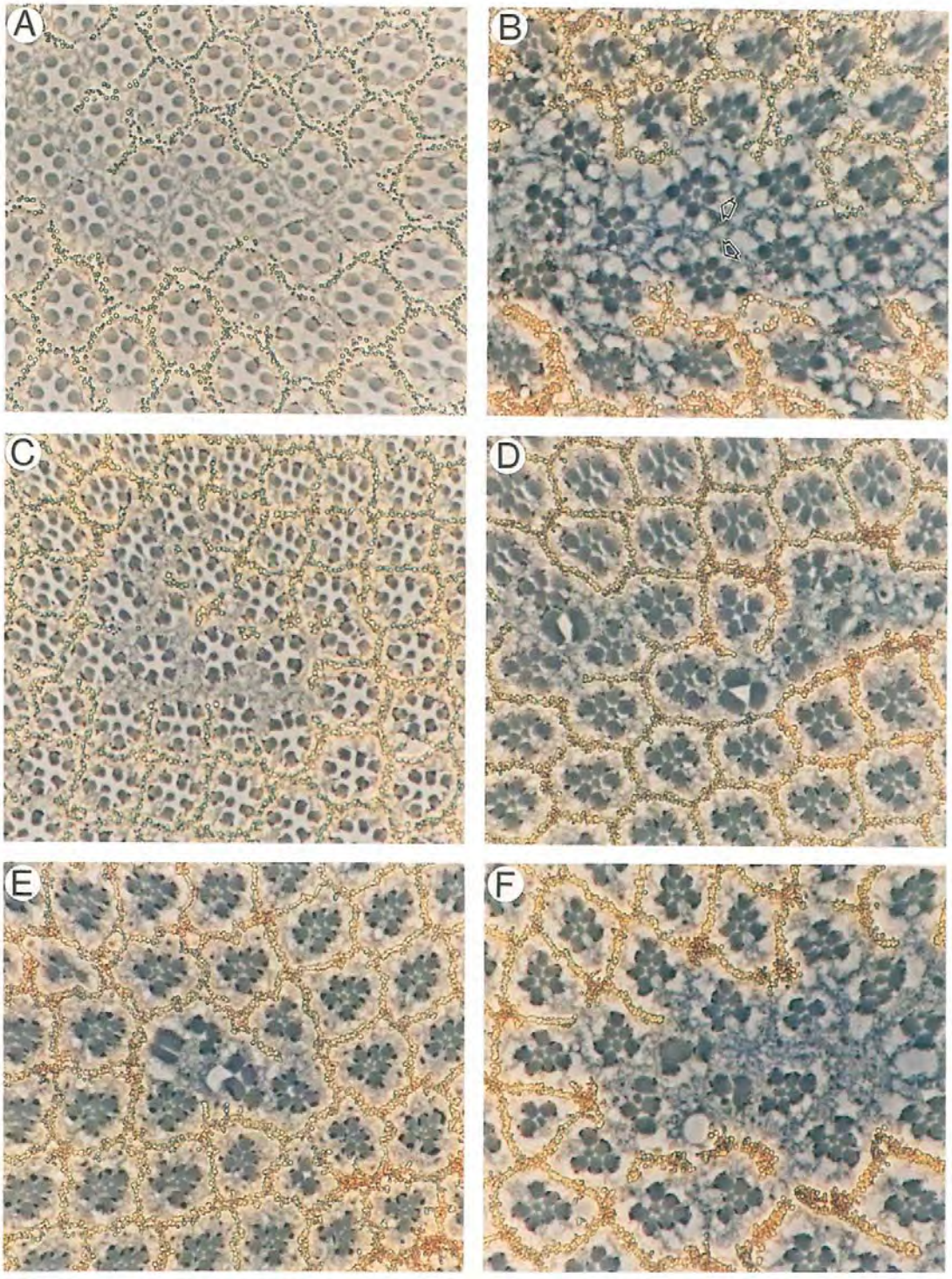

Figure 5. The Requirement for the *E(sev)* Loci during Photoreceptor Development

Animals heterozygous for an *E(sev)* mutation were X-irradiated in order to generate clones of cells homozygous for the *E(sev)* mutation. The homozygous *E(sev)* cells are marked by the absence of a *white*$^{+}$ gene. The *white* gene is required for the formation of pigment granules in both photoreceptors and pigment cells. The pigment granules of the photoreceptor cells are apparent as dark structures near the rhabdomere of each

E(sev)1A, *E(sev)2A*, and *E(sev)2B*, were very effective suppressors and restored the eye to a nearly normal appearance. Mutations at a fourth locus, *E(sev)3C*, were less efficient in their suppression, but were clearly distinguishable from *Elp*/+ flies.

These results suggest that at least four of the *E(sev)* loci encode proteins that somehow participate in a signaling pathway that is shared by at least two PTK receptors. The same four loci are required for the formation of photoreceptor cells. These results suggest that the products encoded by these four loci may be functionally linked, perhaps as parts of a single pathway. We therefore chose to concentrate on the molecular cloning of two of these four loci.

The *E(sev)3C* Locus Is the *Ras1* Gene

The *E(sev)3C* locus is represented by two mutant alleles. Recombination mapping of the alleles placed the locus on the right arm of the third chromosome near *curled*. The position was further refined by complementation mapping using deletions for the region. Two deletions were particularly informative. The chromosome carrying *Df(3R)by10* is deleted for the 85D8–11 to 85E10–13 region and is unable to complement the lethality of either *E(sev)3C* mutation. The chromosome carrying *Df(3R)by62* is deleted for the region 85D17–22 to 85F13–16 and rescues the lethality of either allele (Kemphues et al., 1980). These results place the *E(sev)3C* locus in the chromosomal region 85D8–22.

One gene that is both believed to play a role in signal transduction and that is known to map to this region is *Ras1*, the Drosophila homolog of the c-*H-ras1* gene of vertebrates (Neuman-Silberberg et al., 1984). We sought in two ways to test directly whether the *E(sev)3C* mutations mapped to *Ras1*. Our initial approach was to place a 12 kb DNA fragment containing the *Ras1* gene into the germline by P element–mediated transformation and test whether this construct would rescue the lethality of animals carrying *E(sev)3C* mutations. The P element carrying *Ras1*, P[*w*,*Ras1*A], was capable of rescuing the lethality of $E(sev)3C^{e1B}/E(sev)3C^{e2F}$ flies. This result indicates that the *E(sev)3C* gene lies within the 12 kb transformation fragment.

The *E(sev)3C* mutations were then localized to the *Ras1* gene itself by sequencing the *Ras1* genes from the *E(sev)3C* mutant chromosomes. The sequence of the entire coding sequence of each allele was determined and compared with a previously published sequence of *Ras1*. Both mutant alleles contained changes that alter the amino acid sequence of the resulting *Ras1* protein (Figure 8). The *Ras1* allele from the $E(sev)3C^{e1B}$ chromosome contains a point mutation that changes glutamate-62 to a lysine. The $E(sev)3C^{e2F}$ allele contains a point mutation that changes aspartate-38 to an asparagine. We conclude from these results that *E(sev)3C* is *Ras1*. The two mutant alleles have therefore been renamed $Ras1^{E62K}$ and $Ras1^{D38N}$.

The *E(sev)2A* Locus Encodes a Potential Guanine Nucleotide Exchange Factor

E(sev)2A was the second gene that we sought to identify. This locus is represented by seven mutant alleles. The mutations differ significantly in their ability to enhance the sev^{84} phenotype and to suppress the *Elp* phenotype. When strong alleles, such as $E(sev)2A^{e2H}$ and $E(sev)2A^{e4G}$, are crossed to each other, viable *E(sev)2A*/*E(sev)2A* progeny are not observed. However, combinations of weak alleles, such as $E(sev)2A^{e0D}$ and $E(sev)2A^{e1A}$, are generally viable, but the mutant progeny have rough eyes (data not shown).

Recombination mapping indicated that the *E(sev)2A* locus is near the *black* gene (polytene position 34D4–6). Rogge et al. (1991) have recently reported the isolation of a dominant allele of the gene *l(2)34Ea* that maps adjacent to *black* (Woodruff and Ashburner, 1979; M. Ashburner and J. Roote, personal communication). This mutant allele was identified by its ability to suppress a particular mutant allele of *sev*. The locus was renamed *Son of sevenless* (*Sos*). These results suggested the possibility that the *E(sev)2A* alleles might also be allelic to *l(2)34Ea* mutations. Crosses between *E(sev)2A* alleles and two alleles of *l(2)34Ea* confirmed this hypothesis (data not shown). We will therefore refer to the *E(sev)2A* locus as *Sos*.

The extensive genetic analysis available for the 34D region suggested that *Sos* is the first essential gene distal to *black*. This places *Sos* in the vicinity of polytene band 34D4. We isolated the DNA from this region by chromosome walking. The starting point for the walk was a hobo transposable element inserted in polytene band 34D1 (Blackman et al., 1989). We isolated overlapping cosmid

photoreceptor, but pigment granules are not present in every section. (A) shows a clone of *white* cells in the absence of *E(sev)* mutations. The spacing and construction of the ommatidial clusters is normal. (B) shows a clone of cells homozygous for the $E(sev)3D^{e0Q}$ mutation. Most of the ommatidia are phenotypically wild-type, but occasionally ommatidia contain an extra photoreceptor (see arrows). The remaining panels show the phenotypes of clones of cells that are homozygous for different *E(sev)* mutations: (C), $E(sev)1A^{e0P}$; (D), $E(sev)2A^{e2H}$; (E), $E(sev)2B^{e0A}$; (F), $E(sev)3C^{e1B}$. Analysis of a number of mosaic ommatidia, as judged by the presence of at least one wild-type and one mutant pigment cell, indicates that cells homozygous for any of these mutations are greatly underrepresented as photoreceptors. Approximately half of the photoreceptors from mosaic ommatidia are expected to be homozygous for *white* in the absence of *E(sev)* mutations. The fraction of photoreceptors from mosaic ommatidia that were homozygous for the *E(sev)* mutations were as follows: $E(sev)1A^{e0P}$, 0/384; $E(sev)2A^{e2H}$, 4/345; $E(sev)2B^{e0A}$, 2/411; $E(sev)3C^{e1B}$, 15/798. Similar results were obtained for clones homozygous for the $E(sev)3C^{e2F}$ mutation. The analysis of other alleles of the *E(sev)2A* locus was more complex. Clones of cells homozygous for the $E(sev)2A^{e4G}$ allele were similar to those for the $E(sev)2A^{e2H}$ allele. However, clones homozygous for the $E(sev)2A^{e0D}$ allele were more normal. Many ommatidia lacked one or two photoreceptors, but homozygous $E(sev)2A^{e0D}$ photoreceptors were seen. Since the $E(sev)2A^{e2H}$ and $E(sev)2A^{e4G}$ alleles both contain premature termination codons and produce substantially truncated proteins, we conclude that this is the null phenotype for this locus (see Figure 10). The phenotype of clones of cells homozygous for the $E(sev)2A^{e0D}$ mutation is similar to that observed by Rogge et al. (1991) for an allele of this locus, called $Son\ of\ sevenless^{X122}$ (see main text). When tested for lethality in trans to other *E(sev)2A* alleles, the $E(sev)2A^{e0D}$ mutation has a weaker effect than either $E(sev)2A^{e2H}$ or $E(sev)2A^{e4G}$. Furthermore, the $E(sev)2A^{e0D}$ mutation is a missense mutation (see Figure 10). We conclude that $E(sev)2A^{e0D}$ and the previously reported allele, $Son\ of\ sevenless^{X122}$, retain partial activity.

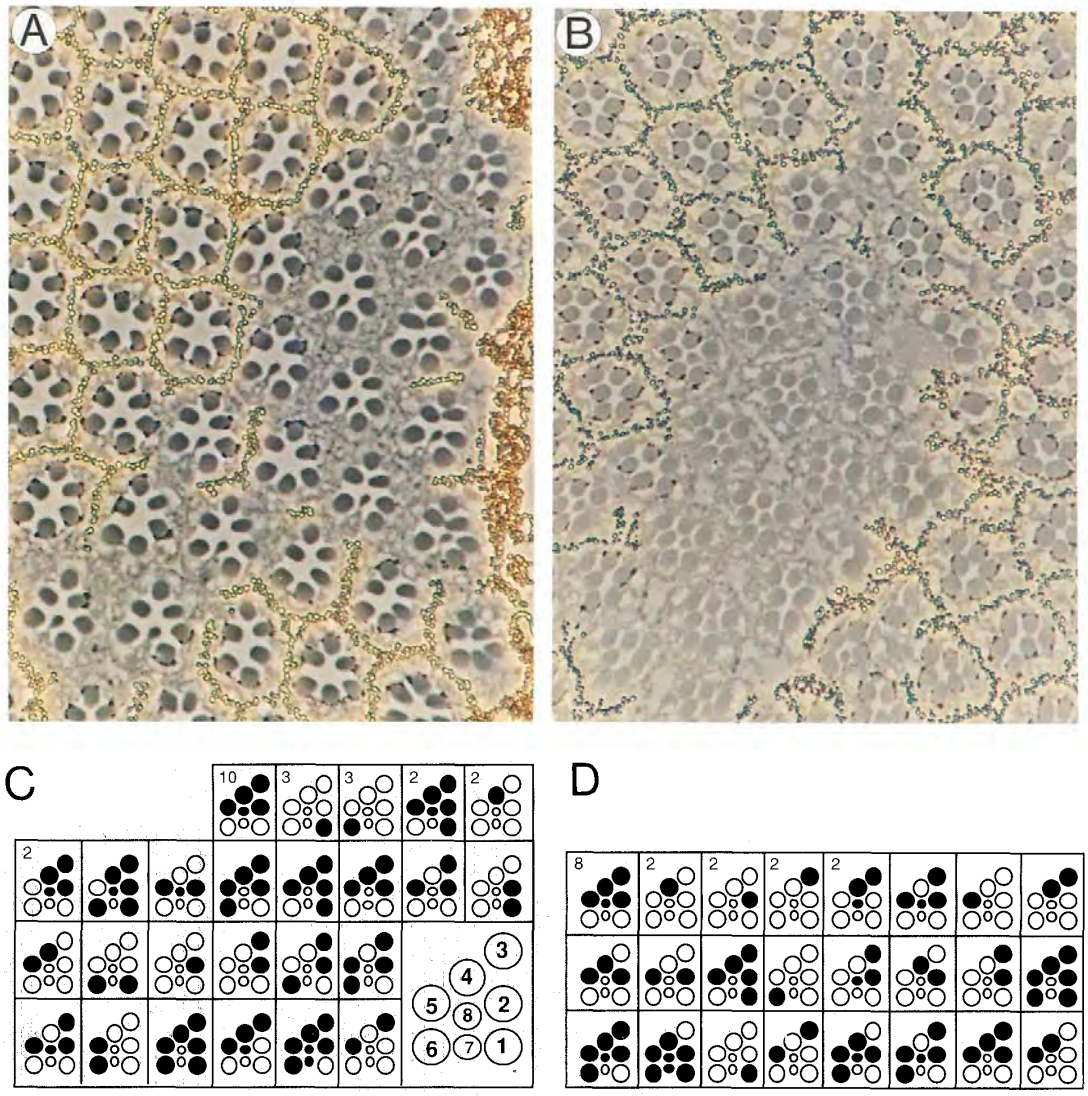

Figure 6. The *E(sev)1A*^{e0P} and *E(sev)3C*^{e1B} Mutations Act in the R7 Cell

X-irradiation was used to generate marked clones of *E(sev)*$^{+}$ cells in flies that carried the *sev*^{B4} allele and were heterozygous for the *E(sev)* mutation. The cells of the clones were genetically marked by the absence of a functional *white* gene and thus lack pigment granules. If an *E(sev)* mutation exerts its effect in a particular cell, then that cell should tend to be *white* in mosaic ommatidia that are wild-type in construction. (A) and (B) are photomicrographs of such clones for the (A) *E(sev)1A*^{e0P} and (B) *E(sev)3C*^{e1B} alleles. In each case, the presence of the *E(sev)* mutation in the cells outside of the clone blocks the ability of the *sev*^{B4} allele to initiate R7 development. (C) and (D) illustrate the genetically mosaic and phenotypically wild-type classes of ommatidia that were observed for *E(sev)1A*^{e0P} and *E(sev)3C*^{e1B}, respectively. When a given genotypic class was observed more than once, the number of occurrences is indicated in the upper left of the box. Open circles represent *white*, *E(sev)*$^{+}$ photoreceptors, while closed circles represent *E(sev)/+* photoreceptors. The data can be summarized as the fraction of *white* cells for each photoreceptor. For *E(sev)1A*^{e0P}, the data are (41 total): R1, 10; R2, 28; R3, 27; R4, 24; R5, 25; R6, 10; R7, 1; R8, 19. For *E(sev)3C*^{e1B}, the data are (35 total): R1, 4; R2, 23; R3, 21; R4, 22; R5, 22; R6, 6; R7, 1; R8, 16. In each case, the data are most consistent with the conclusion that the *E(sev)* mutation acts in the R7 cell. Since the extent of enhancement by the *E(sev)* alleles varies with very slight changes of the incubator temperature, we discarded clones when more than one phenotypically wild-type ommatidium that was not part of the clone was apparent in the sectioned region. Occasionally, severely defective ommatidia were seen along the edge of the clones. These ommatidia presumably contained homozygous *E(sev)* cells that were generated by the mitotic recombination.

clones representing the approximately 150 kb of DNA in the 34D1–8 region. The approximate position of the *Sos* gene within the cloned DNA was determined by analyzing the breakpoints of several chromosomal rearrangements. This analysis indicated that at least part, if not all, of the *Sos* gene is contained within an 8 kb interval corresponding to coordinates 110 to 118 (see Figure 9). This prediction was tested by placing a 10 kb BglII–XbaI DNA

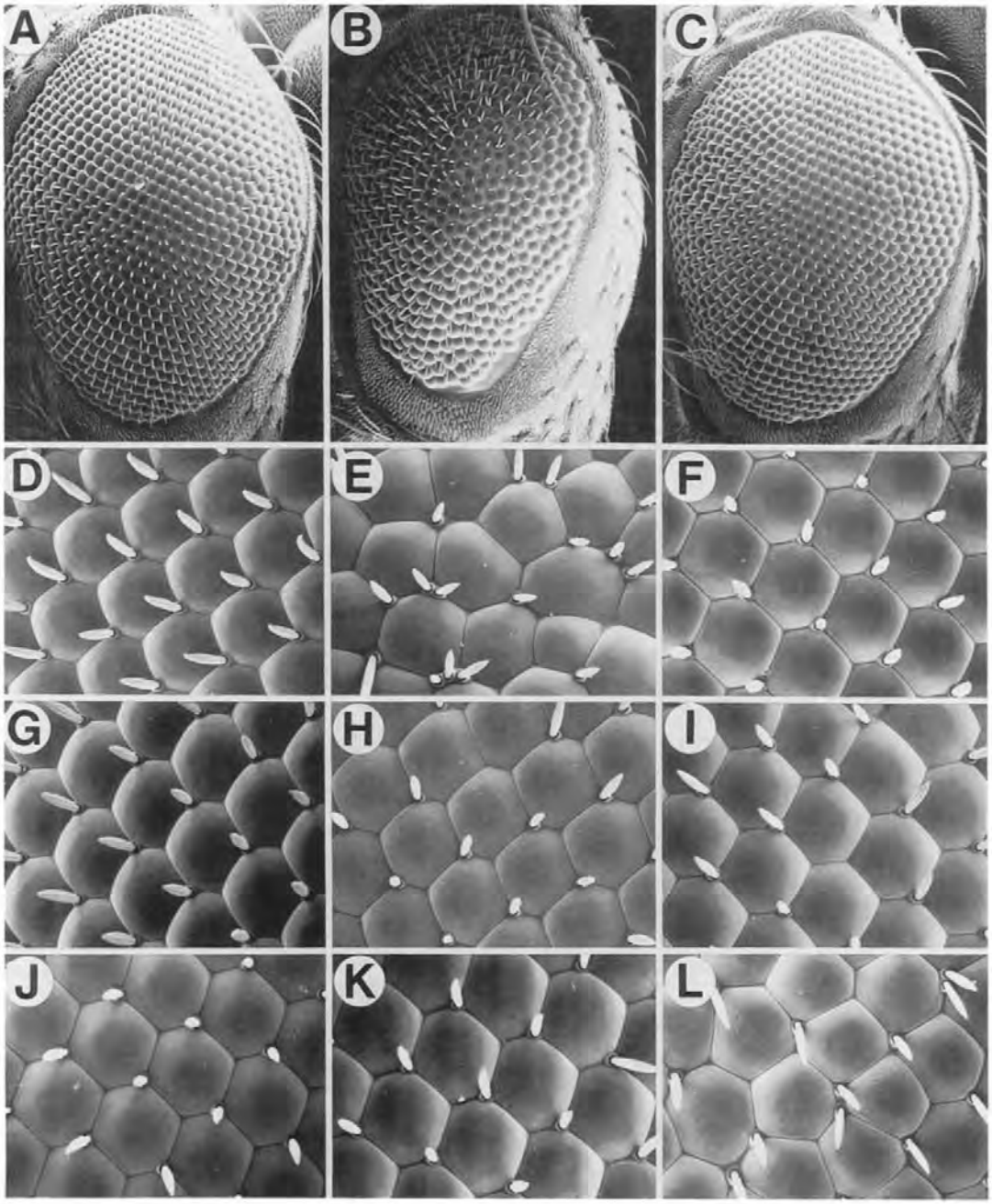

Figure 7. The Effect of *E(sev)* Mutations on the Phenotype of the *Elp* Mutation

All of the eyes shown except those in (A) and (D) are from female flies heterozygous for the sev^{d2} mutation. (A) Scanning electron micrograph (SEM) of an eye from a female wild-type (CantonS) fly. (B) SEM of an eye from a fly heterozygous for the *Elp* mutation. The *Elp* mutation causes reduction in eye size and roughening of the eye surface (Baker and Rubin, 1989). (C) SEM of an eye from a fly heterozygous for both $E(sev)2A^{e2H}$ and *Elp*. The presence of the *E(sev)* mutation strongly suppresses the phenotype of the *Elp* mutation. (D) to (L) show higher magnification SEMs that illustrate the effect on the *Elp* phenotype by different *E(sev)* mutations. (D) is an SEM of an eye from a wild-type (CantonS) female fly. (E) is an SEM from a fly heterozygous for the *Elp* mutation. The flies in (F) to (L) were heterozygous for the *Elp* mutation and the indicated *E(sev)* mutation: (F), $E(sev)2A^{e2H}$; (G), $E(sev)2A^{e4G}$; (H), $E(sev)3C^{e1B}$; (I), $E(sev)3C^{e2F}$; (J), $E(sev)1A^{e0P}$; (K), $E(sev)2B^{e0A}$; (L), $E(sev)3B^{e1E}$. Since *Elp* disrupts the normal hexagonal array of the ommatidial lenses and bristles, the suppression of *Elp* can be scored as the return of the normal hexagonal array. $E(sev)1A^{e0P}$, $E(sev)2A^{e4G}$, $E(sev)2A^{e4G}$, and $E(sev)2B^{e0A}$ largely reverse the effects of the *Elp* mutation. The mutations $E(sev)3C^{e1B}$ (H) and $E(sev)3C^{e2F}$ (I), partially suppressed the *Elp* phenotype. Note that there are still some irregularities in the shapes of the lenses and in the placement of the bristles. Mutations at the remaining *E(sev)* loci had no effect (see [L] for an example).

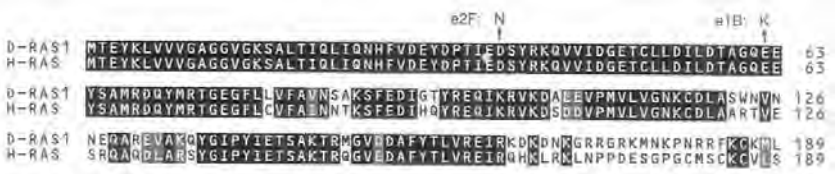

Figure 8. The *E(sev)3C* Locus Is *Ras1*

The sequence of the Drosophila *Ras1* protein (Brock, 1987) is compared with that of the Human p21$^{H\text{-}ras}$ (Capon et al., 1983). Identical residues are boxed in black. Similar residues are boxed in gray. The following amino acids were considered similar: E, D; V, L, I, M; A, G; F, Y, W; S, T; Q, N. The mutation present in the *E(sev)3C*^{e2F} allele changes the GAC aspartate-38 codon to an AAC asparagine codon. The mutation present in the *E(sev)3C*^{e1B} codon changes the GAG glutamate-62 codon to an AAG lysine codon. No other changes were observed.

fragment (coordinates 110 to 120) into the germline by P element–mediated transformation. This P element, P[*w*, *Sos*A] is capable of rescuing the lethality of animals carrying either strong allele, *E(sev)2A*^{e2H} or *E(sev)2A*^{e4G}, and a deletion of the *Sos* gene, *Df(2L)b*^{80c1}. This indicates that all essential functions of *Sos* are contained within this 10 kb region.

We isolated eye-antennal imaginal disc cDNA clones derived from this region. Two classes of nonoverlapping cDNA clones were categorized. One class originates from the distal portion of this interval and from the neighboring XbaI fragment. This class is not fully contained within the 10 kb rescue fragment and was not characterized extensively. The second class of cDNA clones represents an mRNA transcript that spans most of the region implicated by the deletion mapping. The sequences of a 5.3 kb cDNA clone and the genomic region encoding this transcript were determined. The deduced structure of the transcript is shown in Figure 9.

Conceptual translation of the long open reading frame of this transcript from the first in-frame methionine shows that it is capable of encoding a protein of 1596 amino acids (Figure 10). We determined that this open reading frame actually encodes the *Sos* protein by cloning this region from three of the chromosomes containing *Sos* mutations and sequencing the open reading frames (Figure 10). Each mutant allele contains a point mutation that changes the predicted protein sequence. For instance, the strong alleles, *Sos*^{e2H} and *Sos*^{e4G}, introduce termination codons at amino acid positions 579 and 421, respectively. The weak

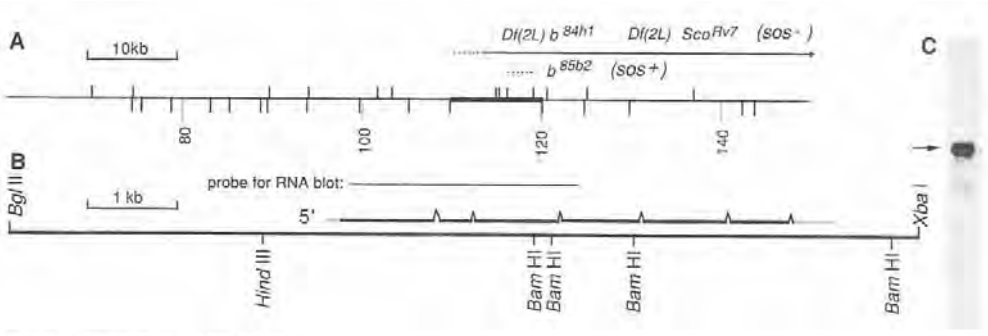

Figure 9. Structure of the *Sos* Gene

(A) A map of the region near *Sos*. The map of the *Sos* region is shown for restriction enzymes XbaI (marks below the line) and BamHI (marks above the line). Distal is to the left. The coordinates are given as distance from the insertion site of the hobo[*ry*] element that served as the entry point for the chromosomal walk. The approximate positions of chromosomal breakpoints were determined by genomic DNA blots and are indicated above the map. The solid bar represents DNA that is deleted from the *Df(2L)b*^{84h1} and *Df(2L)Sco*^{Rv7} chromosomes. These chromosomes are deficient for polytene bands 34D3 to 35A4 and 34D5 to 35D5–7, respectively (M. Ashburner and J. Roote, personal communication). The broken line represents the uncertainty in assigning the position of the distal breakpoint. The nature of the *b*^{85b2} rearrangement is not entirely clear, but there appears to be a breakpoint in the region indicated by the broken line.

(B) The structure of the *Sos* transcript. The region (coordinates 110 to 120) indicated by the bold line in (A) is shown. The deduced structure of the *Sos* mRNA is shown above the map. The exon and intron structure was deduced from a comparison of the genomic sequence of the 6601 bp HindIII to SalI region with the sequence of a 5.3 kb cDNA clone that was isolated from an eye imaginal disc cDNA library. The cDNA clone was incomplete at both ends. The indicated position (nucleotide 6451) of the polyadenylation site was determined from an *Sos* cDNA clone derived from an embryonic cDNA library that included a poly(A) tail. The 5′ end of the mRNA was determined by S1 mapping and primer extension studies and is heterogeneous. There appear to be several start sites; the two major sites map to the 440 to 470 region. In the diagram of the mRNA structure, protein coding regions are indicated by the black line and 5′- and 3′-untranslated segments by the gray line. The 10 kb BglII–XbaI fragment is capable of rescuing the recessive lethality of *Sos* mutations when placed into the genome by P element–mediated transformation.

(C) Size of the *Sos* mRNA. Polyadenylated RNA was extracted from imaginal discs. Two micrograms of RNA was fractionated, blotted, and hybridized to ^{32}P-labeled DNA from the region indicated in (B). A single transcript of approximately 6 kb was detected. Analysis of embryonic RNA revealed a transcript of the same size (data not shown).

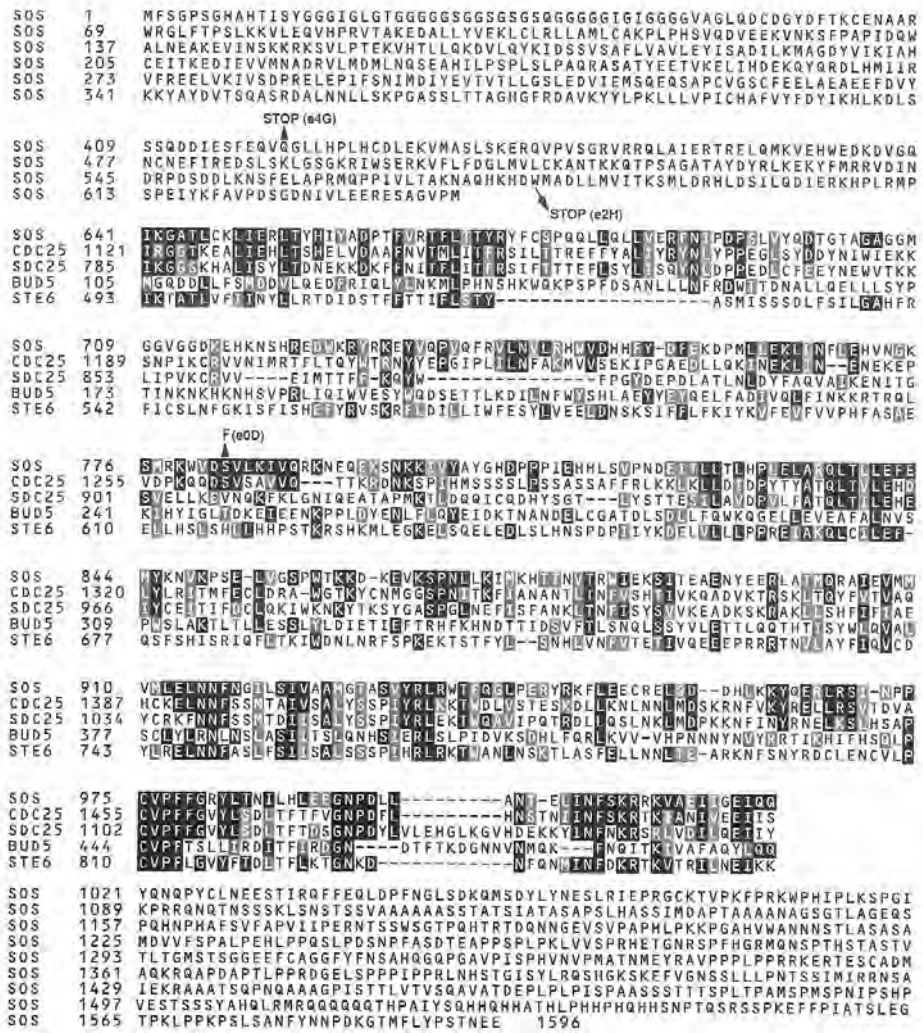

Figure 10. The Predicted Amino Acid Sequence of the *Sos* Protein

A conceptual translation of the long open reading frame of the *Sos* RNA transcript is shown. The translation begins at the first in-frame methionine codon, which is approximately 370 bases from the 5′ end of the transcript (position 841 in the sequenced genomic DNA). The coding regions of three mutant alleles were sequenced. The positions of mutations that change the coding capacity of the transcript are indicated: Sos^{e4G}, CAG codon to TAG; Sos^{e2H}, TGG codon to TGA; Sos^{e0D}, TCC codon to TTC. The Sos^{e4G} allele contains one additional change (C to T at position 1473 in the genomic sequence) that does not affect the protein sequence. The predicted *Sos* protein sequence is compared with the sequences of four related proteins: the S. cerevisiae *CDC25* protein (Camonis et al., 1986; Broek et al., 1987); the S. cerevisiae *SDC25* protein (Damak et al., 1991); the S. cerevisiae *BUD5* protein (Chant et al., 1991; Powers et al., 1991); and the S. pombe *STE6* protein (Hughes et al., 1990). Residues are boxed wherever they are identical or similar to the *Sos* protein sequence. Identical residues are boxed in black. Similar residues are boxed in gray. The following amino acids were considered similar: E, D; V, L, I, M; A, G; F, Y, W; S, T; Q, N. The alignments were constructed using the Genalign program (Intelligenetics, Inc.)

allele, Sos^{e0D}, has a point mutation that changes serine-783 to a phenylalanine. We therefore conclude that this transcript encodes the *Sos* protein.

Comparison of the predicted *Sos* protein sequence with previously reported sequences shows considerable homology with the *CDC25*, *SDC25*, and *BUD5* proteins of Saccharomyces cerevisiae and with the *STE6* protein of Schizosaccharomyces pombe (Figure 10). The *Sos* protein is most similar to the *CDC25* and *SDC25* proteins. These proteins share a homologous domain that is approximately 380 amino acids in length. For the alignment shown, the *CDC25* and *SDC25* proteins are 28% and 22% identical, respectively, to the predicted *Sos* protein in this region.

Genetic studies have suggested that the *CDC25* protein functions by activating *ras* protein activity (Broek et al.,

1987; Robinson et al., 1987). Biochemical studies have demonstrated that the *CDC25* and *SDC25* proteins can act as guanine nucleotide exchange factors to stimulate the conversion of inactive GDP-bound *RAS2* protein to the active GTP-bound form (Jones et al., 1991; Créchet et al., 1990). These studies have further shown that a fragment of the *SDC25* protein that includes the homology to *Sos* is sufficient to catalyze nucleotide exchange by either the *RAS2* protein or by the human c-*H-ras* protein. These results suggest that the region of homology defines a domain that catalyzes guanine nucleotide exchange by *ras* proteins and that the *Sos* protein may also act by catalyzing the activation of a *ras* protein.

Discussion

In this study, we sought to identify genes that are required for signaling by the *sev* PTK. Our strategy was to limit the strength of signaling initiated by Sevenless so that the signal was barely adequate to achieve its function. We reasoned that when the *sev* PTK activity was barely above the threshold necessary for R7 cell formation, small reductions in the abundance or activity of other elements of the pathway might lower the signal strength sufficiently to cause a mutant *sev* phenotype. Our hope was that this system would be so sensitive to the levels of components of the pathway that mutations which inactivate only one copy of a gene that encodes a downstream element of the pathway would result in the absence of the R7 cell. Since the other copy of the gene would remain functional, these loci, *Enhancers of sevenless*, could be identified even if their function is essential for producing a fertile fly.

Analysis of the first 20 *E(sev)* mutations identified seven *E(sev)* loci. For the two best characterized loci, *Sos* and *Ras1*, the mechanism of enhancement is inactivation of one copy of an essential gene. The nature of the *E(sev)* mutations in the other five loci has not been determined. However, for at least two loci, *E(sev)3A* and *E(sev)3B*, the frequency of *E(sev)* alleles is consistent with the proposal that these mutations are loss-of-function alleles. Furthermore, our assumption that many of the genes whose products are required for normal signaling would be essential for viability appears to be correct for at least four of the seven *E(sev)* loci. We do not have enough alleles of the other loci to determine whether they are essential genes. The distribution of alleles among the *E(sev)* loci suggests that we have identified most, but probably not all, of the loci that can be easily mutated to enhance the sev^{B4} phenotype.

This screen was designed to identify genes that might encode proteins that participate directly in signaling by Sevenless. Mutations in several other classes of genes might also be capable of producing an *E(sev)* phenotype. For example, mutations in genes whose products regulate the expression or stability of the temperature-sensitive *sev* protein might reduce the Sevenless-mediated signal. Another possible class of *E(sev)* mutations could affect the abundance of the ligand that activates the *sev* protein. It is interesting to note that a mutation that inactivates one allele of the *bride of sevenless* gene, the best candidate to be that ligand (Krämer et al., 1991), does not show a strong enhancement of the sev^{B4} phenotype in our assay (data not shown). This failure to enhance the sev^{B4} phenotype might be expected if the ligand is presented in considerably greater quantity than is necessary to saturate the *sev* protein ligand-binding sites. Some of the *E(sev)* mutations might also be expected to limit signaling by pathways that are parallel to the Sevenless-mediated pathway and are also essential for the formation of the R7 cell. We do not know whether such signaling pathways exist.

A Role for the *Ras1* Protein in Sevenless Signaling

The observation that the *E(sev)3C* locus is the *Ras1* locus, a gene whose product is closely related to the vertebrate $p21^{ras}$ proteins (Neuman-Silberberg et al., 1984; Brock, 1987), suggests that at least some of the signals initiated by the activation of either the *sev* or *Elp* PTKs are transduced via activation of *Ras1* protein. Evidence for the involvement of *ras* proteins in signaling by other PTKs comes from several sources. The earliest indication that Ras activity is necessary for the action of PTKs came from experiments showing that the injection of monoclonal antibodies that inhibit Ras activity blocks the ability of oncogenic PTKs to transform cells (Smith et al., 1986). Similarly, the expression of dominant interfering *ras* proteins can block the action of $pp60^{v\text{-}src}$ (Feig and Cooper, 1988). These results are consistent with the idea that Ras might be part of the signaling mechanism initiated by PTKs. Additional evidence for the association of Ras activity with signaling by PTKs comes from recent studies of vulval development in the nematode Caenorhabditis elegans. A PTK, the product of the *let-23* gene, is essential for the decision by vulval precursor cells to adopt a primary vulval fate (Aroian et al., 1990). Mutations that reduce the activity of a *ras* protein, the product of the *let-60* gene, also prevent the vulval precursor cells from adopting a vulval fate (Han and Sternberg, 1990; Beitel et al., 1990). Furthermore, dominant mutations that activate the *let-60* protein, or the overexpression of the normal *let-60* protein, cause too many of the vulval precursor cells to commit to the vulval fate and eliminate the need for the *let–23* PTK. These data suggest that the function of the *let-23* PTK in vulval development is to activate the *let-60* (*ras*) protein. However, there is no evidence that the *let-23* and *let-60* gene products act in the same cell, a requirement for their proposed relationship. All of these results are consistent with the hypothesis that activation of *ras* proteins is controlled by PTK activity and is crucial for proper signaling by many PTKs. Our results are, however, also consistent with the hypothesis that *Ras1* protein acts in a separate pathway that is merely required for the action of the Sevenless-mediated pathway. Biochemical analysis will be required to address whether Sevenless activation leads to Ras1 activation.

A Model for *Ras1* Protein Activation

The activity of a *ras* protein is regulated by the guanine nucleotide that is bound to the protein (reviewed by Bourne et al., 1990, 1991). The GTP-bound state is active, while

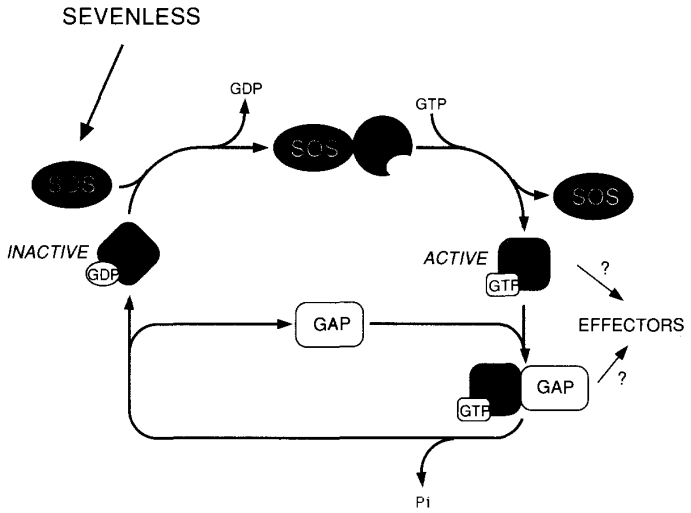

Figure 11. A Model for the Action of the *Sos* and *Ras1* Proteins

See text for description.

the GDP-bound state is not. The ratio of GTP:ras to GDP:ras is determined by two reactions. An active GTP:ras molecule is inactivated by the intrinsic GTPase activity of the *ras* protein. This inactivation reaction is greatly stimulated by the presence of a GTPase activating protein, rasGAP (Trahey and McCormick, 1987; Gibbs et al., 1988). An inactive GDP:ras molecule is activated by the exchange of the bound GDP molecule for a GTP molecule. The activation reaction has only been extensively characterized in budding yeast, where the *CDC25* protein acts as a guanine nucleotide exchange factor to catalyze the reaction (Jones et al., 1991). The in vivo catalyst for guanine nucleotide exchange by Ras in metazoan organisms is not certain, although exchange-promoting activities have been detected in relatively crude extracts of cells and, in one case, purified to near homogeneity.

The discovery that *E(sev)2A*, that is, *Sos*, encodes a protein that is related to the *CDC25* protein suggests a possible biochemical link between the Sevenless and Ras1 activity (see Figure 11). If the similarity between the *CDC25* protein and the *Sos* protein is indicative of similar activities, then our genetic results are consistent with a model in which the activation of the *sev* PTK by ligand binding stimulates the activity of the *Sos* protein. The activated *Sos* protein would then stimulate *Ras1* protein activity by promoting the conversion of GDP:Ras1 to GTP:Ras1. This model requires that the *Ras1* and *Sos* proteins each perform their roles in signal transduction in the developing R7 cell. We have demonstrated this directly for *Ras1*, while Rogge et al. (1991) have shown that the dominant activated Sos^{ic2} allele acts in the R7 cell. The mechanism for stimulation of *Sos* protein activity by Sevenless could be achieved directly through tyrosine phosphorylation of *Sos* protein by the *sev* PTK or indirectly. Alternately, activation by Sevenless could be indirect. If so, then the products of the remaining *E(sev)* loci are the prime candidates for the roles of intermediaries.

Our experiments cannot exclude the possibility that the *Sos* protein might be a constitutive activator of Ras1 and that Sevenless could act solely by inhibiting the activity of a negative regulator such as rasGAP. Biochemical studies will be required to determine whether *Sos* protein activity is actually regulated by Sevenless activity. The available data do, however, permit the conclusion that the level of *Sos* protein activity can be a limiting step in the decision by the presumptive R7 cell to become an R7 cell. The observations that the inactivation of one copy of the *Sos* gene decreases the effectiveness of signaling by Sevenless, while a dominant allele, which presumably elevates *Sos* activity, increases signaling show that small changes in the level of *Sos* function can be critical.

Only two of the seven *E(sev)* loci have been molecularly characterized. The finding that two genes encode proteins well-suited to participate in signaling suggests that this genetic screen is capable of identifying many components of the signaling pathway initiated by Sevenless. What are some of the other participants that we might expect to find? Since the targets for *ras* protein action are undefined, the most interesting possibility is that one of the proteins might be an effector molecule that is regulated by the *Ras1* protein. Since *ras* proteins appear to participate in the signaling by many PTKs, the strongest candidates for a gene encoding a Ras1 effector molecule are probably the remaining two *E(sev)* loci that also affect the Ellipse pathway. The products of the *E(sev)* loci that affect signaling by the *sev* PTK but not by the *Elp* PTK are candidates for pathway components that differentiate the cellular response to Sevenless activation from the response to the activation of other PTKs.

Experimental Procedures

Genetics

Fly culture and crosses were performed according to standard procedures. The mutagenesis screen for *Enhancers of sevenless* mutations was performed as follows. Male sev^{42} flies that were isogenic for the second and third chromosomes were fed 25 mM ethylmethanesulfo-

nate as described (Lewis and Bacher, 1968) and mated to sev^{d2}; *CxD*, *D/TM3*, *Sb*, *ry*, P[*ry*,sev^{84}] females. The F1 progeny were reared at 22.7°C, and *Sb* individuals were assayed by the reduced corneal pseudopupil method for the presence of R7 photoreceptors (Franceschini and Kirschfeld, 1971). Approximately 30,000 *Sb* F1 progeny were screened. Individuals that displayed abnormal reduced corneal pseudopupils were crossed to sev^{d2}; *CxD*, *D/TM3*, *Sb*, *ry*, P[*ry*,sev^{84}] flies and their progeny were examined for R7-minus reduced corneal pseudopupils. These individuals were used to map the *E(sev)* mutation to a chromosome and then balance the mutation. The chromosomes used for both segregation analysis and for balancing were *FM7c*, *CyO*, and *TM3*.

The X-linked mutations were mapped meiotically with the markers *y*, *cv*, *f*, and *car*. Females heterozygous for the *E(sev)*, sev^{d2} chromosome and a *y*, *cv*, sev^{d2}, *f*, *car* chromosome were crossed to sev^{d2}; *CxD*, *D/TM3*, *Sb*, *ry*, P[*ry*,sev^{84}] males. Individual virgin females were collected and scored for the *E(sev)* phenotype. These females were then mated to *y*, *cv*, sev^{d2}, *f*, *car* males, and their progeny were scored for the presence of the markers. Similar mapping crosses were performed for the second and third chromosome *E(sev)* mutations using sev^{d2}; *b*, *pr*, c, *px*, *sp* and sev^{d2}, *th*, *st*, *cu*, *sr*, e, *ca* flies, respectively. However, in these cases, the *E(sev)* phenotypes were more difficult to score, and we therefore only backcrossed females that were obviously *E(sev)*. In all cases, approximately 50 to 100 potentially recombinant chromosomes were scored. We estimate an error rate of approximately 2% in scoring the *E(sev)* phenotpye. The *E(sev)2A* locus was further mapped by the failure of mutant alleles to complement chromosomes carrying either $l(2)34Ea^{DM7}$ or $l(2)34Ea^{SF15}$ (Woodruff and Ashburner, 1979; M. Ashburner and J. Roote, personal communication).

Histology

Fixation and sectioning (2 mm) of adult Drosophila eye was performed as described (Tomlinson and Ready, 1987). Scanning electron microscopy was performed as described (Kimmel et al., 1990).

Mosaic Analysis

Clones of cells homozygous for *E(sev)* mutations were generated by X-ray-induced mitotic recombination between heterozygous $E(sev)^+$ and *E(sev)* mutant chromosomes. Clones were visualized by marking the $E(sev)^+$ chromosome with the cell-autonomous $white^+$ gene. Clones were identified as *white* patches in otherwise pigmented eyes. For *E(sev)* mutations on the autosomes, the $white^+$ gene was provided on a transposable element. The following elements were used: 2L, P[(*w*, *ry*)A-R]4-2 in 39E-F; 2R, P[(*w*, *ry*)A-R]042 in 49D; 3L, P[*w*]33 in 70C; 3R, P[(*w*, *ry*)D]3 in 90E (Hazelrigg et al., 1984; Levis et al., 1985; our unpublished data). For the X chromosome, the *E(sev)* chromosome was marked with *white* and the $E(sev)^+$ chromosome was marked by the normal chromosomal copy of $white^+$. All flies carried one copy of the sev^+ gene provided either at its normal chromosomal location or on an autosome as part of a P element. Flies were irradiated as first instar larvae (1000R). The flies were reared at 25°C. The frequency of clones was approximately 1 in 100 flies.

Clones of wild-type cells were generated in a similar manner, except that the *E(sev)* chromosome rather than the $E(sev)^+$ chromosome was marked by the $white^+$ gene. The $E(sev)^+$ cells are therefore marked as *white*. For the *E(sev)1A* locus, $E(sev)1A^{e0P}$, sev^{d2} females were crossed to w^{1118}, sev^{d2}; *CxD*, *D/TM3*, *Sb*, *ry*, P[*ry*, sev^{84}] males. For the *Ras1* locus, w^{1118}, sev^{d2}; $Ras1^{E62K}$/*TM3* females were crossed to w^{1118}, sev^{d2}; P[*ry*, sev^{84}]A males. P[*ry*, sev^{84}]A is a second chromosome insert. The flies were irradiated as above and reared either at 22.7°C (for *E(sev)1A* experiments) or at 23.5°C (for *Ras1* experiments). The difference in temperature is due to a slight difference in the level of expression from the two P[*ry*, sev^{84}] inserts. Progeny of these crosses that carried the *E(sev)* mutation and the P[*ry*, sev^{84}] insert were examined for clones and sectioned as described above.

Generation of Temperature-Sensitive *sev* Alleles

Site-directed mutagenesis was used to generate the amino acid substitutions shown in Figure 1 (Sambrook et al., 1989). The 17.5 kb DNA fragment containing the mutated *sev* allele was placed in a modified Carnegie 20 P-transformation vector and injected into sev^{d2}, ry^{506} embryos as previously described (Karess and Rubin, 1984). The 17.5 kb fragment consists of a 15 kb EcoRI fragment that has previously been shown to be providing full *sev* function and an additional 2.5 kb 5′ of the *sev* gene (Hafen et al., 1987). The transformants were assayed by sectioning and by the reduced corneal pseudopupil method. The *TM3*, *Sb*, *ry*, P[*ry*, sev^{84}] chromosome was generated by transposition of the P[*ry*, sev^{84}] element.

Isolation and Transformation of the *Ras1* and *Sos* Regions

The *Ras1* region was cloned from a library of Drosophila genomic DNA cloned into the λFixII (Stratagene, Inc.) vector, using a 700 bp NcoI–EcoRI fragment as a probe (Neuman-Silberberg et al., 1984). A 12 kb XhoI–NotI fragment was cloned into the pW8 transformation vector (Klemenz et al., 1987) and injected into w^{1118}, sev^{d2} embryos as previously described (Karess and Rubin, 1984). The XhoI site lies approximately 4.5 kb 5′ of the initiating methionine of the *Ras1* gene. The NotI site is derived from the vector polylinker sequences and corresponds to a position approximately 5 kb 3′ of the *Ras1* termination codon. The *Ras1* alleles from the *E(sev)3C* mutant chromosomes were cloned as 5.7 kb EcoRI fragments into the λZapII (Stratagene, Inc.) vector. The mutant alleles were then cloned into pBluescript(+) or (−) for sequencing. The mutant alleles could be distinguished from the balancer-derived alleles on the basis of a sequence polymorphism in a noncoding region.

The *Sos* region was cloned by chromosome walking using a cosmid library prepared by J. Tamkun. The starting point was the Hobo[ry^+] element inserted in polytene band 34D1 (Blackman et al., 1989). A library of genomic DNA was constructed in λFixII and screened with a probe from the *rosy* gene. The walk was oriented and monitored by hybridization of cosmid DNAs to wild-type and *Sos* deficiency chromosomes. Genomic blot analysis of deficiency chromosomes was performed by standard techniques using probes from the *Sos* region (Sambrook et al., 1989). The *Sos* alleles from mutant chromosomes were cloned into λFixII as an approximately 18 kb BglII fragment that includes the entire gene. The alleles from the mutant chromosomes were distinguished from the balancer-derived alleles on the basis of polymorphic HindIII sites. The mutant alleles were subcloned into plasmid vectors for sequencing. The *Sos* transformation construct was made by inserting the 10 kb BglII–XbaI fragment into BamHI-XbaI-cut pW8. The resulting plasmid was injected into w^{1118}, sev^{d2} embryos as previously described (Karess and Rubin, 1984).

The 10.3 kb XbaI fragment that contains the entire *Sos* gene was used to screen an eye imaginal disc cDNA in λgt10 constructed by Dr. A. Cowman. A single cDNA λsosT was then subcloned into pBlueScript(−) for sequencing.

Transcript Analysis

Polyadenylated RNA was prepared from imaginal discs and from embryos, electrophoresed through a 1% agarose–formaldehyde gel, and blotted onto nitrocellulose by standard methods (Sambrook et al., 1989). The blot was probed with the fragment of the sosT cDNA shown in Figure 9.

Nuclease S1 analysis and primer extension were performed exactly as described (Sambrook et al., 1989). The probes used for S1 analysis were uniformly labeled single-stranded probes corresponding to the reverse complements of nucleotides 0 to 610 and 0 to 731. The primers used correspond to nucleotides 581 to 610 and 706 to 731.

DNA Sequencing

All DNA sequences were determined using the dideoxy chain termination method (Sanger et al., 1977) using Sequenase (U.S. Biochemical). Templates for the determination of the 6601 bp genomic *Sos* sequence were generated by sonication of plasmid DNA and insertion of the sonicated fragments into the vector M13mp10. The entire sequence was determined on both strands. The sequences of *Sos* cDNAs were determined on one strand using templates generated from plasmids containing EcoRI fragments inserted into the pBlueScript(−) vector using an Erase-a-Base kit (Promega). Mutant alleles of *Sos* were sequenced on one strand using oligonucleotide primers spaced approximately every 200 bp. The exons of the mutant *Ras1* alleles were sequenced on one strand using oligonucleotide primers.

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