Mutations Modulating Raf Signaling in *Drosophila* Eye Development

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Manuscript received April 6, 1995

Accepted for publication September 21, 1995

ABSTRACT

The R7 fate is specified during *Drosophila* eye development by an inductive signal transduced intracellularly via the Raf kinase. We have performed a genetic screen for dominant mutations that alter the efficiency with which cells respond to a constitutively activated Raf kinase. Such mutations may affect genes involved in signal transduction downstream of Raf. We have isolated 44 mutations that define eight genes. One of these encodes a mitogen-activated protein kinase homologue; another is a putative target gene of this signaling pathway. We present the results of this screen in detail, as well as a preliminary genetic analysis of the six loci still to be characterized molecularly.

THE Raf serine/threonine kinase has been shown to play a critical role in the signal transduction pathways activated by receptor tyrosine kinases (RTKs) across a broad phylogenetic spectrum. Within these pathways, Raf acts to couple Ras activation to the mitogen-activated protein kinase (MAPK) cascade, which consists of the protein kinases MEK (MAPK kinase) and MAPK (MARSHALL 1995). The roles of these proteins in Raf signaling have been well established by both biochemical and genetic studies. Less well understood are the roles of other Raf-binding proteins, such as 14-3-3 proteins (FANTI *et al.* 1994; FREED *et al.* 1994; FU *et al.* 1994), hsp90 (STANCATO *et al.* 1993) and immunophilins (STANCATO *et al.* 1994), and the extent to which signal transduction via Raf is further regulated by as yet uncharacterized proteins acting in this or parallel pathways.

One approach to identify new components of any given biological process is to search for mutations that dominantly modify the effects of another mutation disrupting the same process. This allows such mutations to be recovered in a simple one-generation (F_1) screen. More importantly, by sensitizing only a single biological pathway, one can create conditions in which even a slight reduction in gene activity (*e.g.*, due to the loss of only one functional copy of the gene) can result in a detectable phenotype. This is of particular utility when the gene is involved in many other processes, and a more severe loss of function may therefore produce a less informative phenotype, such as lethality. Simon *et al.* (1991) successfully used such an approach to identify components of the RTK signal transduction pathway involved in the induction of the R7 fate during *Dro-*

sophila eye development. Starting with a hypomorphic *sevenless* (*sev*) allele encoding a mutant RTK barely adequate for signaling, they isolated several dominant *Enhancer of sev*, or *E(sev)*, mutations that further reduced signaling to levels below the threshold required for R7 development. Three of the *E(sev)* loci have been shown to encode proteins acting between Sev and Raf in this pathway: Drk, Sos and Ras1 (SIMON *et al.* 1991, 1993; OLIVIER *et al.* 1993). We wished to extend these studies to identify components acting downstream of Raf.

An analogous screen starting with a hypomorphic *raf* allele would not be feasible, since Raf, unlike Sev, is also required in vital processes. A further reduction in the strength of signaling via an already mutated Raf kinase might result in lethality, precluding the isolation of *E(raf)* loci. An alternative approach would be to start with a *raf* allele that is just below the activity required for viability and then isolate *Su(raf)* mutations that dominantly suppress this lethality. Such suppressor mutations may represent loss-of-function mutations in genes encoding negative regulators of Raf function or gain-of-function mutations in genes involved in the positive regulation of Raf signaling. Two groups have recently performed such a screen and isolated gain-of-function mutations in a gene encoding a *Drosophila* MEK homologue (*D-MEK*) (TSUDA *et al.* 1993; LU *et al.* 1994).

We have conducted a complementary screen, in which we have isolated mutations that dominantly modify the phenotype of a gain-of-function *raf* allele affecting only R7 development. The main advantage of this approach is that, by specifically addressing R7 development, it has been possible to avoid complications with lethality, and thus screen simultaneously for both suppressor, or *Su(Raf)*, and enhancer, or *E(Raf)*, mutations. Thus, both positive and negative regulators can potentially be identified with greater efficiency by the recovery of loss-of-function alleles. We have identified six *Su(Raf)* loci and two *E(Raf)* loci. The *Su(Raf)* loci in-

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clude *rolled* (*rl*), encoding a Drosophila MAPK (BIGGS and ZIPURSKY 1992; BIGGS *et al.* 1994), and *phyllopop* (*phyl*), a putative target gene of the Raf pathway in R7 development (CHANG *et al.* 1995; DICKSON *et al.* 1995). Here we describe this screen in detail and present a preliminary genetic analysis of the other loci identified.

MATERIALS AND METHODS

Mutagenesis: *Su(Raf)* and *E(Raf)* mutations were isolated by virtue of their ability to dominantly modify the rough eye phenotype in transgenic flies carrying the activated Raf construct *Raf^{torY9}*. The *Raf^{torY9}* fusion is identical to the *Raf^{tor}* fusion described in Dickson *et al.* (1992), except that it contains the weakly activating Y9 mutation in the torso extracellular domain (SPRENGER and NUSSLEIN-VOLHARD 1992). Two different *Raf^{torY9}* transgenic lines were used in this screen. The first was a second chromosomal insertion of a construct in which *Raf^{torY9}* is expressed under the control of a single *sev* enhancer and the heat-inducible *hsp70* promoter. This insertion is lethal in homozygotes. Flies carrying this chromosome balanced over the *CyO* balancer were used for the first rounds of mutagenesis and the *Cy⁺* progeny scored. Subsequent rounds were performed using flies homozygous for a chromosome 2 carrying a *Raf^{torY9}* fusion expressed under a duplicated *sev* enhancer and the *sev* promoter (B. DICKSON and E. HAFEN, unpublished data). The two *Raf^{torY9}* constructs produce phenotypes of identical strength in heterozygotes. The first number of each allele indicates the round of mutagenesis in which the allele was isolated: numbers from 1–7 were isolated using the first construct, 8–22 with the second. The *phyl* alleles 3G6, 17L1 and 20Q1 have also been referred to as *phyl¹*, *phyl²* and *phyl³*, respectively (DICKSON *et al.* 1995).

The first rounds (1–7) were performed by mutagenizing *w¹¹¹⁸* males isogenized for chromosome 2; subsequent rounds (8–22) were performed with males reisogenized for the major autosomes. Males were treated with EMS, as described by LEWIS and BACHER (1968), and crossed to *Raf^{torY9}* transformants. The efficiency of mutagenesis, as estimated by the induction of sex-linked lethal mutations, was consistently ~0.60 lethal hits per major chromosomal arm.

Potential modifier mutations detected in the F₁ progeny were retested by backcrossing to *Raf^{torY9}* flies, and stocks were established from those crosses in which a modifier mutation segregated to half of the progeny. Mutations were assigned to a chromosome and balanced using *CyO* and *TM3* balancer chromosomes carrying a *Raf^{torY9}* insertion. Complementation analyses were performed on the basis of recessive lethality at 25°. Semilethal allelic combinations were observed only at the *E(Raf)2A* locus, with rare escapers of the genotypes 14O1/16H1 (18 adults emerged of 270 expected) and 14O1/16T1 (22/232). 16H1/16T1 is fully lethal (0/121).

Mapping of *Su(Raf)* and *E(Raf)* loci: Modifier mutations were first mapped by meiotic recombination using the markers *al c sp b* on chromosome 2, and *h th st cu sr e ca* on chromosome 3. Consistent results were obtained only when a single marker was scored in each cross: a modifier mutation was outcrossed to the multiply marked stock and then backcrossed to a panel of strains carrying *Raf^{torY9}* and just one of the marker mutations. Approximate cytological locations were then determined using *P*-element insertions and deficiencies.

E(Raf)2A was mapped to 2–1.0 ± 0.5 and fails to complement the deficiency *Df(2L)PMF* = 21A;21B7-8. *P*-element alleles of *Su(Raf)2A* and *Su(Raf)3A* have been recovered and used to map these loci cytologically (B. DICKSON, A. VAN DER STRATEN and E. HAFEN, unpublished data). Fine scale map-

ping of *Su(Raf)3B* was performed using *Pw⁺* insertions at 88C, 90E and 92B, as well as Sb at 89B9-10; and *Su(Raf)3C* with insertions at 97F5-8 and 98F11-12. *Su(Raf)3B* mapped to 0.4 ± 0.2 cM from 90E; and *Su(Raf)3C* is 3.2 ± 1.1 cM from 97F5-8 and 3.3 ± 0.8 cM from 98F11-12. None of the deficiencies *Df(3R)P115*=89B7-8;89E7-8, *Df(3R)C4*=89E;90A, *Df(3R)DG2*=89E-F;91B1-2, *Df(3R)P14*=90C2-D1;91B1-2, and *Df(3R)ChaM7*=90F;91F either suppresses *Raf^{torY9}* or shows any obvious phenotype in *trans* to *Su(Raf)3B^{18A2}*, indicating that, unless either *Su(Raf)3B* or the deficiency breakpoints are incorrectly mapped, *Su(Raf)3B^{18A2}* is not a loss-of-function allele. An alternative, though unlikely, possibility is that the removal of a closely linked gene may compensate for loss of *Su(Raf)3B* in these deficiencies. No deficiencies were available in the region to which *Su(Raf)3C* had been mapped.

Interactions with *raf^{HM7}*: Interactions with *raf^{HM7}* were tested by crossing *raf^{HM7},w⁺*/FM7 females to *Su(Raf)/Pw⁺* or *E(Raf)/Pw⁺* males at 18° and comparing the *raf^{HM7};Su(Raf)/+* or *raf^{HM7};E(Raf)/+* progeny to their *raf^{HM7};Pw⁺/+* brothers. The *Pw⁺* insertions were compared to the parental chromosomes to verify that they did not modify the *raf^{HM7}* phenotype themselves.

Histology: Scanning electron micrographs and plastic sections of adult eyes were prepared as described in BASLER *et al.* (1991).

Germline clones: Germline clones were generated using the FLP-FDS technique (HOU *et al.* 1995).

RESULTS

Isolation of modifiers of the *Raf^{torY9}* phenotype: The Raf kinase can be activated either by truncation of its N-terminal domain (STANTON *et al.* 1989) or relocation of the entire protein to the membrane following post-translational modification at an artificial C-terminal CAAX site (LEEVEES *et al.* 1994; STOKOE *et al.* 1994). Similarly, the Drosophila Raf kinase is activated following N-terminal truncation and relocation to the plasma membrane via fusion to the extracellular and transmembrane domains of the Torso protein, a RTK (DICKSON *et al.* 1992). The Raf kinase activity of this fusion protein can be further increased by introducing point mutations in the Torso extracellular domain analogous to dominantly activating mutations in the Torso RTK, *tor^{Y9}* and *tor⁴⁰²¹* (SPRENGER and NUSSLEIN-VOLHARD 1992). We generated transgenic flies carrying such an activated Raf construct (*Raf^{torY9}*) expressed under the transcriptional control of an enhancer element of the *sevenless* (*sev*) gene (BASLER *et al.* 1989). In these transgenic flies, the Raf kinase is constitutively activated in the five cells of the developing eye that choose between the alternative fates of development as an R7 photoreceptor or a nonneuronal cone cell. Normally, Raf is activated in only one of these, the R7 precursor, via the local activation of the *sev* RTK. Activation of Raf is both necessary and sufficient for this cell to select the R7 fate (DICKSON *et al.* 1992). In *Raf^{torY9}* flies, ectopic Raf activity in the cone cell precursors results in some of these cells also developing as R7 cells. As a result, most of the 800 ommatidia in the compound eye contain several instead of just one R7 photoreceptor (Figure 1F), and these abnormal ommatidia disrupt the

FIGURE 1.—Modification of the *Raf^{torY9}* phenotype by *Su(Raf)* and *E(Raf)* mutations. Scanning electron micrographs (A–D) and tangential sections (E–H) of eyes of the following genotypes: (A and E) wild type, (B and F) *Raf^{torY9}/+*, (C and G) *Raf^{torY9}/+; Su(Raf)3A⁹¹/+*, and (D and H) *Raf^{torY9}/+; E(Raf)2A^{16T1}*. Note that the *Su(Raf)* and *E(Raf)* mutations dominantly modify the degree of eye roughening in *Raf^{torY9}* flies. In the case of *Su(Raf)3A*, all ectopic R7 cells are eliminated and the eye regains the smooth texture observed in the wild type.

regular hexagonal ommatidial lattice. The eye thus has a “roughened” external appearance, readily observed in live anaesthetized flies (Figure 1B). The degree of roughening reflects the average number of additional R7 cells per ommatidium and thus provides an indirect measure of the efficiency of Raf signaling within the cone cell precursors. This allowed us to isolate mutations that dominantly modify the degree of roughening and thus potentially affect some step in the pathway between Raf activation and the selection of the R7 fate.

In a preliminary screen of deficiencies uncovering in total ~60% of the *Drosophila* genome, two overlapping deficiencies, *Df(2R)L48* and *Df(2R)trix*, were identified that strongly suppressed the *Raf^{torY9}* phenotype. Three deficiencies weakly enhanced the *Raf^{torY9}* rough eye phenotype: *Df(2L)PMF* and the overlapping deficiencies, *Df(2L)S³* and *Df(2L)ast¹* (Figure 2).

To obtain point mutations at such dominant modifier loci, we subsequently screened ~200,000 flies carrying the *Raf^{torY9}* construct and a set of EMS-mutagenized chromosomes. In this screen we recovered 40 mutations that dominantly suppress the *Raf^{torY9}* rough eye phenotype and four dominant enhancer mutations. The suppressors fall into six *Su(Raf)* complementation groups; the enhancers into two *E(Raf)* groups (Figure 2). Two loci were found to correspond to known genes: the 19 alleles of the *Su(Raf)2B* complementation group were found to be new alleles of the gene *rl*, and the single *E(Raf)2B* allele fails to complement *Star* mutations. We have recently reported a detailed analysis of a second suppressor locus, *Su(Raf)2C*, which we renamed *phyllopod* (*phyl*) (CHANG *et al.* 1995; DICKSON *et al.* 1995).

EMS-induced alleles were recovered for each of three loci whose existence was inferred from the initial deficiency screen: *phyl* is uncovered by the deficiencies *Df(2R)L48* and *Df(2R)trix*, *E(Raf)2A* by *Df(2L)PMF*, and *Star* by *Df(2L)S³* and *Df(2L)ast¹*. These mutations are therefore likely to result in the loss of gene function. Two deficiencies that fail to complement all three *Su(Raf)3A* alleles were not selected in the deficiency screen and upon subsequent retesting showed only a very slight suppression of the *Raf^{torY9}* phenotype. Since all three EMS-induced *Su(Raf)3A* alleles are very strong suppressors (Figure 1, C and G), they are likely to act as antimorphs in their interactions with *Raf^{torY9}*. Similarly, deficiencies that, according to our mapping data, should uncover *Su(Raf)3B*, did not suppress *Raf^{torY9}*, suggesting that the single allele at this locus is also due to an antimorphic mutation. The remaining *Su(Raf)* loci are not uncovered by any of the deficiencies tested. The high frequency at which *Su(Raf)2A* and *rl* alleles were recovered suggests that these are loss-of-function mutations; the nature of the single *Su(Raf)3C* allele recovered could not be determined.

Sections through the eyes of *Raf^{torY9}* flies carrying either a *Su(Raf)* or *E(Raf)* mutation (Figure 1 and data not shown) revealed that, as expected, all *Su(Raf)* mutations do result in a reduction in the average number of additional R7 cells per ommatidium. None of the *E(Raf)* mutations, however, significantly increases the average number of R7 cells. These mutations may produce additional defects that result in further eye roughening independently of R7 development. Indeed, strong *Star* alleles, including the one we isolated, have

FIGURE 2.—Mapping of *Su(Raf)* and *E(Raf)* loci. The known or estimated cytological locations of the six *Su(Raf)* and two *E(Raf)* complementation groups are shown. All loci were mapped to either chromosome 2 or 3. The approximate breakpoints of selected deficiencies are also shown. Deficiencies that both modified the *Raf^{torY9}* phenotype and failed to complement EMS-induced *Su(Raf)* or *E(Raf)* mutations are indicated by ■. The two deficiencies indicated by □ fail to complement *Su(Raf)3A* but do not appreciably suppress *Raf^{torY9}*. The deficiencies indicated by □ completely span the interval to which *Su(Raf)3B* was mapped, but neither modify the *Raf^{torY9}* phenotype nor display an obvious phenotype in *trans* to *Su(Raf)3B^{18A2}*.

a dominant rough eye phenotype even in a wild-type genetic background.

Interactions with a hypomorphic *raf* allele: The gain-of-function construct *Raf^{torY9}* provided a convenient means for isolating interacting mutations. These mutations may, however, interact specifically with this construct (*e.g.*, with either the *sev* enhancer or Torso domain) and not with the Raf kinase itself. We therefore tested each *Su(Raf)* and *E(Raf)* locus for genetic interactions with the hypomorphic *raf* allele, *raf^{HM7}*. The *raf^{HM7}* allele produces reduced levels of the wild-type protein, sufficient for survival at 18° but not at 25° (MELNICK *et al.* 1993). Raised at the permissive temperature of 18°, only 50% of ommatidia in the eyes of hemizygous *raf^{HM7}* males contain an R7 cell (DICKSON *et al.* 1992).

If *Su(Raf)* mutations impair signaling via Raf, we would expect them to enhance the *raf^{HM7}* phenotype. If they are required generally for Raf function, this may result in synthetic lethality (*i.e.*, nonallelic noncomplementation) at 18°. On the other hand, if they are

specifically required for Raf function in the eye, they should enhance the *raf^{HM7}* eye phenotype without affecting the viability of these flies. Conversely, *E(Raf)* mutations, if they relieve negative influences on Raf signaling, would be expected to suppress the *raf^{HM7}* phenotype.

Four of the *Su(Raf)* loci enhance the *raf^{HM7}* phenotype in accordance with these predictions (Table 1). Of these, three (*rl*, *Su(Raf)3A* and *Su(Raf)3B*) are synthetically lethal in combination with *raf^{HM7}*, suggesting that they encode proteins generally required for signal transduction via Raf. In agreement with this prediction, *rl* has been shown to encode a MAPK homologue required in several signaling events involving Raf (BIGGS *et al.* 1994; BRUNNER *et al.* 1994b). The fourth of these loci, *phyl*, enhances only the eye phenotype of *raf^{HM7}*, significantly increasing the number of ommatidia lacking R7 cells (DICKSON *et al.* 1995). This is consistent with our interpretation of *phyl* as a target gene transcriptionally regulated only in R7 and two other photorecep-

TABLE 1
Genetic interactions with *raf^{HM7}*

Locus	Allele tested	No. of <i>raf^{HM7}</i> ; <i>Su</i> /+ or <i>raf^{HM7}</i> ; <i>E</i> /+ males	No. of <i>raf^{HM7}</i> ; <i>Pw</i> ⁺ /+ males	Relative viability (%)	Modification of <i>raf^{HM7}</i> eye phenotype
<i>Su(Raf)2A</i>	<i>3E8</i>	73	136	54	None
	<i>4P5</i>	54	45	120	None
<i>rolled</i>	<i>2L1</i>	0	61	0	—
	<i>6L1</i>	0	63	0	—
<i>phyllopod</i>	<i>3G6</i>	50	53	94	Enhanced
	<i>17L1</i>	156	118	132	Enhanced
<i>Su(Raf)3A</i>	<i>9J1</i>	0	83	0	—
	<i>19F2</i>	0	60	0	—
<i>Su(Raf)3B</i>	<i>18A2</i>	0	174	0	—
<i>Su(Raf)3C</i>	<i>17M1</i>	127	121	105	None
<i>E(Raf)2A</i>	<i>14O1</i>	13	72	18	Enhanced
	<i>16H1</i>	0	135	0	—
	<i>16T1</i>	0	157	0	—
<i>Star</i>	<i>2J1</i>	0	50	0	—

Genetic interactions between *raf^{HM7}* and *Su(Raf)* and *E(Raf)* loci. *raf^{HM7}*, *w^a/FM7* virgins were mated to *Su(Raf)/Pw⁺* or *E(Raf)/Pw⁺* males, and the total number of *raf^{HM7}* progeny surviving to adulthood were scored for each class. For viable combinations, the eyes of both classes were compared for any modification of the mild roughening caused by the *raf^{HM7}* mutation.

tors in response to activation of the Raf pathway. There is at present no evidence for *phyl* acting as a target gene for Raf signaling in any other tissue. The other two *Su(Raf)* loci, *Su(Raf)2A* and *Su(Raf)3C*, show no dominant genetic interaction with *raf^{HM7}*.

Somewhat surprisingly, neither of the *E(Raf)* loci suppresses *raf^{HM7}*, but in fact enhance the hypomorphic *raf* phenotype, resulting in synthetic lethality. The *E(Raf)2A* allele *14O1* appears to be weaker than the other two alleles at this locus, and a small number of flies of the genotype *raf^{HM7}/Y*; *14O1/+* eclose. The *raf^{HM7}* eye phenotype is also slightly enhanced in these flies. Since the *E(Raf)* mutations enhance the phenotypes of both loss- and gain-of-function *raf* alleles, we consider it unlikely that these genetic interactions reflect direct biochemical interactions between the proteins these genes encode and Raf.

Homozygous *Su(Raf)* and *E(Raf)* phenotypes: The recessive phenotypes of *rl*, *phyl* and *Star* mutations have been reported elsewhere (HEBERLEIN and RUBIN 1991; HEBERLEIN *et al.* 1993; BIGGS *et al.* 1994; CHANG *et al.* 1995; DICKSON *et al.* 1995). *rl* appears to be required for the development of all eight photoreceptors, whereas *phyl* is required specifically for R1, R6 and R7. There is only circumstantial evidence for a role of *Star* in R7 development (KOLODKIN *et al.* 1994), but in any case the recovery of a *Star* allele in our screen is more likely due to its dominant rough eye phenotype than a specific interaction with *Raf^{torY9}*.

Surprisingly, three of the *Su(Raf)* genes do not appear to be absolutely required for development either of R7 or any other photoreceptor. The single *Su(Raf)3B* and *Su(Raf)3C* alleles are both viable in the homozygous condition and show no obvious defects in eye development (Figure 3B and data not shown). *Su(Raf)2A* mutations

are homozygous lethal, but patches of homozygous mutant tissue could readily be recovered in heterozygous animals using the FLP/FRT technique to induce site-specific mitotic recombination (XU and RUBIN 1993). Examination of such homozygous *Su(Raf)2A* clones shows that, although required for viability, this gene is dispensable for normal eye development (Figure 3A).

Su(Raf)3A mutations are also lethal in the homozygous state, and we were unable to generate *Su(Raf)3A* mutant clones by mitotic recombination. This suggests that *Su(Raf)3A*, like *raf* and *rl*, may also be required for cell proliferation (NISHIDA *et al.* 1988; BIGGS *et al.* 1994). The requirement of *Su(Raf)3A* in the early proliferative phase of eye development precludes a direct examination of its role in the later stages of eye development during which cell fates are determined.

Finally, *E(Raf)2A* is required for both viability and eye development. Homozygous mutant ommatidia are of variable composition, often lacking either R7 or one or more other photoreceptors, but also occasionally containing extra photoreceptors of either class (Figure 3C).

***Su(Raf)2A* is required for ectopic R7 development:** We were intrigued to find that elimination of one copy of the *Su(Raf)2A* gene severely impairs signaling via *Raf^{torY9}*, whereas elimination of both copies does not appear to affect signaling via the endogenous Raf kinase. One possible explanation of this result would be that *Su(Raf)2A* encodes a protein that interacts specifically with the activated *Raf^{torY9}* fusion protein but not at all with the endogenous Raf. To test this possibility, we generated *Su(Raf)2A* clones in genetic backgrounds in which the Raf pathway is constitutively activated at points further upstream: at *Sev* in *Sev^{S11}* transformants (BASLER *et al.* 1991) and at *Ras1* in *Ras1^{V12}* transformants (FORTINI *et al.* 1992). In both

FIGURE 3.—Homozygous *Su(Raf)* and *E(Raf)* eye phenotypes. (A) *Su(Raf)2A* is homozygous lethal. Shown is a tangential section through a clone of homozygous mutant *Su(Raf)2A* cells generated by FLP-induced mitotic recombination in *w, hsFLP1; Su(Raf)2A^{3E8} FRT40A/P[w⁺]30C FRT40A* flies. Larvae were shifted to 37° for 1 hr during the first instar. No pattern defect can be observed within the mutant tissue. (B) The eyes of homozygous *Su(Raf)3C^{17MI}* flies also show no morphological aberrations. (C) An *E(Raf)2A^{16T1}* clone generated in an analogous manner to the clone shown in A. Some homozygous mutant ommatidia contain an abnormal complement of photoreceptors.

cases, the formation of ectopic, but not endogenous R7 cells is completely blocked within the *Su(Raf)2A* clone (Figure 4A and data not shown). This observation argues strongly against a specific interaction between *Su(Raf)2A* and *Raf^{torY9}* and suggests that the protein encoded by *Su(Raf)2A* is indeed important for signaling via the wild-type Raf kinase, at least for the generation of ectopic R7 cells due to constitutive activation of the pathway at or above Raf.

Ectopic R7 cells are also formed in *yan* mutants, in this case independently of the Raf pathway (LAI and RUBIN 1992; TEI *et al.* 1992). It is believed that the Raf/MAPK pathway acts in part to overcome the inhibitory influence on R7 development normally exerted via the Yan protein (BRUNNER *et al.* 1994b; O'NEILL *et al.* 1994; REBAY and RUBIN 1995). The formation of extra R7 cells in *yan* mutants is also independent of *Su(Raf)2A* (Figure 4B), suggesting that in *Su(Raf)2A* mutant tissue the pathway leading to ectopic R7 development is blocked at some point between Raf and Yan.

Maternal effects of *Su(Raf)* and *E(Raf)* mutations: The Raf signal transduction pathway is utilized repeat-

edly during development to induce responses to the activation of several different RTKs. For example, the Torso RTK activates this pathway at the anterior and posterior poles of the early embryo, ultimately leading to their differentiation as the acron and telson, respectively (reviewed in DUFFY and PERRIMON 1995). In this case, all proteins required for signaling are encoded by maternally provided mRNAs. To determine whether any of the *Su(Raf)* and *E(Raf)* loci might also be involved in signaling via Torso, we therefore removed any possible maternally derived mRNAs by generating germline clones for *Su(Raf)2A*, *phyl*, and *E(Raf)2A*. Embryos derived from such germline clones show a variety of defects (Figure 5). Only in the strongest phenotypic class of *Su(Raf)2A* embryos is there any indication that Torso signaling might be disrupted, in that both the anterior head skeleton and posterior Filzkörper are frequently absent (Figure 5B). This phenotype does not mimic torso mutants, however, since segmentation is also severely disrupted. In weaker *Su(Raf)2A* embryos (Figure 5C), as in embryos from *phyl* germline clones (Figure 5D), both head skeleton and Filzkörper are always pres-

FIGURE 4.—*Su(Raf)2A* clones in *Sev^{S11}* (A) and *yan* (B) mutant backgrounds. In *Sev^{S11}* transformants, additional R7 cells can be seen in the pigmented *Su(Raf)2A⁺* tissue on the left of A, but not within the unpigmented *Su(Raf)2A⁻* clone occupying the right half of the panel. The region shown in B lies almost entirely within a *Su(Raf)2A* clone in a *yan* background. *Su(Raf)2A* is not required for the formation of ectopic R7 cells in *yan* mutants. Clones were generated by FLP-induced mitotic recombination in *w, hsFLP1; Su(Raf)2A^{3E8} FRT40A/P[w⁺]30C FRT40A*; *Sev^{S11}/+* and *w, hsFLP1; yan¹ Su(Raf)2A^{3E8} FRT40A/yan¹ P[w⁺]30C FRT40A* animals, respectively.

FIGURE 5.—Cuticular preparations of wild-type embryos (A) and embryos derived from females bearing *Su(Raf)2A*^{3E8} (B and C), *phyl*^{7L1} (D), and *E(Raf)2A*⁶⁰¹¹ (E) germline clones. Embryos lacking maternally derived *Su(Raf)2A* function show a range of phenotypes but can be roughly divided into two classes in an approximate 1:1 ratio, examples of which are shown in B and C. The weaker class (C) probably represents embryos in which the phenotype is partially rescued by a wild-type *Su(Raf)2A* gene provided paternally, whereas the stronger class (B) contains embryos lacking both maternal and zygotic *Su(Raf)2A* function. Severe segmentation defects are seen in both classes. Anterior and posterior structures, the head skeleton (arrowhead) and Filzkörper (arrow), respectively, are invariably present in embryos of the weaker class and often either absent or severely malformed in the stronger class. Weaker segmentation defects are seen in embryos lacking either maternal *phyl* or *E(Raf)2A*. Filzkörper and the head skeleton, though often abnormal, are always present in both cases.

ent, though often malformed. Similarly, embryos lacking maternal *E(Raf)2B* function show no indication of either absent or ectopic activation of the Torso pathway (Figure 5E).

Females homozygous for *Su(Raf)3B* or *Su(Raf)3C* are fully fertile, suggesting that signaling via Torso is also intact in these mutants. A detailed analysis of *Su(Raf)3A* germline clones will be presented elsewhere. Thus, of all the *Su(Raf)* and *E(Raf)* loci, only *rl*, and possibly *Su(Raf)2A*, appear to be required in both Sev and Torso signaling (BRUNNER *et al.* 1994a) (Figure 5B).

DISCUSSION

We have isolated mutations that dominantly modify the rough eye, multiple R7 phenotype caused by ectopic activation of the Raf serine/threonine kinase in the R7 and cone cell precursors. These mutations define six suppressor and two enhancer loci. We have further tested these mutations for genetic interactions with a loss-of-function *raf* allele and performed a preliminary analysis of their recessive phenotypes in a wild-type background.

***Su(Raf)* loci:** The 40 *Su(Raf)* mutations fall into six complementation groups, four of which are represented by multiple alleles. One, and possibly both, of the single-hit mutations appear to be rare antimorphic alleles. We therefore believe we have attained saturation for genes that can be readily mutated to suppress the

Raf^{torY9} phenotype. Clearly, not all genes involved in Raf signaling can be identified in such a screen. In particular, if such a gene is to be identified by a loss-of-function mutation in this screen, a 50% reduction in gene dosage must be sufficient to reduce the efficiency of signaling below the threshold required to transform cone cells into additional R7 cells. We did not, for instance, isolate any alleles of *D-MEK* or *pointed* (*pnt*), both of which are required downstream of Raf in the pathway that induces R7 development (BRUNNER *et al.* 1994a; HSU and PERRIMON 1994, O'NEILL *et al.* 1994). Loss-of-function alleles at these loci do not suppress the *Raf*^{torY9} phenotype (data not shown). Presumably, both D-MEK and Pnt proteins are present in at least a twofold excess above the levels required for Raf signaling in *Raf*^{torY9} flies.

One way in which such nonlimiting factors involved in Raf signaling might nevertheless be identified in such a screen is via the isolation of antimorphic alleles. The products of such alleles might dominantly interfere either with the product of the wild-type allele at the same locus or with any other component of the signal transduction pathway. Although antimorphic alleles are rare, the availability of a simple F₁ screen allows large numbers of flies to be screened to recover such mutations. It appears that at least two of our suppressor mutations, *Su(Raf)3A* and *Su(Raf)3B*, are antimorphic in nature. The fact that *Su(Raf)3C* is represented by a single allele suggests that it too may be an antimorph.

Based on our genetic analysis, three loci emerge as strong candidates for genes encoding proteins that mediate Raf signaling during eye development: *rl*, *phyl*, and *Su(Raf)3A*. All three not only suppress the phenotype caused by constitutive activation of the Raf kinase, but also enhance the phenotype resulting from a partial loss in *raf* function. Furthermore, all three show recessive phenotypes consistent with a role in signal transduction pathways involving Raf. Detailed genetic and molecular analyses have confirmed these predictions for *rl* and *phyl* (BIGGS *et al.* 1994; CHANG *et al.* 1995; DICKSON *et al.* 1995).

It was somewhat disconcerting to find that three *Su(Raf)* loci, *Su(Raf)2A*, *Su(Raf)3B* and *Su(Raf)3C*, appear to play no major role in normal eye development. Four explanations can be advanced as to why these mutations nevertheless strongly suppress the *Raf^{torY9}* phenotype. (1) It is possible that the products of these loci interact specifically with the *Raf^{torY9}* fusion but not the endogenous Raf protein. (2) *Raf^{torY9}* may activate novel signaling pathways not utilized by the endogenous Raf kinase. (3) *Raf^{torY9}* may have artificially high levels of kinase activity, such that downstream components that normally have only a redundant function in signal transduction might now become rate-limiting. (4) These mutations might disrupt the transformation of cone cells into R7 cells but not affect the fate of the R7 cell itself.

Since we are primarily interested in the means by which signaling via the wild-type Raf kinase leads to R7 development, loci identified as suppressors of *Raf^{torY9}* on the basis of either of the first two possibilities would represent unavoidable artifacts of our screening procedure. Our analysis of *Su(Raf)2A* suggests that this locus is not such an artifact, since under certain circumstances it is also required for signaling via the endogenous Raf kinase. Further genetic analysis will be required to distinguish between models (3) and (4) for *Su(Raf)2A* function and to determine the roles, if any, of *Su(Raf)3B* and *Su(Raf)3C* in Raf signaling.

***E(Raf)* loci:** Only four mutations were isolated that dominantly enhanced the *Raf^{torY9}* phenotype: a new *Star* allele and three alleles of *E(Raf)2A*. This screen was performed in a *Raf^{torY9}* background, rather than the weaker *Raf^{tor}* or stronger *Raf^{tor4021}*, in the hope that this intermediate level of activity would facilitate the isolation of both enhancer and suppressor mutations. Given the low frequency at which *E(Raf)* mutations were recovered, however, we must conclude that either very few such loci exist, or that they result in only a subtle increase in eye roughness that escaped detection in our screen.

Loss-of-function *E(Raf)* mutations would be expected to affect a protein that plays a negative role in signal transduction downstream of Raf. One such protein has been identified by other means: the ETS-domain protein Yan (LAI and RUBIN 1992; TEI *et al.* 1992). Loss-

of-function *yan* mutations only slightly enhance the *Raf^{torY9}* phenotype (data not shown), and it is therefore not surprising that we did not recover such mutations in our screen.

Star encodes a transmembrane protein required for the development of the R2, R5 and R8 photoreceptors, as well as in many other process unrelated to eye development (HEBERLEIN and RUBIN 1991; HEBERLEIN *et al.* 1993; KOLODKIN *et al.* 1994). Although dominant genetic interactions have also been reported between *Star* and other components of the Raf signaling pathway, such as *Ras1* (HEBERLEIN *et al.* 1993) and *Sos* (KOLODKIN *et al.* 1994), the biochemical basis of these interactions is at present unknown. Similarly, *E(Raf)2A* appears to be only indirectly involved in signaling via Raf. Like *Star*, it enhances both the loss- and gain-of-function *raf* phenotypes, the latter occurring without an apparent increase in the average number of R7 cells, and the recessive *E(Raf)2A* phenotype is also indicative of a complex requirement for this gene during eye development.

Conclusion: Genetics has proven to be a powerful adjunct to biochemical studies in the analysis of signal transduction pathways. A particularly useful approach has been to isolate mutations that dominantly modify the phenotypic effects of other mutations affecting the same pathway. We have applied this approach to search for genes acting downstream of Raf in the signal transduction pathway controlling induction of the R7 fate during *Drosophila* eye development. Amongst the loci identified in our screen are *rl*, encoding a MAPK, and *phyl*, a putative target gene of the signal transduction cascade. We anticipate that a detailed genetic and molecular analysis of some of the other loci identified in this screen will provide further insights in to the mechanism by which the signal transduced via Raf leads to selection of the R7 fate.

We thank Dr. KATHY MATTHEWS and the Bloomington Stock Center, Indiana, Dr. G. RUBIN and Dr. C. KLÄMBT, for providing fly stocks. M.D. is supported by a postdoctoral fellowship from the Fundación Ramón Areces. This work was financed by a grant from the Swiss National Science Foundation to E.H.

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Communicating editor: T. SCHÜPBACH