Identification of Autosomal Regions Involved in Drosophila Raf Function

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Manuscript received March 23, 2000
Accepted for publication June 26, 2000

ABSTRACT

Raf is an essential downstream effector of activated $p21^{Ras}$ (Ras) in transducing proliferation or differentiation signals. Following binding to Ras, Raf is translocated to the plasma membrane, where it is activated by a yet unidentified "Raf activator." In an attempt to identify the Raf activator or additional molecules involved in the Raf signaling pathway, we conducted a genetic screen to identify genomic regions that are required for the biological function of Drosophila Raf (Draf). We tested a collection of chromosomal deficiencies representing ~70% of the autosomal euchromatic genomic regions for their abilities to enhance the lethality associated with a hypomorphic viable allele of *Draf*, $Draf^{Su2}$. Of the 148 autosomal deficiencies tested, 23 behaved as dominant enhancers of $Draf^{Su2}$, causing lethality in $Draf^{Su2}$ hemizygous males. Four of these deficiencies identified genes known to be involved in the Drosophila Ras/Raf (Ras1/Draf) pathway: *Ras1*, *rolled* (*rl*, encoding a MAPK), *14-3-3ε*, and *bowel* (*bowl*). Two additional deficiencies removed the Drosophila *Tec* and *Src* homologs, *Tec29A* and *Src64B*. We demonstrate that *Src64B* interacts genetically with *Draf* and that an activated form of Src64B, when overexpressed in early embryos, causes ectopic expression of the Torso (Tor) receptor tyrosine kinase-target gene *tailless*. In addition, we show that a mutation in *Tec29A* partially suppresses a gain-of-function mutation in *tor*. These results suggest that Tec29A and Src64B are involved in Tor signaling, raising the possibility that they function to activate Draf. Finally, we discovered a genetic interaction between $Draf^{Su2}$ and *Df(3L)vin5* that revealed a novel role of *Draf* in limb development. We find that loss of *Draf* activity causes limb defects, including pattern duplications, consistent with a role for Draf in regulation of *engrailed* (*en*) expression in imaginal discs.

THE Raf serine/threonine kinase is an essential effector, downstream of Ras, in mediating the transmission of signals that control cellular proliferation, differentiation, and development (reviewed by Avruch *et al.* 1994; Marshall 1994; Moodie and Wolfman 1994). In a simple linear model, Ras, Raf, MEK, and MAPK are sequentially activated following the activation of a cell surface receptor tyrosine kinase (RTK). While the signaling cascade from Raf to MAPK involves direct phosphorylation, it is not known precisely how Raf is activated following Ras activation (reviewed by Morrison and Cutler 1997). It has been proposed that the sole function of Ras in Raf activation is to translocate Raf to the plasma membrane, where Raf activation is regulated by an unknown factor (Leevers *et al.* 1994; Stokoe *et al.* 1994). However, from our analyses of Draf we have proposed that in addition to targeting Raf to the plasma membrane, Drosophila Ras (Ras1) has a second function and activates the "Raf activator," which in turn activates Raf (Li *et al.* 1998). Such results present a more complex picture for Raf activation and predict the presence of a hypothetical factor, the Raf activator, that is also regulated by Ras. Draf is structurally and functionally homologous to mammalian Raf-1. Human Raf-1 is 46% identical in amino acid sequence to Draf and is able to substitute for Draf for viability and signal transduction in Drosophila (Ambrosio *et al.* 1989; Casanova *et al.* 1994; A. Brand, X. Lu and N. Perrimon, unpublished data). Thus, we expect the mechanism of activation of Draf to be evolutionarily conserved.

The Ras1/Draf/MEK/MAPK signaling cassette is commonly used for signaling by a number of Drosophila RTKs, such as Torso (Tor; reviewed by Duffy and Perrimon 1994), the epidermal growth factor receptor (EGFR or DER; Brand and Perrimon 1994), Sevenless (Sev), and the fibroblast growth factor (FGF) receptor homologs Breathless (Btl; Klambt *et al.* 1992) and Heartless (Htl; Beiman *et al.* 1996; Gisselbrecht *et al.* 1996). These RTKs are involved in various developmental decisions. The Tor pathway specifies cell fates at the embryonic termini. Btl and Htl are required for cell migration and differentiation of the embryonic tracheal system and mesoderm, respectively. The Sev pathway functions exclusively in the eye to specify R7 photoreceptor cell fate. In contrast, the EGFR has multiple functions throughout development and is required during oogenesis and embryogenesis, as well as the development of wing vein and the eye. All these Drosophila RTKs activate the Ras1/Draf/MEK/MAPK cassette to mediate signal transduction to the nucleus, and perturbation of Draf activity impedes signaling processes of these RTKs, resulting in visible phenotypes and/or lethality to the animal.

To genetically isolate the potential Raf activator, as

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well as new components of the Raf signaling cascade, we conducted an F_1 screen for modifiers of Draf function. Here we describe an F_1 screen to identify genomic regions on the second and third chromosomes that enhance the lethality of a mutation in *Draf* (*Draf*^{Su2}) that is associated with reduced activity (Lu *et al.* 1994). We demonstrate that some of the deficiencies identify genes known to be involved in Draf signaling, thus validating the specificity of this screen. We also identified regions that were not described in previous screens and potentially contain new components involved in Draf signaling. Interestingly, two of these deficiencies remove Src64B and Tec29A, respectively, raising the possibility that these cytoplasmic tyrosine kinases are directly involved in Raf activation. Finally, we describe a novel role of Draf in limb patterning. We found that reduction in Draf function caused limb defects, including notching of the posterior wing and anterior pattern duplications in the posterior compartment. Such defects are similar to those caused by misregulation of *engrailed* (*en*) in the imaginal discs, suggesting a possible role for Draf in the regulation of *en* expression during imaginal disc development.

MATERIALS AND METHODS

Stocks: The deficiency kit stocks for second and third chromosomes were kindly provided by the Bloomington Stock Center (Bloomington, IN; http://flystocks.bio.indiana.edu/df-kit.htm). The *Draf*^{Su2} allele is the same as Su(*Draf*^{C110})^2 described in Lu *et al.* (1994). Transgenic flies bearing the *hsp70-Src64B*$^{\Delta 540}$ transgene are as described in Kussick *et al.* (1993).

Genetic screens: Virgin females homozygous for *Draf*^{Su2} were crossed to males of each mutant or deficiency-bearing stock. Multiple broods for each cross were made when necessary to ensure scoring of >100 progeny. A mutant was considered as an enhancer of *Draf*^{Su2} if far fewer *Draf*^{Su2}/Y; m/+ males were recovered compared to the number of *Draf*^{Su2}/Y; +/Balancer males. Similarly, a deficiency was considered to enhance *Draf*^{Su2} if far fewer deficiency-bearing males were recovered, relative to the number of balancer-bearing *Draf*^{Su2} males.

Examination of embryos: To determine the effect of *Src64B*$^{\Delta 540}$ overexpression on *tll* expression, one copy of the *hsp70-Src64B*$^{\Delta 540}$ transgene (Kussick *et al.* 1993) was introduced from the father by crossing wild-type females with males homozygous for the *hsp70-Src64B*$^{\Delta 540}$ transgene. Embryos (0- to 1-hr old) were collected on agar plates and allowed to develop for an additional hour at 25°. They were heat shocked by floating the plates in a 37° water bath for 50 min, cooled to 4°, then fixed for *in situ* hybridization using a *tll* probe.

tll mRNA expression in embryos was examined by whole-mount *in situ* hybridization as described in Li *et al.* (1997). Embryos were mounted in Euparal (Carolina Biological Supply) following *in situ* staining or Hoyer's mountant for cuticle preparations.

Generation of partially rescued *Draf* null males: To generate *Draf* null males, *Draf*$^{11\text{-}29}$ females were crossed to males carrying an autosomal insertion of a wild-type *Draf* gene under the control of the heat-shock promoter (Diaz-Benjumea and Hafen 1994) to create a line that was *Draf*$^{11\text{-}29}$/FM7; *hs-Draf*^{BT127}/*hs-Draf*^{BT127}. This line could then be used to generate partially rescued *Draf* males, or outcrossed to a line carrying a homozygous viable *en-lacZ* insertion, to generate partially rescued *Draf* null males in which *engrailed* expression could be assessed. If provided with daily heat shocks from embryonic stages through late pupal stages, this transgene will rescue *Draf*$^{11\text{-}29}$ males to full viability and fertility.

To examine the role of Draf in the development of the imaginal discs, heat shocks were provided daily (2 hr, 37° waterbath) until the beginning of the second larval instar. Then no further heat shocks were provided until 24–36 hr after pupation, when daily heat shocks were again provided until adults eclosed. Imaginal discs were dissected from wandering third instar larvae, fixed briefly in 4% methanol-free formaldehyde, and stained with X-gal according to established procedures. To examine adult structures, legs and wings were mounted in Hoyer's mountant.

RESULTS

Design of a sensitized screen: To genetically isolate the potential Raf activator or molecules involved in the Raf signaling pathway, we conducted an F_1 screen for modifiers of Draf function. Among a series of *Draf* mutant alleles, one particular allele, *Draf*^{Su2}, which carries two point mutations in *Draf*, appeared suitable for such a screen. Unlike most other *Draf* alleles, *Draf*^{Su2} homozygous females or hemizygous males are viable. *Draf*^{Su2} was isolated as a suppressor of *Draf*^{C110}, a hypomorphic mutation associated with late zygotic lethality. *Draf*^{C110} carries a point mutation, R217L, that prevents the Draf:Ras1 interaction (Melnick *et al.* 1993; Hou *et al.* 1995). Arg217 in Draf is equivalent to Arg89 in Raf-1, which is a critical amino acid residue lying in the first Ras-binding domain (RBD) of Raf-1 and is essential for its binding to activated Ras (Fabian *et al.* 1994). In a screen for dominant suppressors of *Draf*^{C110}, Lu *et al.* (1994) isolated Su2 that restores viability to *Draf*^{C110} flies. *Su2* is an intragenic point mutation [P308L] in the second RBD of Draf, also known as the cysteine-rich domain (CRD). *Su2* increases the biological activity of Draf^{C110} without restoring the binding of Draf^{C110} with Ras1 (Li *et al.* 1998). Although flies carrying both the original *Draf*^{C110} mutation, R217L, and the *Su2* mutation, P308L, are fully viable as homozygotes (referred to as *Draf*^{Su2}), they are only 18% viable *in trans* with *Draf*^{C110}, and are lethal with a null allele of *Draf* (Lu *et al.* 1994). This suggests that further reduction of *Draf* activity, such as that resulting from mutation of one copy of its activator or downstream signaling components, should reduce the viability of *Draf*^{Su2} flies. Since the Draf^{Su2} protein does not bind to Ras1, it should rely more on the hypothetical Raf activator for its activity and therefore be more sensitive to changes in the levels of such an activator.

Validity of the screen: To validate the hypothesis this screen was based upon, we tested available mutant alleles of genes that are known to be involved in the Ras1/Draf signaling pathway (Table 1). Since *Draf*^{Su2} encodes a mutant protein that has reduced Draf function, we predicted that it might be most sensitive to changes in the dosage of genes that act close to Draf in the signaling

TABLE 1

***Draf*^{Su2} provides a sensitive genetic background for an F_1 enhancer screen**

Gene (allele) tested	Reference	Percentage of expected *Draf*^{Su2}; m/+ male progeny	Enhancement of *Draf*^{Su2} lethality
Egfr (*Egfr*^{f2})	Price *et al.* (1997)	77 (111)	No
drk (*drk*^{TZ160})	Hou *et al.* (1995)	89 (107)	No
Dos (*Dos*^{R31})	Raabe *et al.* (1996)	117 (78)	No
Sos (*Sos*^{X122})	Rogge *et al.* (1991)	83 (99)	No
Ras1 (*Ras1*$^{\Delta C40B}$)	Hou *et al.* (1995)	0 (64)	Yes
Ksr (*Ksr*^{7M6})	Li *et al.* (1997)	0 (81)	Yes
14-3-3ε (*14-3-3ε*$^{\Delta 24}$)	Chang and Rubin (1997)	27 (79)	Yes
leonardo (*leo*^{P1188})	Li *et al.* (1997)	112 (123)	No
Dsor (*Dsor*^{r1})	Tsuda *et al.* (1993)	0 (68)	Yes[a]
rolled (*rl*^{698})	Eberl *et al.* (1993)	0 (72)	Yes
tailless (*tll*^{L10})	Pignoni *et al.* (1990)	120 (108)	No
huckebein (*hkb*^{A321R1})	Weigel *et al.* (1990)	123 (136)	No

Mutant alleles (m) of genes known to be involved in Draf signaling were tested for their ability to enhance *Draf*^{Su2}. Virgin females homozygous for *Draf*^{Su2} were crossed to *m/Balancer* males. The percentage of expected F_1 progeny is calculated by the number of *Draf*^{Su2}*/Y; m/+* males divided by the number of *Draf*^{Su2}*/Y; +/Balancer* males. The total number of male progeny scored in each experiment is indicated in parentheses.

[a] *Dsor* was tested by crossing virgins of *Draf*^{C110} *Dsor*^{r1}*/Binsc* to *Draf*^{Su2} males. The percentage of expected F_1 progeny is calculated by the number of *Draf*^{C110} *Dsor*^{r1}*/Draf*^{Su2} females divided by the *Draf*^{Su2}*/Binsc* females.

pathway. In support of this hypothesis, mutant alleles of *Ras1* (Simon *et al.* 1991), *Ksr* (Therrien *et al.* 1995), *Dsor* (encoding a MEK or MAPKK; Tsuda *et al.* 1993), *14-3-3ε*, and *rolled* (*rl*, encoding a MAPK; Brunner *et al.* 1994) enhanced *Draf*^{Su2}, causing lethality *in trans* with *Draf*^{Su2} hemizygous males (Table 1). These results indicate that *Draf*^{Su2} can provide a sensitive background for isolating additional components required for Draf function and particularly for its activation.

The autosomal deficiencies that removed the genes *rl*, *Ras1*, and *14-3-3ε* enhanced the lethality of *Draf*^{Su2} hemizygous males. *In(2R)bwV*^{De2L}*Cy*^{R} carries a deficiency of the 41A–B; 42A2–3 interval and removes *rl*. This deficiency behaved as a strong enhancer of *Draf*^{Su2} (Table 2), indicating that the lethality of *Draf*^{Su2} observed *in trans* with *In(2R)bwV*^{De2L}*Cy*^{R} is due to removal of at least *rl*. *Df(3R)by10* deletes the 85D8–12; 85E7–F1 interval and removes *Ras1*. This deficiency strongly enhances *Draf*^{Su2} lethality (Figure 1 and Table 2). Therefore, the lethality of *Draf*^{Su2} observed *in trans* with *Df(3R)by10* is due at least in part to haploinsufficiency of *Ras1*. Finally, *Df(3R)DG2*, which removes 89E1–F4;91B1–2, behaved as a strong enhancer of *Draf*^{Su2}. *Df(3R)C4*, removing 89E; 90A, did not display any enhancement of the phenotype. Two genes located in this region have been previously implicated in Draf signaling: *Suppressor of Ras85D 3-6* (*SR3-6*; Karim *et al.* 1996), which maps to 90D–E, and *14-3-3ε*, which maps to 90F6–7 (Chang and Rubin 1997). A *14-3-3ε* null allele was tested and was found to modestly enhance *Draf*^{Su2} (Tables 1 and 2).

Autosomal regions that enhance *Draf*^{Su2} lethality: In this section we describe the deficiencies that enhance *Draf*^{Su2}. FlyBase (1999) search for some deficiency regions allowed us to further identify some of the genes that interact with *Draf*^{Su2}.

21A1; 21B7–8: This cytogenetic interval is removed by *Df(2L)net-PMF*, which enhances *Draf*^{Su2} lethality. No obvious candidate gene could be identified in this region.

23A1–2; 23C3–5: Df(2L)C144 removes the cytogenetic interval 23A1–2; 23C3–5 and acts as a moderate enhancer of *Draf*^{Su2}. This region contains a previously identified locus *Su(Raf)2A* that suppresses a rough eye phenotype generated by expression of an activated form of Draf (Draf^{Tor}) in the eye and therefore is thought to act positively in regulation of Draf function (Dickson *et al.* 1996). The molecular nature of the *Su(Raf)2A* mutation is not known and mutants were not available for testing in the *Draf*^{Su2} background.

35F6–12; 36A8–9: Draf^{Su2} is enhanced by *Df(2L)cact-255rv64*, which removes 35F6–12; 36D, but not by *Df(2L)H20*, which deletes 36A8–9; 36E1–2, suggesting that the cytogenetic region 35F6–12; 36A8–9 contains an enhancer of *Draf*^{Su2}. The gene *dachshund* (*dac*) is mapped to 36A1–2 and is disrupted by *Df(2L)cact-255rv64*. *dac* was first isolated as a dominant suppressor of an activated *EGFR* mutation, *Ellipse* (*Elp*). It also enhances a partial loss-of-function mutation in *EGFR* during eye development, and therefore is a positive regulator of the EGFR signaling pathway (Mardon *et al.* 1994). Thus, it is possible that the enhancement of *Draf*^{Su2} is due to removing half a dose of *dac*; however, this was not confirmed by using a *dac* allele (Table 2).

36E1–2; 37B9–C1: This cytological interval is defined by two overlapping deficiencies: *Df(2L)TW137*, which removes 36C2–4; 37B9–C1 and behaved as a strong

TABLE 2

Deficiencies enhancing *Draf*^{Su2} and candidate genes

Deficiency	Breakpoints	% expected (*N*)		Candidate gene (allele) tested	Enhancement of *Draf*^{Su2} male lethality
		Draf^{Su2}; Df/+ male progeny	*Draf*^{Su2}; Bal/+ male progeny		
Df(2L)net-PMF	21A1; 21B7–8	20 (48)	68 (59)	—	—
Df(2L)C144	23A1–2; 23C3–5	17 (42)	67 (65)	—	—
Df(2L)sc19-8	24C2–8; 25C8–9	0 (51)	88 (91)	*bowel* (*bowl*$^{l-26}$, *Su(tor)*^{85})	Yes
Df(2L)TE29Aa-11	28E4–7; 29B2–C1	26 (72)	82 (82)	*Tec29A* (*Tec29A*^{k00206})	No
Df(2L)cact-255rv64	35F6–12; 36D	16 (52)	80 (74)	*dachschand* (*dac*^{1})	No
Df(2L)TW137	36C2–4; 37B9–C1	0 (56)	84 (92)	*tailup* (*tup*^{1}, *tup*$^{isl-1}$)	No
In(2R)bw	41A–B; 42A2–3	0 (67)	62 (102)	*rolled* (*rl*^{EMS698})	Yes
Df(2R)44CE	44C4–5; 44E2–4	20 (59)	83 (86)	*E(sina)8 or peanut* (*pnut*^{02502})	No
Df(2R)B5	46A; 46C	33 (81)	62 (112)	*Mef2* (*Mef2*^{X1})	No
Df(2R)CX1	49C1–4; 50C23–D2	18 (105)	81 (152)	—	—
Df(2R)Vg-C	49B2–3; 49E2	28 (86)	90 (116)	*drk* (*drk*^{TZ160})	No
Df(2R)Pu-D17	57B4; 58B	0 (102)	54 (150)	*Egfr* (*Egfr*^{2}, *Egfr*^{k05115})	No
Df(3L)M21	62F; 63D	0 (43)	19 (62)	*Hsp83*	—
Df(3L)GN24	63F4; 64C13–15	29 (58)	63 (80)	*Src64B*$^{\Delta 17}$)	Yes[a]
Df(3L)AC1	67A2; 67D13	38 (76)	68 (85)	—	—
Df(3L)vin5	68A2; 69A1	35 (97)	75 (114)	*brachyenteron* (*byn*^{5})	No
Df(3L)vin7	68C8–11; 69B4–5	40 (95)	70 (109)	*brachyenteron* (*byn*^{5})	No
Df(3R)ME15	81F3–6; 82F5–7	8 (48)	68 (69)	*huckebein* (*hkb*^{A321R1})	No
Df(3R)by10	85D8–12; 85E7–F1	0 (97)	56 (134)	*Ras1* (*Ras1*$^{\Delta C40B}$)	Yes
Df(3R)M-Kx1	86C1; 87B1–5	0 (44)	71 (72)	*seven up* (*svp*^{07842})	No
Df(3R)T-32	86E2–4; 87C6–7	0 (34)	49 (67)	*seven up* (*svp*^{07842})	No
Df(3R)DG2	89E1–F4; 91B1–2	0 (69)	54 (91)	*14-3-3*ε (*14-3-3*ε$^{\Delta 24}$)	Yes
Df(3R)Dl-Bx12	91F1–2; 92D3–6	20 (78)	81 (130)	*branchless* (*bnl*^{06916})	No
Df(3R)B81	99C8; 100F5	0 (63)	70 (87)	*tailless* (*tll*^{L10})	No

The percentage of expected progeny is calculated as the number of male progeny divided by the number of the female progeny of the same genotype. Female progeny have the genotype of *Draf*^{Su2}/+; *Deficiency*/+ or *Draf*^{Su2}/+; *Balancer*/+. *N* indicates total number of flies counted. For example, in case of *Df(2L)net-PMF*, 48 is the total number of *Draf*^{Su2}/y; *Df*/+ males and *Draf*^{Su2}/+; *Df*/+ females, and 20 is the number of males divided by the number of females times 100. Candidate genes were chosen by searching FlyBase for each genomic region that enhanced *Draf*^{Su2}. Alleles for each candidate gene, if available, were tested for enhancement of *Draf*^{Su2} as heterozygotes. —, not available or not tested.

[a] Enhanced as homozygotes.

enhancer of *Draf*^{Su2}, and *Df(2L)H20*, which removes 36A8–9; 36E1–2 and is completely viable as *trans*-heterozygotes in the *Draf*^{Su2} background. The gene *tailup* (*tup*), which maps to 37A2–6, is disrupted by *Df(2L)TW137*. *tup*, also known as *islet* (*isl*), encodes a LIM domain protein (Thor and Thomas 1997) that mediates Tor signaling (Strecker *et al.* 1991). However, a *tup* allele tested did not show enhancement of *Draf*^{Su2} (Table 2).

44C4–5; 44E2–4: This cytological region is removed by *Df(2R)44CE*, which enhanced *Draf*^{Su2}. A candidate enhancer in this region is *peanut* (*pnut*; Neufeld and Rubin 1994), encoding a Septin homolog required for cytokenesis. *pnut* was first identified as *E(sina)8*, which genetically interacts with *seven in absensia* (*sina*; Carthew *et al.* 1994), a gene encoding a nuclear protein required for the correct development of R7 photoreceptor cells in the Drosophila eye. *pnut* has been demonstrated to act positively in the Sev pathway. Loss of *pnut* could therefore enhance *Draf*^{Su2}. However, a *pnut* allele did not show enhancement of *Draf*^{Su2} (Table 2).

46A; 46C: Df(2R)B5 removes region 46A; 46C and behaves as a weak enhancer of *Draf*^{Su2}. No previously identified components of the Ras/Raf signaling pathway are located in this region. *Df(2R)B5* disrupts, among other genes, *Mef2* (Ranganayakulu *et al.* 1995), which encodes the Drosophila homolog of the myocyte enhancing factor 2 and functions during embryonic muscle development (Bour *et al.* 1995; Taylor *et al.* 1995). A loss-of-function allele of *Mef2* did not show enhancement of *Draf*^{Su2} male lethality (Table 2).

49C1–4; 49E2: This cytological interval is defined by two overlapping deficiencies *Df(2R)CX1* and *Df(2R) Vg-C*, both of which behaved as weak enhancers of *Draf*^{Su2}. A gene disrupted by these two deficiencies is *E(EGFR)B56*, which dominantly enhances mutations in *EGFR* [Price *et al.* 1997; *E(EGFR)B5* was not available for testing]. *Df(2R)CX1* additionally disrupted *drk*, a known component of the Ras1/Draf signaling pathway (Simon *et al.* 1993). However, a *drk* allele did not show enhancement (Table 2).

57D11–12; 58A1–2: This cytological region is defined by the overlapping deficiencies *Df(2R)AA21*, *Df(2R)*

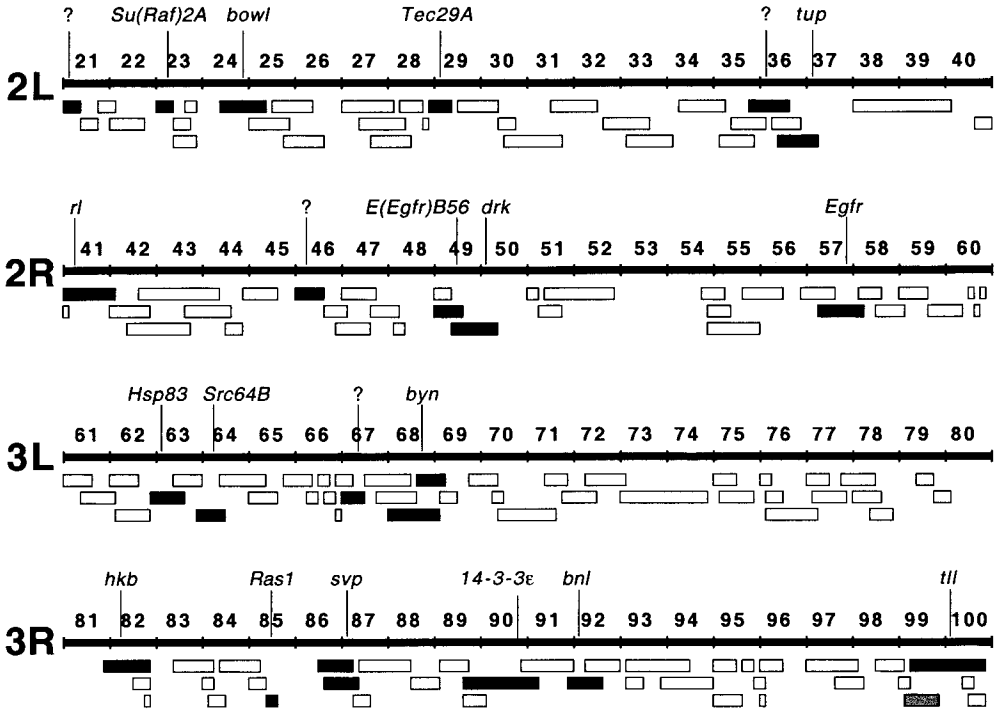

FIGURE 1.—Autosomal deficiencies screened for enhancement of *Draf*^{Su2}. The four major autosomal arms are represented as solid lines. Numbers above each line represent the cytological regions. Boxes below each autosomal arm represent the approximate genomic regions removed by each deficiency tested in this study. Solid and open boxes represent the deficiencies that did and did not enhance *Draf*^{Su2}, respectively. Shaded boxes indicate that the results were uninterpretable due to haploinsufficiency of the deficiency tested. The locations of possible candidate genes are indicated above the respective autosomal arms. However, they may not enhance *Draf*^{Su2} in this study. Regions where no obvious candidate genes were found are indicated by a question mark.

X58-7, and *Df(2R)Pu-D17*. *Df(2R)AA21* and *Df(2R)X58-7* remove 56F9–17; 57D11–12 and 58A1–2; 58E4–10, respectively, and did not enhance the lethality of *Draf*^{Su2} males. However, *Df(2R)Pu-D17*, which deletes the 57B4; 58B region, behaved as a strong enhancer of *Draf*^{Su2}. *Df(2R)Pu-D17* disrupts the *EGFR* gene, which maps at 57F1. However, neither of the two mutant alleles of *EGFR* tested were able to dominantly enhance the lethality of *Draf*^{Su2} males (Table 2).

62F; 63D: This cytological region is removed by *Df(3L)M21*, which behaves as a strong enhancer of *Draf*^{Su2}. This deficiency disrupts *Hsp83*, which has been shown to be required for Draf signaling (VAN DER STRATEN *et al.* 1997; not available for testing).

67A2; 67D13: This cytological region is defined by *Df(3L)AC1*, which behaves as a weak enhancer of *Draf*^{Su2}. There are no obvious candidate genes that might be involved in Draf signaling in this region.

81F3–6; 82F5–7: Df(3R)ME15 removes this region and behaved as a strong enhancer of *Draf*^{Su2}. A candidate gene disrupted by this deficiency is *huckebein* (*hkb*), a Tor target gene involved in the specification of terminal cell fates (BRONNER and JAECKLE 1991). However, this was not confirmed by testing a *hkb* mutation (Table 2).

86E2–4; 87B1–5: This genomic region is defined by the two overlapping deficiencies *Df(3R)M-Kx1*, removing 86C1; 87B1–5, and *Df(3R)T-32*, removing 86E2–4; 87C6–7. Both deficiencies behaved as strong enhancers of *Draf*^{Su2}. A candidate gene in the region is *seven up* (*svp*), which maps to 87B4 and is deleted in both *Df(3R)M-Kx1* and *Df(3R)T-32*. *svp* encodes a member of the steroid receptor gene superfamily and is required for photoreceptor cell fates during eye development (MLODZIK *et al.* 1990). Further, all the components of the Ras1/Draf pathway are required for the function of *svp* (BEGEMANN *et al.* 1995). However, a *svp* allele tested did not enhance *Draf*^{Su2} (Table 2).

91F5; 92B3: This region is defined by deficiencies *Df(3R)Cha7*, removing 90F1–2; 91F5, *Df(3R)Dl-BX12*, removing 91F1–2; 92D3–6, and *Df(3R)H-B79*, removing 92B3; 92F13. *Df(3R)Dl-BX12* behaved as an intermediate enhancer of *Draf*^{Su2}, while the other two did not, thus narrowing the genomic region required for *Draf*^{Su2} function to 91F5; 92B3. A candidate gene in the region is *branchless* (*bnl*), which encodes the Drosophila FGF homolog. *bnl* is located at 92B2–3 and is deleted by *Df(3R)Dl-BX12* (SUTHERLAND *et al.* 1996). Bnl functions as a ligand for the Btl RTK to specify the tracheal branching pattern. However, a hypomorphic *P*-element allele of *bnl* (Table 2) did not enhance *Draf*^{Su2}.

99C8; 100C: This region is defined by the two overlapping deficiencies *Df(3R)B81*, removing 99C8; 100F5, *Df(3R)awd-KRB*, removing 100C; 100D, and *Df(3R)faf-BP*, removing 100D; 100F5. Of these three deficiencies, only *Df(3R)B81* behaved as a strong enhancer of *Draf*^{Su2}. The gene *tll* that maps to 100B1 was a possible candidate; however, a loss-of-function *tll* mutation did not enhance *Draf*^{Su2}.

***Su(tor)*^{85} is allelic to *bowl* and is responsible for the enhancement of *Draf*^{Su2} by *Df(2L)sc19-8*:** *24C2–8; 25A5:* This cytological region is defined by the overlapping deficiencies *Df(2L)sc19-8*, removing 24C2–8; 25C8–9, and *Df(2L)sc19-4*, removing 25A5; 25E5. *Df(2L)sc19-8* behaved as a strong enhancer of *Draf*^{Su2}, while *Df(2L)sc19-4* did not. The gene *bowel* (*bowl*) maps to 24C2–3 and is disrupted by *Df(2L)sc19-8*. *bowl* is a terminal class

gene regulated by the Tor target gene *tll* and encodes a zinc-finger protein that mediates a subset of *tll* functions in specifying the hindgut and proventriculus in the posterior of the embryo (Wang and Coulter 1996). *In trans*-heterozygotes (see Table 2 and below) a *bowl* mutation enhanced $Draf^{Su2}$, indicating that the lethality of $Draf^{Su2}$ observed *in trans* with *Df(2L)sc19-8* is likely due to removal of *bowl*.

A previous screen for suppressors of a gain-of-function *tor* allele identified a number of loci, referred to as *Su(tor)*, that may encode signaling components downstream from the Tor RTK (Doyle and Bishop 1993). We surveyed the existing collection of *Su(tor)* alleles for their abilities to enhance $Draf^{Su2}$. We tested eight *Su(tor)* alleles that were available (17-29-5, 85, 293-12-3, 307-14, 321-16-5, 337-73, 341, and 404-9-1; Doyle and Bishop 1993). Among these, $Su(tor)^{404}$ and $Su(tor)^{85}$ enhanced $Draf^{Su2}$ [*i.e.*, $Draf^{Su2}$/Y; $Su(tor)^{404}$/+ and $Draf^{Su2}$/Y; $Su(tor)^{85}$/+ males died]. $Su(tor)^{404}$ is allelic to *Ras1* (Doyle and Bishop 1993). Because $Su(tor)^{85}$ has been mapped meiotically to map position 16 on 2L, which corresponds to the 24–25 cytological region, we determined if $Su(tor)^{85}$ is the mutation in the 24C2–8; 25A5 region responsible for the enhancement of $Draf^{Su2}$. We found that $Su(tor)^{85}$ is an allele of *bowl* on the basis of its noncomplementation with either *Df(2L)sc19-8* or the $bowl^{L26}$ allele. In addition, $Su(tor)^{85}$ is an embryonic lethal recessive mutation and the embryonic phenotypes associated with loss of $Su(tor)^{85}$ are identical to those of *bowl* embryos (Wang and Coulter 1996). These embryos are missing the denticle bands two, six, and eight.

Src64B genetically interacts with Draf and overexpression of an activated form of Src64B causes ectopic expression of the Tor target gene *tll*: *63F4; 64C13–15: Df(3L)GN24* removes this region and behaves as a moderate enhancer of $Draf^{Su2}$. *Df(3L)GN24* removes the *Src64B* gene, which maps to 64B12–17, that encodes the Drosophila homolog of mammalian *c-src* (Simon *et al.* 1985). The existing *Src64B* mutation is homozygous viable, suggesting that *Src64B* may be functionally redundant, or that it may not be essential for viability. $Src64B^{\Delta 17}$ is associated with a deletion that removes the first two exons of the *Src64B* transcript, and $Src64B^{\Delta 17}$ homozygotes produce no Src64B protein. Interestingly, $Src64B^{\Delta 17}$ is classified as a weak allele on the basis of the observation that animals hemizygous for $Src64B^{\Delta 17}$ ($Src64B^{\Delta 17}$ *in trans* with a deficiency of the region) exhibit a more severe oogenesis phenotype than $Src64B^{\Delta 17}$ homozygotes (Dodson *et al.* 1998). We found no enhancement of $Draf^{Su2}$ by $Src64B^{\Delta 17}$ in the heterozygous situation. However, we found that $Draf^{Su2}$/Y; $Src64B^{\Delta 17}/Src64B^{\Delta 17}$ males are semilethal, 51% (38/74) as viable as *FM7/Y*; $Src64B^{\Delta 17}/Src64B^{\Delta 17}$ siblings. Further, the $Draf^{Su2}$/Y; $Src64B^{\Delta 17}/Src64B^{\Delta 17}$ males that survived had eyes that were slightly small and rough (data not shown), similar to the eye phenotypes due to certain mutations in genes of the Ras/Raf pathway. Finally, when $Draf^{Su2}/FM7$; $Src64B^{\Delta 17}$ females were crossed to *Df(3L)GN24/TM8 Sb* males, no $Draf^{Su2}/Y$; $Df(3L)GN24/Src64B^{\Delta 17}$ progeny were recovered, while *FM7/Y*; $Df(3L)GN24/Src64B^{\Delta 17}$ males survived ($N = 31$), as well as females with or without the *Df(3L)GN24* chromosome ($N = 112$). These results suggest that *Src64B* is a candidate gene responsible for the enhancement of $Draf^{Su2}$ located within *Df(3L)GN24*. There may be another gene deleted by *Df(3L)GN24*, which, in conjuction with $Src64B^{\Delta 17}$, is also required for Draf function (see discussion).

To investigate the involvement of Src64B in Draf signaling, we overexpressed an activated form of Src64B, $Src64B^{\Delta 540}$ (Kussick *et al.* 1993), in early embryos under the control of the heat-shock promoter and examined its effects on the Tor pathway. $Src64B^{\Delta 540}$ lacks the C-terminal negative regulatory domain and acts as a constitutively activated kinase (Kussick *et al.* 1993). Heat-shock induction of $Src64B^{\Delta 540}$ in early embryos caused dramatic defects in the cuticular structures, most notably deletions of the ventral denticle bands (Figure 2E). Such defects are reminiscent of those associated with *tor* gain-of-function mutations. The embryos derived from females carrying a *tor* gain-of-function mutation show expansion of the *tll* expression domains (Figure 2D) and disruption of the cuticular structures in the central region of the embryo (Figure 2C). *tll* is activated at the embryonic termini (Pignoni *et al.* 1990, 1992) and the posterior *tll* expression domain has been used as a molecular "readout" for the strength of Tor as well as Draf activities (Hou *et al.* 1995; Li *et al.* 1997, 1998). To investigate whether the cuticular defects caused by overexpression of $Src64B^{\Delta 540}$ are due to expansion of *tll* expression domains, we examined *tll* mRNA levels in embryos following heat-shock induction of $Src64B^{\Delta 540}$. Indeed, 16% ($N = 36$) of appropriately aged embryos showed marked expansion of *tll* expression domains (Figure 2F). No control embryos showed expansion of *tll* expression under the same heat-shock treatment. Together, these results suggest that Src64B activation can positively influence Tor signaling, raising the possibility that it activates Draf *in vivo*.

Mutation in *Tec29A* partially suppressed a gain-of-function mutation of *tor*: *28E4–7; 29B2–C1: Df(2L)TE29Aa-11* removes the cytogenetic region 28E4–7; 29B2–C1 and behaves as a weak enhancer of $Draf^{Su2}$. *Tec29A*, which is located in this interval, encodes the Drosophila homolog of a cytoplasmic tyrosine kinase of the Tec family. Mammalian Raf-1 can be phosphorylated and activated by the Src tyrosine kinase *in vitro*, but there has been no confirmation of this *in vivo* (reviewed by Brown and Cooper 1996; Morrison and Cutler 1997). Tec proteins share homology with Src in the kinase domain and are regulated by Src (Guarnieri *et al.* 1998; Roulier *et al.* 1998). Although a mutant allele of *Tec29A* did not dominantly enhance the lethality of $Draf^{Su2}$ (Table 2), by examining the genetic interactions between *Tec29A* and *tor*, we found that *Tec29A* mutations partially suppress a gain-of-function allele of *tor* (see below).

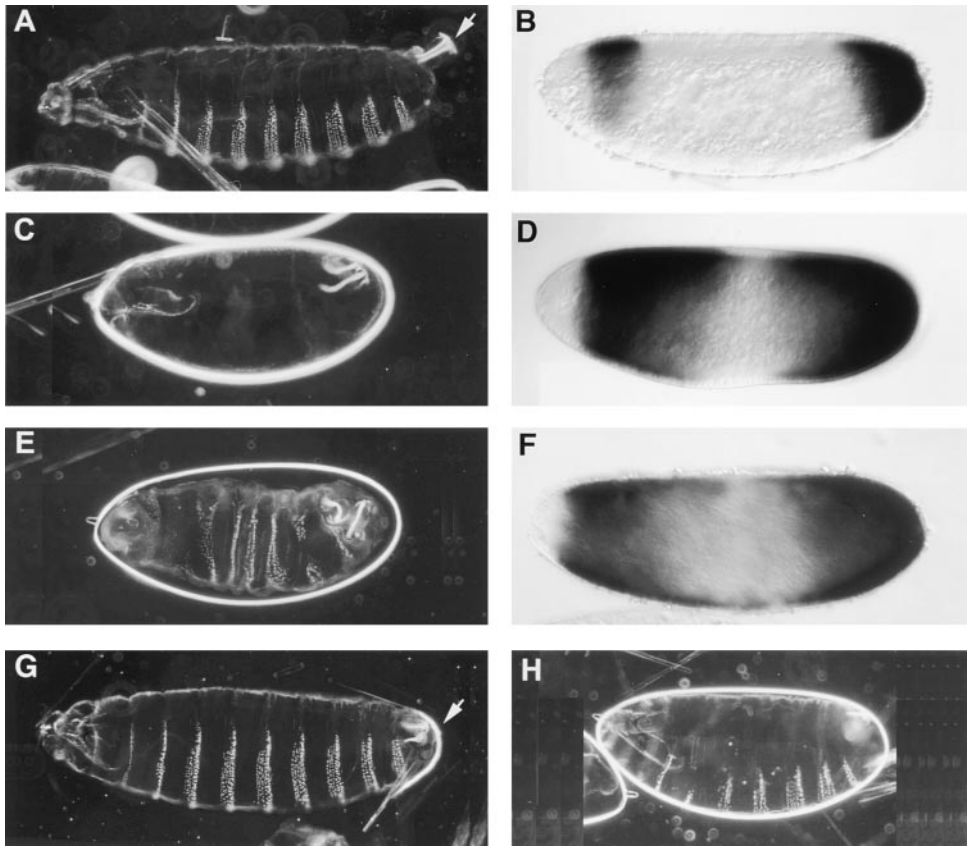

FIGURE 2.—Cuticular phenotypes and *tll* mRNA expression patterns. (A) A wild-type larva, showing eight abdominal denticle bands, the Filzkörper materials (arrow) within the posterior spiracles, and head skeletons. (B) *tll* expression pattern in a wild-type embryo at the precellularization stage. The posterior domain is ~15% of egg length. (C) An embryo laid by a female heterozygous for the gain-of-function mutant tor^{Y9}. Note that all the ventral denticle bands are missing and the Filzkörper is slightly enlarged. (D) Expansion of *tll* in an embryo derived from a $tor^{Y9}/+$ mother. (E) Embryos carrying one copy of the $hsp70\text{-}Src64B^{\Delta 540}$ transgene were heat shocked during early embryogenesis. Note the severely disrupted denticle bands and slightly enlarged Filzkörper materials (compare with C). (F) *tll* expression in embryos carrying one copy of the $hsp70\text{-}Src64B^{\Delta 540}$ transgene following heat-shock treatment. Note the expansion of *tll* expression domains similar to the one shown in D. (G) A larva homozygous for $Tec29A^{206}$. Note the defective mouth parts (see also ROULIER *et al.* 1998) and shortened Filzkörper (arrow). (H) An unhatched embryo from a $tor^{Y9}/Tec29A^{206}$ mother crossed to $Tec29A^{206}/+$ males presumably homozygous for $Tec29A^{206}$. These embryos were recognized as $Tec29A^{206}$ homozygotes because they had defective mouth parts and shortened Filzkörper that are identical to $Tec29A^{206}$ homozygous embryos. They exhibited significantly more ventral denticle bands than those laid by $tor^{Y9}/+$ females.

A mutation in *Tec29A*, $Tec29A^{206}$ is caused by a *P*-element insertion at the 5′ untranslated region of *Tec29A* that greatly reduces *Tec29A* transcripts such that no *Tec29A* mRNA is detectable in $Tec29A^{206}$ homozygous embryos (ROULIER *et al.* 1998). However, in contrast to *Df(2L)TE29Aa-11*, we found that $Tec29A^{206}$ did not cause lethality to $Draf^{Su2}$ hemizygous males in the heterozygous situation. Embryos homozygous for $Tec29A^{206}$ do not hatch, and their cuticles showed defects in the mouth parts and the posterior spiracles. These terminal structures are either defective or missing in embryos mutant for the Tor pathway genes. As has been previously demonstrated (ROULIER *et al.* 1998), the mouth parts of $Tec29A^{206}$ homozygous embryos are missing several components (Figure 2G). In addition, the posterior spiracles of $Tec29A^{206}$ homozygous embryos are shorter than in wild type (arrow in Figure 2G). These observations suggest that *Tec29A* is required for the differentiation of terminal structures and that it may function in the same genetic pathway as Tor and Draf. Since mutations in *Tec29A* disrupt the terminal structures in the embryos, we examined the relationship between *Tec29A* and *tor*. A single copy of the gain-of-function allele tor^{Y9} causes expansion of the expression domains of *tll* and consequently disruptions of the larval cuticular structures. Embryos laid by tor^{Y9} heterozygous females lack all denticle bands and have a severely disrupted head skeleton. Most of them retain intact and often enlarged posterior spiracles (Figure 2C). We found that zygotic homozygosity for $Tec29A^{206}$ partially suppressed the tor^{Y9} maternal effect phenotype. When $tor^{Y9}/Tec29A^{206}$ females were crossed to $Tec29A^{206}/+$ males, all embryos showed a significant increase in the number of ventral denticle bands, suggesting that reducing the maternal amount of *Tec29A* by half suppressed tor^{Y9} ($N > 500$). About one-quarter of the embryos, presumably zygotically null for *Tec29A*, exhibited mostly the $Tec29A^{206}$ mutant phenotype (Figure 2H). However, they did not completely restore all the ventral denticle bands (Figure 2H), suggesting that homozygosity of *Tec29A* does not completely suppress tor^{Y9}. We observed similar partial suppressions of the tor^{Y9} phenotype when we used $tor^{Y9}/Df(2L)TE29Aa\text{-}11$ females in the above cross. These results indicate that $Tec29A^{206}$ is epistatic to tor^{Y9}, consistent with a model in which *Tec29A* functions downstream from, or in parallel, to Tor and is minimally required for a gain-of-function Tor protein to transduce signal.

Since Tec29A is regulated by Src64B during oogenesis (GUARNIERI *et al.* 1998; ROULIER *et al.* 1998) and overexpression of $Src64B^{\Delta 540}$ is associated with an expansion

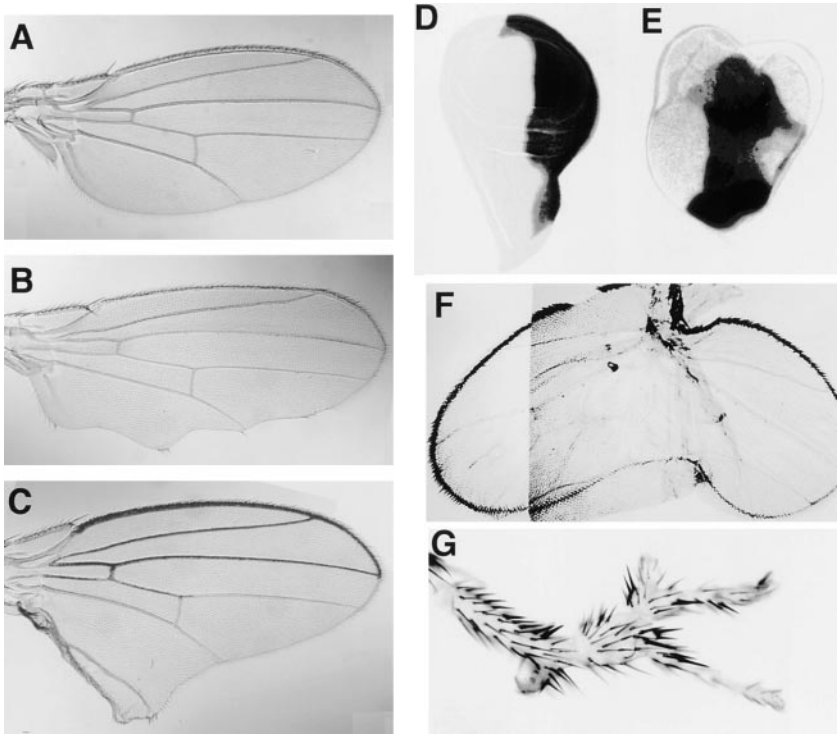

FIGURE 3.—Draf is essential for limb patterning. Adult wing defects exhibited by *Draf*^{Su2}*/Y; Df(3L)vin5/+* males. (A) Wild-type adult wing. (B) A wing from a *Draf*^{Su2}*/Y; Df(3L)vin5/+* adult male showing notching in the posterior compartment of the wing. (C) A wing from a *Draf*^{Su2}*/Y; Df(3L)vin5/+* adult male showing partial duplication of anterior structures in the posterior compartment. (D) A wild-type wing disc showing *en-lacZ* expression pattern. (E) *en-lacZ* expression in a wing disc with insufficient levels of Draf during the second and early third larval instars. Note expansion of en-lacZ staining beyond the normal posterior compartment and the mirror-image duplication of the anterior compartment in the posterior. (F) Mirror image duplication of anterior structures in the posterior compartment due to an insufficient amount of Draf. (G) Leg duplication in a similarly treated partially rescued Draf male.

of *tll*, we examined whether overexpression of *Tec29A* is able to increase Tor signaling. In contrast to Src64B$^{\Delta 540}$, overexpression of *Tec29A* from a heat-shock inducible transgene did not cause a significant change in *tll* expression in the embryo (data not shown). If the activity of Tec29A is regulated by Src64B, overexpression of a wild-type version of the gene may not have significant effects on the animal.

Draf function is required for limb development: *68D6; 69A1:* This cytological region is defined by the overlapping deficiencies *Df(3L)vin5*, which removes 68A2; 69A1, *Df(3L)vin7*, which deletes 68C8–11; 69B4–5, and *Df(3L)vin2*, removing 67F2; 68D6. *Df(3L)vin5* and *Df(3L)vin7* weakly enhanced *Draf*^{Su2} male lethality, whereas *Df(3L)vin2* showed no interaction with *Draf*^{Su2}. Interestingly, about half of the *Draf*^{Su2}/Y; *Df(3L)vin5/+* escaper males (10/25) exhibited wing defects in the posterior part of the wing, showing either notches along the margin (Figure 3B) or pattern duplications in ~30% of animals (Figure 3C). These defects were also found in ~10% of *Draf*^{Su2}/Y; *Df(3L)vin7/+* males. One gene deleted by *Df(3L)vin5* and *Df(3L)vin7*, but not by *Df(3L)vin2*, is *brachyenteron* (*byn*), located at 68E1–4. *byn* encodes a homolog of the mouse *Brachyury* gene, a T-related homeobox gene regulated by Tor and required for specification of the hindgut and anal pads during embryogenesis (SINGER *et al.* 1996). *byn* is activated by *tll* and repressed by *hkb*. Therefore it is possible that *byn* is responsible for the enhancement of these two overlapping deficiencies. However, a strong *byn* allele that we tested did not enhance *Draf*^{Su2} in heterozygotes (Table 2), nor did the *Draf*^{Su2}/Y; *byn/+* males exhibit any wing defects.

Draf^{Su2}/Y; *Df(3L)vin5/+* male escapers exhibit wing notches that are restricted in the posterior compartment and anterior pattern duplications at the expense of posterior pattern elements (see above and Figure 3, B and C). This suggests that *Draf*, together with a gene that is removed by *Df(3L)vin5*, has a function in patterning the imaginal discs.

The wing disc is divided into anterior and posterior compartments, and the cell identity in the posterior is maintained by continued expression of *en* (reviewed by LAWRENCE and STRUHL 1996), but elevated levels of En in the posterior compartment result in partial inactivation of both *en* and *invected* (*inv*), indicating that En has a negative autoregulatory mechanism (GUILLEN *et al.* 1995). *hedgehog* (*hh*) is expressed by cells in the posterior compartment, which by virtue of their En expression are not responsive to Hh. The Hh protein diffuses into the anterior region and, along the anteroposterior (A/P) boundary, activates *decapentaplegic* (*dpp*), which encodes a morphogen that organizes the global patterning of the wing (TABATA *et al.* 1992; BASLER and STRUHL 1994; ZECCA *et al.* 1995).

To determine if the cause of the posterior wing notching and anterior pattern duplications observed in the *Draf*^{Su2}/Y; *Df(3L)vin5/+* male survivors could be explained by an additional reduction in Draf activity, we examined the role of Draf in patterning of the wing and leg imaginal discs using partially rescued Draf null males (see MATERIALS AND METHODS; clones of Draf

null alleles cannot be recovered). When Draf is not provided during the second and third larval instars, the domain of *en-lacZ* expression and the overall levels of *en-lacZ* expression are greatly increased [Figure 3E; this was also confirmed using antibodies directed against En (data not shown)], and an ectopic anterior compartment is induced. When allowed to reach adulthood these animals exhibited the pattern duplications predicted on the basis of *en* expression observed in the discs, that is, duplication of anterior pattern elements in the posterior compartment (Figure 3F). Comparable pattern duplications originating from the posterior compartment were also observed in the legs by withholding Draf during the second and third larval instars (Figure 3G).

Therefore, Draf appears to have a role in negatively regulating *en* expression in imaginal discs. The wing notching observed exclusively in the posterior compartment is also consistent with Draf negatively regulating *en*, since increased expression of En in the posterior compartment serves to partially inactivate *en* and *inv*, which is thought to be required for the determination of posterior cell fates (Guillen *et al.* 1995). These results, together with the observation that ectopic activation of Raf or Draf in the discs results in a marked reduction in En and Hh expression in the posterior compartment (E. Noll, unpublished observations), suggest that *Draf* is essential for limb patterning. On the basis of these observations, the simplest interpretation of the posterior wing notching and duplication in the $Draf^{Su2}$/Y; *Df(3L)vin5*/+ males is that the gene removed by *Df(3L)vin5* further reduces Draf signaling.

DISCUSSION

Previously, genetic screens using EMS-induced lesions have been conducted for modifiers of activated forms of Ras1 or Draf (Dickson *et al.* 1996; Karim *et al.* 1996) or for suppressors of a *Draf* hypomorphic allele (Tsuda *et al.* 1993; Lu *et al.* 1994). These screens have proven to be very fruitful in isolating new components in the Ras/Raf pathway. However, the mechanism of Raf activation remains unclear. To isolate additional components of the Ras1/Draf pathway, possibly including a Draf activator, we conducted a screen for enhancers of a viable allele of *Draf*. We tested 148 autosomal deficiencies in an F_1 screen and found that 23 behaved as dominant enhancers of $Draf^{Su2}$. Some of these deficiencies remove genes or loci known to be involved in the Ras1/Draf pathway. Among the deficiencies that remove genomic regions not previously known to contain genes involved in the Ras1/Draf pathway, two deficiencies remove genes encoding Tec29A and Src64B, respectively. In addition, through this screen, we identified a novel role for Draf in limb development.

Verification of candidate genes for the identified genomic regions that enhanced $Draf^{Su2}$: For each genomic region that enhanced $Draf^{Su2}$, we searched FlyBase and tried to identify a candidate gene that is most likely responsible for the enhancement. For most of these genes, we obtained mutant alleles and determined if they were able to enhance $Draf^{Su2}$ as heterozygotes. As shown in Table 2, in four of the candidate genes, *bowl*, *rl*, *Ras1*, and *14-3-3ε*, the mutant allele behaved as the respective deficiency and dominantly enhanced $Draf^{Su2}$. The rest of the candidate mutations did not dominantly enhance $Draf^{Su2}$ lethality. There are several possible explanations for this. First, the candidate gene selected may not be responsible for the enhancement of $Draf^{Su2}$, but rather the true enhancer gene was not identified because the responsible gene has not yet been identified, or is not an obvious candidate for an enhancer. Second, it is possible that some of the alleles tested are not as strong as a deficiency and therefore are unable to enhance $Draf^{Su2}$ as the deficiency does. Third, mutations in a single candidate gene alone may not be sufficient to enhance $Draf^{Su2}$. It is possible that a second gene located in the same deficiency region has to be mutated in order to observe an enhancement of the lethality. Thus, we cannot rule out a gene as a candidate simply on the basis of the inability of a particular mutant allele to enhance $Draf^{Su2}$.

Are Src64B and Tec29A activators of Draf? *Src64B* and *Tec29A* are removed by two deficiencies that each dominantly enhanced the lethality of $Draf^{Su2}$. They were selected as candidate genes for these two deficiencies because a survey of FlyBase for genes in the regions removed by the deficiencies did not yield other genes more likely to be involved in Draf function. We showed that the $Src64B^{\Delta 17}$ allele in homozygotes enhanced $Draf^{Su2}$, confirming that *Src64B* genetically interacts with $Draf^{Su2}$. We further showed that overexpression of an activated form of Src64B in early embryos can cause activation of the Tor target gene *tll* and cuticular defects similar to those caused by gain-of-function mutations in *tor*. These results are consistent with a role of Src64B in Tor signaling and/or Draf activation (Figure 4). We were unable to demonstrate that Tec29A could enhance Draf using an available mutant allele of Tec29A. However, we obtained indirect evidence suggesting a requirement of Tec29A in Tor signaling. First, $Tec29A^{206}$ homozygous mutant embryos exhibit defects in the terminal structures that are specified by the Tor pathway. Specifically, they showed defective mouth parts and shortened Filzkörper, phenotypes consistent with disruption of Tor signaling. Further, we found that reducing the activity of *Tec29A* suppresses a gain-of-function *tor* allele. Most strikingly, embryos zygotically homozygous for $Tec29A^{206}$ that are derived from tor^{Y9} mothers exhibited mouth parts and Filzkörper indistinguishable from those of $Tec29A^{206}$ embryos. Mutation of *Tec29A* restored most of the ventral denticle bands that would have been deleted due to tor^{Y9}, suggesting that *Tec29A* is genetically epistatic to *tor*. However, many of the em-

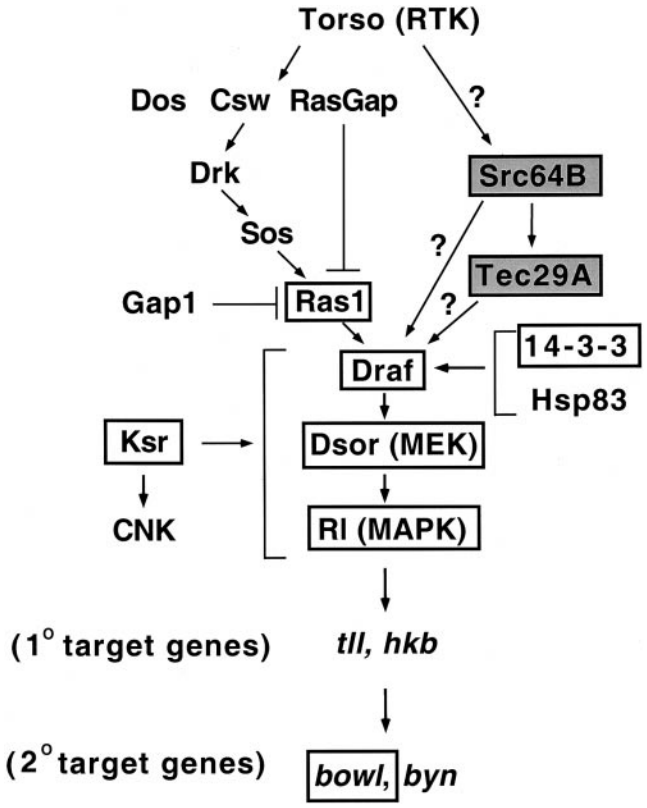

FIGURE 4.—Components of Draf signaling in the Tor pathway. Components of the Tor signaling pathway identified in this and other studies are listed. The relationship between adjacent components is indicated by an arrow (activation); —| (inactivation), or bracket (structural). Molecules that interacted with *Draf*^{Su2} in this study are boxed.

bryos still exhibited minor disruptions in the ventral denticle bands, a defect reminiscent of weak *tor* gain-of-function mutations. This suggests that homozygosity for *Tec29A*^{206} cannot completely suppress *tor*^{Y9}. Possibly, while Tec29A may be required for Tor signaling, *Tec29A*^{206} may not be a null allele and therefore cannot completely suppress *tor*^{Y9}. This would be consistent with the inability of this allele to enhance *Draf*^{Su2}. Alternatively, the maternally contributed Tec29A may be able to partially mediate signaling by the mutant *Tor*^{Y9} protein. Finally, Tec29A may not be an absolute requirement for Tor signaling, but rather functions in a separate pathway that in conjunction with Tor is required for the differentiation of terminal structures.

The likelihood that Src64B and Tec29A are involved in Draf activation is based upon data from *in vitro* studies of mammalian c-Src function. Src kinases can phosphorylate and activate Raf-1 *in vitro*, and the tyrosine residues phosphorylated by Src are important for Raf-1 activation (reviewed by MORRISON and CUTLER 1997; THOMAS and BRUGGE 1997). Tec kinases are very similar to Src kinases in the kinase domain, but lack the C-terminal regulatory tyrosine and the N-terminal myristylation site that are specific for Src family members. Tec kinases interact with and are activated by Src through phosphorylation (RAWLINGS *et al.* 1996). It has been shown in Drosophila that Tec29A is regulated by Src64B and both are required for the growth of ring canals of the egg chamber (GUARNIERI *et al.* 1998; ROULIER *et al.* 1998). Although it has not been documented that Tec can phosphorylate Raf *in vivo*, given the similarities in the kinase domain, it is not unreasonable to propose that Tec could do so. Finally, consistent with our results, the two genomic regions containing Src64B and Tec29A were also identified as required for the function of Corkscrew (Csw) in a similar screen for modifiers of a partial loss-of-function *csw* allele (L. PERKINS, personal communication).

Function of Draf in limb patterning: The proper expression of *en* in the posterior compartment of imaginal discs is essential for maintaining compartmental boundaries and patterning of Drosophila limbs (TABATA *et al.* 1995). Despite much insight into the events required for Hh signaling (TABATA *et al.* 1992; BASLER and STRUHL 1994; ZECCA *et al.* 1995; reviewed by LAWRENCE and STRUHL 1996), little is known about the mechanism(s) by which *en* expression is controlled in the posterior compartment.

We have identified two instances where a further reduction in Draf function, due to the presence of a deficiency, results in defects in posterior pattern elements in the limbs. *Draf*^{Su2}/Y; *Df(3L)vin5*/+ male survivors exhibit notching only in the posterior region of the wing, and partial pattern duplications in the posterior compartment. Since no specific role for Draf has been described in the limbs, we examined what the requirements for Draf were in the imaginal discs. Since clonal analysis with null alleles is uninformative, because *Draf* mutant clones do not develop, we conditionally provided *Draf* to the developing animals in a *Draf* null background.

By withholding Draf during the second and early third larval instars, animals with anterior pattern element duplications in the posterior compartment were frequently observed (Figure 3F). By examining the imaginal discs of these animals, we were able to determine that when there are insufficient levels of Draf, *en* expression is no longer restricted to the normal posterior compartment, which suggests that Draf may act to repress/restrict En expression. Along with ectopic expression of En in the anterior compartment and increased levels of En in the posterior compartment, a new mirror image anterior compartment devoid of en expression was induced (Figure 3E). This observation is consistent with the observations of GUILLEN *et al.* (1995), who found that when En was ectopically expressed, ectopic anterior pattern elements were induced. They also found that ectopic expression of En in the anterior compartment induced expression of high levels of Hh and Dpp, which were responsible for overgrowth and the duplication of anterior pattern elements. Indeed, when Hh was examined in the partially rescued Draf null males, it was found to be widely ectopically expressed (E. NOLL, unpublished observations). The posteriorly restricted wing notching

observed in *Draf*^{Su2}/Y; *Df(3L)vin5*/+ male survivors is also consistent with a requirement for Draf in negatively regulating *en*, since elevated levels of En expression in the posterior compartment partially inactivate both *en* and *inv*, which are necessary for the development and terminal differentiation of posterior fates (Guillen *et al.* 1995; Tabata *et al.* 1995). Taken together, these observations suggest that the *Df(3L)vin5* deficiency contains a gene that participates with Draf in patterning of the limbs.

Specificity of genetic screens using deficiency stocks: One concern regarding screens for enhancement of lethality using deficiency stocks is that the resulting flies are in general less healthy as heterozygotes. Therefore, this could produce enhanced lethality in a nonspecific manner due to the fact that a deficiency usually disrupts multiple genes. In our screen we identified 23 deficiencies that behaved as enhancers of *Draf*^{Su2}, and it is possible that some of these are not true enhancers of *Draf*^{Su2}. However, we believe that at least some of them are specifically required for Draf function. Comparison of our results with those of similar screens performed by others suggests that screens with deficiency stocks can reveal specific interactors. For example, in a similar screen using deficiency stocks for enhancers of a weak allele of *dpp*, W. Gelbart's laboratory identified three deficiencies that maternally dominantly enhanced the lethality of a weak *dpp* allele (Nicholls and Gelbart 1998). None of these three deficiencies were found to enhance the lethality of *Draf*^{Su2}. *dpp* has essential functions in multiple processes during Drosophila development that are mostly distinct from those that require *Draf* function, except perhaps in the embryonic endoderm induction (Szuts *et al.* 1998). Therefore, it is not surprising that no deficiencies were found to commonly enhance both *Draf* and *dpp* mutations. In contrast, L. Perkins' laboratory performed a screen for modifiers of *corkscrew* (*csw*; Perkins *et al.* 1992), a component of Drosophila RTK signaling that shares a common pathway, Draf. They were able to identify a total of 27 autosomal regions required for *csw* function (L. Perkins, personal communication), and 12 of these were also identified in our study as enhancers of *Draf*^{Su2}. This suggests that many deficiencies do not nonspecifically increase the lethality of flies heterozygous for a mutation in an essential gene, but rather can provide a source for identifying specific interacting genes.

We thank E. Hafen, M. Bishop, J. Lengyel, S. K. Beckendorf, and the Bloomington Stock Center (Bloomington, IN) for reagents or strains. We thank L. Perkins for communicating results prior to publication. This work was supported by the Howard Hughes Medical Institute from which N.P. is an Investigator. W.L. is supported by a postdoctoral fellowship from the American Cancer Society.

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Communicating editor: T. Schüpbach