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Developmental and molecular characterization of mutations in the *Drosophila-raf* serine/threonine protein kinase

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SUMMARY

Formation of the tail region of the *Drosophila* larva requires the activities of the terminal class genes. Genetic and molecular analyses of these genes suggests that localized activation of the receptor tyrosine kinase *torso* at the posterior egg pole triggers a signal transduction pathway. This pathway, mediated through the serine/threonine protein kinase *D-raf* and the protein tyrosine phosphatase *corkscrew*, controls the domains of expression of the transcription factors *tailless* and *huckebein*. In this paper, we report the molecular and developmental characterization of mutations in the *D-raf* gene. We show that mutations that alter conserved residues known to be necessary for kinase activity are associated with a null phenotype, demonstrating that *D-raf* kinase activity is required for its role in *torso* signaling. Another mutation, *D-raf*^{PB26}, which prematurely truncates the kinase domain shows a weaker maternal effect phenotype that is strikingly similar to the *corkscrew* maternal effect phenotype, suggesting that a lower amount of kinase activity decreases the terminal signaling pathway. Finally, molecular and developmental characterization of two mutations that affect the late *D-raf* zygotic function(s) implies a novel role for *D-raf* in cell fate establishment in the eye. One of these mutations, *D-raf*^{C110}, is associated with a single amino acid change within the putative *D-raf* regulatory region, while the other, *D-raf*$^{HM-7}$, most likely reduces the wild-type amount of *D-raf* protein.

Key words: *Drosophila*, kinase, pattern formation, signal transduction

INTRODUCTION

Cell identity along the anteroposterior axis of the embryo is under the control of three different maternal systems (Nusslein-Volhard et al., 1987; Nusslein-Volhard 1991; St. Johnston and Nusslein-Volhard, 1992). Patterning of part of the head and the thorax depends upon the morphogen gradient of *bicoid* activity (Driever and Nusslein-Volhard, 1988a,b), while patterning of the abdominal regions is under the control of the *nanos* graded morphogen (Wang and Lehmann, 1991). Whether the terminal system that controls formation of the most anterior head region (acron) and most posterior tail region (telson) functions as a morphogen gradient remains unclear (Casanova and Struhl, 1989).

One of the key molecules of the terminal system is the receptor tyrosine kinase (RTK) encoded by the gene *torso* (*tor*) (Sprenger et al., 1989). *tor* RNA and protein are ubiquitously expressed in early embryogenesis (Sprenger et al., 1989; Casanova and Struhl, 1989). It has been postulated that activation of the *tor* tyrosine kinase activity, presumably by a ligand localized at the egg termini (Stevens et al., 1990), triggers a signal transduction pathway which involves two ubiquitously expressed proteins: the serine/threonine kinase *D-raf*, encoded by the gene *l(1)pole hole* (Ambrosio et al., 1989a) and the non-receptor tyrosine phosphatase *corkscrew* (*csw*) (Perkins et al., 1992). Embryos that lack either *tor* or *D-raf* maternal activities (referred to as *tor* and *D-raf* embryos) fail to develop specific head skeletal structures as well as posteriorly all derivatives of abdominal segments 8 through 10, the posterior midgut, Malpighian tubules and hindgut (Nusslein-Volhard et al., 1987; Ambrosio et al., 1989b). Embryos derived from females lacking *csw* activity in the germline (*csw* embryos) are associated with lesser terminal defects. Head formation is disrupted and, at the posterior termini, the posterior midgut and Malpighian tubules are missing (Perkins et al., 1992; L. Perkins and N. Perrimon, unpublished data).

One of the zygotic targets of the terminal class signal transduction pathway is encoded by the putative transcription factor *tailless* (*tll*), a member of the steroid receptor superfamily (Pignoni et al., 1990). In wild-type embryos, *tll* is expressed in a dynamic fashion at both embryonic ter-

mini (Pignoni et al., 1990, 1992). Unlike the posterior termini where *tll* expression is strictly dependent upon the terminal class signaling pathway, *tll* is expressed at the anterior termini in embryos that lack *tor* or *D-raf* due to the input of the *bicoid* patterning system (Pignoni et al., 1992). The localized expression of *tll*, in combination with the spatially localized expression of at least another gap gene *huckebein* (*hkb*), is required to control the expression of other downstream segmentation genes such as *hunchback, forkhead* and *fushi-tarazu* (Mlodzik et al., 1987; Tautz, 1988; Weigel et al., 1990; Bronner and Jackle, 1991; reviews by Klinger, 1990; Perkins and Perrimon, 1991). *hkb* encodes a putative zinc finger transcription factor (H. Jackle, personal communication) and is expressed at the cellular blastoderm stage in two caps at the egg termini (Bronner and Jackle, 1991). The posterior domains of expression of both the gap genes *tll* and *hkb*, as well as the more downstream segmentation genes at the blastoderm stage, can be used as markers to follow the amount of terminal class activity present in the early embryo (Perkins et al., 1992).

l(1)pole hole encodes the *Drosophila* homologue of the mammalian *Raf-1* serine/threonine protein kinase encoded by the *c-raf-1* gene (Mark et al., 1987; Nishida et al., 1988; Ambrosio et al., 1989a). In vivo experiments have demonstrated that the mammalian *Raf-1* protein is able to rescue *l(1)pole hole* mutations indicating that both the mammalian and *Drosophila* proteins utilize the same substrates (A. Brand, X. Lu and N. Perrimon, unpublished data). Since both proteins have homologous functions, we now refer to *l(1)pole hole* as *D-raf*. To gain further insight into the function of *D-raf*, and therefore *Raf-1* protein in signal transduction, we determined the molecular alterations associated with *D-raf* alleles as well as analyzed in detail the phenotypes of these various mutations.

MATERIALS AND METHODS

Origin of *D-raf* mutations

The ten *D-raf* mutations listed in Table 1 were recovered from various sources. All mutations were shown to be allelic by the following criteria: (1) they all map meiotically to position 0.9 between *yellow* (0.0) and *white* (1.5); (2) they fail to complement with a deficiency of the *D-raf* region (*Df(1)64C18*, Perrimon et al., 1985), as well as in interallelic crosses, and (3) they are rescued by *B13-1* (Ambrosio et al., 1989a), an autosomal P-element transformant that carries the wild-type *D-raf* gene.

D-raf alleles, *C110, EA75, DF903* and *DC817*, were induced on an *M65i* wild-type chromosome (Perrimon et al., 1985). *C2Z2* and *PB26* were recovered on a *y f* chromosome during a screen for *X*-linked zygotic lethal mutations (Perrimon et al., 1985; N. Perrimon, unpublished). *HM-7* and *400B8* were induced on a *y* w^a chromosome (Kramers et al., 1983). *107* was induced on a *w* chromosome and obtained from J. M. Dura (personal communication). *11-29* was obtained from A. Schalet.

Production of germline clones using the 'FLP-DFS' technique

Germline clones of *D-raf* mutations were generated using the 'FLP-DFS' technique (Chou and Perrimon, 1992). To recover females with *D-raf* homozygous germline clones of genotype *D-raf* FRT^{101}/*w* ovo^{D1} FRT^{101}; *F38/+*, progeny from the cross *FM7/D-raf* FRT^{101} with *w* ovo^{D1} FRT^{101}/*Y; F38/F38* were heat shocked for 2 hours at 37°C during larval stages. The *X*-linked dominant female sterile mutation ovo^{D1} was kept as *C(1)DX, y f/Y*; *F38/F38* females crossed to *w* ovo^{D1} FRT^{101}/*Y; F38/F38* males (Chou and Perrimon, 1992). All *D-raf* mutations were recombined with FRT^{101} by conventional genetic techniques to generate the chromosomes necessary for the production of germline clones. All recombinant stocks are kept using the *FM7c* balancer chromosome.

Chromosomes and mutations that are not described in the text can be found in Lindsley and Zimm (1992). Flies were raised on standard *Drosophila* media at 25°C unless otherwise indicated.

Visualization of embryos

In situ hybridizations on whole-mount *Drosophila* embryos using the *tll* and *hkb* DNA probes were performed as described by Tautz and Pfeifle (1989) using the Genius kit (Boehringer Mannheim). Single-stranded sense and antisense, digoxigenin-containing DNA probes were prepared by the PCR labeling technique (N. Patel, personal communication) using T3 and T7 primers (Biolabs). The labeled DNA was boiled for 30 minutes to reduce its size. For visualization, embryos were dehydrated in ethanol and mounted in Euparal (Carolina Biologicals). Immunocytochemistry was performed as described in Smouse et al. (1988). Embryos were dehydrated in ethanol and cleared in methyl salicylate. Antibodies, αhb (obtained from Dr P. MacDonald) and αfkh (obtained from Dr H. Jackle), were used at dilutions ranging from 1:250 to 1:1000. Examination of larval cuticles was done following the procedures of van der Meer (1977). Embryos were photographed on a Zeiss Axiophot microscope using Nomarski optics for in situ hybridizations and antibody stainings, and with dark-field illumination for cuticule preparations.

Visualization of mutant eyes

Adult eyes were prepared for scanning electron micrography as described by Hodgin and Bryant (1978). Plastic sections, 2 μm thick, were prepared as described by Tomlinson and Ready (1987).

Molecular analyses and determination of the molecular lesions

cDNA isolation. Full length *D-raf* cDNAs were isolated from a 9-12 hour embryonic cDNA library cloned into lamda-gt11 (Zinn et al., 1988) using *D-raf* genomic DNA (Mark et al., 1987) as a probe.

PCR amplification of *D-raf* mutations. Genomic DNA was prepared from three or four mutant larvae (in the case of *C2Z2, 400B8, PB26* and *C110*) or adults (*HM-7*) by the method of Junakovic and Angelucci (1986). Conditions for PCR amplification of *D-raf* from mutant genomic DNA were as follows: denature for 1 minute at 94°C, anneal for 2 minutes at 55°C, extend for 3 minutes at 72°C, then repeat for 30 cycles and follow by a 10 minute extension at 72°C. Each PCR reaction mix contained 10 μl 10× PCR buffer (Perkin Elmer Cetus), 10 μl 2.5 mM dNTPs, 400 ng genomic DNA, 1 μg each of forward and reverse primer, 5 units Taq polymerase (Perkin Elmer Cetus) and dH_2O to 100 μl.

The *D-raf* gene was cloned by PCR in three parts. Bases −43 to 367, 348 to 1250, and 1230 to 2545 relative to the translation start site were obtained using 18mer primers flanking these positions. These primer sets roughly define firstly the new 5′ extension to the *D-raf* ORF, secondly the N-terminal regulatory domain of the previously reported ORF (Nishida et al., 1988), and thirdly the kinase domain. PCR products were cloned into pBSK+ using *Bam*HI restriction sites in the primers, and three clones were sequenced from each PCR reaction to control for the possibility of polymerase errors.

Table 1. Characteristics of *D-raf* mutations

Mutation	Mutagen	Stage of lethality		Maternal effect	Classification	Molecular alteration
		D-raf/D-raf	*D-raf/Df*			
C2Z2	EMS	L-P	L-P	Yes	Class 1	E^{516} to V
11-29	Spont.	L-P	L-P	Yes	Class 1	Intragenic insertion
400B8	DCE	L-P	L-P	Yes	Class 1	G^{484} to R
EA75	EMS	L-P	L-P	Yes	Class 1	ND
DF903	EMS	L-P	L-P	Yes	Class 1	ND
DC817	EMS	L-P	L-P	Yes	Class 1	ND
107	EMS	L-P	L-P	Yes	Class 1	ND
PB26	EMS	P	L-P	Yes	Class 2	W^{634} to Stop
C110	X-rays	P-A	P	No	Class 3	R^{217} to L
HM-7	HMS	P-A	P	No	Class 3	No amino acid change*

We have classified *D-raf* mutations into three groups. Class 1 mutations behave genetically as null or amorphic mutations; i.e., $D\text{-}raf^{Class\ 1}/Df$ animals die at the larval-pupal interface like $D\text{-}raf^{Class\ 1}/D\text{-}raf^{Class\ 1}$ animals. Class 1 mutations have the strong maternal effect phenotype shown in Fig. 1. Class 2 and 3 mutations behave genetically as hypomorphic mutations; i.e., the stage of lethality of *D-raf/Df* animals occurs at an earlier stage than in the homozygous animals. The class 2, or $D\text{-}raf^{PB26}$ mutation, has a weaker maternal effect than class 1 mutations. Class 3 reduced activity mutations have no, or very weak maternal effects. $D\text{-}raf^{C110}$ flies have a rough eye phenotype and die either during pupariation or during hatching. $D\text{-}raf^{HM\text{-}7}$ mutant animals are viable at 18°C. Above 25°C, $D\text{-}raf^{HM\text{-}7}$ flies either do not hatch or die soon following emergence. The rough eye phenotype associated with $D\text{-}raf^{HM\text{-}7}$ is present both at 18 or 29°C and is suppressed by the introduction of the *B13-1* P-element transformant. Abbreviations: ethylmethane sulfonate (EMS), 1-2 dichloroethane (DCE), hycanthone methanesulfonate (HMS), Spontaneous (Spont.), Not determined (ND). Stages of lethality: Larval-pupal (L-P), Pupal (P), Pupal to adult (P-A). *See text for discussion.

DNA sequencing. DNA sequencing employed a dideoxy termination protocol (Del Sal et al., 1989) with the following modifications: template was denatured at 70°C for 15 minutes, and annealed with primer at 37°C for 15 minutes. Sequence of multiple *D-raf* clones from each mutant background was compared to our sequence of a wild-type genomic clone (clone originally isolated by Mark et al., 1987). Differences from the wild-type were classified either as mutations when they caused a unique amino acid change or as polymorphisms when they did not cause an amino acid change or when they were shared by multiple mutant backgrounds.

The wild-type *D-raf* genomic sequence. Before sequencing *D-raf* from mutant alleles, we first confirmed the wild-type *D-raf* genomic sequence by sequencing a genomic clone isolated by Mark et al. (1987). A number of differences from the sequence reported by Nishida et al. (1988) were found. Four sequence differences result in amino acid changes: an A instead of C at bp 1582 of the previously reported (Nishida et al., 1988) genomic sequence gives an asparagine instead of a threonine at amino acid residue 161 of the new *D-raf* ORF; and G instead of C at bp 2718 gives an alanine instead of a proline at amino acid residue 496. In addition, two small frame shifts were found relative to the previously reported sequence. The first involves three bases in the beginning of the ORF that were missed as a result of compressions, including an extra C after base 1119, an extra G after base 1126 and an extra C after base 1133, altering the amino acid sequence SPKRQ to STEGDS. The other small frame shift that we found is the result of a C after base 3397, and the absence of the C at base 3408. This change alters the amino acid sequence RRHS to PQAL, which is more homologous to *c-Raf1*. Other sequence differences did not result in amino acid changes: C instead of T at bp 2102, C instead of G at bp 2321, G instead of A at bp 2591, and T instead of C at bp 3160. The *D-raf* genomic sequence differing in these ways from the previously reported sequence (Nishida et al., 1988) was used as the wild-type *D-raf* to which we compared sequence obtained from mutant alleles.

RESULTS

Mutations in *D-raf* define a phenotypic series

We have classified *D-raf* mutations into three classes based upon their maternal effect phenotypes as well as the severity of their zygotic phenotypes (Table 1).

Phenotype of class 1 *D-raf* embryos

Previous analyses (Perrimon et al., 1985; Ambrosio et al., 1989b) have demonstrated that two embryonic phenotypes are observed in embryos derived from females that lack maternal *D-raf* activity. Embryos that lack both maternal and zygotic activities (*D-raf* unrescued embryos) fail to differentiate into structured embryos and degenerate around 7 hours of development (Ambrosio et al., 1989b). These embryos secrete a small amount of naked cuticle (Fig. 1G). Their siblings, which lack maternal activity but have received a wild-type *D-raf* gene from their father (*D-raf* rescued embryos), develop into structured animals missing the most anterior and posterior structures (Fig. 1D,E; Ambrosio et al., 1989b). At the posterior end, all pattern elements posterior to abdominal segments 6 or 7 are deleted. Anteriorly, a portion of the cephalopharyngeal skeleton, the labral sense organ and the medial tooth are deleted. We have classified as class 1 mutations those that exhibit these unrescued and rescued embryonic phenotypes. To follow the development of internal posterior structures in class 1 rescued embryos, we used the marker *fkh* which in germ-band-shortened wild-type embryos is expressed posteriorly in the hindgut, Malpighian tubules and anal pads (Weigel et al., 1989). In class 1 embryos, posterior *fkh* expression is entirely absent (Fig. 2E, F).

To correlate the extent of missing embryonic structures with gene expression at the blastoderm stage, we determined the expression patterns of several zygotic terminal class genes in class 1 *D-raf* embryos. At appropriate cellular blastoderm stages, the posterior domains of expression of *tll*, *hkb*, *hb* and *fkh* are never observed in both rescued and unrescued class 1 embryos (Fig. 3G for *tll* and Fig. 3H for *hb*, for others data are not shown). Additionally, as previously reported (Ambrosio et al., 1989b), the 7th *ftz* stripe is deleted and the 6th stripe is variably

deleted and/or expanded posteriorly. We conclude that the region of the blastoderm fate map deleted in class 1 alleles extends from 0% EL to approximately 20 to 25% EL.

Phenotype of class 2 *D-raf* embryos

That the class 2 reduced activity mutation, *D-raf*^{PB26}, is associated with residual activity is suggested by the obser-

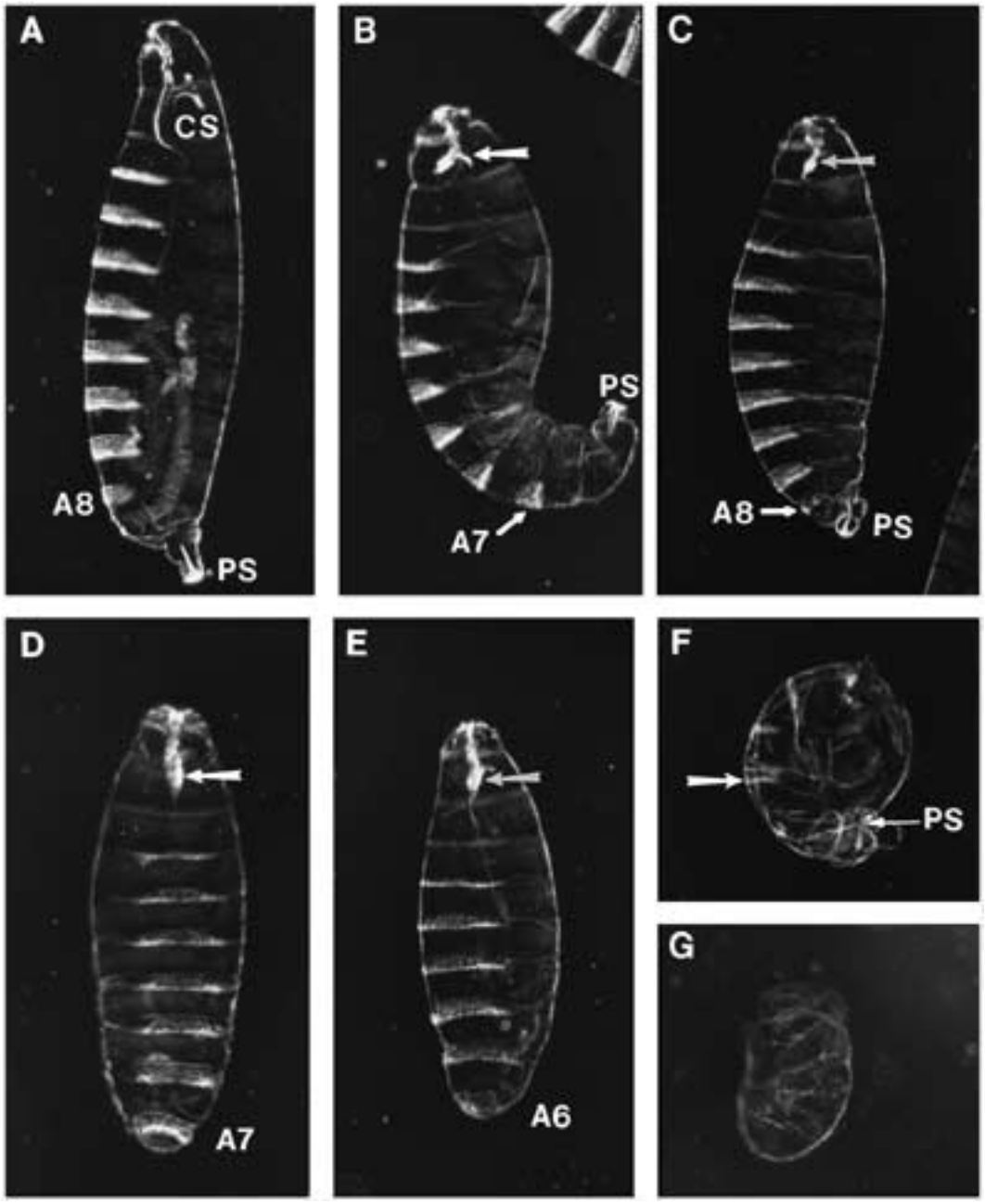

Fig. 1. The cuticle phenotypes of *D-raf* mutations. A through G are dark-field micrographs of a wild-type larva (A), two rescued *D-raf*^{PB26} larvae (B,C), two rescued *D-raf*^{C2Z2} larvae (D,E), unrescued *D-raf*^{PB26} (F) and unrescued *D-raf*^{C2Z2} (G) embryos. Animals in B and C differentiate the posterior spiracles although defects associated with A8 can be detected. A8 is missing in B and considerably reduced in C. Unrescued *D-raf*^{PB26} embryos develop more structures than unrescued *D-raf*^{C2Z2} embryos. The arrow in F points to some differentiated denticle bands that are absent in G, these animals also develop posterior spiracles. In B through E, the arrows point to the truncated head skeleton.

There is some variability in the extent of posterior structures deleted by class 1 mutations, ranging from embryos with all structures posterior to A6 deleted to embryos with all structures posterior to A7 deleted. For example, among *D-raf*^{C2Z2} rescued embryos, 40% are missing cuticular elements posterior to A6 (C) while the remaining 60% are missing structures posterior to A7 (D). A similar variability is observed in embryos derived from *tor*^{XR1} (a protein null mutation, Sprenger et al., 1989) homozygous females (N.P. unpublished; Schupbach and Wieschaus, 1986). Abbreviations: cephalopharyngeal skeleton (CS), posterior spiracles (PS).

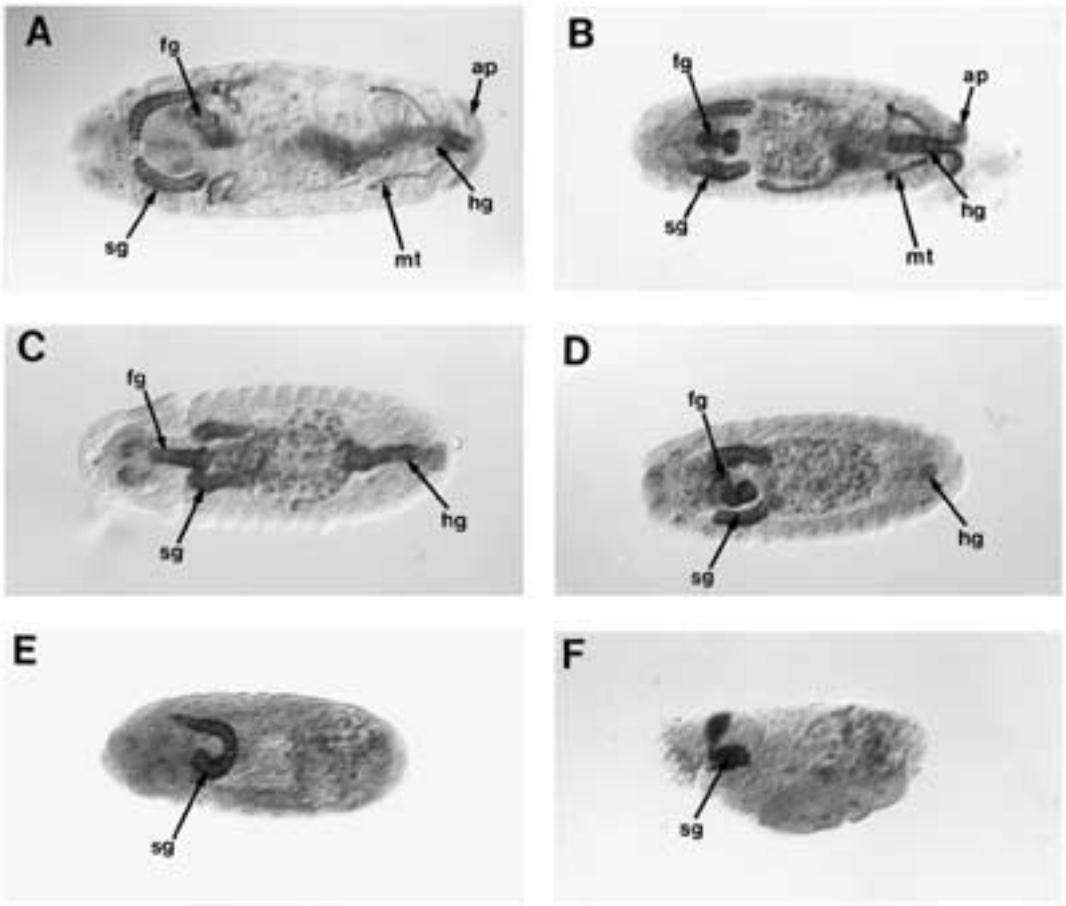

Fig. 2. Expression of *fkh* in *D-raf* mutations. In wild-type germ band shortened embryos (A) antibodies to *fkh* label posterior structures including the hindgut (hg), anal pads (ap) and the extended Malpighian tubules (mt) and anterior structures including part of the foregut (fg) and the salivary glands (sg) (Weigel et al., 1989). In a class 3 mutant embryo, *D-raf*^{C110} (B), *fkh* protein expression appears like wild-type. Two phenotypes are observed from class 2 *D-raf*^{PB26} rescued mutant embryos, embryos in which the hindgut (hg) is present yet reduced in length (C) and embryos in which the hindgut (hg) is almost entirely missing (D). Note that the Malpighian tubules are entirely deleted in *D-raf*^{PB26} embryos. Class 1 mutant *D-raf*^{C2Z2} rescued (E) and unrescued (F) embryos are missing all posterior *fkh*-expressing structures. Abbreviations: anal pads (ap), foregut (fg), hindgut (hg), Malpighian tubules (mt), salivary glands (sg).

vation that these embryos develop considerably more terminal cuticular elements than class 1 embryos (compare Fig. 1B,C to D,E and Fig. 1F to G). Posteriorly, in *D-raf*^{PB26} rescued embryos, the posterior spiracles are present (Fig. 1B,C) and the A8 denticle band is frequently observed and often well differentiated (Fig. 1C). A large fraction (35%) of class 2 rescued embryos are twisted (Fig. 1B), a phenotype also observed in *csw* embryos (Perkins et al., 1992). Unrescued *D-raf*^{PB26} embryos differentiate more cuticular elements than unrescued class 1 embryos (Fig. 1F). To determine whether the most posterior internal structures were affected in *D-raf*^{PB26} animals, we analyzed the *fkh* expression pattern (Fig. 2). Internally, the hindgut is variably present, though always malformed, and the Malpighian tubules are missing. As expected from analyses of both the cuticular and posterior gut structures present in *D-raf*^{PB26} animals, we observed reduced expression of both *tll* and *hb* at the posterior termini of cellular blastoderm stage embryos (Fig. 3). We conclude that the region of the blastoderm fate map deleted in the class 2 allele extends from 0% EL to approximately 10 to 15% EL.

Phenotype of class 3 *D-raf* embryos

Germline clone analysis of the two class 3 hypomorphic alleles indicates that only a small fraction of embryos derived from mosaic females fail to hatch (20% for *D-raf*^{C110} and 16% for *D-raf*$^{HM-7}$). Cuticular preparations of these embryos reveal no consistent cuticular defects and internal posterior structures, detected with antibodies to *fkh*, appeared like wild-type (Fig. 2B). We examined the expression of both *tll* and *hb* in *D-raf*^{C110} (the stronger of the two alleles) embryos derived from mothers carrying homozygous germline clones. Expression of both *tll* and *hb* in these mutant embryos was indistinguishable from wild-type (Fig. 3C,D). In addition, the expected numbers of dead mutant males are detected among progeny derived from germline clones of both *D-raf*^{C110} and *D-raf*$^{HM-7}$. These results indicate that class 3 mutations have no, or a very weak, maternal effect.

Role of *D-raf* in photoreceptor cell fate specification

The eyes of both class 3 alleles are rough and smaller in size when compared to wild-type eyes (Fig. 4). To determine whether defects associated with *D-raf* mutations could be informative about the function of *D-raf* in other developmental processes, we examined the cellular basis of the rough eye phenotype associated with these class 3 muta-

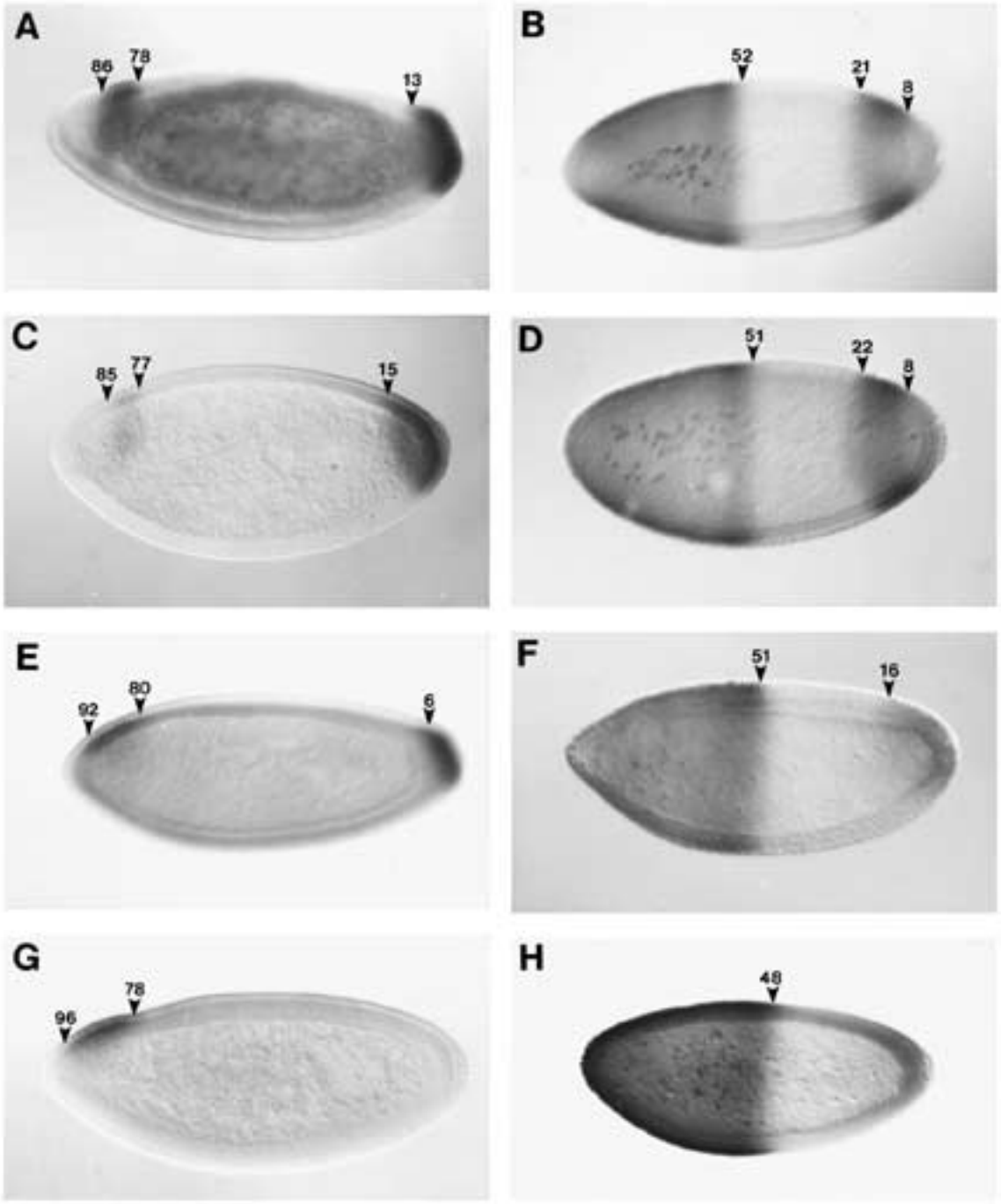

Fig. 3. Expression of *tll* and *hb* in *D-raf* mutations. The *tll* RNA (A,C,E,G) and *hb* protein (B,D,F,H) expression patterns at the blastoderm stages are shown. (A,B) In wild type at the cellular blastoderm stage, *tll* is expressed at the posterior from 0-15% EL and *hb* is initially expressed at the posterior as a polar cap, which resolves into a stripe from approximately 8-21% EL. (C,D) In class 3, *D-raf*^{C110}, mutant embryos, the expression patterns of *tll* and *hb* are similar to wild type. (E,F) In *D-raf*^{PB26} embryos, posterior *tll* and *hb* expression domains are reduced. In *D-raf*^{PB26} embryos, *tll* is expressed from approximately 0 to 6% EL (Fig. 3E), and posterior *hb* expression extends from 0 to 16% EL (Fig. 3F) and is not repressed at the most posterior egg pole. (G,H) In class 1, *D-raf*^{C2Z2} mutant embryos, both *tll* and *hb* posterior expression is completely deleted consistent with previous reports that *tll* is not expressed in embryos that lack *tor* or *D-raf* maternal activities (Pignoni et al., 1992). Expression patterns are designated in per cent egg length (EL), with 0% EL being the most posterior end of the embryo and 100% EL being the most anterior end of the embryo.

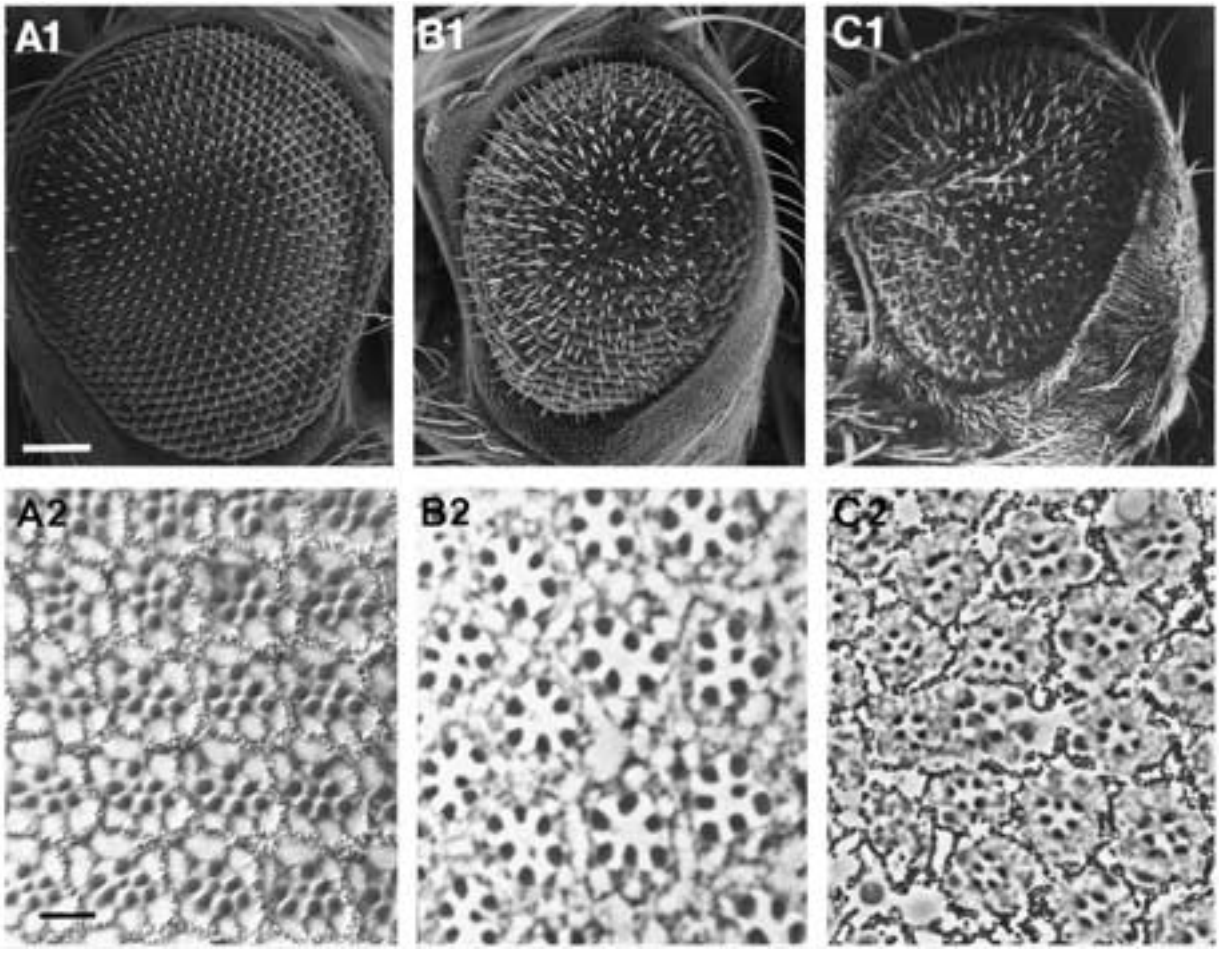

Fig. 4. Role of *D-raf* during eye development. Scanning electron micrographs and retinal sections of wild-type (A1 and A2); *D-raf*$^{HM-7}$ (B1 and B2) and *D-raf*^{C110} (C1 and C2) eyes. Note the regular array of ommatidia in the wild-type compound eye which is composed of approximately 800 ommatidia. In the center of the ommatidia resides the rhabdomeres that contain the photopigments. Although each ommatidia contains 8 photoreceptor cells (R1 to R8), only R1 through R6 and either R7 or R8 are detectable at any plane of section; R7 is positioned more superficially than is R8. In addition to the photoreceptor cells, pigment cells that delimit the photoreceptors are clearly visible. The eyes of both class 3 animals are rough and smaller (B1, C1) with approximately one third of the ommatidia missing. In *D-raf*$^{HM-7}$ eyes, approximately 50% of the ommatidia are missing R7 (B2). In *D-raf*^{C110} animals (C2), the number of photoreceptor cells is variable. In addition, other non-receptor cells are disrupted in class 3 mutants resulting in some cases in fusion of ommatidia. Scale bar, 50 μm in A1 and 10 μm in A2.

tions. When serial sections of *D-raf*^{C110} and *D-raf*$^{HM-7}$ mutant eyes were examined at the level of the R7 photoreceptor, the ommatidia showed a number of abnormalities. The most consistent defect associated with *D-raf*$^{HM-7}$ ommatidia is the absence of the R7 photoreceptor (Fig. 4B2). In contrast, the defects associated with *D-raf*^{C110} eyes are more variable: although the R7 photoreceptor is absent in a fraction of the ommatidia, other photoreceptor and non-photoreceptor cells are also variably absent (Fig. 4C2). Examination of these mutant phenotypes reveals a novel function for *D-raf* in cell fate determination during development of the adult eye.

Structure of the *D-raf* gene

To gain insights into the biochemical properties of the *D-raf* protein, we determined the molecular alterations associated with various *D-raf* mutant alleles. In deciding on the extent of genomic DNA to sequence from *D-raf* mutant alleles, we first re-examined the *D-raf* open reading frame (ORF). The *D-raf* ORF reported by Nishida et al. (1988) predicts a 666 amino acid protein with a relative molecular mass (M_r) of 75,500. Nishida et al. (1988) report the sequences of partial cDNAs that do not extend to the proposed translation initiation site. We have characterized additional cDNAs, including one that extends 1100 base pairs more 5′ (to position 450 of the genomic sequence of Nishida et al., 1988) than the longest cDNA reported by Nishida et al. (1988). From examining this transcribed sequence, an extension of the previously characterized ORF can be found that starts with an ATG at position 1104 of the genomic sequence and shows excellent *Drosophila* codon usage and a much better match to the consensus for translation initiation (Cavener, 1987) than the start site proposed previously (Nishida et al., 1988). A new putative TATA box can be found at position 351 of the genomic sequence. The new ORF predicts a protein 782 amino acids

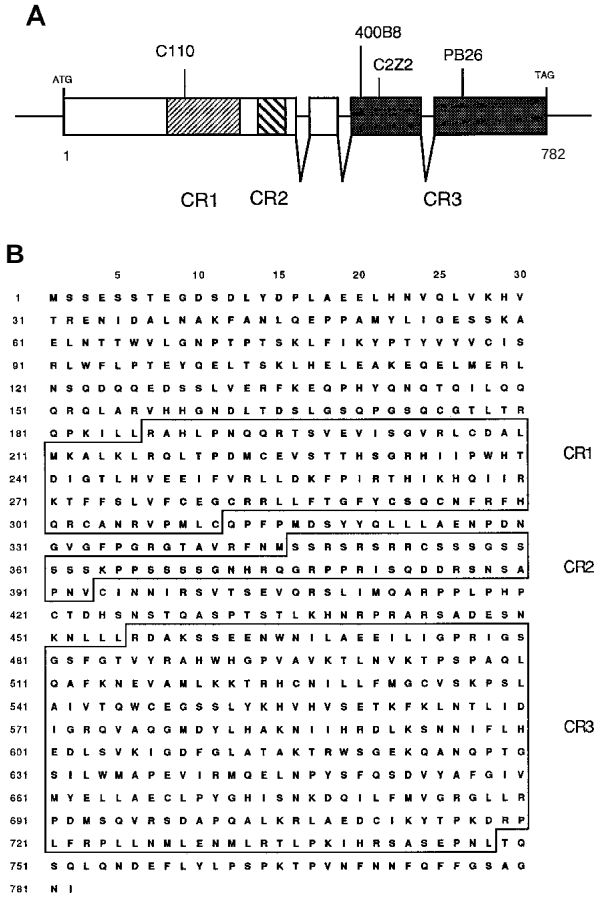

Fig. 5. Structure of the *D-raf* gene and position of the *D-raf* mutations. A is a schematic representation of the *D-raf* gene. The position of the mutations is indicated. B indicates the new sequence of *D-raf* taking into account the extended open reading frame (see text). The three domains that correspond to the conserved regions (CR) 1 through 3 of *D-raf* are indicated. CR1 (amino acids 187 to 311) and CR3 (456 to 748) are readily recognizable upon comparison of the *Raf1* and *D-raf* amino acid sequence; we define the *D-raf* CR2 domain (346 to 393) as spanning a highly serine-rich region as well as the sequence RSNSAP, similar to the sequence RSTSTP in the *Raf-1* CR2 domain suggested by Ishikawa et al. (1988) to be important in *raf* activation. Sequence identity between *D-raf* and *Raf-1* remains unchanged (46%) by the amino terminal extension of the *D-raf* open reading frame.

in length (116 amino acids longer at the N terminus) with a predicted M_r of 89,000 (Fig. 5B). While considerably longer than the human *Raf-1* which is 648 amino acids and 73,000 M_r, the new *D-raf* ORF, with its 3.3 kb transcription unit, fits well with northern data which reveals a 3.2 kb transcript (Mark et al., 1987). Thus, based on the cDNAs isolated and their resulting codon preference and translation initiation consensus, we examined this longer ORF for the presence of molecular lesions in the *D-raf* mutant alleles.

Molecular lesions associated with *D-raf* mutations

To gain information on the *D-raf* protein, we determined the molecular nature of the three hypomorphic class 2 and class 3 *D-raf* mutations, as well as three class 1 null mutations.

We have identified the molecular lesions associated with three class 1 mutations: $D\text{-}raf^{400B8}$, $D\text{-}raf^{C2Z2}$ and $D\text{-}raf^{11\text{-}29}$. $D\text{-}raf^{400B8}$ was found to be a mutation in the third glycine of the G-X-G-X-X-G consensus nucleotide-binding motif, Gly^{484} to arginine (corresponding to kinase subdomain 1 of Hanks et al., 1988). $D\text{-}raf^{C2Z2}$ reveals the mutation Glu^{516} to valine at a highly conserved position that defines subdomain 3, which is thought to be critical to nucleotide-binding (Hanks et al., 1988). Finally, the molecular lesion associated with $D\text{-}raf^{11\text{-}29}$ is a truncation of the *D-raf* protein caused by a 3.6 kb insertion located 751 to 1520 bases from the start of translation (between amino acids 252 and 466).

The intermediate terminal class phenotype that we detect in the class 2 mutant $D\text{-}raf^{PB26}$ suggests that the mutant *D-raf* protein produced has some activity and is able to partially operate in *torso* signaling. We found that $D\text{-}raf^{PB26}$ corresponds to a truncation of the C terminus of the kinase domain as a result of the conserved tryptophan in subdomain 8, Trp^{634}, being mutated to a stop codon. This premature stop eliminates much of kinase subdomain 8 and all of subdomains 9, 10 and 11.

The sequence of *D-raf* from the class 3 mutation $D\text{-}raf^{C110}$ revealed a single amino acid change, Arg^{217} to leucine, in the CR1 region of the N-terminal regulatory domain. $D\text{-}raf^{HM\text{-}7}$, a hypomorphic allele, was previously thought to be the result of an insertion in the *D-raf* promoter (Ambrosio et al., 1989a). Further examination of the DNA from $D\text{-}raf^{HM\text{-}7}$ homozygous flies failed to reproduce this result (data not shown). The sequence analysis of $D\text{-}raf^{HM\text{-}7}$ revealed a single amino acid change, Trp^{238} to arginine, from the wild-type sequence. However, since this change is also observed in other *D-raf* mutations, we consider it a polymorphism (see Table 2). It is likely that $D\text{-}raf^{HM\text{-}7}$ is due to a mutation in sequences 5′ or 3′ to the open reading frame that might affect the efficiency of *D-raf* transcription or translation. If the $D\text{-}raf^{HM\text{-}7}$ phenotype is due to reduced expression of *D-raf*, it suggests that some molecule in the torso signaling pathway is temperature sensitive since $D\text{-}raf^{HM\text{-}7}$ shows temperature sensitivity (see Table 1).

DISCUSSION

Structure of the *D-raf* protein

The *D-raf* gene encodes a protein with a C-terminal serine/threonine kinase catalytic domain, joined to an N-terminal regulatory domain. This regulatory domain in turn comprises two regions, conserved regions 1 and 2 (CR1 and CR2), originally identified on the basis of sequence conservation between human *A-raf* and *c-raf-1* (Heidecker et al., 1990; Fig. 5). CR1 contains a cysteine finger motif (C-X_2-C-X_9-C-X_2-C) reminiscent of the lipid-binding motif of protein kinase C (Lee and Bell, 1986) or of metal-binding domains (Berg, 1986). CR2 consists of a serine-rich region thought to be important in *raf* activation as deletion of CR2 alone (Ishikawa et al., 1988), or the entire N-terminal regulatory domain (Heidecker et al., 1990), gives rise to trans-

Table 2. Nature of *D-raf* mutations

Nucleotide Position	HM-7	400B8	PB26	C222	C110
650					G→T (R→L)
712	T→A (W→R)	T→A (W→R)	T→A (W→R)	T→A (W→R)	
837					C→T
978					G→A
1263			A→T	A→T	
1315				A→C	
1480			C→T	C→T	
1516			A→C	A→C	
1582		G→A (G→R)			
1674			G→A	G→A	
1679				A→T (E→V)	
1890					T→C
1908			T→C	T→C	
1921					T→G
1943					T→A
1952			A→T	A→T	
1959			T→G	T→G	
2006			T→C	T→C	T→C
2099			G→A (A→*)		
2165					C→T
2435			A→G	A→G	A→G

Nucleotide positions are given with respect to the new putative start of translation (bp 1104 of the genomic sequence published by Nishida et al., 1988). Nucleotide positions have been corrected as described in the Materials and Methods. Changes that lead to a novel amino acid are indicated in parenthesis. * indicates a stop codon.

The DNA sequence differences from wild type, and where appropriate the resulting amino acid changes, are indicated. A pattern of polymorphisms was observed that confirms the genetic backgrounds in which different *D-raf* alleles were isolated. For example, *D-raf*^{PB26} and *D-raf*^{C2Z2} were isolated in the same genetic background and share a virtually identical set of polymorphisms. Likewise, *D-raf*$^{HM-7}$ and *D-raf*^{400B8} are similar to each other in their lack of polymorphisms compared to wild type. *D-raf*^{C110} appears to come from a different genetic background altogether. There is one sequence change that does result in an amino acid change (T to A at bp 712), but is shared by four *D-raf* alleles, *D-raf*$^{HM-7}$, *D-raf*^{400B8}, *D-raf*^{PB26} and *D-raf*^{C2Z2} , and for that reason we consider it a polymorphism. Aside from this change, only one other sequence change causing an amino acid difference was found in each of the *D-raf* alleles, *D-raf*^{400B8}, *D-raf*^{PB26}, *D-raf*^{C2Z2} and *D-raf*^{C110}.

forming activity by the human *Raf-1* protein. To gain insight into the function of these various domains, we characterized the alterations associated with various *D-raf* mutations.

Kinase activity is necessary for *D-raf* function

Two of the class 1 mutations, *D-raf*^{400B8} and *D-raf*^{C2Z2}, are each associated with a single amino acid substitution in conserved residues in subdomains of the kinase catalytic region. The severity of the mutant phenotypes of these mutant alleles coupled with the location of their molecular lesions suggests that serine/threonine kinase activity is necessary for transduction of the terminal class signal.

D-raf^{400B8} is a mutation in the third glycine, Gly^{484} to arginine, of the G-X-G-X-X-G consensus nucleotide-binding motif. Examination of the crystal structure of the catalytic subunit of cAMP-dependent protein kinase (Knighton et al., 1991a), like *Raf-1* a serine/threonine kinase, allows us to put this change into context. The basic architecture that this kinase revealed was a bilobal shape with MgATP and peptide substrate found in the cleft between the lobes. The smaller N-terminal lobe is primarily associated with nucleotide binding, while the larger C-terminal lobe, contains the catalytic residues and makes the substrate contacts. The glycine motif is found in the small lobe where it permits a sharp turn between two antiparallel strands at the beginning of a beta sheet under which the nucleotide is buried. A charged residue, like arginine, instead of a glycine would disrupt this structure and the nucleotide-binding fold.

D-raf^{C2Z2} results in a change of Glu^{516} to valine. Glu^{516} of D-raf corresponds to Glu^{91} of cAMP-dependent protein kinase, which is found on the edge of an alpha helix in the small lobe, facing the nucleotide-binding cleft. Based on the structure of cAMP-dependent protein kinase, the highly conserved residues Glu^{516}, Lys^{498} and Asp^{609} of D-raf make up a triad in close contact with the gamma phosphate of MgATP. In *D-raf*^{C2Z2}, the Glu^{516} to valine change, by eliminating one of the carboxyl groups important for this contact, apparently destroys kinase activity. This is similar to a Glu^{91} to alanine mutation in cAMP-dependent protein kinase generated by site-directed mutagenesis as part of a changed-to-alanine mutation strategy, which was one of the few such changes that resulted in less than 1% wild-type kinase activity (Gibbs and Zoller, 1991). These two class 1 mutations have a phenotype similar to *D-raf*$^{11-29}$ which is a truncation of the *D-raf* protein due to a 3.6 kb insertion.

These results indicate that *D-raf* kinase activity is necessary for terminal class activity. In support of these findings, in vitro experiments have demonstrated that D-raf is able to act as a protein kinase in vitro (L. Ambrosio and N. Perrimon, unpublished results).

Partial terminal class activity provided by lowered *D-raf* kinase activity

The intermediate terminal class phenotype associated with

D-raf^{PB26} suggests that the mutant *D-raf* protein produced still has some activity and is thus able to partially transduce the terminal class signal. Interestingly, we found that the *D-raf*^{PB26} mutation is a truncation of the C terminus of the kinase domain. This premature stop eliminates much of kinase subdomain 8 and all of subdomains 9, 10 and 11. Based on the cAMP-dependent protein kinase crystal structure, three C-terminal alpha helices, which normally help cradle the peptide substrate, would be eliminated; however, the small lobe and all residues involved in nucleotide binding would be untouched, as would the putative catalytic residues, in particular the catalytic loop (residues 590-596 in D-raf). In addition, as revealed by the co-crystal of cAMP-dependent protein kinase and a peptide inhibitor (Knighton et al., 1991b) and, in particular, the structure of the substrate consensus recognition site, even the *D-raf*^{PB26} truncated protein should still be able to make contacts from both sides with substrate residues from P+1 to P–3 relative to the phosphorylation site. In other words, enough of a cradle might still remain to weakly hold substrate in place and give some minimal activity. Supporting this hypothesis is the recent characterization of a naturally occurring truncated form of the *Xenopus* activin receptor (XSTK2), a receptor serine/threonine kinase (Nishimatsu et al., 1992). The carboxy-terminal truncated form encoded by the *XSTK2* gene lacks kinase subdomains 8 through 11, yet injection of mRNA into *Xenopus* embryos results in mesoderm induction and duplication of the body axis to a degree similar to that caused by injecting mRNA from the full-length *XSTK8* gene. This suggests that a native serine/threonine kinase with a truncation analagous to mutant *D-raf*^{PB26} can still transduce the activin signal.

Mutations in the CR1 domain affect late D-raf activity

One mutation, *D-raf*^{C110}, is associated with a single amino acid change in the CR1 domain. The CR1 and CR2 domains together are thought to constitute an amino terminal-negative regulatory region, based on the following lines of evidence. (1) Removal of CR1 and CR2, and expression of CR3 alone, yields an activated raf molecule. This is seen in the gag fusion giving rise to *v-raf* and in the transfection studies of Heidecker et al. (1990) and Bruder et al. (1992). (2) Expression of *Raf-1* CR1 and part of CR2 in mammalian cells serves as a dominant negative mutation, blocking *Raf-1* activation (Bruder et al., 1992). Specifically, expression of this region will block *ras*-mediated *Raf-1* activation. Furthermore, site-directed mutagenesis has shown that the cysteine finger motif in CR1 is essential for the dominant negative phenotype of the regulatory domain alone, and for *ras*-mediated activation of the full-length *Raf-1* molecule (Bruder et al., 1992). (3) Insertion into (Heidecker et al., 1990) or deletion of (Ishikawa et al., 1988) the *Raf-1* CR2 domain results in *Raf-1* activation.

The similarity of the cysteine motif in CR1 to the *PKC* lipid-binding motif (Lee and Bell, 1986) or to metal-binding motifs (Berg, 1986) suggests that CR1 may bind a *ras*-generated *Raf-1* ligand. Binding of a ligand at CR1 and serine/threonine phosphorylation at CR2 may result in *raf* activation (Bruder et al., 1992). The *D-raf*^{C110} mutation Arg^{217} to leucine may remove a charged residue important for ligand binding, making it more difficult to activate *Raf-1*. This mutation is not analagous, however, to the *Raf-1* Cys^{168} to serine change created by Bruder et al. (1992) in the cysteine finger, a change with a moderately activating effect.

Role of D-raf in the formation of the terminal class gradient

A morphogen gradient is defined by a set of spatially distinct responses in patterning resulting from the concentration change of a single molecule (Sander, 1959, 1976; Driever and Nusslein-Volhard, 1988b). *D-raf* establishes a morphogen gradient at the posterior end of the embryo since a series of spatially distinct responses, detected by the spatial expression of *tll* and *hb*, are associated with mutations that affect the level of *D-raf* activity. Since *D-raf* is expressed ubiquitously in the embryo (Ambrosio et al., 1989a) and is not a transcription factor, it suggests that *D-raf* is a component of a phosphorylation cascade that controls the activity of a transcription factor whose activation defines the spatial expression of *tll* and *hb*. This putative transcription factor must be present within the entire egg since gain-of-function *tor* mutations activates *tll* in almost the entire embryo (Steingrimsson et al., 1991).

It is of interest to note the similarity between the *D-raf*^{PB26} and the *csw* (Perkins et al., 1992) maternal effect phenotypes. Both appear to result in the same constellation of terminal structures and effects on *tll* and *hkb* spatial expression, supporting the hypothesis that *csw* function is required to up-regulate *D-raf* activity in wild-type embryos. This model is supported by the genetic interactions between *csw* and *D-raf* that have shown that the severity of terminal defects increases in double mutant combinations (Perkins et al., 1992).

Role of *D-raf* in other RTK signaling pathways

Several observations indicate that *D-raf* functions in RTK signaling pathways other than that generated by *tor*. (1) The eye phenotypes associated with class 3 mutations suggest a role of *D-raf* in cell fate determination in the developing eye. Although additional defects are observed in class 3 mutant eyes, they resemble the loss-of-function phenotype associated with the *sevenless* RTK. Indeed, Dickson et al. (1992) have recently provided evidence that *D-raf* is involved in *sevenless* signaling. (2) The *D-raf* unrescued maternal effect phenotype suggests a function for *D-raf* in the *Drosophila* homolog of the EGF-receptor signaling pathway. *D-raf* unrescued embryos fail to differentiate into structured embryos and degenerate during germ band shortening (Ambrosio et al., 1989b), a phenotype strikingly similar to the phenotype associated with *faint little ball* mutations that correspond to molecular lesions in the EGF receptor (Schejter and Shilo, 1989; Clifford and Schupbach, 1992). (3) *D-raf* activity is required for cell division in the imaginal discs (Perrimon et al., 1985; Nishida et al., 1988), which may involve a RTK signaling pathway. Further analyses of the role of D-*raf* in these developmental processes will be necessary to establish definitively the role

of *D-raf* in transduction of the signals received by other RTKs.

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Genbank Accession number of wild-type *D-raf* genomic sequence is L10626.