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Multiple functions of *raf* proto-oncogene during development from analysis of a temperature-sensitive mutation of *Drosophila*

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ABSTRACT **A temperature-sensitive (ts) mutation of *Drosophila melanogaster* for *D-raf*, encoding a serine/threonine protein kinase, was newly induced by EMS-treatment. Temperature-shift experiments on the ts mutant revealed that *D-raf* is required during most of the developmental stages, and confirmed the previously reported roles of *D-raf* in the regulation of cell proliferation and in the determination of cell fates at terminal regions of the embryo (Nishida *et al., EMBO J. 7:* 775-781, 1988; Ambrosio *et al., Nature 342:* 288-291, 1989a). Detailed analysis of cell proliferation demonstrated the role of *D-raf* at other than M-phase in cell cycle. TSP analysis during pupal stages revealed yet another role of *D-raf* in eclosion. Mosaic analysis of an eclosion-defective hypomorphic mutation revealed the tissue responsible for this defect to be the muscle and/or nervous system in the thorax. Molecular lesion associated with the ts mutation was found to be an alteration of an amino acid residue in a highly conserved region that defines the kinase subdomain VIII. Molecular analysis of null mutations also suggested the importance of the kinase domain for the biological functions of *D-raf*. Elucidation of the multi-functional nature of signal transducers is of great importance for our understanding of the molecular mechanisms of development, and the ts mutation for pleiotropic *D-raf* obtained in this study promises to be useful for dissecting signal transduction pathways during development.**

KEY WORDS: *proliferation, signal transduction, terminal system, molecular lesion*

Introduction

Signal transduction plays major roles in the determination of cell fates mediated by specific cell-cell interactions during development. Despite the magnitude of the complexity and specificities of cell-cell interactions, the species of factors involved in signal transduction seems to be rather limited. This may suggest that many of these factors act in multiple signal transduction pathways, and, indeed, many genes encoding signal transduction factors have been found to be expressed in a wide variety of cells. For our understanding of the molecular mechanisms of development, the elucidation of the multi-functional nature of signal transducers would be of great importance.

Raf-1, a serine/threonine protein kinase, plays essential roles in the transduction of transmembrane growth-stimulating signals (for reviews see Li *et al.*, 1991; Rapp, 1991). *Drosophila* homolog of the human c-*raf-1*, *D-raf*, has been cloned and mutants defective for this gene have been identified (Nishida *et al.*, 1988; Ambrosio *et al.*, 1989a). Phenotypes of null mutations revealed roles of *D-raf* in the regulation of cell proliferation and in the determination of cell fates at terminal regions of the embryo. Furthermore, analysis of the hypomorphic mutations of *D-raf* showed additional roles of *D-raf* in the development of the compound eye (Melnick *et al.*, 1993; Y.N. unpublished results). These observations suggest the multi-functional nature of *D-raf* during development.

Genetic analysis using a variety of mutations such as null, hypomorphic and hypermorphic mutations would enable us to dissect multiple functions of pleiotropic genes such as *D-raf*. Temperature-sensitive (ts) mutation is of particular importance, since it makes it possible to analyze gene functions at specific developmental stages. In this article, we report isolation of a temperature-sensitive mutation of *D-raf* and multiple roles of *D-raf* during development as revealed by studies on the ts mutation.

Results

Temperature-sensitive periods

New mutations of *D-raf* were induced with ethylmethane sulfonate (EMS) or X-ray as described in Materials and Methods. An EMS-

Abbreviations used in this paper: EMS, ethylmetane sulfonate; TSP, temperature-sensitive period; ts, temperature sensitive.

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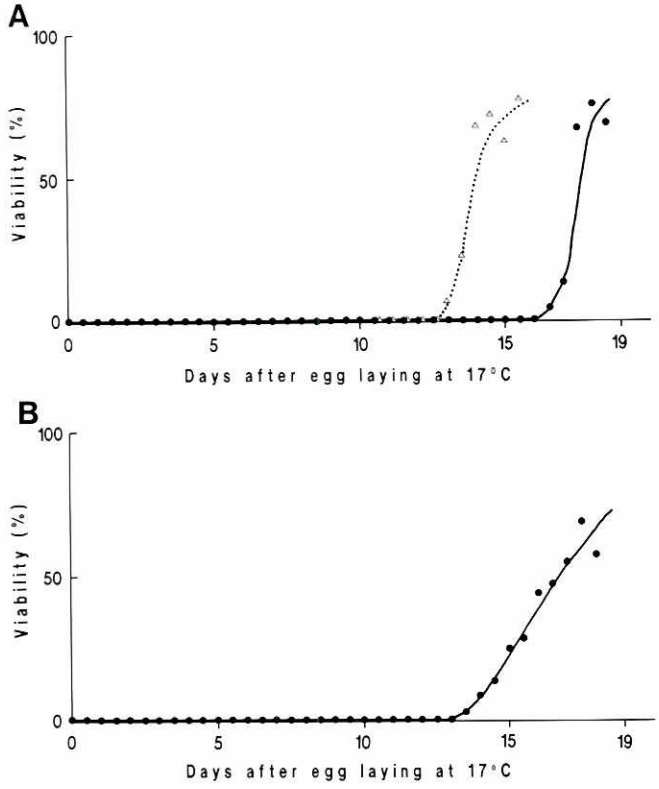

Fig. 1. *D-raf* is required for viability of flies during most developmental stages. (A) *An upshift experiment. Mutant flies were allowed to lay eggs at 17°C and the temperature was shifted up to 28°C at indicated time points. Viability was examined as described under Materials and Methods (closed circles). Pupae which failed to eclose were dissected and formation of adult structures was examined (open triangles).* **(B)** *A 12 h pulse experiment. Mutants reared at 17°C were treated at 28°C for 12 h at indicated time points. At 17°C, hatching, pupation and eclosion took place at 2 days, 10 days and 19 days after egg laying, respectively.*

induced ($D\text{-}raf^{E1}$) mutation and an X-ray-induced ($D\text{-}raf^{X1}$) one were obtained out of about 16,000 and 1,200 chromosomes screened, respectively. $D\text{-}raf^{E1}$ was found to be a temperature-sensitive (ts) mutation; it is fully viable and fertile at 17°C but is lethal at above 20°C. $D\text{-}raf^{X1}$ showed no temperature-sensitivity, and its phenotypes were essentially the same as those of $D\text{-}raf^{1}$ and $D\text{-}raf^{2}$ (Nishida *et al.*, 1988). No difference in the phenotypes was seen between $D\text{-}raf^{X1}/D\text{-}raf^{X1}$ and $D\text{-}raf^{X1}/Df(1)2F1\text{-}3A4$, indicating that $D\text{-}raf^{X1}$ is a null mutation.

The requirement of *D-raf* during development was analyzed by temperature-shift experiments using the ts mutant. The mutant never developed into fully mature adults when the temperature was shifted up from 17°C to 28°C at any time during development except the last quarter of the pupal stage (Fig. 1). The result demonstrated the latest temperature-sensitive period (TSP) at the late pupal stage. A downshift experiment showed that embryos never hatched even when they were transferred to 17°C three h after egg laying at 28°C (data not shown). The result indicates that *D-raf* is required at early phases of embryonic development. To further elucidate the roles of *D-raf* during development, 12 h pulses of treatment at 28°C were given to the mutant at various developmental stages. As shown in Fig. 1B, a single 12 h pulse treatment affected the mutant throughout development except in the late half of the pupal stage. These results clearly demonstrate that *D-raf* is needed during most of the developmental stages.

Cell proliferation

As the major role of *D-raf* has been shown to be in the regulation of cell proliferation (Perrimon *et al.*, 1985; Nishida *et al.*, 1988; Tsuda *et al.*, 1993), temperature-dependency of cell proliferation in $D\text{-}raf^{E1}$ was tested. The mutants die at the late larval or early pupal stages at temperatures above 20°C. Inside normal-looking larvae, the growth of tissues with proliferating cells was affected in a temperature-dependent manner; the higher the temperature, the more severe the effect on the growth of the imaginal discs (Fig. 2).

The temperature-sensitivity of cell proliferation in $D\text{-}raf^{E1}$ was further examined quantitatively by a clonal analysis or, the so-called twin-spot analysis (Postlethwait, 1978; Lawrence *et al.*, 1986; Tsuda *et al.*, 1993). Mitotic recombination in a proliferating cell induced by X-ray irradiation in larva heterozygous for $D\text{-}raf^{E1}$ would cause production of two daughter cells, each homozygous for either $D\text{-}raf^{E1}$ (marked with *mwh*) or $D\text{-}raf^{+}$ (marked with f^{36a}). Comparison of the sizes of two adjacent clones originating from each daughter cell on the wing blade clearly demonstrated a temperature-dependent proliferation defect in $D\text{-}raf^{E1}$ (Table 1). The result suggests a direct correlation of the *D-raf* activity with the proliferation rates and the importance of *D-raf* in the determination of rates of cell proliferation *in vivo*. In a hypomorphic mutant $D\text{-}raf^{C110}$, no apparent retardation in growth of the imaginal discs was observed upon dissection of the mutant larvae, but clonal analysis revealed a mild but statistically significant decrease in the growth rate (Table 1).

The role of *D-raf* in cell cycle was also analyzed using the ts mutant. About 1.7% of the neuroblast cells were found to be at M-phase in brain lobes from the mature larvae grown at the permissive temperature (Table 2). The fractions of M-phase cells decreased when the mutant larvae were transferred to the non-permissive temperature for a prolonged time period. The fraction of M-phase cells did not decrease in the control parental strain, *y w spl sn*, even after 28 h of treatment at 28°C. The result indicates that the M-phase proceeds normally at the non-permissive temperature and that the cell cycle is arrested at a phase or phases other than M-phase in the ts mutant. No aberrant mitotic figure was observed in the mutant reared at 28°C or in the null-function mutants, $D\text{-}raf^{1}$ and $D\text{-}raf^{X1}$ (data not shown).

Maternal effect on embryonic development

It has been shown that the maternal *D-raf* activity is essential for the determination of cell fates at both anterior and posterior ends of the embryo by acting as a transducer of transmembrane signals generated by the *torso (tor)*-encoded receptor tyrosine kinase (Perrimon *et al.*, 1985; Nishida *et al.*, 1988; Ambrosio *et al.*, 1989b; for a review see Perrimon, 1993). To analyze only the maternal effect, females homozygous for $D\text{-}raf^{E1}$ were mated with $+/B^{S}y^{+}w^{+}Y$ males. A portion of the X chromosome including the $D\text{-}raf^{+}$ locus is duplicated on the $B^{S}y^{+}w^{+}Y$ chromosome, so that all the progeny should carry $D\text{-}raf^{+}$. The TSP for this maternal function was determined by a temperature-shift experiment. As shown in Fig. 3, maternal *D-raf* is necessary about 60 min after egg laying at 28°C, or up to about 260 min after egg laying at 17°C. The TSP roughly corresponds to the stages from late cleavage division to cellular blastoderm. These lethal embryos failed to produce cuticle structures corresponding to regions posterior to the seventh abdominal segment.

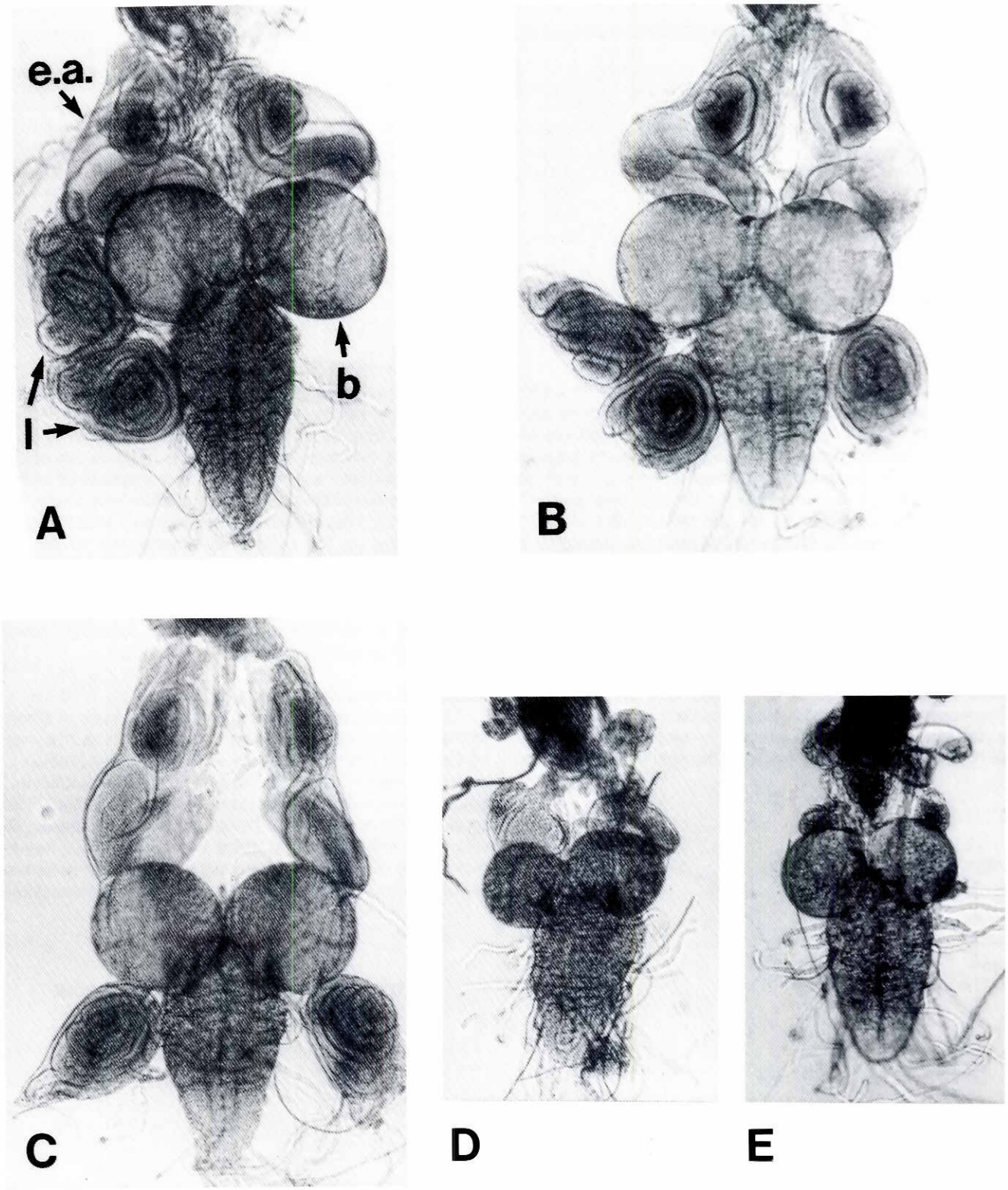

Fig. 2. Temperature-sensitive growth of imaginal discs in a ts mutant of *D-raf*. *y D-raf^{E1} w spl sn/Binsc females were crossed to normal (Canton S strain) males and were allowed to lay eggs at 17°C. 1st instar larvae hatched at 17°C were then reared at either 17°C* **(B)**, *20°C* **(C)**, *25°C* **(A and D)** *or 28°C* **(E)**. *Mature larvae were dissected and their internal organs with proliferating cells were examined. The hemizygous mutant larvae* **(B,C,D and E)** *were distinguished with y from normal y$^+$ larvae* **(A)**. *b, brain lobe; e.a., eye-antennal disc; l, leg discs.*

TABLE 1

TEMPERATURE-DEPENDENT DECREASE OF GROWTH RATES IN A ts MUTANT *D-raf^{E1}*

Genotype	Temperature (°C)	No. of twin spots analyzed	Mean No. of doubling *mwh* (A)	Mean No. of doubling *f^{36a}* (B)	Relative rate (A/B)	Normalized rate
+/M2'; mwh/mwh	25	57	6.63±1.47	7.07±1.51	0.946±0.126	1.00
D-raf^{E1}/M2'; mwh/mwh	17	36	6.07±1.21	7.13±0.89	0.850±0.127	0.90
	20	11	5.47±1.23	7.04±1.24	0.779±0.101	0.82
	25	24	4.16±1.33	6.05±1.14	0.696±0.200	0.74
	28	28	3.97±1.55	6.34±1.13	0.632±0.224	0.67
D-raf^{C110}/M2'; mwh/mwh	25	34	5.92±1.20	7.25±1.23	0.817±0.098	0.86
D-raf^{1}/M2'; mwh/mwh[a]	25	79	3.41±1.07	5.79±0.99	0.588±0.161	0.62

[a]Data from Table 1 of Tsuda *et al.* (1993)

Eclosion

When temperature was shifted up to 28°C within three days after puparium formation, the mutant never developed into mature adult inside the pupal case (Fig. 1A). The mutant developed into an apparently normal adult fly when the temperature was raised after this period (Fig. 1A, open triangles). However, it failed to emerge from the pupal case. This phenotype on eclosion is also seen with *D-raf^{C110}*, a hypomorphic mutation, and most of the *D-raf^{C110}* mutant flies, which are apparently normal except for a rough eye phenotype and a mild effect in wing vein formation, die during or soon after emergence. The temperature-sensitivity of eclosion in *D-raf^{E1}* indicates that *D-raf* is also involved in this process.

As no obvious morphological aberration was observed in these eclosion-defective flies, the tissue responsible for this defect was analyzed by a mosaic analysis with *D-raf^{C110}* using the mitotically unstable ring X chromosome, *w^{VC}* (Hotta and Benzer, 1972). Spontaneous loss of the ring X chromosome in the nuclei during early cleavage divisions in the embryos heterozygous between *y D-raf^{C110} sn* and *w^{VC}* would result in the formation of mosaic animals with clones of *y D-raf^{C110} sn*/O mutant cells. The mutant clones are easily distinguished with the epidermal markers, *y* and *sn*. If a particular part of the body is responsible for this phenotype, the mutant clone would never be found in this part of normally eclosed flies. However, in every body part the mutant epidermal clone was found in these mosaic flies, so the defect is in an internal organ, not in the epidermis. The mutant epidermal clones occurred less frequently in the ventral region of the thorax as compared to the control (Table 3). The contour line intersection for the relative frequencies on the fate map at blastoderm stage embryo (Hotta and Benzer, 1972) indicates the focus for the eclosion defect is the muscle or nervous system in the thorax (Fig. 4). Our preliminary examination of the thorax and leg muscles, however, showed no gross morphological aberration.

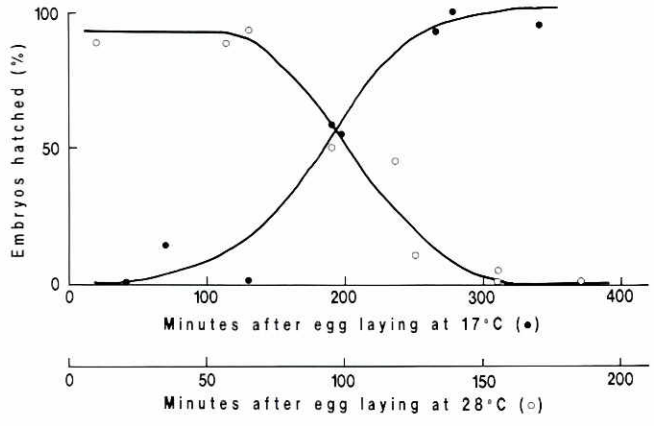

Fig. 3. Temperature-sensitive period for the maternal effect of *D-raf* on development of embryonic termini. *y D-raf^{E1} w spl sn/y D-raf^{E1} w spl sn* females grown at 17°C were mated with *+/B^{S}y^{+}w^{+}Y* males and were allowed to lay eggs at either 17°C or 28°C. At appropriate time points, temperature was upshifted (closed circles) or downshifted (open circles).

Molecular characterization of mutations

To obtain information on the functional domains of *D-raf*, molecular lesions associated with a ts mutation, a hypomorphic mutation and two null mutations were determined (Table 4). The molecular lesion associated with *D-raf^{X1}*, a null mutation, is a deletion of six amino acid residues (residue 591 to 596) in kinase subdomain VI of Hanks *et al.* (1988). Another null mutation, *D-raf^{1}*, was found to be truncation of the D-raf protein by a non-sense mutation at Gln^{412}, and the product of the mutant gene lost the entire sequence of the kinase domain. Thus, the kinase domain is

TABLE 2

TEMPERATURE-SENSITIVITY OF CELL CYCLE IN *D-raf^{E1}*, A ts MUTANT

Strain	Time at (h) 28°C	Total No. of cells	No. of M-phase cells	%
y D-raf^{E1} w spl sn	0	4,888	81	1.66
	3	2,423	40	1.65
	7	3,717	33	0.89
	18.5	3,508	20	0.57
	28	3,581	22	0.61
y w spl sn	0	3,425	61	1.78
	3	3,743	64	1.71
	7	2,067	41	1.98
	18.5	4,242	68	1.60
	28	3,157	53	1.68

TABLE 3

MOSAIC ANALYSIS OF THE ECLOSION DEFECT

Site[a]		Frequency of occurrence of X/O clones (%)		Relative frequency
		D-raf^{C110b} (A)	*D-raf*$^{+c}$ (B)	(A/B) (%)
Head	ANT	20.7	53.7	38.6
	AO	21.1	54.8	38.5
	PO	21.1	54.3	38.9
	IV	22.4	54.8	40.9
	OV	20.7	53.5	38.7
	OC	21.1	55.8	37.8
	PV	20.7	54.1	38.3
	OCC	20.2	52.5	38.5
	VB	21.1	55.0	38.4
	PT	20.7	55.8	37.1
	PR	18.1	53.1	34.1
	PA	21.1	55.2	38.2
Thorax (ventral)	SP	19.8	61.4	32.2
	SN	15.5	57.9	26.8
	I	15.1	59.7	25.3
	II	15.5	57.9	26.8
	III	18.5	57.0	32.5
Thorax (dorsal)	HU	22.4	61.4	36.5
	ANP	22.0	60.7	36.2
	PNP	21.1	61.2	34.5
	PST	22.0	60.7	36.2
	ADC	21.1	62.4	33.8
	PDC	21.1	61.8	34.1
	PPA	20.7	62.0	33.4
	ASA	22.0	61.8	35.6
	PSA	21.6	61.6	35.1
	APA	20.7	61.2	33.8
	SCT	22.0	60.7	36.2
	W	25.4	62.6	40.6
Abdomen	2T	22.8	48.8	46.7
	3T	25.4	48.6	52.3
	4T	25.9	47.7	54.3
	5T	26.7	47.9	55.7
	6T	28.0	46.5	60.2
	GT	33.2	39.3	84.5
	2S	23.7	52.5	45.1
	3S	22.8	48.8	46.7
	4S	26.3	47.9	54.9
	5S	28.4	44.8	63.7
	6S	25.4	40.5	62.7
	GS	30.2	41.3	73.1

[a]Abbreviations: ADC, anterior dorsocentral bristle; ANP, anterior notopleural bristle; ANT, antennae; AO, anteroorbital bristle; APA, anterior postalar bristle; ASA, anterior supraalar bristle; GS, genital sternite; GT, genital tergite; HU, humerus; IV, inner vertical bristle; OC, ocellar bristle; OCC, occiput; OV, outer vertical bristle; PA, palpus; PCD, posterior dorsocentral bristle; PNP, posterior notopleural bristle; PO, postorbital bristle; PPA, posterior postalar bristle; PR, prementum; PSA, posterior supraalar bristle; PST, presutural bristle; PT, ptilinum; PV, postvertical bristle; 2S~6S, 2nd~6th sternite; SCT, scutellum; SP, sternopleural; 2T~6T, 2nd~6th tergite; VB, vibrissae; W, wing; I~III, 1st~3rd legs; [b]N= 232; [c]N= 484.

severely affected in these null mutations. A mutation in the kinase domain was also found in *D-raf*^{E1}, a ts mutation, which reveals the mutation Pro^{637} to serine at a highly conserved position that defines subdomain VIII. *D-raf*^{C110}, a hypomorphic mutation, was found to be a mutation in Arg^{217} to leucine in the CR1 region of the N-terminal regulatory domain. The molecular lesion associated with *D-raf*^{C110} has been also reported recently (Melnick *et al.*, 1993). A number of nucleotide changes and insertions without alteration of amino acid residues were also found in these mutant genes as summarized in Table 4. They would be mostly polymorphic variations.

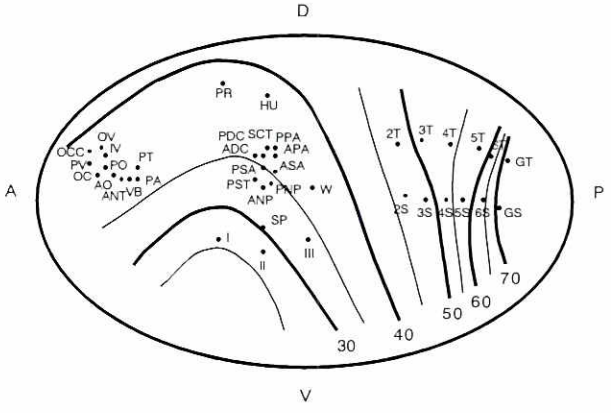

Fig. 4. Focus for the eclosion defect on the fate map of blastoderm embryo. *A, anterior; D, dorsal; P, posterior; V, ventral. Other abbreviations are as in Table 3.*

TABLE 4

MOLECULAR LESIONS ASSOCIATED WITH *D-raf* MUTATIONS

Nucleotide position[a]	Alterations[b]			
	D-raf^{1}	*D-raf*^{E1}	*D-raf*^{X1}	*D-raf*^{C110}
1,750				G→T (R^{217}→L)
1,937				C→T
2,078				G→A
2,334	C→T (Q^{412}→stop)			
2,913~2,930			Δ [18 bp] (Δ[T^{561}KFKLN^{566}])	
2,924				A→G
2,966				C→T
2,990				T→C
3,018				T→G
3,043				T→A
3,106				T→C
3,160		C→T	C→T	C→T
3,206		C→T (P^{637}→S)		
3,232				A→G
3,265				C→T
3,535				A→G
3,767		C→T	C→T	
3,799		insertion [AAGAGAGAGA]	insertion [AAGAGAGAGA]	
3,909				C→A
3,972 [AAACT]		insertion [AAACT]	insertion [AAACT]	insertion

[a]Nucleotide positions are given according to the genomic sequence of *D-raf* reported by Nishida *et al.* (1988). [b]Alterations of amino acid residues associated with nucleotide changes are indicated in parentheses. Amino acid positions are given with respect to the new putative start of translation (Sprenger *et al.*, 1993).

Discussion

Previous studies on the phenotypes of *D-raf* mutants have shown multiple roles of *D-raf* in cell proliferation, the terminal system, and eye development (Perrimon *et al.*, 1985; Nishida *et al.*, 1988; Ambrosio *et al.*, 1989b; Melnick *et al.*, 1993). Analysis of a ts mutation newly induced in this study demonstrated that *D-raf* plays vital roles during most of the developmental stages. Detailed analysis of the temperature-sensitivity of cell proliferation in the ts mutant *D-raf*^{E1} clearly demonstrated the essential role of *D-raf* for the normal rates of proliferation by acting during a phase or phases other than M-phase in cell cycle. To obtain further details on the role of *D-raf* in the regulation of proliferation, we are currently trying to establish cell lines from the embryos of *D-raf*^{E1}.

The temperature-sensitivity of the maternal *D-raf*^{E1} on development of terminal regions of the embryo also confirmed the previous results that *D-raf* is a member of the terminal class genes (Nishida *et al.*, 1988; Ambrosio *et al.*, 1989a,b; Tsuda *et al.*, 1993). TSP for this phenotype was demonstrated to be stages from late cleavage division to cellular blastoderm. This corresponds to the period when *tll*, a putative target gene of the terminal system signal transduction pathway, is expressed at terminal regions of the embryo (Pignoni *et al.*, 1990).

Detailed analysis of the ts mutant during pupal development revealed an additional role of *D-raf* in eclosion. Since the TSP for this phenotype is much earlier than the time of eclosion, *D-raf* is not involved in this process itself. Instead, *D-raf* would be needed for the proliferation or differentiation of cells required for eclosion. Although the eclosion-defective flies showed no obvious morphological aberration, a mosaic analysis of the eclosion-defective hypomorphic mutation *D-raf*^{C110} revealed the focus for this defect in the muscle and/or nervous system of the thorax. Further detailed morphological study is necessary to characterize the defect.

Analysis of the molecular lesions associated with mutations of *D-raf* demonstrated the importance of the kinase domain, which is severely affected in two of the null mutations analyzed. It is surprising that an alteration of amino acid within a highly conserved region at subdomain VIII is associated with the ts mutation *D-raf*^{E1}. The sequence is conserved in most of the protein kinases specific to both serine/threonine and tyrosine, and the similar alteration of the Rous sarcoma virus *src* gene product inactivates the tyrosine kinase activity (Bryant and Parsons, 1984). To determine whether or not the molecular lesion found in *D-raf*^{E1} is responsible for the temperature-sensitivity, we are currently analyzing the temperature-sensitivity of *in vitro* mutagenized wild-type and truncated *D-raf* genes and the human c-*raf-1* gene activated by internal deletion of the N-terminal regulatory sequence (kindly provided by K. Shimizu). Pro^{637} would be of particular importance for maintaining the functional conformation of the kinase, and the alteration of the residue to serine would considerably destabilize the conformation of the protein at normal temperature.

The ts mutation obtained in this study would be useful for further elucidation of the multiple roles of *D-raf* during development.

Materials and Methods

Mutant screening

Fly culture and crosses were performed according to standard procedures at 25°C unless otherwise described. For descriptions of the genetic markers and balancers, see Lindsley and Zimm (1992).

For screening of new alleles of *D-raf*, *y w spl sn*/Y males were fed 0.01 M ethylmethane sulfonate (EMS) or irradiated with X-ray (4,000 R) and mated to virgin *y Df(1)2F1-3A4/Bincs, oc ptg* females. The daughters with the genotype of *y w spl sn/Binsc* were further mated to *Binsc*/Y males. For EMS-induced screening, these crosses were performed at 28°C. About 550 EMS-induced and 21 X-ray-induced recessive lethals were obtained among about 16,000 and 1,200 chromosomes screened, respectively. They were further tested for allelism to *D-raf* by crossing heterozygous females to *y D-raf*^{1}/*B*^{S}*y*$^{+}$*w*$^{+}$Y males.

Temperature-shift experiments

For temperature-shift experiments, batches of flies were kept at a particular temperature inside 50 ml plastic centrifuge tubes (Falcon 2970) with cotton plugs. Slide glasses thickly coated with fly meal were inserted into these tubes and females were allowed to lay eggs for an appropriate period. The slide glasses were transferred to new tubes and kept at a particular temperature. One to two days after hatching of normal embryos, the numbers of hatched and unhatched embryos were counted. For TSP analysis at later stages, 1st instar larvae newly hatched at 17°C were placed in pre-cooled culture tubes (50 larvae per tube), and tubes were transferred to 28°C at an appropriate time.

Cytological preparations of neuroblasts from larval brain lobes were made as described (Guest and Hsu, 1973).

Mosaic analysis

For clonal or twin-spot analysis, *y D-raf*^{E1} *w spl/Binsc, oc ptg; mwh red e* females were mated to *Dp(1; Y; 3)M2', mwh*$^{+}$ *ve*$^{+}$ *FR1, y cv v f*^{36a}/Y; *mwh ve h* males and their progeny were irradiated with X-ray (1,500 R) at 3rd instar. Twin spots formed on wings were analyzed as described (Postlethwait, 1978; Lawrence *et al.*, 1986; Tsuda *et al.*, 1993).

y w spl/w^{VC}, kindly provided by Y. Hotta, was used for a focus assay. *y w spl/w*^{VC} females were crossed with *y D-raf*^{C110} *sn/B*^{S}*y*$^{+}$*w*$^{+}$Y males or *y w spl sn/B*^{S}Y males, and the mosaics were analyzed as described (Hotta and Benzer, 1972).

Molecular procedures

DNAs were extracted from homozygous mutant larvae as described previously (Nishida *et al.*, 1988). The DNAs were digested with *Bam*HI, and 4 to 5 kb fragments were cloned by ligating with the EMBL3 arms generated by digestion with *Bam*HI. Phages were screened with a *D-raf* cDNA probe. Positive clones contained several *Bam*HI fragments, and the fragments hybridizing to the cDNA probe were subcloned into the *Bam*HI site of pGEM3 (Promega Corporation, Wisconsin, USA). The nucleotide sequences were determined using oligonucleotide primers synthesized according to the genomic sequence of *D-raf* (Nishida *et al.*, 1988) by the chain termination method (Sanger *et al.*, 1977).

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