The LAMMER Protein Kinase Encoded by the *Doa* Locus of Drosophila Is Required in Both Somatic and Germline Cells and Is Expressed as Both Nuclear and Cytoplasmic Isoforms Throughout Development

Bokyoung Yun,*,[1,2] Kun Lee,*,[1,3] Robert Farkaš,† Christophe Hitte*,[4] and Leonard Rabinow*,‡

**Waksman Institute, Rutgers University, Piscataway, New Jersey 08855, †Institute of Experimental Endocrinology, Slovak Academy of Sciences, 833 06 Bratislava, Slovak Republic and ‡Laboratoire d'Embryologie Moléculaire, UPRES-A8080, Université de Paris XI, 91405 Orsay, France*

Manuscript received April 3, 2000
Accepted for publication June 21, 2000

ABSTRACT

Activity of the *Darkener of apricot (Doa)* locus of *Drosophila melanogaster* is required for development of the embryonic nervous system, segmentation, photoreceptor maintenance, normal transcription, and sexual differentiation. The gene encodes a protein kinase, with homologues throughout eukaryotes known as the LAMMER kinases. We show here that DOA is expressed as at least two different protein isoforms of 105 and 55 kD throughout development, which are primarily localized to the cytoplasm and nucleus, respectively. *Doa* transcripts and protein are expressed in all cell types both during embryogenesis and in imaginal discs. Although it was recently shown that DOA kinase is essential for normal sexual differentiation, levels of both kinase isoforms are equal between the sexes during early pupal development. The presence of the kinase on the cell membrane and in the nuclei of polytene salivary gland cells, as well as exclusion from the nuclei of specific cells, may be indicative of regulated kinase localization. Mosaic analysis in both the soma and germline demonstrates that *Doa* function is essential for cell viability. Finally, in contrast to results reported in other systems and despite some phenotypic similarities, genetic data demonstrate that the LAMMER kinases do not participate in the ras-MAP kinase signal transduction pathway.

THE LAMMER protein kinase family possesses homologues in all eukaryotes, including Drosophila, mice, *Saccharomyces cerevisiae*, and humans (Yun *et al.* 1994). Although the mammalian members of this family are often referred to as Clks (Cdc2-like kinases), this name is somewhat misleading, since it is based upon amino acid homologies in a limited number of kinase catalytic subdomains and residues. Unlike cdk kinases, LAMMER kinases also possess extensive N-terminal noncatalytic domains and there is no evidence that they require cyclins for their activity. Sequence alignments clearly demonstrate that they form a separate subgroup of the CMGC-V kinases (see http//www.sdsc.edu/kinases/pk_home.html). Despite their ubiquitous presence in eukaryotes, the *in vivo* specificity and function of LAMMER kinases remain at best partially characterized.

LAMMER kinases autophosphorylate on Ser/Thr and Tyr residues ("dual specificity"; Ben-David *et al.* 1991; Howell *et al.* 1991; Lee *et al.* 1996; Sessa *et al.* 1996), although this specificity does not extend to the only known substrates, which are phosphorylated exclusively on Ser residues. Among these are the SR proteins, which are best known for their function in the splicing of pre-mRNAs (see below). All LAMMER family members possess motifs that are nearly 100% identical in catalytic subdomains essential for phosphotransfer and substrate interaction, suggesting that they perform similar functions in widely diverged organisms (Yun *et al.* 1994). All LAMMER family members also possess virtually 100% identity of the motif "EHLAMMERILG" in kinase subdomain X. This motif was recently reported to be essential for kinase activity, but not for binding with substrates *in vitro* (Savaldi-Goldstein *et al.* 2000).

Understanding of LAMMER kinase function within the context of a developing organism depends largely upon analysis of the Drosophila family member, encoded at the *Darkener of apricot* (*Doa*). *Doa* is an essential gene whose mutations were isolated during screens for dosage-sensitive regulatory loci (Rabinow and Birchler 1989). Its mutations suppress the mutagenic effects of several insertions of the retrotransposon *copia*, accompanied by a two- to fourfold increase in its stable transcript levels, apparently due to elevated rates of transcription (Rabinow *et al.* 1993). *Doa* alleles suppress *copia*-induced phenotypes and alter accumulation of host-locus mRNAs

Corresponding author: Leonard Rabinow, Laboratoire d'Embryologie Moléculaire, Bâtiment 445, Université de Paris XI, 91405 Orsay Cedex, France. E-mail: lenny.rabinow@emex.u-psud.fr

[1] These authors contributed equally to this work.

[2] *Present address:* Department of Biological Sciences, Basic Science Institute, Dankook University, Cheonan City, Chungnam 330-714, South Korea.

[3] *Present address:* The Fox Chase Cancer Center, 7701 Burholme Ave., Philadelphia, PA 19111.

[4] *Present address:* CNRS UPR41 Recombinaisons Génétiques, 2, rue du Pr. Leon Bernard, 35043 Rennes Cedex, France.

independently of the orientation of the transposon insertion into the host locus. Therefore, suppression of *copia*-induced phenotypes is strand independent, suggesting either that cellular processes other than splicing are affected by LAMMER kinases or that the suppression of *copia*-induced phenotypes by *Doa* alleles is indirect.

In addition to suppression of *copia* insertion phenotypes, *Doa* mutants possess pleiotropic defects, although homozygotes derived from heterozygous females develop normally and die during early larval stages (Rabinow and Birchler 1989). In contrast, homozygotes obtained from heteroallelic mutant mothers die as embryos with severe defects in embryonic segmentation and nervous system development, revealing a maternal contribution of *Doa* activity to the oocyte. Pleiotropic defects are also visible in heteroallelic adults and larvae or rare homozygotes of two specific genotypes escaping lethality. These include small imaginal discs, degeneration of retinal photoreceptors, and aberrant (extra) wing venation (Yun *et al.* 1994).

Failure of the embryonic nervous system to elaborate and the specific degeneration of retinal photoreceptors in mutants possessing residual *Doa* activity, along with loss of adult cuticular structures such as bristles and occasionally ocelli, suggest the existence of a requirement for higher *Doa* activity in the nervous system relative to other tissues. Since even homozygotes dying as embryos possess residual maternally contributed *Doa* activity, we have hypothesized a general role for LAMMER kinases in the initiation of differentiation. Furthermore, the degeneration of retinal photoreceptors in heteroallelic animals demonstrates a continued requirement for the gene's activity for cellular viability. Thus, we believe that LAMMER kinases' function is essential for cellular viability and differentiation.

Support for the latter role interpretation is derived from the identification of PK12, a tobacco LAMMER kinase, whose transcription and activity are induced in response to the hormone ethylene (Sessa *et al.* 1996), suggesting that this kinase modulates cellular responses in this signaling pathway. Other evidence supporting the hypothesis that LAMMER kinases function in cellular differentiation is provided by the observation that expression of a murine LAMMER kinase in PC-12 cells induces cell cycle arrest and neurite outgrowth (Myers *et al.* 1994).

The LAMMER kinase-encoding locus, *KNS1*, from the yeast *S. cerevisiae*, would be amenable to genetic analysis. However, it is not esssential for vegetative growth, meiosis, or sporulation (Padmanabha *et al.* 1991). Moreover, screens for synthetic lethals in a *kns1* background failed to identify any interacting loci (M. Horn, T. Kinzy and L. Rabinow, unpublished results).

The only LAMMER kinase substrates so far identified *in vivo* are the SR proteins. This conserved eukaryotic protein family influences RNA splice site selection and other aspects of RNA metabolism (for reviews, see Manley and Tacke 1996; Valcarcel and Green 1996; Misteli and Spector 1997). The phosphorylation of SR proteins on Arg-Ser rich domains (RS domains) influences their protein-protein interactions and the specificity of RNA-protein interactions (Cao *et al.* 1997; Tacke *et al.* 1997; Xiao and Manley 1997). Several protein kinases, including those in the LAMMER family, phosphorylate SR proteins *in vitro* (Gui *et al.* 1994a,b; Colwill *et al.* 1996a; Kuroyanagi *et al.* 1998; Tang *et al.* 1998; Wang *et al.* 1998). LAMMER kinase-SR protein interactions were first described in two-hybrid screens in yeast and have also been biochemically characterized (Colwill *et al.* 1996a,b; Nayler *et al.* 1997). We have described reduced SR protein phosphorylation and aberrant intranuclear localization in *Doa* mutants (Du *et al.* 1998), demonstrating that LAMMER kinases function in the regulation of alternative splicing. *Doa* mutants also possess altered sexual differentiation due to aberrant pre-mRNA splicing of *dsx*. Epistasis analysis demonstrates that these effects occur at the same level as mutations in the two SR-related splicing factors *tra* and *tra2*, suggesting that their activity is directly affected by hypophosphorylation due to mutations in DOA kinase. Thus, one *in vivo* "target" of LAMMER kinase function is alternative pre-mRNA splicing, although whether this is a constitutive or regulated requirement remains to be determined.

To further characterize the function of *Doa* and its products, we have examined its expression patterns, as well as requirements for its activity in mosaic animals. Here we report that at least two different isoforms of DOA protein are expressed in all tissues and developmental stages examined. One of these is cytoplasmic while the other is primarily nuclear, as revealed in part through subcellular fractionation experiments. Analysis of *Doa* transcript and protein levels and expression patterns during embryogenesis and in imaginal discs reveals ubiquitous expression, while both somatic and germline mosaic analyses demonstrate that *Doa* activity is essential for cellular viability. Finally, although several pleiotropic phenotypes of *Doa* mutations resemble those of mutations in components of the *ras*-mitogen-activated protein kinase (MAP-K) signal transduction pathway, no genetic interactions were observed in double mutants, suggesting that *Doa* acts independently of this well-characterized signal transduction cascade.

MATERIALS AND METHODS

Drosophila stocks and crosses: *Drosophila melanogaster* were raised on standard cornmeal medium at 25°. *Doa* mutant strains and heteroallelic combinations were previously described (Rabinow *et al.* 1993; Yun *et al.* 1994). Crosses to generate heteroallelic *Doa* flies heterozygous or homozygous for other mutations being tested for interaction used standard chromosomal segregation, replacement, and recombination. Our rationale in tests for genetic interactions between *Doa* and components of the ras-MAP-K pathway and other loci was

TABLE 1

Stocks used in the generation of somatic and germline mosaics

Stock	Source
w ovo; P[w, ovo^{D1} 11.3b]/+	Maryvonne Ninio
yw; P[ry^{+} hs-neo FRT]82B; P[mini-w^{+} hs-πM]87E; e Doa^{γ3B}/TM3, Ser	This study
yw; P[ry^{+} hs-neo FRT]82B; P[mini-w^{+} hs-πM]87E; e Doa^{EMS2}/TM3, Ser	This study
yw; P[ry^{+} hs-neo FRT]82B; P [mini-w^{+} hs-πM]87E; e Doa^{I5}/TM3, Ser	This study
w, hsFLP1; P[ry^{+} hs-neo FRT]82B; P[mini-w^{+} hs-πM]87E	Bloomington Stock Center
yw, hsFLP1; P[ry^{+} hs-neo FRT]82B; P[mini-w^{+} hs-πM]87E; P[ry^{+} y^{+}]96E	Bloomington Stock Center

that eye morphology or *w^{apricot}* pigmentation would be affected if interactions existed. Homozygotes of loci encoding components of the ras-MAP-K cascade were tested wherever possible. Additionally, heteroallelic combinations of *Doa* alleles were tested in addition to heterozygotes in most cases. We reasoned that heteroallelic combinations with smooth eyes (*Doa^{HD}/Doa^{Dem}*) would be roughened (enhanced), or those with rough eyes (*Doa^{HD}/Doa^{105}*) would be smoothened (suppressed), if interactions occurred. A minimum of 65 progeny were examined in evaluating all genetic interactions and complementation tests.

The deficiency chromosome *Df(3R) 3450* (98E3:99A6–8) was examined as a possible deletion of the *Doa* locus, and the *lacZ*-marked *P*-element-induced recessive lethal *l(3)01705* at 98F1–4 from the Berkeley Drosophila Genome Project (Spradling *et al.* 1995) was examined as a possible *Doa* allele. β-Galactosidase expression was examined in embryos, imaginal discs, and larval tissues using assays for activity (Ashburner 1989b) as well as via immunocytochemical staining with a commercial antibody against the enzyme (Cappel).

Generation of somatic mosaic clones: Stocks for the induction of somatic and germline mosaics are listed (Table 1). Somatic clones of three *Doa* alleles (*Doa^{γ3B}*, *Doa^{EMS2}*, and *Doa^{I5}*) were generated using FRT/FLP site-specific recombination (Xu and Rubin 1993). Analysis of multiple alleles was necessitated because it is difficult to genetically define a null *Doa* allele. All alleles of the gene suppress *w^{a}* equally, while several hypomorphs (based on their survival in heteroallelic combinations with others) are lethal in combination with a deficiency of the locus (this report). Virtually all alleles are recessive lethal as homozygotes or in combination with other *Doa* alleles. Only a few have been characterized at the molecular level (Yun *et al.* 1994).

Three alleles were chosen for the generation of mosaic clones on the basis of availability of cytological, genetic, and molecular data, as follows. *Doa^{γ3B}* is a null, because mutants possess only 50% of wild-type *Doa* 2.7-kb mRNA levels encoding an active kinase and because it only poorly complements two *Doa* alleles (*Doa^{Msu2}* and *Doa$^{Rem γA}$*), which complement the vast majority of other *Doa* mutations (C. So, B. Yun and L. Rabinow, unpublished results). The *γ3B* allele is due to a small cytologically visible inversion and possesses a breakpoint directly in the gene, upstream of the region encoding the kinase catalytic domain (Yun *et al.* 1994). This inversion raised the possibility that a second locus might also be mutated in this stock. Therefore, two additional alleles, *Doa^{EMS2}* and *Doa^{I5}*, which do not possess cytogenetically visible lesions (Rabinow and Birchler 1989), were also used. Both are recessive lethal as homozygotes. The *I5* allele does not produce heteroallelic survivors in combination with any other allele, while the *EMS2* allele was discovered to do so with only a single *Doa* allele after the completion of mosaic analysis. Southern blots on genomic DNA demonstrate no gross rearrangements in the *Doa* locus of either the *I5* or *EMS2* strains. Transcript analysis suggests that levels of the 2.7-kb RNA presumably encoding an active form of *Doa* kinase are reduced in *EMS2* relative to wild type (B. Yun, unpublished results).

These three *Doa* alleles (98F) were recombined onto a third chromosome carrying a FRT at 82B. Mutant cell clones were marked in repulsion with *white^{+}* in eyes and were linked to *ebony*. Clones were produced by collecting eggs for 12 hr, aging them for between 24 hr and 6 days, and incubating them in a 38° water bath for 60 min. Cultures were then returned to 25°. Homozygous mutant eye clones were identified as patches lacking pigment, and cuticle clones were identified by homozygosity for *ebony*. A large sample of individuals in which clones had been induced was examined (>50 for each genotype). These experiments were repeated several times, with clones induced at various developmental stages. In examination of mosaic imaginal discs, 6-day-old larvae were heat shocked, returned to 25° for 2–3 hr, and dissected on ice.

Generation of germline mosaic clones: *y w^{-}*; P[FRT], *e Doa^{γ3B}*/TM3 female virgins were crossed with *w^{-}*; P[*w^{+}*, *ovo$^{D1,11.3b}$*]/+ males, a gift of Maryvonne Ninio (Mével-Ninio *et al.* 1994). Approximately 48-hr-old larvae and early pupae were irradiated with a Rigaku RU200 X-ray machine for 7 min (copper anode at 5 kW, 1000 rad total dose). A total of 290 irradiated *y w/w*; P[FRT], *e Doa^{γ3B}*/P[*w^{+}*, *ovo$^{D1,11.3b}$*] virgins were crossed with *y w*; *Doa^{γ3B}*/TM3 in 21 vials. As a control, wild-type females were crossed with male *w^{-}*, *ovo^{0}*: P[*w^{+}*, *ovo$^{D1,11.3b}$*] and F_1 irradiated as above. A total of 279 irradiated *y w/w*; +/P[*w^{+}*, *ovo$^{D1,11.3b}$*] virgins were crossed with +/+ males. Females in 13/21 vials (134 flies) laid eggs, indicating the successful induction of germline clones.

***In situ* hybridization to whole embryos:** The procedure of Pfeifle and Tautz was followed for nonradioactive *in situ* hybridization to transcripts in whole mount embryos (Pfeifle and Tautz 1989), using an antisense RNA probe of a 2-kb *Doa* cDNA (Yun *et al.* 1994). Probes were alkaline hydrolyzed to 150–200 bp as described (Cox *et al.* 1984).

Bacterial fusion proteins and antibody generation: Antibodies against the affinity-purified 85-kD catalytic domain DOA fusion protein (Lee *et al.* 1996) were produced in two rabbits (Lampire Laboratories, Piperville, PA). Sera were tested for reactivity on immunoblots of both bacterially expressed fusion protein and adult Drosophila proteins. Preimmune sera did not recognize either the recombinant protein or those of 105 and 55 kD (not shown). Useful serum from only one of the two rabbits was produced by these criteria.

Anti-DOA fusion sera were affinity purified on Affi-gel 10 and Affi-gel 15 according to instructions (Bio-Rad, Hercules, CA). Nonspecific antibodies were removed by chromatography through a mixture of Affi-gel 10 and 15 coupled with crude bacterial extracts in which the pMAL vector had been expressed. Antibodies specific to the maltose-binding protein

(MBP)-DOA fusion protein were bound on Affi-gel 10 and eluted with 0.1 M glycine, pH 2.5.

An octameric peptide (Tam 1988) of the most highly conserved 11 amino acids in the LAMMER motif (EHLAMMERILG) was also used to generate antibodies (anti-LAMMER) in two rabbits (Research Genetics, Huntsville, AL). Immune sera recognized bacterially expressed DOA and a TrpE-murine Clk1 fusion protein (Ben-David *et al.* 1991), which were not recognized by preimmune sera (not shown). The specificity of the anti-LAMMER antiserum for DOA was also tested on immunoblots of factor-X-digested purified MBP-DOA fusion protein. Digested proteins were blotted and probed with antisera or processed for renaturation kinase assays. Immunoblots demonstrated that the serum possesses DOA-specific immunoreactivity identical to the pattern of kinase activity (not shown).

Histology and immunocytochemistry: Immunohistochemical staining of dissected imaginal discs and embryos was performed as described (Ashburner 1989a). Affinity-purified anti-DOA was used at dilutions between 1/50 and 1/200, except where specifically noted. Crude anti-DOA and crude anti-LAMMER sera were used at dilutions between 1:500 and 1:2000. Anti-ELAV antibody was a gift of Marie-Laure Samson. For diamino-benzidine (DAB) staining, discs were fixed in 4% paraformaldehyde, washed in PBS, permeabilized with PBS + 0.1% Triton X-100 (PBT), blocked for 4 hr in PBT + 2% BSA and 1% normal rabbit serum, incubated with primary antibodies at least 4 hr, and washed with PBT. For HRP labeling, embryos or discs were incubated with biotinylated anti-goat antibody (Vector Laboratories, Burlingame, CA) for 3 hr, washed with PBT + 1% rabbit serum, and incubated with strepavidin-conjugated HRP, followed by washing and staining with 0.1% DAB and 0.02% hydrogen peroxide. For immunofluorescence, tissues were fixed in 4% paraformaldehyde + 1.5% glutaraldehyde in PBS, permeabilized with 0.3% Triton X-100 in PBS, and blocked with PBS containing 2% BSA and 1% normal goat serum. Affinity-purified anti-DOA was used at a dilution of 1:100–200 overnight at 4°. After washing, FITC-conjugated goat anti-rabbit (Jackson Laboratories, West Grove, PA) was added for 2 hr at room temperature, tissues were washed three or four times with PBS-Triton, and mounted in Mowiol. Images were scanned on a Zeiss LSM-410 laser confocal microscope.

Protein preparation and pupal nuclear extracts: Embryos, third instar larvae, 0- to 1-day-old pupae, and 0- to 2-day-old adults were collected and washed in phosphate-buffered saline. Sexed pupal extracts were prepared by sorting third instar larvae prior to pupariation and aging them. Individual tissues were hand dissected. Samples were homogenized in SDS gel loading buffer and boiled. Pupal nuclear extracts were prepared according to Dorsett (1990).

SDS gel electrophoresis, protein transfers, and immunoblots: SDS gel electrophoresis, protein transfers, and immunoblots were performed as described (Ausubel *et al.* 1989), using a 10% separating gel. Separated proteins were transferred to nitrocellulose for immunological detection utilizing the ECL chemiluminescence system (Amersham, Arlington Heights, IL). Protein loading on the gels was controlled by reprobing the blots with anti-α-tubulin monoclonal antibody DM1A (Sigma, St. Louis). Antibody dilutions for immunoblot analyses were similar to those described for immunocytochemical analyses.

RESULTS

Immunoblot analysis of DOA expression during development reveals at least two protein isoforms: Affinity-purified anti-DOA antibodies and crude antipeptide sera directed against the LAMMER motif reveal the size and developmental expression of reacting proteins on immunoblots. Proteins of 55 and 105 kD are detected at all developmental stages (Figure 1, A and B), including 0- to 24-hr embryos, third instar larvae, 0- to 24-hr pupae, and adults, as well as in extracts of 0- to 4-hr embyros, larval eye-antennal imaginal discs, salivary glands, fat bodies, adult heads, and ovaries (not shown). These proteins are recognized by both crude and affinity-purified anti-DOA serum (Figure 1, A and B), as well as the anti-LAMMER serum (Figure 1D, lanes 3 and 4). For unclear reasons, the affinity-purified anti-DOA serum produces relatively stronger signal against the 105-kD isoform than against the 55-kD isoform (Figure 1A; compare lanes 1–3 with 4–6). Additional minor bands are also recognized by these sera, including the affinity-purified one. We believe that some, if not all, of these are degradation products, due to their variability among transfers, although it remains possible that these bands represent minor DOA isoforms.

The 55-kD protein is consistent in size with that of the kinase deduced from the sequence of cDNA clones deriving from a 2.7-kb *Doa* transcript expressed throughout development (Yun *et al.* 1994). *Doa* transcripts large enough to encode a protein of 105 kD exist at many, but not all, stages of Drosophila development, *e.g.*, not in early embryos (Yun *et al.* 1994), but have not been characterized at the level of sequence. However, the 105-kD protein is detected in all stages and tissues examined, including early embryos. Since the 105-kD protein is found in ovary tissue extracts (not shown), it is presumably maternally contributed to the oocyte.

The authenticity of the 105-kD protein as an independent protein isoform, as opposed to being due to post-translational modification, was examined by treating pupal extracts with urea (final concentration 6 M), in addition to SDS, to exclude the possibility that the 105-kD protein is a dimer of the 55-kD protein resistant to SDS and reducing conditions. No differences with control samples were observed (not shown). Also, treatment of embryonic and pupal extracts with either calf-intestinal phosphatase or potato-acid phosphatase for 30 min at 37° did not produce any differences in levels or mobility of the 55- or 105-kD isoforms (not shown). However, this analysis does not eliminate the possibility that these apparently different proteins are not due to other post-translational modifications, for example carbohydrate modification.

***Doa* mutations reduce levels of the 55- and 105-kD proteins:** To confirm that the 55- and 105-kD proteins are both encoded by *Doa*, we examined their levels in extracts of mutant pupae. Pupae were used because selection of rare homozygous or heteroallelic adults expressing higher than average DOA levels might be responsible for the survival of the rare individuals escaping recessive lethality. This phenomenon was in fact observed, since pupal extracts of the *105/HD* genotype

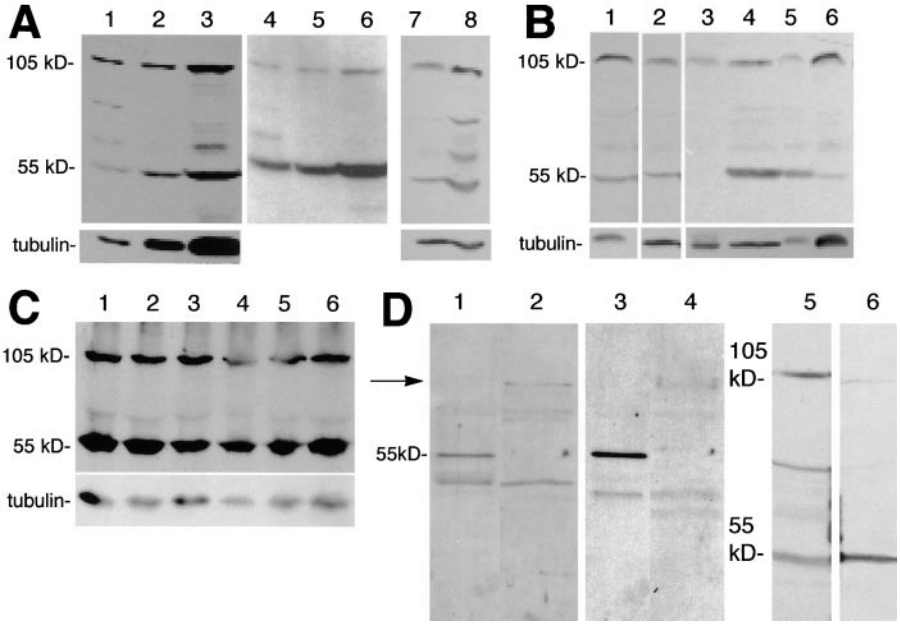

FIGURE 1.—Immunoblot analysis of DOA expression. Samples were obtained from mixed-sex cultures. All transfers were probed with affinity-purified anti-DOA serum, except as noted. Reprobing with anti-tubulin was used as a loading control. (A) DOA expression during development. Lanes 1–3 and 4–6 are the same gel, probed, stripped, and reprobed; *white*^{apricot} genotype. Lanes 7 and 8 are *Ore*^{R} extracts run on a separate gel. Lanes 1 and 4, 0- to 24-hr-old embryos; lanes 2 and 5, third instar larvae; lanes 3 and 6, 0- to 24-hr-old pupae; lane 7, 0- to 24-hr-old pupae; lane 8, 0- to 24-hr-old adults. Lanes 1–3, 7, and 8 were probed with the affinity-purified serum. Lanes 4–6 were reprobed with crude anti-DOA serum. Note the higher signal of the 55-kD relative to the 105-kD protein in lanes 4–6 compared with lanes 1–3. Signals at other molecular weights are variable between experiments. Although they may represent minor DOA isoforms, we hypothesize that they are due to limited proteolysis. (B) Expression of both DOA protein isoforms is reduced in mutants. Extracts were from 0- to 24-hr-old pupae (except lane 4). All samples were transferred from the same gel. Lane 1, w^a; lane 2, *Ore*^{R}; lane 3, *Doa*^{105}/*Doa*^{HD}; lane 4, *Doa*^{105}/*Doa*^{HD} 0- to 24-hr-old adults; lane 5, *Doa*^{RemγA} homozygotes; lane 6, *Doa*^{Msu2} homozygotes. Levels of the 55- and/or the 105-kD DOA isoforms are reduced in the heteroallelic or homozygous mutants relative to wild type. This result was obtained in four independent experiments. Between 27 and 40% of wild-type (w^a and *Ore*^{R}) levels of the 105-kD protein are observed in *Doa*^{105}/*Doa*^{HD} heteroallelic mutants. Lane 2 in B is the same as lane 7 in A. (C) DOA expression is essentially equal in males and females of three wild-type strains. Lane 1, w^a; +/+ males; lane 2, w^a; +/+ females; lane 3, C^S males; lane 4, C^S females; lane 5, *Ore*^{R} males; lane 6, *Ore*^{R} females. (D) The 55-kD isoform is nuclearly localized and the 105-kD isoform is restricted to the cytoplasm in 0- to 24-hr-old pupal extracts. Nuclear (lanes 1 and 3) and total (lanes 2 and 4; nuclear + cytoplasmic) proteins (same gel, stripped and reprobed, 5 μg loaded each lane) are shown. Note the weak signal (arrow) in the total extracts (lanes 2 and 4), which we believe to be the 105-kD isoform. Lanes 1 and 2 were probed with anti-DOA serum; lanes 3 and 4 were reprobed with the antipeptide (anti-LAMMER) serum. In lane 5, 20 μg of total (nuclear + cytoplasmic) protein was loaded. Loading of nuclear extract in lane 6 was normalized to the 55-kD signal (~5 μg loaded; both lanes from the same gel).

possess significantly less DOA protein than wild type, while adults of this genotype escaping lethality possess more nearly normal levels (Figure 1B, lane 3 *vs.* lane 4).

Levels of both the 55- and 105-kD proteins are visibly reduced in at least two of the three *Doa* mutant combinations relative to all three wild-type strains examined (Figure 1B). It is not surprising that DOA protein is detected in the mutant extracts, since activity of the gene is required for viability, and these alleles are necessarily hypomorphic. Since affinity-purified anti-DOA antibodies detect the 105-kD protein, and two of three mutant *Doa* genotypes examined affect its quantity, we conclude that both the 55- and 105-kD proteins are authentic DOA isoforms.

Expression of DOA is equivalent between the sexes: As mentioned, *Doa* alleles subtly alter sexual differentiation, as well as alter the splicing of mRNA from the *dsx* locus, a crucial component of somatic sex determination (DU *et al.* 1998). We therefore examined protein extracts from sexed pupae to determine whether DOA protein levels are regulated as a function of sex. No differences were found in expression of either the 55- or 105-kD isoforms in wild-type animals (Figure 1B, lanes 1–6).

Differential subcellular localization of the 55- and 105-kD isoforms: Immunoblot analysis of nuclear and whole pupal cell extracts was used to determine the intracellular localization of the two DOA isoforms, since our antisera do not differentiate between them. Normalization of the loading of each sample was accomplished via two methods, in which (1) the total amount of protein loaded was equivalent between both samples, as well as (2) the amount of the 55-kD signal was normalized between the two.

The results using crude anti-DOA, affinity-purified anti-DOA, and anti-LAMMER sera show that the 105-kD protein is found only in whole-cell extracts, while it is missing in nuclear fractions (Figure 1D). In contrast, the 55-kD isoform is found in both nuclear and whole-cell extracts. Only ~5 μg of nuclear extract was needed to detect the 55-kD protein, whereas 20 μg of total protein was required to detect the cytoplasmic form, suggesting that the bulk of the kinase is nuclearly localized. This analysis does not exclude the possibility that some of the 55-kD protein is cytoplasmically localized in all cells, or all of it is cytoplasmic in certain cell types.

***Doa* transcript accumulation during embryogenesis and in imaginal discs:** The pattern of *Doa* transcription

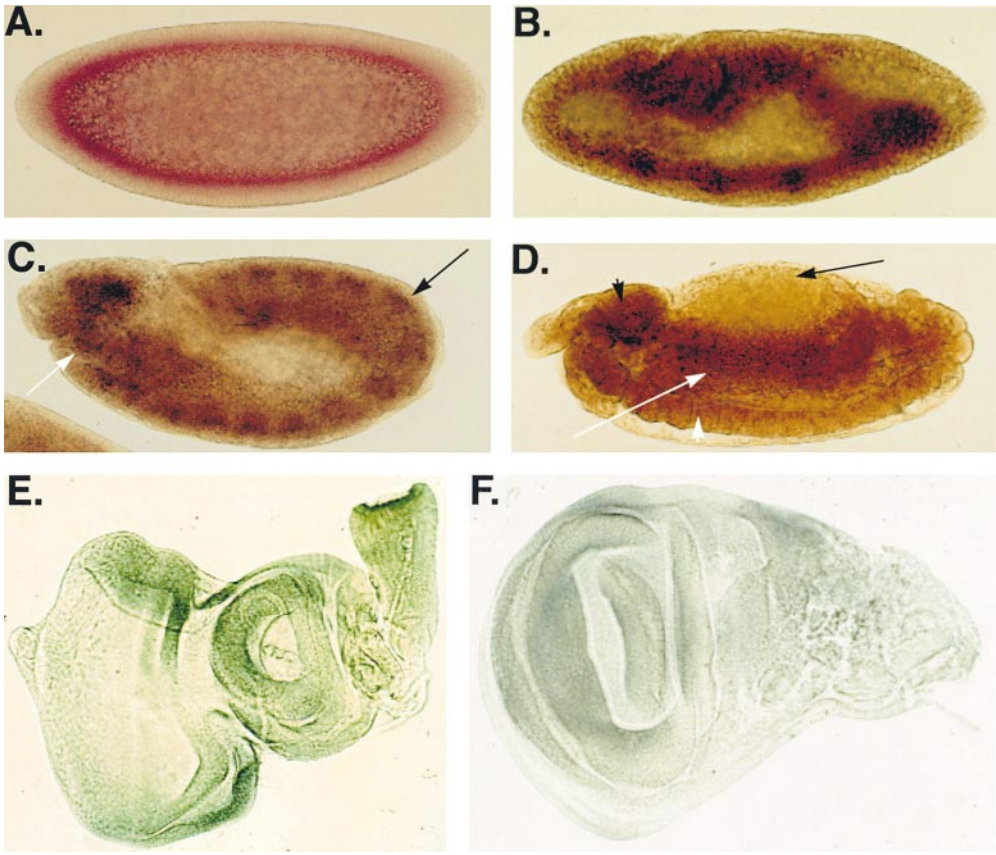

FIGURE 2.—*Doa* transcription patterns in embryos and imaginal discs. Transcript distribution is visualized with a digoxigenin-UTP-labeled *Doa* cDNA probe and subsequent HRP detection. (A) Stage 5 cellular blastoderm; (B) stages 7 and 8, gastrulation; (C) stages 10 and 11, germ band extension; the densely staining segmented areas in the ectoderm may correspond to the tracheal pits (black arrow); the procephalon is labeled with a white arrow. (D) Stage 13, germ band retracted. The lightly stained amnioserosa is indicated with a black arrow, the ventral nervous system with a white arrowhead, the brain with a black arrowhead, and the mesoderm with a white arrow. Low but ubiquitous levels of *Doa* transcripts are observed in (E) eye-antennal and (F) wing imaginal discs obtained from late third instar larvae. Apparent differences in labeling intensity are due to folds in the cellular monolayer of the discs.

was examined during embryogenesis and in imaginal discs by *in situ* hybridization using a 2.0-kb cDNA probe (YUN *et al.* 1994). *Doa* transcripts are ubiquitously expressed in both embryos and imaginal discs, although they are not uniformly distributed (Figure 2).

In the stage 1 precellular blastoderm (stages as per CAMPOS-ORTEGA and HARTENSTEIN 1997), uniform staining of *Doa* transcripts, presumably of maternal origin, was detected throughout the embryo (not shown). Uniform distribution of *Doa* transcripts persists during the cellular blastoderm phase, (stage 5; Figure 2A). Zygotically expressed *Doa* transcripts are detected following the germ band (stages 7–8; Figure 2B). At stages 10–11, when the three layers of the germ band are established, widespread staining includes the presumptive ectoderm and mesoderm (Figure 2C). Densely stained segmented areas were detected in the ectoderm at these stages, possibly corresponding to tracheal pits, where cellular elongation is occurring. *Doa* transcripts are also clearly detected in the procephalon (Figure 2C). Once germ band retraction is completed (stage 13), *Doa* is transcribed most intensely in the ventral and anterior regions of the embryos, while comparatively little transcript accumulates dorsally, *e.g.*, in the amnioserosa (Figure 2D). The ventral staining region includes the ventral cord of the central nervous system, consistent with defects found in the central nervous system (CNS) of *Doa* mutant embryos derived from heteroallelic mutant mothers. Anterior staining is detected in the brain, optic lobe precursor, and clypeolabrum. Dorsal views of the embryo show *Doa* transcripts in tissues surrounding the visceral mesoderm as well as in the brain (not shown). *Doa* transcripts persist in the condensed ventral nerve cord and brain in stage 17 embryos (not shown).

Light but relatively uniform staining is observed in the eye and wing imaginal discs (Figure 2, E and F). Apparently higher localized transcript concentrations in the figure are due to folds in the monolayer of disc cells, doubling the apparent *Doa* transcript signal in these regions.

DOA protein expression during embryogenesis and in imaginal discs: DOA protein expression was examined via immunohistochemical staining with affinity-purified anti-DOA antiserum. Consistent with the pattern of transcript expression and pleiotropic phenotypes, DOA protein is widely expressed (Figure 3) in patterns nearly identical with the transcription patterns observed (*e.g.*, compare Figures 2C and 3B, and 2D and 3C). Unlike the segmental pattern of transcript accumulation imposed on a virtually ubiquitous background (Figure 2C), no segmental variation in the localization of DOA protein was observed (Figure 3B). At stage 17, expression of DOA in the CNS and brain is noticeably heavier than surrounding tissue (Figure 3D). Early embryos (precellular blastoderm) stained with affinity-purified antibod-

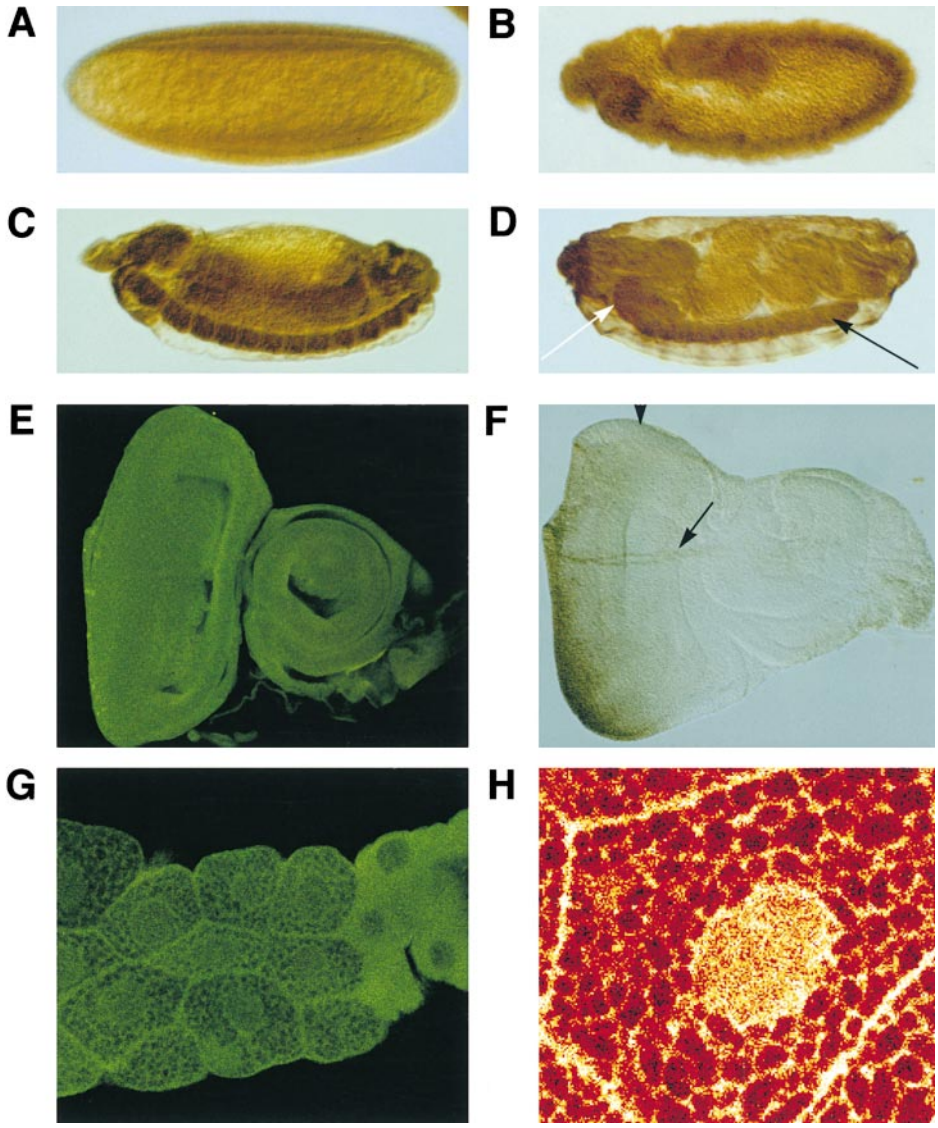

FIGURE 3.—Expression of DOA protein in embryonic and larval tissues. Immunocytochemical staining of DOA expression, as revealed with affinity-purified anti-DOA antibodies. A–D and F were revealed with DAB-coupled secondary antibody; E and G were revealed with FITC, and H with Cy5-coupled secondary antibodies and examined under epifluorescence. (A) Stage 5, 0- to 4-hr embryo, cellular blastoderm; (B) stages 10 and 11, germ band extension and gastrulation; (C) stage 13, germ band retracted; (D) stage 17, terminal embryogenesis. Note the heavily stained condensed nervous system (black arrow) and brain (white arrow). (E) Eye-antennal imaginal disc from late third instar larvae, revealed with a FITC-conjugated secondary antibody to demonstrate ubiquitous staining of the kinase; (F) a third instar eye-antennal imaginal disc stained, revealed with a DAB-conjugated secondary antibody, to demonstrate slightly higher levels of DOA expression posterior to the morphogenetic furrow (arrowhead) and presence of the kinase in Bolwig's nerve (arrow). (G) A portion of a late third instar larval salivary gland (anterior to the right). Note the nuclear and cell membrane staining in the large polytene cells, in contrast to the uniform cytoplasmic labeling and unlabeled nuclei at the anterior end of the gland. Also note the cytoplasmic "mesh" labeled in the posterior cells. This image was obtained using a confocal microscope, and labeled nuclei in some cells are out of the plane of focus and not revealed here. (H) An enlargement of a polytene salivary gland cell, using enhanced contrast to emphasize the cytoplasmic "meshwork" staining pattern.

ies reveal essentially cytoplasmic staining (not shown), but the higher affinity of the purified sera for the cytoplasmic 105-kD DOA isoform demonstrated above (Figure 1) may significantly reduce nuclear staining. Virtually identical embryonic staining patterns were observed using the crude anti-DOA serum, as well as the antipeptide (anti-LAMMER) serum described above, while preimmune sera revealed no labeling of embryos or discs (not shown), demonstrating the specificity of the signals as due to DOA.

DOA protein is ubiquitously expressed in imaginal discs (Figure 3E). Slightly higher DOA protein levels are observed in the differentiating third instar eye imaginal disc posterior to the morphogenetic furrow (Figure 3F), consistent with *Doa*'s demonstrated role in photoreceptor differentiation and maintenance. It should also be noted that relatively higher levels of DOA expression are observed in Bolwig's nerve (Figure 3F, arrow), which innervates the larval visual organ, demonstrating both that relatively higher levels of DOA are found in neural cells as well as the presence of the kinase in axonal cytoplasm.

Figure 3G shows a portion of a late third instar salivary gland stained with affinity-purified anti-DOA. Clear labeling of nuclei, the cell surface, and a cytoplasmic meshwork is observed, easily visible in a higher magnification view of a salivary gland cell (Figure 3H). This mesh may correspond to a component of the cytoskeleton, and the same pattern is revealed when these cells are labeled with antitubulin (R. FARKAŠ and L. RABINOW, unpublished results). However, these cells are producing high levels of glue protein at this stage, which

TABLE 2

Alleles and loci tested for interaction and complementation with *Doa*

A. Alleles tested for complementation of *Doa*

Locus	*Doa* alleles tested	Result
E(gl)3C (3-99)	*γ3B, HD, 105, DEM*	Viable and fertile. No suppression of w^a; eyes, wings, and bristles normal
l(3)01705 (98F4–5)	*γ3B, HD*	Lethal *in trans*; w^a suppressed
	105, Msu, Msu2, DEM	Partial complementation: rough eyes, w^a suppressed to wild type in *trans*-heterozygotes, duplicated scutellar bristles
Df(3R)3450 98E3–99A6–8	*γ3B, HD, EMS2, DEM*	Lethal *in trans*; w^a suppressed
	Msu2, Msu, RemγA	Partial complementation: rough eyes, w^a suppressed to wild type, duplicated scutellar bristles

B. Specific alleles tested for interactions with *Doa*

Locus/allele(s)	Tested with *Doa* heterozygotes	Tested with *Doa* heteroallelics	Su(w^a)?
$sina^1$	*γ3B, HD, DEM, 105, Msu2*	*HD/DEM, HD/105*	No
ras^{e2F}	*γ3B, HD, DEM, 105, Msu2*	*HD/DEM, HD/105*	No
sev^{11}, sev^{l2} [a]	*γ3B, HD, RemγA*		Not done
$svlf^{e22}$	*γ3B, HD, RemγA*		No
rl^1, rl^{Sem}	*γ3B, HD, RemγA, DEM*	*HD/DEM* *HD/105*[b]	No
Elp	*γ3B, HD, DEM, Msu2* *RemγA*	*HD/DEM*	No
R, Df (3L)R	*γ3B, HD, DEM, 105, Msu2*		No
gl^{60j}, gl^2 [c]	*HD, DEM, 105*	*HD/DEM, HD/105*	No

The indicated alleles were tested as heterozygotes (or hemizygotes or homozygotes, where indicated) for enhancement or suppression of *Doa* or other phenotypes. These alleles were combined with heterozygotes or heteroallelic combinations of the *Doa* alleles, as listed.

[a] Performed in hemizygous males.

[b] Roughness of *Sem* allele slightly increased in combination with *HD/105*, attributed to additivity of the two phenotypes.

[c] Also tested as *gl* homozygotes, heterozygous for Doa^{HD}.

is being stored in vacuoles prior to secretion. Thus the entire cytoplasm of the polytene salivary gland cells may be restricted to the "mesh" observed, and all its components might be erroneously thought to co-localize. Further experiments will be required to determine the origin of this mesh.

In contrast to the polytene glue-producing cells, the cytoplasm at the anterior end of the salivary gland in the imaginal ring is intensely and uniformly labeled. Staining in these cells is excluded from the nuclei, suggesting that the intracellular localization of DOA is regulated.

Identification of deletion and enhancer-trap *Doa* alleles: The deficiency chromosome *Df(3R) 3450* (98E3:99A6-8) was examined as a possible deletion of the *Doa* locus, while the *lacZ*-marked *P*-element-induced recessive lethal *l(3) 01705* at 98F1–4 from the Berkeley Drosophila Genome Project (Spradling *et al.* 1995) was examined as a possible insertion in the locus. Complementation tests as well as assays for suppression of $white^{apricot}$ confirm that both lesions reduce *Doa* function (Table 2A). A subset of the *Doa* alleles tested for complementation with both the deficiency and the *P* insertion produced individuals with typical extreme *Doa* phenotypes (rough eyes, suppression of $white^{apricot}$ to wild type, extra wing venation, missing bristles). Interestingly, several alleles defined as hypomorphs based on their ability to provide interallelic complementation failed to survive when combined with *Df(3R) 3450* (*HD*, *DEM*, *EMS2*), suggesting that they were unable to provide sufficient activity for viability during at least one critical developmental period.

β-Galactosidase activity in embryos and imaginal discs of the *l(3)01705* line was detected in only a small set of tissues. However, immunohistochemical staining with anti-β-galactosidase revealed widespread expression (not shown), consistent with the labeling observed with anti-DOA serum.

We also tested an enhancer of *glass*, *E(gl)3C*, which maps by recombination to the same location as *Doa* (Ma *et al.* 1996), as a potential allele. This enhancer was of special interest, since heteroallelism or homozygosity for some *Doa* alleles results in loss of all retinal photoreceptors (Yun *et al.* 1994), similar to the phenotype of *glass* mutants (Moses *et al.* 1989). However, no interaction with *Doa* or suppression of $w^{apricot}$ was observed with *E(gl)3C*, and we conclude that they are not allelic.

An essential role for *Doa* in the soma: To analyze the effects of complete loss of *Doa* function on the survival or differentiation of individual cell types and to determine whether *Doa* mutations act cell autonomously, homozygous mutant *Doa* clones were generated by mitotic recombination in the soma. Clonal analysis was performed using three alleles, $Doa^{\gamma 3B}$, Doa^{EMS2}, and Doa^{I5} (Table 1), which were chosen for reasons explained in MATERIALS AND METHODS.

When somatic recombination was induced in $Doa^{\gamma 3B}/+$ heterozygotes 25–36 hr after egg laying (AEL), no mutant clones were detected in either eyes or cuticles, although +/+ clones were recovered in mosaic eyes. The morphology of eyes and cuticles was normal in these individuals. Cell clones were therefore induced, but the cells did not produce adult cuticle. To determine whether homozygous cell clones would survive and affect the development of specific cell types if induced later during development, mitotic recombination was induced every 12 hr from early first instar to early pupal periods (7 days AEL). When somatic recombination was induced in 4- to 6-day-old larvae (late second through third instar), $\gamma 3B$ mosaic eyes containing +/+ clones were often rough. Sagittal sections of these mosaic eyes revealed randomly missing photoreceptors and pigment cells, although no loss of specific cell types was observed. Even in individuals with apparently normal eyes, sectioning revealed occasional defects in the organization of the retina, presumably due to death of homozygous mutant cells (Figure 4A, black arrow). Ommatidia near homozygous wild-type clones for *Doa* were often missing random photoreceptors (Figure 4A, white arrow). Flies were also recovered with one or more bristles missing in the triple row of the anterior wing margin (Figure 4B), suggesting that genetic ablation of one or more of the progenitor cells in the lineage leading to these bristles had occurred on clone induction. Thus, no $Doa^{\gamma 3B}$ homozygous clones were detected in mosaic eyes or cuticles, indicating that mutant cell clones induced even late in third instar periods die.

To determine how quickly the putative cell death occurred in $Doa^{\gamma 3B}/Doa^{\gamma 3B}$ homozygous cells, develop-

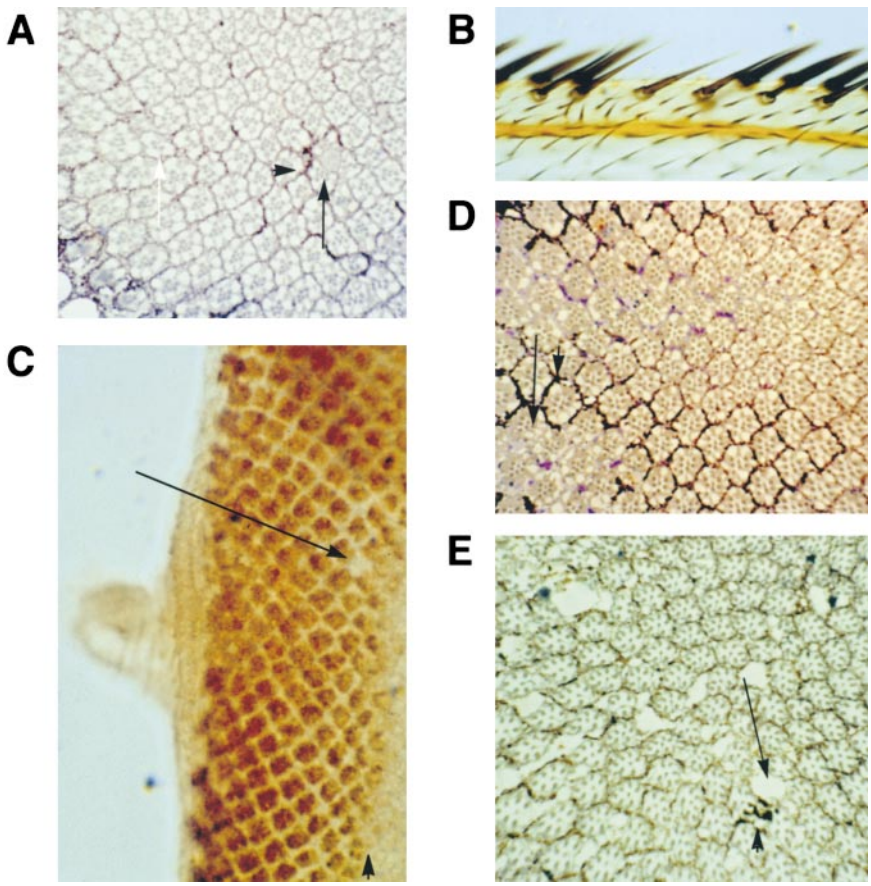

FIGURE 4.—Somatic mosaic analysis demonstrates *Doa* activity is essential for cellular survival. Mosaic cell clones marked in repulsion with $white^{+}$ were generated in backgrounds heterozygous for *Doa* alleles ($\gamma 3B$, *EMS2*, and *I5*), as described. (A) Sagittal section, showing several wild-type clones induced during third instar larval development. One of these is marked with an arrowhead and is recognized as having a higher density of pigment granules than the heterozygous cells, which are the majority visible in this section. No $Doa^{\gamma 3B}$ clone (which would be lacking all pigment granules) is adjacent to the wild-type cells. Note the disorganized area adjacent to the wild-type clone, presumably due to death of the $\gamma 3B$ cell twinspot clone. Also note (white arrowhead) ommatidia lacking one or more photoreceptors, again presumably due to loss of $\gamma 3B$ homozygous cells. (B) The anterior wing margin of a $\gamma 3B/+$ individual in which clones were induced during late third instar/early pupal periods and in which $white^{+}$ clones were observed in the eye. Note loss of approximately two anterior wing margin bristles. These were presumably due to death of $Doa^{\gamma 3B}$ homozygous cells. (C) Immunohistochemical staining of a $Doa^{\gamma 3B}/+$ eye imaginal disc, following mitotic recombination induced in 6-day-old third instar larvae. Discs were dissected 2 hr later and stained with the neuronal anti-ELAV antibody. The missing cluster of differentiating photoreceptors (arrow) is a presumptive homozygous cell clone and is two rows of cells posterior to the morphogenetic furrow (arrowhead). The entire photoreceptor cluster is missing, perhaps due to ablation of the R8 photoreceptor precursor at a time when it could no longer be replaced. Other discs showed only single missing photoreceptor precursors. (D) Sagittal section of a $Doa^{EMS2}/+$ eye containing both *Doa* mutant ($white^{-}$, arrow) and wild-type ($white^{+}$, arrowhead) clones. The *EMS2* mutant clones possess normal organization. (E) Sagittal section of an eye containing wild-type *white* clones induced in a 4- to 5-day-old larva (*e.g.*, arrowhead), accompanied by cell loss of Doa^{I5} homozygous cells. The random disorganization of retinal organization due to death of the *Doa* homozygous cells is similar to that found in *Doa* heteroallelic flies.

ment in mosaic eye discs was examined using a monoclonal antibody recognizing the nuclear neuronal antigen ELAV, which is expressed immediately posterior to the morphogenetic furrow in eye imaginal discs (Robinow and White 1988). Mitotic recombination was induced in 6-day-old larvae, and eye imaginal discs were dissected 2–3 hr later. In 63 eye imaginal discs potentially containing $Doa^{\gamma 3B}/Doa^{\gamma 3B}$ mitotic clones, one or more photoreceptor precursor cells were missing in four (Figure 4C, arrow), one or two rows behind the morphogenetic furrow (Figure 4C, arrowhead), as revealed by lack of ELAV expression interrupting the ordered array of differentiating neuronal cells. The ordered array of photoreceptor clusters was seen without any such defects in an equal number of heat-shocked wild-type eye disc controls (not shown). This finding suggests that homozygous mutant *Doa* photoreceptor precursor cells died almost immediately following clone induction, or were unable to differentiate as neuronal photoreceptor precursors, since the morphogenetic furrow requires roughly 2 hr to generate each row.

When mitotic recombination was induced in 5- to 6-day-old larvae heterozygous for Doa^{I5}, no *white*$^-$ (*i.e.*, homozygous Doa^-) clones were detected in the eye. Moreover, mosaic eyes containing +/+ clones were rough. Sagittal eye sections of these animals show disruptions in the ommatidial pattern (Figure 4E, arrow), near homozygous wild-type cell clones (Figure 4E, arrowhead), identical to the defects observed in *Doa* heteroallelic flies (Yun *et al.* 1994). Occasional anterior wing-margin bristles were also missing (6/98), when mitotic recombination was induced in 4- to 6-day-old Doa^{I5} heterozygous larvae, again suggesting that these disruptions were due to the induction of somatic *Doa* clones in bristle progenitor cells, which then died.

In contrast to the results with the *γ3B* and *I-5* alleles, homozygous cell clones of the *EMS2* allele were recovered in eyes and cuticles when mitotic recombination was induced during first instar and later larval periods. Sagittal eye sections containing these clones show essentially normal retinal organization (*e.g.*, Figure 4D, arrow), adjoining homozygous wild-type clones (Figure 4D, arrowhead). Cuticular *EMS2* clones marked with *ebony* were also recovered (not shown), demonstrating survival of *EMS2* homozygous cells without developmental repercussions. On the basis of the normal appearance of clones both in the cuticle and eye, we conservatively conclude that this allele produces homozygous viable and normal mutant *Doa* clones, probably due to being a hypomorphic as opposed to a null allele.

Synthesizing the data obtained with the *γ3B*, *I5*, and *EMS2* alleles, *Doa* appears necessary for the proper development of all cuticular structures, including the eye, wing, and thorax. This result suggests that the gene is essential for cell viability and is consistent with its ubiquitous expression in embryos and imaginal discs.

***Doa* is essential in the female germline:** A maternal contribution of *Doa* function to the embryo had been deduced from the observation that reciprocal crosses between *Doa* alleles yielding heteroallelic adults produce different percentages of *trans*-heterozygous adult escapers (Rabinow *et al.* 1993), as well as the ability of homozygous embryos from heterozygous females to hatch, while those derived from heteroallelic females are inviable (Yun *et al.* 1994). Finally, RNAs from both 0- to 4-hr-old embryos and ovaries include a large complement of the 2.7-kb and a 1.9-kb *Doa* transcript, and so these RNAs are maternally contributed to the developing oocyte (Yun *et al.* 1994).

To analyze the effects of homozygosity for *Doa* mutations, we induced germline clones to eliminate all maternal contributions (Perrimon 1984). We used $ovo^{D1,11.3b}$ carried on chromosome 3R (Mével-Ninio *et al.* 1994), which was crossed as males to adult female $Doa^{\gamma 3B}$ heterozygotes. Progeny of this cross were irradiated as first instar larvae. Adult female $Doa^{\gamma 3B}/P[w^+, ovo^{D1,11.3b}]$ deriving from the irradiated progeny were crossed with $Doa^{\gamma 3B}/TM3$ males. Among 290 females crossed in 21 vials, females in only 1 vial laid eggs, and these developed normally. These progeny were probably due to leakiness of the $ovo^{D1,11.3b}$ allele (Mével-Ninio *et al.* 1994), since control irradiations generated germline recombinants at much higher rates (see materials and methods). Dissection of 72 of the irradiated $Doa^{\gamma 3B}/P[w^+, ovo^{D1,11.3b}]$ females revealed four individual ovaries even more rudimentarily developed than the minimal development observed in $P[w^+, ovo^{D1,11.3b}]$ heterozygotes, presumably due to the existence of *Doa* clones and the necessity of their activity for development of the germline. Therefore, stem cell clones homozygous for $Doa^{\gamma 3B}$ were generated by X-ray irradiation, but were unable to produce eggs due to a requirement for *Doa* function.

This finding was confirmed by the Perrimon laboratory with a second *Doa* allele during screens for zygotic lethals with maternal-effect phenotypes (Perrimon *et al.* 1996). In these screens, the *l(3) 01705* allele (allelic to *Doa*; see above) was examined for effects on embryonic development in germline clones. No eggs were laid by individuals carrying germline clones homozygous for this allele (N. Perrimon, personal communication). This observation confirms our analysis using the *γ3B* allele and demonstrates that even residual levels of *Doa* activity are insufficient to support normal oogenesis, since the survival of some heteroallelic *Doa* combinations with *l(3) 01705* demonstrates it is a hypomorph (Table 2).

***Doa* acts independently of the *ras*-MAP kinase pathway:** Adult heteroallelic and homozygous *Doa* mutants of various classes display a number of phenotypes similar to those of mutations in components of the *ras*-MAP-K pathway (Dickson *et al.* 1992; Briggs *et al.* 1994; Brunner *et al.* 1994; Diaz-Benjumea and Hafen 1994), which involves signal transduction in part through a cascade of several highly conserved protein kinases (*e.g.*, Neiman 1993; Tsuda *et al.* 1993). As a first attempt at identifying

potential regulators or additional substrates of DOA kinase, we determined whether *Doa* alleles interact with mutations in the *ras*-MAP-K pathway as well as a few loci with related phenotypes. Specific loci, alleles, and combinations tested are listed in Table 2B. The rationale and crosses are described in MATERIALS AND METHODS.

Ras-MAP-K pathway components tested included hypomorphic or null alleles of *ras*, *rolled* (MAP kinase), *sevenless*, *seven-up*, and *sina*. Other hypomorphic or null alleles tested included *glass* and a deficiency including *Roughened*, a GTP-binding protein homologous to mammalian rap (HARIHARAN *et al.* 1991). Hypermorphic alleles of *DER* (*Elp*, the *Drosophila* EGF receptor homologue), *rolled*, *sevenless*, and *Roughened* were also tested. In each case, these mutations were genetically combined as heterozygotes or, where indicated, as homozygotes, with the *Doa* alleles listed in Table 2B. Heteroallelic *Doa* combinations were additionally generated (Table 2B), with heterozygous mutants in the *ras* cascade (Table 2B), to reduce *Doa* activity to minimal levels for examination of modification of *Doa* and *ras*-MAP-K phenotypes. In no case was enhancement or suppression of *Doa* or other phenotypes observed, suggesting that DOA and, by extension, the other LAMMER kinases function independently of the *ras* pathway. It should be noted that several of the same *Doa* alleles that fail to interact with *ras* pathway components interact to various extents with other mutations affecting eye and imaginal development (C. DU and L. RABINOW, unpublished results), as well as with those influencing sex determination (DU *et al.* 1998).

DISCUSSION

The ubiquitous expression of *Doa* mRNA and DOA protein is consistent with the essential role for activity of the gene demonstrated in clonal analysis of both somatic and germline tissues. The finding of low but detectable levels of the kinase in all cells of the eye-imaginal disc, with increased expression posterior to the morphogenetic furrow, is also consistent with previously described phenotypes of *Doa* mutants. These observations both lend support to the hypothesis that DOA kinase is essential for the initiation and maintenance of cellular differentiation, but the exact pathway(s) implicated remain to be identified.

Developmental profiles on immunoblots demonstrate that DOA protein is expressed as 55- and 105-kD isoforms throughout development in all tissues examined. One or both isoforms are reduced in quantity in various mutants, verifying their origin as the *Doa* locus. These isoforms are not interconvertible by treatment with urea or phosphatase, suggesting that they possess different primary structures. The 55-kD protein is essentially nuclear, while the 105-kD protein is cytoplasmically restricted, as demonstrated on immunoblots of fractionated pupal extracts. Expression of the 105-kD protein in early embryos and ovaries suggests that DOA protein as well as RNA is maternally contributed to the developing oocyte. Further studies are required to examine the dynamics of subcellular localization of the 55-kD isoform during development and in different tissues. Determination of the structure and kinase activity of the 105-kD isoform will provide further insight into DOA function, but even this level of analysis will not reveal where and when the kinase is catalytically active. The observation that expression of both kinase isoforms is essentially equal between the sexes suggests that post-translational regulation of *Doa* activity may occur to influence sex-specific splicing, since *Doa* alleles affect sexual differentiation and splicing of *dsx*.

In support of our observations of the existence of multiple DOA isoforms, the Berkeley Drosophila Genome Project (BDGP; see http://www.fruitfly.org/) has recovered multiple DOA cDNAs (clot 2594), as well as sequenced the entire gene (ADAMS *et al.* 2000). Three "subclots" have been recognized. One (subclot 2) corresponds to the cDNAs we reported (YUN *et al.* 1994) and includes additional exons and introns making up a 5′ untranslated region. The structure of the RNAs of this subclot suggests that the second Met codon identified in sequencing the genomic DNA adjoining the 5′ end of our near-full-length cDNA is the beginning of the open reading frame (ORF). A second subclot identified by BDGP (subclot 1) has the capacity to code for a slightly longer version of DOA protein, with the use of an alternative 5′ exon, increasing the coding capacity of these cDNAs by ~70 amino acids at the N terminus of the protein. Whether the mRNAs corresponding to this subclot account for one or more of the additional signals occasionally seen on our immunoblots requires further analysis. However, based on the size of the potential protein product, it would seem unlikely that the mRNA defining subclot 1 is responsible for the 105-kD protein observed. The third subclot listed at BDGP is represented by a single, apparently partial cDNA, since alignment of its putative translation product demonstrates that it lacks virtually all sequences N-terminal to the kinase catalytic domain.

Preliminary analysis of the Berkeley genomic *Doa* sequence has identified several putative ORFs within the large introns of the gene, which is at least ~40 kb in size, on the basis of the span of exons of the mRNA encoding the 55-kD protein. At least one of these appears to be expressed, since we recently recovered a cDNA including one of these alternative exons (L. CHANEY, B. YUN and L. RABINOW, unpublished reults).

Our studies suggest that the kinase is localized to specific cytoplasmic structures, *i.e.*, a mesh and the cell surface in the polytene cells of the salivary gland. DOA is excluded from the nuclei of specific cells in the salivary gland, further suggesting the intriguing possibility that intracellular localization of the kinase is regulated. The generation of antibodies specific for the two different DOA isoforms, as well as additional staining of embry-

onic and larval tissues, will be necessary to further examine the significance of these observations. One obvious implication is that DOA participates in the transmission of signals from the cytoplasm to the nucleus.

Murine Clk1, which encodes a LAMMER kinase, expresses multiple transcripts (Ben-David *et al.* 1991; Howell *et al.* 1991). One of these directs synthesis of a protein colinear with the 55-kD DOA protein (Yun *et al.* 1994). However, in contrast to DOA, the subcellular localization of an epitope-tagged Clk1 protein in transfected cells revealed it only in nuclei (Duncan *et al.* 1995). Other studies in cultured cells using antipeptide antisera directed against C-terminal sequences of four different murine LAMMER kinases, or the anti-LAMMER sera described here, also demonstrated their nuclear localization, with insignificant cytoplasmic labeling (Nayler *et al.* 1997; K. Lee and L. Rabinow, unpublished results). However, in recent reports, mammalian CLK1 and CLK2 were found to phosphorylate and activate a cytoplasmic protein tyrosine phosphatase when coexpressed in cultured cells (Moeslein *et al.* 1999), and CLK3 was also described in the cytoplasm of spermatozoa (Menegay *et al.* 1999). These observations support our observation of cytoplasmic LAMMER kinase. Immunoblot analyses were not reported in these studies, so the number and size(s) of Clk isoforms remain to be determined. Moreover, since cultured cells were used, developmental regulation of nuclear localization of the kinases could not be examined.

The differential function(s) of the two DOA isoforms, if any, have yet to be identified. It is clear that LAMMER kinases phosphorylate SR proteins, both *in vitro* (Colwill *et al.* 1996a,b; Nayler *et al.* 1997) and *in vivo* (Du *et al.* 1998), affecting developmentally regulated alternative splicing. The existence of a cytoplasmic DOA isoform suggests that additional processes are also targets of LAMMER kinase function.

The similarities in phenotypes between *Doa* alleles and mutations in various loci encoding proteins in the MAP kinase cascade, as well as a report suggesting that the murine LAMMER kinase Clk1 influenced MAP kinase pathway activity (Myers *et al.* 1994), led us to examine whether genetic interactions existed. The fact that no interactions were found in our study, in combination with the lack of the isolation of *Doa* alleles in several large screens for modifiers of Ras-MAP-K pathway components (*e.g.*, Dickson *et al.* 1996; Karim *et al.* 1996; Neufeld *et al.* 1998; Rebay *et al.* 2000), led us to conclude that the pathway(s) in which LAMMER kinases function is separate from that of Ras-MAP kinase.

In summary, our data reveal the necessity of DOA activity for cell survival and differentiation. The existence of multiple protein isoforms with localization to specific intracellular compartments and structures raises the distinct possibility that DOA and the other LAMMER kinases possess multiple functions in the relay of as yet unidentified signals during development, to regulate vital cellular processes in addition to the regulation of alternative splicing of pre-mRNAs.

We especially thank Lee Simon for generous help and instruction in tissue sectioning and Dick Leidich for his expertise and assistance with the X-ray machine. Thanks are also due to Maryvonne Mevel-Ninio, who generously provided the P[*ovo*^{D}] on 3R stock prior to publication; Norbert Perrimon for communicating unpublished data; Marie-Laure Samson for the gift of anti-ELAV antibody and helpful suggestions throughout this work; Ernst Hafen, Kevin Moses, and Yosh Hiromi for Drosophila stocks; and Dale Dorsett for the pupal nuclear preparation protocol. We also benefited from the availability of both genomic and EST clone sequences made accessible by the Berkeley Drosophila Genome Project. Cheng Du generously provided the Western blot of DOA protein in extracts of sexed Drosophila pupae. Additional Drosophila stocks were also provided by the Bowling Green and Indiana Drosophila stock centers. This work was supported by grants to L.R. from the National Science Foundation, the Margaret Colyer Research Fund of the Fight for Sight—Research Division of Prevent Blindness America, and the New Jersey Commission on Cancer Research. R.F. was supported in part by grants VEGA 95/5305/272 and SO 95/5305/043 from the Slovak Grant Agency for Science and a supplement from the office for Central European Programs, U.S. National Science Foundation.

LITERATURE CITED

Adams, M. D., S. E. Celniker, R. A. Holt, C. A. Evans, J. D. Gocayne *et al.*, 2000 The genome sequence of *Drosophila melanogaster*. Science **287:** 2185–2194.

Ashburner, M., 1989a *Drosophila: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ashburner, M., 1989b *Drosophila: A Laboratory Handbook*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman *et al.*, 1989 *Current Protocols Molecular Biology*. Greene/Wiley Publishing, New York.

Ben-David, Y., K. Letwin, L. Tannock, A. Bernstein and T. Pawson, 1991 A mammalian protein kinase with potential for serine/threonine and tyrosine phosphorylation is related to cell cycle regulators. EMBO J. **10:** 317–325.

Briggs, W. H., K. H. Zavitz, B. Dickson, A. van der Straten, D. Brunner *et al.*, 1994 The *Drosophila rolled* locus encodes a MAP kinase required in the *sevenless* signal transduction pathway. EMBO J. **13:** 1628–1635.

Brunner, D., N. Oellers, J. Szabad, W. H. I. Biggs, S. L. Zipursky *et al.*, 1994 A gain of function mutation in *Drosophila* MAP kinase activates multiple receptor tyrosine kinase signalling pathways. Cell **76:** 875–888.

Campos-Ortega, J. A., and V. Hartenstein, 1997 *The Embryonic Development of Drosophila melanogaster*. Springer, Berlin.

Cao, W., S. F. Jamison and M. A. Garcia-Blanco, 1997 Both phosphorylation and dephosphorylation of ASF/SF2 are required for pre-mRNA splicing in vitro. RNA **3:** 1456–1467.

Colwill, K., L. L. Feng, J. M. Yeakley, G. D. Gish, J. F. Caceres *et al.*, 1996a SRPK1 and Clk/STY protein kinases show distinct substrate specificities for serine/arginine-rich splicing factors. J. Biol. Chem. **271:** 24569–24575.

Colwill, K., T. Pawson, B. Andrews, J. Prasad, J. L. Manley *et al.*, 1996b The Clk/STY protein kinase phosphorylates SR splicing factors and regulates their intranuclear distribution. EMBO J. **15:** 265–275.

Cox, K. H., D. V. DeLeon, L. M. Angerer and R. C. Angerer, 1984 Detection of mRNA in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. Dev. Biol. **101:** 485–502.

Diaz-Benjumea, F., and E. Hafen, 1994 The sevenless signaling cassette mediates *Drosophila* EGF receptor function during epidermal development. Development **120:** 569–578.

Dickson, B., F. Sprenger, D. Morrison and E. Hafen, 1992 Raf functions downstream of RAS1 in the sevenless signal transduction pathway. Nature **360:** 600–603.

Dickson, B. J., A. van der Straten, M. Dominguez and E. Hafen,

1996 Mutations modulating raf signaling in Drosophila eye development. Genetics **142:** 163–171.

Dorsett, D., 1990 Potentiation of a polyadenylation site by a downstream protein-DNA interaction. Proc. Natl. Acad. Sci. USA **87:** 4373–4377.

Du, C., M. E. McGuffin, B. Dauwalder, L. Rabinow and W. Mattox, 1998 Protein phosphorylation plays an essential role in the regulation of alternative splicing and sex determination in Drosophila. Mol. Cell **2:** 741–750.

Duncan, P. I., B. W. Howell, R. M. Marius, S. Drmanic, E. M. J. Douville *et al.*, 1995 Alternative splicing of STY, a nuclear dual specificity kinase. J. Biol. Chem. **270:** 21524–21531.

Gui, J. F., W. S. Lane and X. D. Fu, 1994a A serine kinase regulates intracellular localization of splicing factors in the cell cycle. Nature **369:** 678–682.

Gui, J. F., H. Tronchere, S. D. Chandler and X. D. Fu, 1994b Purification and characterization of a kinase specific for the serine and arginine-rich pre mRNA splicing factors. Proc. Natl. Acad. Sci. USA **91:** 10824–10828.

Hariharan, I. K., R. W. Carthew and G. M. Rubin, 1991 The Drosophila *Roughened* mutation: activation of a *rap* homolog disrupts eye development and interferes with cell determination. Cell **67:** 717–722.

Howell, B. W., D. E. H. Afar, J. Lew, E. M. J. Douville, P. L. E. Iceley *et al.*, 1991 STY, a tyrosine-phosphorylating enzyme with sequence homology to serine/threonine kinases. Mol. Cell. Biol. **11:** 568–572.

Karim, F. D., H. C. Chang, M. Therrien, D. A. Wassarman, T. Laverty *et al.*, 1996 A screen for genes that function downstream of Ras1 during *Drosophila* eye development. Genetics **143:** 315–329.

Kuroyanagi, N., H. Onogi, T. Wakabayashi and M. Hagiwara, 1998 Novel SR-protein-specific kinase, SRPK2, disassembles nuclear speckles. Biochem. Biophys. Res. Commun. **242:** 357–364.

Lee, K., C. Du, M. Horn and L. Rabinow, 1996 Activity and autophosphorylation of LAMMER protein kinases. J. Biol. Chem. **271:** 27299–27303.

Ma, C., H. Liu, Y. Zhou and K. Moses, 1996 Identification and characterization of autosomal genes that interact with *glass* in the developing *Drosophila* eye. Genetics **142:** 1199–1213.

Manley, J. L., and R. Tacke, 1996 SR proteins and splicing control. Genes Dev. **10:** 1569–1579.

Menegay, H., F. Moeslein and G. Landreth, 1999 The dual specificity protein kinase CLK3 is abundantly expressed in mature mouse spermatozoa. Exp. Cell Res. **253:** 463–473.

Mével-Ninio, M., I. Guénal and B. Limbourg-Bouchon, 1994 Production of dominant sterility in *Drosophila melanogaster* by insertion of the ovo^{p1} allele on autosomes: use of transformed strains to generate germline mosaics. Mech. Dev. **45:** 155–162.

Misteli, T., and D. L. Spector, 1997 Protein phosphorylation and the nuclear organization of pre-mRNA splicing. Trends Cell Biol. **7:** 135–138.

Moeslein, F. M., M. P. Myers and G. E. Landreth, 1999 The CLK family kinases CLK1 and CLK2 phosphorylate and activate the tyrosine phosphatase PTP-1B. J. Biol. Chem. **274:** 26697–26704.

Moses, K., M. C. Ellis and G. M. Rubin, 1989 The *glass* gene encodes a zinc-finger protein required by *Drosophila* photoreceptor cells. Nature **340:** 531–536.

Myers, M. P., M. B. Murphy and G. Landreth, 1994 The dual-specificity Clk kinase induces neuronal differentiation of PC12 cells. Mol. Cell. Biol. **14:** 6954–6961.

Nayler, O., S. Stamm and A. Ullrich, 1997 Characterization and comparison of four serine- and arginine-rich (SR) protein kinases. Biochem. J. **326:** 693–700.

Neiman, A. M., 1993 Conservation and reiteration of a kinase cascade. Trends Genet. **9:** 390–394.

Neufeld, T. P., A. H. Tang and G. M. Rubin, 1998 A genetic screen to identify components of the sina signaling pathway in Drosophila eye development. Genetics **148:** 277–286.

Padmanabha, R., S. Gehrung and M. Snyder, 1991 The *KNS1* gene of *Saccharomyces cerevisiae* encodes a nonessential protein kinase homologue that is distantly related to members of the *CDC28/cdc2* gene family. Mol. Gen. Genet. **229:** 1–9.

Perrimon, N., 1984 Clonal analysis of dominant female-sterile, germline-dependent mutations in *Drosophila melanogaster*. Genetics **108:** 927–939.

Perrimon, N., A. Lanjuin, C. Arnold and E. Noll, 1996 Zygotic lethal mutations with maternal effect phenotypes in *Drosophila melanogaster*. II. Loci on the second and third chromosomes identified by *P*-element-induced mutations. Genetics **144:** 1681–1692.

Pfeifle, C., and D. Tautz, 1989 A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma **98:** 81–85.

Rabinow, L., and J. A. Birchler, 1989 A dosage-sensitive modifier of retrotransposon induced alleles of the *Drosophila white* locus. EMBO J. **8:** 879–889.

Rabinow, L., S. L. Chiang and J. A. Birchler, 1993 Mutations at the *Darkener of apricot* locus modulate transcript levels of *copia* and *copia*-induced mutations in *Drosophila melanogaster*. Genetics **134:** 1175–1185.

Rebay, I., F. Chen, F. Hsiao, P. Kolodziej, B. H. Kuang *et al.*, 2000 A genetic screen for novel components of the Ras/Mitogen-activated protein kinase signaling pathway that interact with the *yan* gene of Drosophila identifies *split ends*, a new RNA recognition motif-containing protein. Genetics **154:** 695–712.

Robinow, S. N., and K. White, 1988 The locus *elav* of *Drosophila melanogaster* is expressed in neurons at all developmental stages. Dev. Biol. **126:** 294–303.

Savaldi-Goldstein, S., G. Sessa and R. Fluhr, 2000 The ethylene-inducible PK12 kinase mediates the phosphorylation of SR splicing factors. Plant J. **21:** 91–96.

Sessa, G., V. Raz, S. Savaldi and R. Fluhr, 1996 PK12, a plant dual-specificity protein kinase of the LAMMER family, is regulated by the hormone ethylene. Plant Cell **8:** 2223–2234.

Spradling, A. C., D. Stern, I. Kiss, J. Roote, T. Laverty *et al.*, 1995 Gene disruptions using P transposable elements: an integral component of the Drosophila genome. Proc. Natl. Acad. Sci. USA **92:** 10824–10830.

Tacke, R., Y. Chen and J. L. Manley, 1997 Sequence-specific RNA binding by an SR protein requires RS domain phosphorylation: creation of an SRp40-specific splicing enhancer. Proc. Natl. Acad. Sci. USA **94:** 1148–1153.

Tam, J. P., 1988 Synthetic peptide vaccine design: synthesis and properties of a high density multiple antigen peptide system. Proc. Natl. Acad. Sci. USA **85:** 5409–5413.

Tang, Z., M. Yanagida and R. J. Lin, 1998 Fission yeast mitotic regulator Dsk1 is an SR protein-specific kinase. J. Biol. Chem. **273:** 5963–5969.

Tsuda, L., Y. H. Inoue, M. A. Yoo, M. Mizuno, M. Hata *et al.*, 1993 A protein kinase similar to MAP kinase activator acts downstream of the raf kinase in Drosophila. Cell **72:** 407–414.

Valcarcel, J., and M. R. Green, 1996 The SR protein family: pleiotropic functions in pre-mRNA splicing. Trends Biochem. Sci. **21:** 296–301.

Wang, H. Y., W. Lin, J. A. Dyck, J. M. Yeakley, Z. Songyang *et al.*, 1998 SRPK2: a differentially expressed SR protein-specific kinase involved in mediating the interaction and localization of pre-mRNA splicing factors in mammalian cells. J. Cell Biol. **140:** 737–750.

Xiao, S. H., and J. L. Manley, 1997 Phosphorylation of the ASF/SF2 RS domain affects both protein-protein and protein-RNA interactions and is necessary for splicing. Genes Dev. **11:** 334–344.

Xu, T., and G. M. Rubin, 1993 Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development **117:** 1223–1237.

Yun, B., R. Farkas, K. Lee and L. Rabinow, 1994 The *Doa* locus encodes a member of a new protein kinase family, and is essential for eye and embryonic development in *Drosophila melanogaster*. Genes Dev. **8:** 1160–1173.

Communicating editor: J. A. Birchler