The *Doa* locus encodes a member of a new protein kinase family and is essential for eye and embryonic development in *Drosophila melanogaster*

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Mutations at the *Darkener of apricot* (*Doa*) locus of *Drosophila* cause roughened eyes and increase transcript accumulation from the retrotransposon *copia* up to fourfold. Cloning of the gene and sequencing of cDNAs reveals that it encodes a putative serine/threonine protein kinase. Sequence data base searches identify it as a member of a novel highly conserved protein kinase family, with homologs in humans, mice, and *Saccharomyces cerevisiae*, not related to each other previously. Family members are characterized by a peptide motif reading EHLAMMERILG at kinase subdomain X, which is virtually 100% identical in all homologs. We therefore refer to this new family as the LAMMER protein kinases. As predicted from its primary sequence, *Doa* protein possesses intrinsic protein kinase activity when expressed in bacteria, as assayed via autophosphorylation. The gene is expressed throughout development, and both stage and tissue-specific RNAs are found. Its function is essential, because maternally deposited or zygotically transcribed mRNA is required for development to larval stages, and defects in segmentation and development of the nervous system are observed in embryos derived from heteroallelic mothers. *Doa* function is also critical to *Drosophila* eye development, because the organization and development of pigment cells, bristles, and photoreceptors are affected in various mutant classes. In the most extreme cases that survive to adulthood, retinal photoreceptors degenerate prior to eclosion. These results demonstrate that the kinase encoded by *Doa* is required at multiple stages of development, for both differentiation and maintenance of specific cell types.

[Key Words: *Drosophila*; *Doa*; protein kinase; LAMMER; eye development]

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Families of conserved protein kinases, with homologs in eukaryotic organisms ranging from yeast to man, regulate crucial cellular processes such as the cell cycle, differentiation, and signal transduction (e.g. Neigeborn and Mitchell 1991; Woodgett 1991; Meyerson et al. 1992; Pelech and Sanghera 1992; Crews and Erikson 1993; Kosako et al. 1991; Neiman 1993; Puziss et al. 1994). In several instances, kinases with high levels of amino acid conservation have been proven to possess functional as well as structural homology, by complementation of mutations in yeast (Jimenez et al. 1990; Lehner and O'Farrell 1990; Leopold and O'Farrell 1991; Ninomiya-Tsuji et al. 1991; Hughes et al. 1993) or *Drosophila* (Siegfried et al. 1992).

Protein kinases are grouped into phylogenetically related families, based upon their amino acid sequences (Hanks et al. 1988; Hanks and Quinn 1991). These families are broadly classified into those that phosphorylate tyrosine residues or those that phosphorylate serine/threonine residues. Kinases in these two classes possess specific amino acid residues within their catalytic domains, allowing the classification of untested members as either tyrosine or serine/threonine kinases. Recently, however, a small number of protein kinases, classified as serine/threonine kinases, based on sequence, autophosphorylate on both serine/threonine and tyrosine residues (for review, see Lindberg et al. 1992). Two members of this "dual-specificity" kinase class, *weel* (Featherstone and Russell 1991; Parker et al. 1992) and MAPK-K (Nakielny et al. 1992), also phosphorylate exogenous substrates on serines/threonines and tyrosines, in addition to autophosphorylating on these residues. However, nim1 protein kinase, which autophosphorylates in vitro with dual specificity, apparently does not phosphorylate its substrate, *weel*, on tyrosines (Parker et al. 1993; Wu and Russell 1993). Thus, further studies on additional dual-specificity kinases are required to understand their function and the significance of their unusual specificity.

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We are investigating the function of the Darkener of apricot (Doa) locus of Drosophila. Doa mutants were isolated in screens for dosage-sensitive modifiers of white\textsuperscript{apricot} (w\textsuperscript{a}) (Rabinow and Birchler 1989). Because this allele is caused by the insertion of the copia retrotransposon into the second intron of the white (w) gene (Bingham and Judd 1981), many loci isolated in screens for modifiers of w\textsuperscript{a} interact specifically with the copia element (Birchler et al. 1989; Rabinow and Birchler 1990). Doa is one of these (Rabinow and Birchler 1989).

Unlike the majority of modifiers of retrotransposon-induced mutations in Drosophila, Doa alleles are dominant in their suppression of w\textsuperscript{a} (Rabinow and Birchler 1989) and at least two other copia-induced mutations (Rabinow and Birchler 1990, Rabinow et al. 1993). Mutant Doa alleles elevate copia transcript accumulation, probably because of increased rates of copia-specific transcription (Rabinow et al. 1993). The product of Doa has a vital role in the fly, because most mutant alleles are recessive lethal. The requirement for Doa function was characterized previously as occurring during early larval stages. However, complex complementation patterns among alleles allow occasional trans-heterozygotes to escape lethality; their survival rate depends on the direction of the cross producing them, demonstrating that Doa function is maternally contributed to the oocyte (Rabinow et al. 1993). Adult trans-heterozygotes display roughened eye morphology and are also frequently missing bristles on the scutellum and head capsule, suggesting a role for Doa in the development of these structures.

These intriguing phenotypes prompted us to examine the role of Doa in Drosophila development more closely and to characterize the gene molecularly. We report here that segmentation and nervous system defects are observed in homozygous mutant embryos derived from heteroallelic mothers. In rare homozygous adult mutants escaping lethality, photoreceptor cells degenerate prior to eclosion. Molecular cloning of Doa reveals that it is expressed in developmentally regulated patterns, including stage- and tissue-specific transcripts. The gene encodes a protein kinase, with highly conserved homologs in humans, mice, Arabidopsis thaliana, and the yeast Saccharomyces cerevisiae, which were not recognized previously as being related to each other. The murine homolog is a dual-specificity protein kinase, suggesting that other family members may also possess this property. Finally, when expressed as a fusion in Escherichia coli, Doa protein autophosphorylates, demonstrating that it possesses intrinsic protein kinase activity.

**Results**

**Retinal photoreceptors are missing in rare Doa homozygotes**

We had observed previously that the highly ordered ommatidial pattern of wild-type Drosophila (Fig. 1A,B), is disorganized in Doa heteroallelic individuals (Fig. 1C,D; Rabinow and Birchler 1989). Detailed observations of these defects reveal ommatidial individuals (Fig. 1C,D; Rabinow and Birchler 1989). Detailed observations of these defects reveal ommatidial of varying size, as well as disorganization of the interommatidial bristles (Fig. 1D). These defects are drastically increased in rare adults escaping lethality in homozygotes of two unusual alleles,

![Figure 1](genesdev.cshlp.org)
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Doa<sup>Msu2</sup> and Doa<sup>vA</sup> [Fig. 1E,F]. These alleles are almost certainly not null, and we presume that the rare homozygotes possess low levels of Doa function, allowing their survival.

The superficial ommatidial disorganization observed in Doa mutants suggested that the gene might be involved in the development of one or more specific cell types. We therefore examined sagittal and transverse sections from flies of the genotypes shown in Figure 1. These reveal defects in both photoreceptors and pigment cells (Fig. 2). Sagittal eye sections of heteroallelic Doa adults reveal random interruption of the normal lattice of pigment cells [Fig. 2C]. Random vacuolization of the retina is seen in the corresponding transverse sections of these flies. Rhabdomeres, the light-gathering organelle of the photoreceptor cells, are not affected in either number or identity in this genotype. Consistent with the effects of Doa mutations as suppressors of w<sup>a</sup>, pigment granules are restored to at least normal levels, as seen in both sagittal and transverse sections of Doa heteroallelic flies, compared to w<sup>a</sup> (Fig. 2B,C).

The most severe effects of Doa mutations on retinal organization are found in homozygotes and in one heteroallelic combination examined. In these flies, photoreceptors are completely lacking in the adult retina, as seen in both sagittal and transverse sections (Fig. 2D). Photoreceptors in the ocelli (the simple eyes) of Doa homozygotes are also affected, although not as dramatically; occasional homozygotes are completely missing one or more ocelli. Pigment granules are randomly distributed in the pigment cell lattice, as might be expected given the variegating phenotype of these flies. The lattice of pigment cells is also randomly disrupted, as in the heteroallelic flies. Transverse sections of the homozygotes further reveal that the sizes of the lamina and optic lobe are reduced, perhaps because of loss of proliferative cues from the photoreceptors.

Photoreceptors degenerate late in development in Doa mutants

Differentiation of pigment and other accessory cells in the Drosophila eye is an inductive process, directed by the photoreceptor precursors. The photoreceptors themselves develop through a series of sequential inductive steps, initiated by photoreceptor precursor 8 [for review, see Cagan and Zipursky 1992]. The dramatic effects of homozygosity for Doa mutations on photoreceptor differentiation could thus be explained either by failure of the photoreceptors to undergo terminal differentiation or by their differentiation and subsequent degeneration prior to eclosion of the adults. We determined when aberrant development occurred by immunohistochemical staining of developing eye–antennal imaginal discs from mutant third-instar larvae and from pupal retinas at 60 hr of pupal development. The results demonstrate that photoreceptor precursors differentiate normally and then degenerate prior to eclosion of adults.

Antigens common to all neuronal species are expressed normally in the eye–antennal imaginal discs of Doa homozygotes (Fig. 3A,B), as revealed with antibodies to the neuronal nuclear protein ELAV (Robinow and
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White 1988) and to horseradish peroxidase (HRP, not shown), which specifically label neurons in insects (Jan and Jan 1982). Expression of chaoptin, a photoreceptor-specific antigen (Zipursky et al. 1984, 1985), is also normal in eye–antennal imaginal discs derived from the rare Doa homozygotes (not shown). Progression of the morphogenetic furrow, behind which ommatidial organization occurs, also appears to be normal. We also examined expression of glass, which encodes a zinc finger DNA-binding protein required for differentiation of photoreceptors (Moses et al. 1989; Moses and Rubin 1991). Mutants at glass lack photoreceptors, similarly to Doa homozygotes, and it was thus possible that the lack of photoreceptors in Doa homozygotes was attributable to altered glass expression. Immunohistochemical staining of eye–imaginal discs from Doa heteroallelic and homozygous mutants using an anti-Glass monoclonal antibody (Ellis et al. 1993) revealed normal levels and distribution of Glass in all genotypes [Fig. 3C, D].

Because larval development of the eye–imaginal discs was normal, pupal retinas were examined at 60 hr of postpupariation development. By this time the determination of the various cell types in the ommatidia is complete (Cagan and Zipursky 1992). No differences were found between pupal retinas derived from wild-type and Doa heteroallelic or homozygous individuals, when stained with antibodies against HRP (which recognizes a general neuronal antigen) or chaoptin (photoreceptor-specific). Because both nonspecific neuronal and photoreceptor-specific gene expression occurs normally in eye discs obtained from Doa homozygotes, the photoreceptor cells must degenerate after differentiation but prior to the eclosion of adults.

Although immunohistochemical staining of eye–antenna-
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tennal imaginal discs from Doa heteroallelic or homozygous larvae shows normal differentiation of all cell types, one nonspecific abnormality is readily observed. Eye–imaginal discs derived from homozygous mutants are significantly smaller than wild type (Fig. 3). This observation also extends to at least the wing and leg imaginal discs, although the size differences are less dramatic. These size differences may reflect a nonspecific effect of Doa mutations, rather than a specific role for the gene in cellular proliferation, because homozygous larvae, pupae, and adults are noticeably smaller than wild type.

Segmentation and nervous system defects in homozygous embryos derived from heteroallelic Doa mothers

Recessive lethality of Doa alleles was initially characterized as occurring at early larval stages (Rabinow and Birchler 1989). However, because the direction of reciprocal crosses influenced the survival rate of Doa trans-heterozygotes, a maternal contribution of Doa function to embryos was suggested (Rabinow et al. 1993). This in turn raised the possibility that defects would be found in Doa mutant embryos derived from mutant mothers. To deplete mutant embryos of as much of the maternal Doa contribution as possible, large numbers of female trans-heterozygotes were produced. These semifertile females were crossed with male heterozygotes of an allele that does not produce any heteroallelic flies. Nonhatching embryos from this cross were scored (for details, see Materials and methods).

Although only 50% of the embryos from this cross were trans-heterozygotes of an inviable heteroallelic combination, only ~20% of them hatched. Thus, maternal contribution of Doa product, its mRNA or modified substrates is essential to embryonic development, although rescue by zygotic transcription of the paternal allele occurs occasionally.

Embryos failing to develop from the above crosses were characterized by examination of cuticles, and immunohistochemical staining of the nervous system (Fig. 3E,F,G,H). Defects in both were observed. Both the stage at which embryonic development arrested and the observed defects were variable among individuals, probably because of the residual maternal contribution of Doa and its perdurance in the embryos.

Molecular cloning of Doa

We cloned Doa, benefiting from the revertant DfdRX1, a transposition of the Dfd locus into 98F1-2 (Hazelrigg and Kaufman 1983), the established location of Doa. Genetic tests demonstrated that the DfdRX1 insertion had generated a Doa allele (for details, see Materials and methods). A wild-type Dfd clone, kindly provided by W. McGinnis (Yale University, New Haven, CT), was used to cross the breakpoint into Doa, and phage clones distal to it were isolated from a wild-type genomic library. A subclone of one of these hybridized to polytene chromosome bands 98F1-2 (Fig. 4). Restriction mapping of DNA from four mutant Doa alleles and two independent revertants demonstrated conclusively that we had cloned the locus, delimiting a minimal region of ~35 kb encoding the locus (Fig. 4; for details, see Materials and methods).

Doa mutations alter expression of a 2.7-kb transcript

The 7-kb EcoRI–SalI genomic fragment encompassing the closely linked DfdRX1 and DoaV38 breakpoints (approximate coordinates –4 to +4, Fig. 4) was used as a probe to isolate phage from a 3- to 12-hr embryonic cDNA library (Poole et al. 1985). The four cDNA clones obtained hybridize to genomic clones covering 37 kb and encompass the breakpoints and insertions described above (Fig. 4). The large size of Doa is consistent with the large number of mutant alleles recovered with several different mutagens (Rabinow and Birchler 1989; Peng and Mount 1990; Kurkulos et al. 1991; Rabinow et al. 1993). Two of the cDNA clones derive from putative introns, based on the lack of open reading frames within them and also because they detect no RNA on Northern blots (not shown). The other two cDNA clones substantially overlap on the basis of restriction maps and DNA sequence, comprising a composite cDNA of 2567 bp.

When the composite cDNA was used to probe Northern transfers, a prominent 2.7-kb RNA was observed throughout development (Fig. 5, lanes 1–13). Because of [1] the nearly ubiquitous expression of the 2.7-kb transcript, [2] the fact that it matches closely in size the composite cDNA encoding a long open reading frame [below], and [3] the fact that several different Doa mutations specifically reduce its accumulation [below], we believe that this transcript encodes the primary form of functional Doa mRNA.

Final confirmation that the 2.7-kb cDNA encodes Doa was provided by examination of RNA isolated from both heteroallelic and heterozygous mutants (Fig. 5, lanes 14–20). RNA from pupal trans-heterozygotes escaping lethality examined on Northern blots shows virtual elimination of the 2.7-kb transcript, compared with heterozygous siblings or wild-type strains (Fig. 5, lanes 14–16).

Interestingly, RNA from heteroallelic adults does not show a strong reduction in this mRNA species [not shown], suggesting a developmentally specific defect in expression in one or both of the alleles (HD105) used to produce these animals. Doa adult heterozygotes of two of three additional alleles examined also show noticeable reductions in the 2.7-kb RNA relative to wild type (Fig. 5, lanes 17,18,19,20).

Developmental analysis of Doa transcription

Analysis of the transcripts hybridizing to the composite cDNA reveal that the transcript pattern of the locus varies significantly during development (Fig. 5). In addition to the 2.7-kb RNA described above, major transcripts of 3.8, 4.0, and 5.5 kb are expressed with precise developmental specificity (Fig. 5). A minor pupal-specific transcript >7 kb is also observed. The higher molecular weight transcripts in Figure 5 may represent splicing in-
intermediates, because genomic DNA subclones that do not contain exons present in our composite cDNA [introns 3 and 4: Fig. 4] detect the 4.0- and 5.5-kb RNAs when used as probes on Northern transfers [not shown]. Alternatively, these RNAs may encode differentially spliced, initiated, or terminated functional messengers.

Tissue-specific transcripts were also observed. One, of 1.9 kb, is restricted to the first 4 hr of embryonic development [Fig. 5, lane 1]. Because Doa alleles have maternal effects, and zygotic transcription does not commence until ~2 hr of development, we determined whether the 1.9-kb transcript was of maternal origin. Analysis of RNA prepared from dissected ovaries of adult females reveals the 1.9-kb and ubiquitous 2.7-kb transcripts [not shown], confirming that the former is maternally contributed to the developing oocyte. The structural basis for its reduced size is unknown.

A second tissue-specific transcript is of particular interest, because of the effects of Doa mutations on retinal development. RNA prepared from total adults shows predominant transcripts of 4.0, 3.8, and 2.7 kb. [Fig. 5, lane 10]. Minor transcripts of 5.5 and 2.9 kb are also found. Adult Drosophila were fractionated into head and body samples, from which RNA was prepared. The ubiquitous 2.7-kb transcript is reduced in quantity in the head fraction, and a novel 2.9-kb transcript is found [Fig. 5, lane 11, overexposed in lane 13]. The 4.0-kb transcript is found in both head and body fractions, whereas the 3.8-kb transcript is found only in samples from adult bodies [Fig. 5, lane 12]. It is possible that the 2.9-kb transcript is actually present throughout the nervous system and is observed only in the head samples because neural cells are highly enriched by this fractionation.

To identify other possible transcription units in the
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Figure 5. Northern analysis of Doa expression during development and in mutants. A 1.8-kb cDNA clone containing the Doa open reading frame was used as the probe. The same transfers were reprobed with a probe for RNA from the ribosomal rp49 gene as a loading control. (Lanes 1–13) RNA from developmentally staged organisms. (Lanes 1–6) Four hour windows during embryonic development. (Lane 1) 0–4 hr; (lane 2) 4–8 hr; (lane 3: 8–12 hr; (lane 4) 12–16 hr; (lane 5) 16–20 hr; (lane 6) 20–24 hr; (lane 7) first-instar larvae; (lane 8) late third-instar larvae; (lane 9) 0–24-hr-old pupae; (lane 10) total adult (mixed sex; no sexed flies; not shown); (lane 11) first-instar larvae; (lane 12) adult head; (lane 13) the same adult head sample as lane 11, overexposed to show the 2.9-kb head-specific transcript. The 2.7-kb transcript is expressed throughout development. The 1.9-kb transcript restricted to 0–4-hr-old embryos is maternal in origin, because zygotic transcription does not commence until ~3 hr of development, and this transcript can also be detected in RNA derived from dissected ovaries (not shown). The RNAs >2.7 kb may represent unspliced precursors to the 2.7-kb RNA (see text for details). (Lanes 14–20) RNA from Doa mutants shows a reduction in the ubiquitous 2.7-kb transcript. (Lanes 14–16) RNA from Doa mutant pupae. (Lane 14) Canton-S; (lane 15) Doa heterozygous mutants (alleles 105 or HD, mixed); (lane 16) Doa heteroallelic mutants (105/HD). Samples in lanes 15 and 16 are derived from siblings originating from a cross between balanced stocks of Doa(l 05) and Doa(1D) [for details, see Materials and methods]. (Lanes 17–20) RNA from adults heterozygous for Doa mutations. (Lane 17) Canton-S; (lane 18) Doa(1D)/TM3; (lane 19) Doa(l 05)/TM6; (lane 20) Dfd(RX1)/TM3. Note that heteroallelism for Doa mutations virtually eliminates expression of the 2.7-kb transcript in pupal samples, whereas in adult heterozygotes, expression of the same transcript is also reduced in two of the three mutant strains examined, relative to wild type (Canton-S).

Doa encodes a protein kinase

To further our understanding of Doa function, we sequenced the two cDNAs yielding a single long composite clone. The deduced amino acid sequence of the 1.5-kb open reading frame contained in the 2567-bp composite cDNA sequence [Fig. 6] reveals perfect matches with the conserved residues and domains of a serine–threonine protein kinase [Hanks et al. 1988; Hanks and Quinn 1991]. A 3′-untranslated region of 1035 bp terminates in a poly(A) tail of 20 nucleotides, indicating that the entire 3′ end of an mRNA was cloned. Each genomic fragment hybridizing to the cDNA was also sequenced to determine the exact exon–intron junctions. The composite cDNA comprises 10 exons [Figs. 4 and 6].

The composite cDNA of 2567 bases nearly matches the size of the highly expressed ubiquitous 2.7-kb RNA and, thus, is nearly full length. However, the conceptual translation product of the cDNA began with a histidine residue [tailed arrow, Fig. 6], indicating that the initiating methionine residue and the 5′ end of the mRNA were missing. To identify the probable initiating methionine residue, we used a probe from the 5′ end of the cDNA to identify the genomic sequences hybridizing to it. The sequence of this fragment revealed an in-frame methionine codon with no open reading frame 5′ to it, 13 amino acids 5′ to the end of the composite cDNA [Fig. 6]. A second methionine codon is found 7 residues downstream of the first; either might be utilized in vivo. The DNA sequence adjacent to the upstream potential initiating methionine (GACA ATG) shows two of four matches with the Dro sophila consensus translation start [C/A AA C/A ATG; Cavener 1987], although the downstream potential initiator [TCAG ATG] shows fewer matches, favoring the upstream methionine as the translational initiator. Although a likely candidate for the initiating methionine was found in an appropriate context and location, this analysis does not eliminate the possibility that yet another intron separates the sequenced cDNA from its actual translation start. The point of Doa transcriptional initiation also may lie nearby, because the composite cDNA is nearly full length compared with the 2.7-kb transcript, and RNA transfers probed with restriction fragments 5′ to the SalI site at +30 [Fig. 5] do not reveal Doa transcripts.

The putative protein kinase catalytic domain lies to-
ward the carboxyl terminus of the deduced 517- amino
acid Doa product, beginning at Y170 and ending at F484
[Fig. 6], assuming translation begins at the AUG 39
nucleotides 5′ to the end of the composite cDNA—the
lysine residue crucial to Mg–ATP binding that is invari-
ant in all protein kinases is K199. The kinase catalytic
domain is delimited with upward-pointing tailed arrows.

As expected, Doa protein (DOA) possesses intrinsic
protein kinase activity. When expressed in E. coli, a puri-
ified fusion protein containing the entire catalytic do-
main autophosphorylates in a filter-renaturation assay
[Fig. 6; for details, see Materials and methods]. The puri-
ified fusion protein, detected with Coomassie blue, is
delimited by upward-pointing tailed arrows in
Figure 6.

The nucleotide and deduced amino acid sequence of a Doa-encoding composite cDNA. Sequence of 2.6 kb is shown. The predicted amino acids are shown below the cDNA sequence. Translation terminates with the codon TGA [nucleotides 1552–1554], yielding a 1055-bp 3′-untranslated region (including a 20-bp poly(A) tract). The downward arrowheads indicate the positions of introns.

Highly conserved protein kinases homologous to Doa in widely diverged species define a new protein kinase family

Searches of computer data bases with the conceptual
Doa translation product and DNA sequence revealed a
number of highly conserved homologs. The first identi-
fied was a murine kinase known as
Clk/STY (Howell et al. 1991) (Fig. 8; Table 1). Clk/STY was independently identified in two
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Figure 7. DOA protein possesses intrinsic protein kinase activity in vitro. A cDNA subclone encompassing the entire Doa catalytic domain was expressed in E. coli as a fusion with maltose-binding protein (MBP) and purified on an amylose column. Following gel electrophoresis and transfer to nitrocellulose membrane, the samples were denatured in guanidine and renatured, before being incubated in kinase assay conditions. [Lane 1] IPTG-induced crude lysate from E. coli harboring the vector used for expression [without Doa]; [lane 2] IPTG-induced crude lysate from the MBP-Doa fusion strain; [lane 3] purified MBP-DOA fusion protein; [lane 4] crude lysate from a Trp-Clk fusion strain, as a positive control. Approximately 3 μg of amylose-column-purified material was loaded in lane 3, compared with 30 μg of crude lysates. DOA and Clk fusion proteins autophosphorylate, demonstrating that they possess intrinsic protein kinase activity. The trp-Clk fusion protein coincidentally has the same molecular mass (84 kD) as the MBP-DOA fusion.

Figure 8. The LAMMER protein kinase family. Alignment of Doa with human clk, pk3-G1 (human), mouse clk, and KNS1 (yeast). Amino acids identical between Doa and any of the other proteins are indicated by hyphens. Gaps in alignment are indicated by periods. The open box represents the LAMMER motif, found only in members of this protein kinase family, which all family members possess in an identical location relative to kinase catalytic domains, as marked.
Table 1. Comparative values of amino acid identity among LAMMER kinases

|       | Doa       | Human clk | pSK-G1    | Mouse clk | AFC1     | KNS1     |
|-------|-----------|-----------|-----------|-----------|----------|----------|
| Doa   | 100 (100) | 62 (46)   | 75 (56)   | 62 (46)   | 48 (36)  | 38 (27)  |
| Human clk | 100 (100) | 67 (55)   | 92 (87)   | 62 (46)   | 40 (29)  |          |
| pSK-G1 | 100 (100) | 64 (54)   | 45 (35)   | 35 (26)   |          |          |
| Mouse clk | 100 (100) | 77 (56)   | 40 (30)   | 41 (30)   |          |          |
| AFC1  | [100]     | [100]     | [100]     | [100]     | [100]    | [100]    |
| KNS1  | [100]     | [100]     | [100]     | [100]     | [100]    | [100]    |

The top number in each case represents amino acid identity in the kinase catalytic domain; the number in parentheses compares the overall amino acid identity between the proteins. The species of origin of the homologs are Doa, Drosophila melanogaster; clk, cdc2-like kinase (human and mouse); pSK-G1, human; AFC1, Arabidopsis thaliana; KNS1, S. cerevisiae. The sequence comparisons with AFC1 and the overall comparison with pSK-G1 were made possible by Judith Bender and Gerry Fink, and Steve Hanks, respectively, who generously provided unpublished data.

AFC1, an LAMMER protein kinase

... further analysis, although their presence in all family members suggests that they may be utilized.

... amino acid identity values of catalytic domains among these protein kinase homologs are the long runs of 100% amino acid identity in domains critical to protein kinase function and that are implicated in interactions with substrates. Examples are found in domain VIb, and the 50 amino acid residues encompassing domains VII, VIII, and part of domain IX (amino acid residues 501–549). In addition to playing a role in the transfer of the γ-phosphate from ATP to the peptide substrate, these regions may be involved in substrate recognition [Taylor et al. 1992].

... extremely high amino acid sequence identity of kinase-related motifs, virtual 100% identity of a motif not related to any known protein kinase or other function is found at the amino terminus of kinase domain X. This motif reads EHLAMMERILG in all homologs except in S. cerevisiae, in which three substitutions occur (Fig. 8, box). A similar sequence containing a single conserved substitution is also found in AFC1 [J. Bender and G. Fink, pers. comm.]. Correlations of the location of this motif with the known three-dimensional structure of other protein kinases [Taylor et al. 1992; DeBondt et al. 1993] places it in an α-helix below the substrate-binding cleft, where it might be reasonably expected to make contact with substrates. Combined with identical spacing and high amino acid identity levels throughout catalytic domains, the LAMMER motif defines a new protein kinase family, and we suggest the name LAMMER kinases to describe it. The LAMMER kinases must play a role in the regulation of a basic cellular function, because they are highly conserved in widely diverged eukaryotes, including fungi, green plants, and animals.

Discussion

Mutants at the Doa locus were characterized previously...
as increasing transcript levels of the copia retrotransposon, probably through altering transcription rates [Rabinow et al. 1993]. Additionally, Doa mutations decrease levels of transcripts truncated in the copia element inserted in \( w^\text{t} \) [Rabinow and Birchler 1989]. Here, we demonstrate an essential role for Doa in Drosophila embryonic development and maintenance of photoreceptor cells. The product of the locus is a protein kinase that possesses the potential for dual specificity, with ho-

rasing the possibility that they may phosphorylate nu-

meller, and proliferation of specific cell types. Differentiation, maintenance, and proliferation of specific localization signals are found in all LAMMER kinases, discs of mutants are smaller because of a reduction in cell number. Because Doa regulates copia transcript lev-

els, it is particularly intriguing that potential nuclear localization signals are found in all LAMMER kinases, raising the possibility that they may phosphorylate nuclear proteins directly.

Defects in the adult eye are not restricted to photore-
ceptors and are also observed in pigment cells and in the organization of interommatidial bristles. These defects are only observable in animals that possess residual Doa activity; thus, these phenotypes may be less severe than those that would be observed in a complete null background. Defects in both embryonic segmentation and differentiation of the nervous system are found in zygotes substantially deprived of both maternal and zy-
gotic Doa function. The variability in the development of these embryos suggests that there must be continued contribution of Doa function from their heteroallelic mutant mothers. Small amounts of residual function may allow cell differentiation and survival past the stage when Doa is actually required, during both imaginal and embryonic development. Further analyses to determine the role Doa plays during embryogenesis and photoreceptor development will require elimination of residual activity using genetic means.

The highly conserved sequence identity and structure of the LAMMER protein kinases suggest that they regu-
late one or more conserved cellular processes. Further analysis of the biological consequences of Doa muta-
tions and the identification of molecules with which it interacts will help to elucidate the role of the LAMMER protein kinases throughout eukaryotes.

Materials and methods

Drosophila culture and genetic crosses

Stocks and crosses were grown on standard medium [cornmeal, molasses, agar]. All crosses were performed at 25°C. Crosses with \( Dfd^{RX1} \) were performed with Doa alleles \( HD, I5 \), and \( CG \), the former two of which have normal cytology. Each allele was crossed as females with two different balanced stocks carrying \( Dfd^{RX1} : \text{Df}(3R)30c76 \text{ Tp}(3;3)Dfd^{RX1}, \text{ Df}^{Rd6}, \text{ Df}^{TM6}, \text{ kindly provided by E. Stephenson [University of Alabama, Tuscaloosa], and Tp}(3;3)Dfd^{RX1}, \text{ Tp}(3;3)S31/TM3, kindly provided by W. McGinn}

n. The Df(3R)30c76 Tp(3;3)Dfd^{RX1}, \text{ Tp}^{Rd6}, \text{ Tp}^{TM6} stock was found to suppress \( w^\text{t} \), a phenotype typical of Doa. Crosses to generate heteroallelic Doa\text{HD}/Doa\text{OS} individuals were performed as described [Rabinow et al. 1993]. The two alleles yielding extremely rare homozygotes, Msu2 and \( \gamma A \), were induced by ethylmethane sulfonate [EMS] mutagenesis and gamma-irradi-

ation, respectively. Neither is homozygous viable, except at extremely low rates when balanced with \( T M 6 \), for unknown rea-

sons. Msu2 and \( \gamma A \) are probably attributable to position effects, based on their variegating phenotype when homozygous, the existence of cytologically visible breakpoints in or near the gene, and their ability to complement the recessive lethality of some but not all other Doa alleles [Csink et al. 1994; B. Yun and L. Rabinow, unpubl.]. Additionally, in combination with Doa alleles allowing survival of trans-heterozygotes at or near Mendelian ratios, both Msu2 and \( \gamma A \) often produce eye-roughening and complete suppression of \( w^\text{t} \) to wild-type pigment levels, both phenotypes found in trans-heterozygotes of mutant Doa alleles (B. Yun and L. Rabinow, unpubl.). The induced revertant \( Doa^{RD} \) was induced during crosses of \( HD \) with the \( \Delta 2-3 \) P-element at \( 9 \beta B \) [Robertson et al. 1988]. The spontaneous re-

vertant, \( Doa^{RD} \), was recovered as a homozygote and occurred on a \( D fd^{HD} \) chromosome in a stock balanced with \( TM 3 \).

A Deformed revertant is a Doa allele

We tested whether a Doa allele had been induced by the \( Dfd^{RX1} \) transposition by performing complementation tests based on the recessive lethality of Doa. Two different stocks of \( Dfd^{RX1} \) were crossed independently with three Doa alleles, two which have normal cytology, to rule out the possible induction of closely linked secondary recessive lethal mutations in both the Doa and \( Dfd^{RX1} \) chromosomes. No \( Dfd^{RX1} \) segregants from either stock survived as trans-heterozygotes with any Doa allele tested. Additionally, \( Dfd^{RX1} \) suppresses \( w^\text{t} \) (one stock tested), a phenotype characteristic of Doa.

Embryonic phenotypes

To obtain embryos as depleted of the maternal Doa contribu-
tion as possible, \( Doa^{HD} / Doa^{OS} \) females were generated and crossed with \( Doa^{AB} / TM 3 \) males. The latter allele does not produce heteroallelic escapers in any combination, causes a dramatic reduction in the 2.7-kb Doa RNA (this report), and does not complement several different classes of Doa alleles, including Msu2 and \( \text{Rem}^\text{A} \) [B. Yun and L. Rabinow, unpubl.]. Emb-

bryos obtained from this cross were collected on fine nylon mesh and treated for antibody staining or cuticle preps, as de-
scribed [Ashburner 1989].

Scanning electron microscopy

Samples for scanning electron microscopy were processed as described by Kretzschmar et al. [1992]. Fixed adult flies were dehydrated through an ascending ethanol series [30%, 50%, 70%, 96%, and 3x 100%], followed by critical point drying and spatter-coating with gold/palladium. Flies were viewed and pho-
tographed in a Jeol JSM 35-C scanning electron microscope op-

erating at 10, 15, or 25 kV.

Histology and immunocytochemistry

Plastic sections were prepared as described by Tomlinson and Ready [1987], as modified by Kimmel et al. [1990]. Adult heads
were dissected in *Drosophila* saline, and holes were made between the eyes to aid penetration of fixatives and embedding components. Heads were fixed in 2% glutaraldehyde in phosphate buffer, postfixed in 1% osmium tetroxide, and dehydrated in ethanol. After infiltration in propylene oxide–Durcupan they were embedded in Durcupan ACM plastic resin [Fluka AG, Buchs, Switzerland]. Plastic sections of 1.5–2 μm were stained with toluidine blue [Basler and Hafen 1988], and mounted in DPX [Fluka AG].

Immunohistochemical staining of imaginal discs and pupal retinas was performed with anti-HRP (Cappel), anti-chaoptin (mAb 24b10), kindly provided by S. Benzer [California Institute of Technology, Pasadena], and anti-ELAV and anti-Glass, kindly provided by E. O’Neill and G. Rubin [University of California, Berkeley]. Dissected discs or pupal retinas were fixed in 4% paraformaldehyde in PBS for 3 hr at room temperature and washed 3X in PBS. The peripodial membrane was removed using heptane–dimethylsulfoxide, and discs were permeablized with PBS + 0.1% Triton X-100 [PBT]. After 4 hr of blocking in PBT + 2% BSA and 1% normal rabbit serum, discs were incubated with primary antibodies overnight. After washing with PBT, secondary biotinylated anti-goat antibody [Vector Laboratories] was added for another 3 hr at room temperature. Staining was performed with strepavidin-conjugated-HRP, followed by extensive washing and incubation with 0.1% diaminobenzidine and 0.02% hydrogen peroxide in 0.1 M Tris-HCl [pH 7.6].

**Clones from 98F1-2 detect DNA polymorphisms in Doa alleles**

The generation of a *Doa* allele by the *Dfd*/*Rx1* reversion allowed us to “jump” into the *Doa* locus, because the precise breakpoint in *DfdRX1* had been mapped [Regulski et al. 1987]. A clone from transposon DNA near the break was used to isolate genomic phage from a *DfdRX1* genomic library, crossing the breakpoint between the two loci. Because *Dfd*/*RX1* is maintained as a balanced stock, only one-half of the cloned phage should reveal the breakpoint. Of four phage isolated, one had a restriction map differing from the wild-type *Dfd* locus, suggesting that it defined the *Dfd*–*Doa* breakpoint. To confirm this, a restriction fragment distal to the putative *DfdRX1* breakpoint was used as a probe to screen a wild-type genomic library, from which four overlapping phage were isolated (Fig. 4). A subclone of phage Doa [approximately coordinates –4 to + 4, Fig. 5] was used as a probe to salivary gland polytene chromosomes of the Canton-S wild-type strain. Chromosomal walking from our entry point isolated additional phage from the 98F1-2 region, using the ends of both genomic and cDNA [see below] clones to probe λ libraries from wild-type *Drosophila*. Six of those characterized span 50 kb at 98F1-2 [Fig. 4]. Restriction maps from five *Doa* mutant alleles were then constructed, based on data from Southern transfers using the wild-type genomic clones as probes, to identify lesions in the gene and aid in defining its structure (Fig. 4). We particularly wished to locate aberrations associated with three-hybrid dysgenically induced alleles. This desire stemmed in part from the existence of two independent revertants, one of which occurred spontaneously [B. Yun and L. Rabinow, unpublished]. A second was induced during crosses to the Δ2–3 element [Robertson et al. 1988]. The finding of DNA polymorphisms coincidentally induced with a *Doa* mutant allele, which were subsequently altered during reversion, would provide unambiguous evidence that we had cloned the gene.

An insertion of ~5 kb was found in the identical location in both the *HD1* and *HD2* alleles (Fig. 4). This is not surprising, as both potentially originated from a single event during mass crosses [Rabinow and Birchs 1989]. Furthermore, this insertion is missing in both revertants described above, presumably through excision of the uncharacterized 5-kb transposable element. A 2-kb insertion was found at a different location in a third dysgenically induced allele, Dem (Fig. 4). We also found a breakpoint in *Doa~rx1*, which closely flanks the *DfdRX1* insertion. This allele is associated with a small chromosomal abnormality at 98F1-2, probably a localized inversion, an interpretation supported by our restriction maps.

**Molecular biology**

Genomic library construction, plaque isolation, and subcloning, Southern blots, probe preparation, and so forth, all made use of standard protocols, [e.g. Ausubel et al. 1989]. The *DfdRX1* genomic library was constructed by Sau3AI partial digestion and cloning of genomic fragments into phage EMBL3 at the BamHI site. DNA sequencing was performed by double-stranded sequencing of plasmids using the dyeodeoxy method [Sanger et al. 1977]. All restriction sites were crossed, and both strands of cDNA and genomic clones were sequenced. Computerized searches of sequence data bases and alignments were generated using GCG software. Polytene chromosome squashes and in situ hybridization were performed using biotinylated DNA probes and alkaline phosphatase detection, essentially as described [Ashburner 1989]. RNA isolation, Northern blots, and RNA probes were performed as described [Rabinow et al. 1993]. Briefly, total RNA was extracted from frozen organs by guanidine-HCl extraction [Cox 1968]. Five micrograms per lane of total RNA was separated on 1.5% formaldehyde–agarose gels and transferred using the Southern method. The RNA was fixed to the filter by both UV cross-linking and baking under vacuum at 80°C for 2 hr. Antisense RNA probes were synthesized with T3 or T7 RNA polymerase. Loading controls were performed by rehybridizing the blots with a probe for rp49 [O’Connell and Rosbash 1984]. Blots were hybridized at 60°C, under described conditions [Dorsett et al. 1989], at a probe concentration of >2 x 10^6 cpn/ml. Filters were washed with 0.1 x SSC, 0.4% SDS, at 75°C after overnight hybridization.

**Expression of Doa protein in *E. coli* and autophosphorylation assays**

A subclone of the composite *Doa* cDNA encoding the entire catalytic domain, starting at K129 to a StyI site in the 3'-untranslated region, was inserted into the *XbaI* site of pMAL-C2 of New England Biolabs. Expression of the fusion protein in *E. coli* was induced with IPTG, and the fusion was recovered on an amylose column, analyzed for purity on an SDS gel, and transferred to a nitrocellulose membrane. Kinase-renaturation was performed as described for murine Clk [Ben-David et al. 1991].

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Note added in proof

The sequence data described in this paper have been deposited into the EMBL/GenBank data library under accession number X78715.

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