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# *Drosophila melanogaster* Homologs of the *raf* Oncogene

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**A murine v-*raf* probe, representing the kinase domain, was used to identify two unique loci in *Drosophila melanogaster* DNA. The most closely related to v-*raf* was mapped by in situ hybridization to position 2F5-6 (*Draf-1*) on the X chromosome, whereas the other *raf*-related gene (*Draf-2*) was found at position 43A2-5 on chromosome 2. The nucleotide and amino acid homologies of *Draf-1* to the kinase domain of v-*raf* are 61 and 65%, respectively. The large amount of a 3.2-kilobase *Draf-1* transcript detected in eggs as a maternal message decreases during embryonic development, and significant steady-state levels are observed throughout the remainder of morphogenesis. We speculate that the *Draf-1* locus plays an important role in early embryogenesis.**

Acutely transforming retroviruses have been the source of the majority of oncogenic sequences identified to date. These oncogenes were acquired by recombination with, and subsequent transduction of, cellular proto-oncogene loci (reviewed in reference, 2–4). Of the four major oncogene classes (*src* family, *ras* family, nuclear oncogenes, and growth factors), members of three classes have been isolated from *Drosophila melanogaster*. Three genes representing the *ras* family (28), four genes representing the *src* family (10, 11, 19, 33, 34) and one representing the c-*myb* proto-oncogene (15) have been identified in *D. melanogaster*. The conservation of proto-oncogene sequences throughout the hundreds of millions of years of evolution since invertebrate speciation attests to the essential roles played by these genes in normal cellular growth and differentiation. Although the relationship between several of these proto-oncogenes and related sequences to known polypeptides has been demonstrated (exemplified by platelet-derived growth factor and *sis*; epidermal growth factor receptor and *erbB*; CSF-1 receptor and *fms*; and insulin receptor and *ros*-related sequences), the mechanism by which they transduce their signal to effect a cellular response is presently obscure (3, 13).

The largest oncogene family, the *src* family, has yielded the greatest number of *Drosophila* homologs. Thus far these phylogenetically ancient homologs have all been representatives of those *src* family members encoding tyrosine-specific protein kinases. We report here the isolation and preliminary characterization of a *Drosophila* gene that corresponds to the proto-oncogene c-*raf* and the identification of a *raf*-related *Drosophila* locus which may represent the recently identified *raf*-related proto-oncogene *pks* present on the short arm of the human X chromosome (23). The v-*raf* product exhibits serine-threonine protein kinase activity (26), and c-*raf* shows significant primary and secondary amino acid sequence homology to protein kinase C; thus the *Drosophila* homolog might be expected to share this specificity, and the conservation of amino acid sequences observed is supportive of this expectation. Our finding extends the number and variety of *Drosophila* oncogene-related sequences.

## MATERIALS AND METHODS

**Hybridization, cloning, and DNA sequencing.** A *D. melanogaster* genomic library made from size-selected partial *Eco*RI-digested DNA ligated into Charon 4A was obtained from Tip Benyajati (Litton Bionetics, Frederick, Md.). Hybridization of plaque lifts (1) was performed under relaxed stringency conditions, as were the Southern blot analyses. Nitrocellulose filters were prehybridized at 60° for 2 h in 5× SCC (1× SSC is 0.15 m NaCl plus 0.015 M sodium citrate)–5× Denhardt solution and then hybridized overnight at 60°C in 5× SSC–1× Denhardt solution–10% dextran sulfate and about $5 \times 10^6$ cpm of nick-translated probe per ml (30) representing the kinase domain of v-*raf* (0.68-kilobase [kb] *Xho*I-to-*Sst*II fragment [21]). The filters were washed repeatedly in 2× SSC–0.1% sodium dodecyl sulfate at 55°C and exposed to X-ray film overnight with an intensifying screen. Subcloning of DNA fragments into pBR322 was done essentially by the method of Maniatis et al. (20). The DNA sequence was determined by the method of Maxam and Gilbert (24) from uniquely labeled restriction site termini after isolation by Seaplaque (FMC Corp. Marine Colloids Div., Rockland, Maine) agarose gel electrophoresis as described previously (22).

**In situ hybridization.** The method of Bonner and Pardue (5) was used for in situ hybridization, with modifications to accommodate the use of biotinylated probes. Biotinylated probes were synthesized by using biotin-11 dUTP (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and the primer extension procedure of Feinberg and Vogelstein (8). Hybridization conditions were 2× SSC, 50% formamide, and 10% dextran sulfate for 24 h at 37°C. Development of the hybrid was performed as described in the ENZO Deteck Kit. Localization on the polytene chromosome was determined by using the alignment of Bridges' chromosome maps, described by Lefevre (16).

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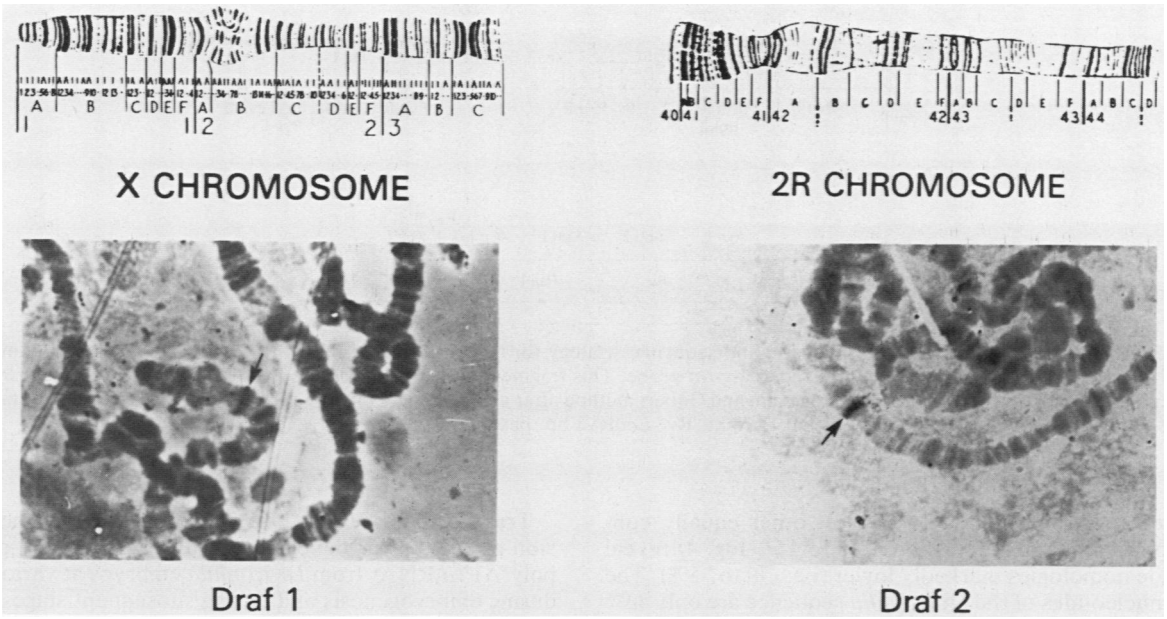


FIG. 1. In situ hybridization to salivary gland chromosome squashes. DNA plasmids containing *Draf-1* (left panel) or *Draf-2* (right panel) sequences were biotinylated and used as hybridization probes to determine the chromosomal location of these sequences. Shown above the squash are representations of the relevant chromosomal regions. The arrows indicate the sites of hybridization at 2F5-6 (*Draf-1*) and 43A2-5 (*Draf-2*); no other sites of hybridization were observed.

**RNA analysis.** Total poly(A)$^+$ mRNA was isolated from staged embryos using the guanidium-cesium chloride method of isolation and selection on oligo (dT) cellulose (9, 20). RNAs were electrophoretically separated on a 1.0% agarose-formaldehyde gel, transferred to nitrocellulose paper, and baked at 80°C for 2 h. Filters were prehybridized with a solution containing 50% formamide, 1 M NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, 0.1% sodium $PP_i$, 1× Denhardt solution, and 100 μg of denatured salmon sperm DNA per ml at 42°C. Hybridization was performed in the same buffer containing 10% dextran sulfate at 42°C, using $10^7$ cpm of nick-translated, 0.95-kb *Pst*I DNA fragment probe. Filters were washed two times at room temperature in 2× SSC–0.1% sodium dodecyl sulfate and three times at 42°C in 0.2× SSC–0.1% sodium dodecyl sulfate before exposure to X-ray film in the presence of an intensifying screen, as previously described (7). Densitometry was done with a Shimadzu CS-930 dual-wavelength scanner.

## RESULTS AND DISCUSSION

**Chromosomal localization of DNA clones.** To determine the position of each of the *raf*-related loci, we labeled subcloned fragments in pBR322 with biotin and used them as probes for in situ hybridization to the polytene chromosomes of larval salivary glands. The *Draf-1* probe hybridized to position 2F5-6 on the X chromosome, while the *Draf-2* locus was mapped to position 43A2-5 on the right arm of chromosome 2 (Fig. 1). Previously described *src* family homologs have been mapped to positions 29A, 64B, 73B (11, 33), and 57F (19). Search of the master list of *Drosophila* clones, breakpoints, and transformants (25) revealed the *Draf-2* locus to be in a region from which head-specific RNA is transcribed (18). The location of the *Draf-1* locus between prune (*pn*; 2E1-2) and giant (*gt*; 3A1) places it in a region of eight known developmentally important genes (29). This fact combined with the seemingly high degree of homology of the *Draf-1* sequence to the murine v-*raf* oncogene led us to concentrate further analyses on this locus.

**Nucleotide and predicted amino acid sequences.** To facilitate the ultimate DNA sequencing of the *Draf-1* locus, a 7.8-kb *Eco*RI-*Hin*dIII fragment containing the *raf* homology was subcloned into pBR322 and mapped with restriction enzymes (Fig. 2). Southern blotting of this DNA after endonuclease digestions localized the region related to the v-*raf* kinase domain to be within the 0.95-kb *Pst*I fragment. DNA sequence analysis was performed on this fragment by the Maxam and Gilbert method (24) using the strategy outlined in Fig. 2. The *Draf-1* sequence was aligned with the v-*raf* sequence by using a forward homology matrix program (Pustell and International Biotechnologies, Inc.). The determined sequence was found to be broken by a putative intron of 65 nucleotides (Fig. 3). The removal of the intervening sequence was necessary to maintain an open reading frame throughout the *Draf-1* kinase domain (Fig. 4) as well as conserve the almost absolute amino acid homology observed between *raf* subfamily members within this region. Flanking the insertion are sequences which closely resemble splice donor (AG/GT) and splice acceptor (polypyrimidine AG/G) consensus sequences (27). These sequences are underlined in Fig. 3. Of the other three *src* family *Drosophila* proto-oncogenes sequenced, both *Dsrc* and *Dash* contained small insertions (61 and 85 base pairs, respectively) within their kinase domains (10). Of the 873 nucleotides of coding region, 533 base pairs (or 61%) are identical between the *Drosophila* and v-*raf* genes (the latter sequence is presumably very closely related to the murine genomic sequence). The first stretch of significant homology (79%) begins at position 40 and ends at position 72 and represents the beginning of the postulated nucleotide-binding domain of the *Draf-1* peptide. The most extensive region of nucleic acid homology begins at position 229. A total of 90% of the next 57 nucleotides are shared between the *Drosophila* and murine sequences. Although the predicted amino acids of this domain are highly

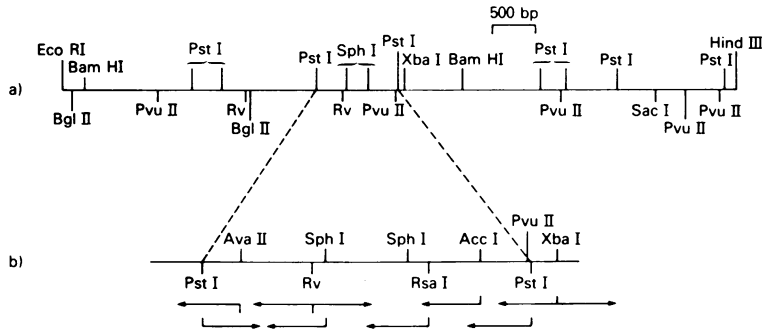


FIG. 2. Restriction map of the *Draf-1* locus (a) and sequence strategy for the v-*raf*-related region (b). Shown is a restriction map of the 7.8-kb *Eco*RI-*Hin*dIII fragment which hybridized to a v-*raf* probe. This fragment was subcloned into pBR322 from a *D. melanogaster* partial *Eco*RI genomic library and sequenced by the Maxam and Gilbert method after end labeling with polynucleotide kinase or terminal transferase. The *Draf-1* locus is shown oriented 5′ to 3′, left to right. Rv, *Eco*RV; bp, base pairs.

conserved (positions 78 to 95, Fig. 4), other equally conserved protein domains (positions 113 to 153, Fig. 4) reveal nucleotide homologies markedly lower (i.e., 70 to 75%). The last 250 nucleotides of the *Drosophila* sequence are only 44% homologous to the murine sequence. The unique quality of individual *src* family members is particularly evident in their carboxy termini. This diversity of carboxy-terminal sequence has been observed between closely related family members (*src* and *abl*) as well as between ancestral homologs (*Dsrc* and *src*, *Dash* and *abl* [10]; and DER and HER [19]).

Within the predicted 291-amino acid sequence (Fig. 4), 190 positions are shared with murine sequence (or 65%), while 163 positions are shared between all the *raf*-related genes thus far sequenced, including the distantly related human X-linked *pks* proto-oncogene (23). Of the remaining 101 amino acid positions, approximately half (52) are either present in one member of the family or represent a conservative amino acid change. The *raf* subfamily contains two known branches, one encoding the homologs of v-*raf* and the other encoding the homologs of *pks*. Consistent with the *Draf-1* sequence representing the v-*raf* homolog is the strong DNA hybridization between these genes and the 24 dissimilarities in amino acid sequence, relative to the *pks* locus, where there are homologies to the *raf* homologs. Also, similar to the *raf* homologs, the *Draf-1* gene is not truncated at its carboxy end as is the *pks* polypeptide. Despite these differences there are extensive regions of protein sequence conservation, suggesting that the *Drosophila* homolog shares functional properties with the vertebrate *raf* and *pks* subfamily members—most likely the serine-threonine protein kinase activity described for the v-*raf* fusion polypeptide (26). Consistent with this hypothesis are the conserved (i) nucleotide-binding region (positions 14 to 35, Fig. 4); (ii) 75 amino acids (positions 77 to 152, Fig. 4) preceding the domain analogous to the phosphate acceptor site (underlined to Fig. 4) of the tyrosine-specific protein kinases (14); (iii) absence of a tyrosine residue at the position occupied by one in the sequence of the tyrosine-specific protein kinases, and its replacement by the serine residue common to all the sequences at position 157 (Fig. 4); (iv) short stretch of a dozen amino acids (position 189 to 201, Fig. 4) which follow the putative phosphate acceptor site. Similar patterns of conservations are also seen between the *abl*, *src*, and *erb*β sequences and their respective *Drosophila* homologs, suggesting that these domains play an important role in the function of these proteins.

**Transcription of *Draf-1*.** To determine whether the expression of *Draf-1* was developmentally regulated, we prepared poly(A)$^+$ mRNAs from *Drosophila* embryos at various times during embryogenesis and during subsequent stages of morphogenesis (Fig. 5). A radioactive probe representing the 0.95-kb *Pst*I *Draf-1* fragment was used to hybridize to Northern blots of these RNAs. Throughout *Drosophila* development a single 3.2-kb *Draf-1* transcript was detected. This message was most prominent during early embryogenesis, and then its steady-state levels dropped markedly and remained relatively low. The amounts of *Draf-1* RNA accumulated varied over a greater than 10-fold range between preblastoderm (maternal RNA) and blastoderm stages and the larval stages of development. This study demonstrates that the accumulation of the *Draf-1* mRNA varies during development and that it shares with the other *src*-related transcripts the characteristic of being a relatively highly abundant maternal messenger. The pattern of rapid embryonic decrease in message levels most closely resembles that of a previously described *src*-related *Drosophila* proto-oncogene (designated *src*-4 and mapped to genomic position 28C (35). Interestingly, the levels of both of these RNAs decline coincidently with the time of onset of zygotic transcription. *Draf-1* differs from the c-*src* homolog *Dsrc* in that the latter locus has been found to be expressed in the form of three noncoordinately transcribed mRNAs of 5.2, 4.8, and 3.1 kb during early embryogenesis and pupal stages of development (17, 32, 35). Multiple spliced and terminated forms of the c-*abl* homolog *Dash* have been identified in unfertilized eggs and ovaries as 5.5- to 6.0-kb and 3.0-kb RNA species (17, 34) and during all but the late larval stage of development (34). Finally, transcripts representing the epidermal growth factor receptor homolog (6.0 kb) were detected in embryonic, pupal, and adult tissues (36). Thus, common to all the *src* family gene products is their presumed participation in early developmental events. During all stages of *Drosophila* development transcripts representing *ras* and *myb* have been detected (15, 17). Thus, we conclude that *Draf-1* is a functional gene whose transcripts predominate during early embryonic development.

**Concluding remarks.** We report here the identification and chromosomal localization of two *Drosophila* loci related to the v-*raf* oncogene. The existence of a unique *Draf-1* locus was expected from the phylogenetic arrangement of the *src* family (21) and the prior characterization of *src*, *abl*, and *erbB* homologs in this organism. The presence of the second, weakly hybridizing *raf*-related locus (*Draf-2*) was, however,

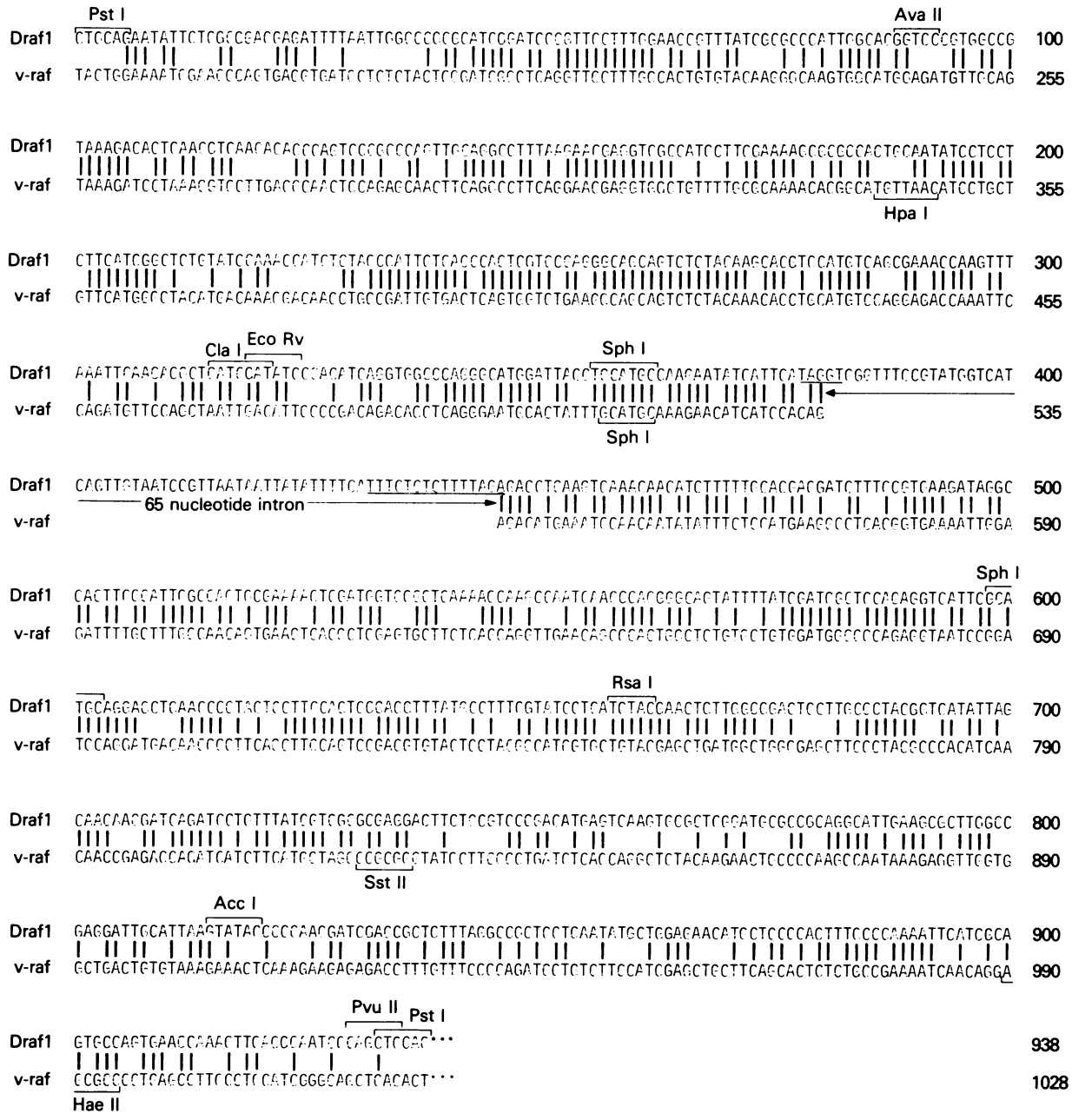


FIG. 3. Nucleotide sequence of the *Draf-1* kinase domain and alignment with the v-*raf* sequence. The DNA sequence of *Draf-1* is numbered arbitrarily from the 5′ *Pst*I site in Fig. 1b. The corresponding v-*raf* sequence begins at nucleotide 156 of the published sequence (21). Vertical bars indicate identities in sequence. The location of a putative 65-basepair intron is shown by the arrow, and flanking consensus 5′ and 3′ splice signals are underlined.

unexpected at the time these experiments were initiated. Subsequently, we have identified and characterized a new *raf*-related gene (*pks*) in the human genome (23) which we suspect represents the human equivalent of the *Draf-2* locus. Should this prove to be the case the common ancestor which gave rise to these two genes predated the chordate-arthropod divergence nearly 800 million years ago. This is in contrast to the presumption that the *yes* proto-oncogene recently diverged from the *Dsrc* locus present in the *Drosophila* genome (32). In this regard it is noteworthy that the *Dsrc* and *Dash* loci both hybridize to a v-*src* probe (31) and share significant nucleotide homologies within their tyrosine-specific kinase domains, suggesting that they diverged from the same primordial ancestor. That this ancestor is different from, or perhaps more recent evolutionarily than, that of the *Draf-1*:*Draf-2* loci might be suggested from observing a possible yeast homolog for the latter sequence.

We sought to determine whether a *raf* homolog existed before the divergence of *D. melanogaster*. To investigate this possibility, *Saccharomyces cerevisiae* DNA was restricted with *Eco*RI or *Hin*dIII, submitted to Southern blot analysis, and hybridized under conditions of reduced strin-

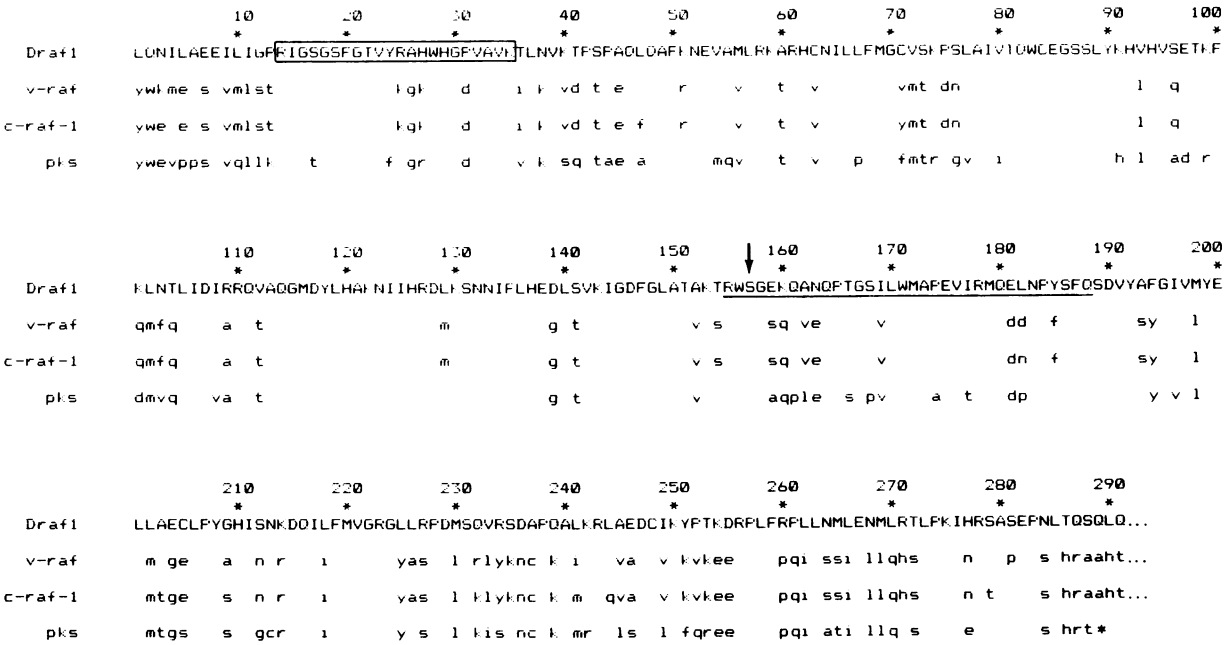

FIG. 4. Alignment of the predicted amino acid sequence of the *Draf-1* kinase domain with that of *raf* and *pks* subfamily members. The numbers designate the position on the *Drosophila* protein. The murine v-*raf*, human c-*raf*-1, and human *pks* sequences correspond to the published sequences truncated for purposes of comparison to the *Drosophila* homolog; thus they begin at amino acid positions 20, 114, and 11, respectively. The termination codon of the *pks* sequence is indicated with an asterisk. Only variations from the *Drosophila* sequence are noted. The putative ATP-binding domain is boxed, and the region equivalent to the tyrosine acceptor site domain is underlined. The arrow points to the serine residue contained in all family members, including the avian *mht/mil* sequence (not shown).

gency to a probe representing the *Draf-1* sequence (0.95-kb *Pst*I fragment). Two fragments (5.7 and 3.0 kb) were detected in the *Hin*dIII-digested DNA which hybridized to the *Draf-1* probe, whereas only a 4.7-kb fragment was detected in *Eco*RI-digested DNA (data not shown). The 0.95-kb *Pst*I fragment probe was further fractionated by *Cla*I digestion (Fig. 2) into two probes representing the nucleotide-binding domain and the presumed kinase active site. Both hybridized to sequences within the 4.7-kb *Eco*RI yeast DNA fragment (data not shown). The exact nature of the sequences which hybridize to the *Draf-1* probe is under investigation.

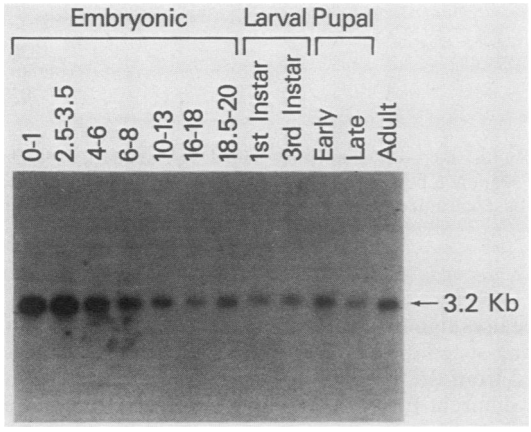


FIG. 5. Expression of *Draf-1* RNA during *Drosophila* development. Total poly(A)$^+$ RNA was prepared from organisms at various stages of development ranging from embryonic to adult. The time points are given as hours after egg laying for the embryonic period. RNA was separated by formaldehyde-agarose gel electrophoresis, transferred to a nitrocellulose filter, and hybridized to a probe representing the 0.95-kb *Pst*I fragment of *Draf-1* (Fig. 1b).

DNA sequence analysis of a portion of the *Draf-1* locus was undertaken to demonstrate unequivocally the ancestral relationship between this *Drosophila* gene and the v-*raf* oncogene. Alignment of the *Draf-1* and v-*raf* nucleotide sequences revealed an average homology of 61% and the existence of a 65-nucleotide intron interrupting the only translatable reading frame. This reading frame encoded a polypeptide with 65% homology to the v-*raf* kinase domain, sharing a number of topographical landmarks with this latter sequence as well as with the other members of the *raf* and *pks* subfamilies. This conservation can be seen (Fig. 6) most readily by the superimposition of their predicted hydrophobic and hydrophilic domains (12). The familiar homology of these sequences significantly decreases after position 224 of the *Draf-1* gene (Fig. 4); however, several interesting features are still identifiable. Of the 16 proline residues (residues implicated in determining the secondary folding of a peptide) predicted in the 291-amino acid sequence of *Draf-1*, half are located in the last 85 amino acids and all but one are shared with all members of the *raf* and *pks* subfamilies. Also conserved is the location of a putative N-glycosylation site (positions 284 to 286, Fig. 4) shared by the *raf* subfamily members (positions 277 to 279, Fig. 4) but lacking in the only member of the *pks* subfamily so far sequenced. Finally, of the five cysteine residues distributed through the *Draf-1* kinase domain only two (positions 84 and 248, Fig. 4) are invariantly located in all members (including the avian homolog, *mht/mil*; not shown) of the *raf* and *pks* subfamilies. We feel that the stability of these primary sequence elements through the millions of years of evolution testifies to their importance to the function of these kinases.

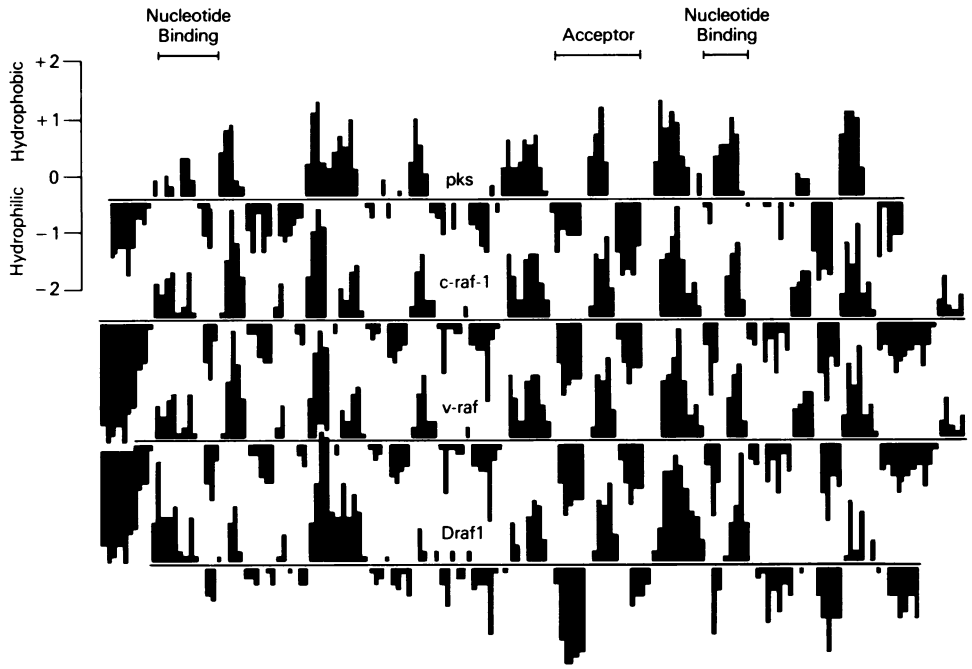


FIG. 6. Hydropathy profiles of *raf* and *pks* subfamily kinase domains. The ordinate is an arbitrary scale with hydrophobic (+) values above zero and hydrophilic (−) values below zero, according to Hopp and Woods (12). The plots for each predicted amino acid sequence were aligned so that their ATP-binding domains overlapped. Also indicated is the region analogous to the tyrosine acceptor domain of the tyrosine-specific protein kinases, as well as a region which represents the last area of primary sequence homology and may be part of the nucleotide-binding site. Amino acid sequences depicted begin at position 1 for *pks* (23), v-*raf* (21), and *Draf-1* and at position 96 for c-*raf*-1 (6).

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