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Tissue Localization of *Drosophila melanogaster ras* Transcripts during Development

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Three *ras* homologs have been identified in *Drosophila melanogaster*. Here we describe the tissue distribution of their transcripts as analyzed by in situ hybridization. The RNAs of the three genes show a similar distribution at every developmental stage examined. In embryos, the transcripts are uniformly distributed. In larvae, *ras* transcripts are restricted to dividing cells (e.g., imaginal disks, gonads, and brain). At the adult stage, several tissues contain *ras* transcripts. The strongest hybridization signals are localized to the adult ovaries and to the cortex of the brain and ganglia, which at this stage are comprised of differentiated, nondividing cells. The tissue distribution of *ras* transcripts in *D. melanogaster* suggests that the *ras* proteins have multiple roles during development which may be related to both the proliferative and differentiated states of the tissues.

The vertebrate p21 *ras* proteins are membrane-associated proteins (33) which have GTP-binding activity (18). They were shown to be involved in a variety of viral (5) and non-virus-associated tumors. In human and rodent tumors, *ras* genes were shown to be activated by a point mutation in codon 12 (21, 27) or codon 61 (29, 36). The GTP-binding activity and the structural homology to other GTP-binding proteins such as transducin (28) have suggested that p21 *ras* may play a role as a transducer of mitogenic signals from the plasma membrane into the cell. Indeed, it appears that mutations which lead to oncogenic activation of *ras* exert their effect by reducing an intrinsic GTPase activity of *ras* (14, 26), similar to the effect of various bacterial toxins or GTP analogs on G proteins (8). Under these conditions, the G proteins transmit the signal continuously without being triggered by the appropriate receptor.

ras genes were found to be highly conserved in evolution and so far are the only oncogenes shown to be conserved not only in *Drosophila melanogaster* (16, 23) but also in yeasts (6, 20) and slime molds (22). In yeasts, the *ras* proteins appear to regulate the adenylate cyclase pathway (31), whereas in vertebrates, they probably participate in a different pathway (3). In addition to the high degree of structural conservation, another feature of *ras* which has been conserved from yeasts to humans is the multigene nature of this family. There are two genes in bakers' yeast (*Saccharomyces cerevisiae*) which show a high degree of homology to *ras* (20) and three *ras* genes in *D. melanogaster* (16) as well as in vertebrates (5, 29).

The most conserved domain of *ras* within each species and between species is the N terminus, which is also responsible for the GTP-binding activity (9, 30). A variety of structural manipulations of *ras* genes have shown that this domain is responsible for interaction with the next component in the presumptive signal transduction pathway (4, 12, 23, 35). Alterations in the variable C-terminal sequences did not affect the function of *ras* in mammalian cells or in yeasts. The role of these sequences and the possibility that they confer a unique function to each of the *ras* genes thus remain open issues.

In *D. melanogaster*, three *ras* genes termed *Dras1*, *Dras2*, and *Dras3* have been isolated and mapped to positions 85D, 64B, and 62B, respectively, on the third chromosome (16). Structurally they differ from each other primarily at the C terminus, and all have a predicted molecular weight of 21,000 (16, 23). Analysis of the transcription pattern of the *ras* genes in *D. melanogaster* has shown that all three are expressed at similar levels in the embryonic, larval, pupal, and adult stages (13). In this paper, we describe the tissue distribution of the three *D. melanogaster ras* transcripts, monitored by in situ hybridization. The results show a similar tissue distribution for the three transcripts. The distribution of *ras* transcripts is correlated with cell division only at the embryonic and larval stages. The presence of *ras* transcripts in differentiated adult neural tissues suggests that the role of p21 *ras* proteins is not restricted to cell proliferation.

MATERIALS AND METHODS

SP6 probes. Labeled single-stranded RNA probes were prepared by incubation of 1-μg linearized SP6 plasmids containing the different *ras* inserts in the standard SP6 reaction mixture (15) in the presence of [α-^{32}P]UTP or [α-^{35}S]UTP. The concentration of cold UTP was adjusted to 5 μM. ^{32}P probes were used directly for Northern or Southern blots. ^{35}S probes were alkali treated before in situ hybridization by incubation for 1 h at 60°C in 0.5 M $NaHCO_3$–0.6 M Na_2CO_3 in 400 μl to reduce the probe size to 50 to 200 base pairs. The alkali treatment was stopped by the addition of 15 μl of 3 M sodium acetate (pH 6.0) and 2.5 μl of glacial acetic acid. The probe was ethanol precipitated and suspended in a small volume of H_2O. Probe sizes before and after alkali treatment were monitored by loading 5×10^5 cpm of probe on 5% acrylamide–urea sequencing gels. Routinely, about 5×10^7 cpm was obtained after [α-^{35}S]UTP labeling of 1 μg of DNA template.

Northern blotting. Samples (2 μg) of adult poly(A)$^+$ RNA were loaded on a 1.5% agarose–formaldehyde gel. Prehybridization and hybridization solutions contained 50% form-

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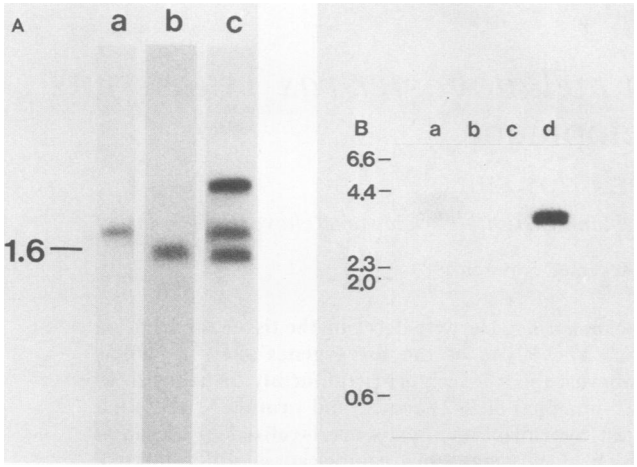

FIG. 1. (A) *ras* transcripts in *D. melanogaster* adults. Samples (2 μg) of adult poly(A)$^+$ RNA were loaded on each lane, transferred to nitrocellulose filters, and hybridized with a ^{32}P-labeled, single-stranded SP6 probes containing the antisense strand of (a) *Dras1*, (b) *Dras2*, or (c) *Dras3*. The size marker is given in kilobases and corresponds to the size of the *Dras2* transcript. (B) The *Dras3* probe is unique. Samples (1 μg) of DNA of *Dras1*, *Dras2*, *Dras3*, and v-*ras*^{H} clones were digested, size fractionated, transferred to a nitrocellulose filter, and hybridized with a ^{32}P-labeled *Dras3* SP6 probe. Lanes contained (a) DNA of the *Dras1* SP6 clone (see text) digested with *Eco*RI and *Hin*dIII to generate a 700-base-pair *Dras1* fragment, (b) DNA of the *Dras2* genomic clone inserted in the *Pst*I site of pBR322 and digested with *Pst*I to yield a 3.5-kilobase *Dras2* fragment, (c) plasmid DNA of the v-*ras*^{H} clone digested with *Bam*HI and *Eco*RI to give a 2.2-kilobase v-*ras*^{H} fragment, and (d) phage DNA of the *Dras3* genomic clone digested with *Eco*RI to yield a 3.5-kilobase *Dras3* fragment. Size markers are given in kilobases.

amide, 5× SSCPE (0.15 M NaCl, 0.015 M sodium citrate, 13 mM KH_2PO_4 [pH 7.0], 1 mM EDTA), 2.5× Denhardt solution, 0.2 mg of boiled salmon sperm DNA per ml, and 0.1% sodium dodecyl sulfate. About 10^7 cpm of probe was added per 10 ml of hybridization solution. Prehybridization and hybridization were carried out at 52°C. The filters were washed in 0.5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 67°C and exposed to X-ray film.

In situ hybridization. The production of 8-μm-thick frozen tissue sections and subsequent fixation and dehydration procedures were as described by Hafen et al. (10). The hybridization solution contained 50% formamide, 0.6 M NaCl, 1× Denhardt solution, 10 mM Tris hydrochloride (pH 7.5), 1 mM EDTA, 0.2 mg of tRNA per ml, 10% dextran sulfate, 10 mM dithiothreitol, and about 5×10^5 cpm of probe per slide. Hybridization was carried out overnight at 45°C. The slides were subsequently washed at 45°C overnight in 50% formamide–0.6 M NaCl–Tris hydrochloride (pH 7.5)–1 mM EDTA–10 mM dithiothreitol, with several changes of the wash solution, and air dried. After immersion in Kodak NTB2 emulsion, the slides were exposed at 4°C for 3 to 10 days, developed, and stained with Giemsa. The slides were subsequently mounted in Permount and examined by bright- and dark-field microscopy with a Zeiss microscope.

RESULTS

***ras* probes.** To increase the sensitivity of hybridization, we prepared strand-specific probes for each of the three *ras* genes. Fragments of genomic clones that were previously shown to contain only the transcribed *ras* sequences or fragments of cDNA clones were subcloned into the SP6 plasmid. For the *Dras1* probe, we used a 700-base-pair *Eco*RI-*Nco*I restriction fragment of a cDNA clone containing 5′ noncoding sequences and 330 nucleotides which code for the N terminus of the protein (16). This fragment was inserted in both orientations to also provide the sense strand as a negative control for hybridization. As a *Dras2* probe, we used a 600-base-pair *Hin*dIII-*Eco*RI fragment from the genomic clone, which contains coding sequences from residue 52 to the C terminus as well as some 3′ noncoding sequences (16). For *Dras3*, we used a 1.5-kilobase *Eco*RI-*Pst*I fragment from the genomic clone. This fragment contains 5′ noncoding sequences as well as a sequence coding for the N-terminal 93 residues (23). To generate the probe, this plasmid was cleaved within an internal *Cla*I site to yield a 1.2-kilobase probe. Again, this probe includes only *Dras3*-transcribed sequences.

To verify that the different probes do not cross-react, ^{32}P-labeled probes representing the antisense strand of each gene were prepared and hybridized with adult poly(A)$^+$ RNA under conditions similar to those of the in situ hybridization (Fig. 1A). *Dras1* and *Dras2* probes each hybridized with a unique transcript. The *Dras3* probe detected three transcripts. Previous hybridizations with a *Dras3* cDNA probe have shown a similar pattern (13). Although the two lower bands of *Dras3* hybridization comigrate with the *Dras1* and *Dras2* transcripts, they are not likely to represent cross-hybridization with these transcripts. Characterization of the *Dras3* genomic clone has shown that its hybridization to the v-*ras*^{H} probe was restricted to the *Pst*I-*Ava*I fragment of *Dras3* (16), although the homology at the amino acid level extends also to the coding sequences located 5′ of the *Pst*I site (23). Since the *Dras3* probe we have generated contains only the sequences 5′ to the *Pst*I site, we do not expect it to cross-hybridize with the other two *Dras* genes. To verify that the *Dras3* probe is unique, we hybridized it to a filter containing cloned DNAs of *Dras1*, *Dras2*, *Dras3*, and v-*ras*^{H} under conditions similar to the in situ hybridization. We detected hybridization only to the *Dras3* DNA band (Fig. 1B), indicating that the probe is indeed unique.

Embryos. Sections of embryos from two stages were examined. We used 10- to 14-h-old embryos, which represent roughly the midpoint of embryogenesis, and 21-h-old embryos, which represent the final stages of embryonic development.

Major structures such as brain, ganglia, and gut are already present at 10 h. Hybridization of the three *Dras* probes to specific organs could not be detected in the two embryonic stages examined. Figure 2 shows the uniform pattern of hybridization observed in the embryonic sections. No hybridization above background could be detected with the sense-strand probe of *Dras1* (data not shown).

Larvae. Three types of tissues can be distinguished during larval development. The majority of larval tissues are comprised of large polytenized cells which are functional only during the larval phase and are histolyzed during the pupal stage (for example, the salivary glands and the larval hypoderm). Another set of tissues includes the imaginal disks, which give rise to the adult integument after metamorphosis but are not required for larval development. The imaginal disks are comprised of small diploid cells which are continuously dividing during the larval stage (19). The third class of tissues includes the brain and thoracic and abdominal ganglia. These organs are functional during the larval period as well as during the adult stage.

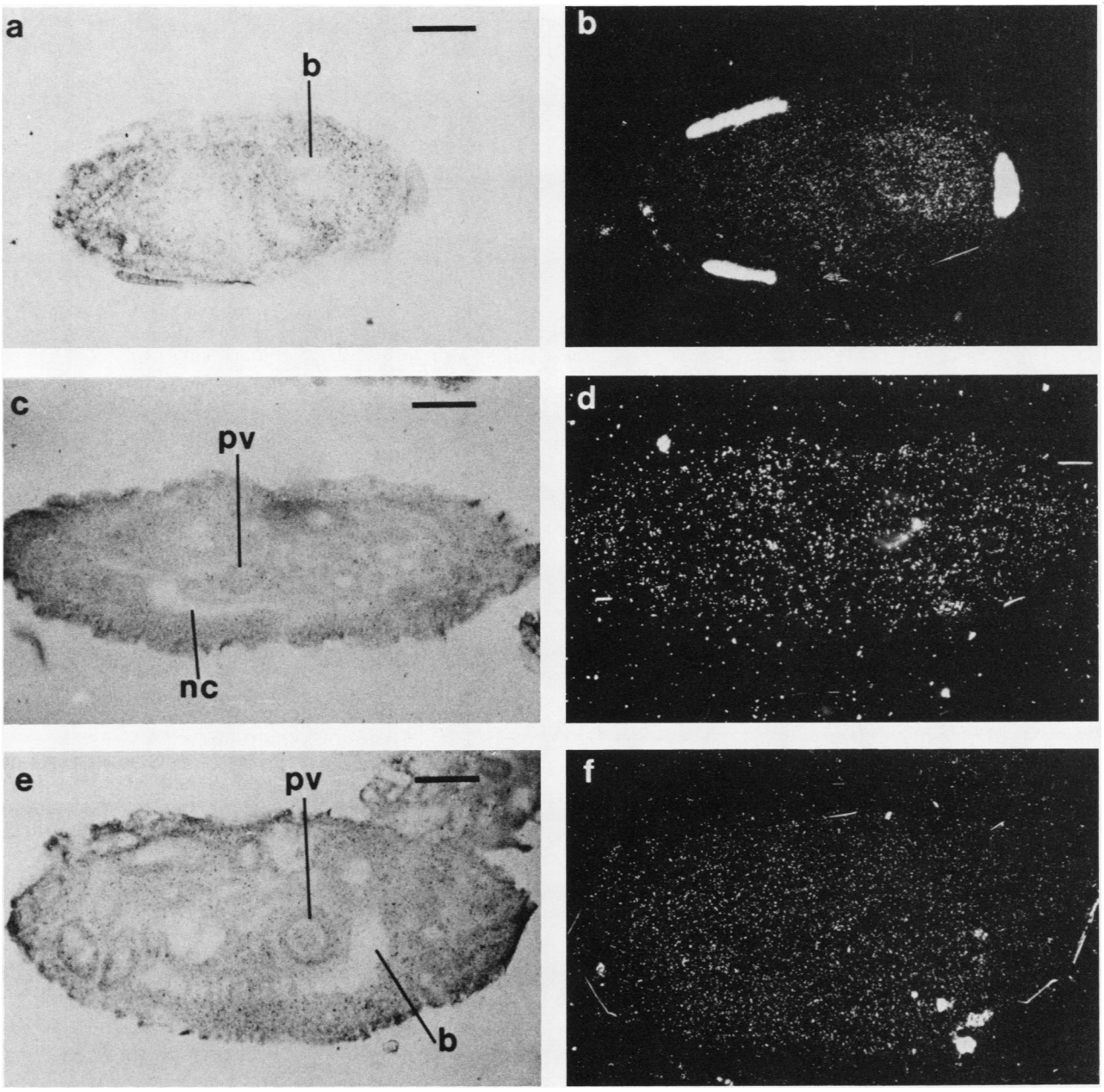

FIG. 2. Localization of *ras* transcripts in embryos. Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography and staining. (a and b) Horizontal section of a 10-h embryo hybridized with a *Dras1* probe. The anterior part is to the right. (c and d) Sagittal section of a 26-h embryo hybridized with a *Dras2* probe. The anterior part is to the left. (e and f) Sagittal section of a 14-h embryo hybridized with a *Dras3* probe. The anterior part is to the right. Abbreviations: b, brain; nc, nerve cord; pv, proventriculus. Bars, 0.05 mm.

The three *ras* probes showed a similar and highly localized distribution of transcripts in the mature larva (Fig. 3 and 4). Only two of the three above-mentioned categories of tissues within the larva exhibited hybridization to the probes. No hybridization to the polytenized tissues, including the salivary glands, fat cells, and hypoderm, was observed. On the other hand, very distinct hybridization to the imaginal disks was detected. It includes, most notably, the eye-antenna disks, the wing disks, the genital disks, and the anlagen of the testes and ovaries. Within the disks, the pattern of hybridization appeared to be uniform.

Distinct hybridization of the *ras* probes was also observed in the cortex of the larval brain. No hybridization was seen in the interior of the brain at the site of the neuropile, where the neural dendrites and axons are concentrated. Since the neuropile contains extentions of brain cortex cell bodies, the lack of hybridization is likely to reflect a lower overall RNA content in the neuropile. The pattern of hybridization appeared uniform throughout the cortex, and the intensity of hybridization to the brain was similar to that of the imaginal disks. The control hybridization with the sense strand of *Dras1* as a probe showed no specific hybridization and demonstrated that the background hybridization in our experiments is low (Fig. 3).

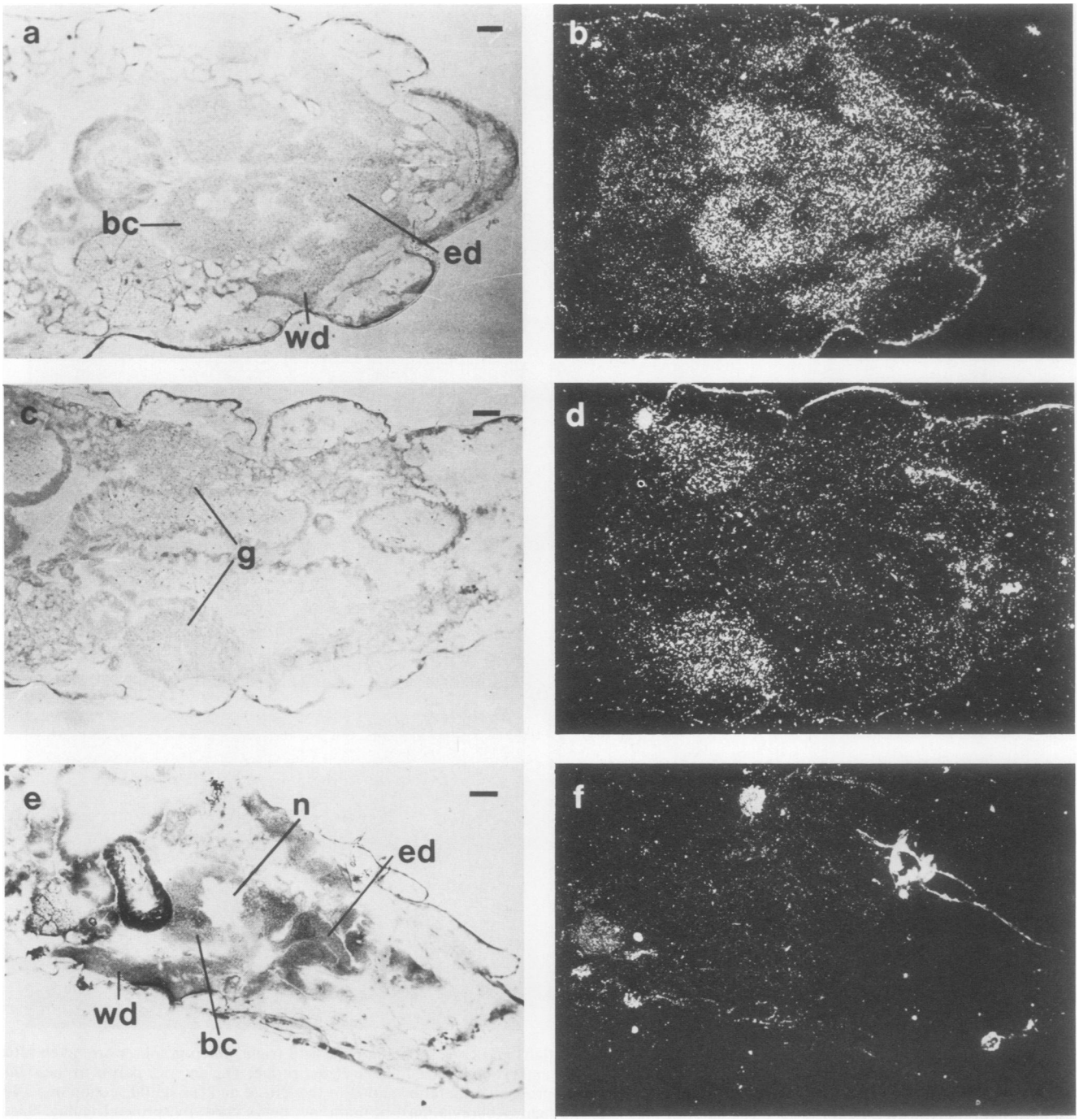

FIG. 3. Localization of *Dras1* transcripts in mature larvae. Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography and staining. (a and b) Frontal horizontal section of a third-instar larva, showing hybridization of the *Dras1* probe with the brain cortex (bc), eye-antenna imaginal disk (ed), and wing disk (wd). (c and d) Horizontal section of the posterior part of a third-instar larva, showing hybridization of the *Dras1* probe with gonad precursor cells (g). The anterior part is to the left. (e and f) Frontal section of a third-instar larva hybridized with a probe representing the sense strand of *Dras1*. No specific hybridization is observed. n, Neuropile. Bars, 0.05 mm.

Adults. The hybridization of the *Dras* probes in adults was localized to several tissues (Fig. 5). It was clear that the transcripts were present at higher concentrations in the cortex of the brain and in the thoracic and abdominal ganglia. They were also detected, however, in other tissues, such as the flight muscles. In the abdomen, the organs with the highest content of transcripts were the ovaries. The exceptionally high levels of *ras* transcripts in the ovaries indicate that the high levels of *ras* RNAs previously found in unfertilized eggs (13) are produced in the ovaries and deposited in the developing eggs, where they are stored as maternal RNA. Figure 5 shows the results with the *Dras3* probe. Similar results were also obtained with *Dras1* and *Dras2* probes.

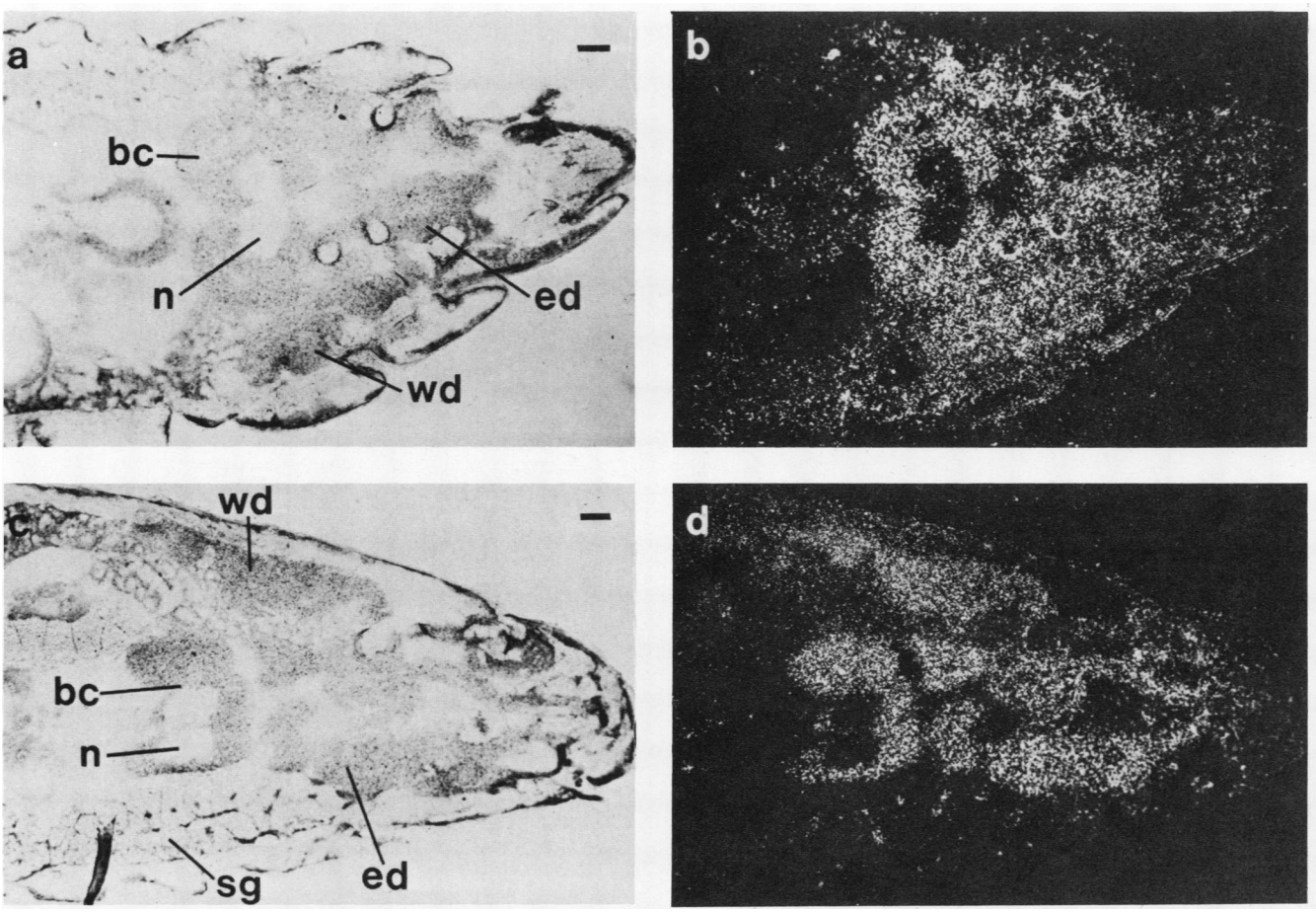

FIG. 4. Localization of *Dras2* and *Dras3* transcripts in mature larvae. Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography. (a and b) Frontal horizontal section of a third-instar larva showing hybridization of the *Dras2* probe with the brain cortex (bc), eye-antenna disk (ed), and wing disk (wd). No hybridization can be detected in the neuropile (n). (c and d) Frontal section of a third-instar larva hybridized with a *Dras3* probe. sg, Salivary gland. Bars, 0.05 mm.

DISCUSSION

We describe here the localization of transcripts of the three *D. melanogaster ras* genes during development. Our studies shed light on some of the issues concerning the function of *ras*. First, it is clear from the results that all three *ras* genes are functional in the same tissues, since the different transcripts have a very similar spatial distribution. The resolution of in situ hybridization does not allow us to determine whether in a given tissue all three transcripts are expressed within the same cells. However, the uniform distribution of hybridization in the different tissues argues against the possibility that each of the three *ras* transcripts is localized to a different subset of cells within the same organ.

Our results raise the question of why the coordinate expression of three *ras* genes is required in a given tissue. Structure-function studies have suggested that the N terminus, which is conserved among all *ras* proteins, is responsible for the activation of the targets of *ras* (4, 12, 23, 35). It thus appears that the different *ras* proteins may be stimulated by different signals but subsequently activate the same targets within the cell. If the different members of the *ras* family in a given species indeed have distinct functions, the specificity would be dictated by the variable residues at the C terminus. Whether these variable sequences determine interaction with different triggering signals such as receptors is not known. If this were the case, then the coexpression of different *ras* proteins within the same tissue that we have observed may broaden the spectrum of signals which can be transduced by *ras*. This hypothesis becomes especially attractive if we consider the possibility of regulation by synergistic effects of different signals, in the same cell or tissue, each signal being transduced by a different *ras* protein.

The developmental profile of *ras* transcripts is complex and suggests that the *ras* proteins have dual or multiple roles during development. With regard to embryogenesis, although *ras* transcripts are present in maternal RNA, it is difficult to determine whether the *ras* proteins have any role in the regulation of the early cycles of nuclear division. The first 12 divisions in the embryo take place without concomitant cytokinesis and give rise to a syncytial blastoderm lacking cell membranes that partition its numerous nuclei from each other. Yet, the transforming function of p21 *ras* in mammalian cells was shown to be tightly associated with its ability to be attached to the plasma membrane (34). Thus, *ras* proteins may not participate in the regulation of the early nuclear divisions in the *D. melanogaster* embryo. Later during embryogenesis, ample cell membranes are formed, and two or three additional cycles of cell division take place (11). *ras* proteins may participate in the regulation of these divisions.

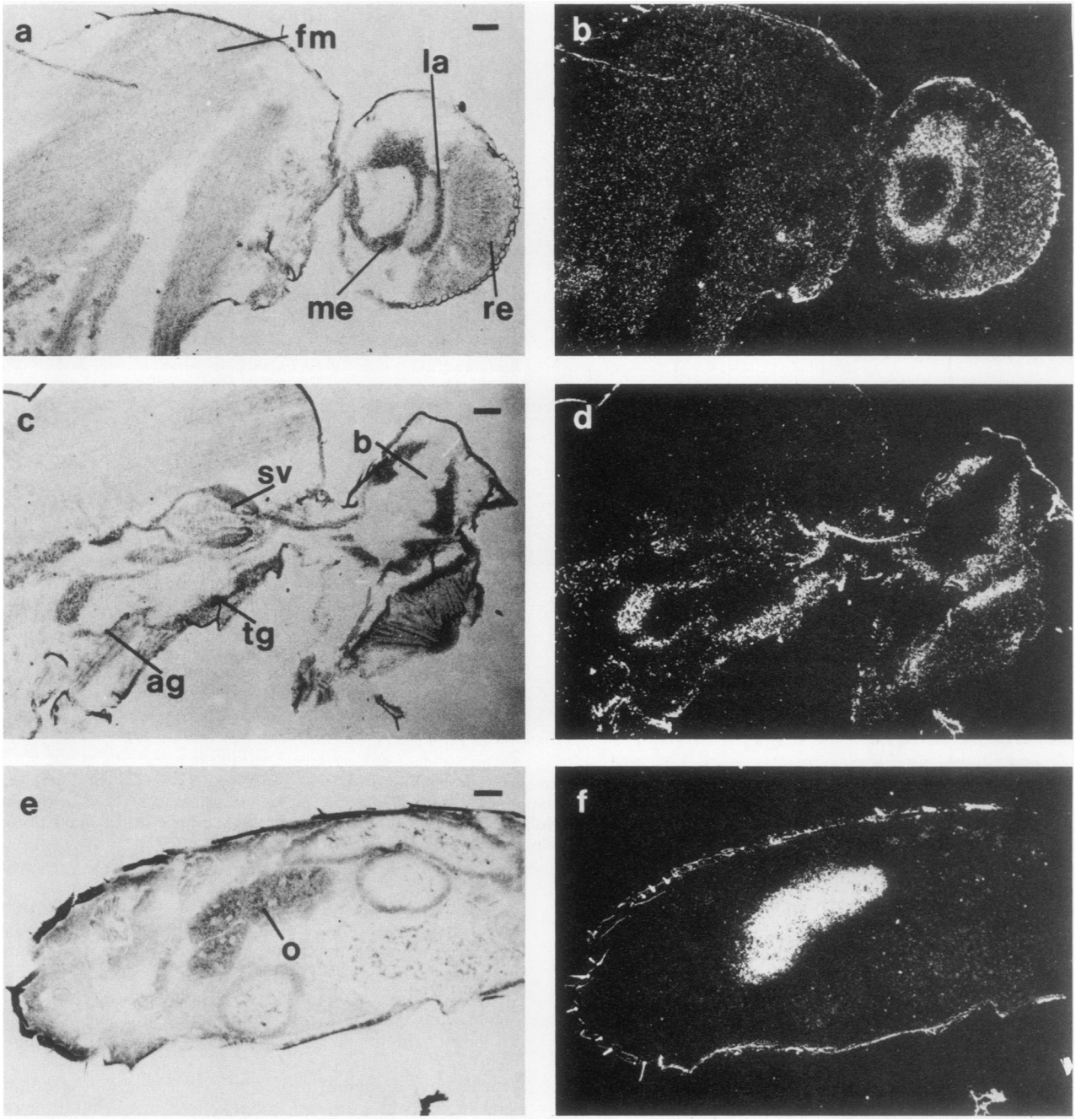

FIG. 5. Localization of *Dras3* transcripts in *D. melanogaster* adults. Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography and staining. (a and b) Sagittal section showing hybridization of the *Dras3* probe to the retina (re), lamina (la), and medula (me) and also to the flight muscles (fm), although less intensely. (c and d) Sagittal section showing hybridization of the *Dras3* probe to the brain cortex (b), thoracic ganglia (tg), and abdominal ganglia (ag). sv, Stomodeal valve. (e and f) Sagittal section of a female abdomen, showing hybridization of the *Dras3* probe to the ovaries (o). Bars, 0.05 mm.

The dramatic concentration of *ras* transcripts in proliferating cells during larval development suggests that the *ras* proteins are essential components in the division of the epithelial cells which constitute the imaginal disks. Our results from the larval stage indicate that *ras* functions may not be strictly limited, however, to proliferating cells. Extensive thymidine pulse-chase experiments have shown that in the larval brain only the neuroblasts at the periphery of the cortex are dividing (32). The two products of this division have different developmental fates. One cell continues to divide as a neuroblast, whereas its sibling undergoes another division whose products differentiate to mother ganglion cells. The position of the mother ganglion cells within the cortex is determined by the stage in which they were generated, older cells being closer to the center (32). Therefore, the uniform distribution of *ras* transcripts within the cortex suggests that the proteins have roles which are not only related to cell proliferation but may also be associated

with the developmental events which occur in the cortex cells. Since the time scale of larval development is several days, it seems unlikely that *ras* RNAs within the cortex represent transcripts remaining from the neuroblast stage. Association of *ras* transcripts with differentiated nervous tissue is more dramatically observed in the adult stage. All three *Dras* transcripts are more abundant in the cortex of the brain and in the thoracic and abdominal ganglia, although hybridization to other tissues is also observed.

The possible association of oncogenes with processes of differentiation has been recently suggested from observations in several cell types and organisms. Both *ras* and *src* oncogenes were shown to have an effect similar to that of nerve growth factor in the induction of differentiation of pheochromocytoma PC12 cells (1, 2, 17). In addition, the tissue distribution of c-*src* transcripts in *D. melanogaster* (24) and of the c-*src* protein in chickens (7, 25) suggests that c-*src* plays a role in the differentiation or maintenance of the nervous system. Likewise, we find that the transcripts of the *D. melanogaster* epidermal growth factor receptor homolog have a tissue distribution similar to that of *Dras*, indicating that this oncogene may also be associated with proliferating as well as differentiated tissues (Schejter et al., submitted).

In conclusion, the broad range of tissues where *ras* transcripts are detected during *D. melanogaster* development suggests a pleiotropic role for these proteins during growth and development. Thus, despite the high degree of structural conservation of *ras* from yeasts to humans, *ras* proteins are likely to have distinct roles in unicellular and multicellular organisms. It appears that the oncogenic or growth-promoting activity of *ras* represents only one facet of its full repertoire of biological activities.

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