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Tissue Localization of *Drosophila melanogaster* Insulin Receptor Transcripts during Development

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The *Drosophila melanogaster* insulin receptor (*Drosophila* insulin receptor homolog [dIRH]) is similar to its mammalian counterpart in deduced amino acid sequence, subunit structure, and ligand-stimulated protein tyrosine kinase activity. The function of this receptor in *D. melanogaster* is not yet known. However, a role in development is suggested by the observations that levels of insulin-stimulated kinase activity and expression of dIRH mRNA are maximal during *Drosophila* midembryogenesis. In this study, a 2.9-kilobase (kb) cDNA clone corresponding to both the dIRH tyrosine kinase domain and some of the 3′ untranslated sequence was used to determine the tissue distribution of dIRH mRNA during development. Two principal mRNAs of 11 and 8.6 kb hybridized with the dIRH cDNA in Northern (RNA) blot analysis. The abundance of the 8.6-kb mRNA increased transiently in early embryos, whereas the 11-kb species was most abundant during midembryogenesis. A similar pattern of expression was previously determined by Northern analysis, using a dIRH genomic clone (L. Petruzzelli, R. Herrera, R. Arenas-Garcia, R. Fernandez, M. J. Birnbaum, and O. M. Rosen, Proc. Natl. Acad. Sci. USA 83:4710–4714, 1986). In situ hybridization revealed dIRH transcripts in the ovaries of adult flies, in which the transcripts appeared to be synthesized by nurse cells for eventual storage as maternal RNA in the mature oocyte. Throughout embryogenesis, dIRH transcripts were ubiquitously expressed, although after midembryogenesis, higher levels were detected in the developing nervous system. Nervous system expression remained elevated throughout the larval stages and persisted in the adult, in which the cortex of the brain and ganglion cells were among the most prominently labeled tissues. In larvae, the imaginal disk cells exhibited comparatively high levels of dIRH mRNA expression. The broad distribution of dIRH mRNA in embryos and imaginal disks is compatible with a role for dIRH in anabolic processes required for cell growth. The apparently elevated expression of dIRH mRNA in nervous tissue during mid- and late embryogenesis coincides with a period of active neurite outgrowth and suggests that dIRH may be involved in this process.

The mammalian insulin receptor and its *Drosophila* homolog are cell surface, transmembrane glycoproteins that possess ligand-dependent protein tyrosine kinase activity (5, 6, 23–25, 34). The molecular cloning of the cDNA encoding the human insulin proreceptor revealed that its kinase domain was homologous to those of the oncogene-encoded protein tyrosine kinases and the cytoplasmic domain of the epidermal growth factor (EGF) receptor (5, 34). This similarity suggested that insulin and its receptor may be directly involved in the regulation of growth and differentiation. While the mitogenic properties of insulin have been studied with a variety of cultured cells (10), little is known about the action of insulin as a growth factor during normal development.

To address this question, we have been studying an insulin receptor homolog in *Drosophila melanogaster* (dIRH). *D. melanogaster* and other insects synthesize a molecule or group of molecules which exhibits immunological cross-reactivity with insulin, as well as similar bioactivity in both mammalian cells and insect larvae (4, 32). The putative receptor for the *Drosophila* insulin-like ligand is homologous to the human insulin receptor (hIR) in both its deduced primary amino acid sequence and subunit structure (6, 22–24). Like hIR, dIRH is a plasma membrane glycoprotein composed of alpha subunits (M_r, 110,000 to 120,000), which bind insulin, and transmembrane beta subunits (M_r, 95,000), which contain protein tyrosine kinase domains and are autophosphorylated on tyrosyl residues (6, 23–25). Similarly, dIRH subunits are derived by processing from a proreceptor with an M_r of 200,000 to 210,000 (6). Unlike hIR, the dIRH oligomer can also include a protein with an M_r of 170,000 which is covalently linked to the alpha subunit (6). This component is phosphorylated on tyrosyl residues in response to insulin and is immunologically related to the human and *Drosophila* beta subunits (6). The significance of this subunit is not known.

The dIRH kinase domain has been localized by in situ hybridization of a genomic probe (24) to position 93E of the polytene chromosomes (unpublished observations). The deduced amino acid sequence of the tyrosine kinase domain of dIRH, compared with a number of *src*-related kinases, is most homologous to that of hIR (24). Overall amino acid sequence identity is 53%, with a region of approximately 70 amino acids which are greater than 90% identical. dIRH is also homologous to the human insulin-like growth factor I receptor (35) and, somewhat less so, to v-*ros*. Less extensive homology has been noted for the kinase domains of the *Drosophila abl* gene and EGF receptor homologs (24).

dIRH specifically binds mammalian insulin. Its protein tyrosine kinase is activated by insulin but not by insulin-like growth factors I and II, EGF, or silkworm prothoracicotropic hormone (6). The latter, interestingly, is homologous to mammalian insulin (21). Thus far, dIRH is the only peptide growth factor receptor homolog in *Drosophila* for which a ligand, albeit a mammalian one, is available.

Previous reports have shown that both the insulin-dependent protein tyrosine kinase activity (25) and the abundance of dIRH mRNA (24) are highest during *Drosophila* midem

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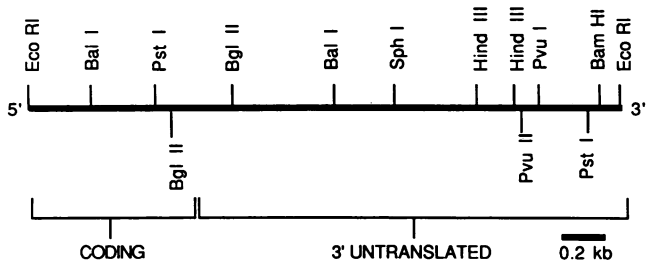

FIG. 1. dIRH cDNA restriction map. The coding sequence corresponds to the C-terminal third of the dIRH beta subunit, beginning at nucleotide 2458 of the previously reported cDNA sequence (22). The 0.65-kb *Eco*RI-*Pst*I fragment was subcloned into Bluescribe to yield an antisense RNA probe containing only the coding sequence (coding sequence probe). The entire clone in Bluescribe was linearized by using *Bgl*II to yield an antisense RNA probe corresponding to the 1.9-kb *Bgl*II-*Eco*RI 3′ untranslated sequence (3′ untranslated probe).

bryogenesis. While dIRH mRNA is present throughout the *Drosophila* life cycle, the elevated level of expression during early development suggests that the receptor may be playing a role in events which occur at this time.

In the present study to analyze the functional role of dIRH, we used in situ hybridization to determine the tissue distribution of dIRH mRNA during development. The pattern found appears to be different from that reported for two other *Drosophila* protein tyrosine kinases, the *Drosophila src* gene, and the *Drosophila* EGF receptor homolog (dERH) but similar to that reported for the *Drosophila ras* gene (12, 28, 30, 31).

MATERIALS AND METHODS

***Drosophila* culture.** Wild-type *D. melanogaster* (Canton S) was maintained at 25°C.

Isolation of dIRH cDNA clone. The dIRH cDNA clone used in this study was isolated from a 3- to 12-h embryo lambda gt10 cDNA library (generously provided by L. Kauvar). Library screening was done according to Maniatis et al. (18). A 2.9-kilobase (kb) cDNA was isolated by hybridization to a 0.8-kb *Bam*HI-*Bgl*II fragment of *Drosophila* genomic clone 18-16 (24) encoding a portion of the tyrosine kinase domain of dIRH. Sequence analysis revealed that the cDNA clone includes nucleotides 2458 to 3586 of the cDNA sequence reported by Nishida et al. (22) and additional 3′ untranslated sequence. The coding region of the cDNA clone corresponds to approximately 60% of the cytoplasmic portion of the dIRH beta subunit, including the tyrosine kinase domain. In addition, it contains approximately 1.9 kb of the 3′ untranslated sequence. The cDNA was subcloned into the *Eco*RI site of the Bluescribe vector (Stratagene Cloning Systems, San Diego, Calif.) for use in subsequent analyses.

Probe synthesis. For in situ hybridization, the dIRH cDNA clone in the Bluescribe vector was linearized by digestion with *Bgl*II to produce a 1.9-kb antisense RNA probe consisting of only the 3′ untranslated sequence (Fig. 1). The 0.65-kb *Pst*I-*Eco*RI coding fragment was subcloned separately into Bluescribe, linearized by digestion with *Eco*RI or *Pst*I, and transcribed to produce antisense or sense RNA probes, respectively. Transcription reactions were carried out according to the instructions of the manufacturers, with α-^{35}S-labeled UTP (New England Nuclear Corp., Boston, Mass.) used to radiolabel RNA. After ethanol precipitation of radiolabeled RNA, the probes were base treated for 40 min at 60°C in 0.2 M carbonate buffer (pH 10.2) to reduce the probe sizes.

For Northern (RNA) blot analysis, the probes were nick translated to a specific activity of 1×10^8 to 2×10^8 cpm/μg of DNA by using [α-^{32}P]dATP and dCTP, according to the method of Maniatis et al. (18).

In situ hybridization. *Drosophila* embryos were collected and allowed to develop for the indicated times at 25°C before dechorionation and fixation by the method of Mitchison and Sedat (20). Late embryos (17 to 20 h) were dechorionated and embedded directly without prefixation. Frozen sections (8 to 12 μm) of embryos, larvae, and adult flies were prepared and collected on polylysine-coated microscope slides. Sections were pretreated and slides were washed after in situ hybridization by the method of Ingham et al. (11). ^{35}S-labeled RNA probes were used at a final concentration of 0.3 to 1.0 ng/μl of a solution containing 50% formamide, 0.3 M NaCl, 10 mM Tris hydrochloride (pH 7.6), 1 mM EDTA, 1× Denhardt solution (0.02% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.], 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin), 10% dextran sulfate, 75 mM dithiothreitol, and 1 mg of yeast RNA per ml. Hybridization was carried out for 16 to 20 h at 50°C. After being washed to remove unbound probe, the slides were subjected to autoradiography by using Kodak NTB2 nuclear track emulsion for 1 to 3 weeks.

Northern analysis. Poly(A)$^+$ RNAs from eggs, staged embryos, and adults were isolated by the method of Kidd et al. (13) and, for some experiments, kindly provided by S. Kidd, Rockefeller University, New York City, N.Y. The RNA (5 μg) was separated on 0.6% agarose gels containing 2.2 M formaldehyde and transferred to Genescreen Plus (New England Nuclear) by using the standard methods. Nick-translated dIRH cDNA probes (5 to 10 ng/ml) were hybridized to blots for 16 to 20 h at 42°C in a solution of 50%

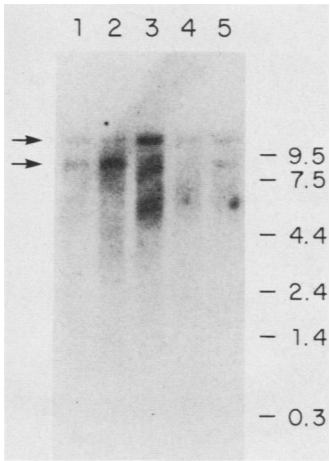

FIG. 2. Northern analysis of dIRH mRNA expression in staged embryos and adults. Poly(A)$^+$ RNAs (5 μg) from unfertilized eggs (lane 1), 0- to 4-h embryos (lane 2), 8- to 12-h embryos (lane 3), male adults (lane 4), and female adults (lane 5) were fractionated on a 0.6% agarose gel containing 2.2 M formaldehyde. After being transferred to Genescreen Plus, the filter was hybridized with the coding sequence probe (Fig. 1) labeled by nick translation. Autoradiography was done for 26 h at −70°C with a Quanta III intensifying screen. The smear in lane 3 in the region of the 8.6-kb transcript is an artifact. The upper arrow points to the 11-kb transcript and the lower arrow points to the 8.6-kb transcript. Standards (in kilobases) are indicated on the right.

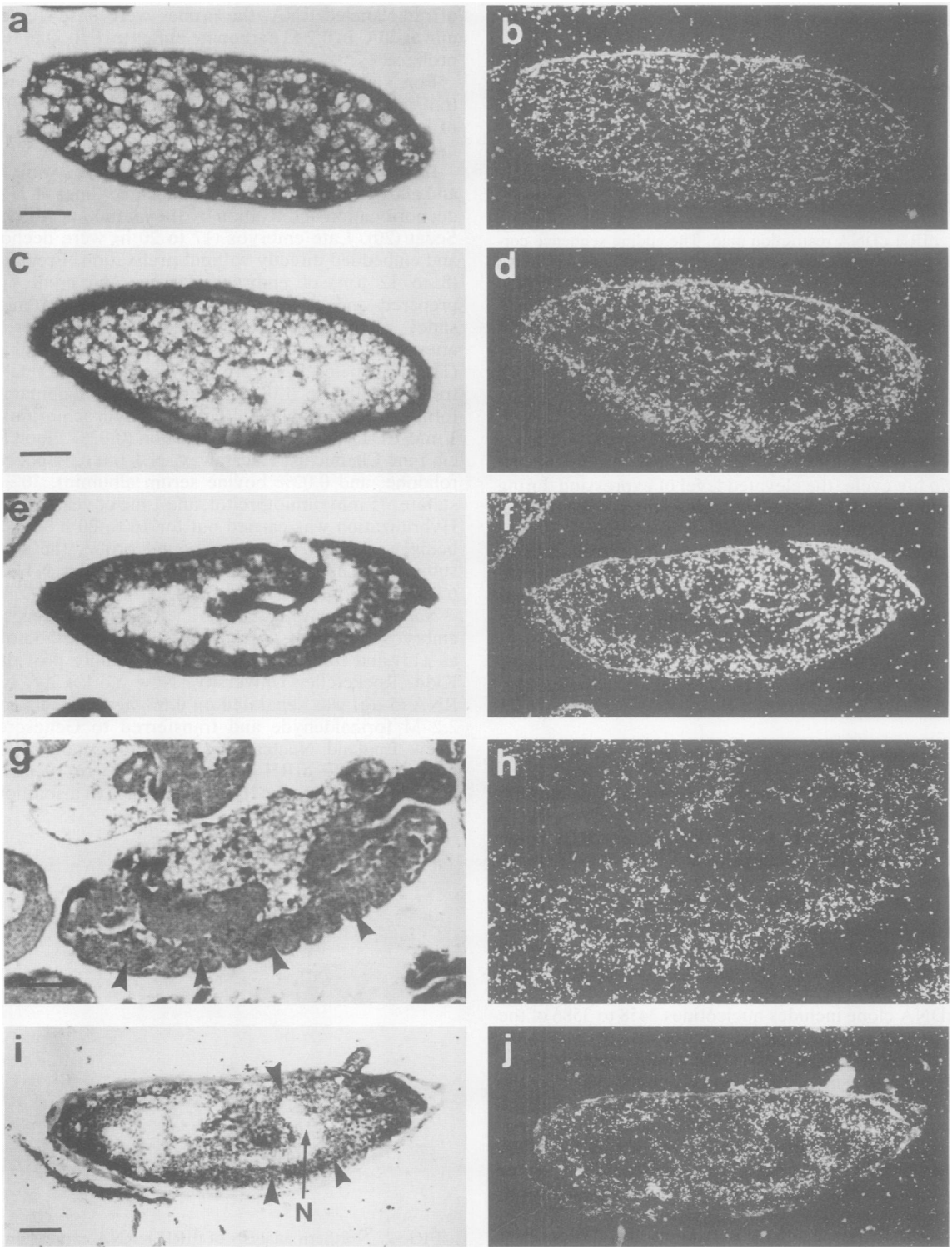

FIG. 3. Localization of dIRH transcripts in *Drosophila* embryos. Bright-field (left) and corresponding dark-field (right) micrographs were taken after autoradiography and staining. The embryos are oriented with anterior ends to the right. (a and b) Syncytial blastoderm embryo (less than 90 min). dIRH transcripts are distributed throughout the embryo. (c and d) Early cellular blastoderm embryo (2 to 3 h). dIRH mRNA appears concentrated over the cortical region where cells are forming. (e and f) Embryo (3 to 6 h) undergoing germ band extension. dIRH mRNA is distributed throughout the cellular portion of the embryo but not in the yolk. (g and h) Embryo (8 to 10 h) having complete germ band retraction. dIRH transcripts are broadly distributed, but more intense hybridization can be detected in the region of the ventral nerve cord (arrowheads). (i and j) Embryo (17 to 20 h). Hybridization is fairly general, but higher levels are observed in the cellular cortex of the brain (arrowheads). The neuropil (N) did not hybridize with dIRH probes. Hybridization was done with antisense RNA corresponding to either the 3′ untranslated probe (panels a to f) or the coding sequence probe (panels g to j). Exposure was for 2 weeks (panels a to f, i, and j) or 1 week (panels g and h). Bar, 0.05 mm.

formamide, 1 M NaCl, 1% sodium dodecyl sulfate, 10% dextran sulfate, 10× Denhardt solution, 0.1% tetrasodium PP_i, and 0.5 mg of denatured salmon sperm DNA per ml. Filters were then washed twice for 5 min each in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature, twice for 30 min each in 2× SSC–1.0% sodium dodecyl sulfate at 60°C, and twice for 30 min each in 0.1× SSC at room temperature.

Materials. α-^{35}S-labeled UTP, [α-^{32}P]dATP, [α-^{32}P]dCTP, and Genescreen Plus were obtained from New England Nuclear. Restriction endonucleases and DNA polymerase I were obtained from New England BioLabs, Inc., Beverly, Mass. T_3 and T_7 RNA polymerases were obtained from Stratagene. RNA ladder molecular weight standards were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Md.

RESULTS

Isolation of dIRH cDNA. The 2.9-kb cDNA clone used in this analysis is shown diagramatically in Fig. 1. It was obtained from a 3- to 12-h embryo lambda gt10 cDNA library. The clone corresponds to the carboxy-terminal third of the beta subunit, which includes the tyrosine kinase domain and approximately 1.9 kb of the 3′ untranslated sequence.

For in situ hybridization studies, single-stranded antisense RNA probes were synthesized after insertion of either the entire cDNA clone or a subclone corresponding to the 0.65-kb *Eco*RI-*Pst*I coding fragment (Fig. 1) into the Bluescribe vector containing T_3 and T_7 RNA polymerase promoters. The construct containing the entire cDNA clone was linearized by using *Bgl*II to obtain an antisense RNA corresponding to only the 3′ untranslated sequence. The coding fragment subclone was linearized by using *Eco*RI to obtain the antisense RNA or by using *Pst*I to obtain the sense transcript which was used as a control for nonspecific hybridization.

Northern analysis. Northern analysis of dIRH transcripts present in poly(A)$^+$ RNAs from staged embryos and adults was performed, and the results are shown in Fig. 2. Two principal transcripts of 11 and 8.6 kb were consistently labeled with the nick-translated 0.65-kb coding fragment used as a probe. The same two species hybridized with the probe containing only the 3′ untranslated sequence, as well as with a probe derived from a genomic clone corresponding to a portion of the dIRH alpha subunit (data not shown). These two RNAs also corresponded in size to those identified earlier in a more detailed Northern analysis, using a genomic clone encoding a portion of the tyrosine kinase domain of dIRH (24). The hybridization of both the 11- and 8.6-kb mRNAs with three distinct probes for the 3′ untranslated, beta subunit, and alpha subunit sequences strongly suggested that these two mRNAs are largely overlapping in sequence and are likely to encode the complete dIRH precursor(s). Multiple smaller transcripts were evident in the RNA isolated from 8- to 12-h embryos (Fig. 2, lane 3). This apparent heterogeneity was also seen with the genomic DNA probe (24) and has been reported for hIR transcripts in placenta and cultured cells (5, 34). Since the genomic sequence encoding the dIRH kinase domain localized to one chromosomal region and since there is only one hIR gene (5, 34), multiple transcripts may reflect alternative RNA processing events. Another possibility is that the mRNA isolated from 8- to 12-h embryos is more easily degraded than mRNA isolated at other times. The smaller transcripts which were observed in RNA from 8- to 12-h embryos (Fig. 2, lane 3) are unlikely to result from cross-hybridization with known *src*-family genes in *D. melanogaster*. The patterns of expression of related genes, such as dERH (6.3 to 6.6 kb [15]), *src* (3.5, 5.0, and 5.5 kb [7, 14, 31, 36]), and *abl* (6.2 kb [14]), are significantly different from that detected with dIRH probes. The *Drosophila abl* and 3.5-kb *src* transcripts, for example, are expressed at their highest levels in unfertilized eggs and 0- to 2-h embryos (14, 31, 36), at which time no lower-molecular-weight mRNAs were detected with dIRH probes (Fig. 2, lanes 1 and 2). Likewise, dERH and the *Drosophila* 5.0- and 5.5-kb *src* mRNAs should be abundant in RNA from 0- to 4-h embryos (14, 15, 31).

There appeared to be developmentally regulated as well as sex-specific differences in the relative abundances of the 11- and 8.6-kb dIRH mRNAs (Fig. 2). In unfertilized eggs and 0- to 4-h embryos, both transcripts were present. Before fertilization, the 11- and 8.6-kb transcripts were present in similar amounts. However, in 0- to 4-h embryos, the 8.6-kb species was relatively increased. This finding suggested either that the 8.6-kb mRNA is more stable than the 11-kb species at this time or that after the initiation of zygotic transcription, the 8.6-kb transcript is preferentially synthesized. By mid-embryogenesis (8- to 12-h embryos), the 11-kb species was more prominent (Fig. 2, lane 3 and legend). In a previous analysis of dIRH mRNAs during each developmental stage, the abundance of the 8.6-kb transcript was found to diminish during later embryogenesis and to become undetectable during larval and pupal development (24).

Adult flies of both sexes contained the 11-kb transcript, but the 8.6-kb mRNA appeared to be expressed at a higher level in females (Fig. 2, lanes 4 and 5). Little if any of the 8.6-kb mRNA was detected in adult males. The presence of the 8.6-kb mRNA in both unfertilized eggs and adult females and its low level or absence in adult males suggested that it

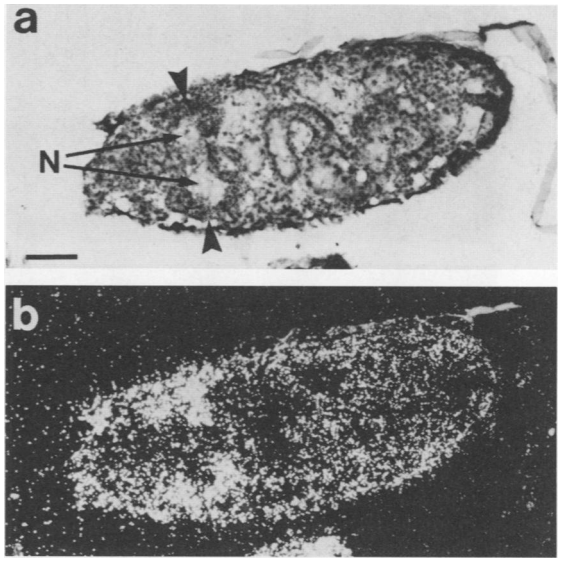

FIG. 4. Localization of dIRH transcripts in 17- to 20-h embryos. Bright-field (a) and corresponding dark-field (b) micrographs of a 17- to 20-h embryo hybridized with antisense RNA corresponding to the 3′ untranslated probe. The anterior end of the embryo is to the left. This horizontal section reveals hybridization in the cellular cortex of both lobes of the brain (arrowheads). The neuropil (N) hybridized poorly. dIRH transcripts were expressed in most cells of the embryo. Exposure was for 2 weeks. Bar, 0.05 mm.

may be synthesized primarily for storage in oocytes as maternally derived RNA.

***In situ* hybridization of dIRH transcripts.** In situ hybridization was carried out by using antisense RNA probes corresponding to the *Eco*RI-*Pst*I coding fragment and the *Bgl*II-*Eco*RI 3′ untranslated sequence (Fig. 1). At all stages examined, both probes gave the same hybridization patterns. A greater intensity of hybridization was sometimes observed with the 1.9-kb 3′ untranslated fragment, most likely because its size was larger than that of the 0.65-kb probe containing only the coding sequence. Control hybridizations with the coding sequence sense strand showed no specific hybridization above background.

In *D. melanogaster*, fertilization is followed by a series of 13 nuclear divisions in the syncytial blastoderm. The nuclei migrate to the periphery of the embryo and are then surrounded by cell membranes to form the cellular blastoderm (2.5 to 3.0 h) (2). A syncytial blastoderm embryo (less than 90 min) is shown in Fig. 3a and b. Hybridization with dIRH probes appeared uniform; autoradiographic silver grains were found throughout the yolk region. Late syncytial blastoderm embryos (1.5 to 3.0 h) exhibited a concentration of grains over the periphery (Fig. 3c and d), where cells were forming after migration of the nuclei to that region. The increased hybridization in the cortex of the embryo at this stage might reflect the redistribution of existing maternally derived transcripts or the activation of transcription by the zygotic nucleus which occurs during cellular blastoderm formation (19).

Gastrulation results in the formation of a primitive mesoderm and rudiments of the anterior and posterior midgut. A process known as germ band extension then occurs, in which cells of the ventral mesoderm and ectoderm (the germ band) extend around the posterior pole and migrate anteri-

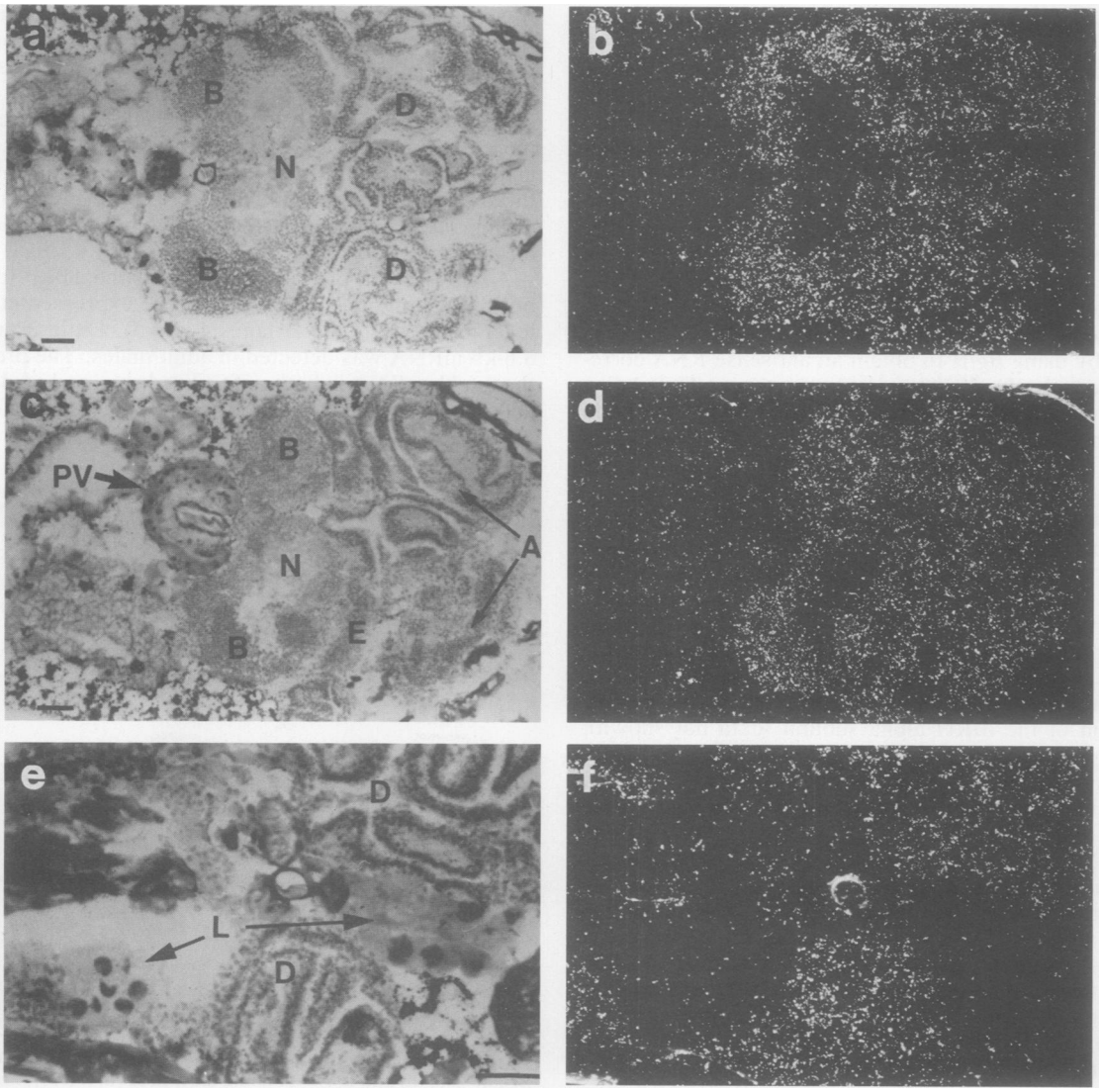

FIG. 5. Localization of dIRH transcripts in third instar larvae. Bright-field (left) and corresponding dark-field (right) micrographs of larvae hybridized with antisense RNA corresponding to the coding sequence probe (a to d) or the 3′ untranslated probe (e to f). The anterior ends of the larvae are to the right. Hybridization to the cellular cortex of the brain (B) (panels a to d) and the imaginal disks (D) (panels a, b, e, and f), including the eye (E) and antenna (A) portions of the eye-antenna disks (panels c and d) is observed. Polytene cells of the larvae (L) (panels e and f), including those of the proventriculus (PV) (panels c and d), hybridized poorly with dIRH probes. Exposure was for 1 week. Bar, 0.05 mm. N, Neuropil.

orly along the dorsal surface of the embryo. Embryonic cells still exhibit mitotic activity at this time, although, with the exception of the precursors of the central nervous system and epidermal sensory organs, most cells undergo only two or three divisions and most mitoses are complete by about 6 h of development. A 3- to 6-h embryo undergoing germ band extension exhibited fairly uniform hybridization over all cells (Fig. 3e and f). The yolk was devoid of grains.

The generalized distribution of dIRH transcripts persisted throughout embryogenesis, as seen in 8- to 10-h (Fig. 3g and h) and 17- to 20-h embryos (Fig. 3i and j). The 8- to 10-h embryo shown in Fig. 3g and h had completed germ band retraction. At this stage, increased hybridization was often found in the ventral region, corresponding to the developing ventral nerve cord (Fig. 3g and h, arrowheads). The embryonic nervous system continued to exhibit a higher level of hybridization relative to that of the rest of the embryo at 17 to 20 h (Fig. 3i and j and Fig. 4a and b). The brain and ventral nerve cord had clearly distinguished cortex and neuropil, with the latter containing primarily the axonal and dendritic processes of nerve cells. The neuropil was unlabeled, whereas the cellular cortex hybridized strongly.

In larvae, expression of dIRH appeared limited to two kinds of tissues: the nervous system and the imaginal disks. As in late embryos, larval brain cortex cells appeared to hybridize uniformly, whereas the neuropil was devoid of label (Fig. 5a to d). Ventral ganglion cells also exhibited strong hybridization (data not shown). The imaginal disks are composed of diploid cells which divide continuously throughout larval development. During metamorphosis, they form the adult epidermis. All imaginal disks, including the genital disks, hybridized with dIRH probes. Several exam-

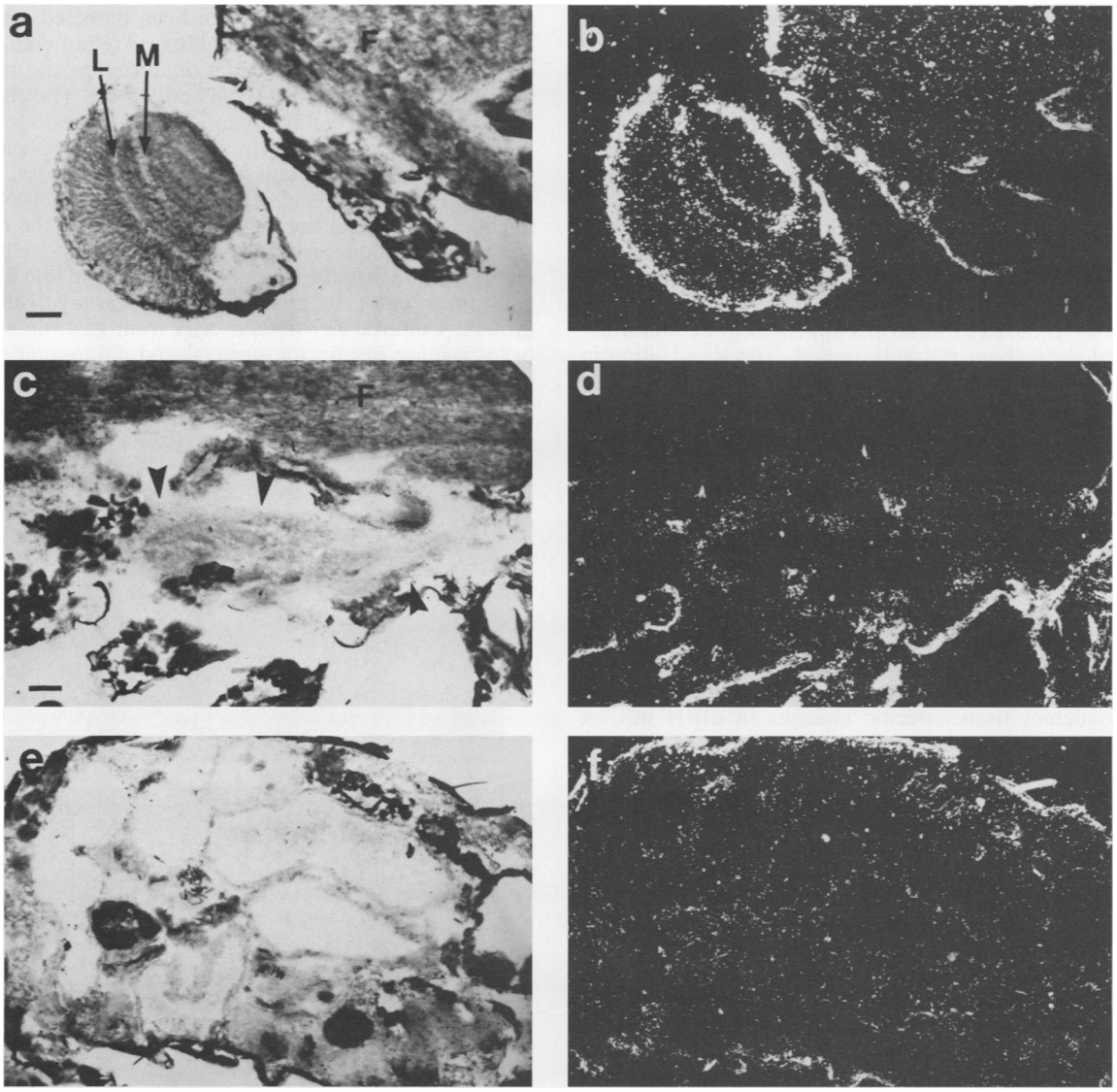

FIG. 6. Localization of dIRH transcripts in adult males. Bright-field (left) and corresponding dark-field (right) micrographs of male adults hybridized with antisense RNA corresponding to the coding sequence probe (a, b, e, and f) or the 3′ untranslated probe (c and d). The anterior ends of the specimens are to the left, except in panels c and d. Hybridization to the cellular cortex of the brain, notably the lamina (L) and medulla (M) (panels a and b) and cells of the thoracic and abdominal ganglia (arrowheads) (panels c and d), is observed. Little hybridization to the flight muscle (F) is observed. No tissue localization was found in the male abdomen (panels e and f). Exposure was for 2 weeks. Bar, 0.05 mm.

ples are presented in Fig. 5. These results suggest that dIRH expression correlates with some general process, such as mitosis, that is common to all disks at this period. The remainder of the larval cells are large, polytene cells destined to be histolyzed during metamorphosis. These hybridized poorly with dIRH probes, as exemplified by cells of the proventriculus (Fig. 5c and d) and larval cells (Fig. 5e and f), which did not exhibit hybridization, in contrast to the imaginal disk cells that surround them.

The highest levels of dIRH transcript expression in adult flies appeared to be in the nervous system and ovaries. The cortex of the brain (Fig. 6a and b) and the cells of the thoracic and abdominal ganglia (Fig. 6c and d and Fig. 7) hybridized well with dIRH probes. Hybridization to ganglion cells was more clearly visible at higher magnification (Fig. 7). Muscle tissue, on the other hand, hybridized only slightly above background, if at all. In the male abdomen (Fig. 6e and f), there was no specific tissue localization. In contrast, in the female abdomen, the ovaries exhibited strong hybridization with dIRH probes (Fig. 8). Nurse cell-oocyte complexes in various stages of development all hybridized (Fig. 8a and b), as did mature oocytes (Fig. 8c and d). In nurse cell-oocyte complexes, hybridization was seen in the cytoplasm of nurse cells (Fig. 8e and f), suggesting that, like other maternally derived mRNAs, dIRH transcripts accumulate there before transfer into the oocyte. A stream of hybridizing RNA was seen (Fig. 8), which may represent dIRH mRNA in the process of extrusion from the nurse cells into the oocyte (arrow). The follicle cells which surround the oocytes did not hybridize with dIRH probes (Fig. 8). In mature oocytes, dIRH transcripts appeared to be uniformly distributed throughout the yolk region. The localization in mature oocytes was similar to that seen in early precellular blastoderm embryos (Fig. 3a and b). Note that elements of the gut as well as other abdominal tissues, including adipose tissue, did not appear to hybridize significantly with dIRH probes (Fig. 8a to d).

DISCUSSION

The pattern of dIRH transcript expression, as revealed by Northern analysis (Fig. 2) (24), is complex, with two principal mRNAs undergoing developmentally regulated changes in abundance. This information can now be considered in light of the experiments reported here using in situ hybridization to detect tissue-specific changes in dIRH mRNA expression.

Expression of dIRH mRNA in developing oocytes and unfertilized eggs indicated that dIRH transcripts are maternally transmitted. If this dIRH mRNA reflects expression of dIRH proteins, it may indicate an important role for dIRH and its ligand in early developmental events. Northern analysis suggested that both the 11- and 8.6-kb transcripts are present at fairly equivalent amounts in unfertilized eggs but that after 2 to 3 h, the 8.6-kb transcript is preferentially accumulated. This increase is transient; the 8.6-kb transcript becomes undetectable later in development (24). During embryogenesis, dIRH transcripts are present throughout the cellular regions of the embryo. We have been unable to establish if the localizations of the 11- and 8.6-kb transcripts differ in early embryos.

In contrast to the 8.6-kb transcript, the level of the 11-kb mRNA began to rise after 4 h of embryogenesis, until it reached its peak at 8 to 12 h (Fig. 2) (24). A significant developmental event that correlates temporally with the peak of dIRH mRNA expression is the growth of the embryonic nervous system (33). Thus, it may be significant that expression of dIRH transcripts in 8- to 10-h embryos appeared to be elevated in the developing nervous system (Fig. 3). Although the cell density of nervous tissue is greater than that of other embryonic tissues and might contribute to the elevated hybridization signal, higher levels of dIRH mRNA expression in nervous tissue persist until adulthood, whereas expression in most other tissues declines.

The localization of dIRH transcripts in nervous tissue during the period of active nervous system development suggests that insulin and its receptor could be important in neuronal growth. Insulin promotes neurite outgrowth in human neuroblastoma cells (26) and cultured cells from chick sympathetic and sensory ganglia (27), but little information is available on the role of insulin in brain development. Insulin receptors are present in chick and rat brains (8, 9), but their function is poorly understood. Marked increases in the insulin-binding capacity of the chick embryo brain during late embryogenesis have been detected, and it has been suggested that these increases correlate with a period of considerable change in brain organization and the establishment of organized electrical activity (9). The correlation of elevated insulin receptor expression with periods of active brain development in two such disparate species again suggests that insulin plays a role in this process. Alternatively, elevated levels of dIRH transcripts in nervous tissue might simply reflect the differentiated state of the neurons.

The expression of dIRH mRNA in most cells of the embryo is consistent with a general metabolic role for insulin and its receptor in anabolic processes involved in cell growth. Cultured *Drosophila* cells from gastrula stage embryos require insulin for survival and differentiation into a variety of cell types (29). Thus, insulin-responsive cells arise early in embryogenesis. The varied developmental fates

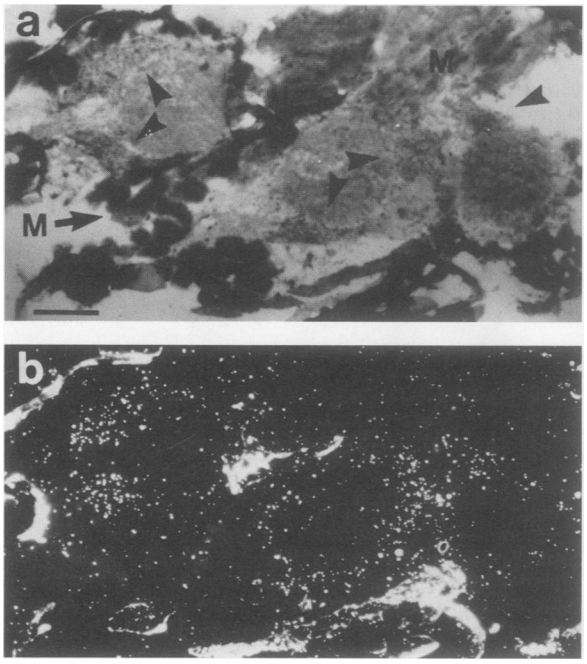

FIG. 7. Localization of dIRH transcripts in ganglion cells of the adult. Bright-field (a) and dark-field (b) micrographs of thoracic and abdominal ganglia hybridized with antisense RNA corresponding to the coding sequence probe. The anterior end of the specimen is to the right. Hybridization to the ganglion cells (arrowheads) is observed. Exposure was for 1 week. Bar, 0.05 mm. M, Muscle.

observed in these culture experiments are in accord with the widespread expression of dIRH mRNA observed in our studies.

The patterns of expression of other protein tyrosine kinase-encoding genes in *D. melanogaster* overlap with the pattern of dIRH expression, yet each is distinct. dERH is the only other *Drosophila* growth factor receptor homolog that has been characterized (17, 28, 37). Its ligand remains unknown. dERH mRNA is present at low levels in oocytes and unfertilized eggs (12, 15). In fact, the pattern of hybridization seen in ovaries is complementary to that of dIRH mRNA in that hybridization to follicle cells is much higher than hybridization to nurse cells and the oocyte itself (12). Increases in dERH mRNA levels are coincident with formation of the cellular blastoderm (12, 15) and remain elevated throughout embryogenesis, declining to low levels thereafter. In contrast, the *src*-related genes of *D. melanogaster* are maternally transmitted (7, 14, 31, 36). Some *src* mRNAs disappear within the first few hours of development, whereas others can be detected until midembryogenesis (7, 14, 31, 36).

In situ hybridization of dERH probes to embryos indicates a uniform distribution of transcripts with no apparent tissue specificity throughout development (12, 28). *Drosophila* c-*src* mRNA also exhibits a broad distribution, with elevated levels detected in the presumptive smooth muscle of the gut (31). In late embryos, c-*src* expression generally is low, but higher levels can be detected in the brain and nerve chord

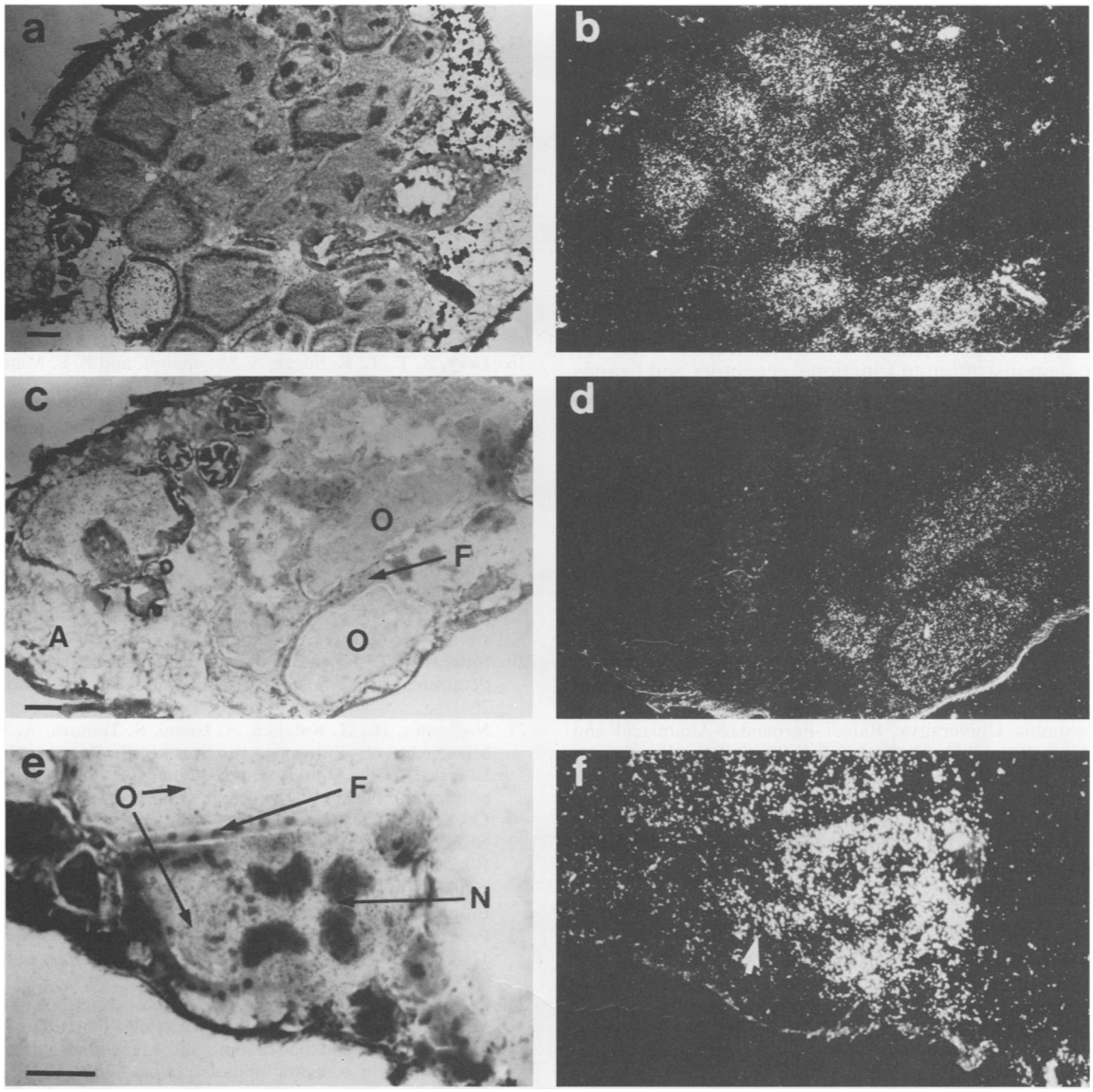

FIG. 8. Localization of dIRH transcripts in ovaries. Bright-field (left) and corresponding dark-field (right) micrographs of female abdomens hybridized with antisense RNA corresponding to the 3′ untranslated probe. The anterior ends of the specimens are to the right. Hybridization to nurse cell-oocyte complexes in various stages of development is observed (panels a and b). dIRH transcripts appear to be distributed throughout the yolk region of mature oocytes (O), whereas the follicle cells (F) which surround them hybridized poorly (panels c to f). Other tissues in the abdomen, including adipose tissue (A), exhibit little hybridization with dIRH probes. Higher magnification of a nurse cell-oocyte complex shows abundant hybridization in the cytoplasm of nurse cells (N) (panels e and f). Exposure was for 1 (panels a to d) or 2 (panels e and f) weeks. Bar, 0.05 mm.

(31). Although subtle differences exist in the patterns of in situ hybridization, the expression of the mRNAs encoding these three protein tyrosine kinases is fairly general throughout much of embryonic development. It is of interest to note that insulin-binding studies in chick embryos reveal a wide distribution of receptors very early in development (12). In all chick and human embryonic tissues examined, c-*src* kinase activity has been detected (16), and EGF binding is widespread during mouse development (1). Thus, expression of elevated levels of tyrosine kinases and receptors bearing tyrosine kinase activity may be a conserved feature of embryonic cells.

In larvae, dERH transcripts are expressed by all imaginal disks, as are dIRH transcripts, but dERH expression in the larval brain is low except for discrete regions thought to contain dividing cells (12, 28). Conversely, c-*src* transcripts are elevated in larval and pupal brain and nerve chords (31). Lower levels of c-*src* mRNA have been detected in imaginal disks, with the exception of the eye-antenna disks, which exhibit abundant expression (31). Thus, in larvae, dIRH mRNA is more broadly expressed than either dERH or c-*src* mRNA, since dIRH mRNA is expressed in nervous tissue as well as in the dividing cells of the imaginal disks.

In adults, dERH transcripts appear to be localized in the cortex of the brain and ganglia (28), as are dIRH transcripts. Localization of c-*src* mRNA in adult tissues other than ovaries (7) has not been reported.

The early and widespread distribution of dIRH mRNA in *Drosophila* embryos is consistent with a role for the insulin receptor in development. However, conclusive evidence awaits the demonstration of functional receptors and ligands at the appropriate stage and in the appropriate tissues, as well as the availability of mutants lacking dIRH expression. A *Drosophila* insulin-like molecule must be present if the receptor is to function as a ligand-activated kinase. In this regard, it is interesting to note that immunoreactive insulin has been detected in chicken eggs (3). Thus, it is conceivable that insulin-activated protein phosphorylation may be among the earliest hormone-activated signal transduction events after fertilization.

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