Expression of *Drosophila* epidermal growth factor receptor homologue in mitotic cell populations

K. L. KAMMERMEYER and SAMUEL C. WADSWORTH

Worcester Foundation for Experimental Biology, 222 Maple Avenue, Shrewsbury, MA 01545, USA

Summary

Expression of the *Drosophila* homologue (DER) of the human epidermal growth factor (EGF) receptor has been studied during development by RNA blot hybridizations and *in situ* hybridizations. One of the hypothetical functions of the protein encoded by this gene is mitotic signal transduction and, therefore, we have searched for evidence of its expression in mitotic cell populations. Increased DER transcript levels were detected in virtually all cells in cellular blastoderm embryos, indicative of the onset of transcription of the gene at this stage of development. These transcripts persist at least until the formation of the ventral furrow at the beginning of gastrulation. Expression of DER has been investigated in populations of nondividing cells, imaginal cells and nervous tissue in third instar larvae. By RNA blot hybridizations, we have shown that imaginal discs contain the bulk of the DER transcripts present in the whole third instar larvae. This result has been confirmed by *in situ* hybridizations. All imaginal discs, representing dividing cells, contain DER transcripts while salivary glands, representing nondividing cells, do not. Within the central nervous system, hybridization of the DER probe is confined to small clusters of cells in the brain cortex. During oogenesis, cell division programs are highly ordered temporally and spatially. Follicle cells express high levels of DER RNA during their mitotic phase of growth but lose these transcripts as they cease division. Nurse cells also express DER transcripts at lower levels even though they are not dividing. However, nurse cell transcripts represent stored maternal RNA species destined for use during preblastoderm embryonic development. These results indicate that the DER gene is expressed in mitotic cell populations during several stages of development and is not expressed in certain populations of nonmitotic cells.

Key words: *Drosophila melanogaster*, expression, epidermal growth factor (EGF), receptor, mitosis, cell populations, DER, cell division.

Introduction

Depending on their locations within the embryo, founder cell populations established at the cellular blastoderm stage of *Drosophila* development follow specific programs of cell division. Most cells destined to establish larval tissues go through only a few mitotic divisions before becoming polytene whereas imaginal cell populations continue to expand mitotically throughout embryonic and larval development. The proper control of initiation and cessation of mitosis is crucial for the execution of these developmental events. We have been studying a *Drosophila* gene that may play a role in cell growth control, a homologue of the vertebrate epidermal growth factor (EGF) receptor (Wadsworth, Vincent & Bilodeau-Wentworth, 1985b; Livneh, Glazer, Segal, Schlesinger & Shilo, 1985). The basis for this hypothesis is the fact that one of the clearly documented functions of the vertebrate EGF receptor is mitogenic signal transduction in response to binding of the endocrine peptide, RGF (Carpenter & Cohen, 1979). As deduced from the nucleotide sequence, the DER protein retains the three functional domains of the vertebrate protein possessing the greatest degree of homology. Alternate splicing of 5' exons has been reported (Schejter, Segal, Glazer & Shilo, 1986). That the three major domains of the gene are structurally conserved gives rise to the hypothesis that the *Drosophila* homologue might be
functionally conserved and serve to transmit mitogenic signals in a manner analogous to the vertebrate receptor.

Expression of the DER gene has been documented during embryonic, metamorphic and adult stages of development (Wadsworth et al. 1985b; Lev, Shilo & Kimch, 1985). At least two transcripts of greater than 6 kilobases (kb) exist in the majority of stages examined although the smaller species seems to predominate in adults. Because the developmental stages examined by RNA blot hybridizations contain a variety of cell types, no information about the functional role of the receptor was provided. One way to approach the question of the functional conservation of the gene would be to ask whether proliferating cells express the DER gene. Therefore, we have examined different populations of mitotic and nonmitotic cells during development by RNA blot hybridizations and in situ hybridizations. Although the survey of developmental stages and tissues is not complete, we report here that DER expression is positively correlated with the mitotic state of specific cell populations during embryonic and larval development and oogenesis. In contrast, DER expression was not observed in certain nonmitotic cell populations. Our results are substantially in agreement with those recently published by Schejter et al. (1986). They reported evidence that the DER gene was expressed generally in embryonic tissues, preferentially in imaginal discs in third instar larvae and neural tissues in adults. On the basis of these results, they proposed functional roles for the DER gene product in proliferating and differentiated cells. We hypothesize that the DER protein might function in mitotic signal transduction in certain cell populations during development. Additional functions of the DER gene product suggested by Schejter et al. (1986) in differentiated cells are not excluded by our results.

**Materials and methods**

**RNA extraction and hybridization**

Extraction of RNA from the various developmental stages and specific tissues was performed as described before (Wadsworth et al. 1985b; Wadsworth, Madhavan & Bilodeau-Wentworth, 1985a). Mass-isolated imaginal discs from third instar larvae were obtained from James Fristrom. RNA gel electrophoresis, blotting and hybridization procedures were performed according to Wadsworth et al. (1985a).

**Anti-sense probe constructions and in vitro transcription**

To construct the DER in vitro transcription template, a 3.5-kb EcoRI–HindIII fragment from the lambda 42 genomic clone (Wadsworth et al. 1985b) was cloned into pSP65 (Melton, Krieg, Rebagliati, Maniatis, Zinn & Green, 1984; purchased from Promega) (Fig. 1). This fragment contains sequences encoding the entire kinase domain and the majority of the extracellular domain. The cloned DNA was truncated at the indicated HindIII site before in vitro transcription with SP6 RNA polymerase according to the instructions provided by the manufacturer. To construct the actin 5C anti-sense clone, the 1.8-kb HindIII fragment from the genomic actin 5C gene (Fyrborg, Kindle & Davidson, 1980) was cloned into the Bluescribe vector (purchased from Stratagene) for in vitro transcription with T7 RNA polymerase (Tabor & Richardson, 1985). H-labelled anti-sense (or sense) RNA probes were used for in situ hybridization experiments and 32P-labelled anti-sense RNA probes were used for RNA blot hybridizations. The DNA template was removed by brief DNase treatment. The probes were extracted with phenol/chloroform in equal volumes, ethanol precipitated and resuspended in distilled water. The probes were hydrolysed to approximately 150 bases before hybridization (Cox, DeLeon, Angerer & Angerer, 1984).

**Tissue preparation and in situ hybridization methods**

To obtain early embryos, eggs were collected for 1 h and aged for 2–3 h. They were then dechorionated with 3% sodium hypochlorite and then permeabilized and devitellinized following the procedure of Mitchison & Sedat (1983). Brains, imaginal discs and salivary glands were dissected from third instar larvae and ovaries were dissected from adult females. The embryos or tissues were transferred to OCT embedding medium (Miles Laboratories) on a cryostat specimen holder, frozen by placing the specimen holder on dry ice and stored at −20°C. Embedded tissue was sectioned in a cryostat at −20°C to −23°C. Sections measuring 8–10 μm were collected on poly-L-lysine-coated slides (Cox et al. 1984) and placed on a slide warmer for 25–45 min. The tissue sections were fixed to the slides and permeabilized according to the method of Hafen, Levine, Garber & Gehring (1983).

For in situ hybridizations, the method of Cox et al. (1984) was modified as follows. The RNA probe, carrier RNA (calf liver tRNA), and poly(A) were heated at 80°C for 3 min in distilled water before adding the other ingredients to yield the following final concentrations: 50% formamide, 0.3 M- NaCl, 10 mM-Tris–HCl (pH 7.5), 1 mM-EDTA, 1× Denhardt’s solution (0.02% each of Ficoll, polyvinylpyrrolidone and bovine albumin), 10% dextran sulphate, 1.5 mg ml⁻¹ tRNA, 0.5 mg ml⁻¹ poly(A), and 0.2–0.3 μg ml⁻¹ of RNA probe. Hybridizations were performed in a volume of 20 μl per slide, under baked coverslips sealed

![Fig. 1. Plasmid pSP65 subclone of Drosophila EGF receptor homologue used to synthesize anti-sense RNA probes. The cloned DNA was truncated at the indicated HindIII site before in vitro transcription with SP6 RNA polymerase as described in Materials and methods.](image-url)
with rubber cement. Hybridization was overnight at 48°C. Coverslips were removed, individual slides were treated with RNase A (20 μg ml⁻¹) in 0.5 M NaCl, 10 mM Tris–HCl (pH 7.5), 1 mM EDTA at 37°C for 30 min, then groups of slides were washed in the RNase buffer at 37°C for 30 min, 2 × SSC at room temperature for 30 min, 0.1 × SSC at 48°C for 30 min and dehydrated in a graded ethanol series. Autoradiography was as described by Pardue & Gall (1975) and slides were stored at 4°C in a light-tight dry chamber. At the appropriate times the slides were developed in Kodak D-19 developer and fixed in Kodak Rapid Fix. The slides were lightly stained with Giemsa and examined by light microscopy.

Results

DER expression in embryonic cells
In this study we chose to investigate DER expression during early embryogenesis before the cessation of mitosis in many of the embryonic cells. At different times during embryogenesis the majority of the cells established at the cellular blastoderm stage undergo mitotic divisions. Apparently only salivary gland cells fail to go through some mitotic divisions after cellular blastoderm formation (Sonnenblick, 1950). If the DER protein were required as a transmembrane signaling protein for the onset of mitosis in these cells, then its expression should be initiated at an earlier developmental stage. Therefore we have searched for evidence of DER expression around the time of formation of the cellular blastoderm stage, the stage at which transcriptional activity in the embryonic nuclei can first be detected.

Shown in Fig. 2 are several examples of the distribution of DER RNA in early embryos detected by in situ hybridization. In Fig. 2A,B, DER RNA can be observed in cells at the periphery of a cellular blastoderm embryo in contrast with the apparent absence of DER RNA in an adjacent preblastoderm embryo as well as the interior of the cellular blastoderm embryo. Longitudinal and cross sections of different cellular blastoderm embryos are shown in Fig. 2C,D and Fig. 2E,F respectively. RNA blot hybridization experiments have documented a net increase in DER RNA levels following cellular blastoderm (Wadsworth et al. 1985; Lev et al. 1985), thus we conclude that the cellular blastoderm RNA sequences detected by in situ hybridization represent transcription of the DER gene. We have found no evidence from these experiments of cellular blastoderm cells that fail to express DER RNA sequences. Shown in Fig. 2G,H is a cross section of an embryo during ventral furrow formation, approximately 1h after the cellular blastoderm stage. It can be seen that the invaginating mesodermal cells have retained DER RNA sequences. These hybridization patterns are typical of embryos at similar stages of development observed during this study.

DER expression in larval tissues
The majority of embryonic cells give rise to the larval tissues. At different times during middle to late embryogenesis, these cells cease to divide and become polytene, increasing in size until they are histolyzed during metamorphosis. Other cells, referred to as imaginal cells, do not differentiate during embryogenesis but they continue to divide mitotically throughout the remainder of embryonic and larval life. During metamorphosis, these cells will differentiate to form the adult epidermis. With the exception of those in histoblast nests, imaginal cells are arranged in imaginal discs which can be mass isolated from third instar larvae. We have purified RNA from imaginal discs and compared the level of DER RNA with that in whole third instar larvae (Fig. 3). The 6-3 and 6-6kb DER RNA species (which cannot be resolved from each other in the photograph) can be readily detected in imaginal disc RNA preparations (Fig. 3A). Upon longer exposure, the same RNA species can be detected in whole third instar larval RNA (Fig. 3B). Quantification of the hybridization levels shown in Fig. 3 revealed an approximately 40-fold enrichment in DER RNA in purified imaginal discs over that present in whole third instar larvae. Thus, DER RNA accumulates preferentially in imaginal discs. The differences in the relative molecular masses of the DER transcripts as determined in this study and previously published values (Wadsworth et al. 1985; Lev et al. 1985) are due to the use of more appropriate size standards. Here we have sized the DER transcripts relative to actin mRNA species by rehybridization of the same blot and relative to full-length Rous sarcoma virus RNA (9.3 kb, Schwartz, Tizard & Gilbert, 1983) electrophoresed in an adjacent lane on the same gel. The consistent appearance of two DER RNA species has been noted before (Wadsworth et al. 1985; Lev et al. 1985).

To extend and confirm these observations, we carried out in situ hybridization on brain–imaginal disc–salivary gland complexes dissected from third instar larvae. Examples of hybridization of the DER anti-sense probe to imaginal discs are shown in Fig. 4A–D. Eye–antennal discs can be easily recognized by their size and morphology. Within the same brain–imaginal disc complexes, grain counts revealed that the hybridization levels detected in imaginal discs are approximately tenfold higher than those in adjacent brain cortex and salivary gland tissue. Similar hybridization results were obtained over all imaginal
Fig. 2. **In situ** hybridization of DER anti-sense probe to early embryos. Panels A,C,E and G, Giemsa-stained sections; panels B,D,F and H, dark-field illumination of the same sections. Autoradiographic exposure was from 5 to 6 weeks.
Fig. 3. Hybridization of DER anti-sense probe to poly(A)-containing RNA from imaginal discs and whole third instar larvae. Approximately 2 \( \mu \)g of RNA was electrophoresed on a formaldehyde–agarose gel and transferred to nitrocellulose. The right-hand side of the figure is a longer autoradiographic exposure of the same blot shown on the left. Size markers for this experiment were actin mRNAs, detected by rehybridization of the block with Act5C probe, and genomic-length Rous sarcoma virus RNA (9-3 kb, Schwartz, Tizard & Gilbert, 1983), detected by hybridization with a \( v-src \) probe. These values for the DER mRNAs should be more accurate than our previously published values (Wadsworth et al. 1985). 

Salivary glands, exemplifying polytene tissue, did not hybridize detectably with the DER anti-sense probe. An anti-sense probe for the DER extracellular domain yielded results identical to those shown in Fig. 4 and a s-sense probe containing the same sequences did not hybridize above background level (data not shown).

During the early stages of oogenesis, a germ-line stem cell undergoes four incomplete divisions to give rise to the oocytes proper and 15 interconnected nurse cells. The complex of nurse cells and the oocyte is then surrounded by a layer of follicle cells. Each of these three cell types has a different physiology and developmental fate. The oocyte is transcriptionally inactive and is dependent upon the nurse cells and follicle cells for its maturation. After four mitotic divisions, the nurse cells cease to divide, become polytene and express a wide variety of maternally inherited components including mRNAs, which are stored in the oocyte for use during the preblastoderm stage of embryonic development. The follicle cells go through four complete cell divisions after associating with the oocyte and nurse cells and cease to divide thereafter. During the vitellogenic stages of oogenesis, the follicle cells are actively engaged in yolk protein uptake from the circulatory system for deposition in the oocyte (King, 1970). During the later stages of oogenesis, the follicle cells are still metabolically active in that they synthesize the vitelline membrane that surrounds the mature oocyte and they deposit the chorion that forms the outermost covering of the oocyte (King, 1970). Cryostat sections of isolated ovaries were hybridized with DER anti-sense probes to determine whether any of these lineages preferentially expressed the DER gene.

These hybridizations revealed two different modes of DER expression in ovaries. First, all vitellogenic follicle cells expressed higher levels of DER RNA than the adjacent nurse cells and oocytes. These results are shown in cross section (Fig. 5A,B) and longitudinal section (Fig. 5C,D). No examples of vitellogenic follicle cells not expressing DER RNA were seen. Preferential expression of DER transcripts in follicle cells relative to the nurse cells that they surround is observed even in young egg chambers (those at the top of Fig. 5E,F). Follicle cells surrounding the more mature oocytes no longer express higher levels of DER RNA (Fig. 5A,B). Thus as follicle cells age beyond their mitotic phase of growth, their complement of DER RNA decreases. An anti-sense probe for the DER extracellular domain yielded results identical to those shown in
Fig. 5 and a sense probe containing the same sequences did not hybridize above background level (data not shown).

Anti-sense actin probes were also hybridized to ovary sections. It can be clearly seen that the localization of actin transcripts is the reverse of the DER transcripts. Actin transcripts are abundant in nurse cell cytoplasm and in the oocyte proper but are present at greatly reduced levels in follicle cells (Fig. 5G,H), in agreement with the fact that actin transcripts are maternally inherited (Madhavan, Bilodeau-Wentworth & Wadsworth, 1985).

Although the expression of DER RNA is relatively high in follicle cells, the levels seen in nurse cells and oocytes are higher than background. In general, maternal mRNA species do not function as message during oogenesis but are translationally inactive until embryogenesis. Although we cannot assess the translational activity of the nurse cell transcripts, we tentatively conclude that the RNA species detected in oocytes and nurse cells by *in situ* hybridization are destined to serve as functional mRNA during embryogenesis. This maternal mode of expression of DER RNA that we have observed by *in situ* hybridization confirms the observation of DER RNA in unfertilized eggs (Lev et al. 1985).

**DER expression in nonovarian tissue**

It has been previously shown that in adults other *Drosophila* src-related genes are expressed almost exclusively in ovarian tissue (Wadsworth et al. 1985). To determine whether this was the case for the DER gene, total RNA from adult males, females and isolated ovaries was hybridized with the DER anti-sense probe. As shown in Fig. 6, DER RNA expression occurs in female non-ovarian tissue and in males. Because there are few dividing cells in adults outside of the reproductive system, these results suggest that DER RNA may also be present in nonproliferating cells in adults.

![Fig. 4. *In situ* hybridization of DER anti-sense probe to sections of brain–imaginal disc complexes (A–D) and salivary gland (E–F). Panels G–H show Act5C anti-sense probe hybridization to a brain section. Panels A, C, E and G, Giemsa-stained sections; panels B, D, F and H, dark-field illumination of the same sections. Autoradiographic exposure was from 5 to 6 weeks in the case of the DER probe. The Act5C experiment was exposed for three days. Symbols: a, eye–antennal disc; i, other imaginal discs; n, brain neuropil; c, brain cortex; v, ventral ganglion.](image-url)
Discussion

Because the DER gene is structurally conserved relative to the human gene, it is possible that it is also functionally conserved. One of the hypothetical functions of the Drosophila protein would be mitotic signal transduction. A prediction of this hypothesis is that the DER gene would be expressed in dividing cell populations. In this report we have provided evidence of a positive correlation between the DER expression and the mitotic state of cells during embryonic and larval development and during oogenesis.

As shown by Lev et al. (1985), DER gene expression increases after the cellular blastoderm stage. In this study we have detected these cellular blastoderm transcripts directly by in situ hybridization and have shown they persist beyond the initiation of gastrulation. It is soon after this stage that mitotic divisions in many embryonic cells begin. Thus those cells poised for the completion of embryonic development contain DER RNA. Schejter et al. (1986) detected DER transcripts in wide variety of cells in 14h embryos with no specific tissue localization noted. Taken together, these results suggest that DER expression is a general feature of the developmental programme of embryonic cells.

In third instar larvae, we have shown that all types of discs associated with the brain-imaginal disc complex, and apparently all cells within the discs, express DER RNA while the majority of the cells in the animal at this stage do not. Schejter et al. (1986) have also demonstrated preferential expression of DER RNA in imaginal discs. Imaginal cells in third instar larvae have continued to divide during the larval lifetime in contrast to the polytene cells which stopped dividing during embryogenesis. The absence of high levels of DER RNA in polytene cells is interpretable if, indeed, expression of an EGF receptor homologue is required only in dividing cells. Although some imaginal cells are arrested in the cell cycle at the end of the third instar larval stage (Fain & Stevens, 1982), they are poised to divide and differentiate to form the adult. Thus the DER RNA that we detect may reflect the recent mitotic state of the imaginal cells and/or the waves of mitoses that the cells will soon undergo during the early stages of metamorphosis.

Within third instar larvae, we have also demonstrated preferential DER expression in minor cell populations in the brain cortex and have tentatively concluded that they correspond to the previously described proliferative centres (White & Kankel,
Fig. 5. *In situ* hybridization of DER and Act5C anti-sense probes to sections of ovary. Panels A,C and E, Giemsa-stained sections hybridized with antisense DER probe; panels B,D and F, dark-field illumination of the same sections; panel G, Giemsa-stained section hybridized with the anti-sense Act5C probe; panel H, dark-field illumination of the same section. Autoradiographic exposure was from 5 to 6 weeks in the case of the DER probe. The Act5C experiment was exposed for three days. Symbols: o, oocyte; n, nurse cell; vf, vitellogenic follicle cells; f, follicle cells surrounding more mature oocytes.
Expression of Drosophila EGF receptor

Fig. 6. Hybridization of anti-sense DER probe to poly(A)-containing RNA isolated from ovaries, females, and males. Approximately 2 µg of poly(A)-containing RNA was electrophoresed on a formaldehyde–agarose gel and transferred to nitrocellulose.

1978). This observation is consistent with our hypothesis that DER expression would be characteristic of dividing cells since they are found in the larval central nervous system and are concentrated within the proliferative centres. However, in contrast to our results, Schejter et al. (1986) found low levels of DER RNA throughout the brain cortex. Although it is difficult to reconcile these differences completely, it is possible that the brain sections examined by Schejter et al. (1986) did not include the proliferative centres. Additional experiments will be required to complete the correlation of DER expression with the proliferative centres in the brain cortex.

Expression of DER RNA has also been documented in mitotic cells during oogenesis. We have shown that follicle cells express DER RNA during the stages in which they are actively dividing (stages 1 and 6 of oogenesis), that these levels appear to remain constant at least through stages 10B which ends 24 h later and that DER RNA levels gradually decline in the follicle cells surrounding more mature oocytes. There are at least two interpretations of this pattern of expression. It could be that as follicle cells cease to divide mitotically, the rate of DER gene transcription declines slowly, thus accounting for the presence of the transcripts in the postmitotic stage. It could also be that the DER transcripts synthesized during the mitotic phase of growth have a long enough half-life to survive at least until stage 10B, i.e. 24 h. Unless DER gene transcription is tightly coupled to the mitotic cycle, a combination of these two hypotheses could readily account for the observed pattern of RNA expression. Unfortunately, neither hypothesis can be readily subjected to a simple test.

Our results have also documented a second mode of DER expression during oogenesis in that DER transcripts accumulate in nurse cells and oocytes, although at a lower level than in follicle cells. We believe that these nurse cell/oocyte transcripts, like classical maternal mRNA species, are translationally masked during oogenesis and activated during embryogenesis. Thus the presence of DER RNAs in nonmitotic nurse cells and oocytes does not necessarily invalidate our working hypothesis that DER expression is correlated with mitotic cell division.

Clearly, the results that we have presented allow us only a glimpse at the functional role of the DER gene during development. In the tissues that we have examined to date, there is a positive correlation between the presence of DER RNA and the recent or impending mitotic state of the cell, providing some basis for suggesting that the Drosophila homologue might serve in mitotic signal transduction. All of the functional domains of the vertebrate EGF receptor are conserved in the DER gene (Livneh et al. 1985) and our hybridization results suggest that these functional domains are encoded by the transcripts present in each of the tissues and developmental stages studied. However, we and Schejter et al. (1986) have presented evidence suggesting that DER RNA may be present in nonproliferating cells. The eventual complete understanding of the function of the DER protein in different cell types will require critical testing of a number of its biochemical and physiological properties.

The expression pattern that we have observed in this study should be useful in future studies on the functional role of the DER gene product. If DER function is required for mitotic cell growth, then it could be predicted that mutations that eliminate receptor function completely would be lethal at several points in the life cycle. Perhaps more approachable in the short run, however, would be the application of anti-sense RNA techniques. If expression of DER anti-sense RNA could be temporally or spatially controlled through selection of the appropriate promoter, then it should be possible to affect the growth of some of the specific cell populations that we have identified.

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