Prepupal differentiation in *Drosophila*: distinct cell types elaborate a shared structure, the pupal cuticle, but accumulate transcripts in unique patterns

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Summary

The components of the pupal cuticle are the main differentiation products synthesized by both the larval and adult epidermis during the prepupal period of *Drosophila* development. The pupal cuticle is formed *in vitro* by imaginal discs in response to a 6 h pulse of 20-hydroxyecdysone (20-HE). We previously described the isolation and initial characterization of four ecdysone-dependent genes (EDGs) whose expression in imaginal discs occurs only in response to a pulse of 20-HE. In this report, we demonstrate that the pattern of temporal and tissue-specific expression of these EDGs *in vivo* is like that expected for genes that encode pupal cuticle proteins. Transcripts of these genes are detected in prepupae only in the epidermis and only when cuticle components are synthesized and secreted. Nonetheless, their temporal and spatial patterns of accumulation differ. EDG-84A-1 transcripts accumulate only in prepupae and only in imaginal cells. EDG-78E and EDG-64CD transcripts accumulate at the same time in both larval and imaginal cells. EDG42-A transcripts appear first in prepupae in imaginal cells and then, after a 2—4 h lag, in larval cells. It is evident that some genes are not restricted in their expression to only larval or imaginal epidermis.

Key words: *Drosophila*, pupal cuticle protein genes, gene expression, imaginal discs, ecdysone-dependent genes.

Introduction

Two major developmental pathways are used during embryogenesis in *Drosophila*: one for larval and one for adult epidermal tissues. Cells of the larval tissues do not divide during larval development but grow in size and form large polytene nuclei. The presumptive adult cells remain diploid and proliferate during larval and early pupal development (Szabad & Bryant, 1982; Roseland & Schneiderman, 1979). These presumptive adult cells occur in clusters known as imaginal discs, imaginal histoblast nests and imaginal rings. During embryonic and larval periods, the larval epidermis is solely responsible for the synthesis and secretion of the components of three larval cuticles. Likewise, the adult cuticle is synthesized exclusively by imaginal cells. During metamorphosis, an intermediate cuticle, the pupal cuticle is produced when the epidermis is a mosaic of larval and imaginal cells. Both the larval epidermis and the imaginal discs synthesize and secrete the pupal cuticle. The anterior half of the pupal cuticle (covering the head, thorax and appendages) is elaborated by imaginal discs whereas the posterior half (covering the abdomen) is produced mainly by larval cells with a small contribution from the patches of abdominal histoblasts and the genital disc. Thus, two distinct tissues, one polytene and larval, the other diploid and imaginal, contribute to the formation of a continuous and apparently uniform extracellular sheet: the pupal cuticle.

The collaboration of juxtaposed larval and adult epithelia to produce the pupal cuticle provides a unique opportunity to study how these two tissues respond to the same hormonal stimulus. Is the same gene network recruited by both the larval and imaginal cells or does each tissue use a separate set of gene products? In this report, we examine the response of the larval and imaginal epidermal cells to a hormonal stimulus that elicits the synthesis and secretion of the components of the pupal cuticle.

Insect cuticles are composed of an outer epicuticle and an inner, chitin-containing procuticle which itself is sometimes divisible into endo- and exocuticles. Procuticles in particular are rich in extractable cuticle proteins (CPs). Low molecular weight pupal cuticle proteins, L-PCPs (PCP15, 19, 21, 22, 23, and 25) are synthesized from about 8 to 12 h after puparium formation (Doctor *et al.* 1985; Fristrom, D. *et al.* 1986) and are deposited in the exocuticle (Wolfgang *et al.* 1986). From about 12 to 20 h, high molecular weight H-PCPs (PCP40, 56, and 82) are produced (Doctor *et al.* 1985) and deposited in...
the endocuticle (Wolfgang et al. 1986). The H-PCPs are detected in anterior cuticles 2–4 h before they are detected in posterior cuticles (Doctor et al. 1985).

With a few exceptions, the pupal cuticle proteins in the anterior are electrophoretically similar to those in the posterior (Doctor et al. 1985). However, electrophoretically equivalent proteins may be encoded by different genes, particularly because CPs belong to multigene families (Snyder et al. 1982). Indeed two proteins have been identified that are limited to the anterior pupal cuticle; PCP15 (Chihara et al. 1982) and a tyrosine-rich PCP, PCP8 (Fristrom, J. et al. 1986; Alexander, King, and Fristrom, unpublished data). In contrast, transcripts complementary to a cuticle protein gene (CP-GART) nested in the major intron of the GART gene, are limited to the posterior prepupal epidermis and have not been detected in imaginal discs undergoing pupal cuticle formation in vitro (Henikoff et al. 1986; unpublished observations).

We have described the isolation and initial characterization of four ecdysone-dependent genes (EDGs) that, based on their in vitro expression in imaginal discs, may participate in the formation of the pupal procuticle (Fechtel et al. 1988). Table 1 summarizes key features of these genes. To clarify the function and regulation of these genes, this paper describes the distribution of their transcripts in polytene and diploid epidermal cells during the in vivo formation of the pupal cuticle.

### Materials and methods

#### Collection of staged prepupae and pupae

Developmentally staged prepupae and pupae were collected from an Oregon R strain of *Drosophila melanogaster* that has been mass cultured in our laboratory since 1965. White prepupae (0 h) were selected by hand and then prepupal and pupal stages were collected following incubation at 25°C for 0, 3, 6, 8, 10, and 14 h from puparium formation. (The prepupal period ends with the eversion of the cephalic complex at pupation, approximately 12 h following puparium formation.)

#### Analysis of transcript accumulation in prepupae and pupae

Isolation of RNA, electrophoresis and transfer to nylon membranes, filter hybridizations and preparation of single-strand RNA probes have been described (Natzle et al. 1986; Fechtel et al. 1988). Restriction maps for each of the EDGs and subclones used in the preparation of labelled probes are depicted in Fig. 1.

### Table 1. Characteristics of the ecdysone-dependent gene set

<table>
<thead>
<tr>
<th>Gene</th>
<th>Plasmid subclone</th>
<th>RNA length (kb)</th>
<th>Stage-specific transcript accumulation</th>
<th>Transcript accumulation ecdysone-dependent in cultured discs</th>
<th>Anti-PCP immune-precipitable translation product</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDG-84A-1</td>
<td>51RX1.5</td>
<td>0.9</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>EDG-78E</td>
<td>57R1.5</td>
<td>0.5</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>EDG-42A</td>
<td>61SR3.2</td>
<td>3.0</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>EDG-64CD</td>
<td>63RS6.6</td>
<td>5.4, 5.0</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>


**Preparation of tissue sections**

Third instar larvae, and 0 to 8 h prepupae, were placed directly in a drop of OCT compound (Miles Scientific, Naperville, IL) and frozen on a block of dry ice. 10 h prepupae and 14 h pupae were dissected from their pupal cases and fixed for 1 h in 4% buffered formalin (0.1 M sodium cacodylate, pH 7.2), then placed in 10% sucrose in phosphate-buffered saline (PBS) for 2 h before freezing. After fixing, 10 h prepupae were broken in two at the junction of the thorax and abdomen. The two pieces were frozen side by side in OCT compound so that transverse sections of both thorax and abdomen of one animal could be examined simultaneously.
Sections (6 μm) were cut at −18°C and fixed to subbed slides (1% gelatin) with 4% buffered formalin for 15 min and then washed in two changes of PBS.

**In situ hybridization to tissue sections**

The methods employed for *in situ* hybridization to tissue sections are combined from those used by Cox *et al.* (1984) and Hafen *et al.* (1983) and have been described (Natzle *et al.* 1988). Single-strand 3H-labeled RNA probes were used with 2 × 10^6 to 1 × 10^7 cts min⁻¹ per slide. Exposure times were 3 to 7 days. To provide internal controls on the hybridization, sections from different stages were mounted on each slide and exposed simultaneously to one probe. Likewise, sequential sections from the same set of specimens were exposed to probes for all four genes. Because sections from different stages were simultaneously exposed to the same probe, comparisons of the amount of hybridization between different sections were simplified.

**Results**

**EDG transcripts are the same size in imaginal disc, prepupal and pupal RNA**

To study the tissue distribution of transcripts complementary to a set of four apparently unique genes that may participate in the formation of the pupal procuticle (Fechtel *et al.* 1988), we used *in situ* hybridization to RNA in tissue sections using single-stranded, genespecific probes. The correct polarity of the single-stranded probes used in this study was determined by hybridizing each 32P-labeled probe to RNA fractionated by size and transferred to nylon membranes. In this manner, the complementary, antisense (experimental) and the identical, sense (control) strands were identified (data not shown). The antisense probes representing each gene are complementary to the same length transcripts from either 8–12 h prepupae or 12–16 h pupae that had been found for hormone-dependent transcripts of cultured imaginal discs (Fechtel *et al.* 1988). Because these subclones hybridize to the same length transcripts in imaginal disc, prepupal and pupal RNA, we conclude that they are suitable probes for *in situ* hybridization experiments to examine the precise spatial and temporal patterns of transcript accumulation of these genes during prepupal and early pupal development.

**Artifactual binding of probes to extracellular cuticle occurs**

One noteworthy artifact that we have encountered in our *in situ* hybridization analysis is the strand-specific binding of hybridization probes to cuticular structures. At low magnification (10×), this artifactual binding can obscure hybridization results. At higher magnification (40–100×), artifactual binding to extracellular cuticular structures is easily distinguished from signal located over cells (Fig. 2). We do not understand the basis for the strand specificity of this artifact, which was encountered only with particular probes and only with certain pupal stages, suggesting that the developmental state of the cuticle affected the degree of non-specific binding of the probe.

**Hybridization of EDG probes is limited to the epidermis**

Only the epidermis secretes a pupal cuticle. Therefore, genes that encode proteins participating in this process must accumulate only in the larval and/or imaginal epidermis. All four EDGs are complementary to transcripts that accumulate only in the larval and/or imaginal epidermis.

EDG-84A-1 hybridizes to transcripts limited to the imaginal epidermis (Fig. 3A,B). No transcripts were detected in the larval epidermis (Fig. 3C). Using precisely timed animals, transcripts hybridizing to this clone were found in 8 h prepupae, and reached maximal accumulation at 10 h. No transcripts were detected in 0–6 h prepupae, or in 14 h pupae (data not shown). (We were not able to obtain adequate frozen sections of 12 h pupae).

EDG-78E transcripts are present in equal abundance in both imaginal and larval epidermis in 10 h prepupae (Fig. 3E–G). The timing of transcript accumulation is like that found for EDG-84A-1: transcripts are detected in 8 h prepupae with maximal accumulation in 10 h prepupae. Transcripts were undetected in 0, 3, 6, and 14 h animals (data not shown).

EDG-42A transcripts were barely detectable above background in imaginal cells of 8 h prepupae (Fig. 4A). Strong signal is seen in the imaginal epidermis in 10 h prepupae but is not detected in the larval epidermis at this time (Fig. 4B). In 14 h pupae, transcripts are abundantly expressed in both the larval and imaginal epidermis (Fig. 4C). Thus, EDG-42A transcripts are present at later stages than those of EDG-84A-1 and EDG-78E and are detected in imaginal epidermis before they are detected in larval epidermis. They are also detected at high levels in pupae.

EDG-64CD transcripts are present in RNA isolated from larval as well as prepupal stages (Fechtel *et al.* 1988). Consequently, we examined sections of larvae and prepupae. EDG-64CD transcripts were detected in larval epidermal cells of mid-third instar larvae and in prepupae in both imaginal and larval epidermal cells. Transcripts are detected in mid-third instar larvae (Fig. 5A) when larval cuticle proteins are being synthesized (Kimbrell *et al.* 1988), are absent 0–6 h after puparium formation (Fig. 5B), and are detected again in 8 h prepupae in both larval and imaginal epidermal cells (Fig. 5C) coincident with the deposition of the pupal exocuticle. Transcripts were still detectable in 14 h pupae, but at lower levels than observed in 10 h prepupae (data not shown).

**Discussion**

The four ecdysone-dependent genes analyzed here were originally isolated as putative pupal cuticle protein genes (Fechtel *et al.* 1988) because both accumulation of their transcripts and the formation of pupal cuticle in imaginal discs *in vitro* occur in response to a 6 h pulse of 20-HE. The *in vivo* temporal and spatial distribution of the transcripts described here provides further evidence
that these four EDGs are involved in the formation of the pupal cuticle. First, their distribution in vivo is limited to cells that form cuticle. Second, they are present only at times when a procuticle is being deposited. Indeed, as shown previously, EDG-78E encodes a low molecular weight polypeptide that, based on its immunological properties (Fechtel et al. 1988) is an L-PEP. Apple and Fristrom (unpublished) have determined that the conceptual amino acid sequence of EDG-78E is that of an L-PCP.

EDG-84A-1 probably also encodes an L-PCP. It specifies a small, 0-9 kb, transcript (Fechtel et al. 1988) whose accumulation in vivo and in vitro occurs with similar timing to transcripts of EDG-78E and the appearance of the L-PCPs themselves. Its conceptual amino acid sequence (Apple and Fristrom, unpublished) is similar to that of a cuticle protein. In contrast, EDG-42A may encode an H-PCP. It specifies a 3-0 kb transcript whose accumulation in vitro is delayed compared to that of EDG-78E transcripts. Maximal accumulation of EDG-42A in vivo occurs in early pupae (Fig. 5), the time at which maximal synthesis of the H-PCPs occurs (Doctor et al. 1985). In addition, there is a 2-4 h lag between the appearance of both EDG-42A transcripts and of H-PCPs in the posterior of the animal. Consequently, three of the four EDGs appear to encode pupal cuticle proteins.

The fourth gene in the set, EDG-64CD, is complementary to transcripts that accumulate during both larval and prepupal development and presumably does not encode a stage-specific pupal cuticle protein. It is possible that EDG-64CD encodes a protein, such as chitin synthetase, required for the synthesis of all cuticles.

One of the major attributes of the development of a holometabolus insect, such as Drosophila, is the separation of cell lineages that lead to the formation of larval (juvenile) structures from those that lead to the formation of imaginal (adult) structures. It is of interest to compare gene expression and regulation during the
differentiation of juvenile and adult forms of like tissues. Wigglesworth (1959) proposed the extreme view that separate sets of genes are used for differentiation of juvenile and imaginal tissues, e.g. for the formation of larval and imaginal cuticles. Willis (1986), however, strongly questioned the validity of such a view. The formation of the pupal cuticle is noteworthy because it is the shared product of larval and imaginal cells. If the extreme view mentioned above were true, we would expect that a gene involved in cuticle formation would be expressed in imaginal cells, or in larval cells, but not in both. Despite some examples of
Fig. 4. Pattern of transcript accumulation for EDG-42A in frozen sections of (A) 8h prepupa, (B) 10h prepupa and (C & D) 14h pupa. Hybridization probes synthesized from the 615R3.2 subclone; A, B & C, antisense strand; D, sense strand. Note the presence of signal only over the thoracic epidermis at 10h (B). By 14h signal is present over both imaginal and larval epidermis. Arrow indicates the junction between larval and imaginal epithelia. Ab, abdomen; Th, thorax; LC, larval cuticle; PC, pupal cuticle; LE, larval epidermis with large polytene nuclei; IE, imaginal epidermis with small diploid nuclei. Bars, 0.22 mm.

differential distribution (e.g. PCP8 and 15, see Introduction), most proteins seem to be present in both the anterior and posterior pupal cuticle, indicating that the genes that encode these proteins are expressed in both larval and imaginal epidermal cells. Nonetheless, it is possible that electrophoretically equivalent proteins could be encoded by separate (or even duplicate) genes that are differentially expressed in larval and imaginal epidermal cells. Alternatively, imaginal cells in histoblast nests or in genital discs could account for the presence of an 'imaginal' protein in abdominal cuticle.

Studies using gene-specific probes can clarify whether a particular gene is expressed in imaginal, or in larval cells, or in both. Indeed, CP-GART is expressed only in larval cells (Henikoff et al. 1986) and EDG-84A-1 is expressed only in imaginal cells (Fig. 3). In clear contrast, transcripts complementary to EDG-78E, EDG-42A, and EDG-64CD accumulate in prepupae in both larval polytene cells and imaginal diploid cells (Figs 3, 4, and 5). Because these genes appear to be unique in the Drosophila genome (Fechtel et al. 1988), detection of their transcripts in both larval and epider-
Fig. 5. Pattern of transcript accumulation for EDG-64CD in frozen sections of (A) mid-third instar larva, (B) 0 h prepupa and (C) 10 h prepupa, imaginal disc, (D) 10 h prepupa, abdominal epidermis. Hybridization probes were synthesized from the 63RS6.6 subclone (only antisense hybridization shown). Note the large polytene nuclei (N) of the larval epidermis (LE). LC, larval cuticle; IE, imaginal epidermis; M, muscle; F, fat body; Bars, 0.02 mm.

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References


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