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Tissue-specific ecdysone responses: regulation of the *Drosophila* genes *Eip28/29* and *Eip40* during larval development

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Summary

The *Drosophila* genes *Eip28/29* and *Eip40* are expressed in Kc cells and are rapidly induced by the steroid hormone ecdysone. The molecular basis for *Eip28/29*'s regulation in those cells has been studied in some detail. To determine how this regulation relates to normal development, we have examined the expression of both genes throughout *Drosophila* development, with special attention to *Eip28/29* and the final larval instar. *Eip28/29* expression is complex; there are tissues in which it is never expressed, others in which it is continuously expressed at a low level and tissues in which its expression is regulated without obvious relationship to endocrine events. However high-level *Eip28/29* expression always correlates with the presence of ecdysone and there is good evidence that *Eip28/29* is directly regulated by the hormone in some tissues and at some stages. Most striking are the induction of *Eip28/29* transcripts in numerous tissues at the last larval molt, their induction in the epidermis at the time of the 'late 3rd transition', their extinction in the same tissue by the premetamorphic ecdysone peak, and their induction by that peak in the lymph gland, hemocytes and proventriculus. These contrasting regulatory behaviors provide a well-defined model for studying the developmental specificity of steroid responses.

***Eip40* appears to be ecdysone-inducible only in the lymph gland and there only at the premetamorphic peak. The similarities been *Eip28/29* and *Eip40* regulation in the lymph gland and Kc cells support the idea that Kc cells are derived from a hematopoietic ancestor.**

Key words: ecdysone, *Eip28/29*, *Eip40*, *rp49*, *Drosophila*, ecdysone-inducible polypeptides, lymph gland, hemocytes, proventriculus, epidermis, midgut, Malpighian tubules, fat body, late 3rd transition.

Introduction

From a developmental perspective, the most remarkable feature of steroid hormone action is the diversity of the stage- and tissue-specific responses that may be elicited by a single hormone. There is no clearer example than that of insect metamorphosis. The steroid molting hormone ecdysone is the signal that triggers metamorphosis and experimentally the hormone stimulates metamorphosis both in intact organisms and (to a lesser extent) in organ cultures (Doane, 1973). Yet the transformations involved are as diverse as death and histolysis (of most larval tissues) and cytodifferentiation (of the imaginal precursors, the histoblasts and imaginal discs). Plainly, it is an important challenge to understand how a target cell's developmental history determines the nature of its response to ecdysone.

Recent results have illuminated some of the fundamentals of ecdysone action. An ecdysone receptor (EcR) has been identified and shown to be a member of the nuclear hormone receptor family (Koelle et al., 1991). That receptor binds to DNA sequences in the vicinity of regulated genes and several examples of those sequences (EcREs) have been studied (Cherbas et al., 1991). The characteristics of both EcR and EcREs reveal an especially close relationship between EcR and that branch of the steroid receptor family that includes the thyroid, estrogen and retinoic acid receptors. Most importantly, it is clear that EcR-EcRE interaction is a necessary and, under some circumstances, a sufficient condition for a gene to be regulated by ecdysone (Cherbas et al., 1991; Koelle et al., 1991).

We assume that, when a responsive cell encounters ecdysone, its initial reaction consists of transcriptional alterations in a set of directly ecdysone-responsive genes (the 'primary set'). If, as seems certain, the response is amplified by the regulatory activities of some of these primary gene products (Ashburner, 1990), the hormone's impact on gene expression will, of course, eventually be much more widespread.

We have defined the primary set of genes in order to frame questions concerning them. For example, we do not know the number of genes included in the primary set. We do know that a subset – the 'early puff sites' in the sali-

vary gland chromosomes of *Drosophila* – is quite small, including fewer than 10 genes (Ashburner, 1990); however, it is unclear whether a majority of responsive genes are represented by puffs. Moreover, we do not know whether most members of the primary set are stage- and tissue-specific. Among the small set of ecdysone-responsive genes that has been studied, a few (notably the early puff products E74, E75 and some of the BR-C genes) appear to be widely ecdysone-responsive (for review, see Andres and Thummel, 1992). In contrast, some others (fbp1, the IMP genes; Natzle et al., 1988; Andres and Thummel, 1992) appear to be tissue- and stage-specific in both their expression and their hormonal inducibility. Clearly, if we are to understand developmentally divergent ecdysone responses, we shall need more fully to understand whether and how the primary sets of target genes differ.

Here we report an example of a primary gene whose behavior is quite different from the examples cited above. Like the early puff genes, *Eip28/29* is expressed in a wide variety of tissues and at many times during development. However, unlike them, it is positively ecdysone responsive in some times and places, negatively responsive in some others and non-responsive in others.

The *Eip28/29* gene was identified by virtue of its immediate response to ecdysone in the Kc line of *Drosophila* cultured cells (Savakis et al., 1980, 1984). Subsequent experiments have demonstrated that it is a member of the primary set in Kc cells (Bieber, 1986) and that it is part of the immediate response to ecdysone in many of the embryonically derived *Drosophila* cell lines. Although we do not know the function of *Eip28/29*, we do know that the gene has been conserved over considerable evolutionary distance; a tomato homolog is regulated by the hormone ethylene (Cordes et al., 1989). In addition, since *Eip28/29* regulation in Kc cells has been the subject of considerable study, we know a considerable amount about the sequence requirements for its proper regulation and expression in those cells (Cherbas et al., 1991; Lee, 1990). The *Eip40* gene was revealed by the same screen that identified *Eip28/29*; it too is a primary gene in Kc cells and it encodes a protein related to γ-cystathionase (Savakis et al., 1984; Rebers, J. and P. Cherbas, unpublished). Both *Eip* genes are expressed in naive Kc cells and their transcripts are moderately abundant (approx. 0.1% of poly(A)+ RNA). Following hormone treatment, both transcripts accumulate to higher levels. In the case of *Eip28/29*, a rapid transcriptional upshift (approx. 15-30 minutes) leads within 3-4 hours to an induced steady-state transcript level, approx. 10-fold higher than basal (Bieber, 1986).

Since the *Eip* genes were identified and have been studied only in cells in culture, it has been a matter of some interest to us to discover their developmental provenance. For example, it is of interest to discover whether there exist normal tissues in which these genes behave as they do in Kc cells. Here we report in the affirmative. Given that the *Eips* are directly ecdysone-responsive in some tissues and at some stages, are they universally so? Here we shall report that, to the contrary, the responsiveness of these genes is highly tissue-and stage-specific. It follows that, in addition to EcR and functional EcREs, the ecdysone response must be controlled by other factors, as yet unknown.

Materials and methods

Cell culture and hormone treatments

Kc167/M3 cells were grown as described previously (Cherbas et al., 1988). Kc-C are untreated cells; Kc-E are cells treated for 4 hours with 10^{-6} M 20-hydroxyecdysone.

Drosophila *culture and staging*

Synchronized *Drosophila* larvae were isolated from a genetically heterogeneous Oregon R population in continuous culture at 25°C on hard agar molasses plates (14% molasses, 3.5% agar, 0.15% tegasept) coated with a paste of brewer's yeast. Eggs were collected at 1 hour intervals, the first two collections being discarded. The embryos were washed in tap water (25°C) and allowed to develop on hard agar plates at 25°C. Newly hatched first-instar larvae were collected from the plates at 15 minute intervals and transferred to fresh plates coated with brewer's yeast for further development. Quasi-synchronous cohorts were collected at 8-hour intervals until pupariation, as described in the Results.

Salivary glands were dissected in Ringers solution (Roberts, 1986), fixed (5 minutes) in 45% acetic acid and stained (5 minutes) in aceto-carmine (La Cour, 1941). Puff stages were determined according to Ashburner (1972). Non-feeding ('wandering') larvae in puff stage 1 were identified by the staining of their midguts when they were reared on a medium containing 0.05% bromphenol blue (Maroni and Stamey, 1983).

Probes

Template-strand RNA probes for *Eip28/29* and *IMP-E1* were prepared by transcription in the presence of ^{32}P- or ^{35}S-labeled nucleotides with T3 (*Eip28/29*) or Sp6 (*IMP-E1*) RNA polymerase, essentially according to Melton et al. (1984). ^{32}P-RNAs were typically labeled at 2×10^9 cts/minute/μg RNA, ^{35}S-labeled RNAs typically at 10^9 cts/minute/μg. ^{32}P-labeled DNA probes for *rp49* were prepared by nick-translation (Rigby et al., 1977); their specific activities were approx. 5×10^7 cts/minute/μg. The following plasmids were used for preparation of these probes.

(1) *Eip28/29*: pBSM45(ScP)B, prepared by insertion of a 540 bp *Sac*I/*Pst*I fragment from the cDNA clone pKc45 (Cherbas et al., 1986) into pBS- (Stratagene Cloning Systems). The insert (bases 108-647 of the mRNA) includes portions of all four exons.

(2) *IMP-E1*: pE1-8.0R1a (Natzle et al., 1988), a generous gift from Jeanette Natzle. It consists of an 8.0 kb genomic fragment of lambda phage IMP-E1 cloned into the *Eco*RI site of pSP64 (Promega Biotec).

(3) *rp49*: HR0.6, containing a 0.6 kb *Eco*RI/*Hin*dIII fragment of the ribosomal protein gene *rp49* cloned into pBR322 (O'Connell and Rosbash, 1984), a generous gift from Michael Rosbash.

RNA isolation

Total nucleic acids were isolated from animals or cells using an SDS-proteinase K treatment for RNA described as method II by Schulz et al. (1989) and modified by the omission of DNAase treatment.

Northern blots

RNA was displayed on 1.5% agarose/formaldehyde gels (Lehrach et al., 1977). Each sample contained 4-10 μg total nucleic acid and ethidium bromide at a concentration of 125 μg/ml. After electrophoresis, gels were photographed in short-wave ultraviolet light, the stained rRNA bands serving as confirmation of RNA loading and integrity. Gels were soaked in 20× SSC (3 M NaCl, 0.3 M sodium citrate) for 1 hour and blotted onto nitrocellulose (Schleicher & Schuell) in 10× SSC according to Thomas (1980). Filters were then baked for 2 hours at 80°C in a vacuum oven.

Hybridization with nick-translated probes was carried out as described by Savakis et al. (1984). When single-stranded RNA probes were used, the hybridization was carried out at 55°C, and washes were at 55°C and 60°C. With either probe the hybridization solution contained approx. 5×10^6 cts/minute ^{32}P/ml. Autoradiography (at −80°C) employed Kodak XAR-5 film with an intensifying screen.

In situ hybridizations to tissue RNAs

Embryos, larvae and pupae were washed several times in PBS (130 mM NaCl, 7mM Na_2HPO_4, 3 mM NaH_2PO_4) and chilled on ice before embedding in OCT compound (Miles). Adult flies were etherized and embedded without washing. Eight 10 μm sections were cut on a Slee cryostat and placed on microscope slides subbed with 0.1% gelatin, 0.01% chromium potassium sulfate and 1 mg/ml poly-L-lysine hydrobromide (Sigma) (Gall and Pardue, 1971). The slides were heated to 50°C for 2 minutes, fixed at 22°C in 4% paraformaldehyde/PBS for 20 minutes, washed for 15 minutes in PBS and dehydrated through a graded series of ethanols. Dried slides were stored for up to several months at room temperature before hybridization.

Intact tissues were dissected from wandering third-instar larvae in TB-1 buffer (Bonner et al., 1984). Tissues were fixed in 4% paraformaldehyde/PBS for 15 minutes, rinsed twice for 10 minutes in PBS, transferred to 70% EtOH and stored overnight at 4°C. The fixed samples were transferred to poly-L-lysine-coated teflon chamber slides (Celline Assoc) in 95% EtOH and allowed to air dry. Dried slides were stored at room temperature for up to several weeks before hybridization. Slides containing sections or whole mounts were prepared for hybridization according to Hafen et al. (1983) with the following modifications. (i) The slides were not treated with HCl prior to 2× SSC heat treatment and (ii) protease treatment consisted of an 8 minute incubation (room temperature) with Pronase E (Sigma; 167 μg/ml). The enzyme was diluted from a 40 mg/ml aqueous stock that had been allowed to autodigest for 5 hours at 37°C. Hybridization, washes and autoradiography were carried out according to Ingham et al. (1985) except that SDS was added to a final concentration of 0.5% in the hybridization solution and hybridization was carried out at 55°C. ^{35}S-labeled probes were included at a concentration of 0.3 ng/μl for each kb of template. Approximately 150 μl of hybridization solution was added to each slide and covered with a 24×60 mm siliconized coverslip. Slides were coated with NTB-2 film emulsion (Kodak; diluted 1:1 with water), exposed at 4°C for 1 week and developed in D-19 developer (Kodak) for 2.5 minutes. Tissues were stained in 250 μg/ml pinocyanol-Cl (Sigma) in 70% EtOH for 2 minutes (Proescher, 1933).

Analysis of in situ hybridization data

On each slide, we included sections from pellets of untreated and ecdysone-treated Kc167/M3 cells (see Fig. 3-1A,B); *Eip28/29* RNA levels in these cells differ by a factor of approx. 10 (Savakis et al., 1984; this paper). The grain densities over these sections served as our dual standards. By reference to them, we scored the grain densities over tissues (by eye) on a scale of 0-5, where score 0 represents undetectable signal, score 2 represents the Kc-C level and score 4, the Kc-E level. Scores 1 and 3 were used for intermediate densities and score 5 for densities in excess of Kc-E. Occasionally fractional values were used to give an accurate representation of comparative densities. Since the scale is probably logarithmic, averages, e.g. Table 1, are approximate geometric means.

Results

The Eip genes are expressed throughout development

We began by investigating the temporal specificity of *Eip* gene expression. RNAs were extracted from whole organisms collected at intervals following synchronization and analyzed by northern blots to display the stage-specific titers of the *Eip28/29* and *Eip40* transcripts (Fig. 1). Since, for *Eip28/29*, the northern blots do not distinguish the short and long form alternatively-spliced transcripts (Schulz et al., 1986) we refer to their combined signal collectively as *Eip28/29* 'transcripts'.

With respect to the *Eip28/29* transcripts, several conclusions appear justified.

(i) Expression occurs at a low and virtually constant level throughout embryogenesis save for an obvious peak at 9-12 hours. The coincidence of this peak with the mid-embryonic ecdysone peak and with the first appearance of ecdysone receptors (Koelle et al., 1991) is suggestive. While the absence of signal in unfertilized eggs (UF) could mean that *Eip28/29* transcripts are not maternally loaded into oocytes, there is evidence for maternal loading (from in situ images of developing oocytes, see below); hence, we suspect that the *Eip28/29* transcripts are unstable in unfertilized eggs collected over a 3 day period. Therefore, the moderately strong signal in 0-3 hour embryos may be due to maternal RNA and cannot be considered evidence of early zygotic expression.

(ii) The larval stages are marked by obvious fluctuations in the titers of the transcripts. The L1 molt (hatching) is accompanied by an obvious increase in titer. Subsequent maxima occur during the L1-L2 and L2-L3 molt cycles. It is possible, but not certain given the resolution of the experiment, that these maxima coincide with the ecdysone peaks that precede each molt. However, there can be little doubt that, during L3, maximal expression occurs during the mid-instar, i.e. prior to the major ecdysone peak that precedes pupariation. Indeed, taken alone, these data are consistent with the idea that ecdysone depresses the *Eip28/29* titer just prior to pupariation.

(iii) *Eip28/29* expression appears to increase slightly at pupariation (cf. 104 hour L3 and 0 hour 'prepupae') and again at about the time of the true pupal molt (12-18 hours following pupariation). Otherwise during the prepupal and pupal periods, titers are low and comparable to those that prevail during embryogenesis.

(iv) In adults, the titer increases following eclosion, then remains relatively high and constant for 2-3 days, dwindling only slightly over approx. 20 days. Comparable blots of RNAs from adult males show a similar pattern but somewhat lower signals relative to rRNA (not shown).

By comparison, the *Eip40* expression patterns are far less variable. In point of fact, during L3, *Eip40*'s titer is more constant than that of the nominal 'loading control' (*rp49*). [Those who employ *rp49* as a control should note that its abundance does not appear to be constant throughout development.] The *Eip40* transcript is maternally loaded, though its titer may still increase slightly early in embryogenesis. Thereafter its titer remains comparatively high and invariant throughout embryogenesis and the larval stages (save for a reduction at the end of L3) and low throughout the prepupal and pupal stages. In adults, its level is comparable to that of *Eip28/29*, both in females (Fig. 1) and in males (not shown).

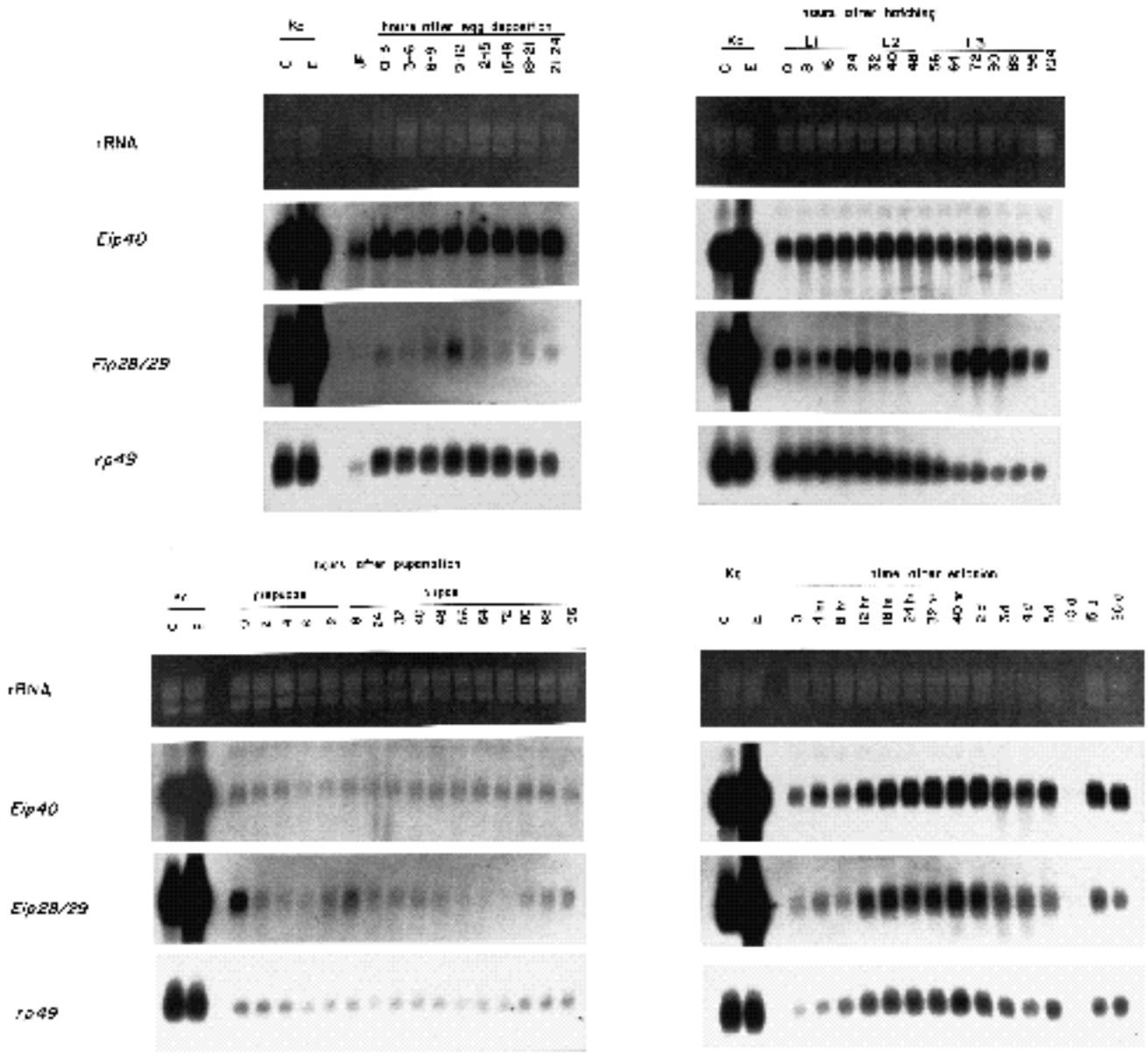

Fig. 1. Temporal variation in Eip gene expression. The relevant portions of northern blots probed for *Eip28/29* and *Eip40.* The four panels represent separate blots spanning the entire life history. All the blots were probed for *Eip28/29,* then washed and probed for *Eip40* and finally – as a loading control – for *rp49* (for details, see Methods). The top segment of each panel shows the image of the ethidium bromide-stained rRNA region. Relative to the rRNA image, there is an obvious decline in *rp49* titers during L3 which, so far as we are aware, has not previously been reported. All Panels: Each lane contained 4 μg total nucleic acids. C, 4 μg total nucleic acids from untreated Kc167 cells. E, 4 μg total nucleic acids from ecdysone-treated Kc167 cells. Embryos: Time 0, oviposition. *UF* refers to unfertilized eggs collected over a 3-day period as they were deposited by virgin females. Larvae: Times are in hours after hatching. In our population, L1 lasted 24±1 hour, L2 24±2 hours, L3 56±6 hours and pupariation occurred at 104±6 hours. Pupae: Cohorts pupariated during a 1 hour window. In our population, the pupal molt occurred approximately 12 hours later. Adults: Cohorts eclosing within a 1 hour window were collected and separated by sex. Samples from females are shown in the figure. The '10 day' sample was degraded.

It is fair to compare the intensities of the *Eip28/29* and *Eip40* signals: the probes were similar in length and specific activity and the autoradiographic exposures were identical. More importantly, it is fair to compare the signal intensities for both genes with the signals (for which Fig. 1 shows only gross overexposures) due to similar loadings of untreated and ecdysone-treated Kc167 cells. Our estimate (based on varied RNA loadings and multiple exposures, not shown) is that the maximal signals due to *Eip28/29* and *Eip40* in whole organisms never exceed 5-10% of those for untreated Kc167 cells.

In our population, a cohort of larvae synchronized at hatching, pupariates over a period of approx. 6 hours. Given this asynchrony, we wondered whether the obvious *Eip28/29* peak in L3 might not, in fact, coincide with the premetamorphic ecdysone peak. To test this possibility, we

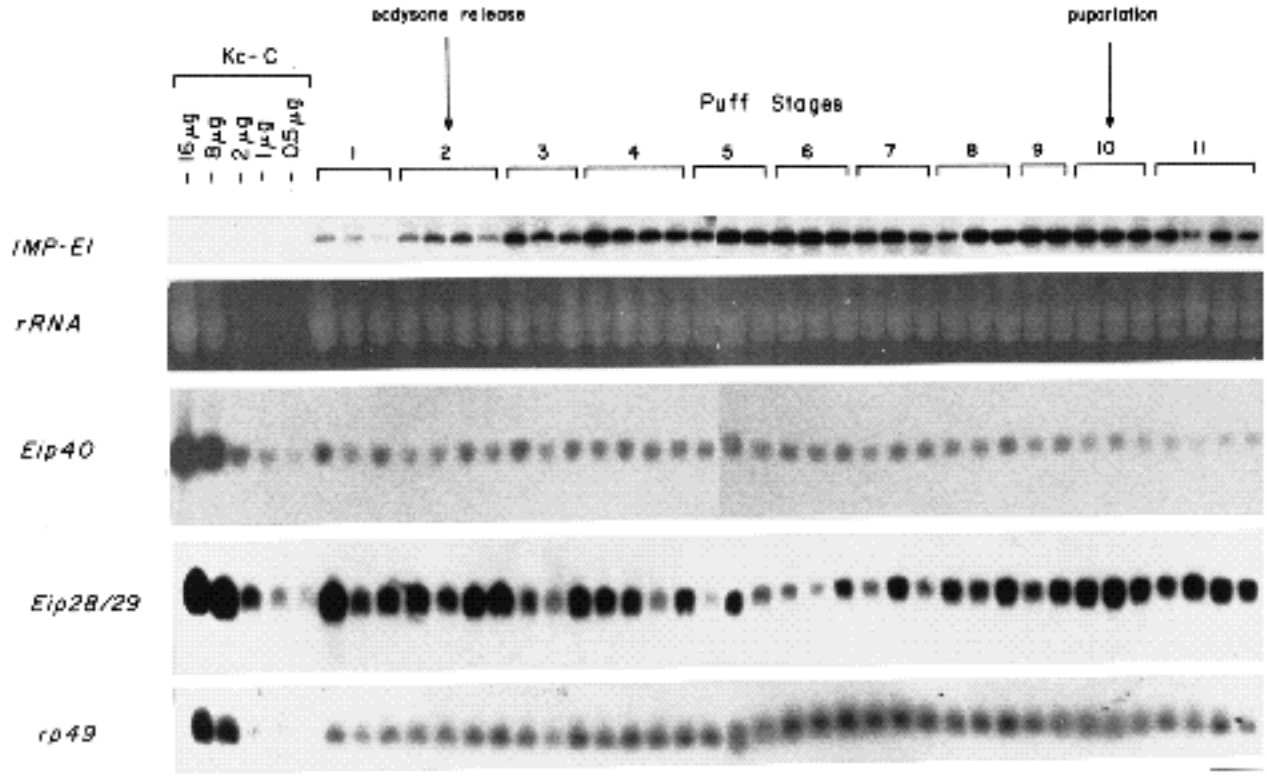

Fig. 2. Eip gene expression in puff-staged, late L3 larvae. Each lane contained total nucleic acids (7-15 μg) from a single late L3 larva. The larvae were staged by examining the puffing patterns in the salivary gland. For comparison, the lanes at the left contained varied amounts of total nucleic acids isolated from untreated Kc167 cells. Probes were identical to those in Fig. 1, except that we also included *IMP-E1* (see text).

examined individual larvae staged by their polytene puffing patterns (Fig. 2). Wandering third instar larvae were selected and their salivary glands processed for cytological analysis. The remainder of each animal was homogenized in extraction buffer and frozen at −80°C for later recovery. In Fig. 2, each lane represents the RNA recovered from a single individual and the lanes were assigned by puff stage (PS). As controls, we have included not only the rRNA pattern and *rp49* but also *IMP-EI*, a gene that is known to be induced (in imaginal discs) by the premetamorphic ecdysone peak (Natzle et al., 1988).

Inspection of Fig. 2 will reveal one of the consequences of studying individuals: by reference to rRNA staining, the maximal titers of *Eip28/29* are substantially higher than in Fig. 1, equivalent to 30-50% or more of the Kc-C level. However, despite the improved temporal resolution, Fig. 2 is fundamentally consistent with Fig. 1. *Eip28/29* transcripts are present throughout the molt and their titers vary substantially between individuals. To the extent that a developmental pattern is present, it consists of a decline immediately following ecdysone release (PS 5-7) followed by recovery at pupariation. *Eip40* appears to vary very little during the early puff stages and to decline gradually during the later ones. These patterns contrast with the behavior of the *IMP-E1* transcripts which are not abundant during the intermolt period (PS 1) but strongly induced during and after PS 2.

In summary, *Eip28/29* is evidently subject to some quantitative regulation and features of its developmental profile suggest that ecdysone may play a role in that regulation. However, the pattern that would be simplest to interpret, simple induction of both *Eip* genes by the premetamorphic ecdysone peak either ubiquitously or in selected tissues, clearly does not occur. If the *Eip* genes are ecdysone-inducible and immediately responsive in some tissues and at some stages, such regulation cannot dominate their overall expression patterns. Finally, if any tissue behaves like Kc cells with respect to basal and induced *Eip* gene titers, that tissue must be small (< approx. 10% of total organismal RNA).

Eip28/29 expression patterns are tissue-specific and diverse

Clearly, 'whole organism' RNA titers are a crude measure that will be informative only under special circumstances. Therefore we proceeded to analyze both the spatial and temporal distributions of the transcripts by in situ hybridization. Although our account will incorporate some relevant observations concerning both *Eip* genes and all developmental stages, henceforth we will concentrate our attention on the manageable problem of *Eip28/29* expression during L3.

Animals were synchronized as for Fig. 1, collected at intervals and sectioned serially for in situ hybridizations. As reference standards, we included on each slide sections prepared from untreated (Kc-C) and ecdysone-treated (Kc-E) Kc167 cell pellets (Fig. 3-1A,B). The inclusion of these

Table 1. *Tissue-specific Eip28/29 expression during L3*

(A)

Age	n	Group I		Group II				Group III			
		VG		PX		SG		MG		MT	
48-L2	5	1.0	[0, 1]	0.4	[0, 1]	0	[0, 0]	2.4	[2, 3]	0.3	[0, 1]
48-L3	5	1.0	[.5, 1.5]	4.0	[4, 4]	2.6	[0, 4]	2.5	[1.5, 3]	0.5	[0, 1]
56	10	1.0	[1, 1]	0.3	[0, 1]	0.2	[0, 1.5]	1.8	[0, 3.5]	0.2	[0, 1]
64	10	0.9	[.5, 1]	1.1	[0, 2]	0	[0, 0]	0.7	[0, 2.5]	0.1	[0, 1]
72	9	1.0	[1, 1]	0.8	[0, 1]	0	[0, 0]	0.5	[0, 1.5]	0.4	[0, 1.5]
80	10	1.0	[1, 1]	1.1	[.5, 1.5]	0.2	[0, .5]	1.1	[0, 3]	2.2	[0, 4]
88	10	1.3	[1, 2]	0.8	[0, 1.5]	0.4	[0, 1]	0.7	[0, 3]	1.9	[0, 4]
96	10	1.0	[0, 1.5]	0.3	[0, .5]	0.1	[0, .5]	0.5	[0, 1.5]	1.6	[0, 4]
WPP	10	0.7	[0, 1]	0.3	[0, 1]	0.1	[0, .5]	0.3	[0, 1]	2.9	[1, 4]

(B)

Age	n	Group IV						Group V					
		AS		EP		FB		PV		LG		HC	
48-L2	5	3.2	[2, 5]	0.2	[0, 1]	0.8	[0, 1]	0.5	[0, 1]	0	[0, 0]	0	[0, 0]
48-L3	5	4.4	[4, 5]	2.4	[2, 3]	1.9	[1, 3]	0.8	[0, 1]	0	[0, 0]	0	[0, 0]
56	10	3.1	[2, 4]	0.7	[0, 2]	0.5	[0, 1]	0.7	[0, 1]	0.2	[0, 1]	0	[0, 0]
64	10	2.3	[1, 3.5]	0.3	[0, 1]	0.6	[0, 1]	0.3	[0, 1]	0	[0, 0]	0	[0, 0]
72	9	1.9	[1.5, 2]	1.3	[0, 2]	1.2	[1, 2]	0.5	[0, 1]	0.1	[0, 1]	0	[0, 0]
80	10	4.0	[4, 4]	3.8	[3, 4]	3.4	[2, 4]	0.5	[0, 1]	0.4	[0, 1]	0.1	[0, .5]
88	10	4.2	[4, 5]	4.3	[3.5, 5]	3.2	[2, 4]	0.7	[0, 3]	0.8	[0, 1.5]	0.5	[0, 1]
96	10	4.0	[4, 4]	3.6	[2, 5]	2.1	[1, 3]	0.6	[0, 2]	0.8	[0, 3]	0.5	[0, 1]
WPP	10	0.1	[0, 1]	0.8	[0, 2]	1.2	[0, 2]	4.3	[3, 5]	3.1	[3, 4]	3.1	[2.5, 4]

The table summarizes all of the in situ hybridization data for synchronized, sectioned L3 larvae. The left-hand column gives the animal's age (hours after hatching). At 48 hours some larvae are L2, some L3; they are described separately. In our population, the mean age of white puparia (WPP) was 104 hours. The second column reports the number of animals studied.

Signal densities were scored as described in Methods. In the data columns, the left-hand value is the mean score. In brackets, we report the minimum and maximum scores recorded. VG, ventral ganglion; PX, pharynx; SG, salivary glands; MG, midgut; MT, Malpighian tubules; AS, anterior spiracle; EP, epidermis; FB, fat body; PV, proventriculus; LG, lymph glands; HC, hemocytes.

standards made it possible to record results on a subjective, but quantitative scale, as described in Methods.

For our analysis of L3, we examined serial sections from 79 individual larvae taken at 8 hour intervals throughout the period from 48-104 hours after hatching. During this period, *Eip28/29* transcripts were detectable, at one time or another, in 11 different tissues: central nervous system, epidermis, fat body, the gland cells of the anterior spiracle, peripheral hemocytes, lymph glands, Malpighian tubules, midgut, pharynx, the posterior half of the proventriculus and salivary glands (Table 1). While each tissue's expression pattern is in some respect unique, all of the tissues can reasonably be combined into 5 'Expression groups'.

Expression group I : central nervous system

Group I comprises only the central nervous system (CNS). The entire ventral ganglion (VG), expresses *Eip28/29* at a constant, low level throughout L3 with little animal-to-animal variation (Fig. 3-2 panels F1,F2; Table 1). There is

no indication that this expression responds in any way to changing hormone levels. *Eip28/29* appears to be expressed in the brain as well, but at levels very near background. Because of the uncertainty associated with estimating these levels, brain expression levels are omitted from Table 1.

Expression group II : pharynx and salivary glands

The group II pattern consists of strong expression immediately following the L2-L3 molt followed by low to undetectable expression later in L3. In our populations, 48 hour larvae were approximately evenly divided between L2 and L3 (mouth hook morphology) and we studied both groups. In five 48 hour-L3 individuals, there was intense hybridization throughout the pharynx (Fig. 3-1C) — hybridization that is not due to cuticular adsorption since the complementary probe fails to hybridize. Similarly 3/5 individuals exhibited intense hybridization throughout the salivary glands (Fig. 3-1D). In the five 48 hour-L2 individuals, both tissues were essentially negative. After the L2-L3 molt, the hybridization intensity in both tissues fell within 8 hours to low levels and, save in the pharynx of one individual, never again approached the Kc-C level (Table 1A).

It seems likely that the abrupt and short-lived appearance of *Eip28/29* transcripts in the group II tissues at the time of the L2-L3 molt is one consequence of the ecdysone peak which precedes that molt (Richards, 1981).

Expression group III : midgut and Malpighian tubules

Group III is more heterogeneous. The common feature is that in both the midgut and the Malpighian tubules, *Eip28/29* expression is highly variable and probably not strictly a function of developmental age.

Throughout the midgut, *Eip28/29* transcripts are expressed at levels in excess of Kc-C in both late L2 larvae and young L3s. From 64 hours onward, the prevailing expression level is quite low to undetectable. However, occasionally and non-reproducibly single intestinal loops (Fig. 3-2 panel G1) or even single cells (Fig. 3-2 panel G2) exhibit intense hybridization. We can discern no pattern to these instances of intense expression. It is possible that they reflect responses to the environment (i.e. food or its metabolic products) rather than a developmental program. As a glance at Table 1 will show, mean scores are essentially meaningless in the face of this idiosyncratic variation.

In contrast, entire sets of Malphighian tubules (MT) tend to behave similarly; but as in the case of the midgut, there is striking larva-to-larva variation (Fig. 3-3 panels J1, J2). This variability persists throughout the instar and almost obscures any underlying pattern. It is clear, however, that there is no peak of expression following the L2-L3 molt and that the highest levels of expression in the MT occur during the final half of the instar (after approx. 80 hours). Although it is possible that this situation reflects the genetic heterogeneity of the larvae, we once again suspect environmental control, probably superimposed on an endocrine change in sensitivity at about 80 hours.

Expression group IV : anterior spiracle, epidermis and fat body

Group IV is especially interesting to us because it includes tissues that together comprise a substantial fraction of the mass of an L3 and which presumably contribute significantly to the northern patterns in Fig. 1. All of the group IV tissues exhibit a well-defined pattern of *Eip28/29* expression: expression is high at the start of L3, then it falls to low levels that continue until approx. 80 hours; after 80 hours expression is once again elevated until it falls precipitously at pupariation. Since it is clear that a number of developmental events occur in the vicinity of 80 hours and since we shall have repeated occasion to refer to this checkpoint, we shall call it the 'late 3rd transition' (see the Discussion).

The gland cells of the anterior spiracle exhibit especially intense hybridization (Fig. 3-1 panels E1,E2) and, in this tissue, high level expression is already evident in some 48 hour-L2 larvae. Nonetheless, it is clear that expression is elevated following the molt and that transcript levels fall between 56 and 72 hours. Following the late 3rd transition, levels of expression become uniformly high (comparable to the Kc-E level) until they fall between 96 hours and pupariation.

The epidermis (Fig. 3-2 panels H1,H2) and the fat body (panels I1 and I2) behave similarly, save that in both cases the initial peak is lower. In the fat body, expression levels are somewhat variable and seldom reach or exceed Kc-E levels. Although we can discern no systematic spatial differences among regions of the fat body, expression within that diffuse tissue tends to be patchy and mean levels can serve only as a general guide.

As in the cases of the group II tissues and the midgut, it seems likely that the early peak of group IV expression is one consequence of the late L2 ecdysone peak. We do not know the endocrine events underlying the late 3rd transition, though they certainly involve ecdysone (see Discussion). The abrupt decline of *Eip28/29* in the group IV tissues between 96 hours and pupariation seems very likely to be a consequence of the premetamorphic ecdysone peak.

Expression group V : posterior proventriculus, lymph glands and hemocytes

In the group V tissues, *Eip28/29* expression is barely detectable prior to metamorphosis; in the interval between 96 hours and pupariation, expression rises to Kc-E levels. Cells of the posterior portion of the proventriculus (Fig. 3-3L) exhibit this expression pattern, while the anterior proventriculus never hybridizes above background levels. Hybridization occurs over all of the lymph glands (Fig. 3-3M) although its intensity is variable and somewhat patchy. Finally, group V includes nests of diploid, circulating hemocytes (Fig. 3-3K) wherever they may be located. It seems very likely that the abrupt appearance of *Eip28/29* in the group V tissues represents induction by the premetamorphic ecdysone peak.

Eip28/29 expression during other developmental stages

Low level expression is detected in early embryos associated with the blastoderm nuclei. At germ band extension, this generalized expression falls to undetectable levels. The peak of *Eip28/29* expression at the time of germ band retraction (Fig. 1) is associated with intense (Kc-E level) hybridization localized to the anterior and posterior midgut primordia. By 12 hours, this specific expression ceases save

for occasional bursts of expression in various regions of the developing midgut.

During the first and second instars, we have observed above-background hybridization in only three tissues: epidermis, fat body and midgut. For the most part, the midgut follows the idiosyncratic pattern characteristic of L3. Many L1 larvae express *Eip28/29* at moderate levels somewhere in the midgut. This expression accounts for the majority of the signal detectable by in situs. In L2, most larvae exhibit high level expression at some unpredictable location in the midgut irrespective of developmental age.

In the epidermis and fat body of L1s, *Eip28/29* expression barely exceeds background levels, but increases near the end of the instar. In L2, epidermal and fat body expression become comparable in intensity to the localized patches of midgut expression and these probably account for the majority of the northern signal.

We could not detect expression (by in situs) in prepupae (after the WPP stage) or in pupae. In adults of both sexes, the crop and fat body exhibited invariant high level expression and the midgut variable high level expression. In females, we observed high level expression in nurse cells of all stages and the ovarian oocytes give a moderately intense and spatially uniform hybridization signal.

Eip40 expression during L3

Our analysis of *Eip40* has been limited in scope: however, it supports the following three conclusions: (i) *Eip40* is expressed at a low but detectable level in virtually all tissues; (ii) it is expressed at the Kc-C level in the fat body, salivary glands and lymph glands throughout L3; (iii) like *Eip28/29*, *Eip40* transcript increases to Kc-E levels in the lymph glands just prior to metamorphosis. Comparable high level expression of *Eip40* has not been observed in any other tissue at any time during L3.

Changes in Eip28/29 expression at the end of L3 are concomitant with early ecdysone effects

Our survey showed that, at about the time of the premetamorphic ecdysone peak, *Eip28/29* expression declines precipitously in group IV tissues and increases dramatically in group V tissues. It is attractive to suppose that both transitions are effected by ecdysone. To determine whether that is likely, we looked at the timing of these events on a more informative time-scale based on the appearance of the early ecdysone-induced puffs in the salivary glands (Ashburner, 1972). Intermolt salivary glands have a stable puffing pattern (PS 1). When the premetamorphic ecdysone peak occurs (5-6 hours prior to pupariation), the puffing pattern changes rapidly, moving through a number of arbitrarily designated stages by WPP (PS 10/11). The well-known salivary gland early puffs (e.g. 74EF, 75B) become detectable during PS 2 and reach their maxima by about PS 5.

We selected late L3 larvae (and some WPP), determined the puffing stage for each (by analyzing a single salivary gland) and used the remainder of the animal for in situ analyses. The proventriculus was removed and studied by whole-mount in situs; the anterior and posterior portions of each animal were frozen for sectioning and in situ examination of other tissues. The results are shown in Table 2 and illustrated in Fig. 4.

Table 2 shows that group IV expression levels are high in larvae designated PS 1-2. The decline in levels appears to require at most the period from PS 3 to PS 8. In the epidermis, the decline appears to be virtually complete by PS 4-5. The same pattern can be seen for the fat body, though it is somewhat obscured by the variability of the data. Conversely, in the group V tissues, expression levels are low during PS 1-2 and they gradually rise from PS 3 to PS 8.

Fig. 4 illustrates the striking pace of these events. The A panels represent PS 1. At PS 1 expression in the posterior proventriculus is barely detectable; in contrast, each of the large epidermal cells (panels A2, A3) is intensely labeled. By PS 3, i.e. within minutes of ecdysone release (panels B) epidermal expression is undetectable and the posterior proventriculus is clearly labeled. By PS 8 (panels D), proventricular hybridization is intense as is that associated with the nests of peripheral hemocytes in the vicinity of the epidermis.

Since the lymph glands (LG) did not survive the whole-mount in situ procedures, this experiment provides no direct evidence concerning their timing. However in the approx. 100 larvae that we have analyzed in sections, the switch from low to high level expression of *Eip28/29* is absolutely correlated among group V tissues.

Hence we are confident that the following events occur concurrently and within minutes of the premetamorphic ecdysone release: (i) the early puffs are induced in the salivary glands; (ii) the *Eip28/29* transcripts disappear from the group IV tissues; and, (iii) the *Eip28/29* transcripts are induced in the group V tissues where they reach their maximal titers within 3-4 hours of the hormone's appearance.

Discussion

Because *Eip28/29* and *Eip40* are ecdysone-responsive in numerous *Drosophila* cell lines of independent origin (Cherbas et al., 1991), we anticipated that they would be similarly responsive in one or more settings during normal fly development. There seems little question that this is true.

Our work is most thorough with respect to L3 when, on prior evidence, we suppose that changes in the endocrine environment include at least three 'events': **(i)** the after-effects of the ecdysone peak antecedent to the L2-L3 molt; **(ii)** a 'late 3rd transition' about half-way through L3 (see below); and **(iii)** the premetamorphic ecdysone peak that occurs approx. 5-6 hours prior to pupariation. Fig. 5 summarizes our interpretation of *Eip28/29* expression during that instar and shows graphically that each episode of moderate- or high-level *Eip28/29* expression follows one or another of these events.

(1) At the time of the L2-L3 molt, *Eip28/29* is ecdysone-responsive in the group II and group IV tissues and in the midgut. Expression is transient and may well be absolutely dependent on ecdysone.

(2) By mid-instar, *Eip28/29* is no longer ecdysone-responsive in some tissues. However, the group IV tissues and the Malpighian tubules do respond to the late 3rd transition and in them expression modulates to higher levels. Specifically in the group IV tissues this leads to high level expression that lasts until pupariation. The temporal pattern

Table 2. *Tissue-specific Eip28/29 expression during early puff stages*

	Group II	Group III		Group IV			Group V	
Puff Stage	PX	MG	MT	AS	EP	FB	HC	PV
1-2	1	3	2	5	5	2	1.5	X
1-2	1	3	4	5	5	2.5	1	+
1-2	1	4	4	5	5	4.5	2	+
1-2	1.5	3	3	5	5	3	1	+/-
1-2 *	2	3	2.5	5	5	2.5	2	+
1-2	1	3.5	5	5	4	2.5	2	+/-
1-2	1	3	3	X	5	3	1	+
1-2	2	3	4	5	5	2	2	+
2-3	2	2	3	5	3	1	3	++
3	1	3	5	5	3	2	2.5	X
3	1	1.5	3	1	1.5	2	2.5	++
3 *	1	2	.5	4	4	1	3	++
3	1	3	5	5	2.5	.5	3	++
4-5 *	1	3	4.5	4	.5	2	3.5	+++
8 *	.5	1	5	1	0	2	5	+++
10	1.5	.5	4	.5	.5	1	4.5	+++
11	1	1	5	1	.5	1	5	X
11	1	.5	4.5	1	.5	2.5	5	X

Results of the experiment illustrated in Fig. 4 and described in the text. Each row reports tissue-specific *Eip28/29* expression for a single puff-staged larva or WPP. [PS 10 and 11 animals were WPP.] Expression levels were determined by in situ hyrbridizations to frozen sections except in the case of the proventriculus which was studied in whole-mount preparations. For the proventriculus '+/–' indicates background hybridization and '+++' indicates very high level expression. 'X' means that the tissue in question was not recovered. The four larvae marked with asterisks are those illustrated in Fig. 4. Other abbreviations are as described for Table 1.

is, in fact, virtually identical to that of glue gene expression in the salivary glands (Hansson and Lambertsson, 1983; Crowley et al., 1984). Our northern data (Fig. 1) are plainly dominated by the large mass of group IV tissues; consequently they show high level expression during the latter half of the instar.

(3) Finally, the premetamorphic ecdysone peak is responsible for terminating expression in group IV and for stimulating a burst of expression in the group V tissues.

Timetables of expression cannot by themselves say how a gene is regulated, but there is good reason to believe that *Eip28/29* is regulated directly by ecdysone during the premetamorphic period. Fig. 4 attests to the near-instantaneous decline of its transcript in epidermis following ecdysone release – a decline that suggests not only tran-

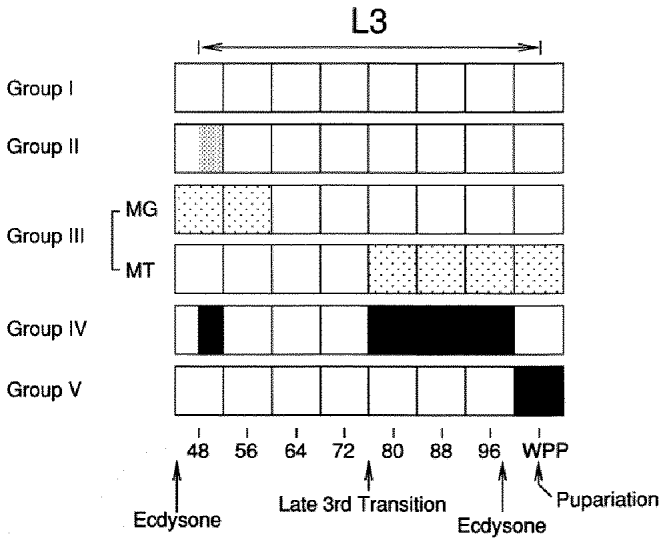

Fig. 5. Summary of tissue-specific patterns of *Eip28/29* expression in L3. A summary and interpretation of the data in Table 1. Each square represents an 8-hour period centered about the time of sampling. The left-most square is subdivided to report separately expression in L2 and L3 individuals. Approximate times for the relevant endocrine events are indicated. Open squares indicate low level or undetectable expression of *Eip28/29*, filled squares indicate high level expression. Intermediate expression levels are indicated by dots of different densities.

scriptional regulation but also transcript destabilization. Table 2 shows that accumulation in the group V tissues parallels the activity of the early puffs in salivary glands. Moreover, we know that ecdysone can induce *Eip28/29* and *Eip40* expression in cultured lymph glands and that both the rate and magnitude of the induction are comparable to those in Kc cells (A. J. Andres and P. Cherbas, in preparation).

The event that we have called the 'late 3rd transition' is emerging as an important landmark in *Drosophila* development. Although ecdysone titer measurements in flies are plagued by the poor synchrony of larval development, numerous studies have reported a small ecdysone peak about mid-way through the third instar, preceding the behavioral switch from feeding to wall-crawling (see Richards, 1981, for review; Berreur et al., 1984). The significance of this peak gains from the situation in *Manduca sexta* (Riddiford, 1986) where the endocrinology is well-defined and a similar (possibly homologous) small ecdysone peak causes both the change to 'wandering' behavior and important changes in epidermal gene expression. In *Drosophila* evidence is accumulating to suggest that significant changes in gene expression occur at or around 72 hours post-hatching (96 hours post-oviposition). These include the switch of alcohol dehydrogenase transcription from a proximal to a distal promoter (Savakis et al., 1986), induction of the *fbp1* gene in fat body (Lepesant et al., 1982) and in the salivary gland both the onset of glue polypeptide synthesis (Beckendorf and Kafatos, 1976) and a cycle of chromosomal puffing (Belyaeva et al., 1981). The salivary gland response includes the appearance of GEBF-I, a transcription factor essential for the ecdysone-induced expression of the glue gene *Sgs-3* (Georgel et al., 1991). Ecdysone can be rate-limiting for each of these events, as shown by the effects of administering exogenous steroid to hormone-deficient mutant strains (Murtha and Cavener, 1989; Hansson and Lambertsson, 1983) and to wild-type strains (Georgel et al., 1991). It is a reasonable hypothesis that all these events – and the induction of *Eip28/29* in the group IV tissues and Malpighian tubules – are among the sequelae of a single, small ecdysone peak at approx. 72 hours post-hatching. Of course, it remains to be determined whether *Eip28/29* induction at this time is a direct effect of the hormone.

The L2-L3 molt is certainly caused by ecdysone and so presumably is the widespread induction (in groups II, IV and the Malpighian tubules) of *Eip28/29* that accompanies the molt. Whether the effects on *Eip28/29* are direct is unknown. One of the tissues that responds at this time, the salivary gland, requires additional comment. In the polytene map, the *Eip28/29* gene is located within the diffuse 71CD region, approx. 40% of the distance between the intense 71C1,2 and 71F1,2 bands (Cherbas et al., 1986). It is a location that coincides, so far as we can tell, precisely with that of a small early puff (Semeshin et al., 1985). This puff, like the other earlies, is induced by the premetamorphic ecdysone peak at the end of L3, i.e. at a time when we can detect no *Eip28/29* RNA in the salivary gland. Although the entire 71C1,2-F1,2 region has been cloned and used to probe salivary gland RNA, no alternative candidate for the responding gene has been detected (Eickbush, 1987). Since puffing can be dissociated from transcription (Richards et al., 1983; Crowley et al., 1984), it is conceivable that the 71CD early puff is a transcriptionally non-productive partial recapitulation of the L2-L3 ecdysone response at *Eip28/29*.

Although the case is clearest for L3, it appears quite possible that *Eip28/29* expression is controlled by ecdysone throughout much of development. The mid-embryonic expression peak clearly evident in Fig. 1 is probably due to the anterior and posterior midgut primordia. In any event, it follows the appearance of ecdysone receptor mRNA (Koelle et al., 1991) and occurs at about the time of a well-established ecdysone peak (Richards, 1981). Similarly, expression in the epidermis and fat body during L1 and L2 is episodic and may be synchronized to the molting cycle.

Quite aside from endocrine regulation our results reveal that *Eip28/29* expression patterns are surprisingly elaborate. Expression has been detected in tissues derived from all germ layers and developmental stages; expression can be **(i)** low-level and unregulated, **(ii)** hormone-regulated, or **(iii)** variable and apparently unrelated to endocrine signals. A full molecular description of *Eip28/29* transcription will need to account for all of these specificities. Finally, there is a significant group of tissues that never express *Eip28/29* at detectable levels. Minimally, this group includes the imaginal discs, muscles, tracheae, and the hindgut and foregut. In this regard, it is important to recall that our in situ procedures were calibrated to detect expression levels comparable to those in Kc cells where *Eip28/29* RNA is relatively abundant; we would have missed examples of very low level expression.

In contrast, *Eip40* expression appears quite simple, at least for L3. During that instar *Eip40* is expressed continuously in virtually all tissues, at the Kc-C level in the fat body, salivary glands and lymph glands, and at low but detectable levels elsewhere. The only regulation that we have observed is its induction (by the premetamorphic ecdysone peak) in the lymph gland. Stated differently, the lymph gland is the only tissue in which both *Eip28/29* and *Eip40* are both induced by ecdysone and it is the only tissue in which Kc-E levels of both transcripts occur. One interesting consequence of this observation is to shed light on the origin of Kc cells.

Like most of the *Drosophila* cell lines in use today, Kc cells originated from heterogeneous primary cultures prepared from dissociated late embryos (Schneider and Blumenthal, 1978; Sang, 1981). A variety of distinct cell types are viable in these cultures, and it has never been possible to designate one as the precursor of the permanent lines that evolve. Because mitotic cell clusters exist within the late embryonic nervous system and because acetylcholinesterase is ecdysone-inducible in Kc cells, we suggested that Kc cells might be neural or glial in origin (Cherbas et al., 1977). No additional evidence supporting that hypothesis has emerged and it is now clear that low level acetylcholinesterase expression is not a specific neural marker (Spindler-Barth et al., 1988; Goulielmos and Alahiotis, 1989; Zador, 1989; Small 1990). However, considerable accumulating evidence is consistent with the idea that Kc cells, and presumably other similar lines, are derived from a transformed hematopoietic cell.

The lymph gland is the hematopoietic tissue of *Drosophila* (Stark and Marshall, 1930; Castiglioni and Raimondi, 1963; Srdic and Gloor, 1978; Shrestha and Gateff, 1982) and its cells are diploid and mitotic throughout the late embryonic period and most of larval life (Madhavan and Schneiderman, 1977). *Drosophila* blood cells are known to proliferate in culture and to form permanent lines (Horikawa and Kuroda, 1959; Gateff, 1978). Expression of the type IV basement membrane collagen gene DCg1 was first identified in Kc cells (Lunstrum et al., 1988; Blumberg et al., 1988); during larval development its expression is confined largely to the lymph gland and peripheral hemocytes (Knibiehler et al., 1987). Expression of the β3 tubulin gene (polytene location 60C) is mesoderm-specific but it is also expressed (and ecdysone-inducible) in Kc cells (Sobrier et al., 1989). The cecropins are antibacterial peptides induced in response to the presence of bacteria. In *Drosophila* they are expressed in the fat body and in a subset of hemocytes. They are also expressed (and inducible by lipopolysaccharide and laminarin) in S2 cells, a line very similar to Kc cells (Samokovlis et al., 1990). Here we have reported that *Eip28/29* and *Eip40* are both ecdysone-regulated only in the premetamorphic lymph gland and that high level *Eip40* is confined to that tissue. In point of fact, we have demonstrated that the regulatory sequences required for appropriate expression of *Eip28/29* in the lymph gland are identical to those required in Kc cells (A. J. Andres and P. Cherbas, in preparation). Finally, we note that ecdysone-induced Kc cells undergo a morphological transformation accompanied by increased motility (Cherbas and Cherbas, 1981) and that both responses are plausible representations, in culture, of the metamorphic behavior of the lymph gland-blood cell system (Whitten, 1964; Srdic and Reinhardt, 1980; Lackie, 1988). To summarize, our hypothesis is that Kc cells are derived from a late embryonic hematopoietic cell, that they faithfully retain numerous markers of that lineage and that, when challenged with ecdysone in culture, they undergo a metamorphic response.

Our investigations began with the question whether the *Eip* genes are ecdysone-responsive during development. Plainly they are and, in the case of *Eip28/29*, a new and interesting element has been added to the inventory of ecdysone-responsive genes. The growing list of these includes numerous examples whose ecdysone-induced expression appears to be ubiquitous and it also includes examples of strictly tissue-specific genes turned on by ecdysone. In the case of *Eip28/29*, the directionality of the ecdysone response is evidently modulated in a tissue- and stage-specific way. In particular, the contrasting behaviors of the group IV and group V tissues provide a setting in which to explore how a steroid response is modulated during development.

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Fig. 3-3. (J) Glancing longitudinal sections of Malpighian tubules from two WPP. J1 shows high level expression, while in J2 there are only patches of moderate expression (arrows). (K) Longitudinal section of integument (WPP) showing hybridization to the hemocytes adhering to the epidermis. Note the low level of hybridization associated with the epidermal cells (arrows) at this stage (cf. Fig. 3-2 panel H2). (L) Longitudinal section of a WPP proventriculus showing intense of hybridization in the posterior half of the tissue. (M) Longitudinal dorsal section of the anterior-most lobes of a WPP lymph gland.

Fig. 3-2. (F) The central nervous system. F1 is a longitudinal section through a brain hemisphere and the ventral ganglion (80 hours). F2 is a cross section through the ventral ganglion only (88 hours). (G) Glancing sections of midgut. G1 details the regional hybridization often seen in only one part of the midgut (in this case 1 of 4 loops) (88 hours). The top arrow indicates a region not expressing Eip28/29. The bottom arrowhead points to a Malpighian tubule which is expression-positive. G2 shows a section in which hybridization was mostly restricted to a single midgut cell (arrow) of a 56 hour L3. (H) Longitudinal sections through the epidermis (88 hours). H1 shows the anterior portion of a larva at low magnification. H2 shows another section of the same age at higher magnification. Note the absence of detectable signal associated with the hemocytes (arrows). (I) Longitudinal sections showing expression in the fat body (88 hours). I1 is a low magnification view. I2, at higher magnification, shows that expression is patchy (arrows indicate regions of low level expression).

Fig. 3-1. Localization of *Eip28/29* transcripts by in situ hybridization. Left-hand panels are bright-field images and right-hand, dark-field. Scale bar, 0.1 mm. (A) Untreated Kc167 cells. (B) Ecdysone-treated Kc167 cells. (C) Longitudinal section of the pharynx (48 hour-L3). (D) Glancing longitudinal section of a salivary gland (48 hour-L3). (E) The gland cells of the anterior spiracles (56 hours). E1 is a longitudinal section demonstrating hybridization to the anterior-most cells and E2 is a cross section.

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Fig. 4. Changes in *Eip28/29* expression associated with an ecdysone peak. The figure illustrates *Eip28/29* expression in tissues from four larvae (A-D) whose developmental ages were determined by puff staging. Larva A, PS 1-2; larva B, PS 3; larva C, PS 4–5; larva D, PS 8. Complete data for the four larvae are given in Table 2. Anterior is to the left and dorsal at the top. Scale bar, 0.1 mm. Row 1 shows whole-mount in situ preparations of the proventriculi. Rows 2 (bright-field) and 3 (dark-field) shows sections which include both epidermal cells (large arrows) and clusters of hemocytes (small arrowheads).