Flies on steroids – *Drosophila* metamorphosis and the mechanisms of steroid hormone action

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Recent studies have provided new insights into the molecular mechanisms by which the steroid hormone ecdysone triggers the larval-to-adult metamorphosis of Drosophila. Ecdysone-induced transcription factors activate large sets of secondary-response genes and provide the competence for subsequent regulatory responses to the hormone. It seems likely that similar hormone-triggered regulatory hierarchies exist in other higher organisms and that Drosophila is providing our first glimpses of the complexities of these gene networks.

Extensive studies over the past decade have demonstrated that steroid hormones exert their effects via nuclear hormone receptors that directly regulate the transcription of target genes. Our detailed understanding of receptor function, however, contrasts with the paucity of information regarding the events that occur downstream from this initial step. Relatively few target genes have been identified and it remains unclear how these genes propagate the hormonal signal to direct the appropriate growth and development of the organism. Only by defining the gene networks that are controlled by hormones can we make the critical connection between the signal and its developmental responses.

This review describes some recent studies of *Drosophila* metamorphosis that have provided new insights into the molecular mechanisms of steroid hormone action. The focus is on the transcription factors induced by the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone), and how these factors transduce and amplify the hormonal signal by coordinating the induction of secondary-response genes. From these studies has emerged a clearer understanding of the mechanisms by which a systemic hormonal signal is refined into stage- and tissue-specific developmental responses.

Polytene chromosome puffing

Pulses of ecdysone direct *Drosophila* through its life cycle, with peak hormone titers signaling the major postembryonic developmental transitions[1]. The most dramatic of these responses occurs at the end of the third larval instar, when a high titer ecdysone pulse triggers puparium formation (Fig. 1). This is followed about 10 h later by an ecdysone pulse that triggers the prepupal–pupal transition. Most larval organs are destroyed by histolysis during prepupal development and are replaced by adult tissues that develop from clusters of imaginal progenitor cells. Remarkably, these divergent developmental pathways are manifested simultaneously, resulting in a complete transformation in form and function – from a crawling larva to the highly motile, reproductively active adult fly. Insights into the mechanisms by which ecdysone initiates this transformation came first from the giant polytene chromosomes present in the larval salivary glands. Transcribed genes in the polytene chromosomes are often represented by puffs, providing an unparalleled opportunity to visualize the effects of a steroid hormone on gene expression. From simple observation of puffing patterns emerged a detailed image of the gene networks induced by ecdysone as the animal changes from larva to pupa[2,3].

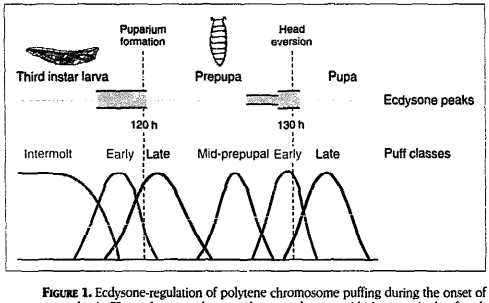

FIGURE 1. Ecdysone-regulation of polytene chromosome puffing during the onset of metamorphosis. The ecdysone pulses are shown at the top, with the magnitude of each pulse represented by the width of the gray bar[1,24]. Developmental time proceeds from left to right, starting at the mid-third instar larval stage when the polytene chromosomes can first be visualized. The dotted line on the left represents puparium formation at about 120 h after egg laying, while the dotted line on the right represents head eversion and the prepupal–pupal transition at about 130 h after egg laying. The activity of intermolt, early, late and mid-prepupal polytene chromosome puffs is represented at the bottom. These patterns are generalizations, which do not represent all members of each puff class[2,3].

Approximately 15 'intermolt' puffs are present when the polytene chromosomes first become large enough to analyze, in mid-third instar larval salivary glands (Fig. 1). These puffs regress in response to the ecdysone pulse that triggers puparium formation as at least six 'early' puffs are rapidly and directly induced by the hormone. The early puffs are only transiently active; they regress after several hours followed by the appearance of more than 100 'late puffs'. A few 'mid-prepupal' puffs are induced as the ecdysone titer drops after puparium formation, after which the prepupal ecdysone pulse re-induces the early–late puffing sequence. Although many of the same early and late puffs are induced by the late larval and prepupal ecdysone pulses, some puffs respond in a stage-specific manner.

Based on a series of detailed studies, Ashburner and colleagues

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derived a model for the genetic regulation of early and late puff induction by ecdysone[3]. They proposed that the ecdysone–receptor complex directly induces the early puffs. The early puff proteins exert two opposing regulatory functions: they repress their own activity, self-attenuating the regulatory response to the hormone, and they induce the late puffs. The model further proposed that ecdysone directly represses late puff activity, preventing their premature induction by the early puff proteins.

As the Ashburner model was proposed, efforts began in the Hogness laboratory to define the mechanisms of ecdysone action at the molecular level. These pioneering cloning efforts resulted in the isolation of the first genes from intermolt puffs[4,5] and the *4F* and *71E* late puff genes [M. Wolfner (1980) PhD thesis, Stanford University]. Although the magnitude of the early puffs led to the hypothesis that their encoded transcripts would be abundant, this was found not to be the case. Molecular cloning of the early puff genes was not achieved until the development of chromosomal walking techniques, which allowed the isolation of *Drosophila* genes based solely on cytogenetic location[6].

Three early puff genes encode ecdysone-inducible transcription factors

Molecular characterization of the 2B5, 74EF and 75B early puffs led to the isolation of the *Broad-Complex* (*BR-C*), *E74* and *E75* genes, respectively. Consistent with their proposed regulatory function, these early genes direct the synthesis of transcription factors. The *BR-C* encodes a family of related protein isoforms, each containing one of four possible pairs of zinc fingers (designated Z1–Z4; Refs 7, 8). The E74A and E74B proteins share an identical ETS DNA-binding domain, and the E75A, E75B and E75C proteins are orphan members of the nuclear-hormone-receptor superfamily[9,10]. Interestingly, the *EcR* ecdysone receptor gene is also induced directly by ecdysone, in parallel with some early mRNAs (Refs 11, 12). All three EcR protein isoforms can function as ecdysone receptors following heterodimerization with the *Drosophila* RXR homolog, USP [M.R. Koelle (1992) PhD thesis, Stanford University; Refs 13, 14].

Although the isolation of the *BR-C*, *E74* and *E75* was based on their expression in late larval salivary glands, subsequent studies showed that these genes are widely expressed during development – at multiple times that correlate with the peaks in ecdysone titer and in target tissues that have distinct developmental fates[15–17]. Furthermore, mutations in the *BR-C* and *E74* have widespread effects on the development of larval and imaginal tissues[18–20]. These observations support the tissue coordination model, which proposed that ecdysone-triggered regulatory hierarchies, similar to that described by Ashburner in the larval salivary gland, dictate the morphological and functional properties of each ecdysone target tissue at each stage in its development[9,15]. According to this model, overlapping combinations of early transcription factors specify stage- and tissue-specific developmental responses through the regulation of distinct sets of secondary-response genes.

Detailed studies of the *BR-C* have demonstrated that the individual functions of this gene are manifested in a more tissue-restricted manner. The *BR-C* is defined by three lethal complementation groups: *br*, *rbp* and *2Bc* (Refs 18, 21). Mutations in each of these functions leads to a characteristic set of lethal defects. For example, *rbp* mutations prevent histolysis of the larval salivary glands, while *br* mutations affect imaginal disc eversion and elongation[18,19]. These effects on specific developmental pathways correlate well with the restricted expression patterns of the Z1 and Z2 BR-C protein isoforms, supporting their assignment to the *rbp* and *br* functions, respectively[8,16]. These observations also suggest that individual BR-C protein isoforms direct distinct developmental responses to ecdysone[16].

Interestingly, *EcR* also appears to contribute to the tissue-specificity of ecdysone responses. The EcR-B1 isoform is expressed primarily in larval cells that are fated to die, while EcR-A is expressed in developing adult structures and tissues[13]. EcR-A is also expressed in a set of neurons in the central nervous system that die shortly after adult eclosion. The death of these cells depends on a decrease in ecdysone titer, suggesting that the specific expression of the EcR-A isoform is required for the appropriate developmental fate of these cells[22]. EcR isoforms might contribute to the tissue-specificity of ecdysone responses by inducing tissue-restricted primary-response genes, such as the BR-C protein isoforms described above. EcR isoforms might also function one level down in the ecdysone hierarchies, by directly regulating the expression of secondary-response genes[3,23].

Pulses of ecdysone are transduced into waves of transcription factors

Although the early puffs are induced at the end of larval development, transcripts from the *BR-C*, *EcR* and *E74B* can be detected more than a day earlier, in mid-third instar larvae[17,24,25] (Figs 1, 2). *EcR* and *E74B* are then repressed in late larvae, as *E74A* is induced (Fig. 2). A simple model to explain these temporal patterns of expression arose from ecdysone dose–response studies using cultured larval organs[11,26]. The *BR-C*, *EcR* and *E74B* promoters are the most sensitive to ecdysone, responding at a critical threshold concentration of about 2×10^{-9} M. In contrast, approximately tenfold higher hormone concentrations are required to repress *EcR* and *E74B* and induce *E74A* transcription. Combined with the available hormone titer data, these dose–response profiles suggest that a low titer pulse of ecdysone in early third instar larvae induces *BR-C*, *EcR* and *E74B* transcription (Fig. 2)[24]. The late larval pulse is of sufficient magnitude to repress *EcR* and *E74B*, induce *E74A*, and further induce the *BR-C* (Fig. 2).

The mid-prepupal period introduces another ecdysone-regulated transcription factor, the βFTZ-F1 orphan receptor[27]. Unlike the early genes, *βFTZ-F1* is repressed by ecdysone, explaining why this gene is active during the brief interval of low hormone titer in mid-prepupae[28]. The rise in ecdysone titer in late prepupae can then repress *βFTZ-F1*, re-induce *E74B* and *EcR*, and later repress their transcription as *E74A* is induced, recapitulating the switch seen in late larvae (Fig. 2). This switch occurs within a much briefer time span, however, suggesting that the hormone titer rises rapidly in late prepupae.

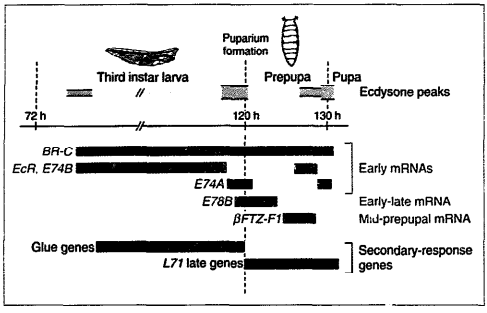

FIGURE 2. Temporal patterns of ecdysone-regulated gene expression during the onset of metamorphosis. The ecdysone pulses are shown at the top, with the magnitude of each pulse represented by the width of the gray bar[1,24]. Developmental time proceeds from left to right, with the major ecdysone-triggered transitions marked by dotted lines. The dotted line on the left represents the second-to-third instar larval molt while the dotted line on the right represents head eversion and the prepupal–pupal transition. Green bars show the timing and duration of primary-response regulatory gene transcription, the purple bar represents *βFTZ-F1* transcription, and the blue bars represent secondary-response gene transcription.

Thus, the profile of the ecdysone titer appears to be transduced into waves of transcription factors, determined by the critical threshold concentrations required to induce or repress each promoter (Fig. 2). This model emphasizes that hormone pulses of different concentration can have different regulatory consequences. In addition, decreases in hormone titer might be significant, allowing the induction of unique transcription factors, such as βFTZ-F1. It is also interesting to note that the rate of increase in ecdysone titer determines the duration of *EcR* and *E74B* expression. These promoters are only active in the window defined by the low hormone concentration required for their induction and the higher concentration required for their repression. Thus, changes in hormone concentration appear to be significant as well as the peak titers that they achieve.

Ecdysone-regulated transcription factors provide the competence for later responses to the hormone

It is well known that target tissues are not always competent to respond to a hormonal signal. Indeed, competence provides a critical means by which hormone responses become temporally and spatially restricted. Studies of *Drosophila* metamorphosis are revealing that the competence to direct an appropriate transcriptional response to ecdysone is provided by the assembly of the proper transcription factors on the target promoter. Genetic studies have shown that at least some of these critical *trans*-regulators are, themselves, ecdysone-regulated proteins.

The *BR-C* appears to play a wide role as a competence factor for ecdysone responses. The *2Bc* function of the *BR-C* is required for the maximal ecdysone-induction of the *BR-C*, *E74A* and *E75A* mRNAs in late third instar larvae (Fig. 3)[29]. Other primary-response genes also depend on the *BR-C* for their maximal induction by ecdysone, including *hsp23* and *hsp27* in the salivary gland[30], *Fbp-1* in the fat body[31] and *Ddc* in the epidermis[32]. It seems likely that the binding of BR-C proteins to these target promoters allows maximal levels of transcriptional induction by the ecdysone–receptor complex. Alternatively, BR-C proteins might act more indirectly by opening the chromatin to allow efficient binding by other *trans*-acting factors[30]. The tissue specificity of these responses could be due, at least in part, to the expression patterns of individual BR-C protein isoforms.

βFTZ-F1 appears to function during prepupal development like the *BR-C*, enhancing the reinduction of the *BR-C*, *E74A* and *E75A* by ecdysone (Fig. 3)[28]. In addition, *βFTZ-F1* is sufficient to direct the stage-specific ecdysone-induction of the *E93* early gene in prepupal salivary glands (Fig. 3). Thus, *βFTZ-F1* expression in mid-prepupae appears to be critical for resetting the system, allowing the appropriate early or late genetic response to ecdysone, as well as providing the competence for stage-specific transcriptional responses to the hormone. Interestingly, *βFTZ-F1* represses its own transcription, ensuring that the competence it provides will be of short duration (Fig. 3)[28]. That this competence factor is, itself, a member of the nuclear receptor superfamily raises the possibility that it might function by interacting directly with the EcR–USP complex.

Competence factors can also contribute to the timing of target-gene induction. An example of this regulation is provided by the *E78B* early-late gene. *E78B* is induced by ecdysone after *E74A* in late third instar larvae (Fig. 2)[33,34]. Experiments with cultured larval organs have shown that *E78B* is a true primary-response gene, in that it can be induced by ecdysone in the absence of protein synthesis. However, unlike the early genes, *E78B* requires ecdysone-induced protein synthesis for its maximal levels of transcription[34]. Thus, the delay in *E78B* induction can be attributed to a requirement for one or more early ecdysone-induced transcription factors for its peak expression. According to this model, the competence factors are not present in advance, as are the *BR-C* products and, thus, dictate a delay in *E78B* induction. Interestingly, *E78B* is submaximally induced in *E74A* mutants, indicating that E74A functions as at least one of these critical *trans*-regulators (Fig. 3)[35].

Making the regulatory connection – glue genes and late genes are induced by the early transcription factors as a secondary-response to ecdysone

Clearly, the ultimate function of steroid-induced transcription factors is to transduce and amplify the hormonal signal by coordinating the induction of secondary-response effector genes. Secondary-response genes in the larval salivary gland are represented by the

intermolt and late puffs in the polytene chromosomes (Fig. 1). Most, if not all, intermolt puffs contain glue genes, which produce a secretion that affixes the animal to a solid surface for metamorphosis[36]. The glue genes are induced indirectly by ecdysone in mid-third instar larvae, after the *BR-C* and *E74B*, and are repressed by the rising ecdysone titer at puparium formation (Fig. 2)[24,25,37,38]. The *L71* late genes encoded within the 71E puff are coordinately induced in newly formed prepupal salivary glands and their transcripts are undetectable 12–14 h later (Fig. 2)[39,40]. These genes encode a family of small, apparently secreted polypeptides that resemble defensins and venom toxins. It is possible that this final secretion from the salivary glands into the space between the imaginal hypoderm and prepupal cuticle might protect the animal against infection during metamorphosis[40].

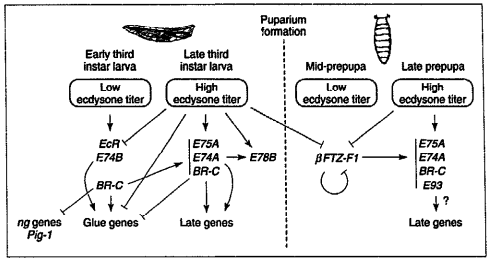

FIGURE 3. Multiple ecdysone-triggered regulatory hierarchies direct the onset of *Drosophila* metamorphosis. This figure summarizes regulatory interactions discussed in the text. Red bars represent repressive effects and green arrows represent inductive effects.

These secondary-response genes differ from the primary-response early genes in two key ways. First, in contrast to the widely expressed early genes, the glue and *L71* genes are expressed exclusively in one tissue at one time during development. Second, these sets of genes are coordinately regulated, apparently responding to common primary-response transcription factors. These secondary-response promoters, thus, provide an opportunity to understand how tissue-specificity is encoded in the regulatory response to a steroid hormone, as well as how batteries of secondary-response genes can be coordinately controlled.

Genetic studies have demonstrated that the *BR-C* and *E74* are required for appropriate glue gene expression in mid-third instar larvae and *L71* expression in prepupae (Fig. 3)[29,35,41]. BR-C proteins bind to critical regulatory sequences of the *Sgs-4* glue gene promoter, and the BR-C Z1 and E74A proteins bind to and regulate an *L71* promoter, arguing that these early ecdysone-induced transcription factors directly regulate their target genes (K. Crossgrove, C.A. Bayer, J.W. Fristrom and G. Guild, unpublished; Refs 25, 42). Double mutant studies suggest that the *BR-C* and *E74A* function together to regulate glue gene and *L71* transcription[43].

The *BR-C* also mediates at least two switches in target gene transcription during the onset of metamorphosis. A set of three salivary-gland-specific *ng* genes and the *Pig-1* gene are clustered with *Sgs-4* at the 3C intermolt puff[44,45]. The *ng* genes and *Pig-1* are expressed before the glue genes in early third instar larvae and are repressed as the glue genes are induced. Interestingly, the repression of *ng* and *Pig-1* transcription, like glue gene induction, is dependent on the *BR-C* (Refs 25, 46; Fig. 3). Similarly, *BR-C* function is required for glue gene repression and *L71* induction at puparium formation[29,41] (Fig. 3). These observations indicate that not all effects of early genes are inductive and that the *BR-C* plays a central role in directing the appropriate switches in secondary-response gene expression during the early stages of metamorphosis.

Future directions and perspectives

It is now clear, as Ashburner and colleagues emphasized when presenting their model[3], that the two-step regulatory hierarchy represented by the puffing response to ecdysone is a vast oversimplification. Ecdysone triggers multiple regulatory hierarchies during the onset of metamorphosis, dictated, at least in part, by dynamic changes in hormone titer (Fig. 3). Furthermore, there are positive cross-regulatory interactions among ecdysone-induced transcription factors that provide the competence for later regulatory responses to the hormone. Although our understanding of secondary-response gene regulation is restricted to the larval salivary gland, it is also clear that the ecdysone hierarchies are not restricted to one stage and tissue. Rather, the larval salivary gland polytene chromosomes have provided a means of identifying the critical regulators in these pathways and a cytogenetic definition that allows their isolation. In this sense, the salivary gland puffing hierarchy has provided critical clues to the general regulatory functions of ecdysone. A more detailed understanding of *EcR* and early gene function should provide further insights into how hormonal signals are refined into stage- and tissue-specific developmental responses.

Given the conservation of developmental regulatory mechanisms extending from flies to humans, it is likely that hormones function via genetic regulatory hierarchies in other higher organisms. Among vertebrates, molecular studies of *Xenopus* metamorphosis are the most advanced, providing striking parallels with insect metamorphosis[47,48]. Unraveling these regulatory codes will provide interesting insights into the similarities and differences by which insects and frogs reorganize their body plan. We can hope that future research will yield not only a more detailed understanding of the elegant regulatory mechanisms that direct metamorphosis, but also reveal the signaling pathways by which hormones manifest their remarkable effects on vertebrate growth, development and homeostasis.

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