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Ecdysone-regulated puff genes 2000

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Abstract

The Ashburner model for the hormonal control of polytene chromosome puffing has provided a strong foundation for understanding the basic mechanisms of steroid-regulated gene expression (Cold Spring Harbor Symp. Quant. Biol. 38 (1974) 655). According to this model, the steroid hormone 20-hydroxyecdysone (referred here as ecdysone) directly induces the expression of a small set of early regulatory genes. These genes, in turn, induce a much larger set of late target genes that play a more direct role in controlling the biological responses to the hormone. The recent characterization of two early puff genes, *E63-1* and *E23*, and three late puff genes, *D-spinophilin*, *L63*, and *L82*, provide further confirmation of the Ashburner model. In addition, these studies provide exciting new directions for our understanding of ecdysone signaling. Overexpression studies of *E63-1* implicate this gene in directing calcium-dependent salivary gland glue secretion. In contrast, overexpression of *E23* indicates that this ABC transporter family member may negatively regulate ecdysone signaling by actively transporting the hormone out of target cells. Finally, genetic studies of the *L63* and *L82* late genes reveal unexpected possible functions for ecdysone in controlling developmental timing and growth. This review surveys the recent characterization of these ecdysone-inducible genes and provides an overview of how they expand our understanding of ecdysone functions during development.

Keywords: Ecdysone; *Drosophila* metamorphosis; Gene regulation

1. Introduction

The isolation and purification of ecdysone and 20-hydroxyecdysone revolutionized the field of insect endocrinology, propelling it into the molecular era (Butenandt and Karlson, 1954). What no one could have anticipated at the time, however, was the more widespread ramifications that this discovery would have on the regulation of eukaryotic gene expression. It was the pioneering work of Ulrich Clever which showed that ecdysteroids could initiate a cascade of gene activation, as visualized by the puffing patterns of the *Chironomus tentans* salivary gland polytene chromosomes (Clever and Karlson, 1960; Clever, 1964). Work by a number of labs confirmed these studies, including studies of puff regulation in a genetic model organism, the fruit fly *Drosophila melanogaster* (Becker, 1959; Ashburner et al., 1974). These studies of puff gene regulation established a new paradigm for thinking about the mechanisms of steroid hormone action — raising the exciting possibility that these hormones could act directly on the nucleus, triggering a complex regulatory cascade of gene expression (Yamamoto and Alberts, 1976).

Through a series of detailed and elegant studies, Ashburner and co-workers proposed a model for the regulation of gene expression by 20-hydroxyecdysone (referred to hereafter as ecdysone) (Fig. 1). Briefly, this model proposed that ecdysone, bound to its specific receptor, directly induces the expression of a small set of early regulatory genes. The protein products of these genes, in turn, repress their own expression and induce a much larger set of late target genes. It was assumed that these late genes would function as effectors that directly or indirectly control the appropriate biological responses to the pulse of ecdysone. Ashburner and colleagues also determined that the late puffs could be divided into two classes, based on their regulation by ecdysone (Ashburner and Richards, 1976). The early-late puffs are induced relatively rapidly after the addition of hormone and require the continuous presence of ecdysone for their activity, much like the early puffs. The late-late puffs, in contrast, are induced at later times and are prematurely induced upon ecdysone withdrawal. This latter result was interpreted to mean that the ecdy-

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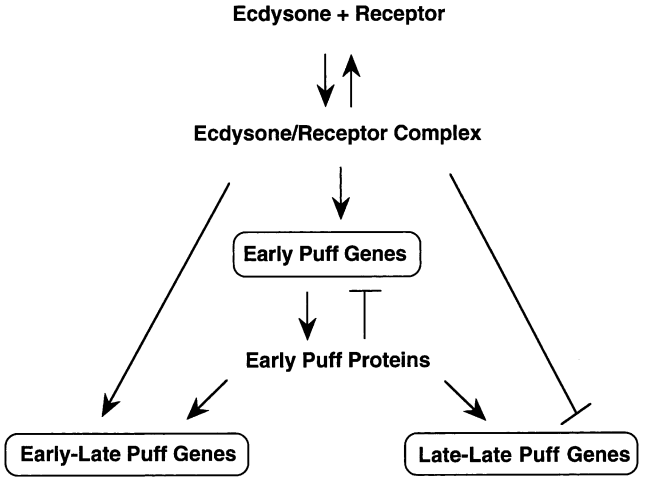

Fig. 1. The Ashburner model for ecdysone-regulated puff gene expression. A modification of the Ashburner model is depicted (Ashburner et al., 1974), in which the early-late and late-late puffs have been included (Ashburner and Richards, 1976). Ecdysone, bound to its receptor, directly induces the early puff genes. These genes express proteins that perform dual regulatory functions — they repress their own expression and they induce the late puff genes. The early-late puff genes are also dependent on direct induction by the ecdysone–receptor complex, resulting in their expression earlier than the late-late puff genes, which are repressed by the ecdysone–receptor complex.

sone–receptor complex might exert a direct negative effect on the late-late gene expression. A schematic representation of the Ashburner model is depicted in Fig. 1, along with the two classes of late puff genes that were not included in the original model.

Molecular studies published over the past decade have provided remarkable confirmation of this model. Several early puff genes have been characterized in detail and shown to encode transcriptional regulators that can directly control late gene expression. A recent book by Zhimulev (1999) surveys the ecdysone-regulated puff genes, and a number of recent reviews have summarized the regulatory connections between these genes (Segraves, 1994; Russell and Ashburner, 1996; Thummel, 1996; Richards, 1997; Henrich et al., 1999). In this review, the focus is on several recent papers that describe the characterization of five other puff genes. Two of these correspond to early puffs, at 23E and 63F, that do not encode transcriptional regulators. Their encoded proteins, however, can perform distinct regulatory functions, broadening our understanding of the role of early genes in the ecdysone cascades. In addition, three late puff genes have been recently characterized, at 62E, 63E, and 82F. The *L63* gene encodes CDK proteins that can clearly exert a regulatory function, indicating that not all late genes will encode effector functions (Stowers et al., 2000). Continued molecular studies of ecdysone-regulated puff genes takes one beyond the Ashburner model, toward a more detailed understanding of the mechanisms by which the steroid hormone ecdysone controls insect development.

2. E63-1: an ecdysone-inducible calcium binding protein that can regulate salivary gland glue secretion

Molecular analysis of the 63F early puff provided the first evidence that not all early puffs encode transcriptional regulators. This work identified a pair of divergently transcribed ecdysone-inducible genes: *E63-1* and *E63-2* (Andres and Thummel, 1995). *E63-2* produces a single 1.2 kb mRNA with no extended open reading frames. Genetic studies indicate that this gene has no essential functions during development, suggesting that it may only be expressed due to its proximity to *E63-1* (Vaskova et al., 2000). In contrast, *E63-1* encodes a calcium-binding protein with four EF hands, most closely related to calmodulin. The regulation of *E63-1* provides a further departure from prior studies of early puff genes, in that it is induced by ecdysone in a tissue-specific manner. Low to moderate levels of *E63-1* are widely expressed in the third instar larvae, prior to the late larval ecdysone pulse. Only in the salivary gland is *E63-1* transcription rapidly and directly induced by the hormone at puparium formation (Andres and Thummel, 1995). This restricted pattern of induction, combined with the known role of calcium-binding proteins in regulating secretion, led to the proposal that *E63-1* might contribute to the physiology of the salivary gland by regulating ecdysone-induced secretion.

Although loss-of-function mutants provide an ideal means of testing this model, inactivation of the *E63-1* gene has no detectable effect on viability or reproduction (Vaskova et al., 2000). In retrospect, this is not surprising, given that other calcium-binding proteins are encoded by the *Drosophila* genome. Consistent with possible functional redundancy in this pathway, recent studies have shown that salivary glands compromised for both calmodulin and E63-1 are defective in glue secretion (T.-V. Do and A.J. Andres, personal communication). In addition, ectopic expression of *E63-1* in transgenic animals is sufficient to trigger glue secretion if the intracellular calcium levels are elevated (A. Biyasheva et al., 2001). Moreover, ecdysone alone can lead to increased levels of intracellular calcium in larval salivary glands, with a detectable increase after 2 h of exposure. Ecdysone thus leads to two responses that can synergistically trigger salivary gland glue secretion — increased levels of *E63-1* expression as well as increased cytoplasmic calcium levels (Fig. 2). Although the time frame for calcium elevation suggests that this is a secondary-response to the hormone, the mechanism by which calcium levels are effected remains to be determined.

E63-1 protein shows dynamic changes in its subcellular distribution as the salivary glands secrete glue, providing further evidence of a possible role in glue secretion (Vaskova et al., 2000). Initially, before the glue is secreted, E63-1 is localized to cell membranes, in the

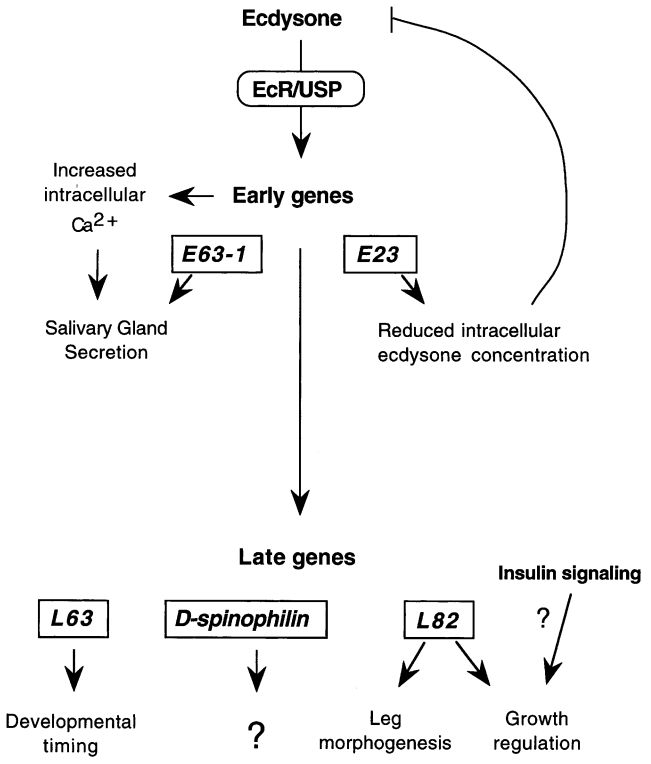

Fig. 2. Summary of proposed functions for ecdysone-inducible genes discussed in the text. Ecdysone can trigger salivary gland glue secretion through two synergistic pathways, by increasing the intracellular calcium concentration and by inducing the E63-1 calcium-binding protein. In contrast, the E23 ABC transporter may negatively regulate ecdysone responses by reducing the intracellular hormone concentration in certain tissues. Three late genes are also discussed in the text: *D-spinophilin*, *L63*, and *L82*. *D-spinophilin* is of unknown function. *L63* mutants result in developmental delays and *L82* is required for proper adult leg morphogenesis and body size.

nuclei, and between the secretory granules. Later, after glue secretion, E63-1 is excluded from the nuclei and remains cytoplasmic, even after the glue is expelled.

So, how might *E63-1* function? Andres and co-workers point out that the N-terminal domain of E63-1 is ~45% identical to myosin light chains while the C-terminal calcium binding domains are ~40% identical to calmodulin (Vaskova et al., 2000). Calmodulin has been shown to act as a light chain for unconventional myosin motors, moving cargoes along a cytoskeletal network. Andres and colleagues propose that E63-1 may exert a similar function in larval tissues, albeit in a redundant fashion with calmodulin or another similar calcium-binding protein. Thus, in the salivary glands, E63-1 could direct secretion of glue granules in response to ecdysone. Support for this model will require further physiological and genetic studies, providing new opportunities to understand the biological roles of calcium in controlling hormone-induced secretory events.

3. The *E23* early puff gene may regulate ecdysone responses by controlling intracellular hormone concentrations

The 23E ecdysone-inducible puff is among the last early puffs described by Ashburner to be characterized at the molecular level. Given the precedent that all but the 63F early puff encoded transcription factors, the assumption was that 23E would encode a similar protein, in keeping with the Ashburner model. In contrast, Garza and colleagues have recently reported that the 23E early puff encodes something quite different — a member of the ABC family of transporter proteins, raising new possibilities for our understanding of the regulation of ecdysone responses (Hock et al., 2000).

E23 directs the synthesis of a 4.5 kb mRNA from an ~20 kb transcription unit located in the center of the 23E early puff (Hock et al., 2000). This transcript is inducible directly by ecdysone in cultured larval organs, and shows a temporal pattern of expression during development that parallels the major pulses of ecdysone. In addition, *E23* is maternally deposited suggesting that it may exert functions before those of the other known early puff genes. The 23E puff shows a response to ecdysone over a wide range, from 10^{-10} to 7×10^{-7} M, at hormone concentrations that are lower than those required to activate the 74EF and 75B early puffs (Ashburner, 1973). It would be interesting to know whether the dose-response of *E23* transcription reflects this regulation. Consistent with this possibility is the observation that *E23* is induced relatively early at the end of larval development, possibly as part of a mid-third instar response.

Efforts are currently underway to isolate *E23* mutants as a means of determining its functions during development. In the meantime, Hock et al. (2000) report gain-of-function studies in which *E23* has been ectopically expressed under the control of a heat-inducible promoter. Induction of *E23* at 12 h intervals throughout the fly life cycle results in lethality during the early stages of metamorphosis, suggesting a specific block in ecdysone signaling during development. More precise studies provide further support for this model. Ectopic expression of *E23* in late third instar larvae and newly formed prepupae leads to complete lethality, whereas expression at times of low hormone titer has less dire effects on viability. Interestingly, these effects on viability are reflected by reduced transcription of the *E23*, *E74*, and *E75* early genes, as well as an ecdysone-inducible *lacZ* transgene, indicating that *E23* can exert global effects on ecdysone-inducible gene expression. It is important to note that these studies were performed in cultured larval organs; therefore, these effects on gene expression cannot be simply attributed to the death of the animal but rather must reflect some tissue autonomous function of the *E23* gene.

These observations thus define *E23* as a negative regu-

lator of ecdysone signaling, fulfilling a central prediction of the Ashburner model that at least one of the early puff proteins should negatively regulate early gene expression (Ashburner et al., 1974). However, the identity of the E23 protein as a member of the ABC family of transporters indicates that it does not exert this effect directly at the level of gene transcription. Rather, Hock et al. (2000) propose an intriguing model to explain their results, postulating that *E23* might control the effective intracellular concentration of ecdysone within target tissues (Fig. 2). This proposal is based on the observation that ABC transporters can transport steroids in both yeast and vertebrate cells (van Kalken et al., 1993; Kralli et al., 1995; Gruol et al., 1999), although the functional consequences of this transport have not yet been defined. *E23* may provide an ideal model system to work out this regulatory pathway.

One mechanism by which *E23* could contribute to early gene repression would be to actively remove the hormonal inducer from target cells (Fig. 2). This function could also increase the dynamic range of hormone concentration inside the cell relative to much slower changes that might take place in the surrounding hemolymph. For example, it has long been known that the ecdysone titer needs to reach a very low level in mid-prepupae in order to allow induction of the βFTZ-F1 competence factor and facilitate appropriate responses to the prepupal pulse of ecdysone (Richards, 1976). Hemolymph titers of ecdysteroids at this stage, however, are higher than the concentration that is required in vitro for these mid-prepupal responses. This discrepancy could be explained by active transport of ecdysone out of target tissues, reducing the effective intracellular hormone concentration. Hock et al. (2000) also propose an intriguing extension of this model, whereby tissue-specific patterns of *E23* expression could define distinct spatial responses to the systemic hormonal signal. This would provide an explanation for the different temporal patterns of early gene expression seen in different ecdysone target tissues (Richards, 1982; Karim and Thummel, 1991; Huet et al., 1993).

Evidence in support of these models must await further characterization of *E23*. Mutant studies will certainly be critical, although Hock et al. (2000) warn that another ecdysone-inducible ABC-transporter coding region maps near *E23*. Localization of the E23 protein will indicate whether it is expressed in a tissue-specific manner, and will also allow a determination of the subcellular localization of the protein. In addition, studies in cultured cells should provide a better understanding of the transporter functions of this protein, and a determination of whether E23 is capable of moving ecdysone across the cell membrane. It is also interesting to note that ABC transporter genes such as *E23* encode half-transporters that function as either homo- or heterodimers. Thus, the potential exists to modulate the substrate specificity or affinity of E23 by generating distinct heterodimer combinations, providing a potential for combinatorial regulation of transporter functions. The exciting possibility is that further studies of this gene could provide a radical shift in the way we think about the hormonal regulation of tissue- and stage-specific responses.

4. Late genes are not the end of the line!

Remarkably, of more than 100 late puffs present in the larval salivary gland polytene chromosomes, molecular characterization of only one late puff had been published prior to 1999, located at 71E. Entry into this locus came from the first molecular screen for differential gene expression, performed by Wolfner and colleagues in the lab of D.S. Hogness at Stanford University (Wolfner, 1980). A series of ecdysone-inducible cDNAs identified in this study were mapped to the 71E locus, leading to the eventual discovery of five pairs of short (~450 bp) divergently transcribed late genes that map in close proximity to one another (Wright et al., 1995). These genes are coordinately expressed in a highly restricted temporal and spatial manner (Restifo and Guild, 1986; Wright et al., 1995). Although no function has yet been ascribed to the *L71* genes, they encode a family of small, apparently secreted polypeptides that resemble defensins and venom toxins. It is thought that this final secretion from the salivary glands into the space between the imaginal hypoderm and prepupal cuticle may protect the animal against infection during metamorphosis (Wright et al., 1995).

Three recent publications have significantly expanded our understanding of the late gene function by reporting the isolation of ecdysone-inducible genes from the 62E early-late puff as well as the 63E and 82F late-late puffs (Stowers et al. 1999, 2000; Keegan et al., 2000). Unlike the *L71* genes, these three late puff genes comprise unusually long and complex transcription units that encode multiple protein isoforms (Table 1). Indeed, these late puff genes most closely resemble early genes such as the *Broad-Complex* (*BR-C*) rather than the *L71* late genes (DiBello et al., 1991). It seems that the notion that late genes encode simple effector functions of ecdysone signaling may be the exception rather than the rule.

5. The 62E early-late ecdysone-inducible puff encodes D-spinophilin

Molecular characterization of the 62E early-late puff revealed at least five size classes of mRNA that arise from a single transcription unit that is at least 30 kb in length (Keegan et al., 2000). Sequence analysis of the cDNA clones indicated that these transcripts encode a protein with a central core of 385 amino acids that is

Table 1
Summary of puff genes

Gene	Corresponding puff	Gene length	mRNA isoforms	Encoded protein
E63-1	63F early puff	23 kb	3	Calcium-binding protein
E23	23E early puff	~20 kb	1	ABC transporter
D-spinophilin	62E early-late puff	≥32 kb	≥5 size classes	Spinophilin/neurabin II
L82	82F late-late puff	53 kb	≥7 isoforms from nested promoters	Unknown function
L63	63E late-late puff	83 kb	≥9 isoforms, 3 nested promoters	CDK

46% identical to the mammalian protein spinophilin/neurabin II. Keegan et al. (2000), accordingly, designate this gene *D-spinophilin*, and point out that it is the only such homolog encoded by the *Drosophila* genome. Although this protein is well conserved throughout evolution, its functions remain largely unknown. The conserved protein domain contains three distinct motifs, a PDZ domain, a protein phosphatase 1 (PP1)-binding domain, and a coiled-coil domain, all of which can mediate higher order protein–protein interactions. The authors point out that PP1 is a serine/threonine phosphatase that has widespread effects on cellular physiology, including muscle contraction, cell division, and gene expression. Thus, at least one mechanism by which D-spinophilin could control developmental responses to ecdysone would be through direct interaction with *Drosophila* PP1. Garza and colleagues are currently pursuing double mutant studies as a test of this hypothesis (D. Garza, personal communication).

Transcriptional regulation of *D-spinophilin* is complex. Some transcripts are present in the mid-third instar larvae, prior to the ecdysone pulse, suggesting that aspects of its regulation are ecdysone-independent. More detailed studies of the temporal patterns of *D-spinophilin* expression are required to confirm this possibility. In addition, different size classes of mRNA are induced by the late larval and prepupal ecdysone pulses, suggesting stage-specific promoter utilization. Antibody stains revealed a widespread pattern of D-spinophilin protein expression in early prepupae, including the salivary glands, fat bodies, central nervous system, and imaginal discs. Moreover, the protein is localized at or near cell membranes in several tissues. These patterns of expression, combined with the known activities of mammalian spinophilin/neurabin I, raise the interesting possibilities that *D-spinophilin* may mediate the cytoskeletal changes associated with adult leg morphogenesis and/or the dramatic remodeling of the central nervous system that occurs during metamorphosis. Regardless, it is clear that genetic studies of this gene will provide important new insights into the functions of this gene family.

6. The *L63* and *L82* late-late genes are unusually long and complex, with both ecdysone-independent and ecdysone-dependent promoters

Molecular characterization of the genes from the classic 63E and 82F late-late puff loci reveal a number of similarities to each other and to the *D-spinophilin* gene. Both genes are usually long and complex (Stowers et al. 1999, 2000). *L63* is ~83 kb in length and contains three nested promoters each of which directs the synthesis of multiple mRNA isoforms. *L82* is ~53 kb in length and directs the synthesis of at least seven mRNA isoforms from distinct promoters. Also like *D-spinophilin*, some of the *L63* and *L82* mRNAs show little or no ecdysone-responsiveness, while the *L63B* mRNAs and *L82A*, *B*, and *F*, appear to be ecdysone-inducible. The temporal profiles of *L63* and *L82* transcription throughout development are remarkably similar, with clear maternal deposition of mRNA, a peak of mid-embryonic expression (in parallel with the embryonic ecdysone pulse), a peak of expression during the early stages of metamorphosis, and high levels of expression in adults. This pattern, however, reflects a summation of all of the encoded mRNA isoforms in whole animals, and very different kinetics may be uncovered by characterization of tissue-specific patterns of expression. Indeed, recent studies have shown that at least one *L82* mRNA isoform is ecdysone regulated in the salivary glands (D. Garza, personal communication). Given the remarkable complexity of these three late puff loci, its seems that the clustering of the short *L71* mRNAs within the 71E puff locus may be more unusual than previously suspected.

7. *L82* encodes an essential protein of unknown function

The *L82* mRNAs encode a protein with two evolutionarily conserved domains, although the functions of these domains remain unknown. It is thus of some interest to note that Stowers et al. (1999) have generated a series of *L82* mutant alleles and that these mutations have clear effects on the fly life cycle. Alleles of increas-

ing severity lead to increasing developmental delays, with null alleles doubling the developmental time of the animals. Stowers et al. (1999) interpret these results as evidence of a possible role for this gene in establishing proper developmental timing, although there are many non-specific ways in which development might be delayed. Determination of when these delays occur and whether they are stage-specific will provide important insights into *L82* function. Moreover, null *L82* mutants die at adult eclosion, with the distal tips of their wings adhering to the pupal case. Mutant animals dissected out of the pupal case, however, can be maintained for several weeks, indicating that they do not have severe developmental defects. These studies establish a foundation for further phenotypic characterization that may provide important clues into the functions of this novel gene family, as well as a possible role for *L82* in the normal progression of the *Drosophila* life cycle.

8. *L63* encodes a family of proteins related to the murine PFTAIRE CDK-like proteins, and is required for proper leg formation and body size

Unlike *L82*, database searches with the *L63*-encoded proteins revealed highly informative sequence identities (Stowers et al., 2000). All L63 proteins contain a 294 amino acid region that is 51% identical to the yeast CDC28 CDK. The L63 proteins also carry both N-terminal and C-terminal extensions from this region, like a murine CDK family member designated PFTAIRE. Moreover, the CDK region of L63 shows its highest identity with PFTAIRE (70%), and L63 and PFTAIRE are 54% identical in the first 24 amino acids of their C-terminal extensions. L63 thus appears to be a functional homolog of PFTAIRE, which has been proposed to play a role in cell differentiation (Lazzaro et al., 1997; Besset et al., 1998).

Consistent with this possible function, and unlike most CDKs, *L63* mutants have no effect on cell division (Stowers et al., 2000). No defects are observed in the fraction of cells undergoing mitosis, in polytenization of the salivary gland polytene chromosomes, or in adult eye development (which depends upon extensive rounds of cell division). Rather, *L63* mutants display defects in adult leg morphogenesis, a well-characterized developmental response to ecdysone (Fristrom and Fristrom, 1993). Stowers et al. (2000) propose that *L63* might control the changes in cell shape that drive leg elongation by phosphorylating critical components of the cytoskeleton. They also postulate that *L63* might be required for proper cell–cell communication during leg elongation. It is important to note that *L63* is the first late ecdysone-inducible gene described to date that is required for leg elongation, and thus provides a critical link in the regulatory cascade that controls this stage- and tissue-specific developmental response to ecdysone. Fitting this gene into the leg morphogenesis program should constitute a significant step forward in our understanding of this pathway.

Quite remarkably, *L63* mutants also display clear changes in overall size, with mutant pupae as much as one third the size of wild type animals (Stowers et al., 2000). This phenotype bears striking resemblance to those associated with defects in the insulin signaling pathway, and raises the exciting possibility that *L63* might provide an integration point between the temporal signaling by ecdysone and growth control (Edgar, 1999; Leevers, 1999). Indeed, it is well known that steroids and other small lipophilic hormones have profound effects on the growth of vertebrate organisms, and that nuclear hormone receptors can sense nutritional signals, much like the insulin pathway. Further studies of *L63* may thus provide important clues regarding how steroid hormones can regulate growth.

By rescuing *L63* mutants with wild type and mutant *L63* transgenes, Stowers et al. (2000) have demonstrated that *L63* function is dependent on its CDK activity. Mutations in two amino acids that are essential for binding cyclin A, G243 and I249, reduce or eliminate the rescuing activity of these transgenes. In addition, their studies identify two other highly conserved amino acids as essential for L63 function — E251, which is essential for human CDK kinase activity, and S359, which is a phosphorylation site required for full CDK activity. Taken together, these observations indicate that L63 acts as a cyclin-dependent protein kinase, like other CDK family members. Interestingly, neither of the true *Drosophila* CDKs (cdc2 and cdc2c) could replace L63 function, consistent with the proposal that this gene does not act during the cell cycle.

9. Conclusions

The molecular and genetic characterization of these five ecdysone-regulated puff genes almost doubles the number of such genes described to date, and provides exciting new directions for future research. This is, however, not the end. An additional late puff gene has been recently identified by Dr I. Zhimulev and colleagues, and was announced at the Ecdysone 2000 Workshop in Rapperswil, Switzerland. This gene, derived from the relatively small late puff at 62C, corresponds to the *kettin* gene (Kolmerer et al., 2000). Mutations in *kettin* lead to defects in molting of the cuticle during the first and second larval instars, elongated stationary late third instar larvae, and cryptocephalic pupae — all classic defects that arise from a block in ecdysone signaling (G.V. Pokholkova, V.F. Semeshin, I.V. Makunin, E.S. Belyaeva and I.F. Zhimulev, personal communication). Moreover, some dominant mutations in *kettin* are flight-

less alleles, indicating a role for this gene in development of the adult thoracic muscles. This function is consistent with molecular studies of *kettin*, which have shown that it encodes a giant modular protein that is associated with the Z-disc of insect muscles (Lakey et al., 1993). This gene may thus play a critical role in muscle integrity and function during development, leading to the observed defects in the ecdysone-regulated muscular contractions required for molting, puparium formation, and head eversion. It will be interesting to learn more about how this gene might contribute to muscle function and development during ecdysone-triggered developmental transitions.

A lesson that clearly emerges from the recent studies of late puff genes is that large polytene chromosome puffs contain long complex genes. These observations support earlier work on the *BR-C*, *E74*, and *E75* early puff genes, all of which map to large puff loci and all of which are giant complex genes (Burtis et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991). Perhaps not surprisingly, this provides further support for the pioneering proposal by Beermann (1952) that polytene chromosomes puffs are visible manifestations of nascent transcription. This is further supported by elegant deletion mapping reported by Stowers et al. (2000) for the *L63* gene, which show a clear correlation between transcription of the ecdysone-inducible isoforms of *L63* and puff formation at 63E.

Another interesting observation that emerges from the characterization of *L63* and *L82* is that their overexpression at different times during the fly life cycle has no apparent effects on development (Stowers et al. 1999, 2000). This, at least superficially, appears to contradict the very strict temporal regulation that is normally conferred by ecdysone, and is consistent with the widespread expression of at least some *L63* and *L82* isoforms. Perhaps the ecdysone-regulated transcripts encoded by these genes are an evolutionary hangover that is no longer essential, but which reveal the more fundamental role of these genes in ecdysone-regulated developmental events. Alternatively, specificity could be conferred by other essential factors that are expressed in a stage-specific manner. In the case of L63, this function could be provided by associated cyclins that might regulate L63 activity. In addition, and perhaps most likely, different *L63* and *L82* isoforms may display distinct tissue-specific responses to ecdysone.

The complexity that emerges from these molecular and genetic studies clearly propels our studies of ecdysone signaling beyond the relatively simple cascades envisioned by the Ashburner model. Not all early puff genes function as direct transcriptional regulators and not all late puff genes encode simple effector functions. Moreover, these recent studies provide a firm foundation for expanding our understanding of the temporal regulation of insect development as well as more general insights into the mechanisms of steroid hormone action.

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References

Andres, A.J., Thummel, C.S., 1995. The *Drosophila* 63F early puff contains *E63-1*, an ecdysone-inducible gene that encodes a novel Ca^{2+}-binding protein. Development 121, 2667–2679.

Ashburner, M., 1973. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. I. Dependence upon ecdysone concentration. Dev. Biol. 35, 47–61.

Ashburner, M., Chihara, C., Meltzer, P., Richards, G., 1974. Temporal control of puffing activity in polytene chromosomes. Cold Spring Harbor Symp. Quant. Biol. 38, 655–662.

Ashburner, M., Richards, G., 1976. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster*. III. Consequences of ecdysone withdrawal. Dev. Biol. 54, 241–255.

Becker, H.J., 1959. Die puffs der speicheldrüsenchromosomen von *Drosophila melanogaster*. I. Beobachtungen zur verhalten des puffmusters in normalstamm und bei zwei mutanten, giant und *lethal-giant-larvae*. Chromosoma 10, 654–678.

Beermann, W., 1952. Chromomeren konstanz und spezifische modifikation der chromosomenstruktur in der entwicklung und organ differentzierung von *Chironomus tentans*. Chromosoma 5, 139–198.

Besset, V., Rhee, K., Wolgemuth, D.J., 1998. The identification and characterization of expression of PFTAIRE-1, a novel Cdk family member, suggest its function in the mouse testis and nervous system. Mol. Reprod. Dev. 50, 18–29.

Biyasheva, A., Do, T.V., Lu, Y., Vaskova, M., Andres, A.J., 2001. Glue secretion in the Drosophila salivary gland: a model for steroid-regulated exocytosis. Dev. Biol. 231, 234–251.

Burtis, K.C., Thummel, C.S., Jones, C.W., Karim, F.D., Hogness, D.S., 1990. The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. Cell 61, 85–99.

Butenandt, A., Karlson, P., 1954. Über die isolierung eines metamorphose-hormons der insekten in kristallisierter form. Z. Naturforsch. Teil B 9, 389–391.

Clever, U., 1964. Actinomycin and puromycin: effects on sequential gene activation by ecdysone. Science 146, 794–795.

Clever, U., Karlson, P., 1960. Induktion von puff-veränderungen in den speicheldrüsenchromosomen von *Chironomus tentans* durch ecdyson. Exp. Cell. Res. 20, 623–626.

DiBello, P.R., Withers, D.A., Bayer, C.A., Fristrom, J.W., Guild, G.M., 1991. The *Drosophila Broad-Complex* encodes a family of related proteins containing zinc fingers. Genetics 129, 385–397.

Edgar, B.A., 1999. From small flies come big discoveries about size control. Nat. Cell Biol. 1, E191–193.

Fristrom, D., Fristrom, J.W., 1993. The metamorphic development of the adult epidermis. In: Bate, M., Martinez Arias, A. (Eds.), The Development of *Drosophila melanogaster*, II. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 843–897.

Gruol, D.J., Vo, Q.D., Zee, M.C., 1999. Profound differences in the transport of steroids by two mouse P-glycoproteins. Biochem. Pharmacol. 58, 1191–1199.

Henrich, V., Rybczynski, R., Gilbert, L.I., 1999. Peptide hormones, steroid hormones, and puffs: mechanisms and models in insect development. Vitam. Horm. 55, 73–125.

Hock, T., Cottrill, T., Keegan, J., Garza, D., 2000. The *E23* early gene of *Drosophila* encodes an ecdysone-inducible ATP-binding cassette transporter capable of repressing ecdysone-mediated gene activation. Proc. Natl. Acad. Sci. USA 97, 9519–9524.

Huet, F., Ruiz, C., Richards, G., 1993. Puffs and PCR: the in vivo dynamics of early gene expression during ecdysone response in *Drosophila*. Development 118, 613–627.

Karim, F.D., Thummel, C.S., 1991. Ecdysone coordinates the timing and amounts of *E74A* and *E74B* transcription in *Drosophila*. Genes Dev. 5, 1067–1079.

Keegan, J., Schmerer, M., Ring, B., Garza, D., 2000. The 62E early-late puff of *Drosophila* contains *d-spinophilin*, an ecdysone-inducible PDZ-domain protein dynamically expressed during metamorphosis. Genet. Res. 77, 27–39.

Kolmerer, B., Clayton, J., Benes, V., Allen, T., Ferguson, C., Leonard, K., Weber, U., Knekt, M., Ansorge, W., Labeit, S., Bullard, B., 2000. Sequence and expression of the *kettin* gene in *Drosophila melanogaster* and *Caenorhabditis elegans*. J. Mol. Biol. 296, 435–448.

Kralli, A., Bohen, S.P., Yamamoto, K.R., 1995. LEM1, an ATP-binding-cassette transporter, selectively modulates the biological potency of steroid hormones. Proc. Natl. Acad. Sci. USA 92, 4701–4705.

Lakey, A., Labeit, S., Gautel, M., Ferguson, C., Barlow, D.P., Leonard, K., Bullard, B., 1993. Kettin, a large modular protein in the Z-disc of insect muscles. EMBO J. 12, 2863–2871.

Lazzaro, M.A., Albert, P.R., Julien, J.P., 1997. A novel cdc2-related protein kinase expressed in the nervous system. J. Neurochem. 69, 348–364.

Leevers, S.J., 1999. Perspectives: cell biology. All creatures great and small. Science 285, 2082–2083.

Restifo, L.L., Guild, G.M., 1986. An ecdysone-responsive puff site in *Drosophila* contains a cluster of seven differentially regulated genes. J. Mol. Biol. 188, 517–528.

Richards, G., 1976. Sequential gene activation by ecdysone in polytene chromosomes of *Drosophila melanogaster* IV. The mid prepupal period. Dev. Biol. 54, 256–263.

Richards, G., 1982. Sequential gene activation by ecdysteroids in polytene chromosomes of *Drosophila melanogaster* VII. Tissue specific puffing. Wilhelm Roux's Arch. Dev. Biol. 191, 103–111.

Richards, G., 1997. The ecdysone regulatory cascades in *Drosophila*. Adv. Dev. Biol. 5, 81–135.

Russell, S., Ashburner, M., 1996. Ecdysone-regulated chromosome puffing in *Drosophila melanogaster*. In: Atkinson, B.G., Gilbert, L.I., Tata, J.R. (Eds.), Metamorphosis. Postembryonic reprogramming of gene expression in amphibian and insect cells. Academic Press, New York, pp. 109–144.

Segraves, W.A., 1994. Steroid receptors and other transcription factors in ecdysone response. In: Recent Progress in Hormone Research, vol. 49. Academic Press, New York, pp. 167–195.

Segraves, W.A., Hogness, D.S., 1990. The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. Genes Dev. 4, 204–219.

Stowers, R.S., Garza, D., Rascle, A., Hogness, D.S., 2000. The *L63* gene is necessary for the ecdysone-induced 63E late puff and encodes CDK proteins required for *Drosophila* development. Dev. Biol. 221, 23–40.

Stowers, R.S., Russell, S., Garza, D., 1999. The 82F late puff contains the *L82* gene, an essential member of a novel gene family. Dev. Biol. 213, 116–130.

Thummel, C.S., 1996. Flies on steroids — *Drosophila* metamorphosis and the mechanisms of steroid hormone action. Trends Genet. 12, 306–310.

van Kalken, C.K., Broxterman, H.J., Pinedo, H.M., Feller, N., Dekker, H., Lankelma, J., Giaccone, G., 1993. Cortisol is transported by the multidrug resistance gene product P-glycoprotein. Br. J. Cancer 67, 284–289.

Vaskova, M., Bentley, A.M., Marshall, S., Reid, P., Thummel, C.S., Andres, A.J., 2000. Genetic analysis of the *Drosophila* 63F early puff. Characterization of mutations in *E63-1* and *maggie*, a putative Tom22. Genetics 156, 229–244.

Wolfner, M., 1980. Ecdysone-responsive genes of the salivary gland of *Drosophila melanogaster*. PhD thesis, Stanford University.

Wright, L.G., Chen, T., Thummel, C.S., Guild, G.M., 1995. Molecular characterization of the 71E late puff in *Drosophila melanogaster* reveals a family of novel genes. J. Mol. Biol. 255, 387–400.

Yamamoto, K.R., Alberts, B.M., 1976. Steroid receptors: elements for modulation of eukaryotic transcription. Annu. Rev. Biochem. 45, 721–746.

Zhimulev, I.F., 1999. Genetic organization of polytene chromosomes. In: Hall, J., Dunlap, J., Friedmann, T., Giannelli, F. (Eds.), Advances in Genetics, vol. 39. Academic Press, New York.