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# The acquisition of competence to respond to ecdysone in *Drosophila* is transcript specific

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**Abstract**

The steroid hormone ecdysone induces a precise sequence of gene activity in *Drosophila melanogaster* salivary glands in late third larval instar larvae. The acquisition of competence for this response does not result from a single event or pathway but requires factors that accumulate throughout the instar. Individual transcripts become competent to respond at different times and their expression is differentially affected in *ecd*$^{1}$, *dor*$^{22}$ and *BR-C* mutants. The induction of early-late transcripts, originally assumed to necessarily follow early transcripts, is partially independent of early transcript activation. Attempts to inhibit the synthesis of regulatory proteins reveal transcript-specific superinduction effects. Furthermore these inhibitors lead to the induction of *βFTZ-F1* and *E93* transcripts at levels normally found in prepupal glands. These studies reveal the complexity of the processes underlying the establishment of a hormonal response. 

*Keywords:* Competence; Ecdysone; Nuclear receptors; *Drosophila*

## 1. Introduction

Insect development is orchestrated by the steroid hormone ecdysone which exerts its effects through members of the nuclear hormone receptor superfamily, key molecules of a highly conserved regulatory mechanism found both in invertebrates and vertebrates. Although there are many studies concerning events during a hormonal response, notably hormone binding to receptor, receptor binding to target DNA sequences, interactions with co-activators or repressors and the induction of both natural and reporter genes (see Mangelsdorf et al., 1995; Glass et al., 1997 for recent reviews), little is known about the events which occur in the target cell prior to the arrival of hormone and which are necessary to organise the response to hormone.

The effects of 20-OH ecdysone (hereafter ecdysone) on *Drosophila* larval and prepupal tissues are perhaps the best characterised physiological responses to a steroid hormone. Molecular studies have provided insights into how ecdysone might induce stage and tissue specific changes in gene expression. Ecdysone acts via a heterodimer composed of the proteins EcR and USP encoded by the *ecdysone receptor* (*EcR*) gene and *ultraspiracle* (*usp*) respectively (Yao et al., 1992), in which the EcR partner is any one of three isoforms which show stage and tissue specificity (Talbot et al., 1993). Equally the early genes induced by ecdysone are complex transcription units consisting of a number of isoforms (Thummel, 1996; Richards, 1997 for reviews) and the combination of isoforms induced and their level of expression are stage and tissue specific (Huet et al., 1993). This suggests that cells are preset to respond to the arrival of hormone and that their ability to respond, or competence, is both stage and tissue specific.

The term ‘acquisition of competence’ was used to describe a change in the prepupal salivary gland between 4 and 6 h after pupariation (Richards, 1976a). At 4 h the early polytene chromosome puffs of the late prepupal response are refractory to hormone treatments while by 6 h they respond as they would normally do so between 8 and 10 h in response to the late prepupal increase in ecdysone

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titre. Competence appeared to result from either a single event or a series of events that occurred almost simultaneously at different loci. A number of factors contributing to this acquisition of competence have since been described. Both *EcR* and *βFTZ-FI* transcripts are dramatically induced in 6 h prepupal glands (Huet et al., 1995). Logically, this increase in *EcR* transcripts should lead to changes in the abundance of the ecdysone receptor complex. The FTZ-F1 protein is localised on prepupal ecdysone sensitive puff loci suggesting that it is necessary for ecdysone regulation in late prepupae (Lavorgna et al., 1993). Furthermore, by expressing *βFTZ-FI* ectopically in late larvae, Woodard et al. (1994) were able to induce the ecdysone induced late prepupal specific gene *E93* (Baehrecke and Thummel, 1995) in larval glands. *βFTZ-FI* activation requires the prior interactions of *DHR3* and *E75B*, further members of the nuclear receptor superfamily active at the end of the larval response to ecdysone (White et al., 1997; Lam et al., 1997). Although these studies on the late larval to late prepupal transition are encouraging, as this transition occurs over a relatively short period (3 to 5 h), it may prove difficult to detect transcript specific elements of competence.

The sequence of events prior to the late larval response occurs over a much longer period. The molecular complexity of events between 86 and 112 h emerged with the analyses of Andres and Huet and their respective co-workers (Andres and Cherbas, 1992; Andres et al., 1993; Huet et al., 1993; Huet et al., 1995; Huet et al., 1996). Hormone titre measurements in this period suffer from the developmental heterogeneity between individual larvae and the timing and extent of a number of small peaks that have been reported are poorly defined (see Andres et al., 1993 for a recent discussion). Karim and Thummel (1992), using mixed late larval tissues, defined class I (*EcR*, *E74B*) and class II (*E74A*, *E75A*, *E75B*) early transcripts on the basis of their induction by different concentrations of ecdysone in vitro. Our studies of salivary gland transcripts (summarised in Fig. 1) showed that in normal development *EcR* and *E74B* (as well as *usp* and *E75C*) increase from 86 h onwards and decline during the late larval response to hormone. Both the class II early transcripts and the early-late transcripts (*E78B*, *DHR3* and *DHR39*) are induced in the first few hours of the late larval response (112-120 h). When salivary glands are selected at 110 h, prior to the late larval response, and are cultured with differing concentrations of ecdysone, *E74A*, *E78B* and *DHR39* are induced with $>10^{-8}$ M ecdysone, *E75A* and *E75B* require $>10^{-7}$ M ecdysone and *DHR3* $10^{-6}$ M ecdysone for normal induction (Huet et al., 1995), which reflects their order of induction in vivo (Fig. 1).

While this suggests a threshold model, in which promoters having varying sensitivities to hormone are triggered sequentially by a steadily rising titre, the dose response curves overlap sufficiently to ensure that the full induction of class I transcripts will lead to the suboptimal induction of class II transcripts, which does not occur in vivo. An alternative is that each increase in hormone triggers a new set of primary induced genes and subsequent protein synthesis alters the cellular response so that a later peak of hormone will have a distinct effect. Finally if transcripts are induced as a secondary response to hormone their induction will require protein synthesis and they will increase some hours after the beginning of a hormone peak.

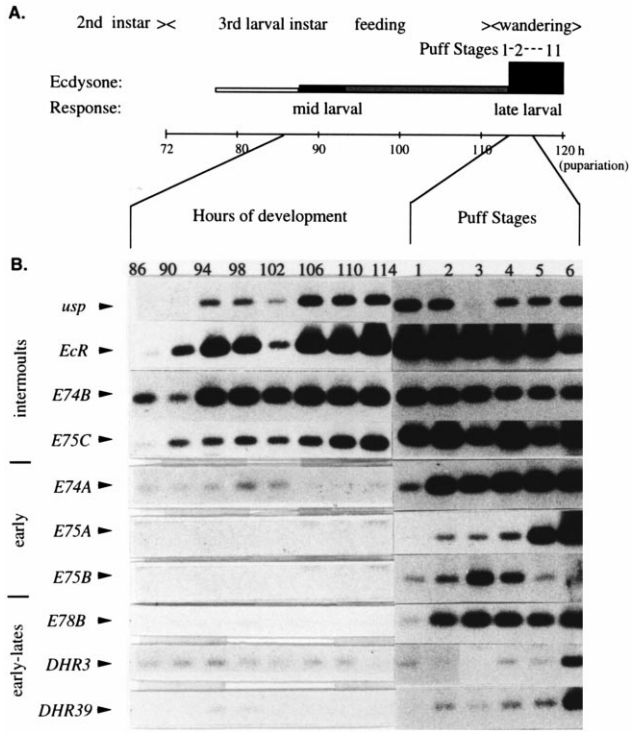


Fig. 1. The sequential activation of ecdysone inducible transcripts in third larval instar salivary glands of *Drosophila melanogaster*. (A) The feeding and wandering phases of the third larval instar are shown with respect to hours after egg laying and the mid larval and late larval ecdysone responses. (B) Transcripts of the different genes analysed by RT-PCR are shown for the mid larval and late larval period, the former staged by hours after egg laying, the latter by puff staging the contralateral lobe of the salivary gland. Each column represents transcripts analysed in the same salivary gland. Data from Huet (1995) and Huet et al. (1995).

Our experimental approach was simple. If transcripts are induced as a consequence of hormone titre alone then it will be sufficient to treat mid instar glands with high levels of hormone so as to induce the class II early transcripts. If developmental age is important, then by treating glands of different ages we might be able to define the period during which competence is acquired. We find temporal differences in the capacity of transcripts to respond to hormone, suggesting that competence is not acquired as the result of a single event.

Dissection of events in the ecdysone response has traditionally used inhibitors of macromolecular synthesis and mutant strains. We therefore cultured glands with hormone and protein synthesis inhibitors to see whether the expression of the different transcripts requires the synthesis of new factors. This technique was used in the initial puffing studies (Clever and Romball, 1966; Ashburner, 1974) and indeed

established the paradigm for early gene activation. However, we found that not only are many larval transcripts subject to a superinduction effect but also that prepupal transcripts are induced heterochronically in the presence of these inhibitors.

The expression of a number of ecdysone induced genes is affected to varying degrees in *ecd*$^{1}$, *dor* or *BR-C* mutant late third instar larvae, suggesting differences in the underlying regulatory pathways (see Huet et al., 1996; Meister and Richards, 1996 for recent examples). *ecd*$^{1}$ mutants are deficient in ecdysone (Garen et al., 1977), *dor* encodes a protein with a zinc finger-like motif (Shestopal et al., 1997) and *BR-C* a family of proteins differing in their 'zinc-finger' motifs (DiBello et al., 1991). In these mutants we found both evidence for differences between early and early-late transcripts and support for the conclusion that competence is a locus-specific state.

## 2. Results and discussion

### *2.1. Differential response of transcripts to ecdysone in 90 and 100 h salivary glands in vitro*

We cultured salivary glands from 90 h and 100 h larvae for 0 to 6 h in the absence or presence of low ($10^{-8}$) or high ($10^{-6}$) levels of ecdysone (Fig. 2A). After analysis of *rp49* transcripts, representative series of glands were analysed for three groups: the intermoult transcripts (*usp*, *EcR*, *E74B* and *E75C*), the early transcripts (*E74A*, *E75A* and *E75B*) and the early-late transcripts (*E78B*, *DHR3*, *DHR39*). To compare maximum transcript levels seen in 90 and 100 h glands with those in 110 h glands, RNAs from both were analysed in parallel (Fig. 2B and data not shown).

In 90 h glands there are only minor changes in the levels of *usp*, *EcR* and *E74B* in culture. Indeed, changes in the levels of these transcripts are the least sensitive to hormone treatments although *usp* and *EcR* are both induced at 100 h by $10^{-8}$ M while with $10^{-6}$ M hormone they are first induced and then repressed, as in 110 h glands (Huet et al., 1995). *E74B* transcripts are already suboptimally induced by 86 h (Fig. 1) and levels are not significantly altered by these hormonal treatments. *E75C* transcripts were difficult to detect and did not appear to respond to ecdysone treatments (data not shown). These results support the conclusion of Hall and Thummel (1998), from a study of *usp* mutants, that expression of the mid third-instar regulatory hierarchy does not require the ecdysone-receptor complex.

The early *E74A* and *E75A* transcripts are induced with $10^{-6}$ M ecdysone at 90 h showing that there are functional ecdysone receptors at this stage. This response is greater at 100 h where both transcripts start to decline by 6 h of in vitro culture, as in late instar glands. *E75B* is also induced suboptimally at 90 h by $10^{-6}$ M ecdysone while at 100 h there is a biphasic response, also characteristic of late instar glands (Segraves and Hogness, 1990).

The early-late transcripts *E78B* and *DHR3* are slightly induced by $10^{-6}$ M ecdysone by 4 and 6 h in 90 h glands and more significantly in 100 h glands, where a clear induction is seen from 2 h onwards, while *DHR39* transcripts are essentially only induced at 100 h. For all three transcripts, the profiles obtained at 100 h resemble those seen with 110 h glands. However, while *E78B* and *DHR3* transcripts accumulate to similar levels, for *DHR39* the peak levels are approximately 50% lower than those in 110 h glands (Fig. 2B). Note that we detect a basal hormone-independent activity of *DHR3* in all glands (see also Fig. 1B), possibly linked to alternate promoter usage. Our RT-PCR assay detects the core region of *DHR3* while multiple RNAs are seen in northern analyses (Koelle et al., 1992).

Both the early and the early-late transcripts respond essentially to $10^{-6}$ M ecdysone, the kinetics and the extent of the response changing between 90 and 100 h. While the early genes respond more readily than the early-lates at 90 h this is far from the massive induction seen by 1 h in 110 h glands. In 100 h glands the response of the early transcripts *E74A*, *E75A* and *E75B*, approaches that of the end of the instar. Peak levels for all three are comparable to those seen in older glands (Fig. 2B), although the low levels after 1 h of culture are equivalent to those seen with $10^{-8}$ M ecdysone in 110 h glands (see Huet et al., 1995). Acquisition of competence for *E78B* and *DHR3* occurs between 90 and 100 h and their response at 100 h resembles that seen in 110-h glands (Fig. 2A,B). In this respect they appear fully competent earlier than the early transcripts. At 100 h, the third early-late transcript *DHR39* is still acquiring competence to respond.

Differences in the response of promoters might simply reflect binding affinities between the ecdysone receptor and the hormone response element, as measured in vitro. However Antoniewski et al. (1996) showed in transgenic *Drosophila* that the absolute binding affinity of the hormone response element was less important than promoter context for *in vivo* activity. Thus, it is likely that the response depends both on binding affinity and on factors binding either to adjacent sequences (either prior to or concomitantly with receptor complex binding) or directly to the receptor complex (as a receptor co-activator or repressor). If receptor levels have reached maximal levels, competence should reflect the accumulation (or inactivation) of such proteins.

### *2.2. Protein synthesis inhibitors cause transcript-specific superinduction in mid instar glands*

The previous experiments eliminate a timing mechanism based on hormone titre alone and suggest that other factors are involved. In an attempt to block de novo synthesis of regulatory proteins, we cultured 90 and 100 hour gland pairs for 2 or 4 h with $10^{-6}$ M ecdysone in the presence or absence of the protein synthesis inhibitor cycloheximide. One lobe was incubated with hormone and inhibitor while the second

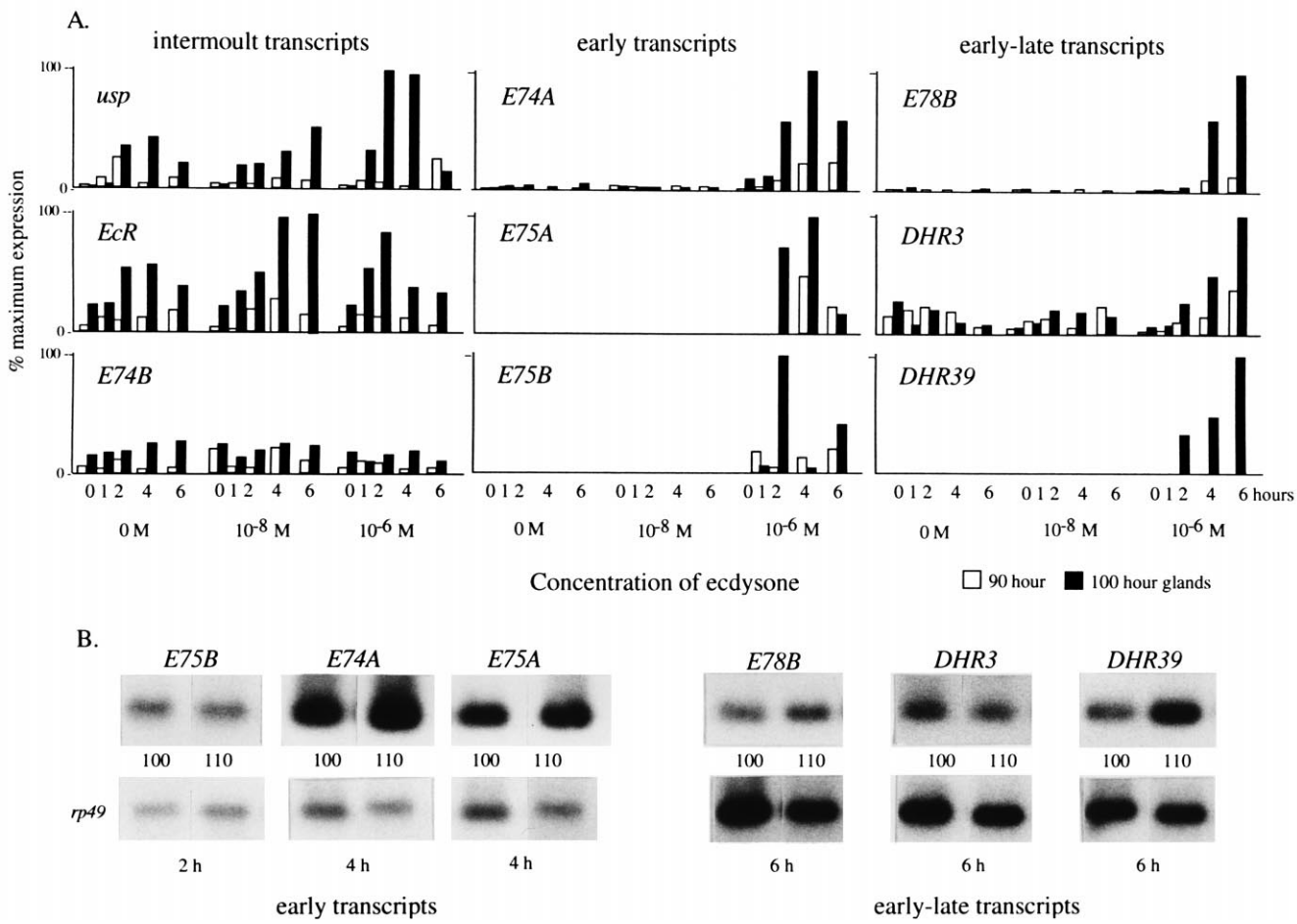


Fig. 2. Ecdysone induction of transcripts in staged salivary glands. (A) Individual 90- and 100-h glands were cultured for 1–6 h in Grace's medium with ($1.8 \times 10^{-8}$, $1.8 \times 10^{-6}$ M) or without (0 M) ecdysone as shown. (□) 90-h glands; (■) 100-h glands. RT-PCR reactions for the different transcripts used aliquots of RNA from the same glands (see Section 4). Transcript levels are expressed as a percentage of the maximum level observed for that transcript, with the exception of *E74B* which uses the *E74A* standard (see Section 4). (B) Maximum transcript levels in 100- and 110-h glands cultured with $1.8 \times 10^{-6}$ M ecdysone for 2 h (*E75B*), 4 h (*E74A* and *E75A*) or 6 h (*E78B*, *DHR3* and *DHR39*). *rp49* transcripts in the same glands are shown below.

lobe was incubated with hormone alone. A further series of 100 hour glands were treated with anisomycin. Although both inhibitors were used in early puffing studies (Ashburner, 1974), subsequently cycloheximide has been used almost exclusively in *Drosophila* studies, while both have been used in vertebrate studies. When transcripts were compared between the two lobes for several pairs of glands (normally 4 or 5), we found two situations. In the first, depending on the pair of lobes, transcript levels were slightly higher either with or without inhibitor so that individual expression ratios (see Section 4) could be greater or less than 1.0 and we concluded that there was no effect of the inhibitor. In the second, transcripts were consistently higher in the lobe with inhibitor. For clarity, only the expression ratios after 4 h of culture are presented (Fig. 3A–C) although in addition the most striking effects of inhibitor are illustrated by two pairs of lobes (Fig. 3D).

In 90-h glands, all intermoult transcripts, especially *EcR* and *E74B*, were higher in the presence of cycloheximide (Fig. 3A). In 100-h glands the effect was less marked although expression ratios were between 2.5 and 3.5 for both inhibitors (Fig. 3A). Unexpectedly, *E75C* transcripts, normally difficult to detect (see above), were dramatically increased in the presence of both inhibitors at 100 h (Fig. 3B,D). There is no effect of cycloheximide for the early transcript *E74A* in 90-h glands, while *E75A* transcripts in the same glands show a 5-fold superinduction. At 100 h both *E74A* and *E75A* ratios are close to 1, as would be expected for an induction that is independent of protein synthesis. In contrast the inhibitors cause a striking superinduction of *E75B* transcripts both at 90 and 100 h (Fig. 3A,D). Effects on *E78B* transcripts are modest, at most we observed a 30% increase in the presence of cycloheximide (Fig. 3A). While both *DHR3* and *DHR39* are increased 5-fold in 90-h glands, in 100-h glands the effect was some 2–3-fold (Fig. 3A,D).

As transcript levels were higher in the presence of inhibitors, we incubated glands with anisomycin in the absence of hormone for 4 h (Fig. 3C). For most transcripts there was no effect (data not shown) while for *usp* and *E75B*, levels were increased some 3-fold. For *E75B*, as levels were negligible compared to those seen with hormone, there is not a significant induction by anisomycin per se. The most strik-

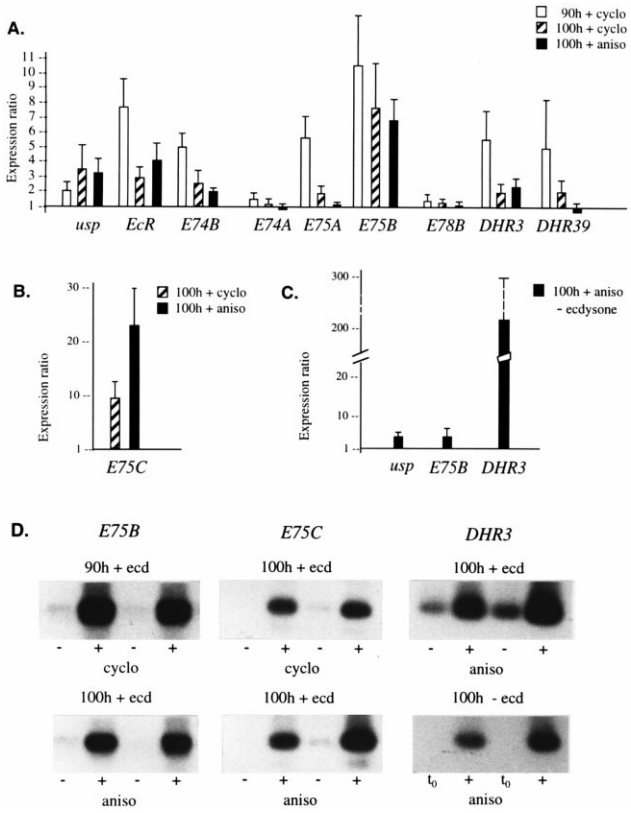


Fig. 3. The effects of inhibitors of protein synthesis on ecdysone regulated transcript levels. Salivary glands were dissected and sister lobes cultured for 4 h in the presence of $1.8 \times 10^{-6}$ M ecdysone with or without cycloheximide (90- and 100-h glands) or anisomycin (100-h glands) as shown. For each point five pairs of lobes were cultured in parallel and data from between 3 and 5 pairs were analysed for all transcripts in terms of the expression ratio (see Section 4). (A) Expression ratios ( ± SE) in 90- and 100-h glands for cycloheximide treatments and in 100-h glands for anisomycin treatments in the presence of ecdysone. (B) Expression ratios ( ± SE) in 100-h glands for *E75C* transcripts for cycloheximide or anisomycin treatments in the presence of ecdysone. (C) Expression ratios ( ± SE) in 100-h glands for anisomycin treatment in the absence of ecdysone for *usp*, *E75B* and *DHR3*. The control lobe ($t_0$) was frozen immediately following dissection. (D) Examples of two pairs of sister lobes from the series analysed in panels A–C. Lobes were from 90- or 100-hour larvae, cultured for 4 h, in the absence ( − ) or presence ( + ) of inhibitor, except for the control lobes ($t_0$) of panel C.

ing result was the massive increase of *DHR3* transcripts which reached levels similar to those seen with $10^{-6}$ M ecdysone (Fig. 3C,D).

We expected that protein synthesis inhibitors would help to distinguish between direct and indirect effects of hormone but instead discovered an extensive superinduction of transcripts. The superinduction effects are stage dependant and decrease as pupariation approaches. They are more pronounced in 90-h glands than in 100-h glands (Fig. 3) while in previous studies with late larval glands (i.e. 110–114 h), including our own (Huet et al., 1995), there has been little evidence for such an effect other than with *E75B* (Segraves and Hogness, 1990). This stage- (and possibly tissue-) dependency may explain discrepancies in results from different laboratories. For example the modest increase seen here in *E78B* transcripts in the presence of cycloheximide in 100-h glands differs from our observation that *E78B* induction was essentially insensitive to cycloheximide treatments in 110 hour glands (Huet et al., 1995) and the finding of Stone and Thummel (1993) that *E78B* induction in mixed late larval tissues was reduced in the presence of cycloheximide.

A number of mechanisms have been suggested to explain the superinduction phenomenon. These include: (i) the existence of labile repressor proteins whose rapid turnover is sensitive to inhibitors; (ii) the protection of transcripts from degradation either by physical association with inhibitors or by the inhibition of labile mRNases; (iii) the inhibition of early protein synthesis to prevent the negative feed-back loop postulated to repress early transcript synthesis (e.g. Ashburner et al., 1974) and (iv) the inhibitor acting as an inducer at concentrations below those necessary to block protein synthesis. In the case of *fos* and *jun* signalling, Edwards and Mahadevan (1992) showed that anisomycin may act through the last three mechanisms but that labile repressors were not involved. In our experiments, as effects are transcript specific, this may reflect different combinations of mechanisms for each locus.

### *2.3. Protein synthesis inhibitors cause heterochronic induction of prepupal specific transcripts in larval glands*

Since cycloheximide treatments lead to the induction of the late prepupal specific 93F puff in larval glands (Richards, 1976b), we looked for the corresponding *E93* transcripts (Baehrecke and Thummel, 1995), in 100 hour glands. We also assayed *βFTZ-F1* transcripts from the mid prepupal specific puff 75CD. *βFTZ-F1* transcripts increased in the presence of inhibitors in the absence of ecdysone (Fig. 4A) and paralleled the induction of *DHR3*

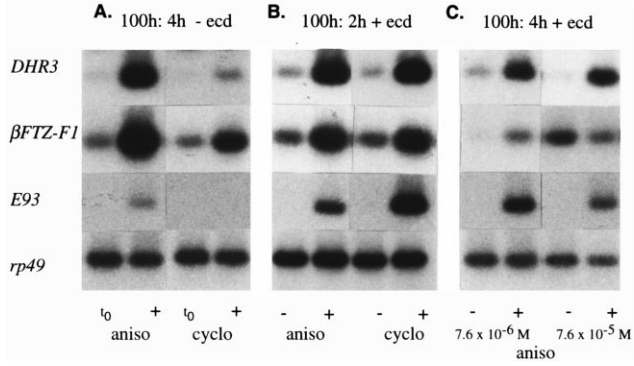


Fig. 4. Protein synthesis inhibitors cause premature induction of *βFTZ-F1* and *E93* transcripts in salivary glands from 100 hour larvae. In each panel *DHR3*, *βFTZ-F1*, *E93* and *rp49* transcripts are shown from a pair of sister lobes. (A) Experimental lobes were cultured 4 h in the absence of ecdysone, in the presence of inhibitor (cyclo-cycloheximide, aniso-anisomycin) while the control lobes ($t_0$) were frozen immediately. (B) Lobes were cultured 2 h with ecdysone, in the absence (–) or presence (+) of inhibitor. (C) Lobes were cultured 4 h with ecdysone, in the absence (–) or presence (+) of $7.6 \times 10^{-5}$ or $7.6 \times 10^{-6}$ M anisomycin as shown.

transcripts in the same glands. In glands treated for 2 h with ecdysone and inhibitor, we consistently found *E93* transcripts which were not present in the sister lobe treated with ecdysone alone (Fig. 4B). Although *E75B*, *DHR3* and *βFTZ-FI* transcripts are also induced in these glands it is most unlikely that the sequential induction of the respective proteins, which precedes *E93* induction in late prepupae (White et al., 1997; Lam et al., 1997), occurs. Presumably the balance of factors necessary for the correct stage specific expression is disrupted, leading to this heterochronic induction.

Edwards and Mahadevan (1992) showed superinduction effects at anisomycin concentrations which do not block protein synthesis completely. We incubated glands with ecdysone and $7.6 \times 10^{-5}$ M or $7.6 \times 10^{-6}$ M anisomycin, the latter being suboptimal for protein synthesis inhibition (Ashburner, 1974). Results for *E93* and *DHR3* were similar for both concentrations (Fig. 4C) suggesting that protein synthesis inhibition is not the determining factor in these inductions. Note that the accumulation of *βFTZ-F1* transcripts is transient and that by 4 h of culture with ecdysone they have already disappeared from some glands (Fig. 4C).

### 2.4. Defects in the ecdysone response are transcript specific in mutant larvae

If competence depends on a single event, or chain of events, then mutations in the ecdysone response will have similar effects on all transcripts. We analysed transcripts in *ecd*$^{1}$, *dor*$^{22}$ or *BR-C* mutant larvae which reach the wandering stage at the end of the third larval instar and then either appear blocked prior to the late larval response (*ecd*$^{1}$ and *dor*$^{22}$) or engage in an aberrant response (*BR-C* alleles). For *ecd*$^{1}$ we compared transcripts from late instar wandering animals cultured at the restrictive temperature (29°C) with those maintained at 20°C. For *dor*$^{22}$ and *BR-C* alleles we compared transcripts in glands of wandering male larvae to those in female sibs carrying a wild type allele. All control larvae were in puff stages 1–6 as assessed by their transcript profiles (see Fig. 1). These analyses reflect the most advanced developmental stage observed for a given transcript in the salivary gland of the mutant.

For *ecd*$^{1}$, as would be expected from the apparent developmental age of the wandering larvae, the intermoult transcripts are at levels comparable to those of the controls (Fig. 5). While there is little evidence of induction of early transcripts, there is a higher expression of both *E78B* and *DHR39* whose expression normally follows that of the early transcripts. The absence of *DHR3* transcripts suggests that more than one regulatory pathway exists for this class. These results are consistent with previous experiments (Andres et al., 1993; Huet et al., 1996) which show that different ecdysone responses are arrested at different stages in *ecd*$^{1}$.

Previous studies with *dor*$^{22}$ have shown that while salivary gland glue synthesis precedes normally in the second

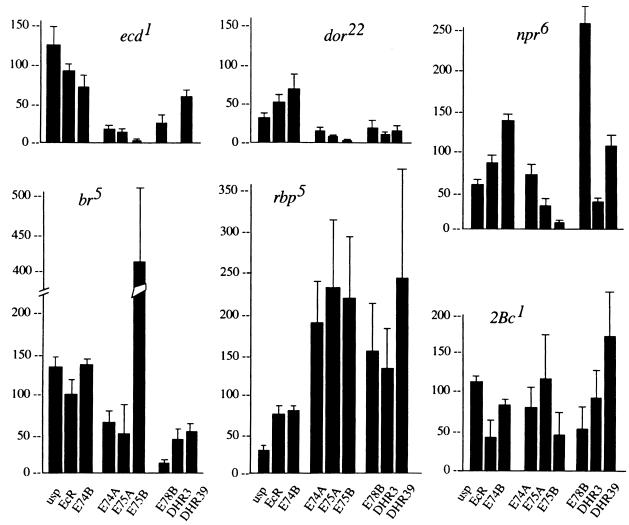


Fig. 5. Expression of ecdysteroid regulated transcripts in *ecd*$^{1}$, *dor*$^{22}$ and the *BR-C* mutants: *npr*$^{6}$, *2Bc*$^{1}$, *br*$^{5}$, *rbp*$^{5}$. For *ecd*$^{1}$, transcript levels in late third instar wandering larvae at 29°C are expressed as a percentage of those in controls raised at 20°C while for the remaining X-linked mutants, mutant males are compared with their female sibs. All transcripts were analysed in RNA extracted from the same individual salivary glands dissected from between five and ten individuals and the percentage expression (±SE) determined as the average expression in the experimental group divided by that of the control group.

half of the third larval instar (Georgel et al., 1991), the ecdysone regulated increase of *hsp27* transcripts in that same period is blocked (Huet et al., 1996). Induction of the early and early-late puffs in *dor*$^{22}$ salivary glands requires exogenous ecdysone (Biyasheva et al., 1985), which will also restore the maturation of the immune response in *dor*$^{22}$ larvae (Meister and Richards, 1996). In the present case, results with *dor*$^{22}$ are similar to those with *ecd*$^{1}$, except for a general reduction in the intermoult transcripts, particularly for *usp*, and the presence of low levels of *DHR3* transcripts (Fig. 5).

The *BR-C npr*$^{6}$ allele is non-complementing with the *br*, *rbp* and *2Bc*$^{1}$ *BR-C* subfunctions and the 74EF and 75B early puffs are reduced in size in mutant larvae (Belyaeva et al., 1981). In *npr*$^{6}$ males intermoult transcript levels are close to normal while *E74A* induction appears closer to controls than in the previous mutants. *E75A* and *E75B* transcripts are lower in these same animals and this may reflect their lower sensitivity to hormone in wild type animals (see above). Using a different *npr* allele, Karim et al. (1993) found reduced *E74A* and *E75A* transcript levels in mixed larval tissues cultured with ecdysone, while *E75B* transcripts were only lower at early timepoints. A more striking result is the level of *E78B* transcripts, which are abundant compared to controls. *DHR3* levels are low, while *DHR39* levels are as in the control larvae. *E78B* and *DHR39* induction in these animals is also coherent with the sensitivity of these genes to ecdysone treatments in wild type glands (see above).

To study the contributions of the three genetically defined

subfunctions of the *BR-C* locus we used the $br^5$, $rbp^5$ and $2Bc^1$ alleles (Fig. 5). Although a considerable effort has been made to correlate molecular events with the *BR-C* subfunctions, as the *BR-C* products are structurally related, in many instances it has proven difficult to implicate one subfunction as the key regulator of a given molecular event (Richards, 1997 for review). Our results are no exception as, for all three alleles, expression of both the early and the early-late transcripts is closer to controls than in the $npr^6$ larvae which lack all *BR-C* functions. Although $br^5$ mutants initiate transcription of both the early and early-late genes, levels are lower than in the controls, with the notable exception of *E75B* which, as in the case of superinductions (Fig. 3), is distinct from that of the other two early transcripts, *E74A* and *E75A*. For $rbp^5$ males, collected early in the wandering phase, intermoult transcripts are reduced and both early and early-late transcript levels are higher than controls. Thus, although emerging later, these males start their wandering at a more advanced developmental stage than their female sibs. This behavioural difference was observed in the transcript profiles of males from two independent collections made several months apart. Finally transcripts in $2Bc^1$, essentially a prepupal lethal, show a profile that is overall closest to that of the control group suggesting that initiation of the response in salivary glands is normal although its progression may be altered as Karim et al. (1993), using whole animals, reported a reduction of *E74A* and *E75A* transcripts in older $2Bc^1$ larvae.

## 3. Conclusions

The present results show that: (i) the response to ecdysone depends on the developmental age of the gland; and (ii) that individual loci evolve constantly in their capacity to respond to hormone. Although there are transcript-specific consequences of treatments with protein synthesis inhibitors, there are clearly serious limits to their use and one must be careful in interpreting such experiments, particularly if only one or a few transcripts are being followed. In the present study the aberrant expression in larval glands of the essentially prepupal transcripts *βFTZ-F1* and *E93* was observed when we re-examined the collection of samples we had previously analysed for the larval transcripts. Finally the expression of early and early-late transcripts is differentially altered in different mutants. In early wandering $ecd^1$, $dor^{22}$ and $npr^6$ larvae, expression of early transcripts is reduced while early-late transcript levels are less affected confirming that induction of these early-late transcripts is not dependant upon early gene expression (Huet et al., 1995). However the corresponding lesions show transcript-specific effects and thus it is essential to follow several transcripts to assess hormonal competence. Quantitative hybridisation to DNA microarrays may facilitate this analysis as our present approach is labour-intensive (results from several thousand RT-PCR reactions are summarised in this report).

The present challenge is to understand the molecular mechanisms underlying this 'acquisition of competence'. Progress is most likely to come from the use of mutants and/or the ectopic expression of genes known to be implicated in the ecdysone responses. It would be surprising if this complexity in the organisation of a hormonal response is restricted to insects and such a biological framework should prove conceptually useful for studies on co-regulators of nuclear receptors in vertebrates. Currently there is rarely a notion of developmental specificity in such studies and responses are most often studied in a given cell line (as representing a tissue) with the underlying idea of using a starting material that is as uniform as possible. It is clear that, if we are to understand the role of hormones in regulating animal development, we must develop models that allow one to study the dynamics of hormonal responses.

## 4. Experimental procedures

### 4.1. Drosophila strains

The *Oregon-R* wild type strain was maintained at 25°C on cornmeal agar medium. Larvae were selected at the second to third instar moult, 72h after egg laying, by anterior spiracle morphology and transferred in groups of 20 to fresh culture medium until they reached the desired age. These larvae leave the food at approximately 110–112 h and pupariate in a 4–8-h period around 120 h.

Larvae from the temperature sensitive *ecd1* strain (Garen et al., 1977) were maintained at 20°C (permissive temperature – control animals) or transferred to 29°C at the beginning of the third larval instar. At 29°C, animals eventually leave the food and then remain blocked in an extended wandering phase. Females from the X-chromosome 2B mutant strains bearing either the $dor^{22}$ *[y* $l(1)t^{187}$*/FM6l-69j/Dp(1)*$y^2$*Y67g]* mutation or the *BR-C* $npr^6$*[y* $l(1)t^{435}$*/FM6l-69j/Dp(1)*$y^2$*Y67g]*, were crossed with Oregon-R males so as to obtain hemizygous males which die as late larvae. In these crosses the *FM6l-69j* carrying males die before the third instar whereas both classes of females are viable and serve as internal controls. These and further *BR-C* strains, $br^5$, $rbp^5$ and $l(1)2Bc^1$ were obtained from Igor Zhimulev, Greg Guild or both.

### 4.2. Salivary gland culture

Individual salivary glands were incubated in 25 μl of culture medium in glass depression slides, covered with grease sealed coverslips and maintained at 25°C. Contralateral lobes were either cultured in parallel with different medium (±hormone or hormone ± cycloheximide etc.) or served as 0 h timepoints. The culture medium (Ashburner, 1972) is modified Grace's medium (50:9:1 Grace's medium (Gibco)/distilled water/ethanol). Cycloheximide ($7 \times 10^{-5}$ M final concentration) or anisomysin ($7.6 \times 10^{-5}$ M final

concentration) were added in the distilled water fraction while 20-OH ecdysone (Simes, Milan) was added in the alcohol fraction as appropriate.

### *4.3. RNA extraction and RT-PCR analyses*

Methods for the micro-RNA extraction and the subsequent RT-PCR protocol have been described (Huet et al., 1993, 1995). Glands were either dissected, transferred to 25 $\mu$l of extraction buffer and frozen at –80°C immediately or cultured prior to transfer to extraction buffer. For each point at least four individual glands were first analysed for *rp49* transcripts to monitor RNA extraction from individual glands which is highly reproducible (see Fig. 4, and Huet et al., 1993). In general this step serves to eliminate the rare cases where a lobe is damaged or lost between dissection, culture and transfer to extraction buffer. We then undertook RT-PCR reactions using the equivalent of 1.6% of the RNA from a salivary gland per transcript. The reproducibility of the assay has been discussed in Huet et al. (1993) – in general we observe at most a 2–3-fold variation between replicate glands. Template concentrations were such that RT-PCR reactions are in the linear range of amplification. Products were separated on 2% agarose gels and analysed after transfer to nylon membranes and hybridisation with a labelled internal oligonucleotide. For *usp*, primers were TACGAGAAGCTCTGGTTGAG and CAATAGCGTGTCCAGGGATT and the internal probe was TCATCGAGCGAAACGATGCT, while for *E93* primers were TGGCCGACTTCAATCTGATC and ATCTAGCTTGTTGGCCACCA and the internal probe CGCCAACTACCTCAGAAGTA. Primers and hybridisation probes for all other transcripts are given in Huet et al. (1993, 1995). Prior to autoradiography, filters were exposed with a Fuji Type Bas-III imaging plate which was scanned using a Fujix Bas 2000 phospho-imager and the relative values plotted after subtraction of background. Comparisons of absolute levels between transcripts are not possible as the efficiency of RT-PCR reactions and probe hybridisation are variable. The only exception is for the *E74A* and *B* isoforms where our RT-PCR assay (Huet et al., 1993) gives the same results as northern analyses using a probe from the *E74* common region (Karim and Thummel, 1991). In consequence we have used the *E74A* maximum as a standard for both isoforms so as to show relative isoform abundance.

For each time/treatment point in Fig. 3 the twin lobes from five pairs of glands were cultured individually, in parallel, with or without cycloheximide or anisomysin. After phospho-imager analyses, we derived the expression ratio for each transcript, defined as the value for transcripts in the treated lobe divided by the value obtained in the sister lobe. In the absence of effect the expression ratio will be 1.0. In extreme cases, when levels of expression in the control gland are close to background, we obtain widely divergent expression ratios as the denominator is close to zero. Expression in Fig. 5 is shown as the percentage of transcript levels in glands from mutant larvae compared to those in wild type sibs.

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