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The role of ecdysone in the induction and maintenance of hsp27 transcripts during larval and prepupal development of Drosophila

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Abstract The *Drosophila melanogaster* small heat-shock gene *hsp27* carries the canonic ecdysone response element (EcRE) at -537. This EcRE has been used extensively both in cultured cells and in vitro for studies of the ecdysone response. We have characterised the developmental expression of *hsp27* in wild-type larval and prepupal salivary glands in parallel with that of the *E74B* and *E74A* primary ecdysone response transcripts, which are induced in the mid and late third larval instar by a minor and major increase in ecdysone titre respectively. The induction of *hsp27* occurs between these two events in larvae and in parallel with that of *E74A* in prepupae. Transcript levels are severely reduced in *ecd1* and dor^{22} (*deep orange*) mutant larvae but are only moderately reduced in larvae mutant for the *Broad-Complex* allele br^{t435}. By culturing salivary glands of different ages with low (1.8×10^{-8} M) or high (1.8×10^{-6} M) concentrations of hormone, we show that the response of an EcRE varies during development and that the timing of the response cannot be predicted solely from its apparent strength in cell line analyses.

Key words *hsp27* · Ecdysone · EcREs · Developmental regulation · Hormonal response

Introduction

The molecular characterisation of nuclear receptors has improved our understanding of the principles of co-ordination of animal development. Extensive analyses in cultured animal cells and in vitro have defined both the domain structure of the receptors and their DNA targets, the hormone response elements. In general these studies show that receptors bind an optimal or consensus six base pair motif (or half-site) arranged as a palindromic repeat or as tandem repeats with variable spacing (see Pfahl 1994, for review). In insects, the steroid hormone ecdysone is used repeatedly in different tissues and developmental stages to activate specific gene programmes. Elements contributing to this specificity may be the absolute hormone titre (Huet et al. 1993) or differential expression of ecdysone receptor (EcR) isoforms (Talbot et al. 1993). An ecdysone response element (EcRE) was first identified upstream of the small heat-shock protein gene *hsp27* using transfection assays in cultured cells (Riddihough and Pelham 1987). In fact, functional ecdysone receptors are heteromers including an EcR isoform (Koelle 1992), and the *ultraspiracle* gene product USP and the in vitro binding experiments that characterised the heteromer (Yao et al. 1992) used the *hsp27* EcRE. This canonical EcRE (GGTTCAaTGCACT) has been used as a standard in the characterisation of other natural EcREs, all of which to date appear to have a lower binding affinity for the receptor (e.g. Cherbas et al. 1991; Ozyhar et al. 1991; Antoniewski et al. 1993 and references therein). However, a number of synthetic elements, notably half-sites arranged as direct repeats, have recently been shown to have a higher binding affinity than the *hsp27* EcRE (Antoniewski et al. 1996).

The small heat-shock genes, including *hsp26* and *hsp27*, were first shown to be ecdysone inducible in Schneider line 3 (S3) *Drosophila* cells and mass isolated late third larval instar imaginal discs, although the effect was modest (two-fold) in the latter (Ireland et al. 1982). Later analyses, which revealed an increase in levels of both *hsp26* and *hsp27* transcripts at the end of the third larval instar, led to the suggestion that these genes were induced by ecdysone during normal development. For *hsp26*, transcript levels remained low in mutant *ecd1* larvae (Garen et al. 1977), which show a reduced level of

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ecdysone when maintained at a restrictive temperature (29°C), but increased 40-fold upon ecdysone feeding (Thomas and Lengyel 1986). From this result, it has been generally assumed that the increase in levels of *hsp27* transcripts at the end of the third larval instar depends on the massive increase in hormone titre occurring a few hours before puparium formation. In view of the central importance of the *hsp27* EcRE, we have undertaken a more detailed developmental study of *hsp27* transcripts in salivary glands during the ecdysone responses characteristic of the mid and late third larval instar and the prepupal period using a micro reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, which enables us to follow up to 60 transcripts in the RNA extracted from a single gland (Huet et al. 1995). We developed this approach because the developmental heterogeneity between larvae is such that the mass collections necessary for Northern analyses of transcripts obscure details of the molecular response.

So as to compare *hsp27* regulation with well characterised early ecdysone-induced transcripts, we analysed transcripts in the same glands from the *E74* gene localised in the 74EF early ecdysone puff. *E74* gives rise to two primary induced transcripts of the ecdysone regulatory hierarchies, *E74B* and *E74A*, which Karim and Thummel (1992) assigned to early class I and early class II respectively, based on their sensitivity to hormone as determined by the culture of tissues extracted from late third instar larvae. In their experiments, 50% induction of *E74B* and *E74A* required 2×10^{-8} M and 10^{-7} M ecdysone respectively. The comparison of these results with their developmental expression (Huet et al. 1993) suggested that class I transcripts are induced by a modest increase in hormone between 86 and 90 h after egg laying, whilst class II transcripts are induced by the massive increase in titre that starts some 6 to 8 h before pupariation. However, analyses of ecdysone titres in this period also suffer from the problem of heterogeneity and our knowledge of their profiles remains sketchy (see Richards 1981; Andres et al. 1993 for discussion). We find that, in larval development, *hsp27* transcripts are induced between *E74B* and *E74A* transcripts but are induced in parallel with *E74A* transcripts in prepupae.

Both *E74* and the ecdysone-regulated *BR-C* (*Broad-Complex*) are involved in the well-characterised larval and prepupal ecdysone responses and, when mutant, lead to changes in the expression of other members of these ecdysone-regulated hierarchies (Fletcher and Thummel 1995). Transcription of the 67B small heat-shock gene cluster (which includes *hsp27*) and its flanking chromatin structure are affected in *BR-C* mutants (Dubrovsky et al. 1994). We have analysed larvae mutant for either *BR-C* mutants or dor^{22}(*deep orange*), an adjacent late third larval instar lethal with an ecdysone deficient phenotype. While *hsp27* expression is moderately reduced in mutants for the *BR-C* allele br^{t435}, it is dramatically reduced in dor^{22}. A similar result was seen using these same mutants to study the ecdysone regulation of the maturation of the diptericin promoter (Meister and Richards 1996) and is the opposite to that seen with *Sgs-3* (Georgel et al. 1991). Finally, we have studied the regulation of *hsp27* transcripts by culturing 90, 100 and 110 h larval glands and 6 h prepupal glands with 1.8×10^{-8} M or 1.8×10^{-6} M ecdysone and show that the response to hormone changes throughout this period.

Materials and methods

Wild-type Oregon-R larvae, maintained at 25°C, were selected at the second to third larval moult at 72 h after egg laying by size and anterior spiracle morphology. These larvae leave the food at approximately 112 h and pupariate in a 4–8 h period around 120 h. From the wandering stage onwards, larvae were staged by puff analysis of one salivary gland lobe. Prepupae were selected at pupariation as 0 h pp and aged as appropriate. After dissection, one salivary lobe was taken for cytological analysis whilst the other was frozen at –80°C in RNA extraction buffer (see Huet et al. 1993). If appropriate, further tissues were frozen in parallel. After puff staging, total RNA was extracted from the contralateral lobe (or other tissues) and aliquots were stocked at –80°C. 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 this restrictive temperature, animals eventually leave the food and then remain blocked in an extended wandering phase. The 2B mutant strains bearing the br^{t435}*[y l(1)*t^{435}*/FM6 l-69j/Dp(1)*y^2*Y67g]* or dor^{22} *[y l(1)*t^{187}*/FM6 l-69j/Dp(1)*y^2*Y67g]* mutations were kindly supplied by Igor Zhimulev of the Russian Academy of Sciences, Novosibirsk. Females from these stocks were crossed with Oregon-R males so as to obtain male larvae hemizygous for the 2B mutations which die as late larvae or prepupae. In these crosses the *FM6 l-69j* carrying males die before the third instar whereas both classes of females (i.e. carrying a wild-type X chromosome and either the *FM6 l-69j* chromosome or the 2B mutant chromosome) are viable and serve as internal controls. Further *BR-C* mutant strains were obtained either from Igor Zhimulev or Greg Guild (or both): br^5*[=l(1)*t^{35}*], rbp*^{5}*[=l(1)*t^{376}*]* and *l(1)2Bc*^{1} *[=l(1)*t^{10}*]*. These were either maintained as br^{t435}above or re-balanced with a *Binsn* X chromosome (Lindsley and Zimm 1992). In the latter case, mutant males were selected by their yellow mouthhooks.

For in vitro analyses, individual wild-type glands were cultured at 25°C in modified Grace's medium [Graces (GibCo)/distilled H_20/2.5% ethanol in the ratio 50/9/1] in a volume of 25 µl per lobe and 20-OH ecdysone (Simes, Milan) was dissolved in the ethanol fraction. Glands were cultured in glass depression slides with a grease-sealed coverslip to avoid dessication.

RT-PCR reactions were performed as described in Huet et al. (1993, 1995) using the equivalent of 1.6% of the RNA from a salivary gland. Products were separated on 2% agarose gels and, after transfer to nylon membranes, hybridised with an internal labelled oligonucleotide. For each time point at least four individual glands were first analysed for *rp49* transcripts (to monitor RNA extraction – data not shown) and for *E74* transcripts. Thereafter, a representative series was assayed for *hsp27* transcripts. The RT-PCR strategy for *hsp27* is shown in Fig. 1A. Unlike *E74A* and *E74B* where primers are located either side of a large intron, for the intronless *hsp27* we used a DNase I pretreatment to eliminate contaminating DNA (Huet et al. 1993). Four treatments were performed in parallel on a standard RNA preparation, included as a control in every RT-PCR experiment (Fig. 1B). The reproducibility of the assay has been illustrated and discussed in Huet et al. (1993; see also Fig. 1C). In general, we observed two- to three-fold variation between replicates for the same stage or treatment. The PCR reactions were in the linear range of amplification but certain autoradiographic exposures reached saturation as was to be expected when comparing transcript levels differing by at least 100-fold between time points (see Huet et al. 1993 for examples). The oligonucleotide primers for *hsp27* were GGGTATACTTGCGCACAAAG and TTACCACTGCTGCACTTGG and the

internal probe TGACGCTGGATCATTCCATG (derived from Southgate et al. 1983).

Results

To test the assumption that increases in *hsp27* transcripts depend on the increase in ecdysone titre characteristic of the end of the third larval instar, we used a micro RT-PCR assay (Fig. 1A and B) to study *hsp27* transcripts in RNA extracted from different tissues of individual larvae. We first analysed the level of these transcripts in salivary glands of four wandering stage larvae of the *ecd1* temperature-sensitive mutant (Garen et al. 1977) maintained at the permissive (20°C) or restrictive (29°C) temperature (Fig. 1C). As previously shown for *hsp26* (Thomas and Lengyel 1986), *hsp27* transcripts are significantly lower in those mutant larvae which are developmentally arrested at the wandering stage. We then investigated the effect on *hsp27* transcripts of the late larval increase in ecdysone levels using tissues from four wild-type larvae staged by their polytene chromosome puffing patterns between PS1 (prior to this ecdysone increase) and PS11 (puparium formation). As can be seen in Fig. 2A, transcript levels are similar in the salivary glands, fat body, wing discs and gut, and show no evidence of ecdysone regulation in this period. Thus, contrary to our initial expectation, *hsp27* appears fully induced in late PS1 animals. This is in contrast to *E74A* transcripts which increase from PS2 in the same salivary glands (see Fig. 3) and in other tissues of these same animals (see Huet et al. 1993 – their Fig. 7). To determine whether incubation with ecdysone would alter transcript levels in PS1 glands, we incubated 110-h glands with 1.8×10^{-6} M ecdysone. In both short and long term culture we did not detect a significant induction of transcript levels, indeed at 6 h transcript levels started to decline (Fig. 2B).

We then used a more extensive set of staged RNAs to follow *hsp27* transcripts in salivary glands from 86 h of development until gland histolysis at 132–4 h (12–14 h prepupae). The results are presented in Fig. 3 in direct comparison with those for *E74A* and *E74B* from the same RNAs. In the mid to late larval period, the *E74B* transcript is present and increases from 86 h onwards. *hsp27* transcripts increase rapidly from 90 h to peak by 94 h (see also Fig. 5A below). This precedes the appearance of the salivary gland specific *Sgs-3* transcripts at 102 h and the induction of *E74A* from 114 h onwards (see Fig. 4). By 114 h, both *E74B* and *hsp27* transcript levels are maximal. Whilst *E74B* transcripts decline slowly from PS2 onwards, *hsp27* transcripts only decline significantly from PS11 onwards in parallel with *E74A*. This decrease may be simply due to a decrease in both ecdysone and receptor levels (see Huet et al. 1995 for discussion) or may be an ecdysone-dependant late event of this response. *E74A*, *E74B* and *hsp27* transcripts are minimal in young (1–6 h) prepupae. In late prepupae, *E74B* induction from 6 h precedes that of both *E74A* and

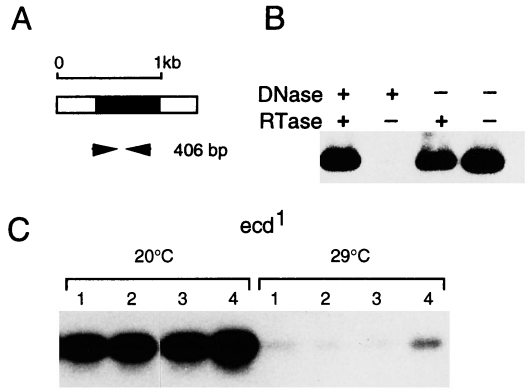

Fig. 1A–C Reverse transcriptase-polymerase chain reaction (RT-PCR) assays and the expression of the heat-shock gene *hsp27* in *ecd1* larvae (mutant for ecdysone production). **A** The location of the reverse transcription and PCR primers (*arrows*) which give rise to a 406-base pair product. The *hsp27* transcription unit is shown as a *box section* with the translated portion shown as a *solid box section*. **B** Elimination of contaminating DNA from RNA samples. Extracts were treated with or without DNase to remove contaminating genomic DNA prior to reverse transcription (*RTase*) and subsequent PCR amplification of the cDNA products. **C** *hsp27* transcripts analysed by RT-PCR in salivary glands from four individual wandering stage *ecd1* larvae (1–4) maintained at the permissive temperature (20°C) or transferred to the restrictive temperature (29°C) at the beginning of the third larval instar. The two- to three-fold variation in transcript levels between larvae maintained at 20°C is typical for the assay (see Materials and methods)

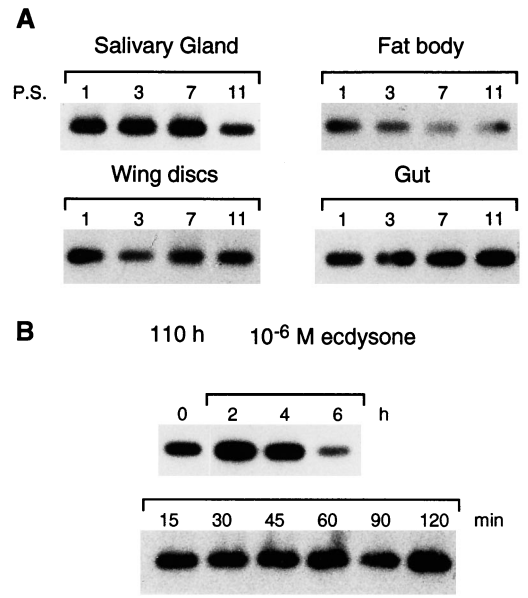

Fig. 2 **A** *hsp27* transcripts in tissues from four late instar larvae staged by the puffing patterns of one salivary gland between PS1 (stage prior to ecdysone increase) and PS11 (puparium formation). Tissues were salivary glands, fat body, wing discs and gut. **B** Individual salivary glands from 110 h; wandering stage wild-type larvae were cultured with 1.8×10^{-6} M ecdysone for long (0 to 6 h) or short (15 to 120 min) term incubations as shown

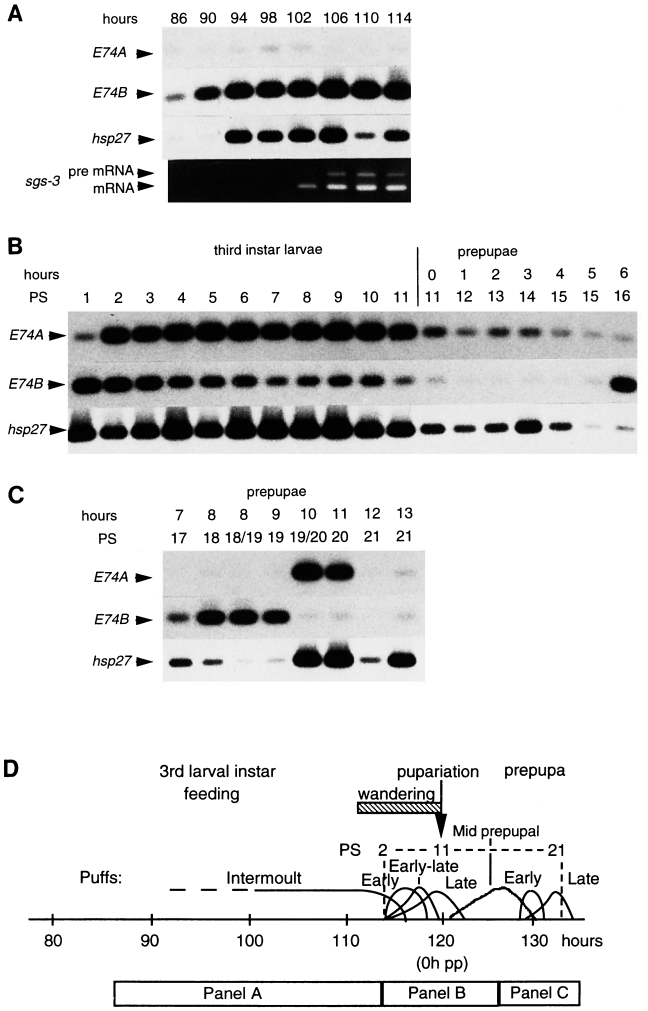

Fig. 3A–C *hsp27* transcripts in individual salivary glands of larvae between 86 and 114 h (**A**), late instar larvae and prepupae puff staged from PS1 to PS16 (approximately from 6 h before to 6 h after puparation – PS11; **B**), late prepupae, 7 to 13 h after puparation (**C**). In each case the *E74A* and *E74B* transcripts detected in the same gland are shown, while for the first period, *Sgs3* transcripts are also shown. Note that the low *hsp27* point at 110 h is probably an artefact as it is not seen in other glands of this age. **D** The major developmental and classical puffing events occurring during the three periods studied in **A** to **C**

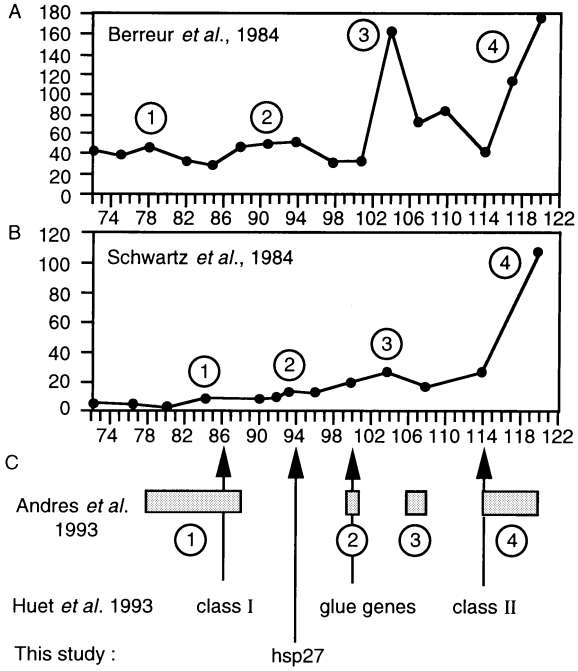

Fig. 4A–C Correlations between peaks in ecdysone titre and transcriptional events in third instar larvae. This figure is adapted from Andres et al. (1993 – their Fig. 7). Fluctuations in hormone titre (pg ecdysteroid equivalents/animal) reported by Berreur et al. (1984; **A**) and Schwartz et al. (1984; **B**) are shown above the gene activations detected by Andres et al. (1993), Huet et al. (1993) and the present study (**C**). Andres et al. (1993) drew parallels between possible hormone peaks (*circled numbers*) from titre studies and the periods (*bars*) known to be important for ecdysone-induction of different classes of genes. *Arrows* mark the time of induction of the different classes in our studies. The inherent difficulty of determining hormone titres in this period is discussed in Richards (1981) and Huet et al. (1993)

hsp27 which are induced in parallel from 10 h onwards during PS19/20 and 20. In this series, both appear to decline at 12 h and then increase at 13 h.

We investigated the role of ecdysone in larval induction by culturing salivary glands from 90- and 100-h larvae with 1.8×10^{-8} M or 1.8×10^{-6} M ecdysone (Fig. 5A). These concentrations are thought to correspond to levels characteristic of the second half of the third larval instar and the puparation peak respectively. At 90 h, non-incubated glands showed no or low levels of transcripts. When such glands were cultured in the absence of hormone, we detected very low levels of transcripts on extended autoradiographic exposure (data not shown). With 1.8×10^{-8} M ecdysone, we observed the maintenance of, or a modest increase in, *hsp27* transcripts, whilst incuba-

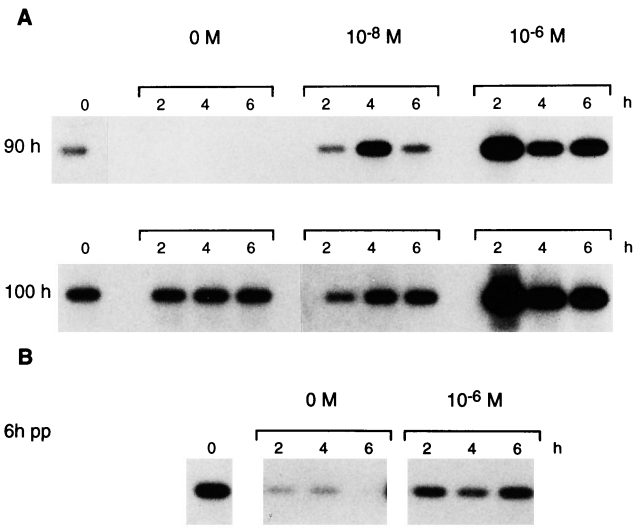

Fig. 5A, B *hsp27* transcripts in individual salivary glands from 90- and 100-h wild-type larvae cultured with 0, 1.8×10^{-8} M or 1.8×10^{-6} M ecdysone for 0–6 h (**A**), and individual salivary glands from 6-h prepupae cultured with 0 or 1.8×10^{-6} M ecdysone for 0–6 h (**B**)

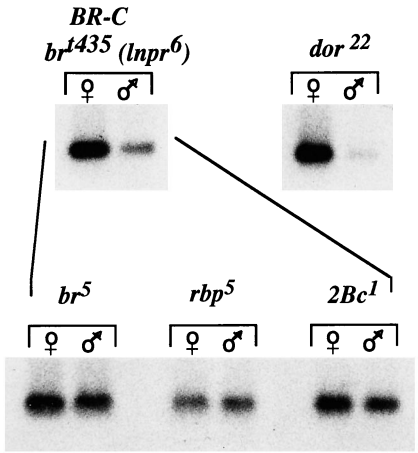

Fig. 6 *hsp27* transcripts in female (control) and mutant males from the strains br^{t435} and dor^{22} (*deep orange*). As br^{t435} is a long non-complementing *Broad-Complex* (*BR-C)* allele, we examined sub-functions with the br^5, $rbp^5$and $l(1)2Bc^1$ strains. For each sample, individual RT-PCR reactions were performed on five animals as for *ecd1* in Fig. 1 (data not shown). Thereafter, 2-µl aliquots from each reaction were pooled to give a total loading sample of 10 µl per lane as for all preceeding figures. Several different strains carrying br^5 or rbp^5were tested and gave essentially the same result (data not shown)

tion with 1.8×10^{-6} M ecdysone led to higher levels of transcripts (Fig. 5A). At 100 h, as expected, there were relatively abundant *hsp27* transcripts in the non-incubated glands. These levels were maintained if glands were cultured in 1.8×10^{-8} M ecdysone or in the absence of hormone, whilst with 1.8×10^{-6} M ecdysone they increased to levels similar to those seen in 114-h glands.

As ecdysone inductions occur from 6 h onwards in prepupae (see *E74B* in Fig. 3B and C) we investigated the effect of ecdysone on 6-h prepupal glands (Fig. 5B). In the presence of 1.8×10^{-6} M ecdysone *hsp27* transcript levels were maintained, whilst in the absence of hormone the transcripts diminished by 6 h. This is in contrast to both *E74A* and *E74B* transcripts which were induced at least fifty-fold by the ecdysone treatment of 6-h prepupal glands (Huet 1995).

In addition to *ecd1*, *BR-C* and the neighbouring *dor* mutant are known to be necessary for larval responses to ecdysone. When males hemizygous for a mutant were compared with their female siblings, we observed that *hsp27* expression was moderately reduced in br^{t435}, whilst expression was considerably lower in dor^{22} males (Fig. 6). As br^{t435} is a long non-complementing allele of *BR-C,* we used the br^5, rbp^5 and $l(1)2Bc^1$ mutants to attempt to localise this effect to one of the genetic subfunctions of *BR-C*. The results (Fig. 6 and data not shown) showed that the effect is not localised to any one of these three subfunctions as there is little or no diminution of *hsp27* transcripts in mutant males from these strains.

Discussion

The observation that *hsp27* transcripts are ecdysone-inducible in *Drosophila* S3 cells (Ireland et al. 1982; Vitek and Berger 1984) was of fundamental importance for the later isolation of an EcRE (Riddihough and Pelham 1987). However, whilst Ireland et al. (1982) observed a 15-fold induction which peaked after the first 8 h, Vitek and Berger (1984) found a 4-fold induction essentially occurring between 18 and 24 h of treatment. More recently, Amin et al. (1991) showed a steady accumulation of transcripts from 2 to 24 h and concluded that the endogenous promoter contains both primary and secondary hormone-responsive elements. Similarly, when the *hsp27* EcRE is used to drive gene expression in transfection studies, although this is considered a primary response as it is largely insensitive to protein synthesis inhibition, the characteristics of the response are poorly defined as the cells are usually treated with hormone for at least 24 h before analysis (see for example, Koelle et al. 1991).

Here, we show that in larval development, the major induction of *hsp27* transcripts occurs well before the classic primary hormonal induction in late larval salivary glands of early puffs, such as 74EF and 75B, which harbour transcripts that increase at least 100-fold by 2 h of treatment (Huet et al. 1993, 1995 for examples). For *hsp27*, the response changes between 90 and 110 h. Whilst 1.8×10^{-8} M ecdysone increases transcript levels at 90 h, by 100 h this concentration of ecdysone no longer affects levels. Equally, the final levels seen with 1.8×10^{-6} M ecdysone treatments increase from 90 to 100 h to reach the maximal levels characteristic of 110-h wandering larvae. Note however, that in vivo the glands would not experience 10^{-6} M ecdysone before 110 h. In glands from 110-h larvae, whilst there may be a modest induction similar to that seen by Ireland et al. (1982) in mass isolated discs, the only clear effect of culture with ecdysone is a reduction by 6 h perhaps reflecting a late ecdysone-induced event, as a similar decrease is seen in PS11 glands in vivo (Fig. 3). Thus, although *hsp27* is ecdysone-inducible at 90 h, this is not a simple trigger response as transcript levels remain ecdysone-sensitive for some 20 h. It is possible that there exists a combination of induction and maintenance phenomena in this period and that if transcription is initiated when titres reach a certain threshold (see Fig. 4), their accumulation relies both on the continued presence of hormone and a gradual increase in titre. By the prepupal stage there are clear changes in the response to ecdysone as, in 6-h prepupae, hormone maintains rather than induces *hsp27* transcript levels. Unlike *E74A*, which is inducible in these glands (Huet 1995), *hsp27* induction from 8 h onwards may require further factors that are not present in the 6-h prepupal glands.

The result with the *ecd1* strain would be coherent with a low residual ecdysone titre at the restrictive temperature which would allow a low level of *hsp27* transcription. In this respect it should be noted that, for *hsp27*, the mutant larvae are arrested at 90 h of salivary

gland development although from their wandering stage behaviour they are normally assimilated with 110-h wild-type animals. In consequence, the interpretation of ecdysone treatments of such larvae is problematic. Expression in the *dor*^{22} males is comparable to that in the *ecd1* strain suggesting a similar developmental blockage, whilst the intermediate levels in *br*^{t435} males suggests that the ecdysone response starts normally. These results with *br*^{t435} and *dor*^{22} resemble those seen with the diptericin promoter (Meister and Richards 1996) and are quite distinct from those seen with *Sgs-3*, where the *br*^{t435} mutant abolishes expression whilst *dor*^{22} has little effect (Georgel et al. 1991). Thus, although caution is necessary in the interpretation of the mutant studies they do provide a means of assessing the independance or overlap of the underlying regulatory hierarchies.

We were unable to localise the *br*^{t435} effect to a subfunction of the *BR-C* by using *br*^{5}, *rbp*^{5}and *l(1)2Bc*^{1} mutants (see Fletcher and Thummel 1995 for a recent discussion of genetic subfunctions of the *Broad-Complex*). This contrasts with mid instar glue gene induction (Guay and Guild 1991) and the later *E74A* induction (Karim et al. 1993) where the *rbp* and *l(1)2Bc* functions respectively are critical and suggests a distinct mode of *BR-C*-dependant regulation for *hsp27* in mid instar glands. Similar results have been obtained recently with an *hsp2*3 fusion gene where a marked reduction of expression was observed with long non-complementing alleles, but little or no effect was seen for these same subfunctions in the salivary gland (E. Dubrovsky, G. Dretzen and E.Berger, personal communication). Equally, Karim et al. (1993) and D'Avino et al. (1995) showed that these same three alleles had little or no effect on late larval glue gene transcript levels when looking at transcripts in wandering larvae 3–4 h prior to pupariation. Thus, it is proving difficult to assign these effects to a single subfunction and may reflect a problem of a partial functional redundancy between the different *BR-C* protein isoforms associated with the subfunctions (see Fletcher and Thummel 1995).

In conclusion, the mere presence of the canonic EcRE in a promoter does not enable one to predict the response to hormone in vivo. The *hsp27* response to ecdysone is modulated throughout development, possibly by changes in both receptor components and gene-specific transcription factors that alter the competence of the EcRE to respond to the hormone-receptor complex. Indeed, as shown recently by Antoniewski et al. (1996), the exact nature of the response element may be less critical for correct developmental expression than the regulatory sequences flanking the EcRE. When they substituted the natural palindromic EcRE of the Fbp1 enhancer by synthetic direct repeat elements, they found that a strong fat body specific response was still obtained in transgenic flies. It is unlikely that these conclusions are restricted to the ecdysone response in insects and our understanding of the hormonal regulation of animal development requires the integration of results obtained in vitro with those from similar detailed developmental studies.

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References

Amin J, Mestril R, Voellmy R (1991) Genes for *Drosophila* small heat shock proteins are regulated differently by ecdysterone. Mol Cell Biol 11: 5937–5944

Andres AJ, Fletcher JC, Karim FD, Thummel CS (1993) Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid regulated transcription. Dev Biol 160: 388–404

Antoniewski C, Laval M, Lepesant J-A (1993) Structural features critical to the activity of an ecdysone receptor binding site. Insect Biochem Mol Biol 23: 105–114

Antoniewski, C, Mugat, B, Delbac, F, Lepesant J-A (1996) Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster*. Mol Cell Biol 16: 2977–2986

Berreur P, Porcheron P, Moriniere M, Berreur-Bonnenfant J, Belinksi-Deutsch S, Busson D, Lamour-Audit C (1984) Ecdysteroids during the larval instar in *l(3)ecd-1*^{ts}, a temperature-sensitive mutant of *Drosophila melanogaster*. Gen Comp Endocrinol 54: 76–84

Cherbas L, Lee K, Cherbas P (1991) Identification of ecdysone response elements by the analysis of the *Drosophila Eip 28/29* gene. Genes Dev 5: 120–13

D'Avino PP, Crispi S, Polito LC, Furia M (1995) The role of the *BR-C* locus on the expression of genes located at the ecdysone-regulated 3C puff of *Drosophila melanogaster*. Mech Dev 49: 161–171

Dubrovsky EB, Dretzen G, Bellard M (1994) The *Drosophila Broad-Complex* regulates developmental changes in transcription and chromatin structure of the 67B heat-shock gene cluster. J Mol Biol 241: 353–362

Fletcher JC, Thummel CS (1995) The ecdysone-inducible *Broad-Complex* and *E74* early genes interact to regulate target gene transcription and *Drosophila* metamorphosis. Genetics 141: 1025–1035

Garen A, Kauvar L, Lepesant J-A (1977) Roles of ecdysone in *Drosophila* development. Proc Natl Acad Sci USA 74: 5099–5103

Georgel P, Ramain P, Giangrande A, Dretzen G, Richards G, Bellard M (1991) *Sgs*-3 chromatin structure and trans-activators: developmental and ecdysone induction of a Glue Enhancer Binding Factor, GEBF-I, in *Drosophila* larvae. Mol Cell Biol 11: 523–532

Guay PS, Guild GM (1991) The ecdysone-induced puffing cascade in *Drosophila* salivary glands: a *Broad-Complex* early gene regulates intermolt and late gene transcription. Genetics 129: 169–175

Huet F (1995) Caracterisation des activations séquentielles de gènes par l'ecdysone au cours du développement de *Drosophila melanogaster.* Ph.D. thesis, Université Louis Pasteur, Strasbourg

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

Huet F, Ruiz C, Richards G (1995) Sequential gene activation by ecdysone in *Drosophila melanogaster:* the hierarchical equivalence of early and early-late genes. Development 121: 1195–1204

Ireland RC, Berger E, Sirotkin K, Yund MA, Osterbur D, Fristrom J (1982) Ecdysterone induces the transcription of four heat-

shock genes in *Drosophila* S3 cells and imaginal discs. Dev Biol 93: 498–507

Karim FD, Thummel CS (1992) Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. EMBO J 11: 4083–4093

Karim FD, Guild GM, Thummel CS (1993) The *Drosophila Broad-Complex* plays a key role in controlling ecdysone-regulated gene expression at the onset of metamorphosis. Development 118: 977–988

Koelle MR (1992) Molecular analysis of the *Drosophila* ecdysone receptor complex. Ph.D. thesis, Stanford University, Stanford, California

Koelle MR, Talbot WS, Segraves WA, Bender MT, Cherbas P, Hogness DS (1991) The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 67: 59–77

Lindsley DL, Zimm GG (1992) The Genome of *Drosophila melanogaster*. Academic Press, San Diego

Meister M, Richards G (1996) Ecdysone and insect immunity: the maturation of the inducibility of the diptericin gene in *Drosophila* larvae. Insect Biochem Mol Biol 26: 155–160

Ozyhar A, Strangmann-Diekmann M, Kiltz H-H, Pongs O (1991) Characterisation of a specific ecdysteroid receptor-DNA complex reveals common properties for invertebrate and vertebrate hormone-receptor/DNA interactions. Eur J Biochem 200: 329–335

Pfahl M (1994) Vertebrate receptors: molecular biology, dimerization and response elements. Semin Cell Biol 5: 95–103

Richards G (1981) The radioimmune assay of ecdysteroid titres in *Drosophila melanogaster.* Mol Cell Endocrinol 21: 181–197

Riddihough G, Pelham HRB (1987) An ecdysone response element in the *Drosophila hsp27* promoter. EMBO J 12: 3729–3734

Schwartz MB, Imberski RB, Kelly TJ (1984) Analysis of metamorphosis in *Drosophila melanogaster:* characterisation of *giant*, an ecdysteroid-deficient mutant. Dev Biol 103: 85–95

Southgate R, Ayme A, Voellmy R (1983) Nucleotide sequence analysis of the *Drosophila* small heat shock gene cluster at locus 67B. J Mol Biol 165: 35–57

Talbot WS, Swyryd EA, Hogness DS (1993) *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73: 1323–1337

Thomas SR, Lengyel JA (1986) Ecdysteroid-regulated heat shock gene expression during *Drosophila melanogaster* development. Dev Biol 115: 434–438

Vitek MP, Berger E (1984) Steroid and high-temperature induction of the small heat-shock protein genes in *Drosophila*. J Mol Biol 178: 173–189

Yao T-P, Segraves WA, Oro AE, McKeown M, Evans RM (1992) *Drosophila ultraspiracle* modulates ecdysone receptor function via heterodimer formation. Cell 71: 63–72