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Ecdysone regulation of the *Drosophila Sgs-4* gene is mediated by the synergistic action of ecdysone receptor and SEBP 3

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The steroid hormone 20-hydroxyecdysone controls both induction and repression of the *Drosophila* 'intermolt gene' *Sgs-4*. We show here that the ecdysone receptor binds to two sites, element I and element II, in the regulatory region of *Sgs-4*. A functional analysis revealed that element II appears to be of no importance for *Sgs-4* expression, while element I proved to be an ecdysone response element that is necessary, but not sufficient, for induction of *Sgs-4* expression. Our results provide no evidence that repression of *Sgs-4* expression is mediated by one of the two receptor binding sites. In the close vicinity of elements I and II, we detected two binding sites of secretion enhancer binding protein 3 (SEBP 3). Like receptor element I, one of these sites also proved to be necessary, but not sufficient, for expression of *Sgs-4*. Therefore, induction of *Sgs-4* requires binding of both ecdysone receptor and SEBP 3 to a complex hormone response unit, which also contains binding sites for a third factor, SEBP 2. The SEBP 2 sites coincide with binding sites of products of the *Broad-Complex* locus, which has been implicated recently with transduction of the hormonal signal. Thus, the available data suggest that induction of *Sgs-4*, and possibly other 'intermolt genes', is a combination of a primary and a secondary response to the hormone.

Key words: *Broad-Complex*/ecdysone receptor/hormone response unit/SEBP/*Sgs-4*

Introduction

The polytene chromosomes of insects, by the formation of puffs at transcriptionally active loci, offer the opportunity to establish the expression status of whole gene networks by a simple cytogenetic analysis (reviewed in Ashburner and Berendes, 1978; Korge, 1987). The discovery that the steroid hormone 20-hydroxyecdysone (hereafter referred to as ecdysone) is able to induce the formation and regression of puffs showed that the primary effect of steroid hormones is to alter the activity of specific genes (Clever and Karlson, 1960; Clever, 1964). Since then, it has been well established that the complex developmental and physiological responses to steroid hormones require the formation of a hormone receptor complex which binds to hormone-responsive sequences in the vicinity of target genes, thereby regulating the transcription of these genes (reviewed in Evans, 1988; Green and Chambon, 1988; Beato, 1989; Lucas and Granner, 1992).

In an extensive study on ecdysone-induced puffing in *Drosophila* salivary glands, Ashburner and his colleagues (Ashburner, 1972, 1973; Ashburner *et al.*, 1974; Ashburner and Richards, 1976) found that ecdysone exerts different kinds of control on a sequence of puffing patterns that marks the larval to prepupal transition (for a review see Andres and Thummel, 1992). Thus, a small group of 'early puffs' is very rapidly induced by ecdysone shortly before puparium formation. The genes residing in three of these puffs, *E74*, *E75* and *Broad-Complex* (*BR-C*), have been cloned and their products identified as DNA binding proteins (Burtis *et al.*, 1990; Segraves and Hogness, 1990; DiBello *et al.*, 1991). Another group of puffs, the 'intermolt puffs', is active throughout most of the third larval instar and regresses at about the time when the 'early puffs' are induced. Several of these puffs contain genes, designated *Sgs* (salivary gland secretion protein) genes, whose products form a glue (Korge, 1975, 1977; Beckendorf and Kafatos, 1976) that serves to attach the pupa to a solid surface (Fraenkel and Brookes, 1953). The 'intermolt puffs' and their genes differ in their response to ecdysone, and they depend to different extents upon the 'early gene' *BR-C*. This is exemplified by the puff at 68C which harbours a cluster of three *Sgs* genes (*Sgs*-3, *Sgs*-7 and *Sgs*-8), and the puff at 3C11-12 which contains the *Sgs-4* gene. In ecdysone-deficient larvae both puffs are properly formed, but surprisingly neither *Sgs-4* nor the 68C genes are transcribed (Hansson *et al.*, 1981; Hansson and Lambertsson, 1983, 1989). Larvae lacking the wild-type *BR-C* function do not produce any 68C transcripts, but they do show expression of the *Sgs-4* gene (Crowley *et al.*, 1984). However, mutations of two subfunctions of the *BR-C*, rbp^+ and $2Bc^+$, reduce the amount of *Sgs-4* transcripts, or at least cause a delay in the appearance of transcripts (Guay and Guild, 1991; Karim *et al.*, 1993; von Kalm *et al.*, 1994). This effect of the *BR-C* on *Sgs-4* expression has been correlated with a direct binding of *BR-C* products to regulatory sequences flanking the *Sgs-4* gene (von Kalm *et al.*, 1994).

Interestingly, the response of 'intermolt puffs' and genes to ecdysone and *BR-C* products changes towards the end of the third larval instar. Thus, regression of the 68C puff in culture is accelerated on induction by ecdysone (Ashburner, 1973), and the puff fails to regress in *BR-C* mutants (Belyaeva *et al.*, 1981; Zhimulev *et al.*, 1982; Crowley *et al.*, 1984). The latter observation and the sensitivity of ecdysone-dependent puff regression to inhibitors of protein synthesis (Ashburner, 1974) point to an indirect nature of this late third instar response. However, studies at the transcript level have shown that ecdysone very rapidly reduces the accumulation rate of 68C puff mRNAs, suggesting a direct interference of the hormone with mRNA production (Crowley and Meyerowitz, 1984). In contrast to the 68C puff, regression of the *Sgs-4*

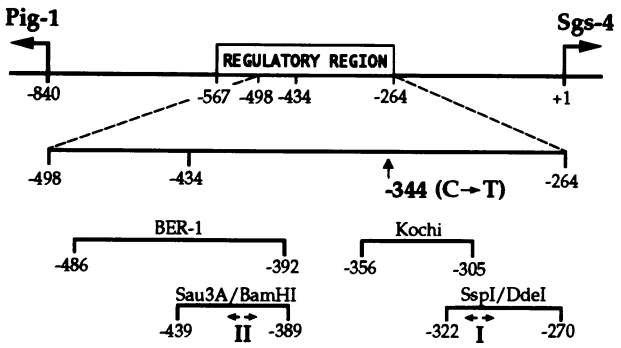

Fig. 1. Arrangement of regulatory important sequences in the intergenic region of *Sgs-4* and *Pig-1*. The regulatory region from −264 to −567 is sufficient to direct stage- and tissue-specific activation of the *Sgs-4* and *Pig-1* promoters (Jongens *et al.*, 1988; Mougneau *et al.*, 1993). The deletions mapped in the non-producer strains BER-1 and Kochi are responsible for the production of no or only minute amounts of *Sgs-4* mRNA in these strains (Muskavitch and Hogness, 1980, 1982). Restriction fragments *Sau*3A−*Bam*HI and *Ssp*I−*Dde*I proved to be potent competitors of EcR binding to the *hsp 27* EcRE. The locations of the half-palindromes of EcR elements I and II are given by horizontal arrows. The vertical arrow marks the position of the C to T transition in the strain Samarkand which leads to reduced expression of *Sgs-4* and dosage effect (Hofmann and Korge, 1987; Hofmann *et al.*, 1987).

puff at 3C11-12 appears to be independent of ecdysone (Ashburner, 1972, 1973) and *BR-C* products (Belyaeva *et al.*, 1981). At the transcript level, however, *Sgs-4* seems to respond in a manner similar to the 68C genes (Hansson and Lambertsson, 1989).

In this study we focus on the question of whether the regulation of the *Sgs-4* gene by ecdysone involves a direct binding of the ecdysone receptor complex to regulatory sequences of the gene. *Sgs-4* shares a common upstream region with *Pig-1*, a gene of unknown function which is transcribed in the opposite direction (Figure 1). Expression of both genes is restricted to salivary glands but the temporal patterns of expression differ, switching from *Pig-1* transcription to *Sgs-4* transcription in mid-third instar larvae (Hofmann and Korge, 1987; Andres *et al.*, 1993). A regulatory region shared by both genes contains an enhancer element (−264 to −434) which is by itself sufficient to specify the correct pattern of *Sgs-4* expression. A second element within this region (−498 to −567) is held responsible for the transcriptional switch between the *Pig-1* and the *Sgs-4* promoters (Jongens *et al.*, 1988; Mougneau *et al.*, 1993).

A major problem facing a molecular analysis of the divergent effects of ecdysone on the transcription of *Sgs-4* and other 'intermolt genes' was, until recently, the inaccessibility of the ecdysone receptor. This inaccessibility proved to be largely due to the fact that the formation of a heterodimer between two members of the steroid receptor superfamily, the product of the *EcR* gene (EcR; Koelle *et al.*, 1991) and the product of the *ultraspiracle* gene (USP; Henrich *et al.*, 1990; Oro *et al.*, 1990; Shea *et al.*, 1990), is required to generate a functional 'ecdysone receptor' (Yao *et al.*, 1992, 1993; Thomas *et al.*, 1993). Moreover, the EcR can occur in three isoforms, EcR-A, EcR-B1 and EcR-B2, which have common DNA and hormone binding domains but different N-terminal regions (Talbot *et al.*, 1993).

Here, we demonstrate that one of the 'intermolt genes', *Sgs-4*, is directly activated by binding of the ecdysone receptor complex to a single, comparatively weak, ecdysone response element (EcRE) which is part of a more complex ecdysone response unit. Hormonal induction requires binding of both the ecdysone receptor and at least one additional transcription factor, secretion enhancer binding protein 3 (SEBP 3), to this response unit. Furthermore, we provide evidence that direct binding of the ecdysone receptor complex to a single putative repressor element is not sufficient to mediate the repressive effect of ecdysone on *Sgs-4* transcript levels at the end of the third larval instar.

Results

EcR from third instar salivary glands binds to the hsp 27 EcRE

To assess the receptor status of salivary glands from third instar larvae and to devise an assay for the detection of EcR binding sites in the *Sgs-4* upstream region, we analysed the binding of nuclear proteins from salivary glands to the *Drosophila hsp 27* EcRE. The *hsp 27* EcRE is a 13 bp sequence which is sufficient to confer hormone inducibility on a heterologous promoter (Riddihough and Pelham, 1987; Cherbas *et al.*, 1991) and is strongly bound by EcR/USP from *Drosophila* S2 cells or embryos (Koelle *et al.*, 1991; Yao *et al.*, 1992). Nuclear extract from embryos forms two specific complexes, C1 and C2, with the *hsp 27* EcRE which migrate with a slightly different mobility, resulting in the formation of a single broad band (Figure 2A). On shorter exposures this band is clearly resolved into two complexes (results not shown). The mobility of both complexes is retarded by the monoclonal antibody AG 10.2 which recognizes all three isoforms of the EcR (Talbot *et al.*, 1993), while unrelated monoclonal antibodies have no effect. Nuclear extract from salivary glands also forms complex C1, but instead of complex C2 a complex of very low mobility, C3, is formed which is also supershifted by the EcR antibody. Establishing the EcR isoform composition of complexes C1, C2 and C3 will require the use of isoform-specific antibodies. However, available data about the size and tissue distribution of EcR isoforms (Talbot *et al.*, 1993) give support to the idea that complex C1 is composed of EcR-A and EcR-B1, while complex C2 is formed by EcR-B2. The size of complex C3 suggests that it is a multimeric complex that might contain additional factor(s) beside EcR and USP (see below).

The regulatory region of Sgs-4 contains two binding sites for the EcR

To identify EcR binding sites in the *Sgs-4* upstream region, we used a mobility shift competition assay. Restriction fragments and synthetic oligonucleotides which cover the *Sgs-4* upstream region from −1 to −613 were tested for their ability to compete with the *hsp 27* EcRE for binding of the EcR from third instar salivary glands. This screening procedure enabled us to identify two short restriction fragments extending from −270 to −322 (Figure 1, *Ssp*I−*Dde*I fragment) and from −389 to −439 (Figure 1, *Sau*3A−*Bam*HI fragment). Both fragments are located within the enhancer element of the *Sgs-4* regulatory region

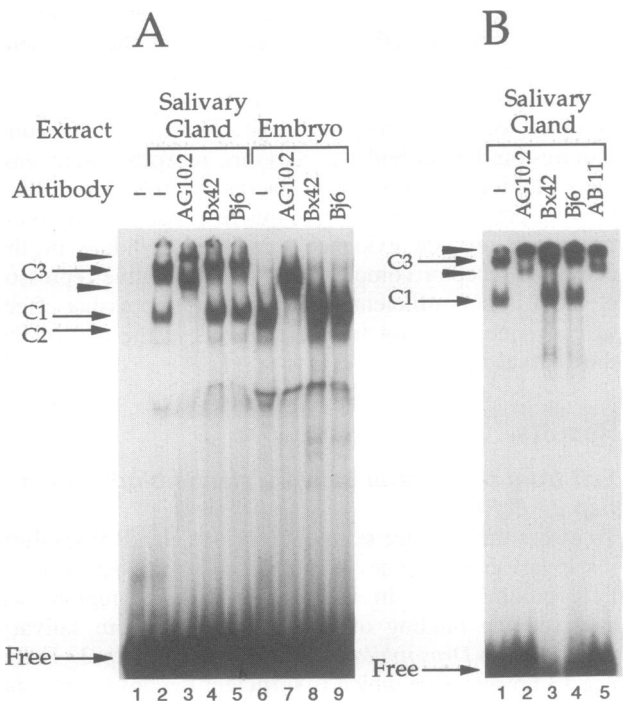

Fig. 2. Binding of the ecdysone receptor to the *hsp 27* EcRE and EcR element I of *Sgs-4*. Radiolabelled oligonucleotide probes containing (**A**) the EcRE of *hsp 27* or (**B**) EcR element I of *Sgs-4* were equilibrated with nuclear extract from embryos or salivary glands in the absence or presence of the indicated monoclonal antibodies. AG 10.2 is an EcR antibody (kindly provided by W.S.Talbot), AB 11 (kindly provided by D.L.King) is an antibody directed against USP, and Bx42 and Bj6 were used as monoclonal control antibodies (kindly provided by H.Saumweber). The mixture was then separated in a mobility shift gel. C1, C2 and C3 are specific protein–DNA complexes formed with nuclear extract from salivary glands and/or embryos. Complexes supershifted by the EcR or USP antibody are marked with arrowheads. Free, unbound probe.

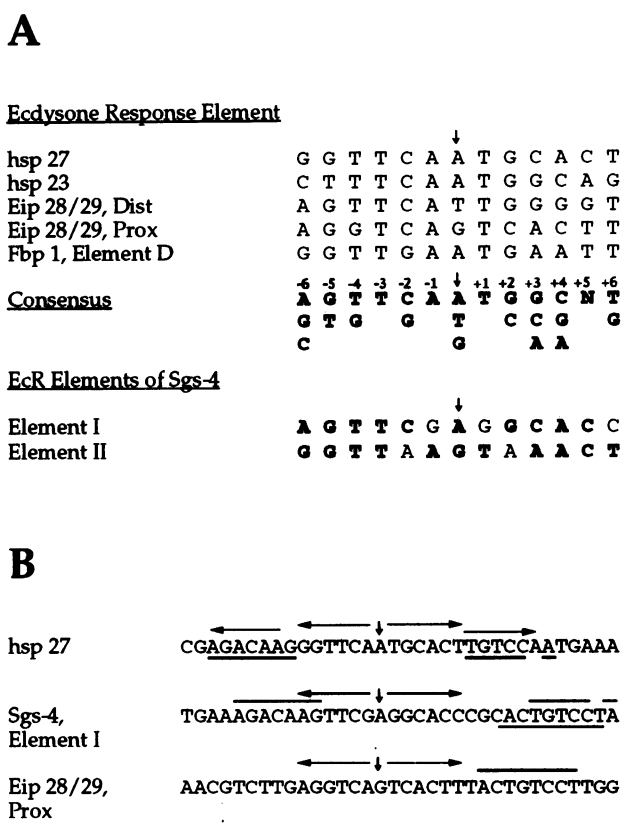

Fig. 3. Comparison of the EcR elements I and II of *Sgs-4* with EcREs of other *Drosophila* genes. (**A**) EcR elements I and II are compared with a consensus sequence derived from five palindromic sequences that have been shown previously to act as ecdysone response elements (*hsp 27*: Riddihough and Pelham, 1987; Cherbas *et al.*, 1991; Martinez *et al.*, 1991; *hsp 23*: Luo *et al.*, 1991; *Eip 28/29*: Cherbas *et al.*, 1991; *Fbp 1*: Laval *et al.*, 1993). Positions coinciding with the consensus sequence are marked by bold letters. (**B**) Flanking sequences of EcR element I of *Sgs-4* are compared with flanking sequences of the *hsp 27* EcRE and the proximal element of the *Eip 28/29* gene. Inverted repeated motifs in the *hsp 27* sequence are indicated by arrows. Stretches of sequence identity on both sides of the central palindromic sequence are pinpointed by thick bars.

(see Introduction). A close inspection of the nucleotide sequence of the competing fragments revealed that both fragments contained imperfect palindromic sequences with homology to the *hsp 27* EcRE and other EcREs (Figure 3A). The palindromic sequence within the *Ssp*I–*Dde*I fragment (EcR element I, −296 to −308) matches in 10 out of 13 positions with the consensus sequence and shows highest similarity to the *hsp 27* EcRE. The palindromic sequence within the *Sau*3A–*Bam*HI fragment (EcR element II, −403 to −415) matches in 11 out of 13 positions with the consensus and shows highest homology to both the *hsp 27* and the *Fbp 1* sequences. Interestingly, the homology of EcR element I with the *hsp 27* sequence and the proximal element of the *Eip 28/29* gene conspicuously extends beyond the 13 bp palindromic sequence (Figure 3B), supporting the idea that conserved flanking sequences might be involved in the function of a specialized class of EcR binding sites. As noted by Martinez *et al.* (1991) for the *hsp 27* sequence, these conserved elements are very similar to half-palindrome sequences that form response elements for members of the glucocorticoid and oestrogen subfamilies of nuclear receptors.

Oligonucleotides bearing EcR elements I and II of *Sgs-4* proved to be potent competitors of EcR binding to the *hsp 27* EcRE, indicating that the palindromic sequences are indeed responsible for receptor binding. EcR binding site I has a higher affinity for the EcR than site II, and both sites bind the receptor with a clearly lower affinity than the *hsp 27* EcRE (Figure 4, lanes 2–5 and lanes 11–14). A radiolabelled oligonucleotide probe containing EcR element I forms the same EcR–DNA complexes with nuclear extract from salivary glands as the *hsp 27* probe (Figure 2B). Complexes C1 and C3 are supershifted not only by the EcR antibody but also by USP antibody AB11 (Khoury Christianson *et al.*, 1992), indicating that also the USP protein contributes to the formation of both complexes.

We tested the effects of various point mutations (Figure 6A) on the binding ability of EcR elements I and II. Insertion of one base pair 3′ to the spacer of the palindromic sequence of EcR element II [Figure 4, mutation E(II)1In] or four base substitutions in the right half of the palindrome [mutation E(II)4T] lead to a complete loss of the ability to compete for EcR binding. In both cases even a 100-fold molar excess of mutated oligonucleotide was not sufficient to cause a significant decrease of EcR binding to the *hsp 27* probe. The clear effects of mutations

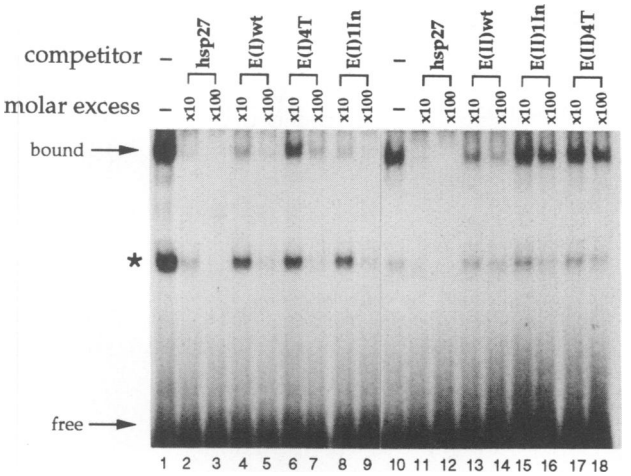

Fig. 4. Specific competition of EcR binding to the *hsp 27* EcRE by wild-type and mutated *Sgs-4* sequences. A radiolabelled hsp 27 oligonucleotide probe was equilibrated with nuclear extract from salivary glands in the absence or presence of unlabelled competitor oligonucleotides. The following oligonucleotides were used as competitors at the indicated molar excess: hsp 27 is the same oligonucleotide as the probe, E(I)wt and E(II)wt contain the wild-type sequence of EcR elements I and II, respectively. E(I)4T, E(I)1In, E(II)1In and E(II)4T contain mutations of EcR elements I or II as shown in Figure 6. The EcR–DNA complex (bound) and the free probe (free) are marked with arrows. The complex marked by an asterisk is not reproducibly formed, and its strength varies very much with the nuclear extract used. Since it also behaves very similar to the EcR–DNA complex, this complex is presumably formed by a degradation product of EcR and/or USP.

of EcR element II confirm our conclusion that this element is responsible for receptor binding.

Insertion of a T 3′ to the spacer of the palindromic sequence of EcR element I has no or little effect on receptor binding [Figure 4, mutation E(I)1In], whereas four base substitutions scattered over the entire palindrome clearly reduce, but do not completely prevent, binding of the EcR to element I [Figure 4, mutation E(I)4T]. The inefficiency of the spacer mutation E(I)1In is not totally unexpected because the introduction of a T in position +1 improves correspondence to the EcRE consensus sequence in a position that is conserved in all known EcREs (Figure 3A). The introduction of four base exchanges, however, clearly affects receptor binding, corroborating our conclusion that EcR element I is responsible for receptor binding.

EcR binding sites I and II are located in the vicinity of binding sites for other secretion enhancer binding proteins

To identify factors involved in the stage- and tissue-specific expression of *Sgs-4*, we tried to identify proteins present in nuclear extracts from salivary glands that specifically bind to the *Sgs-4* regulatory region. Using a mobility shift assay, we were able to detect three factors with different binding specificities, which were designated secretion enhancer binding proteins (SEBP). One of these factors, SEBP 1, binds to a well-defined site within the section of the *Sgs-4* regulatory region that is thought to be responsible for the transcriptional switch between the *Pig-1* and the *Sgs-4* promoters (M.Lehmann, A.Hofmann and G.Korge, manuscript in preparation). Another factor, SEBP 3, binds with high affinity to a site at position −422, just upstream of EcR binding site II, and with lower affinity to a site around position −344, ~40 bp upstream of EcR binding site I (Figure 5A). A change of C at position −344 to a T in the strain Samarkand has been shown to be responsible for a reduction of Sgs-4 protein expression to 40% of the level found in the strain Oregon R. Moreover, this transition seems to affect the mechanism of dosage compensation which normally leads to hyperexpression of the X-chromosomal *Sgs-4* gene in males (Korge, 1981; Hofmann and Korge, 1987; Hofmann *et al.*, 1987). When an oligonucleotide containing the C to T transition at position −344 (Sam 344) is used to compete for SEBP 3 binding to the −422 site, it shows very weak competition at a 10-fold molar excess in comparison with an oligonucleotide (Org 344) lacking this transition (Figure 5A, compare lane 4 with lane 6). Even at a 50-fold molar excess, competition by Sam 344 is weaker than competition by a 10-fold molar excess of Org 344 (Figure 5A, lanes 4 and 7). This suggests that impaired binding of SEBP 3 is responsible for the effects of the C to T transition observed in the strain Samarkand. The strong SEBP 3 binding site around position −422 spans a region from −426 to, at least, −414 (Figure 5B and data not shown), and consequently overlaps with EcR element II. A simultaneous binding of SEBP 3 and EcR in this region should therefore be impossible for steric reasons. The binding of SEBP 3 to the −422 site proved to be sensitive to two base substitutions at positions −422 and −423 (Figure 5B, mutation S3T2).

Figure 5 shows that there is a second factor binding in the region from −399 to −432 that forms a complex migrating with a higher mobility than the SEBP 3 complex. This factor, SEBP 2, binds to several A/T-rich sites within the *Sgs-4* regulatory region. For example, an oligonucleotide representing the region from −447 to −480, that contains at least one strong SEBP 2 binding site, proved to be a specific competitor of SEBP 2 binding to the −399 to −432 region, while SEBP 3 binding was not impaired (Figure 5B, lane 8). A complete presentation of SEBP 2 binding data will be given elsewhere (M.Lehmann and G.Korge, manuscript in preparation). A detailed analysis of SEBP 3 and SEBP 2 binding to the −399 to −432 region revealed that SEBP 2 binds immediately downstream of the SEBP 3 site in a region extending from −396 to −422 (Figure 5B, lane 5) that also contains EcR binding site II. Indeed, mutations that eliminate EcR binding to element II turned out to interfere also with SEBP 2 binding (Figure 5B, lanes 6 and 7), clearly indicating that both binding sites overlap in a way that precludes a simultaneous binding of both proteins.

Both EcR binding site I and the SEBP 3 binding site are necessary for activation of Sgs-4

To determine the functional significance of the factor binding sites identified by mobility shift assays, we constructed plasmids for P-element transformation containing mutations which turn off single sites or different combinations of binding sites. Moreover, we converted EcR element I, EcR element II or both elements of *Sgs-4* to perfect *hsp 27* EcREs, to investigate whether the receptor binding sites of both genes are functionally interchange-

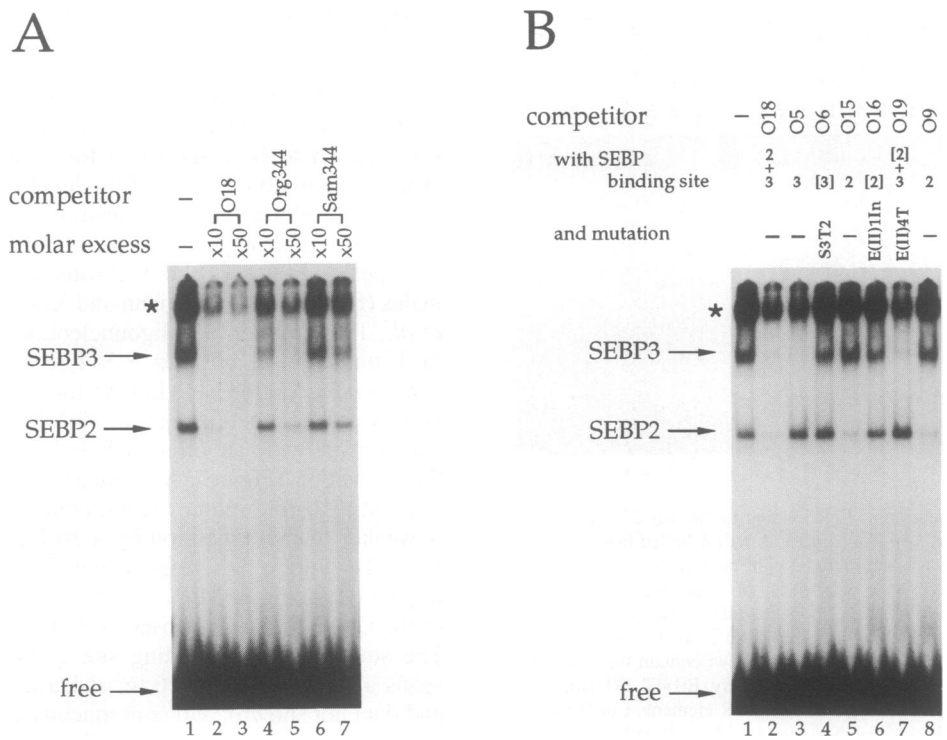

Fig. 5. Binding of SEBP 3 and SEBP 2 to the *Sgs-4* upstream region. ^{32}P-labelled oligonucleotide probe O18, representing the *Sgs-4* sequence from −399 to −432, was incubated with nuclear extract from salivary glands in the absence or presence of an indicated molar excess of cold competitor oligonucleotides. The mixture was then separated in a 5% mobility shift gel. Free probe and protein−DNA complexes formed by SEBP 3 and SEBP 2 are marked with arrows. The complex marked with an asterisk has a less distinct specificity but shows a similar competition behaviour as the SEBP 3 complex, suggesting that SEBP 3 also contributes to the formation of this complex. The following competitor oligonucleotides were used. (**A**) O18 corresponds to the probe; Org 344 represents the Oregon sequence from −329 to −359; Sam 344 represents the respective Samarkand sequence, both oligonucleotides differing only in position −344, which is a C in the Oregon sequence and a T in the Samarkand sequence. (**B**) O18 corresponds to the probe; O5 represents the wild-type sequence from −405 to −438; O6 corresponds to O5 with the exception of a C to T transition at −422 and a A to G transition at −423 (mutation S3T2); O15 represents the wild-type sequence from −396 to −422; O16 corresponds to O15 except that it contains mutation E(II)1In; O19 corresponds to O18 except that it contains mutation E(II)4T; O9 represents the wild-type sequence from −447 to −480. For the sequence of mutations see Figure 6. To facilitate interpretation of the results, for each competitor oligonucleotide the presence of intact or modified SEBP 3 and/or SEBP 2 binding sites is given. Binding sites modified by the indicated mutations are marked by brackets. For example, O19 contains mutation E(II)4T which obviously hits the SEBP 2 site and leaves the SEBP 3 site intact.

able. All mutations were introduced by site-directed mutagenesis into the *Sgs-4* allele of the strain Oregon R comprising the complete transcription unit, 2490 bp of upstream sequence and ~2800 bp of downstream sequence. These sequences contain all elements necessary for high level expression of *Sgs-4*, comparable with the expression of the endogenous allele of the Oregon R strain (Figure 7, lanes 1 and 3). Transformations were carried out with the underproducer strain Kochi;*rosy*^{506} (Krumm *et al.*, 1985), which carries a deletion in the *Sgs-4* upstream region (see Figure 1) that leads to an almost complete loss of expression of the endogenous *Sgs-4* gene (Muskavitch and Hogness, 1980, 1982; Korge, 1981; Figure 7, lanes 2 and 13). Expression of the constructs was quantified by RNase protection assays (Figure 6B).

Mutation E(I)4T, which has been shown to reduce receptor binding to EcR element I *in vitro*, results in a reduction of the amount of *Sgs-4* mRNA to ~30% of the amount produced by the wild-type control construct. When receptor binding is completely prevented by a deletion of EcR element I [construct ΔE(I)], expression of *Sgs-4* is further reduced to <6% of the wild-type level (Figure 7, lanes 3–5 and lanes 14–16). As one would expect from these results, an additional mutation of EcR binding site II in combination with a deletion of element I shows the same effect as the deletion of element I alone (Figure 7, lanes 7 and 18). If element I is converted to a perfect *hsp 27* EcRE, expression of *Sgs-4* is enhanced ~2-fold (Figure 7, lanes 9 and 20) and an elevated level of *Sgs-4* mRNA is still detectable in white prepupae (results not shown). This demonstrates that *Sgs-4* element I can be functionally substituted by the *hsp 27* element. The stronger activity of the *hsp 27* EcRE correlates well with our finding that it also constitutes the better EcR binding site *in vitro*. We conclude from these results that EcR element I acts as a positive EcRE which is necessary for the activation of *Sgs-4* transcription.

In the presence of two transitions that interfere with SEBP 3 binding to the −422 site *in vitro*, we observe an ~5-fold reduction of the amount of *Sgs-4* mRNA (Figure 7, lanes 8 and 19), indicating that the SEBP 3 site is also necessary for an efficient transcriptional activation of *Sgs-4*. It thus follows that neither the SEBP 3 site at −422 nor EcR element I alone are sufficient to effectively activate the *Sgs-4* promoter.

EcR binding site II seems to be without significant influence on the expression of Sgs-4

Four base exchanges in EcR element II that prevent receptor binding and binding of SEBP 2 to the overlapping

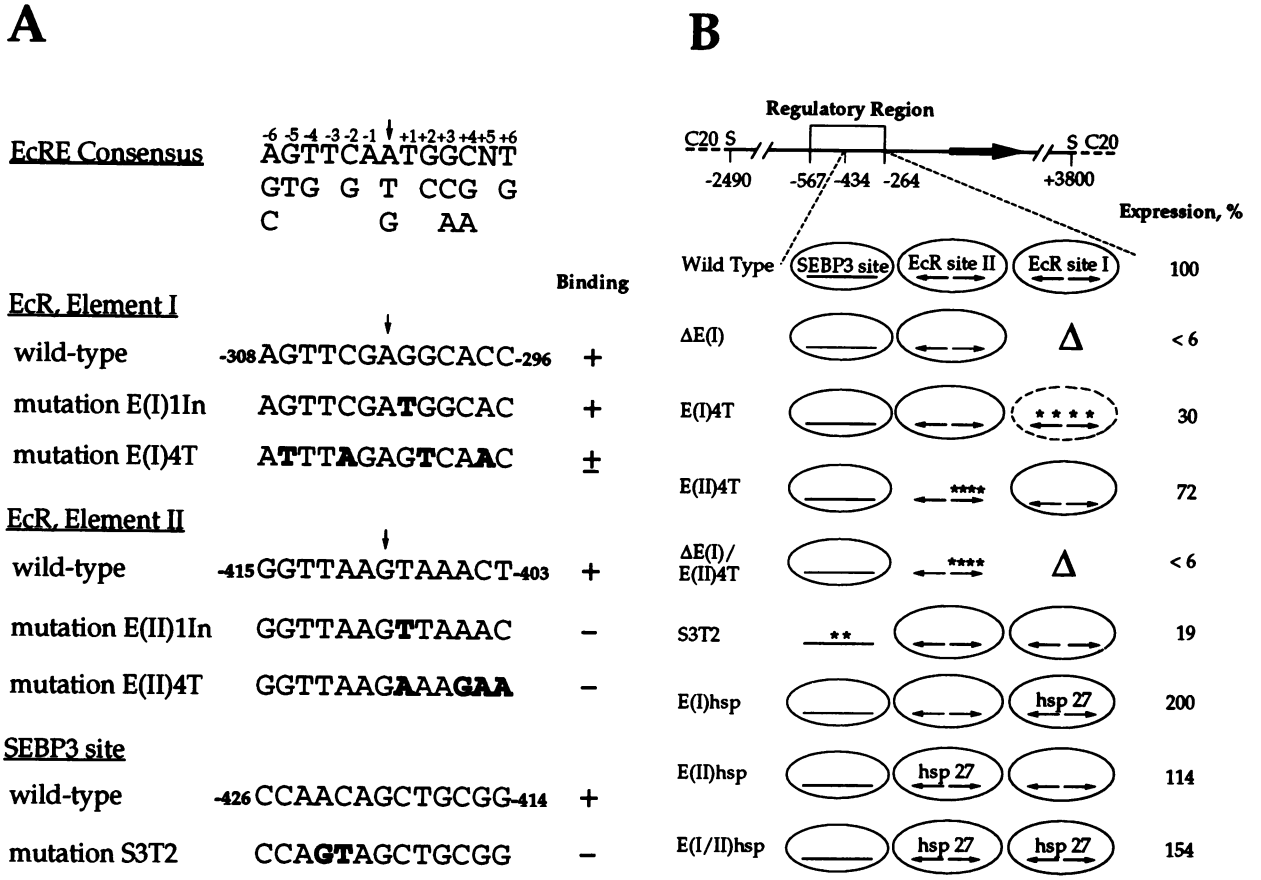

Fig. 6. Functional analysis of the two EcR binding sites of *Sgs-4* and the SEBP 3 binding site at −422. (**A**) Mutations introduced into the wild-type sequence of EcR elements I and II and the SEBP 3 binding site at −422 are indicated by bold letters. For derivation of the EcRE consensus sequence see Figure 3. Specific protein binding of the wild-type and mutated sequences is assessed according to the results of mobility shift competition assays (Figures 4 and 5). (**B**) Plasmids constructed for P-element analysis of *Sgs-4* expression in the presence of different combinations of binding site mutations. Expression of the constructs was measured by determining the ratio of *Sgs-4* to *Sgs-3* transcripts and is expressed as the percentage of expression of the wild-type control construct. The data were derived from six independent RNase protection assays, and for each construct at least three independent strains were tested, with the exception of construct E(II)hsp (two strains) and construct E(I/II) hsp (one strain).

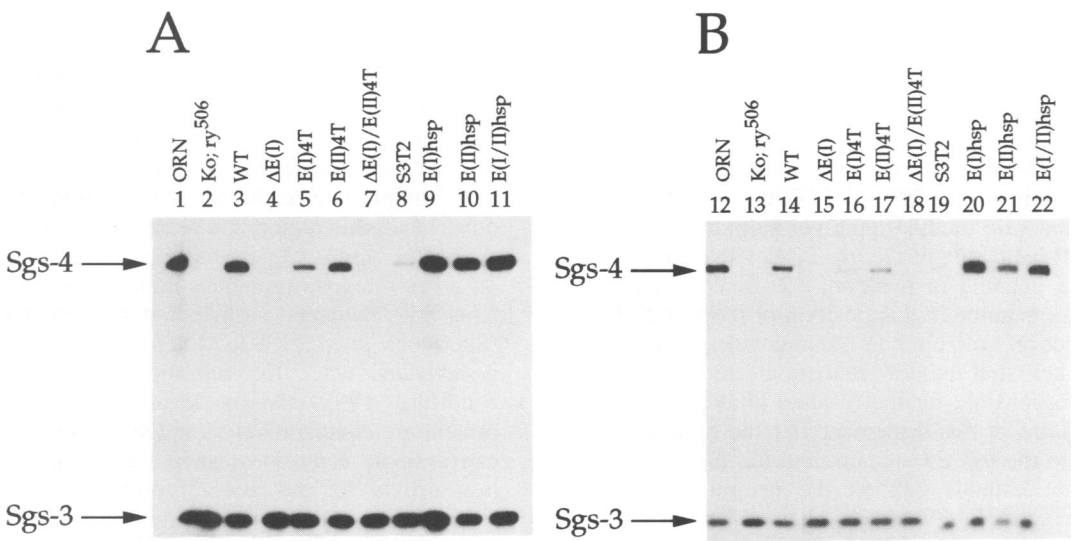

Fig. 7. Expression of *Sgs-4* transgenes carrying different combinations of EcR and SEBP 3 binding site mutations. Salivary glands dissected from mid-third instar larvae (**A**) and late third instar larvae (**B**) of lines transformed with the indicated constructs were analysed for the presence of *Sgs-4* transcripts by an RNase protection assay (see Materials and methods). RNA from two salivary glands each was hybridized to ^{32}P-labelled RNA probes protecting a 231 nucleotide fragment of the *Sgs-4* mRNA and a 52 nucleotide fragment of the *Sgs-3* mRNA (marked by arrows). *Sgs-3* has the same expression pattern as *Sgs-4* (Andres *et al.*, 1993), and transcription from the endogenous *Sgs-3* gene was therefore used as both a loading control and an internal developmental control. To compare the expression level of transgenes with the expression of the endogenous *Sgs-4* allele of Oregon R (ORN), which was used for plasmid constructions, and the endogenous *Sgs-4* allele of Kochi;ry^{506}, the recipient strain for P-element transformation, these two strains were also included in the analysis.

binding site *in vitro* [mutation E(II)4T] do not seriously affect ecdysone induction of *Sgs-4* expression (Figure 7, lane 6). Animals carrying the E(II)4T construct produce ~28% less *Sgs-4* mRNA than wild-type control animals, pointing to an only weak contribution of either the EcR or the SEBP 2 binding site to *Sgs-4* activation. Binding of the EcR to element II as a consequence of a rising concentration of ecdysone receptor complexes, thereby displacing SEBP 3 from its binding site at −422, could provide a mechanism for an ecdysone-induced repression of *Sgs-4* transcription at the end of the third larval instar. If such a mechanism exists, it has to be predicted that switching off element II results in elevated *Sgs-4* mRNA levels in late third instar larvae and prepupae. However, salivary glands from late third instar larvae of transformants carrying the mutation E(II)4T contain rather lower amounts of *Sgs-4* transcript than salivary glands from larvae carrying the wild-type construct (Figure 7, lanes 14 and 17). In white prepupae of both E(II)4T transformants and wild-type controls, *Sgs-4* mRNA is equally reduced to a very low level (results not shown). Hence, EcR element II does not seem to be relevant to the transcriptional repression of Sgs-4.

Discussion

EcR binding sites of the Sgs-4 gene

We have identified two EcR binding sites, element I and element II, in the *Sgs-4* regulatory region. Element I differs in three positions from the EcRE consensus sequence, two of which (position −1 and +1; Figure 3A) are conserved in all EcREs known so far. Therefore, we assume that the two guanines at positions −1 and +1 are responsible for the reduced binding affinity of element I in comparison with the *hsp 27* element. However, our data indicate that, despite these two deviations from the consensus sequence, element I is a functional EcRE. Surprisingly, two additional mismatches to the consensus sequence in positions −2 and +2 apparently did not completely switch off element I [E(I)4T; Figure 4]. Upon re-examination of the mutated sequence, however, we observed that mutation E(I)4T gives rise to a new imperfect palindrome in an inverted orientation, which is shifted upstream by 1 base pair with respect to element I: −295 GGTTGACTCTAAA −307. The left-hand side of this imperfect palindrome matches with the left-hand side of the *hsp 27* EcRE, with the only exception a guanine in position −2. Since it has been shown that a guanine in this position of the *hsp 27* EcRE has no effect on EcR binding (Antoniewski *et al.*, 1993), it is very likely that the new element created by mutation E(I)4T is responsible for the residual binding activity. A striking feature of *Sgs-4* element I is the high sequence homology to the *hsp 27* element and the *Eip 28/29* Prox element that extends beyond the central palindromic structure (Figure 3B). Although it has been shown for the *hsp 27* EcRE that the central palindromic structure is sufficient to confer ecdysone inducibility on a heterologous promoter (Cherbas *et al.*, 1991; Martinez *et al.*, 1991), we speculate that the conserved flanking sequences might be involved in the regulation of certain EcREs by additional factors.

Element II differs from the EcRE consensus sequence in two positions, −2 and +2 (Figure 3A). The fact that element II is an even weaker EcR binding site than element I, and the fact that we could not obtain unequivocal evidence for a function of element II *in vivo*, indicate that positions −2 and +2 are more relevant to the function of EcR binding sites than positions −1 and +1. The data of Antoniewski *et al.* (1993), as well as a sequence comparison of all EcREs including *Sgs-4* element I (Figure 3A), support this view, since they suggest that only a guanine or a cytosine is allowed in position 2. A high conservation of position 2 is also found in the palindromic recognition sequence of different vertebrate steroid hormone receptors, in which the second position is occupied by a guanine. This guanine has been shown by methylation interference to be contacted by the receptor protein through interaction with the N-7 position (Metzger *et al.*, 1988; Klein-Hitpass *et al.*, 1989; Truss *et al.*, 1991). Recently it has also been demonstrated that the guanine in position +2 of two EcREs is involved in the formation of contacts to the receptor protein (Antoniewski *et al.*, 1994).

EcR element I is necessary but not sufficient to mediate the ecdysone response of Sgs-4

Our results provide strong evidence that EcR element I functions as a positive EcRE that is necessary to mediate the ecdysone response of *Sgs-4*: (i) element I binds EcR/USP *in vitro* and shows strong homology to the *hsp 27* EcRE and other EcREs; (ii) a deletion of element I leads to a drastic reduction of *Sgs-4* mRNA levels *in vivo*; (iii) four base exchanges that weaken EcR binding *in vitro* result in a reduced expression of *Sgs-4 in vivo*; and (iv) four base exchanges that convert element I to a perfect *hsp 27* element, i.e. to an element that exerts stronger EcR binding *in vitro*, clearly enhance expression of *Sgs-4 in vivo*. Interestingly, the *Sgs-4* allele of the recipient strain for transformation, Kochi;*rosy*^{506}, carries a deletion that extends into the left half-palindrome of element I (see Figure 1). A comparison of *Sgs-4* mRNA production by the Kochi allele and the transgene bearing a deletion of EcR element I reveals that both genes produce approximately equal amounts of *Sgs-4* mRNA (M.Lehmann, unpublished data). Thus, the effect of the Kochi deletion finds a sufficient explanation in the destruction of EcR element I.

Our data indicate that element I is the only functional EcR binding site in the *Sgs-4* regulatory region. On the other hand, this region has been shown to be sufficient to confer the stage and tissue specificity of *Sgs-4* expression on a heterologous promoter, implicating that it also contains all elements sufficient for hormonal induction (Shermoen *et al.*, 1987; Mougneau *et al.*, 1993). *Sgs-4* is activated when the ecdysone titre (Richards, 1981; Riddiford, 1993) and the levels of EcR transcript and protein are comparatively low (Koelle *et al.*, 1991). How can a single ecdysone response element with relatively low affinity for the receptor protein mediate hormonal induction in such a developmental phase? Our results indicate that this is achieved by cooperation with at least one other transcription factor, SEBP 3. Two point mutations in the SEBP 3 binding site at −422 and −423 lead to a strong, ~5-fold reduction of *Sgs-4* expression. The very low level of expression that remains after switching off either the SEBP 3 site or EcR element I strongly suggests that a synergism of EcR and SEBP 3 is necessary for a full activation of *Sgs-4*. A simple additive

contribution of bound transcription factors to *Sgs-4* induction cannot account for the drastic effects of each single binding site mutation. Such a synergistic interaction of steroid receptors and other transcription factors has been well documented for different vertebrate systems (reviewed in Lucas and Granner, 1992; Renkawitz, 1993; Truss and Beato, 1993). The glucocorticoid and progesterone receptor, for example, are able to act synergistically with many different transcription factors like CACCC-box factor, Sp1, octamer transcription factor or NF 1 (Schüle *et al.*, 1988a,b), and the oestrogen receptor has been shown to interact functionally with Sp1 (Krishnan *et al.*, 1994) and the putative *Xenopus* homologue of NF 1 (Corthésy *et al.*, 1989). Of particular importance for the assessment of *Sgs-4* element I is the observation that the inducibility of a weak glucocorticoid response element (GRE) can be greatly increased by synergizing factors, whereas the activity of a strong GRE is only weakly increased (Schüle *et al.*, 1988a).

A more detailed analysis of the interaction between SEBP 3 and EcR that confers hormone responsiveness upon the *Sgs-4* promoter requires the cloning of the gene that encodes SEBP 3. Obviously SEBP 3 is not identical with a product of the *BR-C* locus: the SEBP 3 site at −422 lies in a region that gives a footprint with salivary gland extract but not with various bacterially expressed *BR-C* proteins (von Kalm *et al.*, 1994), and SEBP 3 is present, although at a reduced level, in a *BR-C* mutant background (M.Lehmann and G.Korge, manuscript in preparation).

Besides SEBP 3 and EcR we detected a third factor, SEBP 2, whose binding sites are scattered over the entire *Sgs-4* regulatory region. It is difficult to assess the contribution of SEBP 2 to the regulation of *Sgs-4*. Mutation E(II)4T, which impairs the SEBP 2 binding site overlapping with EcR element II, has only a slight effect on *Sgs-4* expression. However, there are at least four more binding sites for SEBP 2 in the *Sgs-4* regulatory region, therefore a possible role of this factor may be obscured by redundancy. Like the binding sites of *BR-C* products, the SEBP 2 binding sites are very rich in A/T. Furthermore, the SEBP 2 sites show a striking correspondence with the numerous binding sites for *BR-C* proteins detected in the *Sgs-4/Pig-1* intergenic region (von Kalm *et al.*, 1994), suggesting that SEBP 2 might be a product of the *BR-C*. Surprisingly, however, we detected normal levels of SEBP 2 in a *BR-C* mutant background (M.Lehmann and G.Korge, manuscript in preparation). For this reason, SEBP 2 is also not identical to GEBF-I, a factor which is missing in *BR-C* mutant larvae and which binds to two A/T-rich sequence motifs that have been implicated in the induction of the *Sgs-3* gene (Georgel *et al.*, 1991, 1993).

In summary, we propose a model (Figure 8) in which a complex hormone response unit of 130 bp is responsible for the hormonal induction of *Sgs-4*. This unit contains one functional ecdysone receptor binding site and two SEBP 3 binding sites. Each individual protein bound to one of these sites contributes only weakly to the induction of the *Sgs-4* promoter, but working together the bound proteins bring about high level expression. Thus, our data identify *Sgs-4* as a primary response gene and, in this way, take into account that *Sgs-4* is induced by ecdysone even in the complete absence of the *BR-C* locus (Crowley

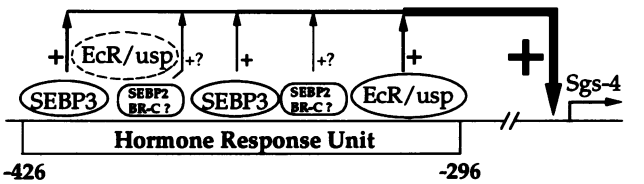

Fig. 8. Model for ecdysone regulation of *Sgs-4*. For details see the text.

et al., 1984). We propose that the primary response is accompanied by a secondary response, mediated by *BR-C* products (von Kalm *et al.*, 1994), which contributes to the correct timing of *Sgs-4* induction, whereas the ecdysone induction *per se* is mediated by the ecdysone receptor complex. This notion is supported by the finding of Karim *et al.* (1993) that *Sgs-4* and three other 'intermolt genes' are induced to a high level by ecdysone towards the end of the third larval instar in all *BR-C* mutant strains they have tested. Recently it has been shown that the fat body-specific gene *Fbp 1* is regulated by an ecdysone-inducible enhancer that contains a single, relatively weak EcR binding site and binding sites for other factors of as yet unknown identity (Antoniewski *et al.*, 1994). The intriguing similarity of the *Fbp 1* enhancer to the *Sgs-4* hormone response unit suggests that our model of a combined primary and secondary response also applies to ecdysone-inducible genes in other tissues that are expressed during the same developmental stage as *Sgs-4*. Interestingly, a similar model, assuming a combined primary and secondary response, has been proposed for the induction of the early genes *E74*, *E75* and *BR-C* which require, besides the ecdysone receptor complex, the *2Bc* subfunction of the *BR-C* for complete induction (Karim *et al.*, 1993).

Functional analysis of EcR element II provides no evidence for a direct repression of Sgs-4 transcription by the EcR

A physical overlapping of EcR element II and the SEBP 3 binding site at −422 results in steric hindrance that would prevent the simultaneous occupancy of both binding sites. It is therefore tempting to speculate that the EcR binds to element II only when its own concentration and that of ecdysone are high, i.e. at the end of the third larval instar, thereby displacing SEBP 3 and turning off *Sgs-4* transcription. Our functional data, however, provide no evidence to support such a simple and obvious model. Four base substitutions that completely abolish the binding ability of element II *in vitro* [mutation E(II)4T] accelerate rather than delay the decline of *Sgs-4* transcripts in both late third instar larvae and white prepupae. Considering the clear effect of mutation E(II)4T on receptor binding *in vitro*, it seems very unlikely that this mutation does not prevent receptor binding *in vivo*, although we cannot absolutely exclude this possibility. It should also be considered that we might have failed to notice a function of EcR element II if ecdysone repression is mediated redundantly by more than one negatively acting element. Early studies on ecdysone-induced puff regression suggested that the *BR-C* mediates at least a part of this response (see Introduction). Interestingly, this notion gains support from the recent finding that the *BR-C* subfunction

$2Bc^+$ is required for the repression of 'intermolt genes' including the 68C genes and *Sgs-4* (Karim *et al.*, 1993). The contribution of *BR-C* products not only to the induction of *Sgs-4* expression but also to its repression indicates that both *BR-C* products and the ecdysone receptor directly contribute to all steps in 'intermolt gene' regulation. It will be interesting to find out how these transcription factors interact with one another and with other factors, like SEBP 3, to elicit the developmental specificity of the ecdysone response.

Materials and methods

Flies and strains

Unless otherwise mentioned, cloned *Sgs-4* gene fragments used in this work were originally derived from the wild-type strain Oregon R (Stanford) of *Drosophila melanogaster*; nucleotide sequences of synthetic oligonucleotides accordingly relate to the *Sgs-4* allele of this strain. Oregon R was also used for the preparation of nuclear extracts. The strain $Ko;ry^{506}$ served as the recipient strain for P-element transformation. This strain was obtained by crossing the strain ry^{506} with the *Sgs-4* underproducer strain Kochi-R (Krumm *et al.*, 1985).

Preparation of nuclear extracts

Third instar larvae and white prepupae were staged according to age, behavioural characteristics and salivary gland morphology. Wandering larvae (collected after 96–108 h of development at 25°C) with high motility and secretory salivary gland cells tightly filled with secretion granulae were classified as mid-third instar larvae, whereas wandering larvae (collected after 108–120 h) with reduced motility and bloated, transparent salivary glands (secretion extruded into the gland lumen) were classified as late third instar larvae. White prepupae were selected by immobility, spiracle eversion and cuticle tanning. Salivary glands from 100 to 200 animals were hand dissected and transferred into 1.5 ml Eppendorf tubes filled with 100 µl ice-cold buffer H (10 mM Tris–HCl, pH 7.5, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM KCl, 0.25 M saccharose). All following steps were carried out at 0–4°C. Nuclei were released from the salivary glands by homogenization with a plastic pestle fitting the Eppendorf tube and pelleted by centrifugation at 3000 r.p.m. for 5 min in an HB-4 rotor (Sorvall). The pellet was rehomogenized in ~100 µl of buffer H and the nuclei were washed twice with 1 ml of buffer H. Nuclei were pelleted as above, except that the speed was 2000 r.p.m. after the first, and 6000 r.p.m. after the second washing step. The nuclei were then carefully resuspended in 10 µl/150 salivary glands of extraction buffer (10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.65 M NaCl, 5% glycerol, 1 mM DTT, 1 mM PMSF, 1 mM benzamidine) and incubated on ice for 5 min. After centrifugation at 18 000 r.p.m. for 10 min in an SS-34 rotor, the supernatant was frozen in liquid nitrogen and stored at −80°C.

Nuclear extracts from 1.5 to 15 h old embryos were prepared on a microscale by an extensive modification of the procedure described by Soeller *et al.* (1988). Embryos were washed with a solution of 0.7% NaCl and 0.04% Triton X-100, and dechorionated for 3 min in 3% sodium hypochloride. The embryos were first rinsed with NaCl/Triton solution, then with distilled water and finally they were quickly blotted dry. All subsequent procedures were carried out at 0–4°C. The dechorionated embryos were homogenized in 1 ml/0.75 g embryos of buffer H supplemented with 1 mM DTT, 1 mM PMSF and 1 µg/ml leupeptin using a glass−glass dounce homogenizer. The homogenate was transferred to a 1.5 ml Eppendorf tube and centrifuged for 5 min at 4000 r.p.m. in an HB-4 rotor. The pellet was rehomogenized in 100 µl and then resuspended in 1 ml of buffer H (supplemented as above). Centrifugation and resuspension steps were repeated once, and nuclei were finally pelleted for 5 min at 6000 r.p.m. in an HB-4 rotor. Nuclei were then extracted for 5 min by resuspension in 50 µl/0.75 g embryos of the same extraction buffer that was used for the extraction of salivary gland nuclei, except that benzamidine was replaced by 1 µg/ml leupeptin. Further operations were carried out as described for the preparation of extracts from nuclei of salivary glands.

Mobility shift DNA binding assay

Binding reactions were carried out in a total volume of 20 µl containing 10 mM Tris−HCl, pH 7.5, 2 mM EDTA, 1 mM DTT, 40–50 mM NaCl, 9% (v/v) glycerol, 1 µg poly(dI−dC)·poly(dI−dC), oligonucleotide competitors as indicated in Results, 0.5–1.0 ng ^{32}P-labelled probe and 1 µl nuclear extract. Binding reactions, carried out for the mobility shifts shown in Figure 5, additionally contained 0.1% NP-40. Supershift experiments were performed by adding 3.5 µl EcR antibody AG 10.2 (Talbot *et al.*, 1993), 2 µl USP antibody AG 11 (Khoury Christianson *et al.*, 1992) or 7 µl control antibodies Bx42 or Bj6 (Saumweber *et al.*, 1990) to the reaction mixture after all other components of the mixture had been mixed. Incubations with antibodies were for 1 h at room temperature, while incubations for all other mobility shift experiments were for 20 min at room temperature. Assay mixtures were loaded onto 5% non-denaturing polyacrylamide gels and run for ~3 h at 150 V in 0.25× TBE (Sambrook *et al.*, 1989), adjusted to a temperature of 4°C and to a pH of 7.5. After electrophoresis, gels were dried for autoradiography.

The following double-stranded oligonucleotides were used as ^{32}P-labelled probes: hsp 27 is a 27 bp EcRE-containing oligonucleotide corresponding to a segment (−552 to −526) of the upstream region of the *hsp 27* gene of *D.melanogaster* (Riddihough and Pelham, 1986, 1987). This oligonucleotide was provided on both ends with a 5′-CTAG overhang to facilitate radiolabelling and to allow subcloning into *XbaI* sites. The probe containing the EcR element I, used for the mobility shift shown in Figure 2B, is a 27 bp oligonucleotide with three unpaired bases at both ends that corresponds to the *Sgs-4* upstream region from −318 to −286 (Hofmann and Korge, 1987). O18 is a 34 bp oligonucleotide with three unpaired bases at both ends that corresponds to the region from −435 to −396. The oligonucleotides were labelled with [α-^{32}P]dCTP by a fill-in reaction with Klenow fragment.

Double-stranded oligonucleotides of wild-type sequence that were used as unlabelled competitors correspond to the *Sgs-4* upstream region as follows: E(I)wt (−315 to −289), E(II)wt (−432 to −399), O5 (−438 to −405), O15 (−422 to −396), O9 (−480 to −447) and Org 344 (−359 to −329).

Double-stranded oligonucleotides of mutated sequence correspond to the following regions: E(I)4T and E(I)1In (−315 to −289), E(II)4T (identical to O19; −432 to −399), E(II)1In (identical to O16; −422 to −396), O6 (contains mutation S3T2; −438 to −405) and Sam 344 (−359 to −329). Sam 344 contains a C to T transition at position −344. Base exchanges and insertions contained in all other mutated oligonucleotides are depicted in Figure 6.

Site-directed mutagenesis

All mutations except ΔE(I) and E(I)hsp were introduced by the method of Kunkel *et al.* (1987) using single-stranded DNA derived from subclones of *Sgs-4* restriction fragments in Bluescript SK(−). Mutations ΔE(I) and E(I)hsp were introduced by the PCR as described by Ausubel *et al.* (1992). EcR elements I and II were converted to perfect *hsp 27* EcREs by the use of single-stranded oligonucleotides, each bearing four base exchanges indicated by bold letters: E(I)hsp, 5′-$^{-286}$GGACAGTG-CGAGTGCATTGAACCTGTCTTTCAC^{-318}-3′; E(II)hsp, 5′-$^{-425}$CA-ACAGCTGCGGTTCAATGCACTAAAGCTGGTG^{-393}-3′. Mutation ΔE(I) was introduced using an oligonucleotide bearing a deletion of EcR element I: 5′-$^{-270}$ATTTTCGTGAAGTGT-Δ-TTCACAAATA-TTTAC^{-328}-3′. All other mutations were generated by oligonucleotides bearing the base substitutions or insertions shown in Figure 6. The presence of the correct mutations was confirmed by DNA sequencing with the chain termination method (Sanger *et al.*, 1977).

Construction of plasmids

Standard methods for manipulating DNA were as described by Sambrook *et al.* (1989). The starting point for construction of plasmids for P-element transformation was plasmid OWSal which contains a 3.5 kb *SalI*−*Hin*dIII restriction fragment subcloned in pBR344 comprising 2.5 kb of the *Sgs-4* upstream and 1 kb of the downstream sequence. A restriction map of this fragment is published in Hofmann and Korge (1987). From OWSal the *SalI*−*XhoI* fragment, the *XhoI*−*Hin*dIII fragment and the *XhoI*−*Eco*RI subfragment of the *XhoI*−*Hin*dIII fragment were isolated and subcloned in pBluescript SK(−) for site-directed mutagenesis (see above). Mutated fragments were reintroduced into OWSal to give the different combinations of binding site mutations. The resulting plasmids were linearized with *Hin*dIII, and the *Sgs-4* upstream region was completed up to position +3800 by the insertion of a 2.8 kb *Hin*dIII fragment isolated from a partial digest of plasmid Ikarus OR6, which contains a *SalI*−*SalI* fragment (−2490 to +3800) comprising the *Sgs-4* gene. The *Hin*dIII site at the downstream end of the *Hin*dIII fragment is a restriction site of the polylinker. The correct orientation of the *Hin*dIII fragment was verified by restriction mapping with *SalI*.

Finally, the whole *SalI*−*SalI* insert (−3490 to +3800 of the *Sgs-4* gene) was excised from the resulting plasmids and cloned into the *SalI* site of the transformation vector Carnegie 20 (Rubin and Spradling, 1983). The presence of the correct mutations in the final product was again verified by sequencing.

The plasmid used for RNase protection analysis of *Sgs-4* mRNA was generated by inserting the *XhoI*−*Hin*dIII fragment (−392 to −1000) of *Sgs-4* into the respective restriction sites of pBluescript SK(−). The plasmid used for the analysis of *Sgs-3* mRNA consists of the *StyI*−*HinfI* fragment (−71 to +52) of *Sgs-3*, isolated from plasmid aDm 2023 (Garfinkel *et al.*, 1983) and subcloned into the *Eco*RV site of pBluescript SK(−).

P-element transformation

P transposons were introduced into the germline of the recipient strain *Ko;ry*^{506} using standard methods (Spradling, 1986; Ashburner, 1989). Homozygous transformant lines were obtained by standard genetic techniques as described by Hofmann *et al.* (1987). The number and location of integrations was determined by *in situ* hybridization to polytene chromosomes following modified procedures of Pardue and Gall (1975). The number of integrations was also confirmed by Southern analysis.

In vitro transcription and RNase protection assay

In vitro transcription reactions were carried out with an RNA transcription kit (Stratagene) according to the instructions of the manufacturer. The plasmid bearing the *XhoI*−*Hin*dIII fragment of *Sgs-4* was digested with *DdeI* and transcribed with T3 polymerase in the presence of [α-^{32}P]rUTP, yielding transcripts that protect a 231 nucleotide fragment of the 3′ end of the *Sgs-4* mRNA. The plasmid containing the *StyI*−*HinfI* fragment of *Sgs-3* was linearized with *Eco*RI and transcribed with T7 polymerase. Transcripts from the linearized plasmid protect a 52 nucleotide fragment of the 5′ end of the *Sgs-3* mRNA.

RNase protection assays were carried out using a lysate ribonuclease protection kit as described by the manufacturer (USB). Briefly, two salivary glands were dissected and immediately transferred into 50 μl lysis solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl). RNA dissolved in 45 μl of lysate was hybridized for 15 h at 37°C with the *Sgs-4* and the *Sgs-3* probes (each present at 1×10^6 c.p.m./assay). After RNase digestion and protease treatment the hybrids were purified and analysed on 7 M urea−7% polyacrylamide gels. The amount of *Sgs-4* mRNA relative to the amount of Sgs-3 *mRNA* was measured by using a Quick Scan Jr densitometer (Helena Laboratories).

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