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The Ecdysone Response Enhancer of the *Fbp1* Gene of *Drosophila melanogaster* Is a Direct Target for the EcR/USP Nuclear Receptor

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The transcription of the *Drosophila melanogaster Fbp1* gene is induced by the steroid hormone 20-hydroxyecdysone and restricted to the late-third-instar fat body tissue. In a previous study we showed that the −68 to −138 region relative to the transcription start site acts as an ecdysone-dependent third-instar fat body-specific enhancer in a transgenic assay. Here we report that seven nucleoprotein complexes are formed in vitro on this enhancer when a nuclear extract from late-third-instar fat body is used in a gel shift assay. Accurate mapping of the binding sites of the complexes revealed a remarkably symmetrical organization. Using specific antibodies, one of the complexes was identified as a heterodimer consisting of the ecdysone receptor (EcR) and Ultraspiracle (USP) proteins. The binding site of the heterodimer as defined by mutagenesis and methylation interference experiments bears strong sequence similarity to the canonical hsp27 ecdysone response element, including an imperfect palindromic structure. The two elements diverge at three positions in both half-sites, indicating that the structure of an active EcR/USP binding site allows considerable sequence variations. In vivo footprinting experiments using ligation-mediated PCR and wild-type or ecdysteroid-deficient larvae show that occupancy of the Fbp1 EcR/USP binding site and adjacent region is dependent on a high concentration of ecdysteroids. These results provide strong evidence for a direct role of the EcR/USP heterodimer in driving gene expression in response to changes of the ecdysteroid titer during *Drosophila* larval development.

At the end of the third larval instar of *Drosophila melanogaster*, an increase in the titer of the steroid hormone ecdysone (ecdysone is used here as a generic term for all ecdysteroids with hormonal activity) triggers the dramatic morphological transformation of a crawling larva into a pupa (39).

From their study of the effects of ecdysone on the pattern of transcription puffs in the polytene chromosomes of the third-larval-instar salivary glands, Ashburner et al. (4) proposed 20 years ago a hierarchical model for the genetic regulation by ecdysone of the cascade of events leading to puparium formation and metamorphosis. According to this model, early puff genes are induced by the binding of an ecdysone-ecdysone receptor (EcR) complex to their regulatory sequences. The products of these puffs were hypothesized to be transcriptional regulators triggering the expression of the late puff genes, which would include some effector genes of puparium formation in salivary glands. The first evidence for the validity of this model at the molecular level came from the cloning and the characterization of the *E74*, *E75*, and *BRC* early puff genes (for a review, see reference 52). As predicted by the model of Ashburner et al., these genes are directly induced by ecdysone, and they encode DNA-binding proteins (1). On the other hand, the *EcR* gene which encodes three isoforms (50) of a protein belonging to the nuclear receptor superfamily has been cloned by Koelle et al. (21). Recent studies (20, 51, 58) have shown that the DNA-binding active form of the EcR is actually a heterodimer composed of the EcR protein and the Ultraspiracle (USP) protein, another *Drosophila* nuclear receptor (15, 35, 48). The observation that the *EcR* gene and the *E74*, *E75*, and *BRC* early puff genes are expressed in most of the ecdysone target larval and imaginal tissues led several authors to emphasize that the Ashburner et al. model could be applicable to every target tissue (7, 34, 46, 53, 56). According to this tissue coordination model, ecdysone activation of overlapping sets of early regulatory genes directs unique patterns of late gene expression in each target tissue at each stage in development.

Although evidence in support of the hypothesis that complex waves of early-expressed regulators determine the tissue- and temporal-specific expression of ecdysone-regulated genes during the third larval instar (16, 18) is accumulating, only limited information is as yet available on the relevant *cis*-acting sequences involved in mediating the transcriptional response of target genes to these factors. A number of ecdysone-responsive genes expressed in various larval and imaginal tissues or in cells in culture have been cloned (1, 47, 49, 55). So far, a high-resolution mapping of the DNA *cis*-regulatory sequences acting as ecdysone response elements (EcREs) has been achieved only in a very few cases. Riddihough and Pelham (40, 41) first identified a functional EcR binding site in a 23-bp element of the *hsp27* promoter. Further studies based on in vitro binding and in vivo cell transfection assays (8, 10, 28, 30, 36, 37, 41) have confirmed this finding and established the 23-bp hsp27 element as a reference EcRE widely used for studies of the EcR (20, 28, 58). This element is characterized by the presence of an imperfect palindrome composed of two

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hexamer half-sites separated by one central nucleotide (see Fig. 4). This structure appears to be critical for EcR binding and in vivo ecdysone response (3, 8, 30, 36, 37). We have shown that the in vitro binding of the EcR requires no more than eight nucleotides on each side of the central base (3), and Cherbas et al. (8) provided evidence in a transfection assay of *Drosophila* cultured cells that a 15-bp element is in fact sufficient for in vivo ecdysone induction. Mutational analyses (3, 30, 36) have identified essential nucleotides for EcR binding to this sequence in keeping with the consensus sequence, RG(G/T)TCANTGA(C/A)CPy, for an EcRE core sequence derived by Cherbas et al. (8) from their study of the EcR binding sites of the Eip28/29 gene.

As a potential target gene of the early ecdysone-induced cascade of regulators, the *Fat body protein 1* (*Fbp1*) gene provides a very valuable system for tackling directly the characterization of functional binding sites of the EcR and other *trans*-acting factors. Previous studies have established that the expression of the *Fbp1* gene is ecdysone dependent and strictly tissue and stage specific. Transcription of its unique mRNA takes place in the larval fat body exclusively and is initiated at the onset of the premetamorphic ecdysteroid peak marked also by a major parallel increase of the early-induced *EcR*, *E74B*, *E75C*, and *BRC* transcripts (2, 23, 25, 38). Mutations impairing accumulation of ecdysteroids at this period abolish the accumulation of the *Fbp1* transcript, which can be restored by supplementing the mutant larvae with 20-hydroxyecdysone (24, 25). The Fbp1 protein, whose role is unknown, shares no homology with any DNA-binding motif, indicating that it plays probably a fat body-specific effector role at puparium formation or at a later stage of metamorphosis. Deletion mapping of the *cis*-acting regulatory sequences of the *Fbp1* gene by germ line transformation has shown that a 70-bp element located in the −138 to −68 region of the promoter contains sequences required for the specific expression of the gene (23). When placed upstream of the heterologous *hsp70* promoter, this element can drive the 20-hydroxyecdysone-dependent expression of an *Escherichia coli lacZ* reporter gene specifically in the fat body of transgenic late-third-instar larvae (23). Hence, this short element exhibits the in vivo properties of a tissue- and stage-specific enhancer and an EcRE.

Using nuclear extracts from late-third-instar fat body and a gel shift assay, we undertook the characterization of the protein-binding properties of the Fbp1 enhancer with a triple aim: (i) to identify the third-larval-instar fat body proteins that bind to the Fbp1 enhancer and that are potentially involved in the specific expression of the *Fbp1* gene, (ii) to investigate the possibility that one of these factors is an EcR and to characterize the binding sequence, and (iii) to identify the constituents of this receptor.

We have shown that several fat body nuclear factors interact with the Fbp1 enhancer in a complex manner and identified one of them as an EcR resulting from the heterodimerization of the EcR and the USP proteins. The in vitro characterization of the EcR/USP binding site and the study of its ecdysone-dependent in vivo occupancy in different genetic backgrounds support the conclusion that the Fbp1 enhancer is a primary functional target of the EcR.

MATERIALS AND METHODS

Mass preparation of fat body from third-instar larvae. Larvae emerging from a 6-h egg collection of the *D. melanogaster* Canton S stock were reared in M medium (100 g of dried yeast per liter, 120 g of sucrose per liter, 17.5 g of agar per liter, 8.3 g of K_2HPO_4 per liter, 9 g of KH_2PO_4 per liter, 1.76 g of methyl hydroxy-4 benzoate per liter, 0.8% propionic acid) at 25°C. When larvae reached the wandering stage, 120 ± 8 h after egg laying, they were collected in water, floated away from the contaminating growth medium by suspension in 2 M NaCl solution, and washed again with water through a 600-μm-pore-size mesh. From this point all operations were carried out at 4°C with media chilled to this temperature. For one standard fat body preparation, 200 g of third-instar larvae was ground with a motor-driven grinding mill, under a continuous flow of FB extraction medium (20 mM sodium glycerophosphate, 10 mM KH_2PO_4, 30 mM KCl, 0.17 M sucrose) from a squeeze bottle. The grindate was passed through a 600-μm-pore-size mesh. Tissue fragments in suspension in the filtrate were collected on a 100-μm-pore-size mesh and transferred to centrifuge tubes with the aid of a squirt of FB extraction medium. After centrifugation (4 min at 3,000 × *g*), floating fat body pieces were collected with a truncated Pipetman tip and put into 1.5-ml Eppendorf tubes. After centrifugation (1 min at 12,000 × *g*), excess FB medium was carefully removed with a Pasteur pipette. By this procedure, approximately 2 to 4 ml of purified fat bodies per 200 g of larvae was obtained.

Fat body nuclear extract. All steps were performed at 4°C. Fat bodies (usually 2 to 4 ml) were homogenized in a Dounce homogenizer in 2 volumes of buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT), 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA; pH 8)] by 20 to 30 strokes with a pestle B. One volume of buffer A supplemented with 1.2 M sucrose was added, and 10 additional strokes were applied. The homogenate was centrifuged (15 min and 5,000 rpm in a Sorvall HB4 rotor). The lipid layer was removed with a tissue, and the supernatant was decanted with a pipette. The pellet was resuspended first in 3 volumes of buffer A with 0.3 M sucrose, and 3 volumes of buffer A with 1.7 M sucrose was added. The homogenate was transferred to a 1.5-ml Eppendorf tube and centrifuged (15 min, at 12,000 × *g*). The pellet was resuspended in 3 volumes of buffer B (10 mM HEPES [pH 7.9], 0.6 M NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 0.5 mM DTT, 0.1 mM EGTA [pH 8]) and gently mixed on a rotary wheel for 30 min. After centrifugation (30 min at 12,000 × *g*), the supernatant was dialyzed overnight against 1 liter of buffer C (20 mM HEPES [pH 7.9], 75 mM NaCl, 20% glycerol, 0.5 mM DTT, 0.1 mM EDTA [pH 8], 0.5 mM phenylmethylsulfonyl fluoride). Buffers A, A–0.3 M sucrose, A–1.2 M sucrose, A–1.7 M sucrose, and B were supplemented with 0.5 mM phenylmethylsulfonyl fluoride and 0.5 μg (each) of leupeptin, chymostatin, aprotinin, antipain, and pepstatin per ml just before use.

Gel shift assay. Three to 5 μg of protein extract, 2 μg of poly(dI-dC), and specific competitor DNA or antibodies, if appropriate, were mixed in binding buffer (25 mM HEPES [pH 7.6], 60 mM KCl, 5% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA [pH 8], 0.75 mM DTT) in a final volume of 16 μl and preincubated for 15 min on ice. All competitions were performed with a 400-fold molar excess of double-stranded oligonucleotides. Assay mixtures that included antibodies contained 2 μl of the DDA2.7 anti-EcR monoclonal antibody (21) or 1 μl of the AB11 anti-USP monoclonal antibody (19). After addition of 2 fmol of probe labeled with ^{32}P at the 5′ end (2.5×10^4 to 7.5×10^4 cpm/fmol), incubation was continued for 15 min at 4°C. Free and complexed DNAs were separated at 4°C in a low-ionic-strength 4% polyacrylamide gel (39:1 cross-linking ratio) containing 25 mM Tris base, 190 mM glycine, 1 mM EDTA, and 2.5% glycerol. The gel was prerun (25 mA for 1 h).

After electrophoresis (25 mA for 3 h), the gel was dried and autoradiographed.

Oligonucleotides were synthesized with a DNA synthesizer (Pharmacia) and purified as recommended by the manufacturer. Probes and competitor oligonucleotides as listed in the figures were obtained by annealing of complementary strands. All gel shift experiments were repeated at least twice.

Methylation interference experiments. The upper strand of the 27-bp hsp27 EcRE (5′AGACAAGGGTTCAATGCACT TGTCCAA3′) and the lower strand of the 33-bp D3 fragment (5′TTGACTCCCGATTGGGTTGAATGAATTTTGCTG3′) were 5′ end labeled and annealed with their complementary strands. The ^{32}P-labeled DNA was partially methylated by a 20-min treatment in 0.5% dimethyl sulfate (DMS) as described by Sakonju and Brown (43). Twenty femtomoles of methylated DNA was incubated with 40 μg of fat body protein extract in a 120-μl reaction mixture under the conditions described above. The mixture was then loaded onto a 4% gel, and the protein-free DNA and EcR/USP-DNA complex were separated by electrophoresis. The EcR/USP-DNA complex was excised from the gel and extracted. Methylated DNA was then phenol-chloroform extracted and purified by using a Nacs column (Pharmacia), precipitated, and subjected to piperidine cleavage by the method of Maxam and Gilbert (32). The DNA was finally dissolved in loading buffer containing formamide and analyzed on a 15% sequencing gel.

In vivo genomic DNA footprinting. The *ecd1^{ts} st ca Drosophila* strain was kept at 20°C. Embryos from 80 to 100 flies were collected over 30-min laying periods and allowed to develop at 20°C until the L1 stage (33 h). In order to help identify the synchronized larvae, groups of 50 newly emerged first-instar larvae were transferred onto standard medium supplemented with bromophenol blue (29) and reared at 20°C until the beginning of the third larval stage (98 h posthatching). At this time, groups of larvae were maintained at 20°C while other groups were shifted to the restrictive temperature of 29°C. Some 44 to 46 h later, fat bodies from 15 to 20 larvae were hand dissected on ice in *Drosophila* Schneider medium (45). Tissues were transferred into new cold medium and kept on ice for 60 min until methylation.

Male larvae hemizygous for the *dor^{22}* (*l(1)t187*) mutation (26) were obtained by crossing *y*, *dor^{22}/FM6*, *l^{69}j* females with Canton S males. The embryos were allowed to develop at 20°C on standard medium supplemented with bromophenol blue. Eight days later, 15 to 20 late-third-instar larvae were hand-dissected to isolate fat body tissues under the conditions described above.

In vivo methylation was carried out at room temperature for 30 to 40 s in a 0.5% solution of DMS prepared in Schneider medium. The methylation reaction was quenched by four washing steps at room temperature in Schneider medium. Tissues were then incubated in 200 μl of extraction buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 10 mM EDTA, 0.2% sodium dodecyl sulfate, 50 μg of proteinase K per ml) at 37°C for 5 to 16 h. Subsequently, the homogenate was extracted four times with 200 μl of phenol-chloroform. DNA was ethanol precipitated, washed in 80% ethanol, and dissolved in 90 μl of water. RNase treatment was omitted. DNA samples were kept on ice until piperidine cleavage.

Naked DNA (control protein-free DNA) was extracted from 16- to 20-h-old Canton S embryos. After deproteinization and purification (13), 10-μg aliquots of DNA were methylated in vitro at room temperature for 1 min in a 0.5% DMS aqueous solution under the conditions described by Maxam and Gilbert (32). The DNA was dissolved in 90 μl of bidistilled water for piperidine cleavage. In vivo- and in vitro-methylated DNA was cleaved by 10% piperidine hydrolysis and purified as previously reported (32).

For ligation-mediated PCR amplification, linkers were ligated to 100 ng of piperidine-cleaved DNA as described by Mueller and Wold (33). A specific set of primers complementary to the *Fbp1* promoter lower strand was designed as follows: primer 1, −285GCTTGTGACCCACAAAAT−268 (Sequenase extension reaction); primer 2, −166CATAATGA GTGAGCGGTTTTTTTAGGAGC−138 (PCR amplification); and primer 3, −154GCGGTTTTTTTAGGAGCTTC−135 (labeling reaction).

Under these conditions, a DNA region downstream of position −135 could be scanned on the lower strand. The annealing temperature of primer 1 was set to 50°C, while the annealing temperatures of primers 2 and 3 were empirically determined and were set to 59 and 62°C, respectively. The exponential amplification and the labeling reaction were carried out with 20 cycles and 3 to 5 cycles, respectively. The labeled amplified products were phenol-chloroform extracted, precipitated with ethanol, and resuspended in 12 μl of formamide-dye mixture. Two microliters of each DNA sample was loaded on an 8% polyacrylamide-urea sequencing gel. The dried gel was autoradiographed without an intensifying screen.

In vivo DMS footprinting analysis of the upper strand of the *Fbp1* promoter was not performed because the DNA sequence of this strand contains very few guanine residues. In particular, the upper strand of the region between −106 and −86, which includes the EcR/USP binding site, does not contain any guanine residue (see Fig. 5).

RESULTS

Fbp1 enhancer binding activities in late-third-instar fat body nuclear extracts. Standard procedures were modified to prepare nuclear extracts from large quantities of pure fat body tissue from synchronized late-third-instar larvae (see Materials and Methods). Seven distinct nucleoprotein complexes were detected when a 74-bp DNA fragment containing the 70-bp Fbp1 enhancer (−138 to −69) and four additional bases (5′AATT3′) for an end-filling labeling reaction was used as a probe in gel retardation assays with these extracts (Fig. 1). The same retardation pattern was obtained with a PCR-amplified 70-bp Fbp1 enhancer as a probe, indicating that the artificial sequence created by the four flanking bases in the 74-bp probe was not involved in the formation of any of the seven complexes (data not shown).

Competition mapping of the binding sites. The formation of complexes 1 to 7 was inhibited in the presence of a 400-fold molar excess of unlabeled 74-bp Fbp1 enhancer, indicating that these complexes are sequence specific (Fig. 1). In order to determine the position of the binding sites involved in the formation of these complexes, we used as competitors a 400-fold molar excess of unlabeled double-stranded oligonucleotides corresponding to various overlapping subregions of the Fbp1 enhancer (Fig. 1).

(i) Complex 1. Binding of complex 1 to the Fbp1 enhancer was inhibited by oligonucleotides D, D2, and D3. Thus, the protein(s) responsible for the formation of this complex must bind to sequences between −85 and −109 (D region). In addition, neither oligonucleotide A nor oligonucleotide B, whose sequences overlap the D region, inhibited complex 1, indicating that its formation involves sequences at the junction between the A and B regions.

(ii) Complexes 2 and 3. Surprisingly, oligonucleotides A and B and all the overlapping oligonucleotides C, C2, D, D2, D3, E,

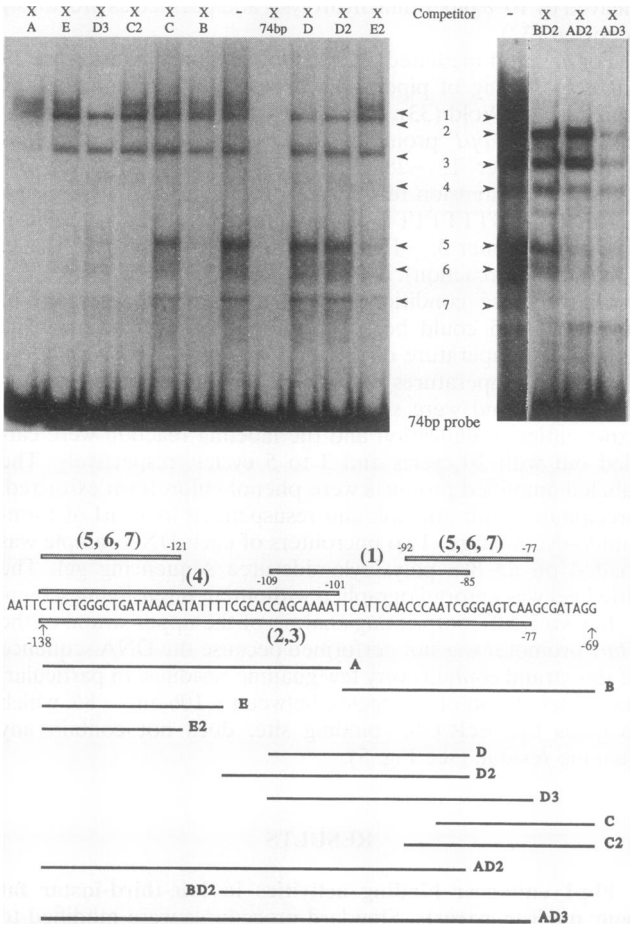

FIG. 1. Analysis of Fbp1 enhancer binding activities in late-third-instar fat body nuclear extracts. A 74-bp DNA fragment containing the 70-bp Fbp1 enhancer (−138 to −69) and four additional bases (5′AATT3′) for an end-filling labeling reaction was used as a probe in a gel shift assay in the absence (−) or in the presence (X) of various unlabeled DNA competitors (indicated above each lane). Nucleoprotein complexes (arrowheads) are referred to as 1 through 7. The nucleotide sequence of the Fbp1 enhancer is numbered (−69 to −138) relative to the transcription start site of the *Fbp1* gene (31). The position and extent of the oligonucleotides used as competitors are indicated (thin lines) below the Fbp1 enhancer sequence. The positions of putative binding regions for the complexes, as deduced from the competition experiments, are indicated (stippled boxes).

and E2 failed to inhibit complexes 2 and 3 (Fig. 1; see also Fig. 2 and 3), suggesting that formation of complexes 2 and 3 requires a very large part of the Fbp1 enhancer sequence. In order to map the binding sites involved in the formation of complexes 2 and 3, we used three additional longer fragments, AD2, AD3, and BD2, as competitors (Fig. 1). Oligonucleotides AD2 and BD2 failed to compete, indicating that the binding sites for both complex 2 and complex 3 are not completely included in either of these two overlapping regions. By contrast, oligonucleotide AD3 competed efficiently for the formation of both complexes 2 and 3, indicating that their binding sites are fully contained in a 62-bp region (AD3) which corresponds to the almost complete 70-bp Fbp1 enhancer sequence.

(iii) Complex 4. The formation of complex 4 was inhibited by oligonucleotide A, but neither oligonucleotide E nor oligonucleotide D2 showed competition activity for this complex. Thus, the complex 4 binding site located in the A region must involve sequences at the junction between the E and D2 regions.

(iv) Complexes 5, 6, and 7. Oligonucleotides A and B each competed for the formation of complexes 5, 6, and 7, indicating that the Fbp1 enhancer carries at least two distinct binding sites responsible for these complexes. Oligonucleotides E and E2 competed for the formation of complexes 5, 6, and 7, indicating that their binding site on the A side is localized within the E2 region, between −121 and −138. The binding site within B is probably located in the region that is common to both oligonucleotide C2 and oligonucleotide D3 (−77 to −92), because these two fragments competed for complexes 5, 6, and 7 while oligonucleotide C or D alone failed to compete. Competition was slightly more efficient with oligonucleotide A than with oligonucleotide B, indicating that affinities of complexes 5, 6, and 7 for the binding site localized in the A region are higher than those for the binding site localized in the B region.

When the competitor oligonucleotides were used separately as radioactive probes in gel shift assays with fat body nuclear extract, they all yielded the retardation patterns expected from the mapping of the different complexes described above (data not shown).

Mutational mapping of the binding sites. The competition experiments described above allowed us to narrow down the putative binding regions for each of the seven complexes. To further define the sequences important for the formation of these complexes, we designed mutations in the middle of these regions. Blocks of four adjacent nucleotides were changed in wild-type oligonucleotides at positions hereafter designated by Greek letter symbols (Fig. 2B). These mutated oligonucleotides were used as competitors.

(i) The δ, ε, and χ sites are involved in the formation of complex 1. In contrast to the corresponding wild-type oligonucleotides, none of the oligonucleotides D3δ, 70ε, and Dχ (Fig. 2B) competed for the formation of complex 1 (Fig. 2A and data not shown), showing that the binding site involved in the formation of this complex includes positions δ, χ, and ε.

(ii) The γ site is involved in the formation of complex 4. The formation of complex 4 was inhibited by oligonucleotide A, but the mutated form Aγ failed to compete (Fig. 2A). In contrast, the mutated forms Aα, Aλ, and Aη (Fig. 2B) still competed (Fig. 2A and data not shown), indicating that the sequence that is essential for the formation of complex 4 is centered around the γ position, between the λ and the η positions (Fig. 2B).

(iii) The αλ and β sites are involved in the formation of the complexes 5, 6, 7, 2, and 3. As described above, both region A and region B of the Fbp1 enhancer carry binding sites for complexes 5, 6, and 7. The three mutated forms Aα, Bβ (Fig. 2A), and Aλ (data not shown) failed to compete for the formation of complexes 5, 6, and 7. On the other hand, mutated oligonucleotides Aγ, Bε (Fig. 2A), and Bφ (data not shown) still competed efficiently for the formation of these complexes. Taken as a whole, these results indicate that two separate binding sites for complexes 5, 6, and 7 are centered around the αλ and β positions. This was confirmed by the fact that the 70α, 70β (Fig. 2B), and 70λ (not shown) mutant forms still inhibited complexes 5, 6, and 7 while the double-mutant form, 70αβ, completely failed to compete (Fig. 2A).

Unexpectedly, the 70α, 70β (Fig. 2A), and 70λ (data not shown) variants of the complete 70-bp Fbp1 enhancer also totally failed to inhibit complexes 2 and 3. This result indicated that αλ and β intact sites are simultaneously required for the binding of these complexes to the Fbp1 enhancer. This conclusion is consistent with the finding that only the AD3

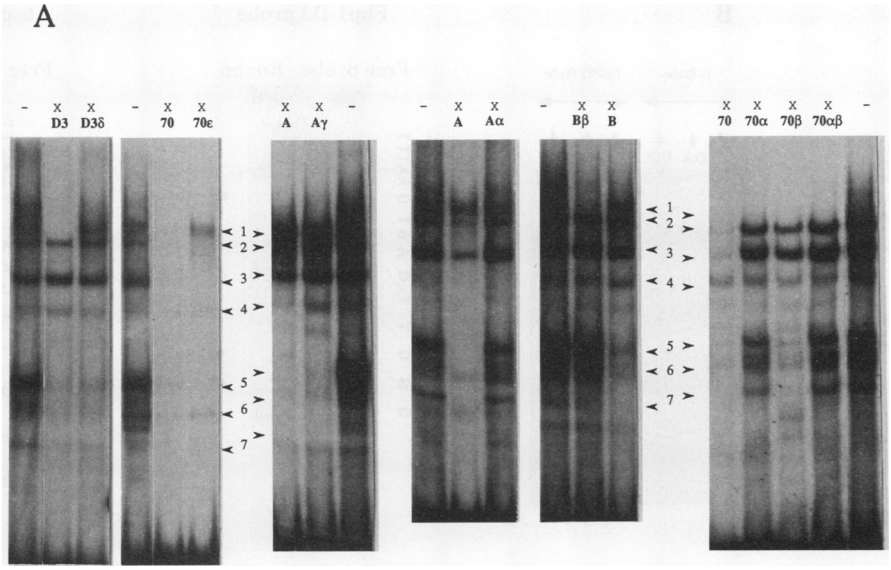

FIG. 2. Mutational mapping of the binding sites involved in the formation of complexes 1 to 7. (A) The 70-bp Fbp1 enhancer was used as a radioactive probe in a gel shift assay as described in the legend to Fig. 1 in the absence (−) or in the presence (X) of wild-type or mutated DNA competitors. (B) Position and sequence of the mutated DNA competitors relative to the Fbp1 enhancer. Mutated DNA competitors are derived from the wild-type DNA competitors indicated by capital roman letters. For each mutated DNA competitor, the block of four altered nucleotides (Greek letter) and the position of this mutation relative to the wild-type sequence (bracket) are indicated. Positions of the binding sites for the complexes 1 to 7 as deduced from the competitions are indicated (hatched boxes).

fragment and the whole 70-bp Fbp1 enhancer inhibit these complexes (Fig. 1). As expected from this finding, the double mutant 70αβ also completely failed to compete for the formation of complexes 2 and 3. In addition, complexes 2 and 3 were inhibited by the mutated forms 70ε (Fig. 2A) and 70γ (data not shown), suggesting that their formation does not require specific intervening sequences between the αλ and β sites.

Involvement of EcR and USP proteins in complex 1 formation. Because functional studies have pointed out that the Fbp1 enhancer acts as an EcRE, we wished to ascertain whether one or several of the factors responsible for the formation of complexes 1 to 7 were an EcR. To address this question we used, as a competitor, an EcR binding site: the hsp27 EcRE (41) (Fig. 3C). Of the seven complexes formed with the 70-bp Fbp1 enhancer, only complex 1 was specifically inhibited by the hsp27 EcRE, as well as by oligonucleotide D (Fig. 3C), which contains the binding site for this complex (Fig. 3A). When used as radioactive probes in the presence of fat body nuclear extract, both oligonucleotide D and oligonucleotide hsp27 EcRE gave rise to a major band of decreased mobility (Fig. 3B). In both cases this band was inhibited by the Fbp1 enhancer and oligonucleotide D3 but not by the mutant forms 70ε, Dχ, and D3δ (data not shown). These results provide evidence that the binding site for complex 1 in the Fbp1 enhancer and the hsp27 EcRE bind the same factor(s) in fat body nuclear extracts. To test whether this factor is an EcR/USP heterodimer (20, 51, 58), binding of the D and hsp27 EcRE probes was carried out in the presence of antibodies raised against the EcR (a gift from D. S. Hogness) or against the USP protein (a gift from F. C. Kafatos). The complexes formed with these two probes were supershifted in the presence of both the anti-EcR and the anti-USP monoclonal antibodies (Fig. 3B). Thus, both the hsp27 EcRE and the D region of the Fbp1 enhancer, hereafter referred to as the Fbp1-D EcRE, bind an EcR/USP heterodimer in fat body nuclear extract. However, quantitative comparison of nucleoprotein complexes in Fig. 3B indicates that the binding affinity of the Fbp1-D EcRE for the EcR/USP heterodimer is lower than that of the hsp27 EcRE.

Identification of guanine contact points between the EcR/USP complex and Fbp1 and hsp27 sequences. The binding site for complex 1 covered by the χ, δ, and ε positions of the Fbp1 enhancer and the hsp27 EcRE share extensive sequence similarity at 12 positions over a 15-bp region that contains an imperfect palindromic structure consisting of two hexamer

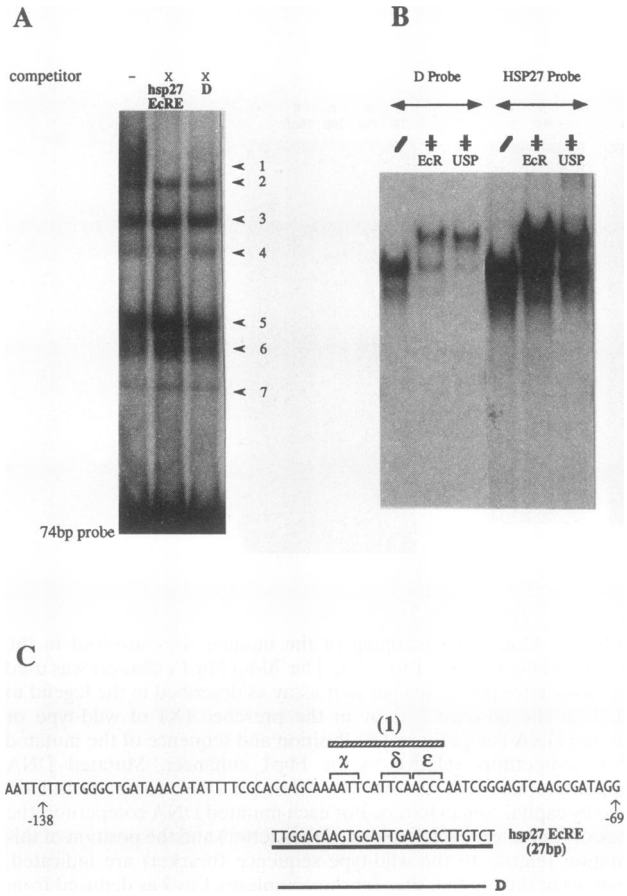

FIG. 3. Complex 1 involves an EcR/USP heterodimer. (A) The 70-bp Fbp1 enhancer was used as a radioactive probe in a gel shift assay as indicated in the legend to Fig. 1 in the absence (−) or in the presence (X) of oligonucleotide D or the hsp27 EcRE as competitors. (B) The D region of the Fbp1 enhancer and the hsp27 EcRE were used as radioactive probes in a gel shift assay with fat body nuclear extract in the absence (/) or in the presence (#) of a monoclonal antibody raised against the EcR or the USP proteins. (C) Position of oligonucleotide D (−85 to −109) relative to the Fbp1 enhancer. Positions (brackets) of the mutations (Greek letters) that abolish the formation of the complex 1 and position of the binding site for this complex (hatched box) as deduced from Fig. 2 are indicated. The nucleotide sequence of the 27-bp hsp27 EcRE is also indicated.

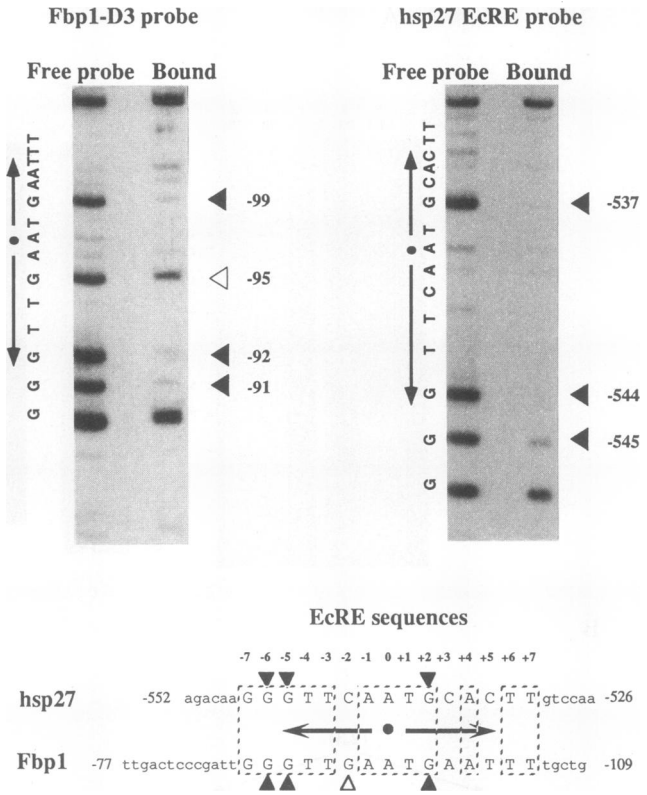

FIG. 4. DMS methylation interference analysis of EcR/USP contact points with the Fbp1 and hsp27 EcREs. DMS methylation interference analysis was performed with the double-stranded oligonucleotide D3 labeled at the 5′ end of the lower strand (left-hand side) and with the double-stranded hsp27 EcRE (41) labeled at the 5′ end of the upper strand (right-hand side). The sequences of both of these DNA fragments are indicated at the bottom. The free probe and the EcR/USP-specific retarded band were the sources of the material in the lanes labeled Free probe and Bound, respectively. Black triangles indicate Gs whose methylation interferes with EcR/USP binding. An open triangle indicates a G whose methylation interferes slightly with EcR/USP binding. Methylation of Gs downstream of −90 in the *Fbp1* sequence and upstream of −546 in the *hsp27* sequence does not interfere with EcR/USP binding (not shown). Sequence comparison of the Fbp1-D and hsp27 EcRE is shown at the bottom. The nucleotide sequence of the 27-bp hsp27 EcRE between −552 and −526 relative to the transcription start site of the hsp27 gene is given (40). The positions of nucleotides relative to the imperfect palindromic structure of the EcREs (arrows) are given above the sequences.

half-sites separated by a single intervening nucleotide (Fig. 4). In vivo and in vitro experiments have provided evidence that, in the case of the hsp27 EcRE, this imperfect palindrome is directly responsible for the binding of the EcR (8, 30, 36, 37).

In order to visualize precisely the bases in the Fbp1-D and hsp27 EcRE sequences that interact with the EcR/USP heterodimer, we performed methylation interference analyses with the D3 and hsp27 EcRE probes. In both cases, methylation of guanines at positions −6, −5, and +2 relative to the central base of the palindromic structure strongly interfered with the binding of the EcR/USP complex, indicating that these three positions are important for the interaction with the EcR/USP complex (Fig. 4). These G positions are strictly conserved between the Fbp1 and hsp27 sequences. In contrast, position −2 is occupied by a G residue in the Fbp1 sequence and a C residue in the hsp27 sequence (Fig. 4). This sequence divergence suggested that the identity of the base at this position is not important for the binding of the EcR/USP complex. Consistent with this supposition, methylation of the G residue at this position in the Fbp1 sequence interfered only weakly with the binding of the EcR/USP heterodimer (Fig. 4). Taken as a whole, these results demonstrate that the critical sequences for EcR/USP binding overlap the imperfect palindromic sequences of the Fbp1-D and hsp27 EcREs.

In vivo protection of the Fbp1-D EcRE is correlated with the expression of the *Fbp1* gene. The characterization of the in vitro binding sites of the EcR/USP heterodimer and other complexes made it possible to examine by ligation-mediated PCR genomic footprinting (33) their in vivo occupancy in correlation with the expression of the *Fbp1* gene (Fig. 5). For this purpose, we used the $ecd1^{ts}$ and dor^{22} mutations that lead to a drastic decrease in the ecdysteroid titer during the third larval instar (6, 11).

In larvae homozygous for the $ecd1^{ts}$ mutation, the *Fbp1* gene

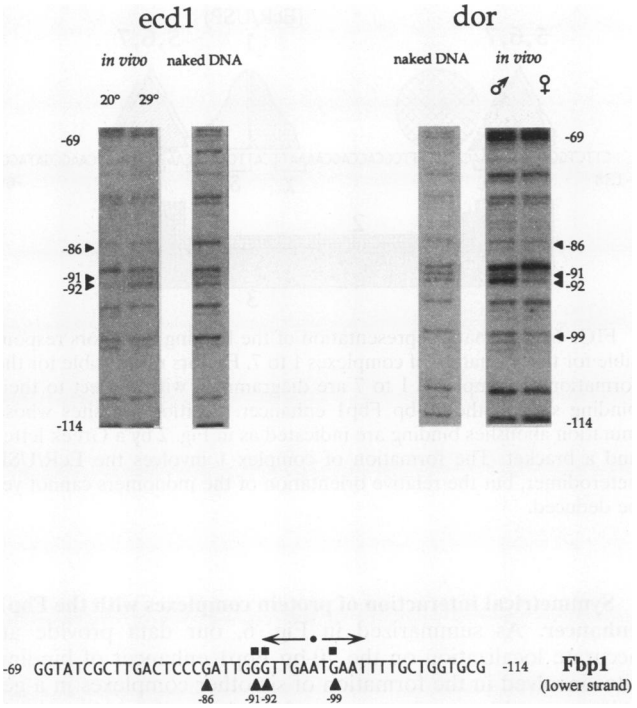

FIG. 5. In vivo DMS footprinting of the Fbp1-D EcRE region (lower strand). Ligation-mediated PCR products revealed with the ^{32}P-labeled primer 3 were analyzed (see Materials and Methods). Naked DNA corresponds to purified DNA cleaved in vitro at guanine-specific locations. All the other lanes correspond to DNA originating from the fat body of late-third-instar larvae treated in vivo with DMS as described in Materials and Methods. The in vivo ecd1 lanes correspond to DNA from in vivo DMS-treated fat body from *ecd1^{ts}* larvae raised at 20°C (permissive temperature) or 29°C (nonpermissive temperature). The in vivo dor lanes correspond to DNA from in vivo DMS-treated fat body from hemizygous *dor^{22}* males or heterozygous *dor^{22}* females. Positions of guanines whose methylation, in vivo, is inhibited are indicated relative to the transcription start site of *Fbp1* (black triangles). In vivo-protected sequences of the Fbp1-D EcRE region are shown at the bottom relative to the imperfect palindromic structure (arrows). Guanines whose methylation interferes in vitro with EcR/USP binding (Fig. 4) are indicated by black squares.

is normally expressed at the end of the third larval instar at the permissive temperature (20°C). Conversely, if these larvae are transferred to the nonpermissive temperature (29°C) at the beginning of the third larval instar, the ecdysteroid titer remains low during this stage (11) and, consequently, the *Fbp1* gene is not expressed (24). We compared the methylation patterns of in vivo DMS-methylated DNA from the fat body of third-instar *ecd1^{ts}* larvae raised at 20 and at 29°C during the third instar. Although the set of primers designed for this genomic footprinting experiment allowed us to scan the region of the *Fbp1* promoter downstream of position −135 (see Materials and Methods), reproducible changes in DMS reactivity of guanine (G) residues were observed only in the −85 to −109 D region (Fig. 3) of the Fbp1 enhancer.

Three G residues (−86, −91, and −92) were protected against the methylation by DMS in the late-third-instar larvae raised at 20°C compared with that in naked DNA (Fig. 5). Consistently, the same protection pattern was obtained when in vivo footprinting experiments were performed with late-third-instar larvae from the wild-type Canton S strain (data not shown). Contrastingly, in *ecd1^{ts}* larvae raised at 29°C, in which there is no expression of the *Fbp1* gene, G residues −86 and −92 became unprotected against DMS, and reactivity of residue −91 was enhanced (Fig. 5).

A similar study was performed with larvae carrying the nonconditional recessive *dor^{22}* mutation located on the X chromosome. In homozygous females and in hemizygous males, this mutation abolishes all ecdysone-induced puffing activity in third-instar larvae (6). In vivo footprinting of the DNA from heterozygous *dor^{22}* females, in which the *Fbp1* gene is normally expressed (25), showed the same protection of guanines −86, −91, and −92 as seen in *ecd1^{ts}* larvae at 20°C. A slight but not reproducible protection of guanine −99 was also observed. In addition, the G residue −90 shows a strong hypersensitivity to DMS compared with the in vitro methylation of naked DNA. In the hemizygous *dor* male larvae, in which *Fbp1* is not expressed (25), the G residues −86, −91, and −99 were consistently more reactive than in the heterozygous females and the G residue −92 was markedly hypersensitive to DMS. The hypersensitivity of G residue −90 was similar to that observed in females.

These results all showed that the in vivo occupancy of the −86 to −99 region by DNA-binding factors is ecdysteroid dependent.

DISCUSSION

An EcR/USP heterodimer present in a late-third-instar fat body nuclear extract binds to the Fbp1 enhancer. This finding is a demonstration that the DNA-binding active form of the EcR in a nuclear extract from a single, homogeneous, ecdysone-responsive larval tissue is a heterodimer between the EcR and USP proteins. The binding site for this heterodimer in the Fbp1 enhancer (Fbp1-D EcRE) contains an imperfect palindromic sequence, as is the case for the hsp27 EcRE (41) (Fig. 4). Either disruption of this sequence by mutagenesis or methylation of guanines −91, −92, and −99 abolishes the EcR/USP binding. This provides a strong indication that the two half-parts of the pseudopalindromic structure participate in the binding of the EcR/USP heterodimer. Further evidence is provided by the fact that oligonucleotides Dχ and 70ε, which are altered at the left and right half-palindromes, respectively, do not compete for the formation of the DNA-EcR/USP complex. Taken together, these results strongly argue that the EcR and USP molecules each recognize one arm of the EcRE, as is the case for other members of the nuclear receptor superfamily (14, 22, 27, 44, 54, 57). In addition, these results imply that EcR and USP monomers cannot each bind a half-palindrome by itself. These conclusions are in complete agreement with the demonstration that in vitro-translated EcR or USP molecules cannot bind alone to the hsp27 EcRE (3a, 51, 58). Because the hsp27 and the Fbp1-D EcREs are not perfectly palindromic, and thus are asymmetric, the question as to whether the EcR and USP molecules bind specifically one or the other arm of the EcR/USP binding site arises.

The fact that the three guanines whose methylation interferes with the EcR/USP binding are strictly conserved in the hsp27 and the Fbp1-D EcREs (Fig. 4) and match the EcRE core consensus sequence RG(G/T)TCANTGA(C/A)CPy first proposed by Cherbas et al. (8) adds support to the relevance of this consensus. However, nucleotides at positions −2, +3, and +5 of the Fbp1-D EcRE (Fig. 4) differ from those at the same position in the hsp27 EcRE sequence and do not match this consensus. The binding affinity of the Fbp1-D EcRE for the EcR/USP heterodimer is lower than that of the hsp27 EcRE, and we have further shown that sequence divergence at

position +5 but not at position −2 was responsible for this lower binding affinity (3). These results, which are in agreement with a mutational analysis of the hsp27 EcRE reported recently (36), provide evidence that EcR/USP binding sites allow important sequence variations that, nonetheless, may have a strong influence on their affinity.

In vivo occupancy of the Fbp1-D EcRE region. The degree of in vivo protection of the −86, −91, and −92 guanine residues against methylation by DMS in the fat body at the end of the third larval instar is correlated with the expression of the *Fbp1* gene. These guanines are protected when the gene is actively transcribed, but they are much less or not at all protected in mutant dor^{22} or $ecd1^{ts}$ third-instar larvae in which it fails to be expressed. The dor^{22} and $ecd1^{ts}$ mutations have pleiotropic effects, one of which is a reduction in the ecdysteroid titer in the larvae. The consequent absence of expression of *Fbp1* in the mutant larvae can be bypassed by adding 20-hydroxyecdysone in their food. The presence of unprotected guanines −86, −91, and −92 in dor^{22} and $ecd1^{ts}$ larvae thus suggests that these bases are involved in the binding of ecdysteroid-dependent factors.

Since methylation of guanines −91 and −92 interferes with the in vitro binding of the EcR/USP heterodimer, their in vivo protection is likely due to the ecdysteroid-dependent interaction of this factor with the EcRE. However, because the synthesis of EcR is known to be ecdysone induced (18), it is not yet clear whether the absence of this protection in the dor^{22} and $ecd1^{ts}$ mutants is due simply to a reduced ecdysteroid titer or to a reduced level of EcR.

In vivo footprinting experiments revealed either no protection or only slight and irreproducible protection of guanine −99 against the action of DMS when *Fbp1* is expressed. This was not expected, because methylation of this residue interferes with the in vitro binding of the EcR/USP heterodimer. Partial or transient in vivo occupancy of the genomic EcR/USP binding sites by the EcR/USP heterodimer could explain this apparent discrepancy between the in vitro and in vivo results.

The slight but reproducible in vivo protection of guanine residue −86 also appears to be ecdysteroid dependent. The EcR/USP heterodimer is probably not directly responsible for this protection. We have shown that eight nucleotides on each side of the central nucleotide of the imperfect palindrome are sufficient for the in vitro binding of the EcR to the hsp27 EcRE (3). We can thus assume that the guanine −86 lies beyond the minimal site required for binding of the heterodimer to the Fbp1 EcRE. Accordingly, methylation interference experiments show that methylation of guanine −86 does not interfere with the in vitro binding of the EcR/USP heterodimer (Fig. 4). In addition, mutation β, which consists of a change in nucleotides −87 to −84, does not prevent EcR/USP binding. It does however prevent the binding of the factors responsible for the formation of the complexes 2, 3, 5, 6, and 7. Thus, in vivo protection of guanine −86 could be due to one or several of these factors. It is interesting to draw a parallel with in vivo changes in DMS reactivity which were first interpreted as glucocorticoid-dependent binding of the glucocorticoid receptor to a target site in the *TAT* gene (5) and have now been shown to result from the glucocorticoid-induced binding of the liver-specific HNF5 factor to an overlapping site (42). Similarly, our in vivo footprinting experiments suggest that a factor(s) distinct from the EcR/USP heterodimer could bind closely to its binding site in an ecdysteroid-dependent manner.

These observations open the way to further work directed at the examination of the potential interactions of these factors with the EcR/USP complex in relation with the in vivo developmental specificities of expression of the *Fbp1* gene.

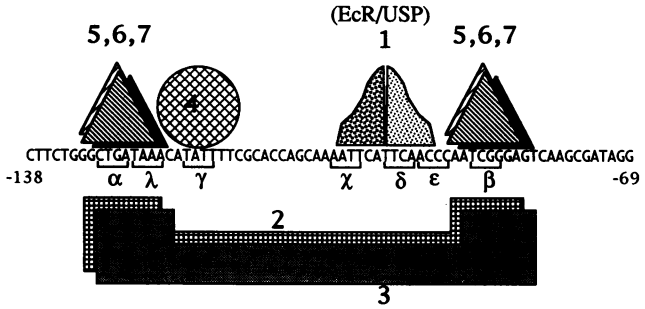

FIG. 6. Schematic representation of the binding of factors responsible for the formation of complexes 1 to 7. Factors responsible for the formation of complexes 1 to 7 are diagrammed with respect to their binding site on the 70-bp Fbp1 enhancer. Positions of sites whose mutation abolishes binding are indicated as in Fig. 2 by a Greek letter and a bracket. The formation of complex 1 involves the EcR/USP heterodimer, but the relative orientation of the monomers cannot yet be deduced.

Symmetrical interaction of protein complexes with the Fbp1 enhancer. As summarized in Fig. 6, our data provide an accurate localization on the 70-bp Fbp1 enhancer of binding sites involved in the formation of six other complexes in a gel shift assay with a nuclear extract from fat bodies of late-third-instar larvae. The nature of the factors responsible for the formation of these complexes remains to be determined. However, it emerges from our analysis that these complexes are organized in a remarkably symmetrical fashion.

(i) Complexes 5, 6, and 7. Our results clearly demonstrate that the same factors involved in the formation of these complexes can bind at two distinct positions, αλ and β.

A simple explanation for the observation that multiple factors bind to the same sequences at each position is that these factors derive from the same protein by proteolysis. Artifactual proteolysis taking place in the fat body nuclear extract appears, however, unlikely, since we used a cocktail of broad-spectrum antiproteases and obtained reproducible retardation patterns with independent batches of fat body nuclear extract. An alternative possibility is that the three factors are related but distinct DNA-binding proteins which share the same sequence specificity for binding. The formal possibility that the formation of complexes 5, 6, and 7 may involve distinct factors having different but overlapping binding sites localized around both the αλ and β positions cannot be dismissed. The resolution of our mutational mapping did not allow us to determine precisely the nucleotides involved in the binding of each factor, a question which could be approached by using methylation interference experiments.

Surprisingly, the αλ and β positions lack any sequence similarity (Fig. 2). Although the binding affinities for the formation of complexes 5, 6, and 7 appear higher for sequences located around the αλ position than for sequences located around the β position, each position is bound with a high sequence specificity. Only oligonucleotides containing the αλ or β position can inhibit complex 5, 6, or 7, and only mutations of these positions abolish the competition. This situation is not without precedent, and several transcription factors such as the C/EBP protein have been shown to bind to very divergent or even completely different sequences (12, 17).

(ii) Complexes 2 and 3. The formation of complexes 2 and 3 involves a long DNA sequence which extends from −138 to −77 and corresponds to almost the complete sequence of the Fbp1 enhancer. Competition results indicate that the forma-

tion of complex 2 or 3 requires the simultaneous binding of a factor(s) to two separate and discrete DNA sites centered on the αλ and β positions. Although sequences between the αλ and β positions do not appear to be involved in the formation of complexes 2 and 3, the distance requirement between these sites remains to be tested. It is possible that factors involved in formation of complexes 2 and 3 could establish a bridge between the two widely separate sites. This would result in looping of the intervening DNA in a manner similar to that achieved by some prokaryotic DNA-binding proteins (9). Another intriguing feature is that the formation of complexes 2 and 3 involves the same sites as those required for the formation of complexes 5, 6, and 7. Competition of complexes 5, 6, and 7 with oligonucleotide A or B does not abolish the formation of complexes 2 and 3. This excludes that complexes 2 and 3 simply result from a simultaneous but independent binding of factors responsible for the formation of complex 5, 6, or 7 to the αλ and β sites. However, this leaves open the possibility that factors responsible for the formation of complexes 5, 6, and 7, when bound to the αλ and β sites borne on the same DNA molecule, interact in a synergistic manner leading to the formation of complexes 2 and 3.

As discussed above for complexes 5, 6, and 7, complexes 2 and 3 could correspond to two proteolyzed forms of the same factor. On the other hand, complexes 2 and 3 could contain two related factors having the same sequence specificity. Again, our mutation mapping suggests that the factors responsible for the formation of complexes 2 and 3 share the same sequence specificity but does not exclude the possibility that these factors have distinct but overlapping binding sites in both the αλ region and the β region.

It is remarkable that the seven complexes we identified interact with the Fbp1 enhancer in a symmetrical manner, with complexes 2, 3, 5, 6, and 7 framing complex 4 and complex 1 (EcR/USP heterodimer) (Fig. 6). This organization may have in vivo functional implications on chromatin structure and accessibility of other factors, such as the EcR/USP heterodimer and factor 4, whose in vitro binding is nonetheless independent from that of any of the other factors.

To our knowledge, the Fbp1 enhancer is the first example of an element shown both to confer a correct developmentally regulated ecdysone inducibility in the context of the whole organism (23) and to bind, in vitro, the EcR/USP heterodimer as shown in this work. In this respect, our results argue strongly in favor of the hypothesis that the EcR/USP heterodimer directly regulates the early expression of effector genes such as *Fbp1* as part of the genetic cascade controlling puparium formation.

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REFERENCES

1. **Andres, A. J., and C. S. Thummel.** 1992. Hormones, puffs and flies: the molecular control of metamorphosis by ecdysone. Trends Genet. **8**:132–138.
2. **Andres, J. A., J. C. Fletcher, F. D. Karim, and C. S. Thummel.** 1993. Molecular analysis of the initiation of insect metamorphosis: a comparative study of *Drosophila* ecdysteroid-regulated transcription. Dev. Biol. **160**:388–404.
3. **Antoniewski, C., M. Laval, and J.-A. Lepesant.** 1993. Structural features critical to the activity of an ecdysone receptor binding site. Insect Biochem. Mol. Biol. **23**:105–114.

3a. **Antoniewski, C.** Unpublished data.

4. **Ashburner, M., C. Chihara, P. Meltzer, and G. Richards.** 1974. Temporal control of puffing activity in polytene chromosomes. Cold Spring Harbor Symp. Quant. Biol. **38**:655–662.
5. **Becker, P. B., B. Gloss, W. Schmid, U. Strähle, and G. Schütz.** 1986. In vivo protein-DNA interactions in a glucocorticoid response element require the presence of the hormone. Nature (London) **324**:686–688.
6. **Biyasheva, Z. M., E. S. Belyaeva, and I. F. Zhimulev.** 1985. Cytogenetic analysis of the X-chromosome region 2B1-2-2B9-10 of *Drosophila melanogaster*. V. Puffing disturbance carried by lethal mutants of gene *dor* are due to ecdysone deficiency. Chromosoma **92**:351–356.
7. **Burtis, K. C., C. S. Thummel, W. Jones, F. D. Karim, and D. S. Hogness.** 1990. The *Drosophila* 74EF early puff contains *E74*, a complex ecdysone-inducible gene that encodes two *ets*-related proteins. Cell **61**:85–99.
8. **Cherbas, L., K. Lee, and P. Cherbas.** 1991. Identification of ecdysone response elements by analysis of the *Drosophila Eip28/29* gene. Genes Dev. **5**:120–131.
9. **Chleif, R.** 1992. DNA looping. Annu. Rev. Biochem. **61**:199–223.
10. **Dobens, L., K. Rudolph, and E. Berger.** 1991. Ecdysterone regulatory elements function as both transcriptional activators and repressors. Mol. Cell. Biol. **11**:1846–1853.
11. **Garen, A., L. Kauvar, and J. A. Lepesant.** 1977. Roles of ecdysone in *Drosophila* development. Proc. Natl. Acad. Sci. USA **74**:5099–5103.
12. **Graves, B. J., P. F. Johnson, and S. L. McKnight.** 1986. Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell **44**:565–576.
13. **Gross-Bellard, M., P. Oudet, and P. Chambon.** 1973. Isolation of high molecular weight DNA from mammalian cells. Eur. J. Biochem. **36**:32–38.
14. **Härd, T., E. Kellenbach, R. Boelens, B. A. Maler, K. Dahlman, L. P. Freedman, J. Carlstedt-Duke, K. R. Yamamoto, J.-A. Gustafsson, and R. Kaptein.** 1990. Solution structure of the glucocorticoid receptor DNA-binding domain. Science **249**:157–160.
15. **Henrich, V. C., T. J. Sliter, D. B. Lubahn, A. MacIntyre, and L. Gilbert.** 1990. A steroid/thyroid hormone receptor superfamily member in *Drosophila melanogaster* that shares extensive similarity with a mammalian homologue. Nucleic Acids Res. **18**:4143–4148.
16. **Huet, F., C. Ruiz, and G. Richards.** 1993. Puffs and PCR: the *in vivo* dynamic of early gene expression during ecdysone response in *Drosophila*. Development **118**:613–627.
17. **Johnson, P. F., W. H. Landschulz, B. J. Graves, and S. L. McKnight.** 1987. Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. Genes Dev. **1**:133–146.
18. **Karim, F. D., and C. S. Thummel.** 1992. Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. EMBO J. **11**:4083–4093.
19. **Khoury Christianson, A. M., D. L. King, E. Hatzivassiliou, J. E. Casas, P. L. Hallenbeck, V. M. Nikodem, S. A. Mitsialis, and F. C. Kafatos.** 1992. DNA binding and heterodimerization of the *Drosophila* transcription factor chorion factor 1/ultraspiracle. Proc. Natl. Acad. Sci. USA **89**:11503–11507.
20. **Koelle, M. R., M. Arbeitman, and D. S. Hogness.** EcR and Ultraspiracle must complex to form hormone- and DNA-binding *Drosophila* ecdysone receptors. Proc. Natl. Acad. Sci. USA, in press.
21. **Koelle, M. R., W. S. Talbot, W. A. Segraves, M. T. Bender, P. Cherbas, and D. S. Hogness.** 1991. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell **67**:59–77.
22. **Kumar, V., and P. Chambon.** 1988. The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell **55**:145–156.

23. **Laval, M., F. Pourrain, J. Deutsch, and J. A. Lepesant.** 1993. *In vivo* functional characterization of an ecdysone-response enhancer in the proximal upstream region of the *Fbp1* gene of *D. melanogaster*. Mech. Dev. **44**:123–138.
24. **Lepesant, J. A., J. Kejzlarovà-Lepesant, and A. Garen.** 1978. Ecdysone-inducible functions of larval fat bodies in *Drosophila*. Proc. Natl. Acad. Sci. USA **75**:5570–5574.
25. **Lepesant, J. A., F. Maschat, J. Kejzlarovà-Lepesant, H. Benes, and C. Yanicostas.** 1986. Developmental and ecdysteroid regulation of gene expression in the larval fat body of *Drosophila melanogaster*. Arch. Insect. Biochem. Physiol. **s1**:133–141.
26. **Lindsley, D. L., and G. Zimm (ed.).** 1992. The genome of *Drosophila melanogaster*. Academic Press, Inc., San Diego, Calif.
27. **Luisi, B. F., W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. B. Sigler.** 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. Nature (London) **352**:497–505.
28. **Luo, Y., J. Amin, and R. Voellmy.** 1991. Ecdysterone receptor is a sequence-specific transcription factor involved in the developmental regulation of heat shock genes. Mol. Cell. Biol. **11**:3660–3675.
29. **Maroni, G., and S. Stamey.** 1983. Use of blue food to select synchronous late third instar larvae. Drosophila Inf. Serv. **59**:142–143.
30. **Martinez, E., F. Givel, and W. Wahli.** 1991. A common ancestor DNA motif for invertebrate and vertebrate hormone response elements. EMBO J. **10**:263–268.
31. **Maschat, F., M. L. Dubertret, P. Thérond, J. M. Claverie, and J. A. Lepesant.** 1990. Structure of the ecdysone-inducible *P1* gene of *Drosophila melanogaster*. J. Mol. Biol. **214**:359–372.
32. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labeled DNA with base-specific chemical cleavage. Methods Enzymol. **65**:499–560.
33. **Mueller, P. R., and B. Wold.** 1989. In vivo footprinting of a muscle specific enhancer by ligation-mediated PCR. Science **246**:780–813.
34. **Natzle, J. E.** 1993. Temporal regulation of *Drosophila* imaginal disc morphogenesis: a hierarchy of primary and secondary 20-hydroxyecdysone-responsive loci. Dev. Biol. **155**:516–532.
35. **Oro, A. E., M. McKeown, and E. M. Evans.** 1990. Relationship between the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. Nature (London) **347**:298–301.
36. **Ozyhar, A., and O. Pongs.** 1993. Mutational analysis of the interaction between ecdysteroid receptor and its response element. J. Steroid Biochem. Molec. Biol. **46**:135–146.
37. **Ozyhar, A., M. Strangemann-Diekmann, H. H. Kiltz, and O. Pongs.** 1991. Characterization of a specific ecdysteroid-DNA complex reveals common properties for invertebrate and vertebrate hormone-receptor-DNA interactions. Eur. J. Biochem. **200**: 329–336.
38. **Paco-Larson, L., Y. Nakanishi, M. Levine, and A. Garen.** 1986. Histochemical analysis of the ecdysterone-regulated expression of the *Drosophila* genes *P1* and *LSP-2*. Dev. Genet. **7**:197–203.
39. **Richards, G.** 1981. Insect hormones in development. Biol. Rev. Camb. Philos. Soc. **56**:501–549.
40. **Riddihough, G., and H. R. B. Pelham.** 1986. Activation of the *Drosophila hsp27* promoter by heat shock and by ecdysone involves independent and remote regulatory sequences. EMBO J. **5**:1653–1658.
41. **Riddihough, G., and H. R. B. Pelham.** 1987. An ecdysone response element in the *Drosophila* hsp27 promoter. EMBO J. **6**:3729–3734.
42. **Rigaud, G., J. Roux, R. Pictet, and T. Grange.** 1991. In vivo footprinting of rat TAT gene: dynamic interplay between the glucocorticoid receptor and a liver-specific factor. Cell **67**:977–986.
43. **Sakonju, S., and D. D. Brown.** 1982. Contact points between a positive transcription factor and the Xenopus 5S RNA gene. Cell **31**:395–405.
44. **Schwabe, J. W. R., D. Neuhaus, and D. Rhodes.** 1990. Solution structure of the DNA-binding domain of the oestrogen receptor. Nature (London) **348**:458–461.
45. **Seecof, R. L., and J. J. Donady.** 1972. Factors affecting Drosophila neuron and myocyte differentiation *in vitro*. Mech. Ageing Dev. **1**:165–174.
46. **Segraves, W. A., and D. S. Hogness.** 1990. The *E75* ecdysone-inducible gene responsible for the 75B early puff in *Drosophila* encodes two new members of the steroid receptor superfamily. Genes Dev. **4**:204–219.
47. **Segraves, W. A., and G. Richards.** 1990. Regulatory and developmental aspects of ecdysone-regulated gene expression. Invertebr. Reprod. Dev. **18**:67–76.
48. **Shea, M. J., D. L. King, M. J. Conboy, B. D. Mariani, and F. C. Kafatos.** 1990. Proteins that bind to *Drosophila* chorion *cis*-regulatory elements: a new C_2H_2 zinc finger protein and a C_2C_2 steroid receptor-like component. Genes Dev. **4**:1128–1140.
49. **Stones, B. L., and C. S. Thummel.** 1993. The Drosophila 78C early late puff contains E78, an ecdysone-inducible gene that encodes a novel member of the nuclear hormone receptor superfamily. Cell **75**:3074–320.
50. **Talbot, W. S., E. A. Swyryd, and D. S. Hogness.** 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell **73**:1323–1337.
51. **Thomas, H. E., H. G. Stunnenberg, and A. F. Stewart.** 1993. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*. Nature (London) **362**:471–475.
52. **Thummel, C. S.** 1990. Puffs and gene regulation: molecular insights into the *Drosophila* ecdysone regulatory hierarchy. Bioessays **12**:561–568.
53. **Thummel, C. S., K. C. Burtis, and D. S. Hogness.** 1990. Spatial and temporal patterns of *E74* transcription during *Drosophila* development. Cell **61**:101–111.
54. **Tsai, S. Y., J. Carlsted-Duke, N. L. Weigel, K. Dahlman, J.-A. Gustafsson, M.-J. Tsai, and B. W. O'Malley.** 1988. Molecular interactions of steroid hormone receptor with its enhancer element: evidence for receptor dimer formation. Cell **55**:361–369.
55. **Urban, P., and C. S. Thummel.** 1993. Isolation and characterization of fifteen ecdysone-inducible *Drosophila* genes reveal unexpected complexities in ecdysone regulation. Mol. Cell. Biol. **13**: 7101–7111.
56. **Urness, L. D., and C. S. Thummel.** 1990. Molecular interactions within the ecdysone regulatory hierarchy. DNA binding properties of the *Drosophila* ecdysone-inducible E74A protein. Cell **63**:47–62.
57. **Wrange, O., P. Eriksson, and T. Perlmann.** 1986. The purified activated glucocorticoid receptor is a homodimer. J. Biol. Chem. **264**:5253–5259.
58. **Yao, T. P., W. A. Segraves, A. E. Oro, M. McKeown, and R. M. Evans.** 1992. Drosophila Ultraspiracle modulates ecdysone receptor function via heterodimer formation. Cell **71**:63–72.