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High level transactivation by the ecdysone receptor complex at the core recognition motif

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ABSTRACT

Ecdysteroid signaling in insects is mediated by the ecdysone receptor complex that is composed of a heterodimer of the ecdysone receptor and Ultraspiracle. The DNA binding specificity plays a critical role of defining the repertoire of target genes that respond to the hormone. We report here the determination of the preferred core recognition motif by a binding site selection procedure. The consensus sequence consists of a perfect palindrome of the heptameric half-site sequence GAGGTCA that is separated by a single A/T base pair. No binding polarity of the ecdysone receptor/Ultraspiracle heterodimer to the core recognition motif was observed. This core motif mediated the highest level of ligand-induced transactivation when compared to a series of synthetic ecdysone response elements and to the natural element of the *Drosophila hsp27* gene. This is the first report of a palindromic sequence identified as the highest affinity DNA binding site for a heterodimeric nuclear hormone receptor complex. We further present evidence that the ligand of the ecdysone receptor preferentially drives Ultraspiracle from a homodimer into a heterodimer. This mechanism might contribute additionally to a tight control of target gene expression.

INTRODUCTION

In insects, development of both larval and imaginal tissues in molting and metamorphosis is controlled to a large extent by the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone). The hormonal signal activates a genetic regulatory hierarchy that controls these coordinate processes of development (reviewed in 1). Ecdysone responsiveness of insect cells is mediated by two members of the superfamily of nuclear hormone receptors, ecdysone receptor (EcR; 2) and Ultraspiracle (USP; 3–5). The activities of the functional EcR complex such as high affinity binding of both DNA and ligand and transcriptional activation of target genes, depend on the formation of a heterodimer of EcR and USP (6–9). USP is the *Drosophila* homolog of the vertebrate RXRs, the receptors for 9-*cis* retinoic acid. RXR is required as an auxiliary factor for high affinitiy DNA binding of nuclear receptors of the non-steroid type subfamily responsive to a variety of ligands, such as the retinoic acid receptors (RARs), the thyroid hormone receptors (TRs), the vitamin D receptors (VDRs) and peroxisome proliferator activated receptor (PPAR; for a review, see 10 and references therein). In addition to the structural similarity, USP and RXR are also functionally similar in that they can substitute for each other to achieve high affinity DNA binding *in vitro* (7,9,11). In analogy to RXR, USP can form heterodimers with multiple partners (12). Despite the similarities, recent studies indicate that USP is responsive to ligands that are classified as juvenile hormones (13).

Studies of the estrogen and glucocorticoid receptor (GR) indicated that the classical steroid hormone receptors recognize their cognate hormone response element (HRE) consisting of half-sites that are arranged as palindromes (PALs) generally as homodimers, with each receptor subunit contacting one half-site (14,15). As a consequence, the protein–protein interface formed between the two equivalent partners of the homodimer is of symmetric nature. In contrast, the preferred responsive elements for heterodimeric receptors formed of RXR and RAR, TR, VDR and PPAR, respectively, consist of a direct repeat (DR) of half-site sequences that are related to the canonical hexamer AGGTCA. Moreover, the spacing of the DRs determines the binding site preference: 1 bp for PPAR/RXR, 3 bp for VDR/RXR, 4 bp for TR/RXR and 5 bp for RAR/RXR (reviewed in 10). Thus, each heterodimer acts preferentially through a unique DR motif. The selective binding of heterodimers to cognate DRs is a consequence of a DNA-supported cooperative and asymmetric dimer interaction within the DNA binding domains (DBDs) or adjacent regions. Both genetic and biochemical studies have shown that the resulting polarity established by the interaction with RAR or TR places the RXR in the 5′ position of the DRs (16–20) and directly links receptor orientation to biological activity. In contrast, a reversed polarity which in addition permits the full responsiveness of RXR to specific ligands has recently been demonstrated for PPAR/RXR (21,22).

Natural ecdysone response elements (EcREs) are characterized in general as imperfect palindromes composed of two half-site sequences related to the hexamer AGGTCA that are separated by one central base pair (23–25, and references therein). However, ecdysone signaling at the promoters of the *ng* genes has been reported to occur through EcREs composed of DRs (26), indicating

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structural variations among natural EcREs. No information is as yet available about binding polarity of the EcR/USP heterodimer to EcREs. In our studies, we have employed an *in vitro* DNA binding site selection procedure (27) to analyze whether nucleotide deviations from the canonical hexamer sequence at both half-sites of a PAL (or eventually of a DR) may contribute to binding polarity of the EcR/USP heterodimer. Interestingly, the determined consensus binding site consists of a perfect palindrome of the heptameric half-site sequence GAGGTCA that is separated by one central A/T base pair. Whereas EcR/USP showed a pronounced binding polarity on DR4, which places USP in the 5′ position, no such polarity was found for the core recognition motif. Achieving the highest affinity binding by a palindromic DNA structure represents a novel feature among heterodimeric proteins of the superfamily of nuclear hormone receptors. Furthermore, the highest level of transactivation by EcR/USP was observed at this consensus binding site when compared to a series of different EcREs. We further demonstrated the impact of EcR-specific ligand on the configuration of dimer formed. In the presence of the EcR agonist muristerone A, USP was driven from a homodimer into a heterodimer with EcR. Thus, the preference for a specific dimer configuration imposed by ligand represents a potential further regulatory mechanism for a tight control of target gene expression.

MATERIALS AND METHODS

Plasmid constructs

Expression vectors for EcR and USP, respectively, were constructed as follows. The cDNA for the *Drosophila* EcR-B1 isoform (2) was amplified by PCR using the primer pair 5′-TCCCCCGGGGAGCTCGGATCCAAGCGGCGCTGGTCG-AAC-3′ and 5′-TCCCCCGGGTCTAGACTATGCAGTCGTC-GAGTGCTC-3′. The region encoding amino acids 2–878 was then subcloned as a *Bam*HI–*Xba*I fragment onto pSCT (28) to generate the expression vector pSCT-EcR[2–878]. Similarly, after PCR-amplification of the cDNA for the *Drosophila* USP (3) using the two primers 5′-AAGGCCTGAGCTCAGATCTGAC-AACTGCGACCAGGAC-3′ and 5′-AAGGCCTTCTAGACTA-CTCCAGTTTCATCGCCAGGCC-3′, pSCT-USP[2–507] was generated by subcloning a *Bgl*II–*Xba*I fragment encoding amino acids 2–507 onto pSCT. The expression plasmid pSCT-EcR[ΔF] encoding a mutant EcR that is truncated for the C-terminal F domain (amino acids 653–878) was constructed by first amplifying an internal fragment of the *EcR* cDNA using primers 5′-CGACA-TATGGGCCAAGACTTTGTTAAGAAGG-3′ and 5′-CGTCC-CGGGTCTAGACTAAACGTCCCAGATCTCCTCG-3′ followed by replacing a *Bsi*WI–*Xba*I fragment on pSCT-EcR[2–878].

Site-directed mutations within the DNA region encoding the DBD of both EcR and USP to generate pSCT-EcR[R290G] and pSCT-USP[R130G] were introduced as reported (29) using the mutagenic primers 5′-GGTTCTTTCGAGGCAGCGTTACG-3′ and 5′-GCTTCTTTAAAGGCACAGTGCG-3′, respectively, and the selection primer 5′-CGGTATCGATACGCGTGATATCGAAT-TCC-3′. The sequence changes resulted in a Arg to Gly substitution at amino acid position 290 of EcR and at position 130 of USP, respectively. Expression plasmids for EcR_{pgr} and USP_{pgr} were generated from pSCT-EcR[2–878] and pSCT-USP[2–507] using the QuikChange site-directed mutagenesis kit (Stratagene) and the mutagenic oligonucleotide 5′-CGCCCTCACCTGTGG-AAGCTGCAAGGTGTTCTTTCGACGC-3′ and 5′-GCGTGTA-CAGCTGTGGAAGCTGCAAGGTCTTCTTTAAACGCAC-3′, respectively. This manipulation altered the P box region of the DBD of EcR and USP to that of the GR (30).

The chloramphenicol acetyltransferase (CAT) reporter plasmid pCAT contains the *Drosophila melanogaster hsp70* promoter (–50 to +200) in front of the *CAT* reporter gene (31). Various double stranded oligonucleotides containing the hexamer half-site motif AGGTCA in either DR or palindromic arrangement, along with the natural EcRE of the *Drosophila hsp27* gene (referred to here as hsp27), were cloned as four tandem copies into the *Bam*HI site at promoter position –50 of pCAT. The sequences are: DR0, 5′-GATCTAGAGAGGTCAAGGTCATGT-CCAAG-3′; DR1, 5′-GATCTAGAGAGGTCAAAGGTCATGT-CCAAG-3′; DR2, 5′-GATCTAGAGAGGTCAAGAGGTCATG-TCCAAG-3′; DR3, 5′-GATCTAGAGAGGTCAAGAAGGTCA-TGTCCAAG-3′; DR4, 5′-GATCTAGAGAGGTCAAGAAAGG-TCATGTCCAAG-3′; DR4m, 5′-GATCTAGAGGGTAAAGAA-AGGTCATGTCCAAG-3′; DR5, 5′-GATCTAGAGAGGTCAA-CGAAAGGTCATGTCCAAG-3′; DR5m, 5′-GATCTAGAGAG-GTCAACGAAAGGTAATGTCCAAG-3′; PAL0, 5′-GATCTAG-AGAGGTCATGACCTTGTCCAAG-3′; PAL1(C+7T), 5′-GATC-TAGAGAGGTCAATGACCTTGTCCAAG-3′; PAL1/A, 5′-GA-TCTAGAGAGGTCAATGACCTCGTCCAAG-3′; PAL1/G, 5′-GATCTAGAGAGGTCAGTGACCTCGTCCAAG-3′; hsp27, 5′-GATCCGAGACAAGGGTTCAATGCACTTGTCCAAT-GG-3′. The β-galactosidase expression vector pA5Cβ-gal used to normalize for transfection efficiencies of *Drosophila* SL-2 cells contains the *Drosophila* actin 5C promoter in front of the *lac*Z gene.

Electrophoretic mobility shift assay (EMSA)

The pSCT-based receptor expression plasmids were used as templates in a T7 RNA polymerase-driven coupled transcription and translation reaction *in vitro* by using a rabbit reticulocyte lysate system as recommended by the supplier (TNT kit, Promega). Relative amounts of expressed receptor proteins were determined by western blot analyses performed according to standard procedures (32) using the monoclonal antibody DDA 2.7 (2) directed against EcR, and AB11 (33) against USP.

For a standard 20 µl EMSA reaction, a total of 2 µl of programmed reticulocyte lysate or of 5 µg of nuclear extract were incubated on ice for 20 min in binding buffer [20 mM HEPES–KOH, pH 7.4, 100 mM KCl, 5% glycerol, 2 mM dithiothreitol (DDT), 0.1% NP-40, 50 µg/ml poly(dI-dC)]. Competitor oligonucleotides or muristerone A (Sigma) at a final concentration of 10 µM were included in the binding reaction mixture as indicated in the text. Approximately 1 ng of annealed oligonucleotide probe, which was labeled by fill-in reaction with T7 Sequenase v2.0 (Amersham Life Science, Inc.) and [α-^{32}P]dCTP to specific activity of ~10^7 c.p.m./µg, was added to the binding reaction and incubated at room temperature for 20 min. The sequences of the hybrid response elements, which contain the two different half-sites AGGTCA and AGAACA, that were used as probes are: DRE4G, 5′-GATCTAGAGAGGTCAAG-AAAGAACATGTCCAAG-3′; PALE1G/A, 5′-GATCTAGAGA-GGTCAATGTTCTCGTCCAAG-3′; PALE1G/T, 5′-GATCTAG-AGAGGTCATTGTTCTCGTCCAAG-3′; PALE1G/G, 5′-GATC-TAGAGAGGTCAGTGTTCTCGTCCAAG-3′; PALE1G/C, 5′-GATCTAGAGAGGTCACTGTTCTCGTCCAAG-3′. For supershifts, antibody was added 10 min after the probe was added.

The reaction products were then separated on a 4% non-denaturing polyacrylamide gel in 0.5× TBE (45 mM Tris, 45 mM boric acid, 0.5 mM EDTA, pH 8.3) that was pre-run for 2 h. After electrophoresis at 10 V/cm for 2 h at room temperature, the gel was dried for autoradiography. PAL1(C+7T) was used as PAL1 probe for all the EMSA experiments described.

Nuclear extracts from *Drosophila* SL-2 cells were performed according to the procedure of Barettino *et al.* (34). Nuclear extracts were dialyzed against 20 mM HEPES–KOH, pH 7.4, 75 mM KCl, 20% glycerol, 1 mM DTT, 0.1% NP-40, 0.2 mM EDTA, 0.5 mM PMSF and stored at –80°C until use.

In vitro DNA binding site selection

The core recognition motif of the EcR/USP heterodimer was determined essentially as described (27). A mixture of 57 bp oligonucleotides was generated by PCR using template oligomers of the sequence 5′-CGCGGATCCCGGGTACC(N)$_{20}$ATCGA-TATCAGATCTGGGCC-3′ containing a central stretch of 20 random nucleotides and the primer pair 5′-CGCGGATCCCGGG-TACC-3′ and 5′-GGCCCAGATCTGATATCGAT-3′, respectively. The amplification reaction was carried out in 50 µl using 1 pmol of random oligomer and 50 pmol of each primer for 25 cycles, with each cycle consisting of 0.5 min at 94°C, 1 min at 65°C and 1 min at 72°C. Double stranded mixed oligomers were separated on a 10% non-denaturing polyacrylamide gel, eluted in gel elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS, pH 8.0), and purified by phenol extraction and ethanol precipitation. The oligomers were end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP and then incubated with *in vitro* translated EcR and USP (2.5 µl each) in 20 µl of binding buffer for 20 min at room temperature in the presence of 10 µM muristerone A. The complexes were separated on a 4% non-denaturing polyacrylamide gel in 0.5× TBE, eluted in gel elution buffer at 50°C for 3 h, and the DNA was recovered by phenol extraction and ethanol precipitation. The DNA was then amplified by PCR and subjected to five additional selection cycles of binding and amplification. Selected oligomers were cloned and subjected to nucleotide sequence analysis.

Cell culture, transfection and reporter gene assay

Drosophila SL-2 cells were grown at 25°C in Schneider's Drosophila Medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin. SL-2 cells were grown in 6 cm plates for 24–26 h to near confluency and cotransfected with 5 µg of either CAT reporter or pCAT control plasmid, 2 µg of pA5Cβ-gal and 3 µg of pBluescriptIISK(+) carrier plasmid by the calcium phosphate precipitation method essentially as described (35). After 16 h, cells were washed once with 1× PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3) and fresh medium containing muristerone A at 1 µM or solvent control was added. The cells were harvested 24–30 h later, and CAT protein was quantified by CAT ELISA according to the specifications of the manufacturer (Boehringer Mannheim). CAT values were normalized for transfection and harvesting efficiency by measuring β-galactosidase activity with an enzyme assay (Promega), and the results reported as average fold activation of the *CAT* reporter gene expression of at least three independent replicates by normalizing for the pCAT control plasmid.

Table 1. Selected DNA binding sites for EcR/USP

A. Nucleotide sequences of selected binding sites.

SSO6 (2x)	tC	**AGGTCA**C**TGACCT**	CTCCTT
SSO5	tG	**AGGTCA**TT**GAACT**	ATTACG
SSO2 (2x)	TGCACA	**AGGTCA**AT**AACCT**	Ca
SSO8	TGCACA	**AGGTCA**ATG*C*A*T*	Ca
SSO9 (2x)	AGG	**AGGTCA**G**TGAAC***C*	CAGC
SSO3	TACAACAAC	G**GGTCA**TT**GAC***at*	cg
SSO13	CGGCG	G**GGTCA**AT**GC***CCT*	CG
SSO4	tG	G**GGTCA**AG**GACCT**	TATGTT
SSO16	ac	c**GGTCA**AT**GACC***A*	CTACCCGC
SSO1 (3x)	CGTGAG	**AG***T***TCA**AT**GC***CT*	Cg
SSO15	TCGCA	**AG***T***TCA**A*C***GACCT**	TT
SSO10 (2x)	CGTGTA	G**GGTCA**TT**GAAC***C*	Tg
SSO20	cG	G**GGTCA**AT**GAC***TG*	GAAGGG
SSO14 (3x)	TACAG	**AGGT***T***AATGAC***AC*	CG
SSO11	GTGGAG	**AGGT***T***AA***C*G**CCC***C*	Ca
SSO7	TCG	G**GGT***T***AT***T*G**TCC***C*	CTAC

B. Consensus binding sequence.

Position	-7	-6	-5	-4	-3	-2	-1	0	+1	+2	+3	+4	+5	+6	+7
%G	58	29	100	83	0	0	0	8	4	96	13	0	0	4	4
%A	25	67	0	0	0	0	100	63	0	4	70	21	21	4	4
%T	0	0	0	17	100	21	0	21	88	0	4	0	4	54	17
%C	17	4	0	0	0	79	0	8	8	0	13	79	75	38	75
Consensus	G	A	G	G	T	C	A	A	T	G	A	C	C	T	C

(**A**) The sequences of 24 cloned EcR/USP binding sites derived by six cycles of selection are shown, aligned for a maximum match to the canonical half-site AGGTCA at the left. The region of the 20 originally random nucleotides is shown by upper case letters, whereas nucleotides from adjacent primer regions are indicated by lower case letters. Letters in bold match the canonical half-site sequence. Deviations from that sequence are indicated by italic letters. The number in parentheses after the sequence designation indicates that some of the binding sites were cloned more than once. (**B**) The deduced consensus binding sequence. The relative distribution of each nucleotide is indicated in percent for every position. Nucleotides were considered significant with at least 50% representation at the indicated position.

RESULTS

Determination of the preferred core recognition motif of EcR/USP

We set out to determine the consensus DNA binding site for the EcR/USP complex by a PCR-assisted selection approach as described in Materials and Methods. As revealed by EMSA, the formation of specific protein–DNA complexes was strictly dependent on the presence of both EcR and USP in the binding reaction (data not shown). After cycle 6, selected oligonucleotides were subcloned and inserts from 24 independent clones were subjected to nucleotide sequence analysis. A hexamer motif identical to or highly related to the canonical sequence AGGTCA was readily detectable, and the cloned sequences were aligned for the best match to this motif (Table 1A). The deduced consensus binding site forms a perfect palindrome of the nucleotide sequence 5′-GAGGTCAATGACCTC-3′ (Table 1B). Thus, the half-site conforms entirely with the canonical sequence but is extended at one end to form a heptamer. Moreover, the central nucleotide that constitutes the spacer between the two half-sites is non-random and consists of an A/T rather than a G/C base pair. A similar consensus sequence was identified after three cycles of selection except for positions –7, +6 and +7, which showed no

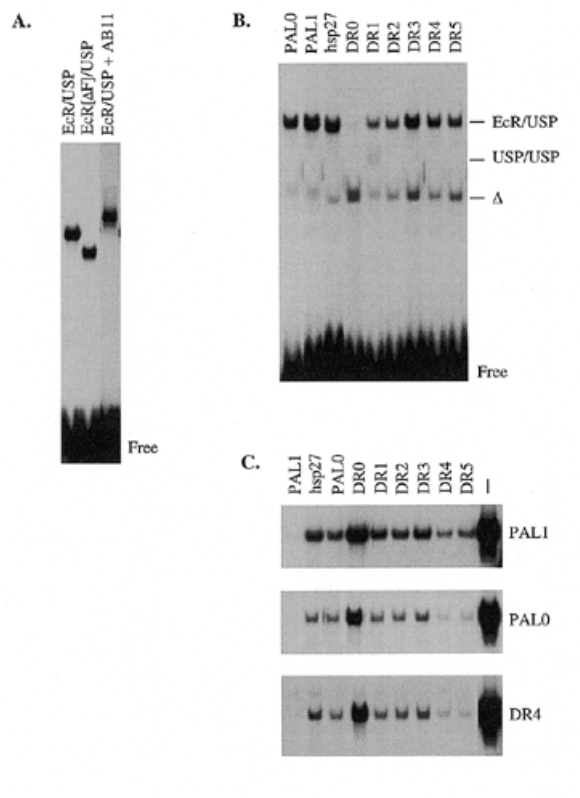

Figure 1. EcR/USP binds to different DNA elements. (**A**) Binding of EcR/USP to PAL1. Proteins were incubated with radiolabeled PAL1 and the reaction products analyzed by EMSA. Receptor protein combinations and mAB AB11 administration are indicated above each lane. The location of free probe is indicated. (**B**) EcR/USP were incubated with radiolabeled probes and the reaction products analyzed by EMSA. The probes used are indicated above each lane. The locations of free probe, probe bound to receptor dimers and unspecific signals of probe bound to protein(s) of the reticulocyte lysate (triangle) are indicated. (**C**) The relative binding affinities of EcR/USP to various DNA elements were determined by competition EMSAs. The radiolabeled probe for each series is indicated at the right hand side. Double stranded oligonucleotides were used as cold competitor at a 10-fold molar excess and are indicated above each lane. The control reaction without any competitor DNA is marked by a dash. Only the signals of probe bound to EcR/USP are shown.

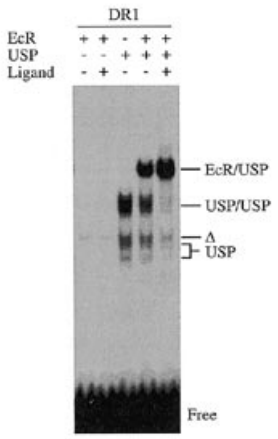

Figure 2. EcR-specific ligand influences the receptor dimer configuration. Protein binding to radiolabeled DR1 was analyzed by EMSA. The combinations of receptor proteins and ligand (muristerone A at 10 μM) are indicated above each lane. The locations of free probe, probe bound to receptor dimer or to monomer and/or degradation products thereof, and unspecific signals of probe bound to protein(s) of the reticulocyte lysate (triangle) are indicated.

obvious nucleotide bias (data not shown). Binding sites composed of half-sites in a DR arrangement were deteceted in no instance among any of the analyzed oligonucleotides.

We next wanted to demonstrate that both receptors are indeed a physical part of a complex with PAL1. When we used *in vitro* translated EcR[ΔF], the retarded complex migrated faster in an EMSA in comparison to a complex specifically formed by full-length EcR and USP (Fig. 1A). Addition of the mAB AB11 directed against USP (33) resulted in a supershift of the complex. Together, these data unambiguously demonstrate that both receptors are indeed constituents of a heterodimeric complex with PAL1.

Relative binding affinities of the EcR/USP heterodimer to various DNA elements

The extensive amino acid sequence identities found in the DBDs of RXR and USP (36,37) suggest that DR motifs might as well constitute DNA binding target sites for the EcR/USP heterodimer. In fact, a subset of synthetic DR type binding motifs has been recently described as DNA binding sites for EcR/USP *in vitro* (38,39) and *in vivo* (39), and natural EcREs composed of DRs have been identified in the promoter regions of the *ng* genes (26). Thus, we compared a series of synthetic DR (DR0–DR5) and palindromic motifs (PAL0, PAL1), along with the natural EcRE hsp27, for their relative affinity to the EcR/USP heterodimer by EMSAs. As shown in Figure 1B, the binding capability of the EcR/USP heterodimer was remarkable in that all the various elements tested were recognized specifically by the receptor dimer. The relative binding affinity of the EcR/USP heterodimer to the various DNA elements was determined by competition EMSAs (Fig. 1C). The following order of decreasing affinity was consistently determined in several independent experiments: PAL1 > DR4 > DR5 > PAL0 > DR2 > DR1 > hsp27, DR3 > DR0.

None of the elements tested constituted homodimer binding sites for either EcR or USP at a detectable level except for DR1. Comparison of the migration position of the most prominent complex formed between DR1 and USP (Fig. 2) to a complex of PAL1 with EcR/USP from *Chironomus tentans* (31 and unpublished results), having a similar molecular weight (62 kDa each) as the *Drosophila* USP (56 kDa), clearly indicated that this complex consists of a homodimer of USP. Two faster migrating complexes represent monomer binding of USP and of a truncated form thereof (compare to Fig. 3B). The binding of USP as a homodimer, like the one of RXR, to a DR1 DNA binding motif has been reported previously (40). But most remarkably, the configuration of the complex formed on a DR1 element was directed to a great extent by the ligand. In the absence of the EcR-specific ligand, complexes of DR1 with both USP homodimers and EcR/USP heterodimers were formed with similar efficiencies. However, the presence of muristerone A at 10 μM in the binding reaction resulted in a shift towards the EcR/USP–DR1 complex (Fig. 2). Only trace amounts of complexes of the configuration USP/USP–DR1 were detectable under these conditions. Thus, ligand is the crucial determinant for the preference of one out of two possible dimer configurations bound to the DR1 element.

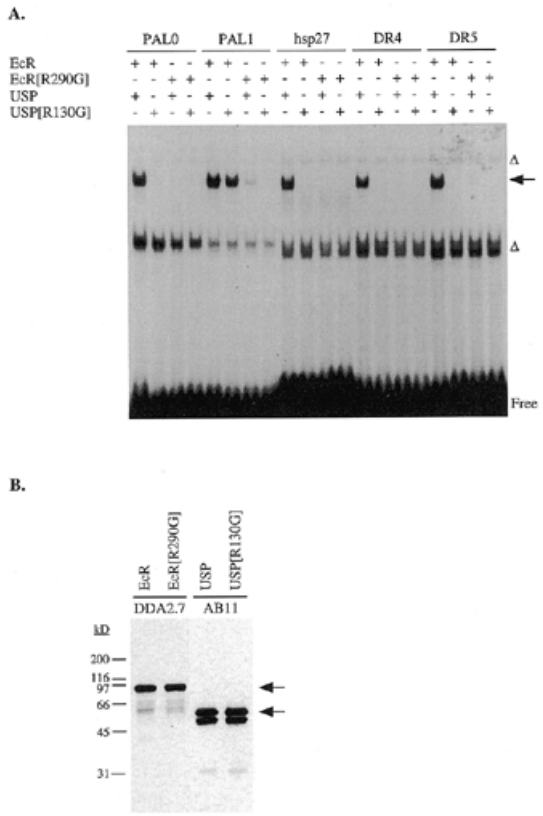

Figure 3. Binding of receptor mutants to various DNA elements. (**A**) Protein–DNA binding was analyzed by EMSA. The receptor combinations and the radiolabeled probe used are indicated above each lane. Muristerone A at 10 µM was included in all reactions. The locations of free probe, probe bound to EcR/USP (arrow), and unspecific signals of probe bound to protein(s) of the reticulocyte lysate (triangle) are indicated. (**B**) Western blot of *in vitro* translated receptor proteins. Expressed receptors and the mAB used for the detection reaction are indicated above each lane. The position and molecular weight of protein markers (in kDa) is indicated at the left hand side. Arrows indicate signals from full-length receptor and correspond to 94.3 or to 55.6 kDa of wild-type and mutant EcR or USP, respectively. Faster migrating protein species originate either from incomplete transcription/translation or from protein degradation.

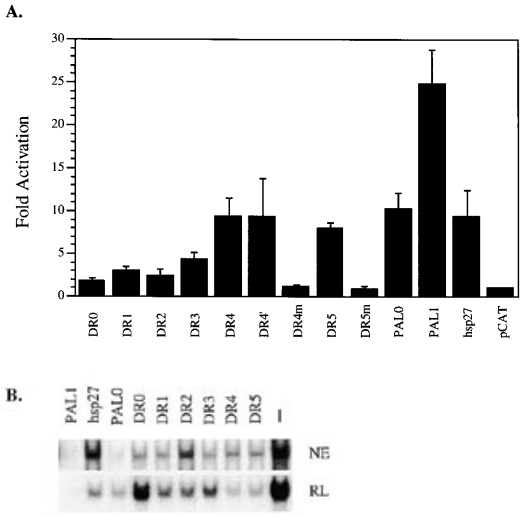

Figure 4. Ligand-dependent transactivation at different EcREs by EcR/USP in *Drosophila* SL-2 cells. (**A**) CAT reporter constructs containing four tandem copies of the indicated EcREs were transiently transfected into *Drosophila* SL-2 cells. DR4′ indicates the inverse orientation of DR4 relative to the transcriptional start site. The fold activation of *CAT* by 1 µM muristerone A, normalized to both pCAT control and transfection efficiency, is indicated. The relative induction levels for the other PAL1 elements are indicated. Each value represents the average of at least three independent transfection experiments. Standard deviations are indicated by bars. (**B**) The relative binding affinities of the endogenous binding activity (NE, nuclear extract) and *in vitro* translated EcR/USP (RL, programmed reticulocyte lysate) to various DNA elements were determined by competition EMSAs. The radiolabeled probe was PAL1. The competitor DNAs were used at a 10-fold molar excess and are indicated above each lane. The control reaction without competitor DNA is marked by a dash.

The EcR-specific ligand apparently drives USP from a homo- into a heterodimeric complex.

Next, we wanted to see whether both EcR and USP contact the various DNA binding elements and if any differences, apart from the binding affinities, do exist for the contacts at these sites. For this purpose, we used receptor mutants EcR[R290G] and USP[R130G], respectively, which displayed impaired DNA binding capabilities. The result of EMSAs using different combinations of wild-type and mutant receptor proteins is depicted in Figure 3. As revealed in a control experiment, both mutant and wild-type receptor showed an identical expression efficiency *in vitro* (Fig. 3B). The mutant phenotype of USP[R130G] was apparent as the respective homodimer had lost its capability to form a complex with DR1 in an EMSA (data not shown). In contrast, the mutant phenotype of EcR[R290G] could only be assessed in combination with the heterodimer partner USP, since we never did observe EcR homodimer binding to any DNA element. None of the elements tested was recognized by a heterodimer in which both receptor partners had been mutated. Reciprocal combinations of wild-type and mutant receptor did not result in the formation of detectable complexes with the various DNA elements tested except for PAL1. However, these heterodimers showed a decreased affinity even to this DNA binding site (Fig. 3A).

Functionality of DNA binding sites in transcriptional activation

To test the different DNA binding sites for functionality in transcriptional activation of a reporter gene, we transiently transfected various reporter plasmids into *Drosophila* SL-2 cells, relying on endogenous expression of EcR and USP. The DNA element PAL1 was the most potent EcRE and achieved a high level of transactivation. Hormone induction gave ~25-fold activation of the reporter gene above background level (Fig. 4A). An intermediate hormone-dependent stimulation of *CAT* of ~10-fold was obtained by using reporter plasmids containing the DNA elements PAL0, hsp27, DR4 and DR5, respectively. The other DNA binding sites tested gave rise to a low but significant activation of *CAT* expression. All the various DNA binding sites analyzed thus belong to the group of functional EcREs. As was tested for DR4, the orientation of the DNA element relative to the TATA box of the *hsp70* minimal promoter did not influence transactivation levels. Equal values of *CAT* stimulation were determined for both possible orientations (DR4, DR4′). Furthermore, base pair substitutions within the 5′ half-site of DR4 (DR4m) and within the 3′ half-site of

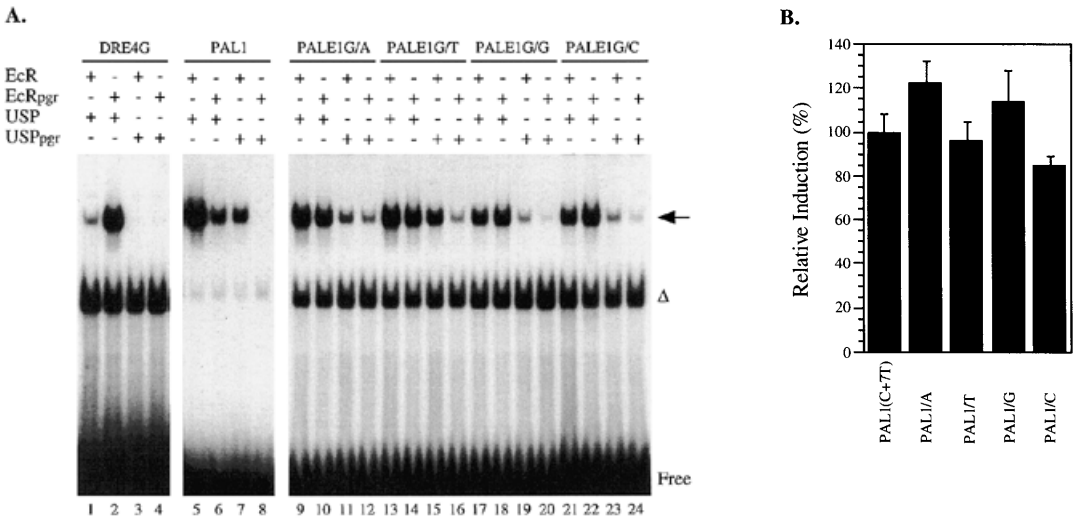

Figure 5. Polarity of the EcR/USP complex to the core recognition motif. (**A**) Receptor species were incubated with labeled probes as indicated above the lanes and the reaction products analyzed by EMSA. Muristerone A at 10 µM was included in all reactions. The locations of free probe, probe bound to EcR/USP (arrow), and unspecific signals of probe bound to protein(s) of the reticulocyte lysate (triangle) are indicated. (**B**) Relative induction levels by different PAL1 variants. Reporter constructs containing four tandem copies of the indicated PAL1 variants were transiently transfected into *Drosophila* SL-2 cells. The induction value of CAT expression by 1 µM muristerone A was arbitrarily set to 100% for PAL1(C+7T). This construct is identical to PAL1/T in respect of the central nucleotide position relative to the transcriptional start site. The relative induction levels for the other PAL1 elements are indicated. Each value represents the average of at least three independent transfection experiments. Standard deviations are indicated by bars.

DR5 (DR5m), relative to the transcriptional start site, identified the nucleotide at the respective mutation site as being crucial for EcRE functionality. In both instances, the C residue of the AGGTCA hexamer half-site was changed to an A. The introduced changes abolished *CAT* reporter gene activation completely (Fig. 4A). The binding affinity of the endogenous EcR and USP to the different EcREs deviated from that determined previously for the receptors translated *in vitro* (Figs 1C and 4B). However, PAL1 was the highest affinity binding site for both binding activities.

Non-polar binding of EcR/USP to the consensus binding site

The previous experiments showed that both receptor partners contact the DNA target site. We thus face the paradox that the heterodimeric EcR/USP complex, which exhibits an intrinsic polarity due to the structural differences of the two receptor proteins, recognizes a consensus DNA binding site that forms a perfect palindrome. The central base pair of PAL1 is the only base pair that potentially could discriminate between binding of one receptor partner to the 5′ rather than to the 3′ half-site relative to the transcription initiation start. Therefore, we determined the EcR/USP binding polarity by using the receptor mutants EcR_{pgr} and USP_{pgr}, both containing a P box of the GR, and hybrid response elements (Fig. 5A). Both heterodimers, EcR_{pgr}/USP and EcR/USP_{pgr}, could form complexes with all the possible variations of hybrid PAL1 elements. The observed differences in binding affinity of the two receptor heterodimers (compare lanes 10 and 11, 14 and 15, 18 and 19, 22 and 23) is rather a consequence of the polarity that was introduced into the hybrid response elements, as no such difference is found on a PAL1 (compare lanes 6 and 7). Moreover, the two EcR and USP receptor variants were expressed *in vitro* at identical efficiencies (data not shown). Therefore, no binding polarity was observed for the EcR/USP complex on the consensus binding site. Surprisingly, the wild-type EcR/USP complex recognized the various hybrid response elements with high affinity (lanes 9, 13, 17 and 21). Again, this finding illustrates the extraordinary flexibility in DNA binding that has been observed previously. Furthermore, the impact of the central base pair of PAL1 on DNA binding affinity is apparent. In agreement to the result of the binding site selection, a A/T base pair dominates over a C/G base pair in terms of binding affinity (compare lanes 9 and 13 with 17 and 21). In contrast to the situation with PAL1, a pronounced binding polarity was found for EcR/USP on DR4. High affinity binding to a hybrid element containing an EcRE half-site at the 5′ and a GRE half-site at the 3′ position was only observed for the EcR_{pgr}/USP but not the EcR/USP_{pgr} complex (compare lanes 2 and 3). This binding polarity thus places the EcR at the 3′ and USP at the 5′ position of a DR4.

To analyze the impact of the central base pair of PAL1 on the transactivation of a reporter gene, five different reporter constructs, containing either the DNA binding site PAL1/A, PAL1/T, PAL1/G, PAL1/C or PAL1(C+7T), were transiently transfected into SL-2 cells. No significant differences in the induction level of the *CAT* reporter gene were found among the different reporter constructs (Fig. 5B). Although the *in vitro* binding studies had demonstrated a clear preference for an A/T base pair at the central position a change to a G/C base pair did not noticeably affect the transactivation potential of EcR/USP at PAL1.

DISCUSSION

High flexibility of ecdysteroid signaling by EcR/USP

EcR/USP showed a high flexibility in DNA binding exceeding that seen for other proteins of the nuclear hormone receptor superfamily. A series of synthetic EcREs was specifically recognized in the order of decreasing binding affinity of PAL1 >

DR4 > DR5 > PAL0 > DR2 > DR1 > hsp27, DR3 > DR0 (Fig. 1C). Antoniewski *et al.* (39) determined a highly similar order, except for hsp27 and DR3, which equally ranked directly after DR5. This difference is most likely due to slight nucleotide variations of the intervening spacer sequences. The impact of specific nucleotides within the spacer sequence on DNA binding affinity has been demonstrated in this paper for PAL1 (Table 1, Fig. 5A) and in previous studies for the binding sites of a number of nuclear hormone receptors (e.g., see 20). Consistent with this view, a strong deviation of the spacer sequences of DR3–DR5 from those used in our study resulted in a different ranking order (38). Our data show that the endogenous binding activities of EcR/USP in SL-2 cells do not match the binding affinities to various EcREs that have been determined for the *in vitro* translated receptors (Fig. 4B). Receptor modifications or interference with other endogenous proteins may contribute to this finding. In any case, PAL1 was the highest affinity binding site. Various receptor protein regions, either within or adjacent to the DBD, determine the DNA-supported dimer interaction, depending on the nature of both the receptor dimer and the cognate HRE (14,15,19,20). Apparently, EcR/USP is able to make use of multiple such interactions in achieving the high flexibility of DNA binding. Both EcR and USP make contact to DNA as was demonstrated with the receptor mutants EcR[R290G] and USP[R130G] that showed an impaired DNA binding capability (Fig. 3A). The DNA binding studies further reveal that the nature of the protein–DNA interaction occurring at PAL1 differs from that seen with the other EcREs. The targeted Arg residue is highly conserved among members of the nuclear receptor superfamily and was shown for several other nuclear receptors to have an impact on DNA binding (14,15,20,41).

The binding affinity of EcR/USP could generally be enhanced 3–4-fold by the presence of muristerone A at 10 μM in the *in vitro* binding reaction (Fig. 2). The increased DNA binding capacity is presumably an indirect effect that originates rather from a stabilization of the heterodimer than from the actual DNA binding process *per se*. Since only the EcR/USP heterodimer is enabled to bind ligand efficiently (6,8), the conformational change that occurs upon ligand binding (42, and references therein) most likely contributes to a more stable heterodimer.

The same DNA elements were demonstrated to permit a ligand-dependent transactivation of a reporter gene (Fig. 4A). In general, a good correlation of DNA binding affinity and transactivation level was observed. PAL1 mediated the highest level of ligand-induced transactivation. Interestingly, hsp27 combined the properties of a rather weak DNA binding site with the intermediate transactivation level obtained for relatively strong binding sites such as DR4 and DR5 (Fig. 4). Identical results were presented in another study (39). Although the molecular basis for this finding remains speculative, it clearly demonstrates that the regulation of hormone signaling comprises aspects of both DNA binding and transactivation. Besides DRs and PALs, inverted PALs have been shown to constitute yet another structural type of functional HREs (43). Ecdysteroid signaling of EcR/USP at these DNA elements remains to be seen. Based on our *in vitro* studies, the structure of natural EcREs is expected to extend beyond those characterized so far. Fine-tuning of ligand-mediated target gene expression could thus be achieved by alternative DNA binding sites.

DNA binding polarity of the EcR/USP complex

The consensus binding site determined in this study is the first report of a perfectly palindromic DNA sequence to constitute the highest affinity binding site for a heterodimeric nuclear hormone receptor (Table 1). The bias in nucleotide composition at the central position suggests a role as contact site for the receptor–DNA interaction, and only this position potentially could discriminate between the individual half-sites, a prerequisite for polar binding. However, the use of EcR and USP receptor mutants that contain the P box of the GR clearly demonstrated that the EcR/USP complex binds to the core recognition motif in a non-polar manner (Fig. 5A), although the central base pair of PAL1 had an obvious impact on DNA binding affinity. Thus, the DNA binding properties of heterodimeric nuclear hormone receptors show a remarkable flexibility. Besides a polar binding to DRs (reviewed in 10) and to inverted PALs (43), the example of non-polar binding to DRs (19) is now extended to PALs by the present study. In contrast, polar binding of EcR/USP to DR4 was observed and placed the EcR in the 3′ position (Fig. 5A). Recently, juvenile hormones were identified as specific agonists for USP (13). But it remains to be seen whether USP is responsive to ligand in a complex with EcR and whether DNA binding polarity of EcR/USP plays a critical role of USP responsiveness.

The central position of the consensus binding site had no significant impact on transactivation, indicating that both orientations of either PAL1/A or PAL1/G are tolerated (Fig. 5B). These results demonstrate that nucleotide deviations from the core recognition motif do not necessarily translate into a reduced transactivation potential. Moreover, as was shown for DR4, the relative orientation of this polar EcRE to the transcription initiation site of a reporter gene had no influence on transactivation (Fig. 4A). Thus, the remarkable flexibility observed for DNA binding extends as well to transcriptional activation. While there are other precedents for binding sites with half-site sequences extending beyond six nucleotides (e.g., see 44), a compelling impact of these additional nucleotide positions on transactivation remains to be seen. With respect to EcREs, a PAL1 element containing the half-site sequence GGTCA was more potent in transactivation than hsp27 (45).

The dimer configuration is influenced by ligand

A potential novel regulatory mechanism for EcR/USP is described. We showed that ligand can influence the nature of dimer that is preferentially formed (Fig. 2). Most likely, the effect of ligand is transmitted by a conformational change of EcR which in turn renders a EcR/USP complex more stable than a USP homodimer. Viewing protein–protein interaction as a dynamic process of continuous on and off reactions, a stabilization of EcR/USP results in a gradual shift from a state of equilibrium and, eventually, in a depletion of USP homodimers. This mechanism would allow for an efficient signal transduction even in the presence of minute amounts of EcR and illustrates the importance of dimerization for the regulation of ecdysteroid signaling. It remains to be seen whether juvenile hormones can counteract the effects of muristerone seen on the EcR/USP complex, e.g. by stabilizing a USP homodimer with altered regulatory properties. The control of target gene expression may thus severely be

influenced by the individual titers of receptor-specific ligand. The influence of ligand on dimer formation appears to be a common phenomenon and was reported previously for other members of the nuclear hormone receptor superfamily (46,47). The possibility of the ligand-induced formation of heterodimers with alternative partners of both EcR and USP and the usage of appropriate EcREs may account for the different ecdysone-mediated biological activities observed at various stages of development (reviewed in 1).

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