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The N-terminus of ecdysteroid receptor isoforms and ultraspiracle interacts with different ecdysteroid response elements in a sequence specific manner to modulate transcriptional activity

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ABSTRACT

The functional insect ecdysteroid receptor is comprised of two nuclear receptors, the ecdysteroid receptor (EcR) and the RXR homologue, ultraspiracle (USP), which form a heterodimer. The dimer recognizes various hormone response elements and the effect of these elements on transcriptional activity of EcR isoforms was determined in vertebrate cells transfected with EcR and USP. Only constitutive activity mediated by the core response elements was preserved after elimination of nonspecific binding sites on the DNA of the vector. The constitutive transcriptional activity was regulated in a complex manner by the N-termini of both EcR and USP, the DBD of USP and the type and number of hormone response elements (HRE). Cooperative effects at oligomeric response elements particularly DR1 depended on the type of ecdysteroid response element and the N-termini of EcR and USP. The DBD of USP abolishes or attenuates synergistic effects. The data show that in the absence of hormone, transcriptional activity is regulated in a complex manner that offers additional possibilities for ecdysteroid receptor mediated gene regulation during development.

1. Introduction

The ecdysteroid receptor is a key regulator for many processes during insect development and metamorphosis. A multitude of DNA binding sites has been localized by genomic mapping [1], which contributes to the complex spatio-temporal pattern of transcriptional regulation of numerous target genes.

In *Drosophila melanogaster*, three EcR-isoforms (EcR-A, EcR-B1 and EcR-B2) are present, which differ only in the length and sequence of their N-termini [2]. The expression of EcR isoforms in a tissue and stage specific manner suggests different functional roles [3]. In fact, each isoform fulfills specific functions during development and in many instances cannot be replaced by the other isoforms [4,5]. By contrast, only one isoform of the heterodimerization partner USP is described in *D. melanogaster* [6].

As a member of the nuclear receptor family, EcR and EcR/USP heterodimer bind to an unusually wide range of ecdysteroid response elements, including perfect (PAL1) and imperfect palindromic repeats (hsp27) and direct repeats (DR) with different spacer lengths [7–9]. The affinities for these elements vary [10], and certainly contribute to the diversification of the ecdysteroid receptor mediated responses. EcR and USP homodimers, as well as the heterodimer EcR/USP [11–13], are able to interact with response elements and modify transcriptional activity of target genes even in the absence of hormone [12]. USP modulates transcriptional properties in two different ways: one involving the DNA binding domain and a second one solely through the ligand binding domain [14], a mechanism, which has also been reported for vertebrate receptors like ER [15].

In this paper we showed that hormonal stimulation of transcriptional activity is not mediated by the core recognition motif, but depends on the presence of additional transcription factor binding sites. We investigated the influence of different core recognition motifs on basal transcriptional activity of EcR isoforms in the absence of hormone. We studied the influence of full length USP, as well as the impact of its N-terminal and DNA-binding domains on constitutive transcription of the EcR/USP heterodimer. The transcriptional activity of the ecdysteroid receptor is routinely measured with reporter assays using the hsp27 ecdysteroid response element [16–19]. We studied several oligomeric response elements with heterologously expressed EcR in vertebrate cells. The effect of each EcR isoform and USP was analyzed. Using this approach, we evaluated the regulatory function of EcR and the EcR/USP heterodimer with core recognition motifs. This experimental paradigm allows for direct comparison of effects because it eliminates the possible influence of promoter context and the

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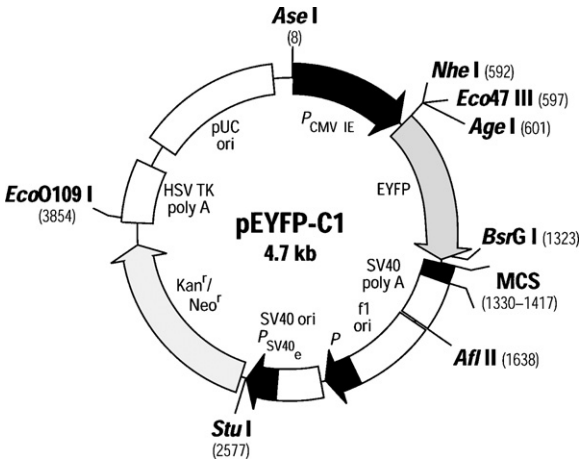

Fig. 1. Scheme of the expression vector pEYFP-C1.

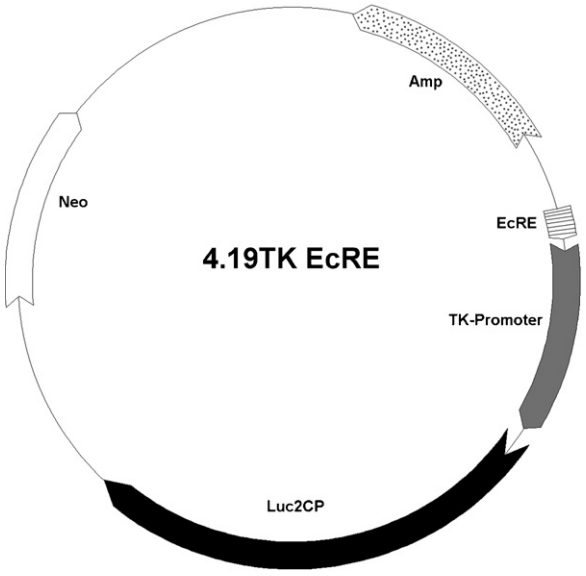

Fig. 2. Scheme of the reporter gene construct pGL4.19TK EcRE.

specific milieu of target cells, which varies among insect tissues and developmental stages.

2. Materials and methods

2.1. Plasmids

The three *Drosophila* EcR isoforms EcR-A, -B1 and -B2 were kindly provided by Dr. A. Ozyhar (Technical University of Wrozlaw, Wrozlaw, Poland). The isoforms were cloned in a pEYFP-C1 vector (Fig. 1) (Clontech, Saint-Germain-en-Laye, France) using *Hind*III and *Bam*HI restriction sites, which results in the expression of full-length EcR isoforms with a fluorescent tag attached to their N-termini [20].

Wild type USP, also provided by Dr. A. Ozyhar, was cloned into pEYFP-N1 (Clontech, Saint-Germain-en-Laye, France) and further modified by Dr. S. Braun (University of Ulm, Ulm, Germany) to express dUSP wt without the YFP-tag [21]. $VP16_{AD}$–USP ΔDBD (USPIII) was cloned between the *Eco*RI and *Hind*III restriction sites into a pVP16 expression vector (Clontech, Saint-Germain-en-Laye, France), replacing the A/B domain of USP wt with the VP16 activation domain [18]. The original A/B domain of USP is replaced by the VP16 activation domain and overcomes the inhibitory effect of this N-terminal domain on the transactivation of reporter genes in vertebrate cells [22]. The *D. melanogaster* USP ΔDBD was constructed by first introducing two *Afl*II restriction sites flanking the DNA binding domain of dUSP using site-directed mutagenesis of pZ7-1 [23]. The 5′ DBD mutation at amino acids 103 and 104 (L and C) changed CTCTGC to CTTAAG. The 3′ DBD mutation occurred at amino acids 169 and 170 (M and K) changing ATGAAG to CTTAAG. Amino acid C104 corresponds to the start of the DBD and K170 to the start of the LBD. The mutations were then verified by sequencing. The mutated dUSP was isolated with PCR using a forward primer tailed with *Not*I at the start codon, and a reverse primer tailed with *Xba*I at the stop codon, removing the 5′ and 3′ UTRs. The sequences of the forward and reverse primers are as follows: 5′-TTTTGCGGCCGCACCATGGACAACTGCGACCAGGAC-3′ (F) and 5′-TTTTTCTAGACTACTCCAGTTTCATCGCCAG-3′ (R).

The insert was then ligated into an empty pBluescript II KS- plasmid (Fermentas, Glen Burnie, MD) and digested with *Afl*II to remove the DBD region. After electrophoresis on a 1% agarose gel to remove the DBD the pBS II KS–dUSP ΔDBD fragment was excised and ligated back together, resulting in a clean transition from the last amino acid in the dUSP A/B domain (L) to the first amino acid of the dUSP LBD (K). The pBS II SK–dUSP ΔDBD construct was digested with *Not*I and *Kpn*I and the dUSP ΔDBD fragment was ligated into the multiple cloning site of the vector pEYFP-N1 (Clontech, Saint-Germain-en-Laye, France). The construct was further modified resulting in the expression of dUSP ΔDBD (Dr. S. Braun, University of Ulm, Ulm, Germany).

The luciferase reporter vector pGL4.19 (Fig. 2) [*luc*2CP/Neo] (Promega, Madison, USA) has been optimized to reduce the number of transcription factor binding sites in the reporter gene and vector backbone. The luciferase of pGL4.19 is destabilized by two different protein degradation sequences (CL1 and PEST) and therefore more responsive to monitor rapid processes. The thymidine kinase (TK) promoter of pGL4.74 [*hRluc*/TK] was digested with *Acc*65I and *Hind*III and ligated into pGL4.19.

DNA sequences of the response elements are indicated in Table 1.

Oligonucleotides were obtained from Sloning (Sloning BioTechnology GmbH, Puchheim, Germany) and cloned in front of the TK promoter, between the *Sfi*I and *Acc*65I restriction sites. The constructs pGL4.19TK ± EcRE were analyzed by DNA sequencing (GATC Biotech, Konstanz, Germany) and their plasmid maps are available upon request.

2.2. Cell culture and transfection

CHO-K1 cells [24] were maintained in DMEM/F12 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FCS) (Sigma, Deisenhofen, Germany). The cells were seeded in six-well plates (Nunc, Wiesbaden, Germany) with 4×10^5 cells per well. After 24 h the cells were transfected with Nanofectin (PAA Laboratories GmbH, Pasching, Austria) according to the manufacturer's instructions. Each well received 3 μg of plasmid DNA (2 μg EcR plasmid + 1 μg EcRE luciferase plasmid or 1 μg EcR plasmid + 1 μg USP plasmid + 1 μg EcRE luciferase plasmid). Four hours after transfection the medium was replaced by fresh DMEM/F12 medium, supplemented with 5% FCS, and 1 h later muristerone A (Sigma, Deisenhofen, Germany), dissolved in ethanol, was added to a final concentration of 1 μM. After 24 h the transfected cells were lysed by shaking in 1× passive lysis buffer (PLB 5×, Promega, Madison, USA; 100 μl per well) and homogenized through multiple uptake in a thin syringe (0.4 mm × 20 mm, Terumo, Leuven, Belgium).

Table 1
DNA sequences of the ecdysteroid response elements.

EcRE	DNA sequence
DR1 1×	5′-AGGTCAAAGGTCA-3′
DR1 3×	5′-AGGTCAAAGGTCATGTCCAAGTAGAGAGGTCAAAGGTCATGTCC AAGTAGAGAGGTCAAAGGTCA-3′
DR12 1×	5′-AGGTCAAGAGGCCAAAGAAGGTCA-3′
DR12 3×	5′-AGGTCAAGAGGCCAAAGAAGGTCATGTCCAAGTAGAGAGGTCA AGAGGCCAAAGAAGGTCATGTCCAAGTAGAGAGGTCAAGAGGCCAAAGAAGGTCA-3′
PAL1 1×	5′-AGGTCAATGACCT-3′
PAL1 3×	5′-AGGTCAATGACCTCGTCCAAGTAGAGAGGTCAATGACCTCGTCC AAGTAGAGAGGTCAATGACCT-3′
hsp27 1×	5′-GGTTCAATGCACT-3′
hsp27 3×	5′-GGTTCAATGCACTTGTCGACAAGGGTTCAATGCACTTGTCGACA AGGGTTCAATGCACT-3′
hsp27 5×	5′-GGTTCAATGCACTTGTCGACAAGGGTTCAATGCACTTGTCGACA AGGGTTCAATGCACTTGTCGACAAGGGTTCAATGCACTTGTCGACAAGGGTTCAATGCACT-3′

2.3. Determination of transfection efficiency

Transfection efficiency was determined by the percentage of fluorescent cells labeled by the YFP-tag of EcR, as measured by fluorescence microscopy. Cell cultures with transfection efficiencies outside 75 ± 5% were discarded. In addition, transcriptional activity was normalized on *Renilla* luciferase activity using the dual luciferase reporter (DRL) assay (Promega, Madison, USA) according to the manufacturer's instructions.

2.4. Western blotting and quantification of receptor protein concentration

Cell extracts (25 μg protein/lane) were separated on sodium dodecyl sulfate (SDS) gels [25], which were then electroblotted on nitrocellulose membranes (BA85, 45-μm pore size, Schleicher and Schuell, Dassel, Germany) and stained with Ponceau S to check transblotting efficiency. The membranes were soaked in blocking buffer (20 mM Tris–HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6, 0.02% Thimerosal) containing 3% milk powder (low fat, <1%) and 1% bovine serum albumin (Sigma, Deisenhofen, Germany) for 1 h. Monoclonal anti-GFP antibody (Clontech, Saint-Germain-en-Laye, France) was diluted 1:500 in the same buffer, and the membranes were incubated overnight. Specific bands were detected with a peroxidise-conjugated secondary antibody (anti-mouse IgG, # A-5906, Sigma, Deisenhofen, Germany), diluted 1:500 in TBS (0.1% Tween 20). Signals were visualized with SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, USA). Bands, with the expected molecular weight, were visualized by the Chemi-Smart 5000 photodocumentation system (Vilber Lourmat, Eberhardzell, Germany) and quantified relative to a standard probe (=100%) using Bio-1D software, and the "rolling ball method" (Bio-1D User Manual, Vilber Lourmat, Eberhardzell, Germany). The linearity of the intensity of specific receptor bands was verified by a calibration curve as described previously [21].

2.5. Determination of transcriptional activity

Activities of the luciferase reporter gene were determined with the dual luciferase reporter (DLR) assay system. Despite comparable transfection efficiencies the concentrations of EcR isoforms vary because of differences in receptor protein stability [19]. Therefore firefly luciferase activities were normalized on receptor protein concentrations as determined by quantification of specific Western blot signals.

2.6. Statistical analysis

At least three independent transfection experiments were performed to measure transcriptional activity. Transcriptional activities obtained with monomeric EcREs were normalized as described in Section 2.5. Transcriptional activities obtained with oligomeric EcREs were normalized and expressed as fold induction of the corresponding EcRE monomer. The results of at least three independent transfection experiments were tested either by Student's *t*-test or by one-way ANOVA followed by a Newman Keuls' test for comparison of individual groups. Results are given as means ± SD.

3. Results

Our intention was to characterize the molecular properties and capabilities of EcR and EcR/USP in a constant environment free of endogenous USP. We used CHO-K1 cells, which were routinely used for heterologous studies of the EcR/USP complex [26]. The rationale for using heterologous cell cultures to test EcR and USP function is discussed extensively by Henrich et al. [27]. These studies will be the basis for an *in vivo* approach, which will allow the discrimination, which receptor property is used and how receptor activity is modulated to adapt ecdysteroid receptor function to the actual physiological requirements of different insect tissues during various developmental stages.

3.1. Elimination of nonspecific binding sites reduces basal transcriptional activity and eliminates hormone inducibility in the absence of an EcRE

The transcriptional activity of EcR isoforms is low when each is cotransfected with the empty luciferase reporter vector pGL4.19TK (Fig. 3). The luciferase enzyme has low stability and does not accumulate during the incubation period, thus allowing for determination of rapid changes in transcriptional activity compared to the previously used pGL3TK vector. The transcriptional activities in the absence of an EcRE are nearly identical for all EcR isoforms with the pGL4.19TK as a reporter vector, but are considerably increased in heterodimers with USP using the pGL3TK vector ($p < 0.01$). Luciferase reporter vectors like pGL3 (Promega, Madison, USA) and EcRE-tK-Luc [17–19] contain several nonspecific transcription factor binding sites in the luciferase gene and the vector backbone, most of which are deleted in pGL4 [pGL4 Luciferase Reporter Vectors, Technical Manual, Promega, Madison, USA]. Enhanced activity was seen with the EcR/USP heterodimer that apparently arise from nonspecific interactions with binding sites in the reporter vector (Fig. 3B).

The deletion of nonspecific consensus transcription factor binding sites also reduced quantitative transcriptional levels of the EcR/USP heterodimers for all three isoforms (Fig. 4). With the luciferase reporter vector pGL4.19TK, none of the EcR/USP wt heterodimers showed an up-regulation of transcriptional activity in the presence of muristerone A, whereas a modest induction was noted with EcR-B1 in the presence of muristerone A when using EcRE-tK-Luc vector ($p < 0.05$).

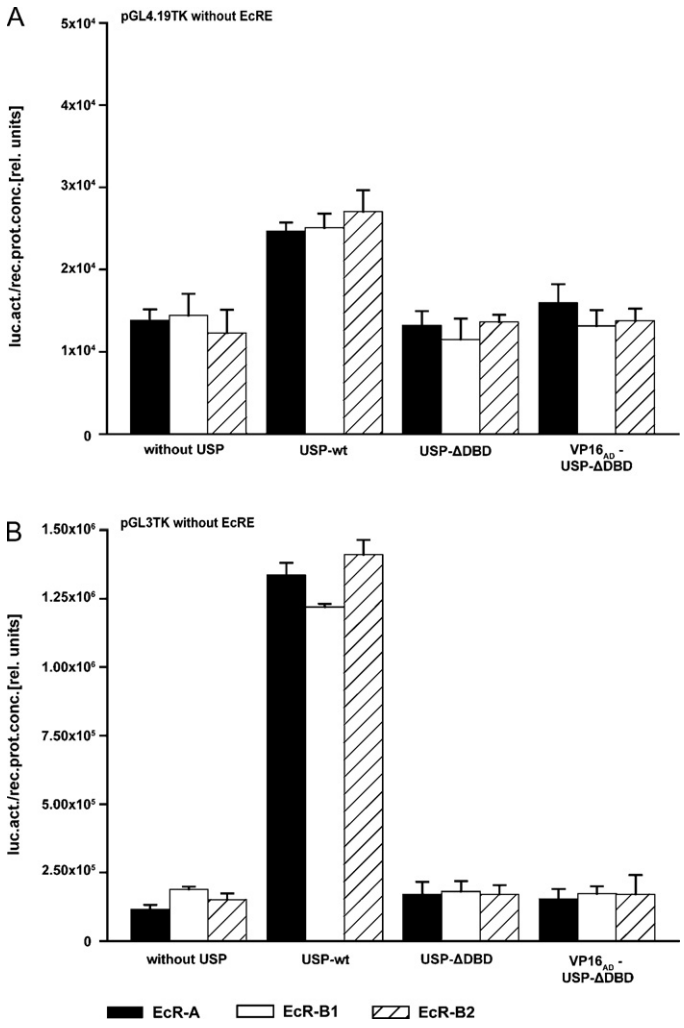

Fig. 3. Non-specific transcription factor binding sites enhance basal transcriptional activity of the ecdysteroid receptor complex. Specific basal transcriptional activity of receptor complexes in the absence of an ecdysteroid response elements (A) without non-specific binding sites (=pGL4.19TK) and (B) with non-specific binding sites (=pGL3TK) ($M \pm$ SD, $n = 3$).

3.2. Transcriptional activity depends on the type of EcRE and is further modulated by the N-terminus of EcR and the DNA binding domain of USP

The pattern of transcriptional activity is similar for all EcR isoforms and is mainly influenced by the type of EcRE (Figs. 5–7). For these studies, luciferase activity was measured as a function of the receptor protein concentration.

Compared to hsp27, PAL1 and DR12 rather low activities were obtained with DR1 independent of the presence of USP (Fig. 5). Generally, luciferase activities of EcR-B2 were about the same or lower in combination with all hormone response elements tested. USP preferentially stimulated EcR-B1 bound to hsp27 and EcR-B2 interacting with hsp27 or PAL1, whereas transcriptional activity of EcR-A was either not affected by USP (hsp27, PAL1) or was even lower with DR12. Deletion of the DBD of USP increased transcriptional activity selectively with EcR-A interacting with hsp27, PAL1 and DR12. Dimerization of EcR-B isoforms with USP ΔDBD reduced the activity of the receptor complex preferentially in the presence of hsp27.

The exchange of the original N-terminus of USP ΔDBD by the VP16 activation domain modified the pattern of transcriptional activity. However, the influence of the N-terminus of USP is of minor importance for EcR-B isoforms. These results demonstrate that basal transcriptional activity is the result of a complex interaction between response element, the N-terminus of both dimerization partners and the DBD of USP.

3.3. Cooperative effects of oligomeric response elements with EcR and USP are most pronounced with direct repeats and are prevented by the DNA-binding domain of USP

Higher relative activity with EcR-A and -B1 and with EcR-B1/USP wt and EcR-B2/USP wt was evoked from a single *hsp27* response element than with three or five tandemly repeated elements (Fig. 6). Interestingly, a suppressive effect between multiple copies of the *hsp27* element and the EcR-B1 and -B2/USP wt heterodimers was subsequently eliminated when the USP DBD was deleted. Oligomeric response elements did not generally elevate, but even reduce transcriptional activity in some cases (Figs. 6 and 7). For all EcR isoforms, interactive effects were most pronounced with the DR1 element (Fig. 7). As seen already with *hsp27* the DBD of USP prevents cooperativity with the exception of EcR-A/USP wt on DR12.

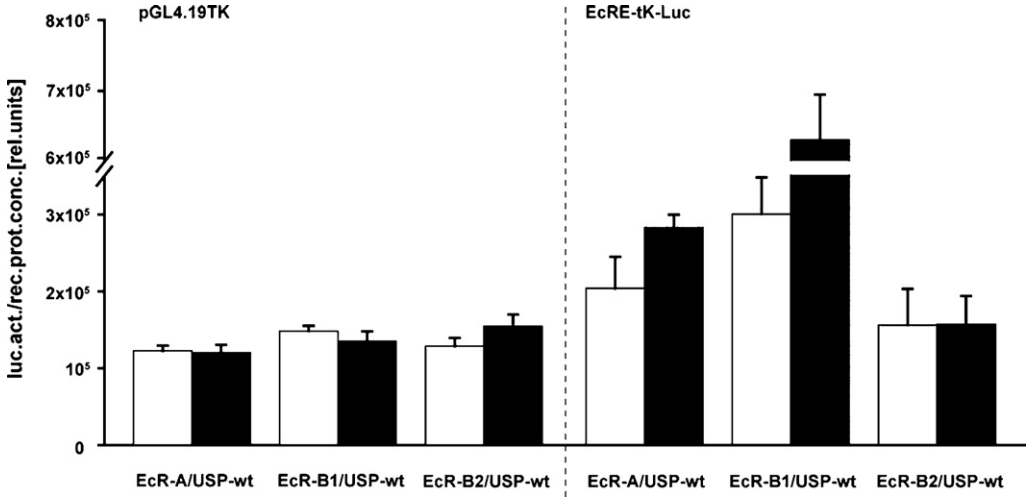

Fig. 4. Non-specific transcription factor binding sites are essential for hormone induced increase in transcriptional activity. Specific transcriptional activity of EcR/USP wt receptor complexes in the presence of pGL4.19TK (most of the non-specific transcription factor binding sites are removed) and EcRE-tK-Luc (many non-specific transcription factor sites present). White bars: without hormone; black bars: with hormone [1 μM muristerone A] ($M \pm$ SD, $n = 3$).

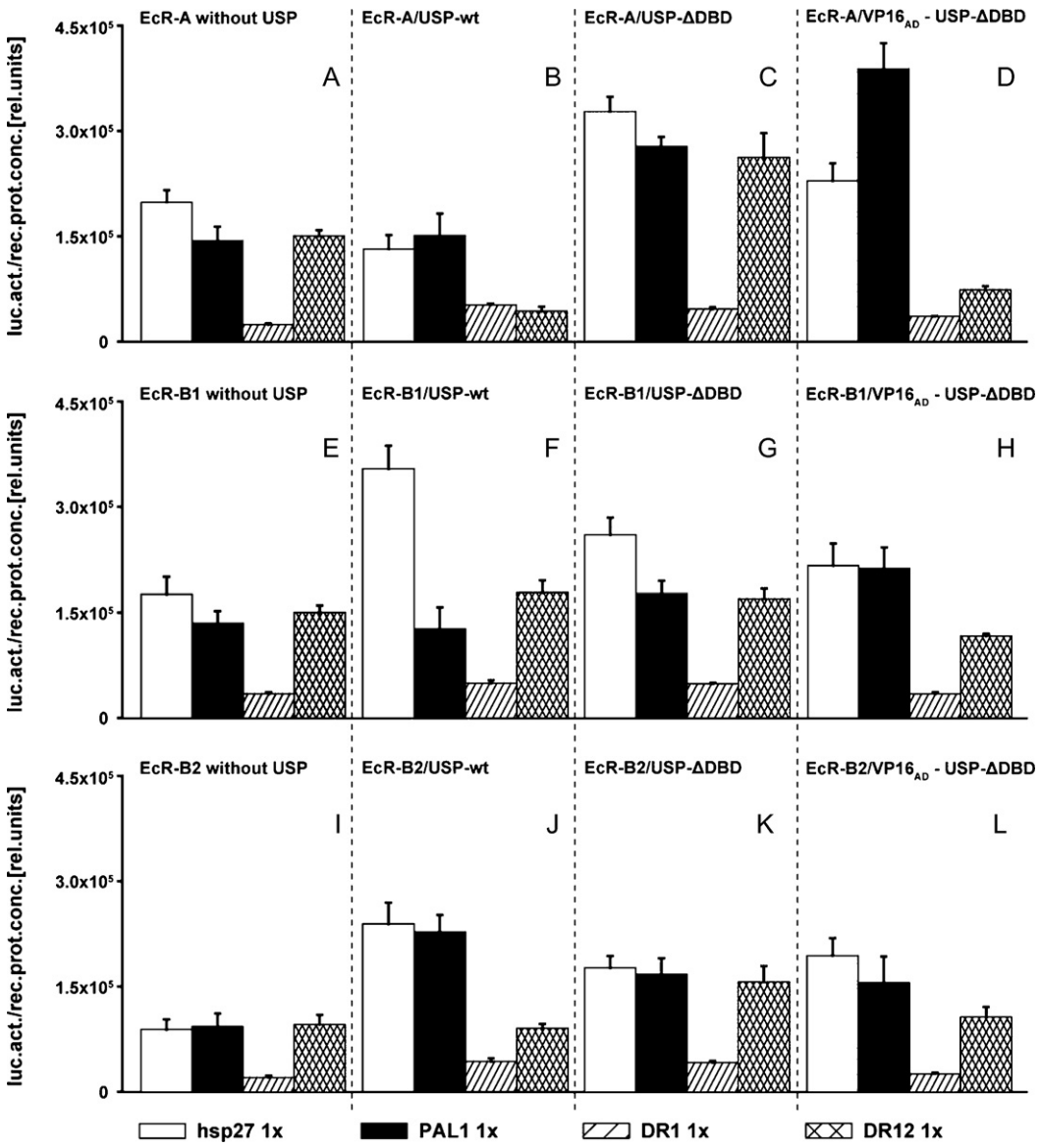

Fig. 5. Specific transcriptional activity is modulated by the AB-domains of EcR and USP, the DBD of USP and the type of ecdysteroid response element. Specific basal transcriptional activities of receptor complexes in the presence of monomeric EcREs (hsp27, PAL1, DR1 and DR12) are compared ($M \pm SD$, $n = 3$).

Comparison of heterodimers with USP ΔDBD and $VP16_{AD}$–USP ΔDBD showed, that in addition to the DBD, the N-terminus of USP also reduce transcriptional activity on oligomeric response elements in some cases (e.g. EcR-A on direct repeats and EcR-B/USP ΔDBD on DR1). Like transcriptional activity of monomeric response elements, cooperativity, as measured by transcriptional activity, depends on the type of hormone response element, the N-termini of both receptors, and the dimerization sites involved.

4. Discussion

A heterologous cell culture system allows for direct comparisons of the ecdysteroid receptor-mediated transcriptional activity using a variety of individual promoters [27]. Because these studies are carried out in a cellular environment that is constant, using cells which display no endogenous ecdysteroid receptor activity, the capability exists to analyze and compare effects of EcR isoforms and modified USP constructs directly. Using this approach these studies revealed that ecdysteroid receptor activity is influenced in distinct ways by several factors: EcRE-promoter context, the number of tandemly repeated response elements, the type of response element, the N-terminal domains of EcR and USP, and the DBD of USP. All of these, therefore, potentially have a bearing on *in vivo* transcriptional activity.

4.1. Influence of the N-terminus of EcR on basal transcriptional activity

Consistent with previous reports, the EcR isoforms displayed different levels of activity that was further affected by the presence of USP, response element type, and response element repeats. When tested alone without USP, generally lower activities are observed with EcR-B2 compared to either EcR-A or -B1. Previous reports have generally indicated that EcR-A displays lower activity levels in vertebrate cells [16,18,28], though if the receptor protein concentration is taken into account, the specific transcriptional activity of EcR-A is about the same as determined for EcR-B1. The N-terminus of EcR-A is more susceptible to proteolytic cleavage ([29]; Schauer, unpublished results) resulting in lower EcR-A concentrations despite comparable transfection effi-

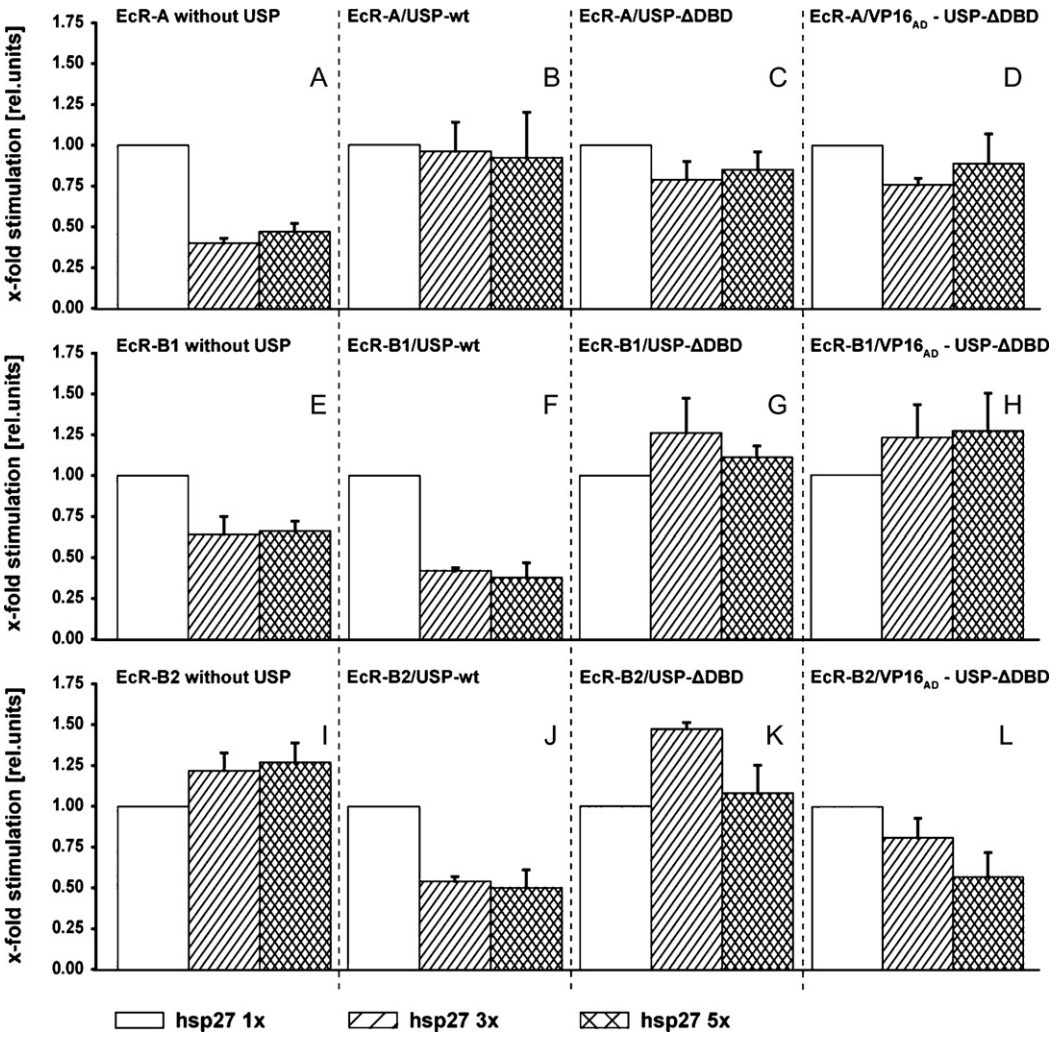

Fig. 6. USP modifies specific transcriptional activity in the presence of oligomeric hsp27. Specific transcriptional activity is expressed as fold induction of the monomeric hsp27 ($M \pm SD$, $n = 3$).

ciencies [19]. The distinct properties of EcR-A illustrate that protein concentration and stability affect measured activity. High levels of EcR-A, such as those observed in certain regions of the CNS at the beginning of metamorphosis [3], thus compensate for the reduced stability of EcR-A *in vivo* even in the absence of USP [12].

4.2. USP selectively modifies transcriptional activity

In contrast to EcR isoforms in the absence of USP, the influence of the N-terminus of EcR on transcriptional activity is more pronounced in the presence of USP wt, especially in combination with *hsp27*. According to Niedziela-Majka et al. [30] the DBD of USP acts as a specific anchor that binds to the 5′ half site of hsp27 and thus determines the orientation of the receptor on the DNA, which may allow a conformational change that thus increases the influence of the N-terminus of EcR on transcriptional activity.

The derepressive effect of removing the USP DBD was seen most obviously with the DR1 element, for which an *in vivo* element that is affected by ecdysteroids has not been verified in the *Drosophila* genome. While the derepressive effect resulting from mutations affecting the USP DBD have been reported for specific ecdysteroid-inducible genes [14], these findings have led to the suggestion that a DR1 element is normally a target for USP-mediated repression of transcriptional activity. The effect of deleting the USP DBD on either single or multiple copies of the canonical hsp27 EcRE was discernible only with EcR-A, though some derepressive effect of USP ΔDBD was seen with EcR-B1 and multiple copies of hsp27 EcRE. The latter observation must be viewed circumspectively from a developmental standpoint, since no examples of such tandemly repeated inverted elements have been reported in the *Drosophila* genome.

The rather low activities reported previously [16,28] for heterodimers with EcR-A compared to EcR-B isoforms interacting with *hsp27*, which were interpreted to be the consequence of an inhibitory region in the N-terminus of EcR-A [16,28] are confirmed by the current study. However, the repressive function of heterodimers with EcR-A and -B1 in the absence of hormone [13] seems to be caused mainly by the DBD of USP, despite reduced dimerization caused by the lack of the dimerization interface in the C-domain [31]. The inhibitory action of the N-terminus of USP [17] is of minor importance at least in the absence of the USP DBD.

Interaction of the N-termini of both dimerization partners EcR and USP with different ecdysteroid response elements modifies the transcriptional capability of the receptor complex. Promoter context-specific modulation of transcriptional activities associated with the N-terminal regions of both dimerization partners were shown previously for the vertebrate nuclear receptors RAR and RXR [32].

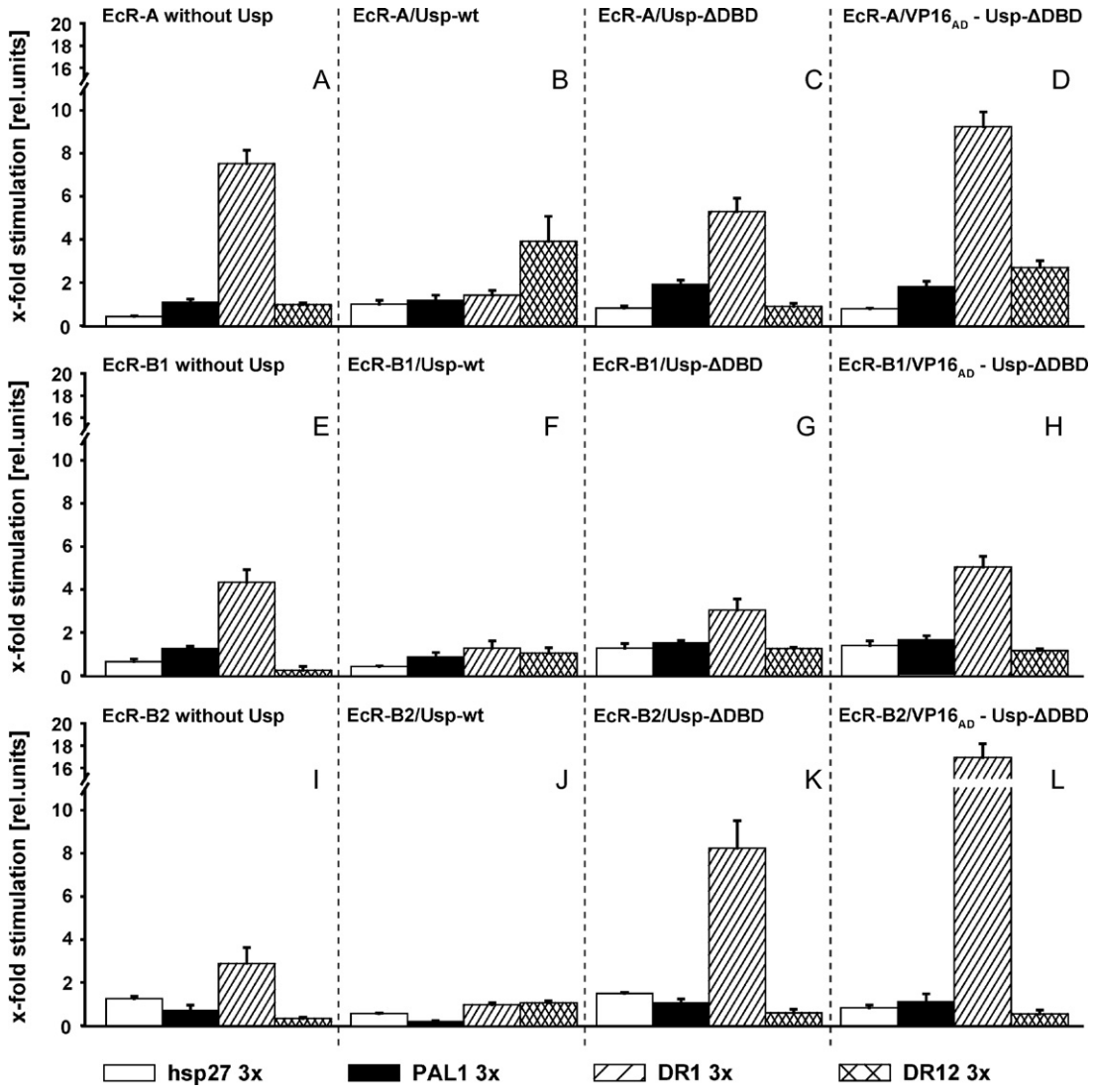

Fig. 7. Transcriptional activity is selectively enhanced with trimeric DR1 in the absence of USP-DBD. Specific transcriptional activity is expressed as fold induction of the corresponding monomeric EcRE ($M \pm$ SD, $n = 3$).

Due to the high plasticity of the EcR DBD [33] a wide variety of different hormone response elements can be bound [7,8,10] and interaction of heterodimers with asymmetric response elements like direct repeats is also possible [34]. Interaction with direct repeats is certainly facilitated by an increased spacer length between the consensus half sites and explains the rather low activity of all isoforms with DR1.

The interaction with different hormone response elements has consequences for dimerization. In solution, nuclear receptors dimerize via the interfaces located in the ligand binding domains, whereas dimerization mediated by the DNA-binding domains takes place only in the presence of DNA [35]. The nature of the response element, therefore, determines the use of the heterodimerization interfaces. Both of these dimerization interfaces were utilized on asymmetric elements like the imperfect palindrome hsp27 or direct repeats. In addition the LBD cooperates with DBDs to enhance the affinity to hormone response elements [36–38]. Symmetric elements such as PAL1 afford only dimerization via the DBD without participation of the ligand binding domain [36].

The type of hormone response element also dictates the orientation of nuclear receptors [38]. Typically symmetrical response elements, like inverted repeats, result in a head-to-head orientation of the protein–protein-interface. Asymmetrical response elements, like direct repeats, result in a head-to-tail orientation [39], although head-to-head arrangements are also reported e.g. for AR [40], demonstrating the high flexibility of nuclear receptor complexes.

4.3. Interaction with hormone response elements alters the conformation of nuclear receptors

Hormone response elements not only are simple docking sites for nuclear receptors, but also modify the conformation of the receptor complex in an allosteric manner and thereby alter the activity seen at specific target genes [41]. Response elements, differing only in a single base pair, can differentially affect receptor conformation as shown recently for GR [42]. In the case of ER, the type of hormone response element with which the receptor associates regulates the structure of the coactivator pocket thereby providing different functional surfaces for interaction with comodulators [43–45]. By analogy to such observations reported for vertebrate receptors therefore, it is reasonable to postulate that altered comodulator interactions arise from the type of response element and is at least partially responsible for the differences in transcriptional activity observed in this study.

4.4. Influence of promoter context on transcriptional activity

These studies showed that ecdysteroid receptor-mediated transcriptional activity not only depends on an interaction between the ecdysteroid receptor and a given hormone response element, but is also modified by surrounding DNA sequences. The importance of flanking sequences adjacent of the hormone response element for regulation of transcriptional activity of the ecdysteroid receptor was outlined previously [7,46], and was shown also for vertebrate receptors such as the androgen receptor [47]. Nevertheless integration of additional flanking sequences or insertion of binding sites for NF-1 and the octamer motif, which is reported to be essential for hormone stimulation [48] did not restore hormone sensitivity [Schauer, unpublished results]. Participation of DNA sequences in addition to the core response element may also be the cause for the inhibition of transcriptional activity in the absence of hormone in insect cells [13,49,50]. The considerable increase in transcriptional activity observed in the presence of full length USP, but not USP ΔDBD, with pGL3TK, which still contains a number of binding sites for transcription factors compared to pGL4.19TK indicates that USP binds to motifs, which have not been identified as typical EcREs interacting with EcR/USP.

4.5. Interaction between multiple hormone response elements

The present study also indicates the possibility that response elements contribute interactively to transcriptional activity. This was evident when testing reporter constructs which lacked an EcRE and were varied in their activity when tested with EcR and/or USP. The arrangement of binding sites within a composite response element is known to affect their regulatory function [51]. Composite hormone response elements may synergistically activate transcriptional activity, when multimerized or tightly linked to other regulatory elements. The cooperativity of nuclear receptors on multiple hormone response elements allows the formation of multimeric receptor complexes and has been shown for thyroid receptor [52]. The influence of the N-terminus of nuclear receptors has been described for vertebrate receptors such as progesterone receptor isoforms [53]. Interaction with DNA shapes the non-structured N-terminus of nuclear receptors into an active conformation [54,55], and is obviously sensitive to even small variations in the sequence of a given hormone response element. Comparison of transcriptional activities of receptor complexes bound to hsp27 and PAL1 reveals that even small variations of the EcRE affect receptor-DNA cooperativity.

Cooperative effects between EcR and USP on transcriptional activity are not detectable with full length USP, but are even more pronounced with USP ΔDBD than in the absence of USP altogether. Dimerization mediated only by the ligand binding domain, without participation of the dimerization interface in the DBD likely increases the flexibility of the receptor complex. The arrangement of the receptor molecule seems to be less important, since opposite effects are observed for DR1 and DR12, although both are commonly associated with head-to-tail arrangement of the receptor proteins. The distance between the EcREs (10–13 nucleotides) is in a comparable range as reported for oligomeric hsp27, routinely used for determination of transcriptional activity [56], and is sufficient to rule out sterical hindrance. According to Jakób et al. [57] the DNA binding domains of EcR/USP interact with 7–8 bp, which means the receptor complex occupies a half side of the core recognition motif and 1–2 additional base pairs, leaving 9–12 unoccupied base pairs between two adjacent response elements.

5. Conclusion

The determination of basic regulatory capabilities of ecdysteroid receptor restricted to the interaction with core recognition motifs in a vertebrate cell culture system revealed a complex pattern of interaction between the N-terminal domain, the dimerization partner and the DNA sequence, that further modulate the transcriptional response. Comparison with *in vivo* experiments will show, which of these differing capabilities of the receptor protein are actually relevant in certain physiological conditions. These capabilities are further diversified *in vivo* by the promoter context and the specific milieu of target cells that depends on the tissue and the developmental stage.

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