Functionality *versus* strength — has functional selection taken place in the case of the ecdysteroid receptor response element?

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Received: 11 July, 2002; accepted: 28 August, 2002

**Key words**: 20-hydroxyecdysone, ecdysteroid receptor, ultraspiracle, nuclear receptor, *Drosophila*, protein–DNA interaction

Nuclear receptors are ligand-dependent transcription factors responsible for controlling differentiation, growth and development of higher eukaryotes. Three amino acids within the recognition α-helix of the DNA-binding domain of the nuclear receptors constitute the so-called “P-box” which determines response element specificity. In the ultraspiracle (Usp) protein, which together with EcR forms the heterodimeric ecdysone receptor, the P-box residues are E19, G20 and G23. Substitution of E19, the most characteristic amino acid for estrogen receptor-like P-boxes, with alanine showed that the mutation did not appreciably alter the affinity of the wild-type Usp DNA-binding domain (UspDBDWT) for a probe containing natural ecdysone response element (*hsp27wt*). Since in many cases E19 contacts a G/C base pair in position –4, which is absent in *hsp27wt*, we analysed the interaction of UspDBDWT, E19A and other P-box region mutants with the *hsp27wt* derivative which contains a G/C instead of an T/A base pair in position –4. UspDBDWT exhibited higher affinity for this element than for *hsp27wt*. Moreover, a different interaction pattern of P-box region mu-

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This work was supported by grants to A. Ożyhar from the State Committee for Scientific Research (KBN, Poland) grant 3 P04B 009 23 and the Center for Biomonitoring, Biotechnology and Protection of Ecosystems of Lower Silesia.

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**Abbreviations**: DBD, DNA-binding domain; 20E, 20-hydroxyecdysone; EcR, product of the *EcR* gene; EcRDBD, DNA-binding domain of the product of the *EcR* gene; EcRE, 20-hydroxyecdysone response element; EMSA, electrophoretic mobility shift assay; GST, glutathione S-transferase; *hsp27wt*, 20-hydroxyecdysone response element consisting of an imperfect palindrome from the promoter region of the *Drosophila hsp27* gene; *hsp27-4G*, *hsp27wt* with base pair G/C in position –4; RE, response element; RXR, retinoic acid receptor; Usp, product of the *ultraspiracle* gene; UspDBD, DNA binding domain of the product of the *ultraspiracle* gene.
20-Hydroxyecdysone (20E) is a steroid controlling larval moulting, metamorphosis and reproduction in insects and other Ecdysozoa (Kozlova & Thummel, 2000; Sluder & Maina, 2001). The hormone acts via its receptor—a heterodimer of two proteins (Yao et al., 1992)—the products of the EcR (Koelle et al., 1991) and ultraspireacle (Oro et al., 1990) genes (EcR and Usp, respectively). EcR and Usp belong to the nuclear hormone receptor superfamily, which comprises ligand-dependent transcription factors with a characteristic domain structure (Evans, 1988). However, ecdysone receptor holds a unique position among other receptors. Although its ligand is a steroid, the functional ecdysone receptor is a heterodimer (Yao et al., 1992) unlike vertebrate steroid hormone receptors (Gehring, 1998). Moreover, it seems to bind its response elements only upon ligand binding (Thomas et al., 1993), which is not true of the receptors heterodimerising with the vertebrate retinoic acid receptor (RXR) (Torchia et al., 1998)—a Usp ortholog. The most conserved part of nuclear receptors is the DNA binding domain (DBD) with two characteristic zinc-binding modules (Freedman et al., 1988). Following the binding of the proper ligand, nuclear receptors interact with specific DNA sequences called response elements (REs). REs are derived from a common consensus sequence which during evolution was duplicated and modified, and now comprise two types of sequences—direct repeats, characteristic for heterodimeric receptors and palindromes, preferred elements for vertebrate steroid hormone receptors (Renaud & Moras, 1995). The duplicated structure of the REs is in consistence with the nature of the nuclear receptors often forming homo- or heterodimers upon binding their REs. The main structure responsible for the specific recognition of the proper RE is the \( \alpha \)-helix of the first zinc-binding module of the DNA-binding domain (Renaud & Moras, 2000). It has been demonstrated that only a few amino acids within the \( \alpha \)-helix are responsible for the recognition of the correct RE. Three of them, which constitute the so-called “P-box”, are the most important ones for the mechanism of RE discrimination (Green et al., 1988; Mader et al., 1989; Nelson et al., 1999).

The proteins in the nuclear receptor superfamily can be divided into subgroups according to their P-box sequences. EcR and Usp belong to the estrogen receptor-like group with the P-box amino acids E19, G20 and G23 (Umesono & Evans, 1989). Unlike vertebrate heterodimeric receptors, which prefer RE organized as direct repeats (Renaud & Moras, 2000), the 20E receptor preferentially mediates transcription through highly-degenerated pseudopalindromes with a single intervening nucleotide (Riddihough & Pelham, 1987; Antoniewski et al., 1993; Antoniewski et al., 1994; Antoniewski et al., 1995; Lehmann & Korge, 1995; Lehmann et al., 1997). Our previous work (Niedziela-Majka et al., 2000) on EcR and Usp DBDs suggested polarity of the UspDBD/EcRDBD heterodimer on the natural pseudo-palindromic 20E RE (EcRE) from the Drosophila hsp27 gene (Riddihough & Pelham, 1987; Ożyhar et al., 1991), with UspDBD acting as a specific anchor that preferentially binds the 5′ half-site of this element locating the heterocomplex in a defined orientation (see Fig. 1 for information on UspDBD and hsp27 EcRE sequences).

Here we demonstrate that mutation of E19, the most characteristic amino acid found in all receptors containing estrogen receptor-related P-box sequences (Umesono & Evans,
1989), did not appreciably alter the affinity of UspDBD and the UspDBD/EcRDBD heterocomplex for hsp27 element. It was unexpected since E19 forms the most comprehensive, structurally conserved and specificity-determining contacts in other receptors (Schwabe et al., 1993; Rastinejad et al., 1995; Zhao et al., 1998; Meinke & Sigler, 1999; Rastinejad et al., 2000; Zhao et al., 2000). In order to determine the basis for the insensitivity of UspDBD towards the mutation of its supposedly most critical P-box amino acid, we analyzed the interaction of wild type UspDBD (UspDBDWT) and its E19A mutant with the hsp27 element and a derivative thereof containing a G/C base pair in position −4 instead of T/A. Our results indicate that the E19 residue of UspDBD seems to be not involved in any hsp27 element sequence-discerning contacts. Interestingly, natural selection tends to favor EcREs which similarly as the hsp27 element contain a T/A base pair in the position −4, although the substitution of the T/A base pair with G/C generates target sequence with higher affinity and distinct functional properties.

MATERIALS AND METHODS

**Bacterial strain and plasmid vector.** The plasmid pGEX-2T (Amersham, Biosciences, Germany) containing lacIq gene was used for expression of DBDs as fusions with Schistosoma japonicum glutathione S-transferase (GST-DBD) (Smith & Johnson, 1988). For GST-DBDs production Escherichia coli strain BL21(DE3)pLysS (Studier, 1991) (Novagen, U.S.A.) was used.

**The construction of DBDs-expression vectors; site-directed mutagenesis.** Construction of the expression plasmids for the wild type Drosophila melanogaster Usp GST-DBD (pGEX-UspDBDWT) was described previously (Niedziela-Majka et al., 1998). PCR-based megaprimer mutagenesis protocol (Barik, 1995) was applied to introduce alanine codons. The plasmid pGEX-UspDBDWT was used as a template and the sequences of the mutated DNA fragments were verified by dideoxy sequencing.

**Overexpression and purification of the wild-type and mutant proteins.** The expression of GST-DBDs and purification of GST-free wild type and mutated UspDBD, were performed as described previously (Niedziela-Majka et al., 1998; Grad et al., 2001). The designations of the respective mutant UspDBD, are based on the amino acid single letter code (e.g., E19A = Glu-19 → Ala).

**DNA-binding assays.** The sequences of the ds oligonucleotides used in the study (see Fig. 1B) are based on the sequence from the D. melanogaster hsp27 gene promoter (Riddihough & Pelham, 1987; Özyhar et al., 1991). Electrophoretic mobility-shift assays (EMSAs) (Fried & Crothers, 1981) were performed under conditions previously elaborated (Grad et al., 2001).

**Protein concentration.** Concentration of the purified proteins was determined spectrophotometrically at 280 nm using absorption coefficients calculated according to Gill and von Hippel (1989).

RESULTS AND DISCUSSION

The E19 residue of nuclear receptor DBDs with estrogen receptor-related P-boxes forms most the defined, structurally conserved and specificity-determining contacts with the response elements. In many receptors E19 accepts a hydrogen bond from the N4 of the cytosine of base pair G-4/C-4 and in many cases it contacts the adenine of the T-3/A-3 base pair through a water molecule and also takes part in other complex interactions responsible for the response element recognition (Schwabe et al., 1993; Rastinejad et al., 1995; Zhao et al., 1998; Meinke & Sigler, 1999; Rastinejad et al., 2000; Zhao et al., 2000) (see Table 1A). Surprisingly, the introduced mutation of E19 did not appreciably alter the affin-
ity of UspDBD for a ds oligonucleotide containing hsp27 EcRE (hsp27\textsubscript{wt}) (Fig. 2, compare lanes 1–3 and 4–6). However, one notable feature of many EcRE elements, including hsp27\textsubscript{wt}, is that a T/A base pair is present at their position –4 instead of a G/C (Riddihough & Pelham, 1987; Antoniewski et al., 1993; Antoniewski et al., 1994; Lehmann & Korge, 1995; Lehmann et al., 1997). To check if E19 exhibits a potential for binding to the –4 position, we substituted the T-4/A-4 base pair in hsp27\textsubscript{wt} with G-4/C-4 to create hsp27\textsubscript{-4G}. This substitution significantly increased the DNA binding affinity of UspDBD\textsubscript{WT} in comparison with its affinity to hsp27\textsubscript{wt} (Fig. 2, compare lanes 1–3 and 7–9). Unexpectedly, the E19A mutant exhibited lower affinity for the hsp27\textsubscript{-4G} probe than to the wild-type sequence (Fig. 3 compare 3A and 3B; Fig. 2, compare the ratio of CIU complexes intensity to the intensity of free DNA (F) in lanes 4, 5, 6 with lanes 10, 11, 12). The above data may indicate that the interaction between the E19 residue and the G-4/C-4 base pair provides a substantial change in free energy, which overcomes some unfavorable interactions between UspDBD and the hsp27\textsubscript{-4G} element, which take place when E19 is absent, i.e. replaced by A. In contrast, removal of E19 did not reduce significantly the affinity of UspDBD for the hsp27\textsubscript{wt} sequence (Fig. 2, compare lanes 1, 2, 3 and 4, 5, 6). Together, these results suggest that in addition to E19 some other amino acids make the hsp27\textsubscript{-4G} se-

Figure 1. Sequences of the macromolecular components used in this study.

A. Amino-acid sequence of wild type UspDBD (UspDBD\textsubscript{WT}). The numbering in bold is relative to the first Zn-coordinating cysteine of the DBD, whereas numbers in parenthesis relate to the residue position relative to the N-terminus of full-length Drosophila melanogaster Usp (Oro et al., 1990). Residues in open circles correspond to the P-box amino acids; residues (19–31, except for C21) from the putative DNA recognition α-helix that were substituted with alanine are boxed. Note that three of the mutant DBDs (F25A, F26A and V29A) could not be analyzed as they were unstable during purification (Grad et al., 2001).

B. Sequences of the oligonucleotides used in electrophoretic mobility-shift assays. The sequences of the ds oligonucleotides are based on the sequence from the D. melanogaster hsp27 gene promoter (Riddihough & Pelham, 1987; Özüyhar et al., 1991). hsp27\textsubscript{wt} contains a 15-bp semi-palindromic EcRE — marked with the arrows. hsp27\textsubscript{-4G} is hsp27\textsubscript{wt} with base pair G/C in position –4; the exchanged nucleotide is underlined. The numbering convention in hsp27\textsubscript{wt} was taken from previous study (Grad et al., 2001). For clearness of presentation only one strand of ds nucleotides is shown.
sequence-specific contacts, which do not take place when \( hsp27_{wt} \) is used as a target sequence. To test this hypothesis we analyzed the interaction pattern of \( hsp27-4G \) with UspDBD mutants where individual amino acids of the putative recognition \( \alpha \)-helix were substituted with alanine (see Fig. 1A). The results presented in Fig. 4 indicate that substitution of the G20, K22, G23, T28 and K31 residues (P-box region amino acids) of UspDBD results in different magnitudes of effect on DNA binding than it was observed for \( hsp27_{wt} \) (compare Fig. 4, lanes 3, 4, 5, 8, 10 and 13, 14, 15, 18 and 20, respectively). No clear differences were observed, however, when other amino-acid residues were mutated (compare Fig. 4, lanes 6, 7, 9, and 16, 17, 19, respectively). Thus, we conclude that the E19 residue of UspDBD seems to be not involved in any \( hsp27_{wt} \) sequence-discerning contacts. In contrast, when a G/C base pair is present at the \(-4\) position of EcRE, E19 creates (possibly

**Figure 2.** Comparison of \( hsp27_{wt} \) and \( hsp27-4G \) interaction with UspDBD and the E19A mutant.

Binding of wild type UspDBD (UspDBD\textsubscript{WT}) (lanes 1–3 and 7–9) and E19A mutant (lanes 4–6; 10–12) to the relevant element — \( hsp27_{wt} \) (lanes 1–6) and \( hsp27_{4G} \) (lanes 7–12) was studied. The proteins and their concentrations are indicated at the top. Monomeric complexes between UspDBD and DNA are indicated by Cl\textsubscript{U}; F, free probe. To estimate the relative binding activities compare the ratio of Cl\textsubscript{U} complexes intensity to the intensity of free DNA in the respective lanes.

**Figure 3.** Comparative DNA-binding of the E19A mutant of UspDBD to \( hsp27-4G \) and to \( hsp27_{wt} \).

EMSAs were performed with the respective elements — \( hsp27_{4G} \) (A) or \( hsp27_{wt} \) (B) and increasing amounts of E19A UspDBD. Protein concentration (in nM) in lanes 1–10 was 0, 8, 16, 32, 60, 120, 240, 400, 600, 800.
in co-operation with other P-box region amino-acid residues) protein–DNA contacts, which are distinct from those of \( hsp27_{wt} \) (summarized in Table 1). Notably, the same seems to be generally true for the \( hsp27_{4G} \) interaction with the UspDBD/EcRDBD heterodimer (Fig. 5). These observations suggest that substitution of the \( hsp27_{wt} \) T-4/A-4 base pair with G-4/C-4 would generate a target sequence with distinct functional properties and possibly with a new specificity. In the case of 20E receptor, most naturally occurring elements have the base pair T-4/A-4 (Riddihough & Pelham, 1987; Antoniewski et al., 1993; 1994; Lehmann & Korge, 1995; Lehmann et al., 1997). The reason of this selection is unclear since binding site selection experiments (Vöglti et al., 1998) have shown that an oligonucleotide with the base pair G-4/C-4 binds the ecdysteroid receptor with the highest affinity. The basis for the T-4/A-4 selection might be associated with the ability to differentiate higher order complexes formation on EcREs with either a T/A or a G/C base pair in position -4. As was shown previously, \( hsp27_{wt} \) serves not only as a target for the UspDBD/EcRDBD heterocomplex but contains all the structural information necessary for the synergistic formation of the homodimeric EcRDBD complex (Niedziela-Majka et al., 2000). Our preliminary results indicate that the \( hsp27_{4G} \) element binds EcRDBD with higher affinity than \( hsp27_{wt} \), but the presence of the G/C base pair at the -4 position appears to exclude the cooperative formation of the EcRDBD homodimeric complexes (data not shown). We therefore suggest that substitution of the T-4/A-4 base pair of \( hsp27_{wt} \) with G/C might generate a more restrictive binding element, which possesses structural determinants that favor the binding of the UspDBD/EcRDBD heterodimer, but at the same time deter the binding of the EcRDBD homodimer. In contrast, a T-4/A-4-containing target (i.e. \( hsp27_{wt} \)) would interact in a clearly synergistic manner either with the UspDBD/EcRDBD heterodimer or with the EcRDBD homodimer (Niedziela-Majka et al., 2000).

Although it is widely, but not universally, accepted that the functional 20E receptor is the heterodimer of Usp and EcR, we hypothesize that subtle nucleotide differences in the EcREs could provide structural basis for the discrimination of the Usp/EcR heterodimer vs. the EcR/EcR homodimer. The implications of this are that both dimers may contact some regulatory elements, for example \( hsp27_{wt} \), yet they may be elements that specifi-

![Figure 4. DNA-binding of UspDBD mutants to \( hsp27_{wt} \) and \( hsp27_{4G} \).](https://example.com/figure4.png)
Table 1. Contacts of the E19 residue of known DBDs with bases of response elements.

Schematic representation of contacts between side chain of the E19 residue of DBDs with estrogen receptor-type P-box and REs based on the respective crystallographic data (A) and results presented in the study (B). In the left column the DBDs of respective receptors are denoted; in the case of dimeric structures the order of the proteins on the RE is kept. On the right the respective oligonucleotides used in the experiments with interactions with E19 marked are shown. W, water molecule. Underlined are half sites of REs, in italics the spacer between half sites. Note that additional contacts in the case of hsp27_4d are possible.

<table>
<thead>
<tr>
<th>DNA binding domain (reference)</th>
<th>response element</th>
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<tbody>
<tr>
<td>ERDBD-ERDBD (Schwabe et al., 1993)</td>
<td>E19</td>
</tr>
<tr>
<td>RARDBD-RXRDBD (Rastinejad et al., 2000)</td>
<td>E19</td>
</tr>
<tr>
<td>RXRDBD-TRDBD (Rastinejad et al., 1995)</td>
<td>E19</td>
</tr>
<tr>
<td>RXRDBD-RXRDBD (Zhao et al., 2000)</td>
<td>E19</td>
</tr>
<tr>
<td>RevErbDBD-RevErbDBD (Zhao et al., 1998)</td>
<td>E19</td>
</tr>
<tr>
<td>NGFI-BDBD (Moenke and Sigier, 1999)</td>
<td>E19</td>
</tr>
</tbody>
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B

UspDBD (this paper) | AGCGACAAAGGTCA A TGGACCTT GTCAATGAA TCGCTGTTTCCAGT G AAGTTAACAGTTA CTTT (hsp27_4d) |
|------------------|------------------|

UspDBD (this paper) | AGCGACAAAGGTCA A TGGACCTT GTCAATGAA TCGCTGTTTCCAGT G AAGTTAACAGTTA CTTT (hsp27_4d) |

Cally bind only the Usp/EcR heterodimer. This hypothesis requires further support by functional experiments, however, a comparison of the natural EcREs clearly indicates that they contain either a T/A (Riddihough & Pelham, 1987; Antoniewski et al., 1993; Lehmann
& Korge, 1995; Lehmann et al., 1997) or a G/C base pair in the –4 position.

Authors thank Ms Miroslawa Ostrowska for her excellent technical assistance.

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