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Expression and purification of 6xHis-tagged DNA binding domains of functional ecdysteroid receptor from *Drosophila melanogaster**

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Two members of the nuclear receptor superfamily, EcR and Ultraspiracle (Usp) heterodimerize to form a functional receptor for 20-hydroxyecdysone — the key ecdysteroid controlling induction and modulation of morphogenetic events through *Drosophila* development. In order to study aspects of receptor function and ultimately the structural basis of the ecdysteroid receptor-DNA interaction, it is necessary to produce large quantities of purified EcR and Usp DNA-binding domains. Toward this end, we have expressed the EcR DNA-binding domain and the Usp DNA-binding domain as proteins with an affinity tag consisting of six histidine residues (6xHis-EcRDBD and 6xHis-UspDBD, respectively) using the expression vector pQE-30. Under optimal conditions, elaborated in this study, bacteria can express the recombinant 6xHis-EcRDBD to the levels of 11% of total soluble proteins and the 6xHis-UspDBD to the levels of 16%. Both proteins were purified to homogeneity from the soluble protein fraction using combination of ammonium sulphate fractionation and affinity chromatography on Ni-NTA agarose. The gel mobility shift experiments demonstrated that the purified 6xHis-EcRDBD and the 6xHis-UspDBD interact specifically with an 20-hydroxyecdysone response element from the promoter region of the *hsp 27 Drosophila* gene.

Nuclear receptors comprise a large family of ligand-dependent transcription factors which display considerable specificity and selectivity in regulating the genetic programs. The receptor proteins have been identified from organisms as diverse as arthropods and mammals. Included in this family are receptors for steroids, retinoids, thyroid hormone, vitamin D,

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Abbreviations: DBD, DNA-binding domain; EcR, product of the *EcR* gene; EcRDBD, DNA-binding domain of the product of the *EcR* gene; 6xHis, six consecutive histidine residues; 6xHis-EcRDBD, DNA-binding domain of the product of the *EcR* gene with six consecutive histidine residues; 6xHis-UspDBD, DNA-binding domain of the product of the *ultraspiracle* gene with six consecutive histidine residues; *hsp 27*, gene for heat-shock protein 27; IPTG, isopropyl-β-D-thiogalactopyranoside; RXR, 9-*cis* retinoic acid receptor; Usp, product of the *ultraspiracle* gene; UspDBD, DNA-binding domain of the product of the *ultraspiracle* gene.

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as well as a large number of proteins whose ligand has not been identified (orphan receptors) [1]. They share a characteristic modular organisation, whereby separable functions are encoded by discrete functional domains, that encompass ligand-binding, DNA-binding, nuclear localisation and transcriptional modulation [2]. The most conserved region is the DNA--binding domain (DBD), responsible for binding of nuclear receptor to specific DNA sequences, called hormone response elements, which identify particular genes as targets for regulation. This receptor region consists of approximately 90 amino-acid residues. It folds stably in isolation and retains DNA-binding specificity identical to that of the whole receptor. The DBD is stabilised by two zinc ions, each of which is coordinated by four conserved cysteines [3]. The detailed analysis of different hormone response elements has led to the identification of three distinct modes of DNA binding that are distinguished by whether receptors interacting with DNA occur as monomers, homodimers, or heterodimers [4]. The best studied homodimeric recognition of response element sequences is exemplified by steroid hormone receptors. Steroid hormone response elements are generally a pseudopalindromic arrangement of two core recognition sequences. Each DBD of the dimer makes analogous contacts with one of the core recognition motifs, resulting in a rotationally symmetric structure formed by the response element and cooperatively acting DBDs [5]. Less characterised, heterodimeric interactions between nuclear receptors and response elements are exemplified by vitamin D receptor, all-*trans* retinoic acid receptor and thyroid hormone receptor. These receptors can also form homodimers, but heterodimerization with 9-*cis* retinoic acid receptor (RXR) greatly increases the efficiency of DNA binding, especially on direct repeat arrangements of core recognition sequences [6]. The heterodimerization creates a new situation, in which an anisotropic heterodimer-DNA complex is formed. This offers an additional combinatorial possibility to control the activity of sets of responsive promoters with a limited number of nuclear receptors in a differentiated way [4, 6].

In *Drosophila melanogaster* a parallel system of heterodimeric receptor has been identified. Two members of the nuclear receptor superfamily, EcR [7] and Ultraspiracle (Usp) [8] heterodimerize [9, 10] to form a functional receptor for 20-hydroxyecdysone — the key ecdysteroid controlling insect moulting [11]. Together, EcR and Usp bind 20-hydroxyecdysone response elements with high affinity, and co-transfection of EcR and Usp into mammalian cells confers 20-hydroxyecdysone responsiveness [10]. Usp is the *Drosophila* homologue of the vertebrate RXR receptors. RXR and Usp share not just the capacity for heterodimer formation but also their selectivity — Usp forms heterodimers with RXR partners and RXR can partially substitute for Usp [10]. Consequently, RXR-containing mammalian cell lines do not require transfection of Usp along with EcR in order to respond to muristerone A which is a potent 20-hydroxyecdysone agonist [10]. It is therefore clear that *Drosophila*, which has been the subject of the most intensive genetic analysis, will be very useful in deciphering of the roles of heterodimeric interactions between nuclear receptors. The *in vivo* use of this system must be accompanied by intensive biochemical, biophysical and structural studies. Perhaps the most important among these is the elucidation of the molecular mechanism by which the target DNA sequences are recognised. Because of the low abundance of the functional receptor for 20-hydroxyecdysone it is desirable to design expression systems suitable for heterologous high-yield production of EcR and Usp proteins, and especially of their DBDs.

Here we report the first successful expression of DBDs of EcR and Usp as proteins with an affinity tag consisting of just six consecutive histidine residues (6xHis). We have also developed methods for their purification. DBDs are soluble and specifically interact with the *hsp 27* palindrome [12, 13] — until now the best characterised 20-hydroxyecdysone response element.

MATERIALS AND METHODS

Bacterial strains and plasmid vectors. The *E. coli* strain NovaBlue (Novagen, U.S.A.) was used as the host strain for cloning, DNA sequencing and production of expression plasmids.

For DBDs production *E. coli* strain BL21-(DE3)pLysS (Novagen, U.S.A.) was used which

contains a plasmid pLysS that provides a small amount of T7 lysozyme cutting a specific bond in the peptidoglycan layer of the *E. coli* cell wall. When produced from the gene cloned in pLysS, relatively high levels of T7 lysozyme can be tolerated by bacteria. On the other side, it has the advantage of facilitating the preparation of cell extracts. Simply freezing and thawing allows the resident T7 lysozyme to lyse the cells efficiently.

The expression of DBDs with an affinity tag consisting of 6xHis tag placed at the N-terminus of the protein was achieved using the plasmid pQE-30 (DIAGEN, Germany). This vector contains an optimised, regulable promoter/operator element consisting of the *E. coli* phage T5 promoter and two *lac* operator sequences. Expression from this promoter/operator element is extremely efficient and can be prevented only by the presence of high levels of *lac* repressor. Since the pQE-30 vector does not contain the *lacI* gene, the host strain BL21(DE3)pLysS was transformed with pREP4 plasmid (DIAGEN, Germany) carrying the $lacI^q$ gene encoding *lac* repressor. This ensures high levels of *lac* repressor and consequently tight regulation of protein expression. This modified strain was designated BL21-(DE3)pLysSpREP4.

Enzymes, biochemicals, and oligonucleotides. Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Boehringer (Mannheim, Germany). Vent polymerase used for the PCR reaction was purchased from New England Biolabs (U.S.A.). T7 DNA polymerase, T7 Sequencing kit and poly(dI-dC) were obtained from Pharmacia Biotech. DNase I, RNase A were from Sigma, Ni-NTA agarose was from DIAGEN (Germany).

All single-stranded oligonucleotides were synthesized by Pharmacia Biotech. The concentrations of the nucleotides were quantified spectrophotometrically [14].

Construction of expression vectors. Standard techniques for DNA manipulations were used [15]. The cDNA clones encoding the full length *Drosophila* EcR [7] and Usp [8], generously provided by Dr D.S. Hogness (Stanford University) and Dr R.M. Evans (Salk Institute, La Jolla), were used as templates for PCR. All PCR-generated fragments in the constructs described below were resequenced.

The DNA fragments encoding DBDs of *Drosophila* EcR (EcRDBD) and Usp (UspDBD) were obtained by PCR. The fragments encoding EcRDBD (amino acids 256–354) and UspDBD (amino acids 94–193) were amplified using primers designed to generate *Bam*HI and *Hin*dIII restriction sites at 5′ and 3′ ends, respectively. The EcR primers were: 5′-gcc cgg *gga tcc* GCG CCA CGG GTG CAA GA-3′ (sense), 5′-gcc cgg *aag ctt* GTC CTT CTC CTT CTG GGC-3′ (antisense). The sequences of the sense and antisense primers for Usp were: 5′-gcc cgg *gga tcc* AAC CAT CCG CTG AGC GGC-3′ and 5′-gcc cgg *aag ctt* GCC TCC GCT GGC GCT GA-3′, respectively. Small letters in the above sequences represent nucleotides added to EcR and Usp coding sequences for cloning purposes whereas the restriction sites are shown in italics. The PCR conditions were 400 μM of each dNTP, 1 μM of each of the primers and 1 U of the Vent DNA polymerase. A UNO Thermoblock (Biometra, Germany) thermal cycler was used to carry out reactions in 50 μl volume using following conditions: 5 min initial denaturation at 94°C followed by: 5 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C and 0.5 min polymerisation at 72°C and 15 cycles of 1 min denaturation at 94°C and 1 min incubation at 72°C for annealing and polymerisation. The final extension was done at 72°C for 2 min. The amplified fragments were cut with *Bam*HI and *Hin*dIII and inserted into *Bam*HI and *Hin*dIII sites of a pQE-30 vector. The constructed plasmids were designated pQE-EcRDBD and pQE-UspDBD and their expression products 6xHis-EcRDBD and 6xHis-UspDBD, respectively.

Small-scale cultures-studies on time course of expression and solubility of 6xHis-EcRDBD and 6xHis-UspDBD. A 3-ml LB starter cultures containing 50 μg/ml carbenicillin, 25 μg/ml kanamycin and 34 μg/ml chloramphenicol were inoculated with pQE-EcRDBD or pQE-UspDBD-transformed *E. coli* BL21(DE3)pLysS-pREP4 (freshly transformed, originating from a single colony). The cultures were grown overnight with shaking at 180 r.p.m. at 37°C and 2 ml of these were used to inoculate 50 ml of LB containing 50 μg/ml carbenicillin, 25 μg/ml kanamycin and 34 μg/ml chloramphenicol. Cultures were grown at 29°C until the absorbance at 600 nm reached a value between 0.5 to 0.6 and after induction by 0.2 mM IPTG the

incubation was continued for 4 h. Aliquots of 1 ml were collected at appropriate time intervals, centrifuged for 2 min at 4°C and 15000 *g*, and pellets resuspended in 150 µl of ice-cold buffer A (10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, 6 mM $(NH_4)_2SO_4$, 5 µM $ZnCl_2$, pH 7.4 at 22°C) and frozen at –80°C. After thawing cell lysates were incubated with DNase I (0.75 µg) and RNase A (1.25 µg) for 1 h on ice and finally centrifuged for 10 min (15000 *g*) at 4°C. The proteins in the supernatant and the pellet (dissolved in 150 µl of sample buffer) were analysed by SDS/polyacrylamide gel electrophoresis (see below).

Growing large-scale bacterial cultures and preparation of 6xHis-EcRDBD. 200 ml of LB medium containing 50 µg/ml carbenicillin, 34 µg/ml chloramphenicol and 25 µg/ml kanamycin were inoculated with 3 ml overnight culture containing *E. coli* BL21(DE3)pLysS-pREP4 transformed with the plasmid pQE-EcRDBD. The culture was grown at 29°C with shaking at 180 r.p.m. until the absorbance at 600 nm reached a value between 0.5 to 0.6, and then IPTG was added to a final concentration of 0.2 mM. After additional 2 h incubation at the same temperature the bacteria were collected by centrifugation for 10 min at 15000 *g* and 4°C. The supernatant was discarded and the sediment was washed by resuspending in 8 ml of lysate preparation buffer A containing 1 mM 2-mercaptoethanol followed by 10 min centrifugation at 15000 *g*. Finally the bacteria were resuspended in 8 ml of lysate preparation buffer A with 1 mM 2-mercaptoethanol and stored in a 50 ml Falcon tube at –80°C until used. The frozen cells were thawed by placing the 50 ml Falcon tube in a 22°C water-bath. The resulting lysate was incubated with DNase I (5 µg/ml) and RNase A (7.5 µg/ml) for 1 h at 0–4°C with gentle mixing. After centrifugation for 1 h at 25000 *g* at 4°C the pellet was discarded and the supernatant was brought at 0–4°C to 40% saturation with solid $(NH_4)_2SO_4$. The 40% precipitate was pelleted by centrifugation for 20 min at 18000 *g* at 4°C and to the supernatant the solid $(NH_4)_2SO_4$ was added to achieve 65% saturation. The sample was stirred on ice for 1 h and after centrifugation for 20 min at 18000 *g* the 65% supernatant was discarded. The 65% pellet was dissolved in 0.5 ml cold buffer B (50 mM Na_2HPO_4, 300 mM NaCl, 6 mM $(NH_4)_2SO_4$, 1 mM 2-mercaptoethanol, 5 µM $ZnCl_2$, 10% by vol. glycerol, pH 7.8 at 22°C). The resulting solution was centrifuged for 10 min at 15000 *g* (4°C) and the clear supernatant was combined with three other 0.5 ml samples obtained in the same way from three 200 ml bacterial cultures. All subsequent chromatographic steps were done at 4–6°C. To remove the excess of $(NH_4)_2SO_4$ the resulting sample (about 2 ml) was chromatographed over a PD 10 column (Pharmacia) equilibrated with buffer B without $ZnCl_2$. The protein containing fractions from PD 10 column were pooled and combined with 1 ml of Ni-NTA-agarose equilibrated with the same buffer. The mixture was kept in a capped polypropylene column (1 cm × 6.5 cm) at 5°C with gentle rotating. After one hour the gel matrix was allowed to settle down and 1 ml fractions were eluted at a flow rate of 0.5 ml/min with equilibration buffer B without $ZnCl_2$ (10 ml) and then with the same buffer containing 50 mM imidazole. The 6xHis-EcRDBD was eluted with equilibration buffer containing 160 mM imidazole. Following analysis of column fractions on Coomassie Blue R 250-stained SDS/polyacrylamide gels, fractions containing 6xHis-EcRDBD were pooled and dialysed against buffer C (10 mM Na_2HPO_4, 150 mM NaCl, 6 mM $(NH_4)_2SO_4$, 1 mM 2-mercaptoethanol, 10 µM $ZnCl_2$, 10% by vol. glycerol, pH 7.4 at 22°C). After centrifugation for 20 min at 15000 *g* and 4°C the supernatant containing 6xHis-EcRDBD was divided into small portions and stored at –80°C.

Growing large-scale bacterial culture and preparation of 6xHis-UspDBD. For the induction and purification of 6xHis-UspDBD 200 ml of LB medium containing 50 µg/ml carbenicillin, 34 µg/ml chloramphenicol and 25 µg/ml kanamycin were inoculated with 3 ml overnight culture containing *E. coli* BL21(DE3)-pLysSpREP4 transformed with the plasmid pQE-UspDBD. The culture was grown at 29°C and the 6xHis-UspDBD protein was purified exactly in the same way as described above for 6xHis-EcRDBD.

Studies of DNA-protein interactions. To determine the ability of the recombinant DBDs to interact specifically with DNA, mobility shift assays [16] were performed using purified preparations of 6xHis-EcRDBD and/or 6xHis-UspDBD. In these experiments synthetic 20-hydroxyecdysone response element from *Dro-*

sophila hsp 27 gene promoter [12, 13] was used. Complementary DNA strands (upper strand: 5'-AGC GAC AAG GGT TCA ATG CAC TTG TCC AAT GAA-3') were annealed at the concentration 80 μg/ml (each oligonucleotide) in 50 mM Tris/HCl, 50 mM NaCl (pH 7.4 at 22°C) by boiling at 90°C for 5 min and cooling to 22°C over a period of 1 h. Double-stranded oligonucleotides were labelled using T4 polynucleotide kinase with [γ-^{32}P]ATP. Nonspecific, nonlabelled double-stranded oligo- nucleotides for competition experiments prepared in a similar way had the following sequence (upper strand shown): 5'-GCC GCC ATG CTG AGA GCT CGG CGA AGT GGT-3'. The indicated amounts (see Fig. 5) of protein(s) and 25 fmoles (about 43000 c.p.m.) of labelled double-stranded *hsp 27* oligonucleotide probe were incubated for 30 min in a final volume of 25 μl of buffer C containing 220 ng of poly(dI-dC) and 0.5 mg/ml of ovalbumin. The receptor-DNA complexes were separated from the free DNA probe immediately after incubation on 5% nondenaturing polyacrylamide gel (20 cm × 16 cm × 0.15 cm) run in 0.25 × TBE (1 × TBE: 90 mM Tris/borate and 2 mM EDTA, pH 8.3). The gel was pre-cooled to 4°C and pre-run at 170 V for 60 min and after applying the samples the electrophoresis was continued at 4°C for 100 min at 200 V. Gels were dried and autoradiographed at –70°C.

SDS/polyacrylamide gel electrophoresis. SDS/polyacrylamide gel electrophoresis [17] was performed to assess the composition and purity of various fractions. Routinely, proteins were separated on 15% polyacrylamide gels and stained with Coomassie Blue R 250. For molecular mass determination proteins from the low molecular mass calibration kit (Pharmacia) were used.

Protein concentration. Protein concentration was determined by the method of Bradford [18] using bovine serum albumin as a standard.

RESULTS

Optimal expression of the soluble 6xHis-EcRDBD and 6xHis-UspDBD in *E. coli*

The pQE-30 constructs bearing inserts coding for 99 amino-acid residues of the EcR and 100 amino-acid residues of the Usp sequences en-

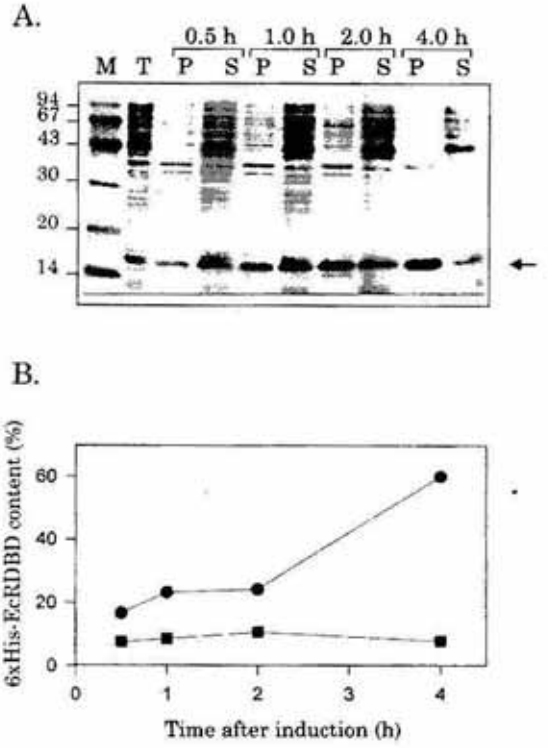

Fig. 1. Time course of appearance and localisation of 6xHis-EcRDBD after induction with IPTG.
A. Electrophoretical analysis of time course of the 6xHis-EcRDBD expression. 50 ml BL21(DE3)pLysSpREP4 bacteria cultures transformed with pQE-EcRDBD plasmid encoding the 6xHis-EcRDBD were prepared and grown as described in Materials and Methods. To analyse samples representing total (T) cellular proteins the BL21-(DE3)pLysSpREP4 cells corresponding to 1 ml culture were harvested before addition of IPTG and solubilized directly in electrophoresis buffer. Specimens corresponding to the soluble proteins (S) and remaining pellet (P) were obtained by lysis of the cells with one cycle of freezing and thawing followed by digestion with DNase I and RNase A and centrifugation. Equal amounts (10 μl) of each fraction obtained 0.5, 1.0, 2.0 and 4.0 h after induction were electrophoresed on a 15% SDS-containing polyacrylamide gel and stained with Coomassie Blue R 250 [17]. The positions of molecular mass markers (M) are shown. The arrow marks the position of the 6xHis-EcRDBD. For more details see Materials and Methods. B. The 6xHis-EcRDBD content in the soluble and insoluble proteins fractions after induction with IPTG. To estimate the 6xHis-EcRDBD content in the soluble fraction and in the pellet appropriate lanes from the Coomassie Blue R 250 stained gel presented in the panel A, were analysed by densitometric scanning. 100% refers to the total protein amount in the respective soluble (■) and pellet (●) fractions obtained 0.5, 1.0, 2.0, and 4.0 h after induction with IPTG.

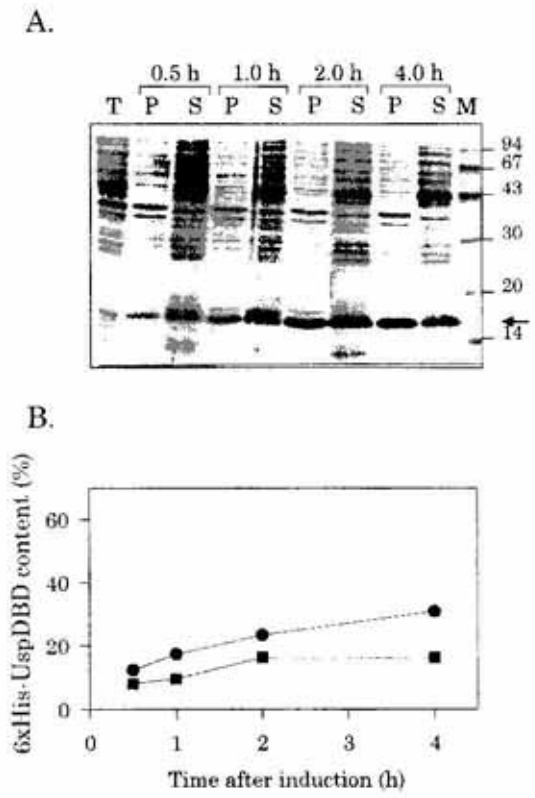

Fig. 2. Time course of appearance and localisation of 6xHis-UspDBD after induction with IPTG.
A. Electrophoretical analysis of time course of 6xHis-UspDBD expression. 50 ml BL21(DE3)pLysSpREP4 bacteria culture transformed with pQE-UspDBD plasmid were grown and analysed as described above for 6xHis-EcRDBD (Fig. 1A). Symbols as in Fig. 1A. B. The 6xHis-UspDBD content in the soluble and insoluble proteins fractions after induction with IPTG. The 6xHis-UspDBD content in the soluble (■) and pellet (●) fractions obtained 0.5, 1.0, 2.0, and 4.0 h after induction with IPTG was analysed as described under Fig. 1B.

compassing the putative DBDs were obtained as described under Materials and Methods. As shown in Fig. 1A and Fig. 2A protein samples obtained from bacteria transformed with pQE-EcRDBD or pQE-UspDBD plasmids and induced with IPTG at 29°C revealed the synthesis of novel proteins of 16.2 kDa and 16.9 kDa, respectively. Although these values were larger then the predicted molecular mass of the 6xHis-EcRDBD (13.598 kDa) and 6xHis-UspDBD (13.551 kDa), it is likely that similarly as in the case of DBDs of glucocorticoid receptor [19] and estrogen receptor [20], the highly basic nature of these proteins could result in aberrant electrophoretic mobility. It is unlikely that expressed proteins were larger than anticipated since the sequence of pQE-EcRDBD and pQE-UspDBD revealed that the appropriate translation initiation and termination codons were intact. Preliminary experiments investigating the localisation of the expressed DBDs showed that they were formed in the insoluble form when induction with IPTG was performed at 37°C (not shown). To maximise the level of the soluble protein, the effect of lowering of the induction temperature [21] was investigated. As shown in Fig. 1A the 6xHis-EcRDBD remains in the soluble protein fraction (lanes S) at all tested time points following induction at 29°C. To define the time course of appearance of the 6xHis-EcRDBD and its content in soluble and insoluble fractions more quantitatively we have performed densitometric scanning analysis of the lanes from the Coomassie Blue R 250 stained gel presented in Fig. 1A. The results of this analysis depicted in Fig. 1B indicate that the 6xHis-EcRDBD comprises about 8% of the soluble proteins 0.5 h after induction. This value did not change appreciably throughout the ensuing 4 h period. In parallel with these changes, the 6xHis-EcRDBD content in the insoluble fraction increases rapidly from 15% (0.5 h) to 61% (4 h). The same type of analysis done for 6xHis-UspDBD shows (Fig. 2B) that its content in the insoluble fraction varies only 2.3-fold from 13% at 0.5 h after induction to 30% at 4 h. A similar trend was evident in the analysis of the 6xHis-UspDBD content in the soluble fraction. At the earliest time points after induction it comprises about 8% and after 4 h increases to value of 16%.

A further decrease in the induction temperature and varying of the IPTG concentration gave no further increase in amount of the soluble DBDs (not shown).

Purification of the soluble 6xHis-EcRDBD and 6xHis-UspDBD

Since DBDs of the EcR and the Usp were expressed with an affinity tag consisting of six consecutive histidine residues placed at the N-terminus, Ni-NTA agarose affinity chromatography was applied as a key step for their purification. This was preceded by bacterial lysate preparation based on information from the previously mentioned studies. Consequently, induction of the BL21(DE3)pLysS-pREP4 *E. coli* transformed with pQE-EcRDBD was performed at 29°C for 2 h. Following in-

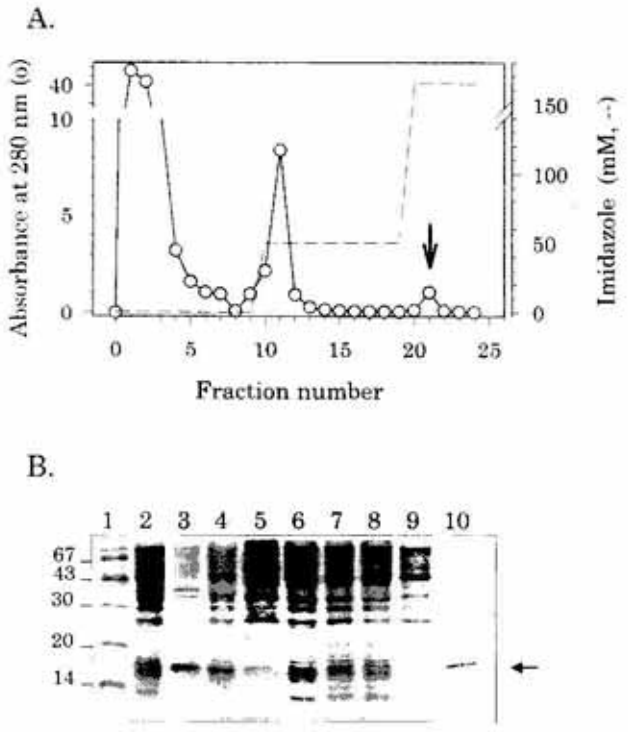

Fig. 3. Purification of 6xHis-EcRDBD.
A. Ni-NTA agarose chromatography. The 40–65% $(NH_4)_2SO_4$ lysate fraction was obtained from BL21(DE3)pLysSpREP4 *E. coli* transformed with pQE-EcRDBD. To remove the excess of $(NH_4)_2SO_4$ the sample (about 2 ml) was chromatographed over a PD 10 column equilibrated with buffer B without $ZnCl_2$. The protein containing fractions from the PD 10 column were pooled and mixed with 1 ml of Ni-NTA agarose equilibrated with the same buffer. The mixture was kept in a capped polypropylene column at 5°C with gentle mixing. The gel matrix was allowed to settle down and the column was operated at 0.5 ml/min, 1 ml fractions were collected. After washing the column with equilibration buffer (fractions 1–10) and with the same buffer containing 50 mM imidazole (fractions 11–20) the 6xHis-EcRDBD was eluted with equilibration buffer containing 160 mM imidazole. Following analysis of the column fractions on SDS/polyacrylamide gels, the fraction marked with an arrow containing the 6xHis-EcRDBD was dialysed, centrifuged and the supernatant was stored at -80°C. For more details see Materials and Methods. B. SDS/polyacrylamide gel electrophoretic analysis of the expression and purification of the 6xHis-EcRDBD. BL21(DE3)pLysSpREP4 strain containing pQE-EcRDBD was grown, induced with IPTG and the resulting cell lysate was subjected to purification procedure consisting of $(NH_4)_2SO_4$ fractionation, PD 10 column gel filtration and Ni-NTA agarose affinity chromatography as described under Materials and Methods. Aliquots of samples from appropriate purification procedure steps were boiled for 5 min in the presence of 1 volume of electrophoresis sample buffer containing 5% (v/v) 2-mercaptoethanol and analyzed by SDS/polyacrylamide electrophoresis gels stained with Coomassie Blue R 250. All specimens which originated from washed pellets were solubilized directly in the electrophoresis sample buffer. *Lane 1*, molecular mass standard proteins; *lane 2*, total proteins (40 µg) from cells grown at 29°C for 2 h, right before induction with IPTG; *lane 3*, proteins (15 µg) in the pellet of induced cells (2 h after induction) after lysis and thawing; *lane 4*, soluble proteins (40 µg) from IPTG induced cells after lysis by freezing and thawing; *lane 5*, protein (40 µg) from 40% $(NH_4)_2SO_4$ pellet; *lane 6*, proteins (40 µg) from 40–65% $(NH_4)_2SO_4$ cut; *lane 7*, proteins (40 µg) from PD 10 column pooled fractions; *lanes 8, 9*, and *10* proteins from Ni-NTA agarose column fractions 1 (40 µg), 11 (20 µg), and 21 (7 µg) (see Fig. 3A). The positions of molecular mass standards (*lane 1*) in kDa are indicated on the left, and the position of the 6xHis-EcRDBD is shown by an arrow.

duction the cells were pelleted and the lysate was prepared as described in Materials and Methods. In order to clarify the lysate and to remove contaminating proteases we apply $(NH_4)_2SO_4$ fractionation as the first step of the purification procedure. After preliminary experiments (not shown) we decided to routinely use 40–65% $(NH_4)_2SO_4$ lysate cut (Fig. 3B, lane 6) containing most of the cellular proteins, including 6xHis-EcRDBD, as starting material for affinity chromatography. In order to remove the excess of $(NH_4)_2SO_4$ a PD 10 gel filtration column was applied. In preliminary experiments the PD 10 column was equilibrated with the buffer B described in Materials and Methods but no $(NH_4)_2SO_4$ was added. In that case however, we have observed considerable variability in recoveries which in some experiments led to irreversible precipitation of the 6xHis-EcRDBD. Since we realised that 6xHis-EcRDBD was stable in the solution obtained after solubilization of 40–65% $(NH_4)_2SO_4$ pellet (i.e. in the presence of $(NH_4)_2SO_4$) we decided to use it as a stabilising agent. The effect of different concentration of $(NH_4)_2SO_4$ on solubility of the 6xHis-EcRDBD was tested. The presence of $(NH_4)_2SO_4$ at 6 mM concentration was found to cause a significant reduction of the amount of the 6xHis-EcRDBD that precipitates and is lost after PD 10 column chromatography (not shown). Consequently, we included 6 mM $(NH_4)_2SO_4$ as a stabiliser in all buffers used for DBDs purification.

After removal of $(NH_4)_2SO_4$ the 6xHis-EcRDBD containing sample (Fig. 3B, lane 7) was subjected to affinity chromatography on Ni-NTA agarose (Fig. 3A). It is well known that proteins with 6xHis tag bind to this resin with an affinity of 10^{-13} M [22]. Thus it is not surprising that the 6xHis-EcRDBD is completely retarded by the column (Fig. 3B, lane 8) and remains bound to it during the washing proce-

dure (Fig. 3B, lane 9) until elution with 160 mM imidazole (Fig. 3B, lane 10). The 160 mM imidazole fraction containing homogeneous 6xHis-EcRDBD was dialysed against buffer C. Between 50 μg and 100 μg of 6xHis-EcRDBD was recovered after removal of the insoluble material by centrifugation.

For the purification of the 6xHis-UspDBD we applied the same procedure consisting of 40–65% $(NH_4)_2SO_4$ fractionation followed by gel filtration on a PD 10 column and affinity chromatography on Ni-NTA agarose column (Fig. 4A and B). However, starting from the same amount of bacterial culture (800 ml), the final yield of the 6xHis-UspDBD was significantly higher. In this case, up to 400–600 μg of homogeneous (Fig. 4B, lane 10) protein could be obtained.

Interaction of the purified 6xHis-EcRDBD and 6xHis-UspDBD with *hsp 27* element

To confirm the identity of each DBD we examined their DNA binding properties using the gel mobility shift assay [16]. Increasing amounts of equimolar mixture of purified 6xHis-EcRDBD and/or 6xHis-UspDBD were incubated with ^{32}P-labelled DNA fragment containing *hsp 27* 20-hydroxyecdysone response element [12, 13], and DNA-protein complexes were resolved by electrophoresis through 5% polyacrylamide gel under nondenaturing conditions. As shown in Fig. 5 (lanes 1–12), with increasing concentration of the DBDs mixture different amounts of two shifted bands labelled (a) and (b) are formed with readily distinguishable electrophoretic mobilities. The complexes are DNA sequence specific, as they are formed in the presence of poly(dI-dC). Furthermore, they can be efficiently competed by 100-fold molar excess of unlabelled *hsp 27* double-stranded oligonucleotide but not with 200-fold molar excess of unlabelled double-stranded oligonucleotide of unrelated sequence (not shown). Although this was not the main goal of this study we tried to define the identity of both complexes. The ^{32}P-labelled *hsp 27* element was incubated with the 6xHis-UspDBD or 6xHis-EcRDBD at DBD concentration equal to the final concentration of both proteins corresponding to lane 10 in Fig. 5. The complexes formed by the *hsp 27* element and 6xHis-EcRDBD or *hsp 27* element and 6xHis-UspDBD were analysed by mobility shift gels

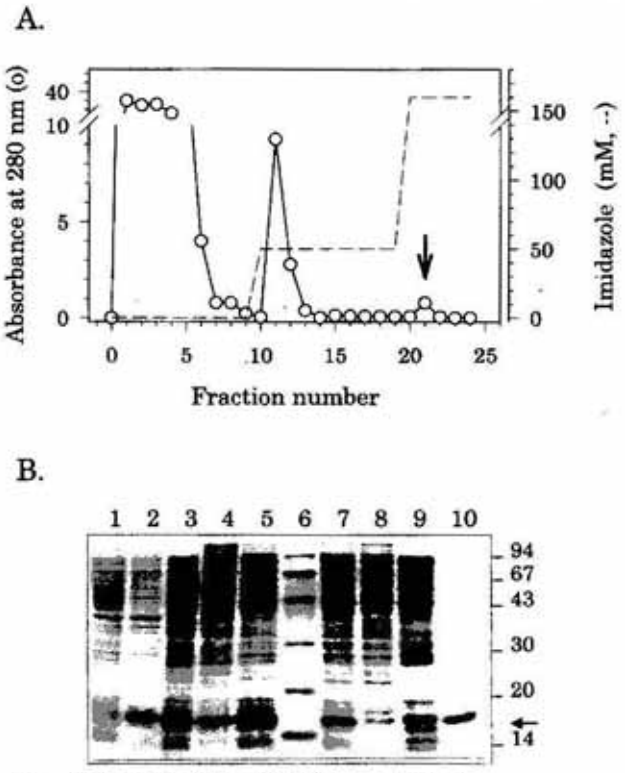

Fig. 4. Purification of 6xHis-UspDBD.
A. Ni-NTA agarose chromatography. The 40–65% $(NH_4)_2SO_4$ lysate fraction was obtained from BL21(DE3)pLysSpREP4 cells transformed with the plasmid pQE-UspDBD as described under Materials and Methods. After removal of the excess of $(NH_4)_2SO_4$ using a PD 10 column the 6xHis-UspDBD containing sample was chromatographed over Ni-NTA agarose column exactly as described for 6xHis-EcRDBD — see Fig. 3A. B. SDS/polyacrylamide gel electrophoretic analysis of expression and purification of the 6xHis-UspDBD. BL21(DE3)pLysSpREP4 cells containing plasmid pQE-UspDBD were grown and IPTG induced as described under Materials and Methods. The cell-free extract was prepared and used as a starting material for purification procedure consisting of $(NH_4)_2SO_4$ fractionation, PD 10 column gel filtration and Ni-NTA agarose affinity chromatography. Analysis of samples from appropriate purification steps was performed by SDS/polyacrylamide gel electrophoresis as described in the legend to Fig. 3B. *Lane 1*, total cell proteins (15 μg) from noninduced cells right before induction with IPTG incubated at 29°C; *lane 2*, proteins (15 μg) in the pellet of IPTG induced cells after lysis by freezing and thawing; *lane 3*, soluble proteins (25 μg) from IPTG induced cells after lysis by freezing and thawing; *lane 4*, proteins (25 μg) from the 40% $(NH_4)_2SO_4$ pellet; *lane 5*, proteins (25 μg) from 40-65% $(NH_4)_2SO_4$ cut; *lane 7*, pooled PD 10 column fractions (25 μg); *lane 8, 9*, and *10*, proteins from the Ni-NTA agarose column fractions 1 (25 μg), 11 (25 μg), and 21 (10 μg) (see Fig. 4A). The positions of molecular mass standards (*lane 6*) in kDa are indicated on the right, and the position of the 6xHis-UspDBD is shown by an arrow.

(Fig. 5, lane 13 and 14, respectively). Only in the case of the 6xHis-EcRDBD two complexes were formed but their mobilities differ clearly from

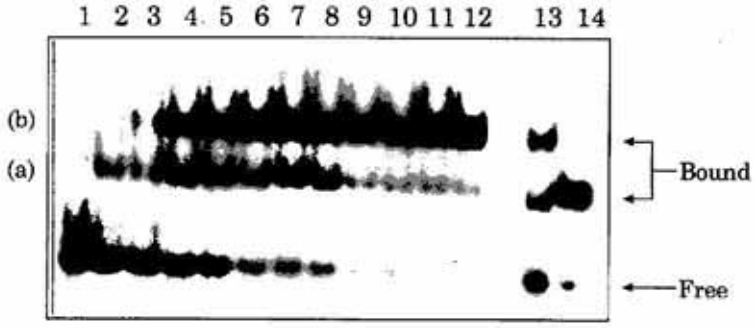

Fig. 5. Binding of the 6xHis-EcRDBD and/or 6xHis-UspDBD to the hsp 27 element.

Amounts indicated below of the purified soluble 6xHis-EcRDBD and/or 6xHis-UspDBD were incubated with a ^{32}P-labelled double-stranded oligonucleotide corresponding to 20-hydroxyecdysone response element from *Drosophila hsp 27* gene promoter [12, 13], and protein-DNA complexes were separated from free DNA by nondenaturing gel electrophoresis as described in Materials and Methods. *Lanes 1–12*, 0.0, 0.39, 0.75, 3.1, 3.9, 5.8, 7.7, 9.7, 13.5, 16.2, 19.3, and 23.1 pmoles of each recombinant DBD, respectively; *lane 13*, 32.4 pmoles of the 6xHis-EcRDBD; *lane 14*, 32.4 pmoles of the 6xHis-UspDBD.

bands (a) and (b) formed in the presence of both DBDs. This result, and the fact that the 6xHis-UspDBD alone forms only one complex, suggest that the band (b) observed in lanes 3–12 represents a heterodimeric complex formed by both DBDs.

DISCUSSION

The steroid hormone 20-hydroxyecdysone plays a key role in the induction and modulation of morphogenetic events through *Drosophila* development [11]. To the best of our knowledge this is the first report describing the expression and purification of the DBDs of the EcR and Usp proteins, which in previous studies have been shown to form a heterodimeric nuclear receptor mediating the action of 20-hydroxyecdysone [9, 10]. The expression of heterologous genes in bacteria is by far the simplest and most inexpensive way for obtaining large amounts of desired polypeptides [23]. However, in many cases the recombinant products were found to be sequestered within large refractory aggregates known as inclusion bodies [24]. Active proteins can be recoverd from inclusion bodies through denaturant-induced solubilization under conditions that favour refolding. Unfortunately refolding is still largely an art and in many cases it reduces the quality and quantity of the expressed protein. For this reason we sought to express the DBDs of the functional ecdysteroid receptor as soluble proteins which can be purified in the absence of any denaturing agents. In preliminary experiments DBDs were recovered as insoluble pellets, however, induction conditions were delineated which allowed their production in a soluble form. Under these conditions BL21(DE3)pLysSpREP4 bacteria can express the recombinant 6xHis-EcRDBD to the level of 11% of total soluble proteins and the 6xHis-UspDBD to the level of 16%. The concentration of the soluble 6xHis-EcRDBD reaches its maximal values shortly after induction (0.5 h) and remains stable during the rest of the tested time period (4 h). At the same time, the content in the insoluble fraction increases dramatically, reaching a value of 61% after 4 h. Although the maximal level of the soluble 6xHis-UspDBD is only slightly higher than of the 6xHis-EcRDBD, its content in the insoluble fraction after 4 h induction reaches only 50% of that for 6xHis-EcRDBD. The above data suggest that for some reason, the 6xHis-EcRDBD, although very efficiently expressed, seems to be less stable in a soluble form. Also in accordance with these observations is its limited solubility in the absence of stabilising 6 mM $(NH_4)_2SO_4$ and considerable variation in the amount of soluble 6xHis-EcRDBD obtained after final dialysis. It should be noted, however, that gel mobility shift experiments demonstrated that purified 6xHis-EcRDBD interacts specifically with *hsp 27* element, with affinity comparable with that displayed 6xHis-UspDBD. Furthermore, it interacts also with 6xHis-UspDBD, changing its mode of interaction with the *hsp 27* element.

In conclusion, the expression system described here provides an efficient method for the recombinant production of the 6xHis-UspDBD and the 6xHis-EcRDBD. Both proteins can be purified in soluble and active form

by Ni-NTA agarose chromatography. This system will be suitable for the production of the quantities of both DBDs for functional and possibly, after some modifications, for structural studies.

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