Functional characterization of two *Ultraspiracle* forms (CtUSP-1 and CtUSP-2) from *Chironomus tentans*

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Abstract:

Two forms, CtUSP-1 and CtUSP-2, of the *Chironomus tentans* homolog of Ultraspiracle (new nomenclature: Chironomus NR2B4) were described and verified as components of the functional ecdysteroid receptor. The two forms differed from each other in the most N-terminal regions of the A/B domain and were tested for several properties. Both forms showed the ability to heterodimerize with CtEcR and interact with a variety of direct repeat and palindromic EcREs, and both conferred specific ligand binding when heterodimerized with EcR. CtUSP-2 showed a twofold higher ponasterone-binding potential than CtUSP-1. Both USP forms demonstrated the ability to activate ecdysteroid-inducible transcription in HeLa cells and the variations in the A/B domain of these forms were not associated with detectable differences in transcriptional activation. Thus, the two forms function similarly. Among species for which USP forms have been reported, *Chironomus* is the most closely related one evolutionarily to *Drosophila*. Despite this proximity, a variety of structural differences were noted in both the A/B and E domains of USP between the two species. The *Chironomus* USP forms lack many of the amino acid residues associated with the ligand-dependent AF2 transactivation function found in all other RXRs and USPs reported so far.

Keywords: Ecdysone receptor; DNA binding; Ligand binding; Transactivation; Nuclear receptors; RXR; Diptera

Article:

1. Introduction

In insects, development of both larval and imaginal tissues during molting and metamorphosis is controlled largely by the steroid hormone 20-hydroxyecdysone (referred to here as ecdysone). The hormonal signal activates a genetic regulatory hierarchy that controls a coordinated developmental process (see Thummel, 1995 for a review). Like a variety of other small, hydrophobic molecules that act as hormonal signals, such as steroids, retinoids, thyroid hormones, and vitamin D3, ecdysone directly controls gene transcription by binding to a nuclear receptor. In turn, these ligand-inducible transcription factors bind to specific, cis-acting hormone response elements in the vicinity of target genes. Ultimately these genes are activated or repressed, either directly by contacting transcription factors of the basal transcriptional machinery or indirectly through intermediary factors.

Nuclear receptors are composed of several modular domains (referred to as A/B, C, D, E and F domains, for review, see Beato, 1989) associated with a variety of functions, including DNA binding (C domain), dimerization (C, D and E domains), ligand binding (E domain), and transcriptional activation (A/B and E domains). Ecdysone responsiveness of insect cells is mediated by two members of the superfamily of nuclear hormone receptors, the ecdysone receptor (EcR, Koelle et al., 1991) and Ultraspiracle (USP, Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990). The activities of the functional ecdysteroid receptors, including high-

affinity binding of ecdysone response elements (EcREs), ligand binding, and transcription of target genes, rely on the formation of a heterodimer of EcR with USP (Yao et al. 1992, 1993; Thomas et al., 1993). USP is the insect homolog of the vertebrate RXRs, the receptors for 9-cis retinoic acid (Mangelsdorf et al. 1990, 1992). Like RXR, USP can form dimers with multiple partners (Sutherland et al., 1995).

The EcR and USP genes of several insect species other than *Drosophila* have been cloned recently (Imhof et al., 1993; Cho et al., 1995; Fujiwara et al., 1995; Kothapalli et al., 1995; Swevers et al., 1995; Dhadialla and Tzertzinis, 1997; Hannan and Hill, 1997; Mouillet et al., 1997), and isoforms for both receptors that show distinct spatial and temporal expression profiles have been reported (Talbot et al., 1993; Jindra et al. 1996, 1997; Mouillet et al., 1997; Tzertzinis et al., 1994; Kapitskaya et al., 1996). However, few of these have yet been characterized biochemically and their properties compared with those of the *Drosophila* EcR/USP heterodimer complex. This issue has taken on added importance recently, since it is known that various insect ecdysone receptors exhibit a range of affinities for nonsteroidal agonists (Dhadialla et al., 1998). Moreover, differences in such properties have been attributed to specific structural regions within the receptor (Suhr et al., 1998).

This study examines the structural, biochemical and functional properties of USP in *Chironomus tentans*. This Diptera is more closely related to *Drosophila* than any of the species for which EcR and USP clones have been obtained so far. A *Chironomus usp* gene has been localized to the chromosomal map region II-14A (Wegmann et al., 1995), a locus that forms a late ecdysone-inducible puff (Clever, 1961). As in some other insects but not *Drosophila*, *Chironomus* has at least two USP forms. They exhibit the same DNA and similar ligand binding properties as well as undistinguishable transactivation capabilities.

2. Materials and methods

2.1. Cloning

The screening of a *C. tentans* prepupal cDNA library with a degenerate oligonucleotide complementary to a region encoding the P box (Umesono and Evans, 1989) within the DNA binding domain (DBD) of nuclear receptors of the RXR heterodimer subfamily (for a review, see Mangelsdorf and Evans, 1995) has been described previously (Imhof et al., 1993). The 32 clones identified in the initial screen were subjected to partial nucleotide sequencing using a primer of the sequence 5'-TGYGAIGGITGYAAIGGITTYTT-3'. The deduced amino acid sequences of the predicted open reading frame from these clones were used to search the Swiss-Prot protein data base for amino acid sequence identities (Wegmann et al., 1995). Apart from the previously described CtEcR (Imhof et al., 1993), six clones displayed substantial similarity to *Drosophila* USP (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990), eleven clones to DHR3 (Koelle et al., 1992), and two clones to *Drosophila* E75A (Segraves and Hogness, 1990). Two CtUSP clones, pMI20, the longest clone, and pMI3 1, were analyzed for sequence (for nucleotide sequence and relationship of clones, see Fig. 2). Other clones included only smaller portions of the sequence found in pMI20.

2.2. Plasmid constructs

Construction of the pSCT-EcR[2-878] expression vector for the *Drosophila* EcR-B1 isoform (Koelle et al., 1991) was described elsewhere (Vögtli et al., 1998). The expression vectors pSCT-CtEcR[2-536], pSCT-CtUSP-1 [2-552] and pSCT-CtUSP-2[2-455] encoding CtEcR (Imhof et al., 1993) and the two forms of *C. tentans* (USP-1 and USP-2), respectively, were constructed in a similar manner. The construction of CtUSP-1 was based on a fusion between pMI31 and pMI20 (for explanation, see Fig. 2). The primer pairs used to amplify the respect-ive coding sequences in a PCR reaction were: 5'-AAGGCCTGAGCTCGGATCCAAGACAGAAAACTTGA-TTG-3' and 5'-AAGGCCTACTAGTTTAGTTTCGATTTATTCGTTCCCG-3' for CtEcR. The amplification of the CtUSP forms relied on the use of a downstream primer (primer 4, for location see Fig. 2) common to both forms, 5'-GCGCGCAAGCTTAAC-CATCTTAATTAAGTTTATGG-3'. The sequence of the CtUSP-1-specific upstream primer 1 (Fig. 2) was: 5'-GCGGATCCTTGAAAAAGGAGAAACCGATG-3', and the sequence of the CtUSP-2-specific upstream primer 2 (Fig. 2) was: 5'-AAGGCCTGAGCTCGGATCCGA-ACTGAGCGGACTACTCGG-3'. The PCR products were then subcloned into pSCT (Rusconi et al., 1990) as a *Bam*HI-*Spe*I DNA fragment for CtEcR and as a *Bam*HI-*Pst*I fragment for the CtUSPs, respectively. Reporter plasmid p(PAL)4-OVEC was constructed by cloning four tandem copies of PAL1(C+7T) (Vögtli et al., 1998)

at promoter position -37 into the β-globin reporter plasmid OVEC (Westin et al., 1987) and used to study ligand-induced transactivation in HeLa cells. The various constructs were verified by nucleotide sequence analysis.

2.3. RT-PCR

Specimens of *Chironomus (Camptochironomus) tentans* embryos, oligopausing larvae, prepupae, pupae, male and female adults were frozen in liquid nitrogen and stored at — 80° C prior to the isolation of total RNA as described (Chirgwin et al., 1979; Ausubel et al., 1992). Approximately ten animals were transferred to a 2 ml microcentrifuge tube containing 0.5 ml guanidium isothiocyanate solution and immediately homogenized with a Polytron homogenizer. After purification by acidic phenol/chloroform extraction and isopropanol precipitation, total RNA was treated with RNase-free DNaseI (Boehringer) at 37*C for 15 min. After heat-inactivation of the DNaseI at 70*C for 5 min and subsequent phenol/chloroform extraction and ethanol precipitation, the concentration of total RNA was determined spectrophotometrically. The purification of mRNA from total RNA was done with an Oligotex mRNA mini kit according to the specifications of the manufacturer (Qiagen). Aliquots of either total RNA or mRNA were used for the RT-PCR reaction by employing a Titan™ one tube RT-PCR system as indicated by the supplier (Boehringer). The sequence of the common region primer 3 (Fig. 2) was: 5'-ACCTCCTGCAGGAGATGTTTGGATCCACTTAA-3'. The Ct USP-1-specific upstream primer 1 (Fig. 2) was: 5'-GCGGATCCTTGAAAAAGGAGAAACCGATG-3' and the CtUSP-2-specific upstream primer 2 (Fig. 2) was: 5'-AAGGCCTGAGCTCGGATCCGAACTGAGCGGACT-ACTCGG-3'. The primer pair for CtL10 (encoding ribosomal protein L10; Galli and Wieslander, 1992; GenBank Accession Number X68332) was: 5'-GAAGCAAAGCGATGTATTGC-3' and 5'-CAATTGGTTAA-CACCATGGC-3'.

2.4. In vitro translation of receptor proteins and DNA binding assay

The pSCT-based receptor expression plasmids were used as templates for the in vitro translation reaction as described (Vögtli et al., 1998). Relative amounts of expressed USP proteins were determined by Western blot analysis performed according to standard procedures (Ausubel et al., 1992) using the monoclonal antibody AB 11 (Khoury Christianson et al., 1992). For the detection of CtUSP forms in *Chironomus* tissue culture cells by Western blotting, samples of cells of the strain established by Wyss (1982) were extracted and processed as described by Rauch et al. (1998). Migrational positions of bands were compared with those of in vitro translated or bacterially expressed CtUSPs (Elke et al., 1997).

DNA binding studies were done by electrophoretic mobility shift assay (EMSA) as described in detail elsewhere (Vögtli et al., 1998). Double stranded oligonucleotides as cold competitor DNA were used at ten-fold molar excess. The sequences of the various oligonucleotides employed are: DR0, 5'-GATCTAGAGAGGTC-AAGGTCATGTCCAAG-3'; DR1, 5'-GATCTAGAGAGGTCAAAGGTCATGTCCAAG-3'; DR2, 5'-GAT-CTAGAGAGGTCAAGAGGTCATGTCCAAG-3'; DR3, 5'-GATCTAGAGAGGTCAAGAAG-GTCATG-TCCAAG-3'; DR4, 5'-GATCTAGAGAGGTCAAGAAAGGTCATGTCCAAG-3'; DR5, 5'-GATCTAGAGAG-GTCAACGAAAGGTCATGTCCAAG-3'; PAL0, 5'-GATCTAGAGAGGTCATGACCTTGTCCAAG-3'; PAL1, 5'-GATCTAGAGAGGTCAATGACCTCGTCCAAG-3'; hsp27, 5'-GATCCGAGACAAGGGTTCAA-TGCACTTGTCCAATGG-3'. For supershifts, 1 μl of undiluted antibody or antiserum was added 10min after the probe was added. Polyclonal antibodies αCtEcRA/B specific for the A/B domain of CtEcR were custom-made by Chiron Mimotopes Pty. Ltd. by immunizing sheep with a synthetic polypeptide of the sequence PNSKLDDGNMSVHMG which corresponds to amino acid position 78–92 of CtEcR (Imhof et al., 1993).

2.5. Ligand binding assay

Ligand binding was measured as described (Turberg and Spindler, 1992). Programmed lysate from the in vitro translation reaction (4 µl of each receptor protein) or unprogrammed lysate as a control were incubated with 2.5 nM [^{3}H]ponasterone A (213 Ci/mmol) in ligand binding buffer (20 mM HEPES-NaOH, pH 7.9, 20 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM 2-mercaptoe-thanol, 1 µg/ml of each aprotinin, leupeptin, and pepstatin) in a final volume of 16 µl. Nonspecific bindingwas determined in parallel by adding 0.1 mM 20-hydroxyecdysone as competitor. After 1 h incubation at room temperature, total as well as nonspecific protein-bound radioactivities (cpm) were measured and specific binding was calculated. Each reaction was done in duplicate

and was terminated by filtration through a nitrocellulose filter. Amounts and quality of the in vitro translated receptors used were routinely checked by quantitative evaluation of Western blots as described by Rauch et al. (1998). Values for [^{3}H]ponasterone A binding were corrected for variations in the abundance of CtEcR and CtUSP-1 or CtUSP-2.

2.6. Insect and cell culture, transfection and transactivation assay

C. tentans strain $2L_{k3}$ was maintained as described previously (Meyer et al., 1983). HeLa cells were grown at 37°C, 6% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, high glucose; AMIMED) supplemented with 10% fetal calf serum and 100 U/ml penicillin/streptomycin. For transcriptional activation of a reporter gene, HeLa cells were seeded in 10 cm dishes to give 60–80% confluency and after growth for 6 h the cells were cotransfected by the calcium phosphate precipitation method according to standard protocols (Ausubel et al., 1992) using 3 μg of each pSCT receptor expression plasmid, 10 μg of $p(PAL)_4$-OVEC reporter plasmid, 1 μg of REF-2 reference plasmid (Wieland et al., 1991), and 5 μg of calf thymus carrier DNA. After 16–18 h, cells were washed twice with 1xphosphate buffered saline (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 (Sigma) at 1 μM or solvent control (ethanol, 0.1% final concentration) was added. The cells were harvested 48 h later and the RNA transcripts of the β-globin reporter gene analyzed by S1 nuclease mapping as described elsewhere (Westin et al., 1987).

3. Results

3.1. Preliminary survey of USP homologs in Chironomus tentans

By use of the monoclonal antibody AB 11, directed against an epitope in the conserved C domain of *Drosophila melanogaster* USP (Khoury Christianson et al., 1992), several cross-reacting bands were revealed on Western blots of extracts from *C. tentans* tissue culture cells, embryos and prepupae (Rauch et al., 1998; Wegmann, 1994, Fig. 1, lane 1). Their apparent molecular weights ranged between 58 and 77 kDa. After phosphatase treatment of the extracts, only the 58 and 60 kDa bands remained (Fig. 1, lane 2) indicating that two USP homologs exist in *C. tentans* and both are phosphorylated (Rauch et al., 1998).

In an extension of the studies by Rauch and coworkers, an additional band was discovered in tissue culture cells, exhibiting an apparent molecular weight of 53 kDa (Fig. 1, lane 5). Its occurrence in these cells is sporadic and its abundance is rare. The band has not been observed so far in whole animal extracts (I. Wegmann, personal communication).

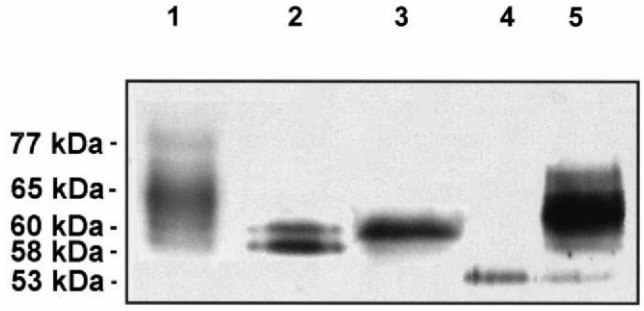

Fig. 1. Comparison of in vitro translated CtUSP-1 (lane 3) and CtUSP-2 (lane 4) with USP homologs present in extracts from *Chironomus* tissue culture cells (lanes 1, 2 and 5) as revealed by Western analyses using an anti-USP-antibody (AB11). The extract in lane 2 has been treated by phosphatase before electrophoresis. Note the comigration of in vitro translated CtUSP-1 with the slower migrating (60 kDa) band clearly visible in dephosphorylated cell extracts (cf. lanes 2 and 3) and the occasional appearance of a faint fast migrating (53 kDa) band comigrating with in vitro translated CtUSP-2 (lanes 4 and 5). Size markers at the left.

The present paper describes the cloning of two cDNA sequences and provides a functional characterization of two USP forms encoded by these clones. Clone CtUSP-1 codes for a protein whose deduced molecular weight

is 61.8 kDa. When expressed by in vitro translation or in *E. coli* and analyzed by Western blotting, the CtUSP-1 encoded protein exhibits an apparent molecular weight of 60 kDa (Fig. 1, lane 3). Thus, it corresponds by size to the 60 kDa USP-homolog detected in tissue culture cells and animals. Clone CtUSP-2 codes for a polypeptide of 51.4 kDa, migrating at a position of 51 kDa when expressed by in vitro translation (Fig. 1, lane 4). The CtUSP-2 product, therefore, corresponds by size to the 53 kDa USP homolog (Fig. 1, lane 5), sometimes detected in extracts of tissue culture cells of *C. tentans*. The low abundance of CtUSP-2 specific RNA, as revealed by RT-PCR using primers 2 and 3 (see Fig. 2 and next section), conforms with the rare occurrence of the 53 kDa homolog in these cells. The cDNA sequence coding for the 58 kDa USP homolog, present in tissue culture cells as well as animals, has not yet been isolated and cloned, although the possibility that this represents a modified form of CtUSP-1 or CtUSP-2 has not been ruled out.

3.2. Description of clones

The insert sizes of the six cDNA clones for *Chironomus* USP were determined, and the longest clone, pMI20, was selected for nucleotide sequencing. A second clone, pMI31 was also sequenced and compared. Its nucleotide sequence (Accession No. AF045891) was identical with that of clone pMI20 in a region of overlap (area printed in bold letters of Fig. 2). However, the two cDNAs differ in the 5' end (Fig. 2, regions printed in italic) so that the N-terminal portion of the deduced protein sequences is different (Fig. 3, see lines designated with CtUSP1 and CtUSP2, N-terminally of "NGS" and "TGR---", respectively). The pMI31 clone encodes a protein of 552 amino acids and includes a 5' sequence of 438 nucleotides not found in pMI20 (Fig. 2, cf. regions printed in italic). This stretch of nucleotides includes a sequence of 124 codons that are in-frame with the remaining common region and the protein encoded by the respective open reading frame has been designated CtUSP-1. Its deduced amino acid sequence shares several regions of similarity with the A/B domains of other insect USPs (cf. Fig. 3). The 5' leader sequence of this clone (Fig. 2, 5' region printed in lower case letters) is preceded by stop codons in all three reading frames. Moreover, the putative AUG start codon lies within a sequence that shows excellent similarity with the consensus *Drosophila* translation initiation site (Cavener, 1987).

The pMI20 clone encodes a second form, CtUSP-2and includes a unique 158 nucleotide 5' sequence (Fig. 2, line USP2, italic letters), that is different from the 5'sequence of the pMI31 clone. This sequence includes a stretch of 27 codons (Fig. 2, line USP2, 5' lower case letter sequence) that are in-frame with the remaining 3'-terminal coding sequence (Fig. 2, upper case letter sequence). The putative start codon (Fig. 2, line USP2: first ATG printed in upper case italic letters) is not preceded by any stop codon, and we cannot formally rule out the existence of other putative start codons lying 5-terminally. The CtUSP-2 specific amino-terminal portion of the A/B domain shows minimal homology with stretches within the homologous domain of CtUSP-1 (Fig. 3).

Since no other USP cDNA clones were obtained thatencoded the A/B domain, we employed RT-PCR and subsequent sequencing to verify the two USP forms. Thestrategy employed a downstream "common" primer (Fig.2, primer 3) in conjunction with an upstream primer specific for one of the USP forms (Fig. 2, primers 1 and 2).The quality of the isolated RNA was monitored by acontrol RT-PCR specific for an RNA species encodingribosomal protein L10 from *Chironomus* (Galli andWieslander, 1992). A 238 nt cDNA fragment expectedfor the A/B domain-coding sequence of CtUSP-1 was amplified from total RNA of prepupal origin and its sequence was confirmed. A 324 nt PCR product that isspecific by length and sequence for CtUSP-2 was obtained from the polyadenylated RNA from larvae andadult males. Genomic PCR with the same pairs of primers yielded amplification products corresponding insize to the RNA-based RT-PCR products. From all theseexperiments, we conclude that CtUSP-1 and CtUSP-2are true forms of *C. tentans* USP and not the result ofa cloning artefact that occurred during library construction.

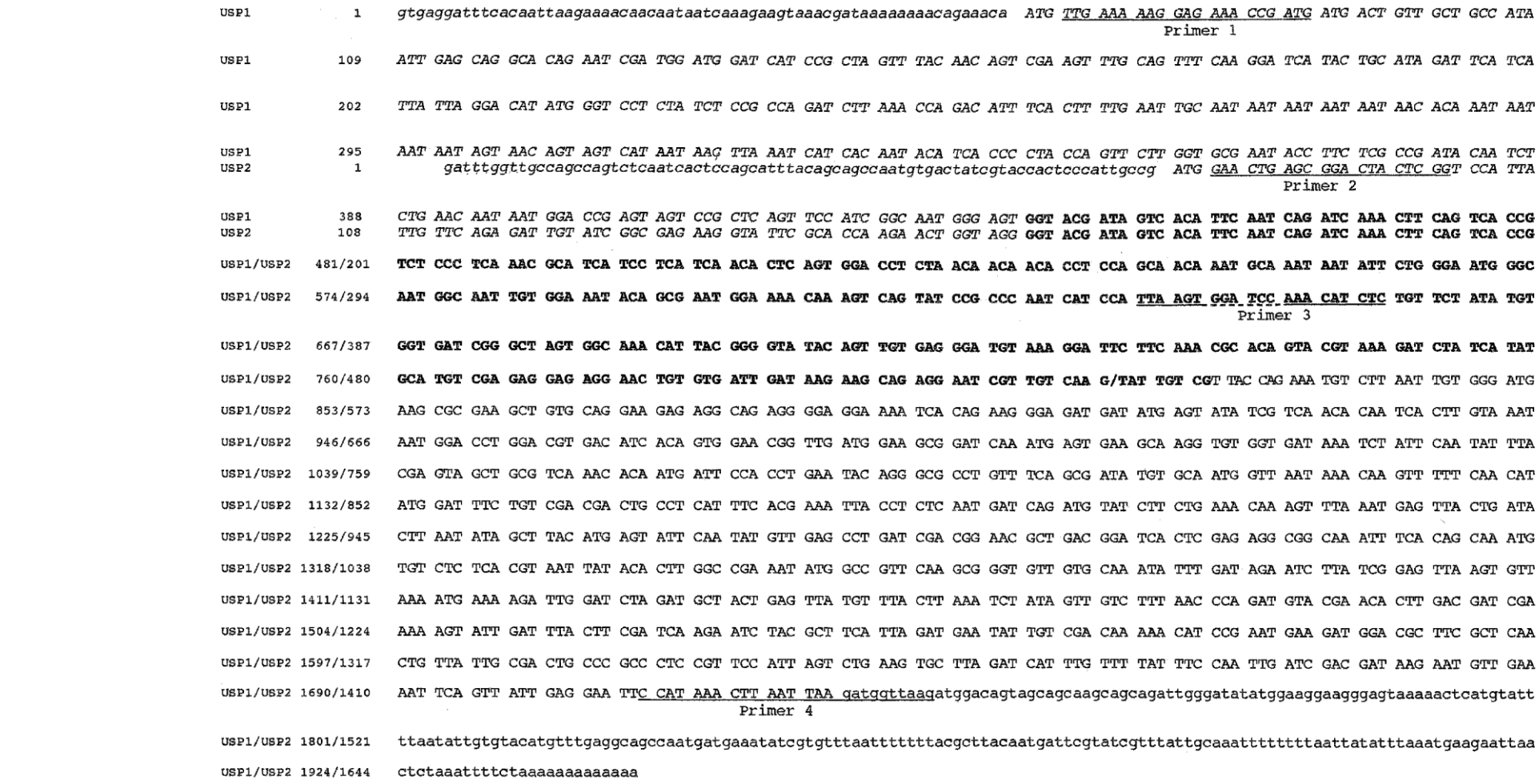

Fig. 2. Description of cDNA clones coding for CtUSP-1 (USP1) and CtUSP-2 (USP2). The nucleotide sequence coding for CtUSP-2 corresponds to the insert of the original cDNA clone pMI20. The nucleotide sequence coding for CtUSP-1 is the fusion product of the 5′ sequence of the original clone pMI31 and the 3′ sequence of pMI20; the fusion site (Bam HI) is indicated by a dotted line. The overlapping sequences of pMI31 and pMI20 are printed in bold letters; the 3′ end of pMI31 coincides with the 3′ end of the bold area. In the third last codon of the common sequence, there is a deviation by one nucleotide (GAT vs. TAT). The respective codon in the CtUSP-1 encoding nucleotide sequence was nevertheless made to be "TAT" corresponding to the situation not only found with pMI20 but also with a third independent clone (pMI45) which otherwise shows the identical sequence in its regions overlapping with pMI31 and pMI20. Sequences printed in lower case letters: non-coding regions. Sequences in italics: regions specific for CtUSP-1 and CtUSP-2, respectively. Underlined sequences: position of primers (1, 2, 3, and 4) used for PCR, RT-PCR, and genomic PCR.

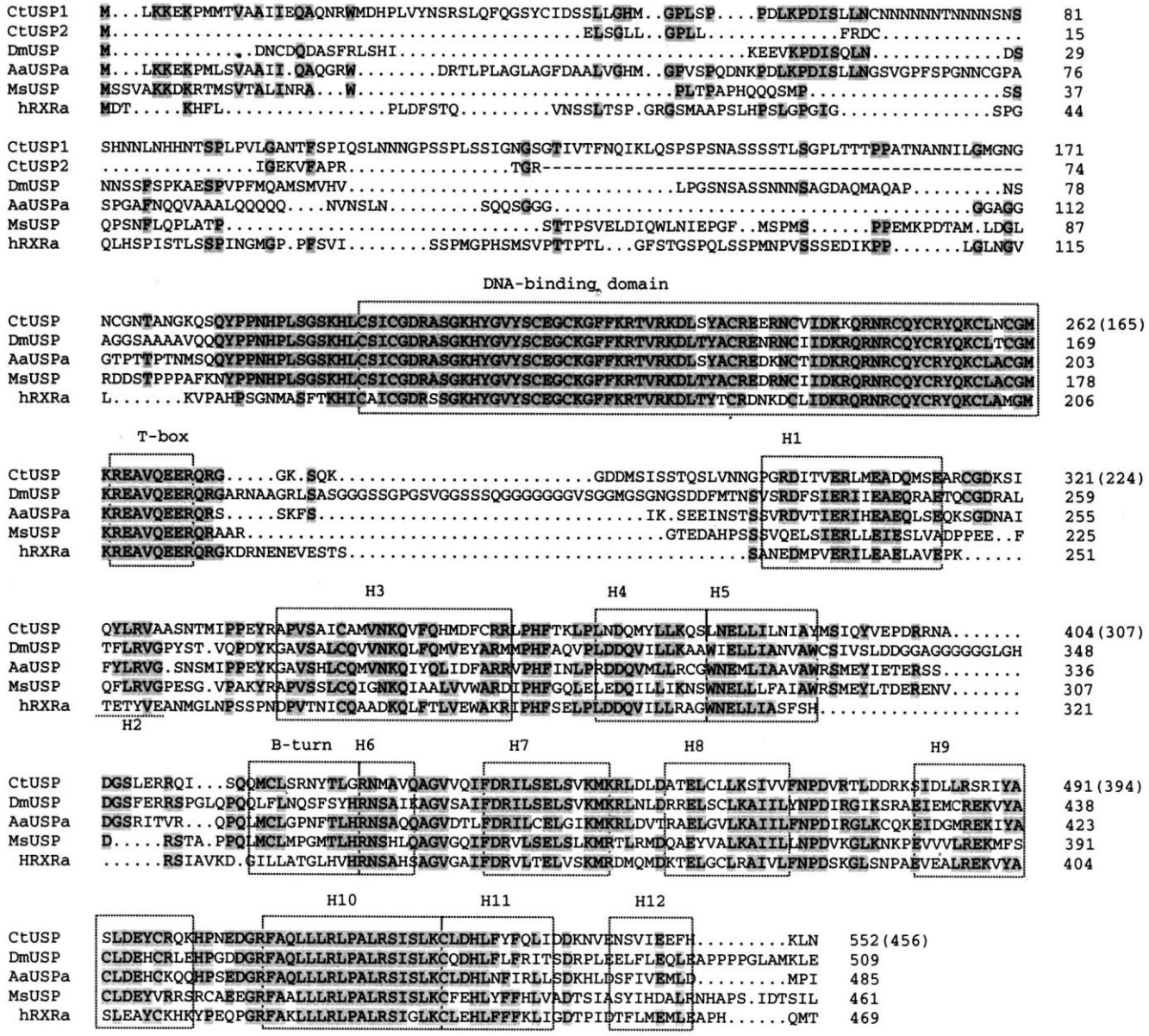

Fig. 3. Deduced amino acid sequences of USP-1 and USP-2 from *Chironomus tentans* and alignment (PAM250 matrix using clustal method) with USP and RXR sequences from other species. The sequence of CtUSP-2 is derived from the nucleotide sequence of pMI20. The sequence of CtUSP-1 is based on the composite nucleotide sequence from pMI31 and pMI20 (see Fig. 2) and it is deposited in the Genbank data base (Accession No. AF045891). Shaded residues are shared by at least three of the listed sequences and boxed regions indicate various domains and subdomains. DNA-binding domain refers to the two zinc finger region, T box refers to an RXR homodimerization region (Wilson et al., 1992), and H1 through H12 refers to subdomains within the ligand binding domain (Wurtz et al., 1996). H2 is only found in human RXR. Dashed line (- - - -) designates the region that is identical between CtUSP-1 and CtUSP-2; this identity continues through the remainder of the sequence. Numbers designate amino acid residues and numbers in paratheses indicate corresponding residue number of CtUSP-2. CtUSP-1, *C. tentans* USP-1; CtUSP-2, *C. tentans* USP-2; DmUSP, *Drosophila melanogaster* USP (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990); AaUSPa, *Aedes aegypti* USPa (Kapitskaya et al., 1996); Ms, *Manduca sexta* USP-1 (Jindra et al., 1997); hRXRα, human 9-*cis* retinoic acid receptor (Mangelsdorf et al., 1990).

3.3. Sequence comparisons of CtUSP with other insect USPs

The CtUSP-1 A/B domain shares several regions of similarity with the analogous domain of other insect USPs (Fig. 3). Beyond clusters of rather general similarity at the very amino terminal end, the CtUSP-1 A/B domain shares additional regions of similarity with corresponding regions of other Diptera i.e., either with mosquito USP (AaUSPa, Kapitskaya et al., 1996) or with fly USP (DmUSP, Henrich et al., 1990). By contrast, the unique portion of CtUSP-2 A/B domain apparently bears little resemblance to the A/B domain of either CtUSP-1 or to that of other insect USPs. This is because it lacks an amino terminal region found in other USP sequences as well as two intervening amino acid stretches that were present in the other USP A/B domains. In fact, a small cluster of amino acids at the very amino terminal end of CtUSP-2 exhibits a reasonable sequence homology to an internal portion of other USP A/B domains.

The sequence of the C domain and of the N-terminal prefinger region, which likely contributes to DNA-binding as well, is virtually identical between CtUSP and those of other USPs (degree of identity: 91-100%). The sequence of the D domain of CtUSP includes a T-box motif that is almost identical among all RXR and USP sequences (Fig. 3, and Chung et al., 1998). This region has been implicated in the regulation of RXR homodimerization. Although USP homodimerization in vitro has been reported with *Drosophila* (Vögtli et al., 1998), with *Chironomus* it could not be observed. The possible role of the T-box region in USP homodimerization (Wilson et al., 1992) has not been explored in either species.

The E domain of CtUSP was aligned with equivalent regions of other insect USP and RXR sequences (Fig. 3). This domain is comprised of up to twelve helical regions (Wurtz et al., 1996). In most of these helical regions, CtUSP showed very strong similarity with other dipteran USPs from *Aedes* and *Drosophila* (see gray shaded areas in Fig. 3). More generally, these helical regions are conserved among most insect USP sequences. For CtUSP, the notable exception seems to be Helix 12. While the CtUSP sequence apparently shows little resemblance to any consensus sequence of H12, it is interesting to note that a glutamic acid residue in the middle of H12 is conserved. For RXR molecules, acidic residues at this position were reported to be important for the ligand-dependent activation function AF-2 in H12 (see Chung et al., 1998; Westin et al., 1998, for discussion and references). In the presence of ligand, H 12 of RXR folds over to form a salt bridge with a basic residue in H4. No such function has yet been attributed to any USP directly, although most USPs including CtUSP contain the respective charged residues in H4 and H12 that could form the postulated salt bridge. CtUSP also resembles the other dipteran USP sequences in non-helical portions of the E domain (gray shaded areas between boxes H1 to H12, Fig. 3). In fact, these regions appear to be highly conserved among USP sequences and to be different from those of RXR (see interhelical regions H1/H3 and H5/H6, Fig. 3), suggesting some as yet unknown functional importance. Among Diptera, *Chironomus* does not share two glycine rich subdomains with *Drosophila* and these glycine rich motifs (in the D domain as well as between H5 and H6d in the E domain) have so far been reported only in *Drosophila* USP.

3.4. DNA-binding properties of CtEcR/CtUSP

We have previously determined the core recognition motif for the EcR/USP heterodimer from *Drosophila* (Vögtli et al., 1998). This PAL1 element was then initially used to assess the DNA-binding properties of the homologous receptor components derived from *Chironomus*. Whereas the individual receptors alone did not bind to PAL 1 when expressed by in vitro translation and assayed under the conditions described in Material and Methods, cooperative binding was observed when both in vitro translated receptor proteins were provided (Fig. 4A, lane 1). Supershift experiments using either the cross-reacting monoclonal antibody AB11 originally directed against DmUSP (Khoury Christianson et al., 1992) or the polyclonal antiserum aCtEcRA/B, which recognizes a short polypeptide region within the A/B domain of CtEcR, indicated that both CtEcR and CtUSP-1 are physical constituents of the observed protein-DNA complex (Fig. 4A, lanes 2 and 3). An identical result was obtained by using the receptor pair CtEcR/CtUSP-2 (data not shown).

We were interested further to see whether CtEcR/CtUSP also shows the remarkable flexibility in DNA binding which has been demonstrated previously for the EcR/CtUSP heterodimer from *Drosophila* (Vögtli et al., 1998). For this purpose, relative binding affinities of the heterodimeric receptor complex to a series of synthetic EcREs with a half-site arrangement either as a direct repeat (DR) or as a palindrome (PAL) were determined by competition EMSAs (Fig. 4B). The various EcREs were used as cold competitor DNA at a tenfold molar excess. The order of decreasing affinity for both receptor complexes, CtEcR/CtUSP-1 and CtEcR/CtUSP-2, regardless of the labeled probe used, was: PAL1 > DR4, DR5, PAL0 > hsp27, DR1 > DR2 > DR3 > DR0. The differences in the A/B domain of CtUSP-1 and CtUSP-2 do not appear to contribute to the DNA-binding affinity of the respective heterodimers (compare upper part of Fig. 4B with lower part of Fig. 4B). Furthermore, the observed affinities reflect those that have been determined previously for the *Drosophila* receptor complex to a large extent (Vögtli et al., 1998).

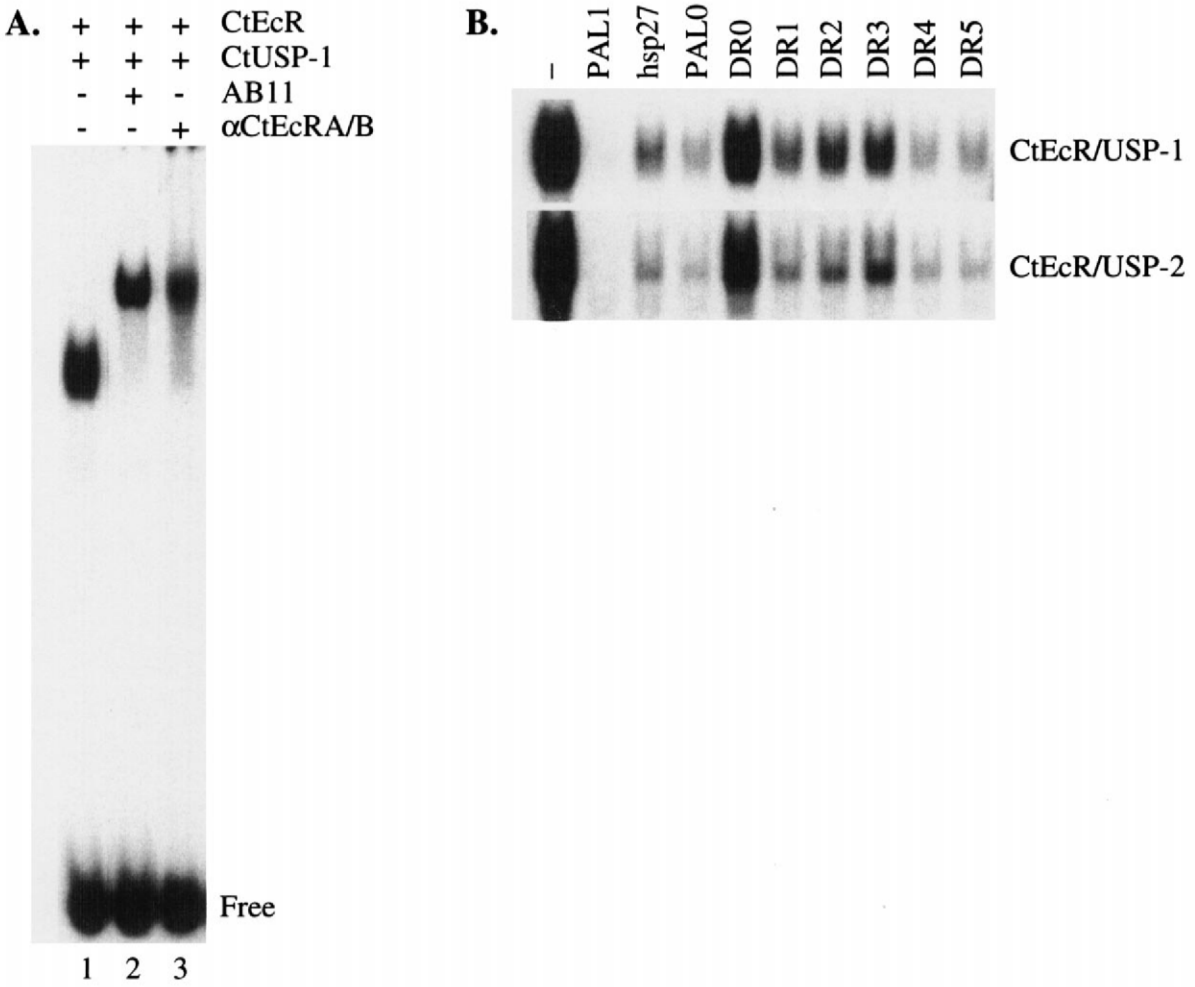

Fig. 4. Binding of CtEcR and CtUSP-1 to DNA. (A) Receptor binding to radiolabeled PAL1 was analyzed by EMSA. The combinations of receptor and antibody or antiserum are indicated above each lane. The location of the free probe is indicated. (B) The CtEcR/USP-1 and CtEcR/USP-2 heterodimer bind to differently structured EcREs. The relative binding affinities to various DNA elements were determined by competition EMSAs. The radiolabeled probe was PAL1. The receptor heterodimer used is indicated at the right. The competitor DNAs were used at a ten-fold molar excess and are indicated above each lane. The control reaction without competitor DNA is marked by a dash.

3.5. Ligand binding of CtEcR/CtUSP

Apart from binding to specific DNA sequences, nuclear hormone receptors must be able to bind their cognate ligand specifically in order to exert their typical effect on target gene expression. We have thus analyzed the ligand binding properties of the *Chironomus* EcR and USP receptors, either alone or as heterodimeric complexes. Whereas no specific binding activity was demonstrated for the individual receptors, both CtEcR/CtUSP1 and CtEcR/CtUSP-2 heterodimers clearly bind ponasterone A (Table 1). Thus, the situation differs from that observed with the homologous receptors of *Drosophila* where EcR alone binds very small but significant amounts of [^{3}H]ponasterone A (M. Grebe and M. Spindler-Barth, unpublished result). However, ligand binding of the heterodimeric complex is much more efficient with both species (Table 1; Rauch et al., 1998; Yao et al., 1993).

Ligand binding is roughly 2.5-fold higher with CtEcR/CtUSP-2 than with CtEcR/CtUSP-1 based on three independent analyses (Table 1). In all cases, expression levels of CtEcR, CtUSP-1, and CtUSP-2 were determined routinely by quantitative evaluation of Western blots (Rauch et al., 1998). The [3H]ponasterone A binding data shown in Table 1 represent values corrected for variations in the abundance of CtEcR and CtUSP-1 or CtUSP-2. Furthermore, reactions containing an excessive amount of smaller fragments than the expected full-length receptor protein were discarded. Specific binding of [^{3}H]ponasterone A by DmEcR/CtUSP-1 was as efficient as the one determined for DmEcR/DmUSP in a control experiment (S. Przibilla and M. Spindler-Barth, unpublished result).

Table 1
Ligand-binding properties of CtEcR and CtUSP

Protein(s)	Binding to [^{3}H]ponasterone A			
	N	Total[a](cpm)	Non-specific[a](cpm)	Specific(cpm)
Lysate	4	57±12	42±16	15
CtEcR	5	52±11	48±26	4
CtUSP-1	2	81±8	74±0	7
CtUSP-2	4	61±16	48±13	16
CtEcR/CtUSP-1	4	502±16	62±3	440
CtEcR/CtUSP-2	5	1119±114	64±15	1055

[a] The values shown correspond to the mean values of the numbers indicated (*N*) of measurements (±standard error) of a total of three experiments; individual measurements were previously corrected for variations in the abundance of CtEcR, CtUSP-1 and CtUSP-2.

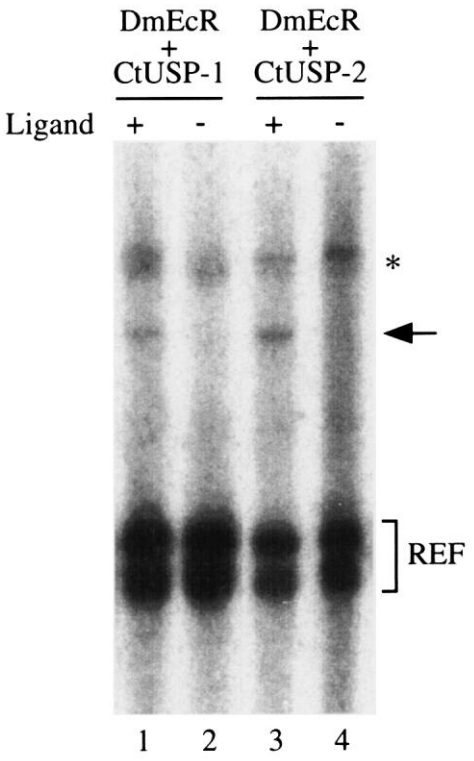

Fig. 5. Transcriptional activation of a reporter gene in HeLa cells by DmEcR/CtUSP-1 and DmEcR/CtUSP-2. The expression plasmids indicated above the lanes were transiently transfected into HeLa cells. Duplicate dishes received either muristerone A at 1 μM (+) or solvent control (−). The transcripts of the β-globin reporter gene were analyzed by S1 nuclease mapping. The arrow marks the position of correctly initiated transcripts. The reference signals (REF) from the control plasmid are indicated. Signals originating from plasmid readthrough transcripts are marked by an asterisk.

3.6. Transcriptional activation of a reporter gene

We next wanted to demonstrate that ligand binding leads to an activated receptor complex that induces the expression of a target gene. In the p(PAL)4-OVEC reporter plasmid used, four tandem copies of a PAL1 EcRE were placed upstream of a minimal *tk* promoter that drives expression of the rabbit P-globin reporter gene. This reporter construct, along with expression vectors for *Drosophila* EcR (DmEcR) and CtUSPs, were cotransfected into HeLa cells. The activation of the reporter gene induced by muristerone A as a most potent ecdysone agonist and ligand was then determined by S 1 nuclease protection analysis of the P-globin mRNA. We were dependent on DmEcR in these experiments because EcR from *Chironomus* proved to be inactive in this transactivation assay because of its A/B domain. As Fig. 5 illustrates, administration of ligand clearly induces expression of the

reporter gene (lanes 1 and 3), whereas no P-globin specific transcripts were detectable in the control (lanes 2 and 4). However, the induction rate appeared to be low when compared to the signal inten sities derived from the internal reference. Transfection of individual expression vectors alone did not evoke detectable signals specific for the reporter gene.

4. Discussion

Through cDNA library screening and RT-PCR, we have identified two forms of USP in *Chironomus tentans*, USP-1 and USP-2. These two forms differ in the most N-terminal portion of the A/B domain, although the USP-2 sequence in this region bears little resemblance to any equivalent region in other USP proteins. Despite these sequence differences, the two forms share both biochemical and functional similarities when tested in vitro or in transfected cells. From a technical standpoint this is an important finding since CtUSP-2 has been employed previously for various in vitro binding studies (Elke et al., 1997; Seibel et al., 1997). Whether CtUSP-1 and CtUSP-2 perform different regulatory functions in vivo, as *Manduca* USP isoforms do (Jindra et al., 1997), remains to be investigated.

Transcripts for both forms, CtUSP-1 and CtUSP-2, have been recovered by RT-PCR from *Chironomus* epithelial cell culture preparations and verified by sequencing (C. Elke, pers. comm.), substantiating our own findings that both are, indeed, true forms. As stated above, CtUSP-1 corresponds by size to the dephosphorylated 60 kDa USP homolog present in these cells whereas CtUSP-2 shares properties with the rare 53 kDa homolog. It appears that CtUSP-2 is encoded by a messenger RNA whose open reading frame extends 5' and that it is produced by usage of the downstream start codon described here. It is a matter of conjecture whether alternative usage of an upstream codon in an adjacent but non-identified cDNA sequence would give rise to the yet missing 58 kDa CtUSP form. However, other explanations for the generation of the 60, 58, and 53 kDa CtUSP forms are also possible, and the presence of undescribed forms resembles the current situation for DmUSP (Henrich et al., 1994).

As noted, we have identified no significant functional differences between the two forms in terms of DNA-binding and transcriptional activation of a reporter gene. The 2.5-fold difference in ligand binding cannot be attributed to differential expression of CtUSP-1 and CtUSP-2 in vitro. Since the ability to heterodimerize seems to be similar (compare upper with lower row in Fig. 4B), it is assumed that CtUSP-1 and CtUSP-2 differ in their affinity to ligand, which assumption is presently being investigated by Scatchard blot analyses (Grebe et al., in preparation). Although examples for intramolecular crosstalk between the A/B and E domains exist (e.g. Onate et al., 1998), it is possible that the longer length of the CtUSP-1 A/B domain hinders correct folding and results in a lower detectable level of ligand-binding compared to CtUSP-2. We have noted previously that in vitro translated *Drosophila* USP binds to a DR1 response element as a homodimer in a ligand-dependent fashion (Vögtli et al., 1998), whereas the CtUSP forms have not shown this property. However, CtUSP-2 expressed in *E. coli* is able to bind to a DR1 element (Elke et al., 1997). The reason for this apparent discrepancy might be the consequence of differences in expression, purification and/or posttranslational modifications. It is unlikely to arise from a difference between the *Chironomus* and *Drosophila* C domains, which share about 92% identity. Rather, the difference likely involves a functional difference involving the dimerization interface of USP, which resides within the E domain (cf. Fig. 3). The variance in DNA binding properties of USP according to varying expression and purification protocols resembles other reported observations. For example, the *sgs*-3 EcRE is recognized by EcR/USP containing nuclear extracts, but not by in vitro translated proteins (Lehmann et al., 1997). In addition, we showed that CtEcR exhibits no ligand binding capacity above background in the absence of USP, whereas *Drosophila* EcR displays minimal but significant binding of ecdysteroid analogues in the absence of USP (M. Grebe and M. Spindler-Barth, unpublished result). It remains to be seen whether this difference is functionally important. While the results of this study suggest that the rare 53 kDa CtUSP-2 form behaves similarly to CtUSP-1, it cannot be ruled out that it plays a modulatory role in vivo.

A careful evaluation of the E domain reveals numerous similarities and a few potentially important structural differences between *Chironomus* USP and other USP sequences, notably a seemingly unusual Helix 12 sequence. Measurements of dissociation constants with nonsteroidal agonists show that the *Chironomus*

ecdysteroid receptor has a much higher affinity for them than does the *Drosophila* receptor (Spindler-Barth et al., 1991). Most of the attention concerning species differences has centered on structural differences in the E domain of insect EcRs. However, a significant role for USP in the stabilization and ligand specificity of the receptor complex through an allosteric effect has not been rigorously tested. The *Drosophila* EcR/mammalian RXR heterodimer responds more robustly to muristerone A, whereas the *Drosophila* EcR/*Drosophila* USP responds more vigorously to ponasterone A (Christopherson et al., 1992) consistent with a specific role for RXR or USP in ligand binding. The development of chimeric EcR and USP receptors for the purposes of testing their function in transgenic Drosophila establishes a strategy for testing variations in receptor structure among species for functional differences. Significantly, a *Drosophila usp* gene whose E domain is substituted by the equivalent *Chironomus usp* domain encodes a protein that functions normally during larval development, but shows impaired function at metamorphosis (Henrich et al., in preparation).

Our conservative description of the CtUSP forms (rather than isoforms) stems from uncertainty about the sequence organization of their gene(s). The differences in the N-terminal region of the A/B domain are precisely the type of differences reported for various EcR isoforms that arise from alternative splicing and the activation of alternative promoters. Moreover, USP probes detect only a single locus on *Chironomus* polytene chromosomes i.e., one which is associated with the late ecdysone-inducible puff II-14 (Wegmann et al., 1995). However, as long as the precise genomic organization of the CtUSP-encoding sequences has not been elucidated we hesitate to use the specific term "isoforms". In fact, we cannot formally rule out the existence of more than one genes or allels or the occurrence of partial allelic differences regarding e.g. the A/B domain. From studies with other genes (C. Elke, pers. communication), we know that our animal stocks as well as our cell cultures of *C. tentans* are highly polymorphic.

Generally, many of the basic functional attributes of the *Chironomus* USP closely resemble those noted for Drosophila USP in earlier studies. In fact, the *Chironomus* USP is able to heterodimerize and induce transcription on in vitro tests with the *Drosophila* EcR, indicating that the two proteins are apparently interchangeable in cells. However, it remains to be determined whether the unique structural properties found in *Chironomus* and other insect USPs reflect important functional differences that impact ecdysteroid receptor function and the course of hormone-regulated development. Such differences, if they exist, provide an important basis for developing a variety of insect control strategies.

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