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Lcusp, an *ultraspiracle* gene from the sheep blowfly, *Lucilia cuprina*: cDNA cloning, developmental expression of RNA and confirmation of function☆

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Abstract

A DNA sequence corresponding to most of the DNA-binding domain of a *Lucilia cuprina ultraspiracle* protein (LcUSP) was amplified by PCR from genomic DNA and cloned. This cloned fragment was used to screen a *L. cuprina* cDNA library and to isolate a full-length LcUSP encoding sequence within a 3800-bp cDNA clone. The conceptually translated amino acid sequence of this open reading frame (467 amino acids) was used in alignment comparisons and phylogenetic analyses to reveal that LcUSP most closely resembles DmUSP relative to other known insect nuclear hormone receptors. An antisense RNA probe specific for the 5′ end of *Lcusp* was used in ribonuclease protection assays to detect significant levels of *Lcusp* RNA throughout *L. cuprina* development. Highest levels were detected in embryos, late third instar larvae, pupae and adult females. This pattern parallels the pattern of expression observed for *Dmusp* RNAs during *Drosophila melanogaster* development. Finally, the *LcUSP* sequence was engineered for expression in mammalian cells and we now report that the cloned LcUSP is functional in vivo and can act as a partner for a chimeric *L. cuprina* ecdysone receptor to form an ecdysteroid-dependent transcription factor in mammalian cells.

Keywords: *Lucilia cuprina*; Sheep blowfly; USP; Ultraspiracle; Ultraspiracle cDNA; Ultraspiracle gene; Nuclear hormone receptor

1. Introduction

It appears that the basic features of the molecular biology underlying the regulation of gene expression by ecdysone are conserved across the class Insecta (see reviews, Henrich and Brown, 1995; Thummel, 1995). Functional ecdysone receptors are heterodimers of two nuclear receptor proteins, EcR (ecdysone receptor) and USP (the product of the *ultraspiracle* gene). USP was first identified in *Drosophila melanogaster* as a homologue of the mammalian retinoid X receptor or RXR (Oro et al., 1990). It was shown that RXR could substitute for USP by forming heterodimers with DmEcR (the EcR from *D. melanogaster*). In a reciprocal way, it was shown that DmUSP could substitute for RXR and form active heterodimers with mammalian nuclear hormone receptors, including retinoic acid receptor, thyroid hormone receptor and vitamin D receptor (Khoury Christianson et al., 1992; Yao et al., 1993; Hatzivassiliou et al., 1997). The *D. melanogaster* genome encodes a single form of USP (Henrich et al., 1990; Oro et al., 1990; Shea et al., 1990) and recently Hall and Thummel (1998) have confirmed that USP is required for ecdysone receptor activity during the development of *D. melanogaster*. They observed that the EcR/USP heterodimer functions during the onset of metamorphosis in *D. melanogaster* and that there is a role for *usp* in stage-specific decisions to moult or pupariate in response to ecdysone pulses during larval development. Unlike the expression of EcR, which correlates with the pulsation of the ecdysone titre during insect development, USP appears to be constitutively expressed during ontogeny.

Homologues of DmUSP have been cloned from a number of insect species: BmUSP, the USP homologue from *Bombyx mori* (Tzertzinis et al., 1994); two isoforms of AaUSP, from *Aedes aegypti* (Kapitskaya et al., 1996); two isoforms of MsUSP, from *Manduca sexta* (Jindra et

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al., 1997); CfUSP, from *Choristoneura fumiferana* (Perera et al., 1998); two isoforms of CtUSP, from *Chironomus tentans* (Vogtli et al., 1999), LmRXR/USP, from *Locusta migratoria* (Hayward et al., 1999) and TmUSP, from *Tenebrio molitor* (Nicolai et al., 2000).

One approach to test for the functionality of putative EcRs and USPs has involved expressing cDNAs, encoding these receptors, in mammalian cells where there are unlikely to be specific inhibitors of the EcR/ecdysteroid system (Yang et al., 1995; Yao et al., 1992). In this way, the single isoform of DmUSP (Yao et al., 1992), both isoforms of tick AamRXR (Guo et al., 1998), both isoforms of AaUSP (Wang et al., 2000) and both isoforms of CtUSP (Vogtli et al., 1999) have been shown to be functional.

In an earlier study (Hannan and Hill, 1997), we showed that *Lucilia cuprina* EcR (LcEcR) could activate target genes in an ecdysteroid-dependent manner in mammalian cells, by acting as a partner for the endogenous mammalian RXR. In this communication we report the cDNA cloning and sequence structure of *Lcusp*, a new *usp*, from *L. cuprina*. We analyse the expression of *Lcusp* RNA during the development of *L. cuprina* and suggest that LcUSP is the natural partner for LcEcR. We also report that LcUSP can partner a chimeric LcEcR to stimulate transactivation of a target gene in mammalian cells in an ecdysteroid-dependent manner.

2. Materials and methods

2.1. Experimental animals

L. cuprina animals were maintained as described before (Hannan and Hill, 1997).

2.2. PCR cloning of a specific probe

Genomic DNA from *L. cuprina* larvae was the template in a polymerase chain reaction (PCR) to prepare a DNA probe specific for an *L. cuprina usp.* Two degenerate oligonucleotides were synthesised based on published sequences of primers used in the cloning of *B. mori usp* (Tzertzinis et al., 1994). The resulting 147-bp PCR product was subcloned into pBluescriptSK+ (Stratagene), sequenced on both strands and used as a template to prepare a ^{32}P-labelled probe essentially as described before (Hannan and Hill, 1997). The probe was used at 10^6 cpm/ml in hybridizations.

2.3. cDNA library screening

We previously prepared an oligo-dT primed cDNA library in *Lambda* ZapII using *L. cuprina* mRNA (Hannan and Hill, 1997). Hybridization with the ^{32}P-labelled probe was conducted under low stringency as previously described (Hannan and Hill, 1997). Twelve positive plaques were purified to homogeneity and converted into pBluescriptSK− plasmids by the in vivo excision method using Exassist (Stratagene) helper phage. A plasmid, pLSP4, derived in this way was selected for further sub-cloning.

2.4. DNA sequence analysis

Dideoxynucleotide sequencing was performed on both strands of double-stranded plasmid DNA and sequence comparisons and conceptual translations were performed using the GCG (Genetics Computer Group Inc.) software package as described previously (Hannan and Hill, 1997).

2.5. Plasmids

Preliminary DNA sequence analysis of plasmid pLSP4 indicated the presence of a protein coding region for a putative LcUSP towards the 5′ end of the cDNA insert. A 2453-bp fragment from the 5′ end of pLSP4 was subcloned into the *Eco*RI site of pBluescriptSK+ (Stratagene) using standard methods (Sambrook et al., 1989) to construct plasmid pBLU1 or into the *Eco*RI site of the mammalian expression vector pSG5 (Stratagene), to construct pSGLcUSP. pBLU1 was digested with *Eco*RI and *Ava*II to provide a 496-bp fragment, which included the 5′ UTR and the sequence encoding the A/B and C domains of *Lcusp*. This fragment was sub-cloned into pBluescriptSK+ (Stratagene) to construct plasmid pLABC. pSGLcEcR, p(EcRE)$_5$CAT and pPGKLacZ were described previously (Hannan and Hill, 1997). pVPLcEcR was constructed as follows. pNLVP16 (a gift from Dr G. Muscat) was digested with *Sal*I and *Xba*I and ligated with the DNA duplex formed by the oligonucleotides SPX5 (TCGACATATAACTTCGCTGCAGA TGCATCCGAGCTCT) and XPS3 (CTAGAGCTCG GATGCATCTGCAGCGAAGTTATATG) to construct plasmid pMOD31. The A/B domain of pSGLcEcR was removed by *Bam*HI and *Pst*I digestion. In its place was ligated a *Bgl*II and *Pst*I 263-bp fragment from pMOD31 to construct plasmid pVPLcEcR. The 263-bp fragment contains a VP16 activation domain (Triezenberg et al., 1988).

2.6. Ribonuclease protection assays (RPAs)

RPAs were conducted as described previously (Hannan and Hill, 1997) with the following modifications. pLABC was linearized with *Hin*dIII and used as a template in a T3 RNA polymerase-based Riboprobe system (Promega) to transcribe a ^{32}P-labelled RNA antisense probe of 584 b in length but specific for 492 b of the 5′ end of *Lcusp*. For each stage of development 10 μg of total RNA were hybridized with 100,000 cpm of

probe essentially as described before (Hannan and Hill, 1997). Protected RNA duplexes were resolved by sequencing gel electrophoresis and detected by autoradiography. Molecular sizes were estimated by comparison with DNA markers from a *Hin*fI digest of pBluescriptSK+, which were ^{32}P-labelled as described previously (Hannan and Hill, 1997).

2.7. In vitro translations

pSGLcUSP was used in a TNT-coupled reticulocyte lysate system (Promega) employing T7 RNA polymerase to transcribe and translate LcUSP in vitro. Methods were essentially as described previously (Hannan and Hill, 1997); [^{35}S]-methionine-labelled LcUSP was resolved by SDS–PAGE and detected by phosphorimaging.

2.8. Cell culture and transient transfections

CV1 monkey kidney cells were maintained as described previously (Yang et al., 1995). Transient transfections were conducted using DOTAP (Boehringer-Mannheim) at 15 μg/ml, essentially as described previously (Hannan and Hill, 1997). Replicate 35-mm dishes of subconfluent CV1 cells were cotransfected with (1) pSGLcUSP or unmodified pSG5 at 1 μg/ml, (2) pVPLcEcR or unmodified pSG5 at 0.2 μg/ml, (3) p(EcRE)$_5$CAT at 1 μg/ml and (4) pPGKLacZ at 1 μg/ml. For induction experiments the ecdysone analogue ponasterone A (a gift from Dr Denis Horn) was added to cells at 1 μM, 6 h after transfection. For control experiments, cells were treated only with carrier ethanol. CAT and β-galactosidase in extracts of cells were measured 48 h after transfection as described previously (Hannan and Hill, 1997). Variations between experiments were controlled by normalizing the level of CAT to β-galactosidase in the same extract. Error bars indicate standard error of the mean.

3. Results

3.1. Isolation of an Lcusp cDNA

PCR amplification from *L. cuprina* genomic DNA was used to prepare a DNA probe specific for part of the DNA sequence of a LcUSP C domain: 147 bp of total DNA encompassing 94 bp of novel *L. cuprina* DNA (see italicized sequence in Fig. 1). Conceptual translation revealed that this DNA encodes a peptide which is identical, except for one residue, to part of the DNA-binding domain of DmUSP (Oro et al., 1990): this residue (D_{129}) is shown in outline in Fig. 1.

Using this probe, we screened an oligo-dT primed cDNA library from late third instar *L. cuprina* larvae

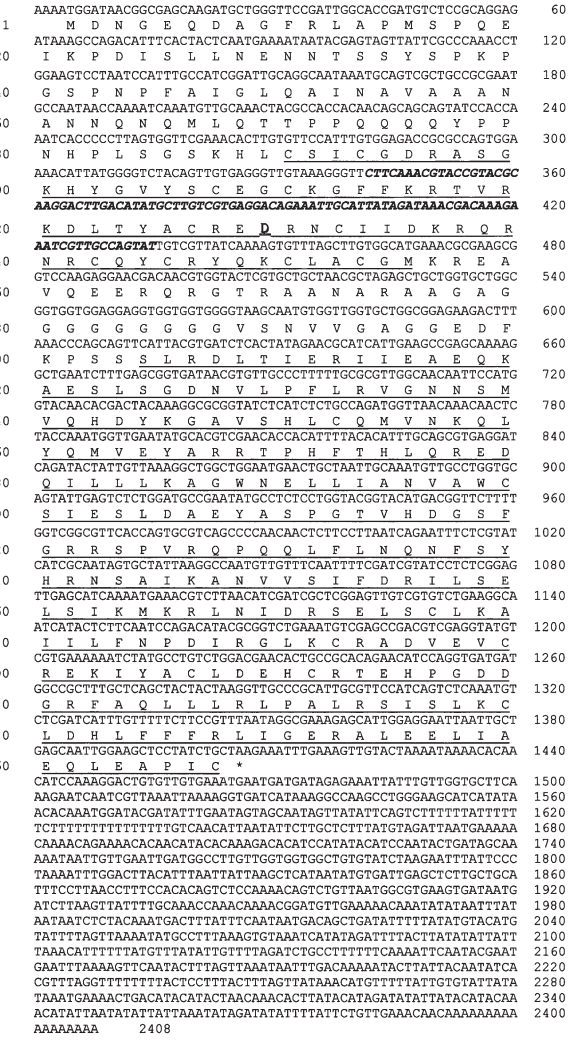

Fig. 1. Novel DNA sequence (2408 bp) and the conceptually translated amino acid sequence from the cDNA clone in pBLU1 encoding an *L. cuprina ultraspiracle* protein (LcUSP). The deduced amino acid sequence of LcUSP falls into domains (A/B, C, D, and E/F) that are characteristic of nuclear hormone receptors (Evans, 1988; Forman and Samuels, 1990) and the amino acid sequence ends with a stop codon at nucleotide 1405. The amino acid sequences of the C (DNA binding) and E (hormone binding) domains are underlined. The 94-bp segment of the C domain (339nt–432nt), corresponding to the novel sequence within the PCR amplified genomic probe, is shown in bold italics; the single residue D_{129} within the C domain which is different for *DmUSP* (Oro et al., 1990) is in outline. The sequence reported in this paper has been deposited in the Genbank database (accession no. AY007213).

(Hannan and Hill, 1997); from one positive plaque we derived plasmid pLSP4 containing a 3800-bp insert. Sequencing revealed that the 5′ portion of pLSP4 encodes an LcUSP which is followed by a long (2.4 kb), apparently untranslated region (UTR). A 2453-bp *Eco*RI fragment was sub-cloned from the 5′ end of the 3800-bp cDNA insert in pLSP4 to construct plasmid pBLU1. As shown (Fig. 1), DNA sequencing revealed 2408 bp of novel sequence for the cDNA insert in plasmid pBLU1 and conceptual translation indicated the presence of a full-length cDNA clone encoding an LcUSP. The

open reading frame (ORF) of 467 amino acids falls into domains (A/B, C, D, and E/F) that are characteristic of nuclear hormone receptors (Evans, 1988; Forman and Samuels, 1990) and it terminates with a stop codon at nucleotide 1405.

3.2. Sequence comparison of LcUSP to RXRs and USPs

The conceptually translated amino acid sequence of LcUSP has significant amino acid identity with sequences of nuclear hormone receptors found in Genbank. Searching via the BLAST software program (Altschul et al., 1990) identified DmUSP (Oro et al., 1990) as the peptide sequence which most closely resembles LcUSP, followed by other insect USPs and then the RXRs (data not shown). To assess these similarities in more detail, a phylogenetic analysis was performed. The protein sequences of cloned insect USPs as well as RXRs from the mouse (Mangelsdorf et al., 1992), the tick (Guo et al., 1998) and humans (Mangelsdorf et al., 1990; Leid et al., 1992) were first aligned using the CLUSTAL W program (Thompson et al., 1994). Xenopus TRα (Yaoita et al., 1990) was selected as an outgroup and the alignment was used to generate an unrooted tree using the parsimony and compatibility method of the PHYLIP program Protpars (Felsenstein, 1989). From this analysis (Fig. 2), it is clear that DmUSP is the most strongly related to LcUSP of these USPs and RXRs. More generally, LcUSP relates more closely to the other dipteran USPs (AaUSP, CtUSP and DmUSP) than the lepidopteran USPs (MsUSP, CfUSP and BmUSP). The dipteran USPs appear further removed from the RXRs than the lepidopteran USPs. The locust LmRXR/USP and the coleopteran TmUSP are closer to the RXRs than the USPs of higher insects.

Next we compared the peptide domains of the conceptually translated LcUSP with those of seven other USP homologues listed in Table 1. We calculated the level of identity for each domain (A/B to E/F) of the other known insect USPs, with respect to the corresponding domain for LcUSP. These data are presented in Table 1 along with the peptide length for each domain. This analysis reveals that the other dipteran USPs (except perhaps CtUSP) are more like LcUSP than the lepidopteran USPs and, importantly, that DmUSP is the most closely related to LcUSP. Between 92% and 100% amino acid identity is observed for the C domains of the eight insect USPs. The next most striking level of amino acid identity is that between the E domains of LcUSP and DmUSP (75%). Also noteworthy is the high level of amino acid identity between the D domains of LcUSP and DmUSP (65%). A 78% level of homology was also observed between the relatively short (23 amino acids) D domain of MsUSP-1 and the corresponding section of the D domain of LcUSP.

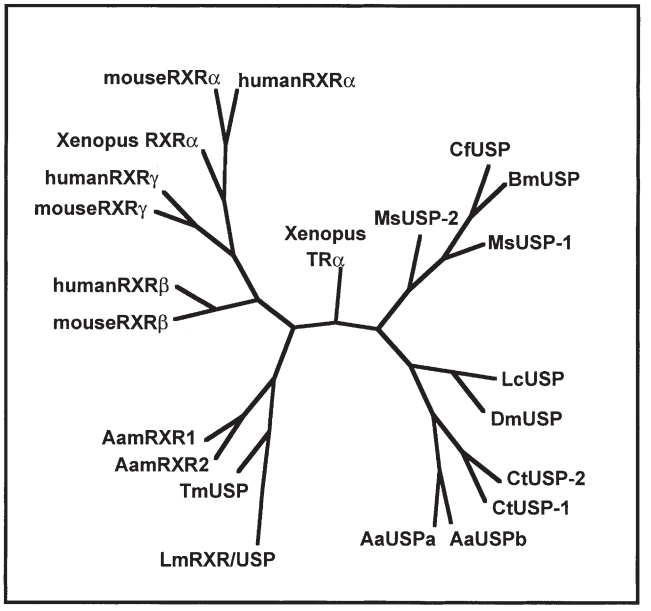

Fig. 2. Phylogenetic tree of the aligned protein sequences of USPs and RXRs. Conceptually translated sequences were aligned by the CLUSTAL W program (Thompson et al., 1994) and analysed by the PHYLIP package of programs (Felsenstein, 1989) using the Protpars parsimony and compatibility method to infer phylogeny and the Drawtree tree-drawing program. The complete sequences were obtained from the Genbank database and the following references, in addition to those given in the legend to Table 1: mouse RXR α, β, and γ (Mangelsdorf et al., 1992), human RXR α (Mangelsdorf et al., 1990), human RXR β (Leid et al., 1992) and human RXR γ (Genbank Accession No. U38480), the tick, *Amblyomma americanum*, Aam RXR 1 and 2 (Guo et al., 1998), Xenopus RXRα (Blumberg et al., 1992). Xenopus TRα (Yaoita et al., 1990) is the outgroup.

3.3. Structural characteristics of the conceptually translated LcUSP

As shown in Fig. 3, we have identified a number of structural features within the ORF of *Lcusp*, which are characteristic of members of the nuclear hormone receptor superfamily. For this analysis we compared LcUSP with the highly homologous DmUSP and with human RXRα for which structures of both the DNA- and ligand-binding domains have been determined (Rastinejad et al., 1995; Bourguet et al., 1995). (1) The A/B or transactivating domains for LcUSP and DmUSP share a similar length and a similar amino acid sequence compared to the equivalent regions for other known RXRs and USPs. Whether this indicates a special regulatory or structural feature specific to the fly USPs remains to be investigated. (2) The C domain contains two C_2C_2-type zinc finger motifs, which are typical of nuclear hormone receptors; hatches in Fig. 3 indicate these cysteine residues. Also indicated are those zinc finger residues which are involved in specific half-site recognition, EGCKG (P-box; Umesono and Evans, 1989) and in recognition of half-site spacing, REDRN (D-box; Umesono and Evans, 1989). (3) At the start of

Table 1
Comparison of the predicted amino acid sequences among insect ultraspiracle protein homologues

Domain	Length (amino acids)									% Identity[a]							
	LcUSP	DmUSP[b]	AaUSPa[c]	CtUSP1[d]	MsUSP[e]	BmUSP[f]	CfUSP[g]	TmUSP[h]	LmRXR/USP[i]	DmUSP[b]	AaUSPa[c]	CtUSP1[d]	MsUSP[e]	BmUSP[f]	CfUSP[g]	TmUSP[h]	LmRXR/USP[i]
A/B	89	103	137 (112)[j]	196 (99)[k]	112 (59)[l]	113	118	84	87	55	33 (50)[j]	49 (35)[k]	30 (49)[l]	28	34	38	39
C	66	66	66	66	66	66	66	66	66	97	95	92	100	98	97	92	96
D	48	64	26	32	23	23	23	23	23	65	50	38	78	61	57	44	48
E/F	264	275	255	258	260	260	260	233	213	75	65	51	49	48	48	49	53
Total	467	508	484 (459)[j]	552 (455)[k]	461 (408)[l]	462	462	406	389	73	65 (66)[j]	57 (54)[k]	55 (60)[l]	53	55	54	57

[a]Identity versus *Lucilia cuprina ultraspiracle* protein homologue (LcUSP) expressed as % of identical matches, within aligned segments.
[b]*Drosophila melanogaster ultraspiracle* protein (Oro et al., 1990).
[c]*Aedes aegypti ultraspiracle* protein homologue (Kapitskaya et al., 1996).
[d]*Chironomus tentans ultraspiracle* protein homologue (Vogtli et al., 1999).
[e]*Manduca sexta ultraspiracle* protein homologue, isoform MsUSP-1 (Jindra et al., 1997).
[f]*Bombyx mori ultraspiracle* protein homologue (Tzertzinis et al., 1994).
[g]*Choristoneura fumiferana ultraspiracle* protein homologue (Perera et al., 1998).
[h]*Tenebrio molitor ultraspiracle* protein homologue (Nicolai et al., 2000).
[i]*Locusta migratoria RXR/ultraspiracle* protein homologue (Hayward et al., 1999).
[j]Data in brackets for second isoform of *Aedes aegypti ultraspiracle* protein homologue, AaUSPb (Kapitskaya et al., 1996).
[k]Data in brackets for second isoform of *Chironomus tentans ultraspiracle* protein homologue, CtUSP2 (Vogtli et al., 1999).
[l]Data in brackets for second isoform of *Manduca sexta ultraspiracle* protein homologue, MsUSP-2 (Jindra et al., 1997).

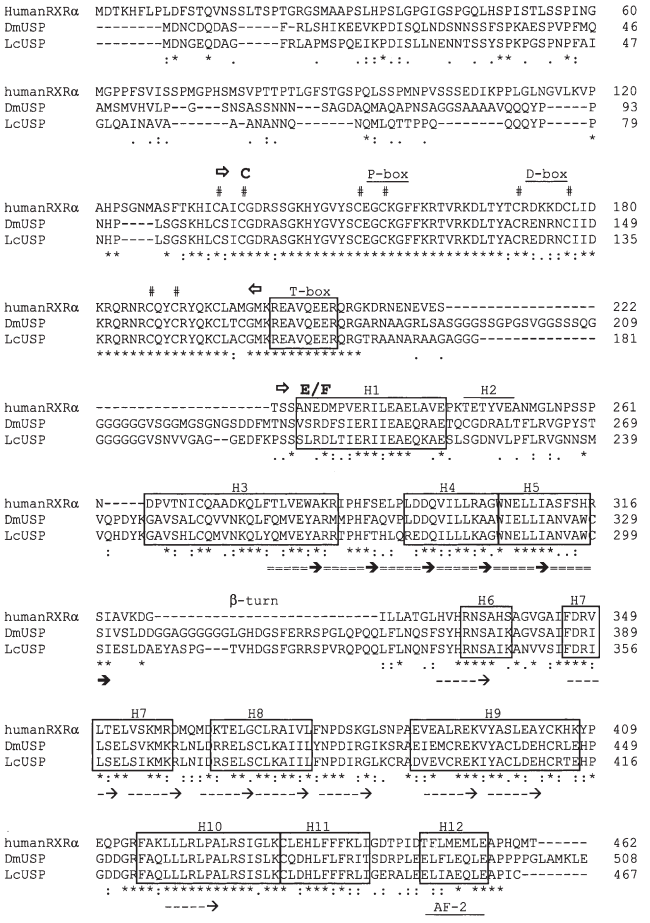

Fig. 3. Alignment of the conceptually translated amino acid sequence of *L. cuprina* USP (LcUSP) with DmUSP and human RXRα. Multiple sequence alignment was performed using the CLUSTAL W program (Thompson et al., 1994). Stars indicate identical residues and dots conserved substitutions. Gaps were introduced to improve the alignment. The limits of the C and E/F domains are indicated by open arrows. Within the C domain, hatch signs indicate the eight cysteine residues of the two zinc fingers. Also shown are the P-box residues (EGCKG) involved in specific half-site recognition and the D-box residues (REDRN) involved in recognizing the half-site spacing (Umesono and Evans, 1989). The positions of the T-box residues (REAVQEER) are shown as a box within the D domain. Within the E/F domain, the positions of the 11α-helices H1 and H3 to H12 are shown as boxes. H2 is only found in human RXR (Wurtz et al., 1996) and a bar is shown above the residues which form H2 in human RXR. The six consecutive heptad repeats which form a putative helix–turn–zipper motif (Maksymowych et al., 1992) are underlined by broad-headed arrows. The nine discontinuous heptad repeats which form a putative regulatory zipper are underlined by small-headed arrows. The positions of the β-hairpin turn and the AF-2 activation domain are also indicated. Numbers in the margin indicate positions of amino acids within each peptide sequence.

the D domain is an 11-amino-acid span which is conserved among other USPs and RXRs (Kapitskaya et al., 1996). This span contains the 8-residue T-box REAVQEER, which forms a heterodimerization surface in RXRs (Zechel et al., 1994). Significantly, the D domains of LcUSP and DmUSP, which have the highest level of identity at the amino acid level (65%), are also substantially longer than the equivalent regions of other known RXRs and USPs. These properties may reflect specific and shared structural features distinguishing the USPs of cyclorapphous flies from those of other insects. (4) The ligand-binding domains (LBDs) of nuclear receptors are composed of up to 12 helical regions (Wurtz et al., 1996). In most of these helices LcUSP shows high homology with DmUSP and human RXRα. Also within helices 3 to 5 of the LBD of LcUSP, we note the presence of a putative helix-turn zipper motif (Maksymowych et al., 1992) consisting of six consecutive heptad repeats. These have been identified previously within the LBDs of human RXRα (Bourguet et al., 1995) and AaUSP (Kapitskaya et al., 1996).

The E domain of LcUSP contains another structural feature which is characteristic of USPs from higher insects (Oro et al., 1990; Tzertzinis et al., 1994; Chung et al., 1998; Vogtli et al., 1999; Jones and Jones, 2000), namely an additional 12- to 27-residue sequence (E_{307}–Q_{330} for LcUSP). This peptide sequence is absent from the LBD of the coleopteran TmUSP (Nicolai et al., 2000) and from the LBDs of RXRs, including the locust LmRXR/USP (Hayward et al., 1999). It corresponds to the β-turn region between helices 5 and 6 of human RXRα and is part of the putative LBS (Bourguet et al., 1995). Tzertzinis et al. (1994) observed that it may represent a flexible border between sub-domains. Alternatively, Jones and Jones (2000) have suggested that this additional insert may contribute to USPs having a larger ligand-binding pocket than occurs in RXR or other nuclear hormone receptors. Interestingly, LcUSP is the only orthologue of DmUSP identified which has such a long stretch of amino acids in the region between helices 5 and 6 of the LBD. This unique feature of the fly LBD pockets raises the possibility that LcUSP and DmUSP may interact with ligands that are different from those of other insect USPs.

The next sub-domain in the LcUSP LBD is a regulatory zipper (Forman and Samuels, 1990) of nine heptad repeats. This corresponds to a region spanning helices 6 to 10 for human RXRα and includes the dimerization interface. A crystal structure for RXR has implicated several of these heptad repeats in intramolecular interactions stabilizing the architecture of the ligand-binding domain (Bourguet et al., 1995). We observed high amino acid conservation for a 19-amino-acid leucine-rich stretch encompassing the ninth heptad repeat and this appears to be characteristic of all USPs and RXRs (Kapitskaya et al., 1996). Finally, the putative AF-2 domain of LcUSP had three out of seven residues identical with the AF-2 domain of human RXRα: these include the two glutamic acid residues considered to be necessary for the ligand-dependent activation function of the AF-2 domain (Westin et al., 1998).

3.4. Developmental expression of Lcusp mRNA

We have conducted RPAs using the putative *Lcusp* cDNA in plasmid pBLU1 to detect *Lcusp*-specific RNAs, which are expressed during the development of *L. cuprina.* To facilitate the analysis, we generated an antisense RNA probe (584 b) using plasmid pLABC; this was 92 b longer than the region of *Lcusp* RNA (492 b), which was to be protected by duplex formation during the RPA. As shown (Fig. 4) a band corresponding to 492 b was observed for most stages (lanes 5, 8, 9 and 10) during *L. cuprina* development. In contrast, no band was observed for a mock hybridization using excess yeast tRNA (lane 4), and in the absence of RNase the full-length 584-b antisense probe was apparent (lanes 2 and 3). Significant levels of *Lcusp* RNA were detected throughout *L. cuprina* development. The most intense bands were observed for the embryonic (lane 5), late third instar (lane 8) and pupal (lane 9) stages of the *L. cuprina* life cycle as well as for adult females (lane 10). Lower levels of *Lcusp* RNA were observed for the first (lane 6) and second (lane 7) larval instars. This pattern of expression of *Lcusp* RNA parallels the pattern of expression of *usp* RNAs observed during development of *D. melanogaster* (Oro et al., 1990; Henrich et al., 1994).

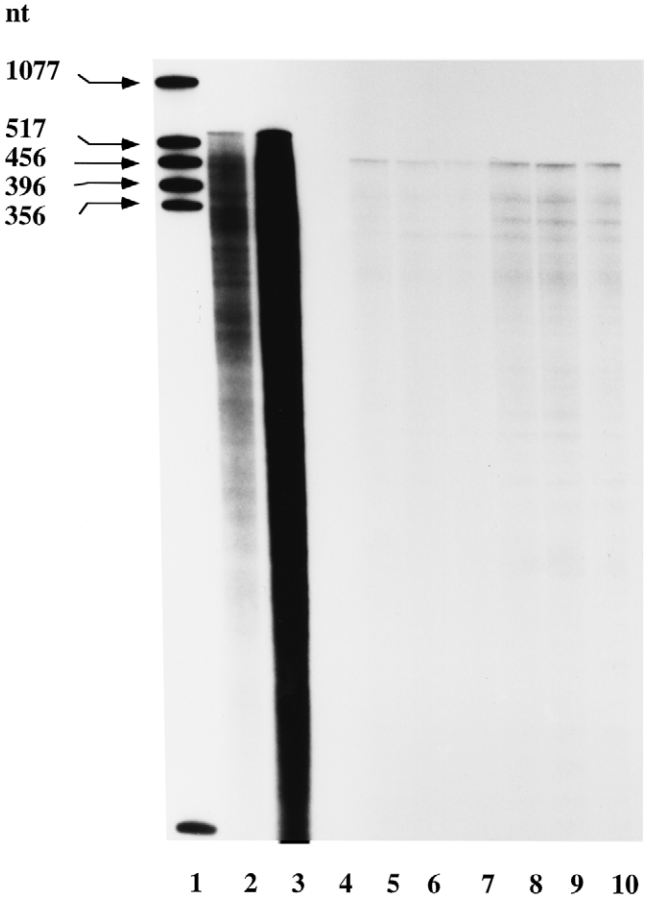

Fig. 4. Developmental expression of *Lcusp* RNAs. Ten micrograms of total RNA, from each stage of the life cycle of *L. cuprina*, were challenged with a ^{32}P-labelled antisense RNA probe (1 fmol), specific for the DNA sequence encoding the A/B and C domains of *Lcusp* cDNA. Samples were subjected to a ribonuclease protection assay (RPA) and resolved on a sequencing gel, loaded as follows: lane 1, molecular size ladder of DNA fragments from a *Hin*fI digest of pBluescript SK+; lane 2, control 584-b antisense probe (0.01 fmol) prior to hybridization in the RPA; lane 3, control 584-b antisense probe (1 fmol) after mock hybridization in the presence of excess yeast tRNA but in the absence of RNases; lane 4, control 584-b antisense probe (1 fmol) after mock hybridization in the presence of excess yeast tRNA and RNases; lanes 5 to 10, total RNAs from *L. cuprina* after probe hybridization and RNase treatment: lane 5, embryos; lane 6, first instar larvae; lane 7, second instar larvae; lane 8, wandering late third instar larvae; lane 9, pupae; lane 10, adults.

3.5. Testing for biological activity of LcUSP in vivo

Before attempting to express the *Lcusp* cDNA in vivo, we confirmed that it could be used to express a full-length translation product in vitro. When the mammalian expression plasmid pSGLcUSP was used in an in vitro transcription and translation system we observed a protein of the predicted size for LcUSP (approximately 51 kDa) as the major product resolved by SDS–PAGE (data not shown). To assess the functioning of LcUSP expressed in vivo, four plasmids were cotransfected into monkey kidney cells (CV1 cells): pSGLcUSP, and pVPLcEcR, along with p(EcRE)$_5$CAT, a specific CAT reporter plasmid whose inducible promoter contained *EcRE*s, and also pPGKLacZ, an independent β-galactosidase reporter plasmid, whose constitutive promoter contained no *EcRE*s. As shown in Fig. 5, treatment of these cells with the ecdysteroid ponasterone A at 1 μM induced significant levels of expression of the specific reporter CAT relative to the independent reporter β-galactosidase. When both pSGLcUSP and pVPLcEcR were transfected into CV1 cells, a 73-fold level of induction was achieved (column 8). In contrast, when pVPLcEcR was transfected into CV1 cells in the absence of pSGLcUSP (column 4), only a 4-fold level of induction was achieved. This low level of activity is presumably due to formation of an active complex by the chimeric LcEcR and the endogenous RXR in CV1 cells. Only background synthesis of CAT was observed in the absence of hormone (columns 1, 3, 5 and 7) and no significant induction occurred when the expression plasmid lacked the *LcEcR* cDNA insert (columns 1, 2, 5 and 6). Overall, these assays indicated that the cloned LcUSP represents an intact USP, which is functional in vivo.

4. Discussion

4.1. Sequence analysis indicates that LcUSP is a homologue of DmUSP

Analysis of the conceptually translated amino acid sequence for LcUSP indicates that it is not only a mem-

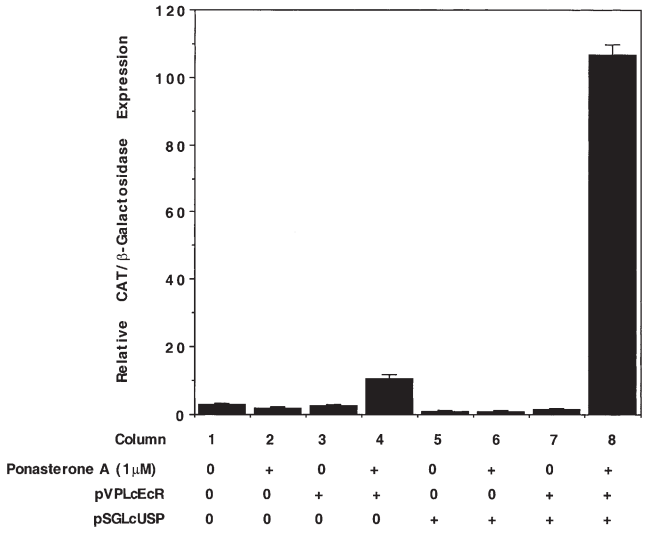

Fig. 5. The *L. cuprina ultraspiracle* protein (LcUSP) can function in vivo in mammalian cells. As described in Section 2, CV1 cells were cotransfected with (1) the *Lcusp* expression plasmid pSGLcUSP (columns 5, 6, 7 and 8) or the parental expression plasmid pSG5 as a control (columns 1, 2, 3 and 4) at 1 μg/ml, (2) the *LcEcR* expression plasmid pVPLcEcR (columns 3, 4, 7, and 8) or the parental expression plasmid pSG5 as a control (columns 1, 2, 5 and 6) at 0.2 μg/ml, (3) $pEcRE_5CAT$ at 1 μg/ml, a *CAT* reporter plasmid, and (4) an independent reporter pPGKLacZ at 1 μg/ml. CAT expression was induced with 1 μM ponasterone A (columns 2, 4, 6 and 8), while control cells received only the carrier ethanol (columns 1, 3, 5 and 7). CAT and β-galactosidase were estimated in extracts of cells 48 h after transfection as described previously (Hannan and Hill, 1997). Variations between experiments were controlled by normalizing the level of CAT to β-galactosidase in the same extract. The average values of three independent experiments are shown and the error bars indicate standard error of the mean.

ber of the nuclear hormone receptor superfamily but is clearly a homologue of the USP from *D. melanogaster*. Within the LcUSP peptide sequence we have identified all domains (A/B, C, D and E/F) and structural motifs which are characteristic of members of the nuclear hormone receptor superfamily (Forman and Samuels, 1990; Wurtz et al., 1996) and which are conserved for other RXRs and USPs (Kapitskaya et al., 1996). Comparison of the LcUSP protein sequence with that of other insect USPs and, in particular, with DmUSP, AaUSP, CtUSP and MsUSP-1 (which have been demonstrated to be functional) gave us confidence that the cloned LcUSP should also be functional. The LcUSP C domain was found to have up to 100% peptide identity with the C domains of the other insect USPs.

The ligand-binding or E/F domains of LcUSP and DmUSP show considerable amino acid identity (75%). By contrast, there is only moderate peptide identity between the E/F domain of LcUSP and the corresponding USP E/F domains from the other insect species. This may point to differences in the ligand-binding properties of the various insect USPs. However, it will only be possible to test this hypothesis if ligands for the USPs are identified. While 9-*cis* retinoic acid is considered to be the native ligand for RXR (Heyman et al., 1992), USP is still considered to be an orphan receptor (Thummel, 1995; Buszczak and Segraves, 1998). Other evidence (Jones and Sharp, 1997) suggests that USP may be a component of the receptor for juvenile hormone (JH). JH was shown to bind to USP (albeit with relatively low affinity) and to stimulate the formation of multimers of a fusion-protein incorporating the ligand-binding domain of DmUSP. Jones and Sharp (1997) raised the possibility that the JH-induced formation of multimeric USP complexes may suppress the formation of EcR/USP heterodimers, thereby influencing ecdysteroid-induced gene transcription during insect development. Recently, Jones and Jones (2000) compared the peptide sequences of six insect USPs and they proposed that the most likely structure of USP should allow for binding of terpenoid-derived ligands (such as JH) and subsequently a vertebrate-receptor type conformational change for coactivator recruitment. Whether this model holds for LcUSP, as well as other insect USPs, remains to be determined.

4.2. Expression of Lcusp RNAs during L. cuprina development parallels the expression of Dmusp RNAs during D. melanogaster development

Our antisense probe was specific for the 5′ UTR and sequence encoding the A/B to C domains of the cloned *Lcusp* described in this communication. Expression of *Lcusp* RNAs during *L. cuprina* development was observed to parallel the expression of *Dmusp* RNAs during *D. melanogaster* development. High levels of *Dmusp* RNA occur during the embryonic, late larval and pupal stages of *D. melanogaster* (Oro et al., 1990; Henrich et al., 1994), while lower levels occur during the first and second larval instars and in adults. The observed pattern of expression of *Lcusp* RNA can also be compared with the pattern of expression of *LcEcR* RNA (Hannan and Hill, 1997); low levels of *LcEcR* mRNA were observed for the first and second instar stages of *L. cuprina* larval development. A parallel expression of RNAs of the two partner proteins LcUSP and LcEcR occurs at other stages and this may indicate a requirement for the mature LcEcR/LcUSP complex during *L. cuprina* development.

However, the appearance of *Lcusp* RNAs may not necessarily predict for functional LcUSP protein during all stages of the life cycle. Henrich et al. (1994) have observed that for *D. melanogaster*, the level of *usp* RNA does not always correlate with the expression of USP protein. They observed that while DmUSP protein generally correlated with the presence of *Dmusp* transcript at most stages of the life cycle, during the second larval instar the level of DmUSP protein was significantly lower without a corresponding lowering of the transcript level. Furthermore, Hall and Thummel (1998) reported that *usp*-mutant larvae rescued through an early lethal

phase developed until the end of the third instar when developmental defects became manifest and USP protein was no longer detectable. They concluded that the EcR/USP heterodimer functions in a critical manner during the onset of metamorphosis in *D. melanogaster*. More recently, Schubiger and Truman (2000) have reported the effect of *usp* mosaics on the progress of metamorphosis in the nervous system. They have proposed a model in which silencing by the unliganded EcR/USP complex and the subsequent release of silencing by ecdysone may be involved in coordinating early development. Understanding the role of *Lcusp* RNAs in the development of *L. cuprina* will require a more detailed study, particularly if other isoforms of LcUSP are found.

4.3. Lcusp can interact with a chimeric LcEcR to form an ecdysteroid-dependent transcription factor in mammalian cells

Of the cloned insect USPs, DmUSP, AaUSPa, AaUSPb, MsUSP-1, CtUSP-1 and CtUSP-2 have been shown to be functional in cells (Yao et al., 1993; Wang et al., 2000; Lan et al., 1999; Vogtli et al., 1999). Also, Swevers et al. (1996) showed that in vitro translated BmUSP and BmEcR formed a functional complex in gel-shift assays and in radio-ligand binding assays. We have now shown that LcUSP can, in concert with a chimeric EcR, permit ecdysteroid-dependent activation of the expression of a reporter gene in mammalian cells. Mammalian cells were chosen as they are unlikely to possess other elements of the insect ecdysteroid/EcR system which may compete with EcRs and *EcREs*. More specifically, CV1 cells were chosen as they are considered (Yao et al., 1993; Suhr et al., 1998) to have little endogenous RXR. It is important to minimize any potential competition between endogenous RXR and the introduced LcUSP in the formation of active heterodimers with exogenous LcEcR. However, in preliminary experiments we found that LcUSP, when partnered by the cloned LcEcR (Hannan and Hill, 1997), provided only marginal ecdysteroid-dependent activation of a reporter gene in mammalian cells (data not shown). We chose to improve the level of activation achievable in mammalian cells by constructing a chimeric LcEcR to partner the cloned LcUSP. More transcriptionally active chimeric EcRs have been constructed employing EcRs from other species by introducing the VP16 activation domain (Christopherson et al., 1992; Yao et al. 1992, 1993; No et al., 1996; Suhr et al., 1998). In all those studies in which CV1 cells were used, some level of induction of the *EcRE* containing reporter genes was observed, in the absence of exogenously introduced partner proteins, USP or RXR. The low but persistent level of endogenous RXR found in CV1 cells can account for this. In fact, the very high level of activation achieved even in CV1 cells, with a chimeric BmEcR, encouraged Suhr et al. (1998) to propose that the chimeric BmEcR could be highly suited for use in single plasmid gene therapy vectors.

Our studies show that a chimeric LcEcR, in which most of the endogenous A/B domain was replaced by a VP16 activation domain, serves as a very good partner for LcUSP in CV1 cells. It allowed a 73-fold level of induction following addition of 1 μM ponasterone A to the cells. However, the chimeric LcEcR also serves as a partner for the endogenous RXR in CV1 cells, as shown by the 4-fold level of induction observed in the absence of exogenously introduced LcUSP. The range of cell types over which LcUSP can be used to effectively partner EcRs to mediate ecdysteroid-dependent regulation of target genes remains to be determined.

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