Cloning and characterization of the ecdysone receptor and ultraspiracle protein from the water flea *Daphnia magna*

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Abstract

cDNAs encoding the ecdysone receptor (EcR) and ultraspiracle (USP) protein were cloned from the water flea *Daphnia magna* (Crustacea: Cladocera). The deduced EcR and USP amino acid sequences showed a high degree of homology to those of other crustaceans as well as insects. We isolated three isoforms of EcR that differ in the A/B domain. Quantitative PCR analysis indicated differing temporal expression patterns of the EcR isoforms during the molting period and demonstrated that the expression of one subtype correlated well with the timing of molt. Using cDNAs encoding EcR and USP, we constructed a *Daphnia* EcR/USP reporter based on a two-hybrid system.

The gene fusions encoded the EcR ligand-binding domain (LBD) fused to the Gal4 DNA-binding domain, and the USP–LBD fused to the Vp16 activation domain. These chimeric genes were transfected with a luciferase reporter gene. Dose-dependent activation of the reporter gene could be observed when transfectants were exposed to Ec and other chemicals known to have Ec-like activities. This two-hybrid system may represent a useful reporter system for further examination of hormonal and chemical effects on *Daphnia* at the molecular level.

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Introduction

Ecdysone (20-hydroxyecdysone; Ec) is known to play a pivotal role in the growth of arthropods. It has been reported that Ec is responsible for embryo development, larval molts, pupation, and metamorphosis in *Drosophila*, *Bombyx mori* and other arthropods (Riddiford 1993, Subramoniam 2000, Sekimoto *et al.* 2006). The Ec is known to regulate a number of biological processes by coordinating with juvenile hormone (JH; Dubrovsky 2005) and cross talk between them has been reported in *Daphnia* and other arthropods (Mu & LeBlanc 2004).

Molecular target of Ec is known as an ecdysone receptor (EcR), which belongs to the nuclear receptor family. EcR is a ligand-dependent transcription factor and it activates transcription of target genes by forming a heterodimer with another nuclear receptor, the ultraspiracle (USP) protein. This heterodimer formation is essential for gene activation in *Drosophila* and other arthropods (Yao *et al.* 1992, 1993, Hall & Thummel 1998).

As in the case in other arthropods, Ec and JH play important roles in cladocerans (Baldwin *et al.* 2001, Mu & LeBlanc 2002). The Ec affects male progeny production and chemicals related to JH increase male production in *Daphnia magna* and other daphnids (Peterson *et al.* 2001, Tatarazako *et al.* 2003, Oda *et al.* 2005). These findings suggest that sex determination in *Daphnia* is closely tied to hormonal systems.

Thus, the investigation of hormonal systems in *Daphnia* will improve our understanding of reproduction, development, and growth. Therefore, it is important to clarify the genetic structure and expression profile of EcR in *Daphnia* for understanding the hormonal effect on *Daphnia*.

Understanding of EcR and USP of *Daphnia* is also important from the ecotoxicological point of view. Small crustaceans such as water fleas play important roles in ecosystems, providing an essential component of fish diets and contributing to water clarity by grazing algae. In light of the importance of small crustaceans to the ecosystem, there has been a considerable effort to evaluate the ecotoxicity of various chemicals on daphnids and to quantify their effects on growth, reproduction, and behavior. In contrast, our understanding of *Daphnia* hormonal system at the molecular level remains limited.

Understanding of EcR and USP may also be important for the development of insecticides. Some insecticides have been developed to target the growth of pests by disturbing hormonal systems (Dhadialla *et al.* 1998); however, their effects on non-target arthropods have not been evaluated fully. In order to develop insecticides that are safe for the environment, it would be desirable to have molecular discrimination between the hormone receptors of pests and non-target arthropods. Cloning of hormone receptors and development of reporter systems may be helpful in understanding the similarities and differences between *Daphnia* and other arthropods.

Table 1 Primer sequences for quantitative real-time PCR

	Forward	Reverse
Gene		
L32	GACCAAAGGGTATTGACAACAGA	CCAACTTTTGGCATAAGGTACTG
EcR-A	CAGGCACATCAACATCAACAAC	GGCGACATGGAATCGACA
EcR-B	CACCACAACCAACTGCATTTC	CCATTAAATGTCAAGATCCCACA
USP	TAGGCCACTCGGGTACTTAA	GAGTGGGTGTTAGGTGGATAA

In this study, we cloned cDNAs encoding EcR and USP from *Daphnia*. We identified EcR subtypes and showed that these subtypes are differentially regulated. In addition, using a reporter system, we analyzed their responses to several chemicals.

Materials and Methods

Chemicals

Ponasterone A, Muristerone A, Tebufenozide, Pyriproxyfen, and Fenoxycarb were purchased from Wako Pure Chemical Industries Ltd, Osaka, Japan. JH and 20-hydroxy Ec were purchased from ICN (Costa Mesa, CA, USA). All chemicals were dissolved in ethanol.

Daphnia strain and culture conditions

The *D. magna* strain (NIES clone) was obtained from the National Institute for Environmental Studies (NIES; Tsukuba, Japan; Tatarazako *et al.* 2003). The strain originated at the Environmental Protection Agency USA and was maintained for more

than 10 years at NIES. Culture medium was prepared using charcoal-filtered tap water maintained at room temperature overnight prior to use. Cultures of 20 individuals per liter were incubated at 24 ± 1 °C under a 14 h light:10 h darkness photoperiod. A 0.01 ml suspension of 4.3 × 10⁸ cells/ml *Chlorella* was added daily to each culture. Water quality (pH and dissolved oxygen concentration) was measured every 2 days by the Environmental Research Center KK (Tsukuba, Japan). Water hardness was between 72 and 83 mg/l, pH between 7.0 and 7.5, and dissolved oxygen concentrations between 80 and 99%.

Cloning of EcR and USP

A *Daphnia* cDNA library (Watanabe *et al.* 2005) was screened with digoxigenin-labeled DNA probes of *Drosophila* EcR or USP (Dr S Kato, Tokyo University, Japan) and partial cDNA clones were obtained. The cDNAs encoding full-length EcR B1 and USP of *Drosophila* were excised from the expression vectors of the genes (Maki *et al.* 2004). The excised DNA fragments were labeled with digoxigenin-11 dUTP using DIG Labeling Kit (Roche Diagnostics). *Daphnia* cDNA libraries

Figure 1 Schematic representation of *D. magna* EcR (DapEcR) structures. Three protein subtypes were encoded by the five cDNAs isolated. Both EcR-A1 and A2 have two types of UTRs, which are indicated as α and β . Open reading frames of the subtypes are indicated in boxes. Different nucleotide sequences are indicated in different patterns.

were screened with the DIG-labeled probes. Hybridization was performed using DIG Easy Hyb (Roche Diagnostics) and detected by DIG Nucleic Acid Detection Kit (Roche Diagnostics). From 2×10^6 independent clones, 10 and 14 clones were isolated as EcR and USP cDNA respectively. Based on the sequences of the cDNAs, we performed 5' rapid amplification of cDNA ends (RACE; Cap Fishing, SeeGene, Seoul, South Korea), followed by PCR. These products were purified by agarose gel electrophoresis, cloned into pGEM-T easy (Promega), and sequenced using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Japan Ltd, Tokyo, Japan). The presence of specific mRNAs was confirmed using PCR with oligonucleotides containing the translational start and stop codons. These nucleotide sequences were submitted to the DNA Data Bank of Japan (DDBJ) web site (Accession number: AB274819-24).

Ligand-binding domain (LBD) amino acid sequences were aligned and analyzed using CLUSTAL W from the DDBJ web site (<http://www.ddbj.nig.ac.jp/search/clustalw-j.html>; Thompson *et al.* 1994), to construct a phylogenetic tree

using the neighbor-joining method (Saitou & Nei 1987). The CLUSTAL W analysis was performed using default settings and relative branch support was evaluated by bootstrap analysis.

Quantitative PCR

For gene expression analysis, *Daphnia* at 2 weeks of age were used. The time of molting was assigned as 0 h and samples were collected every 6 h for the following 72 h. In general, the next molting was observed at 66 h. For embryos, the time of ovulation was assigned as 0 h. The embryos were collected every 6 h for the following 72 h. After collection, *Daphnia* were washed briefly, and then treated with TRIZOL (Invitrogen Corp.) to extract total RNA, according to the manufacturer's protocol. Homogenization was performed using the NS-310E physcotron (Nichion, Tokyo, Japan), after which cDNA was synthesized from total RNA using Superscript II RT (–; Invitrogen Corp.) with random primers at 42 °C for 60 min. The PCR were performed in an

AGGTGCAGAAGATTTGTCGCCACCAGCAGCGTCAATGGCGCGGTGGCTATTCCGTGGACAGTTTCACCGACGCCAAGAAGAAGGATCCCCTACT	100
G R E D L S P P G S V N G G G G Y S V D S F T D A K K K K G P V P	33
CGACAGCAAGAGGAAGTGTCGTGGTCTGCGGTGATCGAGCTTCCGGTTACCACTATAACGCCCTCACCTCGCAAGGATGCAAAGGATTCTTCGGCGGA	200
R Q Q E E L C L V C G D R A S G Y H Y N A L T C E G C K G F F R R S	67
GTAAACGAAAATGCAAGTCTACCAAGTGAACAACGGAACGGATGCGACATTGACATGTACATGAGCGAAAGTGTCAGGAATGTCGACTCAAAAAATG	300
I T K N A V Y Q C K Y G N G C D I D M Y M R R K C Q E C R L K K C	100
CCTCACGGTCGGCATGCGGCCGAATGTTGTGCCCCGAGTACCAAGTGC CGCTCAAAGGGAGGCAAGAAGCGCAAAAGGAGAAGGACCGGCCAAT	400
L T V G M R P E C V V P E Y Q C A V K R E A K K A Q K E K D R P N	133
TCCAGTTCTTTGGGATCACC TGACATGAAAGAGCGGAATCAAGGCCATCCACGACCAACAACAGCAGCAACAACAGCAATGCAGCAGCAACAGC	500
S S S L G S P D M K E P E I A K I H Q Q Q Q Q Q Q Q Q Q Q Q Q	167
AGCAATTACAGCAACATACACGTGTTGGACGAGAACCGATGATCGTTTTCGCGCCCTGCCAACGGTGGAGTCAGCAACGGATCGCCGCTCAAAACCT	600
Q L Q Q H I H V L D E K P M I V C G P A N G G V S N G S P V K P L	200
CAGTCCCGAACAGGAGGAGCTCATCAACCGGCTCGTCTATTCCAGGAAGAATTCGATCAGCGCTCGGAAGAGGACCTGAGGAAAATTCGACGTCGGGC	700
S P E Q E E L I N R L V Y F Q E E F D Q P S E E D L R K I S T S G	233
ATTCACGAGTCGGACGCGGATGCCAAATTAACACATACCGAGATGACGATCTGACGGTGCAACTGACGGTGAATTCGAAAGCGGTTGCCGGTT	800
I H E S D A D A K F K H I T E M T I L T V Q L T V E F S K R L P G F	267
TCGACACGCTCTGCGGGAGGATCAGATCACGTTGCTCAAGCGTGTTCCAGCAGGATGATGATGTTGCGCTGCGCACGGCGCTACGACGCCAACACGGA	900
D T L L R E D Q I T L L K A C S S E V M M L R C A R R Y D A N T D	300
CTCTATCGTCTTCGCAATAATCTGCCCTACACCGCGAGTCGTACAACATGGCCGGCGTCGGTGACACGGCCGATTCTGCTCTTCGCTTCGGCAAGACC	1000
S I V F A N N L P Y T R E S Y N M A G V G D T A D S L F R F G K T	333
ATGTCTCTCATGAAAGTGGACAAATGCCGAGTAGCTCTACTCACGCCATCGTCATCTTTTCGGAACGACCGGGATTGGTGAGGCGAGAAAGTGGA	1100
M S L M K V D N A E Y A L L T A I V I F S E R P G L V E A R K V E K	367
AGATCCAGGAGATTTACTTGAGGCGCTGACGGCTTACGTGATGAACCATCGGTGCGGCCGATGACGGAGTTTGCCAACACTCTTGTGCGTGCTAACCGA	1200
I Q E I Y L E A L Q A Y V M N H R V R P M T E F A K L L S V L T E	400
GTTGCGACGCTCGGCAACCTCAACTCTGAGATGTGCTTTCTCTCAAGCTCAAAAAACAAGAACTGCCGCCCTCTCTCGCCGAAATTTGGGACGTCCAT	1300
L R T L G N L N S E M C F S L K L K N K K L P P F Q L A E I W D V H	433
TCGTGAACGCCATCTCTGCCCTTAGGAAAAAAAAACAAACGAAACGAGCAGCCACGCGTGATTCTACTACACCTACCCCGCGCTCCGTAAACAAA	1400
S	444
GTACTCCGCTCTCAAAAAAAAAAAAAAAAAAAAAAA 1440	

Figure 2 Nucleotide sequence of the *D. magna* EcR common region. Nucleotide sequence of the *D. magna* EcR cDNA common to all subtypes is indicated. Deduced amino acid sequence is also indicated. Dark and light shaded amino acids indicate DNA-binding domain (DBD) and ligand-binding domain (LBD) respectively. Nucleotide sequences that were used for 5'-RACE are indicated in boxes.

ABI Prism 7000 (Applied Biosystems Japan Ltd) using the SYBR-Green PCR core reagents kit (Applied Biosystems Japan Ltd), in the presence of appropriate primers. PCR amplifications were performed in triplicate using the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by a total of 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C). In order to avoid the unexpected degradation of RNA, we did not treat RNA with DNase. Instead, we confirmed that contamination of genomic DNA was negligible by PCR using intron containing primers (data not shown). Gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of non-specific bands. The primers were chosen to

amplify short PCR products of <150 bp; primer sequences are listed in Table 1.

Construction of the reporter system

We used a two-hybrid system to detect chemicals that activate *Daphnia* EcR. DNA encoding the LBDs of EcR and USP were amplified using the following oligonucleotides: *Daphnia* EcR, Forward (F) 5'-GGATCCTCAC-GGTGGCATGCGGCC-3' and Reverse (R) 5'-TTCTAGATGGCGTTACGAATGGAC-3'; *Daphnia* USP, F 5'-AGGATCCTGCAGATGGGCATGAAGCG-3' and R 5'-TTCTAGACCAGTTCTAAGTTTCTGC-3'.

a) subtype A-specific UTR α

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GTCAGTGTATACAGACTCTGAGTAAGAGAGAGAGAGTCTCTGGCATAGAGAAAGAGCAGGGTTTTTTTTCTGTGCGTGTGTGTGCGTGTGTGT 100
GTGTGTGAAGAGTTGGGGTACAAGAAACGAAAAGTTCTTTTAATAATCAACAGTTTTTGGCTTTTTCTGTGTGAGTTGGGGTTTGATTTTGT 200
TTTCACACGTGGAGGCGGTGTCATCGTGAACCTTTGATCGGATATAATTTTTCTCGAATCCCCAGGTGCGAAAACCTCA 281
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b) subtype A-specific UTR β

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AGTTTTCTATGTGCGAGTCCGCCGAGACAGAGCGGACGACTGTGCGTGCCAGTGAAAAGACAAGTTGGGTTCATTTTCTGTGGTTTTTCGGCCCGTT 100
TACATAATTACTGTGTCCAATCAAGAACTGAGTACTAACTCAGGGAAAAATTTGGCTTATTTCAATTCACTCAAAAGT 182
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c) subtype A2-specific region

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GGTGGCGATTTTTTTTTTTTTTTTTTTTATTGAAATTCGTACTTGGCAGCCATTCTTCCCTTACATTTTTCTGTATTACGCAACACGCACATTGGCCAA 100
AAAAATCAGTTAAATAGTGAGAGTGTGCGCGCTCTTAAATCAAAAGAAACAGTGTCGCATATAGAGAGAGAAAGAGAGAGAGAGATCCCGTAT 200
CGGTGGAATGCACCGAAACATGGAAGTGAACAGCTGGAACGTTGCTACTTGGCGTGGCGGTGATTTTTATCGGCCGGCTCGCGTAATCGAATCA 300
M E V E Q L E R S T T G A G R V F Y R P A S R N R I N 27
ACAGTATGGAAGGTGTGGTGATAAGTGGCGCAGAGACTCAGCAACAGCAGCAGAGAATCCGGTGGCATCTTCGCTCTATCGGTGGCCACCATCATCAA 400
S M E G V V I S G A E T Q Q Q Q R I R L A S S V L S V A T I I K 60
AAGGGAACCGCGCAACTCCGATTACCCACGACCACTCTCCAACAACAACAACAACAACACTCTTCTCTTGTCTTCTCTCTCTCTCTCTCTCTCT 500
T E P R N S D S P T T T P L Q Q Q Q Q H N S S S S C S S P S P S S 93
GCTAGTTCGATCCGTCGATCAGCTCCCGCATCAGTACAG 540
A S S I R R S R S R I S T G 107
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d) subtype A-common region

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GGGGAGGCGAGCCCATGGGTAGTTACCCGTACCCGACGTCGCCATCTGCCGAATGCACCCGATCTCCATGTGCTGTACATTATCGTCGCCGTGATC 100
G G S P L G S S P S P T S P S A A (M) H P I S M S L L H S S S P S S 33
TTTCGATCACCACCATCCGACCATCCGATCAATCGTTTACGGAGGAGATCGACGACGCTACGTTGGTAGGGGCGTCGGCCAGCGGATCGGTCCAT 200
S H H H P H H P H Q S F Y G G G S T T P T L V G A S A S G S V H 66
TCGCCGAGCCCGTTGAAACGATCCAGGCACATCAACATCAACAACAACAACAACAAGCAGCAGCGCTATGGAAGAATGGTTACCTTCGCCCGGCC 300
S P S P L K R S R H I N I N N N N S S S S A M E E W L P S P G Q 100
AAATGTCTGTGATTCATGTGCCACCGCTCCGCCAGCAGTTCGTCATGTGATGAGGGCAGACGGCTTTAGAGCCGGCCAGCAACAGCAGTT 400
M S V D S M S P P P P P S S S S M S M R A D G L L E P G Q Q Q Q L 133
GCAACAGCAACCGCGTCCAGCAATGGCCATTCTACCTTACCGCTTCCGGTCATCTCGTCGCAACGGCTATTACCATCGCCATGTGACGGGGCAGC 500
Q Q Q R A S S N G H S T L P P S V I S S S N G Y S P S P M S T G S 166
TACGAGCCGCATTAGCCCTGGCGGTGGAGGAGGCAATT 541
Y E P P F S P G G G G K L 179
specific A-box
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Figure 3 Nucleotide sequence of the *D. magna* EcR-A specific region. Nucleotide sequences of subtype A specific region is indicated with deduced amino acid sequence. As schematically indicated in Fig. 1, four types of cDNAs were identified, which correspond to a+c+d (EcR-A2 α), b+c+d (EcR-A2 β), a+d (EcR-A1 α), and b+d (EcR-A1 β). The first methionine of EcR-A1 is indicated by circle. Amino acid sequence conserved as A box of EcR in many species is underlined.


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CGACGCGTTTTCGACGCTCAACGAGTCACTTGGGTGTGTGTTGTCGATTGTTTTGTTGGTGTGTTCAACACCGTCGGTGCAAAAAAACA 100
ACACAATCGAACGTCAAACAATTCGAAAGAAAAAGTGAATTCAAATTAGTGTGAGGTGCGCTTGTGATTGTGTAGAGGACGCCGATTAGAGCGAA 200
AGTGAATAATCGATTCCCATCGCTGACTGTTGTGATTTTCTAAACCTCATTGTTGGGTGTGTGTCTGTGATCGTTCTCCGTTGTTGCCAGCAAAATA 300
GAAAAAGACGGGCAACCTGGCCGAGTATCTTTACGAGAAAAAAGCAAAATGAATATTGTGCGGCTTTCAACGCTCTTGACATCAACGACA 400
TCGAAAAATTAATTTGGCAAAAAAAGTCTTCGCGAGCTGGAATCGAACCGAGGCTTCTGTCTTTCTGTTGTTTTTCTTCTGTT 500
TCTTTAAAAAATCAAATCCAGTTCATTATGTGGCTGGTGTGAGGCCACGATCGTGATAAGAACACGCACAAGTACAGAGTGTGTGGCAGGGCGAGT 600
GGATGGATTGGTAATGAGCTTTATTTTGTCTGTGCTGCTCACTAGGCAATTAGCTGGTGTATCGTCTTGCCCTGTGTTTCTTGTCTGTTCAATT 700
TTGTATTTTTTTCATTGACAAAAATGACCATGGGCGATCATGGTGAAGTCAGTTCGTCTCTGTGCCGATGACACATCTCCGCTGCGTCCTGGCAG 800
      M T M G D H G E V S S S S S V P M T T S P S A S W Q 26
TCGAACCGCGGCCAATCAACAGCAACAGTTGAAATACCTTACGGTCAGTGACCCGCGGTGCCAACAATGCTGGCCAGTTACTCGTCCCGGTC 900
S N R A A N Q Q Q Q L K Y P Y G H V T R G A N K M L A S Y S L P G Q 60
AAGTGATGCGTAGCAACGCTGTGAGCAGCAGCAGCGCCACGGCCATCGCCATCTCTTTGCATCAGCATGCCATCAGGGATCGAATCAACAATCAAC 1000
V M R S N A V S T T T A N G H R H P L L H Q H A H H G S N Q Q S T 93
CGGTTTGATGCAACCGCGCTTTTCGTGCCAACGGCGCGCAGGCGCGCATCGTTGCTGCCACACGGCGCGCAGCGCTTGACCGGTTTGAC 1100
G L M Q P P S F A A N G G A G G G I V A A T T A G T T V L T G L H 126
CACCACAACCACTGCATTACTCAACAATGGCGCAGTCGTCAGACGAGCATTCACTTCGCTCAGATATTGGCGAAGTGATTAGATCTGTGGG 1200
H H N Q L H L L N N G G S P S D E H S L P S S D I G E V D L D L W D 160
ATCTTGACATTAATGACCGCTCCGTCAGTCCCAATCGTCGACGGCGGTGGACACAGTCCCGCGCTCCCGCAGGCGAGCAGATCGTGTGACACGTCCTC 1300
L D I N G P S V S P Q S S T G G G H S P A V P P G R R S S D T S S 193
      specific B-box
TACTTC 1306
T S 195

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Figure 4 Nucleotide sequence of the *D. magna* EcR-B specific region. Nucleotide sequences of subtype A specific region is indicated with deduced amino acid sequence. Amino acid sequence conserved as B box of EcR in many species is underlined.

The amplified DNA fragments were digested with BamHI and XbaI and cloned into pBIND and pACT (Promega) respectively. They were designated as pBIND-EcR (LBD) and pACT-USP (LBD) respectively. As a control, we also constructed *Drosophila* EcR/USP two-hybrid system. The -corresponding *Drosophila* domains were amplified using the following oligonucleotides: *Drosophila* EcR, F 5'- GG ATCCTCACGGTCGGCATGCGGCC -3' and R 5'- GCTCTAGACTATGCAGTCGTCGAGTGCTCC -3'; and *Drosophila* USP, F 5'- GTCGACTAACCTGCGGCAT GAAGCG-3' and R 5'- TCTAGACTACTCCAGTTTCAT CGCC-3'. The amplified DNA fragments were digested with SalI and XbaI and cloned into pBIND and pACT (Promega) respectively. The cDNA encoding the LxxLL domain of *Drosophila* Taiman protein (1028–1235 amino acid (aa)) was amplified using total RNA prepared from Schneider cells and the primers 5'-GGATCCGTGGCGGTCTGGG AGGACTG-3' and 5'- TCTAGATCAGGCTAGCGTGCT

GCTCAC-3'. The amplified DNA fragment was cloned into pGEM-T easy, digested with BamHI and NotI, then subcloned into pACT using the BamHI and NotI sites. This construct was designated as pact-taiman (LXXLL). All of the inserted sequences were confirmed as being cloned in-frame without any amino acid substitutions or deletions.

For transfections, Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and incubated at 37 °C in a 5% CO₂ atmosphere. The day before transfection, cells were transferred to a 24-well plate. The transfection was performed using FuGENE6 (Roche Diagnostics), according to the manufacturer's protocols. Each well received 0.03 µg pBIND-EcR (LBD), 0.03 µg pACT-USP (LBD), 0.1 µg pACT-taiman (LXXLL), and 0.3 µg pG5luc, which has luciferase gene under the control of GAL4-binding site. Ligand was added to the medium after 24 h and cells were harvested after 48 h. Reporter activities were measured using

<i>Daphnia</i>	89	NQQTGLMQPPSFAANGGAGGIIAATTAGTTVLTGLHHNQLHLLNNGGSPSDEHSLPS	169
fiddler crab	13	MFVLGSGVATLNLSTMGDESCSEVSSSPLTSPGALSPALVSVGVSGVSPPT--SLASSDIGEVDLDFDLNLNSPSP	91
yellow mealworm	1	MKRRWSG--LQAVRVTPESSEVTSSSTL-----VMSPAN--SLASTDIGVDLEFIDLNLGAKSR	60
patato beetle	1	MKRRWNG--FRDAEESSEVTSSS-TL-----VMSPAN--SLASADIGVDLEFIDLNLHQHHA	56
silkworm	61	MRRRWSNNGGFP-LRMLEESSEVTSSS-ALGLPAM-----VMSP--SLASPEYRALEL--WSYDDGITYNT	123
spruce budworm	1	MRRRWSNNGGFTLRMLEESSEVTSSS-ALGLPAM-----VMSP--SLASPEYGGLEL--WGYDDGLSYNT	64
malaria mosquito	2	MKRRWSNNGGFTLRMLDSSSEVTSSAALG-----M-----TMSPN--SLGSPNYDELEL--WSYEDNAYNG	62
fruit fly	1	MKRRWSNNGGF--MRLPEESSEVTSSSNGLVLPSPV-----NMSPS--SLDSHYCDQDL--NLGNEGSGFG	63

specific B-box

Figure 5 Aligned amino acid sequence of EcR-B conserved region. Amino acid sequence of *D. magna* EcR-B was compared with other EcR-Bs and the conserved region is indicated. Identical amino acids are indicated.

Table 2 Conserved amino acids of *Daphnia* ecdysone receptor and ultraspiracle

	DBD	LBD	Total	DBD	LBD
<i>Daphnia</i> EcR	100	100	721	327–399	488–717
Crab EcR	94·5	71·4	518	157–229	283–513
Tick EcR	97·7	69·4	570	189–261	335–566
Honey bee	97·3	73·3	630	250–322	395–626
Silkworm	87·5	55·1	606	207–279	350–585
Fruit fly EcR	87·7	60·8	878	264–336	417–650
Human	67·1	41·4	447	98–170	207–443

Amino acid identity between *Daphnia* EcR and other species is indicated. Identity versus *Daphnia* EcR is indicated as percentage of the consensus length. The total number of amino acids, as well as the DNA-binding domain (DBD) and ligand-binding domain (LBD) positions, are indicated.

the Dual-Luciferase Assay kit (Promega), according to the manufacturer’s protocols. The experiment was repeated thrice and the average values were calculated.

Results

EcR structure

By screening the *Daphnia* cDNA library (Watanabe *et al.* 2005) and combination of 5’ RACE, five types of full-length cDNAs were obtained as schematically indicated in Fig. 1. The deduced amino acid sequences indicated that all five cDNAs encoded identical 39 aa at the C-terminus of the A/B domain, 73 aa of C domain, 102 aa of D domain, and 221 aa of E/F domain (Fig. 2). The five cDNAs differ in most part of A/B domain and 5’ untranslated region (UTR). According to their coding sequences, there were three types of A/B domain. Two of them have identical amino acid sequence except for N-terminal region. The shorter subtype (597 aa) lacks 124 aa at the N-terminus of the longer subtype (721 aa). These two subtypes have two common types of UTRs as indicated in Figs 1 and 3. As BLAST searches indicated similarity between the common amino acid sequence of the two subtypes and those of EcR–A of other species, these two proteins were designated as A1 (597 aa) and A2 (721 aa) subtypes. Characteristic amino acids conserved in the A box of many species could be identified in the two subtypes (Cruz *et al.* 2006).

The other subtype shared no common amino acid sequences with EcR–A in the A/B domain except for 39 aa mentioned above. It had a characteristic 195 aa in the A/B domain and the full-length protein was 629 aa. Nucleotide sequence specific for this subtype is indicated in Fig. 4. The 629 aa subtype showed similarity to EcR–B from other species and EcR–B subtype-specific amino acids were conserved in the *Daphnia* EcR as shown in Fig. 5. Thus, this subtype was designated as EcR–B.

The deduced sequences of *Daphnia* EcR exhibited >87% amino acid identity to the DBD and >60% identity to the LBD of *Drosophila* EcR. Within the DBD domain, the P- and D-boxes showed 100% identity with those of *Drosophila* EcR. *Daphnia* EcR also exhibited 67% amino acid identity to the DBD of human LXR α (Table 2).

Based on the amino acid sequences of LBD, phylogenetic analysis was performed. Amino acid sequences of LBD of 18 different EcR-related genes (Table 3) were obtained from database (National Center for Biotechnology Information: <http://www.ncbi.nlm.nih.gov/>) and analyzed. *Daphnia* EcR showed highest similarity to the fiddler crab *Uca* (Chung *et al.* 1998). As indicated by previous study of the fiddler crab, LBD of this group showed similarity to LBD of human LXR (Fig. 6).

Table 3 Ligand-binding domain sequences of EcR-related genes obtained from the National Center for Biotechnology Information database

Diptera	
<i>Aedes aegypti</i> (yellow fever mosquito):	P49880 (675 a.a.: 334–568)
<i>Anopheles gambiae</i> (African malaria mosquito):	EAA00117 (426 a.a.: 185–419)
<i>Drosophila melanogaster</i> (fruit fly):	P34021 (878 a.a.: 417–650)
<i>Lucilia cuprina</i> (greenbottle fly):	O18531 (757 a.a.: 440–673)
Lepidoptera	
<i>Heliothis virescens</i> (tobacco budworm):	O18473 (576 a.a.: 312–544)
<i>Choristoneura fumiferana</i> (spruce budworm):	AAC36491 (541 a.a.: 285–521)
<i>Bombyx mori</i> (domestic silkworm):	P49881 (606 a.a.: 350–585)
Other insects	
<i>Tenebrio molitor</i> (yellow mealworm):	CAA72296 (491 a.a.: 254–485)
<i>Locusta migratoria</i> (migratory locust):	AAD19828 (541 a.a.: 306–537)
<i>Blattella germanica</i> (German cockroach):	CAJ01677 (570 a.a.: 335–566)
<i>Apis mellifera</i> (honey bee):	XP_394760 (630 a.a.: 395–626)
Crustacea	
<i>Daphnia magna</i> : this work (EcRA2, 721 a.a.):	488–717
<i>Celula pugilator</i> (Atlantic sand fiddler crab):	AAC33432 (518 a.a.: 283–513)
Chelicerata	
<i>Ambryomma americanum</i> (lone star tick):	AAB94567 (EcRA2, 570 a.a.: 335–566)
Veretebrate	
<i>Homo sapiens</i> (human):	Q96R11 (FXR, 486 a.a.: 260–483)
	Q13133 (LXR α , 447 a.a.: 207–443)
	P55055 (LXR β , 461 a.a.: 221–457)
Urochordata	
<i>Ciona intestinalis</i> (transparent sea squirt):	FAA00147 (FXR, 516 a.a.: 293–513)
	BAE06541 (LXR, 557 a.a.: 321–555)

Accession numbers of amino acid sequences of EcR and positions corresponding to LBD are indicated.

Figure 6 Phylogenetic tree of EcRs and related nuclear receptors. Amino acid sequence of *Daphnia* EcR LBD was compared with other EcR LBDs. Bootstrap values for 1000 replicate analyses are shown at the branching points. The bar at the bottom indicates branch length, corresponding to the mean number of differences (0.1) per residue along each branch.

USP structure

By screening the *Daphnia* cDNA library (Watanabe *et al.* 2005) with the *Drosophila* USP probe, we isolated clones exhibiting a high degree of sequence similarity to genes encoding other USPs and retinoid x receptors (RXRs). Using the clone sequences, we performed 5' RACE to obtain full-length USP cDNA and obtained two DNA fragments. The deduced amino acid sequences of these fragments were identical and the only differences observed were in the 5' flanking sequences and the amino acid sequence was identical to the previous study (Wang *et al.* 2007; Supplemental Figure; see supplementary data in the online version of the Journal of Endocrinology at <http://joe.endocrinology-journals.org/content/vol193/issue1>).

USP comprises A/B, C, D, and E/F domains (81, 66, 31, and 222 aa respectively), and as with other nuclear receptors,

the DNA-binding domain exhibits a high level of conservation with other USP/RXRs (Table 2). We also obtained a variant containing a 6 aa deletion in the A/B domain (deleted between amino acids 49 and 54). Different from the fiddler crab that showed highest similarity to *Daphnia* EcR, deletion variant of LBD could not be isolated in this study. The LBD phylogenetic tree indicates a similarity between *Daphnia* USP and USPs in other crustaceans, as well as insects. A dendrogram calculated from alignments of these LBDs is presented in Fig. 7, although the DBD of *Daphnia* USP shows a greater similarity to that of *Drosophila* USP than to human RXR, the LBD shows a greater similarity to the latter than to the former. Overall, these data indicate that *Daphnia* EcR is most similar to EcRs from other crustaceans and that *Daphnia* USP is most similar to mammalian RXRs, rather than to USPs from insects such as the Diptera or Lepidoptera.

Figure 7 Phylogenetic tree of USP and related nuclear receptors. Amino acid sequence of *Daphnia* EcR LBD was compared with other EcR LBD. Bootstrap values for 1000 replicate analyses are shown at the branching points. The bar at the bottom indicates branch length, corresponding to the mean number of differences (0.1) per residue along each branch.

Temporal expression patterns

The fact that there were five types of mRNA coding EcR raised a possibility that expression of EcR subtypes are differentially regulated in a spatio-temporal manner. Thus, we examined expression of the different EcR subtypes between molts in adulthood (> 2 weeks old). We designed primers to discriminate between expression of *EcR-A* and *EcR-B* (Table 1), and observed differences in their temporal expression (Fig. 8). Changes in mRNA expression levels of these subtypes were different from each other. During the molting period, gene expression changes of EcR-B were prominent. Gene expression levels of EcR-B were activated more than 20-fold just before molting, whereas EcR-A was activated only fivefold. Similar to EcR-A, gene expression changes of USP were not drastically changed but it was

notable that temporal gene expression profile of USP was similar to that of EcR-A. Both USP and EcR-A were activated around 60 h, which is 6 h before molting. Although clarification of tissue-specific expression patterns of these subtypes is necessary, this result suggests that EcR-A and EcR-B play a distinct role in molting.

As it is known that *EcR* and *USP* play an important role not only in molting but also in development, we examined *EcR* and *USP* expression during embryogenesis. It was notable that the expression of *EcR-B* was activated in two phases. One was at the beginning of embryogenesis (6 h) and the other was at mid-maturation (45 h) that corresponds to rupture of embryonic membrane, whereas that of *EcR-A* increased only about threefold, 6 h before molting. The increase in *EcR-B* expression correlated closely with molting, suggesting that it relates to molting control. In contrast, we observed small changes in *USP*

Figure 8 Expression of *EcR* (A and B) and *USP* in adult *Daphnia*. Expression levels were examined in *Daphnia* adults (2-week-old) during a molting period. Time at molting was assigned as 0 h and the next molting was observed at 66 h (indicated by arrows). The minimum expression levels were designated as one and the magnitude of changes in expression are indicated for each time point. Bars indicate s.d.

Figure 9 Expression of *EcR* and *USP* in *Daphnia* embryos. Expression levels were examined during embryogenesis. Ovulation was assigned as 0 h. Gene expression levels at each time point were divided by the minimum expression levels during the period and its value was indicated. Bars indicate s.d.

expression (Fig. 9). We detected a decrease of *USP* mRNA at the beginning of embryogenesis that was similar to the result of the previous study (Wang *et al.* 2007).

Response to *Ecs* and construction of a reporter system

In order to confirm the response of *EcR* to *Ec* and related chemicals, we constructed a two-hybrid system as a reporter for ligand-dependent transcriptional activation of *EcR* from *Daphnia* and *Drosophila*. For both organisms, we prepared fusions of DNA encoding the LBD of *EcR* and the DBD of Gal4, as well as the LBD of *USP* and the transcriptional activation domain of VP16. When these chimeric genes were transfected into CHO cells, ligand-dependent transcriptional activation could be detected. This activation was enhanced further by cotransfection of DNA encoding the *Drosophila* Taiman LxxLL motif (Bai *et al.* 2000), a known coactivator of *EcR* in *Drosophila*. The enhancement of the reporter gene was evident when it was transfected with *Drosophila* *EcR* and *USP* (data not shown).

Ligand-dependent transcriptional activation was observed for both *Daphnia* and *Drosophila* *EcR* reporters only when *Ec* analogs such as Ponasterone A, Muristerone A, and Tebufenozide were added to the culture medium (Fig. 10).

Figure 10 Effects of chemicals on EcR/USP interaction. Ecdysone-like activities of chemicals were estimated using a two-hybrid system containing recombinant EcR and USP. Cells were transfected with plasmids, pBIND-EcR (LBD), pACT-USP (LBD), pACT-taiman (LXXLL), and pG5luc and exposed to a chemical for 24 h. As a control, the same system using *Drosophila* EcR and *Drosophila* USP was examined (see Materials and Methods). Concentration of each chemical was 1 μ M. Ec, Ecdysone; Pona, Ponasterone A; Muri, Muristerone A; Teb, Tebufenozide; JH3, Juvenile hormone; Pyri, Pyriproxyfen; Feno, Fenoxycarb. Bars indicate s.d.

Non-ecdysteroidal chemicals such as JH, Pyriproxyfen, and Fenoxycarb did not activate the reporter. These results suggest that the recombinant EcR and USP can interact in a ligand-dependent manner that is specific to Ec.

Using this system, we examined dose-dependent protein-protein interactions of EcR and USP. We observed similar responses to Ec from both the *Daphnia* and *Drosophila* EcR/USP systems (Fig. 11). In contrast, *Daphnia* EcR/USP responded to lower doses of Ponasterone A than *Drosophila* EcR/USP (Fig. 11). Interestingly, the effects of Tebufenozide were different to Ponasterone A with *Drosophila* EcR/USP exhibiting higher expression levels than *Daphnia* EcR/USP at equivalent dosages. Given that these responded similarly to Ec, the different responses to Ponasterone A and Tebufenozide may reflect differences in ligand specificity.

Discussion

Daphnia EcR and USP

In this study, we cloned and characterized EcR and USP from *D. magna*. We found three isoforms of EcR: two subtypes exhibited similarity to *Drosophila* EcR-A and the third showed similarity to *Drosophila* EcR-B. This is the first report showing A/B isoforms in crustaceans. The presence of multiple EcR subtypes has been observed for other species. Three subtypes are also found in *Drosophila*: one EcR-A isoform and two EcR-B isoforms (EcR-B1 and the truncated form EcR-B2; Talbot *et al.* 1993). Characteristic tissue and temporal expression patterns of these isoforms have been reported in *Drosophila* and other arthropods such as silk worms (Kamimura *et al.* 1997) and tobacco hornworms

Figure 11 Dose-dependent responses of the EcR reporter systems in response to chemicals with ecdysone-like activity. Cells were transfected with plasmids, pBIND-EcR (LBD), pACT-USP (LBD), pACT-taiman (LXXLL), and pG5luc. As a control, the same system using *Drosophila* EcR and *Drosophila* USP was also examined. Several doses of chemicals were used to evaluate dose-dependent gene activation. X-axis indicates concentration of chemical and Y-axis indicates fold changes. Bars indicate s.d.

(Jindra *et al.* 1996). These isoforms share common DNA and LBDs, but differ in their N-terminal A/B domains.

We examined temporal changes in the expression of these EcR subtypes, although differences in tissue distribution remain to be determined. Our observation of differential isoform expression in *Daphnia* suggests that, as with other species, these subtypes play distinct roles in daphnids. As the changes in *EcR-B* expression corresponded closely to molting, this isoform may be responsible for the initiation of this process. Although it was difficult to precisely estimate the copy number of mRNA of each subtype, mRNAs of EcR-A and USP could be easily detected by PCR rather than EcR-B in adult. As EcR functions by forming heterodimer

with USP, major EcR heterodimer may be composed of EcR-A and USP and this component may be changed to EcR-B and USP at critical stages such as molting and early development. Precise spatio-temporal analyses may elucidate the role of other EcR subtypes and indicate how their functional differences affect hormonal systems in *Daphnia*.

At the beginning of embryogenesis, transcription status changes drastically and it is difficult to normalize using genes that are stably expressed. Thus, in this study, we showed gene expression changes based on the number of embryos in Fig. 9. Although we examined gene expression changes of several ribosomal proteins and elongation factors that have been used for the control gene, we indicated only the expression change of ribosomal protein L32 in Fig. 9, because gene expression changes were minimum. It was notable that the changes of total RNA, ribosomal protein L32, and USP were less than ninefold, expression levels of EcR-A and EcR-B were changed more than 30- and 140-fold respectively. Thus, the characteristic changes of EcR expression are not essentially affected by normalization.

Daphnia EcR reporter system

In order to confirm that recombinant *Daphnia* EcR could respond to Ec, we expressed these receptors in cell culture and examined their responses. Certain combinations of EcR and USP cannot efficiently activate ligand-dependent transcription in cell culture. When we transfected DNA encoding the full-length receptors fused to reporter genes, weak activation (less than twofold) was detected. However, activation could not be detected in the absence of USP. In order to enhance ligand-dependent activation, we constructed a two-hybrid system for the detection of ligand-dependent protein-protein interactions. Although the doses required for the detection of gene activation were high (μM concentrations), even for Ec, they were consistent with previously reported dosages (Hu *et al.* 2003).

We cotransfected DNA encoding the *Drosophila* Taiman LxxLL motif with our EcR/USP reporter system. In the absence of Tai (LxxLL) expression, transcriptional activation of *Drosophila* EcR/USP could not be detected, although DaphEcR/USP responded to Ec and other Ec analogs (data not shown). Although the molecular interactions of these chimeric genes remain unclear, it has been suggested that *Drosophila* USP exerts an allosteric effect on EcR (Hu *et al.* 2003) and our study indicates that binding of the Taiman LxxLL motif may contribute to EcR conformational stability.

Daphnia EcR response to chemicals

Using our reporter system, we were able to compare the effects of Ec and other chemicals on EcR/USP activity in *Daphnia* and *Drosophila*. We found that *Daphnia* and *Drosophila* responded similarly to Ec, but exhibited different responses to Ponasterone A and Tebufenozide. Ponasterone A activated *Daphnia* EcR/USP at a lower concentration than that

required to activate *Drosophila* EcR/USP. In addition, Ponasterone A is known to cause molting in *D. magna* at a 10-fold lower concentration than Ec. These results support the suggestion that differences in the affinity of EcR for its ligands are responsible for the different concentrations of EC and Ponasterone A required to affect molting (Baldwin *et al.* 2001). Recently, the structure of *Heliothis virescens* EcR/USP was elucidated using X-ray crystallography, and investigation of Ponasterone A and BYI06830 (a non-steroidal, lepidopteran-specific agonist) binding indicated the presence of different ligand-binding pockets for steroidal and non-steroidal ligands (Billas *et al.* 2003). Thus, a possible reason for differences in ligand-binding activity may be differences in the amino acids responsible for non-steroidal ligand binding. As the amino acids responsible for binding BYI06830 in *Heliothis virescens* are conserved in *Daphnia*, the ligand-binding activity of *Daphnia* EcR cannot be explained completely by the composition of amino acid residues responsible for direct ligand binding.

In this study, we cloned EcR and USP from *Daphnia magna* and confirmed that these nuclear receptors interact in a ligand-dependent manner. As interest in the development of environmentally safe insecticides increases, we consider that the *in vitro* reporter system developed in this study may be useful not only for understanding the role of hormones at the molecular level in *Daphnia*, but also for the screening and evaluation of Ec-like chemicals for the purposes of pest control.

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