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.

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Table 1 Primer sequences for quantitative real-time PCR

Gene	Forward	Reverse
L32	GACCAAAGGGTATTGACAACAGA	CCAACTTTTGGCATAAGGTACTG
EcR-A	CAGGCACATCAACATCAACAAC	GGCGACATGGAATCGACA
EcR-B	CACCACAACCAACTGCATTTAC	CCATTAATGTCAAGATCCCACA
USP	TAGGCCACTCGGGTTACTTAAA	GAGTGGGTGGTTAGGTGGATAA

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 $4{\cdot}3\times10^8$ 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

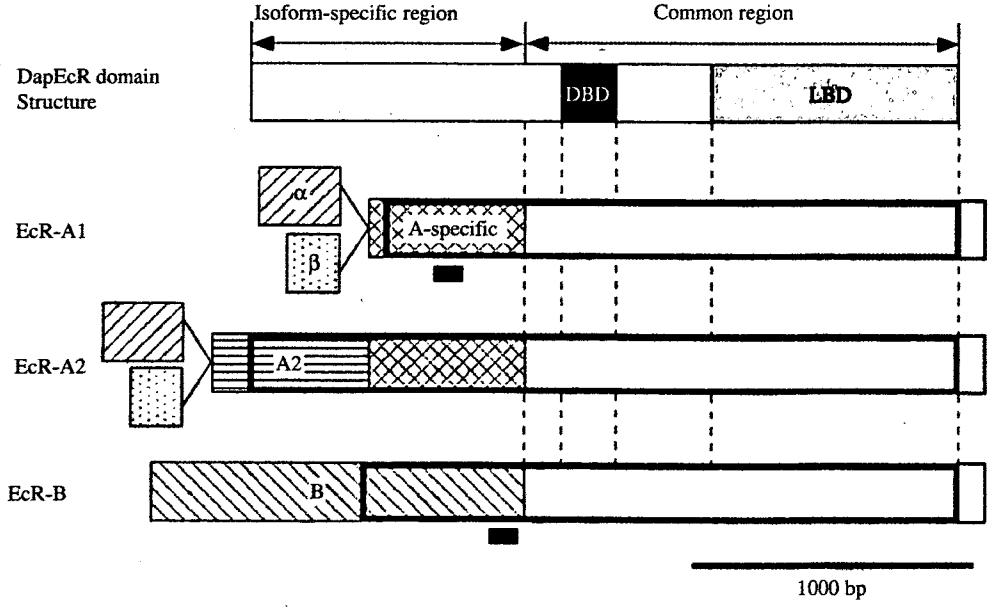

Figure 1 Schematic representation of *D. magna* EcR (DapEcR) structures. Three protein subtypes were encoded by the five cDNAs isolated. Both EcR-A1 and A2 have two types of UTRs, which are indicated as α and β. Open reading frames of the subtypes are indicated in boxes. Different nucleotide sequences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

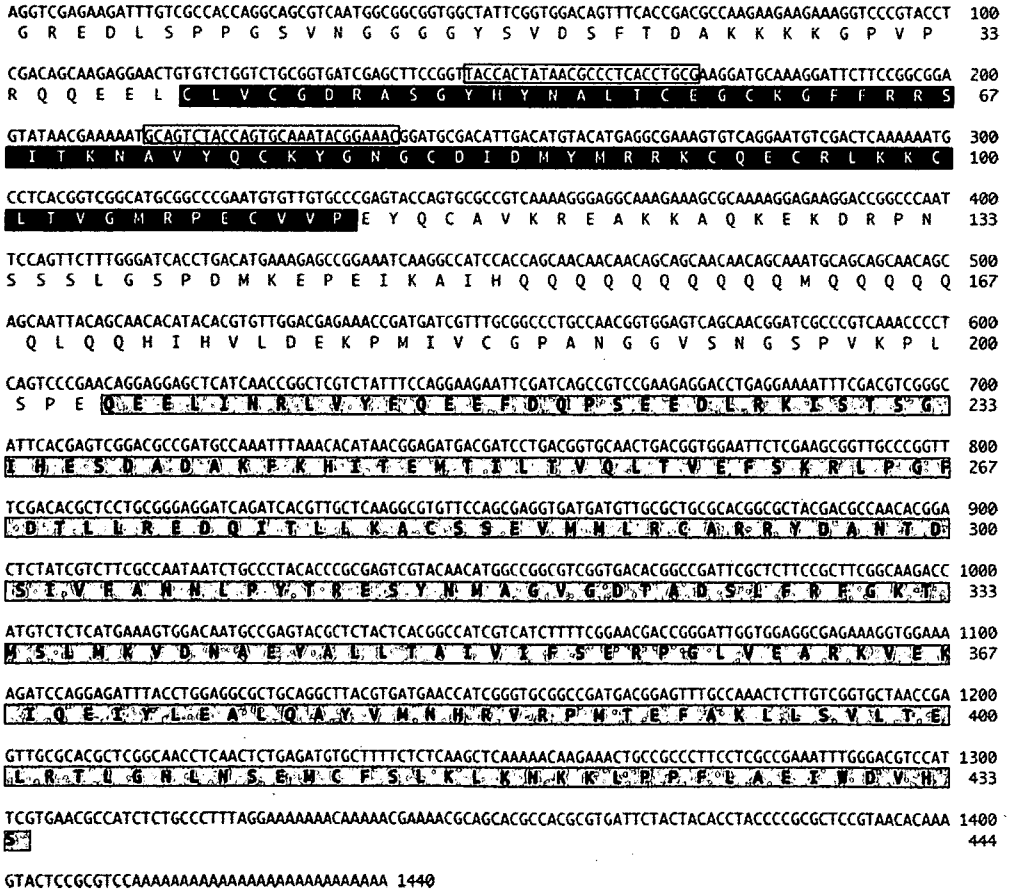

Figure 2 Nucleotide sequence of the *D. magna* EcR common region. Nucleotide sequence of the *D. magna* EcR cDNA common to all subtypes is indicated. Deduced amino acid sequence is also indicated. Dark and light shaded amino acids indicate DNA-binding domain (DBD) and ligand-binding domain (LBD) respectively. 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-GGTCGGCATGCGGCC -3′ and Reverse (R) 5′-TTCTAGATGGCGTTCACGAATGGAC–3′; *Daphnia* USP, F 5′- AGGATCCTGCAGATGGGCATGAAGCG–3′ and R 5′- TTCTAGACCAGTTCTAAGTTTCTGC–3′.

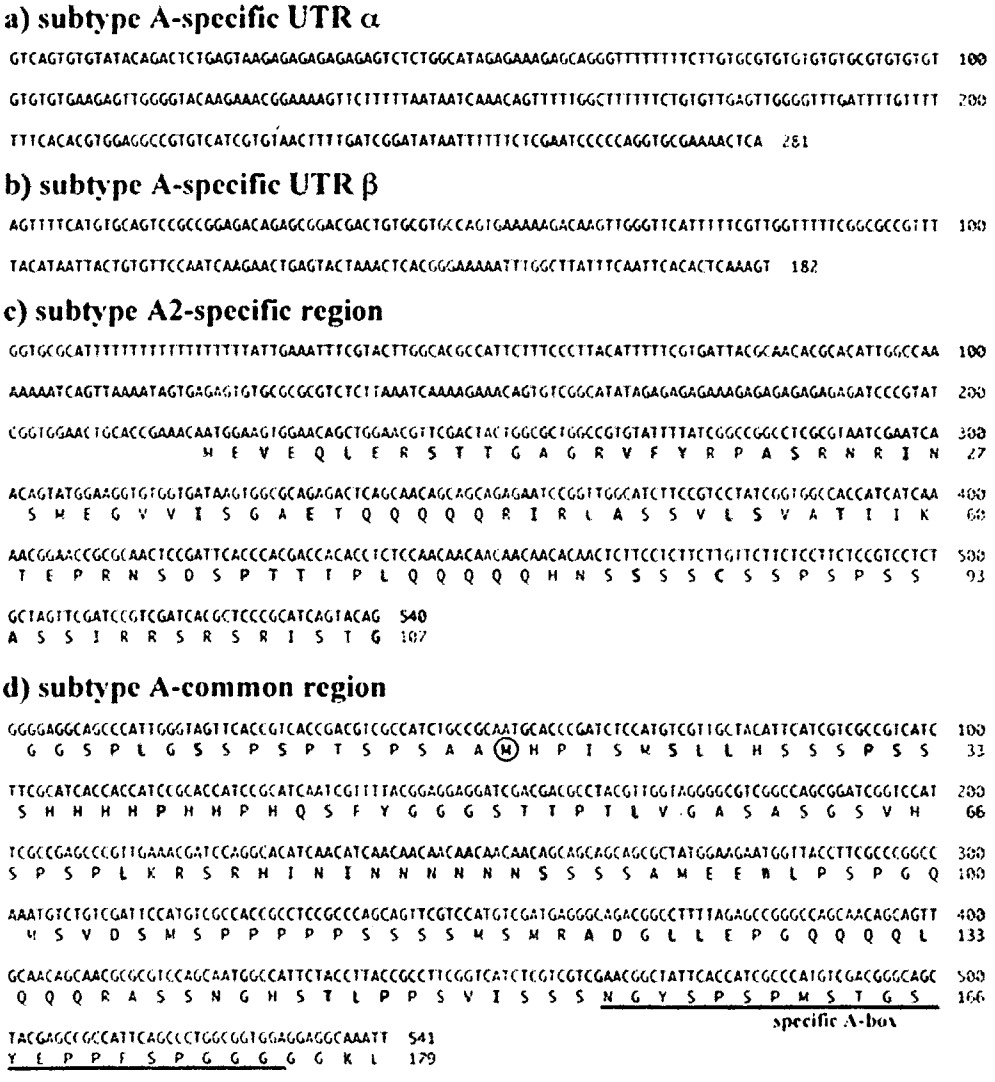

Figure 3 Nucleotide sequence of the *D. magna* EcR-A specific region. Nucleotide sequences of subtype A specific region is indicated with deduced amino acid sequence. As schematically indicated in Fig. 1, four types of cDNAs were identified, which correspond to a+c+d (EcR-A2α), b+c+d (EcR-A2β), a+d (EcR-A1α), and b+d (EcR-A1β). 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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GCAGTCGGTTTTGCGACGTCAACCAGTTCACTTGGGTGTGTGTTGGTCGATTGTTTTTGTTGGTGTGTTCACAAACCGTCGGTGCAAAAAAAAACAAAAA  100

ACACAATCGAACGTCAAACAATTCGAAAAGAAAAAGTGTAATTCAAATTAGTGTCGAGGTGCGCTTGTTTGATTGTGTAGAGGACGCCGATTAGAGCGAA  200

AGTGAAAATCGATTCCCATCGCTGACTGTTGTGATTTTCTAAACCTCATTTGGGGTGTGTGTGTCTGTGATCGGTTCTCCCGTTGTTGGCCAGCAAAATA  300

GAAAAAAGACGGGCAACCTTGGCCGAGTATCTTGTTACGCAGAAAAAAAAAAAGCAAATTGAATATTGTGCGGCTTTCAACGTCCTTGACATCAACGACA  400

TCGAAAATTAAATTGGCAAAAAAAAAAAAAAAAAGTCTTCCGCAGCTGGAATCGAACACGGAGGCTTCTTGTCTTTCTGTTTGTTTTTTTTGCTTTCGTGT  500

TCTTTAAAAAAATCAAATCCACGTTCATTATGTGGCTGGTAGTGAGGCCACGATCGTGATAAGAACACGCACAAGTACAGAGTGTGTGGCGAGGGCGAGT  600

GGATGGATTGGTTAATGAGCTTTATTTTTGTTCTGTGCTGCTCACTAGGCAATTAGCTGGTTGTATCGTCTTGCCCTTGTGTTTCTCTTGTCGTTCATTT  700

TTGTTATTTTTCATTGACAAAAATGACCATGGGCGATCATGGTGAAGTCAGTTCGTCATCTTCTGTGCCGATGACCACATCTCCGTCTGCGTCCTGGCAG  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

TCGAACCGCGCGGCCAATCAACAGCAACAGTTGAAATACCCTTACGGTCACGTGACCCGCGGTGCCAACAAAATGCTGGCCAGTTACTCGCTGCCCGGTC  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

AAGTGATGCGTAGCAACGCTGTGAGCACGACGACGGCCAACGGCCATCGCCATCCTCTTTTGCATCAGCATGCCCATCACGGATCGAATCAACAATCAAC 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

CGGTTTGATGCAACCGCCGTCTTTCGCTGCCAACGGCGGCGCAGGCGGCGGCATCGTTGCTGCCACCACGGCCGGCACGACCGTCTTGACCGGTTTGCAC 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

CACCACAACCAACTGCATTTACTCAACAATGGCGGCAGTCCGTCAGACGAGCATTCACTTCCGTCCTCAGATATTGGCGAAGTTGATTTAGATCTGTGGG 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
                                                                  specific B-box
ATCTTGACATTAATGGACCGTCCGTCAGTCCCCAATCGTCGACGGGCGGTGGACACAGTCCCGCCGTCCCGCCAGGCAGACGATCGTCTGACACGTCCTC 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

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% CO_2 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 pG5*luc*, 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

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Daphnia          89 NQQSTGLMQPPSFAANGGAGGGIVAATTAGTTVLTGLHHHNQLHLLNNGGSPSDEHSLPSSDIGEVDLDLWDLDINGPSVS 169
fiddler crab     13 MFVLGSGVATLNLSTMGDESCSEVSSSSPLTSPGALSPPALVSVGVSVGMSPPT--SLASSDIGEVDLDFWDLDLNSPSPP  91
yellow mealworm   1 MKRRWSG--LQAVRVTPEESSSEVTSSSTTL----------------VMSPAN--SLASTDIGDVDLEFWDLDLNGAKSR  60
potato beetle     1 MKRRWNG-----FRDAAEESSSEVTSSS-TL----------------VMSPAN--SLASADIGDVDLEFWDLDLNHQHHA  56
silkworm         61 MRRRWSNNGGFP-LRMLEESSSEVTSSS-ALGLPPAM----------VMSPE---SLASPEYRALEL--WSYDDGITYNT 123
spruce budworm    1 MRRRWSNNGGFQTLRMLEESSSEVTSSS-ALGLPAAM----------VMSPE---SLASPEYGGLEL--WGYDDGLSYNT  64
malaria mosquito  2 MKRRWSNNGGFTALRMLDDSSSEVTSSSAALG----M----------TMSPN---SLGSPNYDELEL--WSSYEDNAYNG  62
fruit fly         1 MKRRWSNNGGF--MRLPEESSSEVTSSSNGLVLPSGV----------NMSPS---SLDSHDYCDQDL--WLCGNESGSFG  63
                                                                  specific B-box
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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
Daphnia 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 LXRα (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
Aedes aegypti (yellow fever mosquito): P49880 (675 a.a.: 334–568)
Anopheles gambiae (African malaria mosquito): EAA00117 (426 a.a.: 185–419)
Drosophila melanogaster (fruit fly): P34021 (878 a.a.: 417–650)
Lucilia cuprina (greenbottle fly): O18531 (757 a.a.: 440–673)
Lepidoptera
Heliothis virescens (tobacco budworm): O18473 (576 a.a.: 312–544)
Choristoneura fumiferana (spruce budworm): AAC36491 (541 a.a.: 285–521)
Bombyx mori (domestic silkworm): P49881 (606 a.a.: 350–585)
Other insects
Tenebrio molitor (yellow mealworm): CAA72296 (491 a.a.: 254–485)
Locusta migratoria (migratory locust): AAD19828 (541 a.a.: 306–537)
Blattella germanica (German cockroach): CAJ01677 (570 a.a.: 335–566)
Apis mellifera (honey bee): XP_394760 (630 a.a.: 395–626)
Crustacea
Daphnia magna: this work (EcRA2, 721 a.a.: 488–717)
Celuca pugilator (Atlantic sand fiddler crab): AAC33432 (518 a.a.: 283–513)
Chelicerata
Ambryomma americanum (lone star tick): AAB94567 (EcRA2, 570 a.a.: 335–566)
Veretebrate
Homo sapiens (human): Q96RI1 (FXR, 486 a.a.: 260–483)
Q13133 (LXRa, 447 a.a.: 207–443)
P55055 (LXRb, 461 a.a.: 221–457)
Urochordata
Ciona intestinalis (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.

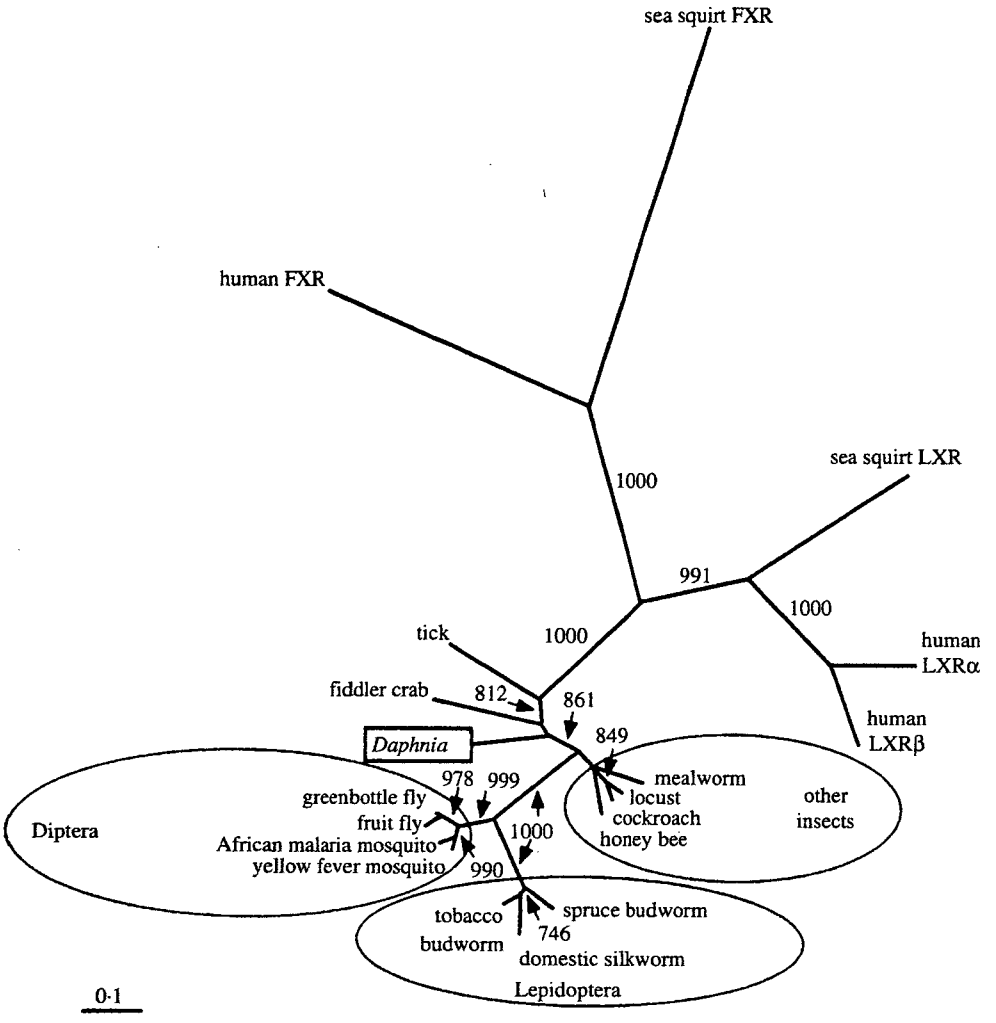

Figure 6 Phylogenic tree of EcRs and related nuclear receptors. Amino acid sequence of *Daphnia* EcR LBD was compared with other EcR LBDs. Bootstrap values for 1000 replicate analyses are shown at the branching points. The bar at the bottom indicates branch length, corresponding to the mean number of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

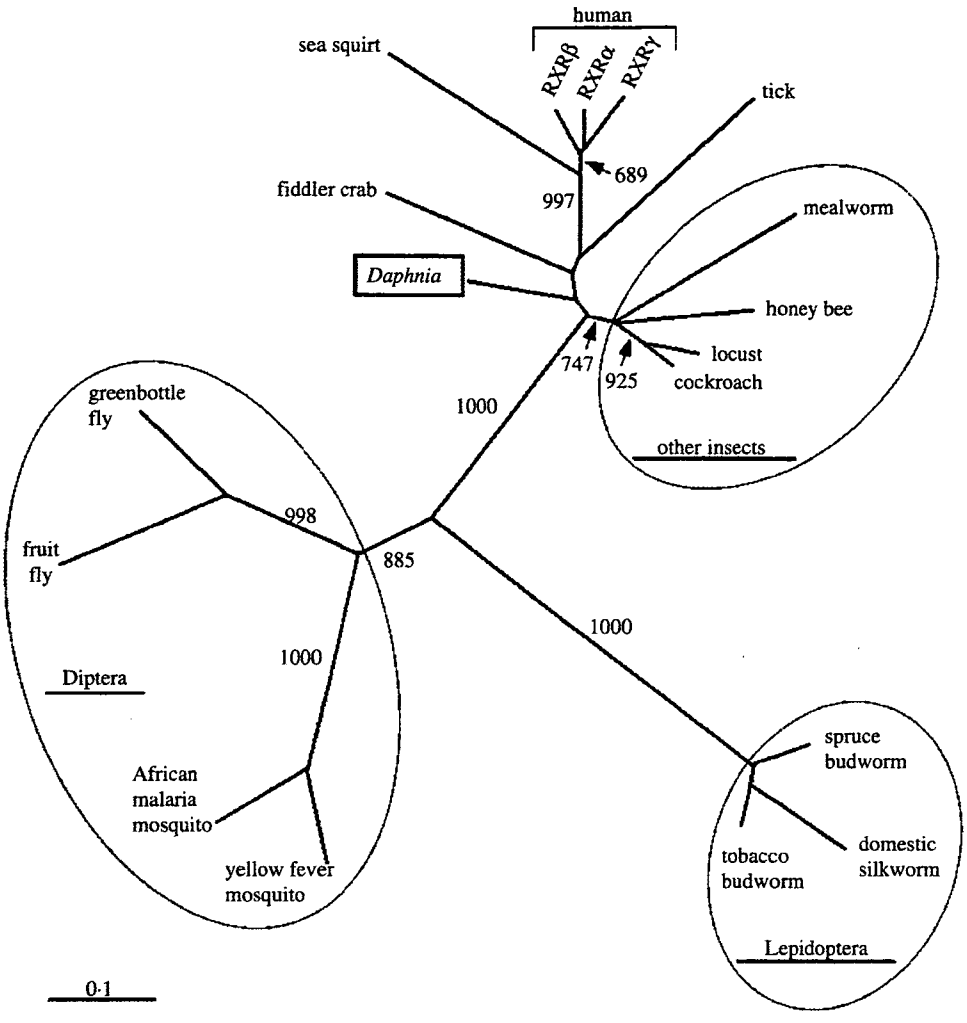

Figure 7 Phylogenic tree of USP and related nuclear receptors. Amino acid sequence of *Daphnia* EcR LBD was compared with other EcR LBD. Bootstrap values for 1000 replicate analyses are shown at the branching points. The bar at the bottom indicates branch length, corresponding to the mean number of differences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

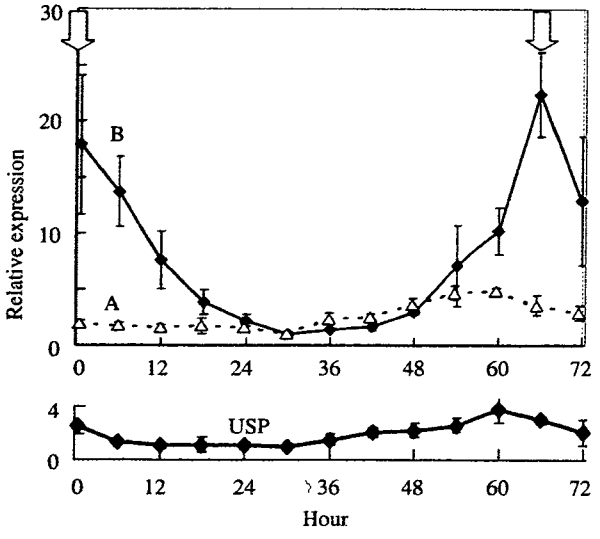

Figure 8 Expression of *EcR* (A and B) and *USP* in adult *Daphnia*. Expression levels were examined in *Daphnia* adults (2-week-old) during a molting period. Time at molting was assigned as 0 h and the next molting was observed at 66 h (indicated by arrows). The minimum expression levels were designated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

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).

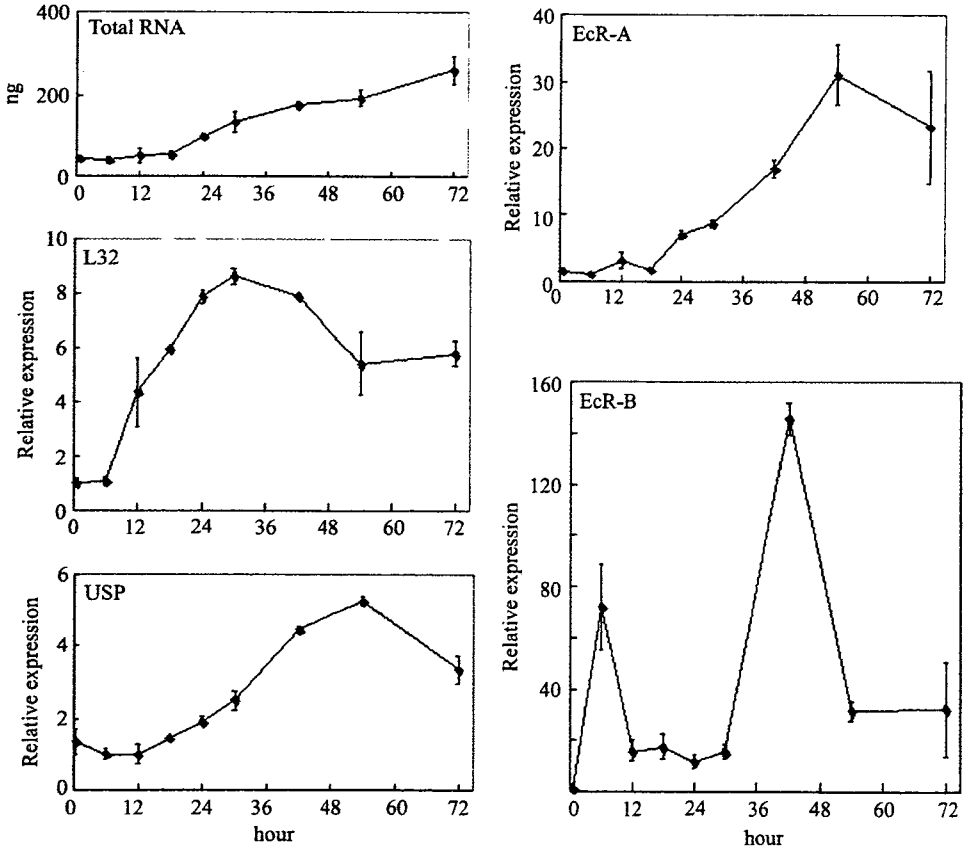

Figure 9 Expression of *EcR* and *USP* in *Daphnia* embryos. Expression levels were examined during embryogenesis. Ovulation was assigned as 0 h. Gene expression levels at each time point were divided by the minimum expression levels during the period and its value was indicated. Bars indicate S.D.

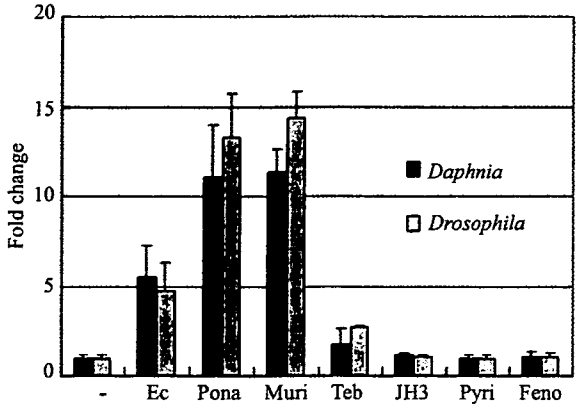

Figure 10 Effects of chemicals on EcR/USP interaction. Ecdysone-like activities of chemicals were estimated using a two-hybrid system containing recombinant EcR and USP. Cells were transfected with plasmids, pBIND-EcR (LBD), pACT-USP (LBD), pACT-taiman (LXXLL), and pG5luc and exposed to a chemical for 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 μM. 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.

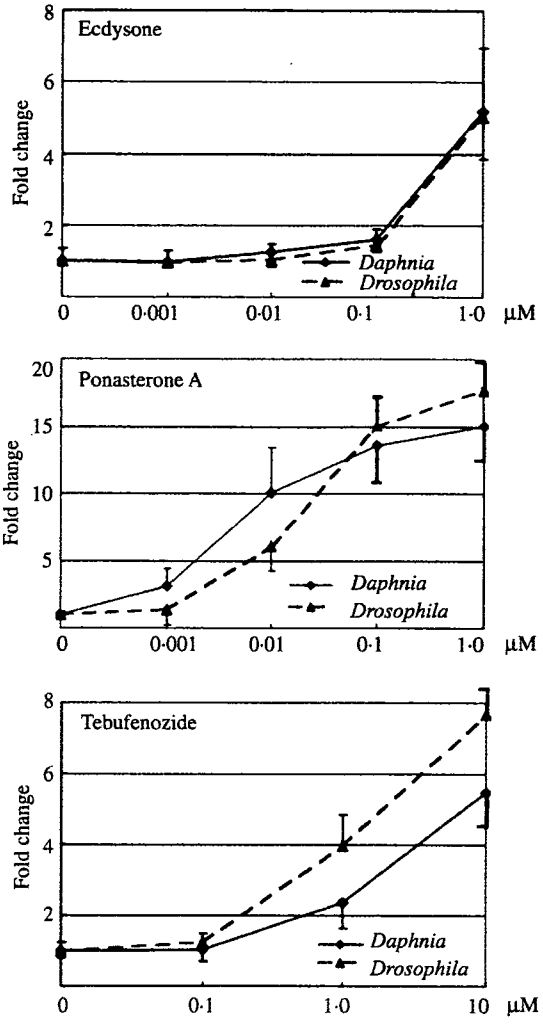

Figure 11 Dose-dependent responses of the EcR reporter systems in response to chemicals with ecdysone-like activity. Cells were transfected with plasmids, pBIND-EcR (LBD), pACT-USP (LBD), pACT-taiman (LXXLL), and pG5luc. As a control, the same system using *Drosophila* EcR and *Drosophila* USP was also 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.

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 crustceans. 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 (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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Environmental Toxicology

DEVELOPMENT OF A *DAPHNIA MAGNA* DNA MICROARRAY FOR EVALUATING THE TOXICITY OF ENVIRONMENTAL CHEMICALS

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Abstract—Toxic chemical contaminants have a variety of detrimental effects on various species, and the impact of pollutants on ecosystems has become an urgent issue. However, the majority of studies regarding the effects of chemical contaminants have focused on vertebrates. Among aquatic organisms, *Daphnia magna* has been used extensively to evaluate organism- and population-level responses of invertebrates to pollutants in acute toxicity or reproductive toxicity tests. Although these types of tests can provide information concerning hazardous concentrations of chemicals, they provide no information about their mode of action. Recent advances in molecular genetic techniques have provided tools to better understand the responses of aquatic organisms to pollutants. In the present study, we adapted some of the techniques of molecular genetics to develop new tools, which form the basis for an ecotoxicogenomic assessment of *D. magna*. Based on a *Daphnia* expressed sequence tag database, we developed an oligonucleotide-based DNA microarray with high reproducibility. The DNA microarray was used to evaluate gene expression profiles of neonatal daphnids exposed to several different chemicals: Copper sulfate, hydrogen peroxide, pentachlorophenol, or β-naphthoflavone. Exposure to these chemicals resulted in characteristic patterns of gene expression that were chemical-specific, indicating that the *Daphnia* DNA microarray can be used for classification of toxic chemicals and for development of a mechanistic understanding of chemical toxicity on a common freshwater organism.

Keywords—*Daphnia magna* DNA microarray Toxicogenomics

INTRODUCTION

Chemicals released into the environment, whether intentionally or unintentionally, are known to have detrimental effects not only on humans but also on various other species, and the toxic effects of chemicals on different species are becoming a great concern, especially in terms of the impact of pollutants on entire ecosystems. To date, however, the number of species that have been examined for the effects of chemical exposure has been limited, and the majority of studies have focused on vertebrates.

Among aquatic organisms, *Daphnia magna* has been used extensively to evaluate organism- and population-level responses of invertebrates to pollutants. In the field, daphnids are widely distributed. They play a central role in the food web and, like other aquatic organisms, are constitutively exposed to multiple chemicals. In the laboratory, *D. magna* is easy to maintain and manipulate because of its short generation time; thus, it has been used as a model organism for aquatic toxicity testing [1,2]. Based on standard and other related toxicological tests, data regarding the effect of a large number of chemicals on daphnids have accumulated [3,4] (http://www.epa.gov/ecotox/).

Recent progress in genomics has provided new approaches to toxicology, termed toxicogenomics. Toxicogenomics has at least three major goals: An understanding of the relationship between environmental exposure and adverse effects, the identification of useful biomarkers of exposure to toxic substances, and the elucidation of the molecular mechanisms of toxicity—that is, an understanding of the biological responses and consequences of exposure [5,6]. Before a phenotype emerges in response to toxic chemicals, cells respond at the level of gene expression to compensate for the stresses caused by chemical exposure. Thus, a phenotype emerges as a result of certain changes in gene expression. Because chemicals do not always affect the same pathways, DNA microarray analysis can potentially provide multiple clues for understanding the molecular pathways that result in phenotypic changes in response to chemicals.

Changes in gene expression in response to chemical exposure can be detected using a DNA microarray, and multiple end points can be analyzed. This approach has been applied to the study of model mammals, and characteristic changes in gene expression profiles in response to certain chemicals have been reported.

In this context, the term ecotoxicogenomics has emerged [7–11] to describe the toxicogenomic approach to ecotoxicology. The application of a toxicogenomic approach to *D. magna* has the potential to increase our knowledge and understanding of ecotoxicity, in particular because our current mechanistic understanding of chemical toxicity in invertebrates is rather limited and useful biomarkers have not been identified.

The purpose of the current study was to establish an ecotoxicogenomic assessment of *D. magna*. We recently analyzed expressed sequence tags for *D. magna* and created a database [12]. Based on this sequence information, we constructed an oligonucleotide-based DNA microarray and explored the acute toxicogenomic response of *D. magna* to several different types of chemical stressors. Copper sulfate ($CuSO_4$), hydrogen peroxide (H_2O_2), pentachlorophenol (PCP), and β-naphthoflavone (βNF) were chosen as model compounds for testing, because

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they have distinct toxic effects. We found that exposure of neonatal daphnids to these chemicals produced alterations in gene expression profiles that were characteristic of each chemical.

MATERIALS AND METHODS

Daphnid strain and culture conditions

Daphnia magna was obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan (NIES clone) [13]. The clone was originally from the U.S. Environmental Protection Agency and has been maintained for more than 10 years at the NIES in Japan. Culture medium was prepared by filtering tap water through charcoal and maintaining it at room temperature overnight before use. Cultures of 20 individuals/L were incubated within the range 24 ± 1°C, with a photoperiod of 14:10-h light:dark. *Chlorella* sp. was added daily to each culture as a 0.01-ml suspension of 4.3×10^8 cells/ml. The water quality (pH and dissolved oxygen concentration) was measured every 2 d by the Environmental Research Center (Tsukuba, Japan). Water hardness, pH, and dissolved oxygen concentrations were 72 to 83 mg/L, 7.0 to 7.5, and 80 to 99%, respectively.

Chemical exposure of neonates

For acute toxicity testing, all offspring were removed from a culture of 20 adult females (age, two to three weeks) 1 d before testing. Neonates (age, <24 h) were exposed to chemicals for 48 h in a static exposure regimen. The chemicals used in this study were $CuSO_4$ (purity, 99.5%), H_2O_2 (purity, 30%), PCP (purity, 99.0%), and βNF (purity, 98%). All chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan). Pentachlorophenol and βNF were dissolved in dimethylformamide to generate a stock solution. Each concentration of chemicals was prepared by diluting a stock solution with fresh culture medium. For the acute toxicity test, 8 to 10 replicates were prepared for each concentration to estimate the median effective concentration (EC50). Immobilization, determined according to Organization for Economic Co-operation and Development test guidelines, was used as the criterion for EC50 [1].

For DNA microarray analysis, the same protocol was used, except that neonates were exposed to chemicals for 24 h and three biological replicates were performed.

Preparation of the DNA microarray

Based on an expressed sequence tag database of *D. magna* (Daphniabase, http://daphnia.nibb.ac.jp), 178 genes were selected for the DNA microarray. Two genes with similarity to the cytochrome P450 genes of other species (*CYP17A1* and *CYP314a1*), the glutathione-*S*-transferase gene, and the ribosomal L8 protein gene were specifically selected; the other genes were randomly selected from the database.

An oligonucleotide corresponding to each gene was designed so that it did not have similarity to other sequences in the database (NGK Insulators, Nagoya, Japan). The oligonucleotides (70 mers) were synthesized by conventional solid-phase synthesis (NGK Insulators, Nagoya, Japan) and spotted onto a Geneslide (Toyokohan, Tokyo, Japan) that had been coated with carbon to facilitate covalent linkage of the DNA. Spotting was performed using an ink-jet printer technique (NGK Insulators). All oligonucleotide sequences are listed in the Gene Expression Omnibus database (accession no. GPL2888; http://www.ncbi.nlm.nih.gov/geo/).

To compare the performance of our DNA microarray with that of conventional DNA microarrays, we also spotted the same set of oligonucleotides onto poly-L-lysine–coated glass using a pin-type spotter (Hokkaido System Sciences, Sapporo, Japan).

Microarray experiments

Neonates (age, <24 h) were obtained as described for the acute toxicity test. Generally, 20 adult daphnids (age, two to three weeks) were cultured in a 2-L flask, and neonates delivered from the culture were used for analysis. Neonates were exposed to chemicals for 24 h in a static exposure regimen and then harvested. Harvested daphnids were briefly washed and homogenized using the physcotron NS-310E (Nichion, Tokyo, Japan). Total RNA was purified with Trizol reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's protocol. To amplify signals from limited sample material, RNA was converted to cDNA, and cRNA was then amplified [14,15] using the Low RNA Input Linear Amp Kit (Agilent Technologies Japan, Tokyo, Japan). Briefly, 500 ng of total RNA were converted to cDNA using a T7-oligo dT primer. The cRNA was then synthesized using T7 RNA polymerase in the presence of either cyanine 3 (Cy3)–cytidine 5′-triphosphate (CTP; PerkinElmer Life Science, Boston, MA, USA) or cyanine 5 (Cy5)–CTP. We prepared larger amounts of RNA from adult daphnids (age, two to three weeks), and these amounts were labeled and used as the cRNA standard. Generally, the cRNA standard was labeled with Cy5, and cRNAs isolated from chemical-treated or control samples were labeled with Cy3. Note that the RNA standard was only used to validate the reproducibility of the DNA microarray and the control RNA prepared from mock-treated neonates. Cyanine- and Cy5-labeled cRNAs were combined and hybridized to the microarray overnight at 42°C in hybridization buffer (5× saline sodium citrate [SSC], 0.5 % sodium dodecyl sulfate [SDS], 4× Denhardt's solution, 10% formamide, and 0.1 mg/ml of denaturated sermon sperm DNA). After hybridization, arrays were washed in buffer (5× SSC, 0.1% SDS) for 10 min at 30°C and scanned by a GenePix4000B (Axon Instruments, Union City, CA, USA). The number of biological replicates was three.

Data analysis

The scanned microarray was analyzed by GenePixPro 5.1 software (Axon Instruments) to obtain signal intensities. The signal intensity of each spot was calculated by subtracting the local median background. Signal intensities were normalized to the 50th percentile per array and used for further analysis. In this study, we used the RNA standard only to confirm the reproducibility of the glass array, and we did not calculate fluorescent ratios of Cy5 and Cy3. Instead, fluorescent intensities of each spot on the microarray were measured directly [16].

To evaluate the quality of microarray platforms, signal intensities of the RNA standard (Cy5) were used, because the same RNA stock was used to obtain Cy5 signals for each type of array. Using normalized values, the standard deviation, coefficient of variation (CV), and Pearson's correlation coefficients of three experiments also were calculated per gene.

For evaluating *D. magna* gene expression and biological reproducibility, signal intensities of Cy3 were used. Signal intensities from three independent experiments were obtained and processed as described for Cy5.

For cluster analysis, the Student's *t* test was performed

using the results of three independent experiments, and genes for which the expression was significantly changed ($p < 0.05$) by chemical exposure were identified. The average signal intensity from three independent experiments was obtained, and fold-changes were calculated by dividing this average signal intensity by the average signal intensity of the control sample. For hierarchical clustering, genes showing more than a twofold change in gene expression were selected and analyzed. Similarity was estimated using Pearson's correlation coefficients, and average linkage was used for the clustering algorithm. Principal component analysis was performed using the same set of genes used for cluster analysis.

Minimum Information About a Microarray Experiment (MIAME) data for the three independent experiments are available as *Supplemental Data* (SETAC Supplemental Data Archive, Item ETC-26-04-003; http://www.setacjournals.org) and have been submitted to the Gene Expression Omnibus database (accession no. GSE3445; http://www.ncbi.nlm.nih.gov/geo/).

Quantitative polymerase chain reaction

Total RNA was purified as described above. The cDNA was synthesized from purified total RNA using Superscript II RT(−) (Invitrogen) and random primers at 42°C for 60 min. The polymerase chain reaction (PCR) reactions were performed in the GeneAmp 5700 sequence detector (PE Biosystems, Tokyo, Japan) using SYBR-Green PCR core reagents (PE Biosystems) in the presence of appropriate primers according to the manufacturer's instructions. Each PCR amplification was performed in triplicate using the following amplification protocol: 2 min at 50°C, and then 10 min at 95°C, followed by a total of 40 two-temperature cycles (15 s at 95°C, 1 min at 60°C).

Gel electrophoresis and melting-curve analyses were performed to confirm the correct amplicon size and the absence of nonspecific bands. Primers were chosen to amplify short PCR products of less than 100 bp. Primer sequences are available on request.

RESULTS

Testing the quality of Daphnia *DNA microarray platforms*

Because spot shape, nonspecific background signal, and slide inconsistency are some of the sources of variation in glass microarray experiments [17], we examined several types of glass slides and printing protocols in an effort to minimize variation in our DNA microarray analysis. We found that printing oligonucleotides onto carbon-coated glass slides using an ink-jet printer gave highly reproducible results. As shown in Figure 1A and C, this platform generated very low nonspecific background and a well-resolved spot shape. On the other hand, the lower quality of background and spot shape on conventional poly-L-lysine–coated slides [18] is shown in Figure 1B and D.

To evaluate the reproducibility of the microarray data, we performed three independent experiments using the same RNA standard. Synthesis of cDNA, cRNA amplification, and hybridization, as described in *Materials and Methods*, were done independently. Because the same RNA standard was used in three independent experiments, the correlation coefficients of signal intensities can serve as an indicator of technical reproducibility. Table 1 shows the correlation coefficients between three independent hybridization experiments using the same

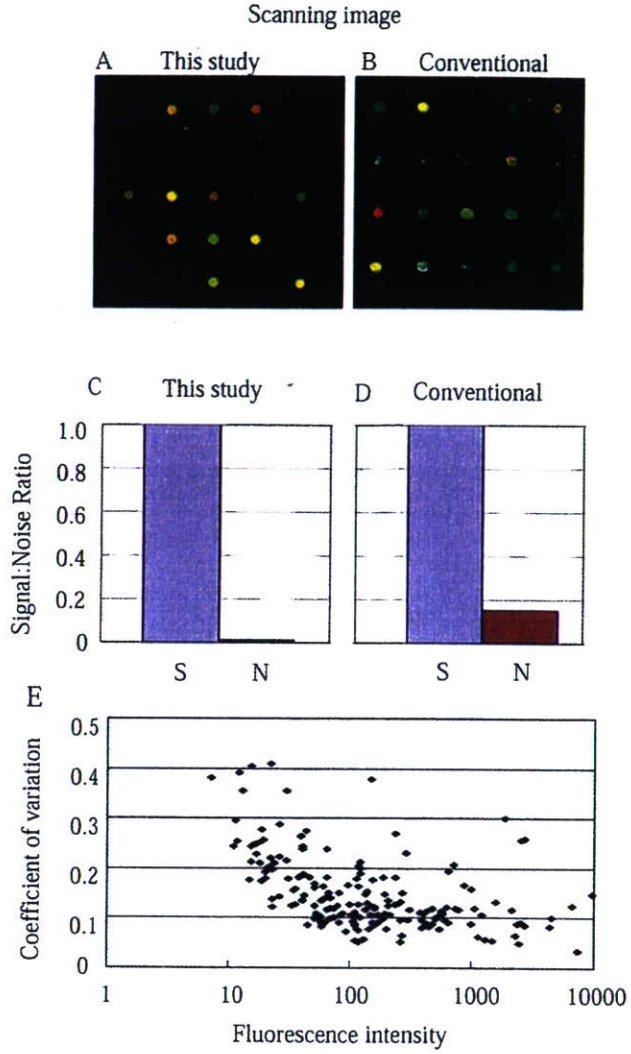

Fig. 1. Scanned image of a *Daphnia* array. (**A**) Part of a scanned image of a representative *Daphnia* microarray used in these studies. (**B**) Part of a scanned image of a conventional microarray, in which a poly-L-lysine–coated slide glass (Matsunami Glass IND, Osaka, Japan) and a pin-type spotter were used for printing the oligonucleotides. (The images in **A** and **B** were obtained using a 4000B GenePix4000B [Axon Instruments, Union City, CA, USA] at a resolution of 10 mm). (**C** and **D**) Signal and noise levels of the two different microarrays. Median signal intensity and background signal were calculated from the images in (**A** and **B**), respectively. The background values were normalized to the median signal intensity, which was set as 1.0. Signal intensity is indicated by blue box, and background level is indicated by brown box. (**E**) Coefficient of variation (CV)and fluorescence intensity CV for three independent experiments. Newborn daphnids were obtained within 24 h of birth, and DNA microarray analysis was performed in the absence of chemical. For each gene, mean fluorescence intensity, standard deviation, and CV of three independent experiments were calculated. Each data point represents a fluorescence intensity corresponding to gene expression level (x axis), and its CV (y axis) are indicated. Median signal intensity was 116.6, and median CV was 0.126. N = noise; S = signal.

RNA (Table 1, Standard). Using our microarray platform, all correlation coefficient values (r^2) were more than 0.99, indicating high reproducibility. Although it is impossible to systematically evaluate all platforms for the highest reproducibility, the combination of improved printing methodology and glass coating likely contributed to the high reproducibility of

Table 1. Correlation coefficients of three independent experiments[a]

		1 vs 2	2 vs 3	3 vs 1
Technical replicates				
Standard		0.995	0.997	0.999
Biological replicates				
Control		0.988	0.998	0.989
$CuSO_4$	5 mg/L	0.970	0.965	0.995
	10 mg/L	0.954	0.974	0.992
H_2O_2	1.2 mg/L	0.930	0.931	0.992
	2.4 mg/L	0.992	0.991	0.994
PCP	75 mg/L	0.992	0.997	0.995
	150 mg/L	0.998	0.998	0.996
βNF	0.5 mg/L	0.982	0.997	0.977
	1 mg/L	0.998	0.999	0.996

[a] Three independent experiments were performed using three glass arrays. The same RNA standard was used for all three technical replicates. After scanning, fluorescent intensities of each spot on the microarray were determined, and correlation coefficients of the fluorescent intensities among the three replicates were calculated as described in *Materials and Methods*. PCP = pentachlorophenol; βNF = β-naphthoflavone.

our system. Thus, we used our *Daphnia* DNA microarray for subsequent experiments.

Testing the reproducibility of D. magna *gene expression data*

Because the data appeared to be highly reproducible using our DNA microarray platform and the RNA standard, we next examined the reproducibility of gene expression profiles of neonatal daphnids using this same platform. Neonates (age, <24 h) were exposed to various chemicals, and their gene expression profiles were examined using DNA microarray analysis. At least 10 neonates were exposed to chemical, and the RNA was pooled and analyzed. To estimate the biological reproducibility, the exposure was repeated three times. Pearson's correlation coefficients of signal intensity for each combination of replicates was calculated. As shown in Table 1, the correlation coefficients between three independent experiments were high (>0.997), indicating that the gene expression profiles in each experiment were very similar. Coefficients of variance for each gene were low (median CV, 0.126) (Fig. 1E). These results indicated that gene expression profiles could be reproducibly obtained from newborn daphnids and that biologically reproducible results from neonatal daphnids exposed to chemicals could be obtained using our DNA microarray tool.

Comparison of data obtained by DNA microarray and quantitative PCR

We next examined whether the data generated by DNA microarray analysis represented actual gene expression changes. Fold-changes caused by chemical exposure estimated by DNA microarray analysis were compared with those obtained from quantitative PCR. For quantitative PCR, 37 genes were selected and analyzed. As shown in Figure 2, DNA microarray results and quantitative PCR results correlated well, although the correlation coefficient for βNF exposure was low.

Chemical exposure and changes in gene expression profiles

To examine changes in gene expression resulting from chemical exposure, we examined the effects of four model

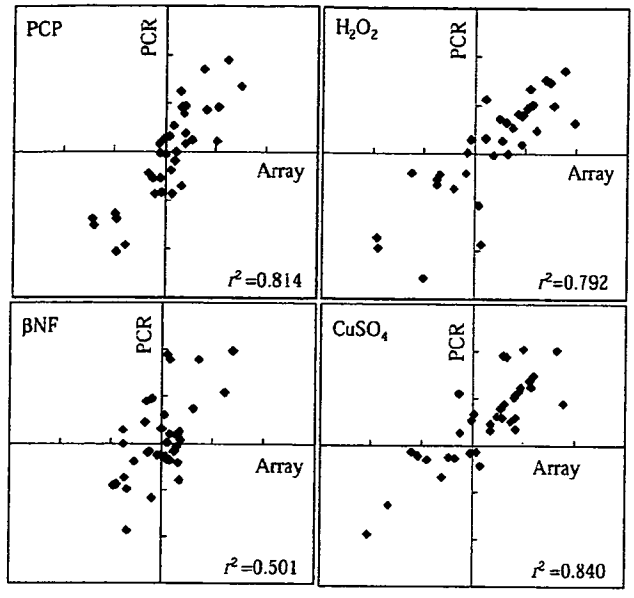

Fig. 2. Comparison of DNA microarray and quantitative polymerase chain reaction (PCR) analysis of changes in gene expression. Neonatal daphnids were exposed to the indicated chemicals, and RNA was prepared as described in *Materials and Methods*. A portion of the RNA was used for DNA microarray analysis, and the other portion was used for quantitative PCR. Fold-changes were calculated by comparing expression levels of chemical-treated samples to those of control (nontreated) samples. The calculated fold-changes were converted to their base-2 logarithm and plotted in a scatterplot. Chemicals used for the exposure are indicated. The *x* axis indicates the fold-changes estimated by DNA microarray analysis, and the *y* axis indicates the fold-changes estimated by quantitative PCR. PCP = pentachlorophenol; βNF = β-naphthoflavone.

chemicals: $CuSO_4$, H_2O_2, PCP, and βNF. These chemicals were selected because each represented a different chemical class. Copper sulfate was selected as a model of heavy metal exposure, H_2O_2 as a model of oxidative stress, PCP as a model of respiratory uncoupling, and βNF as a model for induction of phase I metabolism genes. Median effective concentrations were determined by exposing neonatal daphnids to a range of chemical concentrations and are presented in Table 2. Based on the EC50 of each chemical, we chose two concentrations of each chemical for DNA microarray analysis (Table 2). Following chemical exposure of neonatal daphnids, microarray signal intensities were measured, averaged, and then evaluated for evidence of characteristic changes in gene expression profiles. Genes for which the expression was significantly changed ($p < 0.05$) were selected, and cluster analysis was performed.

Gene expression profiles of neonates exposed to H_2O_2 or βNF showed similar patterns at two different doses, whereas

Table 2. Median effective concentration (EC50) and concentrations of the chemicals used for DNA microarray analysis[a]

Chemical	EC50 (μg/L)	High (μg/L)	Low (μg/L)
$CuSO_4$	13	10	5
H_2O_2	2320	2400	1200
PCP	150	150	75
βNF	2600	1000	500

[a] Chemicals used in the present study and their EC50s are indicated. Based on the EC50, the two indicated concentrations (high and low) were selected for exposure of neonatal daphnids. PCP = pentachlorophenol; βNF = β-naphthoflavone.

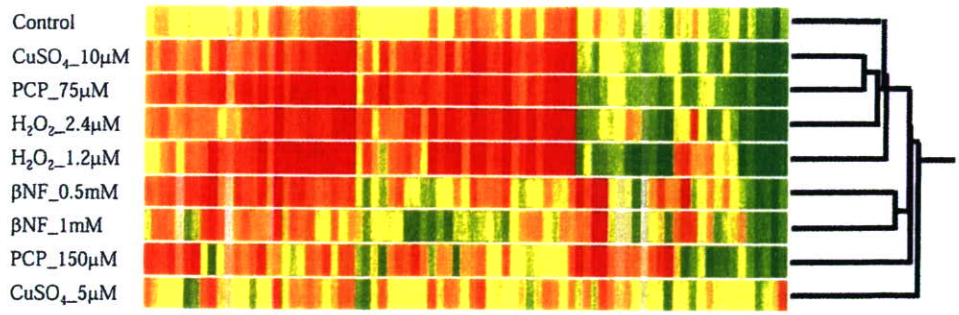

Fig. 3. Two-dimensional hierarchical clustering analysis of gene expression after chemical exposure. Changes in gene expression estimated by DNA microarray analysis are indicated as the fold-change in comparison to control samples. Chemicals and doses used for the exposure are indicated at the bottom. The numbers indicate concentrations of the chemicals in micrograms per liter. Red and green bars indicate induced and repressed genes, respectively. Dendrogram represents correlation of the gene expression profile. PCP = pentachlorophenol; βNF = β-naphthoflavone.

gene expression profiles of neonates exposed to $CuSO_4$ or PCP were different at different doses (Fig. 3). These results indicate that different types and doses of chemicals lead to different gene expression profiles.

To further evaluate whether gene expression profiles differed among daphnids exposed to different chemicals, we performed principal component analyses for each chemical. We found that gene expression profiles after chemical exposure were characteristic for each chemical. Based on two-dimensional scatterplot analysis, higher doses of $CuSO_4$ and H_2O_2 generated similar gene expression profiles, which differed from those generated by PCP and βNF (Fig. 4). These observations are reasonable, because both $CuSO_4$ and H_2O_2 are oxidative stressors, PCP is a respiratory uncoupler, and βNF is an inducer of *CYP* gene expression. These results indicate that the gene expression profiles induced by exposure to a specific type of chemical can be distinguished.

Among the various genes for which expression changed, it also was possible to identify those that displayed chemical-specific responses. For example, the genes encoding cathepsin-L–like protease and glutathione-*S*-transferase had distinct responses to βNF and H_2O_2 (Fig. 5). Changes in the expression of these genes were confirmed by quantitative PCR, as were changes in several other genes in response to specific chemicals (Fig. 6). Evaluation of the characteristic gene expression profiles for a specific chemical could potentially provide valuable information about the mechanisms of toxicity in *D. magna*.

DISCUSSION

DNA microarray platform

We evaluated several methods of constructing DNA microarrays to create a highly reproducible platform. Conventional DNA microarrays often use a cDNA library printed onto a glass slide as the source of DNA, but the use of oligonucleotide DNA microarrays has several advantages [19]. For example, because oligonucleotide synthesis is an established technology, larger amounts of high-quality oligonucleotides can be reproducibly generated for printing onto glass slides. In contrast, when cDNAs are used for a DNA microarray, reproduction and quality control of cDNA synthesis become time-consuming steps in the process [20]. The use of cDNA microarrays also presents a higher risk of cross-hybridization, because the DNA sequence is longer, thus increasing the probability of detecting the expression of similar genes. Furthermore, selecting shorter sequences (50–80 bp), such as oligonucleotides, that represent specific genes is easier than selecting longer sequences (>500 bp) for cDNAs. With the caveat that sequence information should be obtained before the fabrication of a DNA microarray, the DNA microarray that we developed in the present study represents an ideal platform for ecotoxicogenomic assessments.

Generally, to evaluate changes in gene expression using glass DNA microarray platforms, two samples are labeled with different fluorescent substances and hybridized, and a ratio of fluorescent intensity for each gene is then calculated. This two-color method was developed to overcome the poor reproducibility of microarray platforms, because deviation in spotting quality could be minimized by calculating the ratio of fluorescent intensity of the two fluorophores. However, this strategy necessitates the use of control RNA in each experiment. In addition, to calculate ratios optimally, all genes on the array should be expressed in the control sample. In contrast to conventional glass arrays, the DNA microarray developed for the present study was fabricated in a highly reproducible manner, suggesting that the traditional two-color evaluation of DNA microarrays is not necessarily required. Our results also indicate that it is possible to obtain gene expression profiles using one-color evaluation, based on direct measurement of fluorescent intensities. The advantages of single-channel measurement also have been reported by other groups [16]. From a toxicogenomic point of view, one-color evaluation of DNA microarray data is an ideal method, potentially allowing one to easily obtain multiple data points, such as different chemicals, doses, and times of exposure. Two-color DNA microarray analysis would require the same number of control samples as data points, meaning that the scale of the experiment would double. In contrast, using a one-color DNA microarray approach, a single control sample is required, and variations among control samples can readily be estimated.

Feasibility

For evaluating our DNA microarray, we examined performance from both a technical and a biological point of view. From a technical point of view, the data generated by a DNA microarray platform should be highly reproducible. Two major types of DNA microarray platforms currently exist: One uses cDNAs, and the other uses synthesized oligonucleotides as the source of DNA. The cDNA microarray has been conventionally used, but with the aligonucleotide DNA microarray, it is easier to control the quality of the DNA microarray. To fabricate a DNA microarray that was highly reproducible, we used

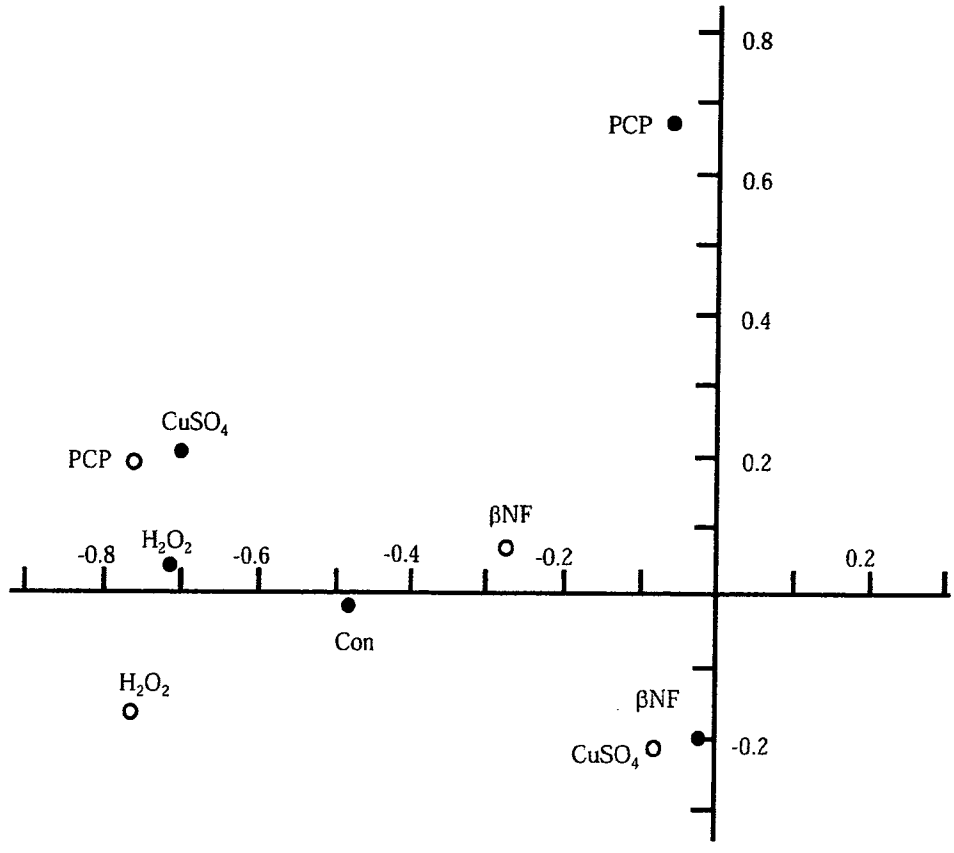

Fig. 4. The results of principal component analysis of the indicated chemicals presented as a two-dimensional scatterplot. The first component and the second component are indicated by the *x* and *y* axis, respectively. Solid circles indicate the gene expression profile at the higher dose of chemical, and open circles indicate the gene expression profile at the lower dose of chemical (see Table 2). PCP = pentachlorophenol; βNF = β-naphthoflavone.

oligo-DNA, because oligonucleotide-based DNA microarrays give more reliable results than cDNA-based microarrays [21].

From a biological point of view, our criterion was that the DNA microarray should show reproducible gene expression patterns in daphnids exposed to different types and doses of chemicals. For this analysis, 10 neonates were sufficient to obtain reproducible gene expression profiles. Because the number of daphnids available for DNA microarray analysis is very limited, this system could be readily implemented in laboratories that study the biology or toxicology of daphnids.

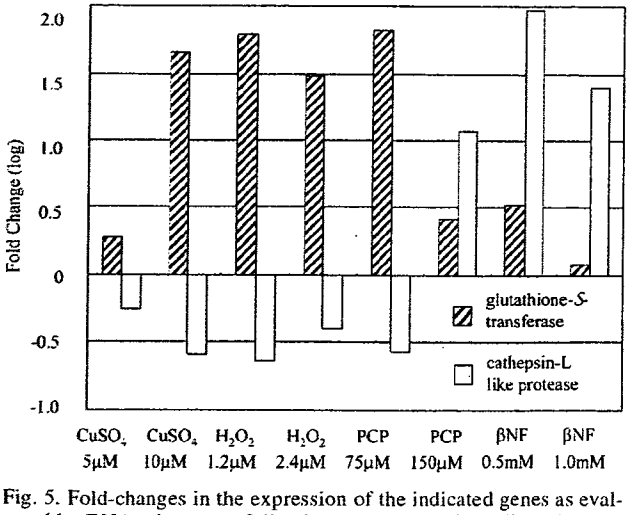

Fig. 5. Fold-changes in the expression of the indicated genes as evaluated by DNA microarray following exposure to the indicated chemicals. Values were converted to their base-2 logarithm and plotted. $CuSO_4$ = copper sulfate; H_2O_2 = hydrogen peroxide; PCP = pentachlorophenol; βNF = β-naphthoflavone.

Using cRNA prepared from adult daphnids as a standard in the present study had two advantages. First, our DNA microarray was based on an expressed sequence tag database populated by information obtained mainly from adult daphnids [12]. Thus, using cRNA, we could reasonably expect all spots to have a certain signal intensity after hybridization. Second, obtaining large amounts of RNA from adult daphnids is much easier than obtaining such amounts from neonates.

Although the number of genes spotted on the array was limited, all but five of these genes were randomly selected. An essential aspect of the use of global normalization is the assumption of random sampling of genes [22]; thus, this normalization method was applicable in our system.

Changes of gene expression

Daphnids were exposed to several chemicals for which the effects have been studied previously in model vertebrate and other organisms. As indicated in Figure 5, a high dose of $CuSO_4$ induced a similar gene expression profile as a high dose of H_2O_2. Both $CuSO_4$ and H_2O_2 are known to induce oxidative stress [23–25], so this result is consistent with previous observations in vertebrate models. This result indicates that certain chemicals have similar effects among vertebrates

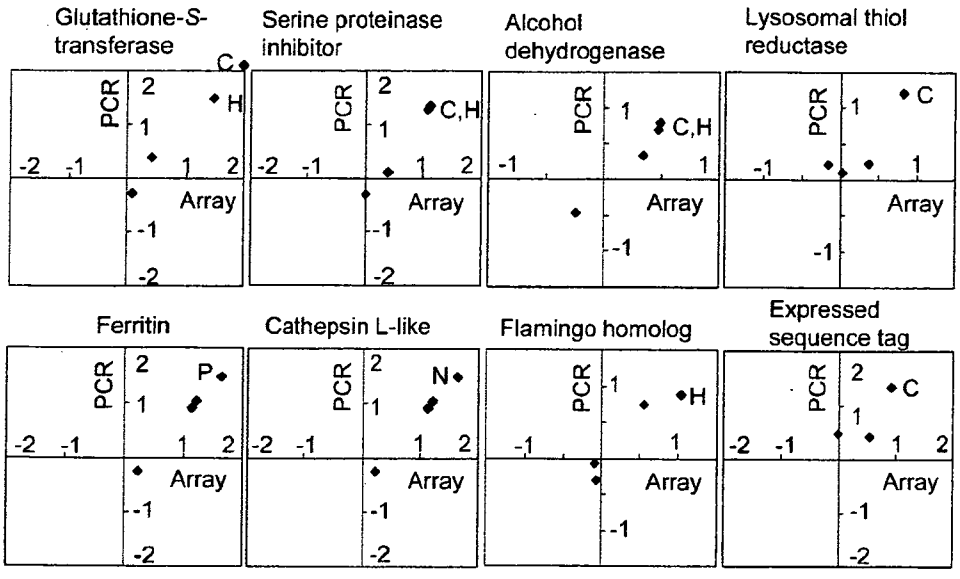

Fig. 6. Fold-changes in the expression of the indicated genes as estimated either by DNA microarray analysis (*x*-axis) or by quantitative polymerase chain reaction (PCR; *y* axis). Chemical exposures that gave maximal change are indicated by abbreviations. C = copper sulfate; H= hydrogen peroxide; N = β-naphthoflavone; P = pentachlorophenol.

and invertebrates. By increasing the number of genes on the *Daphnia* DNA microarray, it may be possible to compare toxic effects between vertebrate model organisms and daphnids in more detail, which may help to facilitate an evaluation of the effects of different chemicals on various species. In addition, taking advantage of common features between traditional vertebrate models and daphnids may help in the development of alternative toxicological tests to animal model testing.

In mammalian cells, the copper/zinc superoxide dismutase gene is activated by H_2O_2 [26] and heavy metals [27]. Copper/zinc superoxide dismutase (SOD1) catalyzes the dismutation of superoxide radicals that are produced during biological oxidations and environmental stress. It is reasonable to expect that *SOD1* would be activated in daphnids, but *SOD1* expression was repressed in the present study. This may be due to the temporal properties of *SOD1* gene expression. In mammals, the induction of *SOD1* gene expression was observed 1 to 2 h after oxidative stress. In contrast, we analyzed gene expression 48 h after chemical exposure. Species differences also may contribute to differences in gene expression between mammals and daphnids.

For $CuSO_4$ or PCP exposure, the gene expression profile at the lower dose was different than that at the higher dose. Although we could not clarify the reason for this variation, a steep dose response to $CuSO_4$ has been reported in *D. magna* [25]. The fact that changes in gene expression caused by a low concentration of $CuSO_4$ were small (faint color changes in Fig. 3) may be a reflection of the characteristic steep dose response to $CuSO_4$. Detailed DNA microarray analysis at different doses and time points may help in our understanding of the relationship between gene expression and toxicity.

Although our results show common aspects in the response to chemical exposure between vertebrates and invertebrates, the DNA microarray also may be useful for detecting effects that are specific to daphnids. For example, we and others have shown previously that some chemicals that mimic juvenile hormone activities can induce the production of more male phenotypes [13,28–30]. Effects of such chemicals cannot simply be interpreted as the activation of genes related to detoxification. By applying DNA microarray analysis to these and other types of chemicals, a mechanistic understanding of how these chemicals mediate their effects may be possible.

It also is notable that DNA microarray analysis took less time compared to reproductive toxicological tests. Although we did not directly compare reproductive toxicity and changes in gene expression profiles in the present study, we expect that the DNA microarray also would detect reproductive toxicity. The general protocol for reproductive toxicity takes 21 d for one set of tests [2], and one obtains only the effective dose of the chemicals of interest. The DNA microarray analysis takes only a few days, and characteristic profiles of chemical effects could be obtained, which in turn may provide additional information regarding the mechanisms of reproductive toxicity.

We have demonstrated the feasibility of a molecular genomic approach to studying toxicity in daphnids using DNA microarrays to analyze changes in gene expression profiles of neonates in response to chemical exposure. Although acute toxicity or reproductive toxicity tests can provide information concerning the hazardous concentrations of chemicals, they provide no information about their mode of action. Our study represents a breakthrough for the evaluation of chemical toxicity on environmental organisms, and our results suggest that chemical effects can be classified according to changes in gene expression profiles. Classification of chemicals according to gene expression profiles or pathways may contribute to estimation of the relative risks of various chemicals. By increasing the number of genes on a DNA microarray, detailed gene expression profiles in response to chemicals can be obtained and new biomarkers and/or new pathways characteristic of environmental chemicals identified.

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