

## Development of an *in vitro* Reporter Gene Assay for Screening the Ecdysone Receptor Agonists in Mysid Crustacean

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**Abstract**—One of steroid hormones, ecdysteroid is a key regulatory factor, controlling the development and molting in arthropod. Molecular target of ecdysteroids is a receptor complex, composed of the ecdysone receptor (EcR) and ultraspiracle (USP). To characterize the transactivation potentials of crustacean EcR by environmental pollutants, we established an *in vitro* reporter gene assay using insect cell line transiently transfected with mysid EcR and USP cDNA expression vectors and a reporter vector containing the synthesized ecdysone response element (EcRE). Comparison of amino acid residues in the ligand-binding domain of mysid EcR with those of other arthropod EcRs showed less similarities (36–45%), implying a functional divergence of the EcR across species. The *in vitro* reporter gene assay showed that treatments of insect cell line with an arthropod molting hormone, 20-hydroxyecdysone, and a plant ecdysteroid, ponasterone A, induced EcR-mediated transcriptional activities in a dose-dependent manner. These results suggest that this *in vitro* reporter gene assay with the mysid EcR/USP complex can be a useful tool to screen candidate compounds that may potentially affect the crustacean EcR signaling cascade.

**Keywords:** ecdysone receptor, reporter gene assay, ecdysteroid, crustacea

### INTRODUCTION

Ecdysteroids, primarily 20-hydroxyecdysone (20E), play crucial roles in initiating and regulating molting and metamorphosis in arthropods. Besides the function as a molting hormone, ecdysteroids are also involved in the control of reproduction and embryogenesis (Subramoniam, 2000). The ecdysone receptor (EcR) is a molecular target of the ecdysteroids. This receptor is a ligand-dependent transcription factor and forms a heterodimer complex with ultraspiracle protein (USP), which is a homologue of vertebrate retinoid X receptor (RXR) (Yao *et al.*,

1992). The EcR/USP complex binds to the ecdysone response element (EcRE) with specific DNA sequences, and consequently regulates the expression of ecdysteroid responsive genes, such as *Broad-Complex*, *E74* and *E75* (reviewed by King-Jones and Thummel, 2005).

In insects, the function of EcR has been well characterized, especially in fruitfly *Drosophila melanogaster* and silkworm *Bombyx mori* (reviewed by Thummel, 1995; Kamimura *et al.*, 1996, 1997). In contrast, the function of crustacean EcR is not fully understood. To date, crustacean EcRs have been sequenced in only limited species, including the decapods, *Celuca pugilator* (Durica and Hopkins, 1996; Chung *et al.*, 1998), *Gecarcinus lateralis* (Kim *et al.*, 2005), *Marsupenaeus japonicus* (Asazuma *et al.*, 2007), water flea *Daphnia magna* (Wang *et al.*, 2007; Kato *et al.*, 2007) and the mysid crustacean *Americamysis bahia* (Yokota *et al.*, 2005). These studies have shown that the EcR/RXR complex functions as a mediator of ecdysteroid signals in crustaceans as well as in insects. In addition, several earlier studies have investigated the adverse effects of environmental chemicals on crustacean molting (DeFur *et al.*, 1999; Zou, 2005). This indicates that the adverse effects may be induced through the disruption of EcR signaling by environmental chemicals.

Among aquatic invertebrate species, mysids have been put forward as suitable test organisms for assessing endocrine disruptors by several agencies, including the US Environmental Protection Agency (USEPA), the American Society for Testing of Materials (ASTM), and Organization for Economic Cooperation and Development (OECD) (reviewed by Verslycke *et al.*, 2004, 2007). However, few studies are available on the development of chemical screening methods based on specific hormone-regulated mechanisms in mysids. As the first-tier screening of chemicals, the reporter gene assay that can evaluate the transactivation potency of target genes by chemically activated nuclear receptors has been proposed. Hence, we attempted to establish an *in vitro* reporter gene assay system using cDNA clones EcR and USP from *A. bahia* (AbEcR and AbUSP).

## MATERIALS AND METHODS

### *Test chemicals*

20E was purchased from Sigma, St. Louis, MO. Ponasterone A (PonA) was obtained from Alexis Biochemicals, San Diego, CA. Test solutions were prepared by dissolving the reagents in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO).

### *Isolation of full-length EcR and USP cDNAs*

Total RNA was extracted from the frozen whole body of adult female mysids by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method using TRIzol<sup>®</sup> reagent (Invitrogen Co., Ltd., Japan) according to the manufacturer's protocol. cDNA was synthesized from total RNA by reverse transcription, using a Marathon cDNA Amplification kit (Clontech). For amplification of full length of AbEcR and AbUSP cDNAs, the forward and

reverse primers were designed based on the previously described from *A. bahia* (Yokota *et al.*, 2005). Full-length DNA fragments were amplified by PCR and cloned into the plasmid pGEM T-Easy vector (Promega). The amplified cDNAs were sequenced using ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

### *Plasmid constructs*

The entire AbEcR and AbUSP cDNA were obtained by digesting pGEM-AbEcR, pGEM-AbUSP with EcoRI, respectively. These AbEcR and AbUSP fragments were then inserted into the EcoRI site of pAc5/V5/HisA (Invitrogen) to yield the expression constructs pAc5-AbEcR and pAc5-AbUSP. Insertion of fragments in constructs was confirmed with restriction enzyme digestion and partial sequencing.

The pGL4-EcRE-TATA-Luc reporter plasmid was constructed by insertion of a complementary oligonucleotide containing four copies of the inverted repeat (IR) site sequence of EcRE located in the upstream region of *Drosophila hsp27* gene into KpnI/XhoI sites of the pGL4.10 vector (Promega). The sequences of these fragments in constructed plasmids were confirmed with ABI PRISM 310 Genetic Analyzer.

### *Cell culture*

*Drosophila* cell line S2 was provided by RIKEN Cell Bank (Tsukuba, Japan). S2 cells were maintained at 28°C in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (100 µg/ml). S2 cells in monolayer culture were pelleted, and resuspended in the above culture medium, approximately twice a week.

### *Transient transfection and luciferase reporter assays*

For transfection,  $3 \times 10^6$  cells/well of S2 cells were seeded into 24-well plates and allowed to grow overnight. After 24 hrs of growth, the complete medium was removed, and cells were washed with Express Five Serum-free medium (Gibco BRL) containing 9 mM L-glutamine. For luciferase assays, 100 ng of pAc5-AbEcR and pAc5-AbUSP, 50 ng of pGL4-reporter constructs, 3 ng of pGL4.74[hRLuc/TK] *Renilla* luciferase control plasmid (Promega) (as an internal control) and 147 ng of empty expression vector pAc5/V5/HisA were transfected using Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions in Express Five Serum-free medium. The transfection was carried out at least in triplicate.

At 15 hrs after transfection, cells were washed with Express Five Serum-free medium and were re-fed with fresh medium containing either vehicle (DMSO) only or  $1 \times 10^{-5}$ ~ $1 \times 10^{-9}$  M 20E,  $1 \times 10^{-5}$ ~ $1 \times 10^{-9}$  M PonA at 28°C for 24 hrs. Luciferase activity was then measured using a Dual-Luciferase Reporter Assay System (Promega) with GloMax 96 Microplate luminometer (Promega). The final luminescence values were expressed as a ratio of the firefly luciferase units to the *Renilla* luciferase unit.

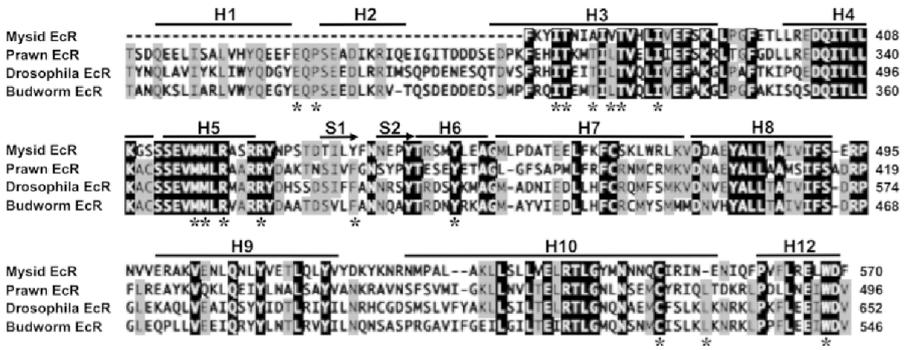


Fig. 1. Amino acid alignment of EcR ligand binding domains from mysid and other species. The major pocket residues conserved for the interaction with PonA in decapod crustaceans and insects are marked (\*). Lines and arrows shown above the sequences denotes  $\alpha$ -helix (H) and  $\beta$ -sheet (S), respectively. Numbers in the right side of the figure represent the amino acid number from the N-terminus of EcR.

Statistical analysis

The experimental data from reporter gene assays were checked for the homogeneity of variance across treatments using a Bartlett test. The data were analyzed by one-way analysis of variance followed by Dunnett’s multiple comparison tests. Statistical difference was considered to be significant at  $p < 0.05$ . All statistical analyses were performed using StatView J 5.0 (SAS Institute Inc., Cary, North Carolina, USA). The 50% effective concentration ( $EC_{50}$ ) of each chemical examined was calculated with GraphPad Prism (version 5.0b, GraphPad Prism Software).

RESULTS AND DISCUSSION

In the present study, full-length cDNA fragments including open reading frames of EcR and USP were amplified by PCR from one of the mysids, *A. bahia*. The isolated AbEcR had an open reading frame (ORF) of 1713 bp, which encoded 570 amino acid residues with a deduced molecular weight of 64.4 kDa. The AbEcR amino acid sequence consists of the five-domain structure that is conserved in all members of the nuclear hormone receptor family. The nucleotide sequence of AbUSP ORF was 1233 bp, which encoded a protein of 410 amino acids with a deduced molecular weight of 46.1 kDa. The AbUSP also had the conserved five-domain structure. Identities of the amino acid sequences of AbEcR with those from other arthropod species were 72% (83% positive) with *Neomysis integer* (ACJ68423), 52% (68% positive) with *C. pugilator* (AAC33432), 49% (65% positive) with *Apis mellifera* (BAF46356) and 47% (63% positive) with *D. magna* (BAF49030). The crystal structure of the ligand binding domain (LBD) of tobacco budworm EcR-USP complex bound to PonA has been provided (Billas *et al.*, 2003). Asazuma *et al.* (2007) indicated that 17 of 24 amino acid residues

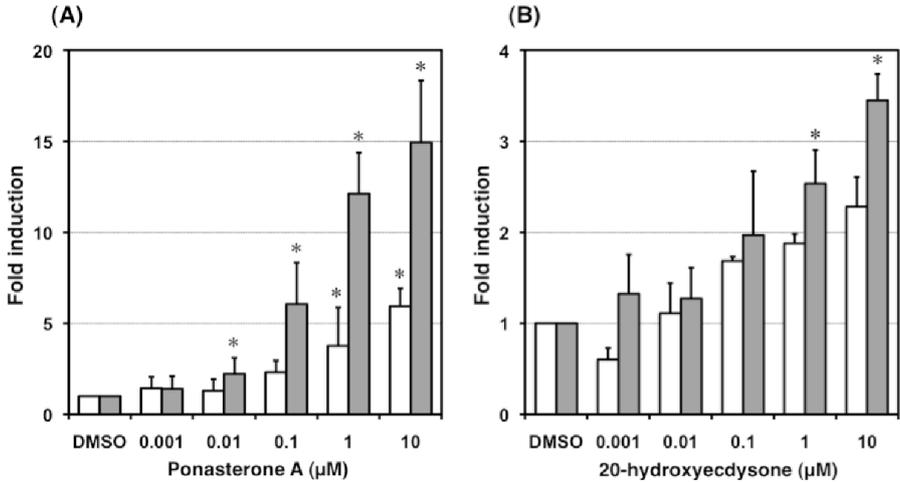


Fig. 2. Transactivation activity of the mysid EcR/USP by PonA (A) and 20E (B). Insect cells were transfected with the AbEcR and AbUSP expression plasmids, pGL4-EcRE-TATA-Luc reporter plasmid, pRL-TK plasmid containing *Renilla* luciferase and empty pAc5 vector, and then treated with a serially diluted PonA or 20E standard solution. White and gray bars mean the cases that non-AbEcR and AbEcR expression vector was transfected in insect cells, respectively. Bars are expressed as the fold induction of transcriptional activity by the treatment of PonA or 20E relative to that by DMSO. Asterisk denotes the detection of a statistical difference from DMSO control cells at the level of  $p < 0.05$ .

of EcR-ligand binding domain involved in the interaction with EcR and PonA were conserved between decapod crustaceans and budworms. To investigate whether these residues are related to ligand binding they were conserved in the AbEcR. We compared the amino acid sequence of ligand binding domain with those of decapod and insect EcRs (Fig. 1). The helical regions of ligand binding domain were well conserved except for the helix H1 and H2. The  $\beta$ -sheet region, S2 also had high similarities. Eleven amino acid residues shared in the major ligand binding pocket interacting with PonA in both budworm and decapod EcRs were conserved in the AbEcR. High conservation of the amino acid residues involved in ligand binding suggests that the AbEcR may have an ability to bind to ecdysteroids. However, the whole sequence of AbEcR ligand binding domain was less similar to those of decapod and insect EcRs sequences (36–45% identities), implying the divergence of EcR ligand profiles across species.

The structure/activity relationship of EcR agonists has not yet been fully characterized. For a better understanding of the ligand selectivity of the EcR/USP heterodimer, the ability to induce EcR-mediated transcription in a luciferase reporter gene assay was examined for arthropod and plant ecdysteroids. This type of reporter gene assay has been widely used to screen ligands of the vertebrate nuclear receptors. In the present study, a reporter vector (pGL4-EcRE-TATA-Luc) was co-transfected with AbEcR and AbUSP expression plasmids (pAc5-

AbEcR and pAc5-AbUSP) into *Drosophila* S2 cells. The reporter plasmid was designed to contain four tandem copies of the *hsp27* EcRE, which functions as a strong binding site for the EcR/USP heterodimer (Yao *et al.*, 1993). To investigate the transcriptional activity of the EcR/USP heterodimer, our reporter gene assay system was treated with PonA or 20E. Dose- and EcR-dependent responses were observed for both chemicals. The EC<sub>50</sub> values of PonA and 20E were 0.2 and 0.39  $\mu\text{M}$ , respectively (Fig. 2). Previous studies showed that the activation of insect, *Drosophila* EcR was expressed in several cell lines, by both of 20E and PonA, and the LOEC or EC<sub>50</sub> values was estimated for each chemical. For example, Baker *et al.* (2000) showed that the EC<sub>50</sub> value of 20E was 0.18  $\mu\text{M}$  when tested in the *Drosophila* EcR luciferase assay. They also demonstrated that PonA was the most potent compound for ecdysteroid activity; the EC<sub>50</sub> value was 0.07  $\mu\text{M}$ . Since the experimental conditions such as cell type, EcRE sequence, incubation time, and positive control widely varied, direct comparison of the data among these studies may be difficult. However, our data is consistent with the previous data on insect EcR showing that PonA has a high potency for activating EcR compared to 20E. These results suggest that this *in vitro* reporter gene assay with the mysid EcR/USP complex may be a useful tool to screen candidate compounds that affect the mysid EcR signaling cascade.

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