General and Comparative Endocrinology 193 (2013) 86–94

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Ecdysone receptor gene from the freshwater prawn *Macrobrachium nipponense*: Identification of different splice variants and sexually dimorphic expression, fluctuation of expression in the molt cycle and effect of eyestalk ablation

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ARTICLE INFO

Article history:
Received 29 March 2013
Revised 2 July 2013
Accepted 8 July 2013
Available online 27 July 2013

Keywords:
Macrobrachium nipponense
Ecdysone receptor
Splice variant
Molting

ABSTRACT

The full-length cDNA of an ecdysone receptor gene (*MnEcR*) from *Macrobrachium nipponense* was cloned and the expression of the gene was investigated. *MnEcR* maintained a relatively low expression level in the early stages of embryos, but from nauplius stage, a steady increase in *MnEcR* expression was detected, it had the highest expression level in zoea stage. *MnEcR* was highly expressed in the hepatopancreas and gills among ten different tissues examined. *MnEcR* was rapidly upregulated in the premolt stage and rapidly downregulated in the postmolt stage. The expression of *MnEcR* was remarkably downregulated after eyestalk ablation in *M. nipponense*. An 18-amino-acid insertion/deletion and a 49-amino-acid substitution were found in the coding region of *MnEcR*, resulting in four splice variants: *MnEcR-L1, -L2, -S1* and-*S2*. The expression of four splice variants of *MnEcR* in gonads was investigated using RT-PCR. Interestingly, the expression patterns of these splice variants differed between males and females. The dominant splice variants in testis were *MnEcR-S1* and *-S2*, while in ovary they were *MnEcR-L1* and *-S2*, indicating specific roles for these splice variants in male and female individuals.

1. Introduction

In crustaceans, molting is the physiological process of removing the old exoskeleton and building a new exoskeleton, and is indispensable for development, growth and metamorphosis (Chang and Mykles, 2011). Ecdysteroids, a steroid hormone, controls molting. The Y-organ is the molting gland where ecdysteroids is produced, while molting is suppressed by molt-inhibiting hormone (MIH) (Covi et al., 2009). MIH, a member of a novel neuropeptide family, is synthesized in the X-organ/sinus gland complex in the eyestalk. Besides MIH, the X-organ/sinus gland complex also secretes several other neuropeptides, including crustacean hyperglycemic hormone (CHH), gonad/vitellogenesis-inhibiting hormone (GIH/VIH) and mandibular organ-inhibiting hormone (MOIH) (Webster et al., 2012). These hormones are involved in many biological processes, such as reproduction, metabolism and osmoregulation. For this reason, monolateral or bilateral eyestalk ablation is performed to accelerate molting and gonad maturation (Kim et al., 2005a; McDonald et al., 2011; Sroyraya et al., 2010; Uawisetwathana et al., 2011).

Ecdysone receptor (EcR), a member of the nuclear receptor family, forms heterodimers with the retinoid-X receptor (RXR) or ultraspiracle protein (USP), the ortholog of RXR in insects, and is activated by Ecdysteroids (Nakagawa and Henrich, 2009). As a transcription factor, the EcR-RXR heterodimer regulates the transcription of molting-responsive genes, such as E75 and chitinase (Kim et al., 2005b; Shechter et al., 2007). EcR has all the conserved nuclear receptor structures, including the A/B, C, D, E and F domains (Renaud and Moras, 2000). The A/B domain is highly variable and contains a transcription activation domain, which is independent of ligand binding. The C domain is the DNA-binding domain, which can bind ecdysone responsive elements (EcREs) in the promoters of molting-responsive genes (Perera et al., 2005). The D domain is the hinge domain and is less conserved. The E domain is the ligand-binding domain, and is more complex than the other domains. It contains a hydrophobic ligand-binding pocket, and mediates heterodimerization and ligand-dependent transcriptional activation function (AF-2). The C-terminal F domain is highly variable (Thomson et al., 2009). .

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http://dx.doi.org/10.1016/j.ygcen.2013.07.014

EcRs have been reported from several crustaceans, including the fiddler crab *Uca pugilator*, the land crab *Gecarcinus lateralis*, the kuruma prawn *Marsupenaeus japonicus*, the water flea *Daphnia magna*, the brown shrimp *Crangon crangon*, the mysid shrimp *Americamysis bahia* and the American lobster *Homarus americanus*. (Asazuma et al., 2007; Chung et al., 1998a; Kim et al., 2005b; Tarrant et al., 2011; Verhaegen et al., 2011; Wang et al., 2007; Yokota et al., 2011). The characterized crustacean EcR and RXR genes typically have many splice variants (Asazuma et al., 2007; Chung et al., 1998a; Tarrant et al., 2011). Most of these variant sequences are in the A/B domain, the hinge domain and the ligand-binding domain, potentially affecting transcription activation, dimerization and ligand binding. For example, four isoforms of RXR of the fiddler crab *U. pugilator* have different expression profiles and distinct DNA binding and ligand binding activities (Wu et al., 2004).

Macrobrachium nipponense is an economically important freshwater prawn in China. Due to its flavor and high nutritive value, it is widely farmed in China. A well-known growth trait of *Macrobrachium* species is that the males grow faster than females, so males are larger than females given the same culture period. The molt cycle in males is shorter than in females in *Macrobrachium* species (Ma et al., 2012; Ventura et al., 2009), but how molt signals differ between males and females remains unknown.

A partial cDNA sequence of EcR from *M. nipponese* was reported (Chen et al., 2009). This paper describes the first full-length cDNA of *MnEcR* and the expression of *MnEcR* in different tissues at different stages of the molt cycle and in response to eyestalk ablation. Four splice variants of *MnEcR* were identified and their expression in males and females was investigated.

2. Material and methods

2.1. Animal culture, observation of molt stages and eyestalk ablation

M. nipponense individuals that were 4–5 cm long were collected from Tai Lake, Jiangsu, China, transported alive, grown in tanks with adequate aeration at 20 °C in a 12 h light and 12 h dark photoperiod and fed twice a day. To collect prawns at different molt stages, the animals were grown individually in glass trays ($30 \times 20 \times 20$ cm) in 120 L tanks (10 trays/tank). The developmental stages of embryos were determined based on the criteria previously described (Chen et al., 2012b). The molt stages of the prawns were identified based on the criteria described by Chan et al. (1988) and Cesara et al. (2006). A piece of the endopodite of the uropods was removed from each prawn and observed under the microscope. Based on the degree of retraction of the epidermal tissue from the cuticle and the morphological changes in setogenesis during the molt cycle, individual prawns were divided into three molt stages: intermolt (C), premolt (D0, D1, D2 and D3) and postmolt (A and B) stages. For the eyestalk ablation experiment, the eyestalks of individual prawns were clipped bilaterally using sterile scissors. Samples from different tissues were collected from prawns at 0, 1, 3 and 7 days after eyestalk ablation. All collected samples were rapidly frozen in liquid nitrogen and then stored at −80 °C.

2.2. Preparation of total RNA and cDNA synthesis

Prawn total RNA was extracted using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's protocol. RNA integrity was verified by electrophoresis on an agarose gel (1.5%). The concentrations were calculated and purity of RNA was determined by use of a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). For all RNA samples, A260/A280 and A260/A230 ratios were in the range 2.0–2.1 and 1.9–2.0, respectively.

To synthesize first-strand cDNA, 2 μg total RNA was first treated with RQ1 RNase-free DNase (Promega, USA) to avoid genomic DNA contamination, and then reverse transcribed by using M-MLV reverse transcriptase (Promega, USA) according to the manufacturer's instructions. The synthesized cDNA was used as template in subsequent PCRs.

2.3. Cloning and sequencing of full-length MnEcR cDNA

A partial cDNA sequence of EcR from *M. nipponese* has been reported (Chen et al., 2009). Another*MnEcR* partial sequence was identified from Roche 454 sequence data (NCBI Sequence Read Archive accession number SRA051767.2) in the GenBank database (http://www.ncbi.nlm.nih.gov). Gene-specific 5′ and 3′ primers were designed for RACE (5′ rapid amplification of cDNA ends) PCR based on these two *MnEcR* partial sequences. RACE cDNA was prepared from total RNA of the testis and ovary of *M. nipponense*, according to the Takara 5′-Full RACE kit protocol. The universal 5′ RACE outer primer and the gene-specific reverse primer MnEcR-5a-Outer were used to perform the first-round PCR (Table 1). The following PCR program was used: denaturation at 94 °C for 3 min, 20 amplification cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 3 min, and a final extension at 72 °C for 10 min. The universal 5′ RACE inner primer and the gene-specific reverse primer MnEcR-5a-Inner were used in the second-round PCR (Table 1). The PCR program in the second round was nearly the same as that in the first round, with the only difference being that the 20-cycle amplification was replaced by a 25-cycle amplification.

The 3′ RACE cDNA was prepared from the total RNAs of the testis and ovary according to the protocol from the Takara 3′-Full RACE Core Set Version 2.0 kit (Takara, Japan). The 3′ RACE PCR was then completed by two rounds of PCR; the universal 3′ RACE outer primer and the gene-specific forward primer MnEcR-3a-Outer were used in the first-round of PCR (Table 1). The PCR program was nearly the same as the 5′ RACE first-round PCR, with the only difference being that the elongation time at 72 °C of 3 min was replaced by 2 min. The universal 3′ RACE inner primer and the gene-specific forward primer MnEcR-3a-Inner were used in the second round of PCR (Table 1). The PCR program in the second round was nearly the same as that in the first round, with the only difference being that the 20-cycle amplification was replaced by one of 30 cycles.

Table 1
Primers for *MnEcR* RACE PCR.

Primer	Sequence (5′ to 3′)
MnEcR-5a-Outer	ACCAGCAGACTCGTAAGAATCCA
MnEcR-5a-Inner	CACTCGACGGTCCTTTTGGCTTAC
MnEcR-3a-Outer	CAGTTCTGCCGGAATTTGTG
MnEcR-3a-Inner	AAAGAGCCGCATAAAGTGGA
Rt-MnEcR-F	GATGCGAGCACAGACTCCAT
Rt-MnEcR-R	CGGCAGAACTGGAAAATGGC
Rt-MnRXR-F	TGGGCATGAAGAGAGAAGCG
Rt-MnRXR-R	CAGGTTGCTCATCTGTAGGGT
Sv-MnEcR-I-F	GTAAGCCAAAAGGACCGTCG
Sv-MnEcR-I-R	CATGTCTGCTTCAGAGGGCT
MnEcR-ORF-F	ATGGAGTTCCCTCGCGACTC
MnEcR-ORF-R	TCAGTATTGGTCGCAACTGTG
Sv-MnEcR-V1-F	CGTCGAGTGCTTCAGTTCCT
Sv-MnEcR-V1-R	AGTCACTTGTATCTTCACCATCG
Sv-MnEcR-V2-F	CCTGTAAGCCAAAAGGACCG
Sv-MnEcR-V2-R	AGTCGTCTTCGTTTAAATCATTGC
MnACTIN-F	CTCCCTGTACGCCTCCGGTC
MnACTIN-R	CTCGCTCGGCGGTGGTAGTG