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Developmental Profiles of the Functional Ecdysone Receptor Transcripts Using RT-PCR

RT-PCR를 이용한 기능적인 Ecdysone Receptor 전사체의 발생단계별 발현 양상

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ABSTRACT

In *Drosophila*, the steroid hormone ecdysone triggers the key regulatory cascades controlling the coordinated changes in the developmental pathway of molting and metamorphosis. Ecdysone action is mediated by heterodimer consisting of the ecdysone receptor(EcR) and *ultraspiracle* proteins (USP). Heterodimers of these proteins bind to ecdysone response element and ecdysone to modulate gene transcription. It is known recently that the functional ecdysone receptors are complexes produced by the heterodimerization between USP and three EcR isoforms. In this study, the transcripts of functional ecdysone receptors during development were analyzed by using a RT-PCR(Reverse Transcription Polymerase Chain Reaction) assay. The transcripts of *usp* and *EcR* isoforms were detected in all developmental stages. This study revealed that the expression patterns of functional ecdysone receptors were correlated with ecdysone titers showing during *Drosophila* life cycle.

Key words : *Drosophila*, ecdysone receptor, *ultraspiracle*, RT-PCR

INTRODUCTION

The nuclear hormone receptor superfamily contains a large number of evolutionarily related transcription factors that mediate the actions of

small molecules such as steriod hormones. Members of this superfamily function by binding to short DNA sequences within gene promoter called hormone response elements. In *Drosophila*, the steroid hormone ecdysone trigger the key regulatory cascades controlling the coordinated changes in the developmental pathway of both larval and imaginal tissues in molting and metamorphosis (Riddiford *et al.*, 1985). The major biological actions of these hormones are mediated by ligand-dependent transcription factors that comprise the steroid/thyroid hormone receptor superfamily. Members of the receptor family share a common modular structure that includes a highly conserved DNA-binding domain and a less conserved carboxyl-terminal regions that contains ligand binding and dimerization functions (Evans, 1988). These receptors achieve physiological function by binding to specific DNA sequences termed hormone response elements, thereby activating or suppressing target gene expression in a ligand-dependent manner(Beato, 1992).

The actions of the ecdysone are mediated by the ecdysone receptor (EcR), a member of the nuclear hormone receptor superfamily(Koelle *et al.*, 1991). *EcR* gene encodes three functional isoforms(EcR-A, EcR-B1, and EcR-B2) that have common DNA- and hormone-binding domains and are distinguished by different N-terminal regions. EcR-A and EcR-B are transcribed from two different promotor, while EcR-B1 and EcR-B2 are produced by alternative splicing (Talbot *et al.*, 1993). The ability of EcR to bind to hormone and to interact with ecdysone response element(EcRE) in the genome depends on the heterodimerization with *ultraspiracle* gene product (USP) (Thomas *et al.*, 1993 ; Yao *et al.*, 1992). USP is the homolog of the mammalian retinoid X receptor, sharing 86% amino acid identity in the DNA-binding domain and 49% in the ligand-binding domains(Oro *et al.*, 1990). Thus the functional ecdysone receptors are the heterodimers between USP and three EcR isoforms.

RT-PCR (Reverse Transcription Polymerase Chain Reaction) is a powerful technique for the analysis of RNA transcripts that are a crucial part of many molecular biology applications. In order to understand the ecdysone action during development, it is required the study about the developmental expression profiles of ecdysone receptor complex. In this study, we investigated the developmental profiles of functional ecdysone receptor transcripts using a RT-PCR assay.

MATERIALS AND METHODS

Drosophila culture and collection of

staging animals

Flies were raised at 25℃ on standard medium containing cornmeal, sugar, yeast, and agar. Several hundred *Drosophila melanogaster* adults (Canton-S strain) transferred to 100-mm petri plates containing an apple medium at 25℃. Embryos were collected at two hour intervals after egg laying. For larval stagings, early first instar larvae were collected as they hatched from agar plates. These larvae were transferred to standard cornmeal media, allowed to develop for the appropriate stage at 25℃. For pupal stagings, wandering third instar larvae were transferred into the vial bottle containing cornmeal media, allowed to develop for the appropriate stage at 25℃. Newly eclosed adult flies were collected and transferred to new vial bottles and incubated for 0-12hr. All animals were frozen in liguid nitrogen, and stored at -70 ℃ until the time of RNA extraction.

RNA extraction

Total RNA were extracted using Micro-Scale Total RNA Separator Kit (Clontech) according to manufacturer's protocol. To calculate the amounts of RNA, the absorbances at 260nm and 280nm were measured. The RNA samples, within the range of 1.7-2.0 value of A260/A280 ratio, were used in cDNA synthesis reaction.

RT-PCR

The primer sets used in RT-PCR were designed by referring to known sequences of each gene, and synthesized from DNA International In. (USA). Primers sequences are given in Table 1 and their position and the predicted size of PCR products derived from RNA are shown in Figure 1. The first strand of cDNA was synthesized using RT-PCR Kit(Clontech) according to manufacturer's protocol. One μg of RNA was used in cDNA synthesis. PCR was carried out using the cDNA template in a DNA Thermal Cycler according to the following protocol : 5μl

Table 1. Oligonucleotide primers used for the RT-PCR analysis

Gene	RTAse/PCR primer	PCR primer
rp49	GTGTATTCCGACCACGTTACA	TCCTACCAGCTTCAAGATCAC
EcR-B1	ATTCGAGAGATCATCGCGACC	ATGAAGCGGCGCTGGTCGAAC
EcR-A	ATTCGAGAGATCATCGCACC	ATGTTGACGACGAGTGGACAA
usp	CGCGCCTTTAGAGTCGGGACC	AAGGGTGCCGTCTCGGC

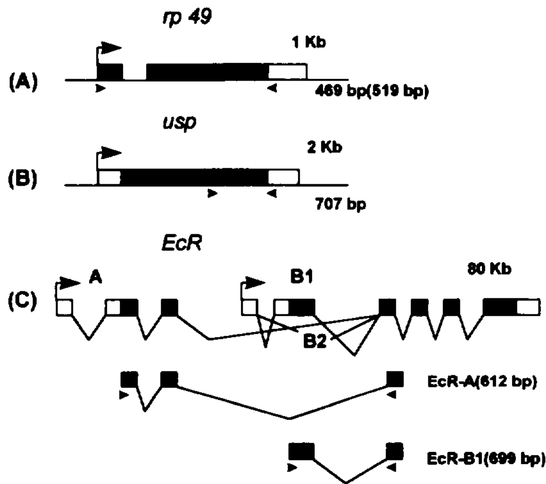

Fig. 1. Gene structure and RT-PCR stratagy. For each gene, transcribed regions are depicted as box segments positioned on the genomic DNA. Coding regions are shown as solid boxes, non-translated regions as open segments. The positions of PCR primers are shown by arrow heads together with the size of the corresponding PCR products. (A)Ribosomal proteins *rp49* (O'Connel and Rosbash, 1984), (B)*usp*(*ultraspiracle*: Henrich *et al.*, 1990), (C)*EcR*(the ecdysone receptor gene: Koelle *et al.*, 1991)

of 10× PCR buffer, 1$\mu\ell$ of dNTP mix (each 10mM), 1$\mu\ell$ of each primer set (15mM), 2 units of Tag DNA polymerase, and added the sterile deionized water to achieve a final volume of 50$\mu\ell$. The amplication was performed for 30 cycles with 45 seconds denaturation at 94℃ and 45 seconds annealing at 60℃, followed by 2 minutes at 72℃. The resulting PCR products were analyzed on 2% agarose gel.

Southern blotting

After electrophoretic separation, the gel was denatured (1.5 M NaCl, 0.5 N NaOH) and then neutralized (1.5 M NaCl, 1.0 M Tris, pH 7.4), and transferred to Hybond-N membrane by capillary transfer method. DNA was fixed to the membrane by baking for 2 hours at 80℃ in a vacuum oven. The tranferred membranes were hybridized under high stringency conditions (50% formamide, 5×SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 2% Blocking reagent solution) with DNA probes at 42℃. The DNA probes were labeled with digoxigenin 11-dUTP using the random primed method (Boehringer Mannheim). After hybridization, the membranes were washed at room temperature in 2 ×wash solution(2×SSC, 0.1% SDS) and 0.5×wash solution(0.5×SSC, 0.1% SDS). The hydridized DNA was detected with NBT and X-phosphate (Boehringer Mannheim).

RESULTS AND DISCUSSION

In *Drosophila* development, at least six pulses of ecdysone are thought to occur, one during each stage of development: embryonic, three larval

instar, prepupal and pupal(Richard, 1981). Ecdysone response is mediated by a hierarchy of transcriptional events. Molecular cloning brought about the isolation of *EcR* gene, encoding a member of the nuclear receptor family(Koelle *et al.*, 1991). However, EcR alone was not sufficient to consititute ecdysonesiveness. Ecdysone response is mediated by a funtional EcR consisting of binary complex of EcR and USP(Thomas *et al.*, 1993 ; Yao *et al.*, 1992). These heterodimer bind a EcRE to regulate target gene transcription by the existence of ecdsyone.

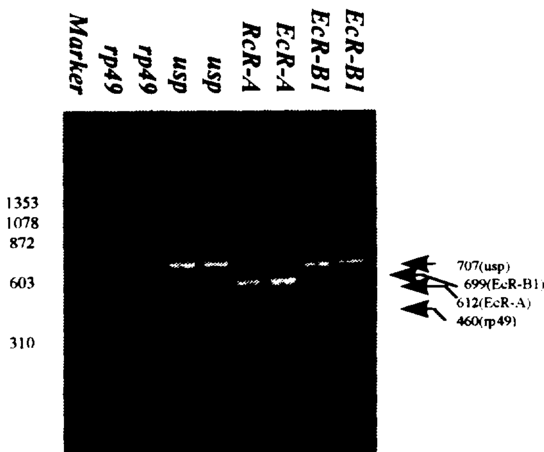

Fig. 2. Agarose gel electrophoresis of RT-PCR products. Total RNAs were extracted from whole animals of pupal stage. RT-PCR was performed described in Materials and Methods. The transcripts of each gene was marked on the right panel.

In order to analyze the expression patterns of ecdysone receptors during *Drosophila* life cycle, their transcripts were analyzed at all developmental stage using RT-PCR. Gene structure

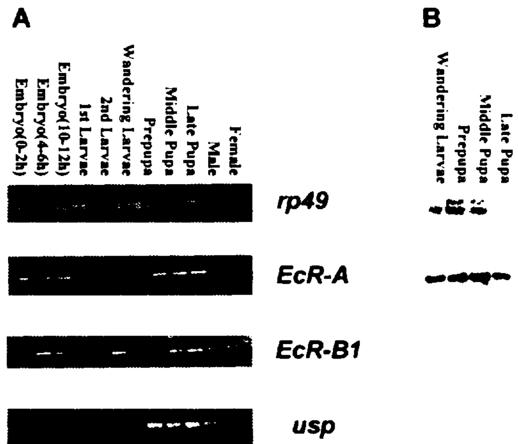

Fig. 3. (A)Analysis of transcripts of ecdysone receptor complex during development. RT-PCR products were separated on 2% agarose gels and photographed after ethidium bromide staining. The products for each gene are described in Fig. 1. (B)Southern blots of each gene using Dig-labeled cDNA probes.

and primer sets used in the RT-PCR are shown in Figure 1. RT-PCR products produced from each gene are consistent with predicted sizes on agarose gel electrophoresis (Figure 2). RT-PCR was performed with each primer in series of the development stages and the products were confirmed by southern blots (Figure 3A and B). The

ribosomal protein 49(*rp49*) served as a standard for RNA extraction and the subsequent RT-PCR assay. *usp* transcripts were detected with nearly equal amount in all developmental stages (embryogenesis, all larval stages, pupal stage, adult) investigated (Figure 3A). This result is consistent with previous report that USP play pleiotropic function during various developmental stages. Phenotypic analysis of *usp* mutant files has revealed that *usp* is required in development stages and multiple tissue during the *Drosophila* life cycle(Oro *et al.*, 1992). *usp* has been shown to be essential for embryogenesis, larval development, pupation, and other development events (Oro *et al.*, 1992). Also, in the profile of *usp* transcription and translation assay, *usp* expression is not confined to developmental periods and cell types associated specifically with major ecdysteroid-induced events. USP is expressed in many tissues throughout development with fluctuations in mRNA and protein levels(Henrich *et al.*, 1994; Kim *et al.*, 1995).

Also EcR isoforms also are expressed simultaneously with *usp* in all developmental stages. The expression of EcR-A was maintained in high level during embryogenesis, decreased during larval stage, and increased after prepupal stage. While EcR-B1 was expressed with higher level during larval stages comparing to EcR-A (Figure 3A). This result suggests that the functional ecdysone receptor are ubiquitous throughout *Drosophila* life cycle, but their expression level is regulated according to specific developmental stage. Especially, the expression levels of each EcR isoform were different during life cycle. These differential expression of EcR isoforms resulted in the ecdysone receptor complexes consisting of the different combination of EcR isoforms(EcR-A and EcR-B1) and USP. Talbot *et al.* (1993) reported that EcR-B1 and EcR-A have independent and quite distinct spatial and temporal expression patterns during development, as shown by studies using specific monoclonal antibodies. They showed that the larval tissues contain more EcR-B1 than EcR-A, imaginal discs contain much more of isoform A than B1.

Our RT-PCR results described only the expression patterns of the functional ecdysone receptor transcripts over all developmental stages in whole animal. Therefore, in order to investigate the spatial and temporal expression pattern of each ecdysone receptor isoforms, it is required RT-PCR using individual tissues during development. We suggest that this RT-PCR method should be useful for the analysis of RNA transcripts of low abundance or RNA isolated from small amounts of cells.

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적 요

노랑초파리에서 스테로이드 호르몬인 ecdysone은 탈피와 변태의 발생과정에서 coordinated changes의 key regulatory cascades을 시발한다. Ecdysone의 작용은 Ultraspiracle 단백질 (USP)과 ecdysone receptor(EcR)의 이합체에 의해 매개된다. 이들 단백질의 이합체는 ecdysone response element(EcRE)에 결합하여 유전자의 전사를 조절한다. 최근에 기능적인 ecdysone receptor는 USP와 ECR isoforms 간에 형성된 이합체 복합체라는 사실이 알려지게 되었다. 본 연구에서는 RT-PCR(Reverse Transcription Polymerase Chain Reaction) 방법을 이용하여 기능적인 ecdysone receptor 전사체의 발생단계별 양상을 조사하였다. *usp*과 *EcR* 전사체들은 비록 양적인 차이는 있지만 분석된 전 발생단계에서 검출되었다. 본 연구결과는 기능적인 ecdysone receptor의 유전자의 발현은 초파리 생애에서 관찰되는 ecdysone titer와 밀접하게 관련되어 있음을 암시해 주었다.