

U-shaped Gene Induced by Ecdysone Signal Pathway in *Drosophila*

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Keywords: *Drosophila*; Ecdysone; U-shaped gene; Hemocyte

Abstract. The *Drosophila* U-shaped (*ush*) gene play important role in hemocyte differentiation. However, the mechanism of how the *ush* gene is mediated is still unclear. In this study, we reveals that *ush* gene expression increased during molting and metamorphosis stage of the third instar larva, 20-hydroxyecdysone (20E) treatment can up regulate *ush* expression, the silence of *USP1* and *EcR-A* led to a reduced transcript level of *ush* gene in S2 cells. Those results indicated that the ecdysone signaling transduction pathway can mediate *ush* expression through its receptor.

Introduction

The insect hemocytes generation mainly happened in two periods during the development [1]. The first is embryonic period, the larval hemocytes was generated in the mesoderm [2]. The second is larval developmental stage. For now, the molecular mechanism of how insect hemocytes differentiated during embryonic period have been fully understand. Several transcription factors play important role, such as *serpent* (*srp*), *lozenge* (*lz*) and U-shaped (*ush*). During the larval developmental stage, the molecular mechanism of hemocytes differentiation is more complicate. The Notch, Wnt and Hedgehog (HH) signal transduction pathway were involved in regulating these transcription factors *srp*, *lz* and *ush*, and then mediate the larval hemocytes differentiation [3].

The *ush* gene is member of the FOG family. Current studies have found that all FOG members function relies on the GATA transcription factor family [4]. The *Drosophila* *ush* protein contains 1191 amino acids and 9 zinc finger domain, it can interact with *srpNC* to regulate the fetal hemocytes differentiation [5]. During the larval stage, *ush* gene only express during third instar larvae [6]. After *Drosophila* *ush* gene was knocked down, the *Drosophila* lamellocytes number in lymph gland increased significantly, caused lymph gland enlargement, those results indicate that *ush* can suppress lamellocytes differentiate into other hemocytes [7]. The *ush* gene mainly express in *drosophila* plasmatocytes, and may be associated with the maintenance of plasmatocytes function [8]. However, some reseach indicated that the knock down of *drosophila* *ush* gene resulting in the prohemocytes numbers decreased, and the increased expression of *ush* gene suppressed crystal cells and plasmatocytes differentiation. *Ush* can suppressed prohemocytes differentiation and maintain the prohemocytes numbers in lymph gland [9].

For now, how *ush* gene is regulated during the larval hemocytes differentiation is not completely understood. In this study, we used *drosophila* as model animal and investigated the expression profile of *ush* gene utilizing real-time PCR, RNAi and hormone treatment methods. The results demonstrate that the *ush* gene can be induced by the ecdysone signal pathway through the the ecdysone receptor *EcR-A* and its heterodimer *USP1*.

Materials and Methods

Insect Maintenance. The *Drosophila* were feed using the artificial food containing agar, glucose, antiseptic reagents, cornmeal and yeast, under the 14:10 h light and dark conditions at 25 ± 1 °C.

Quantitative real-time PCR

The total RNA of *Drosophila* and the S2 cells was isolated and was reverse transcribed into the first strand cDNA as the PCR template according to the manufacturer's instructions.

Based on the *Drosophila* *ush* gene sequence, we designed the primers for the real-time PCR, *ush*F: 5'-gcaactatgtctccacactac-3', the reverse primer *ush*R: 5'-ctgcttcaccgtagatataca-3' (the length is 171 bp). The *act5C* gene was designed as internal standardization with the primers *act5C*F: 5'-ctggcttcgctgtccacctt-3' and the reverse primer *act5C*R: 5'-gcactttcgtgctgcttcc-3'. The real-time PCR tube with 10 µl volume containing TaqTM, 2 µl primers (1 µmol/L) and 1 µl cDNA (1:10 diluted). The PCR was programmed: one cycle at 94 °C for 3 min; 32 cycles at 94 °C for 15 s, 62 °C for 15 s. We used the comparative CT method to analyze the results.

Cell Culture and Hormonal Regulation of *ush* gene. The S2 cells from *Drosophila* were cultured according to the instructions. The 20E (Sigma) which can act similar with ecdysone was diluted and added to the S2 cells, The final concentration was 1 µM 20E. The S2 cells were cultured for various times after 20E was added. The control cells using equal volume of DMSO. Then detected the *ush* gene expression levels by real-time PCR. Three independent experiments were performed and analyzed to construct a graph.

RNAi in S2 Cell Line. In order to investigate whether ecdysone receptor ECR and USP can mediate *ush* expression, we designed the *EcR-A* and *USP1* gene dsRNAs using the RNAi kit (Ambion, Austin, Texas). All the primers for dsRNAs had a T7 binding sequence (5'-gcgtaatacagactcactatagg-3') on the 5' end. The *EcR-A* dsRNA primers, *EcRi*F: 5'-T7-aacaacaattagctgcctct-3', *EcRi*R: 5'-T7-gtccttcttgcctcttcttcc-3'; the *USP1* dsRNA primers, *USP1i*F: 5'-T7-caacggttctgatgacttca-3' *USP1i*R: 5'-T7-gtaatgcggaaga ggaaca g-3'; the green fluorescent protein (GFP) was designed as control; the primers for GFP dsRNA was *GFPi*F: 5'-T7-tggtcccaattctcgtggaac-3' *GFPi*R: 5'-T7-cttgaagttgaccttgatgcc-3'. The dsRNA quantity was detected by 260 nm spectrophotometry. The dsRNA quality was determined by 1.2% agarose electrophoresis. after the cells was transfected 10 h, the 20E was added, continue culture for 6 h, the total RNA was isolated for the real-time PCR analysis. Cells transfected with dsGFP was set as inner control. Three independent experiments were performed and analyzed to construct a graph.

Results

Ush Gene Expression Increased During Molting and Metamorphosis. In order to detect how *ush* gene express during the larval stage, we isolated the total RNA from the third instar at 0 h to 60 h. Real-time PCR results showed that the *ush* mRNA level has two peaks at 0 h and 60 h when the larvae body underwent molting and metamorphosis (Fig. 1).

20E Upregulated the Expression of *Pvr* in S2 Cells. In order to detect how the 20E affect the expression of *ush*, we used 20E treat the S2 cells for 1 h, 3 h, 6 h and 12 h respectively. Then total RNA was isolated and real-time PCR was performed to detect the *ush* gene expression profile. Results showed that the *ush* mRNA level began to increase from 3 h until 6 h after 20E treatment, then the mRNA level dramatically decreased at 12 h (Fig. 2). Those results indicated that *ush* gene can be induced by insect ecdysone.

Silence of EcR-A and USP1 Blocking the Expression of Pvr. In order to determine how the ecdysone receptors EcR and USP1 affect the *ush* gene expression, EcR-A and USP1 were silenced using RNAi method in S2 cells. Real-time PCR results indicated that the silence of EcR-A and USP1 lead to the significant suppression of *ush* gene induced by 20E compared to the control transfected with dsGFP. This results certified that the *ush* gene involved in the ecdysone signal pathway and it acts downstream of the ecdysone receptors (Fig. 3).

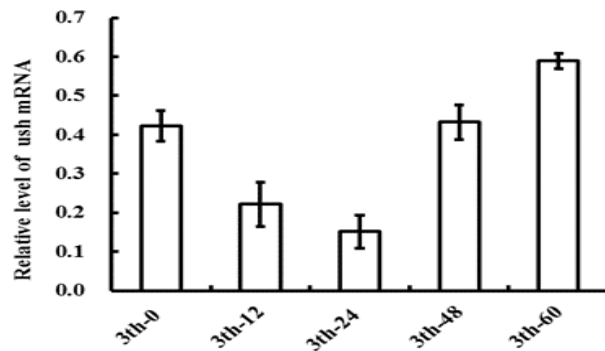


Figure 1. Real-time PCR analyze the *ush* gene expression during the third instar larval from 0 to 60 h ; *act5c* was set as the internal control. Error bars indicate the standard deviation from three independent experiments.

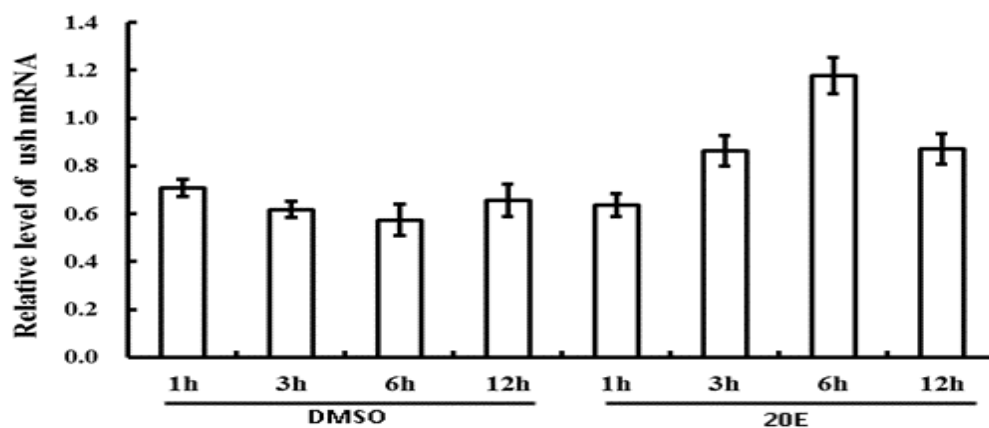


Figure 2. Real-time PCR analyze the *ush* gene expression from S2 cells after 20E treatment. The *ush* expression was measured at 1, 3, 6, and 12 h after hormonal treatment. Control was treated equal dilution of DMSO. Error bars indicate the standard deviation from three independent experiments.

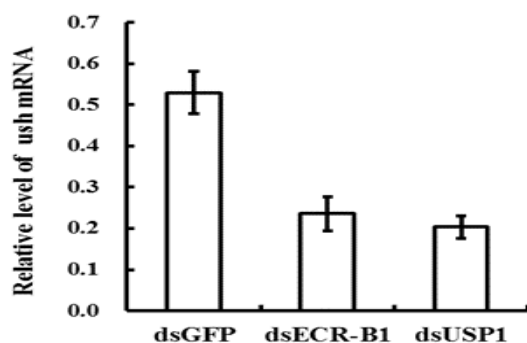


Figure 3. Real-time PCR analyze the *ush* gene expression from S2 cells after EcR-A and USP1 were silenced. Error bars indicate the standard deviation from three independent experiments.

Discussion

In this study, we take *Drosophila* as model animal and used quantitative real-time PCR and RNAi methods to investigate the *Drosophila* *ush* gene expression profile. The results showed that the *ush* gene expression increased during insect molting and metamorphosis stage, ecdysone can induce *ush* gene abundantly expressed, RNAi research results showed that the silenced of EcR-A and USP1 can block the *ush* gene expression. Based on those results, we can conclude that *Drosophila* *ush* gene involved in the ecdysone signal transduction pathway and it can be induced by ecdysone through ecdysone receptor EcR and USP.

Insect larvae hemocytes differentiation is a complex process, this process is not only mediated by some transcription factors, such as *srp*, *lz* and *ush*, but also regulated by Notch, Wnt and Hedgehog (HH) signaling pathway [10-13]. The *ush* gene belong to FOG family, the current studies found that all the FOG members function depends on the GATA transcription factors [3]. The *ush* can also interact with the *srp* to mediate the insect hemocytes differentiation [14, 15]. In this study, we found that the *ush* gene not only express during molting and metamorphosis when hemocytes undergo differentiation, but the *ush* gene also express during feed stage. It is unclear what roles the *ush* play during the feed stage when hemocytes not undergo differentiation. *Drosophila* GATA transcription factors has five isomers, whether *ush* play roles through acting with other isomers need further investigate.

Although the specific role of *ush* on larvae hemocytes differentiation is still unclear, but the knockdown of *ush* gene resulting in the destruction of *Drosophila* hemocytes differentiation [15]. This results suggest that *ush* play an important role in larval hemocytes development. Our study founded that 20E can induce the expression of *ush* through ecdysone receptor, this suggest that the *ush* is a downstream member in the ecdysone signal transduction pathway, ecdysone may mediate larval hemocytes differentiation through up regulating *ush* gene.

Acknowledgements

This research was supported by the Scientific Funds for Outstanding Young Scientists of Shandong Province Award [BS2013SW030], the Project of the Shandong Province Higher Educational Science and Technology Program (J12LK01) and the Natural Science Foundation of Shandong Province (ZR2012HL42)

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