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Cloning, Characterization and Promoter Activity of the Yolk Protein Gene, Bdyp1, in the Oriental Fruit Fly Bactrocera dorsalis 【Research report】

東方果實蠅卵黃原蛋白基因 (Bdyp1) 之選殖及其序列特徵與啟動子活性之分析【研究報告】

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Abstract

In the present study, we cloned a yolk protein gene, Bdyp1, of the oriental fruit fly Bactrocera dorsalis (Hendel) and its 2.7-kb 5′-flanking sequence (GenBank accession no. EU130922). The Bdyp1 gene comprises a 31-bp 5′- untranslational region (5′-UTR), three exons, and a 141-bp 3′-UTR. The 2.7-kb 5′-flanking region of Bdyp1 contains two putative TATA boxes close to the initiation site, six potential ecdysone response elements (EcREs), and numerous binding sites for various transcriptional factors, such as GATA, Doublesex, MAB-3, AEF-1, BBF-2, and so forth. Transient transfection analysis of the Sf21 cells shows that the 2.4-kb frame of the 5′-flanking region of Bdyp1 exhibits the greatest activity in response to 10-2 nM of 20-hydroxyecdysone (20E). Promoter- deletion analysis reveals that 20E is most likely only interacting with the first two EcREs. In addition, the 20E-induced elevation of the promoter activity is suppressed by the addition of juvenile hormone III either before or after the 20E treatment. This is the first demonstration to show that JH is capable of suppressing the action of 20E on stimulating yolk protein gene expression.

摘要

本研究選殖得東方果實蠅 (Bactrocera dorsalis (Hendel)) 卵黃原蛋白基因 1 (yolk protein gene 1, Bdyp1) 及其 2.7 kb 之上游區域 (GenBank accession no. EU130922)。Bdyp1 基因由一段長 31 bp 之 5 端非轉譯區域 (5′-untranslational region, 5′-UTR)、三個外顯子 (exon) 及一段長 141-bp 之 3 端非轉譯區 (3′-UTR) 所構成。分析 Bdyp1 上游 2.7 kb 序列顯示，在靠近基因起始位 (initiation site) 處具有兩個類似TATA box的區域，6 個可能是蛻皮激素受器反應位 (ecdysone response element, EcRE) 分布在不同的位置，以及許多如雌性雙性基因 (female specific doublesex, dsxF)、GATA factor、E75、adult expression factor (AEF) 等等調控組織或性別表現專一性之轉錄因子 (transcription factor) 作用位置。細胞轉染 (cell transfection) 2.4 kb 上游 DNA 片段入 Sf21 細胞，激素測試結果顯示在 10-2 nM 蛻皮激素的刺激下啟動子的活性最高；進一步經啟動子刪除 (promoter deletion) 分析，結果顯示蛻皮激素作用主要的結合位 (binding site) 可能僅為調控區最靠近起始位之兩個 EcRE。另外，本研究首次發現蛻皮激素誘發啟動子活性的現象會因為處理青春激素 (無論在施用蛻皮激素之前或後) 而受到抑制。

Key words: yolk protein gene promoter, juvenile hormone, 20-hydroxyecdysone, Bactrocera dorsalis, cis-regulation

關鍵詞: 卵黃原蛋白基因啟動子、青春激素、蛻皮激素、轉錄因子、東方果實蠅。

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Cloning, Characterization and Promoter Activity of the Yolk Protein Gene, *Bdyp1*, in the Oriental Fruit Fly *Bactrocera dorsalis*

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ABSTRACT

In the present study, we cloned a yolk protein gene, *Bdyp1,* of the oriental fruit fly *Bactrocera dorsalis* (Hendel) and its 2.7-kb 5'-flanking sequence (GenBank accession no. EU130922). The *Bdyp1* gene comprises a 31-bp 5'-untranslational region (5'-UTR), three exons, and a 141-bp 3'-UTR. The 2.7-kb 5'-flanking region of *Bdyp1* contains two putative TATA boxes close to the initiation site, six potential ecdysone response elements (EcREs), and numerous binding sites for various transcriptional factors, such as GATA, Doublesex, MAB-3, AEF-1, BBF-2, and so forth. Transient transfection analysis of the *Sf*21 cells shows that the 2.4-kb frame of the 5'-flanking region of *Bdyp1* exhibits the greatest activity in response to 10^{-2} nM of 20-hydroxyecdysone (20E). Promoter-deletion analysis reveals that 20E is most likely only interacting with the first two EcREs. In addition, the 20E-induced elevation of the promoter activity is suppressed by the addition of juvenile hormone III either before or after the 20E treatment. This is the first demonstration to show that JH is capable of suppressing the action of 20E on stimulating yolk protein gene expression.

Key words: yolk protein gene promoter, juvenile hormone, 20-hydroxyecdysone, *Bactrocera dorsalis*, *cis*-regulation

Introduction

Vitellins (Vns) or yolk proteins (YPs) are major nutrients for embryonic development in most animals. In insects, yolk proteins are generally synthesized in fat body, secreted into hemolymph, subsequently taken up by the growing oocytes via receptor-mediated endocytosis, and finally stored in specialized organelles, *i.e.* yolk granules (Hagedorn *et al*., 1998; Sappington and Raikhel, 1998; Giorgi *et*

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al., 1999).

The expression of YP genes displays a tissue-, sex- and temporal-specific pattern in higher dipterans, *i.e.* cyclorrhaphan insects. While YPs are synthesized in the fat body of adult females of most of these insects (Bownes and Pathirana, 2002), they are synthesized in follicular cells of the ovary of some other dipterans, such as *Drosophila melanogaster* (Yan *et al*., 1987), *Anastrepha suspensa* (Handler, 1997) and *Calliphora erythrocephala* (Martinez and Bownes, 1994). Moreover, YPs are expressed in both fat body and ovary of *Dacus oleae* (Levedakou and Sekeris, 1987), *Ceratitis capitata* (Rina and Mintzas, 1988).

Juvenile hormone (JH) and ecdysteroids are key hormones that are involved in the regulation of yolk protein synthesis and vitellogenesis in most insects (Hovemann *et al*., 1981; Giorgi *et al*., 1999). In contrast to the major regulatory role of JH on vitellogenesis for lepidopterans, ecdysteroids carry out the most important role in the regulation of dipteran reproduction by triggering the expression of ecdysone-responsive genes, such as E74, E75, Broad-complex, ecdysone receptor (EcR), ultraspiracle (USP), and so on (Raikhel *et al*., 2002; Attardo *et al*., 2005), which are involved in the regulatory cascade of the vitellogenesis and oogenesis (Richard *et al*., 1998, 2001). In addition, proteinous nutrients are also essential for promoting the YP synthesis via actuating the JH synthesis in the corpora allatum-cardiacum complex and the ecdysteroids synthesis in the ovary (Bownes, 2005). Yolk protein synthesis fails to be sustained in adult females of *Musca domestica* when they are maintained on a protein deficient diet. However, YP synthesis can be resumed by treatment of these flies with JH and ecdysteroids (Adams and Gerst, 1992). A similar phenomenon has also been observed in *Drosophila* (Bownes *et al*., 1987; Brownes, 1989).

The actions of JH or ecdysteroids in controlling YP synthesis are mediated by diverse hormone-responsive proteins (transcription factors), such as E74, E75, Broad-complex, EcR, USP, and so forth (Raikhel *et al*., 2002; Attardo *et al*., 2005; Wang *et al*., 2005). Moreover, exact expression of sex determination genes is also important in regulating YP synthesis (Bownes, 2005). All these factors interact with the elements responsible on the gene to regulate their expression. For example, in *Drosophila,* the fat body enhancer (FEB) in the intergenic region of *Dmyp1* and *Dmyp2* determines the fat body-, stage- and sex-specific expression of these two genes (Garabedian *et al*., 1987), and ovarian enhancer 1 and 2 (OE1 and 2) control the correct temporal and spatial pattern of *yp1* and *yp2* expression in folliclular cells (Logan *et al*., 1989; Logan and Wensink, 1990). Many transcription factors that interact with *Drosophila* enhancers have been identified and implicated in the control of *yp* expression. For example, the adult enhancer factor-1 (AEF-1) and CCAAT/enhancer-binding proteins (C/EBP) as suppressors (Abel *et al*., 1992; Falb and Maniatis, 1992) and the box-B binding factor-2 (BBF-2) as an activator of *Drosophila* alcohol dehydrogenase (*Adh*) gene act in concert to control the fat body-specific expression of *yp* of this fly (Abel *et al*., 1992).

The oriental fruit fly, *Bactrocera dorsalis* (Hendel), one of the most destructive pests in many Asian countries including Taiwan, causes serious damage to a number of fruit cultures. For many years, insecticides have been used to eradicate *B. dorsalis*; however, too much dependence on chemical insecticides has resulted in the development of severe resistance in this fly. Seeking an alternative method to control *B. dorsalis*, we attempted to isolate a sex-specific gene, such as the yolk protein gene, which could be used in a transgenic system for culturing insects carrying a dominant lethal gene to be released in the field for

potential pest control.

In the present study, we cloned a *B. dorsalis* yolk protein gene, *Bdyp1* (GenBank accession no. EU130922) and its upstream flanking region, and analyzed its structural characters; moreover, we discovered that the prompting effect of 20-hydroxyecdysone (20E) on *Bdyp1* promoter activity could be suppressed by JH.

Materials and Methods

Experimental insects

Oriental fruit flies, *B. dorsalis*, used in this study were maintained in the laboratory as described based on Chen *et al*. (2008). Larvae of *B. dorsalis* were fed an artificial diet and adults were maintained on the mixture of sugar: yeast extract: peptone = 3: 1: 1 with *ad libitum* of water. The rearing condition was maintained at 28 ± 1°C with 12-h light and 12-h dark.

Extraction, cloning and sequencing of genomic DNA

Genomic DNA was extracted from adult females ground with liquid nitrogen, homogenized and digested in the grinding buffer (0.1 M NaCl, 0.1 M Tris-HCl, 0.1 M EDTA, 0.05% SDS, 0.2 M sucrose, and 10 mg/mL proteinase K) at 55°C for 30 min, and then the DNA was purified with phenol/chloroform.

Isolation of *Bdyp1* was carried out with genomic DNA walking using a GenomeWalker™ Universal Kit (CLONTECH Laboratories, Inc., Palo Alto, CA, USA) as per the manufacturer instructions. In brief, genomic DNA was digested with *Eco*RV, *Stu*I, *Pvu*II, and *Dra*I, separately, purified with phenol/chloroform, and then ligated overnight using the adaptors provided in the kit. The adaptor-ligated DNA fragments were used as templates for PCR. The DNA sequences were amplified with a primer of the adaptor and a gene-specific primer designed based on the sequence of *Bdyp1* cDNA (GenBank accession no. AF368053). PCR was performed as follows: 94°C for 5 min; 7 cycles of 94°C for 20 sec and 72°C for 3 min; 32 cycles of 94°C for 20 sec and 67°C for 3 min; and terminated with a final extension at 67°C for 4 min. Nested PCRs were performed as follows: 94°C for 3 min; 35 cycles of 94°C for 20 sec, 60°C for 40 sec and 67°C for 3 min; and a final extension at 67°C for 7 min. The nested-PCR products of each sample were subcloned into pCR II vector (Invitrogen, Carlsbad, CA, USA). DNA were sequenced by dideoxy chain termination using ABI PRISM 377 DNA autosequencer (DNA Sequencing Core Facility, Biotechnology Center, National Chung Hsing University).

Genomic DNA sequence analysis

Determination of exons/introns was performed using either the Genetics Computer Group (GCG) software program Wisconsin Package Version 10.0 (Genetics Computer Group, Accelrys, Inc., San Diego, CA, USA) or the computational bio-informatics analysis program of the NHRI Bioinformatics Services (http://bioinfo.nhri.org.tw/). Searching for putative binding sites of specific transcription factors was done using published consensus sequence databases such as the FINDPATTENS program (GCG) and the TRANSFAC program (TESS, http://www.ebi.ac.uk/). Prediction of the putative promoter sequence was performed using the Promoter 2.0 program on CBS prediction servers (http://www.cbs.dtu.dk/) (Knudsen, 1999), and the GENSCAN web server (http://genes.mit.edu/GENSCANinfo.html). NetGene2 (CBS) (Brunak *et al*., 1991; Hebsgaard *et al*., 1996) was used to identify the exons and introns.

Construction of fusion genes

Four different lengths of *Bdyp1* 5'-flanking region, *i.e.* from +28 to -356, -657, -1385 and -2651 base pairs, respectively, were amplified with *pfx* polymerase (Invitrogen) using the primers shown in Table 1, and the PCR program as follows:

Table 1. Oligonucleotide primers are used in constructing the reporter plasmids for analysis of *Bdyp1* promoter activity

Name	Sequence	Position (nt) on the *Bdyp1*	Purpose
transR	5'-TGATCACGTTTGCAAATCGGTTTG-3'	+6 to +28	Revise primer for all constructs
yp2.7	5'-CTGGTATCGAGTAGAACAAGTTCTTCAAA-3'	-2683 to -2655	Forward primer for yp2.4 construct
yp1.4	5'-CAAAACAACCATTGCATAACGG-3'	-1389 to -1368	Forward primer for yp1.4 construct
yp0.7	5'-GCTACCCTACCTAGGTTCTAAGCT-3'	-661 to -638	Forward primer for yp0.7 construct
yp0.4	5'-ACGTAAGTTATCTCTTAGAGGCCC-3'	-388 to -365	Forward primer for yp0.2 construct

94°C for 5 min; 33 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C, 3 min; termination with a final extension at 68°C for 20 min. After PCR amplification, the 2.7- and 0.4-kb fragments were further digested with *Pvu*II and *Pst*I to obtain the final products, *i.e.* 2.4- and 0.2-kb fragments, respectively. Each product was subcloned into the *Sma*I-*Hind*III sites in the luciferase reporter plasmid (pGL3-basic vector; Promega Corp., Madison, WI, USA). Four constructs were named according to the size of the flanking region as pGL{yp2.4-luc}, pGL{yp1.4-luc}, pGL {yp0.7-luc} and pGL{yp0.2-luc}, respectively. The sequence of all constructs were verified by DNA sequencing. A construct of the pGL3 vector only was used as the control.

Cell culture

The insect cell line, *Spodoptera frugiperda* IPLB-Sf21-AE (*Sf*21), is able to respond to the stimulation of ecdysone (Chen *et al*., 2002); and the cell line was therefore adopted for the assay of *Bdyp1* promoter activity.

The cell line was cultured in Grace's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Israel) and 1% of antibiotics, including penicillin (100 unit/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL) (Invitrogen), at 28°C in a humidified culture chamber.

Transient transfection and luciferase assay

Luciferase assays were performed by the Dual-Luciferase® Reporter Assay System (Promega) according to the manufacturer's protocol. For the reporter enzyme assay, *ca.* 10^5 *Sf*21 cells were plated per well in a twenty four-well plate and cultured for 2 h before transfection. The cells in each well were co-transfected with 0.5 μg of test plasmid, *i.e.* firefly luciferase reporter plasmid recombined behind the indicated promoters, and 0.05 μg of the *Renilla* luciferase plasmid (as an internal control reporter) in 2 μL of liposome (Cellfectin Reagent; Invitrogen). For luciferase activity assays, the cells were lysed in 1X lysis buffer; and 20 μL lysate was used to determine the luciferase activity expressed as related in relative light units (RLU) as per the manufacturer's protocols. Protein concentration of lysate was determined and normalized with the protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as the standard.

Hormonal treatments

The hormones, JH III and 20E, used for experiments were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Ten micrograms of 20E (95%) and JH III (75%) were dissolved in 99 and 140.7 μL of

ethanol (99.5%), respectively, to make stock solutions with 2 x 10^{-1} M final concentration, and appropriate amounts of the stock solution were added for the experiments that followed. All control groups contained an equivalent amount of ethanol.

For hormonal treatment, the medium of transfected cells was removed after 5-h transfection incubation at 28˚C, and replaced with the medium containing a serial concentration of JH III or 20E. After 12-h incubation, the hormone-containing medium was replaced with normal medium, and the cells were further incubated for 48 h. Finally, these cells were harvested and lysed with 1X lysis buffer, and the aliquots of lysate were measured for luciferase activity. At least three replicates were performed for all transfection experiments, and six measurements were taken for each replicate.

To examine the interactions of JH III and 20E to the *Bdyp1* promoter activity, the *Sf*21 cells transiently expressing pGL {yp2.4-luc} were assayed. Before transfection, *Sf*21 cells were incubated in FBS-free Grace' medium at 28˚C for 2 h, and then co-transfected with pGL{yp2.4-luc}/pRL-TK. At the same time, control tests were conducted by co-transfecting the cells with pGL3/pRL-TK. After 5-h transfection, the cells were washed twice with fresh medium and replaced with fresh medium containing either 10^{-2} nM 20E or 10^{-3} nM JH III or both in sequence following the treatment schedule shown in Fig. 7A. After 18-h incubation, the first medium was removed; the cells were washed twice with fresh medium and replaced with the second medium for another 18 h. At the end of the treatments, the cells were collected and lysed for the luciferase activity assay.

Statistical analysis

For the transfection experiments, the luciferase activities were evaluated and transformed to RLU/mg protein sample, the values are shown as means ± S.E. Means for the experiments with multiple variables were analyzed by ANOVA followed by the Tukey HSD test to show the statistical differences between individual means. In addition, Dunnett's test was applied when the statistical analysis involved comparing multiple means with its control. Statistical analyses were preformed with the SAS program (SAS Institute Inc., Cary, NC, USA).

Results

Structural organization and characteristics of *Bdyp1*

A genomic DNA fragment, *ca.* 4.3 kb, containing the *Bdyp1* was obtained by PCR genomic DNA walking (Fig. 1). An alignment with the full-length *Bdyp1* cDNA revealed that this genomic DNA fragment included the *Bdyp1* and its 5'-flanking region. Regarding the *Bdyp1*, an initiator, *i.e.* ACAGT, was noted at position 2 to 6; and for the coding region, the start codon for translation (the first ATG) was located at 34-36 bp, followed by an open reading frame (ORF) spreading over three exons and ending up with a 141-bp downstream untranslational region. A poly A signal sequence, *i.e.* AATAAA, was located at 35-40-bp upstream of the end of the gene (Fig. 1). Two introns of 67 and 76 bp, respectively, were located within the protein coding region, and consensus splicing signals, *i.e.* GT-AG, were noted at the boundaries of both introns.

In the 5'-flanking region, two putative TATA boxes, *i.e.* TATAAA, were located at -23 to -28 and -140 to -145, respectively (Fig. 1). In addition, various putative regulatory elements of transcription were identified as well (Fig. 2). Among them, six putative ecdysone response elements (EcREs), which mediate 20E action on the control of gene expression, were noted scattered between +10 and -1474 bp. Also, elements responsible for the determination of gene specific expression in fat body,

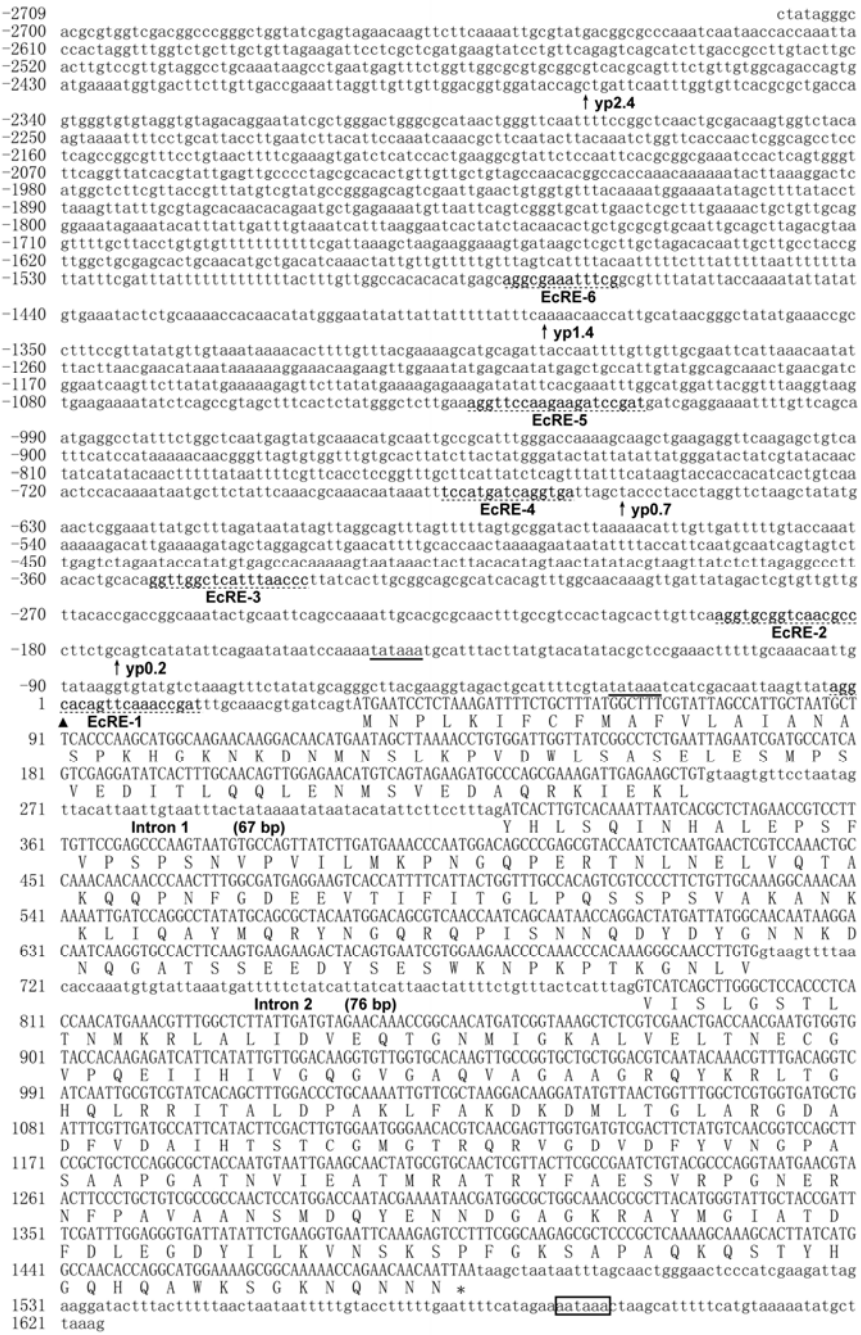


Fig. 1. Nucleotide sequence of *Bdyp1*. The nucleotide sequence of the coding strand together with the deduced amino sequence of Bdyp1 is indicated. Numbers on the left indicate the nucleotide position relative to the transcription initiation site, designed as +1. The upper- and lower-case letters represent the exon sequences and the flanking region and intron sequences, respectively. The putative TATA boxes are underlined. The stop codon is depicted by asterisks. The polyadenylation signal (AATAAA) is shown boxed. Positions marked with yp2.4, yp1.4, yp0.7, and yp0.2 represent the locations for promoter deletion analysis. The putative ecdysone response elements (EcRE) are marked with dashed underlines.

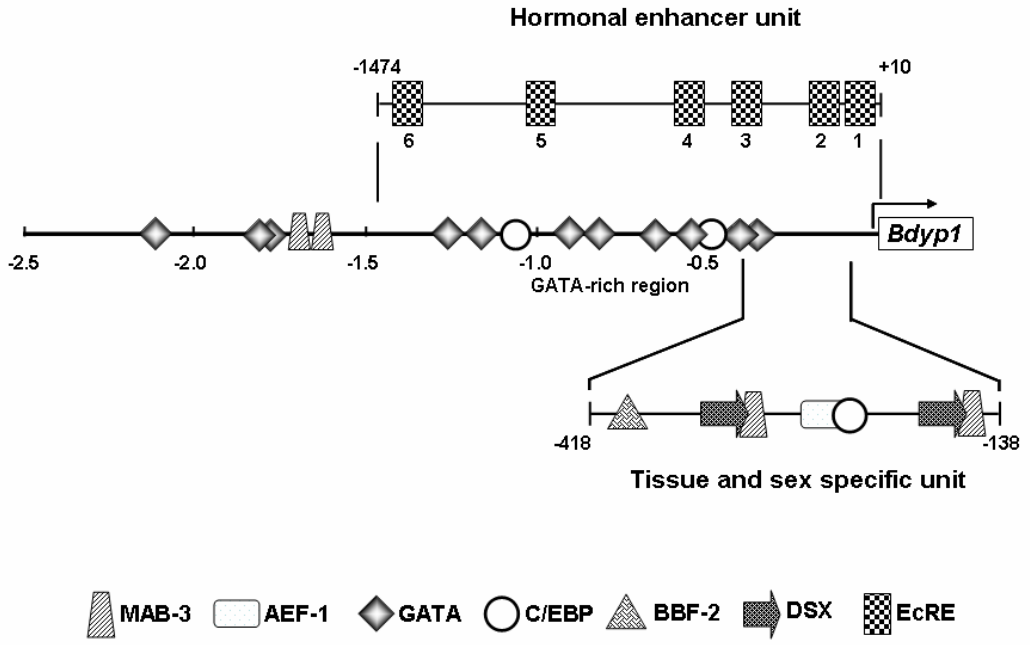


Fig. 2. Analysis of the 5'-flanking region of *Bdyp1*. Schematic illustration represents the regulatory region of *Bdyp1*. Numbers refer to the nucleotide positions relative to the transcription start site. Binding sites in the 5'-flanking region of *Bdyp1* for transcription factors are marked with different shapes underneath the picture. EcRE, ecdysone response element; DSX, the binding site for doublesex protein; BBF-2, the binding site for box-B binding factor-2; C/EBP, the binding site for CCAAT/enhancer-binding proteins; GATA, the binding site for GATA; AEF-1, the binding site for adult enhancer factor-1; MAB-3, the binding site for MAB-3.

including *D. melanogaster* B-box binding factor 2 (BBF-2), CCAAT/ enhancer-binding protein (C/EBP) and GATAa/b, and the regulatory elements for female-specific expression, such as adult enhancer factor-1 (AEF-1) and doublesex (DSX-A and C), were identified at various locations as shown in Fig. 2.

Bdyp1 promoter activity in response to 20E and JH in *Sf*21 cells

To examine the effects of 20E and JH on the promoter activity, the promoter activity of the 2.4-kb upstream region of *Bdyp1* was assayed in the *Sf*21 cells transiently expressing pGL{yp2.4-luc}. As shown in Fig. 3, treatments with various concentrations of 20E elevated the *Bdyp1* promoter activity; nevertheless, their luciferase activities did not display a dose-dependent response. Within the range of 10^{3} to 10^{-4} nM 20E, 10^{-2} nM 20E treatments showed the highest promoter activity, an approximate 4.2-fold increase over the control. Regarding the response to JH, the promoter activity decreased with the increase of JH III using the same range of concentrations. In addition, when JH III concentrations were lower than 10^{-1} nM, *i.e.* 10^{-2} to 10^{-4} nM, the promoter activities increased significantly to 1.45-, 1.61-, and 1.49-fold, respectively, in comparison with the control; on the contrary, with JH III above 10^{-1} nM, the promoter activities were, in general, notably lower than those of the control.

To search for possible hormonal responsible elements in this 2.4-kb region,

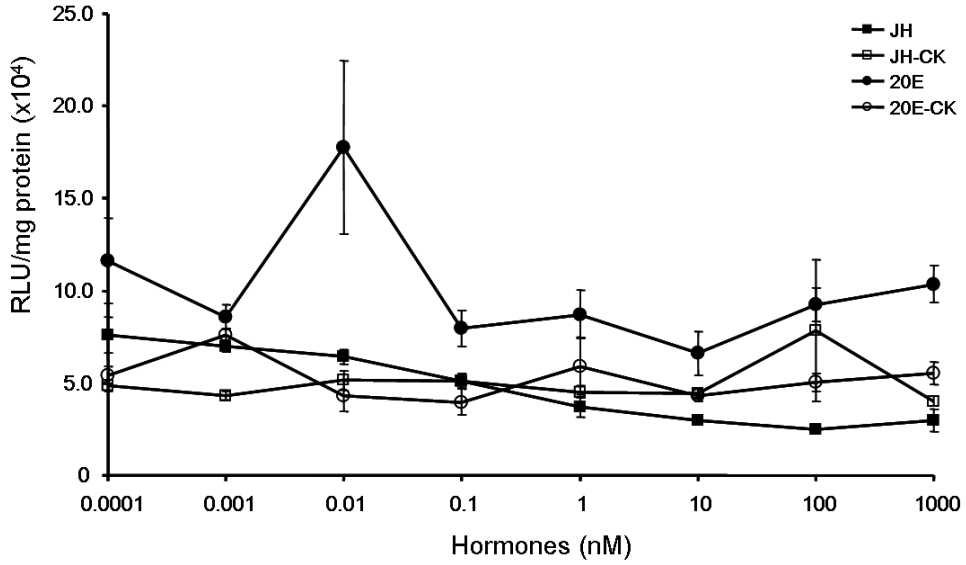


Fig. 3. Transient transfection analysis of *Bdyp1* promoter activity in cells. *Sf*21 cells were co-transfected with pGL{yp2.4-luc} and pRL-TK (as a control of transfection efficiency). After transfection, cells were cultured in medium containing a series of concentrations of 20E (●) or JHIII (○) for 48 h. Data represent the ratios of firefly luciferase to *Renilla* luciferase adctivty (relative luciferase activity, RLU). pGL3, with no promoter region as control (○: 20E treatment; □: JH treatment). Each point represents the means of three replicates of independent transfection experiments; the error bars represent the S.D. of the mean.

various deletion constructs containing 2.4-, 1.4-, 0.7-, and 0.2-kb of the region, respectively, were assayed for promoter activity in response to hormones stimulation. As shown in Fig. 4, by treated with 10^{-2} nM of 20E, little difference of the promoter activity among those with 2.4-, 1.4- or 0.7-kb region was noted; however, when the 5'-flanking region was deleted down to 0.2 kb, the activity was notably lowered to *ca.* 2.9-fold (Fig. 4B). Additionally, 10^{-3} nM of JH III caused these constructs to show *ca.* 1.2- to 1.6-fold elevations of luciferase activities in respect of the control (Fig. 4C).

Interaction of 20E and JH on *Bdyp1* promoter activity in *Sf*21 cells

For investigating the interaction of 20E and JH with the promoter, *Sf*21 cells transiently expressing pGL{yp2.4-luc} were adopted for the practical tests. The fluctuation of the promoter activities with time in response to hormone treatments was first observed in the transient transfected cells. As shown in Fig. 5, the elevation of the promoter activity in reaction to 10^{-2} nM 20E occurred after 12 h, reached a peak at 24 h, and then decreased gradually over time. In addition, a slow increase in the promoter activity was observed in the 10^{-3} nM JH-treated groups, but the maximum activity occurred at 32 h. These results, where the promoter exhibited relative high activities in both 20E- and JH-treatments between 16 h and 32 h, were used to design the time courses (Fig. 6A) for the subsequent experiments.

To determine if JH was able to interact with the action of 20E, the promoter activity was assayed while being

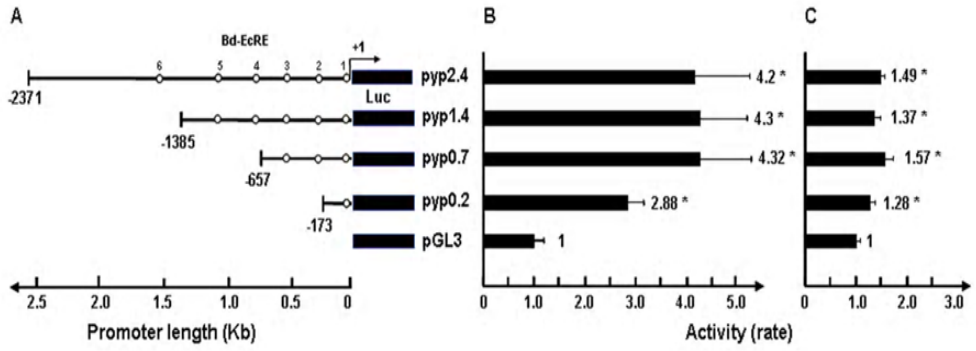


Fig. 4. The effect of different deletions of *Bdyp1* 5'-flanking region on the transcriptional activity in response to 20E and JH. (A) Schematic illustration of the *Bdyp1* promoter region. Putative ecdysone response elements in the reporter constructs are denoted with open circles, and the numbers identify the position relative to the transcription initiation site. EcRE indicates the location of the putative ecdysone responsive element; Luc is the reporter gene, luciferase. (B) *Sf21* cells transfected with the truncated reporter constructs and pRL-TK (*Renilla* luciferase control vector). The transfected cells were cultured for 48 h in the presence of 10^{-2} nM 20E, and then cell lysates were assayed for firefly and *Renilla* luciferase activities. (C) Luciferase activities in *Sf*21 cells treated with 10^{-3} nM of JH III for 48 h. The numbers beside the bars indicate the induction fold relative to the control (pGL3). The transfection efficiency, assessed by *Renilla* luciferase activity from pRL-TK, is similar in each assay. Asterisks (*) indicate the significant difference between test groups and the control based on statistical analysis.

treated with 10^{-2} nM 20E and/or 10^{-3} nM JH III. Fig. 6B indicates that 20E triggered a maximal elevation on the gene expression up to 4.2 fold over the control, while JH III caused a weak increase, up to 1.6 fold. It is worth noting that the treatment of JH, either before or after 20E, resulted in a significant suppression of the 20E-induced promoter activity down to only 1.6- to 1.8-fold over the control.

Discussion

Structure of *Bdyp1* and features of its 5'-flanking region

Bdyp1 is an ortholog YP gene of other known higher dipterans that share several similar features. Generally, the TATA box in the core promoter is the TFIID (a transcription factor composed of several TFIID-associated subunits of eukaryotes) binding site for initiating the transcription of genes. The TATA box is usually located at 25-30 bp upstream of the transcriptional initiation site (Arnosti, 2002; 2003). From this aspect, the first TATA box, located at -23 to -28, of *Bdyp1* could be the functional one. In addition, *Bdyp1* has a short 5'-UTR (31 bp), similar with *Dmyp1* (30 bp) and *Dmyp3* (61 bp) of *D. melanogaster* (Hung and Wensink, 1983; Garabedian *et al*., 1987). Two short introns and conserved splicing sequences of *Bdyp1* occurred as well in *D. melanogaster* (Hung and Wensink, 1983; Garabedian *et al*., 1987) and *C. capitata* (Rina and Savakis, 1991) YP genes. These conserved features imply that YP genes in higher dipterans might co-evolve from a common ancestor and that they have similar post-transcriptional modification processes.

Sequence analysis revealed that the 2.7-kb 5'-flanking region of *Bdyp1* contained a variety of potential regulatory elements known to be involved in the regulation of sex-, tissue- and stage-specific expression of genes. In *D. melanogaster*, female- and fat body-

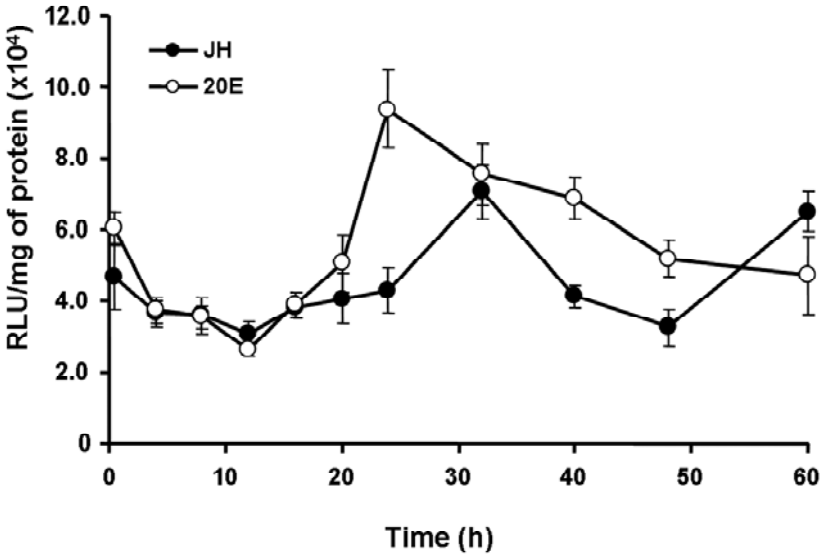


Fig. 5. The time-course of luciferase activity in transfected *Sf*21 cells in response to treatments of 20E and JH. Cells were transfected with pGL{yp2.4-luc} and pTK-RL (the plasmid for internal control). After transfection, the cells were grown in Grace's medium containing 10^{-2} nM 20E or 10^{-3} nM JH III. Cells were collected at 0.5, 4, 8, 12, 16, 20, 24, 32, 40, 48, and 60 h, respectively, after hormone treatment. The reporter activity was quantified and plotted. Data are given in relative light unit (RLU) per mg protein ± S.D.

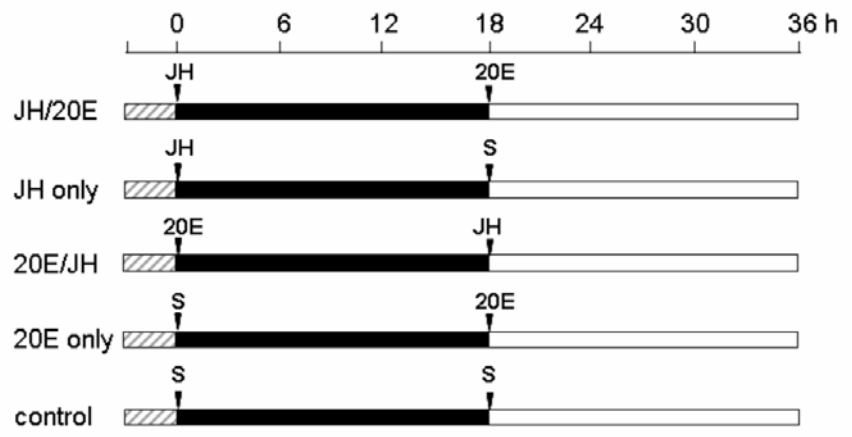
specific expression of YP genes are controlled by transcription factors, including DSX, AEF-1 and C/EBP (An and Wensink, 1995a, b). Among them, DSX is expressed in two forms, *i.e.* DSX^F and DSX^M. DSX^F acts as a regulator that initiates the *yp* expression in female fat body, while the DSX^M acts as a suppressor of this gene in males (Burtis *et al*., 1991; Coschigano and Wensink, 1993; Garrett-Engele *et al*., 2002). Similar actions of DSXs also occur in *M. domestica* (Hediger *et al*., 2004). Moverover, the DSX binding sites were present in the regulatory region of *Bdyp1* (Fig. 2), and Chen *et al*. (2008) reported that the knockdown of DSX^F by double strand dsx^f injection resulted in the decrease of YP synthesis in *B. dorsalis* female adults. These results seem to indicate that DSX^F takes part in the regulation of *Bdyp1* expression, probably by interacting with DSX binding sites in the 5'-flanking region.

In addition, several other transcription factors, such as AEF-1, C/EBP as well as GATA, are also important in the regulation of temporal and spatial expression of genes in insects (An and Wensink, 1995b; Tortiglione and Bownes, 1997; Attardo *et al*., 2003; Hutson and Bownes, 2003; Park *et al*., 2006). Similarly, there were several AEF-1, DSX, C/EBP, and GATA binding sites identified in the 5'-flanking region of *Bdyp1* (Fig. 2), implying that these transcription factors play a role in controlling *Bdyp1* specifically expressed in fat body and ovary of *B. dorsalis* female adults.

Activation by 20E and suppression by JH of *Bdypl* transcription

Insect yolk protein synthesis and its uptake by the developing oocytes during vitellogenesis and oogenesis are mainly regulated by JH and 20E. JH can promote the Vg gene expression in females of *M.*

(A)



(B)

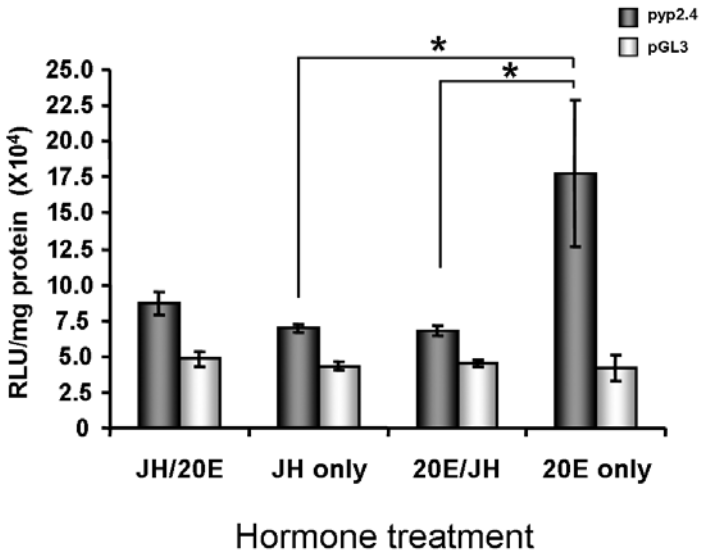


Fig. 6. *Bdyp1* promoter activity in response to 20E and JH in *Sf*21 cells. (A)Schematic illustration for the experimental design to analyze *Bdyp1* promoter activities treated with various hormones. *Sf*21 cells were co-transfected with pGL{yp2.4-luc} and pRL-TK (*Renilla* luciferase control vector). Time schedule, after two hours of cell transfection incubation in Grace's medium (▨), is marked at the top of the panel. The medium containing hormone (10^{-2} nM of 20E (20E), 10^{-3} nM of JH III (JH) or ethanol of the pertinent concentration (S)) was changed at the time indicated with inverted triangles. The cells were cultured first in a hormone containing medium (group "20E/JH" is 10^{-2} nM of 20E; group "20E only" is solvent; group "JH/20E" and group "JH only" are 10^{-3} nM of JH III) for 18 h. Then these cells were washed twice with fresh medium before being cultured in the secondary hormone containing medium (group "20E/JH" is 10^{-3} nM of JH III; group "JH only" is solvent; group "JH/20E" and "20E only" are 10^{-2} nM of 20E) for another 18 h. (B) Data represent the ratios of firefly luciferase to *Renilla* luciferase activity (relative luciferase activity, RLU). Each column represents the means of three replicates of independent transfection experiments; the error bars represent the S.D. of the mean. Asterisks (*) indicate the significance based on statistical analysis.

domestica (Agui *et al*. 1991) as well as in females of *D. melanogaster* (Bownes *et al*., 1987). In *Bdyp1* promoter, however, treatment of JH alone only exhibited a very small elevation in promoter activity (Fig. 3). In contrast, 20E significantly induced *Bdyp1* promoter activity (Fig. 3). These results suggest that 20E, but not JH, plays the critical role in the stimulation of *Bdyp1* expression.

In *Drosophila*, the YP gene, *Dmyp1*, synthesis is up-regulated by JH, but no JH responsive element occurred in the 5'-flanking region (+38 to -887) (Bownes *et al*. 1996). In contrast, as shown in Fig. 3, the activity of a promoter such as yp2.4 was inhibited by JH at concentrations higher than 0.1 nM. Whether this JH-inhibitory phenomenon implies that JH responsive elements might exist in the up-stream region of *Bdyp1* remains to be resolved.

In *Drosophila*, the YP genes in the fat body of different sexes, tissues, and even different regions of the body have diverse sensitivity to JH (Bownes and Rembold, 1987; Bownes *et al*., 1996), implying that the fluctuation of endogenous JH titers may control their development and reproduction. Additionally, ecdysteroid responsive transcription factors, such as the early response gene E74/75, EcR/USP complex and BR, especially EcR, display a striking difference in sensitivity to the physiological dose of 20E in different species and even in different tissues (Deitsch *et al*., 1995; Wang *et al*., 2000). Therefore, we speculate that the *Bdyp1* expression may not be directly regulated simply by 20E and JH; nevertheless, these details need to be further investigated.

It is well known that EcREs, the binding sites of EcR/USP complex, are located in the 5'-flanking regions of many 20E-regulated genes such as *AaVg*, *Dmhsp27* and *DmFbp-1* (Riddihough and Pelham, 1987; Antoniewski *et al*., 1994; 1996; Miura *et al*., 1999; Martín *et al*., 2001). There six putative EcREs were noted in the 5'-flanking regions of *Bdyp1*, and analysis of the activity of various lengths of promoter suggests that the functional element is likely located before -657 bp (Fig. 4). Based on these results, we speculate that EcRE-1 and 2 play a major role in mediating 20E action.

Interaction of JH and 20E in the transcription of *Bdyp1*

The effect of JH and 20E on the regulation of reproduction depends primarily on the balance between these two hormones (Gruntenki and Rauschenbach, 2008); yet the interaction of the hormones on the regulation of YP synthesis has not been clearly explained. Soller *et al*. (1999) reported that JH stimulates YP synthesis in *Drosophila* fat body/follicular cells and these proteins are taken up of by oocytes, but 20E controls the oocyte development at the pre-vitellogenic stage. However, Richard *et al*. (1998, 2001) advanced that the early vitellogenesis in *Drosophila* fat body is initiated by JH, while YP synthesis in the late stage of oogenesis is mainly stimulated by 20E. Moreover, in *Drosophila*, YP synthesis in fat body is initiated by JH (Bownes and Blair, 1986; Bownes *et al*., 1987; Soller *et al*., 1997), but there is no JH responsive element mapped in its 5'-flanking region (Bownes *et al*., 1996).

As to our studies on *B. dorsalis* in transient transfected cells, JH alone could only slightly induce *Bdyp1* transcription, while 20E extensively prompts the *Bdyp1* promoter activity (Fig 3 and 4), indicating that 20E plays the main role in the regulation of *Bdyp1* expression. In addition, an investigation into the interaction between JH and 20E as a result of the activity of *Bdyp1* promoter in the transient transfected cells reveals, rather unexpectedly, that the elevation of *Bdyp1* promoter activity by 20E is notably suppressed by the addition of JH into the medium either before or after 20E treatment (Fig. 6).

In conclusion, we have cloned and

characterized the *Bdyp1* promoter and putative 5'-flanking regulatory sequence of *B. dorsalis*. In addition, we have shown that JH can weakly induce the YP gene expression, but that 20E plays the major role in promoting the accumulation of *Bdyp1* mRNA. It is worth noting that in this paper it was for the first time demonstrated that JH is able to suppress the 20E-induction of the yolk protein synthesis in cells. Currently, we are further investigating on how these two hormones interact on the regulation of yolk protein gene expression in *B. dorsalis*.

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東方果實蠅卵黃原蛋白基因 (*Bdyp1*) 之選殖及其序列特徵與啓動子活性之分析

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摘　要

本研究選殖得東方果實蠅 (*Bactrocera dorsalis* (Hendel)) 卵黃原蛋白基因 1 (*yolk protein gene 1*, *Bdyp1*) 及其 2.7 kb 之上游區域 (GenBank accession no. EU130922)。*Bdyp1* 基因由一段長 31 bp 之 5 端非轉譯區域 (5'-untranslational region, 5'-UTR)、三個外顯子 (exon) 及一段長 141-bp 之 3 端非轉譯區 (3'-UTR) 所構成。分析 *Bdyp1* 上游 2.7 kb 序列顯示，在靠近基因起始位 (initiation site) 處具有兩個類似 TATA box 的區域，6 個可能是蛻皮激素受器反應位 (ecdysone response element, EcRE) 分布在不同的位置，以及許多如雌性雙性基因 (female specific doublesex, dsx^F)、GATA factor、E75、adult expression factor (AEF) 等等調控組織或性別表現專一性之轉錄因子 (transcription factor) 作用位置。細胞轉染 (cell transfection) 2.4 kb 上游 DNA 片段入 *Sf*21 細胞，激素測試結果顯示在 10^{-2} nM 蛻皮激素的刺激下啟動子的活性最高；進一步經啟動子刪除 (promoter deletion) 分析，結果顯示蛻皮激素作用主要的結合位 (binding site) 可能僅為調控區最靠近起始位之兩個 EcRE。另外，本研究首次發現蛻皮激素誘發啟動子活性的現象會因為處理青春激素 (無論在施用蛻皮激素之前或後) 而受到抑制。

關鍵詞：卵黃原蛋白基因啟動子、青春激素、蛻皮激素、轉錄因子、東方果實蠅。

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