Identification and Characterization of a Juvenile Hormone (JH) Response Region in the JH Esterase Gene from the Spruce Budworm, *Choristoneura fumiferana*"}

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Using a differential display of mRNA technique we discovered that the juvenile hormone (JH) esterase gene (Cfjhe) from *Choristoneura fumiferana* is directly induced by juvenile hormone I (JH I), and the JH I induction is suppressed by 20-hydroxyecdysone (20E). To study the mechanism of action of these two hormones in the regulation of expression of this gene, we cloned the 1270-bp promoter region of the Cfjhe gene and identified a 30-bp region that is located between −604 and −574 and is sufficient to support both JH I induction and 20E suppression. This 30-bp region contains two conserved hormone response element half-sites separated by a 4-nucleotide spacer similar to the direct repeat 4 element and is designated as a putative juvenile hormone response element (JHRE). In CF-203 cells, a luciferase reporter placed under the control of JHRE and a minimal promoter was induced by JH I in a dose- and time-dependent manner. Moreover, 20E suppressed this JH I-induced luciferase activity in a dose- and time-dependent manner. Nuclear proteins isolated from JH I-treated CF-203 cells bound to JHRE and the binding was competed by a 100-fold excess of the cold probe but not by 100-fold excess of double-stranded oligonucleotides of unrelated sequence. JH I induced/modified nuclear proteins prior to their binding to JHRE and 20E suppressed this JH I induction/modification. These results suggest that the 30-bp JHRE identified in the Cfjhe gene promoter is sufficient to support JH induction and 20E suppression of the Cfjhe gene.

Although the biological actions of Juvenile hormones (JHs) in insect development and reproduction are well documented, the molecular mechanisms underlying JH action are poorly understood (1–5). Several possible modes of actions have been proposed for JH. Direct action of JH in regulation of genes such as jhp21 (6), juvenile hormone esterase (7, 8), calmodulin (9), vitellogenin (10), and several others have been shown (11, 12). Through indirect action, JH was shown to modulate 20-hydroxyecdysone (20E) action by affecting the expression of genes in a 20E-induced cascade (13, 14). Totally different from the above genomic actions, in ovarian follicular epithelium, JH acts through a membrane receptor to bring about rapid enzyme activation without the need for new transcription (15).

Numerous attempts have been made to identify JH receptors. Palli et al. (16) used human retinoic acid receptor CDNA as a probe and identified a steroid/thyroid superfamily member from *Manduca sexta*. Further characterization of this cDNA revealed that this is not a JH receptor but, rather, an ecdysone-induced transcription factor that plays a critical role in ecdysone signal transduction and is related to *Drosophila* hormone receptor 3 (17), subsequently named *Manduca* hormone receptor 3 (18). The 29-kDa nuclear protein identified in *M. sexta* epidermis turned out to be a low affinity JH-binding protein (19, 20).

The mammalian retinoid X receptor (RXR) forms a heterodimer with several nuclear receptors including the farnesoid X-activated receptor (FXR). JH III but not JH acid or methoprene can bind/activate the FXR and RXR heterodimer (21). Methoprene and methoprene acid but not JH III can activate RXR (22). These two studies suggested that RXR or its insect homologue ultraspiracle (USP) could play an important role in signal transduction of JH or JH-related compounds. Jones and Sharp (23) showed that both JH III and JHB3 bind to a USP homodimer from *Drosophila melanogaster*. Subsequent studies showed that USP from *D. melanogaster* can bind to the DR12 response element and a reporter gene placed under the control of the DR12 response element fused to the jhe core promoter was induced by JH III (24).

A *D. melanogaster* mutant tolerant to methoprene (Met) was identified (25). An 85-kDa protein isolated from *Met* flies showed a 6-fold lower affinity than the wild-type protein for JH III (26). The *Met* gene was cloned and found to be a member of the basic helix-loop-helix-PER-ARNT-SIM (PAS) family of transcriptional regulators (27). *Met* is not a vital gene, as shown by the production of a null mutant allele that is viable (28), but this finding could reflect functional redundancy of *Met*. The *Met* gene product was detected in several tissues, including known JH response tissues (29).

To clone JH receptor cDNA, several approaches, including characterization of JH response gene promoters to identify JH response elements to be used for screening expression libraries to isolate JH receptors, are being pursued. Several JH-responsive genes, including calmodulin, vitellogenin, and diapause...
protein have been identified (9, 11, 30). Another JH response gene, jhp21, was identified in Locusta migratoria, and a response element has been identified in the promoter region of this gene (6). Characterization of protein that binds to this response element lead to the hypothesis that this protein is a transcription factor activated by JH and that participates in the response region. The nuclear proteins that were exposed to JH specifically bound to this 30-bp JH responsive region and were precipitated 20E. The two repeat elements found in this promoter are boxed. Perfect palindromic repeat elements are underlined with inverted arrows. Two nuclear receptor half-sites are underlined. A putative fat body consensus element is double-underlined. GATA elements are shown in bold.

**EXPERIMENTAL PROCEDURES**

**CF-203 Cells**—CF-203, a continuous cell line was developed from the midgut of C. fumiferana (33). These cells were grown in SF900 (In-vitrogen, Rockville, MD) medium supplemented with 10% fetal bovine serum. The cells grow attached to the substrate and were subcultured by trypsinization for a short time using 0.05% trypsin solution (Invitrogen, Rockville, MD) medium supplemented with 10% fetal bovine serum. The cells were treated with trypsin for about 10 s and were washed twice with neat SF900 medium. Then, 200 μl of reporter lysis buffer was added to each well and the cells were collected and assayed for luciferase reporter activity using the Luciferase™ reporter assay system from Promega (Madison, WI).

**Preparation of Nuclear Extracts**—The cells were collected and centrifuged for 5 min at 4°C. The pellet was disrupted in homogenization buffer (20 mM Tris, pH 7.0, 50 mM KCl, 300 mM sucrose, 1 mM EDTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride, homogenized, and centrifuged at 10,000 × g for 60 min at 4°C. The pellet was resuspended in nuclear lysis buffer (10 mM HEPES, pH 7.6, 100 mM KCl, 100 mM EDTA, 10% (w/v) glycerol, 3 mM MgCl2, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 0.4 mM (NH4)2SO4). After incubation for 30 min on ice, the lysate was centrifuged at 14,000 × g for 60 min at 4°C. The nuclear proteins were precipitated by adding 0.3 μl (NH4)2SO4, followed by incubation at 4°C for 30 min. After centrifugation at 14,000 × g for 30 min at 4°C, the precipitate was dissolved in nuclear dialysis buffer (25 mM HEPES, pH 7.6, 0.1 mM EDTA, 40 mM KCl, 10%
Identification of the JH Response Region—We prepared six 5’ to 3’ truncations of the 1270-bp Cfjhe promoter region and cloned them into pGL3-basic luciferase reporter vector (Promega Corp.). The nucleotide sequence is numbered relative to

**Results**

**Identification Cfjhe Promoter Region**—To identify JH and 20E response regions in the Cfjhe promoter, we screened a *C. fumiferana* genomic library with Cfjhe gene cDNA as a probe and identified six genomic clones with an average insert size of 20 kb. Restriction digestion of phage DNA followed by Southern blotting and hybridization using the Cfjhe gene cDNA as a probe identified a genomic fragment containing a 1270-bp region that is located upstream to the transcription start site. This genomic fragment was subcloned into pBSK—(Stratagene Cloning Systems, La Jolla, CA) and sequenced completely from both directions (Fig. 1). The sequence of this genomic fragment showed a conserved transcription start site at 27 bp upstream from the JHJ mRNA translation start site (8) and a typical TATA box at 22 bp upstream to the transcription start site. A search of the 1270-bp sequence (−1 to −1270) that is present upstream to the transcription start site using Patch, Match, and AliBaba2 programs available at TRANSFAC public site (transfac.gbf.de) showed a match with half-sites of several known response elements (REs), including hsp27EcRE, TATA-binding protein, FXRRE, RXRRE, and E74RE. However, when considered together, both half-sites of none of these REs showed a significant match with the 1270-bp sequence. Some of the putative REs are shown in Fig. 1. This sequence also contained a repeat of 161-bp sequence separated by 93-bp sequence (boxed in Fig. 1). This 1270-bp sequence showed 61% identity with the 1200-bp nucleotide sequence found in the 5’ flanking region of *Trichoplusia ni* jhe (36). But, a hormone response element that is similar to DR-1 RE identified in *T. ni* jhe promoter is not found in the Cfjhe promoter.

**Genomic Library Construction and Screening**—A genomic library was constructed using genomic DNA isolated from the spruce budworm larvae and EMBL3 genomic library construction kit form Promega (Madison, WI) following the manufacturer’s protocol. A library containing 500,000 clones with an average insert size of 20 kb was prepared. The genomic library was screened using Cfjhe cDNA as probe and following the methods described for cDNA library screening (35).

**Identification of the JH Response Region**—We prepared six 5’ to 3’ truncations of the 1270-bp Cfjhe promoter region and cloned them into pGL3-basic luciferase reporter vector (Promega Corp.). The nucleotide sequence is numbered relative to...
the transcription start site of CjJHE mRNA. The 27-nucleotide sequence that is present between transcription and translation start site of CjJHE mRNA was included in all constructs. These six constructs were assayed in CF-203 cells. The constructs that contained the CjJHE promoter region from −1270 to +1, −1013 to +1, −956 to +1, and −641 to +1 showed 10- to 15-fold induction of luciferase reporter activity in the presence of JH I when compared with the activity in the presence of Me2SO (Fig. 2). On the other hand, the constructs that contained the CjJHE promoter region from −424 to +1 showed only 2-fold induction, and the constructs that contained −74 to +1 did not show any induction of reporter activity in the presence of JH I. These results showed that the JH I response region in the CjJHE promoter is located between −641 and −424 bp upstream from the transcription start site. The steroid hormone 20-hydroxyecdysone (20E) did not induce reporter activity through any of the constructs tested, but it was able to prevent JH I-induced reporter activity when both hormones were administered simultaneously. These results showed that the 217-bp region of the CjJHE promoter that lies between −641 and −424 was able to support both JH induction and 20E suppression as observed for CjJHE gene (8). Although, the construct containing −424 to +1 showed a small increase in reporter activity in the presence of JH I, further analysis of this region did not show any JH I-mediated response. As will be shown later, the only recognizable hormone response element (a half-site) in this region, AGGTCA, present at −208 appears to play no role in JH I induction of the reporter gene regulated by the 1270-bp CjJHE promoter.

The 217-bp fragment that lies between −641 and −424 was further analyzed by preparing three truncations containing lengths of 217 bp (−641 to −424), 150 bp (−574 to −424), and 75 bp (−499 to −424). Each of these three fragments was fused with the CjJHE gene core promoter (which includes a 46-bp sequence containing the TATA box and the transcription start site, −30 to +15) and cloned into pGL3-basic vector. These three truncation constructs were assayed in CF-203 cells. As shown in Fig. 3, only the construct containing the 217-bp CjJHE promoter fragment showed induction by JH I suggesting that the JH response region in the CjJHE promoter is located between −641 and −574 bp upstream from the transcription start site.

We prepared three truncations of the 67-bp fragment that lies between −641 and −574. Three fragments of the CjJHE promoter 67 bp (−641 to −574), 50 bp (−624 to −574), and 30 bp (−604 to −574) were fused to the CjJHE gene core promoter (−30 to +15) and cloned into pGL3-basic vector and assayed in CF-203 cells. All three constructs showed 10- to 15-fold induction of reporter activity in JH I-treated cells when compared with the reporter activity in Me2SO-treated cells indicating that the 30-bp region between −604 and −574 is sufficient for a JH induced response observed for CjJHE gene (Fig. 4). This sequence contains direct repeat elements separated by a four-nucleotide spacer and shows 100% identity with consensus direct repeat 4 (DR4) element (Fig. 1). This 30-bp region is designated as a putative juvenile hormone response element (JHRE).

**JHRE Is Sufficient to Support JH Induction and 20E Suppression**—To test if the 30-bp fragment that supported JH I induction is also sufficient for 20E suppression of JH I-induced reporter activity, we transfected pGL3JHRE(1270) (where the luciferase reporter was regulated by the 1270-bp fragment) or

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**FIG. 3. Identification of JH response region in a 217-bp fragment of CjJHE gene.** To map the minimal JH response region in the 217-bp fragment (−641 to −424), three truncations of this region containing 217 bp (−641 to −424), 150 bp (−574 to −424), and 75 bp (−499 to −424) fragments were fused to CjJHE core promoter (−30 to +15) and cloned into the pGL3 vector. *A*, schematic of constructs; *B*, the three constructs were transfected into CF-203 cells, and the cells were exposed to 1 μM JH I. At 24 h after adding ligand, the luciferase activity was assayed. Mean ± S.D. (n = 3) are presented. The construct containing the entire 217-bp fragment of CjJHE promoter, showing 15-fold induction of luciferase activity, was the only one showing induction thus indicating that the JH response region is within the 67-bp fragment, which is located between −641 and −574 of CjJHE promoter.
pGL3JHRE (30) (where the luciferase reporter gene is regulated by 30-bp JHRE fused to the Cfjhe gene core promoter, −30 to +15) or pGL3 alone into CF-203 cells. The transfected cells were exposed to Me2SO, JH I, 20E, or JH I plus 20E. As shown in Fig. 5A, the 30-bp region from the Cfjhe promoter is sufficient to support JH I induction as well as 20E suppression of JH I-induced reporter activity. The luciferase reporter in pGL3 vector was not induced by either JH I or 20E or both in combination.

Dose response and Time Course of JH I Induction—The JH I induction of reporter activity through the 30-bp Cfjhe promoter is both JH I dose- and exposure time-dependent. The induction of reporter activity started at as low as 40 nM JH I and increased with the increased concentration of JH I and reached peak levels at 1000 nM concentration of JH I (Fig. 5B). There was a slight decrease in reporter activity at higher concentration of JH I (5000 nM). This may be due to the cytotoxicity of hormone to the cells. The induction of reporter activity increased with an increase in time and reached peak levels by 24 h after adding the hormone (Fig. 5C). There was a decrease in reporter activity by 48 and 72 h after adding hormone.

Nuclear Proteins Isolated from CF-203 Cells Specifically Bind to JHRE—To determine whether JHRE from the Cfjhe promoter binds to nuclear proteins that are present in CF-203 cells, we performed an electrophoretic mobility shift assay (EMSA). Nuclear extracts were prepared from JH I-treated and Me2SO-treated CF-203 cells, and the nuclear proteins were incubated with end-labeled double-stranded oligonucleotides synthesized based on 30-bp JHRE sequence (Table I). As shown in Fig. 6A, the nuclear proteins isolated from JH I-treated CF-203 cells bound to the JHRE, and the binding was competed by a 100-fold excess cold probe but not by a 100-fold excess double-stranded oligonucleotides of unrelated sequence indicating that the binding is specific. To determine if nuclear proteins that bind to JHRE need prior exposure to JH, we cultured CF-203 cells in the presence of Me2SO or 1 μM JH I for 3, 6, 12, and 24 h, and the nuclear proteins were isolated and assayed by EMSA. Nuclear proteins from the cells that were exposed to JH I, but not from the cells that were exposed to Me2SO, bound to JHRE suggesting that JH I induces/modify one or more nuclear proteins that bind to JHRE (Fig. 6B). To test whether 20E suppresses JH I induction/modification of nuclear proteins prior to their binding to JHRE, we cultured CF-203 cells in medium containing Me2SO or 1 μM JH I or 1 μM each of JH I and 20E for 24 h, the nuclear proteins were isolated and analyzed for their binding to JHRE by EMSA. As shown in Fig. 6C, nuclear proteins isolated from JH I-treated cells bound to JHRE, but the nuclear proteins isolated from Me2SO, 20E, or 20E plus JH I treated cells did not bind to JHRE. These data show that JH I induces/modify nuclear proteins prior to their binding to JHRE and that 20E can suppress this action of JH I.
Identification of Nucleotides Critical for Binding of 30-bp JHRE to Nuclear Proteins

To further characterize JHRE and to identify nucleotides critical for binding to nuclear proteins, we produced mutant versions of 30-bp JHRE (Table I) and used them in competition experiments. Nuclear proteins isolated from CF-203 cells bound to the 30-bp JHRE and the binding activity was quantified. Mean ± S.D. (n = 3) values are presented.

**Fig. 5.** (A) the 30-bp region from the Cfjhe promoter is sufficient to support both JH induction and 20E suppression of reporter gene expression. CF-203 cells were transfected with pGL3JHRELuc containing either the 1270-bp or 30-bp Cfjhe promoter. The transfected cells were grown in the medium containing 1 μM JH I, 1 μM 20E, or 1 μM each of JH I and 20E for 24 h, the cells were harvested, and luciferase activity was quantified. Mean ± S.D. (n = 3) values are presented. (B) dose response of JH I induction of luciferase expression under the control of 30-bp Cfjhe promoter. CF-203 cells were transfected with the pGL3 vector containing the 30-bp Cfjhe promoter, the cells were exposed to various concentrations of JH I, and the luciferase activity was measured at various times after addition of hormone. The reporter activity increased up to 24 h after adding hormone, and then started to decrease. Mean ± S.D. (n = 4) are presented.

**Fig. 6.** Nuclear proteins isolated from JH I-treated CF-203 cells bind to the 30-bp fragment of the Cfjhe promoter. Nuclei isolated from JH I-treated and Me2SO-treated CF-203 cells were incubated with 32P-labeled double-stranded 30-bp oligonucleotides corresponding to the -604 to -574 JH response sequence of the Cfjhe promoter. The DNA-protein complexes were separated on 6% polyacrylamide gels. (A) 32P-labeled 30-bp probe alone, probe incubated with nuclear proteins isolated from JH I-treated cells, and 100X excess cold probe or 100X excess double-stranded oligonucleotides of unrelated sequence were analyzed. The nuclear proteins isolated from JH I-treated CF-203 cells bound to the 30-bp fragment and 100X excess cold probe but not nonspecific oligonucleotides competed for the binding. (B) The nuclear proteins isolated from CF-203 cells that were grown in the presence of Me2SO or JH I for 3 or 6 or 12 or 24 h were analyzed to assess their binding to the 30 bp probe. The nuclear proteins isolated from CF-203 cells that were grown in the presence of Me2SO did not bind to JHRE, but the nuclear proteins isolated from CF-203 cells that were exposed to JH I for 3, 6, 12, or 24 h bound to JHRE. C, the nuclear proteins isolated from CF-203 cells, which were grown in the presence of Me2SO, JH I, 20E, or JH I plus 20E for 24 h, were analyzed to assess their binding to 30-bp JHRE probe. The nuclear proteins isolated from CF-203 cells that were grown in the presence of Me2SO did not bind to JHRE, but the nuclear proteins isolated form CF-203 cells that were exposed to JH I plus 20E or 20E alone did not bind to JHRE.

**Identification of Nucleotides Critical for Binding of 30-bp JHRE to Nuclear Proteins—**To further characterize JHRE and to identify nucleotides critical for binding to nuclear proteins, we produced mutant versions of 30-bp JHRE (Table I) and used them in competition experiments. Nuclear proteins isolated from CF-203 cells bound to the 30-bp JHRE and the binding...
was competed by 25-fold excess cold probe (Fig. 7A). A 25-fold excess of a 30-bp oligonucleotide containing a mutation changing G to A in the left half-site (M1, Table I) or in both the half-sites (M2, Table I) or in the right half-site (M3, Table I) showed reduced competition (Fig. 7B). These results showed that G residues present in repeat elements, truncated oligonucleotides where some of the nucleotides at the 5′ end of JHRE were eliminated were synthesized (M5–M8, Table I). These truncated oligonucleotides showed reduced competition as more nucleotides were eliminated (Fig. 7). Mutant 8, in which all nucleotides upstream to the repeat elements were eliminated, showed significantly less competition when compared with the cold probe. The 30-bp JHRE we identified for JH I induction of a reporter gene placed under the control of JHRE, we prepared three mutants of pGL3JHRE(1270) where G residues in each AGATTA element (G-588A and G-598A) were changed to A (G-208A). Analysis of mutant and wild type (WT) constructs in CF-203 cells showed that both mutants where G changed to A (G-208A) reduced JH I induction of reporter gene by 70% when compared with WT (Fig. 9). The suppression by 20E worked in these mutants, because 20E was able to reduce JH I-induced binding of nuclear proteins to JHRE suggesting that the two AGATTA direct repeat elements with a 4-nucleotide spacer is probably the binding site for nuclear proteins. Consequently, we prepared six additional mutants of JHRE (M9–M14) changing conserved nucleotides in the AGATTA element to non-conserved residues (the consensus sequence for DR4 is RGRNYANNNNRGRNYA, where R = A or G, N = A, G, T, or C, and Y = A or T). In mutant 14, the AGATTA was changed to AGGTCA, a common half-site found in hormone response elements. Analysis of these mutants by EMSA showed that Mutants 10, 11, and 12 showed significantly less competition compared with the other mutants. These three mutants had changes in conserved residues of the DR4 element (mutant 10; G in AGATTA was changed to A, mutant 11; the sixth residue, A in AGATTA was changed to T and mutant 12; the third residues in AGATTA changed to T) (Fig. 8, A and B). Mutant 14, where the AGATTA elements were changed to AGGTCA, competed well with the probe for binding of nuclear proteins (Fig. 8, A and B). These data suggest that the DR4 element present in the 30-bp JHRE is responsible for binding to nuclear proteins isolated from CF-203 cells.

To determine whether the G residue in the AGATTA element that is critical for binding of nuclear proteins is also important for JH I induction of a reporter gene placed under the control of JHRE, we prepared three mutants of pGL3JHRE(1270) where the G residues in each AGATTA element (G-588A and G-598A) as well as the G residue in AGGTCA found at -208 were changed to A (G-208A). Analysis of mutant and wild type (WT) constructs in CF-203 cells showed that both mutants where G residues in AGATTA element was changed to A (G-588A and G-598A) reduced JH I induction of reporter gene by 70% when compared with WT (Fig. 9). The suppression by 20E worked in these mutants, because 20E was able to reduce JH I-induced
reporter activity. On the other hand, changes to G residues in AGGTCA at −208 (G-208A) did not effect JH I induction or 20E suppression. These data suggest that the G residues in the DR4 element present in the Cfjhe promoter are important for JH I induction of this gene.

**DISCUSSION**

The major contribution of current study is the identification of a 30-bp JHRE within the Cfjhe promoter that is sufficient for JH induction and 20E suppression of this induction observed for Cfjhe gene. Several lines of evidence support this conclusion. First, when the luciferase gene was placed under the control of this 30-bp JHRE and the Cfjhe core promoter (−25 to +15), JH I induced reporter activity, and, in turn, 20E suppressed this JH I-induced reporter activity. Second, nuclear proteins isolated from CF-203 cells exposed to JH I specifically bound to this 30-bp JHRE. Third, other regions of the 1270-bp Cfjhe promoter did not support JH I induction. Taken together, these data conclusively show that the identified 30-bp JHRE is responsible for the JH induction and 20E suppression observed for the Cfjhe gene (8).

The 30-bp JHRE contains two direct repeats of AGATTA with a four nucleotide spacer and these elements show 100% similarity with the consensus DR4 element (RGRNNYNRRGNYA, where R = A or G, N = A or G or T or C and Y = A or T). Mutagenesis experiments showed that changing the competition of Me₂SO, 1 μM JH I, 1 μM 20E, or 1 μM each of JH I and 20E for 24 h, the cells were harvested, and luciferase activity was quantified. Mean ± S.D. (*n* = 3) values are presented.

*Fig. 9. Mutation of the G residue in AGATTA element reduces JH I response through this element. CF-203 cells were transfected with pGL3JHRE (1270) or its mutant versions. G in the first AGATTA element was changed to A (G-598A), G in the second AGATTA element was changed to A (G-598A), or G in AGGTCA found at −208 (underlined in Fig. 1) was changed to A (G-208A). The transfected cells were grown in medium containing Me₂SO, 1 μM JH I, 1 μM 20E, or 1 μM each of JH I and 20E for 24 h, the cells were harvested, and luciferase activity was quantified. Mean ± S.D. (*n* = 3) values are presented.*
Juvenile Hormone Response Elements

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