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Identification and Characterization of a Juvenile Hormone Response Element and Its Binding Proteins*

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Abstract

Juvenile hormones (JH) regulate a wide variety of developmental and physiological processes in insects. Comparison of microarray data on JH-induced genes in the fruit fly, *Drosophila melanogaster*, L57 cells and in the honey bee, *Apis mellifera*, identified 16 genes that are induced in both species. Analysis of promoter regions of these 16 *D. melanogaster* genes identified DmJHRE1 (*D. melanogaster* JH response element 1). In L57 cells, the reporter gene regulated by DmJHRE1 was induced by JH III. Two proteins (FKBP39 and Chd64) that bind to DmJHRE1 were identified. FKBP39 and Chd64 double-stranded RNA inhibited JH III induction of a reporter gene regulated by DmJHRE1. FKBP39 and Chd64 proteins expressed in yeast bound to DmJHRE1. Two-hybrid and pull-down assays showed that these two proteins interact with each other as well as with ecdysone receptor, ultraspiracle, and methoprene-tolerant protein. Developmental expression profiles and JH induction of mRNA for FKBP39 and Chd64 proteins and their interaction with proteins known to be involved in both JH (methoprene-tolerant protein) and ecdysteroid action (ecdysone receptor and ultraspiracle) suggest that these proteins probably play important roles in cross-talk between JH and ecdysteroids.

Insect development is regulated by the combined action of ecdysteroids and juvenile hormones. A great deal is known about the biological and molecular action of 20-hydroxyecdysone (20E).[2] The 20E receptor is a heterodimer composed of two nuclear receptor superfamily members, ecdysone receptor (EcR) and ultraspiracle (USP). The unliganded EcR·USP receptor complex binds to a short palindromic DNA response element, the EcRE, repressing the transcription of target genes (1). When bound to 20E, the receptor complex is converted into a transcriptional activator (2, 3). The mechanism of juvenile hormone (JH) action remains poorly understood. During larval development, JH modulates 20E action so that a repetition of a larval molt rather than metamorphosis occurs. For this reason, JH is referred to as the “status quo” hormone (4, 5). At the molecular levels, JH is known to modify or suppress the expression of genes involved in 20E signal transduction (6–8). JH is also known to exert its action through direct influence on gene expression independent of 20E (9–11). The involvement of a cell surface-initiated signal transduction

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[2]The abbreviations used are: 20E, 20-hydroxyecdysone; JH, juvenile hormone; JHRE, juvenile hormone response element; X-gal, 5-bromo-4-chloro-3-indolyl-*β*-D-galactopyranoside; EcR, ecdysone receptor; USP, ultraspiracle; GST, glutathione *S*-transferase; LC, liquid chromatography; MS/MS, tandem mass spectrometry; dsRNA, double-stranded RNA; ORF, open reading frame; qRT, quantitative reverse transcription; RNAi, RNA interference.

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mechanism requiring calcium and protein kinase C in JH action was reported (12–17), and a JH-binding protein in the membranes of *Locusta migratoria* follicle cells was found (15). Currently, there is inconclusive experimental evidence to determine whether the JH-dependent induction of gene expression requires only hormone binding to the cell surface or whether induction is mediated through a receptor-dependent mechanism requiring hormone entry into the cell.

Many attempts have been made to identify JH receptors. Jones and Sharp (18) showed that JH III binds to *Drosophila melanogaster* USP (DmUSP), a homologue of the vertebrate retinoid X receptor, inducing conformational changes and homo-oligomerization activity. Moreover, DmUSP-specific binding to the DR12 response element transduces JH III signaling in a transfection system (19). However, the estimated dissociation constant for binding of JH III for the USP receptor is much greater than the concentration of JH typically found in either whole body or serum (20). Another candidate gene for JH receptor is the methoprene-tolerant gene (*Met*) found in *D. melanogaster* (DmMet). DmMet is a member of the basic helix-loop-helix-PAS family of transcriptional regulators. Met is involved in the molecular action of JH through binding to JH and/or through partnering with itself or other proteins to control transcription of one or more downstream effector genes. The Met mutants are resistant to the toxic and morphogenetic effects of JH and JH agonist insecticides, such as methoprene (21–28). However, the product of *DmMet* is not vital, as shown by the production of null mutants that are viable (27, 29).

Several approaches are being pursued to identify JH receptor. Among them is identification of JHRE to be used for screening expression libraries to isolate JH receptor. Several JH-responsive genes have been identified (30–37). In addition, a JH response gene, *JHP21*, was identified from *Locusta migratoria*, and a JHRE has been identified in the promoter region (33, 38). Characterization of the protein complex (including a 35-kDa protein, a transcription factor) that binds to this response element suggests that JH induces the association of preexisting proteins to form an active complex, which binds to the JHRE upstream of *jhp21* and regulates its transcription (38–40). Kethidi *et al.* (17) identified a 30-base pair sequence (*Choristoneura fumiferana* juvenile hormone esterase; CfJHRE), located in the 5′ promoter region of the spruce bud-worm JH esterase gene (from −604 to −574) that showed 100% similarity with the consensus DR4 element (RGRNYANNNNRGRNYA, where R represents A or G, N is any base, and Y is C or T). This element, CfJHRE, is sufficient to support both JH-dependent induction and 20E suppression of this induction.

To study the molecular mechanisms of JH action, microarray analyses were performed, and the genes that are induced by JH in cultured *Drosophila* L57 cells and honey bee *Apis mellifera* have been identified[3] (41). Comparative analysis of JH-induced genes identified 16 genes that are induced by JH in both fly cells and bee brains. The promoter regions of 16 *D. melanogaster* genes were then used to identify a *cis*-acting DNA regulatory element, JHRE. The JHRE was used in a DNA affinity column to identify two proteins, FKBP39 and Chd64, that bind to the JHRE. These two proteins were expressed in yeast bound to the JHRE. Two-hybrid assays as well as an *in vitro* GST pull-down assay showed that these two proteins not only interact with each other but also interact with other proteins, DmEcR, DmUSP, and DmMet, known to be involved in 20E and JH action.

[3]S. R. Palli, unpublished observation.

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EXPERIMENTAL PROCEDURES

Chemicals

JHIII and 20E were purchased from Sigma. All ligands were applied in Me_2SO, and the final concentration of Me_2SO was maintained at 0.001%. Trypsin (modified, sequencing grade, lyophilized) was purchased from Promega (Madison, WI). Protease inhibitor mixture and dithiothreitol were purchased from Sigma. Other commonly used reagents were purchased from Fisher unless otherwise indicated.

Computational Identification of cis-Regulatory Motifs

The MEME algorithm (42) was used for uncovering common motifs present in the promoter regions of 3 kb upstream of the transcription start sites in 16 JH-induced genes in *Drosophila* L57 cells. MAST (motif alignment and search tool; available on the World Wide Web) was used to search whether the identified *cis*-regulatory motif exists at 3 kb upstream of the transcription start site in homologs of *A. mellifera* in these 16 JH-induced genes.

Preparation of L57 Cell Nuclear Extracts and Yeast Cell-free Extracts and Electrophoretic Mobility Shift Assays

Methods recently were followed for the preparation of nuclear extracts from L57 cells (17). For yeast cell-free extract preparation, yeast cells harboring pGildaFKBP39 and pGildaChd64 (see below) were grown in synthetic dropout (SD) medium containing 2% galactose and 1% raffinose lacking uracil and histidine (SD/−His/−Ura) and pB42ADFKBP39 and pB42ADChd64 (see below) in minimum SD media lacking uracil and tryptophan (SD/−Trp/−Ura) overnight at 30 °C for 16 h. Expression of the proteins was induced by diluting the culture 10-fold in 100 ml of respective SD medium and growing cells for 16 h at 30 °C. Cells were collected by centrifugation and washed with cold water. After resuspending the pellet in an extraction buffer containing 50 mM Tris-HCl, pH 7.5, 600 mM KCl, 10% sucrose, 5 mM β-mercaptoethanol, and Roche Applied Science protease inhibitor mixture tablets, cells were homogenized with acid-washed glass beads in a Bead-beater (2000 GENO/GRINDER; Spexcertiprep Inc., Metuchen, NJ) four times at the highest setting for 30 s each. The sample was kept on ice for 30 s between the two bead beatings. Cell-free extracts were obtained following centrifugation at 12,000 × *g* for 1 h at 4 °C to remove cell debris. The supernatant was collected and snap-frozen with liquid nitrogen and stored at −80 °C until use. The electrophoretic mobility shift assay method described by Kethidi *et al.* (17) was followed.

Construction of DmJHRE1 Reporter Plasmids

The following oligonucleotides were synthesized, annealed, and kinased: 5′-TCGACTCGCGCACTCTCTCTCTCTCCCGCTCTC-3′; 5′-TCGAGAGAGCGGGAGAGAGAGAGAGTGCGCGAG-3′. The oligonucleotide contains the 29-bp *DmJHRE1* sequence. Flanking the 29-bp DmJHRE1 sequence are SalI/XhoI restriction enzyme sites (underlined). For pMK12(DmJHRE1·1F) and pMK22(DmJHRE1·1R) construction, pMK43.2 as described (43) was digested with SalI, and the oligonucleotides were ligated to the digested vector. Sequencing analysis shows that pMK12(DmJHRE1·1F) and pMK22(DmJHRE1·1R) constructs have a 1× forward or reverse insert, respectively. In both constructs, a minimal promoter (*Adh* distal promoter) and β-galactosidase cassette are present downstream of DmJHRE1.

For pGL3(DmJHRE1·1F3R) and pGL3(DmJHRE1·6F) construct preparation, the 30-bp CfJHRE element in pGL3JHRE (17) was digested with XhoI and HindIII and replaced with the *cfjhe* gene core promoter, −30 to +15, to get the pGL3MP construct. The resulting construct pGL3MP was cut with XhoI and dephosphorylated with calf intestinal alkaline

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phosphatase, and then the oligonucleotides were ligated to the digested vector. Sequencing analysis shows that the pGL3(DmJHRE1·1F3R) and pGL3(DmJHRE1·6F) contain 1× forward plus 3× reverse inserts and 6× forward inserts, respectively. In both constructs, a firefly (*Photinus pyralis*) luciferase cassette is present downstream from the multiple copies of DmJHRE1.

Insect Cell Culture and Transfection

Methods recently described were followed for L57 cell culture as well as for cell transfection (16). For β-galactosidase activity, 5 μl of lysates were assayed using the Applied Biosystems Gal-Screen® chemiluminescent reporter gene assay system (Foster City, CA). For luciferase activity, 20 μl of cell lysates were assayed using the Promega Luciferase™ reporter assay system.

Affinity Purification and Identification of DmJHRE1-binding Proteins

Nuclear proteins isolated from JH-treated L57 cells were used for affinity purification of DmJHRE1-binding proteins. Three liters of L57 cells treated with JH III for 24 h were transferred from culture flasks (225 cm^2) to 50-ml conical centrifuge tubes and centrifuged for 5 min at 450 × *g*. The pellets were collected, and 5 volumes of lysis buffer (10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, and 10 mM KCl) containing freshly prepared dithiothreitol (final concentration of 1 mM) and 100× diluted Sigma protease inhibitor mixture were added, and the pellets were resuspended gently. After incubation at 4 °C for 15 min, the lysates were centrifuged at 4 °C for 15 min at 420 × *g*. The pellets were then transferred into glass tissue grind tubes and homogenized on ice slowly with 10 up-and-down strokes. Then the disrupted cells were centrifuged for 20 min at 10,500 × *g*. The crude nuclear pellets were resuspended in 0.6× (v/v) extraction buffer (20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, and 25% (v/v) glycerol) containing freshly prepared dithiothreitol (final concentration of 1 mM) and 100× diluted Sigma protease inhibitor mixture and incubated for 30 min with gentle shaking. The extracts were centrifuged for 5 min at 21,000 × *g*. The supernatants were collected and snap-frozen with liquid nitrogen and stored at −80 °C until use.

Complementary oligonucleotides synthesized based on the 29-bp DmJHRE1 sequence (5′-CTCGCGCACTCTCTCTCTCTCCCGCTCTC-3′) were phosphorylated using T4 polynucleotide kinase and annealed to produce double-stranded oligonucleotides and then ligated with T4 DNA ligase. The oligonucleotide multimers were coupled to commercial CNBr-activated Sepharose 4B resin (GE Healthcare) following the manufacturer's protocol. The prepared DmJHRE1 DNA affinity column was used to purify DmJHRE1 binding proteins using the methods previously described (44). From here on, all operations were performed at 4 °C. DmJHRE1 DNA affinity resin (3 ml) was equilibrated in a Bio-Rad Econo-Column with buffer Z (25 mM Hepes (pH 7.8), 12.5 mM $MgCl_2$, 1 mM dithiothreitol, 20% (v/v) glycerol, 0.1% (v/v) Nonidet P-40) containing 0.1 M KCl. Crude nuclear extracts derived from 3 liters of *D. melanogaster* L57 cells were resuspended in buffer Z containing 0.1 M KCl and were combined with 500 μg of sonicated calf thymus DNA and incubated for 15 min. The solution (protein/DNA mixture) was passed through the *DmJHRE1* DNA affinity resin at 20 ml/h by gravity flow. The resin was washed four times with 5 ml of buffer Z containing 0.1 M KCl. After the buffer reached the resin bed, 3.2 ml of buffer Z containing 1.0 M KCl was added to the column, and the resin was thoroughly mixed with the buffer. The resin was allowed to stand for 30 min, the proteins were eluted, and the major elution fraction of DmJHRE1 DNA-binding proteins (3.2 ml) was collected. 3.2 ml of buffer Z containing 0.5 M KCl added to the column and the second elution fraction of DmJHRE1 DNA-binding proteins (3.2 ml) was collected. The affinity column was regenerated by washing with 5 mM Tris-HCl (pH 7.6), 2.5 M NaCl, 0.5 mM EDTA (60 ml),

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10 mM Tris-HCl (pH 7.6), 0.3 M NaCl, and 1 mM EDTA (40 ml). The DmJHRE1 DNA-binding protein fractions were then combined and diluted to 0.1 M KCl with buffer Z without KCl, mixed with sonicated calf thymus DNA, and reapplied to the affinity resin. The same procedure described above was executed two times for further purification. Finally, the eluted fractions of the DmJHRE1 DNA-binding proteins were concentrated with Millipore (Billerica, MA) Microcon YM-10 centrifugal filters to 30 μl.

The eluted samples from the DmJHRE1 affinity column were then digested with trypsin and LC-MS/MS analysis as previously described (45). The LC-MS/MS data were submitted to the MASCOT server for MS/MS ion search (46). The peak lists from the LC-MS/MS spectra were generated by the MASCOT script embedded in the Analyst QS software. The typical parameters used in the MASCOT MS/MS ion search are as follows: *Drosophila* (fruit fly), maximum of two trypsin mis-cleavages, cysteine carbamiodomethylation, methionine oxidation, a maximum of 100 ppm MS error tolerance, and a maximum of 0.3 Da MS/MS error tolerance.

Double-stranded RNAs (dsRNAs) Preparation and Transfection

cDNA clones for the identified proteins were purchased from the *Drosophila* Genomics Resource Center, Indiana university (available on the World Wide Web). Using the plasmids as DNA template, the individual DNA fragments (*fkbp39*, 377 bp; *chd64*, 266 bp) for the proteins to be knocked down were amplified by PCR. Each primer used in the PCR contained a 5′ T7 RNA polymerase binding site (TAATACGACTCACTATAGGG) followed by a gene-specific sequence: *fkbp39* gene, forward primer (5′-AGCGTTTCTCTGTTGGGCTA-3′) and reverse primer (5′-TGCTCCTTGCCAGATTTCTT-3′); *chd64* gene, forward primer (5′-CTTCTTCGAGGTGCTCAAGG-3′) and reverse primer (5′-CAGGCAGATAACCACCGAGT-3′). The PCR products were gel-purified using the QIAquick gel extraction kit from Qiagen (Valencia, CA). The purified PCR products were used as templates by using a HiScribe RNAi transcription kit from New England BioLabs (Ipswich, MA) to produce the dsRNA according to the protocols provided by the manufacturer. The dsRNA was stored at −20 °C.

Drosophila S2 cells were grown in 25-cm^2 cell culture flasks using Schneider's *Drosophila* medium from Invitrogen. S2 cells were transfected with SuperFect® lipid (Qiagen Inc., Valencia, CA) using an adaptation of the manufacturer's protocol. Briefly, cells were seeded and allowed to settle overnight, and dsRNA was complexed with transfection reagent in Schneider's *Drosophila* medium without serum. The complex was incubated at room temperature for 20 min and then added to the cells from which normal growth medium had been removed. After a 4-h incubation, an equal volume of Schneider's *Drosophila* medium plus 20% FBS was added to the cell/complex mixture. 72 h after the initiation of transfection, the ligands were added to the transfected cells, and the cells were harvested at 24 h after adding the ligands. The luciferase reporter activity was assayed using the Promega Luciferase™ reporter assay system.

Yeast, Insect Cell, and Mammalian Cell Two-hybrid Assays

For the yeast two-hybrid interaction assay, we used the Clontech Yeast Matchmaker LexA system (BD Biosciences Clontech) with the LexA DNA-binding domain plasmid, pGilda, and the B42 activation domain plasmid, pB42AD, and *lacZ* reporter plasmid, p8op-lacZ, which carries the *lacZ* gene under the control of eight LexA operators and the minimal TATA region from the *Gal1* promoter. *DmEcR*, *DmUSP*, and *DmMet* ORFs as well as the identified protein ORFs (*FKBP39* and *Chd64*) were generated by PCR with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) from available cDNAs. (The cDNAs of

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FKBP39 and *Chd64* are from the *Drosophila* Genomics Resource Center.) Full-length *FKBP39* and *Chd64* PCR products were inserted into the plasmid pGilda as EcoRI/XhoI and EcoRI/NotI restriction fragments, respectively. The *FKBP39* (full-length) and *Chd64* (amino acids 52–188) PCR products were inserted into the plasmid pB42AD as EcoRI/XhoI restriction fragments, respectively.

Yeast EGY48 strain harboring the reporter genes *LEU2* and *lacZ* (β-galactosidase) were transformed with plasmids pGildaEcR, pGildaUSP, pGildaMet, pGildaFKBP39, pGildaChd64, pB42ADEcR, pB42ADUSP, pB42ADMet, pB42ADFKBP39, and pB42ADChd64, individually or in combination with the Frozen-EZ Yeast Transformation II kit from Zymo Research Corp. Transformed cells were grown on minimum plates for 3–4 days at 30 °C. Colonies from each plate were transferred to SD/−His/−Leu/−Trp/−Ura plate with X-Gal and incubated at 30 °C; β-galactosidase activity was determined by the time required for colonies to turn blue in colonies derived from the colorless X-Gal.

For the insect two-hybrid interaction assay, we used the modified Clontech Mammalian Matchmaker assay system (BD Biosciences Clontech). We replaced the promoter of the constitutive SV40 early promoter P_{sv40e} in both the GAL4 DNA-binding domain plasmid (pM) and the VP16 activation domain plasmid (pVP16) by an *Autographa californica* nucleopolyhedrovirus (AcMNPV) IE1 promoter, and the resulting plasmids were designated as pIE1M and pIE1VP16, respectively. The reporter plasmid was pG6KZ-Luc (a gift from RheoGene, Inc.), which contains six consensus GAL4 binding sites and a synthetic minimal promoter upstream of the *firefly* luciferase gene. *DmEcR*, *DmUSP*, and *DmMet* ORFs as well as the identified protein ORFs (*FKBP39* and *Chd64*) were generated by PCR with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen) from available cDNAs. (The cDNAs of *FKBP39* and *Chd64* are from the *Drosophila* Genomics Resource Center.) To prepare pIE1VP16FKBP39 and pIE1VP16Chd64 fusion protein constructs, full-length *FKBP39* and *Chd64* were excised out of pB42ADFKBP39 and pGildaChd64 vectors using EcoRI/XhoI and EcoRI/BamHI restriction enzymes, respectively, and then cloned into the EcoRI/SalI and EcoRI/BamHI restriction sites in the basic pIE1VP16, respectively. To get pIE1MFKBP39 and pIE1MChd64 fusion protein constructs, full-length *FKBP39* and *Chd64* were excised out of pIE1VP16FKBP39 and pIE1VP16Chd64 using EcoRI/XbaI restriction enzymes and then cloned into the same restriction sites in pIE1M vectors, respectively. All constructs retain the stop codon from the wild-type ORF. A previously established procedure was followed for the transient transfection (17). Briefly, various combinations of pIE1M and pIE1VP16 were transfected into the *Drosophila* S2 cells with the reporter gene pG6KZ-Luc by SuperFect® lipid (Qiagen Inc., Valencia, CA). A second reporter, *Renilla* luciferase, expressed under the *Autographa californica* multicapsid nucleopolyhedrovirus IE1 promoter, was cotransfected into cells and used for normalization. After 4 h of transfection, 50 μl of Schneider's *Drosophila* medium from Invitrogen containing 20% fetal bovine serum and 1 μM ligands were added. All ligands were applied in Me_2SO, and the final concentration of Me_2SO was maintained at 0.1% in both controls and treatments. Twenty-four hours after transfection, the medium was discarded, and the cells were lysed in 25 μl of passive lysis buffer (Promega). Ten microliters of extract were transferred to 96-well opaque plates, and the f*irefly* luciferase and *Renilla* luciferase reporter activities were measured using the Dual Luciferase™ reporter assay system from Promega and Fluoroskan Ascent FL (ThermoLab Systems, Helsinki, Finland). All of the transfection experiments were performed in triplicate, and the experiments were repeated at least three times.

The FKBP39 and Chd64 protein coding regions were cloned into Clontech Mammalian Matchmaker assay system vectors, pM and pVP16 (BD Biosciences Clontech). Transfections and the luciferase reporter assays were performed as previously described (47).

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Protein Expression in Vitro and in Vivo in Escherichia coli and GST Pull-down Experiments

The entire FKBP39 (residues 1–357), Chd64 (residues 1–188), and DmMet (residues 1–716) and the N-terminal A/B region-deleted DmEcR (residues 224–878) and USP (residues 172–508) in the pIE1M vector were cut with EcoRI and XbaI and then cloned into the same restriction sites in pACT (Promega). *In vitro* protein synthesis was performed using the TNT® T7 quick coupled transcription/translation system utilizing the T7 promoter (Promega) according to the manufacturer's instructions using 2 μl of [^{35}S]methionine, 1000 Ci/mmol (GE Healthcare). To monitor the *in vitro* reaction, 3-μl aliquots taken out from the TNT reaction were visualized by means of SDS-PAGE and autoradiography to check the corresponding protein bands (molecular weight and size and the relative amount of protein synthesized) after overnight exposure to a PhosphorImager® screen (GE Healthcare).

To express GST fusion proteins for GST pull-down experiments, DmEcR, DmUSP, and DmMet cDNA in pIE1M vector were cultured with EcoRI and XbaI and cloned into the same restriction site in pACT (Promega) and then double digested with EcoRI and NotI and cloned into pGEX-5×-1 expression vector (GE Healthcare) using BL21 Star™ (DE3)pLysS One Shot Chemically Competent Cells from Invitrogen with appropriate antibiotics. A single colony from a fresh plate was inoculated into 4 ml of LB or 2× YTA medium at 37 °C for 5 h (A_{600} 0.6–0.8), and isopropyl 1-thio-β-D-galactopyranoside was then added to the culture to a final concentration of 0.5 mM. The culture was allowed to grow at 30 °C with vigorous shaking for about 4 h. Cells were harvested by centrifugation at 3500 rpm at 4 °C for 5 min on a Beckman bench top centrifuge and then washed once in ice-cold PBS. Cells were quick frozen in liquid N_2 and then stored at −80 °C until use.

To isolate GST fusion proteins, frozen cells were thawed on ice and resuspended in 0.4 ml of PBS containing complete protease inhibitor from Roche Applied Science. The cells were briefly sonicated on ice, Triton X-100 was added into the sample at the final concentration of 0.1% (v/v), and the sample was incubated on ice for 30 min. Cell debris removed by centrifugation at 3500 rpm for 10 min at 4 °C. 0.2-ml aliquots were quick frozen in liquid N_2 and then stored at −80 °C until use.

For pull-down experiments, bacterially expressed GST fusion protein was bound to glutathione-containing MagneGST™ particles (Promega), and then the binding of [^{35}S]methionine-labeled protein with GST fusion protein was conducted using the MagneGST™ pull-down system (Promega) as described in the manufacturer's manual with some modification. Briefly, 5 μl of particles carrying bait proteins (GST fusion protein or GST alone) were combined with 20 μl of prey protein (the TNT® T7 quick coupled transcription/translation reaction). After capturing, the particles were washed, and the prey proteins were then eluted by suspending in 1× SDS sample buffer. The released proteins were displayed through SDS-PAGE. Gels were dried, and prey proteins bound to the bait protein were assessed by autoradiography (Typhoon InstantImager; GE Healthcare).

Quantitative Reverse Transcriptase (qRT)-PCR

Rearing and staging, dissection, and tissue culture of *D. melanogaster* Canton-S flies were described previously (48). Briefly, for the JH *in vitro* experiment, late third instar *D. melanogaster* larvae (96–102 h after egg laying) were collected, and the larval abdominal tissues (including most of the larval organs, including epidermis, fat body, alimentary canal, muscles, salivary glands, central nervous system, and imaginal discs) were dissected in *Drosophila* Schneider medium. The tissues were transferred to fresh *Drosophila* Schneider medium and incubated at 25 °C for 3 h. The tissues were then transferred to *Drosophila* Schneider medium supplemented with 1 μM JH III for 3 or 12 h. At the end of hormone

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exposure, the tissues were quick frozen in liquid nitrogen and stored at −80 °C. RNA isolation and cDNA preparation and qRT-PCR analysis were performed as described previously (48). Briefly, qRT-PCR was carried out using the MyiQ single color real time PCR detection system (Bio-Rad). Amplification was performed in a mixture (20 μl) containing 1 μl of cDNA, 1 μl of forward primer (5 pM stock), 1 μl of reverse primer (5 pM stock), 200 nM fluorogenic probe, 0.2 μl of 50× SYBR Green 1 (Molecular Probes, Inc., Eugene, OR), 2 μl of 10×PCR buffer (200 mM Tris-HCl, pH 8.4, and 500 mM KCl), 3 mM $MgCl_2$, 0.125 mM dNTPs, and 0.1 μl of Plantinum® Taq (Invitrogen). An initial incubation at 95 °C for 3 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 min, was used. CG7939 (RP49) RNA levels were analyzed simultaneously in all samples. CG7939 (RP49) levels were used for normalization. RP49 standards included in each amplification run were used to calculate relative quantities. Three independently collected tissue samples were used for each time point. The primers used for PCR are from the German Cancer Research Center Genome RNAi data base (available on the World Wide Web) and/or the *Drosophila* RNAi Screening Center at Harvard Medical School (available on the World Wide Web) of predesigned RNAi probes (Table 1).

Statistical Analysis

Statistical analyses of the data were performed by *t* test or one-way analysis of variance with Tukey's post-test using GraphPad Prism version 3.00 for Windows (San Diego, CA). The results were expressed as means ± S.E. and considered significantly different at $p \leq 0.05$.

RESULTS

Identification of Genes Induced by JH in both Fly Cells and Honey Bee

Sixteen genes that are induced by JH in both *Drosophila* L57 cells and *A. mellifera* were identified by comparing the microarray data generated using RNA isolated from JH-treated fly cells and bee brains (Table 2). Four of these genes function in transport, three of them in lipid metabolism, two of them in signal transduction, two of them in protein binding, and one in cytoskeleton organization. The function of the other four genes is not known (Table 2).

Computational Identification of cis-Regulatory Motifs

The MEME algorithm was used to identify common motifs present in the promoter regions of 16 JH-induced genes of *D. melanogaster*. Three putative *cis*-regulatory elements (motifs) were identified in this cluster (Table 3*A*). Motif 1 containing 29 nucleotides was shared by 13 of the 16 genes (Table 3*B*); Motif 2 containing 15 nucleotides was also identified in 13 of the 16 genes and Motif 3 containing 29 nucleotides was identified in all 16 genes. Motif 1 containing 29 nucleotides was also found in the promoter regions of 12 of 16 JH-induced genes from *A. mellifera.*

Validation of the JHRE Motifs

We first investigated whether nuclear proteins isolated from JH-treated *D. melanogaster* L57 cells bind to these three motifs. Nuclear proteins isolated from JH-treated L57 cells showed specific binding to Motif 1 (Fig. 1); therefore, we designated this motif as DmJHRE1. Nuclear proteins isolated from Me_2SO-treated cells did not bind to DmJHRE1 (data not shown). Nuclear proteins isolated from L57 cells bound to Motif 3 but did not bind to Motif 2 (data not shown). Motif 3 contained TATAA sequence; therefore, we concentrated our further studies on DmJHRE1.

A series of reporter gene constructs that contained DmJHRE1 sequence driving the expression of a bacterial *lac-Z* or firefly *luciferase* genes was prepared. Reporter constructs,

containing 1× DmJHRE1 sequence in the forward or reverse direction, showed 4–5-fold induction of β-galactosidase activity in cells exposed to JH III (Fig. 2*A*). In contrast, exposure of cells to 20E did not affect reporter gene expression (Fig. 2*A*). Similarly, the luciferase gene regulated by DmJHRE1 cloned into pGL3 vector was also induced by JH III in L57 cells, whereas 20E had no effect on this induction (Fig. 2*B*). We also evaluated DmJHRE1 containing reporter gene constructs in *D. melanogaster* S2 and Mbn2 cells. In both cell lines, JH induced reporter activity and 20E had no affect on JH induction (data not shown).

Identification of DmJHRE1-binding Proteins

Specific binding of nuclear proteins isolated from JH-treated L57 cells to the DmJHRE1 suggested that this sequence could be used as immobilized bait to capture proteins that bind to this element. Electrophoresis (SDS-PAGE) of proteins purified from JHIII-treated nuclear extracts, through three consecutive DmJHRE1 DNA affinity column showed three main bands (Fig. 3, *lane 1*). The same procedure was applied to Me_2SO-treated nuclear extracts, but no obvious bands were found (Fig. 3, *lane 2*). These candidate DmJHRE1-binding proteins were subjected to in-solution digestion and identified by LC-MS/MS (Table 4). Two proteins, 39-kDa FK506-binding protein (FKBP39) and 21-kDa calponin-like protein (Chd64), were identified. No significant hits corresponding to the middle band (28 kDa) were detected in two independent purification experiments. It is likely that this protein is a degraded form of FKBP39 or a modified form (phosphorylation and/or glycoslyation) of Chd64.

Functional Analysis of DmJHRE1-binding Proteins

We investigated whether individual purified proteins bind to DmJHRE1. cDNAs coding for FKBP39 and Chd64 were expressed in yeast as fusion proteins of the B42 activation domain in pB42AD expression vector and as fusion proteins of LexA (DBD) in pGilda expression vector. Both proteins, expressed in either pB42AD or pGilda, showed competitive binding to DmJHRE1 (Fig. 4), whereas the control (vector without insert) showed no binding to DmJHRE1 (Fig. 4). RNAi was used to determine whether FKBP39 and Chd64 are involved in JH induction of genes in S2 cells. dsRNA were prepared using FKBP39 and Chd64 and control bacterial *malE* gene as templates. dsRNAs were co-transfected with the reporter plasmid pGL3(DmJHRE1·1F3R) or pGL3(DmJHRE1·6F), the transfected cells were exposed to JH III or Me_2SO, and the reporter activity was quantified 24 h later. As shown in Fig. 5, FKBP39 and Chd64 but not *malE* dsRNA blocked JH III induction of a reporter gene regulated by DmJHRE1, suggesting that these two proteins are required for JH induction of reporter genes regulated by DmJHRE1. There is also some decrease in reporter activity in cells transfected with FKBP39 or Chd64 dsRNA and grown in the medium containing Me_2SO, suggesting that these proteins are also involved in expression of JHRE-containing genes even in the absence of JH. The presence of JH may increase the expression of target genes by changing the interactions of these proteins with co-factors. In control experiments, FKBP39 or Chd64 dsRNA co-transfected with pIE1-luc (the luciferase gene is regulated by constitutively active baculovirus, *A. californica* multicapsid nucleopolyhedrovirus IE1 promoter) showed no decrease in the luciferase gene expression with or without JH exposure (data not shown), suggesting that the RNAi effect of FKBP39 and Chd64 is specific to JHRE-containing genes.

As shown in Fig. 5*B*, in the cells transfected with FKBP39 dsRNA, a 2.5-fold reduction in the FKBP39 mRNA levels when compared with its levels in *malE* dsRNA-transfected cells was observed. In the same cells, the Chd64 mRNA levels were similar to those found in *malE* dsRNA-transfected cells. Similarly, in the cells transfected with *chd64* dsRNA, a 1.75-fold reduction in the *chd64* mRNA levels when compared with its levels in *malE* dsRNA-

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transfected cells was observed (Fig. 5*B*). In the same cells, the FKBP39 mRNA levels were similar to those found in malE dsRNA-treated cells. These data show that the RNAi effects are specific to target genes.

Evaluation of Protein-Protein Interactions Using a Two-hybrid Assay

To gain further insights into the functioning of FKBP39 and Chd64 in JH signal transduction, two-hybrid interaction assays were performed in yeast. The two-hybrid assays showed that FKBP and Chd64 proteins interact with each other because, when pGildaFKBP39 + pB42ADChd64 (FKBP39:Chd64) constructs were transformed into a yeast strain containing leucine and LacZ reporter genes under the control of LexA operator elements, the yeast grew on leucine dropout plates, and the colonies turned blue, showing that these two proteins interacted and induced the expression of both reporter genes (Fig. 6*A* and Table 5). In the reverse direction, the combination containing pGildaChd64 + pB42ADFKBP39 (Chd64:FKBP39) also showed interaction, but the intensity of the *blue color* was less than the *blue color* observed with the other combination (pGildaFKBP39 + pB42Chd64; Fig. 6*A* and Table 5).

As shown in Fig. 6*B*, FKBP39 and Chd64 also interacted with nuclear receptors (EcR and USP) and Met in yeast two-hybrid assays. Differences in intensity of reporter activity were observed depending on the fusion proteins used. For example, when EcR was fused to LexA (DBD), a strong interaction was observed with FKBP39 and Chd64 fused to the B42 activation domain. However, when EcR was fused with B42, only a weak interaction was observed with these proteins. Similar differences were observed in interaction of USP and Met with FKBP39 and Chd64. The interactions of FKBP39 and Chd64 with each other and with EcR, USP, and Met were also tested by performing two-hybrid assays in insect and mammalian cells. In these assays, FKBP39 and Chd64 also interacted with each other in S2 cells (Fig. 7*A*) and in 3T3 cells (data not shown). In addition, FKBP39 and Chd64 also interacted with EcR, USP, and Met in both S2 cells (Fig. 7*B*) and 3T3 cells (data not shown).

GST Pull-down Assay Using in Vitro Synthesized FKBP, Chd64, DmEcR, DmUSP, and DmMet

We tested to determine if the *in vitro* synthesized FKBP (residues 1–357), Chd64 (residues 1–188), and DmMet (residues 1–716) and N-terminal A/B region-deleted DmEcR (residues 224–878) and DmUSP (residues 172–508) are capable of interacting with GST-EcR, GST-USP, or GST-Met. Under the conditions of these experiments, any protein that interacts with each other could be captured with the GST fusion protein using glutathione-linked beads. *In vitro* [^{35}S]methionine-labeled prey proteins were individually mixed with beads containing a GST-fused bait protein in the presence of 20% bovine serum albumin. The recovered proteins were resolved by SDS-PAGE followed by fluorography. ^{35}S-Labeled *in vitro* synthesized FKBP and Chd64 were pulled down by GST-DmEcR, GST-DmUSP, or GST-DmMet fusion proteins used as bait (Fig. 8*A*). These pull-down assays also showed that ^{35}S-labeled DmEcR was pulled down by GST-DmUSP and GST-DmMet. Likewise, ^{35}S-labeled DmMet was pulled down by GST-DmEcR and GST-DmUSP (Fig. 8*B*). When the GST alone was used as a bait protein, *in vitro* synthesized FKBP, Chd64, DmEcR, and DmMet were not pulled down, suggesting that GST tag is not responsible for the observed interactions (Fig. 8, *A* (*lanes 1* and *5*) and *B* (*lanes 1* and *4*)). These data show that FKBP39 and Chd64 can interact with DmEcR, DmUSP, and DmMet.

Developmental Expression and JH Regulation of FKBP39 and Chd64

Three small peaks of FKBP39 *mRNA* were detected at the end of embryonic and first and second instar larval stages. The *FKBP39* mRNA levels started to increase beginning at 120 h

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after hatching and reached the maximum levels by 134 h after hatching. The mRNA levels started to decrease and low levels of FKBP39 mRNA were detected at 192 h after hatching (Fig. 9*A*). The *FKBP39* mRNA was also present in both male and female flies, showing relatively higher amounts in females than in males (Fig. 9*A*). The levels of *Chd64* mRNA were low after hatching, and then the mRNA levels started to increase beginning at 12 h after hatching, and three peaks of *Chd64* mRNA were detected at the end of embryonic and first instar and second instar larval stages (Fig. 9*A*). To determine whether JH regulates the expression of DmJHRE1-binding proteins, we used *D. melanogaster* tissues dissected from late third instar larvae when the *FKBP39* and *Chd64* mRNA levels are low (96–102 h after hatching; Fig. 9*A*). The *D. melanogaster* tissues were dissected under sterile conditions and maintained in Grace's medium for 3 h prior to their exposure to JH III for either 3 or 12 h. Both *FKBP39* and *Chd64* mRNA were not induced by JH III in *D. melanogaster* tissues that were exposed to 1 μM JH III for 3 h (Fig. 9*B*). The *FKBP39* and *Chd64* mRNA increased significantly in the tissues cultured in Grace's medium supplemented with JH III for 12 h when compared with the levels in tissues cultured in Grace's medium supplemented with Me_2SO (Fig. 9*B*).

DISCUSSION

In this study, we identified a new cis-acting DNA regulatory element that confers JH-dependent gene expression. A JHRE, DmJHRE1, shared by 13 genes in the fruit fly and 12 genes in the honeybee of the 16 genes induced by JH in both *D. melanogaster* L57 cells and *A. mellifera* brain was identified. The DmJHRE1 was used in a DNA affinity column; two DmJHRE1-binding proteins, 39-kDa FK506-binding protein (FKBP39) and 21-kDa calponin-like protein (Chd64), were identified in *Drosophila* by LC-MS/MS; and the homologs were also found in *A. mellifera*.

Previously, Kethidi *et al.* (17) identified a 30-base pair sequence (CfJHRE), located in the 5′ promoter region of the spruce budworm JH esterase gene (from −604 to −574) that showed 100% similarity with the consensus DR4 element. The CfJHRE is sufficient to support both JH-dependent induction and 20E suppression of JH induction. In *L. migratoria*, a putative JHRE with the partially palindromic 15-nt sequence, GAGGTTCGAG(A/T)CCT(T/C), was found upstream of a JH-induced gene, *jhp21*, and a transcription factor binding to this element was identified. The binding of a factor to the JHRE was abolished by phosphorylation catalyzed by protein kinase C and was brought to an active state through the action of JH (38). The DmJHRE1 we identified in this study does not show sequence similarity with either of the previously identified JHREs, suggesting that JH may function through diverse response elements.

The hypothesis that guided this work is that given that JH coordinates the regulation of many essential response genes, some of these co-regulated genes may share common response elements and regulatory factors (49). Therefore, JHRE sequences may be evolutionarily conserved in *D. melanogaster* and *A. mellifera*. Sinha *et al.* (50) reported on evolutionary conservation of *cis*-regulatory elements between *D. melanogaster* and *A. mellifera*. Identification of *cis*-regulatory elements is helpful to investigate the molecular mechanism of JH action, because *cis*-regulatory elements could be involved in both temporal and spatial regulation of genes regulated by JH. In this study, we used the MEME algorithm and identified a JHRE, DmJHRE1, shared by 13 of the 16 JH-induced genes identified in *D. melanogaster* L57 cells and also shared by 12 of the 16 JH-induced genes identified in *A. mellifera* (Table 3).

Transient transfection experiments in *D. melanogaster* L57 cells were used to confirm the hypothesis that DmJHRE1 supports induction of genes placed under its control. DmJHRE1

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is also present in other JH-induced genes in *Drosophila* (a list of JH-induced genes is given in Ref. 40). We extended the analysis to more evolutionarily divergent insect species, whose genomes have been sequenced and are available in public data bases. DmJHRE1 is present in the promoters of some genes in *A. mellifer* and *Anopheles gambiae*, indicating that DmJHRE1 is conserved during evolution.

Two DmJHRE1-binding proteins, 21-kDa calponin-like protein (Chd64) and 39-kDa FK506-binding protein (FKBP39), were identified. Previous RNAi experiments showed that *FKBP39* is involved in Wg signaling (51). The homolog of FKBP39 in fall armyworm *Spodoptera frugiperda* was identified as a nuclear DNA-binding protein associated with a nuclear kinase that specifically phosphorylates it in the presence of Mg^{2+} and ATP (52). Nuclear FKBPs could help to maintain kinase function by maintaining the proper protein-protein interactions in the kinase complex, and this complex in turn might play a role in signal transduction by aiding in proper folding and stability of short lived labile transcription factors (52). The FKB39 homologs were also found in *A. mellifera* (accession code XP_001121759), *Bombyx mori* (accession code AAY86706), and *Aedes aegypti* (accession code EAT45400).

A homolog of FKBP46 was found in the EcR complex (which includes EcR, USP, and FKBP46) in the nucleus of prothoracic glands of *Manduca sexta*. The developmental expression patterns of EcR and USP changed in concert with the hemolymph ecdysteroid titer, whereas the expression of FKBP46 remained constant throughout development (53). In this study, a FKBP was identified in the nuclear proteins isolated from *Drosophila* L57 cells. We did not find DmEcR and DmUSP in the purified complex, because L57 is a mutant cell line developed from *D. melanogaster* Kc cells by parahomologous gene targeting to inactivate EcRB1 and EcRB2 isoforms (54). These cells lost 90% of the 20E response (55, 56). The lack of USP in the complex was not known, but our two-hybrid assay in yeast and insect cells showed that both DmEcR and DmUSP interacted with FKBP, suggesting that FKBP39 may form a complex with Chd64, DmEcR, and DmUSP.

The homolog of Chd64 was also found in *A. mellifera* (accession code XP_392114). It is interesting that both FKBP39 and Chd64 interact with DmMet, and DmMet interacts with both DmEcR and DmUSP. Since these two DmJHRE1-binding proteins interact with proteins involved in both JH action (Met) and 20E (EcR and USP) action, it is tempting to speculate that these proteins may play an important role in cross-talk between JH and 20E.

We did not detect any specific high affinity JH binding to these two DmJHRE1-binding proteins (FKBP39 and Chd64) expressed in bacteria as GST fusion proteins or in yeast as LexA or B42 fusion proteins. Electrophoretic mobility shift assay studies showed that both of these proteins expressed in yeast can bind to DmJHRE1. Therefore, these two proteins are probably involved in cross-talk between JH and 20E by binding to DNA in complex with other transcription factors involved in JH and 20E action. Studies on developmental expression of *FKBP39* and *Chd64* showed peaks of mRNAs of these proteins at the end of embryonic and first and second instar larval stages. The *FKBP39* and *Chd64* mRNA also increased significantly in the tissues cultured in Grace's medium supplemented with JH III for 12 h. The developmental expression and JH induction of mRNAs support the above hypothesis that these proteins may be part of multi-protein complexes that mediate cross-talk between JH and 20E during molting. However, further studies are needed to determine the role of JH in formation of these multiprotein complexes as well as to regulate the affinity of these complexes to JHRE.

The prevailing hypothesis is that JH is carried by JH-binding protein (JHBP) from the synthesis site (corpora allata), transporting through the hemolymph to the target cells. The

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events that occur after JH reaches target cells remain unknown. In this study, additional insights are found in the JH signal transduction pathway. In the presence of ecdysteroids (mainly 20E), the EcR heterodimerizes with other members of the nuclear receptor superfamily, noticeably with the USP and other cofactors (43, 57–60). The DmEcR/DmUSP can heterodimerize with FKBP (52) and other cofactors and bind to the ecdysone response elements (EcRE) present in the promoter regions of ecdysteroid response genes and regulate their transcription during metamorphosis (Fig. 10). However, during molting, higher levels of JH plus 20E may lead to an increase in the expression of JH response genes that contain JHRE, because JH may influence the strength of the interactions of FKBP39 and Chd64 with EcR·USP·Met, and these complexes may bind to response elements such as DmJHRE1 to regulate the expression of genes containing these response elements (Fig. 10). Our previous studies showed that JH mediated activation of *C. fumiferana* JH esterase by dephosphorylation of some unidentified nuclear proteins, and protein kinase C-mediated phosphorylation prevented binding of nuclear proteins to JH-responsive promoters, resulting in suppression of JH action (16, 17). FKBP39 may be one of the proteins involved in binding to JHRE, depending on its phosphorylation state. Further experiments are needed to investigate the strength of these interactions between various components. Identification of proteins that bind to JH and participate in multiprotein complexes that bind to JHRE will help in understanding JH action. Studies in progress in our laboratory and other laboratories around the world should help in elucidating the action of this important hormone.

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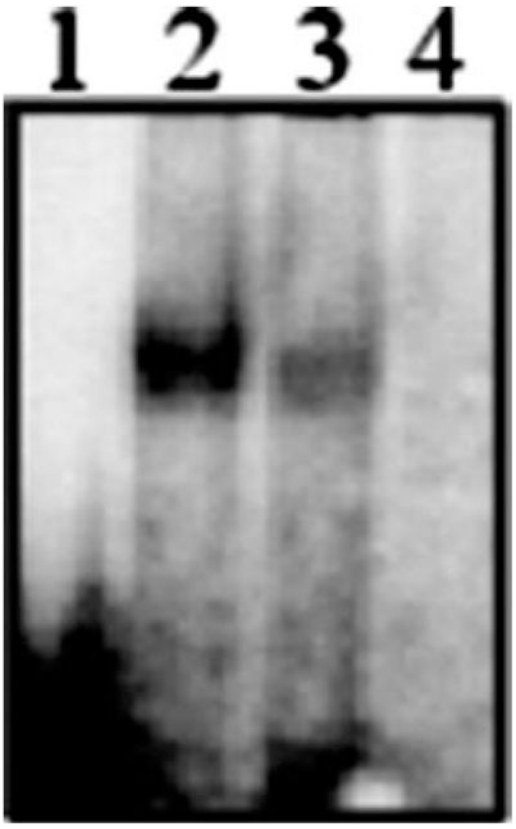

FIGURE 1. Nuclear proteins isolated from JH III-treated *Drosophila* L57 cells bind to *DmJHRE1*

Nuclear proteins were isolated from L57 cells that were grown in the medium containing 1 μM JH III for 24 h. The nuclear proteins were incubated with ^{32}P-labeled double-stranded 29-bp oligonucleotides corresponding to the *DmJHRE1* sequence. The DNA-protein complexes were separated on 6% polyacrylamide gels. Specific competition was performed by adding 100-fold excess cold double-stranded 29-bp oligonucleotides corresponding to DmJHRE1 sequence. Nonspecific competition was performed by adding a 100-fold excess of cold double-stranded 30-bp oligonucleotides of unrelated sequence. *Lane 1*, probe only; *lane 2*, probe plus protein; *lane 3*, probe plus protein and 100× nonspecific probe; *lane 4*, probe plus protein and 100× cold probe.

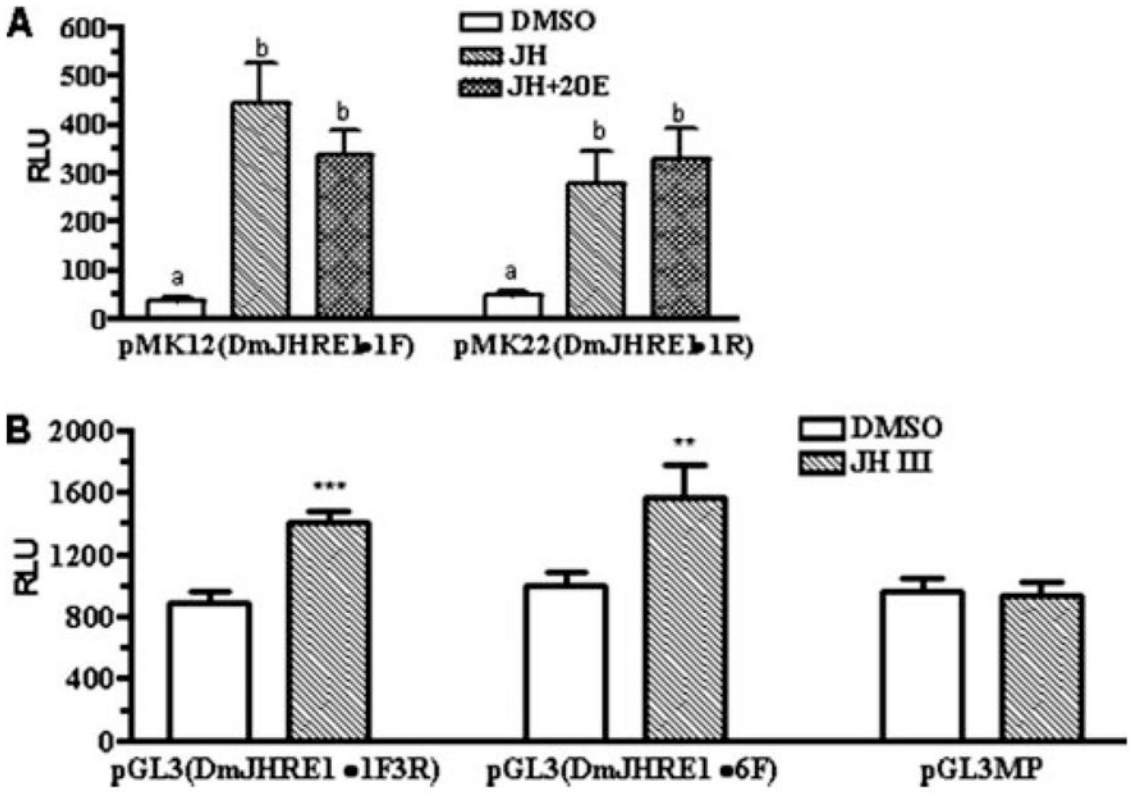

FIGURE 2.

A, the *DmJHRE1* promoter functions in *Drosophila* L57 cells. Cells were transfected with pMK12 (DmJHRE1·1F) (with 1× DmJHRE1) and pMK22 (DmJHRE1·1R) (with 1× DmJHRE1 reverse) DNA. The transfected cells were exposed to 1 μM JH III or 1 μM JH III plus 1 μM 20E. The β-galactosidase activity was quantified at 24 h after the addition of the hormone. The values are mean ± S.E. ($n = 3$) obtained by normalization of the β-galactosidase activity against the constitutive expression of the *Renilla* luciferase activity (pIE1RLuc), whose activity is proportional to cellular mass. Individual mean values with the same *letter* were not significantly different within specific comparable groups (analysis of variance, $p \leq 0.05$). *B*, *DmJHRE1* promoter functions in *Drosophila* L57 cells. Cells were transfected with pGL3(DmJHRE1·1F3R) (with 1× DmJHRE1 forward plus 3× DmJHRE1 reverse sequences) and pGL3(DmJHRE1·6F) (with 6× DmJHRE1 forward sequences) DNA. The transfected cells were exposed to 1 μM JH III. The luciferase activity was quantified at 24 h after the addition of the hormone using the Promega Dual Luciferase™ reporter assay system as described under "Experimental Procedures." The values are mean ± S.E. ($n = 3$) obtained by normalization of the firefly luciferase activity against *Renilla* luciferase activity (pIE1RLuc). The *asterisks* denote significant differences from control values (unpaired *t* test; **, $p \leq 0.01$; ***, $p \leq 0.001$).

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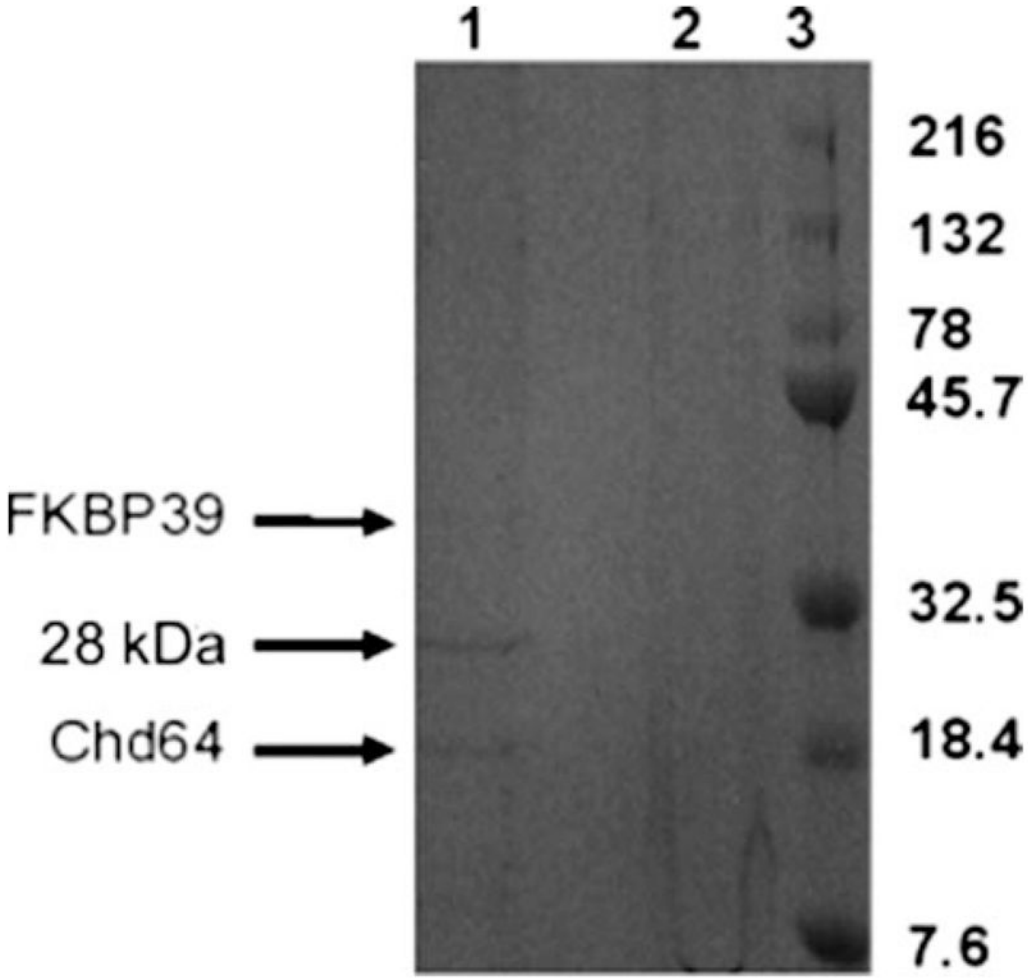

FIGURE 3. Gradient (4–20%) SDS-PAGE electrophoresis of purified DmJHRE1-binding proteins stained by SYPRO® Ruby from Invitrogen

Lane 1, nuclear proteins from JH-treated *Drosophila* L57 cells eluted from the third DmJHRE1 DNA affinity column; *lane 2*, nuclear proteins from Me_2SO-treated *Drosophila* L57 cells eluted from the third DmJHRE1 DNA affinity column; *lane 3*, Kaleidoscope prestained standards from Bio-Rad. The JHRE-binding proteins corresponding to 39-kDa protein (FKBP39) and 21-kDa protein (Chd64) were identified by in-solution digestion (see Table 4). No significant hits corresponding to the middle band (28-kDa protein) were detected in two independent purification experiments.

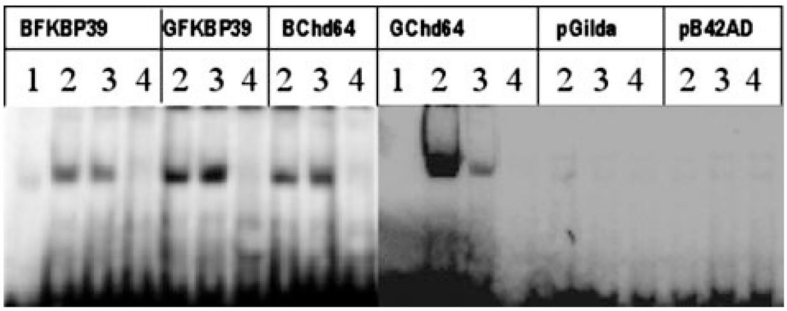

FIGURE 4. DmJHRE1-binding proteins expressed in yeast cells bind to *DmJHRE1*

DmJHRE1-binding proteins expressed in yeast were extracted as described under "Experimental Procedures." The yeast-expressed proteins were incubated with ^{32}P-labeled double-stranded 29-bp oligonucleotides corresponding to the *DmJHRE1* sequence. The DNA-protein complexes were separated on 6% polyacrylamide gels. Specific competition was performed by adding a 100-fold excess of cold double-stranded 29-bp oligonucleotides corresponding to DmJHRE1 sequence. Nonspecific competition was performed by adding 100-fold excess cold double-stranded 30-bp oligonucleotides of unrelated sequence. *BChd64*, protein extracts from yeast transformed with pB42ADChd64; *BFKB39*, protein extracts from yeast transformed with pB42ADFKBP39; *GChd64*, protein extracts from yeast transformed with pGildaChd64; *GFKBP39*, protein extracts from yeast transformed with pGildaFKBP39; *pB42AD*, protein extracts from yeast transformed with basic pB42AD; *pGilda*, protein extracts from yeast transformed with basic pGilda. *Lane 1*, probe only; *lane 2*, probe plus protein; *lane 3*, probe plus protein and 100× nonspecific probe; *lane 4*, probe plus protein and 100× cold probe.

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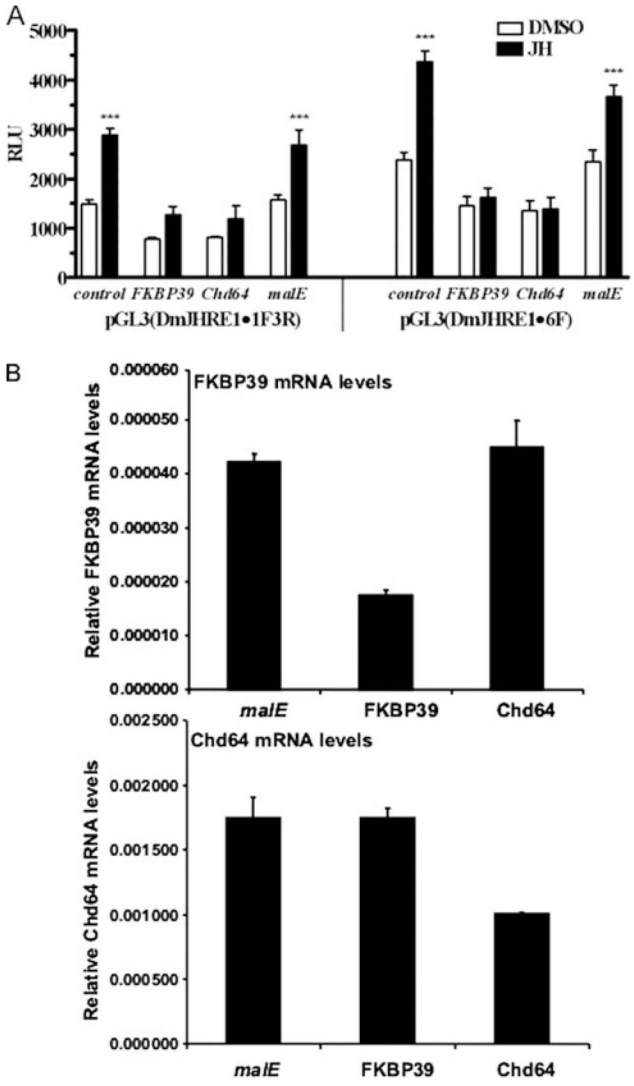

FIGURE 5.

A, FKBP39 and Chd64 dsRNAs suppress JH III induction of the luciferase gene regulated by DmJHRE1. *Drosophila* S2 cells were plated and transfected with dsRNAs and the DmJHRE1 reporter construct (0.5 μg/well on a 96-well/plate). Three days after transfection, 1 μM JH III or Me_2SO was added. The luciferase activity was measured at 24 h after the addition of hormones. Control wells received no dsRNA, and maltase wells received dsRNA made against bacterial *malE* gene. The values are mean ± S.E. obtained by normalization of the *firefly* luciferase activity against *Renilla* luciferase activity. All of the transfection experiments were performed in triplicate, and the experiments were repeated at least three times. The *asterisks* denote significant differences from control values (unpaired *t* test; ***, $p \leq 0.001$). *B*, FKBP39 and Chd64 dsRNAs knock down FKBP39 and Chd64 mRNAs levels. *Drosophila* S2 cells were plated and transfected with FKBP39, Chd64, or *malE* dsRNAs (2.0 μg/well of a 24-well plate). Sixty hours after transfection, the cells were harvested, total RNA was isolated, the RNA was converted to cDNA, and the levels of FKBP39 and Chd64 mRNA were quantified using quantitative real time reverse transcriptase PCR. The rp49 mRNA levels measured in the same samples were used for normalization. Mean ± S.E. (n = 3) values of relative levels of FKBP39 or Chd64 mRNA in comparison with rp49 mRNA levels are shown.

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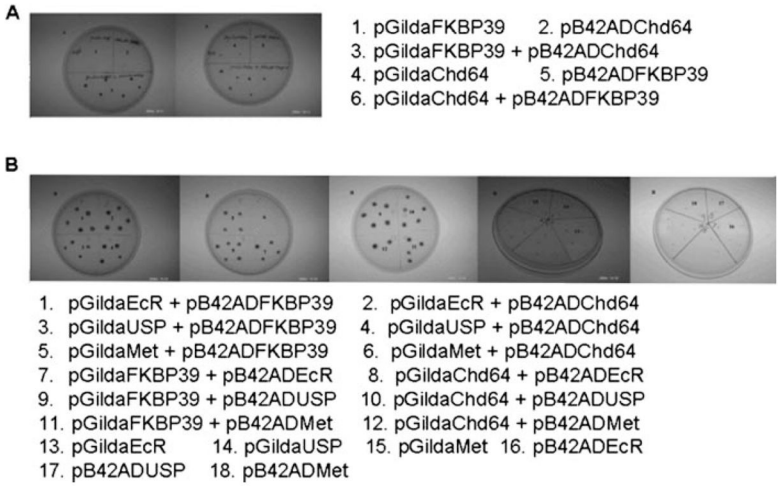

FIGURE 6. Interactions between FKBP39, Chd64, EcR, USP, or Met as determined by the yeast two-hybrid assay

The two-hybrid vectors pGilda and pB42AD contained the LexA DNA-binding domain and the B42 activation domain, respectively. Yeast EGY48 cells were transformed with either one or two plasmids as follows. *A*, pGildaFKBP39 (*1*), pB42ADChd64 (*2*), pGildaFKBP39 + pB42ADChd64 (*3*), pGildaChd64 (*4*), pB42ADFKBP39 (*5*), and pGildaChd64 + pB42ADFKBP39 (*6*). *B*, pGildaEcR + pB42ADFKBP39 (*1*), pGildaEcR + pB42ADChd64 (*2*), pGildaUSP + pB42ADFKBP39 (*3*), pGildaUSP + pB42ADChd64 (*4*), pGildaMet + pB42ADFKBP39 (*5*), pGildaMet + pB42ADChd64 (*6*), pGildaFKBP39 + pB42ADEcR (*7*), pGildaChd64 + pB42ADEcR (*8*), pGildaFKBP39 + pB42ADUSP (*9*), pGildaChd64 + pB42ADUSP (*10*), PGildaFKBP39 + pB42ADMet (*11*), pGild-aChd64 + pB42ADMet (*12*), pGildaEcR (*13*), pGildaUSP (*14*), pGildaMet (*15*), pB42ADEcR (*16*), pB42ADUSP (17), and pB42ADMet (*18*). The expression of the two-hybrid target gene, β-galactosidase, was monitored by filter assays using X-gal as the enzyme substrate. Expression of β-galactosidase turned yeast colonies blue in the presence of X-gal, indicating physical interactions between the two proteins tested.

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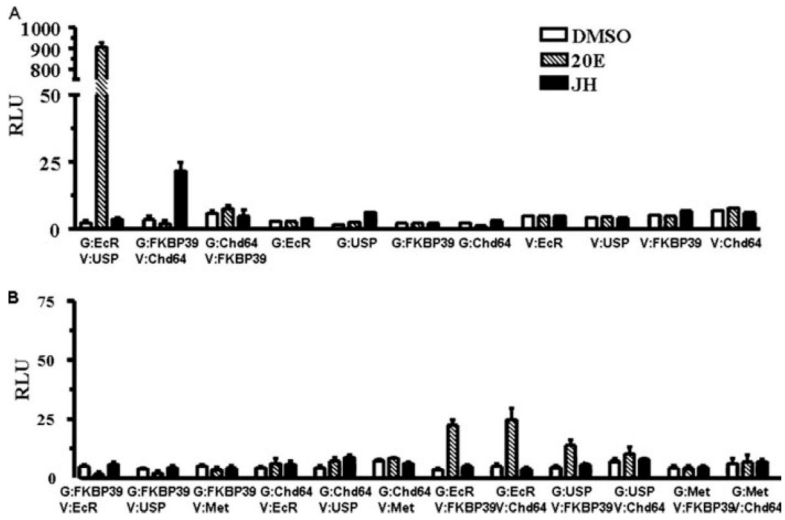

FIGURE 7. Interactions between FKBP39, Chd64, EcR, USP, or Met determined by *Drosophila* S2 cell two-hybrid assay

The two-hybrid vectors pIE1M and pIE1VP16 contained the *GAL4* DNA-binding domain and the VP16 activation domain, respectively, and the reporter vector pG6KZ-Luc contained the 6×GAL4 response elements with the synthetic minimal promoter and the *firefly* luciferase gene. The transfected cells were grown in the medium containing 0 (Me_2SO-treated) or 1.0 μM JHIII or 20E. The cells were harvested at 24 h after adding ligand, and the reporter activity was measured using the Promega Dual Luciferase™ reporter assay system as described under "Experimental Procedures." The relative light unit (*RLU*) value presented as mean ± S.E. was obtained by normalization of the *firefly* luciferase activity against *Renilla* luciferase activity. All of the transfection experiments were performed in triplicate, and the experiments were repeated at least three times. *A*, interactions of EcR with USP and FKBP39 with Chd64. *B*, interactions of FKBP39 and Chd64 with EcR, USP, and Met.

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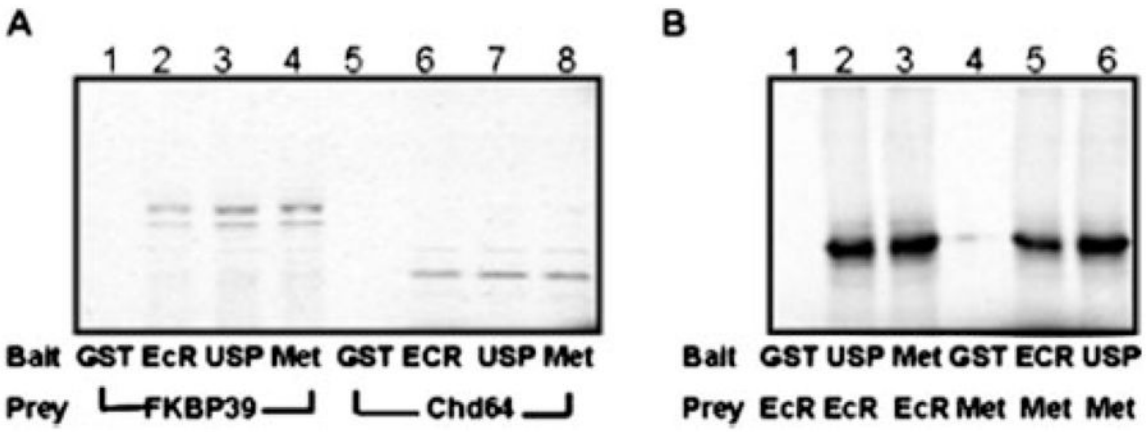

FIGURE 8. GST pull-down experiments using *in vitro* synthesized DmEcR, DmMet, FKBP39, and Chd64 proteins

The *in vitro* radiolabeled FKBP39 (*FKBP*; predicted 39.3-kDa protein), Chd64 (*Chd*; predicted 20.6-kDa protein), DmEcR (*EcR*; predicted 71.8 kDa protein), and DmMet (*Met*; predicted 78.7-kDa protein) were incubated with glutathione particle-bound GST fusion protein (EcR, USP, and Met) or GST protein alone (predicted 26-kDa protein) as described under "Experimental Procedures." Proteins released from glutathione particles were resolved using 12.5% SDS-PAGE (*A*) or 7.5% SDS-PAGE (*B*) followed by autoradiography.

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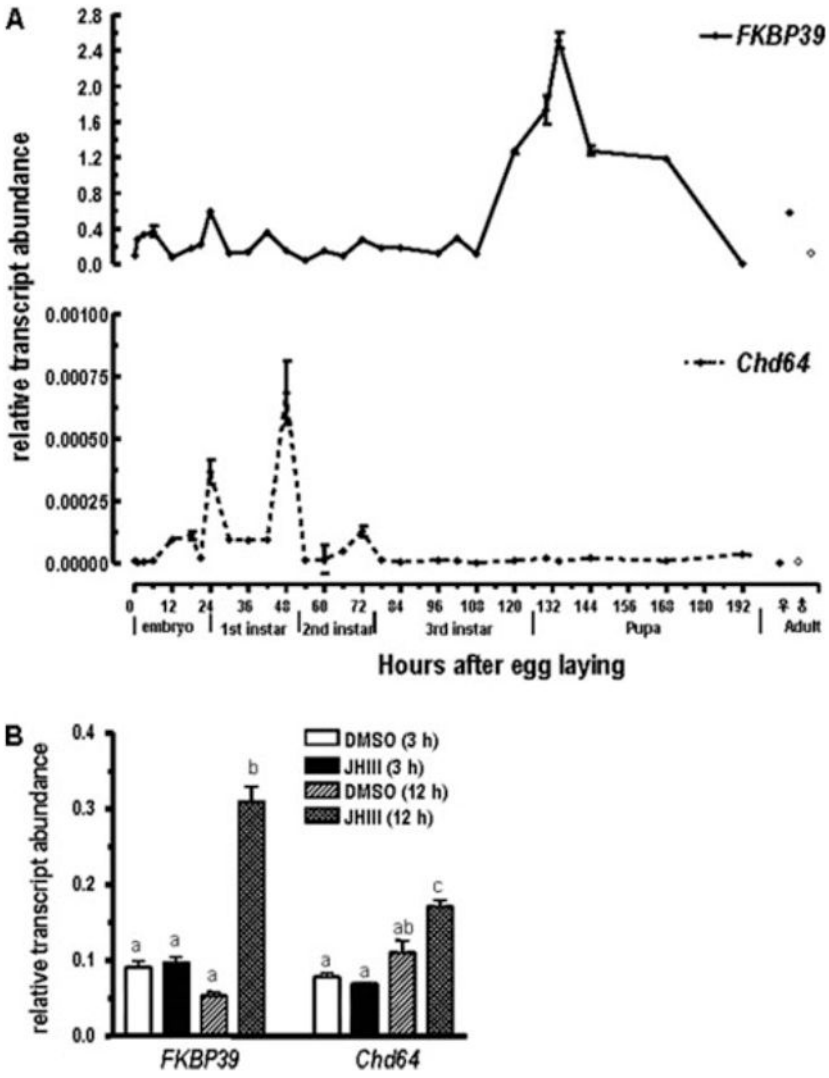

FIGURE 9.

A, developmental changes in *D. melanogaster* DmJHRE1-binding proteins, FKBP39, and Chd64 mRNA levels. Total RNA was isolated from staged insects throughout *D. melanogaster* development. The RNA was converted to cDNA, and the cDNA was used as a template to quantify mRNA of FKBP39 and Chd64 by qRT-PCR. RP49 RNA was used for normalization as well as a standard for qRT-PCR. About 6–30 individually staged insects were pooled for each sample. The mean and S.E. of three samples are presented. *B*, effect of JHIII on expression of DmJHRE1-binding proteins, FKBP39, and Chd64 mRNA levels, respectively, in 3 and 12 h of exposure. *Drosophila melanogaster* tissues were dissected from late third instar larvae prior to puparium formation and cultured in Grace's medium for 3 h. The tissues were then transferred to Grace's medium supplemented with 0.1% Me_2SO, 1 μM JH III. The tissues were harvested at 3 or 12 h after the addition of hormones, total RNA was isolated, and qRT-PCR was used to quantify mRNA levels. Tissues isolated from four larvae were pooled for each sample. Mean and S.E. of three samples are presented. Statistical analysis was performed as described under "Experimental Procedures." Means with the same *letter* within the same gene group are not significantly different ($p \leq 0.05$, analysis of variance).

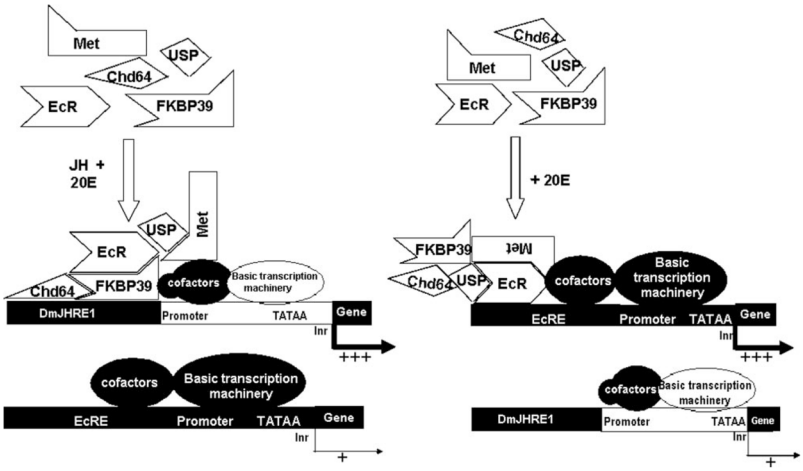

FIGURE 10. Model of the signal transduction pathway for the JH-regulated genes that contain DmJHRE1 JH response element

In the presence of high JH and 20E levels (larval molting stage), the mRNAs for FKBP39 and Chd64 are induced, and these proteins interact with EcR, USP, and Met and bind to the DmJHRE1 sequence. This could result increased expression of DmJHRE1-containing genes and reduction in expression of ecdysone response genes due to a decrease in availability of EcR and USP. 20E increases in the absence of JH (metamorphosis), the FKBP39 and Chd64 levels are low, and EcR heterodimerizes with the other members of the nuclear receptor superfamily, especially with the USP and other cofactors (43, 55–58), and induces the expression of ecdysone-response genes.

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TABLE 1

Primers used for qRT-PCR analyses

The primers used for PCR are from the German Cancer Research Center Genome RNAi database and/or the *Drosophila* RNAi Screening Center at Harvard Medical School of predesigned RNAi probes. NA, not available.

Amplified gene	Primers	Size	Code
		bp	
FKBP39 (CG6226)	5′-TGCACAGCTTTCAGTTCCAC-3′ (forward) 5′-CCTCCTGAAGGGAAAACCAT-3′ (reverse)	204	DRSC33030
Chd64 (CG14996)	5′-AGATAACCACCGAGTTCAGA-3′ (forward) 5′-GAGCTGGCCCAGGAGAG-3′ (reverse)	298	DRSC08649 HFA08649
RP49 (CG7939)	5′-ACAAATGGCGCAAGCCCAAGG-3′ (forward) 5′-ATGTGGCGGGTGCGCTTGTT-3′ (reverse)	109	NA

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TABLE 2

Homologs of JH up-regulated genes newly identified in fruit fly *D. melanogaster* by microarray analysis with $r > 1.5$ and $p < 0.001$

Gene name/symbol	*r* value	Cellular component	Functions
Vha26	1.64	Plasma membrane (ATPase complex)	Proton transport
nrv2	2.20	Plasma membrane (ATPase complex)	Sodium ion transport
Flm	1.56	Unknown	Cytoskeleton organization and biogenesis
CREG	1.51	Nucleus	Protein binding to negatively regulate transcription
CG7842	1.95	Mitochondrion	Fatty acid biosynthesis (*S*-malonyltransferase)
CG7627	1.51	Plasma membrane (ATPase complex)	Extracellular transport in response to toxin
I(2)44DEa	1.63	Unknown	Fatty acid metabolism (acetate-CoA ligase)
CG8271	1.74	Plasma membrane	Cation transport via monocarboxylate porter activity
CG13868	2.11	Unknown	Unknown
CG12090	1.65	Unknown	Unknown
CG32066	1.62	Unknown	Unknown
Cad87A	1.88	Plasma membrane	Signal transduction by calcium-dependent cell-cell adhesion
CG9924	1.83	Unknown	Protein binding
CG2316	2.09	Plasma membrane (ATPase complex)	Lipid metabolism and lipid transport
CG11148	1.70	Unknown	Unknown
Rab1	1.66	Unknown	Small GTPase mediated signal transduction

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TABLE 3

Putative *cis*-regulatory elements identified in JH up-regulated genes shared between *D. melanogaster* cells and *A. mellifera* using the MEME algorithm (42)

A, sequences of three identified putative *cis*-regulatory elements. B, shared *cis*-regulatory motif of DmJHRE1 was identified at 3 kb upstream of transcription start site in 13 *D. melanogaster* JH-induced genes; C, shared *cis*-regulatory motif of DmJHRE1 was identified at 3 kb upstream of transcription start site in 12 homologs of *A. mellifera* in these 16 JH-induced genes by MAST (motif alignment and search tool; available on the World Wide Web).

(A) Motifs identification

motifs	width	best possible match (consensus sequence)
#1 (DmJHRE1)	29	CTCTCGCACTCTCTCTCTCCCGCTCTC
#2	15	CACACGCACGAACGC
#3	29	AATATAAATATATATATTTACTTAGTA

(B) DmJHRE1 consensus sequence

CTCTCGCACTCTCTCTCTCCCGCTCTC

Gene identity	specific sequences in fruit fly *Drosophila melanogaster*
l(2)44DEa	**CTCTCTCTCTCTCTCTCTCCCGCTCTT**
CG9924	**CTCTCTCTCTCTCTCTCTCTCTCTATC**
CG32066	**CTCTCTCTCTCTCTCTCTATCTCTTTT**
CG8271	**CAAGCGCACTCTCTCACTCACTCGCTGGC**
CG7627	**GTTTCTCACTTTCTCCCTGTTCCACTCAC**
Rab1	**CCCGCGCATTTTCCAACACTGCCGCTAAT**
Fim	**CTCTCTCGCGCCGACTTTCTTTCGCTCTC**
CREG	**CTCTTTCATTTTCTCTGTGCGCCGCATTC**
CG7842	**CTCTCTCACTTATCCGCTCTTACTCTCAG**
Vha26	**CTCGCGCTCCTCGTGTAGGCGCCGCTGTT**
CG12090	**GGCGCGCACCCTGTCCCTCAACAGCGAGT**
CG13868	**CCTTCGCTCGCTGCCGAACGGCCGCCTGG**
CG2316	**CCCGCGCAGTCCTTACCTCCCTGGAACAC**

(C) DmJHRE1 sequence found in 12 homologs in A. mellifera of JH-induced genes by MAST.

Fly base ID	homologs (scaffold) in bee	specific sequences in bee *Apis mellifera*
Vha26	NW_001260156	**CCATATCTGGTTGAAATACTGCAGAAGTT**
nrv2	NW_001253330	**GAGGGGCAGAGAGAAAGAGAAAGTGGCAA**
CREG	NW_001253054	**GCATTTCTTGCTGTAAAATAAACGCCGGC**
CG7627	NW_001253024	**TATACGCACAGCCTAGCGTCCGATTAACC**
l(2)44DEa	NW_001253556	**AATATCAACTGCTGTTAAACCAGAAATTG**
CG8271	NW_001253305	**GACAGTTTGCCAGCTAAAGCAAACGAAGG**
CG13868	NW_001253020	**GTTTCTCTGCTTTTATTTCACAAATTTTG**
CG32066	NW_001253009	**CTATTTTAACGATATACTAAAAGAGAGCT**
Cad87A	NW_001253475	**CTCTCTCTCTCTTTATCTCTCTCTCTCTC**
CG2316	NW_001253448	**CTTGTATGCTGTACGAATACAAGAAAAGC**
CG11148	NW_001253522	**GCCGTACTTTCTTACCTTCGTTCACTTTT**

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(C) DmJHRE1 sequence found in 12 homologs in A. mellifera of JH-induced genes by MAST.

Fly base ID	homologs (scaffold) in bee	specific sequences in bee *Apis mellifera*
Rab1	NW_001253372	**GAATCTTTTTCATTAAATGTATCTCTTAT**

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TABLE 4

The *DmJHRE1* DNA-binding proteins from *D. melanogaster* L57 cells identified by LC-MS/MS

Gene identity	Protein name (gene name/symbol)	Theoretical *M*	Sequence coverage
		Da	%
CG14996	Calponin-like protein (*Chd64*)	20,643	17
CG6226	FK506-binding 39k (*FKBP39*)	39,342	10

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TABLE 5

Summary of interactions by yeast two-hybrid assay

The interactions were classified based on time (h) required to form blue colonies: ++++, 0–5 h; +++, 6–12 h; ++, 13–48 h; +, 49–72 h; −, no growth or blue colonies observed. ND, not determined.

	pB42ADFKBP39	pB42ADChd64	pB42ADEcR	pB42ADUSP	pB42ADMet
pGildaFKBP39	ND	+++	++	+++	+++
pGildaChd64	+	ND	++	+++	+++
pGildaEcR	+	+	ND	++	++
pGildaUSP	+	+	++++	ND	++
pGildaMet	−	−	++	++	ND