The Drosophila FTZ-F1 Nuclear Receptor Mediates Juvenile Hormone Activation of E75A Gene Expression through an Intracellular Pathway

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**Background:** JH is a critical insect hormone, but its mechanism of action is contentious.
**Results:** JH activates E75A through an intracellular pathway utilizing FTZ-F1 and GCE, which form a transcriptionally active heterodimer.
**Conclusion:** FTZ-F1 functions as a competence factor that facilitates JH activation of gene expression.
**Significance:** As a competence factor for multiple insect hormones, FTZ-F1 could be a mediator of hormonal cross-talk.

Juvenile hormone (JH) regulates a wide variety of biological activities in holometabolous insects, ranging from vitellogenesis and caste determination in adults to the timing of metamorphosis in larvae. The mechanism of JH signaling in such a diverse array of processes remains either unknown or contentious. We previously found that the nuclear receptor gene E75A is activated in S2 cells as a primary response to JH. Here, by expressing an intracellular form of JH esterase, we demonstrate that JH must enter the cell in order to activate E75A. To find intracellular receptors involved in the JH response, we performed an RNAi screen against nuclear receptor genes expressed in this cell line and identified the orphan receptor FTZ-F1. Removal of FTZ-F1 prevents JH activation of E75A, whereas overexpression enhances activation, implicating FTZ-F1 as a critical component of the JH response. FTZ-F1 is bound in vivo to multiple enhancers upstream of E75A, suggesting that it participates in direct JH-mediated gene activation. To better define the role of FTZ-F1 in JH signaling, we investigated interactions with candidate JH receptors and found that the bHLH-PAS proteins MET and GCE both interact with FTZ-F1 and can activate transcription through the FTZ-F1 response element. Removal of endogenous GCE, but not MET, prevents JH activation of E75A. We propose that FTZ-F1 functions as a competence factor by loading JH signaling components to the promoter, thus facilitating the direct regulation of E75A gene expression by JH.

The life cycle of holometabolous insects is governed by two hormone signals that trigger developmental changes: the steroid 20-hydroxyecdyson (ecdysone)3 and the sesquiterpenoid juvenile hormone (JH). Ecdysone is produced in the prothoracic gland and released in pulses that initiate developmental transitions, and JH is secreted from the corpora allata and is thought to determine the outcome of each transition. High JH titer results in a simple cuticle shedding during larval molts, whereas the absence of JH initiates a complete restructuring of the body plan during metamorphosis.

The titer of JH is tightly regulated during development through both biosynthesis and degradation. One of the major pathways of JH degradation occurs through juvenile hormone esterase (JHE), which converts JH to JH acid by methyl ester hydrolysis (1). Consistent with its role as a major regulator of JH titer, JHE exhibits an exceptionally high binding affinity for JH, allowing the protein to dramatically reduce the concentration of JH circulating through insect hemolymph (2, 3). Manipulation of JHE activity has been used to alter development in several insect species. Chemical inhibition of JHE in dipteran, lepidopteran, and coleopteran species delays pupariation (4). Conversely, overexpression of JHE in lepidopteran species mediates anti-JH activity, manifested as developmental defects in larval molting (5). These experiments have shown that JHE plays a crucial role in regulating insect development and is a powerful tool for altering the concentration of JH in vivo.

JH participates not only in developmental regulation but in various aspects of insect adult life as well. In Drosophila females, JH initiates yolk protein synthesis both in the fat body and ovary and regulates its uptake into a growing oocyte (6–8). In males, JH stimulates the synthesis of accessory gland proteins (9, 10) and controls courtship behavior and sexually dimorphic locomotor activity (11). In addition to reproduction, in many species JH has been implicated in a wide range of other

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3 The abbreviations used are: ecdysone, 20-hydroxyecdysone; bHLH, basic-helix-loop-helix; PAS, Per-AhR/Arnt-Sim; JH, juvenile hormone; JHE, juvenile hormone esterase; JHRE, juvenile hormone response element; FTZ-F1 response element; S2, Drosophila Schneider 2 cell line; TSS, transcription start site; AF-2, activation function 2; H12, helix 12.
biological processes, such as pheromone production, caste determination, diapause, migration, etc. (12).

The pleiotropic nature of JH is one of the reasons why the mode of its action is still debatable (13–15). There is a strong case that some JH-induced signaling events exemplify a rapid “non-genomic” biological response. In Drosophila, treatment of larval salivary glands with JH causes ultrastructural changes of mitochondria known as swelling, which are transcription-and translation-independent (16). When JH is applied in vitro to the ovary of Locusta migratoria, Tenebrio molitor, or Rhodnius prolixus, it creates spaces in the follicular epithelium by reducing the volume of the follicle cells, thus allowing vitellogenin access to the growing oocyte (12, 17). The development of these spaces, termed patency, is mediated by JH binding to the plasma membrane and activation of a downstream regulatory cascade that involves protein kinase C (PKC) and Na+/K+ ATPase.

On the other hand, it is widely accepted that JH could act by regulating gene expression. A number of nuclear proteins (USP, MET, GCE, FKBP39, Chd64, Kr-h1, and FISC) have been identified as critical for JH transcriptional regulation (18–22), although there is no complete understanding of the mechanism (23, 24). Several studies suggest that some actions of JH are mediated by plasma membrane receptors that activate the PKC signaling pathway, as is the case in Drosophila males, where JH stimulates protein synthesis in the accessory glands (9). Another role for PKC in regulating gene expression was proposed in studies of JH signaling in L. migratoria and Choristoneura fumiferana (25, 26). In this case, PKC-mediated phosphorylation reduced the binding of corresponding transcription factors to putative JH response elements, resulting in suppression of JH action.

Another set of studies provides strong evidence that JH modulates gene expression by acting through intracellular receptors, essentially following the paradigm established by steroid hormones, such as ecdysones. Steroid hormones pass through the plasma membrane and bind to nuclear receptors, a superfamily of proteins responsible for regulating major developmental, reproductive, and metabolic processes in all eukaryotic organisms. Nuclear receptors bind to DNA and directly alter transcriptional activity because RNA interference (RNAi)-mediated silencing events depend on MET, which forms a functional complex with its partner FISC, binds JHRE, and activates transcription in the presence of JH (22).

The Drosophila homolog of MET produced in vitro can bind JHIII (a natural form of JH found in most insect species) with nanomolar affinity, and when fused to Gal4 DBD, it displays JH-dependent transcriptional activation of a reporter gene (33). In vivo, JH and MET orchestrate the timing of certain differentiation events because either JH deprivation or MET knock-out causes heterochronic phenotypes in the developing visual system during Drosophila metamorphosis (34). However, the fact that Met-knock-out flies are viable indicates that there is more than one receptor for JH. Recently, a paralog protein encoded by the Drosophila gene germ-cell expressed (gce) was identified and proposed to perform as an intracellular receptor for JH that can functionally substitute for MET (35). Indeed, ectopic GCE can rescue some of MET preadult functions (e.g. restore sensitivity of Met knock-out animals to methoprene, a synthetic analog of JH) (21). However, the redundancy of GCE and MET is not complete but rather stage- and tissue-specific. These observations demonstrate that much information is missing with regard to the nature and number of signaling pathways utilized by JH to regulate diverse biological functions.

In order to better understand the molecular mechanisms underlying JH signaling, we previously conducted a search for JH-regulated genes in Drosophila (36). We found that in S2 cells, JH can activate the E75A nuclear receptor gene (37), which plays a critical role during larval development and at the onset of metamorphosis (38). The activation occurs within 30 min of hormone administration; it is specific and does not require protein synthesis. In this report, we have investigated the molecular pathway of JH signaling for E75A and found that JH must enter the cell to activate this gene. We also provide evidence that the orphan nuclear receptor FTZ-F1 is an essential component of JH signaling that interacts with the candidate JH receptors MET and GCE. FTZ-F1 directly binds to the regulatory regions of E75A and could function as a competence factor to facilitate the direct regulation of gene expression by JH.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Cell Culture, and Cell Transfection**—The pAc5.1/V5-His and pMt/V5-His vectors (Invitrogen) were both used for the expression of proteins described in the transfection assays. Coding regions for Manduca sexta JH esterase and Drosophila melanogaster FTZ-F1, MET, and GCE were amplified by PCR using corresponding cDNA clones as templates and inserted into the expression vector. The JHE cDNA was a gift from Dr. B. Hammock (University of California, Davis), and the FTZ-F1 cDNA was acquired from the Drosophila Genomics Resource Center (expressed sequence tag clone RE02257). Mutant forms of MsJHE were created with the mutagenesis kit (Stratagene) according to the manufacturer’s manual. All inserts were verified by DNA sequencing, and correct expres-
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Drosophila S2 cells were cultured in Schneider’s medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) at 25 °C. JH III (a natural form of JH; Sigma) and methoprene (a synthetic analog of JH) were dissolved in ethanol, and 1 \( \times 10^{-3} \) mM stock solutions were kept at −20 °C. If not otherwise stated, hormones were added to a 1 \( \times 10^{-6} \) M final concentration. Control cells were treated with an equal volume of ethanol.

Transient transfection of S2 cells with expression plasmids was performed by the calcium phosphate method as described previously (39). In each transfection, pAc-GFP or pAc-lacZ expression construct was included as a control of transfection efficiency.

Luciferase and \( \beta \)-Galactosidase Reporter Assays—FTZ-F1, MET, and GCE expression constructs were transfected in S2 cells along with the FIREx3-Tk-Luc reporter construct (a gift from Dr. H. Ueda at Okayama University) and pAc-lacZ (Invitrogen). Protein extracts from S2 cells were quantified using a Bradford assay (Bio-Rad) and standardized by diluting with 1× reporter lysis buffer to yield equivalent amounts of total protein. Luciferase activity was measured using the luciferase assay system (Promega) by using absorbance at 420 nm for 10 min.

RNA Extraction and Northern Blot Hybridization—Total RNA was isolated from S2 cells using the RNeasy minikit (Qiagen). All RNAs were quantified by optical measurements, and 10 \( \mu g \) of each sample was fractionated on a 1% agarose-formaldehyde gel and transferred to a nylon membrane (MSI). Blots were prehybridized at 68 °C for 60 min in a buffer composed of 40 mM sodium phosphate, pH 6.6, 5 × Denhardt’s solution, 5 × SSC, 1% SDS, 200 \( \mu g/ml \) shared salmon sperm DNA (Sigma). Blots were hybridized with specific \( ^32P \)-labeled probes (2–10 \( \times 10^5 \) cpm/ml) at 68 °C for 16–24 h in the same buffer supplemented with 10% dextran sulfate. Probes were labeled by PCR using primers listed in Table 1. After hybridization, blots were washed in 0.1× SSC, 0.1% SDS and exposed at −80 °C with Kodak BioMax MS film.

Double-stranded RNA-induced Gene Silencing—To produce double-stranded RNA (dsRNA) for RNAi, individual DNA fragments containing protein coding sequences were amplified by PCR and cloned in both orientations into the pGM-T Easy cloning vector (Promega). The following primer pairs were used: dERR (5′-CTT GCA CAT CAA ACA GGA GG-3′ and 5′-CAA TGA CGC TGA CCA ATT CC-3′); dHNF-4 (5′-ATC GGA GAG CCA CAT AAT GC-3′ and 5′-GCA GGT TAT CGA TGA TAC GG-3′); DHR39 (5′-AGA TGC CAA ACA TGT CCA GC-3′ and 5′-AGT CCA TAC TCA GGT TCT GC-3′); DHR78 (5′-GCG TTA AGG TTG AGA AGT CTG-3′ and 5′-TAC CAT TTC GCT CCA GTT GG-3′); DHR96 (5′-GGA GCA GAA GCT GAT CAA GC-3′ and 5′-GGT ACT CAA TGC GGA TCA CC-3′); EcR (5′-ATG AGA AGC AGA GCC AAA CGG-3′ and 5′-AGC TGT GGC TGT GGT TGA ATC-3′); FTZ-F1 (5′-GGA AGT TGC TGA TGA TGC GC-3′ and 5′-TGC AGT TGG TCC TGC AGC GC-3′); USP (5′-TAA AGT GCG CTG CTC CAT CG-3′ and 5′-GAA GTC ATC AGA ACC GTT GC-3′); MET (5′-TGA GGC AAG GAG AGT GAG CG-3′ and 5′-TCC TCC TCC AGA TCA CTG CC-3′); GCE (5′-AGT TCA TAT GAG GGC TGG TCC AC-3′ and 5′-ATT CCG ATC CGA ATC TG-3′). Generated clones were used as templates to synthesize sense and antisense single-stranded RNA using the T7 MEGAscript Kit (Ambion). Equal amounts of complementary strands of RNA were resuspended in H₂O and annealed at a heating/cooling cycle to yield a dsRNA.

RNAi knockdown of gene expression in S2 cells was performed according to the protocol developed by Clemens et al. (40). Briefly, 2–3 \( \times 10^6 \) Drosophila tissue culture cells were plated into a 25-cm² flask (Corning Glass) and cultured at 25 °C until becoming about 70% confluent. Then FBS-containing medium was replaced with 2 ml of serum-free medium, and 40 \( \mu g \) of dsRNA was added and mixed by gentle swirling. After a 30-min incubation, 4 ml of medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 \( \mu g/ml \) streptomycin was added. Three days later, cells were subjected to hormonal treatment, and RNA samples were extracted and tested by Northern blot hybridization.

GST Pull-down Assay and Western Blot Hybridization—GST pull-down assays were performed as described by Godlewska et al. (41). Plasmids encoding GST-MET or GST-GCE fusion proteins (a kind gift of Dr. T. Wilson (Ohio State University)) were co-transfected with the V5-tagged FTZ-F1 construct in S2 cells. After 24 h of transfection, cells were treated for 15 min with methoprene or an equivalent volume of solvent ethanol and then subjected to lysis in a buffer composed of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 5 mM EGTA, 1 mM dithiothreitol (DTT). Complete protease inhibitor mixture, and PhosSTOP phosphatase inhibitor mixture tablets (Roche Applied Science). Lysates were incubated for 30 min on ice, cleared by centrifugation at 15,000 \( \times g \) for 15 min at 4 °C, and incubated with glutathione-Sepharose beads from 3 h to overnight at 4 °C. Then beads were washed five times with the lysis buffer, all at 4 °C, and bound proteins were recovered by boiling in Laemmli sample buffer and analyzed by SDS-PAGE.

For Western hybridization, proteins were electrotransferred onto nitrocellulose. Blots were blocked for 30 min at room temperature in PBST buffer (1× PBS, 0.05% Tween 20) supplemented with 5% nonfat dry milk and then incubated overnight at 4 °C in PBST, 5% milk with anti-V5 (1:10,000) or anti-GST (1:2500) antibody. Proteins were revealed with peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.) and the enhanced chemiluminescence (ECL) method (Pierce).

Chromatin Immunoprecipitation—Immunoprecipitation of cross-linked DNA from S2 cells was performed as described (39). 95–100% confluent cells were treated with either ethanol or 1 \( \times 10^{-6} \) M methoprene for 1 h. Cells were then cross-linked with 1% formaldehyde for 10 min at room temperature, followed by quenching with 0.125 M glycine. Cells were washed with 1× PBS, and plasma membranes were lysed with swelling buffer (25 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.1% Nonidet P-40 plus added before use 1 mM DTT, 1× Complete protease inhibitor mixture from Roche Applied Science). Nuclei were resuspended in sonication buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1%
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sodium deoxycholate, 0.1% SDS plus protease inhibitors) and sonicated to yield average DNA fragments of 200–1000 bp. After centrifugation to remove cell debris (25 min at 5000 rpm), chromatin concentration was quantified by A260 and equalized among samples with sonication buffer.

Chromatin was pre-cleared for 1 h at 4 °C with salmon sperm DNA/Protein A-agarose beads (Millipore) and incubated overnight at 4 °C with either nonspecific IgG (goat anti-rabbit, Jackson Immunoresearch) or anti-FTZ-F1 antibodies, which were kindly provided as a gift by Dr. C. Wu (NCI, National Institutes of Health, Bethesda, MD). Immunoprecipitated protein-DNA complexes were retrieved with Protein A-agarose, washed, and then recovered from the beads with elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). Cross-linking was reversed overnight at 65 °C, followed by RNase and proteinase K treatment. DNA was purified with phenol/chloroform followed by ethanol precipitation. Purified DNA pellets were dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and used for real-time PCR analysis.

**Computational Analysis**—Conservation analysis upstream of the E75A transcription start site was performed with the EvoPrinterHD server (42). EvoPrinterHD takes as input a genomic query sequence from D. melanogaster, and reference sequences are assembled from the available genomes of 11 other Drosophila species. Each reference sequence is broken into fragments three different ways (two staggered sets of 11-mers and a set of 9-mers) and aligned to the query sequence. All reference/query alignments are then overlapped into a composite alignment showing conservation among the 12 species. The composite alignment shows the query sequence with absolutely conserved nucleotides highlighted and nucleotides conserved in 11 of 12 species colored according to the species lacking the sequence. For E75A analysis, the first 32 kb of upstream non-coding region containing five experimentally identified enhancers was submitted to EvoPrinterHD. By extracting discrete sequences (≥9 nucleotides long) conserved in at least 11 Drosophila species, we generated a data base of highly conserved elements (supplemental Table S1).

Putative FTZ-F1 recognition elements (F1REs) were identified and mapped using the Pattern Locator server, which allows for direct screening of any chosen nucleotide pattern (43). As a query sequence, we used a consensus compiled from a set of experimentally determined F1REs (44). Only the first nine nucleotides of this consensus, (C/G/T)(C/G)AAGG(A/C/T)(C/T)(A/G), were used in the search because the remaining three nucleotides at the 3’-end have a negligible impact on FTZ-F1 binding (45). The query pattern was searched against the database of highly conserved nucleotides that we generated for sequences upstream of the E75A transcription start site (see above).

**Quantitative PCR and RT-PCR**—Quantitative real-time PCR was performed with FastStart Universal SYBR Green PCR Mix (Qiagen) using 300 μM primers. Thermocycling (95 °C for 15 s, 60 °C for 1 min) was performed for 40 cycles using the Applied Biosystems 7300 real-time PCR system. For quantitative PCR following chromatin immunoprecipitation, enrichment was measured as a percentage of input DNA taken prior to immunoprecipitation using the comparative Ct method (46). For quantitative RT-PCR following FTZ-F1 transient transfection assays, RNA was reverse transcribed with Transcriptor (Roche Applied Science) according to the manufacturer’s manual, and cDNA levels were quantified by quantitative PCR using the comparative Ct method. Amplicon specificity was confirmed using dissociation curves and separation of post-PCR samples on 2% agarose gel. Primer sequences are available upon request.

**RESULTS**

**JHE Blocks Hormonal Activation of E75A**—JH esterase is naturally designed to efficiently metabolize and inactivate juvenile hormone. To determine whether JHE can block hormonal activation of E75A, we ectopically expressed JHE in S2 cells by transfecting an expression plasmid bearing the V5-tagged JHE open reading frame under a constitutive actin promoter. Using Western blot to detect the presence of the V5 epitope, we observed high levels of JHE protein in the experimental cells but not in control ones transfected with an empty vector (pAc-Vector) or a plasmid encoding M. sexta JHE (pAc-MsJHE) and 24 h later treated with 1 μM JHIII for the periods of time shown above each lane. All transfections included the GFP expression plasmid as a control for transfection efficiency. Each sample was then divided into two aliquots for analysis. A, expression of JHE and GFP proteins was confirmed by Western blot hybridization with anti-V5 antibody. B, E75A and rp49 (control for loading) transcript levels were measured by Northern hybridization using specific radiolabeled probes.

![FIGURE 1. JHE suppresses JH activation of E75A](image-url)

**FIGURE 1.** JHE suppresses JH activation of E75A. S2 cells were transfected with an empty vector (pAc-Vector) or a plasmid encoding M. sexta JHE (pAc-MsJHE) and 24 h later treated with 1 μM JHIII for the periods of time shown above each lane. All transfections included the GFP expression plasmid as a control for transfection efficiency. Each sample was then divided into two aliquots for analysis. A, expression of JHE and GFP proteins was confirmed by Western blot hybridization with anti-V5 antibody. B, E75A and rp49 (control for loading) transcript levels were measured by Northern hybridization using specific radiolabeled probes.

To confirm that the prevention of E75A JH activation by JHE is a specific result of the enzymatic degradation of JH, the experiment was repeated using two mutant forms of JH esterase. Based on site-directed mutagenesis, molecular modeling,
and the crystal structure of *M. sexta* JHE (47–49), it was established that JHE contains a catalytic triad made up of serine (Ser226), glutamate (Glu357), and histidine (His471). We generated two inactive forms of JHE either by changing the serine codon AGC into the glycine codon GGC (JHES226G) or by complete removal of the serine codon (JHES226Del). To test whether mutant JHE could still block E75A hormonal activation, all three enzymes (JHEWT, JHES226G, and JHES226Del) were expressed in S2 cells. Although each protein was expressed at approximately equal levels (see Fig. 2, Western), only JHE containing the catalytic serine residue was able to block E75A activation (see Fig. 2, Northern).

In both experiments (Figs. 1 and 2), JHIII was added to a final concentration of 1 μM, and we found essentially a complete abrogation of E75A expression with JHE. Thus, in a cell culture system in which E75A induction occurs at nanomolar concentration of JH (37, 50), ectopic JHE is able to effectively metabolize JHIII and eliminate hormone-dependent gene activation.

**JH Activation of E75A Occurs through an Intracellular Signaling Mechanism**—One of the characteristic features of all known JH esterases is a signal peptide of about 17–23 amino acids at the N terminus (1, 51). The presence of this signal defines JHE as a secretory protein that conforms to a common observation that *in vivo* JHE is primarily found in insect hemolymph. In our experiments, we also noticed that between 10 and 25% of total JHE activity in the culture flask was found in cells, and the remainder was present in the culture medium, indicating that JHE synthesized in the cells is in fact secreted (data not shown).

To determine whether JH action is initiated at the cell surface or through an intracellular mechanism, we designed a JHE-coding plasmid in which the signal peptide sequence needed for secretion has been removed, so upon transfection this expression vector gives rise to a new form of JHE that is exclusively intracellular (JHEIC). Fig. 3A shows that the full-length protein (JHEWT) is indeed present in both the cell culture medium and the cell extracts; by contrast, the N-terminally truncated protein (JHEIC) is present in cell extracts but absent from the culture medium.

The prediction was that if hormone-dependent E75A transcription requires that JHIII enters the cell, ectopic JHEIC will...
metabolize the hormone and thereby prevent E75A induction. However, if the induction is initiated by hormone binding to the cell membrane receptor, JHIII will be inaccessible to JHEIC, and E75A expression will still occur. As expected, transfection of empty vector has no effect on the E75A response to JH, whereas overexpression of JHEIC blocks the response (Fig. 3, A and B). Expression of JHEIC produces a protein of approximately equal intensity but of lower molecular weight, confirming the missing N-terminal region. Importantly, expression of JHEIC blocks E75A JH activation to the same degree as the wild-type protein, demonstrating that JH must enter the cell in order to activate E75A. We therefore conclude that JH activation of E75A occurs through an intracellular mechanism.

The FTZ-F1 Nuclear Receptor Is Required for JH Activation of E75A—The classical paradigm of steroid hormone action is that hormones modulate gene expression via specific intracellular receptors (i.e., members of the nuclear receptor superfamily). Based on this model and the finding that JH induction of E75A occurs through an intracellular pathway, we hypothesized that some nuclear receptors could be involved in JH signaling. The Drosophila genome encodes 18 members of the nuclear receptor superfamily (27). To determine which of these genes is expressed in S2 cells and whether any of them respond to JH activation, we used Northern blot hybridization with probes specific to all 18 nuclear receptor genes (Table I). Nine probes did not produce any signal, whereas the other nine detected specific transcripts in total RNA samples extracted from cells cultured in the presence or absence of JH (Fig. 4). DHR59, DHR39, DHR78, DHR96, and FTZ-F1 genes displayed continuous expression; their transcripts were abundant in all samples independent of hormone treatment. The E75A mRNA was the only one that was rapidly and strongly induced upon hormone treatment, although the expression of four other nuclear receptor genes also displayed some JH dependence. EcR and usp transcript levels declined, and dHNF4 and dERR transcript levels increased during the 4-h period of hormone treatment. However, determination of whether these changes represent a primary hormone response requires further analysis.

![FIGURE 4. Nuclear receptor gene expression in S2 cells.](image-url)

To determine whether any nuclear receptors are required for JH activation of the E75A gene, we used the RNAi approach. S2 cells were transfected with dsRNA targeting each of the expressed nuclear receptor genes (DHR39, DHR78, DHR96, dHNF4, FTZ-F1, dERR, EcR, and usp), and 3 days later, cells were treated with JHIII. Hybridizations with the dHNF4, DHR78, and FTZ-F1 probes confirmed the efficiency and specificity of the knockdown (Fig. 5). Nuclear receptor transcript levels were reduced by about 80% in cells transfected with corresponding dsRNA. Hybridization with the E75A probe showed the role of each nuclear receptor in JH activation. In the absence of dsRNA, E75A exhibits characteristic expression; this expression is unaffected by dsRNA-mediated silencing of dHNF4 or DHR78 nuclear receptor genes (compare E75A expression in lanes 1–4 versus lanes 5–12 in Fig. 5). By contrast, knockdown of FTZ-F1 abrogates JH activation of E75A (Fig. 5, lanes 13–16). Knockdown of the remaining nuclear receptors has no effect on E75A JH activation (data not shown).
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To look more carefully at the requirement of FTZ-F1 for JH activation of E75A, we performed a complementary experiment and measured the effect of ectopic FTZ-F1 protein on E75A expression. S2 cells were transfected with an empty vector (control) or a plasmid carrying the FTZ-F1 open reading frame under an inducible metallothionein promoter. After 24-h incubation in the presence of Cu2+, cells were treated with methoprene. Total RNA was extracted, and E75A expression was studied by Northern blot hybridization and by quantitative RT-PCR (Fig. 6). Ectopic FTZ-F1 expression was readily detectable in cells transfected with the pMt-FTZ-F1 construct but not in control cells. Methoprene activation of E75A is apparent in both groups of cells; however, it is stronger in the presence of ectopic FTZ-F1 (Fig. 6A). A more quantitative approach with quantitative RT-PCR shows that in control cells, E75A expression is elevated just under 3-fold as a response to methoprene. In the presence of FTZ-F1, E75A expression reaches nearly 4-fold, an increase of about 45–50% (Fig. 6B). Thus, ectopic FTZ-F1 is able to potentiate the inducibility of the E75A gene by JH. These results clearly show that the orphan nuclear receptor FTZ-F1 is an essential component of E75A JH activation in S2 cells.

In Vivo FTZ-F1 Binding Upstream of the E75A Transcription Start Site—FTZ-F1 is a DNA-binding transcription factor (52), and its binding to the target genes has been documented (53–56). Because our data show that FTZ-F1 is necessary for the expression of E75A induced by JH, we suggested that FTZ-F1 exerts its function through direct interaction with the E75A regulatory DNA. In the past, the presence of the FTZ-F1 protein at the 75B puff (the cytological location of the E75A transcription unit) was shown by antibody staining of late prepupal salivary gland polytene chromosome spreads (53). However, specific FTZ-F1 binding sites have never been identified and/or mapped for E75A. Previously, we characterized the chromatin structure of the 60 kb of non-coding region upstream of the E75A TSS and identified five enhancers (39). To determine whether FTZ-F1 binds in vivo to any of these enhancers, we first conducted a computational search for putative F1REs. Based on the assumption that functionally important regulatory elements are often conserved during evolution, we employed the EvoPrinterHD program to identify sequences conserved across the 12 Drosophila species. As an input, we submitted the 32-kb region upstream of the E75A TSS, which contains all five enhancers (Fig. 7A). In this region, ~10% of nucleotides and 127 discrete elements are conserved in at least 11 of 12 Drosophila genomes (for the complete data set, see supplemental Table S1). The remaining non-coding region was excluded from analysis because we previously did not find any enhancers in this region and because conservation is negligible (less than 1%; data not shown). Then we used Pattern Locator to search the database of highly conserved nucleotides for the F1RE consensus sequence and identified eight putative FTZ-F1 binding sites (Table 2). Seven of these sequences are distributed among five enhancers, whereas one sequence was identified in a region between enhancers III and IV. The seven sequences located within enhancers represent likely functional F1REs and are designated A–G (Fig. 7A).

To determine which of the putative F1REs is bound in vivo by FTZ-F1, chromatin immunoprecipitation was performed in S2 cells using either anti-FTZ-F1 antibodies or unrelated IgG. Enrichment at each of the seven candidate F1REs was quantified by quantitative PCR (Fig. 7B). As expected, very little enrichment is observed using nonspecific IgG. By contrast, six sites show enrichment by FTZ-F1 antibodies, which is significantly higher than IgG (p < 0.05), although the strength of enrichment varies at each site. An exception was observed at
site D, where FTZ-F1 enrichment is comparable with that of IgG. Thus, upstream of E75A there are at least six F1REs that represent in vivo binding sites for FTZ-F1. As a control, we also measured enrichment within the first intron of the white gene, where there are no conserved F1RE sequences and thus FTZ-F1 is predicted to be absent. We also measured enrichment at a sequence matching the F1RE consensus found outside of the five upstream enhancers (denoted by an asterisk in Fig. 7B). Enrichment by FTZ-F1 antibodies at both of these sites is essentially identical to enrichment by nonspecific IgG. Finally, in order to determine whether FTZ-F1 is also bound in vivo to E75A during the JH response, immunoprecipitated samples that were first treated with methoprene for 1 h were prepared in parallel. The addition of methoprene did not significantly alter the level of enrichment at any of the F1REs. These observations show that FTZ-F1 is bound to each E75A enhancer in vivo prior to the JH activation and that it remains bound during the JH response of E75A in S2 cells.

Heterodimerization of FTZ-F1 with MET and GCE—Previous studies suggest that the FTZ-F1 nuclear receptor functions as a ligand-independent competence factor that relies on protein-protein interactions to facilitate a recruitment of transcriptional regulators to their target promoters (56–59). Because our data indicate that FTZ-F1 is required for JH activation, and it is bound to E75A prior and during hormonal response, we suggested that FTZ-F1 may directly interact with the JH receptor. Because MET and its paralog GCE have been identified as primary candidates for the JH receptor in Drosophila, we tested whether these proteins could form heterodimers with FTZ-F1 in a GST pull-down assay. GST-MET and GST-
GCE fusion proteins were co-expressed in S2 cells with the V5-tagged FTZ-F1 receptor. Complexes were formed in cells cultured either in the presence or absence of a ligand, 1 \mu M methoprene. Whole cell extracts were made, and protein expression was tested by Western blot analysis using anti-V5 and anti-GST antibodies; GST-MET, GST-GCE, and V5-FTZ-F1 proteins were all present at similar levels in lysates prepared from transfected cells independently of hormone treatment (Fig. 8). From these extracts, complexes containing GST-fused proteins were pulled down using glutathione-Sepharose beads. Cells in which GST-fused proteins were not co-expressed served as a negative control for the FTZ-F1 pull-down. As shown in the Fig. 8A, FTZ-F1 does not stick to Sepharose beads, but it does bind to GST-MET and GST-GCE. The binding of FTZ-F1 to MET is further boosted by the addition of methoprene, whereas the binding of FTZ-F1 to GACE appears to be less hormone-dependent (Fig. 8B). When co-expressed with the GST protein alone as an additional control, FTZ-F1 did not show up in the pull-down eluate (data not shown). Thus, FTZ-F1 specifically interacts with both of the JH receptor candidates, MET and GACE.

**FIGURE 8.** **FTZ-F1 forms heterodimers with MET and GCE.** A, S2 cells were co-transfected with plasmids encoding V5-FTZ-F1 and GST-MET or GST-GCE. After 24-h transfection, cells were treated with ethanol or 1 \mu M methoprene for 15 min. Cells were lysed, and GST pull-down was performed with glutathione-Sepharose beads. Protein expression from pull-down and from input lysates was detected by Western blot hybridization using anti-V5 or anti-GST antibody. B, relative band intensities from GST pull-down experiments were quantified and plotted. Binding levels of V5-tagged FTZ-F1 are shown relative to the control lane with no GST protein expressed. Data are shown as the mean ± S.D. (error bars) and are the result of at least three independent experiments.

**FIGURE 9.** MET-FTZ-F1 and GACE-FTZ-F1 heterodimers activate transcription through the F1RE. A, schematic illustration of the reporter plasmid used to detect transcription activation. Three copies of the FTZ-F1 response element (F1RE x3) are located upstream of the thymidine kinase minimal promoter (Ik) and the luciferase open reading frame (Luc). B and C, S2 cells were transfected with F1RE3-tk-Luc reporter plasmid, lacZ plasmid, and expression plasmids encoding the indicated proteins: wild type FTZ-F1, truncated FTZ-F1 (Δ), MET (B), or GACE (C). After transfection, cells were treated with ethanol (white bars) or 5 \mu M methoprene (gray bars) for 24 h. Luciferase activity of cell lysates was normalized to \beta-galactosidase activity and is shown relative to ethanol-treated sample with no ectopic protein expression. +, 10 \mu g of transfected plasmid; †, increase of transfected plasmid from 0.5 to 5 and 10 \mu g. Data are shown as the mean ± S.D. (error bars) and are the result of at least three independent experiments.
When expressed individually, neither FTZ-F1, MET, nor GCE proteins are able to significantly boost hormonal activation. However, when FTZ-F1 and MET are expressed together, the methoprene-dependent activation of the luciferase reporter is significantly elevated and is dependent on the level of ectopic MET (Fig. 9B). When FTZ-F1 and GCE are co-expressed, the reporter also displays a significant increase in activity, which is dependent on the level of GCE (Fig. 9C). However, unlike MET-FTZ-F1, the GCE-FTZ-F1 heterodimer can activate transcription without methoprene, and the addition of hormone has only a minor effect on activation. Despite this difference, the data imply that MET-FTZ-F1 and GCE-FTZ-F1 heterodimers can mediate transcriptional activation through the F1RE.

To validate the ability of MET and GCE to form functional heterodimers with FTZ-F1, we generated a truncated form of FTZ-F1 with a deletion of helix 12 (H12; 15 amino acid residues from the C terminus) of the activation function 2 (AF-2) domain. Because nuclear receptors interact with co-activator proteins through AF-2 using H12 as the heterodimerization surface, we reasoned that deletion of H12 should abrogate interaction of MET and GCE with FTZ-F1ΔH12. Indeed, when co-expressed with MET or GCE, the truncated form of FTZ-F1 failed to activate the reporter construct (Fig. 9, B and C). We conclude that MET-FTZ-F1 and GCE-FTZ-F1 heterodimers are formed via AF-2.

**GCE, but Not MET, Is Required for JH Activation of E75A**—Having determined that both MET and GCE could heterodimerize with FTZ-F1 and activate transcription through the F1RE, next we asked whether MET, GCE, or both are required for JH activation of E75A. First, using Northern blot hybridization, we confirmed that Met and gce genes are expressed in S2 cells (Fig. 10, lanes 1–4). Then we knocked down their expression by exposing cells to dsRNA targeting Met, gce, or both. All cells were treated with JHIII, and the mRNA levels of E75A were assessed by Northern blot. Hormone-mediated transcriptional activation of E75A was not affected in the Met RNAi cells (Fig. 10, lanes 5–8), whereas cells treated with gce or gce and Met dsRNA displayed a sharp reduction in JH activation (Fig. 10, lanes 9–16). These results suggest that GCE, but not MET, is required for JH activation of the E75A gene in S2 cells.

**DISCUSSION**

**Intracellular Signaling by JH**—One of the striking features of juvenile hormone is its wide range of effects on insect development and physiology (12). During preadult development, JH supports larval growth and, together with ec dysone, orchestrates molting and metamorphosis (23, 60). In adults, JH regulates reproductive maturation and affects various aspects of insect behavior (50). The pleiotropic nature of JH creates certain difficulties for understanding the molecular mechanism of its action (15, 24). Some JH functions are apparently initiated at the cell surface level, triggering a membrane-receptor-mediated signal transduction pathway, as is the case with vitellogenesis in females and accessory gland protein synthesis in males. Based on the lipophilic nature of JH, the alternative suggestion is that JH follows the paradigm set up by steroid hormones and acts through an intracellular receptor. Much effort has focused on the latter model, yielding one candidate receptor, MET. The MET protein is a critical component of antimetamorphic regulation by JH in the red flour beetle *T. castaneum* (30). In *Drosophila*, at least some non-vital functions of JH during fly metamorphosis are mediated through MET (34). However, the overall consensus is that JH acts through multiple pathways by utilizing more than one receptor, which highlights the importance of characterizing the specific mechanism of regulation for JH-responsive genes.
We previously identified the \textit{E75A} gene as JH-inducible in the S2 cell line (37). An increase in \textit{E75A} transcript abundance is evident after only 30 min of JH treatment and can occur in the presence of a protein synthesis inhibitor; both of these features indicate a primary response to hormone. In this study, we determined the pathway utilized by JH by taking advantage of the powerful enzymatic activity of JHE that specifically removes intracellular JH from S2 cells. This approach allowed us to determine unequivocally that the presence of JH within the cell is the determining factor for the JH activation of \textit{E75A}. We therefore conclude that JH regulation must occur by utilizing an intracellular receptor pathway.

Members of the nuclear receptor superfamily are key regulators of physiology and represent the classical model of intracellular regulation of gene expression. Nuclear receptors directly alter gene expression by entering the nucleus and binding to DNA response elements in the presence of ligand. In vertebrates, nearly 50 receptors have been identified, and many have been associated with a wide range of small, lipophilic ligands. \textit{Drosophila} represents a much simpler system, with only 18 nuclear receptor genes in the genome and only two known physiologically active lipophilic hormones, ecdysone and juvenile hormone (27). Here, we explored the possibility that JH activation of \textit{E75A} requires a nuclear receptor by individually removing expression of each nuclear receptor gene in S2 cells. From this screen, we identified the orphan nuclear receptor FTZ-F1 as essential to mediating the response to JH. We also showed that FTZ-F1 is bound \textit{in vivo} to the \textit{E75A} gene prior to and during the JH response. Our finding that a nuclear receptor required for JH activation is present in the nucleus and physically bound to \textit{E75A} strongly suggests that the activation of \textit{E75A} occurs through direct regulation of gene expression and complements our findings from the JHE experiments.

\textit{FTZ-F1 Provides Competence for Transcriptional Activation by JH—} FTZ-F1 is an essential component of JH signaling but does not appear to be a JH receptor. No ligand has been associated with FTZ-F1, and studies of FTZ-F1 and related mammalian homologs (LRH-1 and SF-1) suggest that it is not likely to be a hormone-binding receptor. LRH-1 and SF-1 possess constitutively active ligand-binding domains even in the absence of a ligand, although fortuitously bound bacterial phospholipids were identified in the crystal structures of corresponding ligand-binding domains (61, 62). FTZ-F1 itself possesses a ligand-binding pocket that is too small to bind a ligand and still has the AF-2 in the active conformation, suggesting that the FTZ-F1 ligand-binding domain is constitutively active and does not require ligand binding (63).

How does FTZ-F1 mediate gene activation in response to a hormonal signal? Although FTZ-F1 exhibits widespread tissue distribution, its temporal profile is highly restricted. The timing of expression is always found to follow the ecdysone peak; it is transiently expressed before each larval ecdisis and before pupation (64, 65). Genetic analysis showed that FTZ-F1 is necessary for larval molting and early metamorphosis (58, 64). In \textit{Drosophila}, the molecular mechanism of FTZ-F1 action was primarily studied during the ecdysone-activated larva-to-pupa transition, when it serves as a stage-specific competence factor. Its expression in mid-prepupa is both necessary and sufficient to define the temporal specificity of ecdysone signaling (57, 58, 66). In the mosquito \textit{Aedes aegypti}, the AaFTZ-F1 receptor has also been characterized as a competence factor for the ecdysone-activated vitellogenesis. Interestingly, the competence to ecdysone in mosquito fat body is achieved through JH-mediated posttranscriptional control of AaFTZ-F1 (56). In this report, we have explored several features of FTZ-F1 and its role in JH regulation. Our data are reminiscent of FTZ-F1-mediated competence for ecdysone signaling, leading us to conclude that FTZ-F1 can function as a competence factor for JH induction as well.

First, transfection of S2 cells with dsRNA complementary to \textit{FTZ-F1} inhibits transcription of the \textit{E75A} gene in response to JH. Similarly, the knockdown of FTZ-F1 either with dsRNA or a hypomorphic mutation completely blocks ecdysone activation of \textit{E75A} in late prepupae (58, 66). Second, the ectopic FTZ-F1 protein has only a minor effect on gene expression by itself, but it does enhance the JH induction of the \textit{E75A} gene in S2 cells and the ecdysone induction of \textit{E75A} in flies (57). Third, the proposed mechanism by which FTZ-F1 could exert the competency function is through protein-protein interactions with the hormone-receptor complex at the DNA regulatory elements of target genes. Indeed, during the prepupal ecdysone pulse, \textit{Drosophila} FTZ-F1 is bound to many hormone-regulated puffs in the salivary gland polytene chromosomes, including sites that are bound by the EcR-USP heterodimer (53, 67). Moreover, in adult mosquito, AaFTZ-F1 potentiates ecdysone activation by facilitating the recruitment of EcR-USP and its co-activator FISC to ecdysone-inducible target promoters (56).

In this study, we have demonstrated that in S2 cells, FTZ-F1 is bound \textit{in vivo} to multiple F1REs of the \textit{E75A} enhancers prior to and during the JH response. We have also found that FTZ-F1 interacts with the JH receptor candidates MET and GCE. It has to be noted, however, that although both MET/FTZ-F1 and GCE/FTZ-F1 can activate transcription through the F1RE, only GCE is required for JH activation of the \textit{E75A} gene in S2 cells. This observation is consistent with previous data showing that both MET and GCE can promote JH action in \textit{Drosophila}, but their functions are not completely redundant because gce is a vital gene, and \textit{Met} function appears to be dispensable for fly development (21). Our study strongly suggests that FTZ-F1 could provide competence for the JH activation of \textit{E75A} by loading JH signaling components to the promoter through protein-protein interactions.

The ability of FTZ-F1 to interact with receptors for both ecdysone and JH raises an interesting possibility that the FTZ-F1 protein could be involved in the hormonal cross-talk during fly development (60). It has been long known that the JH treatment of \textit{Drosophila} does not produce supernumerary larval molts and/or prevent pupariation. However, the presence of JH was shown to inhibit ecdysone activation of late prepupal puffs (68). Importantly, the critical period for the inhibition coincides with the stage-specific FTZ-F1 expression suggesting that it is required for the antagonistic actions of the two signaling pathways.

In summary, the work presented here shows that JH activates expression of the \textit{E75A} gene via an intracellular pathway that involves the FTZ-F1 nuclear receptor and the GCE bHLH-PAS
transcription factor. Our data suggest that FTZ-F1 functions as a competence factor that facilitates JH activation of gene expression.

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