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Molecular and Cellular Endocrinology 148 (1999) 95–108

MCE
Molecular and Cellular Endocrinology

Spruce budworm (*Choristoneura fumiferana*) juvenile hormone esterase: hormonal regulation, developmental expression and cDNA cloning

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Received 1 July 1998; accepted 23 October 1998

Abstract

We have used the differential display of mRNAs technique to identify *Choristoneura fumiferana* genes that are induced by juvenile hormone I (JH I). Of the six PCR products identified, one bound to a 2.8-kb mRNA from CF-203 cells whose abundance increased when the cells were grown in the presence of JH I. The same 2.8-kb mRNA decreased to undetectable levels when the CF-203 cells were grown in the presence of 20-hydroxyecdysone (20E). The PCR fragment probe also detected a 2.8-kb mRNA in the *C. fumiferana* larval tissues. This 2.8-kb mRNA was present on the first day of the first, third, fourth, fifth and sixth larval and pupal stadia, but was conspicuously absent on the first day of the second larval stadium, as well as during the intermolt periods of the first to fifth instar larval stages. In the sixth instar larvae the 2.8-kb mRNA was detected in the fat body, epidermis and midgut during the intermolt period. The PCR fragment was used as a probe to screen a cDNA library. The deduced amino acid sequence of this 2.8-kb cDNA clone showed similarity with the deduced amino acid sequences of *Heliothis virescens* juvenile hormone esterases (HvJHE). The deduced amino acid sequence of the cDNA clone contained all five functional motifs that are present in most of esterases, proteases and lipases. The cDNA clone was expressed in the baculovirus expression system, producing a protein that showed JHE activity.

Keywords: Juvenile hormone; Juvenile hormone esterase; cDNA cloning; Gene expression; Baculovirus; Spruce budworm; *Choristoneura fumiferana*

1. Introduction

Juvenile hormone (JH) has a dual role in insects, preventing metamorphosis and regulating the maturation process of the reproduction system. During insect development, it modulates metamorphosis by altering the action of 20-hydroxyecdysone (20E), as a result of which the development of pupal and adult characters are inhibited in larvae and pupae respectively. At a critical stage in the final molt, JH disappears, permitting the pupae to metamorphose into adults (Riddiford, 1996). In the adult, however, the JH titer increases again, which results in the control of a wide variety of functions related to reproductive maturation (Wyatt and Davey, 1996). In addition to decreased biosynthesis, JH titer is lowered during larval development by the ester hydrolysis of the JH by JH esterase (JHE) (Hammock, 1985). Thus, JHE plays a crucial role in the regulation of JH titer, which in turn regulates a variety of developmental events in insects.

In most lepidopteran insects studied so far, JHE is synthesized primarily in the fat body and released into the hemolymph, where it hydrolyzes the conjugated methyl ester of JH into JH acids (Weirich et al., 1973). JHE is present in low quantities in the insect hemolymph, but since it has a high affinity for JH, even a trace amount of JH can be rapidly degraded by JHE

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0303-7207/99/$ - see front matter
PII: S0303-7207(98)00228-7

(Ward et al., 1992). In addition, JHE is induced by JH in a feedback mechanism (Jones et al., 1987a).

Because JHE plays an important role in the regulation of JH titer in insects as well as in the development of insecticides based on JHE, this enzyme has been extensively studied in many orders of insects. JHE proteins have been isolated and purified from *Trichoplusia ni* (Yuhas et al., 1983; Rudnicka and Jones, 1987; Wozniak et al., 1987; Hanzlik and Hammock, 1987), *Manduca sexta* (Coudron et al., 1981; Venkatesh et al., 1990), *Heliothis virescens* (Hanzlik et al., 1989), and *Leptinotarsa decemlineata* (Vermunt et al., 1997a). The molecular weight of JHE ranges from 57 to 120 kDa depending on the species and quaternary structure (Vermunt et al., 1997a). There have been two reports on complete JHE cDNA clones isolated from *H. virescens* (Hanzlik et al., 1989) and *L. decemlineata* (Vermunt et al., 1997b). In *T. ni*, partial sequence of a JHE gene has been reported (Venkataraman et al., 1994), while a cDNA clone encoding a JHE-related protein (JHER) has been reported (Jones et al., 1994b). The JHE and JHER from *T. ni* were believed to be structurally distinguishable based on certain residues predicted to function at the active site but were more related to each other than to any other esterase (Jones et al., 1994b). The *H. virescens* JHE cDNA has been inserted into the genome of *Autographa californica* nuclear polyhedrosis virus (AcNPV) and active JHE has been produced by the recombinant virus (Hammock et al., 1990; Ward et al., 1992; Bonning et al., 1995).

In this paper we report the cloning of JHE cDNA from the spruce budworm, *Choristoneura fumiferana*. We performed differential display of mRNAs isolated from FPMI-CF-203 (CF-203) cells grown in the presence or absence of JH I. One of the six PCR fragments isolated was found to hybridize with a 2.8-kb mRNA, the abundance of which increased in the presence of JH I and decreased in the presence of 20E. We used this PCR product as a probe to study the expression of its mRNA throughout the larval development and to isolate a cDNA clone. Sequence analysis showed that this mRNA coded for *C. fumiferana* JHE (*Cf*JHE). We also expressed this cDNA in a baculovirus expression system and produced a functional protein.

2. Materials and methods

2.1. Experimental animals

Spruce budworm (*Choristoneura fumiferana* Clem., Lepidoptera: Tortricidae) eggs were collected within 1 h after oviposition and maintained at 22°C and 70% relative humidity. Under these conditions, the eggs hatched in 8 days. A total of 7 days after hatching, the first instar larvae moulted into second instar stage and entered diapause. The diapausing second instar larvae were stored at 2°C for 20–25 weeks to satisfy the diapause requirement. At the end of diapause, the larvae were reared on an artificial diet at 22°C, 70% relative humidity and a photoperiod of 12-h light and 12-h darkness. Newly molted sixth instar larvae were selected within 30 min after molting, when the head capsule was still white, and were staged from that time onwards. Whole larvae from the first to fifth instar stages, and pupae, as well as the epidermis, fat body and midgut of the sixth instar larvae were used for RNA extraction for Northern blot assays.

2.2. Cell culture and hormone treatment

The CF-203 cells developed from *C. fumiferana* midgut cells (Sohi et al., 1993) were grown in 25-cm^2 Falcon plastic flasks using Insect-Xpress medium (BioWhittaker, Walkersville, MD) supplemented with 2.5% (v/v) fetal bovine serum (FBS). The cultures were seeded with 1.5×10^5 cells per ml of medium, and juvenile hormone I (JH I, 1×10^{-6} M), the protein synthesis inhibitor, cycloheximide (CHX, 10 mg/ml) (Sigma, St. Louis, MO) or 20-hydroxyecdysone (20E, 4×10^{-6} M) (Sigma) was added to the culture a day after the cells were set up. All these chemicals were dissolved in dimethylsulfoxide (DMSO, Sigma); the concentration of DMSO was maintained at 0.1% in all treatments and controls. The cells were harvested at predetermined time intervals and were rapidly frozen in liquid nitrogen and stored at −70°C. Each treatment was replicated three times.

2.3. Differential display of mRNAs

Differential display of mRNAs was performed as described by Liang et al. (1993). Total RNA was isolated from CF-203 cells grown in the presence or absence of 1×10^{-6} M JH I (Ayerst, Montreal, PQ, Canada) for 6 h. Ten-microgram samples of RNA were digested with DNAse, and the cDNAs were synthesized using the reverse transcriptase, Superscript (Life Technologies, Gaithersburg, MD), and T12MN and AP1 primers, and [^{35}S]dATP. The PCR products were resolved on a 6% polyacrylamide-urea denaturing gel and the bands showing the differential expression were excised and used as templates for reamplification. The reamplified PCR products were separated on a 1.2% low melting point agarose gel. The DNA bands were excised, labeled with α-[^{32}P]dATP (Feinberg and Vogelstein, 1984), and used as probes for Northern hybridization and cDNA library screening.

2.4. Northern blot analysis

Total RNA was isolated using the guanidinium isothiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987) either from insect tissues dissected under a microscope or from the cultured cells. Ten micrograms of total RNA were separated on a formaldehyde-agarose gel (1%) and transferred to a Hybond N nylon membrane. The CfJHE PCR product or cDNA fragment was labeled with α-[^{32}P]dATP using the random prime method (Feinberg and Vogelstein, 1984). Hybridization and washes were conducted as described by Palli et al. (1997). Quantitative analysis was performed by using InstantImager™ Electronic Autoradiography (Packard, Meriden, CT).

2.5. cDNA library construction and screening

A cDNA library was constructed in Uni-ZAP XR vector (Stratagene, La Jolla, CA) using mRNA from CF-203 cells and a cDNA synthesis and cloning kit (Stratagene). Screening of the cDNA library was performed using the random prime labeled PCR product isolated using differential display of mRNAs technique.

2.6. Sequencing and computer analysis

The longest cDNA clone isolated from the cDNA library was sequenced in both directions using Cy5™ AutoRead™ Sequencing Kit (Pharmacia, Piscataway, NJ) and ALFexpree DNA Sequencer (Pharmacia). Sequence analysis was performed using the MacVector DNA Analysis Program (International Biotechnologies, New Haven, CT). Sequences were compared to the sequences in GenBank at the National Center for Biotechnology Information using the BLAST Network Service (Altschul et al., 1990). Amino acid sequence alignment was made by using Clustal Alignment Program (Higgins and Sharp, 1988).

2.7. Construction of recombinant baculovirus and infection

The recombinant baculovirus expressing the *C. fumiferana* JHE cDNA was constructed by using the BAC-TO-BAC™ Baculovirus Expression System from Life Technologies (Gaithersburg, MD) following the manufacturers instructions. The CfJHE cDNA was first cloned into the mini-Tn7 element of a pFASTBAC donor plasmid at the sites of *EcoR* I and *Xho* I. The recombinant plasmid was then transformed into DH10BAC cells containing a helper plasmid and *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) bacmid. The mini-Tn7 element carrying the CfJHE cDNA was transposed from the donor plasmid into the bacmid with the help of the helper plasmid. The recombinant bacmid with the inserted CfJHE cDNA was selected based on disruption of the β-galactosidase peptide gene (*lacZ'*) and confirmed by PCR. Expression of the CfJHE cDNA was under control of the polyhedrin promoter in this polyhedrin-minus recombinant virus.

Spodoptera frugiperda SF-21 cells (Vaughn et al., 1977) were cultured in Grace's medium (Grace, 1962) supplemented with 10% FBS and 0.25% trytose broth at 1×10^6 cells per well in six-well plates. The cells were incubated with a transfection mixture containing 300 ng of the recombinant bacmid DNA and 10 μl CELLFECTIN™ reagent (Life Technologies, Gaithersburg, MD) for 5 h at 28°C with gentle shaking. After incubation, the transfection mixture was removed, and 2 ml per well of fresh Grace's medium was added, and the cells were inoculated at 28°C for a further 4 days. The recombinant baculovirus was then harvested and used to infect SF-21 cells cultured in 15-ml flasks for studies on expression of mRNA and protein. The titer of the recombinant virus was determined and a multiplicity of infection (MOI) of 0.06 pfu was used in all experiments.

While the medium from these infected cultures was collected for measurement of enzyme activity, the cells were harvested and used for analysis of mRNA expression at 0, 1, 6, 12, 24, 48, 72, and 96 h post infection (h.p.i.).

2.8. JHE activity assay

Assays for JHE activity were conducted as described by Hammock and Sparks (1977), and Share and Roe (1988). Media were collected after inoculation and concentrated 30 times using a Millipore Ultrafree-15 centrifugal filter (Millipore, Bedford, MA). One hundred microliters of the concentrated media and 1 μl of substrate [^{3}H]-JH III (19.5 Ci/mmol, DuPont, Mississauga, ON) were incubated at 30°C for 1 h. The reaction mixture was then partitioned between an organic isooctane phase and an aqueous methanol-water-ammonium hydroxide (10:9:1) phase. Radioactivity in the organic phase and aqueous phase was counted. The JHE activity is presented as pmoles JH III metabolized/min per ml.

3. Results

3.1. Identification of JH inducible gene

RNA isolated from CF-203 cells grown in the presence and absence of JH I was analyzed by the mRNA differential display technique. Six bands that showed increased-expression in the presence of JH I were excised from the gel and reamplified by using PCR (data

not shown). The PCR products were then labeled and used to hybridize Northern blots containing RNA isolated from CF-203 cells grown in the presence of JH I for 0, 1, 3, 6, 12 and 24 h. One of the six PCR products bound to a 2.8-kb mRNA; the abundance of this mRNA increased after the cells were exposed to JH I for 1 h. For the reasons explained below we have designated this cDNA as *C. fumiferana* juvenile hormone esterase (CfJHE) cDNA. Other PCR product probes bound to mRNA of different sizes but did not show any JH I induction and were therefore not studied further.

3.2. Hormonal regulation of CfJHE mRNA

The PCR product that bound to the 2.8-kb mRNA was used as a probe to further study the regulation of the mRNA by JH I and 20E. CF-203 cells were grown in the Insect X-Press media containing no hormone, or 1×10^{-6} M JH I or 10 mg/ml CHX, or both 1×10^{-6} M JH I and 10 mg/ml CHX. The cells were collected at 1, 3, 6, 12 and 24 h post treatment (h.p.t.). Total RNA was isolated and analyzed on Northern blot using the PCR product as a probe. The probe bound to a 2.8-kb mRNA present in CF-203 cells and the mRNA levels remained unchanged in the cells grown in the medium containing no hormone (Fig. 1A). When the cells were grown in the presence of 1×10^{-6} M JH I, the mRNA level started to increase within 1 h.p.t. and reached its maximum level by 3 h.p.t., followed by a decrease to background level by 24 h.p.t. CHX also caused an increase in the mRNA level within 1 h.p.t. and the mRNA increased to maximal levels by 3 h.p.t. The mRNA expression then decreased and reached background level by 12 h.p.t. In cells grown in the presence of both JH I and CHX, the mRNA level also started to increase within one h.p.t. and reached the maximum level by 3–6 h.p.t. But the mRNA concentration did not decrease to background level until 24 h.p.t. Furthermore, in the presence of both JH I and CHX, the increase in the mRNA level seemed to be additive, higher levels of the mRNA being detected during 1 to 6 h.p.t. than in the presence of either JH I or CHX alone. The increase in the 2.8-kb mRNA level in response to JH I treatment was dose dependent (Fig. 1B,C). The mRNA levels started to increase at 1×10^{-8} M JH I

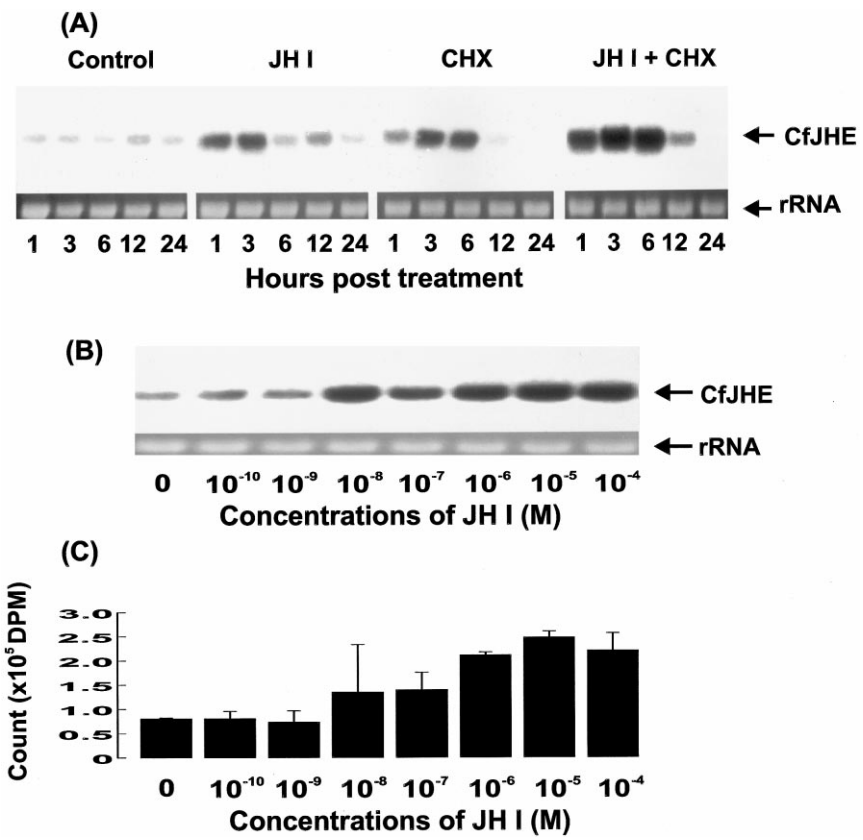

Fig. 1. JH I increases the CfJHE mRNA levels in CF-203 cells. (A) The top panels show the Northern blots hybridized with the CfJHE PCR fragment probe. Each lane contains 10 μg of total RNA extracted from CF-203 cells untreated or treated with 10^{-6} M JH I or/and 10 mg/ml CHX. The bottom panels show ribosomal RNA stained with ethidium bromide. (B) Dose response of JH I induction of CfJHE mRNA. The top panel shows the Northern blot hybridized with the CfJHE PCR fragment probe. Each lane contains 10 μg of total RNA extracted from CF-203 cells treated with 1×10^{-10}–1×10^{-4} M JH I and collected at 3 h.p.t. The bottom panel shows ribosomal RNA stained with ethidium bromide. (C) Radioactivity in the 2.8-kb band was quantified using Instant Imager. The data are the average of two replicated assays.

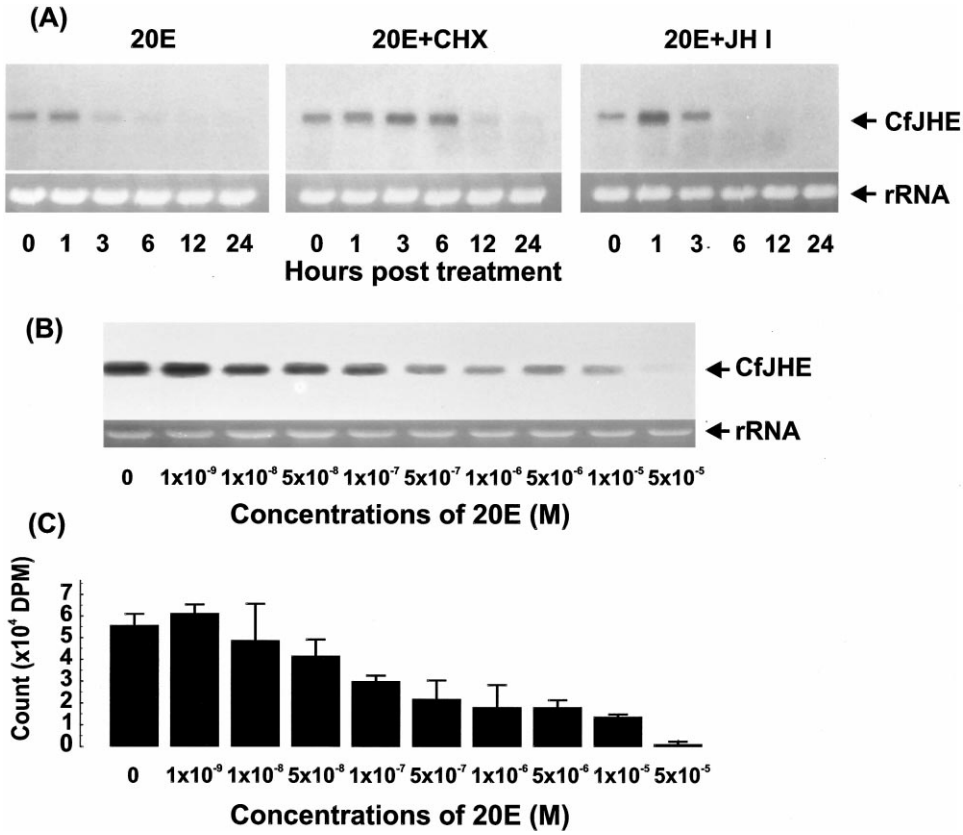

Fig. 2. 20E suppresses the CfJHE mRNA levels in CF-203 cells. (A) The top panels show the Northern blots hybridized with the CfJHE PCR fragment probe. Each lane contains 10 μg of total RNA extracted from CF-203 cells treated with 4×10^{-6} M 20E, or 4×10^{-6} M 20E and 10 mg/ml CHX, or 4×10^{-6} M 20E and 1×10^{-6} M JH I. The bottom panels show ribosomal RNA stained with ethidium bromide. (B) Dose response of 20E suppression of CfJHE mRNA. The top panel shows the Northern blot hybridized with the CfJHE PCR fragment probe. Each lane contains 10 μg of total RNA extracted from CF-203 cells treated with 1×10^{-9}–1×10^{-4} M 20E and collected at 6 h.p.t. The bottom panel shows ribosomal RNA stained with ethidium bromide. (C) Radioactivity in the 2.8-kb band was quantified using Instant Imager. The data are the average of three replicated assays.

and the maximum effect was seen at 1×10^{-5} M. Since the JH I used in these experiments was a mixture of 4 isomers, only one of them being the natural JH I (10R, 11S cis-epoxy isomer), the actual dosages were 60–70% less than the dosages used.

To determine if 20E would regulate the mRNA levels of this gene, we grew CF-203 cells in the medium containing: (i) 4×10^{-6} M 20E, or (ii) 4×10^{-6} M 20E and 10 mg/ml CHX, or (iii) 4×10^{-6} M 20E and 1×10^{-6} M JH I. The cells were collected at 0, 1, 3, 6, 12, and 24 h.p.t. Total RNA was isolated and analyzed on Northern blots using the PCR product as a probe. As shown in Fig. 1A, the probe bound to a 2.8-kb mRNA present in CF-203 cells and the mRNA levels remained low and constant throughout the 24 h of culture in the hormone free medium. The protein synthesis inhibitor, CHX, used alone in culture enhanced the mRNA level to a peak by 3 h.p.t., and then the mRNA level declined again to background levels. When the cells were grown in the presence of 4×10^{-6} M 20E, however, the mRNA level started decreasing at 3 h.p.t. and become undetectable by 12 h.p.t. (Fig. 2A). In the presence of both 20E and CHX, the mRNA first increased during 3 to 6 h.p.t., but then decreased to below background levels by 12 h.p.t.

As indicated above, JH I caused an increase in the mRNA level within the 1st h of treatment, followed by a decline to background levels beginning at 3 h.p.t. (Fig. 1A). When 20E was included in the medium along with JH I, however, the mRNA level increased during the 1st h, as expected from the stimulating action of JH I, but that increase was short lived. By 3 h.p.t., the mRNA levels had returned to near background levels, and thereafter the mRNA levels fell below background to become undetectable by 12 h.p.t. (Fig. 2A). The 20E suppression of the mRNA level was dose dependent (Fig. 2B,C). The mRNA level started to decrease at 1×10^{-7} M 20E and reached the maximum effect when the concentration of 20E was 5×10^{-5} M.

3.3. Developmental expression of CfJHE

Developmental expression of the 2.8-kb CfJHE mRNA throughout the insect development from em-

bryo to pupal stage was also examined using Northern blot hybridization technique. The 2.8-kb mRNA could not be detected during the embryonic stage (Fig. 3A). On the day of hatching to the first instar stage, first day of the first stadium, the mRNA was present (Fig. 3A). However, the mRNA level decreased the following day and reached undetectable levels by day 3. The 2.8-kb CfJHE mRNA transcript could not be detected on the day of molting to the second instar larval stage, i.e. last day of the first stadium (Fig. 3A). The 2.8-kb CfJHE mRNA was conspicuously absent throughout the second instar stage when the larvae were in diapause (Fig. 3B). The mRNA appeared as a pulse on the days of larvae molting to the third and fourth instar stages (day 1 of the third and fourth stadium), followed by a decrease to undetectable levels by day 3 (Fig. 3B).

Similarly, the mRNA was detected again on the day the larvae molted into the fifth instar stage (day 1 of the fifth stadium), but by the following day the mRNA rapidly declined to reach undetectable levels (Fig. 4A). During the intermolt period of the fifth instar larvae, the mRNA was not present. On the day of ecdysis into the sixth instar stage the mRNA was present at background levels in the fat body and the midgut, but at a high level in the epidermis (Fig. 4B). This increased level of mRNA detected in the epidermis returned to background levels in the subsequent 12 h. The mRNA expression in the epidermis started to increase again at 24 h after ecdysis and remained at an almost constant level until head capsule slippage. In the fat body, the mRNA level increased after 24 h and reached maximum levels by 60 h, followed by a gradual decrease. Of these three tissues, the fat body had the highest levels of mRNA during the intermolt period, whereas the midgut appeared to have the lowest levels of mRNA. By the time the insect became a pharate pupa, the mRNA in all these three tissues had decreased to background levels (Fig. 4B).

Again, on the day after ecdysis to the pupa (day 1 of pupal stadium) a high level of the 2.8-kb mRNA was detected, followed by a decrease the next day (Fig. 4C). Another peak of the mRNA levels was observed at 48 h after ecdysis and then the mRNA level decreased to the background level beginning at 72 h after ecdysis to the pupa.

Thus, the 2.8-kb CfJHE mRNA appeared to increase on the day of molting (day 1 of each stadium) during all larval stages excepting the second instar stage, and decrease to undetectable levels during intermolt periods of all larval stages excepting the sixth instar stage. In the sixth larval instar as well as in the pupae, high levels of the mRNA were detected during the intermolt periods as well.

3.4. Cloning of CfJHE cDNA

In order to clone and identify the gene that encodes the 2.8-kb CfJHE mRNA, the PCR product was used as a probe and a cDNA library made from mRNA isolated from CF-203 cells was screened. Six positive

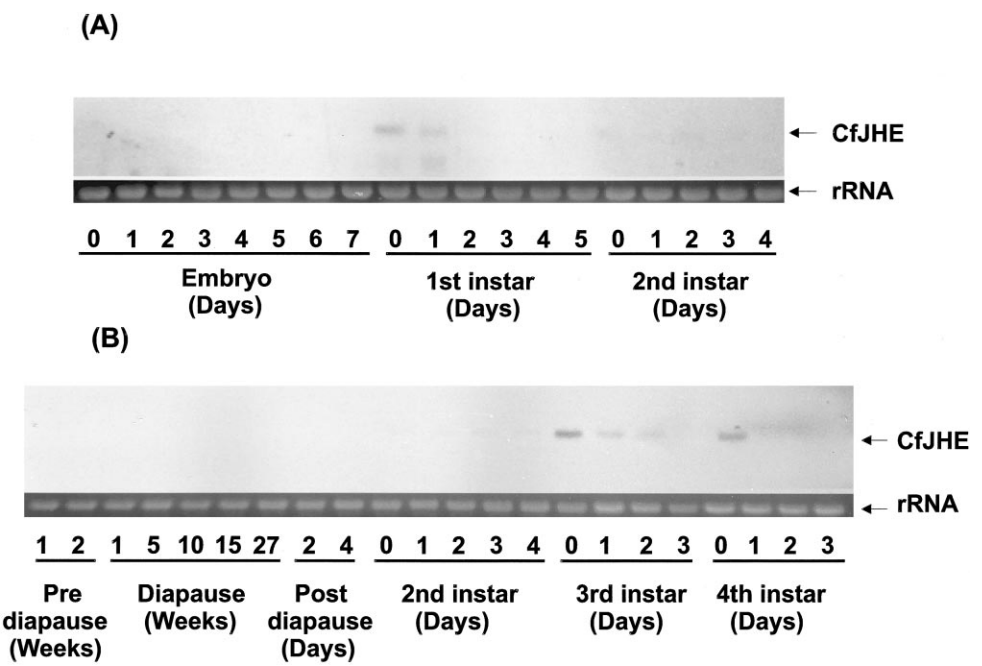

Fig. 3. Expression of CfJHE mRNA in *C. fumiferana* from embryo to the fourth instar larvae. (A) embryo to second instar larvae prior to diapause; (B) diapause and post-diapause second instar to fourth instar larvae. The top panels show the Northern blots containing 10 μg of total RNA extracted from whole larvae and hybridized with the CfJHE PCR fragment probe. The bottom panels show ribosomal RNA stained with ethidium bromide.

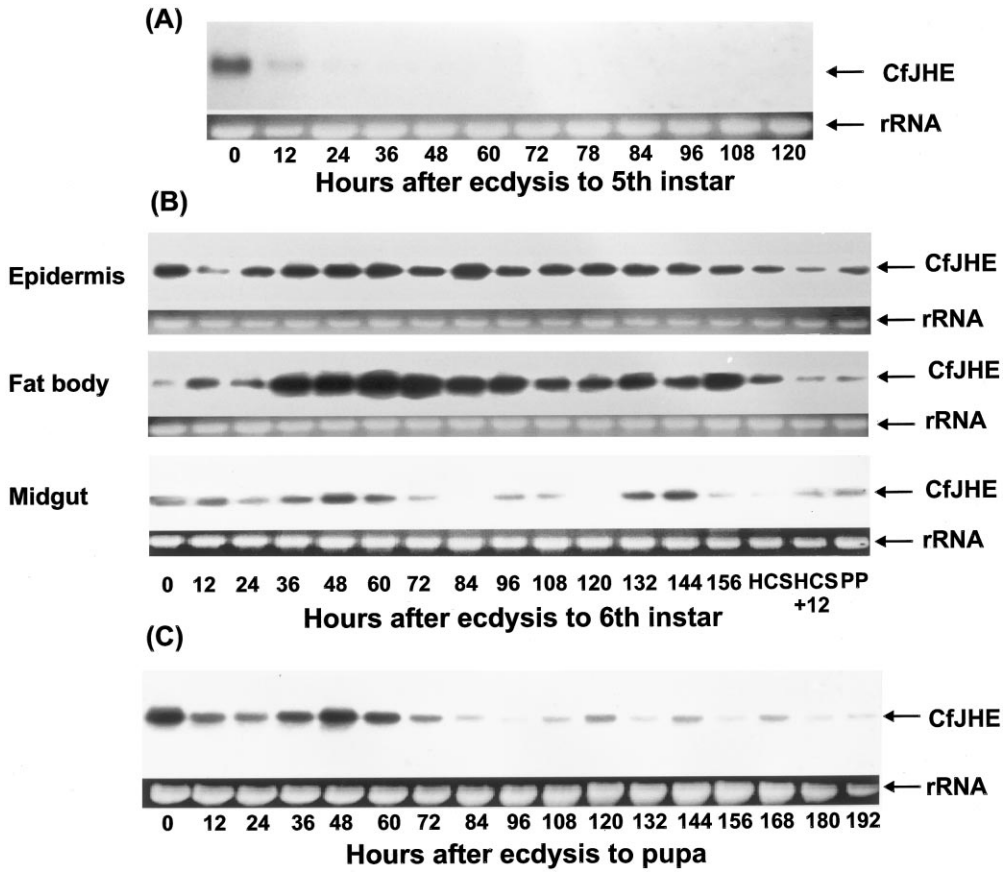

Fig. 4. Expression of CfJHE mRNA in *C. fumiferana* from the fifth instar larvae to pupa. (A) fifth instar; (B) sixth instar; (C) pupa. The top panels show the Northern blots containing 10 μg of total RNA extracted from whole larvae (A and C) or different tissues (B) and hybridized with the CfJHE PCR fragment probe. The bottom panels show ribosomal RNA stained with ethidium bromide. HCS, head capsule slippage; PP, pharate pupa.

clones were isolated. These cDNA clones contained inserts ranging in size from 1.0 to 2.8 kb. Restriction enzyme mapping and Southern hybridization showed that all the clones were copies of the same mRNA. Both strands of the longest cDNA (2.8 kb) were sequenced. The cDNA had a 2787-nucleotide sequence (Fig. 5) with the longest open reading frame (ORF) being 1,677 nucleotides long extending from 13 to 1,689. An ATG initiation codon was located at nucleotide 13 from the 5′-end. A stop codon TGA was at 1,690 nt. The longest ORF had 559 codons and encoded a protein of 61 307 Da. The estimated pI of the predicted protein was 7.47. The 3′ end of the cDNA had a long untranslated region of 1198 nucleotides and included a putative AATAAA polyadenylation signal starting at nucleotide 20 upstream to the poly (A) region.

3.5. Sequence comparison

The deduced amino acid sequence of the CfJHE cDNA showed 47% similarity with the deduced amino acid sequence of JHE cDNA cloned from the tobacco budworm, *H. virescens* (HvJHE) (Hanzlik et al., 1989) (Fig. 6A), and 41% similarity with a JHE-related protein cDNA cloned from the cabbage looper, *T. ni* (TnJHER) (Jones et al., 1994b). The deduced amino acid sequence of CfJHE showed 10% similarity with JHE from the Colorado potato beetle, *L. decemlineata* (LdJHE) (Vermunt et al., 1997b). LdJHE also showed only 10% and 9% similarities with HvJHE and TnJHER, respectively. As shown in the phylogenetic tree (Fig. 6B), CfJHE, HvJHE and TnJHER have closer relationships of evolution with each other than with LdJHE. The five functional motifs that have been identified as required for catalytic activity of JHEs and other esterases (Sussman et al., 1991; Ollis et al., 1992; Ward et al., 1992; Cygler et al., 1993), were all present in the CfJHE deduced amino acid sequence (Fig. 7). These functional motifs were found to align very well in the three species, CfJHE, HvJHE and TnJHER (Fig. 6A). For the DQ motif, CfJHE had a methionine, whereas TnJHER had an alanine, instead of a glutamine as in HvJHE and LdJHE. The functional motif regions seemed to be more conserved than other regions because the amino acid similarities in the core motif

regions were higher when compared to overall similarities. For example, the amino acid similarity of all five motifs between CfJHE and HvJHE was 71%.

3.6. Expression of CfJHE cDNA in a baculovirus expression system

We have expressed the CfJHE cDNA using a baculovirus expression system to perform functional analysis of the CfJHE cDNA product. A recombinant AcMNPV expressing the CfJHE cDNA under the control of the polyhedrin promoter was constructed and designated as AcMNPV-CfJHE. SF-21 cells were inoculated with AcMNPV-CfJHE and the expression of CfJHE cDNA in AcMNPV-CfJHE was detected by monitoring CfJHE mRNA on Northern blots hybridized with the CfJHE cDNA probe and CfJHE protein on SDS-PAGE. SF-21 cells inoculated with AcMNPV-CfJHE showed a 2.8-kb mRNA beginning at 24 h.p.i. (Fig. 8A, left). The mRNA reached maximum levels by 48 h.p.i. The cells inoculated with another recombinant virus (AcMNPV-35 kDa) that did not contain the CfJHE cDNA did not show any CfJHE mRNA (Fig. 8A, right). For analysis of the protein, the culture medium was collected at 1, 24, 48, 72 and 96 h.p.i. and the proteins were resolved on SDS-PAGE. The SDS-PAGE gel analysis showed a 61-kDa CfJHE protein present after 24 h.p.i. (data not shown).

To test the activity of the expression product of CfJHE cDNA, JHE activity was assayed using [^{3}H]-labeled JH III as substrate. SF-21 cells were inoculated with either AcMNPV-CfJHE or AcMNPV-35 kDa, or mock inoculated. The media from the three treatments were collected at 1, 24, 48, 72, and 96 h.p.i. and the enzyme activity was assayed. As shown in Fig. 8B, JHE activity increased from 0.08 pmoles JH III metabolized

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   1 TCAGCAGCAGAGATGGTCTCGAGTGTGAAATCTGTCGCGGTCGCGCTGCTCGTTGCCGTATGTGGTGCGGCGTCGGAGAGGTTGCGCGGCGCGCGTTGCGCA
                 M  V  S  S  V  K  S  V  A  V  A  L  L  V  A  V  C  G  A  A  S  E  R  L  R  G  A  R  C  A   30
 103 CGGAGGCGGGCTGGGTGTGCGGCGCGTGCCCGCGCGGGCGGCGACTACGCCAGCTTCCTCGCCGTGCCCTACGCCAAGCAGCCGCTGGCGGCGCTGCGCTTC
      R  R  R  A  G  C  A  A  R  A  R  A  G  G  D  Y  A  S  F  L  A  V  P  Y  A  K  Q  P  L  A  A  L  R  F   64
 205 CAGGAGCTTCAACCGGCCGAGCCTTGGAAAGGTTGTCGCGACGCCACCAAGCCGGGTCCGATTTGCCCGCAGCGGGACTTGTTCTACGGGCCGACAATGCGC
      Q  E  L  Q  P  A  E  P  W  K  G  C  R  D  A  T  K  P  G  P  I  C  P  Q  R  D  L  F  Y  G  P  T  M  R   98
 307 GCGAGCGAGATGAGCGAAGAGTGTATCCACGCCAACGTGTACATGCCGCTCCAATACCTCCCGGCGGCGAGCGAGGGCGCCGCGCCGCGCAACCGCACGCTG
      A  S  E  M  S  E  E  C  I  H  A  N  V  Y  M  P  L  Q  Y  L  P  A  A  S  E  G  A  A  P  R  N  R  T  L  132
 409 CTGCCGGTGCTGGTGTACATCCACGGCGGCGGGTTCGGCTTCGGCTCCGGCGACCCCGACCTGCACGGCCCTGAGTATCTCATGGGCGACAACGTTGTTGTC
      L  P  V  L  V  Y  I  H  G  G  G  F  G  F  G  S  G  D  P  D  L  H  G  P  E  Y  L  M  G  D  N  V  V  V  166
 511 ATCACATTTAACTACCGGCTTGGGCCGTTTGGTTTTCTCTCTCTGAACTCGTCGAGCGTCCCGGGCAACGCGGGGCTGCGTGATATGGTGACGCTGCTACGC
      I  T  F  N  Y  R  L  G  P  F  G  F  L  S  L  N  S  S  S  V  P  G  N  A  G  L  R  D  M  V  T  L  L  R  200
 613 TGGGTGTGCCGCAACGCGCGGGCCTTTGGCGGCGACCCTGGCGACGTCACCCTGGCGGGCCAGAGCGCCGGAGCCGCCTCCGCCCATATACTCTCACTGTCT
      W  V  C  R  N  A  R  A  F  G  G  D  P  G  D  V  T  L  A  G  Q  S  A  G  A  A  S  A  H  I  L  S  L  S  234
 715 GAAGCCGCACGGGGACTCTTCAAAAGGGCTATACTGATGAGCGGTACGAGCACACGAAGTTTCTTCTCAACATCACCGGCTTATGCCCAGTACGTGGCACAG
      E  A  A  R  G  L  F  K  R  A  I  L  M  S  G  T  S  T  R  S  F  F  S  T  S  P  A  Y  A  Q  Y  V  A  Q  268
 817 CTCTTCCTTACCAACCTCGGCATCAACGGCACCGACCCCGAGGAGGTGCACCGCCTGTTGATCGACACACCAATAGAGAAGATTATCGATGCCCAAAACGAC
      L  F  L  T  N  L  G  I  N  G  T  D  P  E  E  V  H  R  L  L  I  D  T  P  I  E  K  I  I  D  A  Q  N  D  302
 919 CTTCAAGACGTTATCGGTATCACAGTTTTTGTTCCGGTCGTCGAATCCGCGCATCCCGGTGTGGAAATAATCCTTGATAACGACCCTGAAATTCTGCAATCT
      L  Q  D  V  I  G  I  T  V  F  V  P  V  V  E  S  A  H  P  G  V  E  I  I  L  D  N  D  P  E  I  L  Q  S  336
1021 CAGGGACGAGGCAAAGACATACCATTCTTAATGGGCTTTACGAACGCAGAATGCCAGACGTTCCGGCCCCGATTCGAATCTATTGACATCATAAGTCGGATC
      Q  G  R  G  K  D  I  P  F  L  M  G  F  T  N  A  E  C  Q  T  F  R  P  R  F  E  S  I  D  I  I  S  R  I  370
1123 GCCGAACAGCCTGTGCTGGTGATGCCCCCGCCGGCAATTTACGTCACGCCGTTGGACGAGCTCCCCAACAAAATTGGGCAAATTGTACAACGATATTTTAAC
      A  E  Q  P  V  L  V  M  P  P  P  A  I  Y  V  T  P  L  D  E  L  P  N  K  I  G  Q  I  V  Q  R  Y  F  N  404
1225 GGAACACCGAATTTAAACAAGTTTATAAAGTTGTGTTCGGAGTCTTACTTCGTGTACCCCACTTTAAAGCTGGCTGAAATGAGAGCCGCGAACGGGGGTGCT
      G  T  P  N  L  N  K  F  I  K  L  C  S  E  S  Y  F  V  Y  P  T  L  K  L  A  E  M  R  A  A  N  G  G  A  438
1327 CCAGTGTACTTGTACCGGTTCACGTACGAGGCCGACTACAGCGTGTTCCAGGAAGGGTTGCGGCTCAAATATCGTGGCACGGGGCACTCGGAGGACCTCACC
      P  V  Y  L  Y  R  F  T  Y  E  A  D  Y  S  V  F  Q  E  G  L  R  L  K  Y  R  G  T  G  H  S  E  D  L  T  472
1429 CTACTGTTCCGAGCCAACCACGTGCTCGGGGAGAGGGCTATATCCCCGAGAGATCATAAGATGGTCGACGCCATGACGACGTACGTCACTAACTTCATGCTG
      L  L  F  R  A  N  H  V  L  G  E  R  A  I  S  P  R  D  H  K  M  V  D  A  M  T  T  Y  V  T  N  F  M  L  506
1531 CACAGTAACCCAACGAAGGGAGTAGCAGGGTGGCCAGCAGTGAAGCGTGAGAAGTTACAGTATCAAAACATAATTACTCCCGAGCTTGTGACCACCGAAGTG
      H  S  N  P  T  K  G  V  A  G  W  P  A  V  K  R  E  K  L  Q  Y  Q  N  I  I  T  P  E  L  V  T  T  E  V  540
1633 AGCCGGGATCTGCGCGACACCATGACGTTCTTTGATAACATTTACAGCAGTTCCAGCTGAATTTCAGACGTAGGAAGAAGAAAAGACTTTATTTCCAAAGTG
      S  R  D  L  R  D  T  M  T  F  F  D  N  I  Y  S  S  S  S  ***                                          559
1735 TTTGTGTTGTAATTATTAAAAAAAGTGTTAATGTTGTGGTTCTCTTATATAAGTAAGTAGTGTGTTTTGATTGATTGATTGTGTTTAATTGAGGAGACGTGC
1837 GACTGATTTAGTGGTAAGCTATGACTTAAGTTATTTTGGTAAGCGCTAGTAAAGACTGTTTAGGAAGAATTGATCGTTGTTTATTAATAAGAAAAATAAAAG
1939 GAAATAATGGTGAATGAAGGGTTTCGTGCATACAGGATGGTATGTATTTGATATCCAAGAGCCAATTTTTCTTACTTTGCAATTCTCTAACGATGGCAGTTT
2041 GTATATTGGAGTTTCCTAAAACAAAGAAATTTTTTACAAGCACTTTATTTTTAGTTAAAAATTTAATTTGATTGCCACCCCATGCCTGGTTACCTAAAGCCT
2143 GTTTCACAAATAGTGAGCCCTCTTTAGAAAACAAATTCTTAAAATGCAATATATACTTCTAACGATTTATTATTATTTATTACGACAACATGGATGCAATGA
2245 TCCATCATCTTGTAGAGATAGTAAGAAAACTTGGTTCCTGTATACAAATGCACCTTGTTTAACAGAGGTACTGCTAGATCTCGCTAACTCAATTAAGATCCA
2347 CACAATGAGTCCGTGACAGGGCGGAGTTCAAAATATATCTACATCTACATTGTTAACCCATACAAGACGAATGAGTAAAAAAAACTGTTAGCCTTAAAATAG
2449 TATGTAAAATTGTCGGTCAAAGATGTTTTCGAACTGTTTACATAAAACTGATTATAGAACTTTTTGAACTCCGCCCAACTACTGTTACGGCTTCTTGGATTT
2551 TTTGTTTAAAATAATAATTGTAAATATAAATATTTTTTTTGGGTAACTAGAACTAGAAGGAAGAGAAGATTGAACAACACAAACTGATGGTTGAAAGTTGTA
2653 TCTTAAGAAAAGGCAGGAAAGGAAAGGGCAATTGCATTTTATTTTCTTTGTGATGTACAAATACGAATGTTCTTGGTTTATGAAAAAAAAAGCTTAAATAAA
2755 AGTAACTTGATGACAAAAAAAAATAAAAAAAAA
```

Fig. 5. Nucleotide sequence and deduced amino acid sequence of the CfJHE. The stop codon TGA is marked with asterisks. The putative polyadenylation signal AATAAA is double underlined. The amino acids of the predicted signal peptide are underlined. The catalytic residues of functional motifs are given in bold.

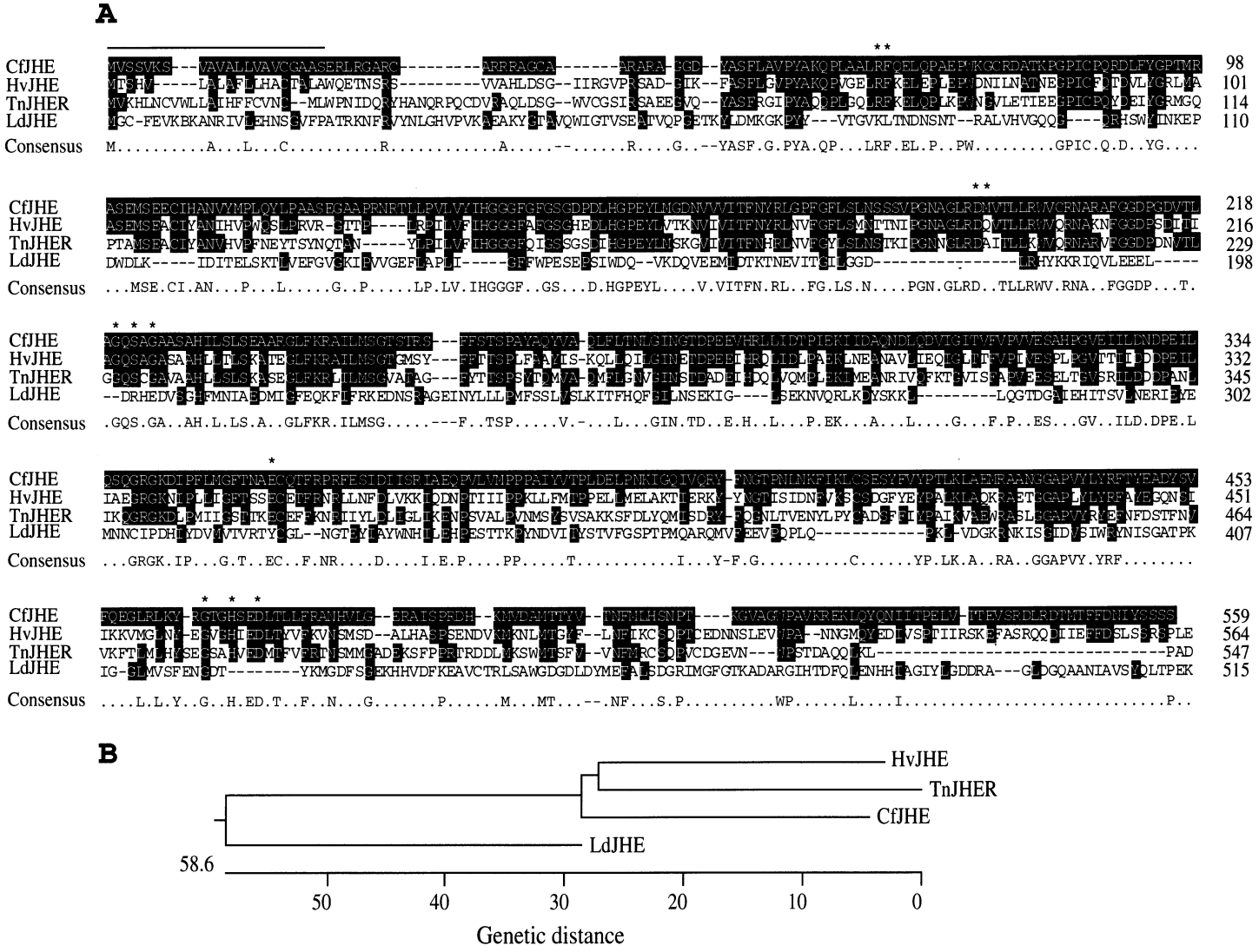

Fig. 6. Alignment (A) and phylogenetic tree (B) of the deduced amino acid sequences of the CfJHE (this study), HvJHE (Hanzlik et al., 1989), TnJHER (Jones et al., 1994b), and LdJHE (Vermunt et al., 1997b). The multiple sequence alignment was performed using Clustal Alignment Program (Higgins and Sharp, 1988). The residues that match CfJHE exactly are shaded with solid black. The consensus residues are given in lines below the alignment panels when at least three of four residues match, otherwise, shown as dots. The predicted signal sequence and the core amino acids of functional motifs are marked with a line and asterisks, respectively.

$min^{-1}ml^{-1}$ medium at 1 h.p.i. to 0.3 pmoles JH III metabolized $min^{-1}ml^{-1}$ medium at 96 h.p.i. in the medium in which SF-21 cells inoculated with AcMNPV-CfJHE were growing, whereas JHE activity remained at the background level in both the controls. These data show that the CfJHE cDNA that we have cloned codes for a functional JHE protein.

4. Discussion

4.1. Confirmation of cloned cDNA as CfJHE

We have used mRNA differential display to identify JH-inducible genes in CF-203 cells. One of the six PCR products isolated bound to a 2.8-kb mRNA whose abundance increased in the presence of 1×10^{-6} M JH I. This PCR product was used as a probe to isolate a cDNA clone from a cDNA library made from CF-203 cells. The deduced amino acid sequence of this cDNA showed high similarity with HvJHE sequence (Hanzlik et al., 1989). The primary structure of the deduced amino acid sequence of the cDNA contained all five functional motifs that have been demonstrated to be required for JHE (Ward et al., 1992), and other esterases and serine proteases (Steitz and Shulman, 1982; Sussman et al., 1991) to function. Expression of the cDNA in the recombinant baculovirus system yielded a protein that showed JHE activity. Taken together, these data clearly show that the cDNA we have cloned encodes *C. fumiferana* JHE.

RF motif

CfJHE	QPLAAL**RF**QELQP	42	
HvJHE	QPVGEL**RF**KELEP	49	(62%)
TnJHER	QPLGQL**RF**KELQP	79	(77%)
LdJHE	PVKAEA**KY**GTAVQ	44	(8%)

DQ motif

CfJHE	PGNAGLR**DM**VTLLRWVCRN	173	
HvJHE	PGNAGLR**DQ**VTLLRWVQRN	175	(90%)
TnJHER	PGNNGLR**DA**ITLLKWVQRN	205	(74%)
LdJHE	SIWDQVK**DQ**VEEMIDTKTN	161	(16%)

GxSxG motif

CfJHE	TLA**G**Q**S**A**G**AA	201	
HvJHE	TIA**G**Q**S**A**G**AS	203	(80%)
TnJHER	TLG**G**Q**S**C**G**AV	233	(70%)
LdJHE	not present		

E motif

CfJHE	GFTNA**E**CQTFRPRFE	332	
HvJHE	GFTSS**E**CETFRNRLL	334	(69%)
TnJHER	GSTTK**E**CEFFKNRII	364	(40%)
LdJHE	GLNGT**E**YIAYWNHIL	328	(13%)

GxxHxxD/E motif

CfJHE	YR**G**TG**H**SE**D**LTLLF	446	
HvJHE	HE**G**VG**H**IE**D**LTYVF	448	(57%)
TnJHER	SE**G**SA**H**VE**D**MTFVF	479	(43%)
LdJHE	FS**G**EK**H**HV**D**FKEAV	431	(21%)

Fig. 7. Alignment of catalytic motifs of the CfJHE (this study), HvJHE (Hanzlik et al., 1989), TnJHER (Jones et al., 1994b), and LdJHE (Vermunt et al., 1997b). The catalytic amino acids in the motifs are given in bold form. The positions of underlined amino acid residues in the mature proteins are given at the right. Given in the parentheses are the percentages of amino acid similarities of the motifs between CfJHE and HvJHE or TnJHER or LdJHE.

The cloned CfJHE cDNA was 2887 nucleotides long. The data from the Northern blots showed that the PCR product probe hybridized to a single mRNA 2.8-kb in size that was present in both the in vitro cultured CF-203 cells (Figs. 1 and 2) and the tissues directly dissected from the larvae (Figs. 3 and 4). Expression of the CfJHE cDNA in AcMNPV also showed a 2.8-kb mRNA (Fig. 8). These results indicate that the CfJHE cDNA that we have cloned is a full-length copy of JHE mRNA.

4.2. Characterization of CfJHE cDNA

The deduced amino acid sequence of the HvJHE cDNA cloned by Hanzlik et al. (1989) included a signal peptide of 19 amino acid residues, whereas in LdJHE cDNA, a signal peptide of 23 amino acids was predicted (Vermunt et al., 1997b). In TnJHER cDNA, no signal peptide was recognized (Jones et al., 1994b). By aligning the CfJHE sequence to the deduced amino acid sequence of HvJHE, a putative 21-amino acid signal peptide (1–21 in Fig. 1) was predicted for CfJHE. This signal peptide has a hydrophobic core of nine non-polar amino acids (from Val_8 to Val_{16}) preceded by a basic residue (Lys_6). The results from the expression of CfJHE cDNA in the baculovirus expression system also provided supporting evidence for the existence of a signal peptide. The Northern blot showed that the CfJHE mRNA appeared between 12 and 24 h.p.i., and JHE activity was detected in the medium from the infected cell cultures beginning at 24 h.p.i. These data suggest that CfJHE cDNA included a signal peptide sequence that aids in transporting the protein out of cells. Therefore, the mature protein of CfJHE is expected to have 538 amino acids with a predicted molecular weight of 59.36 kDa.

Five functional motifs, which have been demonstrated to be required for HvJHE activity (Ward et al., 1992), were all found in the deduced amino acid sequence of the CfJHE cDNA. The motif $G \times S \times G$, which has been considered as the active site of all serine hydrolyases, including proteases, esterases, and lipases (Brenner, 1988), was located near the center of the

peptide, and there was only one such active site in the entire sequence. Three amino acid residues, serine, histidine and aspartate (or glutamate) have long been suggested to form a catalytic triad in many serine proteases and estereases (Steitz and Shulman, 1982; Sussman et al., 1991). Studies on the catalytic mechanism of cloned HvJHE using site-directed mutagenesis by Ward et al. (1992) showed that in the HvJHE, Ser_{201}-His_{446}-Glu_{332} catalytic triad, Asp_{173} and Arg_{47} were essential for the efficient functioning of JHE. In the CfJHE, such a catalytic triad is also present and the positions of these amino acids are exactly the same as in the HvJHE (Fig. 7). The conservation of this catalytic triad in CfJHE indicates the importance of these amino acids to the catalytic activity of JHEs.

The deduced amino acid sequence of CfJHE cDNA showed 47% similarity with HvJHE and all functional motifs are very well conserved. TnJHER also shared $>40\%$ similarity with CfJHE and HvJHE. This protein, however, has not been considered to be a JH-specific esterase because TnJHER has a cysteine residue in its serine catalytic site, instead of alanine as in other JHEs (Jones et al., 1994b). Another JHE sequence reported recently from *L. decemlineata* (Vermunt et al., 1997b) showed only limited amino acid similarity of around 10% with lepidopteran JHEs. Even in the functional motif regions, the amino acid similarities between LdJHE and lepidopteran JHEs are very low (8–22%). No serine catalytic site was present in LdJHE. It remains to be demonstrated whether LdJHE is a JH-specific esterase.

4.3. Hormonal regulation and developmental expression of CfJHE mRNA

Earlier studies have indicated that the fluctuation of JHE titer in insect hemolymph is temporally controlled (Kramer et al., 1977; Wing et al., 1981). The two major findings of our study are that: (1) both JH I and 20E regulate CfJHE mRNA levels in CF-203 cells; and (2) CfJHE mRNA levels peak on the day of molting to all larval and pupal stages except to the second instar

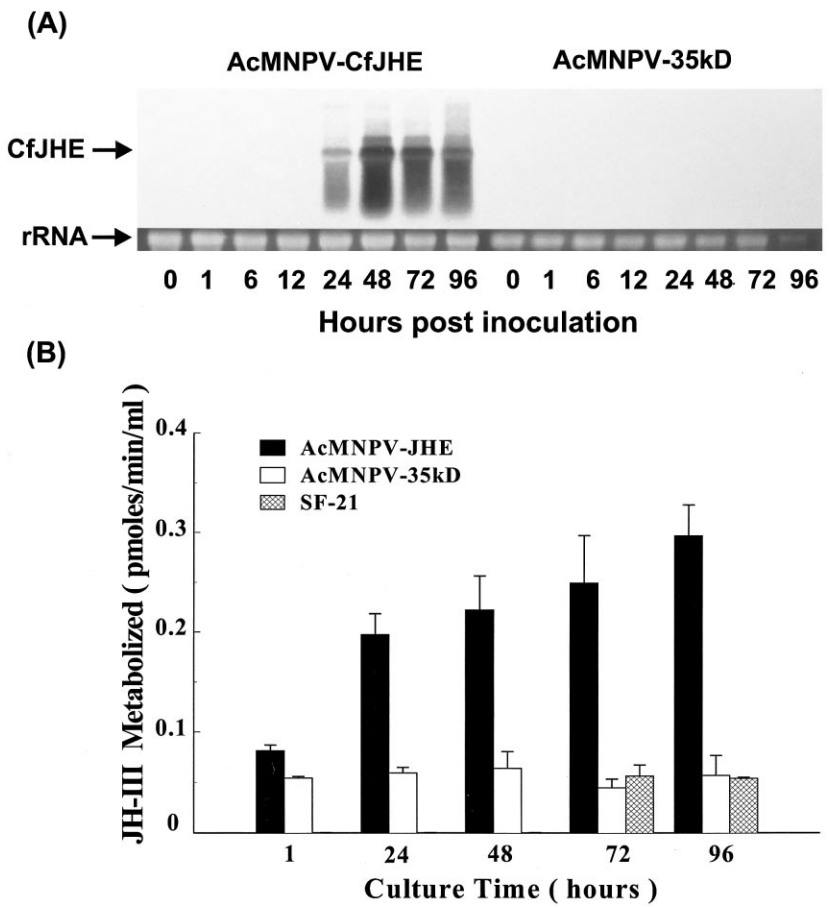

Fig. 8. Expression of the CfJHE cDNA in SF-21 cells inoculated with recombinant AcMNPV-CfJHE. (A) The top panel shows the Northern blot containing 10 μg of total RNA from the cells infected with AcMNPV-CfJHE (left) or AcMNPV-35 kDa (right) hybridized with the CfJHE cDNA probe. The bottom panel shows ribosomal RNA stained with ethidium bromide; (B) JHE activity assays were conducted with the medium from the SF-21 cells uninfected or infected with AcMNPV-CfJHE or AcMNPV-35 kDa. JHE activity is presented as pmoles JH III metabolized $min^{-1}ml^{-1}$ 10x concentrated medium. Each bar represents mean ± SE ($n = 3$).

larval stage (i.e. first day of all larval and pupal stadia except the second stadium). The present data indicate a reciprocal effect of the two hormones, JH I causing an increase and 20E causing a decrease in the mRNA level of CfJHE. Both these observations are evident even in the presence of the protein synthesis inhibitor, CHX, indicating that this effect may be direct. This is one of the rare examples where the expression of an insect gene has been shown to be regulated by both JH I and 20E. Whether or not this regulation occurs at the transcriptional level or at the post transcriptional level remains to be determined. It is conceivable that JH I could be stabilizing the CfJHE mRNA. Previous studies in *T. ni* showed that exogenous treatment with a JH analog resulted in an increase in the level of JHE mRNA (Jones et al., 1994). Wroblewski et al. (1990) also showed an increase in the level of JHE mRNA in the fat body and integument of *M. sexta* after epofenonane treatment. CHX alone appears also to increase the CfJHE level. This may be due to that it inhibits protein synthesis, resulting in the accumulation of mRNA. Alternatively, CHX may be stabilizing CfJHE mRNA.

As is shown in Fig. 2, CfJHE mRNA expression is completely suppressed by 20E treatment in CF-203 cells. This suppression is dose dependent and occurs even in the presence of the protein synthesis inhibitor, CHX, indicating that the action is direct and not mediated by another protein. We are convinced that this 20E suppression of CfJHE expression is real and is not due to the toxic effect of 20E on CF-203 cells or any other artifacts, because when the same Northern blots (Fig. 2) were hybridized with cDNA probes of two other ecdysone-induced transcription factors, *Choristoneura* hormone receptor 3 (CHR3) (Palli et al., 1996) and *Choristoneura* hormone receptor 75 (CHR75) (Palli et al., 1997), the CHR3 and CHR75 mRNA levels progressively increased from 1 to 3 h.p.t., and reached maximum levels by 6–12 h.p.t. after which they decreased by 24 h.p.t. This CHR3 and CHR75 mRNA induction pattern is exactly opposite to the suppression effect of CfJHE mRNA that we observed. The biological significance of this 20E suppression of JHE mRNA is not readily apparent and more work needs to be done in vivo to elucidate its significance. Whereas the CfJHE mRNA levels decreased prior to pupation during the prepupal peak of ecdysteroids (Palli et al., 1995), the mRNA of the two ecdysone induced transcription factors, CHR3 and CHR75, increased, suggesting that some of these transcription factors may play a key role in the 20E suppression of JHE mRNA.

The appearance of a peak of CfJHE mRNA on the day of molting to all larval stages, except the second instar, is another interesting observation. All larval instars feed, grow and, in response to an increase in the ecdysteroid titer, molt to the next stage, except for the second instar larvae, which do not feed, show no ecdysteroid peak and undergo obligatory overwintering diapause. After termination of diapause, the second instar larvae feed, grow and in response to ecdysteroid secretion, the larvae molt into third instars. Therefore, it appears that the CfJHE mRNA peaks that are present at the beginning of each larval stage may be a result of high JH present at the beginning of each larval stadium. For example, in *M. sexta*, Baker et al. (1987) observed maximum levels of JH and JH acid just following ecdysis to the last stadium and at the prepupal stage.

Another feature of the developmental expression of JHE is tissue specificity. In *M. sexta*, the fat body secretes the major proportion of JHE compared to other tissues like the epidermis, and therefore could be the major source of hemolymph JHE (Wroblewski et al., 1990). Our investigation on the expression of CfJHE mRNA in various tissues in the last instar larvae of spruce budworm showed similar results. The fat body had the highest level of the CfJHE mRNA, but the epidermis and midgut showed lower levels (Fig. 4). These results are in agreement with the earlier conclusion that the JHE is synthesized and secreted primarily by the fat body (Wing et al., 1981). In addition, JHE mRNA induction by JH was higher in the integument than in the fat body of *M. sexta* (Wroblewski et al., 1990). In the CF-203 cell line derived from the midgut (Sohi et al., 1993), JH I treatment resulted in the increase in the level of CfJHE mRNA above background levels (Fig. 1A). It appears therefore that there is a tissue-specific response to JH. The tissue with a lower endogenous level of JHE mRNA appears to be more sensitive to JHE mRNA induction by JH.

4.4. JHE for improvement of baculovirus

Because of its important role in regulation of JH titer and developmental events in insects, one of the ultimate objectives of JHE study is to develop a recombinant virus containing JHE cDNA as a viral insecticide for pest management. (Hammock et al., 1990) first explored this potential in studies on the expression of HvJHE cDNA in a recombinant baculovirus system. Infection of *H. virescens* larvae with recombinant AcMNPV expressing modified JHE resulted in 50% reduction in feeding damage when compared with larvae with wild-type AcMNPV (Bonning et al., 1995, 1997). In this study we constructed the recombinant AcMNPV expressing CfJHE cDNA and showed JHE activity in the medium from the infected SF-21 cell cultures beginning at 24 h.p.i. The expression of CfJHE in CfMNPV and assessment of the pest control potential of this recombinant virus is currently in progress.

Acknowledgements

We thank Dr Lynn M. Riddiford of University of Washington for her comments on the manuscript. This research is supported in part by the National Biotechnology Strategy Fund, and the Science and Technology Opportunities Fund of Canadian Forest Service to Subba R. Palli, and a grant from the Natural Sciences and Engineering Research Council to Kenneth G. Davey.

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