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An ecdysone-inducible putative "DEAD box" RNA helicase in the spruce budworm (*Choristoneura fumiferana*)

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Abstract

RNA helicases are a family of enzymes that unwind nucleic acid duplexes, such as RNA/RNA and RNA/DNA, in a 3′ to 5′ direction into single-stranded polynucleotides. A putative RNA helicase cDNA (*CfrHlc64*) was isolated from the spruce budworm, *Choristoneura fumiferana*. *CfrHlc64* was 1998 nucleotides in length, and the deduced protein had 565 amino acids with a predicted molecular mass of 64 kDa. It contained eight functional motifs conserved in the "DEAD box" family of RNA helicases. The deduced amino acid sequence showed 10–50% identities to homologues of other species from bacteria to human. In vitro expression of the cDNA resulted in recombinant proteins of 64 kDa as expected from the deduced amino acid sequence. Northern blotting and RT-PCR analyses revealed the presence of *CfrHlc64* mRNA in all developmental stages from embryo to adult. Higher levels of *CfrHlc64* mRNA were detected in the fat body and midgut than in the epidermis of sixth instar larvae. The *CfrHlc64* protein was distributed mainly in the fat body. Female adults expressed *CfrHlc64* mRNA at higher levels than male adults. The nonsteroidal ecdysone agonist, tebufenozide, enhanced the expression of *CfrHlc64* in a dose-dependent manner.

Keywords: Nucleic acid duplex; RNA unwinding; mRNA splicing; Ribosome biogenesis; Translation; Ecdysone; Tebufenozide

1. Introduction

RNA helicases are a superfamily of enzymes that unwind double-stranded RNA or DNA/RNA hybrids. They have been isolated from a wide range of prokaryotic and eukaryotic organisms (Lüking et al., 1998; de la Cruz et al., 1999). Most of these enzymes share extensive homology in their primary amino acid sequences and contain eight conserved functional motifs, one of which contains a four-amino acid sequence Asp-Glu-Ala-Asp (DEAD), forming a "DEAD box" family (Linder et al., 1989). "DEAD box" RNA helicases usually contain a common core region that includes the eight highly conserved motifs, which are involved in RNA helicase activity like ATP-binding and hydrolysis as well as unwinding of double-stranded RNA, whereas the N- and C-terminal parts of the helicases are more variable and may be responsible for individual protein functions. "DEAD box" RNA helicases are involved in many RNA-related processes, such as transcription, translation, pre mRNA splicing, RNA maturation, RNA export, RNA degradation and ribosome biosynthesis (Lüking et al., 1998; de la Cruz et al., 1999).

Several RNA helicases have been identified from *Drosophila melanogaster*, including *Hlc* (de Couet et al., 1995), *hel* (Eberl et al., 1997), *eIF-4a* (Dorn et al., 1993), *vasa* (Lasko and Ashburner, 1988) and *mle* (Kuroda et al., 1991). These helicases appear to be involved in different cellular processes. The first helicase identified from *D. melanogaster* was the product of the maternally expressed gene *vasa* and plays a role in the formation of the embryo body plan (Lasko and

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Ashburner, 1988). A *vasa*-like gene was also isolated from *Bombyx mori* and it was associated with germ anlage (Nakao, 1999). An enhancer (HEL) of *white* variegation was found in *D. melanogaster* to be similar to RNA helicase (Eberl et al., 1997). The HEL protein was associated with salivary gland chromosomes and located in cell nuclei of embryos and ovaries, but disappeared in mitotic domains of embryos as chromosomes condensed. Deletion or mutation of the *hel* gene enhances *white* variegation but this can be reversed by a transformed copy of hel^{+}. Kuroda et al. (1991) reported that *Drosophila* maleless protein (MLE) is highly homologous to human RNA helicase A and the bovine nuclear helicase II. The maleless gene (*mle*) is required for X chromosome dosage compensation and is essential for male viability. Another *Drosophila* RNA helicase gene *pit* was required for cell growth and proliferation (Zaffran et al., 1998). Mutation in this gene produces small larvae that cannot grow beyond the first instar larval stage.

In this paper we report a putative RNA helicase cDNA (*CfrHlc64*) isolated from a lepidopteran species, the spruce budworm (*Choristoneura fumiferana*). *CfrHlc64* showed amino acid identities with members of the "DEAD box" family from different organisms. We will show temporal, spatial and enhanced expression of this gene in the spruce budworm.

2. Materials and methods

2.1. Experimental insects

Spruce budworm (*Choristoneura fumiferana* Clem., Lepidoptera:Tortricidae) eggs were maintained at 22 °C and 70% relative humidity (RH) and allowed to hatch into first instar larvae on the needles of balsam fir. The first instar larvae molted into second instar in 6 days. The second instar larvae were maintained at 16 °C for 1 week, and then the diapausing second instar larvae were stored at 2 °C for 27 weeks to satisfy the obligatory requirement for diapause. At the end of this period, the larvae were moved from 2–16 °C for 1 week and then placed on artificial diet (McMorran, 1965) at 22 °C, 70% RH and a photoperiod of 12-h light and 12-h darkness and reared until they reached the adult stage.

2.2. Treatment of insects with tebufenozide

The nonsteroidal ecdysone agonist, tebufenozide (RH5992), was applied to 2-day old sixth (last) instar insects using topical application (Hinkle et al., 1985; Retnakaran et al., 1997). A total of 0.5 μl of 10^{-3} M of RH5992 in acetone was applied to each insect. The insects were harvested at 1, 3, 6, 12 and 24 h post treatment. For dose response assays, insects were treated with 0.5 μl of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} M of RH5992 and then harvested at 6 h post treatment. The controls were treated with acetone alone.

2.3. cDNA library screening and sequence analysis

A cDNA library constructed in Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA) using mRNA isolated from the spruce budworm cell line (CF-203) was screened with anti-*Rst(1)JH* antibodies [*Rst(1)JH* was formally called met gene, Wilson and Fabian, 1986; Ashok et al., 1998]. CF-203 cell line was derived from the midgut tissues of the spruce budworm (Sohi et al., 1996) and is responsible to JH action in term of increased expression of JH esterase (Feng et al., 1999). The antibodies were diluted to 1:1000 before use. The sheep anti-rabbit IgG alkaline phosphatase conjugate was used as the second antibody at 1:2000 dilution. Color development of alkaline phosphatase reaction was performed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates. Positive plaques were selected after three-rounds of screening and plasmid cDNA was isolated.

Sequencing was performed using ALFexpress™ AutoRead™ Sequencing Kit and ALFexpress™ DNA Sequencer (Amersham Pharmacia Biotech). Sequences were analysed using the MacVector DNA Analysis Program (International Biotechnologies Inc., New Haven, CT, USA). Alignment of amino acid sequences was performed using the Clustal Alignment Program (Higgins and Sharp, 1988).

2.4. Reverse transcription PCR

Total RNA was isolated from tissues by using the guanidinium isothiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987). Reverse transcription PCR was carried out according to the instruction manual of Superscript Reverse for RT-PCR (Life Technologies, Rockville, MD, USA) by using 0.5 μg of RNA per reaction and *CfrHlc64* specific primers. The forward primer was 5′-TCTGGTTCAGTGCTGTCGTTTG-3′ and the reverse primer was 5′-TATTAGGTGTCGCCCGTTGC-3′. The RT-PCR samples were denatured at 94 °C for 3 min, followed by 28 cycles of 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min for each cycle. PCR products were separated in 0.9% agarose gels and stained with ethidium bromide. Actin-specific primers were used to amplify actin transcript as an indicator of equal amount of RNA per reaction.

2.5. Protein expression in bacteria

CfrHlc64 cDNA was cloned into the pPROEX HTa expression vector (Life Technologies) in fusion with a

sequence of TEV protease cleavage site and 6xHis tag. *E. coli* cells (XL1-Blue) were transformed with the recombinant plasmid DNA. Expression of the recombinant protein was induced by adding IPTG at a final concentration of 1 mM. The recombinant protein was purified using His tag affinity Ni-NTA silica resin (QIAGEN Inc. Chatsworth, CA, USA) following manufacturer's instructions.

2.6. SDS-PAGE

SDS-PAGE was performed according to Laemmli (1970). Protein extracts were denatured at 100 °C for 5 min in an equal volume of 2× protein loading buffer (0.1 M Tris buffer, pH 6.8, 4% SDS, 0.2% β-mercaptoethanol, 40% glycerol, and 0.002% bromphenol blue). Proteins were separated in 12% acrylamide gels in Tris–glycine–SDS buffer (10 mM Tris, 50 mM glycine, 0.1% SDS, pH 8.0) and stained with Coomassie Blue R-250 after electrophoresis.

2.7. Antibody production

Polyclonal antibodies were produced against the recombinant *CfrHlc64* protein. The recombinant protein was cleaved with TEV protease to remove the 6xHis tag and resolved in a SDS-PAGE gel. After electrophoresis and staining, the 64 kDa-protein band was excised and the protein was eluted by electrophoresis inside a dialysis tube. The protein was mixed with Freund's adjuvant and then injected into a rabbit. Eighty micrograms of protein was used in each of three boosters in 14-day intervals. Antiserum was collected after administering three-booster immunizations. Serum from the same rabbit collected prior to immunization was used as the control.

2.8. Western blotting

Immunoblotting was conducted as described by Sambrook et al. (1989). After electrophoresis, proteins were transferred from SDS-PAGE gels to Hybond C nylon membranes. The anti-*Rst(1)JH* antibodies were used as primary antibodies at 1:1000 dilution. The sheep anti-rabbit IgG alkaline phosphatase conjugate (Sigma Chemical Company, St. Louis, MO, USA) was used as the secondary antibody at 1:2000 dilution. Color development of alkaline phosphatase reaction was performed using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates.

2.9. Immunocytochemistry

Staged entire sixth instar larvae were fixed in 4% paraformaldehyde, dehydrated in a series of ethyl alcohol at different concentrations and embedded in paraffin. Five micron-thick paraffin sections were made and deparaffinized, and rehydrated in ethyl alcohol–water series. The sections were incubated in blocking solution (containing 1% BSA in PBS) for 30 min at 37 °C. After three washes in PBS, the sections were incubated in 0.2 M glycine solution for 30 min at 22 °C followed by incubation in the anti-*CfrHlc64* antibodies for 60 min at 37 °C. The dilution of the primary antibodies was 1:500. The sections were then washed three times in PBS and incubated in the secondary antibody, fluorescein-labeled sheep anti-rabbit IgG F(ab)2 fragment (Boehringer Mannheim, Montreal, Canada), at a dilution of 1:500 for 60 min at 37 °C. The sections were washed three times in PBS and then were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) for 30 min and examined under a fluorescence microscope. The photographs were taken as double exposures using fluorescein and DAPI filters.

2.10. Northern blotting

Ten micrograms of total RNA per lane were separated on a formaldehyde–agarose (1%) gel. The RNA was visualized by staining with ethidium bromide and photographed under UV light. The RNA was then transferred to a Hybond N nylon membrane (Amersham Life Science). The blots were hybridized with a cDNA probe labeled with α-[^{32}P]dATP. Hybridization and washes were conducted as described by Palli et al. (1998).

3. Results

3.1. Isolation and sequence of C. fumiferana RNA helicase cDNA (CfrHlc64)

Ten positive clones were isolated from a total of approximately 10^6 plaques after anti-*Rst(1)JH* antibody was used to screen the CF-203 expression library. Sequencing and Blast search in the GenBank database showed that three out of ten positive clones were identical and encoded an RNA helicase, *C. fumiferana* 64 kDa RNA helicase, (*CfrHlc64*) (Fig. 1). Four other clones were identical and encoded another "DEAD box" RNA helicase (data not shown). One clone encoded nuclear exportin and the last two clones encoded two unknown proteins.

Complete sequencing of *CfrHlc64* revealed that it was 1998 bp in length (Fig. 1). Its longest open reading frame (1698 nts) encoded a polypeptide of 565 amino acids with a predicted molecular mass of 64 kDa and a pI of 9.6. All of the eight functional conserved motifs of "DEAD box" RNA helicases were found in the deduced amino acid sequence of *CfrHlc64* (Fig. 1). There was a four-amino acid sequence Asp-Glu-Ala-Asp

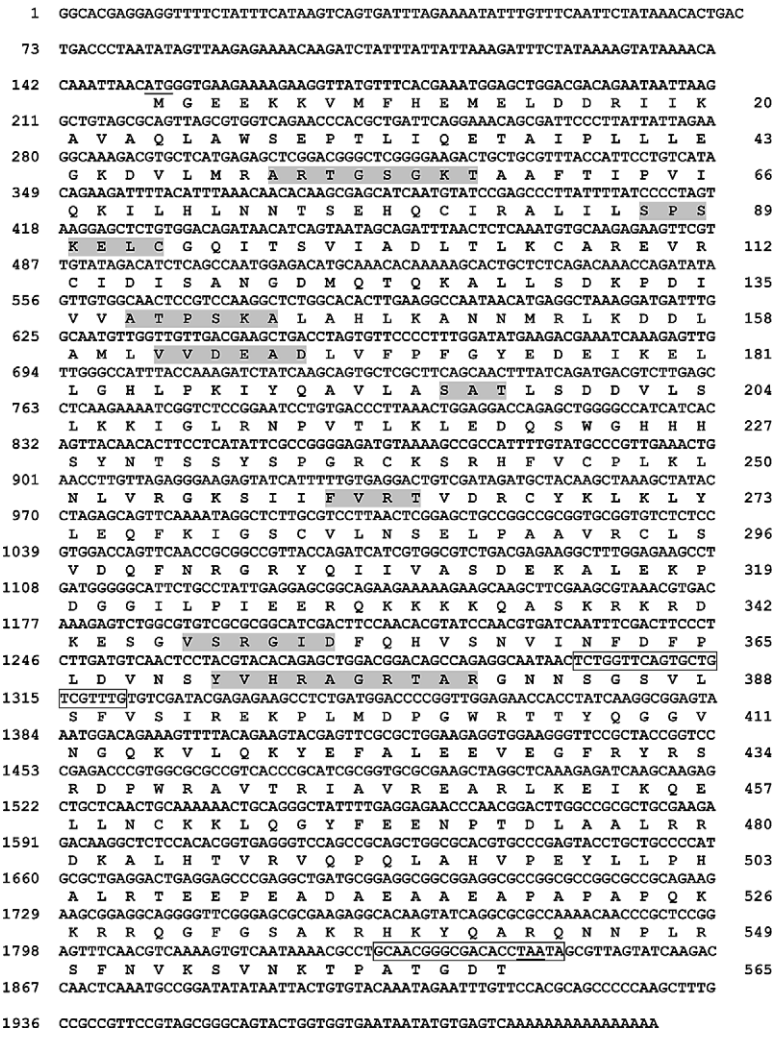

Fig. 1. Nucleotide and deduced amino acid sequences of *CfrHlc64*. Eight functional motifs are highlighted in grey. The start and stop codes are underlined. Forward and reverse primers for RT-PCR are boxed. The numbers on the left are for nucleotide sequence, while the numbers on the right are for amino acid sequence. The GenBank accession number for this sequence is AY460342.

(DEAD) in motif II, indicating that *CfrHlc64* was a member of the "DEAD box" family of RNA helicases.

Sequence alignment analysis indicated that the deduced amino acid sequence of *CfrHlc64* shared 10–50% identities with RNA helicases from other species. Amino acids in motifs I, II, and III were highly conserved in all RNA helicase sequences (Fig. 2A), whereas other regions of the sequences were less well conserved. Fig. 2B shows a phylogenetic tree of 29 RNA helicases, in which two major groups can be found. Among RNA helicases of other species, *CfrHlc64* was most similar to the *Drosophila* RNA helicase, *Hlc* (50% amino acid identity) (de Couet et al., 1995; Miklos et al., 1997; Maleszka et al., 1998; GenBank accession number: T08433) and *Anopheles* homolog (43% identity) (GenBank accession number: XP_309500). These three insect RNA helicases and the homologues of vertebrates, fungi, yeast, nematode, and plants form one group. Two members (human NOH61 and yeast Dbp9b) of this group have been suggested to be involved in ribosomal biogenesis. Biological functions of the other members in this group are not clear. Another group includes six *Drosophila* RNA helicases, *eIF-4A, vasa, ME31B, Hel, pit* and *abstrakt*, and several bacterial RNA helicases. *CfrHlc64* showed only low similarities to the members of this group, for example, 22% identical to *eIF-4A* (Ray et al., 1985; Abramson et al., 1987; Dorn et al., 1993), 21% to *hel* (Eberl et al., 1997) and ME31B (de Valoir et al., 1991), 16% to *vasa* (Lasko and Ashburner, 1988; Hay et al., 1988) and 10% to *mle* (Kuroda et al., 1991). *CfrHlc64* also showed low similarities (15–22%) to several cold shock induced proteins of *E. coli* (Jiang et al., 1996; Jones et al., 1996; Lee et al., 1994; GenBank accession numbers: NP_755783, Q8XA87). These proteins are localized in the ribosomal fractions and can be up-

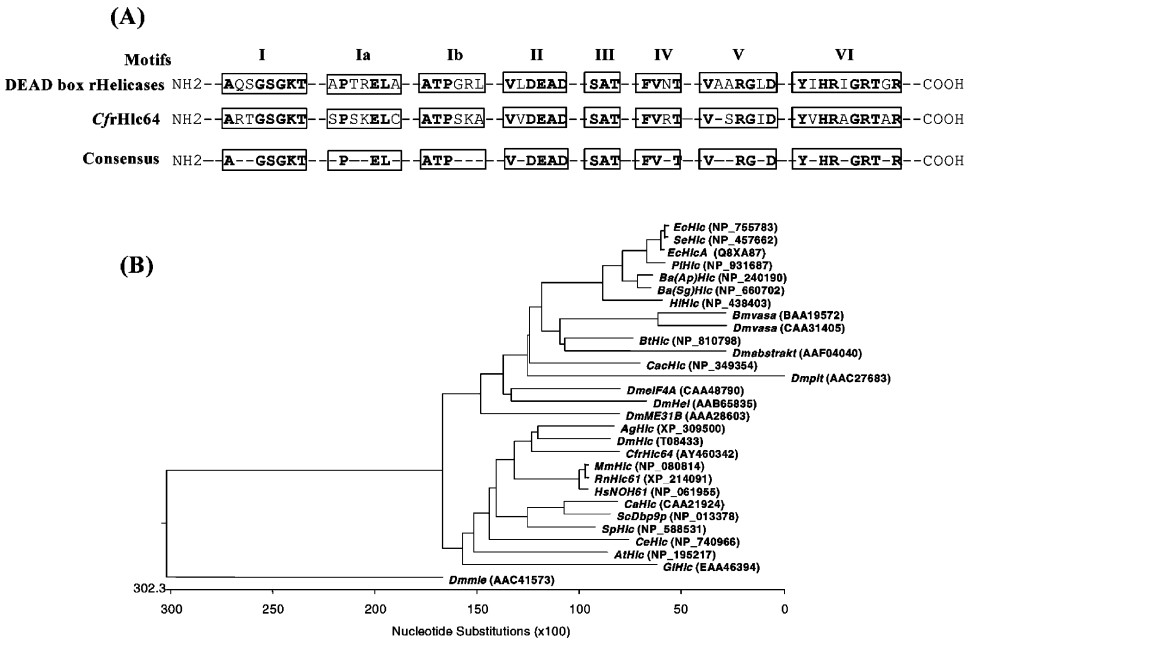

Fig. 2. (A) Alignment of functional motifs of *CfrHlc64* and other RNA "DEAD" box helicases. The conserved amino acid sequence of eight functional motifs of the "DEAD" box RNA helicase family is cited from Aubourg et al. (1999). The residues that match the consensus residues are bolded. (B) Phylogenetic tree of 29 RNA helicases from different species. *CfrHlc64*: *C. fumiferana* 64 kDa helicase; *DmHlc*: *D. melanogaster* helicase; *HsNOH61*: *H. sapiens NOH61*; *MmrHlc*: *M. musculus* RNA helicase; *AgHlc*: *Anopheles gambiae* helicase; *ScDbp9p*: *Saccharomyces cerevisiae Dbp9p*; *SpHlc*: *Schizosaccharomyces pombe* helicase; *CaHlc*: *Candida albicans* helicase; *AtHlc*: *Arabidopsis thaliana* helicase; *DmeIF4A*: *D. melanogaster* eukaryotic translation initiation factor 4A; *DmHel*: *D. melanogaster* RNA helicase; *DmME31B*: *D. melanogaster ME31B*; *Dmpit*: *D. melanogaster pitchoune*; *Dmvasa*: *D. melanogaster vasa*; *Bmvasa*: *B. mori vasa*; *Dmpit*: *D. melanogaster pitchoun*; *Dmabstrakt*: *D. melanogaster abstrakt*; *Dmmle*: *D. melanogaster maleless*; *RnHlc61*: *Rattus norvegicus* 61 kDa RNA helicase; *CaHlc*: *Candida albicans* helicase; *SpHlc*: *Schizosaccharomyces pombe* helicase; *CeHlc*: *Caenorhabditis elagans* helicase; *AtHlc*: *Arabidopsis thaliana* helicase; *GlHlc*: *Giaria lamblia* helicase; *Ba(Ap)Hlc*: *Buchnera aphidicola str. Sg (Schizaphis graminum)* helicase; *BtHlc*: *Bacteroides thetaiotaomicron* helicase; *Ba(Ap)Hlc*: *Buchnera aphidicola str. APS (Acyrthosiphon pisum)* helicase; *PlHlc*: *Photorhabdus luminescens* helicase; *CacHlc*: *Clostridium acetobutylicum* helicase; *HiHlc*: *Haemophilus influenzae* helicase; *EcHlc*: *Escherichia coli* cold-shock helicase; *EcHlcA*: *Escherichia coli* cold-shock helicase A; *SeHlc*: *Salmonella enterica* helicase. GenBank accession numbers for these sequences are included in brackets.

regulated by cold shock. However, *CfrHlc64* did not contain a cold box (TGACGTACAGA) that is usually present in these genes (Jiang et al., 1996; Lim et al., 2000) and was not cold inducible in our preliminary experiments (data not shown).

3.2. In vitro expression of CfrHlc64 in bacterial system

The *CfrHlc64* cDNA was expressed into a recombinant protein using the pPROEX™ HTa vector in fusion with 6xHis and TEV protease cleavage spacer sequences (Fig. 3). The molecular mass of the recombinant protein purified with His-tag affinity Ni-NTA silica resin was around 68 kDa. Based on the size (approximately 4 kDa) of the fused 6xHis and TEV protease cleavage spacer sequences, the molecular mass of the recombinant protein was thus estimated to be 64 kDa, the same as that of the deduced amino acid sequence of the cDNA, indicating that the cloned cDNA did encode a 64 kDa protein. The recombinant protein immunologically cross-reacted with the anti-*Rst(1)JH* antibodies (Fig. 3).

3.3. Developmental expression of CfrHlc64

Developmental expression of *CfrHlc64* from embryos to adults was examined using Northern blotting and RT-PCR (Figs. 4 and 5). Northern blotting

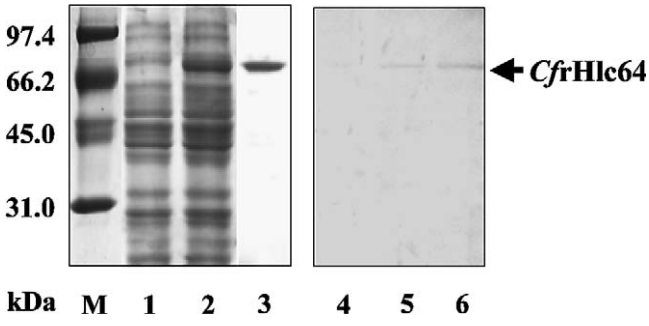

Fig. 3. Expression of *CfrHlc64* in bacterial expression systems. The left panel shows the 12% SDS-PAGE gel stained with Coomassie Blue R-250. The right panel shows the Western blot immunoblotted with anti-*Rst(1)JH* antibody. Five micrograms of protein were loaded into each lane. Lanes 1 and 4: without IPTG induction; Lanes 2 and 5: induction with 1 mM IPTG; Lanes 3 and 6: purified 6xHis tag *CfrHlc64* protein.

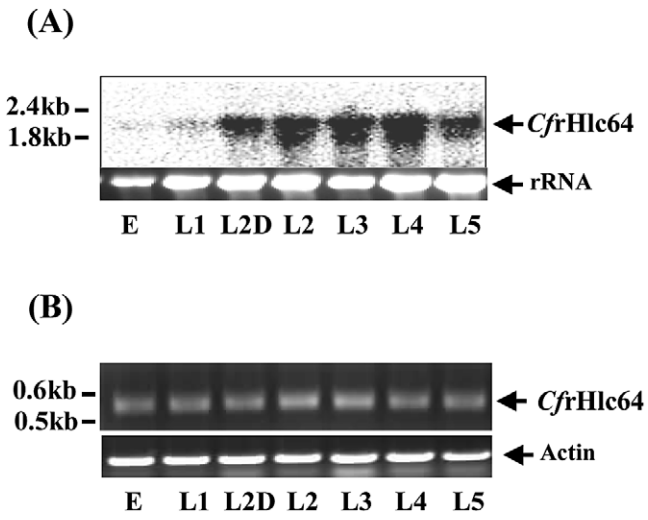

Fig. 4. Developmental expression of *CfrHlc64* mRNA in *C. fumiferana*. (A) Northern blot containing 10 μg of total RNA hybridized with a ^{32}P-labeled *CfrHlc64* cDNA as a probe. The estimated size of *CfrHlc64* was 2 kb. rRNA stained with ethidium bromide indicates equivalent loading of total RNA. (B) Reverse transcription PCR with *CfrHlc64*-specific primers that amplified a fragment of 540 bp. Actin bands amplified with actin-specific primers indicate equal use of RNA in the RT-PCR reactions. E, embryos; Ln, nth instar larva; D, diapause.

revealed an approximate 2 kb transcript throughout development from embryo to the fifth instar (Fig. 4A). The size of this transcript was the same as the calculated size of the *CfrHlc64* cDNA, suggesting that the cloned cDNA represented a full-length mRNA. Relatively low levels of *CfrHlc64* mRNA expression were detected by Northern blotting in embryos and first instar larvae, whereas high levels of *CfrHlc64* mRNA were detected in larvae from the second to fifth instars (Fig. 4A). By using RT-PCR analyses, all the samples from embryo to fifth instar larvae showed same intense cDNA bands of *CfrHlc64* (Fig. 4B).

Northern blots showed that the fat body and midgut of sixth instar larvae contained higher levels of *CfrHlc64* transcripts than the epidermis (Fig. 5A). These differences were also detected by RT-PCR, which detected a most intense band for the fat body (Fig. 5B). Female adults had higher levels of *CfrHlc64* transcripts than the male adults when whole animals were tested (Fig. 5C and D).

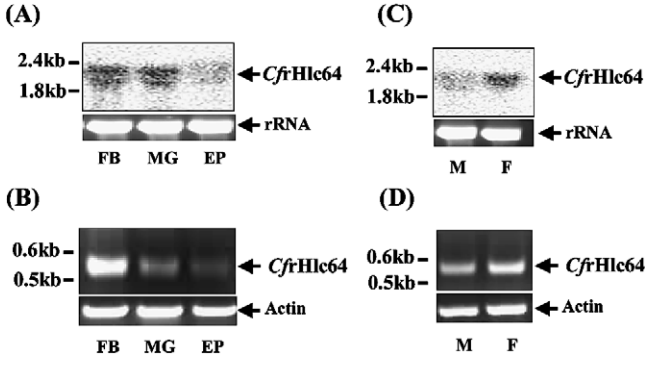

Fig. 5. Tissue-specific expression of *CfrHlc64* mRNA (A and B) and expression of *CfrHlc64* mRNA in male and female adults (C and D). (A) and (C): Northern blot containing 10 μg of total RNA hybridized with a ^{32}P-labeled *CfrHlc64* cDNA as a probe. rRNA stained with ethidium bromide indicates equivalent loading of total RNA. (B) and (D): Reverse transcription PCR with *CfrHlc64*-specific primers that amplified a fragment of 540 bp. Actin bands amplified with actin-specific primers indicate equal use of RNA in the RT-PCR reactions. FB, fat body; MG, midgut; EP, epidermis. M, male; F, female.

3.4. *Spatial distribution of CfrHlc64* protein

Distribution of *CfrHlc64* in the fat body, midgut and epidermis was examined using immunohistochemical staining. The results indicated that fat bodies that were close to the epidermis and midgut contained high levels of *CfrHlc64* protein (Fig. 6A and D). This observation is consistent with the results of Northern blotting and RT-PCR analysis (Fig. 5A and B). The epidermis also contained high levels of the protein (Fig. 6A). No *CfrHlc64* was detected in the midgut cells (Fig. 6D). The *CfrHlc64* protein appeared to be present in the cells whose nuclei were stained strongly by DAPI (Fig. 6A, B, D, and E).

3.5. *Induced expression by ecdysone agonist tebufenozide*

To test whether or not *CfrHlc64* was inducible by ecdysone and juvenile hormone, the nonsteroid ecdysone agonist tebufenozide, RH5992, and methoprene were used to treat 2-day-old sixth instar larvae. The RH5992 treated larvae entered a precocious and incomplete molting at day 2 post treatment, resulting in an extra but abnormal instar stage. The insects died around day 5 post treatment. The methoprene treated larvae grew much bigger than the control animals and stayed at sixth instar for 5–10 days more than the control before they died or molted into pupa depending on the concentrations used. RH5992 enhanced the expression of *CfrHlc64* starting from 3 to 12 h post treatment before any morphological changes were visible (Fig. 7A), whereas methoprene did not have any effect on the expression of *CfrHlc64* (data not shown). The up-regulation of the *CfrHlc64* expression by RH5992 was dose-dependent from 10^{-6} to 10^{-3} M of RH5992 and the highest level of expression, as measured by RT-PCR, was detected at 10^{-3} M of RH5992 at 6 h post treatment (Fig. 7B).

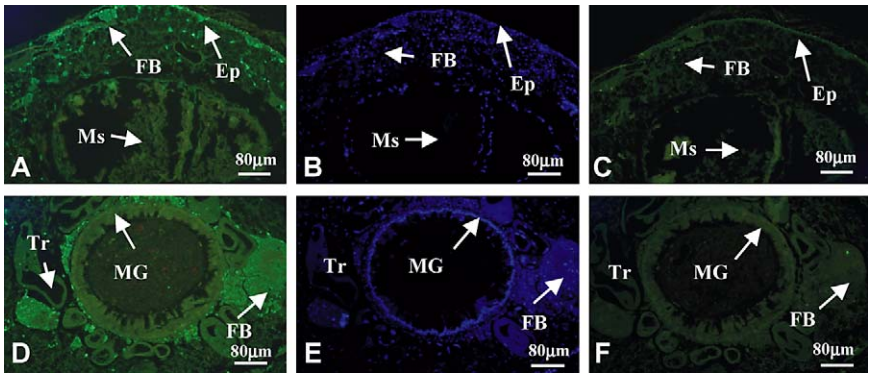

Fig. 6. Cross sections of sixth instar larvae showing the distribution of *CfrHlc64*. The green fluorescence indicates the presence of *CfrHlc64*. (A–C): epidermis and fat body; (D–F): midgut and fat body. (A and D): stained with anti-*CfrHlc64* antibodies and exposed to fluorescein filter. (B and E): exposed to DAPI filter. (C and F): negative control stained with pre-immune serum. Ep, epidermis; FB, fat body; MG, midgut; Tr, trachea; Ms, muscle.

4. Discussion

One of interesting findings in the present study is that expression of RNA helicase *CfrHel64* was induced by RH5992. Identification of *CfrHlc64* using anti-*Rst(1)JH* antibody (Pursley et al., 2000) apparently was spurious though fortuitous. In this study we found no evidence that links this RNA helicase to JH action. However, we found that the ecdysone agonist RH5992 enhanced the expression of the *CfrHlc64* gene in a dose-dependent manner, although this result needs to be further confirmed using more accurate quantitative approaches. Ecdysone is a molting hormone that triggers the molting process by activating expression of a series of genes, such as several early transcription factors, HR3, E74, E75, Broad-complex, E63-1 and E23, etc. (Thummel, 2002; Riddiford et al., 2000). RNA helicases play important roles in post-transcriptional processing and translation of mRNA transcripts. Increased expression of *CfrHlc64* by RH5992 might facilitate post-transcriptional processing and translation of mRNA of these ecdysone-inducible genes.

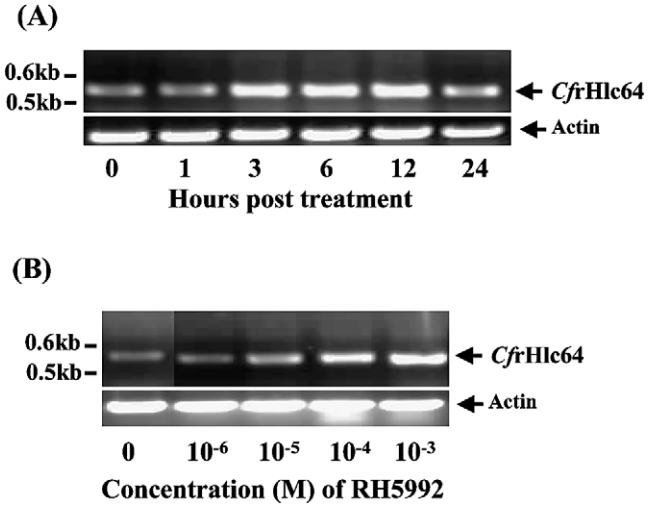

Fig. 7. Induced expression of *CfrHlc64* mRNA by RH5992 in sixth instar larvae of *C. fumiferana*. (A) Reverse transcription PCR with *CfrHlc64*-specific primers that amplified a fragment of 540 bp. Total RNA was extracted at 0, 1, 3, 6, 12 and 24 h post treatment with 10^{-3} M RH5992; (B) Reverse transcription PCR with *CfrHlc64*-specific primers that amplified a fragment of 540 bp. Total RNA was extracted at 6 h post treatment with RH5992 at 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M. Actin bands amplified with actin-specific primers indicate equal use of RNA in the RT-PCR reactions.

The present study has not identified cellular roles for *CfrHlc64* in the spruce budworm. Expression of *CfrHlc64* was detected in all developmental stages from embryos to adults and the protein was found in the epidermis and the fat bodies around the epidermis and midgut. These results might imply that this protein might be required for many physiological processes. *CfrHlc64* is the second lepidoptera RNA helicase reported so far and it shows only 15% identity to *B. mori* RNA helicase *vasa*, which is suggested to be involved in germ anlage development (Nakao, 1999). *CfrHlc64* showed high identities to *D. melanogaster Hlc* (50%), which is localized in flightless region of the *Drosophila* genome (de Couet et al., 1995; Miklos et al., 1997; Maleszka et al., 1998) and *A. gambiae* homolog (43%). However, the biological roles of these two insect RNA helicases have not been reported. Relatively close to these three insect helicases are the human nucleolar RNA helicase, NOH61 (Zirwes et al., 2000), and the yeast RNA helicase, Dbp9p (Daugeron et al., 2001). Both NOH61 and Dbp9p are localized in the nucleolus and are essential proteins that are involved in 60S-ribosomal-subunit biogenesis.

CfrHlc64 also showed low sequence identities (10–22%) to several other *Drosophila* DEAD box RNA helicases, such as *eIF4A* (Ray et al., 1985; Abramson et al., 1987; Dorn et al., 1993), *vasa* (Lasko and Ashburner, 1988; Hay et al., 1988), *hel* (Eberl et al., 1997), *mle* (Kuroda et al., 1991), *pit* (Zaffran et al., 1998) and

ME31B (de Valoir et al., 1991), but appeared to belong to a different group (Fig. 2B). These genes are involved in diverse cellular processes. *CfrHlc64* and these sequences were conserved only in the functional motifs I, II, and III, which are involved in ATP binding and hydrolysis and RNA unwinding (Aubourg et al., 1999), but other regions were variable. Because these un-conserved regions may be involved in specific cellular functions in various species, *CfrHlc64* may have different cellular functions from these helicases. The cellular roles of *CfrHlc64* need to be further investigated.

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