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Identification and developmental expression of the mitochondrial phosphate transport protein gene from the spruce budworm, *Choristoneura fumiferana*

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Abstract

Phosphate transport protein (PTP) is a mitochondrial inner membrane protein responsible for the translocation of inorganic phosphate into the mitochondrial matrix. A full length cDNA clone encoding the PTP was isolated from the spruce budworm, *Choristoneura fumiferana*. The deduced amino acid sequence of the longest ORF of CfPTP cDNA showed high similarity with the amino acid sequences of PTPs cloned from several species. Phylogenetic tree analysis indicated that CfPTP occupied an intermediate position between vertebrates on the one side and yeast and nematodes on the other side. Studies on the developmental expression of CfPTP mRNA showed that higher levels of mRNA were present during the feeding and growing stages than during molting periods.

Keywords: Phosphate carrier; Membrane transport; Mitochondria; cDNA cloning; Gene expression; Molting

1. Introduction

Phosphate transport protein (PTP) is a major protein of the inner mitochondrial membrane and is responsible for the translocation of inorganic phosphate into the mitochondrial matrix for oxidative phosphorylation in energy metabolism of cells (LaNoue and Schoolwerth, 1979). PTP has been isolated and purified to homogeneity from bovine heart (Kolbe et al., 1984) and rat liver mitochondria (Kaplan et al., 1986) by chromatographic methods. The protein has also been functionally reconstituted in proteoliposomes (Kaplan et al., 1986; Stappen and Kramer, 1993). The PTPs are 33 to 34 kDa in size and they most likely act as a dimer in the inner membrane of mitochondria (Kramer, 1996). The sequence of an amino-terminal fragment (Kolbe and Wohlrab, 1985) and partial sequence (Aquila et al., 1987) of the bovine heart PTP have been determined by chemical protein sequence analysis. The complete amino acid sequence of the PTP was deduced from the nucleotide sequence of cDNA clones from bovine heart (Runswick et al., 1987) and rat liver (Ferreira et al., 1989), whereas the PTP sequences from yeast (Phelps et al., 1991) and human heart (Dolce et al., 1994) were from genomic clones. The bovine and human PTP genes have a similar structure consisting of nine exons separated by eight introns (Palmieri et al., 1993; Dolce et al., 1994). The PTP belongs to a superfamily of mitochondrial carrier proteins, including ADP/ATP carrier (AAC), uncoupling protein (UCP) and oxoglutarate carrier (Runswick et al., 1987, 1990; Dietmeier et al., 1993; Palmieri et al., 1993). All these carrier proteins are not only structurally homologous to each other, but also have a similar targeting mechanism as well as a submitochondrial location (Dietmeier et al., 1993). They are located in the inner membrane of mitochondria and contain three-fold repeated sequences that are conserved. Each repeated element contains approximately 100 amino acids and is composed of two hydrophobic membrane-spanning α-helices linked by an extensive hydrophillic domain (Runswick et al., 1987).

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Several models on the topography of the PTP in the mitochondrial membrane have been described. Aquila et al. (1985, 1987) suggested that it was made up of six transmembrane α-helices and a β-strand spanning across the inner mitochondrial membrane. The N-terminal end of the protein was located in the matrix side of the membrane and the C-terminal end was at the cytosol side. Based on the studies on PTP from rat liver mitochondria, Ferreira et al. (1989) suggested that it consisted of six hydrophobic α-helices connected by five extra-membrane hydrophillic loops that were membrane-spanning with both the N-terminal and C-terminal regions of the peptide chain being oriented towards the matrix side of the inner membrane. Capobianco et al. (1991) and Palmieri et al. (1993) proposed a similar model in which six membrane-spanning α-helices cross the membrane but the amino and the carboxyl termini were both located at the cytosol side of the membrane. This topology is the same for other mitochondrial carrier proteins.

Several reviews on mitochondrial PTP have been published with emphasis on the transport system (LaNoue and Schoolwerth, 1979; Kramer, 1996), molecular aspects (Wohlrab, 1986), and comparison of PTP with other mitochondrial carriers (Aquila et al., 1987), but few studies have been reported on the developmental expression of the PTP mRNA with the exception of Dolce et al. (1996) who investigated the tissue-specific expression of two isoforms of the mitochondrial PTP in bovine tissues.

In the present paper, we describe the isolation, sequencing and characterization of a full length PTP cDNA clone (CfPTP) from a lepidopteran insect, *C. fumiferana*. We compare CfPTP with the PTPs from other species and discuss their phylogenetic relationships. In addition, we studied the developmental expression of the CfPTP mRNA and discuss its role in development of *C. fumiferana*.

2. Materials and methods

2.1. Experimental animals

Spruce budworm (*Choristoneura fumiferana* Clem., Lepidoptera: Tortricidae) eggs were collected within one hour after oviposition and maintained at 22°C and 70% relative humidity. Under these conditions, the eggs hatched in eight days. After five days, the first instar larvae molted into second instars and entered diapause. The hibernacula of the diapausing larvae in cheese cloth sheets were stored at 2°C for 20–25 weeks to satisfy the diapause requirement. At the end of diapause, the larvae were reared on artificial diet at 22°C, 70% relative humidity and a photoperiod of 12 hr light and 12 hr darkness. Newly molted fifth and sixth instar larvae were selected within 30 min after molting, when the head capsule was still white and were staged from that time onwards. Morphological markers, such as head capsule slippage, were used to select larvae that were at the same developmental stage and age. Whole larvae from first to fourth instars and various tissues, such as epidermis, fat body and midgut, that were dissected aseptically from the fifth or sixth instar larvae were used for RNA extraction for Northern blot assays.

2.2. Isolation of RNA and cDNA library construction

Total RNA was isolated from first instar and diapausing second instar *C. fumiferana* using the guanidine isothiocyanate-phenol-chloroform extraction method (Chomczysnki and Sacchi, 1987). The RNA was quantified spectrophotometrically, and the poly $(A)^+$ RNA was purified using biotin labeled oligo(dT) and streptavidin Magne Sphere™ particles (Promega, Madison, WI).

A cDNA library was constructed using pooled RNA from first instar and diapausing second instar larvae of *C. fumiferana* as described in Palli et al. (1998).

2.3. Random in vivo mass excision

For random in vivo mass excision one μl of a cDNA library (2×10^5 pfu/μl) and one μl of ExAssist helper phage ($> 1 \times 10^6$ pfu/μl) were added to 200 μl of XL1–Blue MRF™ cells (Stratagene, La Jolla, CA), grown till the OD_{600} reached 5.0 and incubated at 37°C for 15 min. Three milliliters of LB media was then added to the phage/cell mixture and incubated for 2.5 hr at 37°C with gentle shaking. The cells were heat-shocked at 70°C for 15 min, centrifuged at 4000***g*** for 15 min after which the supernatant containing the phagemid was collected. Ten microliters of phagemid was added to 200 μl of freshly grown SOLR™ cells (Stratagene, La Jolla, CA) and incubated at 37°C for 15 min. Different dilutions of the mixture were plated on LB agar plates supplemented with 50 μg/ml ampicillin and the plates were incubated at 37°C overnight. Single colonies were screened by running them on an agarose gel. Ten colonies that had inserts greater than 1 kb were grown overnight in 2 × YT media containing 50 μg/ml ampicillin. Plasmid DNA was isolated from these clones using QIAprep 8 plasmid kit (QIAGEN Inc., Chatsworth, CA).

2.4. Sequencing and sequence analysis

The cDNA of these clones with inserts greater than 1 kb were sequenced on the ends using Cy5™ AutoRead™ Sequencing Kit (Pharmacia Biotech Inc., Piscataway, NJ) and ALFexpress DNA Sequencer (Pharmacia Biotech Inc., Piscataway, NJ). The cDNA clone whose deduced amino acid sequence showed high similarity with PTP was sequenced completely on both strands.

Sequence analysis was performed using the Mac Vector DNA analysis program (International Biotechnologies Inc., 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).

2.5. Northern blot hybridization

Ten micrograms of total RNA was separated on a formaldehyde–agarose gel (1%) and transferred to a Hybond N membrane (Amersham Life Sciences Inc. Arlington Heights, IL, U.S.A.). The RNA was cross-linked to the membrane using a UV cross-linker (Stratagene, La Jolla, CA). The membranes were prehybridized for at least three hours at 42°C in a solution containing 50% formamide, 4 × SSC (1 × SSC: 1.5 M NaCl, 0.15 M Na citrate), 10 × Denhardt's solution (Denhardt, 1966), 50 mM $NaPO_4$ (pH 7.0) and 250 μg/ml denatured herring sperm DNA. The insert from CfPTP cDNA clone was labeled with [^{32}P]dATP using the random primed labeling method (Feinberg and Vogelstein, 1984) and used as a probe for Northern hybridization. Hybridization reaction was carried out for 24 hours at 42°C in a solution containing a fresh aliquot of the above prehybridization solution plus 10% dextran sulfate and the [^{32}P]-labeled probe. Membranes were then washed at 65°C with three changes, 30 min each, of 2 × SSC containing 0.1% SDS, followed by 20 min each in 0.5 × SSC containing 0.1% SDS and 0.1 × SSC containing 0.1% SDS. Embryonic RNA was isolated from precisely staged groups of egg masses collected at specific time points. First to sixth instar RNA was isolated from staged larvae collected at specific time points.

3. Results

3.1. Identification and sequencing of CfPTP cDNA clone

The cDNA inserts from ten clones randomly picked from the cDNA library were sequenced on both ends. The deduced amino acid sequence of one of the cDNA clones showed high similarities with the deduced amino acid sequences of mitochondrial phosphate transport proteins (PTP) cloned from several species (see below). Because of its high similarity with PTPs, this cDNA clone was designated as *C. fumiferana* phosphate transport protein (CfPTP). The cDNA was then sequenced completely on both strands.

The nucleotide and deduced amino acid sequences of CfPTP cDNA are presented in Fig. 1. The cDNA is 1767 nucleotides in length with 5′- and 3′-untranslated regions of 61 and 656 bp, respectively. The longest open reading frame (ORF, bases 62–1108) encodes for 349 codons. Based on this 349-codon ORF, a 38 kDa protein was predicted. Two putative polyadenylation signal sequences (AATAAA) were located at 416 and 650 bases, respectively, downstream from the termination codon (TAA). The cDNA was terminated at its 3′-end by a poly$(A)_{18}$ sequence.

3.2. Sequence comparison with the PTPs of other species

A GenBank search was performed to compare the deduced amino acid sequence of the CfPTP with the PTP sequences of other species. Fig. 2(A) shows the alignment of the deduced amino acid sequence of the CfPTP with the deduced amino acid sequences of PTPs from human (*Homo sapiens*, Dolce et al., 1994), bovine (*Bos taurus*, Runswick et al., 1987), Norway rat (*Rattus norvegicus*, Ferreira et al., 1989), nematode (*Caenorhabditis elegans*, GenBank, accession no: P40614), and yeast (*Saccharomyces cerevisiae*, Phelps et al., 1991). The highly conserved amino acids among these PTPs are given in lines below the alignment of the PTP sequences. The amino acids that are identical to those of CfPTP are highlighted in black. Analysis of sequence-pair distances revealed 33.4, 62.2, 62.5, 63.3, and 65% overall amino acid identities between this CfPTP and the PTPs of *S. cerevisiae, R. norvegicus, H. sapiens, B. taurus* and *C. elegans*, respectively. Phylogenetic tree analysis indicated that CfPTP falls between vertebrates (human, rat and bovine) on the one side and yeast as well as nemotodes on the other side [Fig. 2(B)]. Also, it was closer to *C. elegans* than *S. cerevisiae*. Comparing the import sequences of *B. taurus* (amino acids 1–49, Runswick et al., 1987) and *R. norvegicus* (amino acids 1–44, Ferreira et al., 1989) with CfPTP showed that the import sequence of CfPTP probably consists of 33 amino acids (1–33).

Further analysis on the deduced amino acid sequence of the CfPTP cDNA revealed that there are three internal repeats (Fig. 3). The average length of these repeats is 96 amino acids. Hydrophobicity analysis of the amino acid sequence indicated that each repeated segment contains two hydrophobic regions for a total of six regions in the protein (I and II, III and IV, V and VI). The most highly conserved residues within CfPTP are distributed in these hydrophobic regions of each repeated segment (Fig. 3). The hydrophillic regions between the predicted transmembrane segments in each repeat as well as amino acids on the 5′- and 3′-terminals are poorly conserved.

Comparison of CfPTP with two members of the mitochondrial carrier protein superfamily, the uncoupling protein (UCP, Aquila et al., 1985) and the ADP/ATP carrier protein (AAC, Battini et al., 1987), showed that the deduced amino acid sequence of CfPTP is homologous to both UCP and AAC, which also contain three repeat sequences (Fig. 3).

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1    GGCACGAGGTCAAGCGCGGTGTGGAGTGTCGATCGGTAAAATAGGATTTTAAGACATAATA
62   ATGTTCTCCTCACTTCTGGAAGCGGCCAAAAGCTCGCCGTTCCACGGGCCGCTTACTCCG
       M  F  S  S  L  L  E  A  A  K  S  S  P  F  H  G  P  L  T  P    20
122  GCGCGCTGCGATGCGCCCGCTCAGGGGATGGCTGCGTCGGCGGCGCCAGTCGGAGACTCC
       A  R  C  D  A  P  A  Q  G  M  A  A  S  A  A  P  V  G  D  S    40
182  TGCGAGTTCGGCTCCACCAAGTACTTCGCGCTATGCGGGCTGGGCGGTATCCTCTCATGC
       C  E  F  G  S  T  K  Y  F  A  L  C  G  L  G  G  I  L  S  C    60
242  GGTATCACCCACACGGCGGTGGTACCCCTCGACCTGGTGAAATGCCGGCTGCAGGTGGAC
       G  I  T  H  T  A  V  V  P  L  D  L  V  K  C  R  L  Q  V  D    80
302  GCCGACAAATACAAGAACGTGGTCAACGGCTTCCGCGTGTCCGTGCGCGAGGAGGGCCTC
       A  D  K  Y  K  N  V  V  N  G  F  R  V  S  V  R  E  E  G  L   100
362  CGCGGACTCGCCAAGGGCTGGGCGCCCACTTTCATCGGGTACTCGCTACAGGGTCTCTGC
       R  G  L  A  K  G  W  A  P  T  F  I  G  Y  S  L  Q  G  L  C   120
422  AAGTTCGGCCTTTACGAGGTGTTCAAAGTACAGTACAACAATATGCTGGATGAAGAGACG
       K  F  G  L  Y  E  V  F  K  V  Q  Y  N  N  M  L  D  E  E  T   140
482  GCGTACACTTATCGTACGTTCGTGTACCTCGCTGCCTCGGCGTCGGCTGAGTTCTTCGCT
       A  Y  T  Y  R  T  F  V  Y  L  A  A  S  A  S  A  E  F  F  A   160
542  GACATCGCACTGTCGCCGCTCGAGGCCGCTAAGGTCCGCATCCAGACCATGCCCGGGGTT
       D  I  A  L  S  P  L  E  A  A  K  V  R  I  Q  T  M  P  G  V   180
602  CGCCAACACACTGCGCGACGCGTGGCCCAAGATGGTCCAGAACGAGGGGCGTGGGCACGT
       R  Q  H  T  A  R  R  V  A  Q  D  G  P  E  R  G  A  W  A  R   200
662  TCTACAAGGGCGCTGGTGCCGCTCTGGGGCCGACAGATCCCCTACACCATGATGAAGTTC
       S  T  R  A  L  V  P  L  W  G  R  Q  I  P  Y  T  M  M  K  F   220
722  GCCTGCTTCGAGAAGACCGTCGAGCTGCTTTACAAGCACGTGGTGCCCAAACCGCGCGCG
       A  C  F  E  K  T  V  E  L  L  Y  K  H  V  V  P  K  P  R  A   240
782  GAGTGCTCCAAGGGCGAGCAGCTGGTGGTGACGTTCGCCGCGGGCTACATCGCCGGCGTG
       E  C  S  K  G  E  Q  L  V  V  T  F  A  A  G  Y  I  A  G  V   260
842  TTCTGCGCCATCGTGTCGCACCCGGCCGACACCGTCGTCTCCAAGCTGAACCAGGACAAG
       F  C  A  I  V  S  H  P  A  D  T  V  V  S  K  L  N  Q  D  K   280
902  ACGGCGACTGTGGGCTCGATCGTGGGCAAACTCGGCTTTGCGGGCGTGTGGAAGGGTCTC
       T  A  T  V  G  S  I  V  G  K  L  G  F  A  G  V  W  K  G  L   300
962  GGACCCAGGATCATCATGATCGGTACCCTCACCGCGCTGCAGTGGTTCATCTATGACGCC
       G  P  R  I  I  M  I  G  T  L  T  A  L  Q  W  F  I  Y  D  A   320
1022 GTCAAGGTGTGGCTGAGGATGCCCCGCCCACCGCCAGCGGAGATGCCAGAGTCTATGCGC
       V  K  V  W  L  R  M  P  R  P  P  P  A  E  M  P  E  S  M  R   340
1082 AAGCGACTAGAAGCCGAGGGCAAGCTGTAAACTAGTACCAAGACACTACCCTACAGTACT
       K  R  L  E  A  E  G  K  L                                     349
1142 AAATGATTAAGCAACCGACACATACTTACTATCACAAACTTGACTCAAATTCAAGTGAGT
1202 GTTAAATATTTAGTACTATAGGAAGGATAGAATAATAACGCCGCAACCACGACCAGCCTG
1262 CGTACAGGAACTGTTCAAACACGGAGGAACTCCGGAATTGCCTGAATTACTGAGACGTCG
1322 TTAGGATACGACATGTTTAATGTAATATTTACATTCCCATGTTAAGTTGTAATACAAGCG
1382 GAGATGCAGTTGCTGCGTTCTACGTGTTTGCGTCGTGGACCGGTTGGAATGTTCCGTACA
1442 CCGCCACCATTGTATTAAATAGATACCCAATGTGATTACCAATGTTACAGTATATAAGCG
1502 TAAACGATCATGTTAATTTATTTTTAAATAAATTATATTTTTATTTATTTATTCCGTTTA
1562 TTTAATCGAAATCTTAAAATTTTGTGTGGCAGGAATAGCTTTTCAAATTCTTATTGACGT
1622 TGAAAAAGGTACTTAATGTACTTGTTAAAATTGAACTGCCAGCCTGTCTTTCCATTCTGT
1682 TCTTCGCACGGAAGAATTAATGTAAGTAGTTGATCGTTGAATGAGAAACGATAATAAATA
1742 CATCTCACAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequence of the *C. fumiferana* phosphate transport protein (CfPTP) cDNA. Numbers on the right refer to the deduced amino acid and those on the left refer to the nucleotide sequences. The predicted import sequence is underlined. The polyadenylation sites are double-underlined and the stop codon is dot-underlined. The GenBank accession number for this sequence is AF062383.

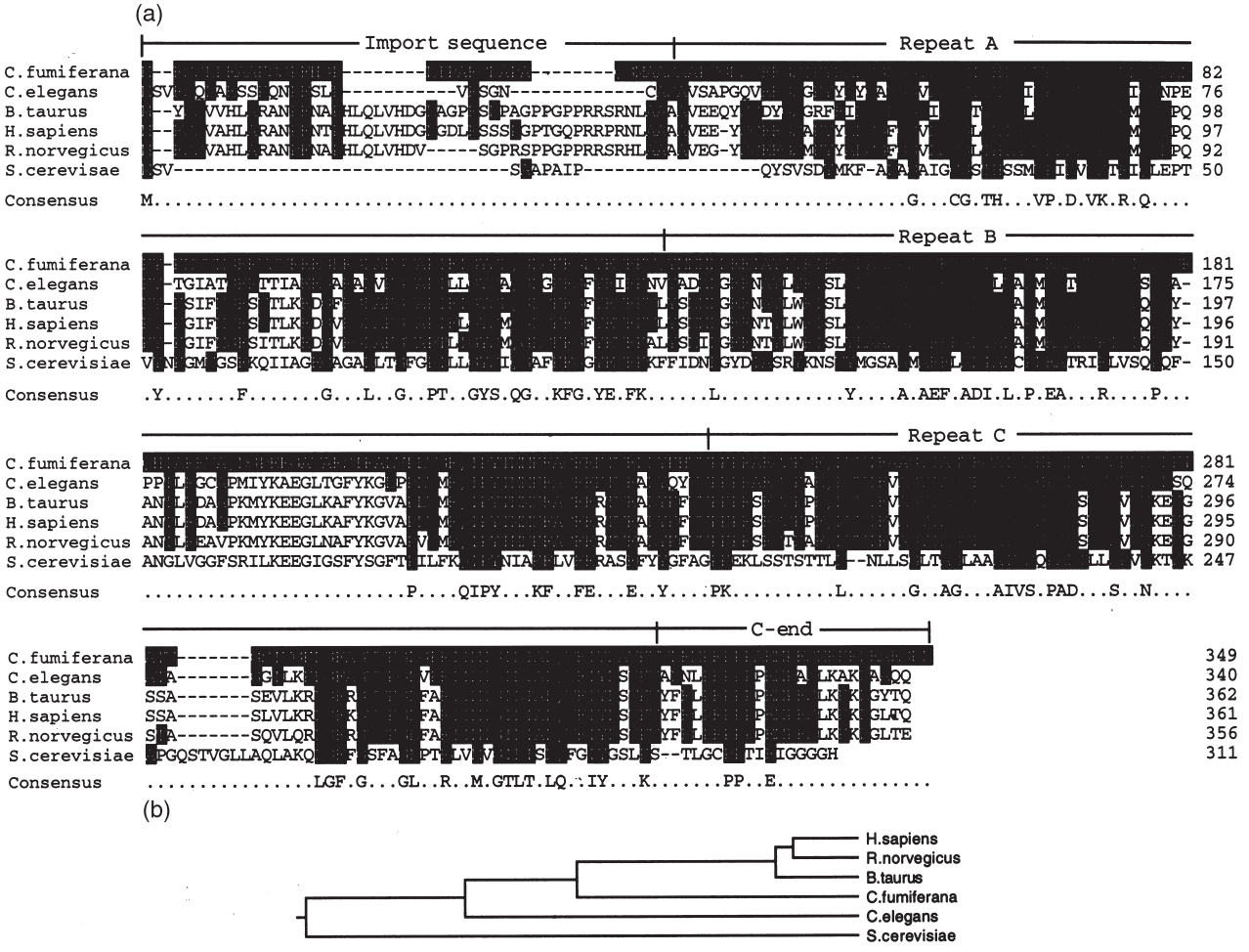

Fig. 2. Alignment (A) and phylogenetic tree (B) of the deduced amino acid sequence of the PTPs of *C. fumiferana* (this study), *C. elegans* (GenBank, accession no: P40614), *B. taurus* (Runswick et al., 1987), *H. sapiens* (Dolce et al., 1994), *R. norvegicus* (Ferreira et al., 1989), *S. cerevisiae* (Phelps et al., 1991). Multiple sequence alignment was performed by using the Clustal program (Higgins and Sharp, 1988). The residues that match *C. fumiferana* exactly are shaded with solid black. The consensus residues are given in lines below the alignment panels when all residues match.

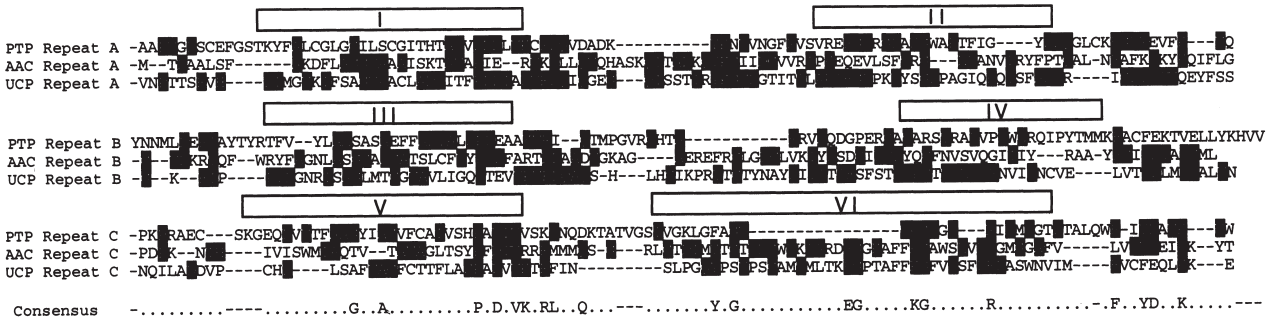

Fig. 3. Repeated segments of deduced amino acid sequences of the CfPTP, AAC (Battini et al., 1987) and UCP (Aquila et al., 1985). The predicted transmembrane regions for CfPTP are indicated by bars and Roman numerals I–VI. The consensus residues are given in the bottom line (5 out of 9 residues match). The N-terminal end (33 amino acids) and C-terminal end (25 amino acids) of the CfPTP are not shown.

3.3. Developmental expression of CfPTP mRNA

Temporal and spatial changes in expression of CfPTP mRNA were investigated by Northern blot analysis. The CfPTP cDNA probe bound to a 1.8 kb mRNA (Figs. 4–8). The CfPTP mRNA was first detected during embryogenesis and first-instar larvae (Fig. 4). High levels of CfPTP mRNA were detected as soon as the eggs were laid. Then the mRNA levels decreased and these low levels were maintained throughout the embryonic

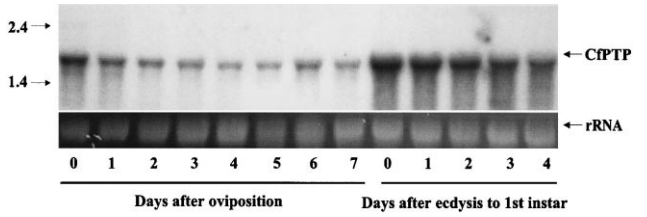

Fig. 4. Developmental expression of CfPTP mRNA during embryogenesis and 1st instar larvae. The top panel shows the Northern blot containing total RNA (10 μg) from embryos and 1st instar larvae hybridized with the 1.8 kb CfPTP cDNA probe. The bottom panel shows ribosomal RNA stained with ethidium bromide. RNA sizes (in kb) from an RNA ladder (GIBCO–BRL) are given on the left.

development (Fig. 4). The CfPTP mRNA levels increased after the first instar larvae hatched from the eggs. As the first instar larvae grew older (day 3 and 4), the mRNA levels began to decrease when the insects approached moulting.

Low levels of CfPTP mRNA were detected during prediapause and diapause (Fig. 5). The CfPTP mRNA levels started to increase when the insects came out of diapause and reached peak levels during the intermoult period (day 0 and 1) of the second instar stage, when the larvae started to feed. During the moulting stage between the second- and third-instars, the mRNA levels decreased again. Such fluctuation of the CfPTP mRNA levels was repeatedly detected in the third- and fourth-instar larvae. For example, during the growth phase (day 1 and 2 of third- and fourth-instars) the larvae showed the presence of higher levels of CfPTP mRNA than during the moulting phase (day 3) (Fig. 5).

The CfPTP mRNA was present during all stages of the 5th instar larvae (Fig. 6). It started to increase soon after the larvae molted from 4th to 5th instar and reached maximum levels during 24–36 hr after ecdysis to the 5th instar larvae. The high level expression of the CfPTP mRNA was maintained for two days, followed by a gradual decrease beginning at 72 hr after ecdysis to the 5th instar. The changes of the CfPTP mRNA level were parallel in both midgut [Fig. 6(A)] and total insect tissues [Fig. 6(B)].

In the epidermis of the 6th instar larvae [Fig. 7(A)],

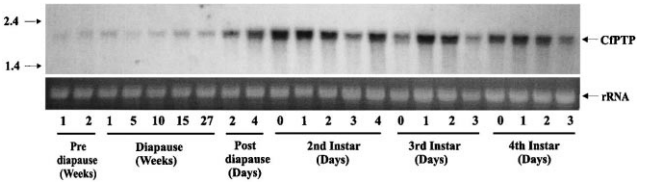

Fig. 5. Developmental expression of CfPTP mRNA during diapause and 2nd to 4th instar larval stages. The top panel shows the Northern blot containing total RNA (10 μg) from diapause and 2nd to 4th instar larvae hybridized with the 1.8 kb CfPTP cDNA probe. The bottom panel shows ribosomal RNA stained with ethidium bromide. RNA sizes (in kb) from an RNA ladder (GIBCO–BRL) are given on the left.

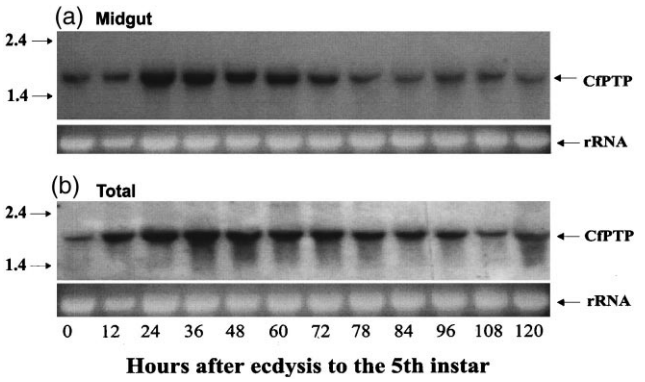

Fig. 6. Developmental expression of CfPTP mRNA in midgut and whole insects during the 5th instar larval stage. The top panels show the Northern blots containing total RNA (10 μg) from the 5th instar larvae hybridized with the 1.8 kb CfPTP cDNA probe. The bottom panels show ribosomal RNA stained with ethidium bromide. RNA sizes (in kb) from an RNA ladder (GIBCO–BRL) are given on the left.

the CfPTP mRNA started to increase at 24 hr after ecdysis to the 6th instar, and was maintained at a relatively constant level until 84 hr after ecdysis. The mRNA level then gradually decreased and finally none could be detected at the pharate pupal stage. In the fat body of the 6th-instar larvae [Fig. 7(B)], a relatively low level of CfPTP mRNA was present during 12–60 hr after ecdysis to the 6th instar. Only a trace amount of the mRNA was detected between 72 and 120 hr after ecdysis. After 120 hr, the mRNA was undetectable. In contrast with the fat body, the midgut of the 6th-instar larvae contained relatively higher levels of the CfPTP mRNA during 12–96 hr after ecdysis [Fig. 7(C)]. Starting from 108 hr after ecdysis, the mRNA levels gradually

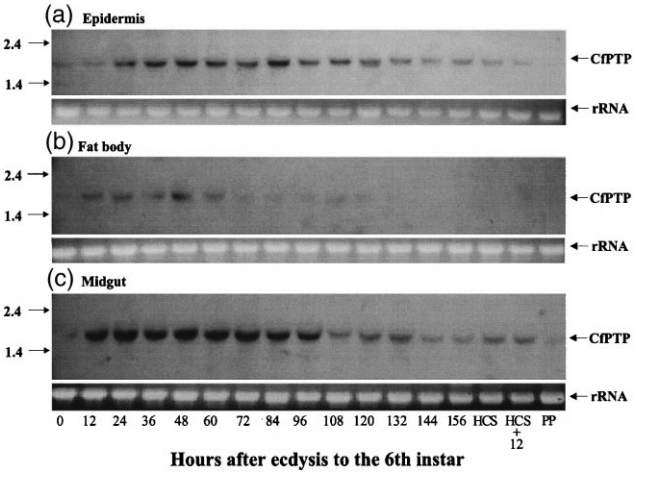

Fig. 7. Developmental expression of CfPTP mRNA in epidermis (A), fat body (B), and midgut (C) of the 6th instar larvae. The top panels show the Northern blot containing total RNA (10 μg) from 6th instar larvae hybridized with 1.8 kb CfPTP cDNA probe, and the bottom panels show ribosomal RNA stained with ethidium bromide. RNA sizes (in kb) from an RNA ladder (GIBCO–BRL) are given on the left. HCS, head capsule slippage; PP, pharate pupa.

decreased and no CfPTP mRNA was detected at the pharate pupal stage.

No CfPTP mRNA could be detected until 132 hours into the pupal stage. Low levels of the mRNA were detected at later stages (for example, 132–192 hr after ecdysis to the pupa) (Fig. 8).

In summary, the fluctuation of CfPTP mRNA levels showed correlation with insect development from embryo to pupa. During feeding and growth periods of each larval stage, high levels of CfPTP mRNA were detected; by contrast, during the molting periods, lower levels of CfPTP mRNA were present. A relatively higher level of CfPTP mRNA was found in the midgut and the epidermis than in the fat body. Thus, CfPTP mRNA levels showed both temporal and spatial regulation of the CfPTP gene during *C. fumiferana* development.

4. Discussion

In this study, we report the isolation, sequencing of cDNA and developmental expression of mRNA for CfPTP. The identity of the cloned cDNA was confirmed by comparing the cDNA sequence with the sequences of PTPs from other species. Since the cDNA clone is 1767 nucleotides in length and cDNA probe binds to a 1.8-kb mRNA (Figs. 4–7), we believe that this is a full length cDNA clone. This is the only second invertebrate and first insect PTP cDNA cloned.

PTPs of mammalian mitochondria are synthesized with a cleavable presequence, unlike the other members of the mitochondrial carrier family (Palmieri et al., 1993). As compared to the PTPs from other sources, the deduced amino acid sequence from the CfPTP cDNA shows a 33-amino acid import sequence at the N-terminal end. The length (33 amino acids) of the import sequence falls within the range of 10 amino acids in the case of yeast PTP (Phelps et al., 1991) and to 49 amino acids in the cases of human (Dolce et al., 1994) and bovine (Runswick et al., 1987) PTPs. In general, it appears that PTPs of vertebrates have longer import sequences.

High amino acid similarity of PTPs from different species indicate that PTPs are phylogenetically well conserved over a long time period in history. The conserved regions are mostly distributed in the hydrophobic segments which are probably transmembrane regions. This implies that these regions may be important for its function of transporting phosphate into mitochondria.

PTP is present in mitochondria where energy is captured as ATP during oxidative phosphorylation for use in cell growth and development. PTP is responsible for the transport of inorganic phosphate into the mitochondrial matrix where phosphate is used for this process. Thus, the presence of PTP or PTP mRNA in insect tissues should reflect a state of active growth and development of insects requiring a high respiratory metabolic rate. We have examined the levels of the CfPTP mRNA in the spruce budworm throughout the development, from the embryonic to the pupal stages and in three diverse types of larval tissues, the epidermis, the fat body and the midgut. Although the 1.8 kb mRNA detected by CfPTP probe was present during most of the developmental stages except in the early pupal stages, the mRNA levels fluctuated with the developmental stages. The CfPTP mRNA levels started to increase soon after larval ecdysis and peaked at 24 hr after ecdysis. The relatively high levels of the mRNA were maintained throughout the feeding and growth periods of the intermoult phase following which the mRNA levels decreased prior to and during the moult periods. It stands to reason that when larvae are actively feeding and growing, they require more energy (ATP) and this is reflected in higher levels of expression of CfPTP gene. Increased expression of PTP would mean that higher levels of phosphate being transported into the mitochondria for use in ATP synthesis. This leads to the question as to what regulates the expression of CfPTP gene in the spruce budworm larvae. The fluctuation of CfPTP mRNA levels is similar to many of the larval intermoult genes of tobacco hornworm (*Manduca sexta*), which code for cuticular proteins and insecticyanin that are made during the intermoult feeding period (Riddiford et al., 1986; Rebers and Riddiford, 1988; Hiruma et al., 1991; Riddiford, 1991). These genes are also expressed during the feeding and growth periods, then are suppressed during the initiation of the moult prior to and during the formation of the epicuticle. It has been known that these genes are regulated by both ecdysteroid and juvenile hormone (Riddiford et al., 1986; Rebers and Riddiford, 1988; Hiruma et al., 1991; Riddiford, 1991). However, dose-response and time-course Northern blots for CfPTP mRNA from the spruce budworm midgut cells treated with either ecdysone (20-hydroxyecdysone, 20E) or ecdysone agonist (RH-5992) indicated that the CfPTP mRNA levels did not change in the presence of 20E or RH-5992 (data not shown). Thus, expression of the CfPTP mRNA in the spruce budworm is probably not regulated directly by 20E. Instead, expression of the

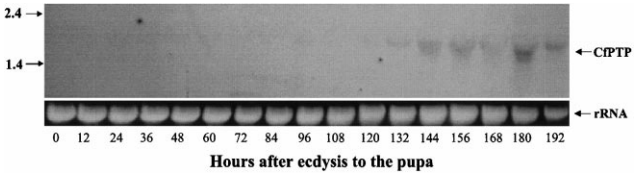

Fig. 8. Developmental expression of CfPTP mRNA during the pupal development. The top panel shows the Northern blot containing total RNA (10 μg) from pupa insects hybridized with the 1.8 kb CfPTP cDNA probe, and the bottom panel shows ribosomal RNA stained with ethidium bromide. RNA sizes (in kb) from an RNA ladder (GIBCO–BRL) are given on the left.

CfPTP gene may be growth-regulated, similar to the expression of the human AAC gene, which has been shown to be growth-regulated (Battini et al., 1987). The mRNA level of human AAC gene increased when quiescent cells were stimulated by either serum, platelet-derived growth factor, or epidermal growth factor. Furthermore, the cycle of expression of the CfPTP gene probably coincides with the cycle of mitochondrial maturation. During mitochondrial maturation in cockroaches, synthesis of mitochondrial proteins are regulated by hypertrehalosemic hormone (HTH) (Keeley et al., 1994) which is a small peptide that regulates heme synthesis for mitochondrial cytochromes and gene expression of cytochrome P450 during mitochondrial maturation. The decrease in CfPTP mRNA during molting periods coincides with an increase in ecdysteroids (Palli et al., 1995), as well as increase in mRNA levels of ecdysone receptor (Kothapalli et al., 1995) and ecdysone induced transcription factors, *Choristoneura* hormone receptor 3 (Palli et al., 1996) and *Choristoneura* hormone receptor 75 (Palli et al., 1997), indicating that ecdysteroids, may be involved in the suppression of CfPTP mRNA. The precise mechanism by which CfPTP mRNA levels are regulated is currently under investigation.

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