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Molecular Characterization of Prothoracicotropic Hormone (PTTH) from the Giant Silkmoth *Antheraea pernyi:* Developmental Appearance of PTTH-Expressing Cells and Relationship to Circadian Clock Cells in Central Brain

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Using a PCR strategy, we have cloned the cDNA for prothoracicotropic hormone (PTTH) from the giant silkmoth, *Antheraea pernyi*. The *A. pernyi* PTTH cDNA encodes a preprohormone of 221 amino acids that is 51 and 71% identical at the amino acid level with *Bombyx mori* and *Samia cynthia ricini* PTTHs, respectively. Bacterially expressed, recombinant *A. pernyi* PTTH stimulates adult development when injected into debrained pupae. PTTH protein (ca. 30 kDa by Western blot) and mRNA (ca. 0.9 kb by Northern blot) are expressed in brain. Immunocytochemistry and *in situ* hybridization show that PTTH protein and mRNA are colocalized in L-NSC III from Day 4 of embryogenesis through adult life, with little variation in either protein or mRNA levels at the various ecdyses. A pair of cells expressing immunoreactivity for the circadian clock protein PER is located in the same region as PTTH-expressing L-NSC III in *A. pernyi* brain. However, double-label immunocytochemical studies show that PTTH and PER are located in different cells. The close anatomical location between PTTH- and PER-expressing cells suggests routes of communication between these two cell populations that may be important for the circadian control of PTTH release.

INTRODUCTION

Prothoracicotropic hormone (PTTH) is a critical neurohormone regulating postembryonic development in insects. Based on studies in Lepidoptera, PTTH is produced by a pair of lateral neurosecretory cells (L-NSC III) in the protocerebrum (Kawakami *et al.,* 1990; Westbrook and Bollenbacher, 1990). After release from axonal terminals in the corpora allata, PTTH stimulates the prothoracic glands to synthesize and release ecdysone (Bollenbacher and Granger, 1985). Ecdysone, in turn, exerts a direct effect on target tissues, regulating growth, molting, and metamorphosis. Thus, PTTH, whose existence was first proposed in 1922 by Kopec, functions as a classical neuroendocrine-releasing factor.

It has only been within the past 6 years that peptide analysis and molecular cloning studies have elucidated the dimeric structure of PTTH from the commercial silkmoth *Bombyx mori* (Kawakami *et al.,* 1990; Kataoka *et al.,* 1991; Adachi-Yamada *et al.,* 1994; Ishibashi *et al.,* 1994). *Bombyx* PTTH is a 30-kDa glycoprotein consisting of two identical subunits linked by a disulfide bond. Each monomeric PTTH subunit is generated by proteolytic cleavage of a precursor molecule, PTTH preprohormone. Importantly, PTTH mRNA is expressed in L-NSC III in *Bombyx* brain, and bacterially expressed, recombinant PTTH from *Bombyx* initiates adult development in debrained pupae (Kawakami *et al.,* 1990). In spite of its essential function in insect development, PTTH has not yet been cloned and functionally characterized in other insects.

In the giant silkmoth, *Antheraea pernyi,* PTTH release is under the control of a circadian clock. This is strikingly evident for the photoperiodic termination of pupal diapause in this species (Williams and Adkisson, 1964a,b). Exposure of diapausing pupae to daylengths of greater than 13.5 hr of

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light per day stimulates PTTH release and subsequent adult development, while shorter photoperiods maintain the diapausing state in unchilled pupae. The photoreceptive mechanism and circadian clock that control diapause termination in *A. pernyi* are centered in the brain (Williams and Adkisson, 1964a,b). Moreover, it has been postulated that PTTH-containing cells and circadian clock cells are one in the same in lepidopteran brain (Williams, 1969).

Genetic analysis of neural circadian clocks in *Drosophila melanogaster* has identified two clock genes, *period* and *timeless*, that are essential elements of a circadian timing mechanism (for review, see Reppert and Sauman, 1995). A homolog of *period* was recently cloned from *A. pernyi* (Reppert *et al.*, 1994). By expressing the silkmoth *per* cDNA in *per*^{o} transgenic flies, it was shown that the silkmoth homolog can function as a circadian clock element in *Drosophila* (Levine *et al.*, 1995). Thus, *per* appears to be a circadian clock gene in moths, and, as such, it should be expressed in circadian clock cells in central brain.

Because of our interest in the circadian regulation of PTTH release in *A. pernyi*, we used sequence information from *Bombyx* PTTH, along with reverse transcription PCR (RT–PCR), to clone a functionally active PTTH from this giant silkmoth. We then localized the expression of PTTH protein and mRNA to L-NSC III in *A. pernyi* brain, examined the presence of PTTH-expressing cells from embryonic through adult development, and assessed the anatomical relationship between PTTH-expressing and *per* protein (PER)-expressing cells.

MATERIALS AND METHODS

Animals

Diapausing pupae of *A. pernyi* were purchased from Worldwide Butterflies Ltd. (Sherborne, England) under U.S. Department of Agriculture permit No. 929010. Cocoons were stored in darkness at 4°C until use. To terminate diapause and initiate adult development, pupae were removed from cocoons and placed in environmental compartments at 24°C with the daily lighting cycle consisting of 17 hr of light and 7 hr of darkness (17L:7D) (Williams and Adkisson, 1964b). Adult development was completed within 3–4 weeks, followed by adult eclosion. To facilitate oviposition, mating pairs were placed into paper bags, and the fertilized eggs were collected at the beginning of light phase for two consecutive days. Larvae were fed freshly cut oak leaves and reared under the same environmental conditions as above.

PCR

Poly(A)$^{+}$ RNA was isolated from brains of developing pupae, primed with oligo(dT), and reverse transcribed. For PCR with degenerate primers, the first-strand cDNA was subjected to 30 cycles of amplification with AmpliTaq DNA polymerase (Perkin–Elmer Cetus) and two oligonucleotide primers (each at final concentration of 200 n*M*). Each reaction cycle consisted of 45 sec at 94°C, 2 min at 45°C, and 2 min at 72°C. For PCR with specific primers, cDNA was amplified through 35 cycles, each consisting of 45 sec at 94°C, 45 sec at 60°C, and 2 min at 72°C. The amplified DNA was separated on an agarose gel and paper-eluted as previously described. The DNA was then subcloned into pCR II using a TA Cloning Kit (Invitrogen), pBluescript (Stratagene), or pGEX-4T-1 (Pharmacia).

Rapid Amplification of cDNA Ends (RACE)

5′-RACE was performed using the 5′-AmpliFINDER RACE Kit (Clontech). First-strand cDNA was generated by reverse transcription of poly(A)$^{+}$ RNA from pupal brains using primer P3 (5′-CGT ACG GAA AGA GAC ACG ATG ATT GTC G-3′) (Fig. 1A). The AmpliFINDER anchor was ligated to the cDNA ends using T4 RNA ligase. The cDNA was then amplified using an anchor primer (complementary to the AmpliFINDER anchor) and primer P4 (5′-GAC ATG TCT CGG GAA AGC ATT CTC GCC-3′) nested immediately upstream of P3 (Fig. 1A). The amplified cDNA was separated on an agarose gel. A single band was paper eluted, subcloned into pCRII, and sequenced.

3′-RACE was performed using the 3′-RACE System (GIBCO/BRL). First-strand cDNA was generated using an Adapter Primer which is an oligo(dT) primer engineered to contain three restriction endonuclease sites at the 5′ end. The cDNA was then amplified by PCR using an Anchor Primer complementary to the specific 5′ sequence of the Adapter Primer and primer P5 (5′-CGA CAA TCA TCG TGT CTC TTT CCG TAC G-3′) (Fig. 1A). The cDNA was separated on an agarose gel. A single band was paper eluted, subcloned into pCRII, and sequenced.

Antibody Production and Affinity Purification

Polyclonal antiserum was generated against a synthetic peptide corresponding to a fragment of the predicted amino acid sequence of *A. pernyi* PTTH (Fig. 1B). The sequence of the 21-residue peptide (TNETVDFGENAFPRHVESRNC), designated PTT-1, was selected based on the Antigenicity Index of Jameson-Wolfe (GCG program). PTT-1 was conjugated to keyhole limpet hemocyanin and injected with Freund's incomplete adjuvant into two rabbits (HRP, Inc.). The antiserum was subjected to immunoaffinity chromatography with the PTT-1 peptide immobilized on the SulfoLink affinity column (Pierce). The specificity of the affinity-purified antibody (274/IV-A) was tested by immunocytochemistry and Western blotting (see results).

Western Analysis

Proteins for Western blotting were extracted from tissues by homogenization in phosphate-buffered saline (PBS) followed by centrifugation (16,000*g* for 20 min at 4°C) to eliminate cellular debris. An aliquot from each sample was removed for protein quantification using the Bradford assay (Bio-Rad). Protein extracts were mixed with SDS–PAGE sample buffer (final concentration, 1% β-mercaptoethanol), boiled for 10 min, and immediately loaded on the gel. An equal amount of protein from each tissue was subjected to 12% SDS–PAGE followed by semidry electroblotting (Millipore) transfer to a nitrocellulose membrane. Membranes with transferred proteins were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; 1 hr at room temperature [RT]), and incubated with primary anti-PTTH antibody (1:10,000 dilution in TBS supplemented with 0.05% Tween 20, 1% bovine serum albumin, TBS–TB) with gentle agitation overnight at 4°C. Following washing with TBS–TB (3× 15 min at RT), the membranes were incubated with

goat anti-rabbit IgG–alkaline phosphatase (AP)-conjugated secondary antibody (1:20,000 in TBS–TB, 1–2 hr at RT) and washed thoroughly with TBS–TB (3× 15 min at RT). Following the final wash, the blots were equilibrated in AP detection buffer (0.1 *M* Tris·Cl, pH 9.5, 0.1 *M* NaCl, 50 m*M* $MgCl_2$) for 10 min at RT. The AP enzymatic activity was visualized by colorimetric reaction using the bromochloroindolyl phosphate and nitroblue tetrazolium (BCIP/NBT) substrate system.

Northern Analysis

Approximately 1.5 μg of poly(A)$^+$ RNA from *A. pernyi* pupal brains was subjected to electrophoresis through a 1% agarose–formaldehyde gel. The separated RNA was transferred onto a GeneScreen Plus nylon membrane (New England Nuclear) and hybridized with ^{32}P-labeled PTTH probe (nt 376–699 of the PTTH cDNA; sp act $>10^9$ cpm/μg). The hybridization cocktail consisted of 50% formamide, 1 m*M* NaCl, 1% SDS, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA. The membranes were hybridized with gentle shaking overnight at 45°C. The final washing step was in 2× SSC, 1% SDS at 65°C for 1 hr. Membranes were exposed to X-ray film with an intensifying screen at −80°C for 1–3 days.

Expression and Purification of Recombinant PTTH

The recombinant *A. pernyi* PTTH was expressed and purified using the glutathione *S*-transferase (GST) Gene Fusion System (Pharmacia Biotech). The cDNA fragment corresponding to the active PTTH hormone was amplified by PCR and subcloned into pGEX-4T-1 (Pharmacia Biotech) in-frame and downstream from the sequence coding for GST and a thrombin proteolytic cleavage signal. JM101 *Escherichia coli* cells were transformed with the pGEX–PTTH construct, and the expression of GST–PTTH fusion protein was induced with IPTG (final concentration 0.1 m*M*) for 5 hr at RT. The fusion protein was purified on glutathione Sepharose 4B affinity chromatography columns followed by thrombin digestion according to the manufacturer's protocol. Aliquots of the samples were removed from each extraction and purification step for subsequent SDS–PAGE and Western blot analysis. The expected molecular weight and purity of the eluted recombinant PTTH after proteolytic cleavage was confirmed by Western analysis with the anti-PTTH antibody. The final protein concentration was determined by the Bradford method.

PTTH in Vivo Bioassay

Both chilled and unchilled diapausing pupae of *A. pernyi* were used to test the biological activity of purified recombinant PTTH. The pupae were surgically debrained under sterile conditions and injected with 5, 25, or 125 ng of recombinant PTTH in 10 μl of sterile insect Ringer's solution. Control animals were injected with the same volume of the vehicle solution. Sham-operated pupae in which the brain was surgically removed and put back in the head served as an additional control. Eight to 10 animals were used for each experimental condition; at least 6 pupae were used for each control injection or sham procedure. After injections, pupae were placed in environmental compartments at 24°C under either short-day (12L:12D) or long-day (17L:7D) lighting conditions. The pupae were monitored daily for adult development and eclosion. After 5 weeks, the remaining pupae were water anesthetized, surgically open, and examined for signs of adult development.

Immunocytochemistry

Brains of fifth instar larvae, pupae, and adults were dissected from CO_2-anesthetized animals under sterile Ringer solution and immediately fixed in modified Bouin–Hollande solution (Levine *et al.*, 1995) overnight at 4°C. Developing embryos, starting 24 hr postoviposition, were manually removed from the chorion, surrounding yolk, and extraembryonic membranes at 12 hr intervals, and fixed as above. Standard histological techniques were employed for tissue dehydration, embedding in paraplast, sectioning (4–7 μm), deparaffinization, and rehydration. To remove residual heavy metal ions from the fixed tissue, the sections were treated with Lugol's iodine followed by 5% sodium thiosulfate. After thorough washing with distilled water and PBS supplemented with 0.2% Tween 20 and 0.1% bovine serum albumin (PBS–TB), the sections were blocked with 10% normal goat serum in PBS–TB (30 min at RT), and incubated with affinity-purified *A. pernyi* anti-PTTH antibody (1:4000 dilution in PBS–TB) in a humidified chamber overnight at 4°C. Following rinsing with PBS–TB (3× 10 min at RT), samples were incubated with goat anti-rabbit IgG–horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch; 1:1000 in PBS–TB, 1 hr at RT). The HRP enzymatic activity was stained with hydrogen peroxide (0.005%) and 3,3′-diaminobenzidine·4HCl (0.25 m*M* in 0.05 *M* Tris·Cl, pH 7.5) as chromogen. Stained sections were dehydrated and mounted in AccuMount-60 mounting medium.

For double-labeling experiments, the primary antibodies (rabbit anti-PTTH and rat anti-PER) as well as the corresponding secondary antibodies (goat anti-rabbit IgG–alkaline phosphatase, AP, and goat anti-rat IgG–HRP) were combined and applied to the brain sections under the same conditions as described above for single antibody labeling. The AP activity was visualized using naphthol AS-MX phosphate (0.24 m*M*) and fast blue BB salt (0.5 m*M*) substrate/chromogen system supplemented with levamisole (3 m*M*). Stained slides were mounted in aqueous chrome–glycerin–gelatin mounting medium. The rat antibody against *Drosophila* recombinant PER protein (Liu *et al.*, 1991) employed here in the double-labeling experiments exhibited virtually identical staining pattern with a rabbit antiserum directed against synthetic peptide based on the silkmoth PER amino acid sequence (not shown).

In control experiments, the primary antibodies were replaced with normal goat serum. As an additional control for binding specificity, the anti-PTTH antibody was preincubated with 100 molar excess of antigen prior to immunological staining. In both cases, no significant staining was observed above background.

Brain Wholemount Immunofluorescence

Larval, pupal, and imaginal brains were dissected as described above for immunocytochemistry, and fixed immediately in aqueous Bouin fixative for 2–4 hr at 4°C. Samples were rinsed briefly in PBS and the neurilemma was manually removed under a dissecting microscope. Brains were then permeabilized in PBS containing 2% Tween 20 (PBS–Tw) with several changes overnight at 4°C. Following blocking with 20% normal goat serum in PBS–Tw (2 hr at RT), brains were incubated with the primary anti-PTTH antibody (1:1000 in PBS–Tw) for 24–48 hr at 4°C. After thorough washing with PBS–Tw (3× 20 min at RT), samples were incubated with

Cy3 fluorophore-conjugated goat anti-rabbit IgG secondary antibody (Jackson ImmunoResearch; 1:500 in PBS–Tw, 2 hr at RT), rinsed thoroughly in PBS–Tw (3× 30 min at RT), mounted in 75% glycerol, and viewed under a Nikon microscope equipped with Nomarski optics and epifluorescence.

In Situ Hybridization

Digoxigenin-labeled antisense probe and sense (control) probe were generated by subcloning the 5′-RACE-amplified PTTH cDNA fragment into pBluescript followed by *in vitro* transcription in the presence of digoxigenin–UTP driven from Sp6 and T7 RNA polymerase promoters, respectively. The efficiency of digoxigenin incorporation and sensitivity of the labeled probes were assessed by detection on nylon membranes.

Dissected brains and developing embryos of *A. pernyi* were fixed in freshly made paraformaldehyde solution (5% in 0.1 *M* sodium phosphate buffer, pH 7.5) for 2–6 hr at RT or overnight at 4°C. Samples were washed thoroughly with the same buffer and processed for sectioning as described above for immunocytochemistry, but omitting the Lugol's iodine and sodium thiosulfate treatments. Rehydrated sections on Vectabond (Vector Labs)-coated slides were treated with 0.2 *N* HCl (20 min at RT), acetylated with 0.25% acetic anhydride in 0.1 *M* triethanolamine, dehydrated, and hybridized with digoxigenin-labeled RNA probes in a hybridization cocktail (final concentration 0.5 ng/μl) overnight at 55°C. The hybridization solution consisted of 50% deionized formamide, 10% dextran sulfate, 2× Denhart's solution, 50 m*M* sodium phosphate buffer, pH 7.0, 2× SSC, 0.1% SDS, 5 m*M* EDTA, 0.1% sodium pyrophosphate, 500 μg/ml sonicated salmon sperm DNA, 500 μg/ml yeast tRNA.

Following hybridization, the sections were washed with 2× SSC supplemented with 0.05% sodium pyrophosphate and 1 m*M* EDTA (SSC–NE; 2× 30 min at RT), digested with RNase A (10 μg/ml in 10 m*M* Tris·Cl, pH 8.0, 0.5 *M* NaCl), and rinsed again with 2× SSC–NE (30 min at RT). The final washes were done with 0.1× SSC–NE (2× 30 min at 55°C, and 2× 20 min at RT). The immunocytochemical detection of hybridized probes with Fab fragments of sheep anti-digoxigenin antibody directly conjugated to AP (Boehringer and Mannheim; 1:500 dilution in PBS–TB) was performed under virtually the same conditions as described above for immunocytochemistry. The AP activity was detected with the BCIP/NBT substrate system. Dehydrated slides were mounted in Accu-Mount-60 mounting medium.

For double-labeling experiments with anti-PTTH antibody, the hybridized brain sections, following the AP staining, were washed thoroughly in PBS–TB, blocked with 10% normal goat serum in PBS–TB (30 min at RT), and incubated with primary anti-PTTH antibody (1:2000 in PBS–TB) overnight at 4°C. Slides were then washed with PBS–TB (3× 10 min) and the binding of the primary antibody was detected with goat anti-rabbit IgG–Cy3-conjugated secondary antibody (1:500 in PBS–TB, 1 hr at RT).

DNA Sequencing

Nucleotide sequences were determined using the dideoxynucleotide chain termination method using Sequenase II (United States Biochemical). Sequencing template was double-stranded plasmid. Primers were synthetic oligonucleotides, either vector specific or derived from sequence information.

Computer Sequence Analysis

All DNA and protein analyses were performed using the Wisconsin Package software of the Genetics Computer Group (1991), Program Manual for the GCG Package, Version 7 (Madison, WI).

RESULTS

Cloning of PTTH from A. pernyi

A fragment of putative PTTH was isolated from mRNA obtained from developing pupal brains by RT–PCR. Degenerate primers used for PCR amplification were designed based on amino acid residues conserved between *Bombyx* PTTH (Kawakami *et al.*, 1990; Kataoka *et al.*, 1991) and *Samia cynthia ricini* putative PTTH (Kataoka and Suzuki, 1995; GenBank Accession No. L25668); *S. c. ricini* PTTH has not been functionally characterized. One pair of primers, corresponding to the peptide sequences 5′ AI(P/Q)DPPC (amino acid residues 124–130 of *Bombyx* PTTH preprohormone) and 3′ WR(Y/F)KLE (residues 195–200), amplified a 195-bp cDNA fragment, designated BL-12, that shows 50 and 70% amino acid identity with the corresponding regions of *Bombyx* PTTH and *S. c. ricini* PTTH, respectively.

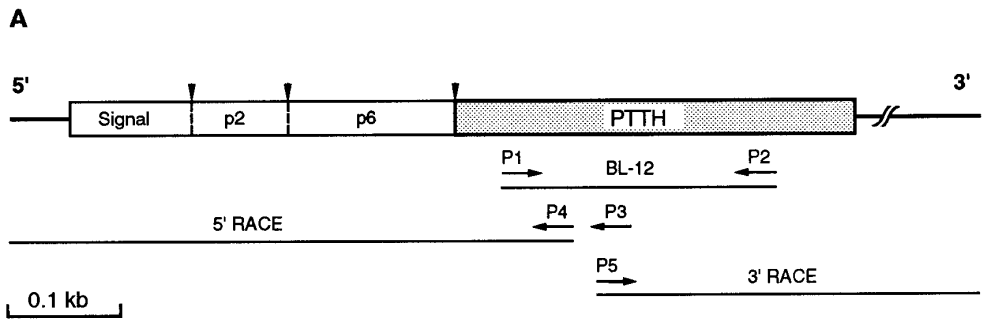
5′-RACE PCR delineated the 5′ portion of the *A. pernyi* PTTH cDNA. The specific primer for reverse transcription, as well as the nested primer for PCR amplification, were based on the sequence of the original BL-12 clone (Fig. 1A). A 474-bp fragment (designated 5′-PTTH) was amplified and subcloned. Sequence analysis confirmed that the 3′ end of the 5′-PTTH clone overlaps with the 5′ end of the BL-12 clone, indicating that both clones are fragments of the same transcript.

The 3′ region was determined by 3′-RACE using one round of PCR amplification. Sequence analysis of 3′-RACE clones revealed a 406-bp fragment (designated 3′-PTTH) overlapping at its 5′ end with the original BL-12 clone (Fig. 1A). The three overlapping clones (5′-PTTH, BL-12, and 3′-PTTH) define the cDNA (903 bp) of the *A. pernyi* putative PTTH preprohormone (Fig. 1B).

PTTH Structure

The PTTH cDNA contains an open reading frame encoding a protein of 221 amino acids. Computer search of the GenBank database revealed that the predicted protein encoded by the silkmoth cDNA has the highest homology with putative *S. c. ricini* PTTH preprohormone, followed by the PTTH precursor of *Bombyx*. Using the BestFit program, the overall identity of the *A. pernyi* protein with the PTTH preprohormones of the two other moth species is 71% (vs *S. c. ricini*) and 52% (vs *Bombyx*) (Fig. 1C). When only the active hormonal subunits are compared, the amino acid identity is 68 and 51%, respectively.

A proteolytic cleavage signal immediately preceding the hormonal portion of the PTTH preprohormone is conserved among all three silkmoth species (Fig. 1C). Unlike the *Bom-*



B

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                                                                                ggtagtctatcctggaggttcaaacaacacata      0

ATGATATCAAGATCTATCGTTATATTACTAGCATGCTCGGGGGTGTTGATTATAATGGAAGCACTAATGCCTAGAACAATGGCAATGAAA    90
M  I  S  R  S  I  V  I  L  L  A  C  S  G  V  L  I  I  M  E  A  L  M  P  R  T  M  A  M  K     30

AGTACTAGAAACATACATGAATTCATGATTGAAGATCAAAGGACACGCAAGAAACACAATTACATGTTTCAAAGAGATAGGAACAACGAT   180
S  T  R  N  I  H  E  F  M  I  E  D  Q  R  T  R  K  K  H  N  Y  M  F  Q  R  D  R  N  N  D     60

ATTTTACGGAATAAAAATAATGGTCTGATGTATGATATGGAATCTTTAGAAATAGATTCAAGCCCTGAAGATTATTCAAACCTCGTGATG   270
I  L  R  N  K  N  N  G  L  M  Y  D  M  E  S  L  E  I  D  S  S  P  E  D  Y  S  N  L  V  M     90

GATTATGCAAACATGAAAAAGAACGATGTCTTTTTATTGGATAATTCCATAGAAACTAGAACGCGAAAAAGAGGCAATATTAAGCGTCAA   360
D  Y  A  N  M  K  K  N  D  V  F  L  L  D  N  S  I  E  T  R  T  R  K  R  G  N  I  K  R  Q     120

AACATTCCTGATCCGCCTTGCAGCTGTGAGTATACAAATGAAACTGTAGATTTCGGCGAGAATGCTTTCCCGAGACATGTCGAATCTAGA   450
N  I  P  D  P  P  C  S  C  E  Y  T  N  E  T  V  D  F  G  E  N  A  F  P  R  H  V  E  S  R     150

AATTGTAGTGAGTTACGACAATCATCGTGTCTCTTTCCGTACGTTTGCAAGGAAACCTTATATGATATAAGCGTTTTAAAACGAAGACAA   540
N  C  S  E  L  R  Q  S  S  C  L  F  P  Y  V  C  K  E  T  L  Y  D  I  S  V  L  K  R  R  Q     180

TCAACGACGCAACCTTCTGAAAAAGTACCAAACGAATTGAAGTTTAGGTGGATTGCTGAAAAATGGCAGATCAGTGTTGGATGTGTATGC   630
S  T  T  Q  P  S  E  K  V  P  N  E  L  K  F  R  W  I  A  E  K  W  Q  I  S  V  G  C  V  C     210

ACCAGGGACTACCGAGATACTATTAATCAAGATTAAaacccaaaattattcagacgattaattaataattataatgtatcatgaaattct   720
T  R  D  Y  R  D  T  I  N  Q  D  *                                                            221

aacaatattgaagattacaaatatgatactaatgctttgtttttggtagctgatattttacttaattaatgaaacatgtaaaaagtaaat   810
gaataatatatttgacttatgaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa                            871
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C

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p  MISRSIVILL ACSGVLIIME ALMPRTMAMK STRNIHEFMI EDQRTRKKHN YMFQRDRNND ILRNKNN.GL MYDMESLEID     79
r  MISRSIVILL VCIGALIIIQ SLMPKTMAMR NTRNIDEFMI EDQRTRKKHN YVLQRPRNNE LLRKKNY.DL MYNMEASDLD     79
m  MITRPIILVI LCYAILMIVQ SFVPKAVALK RKPDVGGFMV EDQRTHKSHN YMMKRARNDV LGDKENVRPN PYYTEPFDPD     80

c  MI-R-IV--- -C---L-I-- -L-P---AM- ----I--FMI EDQRT-K-HN Y---R-RN-- -----N---- -Y--E--D-D

p  SSPEDYSNLV MDYANMKKND VFLLDNSIET RTRKRGNIKR ....QNIPDP PCSCEYTNET VDFGENAFPR HVESRNCSEL    155
r  SNPEEFSNLL LDYDNMKKNN VVLLDNSIET RTRKRGDLRR EKHNQAIQDP PCSCGYTQTL LDFGKNAFPR HVVTRNCSD.    158
m  TSPEELSALI VDYANMIRND VILLDNSVET RTRKRGNIQV E..NQAIPDP PCTCKYKKEI EDLGENSVPR FIETRNCNKT    158

c  --PEE-S-L- -DY-NM--N- V-LLDNSIET RTRKRG---- ----Q-I-DP PC-C-Y---- -DFG-N--PR -V--RNC---

p  RQSSCLFPYV CKETLYDISV LKRRQSTTQP SEKVPNELKF RWIAEKWQIS VGCVCTRDYR DTINQD.... .......... .  221
r  QQQSCLFPYV CKETLYDVNI LKRRETSTQI SEEVPRELKF RWIGEKWQIS VGCMCTRDYR NSTEDYQPRL LTKIIQQRDL S  239
m  QQPTCRPPYI CKESLYSITI LKRRETKSQE SLEIPNELKY RWVAESHPVS VACLCTRDYQ LRYNNN.... .......... .  224

c  -Q--C--PYV CKE-LY-I-I LKRR----Q- S--VP-ELKF RWI-E---IS V-C-CTRDY- ---------- ---------- -
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FIG. 1. Molecular cloning of *A. pernyi* PTTH. (A) PCR-based cloning strategy. The top bar depicts the structure of *Bombyx* PTTH preprohormone. The parts of the cDNA corresponding to the signal, p2, p6, and PTTH peptides are represented by boxes. The proteolytic cleavage sites are marked by arrowheads. Degenerate primers P1 and P2 (arrows) based on *Bombyx* PTTH amino acid sequences successfully amplified a 195-bp fragment (BL-12) of a PTTH homolog from *A. pernyi* pupal brain mRNA. The 5′ end of the *A. pernyi* cDNA was obtained by 5′-RACE. The 3′ end was cloned by 3′-RACE. (B) Nucleotide sequence of *A. pernyi* PTTH cDNA with the deduced amino

byx PTTH precursor, which has two additional putative proteolytic signals, both the *A. pernyi* and *S. c. ricini* precursors have only one additional cleavage site (Fig. 1c). Importantly, the seven cysteine residues in the sequence of the active hormone subunit, the first of which is necessary for the disulfide linkage between monomers and the next six which are important for intrachain disulfide linkages, are all conserved among the proteins of the three silkmoths. Sequence analysis of the *A. pernyi* PTTH also revealed two consensus sequences for N-linked glycosylation. The same number of glycosylation sites is found in *S. c. ricini* PTTH, but only one putative glycosylation site is found in *Bombyx* PTTH (Fig. 1C).

Expression and Purification of Recombinant A. pernyi PTTH in E. coli

To obtain purified *A. pernyi* PTTH for subsequent testing of its prothoracicotropic activity, we expressed the recombinant protein (active hormone subunit) in bacterial cells followed by affinity chromatography purification. Western analysis using the anti-PTTH antibody (274/IV-A) revealed a major PTTH-immunoreactive band with an apparent molecular weight between 41 and 43 kDa which corresponds to the expected molecular mass of the GST–PTTH fusion protein (40.9 kDa)(Fig. 2A). The additional bands with lower molecular weight most likely represent degradation products of the full-length fusion protein. Alternatively, they might be due to incomplete transcription and/or translation of the GST–PTTH gene construct. Control cells transformed with the pGEX plasmid lacking the PTTH insert expressed the GST protein, but their lysates failed to generate any signal on Western blots using the anti-PTTH antibody (not shown). The GST–PTTH fusion protein was purified from the cell lysates on glutathione Sepharose 4B affinity chromatography columns, and the PTTH portion was liberated from the fusion protein by thrombin proteolytic cleavage. The purified recombinant silkmoth active PTTH subunit exhibited a molecular weight of about 12,000 Da, comparable to the expected molecular mass of 11,770 Da (Fig. 2A).

Recombinant PTTH Exhibits Prothoracicotropic Activity in a Pupal Bioassay

The purified recombinant PTTH was tested for its biological activity using a standard pupal bioassay (Ishizaki and Suzuki, 1980; Ishizaki *et al.*, 1983). Diapausing pupae of *A. pernyi* were surgically debrained and injected with the recombinant protein. The treated pupae were exposed to either short-day (12L:12D) or long-day (17L:7D) conditions. In both instances, most of the pupae injected with recombinant PTTH underwent metamorphosis and completed adult development within 3–4 weeks after injection, followed by adult eclosion (Fig. 2B). In contrast to PTTH-injected animals, the control debrained pupae injected with vehicle remained in diapause and did not exhibit any morphological signs of adult development when examined 5 weeks after the injection. In sham-operated animals, only pupae maintained under long-day conditions (17L:7D) underwent metamorphosis and subsequent adult eclosion (Fig. 2B); this is the same as that expected for unoperated animals housed in long days (Williams and Adkisson, 1964b). These results clearly indicate that the cloned *A. pernyi* PTTH cDNA encodes a functional hormone.

PTTH Protein and mRNA Are Expressed in Brain

The anti-PTTH antibody was also used to examine protein extracts from several different tissues and developmental stages of *A. pernyi* (Fig. 3A). Under mild reducing conditions (1% β-mercaptoethanol), two distinct bands of estimated molecular weights of ca. 30 and 60 kDa were detected in both homogenates of adult heads and hemolymph. As expected, no signal was observed in protein extracts from indirect flight muscles (Fig. 3A) and nonbrain nervous tissue (ventral nervous cord including suboesophageal ganglion; data not shown). In contrast to the adult head homogenates and hemolymph, a single immunoreactive band at ca. 30 kDa was obtained with the protein preparations from the dissected brains of the last larval instar, developing pupae, and adults (Fig. 3A). When strong reducing conditions were used (10% β-mercaptoethanol) and the brain protein concentration was diluted 1:5, distinct bands of equal intensity were detected at 30 and 15 kDa (data not shown); under nonreducing conditions, only the 30-kDa band was detected for brain protein extracts (of similar dilution) run on the same gel. This result strongly suggests that the 30-kDa band is the dimeric form of PTTH and the 15-kDa band is the monomeric form. The result also shows that the homodimer is very stable (see also Ishibashi *et al.*, 1994). The difference in molecular weight between the recombinant protein (12 kDa) and the endogenous mono-

acid sequence of the PTTH preprohormone. The nucleotide and the amino acid sequences are numbered from the initial methionine, as indicated to the right of each line. The proteolytic cleavage signals are double underline. The shaded boxed area represents the putative active hormone sequence. The PCR primers used to clone the original cDNA fragment (BL-12) are shown by arrows. The sequence of the 21-residue synthetic peptide used for antibody generation is shown as the open box. The asterix marks the stop codon. The cDNA sequence has been deposited in GenBank under Accession No. U62535. (C) Sequence alignment of the PTTH preprohormone proteins from three silkmoth species, *Antheraea pernyi* (*p*), *Samia cynthia ricini* (*r*), and *Bombyx mori* (*m*). To maximize homologies, gaps represented by dots have been introduced in the sequences. The putative proteolytic cleavage signals are indicated by open boxes. The active hormone portions of the PTTH precursors are printed in bold. The consensus sequences for N-glycosylation of an asparaginyl residue are underlined.

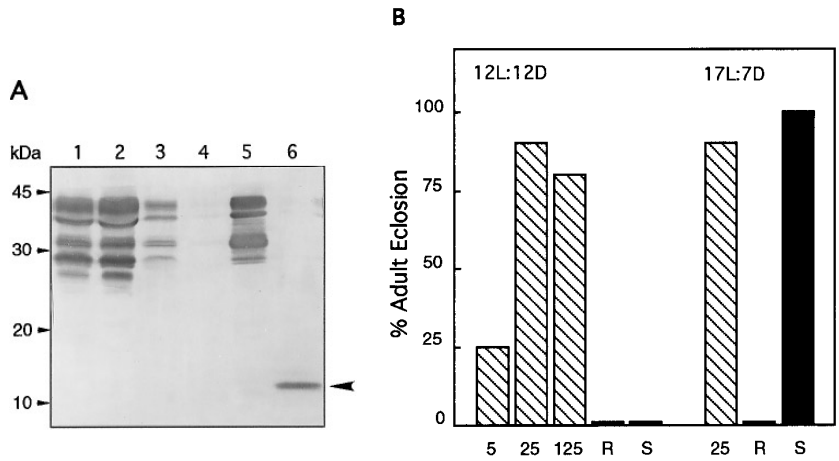


FIG. 2. Expression, purification and functional activity of recombinant *A. pernyi* PTTH. (A) Expression and purification of recombinant pernyi PTTH in bacteria. The immunoblot, probed with the 274/IV-4 antibody against *A. pernyi* PTTH, illustrates the expression and purification of recombinant *A. pernyi* PTTH peptide in JM101 *E. coli*. Lane 1, cell homogenate; 2, cleared sonicate; 3, flowthrough following binding to a glutathione Sepharose 4B column; 4, first wash of the fusion protein bound to the affinity column; 5, eluate from the glutathione Sepharose 4B; 6, purified PTTH after thrombin digest of the GST–PTTH fusion protein. (B) Recombinant PTTH exhibits prothoracicotropic activity in a pupal bioassay. Debrained diapausing pupae of *A. pernyi* were injected with the purified recombinant PTTH and placed in environmental compartments maintained at 24°C in either short or long light–dark cycles as indicated at the top of the diagram. The dose of PTTH injected (ng of protein per pupa) is indicated beneath each column. R, control debrained pupae injected with vehicle (insect Ringer's solution); S, sham operation without any injection.

mer (15 kDa) probably reflects glycosylation of the endogenous protein.

We performed Northern analysis to get an estimate of the size of the endogenous PTTH transcript. Hybridization of pupal brain poly(A)$^+$ RNA with a ^{32}P-labeled cDNA probe corresponding to the entire coding region of the active protein subunit of the *A. pernyi* PTTH cDNA (nt 343–666; Fig. 1B) revealed a single distinct band (Fig. 3B). The size of the hybridized transcript was 0.9 kb, the same as that of the cloned *A. pernyi* PTTH cDNA (903 bp). Thus, the cloned cDNA may represent the full-length mRNA of *A. pernyi* PTTH preprohormone.

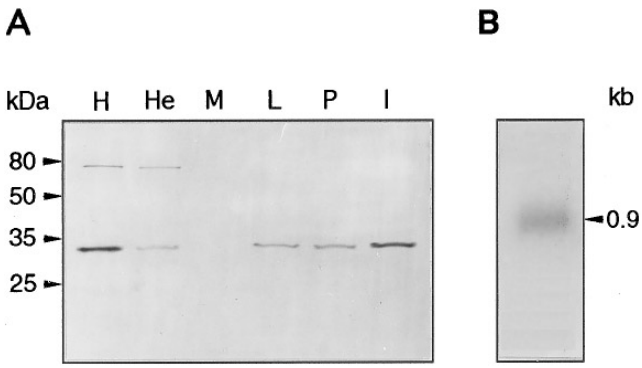


FIG. 3. PTTH protein and mRNA are expressed in brain. (A) Western blot analysis of *A. pernyi* protein extracts with the 274/IV-A antibody against *A. pernyi* PTTH. Equal amounts of protein from several different tissues and developmental stages of *A. pernyi* were loaded in each lane. H, adult heads; He, adult hemolymph; M, indirect flight muscles; L, P, I, larval, pupal, and adult central brain, respectively. (B) Northern blot of PTTH transcripts in *A. pernyi* brain shows a single band at 0.9 kb. Approximately 1.5 μg of poly(A)$^+$ RNA isolated from pupal brains was loaded.

PTTH Protein and mRNA Are Colocalized in L-NSC III

The 274/IV-4 anti-PTTH antibody stained two pairs of L-NSC III (Fig. 4A). The PTTH-like immunoreactive neurons of *A. pernyi* are contralateral, sending their axonal projections to the nervi corporis cardiaci on the opposite side, similar to that reported for *Bombyx* PTTH neurons. Identical to the antibody staining, *in situ* hybridization with a specific digoxigenin-labeled cRNA probe corresponding to the 5′-PTTH cDNA clone (Fig. 1A) also revealed a hybridization signal restricted to two pairs of L-NSC III in adult *A. pernyi* brain (Fig. 4B).

To determine whether the two pairs of neurons detected by immunolabeling are identical to the cells visualized by *in situ* hybridization, we performed double-labeling experiment with both the anti-PTTH antibody and specific PTTH cRNA probe. Two neurons in each brain hemisphere as well as their axonal projections were clearly demarcated by immunofluorescence staining for PTTH (Fig. 4C). The same two cells also exhibited a specific hybridization signal with

the digoxigenin PTTH antisense cRNA probe (Fig. 4D). In contrast to antibody labeling, no hybridization signal was detected in the axons of the stained cells.

PTTH Protein and mRNA Are Expressed in L-NSC III throughout Most of A. pernyi Development

We next examined the expression of the *A. pernyi* PTTH gene at several developmental stages (n = 2–6 animals/stage). PTTH immunoreactivity was first detected in two pairs of dorsolateral brain neurons at Day 3 of embryogenesis; embryogenesis lasts 11 to 12 days in *A. pernyi* (unpublished). The following day (Embryonic Day 4), the staining intensity increased significantly and remained at relatively high levels throughout embryonic life (Fig. 5A). Similarly, the PTTH transcript was detected as a discrete signal in the same neurons from Day 4 without any subsequent fluctuations (Fig. 5B).

During larval development, both PTTH protein and mRNA were detectable immediately following egg hatching with no apparent decrease in staining intensity (Figs. 5C and 5D), and remained relatively constant during all subsequent larval instars, including the wandering and prepupal stages of the fifth instar (Figs. 5E and 5F) (examined daily through the fifth instar and pupal ecdysis; data not shown).

Surprisingly, both peptide and PTTH mRNA were detected in the two pairs of dorsolateral neurosecretory cells in the brains of diapausing pupae, maintained in constant darkness at 4°C for a period of 8 weeks (Figs. 6A and 6B). The L-NSC III of the diapausing pupae exhibited relatively strong cytoplasmic immunolabeling. However, virtually no PTTH was detected in their axonal projections (Fig. 6A) which contrasts dramatically with the strong axonal staining of the same cells in developing pupal brain (see below). On the other hand, the PTTH mRNA levels, of diapausing pupae appeared to be substantially lower compared to nondiapausing conditions, but were still clearly detectable in the two pairs of dorsolateral neurons (Fig. 6B). In the case of nondiapausing pupae examined at 12-hr intervals through adult ecdysis (n = 2 animals/12-hr interval), robust antibody staining was observed in L-NSC III and their axonal projections from the beginning of pupal development (Fig. 6C), throughout metamorphosis, and in pharate adults (data not shown). Similarly, PTTH mRNA was readily detected in the same cells during all stages of pupal development (Fig. 6D). Aside from the diapausing condition, no dramatic changes in the levels of either PTTH protein or mRNA were detected during the entire pupal stage. Following adult eclosion, both PTTH protein and mRNA remained in L-NSC III at relatively high levels during the entire imaginal stage (Figs. 4A–4D).

PTTH and PER Are Expressed in Different Cells

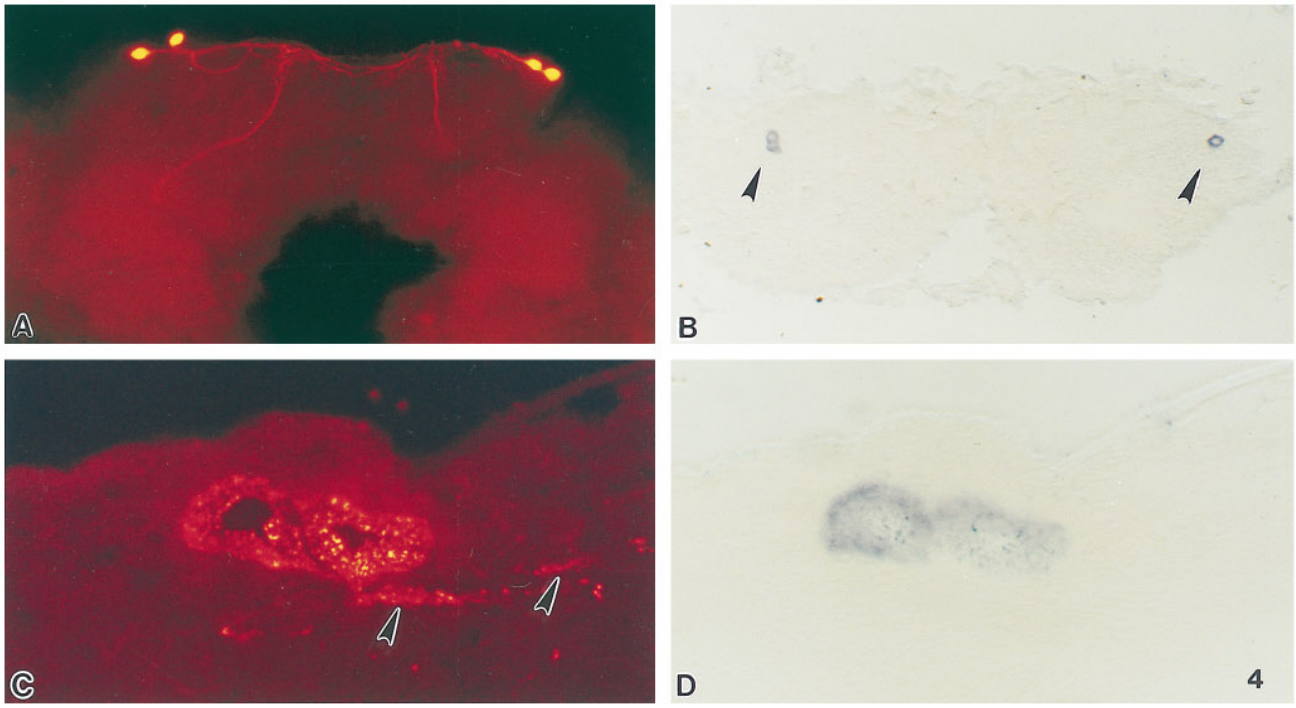
We also examined the anatomical relationship between PTTH-expressing cells and PER-expressing cells in *A. pernyi* brain. This was of interest because PER-expressing cells in *A. pernyi* brain should be circadian pacemakers cells. Thus, an association between circadian clock cells and PTTH-producing cells, whose release is under circadian control, could be examined.

Regardless of the time of day examined, the anti-PER antibody specifically labeled two pairs of dorsolateral neurons in each brain hemisphere, one pair being localized more lateral and the other more medial (data not shown, and see below). The lateral PER-positive neurons are found in the same region of adult *A. pernyi* central brain as the PTTH-producing cells, suggesting that the two peptides are coexpressed. However, double-label immunocytochemical studies showed that PTTH and PER are not expressed in the same cells (Fig. 7A), but that the cell bodies of PTTH and PER neurons are touching each other (Fig. 7B). Interestingly, although the PER immunoreactive cells reside in close vicinity to the contralateral PTTH neurosecretory cells, they send their axonal projections ipsilaterally to the nervi corporis cardiaci (Fig. 7C).

To further show that the two cell populations (PTTH and PER) do not partially overlap, the colorimetric reactions for alkaline phosphatase and horseradish peroxidase were developed sequentially and the number of positive cells throughout the entire brain was determined after each reaction. The resulting numbers obtained with the respective enzymes used in the double labeling experiment (n = 4 PTTH cells; n = 8 PER cells) were the same as the total cell numbers obtained in parallel single labeling experiments for each of the respective antibodies (anti-PTTH and anti-PER). In addition, double immunofluorescent labeling experiments using secondary antibodies coupled to Texas red

FIG. 4. PTTH protein and mRNA are colocalized in L-NSC III. (A) Wholemount immunofluorescence of adult *A. pernyi* brain stained with anti-PTTH antibody (274/IV-A). Two pairs of contralateral L-NSC III and their axonal projections are clearly visible. Original magnification, 80×. (B) Nonradioactive *in situ* hybridization with specific PTTH cRNA antisense probe detected two cells in each brain hemisphere (arrowheads). Original magnification, 80×. (C and D) A pair of micrographs illustrating simultaneous detection of PTTH peptide and mRNA, respectively, in the same section of *A. pernyi* brain. Note the absence of mRNA in the axons, as opposed to the peptide staining (arrowheads). Original magnification, 635×.

FIG. 5. PTTH peptide and mRNA expression during embryonic and larval development. PTTH immunoreactivity in brain sections of Day 4 developing embryo (A, 300×), freshly hatched first instar larva (B, 160×), and last instar wandering larva (C, 250×). PTTH mRNA distribution in brains of Day 7 embryo (D, 215×), Day 1 first instar larva (E, 215×), and wandering stage of fifth larval instar (F, 200×), as detected by nonradioactive *in situ* hybridization.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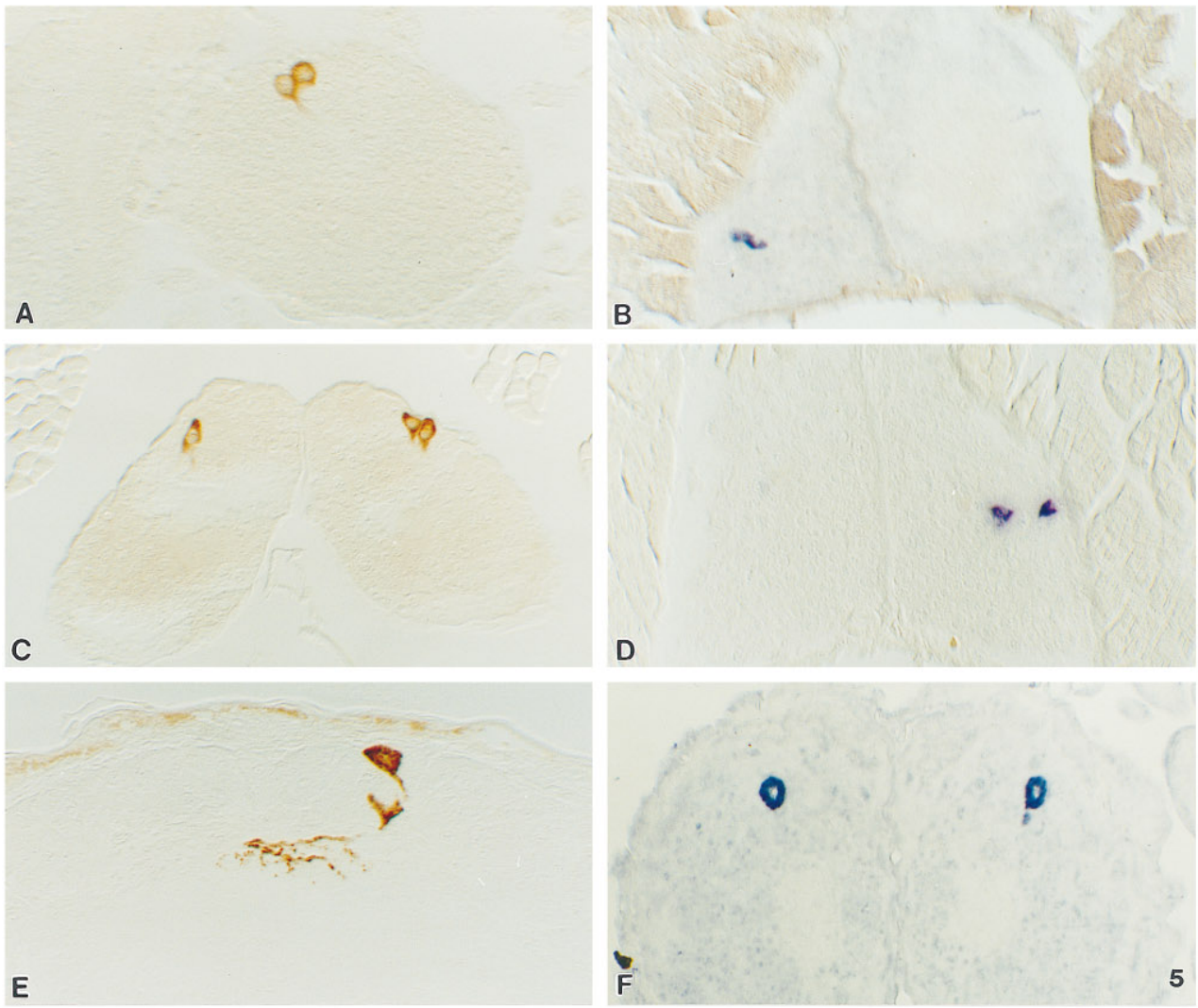
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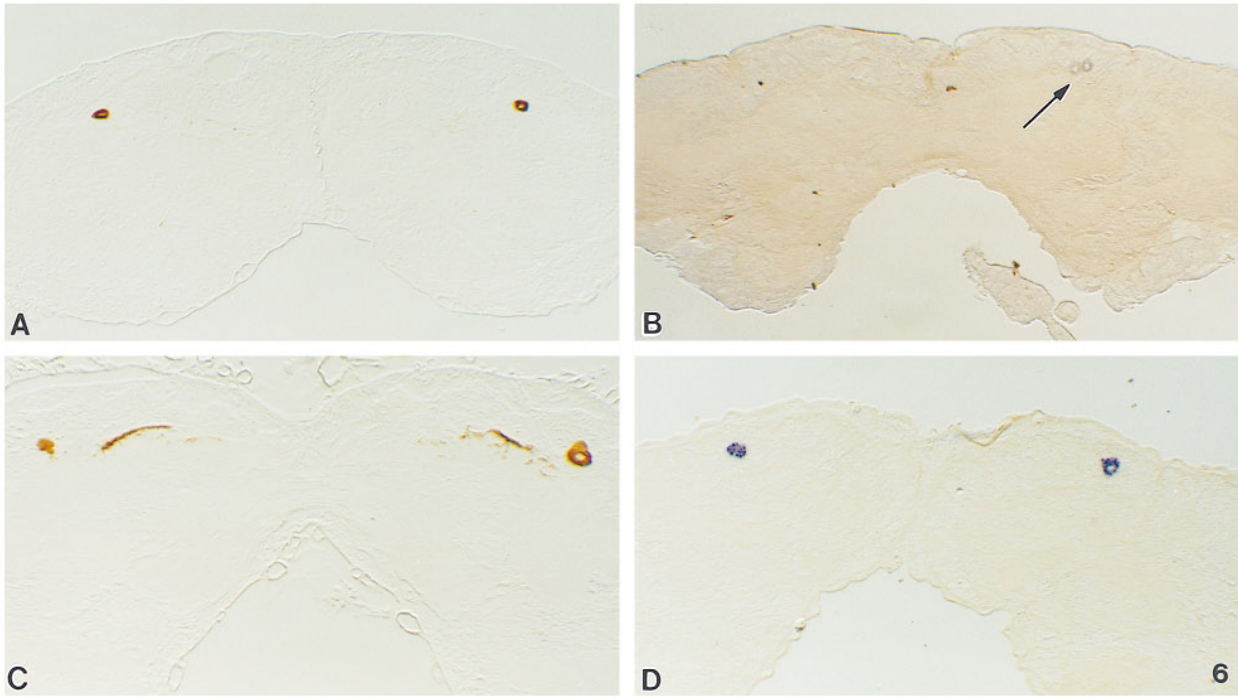
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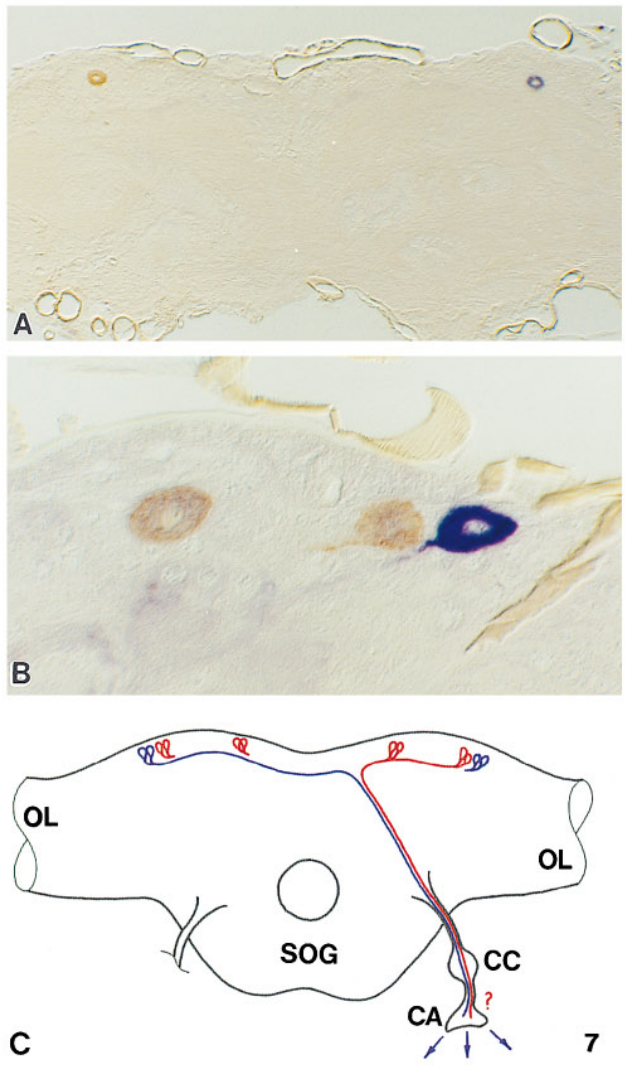
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(PTTH) or Cy2 (green; PER) fluorophores, clearly confirmed that PTTH- and PER-expressing cells represent two populations of completely nonoverlapping neurosecretory cells (data not shown).

DISCUSSION

The results clearly show that we have cloned functionally active PTTH from the giant silkmoth, *A. pernyi.* The *A. pernyi* PTTH preprohormone shares structural features with *Bombyx* and *S. c. ricini* PTTH precursors that appear to be critical for functional activity. First, there is an appropriately placed proteolytic cleavage site within the *A. pernyi* PTTH preprohormone which on activation would liberate the active hormone subunit. Second, all seven cysteine residues found in *Bombyx* and *S. c. ricini* PTTHs are conserved in the *A. pernyi* hormone. Based on biochemical analysis of *Bombyx* PTTH, these cysteine residues are necessary for between and within chain disulfide bonds that are essential for full prothoracicotropic activity (Ishibashi *et al.,* 1994).

A. pernyi PTTH protein and mRNA are also expressed in L-NSC III which, based on extensive studies in several Lepidoptera, is where PTTH neurosecretory cells should reside (Kawakami *et al.,* 1990; Westbrook and Bollenbacher, 1990). Most importantly, recombinant *A. pernyi* PTTH elicits potent prothoracicotropic activity by consistently stimulating adult development in debrained *A. pernyi* pupae. We do not know whether the bacterially expressed *A. pernyi* PTTH formed homodimers. Even if it did not, the monomeric subunit alone has been shown in *Bombyx* to have substantial functional activity (ca. 50% of the functional activity of the homodimer) as long as the intrachain disulfide bonds are maintained (Ishibashi *et al.,* 1994).

Immunocytochemical studies of PTTH immunoreactivity in L-NSC III of the tobacco hornworm *Manduca sexta* and *Bombyx* show that protein levels in these neurons do not undergo dramatic variations at the various ecdyses that occur throughout metamorphosis (O'Brien *et al.,* 1988; Westbrook and Bollenbacher, 1990; Westbrook *et al.,* 1993; Dai *et al.,* 1995). This contrasts with the burst of PTTH levels in the hemolymph at these developmental stages. Moreover, recent studies in *Bombyx* show that mRNA levels also vary little with pupal ecdysis (Adachi-Yamada *et al.,* 1994). Our results extend those studies by showing no large variations in either PTTH protein or mRNA in L-NSC III during larval, pupal, or adult ecdyses. Remarkably, the PTTH gene appears to be transcribed at low levels even in chilled diapausing pupae. Taken together, the results to date suggest that there are no large changes in transcriptional activity of the PTTH gene that accompany the bursts of secretory activity. The primary regulation of PTTH levels in hemolymph appears to be at the level of synaptic release.

Previous studies have identified PTTH-like immunoreactivity in *Manduca* as early as 30% of embryonic development (Westbrook and Bollenbacher, 1990). Our results in *A. pernyi* show a similar pattern of appearance of PTTH immunoreactivity in L-NSC III, but we also document a concurrent appearance of PTTH mRNA in the same cells. In addition, no PTTH-expressing cells outside of the L-NSC III were found during embryogenesis or larval development in *A. pernyi.* This contrast with results in *Manduca* where PTTH-like immunoreactivity is transiently expressed in other brain neurons and neurons in the frontal and subesophageal ganglia during embryonic and larval stages (Westbrook and Bollenbacher, 1990). The discrepancy between our result in *A. pernyi* and the results of others in *Manduca* could reflect species differences in the patterns of PTTH expression. Because no PTTH mRNA data are available to confirm the immunocytochemical staining, it is also possible that the staining in other *Manduca* neurons is due to cross-reactivity of the anti-PTTH antibody with other peptides. The physiological role of PTTH during embryogenesis is currently not known; potential embryonic functions have been discussed previously (Westbrook and Bollenbacher, 1990).

The photoperiodic termination of diapause in *A. pernyi* is controlled by a circadian clock that regulates PTTH release (Williams and Adkisson, 1964a,b; Williams, 1969). Truman (1971) has postulated that the same circadian clock controls both the adult eclosion rhythm and the termination of diapause in *A. pernyi* because based on brain lesion and transplantation studies the clock mechanism for each resides in the same region of central brain. In addition, each circadian event exhibits a similar response to photoperiod. Thus, a primary goal of our study was to determine whether PTTH-expressing cells are actual circadian clock cells. Putative circadian clock cells in *A. pernyi* brain were identified by their ability to express PER which appears to be an essential clock element in *A. pernyi,* as it is in *Drosophila* (Levine *et al.,* 1995).

FIG. 6. PTTH peptide and mRNA expression during pupal development. PTTH immunoreactivity in brain sections of *A. pernyi* diapausing (A, 80×) and developing (B, 100×) pupae. (C and D) Same pupal stages as above for A and B, hybridized *in situ* with digoxigenin-labeled antisense cRNA probes. Original magnifications, 80 and 100×, respectively.

FIG. 7. PTTH and PER are expressed in different cells. Double immunostaining of adult *A. pernyi* brain sections with antibodies against PTTH (blue) and PER (brown). (A) Low-magnification micrograph illustrates that the PTTH- and the lateral PER-expressing cells are localized in the same dorsolateral region of central brain. Original magnification, 100×. (B) High-power view of a similar preparation shows PTTH and PER immunoreactive cells in the same brain hemisphere. Original magnification, 400×. (C) Schematic diagram illustrating the topography of both PTTH-producing (blue) and PER-producing (red) cells and their axonal projections in the adult *A. pernyi* brain–retrocerebral complex. CA, corpus allatum; CC, corpus cardiacum.

Using anti-PER antibodies, we have localized PER to four pairs of neurosecretory cells in *A. pernyi* brain. These appear to be actual circadian clock cells because they show daily *per* mRNA oscillations and also stain with an antibody against another clock element *timeless* recently cloned from *Drosophila* (unpublished data). Although PTTH-expressing cells and the lateral pair of PER-expressing cells are located just next to each other, our double-label immunocytochemical results clearly show that PTTH and PER reside in different cells. However, the close proximity of PTTH- and PER-expressing cells and the extensive dendritic arborization found for neurosecretory cells in this region suggests a route of communication between these two cell populations (Carrow *et al.*, 1984). In addition, there could be axonal communication between PER- and PTTH-expressing cells either along their course or at the level of the terminals. Further ultrastructural and cell lesion studies should help establish the functional relationship between PER- and PTTH-expressing cells in *A. pernyi* brain.

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