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cDNA Cloning and Nuclear Localization of the Circadian Neuropeptide Designated as Pigment-Dispersing Factor PDF in the Cricket *Gryllus bimaculatus*[1]

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Pigment-dispersing factor (PDF) was recently reported to be a principal circadian neuromodulator involved in transmitting circadian rhythms of daily locomotion in insects. In *Drosophila*, PDF functions in some of the neurons expressing the clock genes *period*, *timeless*, *Clock*, and *cycle*, and those clock genes in turn regulate *pdf* gene expression. In the present study, we cloned a cDNA encoding PDF in the brain of a nocturnal insect, the cricket *Gryllus bimaculatus*, and found that an isolated clone (310 bp) codes for an extraordinarily short precursor protein with no definite signal sequence, but a nuclear localization signal (NLS)-like sequence instead. The cricket PDF exhibits high sequence identity (78–94%) and similarity (89–100%) to insect PDFs and also to crustacean β-PDH peptides. In the optic lobes of *G. bimaculatus* there are PDF-immunoreactive neurons in both the medulla and lamina neuropiles. Among the strongly immunoreactive lamina PDF neurons, on electron microscopy we identified cells exhibiting distinct staining that is not only cytoplasmic but also nuclear. When GFP-fused PDF precursor proteins were expressed in COS-7 cells, distinct translocation of the fusion protein into the nucleus was observed. This is the first finding of PDF peptide in the nucleus, which suggests a fundamental role of PDF peptide *per se* in the circadian clock system.

Key words: circadian rhythm, clock gene, neuropeptide, nuclear localization signal, pigment-dispersing factor.

Circadian clocks organize the temporal framework for physiological and behavioral functions in living organisms and consist of a circadian pacemaker center, in which the heterodimer products of the clock genes *clock* and *cycle* act as transactivation factors for the clock gene *period* or *timeless*. The resulting PERIOD and TIMELESS proteins accumulate in the cytoplasm where they form another heterodimer, which is translocated to the nucleus to negatively regulate the two genes by interfering with production of the CLOCK:CYCLE heterodimer. Such a negative-feedback loop was originally discovered in *Drosophila* (*1, 2*), and has now also been found in the circadian clocks of mice and humans (*1*).

Pigment-dispersing factors (PDFs) are close insect homologues of the β-subfamily of crustacean pigment-dispersing hormone (β-PDH) neuropeptides (*3*), and are abundant in the accessory medulla of the optic lobe of orthopteroid insects (*4*). In addition to earlier observations that PDF is involved in the regulation of insect circadian rhythms (*5–7*), a number of recent examinations of this aimed at clarifying the function of PDF in the circadian system of the fruit fly *Drosophila melanogaster* and utilizing various types of mutants have generated several lines of evidence that PDF acts as a neuromodulator functioning in the output pathway of the pacemaker regulating behavior arising from the dorsal central brain (*8–10*). Moreover, immunoreactivity to β-PDH exhibits daily cycling within the terminals of axons projecting from some of the so-called lateral neurons that are required to express circadian rhythms (*11*), either to the fly's optic neuropiles (*12*) or from the small ventral lateral neurons (s-LNvs) (*9*). PDF is thus judged to be a neuropeptide functioning downstream of the clock machinery as a principal circadian transmitter that organizes daily locomotion or other behavior.

Different groups of lateral neurons co-express immunoreactivity to PDF, and the clock genes *period* (*13*), *timeless* (*14, 15*), and *doubletime* (*16*). Recent results indicated that

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Abbreviations: cRACE, circular or concatemeric first-strand cDNA mediated RACE; DMEM, Dulbecco's modified Eagle's medium; EM, electron microscopy; GFP, green fluorescent protein; La, lamina; Me, medulla; NLS, nuclear localization signal; PAP, PDF-associated peptide; PCR, the polymerase chain reaction; PDF, pigment-dispersing factor; PDH, pigment-dispersing hormone; RACE, the rapid amplification of cDNA ends; RT-PCR, reverse transcription PCR.

the *pdf* gene and PDF are regulated by these clock genes, including *Clock* and *cycle* (*8–10, 17*). On the other hand, even though PDF immunoreactivity exhibits daily cycling at the terminals of s-LNvs, neither PDF immunostaining in cell bodies nor *pdf* mRNA levels exhibit such circadian rhythmicity (*9*).

These findings therefore raise questions as to how PDF is regulated by the clock, why *pdf* mRNA levels fail to show rhythmicity, and whether PDF is translocated to the nucleus and functions in the pacemaker clock mechanism or not. In particular, determination of PDF localization within the lateral neuron cell body is crucially important for clarifying its specific functions, if any, within the nucleus, given that a modulatory relationship between the *pdf* gene and clock genes or their products is suggested (*8–10, 17*), but with no elucidation of detailed molecular mechanisms.

To date, circadian clocks have been investigated mainly in diurnal animals such as *Drosophila* and humans. However, many species are nocturnal, and perhaps different systems of modulation either generate or edit diurnal and nocturnal behavioral profiles. In the present study, we have carried out the cDNA cloning of PDF from adult brains of the cricket *Gryllus bimaculatus*, whose behavioral activity manifests nocturnal rhythmicity. The cDNA obtained exhibits a unique preprosequence including an ordinary PDF sequence, with very short putative signal and PDF-associated peptide (PAP) sequences. Computer-assisted sequence analysis suggested the presence of a nuclear localization signal (NLS)–like sequence, MARRARFE, at the N-terminus, and this prompted us to explore the translocation of PDF peptide to the nucleus of PDF neurons. Electron microscopic observations clearly revealed the cells whose nuclei are immunostained with anti-PDF, suggesting that PDF peptide is involved in the circadian clock mechanism.

EXPERIMENTAL PROCEDURES

Animals—All the crickets, *Gryllus bimaculatus*, were maintained with a light/dark (LD) 12:12 photoperiod at 27°C. Three adults were collected every 3 h for a full day, and the entire bodies were immediately frozen in liquid nitrogen to obtain heads, which were then stored at –80°C. For immunocytochemistry, crickets were dissected at 12:00 am, 3 h after lights "on" (ZT3:00).

3′ RACE—mRNAs were purified using a QuickPrep® *Micro* mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions. For the extraction of mRNAs, the frozen heads of crickets were homogenized in the extraction buffer. The mRNAs obtained (1 μg) were reverse-transcribed by the polymerase chain reaction (PCR) using AMV reverse transcriptase (Promega, Madison, WI) and d(T)$_{17}$-adapter primer (5′-GGCCACGCGTCGACTAGTAC-T$_{17}$-3′) as described (*18*). After phenol extraction followed by ethanol precipitation, the resulting cDNA pellet was used as a template for 3′ RACE. The primers were all obtained from Hokkaido System Science (Sapporo), and those for 3′ RACE were as follows: PDF specific primer GB-PDF [5′-AAGCGCAACTC(A/C/G/T)GA(A/G)(A/C)T(A/C/G)ATCAACTCN-3′] and adapter primer Ad (5′-GGCCACGCGTCGACTAGTAC-3′). The GB-PDF primer was designed based on the nucleotide sequence homology among mRNAs clarified for β-PDH and PDF (*19–23*). The PCR mixture (100 μl) included 1 μl of cDNA, 20 pmol of Ad primer, 20 pmol of GB-PDF primer, 200 μM dNTP, 2.5 mM $MgCl_2$, and 2.5 units of PLATINUM *Taq* DNA polymerase (GIBCO BRL). PCR-I (30 cycles) was performed as follows: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1.5 min, and final extension for 10 min at 72°C, using an Geneamp PCR system 2400 (Perkin Elmer). Single-primer control PCRs were also carried out in parallel. Two primer-specific PCR products were identified on a 2% agarose gel, and recovered by phenol extraction followed by ethanol precipitation. The gel-purified PCR products were subcloned into the pCR 2.1-TOPO vector (Invitrogen, Carlsbad, CA). Plasmid DNA containing *pdf* was identified by sequencing analysis with a dye terminator cycle sequencing kit (Amersham Pharmacia Biotech).

cRACE for Determination of the 5′ End of PDF mRNA—For amplification of the 5′ end of PDF cDNA, the cRACE method was performed as described by Maruyama *et al.* (*24*). First-strand cDNA of PDF mRNA was synthesized using mRNA (0.5 μg) for a 5′ Full RACE Core Set kit (TaKaRa). The primer utilized was a PDF gene–specific oligonucleotide, GB-P (5′-ACATAGTACAGATTC-3′), which was phosphorylated with T4 polynucleotide kinase. The resulting cDNA was cyclized with T4 RNA ligase and used as a template for cRACE. The first PCR was carried out with gene specific primers GB-S1 (5′-CTTGGATTACTAAACCAGAGA-3′) and GB-A1 (5′-CTTGCGCCCTGCATCAT-3′). The reaction mixture (100 μl in total) consisted of 1 μl of cDNA, 20 pmol of each primer, 200 μM dNTP, 2.5 mM $MgCl_2$, and 2.5 units of PLATINUM *Taq* DNA polymerase. The reaction was carried out for 30 cycles under the following conditions: denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 1.5 min, and final extension for 10 min at 72°C. A 1 μl aliquot of the resulting PCR product was used for the second nested PCR. The nested PCR was performed in a manner similar to the first PCR of cRACE, except for the use of 20 pmol each of the primers GB-S2 (5′-CCAGAGAAATACATCAAACAACAC-3′) and GB-A2 (5′-CACTTTAGGAAGACCTAAGAG-3′). The PCR products were analyzed on a 2% agarose gel and two primer-specific products were excised. These gel-purified PCR products were subcloned into the pCR® 2.1-TOPO® vector for sequencing analysis.

Based on the sequence information obtained on 3′ RACE and cRACE analyses, reverse transcription PCR (RT-PCR) was performed to amplify the full-length PDF cDNA. The PCR conditions (30 cycle) were as for PCR in the 3′RACE method, except for the primers (20 pmol), GB-amp (5′-GGAGGTAGCAAGCTGGTTGCTG-3′) and GB-A3 (5′-CTTTATTACATAGTACAGATTCAATACG-3′). The PCR products were treated as described above for sequence analysis.

Construction of a cDNA Library—A cDNA library of *G. bimaculatus* heads was constructed using a mRNA (5 μg), a cDNA synthesis kit and a λgt 10 cloning system (Amersham Pharmacia Biotech). First-strand cDNA was synthesized from mRNA and oligo (dT)$_{12\text{-}18}$ primer. Linkers containing a *Not*I restriction site and an *Eco*RI site were added to cDNAs at the 5′ and 3′ ends, respectively. These cDNAs were cloned into the *Not*I/*Eco*RI site of the λExCell vector (Amersham Pharmacia Biotech). *Escherichia coli* NM522 was transformed with the recombinant plasmids to construct a cDNA library. The completed library contained 1 × 10^5 primary recombinants.

cDNA Cloning—Based on the sequence data obtained with the RACE methods, a *pdf* full-length probe was synthesized by PCR with the GB-amp and GB-A3 primers using the cDNA pellet prepared for 3′ RACE as a template. This PCR product of about 250 bp was purified by 2% agarose gel electrophoresis and ^{32}P-labeled by random priming according to the standard procedure (*25*). Hybridization of the replica nitrocellulose filters was performed at 65°C for 12 h in a mixture of 5× standard saline citrate (1× SSC: 1.0 M sodium chloride, 0.1 M sodium citrate), 50 mM sodium phosphate (pH 6.5), 0.5% SDS, 2× Denhardt's solution (100× Denhardt's solution: 0.1% Ficoll 400, 0.1% polivinylpyrrolidone, and 0.1% bovine serum albumin), 50% formamide and 100 μg/ml sonicated salmon sperm DNA. The membranes were finally washed twice for 30 min at 65°C in 0.2× SSC and 0.1% SDS. Hybridization-positive phage plaques were purified, and the recombinant pExCell phagemids were rescued from the bacteriophage λExCell clones by *in vivo* excision according to the manufacturer's instructions for DNA sequencing.

Plasmid Construction of GFP Fusion Proteins with a PDF Precursor—To construct NLS-GFP fusion proteins, the full-length *pdf* cDNA in the pCR® 2.1-TOPO® vector was used as a template for PCR, as described for 3′ RACE. Sense primer P1 (5′-CCCAAGCTTCCAAGCTGCTCGACAAGGAG-3′) was designed in the 5′ UTR region of *G. bimaculatus* PDF precursor cDNA to add a *Hin*dIII restriction enzyme site at its 5′ end. Antisense primer P2 (5′-CGGGATCCCCCTGCATCATTCAGCAC-3′) with a 5′ end *Bam*HI site was designed in the C terminal peptide region excluding codons for Arg, Lys, and stop. The DNA fragment corresponding to the PAP region was amplified with P1 and P3 (antisense primer: 5′-CGGGATCCGTGGACACACATTAATGG-3′). The amplified DNA fragments were purified by gel electrophoresis (2% agarose), and digested simultaneously with both *Hin*dIII and *Bam*HI for subcloning into the pEGFP-N3 vector (Clontech; Palo Alto, CA) treated with *Hin*dIII and *Bam*HI. The constructed fusion proteins were PAP-PDF-GFP and PAP-GFP, in which NLS was positioned at the N terminus of the proteins. When vector pEGFP-C3 (Clontech) was used, proteins GFP-PAP-PDF and GFP-PAP were constructed. NLS is located at the C terminal end of GFP. In this case, a 13-amino acid linker (KLLDKEVASWLLS) was inserted between GFP and PAP. The cDNA sequences of these constructs were examined as described above.

To determine whether the putative NLS of the *G. bimaculatus* PDF precursor causes localization of the GFP fusion protein to the nuclei, plasmids in which three Arg residues in NLS were substituted by Ala were constructed. Site-directed mutageneses were performed by the overlap extension PCR method. For Arg→Ala replacement, a cDNA was amplified with primers P1 and P4 (antisense primer: 5′-GGCTTCAAATGCTGCTGCCGCAGCCATACTCAGCA-3′) using a full-length *pdf* cDNA in the pCR® 2.1-TOPO® vector as a template. Another fragment was also amplified with primers P5 (5′-AGTATGGCTGCGGCAGCAGCATTTGAAGCCAATGC-3′) and P2. P5 is a sense primer having a sequence complementary in part to P4, causing mismatched annealing. These DNA fragments were mixed for PCR amplification with external primers P1 and P2. The obtained DNA fragment was treated with *Hin*dIII and *Bam*HI for insertion into GFP fusion vector pEGFP-N3 or pEGFP-C1, and the resulting mutated constructs, PAP(NLS/3A)-PDF-GFP and GFP-PAP(NLS/3A)-PDF, were examined by sequencing.

Transient Expression of GFP-Fused Proteins in COS-7 Cells—Monkey kidney epithelial COS-7 cells were cultured in dishes with a piece of cover glass. COS-7 cells were cultured at 37°C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (Life Tech., MD). Transient transfection of GFP-fused proteins into COS-7 was carried out according to the DEAE-dextran method. When the cell density reached 80% confluency, the medium was replaced by another medium (0.5 mg/ml DEAE-dextran/DMEM) containing the plasmid (2 μg/ml) coding GFP fusion protein. After incubation for 30 min, chloroquine in DMEM (50.6 μg/ml) was added for additional incubation for 3 h. The medium was then replaced with 10% DMSO/DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum, followed by standing for 2 min at room temperature. After aspiration of the DMSO solution, DMEM was added, followed by culture for 48 h in a CO_2 incubator. Transfected cells on the cover glass were then fixed in 4% paraformaldehyde for observation under a Zeiss LSM510 laser scanning confocal microscope.

Immunocytochemistry—For anatomical analysis of PDF immunoreactive cells, confocal microscopy was first performed after whole-mount immunostaining. Dissected brains, containing the subesophageal ganglion and optic lobes with the retina, were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 16 h. After washing with PBS, they were incubated with a rabbit polyclonal antibody raised against *Uca* β-PDH (1:3500) (*26*) or *G. bimaculatus* PDF (Tomioka *et al.*, to be reported elsewhere) for 48 h at 4°C. FITC or Cy3 conjugated goat anti-rabbit secondary antibodies were used (Jackson Immunoresearch, Westgrove, PA). For washing between all steps, PBS with 1% Triton X-100 was used. Preparations were embedded in Vectashield (Vector Laboratories, Burlingame, CA) and then scanned under a Zeiss LSM510 laser scanning confocal microscope.

To localize the PDF cells and their neurites precisely in the neuropile, Vibratome sections of 80 μm thickness were immunostained through a free-floating reaction by means of the indirect peroxidase-anti-peroxidase technique (*27*). Sections were incubated with a rabbit polyclonal antibody against β-PDH (1:3,500) for 48 h at 4°C, and then with a swine anti-rabbit secondary antibody (DAKO, Glostrup, Denmark), followed by a rabbit antibody to horseradish peroxidase conjugated horseradish peroxidase (DAKO). Sections were finally reacted with DAB and H_2O_2, and then mounted in glycerol gelatin (Sigma, Saint-Louis, MS) for observation by light microscopy with an Olympus BX50.

Immuno-Electron Microscopy—To identify the PDF cells, and their neurites and varicosities, and to localize PDF peptide by electron microscopy (EM), the pre-embedding method for immuno-EM was applied. Adult crickets were decapitated and dissected in a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer. After fixation, horizontal slices of the brains and optic lobes were cut, 80 μm thick, with a Vibratome. Immunocytochemistry was performed on free-floating sections by means of the indirect peroxidase-anti-peroxidase tech-

nique, as described above. After postfixation with osmium tetroxide (0.5%, 1 h) and dehydration in an ethanol series, sections were embedded in Epoxy resin between acrylate sheets. Immunostained profiles in the embedded sections were photographed by light microscopy prior to ultrathin sectioning. Sections, 60–70 nm thick, were cut from the surface of the Vibratome slice, observed and photographed by EM without further staining.

RESULTS AND DISCUSSION

Isolation of a Gryllus bimaculatus PDF cDNA Encoding a PDF Precursor of Extremely Small Size—To be sure of obtaining target mRNAs, the heads of adult crickets, *Gryllus bimaculatus*, were collected every 3 h, *i.e.*, a total of eight times a day. All animals were previously entrained to light with a daily cycle of L12:D12. Although we initially attempted to excise brains from adult heads, it proved difficult to obtain sound mRNA in this way, presumably because of degradation during the prolonged dissection. Thus, the entire body was first frozen to obtain samples of intact heads. The frozen heads were then used directly for the extraction of mRNA. We adopted the procedure developed for direct isolation of polyadenylated RNA without purifying the total RNA. Isolated mRNAs are all converted to cDNAs by the RT-PCR method, in which AMV reverse transcriptase catalyzes the polymerization of DNA using mRNA as a template. This enzyme has intrinsic RNase H activity that degrades mRNA, and thus the method eventually affords a mixture of single-strand cDNAs.

We screened a PDF clone for this cDNA mixture. A PDF-specific sense primer was designed by referring to the results of sequence alignment analyses for both amino acid and nucleotide sequences. The N-terminal portion of PDF including the dibasic processing site (KR), namely residues −2 to +7, was chosen to design a PDF-specific sense primer (GB-PDF). The first PCR with primer GB-PDF and adapter primer Ad lacking $d(T)_{17}$ yielded no distinct gel-bands, and using the resulting PCR product as a template, the second PCR was carried out, to which yielded several products. A faint gel-band containing a two primer-specific PCR product of about 220 bp was subsequently isolated for sequence analysis. The nucleotide sequence determined encodes a PDF peptide with the amidation sequence in addition to the 3′-UTR non-coding region (Fig. 1). It was found that the open reading frame encoding PDF is highly conserved among the *pdf* and *pdh* genes, while the 3′-UTR region was unique in length and sequence. Compared with the 3′-UTR regions of *Drosophila* and other insect *pdf* genes, that of *G. bimaculatus* was very short (*ca.* 41% the length of *Drosophila* 3′-UTR) and also had a quite dissimilar nucleotide sequence.

To isolate the 5′-end of the *pdf* cDNA, we used the cRACE method. Based on the sequence information obtained on 3′ RACE, the antisense primer for RT-PCR against PDF mRNA was set in the 3′-UTR region (Fig. 1). The single-strand cDNA obtained was circularized by self-ligation catalyzed by T4 RNA ligase, and then with this cDNA template the PCR was carried out with gene-specific primers, sense GB-S1 (assigned to the 3′-UTR region) and antisense GB-A1 (assigned to the PDF C-terminal region). GB-S1 and GB-A1 are adjacent, being separated by only 14 nucleotides. Subsequent nested PCR was performed using the first PCR product, and primers GB-S2 and GB-A2, which were set at the inner sides of GB-S1 and GB-A1 in the circularized cDNA. Sequencing analysis afforded the complete sequence data including the 5′-UTR, signal region and PAP peptide region. Although we can not fully exclude another possible initiator codon near the 5′ end of the cDNA, we assigned the initiator codon as shown in Fig. 1 for the following reasons. (i) The selected ATG codon and its adjacent nucleotide sequences nicely fulfilled the criteria for Kozak's consensus motif (*28*). (ii) We found no additional ATG codon in the upstream region of any cDNA clones isolated on RT-PCR analysis. (iii) The three-amino-acid sequence at the putative N-terminal (Met-Ala-Arg) was similar to the N-terminal sequences of *Drosophila* PDF and *Panaeus* β-PDH precursors. Then the full-length PDF cDNA was isolated by RT-PCR using a primer set for both 5′-UTR and 3′-UTR. Eventually, the full length cDNA clone was found to comprise 310 bp with a precursor protein of 43 amino acid residues (Fig. 1). It should be noted that this size of PDF precursor is considerably smaller than those of other insects, which contain, for example, 102 amino acid residues in *Drosophila melanogaster* (*19*), and 89 amino acid residues in *Romalea microptera* (GenBank).

Highly Conserved Primary Structure of the G. bimaculatus PDF Peptide—It was found that the clarified sequence

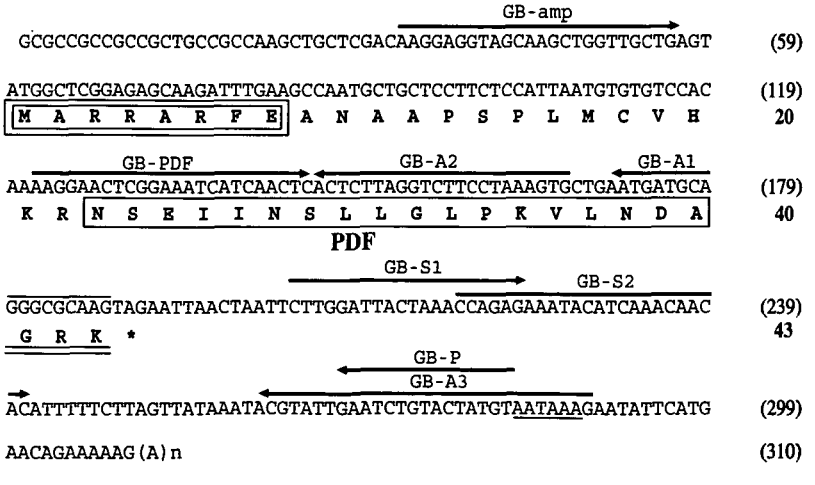

Fig. 1. **Nucleotide and deduced amino acid sequences of the *Gryllus bimaculatus* PDF-precursor.** Arrows indicate the primers used for the 3′ RACE or cRACE method. The putative polyadenylation site is underlined. A potential amidation site is double-underlined. The corresponding PDF peptide sequence is boxed, and the NLS-like sequence is double-boxed.

of *G. bimaculatus* PDF, NSEIINSLLGLPKVLNDA-amide, is identical with that of *Acheta domesticus* (*29*) (Fig. 2). The structure of *G. bimaculatus* PDF itself exhibits a high level of sequence identity (78–100%) and similarity (89–100%) when compared with the PDFs of other insects. For the PDFs of *Periplaneta americana* and *R. microptera*, only one residue substitution was found, namely, Leu/Ile[4] and Leu/Val[14], respectively. As for *Drosophila* PDF (*19*), four residue substitutions were found; *i.e.*, Leu/Ile[4], Ser/Gly[10], Asn/Val[14], and Met/Leu[15]. The Ser[10] residue of *Drosophila* PDF is uncommon, since Gly[10] is conserved in all other animals.

The amino acid sequences of insect PDFs are almost the same as those of crustacean PDHs, especially the so-called β-PDH subfamily (*3, 20–23, 30–34*). As shown Fig. 2, 8 of 18 peptide amide residues are conserved in all PDF/β-PDH peptides, *i.e.* NSE: Asn-Ser-Glu-(1-3), INS: Ile-Asn-Ser-(5-7), L: Leu-9, and A: Ala-18. Structurally homologous substitutions such as Leu↔Ile, Met↔Leu, and Asp↔Glu are seen at positions 4/8/11, 15, and 17, respectively. For instance, comparison between *Uca pugilator* β-PDH and *G. bimaculatus* PDF reveals only three structurally insignificant residue substitutions: Leu/Ile[4], Ile/Leu[8], and Met/Leu[15]. The sequence identity and similarity of all PDF/β-PDH peptides are also very high (61–89% and 89–100%, respectively).

The G. bimaculatus PDF Precursor Protein Contains a Nuclear Localization Signal (NLS)-Like Sequence—The cDNA clone obtained encoded a *G. bimaculatus* PDF precursor with 43 amino acid residues, constituting the third such gene identified for an insect PDF precursor. As indicated above, this precursor is very short compared with those of *D. melanogaster* (number of amino acid residues (n = 102) and *R. microptera* (n = 89). Given that the C-terminal PDF region has exactly the same peptide size, the difference in size of the corresponding precursors means that the combined length of the putative signal and PAP regions of the *G. bimaculatus* PDF precursor is extremely short. The number of amino acid residues is only 20 (Fig. 3), while those for *D. melanogaster* and *R. microptera* are 80 and 66, respectively. β-PDH cDNAs from crustaceans also encode relatively long polypeptides (about 60).

This structural abnormality correlates moreover with the fact that the *G. bimaculatus* PDF precursor is devoid of the ordinary signal sequence at the N-terminus. When analyzed by the computer-assisted signal peptide prediction method for eukaryotic sequences (*35, 36*), no distinct sequence was indicated to be a signal (data not shown). In the same analysis, the *D. melanogaster* PDF precursor was predicted to have a 24-peptide signal sequence, while that for *R. microptera* was a 32-peptide (Fig. 3). By comparison, crustacean β-PDH precursors have been shown to contain an average 22-peptide long signal peptide (Fig. 3). From these results we suspected that the nucleotide sequence had been misread, so we carefully analyzed the cDNA clone from the library.

We screened the cDNA library using a 254 bp ^{32}P-labeled

Insect PDFs

Gryllus bimaculatus	NSE	I	INS	L	L	GLPKVLND	A	-NH$_2$
Acheta domesticus	NSE	I	INS	L	L	GLPKVLND	A	-NH$_2$
Romalea microptera	NSE	I	INS	L	L	GLPKLLND	A	-NH$_2$
Periplaneta americana	NSE	L	INS	L	L	GLPKVLND	A	-NH$_2$
Drosophila melanogaster	NSE	L	INS	L	L	SLPKNMND	A	-NH$_2$

Crustacean β-PDHs

Uca pugilator	NSE	L	INS	I	L	GLPKVMND	A	-NH$_2$
Cancer magister	NSE	L	INS	I	L	GLPKVMND	A	-NH$_2$
Procambarus clarkii	NSE	L	INS	I	L	GLPKVMNE	A	-NH$_2$
Carcinus maenas	NSE	L	INS	I	L	GLPKVMND	A	-NH$_2$
Pandalus jordani	NSE	L	INS	L	L	GLPKVMTD	A	-NH$_2$
Pacifastacus leniusculus	NSE	L	INS	I	L	GLPKVMND	A	-NH$_2$
Penaeus aztecus	NSE	L	INS	L	L	GIPKVMND	A	-NH$_2$
Orconectes immunis	NSE	L	INS	I	L	GLPKVMNE	A	-NH$_2$
Orconectes limosus	NSE	L	INS	I	L	GLPKVMNE	A	-NH$_2$
Callinectes sapidus I	NSE	L	INS	I	L	GLPKVMND	A	-NH$_2$
Callinectes sapidus II	NSE	L	INS	L	L	GISALMNE	A	-NH$_2$
Penaeus vannamei 1, 2	NSE	L	INS	L	L	GIPKVMND	A	-NH$_2$
Penaeus vannamei 3	NSE	L	INS	L	L	GLPKVMND	A	-NH$_2$
Penaeus japonicus I	NSE	L	INS	L	L	GIPKVMTD	A	-NH$_2$
Penaeus japanicus II	NSE	L	INS	L	L	GLPKFMID	A	-NH$_2$

Fig. 2. **Comparisons between the primary structures of mature insect pigment-dispersing factors (PDFs) and crustacean β-pigment-dispersing hormones (β-PDHs).** PDFs from insects: *Gryllus bimaculatus, Acheta domesticus* (*29*), *R. microptera* (*30*), *P. americana* (*3*), and *D. melanogaster* (*19*). PDHs from crustaceans: *Uca pugilator* (*31*), *Cancer magister* (*34*), *Procambarus clarkii* (*32*), *Carcinus maenas* (*20*), *Pandalus jordani* (*3*), *Pacifastacus leniusculus* (*3*), *Penaeus aztecus* (*3*) *Orconectes immunis* (*3*), *Orconectes limosus* (*21*), *Callinectes sapidus* (*22*), *Penaeus vannamei* (*23*), and *Penaeus japonicus* (*33*).

Insect PDF precursors

Gryllus bimaculatus	MARRARFEANAAPSPLMCVH	KR	NSEIINSLLGLPKVLNDA	GRK	
Drosophila melanogaster	MARFTYLVALVLLAICCQWGYCGA	MAMPDEERYVRKEYNRDLLDWFNNVGVGQFSPGQVATLCRYPLILENSLGPSVPIR	KR	NSELINSLLSLPKNMNDA	GK
Romalea microptera	MTAMAVSGKLLTALVLSTYILGLALTIQATQY	EEDKYQENEVKYGRELASWLAQLAHKNEPAICAH	KR	NSEIINSLLGLPKLLNDA	GRK

Crustacean β-PDH precursors

Carcinus maenas	MRSAVIVTMLVVVALAALLTQG	QDQDLKYQEREMVAELAQQIYRVAQAPWAGAVGPH	KR	NSELINSILGLPKVMNDA	GRR
Orconectes limosus	MRSAMVVLVLVAMVAVFTRA	QEQDLKYPEREVVAELAAQIYGWPGSLGTMAGGPH	KR	NSELINSILGLPKVMNEA	GRR
Callinectes sapidus I	MRSSVIVAVLVVVALAALLTQG	QEQELKYPEREMVAELAQQIYRVAQAPWAAAVGPH	KR	NSELINSILGLPKVMNDA	GRR
Callinectes sapidus II	MRSGVFVAVLVVVVFALLTQG	QEQELHVPEREAVANLAARILKIVHAPHDAAAGVPH	KR	NSELINSLLGISALMNEA	GRR
Panaeus vannamei 1	MRSAVVVALLVMVAMSLQLTAA	QEDQEDLKYFEREVVAELAAQILRVAQGPSAFVAGPH	KR	NSELINSLLGIPKVMNDA	GK
Panaeus vannamei 2	MARCFVVLAFLALAAMSLQVATA	QDDQDDLKYFEREVVAELAAQILRVAQGPSAFVAGPH	KR	NSELINSLLGIPKVMNDA	GK
	Signal	**PAP**		**PDF/β-PDH**	

Fig. 3. **Comparisons between the primary structures deduced from the nucleotide sequences of precursor proteins encoding insect pigment-dispersing factors (PDFs) and crustacean β-pigment-dispersing hormones (β-PDHs).** PDF precursors from insects: *D. melanogaster* (*19*), and *R. microptera* (GenBank). PDH precursors from crustaceans: *Carcinus maenas* (*20*), *Orconectes limosus* (*21*), *Callinectes sapidus* (*22*), and *Penaeus vannamei* (*23*).

cDNA probe for confirmation of the sequence. This probe covers the entire sequence of the precursor protein. If the PDF mRNA were in a specific secondary structure, the mRNA may be misread on RT-PCR, yielding a cDNA clone with a mistranslated sequence. It was found, however, that such a misreading of the mRNA was unlikely, since the cDNA clone obtained from the library using the probe exhibited exactly the same nucleotide sequence. Furthermore, to determine whether or not the assigned ATG codon is appropriate as an initiator codon, the nucleotide sequence was carefully checked according to the procedure of Kozak (*28*). We recognized the presence of A at position –3 and G at position +4, ATG being numbered +1 to +3, and thus concluded that the particular ATG codon possessed the residue characteristics of an initiator.

The initiation sequence of the *G. bimaculatus* PDF precursor is MARRARFEAN---, which includes three arginines (R). We noticed that the sequence MARRARFE resembles the sequences of nuclear localization signals previously reported. For instance, most of the precursors of the clock protein PERIOD in insects have the NLS sequence MLKKHR (*37*). Although the exact designation of an NLS sequence is thought currently to be difficult, an abundance of basic amino acids arginine (R) and lysine (K) appears to be one of the characteristic features of NLSs. When we calculated the score that distinguishes the tendency of a peptide to be translocated to either the nucleus or cytoplasm, using the network system (*38*), the 1–22 initiation sequence of the *G. bimaculatus* PDF precursor was judged to have a higher likelihood of being in the nucleus (about 48%) than in the cytoplasm (about 22%).

Nuclear Localization of the PDF Peptide in PDF Neurons on Immuno-EM—Since translocation of the *G. bimaculatus* PDF precursor to the cell nucleus was strongly suggested by the results from our sequence analyses, we proceeded to localize the expression of the PDF peptide itself, to see whether it was in the nucleus of the PDF cells. To this end, we first tried to locate somata showing PDF expression. Confocal studies revealed that the general structure for the PDF cells of *G. bimaculatus* was similar to that previously reported (*4*). We identified two groups of immunoreactive cell clusters in the optic lobe; *i.e.* PDFMe cells in the medulla and PDFLa cells in the lamina. The PDFLa cells were further divided into two subgroups, PDFLa dorsal cells with cell bodies located in the dorsal region in the cleft between the lamina and medulla neuropiles, and PDFLa ventral cells in the ventral region (data not shown). These exhibit dense arborization in the optic lobe and central brain.

Light microscopic examination of Vibratome slices revealed the precise location of PDF cells and their arborization. The PDFMe cells gave rise to dense arborization in the accessory medulla, and to neurites in the first chiasma between the lamina and medulla. They also extended axons into the central brain. PDFLa cells extended their axons beneath the lamina to form a varicose network in the overlying neuropile. Axons from two groups of PDFLa cells gathered in an equatorial region at the edge of the lamina and extended further proximally. Although all of about 13 PDFMe cells exhibited strong immunoreactivity (Fig. 4A), differing immunoreactivity intensities were observed among about 150–200 PDFLa dorsal and ventral cells. An inner cell group was strongly immunoreactive, whereas PDFLa cells situated in a more peripheral location showed rather faint immunoreactivity (Fig. 4B). There were about twice the number of such peripheral cells as strongly immunoreactive inner cells. Since the immunostaining was conducted on Vibratome slices, these variations did not reflect differences in antibody penetration.

In Vibratome slices embedded for EM between acetate sheets, immunoreactive cells within 5–7 μm of the surface of the slices were visible. On light microscopy (Fig. 5A), we could clearly identify the two differentially immunostaining groups of somata of the PDFLa inner cells: namely, cells with fully immunoreactive somata and cells exhibiting immunoreactivity in their perikaryon. On EM, it was found that although the cytoplasm of all these cells showed similar levels of immunostaining, the nuclei of about 70% of the PDFLa inner cells were also strongly immunoreactive, so that both the nucleus and perikaryon were stained (Fig. 5B). There were a great many minute immunoreactive puncta throughout the nucleus, except for the interior of the nucleolus. We believe this is the first report that the nuclei of PDF cells exhibit PDF-like immunoreactivity. On the other hand, it should also be noted that the nuclei of the remaining approximately 30% cells were not stained by the antibody, showing immunoreactivity only in their perikaryon. Figure 5B shows that the two types of cells, with and without nuclear immunostaining, lie adjacent to

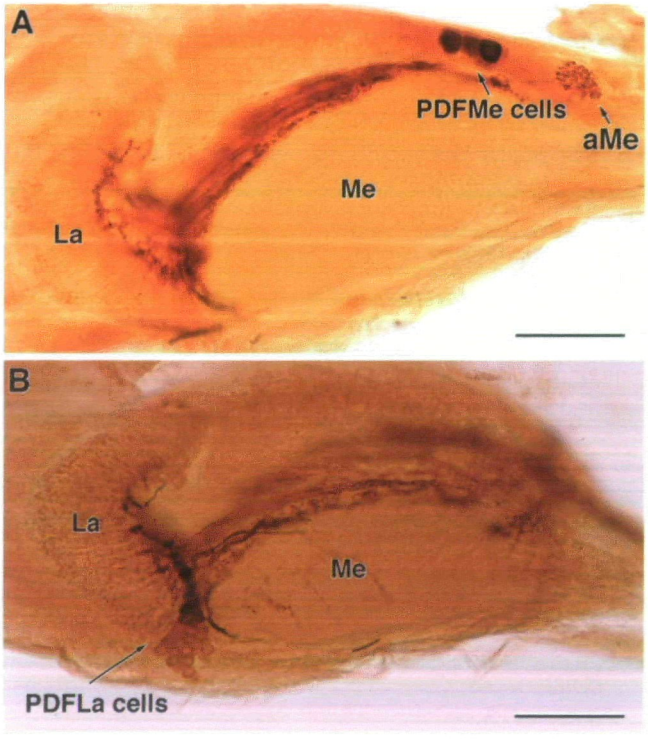

Fig. 4. **Immunocytochemistry of the optic lobes in *G. bimaculatus* illustrating the first (lamina) and second (medulla) neuropile regions.** Medulla (PDFMe) (A) and lamina (PDFLa) PDF cells (B) in horizontal Vibratome slices of the optic lobe immunostained by means of the indirect peroxidase-anti-peroxidase technique. PDFMe cells are located in the anterior cortex of the medulla, and PDFLa cells in the posterior cleft between the lamina and medulla. A rabbit polyclonal antibody raised against *G. bimaculatus* PDF (1:3,500) was used. aMe: accessory medulla, La: lamina, Me: medulla. Scale bars, 100 μm.

each other.

Nuclear Translocation of GFP-PDF Fusion Proteins—In order to examine the nuclear localization of PDF peptide, we performed subcellular localization studies on wild-type and mutant PDF precursor proteins in living cells. We constructed a series of expressing plasmids that consist of a gene encoding an enhanced version of the green fluorescent protein (GFP) at the either the N- or C-terminus of the PDF precursor protein. These constructs were transfected transiently into COS-7 cells and monitored for GFP expression 48 h posttransfection. As reported previously (*39, 40*), GFP alone was equally distributed between the cytoplasmic and nuclear compartments (data not shown). With the fusion proteins in which GFP was connected with PAP-PDF or PAP peptide at their C terminus, clear nuclear localization was observed. PAP-PDF-GFP and PAP-GFP were found predominantly in the cell nucleus, as shown in Fig. 6A, which depicts the localization of the PAP-PDF-GFP fusion protein in a COS-7 cell (data for PAP-GFP not shown). It should be noted that PAP-PDF-GFP and PAP-GFP contain the NLS-like sequence exposed in their N-terminal PAP region.

When GFP was fused to the N-terminus of the PAP-PDF protein, the resulting GFP-PAP-PDF aggregated in the nuclear envelopes. As shown in Fig. 6B, the protein appears to cluster on the membrane surface, although a part of the protein is seen in the nucleus. Almost the same translocation profile was observed for the GFP-PAP fusion protein (data not shown). In these GFP fusion proteins, the NLS-like sequence in the PAP region is not exposed at the N-terminus. The structural disparity between *PAP*-PDF-GFP/*PAP*-GFP and GFP-*PAP*-PDF/GFP-*PAP* appears to be a cause of their different translocation. No distinct nuclear translocation of PAP(NLS/3A)-PDF-GFP and GFP-PAP-(NLS/3A)-PDF was observed. As observed for GFP alone, these fusion proteins were equally expressed in both the cytoplasm and nucleus (Fig. 6, C and D). Since these fusion proteins consist of the MAAAAAF sequence instead of MARRARF, the results strongly suggest that the particular sequence is a genuine NLS sequence.

The present results show that *G. bimaculatus* PDF mRNA encodes a precursor protein that is likely to be translocated into the cell nucleus, and that immunoreactivity to PDF peptide is indeed expressed in the nucleus of most of the lamina's PDF inner cells. The nuclear localization of the PDF peptide was also suggested for the PDF cells in the medulla, although we could not determine the precise numbers of cells with and without nuclear immunostaining. Our findings prompt us to investigate what appears to be a novel aspect of the biological functions of PDF peptide. The PDF peptide in *Drosophila* was recently reported to transmit the rhythm of daily locomotor activity *via* output pathways from the circadian clock (*8–10*). Our confocal study revealed immunoreactivity in the axons and terminals of the *G. bimaculatus* PDF cells, suggesting a similar role for PDF in this species. In the light of the molecular data and subcellular expression patterns presented here, however, it now seems likely that the PDF peptide in *G. bimaculatus* has another, possibly additional role, in the nuclei of cells that are presumed to constitute the circadian

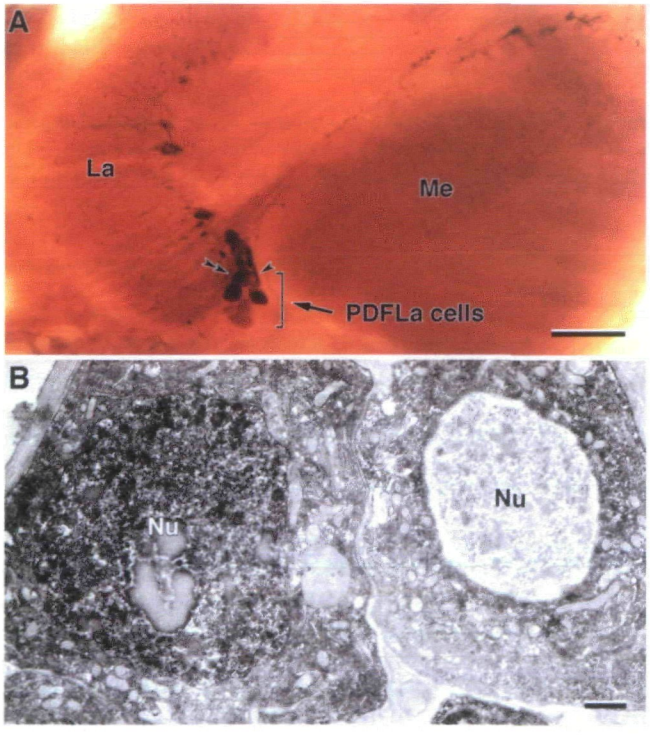

Fig. 5. **PDF neuron somata observed by immuno-EM.** (A) Light micrograph prior to ultrathin sectioning of a Vibratome slice immunostained for EM, revealing two groups of PDFLa-immunoreactive neurons (single and double arrowheads). (B) EM of the region of cell groups indicated by arrowheads in (A). The right immunostained cell, indicated by a single arrowhead in (A), shows that the cytoplasm of the perikaryon is stained but the nucleus is not. The cell on the left, indicated by the double arrowheads in (A), has a nucleus that is stained as well as the surrounding perikaryon. A rabbit polyclonal antibody raised against *G. bimaculatus* PDF (1:3,500) was used. Scale bars, 50 μm (A); 1 μm (B).

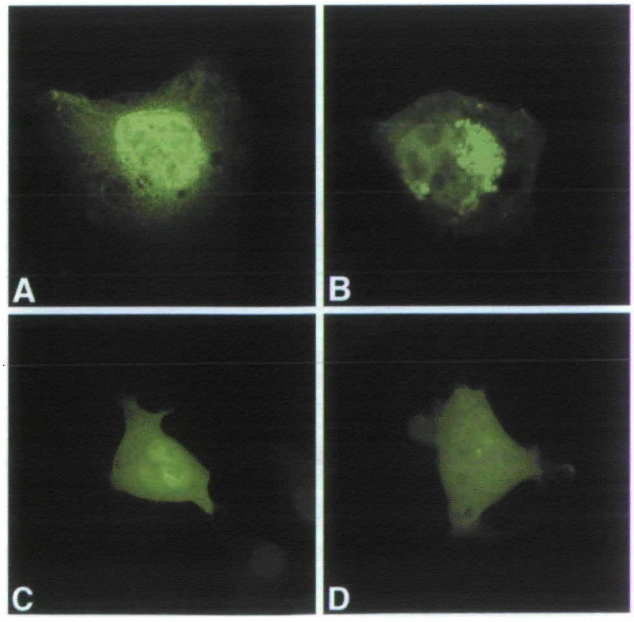

Fig. 6. **Laser scanning microscopy of COS-7 cells expressing GFP-fused PDF precursor proteins.** (A) PAP-PDF-GFP, (B) GFP-PAP-PDF, (C) PAP(NLS/3A)-PDF-GFP, and (D) GFP-PAP(NLS/3A)-PDF.

clock system in this species. There are thus a total of three functions now identified with differing degrees of certainty for the PDH family of peptides: pigment migration in crustaceans (*3*); transmission of cyclical information *via* output pathways from the insect's circadian clock, proposed to act *via* glial cells (*13*); and now in a nocturnal orthopteroid, an unknown function within the nuclei of circadian cells. The elucidation of such a nuclear function of PDF would stimulate investigation for understanding the details of the molecular mechanism of the central circadian oscillator systems.

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