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Molecular Cloning of *Bombyx* Cerebral Opsin (Boceropsin) and Cellular Localization of Its Expression in the Silkworm Brain

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We have cloned a cDNA for a novel opsin from the larval brain of the silkworm *Bombyx mori* in which the photoperiodic photoreceptor had been supposed to reside in the cephalic central nervous system (CNS). Its deduced amino acid sequence was composed of 381 amino acids and included amino acid residues highly conserved in insect visual pigments. This opsin belonged to the long wavelength photoreceptor group of insect opsins and showed the greatest degree of homology (84%) with the green visual photoreceptor in the sphingid moth. We have designated this *Bombyx* cerebral opsin as Boceropsin. Southern blotting experiments indicated that the Boceropsin gene is present in a single copy, and RT-PCR analysis revealed that Boceropsin mRNA is expressed in the larval brain but not in the subesophageal ganglion (Sg) or thoracic ganglion (Tg). Immunohistochemical analyses demonstrated that Boceropsin protein is present bilaterally in some defined cells localized in the brain of *Bombyx* larvae. This is the first report of expression of an opsin-based protein in CNS of an insect. The possibility that the Boceropsin functions as the photoperiodic receptive pigment in the silkworm is also discussed.

Key Words: **extraocular photoreceptor; photoperiodic photoreception; opsin; brain; *Bombyx mori*.**

Insects have developed highly specialized systems to adapt to seasonal changes. They use the annual cycle of changes in daylength as a calendar to synchronize their behavioral and physiological functions with the seasons. This photoperiodic response system has several principal components: a photoreceptor that interprets photic input, a clock that measures the photoperiod, memory that memorizes the photoperiodic signal, and a neurosecretory system that translates the memorized information into endocrine secretion (1). In insects, extraocular photoreceptors have been postulated to function in photoperiodic photoreception and to be brain-centered (2–5). However, few of the cellular localizations of the photoreceptor have not been determined in any insect and few of the photopigments involved have been elucidated at the molecular level.

In vertebrates there is increasing evidence for the existence of opsin-based photopigments in extraocular photoreceptors. Okano *et al.* (6) identified pinopsin as the chicken pineal photoreceptive molecule. Yoshikawa *et al.* (7) reported the expression of a photopigment P-opsin in the anterior nucleous preopticus of the toad. An opsin, melanopsin, was identified in photosensitive dermal melanophores of *Xenopus lavevis* (8). Recently, vertebrate ancient (VA) opsin of Atlantic salmon was shown to be expressed in the pineal organ and bilateral columns of subependymal cells of the deep brain (9). In insects, however, little evidence has been reported for the occurence of opsin-based photopigment in extraocular photoreceptive sites.

The silkworm, *B. mori,* shows the photoperiodic response for the maternal determination of embryonic diapause during the egg and larval stages (10). The photoreceptor involved in diapause induction has been demonstrated to be cephalic but extraocular (11, 12). *In vitro* culture experiments using isolated brain–subesophageal (Sg) complex preparations demonstrated that the photoreceptor of the photoperiodic clock is localized in the complex (13). The silkworm brain was found to have a GABAergic neural center controlling the diapause hormone secretion from the neurosecretory cells localized in Sg via circumesophageal connections (14, 15). It was demonstrated that dietary carotenoid or vitamin A is essential for the photoperiodic induction of the embryonic diapause in

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the silkworm (16) as reported in other insects (17, 18), and the silkworm brain was found to contain both retinal and 3-hydroxyretinal, which are chromophores of insect visual pigments (19). These observations suggested that there may be a retinal opsin-based pigment that functions as the photoperiodic photoreceptor in the silkworm brain. Here we report cloning of cDNA for an opsin in the silkworm brain and detection of the expression of this opsin (Boceropsin) in some cells of the brain by immunohistochemistry.

MATERIALS AND METHODS

cDNA cloning. A Chinese race (*Daizo*) of the silkworm, which shows a sensitive photoperiodic response during larval stages (12), was reared on mulberry leaves. Approximately 500 brains were dissected out carefully from newly ecdysed 5th instar larvae, frozen immediately in liquid nitrogen, and kept at −80°C until use. Total RNA was extracted from the brains by the acid guanidinium isothiocyanate phenol–chloroform (AGPC) method using Isogen (Nippon gene, Toyama, Japan).

Cloning was performed according to the method of Shimizu *et al.* (20). cDNA was made from the total RNA template using a first-strand cDNA synthesis kit (Life Sciences, Inc., St. Petersburg, FL). Reverse transcription of total RNA from the brains was performed using 5 μg of total RNA and 25 units of avian myeloblastosis virus (AMV) reverse transcriptase in a 25 μl cDNA reaction mixture. Aliquots of 1 μl of the cDNA solution were used for amplification with 1.5 units of Taq DNA polymerase (Gene Taq, Wako Chemical Corp.) using primers SF and SR on a DNA thermal cycler model 9600 (Perkin–Elmer). Two degenerate primers were designed on the basis of highly conserved regions [(E/D/A)QAKKM and (D/N)P(I/F)VY-(G/A)] of invertebrate opsins: SF [5′-CCCGAATTCG(M) (N)-CA(R)GC(N)AA(R)AA(R)ATG-3′] and SR [5′-CCCAAGCTTAT (N) (S)C(R)TA(N)AC(D)A(W) (N)GG(R)T-3′]. PCR was performed for 30 cycles of 30 s template denaturation at 94°C, 30 s primer annealing at 45°C, 1.5 min primer extension at 72°C. The PCR products were electrophoresed on 1% agarose gels, and the amplified DNA band was purified. The products (ca. 200-bp band) were cloned into pT7 Blue vector (Novagen: Takara Co., Ltd.) for sequencing. The sequencing reactions were carried out by the cycle sequencing method and analyzed with an ABI-373 automatic DNA sequencer (Applied Biosystems) according to the manufacturer's protocol. All sequences were determined in both directions.

The 3′-terminal sequence was cloned by the 3′-RACE (rapid amplification of cDNA ends) method. First-strand cDNA was synthesized using 10 μg of total RNA as described above. The resulting cDNA was amplified using the primers APT (5′-CGAGGTC-GACGGTATCGAAAGCTTGATTTTTTTTTTTTTTTT-3′) and BR1 (5′-GCTAAGGTGGCACTTATGAC-3′;1018 to 1027: Fig. 1). PCR was performed for 30 cycles of 30 s template denaturation at 94°C, 30 s primer annealing at 57°C, 1.5 min primer extension at 72°C. The products of this PCR were further amplified using a second set of primers APT and BR2 (5′-TGGTTCATGGCTTGGACACC-3′; 1048 to 1067). Finally the PCR products were amplified using a third set of primers APT and BR3 (5′-CAACTACACAGGAATATTGG-3′; 1080 to 1099). The amplified ca. 0.5-kb fragment was purified, cloned into pT7Blue, and sequenced as described above.

The 5′-terminal portion of the cDNA was cloned by the 5′-RACE method (5′ RACE System for Rapid Amplification of cDNA Ends, Gibco BRL, Gaithersburg, MD) using the primers GSBP1 (5′-CAAAGGACTGATCGGAGCGC-3′; 1122 to 1103), GSBP2 (5′-CCAATATTCCTGTGTAGTTG-3′; 1099 to 1080)) and nested GSBP3 (5′-GGTGTCCAAGCCATGA ACCA-3′; 1067 to 1048).

Finally, the complete cDNA coding sequence was amplified from the brain cDNA using two primers, B5-1 (5′-CAAGCTTCATC-CCCTTTCAA-3′; 125 to 144) and B3-1 (5′-CACACAGCCATGTC-GAAGTG-3′; 1520 to 1501), which were based on the sequences obtained by 5′- and 3′-RACE, respectively. The PCR products were amplified using a second set of nested primers, B5-2 (5′-GTGAAA-CAAGTAGAGATGTC-3′; 157 to 176) and B3-2 (5′-TTAGGAGGAG-GAGCAGCCTCATC-3′; 1478 to 1459). The amplified ca. 1.3-kbp fragment was subcloned and sequenced. A phylogenetic tree was constructed by the neighbor-joining (NJ) method using Clustal W (20).

Southern blotting analysis. A 461-bp cDNA probe was generated with primers BR1 and B3-2 by PCR amplification using subcloned cDNA as the template. Probes were labeled with random primers and Klenow fragment of *Escherichia coli* DNA polymerase using a random primer labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ) to obtain fluorescein-labeled DNA probes.

Total genomic DNA was isolated from the fatbody of the silkworm larvae according to the standard method. DNA was digested with *Bam*HI and *Hin*dIII, and aliquots of 2 μg were separated on 1% agarose gels, transferred onto nylon membranes (Hybond-N+, Amersham Pharmacia Biotech), and immobilized by UV cross-linking. Hybridization and detection were performed according to the protocol supplied with the detection kit (RPN3510; Amersham Pharmacia Biotech). Membrane-bound digested DNA was hybridized in rapid-hybridization buffer and the membranes were washed in 2× SSC for 20 min, 1× SSC for 15 min, and 0.5× SSC for 15 min (each buffer contained 0.1% SDS). Following stringent washing, the membranes were incubated with blocking agent and then with anti-fluorescein-AP. The chemiluminescence of the hybridizing band was detected using an ECL instant camera (Amersham Pharmacia Biotech).

One-step RT-PCR analysis. Brains, Sg, and first thoracic ganglia (Tg) of 5th instar larvae were dissected out and 50 of each tissue were collected together and analyzed immediately. mRNA was isolated from the tissues using a mRNA purification kit (Amersham Pharmacia Biotech); the procedures included disruption by the guanidinium isothiocyanate method and selective isolation of mRNA by oligo(dT)-cellulose chromatography. The polyadenylated mRNA was eluted with 200 μl of 10 mM Tris–HCl (pH 7.5) containing 1 mM EDTA. After DNAase treatment of the eluate, mRNA was precipitated by adding 95% ethanol, collected by centrifugation, and finally redissolved in 10 μl of DEPC-treated water.

One-step RT-PCR was performed using a kit purchased from Qiagen (Chatsworth, CA) according to the manufacturer's protocol. RT-PCR analysis was performed with primers BR1 and B3-2. The reaction mixture (50 μl) contained reverse transcriptase, Taq DNA polymerase, dNTP mixture (400 μM of each dNTP), 0.6 μM oligonucleotide primers, 2.5 mM $MgCl_2$, RNase inhibitor (10 units) and 1 μl of mRNA solution obtained by the method described above. As a control, 1 μl of DEPC-treated water was used instead of mRNA. First, reverse transcription reaction was performed at 50°C for 30 min, then the mixture was incubated at 95°C for 15 min to activate Taq DNA polymerase, and PCR amplifications were carried out. PCR was performed for 30 cycles of 1 min template denaturation at 94°C, 1 min primer annealing at 45°C, 1 min primer extension at 72°C. The resultant PCR products were visualized by ethidium bromide staining after 1% agarose gel electrophoresis. The amplified fragments of approximately 460 bp were purified, cloned, and sequenced.

Immunohistochemistry. One peptide corresponding to the carboxy-terminal 25 amino acids (CHSTTTDEASSVASGTTVMEEKPTA) of the deduced sequence was synthesized by Biomedical Center of Takara Shuzo Co., Ltd. (Shiga Pref., Japan) and a polyclonal antibody against the peptide conjugated with maleinimide-bovine serum albumin (BSA) was raised in mice.

Immunocytochemistry was performed according to the method described by Ichikawa *et al.* (14) with slight modifications. Brains and Sg of 5th instar female larvae were dissected out, and isolated organs were fixed in 4% paraformaldehyde in 0.2 M in phosphate

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AGTAGCCTCCACTGCCTCGGCAATCCTGCCACGCCACGCACCACGAGATTGGTTAGCTACAATAAAGACTGGAGTAGTTCGTTAAACGAT   90
TTTCTTCAGTATTTTGTAATAACCATTCTCAACACAAGCTTCATCCCCTTTCAAAATTTACCTTTCGTGAAACAAGTAGAGATGTCGATA  180
                                                                          M  S  I    3
TCTATGGACGCCGGTCCAGGCTTCGCAGCCCTACAGTCATGGAGTAGCCAAGTGGCAGCATTTGGAAACTCCAACCAAACCGTGGTGGAC  270
S  M  D  A  G  P  G  F  A  A  L  Q  S  W  S  S  Q  V  A  A  F  G  N  S  N  Q  T  V  V  D   33
AGAGTTTCGCCAGAAATGTTACATTTGATCGACGCTTACTGGTATCAATTTCCGCCCATGAATCCCTTATGGCATGCGCTATTGGGTTTT  360
R  V  S  P  E  M  L  H  L  I  D  A  Y  W  Y  Q  F  P  P  M  N  P  L  W  H  A  L  L  G  F   63
ACCATTGGAGTCCTCGGTTTCATTTCAATGATGGGCAACGGTATGGTCATTTACATCTTCATGACAACAAAGAATCTGAAAACACCATCG  450
T  I  G  V  L  G  F  I  S  M  M  G  N  G  M  V  I  Y  I  F  M  T  T  K  N  L  K  T  P  S   93
AACTTACTCGTAGTGAACTTAGCATTTTCTGATTTCCTAATGATGTGTGCCATGTCTCCAGCTATGGTAATTAATTGTTACAATGAAACT  540
N  L  L  V  V  N  L  A  F  S  D  F  L  M  M  C  A  M  S  P  A  M  V  I  N  C  Y  N  E  T  123
TGGGTTTTCGGACCATTCGCGTGTGAGCTATACGGTTGCGCTGGATCGCTATTTGGGTGTGCTTCAATTTGGACGATGACAATGATCGCT  630
W  V  F  G  P  F  A  C  E  L  Y  G  C  A  G  S  L  F  G  C  A  S  I  W  T  M  T  M  I  A  153
TTCGACCGCTATAATGTTATTGTGAAGGGAATTGCGGCAAAACCAATGACAAACAACGGAGCTCTTCTACGAATTCTCGGAATCTGGGCC  720
F  D  R  Y  N  V  I  V  K  G  I  A  A  K  P  M  T  N  N  G  A  L  L  R  I  L  G  I  W  A  183
TTCTCGTTGGCATGGACAGTGGCACCTTTCTTCGGTTGGAACAGATACGTACCTGAAGGTAATATGACTGCATGCGGTACTGACTACCTG  810
F  S  L  A  W  T  V  A  P  F  F  G  W  N  R  Y  V  P  E  G  N  M  T  A  C  G  T  D  Y  L  213
ACCAAAGATTGGTTTAGCCGAAGCTACATCGTTGTCTATTCCGTTTTTGTTTACTTTGCCCCGTTGCTTCTGATCGTCTACTCTTATTAC  900
T  K  D  W  F  S  R  S  Y  I  V  V  Y  S  V  F  V  Y  F  A  P  L  L  L  I  V  Y  S  Y  Y  243
TACATTGTACAGGCTGTGTCTGCTCACGAAAAAGCAATGAGGGAACAAGCGAAGAAAATGAACGTGGCATCTCTTAGATCGTCAGAAGCT  990
Y  I  V  Q  A  V  S  A  H  E  K  A  M  R  E  Q  A  K  K  M  N  V  A  S  L  R  S  S  E  A  273
GCTAACACTAGCACTGAATGTAAGCTAGCTAAGGTGGCACTTATGACAATTTCCCTGTGGTTCATGGCTTGGACACCTTACTTAGTCATC 1080
A  N  T  S  T  E  C  K  L  A  K  V  A  L  M  T  I  S  L  W  F  M  A  W  T  P  Y  L  V  I  303
AACTACACAGGAATATTGGAAAGCGCTCCGATCAGTCCTTTGGCTACTATCTGGGGCTCACTCTTTGCCAAAGCTAATGCTGTATATAAT 1170
N  Y  T  G  I  L  E  S  A  P  I  S  P  L  A  T  I  W  G  S  L  F  A  K  A  N  A  V  Y  N  333
CCGATTGTATATGGTATCAGCCACCCTAAATACCAAGCTGCTCTGTACAAAAGATTCCCAGTGCTTCAATGCCACTCAACGACTACTGAT 1260
P  I  V  Y  G  I  S  H  P  K  Y  Q  A  A  L  Y  K  R  F  P  V  L  Q  C  H  S  T  T  T  D  363
GAGGCCAGCTCTGTAGCTTCGGGCACTACTGTCATGGAAGAGAAACCGACAGCATAAGACGTCTACCTACTTCGTTTTTATGTCACATTT 1350
E  A  S  S  V  A  S  G  T  T  V  M  E  E  K  P  T  A  *                                    381
AAATTAGCTTAAAATCTTTTTGTATATACTGTTCGTATTATTTAACGATATAACATAAATGCTTCAAGTCAACAAATTCATATTGTGCAT 1440
GTTATTATATAATGTTAAGATGAGGCTGCTCCTCCTAAATTTAAAATTAATTGATTTATTCACTTCGACATGGCTGTGTGTAATGGAAAA 1530
ATGAATTATATCATAATTAATCTAAATAAAGCAATGTGGAATTCAACCACGT(polyA)                                 1582
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FIG. 1. Nucleotide and deduced amino acid sequences of Boceropsin (GenBank Accession No. AB064496). The amplified fragment produced by the first PCR using degenated primers (SF and SR) is shaded. The translational stop-codon is indicated by the asterisk, and the consensus sequence for polyadenylation signal is double-underlined (AATAAA). The predicted seven transmembrane segments are underlined. The 323th **K**-residue is the lysine residue binding the chromophore.

buffer (pH 7.4) for 24 h at 4°C. They were rinsed for 24 h in 0.1 M phosphate-buffered saline (pH 7.4) containing 0.2% Triton X-100 (PST) and incubated for 3 days at 4°C in the presence of the mouse antiserum diluted 1:200 in PST containing 0.25% BSA. Samples were washed in PST and soaked for 4 h in Alexa TM 488 anti-mouse IgG (H+L) antiserum (Molecular Probes, Inc., Leiden, The Netherlands) diluted 1:100 in PST. They were washed in phosphate-buffered saline, dehydrated with ethanol, and cleared with methylsalicylate. Immunoreactive cells were examined in whole-mount preparations and photographed with an Olympus BX-FLA (Olympus Inc., Japan). As controls for the specificity of the immunocytochemical procedure, samples were incubated without the primary antiserum or incubated with the primary antiserum inactivated by adding the synthetic 25-amino acid peptide.

RESULTS

Cloning of cDNA encoding opsin in the silkworm brain. To clone cDNA encoding opsin in the silkworm larval brain, PCR was performed using degenerate oligonucleotide primers (SF and SR) corresponding to amino acid sequences highly conserved in invertebrate visual pigments. These primers successfully amplified cDNAs of the visual pigments in the silkworm compound eyes (20). Amplification of DNA fragments of approximately 200 bp in length was observed by agarose gel electrophoresis. The nucleotide sequences of the fragments were determined using subclones, and were found to have the sequence (207 bp) of a novel opsin-like protein (Fig. 1).

Using primers based on the nucleotide sequences of the cloned fragment, we cloned the 5′- and 3′-portions of the cDNA. To determine whether these RACE products were actually parts of a single transcript, PCR was performed using primers corresponding to the sequences of the 5′- and 3′-untranslated regions obtained by RACE. We amplified one full-length cDNA fragment

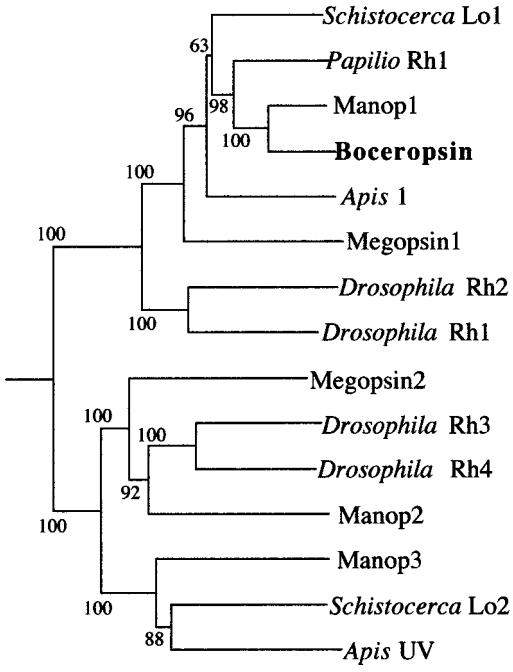

FIG. 2. Phylogenetic tree of insect opsins by the neighbor-joining method as an outgroup of rat opsin (S516779). Numbers at branching points indicate bootstrapping probabilities based on 1000 replications. Accession No: *Apis* 1, U26026; *Apis* UV, U70841; *D. pseudoobscura* Rh1, X65877; *D. pseudoobscura* Rh2, X65878; *D. pseudoobscura* Rh3, X65879; *D. pseudoobscura* Rh4, X65880; Manop1, L78080; Manop2, L78081; Manop3, AB001674; Megopsin1, AF189714; Megopsin2, AF189715; *Papilio xuthus* Rh1, AB007423; *Schistocerca* Lo1, X80071; *Scistocreca* Lo2, X80072.

of 1322 bp. The full-length cDNA contained a possible open-reading frame (ORF) encoding a putative protein of 381 amino acids (Fig. 1).

This deduced protein showed a high degree of homology with insect visual pigments (21). A database search indicated that this protein showed the highest degree of homology, 84%, with Manop1 (377 amino acids), one of the visual opsins found in the moth *Manduca sexta*. Similarities with insect visual pigments included a lysine (Lys-327) in the seventh transmembrane domain to serve as a site for the Schiff-base linkage of the chromophore and a pair of cysteines (Cys-131 and Cys-208) in the second and third extracellular loops to stabilize the tertiary structure by a disulfide bridge. An asparagine residue (Asn-28) susceptible to glycosylation was present in the N-terminal region. The C-terminal region of this protein was rich in serine (S) and threonine (T) residues as potential phosphorylation sites. Similarly to other opsins, this protein was predicted to have seven transmembrane segments (Fig. 1). We named this protein Boceropsin because it was isolated from the *Bombyx* cerebral ganglion (brain).

Figure 2 shows the molecular phylogenetic relationship between Boceropisn and other insect opsins. The insect opsins are divided into two groups: the long wavelength photoreceptor group including *Drosophila* Rh1 and Rh2, and the short wavelength photoreceptor group including *Drosophila* Rh3 and Rh4 (22). This phylogenetic tree shows that Boceropsin belongs to the short wavelength photoreceptor group.

Shimizu *et al.* (20) isolated two distinct fragments encoding two opsins, designated as Bomopsin1 and Bomopsin2 from the compound eyes of *B. mori*. The fragment of Bomopsin1, which was assumed to be a green receptor, consisted of 207 nucleotides encoding a sequence of 69 amino acids. The corresponding region of Boceropsin shows 85 and 96% homogeneity with Bomopsin 1 at the nucleotide and amino acid level, respectively.

Southern blotting analysis. A probe specific to the Boceropsin sequence was used for genomic Southern blotting analysis to determine the copy number of the gene. Digestion of the genomic DNA with *Bam*HI and *Hin*dIII was followed by hybridization with a Boceropsin cDNA-specific probe. Southern blotting analysis of digested genomic DNA revealed a single band in each lane (Fig. 3). This result indicated that the Boceropsin gene of the silkworm is present as a single copy.

RT-PCR analysis of Boceropsin mRNA expression. To investigate transcription of the Boceropsin gene in the brain, Sg and Tg, RT-PCR analysis was performed using mRNA isolated from these ganglia of the silkworm larvae. Amplification of a 461-bp fragment was detected in the brain as expected, but not in Sg or Tg (Fig. 4). The fragment was found to correspond to a partial sequence of the Boceropsin cDNA. Minor amplified bands of approximately 900 bp were found in three ganglia, but not in the water control.

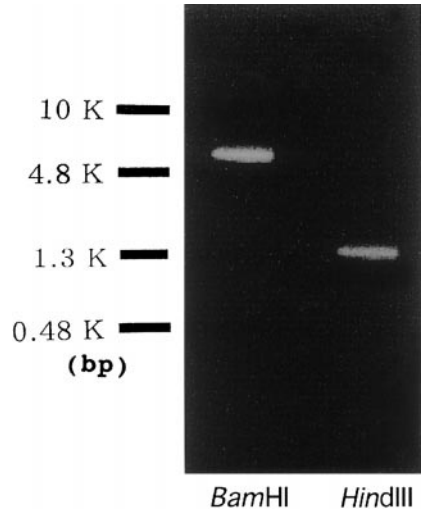

FIG. 3. Southern blotting analysis of the silkworm gene. Silkworm genomic DNA digested with *Bam*HI and *Hin*dIII was electrophoresed and hybridized with the 461-bp Boceropsin-specific probe. The sizes of markers are indicated on the left.

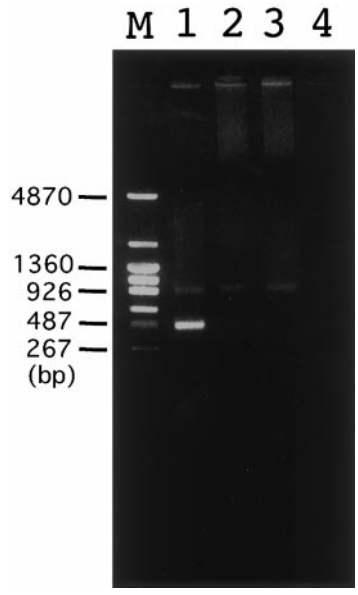

FIG. 4. RT-PCR analysis using mRNA isolated from brains (lane 1), subesophageal ganglia (lane 2), and first thoracic ganglia (lane 3) and water control (lane 4). PCR products were separated on a 1% agarose gel and the gel was stained by ethidium bromide. The sizes of markers are indicated on the left (lane M).

The detected bands were derived from cDNA and not from contaminating genomic DNA because we performed DNAase treatment of the mRNA solution and no bands were detected when reverse transcription (RT) was omitted (data not shown). These observations indicated that Boceropsin mRNA was expressed in the brain, but not in Sg or Tg.

Immunohistochemical localization of Boceropsin. Whole-mount immunohistochemistry was performed to localize the Boceropsin-producing cells in the silkworm brain. Some somata (15–20 mm in diameters) in the brain showed strong immunoreactivity to the mouse anti-Boceropsin antiserum (Figs. 5A–5D). In the anterior protocerebrum, there were two groups of bilateral Boceropsin-immunoreactive cells: dorsal anterior protocerebral (DAP) and ventral anterior protocerebral (VAP) cells. DAP cells had large somata and showed intense immunostaining compared to other immunoreactive cells. Boceropsin immunoreactivity was also found bilaterally in dorsal posterior protocerebral (DPP) cells and lateral posterior tritocerebral (LPT) cells. We found no Boceropsin-immunoreactive cells in Sg (data not shown). When samples were incubated without the primary antiserum or incubated with the primary antiserum inactivated by preincubation with an excess of the synthetic peptide, no staining was seen in the brain (data not shown).

DISCUSSION

We isolated one opsin (Boceropin) cDNA from the silkworm brain, which has been suggested previously to contain a photoperiodic receptor (13). The molecular phylogenetic tree showed that Boceropsin belongs to the long wavelength receptor group of insect opsins

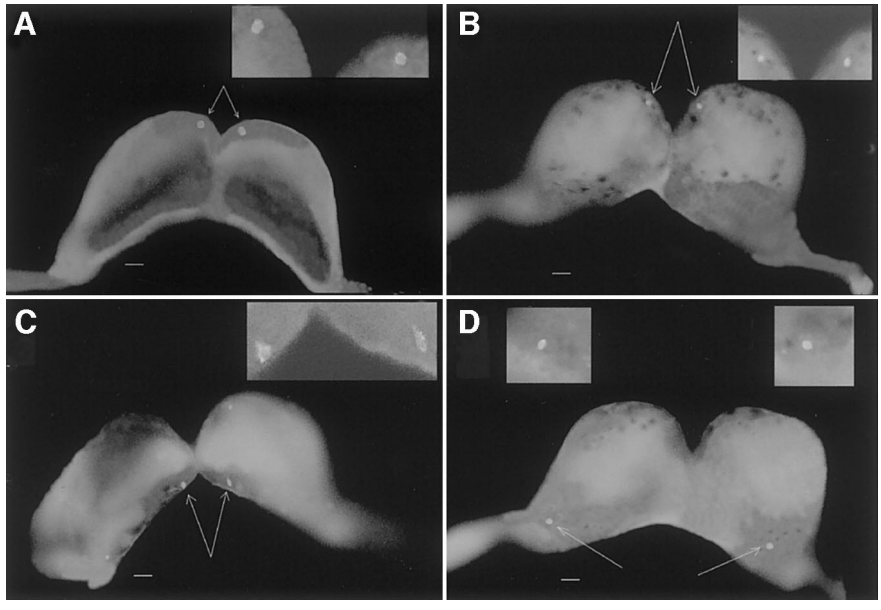

FIG. 5. Whole-mount immunohistochemical analysis of the *Bombyx* brain. Brains of 1-day-old fifth instar larvae were immunostained with Boceropsin antibody. Photographs (A–D) were taken at different planes to follow the immunoreactive cells indicated by arrows. Dorsal anterior protocerebral (DAP: A) and ventral anterior protocerebral (VAP: B), dorsal posterior protocerebral (DPP: C) and lateral posterior tritocerebral (LPT: D) cells were stained in the brain. Scale bars: 50 mm.

(Fig. 2). Boceropsin showed the highest degree of homology to Manop1, one of the *M. sexta* visual pigments. Chase *et al.* (23) suggested that Manop1 may be the opsin P520 identified by biochemical methods. These observations suggested that Boceropsin may function as a green-sensitive photopigment in the silkworm brain.

In principle, a properly conducted intensity-compensated action spectrum should correspond with the absorption spectrum of the pigment molecule involved. Action spectra for the photoperiodic response have been determined in some insect species and found to differ among different insects (1). For example, the work of Lees (24) with the aphid *Megoura viciae* showed maximal sensitivity in the blue region (450–470 nm) as reported in the silkmoth *Antheraea pernyi*, but that for the parasitic wasp *Nasonia vitripennis* was found to have the maximum in the green region (554–586 nm) (25). These diversities in the spectral sensitivity can be explained by the possible involvement of opsin-based photopigments absorption characteristics of which change markedly with the kinds of chromophores present and variations in opsin structure (21).

In a preliminary experiment, Kogure (10) observed that the silkworm was sensitive to blue-green (350–510 nm) light but not to red light (>600 nm). Nakajima (26) carried out "day interruption" experiments using different wavelengths of light during the photoperiodic sensitive stage and examined the interruption effects on the diapause incidence of the next generation. He found that the 500–550 nm wavelength region was most effective to accomplish a long-day photoperiod. This pronounced green sensitivity in the *Bombyx* photoperiodism suggested that Boceropsin, which has been assumed to be a green receptor on the basis of the molecular phylogenetic tree, is involved in photoperiodic photoreception.

In some insects, photoperiodic photoreceptors were reported to reside within the brain. Using *A. pernyi*, Williams (27, 28) suggested that the dorsal region of the protocerebrum containing the lateral neurosecretory cells is essential for photoperiodic reception. Lees (29) identified the site of the photoperiodic receptor controlling the production of sexual and parthenogenetic females of the aphid *M. viciae* by the micro-illumination method. He found that photosensitivity was confined to the central region of the cephalic dorsum covering the protocerebrum region. A histological study also suggested that the photoperiodic photoreceptor in the aphid might be located in the dorsal anterior of the protocerebrum lateral to Group I neurosecretory cells (30). Recently, Gao *et al.* (31) attempted to identify the brain photoreceptor in *M. viciae* immunocytochemically using antibodies directed against invertebrate and vertebrate opsins and phototransduction proteins. They found that some antibodies labeled lateral parts of the protocerebrum in addition to an anterior ventral neurophil region. Further, multilayer structures that resemble the brain lamellar photoreceptors of some invertebrates were found in the near vicinity of Group I neurosecretory cells in the aphid (32). Cymborowski and Korf (33) investigated S-antigen (arrestin)-immunoreactivity as a marker for the extraocular photoreceptor in the blowfly *Calliphora vicina* and found one pair of the immunoreactive cells localized in the dorsal anterior protocerebrum region. These reports suggest the localization of the extraocular brain photoreceptor in the protocerebral region in insects. Thus, it is reasonable to speculate that the dorsal anterior (DAP) and/or ventral anterior (VAP) cells are the most likely candidates for the photoperiodic receptor in the silkworm.

A major function of extraocular photoreceptor is to receive the photic information of the time of day or daylength and connect the information to the neuroendocrine system (34). In the silkworm, the photoperiodic signals are received by the mother moth during her embryonic and larval stages and the information is kept in an as yet undefined neural region until the pupal stage when diapause hormone should be secreted. In this connection, it was reported that microsurgical operation at the early pupal stage of the cortex of the anterolateral area on the ventral side of protocerebrum, which is in the vicinity of DAP and VAP cells, disturbed the control of diapause hormone secretion (35). We also performed surgical microexcision of the anterior protocerebrum in the brain of the diapause egg-producers at the pupal stage and confirmed the disturbance of diapause hormone secretion (unpublished observation). These observations suggested that the components responsible for the photoperiodic response concerning the diapause phenomenon reside in this confined region and strengthened the idea that DAP and/or VAP cells are the photoperiodic receptors.

Within the imaginal optic lobes of homo-metabolous insects, the remnants of the larval stemmata photoreceptors were observed (36, 37). They were reported to retain the morphological and photoreceptive functions there. In the silkworm, the photoreceptor cells of the larval stemmata move into the brain during larval-pupal ecdysis (38). We confirmed by anatomical inspection that the larval stemmata of the silkworm does not move to the brain during larval–larval ecdysis (data not shown), and so it is unlikely that these Boceropsin-immunoreactive cells originated from the stemmata photoreceptors.

In some insects, extraocular photoreceptors are known to function in the circadian rhythm as well as photoperiodism and to be brain-centered (34). It was suggested that opsin-based photopigments are not involved in insect circadian photoreception because carotenoid-deprived *Drosophila peudoobscura* (39) and *B. mori* (40) exhibited normal rhythm, although the

photoperiodic response was lost in the latter (16). Recently, a flavoprotein cryptochrome (CRY) has been proposed as the photoreceptive pigment in the circadian photoreception of *Drosophila* (41, 42). Boceropsin is considered not to be involved in the circadian photoreception of the silkworm.

The present study provided a molecular and morphological basis for the further physiological experiments required for definite functional identification of Boceropsin expressed in the silkworm brain. An interesting question to be addressed in future investigations is whether there is a family of insect cerebral opsins "Insceropsin" including Boceropsin that function as extraocular photopigments in the insect brain.

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