Cloning of genes encoding the visual pigments in the silkworm, *Bombyx mori*

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(Received 17 October 1997; Accepted 25 November 1997)

Abstract

The polymerase chain reaction (PCR) was performed with cDNA from the silkworm compound eye and primers designed to amplify a region of the opsin-encoding cDNA. Two distinct fragments which encode two opsins, designated Bomopsin1 and Bomopsin2, were isolated. The fragments of Bomopsin1 and 2 consisted of 207 nucleotides (nt) encoding a sequence of 69 amino acids (aa), and 213 nt encoding 71 aa, respectively. The deduced amino acid sequences of Bomopsin1 and 2 showed 100–68% identity and 94–63% identity to the corresponding region of other insects opsin. The high identities between *Bombyx* and *Manduca* opsins suggest that Bomopsin1 is a green-sensitive pigment and Bomopsin2 is a UV-sensitive pigment in the silkworm eye.

Key words: Opsin gene, *Bombyx mori*, compound eye, visual pigment

INTRODUCTION

Vision is an essential sense for most animals. Opsin forms the protein component of visual pigments in the eyes, and opsins are ubiquitous in the animal kingdom (Lythgoe, 1979). The opsin gene family thus provides an excellent opportunity to study gene evolution in the divergence of biological functions (Iwabe et al., 1996). The nucleotide sequences and amino acid sequences of over 20 vertebrate opsins have been reported (Hisatomi et al., 1994; Okano et al., 1995). Invertebrate opsin-encoding genes from cephalopods (Hara-Nishimura et al., 1993), crustacea (Hariyama et al., 1993; Sakamoto et al., 1996) and some insects have been reported.

Of the holo-metabolic group of insects, the opsin-encoding gene sequences of the fruitflies *Drosophila melanogaster* (O'tousa et al., 1985; Zuker et al., 1987; Montell et al., 1987) and *D. pseudoobscura* (Carulli and Hartl, 1992), the blowfly *Calliphora erythrocephala* (Huber et al., 1990), the tobacco hornworm *Manduca sexta* (Chase et al., 1996), the ants, *Camponotus abdominalis* and *Cataglyphis bombycina* (Popp et al., 1996), and the honeybee *Apis mellifera* (Chang et al., 1996; Bellingham et al., 1997)

have been reported. The opsin-encoding genes of the mantid *Spododromantis* sp. (Towner and Gartner, 1994) and locust *Schistocerca gregaria* (Gartner and Towner, 1995), which belong to the semi-metabolic group of insects, were also reported. These opsin-encoding nucleotide sequences differ from each other and are diverse among insect species (Gartner and Towner, 1995).

The silkworm, *Bombyx mori*, has been raised by humans since antiquity and has become thoroughly domesticated (Tazima, 1987). *B. mori* differs from wild lepidopteran species in some behavioral characteristics; e.g., *B. mori* has lost the ability to fly. It is of interest to determine how the domestication of *B. mori* has influenced the structure of this insect's visual pigment genes. In the present preliminary study, we examined silkworm cDNA encoding an essential part of opsin, which has the lysine-residue binding a chromophore and is responsible for the spectral tuning of the visual pigment. We then compared the nucleotide and amino acid sequences of this silkworm cDNA with those of other insect opsins.

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MATERIALS AND METHODS

Animals. Commercial silkworms (Kinshu × Showa) were reared on mulberry leaves. About 100 adult retinas were dissected from newly eclosed moths, quickly frozen in liquid nitrogen and stored at -80°C until use.

Preparation of cDNA and amplification by PCR. Opsin cDNA fragments were amplified using the polymerase chain reaction (PCR) method described previously (Sakamoto et al., 1996; Shimizu et al., 1995) with slight modification. The total RNA of the retinas was prepared by guanidinium thiocyanate extraction followed by centrifugation through a cushion of CsCl. cDNA was made from this total RNA template using an oligonucleotide primer [T-Primer; 5'-GCCGAATTCGTCGACAAGCTTTTTTTTTT-3']. The reaction mixture was composed of 10 μg of denaturated total RNA template, 20 pmol of T-primer, 1,000 units of reverse transcriptase (SuperScript RT, 8053SA, Gibco/BRL), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 10 mM dithiothreitol and 0.5 mM each of the four dNTPs in a total volume of 100 μl , and incubated at 42°C for 2 h.

For the PCR, the reaction mixture contained 50 pmol of each primer, 0.2 mM of each dNTPs, 1 unit of *Taq* DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl_2 , 0.1% Triton X-100, 5 μl of cDNA solution and sterilized pure water. The final volume was adjusted to 50 μl . To amplify the DNA, thermal cycling was performed for 40 cycles of denaturation (94°C for 1 min), annealing (45°C for 1.5 min), and extension (72°C for 3 min). The PCR products were cloned into pUC 18 plasmid vector for sequencing. We designed the following two degenerated oligonucleotide primers corresponding to amino acid sequences [(E/D/A)QAKKM and (D/N)P(I/F)VY(G/A)] which are highly conserved in invertebrate visual pigments: primer F (5'-CCC GAATTCG(AC)(AGCT)CA-(AG)GC(AGCT)AA(AG)AA(AG)ATG-3' and primer R (5'-CCCAAGCTTAT(AGCT)(GC)C-(AG)TA(AGCT)AC(AGT)A(AT)(AGCT)GG(A-G)T-3').

Sequencing and data analysis. The sequencing reactions were carried out by the cycle sequencing method, according to the manufac-

turer's protocol (Applied Biosystems). All sequences were determined in both directions with the use of an ABI-373S automatic DNA sequencer (Applied Biosystems). The alignment of the amino acid sequences and the construction of a phylogenetic tree by the neighbor-joining method (Saitou and Nei, 1987) were carried out using software programs of Clustal W (Thompson et al., 1994). The corresponding region of octopus opsin was used as the outer group.

RESULTS

The PCRs were carried out using degenerated oligonucleotide primers corresponding to amino acid sequences highly conserved in invertebrate visual pigments. Amplification of a DNA fragment of approximately 260 nt in length was observed by electrophoresis on an agarose gel. Using this amplified DNA band, we tried to clone the fragment of cDNA encoding the visual pigments of the silkworm.

The nucleotide sequence of the fragment was determined from about 30 clones, and two kinds of fragments were found to encode two distinct opsins, designated Bomopsin1 and Bomopsin2. The fragments of Bomopsin1 and Bomopsin2 were composed of 207 nt and 213 nt, respectively. In addition to the nucleotide-length difference, the two opsin sequences were relatively divergent, being only 43% identical to each other after a match of the alignments. The nucleotide sequences of the isolated fragments were compared with opsins of *Manduca sexta* (Fig. 1). Bomopsin1 and Bomopsin2 were found to be closely related to *Manduca* opsins Manop1 (83% identical) and Manop2 (76% identical) (Chase et al., 1996), respectively.

The deduced amino acid sequences of Bomopsin1 and 2 were aligned with other insect opsins (Fig. 2). The alignment of amino acid sequences was carried out using a software of Clustal W. There was no amino acid replacement (100% identity) between Bomopsin1 and Manop1, at least in the region compared. Four amino acid replacements were found between Bomopsin2 and Manop2, but there was still a high identity (94%) between these two opsins. Bomopsin2 shared identities of 63%, 75% and 72% with locusta Schiop2, Drosop3 and Drosop4 (Gart-

(A)

	10	20	30	40	50	60
Bomop1	AACGTCGCCTCTCTCAGATCATCTGAAGCAGCCAAACACCAGCGCTGAATGCAAACCTCGCC					
Manop1	AATGTGGCCTCCCTCAGATCTTCAGAAGCGGCAAAACACCAGCGCTGAATGCAAACCTAGCC					
	** ** *					
	70	80	90	100	110	120
Bomop1	AAGGTGGCATTGATGACAATTTCCCTGTGGTTCATGGCTTGGACACCTTATTTGGTTATA					
Manop1	AAGGTGCATTAATGACCATTTCCTTATGGTTCATGGCGTGGACACCATACTTGTTCATC					
	***** *					
	130	140	150	160	170	180
Bomop1	AACTATACAGGGGTGTTTGAAAGCGCTCCGATCAGTCCTCTGGCCACCATTGGGGATCG					
Manop1	AACTATACCGGAGTGTGTTGAAAGCGCACCCATTAGCCCTCTGGCCACTATTGGGGCTCC					
	***** *					
	190	200	207			
Bomop1	CTCTTTGCCAAAGCTAACGCTGTGTAT					
Manop1	CTCTTCGCCAAGGCTAACGCCGTCTAC					
	***** *					

(B)

	10	20	30	40	50	60
Bomop2	AACGTGGAATCTTTGCGAGCGAATCAGAGTGGGGCTCTCAATCCGCCGAAATTAGAATA					
Manop2	AACGTAGAATCTCTAAGAGCTAATCAGGGCGGGTCATCAGAGTCGGCAGAGATCAGAATA					
	***** * ***** * * * * * * * * * * * * * * *					
	70	80	90	100	110	120
Bomop2	GCGAAAGCGGCGCTTACTGTATGTTTCTTATTTGTGGCATCCTGGACACCATAACGGAGTG					
Manop2	GCAAAAGCAGCACTCACTGTTTGTCTTCTGTTTCGTGGCCTCGTGGACACCCTACGGCGTT					
	** ***** *					
	130	140	150	160	170	180
Bomop2	ATGGCACTGATAGGTGCTTTTGGAGACCAGCAACTCTTGACTCCGGGGGTAACGATGATA					
Manop2	ATGGCTCTCATAGGCGCGTTTGGAAATCAACAACCTTCTTACTCCTGGGGTTACGATGATT					
	***** *					
	190	200	213			
Bomop2	CCCGCCGTCGCGTGTAAGCTGTGCTTGTATA					
Manop2	CCTGCAGTGGCGTGTAAGCTGTAGCGTGATT					
	* * * * * * * * * * * * * * * * * * * *					

Fig. 1. Comparison of nucleotide sequences of cDNA fragments encoding one region of the silkworm Bomopsins, (A) Bomopsin1 (Bomop1) and (B) Bomopsin2 (Bomop2), with the *Manduca sexta* opsins, (A) Manop1 and (B) Manop2 (Chase et al., 1996). The conserved nucleotides are indicated by asterisks.

ner and Towner, 1995), respectively. It was found that 18 aa, which included the lys-residue (66K in Fig. 2) binding a chromophore, were conserved among all opsin fragments compared. A hypothetical phylogenetic tree was constructed to examine the relatedness among the deduced amino acid sequences (Fig. 3). The tree showed that insect visual pigments have evolved along two lines. The phylogenetic positions of locust Shio, mantid Camop and Hymenoptera opsins (Apisop and Camop) were ambiguous be-

cause of the low bootstrap probabilities on the branch. In each group, however, *Bombyx* and *Manduca* opsins, and *Drosophila* opsins made a clade with each other with high bootstrap probabilities.

DISCUSSION

The diversity in spectral sensitivities in animal vision is mediated through variation in the photosensitive visual pigments (opsins) of the eye (Lythgoe, 1979). Shifts in the absorption of

	1	20	40	60	71
Bomop1	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Manop1	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Schiop1	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Sphop	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Apisop	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Camop	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Drosop1	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Drosop2	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Bomop2	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Manop2	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Schiop2	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Drosop3	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG
Drosop4	NVASLRSEAA	N-TSAECKLAKVAL	MTISLWFM	AWTPYL	VINYTG

Fig. 2. Comparison of deduced amino acid sequence of silkworm Bomopsins (Bomopsin1 and Bomopsin2) fragments with those of other insect opsins. The opsin nomenclature is as follows. Schiop1, 2, *Schistocerca gregaria* opsins (Gartner and Towner, 1995); Sphop, *Sphdromantis* sp. opsin (Towner and Gartner, 1994); Apisop, *Apis mellifera* opsin (Bellingham et al., 1997); Camop, *Camponotus abdominalis* opsin (Popp et al., 1996); Drosop1 (Rh1), 2 (Rh2), 3 (Rh3), 4 (Rh4), *D. melanogaster* opsins (Gartner and Towner, 1995). Alignment of the amino acid sequences was carried out using the software program Clustal W. The conserved amino acids are indicated by asterisks. The 66th K-residue is the lysine-residue binding the chromophore.

visual pigments are the result of interactions within the binding pocket of the opsin between

Fig. 3. Phylogenetic tree of insect opsins constructed based on amino acid identities. The neighbor-joining (NJ) method was employed using a software program Clustal W. Octopus opsin (Genebank: X07797) was used as the outer group. The value given to each branch is the bootstrap probability (in %).

amino acid side chains and the retinal chromophore (Okano et al., 1995). The DNA fragments which were cloned in the present study encode a region of opsin protein in which the lysine-residue binding a chromophore (3-OH-retinal) of the silkworm is present (Hasegawa and Shimizu, 1988). The genomic DNA that encodes this region, which is responsible for the spectral tuning of visual pigments (Neitz et al., 1991), corresponds to the exon-4 of vertebrates opsin-encoding gene. Consequently, studies focusing on this region of opsin genes should give information about the evolutionary relationship among the visual pigments of animals (Hisatomi et al., 1994).

Eguchi et al. (1985) reported that there was no difference concerning the structures and functions of the compound eyes between *B. mori* and *B. mandarina* which is the most closely related wild silkworm to *B. mori*. This finding implies that visual pigments function normally in the eyes of the silkworm. Eguchi et al. (1985) suggested in an electrophysiological study the presence of at least two visual pigments (green and UV-sensitive pigment) in the silkworm compound eyes. In *Manduca*, three opsins (P520, P450 and P357) have been characterized by difference spectra taken from the retinal slices and extracts (Bennett and Brown, 1985; Bennett et al., 1997). Chase et al. (1996) suggested that Manop1 and Manop2 may be the opsin P520 and UV-sensitive photopigment P357 of *Man-*

duca, respectively. The high identities between Bomopsins and Manops imply that Bomopsin1 may be a green-sensitive pigment and that Bomopsin2 may be a UV-sensitive pigment in the silkworm eyes.

The phylogenetic tree showed that there exist at least two distinct groups of insect opsins: insect visual pigments have evolved along two lines, as expected from the difference in sequence length (Fig. 3). Bomopsin1 belongs to the same group of Drosop1 (Rh1) and Drosop2 (Rh2) showing maximum absorbance around 450–480 nm, and Sphop, which is speculated to be a 500–520 nm absorbance pigment (Towner and Gartner, 1994). Bomopsin2 belongs to another group in which Drosop3(Rh3) and Drosop4 (Rh4) exhibiting spectral sensitivity in the UV range are clustered (Gartner and Towner, 1995). These lines of evidence also support the idea that Bomopsin1 and Bomopsin2 are long and short wavelength-sensitive pigments, respectively.

Among the opsins compared, 18 aa residues were conserved (Fig. 2); these amino acids are probably essential in the primary structure for the function of insect visual pigments. It is interesting that a 69 aa sequence of Bomopsin1 was completely identical to that of Manop1, though the nucleotide sequences were different from each other (83% identical): all nucleotide substitutions found between two opsins were synonymous. This finding suggests that this amino acid sequence has been highly conserved to maintain the function of one opsin in the lepidopteran moths. To elucidate the visual function of the Bomopsins, studies to identify the full length of the cDNA encoding the Bomopsins are under way in our laboratory.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-Aid for Scientific Research (No. 08640861) from the Ministry of Education, Science, Sports and Culture of Japan. The authors thank to Professor Russell G. Foster (Imperial College, UK) for discussions on the work in this paper.

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