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Preferential Expression of the Gene for a Putative Inositol 1,4,5-Trisphosphate Receptor Homologue in the Mushroom Bodies of the Brain of the Worker Honeybee *Apis mellifera* L.[1]

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A gene expressed preferentially in the mushroom bodies of the brain of the worker honeybee *Apis mellifera* L. was identified by the differential display method and its cDNA was isolated. The cDNA fragment of 534 bp (clone A1) contained an open reading frame encoding 177 amino acid residues having 78, 72, 70, 59 and 55% sequence identities with the inositol 1,4,5-trisphosphate (IP3) receptors of *Drosophila melanogaster*, *Xenopus laevis* and humans (types 1, 2 and 3), respectively, suggesting that it encodes a putative IP3 receptor homologue of the honeybee. *In situ* hybridization revealed that the gene encoding clone A1 was expressed preferentially in the mushroom bodies and not in the optic lobes, antennal lobes and central bodies; in the mushroom body, it was expressed strongly in the large type Kenyon cells and weakly in the small type Kenyon cells. Reverse transcription polymerase chain reaction analysis showed that the gene was expressed strongly in the head and weakly in the antennae, legs, thorax, and abdomen. These results suggest that the A1 gene product plays a crucial role in neural transmission in the mushroom bodies of the worker bee brain.

The honeybee is a social insect and its society is composed of a queen, drones and workers, each with their own physiological states and behaviours (1). Various exquisite communications are performed by these colony members. It is well known, for example, that worker bees can communicate by dance language; workers which have been foraging for nectar in flowers return to the hive and perform a dance that contains information related to both the distance and the direction of the food source which is understood by other foragers (1, 2). However, nothing is known about molecular basis of such highly advanced behaviors of the honeybees.

The insect central nervous system consists of a brain and a central nerve cord. The paired mushroom bodies (corpora pedunculata) of the brain are generally considered to be the main association, decision-making and memory centers (3-5). The mushroom bodies of the worker honeybee are characteristically well-developed compared with those of other insects. The ratio of the volume of the mushroom bodies to the volume of the worker bee brain is about 12%, whereas those of *Musca domestica* (fly) and *Schistocerca gregaria* (locust) are only about 2% (6). There are two calyces in each mushroom body, which each consist of two morphologically distinct types of intrinsic neurons, large type (diameter 6-7 mm) and small type (diameter 4-5 mm) Kenyon cells, whose fibers have distinct projection patterns (6-8). By contrast, in *Drosophila*, only one calyx is present in each mushroom body, and it contains neurons that are morphologically indistinct (9). The cellular bases of decision-making and memory are thought to be associated with chemical transmitters operating in complex local synaptic circuits of the mushroom bodies, and the ability of the worker bee to perform highly advanced behaviors may be due to the increased complexity, in structure and function, of the mushroom bodies.

To identify the gene(s) involved in the highly advanced behaviors of the honeybees, we tried to isolate gene(s) that are expressed preferentially in the mushroom bodies of the worker bee brain by the differential display method (10-12).

[1] The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL and GenBank databases under the accession number AB006152.

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Abbreviations: PCR, polymerase chain reaction; RT, reverse transcription; IP3, inositol 1,4,5-triphosphate; DIG, digoxygenin.

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MATERIALS AND METHODS

Identification of differentially expressed RNAs using differential display. Mushroom bodies and optic lobes were dissected out from heads of worker bees under a microscope, using fine scissors and tweezers, and the total cellular RNA was extracted by guanidium isothiocyanate-phenol chloroform extraction (13). Differential display was performed essentially as described previously (10-12). Briefly, samples of 2 μg RNA from mushroom bodies and optic lobes were each treated with 0.5 units of RNase-free DNase I in 20 mM Tris/HCl, pH 8.4, containing 50 mM KCl, 0.5 mM dNTPs, 10 mM dithiothreitol and 2.5 mM $MgCl_2$ in a total volume of 40 μl, and then reverse transcribed with or without 200 units of Super Script II (Stratagene) with 40 pmole of an anchored oligo(dT) primer with an *Bam* HI site (Bam-TG primer, 5′-CCCGGATCCT$_{15}$G). The resulting samples were amplified by the polymerase chain reaction (PCR) with 20 combinations of arbitrary 10mers with a *Hind* III site (*Hind*-1 to 20 primers, 5′-CGGGAAGCTTN$_{10}$-3′ where N is degenerate in all bases) and *Bam*-TG primer. The reaction mixtures (10 μl) contained 1/10th by volume of the reverse transcription products, 0.2 μl of 20 μ M Bam-TG primer, 0.2 μl of a *Hind*-1 to 20 primer at 20 mM, 1 μl of 10 × PCR buffer, 0.5 μl of [α-^{32}P]dCTP (> 37 GBq/μM), 0.05 μl of *Taq* Gold polymerase (5 units/μl, Perkin Elmer). The PCR conditions were: (95°C × 10 min + 37°C × 5 min + 72°C × 5 min) × 1 cycle + (95-°C × 15 sec + 55°C × 1 min + 72°C × 1 min) × 25 cycles + 72°C × 5 min. The PCR products were separated on a denatured 6% polyacrylamide gel and exposed to Kodak X-ray film. Reactions without reverse transcriptase were used as control experiments to detect bands amplified from a genomic DNA contaminating the RNA preparations. To ensure that the electrophoresis profile was reproducible, duplicate reverse transcription (RT)-PCR reactions were performed.

Subcloning and sequencing on PCR products. Bands of interest were excised, the gel was boiled in TE (1 mM Tris/HCl, pH 7.4 and 1 mM EDTA) and the DNA was reamplified by PCR with the primer combination used in the differential display method under the same PCR conditions except that the concentration of the dNTPs was 200 μM, the first cycle was omitted and no radioisotope was used. The reamplified DNA was subcloned into a pGEM-3zf(+) vector at the *Sma* I site and transfected into *Escherichia coli* JM109. The nucleotide sequence of each strand was determined by the chain-termination method (14) using Sequenase (Stratagene).

In situ hybridization. In situ hybridization was carried out as described previously (15) with minor modifications. Frozen sections (10 μm) of the worker bee brain were fixed with 4% paraformaldehyde in phosphate buffered saline, and then hybridized with digoxygenin(-DIG)-11-UTP-labeled single-strand sense or antisense RNA probes. The DIG-labeled RNA probes were prepared using DIG RNA Labeling Kit (Boeringer Mannheim). A 513 bp (+22 to +534) fragment of clone A1 was amplified by PCR and subcloned into a pGEM-3Zf(+) plasmid at the SmaI site to remove sequences corresponding to the primers used in the differential display method, and was used as a template to generate RNA probes. This plasmid was linearized with *Eco* RI and *Bam*HI, and transcribed using SP6 RNA polymerase and T7 RNA polymerase to generate 548 and 587 b antisense (cRNA) and sense probes, respectively.

RT-PCR. mRNA (0.1μg) extracted from whole head, thorax, abdomen, legs and antennae using QuickPrep Micro mRNA Purification Kit (Pharmacia) were reverse transcribed using Superscript Preamplification System for First Strand cDNA Synthesis (Gibco BRL). The primers used for PCR were +1 to +23 and +512 to +533 of the A1 clone. The PCR product (533 bp) was analyzed by agarose gel electrophoresis. As a control, two primers for a partial cDNA for honeybee cytoskeltal actin isolated previously (16) were used to generate a 393-bp PCR product.

RESULTS

To identify gene(s) expressed preferentially in the mushroom bodies of the worker bee brain, we employed the differential display method (10-12) using total RNAs from mushroom bodies and optic lobes of the worker bees. Optic lobes were used as control organs to detect genes expressed ubiquitously in neural tissues. By screening approximately 1,000 bands on the sequence gels with 20 combinations of arbitrary primers (*Hind*-1 to 20 primers) and *Bam*-TG primers, seven candidate bands were identified. Among them, a band termed A1 was detected almost specifically in the mushroom bodies by a combination of the *Hind*-1 primer (5′-CGGGAAGCTTATCGACTCCAAG-3′) and the *Bam*-TG primer. The band was excised from the gel and the DNA was reamplified by PCR with the same primers, and subcloned into a pGEM vector. We determined the nucleotide sequences of two independent clones and found that they were the same. The isolated clone was 534 bp long (clone A1) and contained an open reading frame encoding 177 amino acid residues, as shown in Fig. 1.

We searched the databases and found that it shared 78, 72, 70, 59 and 55% sequence identities with parts of the cytoplasmic domains of IP3 receptors from *Drosophila melanogaster* (17, 18), *Xenopus laevis* (19) and humans (types 1, 2 and 3) (20, 21), as shown in Fig. 2. Although we have not yet isolated a full-length A1 cDNA clone, the high homology between these sequences strongly suggests that the A1 clone is part of an IP3 receptor cDNA of the honeybee.

To confirm that the A1 gene is expressed preferentially in mushroom bodies, we performed *in situ* hybridization analysis using frozen sections of the worker bee brain with a DIG-labeled antisense RNA probe. As shown in Fig. 3A and B, expression of the A1 gene was clearly detected in the mushroom bodies but not in any other parts of the brain, including the optic lobes, antennal lobes, and central bodies. In the mushroom bodies, the A1 gene was expressed strongly in the cell bodies of the large type Kenyon cells that are located at both edges of the inside and outside of each calyx, and weakly in the cell bodies of the small type Kenyon cells that are located in the inner core and on the undersurface of the calyces (6), as shown in Fig. 3C. No significant hybridization was observed in any part of the brain in control experiments with sense RNAs as probes (data not shown).

We next examined the expression of the A1 gene in other body parts of the adult worker bees by RT-PCR using two primers corresponding to +1 to +23 and +512 to +533 of the clone A1. As shown in Fig. 4A, a discrete band corresponding to 533 bp was detected strongly in the heads and weakly in the thorax, abdomen, legs and antennae of the worker bees. No bands were detected in any body parts when the RT reaction

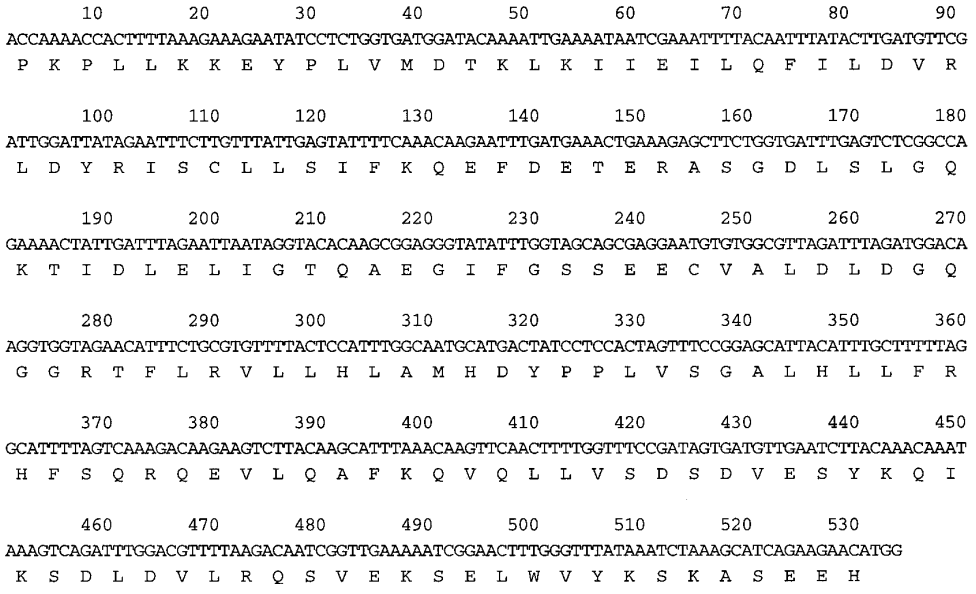

FIG. 1. Nucleotide and predicted amino acid sequences of clone A1. *Hind*- 1 and *Bam*-TG primer sequences found in the 5′- and 3′-flanking regions, respectively, have been omitted.

was omitted (Fig. 4B), indicating that the bands were due to mRNA and not to DNA contaminating the RNA preparation. The gene for cytoskeletal actin used as a control (16) for mRNA preparation and the RT reaction was detected almost equally in all organs examined, as shown in the bottom panels of Fig. 4. Therefore, the A1 gene is predominantly expressed in the head of the worker bees.

DISCUSSION

Our results suggest that the A1 gene product (a putative IP3 receptor homologue of the honeybee) plays a crucial role in neural transmission in the mushroom bodies of the worker bee brain, and is needed more in the large type Kenyon cells than in the small type Kenyon cells.

FIG. 2. Comparison of the amino acid sequences of the A1 gene product and the IP3 receptors of *Drosophila melanogaster* (17, 18), *Xenopus laevis* (19) and humans (types 1, 2 and 3) (20, 21). Gaps were introduced to obtain maximal matching. Numbers indicate the positions of amino acid residues of each protein. Residues that are present in all of these proteins are shown by asterisks.

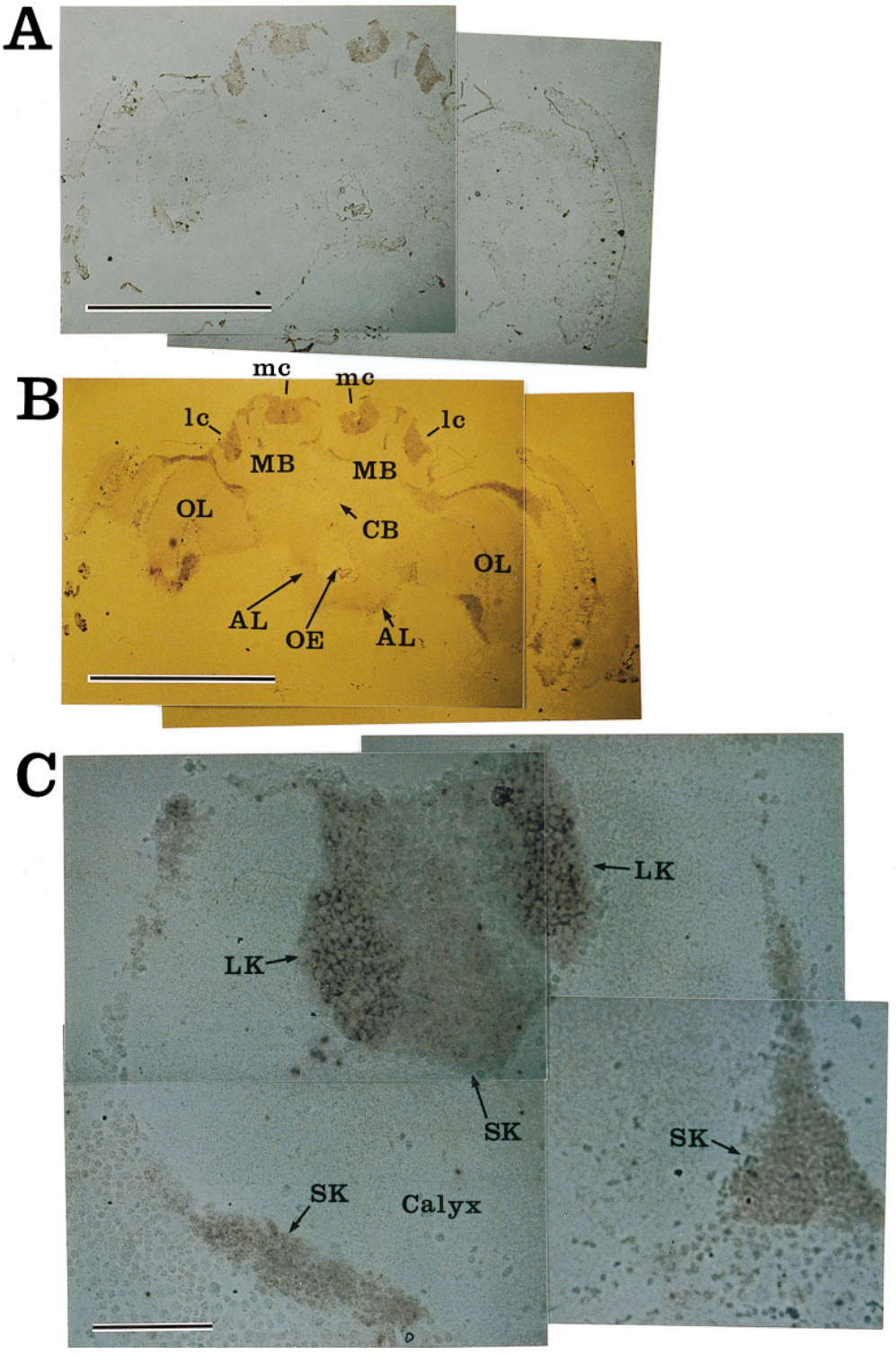

FIG. 3. *In situ* hybridization detection of mRNAs of the A1 gene in the brain of the worker bees. (A) Horizontal section of an adult brain showing hybridization to the Kenyon cells of the paired mushroom bodies. (B) The same section shown in (A) was stained with hematoxylin to locate the cell nuclei. MB, mushroom bodies; OL, optic lobes; AL, antennal lobes; CB, central body; OE, oesophagus; mc, median calyx; lc, lateral calyx. (C) High magnification of a right lateral calyx of a mushroom body showing strong hybridization in the large type Kenyon cells (LK) and weak hybridization in the small type Kenyon cells (SK).

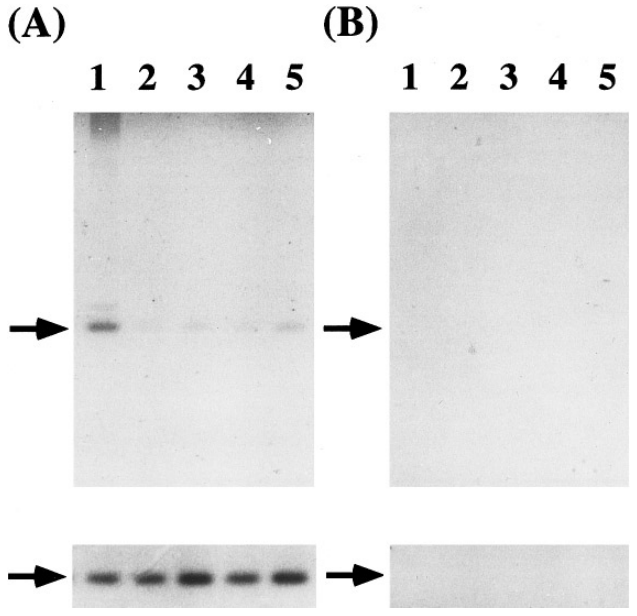

FIG. 4. Expression of the A1 gene in various body parts of the adult worker bee examined by RT-PCR. (A) To detect the A1 gene (top panel) and the cytoplasmic actin gene transcripts (bottom panel), mRNA was extracted from whole head (lane 1), thorax (lane 2), abdomen (lane 3), legs (lane 4) and antennae (lane 5), respectively, and examined by RT-PCR as described under Materials and Methods. (B) The experiment described in (A) was repeated without the RT reactions. Bands of the expected sizes (533 bp and 393 bp in the top and bottom panels, respectively) are indicated by arrows.

The IP3 receptor is a membrane protein that is required for mobilizing Ca^{2+} from intracellular stores in response to IP3-mediated extracellular signals (22, 23). Although cDNAs for IP3 receptors have been isolated from various organisms, only the *Drosophila* homologue has been identified in insects (17, 18). The gene encoding *Drosophila* IP3 receptor is thought to be a single copy gene and is expressed in the eyes, head, antennae and legs of adults (17). Relatively high levels (about 4-fold) of expression in the antennae suggest a role for this gene product during olfactory transduction. In the head, its expression is observed throughout the cortex of the central brain, and is not restricted to mushroom bodies, unlike the A1 gene (17). Thus, if the A1 gene encodes an IP3 receptor homologue of the honeybee, its preferential expression in the mushroom bodies of the brain is unique. There are several possible explanations for this restricted expression pattern. The first is that the gene for the IP3 receptor is also present as a single copy in the honeybee and that its expression is highly up-regulated in the mushroom bodies. Another is that there are two genes encoding IP3 receptor isoforms in the honeybee and that one is preferentially expressed in the mushroom bodies. It is also possible that there are two genes for IP3 receptors in both species, and those for the widely expressed type of the honeybee and for the mushroom body-preferential type of *Drosophila* have not yet been identified. To test these possibilities is necessary to understand the functions of the IP3 receptors in insects.

In *Drosophila*, a role for the mushroom bodies in olfactory learning was identified by mutant analyses (24), and some learning-related genes such as *dunce* (25) and *rutabaga* (26) are expressed predominantly in the mushroom bodies. However, expression of the gene for the *Drosophila* IP3 receptor is not restricted to the mushroom bodies, and its function in learning has not been analyzed genetically (17, 18). We expect that the A1 gene product is involved in function(s) of mushroom bodies of the honeybee such as learning and memory, because an association between olfactory memory and the mushroom bodies has also been suggested in the honeybee (5).

Three types of IP3 receptor have been identified in mammals and each type is expressed in a tissue-specific manner. In particular, the type 1 receptor is expressed predominantly in the cerebellum of the brain (20). An apparent morphological similarity is observed between the neural networks of the insect mushroom bodies and the vertebrate cerebellum (27) and the sequence identity between the A1 protein and the mammalian type 1 receptor was high, so the function of the A1 protein might be related to that of the mammalian type 1 receptor. The A1 gene product may also function in organs other than the brain, as weak expression was observed in the thorax, abdomen, legs and antennae.

The A1 gene is expressed differently by the two types of Kenyon cells. Although Kaiser and his colleagues demonstrated subdivisions of the *Drosophila* mushroom bodies by analyzing enhancer-trap expression patterns (28), the genes have not yet been isolated and thus the expression of their homologues in the honeybee brain has not yet been analyzed. Our results suggest that gene activity differs between the two types of Kenyon cells. To further characterize the function of the A1 gene product, its full length cDNA must be isolated and analyzed.

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