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Identification and punctate nuclear localization of a novel noncoding RNA, Ks-1, from the honeybee brain

MIYUKI SAWATA,[1]* DAISUKE YOSHINO,[2]* HIDEAKI TAKEUCHI,[3] AZUSA KAMIKOUCHI,[1] KAZUAKI OHASHI,[2] and TAKEO KUBO[1,3]

[1]Department of Biological Sciences, Graduate School of Science, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan
[2]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan
[3]Bio-oriented Technology Research Advancement Institution (BRAIN), 3-18-19, Toranomon, Minato-ku, Tokyo 105-0001, Japan

ABSTRACT

We identified a novel gene, *Ks-1*, which is expressed preferentially in the small-type Kenyon cells of the honeybee brain. This gene is also expressed in some of the large soma neurons in the brain and in the suboesophageal ganglion. Reverse transcription–polymerase chain reaction experiments indicated that *Ks-1* transcripts are enriched in the honeybee brain. cDNA cloning revealed that the consensus *Ks-1* cDNA is over 17 kbp and contains no significant open reading frames. Furthermore, fluorescent in situ hybridization revealed that *Ks-1* transcripts are located in the nuclei of the neural cells, accumulating in some scattered spots. These findings demonstrate that *Ks-1* encodes a novel class of noncoding nuclear RNA and is possibly involved in the regulation of neural functions.

Keywords: Kenyon cells; mushroom bodies; neuron; nuclear RNA

INTRODUCTION

The honeybee (*Apis mellifera* L.) is a eusocial insect, and colony members exhibit a large variety of social behaviors that are used to divide colony tasks between them (Winston, 1987). A honeybee colony is composed of three types of adults: a queen, workers, and drones. While drones and queens engage in the reproductive roles, workers engage in social labors such as brood rearing, comb building, and foraging. These labors are further divided among workers in an age-dependent manner (age-polyethism): young workers (usually less than 12 days old) secrete bee milk to rear the brood, while older workers (usually 14–30 days old) forage for nectar and pollen (Robinson et al., 1987; Winston, 1987).

Another prominent biologic characteristic of the honeybee is its use of the dance language: a forager just coming back to the hive from a feeding place informs other foragers of the direction and distance to the place by a waggle dance (Frisch, 1967; Winston, 1987). This behavior occurs only in the honeybee species, strongly suggesting that a unique brain function for communicating using dance language was acquired during the evolution of the honeybee. The molecular basis underlying the social and highly advanced behaviors of the honeybee, however, is not known.

Mushroom bodies (MBs) are prominent paired neuropils located in the protocerebral hemispheres of the insect brain (Strausfeld et al., 1998). The MBs are composed mainly of intrinsic interneurons, termed Kenyon cells (Mobbs, 1982). They receive input from sensory afferent neurons in their dense dendritic fields (calyces), and then forward output to peripheral efferent neurons, innervating their bundled axons (peduncles and/or lobes; Mobbs, 1982). Olfactory learning and memory functions have been assigned to this neuropil (Heisenberg, 1998). Local cooling of the honeybee MBs impairs olfactory memory formation (Erber et al., 1980). Ablation of the MBs impairs olfactory learning and memory in *Drosophila melanogaster* (de Belle & Heisenberg, 1994; Heisenberg et al., 1985). These observations are further supported by the fact that a genetic disruption of the cyclic AMP system in the MBs impairs learning (for review, see Davis, 1996). In addition, mutant analysis indicates that the MBs are essen-

Reprint requests to: Takeo Kubo, Department of Biological Sciences, Graduate School of Science, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; e-mail: stkubo@mail.ecc.u-tokyo.ac.jp.

*These two authors contributed equally to this work.

tial for courtship behavior in *Drosophila* (Hall, 1994). In the American cockroach, *Periplaneta americana*, ablation of MBs affects place memory and motor control (Mizunami et al., 1998b, 1998c). Furthermore, individual output neurons from MBs are often responsible for integrating multimodal stimuli, suggesting that the MB function is also important for sensory integration (Erber, 1978; Li & Strausfeld, 1997).

In Hymenoptera, including the honeybee, MBs are highly organized (Strausfeld et al., 1998). The ratio of the volume of the MBs in the whole brain is about 15% in the honeybee, whereas it is only 2 to 3% in the fruit fly, *D. melanogaster* (Mobbs, 1985). The calycal fields of the honeybee MBs are markedly enlarged, forming a two-cup-like structure in each hemisphere (Strausfeld et al., 1998). Furthermore, the number of Kenyon cells differs between colony members of the honeybee: The number of Kenyon cells in the drone is about 74% of that in the workers (Mobbs, 1982). Withers et al. (1993) reported that the proportion of the calyces and peduncles of the MBs alters with the division of labor of workers. Therefore, molecular analysis of the molecules operating in the MBs might lead to new insights into the molecular basis underlying the honeybee behavior.

Subdivision of the Kenyon cells of the insect MBs has attracted much recent attention because such subdivision might partially explain how these neuropils process plural modalities and perform a variety of functions (Strausfeld et al., 1998, 2000). Subdivision of Kenyon cells has been reported in some insect species, including the fruit fly (Yang et al., 1995), cockroach (Mizunami et al., 1998a), cricket (Schürmann et al., 2000), and some Hymenopteran insects (Goll, 1967; Mobbs, 1982). The honeybee MBs consist of two types of Kenyon cells, termed large- and small-type Kenyon cells based on the diameter of their cell bodies (9–11 μm and 6–7 μm, respectively; Mobbs, 1982). Several genes are preferentially expressed in either type of Kenyon cell. We previously demonstrated that the genes for the inositol 1,4,5-trisphosphate receptor (IP_3R) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), both of which are components of a Ca^{2+}-mediated signal transduction system, are preferentially expressed in the large-type Kenyon cells (Kamikouchi et al., 1998, 2000). The Mblk-1 gene, which encodes a putative transcription factor, is also preferentially expressed in the large-type Kenyon cells (Takeuchi et al., 2001). A royal jelly protein gene (*RJP-3*) is preferentially expressed in a defined population of Kenyon cells (Kucharski et al., 1998). These findings suggest that these two types of Kenyon cells have different physiologic characteristics and might contribute to distinct neural circuits.

The present article reports identification of a novel gene, *Ks-1*, which is preferentially expressed in the small-type Kenyon cells and some other restricted neural cells in the honeybee brain. The transcript does not contain any significant open reading frames (ORFs) and exists exclusively in the nuclei, suggesting that it has a unique function in the molecular mechanisms in the honeybee brain.

RESULTS

Identification of *Ks-1*, which is expressed in subsets of neurons of the honeybee brain

During the search for differentially expressed genes in the MBs of nurse and forager honeybees using the differential display method (Kamikouchi et al., 1998), we identified a gene, termed *Ks-1* (*Kenyon cell/small-type preferential gene-1*), which is preferentially expressed in the small-type Kenyon cells of the honeybee brain. Although northern blot hybridization failed to show clear labor specificity in the expression of the gene (data not shown), the gene is intriguing because of its restricted expression in the honeybee brain (Fig. 1).

When in situ hybridization using frozen sections of the worker brain and digoxigenin (DIG)-labeled *Ks-1* riboprobes was performed, preferential expression was detected in the MBs (Fig. 1A). In the MBs, a strong signal was detected in the small-type Kenyon cells, whose cell bodies are located in the inner-core and outer-bottom regions of the calyx (Fig. 1D). In contrast, the large-type Kenyon cells, whose cell bodies are located in the inner-lateral and outer-top regions of the calyx, express *Ks-1* less strongly (Fig. 1D,E). There was no significant staining in control experiments using sense riboprobes (Fig. 1B).

Furthermore, preferential expression was also observed in other brain regions and in the suboesophageal ganglion. These regions are positioned anterior to the peduncles (Fig. 2A), in the midline of the dorsal anterior part of the protocerebrum (Fig. 2B), around the antennal lobes (Fig. 2C), in the ventral edge of medulla and lobula (Fig. 2D), between the lateral calyx and the optic lobe (Fig. 1D), between the lateral protocerebral lobes and the dorsal lobes of the deutocerebrum (Fig. 2E), in the lateral somatal cortex of suboesophageal ganglion (Fig. 2F), posterior to the peduncles (Fig. 2G), and in the ventral somatal cortex of suboesophageal ganglion (Fig. 2H). All the *Ks-1* signals were detected in the cortex, indicating that they come from neurons but not glial cells. A common characteristic of the *Ks-1*-positive cells is the large soma (approximately 15–20 μm in diameter), although not all of the large soma neurons in these regions expressed *Ks-1* (Figs. 1D, 2A–H). These results suggest that *Ks-1* functions in a restricted neural circuit in the honeybee central nervous system. Furthermore, the signal was detected in the center of these neuronal large somata, suggesting that the *Ks-1* transcripts were localized in the nuclei of these cells (Figs. 1D, 2A–2H).

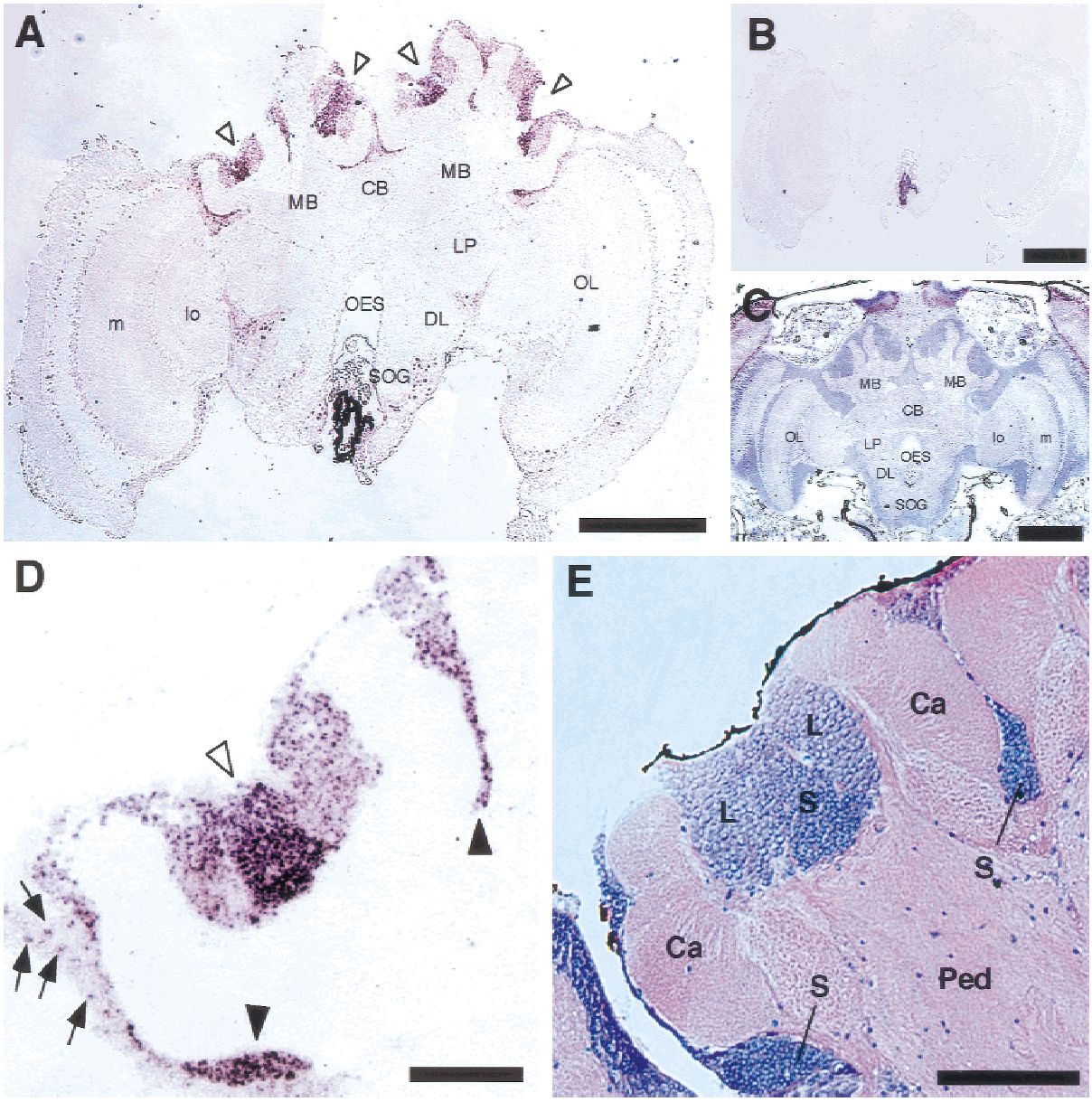

FIGURE 1. Expression of *Ks-1* in the worker brain. **A:** Frontal section of a worker brain hybridized with DIG-labeled antisense riboprobe for a part of the *Ks-1* cDNA (region II; see Materials and Methods). **B:** Control experiment with DIG-labeled sense riboprobe (region II). **C:** Frontal section of a worker brain stained with hematoxylin. **D:** High-magnification micrograph of the lateral calyx hybridized with DIG-labeled antisense riboprobe (region I). **E:** Lateral calyx stained with hematoxylin and eosin. MB: mushroom body; OL: optic lobe; CB: central body; LP: lateral protocerebral lobe; DL: dorsal lobe; SOG: suboesophageal ganglion; OES: oesophagus; lo: lobula; me: medulla; L: large-type Kenyon cells; S: small-type Kenyon cells; Ca:calyx; Ped:peduncle. Small-type Kenyon cell bodies that are located in the core of the inner side are indicated by white arrowheads in **A** and **D**. Small-type Kenyon cell bodies that are located outside surface of the calyces are indicated by black arrowheads in **D**. Large neuronal somata with intense signals are indicated by small arrows in **D**. Bars indicate 500 μm in **A** to **C** and 100 μm in **D** and **E**.

Ks-1 expression in the drone and queen brains

To examine whether *Ks-1* expression is related to the sex and/or caste of the honeybee, in situ hybridization was performed using the brain and suboesophageal ganglion sections of the queens and drones. In both cases, *Ks-1* signals were detected preferentially in the small-type Kenyon cells and in some large soma neurons (Fig. 3A–D), as in the case of workers, suggesting that the *Ks-1* functions irrespective of the sex and/or caste of the honeybee. The number of *Ks-1*-positive large soma neurons that are positioned between the lateral calyx and the optic lobes, however, was increased in the drones (Fig. 3D) compared with the workers or queens (Figs. 1D and 3C). This was confirmed

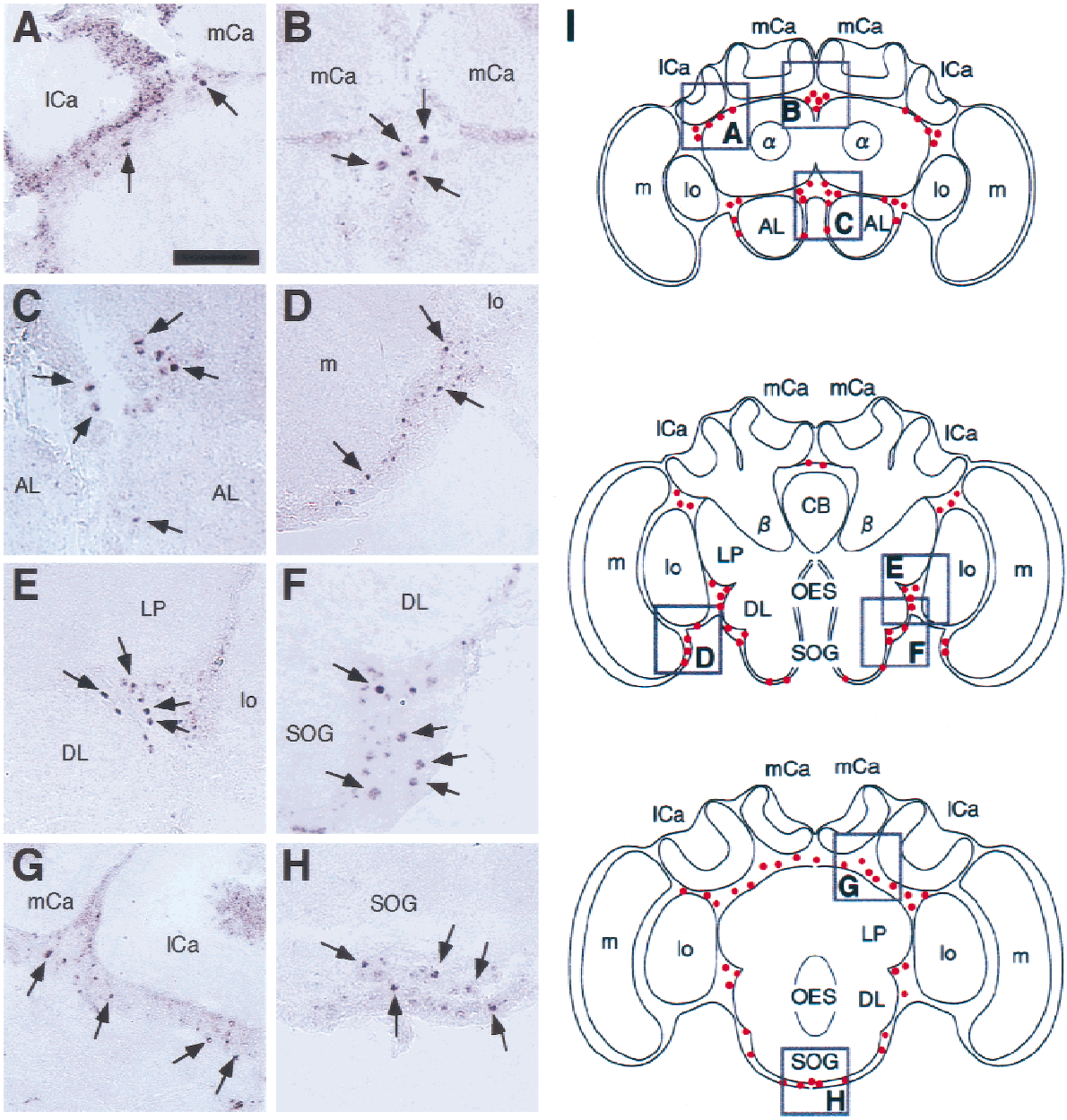

FIGURE 2. Expression of *Ks-1* in the large soma neurons of the worker brain and suboesophageal ganglion. **A–H**: In situ hybridization of *Ks-1* antisense probe to a section of worker bee brain and suboesophageal ganglion. Each view shows a different area of several preparations: anterior to the peduncles (**A**), the midline of the dorsal anterior part of the protocerebrum (**B**), around the antennal lobes (**C**), in the ventral edge of medulla and lobula (**D**), between the lateral protocerebral lobes and the dorsal lobes of the deutocerebrum (**E**), in the lateral somatal cortex of the suboesophageal ganglion (**F**), posterior to the peduncles (**G**), and in the ventral somatal cortex of the suboesophageal ganglion (**H**). Probes used in each panel correspond to region I (**A**), II (**F**), III (**D** and **E**), and IV (**B**, **C**, **G**, and **H**) of the *Ks-1* transcript (see Materials and Methods). Large neuronal somata with intense signals are indicated by arrows. **I:** Schematic representation of the positions of *Ks-1*-positive large neuronal somata (red) in the worker brain and suboesophageal ganglion. Frontal views of anterior (upper), middle (middle), and posterior portions of the sections (lower) are shown. Areas corresponding to **A–H** are boxed. mCa: median calyx; lCa: lateral calyx; AL: antennal lobe; CB: central body; LP: lateral protocerebral lobe; DL: dorsal lobe; SOG: suboesophageal ganglion; OES: oesophagus; lo: lobula; m: medulla; α: α-lobe; β: β-lobe. The bar indicates 100 μm.

using various specimens (data not shown). These results suggest that a drone has more *Ks-1*-positive neurons in these regions, and thus *Ks-1* might be partly involved in the brain functions that are enhanced in the drone.

Ks-1 expression in other parts of the honeybee body

To examine whether *Ks-1* is expressed in other parts of the honeybee body, total RNA was extracted from the

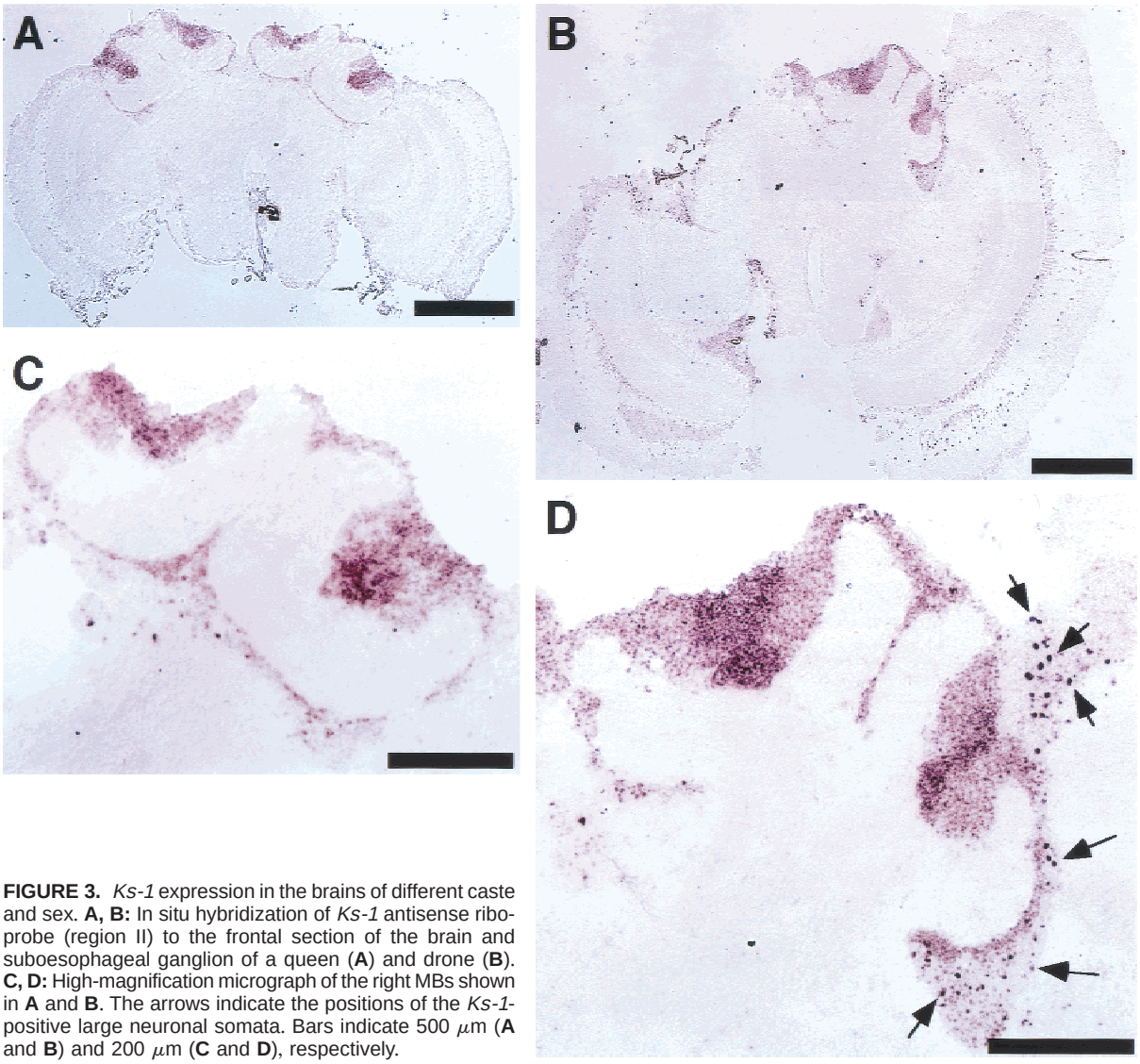

FIGURE 3. *Ks-1* expression in the brains of different caste and sex. **A, B:** In situ hybridization of *Ks-1* antisense riboprobe (region II) to the frontal section of the brain and suboesophageal ganglion of a queen (**A**) and drone (**B**). **C, D:** High-magnification micrograph of the right MBs shown in **A** and **B**. The arrows indicate the positions of the *Ks-1*-positive large neuronal somata. Bars indicate 500 μm (**A** and **B**) and 200 μm (**C** and **D**), respectively.

MBs, heads, thoraxes, and abdomens of the workers and subjected to reverse transcription–polymerase chain reaction (RT-PCR) using *Ks-1*-specific primers. The most intense band was detected from the MBs, whereas a less intense band was detected from the heads, and very weak expression was observed in the thoraxes or abdomens (Fig. 4). In contrast, bands of almost the same intensity were detected for the actin gene (Ohashi et al., 1996) in all four samples. There were no significant bands detected in the samples that were not treated with reverse transcriptase. Thus, the *Ks-1* transcript was enriched in the MBs in the worker body.

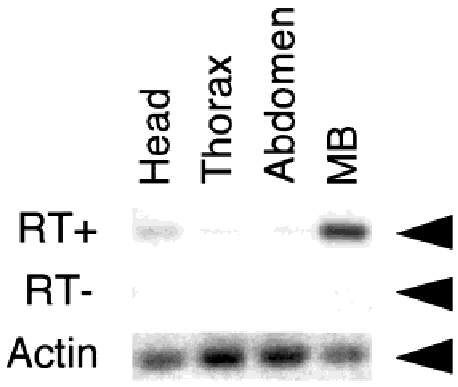

FIGURE 4. Expression of *Ks-1* in various worker body parts. Upper panel: RT-PCR analysis to compare *Ks-1* expression between heads, thoraxes, abdomens, and MBs. Total RNA was extracted from worker heads, thoraxes, abdomens, and MBs and subjected to RT-PCR using *Ks-1*-specific primers. Middle panel: A control experiment without RT reaction. Lower panel: RT-PCR using cytoplasmic actin-specific primers. The positions of bands for the *Ks-1* transcript and the actin mRNA are indicated by arrowheads.

Identification of a 17.5-kb sequence of the *Ks-1* transcript

Next, we determined the sequence of the *Ks-1* transcript. The length of the *Ks-1* transcript was estimated

to be more than 6 kb by northern blot hybridization (data not shown). Therefore, we repeated the screening of the random-primed worker MB cDNA library several times using the most 5′ end of the previously isolated cDNA as a probe (cDNA walking). We finally isolated 32 cDNA subclones and obtained a 17.5-kb consensus cDNA sequence (DDBJ/EMBL/GenBank accession no. AB071139) by assembling sequences of seven representative subclones (Fig. 5A). We confirmed that every part of the consensus *Ks-1* transcript is expressed as a continuous RNA by RT-PCR in which the primers were designed to amplify six fragments of the *Ks-1* cDNA that overlap with each other to cover the entire *Ks-1* cDNA sequence (Fig. 5B). Most major PCR products were detected at the predicted sizes in every PCR experiment (Fig. 5B), suggesting that there is no apparent splicing variant of the *Ks-1* transcript. It is unknown whether this 17.5-kb transcript is polyadenylated. Minor sequence differences, including nucleotide exchange and deletion and insertion of a few nucleotides, however, were sometimes observed among isolated subclones (data not shown). Such differences were located throughout the sequence, suggesting the heterogeneity of the *Ks-1* transcript.

Determination of *Ks-1* transcription initiation site

The 5′ ends of the six cDNA subclones isolated in the last screening were located within approximately 150 nt (Fig. 5A). Furthermore, the further screening failed to isolate any cDNA subclones extending to the 5′ upstream region of the above cDNA subclones. These results suggested that they represent the 5′ ends of the *Ks-1* transcript. To test this possibility, we isolated an *Eco*RI-digested genomic fragment of approximately 6.1 kbp that overlaps 1.7 kb with the 5′ end of the consensus *Ks-1* cDNA (Fig. 5C), and tried to locate the transcription initiation site using RT-PCR and primer extension.

RT-PCR methods were performed using primer sets of a common antisense primer (+596 to +615 of the consensus *Ks-1* cDNA) and four kinds of sense primers (primers 1s to 4s) that are located around the 5′ end of the consensus cDNA (Fig. 5D). Bands of the predicted sizes were detected with primers 1s to 3s, which were positioned at +54 to +73 and −212 to −193 of the 5′ end of the consensus cDNA, in an RT-reaction-dependent manner. The intensity of the bands, however, decreased as the more upstream 5′ primers were used (Fig. 5D). At last, no significant band was detectable with primer 4s, which was located the most 5′ upstream from the 5′ end of the consensus cDNA (−320 to −301; Fig. 5D), even when the number of PCR cycles was increased to 50 (data not shown). Furthermore, no bands of predicted sizes were detected when both of the sense and antisense primers were designed upstream of the 5′ end of the consensus cDNA, suggesting that the upstream regions were not expressed (data not shown). These results suggest that multiple transcription initiation sites are located within the regions corresponding to primers 1s to 4s (+73 to −320).

A primer extension study was performed to more precisely identify the position(s) of transcription initiation site(s) using the antisense primer corresponding to +43 to +72 of the consensus *Ks-1* cDNA (Fig. 5D). As shown in Figure 5E, a band of approximately 80 bases was detected, suggesting that at least one major transcription initiation site exists around 10 bp upstream of the 5′-end of the consensus cDNA, although other minor transcription initiation sites remain to be determined.

Ks-1 transcript contains no significant ORF

To reveal the protein encoded by the *Ks-1* transcript, ORFs within the consensus cDNA were investigated. Unexpectedly, termination codons were located all over the sense strand of the cDNA, and no significant ORF that could encode proteins was observed in any of the three reading frames (Fig. 6A). The longest putative ORF (No. 1; +15147 to +15485) encoded 113 amino acid residues, and there were a total of seven ORFs that could encode more than 67 amino acid residues (201 nt; Table 1). Among them, the No.6 ORF was the most probable because it started with the most 5′-proximal ATG codon, which had relatively good consensus to the translation initiation sequence proposed by Kozak (1987; Fig. 6A; Table 1).

To examine whether this ORF actually encodes a protein, we analyzed the corresponding ORF in the *Ks-1* counterpart from another honeybee species, *Apis cerana* F., assuming that the ORF should be conserved among these species if it is functional. For this, we isolated a partial *A. cerana* cDNA subclone including the sequence corresponding to +1 to +242 of the *A. mellifera Ks-1* cDNA. This ORF was not conserved; +122 and +123 nt of *A. mellifera Ks-1* cDNA were deleted in *A. cerana Ks-1*cDNA, causing a frame shift and resulting in early termination of the ORF in *A. cerana* (Fig. 6B). This finding suggests that the No. 6 ORF does not encode a protein. The other putative ORFs in *A. mellifera Ks-1* cDNA had no proper Kozak consensus sequences, and none of them had significant sequence similarity with any other proteins registered in the databases available up to November 1, 2001. The presence of minor sequence heterogeneity between *Ks-1* cDNA subclones did not influence these conclusions (data not shown). In addition, the overall nucleotide sequence of the *Ks-1* transcript had no significant homology to any nucleotide sequence registered in the databases, including genomic or EST databases.

Despite the absence of a significant ORF, the overall nucleotide sequence of the *Ks-1* transcript was well

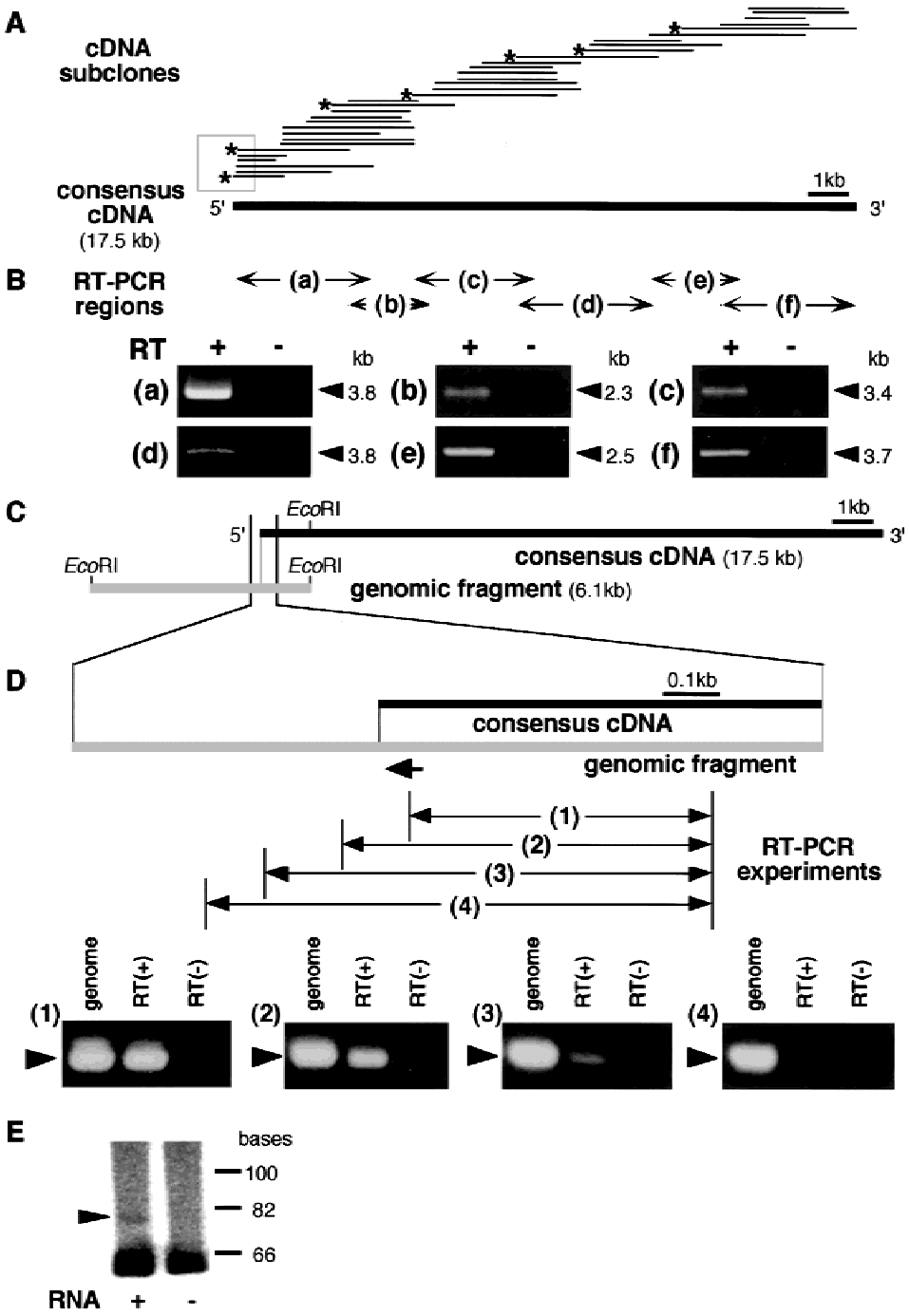

FIGURE 5. cDNA cloning and analysis of the transcription initiation site(s) of the *Ks-1*. **A:** Overview of *Ks-1* cDNA contig. Horizontal lines indicate 32 cDNA subclones isolated by the cDNA walking. The solid bar indicates the full-length consensus *Ks-1* cDNA derived from seven representative subclones indicated by stars. Notice that the 5′ ends of the six subclones (boxed) are in the narrow range. **B:** RT-PCR analysis indicates that the whole *Ks-1* cDNA is expressed continuously. Upper: The horizontal arrows indicate the RT-PCR regions (a–f) designed to amplify six fragments of the *Ks-1* cDNA that overlap with each other to cover the full-length cDNA. Lower: RT-PCR experiments. a to f correspond to the region indicated above. PCR was performed with (+) or without (−) RT reactions and subjected to agarose gel electrophoresis followed by staining with ethidium bromide. The positions of the signals are indicated by arrowheads with predicted sizes in kilobases **C:** Structure of the *Ks-1* genomic fragment and the relation to the consensus cDNA. The upper solid and the lower shadowed horizontal lines indicate the consensus *Ks-1* cDNA and a genomic *Eco*RI fragment, respectively. **D,** Upper**:** Magnified view of **C** around the most 5′ part of the consensus cDNA. The RT-PCR regions amplified with sense primers 1s to 4s (1 to 4), respectively, are indicated by horizontal arrows. The primer used in primer extension is indicated by an arrow below the genomic fragment. Lower: RT-PCR experiments. 1 to 4 correspond to the region indicated above. PCR was performed with (+) or without (−) RT reactions and then subjected to agarose gel electrophoresis. The *Eco*RI genomic fragment was also used as a template for a positive control (lane; genome). The arrowheads indicate the positions of the signals. **E:** Primer extension was performed with the primer indicated in **D** and with (+) or without (−) total worker MB RNA. The position of a band (primer extension product) is indicated by an arrowhead.

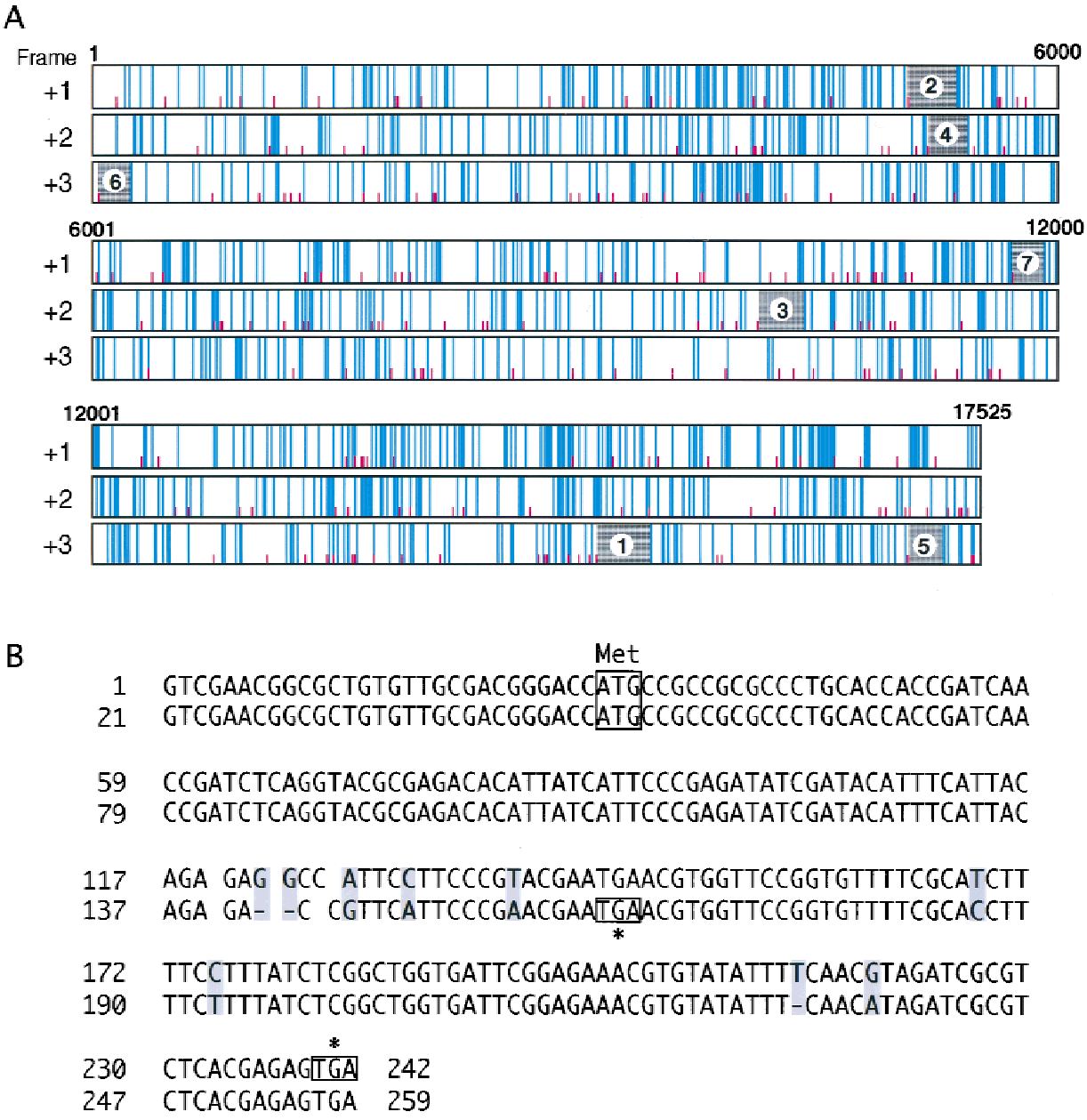

FIGURE 6. ORF analysis in the consensus *Ks-1* cDNA. **A:** Graphic representation of ORFs in the consensus *Ks-1* cDNA. Termination codons (TAA, TAG, and TGA) and the initiation codon (ATG) are indicated by blue and pink vertical lines, respectively, in each of three reading frames of the whole sense strand. The longest putative ORFs are indicated by shaded boxes starting from the nearest ATG to the last termination codon. The numbers indicate the ORF number assigned to each ORF in order of their length (see Table 1). **B:** Comparison of the nucleotide sequences of *A. mellifera* (upper line) and an *A. cerana Ks-1* (lower line) homolog corresponding to the +1 to +242 nt positions of *A. mellifera Ks-1*, which covers the No.6 ORF. The initiation Met codons (ATG) and termination codons (TGA) are boxed. Dashes are introduced in *A. cerana* cDNA corresponding to +122, +123, and +214 of the *A. mellifera Ks-1* cDNA to obtain maximum matching. Nucleotides that differed among these two species are shaded.

conserved between *A. mellifera* and *A. cerana* (Fig. 6B). Comparison of the nucleotide sequences corresponding to +1 to +242, +5198 to +5585, +8055 to +8533, +9960 to +10494, +12998 to +13386, and +15929 to +16279 of *A. mellifera Ks-1* cDNA between these two honeybee species indicated high sequence similarities (96%, 93%, 90%, 90%, 81%, and 90% identities, respectively; data not shown), suggesting that the overall nucleotide sequence is important for the *Ks-1* transcript. Thus, we concluded that the *Ks-1* transcript does not encode a protein, but functions as an RNA.

Ks-1 transcripts are enriched in the nuclei

In situ hybridization signals for the *Ks-1* transcripts were observed in the center of some neural large somata,

TABLE 1. Longest ORFs for the *Ks-1* transcript.

ORF no.[a]	Frame	Start	Stop	Length (bp)	Initiator sequence[b]
1	3	15147	15485	339	t a t A a a **A T G** a
2	1	5089	5364	276	a a g c g g **A T G** t
3	2	10169	10444	276	a a a c C t **A T G** t
4	2	5207	5455	249	a t a A a t **A T G** c
5	3	17094	17321	228	c g g t g g **A T G** c
6	3	30	239	210	G g g A C C **A T G** c
7	1	11740	11940	201	t a g t t g **A T G** G
					Consensus Kozak's rule: G C C A/G C C **A T G** G

[a]The ORF no. column shows the assigned numbers of seven putative ORFs ordered in length. They correspond to the numbered ORFs shown in Figure 6A.

[b]The initiator sequence column shows the −6 to +4 sequence of the putative initiator site. The start codon (ATG) is shown in bold. The nucleotides that match with the Kozak's consensus are shown by upper case.

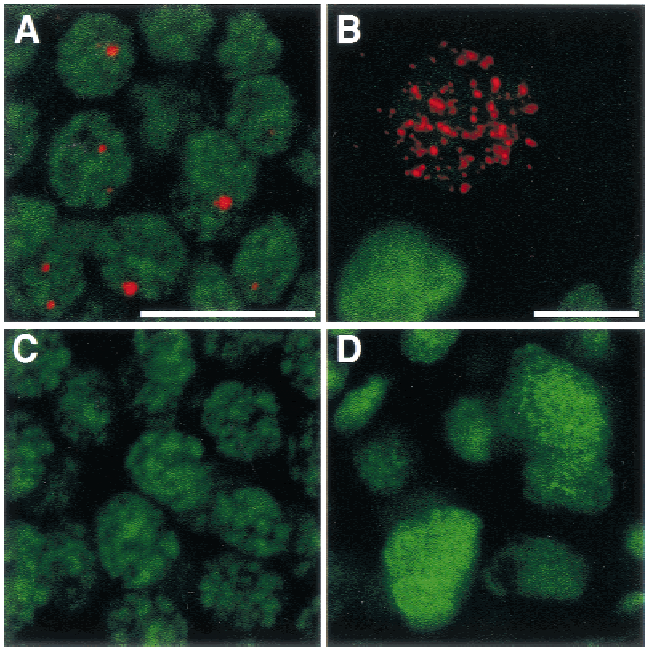

FIGURE 7. Subcellular localization of the *Ks-1* transcript. Fluorescent in situ hybridization of the *Ks-1* transcript using antisense (**A, B**) or sense (**C, D**) riboprobes to the frontal section of the worker brain and suboesophageal ganglion. Probes used in each panel correspond to regions V (**A, C**) and I (**B, D**) of the *Ks-1* transcript (see Materials and Methods). **A, C**: High-magnification micrograph of the small-type Kenyon cell bodies. **B, D:** High-magnification micrograph of the large neuronal somata in the ventral somatal cortex of suboesophageal ganglion. Signals for the *Ks-1* transcript are depicted in red. Chromosomal DNAs are counterstained with SYTOX Blue (green). Bars indicate 10 μm.

which suggests nuclear localization of the transcripts (Fig. 2). Therefore, we analyzed the subcellular localization of the *Ks-1* transcripts in more detail using fluorescent in situ hybridization of DIG-labeled *Ks-1* antisense riboprobes to sections of worker brain and suboesophageal ganglion. The signals were detected as a few (one to five) dots in each nucleus of the small-type Kenyon cells (Fig. 7A). On the other hand, the actin mRNA, analyzed as a control protein coding mRNA, were detected mainly in the cytosol (data not shown). No significant staining was observed when sense probes were used (Fig. 7C). These results indicated that the *Ks-1* transcripts were located almost exclusively in the nuclei of the small-type Kenyon cells.

Nuclear localization of the *Ks-1* transcripts was also observed in the large soma neurons (Fig. 7B). The number of punctate signals per nucleus was increased (approximately 10 to 50) in these large soma neurons in comparison with that in the small-type Kenyon cells. We performed the same experiments using three kinds of riboprobes corresponding to +122 to +589, +8037 to +8551, and +15909 to +16299 of the *Ks-1* transcript and obtained essentially the same results (data not shown). Along with the fact that the *Ks-1* transcript contains no significant ORF, its nuclear localization further supports that the *Ks-1* transcript encodes no protein and functions as an RNA molecule.

DISCUSSION

Preferential *Ks-1* expression in the small-type Kenyon cells

The present study demonstrates that *Ks-1* expression is enriched in the MBs of the honeybee brain among various body parts (Fig. 4). In the MBs, *Ks-1* is expressed preferentially in the small-type Kenyon cells (Figs. 1 and 3). Therefore, *Ks-1* is suggested to be involved in honeybee MB functions, especially in those characteristic of the small-type Kenyon cells.

The honeybee Kenyon cells can be divided into five subtypes, KI to KV, based on their morphology. The large-type Kenyon cells include KI and KII, and small-type Kenyon cells KIII to KV (Mobbs, 1982). Calyces in the honeybee can be divided into three regions: lips, collars, and basal rings, each of which is innervated by afferent neurites conveying olfactory (lips), visual (collars), and various sensory inputs including olfactory and visual inputs (basal rings). The small-type Kenyon cells extend their dendrites mainly to the basal ring (KIII, KIV, and KV) and also to the remaining two compartments (KV; Mobbs, 1982), suggesting that each subtype can receive multiple sensory inputs. In contrast, dendrites of the large-type Kenyon cells extend to the lip (KI) or collar (KII) and receive visual or olfactory sensory inputs independently. Thus, the small-type Kenyon cells seem to contribute to the simultaneous processing and integration of multiple sensory inputs. The *Ks-1* function might be related to such small-type Kenyon cell-specific characteristics.

Ks-1 expression in the other neuronal cell types

Ks-1 is also expressed in some large soma neurons as well as the small-type Kenyon cells in the brain and suboesophageal ganglion (Fig. 2). The positions of some of *Ks-1*-expressing large somata coincide with the regions where the cell bodies of the extrinsic MB neurons exist (Mobbs, 1982; Rybak & Menzel, 1993). In addition, a connection between extrinsic neurons and small-type Kenyon cells is suggested by iontophoretic application of cobalt ions into the α-lobe, in which small subsets of extrinsic neurons and small-type Kenyon cells are labeled (Rybak & Menzel, 1993). Therefore, it is possible that these large soma neurons are extrinsic MB neurons and *Ks-1* is involved directly or indirectly in a connection between Kenyon cells and the large soma neurons. Recently, some cell adhesion molecules such as cadherin were reported to be expressed symmetrically in neurons connecting to each other (Redies, 2000). Considering its nuclear localization (see below), it might be possible that *Ks-1* is involved in the regulation of expression of such cell adhesion molecules in both the intrinsic and extrinsic MB neurons. If this is the case, *Ks-1* could contribute to establish a discrete neural circuit of the protocerebrum.

On the other hand, some of the regions where *Ks-1*-expressing large somata exist (the dorsal pars intercerebralis, anterior part to the peduncles of the lateral calyx, and the lateral and ventral somatal cortex of the suboesophageal ganglion) overlap with regions where the cell bodies of the FMRF amide (Phe-Met-Arg-Phe-NH_2)-like immunoreactive neurosecretory cells exist (Eichmuller et al., 1991). If this is the case, *Ks-1* might contribute to the cellular functions of certain types of neurosecretory cells as well.

Does the *Ks-1* transcript function as a nuclear RNA?

The *Ks-1* transcript contains no significant ORF in its entire 17.5-kb sequence, but its overall nucleotide sequence is well conserved among the honeybee species (Fig. 6). Along with its nuclear localization (Fig. 7), we propose that the *Ks-1* transcript does not encode a protein, but functions as an RNA. So far, several noncoding RNAs have been reported (for review, see Eddy, 1999). Among them, *Xist* (Brown et al., 1992) and *roX* RNAs (Amrein & Axel, 1997; Meller et al., 1997), which are involved in dosage compensation in mammals and *Drosophila,* respectively, are particularly interesting as they have characteristics similar to the *Ks-1* transcript. Both are localized in the nuclei (Brown et al., 1992; Meller et al., 1997) like the *Ks-1* transcript and the length of *Xist* is 19 kb (Hong et al., 2000), which is similar to that of the *Ks-1* transcript.

Dosage compensation includes regulation of gene expression via changes in the chromatin structure (Kelley & Kuroda, 2000; Meller, 2000). For example, inactivated mammalian X chromosome is hypermethylated in their CpG islands (Heard et al., 1997) and has hypoacetylated histones (Gilbert & Sharp, 1999). In *Drosophila*, *roX* RNA associates with male-specific lethal proteins (Meller et al., 2000), which include histone H4 acetyltransferase (Smith et al., 2000) and histone H3 phosphorylase (Wang et al., 2001), and are involved in male-specific hypertranscription of the X chromosome (Akhtar & Becker, 2000; Kelley & Kuroda, 2000). By analogy, we suggest that the *Ks-1* transcript is also involved in regulation of gene expression via changes in chromatin structure.

However, *Ks-1* possesses some characteristics distinct from the above noncoding RNAs: (1) There is no sequence homology between them, (2) *Ks-1* is expressed in a sex-independent manner, and (3) *Ks-1* is expressed in a restricted region of the central nervous system, suggesting that the target genes regulated by *Ks-1*, if any, are different from those of the above RNAs. Target genes should also be expressed in a neural cell-type-specific manner. We assume that the numbers of target genes in the small-type Kenyon cells and large soma neurons are approximately 1 to 5 and 10 to 50, respectively, as judged by the number of signals detected by in situ hybridization (Fig. 7).

Recently, a novel *Drosophila* nuclear structure, "Omega speckles" was reported to contain a noncoding RNA that is transcribed from the 93 D heat shock (*hsr-omega*) locus (Lakhotia et al., 1999; Prasanth et al., 2000). Omega speckles are localized in the interchromatin domains and the numbers vary according to cell type (Prasanth et al., 2000). The *Ks-1* transcript is also distinct from *hsr-omega* RNA, as the latter exists in all cell types (Mutsuddi & Lakhotia, 1995; Prasanth et al., 2000). The similar but distinct features of the *Ks-1* transcript compared with other noncoding RNAs clearly indicate that it is a novel class of RNA molecule that possibly functions in a restricted neural cell type.

Recently, the technique for sperm-mediated transformation of the honeybee was reported by Robinson et al. (2000). This technique would be helpful to investigate the in vivo *Ks-1* function in the honeybee.

Possible mechanism of nuclear localization of the *Ks-1* transcript

The punctate localization of the *Ks-1* transcripts in the nuclei reminds us of certain nuclear structures, such as PcG domains (Satijn et al., 1997), interchromatin granule (ICG) speckles (Spector, 1993), coiled bodies (CBs; Bohmann et al., 1995), and PML nuclear bodies (Koken et al., 1994). PcG domains are suggested to represent some gene-silencing compartments in euchromatin (Platero et al., 1995; Lamond & Earnshaw, 1998). ICG

speckles are suggested to be involved in the storage and/or reassembly of splicing factors (Spector, 1996). They exist in the interchromosomal domain and disperse accompanying with high transcriptional activity (Zeng et al., 1997). CBs are involved in snRNP maturation and/or transport and disperse when transcription and splicing are blocked (Matera, 1999). The function of PML nuclear bodies is unclear, but is related to certain diseases, including cancer, autoimmunity, neurodegenerative disorders, and viral propagation (Doucas, 2000). Whether *Ks-1* transcripts are associated with some of these nuclear structures requires further investigation.

We report that there were fewer signals for *Ks-1* transcript in small-type Kenyon cells than in the large soma neurons (Fig. 7). This might reflect the different cell physiology and/or activity between them. Possibly, the *Ks-1* transcript associates with some nuclear structure that can move dynamically in response to cell activity, such as ICG speckles (Zeng et al., 1997) or CBs (Carmo-Fonseca et al., 1992).

How is the *Ks-1* transcript conserved in insects?

Finally, to what extent are the structure and function of *Ks-1* conserved among insect species? We could not find any nucleotide sequences that were similar to the *Ks-1* transcript in the computer database, including the *Drosophila* genome. Instead, we identified a *Ks-1* homolog in another honeybee genus, *A. cerana*, as judged by gene expression patterns and nuclear localization of the transcripts (data not shown) as well as partial nucleotide sequence. Therefore, *Ks-1* might exist only in a restricted insect species including the honeybees. Alternatively, a secondary and/or ternary structure, but not the nucleotide sequence, might be important for *Ks-1* function. In this case, a less structurally related *Ks-1* homolog might exist in some other insect species. Identification of the *Ks-1* homolog in various insect species is important to clarify its role in insect brain function.

MATERIALS AND METHODS

Animals

The honeybee *A. mellifera* L. colonies kept in The University of Tokyo were used throughout the experiments. A few *A. mellifera* L. and *A. cerana* F. colonies were also purchased from the Kumagaya beekeeping company (Saitama, Japan) and Fujiwara beekeeping company (Morioka, Japan), respectively.

Differential display procedure and identification of *Ks-1*

Differential display was performed as described previously (Miura et al., 1999). One microgram of total RNA samples from MBs of nurse bees and foragers, which were collected according to their behaviors, was treated with 1.0 U of RNase-free DNase I and then used for cDNA synthesis.

When 5′-CGGGAAGCTTATCTTTCTACCC-3′ was used as an arbitrary primer, a 293-bp band (*Ks-1*) was detected preferentially in the forager bee lane. The band was excised from the gel and the extracted cDNA was reamplified using PCR with the same primer combination as in the differential display. The cDNA was subcloned into a pGEM-3zf(+) vector (Promega; Madison, Wisconsin) at the *Sma*I site and transfected into *Escherichia coli* JM109. The sequences of both strands were determined using an ABI 373A DNA sequencer with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems; Foster City, California).

In situ hybridization analysis

In situ hybridization was performed essentially as described previously (Kamikouchi et al., 2000). Frozen sections (10 μm thick) of unfixed honeybee brain and suboesophageal ganglion were collected on 3-aminopropyltri-ethoxysilane (APS)-coated slides. The sections were fixed in 4% paraformaldehyde in phosphate buffer, and then hybridized with digoxigenin (DIG)-11-UTP-labeled sense or antisense riboprobes. The DIG-labeled riboprobes were prepared with a DIG RNA labeling kit (Roche, Basel, Switzerland) using five *Ks-1* fragments (region I–V, +122 to +589, +8037 to +8551, +12980 to +13406, +14598 to +15201, and +15909 to +16299, respectively) subcloned into pGEM-T Easy vectors (Promega) as templates. After posthybridization washes, DIG-labeled RNA was detected immunocytochemically with alkaline phosphatase-conjugated anti-DIG antibody using a DIG Nucleic Acid Detection Kit (Roche).

To examine the subcellular localization of the *Ks-1* transcript, DIG-labeled riboprobes were detected with an HNPP Fluorescent Detection Set (Roche). After counterstaining with 0.1 μM SYTOX Blue nucleic acid stain (Molecular Probes; Eugene, Oregon) in PBS, the signals were detected using a confocal laser-scanning microscope (LSM510; Zeiss, Germany).

cDNA cloning of the *Ks-1* transcript

To isolate a *Ks-1* cDNA subclone, we used the differential display product to screen the oligo-dT-primed MB λZAP-II cDNA library. In this first screening, GeneTrapper cDNA Positive Selection System (Invitrogen) was used to concentrate positive clones. Briefly, a phage cDNA library was converted to single-strand DNA, and subjected to hybridization with 3′-biotinylated *Ks-1* oligonucleotide probe (5′-CGTGAACGTG TCTATGCA-3′, +27 to +44 of the differential display product). Hybrids were then captured on streptavidin-coated paramagnetic beads, and retrieved using a magnet. After releasing from the oligonucleotide probe and paramagnetic beads, single-strand DNAs were recovered, converted to double-strand DNAs, and used to transform *E. coli* by electroporation. Transformants were then subjected to colony hybridization, in which +23 (+16902 of consensus cDNA) to +269 of the differential display product was used for probe synthesis.

To identify the full-length *Ks-1* cDNA, each overlapping cDNA subclone was isolated by screening the random-primed

MB λZAP-II cDNA library five times using the most 5′ end of the cDNA fragment obtained by the last screening (cDNA walking). All the isolated subclones were sequenced partially from the both ends to make a contig. Finally, seven subclones were selected, which covered the full-length *Ks-1* cDNA, and the sequences of both strands were determined. A consensus *Ks-1* cDNA was obtained by assembling these seven cDNA subclones into a contig using SEQUENCHER 4.1 software (Gene Codes Corporation; Ann Arbor, Michigan).

Isolation of a genomic clone

A honeybee genomic library (a gift from Dr. Paul Ebert, Queensland University, Australia) was screened by plaque hybridization using +122 to +589 of the consensus *Ks-1* cDNA as a probe. A single positive clone was isolated from 2×10^5 plaques. The phage DNA of approximately 11.9 kbp was extracted, digested with *Eco*RI, and the resulting four *Eco*RI fragments were each subcloned into pBluescript using λZAPII vector and Gigapak III Gold Packaging Extract (Stratagene; La Jolla, California). The sequences of both strands of these fragments were determined using an ABI 377 DNA sequencer with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). The order of these fragments was determined as judged from the size of the PCR product using primers corresponding to the most 3′ and 5′ regions of these fragments and the genomic clone as a template. Comparison of these sequences with the consensus *Ks-1* cDNA revealed that a 6.1-kbp fragment overlaps with the 5′ end of the consensus *Ks-1* cDNA and approximately 1.7 kbp of the nucleotide sequence from the 5′ end of the consensus cDNA was included in this genomic fragment.

RT-PCR

1. To examine body-part-specific *Ks-1* expression, total RNA samples extracted from the worker bee head, thorax, abdomen, and MBs, were treated with 1.0 U/1 μg RNA of DNase I and reverse transcribed with random primers using Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen). PCR was performed using 1/100 volume of head-, thorax-, or abdomen-derived and 1/250 volume of MB-derived RT products as templates with gene-specific primers for *Ks-1* (+122 to +146 and +565 to +589) and cytoplasmic actin gene (+2 to +21 and +376 to +395 of the previously isolated cDNA fragment). The PCR conditions were: (94 °C × 30 s + 55 °C × 30 s + 72 °C × 1 min) × 28 cycles for *Ks-1* and 22 cycles for actin gene. PCR products were then subjected to agarose gel electrophoresis and detected using SYBR Green I nucleic acid gel stain (Molecular Probes).

2. To confirm that the whole *Ks-1* transcript was expressed, primers were designed to amplify six parts of the *Ks-1* cDNA that overlapped with each other to cover the entire consensus cDNA sequence. The primers used were: (a) +122 to +146/+3965 to +3982, (b) +3310 to +3327/+5586 to +5603, (c) +5173 to +5197/+8531 to +8555, (d) +8015 to +8029/+11830 to +11848, (e) +11796 to +11813/+14283 to +14302, (f) +13797 to +13818/+17474 to +17491 of the *Ks-1* consensus cDNA. Total RNA extracted from worker bee MBs was treated with DNase I and reverse transcribed with random primer. PCR conditions were as follows: (94 °C × 45 s + 60 °C × 45 s + 72 °C × 3 min) × 35 cycles for fragment (a), 94 °C × 1 min + (94 °C × 1 min + 60 °C × 1 min + 72 °C × 6 min) × 35 cycles for (b) and (c), and 94 °C × 1 min + (94 °C × 45 s + 60 °C × 45 s + 72 °C × 3 min) × 30 cycles for (d) to (f). PCR products were then subjected to agarose gel electrophoresis.

3. To examine the putative transcription initiation site of *Ks-1*, total RNA extracted from the worker MBs was treated with DNase I and reverse transcribed with random primers. PCR was performed using primer sets of a common 3′ primer (+596 to +615 of the consensus *Ks-1* cDNA) and four kinds of 5′ primers (primers 1s to 4s), which correspond to 1s +54 to +73, 2s −70 to −51, 3s −212 to −193, and 4s −320 to −301, numbered starting from the 5′ end of the consensus *Ks-1* cDNA. The PCR conditions were: (94 °C × 30 s + 60 °C × 30 s + 72 °C × 2 min) × 40 cycles.

Primer extension

Primer extension was performed to identify the position(s) of transcription site(s) using Primer Extension System-AMV Reverse Transcriptase (Promega). Total RNA (100 μg) extracted from worker MBs was treated with DNase I and reverse transcribed with a 30-base antisense primer corresponding to +43 to +72 of the consensus cDNA, whose 5′ end was labeled with ^{32}P. After subjecting the product to 7-M urea-polyacrylamide gel electrophoresis, the band was detected by autoradiography.

Isolation of partial cDNA subclones for *A. cerana Ks-1* homolog

Total RNA extracted from *A. cerana* worker MBs was treated with DNase I and reverse transcribed with random primers. PCR was performed using primers corresponding to −20 to −1 and +1068 to +1087, and *Pyrobest* DNA Polymerase (TaKaRa; Shiga, Japan). The following PCR conditions were used: (94 °C × 15 s + 55 °C × 30 s + 72 °C × 1 min 20 s) × 32 cycles + 72 °C × 5 min. The PCR products were subcloned into pGEM-T vectors (Promega), and the sequence corresponding to +1 to +242 of *A. mellifera Ks-1* cDNA was determined on both strands using an ABI 3100 DNA sequencer with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

cDNA subclones for an *A. cerana Ks-1* homolog corresponding to +5198 to +5585, +8055 to +8533, +9960 to +10494, +12998 to +13386, and +15929 to +16279 of *A. mellifera Ks-1* cDNA were also amplified by PCR, subcloned, and sequenced.

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