Identification and analysis of the minimal promoter activity of a novel noncoding nuclear RNA gene, *AncR-1*, from the honeybee (*Apis mellifera* L.)

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ABSTRACT

Previously, we identified a gene for a noncoding nuclear RNA, termed *Ks-1*, that is expressed preferentially in a restricted set of neurons in the honeybee brain. In the present study, we identified another novel gene, termed *AncR-1*, whose transcripts were localized to nuclei in the whole cortex region of the honeybee brain, as a candidate novel noncoding nuclear RNA gene. RNA fluorescent in situ hybridization revealed that *AncR-1* and *Ks-1* transcripts were located in a distinct portion of a single neural nucleus, suggesting that they have distinct functions in brain neurons. cDNA cloning revealed that the *AncR-1* transcripts were up to 7 kb in size, had mRNA-like structures, and were alternatively spliced. The reporter assay using *Drosophila* SL-2 cells demonstrated that a TATA box-like sequence located –30 bp upstream of the 5' end of *AncR-1* cDNA had promoter activity. None of the alternatively spliced *AncR-1* cDNA variants contained significant open reading frames, strongly suggesting that *AncR-1* transcripts function as novel noncoding nuclear RNAs. Furthermore, in situ hybridization revealed that *AncR-1* was expressed not only in the brain but also in the sex organs in the queen and drones and in the hypopharyngeal glands and oenocytes of the worker bees, suggesting that *AncR-1* is involved in diverse organ functions. Some of the *AncR-1* transcripts enriched in the nuclei of the hypopharyngeal glands were polyadenylated, indicating the presence of mRNA-like *AncR-1* transcripts in the nuclei.

Keywords: honeybee; noncoding RNA; nuclear localization; tissue/organ-specific expression; brain

INTRODUCTION

The honeybee (*Apis mellifera* L.) is a eusocial insect and colony members exhibit a variety of social behaviors (Winston 1987). A honeybee colony consists of three types of adults: a queen (female reproductive caste), workers (female labor caste), and drones. In the colony, the queen and drones are engaged in reproduction, and workers perform various social labors to maintain the colony activities, such as nursing, comb building, colony defense, and foraging. Worker bees usually perform these labors in an age-dependent manner (age-polyethism): young workers (usually younger than 12 d old) secrete royal jelly to rear the broods, whereas older workers (usually older than 14 d) forage for nectar and pollen (Robinson et al. 1987; Winston 1987). Another prominent social behavior of the honeybee is the

use of dance language: A forager just coming back to the hive from a food source informs other foragers of the direction and distance of the food source by dance language (waggle-dance; Frisch 1967; Winston 1987). The use of a symbolic language in only honeybees among insects, and the variety of the honeybee social behaviors suggest that unique brain functions might have been acquired during their evolution. The molecular basis of the advanced honeybee social behaviors, however, remains largely unknown.

We previously identified a novel noncoding nuclear RNA gene, termed *Ks-1*, that is expressed preferentially in the mushroom bodies (MBs) of the honeybee brain, and whose transcripts are localized to the nuclei (Sawata et al. 2002). MBs are insect brain regions that are important for learning and memory (Heisenberg 1998; Mizunami et al. 1998) and might be involved in sensory integration (Erber 1978; Li and Strausfeld 1997), and are especially well developed in the honeybee (Strausfeld et al. 1998). Because *Ks-1* RNA is enriched in the MBs in a cell-type-preferential manner, we proposed that it has an important role in MB function, and might underlie honeybee social behavior. *Ks-1* is conserved among two honeybee species, but not in other insect spe-

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cies, including *Drosophila*, further supporting the idea that *Ks-1* function is related to the advanced honeybee brain functions (Sawata et al. 2002).

Here we report the identification and characterization of another novel non coding nuclear RNA gene, termed *AncR-1*, from the honeybee brain. *AncR-1* is expressed in sex and some secretory organs as well as in the brain in the honeybee, indicating that there are distinct classes of noncoding nuclear RNAs in the honeybee.

RESULTS

Identification of *AncR-1*, a novel candidate for a noncoding nuclear RNA gene

The findings on *Ks-1* prompted us to screen for novel noncoding nuclear RNA genes expressed in the honeybee brain as candidate genes that are important for honeybee social behavior. We first searched for honeybee genes whose transcripts were restricted to the nuclei by in situ hybridization, using cDNAs previously identified as candidates for genes expressed region preferentially and/or behavior dependently in the honeybee brain by a combination of the differential display method and cDNA microarray analysis (Takeuchi et al. 2002; H. Takeuchi, R.K. Paul, T. Fujiyuki, K. Shirai, Y. Matsuo, Y. Fujinawa, A. Kato, A. Tsujimoto, and T. Kubo, pers. commun.).

Among ~100 genes examined by in situ hybridization, one, termed *AncR-1* (*Apis noncoding RNA-1*), was identified as a candidate, because its in situ hybridization signals in the worker brain seemed to be localized in the nuclei, based on the round-shaped signals and their scattered pattern in the whole brain cortex regions where there are neuronal cell bodies (Fig. 1A,C). To confirm the nuclear localization of the *AncR-1* transcripts, we further analyzed their subcellular locations by RNA fluorescent in situ hybridization (RNA FISH). The signals were detected within the nuclei as discrete spots using *AncR-1* antisense probes (Fig. 1E). In contrast, no significant signals were observed with sense probes (Fig. 1D), indicating that the signals were due to *AncR-1* transcripts. RNA FISH signals for *AncR-1* and *Ks-1* transcripts were detected in distinct nuclear portions in the small-type Kenyon cells of the MBs (Fig. 1F), suggesting that they have distinct functions in the honeybee brain neurons.

Identification of *AncR-1* cDNAs

cDNA cloning was performed to identify the full-length *AncR-1* transcripts (Fig. 2A). As only a partial 270-bp

FIGURE 1. *AncR-1* expression in the worker brain and the subcellular localization of the transcripts. (A,B) In situ hybridization of DIG-labeled *AncR-1* antisense (A) or sense (B) riboprobe to the frontal section of the worker brain and subesophageal ganglion. (C) High-magnification micrograph of the boxed MB region of A. Note that each signal has a round-shaped figure. (MB) mushroom body; (OL) optic lobe; (SOG) subesophageal ganglion. (D,E) FISH of DIG-labeled *AncR-1* sense (D) or antisense (E) riboprobe to the worker brain section. High-magnification micrographs of the cell bodies of small-type Kenyon cells are shown. Kenyon cells are MB intrinsic neurons (Mobbs 1982). Signals for the *AncR-1* transcripts (colored in red) are detected in the nuclei where chromosomal DNAs are counterstained and colored in blue. (F) FISH of biotin-labeled *Ks-1* antisense riboprobes merged with E. Signals for the *Ks-1* transcripts (colored in green) are detected in different positions from those of *AncR-1* transcripts in the nuclei. Bars indicate 500 μ m in A and B, 50 μ m in C, and 10 μ m in D–F.

AncR-1 cDNA fragment was identified by the differential display method, we first used this fragment to search for honeybee brain ESTs (Whitfield et al. 2002) and obtained a contig sequence (contig 1295; the sequence is available at http://titan.biotec.uiuc.edu/bee/honeybee_project.htm).

We then isolated 45 distinct cDNA subclones by the rapid amplification of cDNA ends (RACE) method (28 clones from 5'-RACE and 17 from 3'-RACE). These EST and cDNA sequences were assembled into a final ~7-kb cDNA contig (Fig. 2A). To confirm that the RNA corresponding to the cDNA contig was actually expressed, reverse transcription-polymerase chain reaction (RT-PCR) was performed using an upstream primer designed for the most 5' end and a downstream primer designed for near the 3' end of the cDNA contig (Fig. 2B). As a result, a predicted cDNA of 6.9 kb (*AncR-1a*; accession no. AB125322) was amplified depending on the RT reaction, demonstrating the existence of continuous transcripts corresponding to the cDNA contig (data not shown). At the same time, other RT-PCR clones of 6.8 and 2.7 kb (*AncR-1b* and *c*; accession nos. AB125323 and AB125324, respectively) were also isolated and their nucleotide sequences were determined. Some of the 5'-RACE subclones were spliced differently (Fig. 2A), and these splicing events were confirmed in the shorter RT-PCR clones. *AncR-1c* had an additional splice that was not present in any of the RACE clones (Fig. 2B). In addition, comparison of the cDNA sequences to the genomic sequence

(derived from *A. mellifera*-whole genome shotgun [WGS] sequences in the NCBI Trace Archive) revealed that one more splice had occurred at the 5′ region of the cDNAs (Fig. 2C). All of these spliced-out regions possessed the consensus splice donor and acceptor sequences in both their 5′ and 3′ ends (5′-GT...AG-3′). We also investigated the possibility that *AncR-1* was a host gene for intron-encoded small nucleolar RNAs (snoRNAs; Bortolin and Kiss 1998; Smith and Steitz 1998). This possibility was ruled out, however, because there were no conserved sequences and structures for the typical snoRNAs (box C/D or box H/ACA snoRNAs; Filipowicz and Pogacic 2002) in any of these spliced-out regions. Some of the 3′-RACE subclones possessed long poly(A) sequences (> ~50 bp) that were associated with possible polyadenylation signals at their 3′ ends, indicating that the transcripts from which these subclones were derived were polyadenylated (Figs. 2A, 8B, below). Although all of the other 3′-RACE subclones possessed shorter ~30-bp poly(A) stretches associated with possible polyadenylation signals (data not shown), it is not clear whether they actually represent polyadenylated transcripts, as these shorter poly(A) stretches are expected to be derived from the oligo (dT)₃₀ sequences in the RT primers used in that experiment. These results indicate that some of the *AncR-1* transcripts are processed to mRNA-like structures after transcription.

Analysis of the major *AncR-1* transcript

Because multiple alternative splicing events seemed to occur in the posttranscriptional processing of the *AncR-1* transcripts, we next analyzed the rough structure of the major transcript by Northern blot analysis (Fig. 2D). When a part of the region common among the *AncR-1* cDNAs was used as a probe, a major band of ~6 kb was detected (Fig. 2D, lane a). A band of this size was not detected with a probe for the commonly spliced region among *AncR-1* cDNAs

(Fig. 2D, lane b), but was detectable with probes for other alternatively spliced regions (Fig. 2D, lanes c–e). Therefore, it is likely that the major transcript contained these alternatively spliced regions.

Analysis of the minimal promoter region of *AncR-1* and determination of a transcription initiation site

The most 5' end of the *AncR-1* cDNA contig was consistent with the 5' ends of six subclones isolated by four independent 5'-RACE experiments (Fig. 2A), suggesting that the 5'-end of these clones corresponded to the transcription initiation site of *AncR-1*. To test this possibility, we first analyzed the expression of the immediate upstream sequence of the 5' end of the *AncR-1* cDNA contig by Northern blot analysis (Fig. 3B). When a probe corresponding to –264 to +87 bp of the cDNA contig was used, a 6-kb band was detected (Fig. 3B, lane 1). In contrast, no band was detected with probes corresponding to –264 to –4 and –264 to –62 bp (Fig. 3B, lanes 2,3), indicating that the immediate upstream sequence of the *AncR-1* cDNA contig was not expressed.

We hypothesized that if the 5'-end of the cDNA contig corresponds to a transcription initiation site, there might be a promoter for *AncR-1* in the upstream region. To test this, we ligated the *AncR-1* 5' upstream sequences with the luciferase gene, introduced the constructs into *Drosophila* SL-2 cells, and examined their promoter activities (Fig. 3C). When a construct containing the –875 to +87 sequence was used, approximately 17 times greater reporter activity was detected compared with that containing the +1 to +87 sequence. As there was only a slight loss of reporter activity when the upstream regions were shortened to –364 to +87 and –89 to +87, we concluded that the minimal promoter for *AncR-1* was within the –89 to –1 sequence. Because there was a TATA box-like sequence (TATATAAA) at the –30 position of the cDNA 5' end (Fig. 3A), we examined whether this TATA box-like sequence was responsible for the minimal promoter activity. When a construct in which TATATAAA (–30 to –23) was converted to TACATAAA was transfected into *Drosophila* SL-II cells, reporter activity decreased to approximately 30% (Fig. 3C), demonstrating that the TATA box-like sequence was necessary for minimal promoter activity.

FIGURE 3. Analysis of the transcription initiation site of *AncR-1*. (A, Upper) Schematic representation of the most 5' part of *AncR-1* cDNA and the surrounding genomic region. A TATA box-like sequence was found at the –30 position from the 5' end of the cDNA. (Lower) Regions used as Northern probe templates in B are shown with horizontal arrows (1–3). Regions inserted into the reporter vector for the promoter assay in C are shown with horizontal arrows (4–7). Stars show the relative position of the TATA box-like sequence. Sequence positions are indicated with reference to the position of the 5' end of the cDNA. (B, Upper) Northern blots to analyze the expression of the 5' upstream region of *AncR-1*. (Lanes 1–3) The results from three different experiments using ³²P-labeled cDNA probes for corresponding regions (1–3) indicated in A. Total MB RNA (5 µg) was used in each experiment. The arrowhead indicates the predicted band position according to the size of major transcript. No bands were detected in lanes 2 and 3. (Lower) Dot blot analysis as control experiments. Dot blots of the DNA fragment of region (1) for positive controls were hybridized with the same probes used in Northern blot analysis. (C) Promoter assay of the 5' upstream region of *AncR-1*. Reporter constructs, to which the different regions (4–7) indicated in A were introduced, were transfected to *Drosophila* SL-II cells. Luciferase activity normalized to the β-galactosidase activity ($n = 3$) is plotted in arbitrary units. The promoter activity attributed to the (5) region decreased when a mutation was introduced at –28 to destroy the TATA box-like sequence. (–) Reporter vector without any sequence insertion. Error bars show the standard deviation.

We concluded that the 5' end of the *AncR-1* cDNA contig represented a transcription initiation site, and therefore full-length *AncR-1* transcripts were identified.

Analysis of ORFs in *AncR-1* cDNAs

To examine whether *AncR-1* encodes a protein, open reading frames (ORFs) within *AncR-1* cDNAs were investigated. In each case of the three alternatively spliced full-length cDNAs obtained by RT-PCR (*AncR-1a* to *c*), termination codons appeared frequently in all three frames of the sense strand, and there was no significant ORF that could encode a protein (Fig. 4A). The longest putative ORF (No. 3) could encode 162 amino acid residues (without a first Met), and there were a total of three ORFs that could encode > 66

FIGURE 4. ORF analysis in full-length *AncR-1* cDNAs. (A) Graphic representation of ORFs in three variant full-length cDNAs obtained by RT-PCR. 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 strands. The longest putative ORFs are indicated by shaded boxes. The numbers indicate the ORF number assigned to each ORF in order of the start site (see Table 1). Due to the alternative splicing events, the No. 1 ORF was truncated to < 201 nt in *AncR-1c* (therefore, not shown in the figure), the No. 2 ORF appeared in *AncR-1c*, and the frame and position of the No. 3 ORF was changed between variants. The 3-bp sequences were inserted in the No. 1 ORF in *AncR-1b* compared with *AncR-1a*. (B) Comparison of the nucleotide sequences of *Apis mellifera* *AncR-1* (upper line) and an *Apis cerana* *AncR-1* homolog (lower line) corresponding to the +151 to +398 nt positions of *A. mellifera* *AncR-1*, which covers the No. 1 ORF. The initiation Met codons (ATG) and termination codons (TGA and TAA) are boxed. Dashes are introduced in the *A. mellifera* *AncR-1* sequence after +340 and +387, and in the *A. cerana* sequence corresponding to +279 and +372 of *A. mellifera* *AncR-1* to obtain maximum matching. Nucleotides that differed among these two species are shaded. The ORF structure was not conserved between honeybee species.

amino acid residues (201 nt; Fig. 4A; Table 1). Among them, No. 1 was the most likely to encode a protein because it was located relatively near the 5' end of the cDNAs, and the sequence around its first ATG had relatively good consensus to the translation initiation sequence proposed by Kozak (1987; see our Table 1), although this ORF length was shortened from 222 and 225 nt in *AncR-1a* and *b*, respectively, to 171 nt in *AncR-1c* due to the alternative splicing, associated with a sequence exchange at the 3' end of the ORF in *AncR-1c*.

To assess whether this No. 1 ORF actually encodes a protein, we set the same criterion as used previously when determining if *Ks-1* RNA was coding or noncoding (Sawata

et al. 2002): We analyzed the corresponding ORF in the *AncR-1* counterpart from another honeybee species, *Apis cerana* F., assuming that the ORF would be conserved among this species if it is functional. For this, we isolated a partial *A. cerana* *AncR-1* cDNA subclone, including the sequence around the No. 1 ORF of the *A. mellifera* *AncR-1* cDNA by RT-PCR using *A. cerana* MB total RNA. The No. 1 ORF was not conserved; two-nucleotide exchanges in the ORF resulted in early termination of the ORF in *A. cerana* *AncR-1* cDNA (Fig. 4B). This finding suggests that the No. 1 ORF does not encode a protein. Other putative ORFs in *A. mellifera* *AncR-1* cDNAs had no proper Kozak consensus sequences, and none of them had significant sequence similarity with any other protein coding sequences registered in the databases. In addition, the overall sequences of the *AncR-1* transcripts had no significant homology to any sequence of other species registered in the databases, except an EST sequence derived from *A. cerana* hypopharyngeal glands (accession no. CB350391). The *A. cerana* EST sequence partially overlapped with the No. 3 ORF region of *A. mellifera* *AncR-1* cDNAs, but the No. 3 ORF structure was disrupted in the *A. cerana* EST due to the lack of an initiation codon and frame shifts (data not shown).

Despite the absence of a conserved ORF, nucleotide sequences of the *AncR-1* transcript were well conserved between *A. mellifera* and *A. cerana* (Fig. 4B). Comparison of the nucleotide sequences of the transcripts corresponding to +30 to +533 of the *AncR-1a* be-

tween these two honeybee species indicated 96% sequence similarity. The *A. mellifera* *AncR-1* transcripts (*AncR-1a* to *c*) commonly possessed well-conserved 14-time repeats of a 35-mer sequence in their 3' regions around the No. 3 ORF (Fig. 2E). These observations suggest that the nucleotide sequence is significant for the function of *AncR-1* transcripts. Thus, we concluded that the *AncR-1* transcripts do not encode a protein, but function as RNA molecules.

Analysis of *AncR-1* expression in the adult bees

Next, we investigated *AncR-1* expression in the body of three types of adult honeybees. When Northern blot analy-

TABLE 1. Longest ORFs in the *AncR-1* transcripts

ORF No. ^a	cDNA ^b	Frame	Start	Stop	Length (bp)	Initiator sequence ^c
1	AncR-1a	+3	174	395	222	t C a G C g A T G c
1	AncR-1b	+3	174	398	225	G C a G C g A T G c
2	AncR-1c	+3	501	725	225	a a t G t g A T G c
3	AncR-1a	+1	5041	5529	489	t t a c t g A T G c
3	AncR-1b	+1	4969	5457	489	t t a c t g A T G c
3	AncR-1c	+3	867	1355	489	t t a c t g A T G c
Consensus Kozak's rule:						<div style="text-align: center;"> A G C C C C A T G G G </div>

^aThe assigned numbers for three ORFs ordered by the start position from the 5' end. The same number was assigned to the corresponding ORFs in the three variants of full-length cDNA. They correspond to the numbered ORFs shown in Figure 4A.

^bThe full-length cDNAs in which each ORF exists.

^cThe -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 capitalized.

sis was performed using total RNAs extracted from the head, thorax, and abdomen of the queen, workers, and drones, *AncR-1* expression was detected in every body part in the queen, but only in the head and thorax in workers and in the thorax and abdomen in drones, indicating that *AncR-1* was expressed differentially between the three types of bees (Fig. 5). Because the differences were observed in the head and abdomen, we next performed in situ hybridization to further analyze *AncR-1* expression in these body parts. When the expression in the worker head was investigated, the signal was detected mainly in the hypopharyngeal glands as well as in the brain (Fig. 6A). *AncR-1* was also expressed in the sex organs of the queen and drone: The clearest signals were detected in the follicle and nurse cells of the ovary and spermatheca in the queen abdomen (Fig. 6E,G), and in testis cells other than the maturing sperm, endophallus, and mucus glands in the drone abdomen (Fig. 6I,K,M). In the worker abdomen, signals were detected in

the oenocytes of the fat body (Fig. 6C). Additional signals were detected in the ventriculus of the drone (Fig. 6O), queen, and workers (data not shown). We also observed signals in the most dorsal region of the abdomen in every type of bee, although we could not identify the tissue (data not shown). Malpighian tubules seemed to slightly express *AncR-1*, at least in the queen and worker abdomen (data not shown). Signals were located in the nuclei in all cases, based on the round-shaped signals and comparison with neighboring sections stained with hematoxylin-eosin (data not shown). In contrast, signals were not detected, for example, in the muscles or rectum of

any bee type (data not shown), indicating that *AncR-1* was expressed in a tissue/organ-specific manner.

Because there were at least three splice variants for the *AncR-1* transcripts expressed in the worker MBs (Fig. 2B), we investigated the presence of each splice variant in some of the *AncR-1*-expressing tissues/organs by RT-PCR experiments designed to amplify AncR-1a-, b-, or c-specific fragments (Fig. 7A). Variant-specific PCR products were detected in all organs tested in an RT-dependent manner (Fig. 7B), suggesting that the splicing of *AncR-1* transcripts is common to these tissues/organs.

Verification of polyadenylation of nuclear-localizing *AncR-1* transcripts

cDNA cloning revealed that some of the *AncR-1* transcripts underwent polyadenylation at their 3' ends, like mRNA. Because polyadenylation usually stimulates export of mRNA from nuclei to cytoplasm (Huang and Carmichael 1996), the nuclear localization and the polyadenylation of *AncR-1* transcripts seemed conflicting. Therefore, we examined whether the polyadenylated *AncR-1* transcripts actually exist in the nuclei. Total RNA was extracted from the nuclear fraction of the hypopharyngeal gland cells. Hypopharyngeal glands were selected as a source of nuclear *AncR-1* transcripts, as the size of the nuclei is relatively large (~15 μ m) and it was easy to prepare a pure nuclear fraction. To confirm that the nuclear fraction was properly prepared, we compared the contents of the *actin* transcripts, most of which is present in the cytoplasm, and the *AncR-1* transcripts in the nuclear fraction and whole hypopharyngeal gland cell fraction by RT-PCR. The bands for the *AncR-1* transcripts appeared at 22 PCR cycles both in the nuclear and whole-cell fractions, whereas those for the *actin* transcripts appeared at 32 and 23 PCR cycles in the nuclear and whole-cell fractions, respectively, indicating that the cyto-

FIGURE 5. Distribution of *AncR-1* expression among the body parts of each type adult honeybee. *AncR-1* expression in the head, thorax, and abdomen of the queen, workers, and drones was analyzed by Northern blot analysis. Total RNAs extracted from the head (H), thorax (T), and abdomen (A) of each type of adult bee were subjected to Northern blot analysis using *AncR-1*-specific probes. The arrowhead indicates the predicted band position according to the size of the major transcript. 18S rRNA images are shown as loading controls.

FIGURE 6. *AncR-1* expression in the various tissues/organs of adult honeybees. (A–P) In situ hybridization of *AncR-1* antisense (A, C, E, G, I, K, M, O) or sense (B, D, F, H, J, L, N, P) riboprobe to the frontal section of the worker head (A, B), or the longitudinal section of the worker (C, D), queen (E–H), or drone (I–P) abdomen. Each view shows a partial magnification micrograph of a different tissue/organ. (A, B) The hypopharyngeal gland. (C, D) The fat body on the roof of the abdomen. Arrows in C show the signals in the middle of oenocytes. (E, F) The ovary near the oviduct. Arrows and arrowheads in E show the signals in nurse cells and follicle cells, respectively. (G, H) The spermatheca with duct. Arrows and arrowheads in G show the signals in the duct and on the surface of the spermatheca, respectively. (I, J) The testis of an immature drone. (K, L) The bulb of an endophallus. Arrows in K show the layered signals. (M, N) The mucus gland. Arrows in M show the signals in the inner most cells. (O, P) The ventriculus. Arrows in O show the signals in the glandular epithelium. (Q) Schematic representation of the longitudinal abdomen section of each type adult bee. Areas corresponding to panels A, C, E, G, I, K, M, and O are boxed in red. Dorsal is top and apical is left. (e) Egg; (f) follicle cell; (n) nurse cell; (lp) lateral plate of endophallus bulb; (m) mucus; (v) ventriculus; (r) rectum. Bars indicate 20 μ m in C and I and 100 μ m in the other panels.

plasmic RNAs were efficiently removed through the nuclear fractionation and the nuclear fraction was enriched with *AncR-1* transcripts (Fig. 8A).

When the 3'-RACE was performed using this nuclear total RNA, two of the six resulting 3'-RACE products had long (> ~50 bp) poly(A) sequences associated with possible polyadenylation signals, indicating that they represented polyadenylated *AncR-1* transcripts. The other four 3'-RACE products had shorter (~30 bp) poly(A) stretches reflecting

the oligo (dT)₃₀ sequences in RT primers and thus it was not certain whether they actually represented polyadenylated transcripts, although they were also associated with possible polyadenylation signals (Fig. 8B). All of these putative polyadenylation sites were mapped within 138 bp (between +6366 to +6503 bp of the *AncR-1c*), coincident with the fact that the putative polyadenylation sites for three of the six 3'-RACE products that were isolated during the cDNA cloning procedure and had long poly(A) sequences were also located within the region (Fig. 8B). These results indicated that some of the *AncR-1* transcripts localizing to the nuclei are actually polyadenylated. In addition, the multiple polyadenylation sites are located at the 3' terminal region of these nuclear *AncR-1* transcripts.

DISCUSSION

Identification of a novel noncoding nuclear RNA

In the present study, we identified a novel noncoding nuclear RNA gene, *AncR-1*, by a screening based on the criterion that the transcripts were localized in the nuclei (Fig. 1). The *AncR-1* transcripts are processed to mRNA-like structures after transcription (Figs. 2A–C, 8B). So far, several mRNA-like noncoding RNAs are reported to be functional in some biologic phenomena like imprinting and meiosis (Erdmann et al. 2001). Among them, the most well analyzed ones are those involved in dosage compensation in *Drosophila* (*roX* RNAs; Amrein and Axel 1997; Meller et al. 1997) and mammals (*Xist* RNA; Brown et al. 1992), both of which localize to the nuclei and function in transcriptional regulation (Kelley and Kuroda 2000; Meller 2000). Human *NTT* expressed in activated CD4+ T cells produces another mRNA-like noncoding RNA localized to the nucleus (Liu et al. 1997). Recently, a large number of putative mRNA-like noncoding RNAs were identified through the extensive EST project in mouse (Numata et al. 2003). Together, these findings suggest that there might be many noncoding nuclear RNAs that are functional in various biologic phenomena. *AncR-1* RNA as well as *Ks-1* RNA might be one of those functional noncoding nuclear

FIGURE 7. Expression analysis of *AncR-1* splice variants in different adult organs. (A) Schematic representation of the RT-PCR regions used to detect *AncR-1* splice variants. Structures of *AncR-1a*, *b*, and *c* are presented as in Figure 2B. The horizontal bars (a–c) below splice variants represent splice-variant-specific RT-PCR regions used in the RT-PCR analysis in B; thick and thin parts correspond to exonic and intronic regions, respectively. (B) RT-PCR analysis to examine the presence of each of the three splice variants in the worker MBs (lanes 1,2) and hypopharyngeal glands (lanes 3,4), queen ovaries (lanes 5,6), and drone sex organs (lanes 7,8). (a–c) indicate the results with primer sets to amplify splice variant-specific RT-PCR regions (a–c) shown in A, respectively. Odd and even lanes show RT+ or – experiments, respectively. The black arrowheads indicate the band positions for predicted RT-PCR products.

RNAs that is unique to the honeybee. The screening based on the nuclear localization of the transcripts, which was used in our experiments, is a potentially general and effective criterion for searching for novel noncoding RNAs in various organisms, as prediction of noncoding RNA genes from the genomic information remains difficult. The information on the sequence, structural details, and promoter elements of known noncoding RNAs, however, might support the prediction.

Possible mechanism of nuclear localization of *AncR-1* transcripts

AncR-1 transcripts are localized in the nuclei as determined by in situ hybridization and nuclear RNA fractionation (Figs. 1, 6, 8A). On the other hand, some of the nuclear *AncR-1* transcripts are polyadenylated at their 3' ends (Fig. 8). The proper 3'-end formation, including the polyadenylation, facilitates nuclear export of mRNA (Huang and Carmichael 1996; Cullen 2003). Considering that *AncR-1* is likely to be transcribed by pol II (see below) and some of the transcripts are spliced like mRNA, it is natural to assume that the 3'-end processing of *AncR-1* transcripts occurs in a manner similar to mRNA. If this is the case, some unknown sequence elements in the *AncR-1* transcript and factors that bind to the sequence might function to retain the transcript in the nuclei and properly transport them to specific nuclear sites to function. Further studies are needed to examine this interesting possibility and to identify the elements and/or factors needed for the nuclear localization of the *AncR-1* transcripts.

Possible *AncR-1* functions

AncR-1 was expressed in the whole worker brain (Fig. 1A), suggesting its role in neural functions. Whatever the function of *AncR-1* is, it should be different from that of *Ks-1*, because not only the expression pattern in the brain but also the localization of the transcripts in the nuclei are distinct from those of *Ks-1* (Fig. 1F).

Based on Northern blot analysis, the *AncR-1* expression seemed to depend on the sex in the head, and on the caste in abdomen (Fig. 5). The *AncR-1* expression pattern was almost the same between the worker and drone brains (data not shown); therefore the expression in the hypopharyngeal glands (Fig. 6A), which is unique to the worker bee (Winston 1987), could be a reason for the difference in the Northern blot signals in the worker and drone heads. In the abdomen, *AncR-1* was expressed in the sex organs in the queen and drones (Fig. 6), suggesting that *AncR-1* could be involved in the reproductive system in the honeybee.

AncR-1 seems to have a counterpart in *A. cerana* (Fig. 4B), but not in other animal species, including *Drosophila*, suggesting that *AncR-1* might also be characteristic of the honeybee species, like *Ks-1*. In this aspect, it is interesting that *AncR-1* is expressed not only in the brain but also in the reproductive organs, hypopharyngeal glands, and fat body oenocytes (Fig. 6), all of which are involved in physiologic differences related to the division of roles among the colony members: (1) The queen has well-developed ovaries, whereas the worker ovaries are reduced (Winston 1987); (2) the hypopharyngeal glands of young nurse bees produce royal jelly proteins, but produce α -glucosidase in the older foragers (Kubo et al. 1996; Ohashi et al. 1997); (3) the fat body oenocytes, which biosynthesize hydrocarbons (Fan et al. 2003), are well developed over the worker-specific wax glands as well as beneath the roof of the worker abdomen (Dade 1994). Thus, regulation of *AncR-1* expression might be related to the caste and/or sex-dependent development and/or differentiation of these organs.

Splicing variants and the repetitive sequence in *AncR-1* transcripts

In the course of cDNA cloning, various splicing variants of *AncR-1* transcripts were found (Fig. 2A,B), although they seem to be too minor to be detected by Northern blot analysis (Fig. 2D). The existence of variants suggests that the function of *AncR-1* transcript as an RNA might be partially regulated by the splicing. The three splice variants were not organ specific (Fig. 7), however, suggesting that *AncR-1* has diverse functions in an organ rather than organ-specific function. It might be interesting to examine whether these variants are cell type and/or tissue specific. It is also possible that splice variants other than *AncR-1a*, *b*, and *c* (Fig. 2A,B) have tissue/organ-specific expression patterns. Further investigation is needed to clarify the significance of *AncR-1* transcripts' splicing.

These splicing sites were located at relative 5' regions of the splice variants, and the 3' regions include a common 35-mer repetitive sequence (Fig. 2A,B). *Xist* RNA also contains repetitive sequences and one of them was required for transcriptional repression by *Xist* RNA (Wutz et al. 2002). Therefore, the repetitive sequence might also have an important role in the function of *AncR-1* RNA, which is common to the variants. An interference of this repeat, for example, using antisense peptide nucleic acid oligomers against the repeat sequence (Tyler et al. 1999; Beletskii et al. 2001), might provide a clue to its function.

Regulation of *AncR-1* expression through the TATA box

A TATA box-like sequence at the -30 site of the *AncR-1* cDNA 5' end acted as a minimal promoter in the reporter

assay (Fig. 3C). We used *Drosophila* cells in the assay, but this element is also very likely to be active in the honeybee because of its precise position in relation to the cDNA 5' end. Generally, TATA boxes are observed upstream of the genes that are transcribed by pol II and expressed in developmental stage-specific and tissue-specific manners (Hochheimer and Tjian 2003). Considering that the *AncR-1* possesses a TATA box-like sequence at the 5' upstream region and *AncR-1* transcripts are processed to the mRNA-like structures, it is likely that *AncR-1* is transcribed by pol II. This is also consistent with the fact that *AncR-1* is expressed in a tissue/organ-specific manner among honeybee colony members (Fig. 6). It should be emphasized that other regulatory elements that are probably present upstream of the TATA box and function only in the honeybee were not identified in our reporter assay using S2 cells. Further studies on the regulation of *AncR-1* expression as well as functional analysis of *AncR-1* RNA, using

electroporation to introduce the genes (Kunieda and Kuba 2004), could aid in the understanding of the biologic significance of noncoding nuclear RNAs and the molecular basis that underlies honeybee sociality.

FIGURE 8. Identification of polyadenylated *AncR-1* transcripts in the nuclear RNA fraction. (A) RT-PCR to examine the content of *actin* mRNA and *AncR-1* transcripts in total RNA prepared from the hypopharyngeal gland nuclear or whole-cell fractions. PCR products with (RT+) or without (RT-) RT reactions were analyzed by agarose gel electrophoresis at indicated PCR cycles. The black arrowheads indicate the band positions for each predicted product. (B) Multiple polyadenylation sites for the nuclear *AncR-1* transcripts. 3'-RACE was performed using RT primer containing the oligo (dT)₃₀ sequence, one of two gene-specific primers corresponding to +6181 to +6208 and +6276 to +6303 of *AncR-1a*, and total RNA extracted from the nuclear fraction of the hypopharyngeal glands, and putative polyadenylation sites for the resulting six 3'-RACE products were mapped at the 3' terminal regions of *AncR-1* transcript. The nucleotide sequences corresponding to +6151 to +6861 of *AncR-1a* and an additional 84-nt sequence determined by 3'-RACE performed during the cDNA cloning procedure are shown contiguously. The arrows indicate the positions of the gene-specific primers. The arrowheads above or below the sequence indicate the putative polyadenylation sites for six 3'-RACE products derived from nuclear RNA or six of 17 3'-RACE products isolated during the cDNA cloning procedure, respectively. The black or white arrowheads indicate putative polyadenylation sites associated with long poly(A) or RT-primer-derived short poly(A) stretches, respectively. The canonical (AATAAA) and the next two probable (AT TAAA and TATAAA) polyadenylation signals (Graber et al. 1999; Beaudoin et al. 2000) appearing within 50 nt upstream of the putative polyadenylation sites are underlined.

MATERIALS AND METHODS

Animals

The honeybee *A. mellifera* L. colonies purchased from the Kumagaya bee keeping company and kept in The University of Tokyo were used throughout the experiments. An *A. cerana* F. colony was purchased from the Fujiwara bee keeping company.

In situ hybridization

In situ hybridization was performed essentially as described previously (Kamikouchi et al. 2000). Frozen sections (10 μ m thick) of unfixed honeybee brain, head, and abdomen were collected on 3-aminopropyltri-ethoxysilane (APS)-coated slides. The sections were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS), pretreated, and then hybridized with digoxigenin (DIG)-11-UTP-labeled sense or antisense riboprobes. The DIG-labeled riboprobes were synthesized by T7 and SP6 RNA polymerases with a DIG RNA labeling mix (Roche) using the 270-bp region from a differential display (+5327 to +5596 of *AncR-1a*) subcloned into a pGEM-T vector (Promega) as a template. After hybridization, slides were washed in 50% formamide, 2 $\times$ SSC for 30 min at

50°C, treated with 6.7 µg/mL Ribonuclease A (Sigma-Aldrich) in TNE (10 mM Tris-HCl, 1 mM EDTA, 500 mM NaCl at pH 7.6) for 10 min at 37°C, and washed for 20 min at 50°C in 2× SSC and 0.2× SSC for 2× 20 min. Following treatment with 1.5% blocking reagent (Roche) in TN buffer (100 mM Tris-HCl, 150 mM NaCl at pH 7.5) for 1 h, DIG-labeled riboprobes were detected immunocytochemically with alkaline phosphatase-conjugated anti-DIG antibody using a DIG Nucleic Acid Detection Kit (Roche).

To examine the subcellular localization of *AncR-1* and *Ks-1* transcripts, brain sections were hybridized with a mixture of DIG-labeled *AncR-1* and biotin-16-UTP-labeled *Ks-1* riboprobes. The biotin-labeled *Ks-1* riboprobes were prepared by in vitro transcription with a Biotin RNA Labeling Mix (Roche) using a 468-bp (+122 to +589) fragment subcloned into a pGEM-T Easy vector (Promega) as a template. DIG-labeled riboprobes were detected with an HNPP Fluorescent Detection Set (Roche), and biotin-labeled riboprobes were detected using TSA Biotin System (NEN Life Science Products) and Alexa Fluor 488 conjugated streptavidin (Molecular Probes). After counterstaining with 1 µM TO-PRO-3 iodide (Molecular Probes) in PBS, the signals were examined using a confocal laser-scanning microscope (FV300-BX, Olympus).

cDNA cloning

To identify the whole length of *AncR-1* cDNA, RACE methods were performed repetitively using the SMART RACE cDNA Amplification Kit (Clontech). Total RNA extracted from worker MBs was used for the cDNA template synthesis. The RACE PCR products were subcloned into pGEM-T easy vectors (Promega), and several clones were isolated in each experiment and partially sequenced from both ends to make a contig.

To obtain the full-length *AncR-1* cDNAs, a primer corresponding to the most 5' end (5'-GACATTTCTCCTGAGTCGTCTTCGAA CGGAC-3') and a primer corresponding to the 3'-end (5'-TAGTGC GATTTAGAGCTGTACAAGTTTC-3'), designed between the last two putative polyadenylation sites of the cDNA contig, were used in an RT-PCR experiment. Total RNA (1 µg) extracted from worker MBs was treated with 1.0 U of DNase I and reverse transcribed with random primers using the Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen). PCR was performed using Advantage 2 Polymerase Mix (Clontech), and the products were analyzed by agarose gel electrophoresis. Two bands of ~7 kb and ~2.7 kb were detected in an RT-dependent manner. *AncR-1a* and *b* were isolated from the whole PCR products, and *AncR-1c* was isolated from a ~2.7-kb product extracted from the excised gel. The sequences of both strands of these subclones were then determined.

Sequencing was performed using an ABI 3100 DNA sequencer with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequence analyses and assemblies were done using SEQUENCHER 4.1 software (Gene Codes Corporation).

Northern blotting

To analyze the structure and 5' end of *AncR-1* transcript, 5 µg of total RNA extracted from MBs was electrophoresed on 1% agarose gel with formamide, transferred to a positively charged nylon membrane, and dry-fixed. [$\alpha^{32}\text{P}$]-dATP-labeled cDNA probes

were synthesized with a Strip-EZ DNA kit (Ambion) using partial PCR fragments of *AncR-1* cDNA or genomic DNA as templates. The template regions used in Figure 2D were: (lane a) +1 to +236, +649 to +762, and +813 to +853, (lane c) +358 to +561, (lane d) +1216 to +1416, (lane e) +2424 to +2603 of the *AncR-1a*, and (lane b) +78 to +228 of WGS sequence 173361510. Hybridization was performed using ExpressHyb Hybridization solution (Clontech). After washing, radioactivity on the membrane was detected using a Bioimaging analyzer (Fujifilm).

To examine *AncR-1* expression in the body parts of the queen, workers, and drones, total RNA (20 µg each) extracted from the head, thorax, or abdomen of each bee type was electrophoresed on 1.2% agarose gel with formamide, blotted to the membrane, and hybridized with the probe for the 270-bp region detected by differential display.

Reporter assay

Luciferase reporter vectors containing different amounts of *AncR-1* upstream and cDNA 5'-end regions were prepared by amplifying the corresponding sequences by PCR and ligating each of these PCR products to the MCS of the pGL3-basic vector (Promega) containing the promoter-less firefly luciferase gene.

To create a TATA-box mutated vector, the corresponding original reporter vector was used as a template for the mutating PCR, in which the insert region with the flanking vector sequences were amplified into two kinds of mutated PCR fragments that overlapped each other. The primers used were 5' vector primer (RVprimer2; sited outside of the MCS) in combination with TATA-mutating antisense primer (5'-TTTGCGTACTCCCTAGC TTG-3'), and 5' MCS MluI site mutating primer (5'-GCTCTTAC TCGTGCTAGCCC-3') in combination with a 3' vector primer (GLprimer3). Single-stranded PCR fragments were mutually annealed to some extent to a hetero hybrid, double stranded by *Ex Taq* (TaKaRa) and reamplified by PCR with RVprimer2 and GLprimer3. The resulting PCR product was digested with MluI to select only the TATA-mutated, but not the MluI site-mutated fragments, and with XhoI, and ligated to the MCS of the pGL3-basic vector using these enzyme sites. Unexpectedly, we obtained a mutant vector in which the sequence 5'-TA_TATAAA-3' that corresponded to the TATA box-like sequence was changed into 5'-TACATAAA-3'. No other insert sequence was changed compared with the original construct.

Transfection and luciferase assay were performed as described previously (Park et al. 2003). A mixture of plasmid DNA (0.45 µg) consisting of the luciferase reporter vector (400 ng) and an *actin* 5C-β-galactosidase reporter vector (50 ng) was transfected in each experiment. Reporter gene activity was assayed 46 h later. The efficiency of transfection was normalized by β-galactosidase activity.

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

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 +1 to +29 and +534 to +561 of *AncR-1a*, and *Pyrobust* DNA Polymerase (TaKaRa). The following PCR conditions were used: (94°C × 30 sec + 50°C × 30

sec + 72°C × 1 min 30 sec) × 30 cycles + 72°C × 5 min. The PCR products were subcloned into pGEM-T Easy vectors (Promega), and the sequence was determined on both strands.

Preparation of nuclear total RNA and isolation of *AncR-1* 3' cDNA subclones

Hypopharyngeal glands of 30 worker bees were collected in 875 µL of hypotonic buffer B (15 mM HEPES-NaOH, 10 mM KCl, 5 mM MgCl₂ at pH 7.6) and homogenized with 10 strokes of a tight pestle in a 1-mL Dounce homogenizer. To remove unbroken tissue and cells, the homogenate was filtered through a rough nylon mesh, kept on ice for 15 min, and the supernatant was filtered again through a 41-µm nylon mesh. Buffer B (125 µL), containing 2.0 M sucrose, was added to make the final sucrose concentration 0.25 M. Nuclei were collected by centrifugation at 800g for 10 min, washed once in buffer B containing 0.25 M sucrose, and resuspended in 1 mL of buffer B containing 1.65 M sucrose. The nuclei were pelleted through a cushion of 500 µL of buffer B containing 2.0 M sucrose at 16,000g for 1.5 h, washed once in buffer B containing 0.25 M sucrose, and resuspended in 250 µL of the same buffer. The nuclear integrity was checked under a microscope. Total RNA was extracted from the resulting nuclear fraction using TRIzol LS reagent (Invitrogen), and used for a cDNA template synthesis for 3'-RACE methods, which were performed as described above. Two RACE PCR experiments were performed using gene-specific sense primers corresponding to +6181 to +6208 and +6276 to +6303 of *AncR-1a*, and three distinct *AncR-1* 3' subclones were isolated in each experiment.

RT-PCR

For RT-PCR experiments to check the quality of the total nuclear RNA, control total RNA of whole hypopharyngeal gland cells was prepared in addition to the nuclear RNA. The RNA samples were treated with DNase I and reverse transcribed with random primers. PCR was performed using adequately diluted RT products as templates to adjust the content of *AncR-1* cDNA between the samples, and gene-specific primers for *AncR-1* (+1 to +29 and +60 to +87) or the cytoplasmic actin gene (+2 to +21 and +376 to +395 of the previously isolated cDNA fragment; Ohashi et al. 1996; accession no. AB023025). The products were analyzed by agarose gel electrophoresis.

To examine the tissue/organ-specific distribution of splice variants of *AncR-1* transcripts, total RNAs extracted from MBs and hypopharyngeal glands of worker bees, queen ovaries, and sex organs of drones, including testis, mucus glands, and endophallus, were used for first strand synthesis with random primers. Primer positions used to detect each variant (*AncR-1a*, *b*, or *c*) fragment were: +3865 to +3884 and +4005 to +4034 of *AncR-1a*, +3868 to +3887 and +3970 to +3991 of *AncR-1b*, and +542 to +569 and +654 to +681 of *AncR-1c*.

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