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Bombyxin gene expression in tissues other than brain detected by reverse transcription-polymerase chain reaction (RT-PCR) and in situ hybridization

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Abstract. Bombyxin is a 5 kDa insulin-related peptide produced in four pairs of medial neurosecretory cells in the brain of the silkmoth *Bombyx mori*. We demonstrate here the presence of bombyxin mRNA in tissues other than brain: ganglia, epidermis, testis, ovary, fat body, silk gland, Malpighian tubule, midgut, and hindgut of the *Bombyx* fifth instar larvae. Bombyxin mRNA was detected by Oligotex reverse transcription-polymerase chain reaction (RT-PCR), a rapid and simple procedure of reverse transcription-PCR, and in situ hybridization. The Oligotex RT-PCR method effectively eliminated the contaminating DNA in RNA samples and amplified bombyxin mRNA efficiently. In situ hybridization of the *Bombyx* ovary clearly demonstrated the localization of the bombyxin mRNA in the ovariole. The present study is the first demonstration of expression of brain neurosecretory peptide in tissues other than the central nervous system in insects at RNA level.
Key words. Insulin; insulin-like growth factor; *Bombyx mori*; ovary; neurosecretory peptide; Oligotex reverse transcription-polymerase chain reaction.

Bombyxin is a 5 kDa brain neurosecretory peptide of the silkmoth *Bombyx mori*, belonging to the insulin peptide family [1]. The bombyxin molecule is a heterodimer of A and B chains whose amino acid sequences show homology with insulin. The A and B chains of bombyxin are connected by disulfide bonds in exactly the same manner as in insulin [2] and form an insulin-like core structure (Nagata et al., pers. commun.). The genes encoding bombyxin consist of at least four gene families, A, B, C and D, and each family comprises multiple intronless gene copies [3–6] (Kondo et al., unpubl. data). The major site of bombyxin gene expression is four pairs of medial neurosecretory cells of the brain [3]. Besides bombyxin in *Bombyx*, bombyxin-related peptide genes and cDNAs have been characterized from the saturniid moth *Samia cynthia ricini* [7] and the hornworm *Agrius convoluvuli* [8], and their expression site has been localized to the medial neurosecretory cells in the brain. These bombyxin-related peptides derive from multiple gene copies in the genome. Bombyxin and bombyxin-related peptides thus have an intriguing feature with respect to the number of gene copies and the expression site of the gene in contrast to vertebrate insulin, which has one or two gene copies and is produced mainly in the gastroenteropancreatic organ. Though bombyxin was discovered due to its prothoracicotropic activity in *Samia*, it does not show such tropic activity in *Bombyx* itself [9]. The physiological function of bombyxin is therefore still obscure, but its insulin-like structure suggests important roles in *Bombyx*.

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Bombyxin mRNA has not been detected by northern hybridization in tissues other than brain [5]. This does not necessarily exclude the possibility that bombyxin is expressed in tissues other than brain, because northern hybridization seems not to be sensitive enough to detect minute amounts of mRNA which may still be functional in tissues, as revealed for insulin-like growth factors (IGFs) in vertebrate peripheral tissues [10]. Thus, elucidation of spatial localization of bombyxin mRNA in tissues other than brain may give an indication of the physiological function(s) of bombyxin in *Bombyx*. In order to detect the expression of the bombyxin gene in tissues, we employed a sensitive detection method of reverse transcription-polymerase chain reaction [11] (RT-PCR) and in situ hybridization which would detect, if expressed, a minute amount of bombyxin mRNA in those tissues.

In a highly sensitive detection method like RT-PCR, there is always a possibility to give a false positive result because of the contamination of genomic DNA in the RNA sample [11]. In order to avoid such a possibility, we developed a procedure of RT-PCR performed on Oligotex-dT30. This simple procedure, Oligotex RT-PCR, permitted the specific and efficient amplification of bombyxin mRNA in tissues other than brain such as ganglia, epidermis, testis, ovary, fat body, silk gland, Malpighian tubule, midgut and hindgut. Among these tissues, the expression site in the ovary was localized by in situ hybridization.

Materials and methods

Larvae of a racial hybrid of *Bombyx mori*, Kinshu × Showa, were reared on an artificial diet (Silk-

mate: Nihon Nosan Kogyo, Japan) at 25 ± 1 °C under a photoperiodic regime of 12 h light and 12 h dark.

Total RNA was prepared from ganglia, epidermis, testis, ovary, fat body, silk gland, Malpighian tubule, midgut and hindgut of the feeding fifth instar larvae. The RNA (1 µg) was reverse transcribed in a 20 µl solution of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.5 mM $MgCl_2$, 1 mM of all dNTPs, 0.001% (w/v) gelatin and 1 unit/µl RNase inhibitor (Takara Shuzo, Japan), using 10 units of RAV-2 reverse transcriptase (Takara Shuzo) in the presence of 0.1 mg of Oligotex-dT30 Super (Takara Shuzo, Nippon Roche, Japan). The reaction mixture was incubated for 60 min at 42 °C, boiled for 5 min and chilled quickly on ice. The Oligotex-dT30-cDNA was separated by centrifugation at 14,000 rpm for 5 min at 4 °C and washed twice with 1 ml of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA. The washed Oligotex-dT30-cDNA was suspended in a 100 µl solution of 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM of all dNTPs, 0.001% (w/v) gelatin. The solution also contained 1.25 µM of each of the primers 5′-CCGAATTCAGTAATGTGGG(C/T)GTCAAC-3′ and 5′-AAGGATCCTACGACAGAAGCACGTC-3′ for the family A or 5′-CCGAATTCGAGG(A/C)(G/C)CAGGAGGTAGCGC-3′ and 5′-AAGGATCCGGAAACA(A/G)CATTCGTC(C/T)ACTAC-3′ for the family B. We employed degenerate primers to achieve the amplification of all the bombyxin transcripts because those sequences differ slightly even within the same family. Amplification was performed by PCR with 2.5 units of Taq DNA polymerase (Cetus, USA) using a DNA Thermal Cycler (Perkin Elmer, USA) with the thermal profile of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min.

Four µl of the RT-PCR reaction was electrophoresed on 3% NuSieve (3:1) agarose gel (FMC BioProducts, USA) and transferred to a nylon membrane filter in 0.4 N NaOH. Hybridization was done with the ^{32}P-labeled DNA probe at 65 °C in a solution of 6 × SSC (1.8 M NaCl, 0.18 M sodium citrate), 1% SDS, 1 × Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone and 0.02% bovine serum albumin), 0.1 mg/ml denatured sonicated salmon sperm DNA and 10% (w/v) dextran sulfate. Family A and B transcripts were detected using the bombyxin A-1 gene (position 505–1045 in fig. 1 of ref. 4) and bombyxin B-1 gene (position 98–666 in fig. 2 of ref. 5), respectively. The filter was washed with 2 × SSC containing 0.1% SDS at 65 °C and then with 0.2 × SSC containing 0.1% SDS at 65 °C. The experimental conditions did not allow hybridization between the family A and B sequences [5].

The ovaries of day 3 fifth instar larvae were fixed for 30 to 40 min in a fixative containing 85% ethanol, 4% formaldehyde and 5% acetic acid on ice, and soaked in 10 mM phosphate buffered saline (PBS), pH 7.4, containing 15% sucrose at 4 °C overnight. After serial dehydration, the ovaries were embedded in paraffin wax (Paraplast Plus, Oxford Labware, USA) and sectioned at 10 µm. After dewaxing and hydration, the sections were treated with 0.2 N HCl for 20 min followed by proteinase K (1 ng/ml in 20 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$) at 37 °C for 30 min, and fixed again with 4% paraformaldehyde in PBS at room temperature for 15 min. The sections were washed with PBS and hybridized with the bombyxin family B fragment (position 370–543 in fig. 2 of ref. 5) labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Germany) at 37 °C in the presence of 50% formamide, 5 × SSC, 1 × Denhardt's solution and 5% (w/v) dextran sulfate. After washing with 50% formamide containing 5 × SSC and then with PBT (PBS containing 0.05% Tween 20) at room temperature, the sections were treated with 5% sheep serum (Cappel, USA) at 4 °C overnight and developed using DIG nucleic acid detection kit (Boehringer Mannheim, Germany) in the presence of 1 mM levamisole, a potent inhibitor of lysosomal phosphatases.

Results and discussion

The applicability of RT-PCR [11] was examined by amplification of mRNA transcribed from the bombyxin family A genes in the *Bombyx* fat body, which has been presumed to be an expression site of the bombyxin genes. We first performed conventional RT-PCR without reverse transcriptase in order to examine the genomic DNA contaminating the RNA preparation. The amplified products were found at 25 cycles, and the signal band became more intense with increased cycle

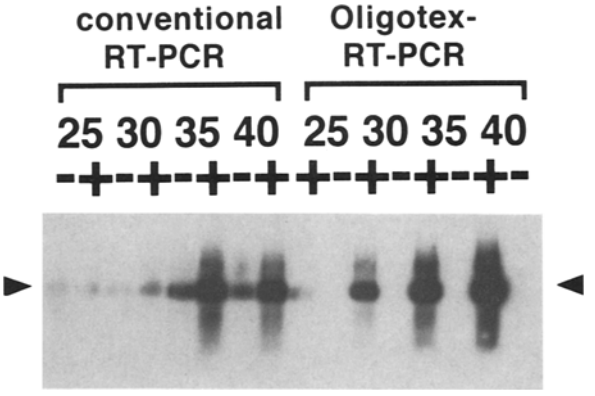

Figure 1. Oligotex RT-PCR analysis of bombyxin family A transcripts in the *Bombyx* larval fat body. Duplicate RNA samples were incubated in the presence (+) or absence (−) of reverse transcriptase and amplified for 25, 30, 35 or 40 cycles as indicated above each lane. The washing step of the Oligotex-cDNA was excluded in conventional RT-PCR, but not in Oligotex RT-PCR. The amplified products were electrophoresed on 3% NuSieve (3:1) agarose gel and hybridized with the bombyxin gene A-1 as a probe. Arrowheads indicate the amplified bombyxin family A cDNAs (~250 bp). Note that (−) samples of the Oligotex RT-PCR show no amplification even after 40 cycles, whereas those of the conventional RT-PCR show amplifications of genomic sequences over 20 cycles.

number (fig. 1). This clearly showed the presence of bombyxin sequences derived from the contaminating DNA in the RNA sample.

Since RT-PCR is an extremely sensitive method of expression analysis, even a minuscule amount of contaminating genomic DNA in an RNA preparation often gives signal bands [11]. The molecular size of the product amplified from mRNA is usually smaller than that from the contaminating genomic sequence because of the presence of intron(s). Bombyxin genes so far characterized are, however, intronless [3–6], which means that the molecular size (~250 bp) of the products amplified from the genomic sequence is the same as that from mRNA as shown in figure 1, and therefore a simple comparison of the molecular size of the products cannot distinguish between the products amplified from bombyxin mRNA and the contaminating genomic sequence. Accordingly, it was necessary to eliminate completely the contaminating DNA for the detection of bombyxin gene expression by RT-PCR.

A thorough DNase treatment of the RNA preparation can be applied to avoid the false-positive result from amplification of genomic sequences, but the DNase treatment frequently destroys the RNA in question due to contaminating RNase activity in the DNase. Therefore, we tried to eliminate the contaminating DNA from the RNA sample using Oligotex-dT30. Oligotex-dT30 is extensively used for separation of polyA RNA [12] and is effective in removing DNA contamination. Such separation of polyA RNA, however, has the disadvantage that the resulting polyA RNA must be concentrated by ethanol precipitation, which means reduced recovery of mRNA, and a relatively large amount of tissue is required. We therefore applied Oligotex-dT30-linked polyA RNA directly to reverse transcription without separating polyA RNA from Oligotex-dT30. After reverse transcription, the resulting Oligotex-dT30-cDNA was thoroughly washed to eliminate the contaminating DNA and then used directly as the template for PCR. This procedure, Oligotex RT-PCR, avoided the false positive amplification of bombyxin genomic sequences. As shown in figure 1, there was no amplification without reverse transcriptase even after 40 cycles of PCR. In addition, the signals obtained from Oligotex RT-PCR were stronger than those from conventional RT-PCR. This efficient amplification may be attributed to the thorough washing of Oligotex dT-cDNA, separating contaminants which possibly suppress the DNA polymerase activity in PCR. This procedure therefore appeared to ensure specific and effective amplification of bombyxin mRNA and was applied to examine bombyxin gene expression in tissues other than brain.

As shown in figure 2, both bombyxin family A and B mRNA have been detected in all the tissues examined: ganglia, epidermis, testis, ovary, fat body, silk gland, Malpighian tubule, midgut and hindgut. We prepared RNA at least twice from each tissue and repeated the RT-PCR at least 5 times for each tissue in order to confirm that the results are reproducible and not due to

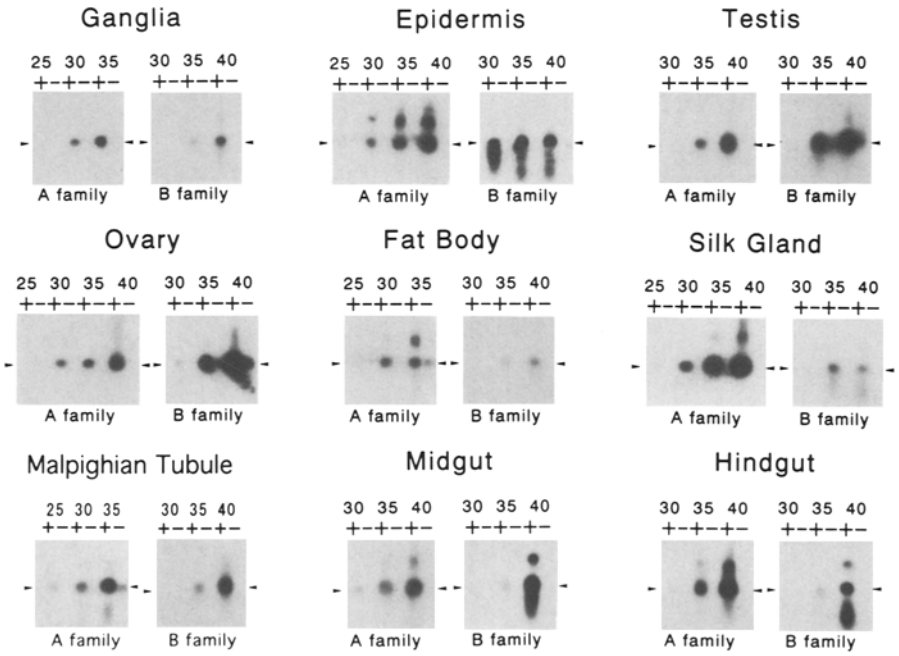

Figure 2. Bombyxin family A and B transcripts in various tissues detected by Oligotex RT-PCR. Duplicate RNA samples were incubated in the presence (+) or absence (−) of reverse transcriptase, and amplified for 25, 30, 35, or 40 cycles as indicated above each lane. Arrowheads indicate the amplified bombyxin cDNAs detected by hybridization using the bombyxin gene A-1 (A family) or B-1 (B family) as a probe.

contaminations. In the RNA samples from ganglia, epidermis, silk gland, midgut and hindgut, the signals of transcripts increased their intensity in accordance with the number of PCR cycles, and no signal was detected after the reactions without reverse transcriptase. Therefore, the signals obtained in the presence of reverse transcriptase were considered to be the amplified products from the bombyxin mRNAs but not from their genes. In testis, ovary, fat body and Malpighian tubule, faint bands appeared without reverse transcriptase. It was possible that the contaminating DNA in the RNA samples could bind nonspecifically to the latex beads, the substrate for binding oligo-dT, and were not washed out through the procedure as described, and thus were amplified by PCR. The intensity of the false positive signals in those tissues, however, was much lower than that of signals obtained in the presence of reverse transcriptase. Therefore, we concluded that the bombyxin family A and B genes are actually transcribed in all the tissues examined. The amount of bombyxin mRNAs in all the tissues examined, however, appeared to be very low because of the lack of hybridization signals in northern analysis [6] when 10 μg of each total RNA was hybridized to the ^{32}P-labeled probes.

In the RT-PCR, upper ($\sim$500 bp) and lower (100–200 bp) bands other than for bombyxin cDNA were occasionally found in epidermis, fat body, silk gland, midgut and hindgut. The reason for these signals is not obvious at present, but it is possible that the upper and lower bands are PCR artefacts and incompletely amplified bombyxin cDNAs, respectively. In the present experiments, we did not quantitate bombyxin mRNAs using an internal control in the PCR reaction because simultaneous amplification of an internal control and target genes often results in lower levels of PCR products due to competition, which varies from sample to sample [13]. In fact, we got no reproducible result when we quantitated the bombyxin mRNAs using an internal control (data not shown).

We further confirmed the expression of bombyxin mRNA by identifying the bombyxin transcripts in the larval ovary, as an example of the tissues, by in situ hybridization (fig. 3). The in situ hybridization was repeated three times and gave a reproducible result. The hybridization signals were detected in serial sections of the ovary, which definitely confirmed the expression of bombyxin mRNA in the ovary.

The expression showed spatial specificity, indicating that the positive signal in the RT-PCR is not a leaky expression. The most intense signals were found in two or three cell layers at the outer surface of each ovariole. The cells at the inner surface of an ovariole and the cell layers beneath the ovarial capsule also gave a positive, but rather less intense, hybridization signal. There was no signal in the interstitial cells between the cell layers in the outer and inner surface of an ovariole and between ovariole and ovarial capsule. This suggests that bombyxin(s) could possess their own function in the tissues, though their role remains to be determined. The presence of bombyxin molecules has been demonstrated in developing embryos shortly after oviposition but before appearance of neurosecretory cells in the brain

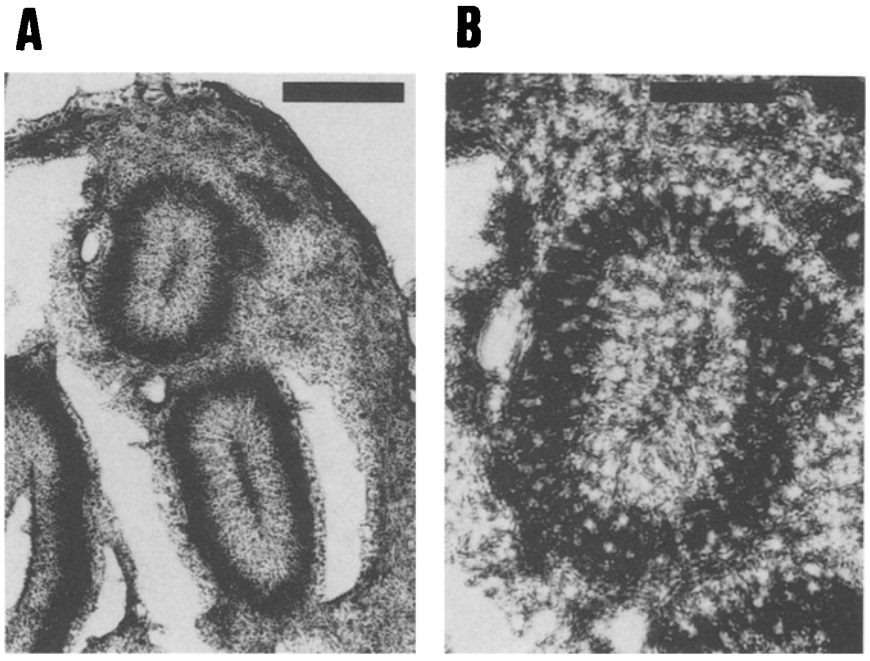

Figure 3. In situ hybridization for localization of the bombyxin transcripts (dark grays) in a section of a *Bombyx* larval ovary (*A*) and a magnified picture showing an ovariole (*B*). The bombyxin B-1 fragment labeled with digoxigenin-11-dUTP was used as a probe. Bar: 100 μm (*A*); 50 μm (*B*).

[14]. It is thus probable that bombyxin(s) in the ovariole are transferred to the embryo.

The presence of insulin-related molecules in the brain has been demonstrated in various insects [15]. In insects such as *Apis mellifera* [16] and *Locusta migratoria* [17], the insulin-related molecules exist in various tissues besides the brain. In addition, insulin-like activity of the insulin-related peptides has been shown in various insects such as *Tenebrio molitor* [18], *Locusta* [17] and *Apis* [19]. At present, no evidence has been presented for bombyxin to correspond to one of those insulin-

related peptides. Nor is there evidence that bombyxin has an insulin-like activity in *Bombyx*. The insulin-like structure of bombyxin family peptides, however, indicates the presence of the insulin-related peptides that comprise bombyxin family peptides.

Bombyxin exhibits a high degree of sequence similarity to insulin as well as to IGFs [3, 4]. The highest degree of similarity appears in the A chain between bombyxin and IGF-II, even higher than that between insulin and IGF-II. IGF-I mRNA is ubiquitously expressed in tissues including liver, uterus, lung, ovary, kidney, heart, testes, pancreas, stomach/intestine, skeletal muscle, mammary gland, brain, spleen, placenta, cartilage and pituitary, while IGF-II mRNA is present in tissues of fetal animals and in brain and spinal cord of adults [10]. The richest source of IGF-I is adult rat liver, which contains approximately 30 times more IGF-I mRNA than the highest level in other tissues [20]. IGFs are multifunctional, inducing mitosis and differentiation in a wide variety of cells and tissues [10]. The present study demonstrates the ubiquitous expression of bombyxin mRNA in the *Bombyx* larvae. The in situ hybridization suggested that the range of action of bombyxin produced in the ovary is local, like that of IGFs. Along with the recent reports that bombyxin induces meiosis in *Bombyx* ovarian cells in vitro [21] and causes cessation of cell division followed by morphological changes in BM-N4 cells, a cell line derived from a *Bombyx* ovarian tissue [22], our results suggest that bombyxin plays important roles in growth, development, and differentiation of insects, similar to vertebrate growth factors such as IGFs.

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Note added in proof: The data referred to above as Kondo et al., unpubl. data, has been published: Kondo H., Ino M., Suzuki A., Ishizaki H. and Iwami M. (1996) Multiple gene copies for bombyxin, an insulin-related peptide of the silkmoth *Bombyx mori*: structural signs for gene rearrangement and duplication responsible for generation of multiple molecular forms of bombyxin. J. Molec. Biol. **259:** 926–937

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