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Molecular Characterization of a cDNA from the Silk Moth *Bombyx mori* Encoding *Manduca sexta* Allatotropin Peptide

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ABSTRACT—Allatotropin is a 13-residue amidated neuropeptide isolated from pharate adult heads of the tobacco hornworm, *Manduca sexta* and strongly stimulates biosynthesis of juvenile hormones in adults, but not larval, lepidopteran corpora allata. From a *Bombyx mori* midgut cDNA library, a cDNA that encodes a 130-amino-acid polypeptide containing *M. sexta* allatotropin sequence was isolated. The *B. mori* allatotropin cDNA consists of 1196 nucleotides. The encoded allatotropin peptide is identical to that isolated from *M. sexta* and that predicted from *Pseudaletia unipuncta*, with 84% and 81% identity in the amino acid sequence of the allatotropin peptide precursor, respectively. *M. sexta* allatotropin is flanked by two different endoproteolytic cleavage sites within the precursor of the *B. mori* allatotropin peptide. Evidence from northern blotting of *B. mori* tissues showed that the allatotropin gene is expressed in the cells of midgut, head and integument with different transcription amount, but not in the fat body and silk gland. Midgut has also a number of allatotropin-immunoreactive cells and nerve fibers. These results will provide valuable information in understanding the AT gene of insects.

Key words: Allatotropin cDNA, allatotropin gene expression, allatotropin-immunoreactive cells, midgut, *Bombyx mori*

INTRODUCTION

Juvenile hormone (JH) is a sesquiterpenoid hormone that plays a central role in metamorphosis, reproduction and adult sexual maturation of insects (Engelmann, 1970). The successful development and sexual maturation of insects are dependent on their ability to control JH levels precisely at all stages of the life cycle (Taylor *et al*., 1996). JH hemolymph titer is regulated mainly by the synthetic activity of the corpora allata (Feyereisen, 1985; Tobe and Stay, 1985).

Biosynthesis of JH by corpora allata is controlled by stimulatory (allatotropin, AT) or inhibitory (allatostatin, AST) neuropeptides (Kramer *et al*., 1991; Pratt *et al*., 1991; Bellés *et al*., 1994; Duve *et al*., 1994; Hayes *et al*., 1994; Stay *et al*., 1994; Woodhead *et al*., 1994; Davis *et al*., 1997). These two neuropeptides are produced by some neuronal cells in the brain and thereafter transported to the retrocerebral complex via the nervi corporis cardiaci in *B. mori* (Park *et al*., 2001). The AT stimulates the glandular cells of the corpora allata to synthesize JH and then release it into hemolymph, whereas AST inhibits them to produce JH. Although allatotropic factors had been purified or identified from the brains of *M. sexta* and *Galleria mellonella* and the suboesophageal ganglia of *Gryllus bimaculatus* and *Acheta domestica* (Gadot and Applebaum, 1985; Bogus and Scheller, 1991; Lorenz and Hoffmann, 1995), more knowledge was necessary on insect AT

The presence of more than one allatotropic factor with a molecular mass of 0.7 to 20 kDa was described in different insect species (Rembold *et al*., 1986; Ferenz and Diehl, 1983). However, it appeared that a large discrepancy in molecular mass between allatotropic factors found in different developmental stages and/or insect species was present. A large AT with Mr. of 20 kDa has been identified in the larval brain of *G. mellonella* (Bogus and Scheller, 1994, 1996), whereas *M. sexta* AT (Mas-AT), an α-amidated tridecapeptide, has been first purified from the head extract of the pharate adult (Kataoka *et al*., 1989). Mas-AT stimulates JH biosynthesis by adult CA, but this has no discernible effect on the rate of JH biosynthesis of larvae or pupae of *M. sexta*. This description suggests that the activity of

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Mas-AT may be restricted to the adult stage of the order Lepidoptera (Kataoka *et al*., 1989).

There were some studies on localization of Mas-AT-immunoreactive cells in different tissues throughout postembryonic development of *M. sexta*, *Drosophila melanogaster*, *Lacanobia oleracea* and *B. mori* (Veenstra and Hagedorn, 1993; Zitnan *et al*., 1993a, 1995; Duve *et al*., 2000; Rudwall *et al*, 2000; Park *et al*., 2001). These studies suggested additional roles for Mas-AT. It has been mentioned that Mas-AT has a cardioacceleratory role in *M. sexta* adult (Veenstra *et al*., 1994) and an inhibitory role in midgut ion transport in *M. sexta* larvae (Lee *et al*., 1998a).

There is little doubt that the peptidergic gut innervation and peptidergic midgut endocrine cells occur in most insects (Zitnan *et al*., 1993b, 1995; Lundquist *et al*., 1994; Nässel *et al*., 1995). In *Galleria mellonella* frontal, ingluvial and proventricular ganglia contain 2 to 4 AT-labeled neuronal cells, and the midgut has endocrine cells with AT-immunoreactivity. There is also a labeling of AT in the neuronal cells of frontal and proventricular ganglia and in the endocrine cells of midgut in *M. sexta*, as well as in the nervous midgut plexus of *Lymantria dispar* (Žitňan *et al*., 1993b).

It has been demonstrated that AT cDNA shows varying structural characteristics with differences in insect species such as *M. sexta* (Taylor *et al*., 1996), *Aedes aegypti* (Veenstra and Costes, 1999) and *P. unipuncta* (Truesdell *et al*., 2000). We describe molecular characterization of *B. mori* cDNA encoding the Mas-AT, making a comparison between *B. mori* AT (Bommo-AT) cDNA and AT cDNA of the insect species described previously.

MATERIALS AND METHODS

Animals

Cold-treated eggs of the silk moth *Bombyx mori* (Lepidoptera) were supplied from the National Institute of Agricultural, Science and Technology (Suwon, Korea) and hatched to the first instar larvae about 10 days after the onset of incubation at the temperature of 27–28°C and relative humidity of 60–70%. Larvae were reared on an artificial diet under a long-day photoperiod regimen (17-hr light and 7-hr dark). They were normally metamorphosed to pupae and then to adults.

RNA isolation and cDNA synthesis

Total RNA was isolated from the 3rd instar larval midguts using the TRI reagent-RNA isolation reagent (Molecular Research Center, INC) (Sambrook and Russell, 2001). About 20 midguts of the 3rd instar larvae were homogenized in the TRI reagent with several strong strokes and then placed it at room temperature for 5 min. For the RNA elution 0.2% chloroform was added. The mixture of homogenized midguts was again placed at room temperature for 5 min and subsequently centrifuged with 12,000xg at 4°C for 15 min. The supernatant was transferred into a new 1.5ml-eppendorf tube and then the isopropanol was added. Centrifugation was again performed with 12,000xg at 4°C for 5 min and the supernatant was discarded. The pellet was washed in 75% ethanol while gently flicking it and then dissolved in the TE buffer containing 10mM Tris-Cl, pH 8.0 and 1mM EDTA.

The cDNA for the templates of the reverse transcriptase-PCR (RT-PCR) was synthesized using a SMART cDNA synthesis kit (Clontech). Degenerate primers were designed, as described in Taylor *et al*. (1996). Four primers for RT-PCR (AT1:5'-CTTCAAGAACGTCGAAATGATG-3'; AT2:5'-AATGATGACYGCNAGAGGCTTC-3'; AT 3:5'-GCAACAGCGACCCACGCG-3';AT4:5'-CTTTCAGATTTAAACCACG AC-3'; Y=A,T,G,C; N=C,G) and two primers for 5'-RACE PCR (primer A:5'-CCACG AACCTGGCGAAGTC-3'; primer B:5'-CGAATGCACCGGACGTCTTG-3') were used.

PCR and cDNA cloning

The RT-PCR reactions were performed with the murine leukemia virus (MLV)-reverse transcriptase (BRL) in *Taq* polymerase buffer (Takara) supplemented to a final concentration of 3.5mM $MgCl_2$, 2.5mM dNTPs and 0.2M of each primer in 50 μl. The mixtures were overlaid with mineral oil and then amplified in a thermal cycler (Perkin Elmer) for 30 cycles (95°C for 1 min; 57°C for 1 min; 72°C for 90 s), preceded by a 4-min denaturation step at 94°C and followed by a 5-min final extension at 72°C. The electrophoresis was performed on the Mupid-21 Kit (50V). The DNA was eluted from the sliced band with a DNA Purification Kit (UltraCleanTM 15, MO BIO). After determination of the concentration of the PCR product, ligation and transformation reactions were performed with a pGEM-T Easy Vector (Promega) System. The Miniprep kit (Wizard, Promega) was used only for sequencing.

Autosequencing for cDNA and searching for the similarity

PCR was performed in the following mixtures; Big Dye, Primer T7, SP6 1 pmol each, plasmid DNA, bringing to total 10 μl on the GeneAmp PCR system 9700 (PE Applied Biosystem). A 1.1kb cDNA clone encoding the Mas-AT was analyzed with a 310 Genetic Analyzer (ABI Prism, Perkin-Elmer). The cDNA sequence similarity searching was performed using the BLAST from the NCBI data base through internet access.

PCR for 5'-RACE

As a full length of Bommo-AT cDNA was not detected in the RT-PCR, two new oligonucleotides (antisense primer A: 5'-CCACGAACCTGGCGAAGTC-3'; antisense primer B: 5'-CGAATGCACCGGACGTCTTG-3') were designed based on the *B. mori* cDNA sequence to find the 5' end of Bommo-AT cDNA by 5'-RACE. The 5'-RACE procedure was performed using a 5'-RACE system for the RACE kit (SMART, Clontech). PCR was conducted under the conditions of a cycle of 94°C for 2 min, 30 cycles of 94°C for 1 min, 59°C for 2 min, and 72°C for 2 min.

Northern blotting

Sense and antisense allatotropin RNA probes were synthesized from the cDNA clone. The probes were transcribed with a digoxigenin-11-UTP RNA labeling mix (Boehringer-Mannheim) using T7 RNA polymerase (Promega). Total RNAs were isolated from the midgut, head, integument, fat body and silk gland of the 3rd instar larva and electrophoresed with 5μg/ml total RNAs per lane. After electrophoresis, the gel was equilibrated in 20XSSC for 2X15 min to remove the formaldehye. The RNA was blotted in the gel by capillary transfer overnight at room temperature with 20XSSC. The probe was prehybridized in a solution at 68°C for 1.5 hr, denatured in boiling water for 10 min, and then immediately placed on the ice. After the discard of the prehybridization solution and then the addition of the hybridization solution containing the DIG-labeled probe, the membrane was washed twice in 2XSSC containing 0.1% SDS at room temperature (15 minX2). Another wash of the membrane continued in 0.5XSSC containing 0.1% SDS at room temperature (15 minX2). The membrane was equilibrated in washing buffer and blocked by gently agitating it in the blocking solution for 30-60 min. The blocking solution was removed and the membrane was incubated in the antibody solution for 30 min. The anti-DIG-POD solution was added to the blocking solution and then mixed gently. The membrane was washed to remove the unbound antibody, followed

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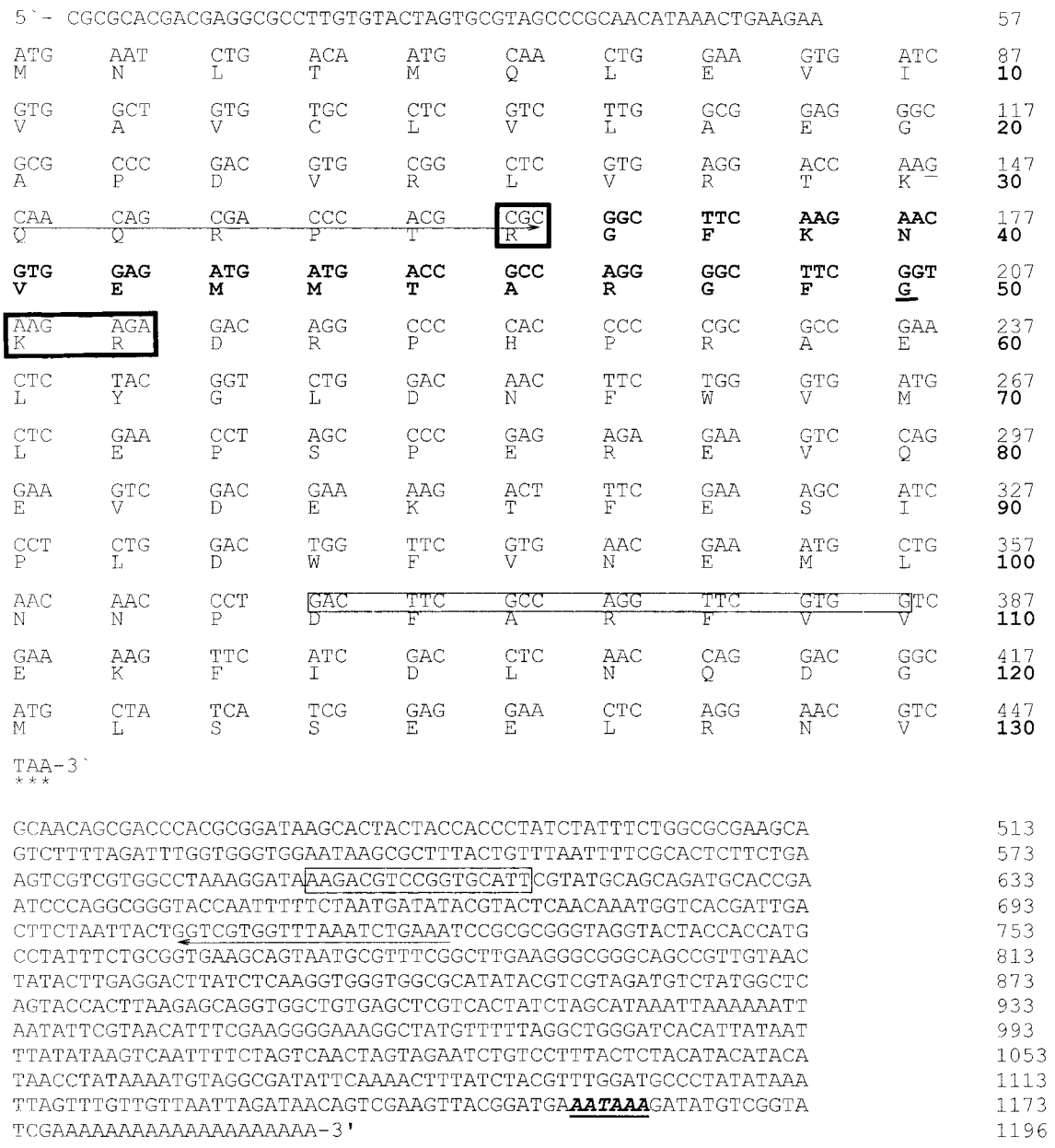


Fig. 1. Nucleotide sequence of the Bommo-AT cDNA and deduced amino acid sequence of the AT peptide precursor. The sequences are numbered at the right. The amino acid sequence for the Mas-AT peptide is shown in bold type. Two proteolytic cleavage sites are surrounded by thick rectangles. Glycine residue required for amidation is single underlined. Potential polyadenylation signal (AATAAA, Whale and Keller, 1992) is shown bold underlined. RT-PCR oligonucleotide primer sequences are underlined with arrows and 5'-RACE primer sequences are surrounded by rectangles.

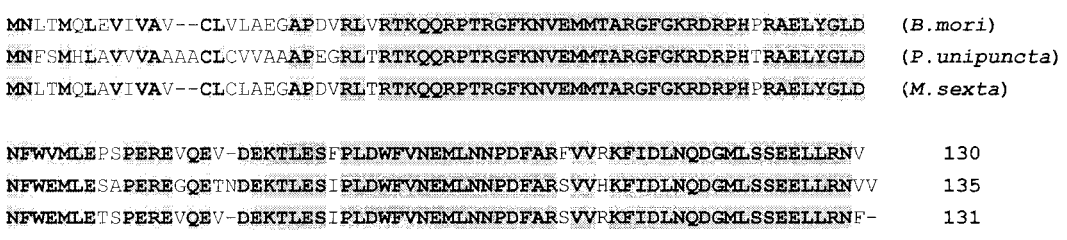


Fig. 2. Comparison of AT precursor peptides from *B. mori*, *M. sexta* (Taylor *et al.*, 1996) and *P. unipuncta* (Truesdell *et al.*, 2000). The common homology from three moths is shown in shaded bold-type letters. Translated portions are conserved with high homology.

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by equilibration in a detection buffer for 2 min. After the pour-off of the detection buffer and the addition of the color substrate solution to the membrane, the membrane was washed in distilled water and then a photo of the desired spots or bands was taken with a polaroid camera.

Wholemount immunocytochemistry

Tissue preparation and wholemount immunocytochemistry were performed according to Lee *et al*. (1998b) and Kim *et al*. (1998). Following anaesthetic treatment of the third instar larvae at 4°C for 1 hr, foreguts, midguts and hindguts were isolated in 0.1M sodium phosphate buffer (pH 7.4) (PB) and then fixed in 4% paraformaldehyde (PFA) in 0.1M PB for 5 hr at 4°C. The fixed tissues were immersed in 0.01M phosphate-buffered saline (PBS) with 1% Triton X-100 at 4°C overnight. Blockage of peroxidase activity was performed in 10% methanol in 3% H_2O_2 for 25 min. Washes in 0.1M Tris-HCl buffer (pH 7.6–8.6) containing 1% Triton X-100 and 4% NaCl were followed by incubation with a primary antiserum (anti-Mas-AT, Veenstra *et al*., 1994), diluted to 1: 1,500 in dilution buffer (0.01M PBS with 1% Triton X-100 and 10% normal goat serum), for 4-5 d while shaking gently. After washes in 0.01M PBS with 1% Triton X-100, tissues were incubated in peroxidase-conjugated swine anti-rabbit IgG (DAKO), diluted to 1:200 for 2 d at 4°C. Following preincubation in 0.03% diaminobenzidine (DAB, Sigma) in 0.05M Tris-HCl buffer for 1 hr at 4°C, tissues were treated with 0.03% DAB in 0.05M Tris-HCl buffer containing 0.01% H_2O_2 for 5-10 min. After being rinsed in a 0.05M Tris-HCl buffer, tissues were mounted in glycerin, examined and photographed with a Zeiss interference microscope. As a specificity control, immunocytochemistry was performed on whole foreguts, midguts and hindguts of larvae with anti-Mas-AT preincubated with 50 nmol synthetic Mas-AT per ml diluted antiserum (diluted to 1:1,500) for 24 hr.

RESULTS

Characterization of Bommo-AT cDNA

Bommo-AT cDNA was synthesized and two PCR products were made. Using a vector system, recombinant DNA was produced and the desirable trasnformation was properly carried out. The complete Bommo-AT cDNA sequence consists of the 1196 nucleotides with 390 coding sequences (Fig. 1). It contains 57 nucleotides of 5'untranslated region upstream of a single open reading frame beginning at position 58 and ending at position 448 with the translation stop codon TAA. The 3'untranslated region that follows is A/T-rich (60%) and extends for 749 nucleotides. The 3'untranslated region contains a 20-nucleotide poly-A tail and a consensus sequence directing polyadenylation (AATAAA) beginning at position 1156 and 15 bases upstream. The encoded Bommo-AT peptide located between Arg^{36} and Gly^{50} is identical to the sequence of Mas-AT (Kataoka *et al*., 1989) and to that predicted from Mas-AT cDNA (Tay-

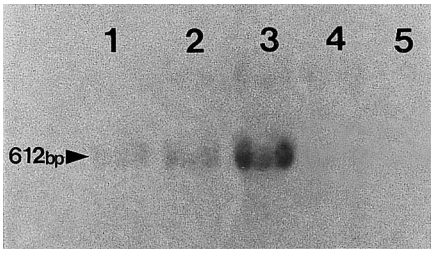


Fig. 3. Northern analysis for expression of Bommo-AT gene in the tissues. The amount of total RNA is 5μg/lane. The total RNAs in lanes 1-5 are from integument (1), head (2), midgut (3), fat body (4), and silk gland (5), respectively. The Bommo-AT gene is strongly expressed in the midgut with mild expression in the head and lowest expression in the integument, but the gene is not expressed in the fat body and silk gland.

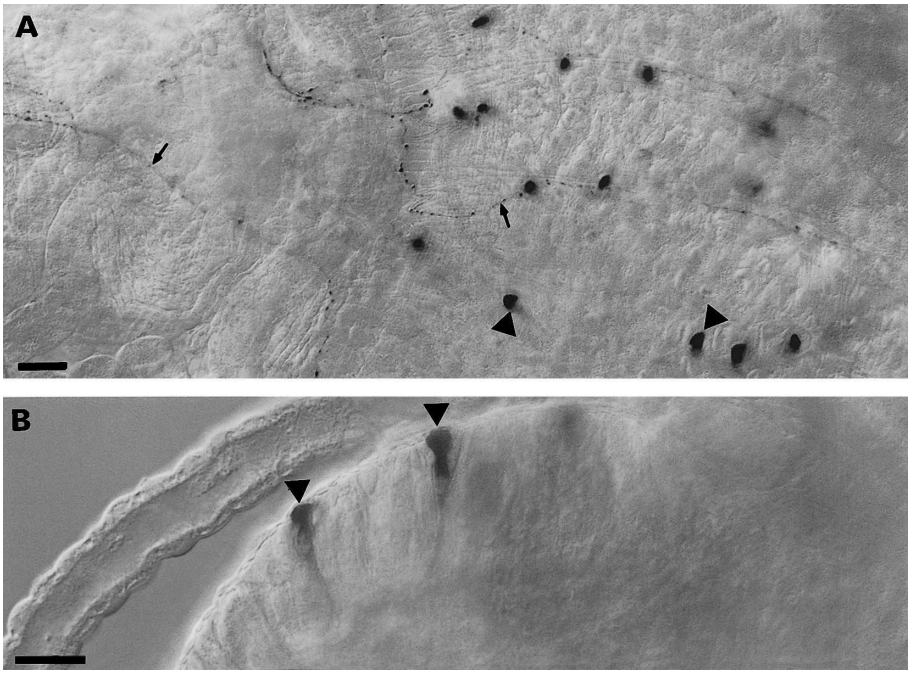


Fig. 4. Mas-AT-immunoreactivity in the larval midgut (whole-mount preparations). A. Midgut of third instar larva showing Mas-AT-immunoreactive cells (arrowheads) and nerve fibers (arrows). B. Another midgut of third instar larva showing two intensively-stained cells.

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lor *et al*., 1996). The Bommo-AT peptide is flanked by two potential endoproteolytic cleavage sites of Arg^{36} and Lys^{51}-Arg^{52}), and ends with a glycine residue, the signal for carboxy-terminal amidation by peptidyl-glycine-α-amidating monooxygenase (Eipper *et al*., 1992). Endoproteolytic cleavage of these sites would result in the production of two additional peptides of 15 and 78 amino acids. This suggests that the Bommo-AT is a secreted peptide after processing from a large precursor peptide. The basic organization of the Bommo-AT peptide precursor, which consists of 130 amino acid sequence, is similar to that of two moths, *M. sexta* with 84% amino acid identity (Taylor *et al*., 1996) and *P. unipunctata* with 81% amino acid identity (Truesdell *et al*., 2000) (Fig. 2). However, the amino acid identity of the AT peptide precursor between *B. mori* and *Aedes aegypti* decreases to less than 70%.

Detection of AT gene expression by northern blotting

For investigation of the expression of the Bommo-AT gene in the tissues, northern blotting was performed using an RNA probe. The total RNAs isolated from the midgut, head, integument, fat body and silk gland of the third instar larva were investigated. The midgut showed the strongest expression of the AT gene, while there was no detection in the fat body and silk gland (Fig. 3). The Bommo-AT mRNA was also included in both head and integument, with its lowest level in the integument and a mild level in the head.

Mas-AT-immunoreactivity in the midgut

There were Mas-AT-immunoreactive cells (Fig. 4A, B, arrowheads) of the endocrine type in the midguts of the third instar larvae, but not in the foreguts or hindguts. Labeled axons could also be detected in the wall of the midguts (Fig. 4A, arrows).

DISCUSSION

Structure of Bommo-AT cDNA

It has been demonstrated that in *B. mori* the Mas-AT neuropeptide is produced in the brain and ventral ganglia (suboesophageal, thoracic and abdominal ganglia)(Park *et al*., 2001). The *B. mori* brains contain this neuropeptide in the larval stages with no Mas-AT neuropeptide during the pupal and adult stages. In the brain of early larval life, very limited number of cells (3 pairs) produce the Mas-AT. However, a larger number of Mas-AT-immunoreactive cells (10 pairs) are located in the brain of late larval stages. The larval midgut, which has been used for molecular characterization of Bommo-AT cDNA, contains a much larger number of the AT-immunoreactive cells than the larval brain, although expression patterns of Mas-AT mRNA in the midguts at various developmental stages remains to be investigated.

The Bommo-AT is identical to the AT identified in *M. sexta* (Talyor *et al*., 1996) and *P. unipuncta* (Truesdell, *et al*., 2000), although the pattern of the AT mRNA transcription or translation appears to vary greatly between *B. mori* and other moths. The Bommo-AT cDNA has very conserved sequences to that of *M. sexta* (Taylor *et al*., 1996) and *P. unipuncta* (Truesdell, *et al*., 2000) with 84% and 81% amino acid identity, respectively. However, it has smaller conserved sequences to the mosquito *A. aegypti* (Veenstra and Costes, 1999) with less than 70% amino acid identity.

The Mas-AT within the Bommo-AT precursor peptide is flanked by two potential endoproteolytic cleavage sites of Arg^{36} and Lys^{51}-Arg^{52}, and it has also an end by a glycine residue, the signal for carboxy-terminal amidation by peptidyl-glycine-α-amidating monooxygenase (Eipper *et al*., 1992), as in *M. sexta* and *P. unipuncta*. Endoproteolytic cleavage of these sites would result in the production of two additional peptides of 15 and 78 amino acids. As with many other neuropeptides (Sossin *et al*., 1989), therefore, it appears that Mas-AT is translated from a transcript and derived from a polyprotein (Taylor *et al*., 1996).

Expression of Bommo-AT gene in the tissues

Northern blotting was performed to investigate the expression of the AT gene in the cells of midgut, head, integument, fat body and silk gland of third instar larva of *B. mori*, using a RNA probe which was synthesized by the RT-PCR. The Bommo-AT gene was expressed in the midgut, head and integument, whereas there was no detection in the fat body and silk gland (Fig. 3). The midgut showed the strongest expression of the AT gene, with the lowest expression in the integument and mild expression in the head. The function of Bommo-AT in the midgut cells remains to be determined, but it could not be excluded that the AT might function as a myoregulatory peptide in the midgut. It is suggested that Bommo-AT mRNA expressed in the head is made for stimulation of JH biosynthesis by the CA. However, functional characteristics of the Bommo-AT made in the integument cells remains to be investigated.

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