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Expression of the allatostatin gene in endocrine cells of the cockroach midgut

(neuropeptide/immunocytochemistry/*in situ* hybridization/juvenile hormone/*Diploptera punctata*)

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ABSTRACT **Cockroach allatostatins are neuropeptides that have been isolated from the brain of *Diploptera punctata* and shown to inhibit juvenile hormone production by the corpora allata. Enzyme-linked immunoassay and immunocytochemistry with antisera to two allatostatins, ASB2 (AYSYVSEYKRLPVYNFGL-NH_2) and ASAL (APSGAQRLYGFGL-NH_2), revealed that allatostatins were located not only in the insect brain but also in several peripheral tissues including the cockroach midgut and hindgut. Allatostatin-like immunoreactivity was found in nerve fibers of the stomatogastric nervous system as well as in intrinsic endocrine cells of the midgut. Midgut extracts were shown to be biologically active in an allatostatin bioassay and to contain several allatostatin-like peptides, including the octadecapeptide ASB2, which was identified by mass spectrometry following HPLC purification. Reverse transcription of brain mRNA followed by PCR with degenerate oligonucleotides for ASB2 and ASAL yielded a 338-bp fragment of the allatostatin gene that encoded six allatostatins. *In situ* hybridization with this probe confirmed that an allatostatin gene is expressed in intrinsic endocrine cells of the midgut. Reverse transcription of midgut mRNA followed by PCR and sequencing of the product revealed that the same gene is expressed in the midgut and in the brain. Allatostatins are thus an example of insect "brain–gut peptides" and we suggest that their function may not be restricted to the regulation of juvenile hormone production.**

The control of juvenile hormone (JH) production by the corpora allata (CA) is crucial to normal development, metamorphosis, and reproduction in most insect species. The activity of the corpora allata is regulated by humoral factors and by innervation of neurosecretory cells in the brain (1). Several types of neuropeptides have been shown to control JH synthesis in short-term *in vitro* assays, either by stimulating (allatotropins; ref. 2) or inhibiting (allatostatins; refs. 3–7) rates of JH production. Allatostatins from the cockroach *Diploptera punctata* have a common C-terminal -Phe-Gly-Leu-NH_2 and inhibit JH synthesis at specific developmental stages (8–10). Recently, Donly *et al.* (11) reported the conceptual sequence of an allatostatin precursor polypeptide. Its cDNA sequence was obtained by PCR amplification of brain cDNA of virgin female *D. punctata* with degenerate primers to the octadecapeptide allatostatin ASB2 (AYSYVSEYKRLPVYNFGL-NH_2) (5), followed by one-sided PCR of the remainder of the cDNA (11). The allatostatin precursor polypeptide contains 13 putative peptides, including seven allatostatins that have been isolated from brain extracts and shown to inhibit JH synthesis by the corpora allata (3–6). In adult female *D. punctata*, sensitivity of the CA to low (10 nM) concentrations of allatostatin ASAL (APSGAQRLYGFGL-NH_2) can be acquired in a few hours (8), and the level of inhibition is dependent on both the sequence of the allatostatin and its concentration (5, 8, 12). While several allatostatins may be required for the precise regulation of the corpora allata in response to physiological and environmental cues, the occurrence of multiple allatostatins may also be related to a multiplicity of functions.

Five neuropeptides with sequence homology to cockroach allatostatins were isolated from the blowfly *Calliphora vomitoria*. These peptides inhibit JH synthesis by cockroach CA but are without allatostatic activity in the blowfly (13). The presence of these peptides in neurons projecting to several peripheral tissues suggests that members of this family of allatostatins may have functions unrelated to the regulation of JH synthesis in blowflies. Similarly, a myotropic peptide from the locust *Locusta migratoria* (14) is homologous (15) to an allatotropin of the tobacco hawkmoth *Manduca sexta* (2) but is devoid of allatotropic activity in the locust (F. Couillaud and R.F., unpublished results). In turn, the *M. sexta* peptide has cardioacceleratory activity in addition to its activity as a stimulator of JH synthesis (16).

We report here that allatostatins can be detected in the cockroach midgut by immunocytochemistry as well as by isolation of peptides and of the gene transcript. The midgut allatostatins are of two origins, nerves of the stomatogastric nervous system and intrinsic endocrine cells situated in the midgut epithelium. Peptidergic innervation and peptidergic midgut endocrine cells in insects have until now been studied mainly by immunocytochemistry (17). Our results establish that midgut endocrine cells express the allatostatin gene.

MATERIALS AND METHODS

Allatostatin ELISA and Bioassay. Antisera to bovine thyroglobulin conjugates of two allatostatins, ASAL (APSGAQRLYGFGL-NH_2) and ASB2 (AYSYVSEYKRLPVYNFGL-NH_2), were produced in rabbits and used in a competitive ELISA according to the general procedure of Kingan (18). Specifically, 10 ng of peptide–bovine serum albumin (BSA) conjugate in 0.05 ml of 0.1 M sodium bicarbonate (pH 9.2) was coated onto the wells of an ELISA plate for 2 hr; the plate was washed with phosphate-buffered saline (PBS)/0.05% Tween 20, pH 7.4, and blocked with PBS/1% BSA. The sample or a known amount of standard peptide was added in 50 μl, followed by the primary antiserum (1:10,000 dilutions for ASAL and ASB2, respectively) also in 50 μl. The plate was incubated for 2 hr and then washed twice with PBS/0.05% Tween 20, pH 7.4. The secondary antiserum (goat anti-rabbit IgG conjugated to calf intestinal alkaline phosphatase, Pierce) was then added and the hydrolysis of *p*-nitrophenyl phosphate was read during 20 min in a Molecular Devices Thermomax plate reader. The allatostatin bio-

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Abbreviations: JH, juvenile hormone; CA, corpora allata.

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assay on CA from 10-day-old, mated females of *D. punctata* was performed as described (4).

Purification of Allatostatins. Extraction of the tissues, purification of the peptides by C_{18} Sep-Pak, and reversed-phase HPLC were performed as described (4, 12). HPLC fractions were assayed in the ELISA for allatostatins. The elution time of ASB2 and ASAL was determined on the same column by ELISA with authentic peptides, and blank runs following the calibration runs were monitored by ELISA to ensure that there was no contamination of the columns. The fractions with the same retention time as authentic ASB2 were collected and analyzed by matrix-assisted laser desorption mass spectrometry on a Finnigan-MAT (San Jose, CA) Lasermat instrument at the Harvard Microchem mass spectrometry facility of Harvard University (Cambridge, MA).

Immunocytochemistry. Midguts were dissected from mated female *D. punctata* and fixed overnight at 4°C in 4% paraformaldehyde in PBS (0.01 M, pH 7.2). The washed tissue was incubated 18–24 hr at room temperature in rabbit anti-ASAL or rabbit anti-ASB2 diluted 1:2000 in PBS/0.5% Triton X-100/5% normal goat serum. Rhodamine-conjugated goat anti-rabbit antiserum (Boehringer Mannheim) was used as the secondary antibody and the midguts were incubated in a 1:200 dilution in PBS/0.5% Triton X-100 overnight at room temperature. After washing and then mounting in 80% glycerol in 0.05 M sodium carbonate/bicarbonate buffer (pH 9.5), preparations were examined and photographed with a Nikon epifluorescence microscope.

Paraffin sections of midguts fixed as above were cut at 5 μm. The sections were mounted on poly(L-lysine)-coated slides and stained by the indirect peroxidase-antiperoxidase (PAP) method. The primary antisera were diluted 1:2000 in PBS and applied overnight at 4°C. Goat anti-rabbit IgG (Sigma) was used at a dilution of 1:50 and PAP was used (Sigma) at 1:100; 10 mg of 3,3′-diaminobenzidine tetrahydrochloride per ml with 0.1% H_2O_2 was used as a chromogen. The sections were dehydrated, cleared in xylene, and mounted for examination.

Controls included preadsorption of the antisera with authentic peptide and deletion of the primary antisera. No specific immunostaining was seen in either of the controls.

Molecular Probe for the Allatostatin Gene. Brains were dissected from male and female *D. punctata* and immediately frozen on dry ice. The brains were homogenized and poly(A)$^+$ RNA was isolated using oligo d(T)-cellulose (Micro Fasttrack mRNA Isolation Kit, Invitrogen). First strand cDNA was synthesized with Superscript RNase H$^-$ reverse transcriptase (GIBCO/BRL). PCR was performed with degenerate primers: GCNTACT/AG/CITAC/TGTIA/TG/CIGAGTACAA and GAA/GTACAAGA/CGIC/TTC/GCCIGTITACAAC/TTTC/TGG as sense-directed primers and CCCAGA/T/GCCGAAA/T/GCCA/GTA as antisense-directed primer, corresponding to the reverse-translated DNA sequence of amino acids 1–9 and 7–17 of ASB2 and 9–13 (and the assumed Gly precursor of the C-terminal amide) of ASAL, respectively. The primers were added at a concentration of 100 ng/μl to a standard PCR reaction mix (50 μl volume) containing 5 μl of cDNA, 1× PCR buffer (0.2 mM dNTPs/50 mM KCl/10 mM Tris, pH 8.3/2.5 mM $MgCl_2$/0.1% gelatin), and 2.5 units of Ampli-Taq DNA polymerase (Perkin–Elmer/Cetus). The reactions were performed at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min, including a 2-min prior denaturation step at 94°C and a 5-min postextension step at 72°C.

Midgut poly(A)$^+$ RNA was isolated as described (19). PCR on midgut cDNA was performed as described above, using an annealing temperature of 55°C with the following primers: GGTCTCGGCAAGAGAAGCAAAATGT and GACGCTGTGCTCCAGATGGTGCTC. The gel-purified PCR products (Sephaglas Band-Prep Kit, Pharmacia) were cloned into the pCR II vector (Invitrogen) and sequenced from both ends with the dideoxynucleotide chain-termination method using the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical).

***In Situ* Hybridization.** Digoxigenin-labeled antisense RNA transcripts were synthesized from *Xba* I and *Bam*HI linearized PCR II plasmids in the presence of digoxigenin-11-UTP (Boehringer Mannheim). The resulting transcript was precipitated with ethanol and sized to ≈150 bp by alkaline hydrolysis. Midguts from day 4 mated female *D. punctata* were fixed in 4% paraformaldehyde in PBS (130 mM NaCl/7 mM Na_2HPO_4/3 mM NaH_2PO_4, pH 7.4) at room temperature for 1 hr. The tissue was prepared for hybridization as described by Hafen *et al.* (20) with the exception that a 1 μg/ml proteinase K digestion was substituted for the Pronase treatment and the tissue was acetylated in 0.25% (vol/vol) acetic anhydride in 0.1 M triethanolamine for 10 min. The midguts were prehybridized in 50% formamide/0.6 M NaCl/1× Haase mix (10× Denhardt's solution/100 mM Tris, pH 7.5/10 mM EDTA)/1 mg of salmon sperm DNA per ml/1 mg of yeast total RNA per ml at 50°C for 1–2 hr and hybridized overnight in the same solution containing 10% dextran sulfate and the digoxigenin-labeled riboprobe. After hybridization, the tissue was washed in 50% formamide/3 M NaCl/0.1 M Tris, pH 6.8/0.1 M Na_2HPO_4/50 mM EDTA for 1–2 hr, RNase treated (20 μg of RNase A per ml and 1 unit of RNase T1 in 0.5 M NaCl/10 mM Tris·HCl, pH 8/1 mM EDTA) for 30 min at 37°C, and washed in 2× SSC for 30 min at room temperature, 0.1× SSC at 50°C, and then in PBST. To detect the digoxigenin-labeled RNA, a primary anti-digoxigenin antibody, a secondary biotinylated antibody, and streptavidin-conjugated Cy3 (Jackson ImmunoResearch) were used. Digoxigenin-labeled sense transcripts were used in control experiments that were negative. The tissue was examined using a Bio-Rad MRC 600 laser-scanning confocal microscope equipped with a krypton-argon laser and a YHS filter cube. Stacks of optical sections were acquired at 2-μm steps with three scans of each step.

RESULTS

Immunocytochemical Demonstration of Allatostatin-Like Material in the Midgut. Allatostatin-like material was found by immunocytochemistry in the brain–corpora cardiaca–corpora allata axis as expected but was also widely distributed in neural and nonneural tissues, such as the hindgut and the antennal heart muscle. Antisera against ASAL (APSGAQRLYGFGL-NH_2) and ASB2 (AYSYVSEYKRLPVYNFGL-NH_2) both revealed the presence of allatostatin-like material in the midgut (Fig. 1). In addition to nerve fibers rich in allatostatin-like material, small but rather numerous intrinsic cells of the midgut also stained with the antisera. The nerve fibers formed a grid pattern at the anterior (Fig. 1*B*) and posterior ends of the midgut but were uniformly longitudinal in the middle portion of the midgut (Fig. 1*C*). These nerve fibers of the stomatogastric nervous system were traced through the esophageal nerve (Fig. 1*A*), recurrent nerve, and the frontal ganglion to the brain. They may originate in one or two clusters of 6–10 cells in the tritocerebrum (not shown). The intrinsic cells of the midgut stained with the antisera are pyramidal, with their narrow end extending to the lumen of the gut and their broad end in contact with the basal lamina on the hemocoel side (Fig. 1 *D* and *E*). No difference in the staining pattern—number of cells or relative distribution—was found with the two antisera.

Allatostatin Bioactivity in the Midgut and Its Purification and Characterization. Midguts from 10-day-old mated females were extracted in acid ethanol and partially purified on a C_{18} Sep-Pak cartridge (4). This material was tested for biological activity in a standard radiochemical assay for JH

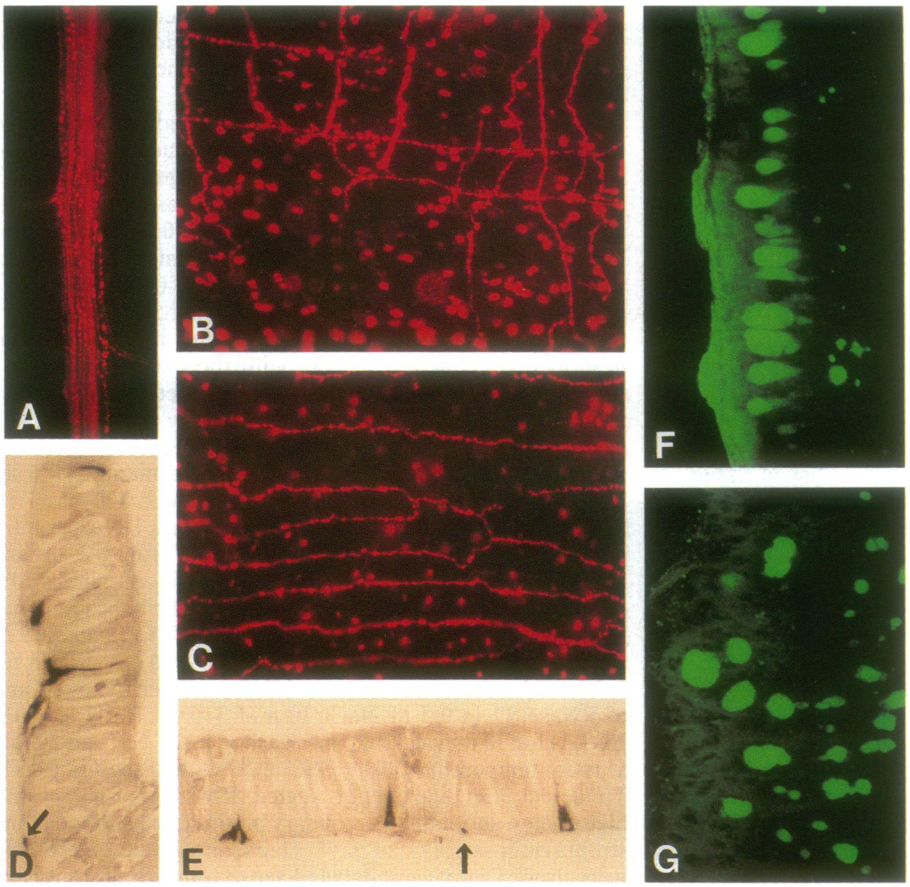

FIG. 1. Immunocytochemistry (*A–E*) and *in situ* hybridization (*F* and *G*) of allatostatins in the *D. punctata* midgut. (*A*) Whole mount of esophageal nerve stained with anti-ASB2 serum. (×210.) (*B* and *C*) Whole mount of the midgut showing the anterior (*B*) and middle (*C*) parts stained with anti-ASB2 serum. (×120.) (*D* and *E*) Paraffin sections of the midgut stained with anti-ASB2 serum showing the pyramidal shape of the endocrine cells traversing the midgut epithelium and immunoreactive axons (arrow). Lumen is right (*D*) and up (*E*). (×180.) (*F* and *G*) *In situ* hybridization of allatostatin transcript in endocrine cells of the midgut. Midgut whole mounts were viewed on a confocal microscope. (*F*) Longitudinal section of the edge of the midgut, 68-μm stack of optical sections (i.e., the "fold" of the flattened tube, lumen situated at the right, staining on the left is a background artefact caused by the edge effect). (*G*) Surface view of the midgut (i.e., the top of the flattened structure, lumen situated underneath the plane of view, as in *B*, 30-μm stack of optical sections). (×270.)

synthesis with CA from 10-day-old mated females. The midgut extract caused a dose-dependent inhibition of JH synthesis with a maximal inhibition of about 85% as did brain extracts prepared and assayed in the same way (Fig. 2). The brain and the midgut contained 21.7 and 9.5 allatostatin units (quantity of allatostatin that causes 50% inhibition of JH synthesis in the standard assay; ref. 4) per insect, respectively. Immunocytochemistry and biological activity, while suggestive, did not firmly establish the presence of allatostatins in the midgut. Sensitive ELISAs to ASAL and ASB2 were developed (Table 1) and used to monitor the separation of midgut extracts by HPLC. Both ELISAs preferentially recognized the amidated C terminus of the allatostatins. The Sep-Pak fraction active in the ELISAs and bioassay (i.e., the fraction eluting at 18–35% acetonitrile in water) was separated on reversed-phase HPLC, eluted with a gradient of acetonitrile with 0.1% trifluoroacetic acid on a C_{18} column.

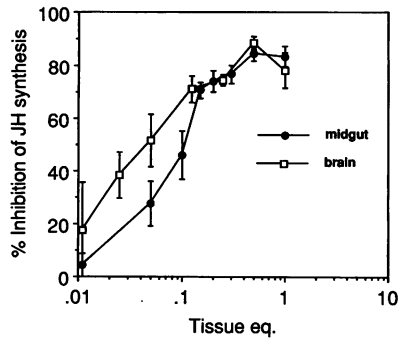

FIG. 2. Inhibition of JH synthesis by midgut and brain extracts. The activity of C_{18} Sep-Pak-purified midgut or brain extracts was assayed on corpora allata from 10-day-old adult females. Results are expressed as a mean (±SE) percentage inhibition of JH release for 10 (brain) or 8–28 assays (midgut).

Several ELISA-positive peaks were observed with both assays (not shown). The fractions with longest retention time, coeluting with ASB2, were negative in the ASAL ELISA, were positive in the ASB2 ELISA, and were also positive in the allatostatin bioassay. These were pooled and further purified with a gradient of acetonitrile with 1% formic acid/0.3% triethylamine on a RP-4 column. A peak of ASB2 ELISA-positive material eluted with the same retention time as authentic ASB2 (Fig. 3). The two fractions of this peak, estimated to represent about 15 pmol of peptide, were pooled and analyzed by matrix-assisted laser desorption mass spectrometry (21), which gave a $[M + H]^+$ ion at m/z 2197.8 corresponding to the formylated peptide (result not shown).

Molecular Cloning of an Allatostatin Precursor Gene. Because the known allatostatin peptides were clearly homologous, with a common Tyr-Xaa-Phe-Gly-Leu-NH_2 C terminus

Table 1. Sensitivity and selectivity of the allatostatin ELISAs

Parameter	ASB2 assay	ASAL assay
ED_{50}, fmol*	49.2 ± 3.7	15.6 ± 0.9
Slope†	0.77 ± 0.02	0.99 ± 0.02
Ratio of ED_{50} values for competing peptides		
ASB2	1	769
ASAL (allatostatin 1)‡	53.1	1
Allatostatin 2‡	≫500§	131
Allatostatin 3‡	≫500§	417
Allatostatin 4‡	≫500§	96.1
ASB2-(11–18)	1.01	71.8
ASB2-(1–9)	≫500§	≫1500§

*fmol of standard required for 50% displacement of binding to conjugate.
†Slope of dose–response curve at 50% displacement.
‡Woodhead *et al.* (3).
§No displacement observed.

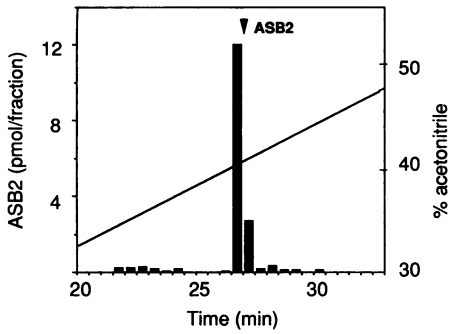

FIG. 3. Purification of ASB2 from midgut extracts by HPLC and ELISA. Extracts from 500 midguts of post-vitellogenic females were purified on C_{18} Sep-Paks and separated by RP-18 HPLC with an acetonitrile/trifluoroacetic acid gradient. The fractions with the same retention time as ASB2 and positive in the ASB2 ELISA were pooled and separated on a RP-4 HPLC column with a 10–50% acetonitrile gradient containing 1% formic acid and 0.3% triethylamine over 35 min. Fractions were collected every 30 sec and a 5% aliquot of each fraction was assayed by the ASB2 ELISA. The arrowhead indicates the retention time of authentic ASB2 under the same conditions.

(3–5), we felt that allatostatins might be encoded by a common precursor. Thus a combination of degenerate forward and reverse primers for two of the longest peptides that we had sequenced (ASB2 and ASAL) was expected, in one combination, to yield a PCR product from brain cDNA. This strategy proved successful, and we cloned and sequenced two PCR products of 320 bp and 338 bp with two forward primers based on the ASB2 sequence and a reverse primer based on the ASAL sequence (Fig. 4). No other primer combination gave a PCR product. The shorter clone was entirely contained in, and identical to, the longer clone. The deduced amino acid sequence was that of an authentic allatostatin precursor: In addition to the sequence of ASB2 and ASAL, it contained the sequence of the octapeptide allatostatin 4 (DRLYSFGL-NH_2) (3) and of three new allatostatins, two octapeptides and one nonapeptide. All of these peptides had the Tyr-Xaa-Phe-Gly-Leu-Gly sequence preceding a Lys-Arg dibasic cleavage site. In addition, the precursor fragment also had a 31-amino acid portion with 16 acidic (Glu/Asp) residues (Fig. 4).

***In Situ* Hybridization of Allatostatin Transcripts in Midgut Cells.** To characterize the intrinsic midgut cells detected by immunocytochemistry, digoxigenin-labeled antisense RNA transcripts of the allatostatin precursor clone were hybridized to whole mounts of midguts. The hybrids were detected by anti-digoxigenin antibodies, secondary biotinylated antibodies, and Cy3-conjugated streptavidin and then analyzed on a confocal microscope. Results showed that the allatostatin gene was expressed in intrinsic cells of the midgut that correspond in number and shape to those detected by immunohistochemistry (Fig. 1 *F* and *G*).

Sequence of Midgut Allatostatin Precursor Fragment. The sequence of the allatostatin precursor clone from the brain provided nondegenerate primers for PCR amplification of the allatostatin cDNA from the midgut. Reverse transcription of midgut poly(A)$^+$ RNA followed by PCR with these primers yielded a 271-bp product as expected from the sequence (result not shown). This PCR product was cloned and its sequence was identical to that of the brain cDNA (Fig. 4).

DISCUSSION

Peptides comprising a family of cockroach allatostatins were isolated from the brain–corpora cardiaca–CA complex (3–6, 22) based on the ability of these peptides to inhibit JH synthesis in a short-term bioassay. It was widely assumed at the time of their isolation by this laborious procedure that allatostatins were produced in neuroendocrine cells of the brain, although biological activity was also found in other parts of the nervous system (23). Our study clearly shows that cockroach allatostatins are distributed beyond the brain–corpora allata axis and that other cells in addition to four neuroendocrine cells of the pars intercerebralis (11) express the allatostatin gene. Allatostatin-like immunoreactivity is present in neurosecretory cells in the brain and in neuroendocrine axons projecting to the corpora cardiaca and CA (24) and in cells of subesophageal and terminal abdominal ganglia (25). It is also present in brain interneurons (24) and in neurons projecting to several peripheral tissues, such as the antennal heart muscle and the hindgut in *D. punctata* (25, 26). This distribution suggests that members of the allatostatin family of neuropeptides may have functions unrelated to the regulation of JH synthesis. Hertel and Penzlin (27) first showed that allatostatin antagonized the excitatory effect of proctolin on the antennal heart of another cockroach, *Periplaneta americana*. An inhibitory effect of allatostatins on hindgut contraction and a proctolin-antagonistic activity was also shown in *D. punctata* (25). Most recently, Duve and Thorpe (28) showed that Leu-callatostatins 1 and 3, peptides from the blowfly that share a homologous Tyr-Gly-Phe-Gly-Leu-NH_2 C terminus with ASAL, are potent inhibitors of peristalsis of the hindgut of *C. vomitoria*. Leu-callatostatins are not found in terminal arborizations within the corpus allatum (13, 28), and they are devoid of allatostatic activity in the blowfly (13). However, Leu-callatostatin immunoreactivity was found in endocrine cells in a narrow part of the midgut immediately anterior to the point of evagination of the Malpighian tubules (28). It appears that peptides belonging to the cockroach allatostatin/callatostatin family are widespread inhibitory neurotransmitters, neuromodulators, and neurohormones. An inhibitory activity toward JH synthesis has so far only been demonstrated in cockroaches.

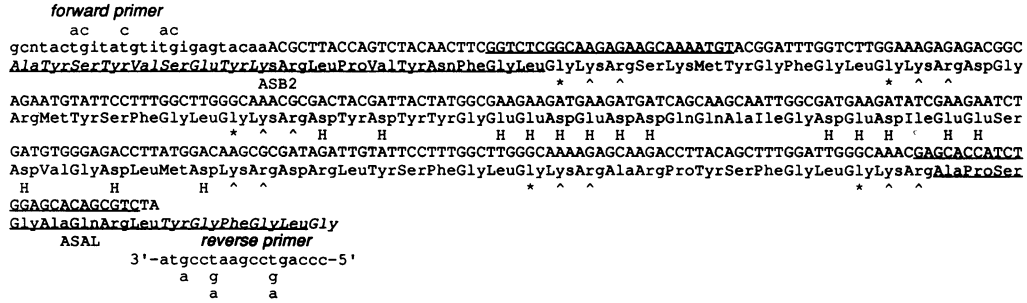

FIG. 4. Nucleotide and deduced amino acid sequence of the allatostatin cDNA fragment. The sequence of the most distal PCR primers is shown in small capital letters. The sequence of the nondegenerate PCR primers used to amplify the midgut allatostatin cDNA is underlined. C-terminal Gly residues that are converted posttranslationally to amides (*), dibasic cleavage sites (^^), and acidic residues (H) of the allatostatin precursor fragment are marked and the ASB2 and ASAL sequences are underlined.

The amount of allatostatin found in the midgut by bioassay is about one-half of the activity found in the brain. The octadecapeptide ASB2 has an internal Lys-Arg potential cleavage site and the C-terminal product of ASB2 cleavage at that site is considerably less potent as an inhibitor of JH synthesis (5). In contrast, Leu-callatostatin 3, the cleavage product of the hexadecapeptide Leu-callatostatin 1 at a dibasic cleavage site homologous to that of ASB2, is considerably more potent than its parent peptide in inhibiting peristalsis of the ileum in the blowfly (28). It was possible that the allatostatin precursor peptide would be cleaved at that site in the midgut of the cockroach. However, the ELISA and bioassay-positive peptide that coeluted with ASB2 on HPLC was identified as the authentic ASB2 octadecapeptide. A full characterization of all midgut allatostatin peptides should now be possible in *D. punctata*, because the products of the allatostatin precursor can be deduced from the cDNA sequence (11).

The possibility remained that the allatostatin we had isolated from the midgut was present in stomatogastric nerves originating in the brain or that the immunocytochemical demonstration of midgut endocrine cells was an artefact (false positive identification by the antisera; ref. 29). Indeed, ELISA and bioassay easily detected allatostatin activity in the hindgut, which is lined with allatostatin-immunoreactive nerve fibers but is devoid of intrinsic allatostatin-immunoreactive cells (G.C.U. and R.F., unpublished data). To resolve this ambiguity and prove that the intrinsic endocrine cells were producing allatostatins, we had to use a molecular probe for the allatostatin gene. A partial allatostatin cDNA was cloned and the nucleotide sequence of this allatostatin precursor fragment was identical to that reported recently by Donly *et al.* (11). Southern analysis of genomic DNA from *D. punctata* revealed that the allatostatin gene is a single copy gene (11). The evidence we present here, *in situ* hybridization, reverse transcription PCR from midgut mRNA, and sequence of the midgut PCR product, thus clearly demonstrate that the same allatostatin gene is expressed in the central nervous system and in the intrinsic endocrine cells of the midgut. It is still possible that transcription and post-translational processing may vary in different cell types. However, the presence of authentic ASB2 and the presence of several allatostatin-like peptides in midgut extracts suggest that such differences, if they occur, are minor.

Our work adds the allatostatin family of peptides to the list of (mostly vertebrate) neuropeptides detected previously by immunocytochemistry in midgut endocrine cells of cockroaches and of other insects (review in ref. 16). The recent description of [Leu]callatostatin-like immunoreactivity in endocrine cells of the blowfly (28), which is evolutionarily very distant from the cockroach, suggests that peptides of the cockroach allatostatin/callatostatin family may be found in midgut endocrine cells of most insects. As pointed out by Brown and Lea (30), only immunocytochemical and morphological similarities to the gut endocrine system in vertebrates lead us to call these midgut cells "endocrine." The ultrastructure of midgut endocrine cells has been described in other cockroaches, *Nauphoeta cinerea* (31), *P. americana* (32), and *Blaberus craniifer* (33), in which at least 10 cell types were distinguishable by the shape and size of their secretory granules. Ultrastructural images of exocytosis of secretory granules from endocrine cells of *P. americana* were presented by Endo and Nishiitsutsuji-Uwo (34). A physiological demonstration of neurohormone release from these midgut cells is still lacking, however, and we can only speculate about the functions of the endocrine cells of the midgut that express the allatostatin gene. Midgut allatostatins may inhibit the CA in response to starvation (1) or play a role in digestion or gut motility, but these "brain–gut peptides" may have other functions as well.

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1. Feyereisen, R. (1985) in *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, eds. Kerkut, G. A. & Gilbert, L. I. (Pergamon, Oxford), Vol. 7, pp. 391–429.
2. Kataoka, H., Toschi, A., Li, J. P., Carney, R. L., Schooley, D. A. & Kramer, S. J. (1989) *Science* **243**, 1481–1483.
3. Woodhead, A. P., Stay, B., Seidel, S. L., Khan, M. A. & Tobe, S. S. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5997–6001.
4. Pratt, G. E., Farnsworth, D. E., Siegel, N. R., Fok, K. F. & Feyereisen, R. (1989) *Biochem. Biophys. Res. Commun.* **163**, 1243–1247.
5. Pratt, G. E., Farnsworth, D. E., Fok, K. F., Siegel, N. R., McCormack, A. L., Shabanowitz, J., Hunt, D. F. & Feyereisen, R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2412–2416.
6. Woodhead, A. P., Khan, M. A., Stay, B. & Tobe, S. S. (1994) *Insect Biochem. Mol. Biol.* **24**, 257–263.
7. Kramer, S. J., Toschi, A., Miller, C. A., Kataoka, H., Quistad, G. B., Li, J. P., Carney, R. L. & Schooley, D. A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 9458–9462.
8. Pratt, G. E., Farnsworth, D. E. & Feyereisen, R. (1990) *Mol. Cell. Endocrinol.* **70**, 185–195.
9. Weaver, R. J. (1991) *J. Insect Physiol.* **37**, 111–118.
10. Stay, B., Joshi, S. & Woodhead, A. P. (1991) *J. Insect Physiol.* **37**, 63–70.
11. Donly, B. C., Ding, Q., Tobe, S. S. & Bendena, W. G. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8807–8811.
12. Pratt, G. E., Farnsworth, D. E., Siegel, N. R., Fok, K. F. & Feyereisen, R. (1991) in *Insect Neuropeptides*, eds. Menn, J. J., Kelly, T. J. & Masler, E. P. (Am. Chem. Soc., Washington, DC), pp. 177–192.
13. Duve, H., Johnsen, A. H., Scott, A. G., Yu, C. G., Yagi, K. J., Tobe, S. S. & Thorpe, A. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2456–2460.
14. Paemen, L., Tips, A., Schoofs, L., Proost, P., Van Damme, J. & De Loof, A. (1991) *Peptides* **12**, 7–10.
15. Veenstra, J. A. & Hagedorn, H. H. (1993) *Arch. Insect Biochem. Physiol.* **23**, 99–109.
16. Veenstra, J. A., Lehman, H. K. & Davis, N. T. (1994) *J. Exp. Biol.* **188**, 347–354.
17. Zitnan, D., Sauman, I. & Sehnal, F. (1993) *Arch. Insect Biochem. Physiol.* **22**, 113–132.
18. Kingan, T. G. (1989) *Anal. Biochem.* **183**, 283–289.
19. Cariño, F., Koener, J. F., Plapp, F. W., Jr., & Feyereisen, R. (1992) in *Molecular Mechanisms of Insecticide Resistance*, eds. Mullin, C. A. & Scott, J. G. (Am. Chem. Soc., Washington, DC), pp. 31–40.
20. Hafen, E., Levine, M., Garber, R. L. & Gehring, W. J. (1983) *EMBO J.* **2**, 617–623.
21. Chait, B. T. & Kent, S. B. (1992) *Science* **257**, 1185–1194.
22. Stay, B., Woodhead, A. P., Joshi, S. & Tobe, S. S. (1991) in *Insect Neuropeptides*, eds. Menn, J. J., Kelly, T. J. & Masler, E. P. (Am. Chem. Soc., Washington, DC), pp. 164–176.
23. Rankin, S. M. & Stay, B. (1987) *J. Insect Physiol.* **33**, 551–558.
24. Stay, B., Chan, K. & Woodhead, A. P. (1992) *Cell Tissue Res.* **270**, 15–23.
25. Lange, A. B., Chan, K. K. & Stay, B. (1993) *Arch. Insect Biochem. Physiol.* **24**, 79–92.
26. Woodhead, A. P., Stoltzman, C. A. & Stay, B. (1992) *Arch. Insect Biochem. Physiol.* **20**, 253–263.
27. Hertel, W. & Penzlin, H. (1992) *Acta Biol. Hung.* **43**, 113–125.
28. Duve, H. & Thorpe, A. (1994) *Cell Tissue Res.* **276**, 367–379.
29. Veenstra, J. (1988) *Neuropeptides* **12**, 49–54.
30. Brown, M. R. & Lea, A. O. (1990) in *Advances in Disease Vector Research*, ed. Harris, K. F. (Springer, New York), Vol. 6, pp. 29–58.
31. Cassier, P. & Fain-Maurel, M. A. (1977) *Arch. Zool. Exp. Gen.* **118**, 197–209.
32. Nishiitsutsuji-Uwo, J. & Endo, Y. (1981) *Biomed. Res.* **2**, 30–44.
33. Andries, J. C. & Tramu, G. (1985) *Cell Tissue Res.* **240**, 323–332.
34. Endo, Y. & Nishiitsutsuji-Uwo, J. (1982) *Cell Tissue Res.* **222**, 515–522.