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Molecular characterisation of cDNAs from the fall armyworm *Spodoptera frugiperda* encoding *Manduca sexta* allatotropin and allatostatin preprohormone peptides☆

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Abstract

Allatotropin (AT) is a 13-residue amidated neuropeptide, first isolated from pharate adult heads of the tobacco hornworm, *Manduca sexta* (Manse-AT), which strongly stimulates the biosynthesis of juvenile hormones (JH) in the corpora allata (CA) of adult moths. In *Spodoptera frugiperda*, a cDNA that encodes 134 amino acids, including an AT peptide, has been cloned. The *S. frugiperda* allatotropin mature peptide (Spofr-AT) [GFKNVEMMTARGFa] is identical to that isolated from *M. sexta*. The basic organization of the Spofr-AT precursor is similar to that of *Agrius convolvuli*, *M. sexta*, *Pseudaletia unipuncta*, and *Bombyx mori* with 83–93% amino acid sequence identity. The Spofr-AT gene is expressed in at least three mRNA isoforms with 134, 171 and 200 amino acids, differing from each other by alternative splicing.

All allatostatins (AS) have an inhibitory action on the JH biosynthesis in the CA. A cDNA that encodes 125 amino acid residues including one copy of the Manse-AS peptide has been cloned from *S. frugiperda* (Spofr-AS; QVRFRQCYFNPISCF). The basic organization of the Spofr-AS precursor is similar to that of *P. unipuncta* with 85% amino acid sequence identity.

Using one step RT-PCR for semi-quantification of the gene expression, we showed that the three mRNAs of the Spofr-AT gene and the Spofr-AS gene are expressed in brains of last instar larvae, prepupae, pupae, and adults of both sexes of *S. frugiperda* with variable intensity.

Keywords: Lepidoptera; Allatostatin; Allatotropin; Hormone processing; Prohormone; Neuropeptide

1. Introduction

The development and reproduction of insects are regulated, to a large extent, by juvenile hormones (JH). During the larval stages these hormones control moulting and metamorphosis, whereas in adult insects they are involved in the regulation of vitellogenesis in females and spermatogenesis and growth of the accessory reproductive glands in males (Koeppe et al., 1985; Nijhout, 1994; Riddiford, 1994; Gäde et al., 1997). Juvenile hormones are synthesized and released from the corpora allata (CA), which are present in all insect stages. During the last decade, interest has focused on factors that regulate JH biosynthesis by CA (Stay et al., 1994; Hoffmann et al., 1999; Bendena et al., 1999; Stay, 2000). Depending upon species and development stages, the signals may be either stimulatory (allatotropin, AT) or inhibitory (allatostatin, AS), and they may reach the glands via haemolymph or via nervous connections.

Since 1989, more than 60 neuropeptides that inhibit JH production by the CA in homologous or heterologous bioassays in vitro have been isolated from brains of several insect taxa (moths, cockroaches, locusts, crickets, flies and bees), and also from crustaceans (for review see Bendena et al., 1997; Gäde et al., 1997; Weaver et al., 1998; Hoffmann et al., 1999). The allatostatins form three different peptide families. Members of the allatostatin A family are characterised by a common C-terminal

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Y/FXFGL/I-a pentapeptide sequence. Some members of the allatostatin A family were deduced from prohormone gene sequences (Donly et al., 1993; Ding et al., 1995; East et al., 1996; Vanden Broeck et al., 1996; Veenstra et al., 1997; Bellés et al., 1999; Davey et al., 1999; Lenz et al., 2000; Meyering-Vos et al., 2001). In some species these peptides have no effect on the CA of the source insect, but instead exhibit myo-inhibiting properties (Duve and Thorpe, 1994). The second family consists of peptides, which were at first isolated from *Gryllus bimaculatus* and all have a common amino acid (Trp) at positions two and nine (allatostatin B family; W^2W^9-amide peptide family; $W(X)_6W$-amides) (Lorenz et al., 1995). A B-type allatostatin preprohormone from *Drosophila melanogaster* (DAP-B) has recently been cloned, which encodes 211 amino acid residues and contains one copy each of five putative allatostatin-B peptides (Williamson et al., 2001a).

Within the Lepidoptera, a non-amidated allatostatin was first characterised from the tobacco hornworm, *Manduca sexta*. This peptide, *M. sexta* allatostatin (C-type allatostatin; Manse-AS; pEVRFRQCYFNPISCF-OH) inhibits JH biosynthesis in vitro by the CA of larvae and adult females of *M. sexta*, and adult females of *Heliothis virescens*, *H. zea* and *Lacanobia oleracea* (Kramer et al., 1991; Audsley et al., 1999, 2000; Edwards et al., 2001; Teal, 2002). Manse-AS did not affect the CA of two orthopteroid species, *Periplaneta americana* and *Melanoplus sanguinipes*, or the CA of the beetle *Tenebrio molitor* (Kramer et al., 1991). The peptide shows no sequence similarity to members of any other allatostatin family. In *Pseudaletia unipuncta*, a cDNA has been characterised, which encodes 125 amino acid residues including the 15-residue peptide of the allatostatin C-type (Jansons et al., 1996). The Pseun-AS did not inhibit JH biosynthesis in 6th instar larvae or newly emerged (0 day) adults, but inhibited the CA activity of 5 day-old adult females of *P. unipuncta* by 60%. Expression of the gene was low in 6th instar larvae, prepupae and early pupae, but relatively high in late pupae and in 1 day- and 3 day-old adults of both sexes (Jansons et al., 1996). In *Spodoptera littoralis* (Audsley et al., 1999) and in *L. oleracea* (Audsley et al., 1998), a peptide that seems to be identical with Manse-AS was detected in larval brain extracts. Very recently, the allatostatin C-type preprohormone has been characterised from *D. melanogaster* (drostatin-C). The drostatin-C peptide differs in only one amino acid residue (F to Y in position 4) from the Manse-AS. The gene was strongly expressed in larvae and adult flies, but less in pupae and embryos (Williamson et al., 2001b).

In the fall armyworm *S. frugiperda*, synthetic Manse-AS did not affect the in vitro rate of JH secretion from the CA of adult moths. However, when the CA had previously been activated by Manse-AT, addition of Manse-AS reduced JH biosynthesis by about 70%. This allatostatic effect of Manse-AS on allatotropin-activated glands was dose-dependent and reversible (Oeh et al., 2000). However, until now, Manse-AS has not been detected in *S. frugiperda*.

To date, only one allatotropin is known (GFKNVEMMTARGFa), which has been demonstrated to stimulate the CA. It was first isolated from the head of pharate adults of *M. sexta* (Kataoka et al., 1989), for which the gene encoding the peptide has also been cloned (Taylor et al., 1996). This gene is expressed in at least three mRNA isoforms that differ from each other by alternative splicing regulated in a tissue-specific manner (Horodyski et al., 2001). Manse-AT has also been isolated from larval and adult brains of *L. oleracea* (Audsley et al., 2000). From the mosquito *Aedes aegypti* an allatotropin immunoreactive peptide has been isolated and its structure determined as APFRNSEMMTARGFa. Furthermore, the cDNA encoding this peptide has been identified (Veenstra and Costes, 1999). A cDNA isolated from the true armyworm, *P. unipuncta*, encodes a 135 amino acids sequence containing the Pseun-AT which is identical to that isolated from *M. sexta*. The AT mRNA was highly expressed in older pupae and 3- and 5-day-old adults of *P. unipuncta* (Truesdell et al., 2000). A precursor, which encodes a 130 amino acid polypeptide, has been cloned from a midgut cDNA library of *B. mori*, and this precursor also contained the Manse-AT peptide sequence (Park et al., 2002).

A peptide that strongly stimulates JH biosynthesis in vitro by the adult female CA was recently isolated from methanolic brain extracts of adult *S. frugiperda* and was identified as *M. sexta* allatotropin (Manse-AT) (Oeh et al., 2000). Injections of Manse-AT twice daily into penultimate and last instar larvae of *S. frugiperda* drastically reduced their weight gain and increased mortality, shortened the life span of the adult females and decreased the total number of deposited eggs (Oeh et al., 2001). Functional assays and distributional studies support additional roles for Manse-AT to that of JH regulation. Manse-AT seems to function as a cardioacceleratory peptide in adult *M. sexta* (Veenstra et al., 1994) and *P. unipuncta* (Koladich et al., 2002) and it inhibits midgut ion transport in day 2 fifth instar larvae of *M. sexta* (Lee et al., 1998). Multiple functional activities are suggested by the distribution of Manse-AT immunoreactive cells in neural tissues of various insect species (Veenstra and Hagedorn, 1993; Veenstra et al., 1994; Žitnan et al., 1993, 1995; Persson and Nässel, 1999; Rudwall et al., 2000; Tu et al., 2001).

In the present study we confirm the structure of the recently identified Manse-AT in *S. frugiperda*, and elucidate the existence of a C-type allatostatin in this species by cloning the cDNAs, which encode the precursors of Spofr-AT and Spofr-AS, respectively. Both the genes were variably expressed in the brains of larvae, pupae and adult moths.

2. Materials and methods

2.1. Animals

Pupae and eggs of *S. frugiperda* were provided by Bayer AG (Leverkusen) and reared at 27 °C and 70% relative humidity under a L 16: D 8 photoperiod as previously described (Oeh et al., 2000). Larvae and adults were reared as described by Oeh et al. (2000). Under these conditions, the 6th (last) larval stage lasts for about 4 days. Each pupa was individually kept in a separate compartment of assortment boxes (9 × 32 × 36 - mm per compartment; Licefa, Bad Salzuflen, Germany) until emergence. Freshly emerged females were kept in 20 × 20 × 10 - cm plastic boxes and fed with 10% sugar solution until dissection.

2.2. Preparation of the cDNA by RACE

Brains (cerebral and subesophageal ganglia) of 530 female adult fall armyworms, 2–3 days old, were dissected in modified cricket saline (Lorenz et al., 1997), frozen immediately in liquid nitrogen and stored at −80 °C until use. Total RNA was extracted with Invertebrate RNA Kit (Peqlab Biotechnologie GmbH). The mRNA was extracted with Oligotex® mRNA Mini Kit (Qiagen GmbH). The Smart™ RACE cDNA amplification kit (Clontech) was used to amplify the cDNA. 1 μg mRNA was added as a template for each RACE reaction. A 3′-RACE was carried out using degenerate oligonucleotide primers ATf4 (5′-GGN TTY AAR AAY GTN GAR ATG ATG A-3′) corresponding to nucleotide positions 397–421 of Fig. 1 and ASf5 (5′-GTN MGN TTY MGN CAR TGY TAY TTY AA-3′) corresponding to nucleotide positions 543-569 of Fig. 4, respectively, in combination with the universal primer (5′-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT-3′) that recognises the primer binding sequence, introduced by the Smart RACE kit components. The cDNA was further amplified by using gene specific primers ATf7 corresponding to nucleotide positions 397–421 and ATr6 corresponding to nucleotide positions 418–444 of Fig. 1 to get the 5′ end and the 3′ end of the AT preprohormone and ASf6 corresponding to nucleotide positions 543–569 and ASr10 corresponding to nucleotide positions 569–593 of Fig. 4 in order to get the 5′ end and the 3′ end of the AS precursor, respectively. The PCR program was 95 °C for 2 min, followed by 10 cycles of 94 °C for 30 s, 68 °C for 45 s decreased with 1 °C per cycle, followed by 35 cycles of 94 °C for 30 s, 58 °C for AS or 60 °C for AT for 45 s, 68 °C for 1.5 min, and a final extension step of 68 °C for 10 min.

2.3. Cloning and sequencing

The PCR products were eluted from low melting point agarose gel (Biozym) with GFX™ purification kit (Pharmacia), and ligated into plasmids with the pGEM-T easy system kit (Promega). The vector with the inserts was grown in *E. coli* JM109 (Promega) or XL1-blue (Stratagene), respectively. Plasmid DNA was purified using QIAprep® Spin Miniprep kit (Qiagen). The templates were sequenced by MWG Biotech (Ebersberg, Germany) or GATC (Konstanz, Germany). Sequences were analysed with the GCG software (Wisconsin Package).

2.4. RT-PCR analysis

For RT-PCR 20 ng mRNA from brains of 6th instar larvae (<12 h), pre-pupae (PP), young pupae (P0), 10-day-old pupae (P10), 2-day-old adult males (M2) and 2-day-old adult females (F2) were used with specific primers ATf7-ATr9 corresponding to nucleotide positions 397–421 and 598–621 of Fig. 1A and primers ASf6-ASr9 corresponding to nucleotide positions 543–569 and 644–670 of Fig. 4, respectively, in 10 μl of the RT-PCR mixture (TITANIUM® One Step RT-PCR Kit, CLONTECH Laboratories, Inc.). The PCR program for amplification of the fragment was 50 °C for 1 h followed by 5 min at 94 °C, 35 cycles of 94 °C for 30 s, 60 °C for AT or 58 °C for AS for 45 s, 68 °C for 1 min, and a final extension step of 68 °C for 2 min. The DNA products were analysed using 1.5% agarose gel at 8 V/cm for 1.5 h, transferred to Hybond-N^+ membrane (Amersham) and hybridised to the probe (ATf7-ATr9) 225 bp corresponding to positions 397–621 of Fig. 1 and probe (ASf6-ASr9) 127 bp corresponding to positions 543–670 of Fig. 4, respectively, labelled by the PCR method with Dig dUTP (Roche). As a control the same quantities (20 ng) of mRNA from different developmental stages were used as a template in combination with two specific primers ACTf1 corresponding to nucleotide positions 1–25 and ACTr2 corresponding to nucleotide positions 469–493 from the nucleotide sequence of *S. littoralis* mRNA for beta-actin (partial; EMBL Nucleotide Sequence Data Base, accession number Z46873), which yields a 494 bp amplified fragment. Expressions of AT and AS were normalized relative to that of beta-actin. A negative control for genomic DNA has been done using RT-PCR program strategy 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 61 °C for 45 s, 68 ° for 1 min, and a final extension step of 68 °C for 2 min. Densitometry was preformed using an ImageMaster® VDS (Pharmacia Biotech) and the ImageMaster 1D Database software. A series of 5 to 20 ng mRNA (Spofr-AT and Spofr-AS from 6th instar larvae and 2-day-old adult females, respectively) was used to produce a standard curve by RT-PCR.

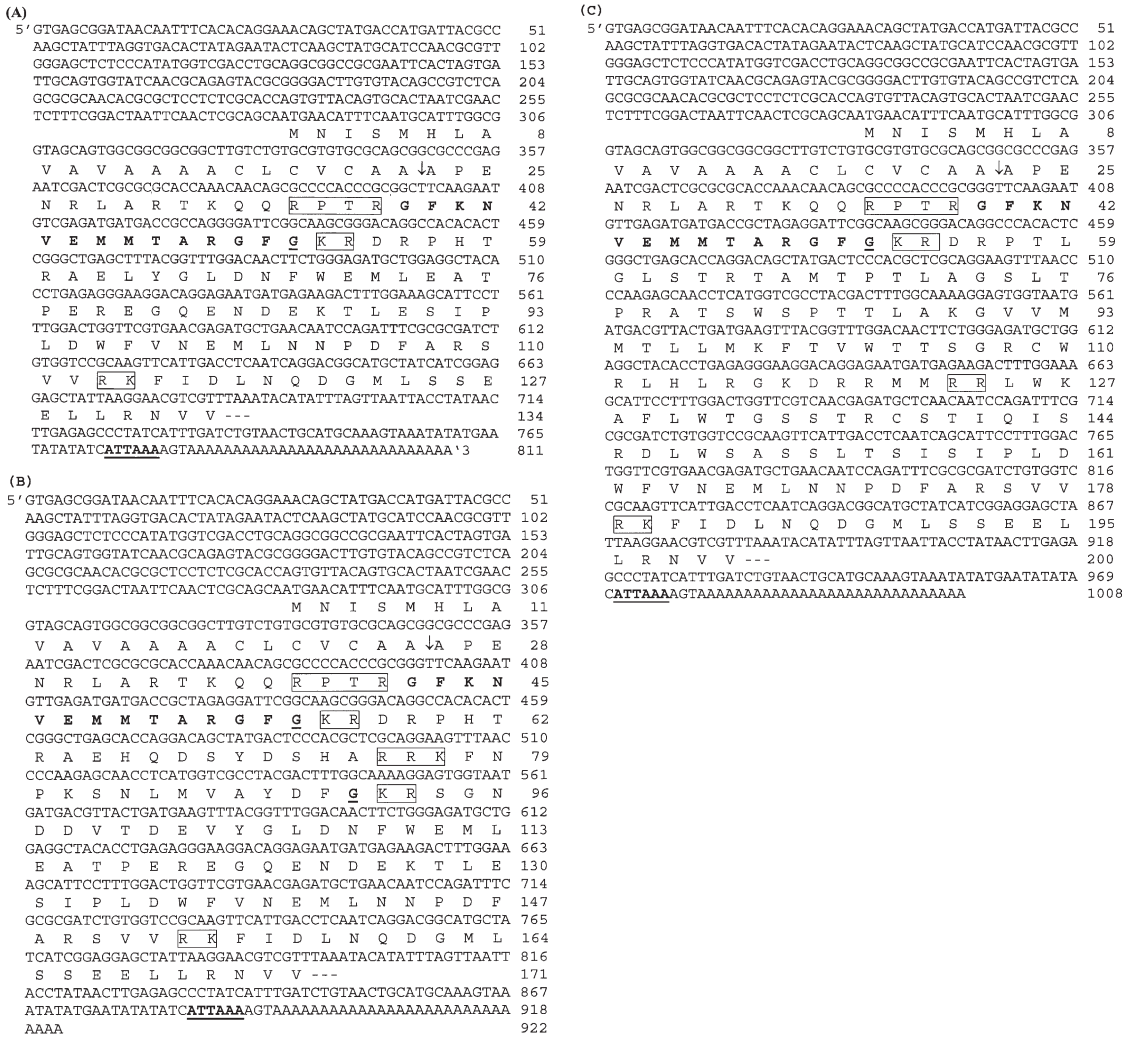

Fig. 1. Spofr-AT sequence data. (A) Nucleotide and the deduced amino acid sequences of the *S. frugiperda* AT cDNA. The sequences are numbered at the right. The Spofr-AT amino acid sequence is shown in bold type. Possible proteolytic cleavage sites are in boxes and the glycine residue required for amidation is underlined. The potential polyadenylation signal is shown in bold type and underlined; --- represents the stop codon. A signal peptide cleavage site is indicated by a downward arrow. (B) Nucleotide and deduced amino acid sequences of the second Spofr-AT precursor mRNA. (C) Nucleotide and deduced amino acid sequences of the third Spofr-AT precursor mRNA.

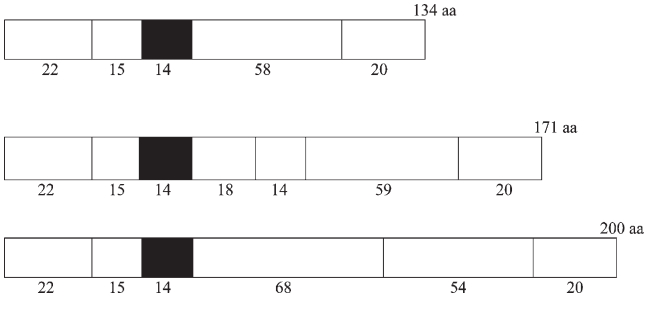

Fig. 2. The organisation of the predicted peptides encoded by the three Spofr-AT mRNAs. All three predicted forms contain a 22-residue signal peptide, a 15-residue peptide, the 14-residue Manse-AT peptide, including the glycine to be amidated, and a 80, 117 or 146-residue C-teminal peptide, which may be further processed.

3. Results

3.1. S. frugiperda *allatotropin (AT) cDNA*

The *S. frugiperda* AT gene is expressed in at least three mRNA isoforms. The cDNA AT sequences with 811, 922 and 1008 nucleotides, respectively, are shown in Fig. 1A–C. The cDNAs contain a 5′ untranslated region of 282 nucleotides and an open reading frame of 134, 171 and 200 amino acids, respectively, corresponding to nucleotides 283–684, 283–795 and 283–882. The assigned initiator codon is located at position 283–285. The stop codon is followed by a 124-nucleotide 3′ untranslated region including the 29-nucleotide poly (A) tail. A consensus polyadenylation signal (ATTAAA) is found after position 773, 884 and 970, respectively,

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Spofr-AT MNISMHLAVAVAAAACLCVCAA↓APENRLARTKQQRP  36
Bommo-AT **LT*Q*E*I**V--**VLAEG**DV**V*******   34
Pseun-AT **F******V*********V*****G**T*******  36
Manse-AT **LT*Q***I**V--***LAEG**DV**T*******   34
Agrco-AT **LT*Q**MI**V--****AEG**DV**********   34

Spofr-AT TRGFKNVEMMTARGFGKRDRPHTRAELYGLDNFWEM   72
Bommo-AT ***********************P**********V*  70
Pseun-AT ***********************************   72
Manse-AT **********************P*************   70
Agrco-AT **********************P*************   70

Spofr-AT LEATPEREGQE-NDEKTLESIPLDWFVNEMLNNPDF  107
Bommo-AT **PSP***V**-V****F******************  105
Pseun-AT **SA*******T************************  108
Manse-AT **TS****V**V-************************  105
Agrco-AT **TS****V**VV************************  106

Spofr-AT ARSVVRKFIDLNQDGMLSSEELLRNVV           134
Bommo-AT **F**E*****************-***-           130
Pseun-AT *****H*********************           135
Manse-AT *************************F           131
Agrco-AT *************************             132
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Fig. 3. Alignment of the AT precursor peptides of *S. frugiperda* (Spofr-AT), *B. mori* (Bommo-AT) (Park et al., 2002), *P. unipuncta* (Pseun-AT) (Truesdell et al., 2000), *M. sexta* (Manse-AT) (Taylor et al., 1996), and *A. convolvuli* (Agrco-AT) (H. Kataoka, unpublished). Asterisks represent amino acids identical to each of the precursors. The mature AT peptide is shown in bold type. A signal peptide cleavage site is indicated by a downward arrow.

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5′CTAATACGACTCACTATAGGGCAAGCAGTGGTAACAACGCAGAGTACGCGG   51
  GGTCATTTGCTTTCAAAAACCTTCGAGGGAGACCACGGAAGCACCAACTAC  102
  AACCTTCCGTAGTTACTAGTAAAAGTGATCCGTACTTTATTTCACGTAAAC  153
  GAAACTCAGTTAGCTGCTGAATTTATTTCGACGTGCAGATTTATAAAGTTC  204
  GAAGGATCACAATGAAAACGAGCGCGTACAACGTGTACCTGGGAGTCGTGG  256
              M  K  T  S  A  Y  N  V  Y  L  G  V  V     13
  CCGCCATGTTGGCTCTACTGTTCGTCACAATTAATGCTGCGCCAATGGAGG  307
  A  A  M  L  A  L  L  F  V  T  I  N  A↓A  P  M  E     30
  CGGACGATGAGACGGCTGAGAACACCCTCGTGGCGCATCCCGATGGTGACA  358
  A  D  D  E  T  A  E  N  T  L  V  A  H  P  D  G  D     47
  TGGAGCTCTCAGGCCCCTGGGATGCTATCAACACTGCCGCTCTACGCAAAC  409
  M  E  L  S  G  P  W  D  A  I  N  T  A  A  L  R  K     64
  TGCTGCTGCAACTTGATGCAGAGGACAGGATGGGCGGGGTGACCCGCTCGT  460
  L  L  L  Q  L  D  A  E  D  R  M  G  G  V  T  R  S     81
  GGCCCCAAGCTGAGCCCCGCGGTTGGGGTCTGCGGGCGTTGGACAGCCGTC  511
  W  P  Q  A  E  P  R  G  W  G  L  R  A  L  D  S  R     98
  TGGCGCGGCAGTGGAGGGCAGACAAGCGGCAGGTGCGATTCCGCCAGTGCT  562
  L  A  R  Q  W  R  A  D  K  R  Q  V  R  F  R  Q  C    115
  ACTTCAACCCAATTTCCTGCTTCCGCAAGTGAAAACAGCACCAACTCAACG  613
  Y  F  N  P  I  S  C  F  R  K ---                     125
  ACGCATCGACCCTTTGACCTAGGGTAGCAAGAACGAATAAAACGTCGCCAT  664
  AATCTCCCGAAAAAAAAAAAAAAAAAAAAAAAAAAAA′3              699
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Fig. 4. Nucleotide and the deduced amino acid sequences of the *S. frugiperda* AS cDNA. The sequences are numbered at the right. The amino acid sequence of the Spofr-AS is shown in bold type. Possible dibasic proteolytic cleavage sites are in boxes. The possible site for cleavage of the signal sequence is marked with a downward arrow. The potential polyadenylation signal is shown in bold type and underlined; --- represents the stop codon.

which is three nucleotides upstream from the poly (A) tail.

The *S. frugiperda* neuropeptide precursor contains a peptide sequence identical to Manse-AT. The Spofr-AT mature peptide is located between amino acid residues Arg^{38} and Lys^{53} on each of the three precursors (Fig. 1A–C and 3), flanked by potential Arg and Lys-Arg endoproteolytic cleavage sites and ends with a glycine residue, the signal for carboxy-terminal amidation by peptidyl-α-amidating monooxygenase (Eipper et al., 1992). The Spofr-AT sequence is present as a single copy. Recognition of all possible proteolytic cleavage sites would result in the production of three additional peptides of 15, 58 and 20 amino acids on the first precursor (134 amino acids), five additional peptides of 15, 18, 14, 59 and 20 amino acids on the second AT mRNA precursor (171 amino acids), and four additional peptides of 15, 68, 54 and 20 amino acids on the third AT mRNA precursor (200 amino acids) (Fig. 2). The cleavage site in the second mRNA at amino acid position 75 can also be RR instead of RK, which might be supported by the H just in front (HARR). This would imply a Lys residue as the first amino acid of the following peptide (15-residue peptide). The 14- or 15-residue peptide of the second isoform is of special interest as that peptide ends with a glycine, the signal for carboxy-terminal amidation, which is characteristic for many bioactive peptides. The peptide shares eight of 14 (15) identities with the Manse-AT-like III peptide predicted in *M. sexta* (Horodyski et al., 2001). The basic organisation of the Spofr-AT peptide precursor (134 amino acids) is similar to that of *M. sexta* (Taylor et al., 1996), *P. unipuncta* (Truesdell et al., 2000), *A. convolvuli* (H. Kataoka, unpublished), and *B. mori* (Park et al., 2002) with 84, 93, 85 and 83% amino acid sequence identity, respectively (Fig. 3).

3.2. S. frugiperda *allatostatin (AS) cDNA*

The complete Spofr-AS cDNA sequence consists of 699 nucleotides as shown in Fig. 4. It contains a 5′ untranslated region of 215 nucleotides upstream of an open reading frame of 125 amino acids. The assigned initiator codon is located at position 216–218. It is possible that the translation initiation occurs at methionines further downstream (Met^{16}, Met^{29}). Initiation at these sites would, however, yield a precursor without an appropriate signal peptide. The open reading frame is followed by a 106-nucleotide 3′ untranslated region including the poly (A) tail. A consensus polyadenylation signal (AATAAA) is found after position 648, which is 19 nucleotides upstream from the poly (A) tail.

The *S. frugiperda* neuropeptide precursor contains a peptide sequence identical to Manse-AS. This Spofr-AS is located at the carboxy-terminus between Arg^{108} and Arg^{124} and is flanked by potential Lys-Arg and Arg-Lys endoproteolytic cleavage sites. The Spofr-AS gene is

present as a single copy. Within the amino-terminus of the precursor a single potential signal cleavage site downstream Ala[26] (Von Heijne, 1986) could be found (Fig. 4). The signal peptide would then contain 18 out of 26 apolar residues and is followed by an 80 amino acids sequence (Ala[27] to Asp[106]). Endoproteolytic cleavage of this internal peptide at Arg[63]/Lys[64] could result in two peptides of 36 and 42 amino acids. These peptides share sequence similarity with those in the Pseun-AS precursor (Jansons et al., 1996). Alignment of the Spofr-AS precursor peptide with that for *P. unipuncta* (Jansons et al., 1996) shows that the C-termini, containing the AS sequence and the processing sites, are identical (Fig. 5). The two precursors are equally long and exhibit 85% amino acid sequence identity. Comparison between Spofr-AS precursor and the respective precursor from *D. melanogaster* (Williamson et al., 2001b) shows that the mature AS peptides differ in one amino acid (change of Phe to Tyr), whereas nearly no sequence homology exists in the other parts of the precursor.

3.3. Spofr-AT gene expression by RT-PCR

RT-PCR analysis of the mRNA from the brain of different developmental stages was done to compare the expression of the three mRNAs of the Spofr-AT gene. The specific primers ATf7 and ATr9 were designed to yield a product of 225 bp, 323 bp, and 420 bp, respectively, of the three mRNAs. The probe of 225 bp, which was used for hybridisation, included the allatotropin encoding sequence (Fig. 1). The Spofr-AT gene was expressed in brains of all developmental stages of *S. frugiperda* studied here (Fig. 6B). The shortest (first Spofr-AT) mRNA was highly expressed in larvae of the 6th larval stage, in 2-day-old adult males and in 2-day-old adult females, whereas it was expressed at a lower level in the prepupal stage and in young pupae. The second Spofr-AT mRNA was highly expressed in the 6th larval stage, whereas no expression was detected in 10-day-old pupae. The expression of the third mRNA was found only in 2-day-old adult females and males and in young pupae. In summary, the RT-PCR analysis based on 20 ng mRNA revealed that the Spofr-AT gene is strongly

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Spofr-AS    MKTSAYNVYLGVVAAMLALLFVTIN   25
Pseun-AS    ***NVC****AI***T**M*F***R   25
Drome-AS    *MKFVNILLCYGLLLT*FFALSEAR   25

Spofr-AS    A↓APMEADDETAENTLVAHPDGDMEL  50
Pseun-AS    ******E**Q*D***********M    50
Drome-AS    PSGA*TGPDSDGLDGQDAE*VRGAY   50

Spofr-AS    SGPWDAINTAALRKLLLQLDAEDRM   75
Pseun-AS    T****T****************E**   75
Drome-AS    G*GY*MPAN*IYPNIPMDRLNMLFA   75

Spofr-AS    GGVTRSWPQAEPRGWGLRALDSRLA   100
Pseun-AS    *R*S*****************G***   100
Drome-AS    NYRPTYSAYLRSPTY*NVNELY**P   100

Spofr-AS    RQWRADKRQVRFRQCYFNPISCFRK   125
Pseun-AS    ************************   125
Drome-AS    ES----*****Y************   121
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Fig. 5. Alignment of the AS precursor peptides of *S. frugiperda* (Spofr-AS), *P. unipuncta* (Pseun-AS) (Jansons et al., 1996), and *D. melanogaster* (Drome-AS) (Williamson et al., 2001b). Asterisks represent amino acids identical to each of the precursors. The mature AS peptide is shown in bold type. A signal peptide cleavage site is indicated by a downward arrow.

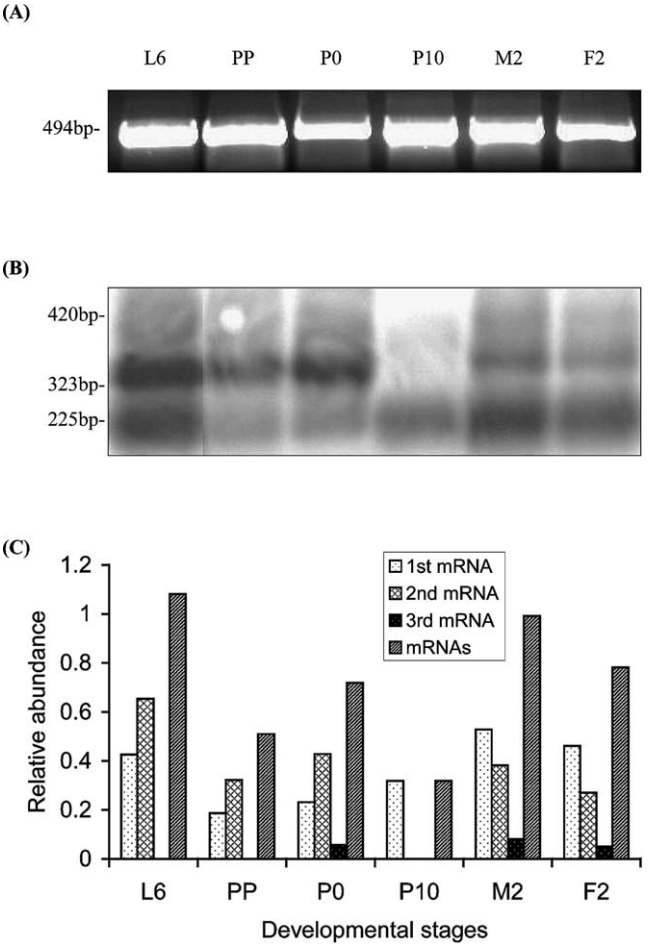

Fig. 6. Expression of Spofr-AT gene in the brains of the sixth larval stage (L6), prepupal stage (PP), young pupal stage (P0), 10-day-old pupae (P10), 2-day-old adult males (M2), and 2-day-old adult females (F2) as measured by RT-PCR. (A) Agarose gel separation of beta-actin control stained with ethidium bromide using 20 ng mRNA as a template in one step RT-PCR; (B) Blotting results for the expression of the three different mRNAs of Spofr-AT gene in the brains; (C) Densitometric quantification of the signals from (B).

expressed in the 6th larval stage (L6) and in 2-day-old adult males (M2) and females (F2), whereas expression is lower in pre-pupae (PP) and in 10-day-old pupae (Fig. 6C). No fragment was visualised in the controls without reverse transcriptase reaction. A beta-actin standard fragment of ACTf1-ACTr2 with 494 bp can be visualized with 20 ng mRNA from each of the developmental stages (Fig. 6A) and this expression strength was used to normalize the data.

3.4. Spofr-AS gene expression by RT-PCR

RT-PCR analysis of the mRNA from the brain of different developmental stages was done to compare the expression of the Spofr-AS gene. The specific primers ASf6 and ASr9 were designed to yield a product of 127 bp of the mRNA corresponding to nucleotide positions 543–670 of Fig. 4. The digoxigenin labelled probe of 127 bp, which was used for hybridisation, proved to include the mature peptide sequence for Spofr-AS gene. RT-PCR analysis shows that the Spofr-AS gene is expressed in the brain of most of the development stages studied (Fig. 7B), with highest expression in 2-day-old adult females (F2). The gene was only slightly expressed in the young pupae (P0) and the 10-day-old pupae (P10) (Fig. 7C). Control reactions were done as described for Spofr-AT.

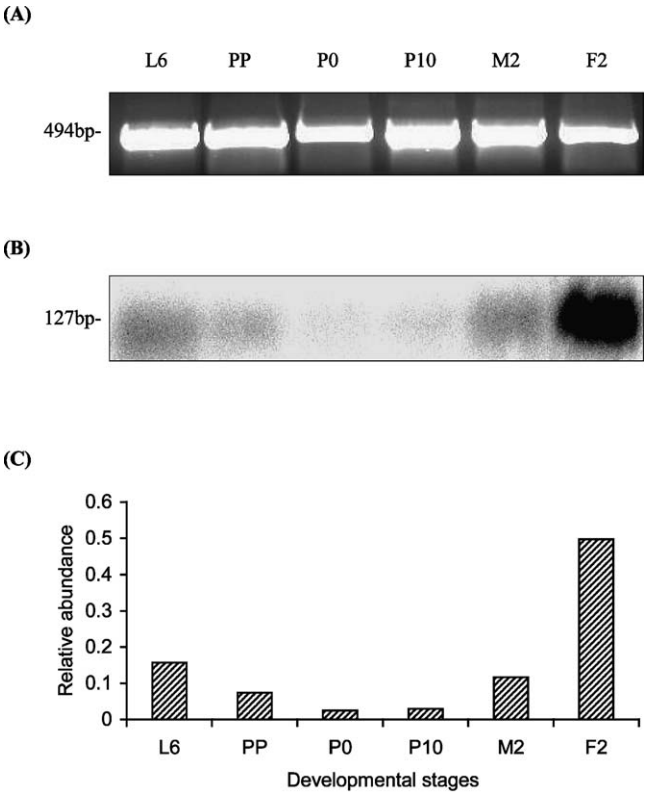

Fig. 7. Expression of Spofr-AS gene in the brains of the sixth larval stage (L6), prepupal stage (PP), young pupal stage (P0), 10-day-old pupae (P10), 2-day-old adult males (M2), and 2-day-old adult females (F2) as measured by RT-PCR. (A) Agarose gel separation of beta-actin control stained with ethidium bromide using 20 ng mRNA as a template in one step RT-PCR; (B) Blotting results for the expression of Spofr-AS gene in the brains; (C) Densitometric quantification of the signals from (B).

4. Discussion

Hitherto, the true armyworm, *P. unipuncta*, was the only lepidopteran species where the cDNAs encoding an allatostatin and an allatotropin had been characterised (Jansons et al., 1996; Truesdell et al., 2000). In this study a second cDNAs encoding *M. sexta* allatotropin and *M. sexta* allatostatin have been characterised for the fall armyworm, *S. frugiperda*, an important agricultural pest.

Factors with allatotropic activity exist in a number of insect species. A stimulating action of JH biosynthesis has been observed, for example, in larvae of the wax moth *Galleria mellonella* (Bogus and Scheller, 1996), in adult locusts *(Locusta migratoria;* Lehmberg et al., 1992 and *S. gregaria;* Veelaert et al., 1996), crickets (*G. bimaculatus;* Lorenz and Hoffmann, 1995) and cockroaches (*Diploptera punctata;* Unnithan et al., 1998), in adult females of the linden bug *Pyrrhocoris apterus* (Hodková et al., 1996), and in the brain of female *Phormia regina* (Tu et al., 2002). So far, however, none of these allatotropic factors has been identified and the *M. sexta* allatotropin (Manse-AT) remains the allatotropin isolated and characterised to date. Allatotropin related peptides have been isolated from the male accessory reproductive glands of *L. migratoria* (Paemen et al., 1991), from the brain of the Colorado potato beetle, *Leptinotarsa decemlineata* (Spittaels et al., 1996), and from abdominal ganglia of the mosquito *A. aegypti* (Veenstra and Costes, 1999). This indicates that allatotropin homologues may be generally present in insects, but have other functions than stimulating JH biosynthesis.

Recently, a peptide from methanolic brain extracts of adult *S. frugiperda*, which strongly stimulates JH biosynthesis in vitro by the CA of adult females was reported. Using HPLC separation, followed by Edman degradation and mass spectrometry, the peptide was identified as Manse-AT (Oeh et al., 2000). The present work confirms the structure of Manse-AT from brains of *S. frugiperda* by molecular techniques. As with many other neuropeptides (Sossin et al., 1989), Spofr-AT is translated from a rare transcript and derived from a polyprotein with a number of products contained on a single precursor. As in Manse-AT (Taylor et al., 1996; Horodyski et al., 2001), the Spofr-AT gene is expressed as at least three mRNA isoforms that differ from each other by alternative splicing. The second Spofr-AT mRNA contains (in addition to Manse-AT) a 14 or 15 amino acid residue peptide, which is flanked by potential endoproteolytic cleavage sites and ends with a glycine residue, the signal for carboxy-terminal amidation. This pep-

tide shows homology to the Manse-AT-like peptides I–III in *M. sexta* (Horodyski et al., 2001). Other than in Spofr-AT, there is no evidence that the cleavage sites flanking the Manse-AT-like peptide are used, and it is difficult to speculate as to the function of this peptide. The basic organisation of the Spofr-AT peptide precursor (134 amino acids) is similar to that in other lepidopteran species.

The results of our semi-quantitative one-step RT-PCR expression studies indicate that the three mRNA isoforms may be differently expressed in brains of larvae, pupae and adults of *S. frugiperda*. Generally, the Spofr-AT gene was strongly expressed in young last instar larvae and in 2-day-old adults, whereas expression was lower in the pupal stage. Efforts at an experimental proof of these results, using transcript-specific probes in in situ hybridisation, are in progress. Whether the Spofr-AT transcripts are alternatively spliced in a tissue-specific manner, as in *M. sexta* (Horodyski et al., 2001), is not yet known.

In a previous paper, it was demonstrated that synthetic Manse-AS may down-regulate higher rates of JH biosynthesis in the CA of adult females of *S. frugiperda* maintained by the allatotropin (Oeh et al., 2000). This inhibitory effect of Manse-AS on allatotropin activated glands was dose-dependent and reversible. However, allatostatin was not isolated from the brains of *S. frugiperda* females by HPLC separation. The current study strongly suggests the presence of Spofr-AS in the brains of adult females by molecular cloning of the preprohormone. The preprohormone is 125 amino acid residues long and contains one copy of a peptide sequence, which is identical to Manse-AS. The basic organisation of the precursor is similar to that of the only other known lepidopteran AS precursor from *P. unipuncta*, with 85% amino acid sequence identity. Williamson et al. (2001b) have demonstrated the existence of a C-type allatostatin preprohormone in *Drosophila*. The drostatin-C sequence differs in only one amino acid residue from the Manse-AS, although, the preprohormone shows little homology. Whether drostatin-C acts as an allatostatin in *Drosophila* itself, has yet to be determined. It is likely, however, that all three types of insect allatostatins may be regarded as generally inhibitory neurohormones and block specific endocrine organs, but they may also act on various muscle tissues in an insect (Williamson et al., 2001b). Our preliminary expression studies by RT-PCR indicate a developmentally regulated expression of Spofr-AS in the brain, but further studies are necessary to unequivocally demonstrate a time- and/or tissue-specific expression of the peptide in *S. frugiperda*. Our in vitro experiments on the regulation of JH biosynthesis in *S. frugiperda* have clearly shown an interaction between Spofr-AT and Spofr-AS, at least in adult females. Such a dual regulatory mechanism would allow a more precise control of hormone production than a single ‘on-off′ mechanism (McNeil and Tobe, 2001). Injections of Manse-AT and Manse-AS into adult females of *S. frugiperda* affected their life span, as well as the total number of eggs deposited. The oviposition rate was reduced with Manse-AT alone and Manse-AS plus Manse-AT, whereas egg deposition in Manse-AS injected females on a per day basis was not affected during their short life span (Oeh et al., 2001). These results indicate a rather sophisticated role of both hormones in adult development and reproduction of *S. frugiperda*. We hope that further experiments formulated within an ecological context, for example, on the life history strategy of populations (McNeil and Tobe, 2001), will yield a better understanding of the modes of actions of allatotropic and allatostatic neuropeptides.

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