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Molecular Cloning and Expression Analysis of cDNAs Encoding Androgenic Gland Hormone Precursors from Two Porcellionidae Species, *Porcellio scaber* and *P. dilatatus*

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ABSTRACT—Male sexual characteristics in Crustacea are induced by androgenic gland hormone (AGH), which is produced by the male-specific androgenic gland. Recently, AGH in the terrestrial isopod *Armadillidium vulgare* was characterized and its cDNA cloned, the first example in which the structure of AGH was elucidated. We report here the molecular cloning of cDNAs encoding AGH precursors from two additional terrestrial isopods, *Porcellio scaber* and *P. dilatatus*. cDNA fragments encoding *Porcellio scaber* AGH (Pos-AGH) and *P. dilatatus* AGH (Pod-AGH) were amplified by RT-PCR using degenerate oligonucleotide primers designed based on the amino acid sequence of *A. vulgare* AGH (Arv-AGH). Subsequently, full length cDNAs were obtained by 5'- and 3'-RACE. Both AGH precursors consisted of a signal peptide, B chain, C peptide and A chain, and exhibited the same organization as that of Arv-AGH. The amino acid sequences of the A and B chains, which comprise mature AGH peptide, were highly conserved among the three species, while that of the C peptide showed only low sequence similarity. In Northern blot analysis, each cDNA fragment used as a probe specifically hybridized with a single band (0.75 kb) in mRNA extracted from whole male reproductive organs. In analysis of the tissue-specific gene expression of these two AGHs by RT-PCR, it was revealed that both AGH transcripts were detected only in cDNA synthesized using total RNA from the testis carrying the androgenic glands, but not in that from testis only, seminal vesicle, vas deferens, or hepatopancreas.

Key words: androgenic gland hormone, Crustacea, terrestrial isopod

INTRODUCTION

The androgenic gland (AG) which is responsible for the development of male characteristics in crustaceans was first discovered by Charniaux-Cotton (1954) in the amphipod *Orchestia gammarella*. Since then, its role has been investigated using biological methods such as the implantation of AGs and the injection of AG extract. In the terrestrial isopod *Armadillidium vulgare*, the implantation of AGs and the injection of AG extract into juvenile females induced the gonadal masculinization of juveniles (reviewed by Katakura, 1984). Similarly, AG implantation into young females in the giant freshwater prawn *Macrobrachium rosenbergii* transformed these genetic females to phenotypic reproductive males (Nagamine *et al*., 1980). These phenomena were considered to be due to the effects of an androgenic gland hormone (AGH) produced by the AGs.

Biochemical studies on AGH have been performed mainly in *A. vulgare* because of the difficulty in establishing quantitative methods for measuring AGH activity in other species (reviewed by Katakura, 1984) and the narrow species-specificity of AGH activity (Martin and Juchault, 1999). AGH *in A. vulgare* (Arv-AGH) was of peptidic nature and was purified by several groups (Hasegawa *et al*., 1987; Martin *et al*., 1990; Nagasawa *et al*., 1995; Okuno *et al*., 1997). Recently, Arv-AGH was isolated from normal AGs and its cDNA cloned (Okuno *et al*., 1999), while the complete amino acid sequence of a precursor of Arv-AGH (pro-Arv-AGH) was determined using AGs from *A. vulgare* which hypertrophied due to symbiosis by *Wolbachia* (Martin *et al*., 1999). The pro-Arv-AGH estimated by both groups consists of 123 amino acid residues and has eight cysteine residues and one potential *N*-glycosylation site. The overall structural

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organization of pro-Arv-AGH consisting of B chain, C peptide (connecting peptide), and A chain each linked by dibasic amino acid residues, Lys-Arg, is similar to that of proinsulin, although there is no similarity in terms of amino acid sequence or peptide chain length. The mature Arv-AGH resulting from post-translational processing by removal of the C peptide is a heterodimer consisting of A and B chains interlinked by disulfide bonds (Martin *et al*., 1999; Okuno *et al*., 2002).

Three kinds of polyclonal antibodies were raised against the different components of the pro-Arv-AGH, e.g. the whole molecule of the recombinant pro-Arv-AGH expressed by *Escherichia coli*, the N-terminal nonapeptide of the B chain, and the N-terminal octapeptide of the A chain (Okuno *et al*., 2001). All of these antibodies absorbed AGH activity in AG extracts (Okuno *et al*., 2001). The AG strongly immunostained with all antibodies, while the testis, the seminal vesicle and the vas deferens did not show immunostaining (Hasegawa *et al*., 2002). These results indicated that these three antibodies recognized the AGH molecule in a highly specific manner. The antibody raised against recombinant pro-Arv-AGH expressed by *E. coli* also cross-reacted with the AGs of several terrestrial isopod species, e.g. *Armadillidium nasatum*, *Porcellio scaber*, *P. dilatatus*, *P. laevis*, *and Alloniscus balssi* (Hasegawa *et al*., 2002). These results indicate that AGHs of the terrestrial isopod species harbor amino acid sequences similar to that of Arv-AGH.

In order to clarify the molecular diversity of AGH in relation to the degree of immunostaining and hormonal activity, we conducted the cloning of cDNAs encoding AGH precursors from two Porcellionidae species, *P. scaber* and *P. dilatatus*, by using polymerase chain reaction (PCR). Here, we describe the cloning and its AG-specific gene expression in both species.

MATERIALS AND METHODS

Animals

Two Porcellionidae species, *P. scaber* and *P. dilatatus*, were collected from natural populations on the campus of Keio University, Yokohama, Japan. Whole male reproductive organs were dissected out and subsequently divided into the testis carrying the androgenic gland, testis only, seminal vesicle, and vas deferens. The hepatopancreas was also dissected out from all individuals. Tissues were frozen and stored at –80°C until use.

Reverse transcription (RT)-PCR

Total RNAs from whole male reproductive organs of *P. scaber* and *P. dilatatus* were prepared using ISOGEN (Nippongene, Japan) according to the instructions of the manufacturer. First strand cDNA was synthesized with 1 μg of each total RNA using a Ready-To-Go™ T-primed First-strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, USA) according to the manufacturer's protocol. For RT-PCR, two degenerate oligonucleotide primers (Arv-AGH-F and -R in Table I) were designed based on the amino acid sequences corresponding to the positions 97-103 and 120-126, respectively, of the Arv-AGH precursor (Okuno *et al*., 1999). The first strand cDNA of each species was used as a template and each amplification was primed by a pair of the primers Arv-AGH-F/Arv-AGH-R (Fig. 1). The following program was used for PCR amplification: 40 cycles of 30 sec at 94°C (3 min and 30 sec only for the first cycle), 30 sec at 48°C, and 1 min 30 sec at 65°C (8 min and 30 sec only for the last cycle).

Table I. Nucleotide sequences of primers used in PCR

Primer	Sequence
Arv-AGH-F	5'-GCCTTYTAYCARGARTGYTG-3'
Arv-AGH-R	5'-ACGGTNGTNCKRTTRCAYTT-3'
Por-AGH-F	5'-GAGTGYTGCAACATTCGAACCGAA-3'
RTG	5'-AACTGGAAGAATTCGCGGCCG-3'
Por-AGH-R	5'-GTTGCAYTTATGTTCGGTTCGAATGTT-3'
Por-AGH-NR	5'-GCAYTTATGTTCGGTTCGAATGTTGCA-3'
AP-1	5'-CCATCCTAATACGACTCACTATAGGGC-3'
AP-2	5'-ACTCACTATAGGGCTCGAGCGGC-3'
Pos-AGH-F	5'-GGCTCCACGAGAAGAAACAAAAG-3'
Pos-AGH-R	5'-CTAACAAAGAAGATTATGACTTCCGAAATT-3'
Pod-AGH-F	5'-CTGAGAGAAAGATCAAAATAGCA-3'
Pod-AGH-R	5'-CAAAGAAGATTATAACTTCTGAAAAC-3'
actin-F	5'-TGTAYGCCTCTGGYCGYACC-3'
actin-R	5'-CVACRTCRCACTTCATGATGS-3'

3'-Rapid amplification of cDNA ends (3'-RACE)

A common specific primer (Por-AGH-F in Table I) was designed based on the nucleotide sequences of the Pos- and Pod-AGH cDNA fragments amplified by RT-PCR. The first strand cDNA of each species was used as a template and each amplification was primed by a pair of primers Por-AGH-F/3'-adaptor primer, RTG (Fig. 1). The following program was used for each PCR amplification: 40 cycles of 30 sec at 94°C (3 min and 30 sec only for the first cycle), 30 sec at 55°C, and 1 min at 72°C (7 min and 30 sec only for the last cycle).

5'-RACE

Poly $(A)^+$ RNA of each species was purified from each total RNA of whole male reproductive organs using Oligotex-dT 30 super (Takara, Japan) according to the instructions of the manufacturer. First and second strand cDNAs for 5'-RACE were synthesized from 1 μg of each Poly $(A)^+$ RNA using a Marathon™ cDNA synthesis kit (Clontech, USA) according to the manufacturer's protocol. A Marathon™ cDNA adaptor (Clontech) including 5'-adaptor primer sequences (AP1 and AP2 in Table I) was ligated to both ends of the second strand cDNAs. Two specific primers (Por-AGH-R and -NR in Table I) were designed based on the nucleotide sequences of the Pos- and Pod-AGH cDNA fragments amplified by RT-PCR. cDNA fragments encoding the 5'-regions of the Pos- and Pod-AGH were amplified by two rounds of PCR. In the first PCR, the each second strand cDNA added with the adaptor sequence was used as a template and each amplification was primed by a pair of the primers Por-AGH-R/AP1 (Fig. 1). In the second PCR, the first PCR products were used as templates and each amplification was primed by a pair of each nested primers Por-AGH-NR/AP2 (Fig. 1). Amplifications were performed with a cycle protocol as follows: 5 cycles of 5 sec at 94°C (3 min and 5 sec only for the first cycle) and 3 min at 72°C, 5 cycles of 5 sec at 94°C, 10 sec at 70°C and 3 min at 72°C, and 27 cycles of 5 sec at 94°C, 10 sec at 68°C and 3 min at 72°C (10 min only for the last cycle).

Confirmation of the nucleotide sequences of Pos- and Pod-AGH cDNAs

The nucleotide sequences of Pos- and Pod-AGH cDNAs

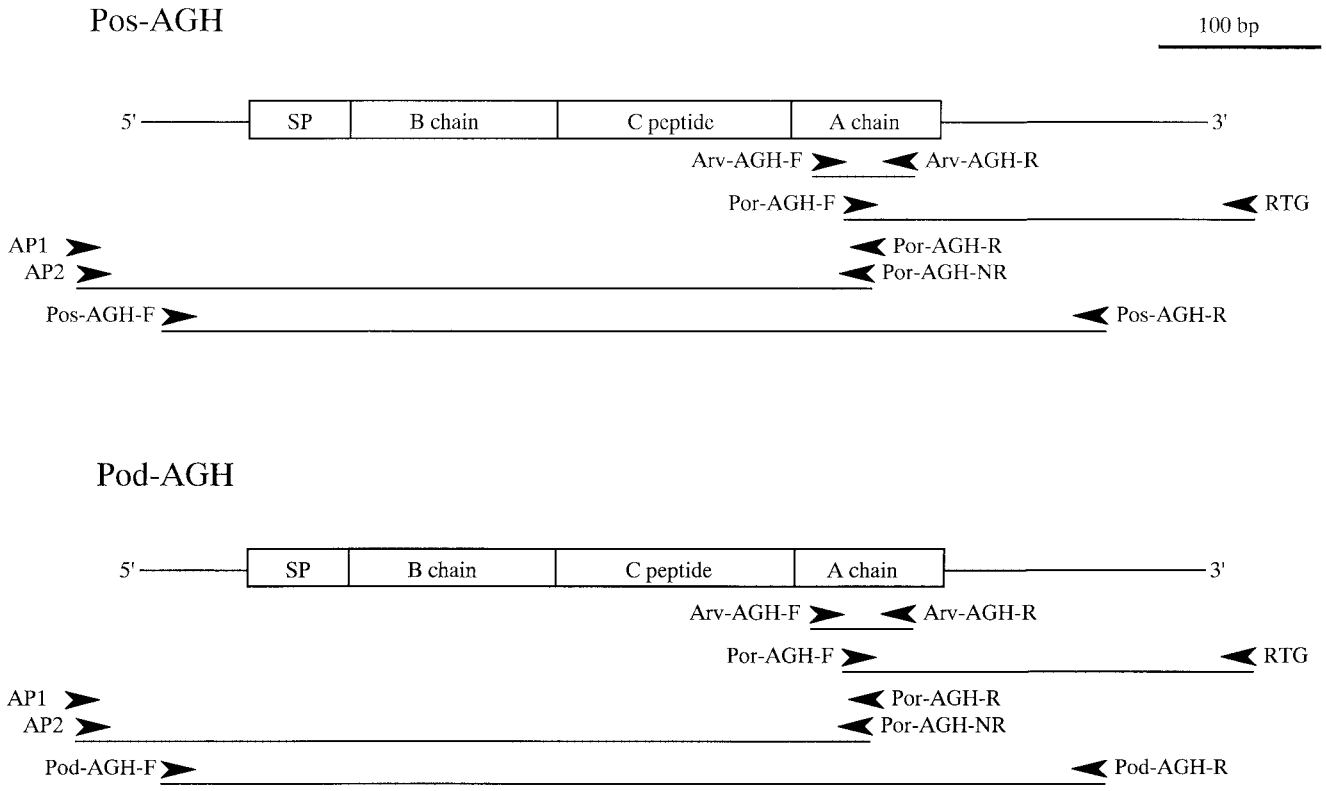

Fig. 1. Schematic representation of the structures of cDNAs encoding *Porcellio scaber* AGH (Pos-AGH) and *P. dilatatus* AGH (Pod-AGH), and the locations of oligonucleotide primers. Arrowheads represent the primers, and lines under the arrowheads indicate the cDNA fragments which were amplified. Boxes represent the open reading frame. Thin lines indicate the 5'- and 3'-untranslated regions. SP is the abbreviation for signal peptide.

obtained by 3'- and 5'-RACE over the coding regions (nucleotide positions 24–568 in Fig. 2A and 23–566 in Fig. 2B, respectively) were confirmed by PCR amplifications using specific primers Pos-AGH-F/-R (Fig. 1) for Pos-AGH and Pod-AGH-F/-R (Fig. 1) for Pod-AGH.

Nucleotide sequence analysis

All PCR products were ligated into pCR 2.1 TOPO vectors (Invitrogen, USA) using a TOPO TA Cloning Kit (Invitrogen) according to the instructions of the manufacturer. Both strands of the plasmid DNAs were sequenced on a Long-Read Tower DNA sequencer (Amersham Pharmacia Biotech) using a Thermo Sequenase Cy 5.5 Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech).

Northern blot analysis

Poly $(A)^+$ RNA from whole male reproductive organs of each species was prepared as described in 5'-RACE. Each poly $(A)^+$ RNA (1 μg) was run on 1.5% formamide-agarose gels and transferred to a Hybond N^+ membrane (Amersham Pharmacia Biotech). The cDNA fragments of Pos- and Pod-AGHs (corresponding to positions 24–568 and 23–566, respectively, in Fig. 2) were labeled with alkaline phosphatase using an AlkPhos Direct Labelling and Detection System with CDP-Star (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Hybridization and signal detection were performed according to the manufacturer's protocols.

Tissue-specific gene expression of Pos- and Pod-AGHs

Total RNAs from the testis carrying the androgenic glands, testis only, seminal vesicle, vas deferens, and hepatopancreas of each species were prepared as described in RT-PCR. Each total RNA was treated with DNase I (Takara) at 37°C for 1 hr. First strand cDNA of each tissue was synthesized as described in RT-PCR. The first strand cDNAs were used as templates and each amplification was primed by a pair of the primers Pos-AGH-F/-R for Pos-AGH or Pod-AGH-F/-R for Pod-AGH. In order to ascertain the amount of total RNA in each tissue, an actin cDNA fragment of each species was amplified with two degenerate oligonucleotide primers (actin-F and -R in Table I), which were designed based on the amino acid sequences of two conserved regions of the vertebrate and invertebrate actins. The following program was used for PCR amplification: 35 cycles of 30 sec at 94°C (3 min and 30 sec only for the first cycle), 30 sec at 55°C, and 30 sec at 72°C. The PCR products were separated on a 2.0% agarose gel and stained with ethidium bromide. The PCR products were subcloned and sequenced as described above.

RESULTS AND DISCUSSION

Amplification of cDNA fragments for Pos- and Pod-AGHs were performed by RT-PCR using degenerate oligonucleotide primers (Arv-AGH-F and Arv-AGH-R in Fig. 1) designed based on the amino acid sequence of the Arv-AGH precursor (Okuno *et al.*, 1999). Subsequently, the 5'- and 3'-regions of the two cDNAs were amplified by 5'- and 3'-RACE using specific primers based on the nucleotide sequences obtained by RT-PCR. In order to confirm the nucleotide sequence of each cDNA, PCR was performed in

order to amplify each cDNA fragment including the full-length open reading frame (ORF). Each nucleotide sequence was determined by analyzing at least five independent clones in order to avoid PCR errors.

The nucleotide and deduced amino acid sequences of the two cDNAs encoding the Pos- and Pod-AGH precursors are shown in Fig. 2. The Pos-AGH precursor cDNA consisted of 672 bp comprising a 5'-untranslated region (68 bp), an ORF (435 bp), a stop codon (TGA), and a 3'-untranslated region (166 bp). The Pod-AGH precursor cDNA consisted of 672 bp comprising a 5'-untranslated region (67 bp), an ORF (438 bp), a stop codon (TGA), and a 3'-untranslated region (164 bp). In both cDNAs, the 3'-untranslated region contained a consensus polyadenylation signal (AATAAA) 15 bp upstream from the poly (A) tail. The ORFs of Pos- and Pod-AGHs were conceptually translated into putative peptides consisting of 145 and 146 amino acid residues, respectively. Both N-terminal segments Met^{-21}-Ala^{-1} in the Pos-AGH precursor (Fig. 2A) and Met^{-21}-Ala^{-1} in the Pod-AGH precursor (Fig. 2B) included a high proportion of hydrophobic amino acid residues (14/21 and 15/21, respectively) and were therefore likely to constitute a signal peptide (Von Heijne, 1986).

The deduced amino acid sequences of the Pos- and Pod-AGH precursors were highly similar (87% identity). Although 19 amino acid residues differed between the Pos-

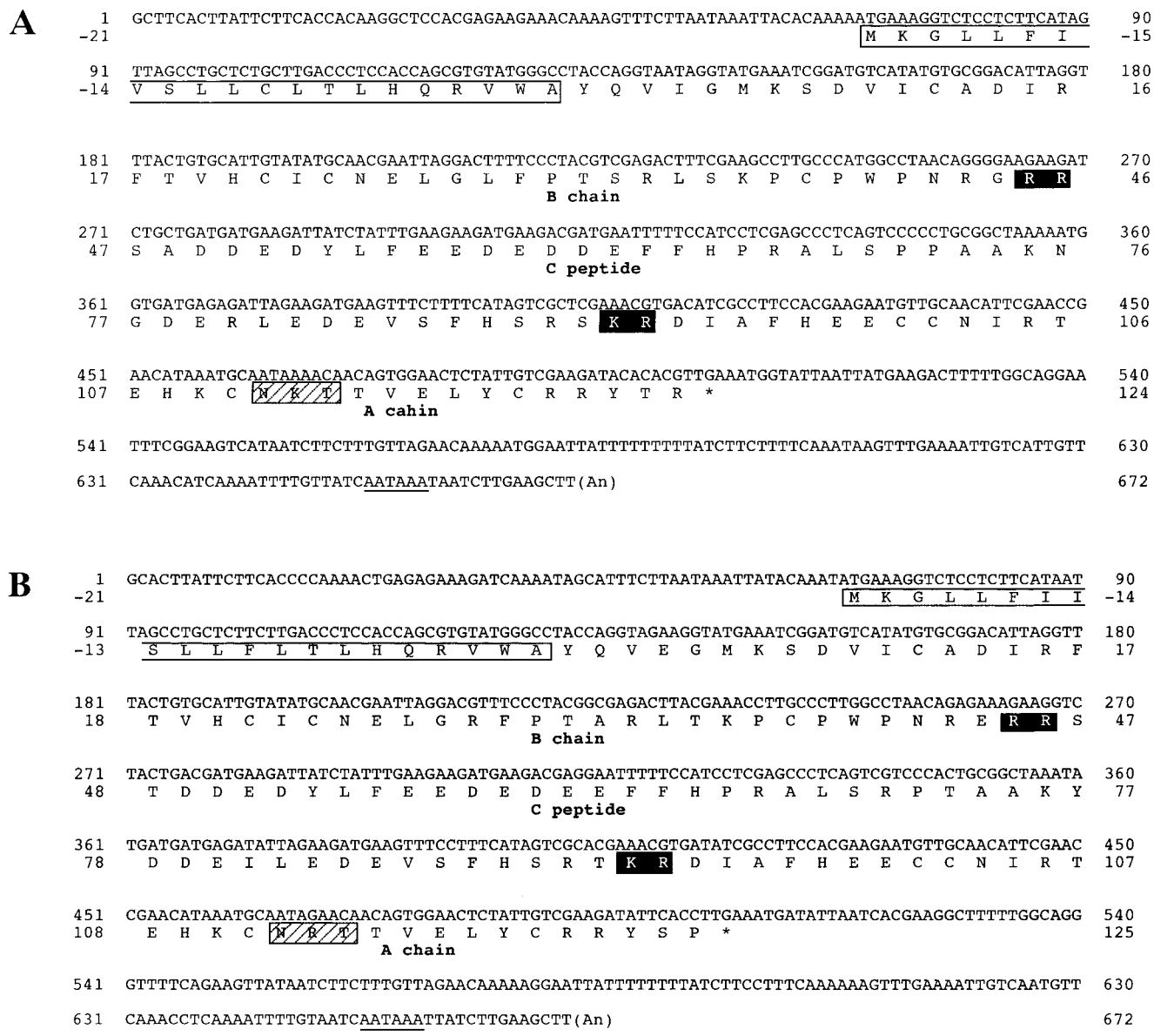

Fig. 2. Nucleotide and deduced amino acid sequences of cDNAs encoding Pos-AGH (**A**) and Pod-AGH (**B**) precursors. Amino acid numbering is commenced at the N-terminal residue of each propeptide of the Pos- and Pod-AGHs. Open and filled boxes indicate the signal peptide and a putative dibasic cleavage site, respectively. Shaded boxes indicate putative *N*-linked glycosylation motifs. Each polyadenylation signal sequence is underlined. The nucleotide sequences of the cDNAs encoding Pos- and Pod-AGHs have been deposited in the DDBJ/EMBL/GenBank databases (accession nos. AB089810 and AB089811, respectively).

and Pod-AGH precursors, 17 of the 19 amino acid residues were interconvertible by single nucleotide substitution. The high structural similarity between the Pos- and Pod-AGH precursors is consistent with the taxonomically close relationship of the two species, both belonging to the same Porcellionidae family. The Pos- and Pod-AGH precursors were also similar to the Arv-AGH precursor (65% and 63%, respectively), although these values were lower than that between the Pos- and Pod-AGH precursors. These results are highly reasonable, given that *A. vulgare* is a member of the Armadillidiidae family, but not of the Porcellionidae family.

Implantation experiments on hormonal activity have been performed previously (Martin and Juchault, 1999). The species-nonspecific nature of AGH was observed between *P. scaber* and *P. dilatatus*, when each AG was implanted into the opposite species. In contrast, AG from *A. vulgare* could not masculinize *P. scaber* and *P. dilatatus*, while both AGs from *P. scaber* and *P. dilatatus* affected partial masculinization in *A. vulgare*. These results concerning the species-specificity of AGH activity agree well with the present results in terms of the degree of sequence identity among the three species.

The alignment of the Pos- and Pod-AGH precursors with the Arv-AGH precursor is shown in Fig. 3. The organization of the Pos- and Pod-AGH precursors was the same as that of the Arv-AGH precursor; the Pos- and Pod-AGH precursors also consisted of B chain (44 amino acid residues), C peptide (45 and 46 amino acid residues, respectively), and A chain (31 amino acid residues). Both A chains were longer than that of Arv-AGH by two amino acid residues at the carboxyl (C)-terminus. Eight Cys residues in the Pos- and Pod-AGHs were located at completely identical positions with those in Arv-AGH (Fig. 3). It was previously reported that four Cys residues of each chain of Arv-AGH form two intrachain and two interchain disulfide bridges (Martin *et al*., 1999; Okuno *et al*., 2002), suggesting that Cys residues of the Pos- and Pod-AGHs form disulfide bridges in the same manner. Pos- and Pod-AGHs possess two typical proteolytic cleavage motifs (R-X-K/R-R) at positions between B chain and C peptide, and between C peptide and A chain (Hosaka *et al*., 1991). The A chains of Pos- and Pod-AGHs possessed a putative *N*-linked glycosylation motif (N-X-S/T). Positions of their proteolytic cleavage motifs and their *N*-linked glycosylation motif were identical with those of Arv-AGH (Fig. 3). The C-terminal end of the B chain of Pos-AGH is likely amidated, as a putative amidation signal sequence (G-R-R) exists at that position (Eipper *et al*., 1992).

The comparison of amino acid sequences of Pos- and Pod-AGHs with that of Arv-AGH revealed that sequence identities of A and B chains were very high (75–82%), whereas C peptide showed much lower sequence identity (44%). These values in C peptide were even lower than those in the signal peptides (57 and 48%, respectively). This observation suggests that C peptide may not be functional. The structural organization of the AGH prohormone consisting of B chain, C peptide, and A chain in this order is similar to those of the insulin superfamily peptides. Similar structural divergence is also observed in the insulin superfamily (Steiner *et al*., 1985); the amino acid sequences of A and B chains among the various species were highly conserved. These results coincided well with the previous finding that antibody raised against recombinant pro-Arv-AGH expressed by *E. coli* cross-reacted with the androgenic gland of *P. scaber* and *P. dilatatus* (Hasegawa *et al*., 2002).

In Northern blot analysis using poly $(A)^+$ RNA from whole male reproductive organs of each species, a single band was detected using a specific cDNA probe for each species (Fig. 4A). The sizes of these bands (approximately

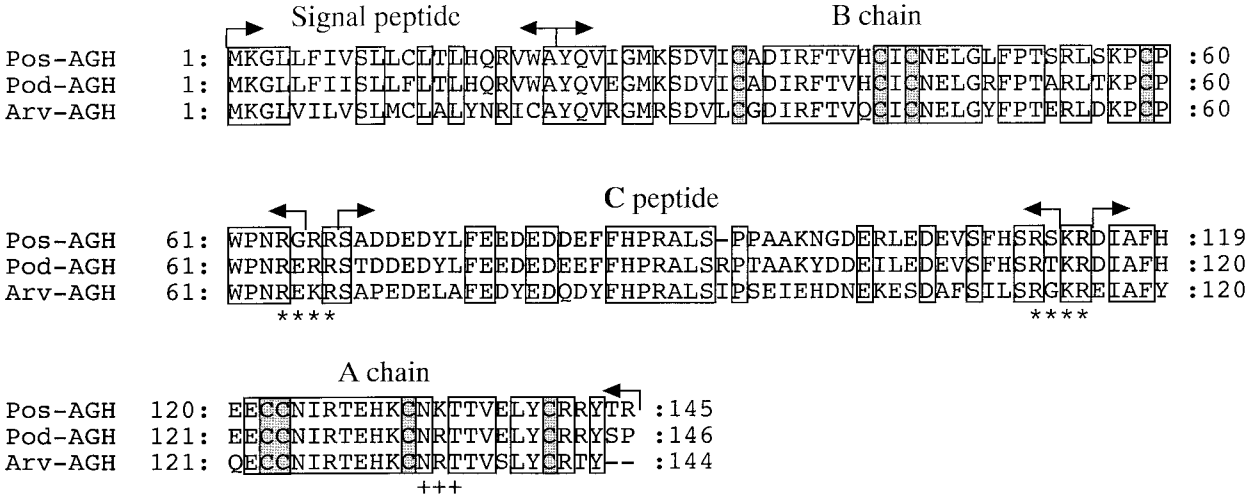

Fig. 3. Alignment of Pos-, Pod- and Arv-AGH precursors. Sequences are from *Porcellio scaber* (Pos-AGH), *P. dilatatus* (Pod-AGH) and *Armadillidium vulgare* (Arv-AGH; Okuno *et al*., 1999), and are aligned using the CLUSTAL W program (Thompson *et al*., 1994). Open boxes indicate the amino acids conserved among all precursors. Gray boxes indicate the positions of conserved Cys residues. The typical proteolytic cleavage motifs (R-X-K/R-R) and the putative *N*-linked glycosylation motif (N-X-S/T) are indicated by asterisks and symbols (+), respectively.

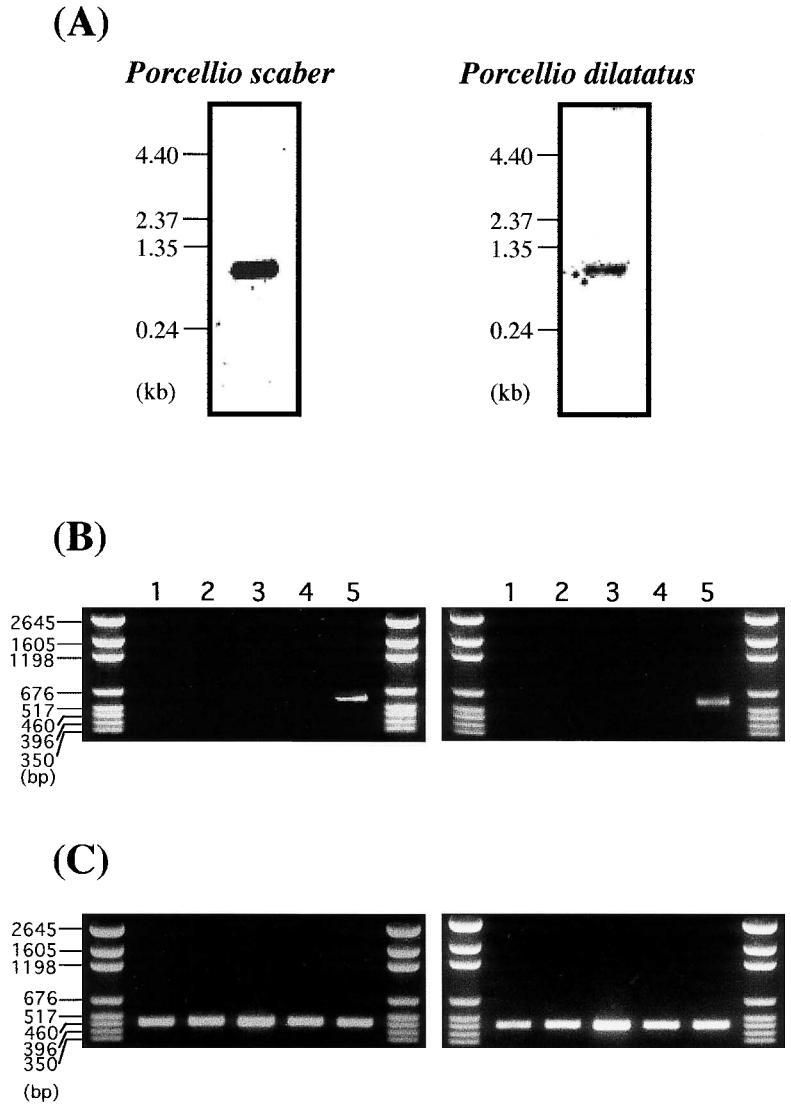

Fig. 4. Northern blot analyses examining the number and size of each AGH transcript (**A**), and RT-PCR analyses for gene expression of each AGH in various tissues (**B**). In Northern blot analyses, poly $(A)^+$ RNA prepared from whole male reproductive organs of each species hybridized with each AGH cDNA probe labeled with alkaline phosphatase. In RT-PCR analyses, first strand cDNAs synthesized using total RNA from the hepatopancreas (lane 1), seminal vesicle (lane 2), vas deferens (lane 3), testis only (lane 4) and testis together with the androgenic glands (lane 5) were used as templates. The expected sizes of the amplified cDNA fragments of Pos- and Pod-AGHs were 545 bp and 544 bp, respectively. As a control, an actin cDNA fragment was amplified using degenerate primers in tissues of each species (**C**). Left and right figures show results for *Porcellio scaber* and *P. dilatatus*, respectively.

0.75 kb) were in good agreement with those of the Pos- and Pod-AGH precursor cDNAs (672 bp). These results indicated that Pos- and Pod-AGHs are translated from a single mRNA species.

The tissue-specific gene expression of Pos- and Pod-AGHs was examined by RT-PCR using specific primers. Both PCR products were detected only in cDNA synthesized from the testis carrying the androgenic glands, but not from the testis only, seminal vesicle, vas deferens, or hepatopancreas (Fig. 4B). The reason why the androgenic gland only was not examined was due to the difficulty in making a complete separation between the testis and the androgenic gland. These results are in good agreement with the previous finding that AGH-producing cells detected immunohistochemically were localized only in the androgenic gland of each species (Hasegawa *et al.*, 2002). This androgenic gland-specific expression of both AGH transcripts also supported the hypothesis that each cDNA encodes a prepro-AGH in each species. The definite conclusion should be possible after a recombinant active peptide is obtained.

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