Temporal Silencing of an Androgenic Gland-Specific Insulin-Like Gene Affecting Phenotypical Gender Differences and Spermatogenesis

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Androgenic glands (AGs) of the freshwater prawn *Macrobrachium rosenbergii* were subjected to endocrine manipulation, causing them to hypertrophy. Transcripts from these glands were used in the construction of an AG cDNA subtractive library. Screening of the library revealed an AG-specific gene, termed the *M. rosenbergii* insulin-like AG (*Mr-IAG*) gene. The cDNA of this gene was then cloned and fully sequenced. The cysteine backbone of the predicted mature Mr-IAG peptide (B and A chains) showed high similarity to that of other crustacean AG-specific insulin-like peptides. *In vivo* silencing of the gene, by injecting the prawns with *Mr-IAG* double-stranded RNA, temporarily prevented the regeneration of male secondary sexual characteristics, accompanied by a lag in molt and a reduction in growth parameters, which are typically higher in males of the species. In terms of reproductive parameters, silencing of *Mr-IAG* led to the arrest of testicular spermatogenesis and of spermatophore development in the terminal ampullae of the sperm duct, accompanied by hypertrophy and hyperplasia of the AGs. This study constitutes the first report of the silencing of a gene expressed specifically in the AG, which caused a transient adverse effect on male phenotypical gender differences and spermatogenesis. **(*Endocrinology* 150: 1278–1286, 2009)**

Ever since it was first proposed as the source of a hypothetical masculinizing hormone in crustaceans, the androgenic gland (AG) has been studied thoroughly in many crustacean species. The consensus emerging from these studies is that the AG plays a unifying role in the bewilderingly varied sex differentiation mechanisms in crustaceans (1–5).

The AG constitutes a feature unique to male crustaceans in that it is an organ regulating sex differentiation separated from the gametogenic organ (unlike the single organ of vertebrate species). This separation enables manipulation of sex differentiation without affecting the gonads (6). In decapod male crustaceans, there are two AGs, each attached to the ejaculatory region of a vas deferens.

In research spanning several decades, the functioning of the AG was investigated in a number of crustacean species by following the morphological and physiological effects of AG removal or transplantation on primary and secondary sex characteristics. In the amphipod *Orchestia gamarella*, for example, bilateral AG ablation decreased spermatogenesis and prevented the development of secondary male characteristics (7). In the crayfish *Procambarus clarkii*, injection of AG extracts accelerated the development of external male characteristics (8). In the giant freshwater prawn, *Macrobrachium rosenbergii*, a degree of masculinization was recorded in AG-implanted females (9). In the same species, fully functional sex reversal from males to neo-females (10) and from females to neo-males (11) was achieved by bilateral AG ablation and transplantation, respectively. The possibility of sex reversal has economical implications for the farming of this sexually dimorphic species because males grow faster than females (12).

It is currently widely accepted that the AG of decapod crustaceans secretes the hormone(s) responsible for male differentiation, with a high probability of such a hormone(s) being proteinaceous in nature (13). This premise is supported by a histological study in the shore crab *Phachygrapsus crassipes* (14).

Multicellular organisms express various insulin-like peptides differentially. The insulin-like peptides discovered in inverte-

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.
Copyright © 2009 by The Endocrine Society
doi: 10.1210/en.2008-0906 Received June 17, 2008. Accepted October 28, 2008.
First Published Online November 6, 2008

Abbreviations: AG, Androgenic gland; CHH, crustacean hyperglycemic hormone; dsRNA, double-stranded RNA; GFP, green fluorescent protein; hAG, hypertrophy and hyperplasia of the androgenic gland; *Mr-IAG*, *Macrobrachium rosenbergii* insulin-like androgenic gland gene; RNAi, RNA interference; T7P, T7 promoter site at the 5′ of one primer; UTR, untranslated region; XO-SG, X-organ sinus gland complex.

brates to date are not confined to carbohydrate metabolism: they play a variety of roles in metabolism, growth, and reproduction. Because Crustacea are believed to be the ancestors of insects (15), cumulative evidence for AG-specific insulin-like peptides may thus imply that these peptides have evolved in the context of sex differentiation in arthropods.

Most insulin-like genes, with the exception of IGFs, encode a single pre-pro-peptide with a signal peptide and contiguous B, C, and A peptides. The pro-peptide is processed into its active form by the formation of two interchain disulfide bridges linking the A and B peptides and an intrachain disulfide bridge linking two cysteine residues within the A chain, followed by proteolytic cleavage of the C peptide (16). Insulin-like peptide families have been reported for a variety of invertebrates. In the nematode *Caenorhabditis elegans*, 10 insulin-like peptides have been divided into three distinct families. Insulin-like peptides from two of these families probably contain an additional disulfide bond (17), as was suggested to be the case for the three isopod insulin-like AG factors described below (18). Seven IGFs are expressed in the brain and digestive system of the freshwater snail *Lymnaea stagnalis*; these proteins function in shell and body growth and in energy metabolism (19). Three insulin-like prothoracicotropic hormones (bombyxin I, II, and III), expressed in the brain of the silkworm *Bombyx mori*, regulate molt (20). In Crustacea the first AG hormone to be reported was found in isopods; it was assigned to the insulin family of hormones because it possesses B and A chains with a skeleton of conserved cysteine residues, separated by a C peptide that is present in the pro-hormone but undergoes cleavage to contribute to the mature hormone (18, 21–25). Extensive efforts to discover orthologous genes, through the use of sequence similarity and RT-PCR, in the important group of decapod species have come to nothing. Only recently did the establishment of an AG cDNA subtractive library in the decapod crayfish *Cherax quadricarinatus* reveal an insulin-like gene (26).

Because the AG has an enormous effect on primary and secondary male characteristics, it is considered not only as a male sex differentiation-regulating organ but also as an organ responsible for the maintenance of male morphological and anatomical features. Sun *et al.* (27) hypothesized that two polypeptides (~16 and ~18 kDa) found in the AG of sexually mature *M. rosenbergii* males could be AG hormones. They based their premise on size similarity to the AG hormone found in Isopoda (for which the pre-pro AG hormone is ~17 kDa) but provided neither functional nor structural evidence.

The discovery of the RNA interference (RNAi) phenomenon has revolutionized functionality assays of newly discovered genes in many fields (28). Recently, RNAi was applied by injecting different decapod crustaceans with double-stranded RNA (dsRNA) specific to the gene of interest to clarify the function of a number of gene products (29–32). Among those genes is the gene expressing a crustacean hyperglycemic hormone (*CHH*), which was silenced by *in vivo* dsRNA injections into adult shrimps. The silencing of the *CHH* gene reduced both CHH and hemolymph glucose levels (30). Another gene that has been silenced *in vivo* is the molt-inhibiting hormone gene, demonstrating a pleiotropic activity because the molt-inhibiting hormone silencing also implied a gonad-stimulating activity (32). The insulin gene was successfully silenced in mice through administration of small interfering RNA both *in vivo* and *in vitro* (33). However, the present study is the first instance in which the functionality of a conceptually novel insulin-like peptide has been elucidated *in vivo* through an RNAi assay.

In this study we identified a novel gene encoding an insulin-like peptide that is specifically expressed in the AG cells of *M. rosenbergii*, termed *M. rosenbergii* insulin-like AG (*Mr-IAG*) (data bank accession no. FJ409645). Silencing of its mRNA through RNAi temporarily prevented the regeneration of male secondary sexual characteristics (typical male growth patterns and presence of the *appendix masculina*), accompanied by the arrest of testicular spermatogenesis, the absence of spermatozoa in the sperm duct, and hypertrophy and hyperplasia of the AG (hAG).

Materials and Methods

Animals

M. rosenbergii males were maintained at Ben-Gurion University of the Negev under the following conditions. Food comprising shrimp pellets (Rangen Inc., Buhl, ID; 30% protein) was supplied *ad libitum* three times a week. Water quality was ensured by circulating the entire volume through a bio-filter maintaining all the water physicochemical parameters as described before (34).

Construction of a cDNA library of the AG by using suppression subtractive hybridization

Endocrine manipulation was achieved by removal of the X-organ sinus gland complex (XO-SG), causing hAG, as described previously for other crustacean species (34–36). This endocrine manipulation facilitates the extraction of the AGs because it promotes AG growth and, therefore, makes the glands easier to handle. The procedure was performed on 15 sexually mature male prawns (each weighing 35–80 g). Total RNA isolation, cDNA preparation, and preparation of the subtraction library of the AG were performed as previously described by Manor *et al.* (26), with the cDNA from 30 hAGs as the tester and the cDNA from other peripheral glands (a mix of mandibular and Y-organs) as the driver. After two hybridization cycles, unhybridized cDNAs, representing genes that are expressed in the AG but are absent from the driver, were amplified by two PCRs. The primary (24 cycles) and secondary (12 cycles) PCRs were performed according to recommendations in the manufacturer's manual (Fermentas, Ontario, Canada), and the PCR products were cloned into the pGEM-T easy vector (Promega Corp., Madison, WI) electroporatically transformed into competent bacteria. Clones containing the inserts were isolated and grown overnight. Plasmid DNA was purified using a miniprep kit (QIAGEN, Hilden, Germany), and the inserts were sequenced.

Sequencing of *Mr-IAG* factor

The complete *Mr-IAG* sequence was obtained by 3′ and 5′ rapid amplification of cDNA ends, performed with the Clontech Smart Race kit (Clontech Laboratories, Inc., Mountain View, CA), according to the manufacturer's protocol. PCR was performed with the gene-specific forward primer 5′-GAGCAGGGAA GAAGCGAACAATATGCTG-3′ (nt 575–602) and a reverse primer from the 3′ Race kit, the gene-specific reverse primer 5′-GCCAAGTATAGGACAGGGACGGGATGAT-3′ (nt 768–795; see Fig. 2) and the Universal Primers Mix (BD Biosciences, San Jose, CA) provided in the 5′ Race kit. The PCR products were cloned and sequenced as described previously.

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Bioinformatic analyses

To enhance the quality of the selected expressed sequence tags, the obtained cDNA sequences were first stripped of low-quality, vector and primer sequences using Sequencher software (Gene Codes Corp., Ann Arbor, MI), followed by clustering and assembly. The resulting contigs and singlets were unified, and their sequences were compared with those in the Universal Protein Resource database (Swiss-Prot + TrEMBL from 18.5.05; European Bioinformatics Institute, Cambridge, UK), using a local installation of National Center for Biotechnology Information's Basic Alignment Search Toolx algorithm. The full length of one of the cDNA sequences, *Mr-IAG*, was computationally translated with the Ex-PASy Proteomics Server (http://ca.expasy.org/tools/dna.html), and the most likely frame was selected (5′→3′ Frame 2). The deduced amino acid sequence was further assessed by SMART (http://smart.embl-heidelberg.de/smart) and CBS Prediction Servers (http://www.cbs.dtu.dk/services). Multiple sequence alignment of the predicted Mr-IAG B and A chain sequences with the B and A chain sequences of the three AG hormones known in Isopoda (18, 24) and with the *C. quadricarinatus* insulin-like AG factor, designated Cq-IAG (26), was performed with CLUSTAL W (37). A phylogram of the five crustacean insulin-like mature sequences was created with a random number generator seed of 111 and 1000 bootstrap trials, using CLUSTAL_X (38), and viewed by TreeView (39).

Mr-IAG tissue-specific expression

RT-PCR

cDNA was prepared by a reverse-transcriptase reaction containing 1 μg total RNA, extracted from mature males (AG, hAG, sperm duct, testis, peripheral glands, muscle, hepatopancreas, and thoracic ganglia), and Moloney murine leukemia virus reverse-transcriptase H minus (Promega), according to the manufacturer's instructions. The cDNA was then amplified by PCR, as previously described by Manor *et al.* (26), and *Mr-IAG* tissue-specific expression was demonstrated using forward (5′-GACAGC GTGAGGAGAA GTCC-3′, nt 627–646) and reverse (5′-TATAGGACAGGGACGGGATG-3′, nt 770–789) *Mr-IAG* specific primers. *M. rosenbergii* β-actin, accession no. AF221096 (40), was adopted as a positive control using specific forward (5′-GA GACCT-TCAACACCCCAGC-3′) and reverse (5′-TAGGTGGTCTCGT-GAATG CC-3′) primers. *M. rosenbergii* sperm duct specifically expressed gene *Mar-SRR*, accession no. DQ066890 (41), was used as the internal control using specific forward (5′-TCTCTGAAGCTGCAAGT-GAT TTAC-3′) and reverse (5′-AATCT GGGTCATTCTCCTGAT TGG-3′) primers. RT-PCR products were electrophoresed in 1.2% agarose, and visualized by exposure to UV light and ethidium bromide.

Northern blot analysis

Total RNA was isolated from the sperm duct, testis, muscle, hepatopancreas, and hAG of mature males. Five micrograms of RNA from each organ were electrophoresed on a 1% agarose formaldehyde gel, transferred to a nitrocellulose membrane, and UV cross-linked. The membrane was prehybridized overnight (42) and radiolabeled with a ^{32}P probe prepared by adding γ^{32}P-deoxycytidine triphosphate (Amersham Biosciences Inc., Piscataway, NJ), together with a *Mr-IAG* PCR product (nt 680–842), to a random priming labeling mix (Biological Industries, Beit Haemek, Israel). The blot was incubated overnight in hybridization buffer containing ^{32}P-labeled DNA. The membrane was washed as described by Shechter *et al.* (42) and exposed to BioMax MS Kodak film (Eastman Kodak Co., Rochester, NY) with intensifying screens at −80 C for 2.5 h. rRNA was visualized with ethidium bromide and UV light.

In situ hybridization

hAGs were dissected out together with the attached terminal ampullae from endocrinologically induced males 9 d after induction. Tissue samples were fixed in modified Carnoy's II for 48 h and dehydrated gradually through a series of increasing alcohol concentrations. Tissues were cleared and embedded in Paraplast (Kendall, Mansfield, MA) according to conventional procedures. Sections of 5 μm were cut onto silane-coated slides (Menzel-Gläser, Braunschweig, Germany). Consecutive sections were used for hybridization with sense and antisense probes, and for morphological observations (staining hematoxylin and eosin). Digoxigenin-labeled oligonucleotides for antisense and sense probes corresponding to nucleotides 29–1745 of *Mr-IAG* cDNA were synthesized using SP6 and T7 RNA polymerases, and the probes were hydrolyzed to reduce their length to approximately 200 bases, as described in the *Digoxigenin Application Manual* (Roche Applied Science, Indianapolis, IN). Hybridization was performed as described previously by Shechter *et al.* (42), with the slight modification of adding 100 μg/ml tRNA to the hybridization solution.

dsRNA preparation

PCR products of plasmids containing green fluorescent protein (GFP) or *Mr-IAG* open-reading frame, primed by two gene-specific primers with T7 promoter site at the 5′ of one primer (T7P) (5′-TAATAC GACT-CACTATAGGG-3′), were used as templates for RNA synthesis. Primer pairs used were as follows. For *Mr-IAG* sense RNA synthesis: primer T7P forward (5′-T7PATGGGATA CTGGAATGCCGAG-3′) *vs.* primer reverse (5′-CTGGAACTGCAGGTGTTAACG-3′). For *Mr-IAG* antisense RNA synthesis: primer forward (5′-ATGGGATACTGGAATGC-CGAG-3′) *vs.* primer T7P reverse (5′-T7PCTGGAACTGCAGGTG TTAACG-3′). For GFP sense RNA synthesis: primer T7P forward (5′-T7PATGGTGAG CAAGGGCGAG-3′) *vs.* primer reverse (5′-TGTA-CAGCTCG TCCATGCC-3′). For GFP antisense RNA synthesis: primer forward (5′-ATGGTGAGCAAGG GCGAG-3′) *vs.* primer T7P reverse (5′-T7PTGTACAGCTCGTCCATGCC-3′). PCR amplicons were electrophoresed on a 1.2% agarose gel, visualized with ethidium bromide and UV light, excised from the gel, and purified with a QIAquick PCR Purification Kit (QIAGEN). Single-stranded RNA was synthesized with MEGAscript T7 kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. RNA was quantified and diluted to 1 μg/μl, and then the two strands were hybridized by heating to 70 C for 10 min, followed by room temperature incubation for 10 min. dsRNA quality was assessed on an agarose gel; dsRNA was kept at −20 C until used.

In vivo Mr-IAG silencing

For the preliminary short-term *in vivo* dsRNA injection experiment, 46 $PL_{130-140}$ *M. rosenbergii* males (each weighing 0.6–2.1 g) were selected according to the presence of genital papillae on the fifth walking legs and appendices masculinae on the second pleopods. The post-larvae were assigned to one of three treatment groups: *Mr-IAG* dsRNA injected (n = 16), GFP dsRNA injected (n = 15), and vehicle injected (n = 15). Each animal was housed in a separate floating fenestrated plastic container (80 cm^2). Before the start of the experiment, the second pleopod

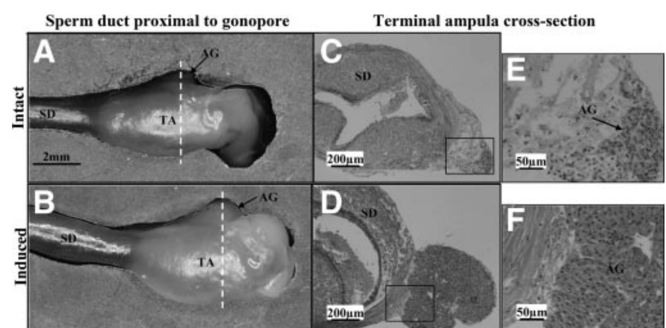

FIG. 1. Effects of endocrine manipulation through bilateral removal of the XO-SG complex on the reproductive system of the *M. rosenbergii* male. Proximal parts of the male reproductive systems of intact (A) and endocrinologically induced (B) *M. rosenbergii* males; hematoxylin and eosin-stained 5-μm cross-sections of the terminal ampullae (TA) of the intact (C) and endocrinologically induced (D) males, with the AG area magnified (E and F, respectively). *Dashed lines in left panel* denote plane of section. SD, Sperm duct.

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nt 1     GGTTATTCCAAGAGGGGCCAAGACTCTGGGATCACACCTCGAACGGCTCTGTCCCTTCCC
nt 61    CTCGTCCGTTTAACCGGTGTTTTCTAGCCACGCTCTCAACACCTAAAAATTCCCTCTCTT
nt 121   GCTTTCTGGCCAGCCTTGCAGTCATCCTTGAAATTCCCTCTTCCTTATATTTCGGGACAT
nt 181   AACATTCTTCTCTCCGGCCTTTTCATATCGAAGTGAAACAAATCAACTACAGAATGGGAT
aa 1                                                            M  G  Y
nt 241   ACTGGAATGCCGAGATCAAGTGTGTGTTGTTCTGCTCACTCGTAGCATCGCTTCTCCCTC
aa 4      W  N  A  E  I  K  C  V  L  F  C  S  L  V  A  S  L  L  P  Q
nt 301   AACCTTCTTCGAGCTATGAGATCGAATGCCTCTCCGTTGACTTTGACTGCGGCGACATAA
aa 24     P  S  S  S  Y  E  I  E  C  L  S  V  D  F  D  C  G  D  I  T
nt 361   CGAACACCCTTGCCTCCGTCTGCCTGAGACACAACAACTACATCAACCCAGGACCCACCT
aa 44     N  T  L  A  S  V  C  L  R  H  N  N  Y  I  N  P  G  P  T  Y
nt 421   ACGTTTCCAAAGAGCGACGATCTGCTGACATCTATACCGTTCCTTCTACGAAGTCTCCAT
aa 64     V  S  K  E [R  R] S  A  D  I  Y  T  V  P  S  T  K  S  P  S
nt 481   CGCTCGCCCACCCGAGAGCTACCCACTTGACCATGGCTGACGAAGAAACTCAGAAGGTAT
aa 84     L  A  H  P  R  A  T  H  L  T  M  A  D  E  E  T  Q  K  V  S
nt 541   CTAAGGTGGAGGAGGAGATTCAGCACATGACGCTGAGCAGGGAAGAAGCGAACAATATGC
aa 104    K  V  E  E  E  I  Q  H  M  T  L  S  R  E  E  A  N  N  M  L
nt 601   TGCATTCGAAGCGTCGCTTCCGGAGGGACAGCGTGAGGAGAAGTCCAAGGGAGGAATGCT
aa 124    H  S [K  R] R  F  R  R  D  S  V  R  R  S  P  R  E  E  C  C
nt 661   GCAACAACGCCTCTTTCAGACGCTGCAACTTCGAGGAAGTCGCCGAATATTGCATCGAGC
aa 144    N  N  A  S  F  R  R  C  N  F  E  E  V  A  E  Y  C  I  E  L
nt 721   TGCGTCCCGGCGTTAACACCTGCAGTTCCAGGTAGGAGGTCTCAAGGATCATCCCGTCCC
aa 164    R  P  G  V  N  T  C  S  S  R  *
nt 781   TGTCCTATACTTGACAGGAGATGCTCAAAGTCAAATCACCGTCTTCGAGTCATGATGTGG
nt 841   AATGACCTTCAGCTAAAGCTGCCTTTTGGCTTTCCTCACAGTCAACTAAAAACAATTTTT
nt 901   TTTATCCTACCGTTACCTTCAGATAAATTATTCCTTTGTCTCAGCTTTAATTTCGGCTAA
nt 961   AGCTTTTTTTTTTGTTCTACCCATGCATTCAGCTAAAGCTTTCTTTTGTTTCGCCTTTAA
nt 1021  ATTCAACACTCCTCTGCCTTACCCTTATTTCAGCTAATGGCTTCTTTTTATTTTACCATT
nt 1081  ACCATCCACAAAGCTTTGTTTTGTCTTACCCTCAGCTGAAACGTTTGTTTGTCTCACCTT
nt 1141  TACCCTCAGCTAAAACTTTCTTTTGTCTTCCCGCTGCTTTAGTAAATGCTTTCTTCTGTC
nt 1201  ACACTTTTACTTTCAGCTAGGGATTCTTTTTTTTTTTTTGCCACTTTTACCTTCAGCTAA
nt 1261  AGGGTACTATTGTCTCACCCTTGCCTTCTGCTAAAGGTTCCTTTTGTCCCACCCTTGCCT
nt 1321  TCAACTAAAGGTTCCTTTTGTCTCACCCTTGCCTTCAGCTAAAGGTTCCTTTTGTCTCAC
nt 1381  CCTTGCCTTCAGCTAAAGGTTCCTTTTGTCTCACCCTTGCCTTCAGCCAAAGGTTCCTTT
nt 1441  TGTCTCACCCTTGCCTTCAGCTAAAGGTTCCATTTGTCTCACCTTTGCCTTCAGCTAAAG
nt 1501  GTTCCTTTTGTCTCACCCTTGCCTTCAGCTAAAGGTCCCTTTTGTCTCACCCGTGCATCC
nt 1561  AACTAAAGGTTCCTTTTACCTCTCTTTTATCTTTAACTAAAGTTTTTTGTTTTTGTATCC
nt 1621  TTGCCTTCAGCCAAACGTTCTTTTGTTTTATCTTTACACGCAACAACATCTAGACATTTC
nt 1681  CAAACATTAAGCATATTGCATTATTATTGGTGATTCTTGTCGATGTTTCCGAAAAATTGT
nt 1741  TTGATACATCAGTTATACGTCAAATAAATGCTTTTGAGAACCCGGAAAAAAAAAAAAGAAA
nt 1801  AAAAAAAAAAAAAAAAAAAAAAAA
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FIG. 2. *Mr-IAG* cDNA sequence and deduced Mr-IAG protein according to the predicted open-reading frame. Putative signal peptide is shown in *bold type*. The putative B and A chains are *underlined*, the putative C peptide is *italicized and shaded*, the predicted arginine C-proteinase cleavage sites are *boxed*, and the stop codon is marked with an *asterisk*. In the 3′ UTR, 9.6 tandem repeats of 34 nucleotides are *underlined* (differentially *dashed and dotted*); nucleotides that deviate from the consensus sequence are shown in *bold type*.

of each animal was removed, and the presence of the *appendix masculina* was confirmed under a light microscope. The pleopods were fixed, stained with eosin, and photographed. Twice a week (over a period of 18 d), each animal was injected with 5 μg dsRNA/g or 5 μg GFP dsRNA, or a similar volume of vehicle (a total of 12 injections). Molts were recorded daily, and weight accumulation was documented. Regeneration of the *appendix masculina* was viewed under a dissecting stereoscope.

For the long-term *in vivo* experiment, the same procedure was repeated with 36 PL_{70-80} males (each weighing 0.25–1.6 g), assigned to two equal-sized groups: *Mr-IAG* dsRNA injected (n = 18) and vehicle injected (n = 18). The injection regime comprised a total of 22 injections, with the injections being given three times a week over a period of 55 d. dsRNA was injected into the sinus between the third and fourth walking legs

Statistical analysis

To evaluate the effect of dsRNA injection on the rate of *appendix masculina* regeneration and on the molt interval, a Cox proportional hazards regression model (43) was used. The model is expressed by: $\mu(t; z) = \mu_0(t)\exp(\Sigma\beta_i z_i)$, where $\mu(t,z)$ is the *appendix masculina* regeneration rate, and $\mu_0(t)$ is the baseline hazard function that can change over time (t). The regression coefficient to be estimated, β_i, represents the independent effect of dsRNA injection on *appendix masculina* regeneration rate. These analyses were performed with S-PLUS 2000 (Mathsoft, Needham, MA). The effect of dsRNA injection on molt increment was statistically analyzed by a paired t test using STATISTICA 6.1 software (StatSoft, Inc., Tulsa, OK).

Results

The reproductive tract of endocrinologically induced males did not seem morphologically different from that of intact males (Fig. 1A); however, the AG of the endrocrinologically induced males was visibly enlarged (Fig. 1B). In the intact male, a cross-section of the terminal ampullae with the adjacent AG showed loose connective tissue with a small cluster of glandular cells (Fig. 1, C and E). In the endocrinologically induced male, the AG showed an increased number of glandular cells occupying the loose connective tissue (Fig 1, D and F). The increase in cell number was accompanied by increase in cell size (12.30 ± 0.59 μm and 8.96 ± 1.08 μm in endocrinologically induced and intact males, respectively) as a result of hypertrophy of the AG in the endocrinologically induced males (Fig. 1, E and F).

A total of 30 enlarged hAGs from mature *M. rosenbergii* males was used to construct a cDNA subtractive library. Bioinformatic analysis of 20 DNA expressed sequence tags from this library revealed a 729-bp DNA sequence with high similarity to the A chain of the putative Cq-IAG (26). By means of 5′ and 3′ Race, the full sequence of the cDNA termed *Mr-IAG* was found to be 1824-bp long (Fig. 2). The predicted untranslated regions (UTRs) included 233 bp in the 5′-UTR and 1070 bp in the 3′-UTR (Fig. 2; nt 1–233 and 755-1824, respectively), with 9.6 tandem repeats of a 34-nucleotide consensus sequence being found in the 3′-UTR (Fig. 2; nt 1250–1577). The predicted proteinaceous product of this cDNA is 173 aa with a predicted mass of 19.76 kDa. Mr-IAG has structural homology to the insulin-like family of proteins, namely a signal peptide in its N′ terminus (27 aa), six cysteine residues aligned with other IGFs, and B and A chains (42 and 46 aa, respectively) separated by a C peptide (58 aa) with predicted arginine C-proteinase cleavage sites at both its N′ and C′ termini (Fig. 2).

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The predicted B and A chains that form the putative mature protein, with a predicted mass of 9.98 kDa, were compared with those of other insulin-like factors that were also shown to be specifically transcribed in crustacean AGs (Fig. 3). The skeleton of cysteine residues, typical of the insulin family of hormones, was conserved in all five sequences and was thus schematically marked for predicted disulfide bonds (Fig. 3A). Two published sequences of the genus *Porcellio* (*Porcellio dilatatus*, accession no. BAC57013 and *Porcellio scaber*, accession no. AAO11675) shared high-sequence similarity (90.8% similarity in 76 aa, calculated by LALIGN server). A third member of the Isopoda, *Armadillidium vulgare* (accession no. BAA86893) exhibited relatively high-sequence similarity with the aforementioned two isopods (81.3% similarity in 75 aa with *P. dilatatus* and 78.7% similarity in 75 aa with *P. scaber*) and lower similarity with the two decapod sequences: Cq-IAG from *C. quadricarinatus* (25.0% similarity in 72 aa) and Mr-IAG from *M. rosenbergii* (28.9% similarity in 75 aa). The sequences of the decapod species shared little sequence similarity among themselves (29.1% in 86 aa) or with those of the two isopods *P. dilatatus* and *P. scaber*.

The sequences were subjected to the Clustal W algorithm, and a phylogram was calculated (Fig. 3B). The phylogram emphasizes the sequence similarity of the two isopods *P. dilatatus* and *P. scaber*, and the larger distance between them and the decapods. The sequence of the third isopod species *A. vulgare* occupies another branch, closer to the decapod species' sequences but, nevertheless, quite remote from them.

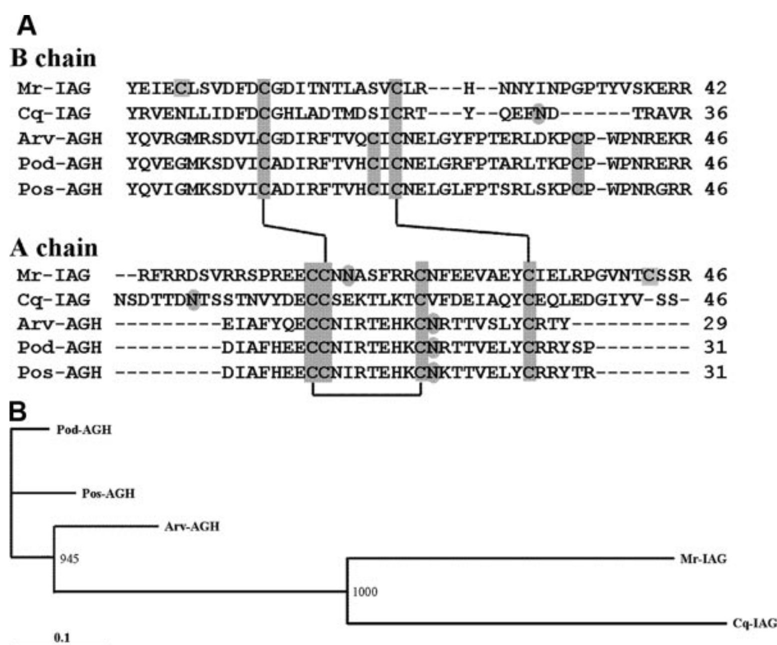

FIG. 3. Mr-IAG predicted B and A chain regions aligned and compared with other known Crustacean AG insulin-like sequences. A, Multiple sequence alignment of predicted mature AG specific factors from two decapods (*M. rosenbergii* and *C. quadricarinatus*) and mature AG hormones from three isopods (*A. vulgare* and predicted in *P. scaber* and *P. dilatatus*). The conserved cysteine residues are *boxed* where the two predicted interchain and one intrachain disulfide bonds are linked. Nonconserved cysteine residues in Mr-IAG are *boxed*. Predicted N-glycosylation sites are *circled*. B, A phylogram of all the known crustacean AG insulin-like sequences with distances (calculated by CLUSTAL_X 1.83, presented by TreeView 1.6.6). *Bar* represents the number of substitutions per site.

The *Mr-IAG* transcript was shown by RT-PCR to be exclusively expressed in the AG of sexually mature *M. rosenbergii* males (Fig. 4A). RT-PCR showed no signal of this gene transcription in females (data not shown) or any other mature male tissues, including sperm duct, testis, thoracic ganglia, muscle, and hepatopancreas. In all the tissues examined, primers for β-actin of *M. rosenbergii* (accession no. AF221096) were used as a positive control for the RT-PCR procedure. The gene *Mar-SRR* (accession no. DQ066890), which was previously reported to be specifically transcribed in the sperm duct of *M. rosenbergii* (41), was also used as an internal positive control for unique transcription in the sperm duct and not in the AG. The size and AG specificity of the *Mr-IAG* transcript were further assessed using Northern blot analysis. The transcript exhibited a single band of approximately 1.9 kb exclusively in the AG, with no signal being detected in the sperm duct, testis, muscle, or hepatopancreas of the mature *M. rosenbergii* male. rRNA served to ensure the presence of RNA in the samples and was visible in all sampled tissues (Fig. 4B). Localization of *Mr-IAG in situ* further confirmed its AG-specific expression (Fig. 4C). A strong, specific signal was detected exclusively in AG cells using an antisense probe. No signal was detected when the sense-strand probe was used.

A short-term preliminary RNAi experiment (silencing via *in vivo* dsRNA injection), using GFP-dsRNA-injected and vehicle-injected control groups, revealed that the two control groups did not differ significantly from one another. Therefore, the long-term *in vivo* dsRNA injection assay included only a vehicle-injected control group and the *Mr-IAG*-dsRNA-injected group. In the long-term experiment, all individuals had molted at least three times by the end of the injection period (d 55), thus statistical analysis was applied only for the first three molt events. Figure 5A shows that cumulative molt events were significantly different between groups (Cox proportional hazards regression model; second molt $z = 2.59$, $P = 0.0097$; third molt $z = 3.07$, $P = 0.0021$). From the time of the first molt event, the *Mr-IAG*-dsRNA-injected group lagged in molt intervals behind the vehicle-injected group, with the most marked lag being that between the first and second molts (lowest exponent coefficient; Fig. 5A). The lag was sustained even after the end of the injection period. Weight accumulation was lower in the *Mr-IAG*-dsRNA-injected group, and this difference became statistically significant by the third molt event (paired *t* test, $P = 0.0224$; Fig. 5B).

The second pleopods of representative individuals from the *Mr-IAG*-dsRNA-injected group and the vehicle-injected control group were removed at the start (Fig. 6, Aa and Ab, respectively) and at the end of the repeated injection period of the long-term experiment (Fig. 6, Ae and Af, respectively). The pleopods along with their second molt exuviae were stained with eosin (Fig. 6, Ac and Ad, respectively). Figure 6A demonstrates the lack of regeneration of the *appendix masculina* in the animals injected with *Mr-IAG* dsRNA (Fig. 6, Ac and Ae, *top row*), in contrast to the regeneration observed in the vehicle-

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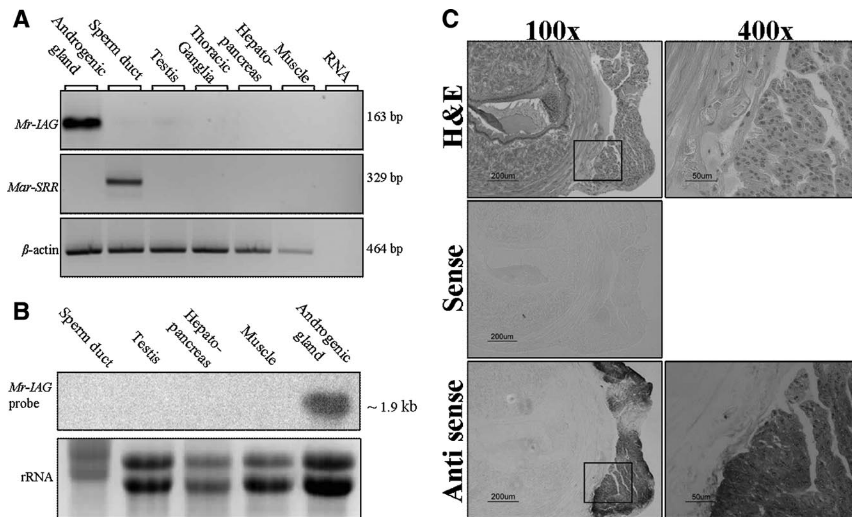

FIG. 4. *Mr-IAG* tissue-specific expression in mature *M. rosenbergii* males. A, *Mr-IAG* cDNA is specifically expressed in the AG, as shown by RT-PCR (*top*) along with *Mar-SRR* specific expression in the sperm duct (*middle*). RNA from the AG (*top and bottom*) and from sperm duct (*middle*) was used as a negative control. *M. rosenbergii* β-actin (*bottom*) was used as a positive control. B, Northern blot analysis showing an approximate 1.9-kb band exclusively in the AG (*top*). Each lane was loaded with 5 μg total RNA, as indicated by the rRNA bands (*bottom*). C, Top to bottom, Hematoxylin and eosin (H&E)-stained sense and antisense probes hybridized with *Mr-IAG in situ*, in 5 μm cross-sections of the sperm duct along with AG cells of a mature *M. rosenbergii* male. Hematoxylin and eosin and antisense are also shown at higher magnitude (×400; *boxed areas* ×100).

injected individuals (Fig. 6, Ad and Af, *bottom row*).The appendices masculinae on the right legs (which were not removed from the animals of both the groups, as control) remained intact. The appendices masculinae of the left leg regenerated exclusively in the vehicle-injected animals. By the 44th day, the appendices masculinae of all 18 control prawns had regenerated, whereas in the group of *Mr-IAG*-dsRNA-injected animals, the organs of only two individuals (out of 16) had regenerated by the end of the repeated injection period (by d 32 and 53 in those two animals). The lag in the regeneration of the appendices masculinae of the animals injected with *Mr-IAG* dsRNA was statistically significant and consistent over the entire injection period (Cox proportional hazards regression model; $z = 4.03$; $P < 0.001$). By 55 d after termination of the injections, the appendices masculinae of all the animals injected with *Mr-IAG* dsRNA had regenerated (Fig. 6B). Four weeks after the termination of the injection period, the differences between the groups were no longer statistically significant.

Serial dorsoventral sections of two representative individuals from the group of animals injected with *Mr-IAG* dsRNA and two from the vehicle-injected group were stained with hematoxylin and eosin (Fig. 7). Both individuals from the *Mr-IAG*-dsRNA-injected group showed a clear arrest of spermatogenesis, as indicated by the absence of spermatozoa in all sections of the sperm ducts and testes (Fig. 7, A and E). Overall, the testes of the animals receiving the *Mr-IAG* dsRNA injections were smaller than those of the vehicle-injected group (Fig. 7, E and F, respectively). The two individuals from the vehicle-injected group showed active spermatogenesis, as seen by the presence of many spermatozoa in the testis lobules and in the sperm ducts (Fig. 7, F and B). The spermatogenic arrest observed in the animals injected with *Mr-IAG* dsRNA was accompanied by hyperplasia and hypertrophy of AG cells (Fig. 7C). The AG cells of the two prawns from the vehicle-injected group were fewer in number and smaller in size than those of the animals injected with the *Mr-IAG* dsRNA (Fig. 7, C and D). The number and size of the AG cells in the two vehicle-injected animals resembled those of intact males of the same age (data not shown).

Discussion

It has previously been demonstrated that XO-SG removal leads to endocrinological induction of hAG in *M. rosenbergii*, as in other decapod crustacean species (34, 36). Histologically, hypertrophy of the AG is evident from a greater abundance of glandular cells and an increase in their volume (cell size). Such hAGs were used for the construction of a *M. rosenbergii* AG cDNA subtractive library. Scanning the clones of this hAG-enriched cDNA library revealed a cDNA encoding an insulin-like factor, termed *Mr-IAG*, based on its similarity to the *Cq-IAG* sequence, the first AG-specific insulin-like factor to be found in decapods (26).

Previous reports of partial purification of peptides from *M. rosenbergii* AG revealed two polypeptides at approximately 16 and approximately 18 kDa (27). Neither of these peptides is likely to be the Mr-IAG because for Mr-IAG the predicted mass is approximately 9.98 and approximately 19.76 kDa for the mature and pre-pro-peptide, respectively, unless posttranslationally modified. In terms of its relatively higher abundance in the AG than in the vas deferens of *M. rosenbergii*, the approximate 9.98-kDa polypeptide extracted by Narksen *et al.* (44) should be further analyzed to determine whether it is related to the mature Mr-IAG.

The low-sequence similarity between the two decapod IAGs found thus far (Cq-IAG and Mr-IAG) indicates their evolutionary distance; nevertheless, all five crustacean insulin-like sequences determined to date share the typical skeleton of conserved cysteine residues of insulin-like proteins. In this regard, it is important to note that the three more closely related isopod species have two additionally conserved cysteine residues in their AG hormones (aa 21 and 38 of the B chain), enabling the formation of an additional intrachain disulfide bond (18). Another two, nonconserved cysteine residues are found exclusively in the Mr-IAG sequence (aa 5 of the B chain and 43 of the A chain), suggesting that a third interchain disulfide bond might occur in the putative protein. The occurrence of an additional two cysteine residues that might form a fourth disulfide bond in insulin-like peptides have been reported for the molluscan insulin-related peptide VII from *L. stagnalis* (19), and type β-insulins have been isolated from *C. elegans* (17).

Further research is needed to address the unusual length of *Mr-IAG* compared with cDNAs encoding insulin-like peptides.

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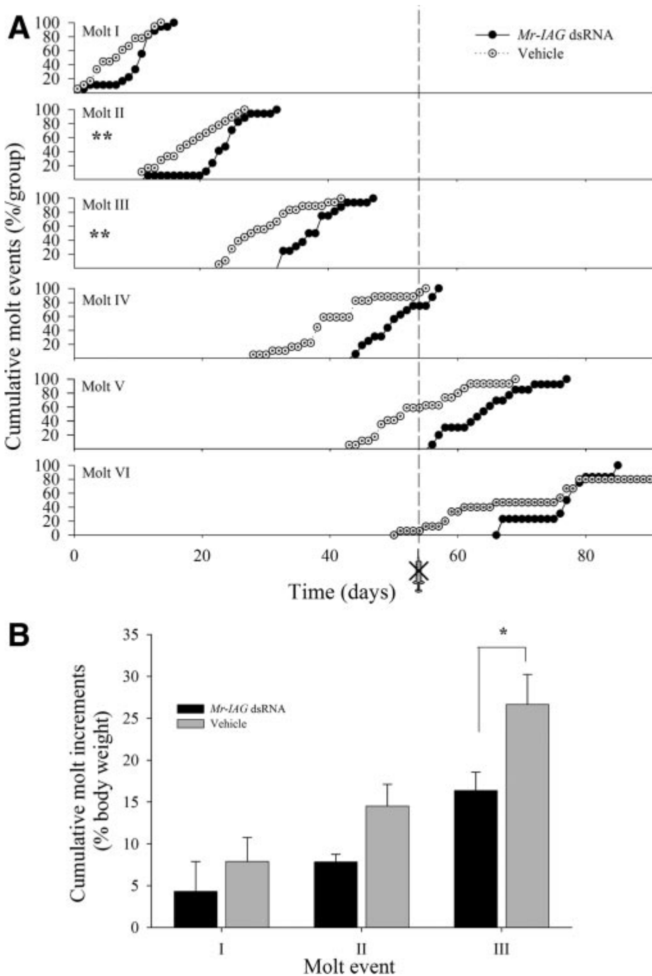

FIG. 5. Effects of *Mr-IAG* dsRNA injection on molt and growth of young *M. rosenbergii* males. A, Cumulative molt events of vehicle-injected (○, n = 18) and *Mr-IAG*-dsRNA- injected (●, n = 18) groups in the *in vivo* assay. Molted individuals are represented as a percentage of each group. The end of the repeated-injection period is marked as ⁑(d 55). Statistically significant differences (Cox proportional hazards regression model) are indicated with *asterisks* ($P < 0.001$). B, Cumulative molt increment of vehicle-injected (□, n = 18) and *Mr-IAG*-dsRNA- injected (■, n = 18) groups during three molt events. Cumulative molt increment is expressed by 100 × weight after molt/weight at start of experiment. *Bars* represent SEM. *Asterisk* represents the statistically significant difference observed at the third molt event (paired *t* test, $P = 0.0224$).

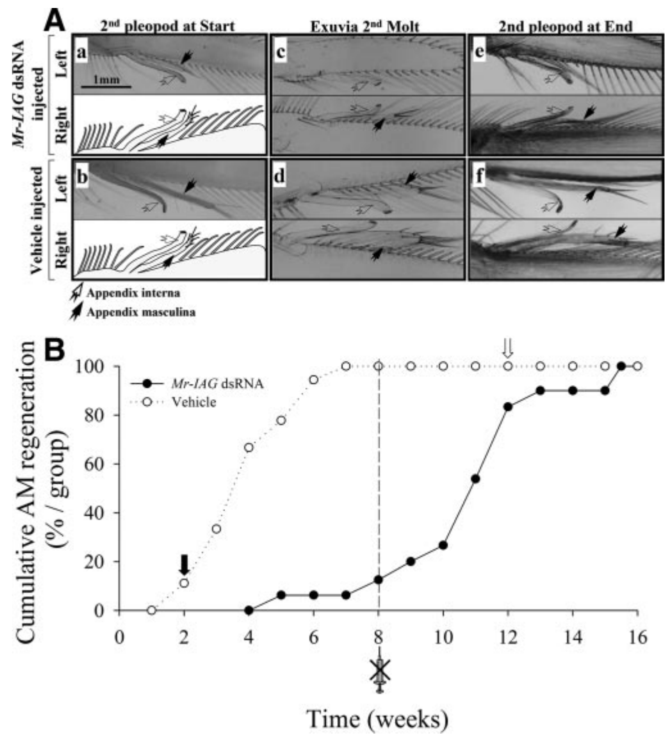

FIG. 6. Effects of *Mr-IAG* dsRNA injections on regeneration of *appendices masculinae* in young *M. rosenbergii* males. Aa and Ae, Left and right second pleopods of representative individuals from the group of animals injected with *Mr-IAG*-dsRNA before injection (a) and at the end of the experiment (e). Ab and Af, As for Aa and Ae for representative individuals from the vehicle-injected group. The exuviae from the second molt of each individual are shown in the *middle column* (c and d). B, Regeneration of the *appendix masculina* (AM) as a percentage of the population over 16 wk in *Mr-IAG*-dsRNA-injected (●, n = 18) and vehicle-injected (○, n = 18) groups. The end of the repeated injection period is marked as ⁑(d 55). *Bold black arrow* represents the point at which statistical differences between the two groups first appeared, and the *white arrow*, the point from which there was no statistical difference (*i.e.* recovery of the *appendices masculinae* in the *Mr-IAG* dsRNA-injected group).

To the best of our knowledge, all known insulin-like peptides (excluding IGFs) are encoded by mRNA that does not exceed 1300 nucleotides (data gathered from 30 insulin-like peptides). Given the fact that most insulin-like peptides do not possess a tandem repeat in the 3′-UTR, as was observed for the uniquely long *Mr-IAG* and *Cq-IAG*, it is possible that the tandem repeat is responsible for their relatively long transcripts. The findings might indicate a posttranscription regulatory mechanism involving the 3′-UTR tandem repeats that is unique to the AG-specific insulin-like peptides of decapod crustaceans. Such 3′-UTR tandem repeats have been shown to mediate translation repression of *tra-2* mRNA in *C. elegans*, repression that is required to permit spermatogenesis in hermaphrodites of the species (45).

Evidence for the importance and evolutionary conservation of insulin-like peptides in the context of sex differentiation is scarce. The small amount of data that is available comes from scattered reports on tissue-specific expression in male sex-differentiating glands in taxa as far removed from each other as Crustacea [AGs (18, 21, 26)] and Mammalia [Leydig cells (46)]. Moreover, evidence for the necessity of insulin-like peptide receptors for normal development of male mice testis (47) strengthens the notion that insulin-like factors might have a role in sex differentiation, and the results reported in the present study add valuable information in this respect.

The arrest of spermatogenesis in the *Mr-IAG*-silenced individuals implies that Mr-IAG is involved in the maintenance of male reproductive activity because it shares effects that are somewhat similar to those seen in mice lacking the androgen receptor in their Leydig cells (48). Moreover, *Mr-IAG* silencing inhibited growth and the development of male secondary sex characteristics. The delay in growth parameters of *Mr-IAG*-silenced individuals implies that Mr-IAG might also act as a growth regulator, although its predicted structure does not resemble that of an IGF. The delay in molt intervals suggests that Mr-IAG could be a factor regulating ecdysteroid levels throughout the molt cycle, as is the case for several reported insulin-like peptides in insects (49–51).

The hypertrophy and hyperplasia observed in the AG cells after *Mr-IAG* silencing suggests that Mr-IAG might convey a feedback inhibition via an autocrine route, similar to insulin (52), or it might induce a sinus-gland-borne AG-inhibiting hor-

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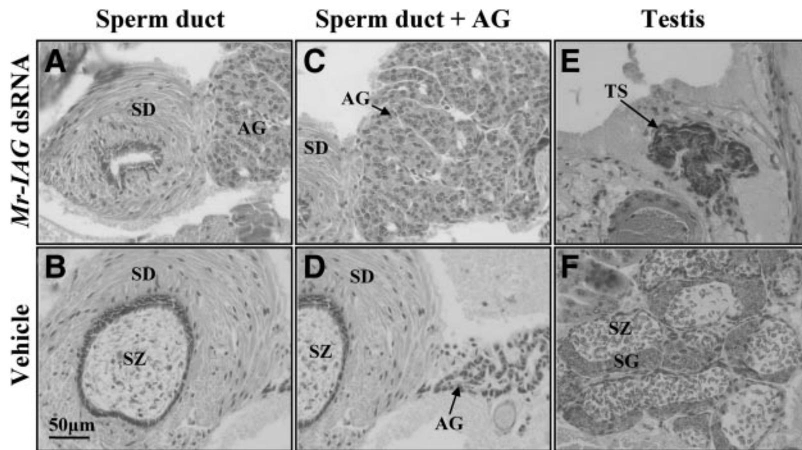

FIG. 7. Representative dorsoventral hematoxylin and eosin-stained sections in *Mr-IAG* silenced and control males. Dorsoventral sections of the fifth walking leg base (A–D) and the cephalothorax (E and F) of representative individuals from the *Mr-IAG*-dsRNA-injected group (A, C, and E) and the vehicle-injected group (B, D, and F). Cross-sections of the terminal ampulla region of the empty sperm duct (SD) shows hyperplasic and hypertrophied AG cells in an individual from the *Mr-IAG*-dsRNA-injected group (A and C), vis-à-vis the spermatozoa-filled sperm duct with normal AG cells in a vehicle-injected individual (B and D). Dorsal sections showing inactive testis lobules (TS) in an individual of the *Mr-IAG*-dsRNA-injected group (E), vis-à-vis the active testis lobules in the vehicle-injected individual (F), containing both spermatogonia (SG) and spermatozoa (SZ).

mone by a long feedback loop, as is the case for ecdysone in the Y-organ and methyl farnesoate in the mandibular organ, whose synthesis is inhibited by sinus gland factors (34). The hAG might be compensatory, resulting in more glandular cells expressing the deficient factor, as reported in endocrine disorders such as congenital adrenal hyperplasia (53), multiple endocrine neoplasia (54), and goiter (55). In goiter syndrome, for instance, thyroxine deficiency results in elevated levels of TSH, which stimulates thyroid enlargement (55).

We hypothesize that a complete functional sex reversal from males to neo-females might be achievable by manipulating *Mr-IAG* at earlier stages, thereby bearing applied potential for the establishment of all-male populations (56) and improving economical performances of prawn farms (12). However, the importance of this study lies not only it its tremendous applied merit but also in the demonstration, for the first time, of a gender-specific insulin-like gene expressed in an endocrine gland (the AG) and involved in a variety of male-related phenomena, such as spermatogenesis, maintenance of secondary sex characters, and sex-specific growth patterns. All the evidence presented here suggests that *Mr-IAG* encodes a pivotal decapod crustacean AG hormone, whose mechanism of action should be further elucidated. In the long term, a "gain-of-function" type of experiment will be required for the final proof of an AG hormone.

Acknowledgments

We thank Mr. Oren Levy and Mr. Ofek Landwirt for prawn maintenance, Dr. Isam Khalaila for sharing his surgical skills, Dr. Ofer Ovadia for advising with statistical analyses, Dr. Shmuel Parnes for his valuable suggestions while reading the manuscript, and Ms. Inez Mureinik for styling the manuscript.

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This study was supported in part by a grant from the European Community (FP6-2003-NEST-A/STREP 4800-PHARMAPOX).

Disclosure Statement: T.V. and A.S. are inventors on a provisional patent (Israel). R.M., E.D.A., S.W., S.R., and L.G. have nothing to declare.

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