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Organization and repression by juvenile hormone of a vitellogenin gene cluster in the crustacean, *Daphnia magna* ☆,☆☆

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Abstract

Two *Daphnia magna* vitellogenin (VTG) genes in neighboring but opposite orientations were identified. One was the gene for DmagVTG1, a previously characterized VTG polypeptide with a superoxide dismutase (SOD)-like domain at its NH_2-terminus [Kato et al., Gene 334 (2004) 157–165]. Both genes had a 17-exon and 16-intron structure in the same configuration. DmagVTG2, a polypeptide encoded by the other gene, also had a SOD-like domain at its NH_2-terminus. The amino acid sequences of the two VTG domains were highly homologous (95.5% identity), while those of the SOD-like domains were less homologous (62.4% identity). The VTG domains are phylogenetically related to insect VTGs while the SOD-like domains are related to viral and bacterial SODs. The intergenic region of 2.6 kb between the two genes contains sequences resembling known juvenile hormone (JH)-responsive and ecdysone-responsive elements. JH agonists, pyriproxyfen and fenoxycarb, strongly repressed the expression of VTG genes in neonate daphnids.

Keywords: Vitellogenesis; Reproduction; Yolk protein; Pyriproxyfen; Water flea; Endocrine disrupters

Vitellogenin (VTG), a major lipoprotein in many oviparous animals, is a precursor of major yolk protein vitellin (VTN). Secretion of VTG has been shown to occur in the liver in vertebrates, the intestine in nematodes, and the fat body in insects, and then taken up by developing oocytes. During these processes, VTG and VTN are modified through cleavage, glycosylation, lipidation, and phosphorylation (reviewed in [1,2]). Both serve as storage proteins providing amino acids, carbohydrates, lipids, and phosphates to the developing embryo [3]. They also serve as trace mineral-transporting proteins [4]. Accumulation of VTG or VTN in oocytes, a process known as vitellogenesis, is one of the key events in the process of ovarian maturation, and is usually under the control of several hormones and has been extensively studied as a model system for the hormonal control of genes at the molecular level. Its regulatory mechanisms have been well defined in oviparous vertebrates and insects [5]. In crustaceans, much less is known about its regulation and associated reproductive function, although many species are important targets of fishery and aquaculture development.

Several anthropogenic chemicals not specifically designed to have hormonal activity, such as some pesticides

☆ The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank Data Bank under Accession Nos. AB252737 and AB252738.

☆☆ *Abbreviations:* VTG, vitellogenin; VTN, vitellin; cDNA, DNA complementary to RNA; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SOD, superoxide dismutase; ORF, open reading frame; UTR, untranslated region(s); bp, base pair(s); kb, kilobase pair(s); aa, amino acid(s); kD, kilodalton; LLT, large lipid transfer; JH, juvenile hormone; JHA, JH agonist; JHR, JH receptor; 20E, 20-hydroxyecdysone; EcR, ecdysone receptor; JHRE, JH responsive element; EcRE, ecdysone-responsive element.

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and plasticizers, exhibit estrogen-like activities in vertebrates. Abnormal production of VTG by male fish after exposure to estrogenic xenobiotics is well documented [5]. Elevation of VTG in male medaka causes significant reproductive impairment [6]. Thus, VTG and VTN also serve as important biomarkers for study of potential endocrine disrupters. In arthropods, including insects and crustaceans, ecdysteroid molting hormone and sesquiterpenoid juvenile hormone (JH) play crucial roles in endocrine regulation. Further, 20-hydroxyecdysone (20E), the major biologically active ecdysteroid, binds to the ecdysteroid receptor (EcR) forming a heterodimer with ultraspiracle (USP), another member of the nuclear hormone receptor superfamily [7]. Since the early 1970s, various kinds of JH agonists (JHAs) have been developed and used as insecticides. Pyriproxyfen and fenoxycarb are highly active compounds used for the control of Dipteran and Lepidopteran pests [8]. Although JHAs generally have low acute toxicity to fish, birds, and mammals [9], these chemicals have toxicity not only to non-target insects [8–10] but also to crustacean species [11–13]. Because cladoceran crustaceans (water fleas) such as *Daphnia* are important members of freshwater ecosystem food webs and because they are the oldest and most widely used test organisms in aquatic toxicology, whether or not hormonally active xenobiotics, such as JHA, have adverse effects upon their endocrine processes is of particular interest.

We previously isolated *Daphnia magna* cDNA encoding a vitellogenin chain, designated DmagVTG1, which is a polypeptide containing an NH_2-terminal domain resembling Cu/Zn-type superoxide dismutase (Cu/ZnSOD), which contains Cu(II) and Zn(II) in its active site. In the present work, we analyzed the genomic sequence of the region around *DmagVTG1* and found another VTG gene (*DmagVTG2*). Organization and expression of the two VTG genes were investigated in conjunction with the actions of JH and JHA.

Materials and methods

Materials. Daphnia magna Straus was obtained from the National Institute for Environmental Studies of Japan. JH and JHAs; pyriproxyfen, fenoxycarb, and methoprene purchased from Wako Pure Chemicals (Osaka, Japan) and methyl farnesoate from Echelon Biosciences, Inc. (Salt Lake City, Utah, USA), were solubilized in DMSO before use. Exposure of daphnids to JH or JHA was conducted at 20 °C under a 16 h:8 h L:D photoperiod in 400 ml of M4 media [14] in 500-ml Erlenmeyer flasks. Final concentration of DMSO was adjusted to 0.01% (w/v).

Isolation of DmagVTG1 and DmagVTG2 genomic DNA, and DmagVTG2 cDNA. The 3′-region (338 bp: from nucleotides 5968 to 6305) of *DmagVTG1* cDNA [15] was amplified by polymerase chain reaction (PCR) and used as a probe for screening of the *D. magna* gDNA library constructed previously [16]. A DIG DNA Labeling Kit and a DIG Nucleic Acid Detection Kit (Roche Diagnostics Japan, Tokyo) were used for probe labeling and detection, respectively. Among the ten positive clones identified, two were shown to contain two VTG genes (*DmagVTG1* and *DmagVTG2*). A cDNA fragment with a deduced sequence of 698 bp encoding the SOD-like domain of DmagVTG2 was amplified by PCR from a *D. magna* cDNA library [17] and used to screen full-length cDNA according to the method described previously [15]. One positive clone, λ-c*DmagVtg2*, was isolated and subsequently analyzed. Applied Biosystems *Taq* DyeDeoxy Terminator Sequencing Kit and an ABI 377 automated sequencer were used for DNA sequencing. A few discrepancies that were probably due to polymorphism (six single-nucleotide substitutions in the case of *DmagVTG1* and five in the case of *DmagVTG2*) were found between the nucleotide sequences of the cloned cDNAs and those deduced from the corresponding genomic sequences.

Preparation of total RNA and protein fractions. Daphnids were homogenized in ISOGEN (Nippongene, Japan) and total RNA and protein fractions were prepared according to manufacturer's instructions. Eggs were removed from the brood chamber before homogenization.

Quantitative real-time PCR. Total RNA fractions were treated with RQ1 RNase-free DNase I (Promega Japan, Tokyo). mRNA was converted to cDNA using SuperScript III Reverse Transcriptase (Invitrogen Japan, Tokyo) with the aid of an Oligo $(dT)_{18}$ primer. The obtained cDNA was treated with RNase H (Invitrogen) and then used as templates for PCR.

Quantitative real time PCR was performed using the Quantitect SYBR Green PCR Master Mix (QIAGEN Japan, Tokyo) and PCR primers were designed as follows: DmagVTG1 forward primer, 5′-GCTACCCACGT CAAGTAATG-3′; DmagVTG1 reverse primer, 5′-GCTGCCGTAG TCTCAA-CAGAA-3′; DmagVTG2 forward primer, 5′-GCACTCTT TCTCGTTATTGCTG-3′; DmagVTG2 reverse primer, 5′-GATCTTGA CTGGGCTATTGATT-3′; β-actin forward primer, 5′-ACCATGTACC CAGGCATTGCTGA-3′; and β-actin reverse primer, 5′-GACAGG GAAGCCAAGATGGATC-3′. The reaction mixtures were held at 50 °C for 5 s, denatured at 95 °C for 15 min, and then subjected to 45 cycles, each consisting of denaturation at 95 °C for 15 s followed by extension at 60 °C for 1 min using an ABI Prism 7700 sequence detection system (Applied Biosystems) with default parameters. After amplification, single products of the predicted size were confirmed by polyacrylamide gel electrophoresis. The threshold cycle (C_t) used to assess the relative levels of *DmagVTG1* and *DmagVTG2* mRNAs normalized to mRNA level of actin measured in the same experiment.

Results

Organization of D. magna VTG genes

Two genomic clones designated λ-g*DmagVtg1-2′* and λ-g*DmagVtg1′-2* and containing two VTG genes oriented in opposite directions were isolated. Clone λ-g*DmagVtg1-2′* contained the complete genomic sequence corresponding to the previously isolated *DmagVTG1* cDNA encoding a VTG with an SOD-like domain at its NH_2-terminus and the 5′-terminal portion of another gene, designated*DmagVTG2*, which encodes another VTG also having an SOD-like domain at its NH_2-terminus. Clone λ-g*DmagVtg1′-2* contained the complete *DmagVTG2* gene and the 5′-terminal portion of the *DmagVTG1* gene. Combining the genomic sequences of these two clones, a nucleotide sequence of 19,313 bp containing the complete *DmagVTG1* and *DmagVTG2* genes was determined. With the aid of the obtained sequence information, a clone carrying *DmagVTG2* cDNA was isolated as described in 'Isolation of DmagVTG1 and DmagVTG2 genomic DNA, and DmagVTG2 cDNA.' The nucleotide sequence of *DmagVTG2* cDNA comprised 6229 bp consisting of a 43-bp 5′-untranslated region (UTR), a 6009-bp ORF starting at the putative first Met codon, ATG, and ending with a stop codon, TAA, a 138-bp 3′-UTR, and a 39-bp poly-A tail. A possible poly-A signal sequence,

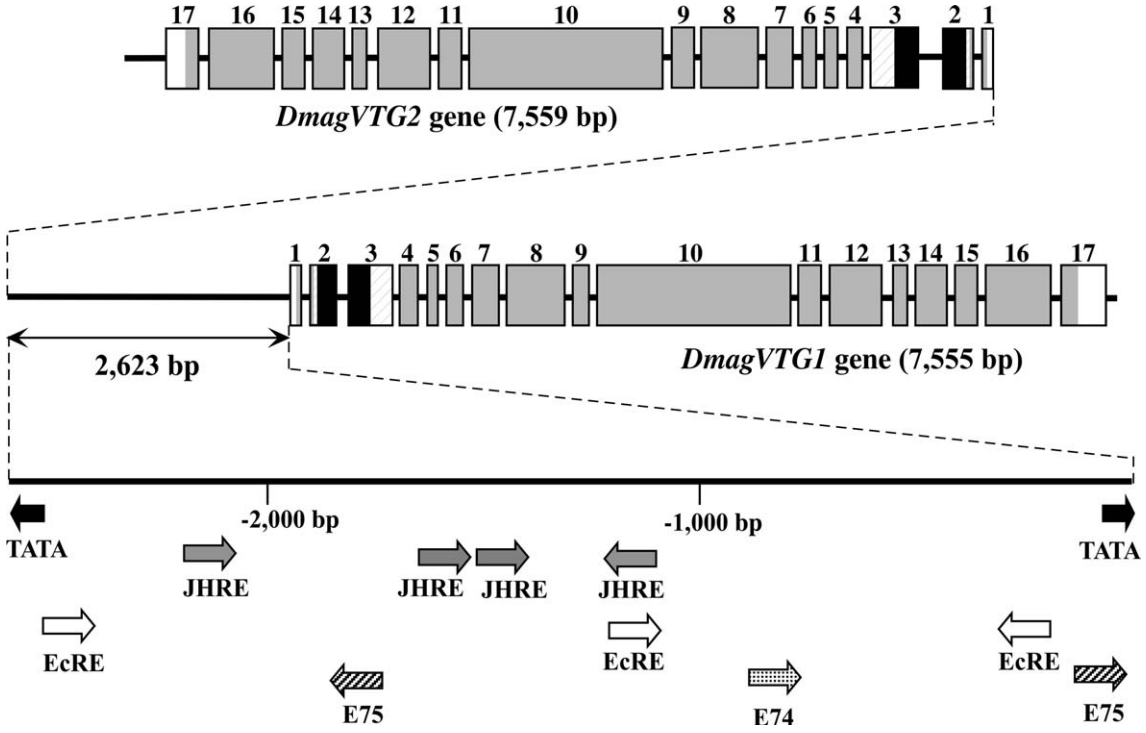

Fig. 1. Map of the *DmagVTG1 and DmagVTG2* genes. Upper: boxes numbered 1 to 17, represent Exons 1 to 17. Gray regions of the boxes encode the signal peptide and the VTG domain. Black regions encode the SOD-like domain. Hatched regions encode sequences linking the above two regions. Open regions represent untranslated regions. Black lines between the boxes indicate introns and an intergenic region. Lower: possible binding sites for transcription factors located in the intergenic region. TATA, TATA box sequence; JHRE, juvenile hormone-responsive element; EcRE, ecdysteroid-responsive element; E74 and E75, binding sites for transcriptional factors, E74 and E75, respectively. Directions of arrows indicate 5′ to 3′ orientation of the DNA strand on which the binding site sequences are located. Exact positions of these and GATA binding sites are shown in Table 1.

AATAAA, was located 21 bp upstream from the poly-A sequence. The deduced aa sequence of DmagVTG2, the product of the *DmagVTG2* gene, showed 91.5% identity as a whole with that of DmagVTG1. Both *DmagVTG1* and *DmagVTG2* consisted of 17 exons and 16 introns in the same configuration (Fig. 1). Introns and exons in each gene were named Intron 1 to Intron 16 and Exon 1 to Exon 17, respectively, with numbering starting from the upstream. Positions of the introns and exons are exactly conserved between the two genes. Introns were found to be very short in length, with an average of 81 bp, which is similar to that observed in *D. magna* hemoglobin genes [16]. The sequence encoding the VTG domain was separated by Intron 3 from the sequence encoding the linker peptide between VTG and SOD-like domains.

Features of the region encoding the signal peptide and the SOD-like domain

The genomic sequence for the signal peptide and that for the SOD-like domain each contains one intron, Intron 1 and Intron 2, respectively. In this region, homology

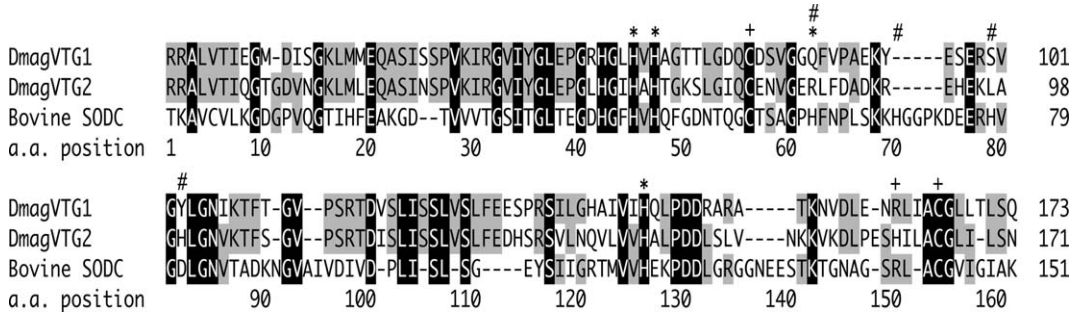

Fig. 2. An alignment of the aa sequences of the SOD-like domains and bovine Cu/ZnSOD. Sequences used in this alignment and their accession numbers in the GenBank/EMBL/DDBJ or SWISS-PROT database are as follows: DmagVTG1 (GenBank Accession No. AB114859), DmagVTG2 (this study), bovine SODC (*Bos taurus* Cu/ZnSOD, GenBank Accession No. M81129). Gaps inserted to optimize the alignment are denoted by dashes. The aa number of the last residue in each sequence is shown to the right. Residues conserved in all of the three sequences are shaded in black. Residues conserved in any two sequences are shaded in gray. The two Cys and one Arg residues, which are important for maintenance of the structure and activity of SOD [19] are denoted by +. The residues involved in the binding of Cu(II) and Zn(II) [18] are denoted by asterisks and sharps, respectively.

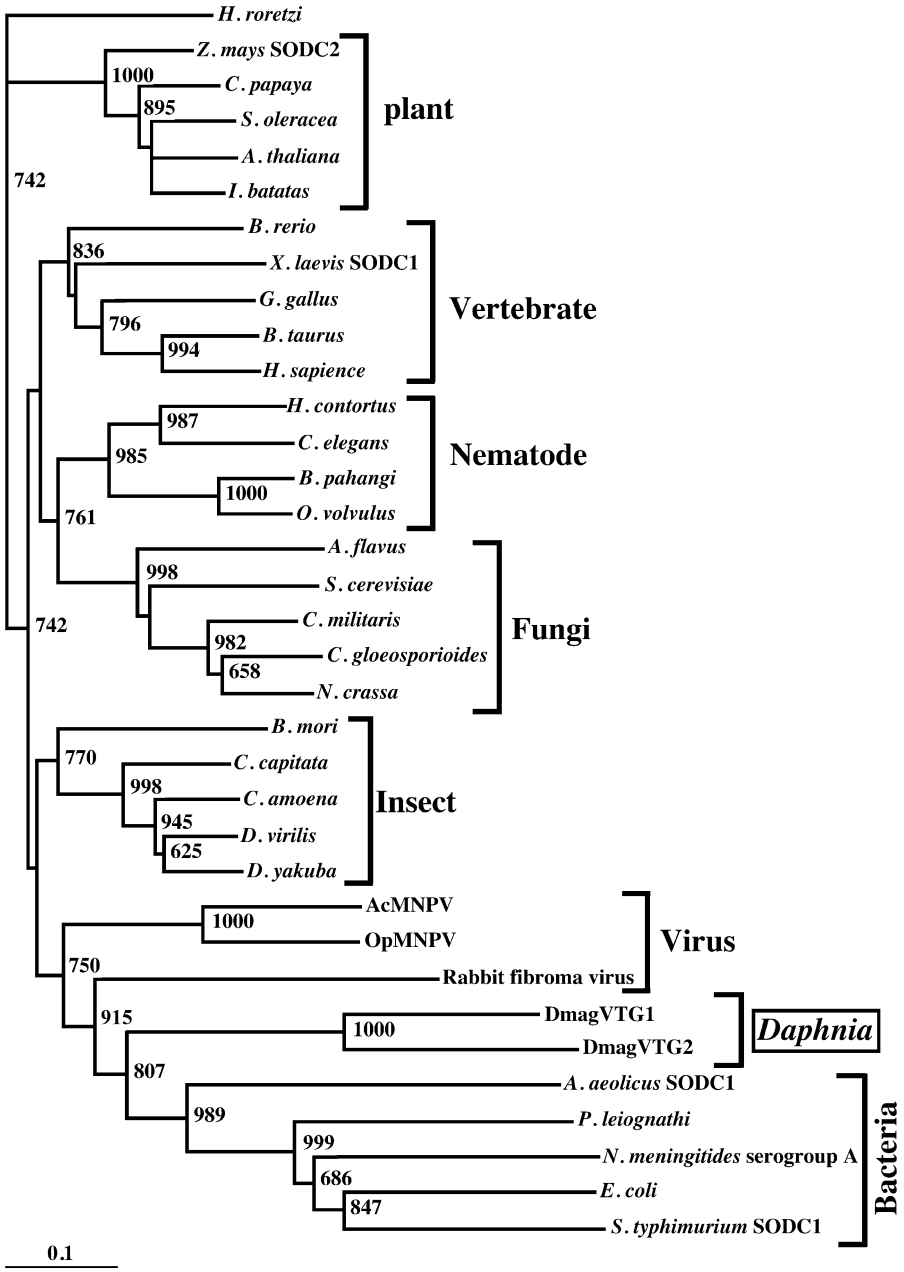

Fig. 3. A phylogenetic tree of the aa sequences of the SOD-like domain of DmagVTGs and various cytosol-type Cu/ZnSOD (SODC). Sequences used in this analysis and their accession numbers in the GenBank/EMBL/DDBJ or SWISS-PROT data bases are from the following species: plants, *Zea mays* SODC2 (maize, SWISS-PROT Accession No. P11428), *Carica papaya* (papaya, SWISS-PROT Accession No. O65768), *Spinacia oleracea* (spinach, SWISS-PROT Accession No. P22233), *Arabidopsis thaliana* (mouse-ear cress, SWISS-PROT Accession No. P24704), *Ipomoea batatas* (sweet potato, SWISS-PROT Accession No. Q07796); vertebrates, *Brachydanio rerio* (zebrafish, SWISS-PROT Accession No. O73872), *Xenopus laevis* (African clawed frog, SWISS-PROT Accession No. P13926), *Gallus gallus* (chicken, SWISS-PROT Accession No. P80566), *Bos taurus* (bovine, GenBank Accession No. M81129), *Homo sapiens* (SWISS-PROT Accession No. P00441); nematodes, *Haemonchus contortus* (barber pole worm, SWISS-PROT Accession No. Q27666), *Caenorhabditis elegans* (SWISS-PROT Accession No. P34697), *Brugia pahangi* (filarial nematode worm, SWISS-PROT Accession No. P41962), *Onchocerca volvulus* (SWISS-PROT Accession No. P24706); fungi, *Aspergillus flavus* (SWISS-PROT Accession No. Q8X1S6), *Saccharomyces cerevisiae* (baker's yeast, SWISS-PROT Accession No. P00445), *Cordyceps militaris* (SWISS-PROT Accession No. Q8J0N2), *Colletotrichum gloeosporioides* (anthracnose fungus, SWISS-PROT Accession No. O94178), *Neurospora crassa* (SWISS-PROT Accession No. P07509); insects, *Bombyx mori* (silk moth, SWISS-PROT Accession No. P82205), *Ceratitis capitata* (Mediterranean fruit fly, SWISS-PROT Accession No. P28755), *Chymomyza amoena* (SWISS-PROT Accession No. Q07182), *Drosophila virilis* (fruit fly, SWISS-PROT Accession No. P10791), *Drosophila yakuba* (fruit fly, SWISS-PROT Accession No. Q9U4X3); virus, Autographa californica nuclear polyhedrosis virus (AcMNPV, SWISS-PROT Accession No. P24705), Orgyia pseudotsugata multicapsid polyhedrosis virus (OpMNPV, SWISS-PROT Accession No. O12933), rabbit fibroma virus (GenBank Accession No. AF170722); bacteria, *Aquifex aeolicus* (SWISS-PROT Accession No. O67149), *Photobacterium leiognathi* (SWISS-PROT Accession No. P00446), *Neisseria meningitides* serogroup A (SWISS-PROT Accession No. P57005), *Escherichia coli* (SWISS-PROT Accession No. P53635), *Salmonella typhimurium* SODC1 (SWISS-PROT Accession No. P53636), and *Halocynthia roretzi* (Sea squirt, SWISS-PROT Accession No. P81926). *H. roretzi* SOD-C sequence was used as an outgroup sequence. The tree was constructed using the CLUSTAL X37 program [39] using default parameters. Bootstrap values for 1000 replicate analysis are shown at branching points. The bar at the bottom shows the branch length corresponding to the mean number of differences (0.1) per residue along each branch.

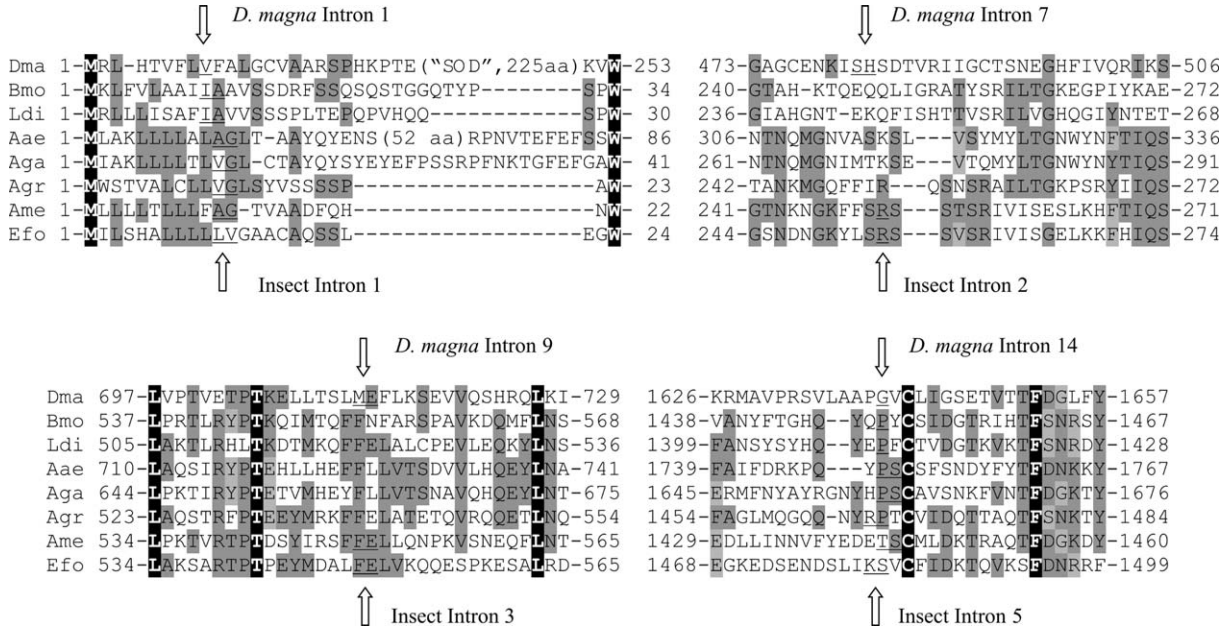

Fig. 4. Intron positions shared by VTG genes of insects and *D. magna*. aa sequences around the conserved intron positions in insect and *Daphnia* VTG genes are shown. Numerals of the first and the last residues of the sequences indicate the positions relative to the initiator methionine of each protein. Arrows and underlined aa residues denote the exact positions of introns. In instances where two residues are separated by an intron, both residues are underlined. Abbreviations and accession numbers are as follows: Dma, *D. magna* (this study); Bmo, *Bombyx mori* (moth, GenBank Accession No. D30733); Ldi, *Lymantria dispar* (moth, GenBank Accession No. U90756); Aae, *Aedes aegypti* (mosquito, GenBank Accession No. U02548); Aga, *Anopheles gambiae* (mosquito, GenBank Accession No. AF281078); Agr, *Anthonomous grandis* (boll weevil, GenBank Accession No. M72980); Ame, *Apis mellifera* (honeybee, GenBank Accession No. AJ517411); Efo, *Encarsia formosa* (parasitoid wasp, GenBank Accession No. AY553878). Residues conserved in all sequences are shaded in black. Residues conserved in more than three sequences are shaded in gray.

between the nucleotide sequences of the two VTG genes was relatively low (63.3% identity). Identity between the aa sequences of the signal peptides was 56.3% and that of the SOD-like domains was 62.4%. An alignment of the aa sequences of the two SOD-like domains with that of bovine Cu/ZnSOD is shown in Fig. 2. The sequences of the SOD-like domains of DmagVTG1 and DmagVTG2 showed 30.9% and 29.0% identities, respectively, with bovine Cu/ZnSOD. As in DmagVTG1, all Zn-binding residues (marked by sharps in Fig. 2, [18]) were substituted or deleted in the SOD-like domain of DmagVTG2. In addition, an Arg residue at the 150th position known to be important for Cu/ZnSOD activity [19] was substituted by His in DmagVTG2.

A phylogenetic tree constructed from the aa sequences of the SOD-like domains of DmagVTGs and known Cu/ZnSODs is shown in Fig. 3. Known Cu/ZnSODs grouped into 7 separate clusters: plant, vertebrate, nematode, fungi, insect, virus, and bacteria. The *D. magna* SOD-like domains were most closely related to the virus and bacteria clusters.

Features of the sequences encoding the VTG domain

In contrast to the genomic sequences for the signal peptide and the SOD-like domain, those for VTG domains were highly conserved between the two genes. Identity of nucleotide sequences (6240 bp in *DmagVTG1* and 6237 bp in *DmagVTG2*) was 93.1%. Even introns of these genes showed high homology in this region. Among the 13 introns contained in this region, 6 introns (Intron 4 to Intron 7, Intron 12, and Intron 13) showed sequence identities higher than 90%. The lengths of the exons contained in this region were exactly conserved between the two genes. An extremely long exon (Exon 10), of 1767 bp, was located in the midst of this region. Identity of the aa sequences of the two VTG domains was 95.5%. Phylogenetic analysis of the aa sequence of the VTG domain of DmagVTG2 gave the same result as that of DmagVTG1 [15]; that is, it grouped with the cluster of insect VTGs rather than to the decapodan crustacean VTGs (data not shown). All insect VTG genes characterized to date contain six introns except for those of mosquitoes, which contain only two positions of the 1st and 5th introns, are conserved in all of insect VTG genes [20]. The positions of these two introns are also conserved in *D. magna* VTG genes (Intron 1 and Intron14, Fig. 4). In addition, the 2nd and 3rd intron positions conserved in two insects (*Apis mellifera* and *Encarsia formosa*) are also conserved in *D. magna* VTG genes (Intron 7 and Intron 9). The decapodan crustacean VTG gene reported to date [21] shares only one intron with *D. magna* VTG genes (Intron 6).

Features of the intergenic region

Assuming that the 5′ terminus of the isolated cDNA coincides with the transcription start site, a non-transcribed region of 2623 bp was located between the

Table 1
Possible *cis*-regulatory elements located in the intergenic region between the *DmagVTG1* and *DmagVTG2* genes

Binding sites in other organisms: **consensus sequence or its complementary sequence**[a]	Homologous sequences in the *D. magna* intergenetic region	Position[b]
JHRE, budworm JHE gene: **RGRNYA**[N]$_4$**RGRNYA or TRNYCY**[N]$_4$ **TRNYCY**	AGA **AGGTTA** TAAG **GGAATA** TTG	−2199
	AAA **AGACAA** ACTCAT **AGAAAA** ATT	−1658
	AGT **AGACTA** GATCTT **AGGTTA** ATA	−1487
	CAA **TAATCT** AAAGCCA **TTATCT** GAC	−1176
EcRE, fly 20E-responsive gene: **RGKTCANTGAMCY**	GTG **AGGTCA** TCTAAATCTTATCACTTA **TGAACA** GCT	−2456
	ATC **AGTTCA** ATAATCTAAAGCCATTATC **TGACTT** TCT	−1171
	ACC **AAGTCA** TTTTGTC **TGACCT** GGC	−260
E75 binding site, mosquito VTG gene: **ATATGGGGCA or TGCCCCATATT**	CTA **CGCCCCATAAT** ATT	−1751
	GAA **AATATAGCGCA** GAG	−141
E74 binding site, mosquito VTG gene: **CATCAGGAAGC or GCTTCCTGATG**	TTT **CAGCAGGAAAA** TAA	−826
GATA binding site: **HGATAR** or **YTATCD**	ATC **TTATCA** CTT	−2466
	TTG **TTATCA** TAT	−2421
	TCT **TGATAA** AAT	−2387
	GCT **TTATCA** AAT	−2234
	TGC **CGATAG** CGG	−2131
	TTG **AGATAG** GCT	−1341
	CCA **TTATCT** GAC	−1176
	TTG **TGATAA** ACA	−1066
	GAT **TTATCT** ACA	−1027
	TTC **TTATCA** CTT	−729

[a] R = A or G, Y = C or T, K = G or T, M = A or C, H = A, C, or T, D = A, G, or T, N = A, C, G, or T.
[b] The positions of the first nucleotide of the motifs relevant to the first nucleotide of Exon 1 of the *DmagVTG*1 gene are shown. The core sequence of each response element is shown in bold.

DmagVTG1 and *DmagVTG2* genes. A typical TATA box sequence, 5′-TATAWAW-3′ (W = A or T; [22,23]) was found in the 5′ flanking region of each gene. The distance between the TATAWAW motif and the presumed transcription start site was 52 and 37 bp in *DmagVTG1* and *DmagVTG2*, respectively. The transcriptional silencing and/or activation of VTG genes in various organisms are regulated by *cis*-regulatory elements located in the 5′-flanking regions. Table 1 and Fig. 1 summarize the possible *cis*-regulatory elements found in the 2623 bp intergenic region. Interestingly, four sequences homologous with the JH-responsive element (JHRE) involved in transcriptional regulation of the budworm JH esterase gene [24] were found. Sequences homologous with the ecdysteroid-responsive element (EcRE) identified in fruit fly *Drosophila* [25] and those homologous with the binding sites of transcriptional factors E74, E75, and GATA responsible for ecdysteroid-dependent regulation of mosquito VTG genes [26] were also found in this region.

Pyriproxyfen represses expression of the DmagVTG genes

Because the intergenetic region of the *D. magna* VTG genes contains JHRE-like sequences as described above, effects of pyriproxyfen, a JHA, on expression of the *DmagVTG1* gene were examined. While control group daphnid neonates ovulated once during the 216 h test period, those exposed to 15.6 nM of pyriproxyfen did not and the production of male offsprings was induced as described by Tatarazako et al. [27]. Relative amounts of *DmagVTG1* mRNA and DmagVTG polypeptide produced during the test period are shown in Figs. 5 and 6, respectively. In the control group, the amount of *DmagVTG1* mRNA (equal to 1 at time 0) began to increase at 72 h and reached 25,000 (Fig. 5, control). In the test group, the amount of *DmagVTG1* mRNA began to increase at 96 h and increased gradually up to 2000 (Fig. 5, pyriproxyfen). Thus, pyriproxyfen drastically inhibited accumulation of *DmagVTG1* mRNA. At a concentration of 0.2 nM, 50% inhibition was achieved at 120 h (data not shown). Accumulation of *DmagVTG2* mRNA was also significantly inhibited by exposure to 15.6 nM of pyriproxyfen (Table 2).

The increase of DmagVTG polypeptides was delayed compared with that of mRNA in both the control and test groups. DmagVTG polypeptides were first detected at 120 h and gradually increased in the control group. In the test group, DmagVTG1 polypeptides were first detected at 168 h and in much smaller amounts than in the control group (Fig. 6).

Repression of expression of the DmagVTG1 gene by various JHAs

The effects of various JH-related compounds on *DmagVTG* mRNA levels after 120 h of exposure are summarized in Table 2. Fenoxycarb showed almost the same

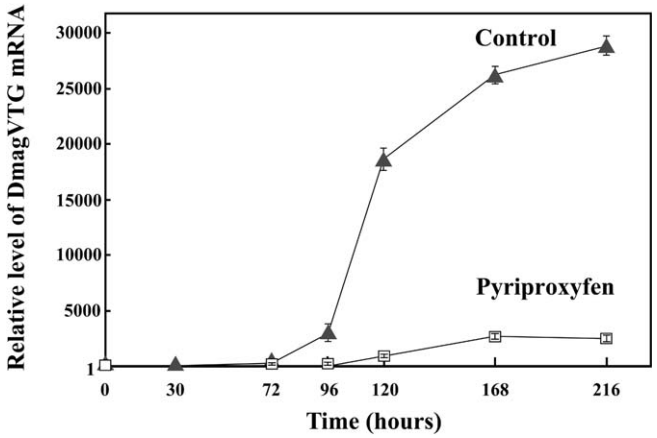

Fig. 5. Effect of pyriproxyfen on accumulation of *DmagVTG1* mRNA during the growth of *D. magna* neonates. *Daphnia* mothers were inspected individually and ones with neonates in the brood chamber were isolated and cultured in separate flasks. More than forty daphnid neonates born from a single mother were collected within 1 h after release from the brood chamber and divided into two groups (time 0). One group (test group) was cultured in the presence of pyriproxyfen at 15.6 nM, and the other group (control group) was cultured in the absence of pyriproxyfen. Food (*Selenastrum capricornutum*) was added to each flask at 1×10^5 cells/ml daily until 72 h, and twice daily thereafter. Daphnids that were of extremely large or small size in each group were eliminated from the flask. Four daphnids (0–72 h) or two daphnids (96–216 h) were taken from the culture and total RNA fractions were prepared. The relative amount of *DmagVTG1* mRNA quantified by real-time PCR and normalized by that of actin mRNA in each preparation is shown. The relative amount of *DmagVTG1* mRNA at time 0 was defined as 1. The mean value ± standard deviation (bar) of three measurements is shown.

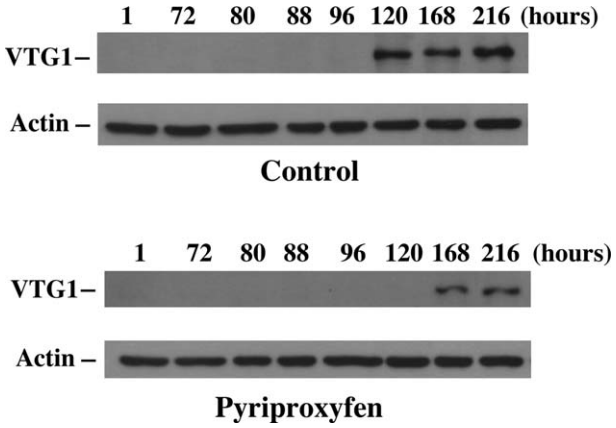

Fig. 6. Effect of pyriproxyfen on accumulation of DmagVTG polypeptide during the growth of *D. magna* neonates. Total protein fractions were prepared from the same daphnid samples used for preparation of total RNA fractions described in the legend to Fig. 5. SDS–PAGE and Western blotting analysis were performed as described previously [15]. Total protein fractions containing 3.75 μg protein were loaded in each well of the gel.

inhibitory activity as pyriproxyfen (99% inhibition at 15.6 nM). Activity of methoprene, another JH agonist, was very low. Crustacean JH, methyl farnesoate, was slightly less active than pyriproxyfen and fenoxycarb (85% inhibition at 15.6 nM).

Table 2
Inhibition of accumulation of VTG mRNAs by exposure to various JH and JHAs

Chemicals [concentration (nM)]	mRNA[a]	% Inhibition[b]
Pyriproxyfen (15.6)	DmagVTG1	96.1 ± 0.4
Pyriproxyfen (15.6)	DmagVTG2	99.4 ± 0.1
Fenoxycarb (15.6)	DmagVTG1	99.0 ± 0.1
Methoprene (15.6)	DmagVTG1	20.2 ± 3.6
Methoprene (156.0)	DmagVTG1	43.2 ± 5.7
Methyl farnesoate (15.6)	DmagVTG1	85.2 ± 0.1
Methyl farnesoate (156.0)	DmagVTG1	99.2 ± 0.1

[a] Relative amounts of VTG mRNA were determined by real-time PCR.
[b] Percentage of inhibition at 120 h as compared to unexposed control, the mean value ± standard deviation of three measurements, is shown.

Discussion

Large lipid transfer proteins (LLTPs), including VTGs and apolipoproteins, are considered to have diverged from a single ancestral molecule. Some of the proposed ancestral exon boundaries in LLTP genes are conserved in VTG genes, although their positions and numbers are irregular [28]. Here, we found that *D. magna* VTG genes and insect VTG genes share 4 exon boundaries. This finding further supports our conclusion that the VTG domains of *D. magna* VTGs are phylogenetically more closely related to insect VTGs than to decapodan crustacean VTGs. In contrast, the phylogenetic analysis of the SOD-like domain in this study showed its close relationship to viral and bacterial Cu/ZnSODs. A viral or bacterial SOD gene may have been transferred horizontally and integrated into an ancestral *D. magna* VTG gene. Homology between the amino acid sequences of the two SOD-like domains is appreciably lower than that between the VTG domains, suggesting that the structural constraint on the SOD-like domains is relatively weak. The SOD-like domain in *Daphnia* may not serve as an SOD but may play a role only in binding and transportation of copper during the embryogenesis, leading to a weakened structural constraint.

In our previous work, we concluded that DmagVTG1 was the most abundant polypeptide in *D. magna* parthenogenetic eggs based on Western blotting analysis using antisera raised against DmagVTG1. Because DmagVTG2 cannot be separated from DmagVTG1 on SDS–PAGE and because of cross-reactivity of the antisera with DmagVTG2, the abundance of each peptide is not clear at present.

The effects of JHA on vitellogenesis in crustaceans vary greatly among species. For example, methoprene inhibited vitellogenesis in the xanthid crab, *Rhithropanopeus harrisii*, but seemed to promote it in the spider crab, *Libinia emarginata* [29,30]. The 5′-flanking regions of crustacean VTG genes have not been analyzed to date. The present results are the first to reveal drastic repression of VTG expression by JH and JHA in cladocerans. Furthermore, four sequences homologous to known JHRE were found in the intergenic region, which may play a role in transcriptional repression

of *D. magna* VTG genes. No sequence homologous with the juvenoid response element playing a role in activation of *Daphnia* hemoglobin gene proposed by Gorr et al. [31] was found in the intergenic region.

Henrich et al. [32] have demonstrated that JH III potentiates EcR-dependent transcription in mammalian cell culture system and that the ligand-binding domain of EcR is a prerequisite component for potentiation by JH III. Maki et al. [33] have suggested that JHs serve as USP ligands that antagonize EcR-mediated ecdysone activity through the recruitment of histone deacetylase complexes in vitro. The presence of sequences homologous to the known EcRE, binding sites for E74, E75, and those for GATA factors suggests that ecdysteroid activates transcription of *D. magna* VTG genes, as in other organisms [26,34] and that the activation is antagonized by JHAs. However, experiments to examine the effect of 20E at environmentally relevant concentrations on the expression of *DmagVTG1* did not produce a clear result.

The effects of JHAs on *Daphnia* include suppression of growth and reduced reproduction [35–37]. Moreover, production of male offspring in parthenogenetically reproducing *D. magna* has also been reported to be stimulated [27,38]. Thus, JHA used as insecticides may cause a disturbance in the basic reproductive strategy of cladocerans in nature, which allows rapid population expansion by parthenogenesis during periods of abundant resources. Removal of cladocerans may cause serious disturbances in freshwater ecosystem food webs. Nevertheless, a biomarker useful for monitoring the effects of JHA on cladoceran reproduction has yet to be developed. The test proposed by OECD requires 21 days to evaluate the effects of chemicals on *D. magna* reproduction [14]. Utilization of *D. magna* VTG gene products as biomarkers may be significant for evaluating the effects of endocrine disrupting compounds (EDC) such as JHA of nanomolar levels on reproduction of cladoceran crustaceans as effects can be detected within 72 to 96 h, allowing the development of a much more rapid detection system.

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