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Full-length sequence, regulation and developmental studies of a second vitellogenin gene from the American dog tick, *Dermacentor variabilis*

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ABSTRACT

Vitellogenin (Vg) is the precursor of vitellin (Vn) which is the major yolk protein in eggs. In a previous report, we isolated and characterized the first Vg message from the American dog tick *Dermacentor variabilis*. In the current study, we describe a second Vg gene from the same tick. The Vg2 cDNA is 5956 nucleotides with a 5775 nt open reading frame coding for 1925 amino acids. The conceptual amino acid translation contains a 16-residues putative signal peptide, N-terminal lipid binding domain and C-terminal von Willebrand factor type D domain present in all known Vgs. Moreover, the amino acid sequence shows a typical GLCG domain and several RXXR cleavage sites present in most isolated Vgs. Tryptic digest-mass fingerprinting of Vg and Vn recognized 11 fragments that exist in the amino acid translation of DvVg2 cDNA. Injection of virgin females with 20 hydroxyecdysone induced DvVg2 expression, vitellogenesis and oviposition. Using RT-PCR, DvVg2 expression was detected only in tick females after mating and feeding to repletion. Northern blot analysis showed that DvVg2 is expressed in fat body and gut cells of vitellogenic females but not in the ovary. DvVg2 expression was not detected in adult fed or unfed males. The characteristics that distinguish Vg from other similar tick storage proteins like the carrier protein, CP (another hemelipoglycoprotein) are discussed.

1. Introduction

Oviparous animals secure resources for the completion of embryo development by depositing large amounts of nutrients into their eggs. Such resources include proteins, lipids, carbohydrates, minerals and other compounds. The major protein is vitellin (Vn). Vitellogenin (Vg), the precursor of Vn, is usually synthesized extra-ovarially, secreted into the hemolymph and taken up by the ovary to be incorporated into growing oocytes via receptor mediated endocytosis. In insects, the vitellogenesis process is well studied. Vg is synthesized in the fat body cells under the regulation of juvenile hormone (JH) and/or ecdysteroids (reviewed by Gilbert et al., 2000; Roe et al., 2008; Tufail and Takeda, 2008). In Crustacea, the mandibular organs produce methyl farnesoate which regulates vitellogenesis and egg production (Laufer and Biggers, 2001; Nagaraju, 2007).

It was originally thought that ticks produced the insect juvenile hormones and like in many insects used JH to regulate vitellogenesis (Pound and Oliver, 1979; Gaber et al., 1983; Khalil et al., 1983). However, Neese et al. (2000) was unable to detect JH in two species, the soft tick *Ornithodorous parkeri* and the hard tick *Dermacentor variabilis*. Current evidence suggests that ecdysteroids and not JH regulate the expression of tick Vg synthesis (Dees et al., 1984; Sankhon et al., 1999; Friesen and Kaufman, 2002, 2004; Seixas et al., 2008; Thompson et al., 2005, 2007; Mitchell et al., 2007; Ogihara et al., 2007). In ticks, the fat body was reported to be the main site of Vg synthesis (Chinzei and Yano, 1985; Diehl et al., 1982; Taylor et al., 1991). Rosell and Coons (1992) suggested both fat body and gut as sources for Vg in the American dog tick, *D. variabilis*. Molecular studies by Thompson et al. (2007) showed that both fat body and gut produced Vg mRNA in *D. variabilis* replete females exclusively. Roe et al. (2008) and Cabrera et al. (2009a) proposed a global model explaining the regulation of reproduction in female Acari (mites and ticks) where ecdysteroids and not JH initiate vitellogenesis.

Several Vg cDNAs have been sequenced and characterized from insects and Crustacea with evidences for the presence of multiple Vg genes in some species (Sappington and Raikhel, 1998; Sappington et al., 2002; Tufail and Takeda, 2008). Much less is known about multiple Vgs in ticks and their tissue source and

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regulation. The first full-length Vg cDNA sequence from a tick was published by Thompson et al. (2007). Donohue et al. (2009) reviewed the different aspects of storage proteins in the Chelicerata including the (common) carrier protein, CP. CP is found in both males and females throughout tick development. Although there have been reports of multiple Vgs in the same tick (Thompson et al., 2007; Boldbaatar et al., 2010), Vg and CP share many common features making their identification problematic.

In this report, we provide a description of a second, full-length Vg in the American dog tick and examine for the first time, the tissue-specific hormonal regulation of multiple Vg messages which includes Vg2 in the same tick species. Our analysis also is explicit in distinguishing Vg from CP.

2. Materials and methods

2.1. Ticks

A pathogen-free line of the American dog tick, *D. variabilis*, was reared as described previously (Sonenshine, 1993). Adult ticks were confined within plastic capsules attached to New Zealand white rabbits (*Oryctolagus cuniculus*) and allowed to feed and mate as required. Rearing conditions were 26 ± 1 °C, 92 ± 6% relative humidity and 14:10 (L:D). All use of animals was conducted at Old Dominion University and approved by the Institutional Animal Care and Use Committee.

2.2. cDNA synthesis and 454 library preparation

The initial Vg2 cDNA fragments were obtained from a cDNA library made from the fat body of replete females as previously described by Thompson et al. (2005). However, the 5′ portion of the gene was obtained unintentionally from a transcriptome sequence made for the synganglia of mixed stage females of the American dog tick. It is not possible to eliminate all trachea and the associated fat body from synganglia dissections. In preparation for sequencing, approximately 50 synganglia including lateral secretory organs and pedal nerves were dissected from female *D. variabilis* that were either unfed, partially fed virgin (attached to the host for 4–5 d), partially fed virgin forcibly detached from the rabbit host and held in culture for 4–5 d, partially fed mated (allowed to mate for ≤1 d), and replete (1 d post-drop off from the host). Tissues from each feeding stage were separately homogenized in TRI Reagent (Sigma–Aldrich, St. Louis, MO), and total RNA was purified according to the manufacturer's recommendations. RNA pellets were rehydrated in 100 μM ATA to prevent degradation (Hallick et al., 1977). Approximately 3 μg of total RNA from each group were pooled, and mRNA was isolated using an Oligotex mRNA isolation kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. Purified mRNA was ethanol precipitated and rehydrated in 2 μl and combined with 10 pmol of modified 3′ reverse transcription primer (5′-ATTCTAGAGACCGAGGCGGCCGACATGT(4)GT(9)CT(10)VN-3′) (Beldade et al., 2006) and 10 pmol SMART IV oligo (5′-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3′) (Zhu et al., 2001). The resulting 4 μl were incubated at 72 °C for 2 min and then combined with the following reagents on ice: 1 μl RNase Out (40 U/μl), 2 μl 5× first strand buffer, 1 μl 20 mM DTT, 1 μl dNTP mix (10 mM each) and 1 μl Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). The reaction was incubated at 42 °C for 90 min then diluted to 30 μl with TE buffer (10 mM Tris HCl pH 7.5, 1 mM EDTA) and stored at −20 °C until further use. To synthesize second strand cDNA, 5 μl of first-strand cDNA was mixed with 10 pmol of modified 3′ PCR primer (5′-ATTCTAGAGGCCGAGGCGGCCGACATGT(4)GTCT(4)GTTCTGT(3)CT(4)VN-3′) (Beldade et al., 2006), 10 pmol of 5′ PCR primer (5′-AAGCAGTGGTATCAACGCAGAGT-3′) (Zhu et al., 2001), 5 μl 10× reaction buffer, 1 μl dNTP mix, 2 μl $MgSO_4$, 0.4 μl Platinum HiFi Taq Polymerase and 34.6 μl H_2O (Invitrogen). Thermal cycling conditions were 94 °C for 2 min followed by 25 cycles of 94 °C for 20 s, 65 °C for 20 s and 68 °C for 6 min. The first PCR reaction was conducted, and 5 μl aliquots from cycles 18, 22 and 25 were analyzed on a 1% agarose gel to optimize the number of cycles. Additional reactions were conducted using the optimized number of cycles to produce sufficient quantities of cDNA for 454 library preparation. The contents were combined, and the cDNA was purified using a PCR purification kit (Qiagen) according to the manufacturer's recommendations. The cDNA library was prepared with appropriate kits (Roche, Indianapolis, IN; Qiagen) for pyrosequencing on the GS-FLX sequencer (Roche) according to the manufacturer's recommendations which were described previously (Margulies et al., 2005). The only deviation from the protocol was prior to titration sequencing where following emulsification PCR, DNA positive beads were enriched to increase the number of reads collected during titration. BLAST searches (Altschul et al., 1997) were conducted using the Vg2 putative sequences using the NCBI nr database (www.ncbi.nlm.nih.gov) to confirm the putative protein function. Putative Vg2 nucleotide sequences were compiled together using the ContigExpress (function) module in Vector NTI (Invitrogen) to obtain the full-length Vg2 cDNA sequence. cDNA translation into amino acids and alignments with other Vgs were performed using Vector NTI.

2.3. In vivo injection of ecdysteroids

20-Hydroxyecdysone (20E) injection into the bodies of partially fed (virgin) females was performed as explained before by Thompson et al. (2005). Briefly, each female received a dose of 1000 ng of 20E on d 4 after attachment to the host. Controls were injected with solvent only. All injections were performed while female ticks were attached to the host. Injected females were collected 1, 2 and 3 d post-injection for RNA isolation and RT-PCR analysis.

2.4. RT-PCR

Whole bodies of tick males (unfed and fed), female (unfed, partially fed, mated pre-ovipositing and ovipositing) and 20E injected females along with their respective controls (previously mentioned) were homogenized separately in TRI reagent, and total RNA was isolated. One microgram total RNA isolated from each target stage was separately reverse transcribed into cDNA using oligo dT and PowerScript reverse transcriptase (Clontech, Palo Alto, CA) according to the manufacturer's recommendations. One microliter of each cDNA was used as a template in a standard 50 μl PCR (Sambrook and Russell, 2001) using oligo dT as a reverse primer and DvVg2 gene specific forward primer (5′-GAAGAGCGAGGATGCTAT-3′) for the detection of the DvVg2 message. The resultant fragment was also purified and sequenced to confirm its identity and was used as a DvVg2 specific probe for Northern blot analysis described later. Detection of DvVg1 expression in injected females was performed using DvVg1 specific forward primer (5′-TCCGTTCGTCCAGAAGGAAG-3′) and reverse primer (5′-TCTTGAGC GAGGTGGTGAGC-3′). The control reaction for the detection of DvVg1 and DvVg2 was conducted using lysozyme specific primers; Lys FP1: 5′-ATGCAGCTGCACGTGCCGCTCGCG-3′ and Lys RP2: 5′-GGTCAGGCGCCAATATATCAA-3′. Five microliter of each reaction were analyzed using agarose gel electrophoresis.

2.5. Tissue dissection and Northern blot analysis

Ovaries, midguts and fat bodies from replete (mated, pre-ovipositing) females were dissected and washed in ice-cold