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Hepatopancreas is the extraovarian site of vitellogenin synthesis in black tiger shrimp, *Penaeus monodon*

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Abstract

The site of yolk protein synthesis in crustaceans has long been a subject of controversy. The vitellogenin gene structure was partially reported only very recently in *Macrobrachium rosenbergii*, after which the hepatopancreas was confirmed as the extraovarian site of vitellogenin synthesis in that species. Ovaries are the most frequently reported as the site of yolk protein synthesis in penaeid shrimp. Using cDNA reversed-transcribed from mRNA isolated from the hepatopancreas of vitellogenic female shrimp, *Penaeus monodon*, we found that its deduced amino acid sequence had high identity of 48% with that from *M. rosenbergii* vitellogenin. A similar location of the intron in the sequenced region of genomic DNA was also found between these two species. We therefore concluded that the hepatopancreas the extraovarian site of vitellogenin synthesis in *P. monodon* in vivo. The partial structure of vitellogenin gene is presented in this study.

Keywords: Crustacean; Penaeid shrimp; Vitellogenesis; Vitellogenin gene; Hepatopancreas; Synthesis site; Vitellin; Genomic DNA

1. Introduction

Vitellogenesis, a process of hormonally regulated synthesis of yolk proteins and their subsequent deposition in ovarian oocytes, is an important reproductive process in oviparous animals. Both the site and the process of yolk protein synthesis in crustaceans are still controversial, due to the divergent experimental methodologies employed or the species concerned. We demonstrated that the hepatopancreas is the synthesis site of vitellogenin (Vg) in the freshwater giant prawn, *Macrobrachium rosenbergii*, through localization of Vg gene expression (Chen et al., 1999).

The yolk proteins mainly consist of Vg and vitellin (Vn) in crustaceans, present, respectively, in extra-oocyte tissues and in oocytes. Vg and Vn are known to be large-molecule compounds. The complexity and heterogeneity of their structure among crustacean species are still disputable, and

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in the past, efforts have mostly been focused on determining their components and moiety. Evidence suggests that yolk proteins are lipoglycoproteins, and similarities in electrophoretic patterns and immunological reactivities between Vg and Vn were further demonstrated in several crustacean species (Zagalsky et al., 1967; Kerr, 1969; Fyffe and O'Connor, 1974 de Chaffoy de Courcelles and Kondo, 1980; Byard and Aiken, 1984; Zagalsky, 1985; Suzuki, 1987; Tom et al., 1987; Lee and Puppione, 1988; Quinitio et al., 1989, 1990; Tirumalai and Subramoniam, 1992; Chang et al., 1993a,b, 1994; Chen and Chen, 1993; Han et al., 1994; Chang and Jeng, 1995; Sagi et al., 1995; Vafopoulou and Steel, 1995; Lee et al., 1997; Qiu et al., 1997; Chen et al., 1999).

Immunohistochemical approaches and tracing of isotope-labeled amino acid incorporation in vitro have often been employed to determine the synthesis site of Vg in crustaceans. Ovaries, hepatopancreas, hemocytes, and fat body/adipose tissues have been reported to date as sites of Vg synthesis in crustaceans (Eastman-Reks and Fingerman, 1985; Yano and Chinzei, 1987; Quackenbush, 1989; Rankin et al., 1989; Suzuki et al., 1989; Browdy et al., 1990; Fainzilber et al., 1992; Shafir et al., 1992; Chen and Chen, 1994; Han et al., 1994; Vafopoulou and Steel, 1995). The ovarian tissues of penaeid shrimp were reported to be the major synthesis site of Vg as revealed by in vitro incubation of ovaries in the presence of labeled amino acids (Yano and Chinzei, 1987; Quackenbush, 1989; Rankin et al., 1989; Browdy et al., 1990; Fainzilber et al., 1992; Shafir et al., 1992). In *Penaeus vannamei* (Quackenbush, 1989) and *Penaeus semisulcatus* (Browdy et al., 1990), yolk synthesis in the hepatopancreas is much lower than that in ovarian tissues. However, in vivo incorporation of labeled methionine into Vg in female *P. semisulcatus* suggested that the hepatopancreas plays an important role in the vitellogenic process than can be deduced from in vitro studies (Shafir et al., 1992). Verification of the synthesis site of Vg in crustaceans requires further elucidation by considering molecular structural correlations between newly synthesized proteins and purified Vn or Vg. The localization of Vg mRNA will clarify the Vg synthesis site in vivo.

We previously reported cDNA encoding a fragment of freshwater prawn Vg which not only revealed the partial primary structure of Vg in crustaceans for the first time but also clearly demonstrated that the hepatopancreas is the site of Vg synthesis in *M. rosenbergii* (Chen et al., 1999). The present study is aimed at determining the site of Vg synthesis in the tiger shrimp, *Penaeus monodon,* through localizing the expression of the Vg gene in vivo. Degenerate primers were designed according to the highly conserved domain sequences of *M. rosenbergii* Vn. RNA isolated from the ovary and hepatopancreas of vitellogenic females was employed in reverse transcription (RT). Homologies of the PCR products compared with the Vg gene of *M. rosenbergii* were then examined to verify the possible site of Vg synthesis in vivo.

2. Materials and methods

2.1. Shrimp

Naturally maturing tiger shrimp, *Penaeus monodon* (85–105 g in body wt.), were collected from Tunkang in southern Taiwan. The vitellogenesis of farm-reared females was induced by the unilateral eyestalk-ablation technique (Tan Fermin, 1991). Both samples were used for RNA isolation and subsequent verification of the Vg synthesis site in vivo.

2.2. RNA isolation and reverse transcription (RT)

RNA was isolated from the ovary and hepatopancreas of both naturally and induced vitellogenic female shrimp by the guanidinium thiocyanate method described by Chomczynski and Sacchi (1987). First-strand cDNA synthesis in RT was performed using a SuperScript II RNase H^- reverse transcriptase (Life Technologies, Inc., MD, USA) to transcribe poly$(A)^+$ RNA with oligo $d(T)_{18}$ as primers. The reaction conditions recommended by the manufacturer were followed.

2.3. Preparations of shrimp DNA

Muscle tissue at 200 mg excised from the abdomen of shrimp was rapidly frozen in liquid nitrogen and crushed to a fine powder. The processed tissue was placed in 2.4 ml digestion buffer (100 mM NaCl, 10 mM Tris–HCl, pH 8, 25 mM EDTA, pH 8, 0.5% sodium dodecyl sulfate, 0.1 mg/ml

proteinase K) and incubated at 65°C for 12–18 h. The digest was deproteinized by successive phenol/chloroform/isoamyl alcohol extractions, recovered by ethanol precipitation, dried and resuspended in 0.1 × TE buffer at 65°C for 30 min, and then stored at 4°C until use for polymerase chain reaction (PCR) (Lo et al., 1996).

2.4. PCR and subcloning

Based on pairwise alignments and phylogenetic comparisons between *M. rosenbergii* Vg amino acid sequences and other animal Vg, primers were designed according to the highly conserved domain sequences of *M. rosenbergii* Vg. Amplification primer pairs for *P. monodon* Vg were as follows: forward 5′-AAGTCCCTTGGCAACATGGG-3′ and reverse 5′-AGTCCTTCAAAACGTGCTCC-3′. Both genomic DNA and cDNA from vitellogenic female shrimp were used as templates in the PCR. The PCR fragments were resolved for length differences by 1.0% agarose gel electrophoresis. The amplified cDNA was cloned into the pGEM-T Easy vector using the reagents and instructions supplied by the manufacture (Promega Corporation, WI, USA). Recombinant bacteria were identified by blue/white screening and confirmed by restriction analysis. Plasmids containing the insert were purified (Promega minipreps) and used as a template for DNA sequencing. PCR reactions were performed for 30 cycles at an annealing temperature of 45°C for 1 min, elongation at 72°C for 2 min, and denaturation at 94°C for 1 min, followed by a 10-min extension at 72°C and cooling to 4°C. The PCR reaction buffer was 50 mM Tris–HCl buffer (pH 9), containing 50 mM KCl and 1% Triton X-100 (Boehringer Mannheim, Germany), 2.5 mM $MgCl_2$, 5 U Taq polymerase, 0.25 mM dNTPs, and 1 μM of each primer.

2.5. Nucleotide sequence analysis

Nucleotide sequence analysis was performed using the dideoxynucleotide chain termination method (Sanger et al., 1977) on a DNA sequencer model 373A (Applied Biosystems, Perkin-Elmer). A sample of 1 μg of plasmid DNA was used for sequencing with a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Perkin-Elmer) and was electrophoresed through 6% denaturing gels. Clones were sequenced with the M13 forward and reverse primers.

3. Results

Only the *Macrobrachium rosenbergii* Vg gene structure has so far been reported for crustaceans; the deduced amino acid sequence of *M. rosenbergii* Vg was aligned with the Vgs from various sources to survey the conserved domain for the purpose of primer design in the cloning of the Vg gene in *Penaeus monodon.* The alignment of amino acid sequences of Vg from prawn, fish, frog, chicken and nematode is shown in Fig. 1. Two highly conserved domains, KSLGNMG and GARFEGL, were therefore employed for the synthesis of forward and reverse degenerate primers and used subsequently in PCR. Total RNA isolated from the hepatopancreas of vitellogenic shrimp was used as the template for first-strand cDNA synthesis in RT. PCR products, respectively, with cDNA and genomic DNA as the templates, were electrophoresed on 1.0% agarose gel (Fig. 2). The former product was estimated to be 600 bp from the mobility on agarose gel compared with that of a 100-bp ladder marker, and the latter was 800 bp. The nucleotide sequences of both products were determined to be 593 and 813 bp, respectively (Fig. 3). Two introns, of 101 and 119 bp, respectively, were found in this sequenced region of genomic DNA. Alignment of the deduced amino acids from *P. monodon* and *M. rosenbergii* is presented in Fig. 4. Identity between these two sequences was calculated to be 48%. We consequently concluded that the hepatopancreas of *P. monodon* is a site of Vg gene expression. In other words, the hepatopancreas is the Vg synthesis site in vivo in this penaeid shrimp.

4. Discussion

In crustaceans, vitellin (Vn) is generally referred to as an intraovarian yolk protein, while vitellogenin (Vg), a yolk precursor, is an extraovarian yolk protein. The fact that Vn originates from Vg is widely accepted, but the transformation process of Vg into Vn is still unclear, since the molecular structures of these yolk proteins

have not yet been elucidated. In insects, Vgs are composed of one large and one small subunit (reviewed in Kunkel and Nordin, 1985; Raikhel and Dhadialla, 1992; Valle, 1993), derived from the cleavage of a single precursor in the fat body (Bose and Raikhel, 1988; Dhadialla and Raikhel, 1990; Heilmann et al., 1993; Yano et al., 1994; Kageyama et al., 1994; Hiremath and Lehtoma, 1997), and in crustaceans, fragmentation of a larger molecule yolk protein into smaller molecules was reported for the amphipod, *Orchestia gammarellus* (Junera and Meusy, 1982); for the terrestrial isopods, *Armadillidium vulgare* (Suzuki, 1987) and *Oniscus asellus* (Vafopoulou and Steel, 1995); and for *Penaeus vannamei* (Tom et al., 1992). Reports on the Vn composition of *Penaeus monodon* are inconsistent to date, with supposedly four to eight polypeptides (Quinitio et al., 1990; Chang et al., 1993a; Chen and Chen, 1993). These discrepancies are possibly due to the different methodologies employed in the process of purification and/or the different stages of ovarian development at the time of Vn extraction. One Vn in the tiger shrimp was presumed to be the yolk protein precursor, which was shown to cross-react with antisera against other components (Chen and Chen, 1993). In *Macrobrachium rosenbergii*, the fragmentation of yolk protein was confirmed not only from the protein level but also from the nucleotide sequences of the Vg gene (Chen and Kuo, 1998; Chen et al., 1999). These two major components of Vn are the products after post-translational modification from the same gene based on an analysis of gene sequencing.

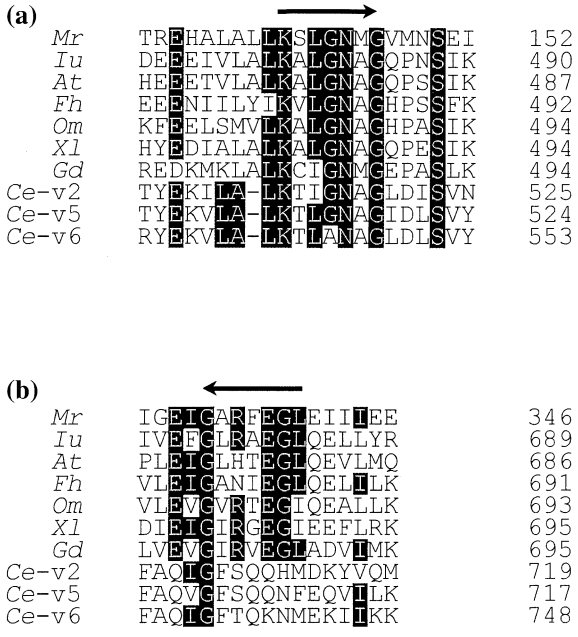

Fig. 1. Amino acid sequence alignments of two vitellogenin fragments from various animal species: freshwater giant prawn *Macrobrachium rosenbergii* (*Mr*) (Chen et al., 1999), silver lamprey *Ichthyomyzon unicuspis* (*Iu*) (Sharrock et al., 1992, GB: M88749), white sturgeon *Acipenser transmontanus* (*At*) (Bidwell and Carlson, 1995, GB: U00455), mummichog *Fundulus heteroclitus* (*Fh*) (LaFleur et al., 1995, GB: U07055), rainbow trout *Oncorhynchus mykiss* (*Om*) (LeGuellec et al., 1988, EMBL: X92804), lanuran *Xenopus laevis* (*Xl*) (Gerber-Huber et al., 1987, GB: M18061), chicken *Gallus domesticus* (*Gd*) (van het Schip et al., 1987, EMBL: X13607), and nematode *Caenorhabditis elegans* (*Ce*) (Speith et al., 1985; v2, EMBL: X56212; v5, GB: M11497; v6, EMBL: X56213). Forward and reverse primers employed were designed, based on the sequences indicated by the solid arrows.

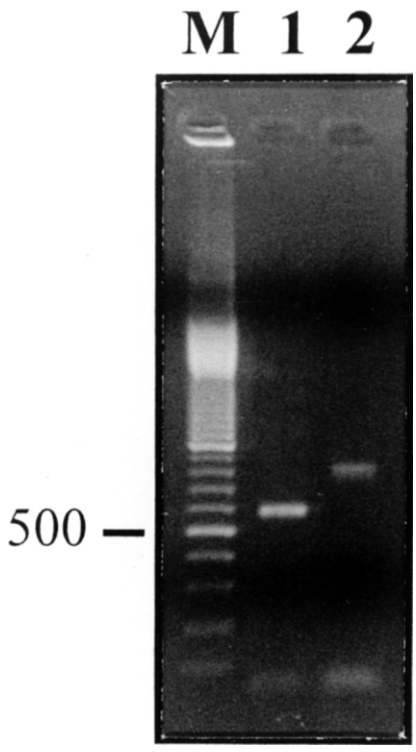

Fig. 2. Electropherogram of PCR products, respectively, using cDNA (lane 1, 593 bp) and genomic DNA (lane 2, 813 bp) as the templates. A 100-bp ladder DNA marker (M) was simultaneously electrophoresed.

Crustacean Vn is generally believed to be a glycolipoprotein (Zagalsky et al., 1967; de Chaffoy de Courcelles and Kondo, 1980; Zagalsky, 1985; Suzuki, 1987; Tom et al., 1987; Quinitio et al., 1990; Tirumalai and Subramoniam, 1992; Chen and Chen, 1993; Chang and Jeng, 1995; Vafopou-

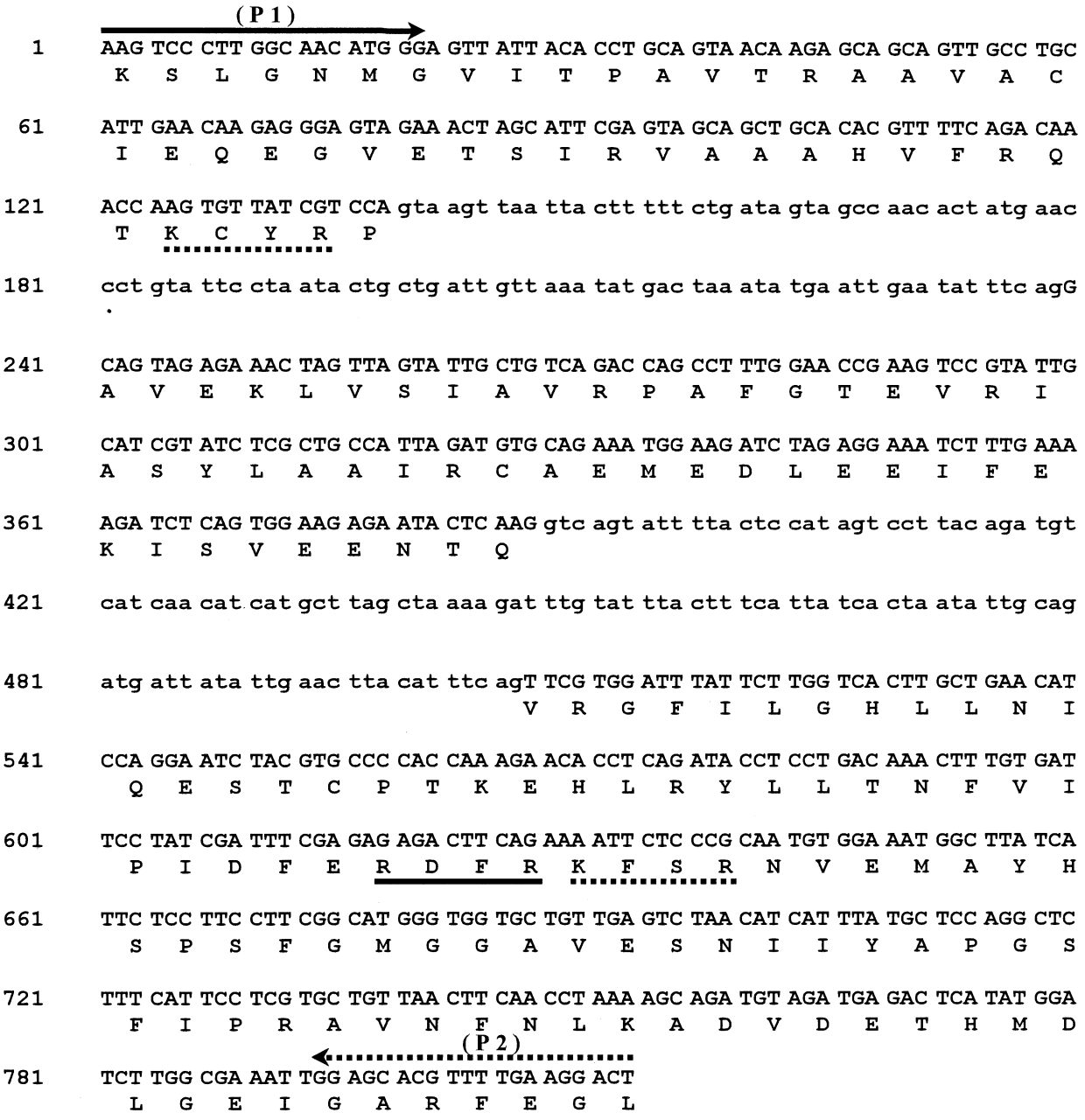

Fig. 3. The nucleotide and deduced amino acid sequences of cDNA encoding a vitellogenin fragment of tiger shrimp, *Penaeus monodon*. Locations of the forward and reverse primers are denoted respectively by solid and broken arrows. The specific preceding motifs for cleavage are underlined. Small letters represent the sequences of introns.

lou and Steel, 1995; Chen and Kuo, 1998). Three potential N-glycosylation sites, NXT, were observed in the revealed Vg nucleotide sequences of *M. rosenbergii* (Chen et al., 1999). In contrast, no such glycosylation site in the corresponding motif of the *P. monodon* Vg gene was detected, even though Vn was identified as a glycolipoprotein (Chang et al., 1993a; Chen and Chen, 1993). Glycosylation may be linked to the remaining domains, which remain to be sequenced.

The synthesis site of yolk proteins in crustaceans has been a subject of controversy, since the Vg gene sequence had not been determined and the molecular relationship between the expressed gene and translated products still remained to be elucidated. The synthesis sites so far

Fig. 4. Alignment of the vitellogenin amino acid sequences from *Penaeus monodon* and *Macrobrachium rosenbergii*. Blanks, represented by dashes, are inserted to maximally align the sequences. Identical residues between these two sequences are in black reverse print.

reported include hemocytes in *Callinectes sapidus* (Kerr, 1968); ovarian tissues in *Uca pugilator* (Eastman-Reks and Fingerman, 1985), *Penaeus japonicus* (Yano and Chinzei, 1987), *P. vannamei* (Quackenbush, 1989; Rankin et al., 1989), *P. semisulcatus* (Browdy et al., 1990), *P. monodon* (Chen and Chen, 1994), and *Callinectes sapidus* (Lee and Watson, 1995); and fat bodies in *Armadillidium vulgare* and *Porcellio dilatatus* (Picaud, 1980; Suzuki et al., 1989). In addition, the hepatopancreas of vitellogenic females is involved to some extent in vitellogenesis in *P. vannamei* and *P. semisulcatus* (Quackenbush, 1989; Shafir et al., 1992). We reported that cDNA reverse-transcribed from the mRNA isolated from a vitellogenic female hepatopancreas encoded a fragments of Vg in *M. rosenbergii.* That was the first time that the Vg gene structure was reported in crustaceans, and the synthesis site of Vg in vivo was also confirmed via verification of the gene expression. Following in vitro incorporation of isotope-labeled amino acids into immunoprecipitable de novo synthesis products, the ovary has been the most frequently proposed site of Vg synthesis in penaeid shrimp. cDNA reverse-transcribed from mRNA isolated from the hepatopancreas of a vitellogenic female *P. monodon* was successfully sequenced. The identities of the deduced amino acid sequences with that of *M. rosenbergii* Vg were as high as 47.8%. Two introns, as in *M. rosenbergii*, were further found in the correspondent region of the genomic DNA. This suggests that the hepatopancreas of *P. monodon* is an extraovarian site of Vg synthesis. Consequently, Vg synthesis in vivo in the hepatopancreas is apparently a common phenomenon among crustacean species.

Most insect Vgs are of the type that is cleaved in the fat body to produce two subunits. The cleavage sites are immediately preceded by a motif, (R/K)X(R/K)R or RXXR (reviewed in Sappington and Raikhel, 1998). Indeed, a 'RQRR' motif is situated just in front of the initiation site of the Vn B polypeptide in *M. rosenbergii*. A similar motif, RDFR, was found in the cDNA sequence of *P. monodon*. Moreover, (R/K)XXR was revealed as the processing motif for the mammalian subtilisin-like protease family (Barr, 1991). Two such domains, KCYR and KFSR, were found in the sequences. As mentioned above, Vg of *P. monodon* was revealed to be composed of four to eight subunits. The cleavage in post-trans-

lational modification seems to be more complicated than that in *M. rosenbergii* and insects. It will be helpful to clarify the possible post-translational modification process of *P. monodon* Vg if the sequence of full-length mRNA is determined. The composition of the Vg component might possibly be suggested from the location of the preceding motif in the sequences.

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