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The vitellogenin cDNA of *Cherax quadricarinatus* encodes a lipoprotein with calcium binding ability, and its expression is induced following the removal of the androgenic gland in a sexually plastic system

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Abstract

Oocyte maturation in decapod crustaceans is a two step process. Primary vitellogenesis is followed by a variable hiatus that lasts up to the onset of secondary vitellogenesis, which is marked by the rapid accumulation of yolk proteins in the oocytes. We have cloned a complete *Cherax quadricarinatus* vitellogenin cDNA. The sequenced cDNA contains a 2584 aa open reading frame which shows sequence similarity to vitellogenins from other crustaceans. The mRNA encodes at least two of the previously identified vitellin components, indicating that the primary translation product is subject to post-translational modification, including proteolytic cleavage. The region close to the 3′ end of the mRNA encodes a previously characterized negatively charged protein (provisionally designated P^{106}). We show here that the negative charge of P^{106} could be due to its ability to bind calcium. Northern blot data show that this gene is expressed as a single 8000 nt transcript and is present in the hepatopancreas of secondary-vitellogenic females. Primary vitellogenic and other tissues examined in male and female animals were negative. In sexually plastic intersex animals, removal of the androgenic gland results in vitellogenin transcription, indicating that the gene is negatively regulated by the androgenic gland.

Keywords: Crustacea; *Cherax quadricarinatus*; Vitellogenin; Calcium binding; Lipoprotein; Androgenic gland; Sexual plasticity

1. Introduction

The decapod crustacean *Cherax quadricarinatus* typically exhibits a gonochoristic mode of reproduction with marked seasonality (Barki et al., 1997). Under optimal conditions, sexual maturity of males and females is attained within 7–9 months (Rouse et al., 1991) when the gonadal rudiment spontaneously auto-differentiates into ovary in the absence of the androgenic hormone (Charniaux-Cotton and Payen, 1988). This hormone is secreted by the androgenic gland, a structure unique to the Crustacea, and has so far been characterized in isopods (Martin et al., 1999; Okuno et al., 1999). In decapod crustaceans the hormone has not yet been characterized; however, the role of the androgenic gland in regulating the development of male characteristics, and feminization—including the onset of vitellogenesis—following its removal was described in a number of decapod crustaceans (Sagi and Khalaila, 2001). In the developing ovary, cells released from the germinative zone complete meiosis and stay in their primary vitellogenic stage until the onset of secondary vitellogenesis, which is marked by oocyte growth and rapid accumulation of yolk proteins.

Intersex *C. quadricarinatus* animals are found in both wild and cultured populations at a frequency of 2–14%. In these animals, a permanently arrested ovary is present contralateral to an active male reproductive system which includes a testis, sperm duct, and an androgenic gland (Sagi et al., 1996a). These intersex individuals are functional males.

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As in other lecithilic oviparous animals, oogenesis in *C. quadricarinatus* is accompanied by a rapid deposition of a large amount of vitellin, the major yolk protein, in the oocytes. Vitellin later serves the developing embryo as an important source of proteins, lipids, and carbohydrates. In some oviparous vertebrates, vitellogenin, the vitellin precursor protein, has been shown to store zinc, magnesium, and calcium (Montorzi et al., 1994, 1995). Vitellogenesis has been well characterized in oviparous vertebrates and insects, but less in crustaceans. Initial studies have shown that crustacean vitellin is a high-density lipoprotein (HDL)[2] complex (Meusy and Payen, 1988). The yolk protein of *C. quadricarinatus* contains six major HDL subunits with approximate molecular masses of 177, 155, 106, 95, 86, and 75 kDa (Abdu et al., 2001). The 106-kDa polypeptide (provisionally designated P^{106}) was found to be negatively charged (Sagi et al., 1999). It is first detected in secondary-vitellogenic ovaries but disappears during early embryogenesis (Abdu et al., 2001). According to immunological studies and peptide mapping, P^{106} does not have any similarity to the other yolk HDL components, and its amino acid composition is different from that of known vitellins from other crustacean species (Abdu et al., 2001).

Crustacean gametogenesis is under hormonal regulation. The neurohormone(s) termed gonad-inhibiting hormone or vitellogenesis-inhibiting hormone negatively regulates vitellogenesis (Charniaux-Cotton and Payen, 1988; Huberman, 2000; Meusy and Payen, 1988). The possible involvement of hormone(s) secreted by the androgenic gland (Meusy and Payen, 1988; Taketomi and Nishikawa, 1996) in the above vitellogenesis regulation process has not been sufficiently investigated. The *C. quadricarinatus* intersex animals exhibit a unique sexual plasticity. Ablating the androgenic gland in intersexes cause a shift in the sexual balance from male to female; secondary vitellogenesis specific HDL appear in the hemolymph (Yehezkel et al., 2000) and large oocytes accumulate yolk polypeptides such as 177-, 155-, and 106-kDa (P^{106}) in the ovaries (Khalaila et al., 1999).

The appearance of vitellins, including P^{106}, in the ovaries of intersex *C. quadricarinatus* following androgenic gland ablation serves as a marker for the onset of gonad maturation in this otherwise permanently arrested ovary. It suggests a unique model in which the onset of secondary vitellogenesis can be manipulated and its regulation studied under controlled sexually plastic conditions. In the present study we investigated the biochemical properties of P^{106}. We cloned and sequenced a cDNA which encodes for secondary vitellogenic yolk proteins, including P^{106}. We studied its site of synthesis and its regulation by the androgenic gland.

2. Materials and methods

2.1. Animals

C. quadricarinatus were raised in our facility at Ben-Gurion University of the Negev. Morphological and anatomical observations were performed as described by Abdu et al. (2000). Intersex *C. quadricarinatus* individuals were andrectomized as described by Khalaila et al. (1999). Intact and andrectomized individuals were dissected and tissue samples were taken for Northern blot analysis.

2.2. Detection of sialic acid residues

The HDL fraction from a secondary vitellogenic ovary was isolated as described by Abdu et al. (2000). Following SDS–PAGE, an electroeluted 106-kDa fraction (Sagi et al., 1999) was analyzed for sialic acid residues by GC/MS. Samples with or without a sialic acid internal standard (*N*-acetylneuramic acid) were hydrolyzed with 0.1 M trifluoroacetic acid in a sealed ampoule at 80 °C for 1 h. The hydrolyzed fraction was lyophilized and then subjected to trimethylsilylation. The samples were subjected to gas chromatography on a 30-m cross-linked and surface-bound dimethylpolysiloxane column (2 min to 100 °C, 100–300 °C, 6 °C/min) connected to a mass spectrometer.

2.3. Migration with calcium on SDS–PAGE and $^{45}Ca^{2+}$ overlay

Two different methods were used to test calcium binding. In the first, 1 mM Ca^{2+} or 0.1 mM EGTA were included in the stacking and separating buffers of the SDS–PAGE (Garrigos et al., 1991). In the second method, bivalent metal ions Ca^{2+}, Mg^{2+}, or Zn^{2+}) or metal chelators (EGTA or EDTA) were added to the sample (not to the gel) before electrophoresis (Garrigos et al., 1991). The $^{45}Ca^{2+}$ overlay was performed as described in Garrigos et al. (1991).

2.4. Cloning procedure

Total RNA was isolated from ovaries and hepatopancreas of primary and secondary vitellogenic *C. quadricarinatus* females using TRIZOL (Gibco BRL). cDNAs were generated using Superscript II RT (Gibco BRL) according to the manufacturer's recommendations. Second strand cDNA synthesis and sequence

[2] *Abbreviations used:* BSA, Bovine serum albumin cDNA, Complementary DNA; cRT-PCR, Circularization-RT-PCR; dNTP, All four Deoxy nucleotide tri-phosphate; DTT-DL, dithiothreitol; EDTA, Ethylenediamine tetraacetic acid; EGTA, Ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GC/MS, Gas chromatography mass spectrometry: HDL, High density lipoprotein; PCR, Polymerase chain reaction; RACE, Rapid amplification of cDNA ends; RT, Reverse transcriptase; SDS–PAGE, Sodium dodecy sulfate–polyacrylamide gel electrophoresis; SSC, Standard saline citrate.

amplification were performed by strand and semi-nested PCR. Forward degenerate oligonucleotide primers were designed to encode the P^{106} amino acid sequence, TAMVVSDTEILTFDGALLR, which was determined by peptide sequencing. The sequence of the first primer (A) was ACIGCIATGGTIGTIWSIGA, which encodes TAMVVSD; the sequence of the second primer (B) was ACIGARATHYTIACITTYGA, which encodes TEILTFD. The reverse primer was dT_{18}. The PCR conditions were 94 °C for 4 min, followed by 35 cycles of 94 °C for 1 min, 42 °C for 1 min, and 72 °C for 3 min, followed by a final extension at 72 °C for 7 min. The PCR products were separated by 1% agarose gel electrophoresis, and the observed DNA band (found only in the secondary vitellogenic hepatopancreas sample) was excised (Concert gel extraction kit, Gibco BRL), and cloned into pGEM-Teasy (Promega). Clones containing inserts were isolated, plasmid DNA was purified and sequenced by dideoxy fluorescent terminator cycle sequencing and an ABI PRISM 377 DNA sequencer (Perkin–Elmer Biosystems, Foster city, CA). The 5′-end of the cDNA encoding P^{106} was cloned by 5′ RACE using a kit and the manufacturer's instructions (Gibco BRL) and cRT-PCR (Couttet et al., 1997).

2.5. Northern blot analysis

Total RNA was isolated from the ovaries and hepatopancreas of primary and secondary vitellogenic *C. quadricarinatus* females and from the hepatopancreas of males as before. Yields were quantified by absorption at 260 nm. RNA was electrophoresed through a formaldehyde 1% agarose gel, transferred to a nitrocellulose membrane, and UV-cross-linked. Blots were prehybridized for 2 h at 42 °C in 50% formamide, 5 × SSC, 0.05 M Na phosphate buffer pH 6.6, 0.1 mg/ml denatured salmon sperm DNA, 5× Denhardt's solution, 0.5% SDS, and 5 mM EDTA. Radiolabeled probes were synthesized by PCR. Blots were incubated overnight in prehybridization buffer containing a ^{32}P-labeled DNA probe encoding to the aa 2348–2584 fragment. The membrane was washed twice in 2 × SSC, 0.1% SDS at 42 °C for 15 min, and then washed again in 1 × SSC, 1% SDS at 42 °C for 10 min. Hybridized membranes were exposed to X-OMAT AR Kodak film with intensifying screens at 70 °C for 24 h. Ribosomal RNA was visualized by ethidium bromide.

3. Results

3.1. Biochemical characterization of the negatively charged P^{106}

Initial analyses of *C. quadricarinatus* vitellin showed that the P^{106} component was likely negatively charged

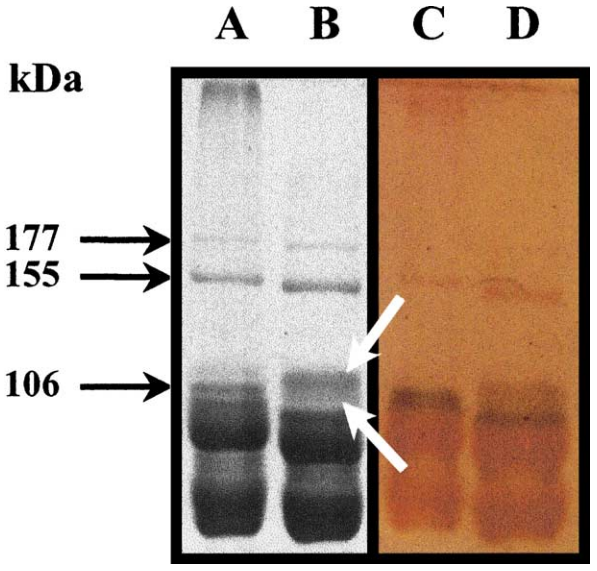


Fig. 1. Detection of calcium-binding polypeptides in the HDL fraction of newly laid eggs of *C. quadricarinatus* by electrophoretic migration in the presence of calcium and of a metal chelator. A and B, egg HDL stained with Coomassie blue; C and D, egg HDL stained with "stains-all". A and C are egg lipoproteins incubated with calcium, B and D incubated with EGTA (lane 2). Arrows indicate the calcium-binding polypeptides.

(Sagi et al., 1999). Similar observations with respect to other proteins have been suggested to be due to the presence of a sialic acid residue, a phosphorus residue, and/or calcium binding capabilities (Campbell et al., 1983; King and Morrison, 1976). To detect sialic acid residues, GC/MS was performed on HDL from newly laid eggs and on purified P^{106}. Neither the GC/MS of the

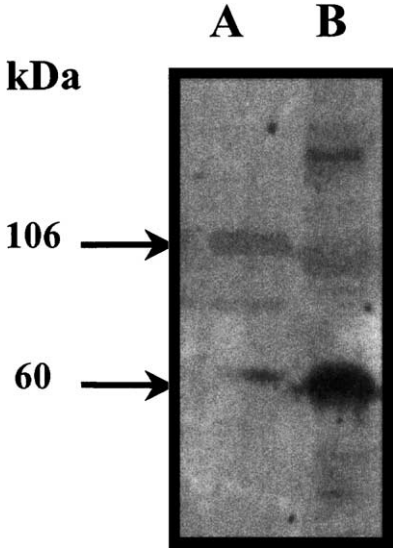


Fig. 2. Detection of calcium-binding polypeptides in the HDL fraction of newly laid eggs of *C. quadricarinatus* by $^{45}Ca^{2+}$ overlay assay. Lane A, autoradiograph of egg HDL; lane B, autoradiograph of sarcoplasmatic reticulum from rabbit muscle (positive control).

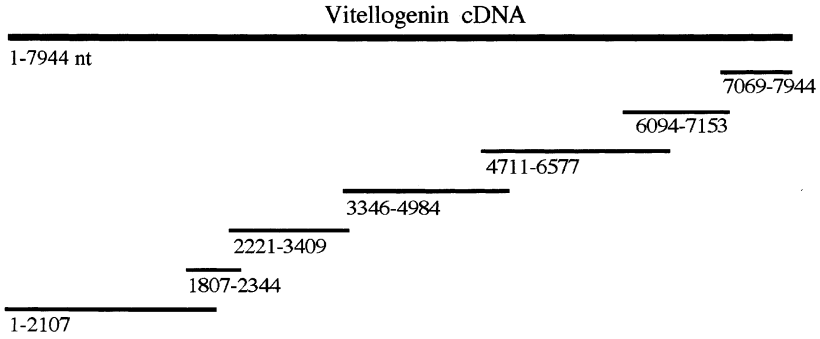


Fig. 3. Schematic presentation of overlapping subclones obtained from full length cDNA by an initial PCR amplification using a P^{106}-encoding primer and oligo(dT), consecutive 5′ RACE reactions, and a final cRT-PCR reaction (nt 1–2107). The resulting single contiguity represents a full length cDNA sequence encoding secondary vitellogenic yolk lipoproteins. Contiguous sequence data within individual clones were obtained by primer-walking.

egg HDL nor that of the purified P^{106} showed sialic acid residue (data not shown). To investigate whether the negative charge of P^{106} confers on this subunit the ability to bind calcium, two tests were performed: electrophoretic migration in the presence of calcium (Fig. 1) and a $^{45}Ca^{2+}$ overlay assay (Fig. 2). Figs. 1A and C show that only one band with an approximate molecular mass of 106 kDa was stained blue when the HDL from newly laid eggs was subjected to electrophoresis in the presence of calcium. In contrast, in the presence of the ion chelator EGTA (Figs. 1B and D) a gel shift was noted in which two polypeptides with approximate molecular masses of 115 and 100 kDa (Fig. 1B white arrows), which were stained blue (Fig. 1D). Identical results were obtained in the presence of the two other bivalent metal ions tested, magnesium and zinc, and in the presence of the ion chelator EDTA (data not shown). The ^{45}Ca overlay test confirmed that P^{106} binds calcium (Fig. 2A arrow), as did the above two polypeptides with approximate molecular masses of 115 and 100 kDa (data not shown). In addition, another much less prevalent polypeptide with an approximate molecular mass of 80 kDa also had the ability to bind calcium (Fig. 2A) as did the positive control 60 kDa polypeptide of sarcoplasmatic reticulum from rabbit muscle (Fig. 2B arrow).

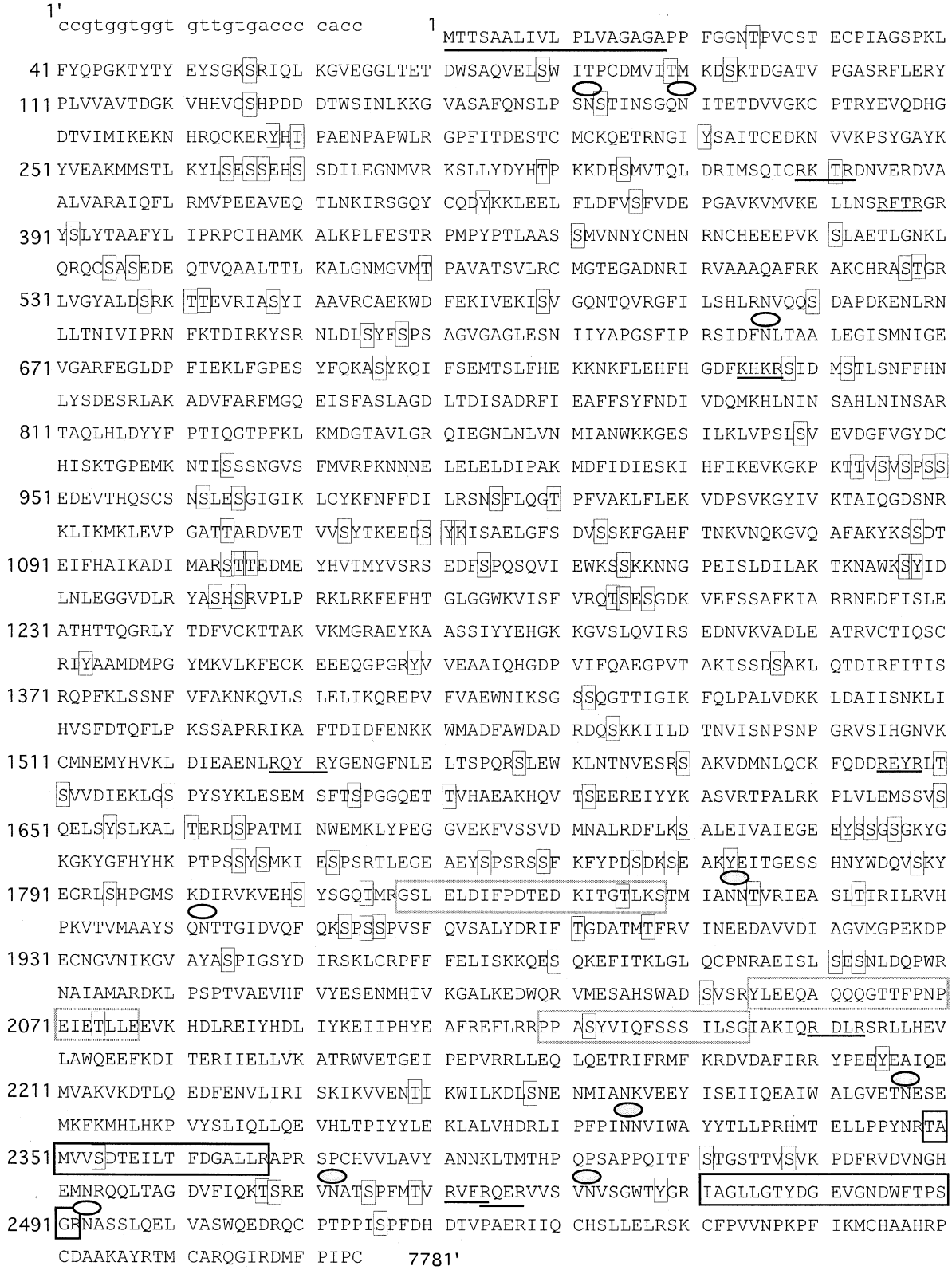
3.2. Isolation of a cDNA fragment encoding P^{106}

Since the N-terminal of P^{106} was found by Edman degradation to be blocked, two degenerate primers, A and B, were designed on the basis of the internal peptide sequence obtained from the 106-kDa polypeptide. A PCR product of about 800 bp was obtained from the hepatopancreas of a secondary vitellogenic crayfish female with primers A and dT_{20}. This fragment was used as a template for a semi-nested PCR using primers B and dT_{20}. Bands from the first PCR reaction and from the semi-nested PCR were cloned and sequenced. Sequence analysis showed that the products of the first and semi-nested PCRs encoded amino acid sequences identical to experimentally determined P^{106} peptide sequences, including the experimentally determined peptide sequence (IAGLLGTYDGEVGNDWFT), which was not encoded by any of the PCR primers. These initial data indicated that a cDNA from a gene encoding the P^{106} protein had been cloned. The full length cDNA was obtained by 5′ RACE and circularization PCR (Fig. 3).

3.3. Molecular characterization of the full length cDNA

A contiguous cDNA sequence was derived from the overlapping cDNA clones (Fig. 3). Conceptual translation in all three reading frames revealed a single large open reading frame of 7752 nt, encoding a 2584 aa polypeptide, including the amino acid sequences observed in the sequenced peptides (Fig. 4). With respect to this reading frame, the cDNA contained a 24 bp 5′ untranslated region and a 107 bp 3′ untranslated region. The 3′ end of the cDNA contained a polyadenylation signal, AATAAA, 12 nucleotides upstream of a 47 bp

Fig. 4. Structure of the full length *C. quadricarinatus* cDNA. The 7944 nt length of the cDNA sequence is represented by nucleotide sequence in the deduced 5′ and 3′ untranslated regions (primed numbering starting at the first nucleotide of the cDNA) and by the conceptual translation of amino acid sequence in the 7752 nt open reading frame (non-primed numbering beginning with the methionine of the deduced AUG initiation codon). The predicted polyadenylation signal in the 3′ untranslated region is underlined. Within the amino acid sequence, predicted structures are indicated as follows: Amino terminus secretion signal peptide and potential subtilisin cleavage sites are underlined. Predicted candidates for Ser, Thr, or Tyr phosphorylation are boxed. Predicted candidate sites for N-linked glycosylation are marked with an oval above Asn. Matches in the deduced amino acid sequence to the experimentally determined peptide sequences to P^{95} and P^{106} are boxed (gray outline, P^{95}; black outline, P^{106}). The nucleotide sequence has been entered into the GenBank/EMBL Data Bank and carries Accession No. AF306784.



poly(A) tail (Fig. 4). The 2584 aa primary translation product had a predicted molecular mass of 291,951 Da. A signal peptide containing 18 contiguous highly hydrophobic amino acid residues was predicted by the eukaryotic signal peptide prediction program CBS (Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark); suggesting that the protein is cleaved between amino acid residues

18 and 19 and exported from the cell. Two partial P^{106} amino acid sequences were found at the carboxyl end of the deduced amino acid sequence at aa 2349–2367 and 2471–2492. Three partial amino acid sequences from the 95-kDa HDL yolk polypeptide were found at aa 1818–1838, 2055–2077, and 2108–2124 (Fig. 4). The deduced amino acid sequence had eight potential cleavage sites for endoproteases of the subtilisin family (R–X–X–R adjacent to predicted secondary structure turns), which are known to cleave vitellogenin (Sappington and Raikhel, 1998). One of these lay between the peptide fragments of the P^{106}) and the 95-kDa proteins. We therefore tentatively assigned amino acids 2132–2584 to P^{106}. The conceptual protein sequence was searched for known domains and motifs using the Reverse Position Specific BLAST algorithm (Altschul et al., 1997) at the NCBI. No known calcium-binding domain was identified. The PEPTIDE STRUCTURE protein secondary structure module (Wolf et al., 1988) of the GCG sequence analysis program suite (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, WI) identified ten potential glycosylation sites. Potential serine, threonine, or tyrosine phosphorylation sites were identified using the NetPhos 2.0 algorithm (Blom et al., 1999). There was no evidence of transmembrane regions.

A BLAST search (Altschul et al., 1997) executed at the NCBI revealed that the deduced sequence had a relatively high similarity to identified vitellogenin genes from other Crustacea; the Kuruma prawn, *Marsupenaeus japonicus* (Tsutsui et al., 2000) (65%), the green tiger shrimp, *Penaeus semisulcatus* (Avarre et al., 2002, Direct submission GenBank Accession No. AY051318) (62%) and the giant freshwater prawn *Macrobranchium rosenbergii* (Okuno et al., 2002) (59%) throughout its length (Fig. 5). The N- and C-termini of the deduced sequence were similar to apolipophorin of *Locusta* (42 and 41%, respectively) (Bogerd et al., 2000). Also, the N-terminal of the deduced amino acid sequence was similar to the apolipoprotein B (40%) and in its C-terminal to type-D domain of von Willebrand factor (40%). Weaker similarity to vertebrate vitellogenins (36–39%) was identified. No significant sequence similarity to the insect vitellogenins was identified.

3.4. Expression and regulation of the vitellogenin gene

Northern hybridization using cloned cDNA probes identified a single band of approximately 8 kb in the hepatopancreas of secondary vitellogenic females, (Fig. 6, Lane C). Hybridization was not detected in the primary and secondary vitellogenic ovaries (Fig. 6, lanes A and B, respectively), nor in hepatopancreas of primary vitellogenic or late secondary vitellogenic females (Fig. 6, lanes D and F), nor in the hepatopancreas of males (Fig. 6, lane E). The hepatopancreas of intact intersex individuals showed no hybridization (Fig. 7, lane C). However, following androgenic gland ablation, the probe hybridized to a single band of approximately 8-kb (Fig. 7, Lane D), the same size as observed in the hepatopancreas of secondary vitellogenic females (Fig. 7. Lane B).

4. Discussion

Oogenesis and its regulation have been well studied in some vertebrate and insect species. Oogenesis in crustaceans, despite their commercial significance, has been less extensively studied. The decapod crustacean, *C. quadricarinatus* has a two step oocyte maturation process. After an indefinite pause, final egg maturation is marked by a rapid accumulation of vitellin proteins—secondary vitellogenesis—which in decapod crustaceans is inhibited by the androgenic gland (Khalaila et al., 1999, 2001; Taketomi and Nishikawa, 1996). Our previous studies on *C. quadricarinatus* oocyte maturation indicate that hemolymphatic vitellogenin, carried in the

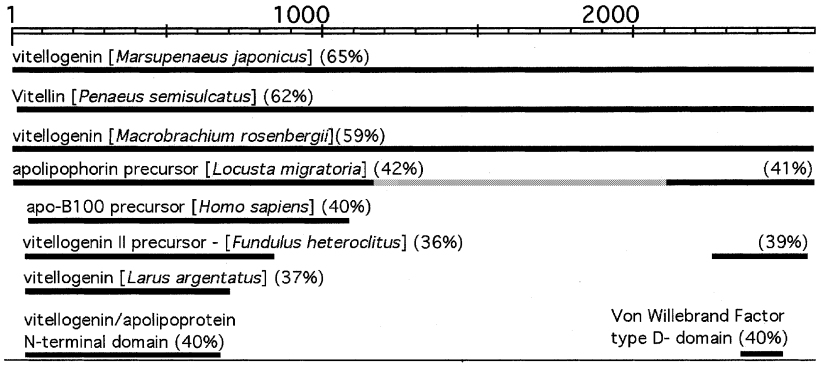


Fig. 5. Similarity of the deduced primary amino acid sequence of cDNA of the secondary-vitellogenic yolk lipoprotein from *C. quadricarinatus* with other sequences. The conceptually translated primary amino acid sequence from the *C. quadricarinatus* vitellogenin mRNA was compared with similar conceptually translated sequences using the BLAST server at the NCBI. The percent sequence similarity is indicated above each match.

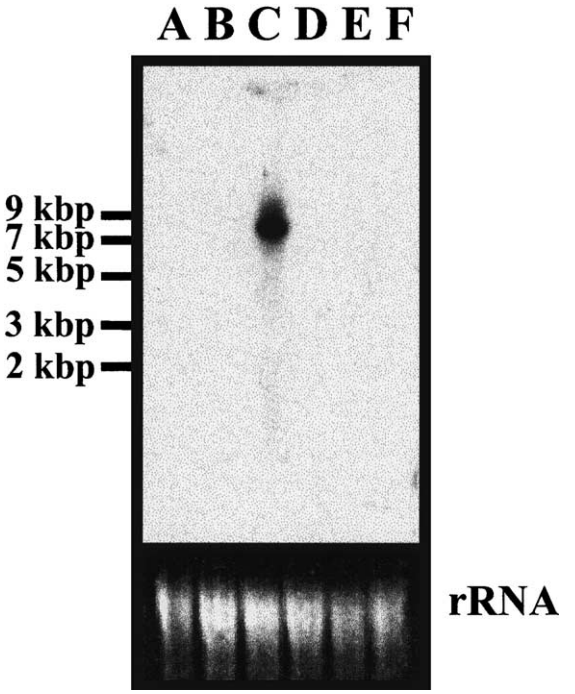


Fig. 6. Northern blot analysis of tissue-specific expression of the secondary vitellogenic yolk HDL. Lane A, RNA from ovary of primary-vitellogenic female; lane B, RNA from ovary of secondary-vitellogenic female; lane C, RNA from hepatopancreas of secondary-vitellogenic female; lane D, RNA from hepatopancreas of primary-vitellogenic female; lane E, RNA from hepatopancreas of male; lane F, RNA from hepatopancreas of post-vitellogenic female. rRNA, ribosomal RNA visualized by ethidium bromide.

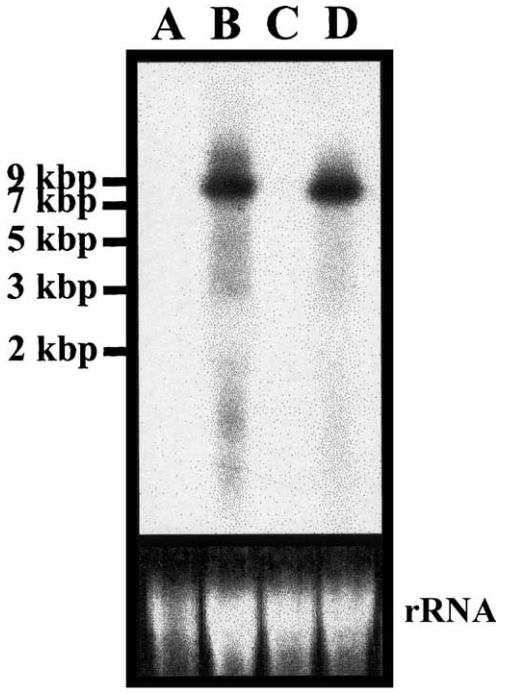


Fig. 7. Northern blot analysis of expression of mRNA of the secondary vitellogenic yolk HDL in the hepatopancreas of intersex individuals and of females of *C. quadricarinatus*. Lane A, RNA from primary vitellogenic female; lane B, RNA from secondary vitellogenic female; lane C, RNA from intact intersex individual; lane D, RNA from androgenic-gland-ablated intersex individual. rRNA, ribosomal RNA visualized by ethidium bromide.

circulation from the hepatopancreas to the ovary, is comprised of four subunits with approximate molecular masses of 208, 196, 177, and 80 kDa (Yehezkel et al., 2000) and the mature yolk protein contains subunits with approximate molecular masses of 177, 155, 106, 95, 86, and 75 kDa (Abdu et al., 2001).

To better understand the regulation of secondary vitellogenesis and the relationship between the large set of different yolk proteins and their precursors, we cloned a gene encoding the most distinctive vitellin protein, the negatively charged P^{106}. The techniques used to produce cDNAs from the 5′ end of the mRNA and the correspondence between the size of the cDNA and the mRNA as determined by Northern blotting indicate that an essentially full length cDNA sequence had been obtained. The 2584 amino acid primary conceptual translation product (predicted molecular mass of 292 kDa) is far larger than needed to encode just the P^{106} polypeptide and all the previously identified P^{106} peptide sequences were found at the carboxy end of the translated sequence. Based upon the peptide sequence data, the sequence 5′ to the P^{106} sequence encodes in addition at least the 95-kDa HDL yolk polypeptide. The deduced amino acid sequence displays relatively high sequence similarity to the vitellogenin gene of the shrimp (kuruma prawn), *M. japonicus* (Tsutsui et al., 2000) and *M. rosenbergii* (Okuno et al., 2002), throughout its length. It is also similar to apolipophorin of the insect *Locusta migratoria* (GenBank Accession No. AJ130944), and the moth tobacco hornworm (Sundermeyer et al., 1996) at the N- and C-termini. The N-terminal was similar to mammalian apolipoprotein B (Cladaras et al., 1986) and the C-terminal to the type-D domain of von Willebrand factor (Ruggeri and Ware, 1993), which is involved in protein multimerization (Jorieux et al., 2000). Based upon the proteins it encodes and the similarity to other studied sequences, the cDNA is clearly from a *C. quadricarinatus* vitellogenin gene. Like the vitellogenins in other arthropod species, the precursor polypeptide is most likely post-translationally cleaved and modified to produce at least some, or perhaps all of the secondary vitellogenic specific hemolymph and oocyte HDLs that we have characterized. Of the different predicted subtilisin cleavage sites, only the KHKR variant site identified at aa 724–727 is conserved among the identified crustacean vitellogenins, corresponding to the predicted site RTKR at aa 727 of the primary translation product of the *M. japonicus* mRNA and the known cleavage site RQRR at aa 724 of the primary translation product of the *M. rosenbergii* cDNA. The location of the different experimentally determined peptide sequences in the conceptual translation product, the subtilisin-like

cleavage sequence RDLR at aa 2133, and the initiation of sequence similarity to the insect apolipophorins at aa 2111 suggest that the P^{106} peptide consists of aa 2134–2584. The predicted molecular weight of this polypeptide is 52 kDa, less than half of the 106 kDa estimated by denaturing gel electrophoresis, suggesting extensive secondary modification, perhaps through glycosylation at some of the five potential sites present in this sequence. Cleavage at the conserved subtilisin like sequence at aa 726 would yield a protein of 80 KDa, perhaps corresponding to the 80 kDa HDL found in the hemolymph (Yehezkel et al., 2000). Although the size correlation is almost exact, the identification is tentative only, as glycosylation at any of the three potential sites identified in this sequence could significantly alter the molecular weight. The sequence between the two potential cleavage sites at aa 727 and aa 2134 would yield a protein with a calculated molecular weight of 159 kDa, which is close to the 177 kDa molecular weight of one of the hemolymph HDL subunits (Yehezkel et al., 2000). If the different hemolymph HDL subunits we have identified (Yehezkel et al., 2000) consist of different protein sequences, rather than being differentially modified versions of the same sequence, then it is unlikely that the cDNA we have cloned encodes all of them. Determining the precise relation between the different polypeptides and their encoding awaits further biochemical characterization of the HDLs.

There has long been a debate over the site of vitellogenin synthesis in crustaceans (Soroka et al., 2000). Recently, four new vitellogenin expression studies showed that the site of vitellogenin synthesis might vary from species to species. In the shrimp *M. japonicus,* the vitellogenin gene is expressed in both the hepatopancreas and the ovary (Tsutsui et al., 2000), in *Penaeus mondon*, the hepatopancreas is the extraovarian source for vitellogenin (Tseng et al., 2001) whereas in the freshwater prawn *M. rosenbergii* it is restricted to the hepatopancreas (Chen et al., 1999; Soroka et al., 2000; Yang et al., 2000). Northern analysis of the *C. quadricarinatus* cDNA indicated a single 8000 nt mRNA band present only in the hepatopancreas of secondary vitellogenic females, indicating that in *C. quadricarinatus*, at least the egg vitellins derived from vitellogenin expression are synthesized in the hepatopancreas and transported to the maturing oocyte. These results are consistent with our previous data suggesting extraovarian synthesis of yolk proteins in *C. quadricarinatus*, including, a strong correlation between hemolymph astaxanthin (prosthetic group of hemolymphatic vitellogenin) and oocyte diameter (Sagi et al., 1996b), and the presence of the secondary vitellogenesis specific, P^{106} crossreactive protein, in the hemolymph as determined by ELISA (Sagi et al., 1999). Moreover, our observation that oocyte yolk globules were more concentrated next to neighboring ovarian walls also suggests the transport of yolk protein from external sources into the oocyte (Abdu et al., 2000).

The *C. quadricarinatus* intersex animals exhibit an inducible sexual plasticity. These animals are normally functional males, bearing testes on one side and an arrested ovary on the other. Ablating the androgenic gland of intersexes causes a shift in the sexual balance from male to female. The block on the ovary is removed and secondary vitellogenesis specific HDL appears in the hemolymph (Yehezkel et al., 2000) and yolk polypeptides appear in large oocytes. The Northern data indicate that the removal of the androgenic gland from intersex individuals results in the expression of the vitellogenin gene. In normal intersex animals, expression of the gene in the hepatopancreas seems to be suppressed by the androgenic gland. The most immediately striking product of the vitellogenin gene is the P^{106} protein. Although a product of secondary vitellogenesis, the protein does not appear to be part of the hemolymphatic vitellogenin complex and was found to be negatively charged (Abdu et al., 2001). The protein was shown to bind calcium. The gel shift on SDS–PAGE (into two polypeptides with molecular masses of 115 and 100 kDa) may be explained by different phosphorylation states, as has been reported for yolk protein 2 in *Drosophila hawaiiensis* (Cohen, 1998) and for the heart membrane protein, phospholamban (Wegner and Jones, 1984).

Bound calcium is an ionic component of many functional proteins. Free calcium ions also play important roles in a number of physiological processes as an intracellular messenger molecule. Calcium is important in the reproductive processes, including oocyte maturation, fertilization, and embryo development, in both vertebrates and invertebrates (Silva-Neto et al., 1996). In egg-laying vertebrates, vitellogenin serves as a calcium store for the development of bones and teeth during embryogenesis. In crustaceans, calcium plays an important role in building up the new cuticle during molting (Mercaldo et al., 1994; Wheatly, 1997). Since the developing crustacean embryo undergoes several molts before it hatches, it is possible that the described calcium-binding P^{106} protein plays a role in transporting and storing calcium to be used during oocyte maturation, fertilization, and embryo development. It is not clear what the biochemical basis of the calcium binding is. The putative P^{106} sequence (aa 2132–2584) did not match any known calcium binding domains. Nevertheless, this sequence is significantly similar to a carboxy region lying at nt 7766–9196 of the Locust apolipophorin cDNA, whereas the sequence lying further towards the amino end of the protein is not. The putative P^{106} also contains sequence similar to Von-Willibrands D-type domain, which is not known to bind calcium. Poly-serine sequences, which are hypothesized to bind bivalent cations in other vitellins (Sappington and

Raikhel, 1998) are completely absent from the P^{106} sequence. Indeed, compared to the rest of the predicted amino acid sequence, this region is serine-poor, 5.0% vs. 9.3%.

Cloning the *C. quadricarinatus* vitellogenin cDNA provides us with an entrance to analyzing the regulation of secondary vitellogenesis in a new model system for a commercially important but relatively unstudied group of animals. The expression of this gene is part of a unique sexually plastic system that may be manipulated to enable the study of its regulation at the molecular level.

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