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Molecular characterization, developmental expression and immunolocalization of clathrin heavy chain in the ovary of the American cockroach, *Periplaneta americana* during oogenesis

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Abstract: Clathrin is the principal protein involved in receptor mediate endocytosis and the main component of the coated vesicles. It is composed of three identical clathrin heavy chains (*CHC*), each with an attached light chain. We characterized the deduced amino acid sequence of the partial cDNA clone of the American cockroach, *Periplaneta americana* (*Pam*) *CHC*. The analysis showed that this sequence is represented as multiple alpha helical repeats occurred in the arm region of the *CHC* and displayed a high level of identity and similarity to mosquitoes and *Drosophila melanogaster CHCs*. This is the first report on *CHC* from a hemimetabolous insect. The amplified *CHC* probe could hybridize two *CHC* transcripts in the current preparations, 6.3 kb and 7.3 kb. The Northern blot analysis confirmed that a 6.3 kb transcript is specifically expressed in ovarian tissues at high levels throughout the ovarian development, especially in previtellogenic ovaries (Days 1-4) but dropped during the vitellogenic period (days 5-7) and ultimately no transcript was detected in fully vitellogenic ovaries (days 9-13). Immunoblot analysis detected an ovary specific *CHC* protein of ~175 kDa that was present in previtellogenic ovaries on the day of female emergence and after initiation of vitellogenesis and onset of *Vg* uptake. Immunocytochemistry localized *CHC* protein to germ-line derived cells, oocytes, and revealed that *CHC* translation begins very early during oocyte differentiation in the germarium. The present work suggested a possible role for clathrin in the early fluid phase endocytosis (pinocytosis) in addition to its role in receptor-mediated endocytosis.

Keywords: Clathrin, Receptor-mediated endocytosis, Vitellogenin, Insect oocyte, *Periplaneta americana*.

1. Introduction

Endocytosis is a carefully orchestrated process required by all cells for nutrition and defense. Through this process, massive amounts of the major extraovarian yolk protein precursors, vitellogenins (*Vgs*), are internalized by oviparous animals during egg maturation (1, 2). This uptake is achieved by a membrane bound receptor through receptor-mediated endocytosis (3). The principal cellular organelles involved in receptor mediate endocytosis are the coated vesicles (4). The major component of these specific organelles is clathrin, a unique protein occurring as a characteristic three legged structure (triskelions or trimmers) which polymerizes into a polyhedral lattice on the cellular membrane (5). Clathrin trimmers are composed of three identical clathrin heavy chains (*CHC*), each with an attached light chain. These triskelions associate with membrane receptor-ligand complexes through adaptor proteins or adaptins (6). The detailed molecular characterization of the major components of coated vesicles is of great importance for understanding the cellular transport machinery in eukaryotes.

In insects, the process of yolk protein uptake by developing oocytes has been the subject of early and intensive research, especially in holometabolous species, like mosquitoes (3, 7, 8) and *Drosophila*

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melanogaster (9, 2). Conversely, studies based on less modified hemimetabolous species, like cockroaches in which ovaries are of panoistic type (no nurse cells associated with oocytes), are scarcer.

So far, complete cDNA for *CHC* has been cloned from four insect species: *Culex quinquefasciatus*, *Aedes aegypti* (10, 11), GenBank accession numbers: XM001864895 and XM001656826.1, respectively, *Drosophila melanogaster* (accession no.: Z14133.1). However, only a partial cDNA clone sequence for the American cockroach, *Periplaneta americana* is available in the GenBank database under the accession number: GQ887261.1.

In an attempt to characterize all the structural elements of the American cockroach, *P. americana* (*Pam*) *Vgs* transport machinery, two major components of the coated vesicles, the ligand-vitellogenin and its receptor (*VgR*), were previously cloned and characterized at both biochemical and molecular levels, including developmental expression, localization and sequencing analysis (12, 13, 14,15, 16, 17). In this study, we address the molecular characterization of the database available partial *Pam CHC*, the most basal insect *CHC* yet examined, and show its comparison to other insects *CHCs*. Based on this analysis, we described the expression pattern, developmental characteristics and cellular distribution of *Pam CHC* during oogenesis in *P. americana*.

2. Materials and Methods

2.1 Insect rearing and sample collection

P. americana stock cultures were maintained in the laboratory as described elsewhere (17). Newly emerged females were collected from the stock colonies, kept separately under constant light conditions at 26°C and used when required. Ovaries, at different developmental periods, were isolated in phosphate buffered saline (1x PBS: 2mM KH_2PO_4, 137mM NaCl, 10mM Na_2HPO_4, 2.7mM KCl, pH 7.4), fixed or frozen immediately in liquid nitrogen and stored at -80°C, until required.

2.2 Poly (A^+) RNA extraction and cDNA construction

Total RNA was extracted from previtellogenic ovaries using Isogen reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Then, mRNA was purified from pooled total RNA using mRNA purification kit (Amersham-Pharmacia, Piscataway, NJ, USA). A total of 1.5µg of mRNA was used to generate ds cDNA using *Avian myeloblastosis* virus reverse transcriptase (20 units) and an oligo (dT) primer [a cDNA synthesis primer (10 lM)] with the dNTP mixture (10mM) from the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). For second strand synthesis, a 20x second strand enzyme cocktail (RNase H, *Escherichia coli* DNA polymerase I and *E. coli* DNA ligase) was used. The ds cDNA constructed library was then used as a template for PCR.

2.3 Cloning of *CHC* probe

The cDNA prepared from female *P. americana* previtellogenic ovaries and two specific primers based on the partial cDNA sequence of *P. americana CHC*, available in the GenBank database under the accession number: GQ887261.1, were used to amplify approximately 218 bp of *Pam CHC* fragment (position: 543 – 761). Briefly, the designed specific primers were as follows: Forward primer 5`-ATTTCAAGGGGCAGTTGATG-3` (position: 543 – 562), and reverse primer 5`-AAACCTAGTGCTGCCTCCAA-3` (position: 742 – 761). The employed amplification conditions were heated to 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 2 min. The amplified fragment (~ 218 bp) was subcloned into pT7Blue vector (Novagen) and sequenced. Sequencing was performed by using an ABI Prism BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA) and a DNA sequence (ABI, Prism 3100 Genetic Analyzer, PE Applied Biosystems). The obtained sequence was checked for homology with the available sequence in the GenBank database for *P. americana CHC* using a BLAST homology search of the NCBI database. Sequence comparisons with other insects *CHC*s were performed using the CLUSTALW program and the GENETYX Ver. 5.1 program (Genetyx Corporation, Tokyo, Japan).

2.4 Northern blot hybridization analysis

For the developmental expression profile assay, ovaries from 1 to 13 days post emergence adults female were used. Aliquots of 15µg of total RNA from ovaries were subjected to electrophoresis using 1% agarose / 0.66M formaldehyde gel in 3-morpholino-propanesulfonic acid (MOPS) buffer. The resolved RNAs were transferred to a Hybond N^+ membrane (Amersham Pharmacia) through capillary transfer. The blots were then hybridized with the *Pam CHC* (PCR amplified) probe (Fig. 1) which was then fluorescein-labeled using a Gene Images Random Prime Labeling Module (Amersham Pharmacia), according to the kit instruction manual. The probe was hybridized based on the protocol from Hybond N^+ (Amersham).

Briefly, the membranes was blocked for 30 min at the 68°C using Express-Hyb™ Hybridization solution (Clontech) and then probed for 1 h at 68°C, followed by repeated washes in 2x sodium chloride–sodium citrate in water (SSC) at 68°C and 0.1x SSC – 0.1% SDS at 55°C. The hybridization blots were visualized and analyzed by BAS 2000 (Fuji Photo Film, Tokyo, Japan). Samples were qualitatively normalized by determining ribosomal RNAs after staining with ethidium bromide.

```
AedesCHC        DHRNLQNLLILTAIKADRSRVMDYINRLDNYDAPDIANIAINNELYEEAFAIFKKFDVNT 1080
CulexCHC        DHRNLQNLLILTAIKADRSRVMDYINRLDNYDAPDIANIAINNELYEEAFAIFKKFDVNT 1067
PamCHC          ----------------------------------ANIAINNQLYEEAFAIFKKFDVNT 24
DmCHC           DHRNLQNLLILTAIKADRTRVMDYINRLENYDAPDIANIAISNQLYEEAFAIFKKFDVNT 1080
                                                  ****************:*.*****

AedesCHC        SAIQVLIEQVHNLERANEFAERCNEPAVWSQLARAQLQQGLVKEAIDSYIKADDPSAYMD 1140
CulexCHC        SAIQVLIEQVHNLERANEFAERCNEPAVWSQLARAQLQQGLVKEAIDSYIKADDPSAYID 1127
PamCHC          SAIQVLIDNVNNLDRAYEFAERCNEPAVWSQLAKAQLQQGLVKEAIDSFIKADDPSAYID 84
DmCHC           SAIQVLIDQVNNLERANEFAERCNEPAVWSQLAKAQLQQGLVKEAIDSYIKADDPSAYVD 1140
                *:*********:***************:***************** **:**:*::*******

AedesCHC        VVETASKNESWEDLVRYLQMARKKARESYIESELIYAYARTGRLADLEEFVSGPNHADIQ 1200
CulexCHC        VVETASKNESWEDLVRYLQMARKKARESYIESELIYAYARTGRLADLEEFVSGPNHADIQ 1187
PamCHC          VVETAHKTESWEDLVRYLQMARKKARESYIESELIYAYARTNRLADLEEFISGPNHADIQ 144
DmCHC           VVDVASKVESWDDLVRYLQMARKKARESYIESELIYAYARTGRLADLEEFISGPNHADIQ 1200
                *********:********.*****************************:*** * *.:**

AedesCHC        KIGDRCFNDKMYEAAKLLYNNVSNFARLAITLVHLKEFQGAVDGARKANSTRTWKEVCFA 1260
CulexCHC        KIGDRCFNDKMYEAAKLLYNNVSNFARLAITLVHLKEFQGAVDGARKANSTRTWKEVCFA 1247
PamCHC          KIGDRCFDDGMYDAAKLLYNNVSNFARLAITLVHLKEFQGAVDGARKANSTRTWKEVCFA 204
DmCHC           KIGNRCFSDGMYDAAKLLYNNVSNFARLAITLVYLKEFQGAVDSARKANSTRTWKEVCFA 1260
                ****************.*********:*********************:** *.***:***

AedesCHC        CVDAEEFRLAQMCGLHIVVHADELEDLITYYQDRGHFEELIGLLEAALGLERAHMGMFTE 1320
CulexCHC        CVDAEEFRLAQMCGLHIVVHADELEDLITYYQDRGHFEELIGLLEAALGLERAHMGMFTE 1307
PamCHC          CVDSEEFRLAQMCGLHIVVHADELEDLINYYQDRGYFEELINLLEAALGLERAHMGMFTE 264
DmCHC           CVDAEEFRLAQMCGLHIVVHADELEDLINYYQNRGYFDELIALLESALGLERAHMGMFTE 1320
                **************:*** ***:*:**:***.************************:***

AedesCHC        LAILYSKYKPAKMREHLELFWSRVNIPKVLRAAEQAHLWSELVFLYDKYEEYDNAVLAMM 1380
CulexCHC        LAILYSKYKPAKMREHLELFWSRVNIPKVLRAAEQAHLWSELVFLYDKYEEYDNAVLAMM 1367
PamCHC          LAILYSKYKPAKMREHLELFWSRVNIPKVLRAAEQAHLWAELVFL--------------- 309
DmCHC           LAILYSKFKPSKMREHLELFWSRVNIPKVLRAAESAHLWSELVFLYDKYEEYDNAVLAMM 1380
                *****:****.************************:**:*******
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Fig. 1(A). Comparison of the deduced amino acid sequence of *P. Americana* clathrin heavy chain (*Pam CHC*) partial cDNA obtained from GenBank database (accession number: GQ887261.1) with other insect species. The *Pam CHC* is aligned with the homologous region of *Aedes aegypti* (accession number: XM001656826.1), *Culex quinquefasciatus* (accession number: XM001864895) and *Drosophila melanogaster* (accession number: Z14133.1). Gaps are indicated by dashes; identical and similar residues are indicated by asterisks and dots.

2.5 Immunoblot analysis

SDS-PAGE was performed according to the method of (18) using 10% polyacrylamide gel. The ovaries at the appropriate developmental stages were homogenized on ice in 1ml of homogenization buffer (10mM Tris-HCl pH 7.4, 1mM EDTA, 1mM DTT, 2.5% SDS and 1mM PMSF), supplemented with protease inhibitor cocktail (Complete, Roche Diagnostics GmbH, Mannheim, Germany), using HG30 Homogenizer (Hitachi, Tokyo, Japan). The homogenates were centrifuged at 1,000 *xg* for 5 min and aliquots of the supernatants were used. Protein content was measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to the instruction of the supplier. Bovine serum albumin was used as a standard protein. Equal amounts of protein extracts (10µg protein equivalent) were separated on 10% SDS-polyacrylamide gels, and either transferred to 0.45µm Immobilon-P membranes (Millipore, Billerica, MA), or duplicate gels of identical protein extracts were assessed for integrity by Coomassie brilliant blue (CBB). Following incubation of membrane, for 1 h at room temperature (RT), with 5% non-fat dry milk in Tris-buffered Saline-Tween (TBST; 50mM Tris-base, pH 8.0, 150mM NaCl. 0.1% Tween 20). The membrane was then, incubated overnight at 4°C, with gentle agitation, with rabbit anti-Clathrin heavy chain antibody (*CHC*), (a kind gift from Prof. Dr. Alexander Raikhel, Department of Entomology, University of California, Riverside, CA, USA), diluted 1: 15.000 in 1% bovine serum albumin "BSA" (Nacalai) in TBST (B-TBST), followed by incubation for 1 h at RT with donkey anti-rabbit IgG HRP-conjugated secondary antibody (Amersham), diluted 1: 50.000 in B-TBST. The immune complexes were detected using the ECL plus detection system (Amersham) and exposed to Autoradiographic Hyperfilm ECL (Amersham).

2.6 Immunocytochemistry

The dissected ovaries were fixed overnight at 4°C in a modified Bouin-Holland solution supplemented with 0.7% mercuric chloride and without acetic acid, then dehydrated and embedded in paraffin. Sections 9µm thick were cut, mounted on poly-L-lysine-coated slides, deparaffinized and rehydrated. The sections were then treated with Lugol's iodine followed by a 7.5% solution of sodium thiosulphate to remove heavy metal ions and washed with distilled water and Tris-buffered saline (pH 7.6), containing 0.3% Tween 20 (TBST) for 5 min each. Sections were blocked with 5% normal goat serum in TBST for 30 min at RT, and incubated with rabbit anti-*CHC*, diluted 1:2000 in blocking

serum, in a humidified chamber overnight at 4°C. In control experiments, the primary antibody was replaced with preimmune rabbit serum. After rinsing with TBST (3x10 min) at RT, the sections were first incubated with a biotinylated secondary antibody (rabbit IgG-Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA), diluted at 1:200 in blocking serum for 1 h at RT, rinsed again with TBST (3x10 min), and then treated with a horseradish peroxidase (HRP) labelled avidin-biotin complex (diluted in TBST) for 50 min at RT. After incubation, sections were thoroughly washed with TBST (3x10 min) and with 0.05 M Tris-HCl, pH 7.5 (1x10 min). The peroxidase activity was finally revealed by using hydrogen peroxide (0.005%) and 3,3'-diaminobenzidine tetrahydrochloride (DAB, 0.25mM in 0.05 M Tris-HCl, pH 5.5) as a chromogen. Stained sections were dehydrated, mounted in a Biolet mounting medium (Kouken Rika, Osaka, Japan).

The stained sections were all examined under a DX50 microscope (Olympus, Melville, NY) equipped with Nomarski (differential interference contrast), and photographed with a charge-coupled device camera (Olympus).

```
        10        20        30        40        50        60
atttcaaggggcagttgatggtgctagaaaggctaatagcacacggacatggaaggaggtt
  F  Q  G  A  V  D  G  A  R  K  A  N  S  T  R  T  W  K  E  V

        70        80        90       100       110       120
tgctttgcatgtgtggacagcgaagagttccgtctggcccaaatgtgtggccttcacatt
  C  F  A  C  V  D  S  E  E  F  R  L  A  Q  M  C  G  L  H  I

       130       140       150       160       170       180
gtagtacacgcagatgaattagaagacctaattaattattaycaggatcgaggatatttt
  V  V  H  A  D  E  L  E  D  L  I  N  Y  X  Q  D  R  G  Y  F

       190       200       210       220
gaagaactgatcaatttgttggaggcagcactaggttt
  E  E  L  I  N  L  L  E  A  A  L  G
```

Fig. 1(B). Nucleotide sequence (lower case letters) and deduced amino acids (upper case letters) of *Pam CHC* PCR amplified fragment which used as probes for Northern blot analysis. Primers positions are underlined.

3. Results and Discussion

3.1 Sequence analysis and alignment

Analysis of the deduced amino acid of the partial sequence of *Pam CHC* (obtained from the GenBank database, accession no.: GQ887261.1) shows that this sequence is represented as multiple alpha helical repeats occurred in the arm region of the clathrin heavy chain. The deduced amino acid sequence of *Pam CHC* displayed a high level of identity and similarity to mosquitoes and *Drosophila melanogaster CHC*s. The *Pam CHC* was 93% identical to *Aedes aegypti* (Aa), 94% identical to *Culex quinquefasciatus*, and 92% identical to *Drosophila melanogaster CHCs.* Alignment of these sequences revealed that this region of the *Pam CHC* peptide is highly conserved in all analyzed species (Fig. 1A). In all insect species, so far studied, the level of conservation in the middle portion of *CHC* was found to be higher than that of the sequence as a whole. This evolutionary conservation possibly reflects the importance of this region in forming the three-dimensional triskelion (19). These obtained results enabled us to confidently amplify the probe for *Pam CHC* expression analysis from this region (Fig. 1B).

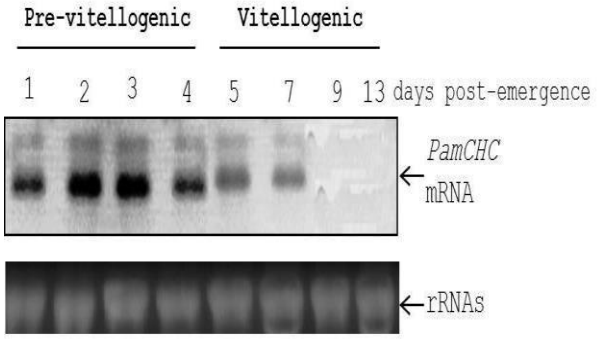

Fig. 2. Developmental expression patterns of *Pam CHC* in the ovary of adult female *P. Americana* during vitellogenic period. Northern blot analyses were performed on total RNA extracted from above-mentioned developmental periods. The transcript signals were probed with fluorescein-labeled cDNA fragments of *Pam CHC* (see Materials and Methods). Ovarian *Pam CHC* transcript (~6.3 kb) is marked by an arrow; the upper band (~7.3 kb) might represent another isoform of *CHC*. Ribosomal RNAs (rRNAs, bottom panel) are shown as internal controls after staining with ethidium bromide. This result is a representative of two to three replicates.

3.2 Developmental expression pattern of *Pam CHC* gene transcript

In order to determine the developmental profile of *Pam CHC* transcript, Northern blot analysis was performed with total RNA extracted from the ovaries during different periods of egg development. The Northern blotting analysis, using a 218bp PCR amplified fragment (Fig. 1A) as a probe demonstrated two transcripts in the current preparations, 6.3 kb and 7.3 kb (Fig. 2). However, the 7.3 kb transcript is much lower than that of 6.3 kb transcript but it follows the same kinetics. Accordingly, the detected 6.3 kb transcript is considered as an (abundant) ovarian-specific *Pam CHC* transcript. Similarly, two *CHC* transcripts were detected in mosquito: 6.5 kb of the mosquito *Aa CHC* transcript was determined to be an ovarian specific transcript, expressed only in oocytes and nurse cells, whereas 7.5 kb *Aa CHC* transcript expressed in the somatic tissues of both sexes (10). The *Pam CHC* ovarian specific transcript was pronounced in the previtellogenic period (days 1-4) but dropped during the vitellogenic period (days 5-7) and ultimately no transcript was detected in fully vitellogenic ovaries (days 9-13). This is in contrast to *Aa CHC* where the transcript level started to increase dramatically from 1 day to 3 days after eclosion and increased further after the onset of vitellogenic periods, reaching its peak by

12h post blood meal (PBM), then decreased considerably by 24h PBM when the endocytotic activity is at its peak (20,11). Recently, (15) reported that the transcription level of *P. americana VgR* gene was pronounced in immature female ovaries of final instar nymphs and in the early previtellogenic period (day 1-2). Thus, it appears that genes coding for receptor-mediated endocytosis are specifically expressed at high levels in the early stages of the ovarian development.

3.3 Developmental profile and cellular distribution of *Pam CHC* protein

In order to analyze the developmental profile of *Pam CHC* protein synthesis, extracts prepared from female ovaries during different stages of development were separated by 10% SDS-PAGE and assayed using an immunoblot analysis (Fig. 3). Consistent with our obtained results from Northern blot analysis, *Pam CHC* protein was detected with an apparent molecular weight of ~175 kDa in ovarian tissues. The *Pam CHC* protein was detected throughout the ovarian developmental periods. However, its level was dramatically higher on days 2 of previtellogenic period and then days 4, 5 of late previtellogenic and early vitellogenic periods. Similarly, *P. americana VgR* protein was also observed early, on the day of adult female emergence. However, *Vg* uptake occurred on day 5, one day after *Vg* first appeared in the haemolymph (15). Together, both *VgR* and *CHC* proteins are produced in the ovary of *P. americana* long before vitellogenesis begins.

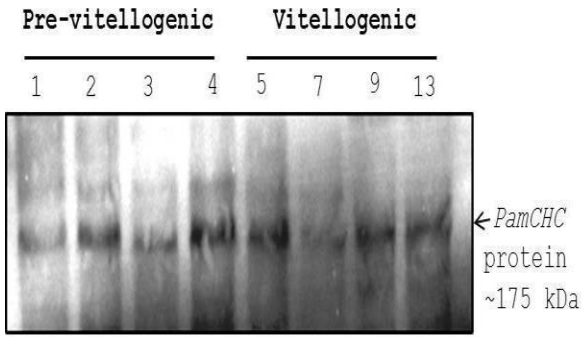

Fig. 3. Developmental expression profile of *Pam CHC* protein in *P. americana* ovaries as revealed by immunoblot analysis. Protein extracts (10µg/ lane) from female adult ovaries at indicted times were separated by 10% SDS-PAGE, electroblotted to Immobilon-P membranes and probed with anti-*CHC* antibody. Arrow on the right indicates the molecular weight (kDa) of the identified *Pam CHC*.

Next, the *Pam CHC* protein was detected by immunocytochemical techniques in the germline cells (oocytes) of the germarium (Fig. 4A). Surprisingly, in early and late previtellogenic ovaries, the *Pam CHC* protein was found to be accumulated primarily in the oocyte cortex adjacent to the follicular epithelium (Fig. 4B-D). No *Pam CHC* protein signal was observed in controls immunostained with preimmune rabbit serum (Fig. 4E). In contrast, previous immune cytochemical study with *P. americana VgR* revealed that *VgR* protein was found to be distributed throughout the oocytes of early previtellogenic ovaries then accumulated in cortex adjacent to the follicular epithelium only in late previtellogenic ovaries (15). Together, our obtained immunocytochemical results suggest a possible role for clathrin in the early fluid phase endocytosis (pinocytosis) in addition to its role in receptor-mediated (*Vg*/*VgR* complex) endocytosis.

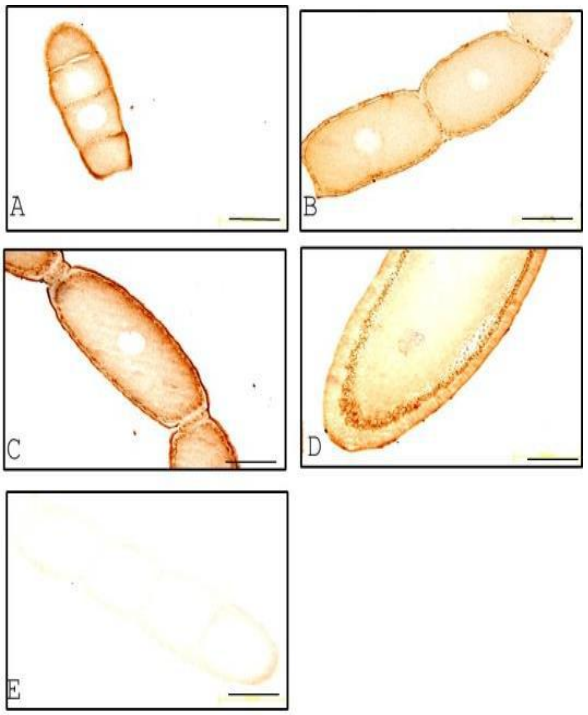

Fig. 4. Immunocytochemical localization of the *Pam CHC* protein in *P. americana* ovariole using 3, 3′-diaminobenzidine tetrahydrochloride (DAB) staining. Immunostaining with *CHC* antibody shows that the *Pam CHC* protein is expressed at high levels in germ-line cells and is also detected in the germarium (A). *Pam CHC* protein is accumulated primarily in the oocyte cortex of the primary oocytes (B–C) and in secondary oocytes (B) just before the start of vitellogenesis. The control immunostained with preimmune rabbit serum (E). Scale bars = 100µm.

4. Conclusion

Two *CHC* transcripts were detected in the American cockroach, *P. americana* during oogenesis, an ovarian specific transcript and the other might be of somatic one, suggesting that *CHC* gene might be represented by two isoforms in this cockroach. However, this suggestion will need further investigation to be confirmed. Both *Pam CHC* transcript and protein are expressed at high levels in the early stages of the ovarian development. Accordingly, a possible role for *Pam CHC* in the early fluid phase endocytosis (pinocytosis) in addition to its role in the receptor-mediated (*Vg*/*VgR* complex) endocytosis was suggested.

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