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Molecular Characterization of a cDNA Encoding Putative Vitellogenin from the Pacific Oyster *Crassostrea gigas*

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ABSTRACT—To elucidate the molecular mechanisms involved in oogenesis, we applied a differential display method to identify genes whose expression was detected only in ovaries containing oocytes. One of the cDNA fragments isolated by mRNA differential display was similar in structure to vitellogenin. Using this fragment, a full-length cDNA encoding putative vitellogenin in the Pacific oyster *Crassostrea gigas* was cloned by RACE (rapid amplification of cDNA ends), and its amino acid sequence was deduced. The open reading frame predicted 1583 amino acid residues. The deduced primary structure of putative vitellogenin in *C. gigas* was shown to be similar to vitellogenins of various other mollusk, fish, crustacean and nematode species, especially in the N-terminal region. Reverse transcription-mediated PCR revealed that mRNA encoding putative vitellogenin was expressed only in the ovary. *In situ* hybridization analysis revealed that putative vitellogenin mRNA was expressed strongly in the follicle cells in the ovary. It is concluded that the follicle cells are the site of putative vitellogenin synthesis.

Key word: follicle cells, mRNA differential display, oogenesis, oyster, vitellogenin

INTRODUCTION

In marine bivalves as in most oviparous animals, a large amount of yolk protein (vitellin) is accumulated in oocytes during ovarian maturation. In vertebrates such as fish, amphibians and birds, yolk protein is synthesized from a precursor, vitellogenin (Vg), produced by the liver, and is transported to the oocytes via the blood circulation system. The relationship between yolk protein and Vg has been extensively studied in *Xenopus laevis* and in the chicken, *Gallus gallus*, and the amino acid sequences of these species have been determined (Gerber-Hunber *et al*., 1987; Van het Schip *et al*., 1987). Vgs have been shown to be present in almost all species of oviparous animals ranging from nematodes to vertebrates, and extensive sequence conservation is seen among these groups (Chen *et al*., 1997). Recently, complete cDNA sequences of Vg have been reported from several fish and crustacean species (LaFleur *et al*., 1995; Mouchel *et al*., 1996; Tsutsui *et al*., 2000; Okuno *et al*., 2002).

In bivalve molluscs, a few biochemical studies of yolk proteins have been carried out (Osada *et al*., 1992; Suzuki *et al*., 1992). In the oyster, vitellin has been isolated and characterized (Suzuki *et al*., 1992; Li *et al*., 1998), but the characterization of Vg has not been reported, and information on bivalve vitellogenesis is still limited. To identify the genes associated with vitellogenesis, we cloned and sequenced more than 100 cDNA fragments using mRNA differential display, which is a method for studying differential gene expression from different sources (Liang and Pardee, 1992), and found that one of the isolated genes was similar to Vg. The objective of the present study was to clone the full-length of the Pacific oyster putative Vg cDNA on the basis of the cDNA fragment obtained by mRNA differential display, to deduce the complete primary amino acid sequence, and to examine the expression of its mRNA.

MATERIALS AND METHODS

Animals and isolation of RNA

Cultured Pacific oysters were collected monthly from March to September 1999 in Gokasho Bay, Mie Prefecture. The gonad was homogenized using ISOGEN (Nippon Gene, Toyama, Japan), and the total RNA was extracted according to the manufacturer's instructions (Chomzynski and Sacchi, 1987).

mRNA differential display

First strand cDNA was synthesized by annealing pd(N)$_6$Primer per 5 µg of total RNA using a First Strand cDNA Synthesis kit

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(Amersham Biosciences Corp., NJ), according to the manufacturer's protocol. The resulting cDNAs were amplified with seven different 10-nucleotide random primers (RA1, 3, 5, 7, 13, 14 and 16) selected from the list of Monna *et al.* (1994). The cycling parameters were as follows : one cycle of 92°C for 1 min, and 40 cycles of 92°C for 1 min, 45°C for 1 min, 72°C for 1 min and lastly one cycle of 72°C for 5 min. The amplified cDNA was then separated on an 8% polyacrylamide gel and stained with ethidium bromide. Complementary DNA fragments whose expression was detected only in the spawning season (July and August) were recovered directly by cutting out the gel slices, and reamplified using the same primer and PCR conditions except an annealing temperature at 55°C. Reamplified PCR fragments were subcloned and sequenced.

Oligonucleotides

The oligonucleotides used as PCR primers are listed here and shown in Fig. 2. Primers for β-actin were based on the nucleotide sequences of sea scallop and oyster (Patwary *et al.*, 1996; Cadoret *et al.*, 1999).

Primer 1. 5'- ACG GGT AAG TAC TGT TGG AC -3'
Primer 2. 5'- GAC GAA TGT TCC GAC GTG GG -3'
Primer 3. 5'- CCC GAT GTC AAT GGC TTA TGG -3'
Primer 4. 5'- GCA GAT GGA AGG ATG TCC ATC AG -3'
Primer 5. 5'- TTC ACA GTC ATG GAG CCC AGC AT -3'
Primer 6. 5'- GAC CCG TCA GAA TTG TTG TCA GAC -3'
Primer 7. 5'- GAC TTC GAA CAA GAG ATG -3'
Primer 8. 5'- GAT ATC GAC ATC ACA TTT C -3'

Rapid amplification of cDNA ends (RACE)

5' RACE was performed using a 5' RACE System (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions (Frohman *et al.*, 1988). First-strand cDNA was synthesized in a 25 μl solution containing 5 μg of total RNA, 2.5 pM of primer 1, and 200 U of Superscript II. PCR with primer 2 and 5' RACE abridged anchor primer was initiated at 94°C for 2 min, followed by 30 cycles of 94°C for 0.5 min, 55°C for 0.5 min, 72°C for 2 min and lastly one cycle of 72°C for 7 min. For 3'RACE, first strand cDNA was synthesized by annealing Not I-d(T)$_{18}$Primer using a First strand cDNA Synthesis kit (Amersham Biosciences) as described above. PCR was conducted with primer 3 and adapter primer using the same conditions but 35 cycles of amplification. The PCR products were subcloned and sequenced. Since these fragments were too long to sequence, PCR walking was performed with the designed primers (primers 4, 5 and 6).

Nucleotide sequence analysis

The cDNA fragments amplified by PCR were subcloned into a pCR2.1 plasmid using a TA Cloning kit (Invitrogen). Both strands of the insert in the plasmid were sequenced on a model 377 DNA sequencer using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA).

Reverse transcription-mediated PCR

Total RNA was prepared from ovary, gill, muscle, mantle tissues of female oysters collected in April, and testis and ovary of the oysters collected in March, May, July and September, and first strand cDNA was synthesized as described above. Twenty-five (Fig. 5A) and 20 (Fig. 5B) cycles of amplification for vitellogenin (primers 4 and 5) and β-actin (primers 7 and 8) using AmpliTaq Gold (Applied Biosystems) were carried out under the following conditions : denaturation at 94°C for 0.5 min, annealing at 54°C for 0.5 min, and extension at 72°C for 0.5 min. At completion of the PCR, fragments were separated on a 1.5% agarose gel and stained with ethidium bromide.

In situ hybridization

For *in situ* hybridization, the digoxigenin (DIG)-labeled sense and antisense RNA probes were transcribed *in vitro* from 807 bp cDNA fragment of oyster putative Vg (region between primers 4 and 5) using a DIG RNA Labeling kit SP6/T7 (Roche Diagnostics, Mannheim, Germany). The ovarian fragments were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 16 hr, embedded in paraffin, and cut into 5 μm serial sections. Deparaffinized sections were treated with proteinase K (10 μg/ml) in PBS at 37°C for 15 min, and then incubated at 42°C for 16 hr in a hybridization buffer (50% formamide, 2 x SSC, 1 μg/μl tRNA, 1 μg/μl Salmon sperm DNA, 1 μg/μl BSA, 10% Dextran sulfate) and 1 mg/ml DIG-labeled probe. After hybridization, the sections were washed three times with 2 x SSC/50% formamide at 42°C for 1 hr and twice with 2 x SSC at room temperature for 15 min. The sections were incubated with the Fab fragment of an anti-DIG alkaline phosphatase-conjugated antibody (1:500 dilution, Roche Diagnostics), and visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics).

RESULTS AND DISCUSSION

Isolation and characterization of cDNA encoding putative Vg

In order to identify the expressed genes in the ovary, transcripts derived from the ovaries during the spawning season and the spent gonads containing no oocytes were examined by mRNA differential display using seven different primers. During this procedure, we identified a cDNA of approximately 1.2 kb in length, which was detected in the ovary during the spawning season (Fig. 1). Sequence analysis of the cDNA fragment followed by a database search revealed a similarity to Vg (Fig. 2: C1184).

Subsequently the 5' and 3' ends of this cDNA were obtained by 5' and 3' RACE with the gene-specific primers, respectively (Fig. 2). The deduced amino acid sequence of

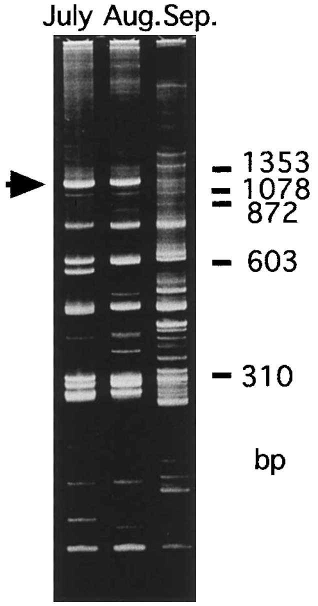

Fig. 1. Differential display of mRNAs from ovary (July and August) and spent gonad (September) of oyster. RNA was reverse transcribed with random primer. Resulting cDNA was amplified with an arbitrary primer (RA16, CCGACAGCTT). A differentially expressed fragment (arrow) was recovered, eluted from the gel slice, and reamplified. Numbers to the right represent molecular size markers.

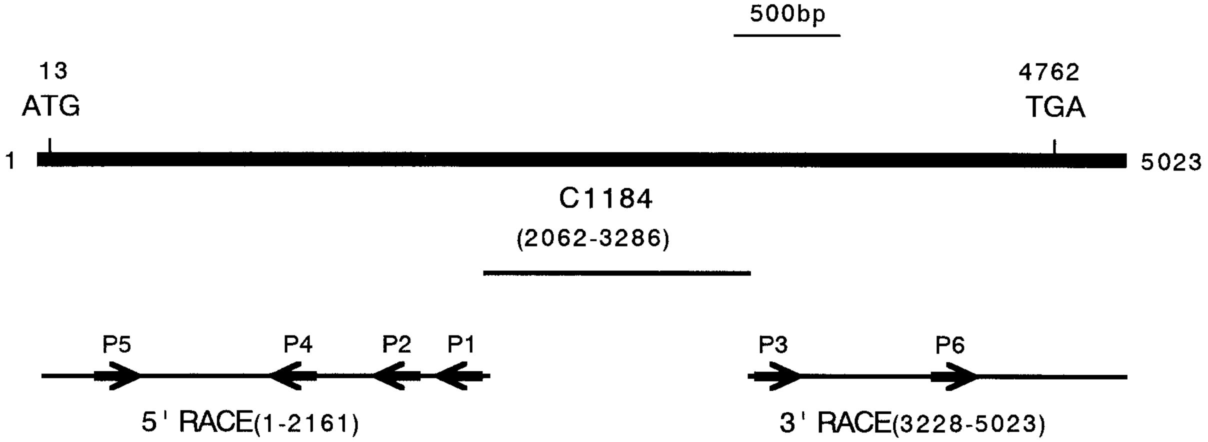

Fig. 2. Cloning strategy and schematic view of *C. gigas* putative vitellogenin cDNA. Location of the specific primers is shown corresponding to oligonucleotides listed in the Materials and Methods. The obtained cDNA is delineated, showing positions of the start codon (13 ATG) and stop codon (4762 TGA). C1184 indicates the PCR product obtained by mRNA differential display.

C. gigas putative Vg cDNA is shown in Fig. 3. The cDNA consisted of 5023 bp, comprising a 5' untranslated region (12 bp), and open reading frame (4749 bp), a stop codon (TGA), and a 3' untranslated region (259 bp). The 3' untranslated region contained a polyadenylation signal (AATAAA) (Fig. 3).

Characterization of the deduced amino acid sequence

The cDNA had an ORF (open reading frame) that encoded 1583 amino acid residues with a predicted molecular mass of 179,191 Da. The deduced amino acid sequence contained a consensus cleavage site, R-X-R-R (Arg^{828} to Arg^{831}), capable of undergoing processing by endoproteases of the subtilisin family (Barr, 1991) that have also been reported in Vgs of insects and crustaceans (Chen *et al.*, 1994; Yano *et al.*, 1994; Tsutsui *et al.*, 2000; Okuno *et al.*, 2002). If this processing site is cleaved by an endoprotease, the resultant two subunits would have calculated molecular masses of 94,000 Da and 85,209 Da. Previous studies have shown that in the Pacific oyster *C. gigas*, two major bands (179 and 110 kD) and several minor bands (Li *et al.*, 1998), and seven protein bands with relative molecular masses of 105, 85, 66, 64, 60, 45 and 41 kD (Suzuki *et al.*, 1992) were recognized in SDS-immunoblot analysis of the vitellin. The cDNA isolated in the present study may correspond to one or some of these proteins. The overall serine content of the oyster putative Vg (11.9%) was higher than in the mosquito (10.1%) and the silkworm Vg (9.8%), which possess polyserine clusters. The deduced amino acid sequence contained 56.2% of serine residues in the region between Ser^{1149} and Ser^{1180}, and this region may be the polyserine domain in *C. gigas* putative Vg. This protein possessed six potential *N*-linked glycosylation sites, which is conserved in vertebrate and invertebrate Vgs.

The deduced amino acid sequence was compared to those of Vgs of the scallop *Patinopecten yessoensis* (partial-length, accession number AB055960), *Caenorhabditis elegans* (Spieth *et al.*, 1991), *Fundulus heteroclitus* (LaFleur *et al.*, 1995), rainbow trout (Mouchel *et al.*, 1996), and kuruma prawn (Tsutsui *et al.*, 2000). The BLAST algorithm indicated homologies of 35% to scallop Vg (residues 24–519), 21% to *C. elegans* vit-5 (residues 24–954), 19% and 24% to *Fundulus* Vg1 (residues 22–285 and 454–953, respectively), 25% and 22% to rainbow trout Vg (residues 24–266 and 567–932, respectively) and 28% to kuruma prawn Vg (residues 559–940). As shown in Fig. 4, the region of *C. gigas* putative Vg which showed similarity to other Vg was limited to the N-terminal and the central region. The C-terminal region showed no significant homology to any of the other reported species. Thus this region appears to possess unique properties compared with Vg of the other known species. Best conserved amino acid motif between invertebrate and vertebrate Vgs (Mouchel *et al.*, 1996) is found in the sequence of *C. gigas* putative Vg (KTIGNAG: positions 603–609). This sequence is speculated to play a highly conserved role in vitellogenesis, such as specific recognition by oocytes (Spieth *et al.*, 1991).

Tissue distribution of oyster putative Vg mRNA

The levels of putative Vg mRNA in various tissues from female oyster and stage-specific expression were measured by reverse transcription-mediated PCR. In April, the oyster contained fully grown oocytes and putative Vg mRNA expression was detected only in the ovary (Fig. 5A). As Fig. 5B shows, putative Vg mRNA expression was detected only in the ovary, and indicated maximum level in March (the early stage of maturation). To determine the distribution of oyster putative Vg mRNA expression in ovary, we performed *in situ* hybridization using DIG-labeled sense and antisense RNA probes, respectively. A strong signal was detected in the follicle cells using an antisense RNA probe (Fig. 6A). In contrast, hybridization with a sense control probe produced no significant signal (Fig. 6B).

The synthesis, secretion and processing of Vgs differ among phyla. Vgs are synthesized by extraovarian tissues such as the liver in vertebrates and the fat body in insects (Sappington and Raikhel, 1998), secreted into the circulatory system, and transported into the ovary. In crustaceans, three tissues have been reported as sites of Vg synthesis:

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   1 TTACACGGCAGAATGTTGCTTCTGCTTGCGGTCTCAACAGCCCTGGTGGCGGCAGCCGCCCAGACTCCCAGTCAAGCCTATGAAAGCAAT
                    M   L   L   L   L   A   V   S   T   A   L   V   A   A   A   A   Q   T   P   S   Q   A   Y   E   S   N     26
     KEYLYEYETQALTGIPMGSTIYSGMKMKSDVRIQFRSRSSATLKMDKLTFAKINDPIESVDPTQQQVPAEMFQPLTGRDAEQMLTDLSRP  116
     INFRYVRGNVKDIHHEADDPEWSVNVKKGLLSMLEMNLEKRKELRKSGVIPQVLRPQSSDEGSMFTVMEPSIVGECETLYRISPWTSTSA  206
     NPWMHITKVRNYQHCLDRPKYFGSMFHLRQCAECVREQSEPLRSASQIRYTLRGNMRQFQIQSAIAESQHVFTPYSAKGGHVATYLNQTL  296
     NLVKEEDVKTSLSEPQSPTKVKSGLQYTSRELEMRDSSSILQQSSDSLSTDKSSPESPQHSSSSSNHHPSPQPPSPQPKTTSNQAADRIR  386
     KLLKMLEVFMKPTIQPEAGPLLMSLLEEIRTADSESLRAVYRECSNGETAATVQDADGHPSICGTTSATEVLVDSILNDQLPQSEAVVAL  476
     GILSLSARPDVLIAKKLLDLTRSSQVSKDRYLKRAAFLCLGSVAGMLREEGWRRSREISRQEEIVKTLISQESRSQTRNELKSKKRELED  566
     LKKREHSIKTKIKQEIVKELQMLMRSTVFDDKILSFKTIGNAGLWEMIPTIRTYIQDKSQPQVLRTQAIYSLRKLARHYPDDIQTTLLPL  656
     YFDQSEKEEVRIGSYLVMTFTEPSRQLLEMVAQSLHRERNPHVGTFVYTHLEQMSNSTYPCLMSWAKNASFAMRFAKKFSPMYHYSRFMH  746
     LSGFNEMHKMGAAAELGLVTTPEEFIPRAGAVNLHTYVFGRSVNFAEIGFNTEGLQTLVSKLVGPLGELTKGKSLVDVLKQRVRRSAESS  836
     QPSDPISQIHKQLKVSPRTSPTPKGHMYMKMMGNELQYITLDGTLVDTLLREGKLLSGVSEQDLKTGLNVEVHRSTMPLEAEIMIPSECG  926
     LPLRLKLHGTAAIKVTGKVGVTGMPSIFEINRPGKQAKELSFNFELRPSVLFQLRGEMEMDAEYFKMGVALKTMAHMETPLSLTASANLP 1016
     KAKFYTKFNIEKLSEKIARLEVSPYTYFKEDPSEITKYPLPRETQEISVAKNAKVFPMSMAYGKQTLGLEIKLSGQAVVRDFEMDVPYYP 1106
     MIGKQEMIVTLSPGTDPQKYVEIQFQLMKRMSQSKQQPTSEESSSSTGLLSWVSSLIGSDQSDQSSPSSSSSSSTSPPKDIRSKDTSLKD 1196
     LLEHLKESQSVHPDGSVVTRNNSMGLIFNVIGIDQSRSIKRHFHISMAAGMNPSPKTTSILLRMNRSPIPTMETKPWNMDVAVNVNLPSR 1286
     LADPSELLSDAYQRELEQQIEVYVRRHGESDYHRWIMLEEPRWESSVLQRLLSLSDTQEVIDEVMNSELSKSEESGTPEDRNIIDLIRKQ 1376
     KQILKEARKIWQRSYDSLRVQDIKAKLSSLIKRSESVRKDLEKDSASVSKSEKSDALRKYCALLQQLIDQRLDEAVRSSRRSLSSKERQD 1466
     IAKSLESSKESLRKILQQQHRSPSKVDSSIHHLALFKSILHRSVKVILKKLKNLRTQNKKINPSNNNSLLNSPRTPCLAINLPKCLVVRL 1556

4681 TGTCAAAGTCAGTGTCAGACCTTTTGCAAAAACAGAAAGATGTGGTTCAGGATATTTCCAGGTCAAAGGAATCAGAGGGATTGAAATCTG
      C   Q   S   Q   C   Q   T   F   C   K   N   R   K   M   W   F   R   I   F   P   G   Q   R   N   Q   R   D   *       1583
4771 TTGATGAGAAAAAAATCAAGGAGGCACTTCTTTTAGCAAAAGAAGTTACTGAAAAGATTCGTCAAGAAAATTCTGGCCCATCTCCTCAAA
4861 ATATCGAGAGGGAAAGACAAAGACTCATGAAATCCGTCATTCAACAACAGCAAATAATGAAATGTTTGGAGCAGCGGGTAAAGAAACCAC
4951 AAACCGCCTCCGGCAAATCTGAAGACCAAATAAAAGAAATTCAGAGAATTATGCAAAAAAAAAAAAAAAAAAA                    5023
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Fig. 3. Deduced amino acid sequence and 5' and 3' untranslated regions of putative Vg cDNA. Positions of nucleotides and amino acids are indicated by numbers on the left and right sides of the figure, respectively. Putative *N*-glycosylation sites are underlined and consensus cleavage sequence for processing by endoproteases of the subtilisin family is framed. The polyadenylation signal is shown in white lettering with black background. The position of DIG-labeled RNA probe used for *in situ* hybridization is indicated by a dotted underline. The nucleotide sequence has been submitted to DDBJ/EMBL/GenBank with the accession number AB084783.

the ovary (Lee and Watson, 1995; Tsutsui *et al.*, 2000), adipose tissue (Okuno *et al.*, 2000) and hepatopancreas (Soroka *et al.*, 2000; Tsutsui *et al.*, 2000; Okuno *et al.*, 2002). Autosynthetic yolk formation is thought to be the main type of vitellogenesis in bivalves and gastropods (Jong-Brink *et al.*, 1983). In marine bivalves, autosynthesis of yolk proteins in the ovary has been postulated, based on the morphological evidence (Pipe, 1987; Dorange and Le Pennec, 1989; Eckelbarger and Davis, 1996). In oyster and other bivalve species, the ovarian acinus is a simple structure containing only developing oocytes and associated follicle cells within a thin germinal epithelium. The function(s) of follicle cells in the bivalve ovary are not well understood, though they are suspected of playing some role in oocyte nutrition. In the present study putative Vg mRNA expression was in the follicle cells in the ovary of *C. gigas*. This suggests that yolk synthesis in *C. gigas* may take place in the follicle cells.

In teleosts, as in other oviparous vertebrates, it is clearly established that the Vg gene expression is regulated by estradiol-17β (E_2), produced by the maturing female ovarian follicles, in the liver (Vaillant *et al.*, 1988; Pakdel *et al.*, 1991; Okumura *et al.*, 2002). In the Pacific oyster, E_2 has been detected in the ovary, and its content shows a synchronous profile with gonadal maturity and decreased in the spawning season (Matsumoto *et al.*, 1997). This is correspondent to the change of putative Vg mRNA levels from early vitellogenic stage to spawning stage, suggesting that E_2 is at least one of the factors which promotes vitellogenesis in oysters. However, further investigation is required in

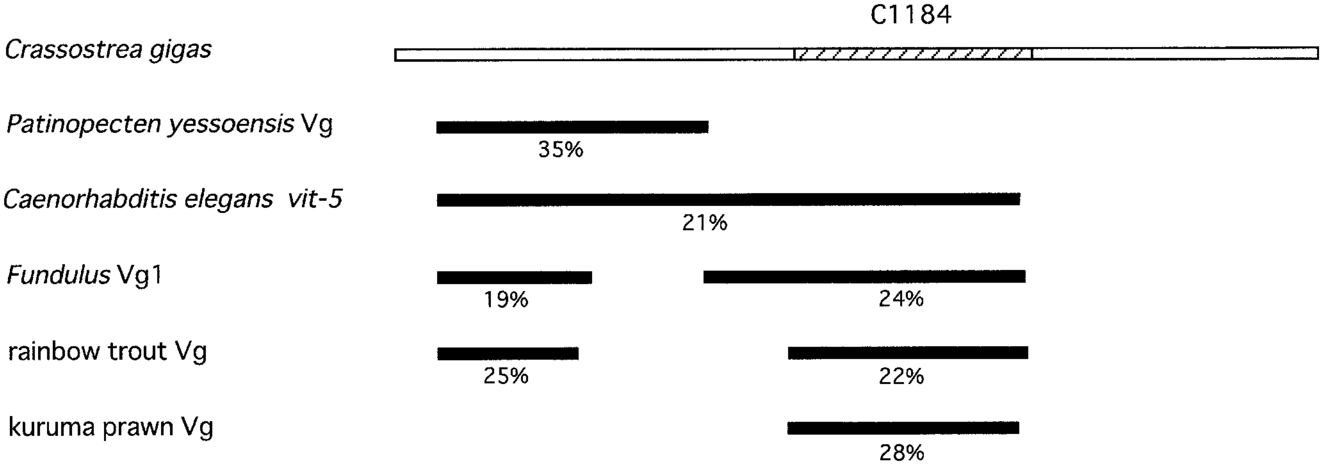

Fig. 4. Alignment of *C. gigas* putative Vg with other animal Vgs: scallop *Patinopecten yessoensis* (partial-length, accession number AB055960); *Caenorhabditis elegans* (Spieth *et al.*, 1991); *Fundulus heteroclitus* (LaFleur *et al.*, 1995); rainbow trout (Mouchel *et al.*, 1996); and kuruma prawn (Tsutsui *et al.*, 2000). The open bar represents the deduced amino acid sequence of *C. gigas* putative Vg. The solid bars indicate the regions of Vg which have homology to those of other animals. Percentage of homology is also indicated.

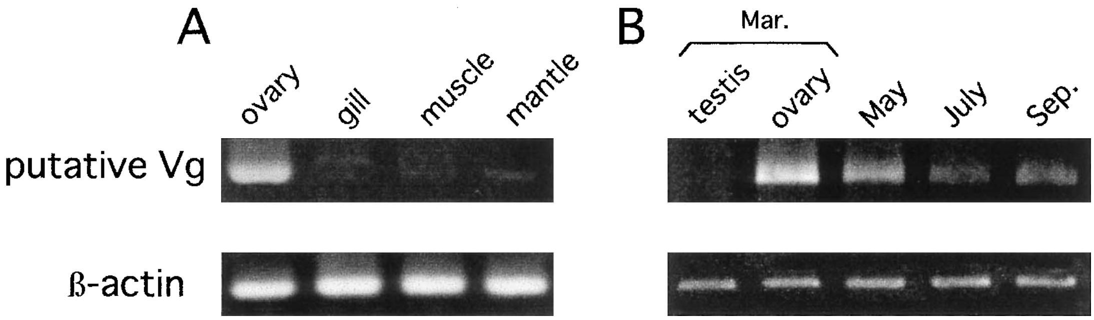

Fig. 5. Distribution of putative Vg mRNA in tissues of oyster. RNA was prepared from ovary, gill, muscle, mantle of female oysters collected in April (A), testis and ovary in March, and ovary in May, July and September (B). β-actin was used as a positive control.

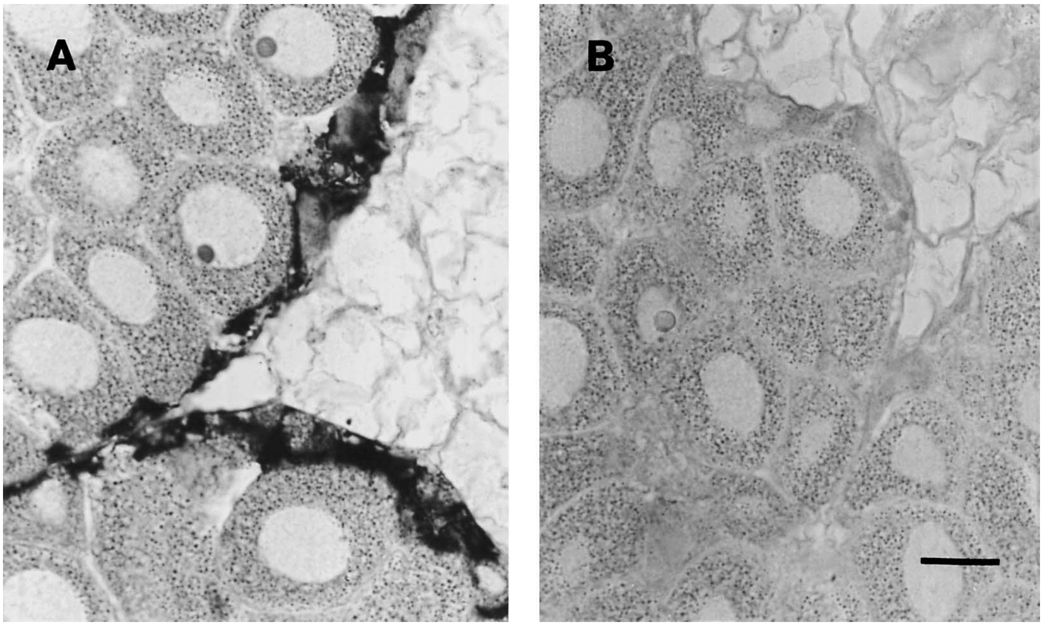

Fig. 6. Localization of putative Vg mRNA in the ovary by *in situ* hybridization. Strong expression of putative Vg was seen in the follicle cells with an antisense probe (A) in the ovary. Hybridization with a sense probe (B) produced no significant signal in the ovary. Bar=25 μm.

order to clarify the estrogen regulatory mechanisms in the oyster.

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