

# Structural and expression analysis of hepatic vitellogenin gene during ovarian maturation in *Anguilla japonica*<sup>☆</sup>

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## Abstract

Vitellogenin (Vtg), the precursor molecule for yolk, is synthesized in the liver under estrogenic control. In all oviparous species, including fish, the process of vitellogenesis is crucial to subsequent embryonic development. This study attempted to obtain the cDNA encoding for Vtg from female Japanese eels, *Anguilla japonica*. Rapid amplification of cDNA ends (RACE) and polymerase chain reaction (PCR) were used to amplify Vtg cDNA prepared from liver extracts. Obtained PCR products were subcloned and sequenced. The overall sequence of eel Vtg cDNA isolated in this study contained 5395 bp nucleotides. This Vtg sequence encodes 1743 amino acids of the precursor molecule, and is entirely composed of the characteristic N-terminal lipovitellin-I region, an internal polyserine domain region, and a c-terminal lipovitellin-II region. The deduced amino acid sequence from these clones shares 34–61% identity with other teleost Vtgs. Northern blot assays of Vtg gene expression following hormonal treatment demonstrated that this Vtg is synthesized in the liver under stimulation by estradiol injection. However, Vtg synthesis may not be enhanced by salmon pituitary homogenate (SPH) induction for the developing ovarian follicles. Notably, the effect of methyltestosterone, following SPH injection, may be more appropriate for the uptake of Vtg by ovarian follicle maturation during the artificial maturation of Japanese female eels.

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**Keywords:** Vitellogenin; Methyltestosterone; Ovarian follicle; Artificial maturation

## 1. Introduction

Japanese eel (*Anguilla japonica*), a catadromous fish, migrates from the sea to freshwater environments during its larval and juvenile stages. Yellow eels remain in the freshwater for 5–12 years, and require significant amounts of energy to develop their gonads as silver eels being their migration toward the sea. The silver eel's complete process of gonadal development has not been observed in wildlife and under rearing conditions. An induction strategy, utilizing serial injections of chum salmon pituitary homogenate, is now routinely applied to generate captivity-bred larvae that can metamorphose into glass eels exceeding 30 cm in length [1]. Therefore, hormonal treatment by injections

or implantations is necessary to induce ovarian development. Although treatment with exogenous SPH is critical for ovarian growth and maturation, multiple crops of developing oocytes are always found in female responders. Of these intraovarian oocytes, only roughly 33% progress to the final maturation stage. The remaining intraovarian oocytes in the ovary are cytological aberrant. It is unknown whether asynchronous oocyte development in these responders results from induction by exogenous SPH. Numerous studies [1,2], and one researcher in the authors' laboratory (unpublished data) has generated Japanese eel eggs and larvae using various induction strategies. However, egg quality and hatchability are marginal with a low larval survival rate.

Vitellogenin (Vtg) is a large phospholipoglycoprotein (200–700 kDa) synthesized in the liver. The process is considered to be principally regulated by ovarian estrogen secreted into the circulatory system either before or during vitellogenesis onset [3], and subsequently internalized by develop-

<sup>☆</sup> The full length cDNA sequence of the Japanese eel vitellogenin has been deposited in Genbank and the accession number is AY775788.

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ing oocytes via receptor-mediated endocytosis in numerous oviparous organisms [4]. Synthesis of Vtg can be induced by exogenous estrogen, and this induction of female-specific protein synthesis occurs in females, males and immature animals. Three major vitellin products: lipovitellin I (LV-I), lipovitellin II (LV-II) and phosvitin (PV) [5], derived from Vtg have been identified in vertebrate oocytes and as a food reserve for developing embryos [4,6,7]. Furthermore, numerous teleost larvae survive solely on the yolk sac after hatching. Therefore, vitellogenesis completion and egg yolk absorption by embryos are crucial for ensuring larvae survival.

Thorough elucidation of the teleost vitellogenesis process is crucial to improving artificial induction methods. Thus, clarification of the molecular expression of the Vtg gene is required. In addition to exogenous gonadotropin, considerable steroid involvement is required to promote oocyte growth and maturation of vitellogenesis. In this study, salmon pituitary homogenate (SPH) and sexual steroids (17 $\beta$ -estradiol and methyltestosterone) were injected weekly to induce vitellogenesis of hepatocytes and ovarian follicles, as well as to derive a full-length cDNA encoding Vtg precursor molecule from *A. japonica* liver by PCR and RACE methods. Based on the cDNA and deduced amino acid sequence, its phylogenetic diversity and the effects of exogenous hormonal treatment on Vtg mRNAs in artificial induction, were discussed.

## 2. Materials and methods

### 2.1. Animals

Pond-cultivated adult female Japanese eels (bodyweight, 450–550 g) were purchased from a local aquaculture farm in Lukang, Taiwan. All these eels were kept in circulated freshwater tanks of 3400 L at 20 °C, and were gradually acclimated to seawater in the same tank. Thereafter, the eels received a weekly intraperitoneal injection of 20 mg/kg bodyweight of SPH suspended in 0.9% saline for 12 weeks. In addition to induce maturation by SPH, this study also received sexual steroids (3 mg/kg bodyweight of 17 $\beta$ -estradiol and methyltestosterone dissolved in propylene glycol) to promote the gonadal and hepatocyte development. Fish were scarified on the seventh day after the final injection. Livers were collected from various treatments of female eels, frozen by immersion in liquid nitrogen. Long-term storage was at –70 °C until preparation of poly(A)<sup>+</sup> RNA.

### 2.2. Histological procedures

Ovarian tissues were fixed with 10% neutral formalin, embedded in paraffin, sectioned to 8  $\mu$ m thicknesses, and stained with hematoxylin-eosin for histological identification of the oocyte developmental stage.

Table 1

Oligonucleotide primers used for reverse transcriptase-polymerase chain reaction experiments

Primer	Sequence
P1	5'-GCTGCAGTCTGGAGTTGCAATCC-3'
P2	5'-CCCTTTTCAATGGCTGCTTCAGC-3'
P3	5'-CTCCTGGGCGGTCTGCCTGAG-3'
P4	5'-CTCCAAGACTGCAGTGCATTC-3'
P5	5'-CCATTGAAAAGGGCAAATTG-3'
P6	5'-GGGCCAGCACTTGGTAGCAAG-3'
P7	5'-GTGCTGGCCCAGGACTGCACC-3'
P8	5'-GACTCCTTCAGGGCAAACACC-3'
P9	5'-CCCTTTGGCCATGAGAGCA-3'
P10	5'-CTTAGGCTGCATGGCTGTCC-3'

### 2.3. cDNA cloning and sequencing

Total RNA was extracted from fragments of frozen tissues using commercial Trizol Reagent (Gibco BRL, New York, NY). Messenger RNA was isolated from total RNA using the FastTrack 2.0 RNA isolation kit (Invitrogen). Messenger RNA (1  $\mu$ g) isolated from livers was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo-dT primer. The strategy entailed first generating a partial cDNA by reverse transcription-polymerase chain reaction (RT-PCR) using one set of degenerate primers (primed with P1 and P2; see Table 1). The PCR was performed in a 50  $\mu$ l volume under the following conditions: a 3 min denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min, and 72 °C for 2 min. Approximately 550 bp product was amplified. The PCR production was gel-purified and T-A cloned into pGEM-T easy vector (Promega), sequence analysis was directly performed by the dideoxynucleotide chain termination method. Clone was sequenced on both strands using SP6 and T7 sequencing primers and internal, specific primers, in an automated fluorescence sequencing system ABI (PE Applied Biosystems, Foster City, CA). Another partial PCR amplicons (primer sets: P3 and P4; P5 and P6; see Table 1) were subcloned and sequenced.

The RACE technique was utilized to clone full-length cDNAs in the 5' and 3' ends using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA). Briefly, 1  $\mu$ g of total RNA was utilized to generate either 5' or 3' RACE Ready cDNA products with specific primers and reagents provided by the kit in the presence of PowerScript reverse transcriptase. In brief, a 50- $\mu$ l PCR reaction mixture composed of 2.5  $\mu$ l 5'-RACE Ready cDNA, 5  $\mu$ l of 10  $\times$  Universal Primer Mix (UPM), 1  $\mu$ l of 10  $\mu$ M gene-specific primer (GSP) and 41.5  $\mu$ l of Master Mix (Clontech) was prepared. Gene-specific primers for the 5' and 3' directions were designed from the Vtg cloned as described above (P7 and P8; see Table 1). 5'-RACE were carried out using GSP1 (primer: P8) and UPM (Clontech) under the following conditions for 30 cycles: 94 °C for 3 min, 94 °C for 1 min, 60 °C for 1 min and 68 °C for 2 min. 3'-RACE were carried out using GSP2 (primer: P7) and UPM under the same conditions as 5'-RACE

amplification. Approximately 515 and 872 bp products were amplified in 5'- and 3'-RACE reaction, respectively. PCR products were gel-purified and T-A cloned into pGEM-T easy vector, then further amplified and purified from plasmid preparations.

The complete coding region of the Japanese eel vitellogenin (Vtg) was amplified from liver mRNA in a RT-PCR using primers targeting the 5' untranslated region (P9) and 3' untranslated region (P10) portions of the cDNA (see Table 1) and expand high fidelity enzyme mix (Roche Applied Science). The PCR was employed under the following conditions: a 3 min denaturation at 94 °C followed by 35 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 2 min.

#### 2.4. Northern blot analysis

Total RNA was extracted from hormone-treated of liver according to the methods described in Chomczynski and Sacchi [8], and was electrophoretically fractionated (5 µg per lane) on 1% agarose gels containing 0.66 M formaldehyde and subsequently transferred to Magna 66 nylon transfer membranes (Micron Separations Inc.). Perhybridization and hybridization to DIG-dUTP (alkali-labile) Vtg-specific probe was performed according to Sambrook et al. [9] using high-stringency conditions. Reactions were detected using anti-AP conjugate and color detection followed by supplier's protocol (Roche Applied Science).

#### 2.5. Statistics

Data are presented as mean ± standard error of the mean. Statistical differences were determined by one-way analysis of variance (ANOVA,  $P < 0.05$ ) followed by Duncan's multiple range tests.

### 3. Results

A 550 bp cDNA fragment was first obtained by RT-PCR from Japanese eel liver total RNA primed with P1 and P2 primers (Table 1). Subsequently, partial PCR amplicons (primer sets: P3 and P4; P5 and P6) were subcloned and sequenced (2.3 and 1.4 kb, respectively). Identities of both cDNA fragments were confirmed by nucleotide sequencing as partial sequences of Vtg. Based on such partial nucleotide sequences, primers P8 and P7 were synthesized for 5'- and 3'-RACE to obtain a full Vtg cDNA sequence. From 3'-RACE, an 872 bp cDNA was amplified from eel poly(A)<sup>+</sup> mRNA primed with P7 (Table 1) and UPM primers. From 5'-RACE, a 515 bp cDNA was obtained with P8 (Table 1) and UPM primers. Overlapping these PCR-amplified cDNA fragments permitted the generation of the entire nucleotide sequence of Japanese eel Vtg cDNA.

Fig. 1 presents the schematic illustration of nucleotide sequence of Japanese eel Vtg cDNA (GenBank Accession Number: AY775788). The overall sequence of eel Vtg cDNA isolated, contained 5395 bp nucleotides, comprising 15 bp of the 5' untranslated region, 5229 bp of the open reading frame (a polypeptide of 1743 amino acids was predicted) and 135 bp of the 3' untranslated region including a 16 bp poly(A) tail. A full-length clone, generated from the specific RT-PCR of liver cDNA using primers P9 and P10 (Table 1), produced a single amplicon of 5.3 kb as expected (Fig. 2). SignalP V1.1 determined that the first 15 amino acids comprised the Vtg signal peptide (<http://www.cbs.dtu.dk/services/SignalP/>) [10]. This predicted site was identical to other vertebrate Vtgs, indicating that the most likely cleavage site for the signal peptide is between Gly(G)15 and Arg(R)16. Two putative polyadenylation signals (AATAAA) are repeated at 25 and 31 bp upstream of the poly(A) trail. A scan of the sequence for post-translational modification sites of putative protein identified 4 potential *N*-glycosylation (Asn-Xaa-Ser/Thr) sites

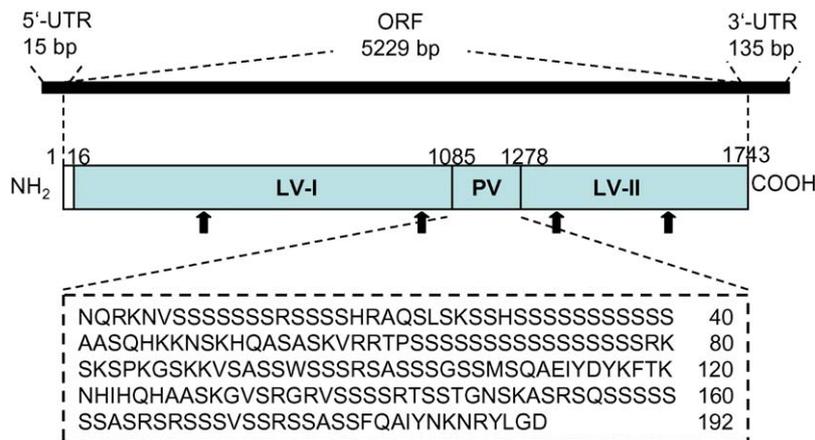


Fig. 1. Schematic illustration of *A. japonica* vitellogenin (Vtg) cDNA (thin black line) and encoding sequence. Three major domains are schematically represented with gray boxes and the number of amino acid residues indicated. Potential *N*-linked glycosylation sites (Asn-Xaa-Ser/Thr) are given by arrows. Signal peptide is portrayed as white area. The dotted box, a well-conserved sequence, found in the phosvitin domains of Vtg. The nucleotide sequence of *A. japonica* Vtg gene obtained from the present study will appear in GenBank under Accession Number AY775788. UTR, untranslated region; ORF, open reading frame; LV-I, lipovitellin-I; PV, phosvitin; LV-II, lipovitellin-II.

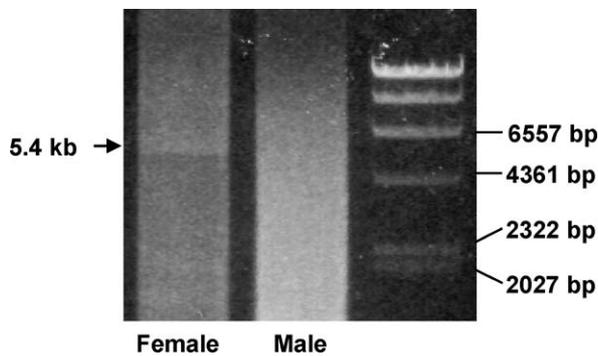


Fig. 2. RT-PCR analysis of the complete coding region of *A. japonica* vitellogenin gene is amplified from P9 and P10 (see Table 1) in female, none in male. The brightest band is about 5.4 kb as indicated with arrow. Marker was the lambda DNA/*Hind* III from promega. Electrophoresis in a 1% agarose gel in TAE buffer.

as expected and was compared with those of other species (Table 2). This Vtg sequence encodes a 1743 amino acid precursor molecule, composed entirely of the characteristic N-terminal lipovitellin-I region (position 16–1071), an internal phosvitin region (position 1072–1256), and a c-terminal lipovitellin-II region (position 1257–1743).

In order to construct a phylogenetic tree for vertebrate Vtgs, a total of 100 bootstrap samples were conducted with programs incorporating the neighbor joining method of ClustalW from the Wisconsin package of the GCG program [11] utilizing the Kimura protein distance method [12] and the PHYLIP program. The Blosum-62-amino-acid substitution matrix was applied to calculate the protein sequence homology [13]. In addition to the Japanese eel, Vtg sequences and accession numbers for other species were obtained from GenBank, including the chicken (*G. gallus*, M18060); African clawed frog (*X. laevis*, Y00354); zebrafish (*D. rerio*, NM170767); rainbow trout (*O. mykiss*, X92804) tilapia (*O. aureus*, AF017250); killifish (*F. heteroclitus*, VgI: U07055 and VgII: U70826); white sturgeon (*A. transmontanus*, U00455); and, lamprey (*I. unicuspus*, M88749). In a phylogenetic tree incorporating these selected vertebrate Vtgs (Fig. 3), the eel Vtg clearly belonged to the teleost cluster and those of other fish Vtgs. In the presence

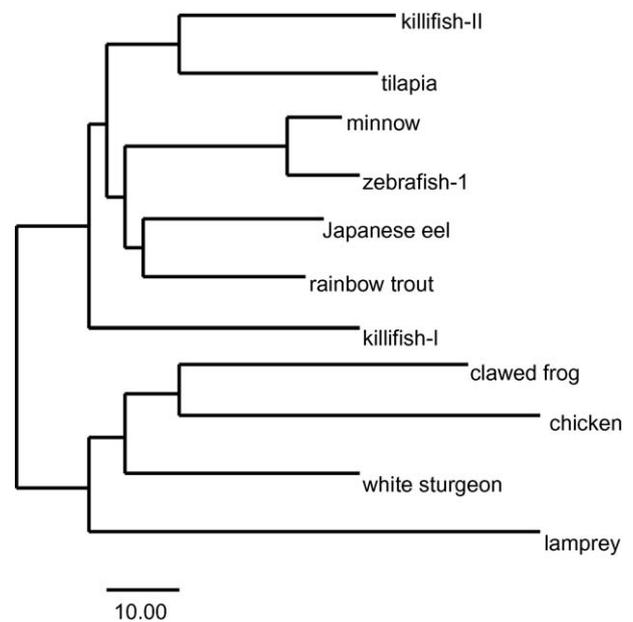


Fig. 3. Phylogenetic tree of the amino acid sequences of vitellogenin in vertebrate animals. The analysis was performed by the method of neighbor-joining, using the distance of Kimura. The numbers beside the branches indicate bootstrap values from 100 replicates.

of the Vtgs protein sequence, phylogenetic analysis results indicate that eel and rainbow trout have a closer evolutionary relationship. Conversely, killifish VtgI diverted from other teleost sequences formed a unique branch on the phylogenetic tree. Many studies utilizing molecular and structural analysis have confirmed the presence of multiple Vtgs in *Xenopus*, chicken and some fish species [14–16]. Thus, further data and evidence may be needed to confirm that multiple Vtgs are also presented in the Japanese eel.

Amino acid composition analysis utilized the SAPS program ([http://www.isrec.isb-sib.ch/software/SAPS\\_form.html](http://www.isrec.isb-sib.ch/software/SAPS_form.html)) [17]. The amino acid composition of Vtg was characterized by an abundance of Ala followed by Ser, Leu, Lys and Val in decreasing order of abundance (Fig. 4).

After 12 weekly injections for artificial induction, experimental fish were scarified and examined according to rear-

Table 2  
Putative post-translational *N*-glycosylation sites of various vertebrates

Species	GenBank accession no.	<i>N</i> -glycosylation sites		
		LV-I domain	PV domain	LV-II domain
Japanese eel	AY775788	1	1	2
Rainbow trout	Z92804	–	1	1
Tilapia	AF017250	–	3	1
Fathead minnow	AF130354	–	–	1
Zebrafish	NM170767	1	–	–
Killifish-I	U07055	6	4	6
Killifish-II	U70826	5	3	8
Sturgeon	U00455	–	4	–
Lamprey	M88749	–	2	1
<i>Xenopus</i>	Y00354	–	1	–

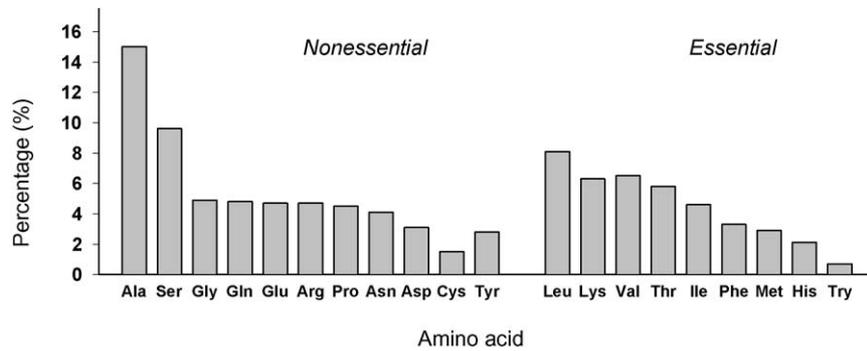


Fig. 4. Essential and nonessential amino acids of Japanese eel Vtg. The analysis was performed by SAPS program ([http://www.isrec.isb-sib.ch/software/SAPS\\_form.html](http://www.isrec.isb-sib.ch/software/SAPS_form.html)).

Table 3

Effects of various hormones treatment on hepatosomatic index and gonadosomatic index of Japanese eel after artificial maturation

Treatment (N)	HSI (g × 100/g B.W.)	GSI (g × 100/g B.W.)	Advanced oocyte after 12 weekly injection
Control (6)	1.22 ± 0.12a	2.92 ± 0.78a	Secondary yolk globule stage
E <sub>2</sub> (7)	2.63 ± 0.90c	5.31 ± 0.65a	Tertiary yolk globule stage
MT (6)	1.69 ± 0.26bc	8.27 ± 2.00a	Tertiary yolk globule stage
SPH (17)	1.58 ± 0.46bc	24.68 ± 14.7b	Migratory nucleus stage
SPH + E <sub>2</sub> (7)	1.85 ± 0.19b	10.64 ± 1.61a	Tertiary yolk globule stage
SPH + MT (7)	1.80 ± 0.30b	33.75 ± 15.63b	Migratory nucleus stage

Data are expressed as the mean ± S.E.M. N = number of individuals. Statistical significance tested by one-way analysis of variance and Duncan's multiple range test: values with the same letter codes are not significantly different at  $P < 0.05$ .

ing conditions, hepatosomatic index (HSI), gonadosomatic index (GSI), and gonadal histology (of the more advanced oocyte). Observational results indicate that treated groups had higher physiological index than those of controls following induction by exogenous hormones (Table 3). The HSI values of the control fish were 1.10–1.38%. Furthermore, E<sub>2</sub>-treated fish (HSI, 1.94–4.24%) has a significantly greater effect ( $P < 0.05$ ) on liver development than various hormone treatments after 12 weekly injections. Although, estrogen induction likely stimulates Vtg synthesis during liver development, this steroid does not have a progressive effect on the gonad development. The GSI values of control fish were 1.60–3.70%. The developmental stage was classified as the secondary yolk globule stage. In E<sub>2</sub>- and MT-treated fish, ovarian follicles were classified as the secondary yolk globule stage, and have similar GSIs (E<sub>2</sub>, 4.30–6.20%; MT, 4.80–10.28%). Under exogenous gonadotropin induction, treatment with SPH (GSI, 7.20–54.20%) alone and SPH plus MT (GSI, 15.70–53.40%) promote completion of vitellogenesis and the most advanced oocytes were at a GVBD stage (46% of intraovarian oocytes in SPH and 76% of these in SPH plus MT; data not shown). In fish treated with SPH plus E<sub>2</sub>, GSI was 7.31–12.07%, whereas, the most advanced oocytes only reached the tertiary yolk globule stage compared with either SPH or SPH plus MT treatment after 12 weekly injections.

To examine eel Vtg expression, Northern blot hybridization was performed, utilizing total RNA prepared from the livers of female individuals with the most advanced oocytes in each treatment. A PCR product of 0.2 kb (data not shown)

corresponding to the Vtg gene was amplified for use as a probe for Northern hybridization analysis. Fig. 5a shows the Northern hybridization patterns of hormone-treated fish liver extracts. Analytical results for RNA revealed a 5.4 kb signal band in Vtg mRNA for E<sub>2</sub>-treated and SPH plus MT-treated fish after 12 weekly injections. However, no alternately spliced Vtg transcripts were detected in liver samples. To understand the regulatory mechanisms of SPH and androgen during early vitellogenesis of artificial induction, a short-term induction also have been performed in this study. In 4 weekly injections for short-term induction, SPH alone and SPH plus MT treatments were compared to examine the expression of Vtg in liver development. Northern blot analysis indicated substantial mRNA transcripts only in the SPH plus MT-treated livers (Fig. 5b). These results suggest that the eel Vtg expression pattern was strictly correlated with a synergistic action on SPH and androgen on Vtg synthesis during either short- or long-term induction. Conversely, no clear expression was detected after SPH and SPH plus E<sub>2</sub> treatments.

#### 4. Discussion

This study isolated and sequenced the cDNA-encoding for the Vtg molecule from Japanese eel liver mRNA using RT-PCR and RACE methods. This Vtg sequence encodes a 1743 amino acid precursor molecule, and is entirely composed of the characteristic N-terminal lipovitellin-I region, an internal phosphitin region (polyserine domain), and a c-

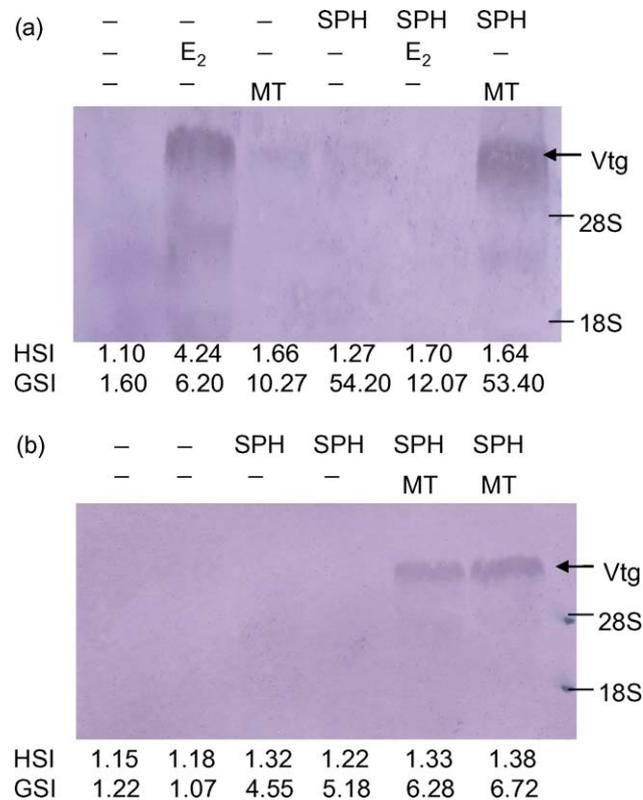


Fig. 5. Northern blot hybridization analysis of *A. japonica* vitellogenin mRNA. (a) Long-term induction (12 weekly injections) with various treatments; (b) short-term induction (4 weekly injections) with SPH alone and SPH plus MT treatments. Ten micrograms of total RNA, selected with individual in each treatment, were loaded on a 1.2% denaturing formaldehyde-agarose gel and separated by electrophoresis at 100 V prepared from various treatments of female eel as indicated on the top of each lane.

terminal lipovitellin-II region, sharing 34–61% identity with other teleost Vtgs. A PROSITE search identified the presence of four potential N-linked glycosylation sites (Fig. 1) in this Vtg: one in the LV-I domain (position 473–476); one in PV domain (position 1085–1088); two in LV-II domain (position 1473–1476 and 1705–1708, respectively). Lim et al. [18], who compared N-linked glycoprotein site with others vertebrate Vtgs, indicated that N-linked glycoprotein is strictly conserved in the PV domain, with few N-linked glycoprotein site in the LV-II domain and none in LV-I. In this study, comparing killifish, zebrafish and Japanese eel Vtg sequences, one or more N-linked glycosylation sites existed in the LV-I domain (Table 2).

Phosvitin is a serine-rich domain containing one or more stretches of serine residues, and the presence of the PV domain was recognized as a characteristic differentiating vertebrate from invertebrate Vtgs. Present in species from the lamprey to the chicken, this PV domain has engendered speculations that the PV phosphate group binds to and transports the metal ion carrier associated with embryonic bone formation [7,19–21]. Evolutionary changes in the PV domain have occurred at a rate faster than that of the LV-I and LV-II region [7]. Therefore, the PV content of teleost

ovaries is variable. The 186 amino acids of Japanese eel PV domain identified in this study is shorter than that for tilapia Vtg1 (195 amino acids) and lamprey (184 amino acids) of their respective PV domain (see [18]). Conversely, the size of PV in zebrafish, rainbow trout and minnow (VtgII) are less than 100 amino acids. Several marine teleosts, including cod [22] and Pacific herring [23,24], have been identified as having very small PV in their ovaries, suggesting that developing eggs can obtain their required phosphate from marine environments. Although the Japanese eel is a catadromous fish that grows in rivers and spawns in the ocean [25], PV content also indicated that the ancestor of the Japanese eel is likely originated from a freshwater environment.

In addition to the serine-rich phosvitin domain, an alanine-rich region was also identified, by PROSITE, at position 642–940 of the eel LV-I domain. This region engenders some questions regarding whether the respective amino acid in Vtg is involved in the process of vitellogenesis or oocyte maturation. Alanine, glutamic acid and leucine have been identified as the most abundant amino acids in rainbow trout [26,27], goldfish [28] and Japanese eel [29] Vtgs. Marine teleost spawn pelagic eggs, and those for barfin flounder [15] and haddock [16] contain a substantial amount of free amino acids compared to that in demersal eggs. *In vitro* studies of marine fishes with pelagic eggs [30], demonstrated that an increase in free amino acids is degraded by proteolysis of LV-I and PV during oocyte maturation and hydration. These processes cause a marked increase in egg size volume and play a significant role in osmoregulation for vigorously adjusted buoyancy in a hyperosmotic environment [31]. In eel Vtg sequence of this study, Ala and Ser had relatively high percentages of nonessential amino acids, and Leu had a relatively high percentage of essential amino acid, respectively. The same composition was observed for haddock LV-I [16] during oocyte hydration. Ala and Ser are the most plentiful nonessential amino acids and are elevated during the vitellogenesis and oocyte maturation stage, whereas Leu, Lys, and Val are the dominant essential amino acids during the stages from oocyte to egg. In addition to osmoregulation for pelagic eggs, these free amino acids may be a primary source of metabolic fuel used for energy by developing marine eggs and larvae. Analysis of the PV domain and amino acids of Vtg indicated that the reproductive action of the female Japanese eel utilizes two different environments for vitellogenesis and oocyte maturation.

Northern blot analysis of liver RNA revealed a 5.4 kb signal band of Vtg mRNA in E<sub>2</sub>-treated fish, and none in SPH-treated fish after 12 weekly injections. Conversely, the similar experiment on female Japanese eels [32] suggests that a transcript 5.8 kb of Vtg (peVg1) mRNA in length existed in estrogen-treated female eels' livers. The discrepancy between results obtained in these observational findings indicates that multiple Vtg genes are also present in the Japanese eel, similar to the existence of multiple Vtgs in some fish species [14–16]. Although the peVg1 is a partial cDNA of the Japanese eel Vtg

[32], multiple Vtg genes may require increased data and evidence in the Japanese eel.

Exocrine hormonal treatments are widely applied to induce maturation in the European eel [33–35] and Japanese eel [36,37]. The few full-grown oocytes and natural spawn were obtained by the use of repeated injections of exogenous gonadotropin, and successfully obtained eel larvae [2,38,39]. Gonadotropin treatment, in the SPH-treated fish, is utilized primarily to promote vitellogenesis in female fish (during the tertiary yolk globule stage to the migratory nucleus stage; GSI values, 7.20–46.90%). However, Northern blot analysis barely detected that Vtg expression in the liver following 4 and 12 weeks, respectively, of induction. Numerous eel studies [40–42] have demonstrated that serum Vtg levels, using a specific antiserum against Vtg, increased in the early Vtg stage and was reduced in the mid and late Vtg stage during SPH treatment. Conversely, Okumura et al. [32] demonstrated that the expression of Vtg in the Japanese eel increased during SPH treatment during the early Vtg to the late Vtg stage and decreased in the migratory nucleus stage. In this experiment, ovarian follicle development in the SPH-treated fish progressed to the migratory nucleus stage after long-term induction, while Vtg synthesis may have been decreased or remained at constant low levels in the liver (Fig. 5a). In addition, all the liver tissues were harvested on day 7 after the final injection, and Vtg mRNA stability may be decreased by SPH treatment. Stimulation of Vtg gene expression, resulting from hormonal treatment, requires further research.

Vitellogenin is synthesized and post-translationally modified under stimulation of  $17\beta$ -E<sub>2</sub>, and by binding to its estrogen receptor (ER) in hepatocytes [43,44]. In this study, the HSI values of the E<sub>2</sub>-treated fish were higher than that of SPH-treated eels ( $2.63 \pm 0.90$  and  $1.58 \pm 0.46\%$ , respectively) after 12 weekly injections. However, ovarian development (GSI values, E<sub>2</sub>: 4.30–6.20%) in long-term artificial maturation was not affected. In the induction of artificial maturation in the European eel [35,45,46], estradiol injection stimulated Vtg synthesis and secretion in the circulation, and did not promote vitellogenesis. These observations indicate that Vtg does not uptake into the ovarian follicle under a lack of endocrine gonadotropin, and that ovarian follicle development was halted or maintained in the tertiary yolk globule stage. Notably, Northern blot analysis and HSI values ( $1.85 \pm 0.19\%$ ) demonstrated that SPH plus E<sub>2</sub> did not have an additive effect on hepatic development (Fig. 5a). Conversely, the pituitary hormone, by some unknown mechanism, may negatively affect Vtg synthesis for E<sub>2</sub> treatment.

Induction of vitellogenesis by androgen has been demonstrated in several teleosts [47–49]. Following long-term induction, treatment with MT alone had no significant effect on the HSI values and Vtg mRNA expressions, and ovarian follicles were maintained in the tertiary yolk globule stage. Although the androgen did not induce hepatic Vtg expression or affect gonadal development in this study, Vtg expression and gonadal development were significantly induced by co-treatment with SPH and MT (Fig. 5a and Table 3). In either

short- or long-term induction (Fig. 5a and b), this synergistic effect of SPH plus MT-treated fish directly affected gonadal development of Japanese female eels, and it can be assumed the potential role of androgen. High doses of androgen stimulate Vtg synthesis in many teleost fish [49,50]. However, numerous studies have also suggested that the androgen can bind to the estrogen receptor [47,51–53] or that androgen serves as an estrogen precursor converted by aromatase [54,55]. Compared with the results of SPH plus E<sub>2</sub> treatment, methyltestosterone, an aromatizable steroid, is not only involved in hepatic aromatase activity in this study, and it also has progress effects in Vtg synthesis and ovarian follicle development by co-treatment with SPH. Although the synergistic effect of co-treatment with SPH and MT induced a stronger hepatic vitellogenic response than SPH or MT alone, it should be noted that crude salmon pituitary homogenate was utilized in this study. In addition to sGTHs, the certain hypophysial components of salmon pituitary extract may involve the pattern of vitellogenesis and steroidogenesis as growth hormone (GH) [56,57]. An *in vivo* study indicated that GH treatment enhanced E<sub>2</sub> receptor levels in hepatocytes [58]. Following the findings derived by numerous studies [51,57], GH and high concentration of androgen were combined to promote the vitellogenic response by estrogen receptor. These observations likely account for the co-treatment results of SPH plus MT in this study, indicating that a close relationship exists between multiple hormones involved in full vitellogenic response of oviparous animal. However, co-treatment of SPH plus E<sub>2</sub> has a contradictory relationship in response to GH and E<sub>2</sub>. As described above, GH enhances E<sub>2</sub> receptor levels and is a potential factor in the E<sub>2</sub> effect of vitellogenesis in silver female eels [56,57,59]. SPH plus E<sub>2</sub> treatment however did not promote sexual maturation, hepatic Vtg synthesis or gonadal development. Therefore, other hypophysial components of the salmon pituitary extract may positively or negatively affect vitellogenesis induction.

In conclusion, a full cDNA-encoding vitellogenin molecule from Japanese eel liver mRNA was cloned using RT-PCR and RACE methods. By utilizing this sequence, the expression of various hormonal treatments in the liver during artificial maturation was examined. Analytical results demonstrated that androgen is directly or indirectly involved in Vtg synthesis [47], and has a potential effect on the SPH treatment of vitellogenesis. The present study also presented a useful strategy for exocrine hormonal treatments for artificial maturation of Japanese female eels. In addition to SPH, participation of sexual steroids must be considered in vitellogenesis and folliculogenesis of gonadal development, particularly for androgen.

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