

## Vitellogenin, a Biomarker for Environmental Estrogenic Pollution, of Reeves' Pond Turtles: Analysis of Similarity for its Amino Acid Sequence and Cognate mRNA Expression after Exposure to Estrogen

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**ABSTRACT.** Vitellogenin (VTG), a biomarker for environmental estrogenic pollution, can be detected in the bloodstream of oviparous animals before morphological and functional abnormalities appear due to exposure to environmental estrogens. Reports observing VTG in turtles have been limited. We therefore cloned and sequenced a partial cDNA of VTG in Reeves' pond turtle, *Chinemys reevesii*. The cloned cDNA fragment possessed the start codon and 2,229 bp, encoding 743 amino acid residues. A sequence of deduced amino acid from the cDNA did not contain a high serine content, such as that which exists in phosvitin. Two N-glycosylation sites were found in the sequence. The sequence was compared to those of two birds (chicken and herring gull), one amphibian (*Xenopus*), and five fishes (carp, zebrafish, eel, haddock, and red seabream). The *C. reevesii* VTG was similar to that of herring gull (78%, value of positives), chicken (76%), *Xenopus* (69%), eel (63%), red seabream (62%), haddock (62%), carp (62%), and zebrafish (61%). The phylogenetic tree showed that *C. reevesii* VTG existed between the amphibian and birds, and it was present far from fish VTGs. A reverse transcription-polymerase chain reaction method was employed to detect the mRNA expression of the *C. reevesii* VTG through the use of primers designed from our sequence. The VTG mRNA expression (292 bp) was proven in the total RNA extraction from the liver of the juvenile turtles which were treated with estradiol-17 $\beta$ . The information herein would be useful for ecotoxicological studies using freshwater turtles and these findings are expected to contribute positively towards wildlife conservation.

**KEY WORDS:** cloning, mRNA expression, similarity, turtle, vitellogenin.

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There has been a large focus on environmental pollution in recent years and many approaches have been employed to assess the level of pollution [6]. Numerous ecotoxicological studies have been performed in various wild or laboratory animals. A series of field studies in alligators (*Alligator mississippiensis*) in Florida, U.S.A., demonstrated that endocrine-disrupting chemicals (EDCs) induced gonadal abnormalities [14-17]. A couple of field studies on other reptilian species such as fresh-water turtles suggest the cause-effect linkage of organochlorine contamination with the occurrence of embryonic abnormalities [2] and the sexual dimorphism in turtles [11]. These cases indicate that we need more sensitive markers before morphological and/or functional abnormalities appear in wildlife in order to ensure adequate conservation. Vitellogenin (VTG) has become recognized as a useful biomarker which indicates the level of environmental pollution with EDCs.

VTG is synthesized in the liver of oviparous animals under estrogenic control, and then is released into the blood stream, incorporated into the oocytes, and subsequently accumulated as egg yolk proteins. Research regarding VTG has been done over the past two decades on various animal species [33]. The VTG protein of birds is cleaved in the

oocytes into phosvitin and lipovitellin [3]. The biochemical, physicochemical, and molecular biological properties of VTG have been well characterized for birds and fish; polymorphism of the VTG gene was found in some species of birds and fish. On the contrary, only a small number of reports have described VTG in reptiles, especially in turtles. The VTG of eastern painted turtle, *Chrysemys picta*, converts into lipovitellin and phosvitin, and these two derivatives are recombined in the oocytes to form a crystalline structure [24]. The molecular weight of the *C. picta* VTG is 210 to 220 kDa, and that of phosvitin is 44 kDa. The *C. picta* lipovitellin is composed of two major subunits, with a molecular weight of 85 and 120 kDa [19]. The phosvitin of *Xenopus* possessed a high serine content [13]. In 1980, Ho *et al.* reported that 50% of the total amino acids in *C. picta* phosvitin were serine [19]. In 1993, a cDNA encoding VTG was cloned from *C. picta* [4] and its cognate mRNA was detected in the liver [5]. The seasonal changes and steroidal regulation of vitellogenesis in *C. picta* have also been studied [8, 9, 12, 17]. Recently, the effects of a pituitary growth hormone on vitellogenesis were examined in *C. picta* [10]. In these reports, the regulatory mechanisms of mRNA expression and protein synthesis of VTG in *C. picta* were studied in detail, but the nucleotide sequence of the turtle VTG has not yet been clarified.

The detection of plasma/serum VTG in male and female indicates the presence of pollution with estrogen mimics. In 1995, Palmer and Palmer prepared a polyclonal antibody

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(pAb) against the VTG of the red-eared turtle, *Trachemys scripta*, and detected the VTG in the serum of male turtles injected with estradiol-17 $\beta$ , diethylstilbestrol as a synthetic estrogen, and DDT (1-chloro-2-[2, 2, 2-trichloro-1-(4-chlorophenyl)ethyl]benzene) as an EDC [26]. Herbst *et al.* reported the isolation of VTG in green turtles (*Chelonia mydas*) and the preparation of monoclonal antibodies (mAbs) against *C. mydas* VTG [18]. In one study, the serum VTG concentration of the captured *C. picta*, which had been distributed in polluted and non-polluted ponds, was determined [21]. Among 28 individual (juvenile and male) captured from the polluted pond and 23 from the non-polluted pond, all of the individuals consistently showed a low levels of the serum VTG. In 2005, Rie *et al.* reported that 42 male captured from polluted pond did not show a significant increase in VTG concentration, in comparison to the VTG concentration of 54 male individuals from the non-polluted pond [29]. These findings indicate that the ecotoxicological significance of turtle VTG has not been established.

Estrogen induces the expression of VTG mRNA before synthesis of VTG protein. This simple principle suggests that detection of VTG mRNA would be superior to that of VTG protein. In addition, modern genetic techniques would provide more sensitive methods than immunological techniques to detect VTG protein. There are numerous reports concerning the mRNA expression of VTG which investigate the effects of estrogens through reverse transcription-polymerase chain reaction (RT-PCR) in birds [25], zebrafish [22], so-iuy mullet [1], and marine mussel [28]. In turtles, however, only northern blotting analysis has been developed [5, 7–10].

Reeves' pond turtles, *Chinemys reevesii*, are widely distributed in Japanese aquatic ecosystems. We purified the VTG protein of *C. reevesii*, prepared a pAb against the VTG, and established a detection method of the VTG with the pAb [30]. The mAbs against the VTG of *C. reevesii* were prepared and used for analyzing the immunological properties of *C. reevesii* VTG and egg yolk proteins [23]. In this study, we presented a nucleotide sequence of partial cDNA encoding the VTG of *C. reevesii* and compared the amino acid sequence among the VTG of birds, fish, and amphibian. In addition, we developed an RT-PCR method to evaluate the mRNA expression of the VTG in the liver of the juvenile male turtles after estrogen exposure in order to contribute to environmental conservation.

## MATERIALS AND METHODS

### *Cloning of cDNA encoding vitellogenin of C. reevesii:*

Total RNA was extracted from the fresh liver of a mature female *C. reevesii* turtle (Shimizu Laboratory Supplies, Kyoto, Japan) by using an Isogen kit (Nippon Gene, Tokyo, Japan). The mRNA was isolated from the total RNA through the use of a PolyA Tract mRNA Isolation System (Promega, Madison, U.S.A.). A cDNA library of *C. reevesii* liver was prepared using a cDNA PCR Library Kit (Takara-Bio, Tokyo, Japan). The 5' adaptor was linked to the cDNA library to perform 5' rapid amplification of cDNA ends (RACE) through the use of a Marathon cDNA Amplification kit (Clontech Laboratories, Palo Alto, U.S.A.). The degenerate primers were designed from the sequence of the VTG of *Xenopus laevis* (GenBank accession no. Y00354), green anole (*Anolis carolinensis*, AF199489), zebrafish (*Danio rerio*, AF406784), quail (*Coturnix coturnix japonica*, AF199490), chicken (*Gallus gallus*, M18060), herring gull (*Larus argentatus*, AY045719), and carp (*Cyprinus carpio*, AF414432) (Table 1). PCR was performed with the library, rTaq polymerase, dNTP mixture, MgCl<sub>2</sub> (Toyobo, Osaka, Japan), and the degenerate primers in 35 cycles, which included denaturing at 94°C for 1 min; annealing at 37°C for 1 min; and extension at 72°C for 1 min. The amplified cDNA was then ligated into a TOPO vector (Invitrogen, Carland, U.S.A.) using a TA cloning technique. One Shot TOP10 *E. coli* cells (TOPO TA Cloning Kit, Invitrogen) were transformed with the vector. Next, the plasmid was purified from the cloned *E. coli* using a QIAprep Spin Miniprep Kit (Qiagen, Tokyo, Japan). The nucleotide sequence of the insert was determined with an Autosequencer Long-Read Tower (Amersham Pharmacia Biotech Japan, Tokyo, Japan) with kits of Thermo Sequenase Cy5 and Cy5.5 Dye Terminators (Amersham). The PCR with VTGSENCE1 and VA800 (Table 1) amplified fragment II (Fig. 1). Fragment II possessed 389 bp. Fragment I was obtained using the 5'RACE method with the AP1 primer and VgaC primer designed from the sequence of fragment II. Fragments II to V were amplified by PCR with the respective primers shown in Table 1.

The phylogenetic relatedness for the VTG was analyzed among *Xenopus vtg b1* (*X. laevis*, GenBank accession no. AB092605), herring gull (*L. argentatus*, AY045719), chicken vitellogenin II (*G. gallus*, NM 001031276), eel

Table 1. Primers used in cloning of vitellogenin gene of *Chinemys reevesii*

| Fragment number | Name      | Orientation | Sequence (5' to 3')                     | Fragment size (bp) |
|-----------------|-----------|-------------|-----------------------------------------|--------------------|
| I               | AP1       | Forward     | CCATCCTAATACGACTCACTATAGGGC             | 538                |
|                 | VgaC      | Reverse     | ATCTGATAGACCTGCTGGGACACAGC              |                    |
| II              | VTGSENCE1 | Forward     | AGAGGAAT(A/T)(C/T)TGAAC                 | 389                |
|                 | VA800     | Reverse     | GC(A/C/G)A(A/G)(C/G/T)TGTTGTCTTGCTTCCAT |                    |
| III             | vgsA      | Forward     | CAGGAGGAATAACCGAGTCTCTATGACC            | 907                |
|                 | VGr1433   | Reverse     | CATTGCCCTTGCTGGCTGCCT                   |                    |
| IV              | VS1380    | Forward     | CT(C/T)CAGCCCATCCATGA(C/G)(A/C)TGGC     | 295                |
|                 | VTGANTI6  | Reverse     | ACAAGC(A/C)A(C/T)CAT(G/T)CGCAC(C/T)TC   |                    |
| V               | VGf1450   | Forward     | GGCAATGCAGGACAACCAGCCAGTA               | 669                |
|                 | VA2600    | Reverse     | C(A/T)GTGTT(A/C/G/T)AC(A/T)CCCAGCGT     |                    |

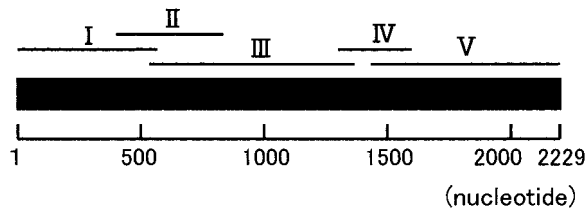


Fig. 1. A schematic drawing of the cloned fragments of cDNA of *Chinemys reevesii* turtle vitellogenin.

(*Anguilla japonica*, AY775788), red seabream VgA (*Pagrus major*, AB181838), haddock vitellogenin A (*Melanogrammus aeglefinus*, AF284035), zebrafish vitellogenin 1 (*D. rerio*, AF406784), and carp vg-B1 (*C. carpio*, AB331884), using the N-J plot [27] with the ClustalX program [32]. The full sequence of amino acid of the VTG of these animals has been clarified.

**Turtles and the injection of estrogen:** The male *C. reevesii* turtles purchased from Shimizu Laboratory Supplies had a carapace length of 8 to 10 cm. Estradiol-17 $\beta$  (Sigma-Aldrich Japan, Tokyo, Japan) was dissolved in a small volume of ethanol and diluted in corn oil (Wako Pure Chemicals, Osaka, Japan). Twelve turtles were injected with the estradiol-17 $\beta$  at 0.5 mg per kilogram body mass in the abdominal cavity. Four turtles were sacrificed by deep anesthesia with diethyl ether 6, 12, and 24 hr after injection. As a control, corn oil was injected into four turtles (designated 0 hr). The liver samples were frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

**mRNA expression of *C. reevesii* vitellogenin in the liver of juvenile turtles:** The total RNA was isolated from the liver of the estrogen-treated male turtles, and treated with RQ1 RNase-Free DNase (Promega). The RT was performed with the treated RNA, Oligo(dT)20, dNTP mixture, and ReverTra Ace $\alpha^{-}$  (Toyobo). The PCR was done with the cDNA, Gene Taq NT polymerase, dNTP mixture (Toyobo), and the primers (vgsA22 and vgaC19) designed from our sequence (Table 2). The  $\beta$ -actin gene was used as an internal control. The primers for  $\beta$ -actin were designed from the sequence of snapping turtle  $\beta$ -actin (*Chelydra serpentina*, GenBank accession no. AF541916). The amplification of cDNA was done in 30 cycles with denaturing at  $94^{\circ}\text{C}$  for 1 min, annealing at  $55^{\circ}\text{C}$  for 1 min, and extension at  $72^{\circ}\text{C}$  for 1 min. After agarose gel electrophoresis and ethidium bromide-staining, the density of the products was determined from a photograph using computer software, NIH image (free distribution). A ratio of the expression of the VTG

mRNA to that of the  $\beta$ -actin mRNA was evaluated using Student's *t*-test between the control (0 hr) and each tested group.

## RESULTS

**Isolation of vitellogenin gene of *C. reevesii* and analysis of the amino acid sequence of *C. reevesii* vitellogenin:** The clone consisted of Fragments I through V presented a partial cDNA of *C. reevesii* VTG with 2,229 bp, encoding 743 amino acid residues. The sequences of nucleotides and deduced amino acids are shown in Fig. 2. The first three nucleotide started as ATG (start codon) and the region of 5' end of the clone did not possess either a leader sequence or an untreated region.

Forty three serine residues (5.8%) were found in the total amino acid residues. The deduced amino acid sequence of the cloned cDNA showed two N-glycosylation sites (Asn<sup>185</sup> and Asn<sup>603</sup>). The region of 1 to 490 amino acids of the deduced amino acid sequences of the part of the *C. reevesii* VTG, two birds, one amphibian, and five fishes were aligned using BLAST in NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) (Fig. 3). The *C. reevesii* VTG was 78% (value in positives) similar to that of herring gull and 76% of chicken, 69% of *Xenopus*, 63% of eel, 62% of red seabream, 61% of zebrafish, 62% of haddock, and 62% of carp, respectively. The phylogenetic relatedness for the VTGs is illustrated with the un-rooted style in Fig. 4. The bird VTGs formed a closely related pair. The fish VTGs formed a closely related cluster. The *Xenopus* VTG was present independently from the VTGs of fish and bird. The *C. reevesii* VTG was present independently from the bird VTGs and the amphibian VTG, and it was located far from the fish VTGs.

**mRNA expression of vitellogenin of *C. reevesii* turtles treated with estrogen:** The expected product (292 bp) was detected in the liver of the turtles 6 hr after the injection of estradiol-17 $\beta$  but not in the liver of the control turtles injected with corn oil (Fig. 5, Panel A). No such products were detected in the liver of intact (no treatment) turtles (data not shown). A time-course of a ratio of the VTG product to the control  $\beta$ -actin product is graphed in Panel B of Fig. 5. The ratio was significantly increased 6 hr after the injection of estradiol-17 $\beta$  ( $P < 0.05$ ). The increased ratio was maintained 24 hr after the injection.

Table 2. Primers used in expression of mRNA of *Chinemys reevesii* vitellogenin

| Gene           | Name   | Orientation | Sequence (5' to 3')    | Position | Product size (bp) |
|----------------|--------|-------------|------------------------|----------|-------------------|
| Vitellogenin   | vgsA22 | Forward     | GGGATTGGAGGTGTCTGCCTTA | 466–487  | 292               |
|                | vgaC19 | Reverse     | GCAACACCAGCGGGTTCAT    | 740–758  |                   |
| $\beta$ -actin | BAF1   | Forward     | TGTGATGGTGGAATGGGTCAG  | 1–22     | 452               |
|                | BAR45  | Reverse     | GGATCTTCATGAGGTAGTCCG  | 432–452  |                   |

10 20 30 40 50 60 70 80 90 100 110 120 130 140 150  
 ATGAGGGGAATCTTACTAGCGCTAGTGTTCACCCCTTGTGGGGAGTGAAAGATGACCTGAGCCTGATTTTAGTAGCAGCAAGACCTACTTGTACAACATATGAGGCCTTACCCTGAATGGACTGCCAGAGCCTGGTGGCAAGAAGCTGGG  
 M R G I L L A L V F T L V G S E K Y D L S L I L V A A R P T C T T M R P Y P E W T A R A W L A R T G  
 160 170 180 190 200 210 220 230 240 250 260 270 280 290 300  
 CTGAGACTGTCTGTAAAGTAGAGATCAGTGTATTGTACAGAGGAATCACCTCCTCAAGATCCGATCTCCACAGCTGGAGGAATACAATGGGATCTGGCCAGAGATCCATTTCATTCGAGCTTCCAAACTCACTCAGCTGATCGCTCGG  
 L R L S C K V E I S V L S Q R N H L L K I R S P Q L E E Y N G I W P R D P F I R A S K L T Q L I A P  
 310 320 330 340 350 360 370 380 390 400 410 420 430 440 450  
 TATCTCACCACCCCTTTAAGTTTGAATACAGCAATGGGCGGTTGGAACAATTTATGCCGAGAGATACCCCGTCACTGATTAACCTAGTGAGAGGAATTATGAACATGTTGCAGATAACTATCAAAAAGTCACAGAAATGCATAC  
 Y L T Q P F K F E Y S N G R V G N I Y A R E D T P V T C I N L V R G I M N M L Q I T I K K S Q N A Y  
 460 470 480 490 500 510 520 530 540 550 560 570 580 590 600  
 GACTTGAAGAGGCTGGGATTGGAGTGTCTGCCTTACAAGTACGTTATCCAGGAAGACAGGAGGAATAACCGAGTCTCTATGACCAAAACCAAGACCTCAACGACTGCCAGGATAAAGTGGTGAAGGATATTGGATGACTTACATC  
 D L Q E A G I G G V C L T R Y V I Q E D R R N N R V S M T K T K D L N D C Q D K V V K D I G M T Y I  
 610 620 630 640 650 660 670 680 690 700 710 720 730 740 750  
 CGCCCCGTCTCTTCTGCCCTTGAAGAAAATATTGAAAAGAACAGCTGCATTACCTACAAATTGAAATATGCAGATTCTGGCACCCCTGATCAGAGAAGCTGTGCCAGGAGTCTATCAGATCTCACCTTCAATGAACCGCT  
 R P C P S C P L K E N I V K G T A A F T Y K L K Y A D S G T L I T E A V S Q Q V Y Q I S P F N E P A  
 760 770 780 790 800 810 820 830 840 850 860 870 880 890 900  
 GGTGTTGGGACATGGAGCAAGACAAGAAGTACCTTGGTTGAAGTGAAGGATGAAGCGGTGAGCCCTCCAGAAATTCAGCTGCAGAACCGTGGAGGCGCTGCTTATCATTTCCACCACCTACTGCTCCAGATGCCAATCAGCTAATC  
 G V A D M E A R Q E L T L V E V K N E R V S P P E I Q L Q N R G G L P Y H F P P L L L Q M P I Q L I  
 910 920 930 940 950 960 970 980 990 1000 1010 1020 1030 1040 1050  
 AAGACGAAAAACCTGAGCAACGGATAGTGAACAATTTGAACAAGTAAATCCAGGACAACCGAGGAGCTCCATGGGATGCTCCATACAAATTCTTAGAGCTTATCAACTGTTTGGGTAGCAAGTCTGATACCTTGGAGTCGCTC  
 K T K N P E Q R I V E T L Q Q V I Q D N Q Q E L H G D A P Y K F L E L I Q L F R V A S P D T L E S V  
 1060 1070 1080 1090 1100 1110 1120 1130 1140 1150 1160 1170 1180 1190 1200  
 TTTAAGCAATTTTCAGATAACCTTATCACAGGCTTGGCTGCTGAGTGAATTAATGCGAGGAGCTGTTGATTGCGTGAAGTTCCTTAAGCTGAGAATTGCAATGATGACCTTAAGTACATTGAGGCACTTGGCTGTTTCTTTT  
 F K Q F S D K P Y H R L W L L S G I T M A G T V D S L K F L K L R I R N D D L K Y I E A L L A V S F  
 1210 1220 1230 1240 1250 1260 1270 1280 1290 1300 1310 1320 1330 1340 1350  
 GGCCTCCATTTAACAAGACGGATGGTCACTGTATTGCAAGTAGCAGCAGATCTAGTGACCAGCTCCGAGCTTGAGAGAATCCATGCTTGCCAGATTGCTTATTTGGGATATGGTCCATGTTAGAGAGATACTGTTCTCTGGCTCA  
 A L H L T K A D G H S I A V A A D L V T S S R L E R I P M L R Q I A Y L G Y G S M V E R Y C S L A S  
 1360 1370 1380 1390 1400 1410 1420 1430 1440 1450 1460 1470 1480 1490 1500  
 TCTTGCCCTACTGAAGCTCTTCAGGCCCTCCATGACCTTGCAAGTGAAGGAGGCAATGAAGATGACATTGTATTAGCTCTGAAGGCCATTGGCAATGCAGGACAACGAGCAGTATCAAGCGCATCCTGAAGTCTTGCCA  
 S C P T E A L Q P L H D L A A E A A S K G N E D D I V L A L K A I G N A G Q P A S I K R I L K F L P  
 1510 1520 1530 1540 1550 1560 1570 1580 1590 1600 1610 1620 1630 1640 1650  
 ATGTTTTCACCTGCCCAATGGCTTTCCAGTTAGAATTTATTTGATGCTGTTTGGCCTTGAGAAAAATAGCCAGGAAAGACCTGCAAAAGTACGGGAAATCTTCTCCAGATCTTTGTGGACCAATCACTTGCTCTACTGTTCTGA  
 M F S P A A M A F P V R I Y I D A V L A L R K I A R K D P A K V R E I L L Q I F V D H S L A P T V R  
 1660 1670 1680 1690 1700 1710 1720 1730 1740 1750 1760 1770 1780 1790 1800  
 ATGATGGCTGTGTGTGTCATCTTGAAGTAAAGCTGCTCTCTCTGTAACAATATGGCCATGGCCTGCTAAAGGAGAGCAGTTTGAGGTGCGCAGTTTCACATATCCCATATGAAGGTTTGGCAATAAGCAGGATCCACAG  
 M M A C V V I F E T K P A L P L V T T M A N A L L K E S S L Q V A S F T Y S H M K V L A I S R I P Q  
 1810 1820 1830 1840 1850 1860 1870 1880 1890 1900 1910 1920 1930 1940 1950  
 CTCTAATGATCTGCTGCTGCGCATTGCGCTTAAGCTACTGAGCCCAAGACTTGACAGGCTGAGCTATGCTTACAGCAAGGTTATTACGTTGGTGGTTATTACCCGGAATATAGAGCTGGTGAATTTGAAGAGTCTATCTCATG  
 L Y N V S A A C S I A L K L L S P R L D R L S Y R Y S K V I H V G G Y Y P E Y R A G A I G R V Y L M  
 1960 1970 1980 1990 2000 2010 2020 2030 2040 2050 2060 2070 2080 2090 2100  
 AACAGTCCAAGCAGTATGTTGCCATCTGCGATAATTATGAAACTGAGAGGTTACTATGCAAAATACCGCAACTGATTTTGTGAGGTGGCTCTCCAGTCACAAGGCCTAACAGAGCTTGTGAGGAAACAGAACATCCCTTTGCTGAATAT  
 N S P S S M L P S A I I M K L R G Y Y A N T A T D F V E V A L Q S Q G L T E L V R K Q N I P F A E Y  
 2110 2120 2130 2140 2150 2160 2170 2180 2190 2200 2210 2220 2230  
 GACACATACAAAAAATGAAGGTGCTTGGGAAAACGCTTCTGGGATGGAAGGAATGCTCCTGAAACCCCTCATATCAGCCTATGTAAGGATTTTGGCCATGAGCTGCGCTTGTGCTGACGTCAAT  
 D T Y K K L K V L G K T L L G W K E L P P E N P L I S A Y V K V F G H E L A F A D V N

Fig. 2. Nucleotide sequence and the deduced amino acids of vitellogenin of *Chinemys reevesii* turtle. The underlined amino acid sequence shows a putative N-glycosylation site.

|                           |                                                                                                       |
|---------------------------|-------------------------------------------------------------------------------------------------------|
| <i>C. reevesii</i> VTG    | MRGILLALVFTLVGSEKYDLSLILVAARPTCTTMR-PYPEWTARAWLARTGLRLSCKVEISVLSQRNHLLKIRSPQLEEYNGIWPDPFIRASKLTQLIA   |
| <i>C. carpio</i> vg-B1    | ..AVV...TVA..A.QQIN.VPEFTPGKTYVYNYEALLGGLPHEG...A.IKVNS..HL.AVTENTF.M.LMD.LIY..A...K...VP.T...SAL.    |
| <i>D. rerio</i> VTG1      | ..AVV...TVA..ACQQFI.VPEFAHDKTYVYKYEALLGGLPQEG...A.IKV.S..L..ATTENTY.M.LMD.L.Y..A..T..K...VP.T...SAL.  |
| <i>A. japonica</i> VTGI   | ..AVV...TLA..AGRQTASTPDPFGTSKYAYKYEAVLLGGLPE.G...A.VKV.S..L..GAA.NTY...LMD..IF..S.T..T...VP.A...AL.   |
| <i>M. aeglefinus</i> VTGA | ..AVV...ALAV.AGQYVNFAPDFASSKTYVYKYEAVLMAGLPEEG..SA..KVL.S..L..NIAENTY...LAD.E.F..S.VL.K...P.NM.KEAL.  |
| <i>P. major</i> VGA       | ..AVV...TLA..AGQPHN.APEF..G.TYVYKYETLLGGLPEEG..KA..KI.S.VL..ATAENTYM..LAE.EIF.LS...KD.L.P.T...SAL.    |
| <i>L. argentatus</i> VTG  | ..GII...AL...Q.F.IDPGFSSRKSHLYSYEGWVNLGLQKESF.KA.V.F.S.L..G..ENTY.....F.....D..T.S..I..AVS            |
| <i>G. gallus</i> VTG II   | ..GII...L...Q.F.IDPGFNSRRSYLYNYEGSMLNGLQDRS.GKA.V...S.L..G.PENAY...V...V...V...D..T.S..I..V.S         |
| <i>X. laevis</i> vtg b1   | ..GII...LLA.A...SQYEPFFSESKTYVYNYEGIILNGIPENG..RS.IK.N..A...GYA..SYM...D.EIK.F..L..KD..T..T..S..IL.   |
|                           | ** *** * * * * *                                                                                      |
| <i>C. reevesii</i> VTG    | PYLTQPFKFEYSNGRVGNIYAREDPVTCINLVRGIMNMLQITIKKSQNAYDLQEAIGGVCLTRYVIQEDRRNRVSMTKTKDLNDCQDKVVKDIGMTY     |
| <i>C. carpio</i> vg-B1    | AQ.QI.I...A..V..KVF.PAGVSP.VL..H...L.I..LNL..T..I.E....AQ...R.H...S..PKA.HITV..S...SH..ERIM..V.LA.    |
| <i>D. rerio</i> VTG1      | AQ.QI.I...A..V..KVF.PAGVSP.VM..H...L.I..LNL..T..I.EM....AQ...R.H...N..PKA.HIIV..S...SH..ERIM..V.FA.   |
| <i>A. japonica</i> VTGI   | AQ.LI.I...A..V..RVF.P.GVSA.VL.VH...L.I..LN...N..V.E....AQ.T.K.D...S..AKAE.IYI..S...N..ERIMQ...A.      |
| <i>M. aeglefinus</i> VTGA | SQ.AT.I...A..V..KV.SP.GIST.VL.IF..VL.I..LN...T..V.E....AQ...S.L.A.T..EKAD.ILL...R...N..E.I...L.LA.    |
| <i>P. major</i> VGA       | AQ.MT.I...T..V..KMF.P.GVSTMVL.VY...L.V..LN...TH.V.E....AQ...K.L.A.A..EKAE.ILL...R...H..E.IM..L.LA.    |
| <i>L. argentatus</i> VTG  | SCF.R.....NS..I...GP..C.DM..I...L.MM.....V.E.....H.....K.S...V...V.Q.N..E..I.SV..A.                   |
| <i>G. gallus</i> VTG II   | SCF.RL.....S..I...P..C.DL.V.I...L.MF.M.....V.E.....I.HA.....K.S.IYV.R.V...N..E..Q.S...A.              |
| <i>X. laevis</i> vtg b1   | EH..R.V...R..Q...F.A..VSETAL.IQ...L.L..L...V...V...DS..S...H.K.....KKA.E.LTVM.SR.FDR.ESR.E.K..VA.     |
|                           | **** * * * * * * * * * * * * *                                                                        |
| <i>C. reevesii</i> VTG    | IRPCPSCLKENIVKGTAFTYKLYADSGTLITEAVSQVQISPFNEPAGVADMEARQELTLVEVKNERVSPPEIQLQNRGGLPYHFPPLLQMPIQL        |
| <i>C. carpio</i> vg-B1    | TER.AE.TERIKSLIE..TYN.IM.P.SA.V...TVEE.H.F....IH.A.Q...K.T.AF..IEKTL.V.IKADYLA..S.Q.E.ATEI..T..H.     |
| <i>D. rerio</i> VTG1      | TEK.AE.TERVKSLE..TLY.IM.P..H.A..A..TVEE.D.F...H.IH.CCM...K.T.AF..IEKTP.V.IKADYMP..S.Q.E.ATEI..T...    |
| <i>A. japonica</i> VTGI   | TET.AH.QQNGKNLR.A..YN.VM.PTAA.A..A..TV.ELH.FT...MT.A.Q....A.TFL.TQ.AA.Q.IQAERYA..S.A.E.ASEI..T...     |
| <i>M. aeglefinus</i> VTGA | TEK.IE.QKEAKNLR.A..YN.V..PVPT.V..LQ.TVNELI.F...LN.A.Q.NTK.S.VFL.IQKTPIA.VNA.YLH..S.IK.E.ASE..T..T.    |
| <i>P. major</i> VGA       | TEK.AK.QQDSKNLR.AT.YN.I..Q.P..IV.L..AVNELI.F...T.MN.A.Q.QTK.S.VFL.IQKAPIV.ITA.YLH..S.K.E.STE..T...    |
| <i>L. argentatus</i> VTG  | .Y...VDMM.GRLI.....S...QS.....V.....LS..T..V...Q.A.L..RS..G.T.D.SM..Y.S.R.Q..SV.P...L..               |
| <i>G. gallus</i> VTG II   | .Y...VDVM..RLT...T..S...QS.....DVS.R.....T...V...Q....RS..G.A.DVPM..Y.S.R.R..AV.P...L..               |
| <i>X. laevis</i> vtg b1   | LET.TQ.KK.VQHLR.S.T....QNK.Q.A..V..SAK...RYA.YY.LY.A.V....I...A.T.SN...ESQV..TK..G.N...WQEEH...FH.    |
|                           | * * * * * * * * * * * * *                                                                             |
| <i>C. reevesii</i> VTG    | IKTKNPEQRIVETLQQVIQDNQQLHGDAPYKFLELIQLFRVASPDLTLESVFKQFSDKPYHRLWLLSGITMAGTVDSLKFLKLIRNDLKYIEALLAVS    |
| <i>C. carpio</i> cg-B1    | M.ISDAPAQ..I.V.KHLVAN.VAMV.E...L..VQ.I..L..STLENI.AIWA..K...AY.R...DALPSV..PVII..I.EKFLAGE.TLP.FIQ.LV |
| <i>D. rerio</i> VTG1      | M.ISDAPAQ...V.KHLVSN.KDMV.D...F..VQ.V..L...LEKI.AIWS..K...VY.R...DALPAV..PVII..I.EKFLAGEFTTP.FIQTLV   |
| <i>A. japonica</i> VTGI   | .RIS.AQAQ...I.NHLVAN.MAAV.E...L..VQ.V..L...NYKSIDAIWA.SKANPAL.R.I.DAVPAI..PVA.Q..EE.FQAG.VTTA.GAQ.LL  |
| <i>M. aeglefinus</i> VTGA | ..IT..QAQ...L.NHLVIN.M.TV.EE..I.....VL.KSNIADIDQIWS..KG..VY.Q.I.DTLPAI..STT.R.IREKLMAR.ISIL..AQ.LV    |
| <i>P. major</i> VGA       | ..VN.VQTQ...I.NHLVTH.M.TV.E..L.....L.S.RFED..MLWS.YRTR.AY.Q.I.DA.PVI..PAA...IREKFLAH..TVA..AQ.LV      |
| <i>L. argentatus</i> VTG  | .....HIV.N...DF.Y.V..R...V..C...A....IWR.A...RY.R...AVSA...TEA...I.S.....INYLQT..S..                  |
| <i>G. gallus</i> VTG II   | .....HIVLN...DF.D.VS.R..VV..C.I.NA.N...IWR.V...RY.R...AVSAS..TET.....N.....NY.QT..T..                 |
| <i>X. laevis</i> vtg B1   | L...A.SQV...HLV.N..ELV..ESTS..F..V..L.TTNQENIQAIW...R.Q..H..VNA.P..S..A...I.QM.H.Q.MTHQ..AT..IL       |
|                           | * * * * * * * * * * * * *                                                                             |
| <i>C. reevesii</i> VTG    | FALHLTKADGHSIAVAADLVTSRLRIPMLRQIAYLVGYGSMVERYCSSLASCPTALQPLHDLAAEAASKGNEDIVLALKAINAGQPASIKRILKFL      |
| <i>C. carpio</i> vg-B1    | V..QMVT..LDT.QLT.SLAMHEKIAKM.A..EVVM.....IA.H.VAVPT.SA.L.R.I.EI...T..NDIRE.T...VL...H...L.P.M.L.      |
| <i>D. rerio</i> VTG1      | I..QMVT..PET.KMT.SLA.HEKFAT..A..EVVM.....LIAK..VAVPT..A.L.R.I.EI..T..I..NDIPE.T...VM...H.S.L.P.M.L.   |
| <i>A. japonica</i> VTGI   | A.VQLV...QPT.EL..SLVFNPKIQTN.I..ELAM.....NKF.AAHP..ADVMSKV..I...IA.A.VTE.T...VM...H...L.T.M.L.        |
| <i>M. aeglefinus</i> VTGA | ASI.MVT..QEA.TIVESLIDNNLIVEN.A...VM...T..AKF..DKIA..S.LIK.I.EKIAR.VAE.E..I.L..VL...H.H.L.T.T.I.       |
| <i>P. major</i> VGA       | ASI.MVS.STEA.QKLEALAVNNKIVES.I..E..IL...T.ISK..AEMAV..A.LIK.IQ..L.D.VA.ADTQE.I.L..VL...H.S.L.P.T.I.   |
| <i>L. argentatus</i> VTG  | ...SMR..EDTVPI..LM...IQKS.V.Q.V.C..S.V.N...QI.A..K....I...DD.I.R.R..KMK...C..M.E.....                 |
| <i>G. gallus</i> VTG II   | LT...LQ..E.TLPI..LM...IQKN.V.Q.V.C..S.V.N...QT.A..K....I...D..I.R.R..KMK...C..M.E...L.L...            |
| <i>X. laevis</i> vtg BI   | M.M.Y.R.SQRAVEI...L..D..VKNA.S.YKS.L.A..L.YK..VDSQ...EA.....EF.....NNAHQEE.T.....Q...                 |
|                           | * * * * * * * * * * * * *                                                                             |
| <i>C. reevesii</i> VTG    | PMFSPAAMAFPVRIYIDAVLALRKIARKDPKAVREILLQIFVDHSLAPTVMACVVFETKPALPLVTMTANALLKES--SLQVASFTYSHMKVLAISR     |
| <i>C. carpio</i> vg-B1    | .GLRT..SSL.L.VQV..I...N..K.E.KL.QPVA..LVL.RA.H.E..V..I.L..S..SVA..SSL.G..KT.T--NMH.V..A...I.S.TRIT    |
| <i>D. rerio</i> VTG1      | .GLRT..N.L.I.VQV..I...N..K.E.KL.QPVA..LVL.RA.H.E..V..I.L..E.SVA.ISSL.G..RT.P--NMH...A...I.S.TRIT      |
| <i>A. japonica</i> VTGI   | .G.GT..A.L.TSVHA...M..NV.KFERRR.QDVA..L.M.RANH.EL..V.SI.L...R.TMGQ..AV...Q..D--N.....A.TR.T           |
| <i>M. aeglefinus</i> VTGA | .L.GS..A.L.TRVHV..I..M.N..K.E.RLIQDMV.L.M.KG.Q.EL..L..I.L...RIG...AL..V.KT.E--N.....S.TR.T            |
| <i>P. major</i> VGA       | .IHGT..ASL.MKVHA..IM...N..K.E.RMIQ.LA..LYM.KA.H.EL..L..ILL...R.MG...L..IVKQ.E--N.....S.TR.T           |
| <i>L. argentatus</i> VTG  | .IS.SS.SDI.TH.Q..IM.....Q...KT.QVY..I..LA.Q..P.E.....AA...R...I..I.VAM...--N.....V....A.SK.T          |
| <i>G. gallus</i> VTG II   | .IS.SS.ADI..H.Q..IT..K...W...KT.QGY..I..LA.Q..P.E.....A...R...A..I..I.VAM...--NM...V....S.SK..        |
| <i>X. laevis</i> vtg BI   | .G..SG.SRL..LQT...M...N..I...R..Q....L.M.RDVH.E..IT..AL...N.GIAL..AV...V.R..KDNM.L.....I.S.SK.M       |
|                           | * * * * * * * * * * * * *                                                                             |

|                           |                                                                                                                                          |
|---------------------------|------------------------------------------------------------------------------------------------------------------------------------------|
| <i>C. reevesii</i> VTG    | IPQLYNVSAACSIALKLLSPRLDRLSYRYSKVIHVGGYPEYRAGAIGRVYLMNSPSSMLPSAIIIMKLRGYANTATDFVEVALQSQGLTELVRKQNIPIF                                     |
| <i>C. carpio</i> vg-B1    | A. DMAA. AG. ANV. I. . M. RK. . . . F. F. RALQLDY. HTPLMI. . A. SA. MI. DAATI. . R. VVA. A. A. L. GA. A. VL. IGV RTE. IQ. ALL. SPAAD     |
| <i>D. rerio</i> VTG1      | A. DMAY. AG. ANV. I. . MNCK. . . . N. . . . RAFKMDY. . TPLMI. . A. SA. MI. DAATI. . R. VVA. A. T. L. GA. A. V. . FGV RTE. IH. ALL. SPAAD |
| <i>A. japonica</i> VTGI   | A. EFAP. A. . NV. V. . . . S. . . . . AT. LDT. STNLM. . . AASAF. V. DAATI. . R. VVA. . A. F. GA. ANVL. FGV RTE. IQ. ALL. TPVAA           |
| <i>M. aeglefinus</i> VTGA | ASHQAS. A. . NV. VRI. . . K. . . . . F. . A. QMDT. SEPMLL. . AASAFYI. DAA. I. . R. FVA. T. . L. GA. A. VL. IGV RTE. . Q. ALL. NPDNE      |
| <i>P. major</i> VGA       | AAIHAS. A. . NV. V. I. . . K. N. . . M. F. . A. . DS. . SPLMI. . AASAFYI. DAATI. . RSFVA. TSA. L. GA. A. VL. . GVRTE. . Q. ALL. NPALI    |
| <i>L. argentatus</i> VTG  | L. YM. . . S. . N. . . . . A. K. . . . . CI. G. FDNKYV. . A. D. FV. . . GT. F. . . . S. . MA. T. GSVL. L. . AGVRVE. . . DVIM. R. . .     |
| <i>G. gallus</i> VTG II   | L. FM. . I. S. . N. . . . . K. . SM. . . . . RADT. FDN. . V. . T. EIFVV. . . RT. F. . . . S. . MANS. GSVL. L. . GIRVE. . ADVIM. R. . .   |
| <i>X. laevis</i> vtg BI   | M. EF. . LAS. . RV. . NIMN. VF. Q. . F. . . AF. . DTFNYPMMG. LALN. VFI. . . NTA. VFALI. V. ECSFGAS. . VF. . . RAE. . Q. VIR. . . Q.      |
|                           | * * * * * * * * *                                                                                                                        |
| <i>C. reevesii</i> VTG    | AEYDTYKKLKVGLKTLGKELPPENPLISAYVKVFGHELAFADVN                                                                                             |
| <i>C. carpio</i> vg-B1    | ESV. RIT. I. RTLRA. AN. . D. . NDQ. . A. V. I. FL. Q. V. . VKID                                                                          |
| <i>D. rerio</i> VTG1      | ESV. RIT. I. RTLRA. TN. . A. . TDK. . A. . . . V. . Q. V. YVNF                                                                           |
| <i>A. japonica</i> VTGI   | GAANRINRM. RVL. A. AD. . AA. GNQ. . A. M. F. L. . Q. I. . LNMD                                                                           |
| <i>M. aeglefinus</i> VTGA | N-A. RIT. MRRVI. A. SQ. RS. . TSK. . ANV. I. FL. Q. I. . NLD                                                                             |
| <i>P. major</i> VGA       | NNA. RMT. M. RVI. A. SELRS. . ART. . A. V. I. F. . Q. I. . NID                                                                           |
| <i>L. argentatus</i> VTG  | . . . P. . . I. EI. . A. . . . . T. A. . . . L. L. . Q. . . YVNI.                                                                        |
| <i>G. gallus</i> VTG II   | . . . P. . . QI. E. . A. Q. . . . T. T. . V. . L. IL. Q. V. . INI.                                                                       |
| <i>X. laevis</i> vtg BI   | S. FSMH. . IVKIL. A. MAY. . A. ANV. . V. . SI. LL. Q. VN. NQAT                                                                           |
|                           | * * * * *                                                                                                                                |

Fig. 3. Comparison of the amino acid sequence of *Chinemys reevesii* turtle vitellogenin with that of carp VTGBI (*C. carpio*), zebrafish VTGI (*D. rerio*), eel VTGI (*A. japonica*), haddock VTGA (*M. aeglefinus*), red seabream VTGA (*P. major*), herring gull (*L. argentatus*), chicken (*G. gallus*), and Xenopus (*X. laevis*).

## DISCUSSION

For the conservation of wildlife and stabilization of environment, development of sensitive indicators is required before functional abnormalities and ecological disorders appear in wildlife. VTG is thus considered to be a good tool for gaining a better understanding of the comprehensive effects of EDCs on wildlife. A variety of information regarding VTG has been accumulated for numerous animal species, but the information for reptiles, especially turtles, has been limited. According to the author's survey, not all of the nucleotide sequences of VTG in any species of turtles have yet been clarified. In this communication, a partial cDNA of VTG in Reeves' pond turtle, *C. reevesii*, was cloned and sequenced.

VTG is a glycoprotein which is composed of lipovitellin and phosvitin [24]. Two N-glycosylation sites were found in the deduced amino acid sequence of the cloned cDNA. One set of 11 and two sets of 25 sequential serine residues are present in a molecular analysis of *Xenopus* phosvitin [13]. *C. picta* phosvitin also contains a high serine content; 50% of the total amino acid residues were serine [19]. Such high serine content was not found in our sequence. This fact suggests that the *C. reevesii* VTG cDNA described herein would not encode a part of phosvitin.

The phylogenetic analysis indicates that the VTG of *C. reevesii* as a reptile exists between bird VTGs and amphibian VTG (Fig. 4). The sequence of other turtles, such as *C. picta*, is of interest in order to clarify the location of the reptilian VTG.

Theoretically, the expression of mRNA occurs before protein synthesis, thus suggesting the possibility that deter-

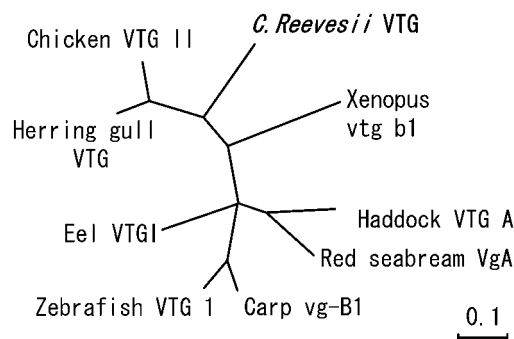


Fig. 4. Phylogenetic relatedness for vitellogenin among *Chinemys reevesii*, chicken, herring gull, *Xenopus*, eel VTGI, zebrafish VTGI, carp VTGBI, and red seabream VTGA, and haddock VTGA.

mining the VTG mRNA may help us to elucidate environmental estrogenic pollution earlier or provide a more sensitive mode of detection than that of VTG protein. In turtles, northern blotting was established in order to detect the mRNA expression of VTG [5, 7–10], but this method is not frequently performed because the usage of radioisotopes and large amounts of RNA extracts are needed. Multiple examination is not available for northern blotting. A RT-PCR method was designed in this study to detect the expression of *C. reevesii* VTG mRNA. Using the primers designed from our *C. reevesii* VTG sequence, a clear amplified product was proven in the RNA extracts of the liver of male *C. reevesii* which were treated with estrogen. The intensity of the mRNA expression of the VTG was significantly increased 6 hr after a single injection of estrogen (0.5

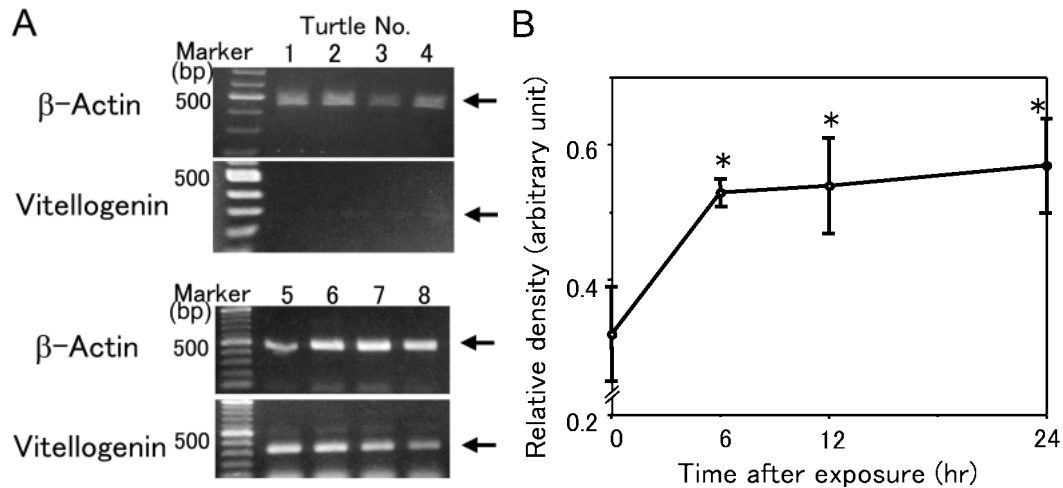


Fig. 5. mRNA expression of vitellogenin of *Chinemys reevesii* after the injection of estradiol-17 $\beta$ . Panel A: mRNA expression of *C. reevesii* vitellogenin in the RNA extracts from the liver of male turtles 0 hr (Turtle No. 1 to 4) and 6 hr (Turtle No. 5 to 8) after the injection of estradiol-17 $\beta$  (0.5 mg/kilogram body mass). The expression of  $\beta$ -actin mRNA was traced as a control. Panel B: The time-course expression of the vitellogenin mRNA in the liver of the turtles after the injection of estradiol-17 $\beta$ . The asterisks denote significant differences in comparison to the ratio of *C. reevesii* VTG to  $\beta$ -actin mRNA at 0 hr ( $P < 0.05$ ).

mg per kilogram body mass) (Fig. 5). Ho *et al.* reported that a significant increase of serum VTG protein was detected 8 hr after a single injection of estrogen (1 mg per kilogram body mass) in male *C. picta* turtles [20]. The RT-PCR method for detecting VTG mRNA is more sensitive than the immunological method for detecting VTG protein. In our recent field study, five out of 360 turtles captured from contaminated rivers showed higher serum VTG concentrations, but the incidence of their VTG concentrations was not statistically significant [31]. The RT-PCR method would be therefore useful to analyze such obscure case. Further studies, such as dose response and precise time-course experiments, should be done to obtain a clear parallel relationship between the mRNA expression and protein synthesis of VTG in *C. reevesii*.

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