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Full Length Research Paper

DNA sequence and prokaryotic expression analysis of vitellogenin from *Antheraea pernyi*

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In this study, the DNA sequence of vitellogenin from *Antheraea pernyi* (*Ap*-Vg) was identified and its functional domain (30-740 aa, *Ap*-Vg-1) was expressed in *Escherichia coli* BL21 (DE3) cells. The recombinant *Ap*-Vg-1 proteins were purified and used for antibody preparation. The results showed that the intact DNA sequence of *Ap*-Vg consisted of 8124 bp including 6 exons (2258, 205, 982, 879, 184 and 829 bp) and 5 introns (84, 78, 410, 1055 and 795 bp) and was highly homologous to the vitellogenin from *Antheraea yamamai*. SDS-PAGE and western blot analysis demonstrated that a 85 KD recombinant protein was successfully expressed in *E. coli* cells and its expression was not remarkably changed under induction by different IPTG concentration. The titre of antibodies raised against rabbits was about 1:7800 which was determined by ELISA.

Key words: DNA sequence, vitellogenin, *Antheraea pernyi*, expression.

INTRODUCTION

Vitellogenin (Vg), the precursor of major yolk protein in insects (Wahli et al., 1981; Kunkel and Nordin, 1985; Sappington et al., 2002), is synthesized in fat body and secreted into the hemolymph, then transported to the developing oocytes through receptor-mediated endocytosis. After being incorporated into oocytes, Vg is deposited as viltellin (Vn) (Kanost et al., 1990; Raikhel and Dhadialla, 1992). In the egg development, Vgs are degraded as the source of lipids, amino acids, carbohydrates, sulphates, phosphates and transported to the embryos (Harnish et al., 1982; Wyatt et al., 1984; Tufail et al., 2005).

The Vgs have been studied extensively in various animals from vertebrates to invertebrates (Nardelli et al., 1987; Chen et al., 1997; Tufail and Takeda, 2008) and some highly conserved structures related with biological functions of Vgs (Raikhel and Dhadialla, 1992; Trewitt et al., 1992; Chen et al., 1997; Lee et al., 2000), such as polyserine tracts, DGXR motif, subtilisin-like endoproteases site (R/KXXR/K) and GL/ICG motif (Taborsky, 1991; Dhadialla et al., 1992; Mayadas and Wagner, 1992; Giorgi et al., 1999; Tufail et al., 2005) are found. The insect Vgs are synthesized mainly in fat body and have the tissue-, stage- and sex-specific characteristics (Raikhel and Dhadialla, 1992; Sappington et al., 2002). However, its synthesis is also found in males of few species according to the reports (Valle, 1993; Piulachs et al., 2003). The biosynthesis of Vgs could be regulated by hormones like juvenile hormone, neuropeptides and ecdysone through receptors (Engelmann, 1983; Wyatt and Davey, 1996; Sappington and Raikhel, 1998; Belles, 2005; Snigirevskaya and Raikhel 2005).

Recently, the cDNA sequences of Vgs from Lepidoptera insects *Bombyx mori* (Yano et al., 1994a; Yano et al., 1994b), *Bombyx mandarina* (Meng et al., 2006), *A. pernyi*

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Abbreviations: Vg, Vitellogenin; ***Ap*-Vg,** vitellogenin from *Antheraea pernyi;* **aa,** amino acid; **bp,** base pairs; **KD,** kilodalton; **IPTG,** isopropylthiogalactoside; **Vn,** viltellin; **cDNA,** complementary DNA; ***Ap*-Vg-1,** N-terminal domain of *Ap*-Vg; **JH,** juvenile hormone; **ELISA,** enzyme linked immuno-sorbant assay; **PCR,** polymerase chain reaction; **dNTP,** deoxynucleoside 5'-triphosphate; **PVDF,** polyvinylidene difluoride; **Ni–NTA,** nickel–nitrilotriacetic acid; **SDS-PAGE,** sodium dodesyl sulfate- polyacrylamide gel electrophoresis.

Table 1. The primers used for PCR.

Primer	Primer sequences
F1 (15-34)	5'-CTCCGAGTGAACAGAAAACG-3'
R1 (705-724)	5'-GCCACACGATGGTGACAATG-3'
F2 (491-510)	5'-TAGACCTTTCCACGCATCGT-3'
R2 (1531-1550)	5'-TCACGATAAACCATCCACAT-3'
F3 (1361-1380)	5'-AAGACAAAGCTCAAGAACTA-3'
R3 (1900-1919)	5'-ATGTCCCAAATTACCAATAG-3'
F4 (1651-1670)	5'-AGTTTACCTTCTACCCTGCG-3'
R4 (3455-3474)	5'-ATTCTGAGATTGTTTACCCA-3'
F5 (3315-3334)	5'-CTTCAACTTCCAGGGATACT-3'
R5 (3663-3682)	5'-TCGGTTTTATGACTTCCTTC-3'
F6 (3556-3575)	5'-GAAGACTTGAGCCCGAACAG-3'
R6 (4312-4331)	5'-AACGGCTGGTACACAGAGAA-3'
F7 (4231-4254)	5'-ACTCTTGAGGCATCGTTGACATC-3'
R7 (4475-4495)	5'-ATGGCATCTTCTTCTGGCTCG-3'
F8 (4406-4426)	5'-AGATAAAGACCTTCAGCAACC-3'
R8 (4877-4896)	5'-CAAACCCTCAGGTGTCAGAT-3'
F9 (4651-4670)	5'-GCAAAGAAGGTATCTGAGGA-3'
R9 (5682-5703)	5'-TTAGGTACATTAAAAATATATT-3'

(Yokoyama et al., 1993; Liu et al., 2001), *Antheraea yamamai* (Liu et al., 2000; Meng and Liu, 2006), *Actias selene* (Yin et al., 2007), *Saturnia japonica* (Meng et al., 2008), *Samia cynthia ricini, Lymantria dispar* (Hiremath and Lehtoma, 1997) and *Philosamia cynthia ricini* (Liu et al., 2003) have been identified while only few DNA sequences of these Vg genes are analyzed. It is reported that the transcription of Vg is regulated by juvenile hormone (JH) through the binding elements located in the promoter of the gene (Segraves, 1994; Wyatt and Davey, 1996; Belles, 2005). To investigate the evolutionary relationship between *Ap*-Vg and other insects' Vg genes and its biological function, we first reported the intact DNA sequence of *Ap*-Vg along with its prokaryotic expression and hope to provide some information for further studies.

MATERIALS AND METHODS

Materials

The experimental animals, *Antheraea pernyi,* were introduced from the sericultural research institute of Shandong Province and reared on the leaves of oak.

Extraction of genomic DNA and total RNA

One gram (1 g) of fat body was collected from female pupae and washed by distilled water, then transferred into liquid nitrogen. Phenol/chloroform method was used for the extraction of genomic DNA (Mahendran et al., 2006) and total RNA was extracted using TRIzol™ Reagent (Invitrogen) according to the instructions. The extracted RNA or genomic DNA was checked by electrophoresis and ultraviolet spectrum.

Cloning of Ap-Vg

Nine pairs of oligonucleotide primers (shown in Table 1) were designed with Primer premier 5.0 software to amplify the whole DNA sequence of *Ap*-Vg gene. PCR was performed using 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 µM of each of primers, 1U *Taq* DNA polymerase (Promega) and 5 ng of DNA. The amplification program consisted of 5 min at 94°C followed by 35 cycles of 94°C for 30 s, 55°C for 35 s and a final elongation step of 72°C for 5 min. Generated DNA fragments were analyzed on 1% agarose gels, then cloned into the PMD-19T easy cloning vector (Takara) and sequenced at Invitrogen, Shanghai.

Construction of recombinant plasmids and protein expression

To investigate the function of *AP*-Vg, the N-terminal domain (30-740 aa, named as *Ap*-Vg-1) with a function of transporting lipids was amplified for protein expression. The oligonucleotide primers Vt F1: 5' CAGGATCCTGGCAAGACGGAAAGGTTT 3' and Vt R1: 5' GACCTCGAG GTTAATTCCAGATTTAAGTGC 3' (restriction enzyme sites are underlined) were designed for PCR amplification according to the open reading frame of the *Ap*-Vg gene. PCR products were digested with restriction enzymes (*Bam*HI and *Xho*I) and ligated to pET28a vector (Novagen, USA). The resulting recombinant plasmids pET28a-*Ap*-Vg were confirmed by DNA sequencing and then transformed into *E.coli* BL21(DE3) (Novagen, USA) for induction by different IPTG concentrations (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mM IPTG, respectively). The recombinant fusion proteins were analyzed by SDS-PAGE.

Preparation of antibodies

Ni–NTA (nickel–nitrilotriacetic acid) affinity chromatography (Qiagen, Germany) was used to purify the recombinant *Ap*-Vg-1 proteins according to the instructions. The New Zealand White rabbits were immunized with 100 µg of purified proteins (homogenized in complete Freund's adjuvant) for three times at 2-week intervals and a boost injection was given for another week with purified proteins (diluted in incomplete Freund's adjuvant). Anti-*Ap*-Vg-1 antiserum was collected seven days after the last immunization (Harlow and Lane, 1999) and stored at −80°C.

Western blotting

Western blotting was carried out according to the method described by Zhu and Wu, 2008). Total proteins collected from *E. coli* cells were subjected to 12% SDS-PAGE and then transferred onto polyvinylidene difluoride (PVDF) membranes (Sigma) by an electrophoretic transfer system (Bio-Rad). Membranes were blocked with phosphate-buffered saline containing 0.1% Tween 20 and incubated with monoclonal anti-His antibody or Anti-*Ap*-Vg-1 antiserum for 2 h at room temperature. Then the membranes were incubated with horseradish peroxidase-conjugated sheep anti-rabbit IgG antibody (Sigma) for 1 h at room temperature. Final detection was done with an horseradish peroxidase-3,3'-diaminobenzidine detection kit (Tiangen). Quantification for total protein was performed using Bradford method (Bradford, 1976).

Enzyme-linked immunosorbent assay (ELISA)

The titer of antibody from the immunized rabbits was determined by

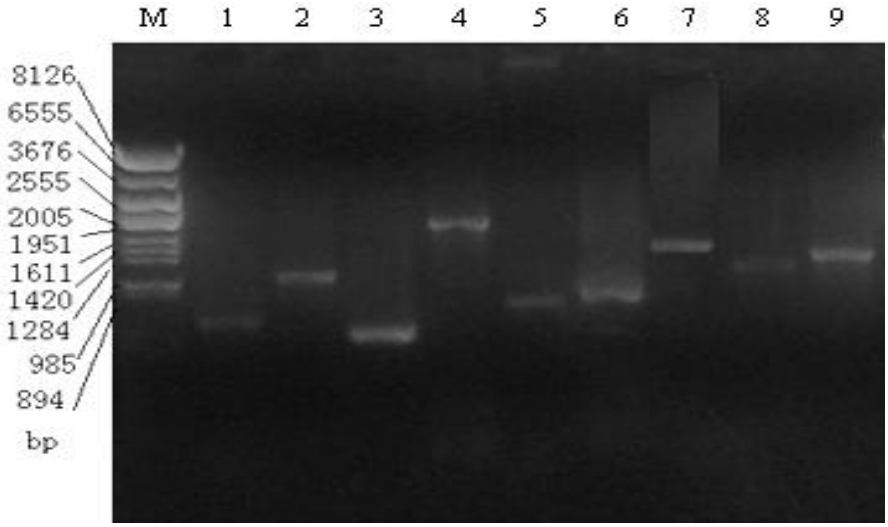

Figure 1. PCR result of *Ap*-Vg DNA. Lanes1-9, the PCR products using primers from F_1R_1 to F_9R_9; M, marker.

ELISA. Briefly, the optimum concentration (0.5 µg per well) of recombinant proteins diluted in 0.05 M carbonate buffer were incubated with blocking solution (5% skimmed milk powder in phosphate-buffered saline). After the plates were washed with phosphate-buffered saline containing 0.1% Tween 20, rabbit serum of different dilutions were added to each well and incubated for 1h at 37°C. Then the plates were incubated with 100 µL of a 1:1000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase for 30 min at room temperature. Finally, 100 µL of TMB/H_2O_2 substrate was added to each well and optical density was measured at 450 nm with Elix 800 Universal micro-plate reader (Biotek Instruments).

Purification of vitellogenin from *A. pernyi*

To demontrate the *Ap-Vg*-1 protein is a part of *Ap*-Vg, the *Ap*-Vg was purified according to our previous studies (Liu et al., 2001) and used for western blot. 2 ml of hemolymph was collected from pupae and *Ap*-Vg was purified by column chromatography with sepharose CL-4B, hydrophobic column chromatography with butl-cellulofine and anion-exchange chromatography with DE52, respectively. The presence of obstained *Ap*-Vg at each step was checked by SDS-PAGE.

RESULTS

PCR result of *Ap*-Vg DNA

Specific primers were designed to identify the intact DNA sequence of *Ap-Vg* according to its cDNA sequence (Liu et al., 2001). As the results obtained show that the PCR products are about 700, 1000, 600, 1900, 750, 800,1300,1000 and 1100 bp, respectively (Figure 1).

Sequence analysis of *Ap*-Vg gene

An intact DNA sequence of 8124 bp (Genbank accession no. EF683091) was obtained by sequencing and six exons (2258, 205, 982, 879,184, 829 bp, respectively), five introns (84, 78, 410, 1055, and 795 bp, respectively) and the conservative cleavage sites of introns are found in it. The primary structure of *Ap*-Vg is highly homologous to other insect Vgs (Chen et al., 1997; Liu et al., 2001), but the DNA sequence is different from each other as far as the number and length of exons and introns are concerned. Seven exons and six introns are found in *A. yamamai, B. mori, L. dispar* and *A. grandis* while six exons and five introns in *A. pernyi* and only three exons and two introns exist in Vg of *A. aegypti* (Table 2). In addition, an exon (31bp followed ATG) found in other insects is absent in *A. pernyi*. Phylogenetic analysis based on the DNA sequence indicated that *AP*-Vg is highly homologous to the Vg from *A. yamamai* (Figure 2), which is similar to our previous studies (Liu et al., 2001; Meng et al., 2008).

Protein expression, antibody preparation and western blot analysis

A 85 KD recombinant protein was detected by SDS-PAGE and the expression was not remarkably influenced by different IPTG concentrations (Figure 3A). The result of western blot analysis showed that a consensus protein band was detected using anti-His antibody or anti-*Ap*-Vg-1 antiserum while no immunoreactive band was found in the control group (Figures 3B and 4). All this indicated that *AP*-Vg-1 is successfully expressed in *E. coli* cells.

ELISA

Anti-*Ag*-Vg-1 rabbit serums of different dilutions (1:6, 1:36,

Table 2. Comparison of Vg genes from various insects.

Species	*A. pernyi*	*A. yamamai*	*B. mori*	*L. dispar*	*A. grandis*	*A. aegypti*
Exon 1	2258	31	31	31	31	31
Intron 1	84	4590	1588	1667	2056	70
Exon 2	205	2227	2242	2143	2003	5214
Intron 2	78	77	91	254	85	57
Exon 3	982	205	208	214	197	1202
Intron 3	410	77	81	781	57	
Exon 4	879	982	985	970	303	
Intron 4	1055	423	88	374	103	
Exon 5	184	879	879	870	170	
Intron 5	795	896	740	3016	59	
Exon 6	829	184	184	202	1692	
Intron 6		806	286	297	87	
Exon 7		829	820	814	977	

Notes: The origins and accession numbers of the Vg genes are: *A. pernyi* (EF683091), *A. yamamai* (AB247378), *B. mori* (D30733), *L. dispar* (U90756), *A. grandis* (M72980), *A. aegypti* (L41842).

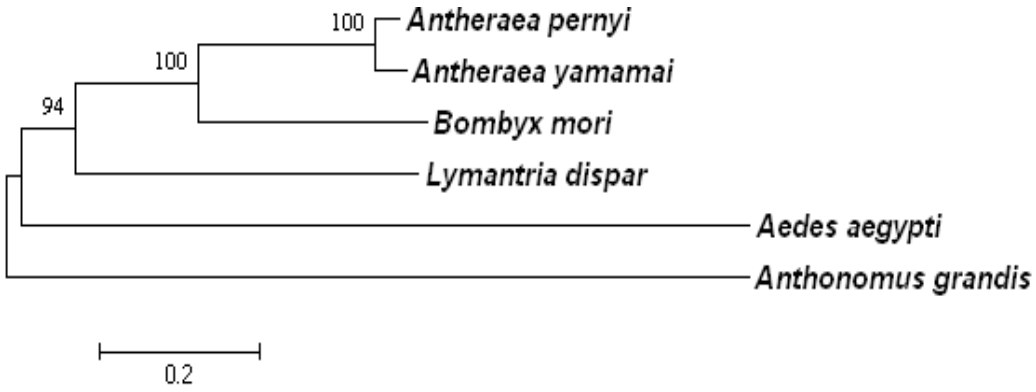

Figure 2. Phylogenetic analysis of *Ap*-Vg based on the complete DNA sequences. The phylogenetic tree was constructed by MEGA (version 4.0) program using the neighbor-joining algorithm method and bootstrap values (1000 repetitions) of the branches are indicated.

1:216, 1:1296 and 1:7776, respectively) were incu-bated with recombinant proteins and the titer of anti-*Ap*-Vg-1 antibodies (about 1:7800) raised against rabbits was determined by ELISA.

Purification of *Ap*-Vg and Western blotting

The *Ap*-Vgs purified from hemolymph were subjected to SDS-PAGE and Western blot. Western blot analysis of *Ap*-Vg using the anti-*Ag*-Vg-1 rabbit serum demonstrated that a protein of about 200 KD was detected (Figure 5), which was in agreement with the size of *Ap*-Vg (Liu et al., 2001). No immunoreactive band was found in the control group using pre-immune rabbit serum. This result demon-strated that the anti-*Ag*-Vg-1 antibody is valid to *Ap*-Vg.

DISCUSSION

Vg is the precursor of vitellin and plays an important role in egg development. During this course, Vgs are processed by proteolytic cleavage, glycosylation, phosphorylation and sulphation at the post-transcriptional level (Raikhel and Dhadialla, 1992; Hagedorn et al., 1998; Giorgi et al., 1999). However, the process of Vg and the number of Vg gene vary in different insects (Tufail and Takeda, 2008). Generally, the Vgs are approximately 200 KD and divided into large subunits (140 - 190 KD) and small subunits (40 - 60 KD) or more smaller subunits by proteolytic cleave (Hiremath and Lehtoma, 1997; Hirai et al., 1998; Tufail et al., 2005, Tufail and Takeda, 2007). Unlike many kinds of Vgs, *Ap*-Vg consists of a large subunit of 200 KD and is secreted without cleavage because of the lack of con-

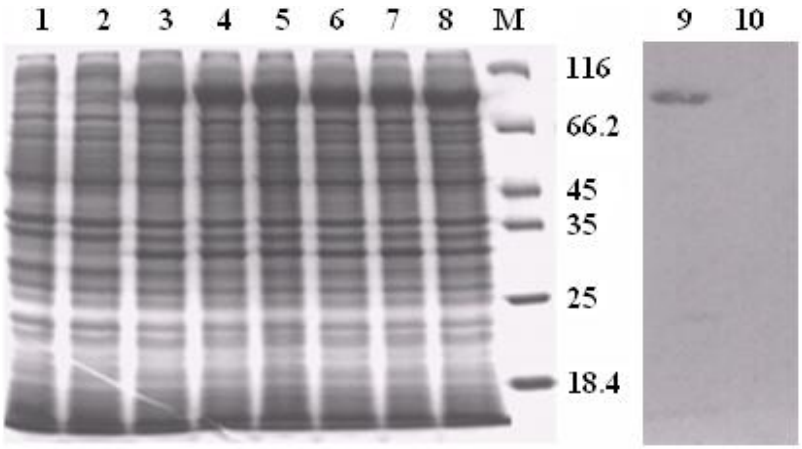

Figure 3. (A) Analysis of recombinant *Ap*-Vg-1 protein on 12% SDS-PAGE gels. The gels were revealed by Coomassie blue R-250 staining. Bacterial proteins were collected after 4 h induction with different IPTG concentration. Lane 1, *E. coli* BL21(DE3); Lane 2, before induction; Lane 3-8, after induction (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mM IPTG respectively); M, molecular weight marker. **(B)** Western blotting of recombinant proteins using anti His-tag antibodies. Lane 9, after IPTG induction; Lane 10, No IPTG induction.

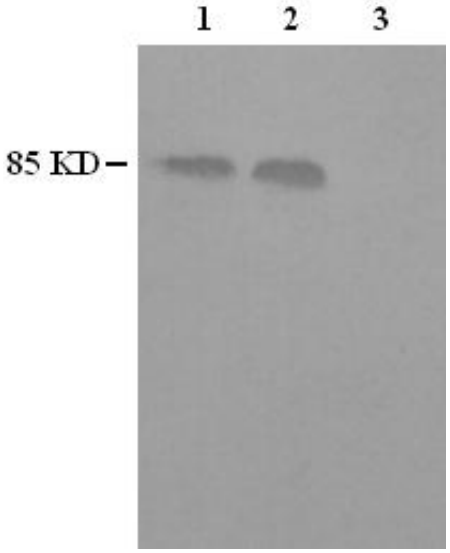

Figure 4. Western blot analysis of recombinant proteins using Anti-*Ap*-Vg-1 rabbit serum. A protein band with a molecular mass of about 85 KD was detected by western blotting. The pre-immunized rabbit serum was used as a control. **Lanes 1 and 2,** Anti-*Ag*-Vg-1 rabbit serum; Lane 3, Pre-immunized rabbit serum.

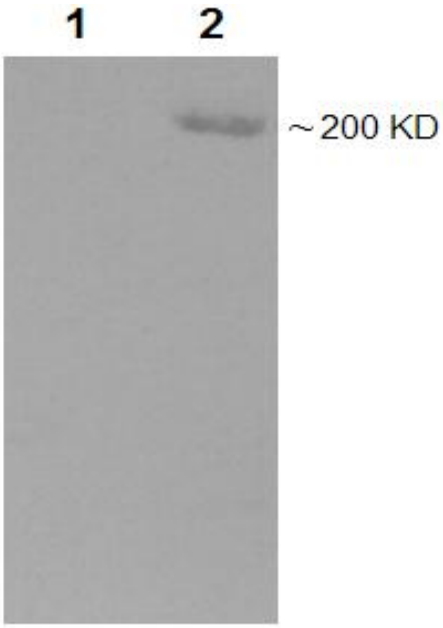

Figure 5. Western blotting of *Ap*-Vg using Anti-*Ap*-Vg-1 rabbit serum. A protein of about 200 KD was detected by western blotting. **Lane 1,** Pre-immunized rabbit serum; **Lanes 2,** Anti-*Ag*-Vg-1 rabbit serum.

sensus R/KXXR/K or RXXR motif which is recognized by convertase (Rouille et al., 1995; Tufail and Takeda, 2007). Meanwhile, only four N-linked glycosylation sites were found in *Ap*-Vg whereas five sites in *A. yamamai*, *B. mori* and *P. cynthia ricini*; this might be related with the function of Vg since glycosylation is important for the synthesis

and secretion of Vgs (Wyatt et al., 1984; Dhadialla and Raikhel, 1990; Don-Wheeler and Engelmann, 1997).

Compared with Vg genes from studied insects, *Ap*-Vg gene (7759 bp) is shorter than *A. yamamai* (12206 bp), *B. mori* (8223 bp), *L. dispar* (11633 bp) and *A. grandis* (7820 bp) except for *A. aegypti* (6574 bp) and the size and number of introns are different from the other Lepidopteran insects. It is hypothesized that introns play a role in regulating gene activity at different developmental stages or controlling local gene expression (Abelson, 1992; Jerry, 2001). So, whether the differences in introns have an impact on the function of Vg still waits to be studied.

The biological properties, protein architecture, biosynthesis and expression regulation of Vgs have been characterized (Rhaikel et al., 2004; Tufail and Takeda, 2008). However, some issues remain to be investigated such as the mechanism of multiple Vgs in some insects and their physiological roles. To determine the role of *Ap*-Vg in the egg development, the prokaryotic expression of its functional domain was performed and purified recombinant proteins were obtained; this might provide a way to figure out the biological functions of *Ap*-Vg.

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