รายงานวิจัยฉบับสมบูรณ์

โครงการการใช้ SiRNA เทคโนโลยีพัฒนาภูมิคุ้มกัน
การติดเชื้อไวรัสในกุ้ง

โดย

ศาสตราจารย์เกียรติคุณสกล พันธุ์ยิ้ม และคณะ

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โครงการการใช้ SiRNA เทคโนโลยีพัฒนาภูมิคุ้มกันการติดเชื้อไวรัสในกุ้ง

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สารบัญ

บทคัดย่อ

Abstract

Shrimp industry in Thailand with export value over 80 billion baths per year had suffered as much as 25% loss from viral diseases. Effective prevention or treatment of viral infected shrimps is not available. This research group aims to develop an effective means to prevent and protect *Peneaus monodon* shrimps from yellow head virus (YHV) infection which usually causes high mortality within 3-7 days. In addition, the group aims to create an infrastructure to study RNA interference (RNAi) in shrimp and to apply siRNA technology for prevention of shrimp mortality from YHV infection.

This research group found RNAi functioning in shrimp lymphoid cells. The ability of dsRNA to inhibit YHV replication was further investigated and found that dsRNA targeting non-structural genes (protease, polymerase, helicase) were clearly more effective than the structural genes (gp116, gp64). Among the nonstructural genes dsRNA protease was the most effective and able to inhibit YHV replication when injecting into shrimp haemolymp. Injection of dSRNA protease not only inhibited YHV replication but also prevented shrimp mortality. This constitutes the first evidence that shrimp mortality from YHV infection may be prevented by the specific dsRNA through RNAi pathway.

The research group cloned essential genes in RNAi pathway (i.e. Argonaute and Dicer). The Argonaute gene encodes 943 a.a. protein whose knock-down impaired RNAi pathway. Two more Argonaute genes whose function is not clear were identified. The cloning and identification of Dicer gene is more complex due to a much larger size. A Dicer gene encoding a minimal 1,786 amino acids was achieved. The gene contained RNA helicase, PAZ, CAT1 and CAT2 RNase III domains and dsRNA binding domain.

Development of viral vector for siRNA delivery employed HPV (Hepato pancreatic virus) whose genome sequence was elucidated. Its single-stranded RNA of 6,321 bases has inverted repeats at both 5′ and 3′ temini which presented difficulties to elucidate the structure. To develop HPV infectious clone we faced an obstacle to find an appropriate host for HPV infection in the laboratory. HPV infectious clone has not been achieved.

This research group produced 14 international publications with high impact factor and 3 manuscripts. There were 22 reseachers; 5 principal investigators, 4 young investigators, 2 Ph.D. students, 9 M.Sc. students and 2 research assistants.

Key words : RNAi, siRNA, *P.monodon*, YHV, dsRNA-protease, Pem-ago, RISC, DICER, mortality, HPV.

บทคัดย่อ

อุตสาหกรรมการเลี้ยงกุ้งซึ่งมีคุณค่าปีละประมาณแปดหมื่นล้านบาท อาจมีการสูญเสียมากถึง 25% จากการติดเชื้อไวรัส ปัจจุบันยังไม่มีวิธีการป้องกันหรือรักษาโรคติดเชื้อไวรัสหัวเหลืองในกุ้งกุลาดำ การศึกษาวิจัยนี้มีจุดประสงค์หลักเพื่อพัฒนาการป้องกันและรักษาโรคติดเชื้อไวรัสหัวเหลืองในกุ้งกุลาดำ ซึ่งโรคดังกล่าวจะทำให้กุ้งติดเชื้อตายภายใน 3-7 วัน จุดประสงค์รองเป็นการสร้างกลุ่มวิจัย นักวิจัยรุ่นใหม่ และนักศึกษาปริญญาโทและเอก ให้มีความรู้ความสามารถในงานวิจัยการใช้ siRNA เพื่อหยุดยั้งไวรัสหัวเหลืองในกุ้ง

กลุ่มวิจัยเลือกใช้เทคโนโลยี siRNA ในการป้องกันและรักษาโรคไวรัส เนื่องจากเป็นเทคโนโลยีใหม่ซึ่งน่าจะหยุดยั้งการเพิ่มจำนวนของไวรัส RNA ที่มีการเปลี่ยนแปลงเร็ว เช่น ไวรัสหัวเหลือง ได้อย่างมีประสิทธิภาพ และน่าจะให้ประสิทธิผลในการป้องกันและรักษา

เทคโนโลยี siRNA ดังกล่าวจะบังเกิดผลเมื่อกุ้งมีระบบ RNA interference ซึ่งในตอนเริ่มต้นปี 2546 ยังไม่มีความชัดเจน จึงได้ทำการทดสอบจนเป็นที่ชัดเจนว่ากุ้งมีระบบ RNA interference ซึ่งจะทำให้เทคโนโลยี siRNA ใช้หยุดยั้งไวรัสหัวเหลืองในกุ้งได้

ได้มีการออกแบบ double-stranded RNA (dsRNA) ให้มีคุณสมบัติปลดปล่อย siRNA ในเซลกุ้งและหยุดยั้งการเพิ่มจำนวนไวรัส เช่น dsRNA helicase dsRNA protease dsRNA polymerase และ dsRNA gp116 dsRNA gp64 โดยพบชนิดที่ดีที่สุด คือ dsRNA protease ซึ่งนอกจากให้ผลดีในเซลกุ้งแล้วยังสามารถป้องกันการติดเชื้อไวรัสหัวเหลืองได้เมื่อใช้ฉีดเข้า haemolymp นับเป็นผลงานวิจัยชิ้นแรกซึ่งป้องกันการติดเชื้อไวรัสหัวเหลืองในกุ้งกุลาดำได้อย่างชัดเจน โดยได้ตีพิมพ์ผลงานวิจัยดังกล่าวแล้ว 2 ฉบับ

นอกจากนี้กลุ่มวิจัยได้ clone ยีน Argonaute ซึ่งเป็นส่วนหนึ่งของ RNA interference ในกุ้ง โดยได้ศึกษาคุณสมบัติเคมีและชีวภาพพบเป็นโปรตีนขนาด 943 กรดอะมิโน ซึ่งยีน Argonaute ดังกล่าวน่าจะเป็นส่วนของกลไก RNA-interference ในกุ้งซึ่งยังไม่มีผู้รายงานการค้นพบมาก่อน และกลุ่มวิจัยได้ clone ยีน Dicer ซึ่งตัด dsRNA ในการปลดปล่อย siRNA ภายในเซลกุ้งได้เกือบเสร็จสมบูรณ์ เนื่องจากยีน Dicer มีขนาดใหญ่มากทำให้ยีนที่ clone ได้ขนาด 1,786 กรดอะมิโน ยังขาดความชัดเจนอยู่

การพัฒนาไวรัสพาหะเพื่อพา siRNA เข้าสู่เซลกุ้งอย่างมีประสิทธิภาพ โดยใช้ไวรัสที่พบในกุ้งแต่ไม่แสดงอาการป่วยโรค เช่น ไวรัส HPV (Hepatopancreatic virus) ได้ดำเนินการค้นหาลำดับนิวคลิโอไทด์ของยีโนมไวรัสขนาด 6,321 bases ซึ่งมีโครงสร้างด้านปลายซับซ้อน จนเป็นผลสำเร็จ และได้พยายามพัฒนา HPV ดังกล่าวให้เป็น infectious clone ยังไม่ประสบความสำเร็จสมบูรณ์ ทำให้การพัฒนาไวรัสพาหะเพื่อพา siRNA ยังต้องทำการทดลองเพิ่มเติมเพื่อให้บรรลุวัตถุประสงค์

โครงการนี้มีผลงานตีพิมพ์ในวารสารนานาชาติ จำนวน 14 ฉบับ พร้อมส่งตีพิมพ์ จำนวน 3 ฉบับ คณะผู้ร่วมวิจัย จำนวน 22 คน เป็นคณะนักวิจัยหลัก จำนวน 5 คน นักวิจัยรุ่นใหม่ จำนวน 4 คน นักศึกษาระดับปริญญาเอก จำนวน 2 คน นักศึกษาระดับปริญญาโท จำนวน 9 คน และผู้ช่วยนักวิจัย จำนวน 2 คน

เนื้อหางานวิจัย

DEVELOPMENT OF siRNA CAPABLE OF INACTIVATING VIRAL REPLICATION IN SHRIMP

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Abstract

RNA interference (RNAi) has been shown to inhibit viral replication in some animals and plants. Whether the RNAi is functional in shrimp remains to be demonstrated. *In vitro* transcribed dsRNA of YHV helicase, polymerase, protease, gp116 and gp64 were transfected into shrimp primary cell culture and found to inhibit YHV replication. dsRNA targeted to nonstructural genes (protease, polymerase, helicase) effectively inhibited YHV replication. Those targeted structural genes (gp116, gp64) were the least effective. These findings are the first evidence that RNAi mediated gene silencing is operative in shrimp cells. The ability of RNAi to prohibit YHV in shrimps was investigated. Haemolymph injection of dsRNA(pro) corresponding to the protease motif of YHV genome resulted in a complete inhibition of YHV replication. The effect of dsRNA lasted for at least 5 days. Injecting sequence-unrelated dsRNA(gfp) or dsRNA(TSV-pol) also resulted in an inhibition of YHV replication but at a comparatively much less extent. Shrimp mortality was monitored for 10 days when more than 90% shrimps receiving no dsRNA died within 8 dpi. However, those receiving dsRNA(pro) showed no mortality. A partial mortality was observed among the shrimps receiving dsRNA(gfp) or dsRNA(TSV-pol). Thus, *P. monodon* possesses the sequence-specific protection to YHV infection, most likely through the RNAi pathway, in addition to sequence-independent protection. It gives a new notion that dsRNA induction of antiviral immunity in shrimp goes through two pathways, sequence-independent and sequence-dependent.

Introduction

Marine shrimp farming is an important aquaculture industry in Thailand. In 1995, the total production of cultured black tiger prawn (*Penaeus monodon*) in Thailand was approximately 225,000 metric tons worth at least $US 1.6 billions. Despite of its tremendous export values, the farming have faced many threats needed to be overcome. Viral diseases are among the most serious obstacle that have severe impact in shrimp farm and hatcheries. In 1996, it was estimated that viral infection costs exceed 500 millions US$ (Flegel et. al 1997).

Yellow head virus (YHV) is listed as one of the most destructive agents causing mass mortality of cultured penaeid shrimp and remains the major problem for shrimp farming industry in Thailand. YHV is an invertebrate virus with positive sense, single stranded RNA genome classified in genus *Okavirus*, family *Roniviridae* and order *Nidovirales* (Sittidilokratna et al 2002, Cowley and Walker 2002). In common with other nidoviruses, YHV possesses similar organization and expression

of the viral replicase gene. The 5 terminal replicase gene of YHV encodes two large overlapping open reading frames that are translated into a large polyprotein, including helicase, polymerase and protease function in genome replication (Cowley, P.J. Walker 2002).

A major defense mechanism in invertebrate including shrimp is relied on innate, pattern recognition immune system. This system comprises cellular response by phagocytes, inducible antibacterial peptides and phenoloxidase cascade (Englemann et al 2005, Little et al 2005). Cellular and humoral mechanisms contribute to the shrimp defense reaction by limiting microbial invasion or for the clearance and killing of invading microbes from tissues and blood circulation (Bachere 2000). A great deal of understanding in innate immunity of shrimp was particularly focused on those targeting to bacterial and fungal infection (Destoumieux-Garzon et al 2001). Whereas, the understanding of shrimp immunity to viral infection is very limit and much lacks behind.

In recent years, the concept of RNA interference (RNAi) has emerged as an ancient conserved mechanism to act as immune system. RNAi is the process by which a gene is post-transcriptionally suppressed using double stranded RNA (dsRNA) to target then destroy their homologous mRNA in a sequence-specific manner (Tuschl et al 1999, Hannon 2002). This phenomenon has been observed as a natural defense against intruding RNA such as viruses or transposon in plants, nematode, insect and mammal (Hannon 2002). The most important characteristics of RNAi is that it is triggered by dsRNA which is cleaved into 21-23 bp so called small interfering RNA (siRNA). The siRNA upon incorporated into muti-component RISC complexes will specifically guide to its complementary target mRNA leading to degradation. In certain organisms lacking adaptive peptide-based immune response such as insect and worm, RNAi acts as a major antiviral immunity (Wilkins et al 2005, Keene et al 2004). RNAi in these invertebrate organisms exerts specific, potent and rapid response thus is in contrast to their broad spectrum innate immunity. The specificity determinants of the RNAi response are siRNA derived and processed from the invading virus or long dsRNA. Thus it is conceivable that RNAi may be a natural adaptive antiviral immunity in those well characterized species (Bagasra and Prilliman 2004).

To date, RNAi has been widely used as a powerful strategy to investigate gene function as well as to develop antiviral agent to combat various viral infection (Capodici et al 2002). This present study aims to investigate in both the primary culture of lymphoid cells as well as in the entire shrimp system whether dsRNA-mediated gene silencing exists in penaeid shrimp if so would it be capable of inhibiting viral replication.

Materials and Methods

Primary cell culture of lymphoid organ

The primary culture of *P. monodon* lymphoid "oka" cells was prepared as described (Assavalapsakul et al 2003). Briefly, Lymphoid tissues "Oka organ" collected from approximate 100 sub-adult shrimps were washed in washing medium (2x Leibovitz's L-15 medium containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 15% fetal bovine serum, 5% lactabumin). The tissue was minced into

small pieces in complete medium (2x Leibovitz's L-15 medium containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 15% fetal bovine serum, 5% lactabumin, 15% shrimp meat extract). The minced tissue was then seeded onto a 24 well plate and allowed to grow to monolayer at 26°C.

Plasmid constructs

Plasmids for in vitro transcription

The following DNA fragments corresponding to specific region of YHV genes were amplified from YHV cDNA template using specific primers: 0.8 kb helicase (5 CAA GGA CCA CCT GGT ACC GGT AAG AC3 and 5 GCG GAA ACG ACT GAC GGC TAC ATT CAC3), 0.7 kb RNA dependent RNA polymerase (5 CCA AAG ATC TCC ATC CA ACCT GTA GAC3 and 5 GGT GCA TTG TAC CAG AAA CCG TCC TC3), 0.45 kb protease (5'ATC GTG CGC GTG TAT GGT GAN CGN GGN GA3 and 5 ACC GTT GAC TGG AGG NAT NCA NGA NAT3), 0.5 kb gp116 (5 TTC GCC AGG ATC CTA AGT GGA ATT CCT GA 3 and 5 ATA CTG AAT TCT ACA TAC AGC CAG GGA CTG 3), 0.5 kb cDNA gp65 (5 AGC TTG GAT CCC ACG ACA GGC ACG TGT T 3 and 5 CCG AGA ATT CAG CGT GAC CAT CGT TGA TG 3). The DNA fragments were cloned into pGEM T-eazy vector (Promega). Recombinant plasmids with the same DNA fragment but opposite orientations were purified by Qiagen DNA purification column.

Plasmids encoding hairpin RNA

To construct recombinant plasmid expressing stem loop YHV-protease RNA, a 0.4 kb cDNA fragment in the coding region of YHV protease gene was PCR amplified with primers (Pro-sense-*Nde* I; 5 CAT ATG GGAATC GAC TAT CGT GAC TGC 3 and Pro-anti-*Pst* I-*Spe* I; 5 CTG CAG ACT AGT ATG CCG ACG ATG TGA GCT CC 3) using Vent DNA polymerase. The fragment was sequentially cloned into pGEM-T easy vector in inverted direction separated with a 0.2 kb DNA fragment of unrelated GFP cDNA amplified with specific primers (GFP-sense-*Spe* I; AAG GCA CTA GTA TGG TGA GCA AGG GCG AGG 3 and GFP-anti-*Pst* I; AAT TGC TGC AGC TGC ACG CCG TAG GTC AG 3). The entire 1 kb insert was excised from pGem T easy backbone by *Nde* I digestion and subcloned into pET-3a vector at the same sites.

Recombinant plasmid expressing stem loop GFP RNA was constructed from two PCR fragments of 0.4 kb amplified with GFP-sense-*Nde* I; 5 AAG GCA CTC ATA TGG TGA GCA AGG GCG AGG 3 , GFP-antisense 1-*Xba* I; TGT TCT AGA ACT CCA GCT TGT GCC CCA GGA TG) and 0.6 kb amplified with GFP-sense-*Nde* I; 5 AAG GCA CTC ATA TGG TGA GCA AGG GCG AGG 3 , GFP-antisense 2- *Xba* I; 5 TGT TCT AGA TTT GCT CAG GGC GGA CTG GGT GCT CAG 3 from GFP cDNA plasmid. The two fragments were joined at *Xba* I site then cloned into pET3a vector at *Nde*I site.

Recombinant plasmid expressing stem loop TSV-polymerase RNA (TSV-pol) was similar constructed as described for GFP stem loop. A PCR fragment from TSV polymerase gene: 0.7 kb amplified with TSV-F-Poly; 5 GTT TCT TGG ACC ATG TGA TGA CG 3 and TSV-R Poly; 5 CTC CAC ATG CAC ATA TCT TCA ATC G

3 was cloned into pGEM-T easy vector. A 0.5 kb fragment was amplified with TSV-stem Poly *Pst* I; 5 AAT TCT GCA GGA CCA TGT GAT GAC GAA CAG 3 and TSV-Stem –Poly *Spe* I; 5 GGG TAC TAG TAT GCT GGT TGA ACC ATT CAC 3 and cloned into the primary recombinant plasmid at *Spe* I and *Pst* I sites. The desired 1.2 kb fragment was excised and subcloned into pET-3a at *Nde*I and *Pst* I site. All plasmid constructions and propagations were performed in *E. coli* DH5□. Nucleotide sequence of the recombinant plasmids was confirmed by automated DNA sequencing.

***In vitro* transcription**

To synthesize dsRNA, two recombinant plasmids with designated insert cDNA but with opposite orientation was linearized by appropriate restriction endonuclease and used as template for *in vitro* transcription using Ribomax kit (Promega). The *in vitro* transcription was followed as described by manufacturer. Equal amount of sense and antisense RNA were annealed to produce dsRNA as described by Worby et al. (2001). Double stranded RNA was quantified by UV spectrophotometry.

dsRNA production in E. coli

The recombinant plasmid was transformed into *Escherichia coli* HT115. The recombinant clone was inoculated into 100 ml 2xYT medium and cultured until OD_{600} of 0.4 at 37 °C. To induce the expression of hairpin RNA of the corresponding gene, IPTG was added to final concentration of 0.4 mM for 4 hr. 1 OD_{600}-ml of bacterial cell was pellet by centrifugation and resuspended in 50 □l Phosphate Saline Buffer containing 0.1% SDS. The sample was boiled for 2 min then snapped cool on ice. To eliminate single-stranded RNA in the loop region of stem loop structure and endogenous RNA from the bacterial host strain, RNase A buffer (300 mM sodium acetate, 10 mM Tris-HCl, pH 8.0) and 1 □g RNAse A was added then incubated for 15 min at 37 °C. Double stranded RNA was extracted from bacterial lysate by TRI reagent (Molecular Research Center) according to manufacturer guideline. Each dsRNA obtained from this preparation appears as a single band in agarose gel electrophoresis corresponded to its expected size. The dsRNA integrity was confirmed by RNase III and RNase A digestion. DsRNA concentration was estimated by $O.D._{260}$ and adjusted to final concentration of 1 □g/□l prior to storage at -80°C until use.

RNA transfection and YHV infection

Double stranded RNAs were introduced into the primary cultured of OKA cells by using Transmessenger RNA transfection kit (Qiagen). To each transfection, OKA cells at 70 % confluent in 24 well tissues culture plate was transfected with 2 □g (unless specified) of indicated dsRNA formulated as described by manufacturer and incubated for 3 hr. The transfected cells were washed once with fresh complete medium then allowed to recover for 40 hr in 26°C incubator. The cells were challenged with YHV at different dilution for 1.5 hr. Excess viruses were removed and fresh complete medium was added then maintained at 26°C until harvesting.

Western blot analysis

200 □l cultured medium was mixed with equal volume of 4x SDS sample buffer then boiled for 10 min at 100°C. Equal amount of protein sample was resolved in 10%SDS-PAGE in Tris-Glycine buffer. Protein was transferred onto a PVDF

membrane (BioRad) using SemiDry transblot apparatus (BioRad). The membrane was incubated with anti-gp116 antiserum in 5% skim milk in Phosphate-buffered saline containing 0.2% Tween-20 (PBST) (dilution 1:2,000) for 1 h at room temperature [13]. Excess antibody was removed by successively washing with PBST then probed with horseradish peroxidase conjugated goat anti-mouse polyclonal antibodies (Sigma Chemical) (dilution 1:8,000). The antigen-antibody complex was detected using the ECL Plus Western Blotting Detection Reagent (Amersham Pharmacia Biotech) following with exposure to X-ray film.

RT –PCR

Total RNA from YHV infected Oka cells and RNA from YHV particle in the culture medium was prepared by using Trizol and Trizol LS reagent (GIBCO, BRL), respectively. First-strand cDNA was synthesized using Improm pt reverse transcriptase (Promega) and oligo dT primer as described by the manufacturer. Determination of YHV RNA level was performed by PCR using primer pairs for helicase gene (0.8kb). When applied, actin cDNA was simultaneously amplified using specific primers (5 GAC TCG TAC GTG GGC GAC GAG G3 and 5 AGC AGC GGT GGT CAT CTC CTG CTC3) for normalization the RNA level. The PCR product was analyzed by agarose gel electrophoresis.

Shrimps rearing

Healthy *P. monodon* juveniles (10-12 g) obtained from commercial farms in Thailand were maintained in 80L tank containing 40 L artificial sea water at 10 ppt salinity with aeration. Before the experiment, the shrimp (4 shrimps/ tank) were acclimated for 3 days at ~ 27-30°C. Half of the water was renewed every 2 days. The shrimps were fed once a day with pellet shrimp diet.

dsRNA injection and YHV challenge

For YHV inhibition assay, shrimps were injected with 25 □g dsRNA (pro), dsRNA (TSV-pol) or dsRNA (gfp) in 50 □l 150 mM NaCl into the haemolymph. The injection was done using 1 ml syringe with 29 gauge needle. 24 hour post injection (otherwise indicated), shrimps were challenged with 50 □l of YHV 10^{-6} dilution in PBS that previously titrated to cause a complete mortality in 3-7 days. For YHV inhibition assay, haemolymph was collected 48 hr after the challenge from individual shrimp for RNA extraction and RT-PCR analysis.

For mortality assay, healthy *P. monodon* juveniles (~5g) were used (9 or 18 shrimps/ group). Shrimps were injected with 25 □g dsRNA in 150 mM NaCl. $YHV10^{-6}$ dilution (50 □l) was injected into haemolymph at 24 hr (otherwise indicated) post dsRNA injection. The mortality was recorded twice a day for 10 days after YHV injection.

Results

Inhibition of YHV replication by long dsRNA in shrimp primary cells

To investigate the effect of introduction long dsRNA into shrimp cells, cDNA corresponding to different regions of YHV genome that may exert crucial role in its replication were selected as template for synthesizing dsRNA *in vitro*. These regions included viral protease (0.45 kb), helicase (0.8 kb) and RNA dependent RNA polymerase (0.7 kb). In addition, unrelated 0.7 kb dsRNA of green fluorescence

protein gene was used as control. These dsRNA (2□g) were transfected into Oka cells then morphological change was examined under microscope. Both morphology and growth rate of cells transfected with these long dsRNAs were similar to the mock transfected cells indicating that introduction of exogenous dsRNA has no deteriorate effect on these cells (Figure 1). Next we investigated whether the presence of dsRNA altered their susceptibility to YHV infection. At 40 hr posttransfection the cells were subsequently infected with different dilution of YHV and morphological change was observed under microscope. Mock transfected cells were highly susceptible to YHV as cytopathic effect (CPE) was observed as early as 48 hr postinfection in cells infected with high level of virus (10^{-3} dilution). CPE were more prominent at 90 hr post infection and its severity was well correlated with the amount of virus used for infection. Similar result was observed in cells transfected with GFP dsRNA although the occurrence of CPE appeared slower comparing to mock transfected cells. In contrast, cells transfected with dsRNA corresponding to helicase, polymerase or protease gene appeared more resistant to YHV. Majority of these cells remained intact and a minor CPE was observed when compared to mock transfected cells at the same titer of infection.

To test whether YHV replication could be inhibited by dsRNA, viral particle in the medium was analyzed by RT-PCR at 48 hr postinfection. As shown in Figure 2A, level of YHV progeny in the culture medium was readily detected in mock transfected cell in all dilution of virus. The intensity of virus was correlated with level of virus used for infection (10^{-3} to 10^{-7}). On the other hand, YHV progenies in samples treated YHV specific dsRNAs were dramatically decreased as the viral progenies could be detected only in sample infected with high dose of virus (10^{-3} to 10^{-4}). Interestingly the level of YHV progenies in cells treated with irrelevant dsRNA (GFP) was clearly

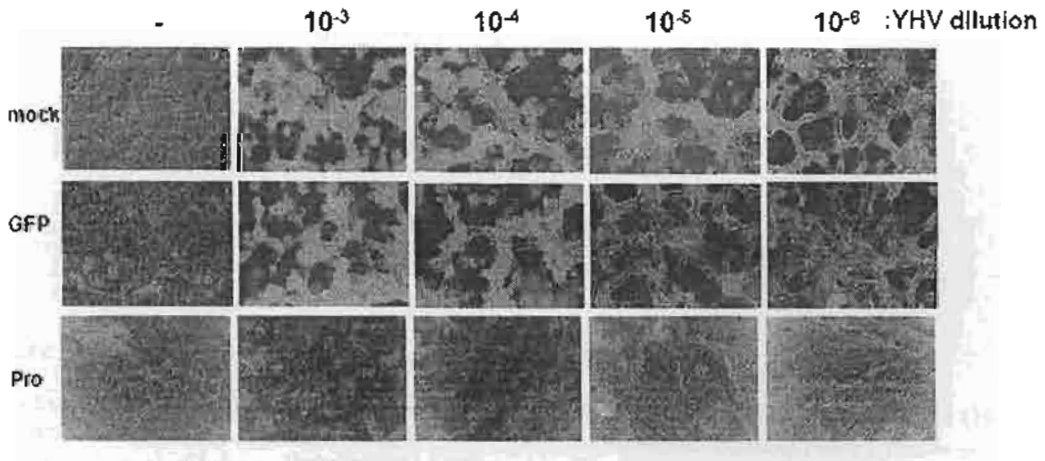
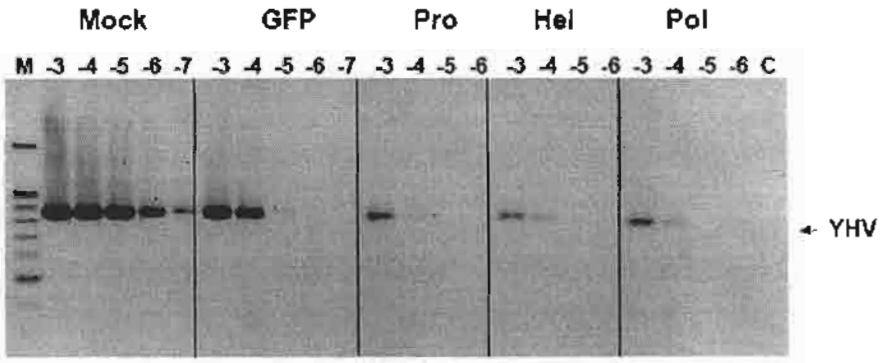


Figure 1. Protection of YHV induced cytopathic effects by YHV specific dsRNA. Oka cells were transfected with dsRNA specific to YHV protease (Pro), unrelated dsRNA for Green fluorescent protein (GFP) or without dsRNA (Mock) then infected with YHV at dilution 10^{-4} to 10^{-6}. Cell morphology was observed under phase contrast microscope at 48 hr post infection compared to the uninfected cells (-).

(A)



(B)

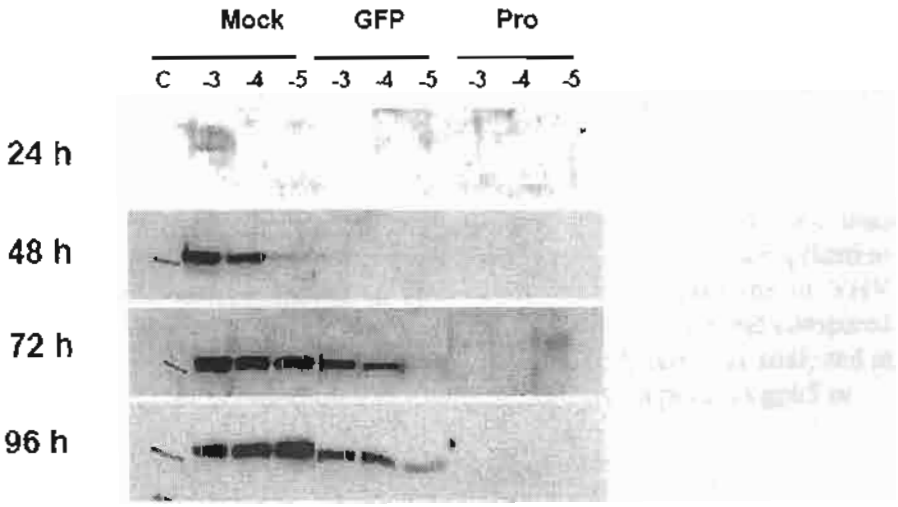


Figure 2. dsRNA inhibits YHV replication in Oka cells.

Oka cells were transfected with dsRNA specific to gene;YHV Protease (Pro), Helicase (Hel) or Polymerase (Pol) or unrelated dsRNA for Green fluorescence protein (GFP) followed by YHV infection at dilution 10^{-3} to 10^{-7} (indicated as -3 to -7) compared to no infection (C). Equal volume of culture medium was collected at 48 hr post infection for determining YHV level by RT-PCR (A). Time course of YHV inhibition of by dsRNA by western blot analysis using anti gp116 of YHV(B). M: 1 kb plus DNA ladder.

higher than those observed in cells treated with YHV specific dsRNA but its level was substantial lower than that from the mock transfected cell. To confirm this result, a similar experiment was performed however YHV progeny in the medium was monitored at different time point postinfection (24, 48, 72 and 96 hr) by western blot analysis using antiserum against gp116 of YHV structural protein. YHV gp116 was readily detected at 48 postinfection in mock transfected cell and its intensity was correlated well with the infection titer (Figure 2B). The signal was further increased when the incubation time was prolonged to 72 or 96 hr particularly for those infected with the lower YHV titer. Gp116 in cells treated with GFP dsRNA were detected in all infection condition albeit with relatively lower level. Indeed the first appearance of the YHV gp116 was observed at 24 hr later compared to the mock transfected control. In contrast, gp116 was not detected in cells transfected with YHV protease dsRNA even at 96 hr postinfection. This data confirmed that YHV replication in Oka cells could be efficiently inhibited by dsRNA specific to its nonstructural genes.

YHV suppression by dsRNA is dose dependent

To determine the potency of dsRNA on YHV inhibition, the primary cell was treated with different amount of dsRNA targeting protease (0.2-1□g)prior to infected with YHV at dilution 10^{-4} and 10^{-5}. The level of YHV RNA in the cell extract was determined and compared. Result in Figure 3 demonstrates that the decreasing amount of dsRNA results in increasing of YHV RNA present in the cells lysate, indicating inverse correlation between YHV inhibition and the amount of dsRNA. While 1 □g of protease dsRNA showed efficient inhibition of YHV, the inhibition was drastically reduced in the presence of 0.2 □g. This data indicates that the efficiency of YHV inhibition by dsRNA occurred in a dose dependent manner.

YHV structural gene is an unfavorable target for suppression

In addition to dsRNA specific to nonstructural genes of YHV, we also investigated whether dsRNA targeted to other region of YHV would block replication of YHV. 0.5kb dsRNAs corresponding to two major structural proteins of YHV (gp116 or pg65) were designed and their inhibitory effect was validated and compared to dsRNA targeted the polymerase gene. Level of YHV in cell lysate was analyzed at 72 hr post infection by RT-PCR. As shown in Figure 4, dsRNA targeted to gp65 or

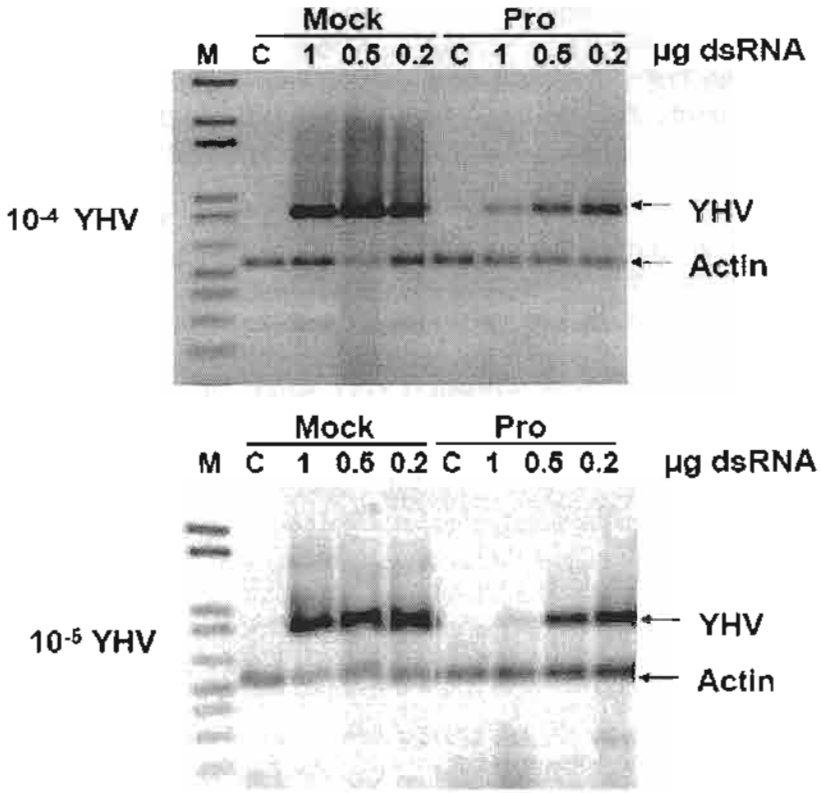


Figure 3. Dose dependent YHV inhibition by specific dsRNA
dsRNA (0.2 – 1 □g) corresponding to YHV protease was tranfected to Oka cells followed by YHV infection at dilution 10^{-4} to 10^{-5}. Cell lysate was collected at 48 hr post infection for analysis of YHV level by RT-PCR. Actin was the internal control for normalizing the RNA level. M: 1 kb plus DNA ladder, Pro: cell transfected with dsRNA, Mock: without dsRNA.

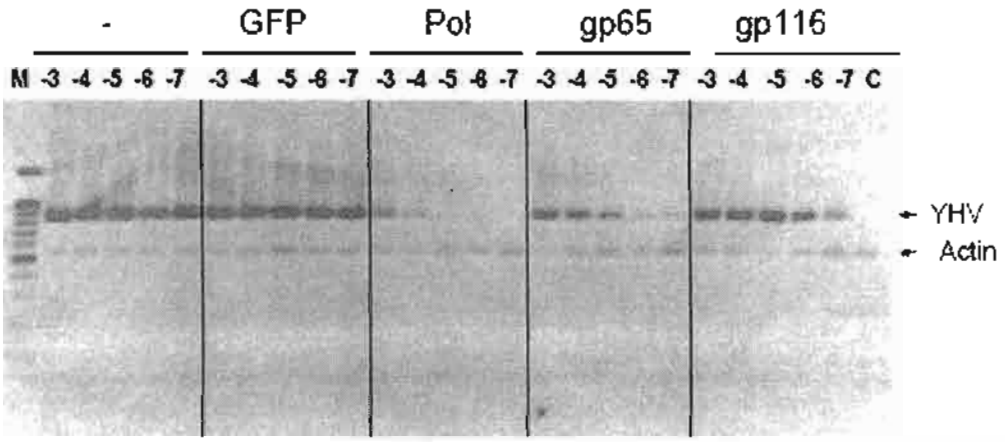


Figure 4. Suppression of YHV replication by dsRNA targeting structural genes
Oka cells were transfected with dsRNA specific to YHV Polymerase (Pol) or structural genes GP65 or GP116 or unrelated dsRNA (GFP) followed by YHV infection at dilution 10^{-3} to 10^{-7} (indicated as -3 to -7). Cell lysate was collected at 72 hr post infection for RT-PCR. Actin for normalizing the RNA level, M: 1 kb plus DNA ladder, C: without YHV.

gp116 could poorly inhibit YHV replication (approximately 2 $\log_{10}$ reduction) compared to the inhibition by dsRNA targeted to polymerase gene. Of the two, dsRNA targeted to gp116 region had the least inhibitory effect on viral replication. This data clearly indicate that target sequence of dsRNA strongly contributed to potency for suppression of YHV replication.

Length of dsRNA affects the RNAi efficiency in Oka cell

The observation that dsRNA targeted to different regions of YHV genome, particular nonstructural and structural genes, showed different potency for YHV inhibition. This led us to further investigate whether size and location of dsRNA are important for this inhibition. To examine the effect of dsRNA length on YHV suppression, 3 shorter dsRNAs for YHV nonstructural genes with approximate size of 100 bp were generated. One of these dsRNA targeted to 3 end of protease whereas the other two targeted to the region corresponding to 5 or 3 end of helicase gene, respectively. The activity of these dsRNAs to suppress YHV replication was determined and compared to their corresponding long dsRNAs as shown in figure 5. Although the 100 bp dsRNAs targeted were capable of inhibiting YHV replication in Oka cells, their efficiency was relatively lower than the longer dsRNA targeting to the same gene. The YHV suppression was irrespective to location in the nonstructural gene these 100 bp dsRNA targeted.

Inhibition of YHV replication in shrimp by dsRNA

To test whether dsRNA could inhibit YHV replication in shrimp, dsRNA 400 bp corresponding to protease region of YHV or unrelated dsRNA with similar size (GFP or Polymerase gene of TSV (TSV-pol)) were injected into the haemolymph prior to a challenge with YHV at dilution 10^{-6}. 48 hr post infection, the level of virus in the haemolymph was monitored by RT-PCR. The level of YHV virus in all control shrimps receiving no dsRNA was clearly detected (Figure 6A). Similar viral load was also observed in the gill (data not shown). In contrast, no YHV was detected in haemolymph of the shrimps injected with 25 □g dsRNA(pro) indicating that dsRNA(pro) prohibits YHV replication. Co-amplification of actin from shrimp haemocyte by RT-PCR in each shrimps was used as internal control. However, actin in moribund shrimps was markedly reduced to barely detectable level (Figure 6 lane

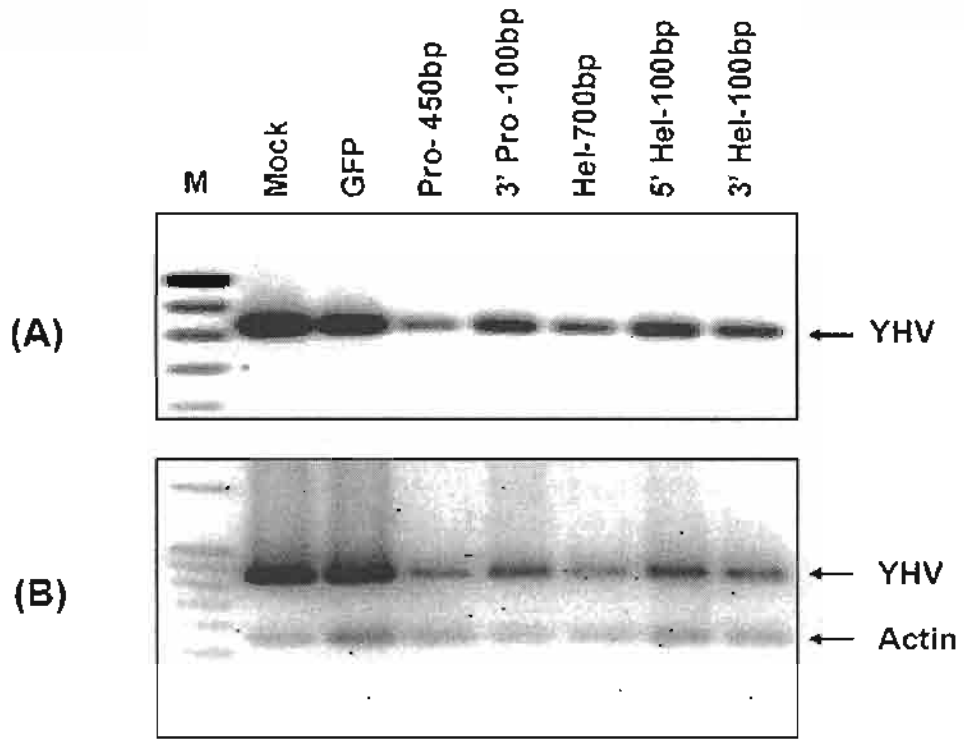


Figure 5. Effect of dsRNA length on YHV inhibition
Prior to YHV infection (at dilution 10^{-4}) Oka cells were transfected with 100 bp dsRNA :3 Pro-100 bp, 5 Hel-100 bp or 3 Hel-100bp. Protection of the cells from YHV infection was compared to its counterpart dsRNA with longer length (450 bp Pro and 700 bp Hel). The level of YHV in culture medium (A) and in cell lysate (B) was determined by RT-PCR at 72 hr post infection. Actin for normalizing the RNA level, M: 1 kb plus DNA ladder.

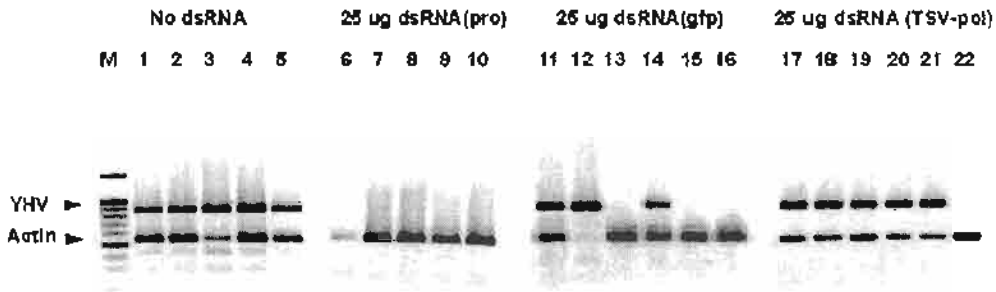


Figure 6. Inhibition of YHV replication by dsRNA
Shrimps (10-12g) were injected into haemolymph with 25 µg dsRNA or 150mM NaCl for 24 hr prior to YHV (10^{-6}) challenge. The viral load in haemolymph of individual shrimps was determined by RT-PCR at 48 hr post YHV challenge by agarose gel electrophoresis (A) and by western blot analysis with anti YHV gp116 specific antiserum (B). 1-5 represents individual shrimps receiving no dsRNA; 6-10 dsRNA(pro); 11-16 dsRNA(gfp); 17-22 dsRNA(TSV-pol). Actin was used as internal control. M is 100 bp DNA ladder. * moribund shrimp.

3, 6, 12 were moribund shrimps). Similar result was observed when RNA of from the gill was used for analysis (data not shown). This could be due to rapid degradation of cellular mRNA in the moribund shrimps. In a separate experiment, we observed a negative correlation between actin mRNA level and moribund stage of shrimps.

The specificity of dsRNA on YHV inhibition was determined in the shrimp receiving equal amount of dsRNA(gfp) or dsRNA(TSV-pol). YHV inhibition was observed in shrimps injected with dsRNA(gfp), however the inhibition was much less efficient. Only ~50% inhibition was observed in the shrimps pre-administrated with dsRNA(gfp). Surprisingly, dsRNA(TSV-pol) from an unrelated shrimp virus TSV, barely prohibited YHV replication (~17%). Hence, this result showed that shrimps were partially protected from YHV infection by unrelated dsRNA. The inhibitory effect of dsRNA on YHV replication was confirmed by western blot analysis. Gp116, YHV specific structural protein, was detected in the shrimps identified positive by RT-PCR except one shrimp with low viral load (Figure 6B lane 14). This is possibly due to lower sensitivity of the detection of western blot as previously shown [16].
The efficiency of YHV inhibition was then examined by varying the amount of injected dsRNA(pro) prior to YHV challenge. As expected, injection 25 □g dsRNA(pro) into shrimp resulted in a complete inhibition of YHV replication. However, decreasing the dsRNA(pro) to 5 or 1 □g resulted in diminished efficiency of YHV inhibition to approximately 80 and 40%, respectively (Figure 7). This result clearly indicated that YHV inhibition of dsRNA(pro) exhibited dose dependent effect.

Longevity of YHV inhibition by dsRNA (pro) in live shrimp

To further investigate the potency of dsRNA(pro) in YHV inhibition, equal amount of dsRNA(pro) (25 □g)was injected into shrimps following by infection with YHV at various time points, from 1 to 5 days. YHV level in haemolymph of the shrimps were monitored at 48 hr post infection by RT-PCR. As expected, YHV level was readily detected in the control shrimps receiving no dsRNA. In contrast, YHV replication was completely inhibited in shrimps administrated with dsRNA(pro) 1-4 days prior to YHV challenge (Figure 8). Although low level of YHV was detected in shrimps with late YHV challenge (5^{th} day), the YHV level however was significantly lower than the control. From this result we demonstrated that the inhibition was prolonged for at least for 5 days post dsRNA. The efficiency of YHV inhibition by dsRNA(pro) was

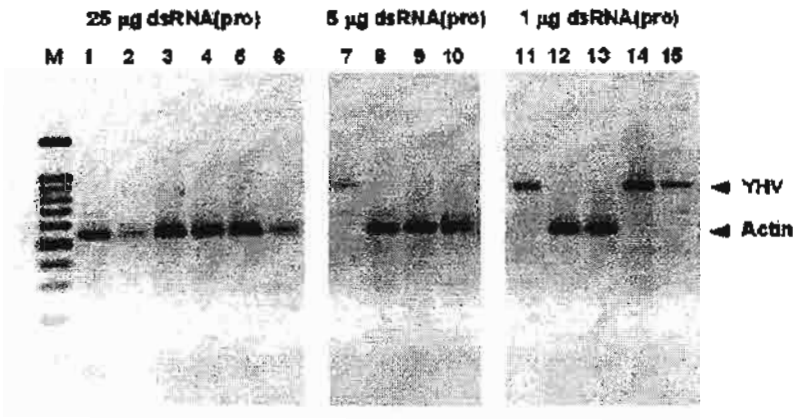


Figure 7. Dose dependent inhibition of YHV by specific dsRNA
Shrimps (10-12g) were injected into heamolymph with 25 □g (1-6), □□g (7-10) and 1 □g (11-15) of dsRNA(pro) and challenged with 10^{-6} YHV as in Fig. 1. M is 100 bp DNA ladder. * moribund shrimp.

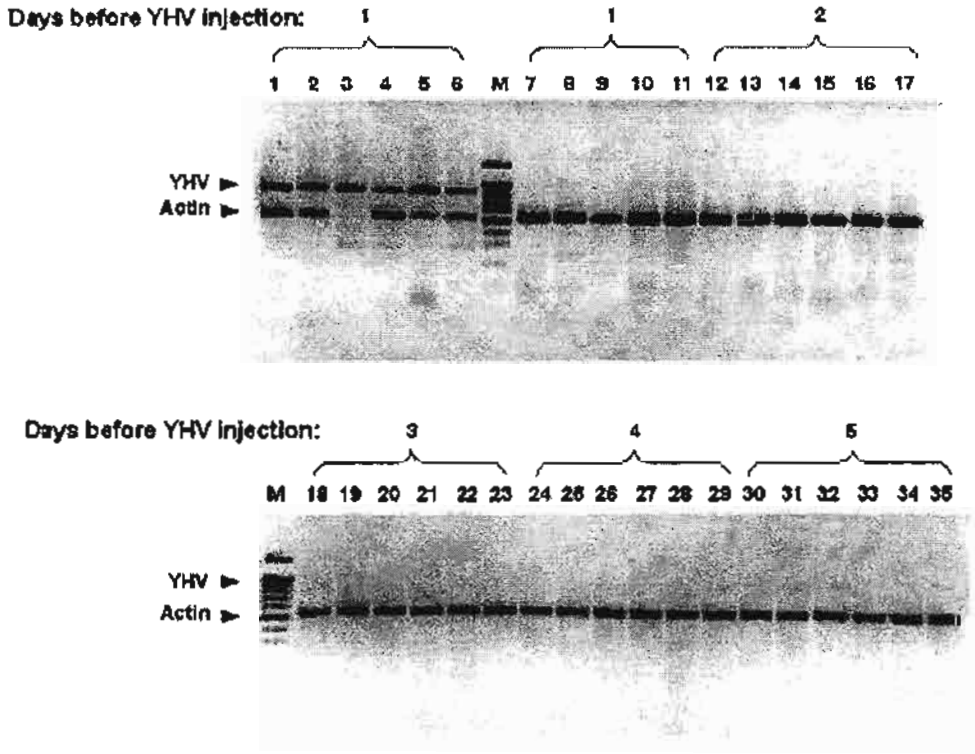


Figure 8. Longevity of dsRNA(pro) effect
Shrimps (10-12 g) were injected with 25 □g dsRNA(pro) and, thereafter, challenged with 10^{-6} YHV at day 1 through day 5. 1-6 represents individual shrimps receiving no dsRNA; 7-11 dsRNA(pro) and challenged after 1 day; 12-17 after 2 days; 18-23 after 3 days, 24-29 after 4 days; 30-35 after 5 days. M is 100 bp DNA ladder. * moribund shrimp.

also tested in shrimps either receiving the dsRNA simultaneously with YHV or receiving dsRNA 30 min post YHV infection. In both cases, YHV inhibition (at 48 hr post infection) in these shrimps was observed (data not shown) implicating that initiation of systemic response to YHV triggered by dsRNA(pro) was immediate process.

dsRNA injection prevents shrimp mortality from YHV infection

Injection in haemolymph with 10^{-6} YHV resulted in a cumulative >90% death of the shrimps in 8 days post infection (Figure 9). Dead shrimps showed high level of YHV in multiple tissues including gill and haemolymph (data not shown). Whereas shrimp injected with dsRNA(pro) showed no difference in mortality from the control at 10 days post infection demonstrating that dsRNA(pro) gave a complete protection against YHV infection. Injection of dsRNA 5 days prior YHV infection also gave a complete protection. No shrimp death was observed 5 days after dsRNA(pro) injection indicating that dsRNA(pro) did not give any cytotoxicity. Shrimps injected with dsRNA(gfp) prior YHV infection resulted in approximately 50% survival rate. All dead shrimps showed the presence of YHV in gill confirming the death arose from YHV infection (data not shown). To analyze the effect of dsRNA(pro) or dsRNA(gfp) on YHV protection compared to the control group (no dsRNA), statistical analysis was performed. Since our sample size and expected frequency are small, Fisher's exact test is more appropriate.

The Fisher's exact test significant are .000 ($P<0.0001$) and .003 ($P<0.01$) for shrimp received dsRNA(pro) and dsRNA(gfp) 1 day prior to YHV infection, respectively. Similarly, the test significant is .000 ($P<0.0001$) for shrimps injected with dsRNA(pro) 5 days prior to the YHV challenge.

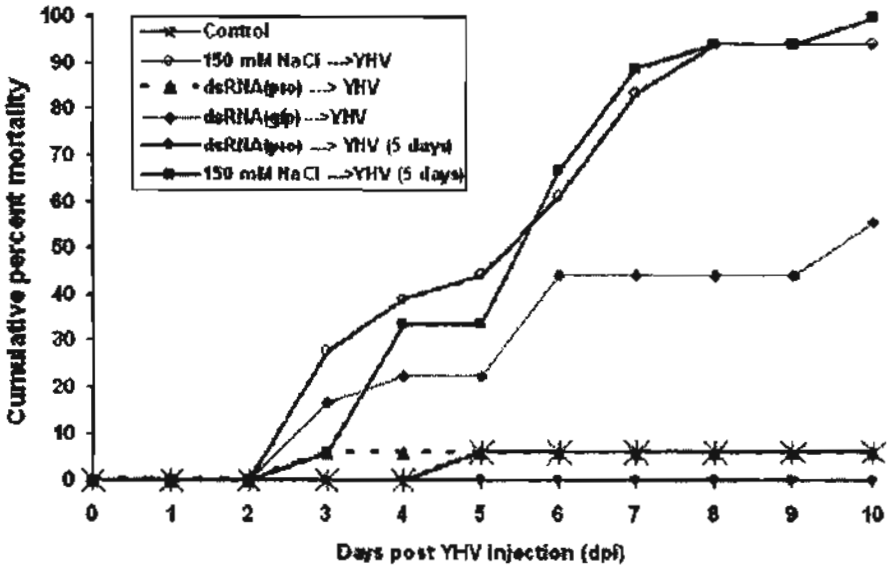


Figure 9. Mortality of shrimps upon YHV infection
Six groups (9 or 18 each) of shrimps were injected into haemolymph with non (control) (•), 150 mM NaCl (□), 25□g dsRNA(pro) (▲) (P<0.0001), 25 □g dsRNA(gfp) (◆) (P<0.01) for 1 day followed by 10^{-6} YHV challenge; or 150 mM NaCl (■), dsRNA(pro) (●)(P<0.0001) 5 days prior to the challenge. Shrimp mortality was recorded twice each day for 10 days. Percent mortality in each experimental groups (except dsRNA(gfp)) was presented as means with error bar of duplicate experiments. Statistical analysis was performed using Fisher's exact test.

Discussion

The result of this study demonstrated that dsRNA administered to the primary lymphoid cell culture (Oka cell) of black tiger shrimp gave protection against YHV infection resembled to RNA interference (RNAi) mechanism in many invertebrate species (Fire et al 1998). In diverse organisms such as *C. elegans*, fruit fly and planaria, long dsRNA has ability to silence the gene function in a sequence specific manner (Fire et al 1998, Sanchez Alvarado and Newmark 1999, Timmons et al 2003). Although components required for RNAi mechanism in shrimp has yet to be identified, it is most likely that dsRNA mediated YHV inhibition occurred though

RNAi pathway. Our finding that dsRNA targeted to nonstructural gene of YHV was more effective than structural gene in suppressing the viral replication should result from copy number of target sequence. Generally the process of viral replication requires replicating enzymes including helicase, polymerase and protease at a lower level compared to the structural proteins thus the mRNA for nonstructural genes of should exist in the infected cells at relatively low abundant (den Boon et al 1996).

Our observation demonstrated that length of dsRNA also contributes to potency of YHV suppression in the shrimp cells. A long dsRNA could generate more diverse pool of effective siRNAs incorporated into RISC complexes than the short

one. This makes one cognate mRNA targeted by a larger pool of siRNA. However, the bona fide of using large fragment of dsRNA should be carefully judged, as a diverse pool of siRNA could compromise the specificity of RNAi by off-target effect (Naito et al 2004, Qiu et al 2005). This phenomenon may explain at least in part the delay in YHV replication observed in cells transfected with irrelevant GFP dsRNA.

The antiviral defense by dsRNA in black tiger shrimp was explored. Introduction of exogenous YHV specific dsRNA in shrimp provided a potent systemic immunity against YHV infection, whereas, unrelated dsRNA even though offered inhibitory effect, its protection was only partial. The systemic inhibition of YHV by specific dsRNA lasted at least for 5 days and was dose dependent. Thus, these results implicate that dsRNA inducing systemic antiviral defense in shrimps is operated through two pathways: sequence-independent antiviral immunity and sequence-specific mechanism. Sequence-specific antiviral protection in shrimps has never been demonstrated. However, sequence independent antiviral defense in shrimp has been reported by Robalino et al (2004) and recently by Westenberg et al (2005). Injection of dsRNA regardless of sequence or its origin resulted in a moderate protection only at low level of viral infection in shrimps (50-60% reduction of mortality rate). The partial YHV inhibition (50-60%) by unrelated dsRNA(gfp) observed in our study, therefore, reinforces that nonspecific response is part of general antiviral immunity in the shrimp. In vertebrate, the non specific antiviral defense by dsRNA modulates through a dsRNA recognition receptor, TLR3, on the cell surface which enables an external immune surveillance imminent viral infection to uninfected cells (Karpala et al 2005). Although TLR3-like receptor was proposed but not demonstrated in shrimp (Westenberg et al 2005), exposure to dsRNA could only be conjectured to go through the TLRs pathways reminiscent to those found in mammals (Schroder, and Bowie 2005).

Our finding that YHV specific dsRNA exerts a potent viral inhibitory effect in shrimp is in consensus with the prominent antiviral response observed in other invertebrates [Dong and Friedrich 2005, Isobe et al 2004). The sequence specific response implicates its intracellular pathway homologous to RNAi. By RNAi pathway, not all viral derived dsRNA would efficiently render the infection. DsRNA (and/or siRNA) specific to a structural gene was not effective in inhibiting viral replication (Westenberg et al 2005). DsRNA targeting a viral nonstructural gene, on the other hand, was shown to trigger the antiviral protection effectively (Lee et al 2002, Valdes et al 2003). Examples are dsRNA targeting baculovirus early gene Ie1 or those targeting protease, polymerase, or helicase of YHV (Valdes et al 2003). These genes are essential but express at low level early in viral replication. Thus, it is conceivable that knocking down an early gene would be more efficient strategy to inhibit viral infection. In addition, knocking down early genes should render any RNAi suppression by viral components ineffective [Li et al 2004], paving the way for efficient viral inhibition.

Taken together our result suggests that at least two mechanisms are operated in systemic antiviral immunity elicited by dsRNA in the shrimp. One of which confers a general antiviral properties thru general non specific pathway resembled to the prominent innate immune response in mammalian, whereas another is mediated through a sequence-specific gene silencing of RNAi. It is most likely that the two pathways are cooperatively functioned in a systemic antiviral defense in shrimp.

When this manuscript has been completed, Robalino et al (2005) demonstrated that dsRNA induced sequence-specific antiviral silencing in addition to nonspecific immunity in a marine shrimp, *L. vanamai* (Robalino et al 2004, Westenberg et al 2005). This finding, although in a different shrimp species, reinforced our new notion that dsRNA induction of antiviral immunity in shrimp goes through two pathways, sequence-independent and sequence-dependent.

Acknowledgements

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CLONING AND FUNCTIONAL CHARACTERIZATION OF GENES ENCODING PROTEIN COMPONENTS OF RNA-induced silencing complex (RISC) OF *PENAEUS MONODON*

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Abstract

Argounate is a family of proteins that are highly conserved across diverse groups of organisms. In eukaryotes, these proteins play key roles in gene silencing processes triggered by small RNA, which is known as RNA interference (RNAi) and related silencing phenomena. Argonaute proteins are defined by the presence of two domains, the RNA-binding PAZ domain and the endonuclease-like PIWI domains. In this study, a cDNA encoding Argonaute protein (Pem-AGO) of the black tiger shrimp *Penaeus monodon* has been cloned. The *Pem-ago* cDNA is composed of 2,829 nucleotides that encoded 943 amino acids Pem-AGO protein. The deduced Pem-AGO exhibited approximately 80% identity to dAGO1 of *Drosophila*. The primary structure and the ubiquitous expression pattern in *P. monodon*'s tissues suggested that Pem-Ago be the member of AGO subfamily of Argonautes that is associated with the RNAi pathway. The dsRNA of *Pem-ago* PAZ domain was able to suppress the expression of *Pem-ago* in the primary culture of *P. monodon*'s Oka cells. This depletion in *Pem-ago* led to the impaired RNAi as the expression of an endogenous gene was rescued from the dsRNA-mediated silencing in these cells. The recombinant PAZ domain and recombinant Pem-AGO were expressed in *Escherichia coli*. These two recombinant proteins displayed RNA-binding activity, and therefore established molecular function of the PAZ domain. Our results has identified Pem-AGO as a member of Argonaute family in penaeid shrimp and implies that Pem-AGO is necessary for RNAi in *P. monodon*.

Keywords Argonaute, Black tiger shrimp, PAZ, PIWI, RNA interference

Introduction

RNA interference, or RNAi, is a gene regulation pathway whereby double-stranded RNA (dsRNA) induced silencing of cognate genes (Hannon, 2002). In eukaryotes, RNAi can act as a natural anti-viral defense (Lu et al., 2005), repress transposition of transposable elements (Sijen and Plasterk, 2003) and be involved in heterochromatin formation (Wassenegger, 2005). RNAi pathway processes long dsRNA into small interfering RNA (siRNA) of ~21 nt by the RNase III enzyme called Dicer. The siRNA is then unwound and one strand of it, referred to as guide strand, is preferentially loaded onto the RNA-induced silencing complex (RISC), which cleaves mRNA target at the fully complementary sequence to the siRNA (Martinez et al.,

2002). The endonucleolytic activity of RISC is believed to be contributed by the Argonaute protein.

Argonaute is a highly conserved family of proteins that is found across diverse groups of organisms ranging from archebacterium to human. Proteins belonging to the Argonaute family play essential roles in several fundamental processes such as nuclear migration and germ-cell formation during embryonic development (Deshpande et al., 2005). Argonaute proteins are implicated in RNA silencing processes that involve short RNA (21-23 nt). RNA silencing could be conducted by several mechanisms, including RNA interference (RNAi) and micro RNA pathway. Argonautes in most eukaryotes are encoded by multigene families. For example, *Caenorhabditis elegans* contains 23 members of Argonaute family (Grishok et al., 2001) *Drosophila melanogaster* has at least 5 Argonaute proteins (Williams and Rubin, 2002) and 8 Argonaute genes were identified in the human genome (Sasaki et al., 2002). Each member of Argonaute family has specialized biological functions. For instances, a member of *C. elegans* Argonaute, RDE1 is required for RNAi but is dispensable for miRNA pathway whereas a mutant of another Argonaute gene, *alg-1*, is miRNA competent but is defective in RNAi (Grishok et al., 2001).

Apart from the involvement in RNAi or related phenomena, some members of the Argonaute family are also associated with developmental control. It has been demonstrated that these two functions of Argonaute proteins may be linked. For example, *AGO1* of *Arabidopsis* was shown to be required for efficient post-transcriptional gene silencing (Fagard et al., 2000), whereas its mutant affected several abnormalities in plant development (Lynn et al., 1999). However, some Argonaute proteins may play essential role in RNAi but are not involved in development such as the RDE-1 of *C. elegans* (Tabara et al., 1999).

Recent studies on biochemical purification of RISC activity have identified Argonaute protein as a core component of this silencing complex (Hammond et al., 2001). Argonaute proteins comprise a highly conserved family whose members are found in diverse organisms across a broad phylogenetic range. The Argonaute family is characterized by the presence of two signature domains, namely PAZ and PIWI domains (Cerutti et al, 2000). The structure of the PAZ domain of *Drosophila*'s Argonaute proteins demonstrated a nucleic acid binding topology that consists of highly conserved residues in all members of the Argonaute family (Lingel et al., 2003 and Yan et al., 2003). The PIWI domain features the tertiary structure that resembles the RNase H family. This RNase H-type structure of the PIWI domain suggests that it contributes the mRNA cleavage activity to the Argonaute protein (Parker et al., 2004, Song et al., 2004).

RNAi has recently been implicated in protection against viruses in the black tiger shrimp, *Penaeus monodon* (Yodmuang et al, 2006). However, a direct evidence for the presence of RNAi pathway in the shrimp is yet to be demonstrated. Identification of the cDNA encoding protein machineries in RNAi pathway will help unravel this question. This study focuses on the identification and characterization of a cDNA encoding Argonaute proteins in *P. monodon*. Both the biological and biochemical functions of *P. monodon*'s Argonaute in RNAi were also examined.

Materials and Methods

RNA extraction and cloning of a cDNA encoding P. monodon's Argonaute (Pem-AGO)

Total RNA was extracted from the lymphoid organ of *P. monodon* by TRI-REAGENT® according to the manufacturer's protocol. Total RNA (about 1 μg) was mixed with 500 nM of oligo-dT (PRT) primer in a final volume of 5 μl of RNase-free water. The mixture was heated at 70°C for 5 min and quickly chilled on ice for 5 min. Then the following components were added to the mixture; 4 μl of 5X Improm-II™ reaction buffer, 2.4 μl of 25 mM $MgCl_2$, 1 μl of dNTP mix (10 mM each), 0.5 μl of 40 unit RNasin ribonuclease inhibitor (Promega), 1 μl of Improm-II™ Reverse-Transcriptase (Promega) and RNase-free water to a final volume of 20 μl. The reaction was incubated at 25 °C for 5 min. Then the temperature was raised to 42°C for 60 min and finally the reverse transcription reaction was inactivated at 70°C for 15 min.

The first strand cDNA was subjected to the amplification of the partial cDNA encoding Argonaute protein using degenerate primers, AGO1 and RISC2-R. The PCR reaction was performed in the volume of 25 μl containing of 17.75 μl of RNase-free water, 2.5 μl of 10X thermophilic polymerase reaction buffer, 2 μl of 2 mM $MgCl_2$, 0.5 μl of dNTP mix (10 mM each), 0.5 μl of 10 μM AGO1 upstream primer, 0.5 μl of 10 μM RISC2-R downstream primer and 1 μl of the first strand cDNA. After incubating the mixture at 94°C for 3 min, 0.25 μl of 1.25 unit Taq DNA polymerase (Promaga) was added. Amplification was performed with 35 reaction cycles, each comprising of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 1 min. The last PCR cycle was followed by a 7 min extension at 72°C for 7 min.

The nucleotide sequence of the partial *Pem-ago* cDNA was used to design a set of specific primers to obtain the remaining sequence of *Pem-ago* cDNA by Rapid Amplification of cDNA ENDs (RACE) strategy. The position and the sequence of the primers used in the cloning steps were shown in figure 1 and table 1, respectively. The oligo-dT-primed first strand cDNA was used as a template for 3 RACE cloning. The PCR reaction was the same as described earlier for the first amplification using degenerate primers, except that 0.5 μl of 10 μM AGO/S1 and 0.5 μl of 10 μM PM-1 primers were used instead of AGO1 and RISC2-R respectively. The amplification step was performed as the previous step, except that the annealing was carried out at 55°C. Then, the nested PCR conducted by AGO/S2 and PM-1 primers was amplified to increase specificity using the same condition, except the annealing temperature at 50°C was used.

For 5 RACE, the first strand cDNA was synthesized from 5 RACE 1 primer and the 3 end of the cDNA was tailed with dATP by the action of terminal deoxynucleotidyl transferase (Promega). The dATP-tailed first strand cDNA was subjected to amplification using 5 RACE 2 gene specific primer, designed from the conserved nucleotide sequence, and oligo-dT primer in the PCR reaction as described in previous steps with the annealing temperature of 55°C.

Amplification of Pem-AGO coding region by Phusion™ DNA polymerase

The first-strand cDNA generated by oligo-dT primer was used as a template to amplify the coding sequence of Argonaute cDNA using 5 and 3 gene specific primers, AGOEx-F and AGOEx-R, that were designed based on the sequence from the start and stop codons of the 5 and 3 RACE products, repectively. The PCR reaction was performed in the volume of 25 µl composing of 12.5 µl of RNase-free water, 5 µl of 5X thermophilic polymerase reaction buffer, 0.75 µl of DMSO, 0.5 µl of dNTP mix (10 mM each), 0.5 µl of 10 µM AGOEx-F upstream primer, 0.5 µl of 10 µM AGOEx-R downstream primer and 1 µl of the first strand cDNA. After incubating the mixture at 98°C for 3 min, 0.25 µl of 1.25 units Phusion™ DNA polymerase was added. Amplification was performed with 35 cycles, each comprising of denaturation at 98°C for 10 sec, annealing at 55°C for 30 sec and extension at 72°C for 90 sec. The last PCR cycle was followed by an extension at 72°C for 10 min. The cDNA was cloned into pUC18 and the recombinant plasmid pUC18/Pem-AGO was transformed into *E. coli* DH5α.

Construction of expression plasmids for expression of recombinant PAZ domain and Pem-AGO in Pichia pastoris

The cDNA coding the PAZ domain and Pem-AGO were amplified from pUC18/Pem-AGO template using specific primers that contain appropriate restriction sites for cloning into pPICZαA expression vector. The location of the primers and restriction sites were shown in figure 2A. The reaction contained 50 ng of DNA template in 1X cloned Pfu buffer, 200 µM each dNTPs 2.5 units of cloned Pfu DNA polymerase 0.5 µM of PAZex-F and PAZex-R primers for PAZ domain cDNA amplification and Argo-F and Argo-R primers for Pem-AGO cDNA. The reaction was hetated to 94°C for 2 min and followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 64°C and extension at 72°C for 4 min and the final extension cycle at 72°C for 7 min.

Expression of recombinant PAZ and recombinant Pem-AGO in P. pastoris

P. pastoris culture in YEPD medium at 1.3-1.5 OD_{600} was collected by centrifugation and the cells were washed once in 1 M ice-cold sorbitol before resuspended in 1 ml of ice-cold 1 M sorbitol. A 40 µl aliquot was dispended into microcentrifuge tube for one transformation reaction. The recombinant plasmid pPICZαA-PAZ and pPICZαA-Pem-AGO were first linearized by digesting with *Dra* I. Then the linearlized plasmids were precipitated with 60 µl of absolute ethanol in the presence of 3 µl of 3 M sodium acetate and 1.5 µl of tRNA and resuspended in 5 µl of sterile distilled water. About 300 ng of linearized recombinant plasmid DNA were mixed with 40 µl of *P. pastoris* competent cells. The mixture was transferred to an ice-cold 0.2 cm electro-cuvette and incubated on ice for 5 min. The cells were pulsed by using BIO-RAD Gene Pulser according to the conditions of 1.5 kV, 25 µF and 200 Ω (time constant should be 4.5). Then 1 ml of 1 M sorbitol was added immediately to the cuvette and the mixture was transferred into a sterile microcentrifuge tube. The tube was incubated at 30°C without shaking for 1 hr, before 1 ml of YEPD medium was added. The culture was further incubated at 30°C with shaking for another 2 hr. The cells were then harvested and 800 µl of YEPD medium was added to resuspend the cell pellet. Then 100 µl of transformed cell was spread on YEPD agar plate

containing 100 μg/ml Zeocin™. The plate was incubated at 30 °C for 2-3 days until visible colonies were formed.

Construction of expression plasmids for expression of recombinant PAZ domain and Pem-AGO in Escherichia coli

The cDNA was amplified from pUC18/Pem-AGO template as described earlier using specific primers that contained appropriate restriction sites for cloning into pET15b expression vector; ExPAZ-F and ExPAZ-R for PAZ domain cDNA and ExAgo-F and ExAGO-R primers for Pem-AGO cDNA amplification (figure 2B). The reaction was set up and carried out in the same condition as described for *P. pastoris* expression plasmid.

Expression of recombinant PAZ and recombinant Pem-AGO in E. coli

The recombinant plasmids were transformed into *E. coli* BL21(DE3)pLysS by $CaCl_2$ method. An overnight culture of *E. coli* BL21(DE3)pLysS harboring recombinant plasmids was diluted 1:100 in LB broth containing 100 μg/ml ampicillin and incubated at 37°C for until OD_{600} of the culture reached 0.5-0.6. To express the recombinant protein under the control of T7-promoter, IPTG was added into the culture at a final concentration of 0.1 mM. The induced culture was incubated at 25°C with shaking at 250 rpm for appropriate time. The cell culture was harvested and analyzed by SDS-PAGE.

Purification of recombinant proteins

Cell pellet harvested from 1 liter expression culture was resuspended with 20 ml of lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM NaCl, 5% glycerol, 10 mM imidazole, 1 mM PMSF and 0.1 mg/ml lysozyme) The lysis of the cells was achieved by sonication with the power level 3 for total sonication time of 3 min before subjected to Cell press (French Press) with 10,000 psi twice. The inclusion protein pellet was separated from the soluble part by centrifugation at 10,000 rpm for 40 min at 4°C. The soluble fraction was purified by using HisTrap™ FF crude column (Amersham) as described by the manufacturer. The soluble fraction of His-tagged recombinant protein was applied into the column at 1 ml/min flow rate. The column was washed with 15 ml of washing buffer (buffer H containing 30 mM imidazole) and the proteins eluted with the gradient of imidazole (100-500 mM) in elution buffer. Each fraction was analyzed on SDS-PAGE or by Western Blot analysis.

Synthesis of double-stranded RNA by in vitro transcription

The small DNA templates for double-stranded RNA (dsRNA) of the PAZ domain were synthesis were amplified using dsPAZF and dsPAZR (Table 1). The PCR product was then ligated to pGEM®-T Easy vector and transformed into *E. coli* DH5α. The direction of the insert fragment was determined by DNA sequencing. The clone that contained the insert in either forward and reverse direction related to the T7 promoter were selected as a template for *in vitro* transcription of the sense or antisense strands. A plasmid DNA template was linearized with an appropriate restriction enzyme (*Sal* I) before subjected to *in vitro* transcription. The reaction was performed in 100 μl reaction mixture containing 20 μl of T7 Transcription 5X Buffer (400 mM of HEPES-KOH (pH 7.5), 120 mM of $MgCl_2$, 10 mM of spermidine and 200 mM of DTT), 30 μl of rNTPs (25 mM each of ATP, CTP, GTP, UTP), 40 μl of

10 μg of linear DNA template, 10 μl of T7 RNA polymerase. The reaction was mixed and incubated at 37°C for 4 hr. Then 10 μl of RNase-Free DNase (1u/ug) was added to the reaction to remove the DNA template following transcription. The sample was incubated at 37°C for 15 min and extracted by phenol/chloroform before isopropanol precipitation.

Equal amount of sense- and antisense RNA was mixed in annealing buffer (100 mM of Potassium acetate, 30 mM of HEPES-KOH (pH 7.4) and 30 mM of MgOAC) and
heated at 90°C for 2 min. The temperature was gradually decreased to 37°C and hold for 1 hr. followed by incubation at room temperature for another 1 hr. The reaction was then treated with 0.1 mg RNase A at 37°C for 10 min followed by phenol/chloroform extraction and isopropanol precipitation.

***In vivo* stem-loop production (Bacterial expression) of double-stranded RNA**

The pET3a recombinant plasmid containing stem-loop construct of protease cDNA of yellow head virus (YHV) or green fluorescent protein (GFP) was grown overnight at 37°C. The 0.5 ml of overnight culture was transfered into 50 ml of 2XYT medium and incubated at 37°C with vigorous shaking until OD_{600} reached 0.6-0.7. Then the culture was induced with 0.4 mM IPTG for 4 hr until OD_{600} reached 1.0. The cell pellet was collected and resuspended in 0.1% SDS in PBS. The sample was boiled for 2 min followed by RNase A digestion to degrade the loop from the dsRNA. The dsRNA was extracted with TRI-REAGENT® and resuspended with 150 mM NaCl.

Preparation of the primary cell culture from the lymphoid (Oka) organ

The method previously described (Assavalapsakul et al., 2003) was followed. The lymphoid organs were collected from 100-150 live sub-adult shrimps. The pooled lymphoid organs were washed five times in washing medium [2X-Leibovitz's L-15 medium containing 15% fetal bovine serum, 5% lactabumin, 200 IU/ml penicillin, and 200 μg/ml streptomycin]. The collected tissue was then transferred to complete medium [2X-Leibovitz's L-15 medium supplemented with 15% fetal bovine serum, 5% (v/v) lactabumin, 15% shrimp meat extract, 200 IU/ml penicillin, and 200 μg/ml streptomycin] and minced into suspension. The minced tissues were seeded onto a 24 well-plate and incubated to confluence in 26°C incubator.

Double-stranded RNA transfection into the lymphoid cells

The administration of dsRNA into the primary culture of Oka cells was performed by using Transmessenger RNA transfection kit (Qiagen). Generally, two micrograms of dsRNA were used in each transfection. The double-stranded RNA was pretreated with specific RNA-condensing reagent (Enhancer R) and Transmessenger transfection reagent as recommended by the manufacturer before evenly dropped onto the surface of the primary culture of Oka cells. After incubation with dsRNA at 26°C for 3 h, the cells were washed with washing medium and further incubated in fresh complete medium in a 26°C incubator. At appropriate time points, total RNA was extracted form the Oka cells, and the expression of particular genes were detected by RT-PCR using primer that were specific to each transcript.

RNA binding assay

The RNA-protein binding was detected by electrophoretic mobility shift assays. A 21 nt single stranded RNA (ssRNA) was label at 5 end with γ-^{32}P ATP by T4 polynucleotide kinase. The RNA-protein binding was allowed to occur in a 15 µl reaction containing 20 mM HEPES (pH 7.4), 3 mM $MgCl_2$, 50 mM KCl, 2mM DTT and 5% glycerol. The amount of ^{32}P-labeled ssRNA was optimized from 10, 100, 200, 500 and 1,000 fmole whereas the amount of the recombinant proteins (recombinant PAZ or Pem-AGO) was varied from 0 to 1 µg. The reaction mixture was incubated for 45 min at room temperature The reactions were analyzed on 5% non-denaturing PAGE with the constant 150 volts for appropriate time. Subsequently, the gel was dried on Whatman paper and the RNA-protein complex was detected by autoradiography.

Results

Cloning and analysis of a cDNA encoding P. monodon's Argonaute (Pem-AGO)

The nucleotide sequences of three overlapping *Pem-ago* cDNA clones were assemble to obtain the virtual transcript of *Pem-ago*. The existence of this *Pem-ago* cDNA was confirmed by the cloning of the entire coding region for *Pem-ago* from lymphoid RNA. The nucleotide and deduced amino acid sequences of Pem-AGO are shown in Figure 3. Similarity search by the blastp program showed that Pem-AGO shared the highest homology, at 77% overall amino acid identity, to Argonaute 1 of *Drosophila* (dAgo1). Similarity between Pem-AGO and other Argonautes is summarized in Table 2. Remarkable degrees of homology were essentially detected in the two conserved domains, in which more than 99% of the amino acid sequences of PAZ and PIWI domains of Pem-AGO could be aligned to the consensus as analyzed by the Conserved Domain Search Service (Marchler-Bauer and Bryant, 2004) via http://www.ncbi.nlm.nih.gov/Structure/ cdd/wrpsb.cgi.

The sequences in Figure 3 illustrated that three *Pem-ago* cDNA variants were obtained. The first type, so called *Pem-ago* or *Pem-ago L1*, was consisted of 2,829 nucleotides that encoded 943 amino acids Pem-AGO protein. The second type, *Pem-ago L2*, contained the same sequence as *Pem-ago* except the deletion of 9 nt at position 227 to 235 of the ORF that resulted in the absence of three amino acid residues at corresponding positions and a substitution of threonine for alanine at position 76 of the encoded protein. The last type of *Pem-ago* variant, namely *Pem-ago S*, possessed a deletion of 81 nt in the coding region for the PIWI domain of *Pem-ago*.

Tissue specific expression of Pem-ago in P. monodon

Expression of *Pem-ago* in different tissues of *P. monodon* was examined by RT-PCR using the primers that amplify the PIWI coding sequence. The result in Figure 4 revealed that the prominent level of *Pem-ago* expression was found in the ovary, gill and lymphoid organ. In addition, *Pem-ago* was also ubiquitously expressed, though at lower levels, in all tissues examined. This ubiquitous expression pattern of *Pem-ago* may reflect its universal role in the shrimp.

***In vitro* transcription and annealing of dsRNA**

Both the sense and antisense single-stranded RNA of the PAZ domain was obtained by *in vitro* transcription. The equal concentration of sense and antisense RNA were used for the annealing reaction to obtain PAZ-dsRNA The annealed dsRNA products showed a major band with several other discrete bands that may represent different multimeric form of the annealing products (Figure 5). In order to prove whether the products of the annealing reaction really existed in the form of dsRNA, these annealing products were treated with RNase A, the enzyme that selectively degrades only single-stranded RNA. The RNase A-treated products appeared as a single band of the smaller size than the major band seen from the untreated products (Figure 5). This result confirmed that the PAZ-dsRNA was obtained.

Silencing of Pem-AGO expression in primary culture of Oka cells by PAZ-dsRNA

To elucidate the potency of PAZ-dsRNA to silence *Pem-ago* expression, the PAZ-dsRNA was delivered into Oka cells by transfection. The level of *Pem-ago* mRNA in the cells was detected by RT-PCR at different time-points after transfection. As shown in Figure 6, the level of *Pem-ago* transcript declined to about 50% in the cells transfected with PAZ-dsRNA comparing with that in the control cells that did not receive any dsRNA. This effect could be observed since 24 h after transfection, and lasted until 72 h. This result demonstrates that PAZ-dsRNA was effective, albeit not absolutely, in silencing *Pem-ago* expression in the Oka cells. Slight repression in *Pem-ago* transcript level was also observed in the cells transfected with irrelevant GFP-dsRNA at every time points examined.

RNAi in Pem-ago depleted cells

To test whether *Pem-ago* is involved in RNAi process, the efficacy of RNAi in *Pem-ago* depleted background was determined in the Oka cells that had been transfected with PAZ-dsRNA and, after 24 h, followed by the dsRNA of endogenously expressed serotonin receptor (5-HTr) gene in comparison with the cells that were only transfected with 5-HTr-dsRNA. The result in Figure 7 showed that 5-HTr-dsRNA could efficiently knockdown the expression of 5-HTr as manifested by barely detectable level of *5-HTr* transcript in the cells administered with 5-HTr-dsRNA alone, whereas high expression level of *5-HTr* was readily detected in the control cells that did not received any dsRNA. However, approximately 40% of *5-HTr* transcript was recovered in the cells into which 5-HTr-dsRNA was introduced at 24 h following the first transfection with PAZ-dsRNA. By contrast, the cells that had received GFP-dsRNA before transfected with 5-HTr-dsRNA at the next 24 h expressed scanty amount of 5-HTr transcript comparable to that in cells transfected solely with 5-HTr-dsRNA. This result indicated that dsRNA-induced gene silencing process was partially impaired in *Pem-ago* depleted cells.

Expression of recombinant PAZ domain in P. pastoris

The recombinant plasmid pPICZαA-PAZ was transformed into *P. pastoris* by electroporation. The recombinant clones harboring integration of the expression cassette into yeast genome were screen by genomic PCR. Five recombinant *P. pastoris* clones were selected for expression. The expression was first induced with 3% methanol for 0-5 days. Since the PAZ cDNA was cloned in-frame with the α-

factor secretion signal, the culture supernatant was used to analyze the secreted recombinant PAZ protein. Figure 8 revealed that comparing to the negative control which were the cells containing pPICZαA integration alone, only an extra faint band at the expected size (~19 kDa) for the His-tagged-PAZ fusion protein was observed. Moreover, the results showed that the amounts of expressed proteins were decreased after 3 days of expression period, especially in positive control that illustrated the large amount of degraded product in day 4 and day 5. Therefore the appropriate period of expression should be less than 3 days.

Recombinant *P. pastoris* clone number 2 was also expressed with various concentration of methanol from 0-5% for the period of 2 days. Both the SAS-PAGE and Western Blot analysis with anti-His antibody showed no positive signal of the expected fusion PAZ domain in the culture medium (Figure 9). The presence of intracellular recombinant PAZ domain was also examined by Western Blot analysis. However, the result showed that the recombinant PAZ was not detectable inside the cells.

Expression of recombinant Pem-AGO in E. coli

Since the expression of recombinant PAZ domain in *P. pastoris* was not successful, the recombinant proteins were expressed in *E. coli* as an alternative. The PAZ and Pem-AGO cDNA were ligated to pET15b expression vector in the same reading frame with the N-terminal six histidine sequence. The expression of recombinant His-tagged Pem-AGO fusion protein in BL21(DE3)pLysS recombinant clones containing pET15b-Pem-AGO was performed in various conditions i.e. induction with 0.1-1 mM IPTG, for 0-20 h at either 25°C or 30°C. The result showed that the optimal condition for expression of recombinant Pem-AGO fusion protein was induction with 0.1 mM IPTG for 2 hr at 25 °C as analyzed by SDS-PAGE and Western Blot analysis (Figure 10A). Most of the recombinant Pem-AOG was accumulated as the inclusion. However, miniature amount of the recombinant protein could be detected in the soluble fraction by Dot Blot analysis (data not shown).

Expression of recombinant PAZ domain in E. coli

The recombinant PAZ domain was also expressed in *E. coli* in the form of hitidine-tagged fusion protein. Expression of the recombinant protein in various conditions showed that the optimal condition for expression was induction with 0.1 mM IPTG at 25°C for 7 h (Figure 10B). Similar to Pem-AGO, small quantity of the recombinant PAZ domain fusion protein was expressed in soluble form.

Purification of recombinant Pem-AGO and PAZ domain proteins

The soluble recombinant proteins harboring the hexa-histidine tag fused to the N-terminus were purified under native conditions by Ni^{2+} affinity chromatography, employing the HisTrap™ FF crude column (Amersham). The fusion proteins were eluted by the gradient of imidazole from 100, 200, 300, 400 and 500 mM and the fractions were analyzed by SDS-PAGE and Western Blot. Figure 11 illustrated that both recombinant Pem-AGO fusion proteins were eluted out in fractions 7 to 9 and Figure 12 showed that the recombinant PAZ domain protein was also eluted from fraction 7 onwards. However, Western Blot analysis using anti-His antibody could also detected some other protein bands that were co-eluted in the same fraction as the

recombinant Pem-AGO whereas a single band of recombinant PAZ was detected by the anitbody.

RNA binding activity of recombinant PAZ domain and recombinant Pem-AGO

Binding of Argonaute protein to siRNA is the crucial step during the incorporation of siRNA into RISC. This binding activity has been demonstrated both structurally and biochemically to be contributed by the PAZ domain of Argonaute protein. Therefore the RNA binding activity of the recombinant PAZ domain as well as the recombinant Pem-AGO was investigated by RNA electrophoretic mobility shift assay. The appropriate ratio between the protein and the siRNA in the binding reaction was determined. The adequate signals were detected when using 1 pmol of ^{32}P-labelled siRNA (AAGCTGACCCTGAA GTTCATC). The clear shift band was observed in the reaction containing 1 pmole of labeled siRNA and 200 to 500 ng of the recombinant PAZ domain as shown in figure 13. The 500 ng of recombinant Pem-AGO was also able to produce a higher molecular weight shift band, although at lower degree.

Discussion

A cDNA encoding a member of Argonaute family from *P. monodon*, so called *Pem-ago*, was successfully obtained by RT-PCR with degenerate primers designed from the conserved sequences among other Argonautes. The encoded protein from this cDNA is classified into the Argonaute family because of the presence of the conserved features, the PAZ and PIWI domains as well as the significant degree of overall homology to its closely related member, dAgo1 of *D. melanogaster*. In *Drosophila*, genome search for the PAZ and PIWI domains has identified five Argonaute-like genes (Williams and Rubin, 2002), which can be divided into AGO and PIWI subfamilies according to the sequence alignment and the construction of phylogenetic tree (Carmell et al., 2002). The *Drosophila* dAGO1 and dAGO2 belong to the AGO subfamily that are expressed ubiquitously in the embryos. By contrast, members of the PIWI subfamily display tissue-specific embryonic expression patterns. The differences in expression pattern suggests that the PIWI subfamily may play tissue-specific role during development rather than a more ubiquitous role in RNAi that is responsible mainly by the AGO subfamily. Since the primary structure as well as the expression of *Pem-ago* in all tissues examined in this study is generally consistent with the AGO subfamily, *Pem-ago* is implicated for its involvement in *P. monodon*'s RNAi mechanism.

To certify this inference, the requirement of *Pem-ago* for RNAi was investigated in the primary culture of *P. monodon*'s lymphoid cells. The Oka cell culture that acquired PAZ-dsRNA by transfection exhibited a considerable suppressed level of *Pem-ago* expression. Comparable level of repression of the rat Argonaute protein, GERp95, was observed in PC12 cells by similar strategy (Thonberg et al., 2004). The requirement of Pem-AGO for RNAi silencing mechanism may explain the incomplete suppression of *Pem-ago* expression by its specific dsRNA since *Pem-ago* expression would be de-repressed at certain time once the amount of Pem-AGO proteins in the cells has been depleted due to the silencing. Another probable explanation is that certain level of *Pem-ago* expression needed to be maintained because it may possibly be essential for cell viability. However, *Argoanute* transcript

could be completely suppressed by loss-of function mutation or gene knockout strategies as has been shown in some species (Williams and Rubin, 2002; Shi et al., 2004), and therefore rule out this possibility. In addition, some lesser extent of silencing of *Pem-ago* expression could also be mediated by unrelated GFP-dsRNA. Similar sequence-independent silencing phenomena have been demonstrated recently in the shrimp (Robalino et al., 2004; Westenberg et al., 2005; Yodmuang et al., 2006).

A substantially impaired RNAi in *Pem-ago*-depleted cells was clearly demonstrated by the recovery of 5-HT receptor transcript from 5-HTr-dsRNA mediated silencing. Moreover, the failure of GFP-dsRNA to direct this impairment suggests the unambiguous influence of *Pem-ago* on the potency of RNAi. Our result is in concurrence with the knockdown of neuropeptide Y (NPY) gene by siRNA in rat OC12 cells that was also impaired after the rat Argonaute GERp95A had been diminished (Thonberg et al., 2004).

Although the Pem-AGO that has escaped the silencing by PAZ-dsRNA may be responsible for the remaining RNAi activity in *Pem-ago*-depleted cells, we can not exclude the possibility that other Argonaute proteins of *P. monodon* also participate in RNAi. Three cDNA variants of *P. monodon*'s Argonaute have been identified in this study. However, the expression of all three *Pem-ago* transcript variants should be diminished by the PAZ-dsRNA in this study because they possessed identical PAZ coding sequence. Since the Argonaute family in many eukaryotes comprises multiple members (Carmell et al., 2002), the existence of additional *Argonaute* members in *P. monodon* that may associate with RNAi and be responsible for partial silencing in *Pem-ago* depleted cells still await further exploration.

In addition to its biological function in RNAi, we have also attempted to assay for biochemical activity of *Pem-ago* in relation to the RNAi mechanism. Recombinant Pem-AGO and recombinant PAZ domain were expressed as N-terminal histidine fusion proteins. The failure in expression of recombinant proteins in *P. pastoris* was probably due to the different codon preference. Moreover, the *Pem-ago* cDNA contained the AT-rich regions that have been shown to cause premature termination in yeast (Henikoff and Cohen, 1984; Scorer et al., 1993). To verify these possibilities, RT-PCR or northern analysis needs to be performed. The expression of recombinant Pem-AGO and recombinant PAZ domain in *E. coli* was successful with small portion of the expressed recombinant protein reside in the soluble fraction, which were then purified based on the affinity to the histidine-tag. The binding of recombinant PAZ and recombinant Pem-AGO to single-stranded siRNA as demonstrated by RNA mobility shift assay established the molecular function of the RNA binding domain in recombinant Pem-AGO. This result also implied that the soluble recombinant proteins adopt the correct fold of the RNA binding motif of this family, which is composed of a six-stranded B-barrel capped by two a-helices and an αβ module on the N- and C terminus, respectively (Ma et al., 2004). This defined structure is specific to RNA binding because both the *D. melanogaster* AGO1 and the PAZ domain of human Ago1 have been demonstrated to bind RNA preferentially over DNA (Yan et al., 2003). The RNA-specific binding property of the PAZ domain of Pem-AGO could be elucidated by using appropriate competitors to compete for the binding.

It has been demonstrated recently that the purified Argonaute 2 from *Drosophila* Schinieder 2 cells extract was the sole protein required for mRNA cleavage in the RNAi pathway (Rand et al., 2004). Rivas et al., 2005 also established

the *in vitro* recombinant human RISC activity that was exclusively composed of purified recombinant Argonaute 2 and siRNA. Accordingly, the function of Pem-AGO in RNAi could be established more concretely by the mRNA cleavage assay in which the complex between siRNA and recombinant Pem-AGO is incubated with the mRNA that is complementary to the siRNA.

In summary, the present study provides compelling evidence on the RNAi-mediated gene silencing in *P. monodon* by having identified *Pem-ago* as the first essential component in RNAi machinery in penaeid shrimp. Our result has also placed crustacean into the list of a diverse range of organisms in which the RNAi plays essential roles.

Acknowledgements

We thank Asst. Prof. Witoon Tirasophon for his kind advice in primary cell culture technique, Dr. Chalermporn Ongvarrasopone for providing the 5HTr dsRNA in the *in vitro* silencing experiment, and Mr. Wanlop Chinnirunvong for his help in the preparation of primary cell culture. The positive control for protein expression, recombinant DerP3.1 protein was given by Miss Nipawan Nuemket and recombinant T-cyaA was provided by Mrs. Busaba Powthongchin.

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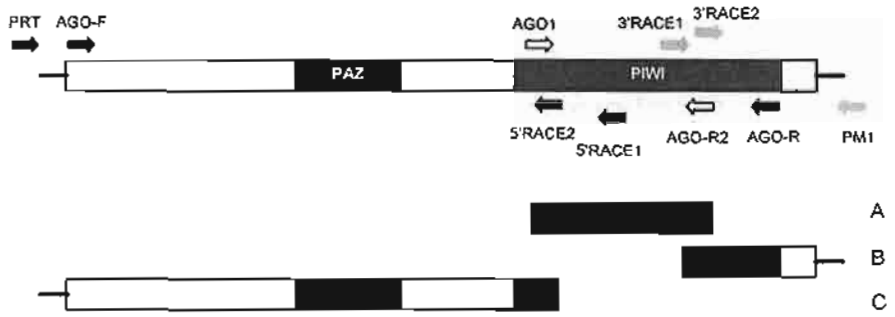

Figure 1. A schematic diagram representing the primers used for cloning of *Pem-ago* cDNA. Three overlapping fragments that were generated by RT-PCR and RACE are illustrated as fragments A, B and C. The primers AGO-F and AGO-R were used to obtain the entire coding sequence of Pem-AGO.

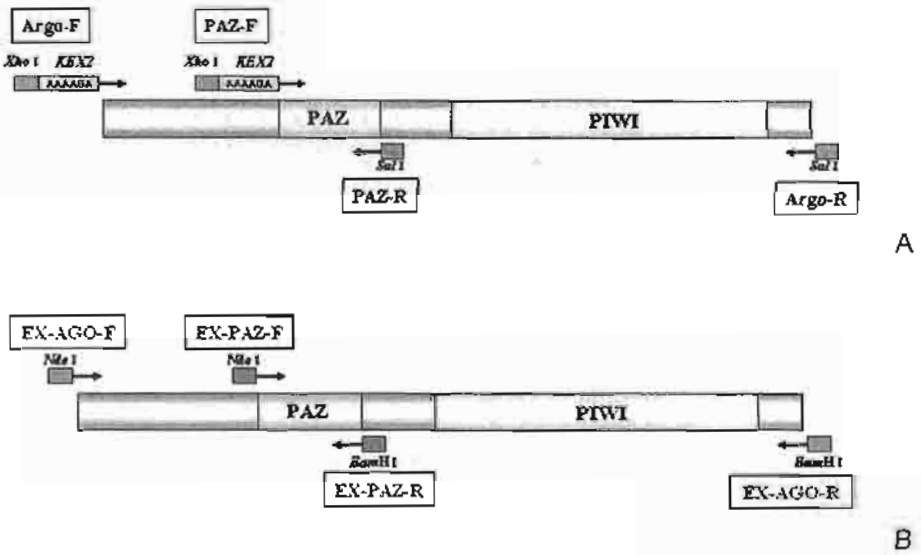

Figure 2. A schematic diagram illustrating the binding sites of primers on Pem-*ago* cDNA template for amplification of Pem-AGO and PAZ domain. The arrows represent the primers and the attached square boxes represent the recognition sequence of restriction enzymes for cloning into pPICZaA vector for expression in *P. pastoris* (A) or into pET15B for expression in *E. coli* (B).

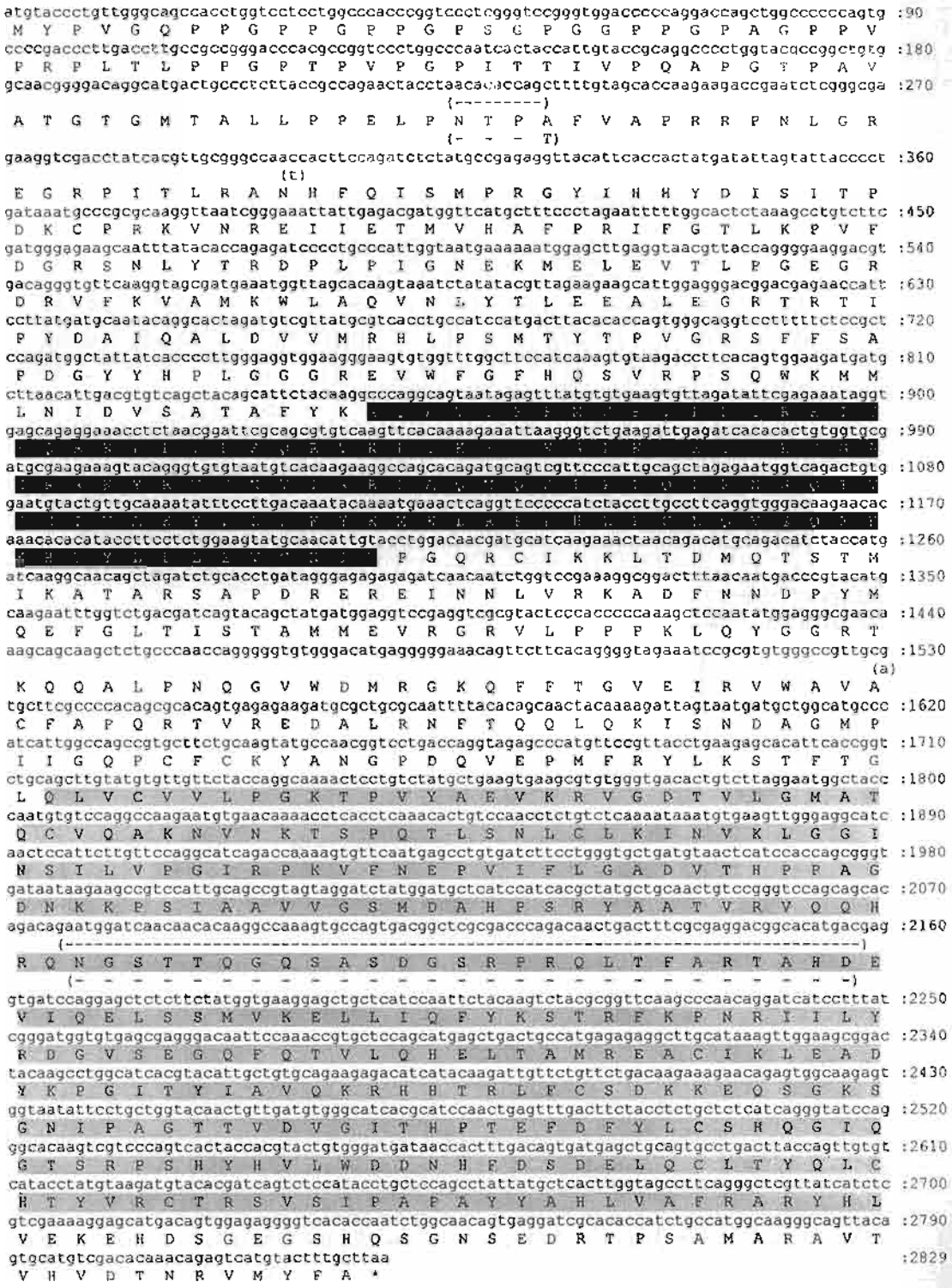

Figure 3. Nucleotide and deduce amino acid sequences of Pem-AGO. The sequence of *Pem-ago* (*Pem-ago-L1*) variant is shown. The deduced amino acid residues are shown in one-letter symbol. The PAZ and PIWI domains are highlighted in black and gray, respectively. The asterisk marks the stop codon. The 9 nt deletion in *Pem-ago-L2* and the 81 nt deletion in the PIWI domain of *Pem-ago-S* are depicted by dashed lines.

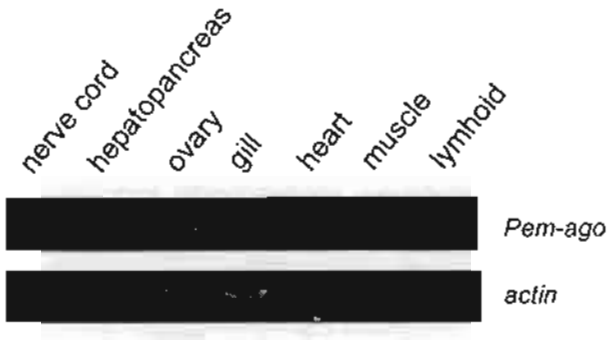

Figure 4. Expression of *Pem-ago* in *P. monodon* tissues. Total RNA was extracted from nerve cord, hepatopancreas, ovary, gill, heart, muscle and lymphoid organ of *P. monodon*. The mRNA of *Pem-ago* in these tissues was detected by RT-PCR. The actin transcript was used as internal control.

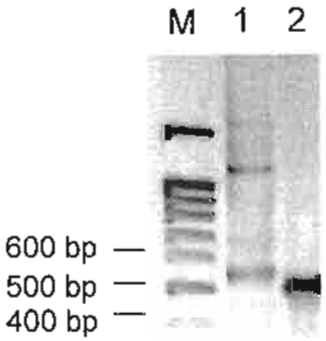

Figure 5. Double-strand RNA of the PAZ domain. Plasmid DNA containing short DNA template in sense and antisense directions of the PAZ coding sequence were transcribed to single-stranded RNA by *in vitro* transcription. Both strands of PAZ *in vitro* transcription products were annealed together to obtain the PAZ-dsRNA product as shown in lane 1. Lane 2 represents dsRNA product in lane 1 after treated with RNase A. The dsRNA was analyzed on 1.5% agarose gel electrophoresis with the 100 bp DNA ladder loaded in lane M.

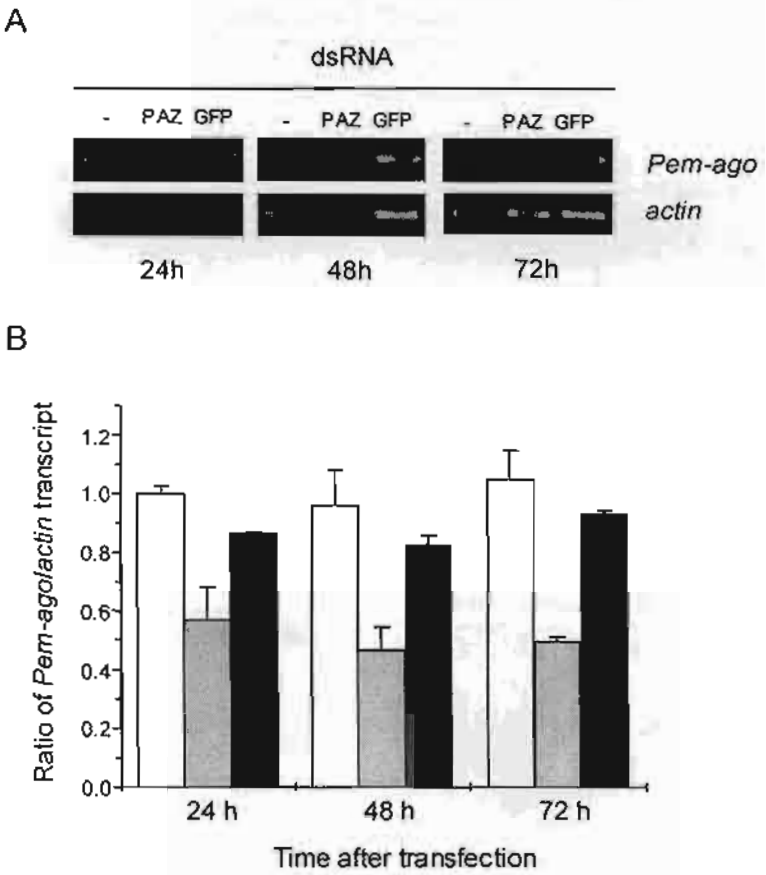

Figure 6. Silencing of *Pem-ago* expression in primary culture of Oka cells. (A) The transcript of *Pem-ago* in the Oka cells that were transfected with 2 □g of either PAZ-dsRNA (PAZ) or GFP-dsRNA (GFP) for 24, 48 and 72 h was detected by RT-PCR and compared with the control cells that did not exposed to any dsRNA (-). (B) Ratio of *Pem-ago* to *actin* transcript calculated from the RT-PCR products in (A).

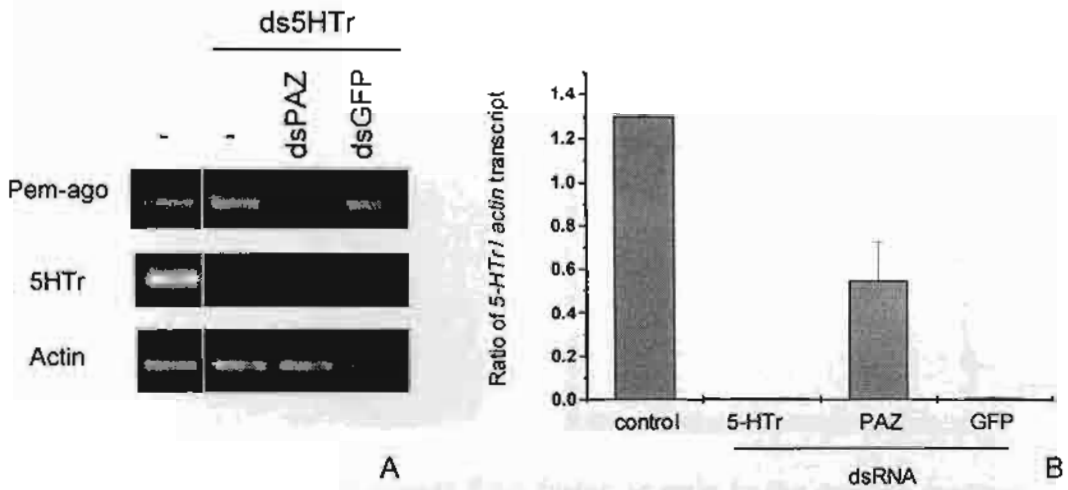

Figure 7. Impairment of RNAi in *Pem-ago* depleted cells. The Oka cells were transfected with 2 □g of either PAZ-dsRNA or GFP-dsRNA 24 h prior to subsequent transfection with 5-HTr-dsRNA. The cells were collected for total RNA extraction at 48 h afterward and the 5-HTr transcript was detected by RT-PCR. (A) RT-PCR of *Pem-ago* and *5HTr* transcripts in the cells that

acquired no dsRNA (-), the cells transfected with 5HTr-dsRNA at 24 h without prior transfection (ds5HTr/-) and the cells transfected with PAZ-dsRNA (ds5HTr/dsPAZ) or GFP-dsRNA (ds5HTr/dsGFP) followed by 5HTr-dsRNA at 24 h later. (B) The graph showing relative amount of *5HTr* to *actin* transcript in the cells in (A) The values represent mean±SEM of two independent experiments in duplicate.

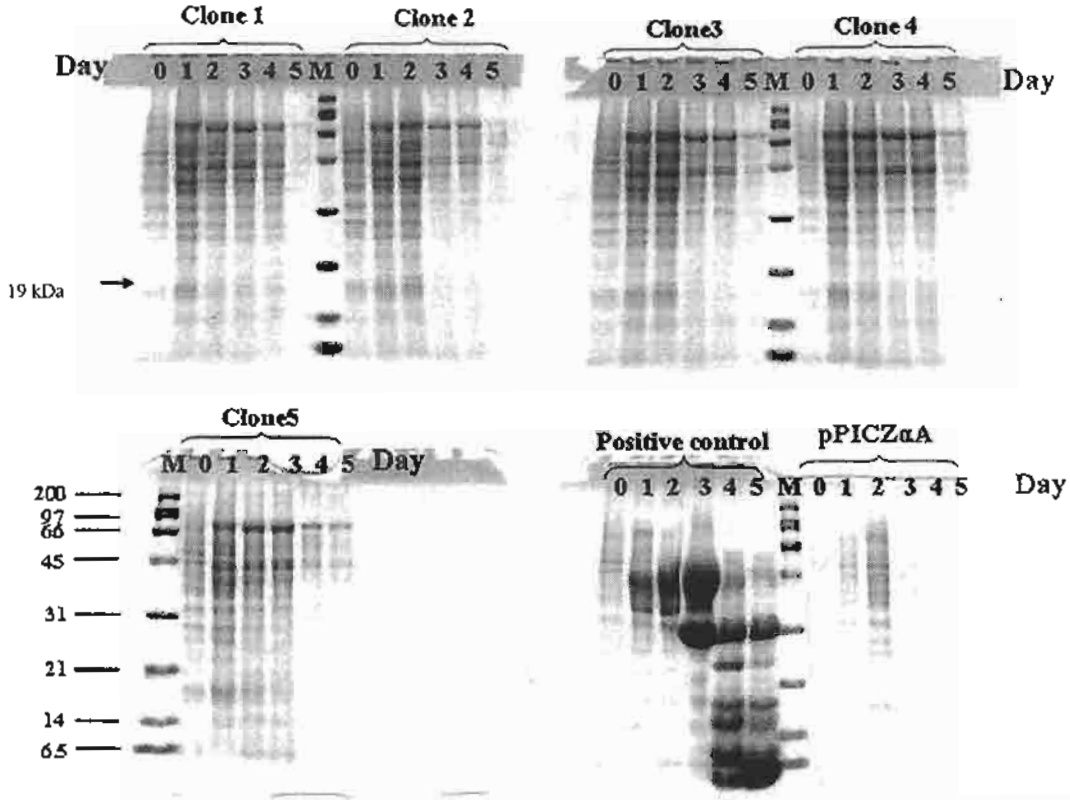

Figure 8. SDS-PAGE analysis of the recombinant His-tagged PAZ fusion protein expression. The figure shows 15% SDS-PAGE with Coomassie blue staining of the 1 ml TCA precipitated culture medium of clones 1 to 5 after induction with 3 %(v/v) methanol for 0-5 days. *P. pastoris* containing integrated recombinant DerP3.1 and pPICZαA were used as positive and negative control, respectively.

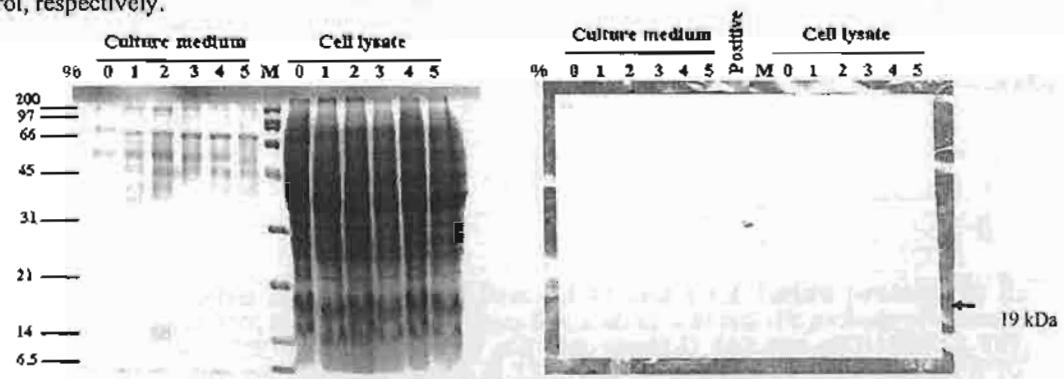

Figure 9. Expression of recombinant PAZ fusion protein in the culture medium and the cell lystae. The figure shows 15% SDS-PAGE with Coomassie blue staining (A) and western blot analysis with anti-His antibody (B) of the 1 ml TCA precipitated culture medium and 6 OD_{600} cell lysate from the *P. pastoris* containing expression cassette pPICZαA-PAZ after induction with 0-5 % methanol for 2 days.

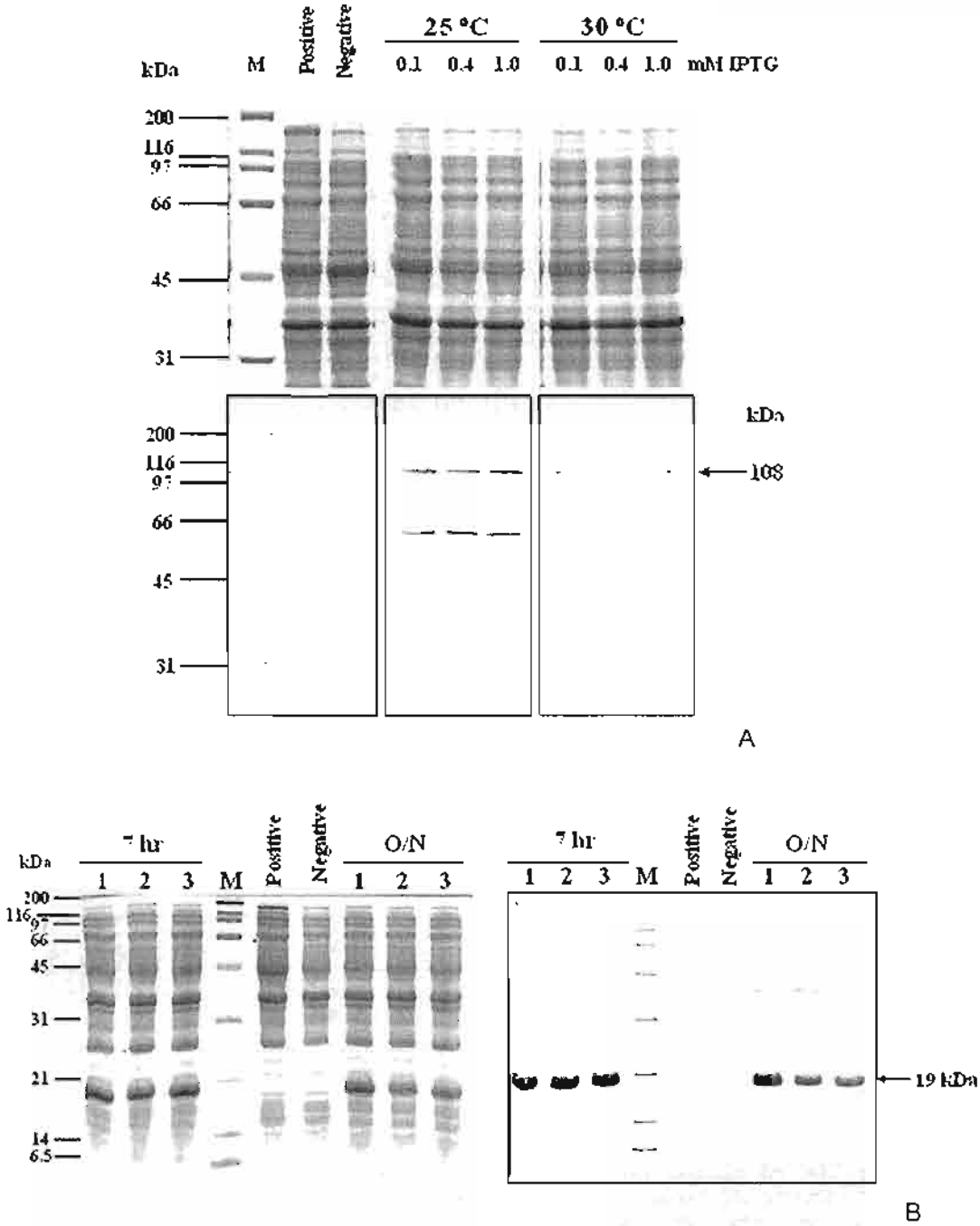

Figure 10. Expression of recombinant Pem-AGO and PAZ fusion proteins in *E. coli*. The figures show 15% SDS-PAGE and Western Blot analysis with anti-His antibody of the crude cell lysate from BL21(DE3)pLysS containing pET-15b/Pem-AGO (A) and pET-15b-PAZ (B). Recombinant Pem-AGO was expressed for 2 h at 25 °C and 30 °C with 0.1, 0.4 and 1 mM IPTG induction. Recombinant PAZ was expressed form clones no.1 to 3 at 25°C with 1 mM IPTG induction for 7 h and overnight (O/N).

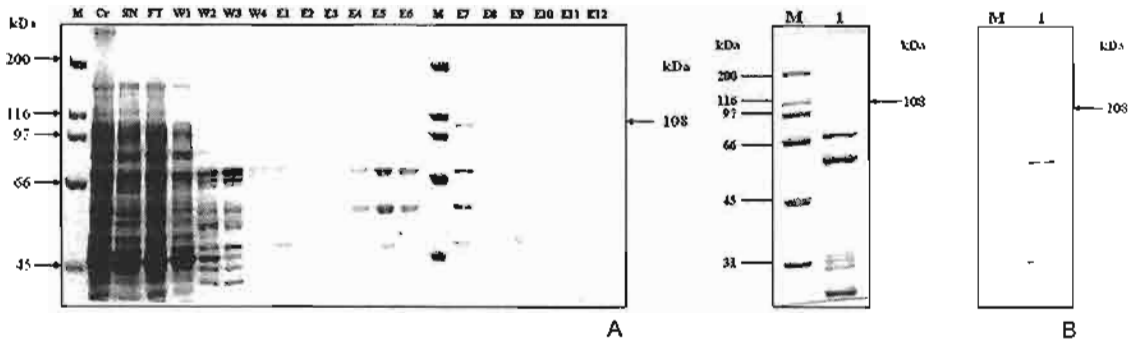

Figure 11. Purification of recombinant His-Pem-AGO fusion protein by Ni^{2+} affinity chromatography. (A) The protein profile on Coomassie blue-stained 8% SDS-PAGE of the purification of recombinant Pem-AGO fusion protein in the soluble fraction by Ni^{2+} affinity chromatography utilizing imidazole gradient step elution.. M: Standard broad range protein marker; Cr: crude cell lysate from BL21(DE3)pLysS harboring pET15b-Pem-AGO; SN: soluble fraction before loading onto the column; FT: flow-through fraction containing unbound protein; W1-W4: wash fractions 1 to 4 (buffer H + 30 mM imidazole) and E1-E12: elution fractions 1 to 12 (buffer H + 100-500 mM imidazole). (B) Western blot analysis of the fraction containing recombinant Pem-AGO fusion protein (lane 1) detected by anti-His antibody.

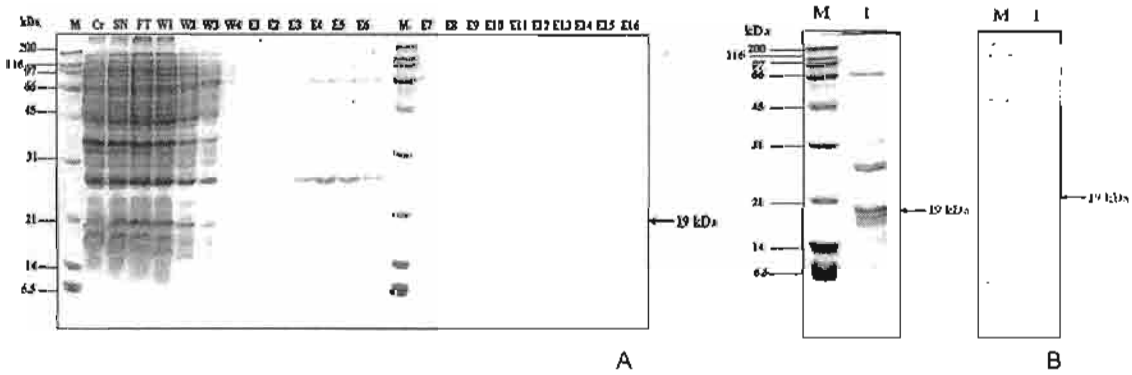

Figure 12. Purification of recombinant His-PAZ fusion protein by Ni^{2+} affinity chromatography. (A) The protein profile on Coomassie blue-stained 8% SDS-PAGE of the purification of recombinant PAZ fusion protein in the soluble fraction by Ni^{2+} affinity chromatography utilizing imidazole gradient step elution.. M: Standard broad range protein marker; Cr: crude cell lysate from BL21(DE3)pLysS harboring pET15b-PAZ; SN: soluble fraction before loading onto the column; FT: flow-through fraction containing unbound protein; W1-W4: wash fractions 1 to 4 (buffer H + 30 mM imidazole) and E1-E16: elution fractions 1 to 16 (buffer H + 100-500 mM imidazole). (B) Western blot analysis of the fraction containing recombinant PAZ fusion protein (lane 1) detected by anti-His antibody.

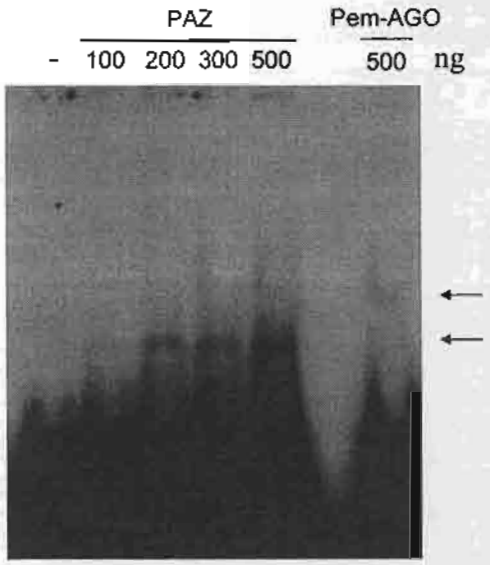

Figure 13. RNA binding activity of recombinant PAZ and recombinant Pem-AGO. Abuout 1 pmole of 21 nt siRNA labeled at the 5' end with □-^{32}P ATP was incubated with either recombinant PAZ or recombinant Pem-AGO fusion protein. The RNA-protein complexes were analyzed on 5% non-denaturing polyacrylamide gel. The amount of each recombinant protein in the reaction was indicated. The arrows depicted the retarded RNA-protein complexes.

Table 1. Primers used in this study and their nucleotide sequences

Primer name	Nucleotide sequence 5 -> 3
PRT	CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTTTTTTTT
PM1	CCGGAATTCAAGCTTCTAGAGGATCC
AGO1	CA(G/A)CCITG(C/T)TT(C/T)TG(C/T)AA(G/A)TA(C/T)GC
AGO-R2	GT(A/G)TG(A/G)TG(AGTC)C(GT)(TC)TT(T/C)TG
3 RACE1	TCTACAAGTCTACGCGGTTC
3 RACE2	ATGAGAGAGGCTTGCATAAA
5 RACE1	GACAGTGTTTGAGGTGAGGT
5 RACE2	CCCTTAATTTCTTTTGTCAACTTGA
AGO-F	TGGAATTCGTCGACAAAAGAATGTACCCTGTTGGGCAGC
AGO-R	ACTCTAGATTAAGCAAAGTACATGACTCTGTTTG
PAZex-F	CCGCTCGAGAAAAGATTTATGTGTGAAGTGTTAGATATTC
PAZex-R	ACGCGTCGACAGATCTAGCTGTTGCCTTGATC
ExPAZ-F	GGAATTCCATATGTTTATGTGTGAAGTGTTAGATATTC
ExPAZ-R	CGGGATCCAGATCTAGCTGTTGCCTTGATC
ExAGO-F	GGAATTCCATATGATGTACCCTGTTGGGCAGC
ExAGO-R	CGGGATCCTTAAGCAAAGTACATGACTCTG
PAZ-F	TTTATGTGTGAAGTGTTAGATATTCGAG
PAZ-R	CATGGTAGATGTCTGCATGTCTGT

Table 2. The percent identity of amino acid sequences of Argonaute proteins

	AGO1 *(A. thaliana)*	alg1	Ago1 *(M. musculus)*	hAGO1	dAGO1	Pem-AGO
AGO1 (*A. thaliana*) (NP_175274.1)	100%					
alg1 (ABA18180.1)	35%	100%				
Ago1 (*M. musculus*) (AAN75579.1)	33%	54%	100%			
hAGO1 (NP_036331.1)	34%	57%	91%	100%		
dAGO1 (NP_725341.1)	36%	61%	61%	64%	100%	
Pem-AGO	36%	61%	63%	66%	77%	100%

The percent identity was obtained by the clustalx program

MOLECULAR CLONING AND CHARACTERIZATION OF DICER cDNA FROM *PENAEUS MONODON*

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Abstract

Dicer belongs to a member of the ribonuclease III superfamily that cleaves double-stranded RNA into small interfering RNAs (siRNAs) of 21-23 nucleotides. These siRNAs are responsible for the sequence-specific degradation of the cognate mRNA. Identification of Dicer cDNA and its protein will provide an insight into an understanding of the mechanism of silencing gene expression. Therefore, the purpose of this study is to clone the full-length cDNA encoding a shrimp Dicer and characterize its function. Based on the highly conserved amino acid sequences of invertebrate Dicer, degenerate primers corresponding to 5 and 3 end of the PAZ domain of Dicer were used to amplify the first stranded cDNA template derived from the reverse transcription of total RNA extracted from lymphoid organs of a black tiger shrimp. The PCR product of about 300 bp was obtained. Sequence analysis demonstrated that the PAZ domain of Dicer from black tiger shrimp has high sequence identity (68%) to that of Dicer from *Drosophila*. In order to clone the 5 and 3 end of this gene, the method of 5 and 3 rapid amplification of cDNA ends (RACE) using gene specific primers based on the PAZ domain were employed. To date, a partial cDNA encoding shrimp Dicer (1786 amino acid) was identified. The Dicer polypeptides consist of the partial part of RNA helicase, PAZ, two tandem catalytic (CAT1 and CAT2) RNase III domains and double stranded RNA binding domain. The putative shrimp Dicer has high sequence similarity to Dicer 1 of *Drosophila melanogaster* and *Anopheles gambiae*. The putative Dicer 1 is found in most tissues examined such as brain and thoracic ganglia, hepatopancreas, ovary, gill. heart, abdominal muscle, and lymphoid implying the existence of RNAi mechanism in all tissues.

Key words: Ribonuclease III, double-stranded RNA, ovary, RNA interference

Introduction

RNA interference (RNAi) is a novel gene regulatory mechanism whereby double-stranded RNA (dsRNA) triggers a sequence-specific mRNA degradation of its homologous mRNA(1). It was first known as post-transcriptional gene silencing or co-suppression in plant, quelling in fungi and RNAi in animals(2). DsRNA, a mediator for potent and specific gene silencing in Nematode worms was discovered in 1998 by Andrew Fire and colleagues(3). RNAi is an important mechanism in several processes including viral defense mechanism, controlling development and regulation of heterochromatin(4).

RNAi can be used as therapeutic purposes such as treatment for viral infection and cancer(5). Similarly, it is possible that RNAi can be used to treat or protect viral infection in shrimp. Viral infection by yellow head virus (YHV) and taura syndrome virus (TSV) has led to high mortality rate in the economically important animals such as *Penaeus monodon* and *Litopenaeus vannamei*, respectively. To date, there is no treatment or protection available for viral infection in these organisms. A single-stranded RNA virus such as YHV and TSV, once inside the cell will become dsRNA which can be the precursor for ribonuclease III (RNaseIII) homolog of Dicer to generate short interfering RNA (siRNA) for sequence-specific mRNA degradation. It is conceivable that RNAi technology can be applied to prevent viral infection in these highly economically important animals. Double-stranded RNA has been shown to induce both sequence-specific antiviral silencing (6, 7) and nonspecific immunity (8) in a marine shrimp. An understanding of the molecular mechanism of dsRNA mediated gene silencing and the roles of the proteins that are involved in RNAi machinery will be essential before using RNAi as a tool for viral protective immunity in shrimp. The key molecular components involved in RNAi mechanism in crustacean have not been identified. One of the key molecular components in these processes is the Dicer enzyme.

Dicer enzyme is belonged to the member of the RNase III superfamily of nucleases. Dicer cleaves dsRNA into small, 21-28 nucleotide short interfering RNAs duplexes (siRNA) containing 2 nucleotides 3 overhang, each strand has 5 phosphate and 3 hydroxyl termini(9, 10). Dicer is a large (220 KDa) multi-domain protein containing four distinct domains: an amino terminal DExH/DEAH box RNA helicase / ATPase domain, PAZ (Pinwheel-Argonaut-Zwille) domain, two tandem catalytic RNase III-like domain (CAT I and CAT II), and a dsRNA binding domain (dsRBD). The dsRBD and RNase III domains are involved in dsRNA binding and cleavage. PAZ domain binds to the 3 termini of dsRNA substrate and is important for Dicer processing activity. Dicer plays a crucial role in siRNA mediated gene silencing by processing dsRNA into siRNAs using its RNase III activity. Interestingly, Dicer can use pre-micro RNA (pre-miRNA) precursor to generate miRNA for miRNA mediated gene silencing. Several organisms have different number of Dicer genes. Human, mouse, worm or yeast has one Dicer gene whereas *Drosophila* has 2 and *Arabidopsis thaliana* has 4. Different Dicer requires specific dsRNA substrate and contributes in different mechanism of gene silencing. An identification of the Dicer cDNA and its protein will provide insights into an understanding of the mechanism of silencing gene expression.

Materials and methods

Materials

Live wild broodstock black tiger shrimp (100-120g) undergoing stage 4 of ovarian maturation were obtained from commercial shrimp farms in the Southern part of Thailand. Ovarian maturation stages were evaluated according to the external observation of ovarian size and color (11). Animals were kept in seawater with continuous aeration.

Cloning the putative Dicer cDNA

Total cellular RNA was isolated from lymphoid and an ovary of a wild broodstock black tiger shrimp undergoing stage 4 of ovarian maturation using TRIzol REAGENT® (Life Technologies, USA) according to manufacturer's protocol. Reverse transcription was performed according to the manufacturer's protocol using PRT-oligo-dT_{12} primer (500ng) (5 CCGGAATTCAAGCTTCTAGAGGATCCT$_{12}$ 3) and ImProm-II™ reverse transcriptase (Promega, USA). The cDNAs were subjected to amplification by PCR using degenerate primers designed from the conserved amino acid sequences of the PAZ domain of Dicer 1 (PAZF: 5 GA(TC)GC(TC)GT(TCG)GT (TCG)ATGCC(TC AG)TGG 3 , PAZR: 5 TC(GA)GG(AGC)AC(GCA)AG(GA)AT(TC) TGCTT 3) from *Drosophila melanogaster* (Dcr-1: **NP 523778**), *Homo sapiens* (**NP 803187**), mouse (**AB 081470**) and *C. elegans* (**S 44849**). PCR products were analyzed by electrophoresis on 1.5% agarose gels. The PCR product of the expected size (500bp) was excised and purified from the agarose gel using Wizard SV gel and PCR cleanup system (Promega, USA), and subsequently cloned into pGEM-T easy vector (Promega, USA) and transformed into *E.coli* strain DH5α. Recombinant clones containing the PCR product were selected and subsequently analyzed by DNA sequencing in both directions using MegaBase DNA sequencing system (Amersham Biosciences, USA). The deduced amino acid sequences derived from the DNA sequencing results were compared and analyzed with the GenBank database.

Rapid amplification of cDNA ends (RACE) was employed to amplify the 3 and 5 regions of the isolated putative Dicer sequences. In order to amplify the 3 end of this gene, PM1, an adaptor primer (5 -CCGGAATTCAAGCTTCTAGAGGATCC-3) and two gene specific primers (3RPAZ: 5 GACCACACATCTGGAAGACTG 3 and nested N3RPAZ: 5 CGTGCCAAACGAGAGAACCTT 3 corresponding to the previously isolated putative Dicer sequences were used. PCR amplification of the 5 end was performed by the 5 RACE method. A poly A tail was added to the 3 -end of the first strand cDNA using terminal deoxynucleotidyl transferase (TdT) (Promega, USA). Two gene specific primers (N5RPAZ: 5 GGCTGATCTTGATTTCTGTAC CA 3 and 5RPAZ: 5 CTTTGTGGATTGAGATGAGTGCA 3) and oligo-dT_{12} primer were used to perform PCR. In addition, a number of gene specific primers were used to amplify the 5 end region.

Sequence analysis of Dicer cDNA and its predicted protein.

The Dicer cDNA clones were sequenced in both directions using an ABI Prism model 377 automated DNA sequencer. The nucleotide sequences were analyzed with the GenBank database and translated into a deduced amino acid sequences by the Bioedit Sequence Alignment Editor program. Sequence comparison to other vertebrates and invertebrate Dicer was performed using the ClustalX program to analyse for the conserved functional domains of Dicer. These amino acid sequences were retrieved from the GenBank database under the following accession numbers; DmDcr-1: **NP524453**, MmDicer: **AF430845**, HuDicer: **AB028449**, BovDicer: **AAR26432.1**, and AgDicer (strain G3): **AA073809.** Nuclear localization signal was analysed by using NucPred program available from ExPASY proteomics tools.

Tissue distribution study

The expression of the putative Dicer sequences was examined in various wild broodstock tissues. Tissues from different organ were collected from one shrimp.

Total RNAs were isolated from ovary, brain and thoracic ganglion, heart, hepatopancreas, muscle, gill, and lymphoid and used as a template for RT-PCR to amplify the putative Dicer. Two gene specific primers (N3RPAZ: 5 CGTGCCAAACGAGAGAACCTT 3 and PmR1: 5 GTAGCATTGTGAGCATT AACACAGCC 3) were used to perform RT-PCR in the presence and absence of reverse transcriptase. Actin was used as an internal control.

Results and discussion

Characterization of Dicer cDNA

Penaeus monodon Dicer cDNA was identified from lymphoid organ and ovary using degenerate primers corresponding to the highly conserved amino acids in the PAZ domain and 3 and 5 RACE method. A partial Dicer cDNA containing 5358 nucleotides was identified (Fig. 1A). The 3 untranslated region contains 64 nucleotides and has a consensus polyadenylation signal (AAUAA). A predicted Dicer polypeptide contains 1786 amino acids with 5 conserved domains including a partial helicase domain, a PAZ domain, two catalytic RNase III domains, and a dsRNA binding domain (dsRBD) (Fig. 1B). A NucPred program predicts a nuclear localization signal between amino acid residue 217 and 227 (GKGRKRRDDVK) suggesting that Dicer may perform function not only in the cytoplasm but also in the nucleus. Several nuclear localization signals have been identified from mouse Dicer(12).

The predicted protein of the *Penaeus monodon* Dicer exhibits highly conserved domains when compares to other invertebrate and vertebrate Dicers. The PAZ domain (Fig. 2A) has 54-68% amino acid sequence similarity (Table 1) and may function to recognize the 3 overhangs of the dsRNA substrate (13). Mutations of the PAZ domain strongly abolish Dicer processing activity(14). The *Penaeus monodon* Dicer protein has two catalytic RNase III domains (Fig. 2B and 2C) with 60-83% sequence similarity (Table 1). The two RNase III domains are proposed to have only a single dsRNA processing center and form intramolecular dimer in which each domain cleaves each strand of dsRNA substrate resulting in 21-23 nucleotides with 2 nucleotide overhang at the 3 end of the Dicer cleavage product. This model for the mechanism of Dicer cleavage (15) has been proposed based on the crystal structure of bacteria *Aquifex aeolicus* RNase III (16) and the study by Zhang et al., 2004 (14). In contrast, bacterial RNase III lacks RNA helicase / ATPase and PAZ domain. It contains only one RNase III domains and dsRBD that are found also in Dicer. Therefore, bacterial RNase III cleaves dsRNA to 11 nucleotides whereas the Dicer cleavage product is 21-23 nucleotides. In addition, the dsRNA binding domain (dsRBD) (Fig. 2D) has 72-94% sequence similarity conserved among the species (Table 1). DsRBD mediates un-specific interaction with dsRNA. The distance between RNase III domain I and PAZ domain is possibly determined the size of the cleavage product. Dicer prefers dsRNA substrate containing 3 overhangs than the blunt-ended dsRNA. Interestingly, another type of Dicer that lacks the PAZ domain is found in *Drosophila* Dcr-2 and *A. thaliana* DCL4. Similar to *Drosophila melanogaster* Dicer-1, *Penaeus monodon* Dicer has the PAZ domain and the highly conserved functional domains (68-94%) suggesting that the putative Dicer is possibly a Dicer –1 protein.

Tissue distribution analysis of Dicer gene

The expression of Dicer was analysed in various tissues of a wild broodstock shrimp using RT-PCR. Dicer is expressed in all tissues examined such as brain and thoracic ganglia, hepatopancreas, ovary, gill. heart, abdominal muscle, and lymphoid suggesting that the RNAi mechanism exists in all tissues.

A.

```
   1  RLNIKESSCTDGCDTESIKSCDLHNGVLSAKNDLLDTAMIQNVKNMTLCN   50
  51  GFEHGSKDDIMDSEKDITSKMNGHVDPSEYDSESEDISDRYKSCESVQSQ  100
 101  ASVETSSKEGSISDTFVPSATSYLPCKADATSAVDETATPSDSAPNEQEL  150
 151  PTCTSGVENTEDPALVSTSGTTQNTTICSDSAAFSNNSNGTQPTTQTVSS  200
 201  EAAAMADTLAMLLPNSGKGRKRRDDVKEKVKVHNPEDPDSVCGLIFVHHR  250
 251  SMAKIIYRLLKELSDIGGDFAWIFPQYTVEAKESVKEDPRAAEAEHKKQE  300
 301  EVLRRFRHHECKILVSTRVLEEGIDVPQCNLVLRFDPPTDYRSYVLSCGR  350
 351  ARGHDTFYFHLITKNQEISFLHDMATYSAFQQVLVSHCGSVEVGTDREVL  400
 401  SSEANAAHAPYLTPAEAAVTMASAIGLLNKYCAKLPSDTFTRLTAMWDVE  450
 451  EIEEAEVEIPKYKCKIMLPINSPLKGTIEGPWQSKVSLAKMAAALECCRR  500
 501  LHQMGELDDQLQPVGKESMKLDDHLCAPPADDQVPEGMPRPGTTKRRQYY  550
 551  YKKVAVCLTGEQPKQGLDLFVYKLDMVLTCPIPDEQNTRGRKIYRPEQSS  600
 601  RSFGIITTKPISQVSGFPVFTRSGEVVVHVQEIERKVNVTQDQLSALQYF  650
 651  HKFTFTHVLRLEKYPIKFDPTNARTAFYIVPLNKFNGSEGIDWEFVKEIQ  700
 701  SEGDPRPVPPQDDARKKFQFQHDLYEDAVVMPWYRNQDQPQYFYVAEICT  750
 751  HLNPQSDFPDAGFETFEKYYLTKYGLQICNLAQPLLDVDHTSGRLNLLTP  800
 801  RHVNRKGVALPTTSEETKRAKRENLQQKQILVPELCTIHPFPASLWRKAV  850
 851  CLPTILYRINALLLADQLRLSVASEVGLGLQTLLPDFSWPPLDFGWSLAD  900
 901  VLRKNQENQEQDDKSNIASEATNSKQKATKTENSECESPTLAAKKKAPKK  950
 951  GNDMEIGTWSNDMAVDPPTPPFDMDTMNGPDEFEIDTFDPNVALPDNLTL 1000
1001  LNGFSGADDDVEGELGADWGTGITERRTKSSCNKDSKGGMFRVGSPSNFE 1050
1051  SDGWDMFGSSGYNGGYGGYGSYDAYSGYGDFQGLADDLEGCESDVSSDMD 1100
1101  DDDKSQTEKLWDEEGTKRRSSMAADEGSSDEEIDLNWPDEDEEVRNEKEK 1150
1151  EFQSFLEDKQKIIKESSCYLAESESLLIEKAHRKLQNESKSSPEKVSHRT 1200
1201  ESSTTEGCEQLSSKGCDNAQQSTSNIIRTVNRDETLALTRPDSEVQCSSW 1250
1251  YSICEEMSFSFDFQPDLLNHPGPSPSVILQALTMSNANDGVNLERLETIG 1300
1301  DSFLKYAITTFLYCTYPQRHEGKLSYLRSKQVSNLNLYRLGKRKGLGECM 1350
1351  VATKFEPHDNWLPPGYFVPRELEEALIDSGVPAGHWNMADLPGLHDLASD 1400
1401  EIRRLVQERSEQIKRSKSEQATSELTATQNPHDLPIFIPYNLLTQHSIPD 1450
1451  KSIADYVEALIGAYLTTCGPRGALLFMSWLGIKVLPCTLESSPEASELIT 1500
1501  YGHLESPQSPLYHCPLTDTRKELDLLLSGYQVFEEKIGYTFRDRSYLLQA 1550
1551  FTHASYYKNRLTGCYQRLEFLGDAVLDYLITRHLYEDKRQHSPGALTDLR 1600
1601  SALVNNTIFASLAVKYDYHKYFRHFSPGLDRVIRDFVKMQEENGHKINEE 1650
1651  YYFMEEDECEAAEDIEVPKALGDVFESVAGAIFLDSGSSLDAVWSVYYTM 1700
1701  MCREIEQFSGVVPKSPIRELLEMEPETAKFGKPERLVDGKVRVSVEIFGK 1750
1751  GSFSGVGRNYRIAKSTAAKRALRHLKKLQMMANQGI               1786
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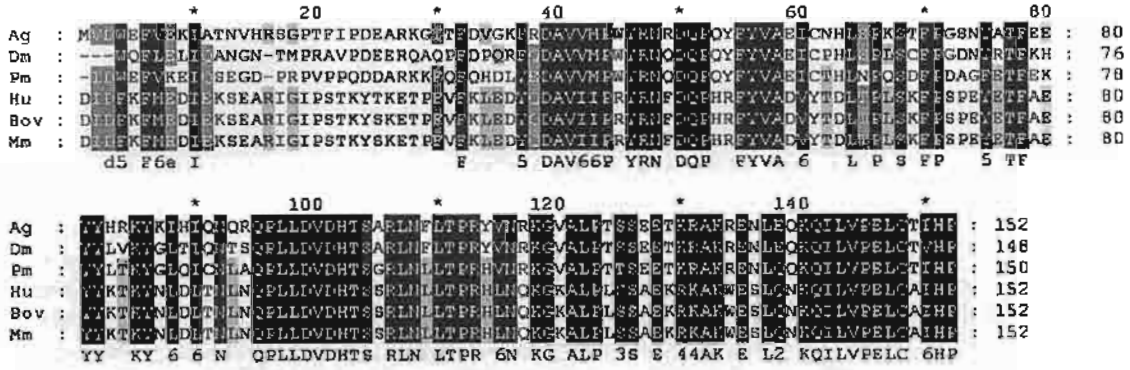
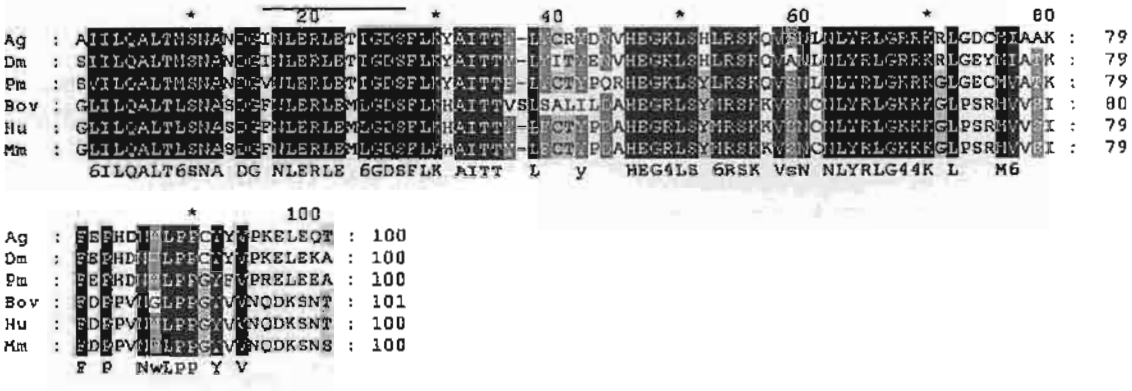
B.

Figure 1. A. *Penaeus monodon* Dicer amino acid sequence. B. Schematic diagram predicted putative domains of the Dicer-1 polypeptides.

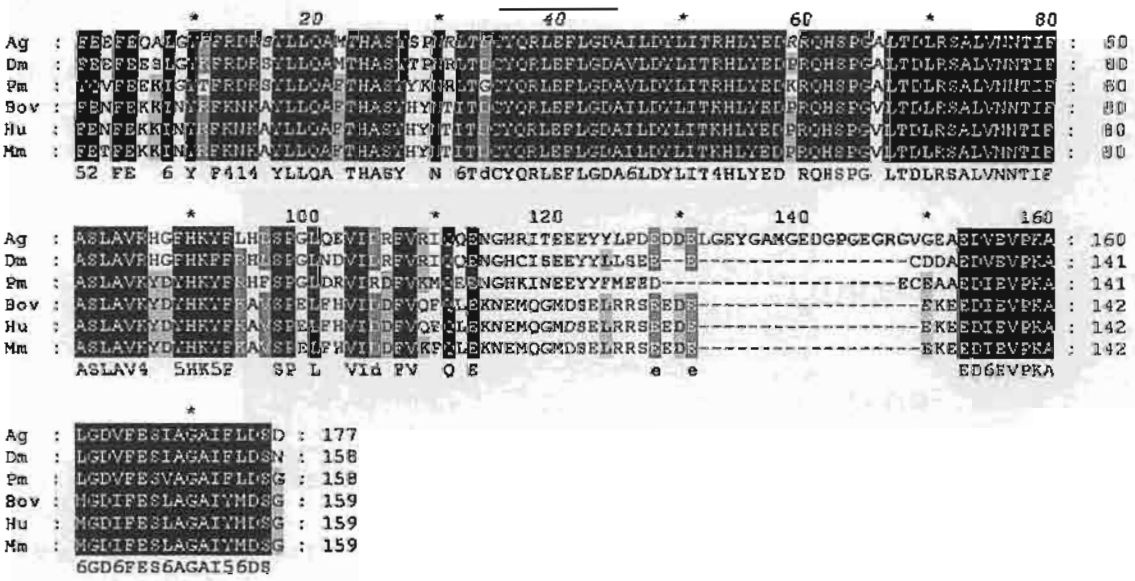
A. PAZ domain



B. RNase III domain I



C. RNase III domain II



D. dsRBD

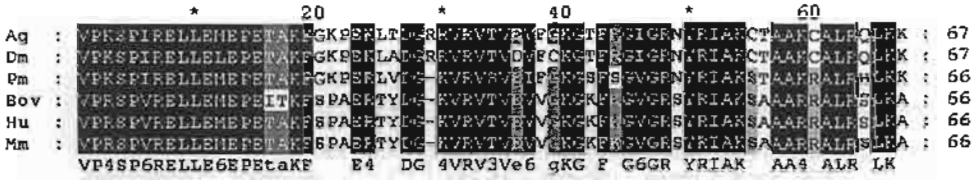


Figure 2. Multiple amino acid sequence alignments of Dicer domains comparing between *Penaeus mondon* (Pm) Dicer and other vertebrates and invertebrate Dicers. The alignments were performed using the ClustalX program. The amino acid sequences of Dicers were retrieved from the GenBank database under the following accession numbers; Dicer from *Anopheles gambiae* (Ag): **AA073809**, *Drosophila melanogaster* (Dm): **NP524453**, *Bos Taurus* (Bov): **AAR26432.1**, *Homo sapiens* (Hu): **AB028449**, and *Mus musculus* (Mm): **AF430845**. In Figure A through D amino acids residues that are hightlighted in black are 100% identity in all species. A. PAZ domain, B. RNase III domain I, C. RNase III domain II, and D. dsRBD. Underlines represent amino acid residues of the consensus catalytic signature sequence.

Table 1. Percent amino acid similarity comparing between Dicer domains of the predicted *Peneaus monodon* and of other species.

Species	% Similarity			
	PAZ	RNaseIII (I)	RNaseIII (II)	dsRBM
Anopheles gambiae (Ag)	67	81	66	82
Drosophila melanogaster (Dm)	68	83	79	94
Bos Taurus (Bovine: Bov)	54	60	68	72
Homo sapiens (Human: Hu)	54	66	69	75
Mus musculus (Mouse: Mm)	54	66	69	75

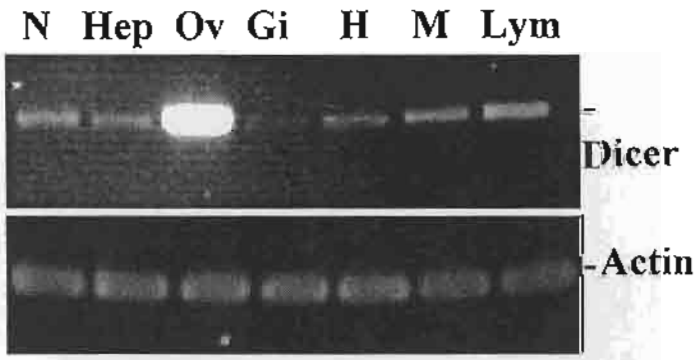


Figure 3. Expression of Dicer in various tissues of a wild broodstock shrimp. N: brain and thoracic ganglia, Hep: hepatopancreas, Ov: ovary, Gi: gill, H: heart, M: muscle, and Lym: lymphoid. Actin was used as an internal control for loading.

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DEVELOPMENT OF A VIRAL VECTOR CAPABLE OF GENERATING A SPECIFIC siRNA IN SHRIMPS

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Abstract

The recently described phenomenon of RNA interference (RNAi) provides a powerful means for silencing gene expression in a sequence-specific manner. It has been applicable for viral protection in a number of organisms through introduction of exogenous specific double-stranded RNA (dsRNA). Research aim is to develop a system for expressing specific dsRNA in the shrimp cells by using hepatopancreatic parvovirus (HPV) as a viral vector.

In order to develop the HPV viral vector for expressing specific dsRNA, the infectious clone of HPV has to be constructed. The complete nucleotide sequence (6,321 b) of HPV genome was firstly determined. The obstacle step is the amplification and cloning of both 5 and 3 genome termini. Taking advantage of its genome self-priming, a single primer with high Tm was used to amplify the inverted repeat ends. The full-length of HPV genome including palindrome termini was then cloned into pGEM-T vector. Infectivity of HPV was evaluated in shrimp primary cultured cells (lymphoid and hepatopancreas) and insect cells (C6/36 and Sf9). The results showed that the viral multiplication was observed only in hepatopancreas cells. A lot of effort has been put into the investigation of the transfection condition for primary cell culture. Unfortunately, the optimal condition has not been achieved. In an attempt to deliver HPV full-length clone into shrimp cells for determination of its infectivity, the direct injection into shrimp as well as the transfection into shrimp zygotes were performed. PCR amplification of HPV gene from treated shrimps and zygotes showed equivocal production of HPV progeny. Furthermore, feeding the treated shrimps to Pl-shrimps did not produce any detectable HPV progeny.

Introduction

Black tiger prawn (*Penaeus monodon*) is an important export food of Thailand. Nowadays, the decrease in shrimp production has been observed in many areas. It could be resulted from growing the shrimp at high density leading to the development of viral infection in the farm. This condition results in vast economic losses. The recently described phenomenon of RNA interference (RNAi) provides a powerful means for silencing gene expression in a sequence-specific manner. It has been applicable for viral protection in a number of organisms through introduction of exogenous specific double-stranded RNA (dsRNA). The DNA-vector-mediated mechanisms have been developed to express substrates that can be converted into siRNA *in vivo* (Brummelkamp et al., 2002). With the plasmid vectors, the phenotypes of genes silencing could be observed by stable transfection of cells. Nevertheless, transient siRNA expression, low and variable transfection efficiency remains the

major obstacle for plasmid vector derived siRNA. To overcome these limitations, several virus vectors have been developed for efficient delivery of siRNA into the target cells and animal model (Lundstrom, 2003, Banerjea et al., 2003). Accumulated literatures have demonstrated that siRNA delivery by viral vector is a promissing application in functional genomic, cancer gene therapy as well as inhibition of viral replication. Therefore, our research's aim is to develop the system for expressing specific dsRNA in the shrimp cells by using one of shrimp virus, hepatopancreatic parvovirus (HPV), which do not cause lethal infection, as a viral vector.

Materials and Methods

Sequence determination of HPV genome

Purification of HPV and isolation of viral DNA

HPV was isolated from infected hepatopancrease of *Penaeus monodon* collected from ponds in southern and central parts of Thailand by urografin ultracentifugation (Sukhumsirichart *et al.*, 1999). For DNA extraction, the purified viral particles were resuspended in NTE buffer (NaCl, Tris-HCl, EDTA, pH 7.4) containing Proteinase K (50 μg/μl final concentration) and incubated at 37°C for 1 h then sarcosyl or SDS (2%) was added and further incubated at 37°C for 2 h. The DNA was extracted directly from this mixture using phenol and chloroform, and then the DNA was precipitated by 2 volumes of absolute ethanol in the presence of 0.3 M sodium acetate. The DNA pellet was washed with 70% ethanol and resuspended in sterile distilled water.

Sequencing and analysis of HPV DNA

The nucleotide sequences of the recombinant clones and the PCR products from primer walking were performed by *Taq* Dye-Deoxy Teminator Cycle Sequencing using an Automate DNA sequencer (PE Applied Biosystems; Model 377 version 3.0 and ABI100 DNA sequencing system [Bioservice unit (BSU), NSTDA and Central Equipment Laboratory, Mahidol University, Thailand].

In addition, the HPV genomic DNA was used as a template for PCR amplification of the HPV genome by using primers HPV12 (5′ GTGAACTT TGTAAATAACTTG 3′) and HPV6 (5′ AAGGGTAAACCACG CACG 3′) and Proof-Reading Expand Long Template *Taq* DNA polymerase (Roche™). The PCR product of approximately 6 kb was then tailed with A nucleotide and ligated to pGEM-T vector using pGEM-T Easy cloning kit (Promega). The recombinant plasmids were screened and a clone HPV10 was selected. The nucleotide sequence of the clone HPV10 was determined on both strands and compared to the original nucleotide sequencing data. DNA sequences of the 5′ and 3′ ends of HPV genome were obtained by cloning of PCR products. Modified PCR with single primer was used to amplify both ends. High Tm primers: SeqGC: 5′ CCACCGCCGCAGCCGCAGTTGCCG 3′ was used for the 5′ end amplification and LeftGC: 5′ GCCGACGCACCGCCCCTAGCT CC 3′ was used for the 3′ end. PCR amplification was performed using *Taq* DNA polymerase (Promega) in the presence of 5% DMSO and 2 M Betaine with increased ramping time. The PCR conditions were 95°C (2 min) and 40 cycles of 95 °C for 10 sec, (3 min ramping from 95 °C to 70

°C), 70 °C for 30 sec, (2.5 min ramping from 70 °C to 74 °C), 74 °C for 1 min and a cycle of 74 °C for 5 min. The amplified products were inserted into pGEM-T Easy vector (Promega). Recombinant plasmids were then transformed and maintained in *Escherichia coli* STBL2 (Invitrogen) at low temperature (22-25 °C) and sequenced using MegaBACE sequencer (Amersham).

Nucleotide sequences of viral DNA at the 3′ and 5′ extremities containing stem-loop structures were performed by using transcription sequencing (CUGA,WAGO company).

All of the DNA sequence data were assembled and analyzed using computer program. The restriction mapping and open reading frame characterization were done using BLAST (http://www.ncbi.nlm.nih.gov:80/gorf/orfig.cgi), DNA strider, BCM Search Launcher, (http://dot.imgen.bcm.tmc.edu: 9331/seq-util.html), DNAsis, Clone manager and GCG. The DNA and deduced amino acid sequences were compared to the data in GenBank/EMBL, and SWISSPORT database using FASTA and BLAST.

Construction and characterization of an infectious plasmid clone of HPV

Plasmid construction

In order to combine 6 kb HPV fragment together with both 5 and 3 termini, unique restriction enzyme, which cut only one site within HPV genome, was selected. The *Hinc*II and *Bgl*II were chosen for being the cloning site of 5 and 3 ends, respectively. In this case, the 3 end clones from the first part do not contain the *Bgl*II site, therefore, PCR amplification using 3ENDLong and 3END3 primers was performed to extend the sequence to cover the *Bgl*II site. Expected 1.3 kb PCR fragment was subsequently cloned into pGEM-Teasy vector and two recombinants (3 -21 and 3 -16) were picked up for further cloning. Firstly, the *Sal*I-*Hinc*II digested DNA fragment containing 5 HPV region was isolated from recombinant clone E8 or E30 and then cloned into the same RE cut pGEM-HPV clone to obtain HPV5 (Figure 1). The 3 HPV end was then put into the HPV5 by using *Sph*I and *Bgl*II digestions. Finally, the full-length of HPV genome was successfully cloned in pGEM-Teasy vector and the sequence of viral genome was verified again by DNA sequencing.

Using bacterial hosts such as STBL2 or SURE, which has been modified for eliminating of DNA recombination, and decreasing the temperature for their growth were done in all cloning steps in order to stabilize the palindromic sequences.

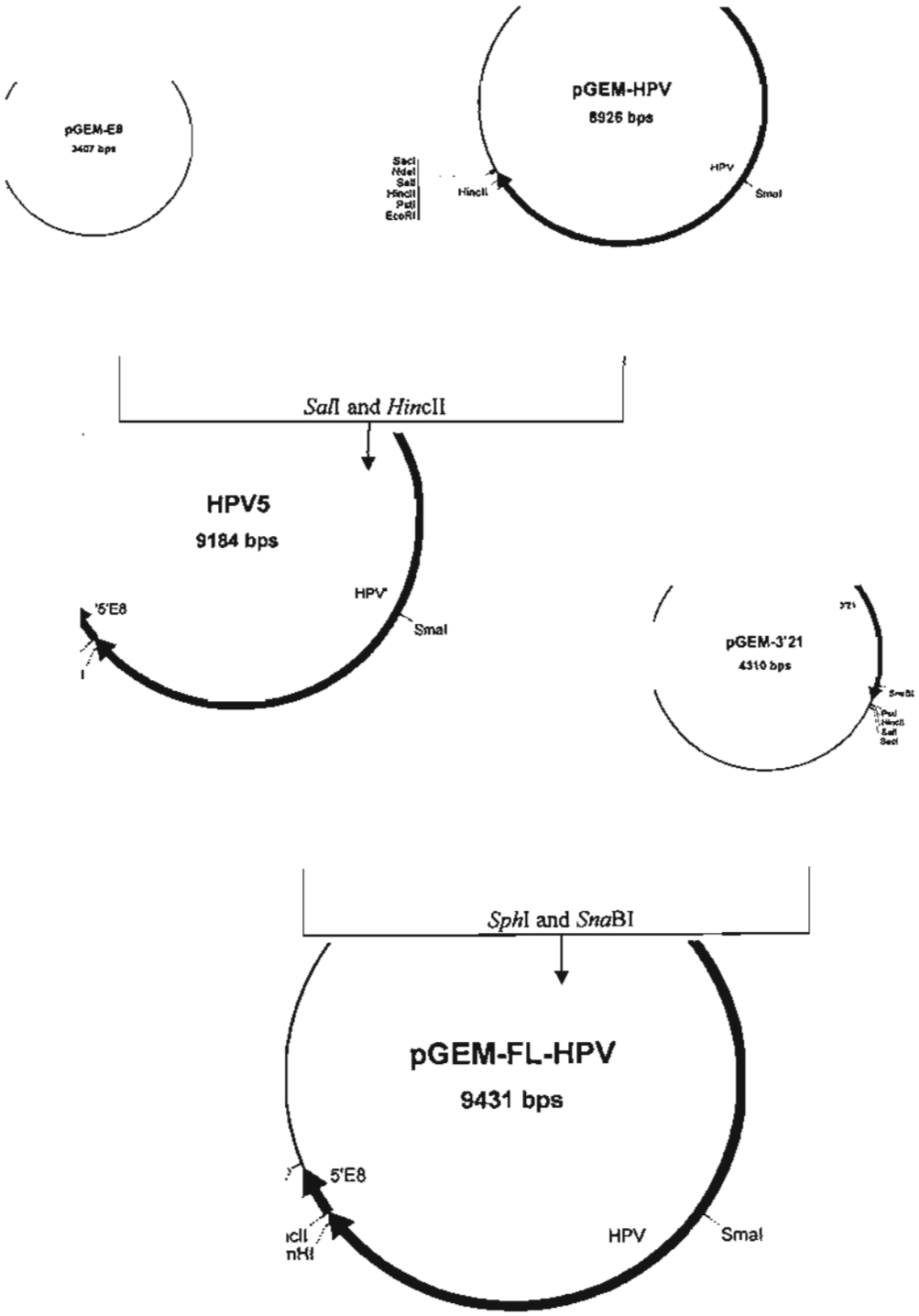


Figure 1 Cloning strategy of HPV genome in pGEM-Teasy plasmid vector

Cell culture

The primary culture of *Penaeus monodon* lymphoid "Oka" cells was prepared as described (Assavalapsakul et al., 2003). Briefly, lymphoid tissues collected from approximately 100 sub-adult shrimps were washed in washing medium (2X Leibovitz's L-15 medium containing 100 IU/ml penicillin, 100 mg/ml streptomycin, 15% fetal bovine serum, and 5% lactalbumin). The tissue was minced into small pieces in complete medium (washing medium containing 15% shrimp meat extract). The minced tissue was then seeded onto a 24-well plate and allowed to monolayer at 26°C.

Base upon the article of Uma A *et al.*, 2002, hepatopancreas was removed from shrimp, put in a Petri dish and cut into small pieces (1 mm^2). After tissue fragments washing in buffer containing 1:10 P/S, cell dissociation was accomplished by mild agitation with a magnetic stirrer for 5 min. The resulting crude cell suspension was filtered through a 60-mesh sieve to remove tissue debris. The filtrate was transferred to centrifuge tube and spin down at 1,200 rpm for 5 min. The cell pellet was subsequently resuspended in culture medium (1X L15 containing P/S and gentamycin) and seed in 24 well plate and incubate at 26°C. In our experiment, cells isolated from hepatopancreas of fasten shrimps provided the attachment and formation of a monolayer within 24 hr after seeding in both 2X L-15 and 1X L-15 culture medium. But we failed to obtain monolayer of cells from normal fed shrimp. It showed suspension cells with four types of cell; E (embryonic), R (resorptive), F (fibrillar) and B (blister-like) cell.

Infectivity test of HPV clone

By injection into shrimps: the full-length HPV clone (5 μg/shrimp) was mix with jetPEI transfection reagent at N/P=5 and used to muscular inject into shrimp (1 g). Twenty days after injection, the treated shrimps were harvested and tissues except muscle were collected and used to feed PL-shrimps. The infectivity of HPV clone was then evaluated by PCR amplification of HPV genome. In order to enhance the sensitivity of HPV detection semi-nested PCR was performed.

By transfection into shrimp zygotes: briefly, fertilized shrimp eggs, within 50 min postspawning, were transferred into a dish containing 1 μg of the HPV plasmid and 1.2 μl of the transfecting reagent jetPEI in 2 ml of sterile seawater. The final reaction mixture was incubated at room temperature for 50 min prior to transfer into a 1l beaker filled with sterile aerated seawater at 28°C for the hatching process. After hatching (12-24 h), the nauplius and unhatched eggs were collected and DNA was then isolated for PCR detection. The infectivity of HPV clone was evaluated by PCR.

Results and Discussion

Sequence determination of HPV genome

The HPV genome of 6,321 bp (Figure 2) was obtained by DNA sequencing using purified HPV genomic DNA and recombinant plasmid (6 kb insert) as templates. The nucleotide sequences at both ends were obtained by modified methods (Materials and Methods). The base composition of the viral (minus) strand of the HPVmon genome was 35.82% A, 17.17% C, 24.49% G, and 22.53% T. The G+C

content was 41.65%, and A+T content was 58.35%. The viral strand of HPVmon has high A+T content, which is similar to the shrimp parvovirus; (IHHNV 56.96%) (Shike *et al.*, 2000), and the mosquito brevidensoviruses; *Aae*DNV, 62.5% (Afanasiev *et al.*, 1991), and *Aal*DNA, 61.8 % (Boublike *et al.*, 1994).

The open reading frames (ORFs) in the nucleotide sequences were determined by computer analysis (Blast, DNA Strider, Clone manager programs). The HPV genome contained three major ORFs (Figure 3) and all of them are found in the complementary strand of the viral genome. The ORF1 (Left ORF) consisted of 1,287 nucleotides while the ORF2 (Mid ORF) contained 1,740 nucleotides and ORF3 (Right ORF) consisted of 2,457 nucleotides. The ORF1 and ORF3 are in the different reading frame to that of ORF2. The internal coding sequences were flanked by non-coding 5′ and 3′ terminal sequences of 222 and 215 nt in length, respectively.

A computer search for putative transcription regulatory sequences upstream and downstream from the ORF1, ORF2, and ORF3 revealed several potential promoters and polyadenylation signals. Upstream from the ORF1, one TATA-like box appropriately spaced with respect to the first ATG codon are present at nt 144 (TATATAT). An appropriately positioned upstream GC-rich region (GGGGCGG, nt 121-127) could act as activator element. A canonical downstream AGAGTC (nt 174-179) transcriptional start signal (Inr-box) and (GTGAAGTT), a downstream promoter element (DPE) is also present. Thus, this region is likely to be a functional promoter. This promoter was named p2, according to its location at map unit 2 (taking the length of HPVmon genome as 100 map units).

The second set of putative transcription regulatory was found upstream from the ORF2. These include TATAGTA (nt 1,380-1,386), a TATA-box; GGCACAG (nt 1,363-1,369), a GC-rich sequence; TCACAG (nt 1,419-1,424), a Inr-box and GGAAAGATGT (nt. 1,443-1,452), a DPE sequence. This putative promoter (p22) very likely controls transcription of the ORF2.

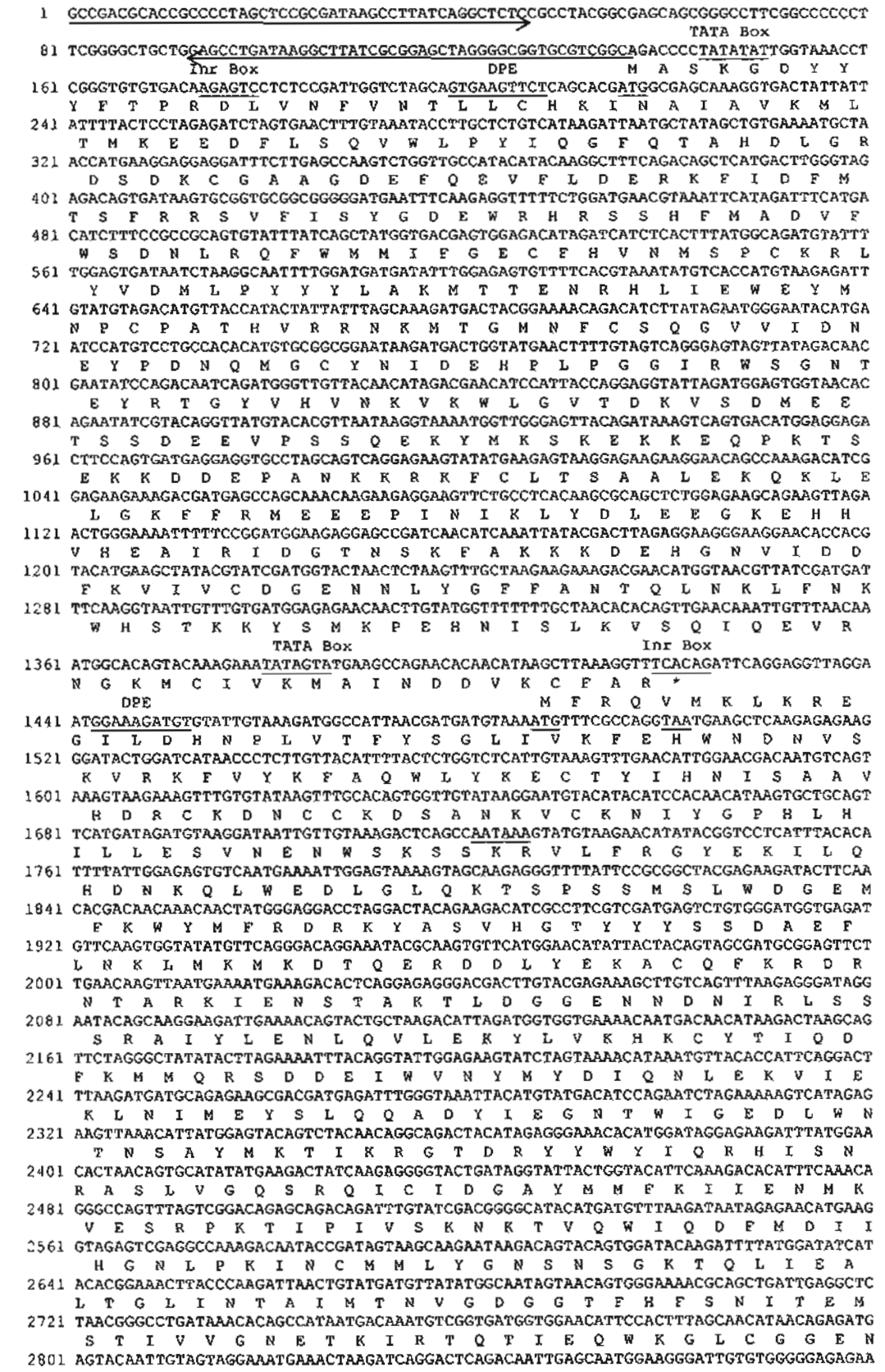
The third set of putative transcription regulatory sequences was found upstream from the right ORF. These included a TATA-box, TATAGCT (nt 3,369-3,375); a GC-rich activator sequence TGTCGGC (nt 3,350-3,356), a Inr-box, TCATGGT (nt 3,425-3,431), and a DPE sequences, CAGACACAGT (nt. 3,455-3,464). This putative promoter (p48) very likely controls transcription of the right ORF3.

The nucleotide sequences of 5′ and 3′ ends were obtained by PCR amplification. Various sizes of amplified PCR products, 200-500 bp, and 100-200 bp were obtained from 5′ and 3′ ends, respectively. All of them were cloned into pGEM-T Easy vector and transformed into STBL2 at low temperature in order to stabilize inverted repeat DNA, which commonly found in terminus of parvovirus genomes. The results revealed that non-coding region of the 3′ extremity of the viral (minus) strand contained 215 nucleotides. The non-coding sequence of the 5′ extremity was 222 nt long. Both extremities contained palindromic sequences capable of forming hairpin-like structures by base pairing, a feature shared by all parvoviruses previously studied (Astell, 1990). The nucleotide sequence at 5′ end (Figure 4a) could be folded into a hairpin-like structure but could not generate a typical Y-, T- or J-form similar to other parvoviruses (Bergoin and Tijssen, 2000). The stem can be formed by base paring between nucleotides 6,152- 6,222 and nucleotides at 6,251-6,321. There was an imperfect palindrome at nts 6,223-6,250 that existed two orientations as reverse-

complement; flip and flop (Fig. 4a). The nucleotide sequence at 3′ terminal region, which obtained from seven independent clones, also formed hairpin-like structure with 44 base-pairs stem length but no flip and flop orientation as found at 5′ end (Fig. 4b). In addition, the analysis of the sequences at the two termini failed to detect any significant sequence complementary between the sequences of 5′ and the 3′ extremities that can qualify as an inverted terminal repeat (ITR). In this respect, the ends of HPV genome resembled the genome of Brevidensovirus and most autonomous vertebrate parvoviruses whose 5′ and 3′ terminal sequences are not complementary (Astell, 1990; Bergoin and Tijssen, 2000).

The most time-consuming step in this experiment was the cloning of the 5′ and 3′ termini of viral genome. However, we were successful in obtaining the sequences of HPV ends from the modified method. By using a self-priming character of all parvoviruses, a single primer was used for the PCR amplification. At 5′ terminus, a specific primer was used to synthesize a new complementary strand in the first round of amplification. Then, the synthesized DNA should fold to form hairpin-like structure at the 5′ end and acted as the primer for the second round of PCR step. The resultant DNA fragment was then served as template for the next step of amplification by using only one primer. Similar to the 5′ end, the hairpin loop at 3′ terminus served as primer for the first round of amplification. The product was then the template for the next cycle by the single primer. As a result of stem-loop structure at both ends, which may lead to PCR amplification failure by normal condition, therefore, two additives such as DMSO (Chakrabarti and Schutt, 2001, Frackman *et. al.*, 1998) and betaine (Henke *et. al.*, 1997) were added in the reaction mixture to destabilize this structure. These two organic solvents reportedly can enhance PCR amplification, particularly for hard-to-amplify high GC templates, which may create secondary structure like hairpins. In addition, ramping time of PCR condition was increased to facilitate the binding between oligonucleotide primer and DNA template much better than template self-priming (Bachmann *et al.*, 2003). High temperature (70 °C) for annealing step was also performed in order to eliminate the forming of hampered structure within DNA template. From these experimental steps, it opens an opportunity for retrieving the sequence data at the end of parvoviruses or a single-stranded virus genome with folded termini.

Both flip and flop were found at 5′ palindromic terminus whereas only one orientation was detected from all seven 3′ end clones. Since only single stranded virion DNA was used in this study, asymmetrical generation of these orientations or encapsidation bias could not be ruled out. The feature of terminal hairpin-like structure can act as the primer for viral DNA replication, which has been proposed in two models. According to replication model of parvovirus like AAV (Cotmore and Tattersall, 1987), the hairpin transfer may result in flip and flop orientations at both ends. Whereas, the modified rolling hairpin model for autonomous parvovirus as in MVM (Astell *et. al.*, 1985) was set to explain asymmetrical generation of flip and flop at both ends. Therefore, HPVmon DNA may replicate by using the latter model.


TATA Box
Inr Box
DPE
TATA Box
Inr Box
DPE

```
      I  T  M  P  M  K  Y  K  E  H  K  T  H  M  F  R  K  P  V  F  L  T  N  Q  H  H
2881  CATAACAATGCCAATGAAGTACAAGGAACACAAGACACACATGTTCAGGAAACCTGTATTTTTGACCAACCAACATCATC
      P  L  V  E  I  S  N  Y  D  D  R  K  A  I  E  N  R  C  F  M  Y  K  V  E  L  G  S
2961  CACTGGTAGAGATATCAAACTATGACGATAGAAAGGCTATAGAGAACAGATGTTTTATGTATAAGGTAGAATTGGGAAGC
      E  A  V  N  A  H  I  K  F  P  N  R  M  I  P  I  K  K  N  P  E  L  T  Q  F  I  L

3041  GAGGCAGTAAATGCACATATAAAATTTCCTAACAGGATGATTCCGATCAAGAAGAATCCAGAACTGACTCAGTTTATATT
        A  C  M  Q  Y  V  H  L  N  Y  M  D  R  A  D  K  K  F  K  I  G  F  F  N  K  L
3121  GGCATGTATGCAATATGTACACTTGAACTATATGGACAGGGCAGATAAGAAGTTTAAGATTGGGTTTTTCAACAAACTTT
      Y  D  M  L  F  E  D  S  *
3201  ATGACATGCTGTTTGAGGACAGCTAAAAATATGTACGAGAACGTTTGACTTTCCCGGGCAACAATAAAGTGATAAGATAA

3281  AATCGTGCGTTTGAGTATCCACATCACACATAGAGGTAGTACACCATGAGTCTATCATGGGAGCAGTCGTGTCGGCAGTA
            TATA Box                                                          Inr Box
3361  GCTGCAGTTATAGCTGTGGTAACTGAAGTGGTCGAATTCATAGTCGATGTCGTGGAAGTGGCTTTCATGGTGGCAGAGGC
                     DPE
3441  TGTACAGGTCGTTGCAGACACAGTCAATTACTTTACTGGCGGCGATAGTGCTGCGAAGAACGATCAGAGCGATCCGGAGA

3521  CGCAGGCAGCTAATGAGGTCAGCAGTACAGCAGAATCGACAAACGTAGGAGGAACTCAGGGCTACATGTTAGGACCCGAA
                                                          M  S  P  T  R  K  G  G  N  Y  Y  A  S
3601  CAGTTGGACGATTACATAGATAGAGCTACAGACATAGACTAATGTCACCTACAAGAAAAGGAGGGAATTATTATGCGAGT
      K  H  F  Q  S  K  R  K  N  K  L  A  R  V  K  D  L  L  A  S  K  K  K  E  R  R  F
3681  AAACACTTCCAAAGTAAGCGAAAGAATAAACTAGCGAGAGTGAAAGATTTACTGGCAAGTAAGAAGAAGGAGAGAAGATT
        K  G  K  G  N  T  L  S  E  K  P  S  T  S  E  W  N  D  P  V  R  Q  R  F  P  E
3761  TAAAGGAAAAGGAAATACTCTAAGTGAGAAGCCAAGTACATCAGAGTGGAACGATCCGGTAAGACAAAGATTTCCAGAAT
      L  E  Q  E  E  R  N  T  F  A  G  L  L  A  I  E  A  A  P  D  Q  R  Q  L  G  R  D
3841  TAGAACAGGAGGAGAGAAATACATTTGCAGGATTACTAGCAATAGAAGCAGCACCAGACCAAAGACAATTAGGGCGCGAT
        N  N  N  Q  L  A  L  V  Q  R  D  T  R  V  A  V  R  Q  S  T  N  R  G  E  A  L  E
3921  AATAACAATCAACTAGCACTAGTACAGAGAGACACAAGAGTAGCAGTAAGACAAAGTACAAACAGAGGAGAAGCATTAGA
        V  V  R  A  A  N  E  A  I  R  S  G  G  D  R  L  A  E  L  V  Q  A  Y  A  S  G
4001  GGTAGTAAGAGCAGCAAACGAAGCAATAAGAAGTGGTGGAGATAGATTAGCAGAATTAGTACAAGCATACGCATCAGGAT
      F  S  D  S  T  E  I  V  E  V  R  Q  E  D  R  V  Q  R  D  I  F  Q  E  E  G  Q  N
4081  TTTCAGACAGCACAGAAATAGTAGAAGTAAGACAAGAAGATAGAGTACAGAGAGACATATTCCAAGAAGAAGGACAGAAT
        L  L  A  I  E  I  A  L  Q  E  P  S  S  V  A  Q  Q  L  D  Q  E  R  T  P  A  V  K
4161  TTATTGGCTATTGAGATTGCATTACAAGAACCAAGCAGTGTAGCGCAACAGTTAGACCAGGAGAGAACTCCAGCAGTCAA
        R  A  L  E  L  T  A  E  E  E  R  I  E  R  I  E  N  A  K  K  Y  I  E  E  V  I
4241  GAGAGCTCTAGAACTAACAGCAGAAGAAGAACGGATAGAACGCATAGAAAACGCTAAGAAATATATTGAAGAAGTCATAG
      E  E  T  N  Q  E  L  Q  E  Q  E  R  Q  E  V  S  A  A  A  E  D  T  M  N  T  E  A
4321  AAGAGACAAATCAAGAACTACAAGAACAAGAGAGACAAGAGGTAAGTGCGGCGGCGGAAGATACGATGAACACCGAAGCA
        P  V  P  M  E  T  S  E  S  G  A  T  A  A  P  Q  Q  R  A  A  A  G  G  G  G  S  G
4401  CCCGTCCCGATGGAAACTTCTGAATCCGGAGCCACCGCCGCACCGCAGCAACGAGCTGCTGCGGCGGCGGCGGCTAGCGG
        G  G  G  E  S  A  G  Y  G  K  N  P  S  D  S  F  Q  R  H  R  N  K  P  V  D  L
4481  AGGCGGAGGAGAATCTGCAGGGTACGGAAAAAACCCCAGCGATTCATTCCAGCGCCACCGCAATAAACCCGTTGATCTCA
      K  H  I  G  D  N  V  Y  V  A  Q  R  V  Y  K  V  E  A  E  C  K  L  V  G  D  K  L
4561  AACACATCGGAGACAACGTATATGTGGCTCAGAGAGTTTATAAGGTAGAAGCTGAGTGTAAACTGGTAGGGGACAAGTTA
        S  W  S  N  T  T  N  S  K  Y  L  R  R  L  L  G  I  N  G  N  S  N  S  G  D  I  K
4641  TCGTGGTCAAACACGACAAACAGTAAATATCTCAGGAGACTGTTGGGAATAAACGGCAACAGTAACTCTGGAGATATTAA
        H  S  F  Y  T  Q  L  S  G  S  I  G  L  G  N  L  A  L  G  N  Y  I  N  S  W  G
4721  GCACAGTTTCTACACACAGCTATCTGGAAGTATTGGTTTGGGAAATCTTGCTCTGGGTAACTACATAAACTCGTGGGGTA
      M  D  N  I  S  K  S  E  D  S  W  A  I  I  A  T  R  G  K  M  N  H  L  Q  A  F  E
4801  TGGACAACATATCCAAGAGCGAGGACAGTTGGGCTATCATAGCCACCAGGGGCAAGATGAACCATCTACAGGCATTTGAG
        M  V  P  Q  Y  Q  G  E  T  V  V  G  Y  T  S  A  P  L  Q  F  G  K  L  L  G  H  V
4881  ATGGTTCCACAATACCAAGGAGAAACTGTAGTGGGATATACAAGTGCACCGCTACAGTTTGGTAAACTTTTGGGACATGT
        Y  Y  P  D  P  K  G  E  E  R  I  K  I  A  S  K  A  D  A  K  E  S  K  M  F  K
4961  ATACTATCCAGATCCCAAAGGTGAAGAAAGGATAAAGATAGCAAGTAAAGCTGATGCCAAAGAATCTAAGATGTTTAAGG
      D  A  M  A  G  Y  L  L  D  D  D  M  N  Q  T  K  V  T  S  E  H  N  H  V  F  A  F
5041  ATGCAATGGCAGGTTATCTATTAGATGACGACATGAACCAGACAAAAGTCACATCAGAACACAACCACGTATTTGCATTC
        T  D  L  R  D  S  P  V  I  S  E  V  A  A  Y  Q  T  N  D  E  P  P  K  I  N  G  I
5121  ACAGACTTGAGAGATTCGCCAGTAATAAGCGAAGTAGCAGCATACCAGACCAACGATGAACCACCAAAAATAAATGGCAT
        G  I  E  Y  Q  G  F  N  L  T  S  D  T  N  A  A  L  I  G  L  M  P  S  N  C  I
5201  TGGAATAGAATACCAGGGATTCAACTTAACATCAGACACAAATGCAGCTCTCATTGGTCTCATGCCAAGTAACTGTATAA
      K  R  R  K  E  I  Q  S  G  M  D  N  V  V  L  W  S  M  K  S  N  R  L  I  D  K  R
5281  AGAGAAGGAAGGAGATACAGTCAGGTATGGACAACGTAGTACTATGGTCCATGAAGAGTAACAGACTCATAGACAAAAGA
        F  W  K  P  E  G  W  T  K  K  S  M  N  G  M  A  K  D  K  V  N  I  T  P  T  T  Y
5361  TTTTGGAAGCCAGAAGGATGGACCAAAAAGAGCATGAACGGTATGGCTAAAGACAAAGTGAACATTACACCAACAACCTA
        D  I  Y  E  E  A  H  V  T  R  T  T  D  Y  A  E  W  A  R  N  E  I  F  Y  D  A
5441  TGATATATATGAAGAAGCTCACGTAACCAGGACAACAGATTATGCAGAATGGGCTAGGAATGAAATATTCTATGATGCAA
      N  T  S  Y  G  S  V  G  P  S  D  I  G  N  F  V  Q  K  Y  N  L  S  D  Q  Y  A  T
5521  ACACTTCATACGGAAGTGTTGGACCAAGTGACATAGGAAATTTTGTACAGAAATACAACTTATCAGACCAATATGCTACA
        D  I  F  F  M  P  Y  V  H  T  Q  R  G  I  I  Q  D  I  V  I  N  F  D  L  T  M  Q
5601  GACATATTCTTTATGCCATATGTTCACACACAGAGAGGCATAATTCAAGACATAGTCATAAATTTTGACTTAACAATGCA
        I  M  V  K  R  I  P  R  Q  V  Y  N  D  F  Y  H  I  N  T  R  A  M  N  P  V  K
5681  AATTATGGTCAAGAGAATTCCACGTCAAGTATATAATGATTTCTACCACATCAACACTAGAGCCATGAACCCAGTTAAAT
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     Y  D  S  A  V  E  R  S  F  G  Y  D  E  I  Y  A  R  S  I  K  I  H  E  N  I  S  G
5761 ATGACAGTGCGGTAGAAAGATCATTCGGATACGACGAAATATATGCAAGATCCATAAAAATACATGAAAACATAAGTGGA
      T  H  G  S  K  Y  A  D  R  G  P  I  S  H  M  E  A  T  K  R  N  S  Y  Q  R  A  Y
5841 ACTCATGGAAGTAAGTATGCAGATAGAGGACCAATAAGTCATATGGAAGCAACAAAGAGGAACTCTTACCAGAGAGCATA
       A  Q  R  R  I  I  L  D  Q  G  V  S  K  M  K  T  R  S  S  A  A  A  E  D  D  I
5921 TGCACAGAGAAGAATAATACTAGATCAGGGTGTATCAAAAATGAAGACAAGAAGTAGTGCGGCGGCGGAAGATGACATTC
     P  E  D  C  D  D  F  L  E  T  S  E  M  D  P  P  P  Q  P  Q  L  P  K  K  K  K  K
6001 CAGAAGATTGTGACGACTTCCTAGAAACTTCTGAAATGGATCCACCGCCGCAGCCGCAGTTGCCGAAGAAGAAGAAGAAA
      Y  R  V  N  V  *
6081 TATAGAGTTAATGTATAATGTTGACATAATACAAATGTATATATTTGAGTTACAATAAAGGTTATAAAAATCACGCGTGC
                                                                          flip/flop
6161 GTGGTTTACCCTTCAGAAGGAGCGATTCTCCGGGGGTAGGTAGGGGGATCTTTGCGATGACACTGCCAGTTGCATATATG
      sequence
6241 CCGTGGGCCATGTCATCGCAAAGATCCCCCTACCTACCCCCGGAGAATCGCTCCTTCTGAAGGGTAAACCACGCACGCGT

6321 G
```

Figure 2 Nucleotide sequence of the HPV genome. Amino acid sequence of putative polypeptides corresponding to major ORFs is shown under the sequence. The putative translation initiation ATG codons and polyadenylation signals (AATAAA) are underlined. The three most likely functional p2, p22, and p48 promoters are indicated with TATA boxes. Inverted repeat sequences are underlines with arrow head. DPE and Inr represent downstream promoter element and transcriptional start signal, respectively.

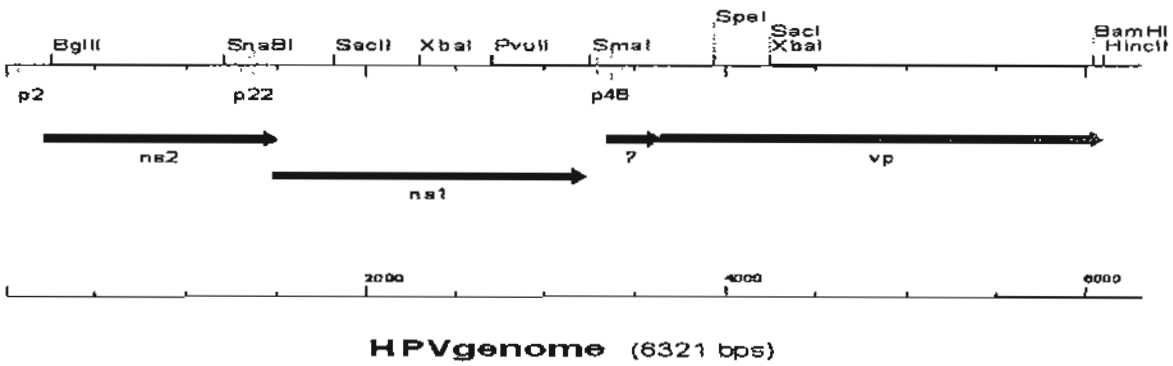

Figure 3 Organization of coding sequences on the plus strand of HPV genome

Three ORFs; left ORF (NS2), mid ORF (NS1) and right ORF (VP) are shown by shaded arrows. Open arrows indicate the three most likely functional p2, p22, and p48 promoters.

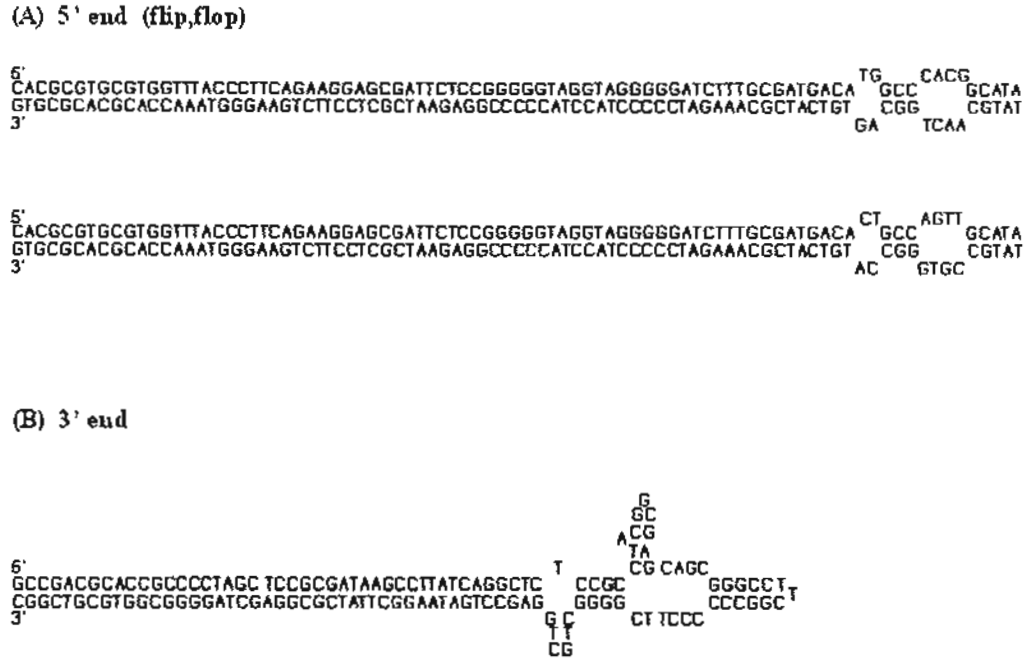

Figure 4 Analysis of the 5 and 3 terminal non-coding sequences of HPV genome. The 5 end exists in flip and flop orientations (A). The 3 end forms only one hairpin-like structure (B).

Construction and characterization of an infectious plasmid clone of HPV

It has been shown that a cloned parvovirus genome transfected into eukaryotic cells can rescue from the plasmid vector and replicate as a wild-type virus (Shull *et al.*, 1988; Ward *et al.*, 2003; Wang *et al.*, 1996). Therefore, the full-length of HPV genome was cloned into plasmid vector, as described in Method, in order to construct the infectious clone.

Infectivity test of HPV

In order to determine the infectivity of the recombinant HPV clone, a suitable cell system for testing has to be investigated. In this case, HPV particles have been used for infection in both cell culture and in shrimp. With a limited information of HPV infection in cell culture as well as a difficulty for preparing shrimp primary cell culture, well established insect cell lines were tested as well.

Two insect cell lines, such as C6/36 and Sf9, and shrimp primary cells isolated from lymphoid organ as well as hepatopancreas were infected with HPV (10^{-3}). Cell and supernatant of each well were collected at various time points. Crude DNA extraction was then performed and the replication of HPV was monitored by PCR amplification with viral specific primers. The results from both of C6/36 and Sf9 showed that the intensity of HPV fragment was decrease after prolong the culture period (Figure 5). It indicated that C6/36 and Sf9 cells are not susceptible for HPV infection. The increasing of HPV genome was observed only in primary hepatopancreas cells (Figure 5). Therefore, the suitable primary cells for further testing with HPV clone are the hepatopancreas cells.

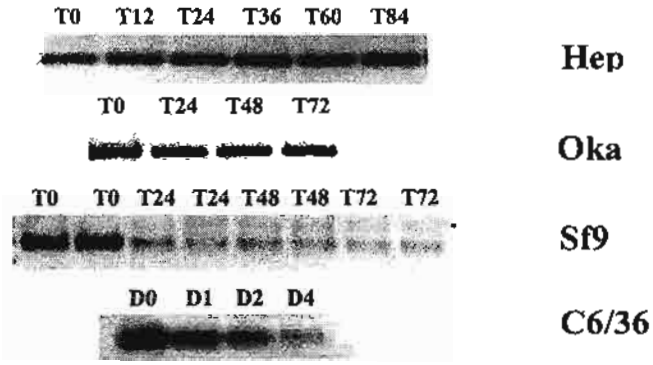

Figure 5 Time-course study of HPV infection in primary shrimp cells isolated from hepatopancreas (Hep) and lymphoid (Oka) as well as insect cells (Sf9 and C6/36). Multiplication of HPV was monitored by PCR amplification of HPV gene. Both cell and supernatant were harvested at each time point (T0-T84= 0-84 h after HPV infection; D0-D4= 0-4 days after HPV infection).

Attempt to investigate the optimal condition for transfection of the plasmid DNA into the shrimp primary cells, an expression plasmid containing functional promoter as well as reporter gene has to be considered. However, little research has been done on functional promoters, which regulate the expression of foreign genes in shrimp cells. In our laboratory, a mammalian viral promoter (CMV) and six putative promoters from shrimp viral pathogens; p2 from HPV, as well as RR1, RR2, VP15, and VP19 promoters from WSSV, were employed for the functional analysis (Phochanukul, 2005). The transient transfection in Sf9 cells showed that luciferase expression was detected from CMV, p1, RR1, and RR2 promoters. Among these promoters, the highest luciferase activity in Sf9 was obtained from RR2 promoter. The conditions of transfection in primary Oka and hepatopancreas cells from *P.monodon* were optimized using liposome-mediated transfection and electroporation methods. CMV and RR2 promoters were applied as positive controls in optimization experiments. However, the expression of luciferase reporter gene using luciferase assay was not detected from all transfection conditions. Besides, the luciferase transcripts were not detected from RT-PCR. The reason of the absence of the luciferase expression was still controversial whether the failure of transfections into primary shrimp cells, or the lack of essential transcription factor(s) in shrimp cells to foster promoter function, or both. Therefore, the optimal condition of DNA transfection into primary shrimp cells has not been figured out.

Infectivity test of HPV clone in shrimps

Several reports have been shown the successful transient expression by direct injection into skeleton muscle of invertebrates (Hansen *et al.*, 1991). According to this knowledge, the full-length HPV clone was used to inject into shrimp to permit the expression of viral proteins essential for viral progeny production. Based on the result of HPV infection in shrimp, the amount of HPV plasmid molecule (50 μg/shrimp) used for muscular injection was approximately 5000 times higher than viral particles at 10^{-4} (10^{9} particles) in order to enhance the effectiveness. Moreover, liposome mediated transfection reagent was used to mix with the plasmid DNA before injection. The infectivity of HPV clone was then evaluated by PCR amplification of

HPV genome. In fact, we could not distinguish the PCR product amplified from injected HPV plasmid and from newly synthesized viral genome, therefore, other set of primer that one binds inside HPV genome and another binds at vector region (HPV/vector) was used (Figure 6A). Result showed that PCR product could be detected from both reactions using HPV primers and using HPV/vector primers. But the intensity of HPV product was much higher than that of HPV/vector indicating that the synthesis of viral progeny from injected HPV clone might be occurred. Nevertheless, we could not detect the infection of HPV in PL-shrimps fed with those injected shrimps (Figure 6B). It might because of the very low level of viral progeny produced from HPV clone that it was not enough to further infect PL-shrimp.

In addition, the difficulty of transient transfection in primary cells is affected by several limitations. The important factor is that the success of DNA delivery depends on cell cycle. It is necessary to introduce DNA vector into nuclear compartment where the transcription machinery is located; so, high expression level of foreign genes was usually obtained in actively dividing cells. To overcome the limitation of low level of active cells in primary cultured cells, the shrimp zygotes, which are continuously dividing cells, were also used for transfection with HPV full-length clone. Liposome mediated transfection according to the protocol of Sun and his colleague (Sun *et al.*, 2005) has been used in this experiment to deliver HPV plasmid into shrimp zygotes. Unfortunately, there was no promising result from this experiment.

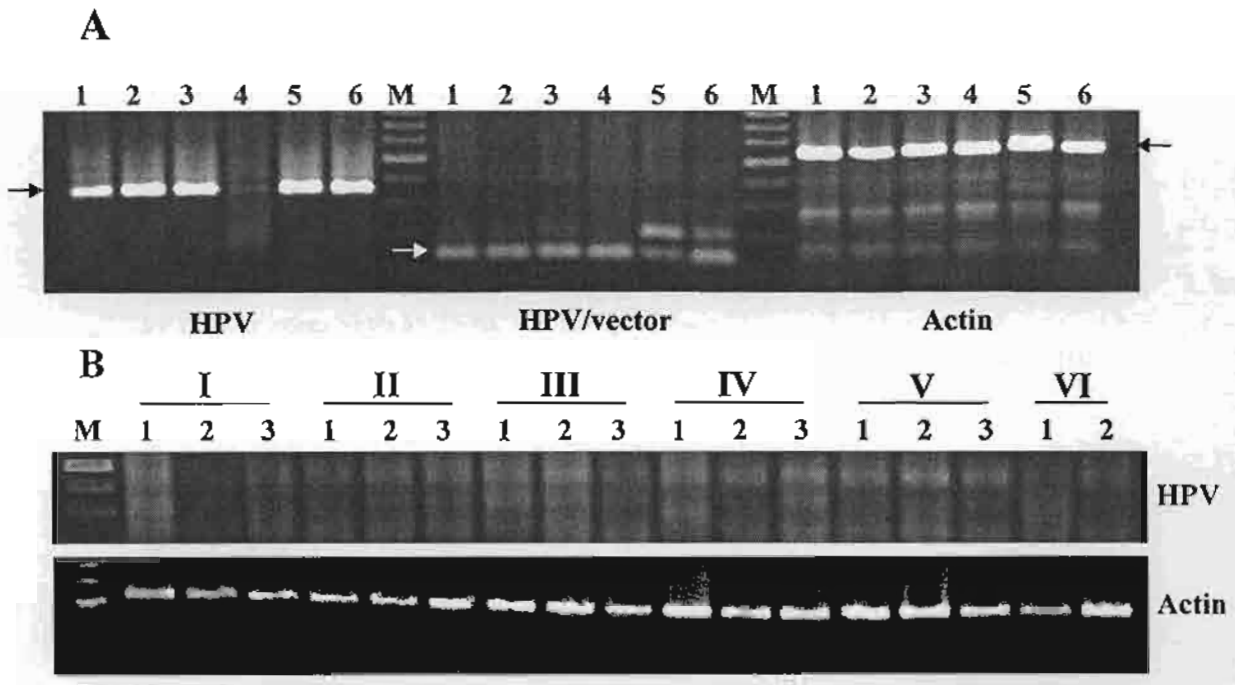

Figure 6 Investigation of HPV production by PCR amplification of HPV genome.
A: Crude DNA extracted from shrimp muscular injected with full-length HPV clone was used to detect the HPV genome. PCR amplification using HPV specific primers (HPV), HPV and plasmid vector specific primers (HPV/vector) and *P. monodon* actin primers (Actin) are shown. Arrows indicate the expected PCR products. Number 1-6 named individual shrimp.
B: Crude DNA extracted from PL-shrimp fed with injected shrimp (I-VI) was utilized as template for PCR amplification using HPV and Actin primers. Three PL-shrimps (1-3) were fed with each injected shrimp (I-VI).

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Research Output
(จากทุนเมธีวิจัยอาวุโส สกว.)

Research Output (จากทุนเมธีวิจัยอาวุโส สกว.)

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1. Therapeutic inhibition of yellow head virus multiplication in infected shrimps by YHV-preotease dsRNA. W. Tirasophon, S. Yodmuang, W. Chinnirunvong and S. Panyim.
2. Identification of Argonaute cDNA from Penaeus monodon and implication of its role in RNA interference. M. Dechklar, A. Udomkit and S. Panyim.
3. Molecular characterization of gonad-inhibiting hormone of Penaeus monodon and elucidation of its role in vitellogenin expression by RNA interference. S. Treerattrakool, A. Udomkit and S. Panyim.

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รายชื่อคณะผู้ร่วมวิจัย

รายชื่อคณะผู้ร่วมวิจัย

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รายชื่อคณะผู้ร่วมวิจัย

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