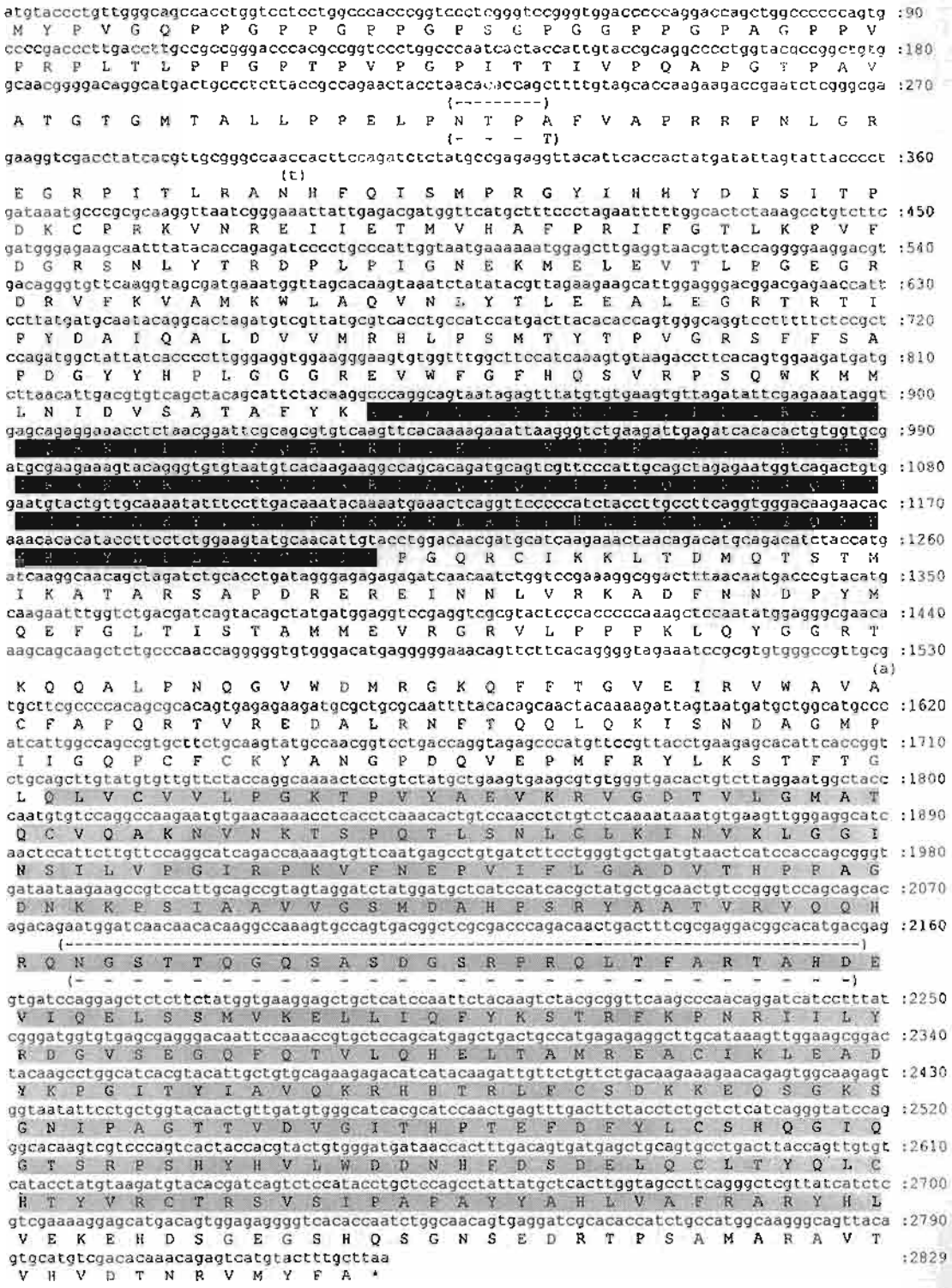

Figure 3. Nucleotide and deduce amino acid sequences of Pem-AGO. The sequence of *Pem-ago* (*Pem-ago-L1*) variant is shown. The deduced amino acid residues are shown in one-letter symbol. The PAZ and PIWI domains are highlighted in black and gray, respectively. The asterisk marks the stop codon. The 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.

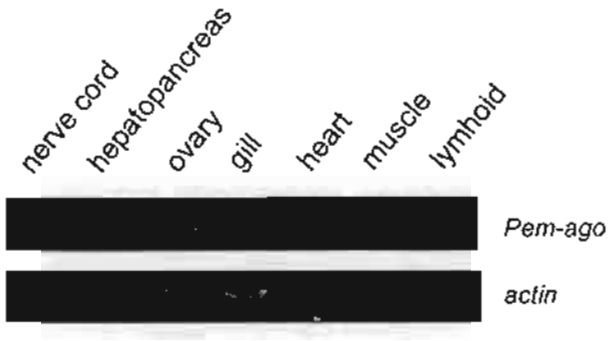

Figure 4. Expression of *Pem-ago* in *P. monodon* tissues. Total RNA was extracted from nerve cord, hepatopancreas, ovary, gill, heart, muscle and lymphoid organ of *P. monodon*. The mRNA of *Pem-ago* in these tissues was detected by RT-PCR. The actin transcript was used as internal control.

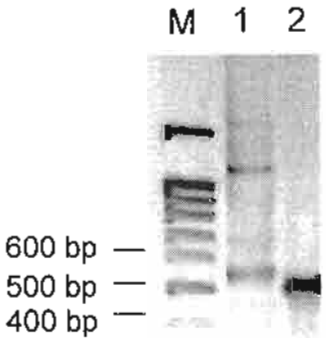

Figure 5. Double-strand RNA of the PAZ domain. Plasmid DNA containing short DNA template in sense and antisense directions of the PAZ coding sequence were transcribed to single-stranded RNA by *in vitro* transcription. Both strands of PAZ *in vitro* transcription products were annealed together to obtain 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.

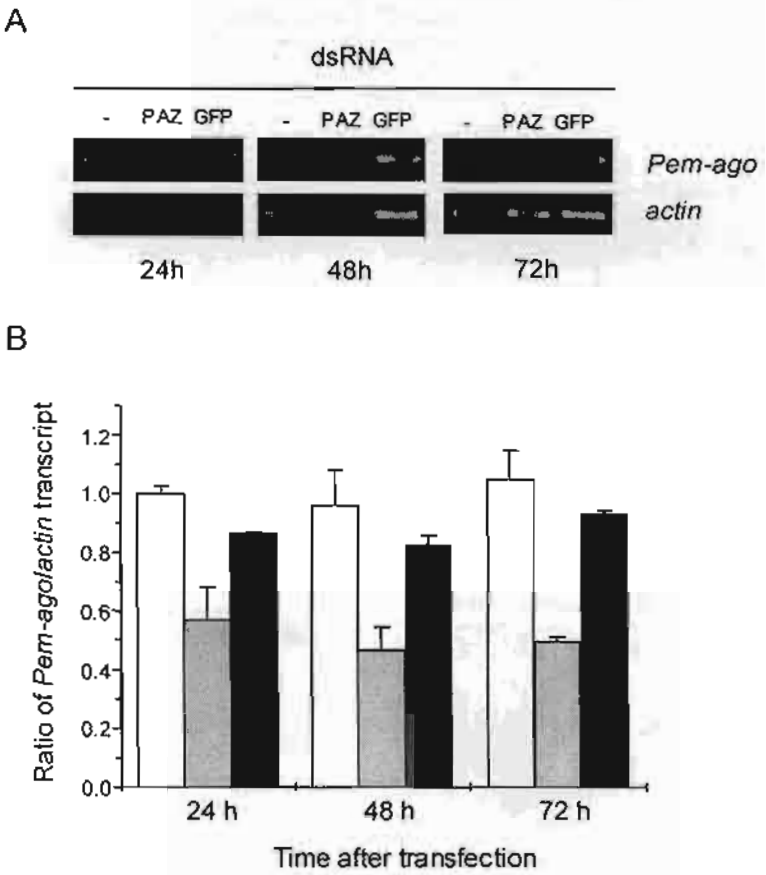

Figure 6. Silencing of *Pem-ago* expression in primary culture of Oka cells. (A) The transcript of *Pem-ago* in the Oka cells that were transfected with 2 □g of either PAZ-dsRNA (PAZ) or GFP-dsRNA (GFP) for 24, 48 and 72 h was detected by RT-PCR and compared with the control cells that did not exposed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

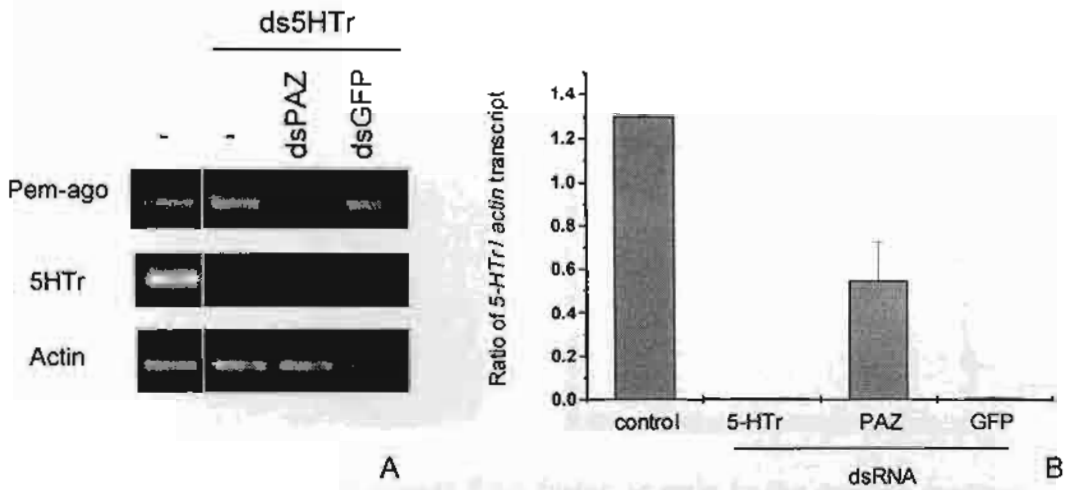

Figure 7. Impairment of RNAi in *Pem-ago* depleted cells. The Oka cells were transfected with 2 □g of either PAZ-dsRNA or GFP-dsRNA 24 h prior to subsequent transfection with 5-HTr-dsRNA. The cells were collected for total RNA extraction at 48 h afterward and the 5-HTr transcript was detected by RT-PCR.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

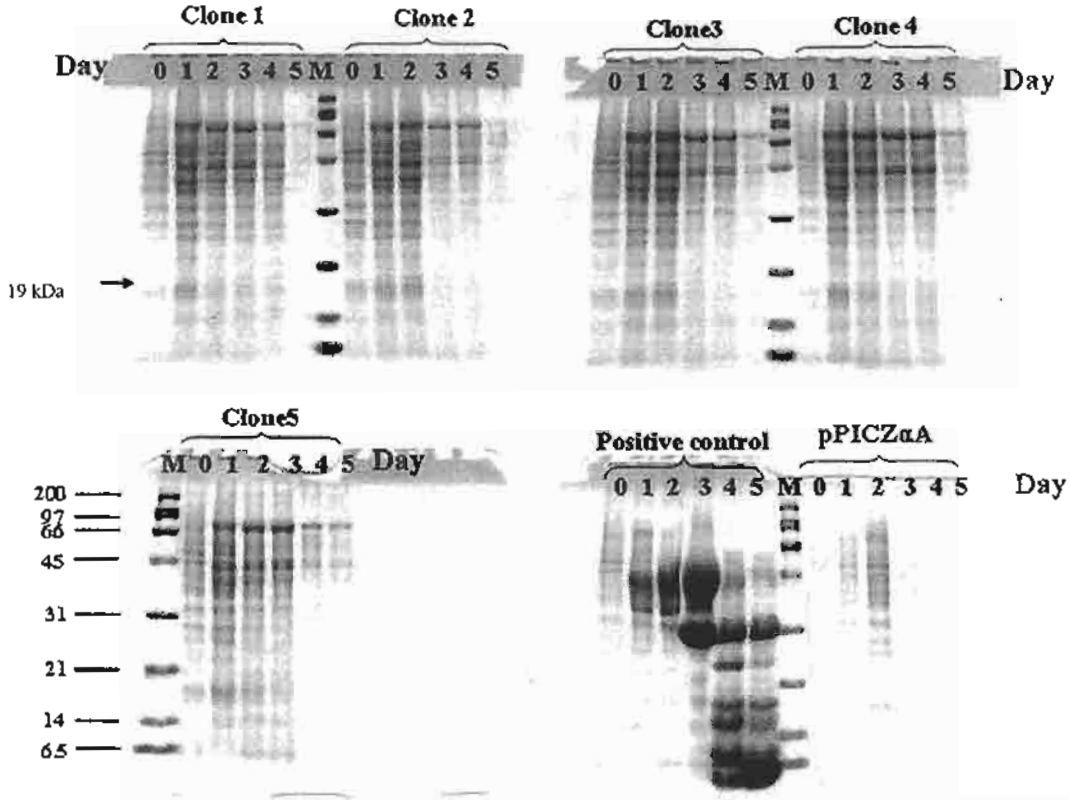

Figure 8. SDS-PAGE analysis of the recombinant His-tagged PAZ fusion protein expression. The figure shows 15% SDS-PAGE with Coomassie blue staining of the 1 ml TCA precipitated culture medium of clones 1 to 5 after induction with 3 %(v/v) methanol for 0-5 days. *P. pastoris* containing integrated recombinant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

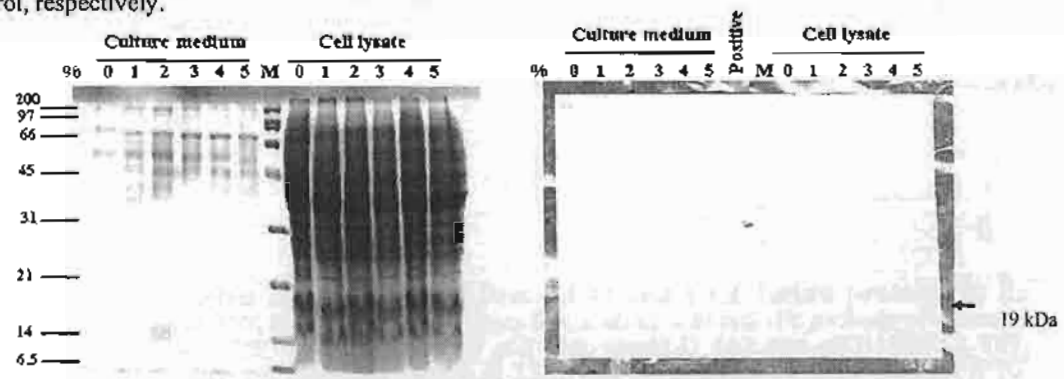

Figure 9. Expression of recombinant PAZ fusion protein in the culture medium and the cell lystae. The figure shows 15% SDS-PAGE with Coomassie blue staining (A) and western blot analysis with anti-His antibody (B) of the 1 ml TCA precipitated culture medium and 6 OD_{600} cell lysate from the *P. pastoris*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

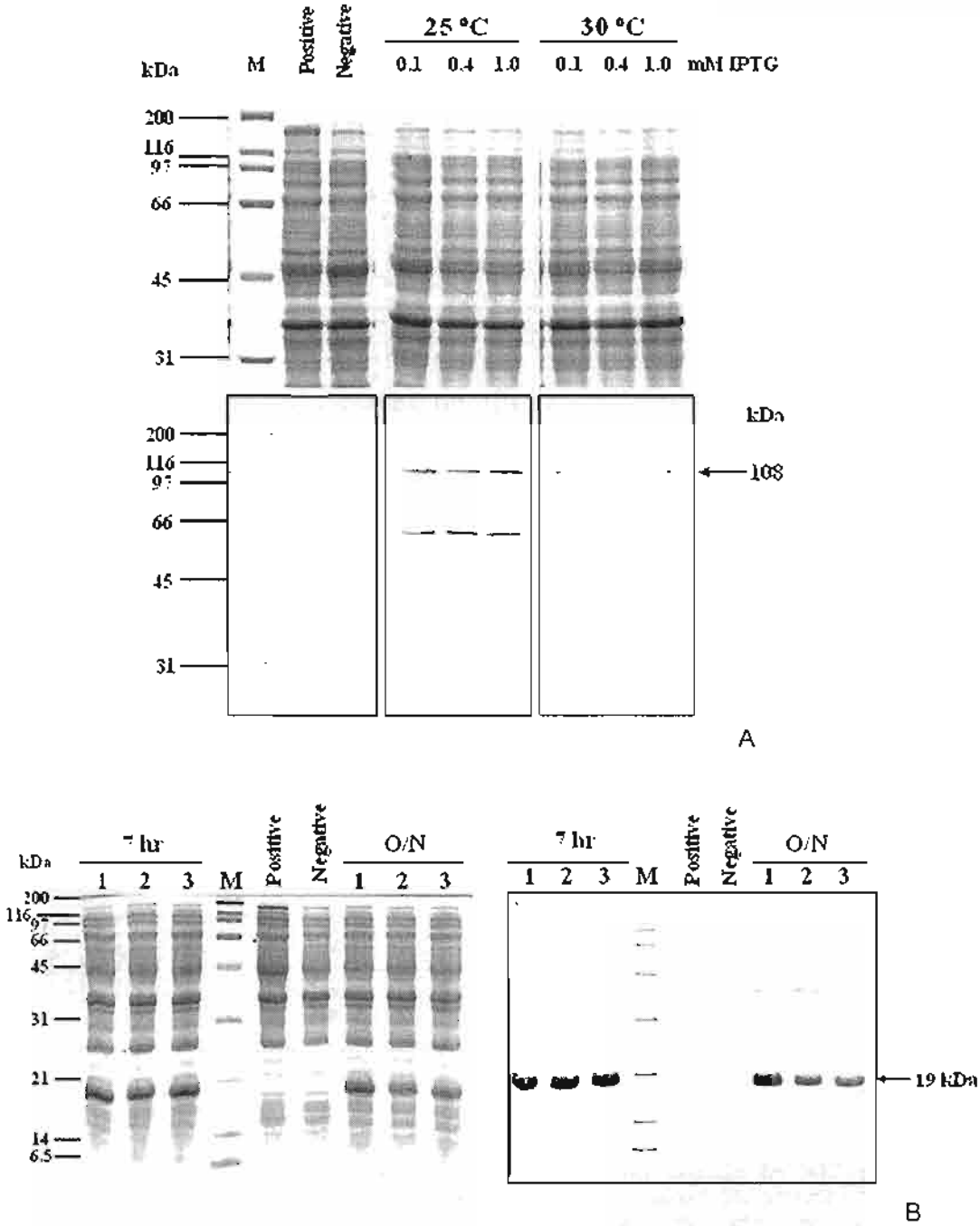

Figure 10. Expression of recombinant Pem-AGO and PAZ fusion proteins in *E. coli*. The figures show 15% SDS-PAGE and Western Blot analysis with anti-His antibody of the crude cell lysate from BL21(DE3)pLysS containing pET-15b/Pem-AGO (A) and pET-15b-PAZ (B). Recombinant Pem-AGO was expressed for 2 h 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).

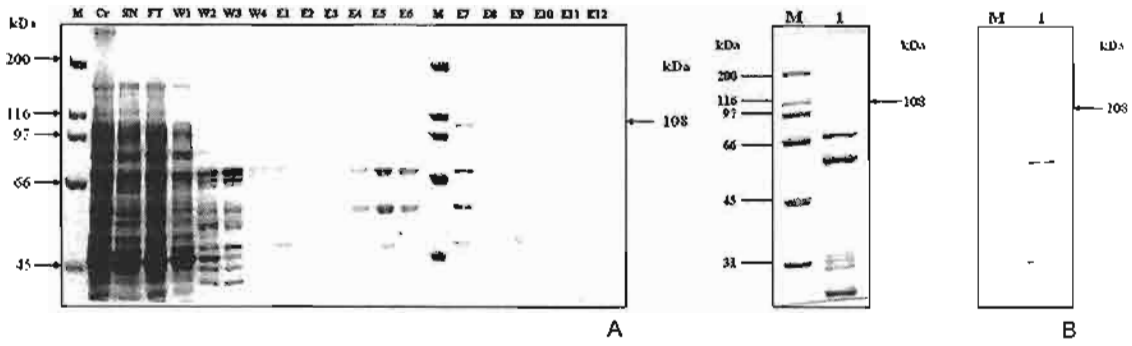

Figure 11. Purification of recombinant His-Pem-AGO fusion protein by Ni^{2+} affinity chromatography. (A) The protein profile on Coomassie blue-stained 8% SDS-PAGE of the purification of recombinant Pem-AGO fusion protein in the soluble fraction by Ni^{2+} affinity chromatography utilizing imidazole 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.

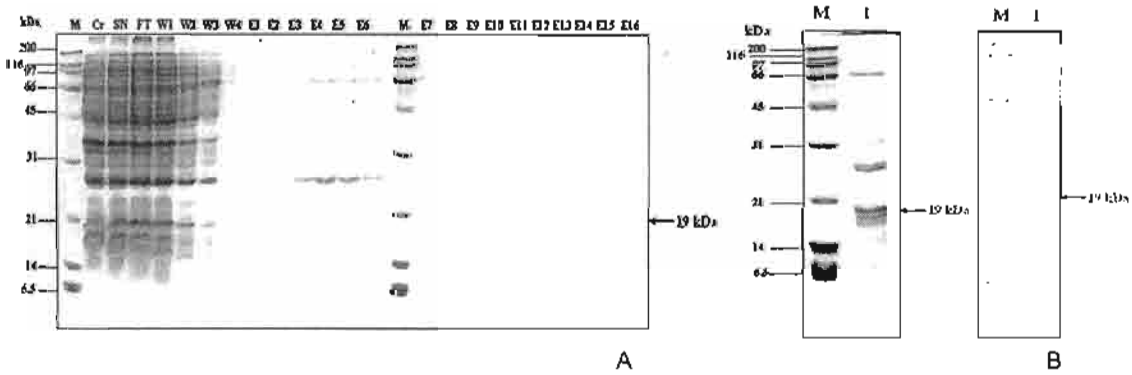

Figure 12. Purification of recombinant His-PAZ fusion protein by Ni^{2+} affinity chromatography. (A) The protein profile on Coomassie blue-stained 8% SDS-PAGE of the purification of recombinant PAZ fusion protein in the soluble fraction by Ni^{2+} affinity chromatography utilizing imidazole gradient 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.

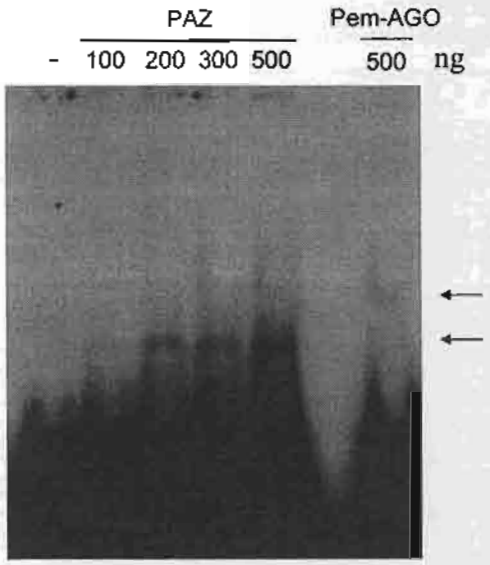

Figure 13. RNA binding activity of recombinant PAZ and recombinant Pem-AGO. Abuout 1 pmole of 21 nt siRNA labeled at the 5' end with □-^{32}P ATP was incubated with either recombinant PAZ or recombinant Pem-AGO fusion protein. The RNA-protein complexes were analyzed on 5% non-denaturing polyacrylamide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

***Chalermporn Ongvarrasopone*, Jarasporn Tangkhabuanbutra, Chalongrat Noree, Mayuree Chanasakulniyom and Sakol Panyim**
Institute of Molecular Biology and Genetics,
Mahidol University, Salaya Campus, Nakhonpathom 73170, Thailand

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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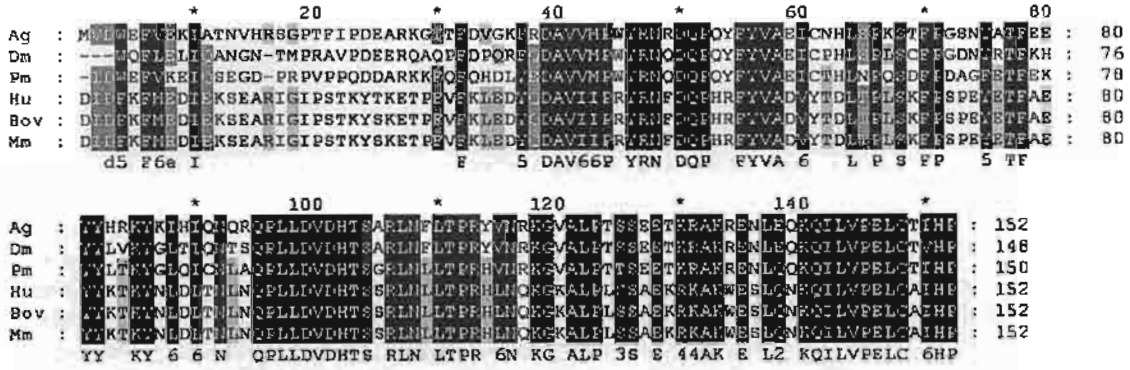
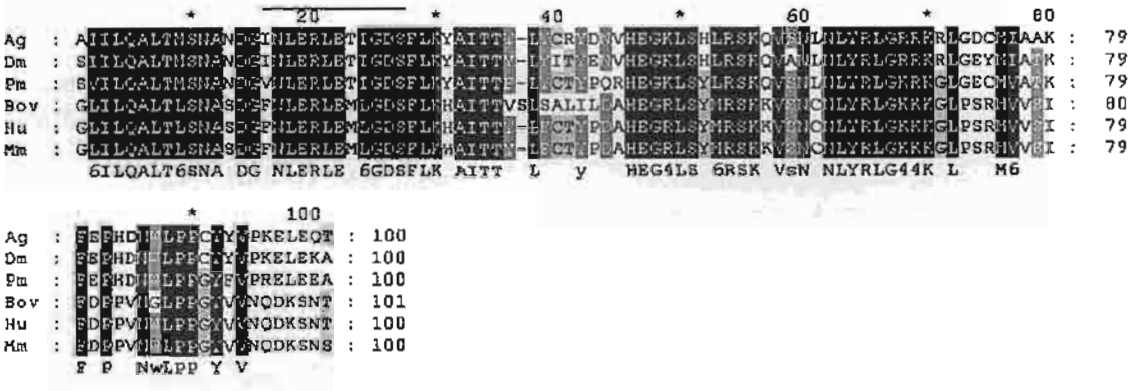
B.

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

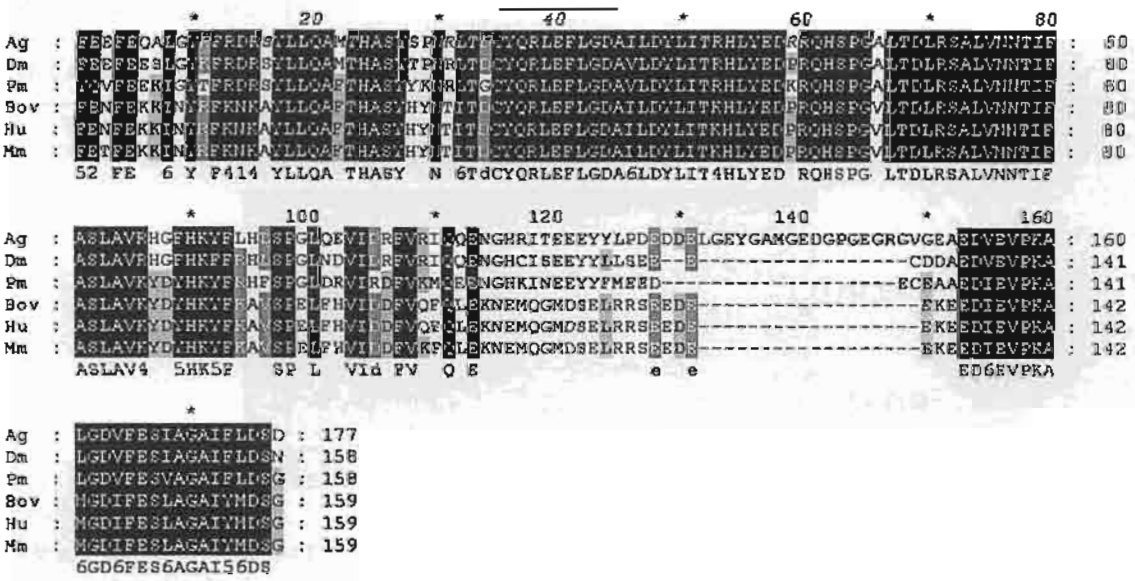
A. PAZ domain



B. RNase III domain I



C. RNase III domain II



D. dsRBD

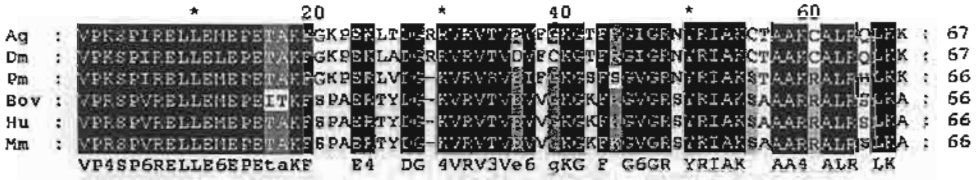


Figure 2. Multiple amino acid sequence alignments of Dicer domains comparing between *Penaeus mondon* (Pm) Dicer and other vertebrates and invertebrate Dicers. The alignments were performed using the ClustalX program. The amino acid sequences of Dicers were retrieved from the GenBank database under the 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

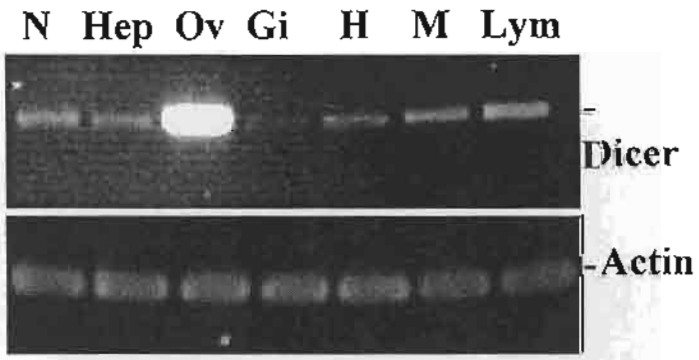


Figure 3. Expression of Dicer in various tissues of a wild broodstock shrimp. N: brain and thoracic ganglia, Hep: hepatopancreas, Ov: ovary, Gi: gill, H: heart, M: muscle, and Lym: lymphoid. Actin was used as an internal control for loading.

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

***Pongsopee Attasart*, Nichanun Phochanukul, Rosukon Kaewkhoa, Chaweewan Chimwai and Sakol Panyim**
Institute of Molecular Biology and Genetics,
Mahidol University, Salaya Campus, Nakhonpathom 73170, Thailand

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.

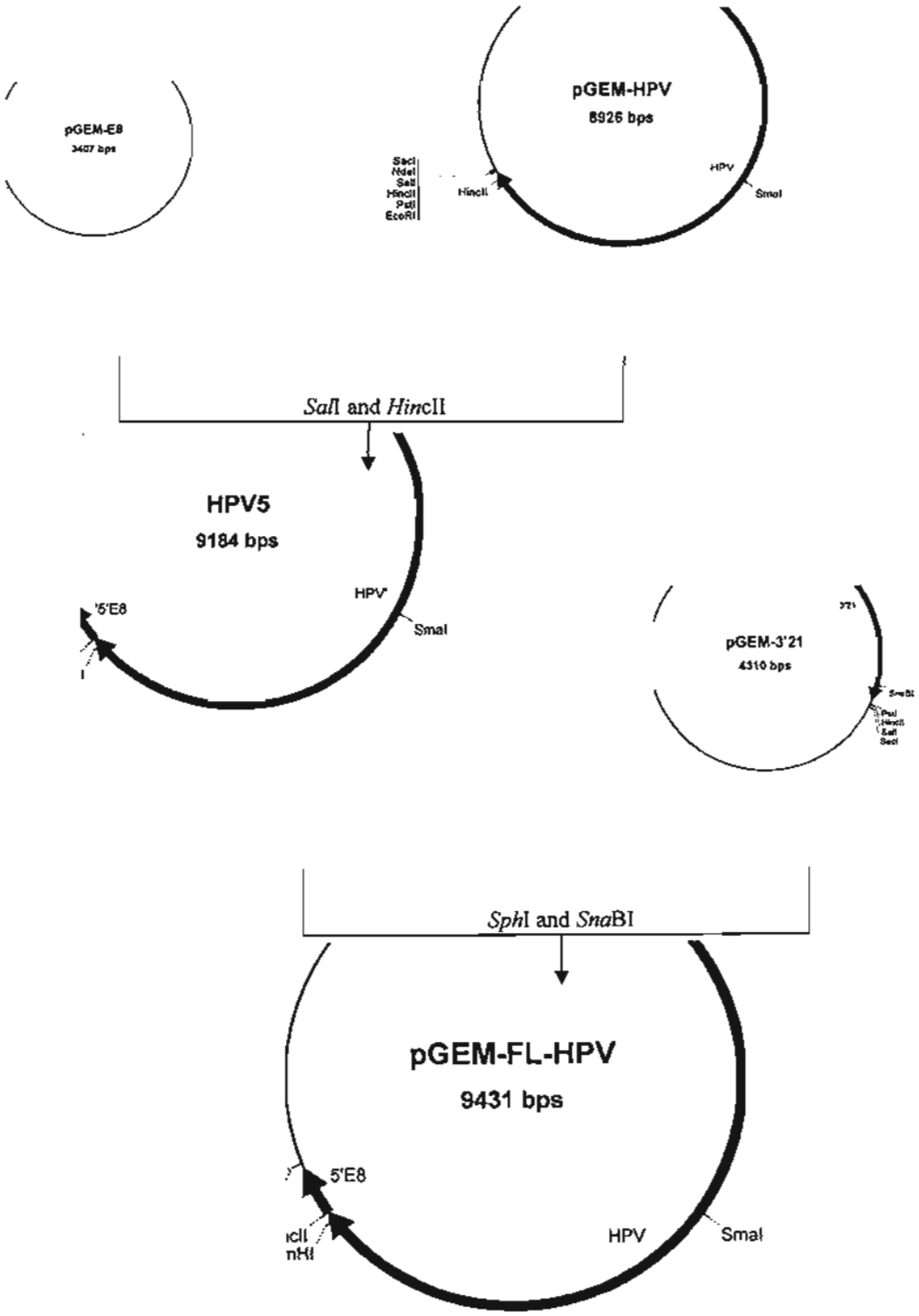


Figure 1 Cloning strategy of HPV genome in pGEM-Teasy plasmid vector

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.

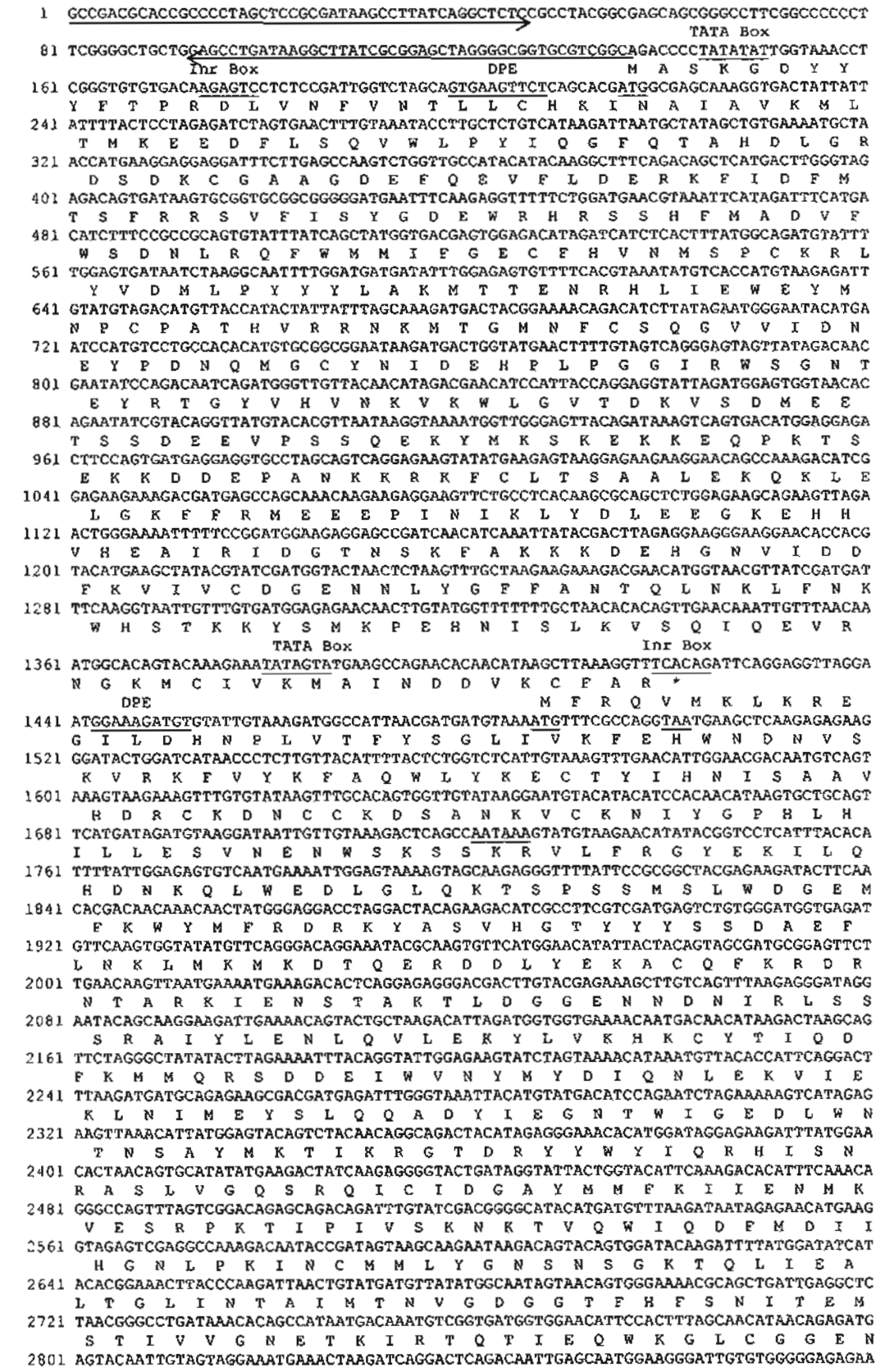
The third set of putative transcription regulatory sequences was found upstream from the right ORF. These included a TATA-box, TATAGCT (nt 3,369-3,375); a GC-rich activator sequence TGTCGGC (nt 3,350-3,356), a Inr-box, TCATGGT (nt 3,425-3,431), and a DPE sequences, CAGACACAGT (nt. 3,455-3,464). This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

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      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.

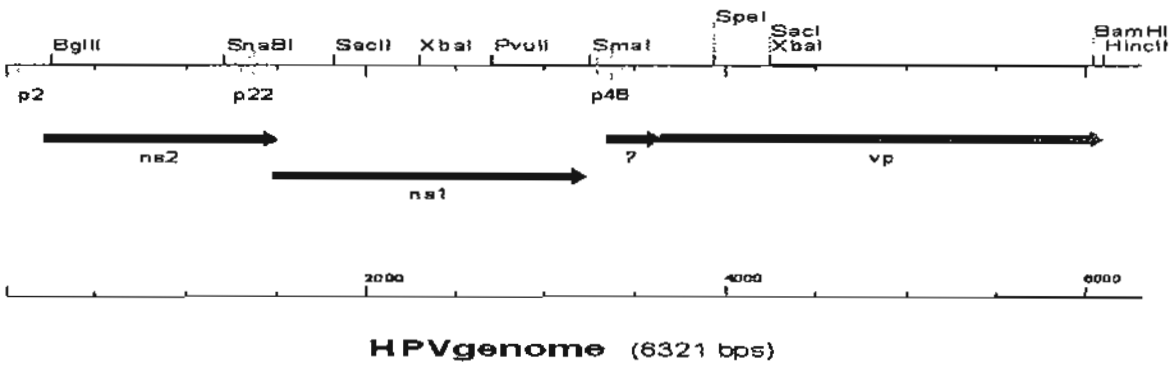

Figure 3 Organization of coding sequences on the plus strand of HPV genome

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.

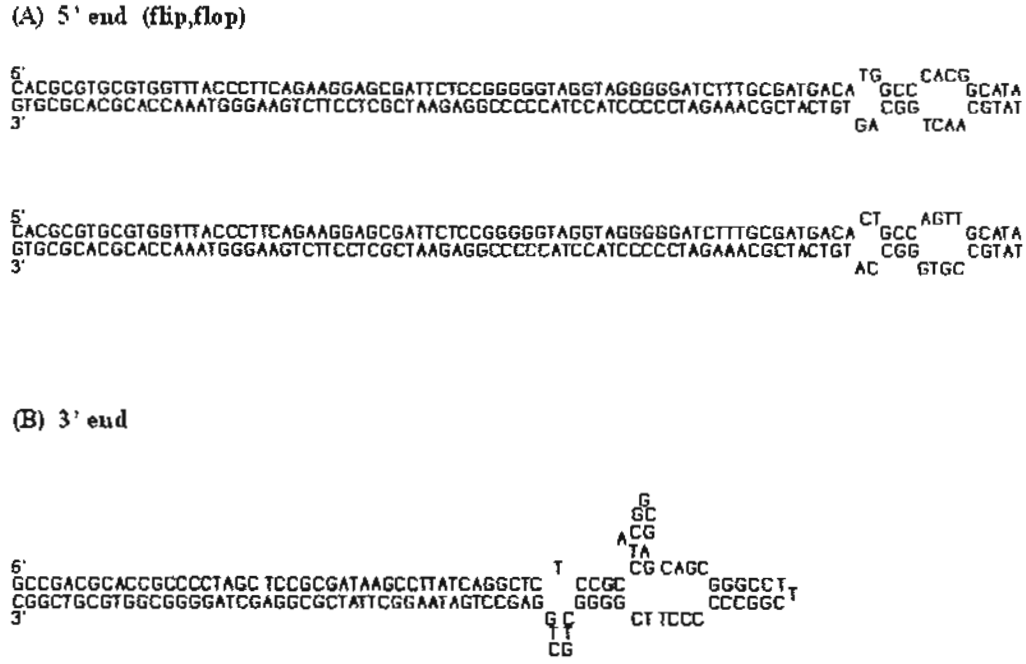

Figure 4 Analysis of the 5 and 3 terminal non-coding sequences of HPV genome. The 5 end exists in flip and flop orientations (A). The 3 end forms only one hairpin-like structure (B).

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.

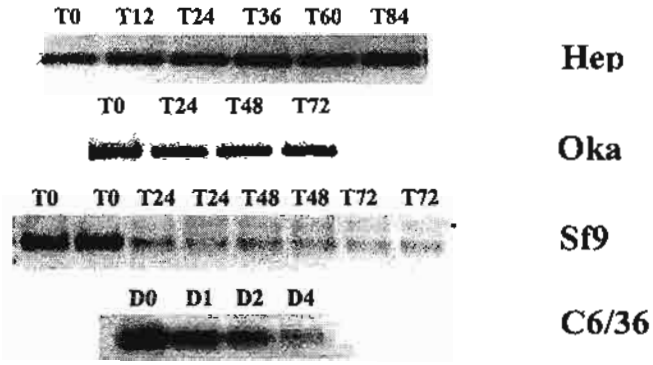

Figure 5 Time-course study of HPV infection in primary shrimp cells isolated from hepatopancreas (Hep) and lymphoid (Oka) as well as insect cells (Sf9 and C6/36). Multiplication of HPV was monitored by PCR amplification of HPV gene. Both cell and supernatant were harvested at each time point (T0-T84=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

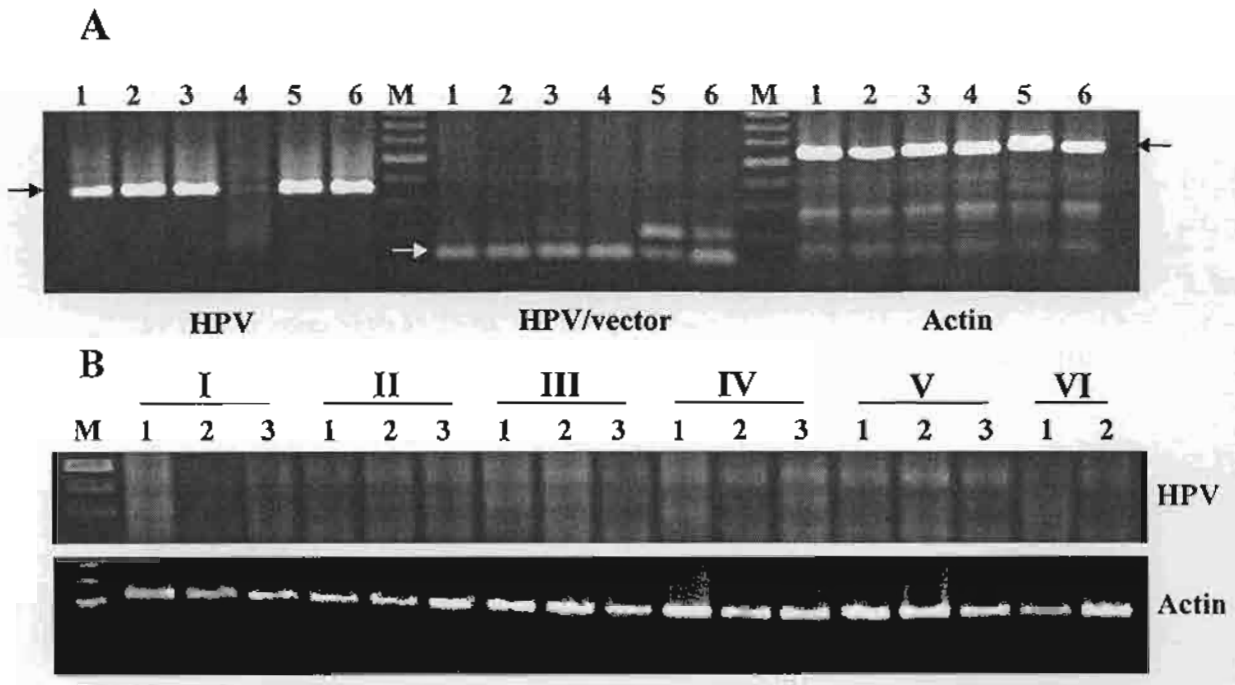

Figure 6 Investigation of HPV production by PCR amplification of HPV genome.
A: Crude DNA extracted from shrimp muscular injected with full-length HPV clone was used to detect the HPV genome. PCR amplification using HPV specific primers (HPV), HPV and plasmid vector specific primers (HPV/vector) and *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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ผลงานเตรียมพิมพ์ในวารสารวิชาการนานาชาติ

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รายชื่อคณะผู้ร่วมวิจัย

รายชื่อคณะผู้ร่วมวิจัย

ชื่อ - นามสกุล	ตำแหน่งวิชาการ				ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน	คณะ/สถาบัน	มหาวิทยาลัย		
1. ดร.สกล พันธุ์ยิ้ม	ศาสตราจารย์	ศาสตราจารย์เกียรติคุณ	คณะวิทยาศาสตร์	มหิดล	หัวหน้าโครงการ	หัวหน้าโครงการ
2. ดร.อภินันท์ อุดมกิจ	ผู้ช่วยศาสตราจารย์	ผู้ช่วยศาสตราจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักวิจัยร่วม	นักวิจัย
3. ดร.วิฑูรย์ ถิระโสภณ	อาจารย์	ผู้ช่วยศาสตราจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักวิจัยร่วม	นักวิจัย
4. ดร.เฉลิมพร องศ์วรโสภณ	อาจารย์	ผู้ช่วยศาสตราจารย์	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักวิจัยร่วม	นักวิจัย
5. ดร.พงโสภี อัตศาสตร์	นักวิจัย 5	นักวิจัย 6	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักวิจัยร่วม	นักวิจัย
6. น.ส.เยาวลักษณ์ รสหอม	นักวิจัย	ศึกษาต่อต่างประเทศ	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักวิจัย	ศึกษาต่อปริญญาเอก ต่างประเทศ ทุน ก.พ.
7. น.ส.สุพรรษา ยอดเมือง	นักวิจัย	ศึกษาต่อต่างประเทศ	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักวิจัย	ศึกษาต่อปริญญาเอก ต่างประเทศ ทุน ก.พ.
8. นายวันชัย อัศวลาภสกุล	นักศึกษาปริญญาเอก	จบการศึกษา	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาเอก	อาจารย์ จุฬาลงกรณ์ มหาวิทยาลัย
9. น.ส.นงลักษณ์ ปล้องทองคำ	นักวิจัย	นักวิจัย	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักวิจัย	นักวิจัย
10. นายวัลลภ ชินนิรันดร์วงศ์	ผู้ช่วยนักวิจัย	ผู้ช่วยนักวิจัย	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	ผู้ช่วยนักวิจัย	ผู้ช่วยนักวิจัย
11. น.ส.วรัญญู วุฒิจันทร์			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	จบการศึกษา
12. น.ส.สุพัตรา ตรีรัตน์ตระกูล			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาเอก	นักศึกษาปริญญาเอก

รายชื่อคณะผู้ร่วมวิจัย

ชื่อ - นามสกุล	ตำแหน่งวิชาการ		คณะ/สถาบัน	มหาวิทยาลัย	ตำแหน่งในโครงการ	สถานภาพปัจจุบัน
	เมื่อเข้าร่วมโครงการ	ปัจจุบัน				
13. นายภาวนัย สุขุมังกูร			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
14. นายจุฑาการ ภูทอง			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
15. น.ส.มนัสวี เดชกล้า			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	จบการศึกษา
16. น.ส.นรากร คุณวีระพงษ์			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
17. นายวีระพงษ์ โฮ			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
18. นายฉลองรัตน์ โนรี			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	ได้รับทุน ก.พ. ศึกษาต่อต่างประเทศ
19. น.ส.มยุรี ชนะสกุลนิยม			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	ได้รับทุน ก.พ. ศึกษาต่อต่างประเทศ
20. น.ส.ณิชนันท์ ภูชนกุล			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	จบการศึกษา ได้รับทุน ก.พ.
21. น.ส.รสสุคนธ์ แก้วขาว			สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	นักศึกษาปริญญาโท	นักศึกษาปริญญาโท
22. น.ส.ฉวีวรรณ ฉิมวัย	ผู้ช่วยนักวิจัย	ผู้ช่วยนักวิจัย	สถาบันอณูชีววิทยาและพันธุศาสตร์	มหิดล	ผู้ช่วยนักวิจัย	ผู้ช่วยนักวิจัย