Construction and evaluation of the versatile recombinant baculoviral vector systems with hybrid promoters designed for the expression of foreign gene in shrimp cells

5.1. Introduction

Studies in shrimp virology and functional genomics have been hampered by the lack of immortalized shrimp cell lines. Moreover, considering the mass mortality of the cultured animals being reported globally due to deadly viruses, establishment of permanent cell lines from shrimp turns out to be of paramount importance to undertake studies on viral morphogenesis, screening anti-viral molecules and for their doses specification. Several investigations have been conducted worldwide to overcome the WSSV havoc through prophylactic strategies (Sudheer et al., 2011; Syed Musthaq and Kwang, 2011). However, lack of a certified shrimp cell line remained all along a major impediment in the development of anti-WSSV drugs (Jose et al., 2012). In the previous chapters, a conclusion could be drawn based on the experience that shrimp cell line development would not be possible unless immortalization had been induced either with oncogenes or any other immortalizing gene. If it is so, a shrimp specific expression vector is the foremost requirement to achieve this target.
Gene transfer refers to the delivery of nucleic acid (gene) encoding a protein to the target cells. Such administration of foreign gene requires a transportation vehicle, called vectors, which carries the gene into the target cell. Various methods are being used to deliver vectors encoding foreign genes into eukaryotic cells, which include both physicochemical (electroporation, bombardment with gold or wolfram microparticles, etc.) and biological (lipid conjugates in the form of liposomes, recombinant viruses, etc.) techniques (Beljelarskaya, 2011). If the foreign DNA (transgene) is introduced into target cell via a viral vector through infection, the process is termed as transduction, which results in transformation or change of target cell genotype. The viral vectors have been proven as the most efficient tools for genetic modification of majority of somatic cells in vitro and in vivo (Sarkis et al., 2000). In this line, many viral vectors have been developed and widely used in gene transfer (transduction) and expression studies in vivo and in vitro. Such viral vectors are adenoviruses (Kozarsky and Wilson, 1993; Huard et al., 1995), retro and lentiviruses (Naldini et al., 1996; Felder and Sutton, 2009; Poluri et al., 2003), adeno associated viruses (Hermonat and Muzyczka, 1984; Carter, 2005) and baculoviruses (Zeng et al., 2009). Among them, the baculovirus has emerged as a promising gene delivery vector in recent years (Lo et al., 2009). However, in every such case, the efficiency of viral mediated transduction and transgene expression depends on the ability of viral particle to transmit their genome into the nucleus.

Baculoviruses, in particular Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) is a large enveloped virus with a double stranded, circular DNA genome of ~130-kb with 154 open reading frames (Ayres et al., 1994). This genome is condensed into a nucleoprotein structure known as a core and is located within a flexible rod shaped capsid, averaging 25-50 nm in diameter and 250-300 nm in length and can expand relatively freely to accommodate even very large recombinant molecules. The core and the capsid are known collectively
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as the nucleocapsid. Membrane enveloped nucleocapsids are referred to as virus particles or virions (Mähönen, 2010a) (Fig. 1).

![Fig. 1. Schematic structure of budded baculovirus (Airenne et al., 2009).](image)

Baculovirus mediated expression system has been widely used for high level gene expression in transduced cells and many such viral expression systems are available in the market in various brand names, an example being Bac-to-Bac baculovirus expression system (Invitrogen). In all such expression systems, the gene of interest is inserted in place of the AcMNPV polyhedrin gene, which is nonessential for viral replication in cell culture (O’Reilly and Miller, 1988). Even though the most studied baculovirus prototype, AcMNPV replicate only in insect cells and fails to replicate in vertebrate cells and it does express the foreign gene in a wide verity of eukaryotic cells including human cells (Condreay et al., 1999). Its specialty is the presence of specific promoters (or hybrid promoters) that drive transcription in such category of variety of cell types (Fig. 2). In addition, Lewin et al. (2005) confirmed that baculovirus promoters carry structural features required by the bacterial RNA polymerase to initiate transcription and the gene expression in *Escherichia coli*. 

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Fig. 2. Schematic diagram of baculovirus-mediated gene delivery in mammalian cells (Kost and Condreay, 2002)

The entry mechanism of baculovirus into eukaryotic cells and the cell surface receptors for its docking have not yet been accurately understood. However, many reports published in this realm suggested that the cellular entry was dependent on electrostatic interaction (Duisit et al., 1999), pH trigger (Dong et al., 2010; Paul and Prakash, 2010), cell surface phospholipids (Tani et al., 2001) and heparin sulfate (Duisit et al., 1999), and the process of trans-membrane transport via adsorptive endocytosis (Volkman and goldsmith, 1985), clathrin-mediated endocytosis and macropinocytosis (Matilainen et al., 2005), and phagocytosis (Laakkonen et al., 2009; Abe et al., 2005). Inside the cell, the intracellular transport is initiated by acid-triggered gp64 fusion (baculovirus envelope protein) and endosomal escape (Kukkonen et al., 2003), followed by
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actin (Salminen et al., 2005) and vimentin (Mahonen et al., 2010b) mediated nuclear transport (Chen et al., 2011). While the nucleocapsids enter into nucleus, the viral DNA is released, and the virus undergoes repeated rounds of transcription and replication, followed by the formation of virions embedded within the proteinaceous structures called occlusion bodies (Ghosh et al., 2002) (Fig 3).

Fig. 3. Baculovirus entry mechanism in eukaryotic cells (Airenne et al., 2009).

Baculoviruses have been studied since 1920s as biopesticides (Black et al., 1997). After a long history, in 1985, first successful in vitro gene transfer by a recombinant baculovirus was accomplished by Carbonell et al. (1985) that made baculovirus a tool in gene transfer technology, especially over expression of cloned genes (O’Reilly et al., 1992). Since then, the recombinant baculoviruses have been successfully used for gene transfer in various eukaryotic animals/cells such as in fishes (Smith et al., 1989; Leisy et al., 2003; Wagle and Jesuthasan, 2003; Wagle et al., 2004), in chicken and duck (Ping et al., 2006; Song et al., 2006), in fruit fly (Oppenheimer et al., 1999; Lee et al., 2000), in honey bees (Ando et al., 2007), in rabbit (Airenne et al., 2000), in monkey (Tani et al., 2001) and in human...
(Hofmann et al., 1995; Kost and Condreay, 2002). Besides, recombinant baculovirus have been used for gene therapy (Luo et al., 2011; Zhao et al., 2012), and for vaccine production (van Oers, 2006; Treanor et al., 2007; Hu et al., 2008; Cox, 2012). More recently, Gamble and Barton (2011) expressed human telomerase reverse transcriptase in primary fibroblasts and extended its replicative lifespan in vivo using recombinant baculovirus successfully. Moreover, the recombinant baculovirus with WSSV Ie1 promoter has been successfully expressed in shrimp cells in vivo (Syed Musthaq et al., 2009; Syed Musthaq and Kwang, 2011) and in vitro (Lu et al., 2005).

As the primary shrimp cells were very sensitive to standard gene delivery systems especially liposome-based transfection and electroporation, for transgenic expression, we employed a transduction method mediated by recombinant baculovirus with shrimp viral promoters for the successful transduction of immortalizing gene(s) in shrimp cells in vitro. Accordingly, recombinant baculovirus AcMNPV constructs have been produced that carry expression cassettes consisting of gene coding GFP as a reporter linked either to WSSV Ie1 or IHHNV P2 promoter for crustacean specific transduction vectors. These versatile transduction systems were designed with a vision to develop cell lines from shrimp by immortalizing gene delivery, and can be applied for developing genetic vaccines to crustaceans against viruses and for developing specific pathogen resistant or multiple pathogen resistant animals.

5.2. Materials and methods

5.2.1. Plasmid vectors used for the experiment, extraction and its purification

P2 complete Fluc pGL3 basic vector containing IHHNV-P2 promoter (Dhar et al., 2007) was kindly received from Dr. Arun K. Dhar, Viracine Therapeutics Corporation, USA. pEGFP-N1 (Clontech) vector containing gene encoding green
fluorescent protein (GFP) was collected from National Centre for Cell Science (NCCS), Pune, India. pFastBac™ 1 containing polyhedrin (PH) promoter and DH10Bac™ (Invitrogen) containing bacmid and helper plasmids were kindly received from Dr. R.B Narayanan, Anna University, Chennai. All plasmids were transformed into respective E. coli host and maintained in glycerol stock at -80 ºC (New Brunswick Scientific, England)

5.2.1.1. P2 complete Fluc pGL3 basic vector

P2 complete Fluc pGL3 basic vector (Dhar et al., 2007), is a modified pGL3-Basic vector (Promega, USA) containing a modified luc coding region of the firefly (Photinus pyralis) luciferase that has been optimized for evaluating transcriptional activity in transfected eukaryotic cells. The P2 promoter region of the infectious hypodermal and hematopoietic necrosis virus (IHHNV) was inserted between Sac I and Xma I sites of pGL3-basic upstream of the luciferase coding sequence (Fig. 4).
5.2.1. 2. pFastBac™ 1 transfer vector

pFastBac™ 1 (Invitrogen) transfer vector into which the gene (s) of interest to be cloned to express in baculoviral expression system is under the control of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin (PH) promoter for high-level expression in insect cells. This expression cassette is flanked by the left and right arms of Tn7 transposon, and also contains gentamicin resistance gene and SV40 polyadenylation signal to form a mini Tn7 for the site-specific transposition properties of the Tn7 transposon (Fig. 5).

![Fig.5. Vector map of pFastBac™ 1 transfer vector](image)

5.2.1. 3. pEGFP-N1 vector

pEGFP-N1 (Clontech) encodes a red-shifted variant of wild-type green fluorescent protein (GFP) under the control of human cytomegalovirus (CMV) immediate early gene promoter for evaluating transcriptional activity in post-transfected eukaryotic cells.
The vector provides dominant selectable marker for resistance to neomycin (G-418) in mammalian or other eukaryotic cells and to kanamycin in *E. coli*. Fusions to the N terminus of EGFP retain the fluorescent properties of the native protein allowing the localization of the fusion protein *in vivo* (Fig. 6).

5.2.1.4. Propagation of *E. coli* containing the plasmid vectors

*E. coli* JM109 with P2 complete Fluc pGL3 basic vector, *E. coli* DH5α with pFastBac™ 1 were propagated in LB ampicillin (100 µg ml⁻¹) and *E. coli* DH5α with pEGFP-N1, in LB kanamycin (30 µg ml⁻¹), all cultures were incubated at 37 °C with shaking at 220 rpm.

5.2.1.5. Plasmid extraction

Plasmid extraction was done using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences) and by following manufacturer’s instruction. Briefly, an aliquot of 2 ml culture after overnight incubation was pelletised at 12,000 x g for 1 min. The pellet was resuspended in 200 µl resuspension solution containing RNase A and lysed by adding 200 µl lysis buffer. An aliquot of 350 µl neutralization solution was added and
centrifuged at 12,000 x g for 10 min to remove the cell debris. Lysate was loaded into GenElute HP Miniprep binding column inserted into a microcentrifuge tube and centrifuged at 12,000 x g for 1 min. Plasmid DNA bound to the column was washed twice with wash solution to remove the endotoxins, salt and other contaminants. To elute the plasmid DNA, the column was transferred to a fresh collection tube, added 100 µl 10 mM Tris-Cl and centrifuged at 12,000 x g for 1 min and stored at -20 ºC. Purity of the plasmid DNA obtained was analysed by agarose gel electrophoresis and by determining the ratio of the absorbance at 260/280 nm in a UV-VIS spectrophotometer (U2800, Hitachi, Japan) and fluorometrically using Qubit® fluorometer (invitrogen™, USA).

5.2.2. DH10Bac™ *E. coli* with baculovirus shuttle vector (Bacmid) and helper plasmid, pMON7124

The baculovirus shuttle vector (bacmid), bMON14272 (136-kb), present in DH10Bac™ *E. coli* contains a low-copy number mini-F replicon, kanamycin resistance marker and a segment of DNA encoding the LacZ peptide from a pUC-based cloning vector into which the attachment site for the bacterial transposon, Tn7 (mini-att-Tn7) has been inserted. The bacmid propagates in *E. coli* DH10Bac™ as a large plasmid that confers resistance to kanamycin and can complement a lacZ deletion present on the chromosome to form colonies that are blue (Lac+) in the presence of a chromogenic substrate X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) and the inducer, IPTG (Isopropyl-beta-D-thiogalactopyranoside). Recombinant bacmids (composite bacmids) are generated by transposing a mini- Tn7 element from a pFastBac™ donor (transfer) plasmid to the mini-att-Tn7 attachment site on the bacmid. DH10Bac™ *E. coli* also contain the helper plasmid, pMON7124 (13.2-kb), which encodes the transposase and confers resistance to tetracycline.
5.2.2.1. Preparation of DH10Bac™ E. coli competent cells

DH10Bac™ E. coli was recovered from the glycerol stock at -80 °C, an aliquot of 10 µl was inoculated into 100 ml LB broth containing kanamycin (50 µg ml⁻¹) and tetracycline (10 µg ml⁻¹), incubated at 37 °C for overnight with shaking (150rpm). An aliquot of 5 ml from overnight grown DH10Bac™ E. coli was further inoculated into 50 ml LB broth containing kanamycin (50 µg ml⁻¹) and tetracycline (10 µg ml⁻¹) and incubated at 37 °C for 2 h at 150 rpm. Transferred the cells to another 50 ml centrifuge tube, centrifuged at 6000 rpm for 20 min at 4 °C. The medium was removed from the pellet, drained off the traces of medium by keeping the tubes in an inverted position on a pad of tissue paper. The pellet was re-suspended by gentle vortexing with 50 ml ice-cold 0.1 M CaCl₂ solution, centrifuged at 6000 rpm for 20 min at 4 °C. The medium was removed from the pellet, drained off the traces of medium by keeping the tubes in an inverted position on a pad of tissue paper. The pellet was re-suspended in 50 ml ice-cold 0.1 M CaCl₂ solution by gentle vortexing. An aliquot of 80 µl above competent cells were mixed with 20 µl 60% glycerol in a 0.5 ml micro centrifuge tube (MCT) and stored at -80 °C. The above competent DH10Bac™ E. coli host was used for constructing recombinant baculovirus shuttle vector (Bacmid).

5.2.3. Crustacean specific putative promoter from WSSV and IHHNV

The WSSV-Ie1 (hereafter Ie1) basic promoter (Liu et al., 2005; Lu et al., 2005) region from -1 to -512 was PCR amplified from P. monodon challenged with WSSV through intramuscular injection (Sudheer et al., 2011). The putative P2 promoter of IHHNV (hereafter P2) was PCR amplified from the P2 complete Fluc pGL3 basic vector (Dhar et al., 2007) containing P2 promoter.

5.2.3.1. Genomic DNA extraction from WSSV infected animal for Ie1 promoter

Genomic DNA was extracted from infected animals using DNAzol® (Chomczynski et al., 1997; Dhar et al., 2001) and by following the method of...
manufacturer (Molecular Research Center, Inc., Cincinnati, Ohio). Briefly, 50 mg gill tissue was collected from dead animal and macerated in 1000 µl DNAzol®, centrifuged at 10,000xg for 10 min. Supernatant was collected into a fresh micro centrifuge tube containing 500 µl ethanol (100%). Gently inverted the sample tube several times to mix and incubated at RT for 3 min, centrifuged at 4000xg at RT to palletize the DNA and supernatant was removed carefully. An aliquot of 1000 µl 70% ethanol was added to the DNA pellet and centrifuged at 4,000xg for 5 min at RT. The ethanol was tipped off and added fresh 1000 µl 70% ethanol and centrifuged at 4,000g for 5 min at RT, supernatant was removed and the DNA pellet was allowed to dry for 15 sec. The DNA was dissolved in 200 µl MilliQ and stored at -20 °C till use.

5.2.3.2. PCR amplification of Ie1 and P2 promoters

The Ie1 basic promoter region from -1 to -512 was PCR amplified using the primer set (enzyme site BamH I is underlined) of F-5’- GGA TCC TCC CTA CGT ATC AAT TTT ATG TGG CTA ATG GAG A-3’ and R- 5’- GGA TCC ACG CGT CGA CCT TGA GTG GAG AGA GAG CTA GTT ATA A-3’ (Lu et al., 2005). P2 complete pGL3 vector (Dhar et al., 2007) was used for the PCR amplification of putative P2 promoter region using the primer set NP602F-5’GGA TCC CTG CGA GCG CTT CGC AG-3’ and NP602R- 5’GGA TCC TAG CAC TTG GAA TAG CCT CTT-3’ (enzyme site BamH I is underlined). The 25µl PCR reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1 µl Taq polymerase (0.5 U µl⁻¹), 1 µl genomic DNA for WSSV-Ie1 promoter and 0.2 µl plasmid DNA (P2 complete PGL3) for IHHNV-P2 promoter (~75 ng), 1 µl of each primer (10 pmol µl⁻¹) and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification was 95 °C for 5 min followed by hold at 80 °C, 32 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% agarose gel electrophoresis, stained with
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5.2.3.3. Cloning with pGEM-T Easy vector

Amplified PCR products of Ie1 and P2 promoters were ligated with pGEM-T Easy vector (Promega, USA) by following the manufacture’s instruction. Briefly, 10 µl ligation mixture containing 0.5 µl pGEM-T Easy vector, 3.5 µl PCR Product, 5 µl ligation buffer, 0.5 µl ligase enzyme and MilliQ was incubated at 4 °C, overnight. This allowed for the ligation of PCR products on pGEMT-Easy vector.

5.2.3.4. Transformation into E. coli DH5α

Thawed the competent cells (E. coli DH5α) by placing on ice for 5-10 min, added 10 µl of each ligation reaction to a sterile 15 ml culture tube already on ice, transferred 50-100 µl of competent cells into the 15 ml tubes (containing ligation mix) on ice, gently flicked the tubes to mix and placed them on ice for 20 min, heat shocked the cells for 90 sec in a water bath at exactly 42 °C, immediately returned the tubes to ice for 2 min, Added 600 µl super optimal broth with catabolite repression (SOC; Composition for 10 ml: 0.2 g tryptone ; 0.05 g yeast extract; 0.005 g NaCl, 100 µl 1M KCl; 50 µl 2 M MgCl2; 200 µl 1 M glucose. MgCl2 and glucose were added just before transformation) to the tubes containing cells transformed with ligation mixture, incubated for 2 h at 37 °C with shaking at 220-230 rpm, plated 100 µl of each transformation culture onto duplicate/triplicate on to LB/ampicillin/IPTG/X-gal plates and incubated the plates overnight (16-18 h) at 37 °C.

5.2.3.5. PCR confirmation of gene insert in the selected clones

The white colonies were selected and patched on ampicillin/IPTG/X-gal plates to reconfirm the transformation. All individually streaked colonies were subjected for colony PCR using vector primers designed from either side of the multiple cloning site of the vector so that whatever be the product formed, primer
could amplify it from either side. The 25 µl reaction PCR reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1µl Taq polymerase (0.5 U µl⁻¹), pinch of colony, 1 µl of T7 and SP6 primers each, and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification of complete genes was 95 °C for 5 min followed by holding at 80 °C, 35 cycles of denaturation at 94 °C for 15 sec, annealing at 57 °C for 20 sec, extension at 72 °C for 1 min, followed by final extension at 72 °C for 10 min. Ten µl of PCR products was analyzed by 1% Agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™ XR+ imaging system, Bio-Rad, USA).

5.2.3.6. Propagation of confirmed colony and plasmid extraction

After confirmation, the transformed *E. coli* DH5α containing cloned vectors were propagated in 10 ml LB ampicillin (100 µg µl⁻¹) medium at 37 °C at 230 rpm. The methodology followed for plasmid exaction was explained elsewhere in this chapter.

5.2.3.7. Restriction digestion of cloned pGEM-T vector with *Bam*H I to release Ie1 and P2 promoters and its purification

The purified pGEM-T plasmid vectors containing Ie1 (pGEMT-Ie1) and P2 (pGEMT-P2) promoter were restriction digested with *Bam*H I enzyme (New England Biolabs, UK) to release the corresponding promoter sequences (Ie1 and P2). An aliquot of 20 µl PCR reaction mixture containing 5 µl plasmid, 0.5 µl enzyme (*Bam*H I -10,000 U ml⁻¹) 2 µl reaction buffer and 12.5 µl MilliQ water was incubated for 1 h at 37 °C. The restriction digestion was confirmed by 1% Agarose gel electrophoresis.

The restricted, released Ie1 (WSSV) and P2 (IHHNV) promoters were gel purified using GenElute™ Gel Extraction kit (Sigma, USA) by following manufacturer’s instruction. Briefly, the agarose gel that contain DNA fragment of appropriate size was excised using X-tracta gel extraction tool (Sigma, USA). The
excised gel slice was taken in a 1.5 ml tube, weighed and added 3 gel volumes (~450 µl) of gel solubilization solution and incubated at 60 °C for 10 min with repeated vortexing in every 2 min. After incubation, added 1 gel volume (~150 µl) of 100% isopropanol, mixed gently until it become homogenous. This solubilized gel solution was loaded into the binding column that was pre treated with column preparation solution, centrifuged at 12,000 x g for 1 min. Added 700 µl wash solution and centrifuged for 1 min at 12,000 x g, repeated the centrifugation and residual wash solution was removed. The binding column was transferred to a fresh collection tube (2 ml MCT) and added 50 µl of preheated (at 65 °C) 10 mM Tris-HCl (pH 9.0), centrifuged at 12,000 x g for 1 min, stored at -20 ºC. The concentration of DNA was measured spectrometrically at 260/280 nm in a UV-VIS spectrophotometre (U2800, Hitachi, Japan) and fluorometrically using Qubit® fluorometer (invitrogen™, USA).

5.2.4. Construction of the versatile recombinant baculoviral vector systems with hybrid promoters

5.2.4.1. Restriction digestion, CIP treatment and purification of pFASTBac™ 1 transfer vector

The pFASTBac™ 1 transfer vector was restriction digested with BamH I enzyme and the methodology followed was same as explained in the previous section (5.2.3.7.). Restriction digested plasmid was CIP (Calf Intestinal Phosphatase) treated to remove the phosphate groups to prevent self ligation. The reaction mixture containing 20 µl plasmid, 0.1 µl CIP enzyme and 5 µl buffer was incubated at 37 °C for 1 h followed by heat inactivation at 65 °C for 20 min. Restriction digested, CIP treated vector was gel purified using GenElute™ Gel Extraction kit (Sigma, USA) and the methodology followed was well explained elsewhere in this chapter.
5.2.4.2. Insertion of crustacean specific viral promoters (Ie1 and P2) into pFASTBac™ 1 vector

The purified Ie1 and P2 promoters were ligated with the restriction digested, CIP treated pFASTBac™ 1 plasmid vector at BamHI restriction region in the multiple cloning site (MCS) downstream to PH promoter to construct two hybrid transfer vector systems such as pBacIe1 and pBacP2 respectively. The 10 µl ligation mixture containing 2 µl CIP treated pFASTBac™ 1 vector, 3 µl gel purified Ie1 or P2 promoter, 1 µl 10X buffer, 0.5 µl T4DNA ligase enzyme and 3.5 µl MilliQ water was incubated at 16 °C, overnight.

5.2.4.3. Transformation of vectors with hybrid viral promoters into E. coli DH5α and its propagation; extraction and purification of the vector systems

To the 10 µl ligation mixture in a 5 ml ice cold screw cap tube, added 50 µl competent E. coli DH5α cells, mixed gently and incubated on ice for 20 min. Heat shock was given for 90 sec at exactly 42 °C. The tubes were returned to ice for 2 min. Added 600 µl super optimal broth with catabolite repression (SOC; Composition for 10 ml: 0.2 g tryptone ; 0.05 g yeast extract; 0.005 g NaCl; 100 µl 1M KCl; 50 µl 2 M MgCl2; 200 µl 1 M glucose. MgCl2 and glucose were added just before transformation) and incubated for 2 h at 37 °C with shaking at 230 rpm. After incubation, 200 µl each was plated onto LB ampicillin (100 µg ml⁻¹) plate containing IPTG and X-gal (LB medium-2 g 100 ml⁻¹; Agar- 2g 100 ml⁻¹) and incubated for 24 h at 37 °C. After PCR confirmation, individual colonies developed (white) were inoculated into 100 ml LB ampicillin broth and incubated at 37 °C with shaking for plasmid extraction. Plasmid extraction was done using GenElute HP Plasmid Miniprep kit (Sigma Life Sciences, USA) and the methodology followed was explained in the previous section.
5.2.5. Insertion of green fluorescent protein (GFP) into the vectors for analysing transcriptional activity of hybrid viral promoter system

Transcriptional activity of hybrid viral promoters in the versatile vectors was analyzed by expressing the green fluorescent protein (GFP) reporter gene. The GFP was inserted downstream to hybrid promoter (PH-Ie1 or PH-P2) in the transfer vector system pBacIe1 and pBaP2 respectively.

5.2.5.1. Gene encoding green fluorescent protein (GFP) and its purification

The gene encoding green fluorescent protein (GFP) was restriction digested from pEGFP-N1 vector with Sal I and Not I enzymes (New England Biolabs, UK) following double digestion. An aliquot of 50 µl reaction mixture containing 5 µl plasmid, 2 µl Sal I enzyme (20,000 U ml⁻¹), 2 µl Not I enzyme (2,500 U ml⁻¹), 4 µl reaction buffer, 0.4 µl bovine serum albumin (BSA) and 36.6 µl MilliQ water, incubated at 37 ºC for 2 h followed by heat inactivation at 65 ºC for 20 min. The restriction digested vector was subjected for gel purification to extract GFP using GenElute™ Gel Extraction kit (Sigma, USA) and the methodology followed was well explained in the previous section.

5.2.5.2. Insertion of green fluorescent protein (GFP) downstream to hybrid viral promoter and its purification

The vectors pBacIe1 and pBacP2 were restriction digested with Sal I and Not I enzymes (New England Biolabs) and purified using GenElute™ Gel Extraction kit. The restriction digested, gel purified GFP gene was inserted to the corresponding restriction sites (Sal I and Not I) (Fig. 7). Ligated products were transfected into E. coli DH5α and the confirmed colonies were propagated for plasmid extraction. The methodology followed was well explained in the previous sections (5.2.3.7 and 5.2.5.1.).
Fig. 7. Construction of transfer vector containing hybrid promoter and GFP. A: GFP cassettes inserted between SalI and NotI site in the transfer vector to construct wild-type baculovirus (control) expressing GFP; B: constructed transfer vector (pBacIe1-GFP) for generating recombinant baculovirus with PH-Ie1 hybrid promoter and GFP reporter; and C: transfer vector (pBacP2-GFP) for generating recombinant baculovirus with PH-P2 hybrid promoter and GFP reporter gene.
5.2.6. Generation of recombinant virus containing hybrid viral promoters and GFP

Bac-to-Bac™ baculovirus expression system (Invitrogen) based on site-specific transposition with Tn7 was used for generating the recombinant baculovirus. Here, the PH-Ie1-GFP and PH-P2-GFP cassettes in the pFastBac™ I transfer vector along with mini-Tn7 transposome element could transpose to the mini-att-Tn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid present in DH10Bac™ E. coli. Colonies containing recombinant bacmids were identified by antibiotic selection and blue/white screening. Since the transposition resulted in disruption of the lacZα gene.
DH10Bac™ transformants produce white colonies. High molecular weight mini-prep DNA was prepared from selected *E. coli* clones containing the recombinant bacmid, and this DNA was then used to transfect insect cells to generate recombinant viral particles (Fig. 8)

5.2.6.1. Transformation of pBaclGFP and pBacP2-GFP transfer vectors containing hybrid promoter system into DH10Bac™ *E. coli*.

To the 10 µl ligation mixture containing pBacl-GFP and pBacP2-GFP transfer vectors in a 5 ml ice cold screw cap tube, added 50 µl competent DH10Bac™ *E. coli* cells, mixed gently and incubated on ice for 20 min. Heat shock was given for 90 sec at exactly 42 °C. The tube was returned to ice for 2 min. Added 600 µl super optimal broth with catabolite repression (SOC; composition for 10 ml: Tryptone-0.2 g; yeast extract-0.05 g; NaCl-0.005 g; 1 M KCl- 100 µl; 2 M MgCl₂-50µl; 1 M glucose-200 µl. MgCl₂ and glucose were added just before transformation) and incubated for 2 h at 37 °C with shaking at 230 rpm. After incubation, 200 µl each was plated onto LB plate containing (LB medium-2 g 100 ml⁻¹; Agar- 2 g 100 ml⁻¹) kanamycin (50 µg ml⁻¹), gentamicin (7 µg ml⁻¹), tetracycline (10 µg ml⁻¹), IPTG (40 µg ml⁻¹), and X-gal (100 µg ml⁻¹), and incubated for 24 to 48 h at 37 °C to select for DH10Bac™ transformants.

5.2.6.2. Propagation of recombinant bacmid DNA in DH10Bac *E. coli*

The individual colonies developed (white) were propagated into 100 ml LB broth containing kanamycin (50 µg ml⁻¹), gentamicin (7 µg ml⁻¹), tetracycline (10 µg ml⁻¹), and incubated at 37 °C with shaking (225 rpm) for plasmid extraction.

5.2.6.3. Isolation of recombinant bacmid DNA from DH10Bac *E. coli*

The recombinant bacmid DNA containing crustacean specific promoter and GFP reporter was isolated and purified using PureLink, HiPure Plasmid Miniprep kit (Invitrogen, Germany) by following manufacturer’s instruction. Briefly,
centrifuged an aliquot of 8 ml overnight culture of DH10Bac containing recombinant bacmid at 15,000xg at RT for 15 min, removed the entire medium. Resuspended the pellet in 0.4 ml of cell suspension buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.2 mg ml⁻¹ RNase A. Added 0.4 ml of cell lysis solution containing 200 mM NaOH and 1% SDS, mixed gently by inverting the capped tube five times and incubated at RT for 5 min. After incubation, added 0.4 ml of neutralization buffer containing 3.1 M potassium acetate (pH 5.5) and mixed immediately by inverting the tube five times. Centrifuged at 15,000xg at RT for 10 min and transferred the supernatant onto the equilibrated column and allowed the solution in the column to drain by gravity flow. The column equilibration was performed by adding 2 ml equilibration buffer containing 600 mM NaCl, 100 mM sodium acetate (pH 5.0) and 0.15% Triton X-100, and the buffer was drained off by gravity. After complete removal of supernatant from the equilibrated column, washed the column two times with 2.5 ml wash buffer containing 800 mM NaCl, 100 mM Sodium acetate (pH 5.0) and allowed the solution in the column to drain off. The recombinant bacmid DNA attached on the column membrane was eluted by adding 0.9 ml of elution buffer containing 1.25 M NaCl, 100 mM Tris-HCl (pH 8.5) and allowed the solution in the column to drain to a 2 ml sterile micro centrifuge tube (MCT). The recombinant bacmid DNA was precipitated by adding 0.63 ml isopropanol, mixed and placed on ice for 10 min, centrifuged at 15,000xg at RT for 30 min. Carefully discarded the supernatant and washed the bacmid DNA pellet with 1 ml of cold 70% ethanol and centrifuged at 15,000xg at RT for 5 min. Carefully drained off the ethanol and air dried the pellet for 10 min at RT, dissolved in 50 µl of TE buffer containing 10 mM Tris-HCl (pH 8.0) and 0.1 mM EDTA. The concentration of recombinant bacmid DNA was calculated fluorometrically using Qubit Fluorometer (Invitrogen) and stored at -20 °C.
5.2.6.4. PCR confirmation of insert orientation in bacmid DNA

The orientation of bacmid DNA-carrying PH-lr1-GFP and PH-P2-GFP fragments (cassettes) were confirmed by PCR amplification using the M13F (5' CCC AGT CAC GAC GTT GTA AA ACG 3') bacmid primer and GFP specific primer (NP266R-5' CAC GAA CTC CAG CAG GAC CAT G 3'). The 25 µl PCR reaction mixture containing 2.5 µl 10x buffer, 2.5 µl dNTP (2.5 mM), 1 µl Taq polymerase (0.5 U µl⁻¹), 0.2 µl bacmid, 1 µl of each primer (10 pmol µl⁻¹) and the mixture was made up to 25 µl with MilliQ. The hot start PCR programme used for the amplification was 95 °C for 5 min followed by hold at 80 °C, 32 cycles of denaturation at 94 °C for 30 sec, annealing at 58 °C for 30 sec, extension at 72 °C for 2 min, followed by final extension at 72 °C for 10 min. An aliquot of 10 µl of PCR products was analyzed by 1% Agarose gel electrophoresis, stained with ethidium bromide, visualized and documented using gel documentation system (Gel Doc™XR+ imaging system, Bio-Rad, USA).

5.2.6.5. Transfection of recombinant bacmid shuttle vector into Sf9 cells to generate recombinant baculovirus

The Sf9 cells at a cell density 1.5 x 10⁶ cells ml⁻¹ grown in TNM-FH medium (Sigma, USA) without antibiotics and serum in 35 mm culture dish was selected for transfection.

To generate the recombinant virus, Sf9 cells were transfected with the confirmed bacmid DNA using Cellfectin® II reagent (Invitrogen) by following manufacturer’s instruction. Briefly, an aliquot of 1 µl (500 ng ml⁻¹) recombinant bacmid DNA was diluted with 100 µl antibiotic and serum free TNM-FH medium and mixed with Cellfectin® II which was previously diluted by adding 8 µl into 100 µl TNM-FH medium (antibiotics and serum free). The lipid-bacmid mixture (transfection mixture) was mixed gently and incubated at RT for 45 min, added drop wise onto the cells and incubated at 28 °C for 6 h. After incubation, the
transfection mixture was replaced with TNM-FH medium containing 15% fetal bovine serum (FBS) and antibiotics. The cells were further incubated at 28 °C until the sign of viral infection (occlusion bodies) and fluorescent signals from green fluorescent protein could be visualized.

5.2.6.6. Isolation, amplification and storage of recombinant baculovirus containing hybrid viral promoters

Recombinant baculovirus released in the TNM-FH medium was collected from each culture dish and re-infected on to another sets of Sf9 cells (1.5 x 10^6 cells ml^-1) to reamplify the viral stock. After reamplification, the medium containing concentrated virus was collected into sterile centrifuge tube and centrifuged at 500xg for 5 min to remove the cell debris. The clear supernatant was transferred to fresh cryovials, covered with aluminum foil to protect from light and stored at -80 °C until transduction experiment was carried out in lymphoid cell culture from *P. monodon*.

5.2.7. Analysis of hybrid viral promoters mediated transcriptional activity in Sf9 cells

Transcriptional activity of the recombinant baculovirus constructs that carry expression cassettes consisting of gene encoding GFP as a reporter linked either to PH-Ie1 or PH-P2 hybrid viral promoter was carried out in Sf9 cells. The hybrid promoter activity of PH-Ie1 or PH-P2 cassettes in the recombinant baculovirus BacIe1-GFP and BacP2-GFP respectively were determined by fluorescence microscopic examination of GFP (reporter gene) signals from the transduced Sf9 cells and by the analysis of the SDS-PAGE separated green fluorescent protein. Activity was compared with that of single promoter (PH) linked GFP cassette (PH-GFP), in recombinant baculoviral vector Bac-GFP as control. Human cell lines such as HeLa and HEp2 were selected to check whether the virus caused any infection in human.
5.2.7.1. Analysis of GFP signals from transduced Sf9 cells

Promoter activity in Sf9 cells transduced with recombinant baculovirus (Bacle1-GFP or BacP2-GFP) consisting of either PH-le1-GFP or PH-P2-GFP hybrid viral promoter cassettes with GFP were carried out. Baculovirus with PH-GFP cassettes, in the Bac-GFP was used as the control. Expression of green fluorescent protein under the control of either of these promoter cassettes was evaluated by microscopic examination using Fluorescence-Inverted phase contrast microscope (Leica DMIL, Germany) with GFP filter, and controlled by image acquisition software (LAS, Leica). Transduced cells were observed in every 3 h for 24 h, subsequently in every 24 h for 3 days for the phenotypic changes and the GFP signals. Once the virus was amplified in Sf9 cells, GFP expression was observed, demonstrating the hybrid promoter activity in these cells.

5.2.7.2. Analysis of hybrid promoter mediated protein expression in transduced Sf9 cells

Transduced Sf9 cell after 72 h was harvested from 35 mm culture dish and protein was extracted using 400 µl of 1x SDS-PAGE lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS) and boiled for 5 min. The extracted protein was subjected to reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) following the method of Laemmli (1970). Briefly, protein extract was (10 µl) mixed with 10 µl of gel loading dye, boiled for 5 min. The protein was separated and analyzed using 4% stacking gel and 15% resolving gel prepared into 10 x 10.5 cm vertical gel plate of miniVE™ mini vertical electrophoresis unit (Hoefer-Amersham, India). Electrophoresis was performed in 1x Tris-glycine SDS (pH 8.3) buffer (tank buffer) at a voltage of 12 mA (EPS 301, Amersham, India). After electrophoretic separation, gel was stained in coomassie brilliant blue stain R-250 (0.025% coomassie brilliant blue R-250, 40% methanol and 7% acetic acid in distilled water), de-stained in de-staining solution I (40% methanol and 7% acetic acid in distilled water) and de-staining solution II (5% methanol and 7%
acetic acid in distilled water), and photographed using Gel Doc™ XR+ imaging system. Protein expression was determined by comparing with the protein profile of un-transduced Sf9 cells (control). Molecular weight of protein band was determined by comparing with that of standards (Genei, India).

5.2.8. Transduction of shrimp cells *in vitro* and *in vivo* with recombinant baculovirus encoding GFP under the control of hybrid viral promoters

Recombinant baculovirus constructs that carry expression cassettes specific to shrimp cells was transduced into primary cell cultures from *Penaeus monodon* and into the whole animal. These experiments were conducted to establish the understanding that the hybrid promoter mediated transcription could be carried out in the cells *in vitro* and *in vivo* (in shrimp).

5.2.8.1. Transduction of shrimp cells *in vitro*

Recombinant baculovirus constructs that carry expression cassettes consisting of gene encoding GFP as a reporter linked to the hybrid promoter either to PH-Ie1 or PH-P2 was transduced into primary cell cultures from *P. monodon*. Cell culture for transduction experiment from various cell types/ tissues of *P. monodon* were prepared in 35 mm culture dish in shrimp cell culture medium (SCCM) by following the methodology explained in the previous chapter (Chapter 1). After 24 h incubation of cell cultures at 25 °C, the medium was replaced with 1 ml SCCM and 1 ml supernatant containing recombinant baculovirus (1:1) and incubated for 6 h at 25 °C, replaced with growth medium (SCCM) containing 15% fetal bovine serum (FBS). Once the virus was amplified in cell culture, GFP expression was observed, demonstrating the hybrid promoter activity in shrimp cells *in vitro*. Expression of green fluorescent protein under the control of either of these promoter cassettes was evaluated by microscopic examination using Fluorescence-Inverted phase contrast microscope (Leica DMIL) with GFP filter,
and controlled by image acquisition software (LAS, Leica). Transduced cell cultures were observed in every 3 h for 24 h, subsequently in every 24 h for 3 days for the phenotypic changes and the GFP signals.

5.2.8.1. Transduction of shrimp cells in vivo

An aliquot of 10 µl viral preparation (~1x10^{-4} pfu) was given intramuscularly to healthy *P. monodon* (Sudheer *et al.*, 2011). The recombinant baculovirus BacIe1-GFP and BacP2-GFP carrying expression cassettes PH-Ie1-GFP and PH-P2-GFP respectively were used for this experiment along with Bac-GFP as control vector (PH promoter only). After injection animals were maintained in the laboratory condition for 45 days in aerated seawater of 15 ‰ salinity and followed strict bio-security norms to prevent the entry of this virus to natural ecosystem. After 45 days, the animals were sacrificed and various tissue/cells of the animals were observed for green fluorescent protein expression using Inverted phase contrast fluorescence microscope (Leica, Germany).

5.3. Results

5.3.1. Construction of the versatile vector systems with hybrid viral promoters

Two recombinant baculoviral transduction vectors, BacIe1-GFP and BacP2-GFP that carried expression cassettes consisting of gene encoding GFP as a reporter linked to the hybrid promoter either to PH-Ie1 or PH-P2 were successfully constructed. The transfer vector pFastBac™1 (Invitrogen) provided the strong polyhedrin (PH) promoter from *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV). Either Ie1 or P2 promoter was inserted at the position 4032 (4032th base in vector map) in continuation with 128-bp sized PH promoter (3904 to 4032) in pFastBac™1 transfer vector to make the hybrid promoter system. The 4032th site of the pFastBac™1 transfer vector was cleaved with BamH I enzyme to insert the shrimp viral promoter le1 or P2 (Fig. 9) with a sequence size 116-bp and 502-bp respectively. le1 and P2 were the crustacean
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specific promoters from white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) respectively. Gene encoding green fluorescent protein (GFP), the reporter gene was inserted at the multiple cloning sites (4037 to 4142) between 4070 and 4090 position by restriction digestion with Sal I and Not I enzymes (Fig. 10), confirming that the GFP was in-frame with hybrid promoter. A total of 20 bases were removed while replacing GFP gene sequence at this position. The results from colony PCR using forward primer of either Ie1 or P2 and the reverse primer of GFP confirmed the alignment of insert in the transfer vector (Fig. 11). The expression cassettes containing hybrid promoter, multiple cloning sites, green fluorescent protein (GFP) were at the position between transposon elements Tn7R and Tn7L allowed the site specific transposition of the expression cassettes along with a gentamicin resistance gene in to the baculoviral genome. Transposon mediated transposition in bacmid was confirmed using M13 (forward) and GFP (reverse) primers, indicating that the inserts were transpositioned in correct orientation (Fig. 12).

5.3.2. Transduction of cell lines in vitro and evaluation of transcriptional activity of hybrid viral promoters in Sf9 cells

The efficiency of newly designed baculovirus-mediated transduction vector in gene transfer was successfully confirmed with Sf9 cells. Baculovirus-derived vector expressing the GFP reporter gene under the control of either PH-Ie1 or PH-P2 hybrid promoters were expressed, suggesting the transcriptional initiation and transduction in Sf9 cells. In the case of both the transduction vectors, expression of GFP was observed in Sf9 cells within 6 h of post transduction. After 6 h of infection with recombinant virus, 10% of the cells expressed green fluorescent protein and this value was increased to 20% within 12 h of post transduction followed by 80% within 24 h and 100% within 32 h (Fig. 13). Moreover, typical baculoviral cytopathic effects (CPE) including the occlusion bodies were observed in 12 h of post infected cells (Fig. 14). Furthermore, the hybrid promoter induced
expression of GFP was confirmed by SDS-PAGE analysis (Fig. 15), resulted in the presence of expressed protein under the control of PH-Ie1 and PH-P2 hybrid promoter cassettes in the cells transduced with recombinant virus BacIe1-GFP and BacP2-GFP respectively. Observed fluorescence intensity from transduced cells suggested that transcriptional activity in the Sf9 cells was more or less similar under the control of hybrid promoters, and high intensity of fluorescent signals was observed at 24 h post transduction. Further, the transduction efficiency and the infectivity of the recombinant virus were found to be 100% within 32 h of post transduction with ~1x10^4 pfu (approximate value, suggested by the manufacturer). Sign of infection and transduction (no GFP signal) were not observed in human cell lines HeLa and HEp2, confirming that the recombinant viral particles would not be infecting human cells (data not shown).

5.3.2. Transduction of shrimp cells in vivo and in vitro, and evaluation of transcriptional activity of hybrid viral promoters

As expected, the recombinant baculovirus BacIe1-GFP and BacP2-GFP containing PH-Ie1 and PH-P2 hybrid promoter cassettes drove the expression of GFP reporter gene in shrimp cells in vitro and in vivo, demonstrated by fluorescent microscopy. However, PH-GFP (Control) expression cassettes under the control of PH promoter in the Bac-GFP virus didn’t express GFP to a detectable level either in in vitro or in vivo experiments. Very late and a feeble transduction efficiency was observed in shrimp primary cells in comparison with Sf9 cells. Transduction efficiency in shrimp cells with both recombinant virus BacIe1-GFP and BacP2-GFP was found to be lower (10-20%) in comparison to the efficiency in Sf9 cells, which was 100%. In vivo experiments with recombinant viral vector proved that the viruses were infective to most of the cell/tissue types tested. The recombinant baculovirus BacIe1-GFP with PH-Ie1 hybrid promoter was infective to gills, nerve ganglion, intestine, muscles, haemocytes and lymphoid organ (Fig. 16 & 17). Whilst, recombinant baculovirus BacP2-GFP with PH-P2 hybrid promoter was
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more infective to hepatopancreas, haemocytes, gills and lymphoid organ (Fig. 18 & 19). In the in vitro experiments, haemocytes, hepatopancreas, lymphoid and heart tissues were used to confirm their susceptibility to the recombinant virus particle. The PH-Ie1 promoter system in BacIe1-GFP virus initiated transcription and expressed GFP in all cell types tested in vitro. Whilst, with PH-P2 promoter system in BacP2-GFP, the virus showed more infectivity to lymphoid cells and found more transduced cells with GFP expression than BacIe1-GFP transduced cells. In both the cases, the expression was observed after 48 h of infection. Control animals were subjected for imaging under fluorescence microscope to avoid misinterpretation from the auto-fluorescence from its exoskeleton and eye stalk (Fig. 20). The shrimp injected with the above recombinant viruses (BacIe1-GFP and BacP2-GFP) survived for 45 days without any mortality (Fig. 21), suggesting the vector system nontoxic to animal which could also be used for immunization (DNA vaccine) against pathogenic bacteria and viruses.

5.4. Discussion

Studies on the development and establishment of shrimp cell lines have been hampered by the lack of effective molecular tools for gene transfer into primary shrimp cell cultures. Because the spontaneous transformation of shrimp cells in vitro and their establishment as permanent cell lines were found impossible to achieve, the induced immortalization was hypothesized to be the only option left to attempt develop shrimp cell lines (Jayesh et al., 2012). Under such a situation, vectors capable of enhanced and long term delivery of immortalizing gene to the primary shrimp cell culture are required to evade the molecular blocks that prevent in vitro transformation. Moreover, primary shrimp cells were found to be very sensitive to standard gene delivery systems especially liposome-based transfection and electroporation. Thus, for the transgenic expression, viral mediated transduction was the better choice amongst all such methods. In this context, this study describes the construction of two recombinant baculovirus vectors with shrimp virus promoters designed to transfer foreign genes in to shrimp cells.
The putative promoters from shrimp viruses (WSSV-Ie1 and IHHNV-P2) such as white spot syndrome virus and infectious hypodermal and hematopoietic necrosis virus have been considered for constructing recombinant baculovirus vectors (BacIe1-GFP and BacP2-GFP). Immediately early (IE) gene Ie1 of WSSV along with ie2, and ie3 were identified in infected shrimps (Liu et al., 2005), wherein Ie1 gene promoter has been considered as an efficient viral promoter to construct expression vectors. Moreover, WSSV Ie1 promoter was found active and control transcription in insect, shrimp, avian and mammalian cells (Prabakaran et al., 2010; Syed Musthaq et al., 2009; He et al., 2008; Gao et al., 2007). Syed Musthaq et al. (2009) constructed a recombinant baculovirus encoding VP28 envelop protein under the control of WSSV Ie1 protein and expressed this vector in shrimp tissue. He et al. (2008) suggested that recombinant baculovirus with WSSV Ie1 promoter was more active than with CMV (cytomegalovirus) promoter for displaying expression of haemagglutination activity of H5N1 virus.

IHHNV P2 promoter was proved to control transcription in insect, fish and crustacean cells (Dhar et al., 2007), that possessed the canonical TATA box (TATATAA). Moreover, Dhar et al. (2007) suggested that, even though the results were highly variable, transient expression of luciferase could be achieved under the control of P2 promoter in the constructed vector P2 complete pGL3. Because, it is located near map unit 2, Shike et al. (2000b) named the promoter as P2. In the present study, the same P2 promoter was used for constructing the recombinant baculovirus; the vector P2 complete pGL3 was kindly given by Dr. Arun K. Dhar, Viracine Therapeutics Corporation, USA.

As the recombinant baculovirus have emerged as a potent tool for protein production (Liu et al., 2010), virus production (Zheng et al., 2010; Lesch et al., 2011), vaccine development (Madhan et al., 2010), cancer therapy (Wang and Balasundaram, 2010), tissue engineering (Lin et al., 2010) and especially used for expression in shrimp (Syed Musthaq et al., 2009). In the present study, baculovirus vectors were used as the backbone to construct recombinant vector with shrimp viral promoters. Additionally, Condrey et al. (1999) described the use of a recombinant baculovirus vector carrying a mammalian expression cassette.
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comprising the cytomegalovirus immediate early (CMV-IE) promoter and the gene for green fluorescent protein (GFP) to direct gene expression in a wide variety of mammalian cell lines as well as primary human cells derived from different tissues. Likewise, studies suggested that recombinant baculovirus vectors carrying GFP reporter gene under the control of WSSV Ie1 promoter were capable of transducing shrimp cells in vivo (Syed Musthaq et al., 2009; Syed Musthaq and Kwang, 2011) and in vitro (Lu et al., 2005).

The hybrid promoter PH-Ie1 and PH-P2 in the recombinant baculovirus vector Bacle1-GFP and BacP2-GFP respectively could control transcriptional initiation in shrimp cells in vitro and in vivo. However, we could transduce only 10-20% shrimp cells with the hybrid promoter system. Further improvisation in transduction has to be carried out in the presence of histone deacetylase inhibitors such as sodium butyrate. Guo et al. (2010) supporting this hypothesis proved that sodium butyrate enhanced the expression of baculovirus-mediated sodium/iodide symporter gene in A549 lung adenocarcinoma cells. Many other earlier reports have also stated that sodium butyrate could significantly enhance baculovirus mediated gene expression in vertebrate cells (Condreay et al., 1999; Airenne et al., 2000). On the contrary, Lu et al. (2005) suggested that the low transduction efficiency might be due to the possible inhibition effect, especially the pH of the medium, on the attachment of baculovirus to the cell membrane. Lu et al. (2005) used L-15 medium for this experiment with a pH above 7.0, however, we used newly designed shrimp cell culture medium (SCCM) with a pH 6.8, a condition well sufficient to amplify the virus.

In conclusion, two recombinant baculoviral transduction vectors (Bacle1-GFP and BacP2-GFP) that carried expression cassettes consisting of gene encoding GFP as a reporter linked to the hybrid promoter either to PH-Ie1 or PH-P2 were successfully constructed and expressed in insect cell line and shrimp cells in vivo and in vitro. Because of its successful expression in shrimp cells without any toxicity, these versatile transduction systems could be used for expression of oncogenes or an immortalizing gene like telomerase reverse transcriptase (TERT) to effect
immortalization of shrimp cells. Moreover, these virus vectors can find application in the development of DNA vaccination and generation of transgenic animals.

Fig. 9. Agarose gel showing — A: Linearized plasmid pFastBac™ 1 digested with BamHI, B: PCR amplified WSSV immediate early gene (Ie1) product of 502-bp size from infected animal, C: WSSV Ie1 promoter (502-bp) released from pGEM-T vector after restriction digestion with BamHI enzyme, D: IHHNV P2 promoter (116-bp) released from PGEM-T vector after restriction digestion with BamHI enzyme.

Fig. 10. Agarose gel showing — A: Green fluorescent protein (GFP) gene restriction digested from pEGFP N1 with SalI and NorI enzyme, B: Colony PCR performed for confirming the alignment of inserted GFP gene in pFastBac™ 1 vector between SalI and NorI enzyme sites.
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Fig. 11. Agarose gel picture from colony PCR for confirming the insert orientation in pFastBac™1 before generating recombinant baculovirus. A: Ie1-GFP alignment confirmation using Ie1 forward primer and GFP reverse primer, B: P2-GFP alignment confirmation using P2 forward primer and GFP reverse primer.

Fig. 12. PCR confirmation of the recombinant bacmid using M13 forward and GFP reverse primers. M: molecular marker of 1-kb size, 1: wild type baculovirus tagged with GFP, 2: recombinant bacmid with P2 promoter, 3: recombinant bacmid with Ie1 promoter.
Fig. 13. Recombinant baculovirus mediated transduction in insect cells (Sf9). A, C, E: phase contrast image of the transduced Sf9 cells with Bac-GFP, BacIe1-GFP and BacP2-GFP respectively; B, D, and F: corresponding image under fluorescence microscope. The green fluorescent signals indicate the active viral transcription inside the cells.
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Fig. 14. Sf9 cells showing cytopathic effect (arrow) after 12h of post infection with recombinant baculovirus. A: infected with Bac-GFP; B: BacIe1-GFP and C: BacP2-GFP virus.

Fig. 15. SDS-PAGE analysis of reporter protein (GFP) synthesis in Sf9 cells infected with recombinant baculovirus (vector): M: molecular marker, C: control cells without infection, 1: protein expression under the control of PH-Ie1 promoter (BacIe1-GFP vector), and 2: protein expression under the control of PH-P2 promoter (BacP2-GFP vector). Arrow indicates green fluorescent protein.
Fig. 16. Transduction of GFP expressing recombinant baculovirus in vivo in susceptible larvae of *P. monodon* 3 days post infection obtained under microscope (10 x magnifications). A, C: bright field image of the animal infected with recombinant baculovirus containing PH-Ie1 hybrid promoter (BacIe1-GFP); B, D: corresponding animals expressing GFP; E: uninfected animal, and F: GFP expression from animal infected with recombinant baculovirus containing PH-P2 hybrid promoter (BacP2-GFP).
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Fig. 17. Expression of GFP from various organ/tissue of *P. monodon* transduced with recombinant baculovirus containing PH-Ie1 hybrid promoter (Bac-Ie1GFP). A, C, E: phase contrast image of heart, muscle and intestine, and B: expression of GFP from heart, D: muscle and F: intestine. Images were taken 3 days post infection under microscope (20 x magnifications).
Fig. 18. Expression of GFP from various organ/tissue of *P. monodon* transduced with recombinant baculovirus containing PH—P2 hybrid promoter (Bac-P2GFP). A, C, E: phase contrast image of heart, lymphoid organ and hepatopancreas, and B: the expression of GFP from heart, D: lymphoid organ and F: hepatopancreas. Images were taken 3 days post infection under microscope (20 x magnifications).
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Fig. 19. Expression of GFP from various gill tissue of *P. monodon* transduced with recombinant baculovirus (BacP2-GFP) containing PH-P2 hybrid promoter. A: phase contrast image of gill tissue; B: expression of GFP from same under fluorescence microscope indicating the viral transcription and successful transduction; C and D: control animal under bright field and fluorescent microscope; low level of auto fluorescence was observed in eyestalk. The images were taken 3 days post infection under microscope (20 x magnifications).
Fig. 20. Transduced _P. monodon_ with wild-type baculovirus containing GFP (Control). A, C, E: phase contrast image of gills, haemocytes and hepatopancreas, and B, D, F: same tissue/cells under fluorescence microscope. Images were taken 3 days post infection under microscope (20 x magnifications).
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Fig. 21. Survival of animals injected with recombinant and wild-type baculovirus