CHARACTERIZATION OF FUNCTIONAL PROMOTERS IN *PENAEUS MONODON* PRIMARY CULTURED CELLS

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CHARACTERIZATION OF FUNCTIONAL PROMOTERS IN *PENEAUS MONODON* PRIMARY CULTURED CELLS

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ABSTRACT

The expression of foreign genes in penaeid shrimp cells has been extensively studied using microinjection, electroporation, and chemical reagents as means of transfection. However, little research has been done on functional promoters which regulate the expression of foreign genes in shrimp cells. Thus, a mammalian viral promoter (CMV) and six putative promoters from shrimp viral pathogens; p1 and p50 promoters from HPV, as well as RR1, RR2, VP15, and VP19 promoters from WSSV, were employed for the functional analysis in this study. The transient transfection in Sf9 cells showed that luciferase expression was detected from CMV, p1, RR1, and RR2 promoters, but not from p50, VP15, and VP19 promoters. Among these promoters, the highest luciferase activity in Sf9 was obtained from RR2 promoter. The conditions for transient transfection in primary shrimp cells (Oka and hepatopancreas cells) were optimized using four different liposome reagents for liposome transfection, as well as using the electroporation method. All essential and crucial parameters were varied according to the manufacturer's recommendations. However, none of the luciferase expression in both transcriptional and translational level was observed from all conditions. The results suggest that the absence of the luciferase expression may be due to 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.

KEY WORDS: PENAEUS MONODON/ PROMOTER/ HEPATOPANCREATIC PARVOVIRUS (HPV)/ WHITE SPOT SYNDROME VIRUS (WSSV)/ SF9

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การศึกษาโปรโมเตอร์ที่สามารถทำงานได้ในเซลล์ปฐมภูมิของกุ้งกุลาดำ
(CHARACTERIZATION OF FUNCTIONAL PROMOTERS IN *PENAEUS MONODON* PRIMARY CULTURED CELLS)

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บทคัดย่อ

ปัจจุบันได้มีการศึกษาถึงการแสดงออกของยีนในกุ้งตระกูล penaeid เป็นจำนวนมาก โดยใช้วิธีฉีดยีนที่ต้องการศึกษาเข้าโดยตรง หรือวิธีที่ใช้กระแสไฟฟ้า รวมทั้งวิธีที่ใช้สารเคมีเพื่อนำยีนที่ศึกษาเข้าสู่เซลล์ ซึ่งการศึกษาถึงการแสดงออกของยีนจำเป็นจะต้องใช้โปรโมเตอร์ที่สามารถทำงานได้ในเซลล์กุ้ง ดังนั้นการทดลองในครั้งนี้ จึงได้นำโปรโมเตอร์จากไวรัสของสัตว์เลี้ยงลูกด้วยนม (CMV) และโปรโมเตอร์จากไวรัสที่ทำให้เกิดโรคในกุ้ง ซึ่งได้แก่ โปรโมเตอร์ p1 และ p50 จากเชื้อไวรัสกุ้งแคระ รวมทั้งโปรโมเตอร์ RR1, RR2, VP15 และ VP19 จากเชื้อไวรัสตัวแดงดวงขาว มาศึกษาการทำงานในเซลล์กุ้ง ข้อมูลจากการทดลองเบื้องต้นในเซลล์แมลง (Sf9) พบว่าสามารถวัดการแสดงออกของยีนรายงานผลได้จากโปรโมเตอร์ CMV, p1, RR1 และ RR2 ซึ่งโปรโมเตอร์ RR2 มีระดับการทำงานสูงสุดเมื่อเปรียบเทียบกับโปรโมเตอร์อื่น สำหรับการศึกษาการทำงานของโปรโมเตอร์เหล่านี้ ในเซลล์ปฐมภูมิจากต่อมน้ำเหลืองและจากตับของกุ้งกุลาดำนั้น ได้มีการทดลองที่สภาวะต่าง ๆ กันโดยวิธีใช้สารเคมีและการใช้กระแสไฟฟ้าเพื่อนำพลาสมิดเข้าสู่เซลล์ปฐมภูมิของกุ้ง โดยพิจารณาถึงปัจจัยต่าง ๆ ที่มีผลต่อประสิทธิภาพในการนำพลาสมิดเข้าสู่เซลล์ อย่างไรก็ตามพบว่าไม่สามารถตรวจสอบการแสดงออกของยีนรายงานผลได้ ทั้งในระดับอาร์เอ็นเอและระดับโปรตีนจากทุก ๆ สภาวะที่ใช้ในการทดลอง ซึ่งอาจจะมีสาเหตุมาจากความล้มเหลวในการนำพลาสมิดเข้าสู่เซลล์ปฐมภูมิของกุ้ง หรือเกิดจากการที่เซลล์กุ้งขาดโปรตีนที่จำเป็นต่อการทำงานของโปรโมเตอร์ หรือเกิดจากทั้งสองปัจจัยร่วมกัน

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
bp	Base pair (s)
BSA	Bovine serum albumin
ºC	Degree Celsius
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalovirus
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	dATP, dCTP, dGTP, and dTTP
et al.	And others
FBS	Fetal bovine serum
g	Gravity or centrifugal force
h	Hour
HPV	Hepatopancreatic parvovirus
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kb	Kilobase pair (s)
kDa	Kilodalton (s)
lacZ	β-galactosidase gene
LB	Luria-Bertani medium
Luc	Luciferase gene
M	Molar
min	Minute (s)
mg	Milligram (s)
ml	Millilitre (s)
mM	Millimolar
nm	Nanometer (s)
nt	Nucleotide (s)

LIST OF ABBREVIATIONS (Continued)

ORF	Open reading frame
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase
RT-PCR	Reverse transcription-polymerase chain reaction
sec	Second (s)
SME	Shrimp meat extract
T_m	Melting temperature
Tris	Tris-(hydroxylmethyl)-aminomethane
µF	Microfarad (s)
µg	Microgram (s)
µl	Microlitre (s)
µM	Micromolar
V	Volt (s)
v/v	volume/volume
WSSV	White spot syndrome virus
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

CHAPTER I
INTRODUCTION

1.1 General background of *Penaeus monodon*

Penaeus monodon, or black tiger shrimp, is an important economic penaeid shrimp in many countries, including Thailand. Thailand is one of the World's leading shrimp producers and frozen shrimp exporters that bring an income roughly 2 billion USD annually (Office of the Prime Minister, 1995). However, the rapid expansion of shrimp aquaculture without proper management has led to the outbreak of shrimp diseases. Thus, viral infection usually causes high mortality of infected shrimps as well as economic problem of shrimp farmers. Several shrimp viral pathogens have been reported (Flegel, 1997). Although some have not yet been identified, most of them have been already characterized such as yellow head virus (YHV) (Tang and Lightner, 1999), white spot syndrome virus (WSSV) (Liu *et al*., 2002; Lo and Kou, 1998), and hepatopancreatic parvovirus (HPV) (Lightner *et al*., 1993; Lightner and Redman, 1985; Sukhumsirichart *et al*., 2005).

1.1.1 White spot syndrome virus (WSSV)

White spot syndrome virus (WSSV) is the causative agent of white spot syndrome in cultivated penaeid shrimps all over the world (Chou *et al*., 1995; Flegel, 1997). The infected shrimps show strong signs of lethargy and a reddish coloration of the body, including discoloration or white spot in the exo-mesoderm under the carapace (Chou *et al*., 1995) followed by shrimp mortality within 2-7 days. This virus is a large double stranded DNA virus (Wang *et al*., 1995) with roughly 300 kb (Yang *et al*., 1997). The electron microscopic studies showed that the virions are enveloped and have a bacilliform shape of ~275 nm in length and 120 nm in width with a tail-like appendage at one end (Wongteerasupaya *et al*., 1995). Although the WSSV genome has been successfully sequenced (van Hulten *et al*., 2001; Yang *et al*., 2001), most open reading frames (ORFs) have no homology to any known proteins or motifs (Yang *et al*., 2001). However, some non-structural proteins and major structural proteins have already been identified. For instance, the known enzymes are ribonucleotide reductase (RR) (Tsai *et al*., 2000), dUTPase, thymidylate synthase, chimeric polypeptide of thymidine kinase and thymidylate kinase (TK-TMK) (Chen and Wang, 1999; Tsai *et al*., 2000), while VP35, VP28, VP26, VP24, VP19, and VP15 structural proteins (Chen *et al*., 2002; Hameed *et al*., 1998; van Hulten *et al*., 2000) have been recognized.

1.1.2 Hepatopancreatic parvovirus (HPV)

Hepatopancreatic parvovirus (HPV) was first reported as viral pathogen in *P. monodon* in 1992 in Thailand. Although shrimps infected with HPV usually show non-specific gross signs of disease, heavy infection can cause poor growth resulting in reduction of shrimp production (Flegel *et al*., 1999). HPV, a minus single-stranded DNA virus, appears icosahedral shape viral particle (Sukhumsirichart, 1998). Recently, complete nucleotide sequence of HPV genome containing ORFs of both structural and non-structural protein-encoding genes has been characterized (Sukhumsirichart *et al*., 2005).

1.2 Primary shrimp cell culture

Although numerous attempts have been undertaken during the past few decades, prawn cell line has not yet been established to date (Toullec, 1999). However, many research groups have paid the attention for development of primary shrimp cell culture proposed from different tissues including ovaries, heart, haemocytes, lymphoid organ, and hepatopancreas (Chen and Wang, 1999; Itami *et al*., 1999; Kasornchandra *et al*., 1999; Uma *et al*., 2002; West *et al*., 1999).

Lymphoid organ of penaeid shrimp locates on the left and the right sides of the antero-dorsal surface of the hepatopancreas (Oka, 1969). The development of Oka cell culture from lymphoid organ has been widely studied (Itami *et al*., 1999; Kasornchandra *et al*., 1999; West *et al*., 1999), and it was successfully developed in Thailand (Assavalapsakul, 2004; Kasornchandra *et al*., 1999). Primary Oka cells exhibit fibroblast-like morphology and the viability can remain up to 3 weeks (Assavalapsakul, 2004).

Like lymphoid organ, preparation of primary cultured cells from hepatopancreas of penaeid shrimps has been established (Toullec *et al*., 1996; Uma *et al*., 2002). The cell attachment and monolayer formation appeared within 24 h and 72 h, respectively (Uma *et al*., 2002). However, crustacean hepatopancreas contains five different cell types including E (embryonic)-, F (fibrillar)-, B (blister-like)-, M (midgut), and R (resorptive)-cells with distinct structure and function (Lehnert and Johnson, 2002). Of five cell types, E-cells are the undifferentiated cells, whereas others are directly derived from E-cells resulting in the degenerating cells. Since the differentiation of E-cells to other cell types could be induced upon feeding, higher portion of E-cells was observed during starvation maintenance (Lehnert and Johnson, 2002).

1.3 Foreign gene expression in penaeid shrimps

Many studies have made much effort to express foreign genes in penaeid shrimps. The common means used for plasmid DNA delivery are direct injection of naked DNA into living shrimps (Arenal *et al*., 2004; Sulaiman, 1995), and gene transfer into shrimp zygotes by microinjection (Cabrera *et al*., 1995; Sun *et al*., 2005), electroporation (Arenal *et al*., 2000; Sun *et al*., 2005; Tseng *et al*., 2000), as well as chemical reagents (Sun *et al*., 2005). The foreign gene expression in Oka and ovarian cells were also done using retroviral-based system (Shike *et al*., 2000). In addition, the introduction of DNA bound with nuclear localization signal (NLS) peptide into *P. schmitti* zygotes was developed to enhance the efficiency of DNA delivery (Arenal *et al*., 2004).

The achievement of foreign gene expression in penaeid shrimps has been obtained from mammalian viral promoters, including CMV (Arenal *et al*., 2000; Arenal *et al*., 2004; Sulaiman, 1995; Tseng *et al*., 2000) and SV40 (Cabrera *et al*., 1995) promoters. Besides, β-actin promoters of carp (Arenal *et al*., 2000) and *P. vannamei* (Sun *et al*., 2005) were also used to regulate gene expression in shrimp zygotes. Additionally, the expression of exogenous gene regulated by long terminal repeat (LTR) of Moloney murine leukemia virus (MoMLV) and Rous sarcoma virus (RSV) in shrimp cells was observed (Shike *et al*., 2000). The success of gene delivery in penaeid shrimps has been examined by detection of reporter gene expression, such as β-galatosidase (Arenal *et al*., 2000; Arenal *et al*., 2004; Cabrera *et al*., 1995; Sulaiman, 1995), luciferase (Shike *et al*., 2000), and bacterial alkaline phosphatase (BAP) (Tseng *et al*., 2000).

However, these promoter activities in shrimp primary cultured cells, except for LTR promoter using viral-based system, have not yet been reported. Hence, studying of functional promoters in shrimp cultured cells will be valuable for further researches about foreign gene expression in shrimp cells. Prior to testing promoter activity, it is necessary to consider about the essential factors including the investigated promoter, the expression cassette harboring promoter region and reporter gene, as well as the means for DNA introduction into cells.

1.4 RNA polymerase II promoter

Eukaryotic cells contain three distinct nuclear RNA polymerases that transcribe different classes of genes. RNA polymerase II is responsible for the synthesis of messenger RNA (mRNA) from protein-encoding genes, and it has been the focus of most studies of transcription in eukaryotes (Cooper, 1996). Transcription with RNA polymerase II is processed by multiple events including the formation of transcription initiation complex around core promoter region (Smale and Kadonaga, 2003). The basic properties of the most common core elements, such as TATA box, initiator (Inr), downstream promoter element (DPE), and TFIIB recognition element (BRE), are described below.

1.4.1 TATA box

TATA box discovered by David Hogness is the first core promoter element identified in eukaryotic protein-encoding genes (Carey and Smale, 1999; Smale and Kadonaga, 2003). The consensus TATAAA is located 25-30 bp upstream of the transcription start site (Fig 1). TATA box is capable of independently causing a low level of transcription by RNA polymerase II. In addition, several studies revealed that a wide variety of A/T-rich sequences can also function as TATA box (Hahn *et al.*, 1989; Singer *et al.*, 1990; Zenzie *et al.*, 1993). Although TATA box is found in most eukaryotic promoters, it is absent in some promoters known as TATA-less promoters.

1.4.2 Initiator element

The initiator element (Inr) is defined as a discrete core promoter element functionally similar to the TATA box and can function independently of a TATA box (Smale *et al.*, 1998; Smale and Baltimore, 1989). The Inr consensus was determined for both mammals (Py Py A(+1) N T/A Py Py) (Javahery *et al.*, 1994; Lo and Smale, 1996; Smale *et al.*, 1990) and *Drosophila* (T C A(+1) G/T T T/C) (Lo and Smale, 1996) when +1 represents the transcription start site as shown in Figure 1. In TATA-less promoters, Inr element plays a critical role to determine the initiation point and to produce basal level of transcription (Latchman, 1998).

1.4.3 Downstream promoter element (DPE)

The DPE was initially identified in *Drosophila* and it is conserved from *Drosophila* to human promoters (Smale and Kadonaga, 2003). Its core sequence (A/G G A/T C/T G/A/C) is located at precisely +28 to +32 relative to the start site in the Inr (Kutach and Kadonaga, 2000) (Fig 1). In *Drosophila*, where the DPE element has been greatly studied, the DPE is found in TATA-less promoters and acts in conjunction with the Inr element to direct specific initiation of transcription (Kutach and Kadonaga, 2000). However, TATA box can function independently of the Inr, while DPE cannot (Burke and Kadonaga, 1996). Thus, DPE and Inr elements function together as a single core promoter unit.

1.4.4 TFIIB recognition element (BRE)

The BRE element discovered by Ebright and colleagues (Langrange *et al.*, 1998) is found in a substantial number of eukaryotic promoters. It prefers a 7-bp sequence (G/C G/C G/A C G C C) located from -32 to -38, just upstream of TATA box (Fig 1) (Langrange *et al.*, 1998).

1.4.5 Binding of transcription factors

The biochemical analyses have revealed that the success of transcription process requires the formation of pre-initiation complex in promoter region (Hampsey, 1998; Orphanides *et al.*, 1996). Such complex contains RNA polymerase II and a number of general initiation factors; including TATA binding protein (TBP), transcription factor TFIIB, TFIID, TFIIF, TFIIE, and TFIIH. The specific binding of these general initiation factors to core promoter elements; such as binding of TBP to the TATA box (Burley and Roeder, 1996), TFIIB to the BRE (Langrange *et al.*, 1998), as well as TAFs of TFIID to the Inr (Smale and Baltimore, 1989) and the DPE (Burke and Kadonaga, 1996), is needed for the transcription machinery. Moreover, RNA polymerase II is assembled to the complex by extensive interaction within promoter region between positions -43 to +24 with relative to the start site (Hahn, 2004). The specific positions for the binding of RNA polymerase II and general transcription factors are shown in Figure 2.

1.4.6 Upstream promoter element and enhancer

In most promoters, both TATA-dependent and TATA–independent promoters, the low basal level activity is dramatically increased by other elements located upstream of the promoter defined as upstream promoter element (UPE) (Goodwin *et al*., 1990). The common UPE elements found in various genes are a GC-rich sequence (Sp1 box) (Dynan and Tjian, 1985) and CCAAT box (McKnight and Tjian, 1986). Not only UPE, but enhancers also affect the expression level. Enhancers located at great distance from the start site can be placed in upstream, downstream, or within a transcription unit, and function in either orientation (Lania *et al*., 1997). The binding of specific factors is needed for such sequences, and their interactions with the basal transcriptional complex result in the increasing of expression level (Lania *et al*., 1997) (Fig 3).

1.5 Functional assay of promoter activity

Several types of functional assay have been used to study transcriptional regulation. The most common is the transient transfection assay, in which plasmids containing the promoter region or interesting sequences are introduced into cultured cells (Carey and Smale, 1999). Typically, the expression of reporter gene placed immediately downstream of promoter is assayed, in either mRNA or protein level, to examine the promoter activity. This strategy is considered to be transient because the plasmids remain as exogenous DNA and rarely integrate into the host genome. Thus, reporter gene expression must be measured within a short period, ranging from 1-3 days after transient transfection to avoid plasmid degradation (Carey and Smale, 1999).

In addition to transient transfection, the stable transfection is another way for promoter studying, in which plasmids transfected into cultured cells are stably integrated into host chromosomal DNA at random locations within the genome. Alternatively, other functional assays can be considered including the *in vitro* transcription that the promoter activity is measured in a cell-free extract.

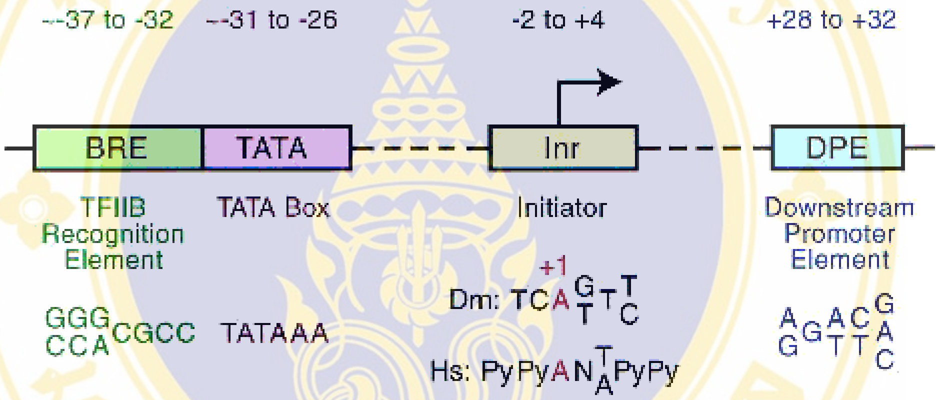


Figure 1: Eukaryotic core promoter (Smale and Kadonaga, 2003)

Four basal core promoter elements, including their consensus sequences and positions in promoter region, are depicted. The Initiator sequence is shown for both *Drosophila* (Dm) and human (Hs), and transcriptional start site is designed as +1. The indicated numbers represent positions of nucleotides based on the transcriptional start site, minus (-) for upstream and plus (+) for downstream position.

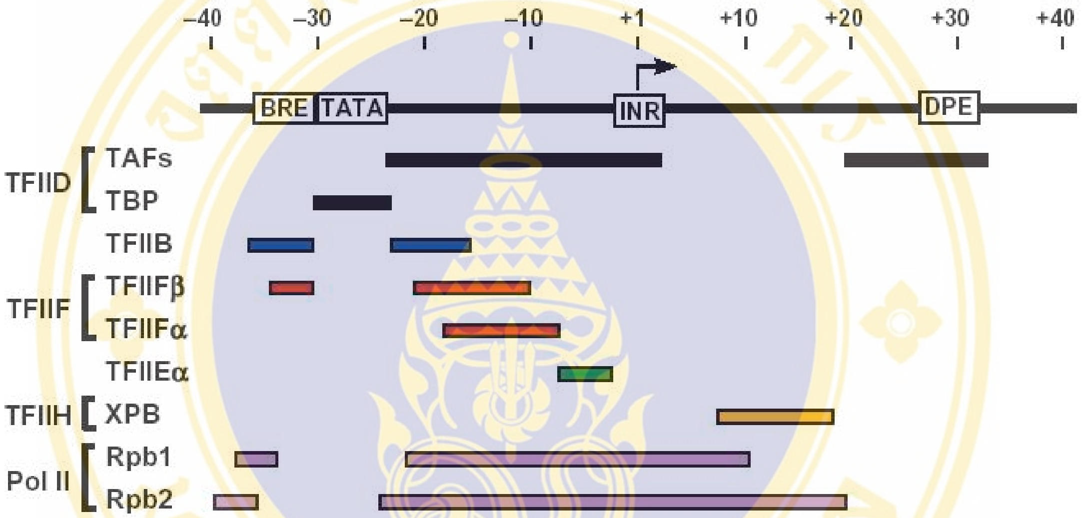


Figure 2: Schematic diagram of the binding positions for transcription factors (Hahn, 2004)

General transcription factors TFIIB, TFIID, TFIIF, TFIIE, TFIIH, and RNA polymerase II (Pol II) including their subunits specifically bind to different positions within promoter region. The numbers represent nucleotide positions at downstream (-) or upstream (+) relative to the start site (+1). The core promoter elements; TATA, BRE, INR, and DPE, are also demonstrated.

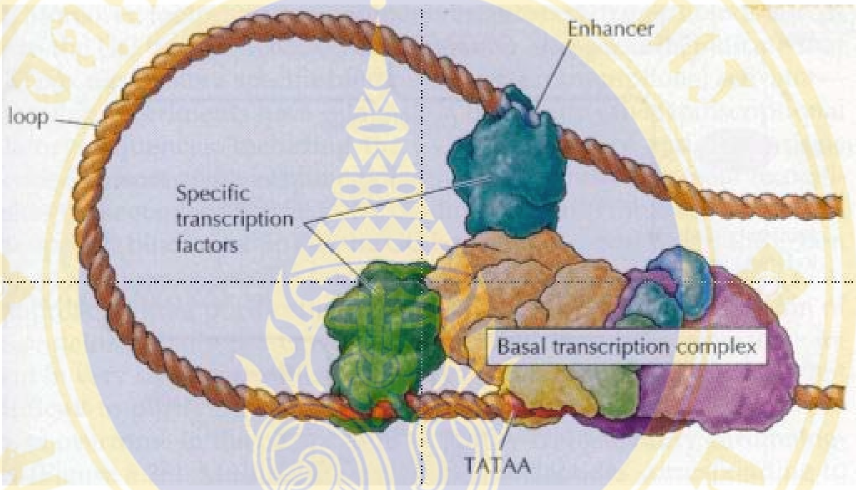


Figure 3: The formation of transcription complex (Cooper, 1996)

The basal transcription complex can be formed at core promoter region. The regulatory proteins bound at upstream promoter element (UPE) and enhancer will interact with the basal complex leading to the increasing of expression level.

Besides, the transgenic assay allows the stable integration of promoter regions into the genome of an animal followed by detection of reporter gene expression in the living system (Carey and Smale, 1999).

1.5.1 Transient transfection

Many techniques have been developed for transient promoter analysis by introduction of plasmid DNA into nuclear compartment of the transfected cells, where the transcription machinery is located. The transfection methods can be classified into two distinct categories: chemical and physical means (Schenborn, 1999).

One common feature of chemical reagents used for transfection is their cationic nature allowing the overall net positive charge of the nucleic acid/reagent mixture (Schenborn, 1999). It is presumably positioned within the proximal area of the outer cell membrane followed by entering into inside the cells. Several chemical reagents have been used for different methods, such as calcium phosphate, diethylaminoethyldextran (DEAE-dextran), and cationic liposomes.

The principle of physical methods of nucleic acid delivery to cells is the penetration of molecules through cell membrane (Schenborn, 1999). Electroporation, using electric field pulses to create reversible holes in the cell membrane, is the most common technique of physical transfection (Heiser, 1999; Schenborn, 1999). Biolistic particle delivery is also used for gene transfer, in which the microscopic gold/DNA particles are projected into the target cells. Microinjection is used to introduce nucleic acids to a small number of cells (Schenborn, 1999).

1.5.1.1 Liposome-mediated transfection

Liposome-mediated transfection, or lipofection, is a technique used to introduce nucleic acids into cells. Several types of synthetic cationic lipids have been designed having the structure of a mono- or polycationic head group chemically linked to a lipophilic moiety (Schenborn, 1999). The cationic region of the molecule associates with the negative charge of nucleic acid. An overall positive or neutral charge of the lipid:DNA complexes is generally correlated with higher transfection efficiency for cultured cells *in vitro* (Schenborn, 1999; Schenborn and Oler, 1999). It is hypothesized that the net neutral or positive charge effectively reduces electrostatic repulsion between the nucleic acid and the negatively charged cellular membrane. The lipid:DNA complexes appear to be endocytosed into cells (Schenborn, 1999; Schenborn and Oler, 1999) (Fig 4).

Although different types of liposomes are commercially available, optimization is specifically needed. Typically, three essential and critical parameters; amount of DNA, ratio of cationic to DNA, and the length of the transfection interval, are required for optimization (Schenborn, 1999). The recommended amounts of DNA and lipid:DNA ratios vary for different lipid reagents. The concentration of DNA and lipid may lead to excessive toxicity at higher concentration and reduce efficiency at lower concentration. Depending upon the specific reagents and conditions tested, 1-24 h is suggested for time intervals. However, longer incubations are generally more toxic (Schenborn, 1999). Paradoxically, higher transfection efficiency may occasionally be associated with higher toxicity (Schenborn and Oler, 1999). Thus, the optimal balance between transfection and toxicity is necessary. Besides, the quality of DNA can affect the transfection efficiency. Impurities such as high EDTA concentration, RNA contamination, and presence of endotoxins can interfere with complex formation between the liposomes and DNA. Presence and absence of serum also have influence to the transfection efficiency. The commercial lipids perform differently in the presence and absence of serum. Hence, it is necessary to select the suitable reagent for each cell types (Schenborn and Oler, 1999).

1.5.1.2 Electroporation

Electroporation is a process in which transient destabilization of cell membrane is induced from high voltage pulse followed by permeability of exogenous molecules (Heiser, 1999; Schenborn, 1999) (Fig 5). The temporary breakdown of the cell is reversible and the pores reclose during recovery after the pulses. The most critical parameters to optimize for any cell type are the magnitude of the voltage and duration of the current pulse that must be sufficient to cause a local breakdown in the cell membrane without irreversible damage (Heiser, 1999; Schenborn, 1999). Combinations of relatively high voltages with a short time constant, or lower voltages with longer time constants, are effective. Excessive voltage and duration of pulses result in cell death. The balance between cell death and efficiency of gene transfer should be determined during the establishment of optimal conditions for electroporation of any cell type. Normally, optimal transfection may occur under conditions that cause approximately 50% cell death (Heiser, 1999; Schenborn, 1999). In transient transfection, gene expression is generally higher in cells electroporated with supercoiled plasmid than those with linearized plasmid (Heiser, 1999). To obtain the maximum transfection efficiency, the highest purity of plasmid DNA is also needed. In eletroporation, high numbers of cells and DNA are required, for instance plasmid DNA can be used at 10-40 μg/ml with 10^6-10^7 cells/ml for transient transfection (Heiser, 1999; Schenborn, 1999).

1.5.2 Luciferase reporter gene assay

The reporter gene will be used to determine the promoter activity from the transfected cells. Most reporter genes such as chloramphenicol acetyltransferase (CAT), β-galactosidase, green fluorescent protein (GFP), and luciferase-encoding genes are derived from insects or prokaryotes that encode enzymatic activities not typically found in most eukaryotic cells (Carey and Smale, 1999). Expression of reporter genes is usually monitored by measuring their enzymatic activities that should be roughly proportional to the transcription frequency from the promoter (Carey and Smale, 1999). Typically, the experimental reporter gene is co-transfected with a control reporter gene serving as an internal control. The experimental variability caused by differences in cell viability or transfection efficiency can be normalized by the internal control (Promega) (Sambrook and Russell, 2001).

The luciferase assay is extremely rapid, simple, relatively inexpensive, sensitive, and posses a broad linear range (Carey and Smale, 1999). The luciferase gene from the firefly *Photinus pyralis* (de Wet *et al*., 1987) encodes a 61-kD enzyme that oxidizes D-luciferin in the presence of ATP, oxygen, and Mg^{++}, yielding the released light as shown in the following reaction.

$$ATP + luciferin + O_2 \longrightarrow AMP + oxyluciferin + PPi + light$$

Luminometers or standard scintillation counters can be used to detect light released by the reaction of the luciferase enzyme. Although the produced flash light rapidly decays, the light signal can be sustained from 5-10 min that permits sufficient time for measurement (Sambrook and Russell, 2001). The amount of luciferase expressed in a transfected cell is a function of the promoter strength, as well as the kinetics of luciferase mRNA transport, translation, and enzyme turnover.

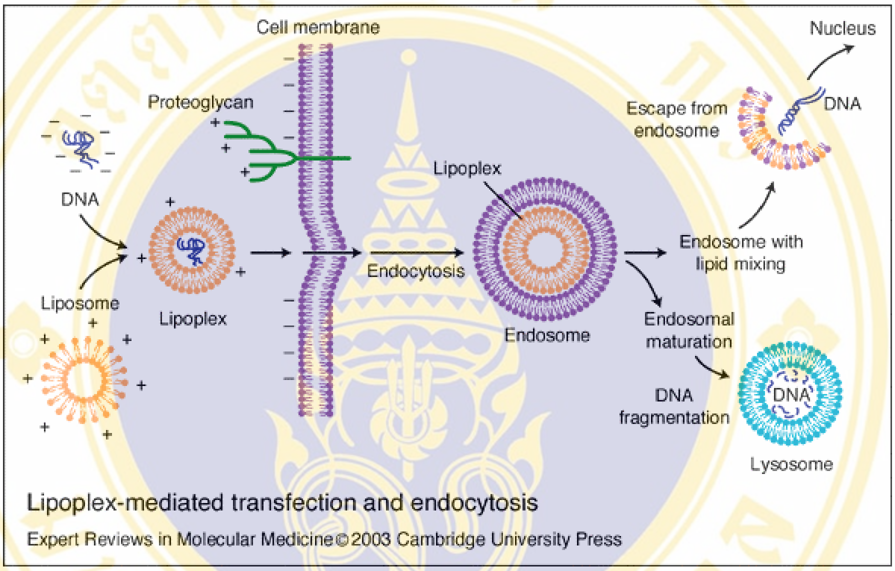


Figure 4: Mechanism of liposome-mediated transfection (Parker *et al.*, 2003)

The negatively charged DNA can be normalized by the positive charge of liposomes leading to the overall positive charge of liposome:DNA complex. The complex is assumed to pass into cell through endocytosis. The released DNA cargo can further move into nuclear compartment where the transcription machinery is located. Alternatively, DNA degradation in lysosome can be occurred.

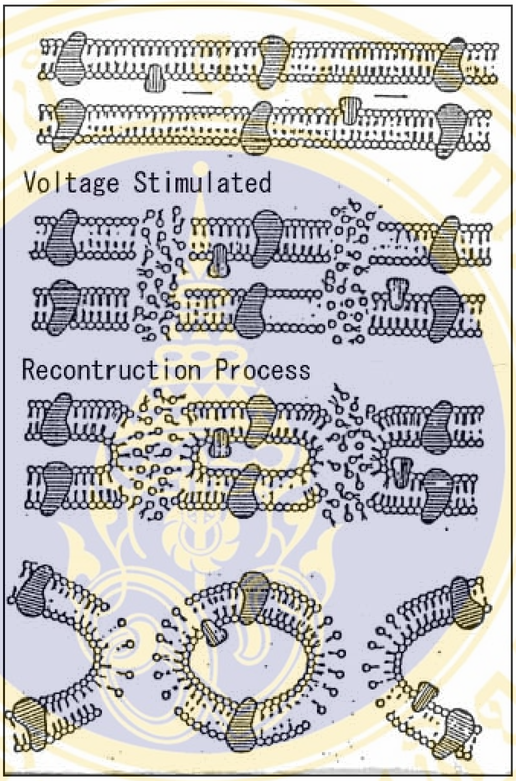


Figure 5: Electroporation mechanism (http://bme.pe.u-tokyo.ac.jp/research/ep/ep-e.htm)

The applied high voltage pulse causes local breakdown of cell membrane and the temporary pores can be formed. The exogenous molecules can move into internal compartment of the electroporated cell.

The time of luciferase expression after transfection can be as short as 16 h or as long as 120 h depending on cell types.

1.6 The investigated promoters in this study

Functional promoter from non-viral pathogen of shrimp (CMV promoter) and putative promoters from shrimp pathogens, such as p1 and p50 promoters from HPV, as well as RR1, RR2, VP15, and VP19 promoters from WSSV, were selected for functional analysis in this study. Based on the consensus sequence of typical eukaryotic promoter (Smale and Kadonaga, 2003), composition of each promoter region is described below.

1.6.1 Human cytomegalovirus (CMV) immediate-early promoter

CMV promoter is the immediate early promoter from human cytomegalovirus presumably encoded for a viral regulatory protein to stimulate transcription in other regions of viral genome (Thomsen *et al.*, 1984). Due to the well characterized promoter, its essential core elements, such as TATA box and CAAT box, have been identified (Thomsen *et al.*, 1984). The TATA box of CMV promoter composes of TATATA consensus sequence, while CAAAT is the sequence in CAAT box. Moreover, the promoter region also includes a 19 bp repeat (CCCCA/GTTGA CGTCAATGGG) and an 18 bp repeat (C/ACTAACGGGACTTTCCAA) having a similar function of promoter-regulatory regions. The CMV promoter sequence with all identified elements was shown in Figure 6.

1.6.2 Putative promoters from HPV

Based on the HPV genome sequence (Sukhumsirichart *et al.*, 2005), two predicted promoter regions, p1 and p50, have been proposed from computer analysis to regulate the expression of non-structural protein and structural protein, respectively.

1.6.2.1 Putative p1 promoter

The hypothetical p1 promoter has been predicted to control the expression of non-structural protein (NS) of HPV (Sukhumsirichart *et al*., 2005). The proposed sequence of p1 promoter represents four core promoter elements, including TATA box, Inr, BRE, and DPE (Fig 7). Initiator region covers AG<u>A</u>GTC, in which the underlined <u>A</u> indicates transcription start site (+1). At the upstream of initiator, TATA box (TATATA) is found at position -29 from the cap site, while BRE (AGACCCC) is positioned immediately upstream of TATA. DPE element (AGTTC) is located downstream of the start site at position +30.

1.6.2.2 Putative p50 promoter

The promoter for structural protein of HPV has been also predicted, called p50 promoter (Sukhumsirichart *et al*., 2005). The core promoter region includes TATA box, Inr, BRE, and DPE elements. The Inr covers TCAGTT with the cap site (+1) at the underlined A. TATA box is placed at position -50 with TATAAA. BRE encompasses ATGCACA located immediately upstream of TATA box, whereas GTACA at position +29 is assigned for DPE element. Besides the basal core promoter, p50 promoter also represents E box and PRE (parvovirus repetitive element) which are the specific elements usually found in promoters of VP genes in parvovirus family (Gu *et al*., 1995; Krady and Ward, 1995). Figure 8 illustrates the sequences in p50 promoter region with the identified core promoter.

1.6.3 Putative promoters from WSSV

The hypothetical promoters of both non-structural and structural proteins from WSSV have been characterized. As for non-structural protein, two putative promotes of ribonucleotide reductase-encoding gene, RR1 and RR2, have been predicted (Tsai *et al*., 2000). In addition, putative promoters of two structural proteins, nucleocapsid protein (VP15) and envelope protein (VP19), have been also proposed (Tsai *et al*., 2004).

1.6.3.1 Putative RR1 promoter

Putative RR1 promoter has been reported as a promoter of ribonucleotide reductase specifically for large subunit (Tsai *et al*., 2000). The core promoter region includes TATA box and Inr element. The Inr sequence harbors TCACTC with the underlined C as the transcription start site (+1). This start site was experimentally confirmed by 5'RACE analysis. TATAAA at position -28 was identified as TATA box of RR1 promoter. Figure 9 shows the sequence in RR1 promoter region with core elements. Although BRE and DPE elements was not proposed in RR1 promoter, their consensus sequences, GGGCCGG for BRE and AGACG for DPE, were found in appropriate positions.

1.6.3.2 Putative RR2 promoter

Like RR1 promoter, putative RR2 promoter has been predicted as a promoter of ribonucleotide reductase, but it was specifically assigned for small subunit instead of large subunit (Tsai *et al*., 2000). The core promoter of RR2 composes of TATA box and Inr in which Inr harbors GCATCA with +1 site at the underlined T, while TATAAA at postition -29 was characterized as TATA box. Similar to RR1 promoter, the transcription start site of this promoter is the actual cap site analyzed by 5'RACE experiment, while BRE (GCATGAG) and DPE (GGTCC) elements were also noticed. (Fig 10)

1.6.3.3 Putative VP15 promoter

VP15 is one of the structural proteins of WSSV forming as nucleocapsid protein of viral particle. The presence of TATA box and Inr element was proposed in region of putative VP15 promoter (Tsai *et al*., 2004). Although the predicted core promoter elements include TATA box (TATAAA) and Inr element (ACAGT), the actual cap site confirmed by 5'RACE is not located in this predicted Inr (Marks *et al*., 2003). Based upon the genuine start site, TATA box is located at position -29 within the A/T rich region (Marks *et al*., 2003). The sequence of VP15 promoter including core promoter elements is shown in Figure 11.

1.6.3.4 Putative VP19 promoter

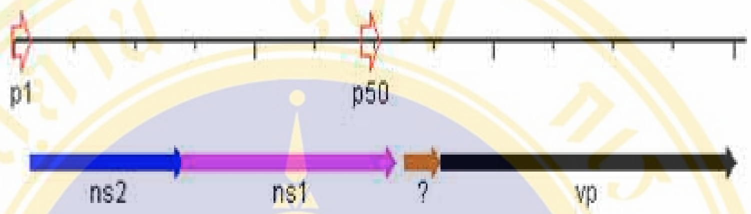
Not only VP15, but VP19 is also one of the structural proteins for WSSV particle that functions as envelope protein. Although putative promoter of VP19 has been proposed with TATA box (TATAAA) and Inr (ACATT) in core promoter region (Tsai *et al*., 2004), 5'RACE analysis showed the genuine start site (+1) is located at different position from the predicted Inr (Marks *et al*., 2003). Moreover, the A/T rich region was also found at 25 bp upstream from this actual start site (Marks *et al*., 2003). Thus, it indicates that these elements are taken as the true core promoter instead of the predicted promoter region (Fig 12).

```
-573 TTAATAGTAA  TCAATTACGG  GGTCATTAGT  TCATAGCCCA  TATATGGAGT
-523 TCCGCGTTAC  ATAACTTACG  GTAAATGGCC  CGCCTGGCTG  ACCGCCCAAC
-473 GACCCCCGCC  CATTGACGTC  AATAATGACG  TATGTTCCCA  TAGTAACGCC
-423 AATAGGGACT  TTCCATTGAC  GTCAATGGGT  GGAGTATTTA  CGGTAAACTG
-373 CCCACTTGGC  AGTACATCAA  GTGTATCATA  TGCCAAGTAC  GCCCCCTATT
-323 GACGTCAATG  ACGGTAAATG  GCCCGCCTGG  CATTATGCCC  AGTACATGAC
-273 CTTATGGGAC  TTTCCTACTT  GGCAGTACAT  CTACGTATTA  GTCATCGCTA
-223 TTACCATGGT  GATGCGGTTT  TGGCAGTACA  TCAATGGGCG  TGGATAGCGG
-173 TTTGACTCAC  GGGGATTTCC  AAGTCTCCAC  CCCATTGACG  TCAATGGGAG
-123 TTTGTTTTGG  CACCAAAATC  AACGGGACTT  TCCAAAATGT  CGTAACAACT
                    CAAT box                                TAAT box
 -73 CCGCCCCATT  GACGCAAATG  GGCGGTAGGC  GTGTACGGTG  GGAGGTCTAT
                                +1
 -23 ATAAGCAGAG  CTCTCTGGCT  AACTAGAGAA  CCCACTGCTT  ACTGGCT
```

Figure 6: Nucleotide sequence of CMV promoter (Thomsen *et al.*, 1984)

CMV core promoter region encompasses TATA box and CAAT box as indicated, including the transcriptional start site (+1). The underline characters represent the 18 bp repeat, while the 19 bp repeat is defined by the double underline characters. The nucleotide numbers are relative to +1.

A.



B.

```
-154 TCCGCGATAA  GCCTTATCAG  GCTCTCCGCC  TAGGCGAGCA  GCGGGCCTTC

-104 GGCCCCCCTT  CGGGGCTGCT  GGAGCCTGAT  AAGGCTTGTC  GCGGAGCTAG
                      BRE      TATA box
 -54 GGCGGTGCG   TCGGCAGACC  CCTATATATT  GGTAAACCTC  GGGTGTGTGA
        Inr                               DPE
  -4 CAAGAGTCCT  CTCCGATTGG  TCTAGCAGTG  AAGTTCTCAG  CACG
        +1
```

Figure 7: Nucleotide sequence of putative p1 promoter (Sukhumsirichart *et al.*, 2005)

Putative p1 promoter region located at 5' end of coding strand in HPV genome includes BRE, TATA box, Inr, and DPE elements.

Panel A: HPV genomic organization

Panel B: Nucleotide sequence of p1 promoter with the nucleotide numbers relative to the putative transcriptional start site (+1)

A.

B.

```
                                                 E-box
-224 AATGCCAATG  AAGTACAAGG  AACACAAGAC  ACACATGTTC  AGGAAACCTG
                PRE
-174 TATTTTTGAC  CAACCAACAT  CATCCACTGG  TAGAGATATC  AAACTATGAC

-124 GATAGAAAGG  CTATAGAGAA  CAGATGTTTT  ATGTATAAGG  TAGAATTGGG
                     BRE       TATA box
 -74 AAGCGAGGCA  GTAAATGCAC  ATATAAAATT  TCCTAACAGG  ATGATTCCGA
                                Inr
 -24 TCAAGAAGAA  TCCAGAACTG  ACTCAGTTTA  TATTGGC
                               +1
```

Figure 8: Nucleotide sequence of putative p50 promoter (Sukhumsirichart *et al.*, 2005)

Putative p50 promoter region placed in the internal position of HPV genome covers BRE, TATA box and Inr. The E box and PRE, regulatory elements, are also displayed.

Panel A: HPV genomic organization

Panel B: Nucleotide sequence of p50 promoter with the nucleotide numbers relative to the putative transcriptional start site (+1)

A.

B.

```
-269 CGGTCCACCC  TCGGAACTTG  ACATCAGGCC  TAGACAGCGG  TCCACCCCCT

-219 AAACTCGAGT  GATCCAGAAA  AATTTTTAAA  AAGTTTTTGA  GGAACAGTTT

-169 TGCACCTGCA  CTACTAATAC  TGCAGCAGCA  GCAGCTGTCA  CCTCACCTTC

-119 TGATTTCTTT  CTACATCAAA  GAACTACGTA  TAATACTATA  TATTTCTTCC
                                               BRE   TATA box
 -69 CCTCCTTCAA  AAATATCCAG  CGACCTTCAC  CGGGCCGGTA  TAAATACAGC
                                 Inr
 -19 TACCGTCCAA  GCCTCTTCAC  TCACATCTTT  CACCCTCCTC  GAGCCGAGAA
     DPE                 +1
 +32 GACGTATAGG  AAAATAAAGC  TT
```

Figure 9: Nucleotide sequence of putative RR1 promoter (Tsai *et al*., 2000)

The core promoter region of putative RR1 promoter composes of BRE, TATA box, Inr, and DPE. The transcriptional start site is shown as +1 with the nucleotide numbers relative to +1.

Panel A: Schematic diagram of *rr1* and *rr2* genes in WSSV genome

Panel B: Nucleotide sequence of RR1 promoter

A.

B.

```
-168 CAAGACCAGA  AGGGTGTATT  TGAGACCGTC  TGCAAAATAA  AGAGCAGAGA

-118 TGGTTTGTGA  ATTGGCCACA  CTGATTCCGA  TACCGACTAA  AAAGGCTGAT
                                            BRE   TATA box
 -68 TGATGGCAAC  CCCCCCCTCC  AGACTCAGCC  GCATGAGTAT  AAATATGGCC
                        Inr                     DPE
 -18 ACTTCTCACA  CCACAGCATC  ATTCCCTCGT  CATCGGTCCT  ACCGTCAACT
                       +1
 +33 TCCATTATTA  CTCC
```

Figure 10: Nucleotide sequence of putative RR2 promoter (Tsai *et al.*, 2000)

Putative RR2 promoter region encompasses BRE, TATA box, Inr, and DPE. The transcriptional start site is shown as +1 with the nucleotide numbers relative to +1.

Panel A: Schematic diagram of *rr1* and *rr2* genes in WSSV genome

Panel B: Nucleotide sequence of RR2 promoter

```
-235 CTTTCCCTAT  GATGTCTGAC  TTGACTCCAG  CCATACCGAA  GGGGGCGTCC

-185 GTATCGACAT  ATAAGGGGTC  GAAAGGGTTG  GGAATAATTC  CCCCATTTTC

-135 TTTTAATCCT  TTGACGCGTA  AAGAGTTTCG  AGACATGGCT  CCTGTATCTA

 -85 CAAAAGAGCT  CGCTTAACCA  CAGTTGCACT  CACCCGGGTA  CAATAAGACA
           TATA box                Predicted Inr
 -35 AAAATTATAA  ATTGGGAAGA  CCGATACAGT  CTTTCATGA
    A/T rich region                         +1
```

Figure 11: Nucleotide sequence of putative VP15 promoter (Marks *et al.*, 2003; Tsai *et al.*, 2004)

The putative VP15 promoter region includes TATA box and Inr element. However, the actual start site (+1) confirmed by 5'RACE is not positioned in the predicted Inr. In addition, the TATA box is located within A/T rich region indicated by the double line characters. The nucleotide numbers are relative to +1.

```
-362 GGACCCAATT  TTCTCAACTT  ATCGGAAAAC  AATTCCCATT  CTTTTGTTAC

-312 CTGTATATTA  TTTGTCTGCA  AAATAGACAC  TACAATATCA  ACATATACCC

-262 TTTCTGGGTC  CTCGCACGGA  TCAGGTCCTA  AAACGCTATA  AGAAGGTCCC
                                          Predicted TATA box
-212 CTCATTTTAT  GAGAAGGTGG  TTCTCCGGTT  ATAAATTCAG  TTCCATCTCC

-162 TTTTAATGAG  GCCGCTGCCG  AAGCGGACGC  CGTGGATCTA  AGATTGTCGT

-112 TAACCCGGCG  TTGAAGAAGA  TATAGTGAAC  AGAATTGACG  GAACATGACC
                                          A/T rich region
 -62 AACTGCGACA  ACTAACGCTG  AGTAAGTCTA  AAAAGACAAA  TAGGTTCGTA

 -12 AGGTGTCCTG  ACAAAAACCG  TACACGATAA  AACAACAGGT  CTTTACGTTA
  Predicted Inr  +1
 +39 CATTGACGTA  CCTCTTCATC  AAACAG
```

Figure 12: Nucleotide sequence of putative VP19 promoter (Marks *et al*., 2003; Tsai *et al*., 2004)

Putative VP19 promoter region includes the predicted TATA box and Inr element. Nevertheless, the experimental start site from 5'RACE is not located in the predicted Inr as shown in the indicated box. The underlined nucleotides represent the start site from different clones. The A/T rich region defined by the double line characters is presumably used as the actual core promoter instead of TATA box placed at the distant position. The nucleotide numbers are relative to +1 (A).

CHAPTER II
OBJECTIVE

2.1 Rationale

Gene transfer in shrimps has been developed from many research groups. However, the suitable and efficient method for DNA introduction and the functional promoter in shrimp cells are the limitation. Although some promoters are functional in shrimp zygotes and living shrimps, the functional promoter in primary shrimp cells has not been reported yet. Therefore, in this study, seven promoters from both pathogens and non-pathogen in penaeid shrimps were functionally investigated in primary shrimp cells. Nevertheless, due to the unavailability of shrimp cell line, Sf9 insect cell line was applied to primarily test promoter activity. Then the promoter function in cultured shrimp cells would be observed.

2.2 Objective

This thesis is aimed to investigate the promoter function in Sf9 insect cell line and in *P.monodon* primary cultured cells.

CHAPTER III
MATERIALS

3.1 Chemicals and enzymes

All chemicals and enzymes used in this thesis are analytical grade from various reliable manufacturers, such as Promega, GIBCO BRL, Sigma, Invitrogen, BIO-RAD, and so on.

3.2 Bacterial strain

Escherichia coli strain DH5α: *supE*44, Δ*lac*U169 (ϕ80 *lac*Z ΔM15), *hsdR*17, *recA*1, *endA*1, *gyrA*96, *thi*-1, *relA*1, obtained from GIBCO BRL, was employed as a host cell for recombinant plasmid.

3.3 Shrimp specimens

Black tiger shrimps (*P. monodon*) with roughly 20 g of each shrimp were purchased from shrimp farms in Chachoengsao, Pathumthani, and Suphanburi provinces of Thailand.

3.4 Virus source

The heamolymph from the WSSV-infected shrimp (*P. monodon*), kindly provided by Dr. Boonsirm Withyachumnarnkul, was used as a source of WSSV.

3.5 Synthetic primers

The primer pairs were specifically designed based upon nucleotide sequences of the desired regions. They were synthesized from Proligo, Singapore. All primers used in this thesis were displayed in Table 1.

Table 1. The list of primers

Name	Sequence	Size (bp)	T_m (ºC)
CMV sense	*Kpn* I 5'AGCGGGGTACCTTAATAGTAATCAATTACGGGGTC3'	35	67
CMV antisense	*Hind* III 5'-AACCCAAGCTTAGCCAGTAAGCAGTGGGTTC-3'	31	67
RR1 sense	*Kpn* I 5'-AACTCGGTACCCGGTCCACCCTCGGAACTTG-3'	31	73
RR1 antisense	*Hind* III 5'-AATCCAAGCTTTATTTTCCTATACGTCTTCTCGG-3'	34	64
RR2 sense	*Kpn* I 5'-AGCGTGGTACCCAAGACCAGAAGGGTGTATTTG-3'	33	70
RR2 antisense	*Hind* III 5'-AACATAAGCTTGGAGTAATAATGGAAGTTGACGG-3'	34	64
p1-ext sense	*Kpn* I 5'-ACACGGGTACCTCCGCGATAAGCCTTATCAG-3'	31	69
p1 antisense	*Hind* III 5'-ATGCTAAGCTTCGTGCTGAGAACTTCACTG-3'	30	64
p50 sense	*Kpn* I 5'-ATAAGGGTACCAATGCCAATGAAGTACAAGGAAC-3'	34	65
p50 antisense	*Hind* III 5'-ATGCTAAGCTTGCCAATATAAACTGAGTCAGTTC-3'	34	63
VP15 sense	*Kpn* I 5'-ACACTGGTACCCTTTCCCTATGATGTCTGAC-3'	31	63
VP15 antisense	*Hind* III 5'-AACATAAGCTTGAAAGACTGTATCGGTCTTC-3'	31	60
VP19 sense	*Kpn* I 5'-ACACGGGTACCGGACCCAATTTTCTCAACTTATC-3'	34	69
VP19 antisense	*Hind* III 5'-AACATAAGCTTCTGTTTGATGAAGAGGTACGTC-3'	33	62
luc sense	5'-ACCAGGGATTTCAGTCGATG-3'	20	60
luc antisense	5'-TGGCAGATGGAACCTCTTGG-3'	20	62

Table 1. The list of primers (Continued)

Name	Sequence	Size (bp)	T_m (ºC)
actin s	5'-GACTCGTACGTGGGCGACGAGG-3'	22	62
actin as	5'-AGCAGCGGTGGTCATCTCCTGCTC-3'	24	65
121F	5'-GCACTTATCACTGTCTCTAC-3'	20	58
276R	5'-GTGAACTTTGTAAATACCTTG-3'	21	41

3.6 Cell line

Sf9 cells, kindly provided by Asst. Prof. Sarawut Jitrapakdee, are a subclone of IPLB-SF-21 cells originally cultured from pupal *Spodoptera frugiperda* tissue widely used in expression vector systems.

COS-1 cell line, kindly given by Dr. Witoon Tirasophon, is originally established from African monkey green kidney. It is derived from CV-1 simian cells transformed by origin-defective mutant of SV40.

3.7 Bacterial growth medium

LB (Luria-Bertani) broth contained 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, and 1% (w/v) NaCl. For agar plate, 1.5% (w/v) of bacto-agar was added into LB broth medium.

3.8 DNA vectors

pGL3-Basic Vector (Promega), kindly provided by Dr. Witoon Tirasophon, is a suitable vector for promoter studying. It harbors firefly luciferase-encoding gene as a reporter gene as well as poly(A) signal region for transcriptional termination (Fig 13).

pGEM-T Easy Vector (Promega) is convenient for cloning of PCR product because of a 3' terminal thymidine added to both ends, which is compatible with a single deoxyadenosine overhang at 3' end of PCR product generated by certain thermostable polymerases (Fig 14).

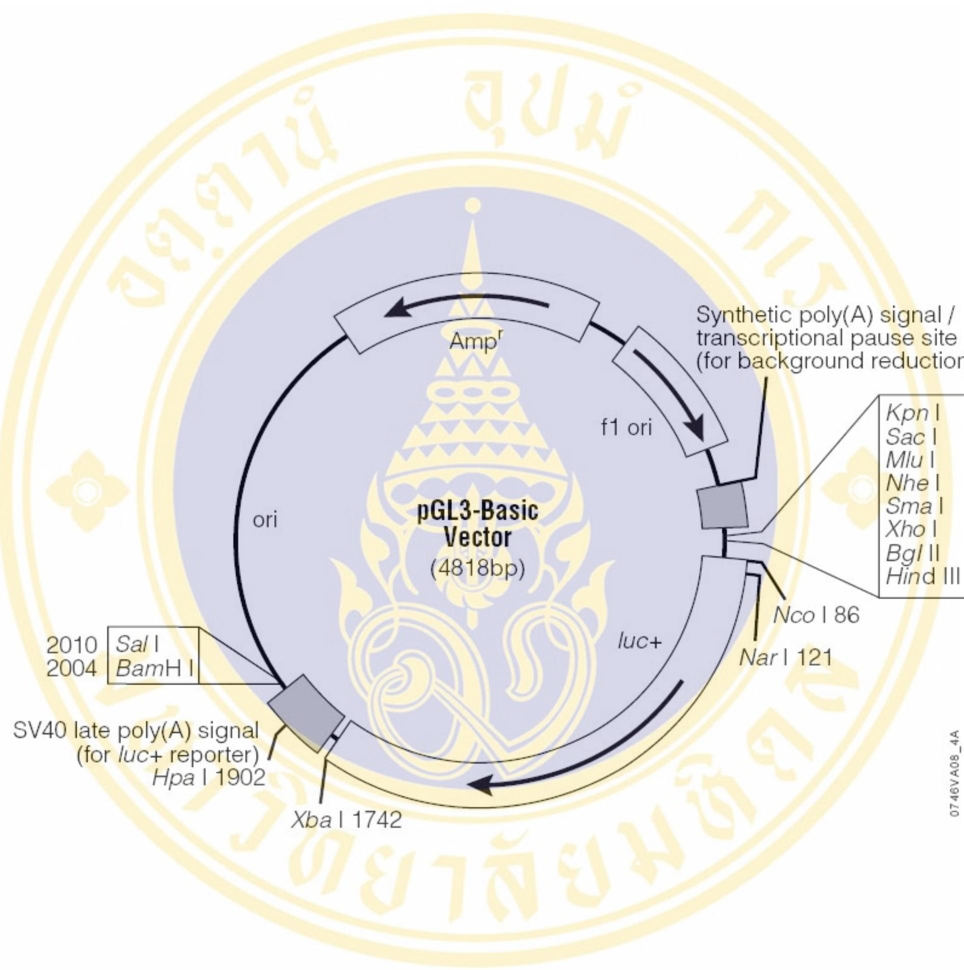


Figure 13: The physical map of pGL3-Basic vector (Promega)

pGL3-Basic vector has the region of firefly luciferase-encoding gene (luc+) and SV40 late poly(A) signal. The expression cassette can be constructed by insertion of promoter region into multiple cloning sites immediately located upstream of the reporter gene.

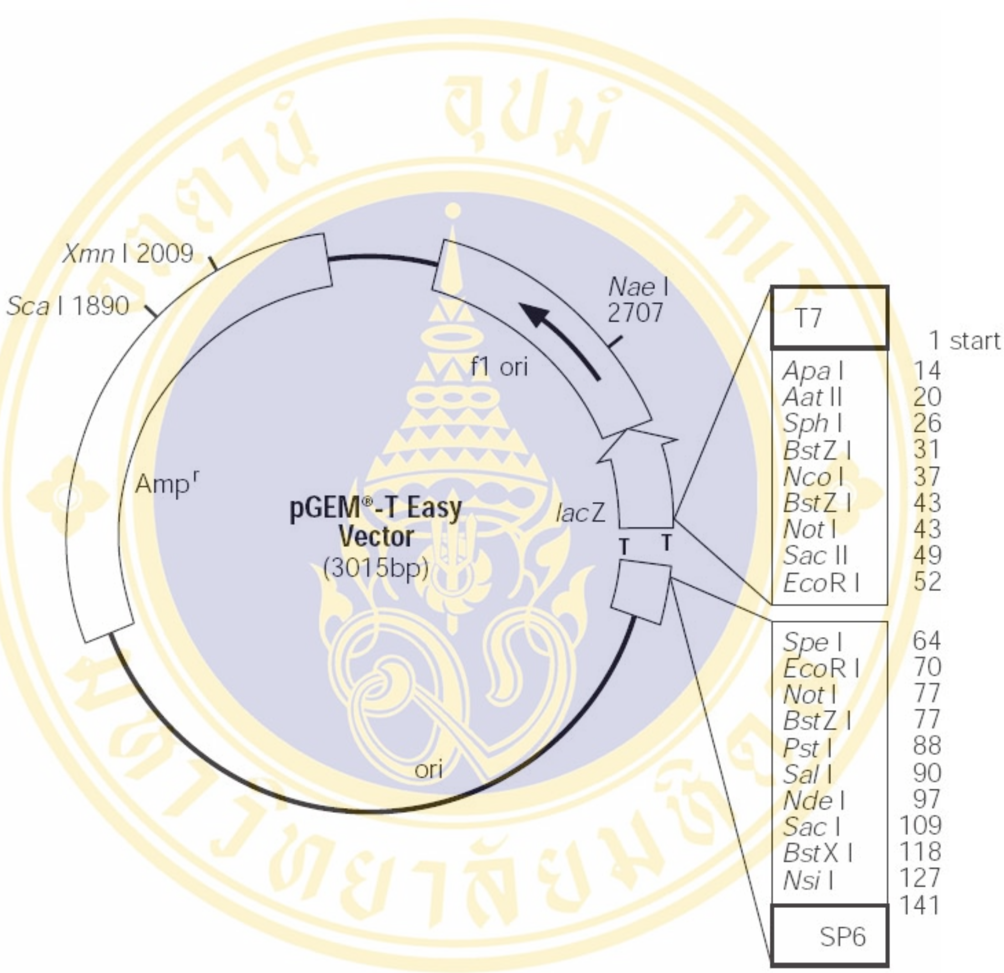


Figure 14: The physical map of pGEM-T Easy vector (Promega)

pGEM-T Easy vector is suitable for cloning of PCR product. It contains a 3' terminal thymidine added to both ends, which is compatible with a single deoxyadenosine overhang at 3' end of PCR product generated by certain thermostable polymerases.

CHAPTER IV
METHODS

4.1 Plasmid DNA extraction

4.1.1 Cetyltrimethylammonium bromide (CTAB) plasmid DNA minipre-parathion (Sal *et al*., 1988)

A single colony of *E.coli* DH5α was inoculated into 3 ml LB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% NaCl, pH 7.5) containing 100 µg/ml ampicillin and grown at 37°C overnight with shaking. The bacterial cell suspension was transferred to a 1.5 ml microtube and centrifuged at 2,700 g for 1 min at room temperature. The pellet was resuspended in 200 µl of STET buffer (8% sucrose, 0.1% Triton X-100, 50 mM EDTA, and 50 mM Tris, pH 8.0) followed by adding 5 µl of lysozyme (50 mg/ml). Then, the mixture was thoroughly mixed and incubated at room temperature for 5-10 min. After incubation, the solution was boiled at 100ºC for exactly 45 sec and subsequently centrifuged at 17,900 g for 15 min at room temperature. The white pellet was removed using toothpick and 20 µl of 5% (w/v) CTAB was added. The mixture was immediately mixed by inversion and centrifuged at 17,900 g for 5 min at room temperature. The DNA pellet was dissolved in 300 µl of 1.2 M NaCl containing 50 µg RNaseA followed by incubation of mixture at 37°C for 30 min. After an equal volume of chloroform was added, the mixture was thoroughly mixed by vortexing and centrifuged at 17,900 g for 3 min at room temperature. The upper aqueous phase was transferred to a new 1.5 ml microtube and 2 volumes of absolute ethanol were added. The solution was mixed by vortexing, incubated at room temperature for 5-10 min, and centrifuged at 17,900 g for 15 min at room temperature. The pellet was washed with 200 µl of 70% (v/v) ethanol and centrifuged at 17,900 g for 3 min at room temperature. Finally, the DNA pellet was air dried and dissolved in 10 mM Tris-Cl pH 8.0 or distilled water.

4.1.2 Plasmid DNA purification using QIAGEN Plasmid Midi Kit

Since nucleic acids purified with QIAGEN Resin are superior or equivalent purity to those prepared by two rounds of purification on $CsCl_2$ gradients, the purified DNA is suitable for the subsequent procedures such as transfection, sequencing, and *in vitro* transcription and translation (QIAGEN). According to the manufacturer's protocol, a single colony of *E.coli* DH5α was inoculated to a starter culture of 2-5 ml LB medium containing 100 μg/ml ampicillin and grown with vigorous shaking at 37° C for 6-8 h. The starter was then diluted in 100 ml of LB medium containing 100 μ g/ml ampicillin and incubated at 37ºC for 14-16 h with vigorous shaking. The cultured cells were transferred to a 250 ml tube and centrifuged at 6,000 g for 15 min at 4ºC to harvest *E.coli* cells. The cell pellet was resuspended in 4 ml of buffer P1 (50 mM Tris-Cl pH 8.0, 10 mM EDTA, and 100 μg/ml RNase A) and vigorously vortexed to resuspend cells. Four millilitres of buffer P2 (200 mM NaOH and 1% (w/v) SDS) were added to the solution. The mixture was gently mixed by inverting 4-6 times followed by incubation at room temperature for 5 min. After that, 4 ml of chilled buffer P3 (3 M potassium acetate pH 5.5) was added. The mixture was immediately mixed but gently by inverting 4-6 times, and incubated on ice for 15 min followed by centrifuged at 20,000 g for 30 min at 4ºC. The supernatant containing plasmid DNA was removed to a 30 ml tube and subsequently centrifuged at 20,000 g for another 15 min at 4ºC. The supernatant was carefully transferred to a new 30 ml tube and loaded to a QIAGEN-tip 100 (QIAGEN) equilibrated with 4 ml of buffer QBT (750 mM NaCl, 50 mM MOPS pH 7.0, 15% (v/v) isopropanol, and 0.15 %(v/v) Triton® X-100). The column was allowed to empty by gravity flow and then washed twice with 10 ml of buffer QC (1 M NaCl, 50 mM MOPS pH 7.0, and 15% (v/v) isopropanol). The bound plasmid DNA was eluted with 5 ml of buffer QF (1.25 M NaCl, 50 mMTris-Cl pH 8.5, and 15% (v/v) isopropanol) by collecting the eluate in a 30 ml tube. Plasmid DNA was precipitated with 3.5 ml (0.7 volumes) of isopropanol. The mixture was mixed thoroughly and centrifuged at 15,000 g for 30 min at 4ºC. The supernatant was carefully discarded and the DNA pellet was washed with 2 ml of 70% (v/v) ethanol followed by centrifugation at 15,000 g for 10 min at 4ºC. The pellet was air dried for 5-10 min and dissolved in a suitable volume of 10 mM Tris-Cl pH 8.0.

4.2 DNA extraction of white spot syndrome virus (WSSV)

The heamolymph from the WSSV-infected shrimp (*P. monodon*) was used as a source of WSSV. Five microlitres of heamolymph were mixed with 5 µl of 10 mM Tris-Cl pH 8.0. Then, one microlitre of 20 mg/ml ProteinaseK was added. The solution was mixed thoroughly, and incubated at 50ºC for 60 min followed heat inactivation of ProteinaseK at 95ºC for 15 min. After centrifugation at 17,900 g for 5 min at room temperature, three microlitres of the extracted DNA were directly subjected as DNA template for PCR amplification.

4.3 DNA extraction from the HPV-infected cultured cells

For 0.5-2 x10^5 cells, the cultured cells were harvested by scraping in 150-200 µl of medium from each well and transferred to a 1.5 ml microtube. Three microlitres of 20 mg/ml ProteinaseK were added and the cell suspension was incubated at 50ºC for 2 h followed by heat inactivation at 95°C for 15 min. The solution was centrifuged at 17,900 g for 1 min at room temperature and 3-5 µl of the supernatant was subsequently used for PCR amplification.

4.4 RNA extraction by TRI REAGENT®

TRI REAGENT® is a complete reagent for the isolation of total RNA. It is a monophase solution with the combination of phenol and guanidine thiocyanate to effectively inhibit RNase activity. For adherent cultured cells, cells were washed once with 1XPBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) The washed cells were directly lysed in the culture plate by adding 1 ml TRI REAGENT® for 5-10x10^6 cells. The solution was mixed by pipetting for several times. The cell lysate was transferred to a 1.5 ml tube and incubated for 5 min at room temperature. Then, 200 µl of chloroform was added to the solution. The mixture was mixed vigorously by vortexing followed by standing for 2-15 min at room temperature. The solution was centrifuged at 12,000 g for 15 min at 4ºC. The colorless upper aqueous phase was transferred to a new 1.5 ml tube. Total RNA in the solution was precipitated by mixing with 0.5 ml of isopropanol and stored at room temperature for 5-10 min. The solution was subsequently centrifuged at 12,000 g for 8 min at 4ºC. The RNA pellet was washed by adding 75% (v/v) ethanol followed by centrifugation at 7,500 g for 5 min at 4ºC. After removal supernatant, the RNA pellet was briefly air dried and resuspended in RNase-free sterile distilled water (DEPC-treated water). The RNA solution was completely dissolved by incubation at 55-60ºC for 10-15 min. The dissolved RNA was stored at -80ºC until used.

The concentration of extracted RNA was examined by measuring the absorbance at 260 nm (A_{260}). The total RNA concentration could be calculated by the following equation.

$$\text{RNA concentration } (\mu g/ml) = 40 \times A_{260} \times \text{dilution factor}$$

The A_{260}/A_{280} ratio was applied to determine the RNA purity. The RNA sample with such ratio about 1.8-2.0 was used in the next process.

4.5 Removal of DNA contamination

The contamination of DNA vector in total extracted RNA was avoided by DNA digestion. The reaction contained total extracted RNA, 3 units of RQ1 RNase-free DNase (Promega), 1X RQ1 DNase reaction buffer, and DEPC-treated water. The mixture was incubated at 37ºC for 30 min. To stop the reaction, 1 µl of RQ1 DNase stop solution was added and the mixture was incubated at 65ºC for 10 min.

4.6 First strand cDNA synthesis

The cDNAs of total mRNAs were synthesized in the mixture containing 500 ng of total RNA with 2µM of oligo(dT) primer and RNase-free sterile distilled water. The primer was allowed to anneal with mRNA at 70ºC for 5 min and the solution was quickly cooled on ice. The following components; 1X ImProm-II™ reaction buffer, 0.5 mM dNTPs, 1µl of ImProm-II™ Reverse transcriptase, and RNase-free sterile distilled water, were added into the previous mixture. The solution was gently mixed before the cDNA synthesis was processed at 25ºC for 5 min, 42ºC for 60 min, and 70ºC for 15 min. Two microlitres of synthesized cDNA were used for PCR amplification.

4.7 Polymerase chain reaction (PCR)

To amplify the target DNA, the reaction was performed by using 5-10 ng of plasmid DNA, or 2 µl of cDNA/RNA hybrid, or 3 µl of crude viral DNA as DNA template. The components of PCR reaction were 0.4 µM of each primer, 200 µM of dNTPs mix, 1X PCR buffer (20 mM Tris-HCL pH 8.8, 2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, and 100 µg/ml nuclease-free BSA), 1.5 units of *Pfu* DNA polymerase, and sterile distilled water. In case of *Taq* DNA polymerase (Promega), the PCR condition was adjusted to 0.2 µM of each primer, 2 mM of $MgCl_2$, 200 µM of dNTPs mix, 1X PCR buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCL), 2.5 units of *Taq* DNA polymerase, and sterile distilled water. The temperature profile for PCR amplification was performed by holding at 94ºC for 2 min, denaturation at 94ºC for 10 sec, annealing at 52-55ºC for 30 sec, and extension at 72ºC for 1-2 min. The annealing temperature was varied depending on the melting temperature of each primer pair. The target DNA was amplified for 30-35 cycles and subsequently held at 72ºC for 5 min. The PCR product was analyzed by agarose gel electrophoresis.

4.8 Restriction endonuclease digestion

The reaction composed of DNA solution, 1X restriction endonuclease buffer, restriction endonuclease (2-5 units for 1µg DNA), and sterile distilled water. The restriction endonuclease buffer was varied depending on the restriction endonucleases used in each reaction. The digestion reaction was incubated at 37°C for 1.30-2 h.

4.9 Purification of DNA fragment from agarose gel using QIAquick Gel Extraction Kit

QIAquick Gel Extraction Kit (QIAGEN) is designed to extract and purify DNA of 70 bp to 10 kb in length from standard or low-melt agarose gels in TAE or TBE buffer. After gel electrophoresis, the desired DNA band was excised from the gel under long-wave UV light. The gel slice was transferred to a 1.5 ml microtube and the process of DNA extraction was conducted following the manufacturer's protocol. The excised DNA fragment was weighed and 3 volumes of buffer QG were added to 1 volume of gel (100 mg ~ 100 µl). The mixture was incubated at 50ºC for 10 min to dissolve the gel. After completely dissolved, 1 gel volume of isopropanol was added and the solution was mixed thoroughly. The sample was loaded to the QIAquick column placed in a 2 ml collection tube and the column was centrifuged at 17,900 g for 1 min at room temperature. The flow-through solution was discarded and 0.5 ml of buffer QG was added to column. The column was centrifuged at 17,900 g for 1 min at room temperature and 0.75 ml of buffer PE was added. The column was centrifuged at 17,900 rpm for 1 min at room temperature. The empty column was subjected to additionally centrifuge at 17,900 rpm for 1 min at room temperature. To elute DNA, a suitable volume of buffer EB was added to the center of column placed into a 1.5 ml microtube. The microtube containing column was centrifuged at 17,900 rpm for 1 min at room temperature and DNA solution was collected in the microtube.

4.10 DNA ligation

The linearized DNA vector was joined with DNA insert by consideration of the molar ratio for both fragments. Such ratio could be varied from 1:3 to 1:6 for molar ratio of vector to insert depending upon the size of each fragment. The low ratio was adopted when the sizes of DNA vector and DNA insert were not greatly different. The two DNA molecules were ligated together by T4 DNA ligase (GIBCO BRL) with 1X T4 DNA ligase buffer (50 mM Tris-HCL pH 7.6, 10 mM MgCl2, 1 mM ATP, 1 mM DTT and 5% (w/v) polyethylene glycol-8000) in 10 µl of final volume. The ligation reaction was incubated at 14-16ºC for overnight.

4.11 Competent cell preparation by simple and efficient method (SEM) (Inoue *et al*., 1990)

About 10-12 colonies (2-3 mm in diameter) of *E.coli* DH5α were inoculated into 250 ml of SOB medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCL, 10 mM $MgCl_2$, and 10 mM $MgSO_4$) in a 2 liter flask and grown at 18°C with vigorous shaking until the absorbance at 600 nm reached 0.6. The culture was cooled on ice for 10 min and then transferred to a 500 ml centrifuge tube. The cell suspension was centrifuged at 3,700 g for 10 min at 4ºC. The supernatant was aspirated and the pellet was resuspended in 80 ml of ice-cold TB buffer (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, and 250 mM KCL). The solution was chilled on ice for 10 min, and centrifuged at 3,700 g for 10 min at 4ºC. The pellet was resuspended in 20 ml of ice-cold TB buffer, and DMSO was then slowly added with stirring to give a 7% final concentration. The culture was incubated on ice for another 10 min. Finally, 100 µl of competent cells was aliquoted into 1.5 ml microtubes, immediately frozen in liquid N_2, and kept at -80°C until used for transformation.

4.12 Plasmid DNA transformation by heat shock method

The competent *E.coli* DH5α cells were transformed with 5-10 ng of plasmid DNA using heat shock method. To transform the ligation product, the ligation reaction was diluted for 20 folds before subsequently transformed to the competent cells. The indicated amount of plasmid DNA was added into 100 µl of the competent cells in a 1.5 ml microtube. The mixture was gently mixed and chilled on ice for 30 min. After that, the mixture was heat shocked at 42ºC for exactly 90 sec and immediately cooled on ice. Then, the transformed cells were diluted in LB medium to 1 ml of final volume and vigorously shaked at 37ºC for 60 min. Finally, 100-150 µl of the transformed cells was spreaded onto LB agar plate (LB medium supplemented with 2% (w/v) agar) containing 100 µg/ml ampicillin. The LB agar plate with the spreaded cells was incubated at 37ºC for 16-18 h. In case of blue-white colony selection, 40 µl of 2% (w/v) X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 4 µl of 100 mM IPTG (isopropyl-thio-β-D-galactopyranoside) were pre-spread on LB agar plate containing 100 µg/ml ampicillin prior to spreading the transformed cells.

4.13 Sf9 (*Spodoptera frugiperda*) insect cell culture and cell counting

The Sf9 insect cell line was grown in a 25 mm^3 cultured flask with 5 ml of the Sf900-II SFM (Invitrogen) containing 100 units/ml penicillin (GIBCO BRL) and 100 µg/ml streptomycin (GIBCO BRL) and incubated at 26±1 ºC under atmospheric condition. The Sf9 cells were passaged every 5-7 day or when the cell confluence reached 100%. The cell number could be determined by using the hemocytometer. The cell suspension was dropped onto the edge between the cover slip and the glass slide. Under the microscope, the number of cells was accurately calculated by counting the cells within the 0.1 mm^3 of chamber

4.14 COS-1 cell culture

COS-1 cells were cultured in complete DMEM (GIBCO BRL) (DMEM supplemented with 10% fetal bovine serum (FBS) (GIBCO BRL), 100 units/ml penicillin, 100 µg/ml streptomycin, and 5 mM L-glutamine) in a 10 mm tissue culture dish. Cells were grown at 37ºC with humidify 10% CO_2 and 95% air atmosphere. When the confluence reached 100%, the cells were washed once with pre-warmed 1X PBS. The cells were dissociated from the plate by incubation with 2.5 ml of 0.25% (w/v) trypsin (GIBCO BRL) in HBSS (1.3 mM CaCl, 5.4 mM KCL, 0.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 136.9 mM NaCl, 4.2 mM $NaHCO_3$, 0.3 mM Na_2HPO_4, 5.5 mM D-glucose, and 8.0 mM HEPES) at 37ºC for 5 min. The cells resuspended in complete DMEM were seeded onto a new tissue culture dish.

4.15 Preparation of primary shrimp cell cultures

4.15.1 Primary Oka cell culture

The procedure for culturing the primary Oka cells from lymphoid organs of *Penaeus monodon* was established by Assavalapsakul *et al*. (2003). The lymphoid organ located at the anterior-ventral surface of hepatopancreas was carefully isolated from the freshly killed *P.monodon* and pooled together in the washing solution (2X Leibovitz's L-15 medium, 1% D-Glucose, 0.5% NaCl, 100 units/ml penicillin, 100 µ g/ml streptomycin, 15% (v/v) fetal bovine serum, 5% (v/v) lactalbumin, and 1 mM L-glutamine). The pooled lymphoid organ was gently washed in the washing medium. The washed tissues were transferred to the working medium (2X Leibovitz's L-15 medium, 1% D-Glucose, 0.5% NaCl, 100 units/ml penicillin, 100 µg/ml streptomycin, 15% (v/v) fetal bovine serum, 5% (v/v) lactalbumin, 1 mM L-glutamine supplemented with 15% (v/v) shrimp meat extract) and then minced into small pieces. The cell suspension was diluted in the working medium and seeded onto a tissue culture plate. The cultured Oka cells were incubated at 26±1 ºC under atmospheric condition. The cell growth was observed under the microscope and the working medium was replaced every 2-3 day.

4.15.2 Primary hepatopancreas cell culture

The process of hepatopancreas cell preparation was modified from Uma *et al.* (Uma *et al.,* 2002). Before isolation of hepatopancreas, the living *P.monodon* was maintained in artificial seawater (salinity 5-10 ppt) with non-feeding condition for 4-5 days. After this time, hepatopancreas was isolated from the freshly killed shrimp and dissected into 1 cm^3 pieces. Then, they were washed in washing solution (1X

Leibovitz's L-15 medium, 1% D-Glucose, 0.5% NaCl, 100 units/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin, 15% (v/v) fetal bovine serum, 5% (v/v) lactalbumin, and 1 mM L-glutamine). The washed tissues were stirred in the washing medium for 5-10 min and subsequently passed through the sieve to remove cell debris. The hepatopancreas cells were cultured in working medium (1X Leibovitz's L-15 medium, 1% D-Glucose, 0.5% NaCl, 100 units/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamycin, 15% (v/v) fetal bovine serum, 5% (v/v) lactalbumin, 1 mM L-glutamine supplemented with 15% (v/v) shrimp meat extract) and seeded onto a tissue culture plate. The cultured hepatopancreas cells were incubated at 26±1 ºC under atmospheric condition.

4.16 Liposome-mediated transient transfection

4.16.1 Transfection with Tfx™-20 Reagent (Promega)

Tfx™-20 Reagent is the mixture of a synthetic, cationic lipid molecule (N,N,N',N'-tetramethyl-N,N'-bis(2-hydroxyethyl)-2,3-di(oleoyloxy)-1,4-butanediammonium iodide) and L-dioleoyl phosphatidylethanolamine (DOPE) (Promega Notes, 1997). The Tfx™-20 Reagent provides an efficient method for the introduction of DNA into eukaryotic cells. However, the optimized transfection condition is necessary to gain the maximum efficiency for specific cell types. The important parameters for optimization are the charge ratio of Tfx™-20 Reagent to DNA, the amount of transfected DNA, and the length of exposure time of cells to the reagents (Promega Notes, 1997). For 24-well tissue culture plate, the charge ratio of Tfx™-20 Reagent to DNA was used at 2:1 and 4:1 ratio, the amount of DNA was varied from 0.25 to 1.0 µg/well, and the cells were exposed to the reagent for 1-10 h. The cultured cells were seeded at 0.5-2.0 x 10^5 cells/well or approximately 80% confluent on the day of transfection.

The transfection protocol was performed following the manufacturer's instruction. For 24-well plate, the indicated amount of DNA was added to 200 µl total volume of medium (without serum and antibiotics). The solution was gently vortexed. The desired amount of Tfx™-20 Reagent was combined to the solution. The mixture was immediately vortexed. The Tfx™-20 Reagent/DNA complex was incubated for 10-15 min at room temperature. During incubation time, the cultured cells were washed once with the medium (without serum and antibiotics), and then the briefly vortexed mixture was applied onto the washed cells (200 µl/well). The transfected cells were returned to the incubator. At the end of the exposure time (1-10 h), one millilitre of the complete medium containing serum and antibiotics was gently overlaid to the transfected cells without removing of the Tfx™-20 Reagent/DNA complex. The cells were continuously incubated until 24-72 h for analysis of the reporter system.

4.16.2 Transfection with Lipofectamine™ 2000 (Invitrogen)

Lipofectamine™ 2000 is the lipid formulation suitable for the transfection of nucleic acids into eukaryotic cells. Certainly, it is essential to optimize the transfection condition with the highest efficiency for each cell type. The critical factors affecting the efficiency of transfection with Lipofectamine™ 2000 are the amount of transfected DNA, the ratio of DNA (in µg) to Lipofectamine™ 2000 (in µl), and the exposure time of the Lipofectamine™ 2000/DNA complex to the cells. For 24-well tissue culture plate, the amount of DNA was used at 0.8-1.0 µg/well, the DNA (in µg): Lipofectamine™ 2000 (in µl) ratio was varied from 1:0.5 to 1:5, and the transfected cells were allowed to incubate with the complex for 6-48 h. Lipofectamine™ 2000 requires high cell density about 90-95% confluent at the time of transfection.

The transfection procedure was performed according to the manufacturer’s instruction. For 24-well plate, the amount of DNA was diluted in 50 µl of medium (without serum and antibiotics), while the appropriate volume of Lipofectamine™ 2000 was diluted in 50 µl of the same medium. After incubation for 5 min at room temperature, the diluted DNA was combined with the diluted Lipofectamine™ 2000. The mixture was gently mixed and incubated for another 20 min at room temperature to form the Lipofectamine™ 2000/DNA complex. During incubation, the cells were washed once with the medium (without serum and antibiotics) and 400 µl of the medium was added to each well. The 100 µl total volume of Lipofectamine™ 2000/DNA complex was applied to each well containing cells and medium. The cultured cells were incubated for the indicated exposure time (6-48 h) and the complex was then replaced with the complete medium (with serum and antibiotics). The transfected cells were continuously incubated until 24-72 h when the reporter gene analysis was assayed.

4.16.3 Transfection with Cellfectin® Reagent (Invitrogen)

Cellfectin® Reagent is a 1:1.5 (M/M) liposome formulation of the cationic lipid N,N^{I},N^{II},N^{III}-Tetramethyl-N,N^{I},N^{II},N^{III}-tetrapalmitylspermine (TM-TPS) and dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water. The reagent is suitable for transfection with various cell types. The optimal amount of Cellfectin® Reagent, DNA concentration, and the incubation time of cells with Cellfectin® Reagent/DNA complex are the essential parameters that should be determined for each cell type. For 24-well format, the amount of DNA was used with 0.2-0.4 µg, the amount of Cellfectin® Reagent was optimized from 0.4 to 3 µl, and the incubation time of complex with cultured cells was varied from 6 to 48 h. The suitable cell density for Cellfectin® Reagent is 60-80% confluent at the time of transfection.

The process of transfection has been guided in the manufacturer's protocol. For each transfection in 24-well plate, the indicated amount of DNA was diluted in 20 µl of the medium (without serum and antibiotics) and the Cellfectin® Reagent was also diluted in the same volume of the medium. Two solutions were combined together, gently mixed, and incubated for 10-15 min at room temperature. The seeded cells were washed once with the medium (without serum and antibiotics) and 160 µl of the medium was added to each well. Then, the Cellfectin® Reagent/DNA complex was applied to each well. The cultured cells were incubated with the complex at different time in the range of optimization (6-48 h). After the end of incubation, the complex was replaced with the complete medium and the cultured cells were further incubated until 24-72 h when the reporter gene assay was reached.

4.16.4 Transfection with jetPEI™-FluoF (PolyPlus-Transfection)

jetPEI™-FluoF is a transfection reagent that ensures effective and reproducible transfection with low toxicity and is specially designed for intracellular tracking experiments using fluorescent labeling. It is a fluorescein-conjugated linear polyethylenimine derivative (excitation at 490 nm and emission at 520 nm). For efficient transfection with jetPEI™-FluoF, the ionic balance of jetPEI™-FluoF cations and DNA anions should be considered. The N/P ratio is the indicator for ionic balance of the complex. It refers to the number of nitrogen residues of jetPEI™-FluoF per DNA phosphate. Practically, the high transfection efficiency can be obtained at N/P = 5-10. For transfection in 24-well plate, the constant 1 µg of DNA was used, while the

N/P ratio was optimized from 5 to 10. Normally, the total volume of the complex solution was 1 ml per well. However, it could be decreased to 0.5 ml per well in order to increase the efficiency. The optimal cell density for transfection with jetPEI™-FluoF was around 50-60% confluence at the time of transfection.

The protocol of transfection was done according to the manufacturer's instruction. For 24-well format, one microgram of plasmid DNA was diluted in 50 µl of 150 mM NaCl, while the appropriate volume of jetPEI™-FluoF solution was also diluted in 50 µl of 150 mM NaCl at the same time. After vortexed gently, the diluted jetPEI™-FluoF solution was added to the diluted DNA solution (important: do not mix solutions in the reverse order). The mixed solution was immediately vortexed and incubated for 15-30 min at room temperature. During complex incubation, the seeded cells were washed once with the complete medium (without antibiotics) and then either 0.9 ml or 0.4 ml of the complete medium was added to each well. The 100 µl of complex solution was applied to each well and the transfected cells were incubated for 24-72 h until the reporter gene activity was determined. Unlike other transfection reagents in this thesis, the complex solution was continuously overlaid onto the cells without replacing with the new complete medium.

4.17 Transient transfection by electroporation method

The cell suspension was diluted in 1 ml of complete medium and mixed with 20 µg of plasmid DNA. The solution was transferred to 0.4 cm electrode of Gene Pulser® Cuvette (BIO-RAD) and chilled on ice. The cuvette containing cell suspension was applied to Gene Pulser®II Electroporation system (BIO-RAD) and processed according to the manufacturer's instruction. The conditions of electroporation were optimized by using the electrical current at 200 µF and varying the voltage from 200-400 V. Both single and multiple pulses were considered in the experiment. In each pulse, the cuvette was immediately chilled on ice. The actual voltage and duration time of electroporation were recorded for all conditions. Then, the electroporated cells were equally distributed to three wells of a 24-well plate and the complete medium was added to make 500 µl of total volume in each well. The cells were incubated for 24-72 h until the activity of reporter gene was observed.

4.18 Luciferase reporter gene assay

4.18.1 Total protein extraction

At the time of reporter gene assay, the medium of cultured cells in each well was totally aspirated and the cells were rinsed once with 1XPBS. All trace of solution was removed and the plate was placed on ice. For 24-well plate, 120 µl of 1X Reporter Lysis Buffer (Promega) was added to each well. The buffer was mixed gently with cultured cells by swirling the plate. The plate was placed on ice for 5 min. Then, the lysed cells were harvested and transferred to a 1.5 ml microtube. The cell lysate was vigorously vortexed and centrifuged at 17,530 g for 10 min at 4ºC. The supernatant was transferred to a new 1.5 ml microtube and total protein concentration was measured.

4.18.2 Determination of total protein concentration

Protein concentration was determined by BIO-RAD Protein Assay based on the method described by Bradford (Bradford, 1976). The standard curve of protein concentration was generated from bovine serum albumin (BSA) prepared at 0, 2, and 4 µg/ml. The standard BSA and protein samples were diluted to 800 µl of total volume. The protein solution was mixed with 200 µl of Dye Reagent concentrate (BIO-RAD). The mixture was incubated at room temperature for 5 min before the absorbance at 595 nm was measured. The protein concentration was calculated according to the standard curve.

4.18.3 Luciferase activity assay

The 20-40 µg of total protein was adopted for the assay of luciferase activity using the luminometer machine (Leader® 450i, Gen-Probe®, USA). The delay of injection and the measuring time were programmed at 2 and 15 sec, respectively. The injectors were washed with luciferase reaction buffer (38.9 mM glycylglycine, 23.3 mM MgSO4, and 7.8 mM γ-ATP) and 111 µM beetle luciferin (Promega) at least 3 times before used. The protein solution was dispensed into a 5 ml luminometer tube (Sarstedt, Germany) and 200 µl of luciferase reaction buffer was injected followed by 200 µl of 111 µM beetle luciferin. The produced light was allowed to count and the value of luciferase activity was recorded.

CHAPTER V
RESULTS

In order to characterize promoter activity, a promoter was used to drive an expression of a reporter gene. In this thesis, pGL3-Basic vector (Promega), a promoter-less vector with luciferase reporter gene, was chosen to harbor the promoter region. The investigated promoters include CMV promoter, putative promoters from HPV (p1 and p50 promoters), as well as putative promoters from WSSV (RR1, RR2, VP15, and VP19 promoters). The DNA region covering regulatory elements of each promoter was amplified and individually cloned into pGL3-Basic vector for construction of expression cassettes.

5.1 PCR amplification of promoter regions

To amplify promoter regions, specific primers that cover all essential regulatory elements of each promoter were designed. The restriction sites of *Kpn* I and *Hin*d III were added at 5' end of sense and antisense primers, respectively, to facilitate the construction of recombinant clones. The primers and DNA templates used in PCR amplification as well as expected sizes of PCR products are shown in Table 2.

Table 2. Primers and DNA templates for PCR amplification of promoter region as well as the size of PCR product

Promoter	Primer pair	DNA template	Fragment size (bp)	Result Figure
CMV	CMV sense-CMV antisense	pUC-CMV-EGFP	609	15
p1	p1ext sense-p1 antisense	pGEM-3'HPV	207	16
p50	p50 sense-p50 antisense	pGEM-HPV	259	17
RR1	RR1 sense-RR1 antisense	Crude DNA of WSSV	338	18
RR2	RR2 sense-RR2 antisense	Crude DNA of WSSV	236	18

Table 2. Primers and DNA templates for PCR amplification of promoter region as well as the size of PCR product (Continued)

Promoter	Primer pair	DNA template	Fragment size (bp)	Result Figure
VP15	VP15 sense-VP15antisense	Crude DNA of WSSV	247	19
VP19	VP19 sense-VP19 antisense	Crude DNA of WSSV	440	20

5.2 Expression cassette construction

In order to construct an expression cassette, it is necessary to consider about an investigated promoter, a reporter gene, and a poly(A) signal. In this case, pGL3-Basic vector is the proper promoter-less vector in which the luciferase reporter gene and poly(A) signal are available. Therefore, an expression cassette can be constructed by insertion of the promoter fragment into multiple cloning sites located upstream region of luciferase reporter gene.

Since all amplified promoters were flanked with *Kpn* I and *Hin*d III sites, the promoter fragments and pGL3-Basic vector were digested with such enzymes followed by DNA fragment purification from agarose gel using QIAquick gel extraction kit (QIAGEN). The purified fragments of individual promoter and pGL3-Basic vector were ligated together and transformed to *E.coli* DH5α with heat shock method. The transformants were grown in ampicillin-containing LB medium followed by plasmid DNA extraction. The positive recombinants of each pGL3-promoter were determined by restriction analysis. The chosen recombinants were subjected to verify the promoter sequence by DNA sequencing before used in the next experiments.

However, five promoters; including p1, RR1, RR2, VP15, and VP19, yielded low amount of the amplified PCR products. Therefore, they were temporary inserted to pGEM-T easy vector (Promega) prior to construction of expression cassettes in pGL3-Basic vector. Blue-white colony screening was performed for pGEM-promoter and white transformants were selected for recombinant analysis. The positive clones from restriction enzyme digestion were submitted to DNA sequencing for promoter sequence confirmation.

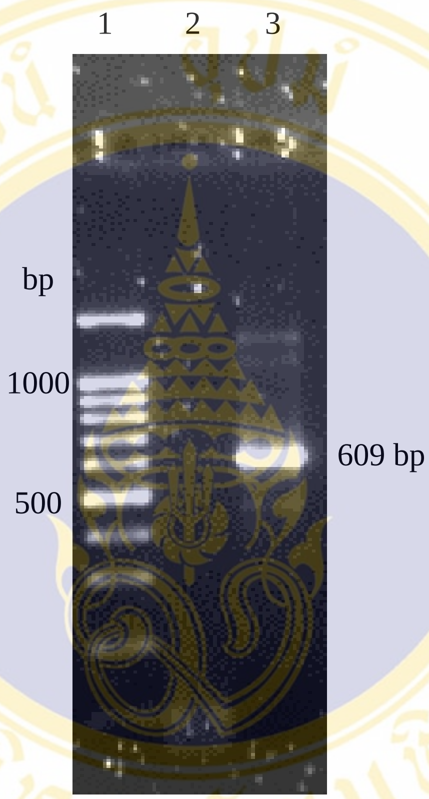


Figure 15: PCR amplification of CMV promoter

CMV promoter region was amplified from pUC-CMV-EGFP using CMV sense and antisense primers. The PCR product was detected by agarose gel electrophoresis stained with ethidium bromide.

Lane 1: 100 bp ladder with 1.5 kb plus

Lane 2: Negative control of amplification (without DNA template)

Lane 3: The amplified product of CMV promoter at 609 bp

Figure 16: PCR amplification of p1 promoter

DNA region of p1 promoter was amplified from pGEM-3'HPV using p1ext sense and p1 antisense primers. The PCR product was detected by agarose gel electrophoresis stained with ethidium bromide.

Lane 1: 100 bp ladder with 1.5 kb plus

Lane 2: The amplified product of p1 promoter at 207 bp

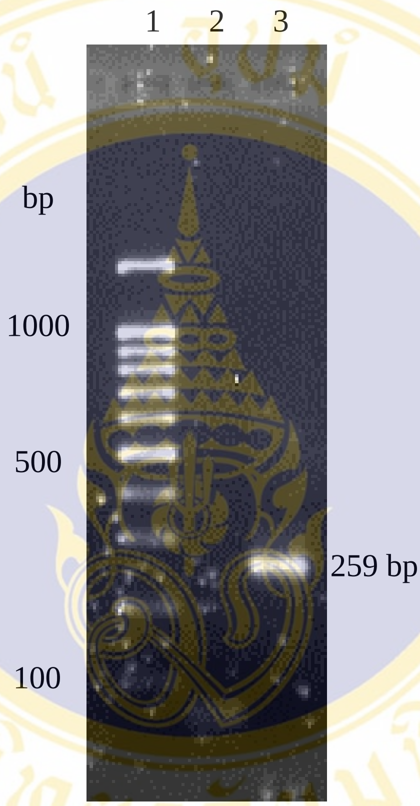


Figure 17: PCR amplification of p50 promoter

DNA region of p50 promoter was amplified from pGEM-HPV using p50 sense and antisense primers. The PCR product was detected by agarose gel electrophoresis stained with ethidium bromide.

Lane 1: 100 bp ladder with 1.5 kb plus

Lane 2: Negative control of amplification (without DNA template)

Lane 3: The amplified product of p50 promoter at 259 bp

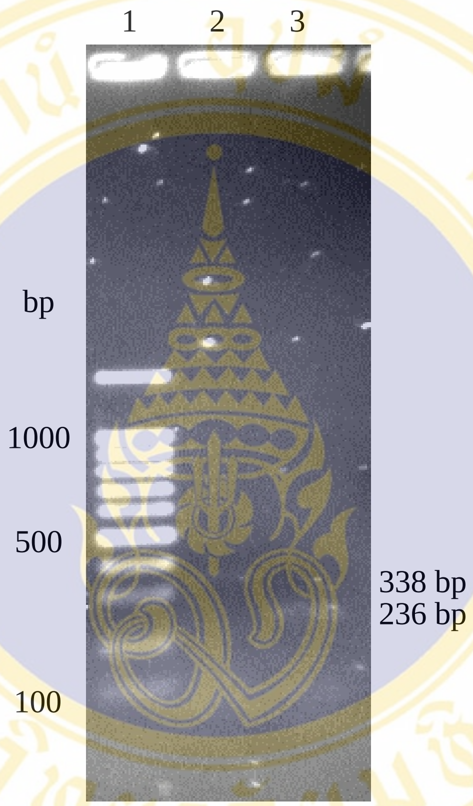


Figure 18: PCR amplification of RR1 and RR2 promoters

DNA regions of RR1 and RR2 promoters were amplified from crude DNA of WSSV virus using RR1 sense and antisense primers for RR1, and RR2 sense and antisense primers for RR2. The PCR products were detected by agarose gel electrophoresis stained with ethidium bromide.

Lane 1: 100 bp ladder with 1.5 kb plus

Lane 2: The amplified product of RR1 promoter at 338 bp

Lane 3: The amplified product of RR2 promoter at 236 bp

Figure 19: PCR amplification of VP15 promoter

DNA region of VP15 promoter was amplified from crude DNA of WSSV virus using VP15 sense and antisense primers. The PCR product was detected by agarose gel electrophoresis stained with ethidium bromide.

Lane 1: 100 bp ladder with 1.5 kb plus

Lane 2: The amplified product of VP15 promoter at 247 bp

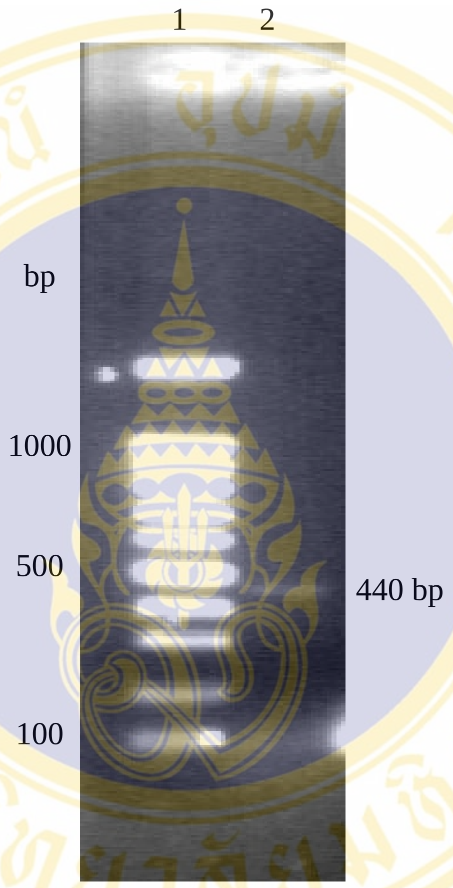


Figure 20: PCR amplification of VP19 promoter

DNA region of VP19 promoter was amplified from crude DNA of WSSV virus using VP19 sense and antisense primers. The PCR product was detected by agarose gel electrophoresis stained with ethidium bromide.

Lane 1: 100 bp ladder with 1.5 kb plus

Lane 2: The amplified product of VP19 promoter at 440 bp

The chosen recombinants were digested with *Kpn* I and *Hin*d III in order to excise the promoter fragment followed by DNA fragment purification from agarose gel using QIAquick gel extraction kit. The purified fragment was subsequently ligated with pGL3-Basic vector digested with *Kpn* I and *Hin*d III leading to the construction of expression cassette (pGL3-promoter). The positive recombinants of pGL3-promoter were examined from restriction analysis and promoter sequences were verified by DNA sequencing before subjected to further procedures. For instances, the construction strategies of pGL3-CMV and pGL3-RR1 are demonstrated in Figure 21 and Figure 22, respectively.

As previously described, the sequences of promoter regions were verified by DNA sequencing. The results from DNA sequencing exhibited none of mutation in promoter sequences, except for p1 promoter. In case of p1 promoter, seven colonies of pGEM-p1 showed point mutation and base deletion at different nucleotide positions (Fig 23). pGEM-p1 clone no.3 with base substitution at position -67 from the transcriptional start site was chosen for subsequent cloning of pGL3-p1. However, the result from sequencing showed that p1 region from pGL3-p1 had sequences as same as those from pGEM-p1 without additional mutation.

5.3 Transient transfection of plasmid DNA into Sf9 insect cell line

In order to investigate whether the amplified promoter regions contain sufficient sequences for basal promoter activity, the expression of reporter gene driven by each promoter would be examined. However, shrimp cell line is unavailable and preparation of primary shrimp cells is quite difficult. Accordingly, Sf9 insect cell line derived from *Spodoptera frugiperda* was adopted to obtain the preliminary data of promoter function before all promoters would be subsequently characterized in shrimp cells.

5.3.1 Conditional optimization for transient transfection in Sf9

To obtain the maximum efficiency of transfection, the transfection condition should be optimized. For transient transfection in Sf9, liposome-mediated transfection was considered as a means of plasmid DNA delivery using Tfx™-20 Reagent (Promega).

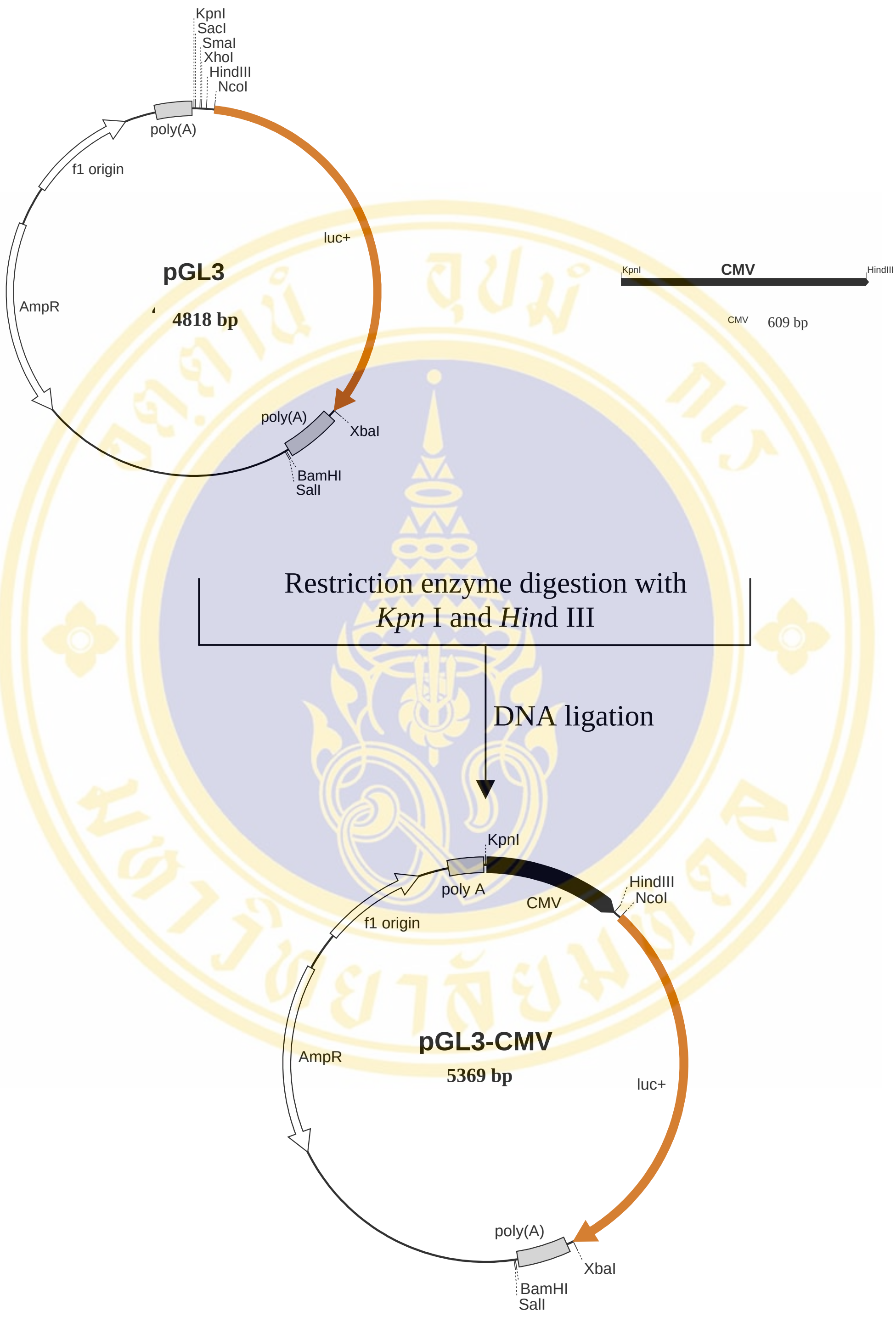


Figure 21: Schematic diagram for construction of pGL3-CMV

pGL3-Basic vector and the amplified CMV region were digested with *Kpn* I and *Hind* III. Both fragments were ligated together with T4 DNA ligase to construct pGL3-CMV.

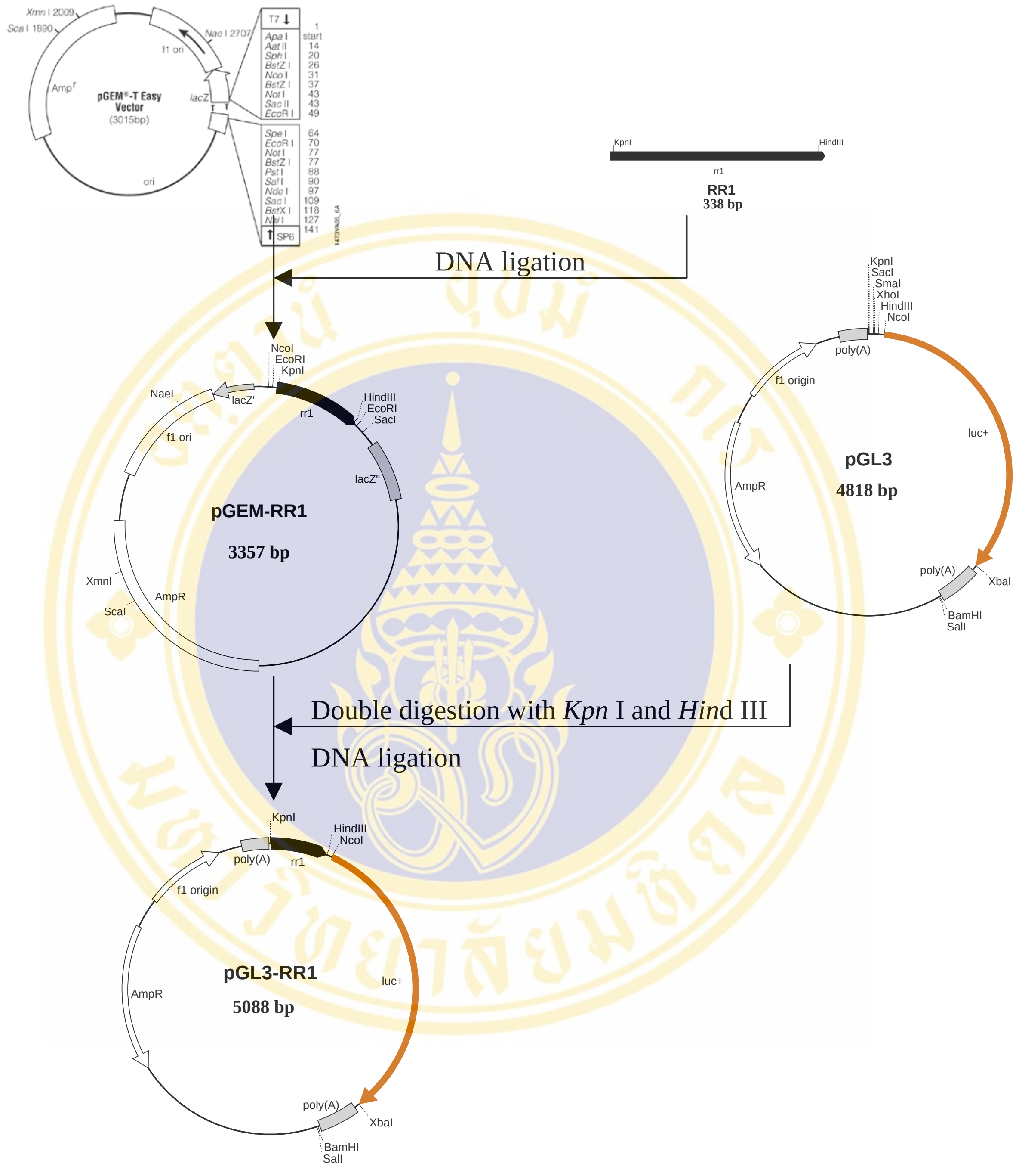


Figure 22: Schematic diagram for construction of pGL3-RR1

The amplified RR1 region was directly ligated with pGEM-T easy vector resulting in pGEM-RR1. pGL3-RR1 was constructed by ligation between pGL3 and RR1 fragments after digestion of pGEM-RR1 and pGL3-Basic vector with *Kpn* I and *Hin*d III.

```
                      1                                           45
     p1_ref     (1)   GGTACCTCCGCGATAAGCCTTATCAGGCTCTCCGCCTAGGCGAGC
 pGEM-p1-no1    (1)   GGTACCTCCGCGATAAGCCTTATCAGGCTCTCCGCCTAGGCGAGC
 pGEM-p1-no3    (1)   GGTACCTCCGCGATAAGCCTTATCAGGCTCTCCGCCTAGGCGAGC
 pGEM-p1-no4    (1)   GGTACCTCCGCGATAAGCCTTATCAGGCTCTCCGCCTAGCCGAGC
 pGEM-p1-no6    (1)   TGATTCTCCGCGATAAGCCTTATCAGGCTCTCCGCCTAGGCGAGC
pGEM-p1-no12    (1)   GGTACCTCCGCGATAAGCCTTATCAGACTCTCCGCCTAGGCGAGC
pGEM-p1-no27    (1)   GGTACCTCCGCGATAAGCCTTATCAGGCTCTCCGCCTAGGCGAGC
pGEM-p1-no28    (1)   GGTACCTCCGCGATAAGCCTTATCAGGCTCTCCGCCTAGGCGAGC
   Consensus    (1)   GGTACCTCCGCGATAAGCCTTATCAGGCTCTCCGCCTAGGCGAGC
                      46                                          90
     p1_ref    (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCCTGATAAGG
 pGEM-p1-no1   (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCCTAATAAGG
 pGEM-p1-no3   (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCCTGATATGG
 pGEM-p1-no4   (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCTTGATAAGG
 pGEM-p1-no6   (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCCTGATAAGG
pGEM-p1-no12   (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCCTAATAAGG
pGEM-p1-no27   (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCCTTATA-GG
pGEM-p1-no28   (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCTTGATAAGG
   Consensus   (46)   AGCGGGCCTTCGGCCCCCCTTCGGGGCTGCTGGAGCCTGATAAGG
                      91                                         135
     p1_ref    (91)   CTTGTCGCGGAGCTAGGGGCGGTGCGTCGGCAGACCCCTATATAT
 pGEM-p1-no1   (91)   CTTGTTGCGGAGCTAGGGGCGGTGCGTCGGCAGACCCCTATATAT
 pGEM-p1-no3   (91)   CTTGTCGCGGAGCTAGGGGCGGTGCGTCGGCAGACCCCTATATAT
 pGEM-p1-no4   (91)   CTTGTCGCGGAGCTTGGGGCGGTGCGTCGGCAGACCCCTATATAT
 pGEM-p1-no6   (91)   CATGTCGGGGAGCTAGGGGCGGTGCGTCGGCAGACCCCTATATAT
pGEM-p1-no12   (91)   CTTGTCGCGGAGCTAGGGGCGGTGCGTCGGCAGACCCCTATATAT
pGEM-p1-no27   (90)   CTTGTCGCGGAGCTAGGGGCGGTGCGTCGGCAGACCCCTATATAT
pGEM-p1-no28   (91)   CTTGTCTCGGAGCAAGGGGCGGTGCGTCGGCAGACCCCTATATAT
   Consensus   (91)   CTTGTCGCGGAGCTAGGGGCGGTGCGTCGGCAGACCCCTATATAT
                      136                                        180
     p1_ref   (136)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
 pGEM-p1-no1  (136)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
 pGEM-p1-no3  (136)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
 pGEM-p1-no4  (136)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
 pGEM-p1-no6  (136)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
pGEM-p1-no12  (136)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
pGEM-p1-no27  (135)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
pGEM-p1-no28  (136)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
   Consensus  (136)   TGGTAAACCTCGGGTGTGTGACAAGAGTCCTCTCCGATTGGTCTA
                      181                    206
     p1_ref   (181)   GCAGTGAAGTTCTCAGCACGAAGCTT
 pGEM-p1-no1  (181)   GCAGTGAAGTTCTCAGCACGAAGCTT
 pGEM-p1-no3  (181)   GCAGTGAAGTTCTCAGCACGAAGCTT
 pGEM-p1-no4  (181)   GCAGTGAAGTTCTCAGCACGAAGCTT
 pGEM-p1-no6  (181)   GCAGTGAAGTT-TCAGCACGAAGCTT
pGEM-p1-no12  (181)   GCAGTGAAGTTCTCAGCACGAAGCTT
pGEM-p1-no27  (180)   GCAGTGAAGTTCTCAGCACGAAGCTT
pGEM-p1-no28  (181)   GCAGTGAAGTTCTCAGCACGAAGCTT
   Consensus  (181)   GCAGTGAAGTTCTCAGCACGAAGCTT
```

Figure 23: Nucleotide sequence alignment of pGEM-p1

Nucleotide sequence in p1 region from pGEM-p1 clone number 1, 3, 4, 6, 12, 27, and 28 were aligned with p1 promoter sequence from HPV genome (p1_ref). pGEM-p1 clone number 3 was chosen for construction of pGL3-p1.

Based on the essential parameters affecting transfection efficiency; cell confluence, amount of DNA, and ratio between DNA and reagent, such factors were optimized in the transfection experiment. pGL3-CMV was used as a positive control of transfection in Sf9.

For a 24-well plate, Sf9 cells were seeded at $0.5x10^5$ and $2x10^5$ cells/well, while the amount of DNA (pGL3-CMV) was applied at 0.75 and 1.0 µg. In addition, the charge ratio of Tfx™-20 Reagent to DNA was used at 2:1 and 4:1. The expression of reporter gene was examined at 48 h post-transfection by luciferase assay. Transfection in Sf9 seeded at $2x10^5$ cells showed higher luciferase activity than those seeded at $0.5x10^5$ cells (Fig 24). Thus, the suitable cell density for transfection into Sf9 in a 24-well format was $2x10^5$cells/well. Using the constant cell number at $2x10^5$ cells, the transfection condition was repeatedly conducted.

At 48 h post-transfection, the highest luciferase activity was noticed from the condition at 1.0 µg of DNA and 2:1 ratio as shown in Figure 25. Hence, this condition; $2x10^5$ seeded Sf9 cells transfected with 1.0 µg DNA at 2:1 charge ratio, was considered as the optimum condition for transfection into Sf9 in this experiment and it was adopted to test all promoter activities in Sf9.

5.3.2 Promoter activity in Sf9 insect cell line

The result from luciferase activity at 48 h after transfection showed the highest luciferase activity from RR2 promoter followed by activity of RR1 promoter, while CMV promoter provided luciferase activity around the half of RR1 (Fig 26). The luciferase activity from p1 promoter was slightly lower than that from CMV promoter. Turning to promoters for structural proteins, the luciferase activity could not be measured from p50, VP15 and VP19 promoters. Three independent experiments were performed and the same manner of promoter activities in Sf9 was observed. However, these promoter activities would be further characterized in shrimp cells.

5.3.3 Transient transfection in Sf9 with HPV infection

It has been proposed that a viral promoter of structural protein may need activators from viral genome itself (Afanasiev *et al*., 1994; Doerig *et al*., 1988; Lorson *et al*., 1996), so putative p50 promoter of HPV may require viral protein for its function. In addition, viral proteins may affect p1 functional activity. In order to verify the influence of viral proteins to HPV promoters, HPV infection during plasmid transfection in Sf9 was conducted. HPV infection was set before transfection (condition I) as well as at the same time of transfection (condition II).

However, the information about HPV infection in Sf9 has not been proposed. To examine the infection of HPV in Sf9, total infected Sf9 cells and supernatant were collected together at 0 (T_0), 24 (T_{24}), and 48 (T_{48}) h after infection. The total DNA was extracted and region of HPV genome was amplified using specific primers (121F-sense and 276-antisense). Figure 27 shows PCR products of HPV genome from two conditions of HPV infection as previously described. Both means depicted the same manner of PCR results that the decreasing of HPV was noticed when the time was increased from T_0 to T_{48}.

The transfected Sf9 cells with HPV infection were collected at 48 h post-transfection and subjected to luciferase assay. The results from luciferase assay (Fig 28) exhibited that p1 promoter had the same activity from the previous result in Sf9 and its activities were not significantly different after Sf9 cells were infected with HPV, while p50 promoter did not show luciferase activity from all conditions.

5.4 Comparison of CMV promoter activity in Sf9 and COS-1 cell lines

The CMV promoter has been typically employed as functional promoter leading to high expression level of reporter genes in various mammalian cell types. However, the results from luciferase assay showed markedly low level of gene expression regulated by CMV promoter in Sf9 cells. In order to determine the cell specificity of CMV promoter, transient transfection of pGL3-CMV into COS-1 and Sf9 cell lines; mammalian and insect cell lines, respectively, was performed. The transfection in Sf9 cells was conducted at the optimum condition using Tfx™-20 Reagent as previously described. In parallel, jetPEI™-FluoF (PolyPlus-Transfection) was used for transfection in COS-1 cells, in which 1.0 µg DNA and N/P ratio (the number of nitrogen residues of jetPEI™-FluoF per DNA phosphate) at 8 were considered as transfection condition. At 48 h post-transfection, the luciferase activity from the transfected COS-1 cells was significantly higher than that from the transfected Sf9 cells (Fig 29).

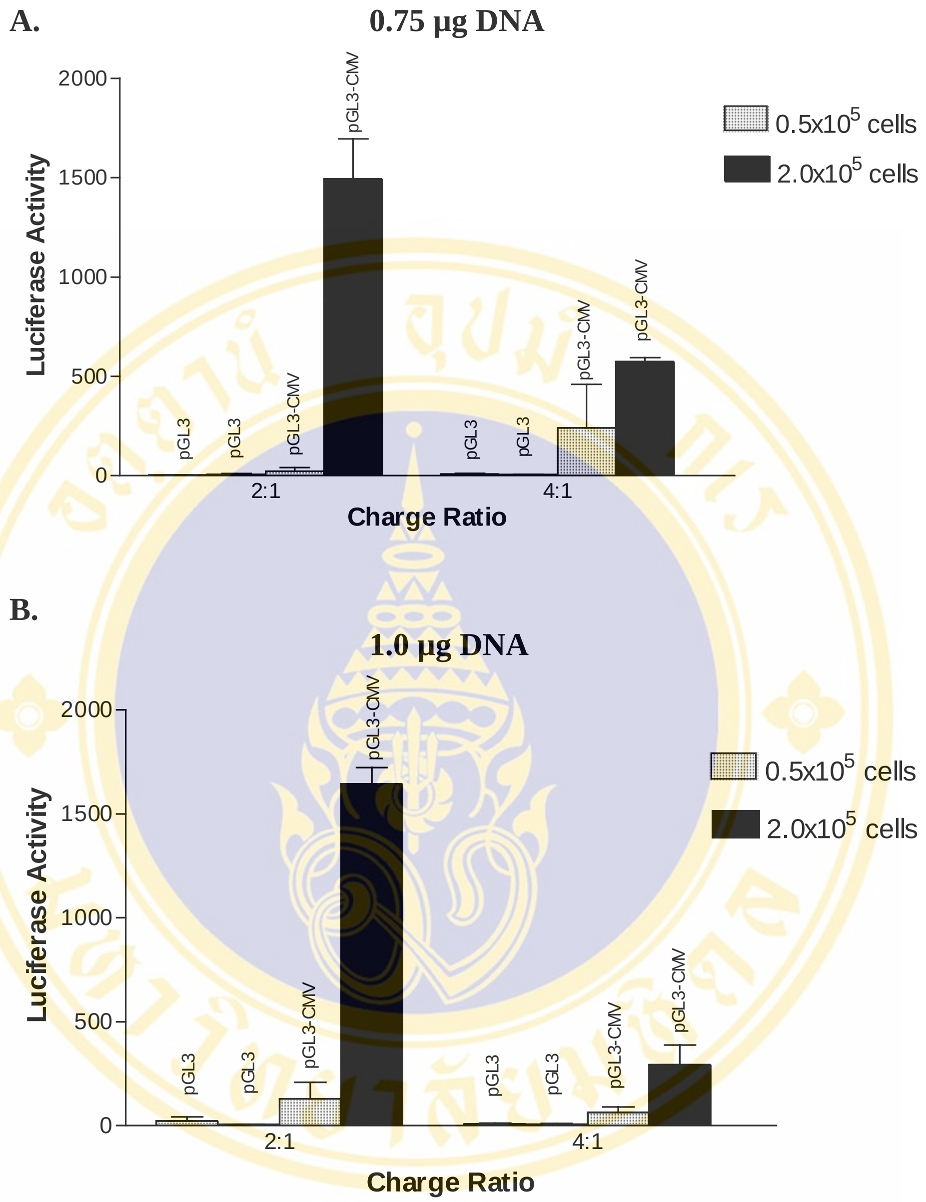


Figure 24: Luciferase assay of promoter activity from optimization of transfection condition in Sf9 cells

Sf9 cells seeded at different confluences ($0.5x10^5$ and $2.0x10^5$ cells) were transfected with 0.75 and 1.0 µg of pGL3-CMV plasmid at 2:1 and 4:1 charge ratio of Tfx™-20 Reagent to DNA. The luciferase assay was performed at 48 h post-transfection and luciferase activity was measured from 10 µg of total protein. The higher luciferase activity could be noticed when $2.0x10^5$ cells were applied. The error bars represent standard deviation of the experiment in triplicate manner.

Panel A: Luciferase activity from Sf9 transfected with 0.75 µg DNA

Panel B: Luciferase activity from Sf9 transfected with 1.0 µg DNA

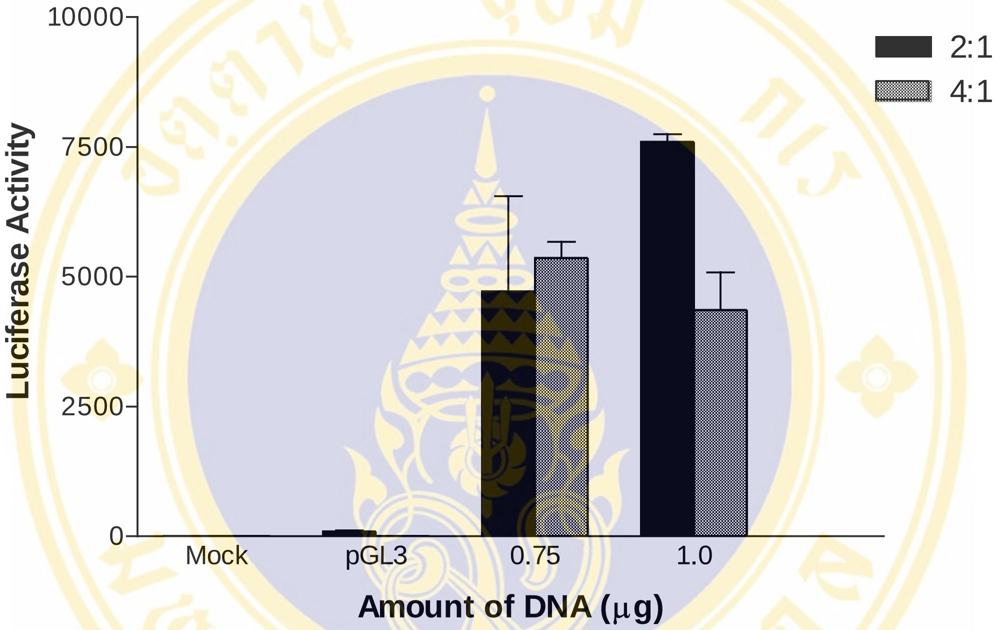


Figure 25: Luciferase assay of promoter activity in Sf9 seeded at $2x10^5$ cells from different conditions

Sf9 seeded at $2x10^5$ cells were transfected with 0.75 and 1.0 µg of plasmid DNA (pGL3-CMV) at different charge ratio of Tfx™-20 Reagent to DNA (2:1 and 4:1). Luciferase assay was performed at 48 h post-transfection and luciferase activity was measured from 20 µg of total protein. The highest promoter activity was detected when 1.0 µg DNA and 2:1 of such ratio were applied. The error bars represent standard deviation of the experiment in triplicate manner.

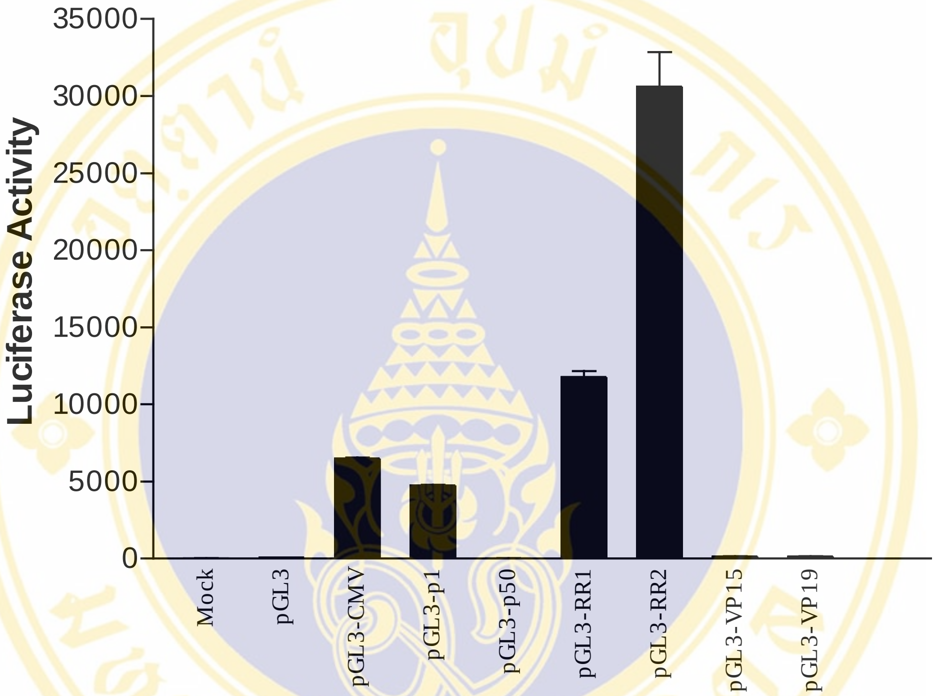


Figure 26: Luciferase assay of different promoters in Sf9 cells

Transfection in Sf9 was performed by using $2x10^5$ cells, 1.0 µg DNA and 2:1 charge ratio of Tfx™-20 Reagent to DNA. Luciferase assay was done at 48 h post-transfection and luciferase activity was measured from 20 µg of total protein. The error bars represent standard deviation from the experiment in triplicate manner.

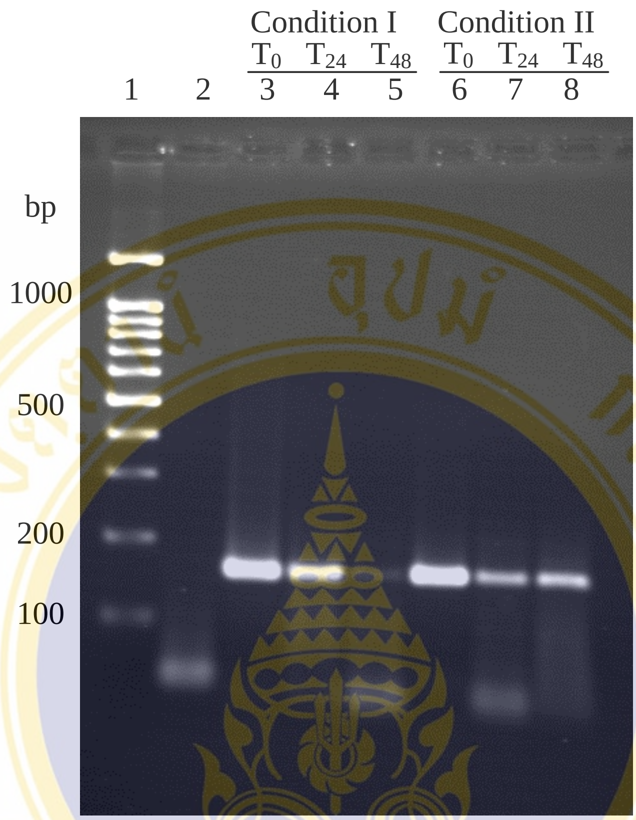


Figure 27: PCR amplification of DNA region from HPV genome in Sf9

The region of HPV genome was amplified from total DNA of HPV-infected Sf9 cells using 121F-sense and 276-antisense primers. Condition I indicates HPV infection for 90 min followed by DNA transfection, whereas condition II represents DNA transfection and HPV infection at the same time. The PCR products were detected by agarose gel electrophoresis stained with ethidium bromide.

Lane 1: 100 bp ladder with 1.5 kb plus

Lane 2: negative control of PCR reaction (without DNA template)

Lane 3: PCR product of HPV from condition I at 0 h after infection

Lane 4: PCR product of HPV from condition I at 24 h after infection

Lane 5: PCR product of HPV from condition I at 48 h after infection

Lane 6: PCR product of HPV from condition II at 0 h after infection

Lane 7: PCR product of HPV from condition II at 24 h after infection

Lane 8: PCR product of HPV from condition II at 48 h after infection

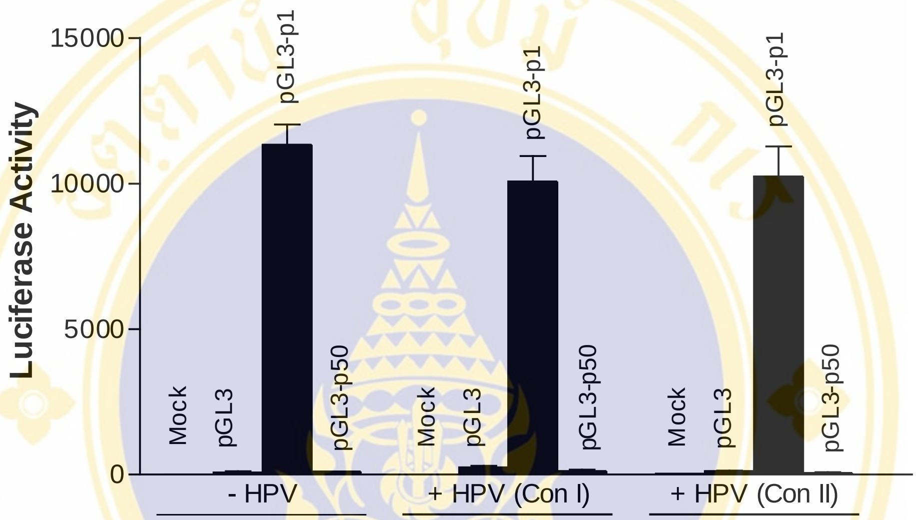


Figure 28: Luciferase assay from transfected Sf9 cells with HPV infection

Transfection in Sf9 was conducted at the optimum condition ($2x10^5$ seeded cells, 1.0 µg DNA and 2:1 charge ratio of Tfx™-20 Reagent to DNA) with HPV infection. Luciferase assay was performed at 48 h post-transfection and luciferase activity was measured from 20 µg of total protein. The error bars represent standard deviation of the experiment in triplicate manner.

- HPV: Transfection without HPV infection

+ HPV (Con I): HPV infection for 90 min followed by transfection

+ HPV (Con II): Transfection and HPV infection at the same time

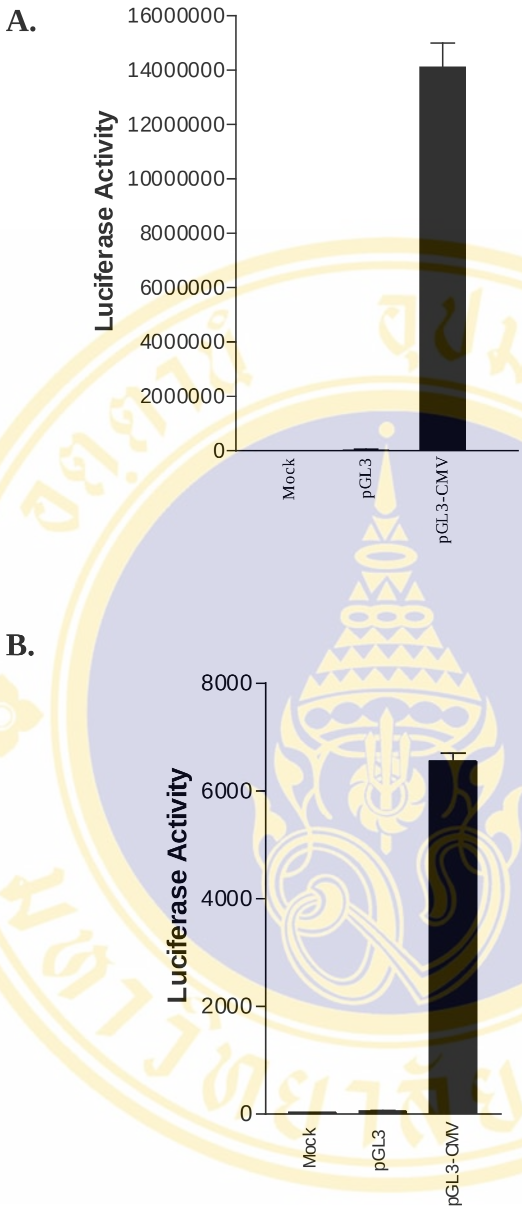


Figure 29: Luciferase assay from CMV promoter in COS-1 and Sf9 cells

Transfection of pGL3-CMV into COS-1 and Sf9 cells was performed using jetPEI™-FluoF (PolyPlus-Transfection) and Tfx™-20 Reagent, respectively. Luciferase activity was measured from 20 µg of total protein at 48h post-transfection. The error bars represent standard deviation of the experiment in triplicate manner.

Panel A: Luciferase activity from CMV promoter in COS-1 cells

Panel B: Luciferase activity from CMV promoter in Sf9 cells

5.5 Transient transfection in primary shrimp cells

Due to unavailable of shrimp cell line, preparation of primary shrimp cells is necessary in order to study gene expression in shrimp cells. In this thesis, Oka primary cells from lymphoid organ and hepatopancreas primary cells from hepatopancreas of *P. monodon* were considered as target cells for promoter studying. Since each cell type requires different conditions of transfection, optimization for the most suitable condition of transient transfection in primary shrimp cells is needed. Both physical and chemical strategies were applied as transfection means. Liposome-mediated transfection was adopted as the chemical method, while the physical way was performed by electroporation. pGL3-RR2 and pGL3-CMV were considered as positive controls in plasmid delivery process because RR2 is the strongest promoter in Sf9 and CMV promoter has been proposed as a functional promoter in shrimp (Arenal *et al*., 2000; Arenal *et al*., 2004; Sulaiman, 1995; Tseng *et al*., 2000).

5.5.1 Liposome-mediated transfection (Lipofection) in shrimp cells

Several liposome reagents are affordable from many companies. Three liposome reagents were considered for introduction of plasmid DNA into shrimp cells, including Tfx™-20 Reagent (Promega), Lipofectamine™ 2000 (Invitrogen), and Cellfectin® Reagent (Invitrogen). Each reagent is the combination of different liposome types. In addition, non lipid-based reagent, jetPEI™-FluoF (PolyPlus-Transfection), which is a stable aqueous solution, was also employed for transfection in shrimp cells. Although, the essential parameters for optimization; such as cell density, amount of DNA, and amount of liposome, are similar, different ways of optimization are specifically needed.

5.5.1.1 Transfection with Tfx™-20 Reagent (Promega)

The manufacturer has guided the optimization for a 24-well plate that the charge ratio of Tfx™-20 Reagent to DNA should be used at 2:1 and 4:1 ratio, the amount of DNA can be varied from 0.25 to 1.0 µg/well, and the cells are incubated with the complex solution for 1 h before the new working medium is added. The recommended cell density is about 80% at the day of transfection.

For condition optimization in Oka cells, all factors were basically varied according to the manufacturer's recommendation. In case of cell density, due to the difficulty in cell counting, the cell confluence was noticed from the turbidity of cell suspension prior to seeding. The confluence was observed under microscope at the time of transfection. pGL3-RR2 was used as DNA vector in this optimization. The amount of plasmid DNA was varied at 0.25, 0.5, 0.75, and 1.0 µg, while both 2:1 and 4:1 charge ratio of Tfx™-20 Reagent to DNA were applied. Oka cells were allowed to incubate with Tfx™-20 Reagent/DNA complex for 1 h followed by adding 1 ml of working medium without removing the complex. At 48 h post-transfection, the transfected Oka cells were collected and the expression of reporter gene was accessed by luciferase assay. However, the luciferase activity could not be detected from any condition.

The condition was further optimized by providing more time for the entry process of complex into cells. The similar conditions from previous experiment at 0.5 and 0.75 µg of pGL3-RR2 as well as 2:1 and 4:1 ratio were applied. The incubation time of complex overlaid onto cells was increased from 1 h to 4, 7, and 10 h before new working medium was added. Even though 2X L-15, the transfection medium, was toxic to cultured cells, higher toxicity was observed from the transfected cells incubated with the transfection complex (Fig 30). The luciferase assay was performed at 48 h after the initial transfection. Like the previous results, none of the luciferase expression was observed. Thus, all transfection conditions with Tfx™-20 Reagent could not show luciferase activity in Oka cells.

5.5.1.2 Transfection with Lipofectamine™ 2000 (Invitrogen)

The optimization of transfection was designed by varying important parameters. According to the manufacturer's protocol, transfection in a 24-well format requires amount of DNA between 0.8-1.0 µg/well, while DNA (in µg): Lipofectamine™ 2000 (in µl) ratio for most cell types locates between 1:2 and 1:3. Nevertheless, this ratio can be additionally varied from 1:0.5 to 1:5 for optimization step. In addition, high cell density about 90-95% confluent at the time of transfection is needed.

Based on the guidance, the experiment for transfection in Oka cells was carefully performed by using 0.8 and 1.0 µg DNA with 1:1, 1:2, 1:3, 1:4, and 1:5 of such ratio. Moreover, both RR2 and CMV promoters were applied for this optimization. The Lipofectamine™ 2000/DNA complex was allowed to overlay onto cells for 6 h before replaced with new working medium. The transfected cells were collected at 48 h post-transfection and subjected to luciferase assay to examine reporter gene expression.

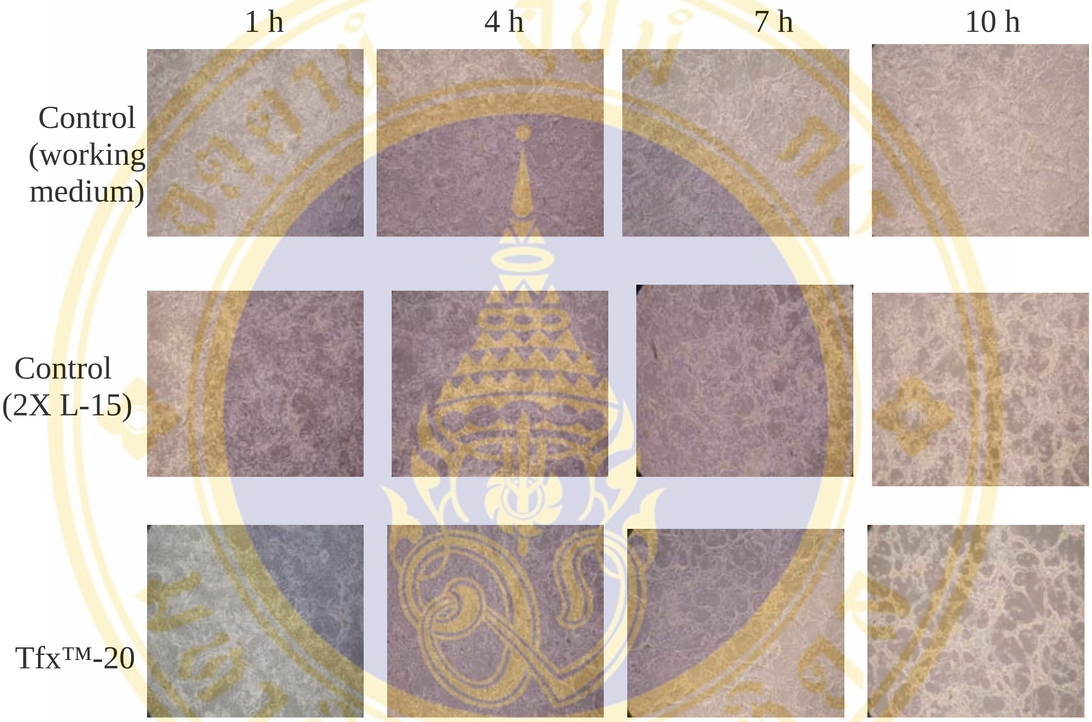


Figure 30: Morphology of primary Oka cells transfected with Tfx™-20

Primary Oka cells were transfected with 0.5 µg pGL3-RR2 at 2:1 charge ratio of Tfx™-20 Reagent to DNA. Complete working medium was added to the overlaid complex at 1, 4, 7, and 10 h after transfection. The controls of Oka cells cultured with working medium and 2X L-15 were exhibited. The photographs were taken at 100X under microscope.

Besides, another experiment was done in a 6-well plate since it provides more space for the fibroblast-like formation of Oka cells. The condition in a 6-well plate was optimized by using 4 µg of pGL3-RR2 plasmid DNA transfected with 1:2 and 1:3 ratios. The complex was replaced after 6 h and luciferase activity was assayed at 48 h after initial transfection. However, the expression of luciferase activity could not be observed from all of these conditions.

To investigate whether the complex had enough time for DNA delivery into inside the transfected cells, the exposure time of cells with the complex was also optimized. The incubation time was increased from 6 h up to 9, 12, 15, and 34 h before the complex was replaced with new working medium. Both RR2 and CMV promoters were applied in this experiment with 1 µg of DNA at 1:2 and 1:3 of the ratio for a 24-well plate. Since Lipofectamine™ 2000 is toxic to cells, the damaged cells were significantly noticed. The expression of luciferase was determined at 48 h after initial transfection. The result from luciferase assay did not show luciferase activity from any condition.

For transfection in hepatopancreas cells, the conditions were optimized by using 0.8 and 1.0 µg of plasmid DNA with 1:1, 1:2, 1:3, and 1:4 of the ratio. pGL3-RR2 and pGL3-CMV were applied for optimization experiment. After Lipofectamine™ 2000/DNA complex was exposed to cells for 6 h, the solution was replaced by new working medium. The transfected cells were collected at 48 h post-transfection and luciferase assay was performed. However, the transfected cells did not showed luciferase activity from these conditions.

5.5.1.3 Transfection with Cellfectin® Reagent (Invitrogen)

In order to optimize the condition in a 24-well plate following the manufacturer's manual, the amount of DNA can be used between 0.2-0.4 µg whereas amount of Cellfectin® Reagent is varied from 0.4 to 3 µl. The experimental condition was done by using constant amount of DNA (pGL3-RR2) at 0.4 µg and applying Cellfectin® Reagent at 0.4, 1.4 and 3 µl. The Cellfectin® Reagent/DNA complex was incubated with cells for 6, 9, 12, 15, and 29 h before replaced with new working medium. Like other regents, Cellfectin® Reagent caused damaged cells due to its toxicity. Moreover, Oka cells seeded with low density easily resulted in cell death. The luciferase expression was assayed at 48 h post-initial transfection. The luciferase activity could not be measured from all conditions in optimization experiments using Cellfectin® Reagent.

Hepatopancreas primary cells were also transfected with Cellfectin® Reagent by using constant amount of DNA (pGL3-RR2 and pGL3-CMV) at 0.4 µg while Cellfectin® Reagent was varied at 0.5, 1, 2, and 3 µl. The Cellfectin® Reagent/DNA complex was incubated with cells for 6 h before replaced with new working medium. The luciferase expression was assayed at 48 h post-transfection. The results did not show luciferase activity from the transfected hepatopancreas cells using Cellfectin® Reagent.

5.5.1.4 Transfection with jetPEI™-FluoF (PolyPlus-Transfection)

For transfection in Oka cells, the experiments were designed for a 24-well plate based on the recommendation from the manual protocol using pGL3-RR2 as a control. The amount of plasmid DNA was constantly used at 1 µg and N/P ratio was applied at 5, 8, and 10. The total volume at 0.5 ml and 1.0 ml of the complex solution overlaid onto cells was considered. Unlike other reagents, the jetPEI™-FluoF/DNA complex solution was continuously incubated with the transfected cells without either replacing or adding of new working medium. The transfected cells were collected at 24, 48, and 72 h after transfection and luciferase assay was performed. Nevertheless, although the conditions of transfection with jetPEI™-FluoF were optimized covering the recommended range, luciferase activity could not be detected from all conditions.

As previously described, jetPEI™-FluoF is conjugated with fluorescent labeling that allows the tracking of complex inside the cell. However, the difference of fluorescence between the transfected cells and non-transfected cells could not be observed because of the auto-fluorescent from Oka cells themselves. Therefore, it could not be concluded whether the complex was passed into intracellular compartment.

The optimization for transfection in hepatopancreas primary cells with jetPEI™-FluoF was performed. pGL3-RR2 was constantly used at 1 µg whereas the N/P ratio was applied at 5 and 8 with 0.5 ml and 1.0 ml total volume of the complex solution. The complex was incubated with cells without replacing of new working medium until reporter gene assay was reached. The transfected hepatopancreas cells were collected at 24 and 48 h after transfection and luciferase assay was performed. Nevertheless, luciferase activity could not be detected from all conditions at both time points. Additionally, the fluorescence conjugated with the reagent could not be noticed from the transfected hepatopancreas cells under microscope.

5.5.2 Electroporation in Oka cells

The important consideration for efficiency of DNA introduction into cells by electroporation method is the mortality rate after electroporation. Theoretically, DNA delivery by electroporation is successful when the mortality of the electroporated cells reaches 50% (Heiser, 1999; Schenborn, 1999). Therefore, such condition causing at least 50% of cell death in Oka cells was optimized by electroporation without DNA vector for condition screening. Table 3 shows the conditions of electroporation in Oka cells. The morphology of the electroporated cells was observed under microscope as shown in Figure 31.

Table 3. Conditional screening for electroporation in primary Oka cells

Condition	Capacity (μF)	Voltage (V)	Pulse
1	200	200	2
2	200	200	3
3	200	300	1
4	200	300	2
5	200	300	3
6	200	400	1
7	200	400	2
8	200	400	3

Of all, five conditions (200μF 200V 3 pulses, 200μF 300V 2 pulses, 200μF 300V 3 pulses, 200μF 400V 1 pulse, and 200μF 400V 2 pulses) causing at least 50% of cell mortality were used for introduction of pGL3-RR2 and pGL3-CMV into cells. The expression of reporter gene was assayed at 48 h after electroporation. However, like the liposome-mediated transfection, the luciferase activity could not be measured from all electroporation conditions in primary Oka cells.

5.6 Reporter gene detection using RT-PCR method

Based on the luciferase assay results, luciferase activity could not be detected from any condition although both chemical and physical methods were applied. However, luciferase assay is the detection means for expression of luciferase reporter gene in translation level. Therefore, the detection of luciferase expression using RT-PCR was performed to clarify the expression in transcription level. Two transfection conditions in Oka cells; transfection with Lipofectamine™ 2000 at 1.0 µg pGL3-CMV and pGL3-RR2, as well as electroporation of pGL3-RR2 at 200 µF, 400V, 2 pulses, were subjected for RT-PCR assay. Contamination of plasmid DNA was avoided by removal with RQ1 RNase-free DNase (Promega). Luciferase transcripts were amplified using luc sense and luc antisense primers, whereas amplification of shrimp β-actin using actin-s and actin-as primers was applied as internal control. The results from agarose gel electrophoresis depicted none of luciferase expression in transcriptional level as shown in Figure 32 and Figure 33 for liposome transfection and electroporation, respectively.

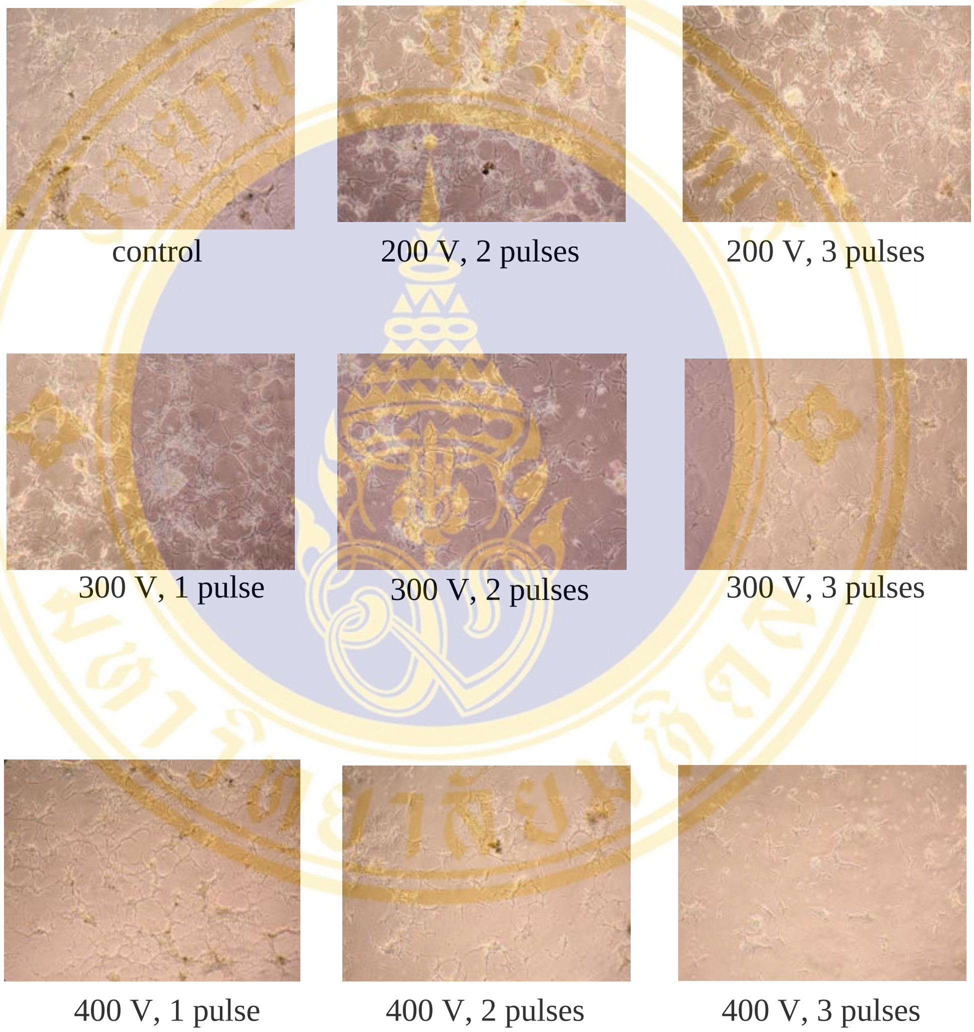


Figure 31: Morphology of primary Oka cells after electroporation

Electroporation of primary Oka cells was constantly performed at 200 µF, while voltage was varied from 200-400 V. Single and multiple pulses were applied as indicated. The photographs were taken at 100X under microscope (64h post-electroporation).

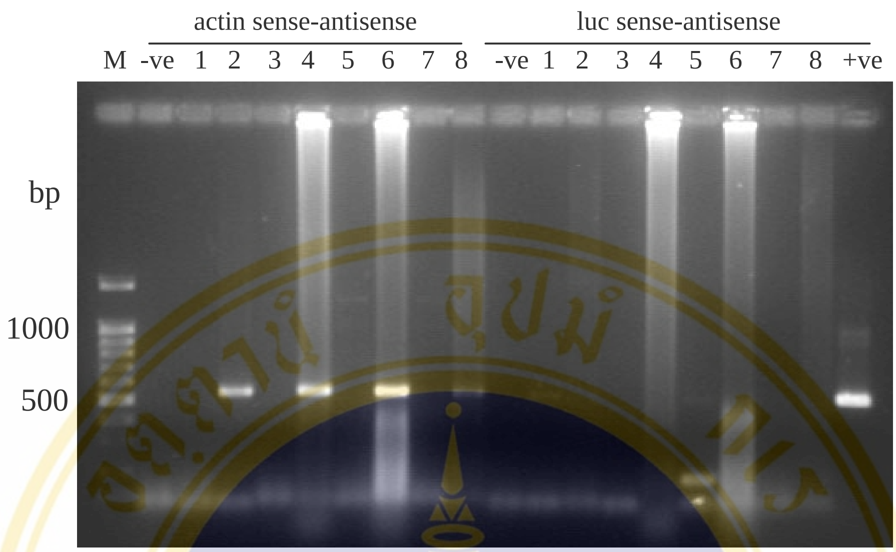


Figure 32: RT-PCR of luciferase transcripts from transfection with Lipofectamine™ 2000 in Oka cells

Total RNA was extracted from Oka cells transfected with 1.0 µg DNA (pGL3-CMV and pGL3-RR2) and 1:2 ratio of DNA (µg) to Lipofectamine™ 2000 (µl) at 24 and 48 h post-transfection. Amplification of actin transcripts was performed as an internal control, whereas luciferase transcripts were amplified using specific primers, luc sense and luc antisense. The PCR products were visualized by agarose gel electrophoresis stained with ethidium bromide.

M: 100 bp ladder with 1.5 kb plus

-ve: Amplification without cDNA template

1: Amplification with cDNA from pGL3-CMV at 24h post-transfection without RT

2: Amplification with cDNA from pGL3-CMV at 24h post-transfection with RT

3: Amplification with cDNA from pGL3-CMV at 48h post-transfection without RT

4: Amplification with cDNA from pGL3-CMV at 48h post-transfection with RT

5: Amplification with cDNA from pGL3-RR2 at 24h post-transfection without RT

6: Amplification with cDNA from pGL3-RR2 at 24h post-transfection with RT

7: Amplification with cDNA from pGL3-RR2 at 48h post-transfection without RT

8: Amplification with cDNA from pGL3-RR2 at 48h post-transfection with RT

+ve: Amplification using pGL3-Basic vector as DNA template (5ng)

(RT: Reverse transcriptase)

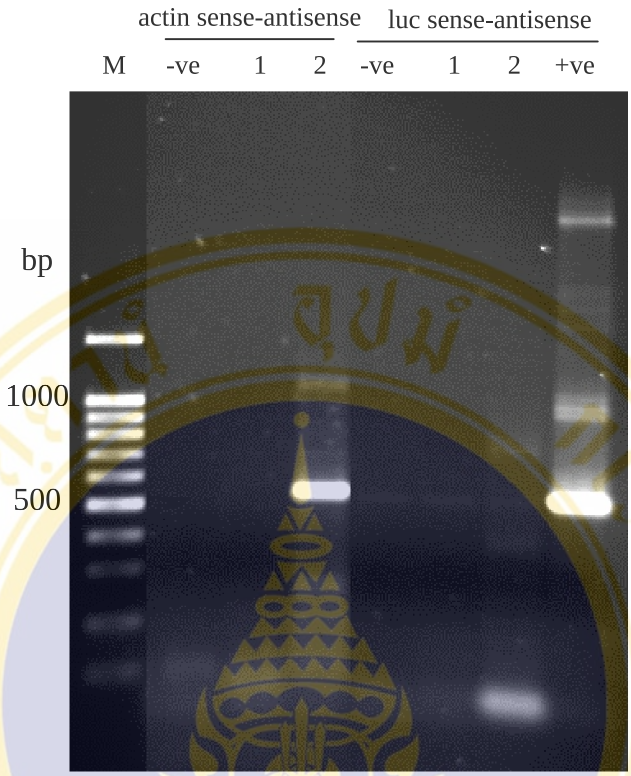


Figure 33: RT-PCR of luciferase transcripts from electroporation in Oka cells

Total RNA was extracted from Oka cells electroporated with 20µg pGL3-RR2 at 200 µF, 400V with 2 pulses. The assay was performed at 24 h post-transfection. Amplification of actin transcripts was used as an internal control using actin sense and antisense primers. The luciferase transcripts were amplified by luc sense and luc antisense primers. The PCR products were detected by agarose gel electrophoresis stained with ethidium bromide.

M: 100 bp ladder with 1.5 kb plus
-ve: Amplification without cDNA template
1: Amplification from cDNA without RT
2: Amplification from cDNA with RT
+ve: Amplification using pGL3-RR2 as DNA template (5ng)
(RT: Reverse transcriptase)

CHAPTER VI
DISCUSSION

Functional activity of several promoters; including CMV promoter, putative p1 and p50 promoters from HPV, as well as putative RR1, RR2, VP15, and VP19 promoters from WSSV, was characterized in this thesis. The expression cassettes containing individual promoter were introduced into cultured cells followed by functional assay. To avoid the difficulty of primary cell preparation due to unavailable shrimp cell line, Sf9 insect cell line was preferably chosen to provide preliminary information about functional activity of investigated promoters. After that, their functions would be further characterized in primary shrimp cells.

The expression cassette used for promoter functional analysis composed of the investigated promoter, reporter gene, and poly(A) signal region. Firefly luciferase-encoding gene was applied as a reporter gene in this study. Although many reporter genes have been affordable such as green fluorescent protein (GFP) that is easily detected under a fluorescent microscope, it cannot be used as a reporter gene in shrimp cells. GFP expression could be interfered by auto-fluorescence since endogenous fluorescent compounds have been reported in *Penaeus vannamei* (Sun *et al*., 2005).

Of all promoters, their regions harbor consensus elements usually found in RNA polymerase II core promoters. A typical core promoter encompasses TATA box, Inr, DPE, and BRE sequences (Carey and Smale, 1999; Smale and Kadonaga, 2003). Such elements have been proposed for p1 and p50 promoters of HPV (Sukhumsirichart *et al*., 2005). In addition, the regulatory elements, E box and PRE, broadly located in some parvovirus promoters (Martyn *et al*., 1990; Murre *et al*., 1989; Ranz *et al*., 1989) were also identified in p50 promoter. However, since the binding positions of RNA polymerase II in promoter region covers -42 to +23 from the start site (Hahn, 2004), the TATA box of p50 promoter located at positions -54 to -49 is not placed within such region (Sukhumsirichart *et al*., 2005). Besides, DPE element was not included in the amplified p50 region in order to avoid the improper translational start sites. Although these start codons (ATG) are not placed within the Kozak context (Kozak, 1989), the mistaken ORF may be occurred. Moreover, the amplified p50 promoter region does not cover other three additional E boxes positioned within 700 bp upstream of TATA box.

In case of WSSV promoters, only TATA box and Inr have been identified for RR1, RR2, VP15, and VP19 promoters (Tsai *et al*., 2004; Tsai *et al*., 2000). However, DPE and BRE sequences were also found in RR1 and RR2 promoters, but not in VP15 and VP19. Moreover, the actual transcriptional start sites of VP15 and VP19 promoters confirmed by 5'RACE (Marks *et al*., 2003) are not located in the predicted Inr (Tsai *et al*., 2004), and the amplified VP15 promoter region does not cover such start site. Besides, VP19 displays odd manner of TATA box that is located in the distant position from typical position (Tsai *et al*., 2004). Thus, the transcription of VP19 was regulated by A/T rich region located at roughly 30 bp upstream of transcriptional start site, not by the unusual TATA box positioned at distant region (Marks *et al*., 2003). This A/T rich region was also proposed in some promoters of VP genes from WSSV (Marks *et al*., 2003).

Unlike promoters from shrimp viral pathogens, CMV promoter does not depict DPE and BRE consensus sequences. Nevertheless, upstream activating elements including CAAT box and CAAT box-like sequences found in some mammalian viral promoters (McKnight and Tjian, 1986) have been recognized in CMV promoter (Thomsen *et al*., 1984).

In order to obtain the optimal condition for plasmid DNA delivery into Sf9, pGL3-CMV was used as a positive control. Although CMV is a functional promoter in mammalian cells (Como *et al*., 1999; Kamahora *et al*., 2000), several studies have showed its activity in Sf9 cells (Promega Notes, 1997; Chan *et al*., 1998; Condreay *et al*., 1999) as well as in penaeid shrimps (Arenal *et al*., 2000; Arenal et al., 2004; Sulaiman, 1995; Tseng *et al*., 2000). The optimal condition for transfection in Sf9 was gained at $2.0x10^5$ seeded cells transfected with 1.0 µg DNA and 2:1 charge ratio. It is slightly different from condition optimized from the manufacturer (Promega) that the highest luciferase activity was measured from $1.8\text{-}2.0x10^5$ seeded cells transfected with 0.75 µg and 2:1 charge ratio (Promega Notes, 1997). Nevertheless, both studies confirm the common characteristic for liposome transfection that there was no proportional dose-response effect with increasing concentrations of DNA and liposomes (Promega Notes, 1997). This optimal condition was adopted as transfection condition to characterize promoter functions in Sf9.

Functional analysis in Sf9 showed that luciferase expression was detected from RR1, RR2, p1, and CMV promoters, but not from p50, VP15, and VP19 promoters. Although the luciferase expression was not normalized with another reporter gene to determine the transfection efficiency, the same manner of all promoter activities in Sf9 was noticed from three independent experiments. In addition, its efficiency may not be affected by either plasmid size or plasmid purity. Even though plasmid size is seemed to affect the transfection efficiency (Kreiss *et al.*, 1999), all expression cassettes were constructed in the same strategy leading to slightly different in plasmid size (4.9-5.3 kb). Moreover, plasmid DNA was purified by QIAGEN Anion-Exchange Resin to ensure highly purified DNA which is suitable for DNA transfection (Sambrook and Russell, 2001; Schenborn and Oler, 1999).

Among these promoters, RR2 is the strongest promoter in Sf9. Even though both RR1 and RR2 are promoters from WSSV, RR2 activity in Sf9 is higher than RR1 and the same result was also proposed by M.S. Hossain *et al.* (Hossain *et al.*, 2004). Besides, this manner was reported by Lin *et al.* (Lin *et al.*, 2002) using Western bolt analysis from WSSV-infected *P.monodon* that the expression level of RR2 was higher than that of RR1 (Lin *et al.*, 2002). Since the transcription of WSSV genes has not been extensively studied and few WSSV specific promoter sequences have been identified (van Hulten *et al.*, 2001), RR2 promoter region may contain binding sites for activators or even supporting the formation of transcription initiation complex.

Although HPV and WSSV are viral pathogens in penaeid shrimps, RR1 and RR2 promoters from WSSV showed higher luciferase expression than p1 from HPV. The difference of virulent degree may affect promoter strength, in which WSSV causes high mortality rate of the infected shrimps (Chou *et al.*, 1995) whereas only growth retardation has been observed from HPV-infected shrimps (Flegel *et al.*, 1999). Therefore, it may be assumed that p1 promoter is intrinsic weakness even though in its host. Moreover, unlike RR1 and RR2, the transcriptional start site of p1 promoter is not experimentally confirmed such as by 5'RACE (Tsai *et al.*, 2000). In addition, specific factors may be needed to activate p1 activity, for instance, some promoters of non-structural proteins in parvovirus require the binding of NS1 to stimulate promoter functions (Doerig *et al*., 1990; Gareus *et al*., 1998).

Like RR1 and RR2, CMV promoter showed higher luciferase expression than p1. The functional activity of p1 promoter has not been previously reported. Moreover, consensus sequences and transcription factors have not been specifically identified for p1 promoter. In contrast, the promoter region of CMV harboring the essential elements as well as sequence-like enhancer has been widely used as functional promoter in many cell types, especially in mammalian cells (Como *et al*., 1999; Kamahora *et al*., 2000). Thus, the basal level of CMV activity measured in Sf9 may be suggested that Sf9 cells sufficiently provide necessary factors at least for basal gene expression of CMV promoter (Chan *et al*., 1998; Condreay *et al*., 1999).

In case of promoters from structural proteins, luciferase expression could not be measured from p50, VP15, and VP19 promoters in Sf9. Specific transcription factor(s) may be needed to activate their functions. For instance, many promoters of structural proteins in parvovirus are regulated by NS1 proteins encoded from its genome and gene expression could be enhanced more than 1,000-fold over the low basal level (Afanasiev *et al*., 1994; Doerig *et al*., 1988; Lorson *et al*., 1996). To clarify whether NS1 is needed for p50 promoter function, transfection of pGL3-p50 to the HPV-infected Sf9 cells was conducted. The luciferase activity could not be detected from transfection with HPV infection. Because PCR result did not show HPV infection in Sf9; thus, the requirement of NS1 for p50 activity could not be concluded. However, instead of HPV infection, co-transfection of pGL3-p50 with NS1-expressing recombinant plasmid into Sf9 can be performed to clarify such issue (Gareus *et al*., 1998; Vanacker *et al*., 1996). For VP15 and VP19 promoters from WSSV, since binding sites for specific proteins in promoter regions have not been identified, the necessary of trans-activator to stimulate their functions is still unknown.

After obtaining the preliminary information about promoter functions in Sf9, characterization of promoter activity in primary shrimp cells was subsequently conducted. In optimization, pGL3-RR2 and pGL3-CMV were adopted as positive controls since RR2 from WSSV, a shrimp viral pathogen, is the strongest promoter in Sf9, while CMV has been proposed as a functional promoter in penaeid shrimps (Arenal *et al*., 2000; Arenal *et al*., 2004; Sulaiman, 1995; Tseng *et al*., 2000) . Primary shrimp cells, Oka and hepatopancreas cells, were transiently transfected with different liposome reagents (Tfx™-20 Reagent, Lipofectamine™ 2000, Cellfectin® Reagent, and jetPEI-FluoF) by optimizing all important parameters such as amount of DNA and liposome, cell confluence, and complex incubation time covering the recommended instruction. Meanwhile, electroporation was also performed in Oka cells. The balance between cell death and efficiency of gene transfer should be considered in electroporation, and optimal transfection may occur under conditions that cause approximately 50% cell death (Heiser, 1999; Schenborn, 1999). Although the high efficient transfection in mammalian cells is generally obtained when electroporated in high-ionic-strength buffer rather than in non-ionic buffer, the suitable medium used in electroporation should be determined specifically in each cell type (Heiser, 1999; Sambrook and Russell, 2001). In the experiment, Oka cells were electroporated from conditions causing at least 50% cell mortality using complete medium containing salt solution. However, the luciferase assay did not show any luciferase expression from the transfected cells at all conditions. Therefore, the alternative way was conducted by detection of luciferase transcripts to examine reporter gene expression in transcriptional level. Like luciferase assay, none of the transcripts was observed from RT-PCR results.

The absence of luciferase expression in shrimp cells may be caused either by the failure of DNA introduction into nucleus where transcription machinery is located or by the lack of some transcription factors in shrimp cells. The amplified RR2 and CMV promoter regions encompass the essential elements for basal activity leading to luciferase expression in Sf9. Several studies have exhibited foreign gene expression regulated by CMV promoter in penaeid shrimps suggesting that transcription factors in shrimp cells sufficiently support at least the basal level of CMV activity (Arenal *et al.*, 2000; Arenal *et al.*, 2004; Sulaiman, 1995; Tseng *et al.*, 2000). In addition, penaeid shrimp is the natural host of WSSV and necessary transcription factors should be available in shrimp cells to foster RR2 function. However, *rr1* and *rr2* have been identified as non-immediate early genes (Liu *et al.*, 2005) suggesting that their promoter functions may depend on the presence of viral protein transcription factor(s). Hence, these factors could be substituted in Sf9 cells leading to successful detection of RR1 and RR2 promoter activities in Sf9 cells (Hossain *et al.*, 2004). Despite the high sensitivity of PCR amplification, RT-PCR results suggest that none of the luciferase transcripts was observed from the transfected cell. Therefore, it may be assumed that the applied conditions failed to deliver plasmid DNA into nuclear compartment of the transfected cells.

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 (Brisson and Huang, 1999; Brunner *et al*., 2002; Tseng *et al*., 1999; Zabner *et al*., 1995). For instance, cell division of Sf9 was observed in roughly 20 h (Braunagel *et al*., 1998). In case of primary shrimp cells, unlike cell line, only 10-15% of Oka cells from *P. stylirostris* exhibited cell dividing during 6-24 h post-initiation of the culture (Shike *et al*., 2000), while the cell division in Oka cells from *P. monodon* was found about 25% at day 3-4 after seeding (Assavalapsakul, 2004). Therefore, this factor was concerned and the transfection in Oka and hepatopancreas cells was performed during 3-4 days and 1-2 days post-seeding, respectively, to ensure the occurrence of cell division. Besides, the transfection means including electroporation and jetPEI™-FluoF, which the plasmid DNA delivery is not absolutely dependent from cell division (Brunner *et al*., 2002) providing small difference in gene expression between G1 and S/G2 phases (Brunner *et al*., 2002), were applied. Nevertheless, luciferase expression was not measured from both methods in shrimp cells. Moreover, another one limitation of transfection into primary shrimp cells is the variability of cell quality was obtained in each preparation. Normally, the fibroblast-like morphology was observed within few days after seeding and then transfection experiment was performed. However, shrimp cells from some preparations showed incomplete fibroblast formation and the cell mortality was noticed earlier than normal cells. Thus, the cell quality may affect the suitable period of transfection. Additionally, lacking of accurate cell number in each transfection experiment may have an influence to transfection efficiency that the same cell density should be repeatedly maintained (Sambrook and Russell, 2001).

Not only this thesis, but H. Shike *et al*. (Shike *et al*., 2000) has also reported that plasmid DNA transfection using various liposome reagents, such as DOTPA

(Boerrhinger Mannheim), Lipofectamine (Gibco BRL), Escort (Sigma), Insectin Plus (Invitrogen), as well as electroporation did not yield expression of foreign genes in shrimp cells (Shike *et al*., 2000). Thus, cultured shrimp cells appear to be troublesome in the introduction and expression of foreign DNA (Shike *et al*., 2000). To avoid difficulty of transient transfection in primary cells, the modified retroviral vectors were applied to achieve foreign gene expression in primary cultured cells from the Oka organ and ovary of *P. stylirostris* (Shike *et al*., 2000).

Presently, many research groups have attempts to express foreign genes in penaeid shrimps in order to understand biological system, to produce shrimp capable of combating diseases, to improve aquaculture production, and so on. Thus, functional promoters in penaeid shrimps are much needed. Until now, functional promoters in penaeid shrimps have been proposed including CMV promoter (Arenal *et al*., 2000; Arenal *et al*., 2004; Sulaiman, 1995; Tseng *et al*., 2000), SV40 promoter (Cabrera *et al*., 1995), LTR promoter (Shike *et al*., 2000), carp β-actin promoter (Arenal *et al*., 2000), and shrimpβ-actin promoter (Sun *et al*., 2005). Moreover, some promoters from shrimp viral pathogens are functional in Sf9 insect cells, such as promoters of immediate early gene (ie1), protein kinases (PK1 and PK2), endonuclease, thymine-thymidylate kinase, ORF89, RR1, and RR2 from WSSV (Hossain *et al*., 2004; Liu *et al*., 2005), as well as p1 promoter from HPV. These promoters should be considered as one of the candidates for further applications in penaeid shrimps. For instance, ie1 promoter derived from immediate early gene does not require viral regulatory proteins for its function and it is also the functional promoter in Sf9 cells. Therefore, it has an outstanding potential to be used for foreign gene expression in further studies.

CHAPTER VII
CONCLUSION

7.1 Seven promoters; CMV promoter, putative p1 and p50 promoters from HPV, as well as putative RR1, RR2, VP15, and VP19 promoters from WSSV, were functionally analyzed in this study. The expression cassettes (pGL3-promoter) that harbor luciferase reporter gene regulated by individual promoter were constructed.

7.2 The optimal transfection condition using Tfx™-20 Reagent (Promega) in Sf9 was obtained at $2x10^5$ seeded cells transfected with 1.0 µg at 2:1 of charge ratio.

7.3 In Sf9, RR2 promoter provided the highest luciferase activity compared with others. The relative promoter strength ordered from higher to lower activity is RR2, RR1, CMV, and p1 promoters, respectively. On the other hand, none of luciferase activity was measured from promoters of viral structural proteins, including p50, VP15, and VP19 promoters.

7.4 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. In liposome transfection, four different liposome types (Tfx™-20 Reagent, Lipofectamine™ 2000, Cellfectin® Reagent, and jetPEI-FluoF) were used for transfection in shrimp cells. The essential parameters, such as amount of DNA, ratio of DNA to liposome, and complex incubation time, affecting transfection efficacy were varied covering the recommended range from the manufacturer. As for electroporation, five conditions leading to at least 50% cell death were used for plasmid delivery in primary shrimp cells. 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.

7.5 The plausible rationale for the absence of luciferase expression may be due to either the failure of plasmid delivery into nuclear compartment, or the lacking of some transcription factors in shrimp cells to stimulate promoter activity, or both.

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