Transient Expression of a Heterologous Gene Driven by Promoters Isolated from Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) of Shrimp

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ABSTRACT

Two putative promoter elements of the infectious hypodermal and hematopoietic necrosis virus (IHHNV) of shrimp were cloned and their abilities to drive heterologous gene expression in shrimp and bacteria were determined. These promoters (herein referred to as P2 and P61) are upstream of the Left (non-structural protein) and Right (capsid protein) open-reading frames (ORFs) contained within the IHHNV genome, respectively. The P2 and P61 promoters were cloned upstream of the firefly Luciferase gene (*Luc*) in the pGL3-Basic (pGL3-B; contains no regulatory element) and pGL3-Enhancer (pGL3-E; contains the SV40 enhancer element) promoter trapping vectors. Luciferase activity was assayed in recombinant bacteria containing these constructs as well as in shrimp tail muscle taken from animals injected intramuscularly with plasmid DNA. In bacteria, *Luc* expression driven by the P61 promoter in pGL3-B was greater than that from the P2 promoter. The SV40 enhancer element contained in pGL3-E based constructs increased bacterial expression driven by the P61 marginally, while expression under P2 control was inhibited. In shrimp, there was no significant difference in luciferase expression driven by these two promoters. The SV40 enhancer element suppressed P61driven Luciferase expression in shrimp. Our data suggest that both P2 and P61 are constitutive promoters, and that these promoters can drive gene expression in prokaryotes and eukaryotes. Having shrimp virus promoters available to crustacean molecular biologists provides new tools for studies in the functional genomics in shrimp.

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INTRODUCTION

Shrimp bioengineering and basic molecular biological research on shrimp have been hampered by the lack of effective tools. There are no shrimp immortalized cell lines, effective promoters for expression of heterologous genes, transposons, regulatory genes, enhancers, or any of the myriad other tools necessary for effective directed manipulation in whole shrimp. Transgenic shrimp have been developed with embryo manipulation (Lu and Sun, 2005), but many of the more directed structure /function research that relies on rapid experiments in shrimp have lagged due to lack of appropriate molecular tools. This study characterizes two shrimp viral promoters that will add to the arsenal of tools for shrimp molecular biological manipulation, perhaps enabling the field to produce an immortalized cell line, identify viral disease control strategies, and provide other benefits that can be derived from the application of modern biotechnological research to this field.

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) is one of the most important viral pathogens that infect penaeid shrimp worldwide (Lightner *et al*., 1996). The virus was first detected in Hawaii in 1981, where it caused lethal disease (up to 90% mortality) in juvenile blue shrimp (*Penaeus stylirostris*) (Bell and Lightner, 1983; Lightner *et al*., 1983). IHHNV is infectious to many other *Penaeus* species (Kalagayan *et al*., 1991; Primavera and Quinitio, 2000). In *P. vannamei* and *P. monodon*, IHHNV causes "Runt Deformity Syndrome" (RDS), which is characterized by reduced growth rates and a variety of cuticular deformities of the rostrum, antenna, thorax, and abdomen (Kalagayan *et al*., 1991; Primavera and Quinitio, 2000).

IHHNV virions are icosahedral, non-enveloped, and measure 22 nm in diameter. The virus contains a linear single-stranded DNA of ~4.1 kb in size (Bonami *et al*., 1990). There are three (Left, Middle, and Right) large open reading frames (ORFs) in the IHHNV genome (Figure 1). The Left ORF most likely represents a non-structural protein, since it contains replication initiator motifs, NTP-binding, and helicase domains (Shike *et al*., 2000). The Right ORF encodes the capsid protein, while the function of the Middle ORF is not known. There are two putative promoters located upstream of the Left (designated

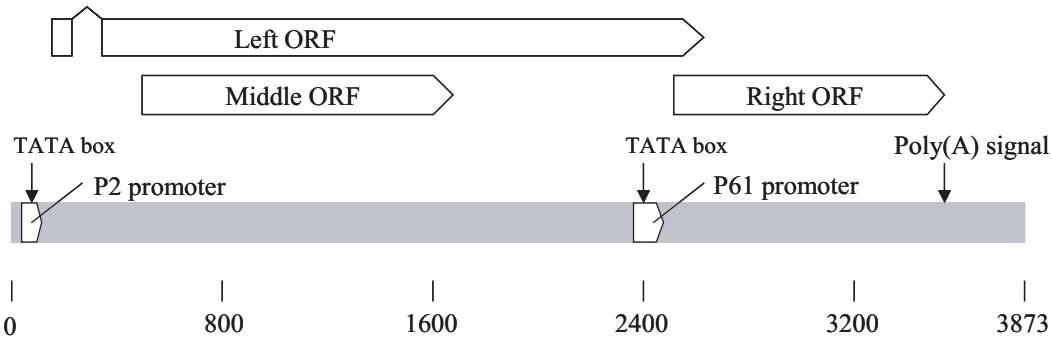


Figure 1. IHHNV genome organization based on the sequence of GenBank accession number AF273615. P2 and P61, the two promoters in this study, are located as indicated. Numbers correspond to the nucleotide number of the sequence ORF.

P2) and the Right (designated P61) ORFs. The genome organization of IHHNV is similar to the densoviruses of the genus *Brevidensovirus* in the family *Parvoviridae* (Shike *et al.*, 2000).

To characterize the IHHNV promoters, the putative P2 and P61 regions were cloned upstream of the firefly luciferase gene (*Luc*) in two transcription reporter vectors, pGL3-Basic and pGL3-Enhancer. These vectors differ in that the latter carries the SV40 enhancer sequence downstream of the reporter gene. The recombinant bacteria were assayed for luciferase activity. The plasmid DNA of P2 and P61 promoter constructs were then used to transfect shrimp tail muscle tissue to determine if *in vivo* luciferase transient expression was observed. The IHHNV promoters were demonstrated to be capable of driving expression of a heterologous gene in both a prokaryotic and its native eukaryotic host (shrimp).

MATERIALS AND METHODS

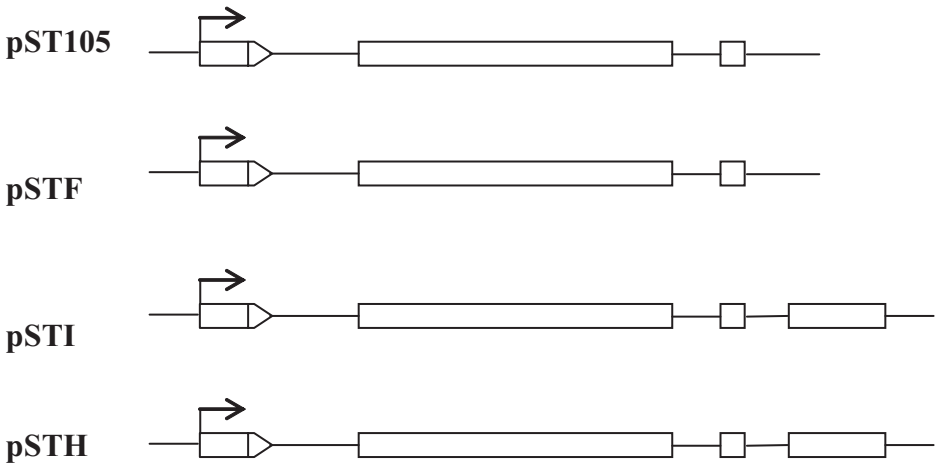
Cloning of IHHNV promoters

The putative IHHNV P61 promoter region (GenBank Accession No: AF273215) was amplified by PCR using the primers: IHHNP61F, 5'GGTACCTCCA GCTGATGGTA AAGCT3' (nucleotides 2347-2371) and IHHNP61R, 5'TTCGTATTCT TGGAAGAGTC CTAG3' (nucleotides 2488-2512) as forward and reverse primers, respectively. The P61 amplicon was cloned in vector PCR2.1-TOPO (Invitrogen, Carlsbad, CA) and reamplified by PCR to generate *Sac*I and *Nhe*I endonuclease restriction sites on the 5' and 3' termini of the amplicon, respectively. To synthesize the putative IHHNV P2 promoter region (nucleotides 21-110) flanked by *Sac*I and *Nhe*I sites, 108-mer sense and antisense oligonucleotides were synthesized (Midland Certified Reagent Co., Midland, TX). The sense and antisense oligonucleotides were annealed and then digested with *Sac*I and *Nhe*I for insertion into the luciferase reporter vectors.

Cloning of luciferase reporter gene

Plasmids constructed for this study were based on pGL3-Basic and pGL3-Enhancer luciferase transcription trapping vectors (Promega, Madison, WI). These vectors contain a modified coding region for firefly (*Photinus pyralis*) luciferase (*Luc*) that has been optimized for evaluating transcriptional activity of unknown promoters in transfected eukaryotic cells. Vector pGL3-Enhancer differs from the pGL3-Basic in that it contains a SV40 eukaryotic enhancer element downstream of the *Luc* gene (Fig. 2). The P2 and P61 promoters were inserted between the *Sac*I and the *Nhe*I sites of pGL3-Basic and pGL3-Enhancer vector upstream of the *Luc* gene using the DNA Ligation kit "Mighty Mix" (Takara, Shiga, Japan) according to the manufacturer's recommendations then transformed into *Escherichia coli* cells (Strain JM109, Invitrogen). Plasmid DNA was isolated from recombinant clones and sequenced for verification. The pGL3-Basic derived clones containing the P2 and P61 promoter were designated pSTI05 and pSTF, respectively, and the pGL3-Enhancer derived clones containing the P2 and P61 promoter were designated as pSTI and pSTH, respectively (Figure 2).

Figure 2. P2 and P61 IHHNV promoter cloning: (A) vector pGL3-Basic, resulting in plasmids pSTI05 and pSTF, and (B) into vector pGL3-Enhancer, resulting in plasmids pSTI and pSTH.



Luciferase assay in recombinant bacteria

Luciferase expression was measured in recombinant bacteria containing P2 and P61 promoter constructs. Triplicate sets of log phase bacterial cultures carrying the control plasmid pUC19 or reporter plasmids (pGL3-Basic, pGL3-Enhancer, pSTI05, pSTF, pSTI, and pSTH) were obtained by growing 100-fold diluted stationary phase cultures in Luria-Bretani (LB) medium containing 100 µg/mL ampicillin at 37ºC in a shaker for 2.5 hours. Bacterial cells (1.5 mL) were pelleted, then resuspended in 0.1 mL bacterial treatment buffer (100 mM $KHPO_4$ (pH 7.8), 2 mM EDTA, 1 µM PMSF (Sigma-Aldrich, St. Louis, MO), and 1 µM benzamidine-HCl (Sigma-Aldrich)). Resuspended bacteria were frozen at -80ºC for 5 min and then thawed in water at room temperature for 5 min. Then 0.3 mL bacterial lysis buffer (Cell Culture Lysis Reagent; Promega, Madison, WI) supplemented with 1.25 mg/mL lysozyme (Research Organics, Cleveland, OH), 2.5 mg/mL BSA (Fisher Scientific, Newark, DE), and 1 µM PMSF, and 1 µM benzamidine-HCl was added and the cells were briefly vortexed and incubated at room temperature for a further 10 min. Cellular debris was removed by centrifugation and 50 µL of the supernatant assayed immediately for luciferase activity while the remaining supernatant was used for total protein determination following Lowry's protocol (Lowry *et al*., 1951) with BSA as protein in the calibration series. The luciferase assay was performed using the Bright-Glo assay system (Promega) according to the manufacturer's protocol. Luminescence measurements were done in white, flat bottom 96-well microplates (Greiner, Bio-One, Longwood, FL) on a Tecan SpectraFluor Plus in the luminometer mode (XFLUOR4 Version 4.50; Tecan US, Research Triangle Park, NC). Readings were converted into luciferase equivalents via a calibration curve prepared by serial dilution of luciferase (Quantilum recombinant luciferase; Promega) into bacterial extract from bacterial cells carrying the control pUC19 plasmid. Luciferase activity in bacteria was normalized to the total protein content and data presented in parts per million.

Luciferase assay in shrimp (*Penaeus vannamei*)

Specific Kona line pathogen-free *P. vannamei* shrimp were purchased from Marine Resources Research Institute, South Carolina Department of Natural Resources (Charleston, SC) and kept at approximately 24ºC using artificial seawater prepared by dissolving Instant Ocean (Aquarium Systems, Mentor, OH) in tap water. Shrimp averaging ~1.25 g were injected in the 4th abdominal segment of the tail muscle with 25-30 µL of a freshly prepared suspension consisting of 10 µg plasmid DNA in 20% glycerol and 0.9% NaCl. Animals were sacrificed at 70 to 72 hr post-injection, immediately frozen in liquid N_2, and then stored at -80ºC until assayed. Approximately 0.25 mg tissue, preferentially including the site of injection, was cut from each shrimp and manually homogenized on ice in a microcentrifuge tube in 200 µL Cell Culture Lysis Buffer (Promega). Tissue debris was removed by centrifugation and 50 µL of the resulting shrimp extract immediately assayed for luciferase activity using the Bright-Glo assay system as described above. The remainder of the tissue extract was used for protein determination according to Lowry (Lowry *et al*., 1951) as described above. A luciferase calibration curve was prepared by using a serial dilution of commercially available luciferase (Quantilum recombinant luciferase, Promega) prepared in tissue extract from sham-injected shrimp. Luciferase activity was normalized to total protein content and presented in parts per billion. The results of two experiments were combined for analysis and data points from shrimp that did not show luciferase activity higher than the background signal plus two standard deviations were excluded from further analysis.

RESULTS AND DISCUSSION

The major impetus for this research was to improve the tools available for expression of heterologous proteins in shrimp. In order to fully characterize these shrimp viral promoters, expression in both shrimp and other eukaryotes as well as in prokaryotic systems needs to be evaluated. In this study, we evaluated both bacteria and shrimp as hosts for exogenous protein expression. Such information would help in future application of these promoters to both shrimp studies and studies in other systems. Expression in other systems, especially bacterial ones, would not be optimal but rather an indication of functionality. Validation of promoters that work in shrimp is a first addition to tools necessary for effective crustacean molecular engineering. Future work will need to be done to optimize the promoters for expression in crustacean hosts as well as to locate other tools like transposons and enhancer elements that are optimally functional in shrimp.

Luciferase expression in bacteria

The promoter activity was determined in *Escherichia coli* JM109 transformed with the luciferase reporter / promoter constructs. The results show that both P2 and P61 promoters are functional in bacteria and that these promoters were influenced by the presence of the SV40 enhancer element. Reporter protein expression driven by the P61 promoter was approximately 1.3 and 4.5-fold higher than the P2 promoter in the pGL3-Basic and Enhancer vectors, respectively (Figure 3). The SV40 enhancer element lowered the activity of the P2 promoter more than twofold to a value lower than the control (pGL3-Enhancer

without a promoter; Figure 3). Since it is unlikely that the eukaryotic SV40 transcriptional enhancer is active in prokaryotes, the significance of differences in expression between the pGL3-Basic and pGL3-Enhancer-based plasmids is unknown.

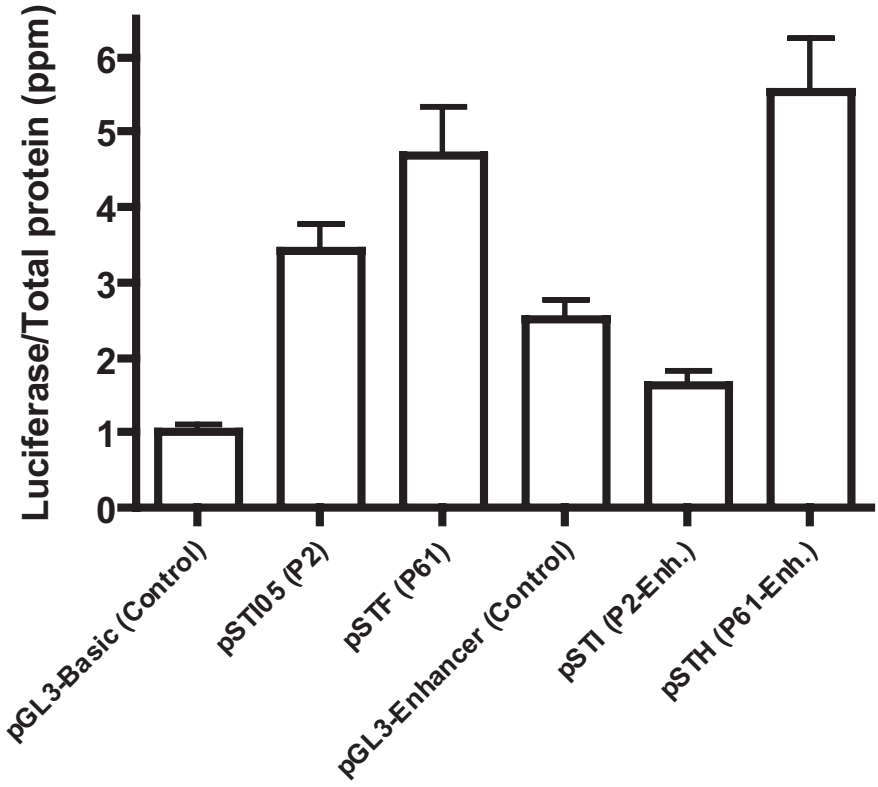


Figure 3. Expression of luciferase (Luc) in recombinant Escherichia coli. Luc expression corrected for background signal by comparison to a plasmid lacking the Luc coding sequence. Error bars represent standard error of the mean.

Luciferase expression in shrimp tail muscle

The transient expression of luciferase in shrimp tail muscle was highly variable (Figure 4). Both the P2 and P61 promoters were functional in shrimp, but the presence of SV40 enhancer element had no significant effect on the P2 promoter and a negative effect on the P61 promoter. Although the mean *Luc* expression value when driven by the P61 promoter was higher than the corresponding mean value of the P2 promoter, there was no significant difference. This is possibly due to observed high individual shrimp variability. Nevertheless, significant numbers of shrimp exhibited luciferase activity when injected with the IHHNV promoter driven *Luc* constructs, clearly demonstrating promoter activity as predicted (Shike *et al*., 2000).

Luc expression in shrimp was more than 10,000-fold lower compared to the *Luc* expression in bacteria even though bacteria are not natural hosts for IHHNV. The difference is probably due to variable efficiency of transformation in bacteria compared to *in vivo* transfection (shrimp). In shrimp, part of the transfection suspension spreads throughout

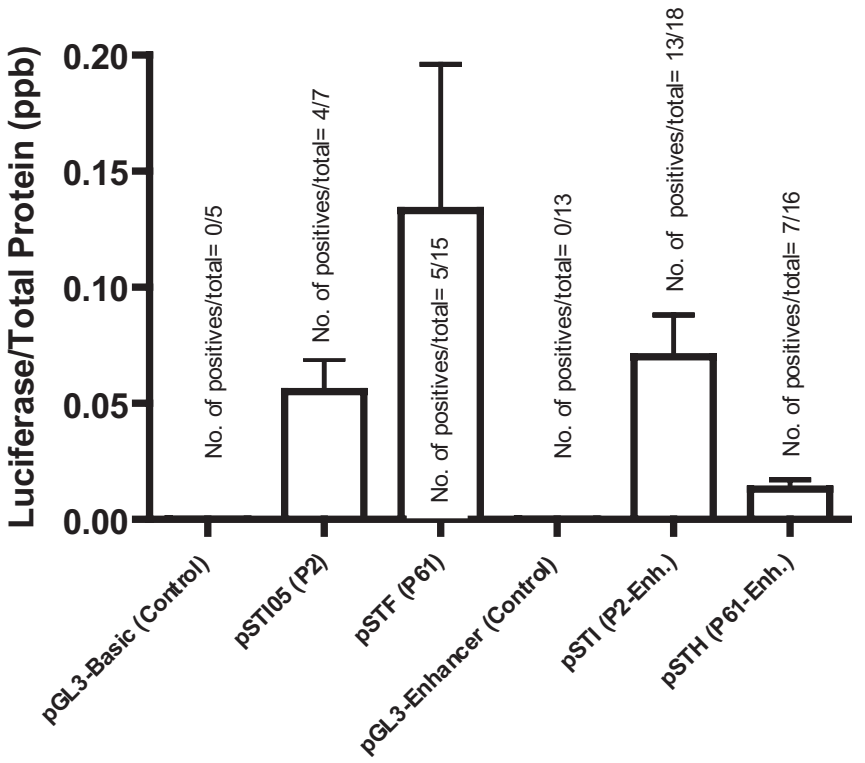


Figure 4. Transient expression of luciferase in shrimp tail muscle 3 days post-transfection. Only samples with a signal greater than background + 2 standard deviations were considered positive. Error bars represent standard error of the mean.

the body immediately upon injection. This was demonstrated by adding a fluorescent protein dye, C-phycocyanin (Martek Biosciences, Columbia, MD), to the injection mix. The dye rapidly spread throughout the shrimp body within two minutes (data not shown). As we collected tissue surrounding the site of injection for luciferase assay, potentially all transfected shrimp cells are not included. In addition, the amount of vector used for transfection, on a target tissue weight basis, and transfection efficiency were much lower than recombinant bacteria.

Our data showed that P2 and P61 are constitutive promoters capable of driving gene expression in both a prokaryote and a eukaryote. The differences in expression between these two promoters in each host system might be due to the difference in recognition of these promoter elements by the cellular transcriptional machinery of the host. In IHHNV, the P2 promoter drives the expression of the non-structural gene (NS-1), whereas P61 drives the expression of the structural gene (Shike *et al*., 2000). Promoters for viral capsid genes are generally stronger than those for non-structural genes. It is possible that during IHHNV replication the P2 promoter transcribes the NS-1 gene first, and that the P61 promoter is then transactivated by the NS-1 protein. In mammalian parvoviruses, such as the minute virus of mice and the rodent parvovirus H-1, there is a temporal order of expression for the structural and non-structural gene promoters. In these viruses, the non-

structural protein is expressed first, and then this non-structural protein transactivates the promoter for the structural gene (Doerig *et al*., 1988; Rhode, 1985). Transactivation of a structural gene promoter by the viral non-structural protein has also been reported in *Aedes aegypti* Densonucleosis virus (Afanasiev *et al*., 1994) and *Junonia coenia* Densonucleosis virus (Giraud *et al*., 1992). The IHHNV promoters will be useful for constructing vectors for the expression of homologous or heterogonous gene(s) in shrimp and potentially other prokaryotic and eukaryotic hosts. This initial research on the IHHNV promoters sets the stage for further characterization of their function at the molecular level in shrimp tissues as well as in exploring their utilities in other eukaryotic systems.

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