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Microarray and RT-PCR screening for white spot syndrome virus immediate-early genes in cycloheximide-treated shrimp

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Abstract

Here, we report for the first time the successful use of cycloheximide (CHX) as an inhibitor to block de novo viral protein synthesis during WSSV (white spot syndrome virus) infection. Sixty candidate IE (immediate-early) genes were identified using a global analysis microarray technique. RT-PCR showed that the genes corresponding to ORF126, ORF242 and ORF418 in the Taiwan isolate were consistently CHX-insensitive, and these genes were designated *ie1*, *ie2* and *ie3*, respectively. The sequences for these IE genes also appear in the two other WSSV isolates that have been sequenced. Three corresponding ORFs were identified in the China WSSV isolate, but only an ORF corresponding to *ie1* was predicted in the Thailand isolate. In a promoter activity assay in Sf9 insect cells using EGFP (enhanced green fluorescence protein) as a reporter, *ie1* showed very strong promoter activity, producing higher EGFP signals than the insect *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) *ie2* promoter.

Keywords: *Penaeus monodon*; WSSV; Microarray; Immediate-early gene; Cycloheximide-insensitive gene

Introduction

White spot syndrome virus (WSSV), the type species of the genus *Whispovirus*, family *Nimaviridae* (Mayo, 2002), is a widespread viral agent in crustacean populations, and it has caused severe mortalities and huge economic losses to the shrimp farming industry, not only in Asia but also globally (Chou et al., 1995; Flegel, 1997; Huang et al., 1995; Lo et al., 2003; Lu et al., 1997; Nakano et al., 1994; Wang et al., 1995; Wongteerasupaya et al., 1995). WSSV has a large (~300 kbp) double-stranded circular DNA genome (Chen et al., 2002; van Hulten et al., 2001; Yang et al., 2001). For the Taiwan isolate (GenBank accession no. AF440570), 532 open reading frames (ORFs) that are at least 60 amino acids long have been identified. Using microarrays, transcripts have been detected for ~90% of these ORFs (Wang et al., in press). The temporal expression of WSSV genes has been investigated both by individual gene studies (Chen et al., 2002; Hossain et al., 2004; Leu et al., 2005; Liu et al., 2001; Lu and Kwang, 2004; Tsai et al., 2000a, 2000b; van Hulten et al., 2000a, 2000b, 2002) and by global analysis (Khadijah et al., 2003; Tsai et al., 2004; Wang et al., in press; Zhang et al., 2004). Some WSSV promoters (namely the genes for PK1, PK2, RR1, RR2, TK-TMK and endonuclease) have also been shown to be active in insect cells (Hossain et al., 2004). However, although these genes exhibit one characteristic of IE (immediate-early) genes (i.e., that they can be successfully transcribed in the absence of viral proteins), neither these genes nor any other WSSV genes have so far been identified as immediate-early genes.

The expression of viral IE genes depends on the host cell machinery and occurs independently of any viral de novo protein synthesis, which means that the IE genes are

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especially important in determining host range (Friesen, 1997). Once expressed, the IE gene products may then function as regulatory *trans*-acting factors and may serve to initiate viral replicative events during infection. In the cascade of viral regulatory events, successive stages of virus replication are dependent on the proper expression of the genes in the preceding stage. For example, during infection by the large DNA viruses, such as baculoviruses and herpesviruses, gene expression is regulated such that the immediate-early (IE or α) genes are transcribed first, followed by the expression of the early (E or β) and late (L or γ) genes, respectively (Blissard, 1996; Blissard and Rohrmann, 1990; Friesen and Miller, 1986; Honess and Roizman, 1974). To study the transcription of viral IE genes, viral infection is induced in the presence of a protein synthesis inhibitor, usually cycloheximide (CHX), which prevents de novo protein synthesis by preventing translation. Translation (but not transcription) of the IE genes is also prevented, and this firmly blocks the infection cycle at the IE stage. Transcription during infection in the constant presence of CHX is the condition that most rigorously defines the IE genes. Here, for the first time, despite the lack of any shrimp cell line and the difficulty of using CHX in vivo, we successfully use CHX as an inhibitor to block de novo viral protein synthesis. A global analysis microarray technique was used to monitor the effect of CHX and suggest candidate genes, and from these candidates, three WSSV immediate-early genes were identified.

Results

Screening for WSSV IE genes using microarrays and CHX treatment

When the WSSV DNA (ORF/gene) microarrays were used to examine viral gene expression, the presence of the protein synthesis inhibitor CHX in the WSSV-infected shrimps was expected to lead to the specific accumulation of RNA transcripts of the viral immediate-early genes. This is because the transcription of all other viral genes requires viral proteins as transcription factors, and the synthesis of these proteins would have been inhibited. Fig. 1 shows scatterplots of normalized florescence intensities (i.e., expression levels) for the 532 WSSV ORFs on the microarrays under conditions of WSSV infection versus mock infection. In the presence of increasing dosages of CHX, the differential expression levels of the WSSV genes increasingly approach the 45° line of equivalence. This is good evidence that the CHX treatment is successfully inhibiting protein synthesis and also further suggests that, for most of

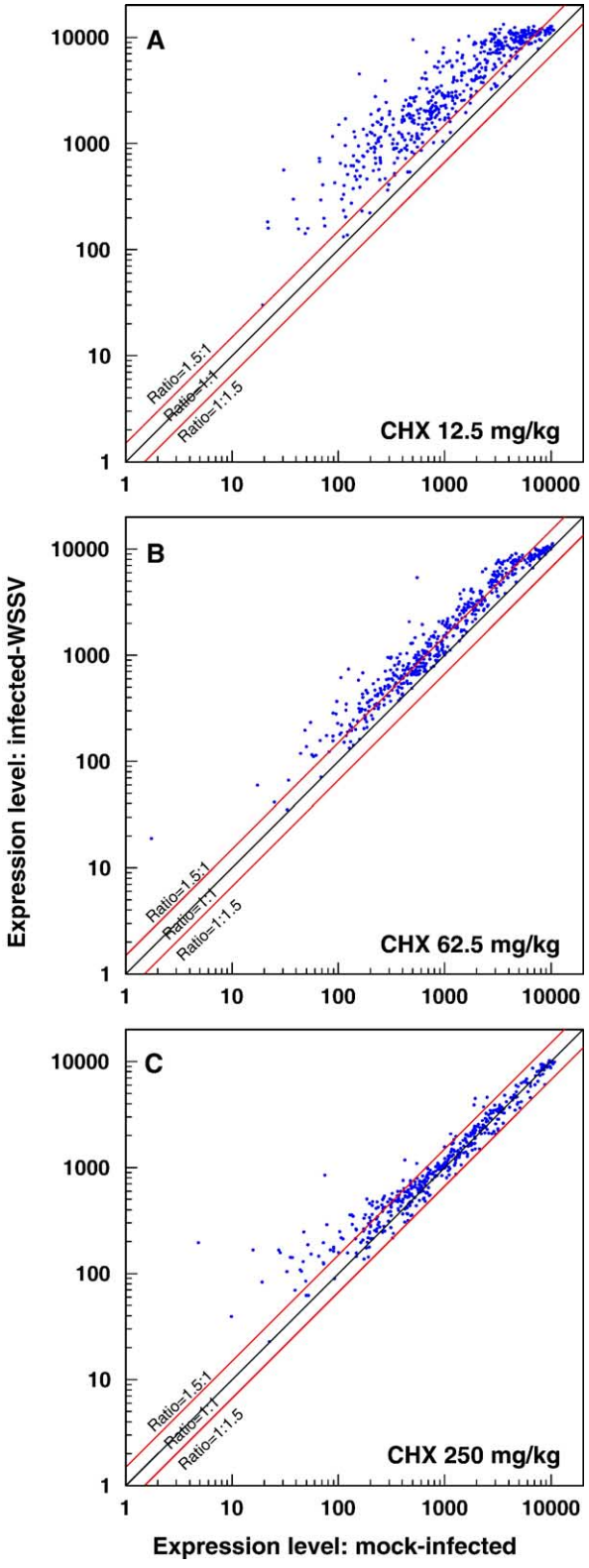


Fig. 1. Scatterplots showing differential levels of normalized Cy3 fluorescence for WSSV infection (vertical axis) versus mock infection (horizontal axis). Each plotted point is based on triplicate microarray results, corresponds to a single WSSV ORF, and represents the ratio of Cy3 fluorescence levels to β-actin expression levels. Proximity to the 45° line of equivalence indicates similar levels of expression under both infected and non-infected conditions. ORFs above the differential expression cut-off line (1.5:1) in graph C were considered to be IE gene candidates.

these 532 WSSV ORFs, WSSV gene transcription does indeed depend on the presence of one or more viral proteins. Sixty ORFs that were relatively unaffected by the highest (250 mg/kg) CHX dosage (i.e., had a differential expression level greater than 1.5:1 in Fig. 1C) were considered to be candidate IE genes.

RT-PCR analysis of CHX-insensitive genes

RT-PCR analysis showed that three candidates, ORF126/ORF128, ORF242, and ORF418, were consistently CHX-insensitive. Since ORF126/128 represents two overlapping genes (Fig. 2A), two additional gene-specific primers for single strand cDNA synthesis, P496 and 128R1, and two primer sets for PCR detection of these single strands, 126F/126SP3, 128F1/128SP1, were designed to distinguish between them (primer sets are listed in Table 1A and B). Fig. 2B shows that the ORF128 transcript was not detected

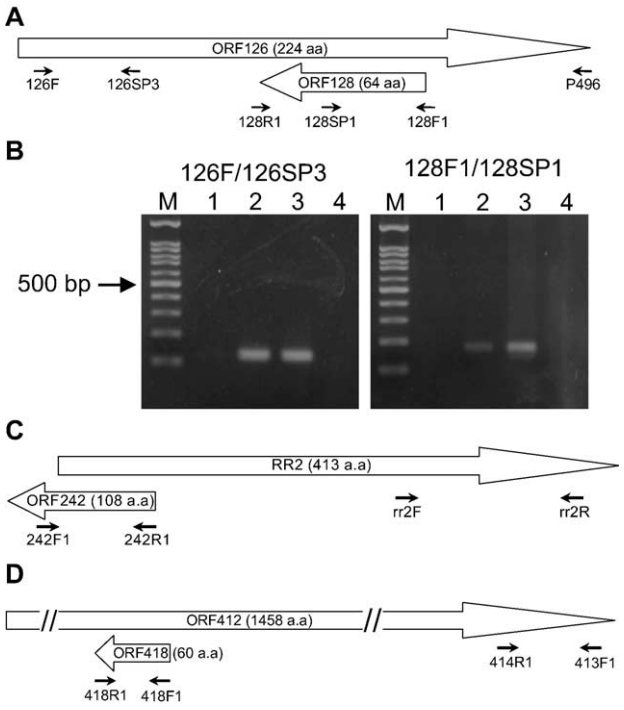


Fig. 2. Determination of CHX-insensitivity for the overlapping ORFs. (A) Schematic representation of the relative position and size of ORF126 and ORF128, showing the locations of the primers used for RT-PCR. (B) Agarose gel electrophoresis results for specifically synthesized single-strand cDNA sequences (ORF128 in lane 1 and ORF126 in lane 2) using the primer sets indicated above the panels. Lane 1: ORF128-specific single-strand cDNA synthesized from the total RNA from CHX-pretreated WSSV-infected shrimp using the ORF128-specific primer 128R1. Lane 2: ORF126-specific single-stand cDNA synthesized from the total RNA from CHX-pretreated WSSV-infected shrimp using the ORF126-specific primer P496. Lane 3: non-specific double-stranded cDNA synthesized from the total RNA from WSSV-infected shrimp with vehicle only (20% ethanol) pretreatment using oligo-dT primer. Lane 4: PCR amplification of total RNA from CHX-pretreated WSSV-infected shrimps to confirm there was no genomic DNA contamination of the RNA samples. (C) and (D) Schematics showing ORF242 overlapping with *rr2*, and ORF418 overlapping with ORF412. M: 100 bp DNA ladders (Lambda Biotech Inc., Taiwan).

by either primer set (lane 1) while the ORF126 transcript (lane 2) was detected by both. We therefore concluded that only ORF126 was CHX-insensitive. Both ORF242 and ORF418 also overlapped with a gene or ORF on the opposite strand (*rr2* and ORF412, respectively; see Figs. 2C and D). The initial identification of these two ORFs on the microarray was therefore checked and confirmed by RT-PCR (Fig. 3: compare ORF242 vs. *rr2* and ORF418 vs. ORF412).

CHX-insensitivity was confirmed by repeated checking of samples from five successive pairs of shrimps, where one shrimp of each pair was pretreated with CHX, and the other was not. In the five successive tests (and also in additional RT-PCR testing; data not shown), since CHX pretreatment never prevented the transcription of these ORFs, they are subsequently referred to as *ie1*, *ie2* and *ie3*, respectively. Fig. 3 presents the RT-PCR results from one of the pairs of tested shrimps and shows that CHX pretreatment does not prevent transcription of these genes (compare lanes 1 and 2). The sequences for all three IE genes are found in all three of the WSSV isolates that have been sequenced. ORF126 (*ie1*, immediate-early gene #1) equates to ORF055 in the Thailand isolate and ORF069 in the China isolate. ORF242 (*ie2*) and ORF418 (*ie3*) equate respectively to ORF187 and ORF359 in the China isolate, but ORFs corresponding to *ie2* and *ie3* have so far not been predicted in the Thailand isolate.

For the non-IE genes *dnapol*, *rr1*, *rr2*, *pk1*, *pk2*, *tk-tmk*, and *endonuc* (endonuclease), and for three latency related genes that correspond to ORFs 151, 366 and 427, respectively, in Khadijah et al. (2003), transcription was usually (but not always) inhibited by the 250 mg/kg CHX pretreatment. Fig. 3 illustrates the successful CHX inhibition of these genes. The occurrence of occasional false-positives for these other genes (data not shown) presumably resulted from different individual in vivo experimental conditions in the CHX-pretreated shrimp, but in any case, even a single instance of transcription suppression by CHX was sufficient to rule out the possibility that the gene in question was an IE gene.

Temporal transcription analysis of WSSV IE genes by RT-PCR

An RT-PCR temporal analysis showed that *ie1*, *ie2* and *ie3* transcripts were first detected at 2 hpi (hours post-infection) and continued to be found through to 72 hpi (Fig. 4). The intensity of the *ie1* and *ie3* PCR product bands both increased over time, reached a maximum at ~18 hpi and continued at a high expression level thereafter. *ie2* first appeared at 2 hpi and its expression levels subsequently remained fairly constant. By comparison, transcripts of the two other WSSV genes, *dnapol* and *vp28* (a WSSV major envelope protein gene), were not detected until 4 hpi.

Table 1
WSSV primers used in this study

(A)

ORF/Gene	Primer sequences (5′–3′)	Amplicon size
126/*ie1*	126F: GACTCTACAAATCTCTTTGCCA 126SP1: CTACCTTTGCACCAATTGCTAG	502 bp
242/*ie2*	242F1: ATACCAACAACCCCAGAA 242R1: ATGGGGCGGGATACAAAA	233 bp
418/*ie3*	418F1: ATGTTGTTCTCGTCTCCAGAAC 418R1: CAAGTTTCTGGCAGAAGGAGG	180 bp
482/*pk1*	pk1F: TGGAGGGTGGGGACCAACGGACAAAAC pk1R: CAAATTG ACAGTAGAGA ATTTTGCAC	512 bp
140/*pk2*	pk2F: GAAGGGCGATTTTGATAGAA pk2R: ATGATTGACTTGGGACTCTC	296 bp
228/*rr1*	rr1F: ATCTGCTAGTCCCTGCACAC rr1R: AAAGAGGTGGTGAAGGCACG	408 bp
243/*rr2*	rr2F: AGGGACGAAGGTCTTCATCG rr2R: GAGGAGGTGCAGTCAGAATT	418 bp
454/*tk-tmk*	tk-tmkF: GAGCAGCCATACGGGTAAAC tk-tmkR: GCGAGCGTCTACCTTAATCC	412 bp
246/*endonuclease*	endonucF: TGACGAGGAGGATTGTAAAG endonucR: TTATGGTTCTGTATTTGAGG	408 bp
039/*dnapol*	dnapolF: TGGGAAGAAAGATGCGAGAG dnapolR: CCCTCCGAACAACATCTCAG	586 bp
486*/latency 1	latency1F: CTTGTGGGAAAAGGGTCCTC latency1R: TCGTCAAGGCTTACGTGTCC	647 bp
425*/latency 2	latency2F: GGGAGAAGGTGAAGACGATG latency2R: AGAAAGCGCGTGCTTTAGCC	266 bp
207*/latency 3	latency3F: CTGTTCTGGCTCTATCTTGC latency3R: CGGTTTCATCTTTGGACGAG	486 bp
480/*vp28*	vp28F: CTGCTGTGATTGCTGTATTT vp28R: CAGTGCCAGAGTAGGTGAC	555 bp
412	413F1: AGCATGTCACTACCGTCCCA 414R1: CGGAACCGCTAACAGCAACA	906 bp
β-*actin*	actinF1: GAYGAYATGGAGAAGATCTGG actinR1: CCRGGGTACATGGTGGTRCC	686 bp
Intergenic	IC-F2: CAGACTATTAATGTACAAGTGCG IC-R3: GAATGATTGTTGCTGGTTAGAACC	1126 bp
126 (128)	126F: GACTCTACAAATCTCTTTGCCA 126SP3: CCTCTTCATCACCTCAATACC	123 bp
126 (128)	128SP1: GAGACTGATCGACATGGACAGTAC 128F1: GTACATCCATATGGATGCCGC	177 bp

(B)

ORF/Gene	Primer sequences (5′–3′)	Usage
126/*ie1*	P496: TTATACAAAGAATCCAGAAATC	ORF126 specific cDNA synthesis
128	128R1: CAGTGGTTCCCATGTCAAGAC	ORF128 specific cDNA synthesis
126/*ie1*	126SP1: CTACCTTTGCACCAATTGCTAG	5′ RACE
126/*ie1*	126SP2: GTACAGTACTGTCCATGTCGAT	5′ RACE
126/*ie1*	126SP3: CCTCTTCATCACCTCAATACC	5′ RACE
126/*ie1*	128SP1: GAGACTGATCGACATGGACAGTAC	3′ RACE
242/*ie2*	242SP1: GCCACTTCTCACACCACAGCATC	5′ RACE
242/*ie2*	242SP2: CTGTTCACCGTTGCTGGTCTG	5′ RACE
242/*ie2*	242SP3: CTCATTATGCAAGTAGATACGTC	5′ RACE
418/*ie3*	418SP1: CTGGCAGAAGGAGGTGGCTTG	5′ RACE
418/*ie3*	418SP2: GGATTGGACGATTCTGGAGATG	5′ RACE
418/*ie3*	418SP3: GGAAGAAGATAATGAGGAAGG	5′ RACE

(A) Gene specific primer sets for the 3 CHX-insensitive genes and for other (control) genes. (B) Specific ORF126 and ORF128 primers used for single strand cDNA synthesis, and primers used for *ie1*, *ie2* and *ie3* 5′RACE, and *ie1* 3′RACE.

* These ORFs are equivalent to ORFs 427, 366 and 151 in the China isolate (see Khadijah et al., 2003).

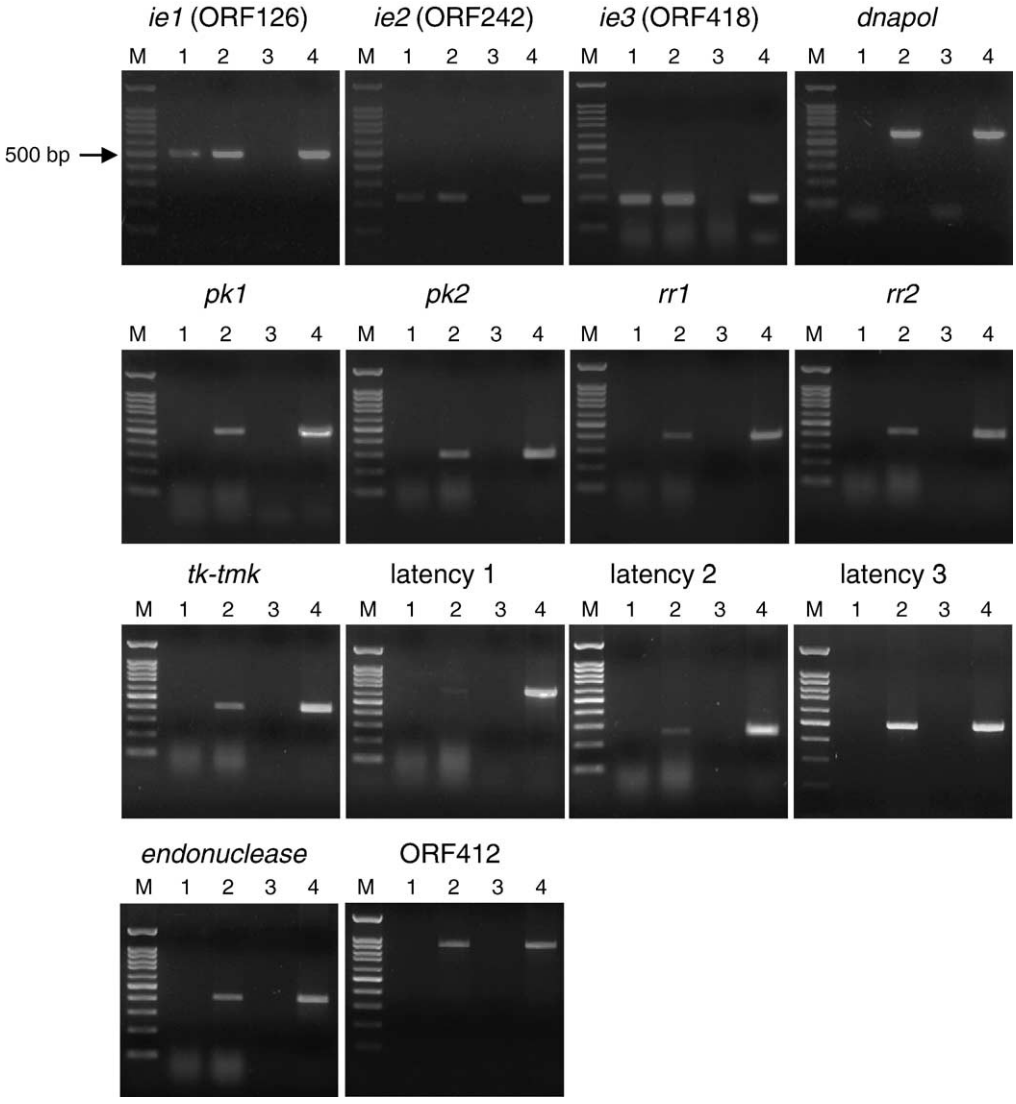


Fig. 3. Agarose gel electrophoresis RT-PCR results for the three WSSV IE genes, *ie1*, *ie2* and *ie3*, and other WSSV genes/ORFs. Results are based on total RNA extracted from the gills of WSSV-infected shrimp at 8 h post-infection. Table 1A lists the primer sets used to amplify the target gene/ORF sequences. Lane 1: CHX pretreatment (250 mg/kg). Lane 2: vehicle only (20% ethanol) pretreatment. Lane 3: as lane 1, but amplification by PCR to confirm there was no genomic DNA contamination of the RNA samples. Lane 4: (Amplicon size reference) PCR amplification of WSSV genomic DNA. M: 100 bp DNA ladder (Lambda Biotech Inc., Taiwan).

Mapping of the 5′ and 3′ termini of the IE gene transcripts

Analysis of the *ie1* 5′ RACE (rapid amplification of the cDNA end) products cloned in pGEM-T Easy vector revealed that in 6 of the first 7 randomly picked clones, the 5′ termini were located 52 nt (G) upstream of the putative ATG initiation codon, while in the other clone, the 5′ terminus was at 51 nt (T) (Fig. 5A). This suggested that the −52 nt G represents the major transcriptional start point. Upstream (−26 nt) of the transcriptional initiation site (at nt −78 to nt −82 relative to the ATG translational start), a putative TATA box (TATAA) was found. NNPP (Neural Network Promoter Prediction) (Reese and EecKman, 1995; Reese et al., 1996) analysis of upstream sequences of the *ie1* putative translation start site identified a high-probability predicted basal promoter region between −92 and −43 away from the putative translation start codon (Fig. 5A). Sequence analysis of the cloned 3′RACE products revealed that poly (A) was added at a site 17 nt downstream of the AATAAA polyadenylation signal (Fig. 5A).

The 5′RACE for *ie2* located the transcriptional start site at 25 nt (C) upstream of the putative ATG initiation codon (Fig. 5B). The initiation site of *ie2* also conforms to the arthropod initiation motif, (A/C/T)CA(G/T)T (Cherbas and Cherbas, 1993). For *ie3* (Fig. 5C), the PCR products of 5′RACE had two different sizes depending on how many hours post-infection the RNA samples were taken (Fig. 5D). Analysis of the 5′RACE products cloned in pGEM-T Easy vector revealed that when the template RNA was taken from shrimp infected by WSSV for 24 h, the 5′ termini of the randomly picked clones were located 110 nt

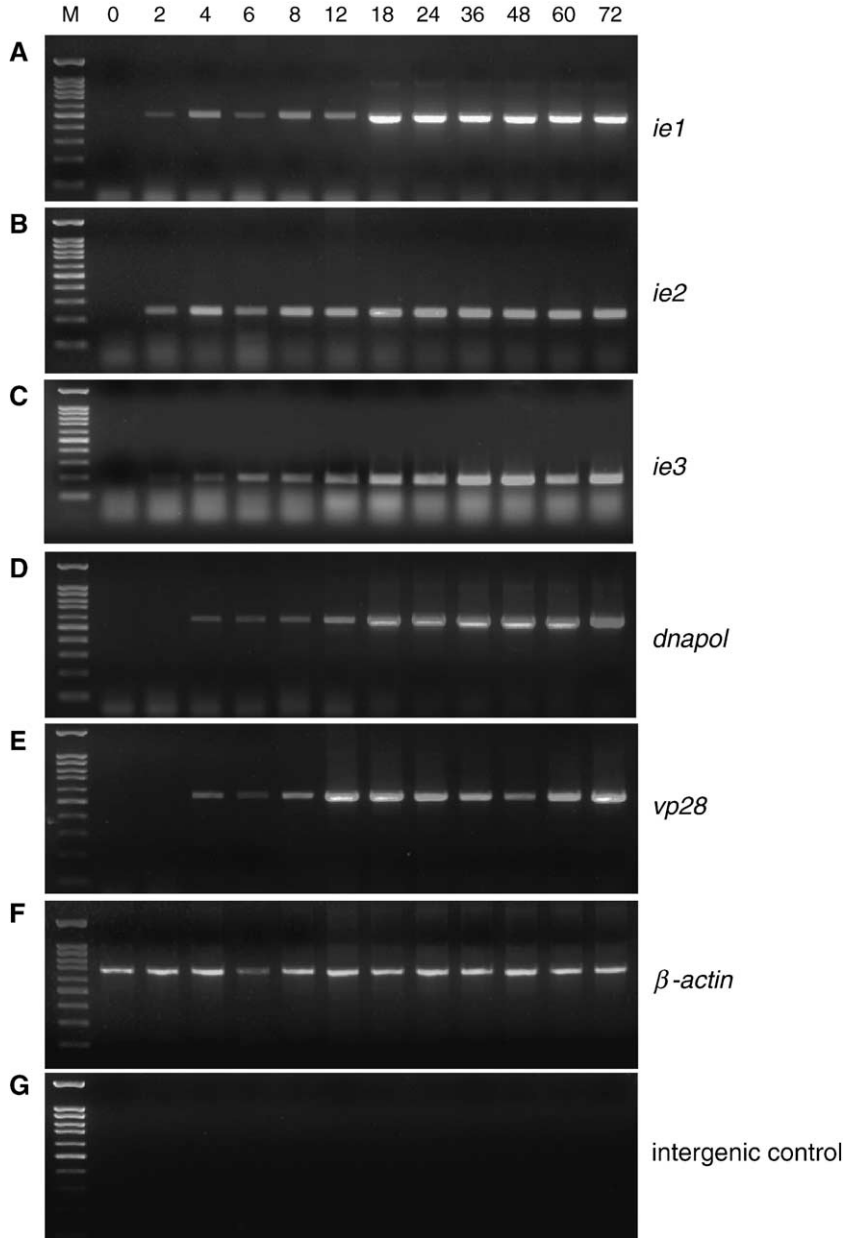


Fig. 4. Temporal transcription analysis of WSSV IE genes. RT-PCR primers were (A)–(C) *ie*-specific (126F/126SP1, 242F1/242R1 and 418F1/418R1, respectively) (D) *dnapol*-specific, and (E) *vp28*-specific. (F) and (G) were internal controls that used shrimp β-actin-specific primers and intergenic primers (IC-F2/IC-R3), respectively. Lane M is a 100-bp DNA ladder (Lambda Biotech Inc., Taiwan), and the other lane headings show hpi.

(T) upstream of the putative ATG initiation codon. For template RNA taken from shrimp infected for 60 h, the 5′ termini were at 435 nt (T) (Fig. 5C). Amplification of the 3′ end of *ie2* and *ie3* (3′RACE using oligo-dT) was unsuccessful, which suggests that these two transcripts might not have a poly (A) tail. We note that while reverse transcription with the kit's oligo-dT primer was unsuccessful here, it nevertheless appeared to succeed in the RT-PCR analysis (Fig. 4), even though the same oligo-dT primer was used to reverse transcribe the first strand cDNA. In fact, however, the low molecular-weight cellular RNAs in the reaction mixture are also capable of serving as primers for cDNA synthesis (Frech and Peterhans, 1994), which means that even without a poly (A) tail, RNA can still be reverse transcribed into cDNA. Similar results were reported in a previous study with WSSV transcripts that lacked a poly (A) tail and here oligo-dT was the primer used for reverse transcription (Tsai et al., 2004).

Fig. 5E shows that the initiation sites of WSSV *ie1*, *dnapol* (DNA polymerase), *rr1* and *rr2* all conform to the arthropod initiation motif, (A/C/T)CA(G/T)T, and that the arrangement of the broad promoter elements (TATA box and CAGT-related initiator) of these four genes are all very similar. By contrast, the *ie2* and *ie3* promoters are TATA-less.

Analysis of the promoters and coding regions of the WSSV IE genes

Results of analysis with the computer program NNPP and the GenBank/EMBL, SWISSPROT, PIR and EMBOSS databases are shown in Figs. 5 and 6. In *ie1*, the region upstream of the transcription initiation codon has two sequences that match the consensus sequences of the baculoviruses, namely the GATA ([A/T]GATA[G/A]) (Kogan and Blissard, 1994) and CGTGC (Harrison and Bonning, 2003) motifs (Fig. 6A). The region also includes two direct repeat sequences (CACACACA and CTCTCTCTCT), repetitions of two short sequences (TTTCTGG and CCAGAAA), and a palindromic sequence (ATTTTTTTAATATAACAAAAAAAT). The *ie1* coding region contains the Cys2/His2-type zinc finger motif X_3-Cys-X_{3-4}-Cys-X_{12}-His-X_{3-4}-His-X_4 (Fig. 5A). Upstream of the *ie2* coding region there are two GATA motifs, a palindromic sequence (AAAAACAGGCAACCTCTTTTT) and a short repeat sequence (CCAGAAA) (Fig. 6B). Upstream of the *ie3* coding region there are three GATA motifs, a palindromic sequence (GTGTTTTCTCTTGAAGAAACAC) and three direct repeat sequences, GCAGCAGCAGCAGCAGCA, CTTCTTCTT and GGAGGAGGA (Fig. 6C). Like many other (>90%) of the identified WSSV ORFs, some of which are highly expressed, the coding region of *ie2* and *ie3* did not contain any recognizable functional motifs.

Promoter activity assay for WSSV IE genes

For this assay, the promoters of the WSSV IE genes were inserted into plasmid constructs and used to drive expression of the EGFP (enhanced green fluorescence protein) gene. The plasmids were then transfected into Sf9 insect cells. At 72 h post-transfection, green EGFP fluorescent signals were observed only in Sf9 cells transfected with the plasmids containing the WSSV *ie1* promoter (pIZΔIE/WSSVie1-1k/V5-EGFP-His and pIZΔIE/WSSVie1-2k/V5-EGFP-His) and with the positive control plasmid (pIZ/V5-EGFP-His) (Fig. 7A). Plasmids containing the promoters of the other two IE genes (pIZΔIE/WSSVie2/V5-EGFP-His and pIZΔIE/WSSVie3/V5-EGFP-His) gave negative results in the Sf9 insect cells (data not shown). We note here the surprising observation that both the 1 kbp and 2 kbp WSSV IE1 gene promoter sequences produced higher intensity EGFP signals than the positive control, insect virus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (OpMNPV) IE2 gene promoter (Fig. 7A, upper panels). Similar results were also found in the Western blot analysis (Fig. 7B). These results were produced consistently in repeated runs of these assays (data not shown).

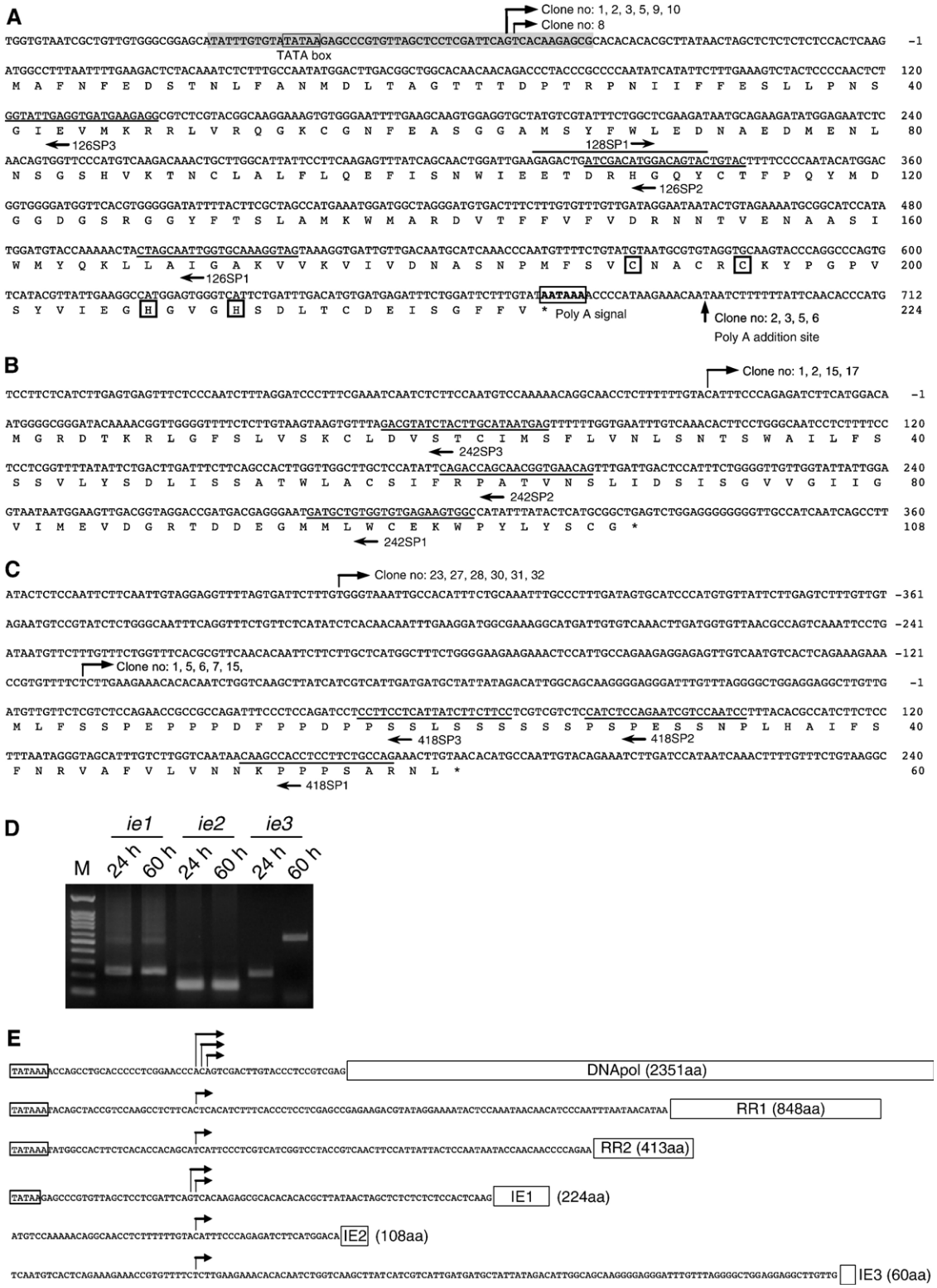
Discussion

Viral IE genes are expressed immediately after primary infection, or as a result of the reactivation of a virus. This class of genes is defined experimentally by their ability to produce transcripts even in the presence of inhibitors of protein synthesis. In this study, a protein synthesis inhibitor, CHX, was used to pretreat *Penaeus monodon* before they were challenged with WSSV. Shrimps were injected with three different dosages of CHX (12.5, 62.5 and 250 mg per kg of body weight), and as expected, as the dosage increased, the number of expressed WSSV genes was reduced (Fig. 1). However, even at the very high dosage of 250 mg/kg body weight-in preliminary tests, unchallenged shrimp treated with this dosage only survived for about 12 h (data not shown)-60 WSSV ORFs still produced relatively high numbers of transcripts in the microarray analysis (Fig. 1C). RT-PCR reduced the number of candidate IE genes to only three that consistently showed CHX-insensitivity (Fig. 3). The reason for the high initial number of candidate genes (and for the false-positives in the RT-PCR results) is presumably that in vivo, translation inhibition by CHX is leaky in the whole organism, and thus some delayed early and late genes would have been transcribed in some cells or tissues. On the other hand, we note too that there may still be other IE genes that were not included in the original 60 candidates because the 1.5:1 differential expression cut-off criterion is somewhat arbitrary.

Several baculovirus immediate-early genes are expressed in a range of insect cell lines early in the infection process, and are normally transcribed by the host cell transcriptional machinery across species (Hegedus et al., 1998; Zhao and Eggleston, 1999). However, the ability to express in insect cells does not necessary mean that a given WSSV gene is an IE gene, and while several WSSV promoters have been shown to be active in Sf9 cells (PK1, PK2, RR1, RR2, TK-TMK and endonuclease genes; Hossain et al., 2004), these promoters are all inhibited by CHX (Fig. 3). These genes therefore cannot be IE genes. Of the three WSSV IE genes reported here, only the WSSV *ie1* promoter was able to drive transient EGFP expression in the non-host Sf9 cells (Fig. 7). The WSSV *ie1* promoter must therefore share the conserved sequences for invertebrate transcriptional factor recognition. We noted above that immediate-early genes are important in determining host range, and the fact that the WSSV *ie1* promoter could be activated even by a non-decapod host transcription factor is consistent with WSSV's ability to infect a wide range of hosts, both crustacean and, possibly, non-crustacean (Flegel, 1997; Lightner, 1996; Lo et al., 1996). On the other hand, in the case of the other two CHX-insensitive genes, *ie2* and *ie3*, whose promoters failed to drive EGFP expression in the transfected Sf9 cells, it seems that some other specific transcriptional factors are required. These factors are presumably decapodal, and are absent in Sf9 cells.

The TATA motif is the principal regulatory element of many baculovirus early promoters. It consists of an A/T-rich motif located 25–31 nucleotides upstream from the transcription initiator (Blissard and Rohrmann, 1991;

Blissard et al., 1992; Dickson and Friesen, 1991; Guarino and Smith, 1992; Pullen and Friesen, 1995a), and together these two components are the basal elements of the RNA polymerase II promoter. The WSSV *ie1* promoter region also conforms to this pattern (Fig. 5A), which suggest that, like most of the insect baculovirus early genes, WSSV *ie1* transcription is mediated by host RNA polymerase II. Fig. 5E shows that WSSV *dnapol*, *rr1* and *rr2* also conform to this pattern. Their transcription start sites match the CAGT/



CAGT-related motif (Chen et al., 2002; Cherbas and Cherbas, 1993; Pullen and Friesen, 1995b), and they are located from 25 to 28 nucleotides downstream of the TATA box. This suggests that these three genes should also be transcribed by host RNA polymerase II. However, these genes are all inhibited by CHX treatment. We therefore conclude that the successful transcription of *dnapol*, *rr1* and *rr2* requires the presence of [a] viral protein transcription factor[s], either in addition to, or as a substitute for, the host transcription factors used by RNA polymerase II.

The promoter activity assay (Fig. 7) suggested that the sequences upstream of the WSSV *ie1* coding region were very effective in activating gene expression in Sf9 cells. The palindromic sequence ATTTTTTTAATATAAC-AAAAAAAT (see Fig. 6A) may function as a transcriptional enhancer (McMurray et al., 1991; Rasmussen et al., 1996), and since it was located further than 1000 nt upstream of the translation start site, this might help to account for the difference in expression levels between pIZΔIE/WSSVie1-2k/V5-EGFP-His and pIZΔIE/WSSVie1-1k/V5-EGFP-His in the transfected Sf9 cells (Fig. 7). Further, the promoter region of *ie1* included several sequences that matched the consensus sequences of the GATA motif (A/T)GATA(G/A). This is potentially important because the GATA motif is recognized as a binding site for host (insect) transcription factors, for example in the promoter of the baculovirus OpMNPV IE gene, *gp64* (Kogan and Blissard, 1994). We also note that although WSSV is distinct from the baculoviruses and it is unable to replicate in insect cells, WSSV *ie1* nevertheless contains the baculovirus early promoter motif (CGTGC, see Fig. 6A). Whether this motif, perhaps in combination with the TATA box and CAGT motif, might also help to account for the very high expression levels of *ie1* in the Sf9 cells (Fig. 7) remains an interesting open question. Lastly, BLAST analysis of the GenBank/EMBL, SWISSPROT and PIR databases predicted that the *ie1* coding region contains the Cys2/His2-type zinc finger motif. This motif has a role in DNA binding and implies that IE1 functions as a transcription factor. Further study should investigate which WSSV genes use IE1 as a transcription factor.

Materials and methods

Viral gene microarray-chip preparation

The WSSV microarrays (chips) were designed from the genome of the *P. monodon* WSSV 1994 Taiwan isolate (WSSV T-1; GenBank accession no. AF440570). Briefly, each chip contains 532 predicted WSSV ORFs and a *P. monodon* β-actin gene partial sequence. From these 532 ORFs, PCR products with amplicon sizes of 200 to 600 bp were spotted on the pre-coated glass slide (U-Vision Biotech Inc., Taiwan) in triplicate for each WSSV ORF, and with 3 replications for the *P. monodon* β-actin gene. The *P. monodon* β-actin acts as a positive control and is used to normalize the data across slides. Details of the viral microarrays are described elsewhere (Wang et al., in press).

Viral gene microarray-target preparation

The WSSV T-1 isolate was used for all the experiments in this study. Shrimps for the CHX (Sigma) treatment and virus challenge were cultured, WSSV-free *P. monodon*, body weight 35–45 g, that were kindly provided by Tung-Kang Marine Laboratory, Taiwan Fisheries Research Institute. During the experiments, shrimps were cultured in eight 1000-l FRP (fiber reinforced plastic) tanks containing sterile 33 ppt (parts per thousand) sea water at an ambient temperature of 25 °C.

WSSV inocula were prepared from pooled tissues from WSSV T-1 infected *P. monodon*. Protocols for viral inocula preparation and challenge trials were as described previously by Tsai et al. (1999). To identify WSSV IE genes, shrimps were treated with CHX (12.5 mg/kg, 62.5 mg/kg and 250 mg/kg of body weight prepared in 20% ethanol) by intramuscular injection 2 h before being challenged. Control shrimps were injected with 20% ethanol only. For the virus challenge trials, the CHX and 20% ethanol pretreated shrimps were mock-infected or infected with WSSV at a dosage of 50 μl per 10 g of body weight by intramuscular injection with 0.9% NaCl solution or viral inocula (prepared in 0.9% NaCl solution), respectively. Gills of the experimentally

Fig. 5. Sequences of the WSSV IE genes. (A) *ie1*. The primers used for 5′RACE and 3′RACE (126SP1, 126SP2, 126SP3 and 128SP1) are underlined. The shaded region between −92 and −43 nt from the translation start indicates the potential basal promoter element as predicted by the NNPP program. The bent arrows indicate the transcriptional start sites as revealed by sequencing seven randomly chosen 5′RACE clones. The TATA and polyadenylation signal (AATAAA) are boxed and boldfaced, respectively. Four boxed amino acid sequences show the Cys2/His2-type zinc finger motif. The poly (A) addition site is indicated by an arrow. (B) *ie2*. The primers used for 5′RACE (242SP1, 242SP2, 242SP3) are underlined. The transcriptional start site was located at 25 nt (C) upstream of the putative ATG initiation codon. (C) *ie3*. The primers used for 5′RACE (418SP1, 418SP2, 418SP3) are underlined. The 5′ termini were located 110 nt (T) and 435 nt (T) upstream of the putative ATG initiation codon depending on whether the RNA templates were taken from shrimp infected for 24 h or 60 h, respectively. (D) Agarose gel electrophoresis of RT-PCR products of 5′ RACE for the 3 WSSV IE genes. RNA templates were taken from shrimp either 24 h or 60 h after infection with WSSV. (E) Sequences upstream of the ATG translation initiation codon for the WSSV DNA polymerase, RR1, RR2 and IE1 genes, showing that the transcription initiation start sites are all located ~26 nt downstream of the TATA box. The transcription start sites identified by 5′RACE are indicated with bent arrows. IE2 and IE3, which are TATA-less, are included for comparison. DNApol data are from Chen et al. (2002) and RR1/RR2 data are from Tsai et al. (2000). Lane M: 100 bp DNA ladder (Lambda Biotech Inc., Taiwan).

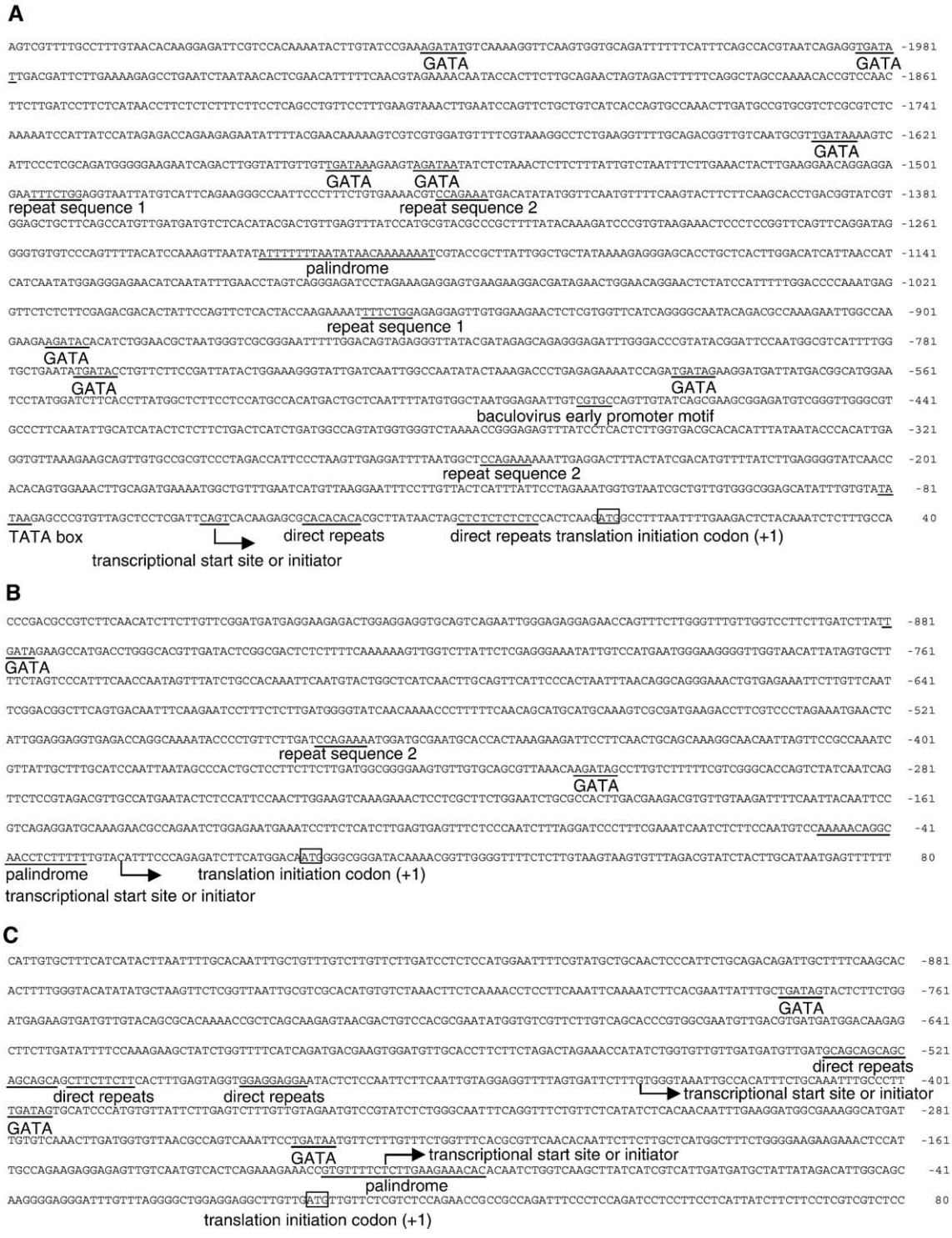


Fig. 6. DNA sequences (2 kbp, 1 kbp and 1 kbp, respectively) of the WSSV *ie1*, *ie2* and *ie3* promoter/enhancer regions, showing the organization and location of predicted regulatory motifs.

challenged shrimps were collected at 8 hpi and immediately frozen in liquid nitrogen until RNA extraction. Total RNA from the gills was isolated by using an RNeasy kit (Qiagen) according to the manufacturer's protocol. For the cDNA targets, RNA samples (20 μg) were fluorescently labeled with Cy3dUTP using a CyScribe First-Strand cDNA Labeling kit (Amersham Bioscience) following the manufacturer's instructions. Before the Cy3-labeled cDNAs were used as microarray targets, they were condensed, and unincorporated free nucleotides were removed using Microcon YM-30 columns (Amicon).

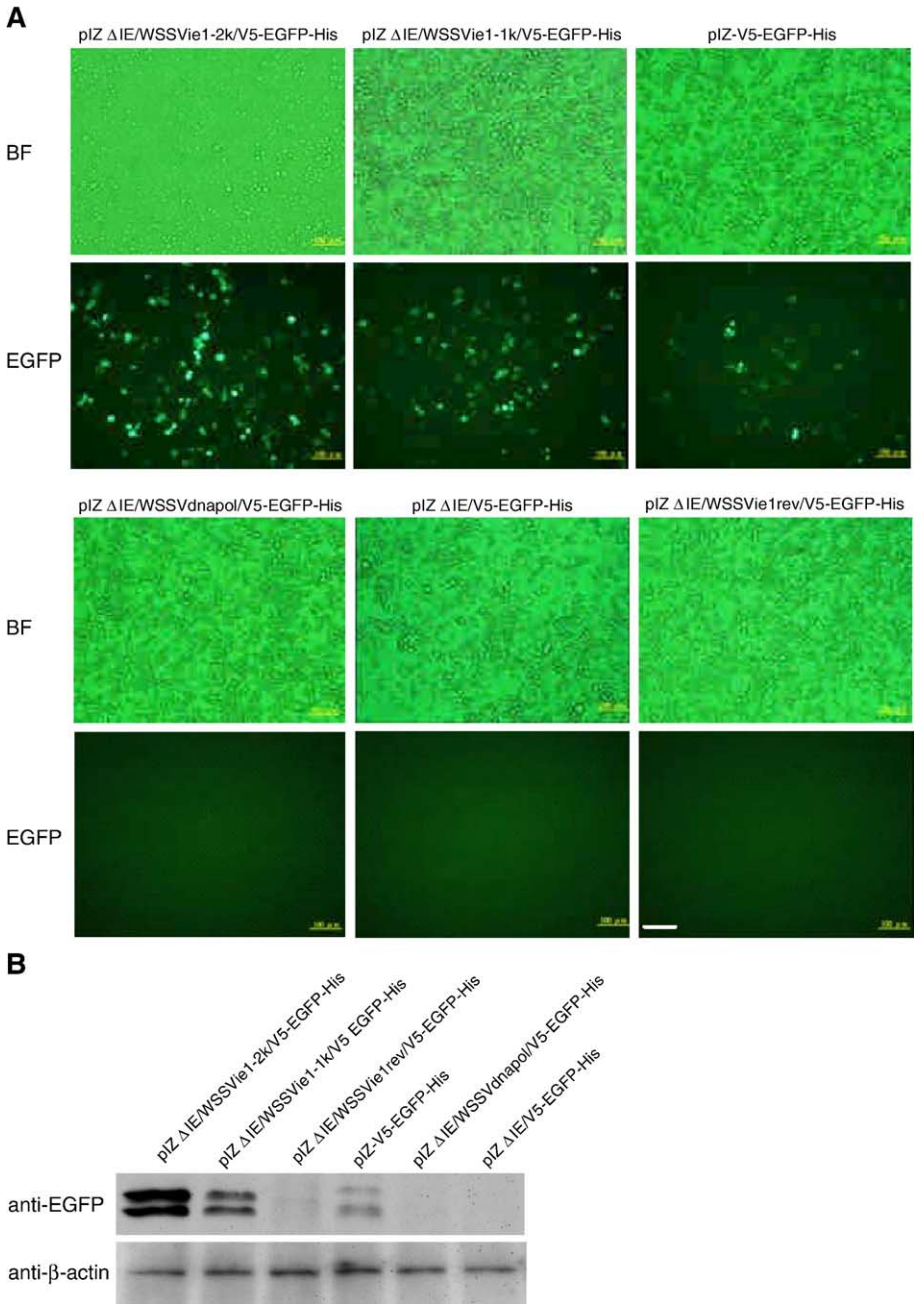


Fig. 7. Promoter activity assay for WSSV IE1 gene. (A) Brightfield (BF) and EGFP green fluorescence signals in Sf9 insect cells transfected with the indicated plasmids for 72 h. Scale bar = 100 μm. The first two panels assay 2 kbp and 1 kbp sequences, respectively, of the WSSV IE1 gene promoter region. The pIZ/V5-EGFP-His is a positive control. The lower three panels are various negative controls: the pIZΔIE/V5-EGFP-His, a plasmid without any IE gene promoter; the pIZΔIE/WSSVdnapol/V5-EGFP-His, a plasmid containing WSSV *dnapol* promoter; pIZΔIE/WSSVie1rev/V5-EGFP-His, a plasmid containing WSSV *ie1* reversed promoter sequence. (B) Western blot analysis of SDS-PAGE-separated cell lysates from the same transfected cells. Total proteins were probed using either anti-EGFP or anti-β-actin (control) antibodies and developed by an ECL chemiluminescence system.

Viral gene microarray-probe/target hybridization, scanning and statistical analysis

After the prepared Cy3-labeled cDNA targets were subjected to hybridization with all the DNA spots in the WSSV microarray, the microarrays were scanned with a confocal laser scanner (GeneTAC™ LS IV Microarray Scanner; Genomic Solutions) and fluorescence intensities were quantified by GenePix 3.0 (Axon Instruments). For those transcripts that bound to the microarray probes, signal intensities were converted to approximate measures of absolute expression by subtracting background signal levels. Signal levels of the positive control *P. monodon* β-actin gene were used to normalize viral gene expression results across different arrays, and the data for each set of triplicate dots were condensed into a single value following the

method described in Chen et al. (2005). WSSV gene expression was determined by plotting the normalized gene expression levels on the CHX-pretreated WSSV-infected chips against the normalized ratios for the corresponding genes in mock-infected chips.

Confirmation of microarray results by RT-PCR analysis of CHX-insensitive genes

For the microarray results at the most effective (highest) level of CHX treatment (250 mg/kg), those ORFs for which the median level of intensity in the infected samples was at least 1.5-fold greater than in the mock-infected samples were subjected to RT-PCR. For the RT-PCR analysis, templates were prepared from samples of the original batches of total RNA (i.e., from the 250 mg/kg CHX-pretreated WSSV-infected shrimps) and from an additional batch of RNA extracted from WSSV-challenged shrimps that were pretreated with 20% ethanol. The RNA samples (20 μg) were treated with RNase-free DNase I (Roche) at 37 °C for 1 h to eliminate any viral genomic DNA contamination in the preparations. An aliquot (~10 μg) of total RNA was used to synthesize the first-strand cDNA by using Superscript II reverse transcriptase (Invitrogen) and oligo-dT primer in a 20 μl reaction mixture. After RT reaction, an aliquot (2 μl) of the reaction product (containing about 1 μg of cDNAs) was subjected to PCR with pairs of specific primers corresponding to those WSSV genes that were expressed in the CHX-pretreated infected shrimps (i.e., the candidate IE genes as shown by the microarray analysis results). As a positive control for CHX treatment, PCR was also run with the gene-specific primer pairs dnapolF/dnapolR, pk1F/pk1R, pk2F/pk2R, rr1F/rr1R, rr2F/rr2R, endonucF/endonucR, latency1F/latency1R, latency2F/latency2R, latency3F/latency3R and tk-tmkF/tk-tmkR (Table 1A). As a quality control to check for WSSV genomic DNA contamination, PCR was also performed on CHX-pretreated WSSV-infected RNA samples that had not been subjected to reverse transcription. PCR product amplified from WSSV genomic DNA served as a PCR positive control and was also used as a relative size marker.

When candidate ORFs on different strands were found to overlap, additional primer sets were designed and used to amplify various non- and partially-overlapping regions to determine which of the ORFs really corresponded to a CHX-insensitive gene. The primers used to distinguish between overlapping genes are listed in Table 1A and B.

Temporal transcription analysis of WSSV immediate-early genes by RT-PCR

WSSV challenged *P. monodon* were sampled at 0 (i.e., immediately before infection), 2, 4, 6, 8, 12, 18, 24, 36, 48, 60 and 72 hpi. Total RNA was extracted from pleopods of the *P. monodon* harvested at each time point, purified with TRIzol Reagent (Invitrogen) and then treated with RNase-free DNase I (Roche) to remove any residual DNA. First strand cDNA synthesis was performed using the oligo-dT primer, and 2 μl (~1 μg) of the cDNA was subjected to PCR in a 50-μl reaction mixture containing an appropriate primer pair (see Table 1A). For comparison, the WSSV *dnapol* and *vp28* gene fragments were also amplified from the same templates by the primer pairs dnapolF/dnapolR and vp28F/vp28R, respectively. A shrimp β-*actin* primer set, actinF1/actinR1 was used as an internal control for RNA quality and amplification efficiency. To confirm there was no WSSV DNA contamination of the RNA samples, a WSSV genomic DNA-specific primer pair, IC-F2/IC-R3, derived from an intergenic region of the WSSV genome, was also used as a quality control. The primer sequences are listed in Table 1A.

Mapping the 5′ and 3′ termini of immediate-early genes transcripts

The 5′ and 3′ regions of the immediate-early genes transcripts were obtained by rapid amplification of the cDNA 5′/3′ ends (Frohman et al., 1988) using a commercial

Table 2
Primers used for constructing transient expression vectors for the promoter activity assay

Plasmid	Primer sequences (5′–3′)
pIZΔIE/WSSVie1-1k/V5-EGFP-His	F: CGGAATTCGAGATCCTAGAAAGAGGAGTG
	R: CCGCTCGAGCTTGAGTGGAGAGAGAGAGC
pIZΔIE/WSSVie1-2k/V5-EGFP-His	F: CGGAATTCGATGATGGTGATGTTTCTAGG
	R: CCGCTCGAGCTTGAGTGGAGAGAGAGAGC
pIZΔIE/WSSVie1rev/V5-EGFP-His	F: CGGAATTCCTTGAGTGGAGAGAGAGAGC
	R: CCGCTCGAGGAGATCCTAGAAAGAGGAGTG
pIZΔIE/WSSVdnapol/V5-EGFP-His	F: TAGAGCTCACTTCTCCTGACACTCTTGACTGAT
	R: GTGGAAGAGGGTGATGGAGCTGGAGATGATCATC
pIZΔIE/WSSVie2/V5-EGFP-His	F: GGGGTACCGTCTTCAACATCTTCTTGTTCG
	R: ATAAGAATGCGGCCGCCATGAAGATCTCTGGGAAATG
pIZΔIE/WSSVie3/V5-EGFP-His	F: CGGAATTCGTCGCACATGTGTCTAAACTTC
	R: CCGCTCGAGCAACAAGCCTCCTCCAGCC

Added restriction enzyme cutting sites are underlined.

5′/3′RACE kit (Roche) with an avian myeloblatosis virus (AMV) reverse transcriptase. The RNA samples used for 5′/3′RACE analysis in this study were isolated from the shrimps at 24 h and 60 h after WSSV infection and then treated with RNase-free DNase I (Roche). The appropriate gene-specific primers used for rapid amplification of the cDNA 5′/3′ ends are listed in Table 1B. The final amplification products were cloned into pGEM-T Easy vector (Promega) and sequenced. The sequences of the inserts were compared with the WSSV genomic DNA sequence.

Analysis of the promoters and coding regions of the WSSV immediate-early genes

Based on the WSSV T-1 strain (GenBank accession no. AF440570), the nucleotide sequences of the ORF and the region upstream of the ORF were analyzed using the computer program NNPP, and the GenBank/EMBL, SWISSPROT, PIR and EMBOSS databases.

Promoter activity assay

For each of the WSSV IE genes that was confirmed by RT-PCR, a transient reporter assay was performed. For this assay, Sf9 insect cells were transfected with promoter assay plasmids that were labeled with the EGFP reporter gene. Expression of the EGFP gene was then driven by the WSSV *ie* promoters.

The plasmid used as a starting point in this assay, pIZΔIE/V5-His, was modified from the plasmid pIZ/V5-His (Invitrogen) by deleting the OpIE2 (OpMNPV *ie2*) promoter located in front of the MCS (multiple cloning sites). To construct the derived plasmids, the EGFP gene was first inserted into the MCS to produce the plasmid pIZΔIE/V5-EGFP-His. Next, part (~1 kbp) of each WSSV IE gene upstream of the ATG was amplified from the WSSV genomic DNA by PCR with primers containing the appropriate restriction endonuclease recognition sites on the 5′ end (see Table 2). Each of the PCR products was then separately purified with the GFX PCR product purification kit (Roche), digested with restriction endonucleases and inserted into the pIZΔIE/V5-EGFP-His MCS in front of the EGFP gene. The resulting plasmids were designated pIZΔIE/WSSVx/V5-EGFP-His, where "x" represents the IE genes selected by the RT-PCR assay. A positive control plasmid, pIZ/V5-EGFP-His, was constructed by cloning the EGFP gene into plasmid pIZ/V5-His, while the pIZΔIE/V5-EGFP-His plasmid served as a negative control. Another negative control plasmid was constructed by inserting the EGFP gene and WSSV *dnapol* promoter region into the ΔIE plasmid to produce pIZΔIE/WSSVdnapol/V5-EGFP-His. For candidate promoters that gave a positive signal, the reversed promoter sequence was used to construct an additional control plasmid designated pIZΔIE/WSSVxrev/V5-EGFP-His.

Lastly, in addition to these 1 kbp forward and reverse plasmids, whenever a plasmid expressed a high level of EGFP, its promoter was further analyzed by constructing an EGFP-reporter plasmid driven by a 2-kbp forward fragment. The fidelity of all these clones was verified by DNA sequencing. The primers used for the construction of these plasmids are listed in Table 2.

For DNA transfection, Sf9 insect cells were seeded into a 24-well plate (3×10^5 cells/well) and grown in Sf-900 II SFM serum-free medium (Invitrogen) overnight at 27 °C. Plasmid DNA was diluted to 1 μg/μl in TE buffer (pH 8.0), and liposome-mediated transfection of the Sf9 cells (1 μg of plasmid DNA per well) was carried out by using the Cellfectin Reagent (Invitrogen) according to the manufacturer's instructions. At 72 h after transfection, EGFP fluorescence signals were observed under an Olympus IX71 inverted fluorescence microscope and photographically recorded using an Olympus DP50 digital microscope camera. Cells were also harvested and lysed at this same time. Cell extracts from each well were adjusted to an equal amount of total protein and assayed for EGFP by Western blotting. For the Western blots, the cell lysate total proteins were separated on 15% SDS-PAGE, transferred to a PVDF membrane (Osmonics) and probed using either anti-EGFP monoclonal antibody (B-2, Santa Cruz Biotechnology) or anti-human β-actin polyclonal antibody (H-196, Santa Cruz Biotechnology). The blots were developed using an enhanced chemiluminescent-light (ECL) detection kit (NEN Life Sciences) using goat anti-mouse IgG or goat anti-rabbit IgG coupled to horseradish peroxidase as secondary antibody.

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