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Cloning, Characterization, and Phylogenetic Analysis of a Shrimp White Spot Syndrome Virus Gene That Encodes a Protein Kinase

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An open reading frame (ORF) that encodes a 715-amino-acid polypeptide was found in an 8421-bp *Eco*RI fragment of the shrimp white spot syndrome virus (WSSV) genome. The polypeptide shows significant homology to eukaryotic serine/threonine protein kinase (PK) and contains the major conserved subdomains for eukaryotic protein kinases. Coupled *in vitro* transcription and translation generated a protein having an apparent molecular mass of about 87 kDa according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. For transcriptional analysis of the *pk* gene, total RNA was isolated from WSSV-infected shrimp at different times after infection. Northern blot analysis with *pk*-specific riboprobe found a major and a minor transcript of 2.7 and 5.7 kb, respectively. Rapid amplification of the 5′ cDNA ends of the major 2.7-kb *pk* transcript showed that there were two transcriptional initiation sites located at nucleotide residues -38(G) and -39(G) relative to the ATG translational start codon. Temporal expression analysis by RT-PCR indicated that the transcription of the *pk* gene started 2 h after infection and continued for at least 60 h. Phylogenetic analysis showed that WSSV protein kinase does not have any close relatives and does not fall into any of the major protein kinase groups.

Key Words: Penaeus monodon; white spot syndrome virus; WSSV Taiwan isolate; WSSV *pk* gene; protein kinase.

INTRODUCTION

Shrimp white spot syndrome (WSS) is one of the most serious diseases faced by the shrimp farming industry all over the world (Chou *et al.,* 1995; Flegel, 1997; Lo *et al.,* 1999). White spot syndrome virus (WSSV), the causative agent of WSS, is a large, double-stranded DNA virus (Wang *et al.,* 1995) that primarily attacks tissues originating from the ectoderm and mesoderm (Wongteerasupaya *et al.,* 1995; Lo *et al.,* 1997). There is little genetic variation among WSSV isolates from around the world (Lo *et al.,* 1999; Chang *et al.,* 2001).

Several WSSV genes have been identified, including genes that encode the ribonucleotide reductase large and small subunits (van Hulten *et al.,* 2000a; Tsai *et al.,* 2000a); nucleocapsid protein VP22 and envelope protein VP25 (van Hulten *et al.,* 2000b);[3] and a novel chimeric protein of thymidine kinase and thymidylate kinase (Tsai *et al.,* 2000b). These studies as well as morphological studies (Wang *et al.,* 1995, 2000a; Wongteerasupaya *et al.,* 1995) have provided evidence that WSSV is a newly isolated virus. However, genome analysis is needed to conclusively establish WSSV's taxonomic position. As part of our continuing work to identify and define the genetic structure of the virus, we report here on a protein kinase gene (the *pk* gene) of WSSV.

The eukaryotic protein kinases (PK) make up one of the largest protein superfamilies, and they all feature a homologous catalytic domain (for a review, see Hanks and Hunter, 1995). These enzymes usually use the γ phosphate of ATP (or GTP) to generate phosphate monoesters, and they use the protein alcohol groups on serine/threonine or the protein phenolic groups on tyrosine as phosphate acceptors (Hunter, 1987; Hanks and Hunter, 1995). They are thus classified into two broad groups, the protein serine/threonine kinases (PSK) and protein tyrosine kinases (PTK).

The protein kinases and the phosphoprotein phosphatases control reversible protein phosphorylation, which is a common mechanism in eukaryotes for regulation of normal cell function such as intracellular protein sorting (Herman *et al.,* 1991), growth, proliferation, differentiation, apoptosis, and stress responses (reviewed in Widmann *et al.,* 1999; Hunter, 2000). Phosphorylation of numerous cellular and viral proteins is also observed in virally infected cells, which suggests that the protein kinases may have a role in regulating a wide variety of viral

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[3] van Hulten *et al.* (2000b) originally identified these proteins as VP26 and VP28, respectively. However, Western blot analysis using antibodies against the recombinant proteins (Lo and Kou, unpublished data) and the N-terminal sequence data given in Wang *et al.* (2000b) both confirm that these two respective proteins are in fact VP22 and VP25.

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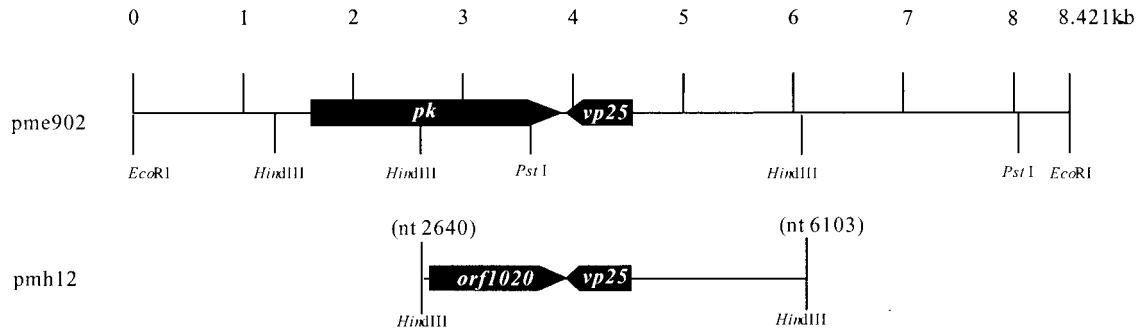

FIG. 1. Restriction enzyme map of the pme902 segment of WSSV DNA showing the location (nt 1662 to 3809) of the WSSV *pk* gene. For reference, both the alignment of the pmh12 fragment (corresponding to nt 2640 to 6103 of pme902) and the location of the partial WSSV *pk* gene (*orf1020*) as well as the envelope protein gene *vp25* are shown.

infections (Leader and Katan, 1988; Burma *et al.,* 1994; Guarino *et al.,* 1992; Kann *et al.,* 1999). Protein phosphorylation in virally infected cells may depend on cellular protein kinases (Prives, 1990) or virally encoded protein kinases (for examples, see Smith and Smith, 1989; Wu *et al.,* 1990; Zhang *et al.,* 1990; Barik and Banerjee, 1992; Lin *et al.,* 1992; Baylis *et al.,* 1993; Bischoff and Slavicek, 1994; Reilly and Guarino, 1994; Li and Miller, 1995). These virally encoded protein kinases possess most, if not all, of the conserved motifs (subdomains I–IX as defined by Hanks *et al.,* 1988) of the catalytic domains found in eukaryotic protein kinases.

Previous sequencing (Liu *et al.,* unpublished data) of a 3.4-kb *Hin*dIII genome fragment from WSSV revealed a 1020-bp open reading frame (ORF) that encodes a 339-amino-acid polypeptide with a high level of homology to the eukaryotic protein kinase catalytic domain, but this protein contained only a portion of the conserved kinase domain and was thus suspected to be a partial WSSV PK. However, a recently published paper (van Hulten and Vlak, 2001) suggested that this 1020-bp ORF encodes the entire PK catalytic domain and therefore may have misinterpreted a number of critical features of this gene. Specifically, less than half of the WSSV PK sequence is shown [although van Hulten and Vlak (2001) correctly give the ORF as 2193 bp/730 aa, they provide sequence data for only 339 amino acid residues out of 730 amino acid residues in total], the sequence of the catalytic domain is incomplete, and its location is incorrect. Further, van Hulten and Vlak (2001) locate subdomains I to V within a region that, as we show in the present paper, is in fact a large (and unique) insertion between subdomains V and VI. They also constructed a phylogenetic tree based on this misinterpretation of the PK catalytic domain sequence data. In this paper, we describe the cloning and characterization of the full-length *pk* gene, the first WSSV-encoded protein kinase gene to be identified. We also investigated and discuss the phylogenetic position of this *pk* gene.

RESULTS AND DISCUSSION

Location and structure of the WSSV *pk* gene

The virus used in this study was isolated from a batch of WSSV-infected *Penaeus monodon* collected in Taiwan in 1994 (Wang *et al.,* 1995) and which is now known as WSSV Taiwan isolate (Lo *et al.,* 1999). From this virus, a plasmid library (referred to as the pmh library, where pm indicates *Penaeus monodon* and h indicates *Hin*dIII) of WSSV *Hin*dIII genomic fragments was constructed (Wang *et al.,* 1995). During the sequencing of a 3.4-kb *Hin*dIII genomic fragment (pmh12), a 1020-nt ORF located at nucleotides 151–1170 of the pmh12 was found. When the deduced amino acid sequence of this 1020-nt ORF was compared with other sequences in GenBank at the National Center of Biotechnology Information by using the BLAST network service (Altschul *et al.,* 1990), it was found to contain only the C-terminal region catalytic domains (subdomains VI to XI) of the eukaryotic protein kinases and therefore did not appear to correspond to the complete coding region of the WSSV *pk* gene. Subsequently, from a plasmid library of *Eco*RI genomic fragments (pme library), a 1020-nt ORF-specific probe was used to identify a plasmid clone with an 8421-nt WSSV genomic fragment (pme902) containing an ORF encoding the full-length WSSV PK (Fig. 1). The putative WSSV PK ORF has two potential in-frame ATG initiation codons at nt 1617 and nt 1662. Thus the presumed PK-coding region was either located between positions nt 1617 and 3809 (2193 nt in length, encoding 730 amino acid residues) or nt 1662 and 3809 (2148 nt; 715 aa residues) of the pme902 DNA fragment. The *vp25* gene (GenBank Accession No. AF272979) coding for a WSSV major envelope protein is adjacent to the *pk* gene and arranged in a tail-to-tail configuration (Fig. 1).

Coupled *in vitro* transcription and translation

To confirm that the entire length of the putative WSSV PK ORF was not interrupted by any overlooked stop codons, nt 1617 to nt 3809 of the pme902 DNA fragment

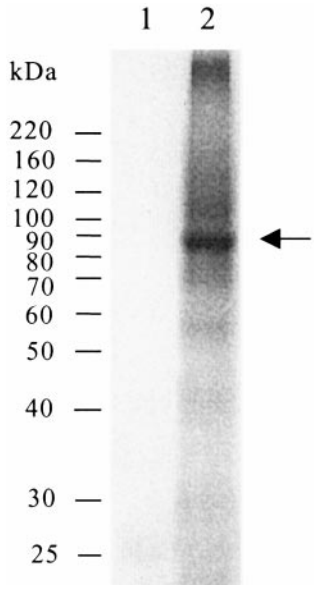

FIG. 2. Coupled *in vitro* transcription and translation product (arrow) of the WSSV *pk* gene from pcDNA3PK (lane 2) and pcDNA3-HA (lane 1, as control). Protein marker size standards (Life Technologies) on the membrane are indicated.

were inserted into the expression vector, which was then used for coupled *in vitro* transcription and eukaryotic *in vitro* translation with rabbit reticulocyte lysate. The translated protein was expected to be a hemagglutinin epitope (MCYPYDVPDYASLA)-tagged polypeptide (HA-tagged PK). As shown in Fig. 2, the synthesized HA-tagged PK had an apparent molecular mass of about 87 kDa according to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). It was also recognizable with a commercial anti-HA antibody (data not shown). At 87 kDa, the apparent molecular mass was in fairly good agreement with the size predicted from the full length PK sequence (i.e., ~80 kDa; this predicted size does not include the HA sequence at the N-terminus).

Major transcripts of the putative WSSV *pk* gene in WSSV-infected shrimp

To determine whether the putative WSSV *pk* gene was expressed during viral infection, total RNAs extracted from WSSV-infected shrimp *P. monodon* at 0, 6, 12, 18, 60 h p.i. (hours postinfection) were analyzed by Northern blot hybridization using a WSSV *pk*-specific riboprobe. A minor transcript of approximately 1.9 kb was present throughout the 60 h of the experiment, but since it was also present at 0 h, it could not have been a WSSV-specific transcript. Two WSSV-specific transcripts were detected: a major transcript of approximately 2.7 kb and a minor transcript of approximately 5.7 kb (Fig. 3). Neither transcript was found until 12 h p.i. The size of the major WSSV *pk* transcript was consistent with the predicted size of the *pk* mRNA after allowing for the presumed PK-coding region (2148 nt) plus stretches of 5′/3′ UTRs (38 nt and 25 nt, respectively; see below) and a poly(A) tail (~500 nt).

Determination of the 5′ terminus of the 2.7-kb *pk* major transcript

The 5′ region of the *pk* transcript was obtained by rapid amplification of the cDNA 5′ ends (5′ RACE) (Frohman *et al.,* 1988) using a 5′/3′ RACE kit (Roche) in which oligo(dT)-anchor primer, anchor primer, and other key reagents were included. The locations of the primers used in this study are shown in Fig. 4. For the first step of 5′ RACE, the appropriate gene-specific primer (5′ RACE-SP6 primer; Fig. 4a) was used for first-strand cDNA synthesis from the total RNA isolated from the shrimp 60 h after artificial infection with WSSV by using an AMV (avian myeloblastosis virus) reverse transcriptase. After the poly(A) head was added to the cDNA products, these cDNAs were used as templates for the first PCR amplification with the 5′ RACE-SP4/oligo(dT)-anchor primer set (Fig. 4a). The first amplification products were then used as a template for the second PCR amplification with the A93R11/anchor primer set. The PCR products from the second amplification formed a single band in an agarose gel at ~166 bp (Fig. 4c). Analysis of 5′ RACE products cloned in pGEM-T Easy vector (Fig. 4d) revealed that all the 5′ termini of the first 14 randomly picked clones were located downstream of the first predicted ATG initiation codon. Contrary to the

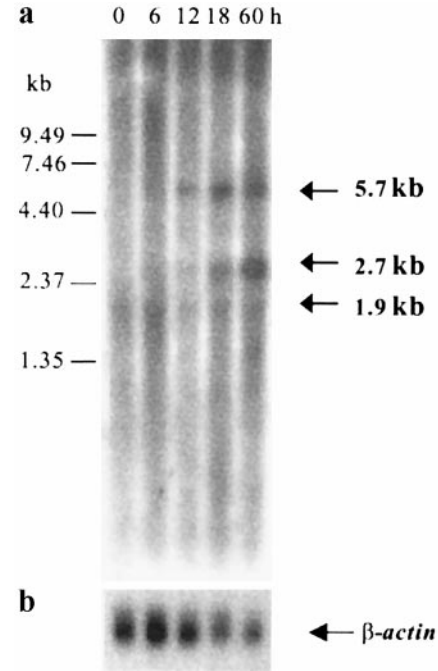

FIG. 3. (a) Northern blot hybridization of total RNA isolated from WSSV-infected *P. monodon* with a *pk*-specific riboprobe. Size standards are by RNA marker (Promega). Lane headings show hours p.i. (b) RNA quality/quantity control; the blot was also hybridized to a *β-actin* riboprobe to control for the amount of RNA loaded.

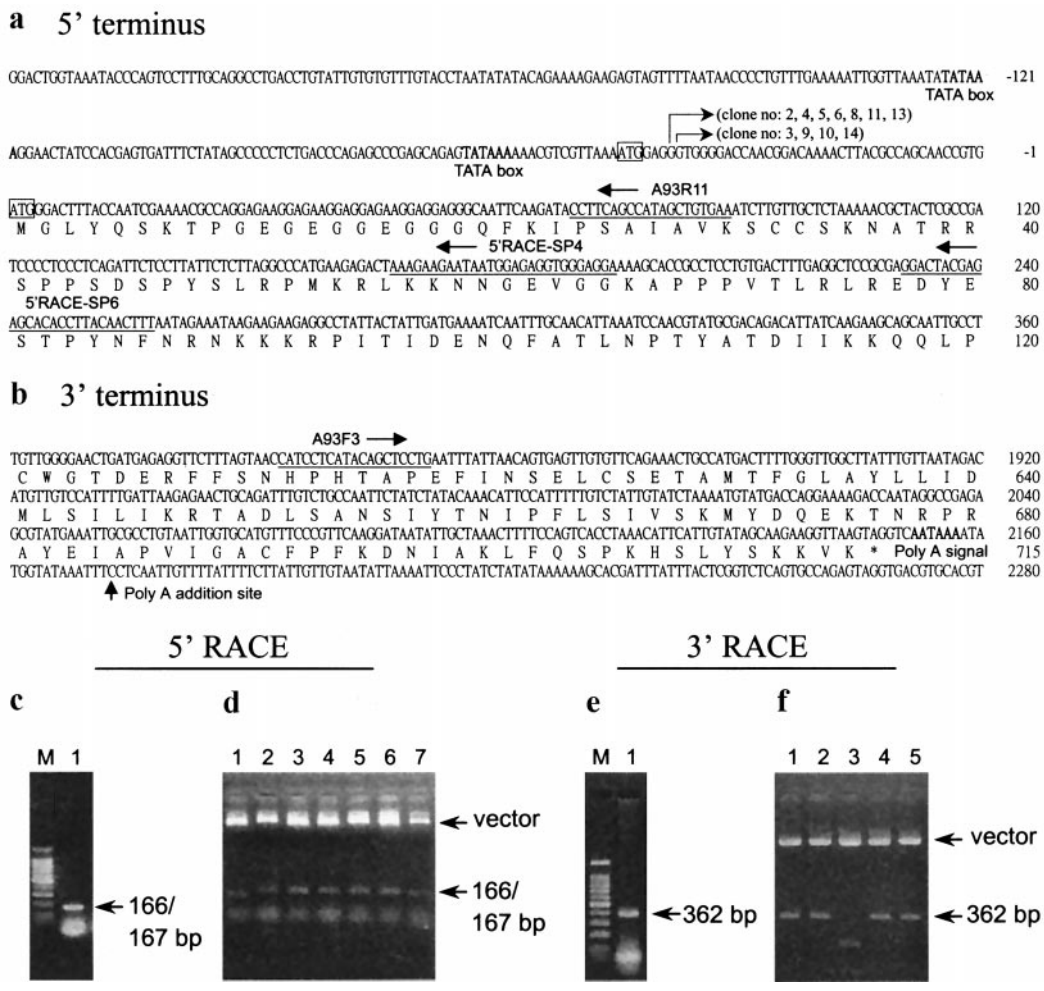

FIG. 4. Determination of the termini of the 2.7-kb major *pk* transcript. The locations of primers used for 5′ RACE (A93R11, 5′ RACE-SP4, 5′ RACE-SP6) and for 3′ RACE (A93F3) are indicated in a and b, respectively. The bent arrows indicate the 5′ termini (transcriptional start points) revealed by sequencing of 11 5′ RACE clones. The first and second ATG initiation codons in the 2193-nt ORF are boxed. (c–f) Agarose gel analysis of RACE products (c, e) and the same RACE products cloned in the pGEM-T Easy vectors (d, f) (arrows). The predicted TATA box and polyadenylation signal (AATAAA) are in boldface type. The poly(A) addition site is indicated by the straight arrow. M in c and e represents a 100-bp DNA marker ladder (Promega).

conclusion reported in van Hulten and Vlak (2001), we therefore conclude that the first predicted ATG initiation codon did not play a role in translation initiation for the WSSV *pk* gene and that the second predicted ATG initiation codon is the translational initiation site. This conclusion is further supported by the fact that the sequences surrounding the putative second translation initiation codon (GTGATGG) conform reasonably well to the eukaryotic translation consensus sequence (Kozak, 1987, 1997).

Of the 14 clones from the 5′ RACE products that were subjected to sequencing, 4 had their 5′ termini at nucleotide residue −38(G) and 7 at −39(G) [relative to the A in the second potential translation initiation codon, which is defined as +1(A)]. The remaining 3 clones were apparently early termination clones (examples: clones 1 and 7 in Fig. 4d). These results suggest that for the WSSV *pk* gene, there are two transcriptional initiation sites, located at nucleotide residues −38(G) and −39(G). Upstream of the transcriptional initiation sites, two putative TATA boxes were found at nt 59 to 64 and at nt 120 to 125.

In our previous study (Tsai *et al.,* 2000a), we found that, for the genes encoding the WSSV ribonucleotide reductase large and small subunits (*rr1* and *rr2* genes), the transcriptional start points were located within a consensus motif (TCAc/tTC). Recently, we found that the same consensus was also present in the WSSV *tk-tmk* gene encoding the novel chimeric protein of thymidine kinase and thymidylate kinase (Tsai *et al.,* 2000b). However, this consensus was not present in the transcriptional start site of the WSSV *pk* gene. Further work is needed to confirm whether there are other WSSV genes that use the same consensus sequence for their transcription initiation sites as the WSSV *pk* gene.

Determination of the 3′ terminus of the 2.7-kb *pk* major transcript

To determine the 3′ terminus of the major WSSV *pk* transcript, 3′ RACE was performed. The first-strand cDNA was synthesized using the oligo(dT)-anchor primer

and AMV reverse transcriptase. The amplification of the 3′ region of the resulting cDNA was carried out by PCR using the A93F3/anchor primer set (Fig. 4b), which yielded a PCR product of 362 bp (Fig. 4e). Sequence analysis of the cloned 3′ RACE products (Fig. 4f) revealed that poly(A) was added at a site 16 nt downstream of the AATAAA polyadenylation signal (nt 2152 to 2157), which was found 3 nucleotides downstream of the translation stop codon (Fig. 4b).

Based on the above results, we conclude that the WSSV *pk* gene consists of 2148 nt with the potential to encode a polypeptide of 715 amino acids with a theoretical size of 80 kDa and a p*I* of 9.36. The WSSV *pk* gene sequence has been deposited with GenBank under Accession No. AF335541.

WSSV *pk* transcription analysis in WSSV-infected shrimp

RT-PCR analysis was used to detect the *pk*-specific transcript in DNase-treated total RNAs from shrimp specimens before infection (0 h) and at 2, 4, 6, 8, 18, 24, 36, and 60 h after artificial infection with WSSV. As a control, PCR was also used to monitor the presence of the viral DNA in the infected shrimp. For the control, the total DNAs were extracted from pleopod tissues (200 mg) from each of the artificially infected shrimp. An aliquot (0.5 μg) of the total DNA of each specimen was subjected to WSSV DNA-specific PCR using a WSSV *pk*-specific primer set. As shown in Fig. 5, the viral DNA was first detected at 2 h and then continued to be found through to 60 h p.i. The relatively small amounts of WSSV DNA observed at 2 h p.i. are likely the result of some of the injected virions reaching the pleopod tissues that were used for the analysis, while the marked increase in intensity at 8 h p.i. probably indicates replication of the viral DNA. This interpretation of the data is only provisional, but it is consistent with what is already known about the WSSV replication cycle, which, in the cuticular epidermis of artificially infected shrimp, has been estimated at 22 h (Chang *et al.,* 1996). This question is further discussed below.

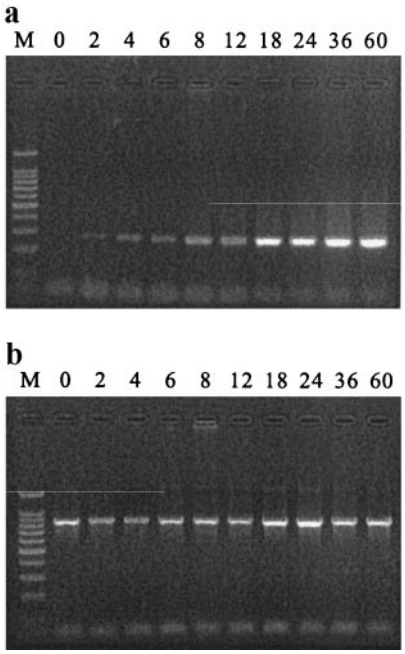

FIG. 5. Amplification of WSSV DNA in artificially infected shrimp using PCR delimited with (a) *pk*-specific primers and (b) decapod 18S ribosomal DNA degenerate primers 143/145 (5′-TGCCTTATCAGCTNTCGATTGTAG-3′/5′-TTCAGNTTTGCAACCATACTTCCC-3′) designed by Lo *et al.* (1996) for template quality/quantity control. M represents a 100-bp ladder DNA marker (Promega). Lane headings show hours p.i.

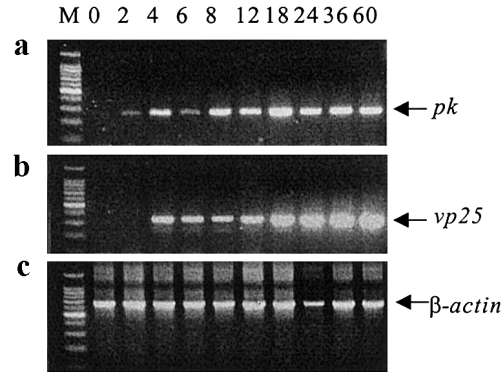

FIG. 6. RT-PCR (a) with *pk*-specific primers, (b) with *vp25*-specific primers, and (c) with *β-actin* degenerate primers (internal control). M represents a 100-bp ladder DNA marker (Promega). Lane headings show hours p.i.

For the RT-PCR analysis, total RNA extracted from the same pleopod tissues (500 mg) of each shrimp specimen was treated with DNase 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 reverse transcriptase (Life Technologies) and oligo(dT) primer in a 20-μl reaction mixture. An aliquot (2 μl) of the reaction product containing about 1 μg cDNAs was then subjected to PCR amplification with the *pk*-specific primer set and other appropriate primer sets (i.e., a primer set for the adjacent *vp25* gene for comparison and a *β-actin* primer set for control). The *pk* transcript was first detected at 2 h p.i. and continued to be found through to 60 h p.i. (Fig. 6a). The *vp25* transcript was first detected at 4 h p.i. and also continued to be expressed through to 60 h (Fig. 6b). Unlike the *pk* gene, the intensity of the *vp25* RT-PCR product band increased significantly over time, especially after 12 h p.i., and during this advanced infection period, the amount of *vp25* transcript was much higher than that of *pk.*

As a quality control, the first-strand cDNAs were subjected to PCR with a WSSV genomic DNA-specific IC-F2/IC-R3 primer set derived from an intergenic region of the WSSV genome. The region delimited by this primer set should not appear in the cDNA, and no RT-PCR products

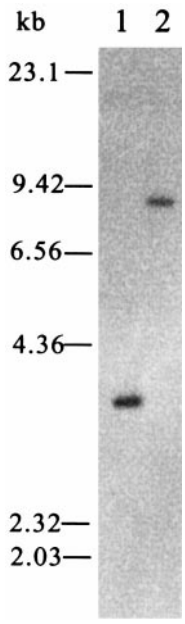

FIG. 7. Hybridization of a DIG-labeled WSSV *pk*-specific probe to Southern blots of WSSV DNA digested with *Hin*dIII (lane 1) and *Eco*RI (lane 2) restriction endonucleases. Size standards are from a Lambda *Hin*dIII DNA marker (Promega).

were in fact yielded (data not shown), thus confirming that no viral genomic DNA was left in the prepared RNA for WSSV *pk* transcription analysis.

Due to the lack of a suitable shrimp cell line, it is impossible to do a temporal analysis of WSSV gene transcription in cells synchronously infected with the virus. However, WSSV, which systematically targets cells originating from the mesoderm and ectoderm (Wongteerasupaya *et al.,* 1995; Lo *et al.,* 1997), is extremely virulent (this is true not only for the Taiwan isolate that we have consistently used since 1994, but also applies to isolates from other laboratories) and in infected individual shrimp it spreads rapidly (Chou *et al.,* 1995) and infects both cuticular epithelial and connective tissue cells at a similar rate. In our previous study (Chang *et al.,* 1996) of WSSV-specific *in situ* hybridization of the tissue sections of experimentally WSSV-infected shrimp, WSSV-positive cells were initially observed at 16 h postinfection in the cuticular epidermis, and by 22 h p.i., WSSV-positive cuticular epidermal cells with hypertrophied nuclei and other obvious cytopathological changes were readily observed in almost all the tissues examined. The sequential progression of the disease is also very similar across different individual shrimp. Thus, when pleopod tissues, which most often showed the highest prevalence of the virus in early infection (Lo *et al.,* 1997; Kou *et al.,* 1998), are used as the source of total RNA in temporal gene expression experiments, the results are generally consistent and reproducible (see, e.g., Tsai *et al.,* 2000a,b). Although this may not be an ideal assay system for temporal WSSV gene transcription analysis, the consistency of the results, whether using the same gene in different sets of RNA samples or different genes in the same set of RNA samples, suggests that this method can provide good estimates of the relative time and expression patterns for WSSV genes.

Screening for WSSV *pk* homologues in the WSSV genome by Southern blot hybridization

Using Southern blot hybridization analysis, the WSSV *pk*-specific probe hybridized only with a 3.4-kb *Hin*dIII fragment and an 8.4-kb *Eco*RI WSSV genomic DNA fragment (presumably pmh12 and pme902, respectively) (Fig. 7). These data suggest that the WSSV genome does not contain other sequences encoding WSSV PK homologues.

Amino acid sequence alignment of WSSV PK

When the deduced amino acid sequence of WSSV PK was compared with other sequences in GenBank at the National Center of Biotechnology Information by using the Blast network service (Altschul *et al.,* 1990), its carboxyl-terminal region (residues 283–715; see Fig. 8) showed homology to the catalytic domain [subdomains I to IX (Hanks *et al.,* 1988)] from a variety of eukaryotic organisms and their viruses. In particular, multiple alignments of the highly conserved regions of each subdomain show that the important residues with assigned functions for subdomains I and II (protein kinase ATP-binding site), subdomain VI (serine/threonine protein kinase active-site site), subdomain VII (Mg^{2+}-binding loop), and subdomain VIII (recognition of peptide substrate) are all conserved in WSSV PK. To take each of the subdomains in turn:

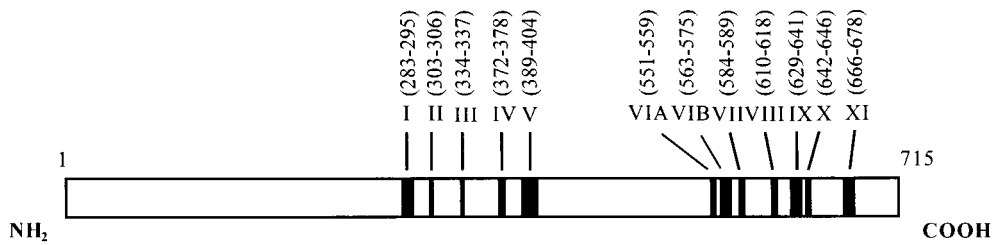

FIG. 8. WSSV PK. Locations of the conserved motifs in catalytic subdomains I to XI.

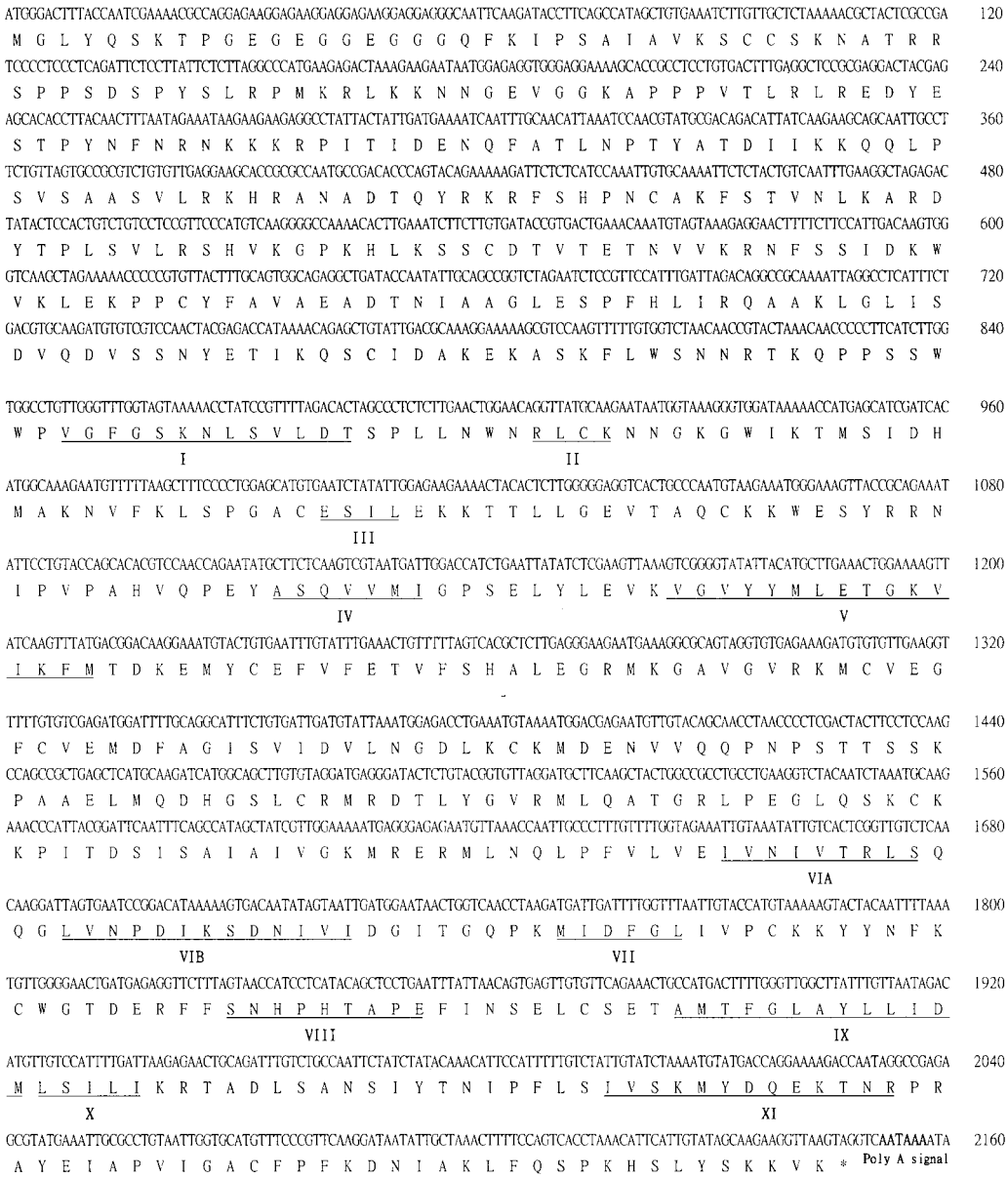

FIG. 9. The nucleotide sequence and deduced protein sequence of the coding region of the WSSV *pk* gene. The sites of the conserved catalytic subdomains are numbered I to XI and underlined.

At the N-terminal extremity of the catalytic domain of WSSV PK (Fig. 9), there is a region (subdomain I; residues 283–295) containing a glycine (G)-rich stretch of residues (284GFGSKN289), and nearby (17 residues downstream) there is the lysine residue (306K) of subdomain II (residues 303–306). The region from subdomains I to II has been shown to be involved in ATP binding (Hanks and Hunter, 1995). In the PROSITE database, this ATP-binding region is one of only two regions that have been selected to build a protein kinase signature. The WSSV PK sequence in this region [283VGFGSKNLSVLDT-x(7)-RLCK306] matches well (although not exactly) the protein kinase signature consensus pattern I: [LIV]-G-{P}-G-{P}-[FYWMGSTNH]-[SGA]-{PW}-[LIVCAT]-{PD}-x-[GSTACLIVMFY]-x-(5,18)-[LIVMFYWCSTAR]-[AIVP]-[LIVMFAGCKR]-K, where K is the amino acid residue that binds ATP. (The convention used throughout this paper for consensus patterns is to list the acceptable alternative amino acids for a given position between square brackets "[ ]" and to place between a pair of curly brackets "{ }" those amino acids that are not acceptable at a given position.)

The sequences of 334ESIL337, 372ASQVVMI378, 389VGVYYMLETGKVIKFM404, and 551IVNIVTRLS559 show homology to subdomains III, IV, V, and VIA, respectively. In subdomain VIB, which is highly conserved

among protein kinases and is used to build the protein kinase signature consensus pattern II, the WSSV PK sequence 563LVNPDIKSDNIVI575 matches well with the protein kinase signature consensus pattern II, [LIVMFYC]-x-[HY]-x-D-[LIVMFY]-K-x(2)-N-[LIVMFYCT](3), where the aspartic acid residue (567D) is probably the residue important for the catalytic activity of the enzyme (Knighton *et al.,* 1991). The residue of WSSV PK at position 569 is K, indicating that it is a serine/threonine-specific protein kinase.

Subdomain VII includes a Mg^{2+}-binding loop that contains a highly conserved Asp-Phe-Gly (DFG) triplet (Hanks and Hunter, 1995). In WSSV PK subdomain VII, the corresponding residues are found in a highly conserved region, 584MIDFGL589. Subdomain VIII plays a major role in the recognition of peptide substrates and contains a highly conserved Ala-Pro-Glu (APE) motif. In WSSV PK, this APE motif is located in a highly conserved region, 610SNHPHTAPE618. In WSSV PK, subdomains IX to XI are not so clearly defined, but three sites were found (amino acid residues 629 to 641, 642 to 646, and 666 to 678) that show significant homology to the corresponding regions of subdomains IX to XI in some of the selected protein kinases (Table 1). The WSSV PK subdomain sequence data presented here, however, should be considered only as preliminary evidence, and further experiments on mutations should be made to confirm the validity of these sites, to precisely determine the location of each subdomain, and to identify which residues are functionally important.

The alignments also clearly demonstrate the overall similarity of the WSSV PK catalytic domain to the other selected protein kinases in terms of the order and spacing of the subdomains (Fig. 8). However, a very large insert (146 residues) occurs in WSSV PK between subdomains V and VI, and this causes the WSSV PK catalytic domain (433 residues) to be considerably larger than most of the other protein kinase catalytic domains, which usually range from 250 to 300 amino acid residues (Hanks and Hunter, 1995). It should also be noted that while the location of the catalytic domain within the protein kinase is not fixed, in most single subunit enzymes it lies near the carboxyl terminus, the amino terminus being devoted to a regulatory role, whereas in protein kinases having a multiple subunit structure, subunit polypeptides consisting almost entirely of catalytic domain are common (Hanks *et al.,* 1988). WSSV PK resembles the single subunit enzymes in that its catalytic domain lies near the carboxyl terminus. The sequence of its long amino terminus (282 residues) upstream of the catalytic domain is unique, however, and more work will be needed to elucidate its function. To date, binding sites for regulatory elements such as Ca^{2+}, cAMP, or phorbol esters/diacyglycerol have not been identified in this region.

Pairwise comparisons

For pairwise comparisons and phylogenetic analysis of the WSSV PK catalytic domain to the PK catalytic domains of other protein kinases, 29 viral PK (including WSSV) and 55 PK from fungi, invertebrates, vertebrates, a bacterium, and a plant were selected. Of the 55 nonviral protein kinases, 21 are representative members of the four major groups of protein kinases designated by Hanks and Hunter (1995), that is, (1) the AGC group, which includes the cyclic-nucleotide-dependent family (PKA and PKG), the protein kinase C (PKC) family, the ribosomal S6 kinase family, and other relatives; (2) the CaMK group, which includes the family of protein kinases regulated by calcium/calmodulin, the Snf1/AMPK (kinase essential for release from glucose repression/AMP-activated protein kinase) family, and other close relatives; (3) the C-M-G-C group, which includes the family of cyclin-dependent kinases, the Erk (MAP) kinase (extracellular signal-regulated kinase) family, the glycogen synthase 3 (GSK3) family, the casein kinase II family, the Clk (Cdk-like kinase) family, and other close relatives; and (4) the "conventional" PTK group. The other 34 nonviral protein kinases fall outside these four major groups (Table 2). As for the 29 viral protein kinases, in addition to WSSV, 9 were from the *Poxviridae,* 8 from the *Baculoviridae,* 11 from the *Herpesviridae,* 2 from the *Phycodnaviridae,* and 1 each from the *Iridoviridae* and *Asfarviridae* (Table 3).

The pairwise identity and similarity (BLOSUM 35) of the WSSV PK catalytic domain to the PK catalytic domains of the selected PKs are summarized in Table 1. The overall homology of the WSSV PK and the protein kinases is not high: approximately 4 to 11% identity and 11 to 26% similarity was shown over the entire length of the catalytic domains. Within the subdomains, however, conservation of amino acid sequences is much higher, with the highest levels of homology [identity(%)/similarity(%)] for the conserved sequence in subdomains I, II, III, IV, V, VIA, VIB, VII, VIII, IX, X, and XI being 46/61, 50/50, 60/80, 42/71, 25/56, 33/66, 61/92, 83/100, 44/66, 42/71, 60/80, and 38/69, respectively.

Phylogenetic analysis

The sequences of the PK catalytic domains of all 84 of the proteins listed in Tables 2 and 3 were included in our original analysis, but it was found that WSSV PK did not cluster with any of the eukaryotic PK or with any of the viral PK (phylogenetic tree not shown). To focus on the relationships among viral PK, most of the eukaryotic PSK were therefore omitted and the phylogenetic tree was reconstructed. In addition, since all of the selected viral PK are PSK, the eukaryotic PTK group was also omitted. Thus in total 38 sequences (29 viral PK and 9 eukaryotic PK) from three of the major groups were used to construct the final phylogenetic trees (Fig. 10). [Note that

TABLE 1

Pairwise Comparison of the Amino Acid Sequence (Identity/Similarity; BLOSUM 35) of the Catalytic Domain of WSSV Protein Kinase (Residues 283–715) with the Consensus Regions for Subdomains I to XI of other Protein Kinases

Protein kinase source	Catalytic subdomains												Entire domain
	I	II	III	IV	V	VIA	VIB	VII	VIII	IX	X	XI	I–XI
1. PKA-Cα (human)	30/46	25/25	60/60	14/42	12/50	33/33	38/76	50/83	33/44	21/50	20/40	15/46	9/19
2. PKG-I (*Bos taurus*)	23/30	25/25	40/60	14/57	12/43	22/33	23/76	50/100	33/44	14/57	20/40	15/46	9/20
3. cPKCα (Norway rat)	30/46	25/25	40/60	0/28	12/43	22/33	30/76	50/83	33/44	28/50	40/40	15/30	9/19
4. βARK1 (human)	23/30	25/25	40/60	14/42	12/31	11/44	38/69	50/83	44/55	14/42	0/20	7/46	8/20
5. S6K (human)	23/38	25/25	60/80	14/42	6/43	22/33	30/76	66/83	33/44	28/50	20/20	23/46	11/22
6. RSK1 (Nt) (human)	30/46	25/25	60/60	28/42	6/43	11/33	30/76	66/83	33/44	21/57	40/40	15/53	10/21
7. DMPK (human)	23/46	25/25	40/60	0/28	12/56	22/44	53/76	50/66	22/44	14/42	20/20	7/30	8/20
8. CaMK2α (Norway rat)	23/46	25/25	40/40	14/42	6/43	22/33	30/84	66/83	22/44	14/42	40/40	23/53	8/19
9. skMLCK (*Oryctolagus cuniculus*)	23/38	25/25	20/60	0/42	12/56	11/44	30/76	83/100	22/33	14/50	0/20	30/53	7/21
10. Mre4 (baker's yeast)	46/53	25/25	60/60	0/42	6/37	22/44	46/84	50/83	33/44	14/50	0/20	15/30	7/19
11. PhKγM (*O. cuniculus*)	23/38	25/25	60/60	0/42	12/50	11/55	38/84	50/83	33/44	14/50	20/60	23/46	8/21
12. Kin1 (baker's yeast)	38/38	25/25	40/60	0/42	12/56	22/44	46/84	83/100	33/44	21/57	40/40	30/53	10/21
13. Snfl (baker's yeast)	30/38	25/25	60/60	0/57	6/43	22/44	30/84	66/83	33/55	21/50	40/40	23/46	8/20
14. Polo (fruit fly)	15/23	25/25	40/60	14/28	0/43	33/44	23/69	66/83	33/44	7/42	20/20	23/38	7/20
15. Cdc5 (baker's yeast)	15/30	25/25	40/40	14/28	6/50	11/33	30/61	66/83	33/44	14/57	20/40	7/38	7/20
16. Cdk2 (mouse)	23/38	25/25	60/80	14/42	18/50	0/33	30/76	66/83	33/44	14/57	0/40	23/53	7/19
17. Erk2 (*B. taurus*)	23/38	25/25	60/60	14/42	12/37	11/44	23/76	66/83	33/55	21/50	0/60	23/38	7/18
18. GSK3α (Norway rat)	30/38	25/25	40/60	14/42	18/43	0/33	30/69	50/66	33/55	14/50	0/60	15/53	7/18
19. CK2α (mouse)	23/53	25/50	60/60	0/57	6/31	0/33	30/76	66/100	22/44	28/50	0/80	15/46	7/17
20. Clk (human)	23/53	25/25	40/60	0/0	6/25	33/33	38/69	50/83	44/55	21/50	0/60	23/53	7/16
21. Ire1 (baker's yeast)	30/61	25/25	40/60	14/28	0/31	33/44	30/76	66/83	33/33	7/42	20/40	30/69	7/17
22. Cdc7 (baker's yeast)	30/53	25/25	40/80	28/71	12/25	0/44	30/61	66/100	33/33	14/57	40/40	7/53	5/14
23. Cot (human)	15/30	50/50	20/20	0/28	12/43	11/44	46/69	66/100	22/44	28/50	0/0	7/46	7/18
24. YpkA (*Yersinia pseudotuberculosis*)	7/23	25/50	20/20	0/28	11/29	11/66	46/69	66/100	33/33	7/21	0/20	7/38	7/18
25. MEK1 (human)	23/46	25/25	40/60	14/42	12/50	22/55	30/76	50/83	22/44	28/57	20/60	23/38	7/18
26. Ste7 (baker's yeast)	23/53	25/25	60/80	0/28	18/43	22/55	38/76	50/83	22/44	21/71	20/60	15/46	8/22
27. Ste11 (baker's yeast)	38/46	25/25	40/80	14/28	18/43	33/44	46/76	50/83	22/44	21/57	0/40	15/46	7/19
28. Nek1 (mouse)	23/38	25/50	40/80	14/28	18/43	11/33	46/76	50/83	22/44	14/50	40/60	15/69	8/21
29. NIMA (*Aspergillus nidulans*)	23/38	25/25	60/80	14/28	12/37	22/44	30/76	66/83	22/44	7/42	40/40	7/38	6/19
30. Fused (fruit fly)	38/38	25/25	40/40	14/57	12/43	22/55	23/76	66/83	33/44	14/42	60/60	7/53	9/19
31. NinaC (fruit fly)	15/30	25/25	20/20	0/14	12/31	11/22	38/84	66/83	33/44	14/50	0/20	23/53	7/17
32. Ste20 (baker's yeast)	23/38	25/25	20/60	14/28	25/43	11/44	53/92	50/83	33/33	21/71	0/40	15/30	9/21
33. Cdc15 (baker's yeast)	30/38	25/25	60/80	14/42	12/50	11/33	38/84	50/83	33/33	14/57	0/40	23/46	8/21
34. Npr1 (baker's yeast)	30/46	25/25	40/40	0/28	6/25	33/44	53/69	66/83	44/66	7/42	20/20	38/46	8/17
35. Pim1 (mouse)	30/38	25/25	40/60	28/57	6/31	11/33	46/84	66/83	22/44	42/71	0/20	7/38	9/22
36. Rna1 (fission yeast)	23/38	25/25	20/60	0/42	18/43	11/44	30/76	66/83	22/55	21/64	0/0	15/69	7/20
37. Esk (mouse)	23/46	25/25	40/60	14/42	0/31	11/44	46/69	66/100	33/44	21/50	40/60	7/30	8/18
38. Elm1 (baker's yeast)	23/23	25/25	40/40	14/57	6/31	11/33	38/69	50/66	33/33	14/50	0/40	15/61	8/18
39. SpWeel (fission yeast)	23/46	25/25	60/60	14/57	18/43	0/33	38/69	50/83	33/55	7/50	20/20	38/46	8/18
40. Weel (Hs) (human)	30/46	25/25	20/20	42/57	12/31	0/33	61/69	33/66	22/33	7/35	0/0	15/38	7/17
41. PKR (human)	23/38	25/50	40/40	14/28	6/56	22/33	38/69	66/83	22/44	21/57	0/40	15/61	7/18
42. Gcn2 (baker's yeast)	23/38	25/25	40/60	28/28	6/37	11/55	38/69	66/83	22/33	21/64	0/40	23/53	6/17
43. CK1α (human)	23/38	25/25	20/40	14/28	0/31	0/55	38/76	83/100	0/22	21/50	20/40	7/23	5/16
44. PKN1 (*Myxococcus xanthus*)	23/38	25/25	20/40	28/42	0/43	0/33	46/76	50/100	33/44	7/42	20/20	7/53	6/18
45. Ykl516 (baker's yeast)	30/61	25/25	40/60	14/42	12/43	33/44	30/84	66/83	22/55	14/42	0/60	15/30	6/17
46. Mos (human)	30/38	25/25	20/60	14/42	0/37	33/55	46/76	50/66	44/55	21/42	0/40	7/38	7/19
47. ZmPK1 (*Zea mays*)	23/38	25/25	40/80	28/42	12/37	11/33	30/84	66/83	33/44	14/71	0/60	7/53	6/20
48. Pelle (fruit fly)	23/30	25/25	40/40	0/42	12/31	11/22	46/76	66/83	22/33	21/71	20/40	7/23	8/19
49. TGFβRII (human)	30/30	25/25	40/60	0/28	6/50	11/33	46/84	66/83	33/44	21/50	0/20	15/61	7/17
50. ACTRII (mouse)	15/15	25/25	40/40	0/28	12/43	11/33	38/76	66/83	33/44	28/50	0/20	15/53	6/16
51. Raf-1 (human)	30/46	25/25	40/80	0/42	6/31	11/22	38/84	66/83	33/44	14/64	0/60	23/46	8/21
52. Sp1A (*Dictyostelium discoideum*)	23/30	25/25	60/60	28/28	18/50	22/33	30/76	66/83	44/55	14/64	0/20	23/61	9/21
53. Src (human)	23/30	25/25	20/60	14/57	25/56	11/44	30/76	66/83	44/44	28/64	0/40	15/53	8/20
54. EGFR (human)	23/46	25/25	20/60	14/42	12/43	22/33	30/76	66/83	22/22	7/57	20/80	23/53	8/19
55. PDGFRβ (mouse)	23/53	25/25	40/60	28/42	12/43	22/55	30/69	66/83	33/33	28/64	20/40	23/61	10/26
56. ASFV-PK (swine virus)	23/46	25/50	20/60	14/42	6/50	11/55	38/84	66/83	22/33	14/57	20/20	7/30	8/21
57. PBCV1-PK (*Chlorella* virus)	15/38	25/25	20/80	0/42	12/37	0/33	46/76	50/66	33/33	28/42	0/40	23/46	7/19
58. FsV-PK (brown alga virus)	23/46	25/25	20/60	28/42	12/50	0/44	38/69	50/83	33/55	0/50	20/20	15/46	6/19
59. IIV6-PK (insect virus)	30/53	25/25	12/25	14/42	5/21	11/33	30/69	50/83	22/33	14/28	20/40	23/46	5/14
60. VACV-PKB1R (human virus)	30/46	25/50	12/12	0/22	0/31	22/55	46/69	50/100	11/22	14/35	0/0	0/15	6/16
61. FWPV (fowl virus)	30/38	25/50	12/25	0/11	6/50	0/44	38/76	50/100	0/33	7/35	0/40	0/0	5/17
62. MYXV-PK (rabbit virus)	15/30	25/50	37/50	11/55	12/31	11/33	46/84	33/66	33/33	7/14	40/40	7/30	7/16
63. MOCV1-PK (human virus)	15/30	25/50	25/50	11/55	12/25	11/33	38/84	33/66	22/22	7/14	20/40	0/38	5/16
64. VAR-PK (human virus)	30/46	25/50	12/12	11/22	0/31	22/55	46/69	50/100	11/22	14/35	0/40	0/15	6/16
65. VACV-PK2 (human virus)	15/30	25/50	25/50	11/55	6/25	22/33	38/84	33/66	33/33	7/21	40/60	7/38	5/15
66. MSEV-PK (grasshopper virus)	7/30	25/50	25/25	11/55	0/17	33/44	38/84	50/66	11/22	0/14	0/20	7/23	4/11
67. LdMNPV-PK (insect virus)	15/23	25/50	20/40	0/28	18/56	11/55	30/76	50/83	22/44	21/42	20/40	7/23	6/19
68. AcMNPV-PK (insect virus)	7/23	50/50	20/40	14/28	6/43	11/33	30/76	50/83	22/44	14/28	20/40	15/23	6/17
69. AfMNPV-PK (insect virus)	7/23	50/50	20/40	14/28	6/43	11/33	30/76	50/83	22/44	14/28	20/40	15/23	6/17
70. OpMNPV-PK1 (insect virus)	15/23	50/50	20/60	14/28	6/43	11/44	23/69	50/83	22/44	14/28	20/40	15/23	6/16
71. HaSNPV-PK (insect virus)	7/23	25/50	20/40	0/28	18/50	11/33	23/76	50/83	22/44	14/42	0/20	15/23	5/17
72. SpltMNPV-PK (insect virus)	15/23	50/50	20/60	28/42	12/56	11/33	30/76	50/83	22/44	7/35	20/40	15/30	6/17
73. SeMNPV-PK1 (insect virus)	15/23	50/50	20/40	0/28	6/50	11/33	30/76	50/83	22/44	14/35	20/60	15/30	5/18
74. BmNPV-PK (insect virus)	7/23	50/50	20/40	14/28	6/43	11/33	30/76	50/83	22/44	14/28	20/40	15/23	6/17
75. HHV1-PK1 (human virus)	30/61	25/50	40/60	0/42	0/31	11/55	46/84	50/66	33/33	14/50	0/20	23/38	6/18
76. SuHV1-PK (swine virus)	23/46	25/50	40/60	14/42	0/37	11/44	30/84	33/66	33/33	14/42	60/60	23/46	6/18
77. HHV1-PK2 (human virus)	23/23	25/25	33/33	12/25	6/31	22/44	38/69	50/66	22/44	14/50	0/60	23/46	6/15
78. HHV2-PK (human virus)	23/23	25/25	33/33	12/25	6/31	22/44	30/69	50/66	22/44	14/50	0/60	23/46	6/14
79. EHV1-PK (equine virus)	30/30	25/25	33/66	12/37	0/18	11/44	38/61	33/66	22/22	14/50	20/40	23/46	5/14
80. EHV4-PK (equine virus)	23/46	25/50	40/60	14/42	6/37	22/44	38/84	50/66	33/33	21/57	40/60	23/38	7/19
81. BoHV1.1-PK (bovine virus)	30/38	25/25	33/50	14/42	0/31	22/44	46/61	50/66	22/33	21/50	0/60	15/23	6/14
82. GaHV2-PK (gallid virus)	30/30	25/25	33/50	14/42	0/18	22/44	46/61	50/66	11/22	21/50	20/60	7/23	5/14
83. CeHV9-PK (simian virus)	7/38	25/50	33/33	0/42	0/31	11/44	38/76	50/66	33/33	21/57	20/80	23/46	5/18

Note. Refer to Fig. 9 for the location of each region and to the text for the abbreviated names.

TABLE 2

Nonviral Protein Kinases Used in the Pairwise Comparison and Phylogenetic Study

Protein kinases	Source	Accession No.
AGC group		
1. PKA-Cα (cyclic AMP-dependent protein kinase catalytic subunit, α-form)	*Homo sapiens*	OKHU2C
2. PKG-I (cyclic GMP-dependent protein kinase, type I)	*Bos taurus*	OKBOG
3. cPKCα (Ca^{2+}-dependent protein kinase C, α-form)	*Rattus norvegicus*	P05696
4. βARK1 (β-adrenergic receptor kinase, type 1)	*H. sapiens*	A53791
5. S6K (70-kDa S6 kinase with single catalytic domain)	*H. sapiens*	NM_003952
6. RSK1 (Nt) (90-kDa S6 kinase, type 1)	*H. sapiens*	NM_002953
7. DMPK (myotonic dystrophy protein kinase)	*H. sapiens*	NP_004400
CaMK group		
1. CaMK2α (Ca^{2+}-calmodulin kinase type II, α subunit)	*R. norvegicus*	A30355
2. PhKγM (skeletal muscle phosphorylase kinase catalytic subunit)	*Oryctolagus cuniculus*	KIRBFG
3. skMLCK (skeletal muscle myosin light chain kinase)	*O. cuniculus*	A35021
4. Mre4 (protein required for meiotic recombination)	*Saccharomyces cerevisiae*	P24719
5. Snf1 (kinase essential for release from glucose repression)	*S. cerevisiae*	AAA35058
6. Kin1 (protein kinase with N-terminal catalytic domain)	*S. cerevisiae*	S52687
C-M-G-C group		
1. Cdk2 (type 2 cyclin-dependent kinase)	*Mus musculus*	NP_058036
2. Erk2 (extracellular signal-regulated kinase, type 2)	*B. taurus*	P46196
3. GSK3α (glycogen synthase kinase 3, α-form)	*R. norvegicus*	P18265
4. CK2α (casein kinase II, α subunit)	*M. musculus*	CAA04753
5. Clk (Cdc-like kinase)	*H. sapiens*	NP_004062
PTK group		
1. Src (cellular homologue of Rous sarcoma virus oncoprotein)	*H. sapiens*	NP_005408
2. EGFR (epidermal growth factor receptor)	*H. sapiens*	NP_005219
3. PDGFRβ (platelet-derived growth factor receptor, type β)	*M. musculus*	P05622
Other protein kinase families (not falling into major groups)		
1. Polo (protein kinase homologue required for mitosis)	*Drosophila melanogaster*	P52304
2. Cdc5 (product of gene required for cell cycle progression)	*S. cerevisiae*	M84220
3. MEK1 (MAP ERK kinase, type 1)	*H. sapiens*	Q02750
4. Ste7 (kinase required for haploid-specific gene expression)	*S. cerevisiae*	P06784
5. Ste11 (protein required for cell-type-specific transcription)	*S. cerevisiae*	S51380
6. Ste20 (product of gene required for pheromone response)	*S. cerevisiae*	S28394
7. Nek1 (NimA-related kinase)	*M. musculus*	P51954
8. NIMA (cell cycle control protein kinase)	*Aspergillus nidulans*	P11837
9. Fused (product of gene required for segment polarity)	*D. melanogaster*	JC4243
10. Weel (Hs) (gene product able to complement *S. pombe* weel mutant)	*H. sapiens*	CAA43979
11. SpWeel ("Wee" size at division kinase; Cdc2 negative regulator)	*Schizosaccharomyces pombe*	AAA35354
12. PKR (double-stranded RNA-dependent kinase)	*H. sapiens*	JC5225
13. Gcn2 (protein required for translational derepression)	*S. cerevisiae*	AAA34881
14. Raf-1 (cellular homologue of retroviral oncogene product)	*H. sapiens*	NP_002871
15. ACTRII (type II receptor for activin)	*M. musculus*	P27038
16. TGFβRII (type II receptor TGF-β)	*H. sapiens*	P37173
17. ZmPK1 (putative receptor protein-serine kinase)	*Z. mays*	P17801
18. CK1α (casein kinase I, type α)	*H. sapiens*	NP_001883
19. PKN1 (protein kinase homologous to eukaryotic kinases)	*Myxococcus xanthus*	A41090
Other protein kinase families (each with no known close relatives)		
1. Mos (cellular homologue of retroviral oncogene product)	*H. sapiens*	TVHUMS
2. Pim1 (proto-oncogene activated by murine leukemia virus)	*M. musculus*	TVMSP1
3. Cot (product of oncogene expressed in human thyroid carcinoma)	*H. sapiens*	P41279
4. Esk ("embryonal carcinoma STY kinase"; dual specificity)	*M. musculus*	AAA37578
5. NinaC (product of gene essential for photoreceptor function)	*D. melanogaster*	B29813
6. Pelle (product of gene required for dorsoventral polarity)	*D. melanogaster*	AAA28750
7. Sp1A (spore lysis A protein kinase)	*Dictyostelium discoideum*	P18160
8. Cdc7 ("cell-division-cycle" control gene product)	*S. cerevisiae*	AAA34458
9. Cdc15 (cell-division-cycle control gene product)	*S. cerevisiae*	S15038
10. Npr1 (product of gene required for activity of ammonia-sensitive amino acid)	*S. cerevisiae*	S63138
11. Elm1 (product of gene required for yeast-like cell morphology)	*S. cerevisiae*	AAA02892
12. Ire1 (required for Myo-inositol synthesis and signaling from ER to the nucleus)	*S. cerevisiae*	A47541
13. YkI516 (putative protein kinase gene on chromosome XI)	*S. cerevisiae*	AAB21999
14. Rna1 (product of gene required for normal meiotic function)	*S. pombe*	A25685
15. YpkA (enterobacterial protein kinase essential for virulence)	*Yersinia pseudotuberculosis*	S30060

TABLE 3

Viral Protein Kinases Used in the Pairwise Comparison and Phylogenetic Analyses

Viral protein kinase	Virus: Species	Virus: Family	Accession No.	Reference
1. ASFV-PK	African swine fever virus	*Asfarviridae*	NP_042813	Baylis *et al.* (1993)
2. PBCV1-PK	*Paramecium bursaria Chlorella* virus 1	*Phycodnaviridae*	U14660	Que and Van Etten (1995)
3. FsV-PK	*Feldmannia* sp. virus	*Phycodnaviridae*	AAC33140	Lee *et al.* (1998)
4. IIV6-PK	Invertebrate iridescent virus 6 (Chilo iridescent virus)	*Iridoviridae*	AAB94478	Bahr *et al.* (1997)
5. VACV-PKB1R	Vaccinia virus	*Poxviridae*	TVVZ9Z	Lin *et al.* (1992)
6. FWPV-PK	Fowlpox virus	*Poxviridae*	NP_039189	Afonso *et al.* (2000)
7. MYXV-PK	Myxoma virus	*Poxviridae*	NP_051734	Cameron *et al.* (1999)
8. MOCV1-PK	Molluscum contagiosum virus subtype 1	*Poxviridae*	NP_043968	Senkevich *et al.* (1996)
9. VARV-PK	*Variola major* virus	*Poxviridae*	AAA60910	Massung *et al.* (1993)
10. VACV-PK2	Vaccinia virus	*Poxviridae*	U32589	Traktman *et al.* (1989)
11. MSEV-PK	*Melanoplus sanguinipes* entomopoxvirus	*Poxviridae*	NP_048244	Afonso *et al.* (1999)
12. LdMNPV-PK	*Lymantria dispar* nucleopolyhedrovirus	*Baculoviridae*	NP_047639	Bischoff and Slavicek (1994)
13. AcMNPV-PK	*Autographa californica* nucleopolyhedrovirus	*Baculoviridae*	NP_054039	Reilly and Guarino (1994)
14. AfMNPV-PK	*Anagrapha falcifera* nuclearpolyhedrosisvirus	*Baculoviridae*	AAB53359	Federici and Hice (1997)
15. OpMNPV-PK1	*Orgyia pseudotsugata* nuclearpolyhedrosisvirus	*Baculoviridae*	NP_046157	Ahrens *et al.* (1997)
16. HaSNPV-PK	*Helicoverpa armigera* nuclearpolyhedrosisvirus	*Baculoviridae*	JC5681	Zhang *et al.* (1997)
17. SpltMNPV-PK	*Spodoptera litura* nucleopolyhedrovirus	*Baculoviridae*	AF039272	
18. SeMNPV-PK1	*Spodoptera exigua* nucleopolyhedrovirus	*Baculoviridae*	NP_037763	Ijkel *et al.* (1999)
19. BmNPV-PK	*Bombyx mori* nuclearpolyhedrosisvirus	*Baculoviridae*	NP_047416	Kamita and Maeda (1997)
20. HHV1-PK1	Human herpesvirus 1 (herpes simplex virus)	*Herpesviridae*	NP_044655	McGeoch *et al.* (1986)
21. SuHV1-PK	Suid herpesvirus 1 (pseudorabies virus)	*Herpesviridae*	D10451	van Zijl *et al.* (1990)
22. HHV1-PK2	Human herpesvirus 1	*Herpesviridae*	NP_044614	McGeoch *et al.* (1986)
23. HHV2-PK	Human herpesvirus 2	*Herpesviridae*	NP_044482	McGeoch *et al.* (1987)
24. EHV1-PK	Equine herpesvirus 1	*Herpesviridae*	NP_041058	Allen and Coogle (1988)
25. EHV4-PK	Equine herpesvirus 4	*Herpesviridae*	NP_045286	Telford *et al.* (1998)
26. BoHV1.1-PK	Bovine herpesvirus type 1.1	*Herpesviridae*	NP_045346	
27. GaHV2-PK	Gallid herpesvirus 2	*Herpesviridae*	NP_057771	Lee *et al.* (2000)
28. CeHV9-PK	Cercopithecine herpesvirus 9	*Herpesviridae*	AAA47887	Fletcher and Gray (1993)

since both the neighbor-joining (NJ) and parsimony trees generated similar results, and since the NJ tree also revealed finer structures within major phylogenetic clades, only the NJ tree (Saitou and Nei, 1987) is shown here.] On the tree, the three major PSK groups (i.e., AGC, CaMk, and C-M-G-C groups), as expected, form three major clades while the viral PK reflect their current phylogenetic grouping. There are two well-supported (bootstrap value greater than 90%) clades within the *Poxviridae* although the relationship between the two poxvirus clades is not resolved. A similar situation is also found in the *Herpesviridae*. This suggests that, within these two viral families, there may have been two different sources for the PK genes. The baculoviruses form a well-supported clade with high bootstrap value, while WSSV, one of the *Iridoviridae* (IIV6), the two *Phycodnaviridae* (FsV and PBCV1), and the type species of the newly defined family *Asfarviridae* (ASFV) (van Regenmortel *et al.,* 2000) all form distinct viral groups of their own. However, the relationships among the major clades, including the eukaryotic PK, constitute an unresolved polytomy. This star phylogeny and the relatively long branch length both suggest that WSSV is a distinct virus (likely at the family level) that does not belong to any of the virus families that are currently recognized.

MATERIALS AND METHODS

Virus, plasmid clone, and sequence analysis

The virus (WSSV Taiwan isolate) was isolated from a batch of WSSV-infected *P. monodon* collected in Taiwan in 1994 (Wang *et al.,* 1995; Lo *et al.,* 1999). From the *Hin*dIII and *Eco*RI libraries of genomic fragments, plasmid clones (pmh12; pme902) carrying the *pk* gene were sequenced on both DNA strands by using universal M13 forward and reverse primers. The internal sequences of the cloned fragments were obtained by automatic sequence walking (Mission Biotech, Taipei, Taiwan) using customed synthesized primers. All of the sequences were confirmed by sequencing both strands completely. Sequence data were compiled and analyzed using three computer programs: GeneWorks 2.5.1 (IntelliGenetics), UWGCG (release 9.0, Genetics Computer Group), and Neural Network for promoter prediction (NNPP; Reese, 1994; Reese and Eeckman, 1995; Reese *et al.,* 1996). The DNA and the deduced amino acid sequences were com-

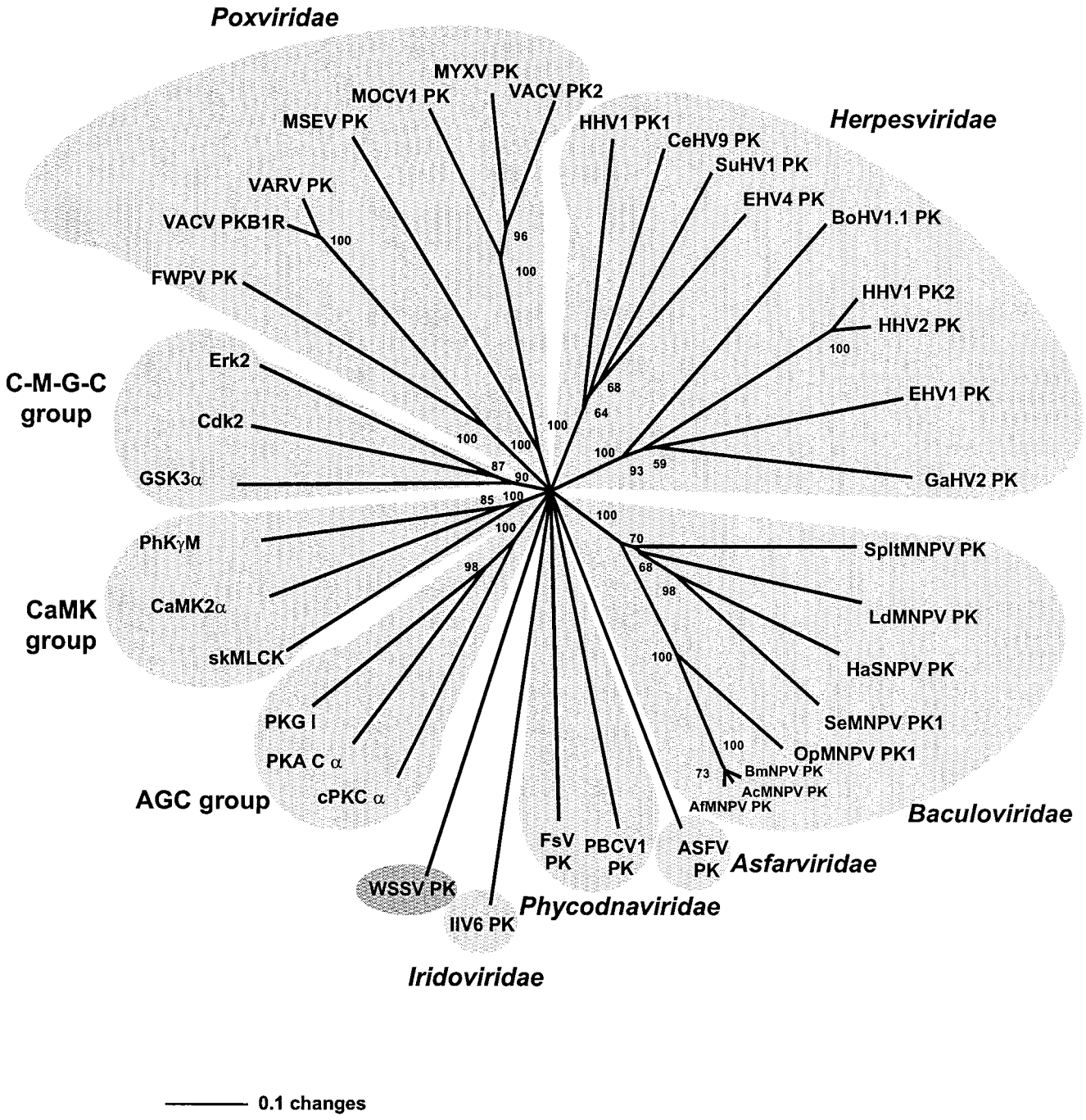

FIG. 10. Unrooted neighbor-joining phylogenetic tree of the WSSV PK catalytic domains of 29 viral PK (listed in Table 3) and 9 eukaryotic PK from three of the major PSK groups (i.e., AGC, CaMk, and C-M-G-C groups; refer to Table 2). Numbers indicate bootstrap values.

pared with the latest GenBank/EMBL, SWISSPROT, and PIR databases using FASTA and BLAST. Alignments of amino acid sequences were made in CLUSTAL_X (Thompson *et al.,* 1997) and edited in GeneDoc (Nicholas *et al.,* 1997).

Coupled *in vitro* transcription and translation

An expression vector with the full-length WSSV *pk* gene was constructed for *in vitro* transcription and translation. The WSSV *pk* gene with additional *Eco*RI and *Not*I restriction endonuclease sites at both ends was amplified from pme902 by PCR with *pk1-Eco*RI/*pk1-Not*I primer sets (5′-CCGAATTCATGGAGGGTGGGGACCAACGGCA-3′/5′--GGGCGGCCGCCTACTTAACCTT-3′; where the underlined bases indicate the restriction sites) and then cloned into pGEM-T Easy (Promega). The resultant plasmid (pPK) with the correct sequence of insertion was cleaved with *Eco*RI and *Not*I and introduced into a modified form of the

pcDNA3 (Invitrogen) based vector. To create this modified form (pcDNA3PK) a hemagglutinin (HA) epitope tag excised from a HA epitope-tagged mammalian expression vector (Clontech) was inserted at the *Hin*dIII and *Bam*HI cloning sites. TNT (Promega) Quick Master Mix (20 μl) was mixed with 1 μl [^{35}S]methionine (1000 Ci/mmol, 10 mCi/ml) and pcDNA3PK (1 μg in 5 μl nuclease-free water). The reaction mixture (25 μl) was incubated at 30°C for 90 min, and an aliquot of the translation product (4.5 μl) was analyzed by 12.5% SDS–PAGE. After electrophoresis, the gel was stained, destained, dried, and exposed to Fuji medical film at room temperature for 17 h. The same reaction but with 0.5 mM methionine instead of [^{35}S]methionine was also carried out for Western blot analysis with PK antibodies.

WSSV *pk* transcription analysis

WSSV-infected shrimp. Since to date no WSSV-susceptible shrimp cell lines have become available, all the RNA for the transcriptional analysis was taken from WSSV-infected shrimp at different times after infection. Healthy (that is, two-step WSSV diagnostic PCR negative; Lo *et al.,* 1996) subadult *P. monodon* (15–20 g) were infected with WSSV by injection using the method described previously by Tsai *et al.* (1999). At various times over the course of the next 60 h, two or three specimens were selected at random and their pleopods were excised. The collected pleopods were immediately frozen and stored in liquid nitrogen until used for RNA isolation.

RNA isolation. For the isolation of total RNA, the frozen pleopods (500 mg) from WSSV-infected *P. monodon* were homogenized in 5 ml TRIzol reagent (Life Technologies) and then subjected to ethanol precipitation according to the manufacturer's recommendations. The total RNA was stored in 75% ethanol at −20°C. Following procedures described in Lo *et al.* (1996), total DNA was extracted from the same tissues and used to check the viral DNA loading in the tissues using PCR with a *pk*-specific primer set *pk*-F2/*pk*-R7 (5′-TTTAGTCACGCTCTTGAGGG-3′/5′-GCCAGTAGCTTGAAGCATCC-3′).

Detection of WSSV pk transcripts in WSSV-infected shrimp by Northern blot hybridization analysis with a pk gene-specific riboprobe. A WSSV *pk*-specific [α-^{32}P]rCTP-labeled riboprobe was used for Northern blot analysis. To generate the riboprobe, the RNA polymerase promoter addition kit Lig'nScribe (Ambion) was used in accordance with the manufacturer's instructions to produce templates from WSSV *pk*-specific PCR products for the *in vitro* transcription. Briefly, the WSSV *pk*-specific fragment was amplified from WSSV genomic DNA by PCR with the primer set *pk*-F2 and *pk*-R4 (5′-TTTAGTCACGCTCTT-GAGGG-3′ and 5′-ACATGCACCAATTACAGGCG-3′, respectively). An aliquot (25 ng) of the WSSV *pk*-specific PCR product was then ligated with T7 promoter adapter (supplied with the kit) using T4 DNA ligase. To generate WSSV *pk*-specific fragments that contained the T7 RNA polymerase promoter, an aliquot (2 μl) of the reaction mixture (10 μl) was used as a template in PCR with a primer set consisting of the PCR adapter primer 1 (supplied with the kit) and *pk*-F2 (5′-GCTTCCGGCTCGTATGT-TGTGTGG-3′ and 5′-TTTAGTCACGCTCTTGAGGG-3′, respectively). An aliquot (3.6 μl) of PCR product (50 μl) was then used to generate the WSSV *pk*-specific [α-^{32}P]rCTP-labeled riboprobe by *in vitro* transcription (Sambrook *et al.,* 1989) in a 20-μl reaction mixture containing 40 U of T7 RNA polymerase (Roche) and 0.02 mCi [α-^{32}P]rCTP for 2 h at 37°C. The reaction mixture was then treated with 200 U RNase-free DNase I for 30 min at room temperature, terminated at 68°C for 15 min, and filtered through a Sephadex G50 column.

For Northern blot analysis, 10 μg total RNA was separated on 1% formaldehyde-containing agarose gel and transferred to a Hybond-N$^+$ membrane (Amersham) (Sambrook *et al.,* 1989). The membrane was prehybridized for 1 h at 65°C in a prehybridization buffer (0.25 M phosphate buffer, 1 mM EDTA, 1% BSA, and 7% SDS) and then hybridized to the WSSV *pk*-specific [α-^{32}P]rCTP-labeled riboprobe for 16 h at 65°C. After washing, the membrane was exposed to Kodak BioMax MR film via an intensifying screen for several days at −70°C and then developed.

Temporal analysis of WSSV pk transcription by RT-PCR. Total RNA in 75% ethanol was centrifuged at 14,000*g* for 30 min at 4°C. The pellet was resuspended in DEPC-water and quantified by spectrophotometry at 260 nm. An aliquot of 10 μg RNA was treated with 200 U of RNase-free DNase I at 37°C for 30 min to remove any residual DNA and then extracted with phenol–chloroform. The DNase-treated total RNA (~10 μg) was denatured by heating at 85°C for 10 min in 10 μl DEPC-water containing 100 pmol oligo(dT) primer (Roche). The first-strand cDNA was synthesized by the addition of 4 μl Superscript II 5× buffer, 1 μl of 100 mM DTT, 1 μl of 10 mM dNTPs, 10 U RNasin (Promega), and 100 U Superscript II reverse transcriptase (Life Technologies). DEPC-water was added to make a final volume of 20 μl. The reverse transcription proceeded at 37°C for 1 h, followed by heating at 95°C for 5 min to stop the reaction. One-tenth of the products of the cDNA reaction (2 μl; ~1 μg) was subjected to RT-PCR in a 50-μl reaction buffer containing 10 mM Tris–HCl, pH 8.8, 1.5 mM $MgCl_2$, 150 mM KCl, 0.1% Triton X-100, 0.2 mM dNTPs, 100 pmol each primer [the primer sets were *pk*-F2/*pk*-R7 (5′-TTTAGTCA-CGCTCTTGAGGG-3′/5′-GCCAGTAGCTTGAAGCATCC-3′) for *pk* and *vp25*-F1/*vp25*-R1 (5′-CAGTGCCAGAGTAG-GTGACG-3′/5′-ATGAAGGAAGAAGATGCGC-3′) for *vp25,* where *vp25* is an envelope gene adjacent to the *pk* gene; it is used here for comparison], and 2 U DyNAzyme II DNA polymerase (Finnzymes). The PCR cycles were as follows: 94°C for 2 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, followed by an elongation at 72°C for 30 min. A *β-actin* transcript was amplified with

the actin-F1/actin-R1 primer set (5′-GAYGAYATGGAGAA-GATCTGG-3′/5′-CCRGGGTACATGGTGGTRCC-3′) and used as an internal control for RNA quality and amplification efficiency. A WSSV genomic DNA-specific primer set IC-F2/IC-R3 (5′-CAGACTATTAATGTACAAGTGCG-3′/5′-GAATGATTGTTGCTGGTTAGAACC-3′) derived from an intergenic region of the WSSV genome was used to confirm that the RNA was not contaminated by any viral DNA.

Southern blot analysis

Southern blot analysis (Southern, 1975) was used to screen the WSSV genome for possible ORFs that potentially encode eukaryotic protein kinase homologues. Since the 1020-nt ORF contains the conserved sequences of the eukaryotic protein kinase subdomains VI to XI, the PCR-generated, DIG-labeled 1020-nt ORF-specific probe was used for this analysis. WSSV DNA extracted from virions purified from WSSV-infected *P. monodon* (Wang *et al.,* 1995) was digested with *Hin*dIII or *Eco*RI restriction enzymes, separated by electrophoresis on a 0.7% agarose gel, transferred to Hybond-N^+ membrane (Amersham), and then hybridized with the DIG-labeled probe. DIG-labeled nucleotides in the blots were detected as described previously (Lo *et al.,* 1999).

Amino acid sequence comparison and phylogenetic construction

Selected protein kinases (29 viral PKs and 55 other PKs from fungi, invertebrates, vertebrates, a plant, and a bacterium) from GenBank were used in the alignment and phylogenetic analyses (Tables 2 and 3). The multiple sequence alignments were done by the multiple sequence alignment program CLUSTAL_X (Thompson *et al.,* 1997). Phylogenetic analysis based on PK sequences was performed using NJ and parsimony methods with the PAUP 4.0b1 program (Swofford, 1998), using CLUSTAL_X (Thompson *et al.,* 1997) to produce input files of aligned protein sequences. One thousand bootstrap replicates were generated to test the robustness of the trees.

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REFERENCES

Afonso, C. L., Tulman, E. R., Lu, Z., Oma, E., Kutish, G. F., and Rock, D. L. (1999). The genome of *Melanoplus sanguinipes* entomopoxvirus. *J. Virol.* **73,** 533–552.

Afonso, C. L., Tulman, E. R., Lu, Z., Zsak, L., Kutish, G. F., and Rock, D. L. (2000). The genome of fowlpox virus. *J. Virol.* **74,** 3815–3831.

Ahrens, C. H., Russell, R. L., Funk, C. J., Evans, J. T., Harwood, S. H., and Rohrmann, G. F. (1997). The sequence of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus genome. *Virology* **229,** 381–399.

Allen, G. P., and Coogle, L. D. (1988). Characterization of an equine herpesvirus type 1 gene encoding a glycoprotein (gp13) with homology to herpes simplex virus glycoprotein. C. *J. Virol.* **62,** 2850–2858.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman. D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410.

Bahr, U., Tidona, C. A., and Darai, G. (1997). The DNA sequence of Chilo iridescent virus between the genome coordinates 0.101 and 0.391: Similarities in coding strategy between insect and vertebrate iridoviruses. *Virus Genes* **15,** 235–245.

Barik, S., and Banerjee, A. K. (1992). Sequential phosphorylation of the phosphoprotein of vesicular stomatitis virus by cellular and viral protein kinases is essential for transcription activation. *J. Virol.* **66,** 1109–1118.

Baylis, S. A., Banham, A. H., Vydelingum, S., Dixon, L. K., and Smith G. L. (1993). African swine fever virus encodes a serine protein kinase which is packaged into virions. *J. Virol.* **67,** 4549–4556.

Bischoff, D. S., and Slavicek, J. M. (1994). Identification and characterization of a protein kinase gene in the *Lymantria dispar* multinucleocapsid nuclear polyhedrosis virus. *J. Virol.* **68,** 1728–1736.

Burma, S., Mukherjee, B., Jain, A., Habib, S., and Hasnain, S. E. (1994). An unusual 30-kDa protein binding to the polyhedrin gene promoter of *Autographa californica* nuclear polyhedrosis virus. *J. Biol. Chem.* **269,** 2750–2757.

Cameron, C., Hota-Mitchell, S., Chen, L., Barrett, J., Cao, J. X., Macaulay, C., Willer, D., Evans, D., and McFadden, G. (1999). The complete DNA sequence of myxoma virus. *Virology* **264,** 298–318.

Chang, P. S., Lo, C. F., Wang, Y. C., and Kou, G. H. (1996). Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. *Dis. Aqua. Org.* **27,** 131–139.

Chang, Y. S., Peng, S. E., Wang, H. C., Hsu, H. C., Ho, C. H., Wang, C. H., Wang, S. Y., Lo, C. F., and Kou, G. H. (2001). Sequencing and ARFLP analysis of ribonucleotide reductase large subunit gene of the white spot syndrome virus found in blue crab *Callinectes sapidus* collected from American coastal waters. *Mar. Biotechnol.,* in press.

Chou, H. Y., Huang, C. Y., Wang, C. H., Chiang, H. C., and Lo, C. F. (1995). Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan. *Dis. Aqua. Org.* **23,** 165–173.

Federici, B. A., and Hice, R. H. (1997). Organization and molecular characterization of genes in the polyhedrin region of the *Anagrapha falcifera* multinucleocapsid NPV. *Arch. Virol.* **142,** 333–348.

Flegel, T. W. (1997). Special topic review: Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand. *World J. Microbiol. Biotechnol.* **13,** 433–442.

Fletcher, T. M., and Gray, W. L. (1993). DNA sequence and genetic organization of the unique short (US) region of the simian varicella virus genome. *Virology* **193,** 762–773.

Frohman, M. A., Dush, M. K., and Martin, G. R. (1988). Rapid production of full-length cDNAs from rare transcripts: Amplification using a single gene-specific oligonucleotide primer. *Proc. Natl Acad. Sci. USA* **85,** 8998–9002.

Guarino, L. A., Dong, W., Xu, B., Broussard, D. R., Davis, R. W., and Jarvis, D. L. (1992). Baculovirus phosphoprotein pp31 is associated with virogenic stroma. *J. Virol.* **66,** 7113–7120.

Hanks, S. K., and Hunter, T. (1995). Protein kinases 6. The eukaryotic protein kinase superfamily: Kinase (catalytic) domain structure and classification. *FASEB J.* **9,** 576–596.

Hanks, S. K., Quinn, A. M., and Hunter, T. (1988). The protein kinase family: Conserved features and deduced phylogeny of the catalytic domains. *Science* **241,** 42–52.

Herman, P. K., Stack, J. H., DeModena, J. A., and Emr, S. D. (1991). A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. *Cell* **64,** 425–437.

Hunter, T. (1987). A thousand and one protein kinases. *Cell* **50,** 823–829.

Hunter, T. (2000). Signaling-2000 and beyond. *Cell* **100,** 113–127.

Ijkel, W. F., van Strien, E. A., Heldens, J. G., Broer, R., Zuidema, D., Goldbach, R. W., and Vlak, J. M. (1999). Sequence and organization of the *Spodoptera exigua* multicapsid nucleopolyhedrovirus genome. *J. Gen. Virol.* **80,** 3289–3304.

Kamita, S. G., and Maeda, S. (1997). Sequencing of the putative DNA helicase-encoding gene of the *Bombyx mori* nuclear polyhedrosis virus and fine-mapping of a region involved in host range expansion. *Gene* **190,** 173–179.

Kann, M., Sodeik, B., Vlachou, A., Gerlich, W. H., and Helenius, A. (1999). Phosphorylation-dependent binding of hepatitis B virus core particles to the nuclear pore complex. *J. Cell Biol.* **145,** 45–55.

Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991). Structure of a peptide inhibitor bound to the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253,** 414–420.

Kou, G. H., Peng, S. E., Chiu, Y. L., and Lo, C. F. (1998). Tissue distribution of white spot syndrome virus (WSSV) in shrimp and crabs. *In* "Advances in Shrimp Biotechnology" (T. W. Felgel, Ed.), pp. 267–271. National Center for Genetic Engineering and Biotechnology, Bangkok.

Kozak, M. (1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J. Mol. Biol.* **196,** 947–950.

Kozak, M. (1997). Recognition of AUG and alternative initiator codons is augmented by G in position +4 but is not generally affected by the nucleotides in positions +5 and +6. *EMBO J.* **16,** 2482–2492.

Leader, D. P., and Katan, M. (1988). Viral aspects of protein phosphorylation. *J. Gen. Virol.* **69,** 1441–1464.

Lee, A. M., Ivey, R. G., and Meints, R. H. (1998). Repetitive DNA insertion in a protein kinase ORF of alatent FSV (*Feldmannia* sp. virus) genome. *Virology* **248,** 35–45.

Lee, L. F., Wu, P., Sui, D., Ren, D., Kamil, J., Kung, H. J., and Witter, R. L. (2000). The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus. *Proc. Natl. Acad. Sci. USA* **97,** 6091–6096.

Li, Y., and Miller, L. K. (1995). Expression and analysis of a baculovirus gene encoding a truncated protein kinase homology. *Virology* **206,** 314–323.

Lin, S., Chen, W., and Broyles, S. S. (1992). The vaccinia virus B1R gene product is a serine/threonine protein kinase. *J. Virol.* **66,** 2717–2723.

Lo, C. F., Ho, C. H., Peng, S. E., Chen, C. H., Hsu, H. C., Chiu, Y. L., Chang, C. F., Liu, K. F., Su, M. S., Wang, C. H., and Kou, G. H. (1996). White spot syndrome baculovirus (WSBV) detected in culture and captured shrimp, crabs and other arthropods. *Dis. Aqua. Org.* **27,** 215–225.

Lo, C. F., Ho, C. H., Chen, C. H., Liu, K. F., Chiu, Y. L., Yeh, P. Y., Peng, S. E., Hsu, H. C., Liu, H. C., Chang, C. F., Su, M. S., Wang, C. H., and Kou, G. H. (1997). Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. *Dis. Aqua. Org.* **30,** 53–72.

Lo, C. F., Hsu, H. C., Tsai, M. F., Ho, C. H., Peng, S. E., Kou, G. H., and Lightner, D. V. (1999). Specific genomic fragment analysis of different geographical clinical samples of shrimp white spot syndrome virus. *Dis. Aqua. Org.* **35,** 175–185.

Massung, R. F., Esposito, J. J., Liu, L. I., Qi, J., Utterback, T. R., Knight, J. C., Aubin, L., Yuran, T. E., Parsons, J. M., Loparev, V. N., Selivanov, N. A., Cavallaro, K. F., Kerlavage, A. R., Mahy, B. W. J., and Venter, J. C. (1993). Potential virulence determinants in terminal regions of variola smallpox virus genome. *Nature* **366,** 748–751.

McGeoch, D. J., Dolan, A., Donald, S., and Brauer, D. H. (1986). Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. *Nucleic Acids Res.* **14,** 1727–1745.

McGeoch, D. J., Moss, H. W., McNab, D., and Frame, M. C. (1987). DNA sequence and genetic content of the *Hin*dIII I region in the short unique component of the herpes simplex virus type 2 genome: Identification of the gene encoding glycoprotein G, and evolutionary comparisons. *J. Gen. Virol.* **68,** 19–38.

Nadala, E. C. B., Tapay, L. M., and Loh, P. C. (1998). Characterization of a non-occluded baculovirus-like agent pathogenic to penaeid shrimp. *Dis. Aqua. Org.* **33,** 221–229.

Nicholas, K. B., Nicholas, H. B., Jr., and Deerfield, D. W., II (1997). GeneDoc: Analysis and visualization of genetic variation. *EMBNEW. News* **4,** 14.

Prives, C. (1990). The replication functions of SV40 T antigen are regulated by phosophorylation. *Cell* **61,** 735–738.

Que, Q., and Van Etten, J. L. (1995). Characterization of a protein kinase gene from two Chlorella viruses. *Virus. Res.* **35,** 291–305.

Reese, M. G. (1994). "Erkennung von Promotoren in pro- und eukaryontischen DNA-Sequenzen durch k stliche Neuronale Netze," Master's Thesis. German Cancer Research Center, Heidelberg, Germany.

Reese, M. G., and Eeckman, F. H. (1995). New neural network algorithms for improved eukaryotic promoter site recognition. *In* "Proceedings of the Seventh International Genome Sequencing and Analysis Conference," Hilton Head Island, SC.

Reese, M. G., Harris, N. L., and Eeckman, F. H. (1996). Large scale sequencing specific neural networks for promoter and splice site recognition. *In* "Biocomputing: Proceedings of the 1996 Pacific Symposium" (L. Hunter and T. E. Klein, Eds.), World Scientific Publishing, Singapore.

Reilly, L. M., and Guarino, L. A. (1994). The *pk-1* gene of *Autographa californica* multinucleocapsid nuclear polyhedrosis virus encodes a protein kinase. *J. Gen. Virol.* **75,** 2999–3006.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* **4,** 406–425.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Senkevich, T. G., Bugert, J. J., Sisler, J. R., Koonin, E. V., Darai, G., and Moss, B. (1996). Genome sequence of a human tumorigenic poxvirus: Prediction of specific host response-evasion genes. *Science* **273,** 813–816.

Smith, R. F., and Smith, T. F. (1989). Identification of new protein kinase-related genes in three herpesviruses, herpes simplex virus, varicella-zoster virus, and Epstein–Barr virus. *J. Virol.* **63,** 450–455.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98,** 503–517.

Swofford, D. L. (1998). PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer, Sunderland, MA.

Telford, E. A., Watson, M. S., Perry, J., Cullinane, A. A., and Davison, A. J. (1998). The DNA sequence of equine herpesvirus-4. *J. Gen. Virol.* **79,** 1197–1203.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997). The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25,** 4876–4882.

Traktman, P., Anderson, M. K., and Rempel, R. E. (1989). Vaccinia virus encodes an essential gene with strong homology to protein kinases. *J. Biol. Chem.* **264,** 21458–21461.

Tsai, M. F., Kou, G. H., Liu, H. C., Liu, K. F., Chang, C. F., Peng, S. E., Hsu, H. C., Wang, C. H., and Lo, C. F. (1999). Long-term presence of white spot syndrome virus (WSSV) in a cultured shrimp population without disease outbreaks. *Dis. Aqua. Org.* **38,** 107–114.

Tsai, M. F., Lo, C. F., van Hulten, M. C. W., Tzeng, H. F., Chou, C. M., Hung, C. J., Wang, C. H., Lin, J. W., Vlak, J. M., and Kou, G. H. (2000a). Transcriptional analysis of the ribonucleotide reductase genes of shrimp white spot syndrome virus. *Virology* **277,** 92–99.

Tsai, M. F., Yu, H. T., Tzeng, H. F., Leu, J. H., Chou, C. M., Hung, C. J., Wang, C. H., Lin, J. Y., Valk, J. M., Kou, G. H., and Lo, C. F. (2000b). Identification and characterization of a shrimp white spot syndrome virus (WSSV) gene that encodes a novel chimeric polypeptide of

cellular-type thymidine kinase and thymidylate kinase. *Virology* **277,** 100–110.

van Hulten, M. C. W., Tsai, M. F., Schipper, C. A., Lo, C. F., Kou, G. H., and Vlak, J. M. (2000a). Analysis of a genomic segment of white spot syndrome virus of shrimp containing ribonucleotide reductase genes and repeat regions. *J. Gen. Virol.* **81,** 307–316.

van Hulten, M. C. W., Westenberg, M., Goodall, S. D., and Vlak, J. M. (2000b). Identification of two major virion protein genes of white spot syndrome virus of shrimp. *Virology* **266,** 227–236.

van Hulten, M. C. W., and Vlak, J. M. (2001). Identification and phylogeny of a protein kinase gene of white spot syndrome virus. *Virus Genes* **22,** 201–207.

van Regenmortel, M. H. V., Fauquet, C. M., Bishop, D. H. L., Carstens, E. B., Estes, M. K., Lemon J. M., Mayo, M. A., McGeoch, D. J., Pringle, C. R., and Wickner, R. B. (Eds.) (2000). "Virus Taxonomy." Academic Press, San Diego.

van Zijl, M., van der Gulden, H., de Wind, N., Gielkens, A., and Berns, A. (1990). Identification of two genes in the unique short region of pseudorabies virus: Comparison with herpes simplex virus and varicella-zoster virus. *J. Gen. Virol.* **71,** 1747–1755.

Wang, C. H., Lo, C. F., Leu, J. H., Chou, C. M., Yeh, P. Y., Chou, H. Y., Tung, M. C., Chang, C. F., Su, M. S., and Kou, G. H. (1995). Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. *Dis. Aqua. Org.* **23,** 239–242.

Wang, C. H., Yang, H. N., Tang, C. Y., Lu, C. H., Kou, G. H., and Lo, C. F. (2000a). Ultrastructure of white spot syndrome virus development in primary lymphoid organ cell cultures. *Dis. Aqua. Org.* **41,** 91–104.

Wang, Q., Poulos, B. T., and Lightner, D. V. (2000b). Protein analysis of geographic isolates of shrimp white spot syndrome virus. *Arch. Virol.* **145,** 263–274.

Widmann, C., Gibson, S., Jarpe, M. B., and Johnson, G. L. (1999). Mitogen-activated protein kinase: Conservation of three-kinase module from yeast to human. *Physiol. Rev.* **79,** 143–180.

Wongteerasupaya, C., Vickers, J. E., Sriurairatana, S., Nash, G. L., Akarajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., and Flegel, T. W. (1995). A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origen and causes high mortality in the black tiger prawn *Penaeus monodon*. *Dis. Aqua. Org.* **21,** 69–77.

Wu, J. Y., Zhou, A. Y., Judd, A., Cartwright, C. A., and Robinson W. S. (1990). The hepatitis B virus-encoded transcriptional trans-activator hbx appears to be a novel protein serine/threonine kinase. *Cell* **63,** 687–695.

Zhang, C. X., Wang, G., Hu, C., and Wu, X. F. (1997). Nucleotide sequence analysis of HaSNPV protein kinase. *Acta Biochim. Biophys. Sin.* **29,** 322–326.

Zhang, G., Stevens, R., and Leader, D. P. (1990). The protein kinase encoded in the short unique region of pseudorabies virus: Description of the gene and identification of its product in virions and in infected cells. *J. Gen. Virol.* **71,** 1757–1765.