

Transcriptional Analysis of the DNA Polymerase Gene of Shrimp White Spot Syndrome Virus

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The white spot syndrome virus DNA polymerase (DNA pol) gene (WSSV *dnapol*) has already been tentatively identified based on the presence of highly conserved motifs, but it shows low overall homology with other DNA pols and is also much larger (2351 amino acid residues vs 913–1244 aa). In the present study we perform a transcriptional analysis of the WSSV *dnapol* gene using the total RNA isolated from WSSV-infected shrimp at different times after infection. Northern blot analysis with a WSSV *dnapol*-specific riboprobe found a major transcript of 7.5 kb. 5'-RACE revealed that the major transcription start point is located 27 nucleotides downstream of the TATA box, at the nucleotide residue A within a CAGT motif, one of the initiator (Inr) motifs of arthropods. In a temporal expression analysis using differential RT-PCR, WSSV *dnapol* transcripts were detected at low levels at 2–4 h.p.i., increased at 6 h.p.i., and remained fairly constant thereafter. This is similar to the previously reported transcription patterns for genes encoding the key enzyme of nucleotide metabolism, ribonucleotide reductase. Phylogenetic analysis showed that the DNA pols from three different WSSV isolates form an extremely tight cluster. In addition, similar to an earlier phylogenetic analysis of WSSV protein kinase, the phylogenetic tree of viral DNA pols further supports the suggestion 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. © 2002 Elsevier Science (USA)

Key Words: *Penaeus monodon*; white spot syndrome virus; WSSV Taiwan isolate; WSSV *dnapol* gene; transcription analysis.

INTRODUCTION

White spot syndrome virus (WSSV) is the causative agent of a disease that has led to severe mortalities of cultured shrimps all over the world (Inouye *et al.*, 1994; Takahashi *et al.*, 1994; Chou *et al.*, 1995; Flegel, 1997). WSSV is an enveloped, ellipsoid, large, double-stranded DNA virus (Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995; Lo *et al.*, 1996b) and it has a wide host range among crustaceans (Lo *et al.*, 1996a; Flegel, 1997, Lo and Kou, 1998). The virus is transmitted both horizontally (Chang *et al.*, 1996; Chou *et al.*, 1998) and vertically (Lo *et al.*, 1997). Even while the molecular data were still limited, the uniqueness of this virus was highlighted by the preliminary WSSV-DNA sequence analysis (Lo *et al.*, 1997), the morphological characteristics, and the general biological properties of the virus (Wongteerasupaya *et al.*, 1995; Lo *et al.*, 1996a). Recent data, including studies on individual genes and analysis of the complete genome sequence, suggest that WSSV is a member of a new virus family (Tsai *et al.*, 2000b; Yang *et al.*, 2001; van Hulst *et al.*, 2001a; Liu *et al.*, 2001).

The size of the WSSV genome has been differently

reported for different isolates: 305,107 bp (GenBank Accession No. AF332093), 292,967 bp (GenBank Accession No. AF369029), and 307,287 bp (GenBank Accession No. AF440570) for viruses isolated from China, Thailand, and Taiwan, respectively. The size differences are mostly due to several small insertions and one large (~12 kb) deletion (Chen *et al.*, 2002). The genome organization and the overall sequences show little variation across these three isolates, reinforcing the early tentative conclusion that there is little genetic variation among WSSV isolates from around the world (Lo *et al.*, 1999; Chang *et al.*, 2001). For the China and Thailand isolates, the analysis of the complete WSSV genome has been published (Yang *et al.*, 2001; van Hulst *et al.*, 2001a). To date, although much of the sequence of the WSSV genome is already known, and the predicted sequences for many genes have already been published, most of the putative WSSV genes have been subjected to sequence analysis only. The few genes that have been studied further are those encoding the ribonucleotide reductase large (RR1) and small (RR2) subunits (Tsai *et al.*, 2000a), two structural proteins (van Hulst *et al.*, 2000, 2001b), a novel chimeric polypeptide of cellular-type thymidine kinase and thymidylate kinase (Tsai *et al.*, 2000b), a basic peptide (Zhang *et al.*, 2001), a protein kinase (Liu *et al.*, 2001), and a nucleocapsid protein with nuclear targeting behavior (Chen *et al.*, 2002). Many genes that are important for the completion of WSSV's infection cycle still remain to be studied. One

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of the most important of these is the DNA polymerase (DNA pol) gene.

Based on the presence of highly conserved motifs, a WSSV gene that codes DNA pol, has already been tentatively identified (Yang *et al.*, 2001; van Hulten *et al.*, 2001a). However, the size of the putative WSSV DNA pol was differently reported in these two studies (2195 and 2351 amino acid residues, respectively), and in addition to showing low overall homology with other DNA pols, the putative WSSV DNA pol is also much larger (2351 amino acid residues vs 913-1244 aa). Since no other information beyond these sequence data has hitherto been provided, here we conduct a transcriptional analysis of the WSSV *dnapol* gene, determine the 5' and 3' terminus of the WSSV *dnapol* transcript, and provide evidence that transcription may be mediated by RNA polymerase II from the host. We also locate the conserved motifs and show that the unusually large size of WSSV DNA pol is due to the extra-large spacer regions between these consensus domains.

RESULTS AND DISCUSSION

Location and structure of the WSSV *dnapol* 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), which is now known as WSSV Taiwan isolate (Lo *et al.*, 1999). From this virus, several plasmid libraries were constructed (Wang *et al.*, 1995; Tsai *et al.*, 2000a; Liu *et al.*, 2001) for sequencing the WSSV genome. From a 28,325 bp contig (pms147, pmshP1, and pmh182), a 7056-nt open reading frame (ORF) was found. When the deduced amino acid sequence of this 7056-nt ORF was compared with other sequences in GenBank by using the BLAST network service (Atschul *et al.*, 1997), it was found to contain the three conserved regions of the exonuclease domain (Exo I, Exo II, and Exo III) and the seven conserved regions of the polymerase domain (Regions IV, II, VI, III, I, VII, and V; Region IV is adjacent to Exo II, as in most cases of *dnapol*) (Fig. 1).

Transcriptional analysis of WSSV *dnapol*

To determine when the *dnapol* mRNA is transcribed in the viral life cycle, and which mRNA is transcribed, we performed a transcriptional analysis of *dnapol* using RT-PCR and Northern blot analysis. RT-PCR analysis was used to detect the *dnapol*-specific transcript in DNase-treated total RNA from shrimp specimens before infection (0 h) and at 2, 4, 6, 8, 12, 18, 24, 36, and 60 h after WSSV infection by intramuscular injection. Primer sets specific to WSSV genes *rr1* and *rr2* were used for transcriptional comparison and a β -actin primer set was selected for template control. After 20 cycles of amplification, the *dnapol* transcript was first detected by RT-

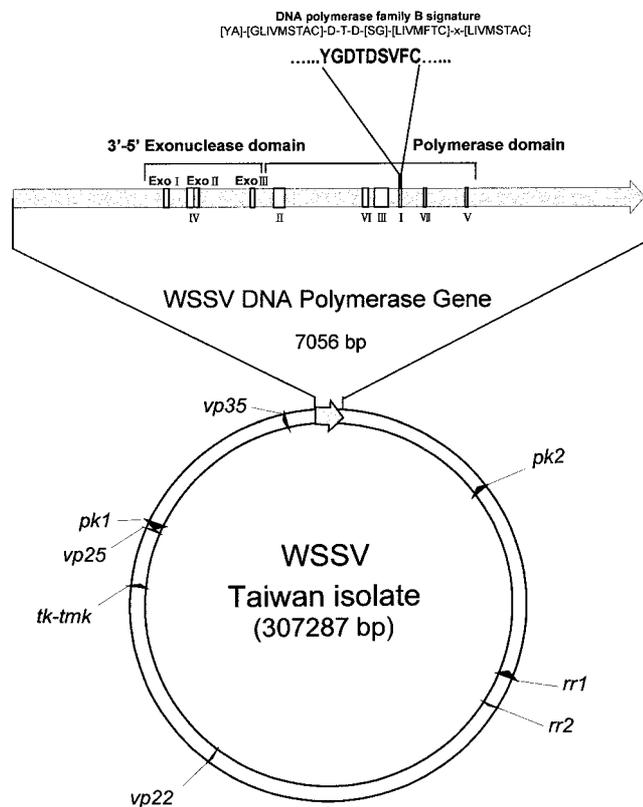


FIG. 1. The relative position of the WSSV *dnapol* gene on the entire WSSV genome. Other published genes [*rr* genes (Tsai *et al.*, 2000a); *tk-tmk* (Tsai *et al.*, 2000b); *vp22*, *vp25* and *pk1* (Liu *et al.*, 2001); *vp35* (Chen *et al.*, 2002)] are shown for reference. A protein kinase gene (*pk2*) that is distantly related to the published protein kinase (*pk1*) is also indicated.

PCR at 6 h.p.i. and continued to be found through to 60 h.p.i. (Fig. 2A). As the number of amplification cycles was increased, the *dnapol* transcript was detectable sooner (at 2 h.p.i.; Figs. 2B, 2C, and 2D) and the intensity of the bands increased, but from 6 h.p.i. (Figs. 2A and 2B) and 2 h.p.i. (Figs. 2C and 2D) the intensity of the *dnapol* RT-PCR product bands remained fairly constant through to 60 h.p.i. The overall transcriptional pattern of *dnapol* was similar to the patterns of *rr1* and *rr2* (Figs. 2E and 2F), and unlike that of the structural protein gene *vp25* (Liu *et al.*, 2001). Positive and genomic DNA contamination controls both gave the expected results (Figs. 2G and 2H), thus confirming RNA template quality and that no viral genomic DNA was left in the prepared RNA.

A DIG-labeled RNA probe derived from the WSSV *dnapol* gene (+2356 to +3111 nt relative to the putative translation initiation codon) was generated by *in vitro* transcription for the detection of the WSSV *dnapol* gene transcript in total RNA extracted from WSSV-infected shrimp. Northern blot analysis with this WSSV *dnapol* gene-specific riboprobe first detected one major transcript of approximately 7.5 kb at 6 h.p.i. (Fig. 3), which is

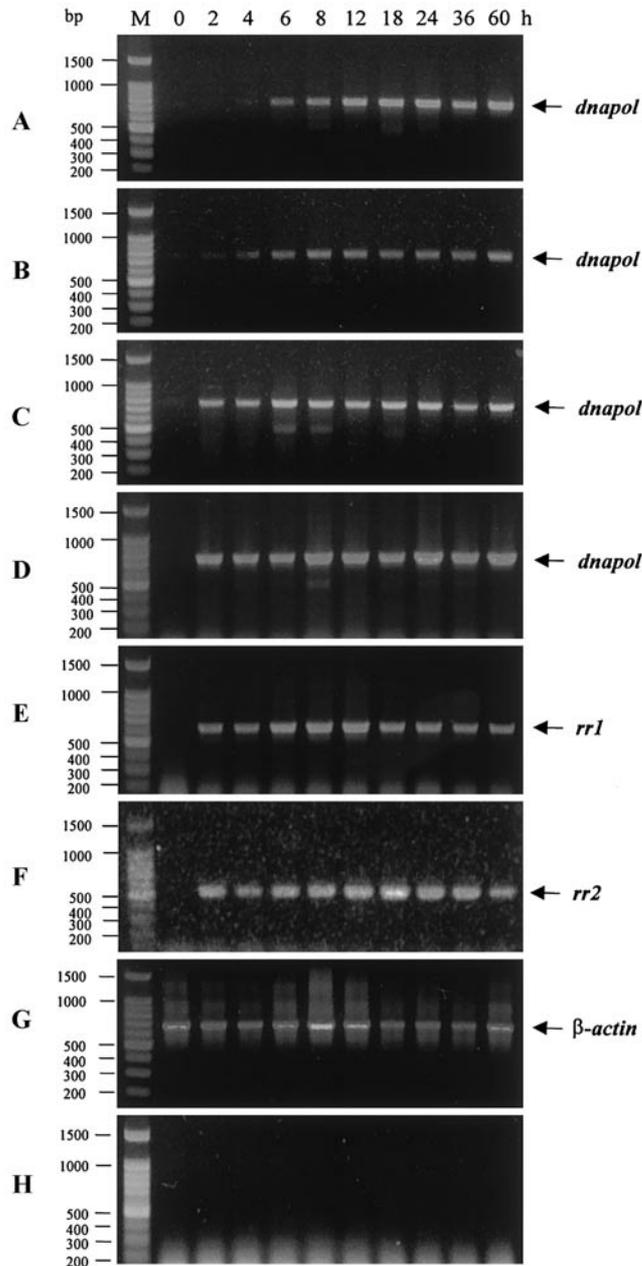


FIG. 2. Temporal transcription analysis of WSSV *dnapol* gene by differential RT-PCR. Differential RT-PCR with WSSV *dnapol*-specific primers after (A) 20 cycles, (B) 25 cycles, (C) 30 cycles, and (D) 40 cycles. RT-PCR (40 cycles) with (E) *rr1*-specific primers and (F) *rr2*-specific primers. Internal controls: total RNA treated with DNase and amplified for 40 cycles with (G) actin-specific primers and (H) intergenic primers. The products were resolved in 1% agarose gel containing 0.5 μ g/ml ethidium bromide. Lane M is a 100-bp DNA ladder (Promega), and the other lane headings show hours p.i.

consistent with the differential RT-PCR result shown in Fig. 2A. Likewise in both differential RT-PCR (Fig. 2A) and Northern blotting (Fig. 3), the transcript was present through to the end of the 60 h experiment. The size of the transcript matched the predicted size of the *dnapol* mRNA after allowing for the presumed *dnapol* coding

region (7056 nt) plus a stretch of 5'/3'-nontranslated regions (5'/3'-NTRs) (see below) and a poly(A) tail.

Mapping 5' end of the *dnapol* transcript

The 5' region of the *dnapol* transcript was obtained by rapid amplification of the cDNA 5' end (5'-RACE) (Forhman *et al.*, 1988) using the 5'/3'-RACE kit (Roche) in which oligo(dT)-anchor primer, anchor primer, and other key reagents were included. The RNA samples used in this study were isolated from the shrimp 24 h after WSSV infection and then treated with RNase-free DNase. The locations of the primers are shown in Fig. 4. For the first step of 5'-RACE, the appropriate gene-specific primer (pol-5'-RACE-sp1 primer; Fig. 4B) was used for first-strand cDNA synthesis from the total RNA by using an avian myeloblastosis virus (AMV) reverse transcriptase. After adding the poly(A) head to the cDNA products, these cDNAs were used as templates for PCR amplification with the pol-5'-RACE-sp2/oligo(dT)-anchor primer set. The PCR products formed a single band in an agarose gel at about 630 bp (Fig. 4B). Analysis of 5'-RACE products cloned in pGEM-T Easy vector revealed that the 5'-termini of 9 of the first 10 randomly picked clones were located 24 to 26 nt upstream of the predicted ATG initiation codon (Fig. 4A). The sequence of these three most likely transcriptional start points (boldfaced) and surrounding nucleotides is CACAGTC. Further, the 5'-terminus of six of these nine clones was at the second A (Fig. 4A), which suggests that this is the major start point for this 60-p.i. RNA sample. In the upstream (\sim 25 nt) of the transcriptional initiation sites, a putative TATA box was found at nt -52 to nt -57 relative to the ATG translational start. The sequences surrounding the putative translation initiation codon (GAGATGA) conform reasonably well to the eukaryotic translation consensus sequence (Kozak, 1987, 1997).

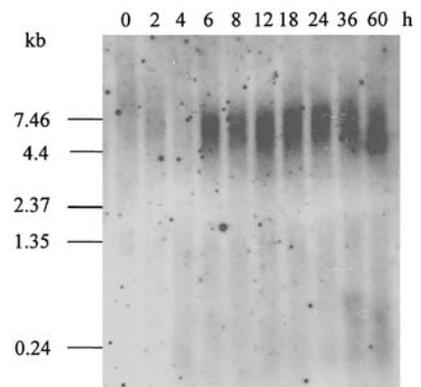
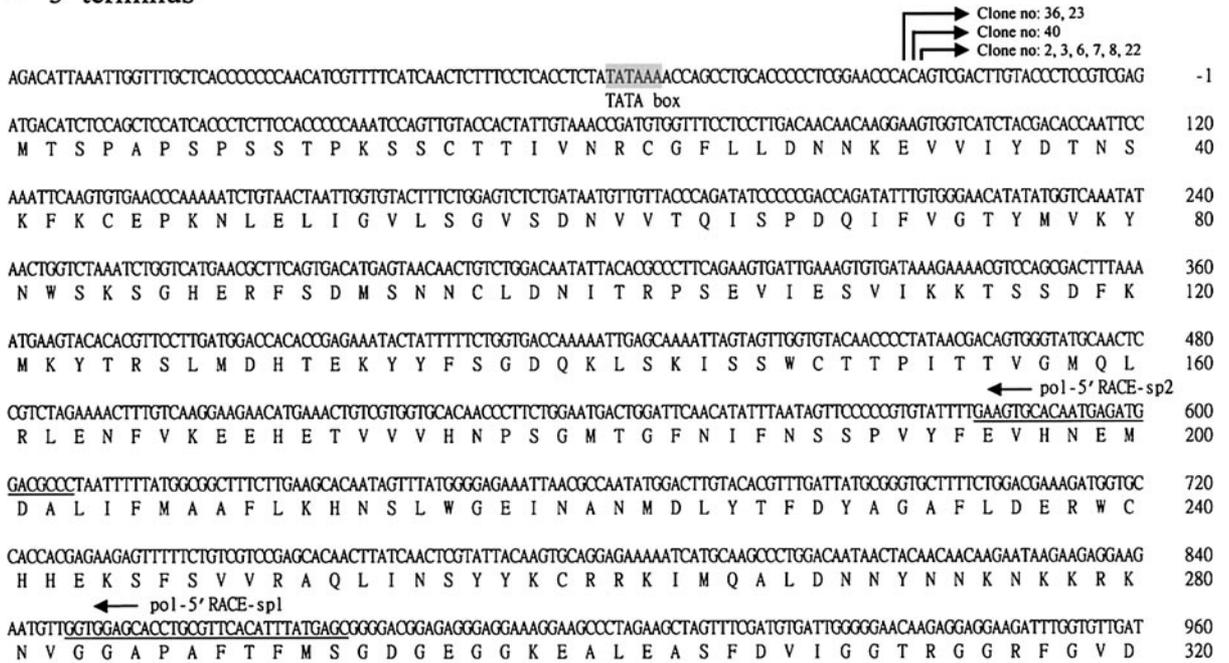
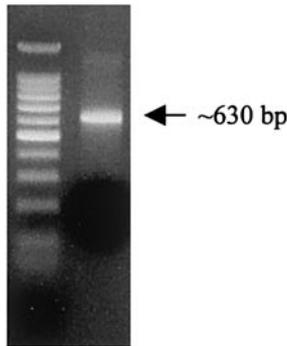


FIG. 3. Northern blot temporal transcription analysis of total RNA isolated from WSSV-infected *P. monodon* using WSSV *dnapol*-specific riboprobes. The transcript is approximately 7.5 kb. The size standards were determined by RNA marker (Gibco-BRL). Lane headings show hours p.i.

A 5' terminus



B M 5'RACE



C

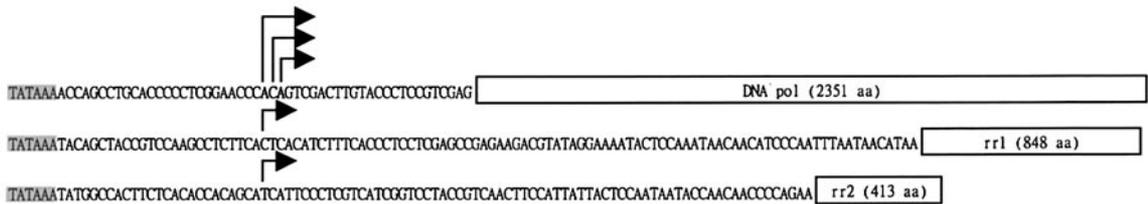


FIG. 4. Mapping the 5' end of the WSSV *dnapol* transcript. (A) The primers used for 5'-RACE (pol-5'-RACE-sp1, pol-5'-RACE-sp2) are underlined. The bent arrows indicate the 5' termini (transcriptional start points) revealed by sequencing nine 5'-RACE clones. The predicted TATA box is shaded. (B) Agarose gel analysis of the 5'-RACE product. Lane M is a 100-bp DNA marker ladder (Promega). (C) Comparison of the tentative basal elements for RNA polymerase II of WSSV *dnapol*, *rr1*, and *rr2* genes. TATA boxes are shaded, and the transcription start sites identified by 5'-RACE are indicated with bent arrows.

Most promoters for RNA polymerase II usually have the TATA box located ~25 bp upstream of the transcription start point (Young, 1991; Nikolov and Burley, 1997). No extensive sequence homology has been reported for the start point, but there is a tendency for the first base of the mRNA to be A, flanked on either side by pyrimi-

dines. This region is called the initiator (Inr), and together with the TATA box, these two components are the basal elements of the RNA polymerase II promoter. The start point itself is thus identified by the Inr and/or by the TATA box close by (Martins *et al.*, 1994; Nikolov and Burley, 1997). Whether a functional TATA box is present or not,

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TTTGAGAACGAAAGATTGGCCCGCAAAATGGCCTAGATGACTCTGTAGATTGTAGTGGCTGAGATGTTGTTTCGGAGGGGATAAACTATTGCCCCAGGAAGTGTAAAAAGGGTAAAAGAT 6717
F E N E R L A R Q I G L D D S V D L M A E M L F G G D K L L A Q E V L K R V K D 2239
pol-3'-RACE-sp1 →
GCTCAAGATAGAAAGTTGGTGAAATCTTTATTGCCCTTAAATATAACCATGACACAAATACAATTATATTTTTGTTTGGAGTCTTTAAGGTTTGTCTCAGAAAACCTGTAGCTGGTATGAGT 6837
A Q D R K L V K S L L P L N Y N H D T N T I I F L F E S L R F A Q K P V A G M S 2279

GTTAGTGAAATAAAAGACGCTGTAGAGGCTGGCCCTTTCTACCACACAGGTAAGTGTGGAAATTATACTGATGAAAGATTTTTTGGACCATTGTATAACATGGATGAACCTTTGTAAC 6957
V S E I K D A V R G L A F S T T T G T V W N Y T D E R F F G P L Y N M D E L C N 2319

GAACGTGTCATGGAATTTGAAATTTGTCCTTTATAACTGGTATTTATCATACGGCAGCAGTAGAATTGGCTGCATGCTATCTTTGTTGTTGTAAGAAATGATAATAAAAAACATG 7077
E R V N G N C K L S F I T G I Y H T A A V E L A A A C L S C V L * 2351

TATAATTTTCATCTGGTTTTTATTTTATATCCTCAAGAGTTGTAGTAGTAGCAGCAGCAGCAGCAATAGCAGCATTATTTTCAGCAGCAGCTTCTTCATTTCTATTGATTGATATCT 7717
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FIG. 5. Mapping the 3' end of the WSSV *dnapol* transcript. The primer used for 3'-RACE (pol-3'-RACE-sp1) is underlined. The polyadenylation signal (AATAAA) is boldfaced. The poly(A) addition site is indicated by the bent arrow.

many insect baculovirus early promoters feature the same conserved transcription initiation sequences, either CAGT or ACGT, at or near the transcription start site (Friesen, 1997). Although WSSV is a shrimp virus rather than an insect virus, the WSSV *dnapol* transcript also matches this pattern in having an Inr (CACAGTC) with a CAGT motif located ~25 bp downstream of the TATA box (Fig. 4C). The structure of the WSSV *dnapol* promoter therefore seems to mimic that of the promoters normally responsive to RNA polymerase II of arthropods (Cherbas and Cherbas, 1993). Furthermore, as noted above, it is the A within this CAGT motif that appears to be the major transcriptional start point. It therefore seems likely that, similar to most of the insect baculovirus early genes that have one or both of these basal elements, WSSV *dnapol* transcription may also be mediated by host RNA polymerase II.

In passing, as Fig. 4C shows, we note that the transcription start sites for WSSV *dnapol*, *rr1*, and *rr2* are all from 25 to 27 nucleotides downstream of the TATA box. The *dnapol* Inr consensus sequence (CAGT) does not exactly match the consensus sequence for the WSSV *rr1* and *rr2* transcription initiation sites, which has previously been tentatively identified as TCAC/tTC (Tsai *et al.*, 2000a), but a provisional, modified a/tCAC/g/tT consensus can be built from these three Inrs. These a/tCAC/g/tT motifs closely match the a/c/t/CAG/tT Inr motifs of arthropods (Cherbas and Cherbas, 1993), in which the CA dinucleotide is the most influential in maintaining levels of CAGT transcriptional initiation (Pullen and Friesen, 1995). The fact that the distance between the TATA box and the start point is almost the same for all three of these genes also suggests that all three may use the same basal transcription factors/cofactors. These speculations still remain to be tested experimentally, but the data shown in Fig. 4C are provocative.

Mapping the 3' end of the WSSV *dnapol* transcript

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

primer and AMV reverse transcriptase. Amplification of the 3' region of the resulting cDNA was carried out by PCR using the pol-3'-RACE-sp1/anchor primer set (Fig. 5) and yielded a PCR product of about 370 bp. 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 (nt 7065 to nt 7070), which was found eight nucleotides downstream of the translation stop codon (Fig. 5).

Amino acid sequence alignment of WSSV DNA pol

When the deduced amino acid sequence of the WSSV DNA pol 7056-nt ORF was compared with other sequences in GenBank, the N-terminal domain was found to contain the three conserved regions of the exonuclease domain (Exo I, Exo II, and Exo III; Bernad *et al.*, 1989) and the seven conserved regions of the polymerase domain (IV, II, VI, III, I, VII, and V; Larder *et al.*, 1987) (Fig. 1). Although WSSV DNA pol is much larger than the other known viral DNA pols, this is due to the expanded spacer regions surrounding the conserved motifs (Fig. 6), while the motifs themselves are still conserved (Figs. 7 and 8). WSSV DNA pol thus has the characteristics of the eukaryotic-type family B DNA pols (Wong *et al.*, 1988; Ito and Braithwaite, 1991).

Phylogenetic analysis

A total of 31 viral DNA pols (family B DNA pols; Table 1) were used to construct phylogenetic trees. Since both the neighbor-joining (NJ) and the 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 viral DNA pols reflect their current phylogenetic grouping (Fig. 9). A similar result was found for a WSSV protein kinase (PK1; Liu *et al.*, 2001). However, the phylogenetic tree of the DNA pols appears to offer better resolution, placing WSSV, the *Adenoviridae*, *Baculoviridae*, and *Poxviridae* in one clade and in the next, the *Iridoviridae*, *Herpesviridae*, and *Phycodnaviridae* in an-

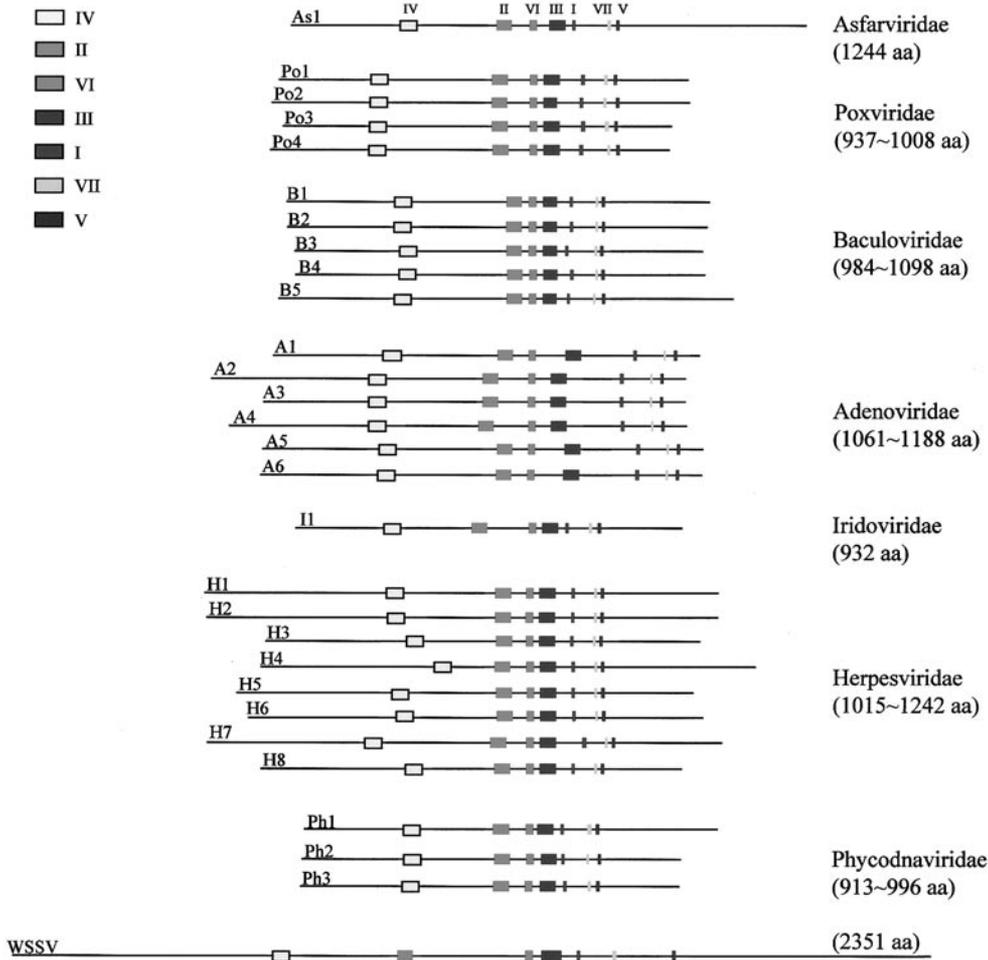


FIG. 6. Protein map indicating proportional lengths of DNA pol (black lines) and relative locations of the seven conserved polymerase regions.

other, while at the next level up, the two clades and *Asfarviridae* form an unresolved polytomy.

As Fig. 9 also shows, the DNA pols from the three sequenced WSSV isolates form an extremely tight cluster, which reflects the closeness of their relationship. The differences among these three isolates are summarized in Table 2. First, it should be noted that while the overall genome size of the China WSSV isolate (Yang *et al.*, 2001) is close to that of the Taiwan WSSV isolate (Chen *et al.*, 2002), i.e., 305,107 bp vs 307,287 bp, the polypeptide encoded by its putative DNA pol ORF (WSV514) is shorter by 246 amino acids (Table 2). Comparison of the two genes shows that a nucleotide (A) addition occurs at +460 relative to the translation initiation codon of the Taiwan isolate. The location of this nucleotide between the 5th and 6th M (ATG) means that the WSV514 ORF uses the 6th M as the translation initiation codon and thus accounts for the 246 aa discrepancy. Second, we note that although the DNA pols of the Taiwan and Thailand isolates are closely related (Fig. 9), with just three differences over 2351 aa (Table 2), the large deletion region (~12 kb; a region including *vp35* shown in Fig. 1) in the genome of the Thailand isolate is

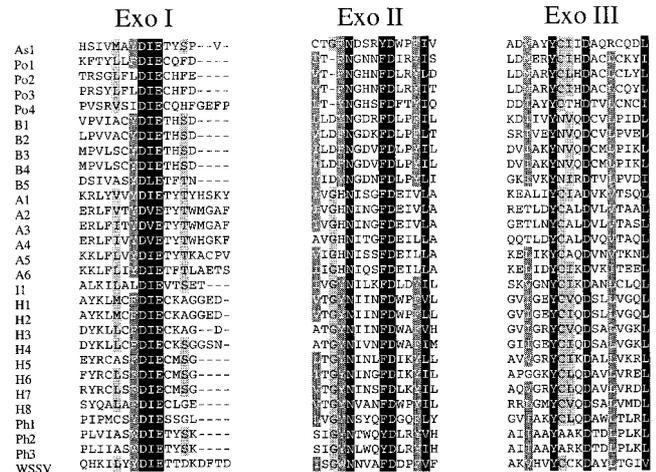


FIG. 7. Alignment of the amino acid sequences of the three exonuclease domains of the DNA polymerase proteins. Gaps, introduced to optimize the alignment, are indicated by dots. Shading is used to indicate the occurrence (black 100%, gray with white letters 80%, and gray with black letters 60%) of identical amino acids. Abbreviations are as for the viruses listed in Table 1.

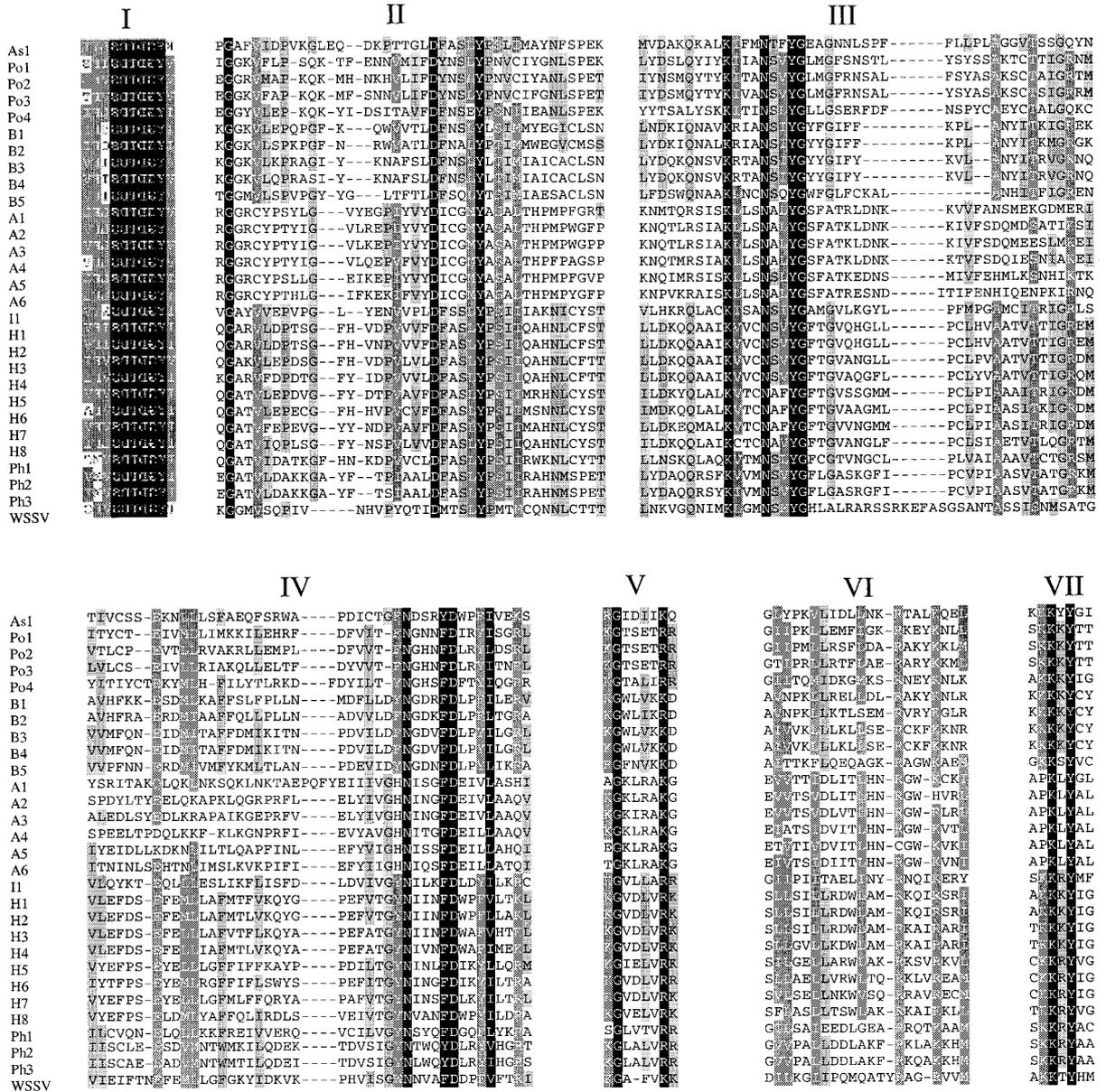


FIG. 8. Alignment of the amino acid sequences of the seven polymerase domains of the DNA polymerase proteins. Gaps, introduced to optimize the alignment, are indicated by dots. Shading is used to indicate the occurrence (black 100%, gray with white letters 80%, and gray with black letters 60%) of identical amino acids. Abbreviations are as for the viruses listed in Table 1.

located not far from the *dnapol* gene. In this region, there are at least 10 genes including a nuclear targeting protein, WSSV VP35 (Chen *et al.*, 2002), and several candidate genes for transcription factors which would normally be actively expressed in the infected shrimp (G. H. Kou and C. F. Lo, unpublished data). To date, the functional relationship between WSSV *dnapol* and the genes in the deletion region have not been elucidated, nor is it known what roles these genes might play in WSSV pathogenesis. Clearly, however, it would be worth investigating other isolates—especially those from Thailand—to see in how many this deletion occurs.

MATERIALS AND METHODS

Virus and genomic plasmid libraries

WSSV collected in 1994 from Taiwan from infected *P. monodon* (Wang *et al.*, 1995) was used to construct WSSV genomic libraries (Lo *et al.*, 1996b; Tsai *et al.*, 2000a; Liu *et al.*, 2001). This virus source has been maintained in our laboratory since 1994 and has the GenBank Accession No. AF440570. It has previously been known simply as the WSSV Taiwan isolate (Lo *et al.*, 1999), but to distinguish it from other WSSV Taiwan isolates, it will henceforth be referred to as the WSSV

TABLE 1
Viral DNA Polymerase Used in the Sequence Alignment and Phylogenetic Construction

Viral DNA polymerase	Virus			
	Species	Family	Accession No.	Reference
1. As1	Africa swine fever virus	<i>Asfarviridae</i>	P43139	Martins <i>et al.</i> (1994)
2. Po1	Fowlpox virus	<i>Poxviridae</i>	DJVZFP	Binns <i>et al.</i> (1987)
3. Po2	Orf virus	<i>Poxviridae</i>	AAB19092	Mercer <i>et al.</i> (1996)
4. Po3	Vaccinia virus	<i>Poxviridae</i>	A24878	Earl <i>et al.</i> (1986)
5. Po4	<i>Choristoneura biennis</i> entomopoxvirus	<i>Poxviridae</i>	S25855	Mustafa and Yuen (1991)
6. B1	<i>Helicoverpa zea</i> nuclear polyhedrosis virus	<i>Baculoviridae</i>	AAA58700	Cowan <i>et al.</i> (1994)
7. B2	<i>Lymantria dispar</i> nuclear polyhedrosis virus	<i>Baculoviridae</i>	JQ1920	Bjornson <i>et al.</i> (1992)
8. B3	<i>Autographa californica</i> nuclear polyhedrosis virus	<i>Baculoviridae</i>	DJNVCP	Tomalski <i>et al.</i> (1988)
9. B4	<i>Bombyx mori</i> nuclear polyhedrosis virus	<i>Baculoviridae</i>	BAA03756	Chaeychomsri <i>et al.</i> (1995)
10. B5	Xestia c-nigrum granulovirus	<i>Baculoviridae</i>	AAC06350	Goto <i>et al.</i> (1998)
11. A1	Duck adenovirus	<i>Adenoviridae</i>	NP_044702	Hess <i>et al.</i> (1997)
12. A2	Human adenovirus type 40	<i>Adenoviridae</i>	NP_040853	Ishino <i>et al.</i> (1987)
13. A3	Human adenovirus type 12	<i>Adenoviridae</i>	NP_040915	Kimura <i>et al.</i> (1981)
14. A4	Canine adenovirus type 2	<i>Adenoviridae</i>	NP_044409	Shibata <i>et al.</i> (1989)
15. A5	Frog adenovirus 1	<i>Adenoviridae</i>	NP_062435	Davison <i>et al.</i> (2000)
16. A6	Turkey adenovirus 3	<i>Adenoviridae</i>	NP_047384	Pitcovski <i>et al.</i> (1998)
17. I1	Lymphocystis disease virus	<i>Iridoviridae</i>	NP_078724	Schnitzler <i>et al.</i> (1987)
18. H1	Herpes simplex virus 2	<i>Herpesviridae</i>	P07918	Tsurumi <i>et al.</i> (1987)
19. H2	Herpes simplex virus 1	<i>Herpesviridae</i>	P09854	Larder <i>et al.</i> (1987)
20. H3	Pseudorabies virus	<i>Herpesviridae</i>	AAA74383	Berthomme <i>et al.</i> (1995)
21. H4	Varicella-zoster virus	<i>Herpesviridae</i>	P09252	Davison and Scott (1986)
22. H5	Guinea pig cytomegalovirus	<i>Herpesviridae</i>	AAA43832	Schleiss (1994)
23. H6	Murine cytomegalovirus	<i>Herpesviridae</i>	P27172	Elliott <i>et al.</i> (1991)
24. H7	Human cytomegalovirus	<i>Herpesviridae</i>	P08546	Kouzarides <i>et al.</i> (1987)
25. H8	Epstein-Barr virus	<i>Herpesviridae</i>	P03198	Bankier <i>et al.</i> (1983)
26. Ph1	<i>Feldmannia sp.</i> virus	<i>Phycodnaviridae</i>	AAB67116	Lee <i>et al.</i> (1998)
27. Ph2	<i>Paramecium bursaria</i> Chlorella virus PBCV-1	<i>Phycodnaviridae</i>	A42543	Grabherr <i>et al.</i> (1992)
28. Ph3	<i>Paramecium bursaria</i> Chlorella virus NY2A	<i>Phycodnaviridae</i>	AAA88827	Grabherr <i>et al.</i> (1992)
29. W1	White spot syndrome virus (Taiwan-1 strain)		AF440570	Present paper
30. W2	White spot syndrome virus (Thailand isolate)		AF369029	van Hulst <i>et al.</i> (2001a)
31. W3	White spot syndrome virus (China isolate)		AF332093	Yang <i>et al.</i> (2001)

Taiwan-1 strain (WSSV T-1 strain). The WSSV T-1 strain was used as the basis for all of the WSSV genome sequence work done in the present study.

Localization and structure of the WSSV *dnapol* gene

Plasmid DNA for sequencing was purified using the QIAprep Miniprep System (Qiagen, Germany) and was sequenced by primer walking on both strands. The nucleotide and the predicted protein sequences were analyzed using GeneWorks 2.5.1 (Oxford Molecular Group, Inc., Campbell, CA). The DNA and the deduced amino acid sequences were compared with GenBank/EMBL, SWISSPORT, and PIR databases using the programs FASTA (Pearson and Lipman, 1988) and BLAST (Altschul *et al.*, 1997). Alignments of amino acid sequences were made in CLUSTAL X (Thompson *et al.*, 1997) and edited in GeneDoc (Nicholas *et al.*, 1997).

Mapping of the 5' end of the *dnapol* transcript

The 5' region of the *dnapol* transcript was determined by rapid amplification of the cDNA 5' end (5'-RACE)

(Forhman *et al.*, 1988) using a commercial 5'/3'-RACE kit (Roche, Germany) according to the instructions provided by the manufacturer. The locations of the primers used in this study are shown in Fig. 4A. Total RNA was isolated from WSSV-infected *P. monodon* as described previously (Tsai *et al.*, 2000a; Liu *et al.*, 2001). The appropriate gene-specific primers (pol-5'-RACE-sp1; Fig. 4A) were then used for cDNA synthesis. Before being subjected to PCR, a poly(A) "head" with terminal transferase was added to the cDNA products in the presence of dATP. The PCR for *pol* was performed using the primer pol-5'-RACE-sp2 and an oligo(dT)-anchor primer. The final products were characterized by subcloning and sequencing, and the resulting sequences were compared with the genomic sequences.

Mapping of the 3' end of the *dnapol* transcript

The 5'/3' region of the *dnapol* transcript was determined by 3'-RACE using a commercial 3'-RACE kit (Roche) according to the instructions provided by the manufacturer. First-strand cDNA was synthesized using

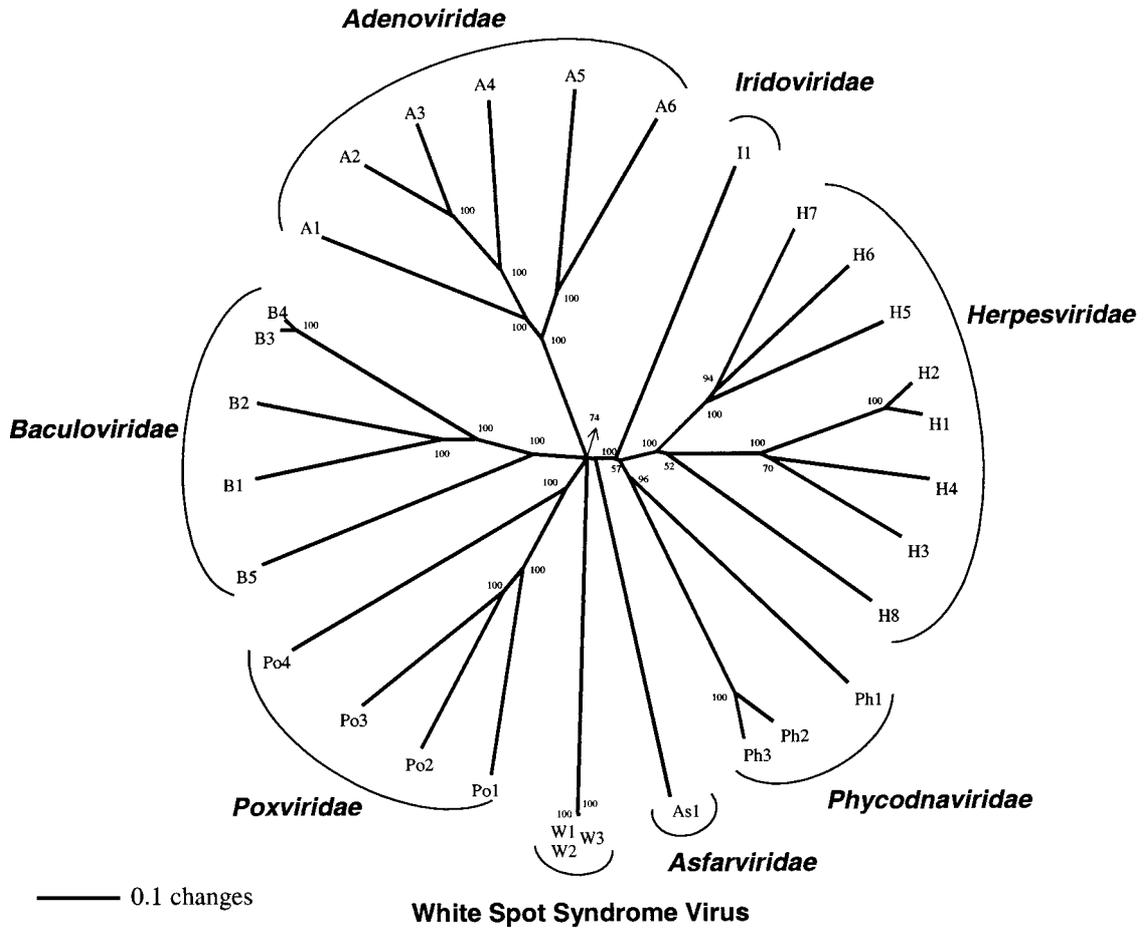


FIG. 9. Unrooted neighbor-joining phylogenetic tree of the WSSV DNA pol. Numbers at branch nodes indicate percent bootstrap support for that node based on 1000 replications. Abbreviations refer to the viruses listed in Table 1. W1, WSSV Taiwan-1 strain; W2, WSSV Thailand isolate; W3, WSSV China isolate.

oligo(dT)-anchor primer. The resulting cDNA was amplified with the anchor and the appropriate primer (pol-3'-RACE-sp1; Fig. 5). The final products were characterized by subcloning and sequencing, and the resulting sequences were compared with the genomic sequences.

WSSV *dnapol* transcriptional analysis

Since to date no WSSV-susceptible shrimp cell lines have become available, all the RNA for the transcrip-

tional analysis was taken from WSSV-infected shrimp at different times after infection using procedures described in Chen *et al.* (2002). Total RNA was isolated as described previously (Tsai *et al.*, 2000a; Liu *et al.*, 2001).

Temporal analysis of WSSV *dnapol* transcription by RT-PCR

The procedure for cDNA synthesis followed the procedure outlined by Chen *et al.* (2002). The cDNA reaction

TABLE 2
The Comparison of the WSSV DNA Polymerase from the Three Different Sources

Virus source (isolate)	Putative DNA pol length (aa)	Differences in amino acid residues (aa ^a)				Reference
		aa 833-836	aa 1386	aa 1680	aa 2069	
Taiwan	2351	GGGG	G	S	L	Present study
Thailand	2351	GGGG	D	N	F	van Hulten <i>et al.</i> , 2001a
China	2195	GGGGG	D	N	L	Yang <i>et al.</i> , 2001

^a The locations of amino acid residues are according to the WSSV DNA polymerase of Taiwan isolate.

products were subjected to PCR with the primer set pol-RTF/pol-RTR (AGTGGGTGGAACAATGTAGC/TCTACAGATTGCTCCTTCTC) for the *dnapol* gene. A β -actin transcript was amplified with the actin-F1/actin-R1 primer set (5'-GAYGAYATGGAGAAGATCTGG-3'/5'-CCRGGTACATGGTGGTRCC-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'-CAGACTATTAATGTACAAGTGC-3'/5'-GAATGATTGTTGCTGGTTAGAAC-3') derived from an intergenic region of the WSSV genome was used to confirm that the RNA was not contaminated by any viral DNA.

Detection of WSSV *dnapol* transcripts in WSSV-infected shrimp by Northern blot hybridization analysis with a *dnapol* gene-specific riboprobe

A WSSV *dnapol*-specific DIG-labeled riboprobe was used for Northern blot analysis. To generate the riboprobe, the RNA polymerase promoter addition kit Lig'nScribe (Ambion, Austin, TX) was used in accordance with the manufacturer's instructions to produce templates from WSSV *dnapol*-specific PCR products for the *in vitro* transcription. Briefly, the WSSV *dnapol*-specific fragment was amplified from WSSV genomic DNA by PCR with the primer set pol-RTF/pol-RTR. An aliquot (25 ng) of the WSSV *dnapol*-specific PCR product was then ligated with T7 promoter adapter (supplied with the kit) using T4 DNA ligase. To generate WSSV *dnapol*-specific fragments that contained the T7 RNA polymerase promoter, an aliquot (2 μ l) of the reaction mixture (10 μ l) was used as a template in PCR with a primer set consisting of the PCR adapter primer 1 (5'-GCTTCCGGCTCGTATGTTGTGTGG-3', supplied with the kit) and pol-RTF. An aliquot (3.6 μ l) of PCR product (50 μ l) was then used to generate the WSSV *dnapol*-specific DIG-labeled riboprobe by *in vitro* transcription (Sambrook *et al.*, 1989) in a 20- μ l reaction mixture containing 40 U T7 RNA polymerase (Roche) and 1 mM NTP labeling mix (Roche) 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.

Total RNA (5 μ g) was separated on 1% formaldehyde-agarose gel and transferred onto a Hybond-N+ membrane (Amersham Pharmacia Biotech, Inc., Piscataway, NJ) (Sambrook *et al.*, 1989). The membrane was prehybridized for 1 h at 68°C in a prehybridization buffer (Roche) and then hybridized with a specific DIG-labeled riboprobe that was added to the buffer. After hybridization for 16 h at 65°C, the membrane was washed for 5 min with wash buffer I (2 \times SSC and 0.1% SDS) at room temperature, and 30 min with wash buffer II (0.1 \times SSC and 0.1% SDS) at 68°C. DIG-labeled nucleotides in the blots were detected as described previously (Lo *et al.*, 1999). The membrane was then exposed to Kodak BioMax

MR film via an intensifying screen for several days at -70°C and the film was then developed.

Amino acid sequence comparison and phylogenetic construction

Thirty-one full-length DNA family B pols from GenBank were used in the alignment and phylogenetic analysis. The multiple sequence alignments were done by the multiple sequence alignment program CLUSTAL X (Thompson *et al.*, 1997) and edited in GeneDoc (Nicholas *et al.*, 1997). Phylogenetic analysis based on the full-length DNA pol sequences was performed using neighbor-joining and parsimony methods with the PAUP 4.0b1 program (Swofford, 1998), using CLUSTAL X 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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