The Journal of Microbiology, April 2007, p. 139-145
Vol. 45, No. 2
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Transcriptional Analysis of the DNA Polymerase Gene of *Bombyx mori* Parvo-like Virus (China Isolate)

Yong-Jie Wang[1,2*], Ke-Ping Chen[2], Qin Yao[2], and Xu Han[2]

[1]*Institute of Fisheries, Anhui Academy of Agricultural Sciences, Hefei 230031, P. R. China*
[2]*Institute of Life Sciences, Jiangsu University, Zhenjiang 212013, P. R. China*

(Received December 26, 2006 / Accepted March 3, 2007)

The *Bombyx mori* parvo-like virus (China isolate) DNA polymerase (*Bm*DNV-3 *dnapol*) gene has been tentatively identified based on the presence of conserved motifs. In the present study, we perform a transcriptional analysis of the *Bm*DNV-3 *dnapol* gene using the total RNA isolated from *Bm*DNV-3 infected silkworm at different times. Northern blot analysis with a *Bm*DNV-3 *dnapol*-specific riboprobe showed a major transcript of 3.3 kb. 5′-RACE revealed that the major transcription start point was located 20 nucleotides downstream of the TATA box. In a temporal expression analysis using differential RT-PCR, *Bm*DNV-3 *dnapol* transcript was detected at low levels at 6 h.p.i., increased from 6 to 36 h.p.i., and remained fairly constant thereafter. Analysis of the predicted DNA polymerase sequence using neighbor-joining and protein parsimony algorithms indicated that the predicted 1115-residue polypeptide contained five motifs associated with DNA polymerases synthetic activities and three additional motifs associated with polymerases possessing 3′ to 5′ exonuclease activity. The molecular phylogenetic analysis of this gene supported the placement of *Bombyx mori* parvo-like virus in a separate virus family.

Keywords: *Bombyx mori* parvo-like virus, DNA polymerase gene, conserved motif, transcription analysis

DNA polymerase is one of the responsible enzymes for replication and repair of DNA along the sequence of a template strand. Genomes of most eukaryotes are populated by DNA copies of parasitic elements known as DNA polymerases which are capable of reproducing themselves in the host genome in a non-Mendelian fashion (Kornberg *et al.*, 1991). The life cycle of viruses is characterized by the production of viral progeny. Among the expressed genes involved in viral-DNA replication, the DNA polymerase protein plays a significant role in determining the level of genomic replication (Liu and Carstens, 1995; Lu *et al.*, 1997). Understanding the biology of DNA polymerases is of great importance because of their increasingly well documented impact on the host genome.

The *Bombyx mori* parvo-like virus, termed as *Bm*DNV-3, is composed of two kinds of different single-stranded linear DNA molecules (VD1 and VD2). The complete nucleotide sequence of the viral genome had been determined (Wang *et al.*, 2006). The GenBank accession numbers are DQ017268. According to ICTV Virus Taxonomy report, *Bm*DNV-3 has not been definitely classified so far. Many characteristic traits of *Bm*DNV-3 were quite similar to those of densovirus, so it was tentatively designated as densovirus (Lu, 1998). But unlike other parvoviruses and densovirus, *Bm*DNV-3 has bipartite genomes and no common terminal palindromic sequences, implying that it is a new type of virus with unique replication mechanisms. In addition, *Bm*DNV-3 has an open reading frame (VD1 ORF4) of its genome codes for a large polypeptide with sequence motifs characteristic of DNA polymerases, and showed an evolutionary relationship with the DNA polymerases involved in protein-primed replication. The VD1 ORF4 replication gene DNA polymerase may have advantages as a tool for resolving *Bm*DNV-3 phylogeny (Wang *et al.*, 2006).

Here we conducted a transcriptional analysis of the *Bm*DNV-3 *dnapol* gene. We also compared its sequence with sequences of other known DNA polymerases, located the conserved motifs, and provided genetic evidence that this ORF encoded a DNA polymerase. This has enabled a preliminary determination of the evolutionary relationship of *Bm*DNV-3 DNA polymerase to other DNA polymerases. The results suggest *Bm*DNV-3 *dnapol* has the characteristics of the eukaryotic-type family B *dnapol*.

Materials and Methods

Sequence alignments and construction of phylogenetic trees
Analyses were performed on alignments from complete DNA polymerase protein sequences and from peptide sequences representing the conserved sequence motifs of the DNA polymerase molecules. Twenty full-length *dnapol* 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). Phylogenetic analysis based on the full length *dnapol* sequences was performed using neighbor-joining and parsimony methods with the PAUP 4.0 program (Swofford, 1998), using CLUSTAL X to produce input files

* To whom correspondence should be addressed.
(Tel) 86-551-516-0958; (Fax) 86-551-516-0958
(E-mail) hfwangyongjie@163.com

of aligned protein sequences.

Virus and total RNA

Since no *Bm*DNV-3 susceptible silkworm cell lines are available so far, *Bm*DNV-3 was propagated in the 5th instar larvae of susceptible silkworm Huaba strains, and all the RNA for the transcriptional analysis was taken from the midgut of inoculated larvae at different times. The midgut was submerged in a fresh 0.05% diethylpyrocarbonate (DEPC) solution before being frozen in liquid nitrogen, and was subjected to RNA purification using an RNA isolation kit (Qiagen).

Northern blot hybridization analysis with a dnapol gene-specific riboprobe

A *Bm*DNV-3 *dnapol*-specific DIG-labeled riboprobe was used for Northern blot analysis. To generate the riboprobe, a probe preparation kit (Takara) was used in accordance with the manufacturer's instructions to produce templates from *Bm*DNV-3 *dnapol*-specific PCR products for the *in vitro* transcription. Briefly, the *Bm*DNV-3 *dnapol*-specific fragment was amplified from the sequence from nts 4451-5414 of the *Bm*DNV-3 VD1 genome by PCR with the primer set (5'-GTATCCCAACTCAACACTCTT-3'; 5'-GGAGCATAAATT AATGCTTCT-3').

Total RNA isolated from midgut, before infection (0 h) and at 2, 4, 6, 12, 24, 36, 48, 60, 72, and 84 h after *Bm*DNV-3 infection, was separated on 1.2% formaldehyde agarose gel and transferred onto a positively charged nylon membranes (Roche, USA). 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 42°C, the membrane was washed for 5 min with wash buffer I (2× SSC and 0.1% SDS) at room temperature, and 30 min with wash buffer II (0.1× SSC and 0.1% SDS) at 68°C. DIG-

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                        Exo I                          Exo II                        Exo III
PLEOS      261  ISKNFITMDLETRN   (...)  323  YLHNFSYFDGI   (...)  448  IKYCELDCI
FLAVE      262  ISNKIMTMDIETKT   (...)  322  YFHNFSSFDSI   (...)  453  IKYCESDVI
NEUIN      334  ENPKIITLDLETRS   (...)  551  YTHNFSYFDGI   (...)  546  IKYCEIDTL
NEUCR      316  QDKKILAFDLETFQ   (...)  377  YCHNFSKFDIN   (...)  511  IIYLEKDIK
PODAN      346  LNSRIGTFDLETFQ   (...)  397  FTHNLGGYDII   (...)  585  LHYLERDLL
ZEAMA      206  NKTKFIVADLETIP   (...)  294  YFHNLGQFDGI   (...)  396  LSYLKQDIM
BRANA      282  KMKAFIVADIETIM   (...)  369  YFHNMSKFDGI   (...)  467  IDYMIQDTR
B1         10   KSAKLLTLDTEFRE   (...)  62   YVHNLD.FDLG   (...)  172  NEYLEYDCR
B2         29   REIKLFTLDTETRG   (...)  80   YIHNLD.FDLS   (...)  191  CEYMEYDCR
Phi29      4    MPRKMYSCAFETTT   (...)  57   YFHNLK.FAGA   (...)  163  UAYIKNDIQ
BmDNV-3    329  KHCLVAYADFEAII   (...)  451  YFHNFKGYDHH   (...)  613  LLYNELDVW
Consensus                D ET               U HN   YD                   Y E D

                      I                               II                            III
PLEOS      537  RYDANSLUPUAMK   (...)  666  SKMLLNSLYGRLG   (...)  739  VVSSIVTASARIYMSK
FLAVE      543  RYDVNSLUPEAMK   (...)  681  SKLLMNSLYGRFG   (...)  758  AIAATVTAEARIBMSK
NEUIN      632  SYDVNSLYPFAMK   (...)  768  SKLLMNSLYGRFG   (...)  835  PISSAIAAYSRIBMSH
NEUCR      625  SFDFNSLYPTAMM   (...)  749  AKLLLNTLYGRTG   (...)  837  SIAAATASWSRILMNK
PODAN      570  YYDVNSLYPFVAK   (...)  793  TKFLLNSLLGRFG   (...)  885  SISAAVTAYARIFMAC
ZEAMA      483  YYDVNSLYPYSML   (...)  617  HKTTMNSLYGRFG   (...)  692  QISAAVTAYARIBMYP
BRANA      555  YYDVNSLYPYVMK   (...)  687  YKTIMNSLYGRFC   (...)  759  QLSAAITAYARIUMYK
B1         265  HLDVNSLYPYVMK   (...)  398  AKLIONALYGKFG   (...)  463  BIASYVTAYARILLYR
B2         286  HVDKNSLYPYVNK   (...)  419  SKLMQNALYGKFA   (...)  484  BISAYITSIARILLFK
Phi29      247  VFDVNSLYPYVMK   (...)  381  AKLMLNSLYGKFA   (...)  427  PMGVFITAWARYTTLT
BmDNV-3    706  YLDVNTMYSYCMK   (...)  843  YKLKNNALFGKDC   (...)  906  QIGFTILELAKLMIYE
Consensus         DVN  Y Y MK               KL  N LYGKF                            AR   Y

                      IV                         V
PLEOS      763  IYYTDTDSIDVDQE (...)  800  IFLAPKVYGGT
FLAVE      782  IFYSDTDSIDINKP (...)  819  IYVSNKAYWAI
NEUIN      859  IYYIDTDGIKVDID (...)  897  ISLGPKVYGGT
NEUCR      857  SAYTDTDSIFVEKP (...)  896  IFLSGKLYLLD
PODAN      912  LYYTDTDSIVTDID (...)  949  FFISAKTYCLI
ZEAMA      714  CYYTDTDSIVVKHP (...)  757  VFLAPKSYMLQ
BRANA      781  CYYTDTDSVVLQDS (...)  818  IFLAPKSYILK
B1         489  IGYCDTDSIACESM (...)  526  IFLQPKFYAER
B2         510  LAYCDTDSCATTTK (...)  547  LIFQPKMYAEK
Phi29      452  IIYCDTDSIHLTGT (...)  493  KYLRQKTYIQD
BmDNV-3    938  MLYTDTDSVIFKFK (...)  994  IGTRAKQYAYS
Consensus         Y DTDS                   I    K Y
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Fig. 1. Amino acid sequence alignment of 11 DNA polymerases. The multiple sequence alignments were done by the multiple sequence alignment program CLUSTAL X (Thompson *et al.*, 1997). The multiple alignment indicates the conserved regions of DNA polymerases; Exonucleasic motifs (Exo I, II, and III) and the five conserved regions (I-V) associated with DNA polymerases synthetic activities are present in all DNA polymerases analysis (Morrison *et al.*, 1991). Consensus show identical residues. Abbreviations are as for the viruses listed in Table 1.

Table 1. Viral DNA polymerase used in the sequence alignment and phylogenetic construction

Virus	Species family	Accession no.	References
*Ac*MNPV	*Autographa californica*	AAA46692	Tomalski *et al.* (1988)
*Oran*NPV	*Orgyia anartoides*	AAC33746.1	Bulach *et al.* (1999)
A1	Duck adenovirus	NP-044702	Hess *et al.* (1997)
A2	Human adenovirus type 40	NP-040853	Ishino *et al.* (1987)
Ph1	*Feldmannia sp.* virus	AAB67116	Lee *et al.* (1998)
Ph2	*Paramecium bursaria*	AAA88827	Grabherr *et al.* (1992)
*Cf*GV	*Choristoneura fumiferana*	AY161135	Direct submission
AGRAE	*Agrocybe aegerita*	O78938	Bois *et al.* (1999)
PICIN	*Pichia inositovora*	CAD91889	Klassen *et al.* (2003)
PLEOS	*Pleurotus ostreatus*	AAK40110	Kim *et al.* (2000)
FLAVE	*Flammulina velutipes*	BAB13496	Nakai *et al.* (2000)
NEUIN	*Neurospora intermedia*	P33538	Chan *et al.* (1991)
NEUCR	*Neurospora crassa*	CAA39046	Court and Bertrand (1992)
PODAN	*Podospora anserina*	Q01529	Hermanns and Osiewacz (1992)
ZEAMA	*Zea mays*	AAR91042	Direct submission
BRANA	*Brassica napus*	NP-862323	Handa *et al.* (2002)
B1	*Bacillus thuringiensis*	NP-829893	Direct submission
B2	*Bacillus thuringiensis* phage	YP-224103	Verheust *et al.* (2005)
Phi29	Bacillus phage phi29	P06950	Direct submission
*Bm*DNV-3	*Bombyx mori* densonucleosis	AAY41819	Wang *et al.* (2006)

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 two days at -70°C and the film was then developed.

Mapping of the 5' and 3' end of the BmDNV-3 dnapol transcript

The RNA samples used in this study were isolated from the 5th instar silkworm 36 h after *Bm*DNV-3 infection and then treated with RNase-free DNase. The 5' region of the *dnapol* transcript was determined by rapid amplification of the cDNA 5' end using a commercial 5'-RACE kit (BD Biosciences Clontech, USA) according to the instructions provided by the manufacturer. From the open reading frames obtained through sequence analysis, the most probable location of the transcripts was predicted. Using DNV-specific primers and 5'-RACE universal primers, PCR amplification was carried out according to manufacturer's instruction of the RACE kit.

The 3' region of the *dnapol* transcript was determined by 3'-RACE using a commercial 3'-RACE kit (BD Biosciences Clontech, USA) according to the instructions provided by the manufacturer. First-strand cDNA was synthesized using oligo(dT)-anchor primer. The resulting cDNA was amplified with the anchor and the appropriate primer.

Temporal analysis of BmDNV-3 dnapol transcription by fluorescence quantitative PCR

Quantitative PCR analysis was used to detect the *dnapol*-specific transcript in RNase-free DNase treated total RNA from silkworm specimens before infection (0 h) and at 2, 4, 6, 12, 24, 36, 48, 60, 72, 84, and 96 h after *Bm*DNV-3 infection. The procedure for cDNA synthesis followed the procedure outlined by Chen *et al.* (2002). The cDNA reaction products were subjected to PCR with the primer set nts 5913-5936; 5'-GTTGACCCAGTAGGATAGGATGAC-3', nts 6210-6187; 5'-CTATTACTCCACCAGCAAAGACGA-3', for the *dnapol* gene. A *Bm*DNV-3 genomic DNA-specific primer nts 2832-2853; 5'-AGGTCACCACCAATGCGTCAA-3', nts 3324-3303; 5'-TCCAGGGTTGTGGTCAGATGA-3' derived from an intergenic region of the *Bm*DNV-3 genome was used to confirm that the RNA was not contaminated by any viral DNA. Copy number was determined by comparison with a standard curve generated from the amplification of the positive control plasmid pUC119.

Results and Discussion

Location of BmDNV-3 dnapol gene

From this virus, several plasmid libraries were constructed for sequencing the *Bm*DNV-3 genome (Wang *et al.*, 2006). A 3345-nt open reading frame (VD1 ORF4) was found, and the deduced amino acid sequence from the first ATG of the ORF predicts an amino acid polypeptide of 1115 residues with a molecular mass of 128.7 kDa. BLAST searches indicated that the *Bm*DNV-3 DNA polymerase protein showed the maximum amino acid sequence identity to DNA polymerases from *Agrocybe aegerita* (25%), followed by *Flammulina velutipes* (22%).

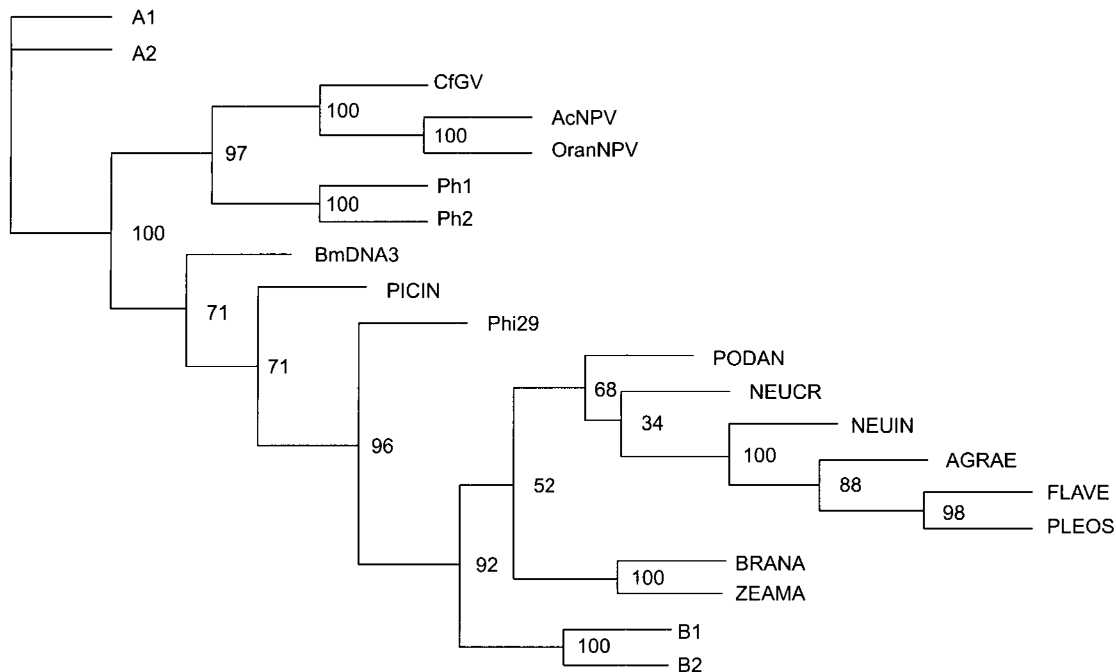

Fig. 2. Phylogenetic analysis of 20 DNA polymerases. Phylogenetic analysis based on the full length *dnapol* sequences was performed using neighbor-joining and parsimony methods with the PAUP 4.0 program (Swofford, 1998), using CLUSTAL X to produce input files of aligned protein sequences. The number at each branch indicates the percent frequency of this grouping after 100 boostrap evaluations. Abbreviations are as for the viruses listed in Table 1.

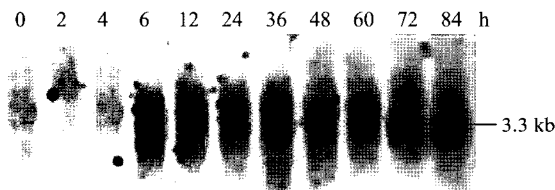

Fig. 3. Northern blot temporal transcription analysis of total RNA isolated from *Bm*DNV-3 infected silkworm using *Bm*DNV-3 *dnapol*-specific riboprobes. Total RNA isolated from midgut, before infection (0 h) and at 2, 4, 6, 12, 24, 36, 48, 60, 72, and 84 h after *Bm*DNV-3 infection, was separated on 1.2% formaldehyde agarose gel and transferred onto a positively charged nylon membranes (Roche, USA). The transcript is approximately 3.3 kb, the size standards were determined by RNA marker. Lane headings show hours p.i.

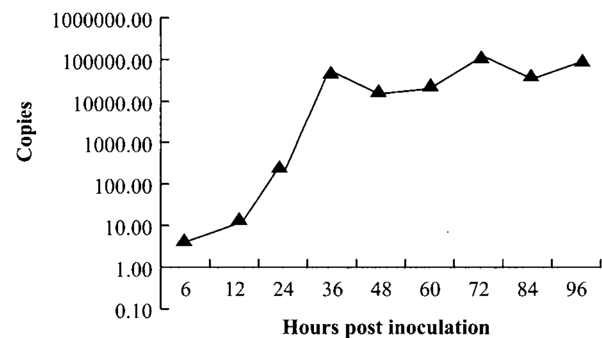

Fig. 4. Temporal transcription analysis of *Bm*DNV-3 dnapol by fluorescence quantitative PCR. Quantitative PCR analysis was used to detect the *dnapol*-specific transcript in RNase-free DNase treated total RNA from silkworm specimens before infection (0 h) and at 2, 4, 6, 12, 24, 36, 48, 60, 72, 84, and 96 h after *Bm*DNV-3 infection. The transcripts were detected at low levels at 6 h.p.i., increased from 6 to 36 h.p.i., and remained fairly constant thereafter.

Amino acid sequence alignment of BmDNV-3 DNA pol

When the deduced amino acid sequence of VD1 ORF4 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). Three conserved regions, Exo I (defined by the DXE consensus), Exo II (Nx_3F/YD), and Exo III (Yx_3D), associated with 3' to 5' exonuclease activity in eukaryotic and prokaryotic organisms, were identified (Morrison *et al.*, 1991). In addition, about 300-aa C-terminal portion is significantly similar to various protein-primed DNA polymerases that belong to the B family of DNA-dependent DNA polymerases. According to the literature (Vladimir and Kapitonov, 2006), the synthetic activities are defined by five conserved motifs Dx2SLYP (motif I), Kx3NSxYG (motif II), Tx2GAR (motif III), YxDTDS (motif IV), and KxY (motif V). These regions (I, II, III, IV, and V) are also involved with enzymatic functions of DNA polymerase, such as the synthetic activity, dNTP-binding and pyrophosphate hydrolysis (Bernard *et al.*, 1990; Wang, 1991). The order of the motifs is the same as that found in other known DNA polymerases. The presence and order of these 8 motifs strongly indicates that the polypeptide product of this gene is a DNA polymerase.

Each viral DNA pols encodes a 1000 to 1400 aa protein

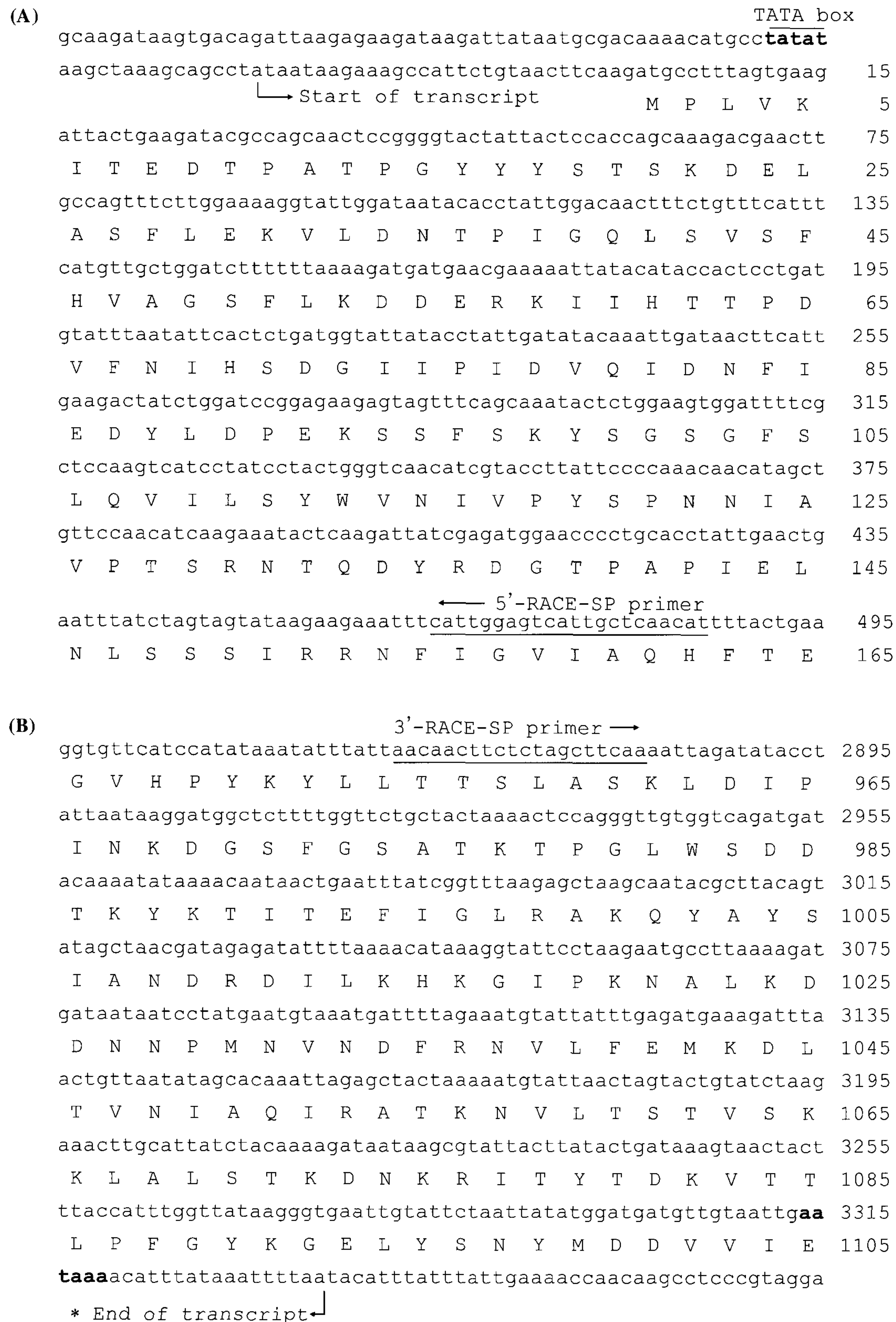

Fig. 5. Mapping the 5' and 3' end of the *Bm*DNV-3 *dnapol* transcript. (A) The primers used for 5'-RACE are underlined. The bent arrows indicate the 5' termini (transcriptional start points) revealed by sequencing nine 5'-RACE clones. The predicted TATA box is boldfaced; (B) The primer used for 3'-RACE is underlined. The polyadenylation signal (AATAAA) is boldfaced. The bent arrows indicate the 3' termini (transcriptional end points) revealed by sequencing nine 3'-RACE clones.

whose 300 aa C-terminal portion is significantly similar to various protein-primed DNA polymerases that belong to the B family of DNA polymerases. Protein-primed POLBs constitute a distinctive group of eukaryotic and prokaryotic DNA polymerases encoded by various phages, vertebrate adenoviruses, and linear plasmids from plant and fungal mitochondria. These polymerases display both 3' to 5' exonucleolytic and 5' to 3' synthetic activities defined by two structurally independent N- and C-terminal domains (Blanco *et al.*, 1996). After inspection of a multiple alignment of *Bm*DNV-3 DNA pol and other POLB polymerases, we found that *Bm*DNV-3 DNA pol conserved motifs had structural characteristics of protein-primed POLB polymerases (Fig. 1). Based on these structural characteristics, we propose the placement of *Bm*DNV-3 DNA pol in the B family of DNA polymerases.

Phylogenetic analysis

A total of 20 viral DNA pols (Table 1) were used to construct phylogenetic trees. To estimate the evolutionary relationship of the *Bm*DNV-3 based on the DNA polymerase protein sequences, we compared the sequence of *Bm*DNV-3 DNA polymerase with other 19 viral DNA polymerase sequences. The phylogenetic tree was constructed using the maximum parsimony method in PAUP Version 4.0 with heuristic search. The phylogenetic tree of the DNA pols appears to offer resolution, placing *Bm*DNV-3, *Ac*MNPV, *Oran*NPV, Ph1, Ph2, and *Cf*GV in one clade (Fig. 2). *Bm*DNV-3 *dnapol* thus has the characteristics of the eukaryotic-type family B *dnapol*. The results obtained in this work could be useful in subsequent studies, such as characterization of the *Bm*DNV-3 DNA polymerase enzymatic activity and analysis of its expression. The phylogenetic relationships established in this work together with previous findings could be important in the understanding of the biological adaptations between virus and its host and contribute to our understanding of *Bm*DNV-3 evolutionary history.

Transcriptional analysis of BmDNV-3 dnapol

A DIG-labeled probe derived from VD1 ORF4 was generated by *in vitro* transcription for the detection of the *Bm*DNV-3 *dnapol* gene transcript in total RNA extracted from *Bm*DNV-3 infected silkworm. Northern blot analysis with this *Bm*DNV-3 *dnapol* gene-specific riboprobe first detected one major transcript of approximately 3.3 kb at 6 h.p.i. (Fig. 3). The band was detected at low levels at 6 h.p.i., increased from 6 to 36 h.p.i., and remained fairly constant thereafter.

RT-PCR primer sets specific to intergenic region of the *Bm*DNV-3 genome were used for transcriptional comparison. After 40 cycles of amplification, *Bm*DNV-3 *dnapol* transcripts were detected at low levels at 6 h.p.i., increased from 6 to 36 h.p.i., and remained fairly constant thereafter (Fig. 4). The genomic DNA contamination controls gave an expected result, thus confirming that no viral genomic DNA was left in the prepared RNA.

To determine when the *dnapol* mRNA was transcribed in the viral life cycle, and which mRNA was transcribed, we performed a transcriptional analysis of *dnapol* using Northern blot and RT-PCR analysis. One major transcript of approximately 3.3 kb at 6 h.p.i. was first detected by northern blot analysis with this *dnapol* gene-specific riboprobe, which is consistent with the quantitative PCR result. Likewise in both the quantitative PCR and Northern blotting, the transcript was increased through to the end of the 36 h.p.i. experiment. The size of the transcript matched the predicted size of the *dnapol* mRNA after allowing for the presumed *dnapol* coding region. The results obtained in this work could be useful in subsequent studies, such as characterization of the *Bm*DNV-3 DNA polymerase enzymatic activity and analysis of its expression.

Mapping 5' and 3' end of the dnapol transcript

The final 5' and 3' end products were characterized by sequencing, and the results were compared with the genomic sequences. Analysis of 5'-RACE products and the viral genome sequence (GenBank accession number DQ017268) revealed that the transcriptional initiation site was located at -25 nt upstream of the predicted ATG initiation codon (Fig. 5), a putative TATA box was found at 27 nt upstream of the transcriptional initiation sites. The TATA box is located on the minus strand between nts 6301 and 6307, which is flanked by a GGCATGT (nts 6309-6315) activator sequence with 5 of its 7 nt identical with the Kozak's consensus sequence (A/GCCAUGG) of translation initiation in eukaryotes (Kozak, 1986). Most promoters for RNA polymerase II usually have the TATA box located -25 bp upstream of the transcription start point (Bensimhon *et al.*, 1983). 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 (nts 2938 to nts 2943), and the translation stop codon was found within the polyadenylation signal sequence (Fig. 5).

Acknowledgements

This work was supported by a grant from Academy Natural Science Foundation of Jiangsu Province, China (project no. BK2006074) and National Basic Research Program of China (project no. 2005CB121000).

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