Identification of the Polyhedrin Gene of Thai Bombyx mori Nucleopolyhedrovirus

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Received 19 Aug 2005
Accepted 5 Jun 2006

ABSTRACT: The full-length of the polyhedrin gene (polh) of Thai Bombyx mori nucleopolyhedrovirus (BmNPV) was cloned and sequenced. The polh sequence contained a 735 bp open reading frame (ORF) encoding a protein of 245 amino acids with a predicted molecular mass of 28.8 kDa. The nucleotide sequence of Thai BmNPV polh shows greater than 98% identity to the sequences of five different BmNPV polh genes that were previously characterized. The high degree of sequence identity with the polh sequences of other BmNPVs suggests that they are orthologues of the BmNPV polh gene in this study. Comparison of Thai BmNPV polh sequence with other polhs of Lepidopteran NPVs (Autographa californica, Helicoverpa armigera, Spodoptera litura and S. exigua) indicated that the nucleotide and amino acid sequences were greater than 65% and 78% identical, respectively.

KEYWORDS: nucleopolyhedrovirus, polyhedrin gene, Bombyx mori, silkworm, Thai.

doi: 10.2306/scienceasia1513-1874.2006.32.421

INTRODUCTION

Bombyx mori nucleopolyhedrovirus (BmNPV) is an infectious agent causing the most destructive disease (grasserie) of the silkworm, B. mori. BmNPV belongs to the genus Nucleopolyhedrovirus, the family Baculoviridae1. Baculoviruses have been detected in over 600 species of arthropod hosts including members of the order Lepidoptera, Diptera, Hymenoptera, Coleoptera, Neuroptera, Thysanura and Trichoptera2. The two morphological subgroups within the NPVs are the single-embedded NPV, in which only one nucleocapsid is present per envelope, and the multiple-embedded NPV, in which several nucleocapsids are packed per envelope3. Polyhedra (occlusion bodies) produced during an epizootic outbreak may persist between seasons in the environment and therefore serve as a reservoir of inoculum to infect subsequent generations of insect hosts4. Polyhedra are mainly composed of a single polypeptide known as polyhedrin (Polh). Polh, which constitutes the crystalline matrix of baculovirus occlusion bodies, plays a significant role in the replication cycle of baculovirus5. Polh is encoded by a gene which is highly conserved among baculoviruses, so it is the most comprehensive option available for estimating the relationship among baculoviruses6.

More than 80% sequence identity has been reported among lepidopteran baculovirus Polhs7. Based on phylogenetic studies using the amino acid sequences of polyhedron proteins, lepidopteran NPVs have been classified into two groups, namely Group I and Group II8. Subsequently, Bulach et al.8 supported these clades and revealed other subclades within Group II by analyzing the polyhedrin and DNA polymerase genes. Evolutionary studies of baculoviruses have been carried out with several additional genes, but systematics of these viruses is still consistent.

The polh genes of NPVs have been characterized in many host insects, such as A. californica, Anticarsia gemmatalis, B. mori, H. armigera, Lymantria dispar, Orgyia pseudotsugata, S. exigua, S. litura, S. frugiperda, S. littoralis, etc9. However, the polh gene of the Thai BmNPV isolate has not been investigated. This paper aimed to study
polh from the Thai BmNPV isolate and determine its relatedness to other BmNPVs. Information on the relationships among BmNPV polh and other NPV polhs is necessary for the understanding of NPV evolution. Moreover, the information is useful for study of the molecular basis of NPVs and their useful applications in construction of recombinant baculoviruses with higher insecticidal activity and for detection of BmNPV for preventive control of grasserie disease.

MATERIALS AND METHODS

Purification of BmNPV and DNA Extraction

BmNPV that was isolated from diseased larvae collected from Udon Thani Sericultural Research Center, Thailand was propagated in silkworm larvae. The polyhedra were purified by 40-65% (w/w) sucrose density gradient centrifugation19. The virions were released from the polyhedra by dissolving with alkaline solution (0.2 M Na₂CO₃, 0.5 M EDTA, 0.34 M NaCl). Genomic DNA was extracted according to the method of O’Reilly et al.11.

PCR Amplification and Cloning of BmNPV polh

A primer set (F: 5'- CCCAAGATGTATAAACC-3' and R: 5'- GCTAACGCGGCCGATGT-3') was designed from the nucleotide sequence of BmNPV T3 (GenBank accession number L33180)12. The PCR amplification cycling was an initial denaturation at 94°C for 5 min, followed by 35 repeated cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min, and the final extension at 72°C for 5 min in a PCT-100 DNA thermal cycler (MJ Research, Inc.). The PCR product was ligated into pGEM-® T vector (Promega) and cloned. The plasmid DNA sequences similar to the consensus TATA and CAAT, which represent important elements of eukaryotic gene promoters were observed at positions -107 and -143. The canonical poly (A) signal AATAAA was present in the 3’end of the Thai BmNPV polh gene at position 1081. The Thai BmNPV polh ORF had the translation initiation codon, ATG, and the termination codon, TAA, as found in other baculoviruses11. The complete sequence of the Thai BmNPV polh overlapped with the lef-2 (late gene expression factor-2) gene in the 5’ flanking region and with orf1629 in the 3’ flanking region. The lef-2 gene was located in the polh upstream region, adjacent to the transcription start site in the same direction and the orf1629 was located in the polh downstream region in the reverse direction.

The orientation of polh, lef-2 and orf1629 in BmNPV are the same as those found in the AcNPV genome10. The position of the orf1629 gene in HaNPV, SeNPV and SNPV is next to polh, similar to BmNPV, but lef-2 is not located in the polh upstream region. In BmNPV, lef-2 is essential for both viral DNA replication and late gene expression22 and orf1629 is essential for BmNPV viability29.

The flanking nucleotides of Thai BmNPV polh and the BmNPV T3 polh fragment (accession number L33180), which was used to design the primers, were compared. The major difference between the two isolates was the deletion of eight nucleotides in the upstream region of Thai BmNPV polh ORF at the position -71 and 3 substitutions at positions A119/G101, C128/T110, C139/T136 were found, while the rest of the nucleotide sequences were almost identical. A previous study using a series of deletions in the upstream region of BmNPV polh (lef-2, orf327, orf453 and bro-e) revealed that the upstream region of polh has no effect on expression from the polh promoter29.

RESULTS AND DISCUSSION

Nucleotide Sequence of Thai BmNPV polh

A 1,440-bp fragment of the Thai BmNPV DNA that contained the full-length coding region of the polh (GenBank accession number AY779044) was successfully amplified and sequenced (Fig 1). The polh ORF consisted of 735 nucleotides that encoded a polypeptide of 245 amino acids with the predicted molecular mass of 28.8 kDa. The sequence contained 228 bp of 5’ UTR and 474 bp of 3’ UTR, respectively. Several characteristics of the Thai BmNPV polh gene sequence were investigated. The immediate upstream sequence of the translation initiation site was AT rich and contained the uniquely conserved transcription start site TAAG motif, which is similar to the consensus sequence of TAAATAAGTATTTT at position -42 to -56 of other baculovirus late gene promoters21. DNA sequences similar to the consensus TATA and CAAT, which represent important elements of eukaryotic gene promoters were observed at positions -107 and -143. The canonical poly (A) signal AATAAA was present in the 3’end of the Thai BmNPV polh gene at position 1081. The Thai BmNPV polh ORF had the translation initiation codon, ATG, and the termination codon, TAA, as found in other baculoviruses11. The complete sequence of the Thai BmNPV polh overlapped with the lef-2 (late gene expression factor-2) gene in the 5’ flanking region and with orf1629 in the 3’ flanking region. The lef-2 gene was located in the polh upstream region, adjacent to the transcription start site in the same direction and the orf1629 was located in the polh downstream region in the reverse direction.

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Fig 1. Nucleotide sequence of Thai BmNPV polyhedrin gene and its flanking regions (1,440 bp, AY 779044). The predicted amino acid sequence is indicated by one-letter code. The sequence at nt -51 initiates the 5' end of the mRNA. The putative transcription initiation motif (TAAG) is underlined. The PCR primers are also shown and the arrows indicate the direction of extension.
Comparison of Thai BmNPV polh With Other NPV polh genes

All BmNPV polh ORFs described in this study contained 735 nucleotides that encoded polypeptides of 245 amino acids. The ORFs of the BmNPV polh of the Thai and Japanese 1 (T3) isolates were identical. Nucleotide and amino acid sequences of Thai BmNPV polh revealed high identity (more than 95%) to those of other BmNPVs and lower identity with AcNPV, HaNPV, ScNPV and SinPV (75.2%, 65.3%, 70.1% and 65.9% for polh gene sequences and 86.1%, 78.0%, 81.6% and 79.6% for Polh protein sequences, respectively; Table 1). This result is similar to a previous report, which indicated that the percent identities of the amino acid sequence of the Canadian BmNPV Polh compared to those of AcNPV, ScNPV, and SinPV were 86%, 82% and 80%, respectively25.

Alignment of the BmNPV Polh sequences showed variation of amino acid sequences near the N-terminus (Fig 2). Comparison of the sequence divergence of nucleotide and amino acid sequences among BmNPVs indicated that BmNPVs found in the Asian countries (Thailand, Japan, Korea and China) were more closely related than the isolate from Canada.

The ORF of BmNPV polh encoded a polyhedrin protein containing 245 amino acids, similar to polh of AcNPV and HaNPV, while the polh ORF of ScNPV and SinPV contained 246 and 249 amino acids, respectively. The amino acid sequence alignment of BmNPV Polh with the other NPVs demonstrated that more differences occur in the N-terminus than the C-terminus (Fig 2). In HaNPV, the amino acid (Histidine/Aspartic acid) of Polh at position 40 was not found. There are many substitutions that make amino acids of BmNPVs differ from other NPVs such as V13/L, L31/W, M107/V, F123/Y, M128/L, R129/K, C147/W, D148/E, P149/E, L187/I, L189/V and L226/I. Interestingly, the amino acid sequences at position 147-149, of all BmNPV studied were different from other NPVs. This sequence may be used to differentiate BmNPVs and NPVs from other host species. There is no report on mutation of amino acids at positions 147-149 of polyhedrin. Therefore, the functionally importance of these amino acid domains is not known. Amino acid content of the putative BmNPV Polh indicated that it is rich in acidic amino acid residues, such as glutamic acid, especially at position 220-223 where four glutamic acids occur consecutively.

The phylogenetic tree of NPV Polh proteins showed that NPV could be divided into two groups (Fig 3). The first group was further divided to 2 subgroups; BmNPV-Canada (I.1) and the Asian BmNPV (I.2) whereas the second group was composed of SinPV, HaNPV, ScNPV and AcNPV. Among the BmNPVs, the Canadian isolate is more distantly related than the Asian isolates (Fig 3). Due to limited vagility of the silkworm, biogeographic variation was observed between different isolates of NPVs in this species, as demonstrated by phylogeographic differences between the Canadian and Asian isolates.

Nucleopolyhedrovirus clades based on the Polh were first described by Zanotto et al.6, who divided NPVs into Group I and Group II. Subsequently, Bulach et al.8 supported these clades and revealed other subclades within Group II by analyzing the combined Polh and DNA polymerase sequences. Our results were concordant with Zanotto et al.6 and Bulach et al.8 where HaNPV, ScNPV and SinPV were allocated into Group II. However, BmNPVs revealed possibly paraphyletic relationships between the Canadian and the Asian isolates.

Table 1. Nucleotide (above) and deduced amino acid sequence (below diagonal) identity of BmNPVs polh. Sequence data of BmNPV polh varieties included 1) Thai (this study, AY779044); 2) Japanese 1 (T3) (L33180); 3) Japanese 2 (M30925); 4) Korean (K1) (U75359); 5) Chinese (X63614); 6) Canadian (M100430); 7) AcNPV (NC_001623); 8) HaNPV (NC_002654); 9) SeNPV (NC_002169) and 10) SinPV (NC_003102).

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Fig 2. Multiple alignments of the deduced amino acids of Thai BmNPV polh (AY779044) and other BmNPV polhs; Japanese 1 (T3) (L33180), Japanese 2 (M30925), Korean (K1) (U75359), Chinese (X63614), Canadian (M100430) and AcNPV (NC_001629), HaNPV (NC_002654), SeNPV (NC_002169) and SINPV (NC_003102). Positions containing different amino acids are shaded.
Harrison and Bonning\textsuperscript{26} constructed a phylogenetic tree of Polh of many lepidopteran NPVs including \textit{Bm}NPV, \textit{Ac}NPV, \textit{Ha}NPV, \textit{Se}NPV and \textit{Sl}NPV. They grouped \textit{Ha}NPV, \textit{Se}NPV and \textit{Sl}NPV into Group II and \textit{Bm}NPV into Group I of the proposed tree of Zanotto \textit{et al.}\textsuperscript{6}. The \textit{Ac}NPV polyhedrin was put on a branch outside of the clade containing the other members of Group I. They suggested that \textit{Ac}NPV may have acquired its \textit{polh} gene by recombination with another virus that is not closely related to other NPVs in Group I\textsuperscript{26}. In addition, Jehle\textsuperscript{27} used a Hidden Markov Model to surmise that the \textit{Ac}NPV \textit{polh} is a chimeric gene which consists of a mosaic of the genome of Group I and II NPVs. From these results, \textit{Ac}NPV can be grouped in both Group I and Group II, depending on the method used for analysis. In this study, \textit{Ac}NPV was also placed in Group I. Since several reports revealed that adding the \textit{Ac}NPV \textit{polh} resulted in distortion and instability to the \textit{polh} gene tree, many other genes were recently employed for phylogenetic analysis of baculovirus\textsuperscript{28}. However, for simple molecular analysis, the \textit{polh} is still useful because a great number of the \textit{polh} gene sequences are available in the GenBank\textsuperscript{29}.

**ACKNOWLEDGEMENTS**

We would like to thank Busara Rawinoo from Department of Agriculture, Ministry of Agriculture and Cooperatives for providing silkworm eggs. This study was financially supported by the Center for Agricultural Biotechnology, Thailand.

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