**p13 gene was first described in Leucania separata multinuclear polyhedrosis virus (Ls-p13) several years ago, but the function of P13 protein has not been experimentally investigated to date. In this article, we indicated that the expression of p13 from Heliothis armigera single nucleocapsid nucleopolyhedrovirus (Hap-p13) was regulated by both early and late promoter. Luciferase assay demonstrated that the activity of Hap-p13 promoter with hr4 enhancer was more than 100 times in heterologous Sf9 cells than that in nature host Hz-AM1 cells. Both Ls-P13 and Hap-P13 are transmembrane proteins. Confocal microscopic analysis showed that both mainly located in the cytoplasm membrane at 48 h. Results of RNA interference indicated that Hap-p13 was a killing-associated gene for host insects H. armigera. The AcMNPV acquired the mentioned killing activity and markedly accelerate the killing rate when expressing Ls-p13. In conclusion, p13 is a killing associated gene in both homologous and heterologous nucleopolyhedrovirus. [BMB Reports 2012; 45(12): 730-735]**

**INTRODUCTION**

Baculoviruses are wildly used for expression of recombinant proteins and are used for biological pesticides. The family *Baculoviridae* is a large and diverse family of occluded viruses with double-stranded DNA genomes. *Baculoviridae* is divided into two genera based on occlusion body morphology. The nucleopolyhedroviruses (NPVs) produce large occlusion bodies containing multi- or single rod-shaped enveloped virions, named MNPV or SNPV in the nuclei of infected cells (1). The other genera, the granuloviruses (GVs), produce small granular occlusion bodies that normally contain a single virion and are only found in Lepidoptera (2). NPVs are further subdivided into two distinct groups (Group I and Group II) based on molecular phylogenies and whether contain a gp64 homologue or not. NPVs in group I show close relation compared to those of Group II (3).

So far, most researches focus on the AcMNPV because of easier manipulation, growth in cell culture and common sets of genes responsible for similar life cycles in all baculoviruses (4). With the availability of the genomes of baculoviruses, more and more novel genes absent in AcMNPV have been reported, such as p13 (5), ld130 family (6) and p49 (7) genes. Understanding the functions of these emerging genes will greatly promote the development of baculoviruses molecular biology and bioprocesses.

**p13 gene was firstly discovered in Leucania separata multinuclear polyhedrosis virus (LsMNPV) from our laboratory in 1995 (5). The Ls-p13 ORF is 861 bp and encodes a 31 kDa leucine zipper-like protein (GenBank: AY394490). About nineteen baculoviruses containing p13 homologues genes have been reported (8-17). p13 is predicted as an early and late gene and encodes a glycosyltransferase of family 8 (10). However, the exact function of P13 protein is unclear.

The work reported focuses on the function of p13, including its expression profile, promoter activity, cellular localization and killing activity. We choose Hap-p13 from Heliothis armigera single nucleocapsid nucleopolyhedrovirus (HaSNPV) and Ls-p13 from LsMNPV to study the killing activity in homologous and heterologous system, respectively.

**RESULTS**

Phylogenetic analysis of P13 proteins

The complete amino acids sequences of p13 genes from eleven baculoviruses were alignment by Pair Distances of ClustalW (Slow/Accurate). All the P13 proteins share the homology of amino acids from 41.4% to 56.9% (18). They are clearly divided into two groups in phylogeny, that is p13 genes of GV and p13 genes of Group II NPVs. p13 genes of GV are closely related while those of Group II NPVs appear to be more divergent (supplementary material).

The Hap-p13 gene was an early and late transcription gene

The p13 promoter contains the core sequences for early
p13 from group II baculoviruses is a killing-associated gene

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Fig. 1. Structure and promoter activity of p13 gene. (A) p13 gene 5’UTR and putative encoded protein structures. (B) Luciferase activity assay of Ha-p13 promoter and hr4 enhancer/Ha-p13 promoter. Hz-AM1 Cells (open triangle) and Sf9 cells (filled triangle) were transfected with plasmid pGL3-Ha-p13 promoter. In another experiment, Hz-AM1 Cells (filled square) and Sf9 cells (open square) were transfected with plasmid pGL3-hr4/Ha-p13 promoter. (C) Western blot analysis. Hz-AM1 cells were infected with HaSNPV-G and lysates were prepared at 8, 18, 28, 38, 48, 60, 72, 90 h p.i. Ha-P13 expression was determined by Western blot using Ha-P13 polyclonal antiserum (upper panel). Actin was used as a control (lower panel). All the results were collected from triplicate experiments.

Fig. 2. Intracellular location of P13 in host and heterologous cells. (A) Ha-P13 location in host Hz-AM1 cells 48h after infection with HaSNPV-G. (B) Ls-P13 location in heterologous Sf9 cells 48 h after infection with rAc-hr5/IE1-Lsp13-G. Green fluorescence indicates the presence of either HaSNPV-G (A-1) or rAc-hr5/IE1-Lsp13-G (B-1) infection. The red fluorescence is the result of Red-labeled goat anti-rabbit antiserum after treatment with polyclonal antibodies against Ha-P13 (A-2) or Ls-P13 (B-2), which represents Ha-P13 or Ls-P13 exclusive localization.

(CAT/AT) and late expression (TTAAG) elements (Fig. 1A) and an hr enhancer is widely located in upstream of p13 promoter (19). To confirm the prediction with experiment, Ha-p13 promoter with or without the hr4 enhancer were inserted into pGL3-Basic reporter vectors, respectively. Luciferase assay indicate that Ha-p13 promoter has activity from early 2 h p.i. to very late 90 h.p.i (Fig. 1B). Activity of Ha-p13 promoter was low both in host Hz-AM1 cells (Fig. 1B, open triangle) and in heterologous Sf9 cells (Fig. 1B, filled triangle). However, when the hr4 enhancer was present upstream, the activity of Ha-p13 promoter increased more than twenty times in host Hz-AM1 cells (Fig. 1B, open square) and more than two thousand times in heterologous Sf9 cells (Fig. 1B, open square). The dramatic variety in the two types of cells might be the effect of some viral or cellular transcript factors act on hr4 enhancer.

On the other hand, the Hz-AM1 cells were harvested at different time after HaSNPV-G infection at 0.1 MOI. The cell extracts were subjected to SDS-PAGE followed by Western blot analysis with anti-HaP13 serum (Fig. 1C). The expression of Ha-P13 was observed from 8 h.p.i to 90 h.p.i, and reached its maximum at 28 h.p.i, then decreased gradually until 90 h.p.i. The results suggest that Ha-P13 expression was an early and late transcription gene and expression consistently during virus infection.

Both Ha-P13 and Ls-P13 proteins are mainly located in cytoplasm membrane at very late stage

To identify the subcellular localization of P13 proteins, the Hz-AM1 cells infected with HaSNPV-G and the Sf9 cells infected with rAc-hr5/IE1-Lsp13-G were fixed at 48 h.p.i., respectively. The cells were then stained with anti-HaP13 or anti-Lsp13 primary antibody and Texas Red-labeled secondary antibodies. Since the green fluorescence could observed in the whole cell infected with HaSNPV-G (Fig. 2A-1) or rAc-hr5/
**p13** from group II baculoviruses is a killing-associated gene

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IE1-Lsp13-G (Fig. 2B-1), it was used as a marker of two kinds of recombinant baculoviruses infection. Laser confocal microscopic analysis, at the settings for eGFP or Texas Red, revealed the expression of Ha-P13 (Fig. 2A-2) or Ls-P13 (Fig. 2B-2) in the virus infected cells (Fig. 2A-1 and B-1), and the P13 localized exclusively to the cytoplasm membrane both in host Hz-AM1 cells (Fig. 2A-3) or heterologous systems sf9 cells (Fig. 2B-3).

**p13** is a killing-associated gene in nature host system

In order to investigate the interference efficacy of dsRNA to Ha-p13, dsRNA-Hap13 was transfected Hz-AM1 cells prior to infection with HaSNPV-G. A dose of 5 μg dsRNA-Hap13 was transfected prior to HaSNPV-G infection could down-regulated most of Ha-P13 expression (Fig. 3A, lane 2).

To investigate the function of Ha-P13 in HaSNPV-G infection, five groups of insect bioassays were performed (N = 50). Co-injected with dsRNA-Hap13 and HaSNPV-G, the larvae mortality decreased with the increase of dsRNA-Hap13 concentration (Fig. 3B). When 5 μg of dsRNA-Hap13 were co-injected, the larvae mortality was decreased from 70% (Fig. 3B, only HaSNPV-G injection) to 10% (Fig. 3B, HaSNPV-G + 5 μg dsRNA-Hap13 co-injection). Few of H. armigera were dead when 5 μg of dsRNA-Hap13 was injected solely (Fig. 3B, 5 μg dsRNA-Hap13 injection) suggesting that dsRNA-Hap13 itself was not toxic to the host.

AcMNPV p35 (Ac-p35) gene is an apoptosis suppressor gene, which is neither present in HaSNPV-G nor in H. armigera genome. When 5 μg dsRNA-Acp35 was co-injected with HaSNPV-G, the mortality was nearly no much reduced compared with HaSNPV-G sole injection (Fig. 3B, HaSNPV + 5 μg dsRNA-Acp35 co-injection vs HaSNPV injection), which elimi-
nated the possibility that dsRNA triggered innate immunity and served to reduce mortality in insects. From above results, we concluded that \( p13 \) gene is a killing-associated gene in its natural host.

\( p13 \) is also a killing-associated gene in heterologous system

Two experimental groups were designed to investigate the effect of Ls-P13 on rAcMNPVs infection in larvae of \( S. \ exigua \). The infection of two recombinant baculoviruses, rAc-G and rAc-hr5/IE1-Lsp13-G (Fig. 4A) used in bioassay were confirmed by fluorescence microscopic (Fig. 4B) and Western blot analysis (Fig. 4C). In group I (Fig. 4D, group I), the mortality of both rAc-hr5/IE1-Lsp13-G and rAc-G reached 100% after five days, but the mortality of rAc-hr5/IE1-Lsp13-G was significantly higher in the early phase.

In group II (Fig. 4D, group II), nearly all of the larvae of \( S. \ exigua \) survived when injected with grace’s medium or Ls-P13 protein. The result demonstrated that Ls-P13 protein itself was not toxic to \( S. \ exigua \). Compared with rAc-G infection, the co-injection of the protein Ls-P13 with rAc-G markedly accelerated the killing process. From above results, we concluded that Ls-P13 could accelerate the killing process of rAcMNPVs no matter it was expressed in vivo or co-injected with rAc-G.

DISCUSSION

The \( p13 \) gene of LsMNPV was first reported in 1995 (5). However, the \( p13 \) function was has been neglected for many years. Our attention was focused on gene \( p13 \) again based on three main reasons. The first is that nearly twenty \( p13 \) genes have been identified in recent years and share no homology with any other gene. The second is that all the reported \( p13 \) genes are present specifically in Group II NPVs and some GV, but not Group I NPVs. Therefore, \( p13 \) can be proposed as a second marker, besides gp64 gene, for differentiating between the two NPV groups in molecular phylogeny (20). The most important reason, however, is that \( p13 \) contains a complex promoter (21), unique motifs, and also encodes a putative glycosyltransferase of family 8 which reported so far in mammals, yeasts and bacteria but not in viruses (22).

\( p13 \) gene contains a GTGTTATA box, a CAT/AT box for the early promoter and a TTAAG box for the late promoter in its 5'-UTR, a mini cistron following the core sequence of the early promoter, and an enhancer hr (n) (n is different among Group II NPVs) at its 5'-UTR, a mini cistron following the core sequence of the early promoter and a TTAAG box for the late promoter in its enhancer/Ha-p13 region. Interestingly, we also found that the hr4 enhancer/Ha-p13 promoter not only has no host specificity, but also increases nearly 100 times activity in heterogeneous Sf9 organisms results in post-transcriptional depletion of the homologue endogenous mRNA (23). Our results showed that dsRNA-Hap13 could specifically knockdown Ha-p13 expression in nature host Hz-AM1 cells. Co-injection dsRNA-Hap13 with HaSNPV-G could decrease the toxicity of HaSNPV-G and the efficacy was dsRNA-Hap13 dose dependent.

On the other hand, as \( p13 \) gene was absent in group I NPVs such as baculovirus AcMNPV, we inserted Ls-p13 gene into AcMNPV to evaluate whether foreign \( p13 \) gene in heterologous system was also killing-associated. Our results clearly indicated that Ls-P13 could accelerate the killing rate of AcMNPV. Although the mechanism of \( p13 \) as a killing-associated gene is unknown, its killing property is attractive and promotes us to further study \( p13 \) functions and its potential applications in biopesticides.

MATERIALS AND METHODS

Cells, insects and viruses

\( Heliothis \) zeas (Hz-AM1) cells and \( Spodoptera \) frugiperda pupal ovarian cell line (Sf9) cells were cultured at 27°C in Grace’s medium (Gibco/BRL), supplemented with 10% fetal bovine serum (Gibco/BRL). Larvae of \( S. \ exigua \) and \( H. \ armigera \) were feed with artificial diet and maintained as previously described (24). Baculoviruses HaSNPV with eGFP droved by polyhedron promoter (HaSNPV-G) and wt-LsMNPV were kindly donated by Dr. Zhihong Hu and Dr. Shengliang Chen (Wuhan Institute of Virology, Chinese Academy of Sciences).

Phylogenetic analysis

The GenBank accession numbers used for alignments are as follows: for \( p13 \) genes, NC 004690 (AhSNPV), AF303045 (HaSNPV), NC 004690 (AhNPV), AF547984 (AoGV), NC 007151 (CcNPV), NC 005068 (CIGV), US3466 (CpGV), U12071 (McNPV), AF499596 (PoGV), AF169823 (SeNPV), AF325155 (SinPV). All the reported \( p13 \) sequences translations were aligned by using the ClustalW algorithm. Genetic distances were estimated by the Kimura two-parameter matrix and phylogenetic tree was generated by MegaAlign method of DNASTAR software.

Plasmids

Plasmid pMal-p2 was obtained from New England Biolabs, United Kingdom. Plasmid pFastBac1 was obtained from Invitrogen. Plasmid plEHR3 containing hr5 enhancer and IE1 promoter fusion sequence was kindly donated by Jarvis et al. (20). Luciferase reporter plasmid pGL3-hr4/Hap13promoter was constructed by inserting the PCR-amplified 5' non-coding region of Ha-p13 gene (including hr4 enhancer and Ha-p13 promoter fusion sequence) from HaSNPV into the BglII and HindIII sites of plasmid sites of the pGL3-Basic vector (Promega). For unique hap13 promoter activity assay, −1 to −175 promoter regions were amplified from pGL3-hr4/Hap13 was inserted into SacI and Smal sites of pGL3-Basic vector to generate the reporter plasmid pGL3-Phap13.
Recombinant baculoviruses construction
AcBac-to-Bac-eGFP expression system (25) was used to con-
struct recombinant AcMNPVs to investigate Ls-P13 function in
heterologous SF9 cells. Briefly, the foreign gene was inserted
into pFastBac1 donor plasmids and transformed into E. coli
dH110Bac containing AcBacmid-eGFP with helper plasmid.
After white/blue selection on kanamycin/gentamicin/tetracy-
cline plates and PCR screening, the resulting Bacmids were
transfected into SF9 cells to yield the rAcMNPVs.

Protein expression, purification and antibody production
Ls-P13, and Hap13 were cloned into the bacterial expression
plasmid pMal-p2 for expression. The foreign genes were fused
with the MalE (maltose binding protein [MBP]) gene of E.coli at
the N-terminus, and the expressed fusion proteins MBP/Ls-P13
and MBP/Ha-P13 were purified by affinity chromatography
taking advantage of the affinity of MBP for maltose. Finally,
the purified fusion proteins were used to inoculate animals for pol-
ycloval antibody preparation.

Luciferase activity and Western blot
Hz-AM1 cells were seeded into 24-well plates at about 60% con-
fluence and maintained at 27°C. Transfection was performed
with 0.8 μg expression plasmid and reporter plasmid us-
ning Cellfectin reagent according to the manufacturer’s proto-
col (Invitrogen). The cells were harvested at different stages af-
fter transfection, and the luciferase activity was measured with
Luciferase Reporter Assay System (Promega) along with the
manufacturer’s protocol. On the other hand, the non-trans-
fected Hz-AM1 cells in the other wells were infected with
HaSNPV-G at 0.1 MOI. The infected cells were lysed at differ-
ent stages and Ha-P13 expression profile was evaluated by
Western blot. Data were collected from the average of triplic-
ate assays of transfections.

Immunofluorescence confocal microscopy
Following infection with HaSNPV-G or rAc-hr5/IE1-Lsp13-G in
48 h, the Hz-AM1 cells or SF9 cells were fixed in 4% paraf-
ormaldehyde in PBS for 20 min at room temperature, per-
meabilized with 0.5% Triton X-100 in PBS for 10 min, fol-
lowed by 30 min incubation in the presence of 1% BSA. The
cells were then incubated with rabbit polyclonal antibodies
against P13 (1 : 100). Following three PBS washes, the cells
were incubated in PBS containing Texas Red-labeled goat an-
ti-rabbit antibodies (1 : 50 dilution in PBS, ZhongShan Biotech
Co., China) for 1 h. After several washes, the cells were ob-
served under a Leica TCS SP1 laser confocal microscopy.

RNAi procedure
Double-stranded RNA (dsRNA) was synthesized in vitro using
the ScriptMAX™ Thermo T7 Transcription Kit (Toyobo) follow-
ing the manufacturer’s instructions. Briefly, the 740 nucleo-
tides fragment (from 21 to 761) of Ha-p13 ORF (831 nt) with
an ATG codon, was amplified by PCR from the constructed ex-
pression vector pMal-p2-Hap13. The primers were flanked for-
ward and reverse T7 promoter sites. The PCR product was
used for the in vitro transcription to obtain the dsRNA, using
the T7 polymerase according to the provided protocol.

5 μg of dsRNA-Hap13 was transfected prior to infection with
HaSNPV-G in Hz-AM1 cells. The interference efficacy of
dsRNA-Hap13 was detected by Western blot. On the other
hand, different amounts of dsRNA-Hap13 were co-injected in
the hemolymph of third instar H. armigera larvae with
HaSNPV-G to study the effect of P13 on viral infection and
dsRNA-Hap13 unique injection as control. To identify whether
dsRNA triggered innate immune response and to reduce mor-
ality in insects, other dsRNA targeting p35 gene of AcCNPV
was used as control. HaSNPV-G (1 × 10^6) was injected with
10 μl in all groups. Dead insects were investigated for GFP flu-
orescence and all results were triplicate.

Bioassay
Two experiment groups of were performed to study Ls-P13 ef-
fact on AcMNPV infection. In group 1, equivalent titer (1 ×
10^6) and volume (10 μl) of rAc-G and rAc-hr5/IE1-Hsp13-G
were directly injected into the hemolymph of third instar lar-
vae of S. exigua and Grace’s medium as a control. In group 2,
the purified MBP/Ls-P13 protein was used to co-inject third in-
star larvae with rAc-G and rAc-G injection was used as
control. Dead insects were counted from the third day and the
experiments were triplicate.

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