

## Phosphorylation of nucleopolyhedrovirus 39K is essential for the regulation of viral gene transcription in silkworm cells

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Received July 12, 2018; revised April 23, 2019; accepted October 21, 2019

**Summary.** – 39K protein was proven to be the most notable hyperphosphorylated protein of *Bombyx mori* nucleopolyhedrovirus (BmNPV), since its amino acid 136 has the highest phosphorylation ratio (16.683) among all of BmNPV phosphorylated proteins (Shobahah *et al.*, 2017). Therefore, we inserted positive (mimicking phosphorylation) and negative (mimicking dephosphorylation) mutants of the highest phosphorylated site of 39K and the wild type 39k into the 39k-ko-Bacmid using the Bac-to-Bac system. These three kinds of recombinant Bacmids along with wild type and knocked-out Bacmid were then transfected into the *Bombyx mori* cells (BmN) and further investigated by qPCR analysis. The results of the qPCR showed that 39K phosphorylation had no significant effect on viral genome replication, unlike the positive mutation that reduced the viral gene transcription drastically compared to the wild type. Thus, phosphorylation of BmNPV 39K protein does not appear to be the essential mechanism for viral genome replication, even though it has an important role in the viral gene transcription.

**Keywords:** BmNPV; 39K; phosphorylation; virus replication; virus transcription

### Introduction

*Bombyx mori* nucleopolyhedrovirus (BmNPV) has a large, circular, double-stranded DNA genome (Zanotto *et al.*, 1993) and is especially pathogenic to *Bombyx mori*. Among the BmNPV open reading frames (ORFs), BmNPV 39k ORF has 834 bp and encodes 277 amino acids. The homolog of 39k in *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV), *pp31* is a delayed-early gene, which is expressed in every phase of virus infection (Guarino and Summers, 1986). Also, PP31 protein is a DNA-binding

protein localized in the nucleus of the infected cell, and related to the formation of virogenic stroma (VS) (Guarino *et al.*, 1992). Besides, Yamagishi *et al.* (2007) have revealed that the deletion of the *pp31* gene led to a significant down-regulation of early and late genes of AcMNPV.

Many studies have shown that protein phosphorylation is essential for regulation of intracellular biological processes. Our previous study showed that 39K protein is the most notable hyperphosphorylated protein of BmNPV, as it has 4 phosphorylated sites with a ratio of: 3.967, 2.287, 1.407 and 16.683-fold, with the latter one having the highest phosphorylation ratio among all of BmNPV phosphorylated proteins. This ratio is even higher than that of host phosphorylated proteins in *Bombyx mori* cells infected by BmNPV at 24 h post infection. (Shobahah *et al.*, 2017). The study of BmNPV 39K phosphorylation is relevant, since 39K promoters have important function in insect cell engineering such as baculovirus expression vector system (BEVS) (Lin and Jarvis, 2013). The function of PP31 and 39K phosphorylation remains

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**Abbreviations:** AcMNPV = *Autographa californica* multinucleocapsid nucleopolyhedrovirus; BmN = *Bombyx mori* cells; BmNPV = *Bombyx mori* nucleopolyhedrovirus

Table 1. The primer sequences for PCR amplification

Name	Primer sequences (5'→3')
39K <i>Bam</i> HI F	Forward: 5'-GCGGATCCTAATTCGGACTGCTTGA-3'
39K <i>Kpn</i> I R	Reverse: 5'-CGGGTACCTTACATCATCATCATCATCATTTCTGACATATTTGTATCTTG-3'
39K 136 Mt. D F	Forward: 5'-GACATCGTCGGATTCAAGCGACAAC-3'
39K 136 Mt. D R	Reverse: 5'-GTTGTCGCTTGAATCCGACGATGTC-3'
39K 136 Mt. A F	Forward: 5'-GACATCGTCGGCTTCAAGCGAC-3'
39K 136 Mt. A R	Reverse: 5'-GTCGCTTGAAGCCGACGATGTC-3'
39K-CF <sup>a</sup>	Forward: 5'-AATGCCTGTCATGCAGGAGCAACGCAAATGTCCAAACGCAAGAAGAAGGTAAGTGTAGGCTGGAGCTGCTTC-3'
39K-CR <sup>a</sup>	Reverse: 5'-ATGAACACAAAATCTGCCAATTCGGGCGTGTTTTTCACCAAATCCCACATATGGGAATTAGCCATGGTCC-3'
M13 F	Forward: 5'-CCCAGTCACGACGTTGTAAAACG-3'
M13 R	Reverse: 5'-AGCGGATAACAATTTACACAGG-3'
<i>lef-3</i> F	Forward: 5'-TCGGATGACCGTTCTACCTCTT-3'
<i>lef-3</i> R	Reverse: 5'-CTTCCAGCAGCATTGAGATTTG-3'
<i>vp39</i> F	Forward: 5'-AGACACCACAAACCCGAACAC-3'
<i>vp39</i> R	Reverse: 5'-TTGATCGCCAACACCACCT-3'
<i>p10</i> F	Forward: 5'-GACACGAATTTTAGACGCCATT-3'
<i>p10</i> R	Reverse: 5'-CGATTCTTCCAGCCCGTTT-3'
<i>39k</i> F	Forward: 5'-TTCGGACTGCTTGACC-3'
<i>39k</i> R	Reverse: 5'-TTATTCTGACATATTTGTATCTTG-3'
<i>β-actin</i> F	Forward: 5'-GCGCGGCTACTCGTTCCTACTACC-3'
<i>β-actin</i> R	Reverse: 5'-TGCCGCAAGCTTCCATACCC-3'
<i>gp41</i> F	Forward: 5'-CGTAGTGGTAGTAATCGCCGC-3'
<i>gp41</i> R	Reverse: 5'-AGTCGAGTCGCGTCGCTTT-3'

<sup>a</sup>These two primers were composed of 39k homology arms and a 20-bp *cat* homologous zone (straight lines). Underlined are restriction enzyme digestion sites of *Bam*HI and *Kpn*I. The bold sequences are the point mutations (39K 136<sup>th</sup> amino acid).

elusive and strongly suggests that phosphorylation might have an important role in promoting protein production.

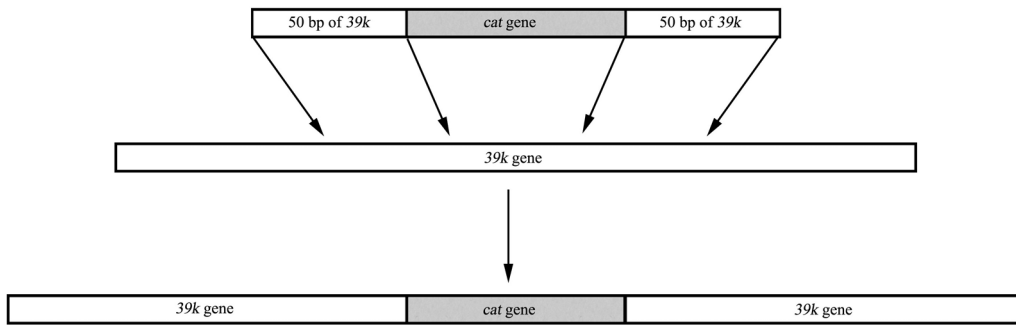
Therefore, wt-Bacmid, 39k-ko-Bacmid, wt-39k-re-Bacmid, 39k 136 positive mutant re-Bacmid and 39k 136 negative mutant re-Bacmid were constructed, transfected into BmN cells and the viral genome replication and transcription levels were determined. This study lays the groundwork for further investigation of the functions of BmNPV 39K phosphorylation in viral genome replication and transcription.

## Materials and Methods

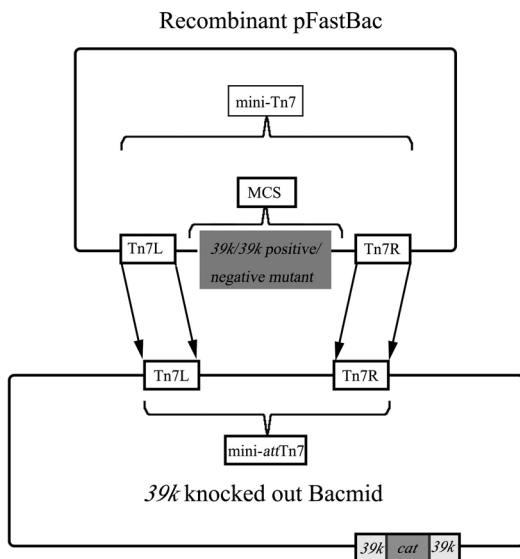
*Construction of pFastBac-39k, positive mutation (pFastBac-39k-S136D) and negative mutation (pFastBac-39k-S136A).* The production of 39k gene was done by PCR using 39K *Bam*HI F and 39K *Kpn*I R primers (Table 1), while fusion PCR was used to produce 39k mutant gene. The first PCR was done using 39K *Bam*HI F and 39K 136 Mt. R primers for the first fragment and 39K 136 Mt. F and 39K *Kpn*I R primers for the second fragment. The second PCR was done using the two fragments as the primers, the rest of the steps were performed as described before in Zhao *et al.* (2016).

*Construction of 39k-knockout Bacmid (39k-ko-Bacmid) and repaired Bacmid.* In order to produce a 39k gene-deficient Bacmid, the 39k gene targeting linear fragment was constructed by PCR using 39K-CF and 39K-CR primers (Table 1) and pKD3 plasmid (containing *cat*) as the template. The 39k-ko-Bacmid was constructed as described before (Zhao *et al.*, 2016). DH10Bac competent cells containing 39k-ko-Bacmid were transformed with the respective pFastBac plasmids carrying modified 39k to obtain the recombinant Bacmids.

*Cell culture, viral transfections, and qPCR analysis of viral DNA replication.* BmN cells, derived from the ovary of silkworm, were cultured at 27°C in Sf-900 medium (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum. Cells in the logarithmic growth phase were transfected with 1 µg DNA of the respective Bacmids (wt-Bacmid, 39k-ko-Bacmid, wt-39k-re-Bacmid, 39k 136 positive mutant re-Bacmid and 39k 136 negative mutant re-Bacmid) using SuperFectin™ II transfection reagent (Shanghai Pufei Biotech, China). The cells were then harvested at 12, 24, 36, 48 and 72 hours post transfection (hpt). The extraction, digestion, purification of viral DNA and the quantitative real-time PCR (qPCR) were done as described before (Li *et al.*, 2016). Primers used for the reaction (*gp41* F and *gp41* R) are shown in Table 1.

(a) 1. PCR amplify 39k-flanked resistance gene (*cat*)2. Transform strain expressing  $\lambda$  red recombinase

## (b)



## (c)

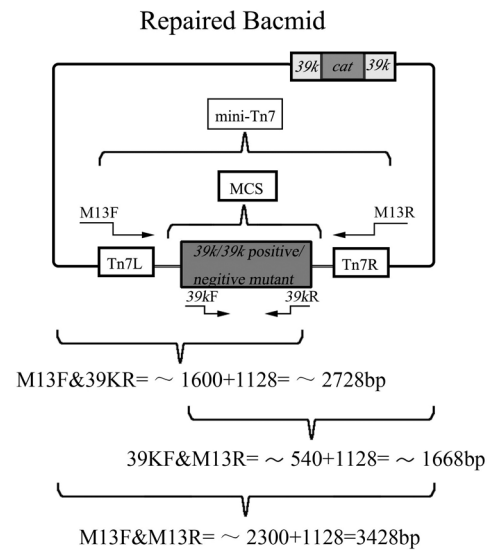


Fig. 1

Construction of 39k knocked out (a) and repaired (b) BmNPV Bacmids and PCR identification (c)

The qPCR analysis of the viral gene transcription at different growth phases. The Bacmids transfection, the extraction of RNA, production of cDNA and qPCR were also done as described before, and primers for qPCR (*lef-3* F&*lef-3* R, *vp39* F&*vp39* R, *p10* F&*p10* R, 39k F&39k R and  $\beta$ -actin F& $\beta$ -actin R) are shown in Table 1 (Li *et al.*, 2016).

## Results

## Confirmation of 39k-knockout and repaired Bacmids

The knocked out 39k construction was prepared using the strategy described in Fig. 1a. The successful construction of 39k-ko-Bacmid was confirmed by PCR, the result of which showed a band size as predicted (1981 bp). Figure

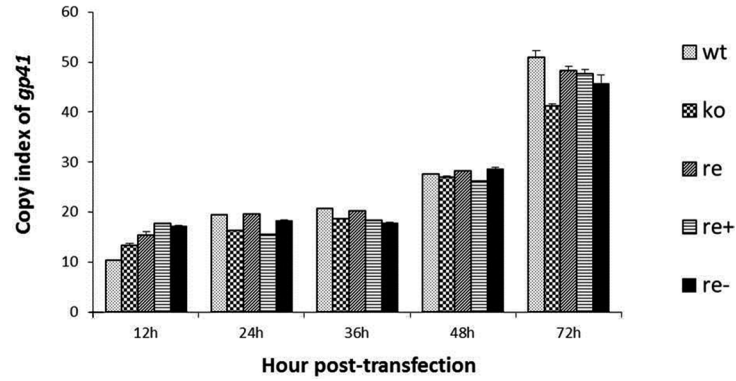


Fig. 2

The qPCR analysis of viral DNA replication

BmN cells transfected with wild type Bacmid (wt), 39k knocked out Bacmid (ko), wild type 39k repaired Bacmid (re), 39k positive mutant repaired Bacmid (re+) and 39k negative mutant repaired Bacmid (re-) were harvested after 12–72 h, respectively. Shown are the values representing the expression of the viral gene *gp41*. Values are expressed as means ± SEM.

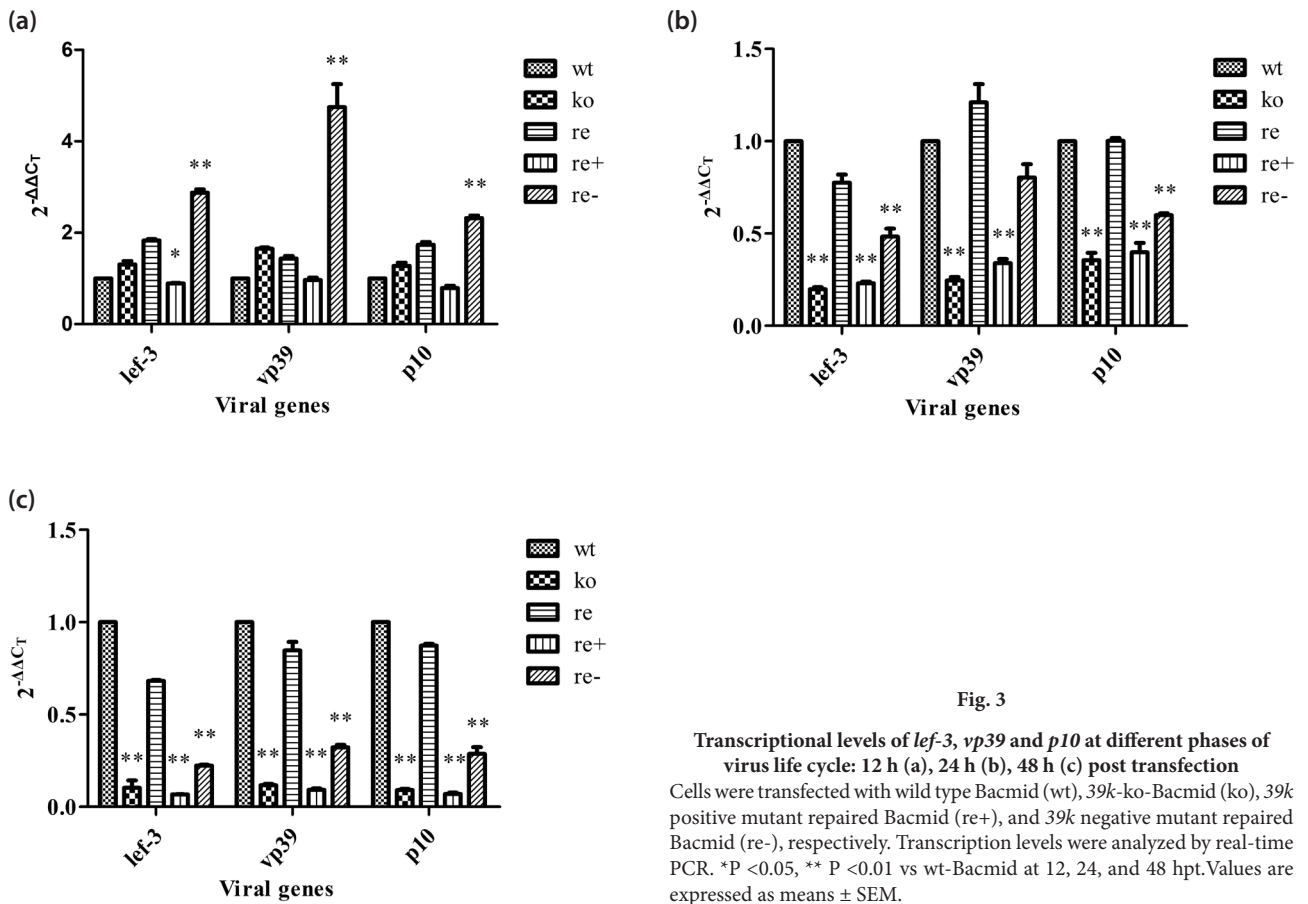


Fig. 3

Transcriptional levels of *lef-3*, *vp39* and *p10* at different phases of virus life cycle: 12 h (a), 24 h (b), 48 h (c) post transfection

Cells were transfected with wild type Bacmid (wt), 39k-knockout Bacmid (ko), 39k positive mutant repaired Bacmid (re+), and 39k negative mutant repaired Bacmid (re-), respectively. Transcription levels were analyzed by real-time PCR. \*P < 0.05, \*\* P < 0.01 vs wt-Bacmid at 12, 24, and 48 hpt. Values are expressed as means ± SEM.

1b describes the strategies of the Bac-to-Bac system, which was used for construction the other recombinant (here: “repaired”) Bacmids. In addition, the predicted lengths of all the amplified products identified by PCR are described in Fig. 1c.

#### *Analysis of viral DNA replication*

In order to confirm that the deletion of the *39k* gene has no impact on viral growth, we performed transfection with the respective Bacmids (wt-Bacmid, *39k*-ko-Bacmid, wt-*39k*-re-Bacmid, *39k* 136 positive mutant re-Bacmid and *39k* 136 negative mutant re-Bacmid), harvested the cells for qPCR and analyzed the transcription of the viral gene *gp41*.

Figure 2 shows that there is no significant difference in the viral replication of the constructed recombinant Bacmids, indicating that neither the deletion of *39k* nor the phosphorylation of 39K had effect on the viral replication.

#### *Analysis of the viral gene transcription at different phases of virus life cycle*

Generally, the transcription of viral genes at each phase of the viral life cycle, i.e. early gene (*lef-3*), late gene (*vp39*), and very late gene (*p10*) had similar tendencies in recombinant Bacmids. The most important observation was that at the 12 hpt (Fig. 3a), the *vp39* and *p10* transcriptional levels in the *39k* positive mutant repaired Bacmid-transfected BmN cells were decreased, but showed a little difference from the level in the wt-Bacmid-transfected cells, this trend was continued in *lef-3* expression with a significant difference ( $P < 0.05$ ), while the negative mutation elevated the transcription of *lef-3*. Nevertheless, at 24 and 48 hpt (Fig. 3b and 3c), the expression of the early (*lef-3*), the late (*vp39*) and very late gene (*p10*) was similarly reduced with a very significant difference ( $P < 0.01$ ), while the negative mutation had only a little reduction impact on the viral transcriptional level.

### Discussion

*39k* is a conserved gene in baculoviruses. Among the BmNPV promoters, 39K promoter has the highest BmNPV-induced transcriptional activity and two regions of 39K promoter have a pivotal role for optimal virus-inducible activity (Cao *et al.*, 2016). Gomi *et al.* (1997) have reported that the deletion of this gene could lead to the remarkable reduction of late gene transcription level. Consistent with their observations, our previous study showed that the deletion of this gene could lead to the extreme significant decrease of viral gene transcription level at different phases of virus life cycle. (Li *et al.*, 2016).

During infection, 39K is a phosphorylated protein with remarkably high phosphorylation ratio (16.683) among all

the BmNPV phosphorylated proteins. Interestingly, PP31 has also been reported to be phosphorylated (Broussard *et al.*, 1996). Accordingly, we presumed that the phosphorylation on BmNPV 39K has a pivotal role for viral activity.

In order to investigate the function of phosphorylation in 39K, we constructed the positive and negative mutants in the highest phosphorylated site of 39K (136<sup>th</sup> amino acid). The creation of mutants were done by fusion PCR with the substitution of serine by aspartic acid and serine by alanine for the positive and negative mutant, respectively. Next we inserted these two mutants and the wild type *39k* into *39k*-ko-Bacmid. These 3 new of repaired Bacmid along with the wild type and knocked out Bacmids were transfected into BmN cells.

Initially, the qPCR was done to unveil the effect of phosphorylation on 39K on the viral replication and transcription. The results showed that the 39K phosphorylation does not have a significant effect on the viral replication. In contrast, the expression of each type of viral gene (*lef-3*, *vp39* and *p10*) had similar response to the 39K phosphorylation site mutation. These gene transcriptions were likely to be lowered in the cells that were transfected with 39K positive mutant re-Bacmid and vice versa, i.e. in the cells transfected with 39K negative mutant re-Bacmid the expression of the respective genes rised.

In conclusion, the phosphorylation of 39K does not affect the viral genome replication, but reduces the viral transcription, suggesting that phosphorylation on 39K tends to alleviate the viral transcription. It remains elusive whether the phosphorylation of 39K is catalyzed by a viral or cellular enzyme. However, our results suggest that 39K phosphorylation, which diminishes the viral transcription, is a mechanism required by the virus to prolong the life of cells so as to harness the cellular material for the production of their viral progeny. Moreover, it is also possible that BmNPV 39K phosphorylation is an important pathway regulated by a cellular enzyme and used for the protection against the onset of virus infection. Further investigation is necessary to verify this hypothesis.

**Acknowledgments.** This work was supported by the Natural Science Foundation of Zhejiang Province (No. LY17C170006), 521 Talent Project of Zhejiang Sci-Tech University and National High-tech R&D program (863 Program) (No. 2011AA100603).

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