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vlf-1 Deletion Brought Ac*M*NPV to Defect in Nucleocapsid Formation

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Abstract. Recent studies have provided direct evidence that the baculovirus very late factor 1 (VLF-I) of *Autographa californica* multicapsid nucleopolyhedrovirus (Ac*M*NPV) was essential for BV production. To elucidate how *vlf-1* deletion blocks BV production we generated a *vlf-1* knockout bacmid by ET-recombination technology on Ac*M*NPV bacmid propagated in *Escherichia coli*. Bacmid DNA transfection and supernatant passage assay revealed that the *vlf-1* knockout bacmid was unable to replicate in cell culture, while *vlf-1* repair bacmid, which was generated by transposition of the *vlf-1* ORF under control of its native promoter into *polyhedrin* gene locus of *vlf-1* knockout bacmid, resumed viral replication ability at wildtype levels. Results of these assays proved the correct construction of the *vlf-1* knockout bacmid. Subsequent electron microscopy revealed that the *vlf-1* knockout bacmid failed to form nueleocapsid in the nuclei of the transfected cells. Instead, intensely electron-dense virogenic stroma characteristic of viral DNA synthesis were observed. Thus, it is demonstrated for the first time that *vlf-1* knockout blocked nucleocapsid formation and the defective nucleocapsid formation resulted in the abolishment of BV and ODV production. Possible roles of *vlf-1* in genome processing are suggested and discussed.

Key words: budded virus, genome processing, nucleocapsid assembly, *vlf-1*

Introduction

Autographa californica multiple nucleopolyhedrovirus (Ac*M*NPV) is the prototype member of the *Baculoviridae* family, a large family of double-stranded DNA viruses with circular covalently closed genomes ranging in size from 80–80 Kb [1,2]. Baculovirus infection cycle is characteristic of early, late and very late phases for gene transcription and protein synthesis. The onset of viral DNA replication and the development of the virogenic stroma coincide with the transcription of late genes [3]. Newly synthesized viral DNA is condensed and packaged into capsid within the virogenic stroma to form nueleocapsid [4].

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vlf-1 is a member of the 29 baculovirus core set genes [1] existing in all the 26 sequenced genomes. VLF-1 was identified as a trans-activator in a temperature – sensitive mutant to regulate the expression of the two very late genes *p10* and *polyhedrin* [5–7]. Additionally by sequence alignment, VLF-1 has been considered as a member of the tyrosine recombinase family which serves an array of biological roles including integration, resolvation, gene regulation, and the decatenation of newly replicated genomes by catalyzing DNA rearrangement [5,8]. A highly conserved R-H-R catalytic triad and a nucleophilic tyrosine in the region near the C-terminus characterize this family. The nucleophilic tyrosine is responsible for cleaving phosphodiester bonds and initiating the strand exchange with substrate DNA. In Ac*M*NPV *vlf-1*, the histidine residue in the R-H-R catalytic triad is

substituted by asparagine as what happens in the eight other known tyrosine recombinase proteins [9]. Mutations in the two arginines (R210, R303) or the tryrosine (Y335) in VLF-1 resulted in nonviable virus production. However, tyrosine residue mutation had no effect on the capability to enhance the very late gene transcription in transient expression assay [10]. This evidence suggests that *vlf-1* could have different active sites for two distinct functions, enhancement of very late genes transcription and genome processing. A recent *vlf-1* knockout test has revealed that *vlf-1* was essential for viral replication [11].

To further examine the role of this protein in viral replication, we utilized Ac*M*NPV bacmid to generate a *vlf-1* knockout mutant with ET – recombination methodology [12] in *Escherichia Ecoli (E.coli)*. Green fluorescent protein (GFP) and Polyhedrin were introduced into this mutant as markers. Necessary of *vlf-1* in viral replication was determined by bacmid constructs transfection and supernatant passage assays in cell culture. The effect of *vlf-1* knockout on nucleocapsid formation was further analysed by electron microscopy.

Material and Methods

Cells, Virus, Bacterial Strains and Primers

The insect *Spodoptera frugiperda* (Sf9) cells were cultured in Grace's medium supplemented with 10% fetal bovine serum at 27°C. The cell line DH10Bac (Invitrogen Life Technology) was used to isolate the bacmid (bMON14272) containing the Ac*M*NPV genome and the helper plasmid pMon7124 encoding a transposase [13]. The plasmid pBAD-gbaA was purchased from Gene Bridges supplying homologous recombination function of bacteriophage λ Red system in *E.coli*. All the primers used in this work is listed in Table 1.

Construction of vlf-1 Knockout AcMNFV Bacmid

To knockout the *vlf-1* from Ac*M*NPV bacmid, we generated a transfer vector in which *vlf-1* was replaced by *zeocin* for antibiotic selection in *E.coli*. The construction strategy of *vlf-1* knockout bacmid is outlined in Fig. 1. First, A 792 bp *Pst*I-*Hind*III fragment containing Zeocin

Table 1. Primers used in this study

Primer	Sequence[a]
5′ 64958 64991	GCG**CTGCAG**TAATCAAATTTATTAAAAACAAAAGGAGAATCGG
3′ 65588 65612	ATC**GGATCC**TGCATAATCATGGCGACGACTCTGT
5′ 63405 63428	ATC**TCTAGA**TTGTCCGGATAGACTTGTTCGCAC
3′ 64010 64033	CGT**AAGCTT**AGCAGTAACCTATACAATAGCGGC
5′ Zeocin	AAT**CTGCAG**AATCTAAGGGGCGGTGT
3′ Zeocin	CGT**AAGCTT**TGCTCACATGTTGGTCT
Primer A	CTGCTCGGTAGACGATGTTCAC
Primer A′	TTGAGTGTGTTATTACGACTGCG
Primer B	GGGAGGGCGTGAATGTAAGC
Primer B′	TGCGCGGCCTGGACGAC
Primer C	AGAATTGAGCAACGACATGG
Primer C′	CTTGACCGAATTGGCGTTC
5′ie-1	GCG**GGTACC**ACATTTTTGTAGGTTATTGAT
3′ie-1	ATC**GTCGAC**AGTCACTTGGTTGTTCACGAT
5′ GFP	AT**CGTCGAC**GTTCAATGCTTTTCAAGAT
3′ GFP	CGT**AAGCTT**TATAGTTCATCCATGCCATG
5′ Polh	CACATAAACTAGACGCCTTGTC
3′ Polh	G**GAATTC**GGGTTGGGACGACGACGATAAT
5′ 63674 63691	ACG**TCTAGA**GGCCGGGCTGACGATAAT
3′ 65138 65156	*TTA***CTCGAG**CAATAAACGACATGGTGGG

[a]The restriction sites (bold) are underlined.

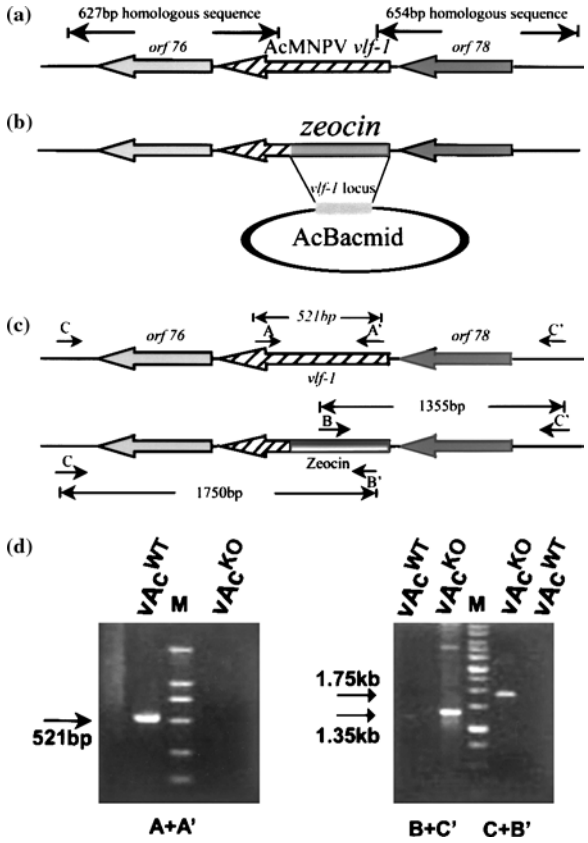

Fig. 1. Strategy for construction of *vlf-1* knockout AC*M*NPV bacmid and confirmation by PCR analysis. (a) Relative location and orientation of ORFs in the *vlf-1* locus of Ac*M*NPV. (b) Construction transfer vector pBSZeocinUD to generate *vlf-1* knockout bacmid by ET – recombination in *E.coli*. A linear fragment containing Zeocin resistant gene under the control of EM7 promoter and flanked by 654 bp upstream and 627 bp downstream sequence to *vlf-1* was excised from pBSzeocinUD, and then cotransformed with bacmid pMON14272 into DH10B cell harbouring pBADgbaA, resulting in the *vlf-1* knockout Ac*M*NPV bacmid (vAc^{KO}) in which Zeocin resistant gene replaced the majority of *vlf-1* ORF. (c) Diagram indicating the relative positions of primers A/A′, B/B′ and C/C′, which were used to confirm the deletion of *vlf-1* and correct insertion of Zeocin resistant gene in *vlf-1* locus. Primers A/A′ was designed to amplify a fragment within the *vlf-1* ORF and used to confirm the absence of *vlf-1* ORF in vAc^{KO}. Primer pairs B/C′ and B′/C were used to examine the recombination junctions. For each primer pair, one primer was specific from the inserted Zeocin gene, and another corresponded to the bacmid sequence, just outside the homologous flanking sequences used for homologous recombination. The lines with arrows indicate the expected size of the PCR products generated from selected primer sets. (d) Ethidium bromide-stained agarose gels showing PCR products, with size of PCR products indicated by side arrows. The used primers are indicated under each panel. M, DNA size marker.

resistance gene under the control of the EM7 promoter was amplified from pPICZαA (Invitrogen Life Science) with primers 5′ Zeo/3′ Zeo (Table 1) and cloned into pBS + which had been digested with *Pst*I and *Hind*III to generate pBSZeocin. Second, a 654 bp fragment homologous to the upstream sequence of *v1f-1* was PCR amplified from Ac*M*NPV genome with primers 5′ 64958–64991/3′ 65588–65612, digested with *Bam*HI and *Pst*I, and then ligated with pBSZeocin which was digested with *Bam*HI and *Pst*I to generate pBSZeocinU. Third a 627 bp *Hind*III–*Xbal* fragment homologous to the downstream sequence of *vlf-1* was also amplified from Ac*M*NPV genome with primers 5′63405–63428/3′64010–64033 and cloned into *Hind*III and *Xbal* digested pUC18 to give pUC-D, at last, the *Hind*III–*Kpn*I fragment from pUC-D, containing downstream sequence fragment D of *vlf-1* was cloned into *Hind*III–*Kpn*I digestd pBSZeocinU to generate

vlf-1 knockout transfer vector pBSzeocinUD. This downstream homologous fragment had been designed to overlap with *vlf-1* ORF cassette 220 bp at 3′ end to keep the potential promoter sequence of unknown ORF76. Therefore the knockout transfer vector contains a 2.2 kb *Bam*HI–*Xbal* fragment, in which *zeocin* replaced the most part of *vlf-1* coding sequence. Finally the 2.2 kb *Bam*HI–*Xbal* fragment was excised from pBSZeocinUD and gel purified, and then suspended in water.

A *vlf-1* knockout Ac*M*NPV bacmid was generated by ET – recombination system according to the manufacturer's instructions. Electro-competent DH10B cell harbouring plasmid pBAD-gbaA which supplied λ Red recombination function was made and electroporated with the 2.2 kb fragment and pMon 14272 DNA. The electroporated cells were incubated at 37°C for 4 h in 1 ml SOC with gentle shaking, then 200 µ1 was spread onto low salt LB agar containing 25 µg/ml Zeocin and 50 µg/ml Kanamycin. Plates were incubated at 37°C for overnight, and colony resistance to Zeocin and Kanamycin were selected and the target colon with *vlf-1* knockout bacmid was confirmed by PCR.

The PCR screening strategy was used to confirm the deletion of *vlf-1* from its locus in Ac*M*NPV and correct insertion of *zeocin*. The relative positions of the primer pairs are showed in Fig. 1c. Primer pairs A/A′ and B/B′ were specific to *vlf-1* and *zeocin* respectively, and primer pairs C/C′ were located far upstream and downstream to the *vIf-1* locus. Prime pairs A/A′ were used to detect the deletion of *vlf-1* from Ac*M*NPV genome, and the PCR product generated by primer pairs B/C′ and C/B′ were used to confirm the correct insertion of *zeocin*, as well as to examine the junction between the upstream or downstream flanking region and *zeocin*.

Construction of vlf-1 Knockout, Repair and Wildtype AcMNPV Bacmid with polyhedrin and gfp

To generate a *vlf-1* repair bacmid and to introduce two marker genes *polyhedrin* and *gfp* into *vlf-1* knockout, repair and wildtype bacmids, we constructed two donor plasmids pFB-ieGPV and pFB-ieGP containing *gpf* and *polyhedrin*. First, a 575 bp *Kpn*I–*Sal*I *ie-1* promoter fragment amplified with primers 5′ ie/3′ ie and a 730 bp *Sal*I and *Hind*III PCR fragment produced by primers 5′ GFP/3′ GFP were cloned into pFastbac1 digested by *kpn*I and *Hind*III to give pFB-ieG. Second, pFB-ieG digested by *Sna*BI and *Eco*RI was cloned with a *Eco*RI digested fragment amplified by primers 5′ Polh/3′ Polh to construct pFB-ieGP. Finally, an *Xba*I–*Xho*I fragment generated by primers 5′ 63674–63691/3′ 65138–65156 containing *vlf-1* ORF under the control of its native promoter was cloned into *Xba*I and *Xho*I digested pFB-ieGP to generate pFB-ieGPV. To prepare bacmid for transposition, the helper plasmid pMON7124 conferring resistance to tetracycline and encodes a transposase was transformed into DH10B harbouring the *vlf-1* knockout bacmid. Then these cells were transformed with donor plasmid pFB-ieGP or pFB-ieGPV to generate a *vif-1* knockout bacmid ($vAc^{kO\text{-}GP}$) (Fig. 2a) or a *vlf-1* repair bacmid ($vAc^{Repair\text{-}GP}$) (Fig. 2b), respectively. In addition, wildtype Ac*M*NPV bacmid ($vAc^{WT\text{-}GP}$) was generated by transforming DH10B cell harbouring bMon14272 and the helper plasmid pMon7124 with donor plasmid pFB-ieGP (Fig. 2c). The transformed cell were incubated at 37°C for 4 h in 1 ml SOC with moderate shaking and then placed onto LB agar containing 25 µg/ml Zeocin, 50 µg/ml Kanamycin, 7 µg/ml Gentamicin, 10 µg/ml Tetracycline, 100 µg/ml X-Gal and 40 µg/ml IPTG. Plats were incubated at 37°C for a minimum of 48 h, and the typical white colonies resistant to Zeocin, Kanamycin, Gentamicin, and Tetracycline were selected and streaked to freshly made plates to verify the phenotype, and then confirmed by PCR. pUC/M13 primer set just outside of the transposition locus were used to verify the transposition success with expected PCR fragment. The relative position of pUC/M13 primers and the expected size of PCR products generated from selected bacmid constructs were illustrated in Fig. 2. Furthermore GFP expression and occlusion body formation in bacmid DNA transfected Sf9 cells confirms the accomplishment of the transposition event.

Transfection of Bacmid DNA to Sf9 Cell

For transfection of Sf9 insect cell, three bacmid constructs DNA were prepared as follows. Each

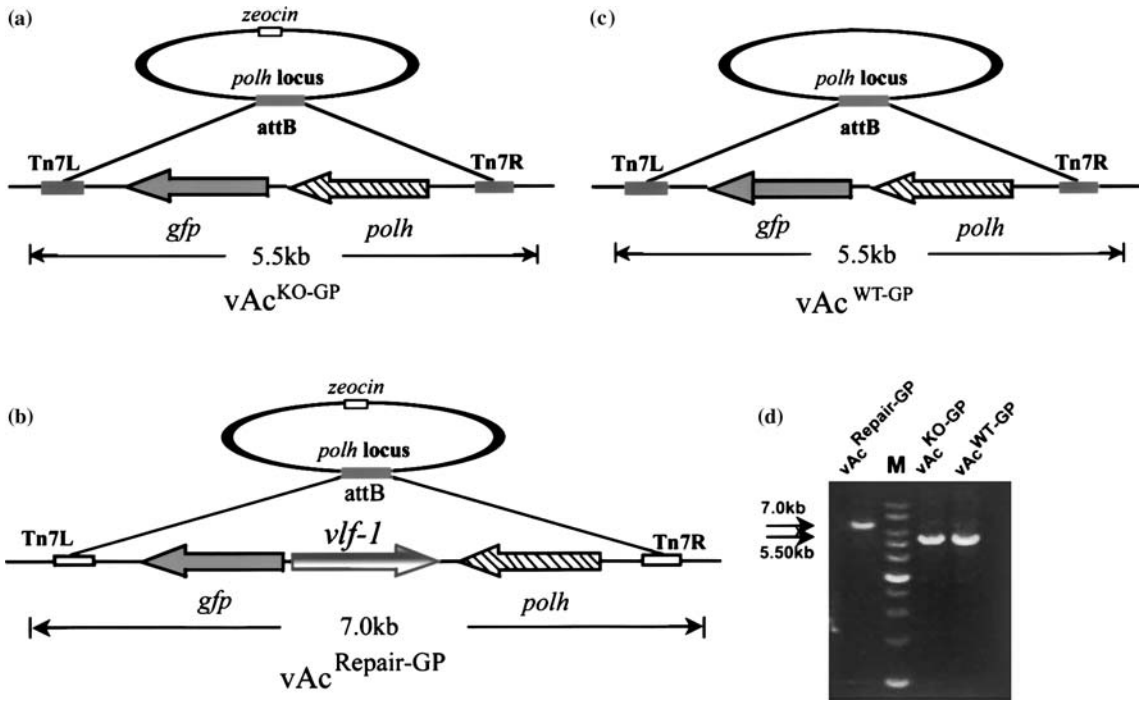

Fig. 2. Strategy for bacmid DNA transposition by donor plasmid and PCR screening. (a) The donor plasmid pFB-ieGP was transposed to the *polyhedrin* locus of *vlf-1* knockout bacmid to generate vAc $^{kO\text{-}GP}$. The *gfp* gene is under the control of the ie-1 promoter and followed by an SV40 polyadenylation signal and *polyhedrin* gene *(polh)* is flanked with its own promoter and polyadenylation signal. The line below the diagram indicated the expected size of PCR product amplified by pUC/M13 primers. (b) The donor plasmid pFB-ieGPV was transposed to the *polyhedrin* locus of *vlf-1* knockout bacmid to generate vAc$^{Repair\text{-}GP}$. The *vlf-1* repair fragment contains it own promoter and polyadenylatin signal. (c) The donor plasmid pFB-ieGP was transposed to the *polyhedrin* locus of wildtype (WT) Ac*M*NPV to generate vAc$^{WT\text{-}GP}$ as a control bacmid. (d) Ethidium bromide-stained agarose gel shows PCR products by pUC/M13 primer pairs. Side arrows indicated the expected size and the template DNAs was indicated under the panels. M, DNA size markers.

bacmid DNA was isolated and electroporated back into DH10B cell on Kanamycin, and colonies screened for sensitivity to tetracycline to ensure that the isolated bacmid was free of the helper plasmid pMON7124. Each resulting *E.coli* strain carrying the helper-free bacmid was used to prepare bacmid DNA for cell transfection. Bacmid DNA was prepared from 0.5–2 l cultures according to Bac-to-Bac manual (Invitrogen Life Technology). Equimolar amounts of bacmid DNA were transfected into Sf9 cells (2×10^6) seeded in a 6-well plate using cationic liposome method (Invitrogen Life Technology). Bacmid DNA was mixed with 20 µl lipofectin in 200 µl Grace's medium and incubated at 27°C for 30 min, then the mixed DNA–lipofectin solution was added to 1 ml Grace's medium and overlaid onto the freshly plated Sf9 cells. After 4 h incubation at 27°C, the mixed solution was removed and the cell was washed 3 times by Grace's medium. Finally the cell was replenished with 2 ml Grace's medium containing 10% fetal bovine serum and incubated at 27°C.

Virus Growth Curve

Sf9 cells were transfected in triplicate with the appropriate bacmid DNA (vAc$^{kO\text{-}GP}$, vAc$^{Repair\text{-}GP}$ or vAc$^{WT\text{-}GP}$) or infected in triplicate with the supernatant of vAc$^{Repair\text{-}GP}$ or vAc$^{WT\text{-}GP}$ at MOI of 5. For titration of BV production at various times post-transfection (p.t.) or post-infection (p.i.), culture medium was harvested by centrifugation (8,000 g, 5 min) to remove cell debris. BV production was determined by endpoint dilution assay ($TCID_{50}$ assay) in Sf9 cells [l4].

Electron Microscopy

Sf9 cells (5×10^6 per 100-mm-diameter tissue culture dish) seperately transfected by $vAc^{WT\text{-}GP}$ and $vAc^{kO\text{-}GP}$ bacmid constructs were harvested at 72 h p.t., and then washed once with Grace's medium lacking fetal serum, pelleted at 1,000 *g* for 5 min. Cell pellets were fixed in 2.5% glutaraldehyde overnight at 4°C, washed 3 times with PBS buffer and post-fixed in 1% osmium tetroxide in PBS buffer for 1 h at 4°C. Cells were washed 3 times with PBS buffer, dehydrated in graded ethanol and soaked in acetone. Infiltration was accomplished by using Sprur (Sigma-Aldrich Co., USA) in gelatin capsules. Thin sections were stained with aqueous uranyl acetate and lead citrate and observed under a JEM-100CXII transmission electron microscope operation at 80 KV.

Results

Analysis of $vAc^{kO\text{-}GP}$, $vAc^{Repair\text{-}GP}$, and $vAc^{WT\text{-}GP}$ Replication in Transfected Sf9 Cells

Sf9 cells were transfected with either $vAc^{kO\text{-}GP}$ $vAc^{Repair\text{-}GP}$, or $vAc^{WT\text{-}GP}$ DNA respectively and examined for viral propagation by the phenotype of GFP expression and occlusion body formation. Scattered fluorescence could be detected in the three transfected cell cultures before 24 h p.t. Clusters of GFP expression appeared in cell cultures transfected with $vAc^{Repair\text{-}GP}$ and $vAc^{WT\text{-}GP}$ at 48 h p.t., indicating virus spread among the cells. In sharp contrast to that, GFP expression in $vAc^{kO\text{-}GP}$ transfected cells showed no increase and remained scattered at 48 and 72 h p.t. (Data not shown), indicating absence of viral transmission beyond the initial transfection. Consistently, the initial occlusion body appeared in $vAc^{Repair\text{-}GP}$ or $vAc^{WT\text{-}GP}$ transfected cells at 48 h p.t. and became prominent at 72 h p.t. There was no any occlusion body detected in $vAc^{kO\text{-}GP}$ transfected cells at 72 h and even 120 h p.t. (Data not shown).

To quantitatively assess the effect of *vlf-1* deletion on virus replication, virus growth curve experiment was performed. The virus titer in $vAc^{kO\text{-}GP}$ transfected cell culture was undetectable even at 144 h p.t. In contrast, the $vAc^{Repair\text{-}GP}$and $vAc^{WT\text{-}GP}$ transfected cell cultures showed parallel

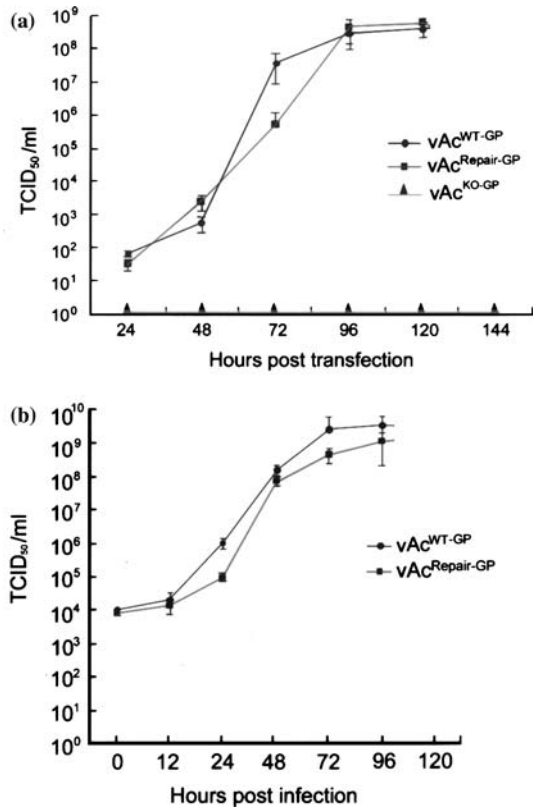

Fig. 3. Virus growth curves. (a) Virus growth curves of $vAc^{kO\text{-}GP}$, $vAc^{Repair\text{-}GP}$ and $vAc^{WT\text{-}GP}$ transfected Sf9 cells. Cells were transfected with related bacmid DNA in triplicate. (b) Virus growth curves of $vAc^{Repair\text{-}GP}$ and $vAc^{WT\text{-}GP}$ infected Sf9 cells at MOI of 5 in triplicate. The cell culture supernatants were harvested at the indicated time points p.t./p.i. and assayed for the production of infectious virus by $TCID_{50}$ assay, each datum point represents the average titer derived from three independent $TCID_{50}$ assay.

virus titers, indicating requirement of VLF-1 in viral infection production (Fig. 3a).

To further verify the infection capability of *vlf-1* knockout bacmid, passage through assay were carried out in Sf9 cell cultures. Bacmid constructs were transfected to Sf9 cells, and then at 120 h p.t. the supernatants were collected and transferred to freshly plated Sf9 cells. As expected, typical clusters of GFP expression and prominent occlusion bodies were observed at 72 h p.i. in the majority of the cells incubated with supernatant of $vAc^{Repair\text{-}GP}$ or $vAc^{WT\text{-}GP}$ transfection(Data not shown), indicating generation of BV and ODV from initial transfection by wildtype and repair bacmid. Conversely, neither GFP nor occlusion body was detected at

72 h p.i. in the cells incubated with $vAc^{kO\text{-}GP}$ transfection supernatant (Data not shown), indicating no infection occurred which suggests lack of infectious viral particles in the transfection supernatant. Additionally, a second growth curve was established to analyse viral replication independent of transfection efficiency. The results of these growth curves revealed that $vAc^{Repair\text{-}GP}$ was the same proficiency as $vAc^{WT\text{-}GP}$ (Fig. 3b). Results of the above transfection and passage through assay demonstrated that deletion of *vlf-1* prevents the production of both BV and ODV.

Electron Microscopy of vlf-1 Wildtype, Knockout and Mock-Transfected Cells

To further elucidate in which step *vlf-1* deletion blocks BV production during its life cycle, we examined $vAc^{WT\text{-}GP}$ and $vAc^{KO\text{-}GP}$ bacmids transfected cells by electron microscope. As expected, typical virogenic stroma (the putative site of nucleocapsids assembly), nuleocapsids and Polyhedrin were observed in the nuclei of $vAc^{WT\text{-}KO}$ transfected cells at 72 h p.t. (Fig. 4a). However, neither nucleocapsid nor occlusion body was observed at 72 h p.t. in the nuclei of $vAc^{kO\text{-}GP}$ transfected cells (Fig. 4b). Compared to mock transfected cells (Fig. 4c), $vAc^{KO\text{-}GP}$ transfected cells showed existance of virogenic stroma like electron dense structure, a fibrillar electron-dense matter characteristic of viral DNA synthesis, and existence of clumps of heterochromatin marginating along the inner nuclear membrane which should normally disperse throughout the nucleoplasm (Fig. 4b). In correspondence with this, cultured cells transfected by $vAc^{kO\text{-}GP}$ became rounded with swelling nuclei under optical microscope at 72 h p.t. These results demonstrated that *vlf-1* deletion led to nucluocapsid assembly failure, though preliminary infection was initiated in $vAc^{kO\text{-}GP}$ transfected Sf9 cells.

Discussion

Early in 1994, *vlf-1* was found to be required for occlusion bodies formation in an occlusion-defective Ac*M*NPV mutant virus [5]. A recent *vlf-1* knockout test has revealed that *vlf-1* was necessary for BV production [11]. This paper reported further *vlf-1* knockout experiments including electron microscope examination. The results provided direct evidence for the first time that *vlf-1* knockout blocked nucleocapsid formation. Whereas the role of *vlf-1* played in nucleocapsid formation was still kept unknown.

As we know, successful nucleocapsid assembly requires synthesis and correct processing of viral DNA and production of structure proteins. It has been reported that *vlf-1* deletion had no effect on viral DNA replication [11]. We also observed a fibrillar electron-dense matter, characteristic of viral DNA synthesis, in $vAc^{kO\text{-}GP}$ transfected cells under electron microscope. It has also been reported that expression of late genes, for example, VP39 (capsid protein), reached the same level in *vlf-1* deleted virus as in wildtype virus [5,11]. Under electron microscope, some kind of tubular structure was found in vlf-1 knockout virus transfected cells (Data not shown), this tubular structure could be empty nucleocapsids and could be another evidence of structure protein production in *vlf-1* knockout virus transfected cells. In the baculovirus life cycle, long-than-unit length genomes were generated during viral DNA replication [15,16]. These multi-unit genomes need to be processed to unit genomes before virion assembly. By sequence alignment, *vlf-1* was considered a member of the tyrosine recombinase family. Proteins of this family are responsible for the decatenation of newly replicated genomes by catalyzing DNA rearrangement [5,8]. Considering the availability of DNA synthesis and structure protein together with *vfl-1* as tyrosine recombinase, we could suggest that *vlf-1* might play a role in resolving the concatameric replicative intermediate into unit genome, which is necessary for nucleocapsid assembly. That is, *vlf-1* deletion might lead to incorrect viral DNA processing or even cease viral DNA processing. This hypothesis might reconcile with the defect in nucleocapsid formation in *vlf-1* knockout virus transfected cells.

If the hypothesis became true, how *vlf-1* works on genome processing is another mystery, although other DNA virus with large genome such as bacteriophage T4 and Simplex Virus type 1 (HSV-1) could provide a model for this mechanism. For example, HSV-1, with a double-stranded DNA of 152 kb have adopted rolling–cycle replication mechanism in its DNA replication [17].

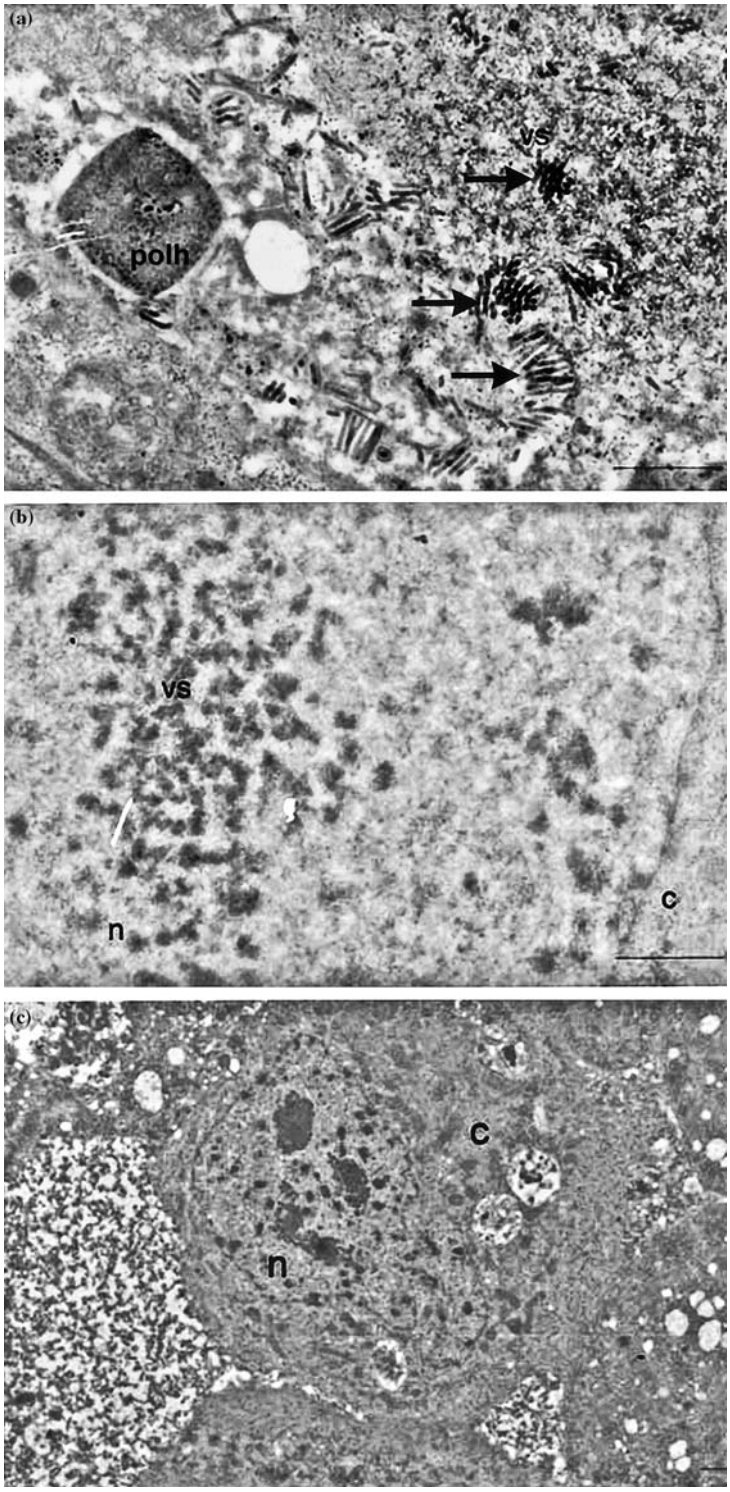

Fig. 4. Electron micrographs of $vAc^{WT\text{-}GP}$, $vAc^{kO\text{-}GP}$ and Mock-transfected cells at 72h p.i. $vAc^{WT\text{-}PG}$ transfected cell at 72h p.t. (A), in which the arrows indicate nucleocapsid; $vAc^{KO\text{-}PG}$ transfected cell at 72h p.t.(B); Mock- transfected cell at 72h p.t. (C), n, nucleus; c, cytoplasm; vs, virogenic stroma; polh, polyhedrin; Bars,1 μm.

Later researches presented more complex on this replication mechanism by recombination. HSV-1 DNA was reported to held together to form a large complex by frequent branches, most of which were Y structure, resulting in a network of replicating molecules [18,19]. These long branched DNA molecules were resolved into unit length for encapsidation by unknown viral protein. Experiments suggested that HSV-1 alkaline nuclease could be the best candidate to execute the function of DNA debranch and processing for packaging [20,21]. *In vitro* test indicated that HSV-1 alkaline nuclease might function at nicks and gaps in replicating DNA to process the replicated genome for encapsidation [22]. Interestingly, *vlf-1* showed high affinity to certain DNA substrates that mimic DNA replicative intermediate structures including Y-forks, three-way junctions, and cruciform DNAs *in vitro* [23]. Moreover, it is intriguing to find that in all sequenced baculovirus genomes there also exists a homolog of alkaline nuclease, which has been proved to involve in the recombination system interacting with DNA-binding protein LEF-3 [24, 25]. Currently, no clue has been obtained of the potential relationship between *vlf-1* and alkaline nuclease regarding their possible role in late viral DNA genome processing and nucleocapsid assembly. It could be possible that both *vlf-1* and alkaline nuclease, perhaps together with other viral coded proteins, be involved in the viral DNA processing.

Two marker genes, *gfp* and *polyhedrin*, were used in the investigation of the *vlf-1* knockout Ac*M*NPV bacmid in this study. The two genes were introduced into the *polyhedrin* locus of Ac*M*NPV genome by transposition in *E.coli*. Phenotypes of GFP expression in cells culture greatly facilitated determination of viral transmission, namely, the production of infectious viral particle, which resulting in the second infection. Additionally, considering the tran-acting relationship between *vlf-1* and *polyhedrin*, we introduced *polyhedrin* back to it native locus in Ac*M*NPV bacmid in which *polyhedrin* ORF was destroyed by inserting of the Tn7 attB transposition sequence. Recovery of polyhedrin formation validated the success repair of the mutant by *vlf-1*. Therefore phenotype of BV production and occlusion body formation in $vAc^{Repair\text{-}GP}$ straightly proves that it was *vlf-1* deletion that resulted in the defect of BV and ODV production in $vAc^{kO\text{-}GP}$ other than any potential second mutation or disruption of regulatory elements located at the *vlf-1* locus.

Electron microscopy brought to an important conclusion that *vlf-1* deletion blocked nucleocapsid formation. It is the defective nucleocapsid formation that resulted in the abolishment of BV and ODV production. And based on this conclusion, a hypothesis was proposed that *vlf-1* might involve in the processing of viral genome mature for capsid filling. Further study on *vlf-1* should envision our understanding on viral genome processing strategy.

Acknowledgments

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