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The autographa californica multiple nucleopolyhedrovirus Ac12: A non-essential F box-like protein that interacts with cellular SKP1 component of the E3 ubiquitin ligase complex

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ABSTRACT

The Autographa californica multiple nucleopolyhedrovirus (AcMNPV) *ac12* gene, which is conserved in ten other baculovirus, codes a predicted 217 amino acid protein of unknown function. In this study, we investigated the role of *ac12* during baculovirus infection, by generating an *ac12* knockout virus. The transfection of the recombinant genome in insect cells resulted in unaltered viral dispersion and occlusion body production when compared to the control bacmid. This finding demonstrates that *ac12* is a non-essential gene. Transmission and scanning electron microscopy (SEM) analyses showed that *ac12* knockout virus produced occlusion bodies morphologically similar to those obtained with the control and capable to occlude virions. However, a slight but significant size difference was detected by SEM observation of purified occlusion bodies. This difference suggests that *ac12* may be involved in regulatory pathways of polyhedrin production or occlusion body assembly without affecting either viral occlusion or oral infectivity in *Rachiplusia nu* larvae. This was evidenced by bioassays that showed no significant differences in the conditions tested. A qPCR analysis of viral gene expression during infection evidenced regulatory effects of *ac12* over some representative genes of different stages of the viral cycle. In this study, we also showed that *ac12* is transcribed at early times after infection and remains detectable up to 72 hours post-infection. The mRNA is translated during the infection and results in a protein that encodes an F-box domain that interacts in vivo and in vitro with S phase kinase associated protein 1 (SKP1) adaptor protein, which is potentially involved in protein ubiquitination pathways.

1. Introduction

Baculovirus are pathogens of insects, mainly lepidopterans, with numerous biotechnological applications. They are employed as bio-insecticides based on their ability to eliminate insect pests with high specificity and their lethality can be increased through the production of recombinant viruses (Beas-Catena et al., 2014). The baculovirus-insect cell system is also used for protein production allows post-translational modifications and reaches high levels of protein expression (Kost et al., 2005; López et al., 2018). Furthermore, because of their diverse tropism and their biosafety, they have been used as tools for vaccine development and transgene expression vectors in mammalian cells (Lykhova et al., 2015; Shoji et al., 1997; Wu et al., 2014).

Baculoviruses are a very diverse group of viruses with a supercoiled, circular double-stranded DNA genome, which ranges from about 80 to over 180 Kbp and contains between 90 and 180 genes (Rohrmann, 2013). Viruses belonging to the genus *Alphabaculovirus* (nucleopolyhedrovirus), whose type species is *Autographa californica multiple nucleopolyhedrovirus*, contain approximately 150 predicted ORFs, many of which have unknown functions. During replication of Autographa californica multiple nucleopolyhedrovirus (AcMNPV) two distinct virion phenotypes with different biological roles are produced, budded virus (BV) and occlusion derived virus (ODV) (Braunagel and Summers, 1994). ODVs are formed by one or many nucleocapsids wrapped in membranes derived from the nuclear envelope and with viral integral membrane proteins. These ODVs finally occlude in a protein matrix of

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Table 1
Primer sequences used in this study. Restriction enzyme sites are underlined. The 50 nt homologous to the 5' and 3' ends of the coding region of *ac12* are in italics.

Primer name	Sequence	AcMNPV nt position
pac12ForBam	5' TAGGATCCCTGTGCACATAAGTTAC 3'	8,374 to 8,390
ac12RevXho	5' CGCTCGAGTATGTATTTATTTCG 3'	9,610 to 9,596
HisRev	5' CGTCAATGGTGATGGTGATG 3'	
Cn12F	5' *CAATAATACTGATGTACAGGCACGCGTGCTATTTATATATAATTTCGCAA*TTTAAGGGCACCAATAACTG 3'	8,947 to 8,996
Cn12R	5' *TGAATTACGAGGACATTGGTTTTTTTGTTTGCATATTAAACAATCGCGCG*TTCCTGTGCGACGGTTAC 3'	9,478 to 9,527
ac12RTR	5' TTATGTATTTATTTCGTATA 3'	9,592 to 9,611
CnfCheckF	5' GATGCCATTGGGATATATCAACG 3'	
ac12RTF	5' CATCAACTGTATTTGCAACG 3'	9,284 to 9,304
iE1F	5' TTAACGCGTCGTACACCAGCG 3'	127,214 to 127,234
iE1R	5' TTATAATAACTTAAATAGTCGTTGGG 3'	127,294 to 127,319
GP64F	5' ACTGCAACGCGCAAATGAAG 3'	109,631 to 109,650
GP64R	5' CGATGGTGATTTCCACGTCC 3'	109,552 to 109,571
POLHF	5' CGCTAAGCGCAAGAAGCACT 3'	4,609 to 4,628
POLHR	5' GCTTCATCGTGTCGGGTTTA 3'	4,753 to 4,762
28SF	5' CTGGCTTGATCCAGATGTTCAG 3'	
28SR	5' GGATCGATAGGCCGTGCTT 3'	
ac12F	5' AAGCGTCGTCATTGTAAATTA 3'	9,075 to 9,095
SfSkp1F	5' CCTAACATAAAACTACAGTCTTCGG 3'	
SfSkp1R	5' GTTACTTCTCCTCACACCATTC 3'	
ac12FSac	5' GGAGCTCGATGAAGCGTCGTCA 3'	9,072 to 9,083

polyhedrin and form structures called occlusion bodies, which initiate the primary infection in the larvae. The BVs obtain their envelope from the plasma membrane, in which viral proteins are inserted, and spread the infection towards the insect tissues.

Baculoviral genes are sequentially transcribed in the viral cycle and they are classified according to the time in which they are transcribed as immediate early, early, late and very late genes. The immediate early and early genes prepare the cell for viral replication and are involved in evasion of antiviral response (Friesen and Miller, 1986). Late transcription requires viral DNA replication and the transcribed genes allow the beginning of the condensation and encapsidation of viral DNA to form the nucleocapsids (Vanarsdall et al., 2006). In very late stages of infection, the nucleocapsids remaining in the nuclei begin to be wrapped in inner membranes and become occluded in large amounts of polyhedrin, thus giving rise to polyhedra.

The AcMNPV genome contains 38 genes that have orthologs in all genera and that are known as "core genes" (Garavaglia et al., 2012; Javed et al., 2017). These genes encode primarily structural proteins and factors necessary for DNA replication and late transcription. In addition, each group of viruses has its own baculovirus genes related to specific pathogenicity (van Oers and Vlak, 2007). The study of the characteristics and functions of viral genes is crucial to understand in detail all the steps of the replication cycle and to optimize the biotechnological applications of baculovirus.

Very little is known about the ORF12 of AcMNPV. Rohrmann in 2013, mentioned that it is conserved in three additional lepidopteran nucleopolyhedrovirus: Plutella xylostella multiple nucleopolyhedrovirus (PlxyMNPV), Rachiplusia ou multiple nucleopolyhedrovirus (RoMNPV) and Lymantria dispar multiple nucleopolyhedrovirus (LdMNPV) (Rohrmann, 2013). Transcriptomic studies of AcMNPV-infected cells showed that despite not having typical patterns of very late gene sequences upstream of the ORF, *ac12* transcripts increase in the last stages of the infection (Chen et al., 2013). However, whether ORF12 is essential to the viral infection cycle remains unknown, as well as at which specific stage it is involved.

The objective of this research was to determine the possible role of ORF12 in the infection of AcMNPV both in cell culture and insect larvae, and to study the presence of the predicted protein during the viral cycle. In this work, we demonstrated for the first time the existence of an Ac12 polypeptide and that this polypeptide is an F-box-like protein that interacts with cellular S phase kinase associated protein 1 component (SKP1) of the E3 ubiquitin ligase complex. Finally, although slight effects on polyhedron size and on the transcription of some viral genes were evident in *ac12* knockout mutant viruses, *ac12* resulted non-essential for virus replication and infectious-polyhedra morphogenesis.

2. Material and methods

2.1. Cell lines and viruses

The Sf9 insect cell line was purchased from Invitrogen and maintained at 27 °C in TNM-FH insect medium (Sigma) supplemented with 10% fetal bovine serum (FBS). Wild type (wt) AcMNPV was obtained from Pharmingen (BD Biosciences). Wt and recombinant baculoviruses were propagated in Sf9 cells.

Bacmid transfections were performed with 1 μg of DNA per 1×10^6 Sf9 cells using Cellfectin II® transfection reagent (Life Technologies) according to the manufacturer's instructions. Viruses were amplified in Sf9 cells and tittered by end-point dilution (O'Reilly et al., 1994).

2.2. Generation of recombinant plasmids and bacmids

The pFBD-PHGFP transfer vector was previously constructed in our laboratory (Alfonso et al., 2012). pFBD-p12-12V5 and pFBD-PHGFP-p12-12V5 were obtained from pFastBac™ Dual plasmid (Invitrogen) or pFBD-PHGFP by inserting *ac12* fused to a V5 tag and the 584 nt sequence upstream the ATG codon that contains its native promoter. Briefly, the *ac12* gene and its promoter region without the complete STOP codon (AcMNPV NC_001623 nt 8,374 to 9,610) were obtained by PCR amplification using DNA extracted from cells infected with wt AcMNPV as a template and the primers pac12ForBam and ac12RevXho (Table 1). The amplified region was cloned into pGEM®-T Easy Vector (Promega) to produce pGEMTp12-12, which was subsequently digested with BamHI and XhoI enzymes. The insert was subcloned into the pIB/V5-His plasmid (Invitrogen) and the p12-12 fragment fused to V5 tag (p12-12-V5) was amplified by PCR with the primers pac12ForBam and HisRev (Table 1) and then cloned into the pGEM®-T Easy Vector. Then, the p12-12-V5 fragment was cloned into pFastBac™ Dual using NcoI and PstI restriction enzymes, with the consequent elimination of the *p10* and *polh* promoters, or into pFBD-PHGFP using NcoI and SpHI restriction enzymes.

Bac-ac12KO bacmid was obtained by homologous recombination in *E. coli* BJ5183, as previously described (Alfonso et al., 2012). The resistance cassette was amplified using primers Cn12F and Cn12R (Table 1). These primers contain 50 nt homologous to the 5' and 3' ends

of the coding region of *ac12* and were designed taking into account not to affect the transcription start site (TSS) of *ac11* (AcMNPV NC_001623 nt 8,953) and polyadenylation site (PAS) of *ac13* (AcMNPV NC_001623 nt 9,516) according to Chen et al. (2013) Resistant colonies were selected in Lysogeny broth (LB) agar containing 50 µg/ml chloramphenicol and 50 µg/ml kanamycin and the presence of the bacmid *ac12*KO was confirmed by PCR. Primers CnfCheckF and ac12RTR (Table 1) were used to verify the correct insertion of the chloramphenicol cassette in the *ac12* locus; primers ac12RTF and ac12RTR (Table 1) amplified a fragment of *ac12* from wild type genomes. The bMON1472.12KO bacmid and pMON7124 plasmid were co-electroporated into *E. coli* DH10 strain and transformants were selected in LB agar medium containing 50 µg/ml chloramphenicol, 50 µg/ml kanamycin and 10 µg/ml tetracycline. Finally, Bac-*ac12*KO and Bac-*ac12*Rep were obtained by Tn7-mediated transposition between bMON1472.12KO and pFBD-PHGFP or pFBD-PHGFP-p12-12V5, respectively, as previously described (Alfonso et al., 2012). Bac-*ac12*V5 was obtained using Bac-to-Bac system (Invitrogen) with pFBD-p12-12V5 as the transfer vector, according to the manufacturer's instructions.

The bacmids were purified from bacteria using Large-Construct purification kit (QIAGEN) following the manufacturer's instructions.

All transfer and intermediate vectors were sequenced to confirm their identity.

2.3. RT-PCR

Sf9 cells were infected with wt AcMNPV at a multiplicity of infection (MOI) of 5 at 4 °C for 1 h and then washed twice with PBS. Fresh TNM-FH medium was added and after 0, 6, 18, 24, 30, 42, 54, 66 and 72 h post-infection (hpi) at 27 °C, total RNA was extracted using TRIZOL Reagent (Life Technologies). Reverse transcription reaction was performed, after DNase I Amplification grade (Invitrogen) treatment, with ac12RTR primer and SuperScript II Reverse Transcriptase (Invitrogen) enzyme. PCR was performed with the same reverse primer and ac12RTF to amplify a 328 bp fragment of the gene.

2.4. RT-qPCR

Sf9 cells were infected with each viral stock, as previously described for RT-PCR, and total RNA was extracted 6, 24, 42 and 66 hpi. A reverse transcription reaction was performed with random hexamers and MMLV enzyme (Promega) after DNase I treatment. For qPCR reactions the primers iE1F, iE1R, GP64 F, GP64R, POLHF, POLHR, 28SF, 28SR (Table 1) were used. Transcription expression levels were normalized using the 28S rRNA gene (Salem et al., 2014). All reactions were performed in triplicates. Oligonucleotide efficiencies were analyzed in each reaction using LinReg (Ramakers et al., 2003), with values higher than 1.8 for all cases. FG Statistics software (Di Rienzo et al., 2000) was used for ratio calculation between each viral gene versus the cellular gene and the results were expressed calculating the relationship between the ratios obtained from Ac12KO or Ac12Rep and AcPHGFP viruses.

2.5. 5' and 3' RACE

Cells were infected with wt AcMNPV at a MOI of 5 and, 48 hpi rapid amplification of cDNA 5' and 3' ends were performed. This procedure was carried out using 5´ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 and 3' RACE System for Rapid Amplification of cDNA Ends, Version E (Life Technologies) following the manufacturer's instructions. As recommended by the manufacturer, an alternative protocol for first strand cDNA synthesis of transcripts with high GC content was assayed to avoid problems because of secondary structure of the target mRNA. Sequences were obtained after cloning in pGEM®-T Easy Vector.

2.6. Western blot

Infected cells were boiled for 5 min in disruption buffer (120 mM Tris–HCl pH 6.8, 4% SDS, 0.02% bromophenol blue, 1.4 M ß-mercaptoethanol, 20% glycerol) and proteins were resolved in 15% acrylamide-bisacrylamide (30:0.8) SDS-PAGE minigels (Bio-Rad). The proteins were blotted onto nitrocellulose membranes and the blots were probed with a mouse monoclonal anti-V5 antibody (1:500) (Invitrogen), followed by an HRP-conjugated anti-mouse antibody (1:1000) (Santa Cruz Biotechnology). Antibody binding was visualized using the reactive SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and G:Box Chemi-XX6 (Syngene).

2.7. Quantification of infectious BVs

Sf9 cells (1×10^6) were seeded in 35 mm-diameter dishes and infected at a MOI of 10 with AcPHGFP, or Ac12KO in triplicates. At 0, 18, 24, 48, 72 and 96 hpi the supernatants were collected, clarified and titered in Sf9 cells by the end-point dilution method.

2.8. Electron microscopy

For transmission electron microscopy (TEM) observations, cells infected with AcPHGFP or Ac12KO were fixed with 2.5% glutaraldehyde, post-fixed in 2% osmium tetroxide and packaged in agar. Samples were dehydrated using ethanol at ascending concentrations (50%, 70%, 96%, and 100%) and acetone (100%). The pre-inclusion in resin was made using one volume of acetone and one volume of Spurr resin. The blocks were then carved and sliced with an ultramicrotome to obtain section pieces (60–90 nm thick) that were collected on copper grids. The samples were contrasted with heavy salts (uranyl; Reynold's method) and visualized in a TEM Zeiss EM 109 T microscope equipped with a Gatan ES1000 W digital camera (Instituto de Biología Celular y Neurociencias "Prof. E. De Robertis", IBCyN, Buenos Aires, Argentina).

For scanning electron microscopy (SEM) observations, polyhedra samples were purified from infected cells with AcPHGFP or Ac12KO as previously described (O'Reilly et al., 1994). The polyhedron samples were dehydrated using acetone at increasing concentrations (50% to 100%) and centrifuging in each step. Then they were attached to poly-L-lysine-coated glass coverslips, covered with gold/palladium and observed in a Philips XL30 TMP New Look microscope at the Museo Argentino de Ciencias Naturales Bernardino Rivadavia, Buenos Aires, Argentina. The occlusion bodies were photographed and measured using ImageJ software (Schneider et al., 2012).

2.9. Bioassays

Laboratory-reared, initially healthy third instar *Rachiplusia nu* larvae were orally infected with 100 occlusion bodies of either AcPHGFP or Ac12KO using the droplet-feeding method. The suspensions consisted of the appropriate occlusion body concentration, 2% sucrose as a phagostimulant and Coomassie brilliant blue dye to visualize the ingestion in the larval guts. The mean intake was previously assessed by weight difference, at 0.16 µl/larva. The healthy controls were fed with water with 2% sucrose and blue dye. Thirty six larvae per treatment were used. Once they ingested the suspensions, the larvae were individually transferred to plastic cups containing artificial diet and their survival was monitored twice a day for 10 days. Times to death and symptom development were recorded. Larval cadavers were observed under a light microscope to corroborate the presence of polyhedra.

2.10. Sequence and phylogenetic analysis

Nucleotide, amino acid alignments and identities percentages were calculated by using Geneious version 11.0.3 (Kearse et al., 2012). A

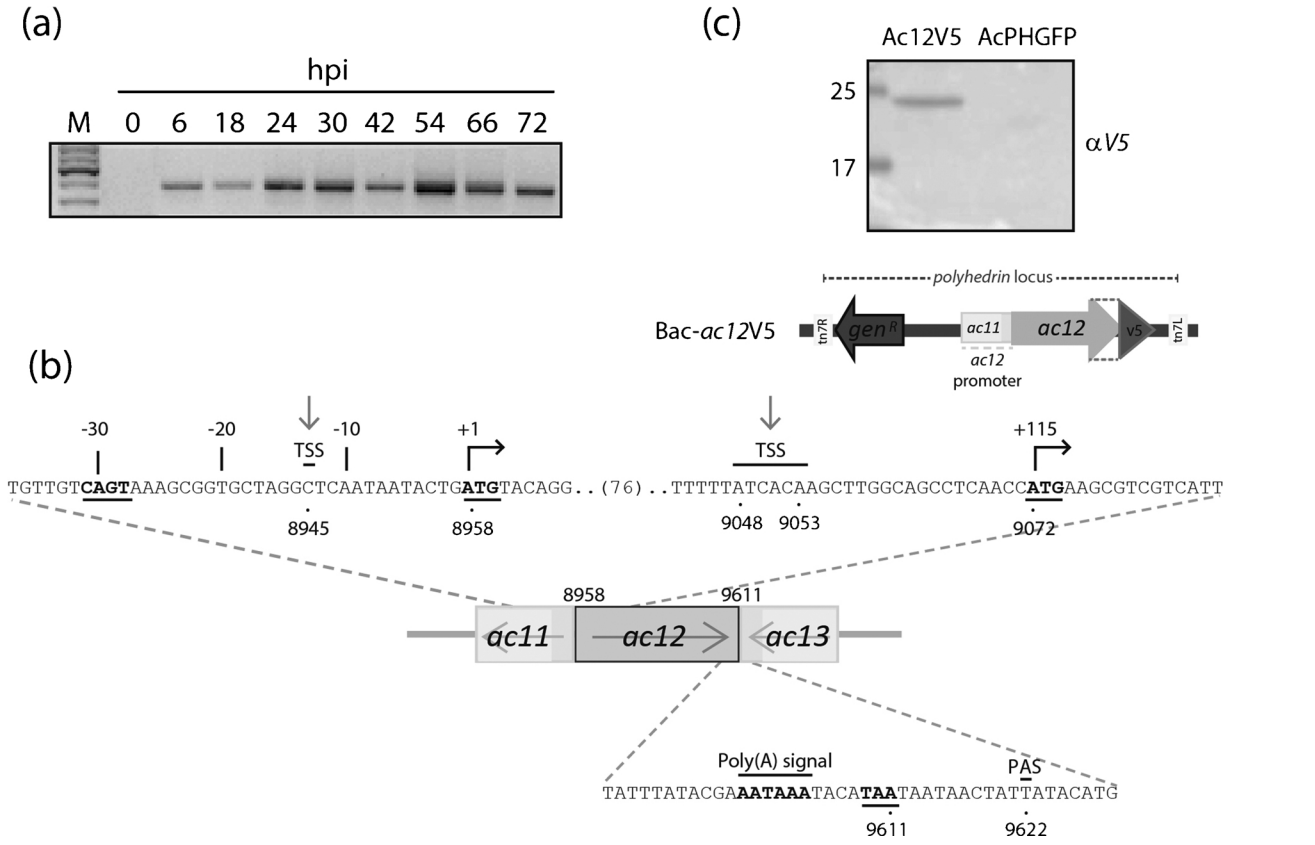

Fig. 1. Transcriptional and translational characterization of *ac12* in infected Sf9 cells. (a) Total RNA from Sf9 infected cells was extracted at indicated hours post infection and RT-PCR reactions were performed for each template. (b) The *ac12* promoter organization showing two transcription start sites (indicated by arrows) obtained by 5′ RACE and their corresponding putative start codons and the *ac12* transcription end site obtained by 3′ RACE are shown. (c) Schematic diagram of the *ac12*V5 bacmid construct and western blot of total lysates of Sf9 cells infected with virus containing V5 tagged Ac12. The *ac12*V5 bacmid contains a second copy of *ac12*, with its own promoter, fused to a V5 tag.

phylogenetic tree of Ac12 and its homologous amino acid sequences were inferred by using the Maximum Likelihood method based on the JTT matrix-based model (Jones et al., 1992). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then by selecting the topology with superior log likelihood value. The analysis involved 10 amino acid sequences. All positions containing gaps and missing data were eliminated. The final dataset contained 148 positions. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

2.11. Yeast two-hybrid assays

The Y2H assays were performed using the Matchmaker TM Gold Yeast Two-Hybrid System (Clontech, Japan) according to the manufacturer's protocol. The Ac12 coding sequence was amplified with the ac12F and ac12RTR primers (Table 1) from AcMNPV infected cells. The *skp1* sequence was obtained from Sf9 cells by PCR with the specific primers SfSkp1F and SfSkp1R (Table 1). The amplicons were cloned first into the pCR®8/GW/TOPO® vector (Invitrogen) and then into both pLAW11 and pLAW10, which express Gal4 DNA activation domain and Gal4 DNA-binding domain, respectively, by using Gateway technology (Invitrogen). Prof. Dr. Richard Michelmore from UC-Davis kindly provided the Y2H vectors. Yeasts carrying each construct were mated and the mating mixtures were plated on SD-Leu-Trp at 30 °C for 6 days. The protein interaction was selected on SD-Leu-Trp-His. SV40 large T-antigen in the prey vector pGADT7-T (AD:T) and murine p53 in the bait vector pGBKT7-53 (BD:p53), which are provided in the Matchmaker kit, were used as a positive control, whereas the AD:T and pLAW10 empty vector (BD) were used as a negative control. Yeast cells expressing each fusion protein of interest were co-transformed with the corresponding AD or BD empty vectors to rule out false positives.

2.12. Polyhistidine pull-down assays

For in vitro and in vivo polyhistidine pull downs, the Ac12 coding sequence was amplified without the complete STOP codon by using the primers ac12FSac and ac12RevXho (Table 1), and cloned into pIB/V5-His plasmid digested with SacI and XhoI upstream and in frame with V5-His tags. The *skp1* sequence with its STOP codon was digested from pCR/GW/TOPO-SKP1 with EcoRI and cloned into the pGADT7 vector in frame with HA tag. Then pGADSKP-1 was digested with BglII and BamHI restriction enzymes and cloned under the *OpIE2* promoter of the pIB/V5-His plasmid. Sf9 cells (1×10^7) were transfected with 10 µg of pIB-Ac12-V5-His, pIB-HA-SKP1 or co-transfected with both plasmids and 48 hpt were washed with ice-cold PBS and resuspended in 2 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 1% NP40, 1 µg/ml Aprotinin, 1 µg/ml Pepstatin). The lysates were centrifuged 10,000 x g for 10 min at 4 °C. In the case of the in vitro assay, the lysate containing the bait protein (Ac12-V5-His) was mixed gently with 20 µl of Ni-NTA slurry (QIAGEN) by shaking on a rotary shaker at 4 °C for 2 h. The mix was flowed through a Thermo Scientific Pierce Spin Column (Thermo Scientific) and washed twice with 1 ml of wash buffer (50 mM NaH2PO4, 300 mM NaCl, 30 mM imidazole, 1% NP40, 1 µg/ml Aprotinin, 1 µg/ml Pepstatin). Then, 700 µl of the prepared prey protein sample (HA-SKP1) was added in the bottom capped column containing the immobilized Ac12-V5-His and mixed at 4 °C for 1 h. The sample was flowed through the column and the process was

repeated with the remaining HA-SKP1 lysate. For the in vivo pull down, the lysate from co-transfected cells (Ac12-V5-His/HA-SKP1) was mixed gently with 20 µl of Ni-NTA slurry by shaking on a rotary shaker at 4 °C for 2 h. The mix was flowed through a Thermo Scientific Pierce Spin Column. In both assays the resin was washed three times with 1 ml of wash buffer and finally the proteins were eluted from the resin with pre-heated disruption buffer. The samples were loaded in SDS-PAGE gels and immunoblotted with anti-HA (1:5000) (Sigma-Aldrich) antibody. The same process was performed replacing Ac12-V5His sample by a lysate of non-transfected Sf9 cells.

3. Results

3.1. Transcriptional analysis, 5' and 3' RACE of ac12

As a first approach to study *ac12*, we assessed temporal expression of *ac12* transcripts in Sf9 infected cells by RT-PCR. A specific amplicon was detectable from 6 until 72 hpi (Fig. 1a), thus showing that *ac12* is transcribed from early time points of the infection. The analysis of the *ac12* sequence showed that the early promoter sequence CAGT (Fig. 1b) and the TATA-like elements (not shown) are located 31 nt, 55 nt and 69 nt upstream of the putative ATG codon (AcMNPV NC_001623 nt 8,958), respectively. Then, we assessed the presence of TSS and PAS by 5' and 3' RACE (Fig. 1b). Two different TSSs were found in this assay. Whereas only one clone presented a start position at nt 8,945 (13 nt upstream of the predicted ATG), most of the clones started at different positions comprised of an ATCACA motif (AcMNPV nt 9,048 to 9,053), upstream of another ATG codon (AcMNPV nt 9,072) and in frame with the first ATG. On the other hand, the PAS was located 11 nt downstream of the STOP codon TAA. These results suggested the presence of two different-sized *ac12* transcripts, with the shorter being most abundantly represented. To avoid secondary structures of RNA messengers that could prevent efficient reverse transcription, we performed many variants of the 5' RACE protocol and in all cases, the downstream TSS at nucleotides 9,048-9,053 remained to be the most frequent.

3.2. Analysis of Ac12 protein

To study the presence of Ac12 protein in the infection event, we constructed a recombinant baculovirus carrying a second copy of *ac12* fused with the V5 epitope sequence and driven by its own promoter region (Fig. 1c). The presence of a specific band was evident in immunoblot assays of insect cells infected with Ac12V5, by using an anti-V5 antibody (Fig. 1c). The molecular weight of the tagged polypeptide coincided with the expected protein translated from the most abundant and smallest detectable transcript (24.9 kDa) (Fig. 1b). Thus, the results indicate that *ac12* transcripts are translated in the infected cells. In accordance with reported proteomic studies, no signal was detected in samples of purified BVs and ODVs (data not shown). This finding confirms that Ac12 is not a structural protein.

3.3. Construction of an ac12 knockout virus

The function of Ac12 was assessed constructing an *ac12* knockout bacmid (Bac-*ac12*KO) by homologous recombination (Material and methods). The expected insertion of the antibiotic resistance cassette was confirmed by PCRs (data not shown). Subsequently, *polyhedrin* and *egfp* were inserted in the genome in order to achieve a complete viral cycle and to follow the infectious process. An identical bacmid without the deletion (Bac-PHGFP) served as control in all the assays (Fig. 2a).

3.4. Replication of ac12 knockout in Sf9 cells

To study the role of *ac12* during baculoviral infection in Sf9 cells, we separately transfected Bac-*ac12*KO and Bac-PHGFP in these cells and measured the viral dispersion by registering eGFP fluorescence signal from 0 to 72 h post-transfection (hpt). Sf9 cells transfected with Bac-*ac12*KO showed a fluorescence pattern similar to those transfected with the control bacmid (Fig. 2b). This result indicates that the deleted genome was able to complete the viral cycle and that the viral progeny infected neighboring cells thus spreading the infection. Besides, all infected monolayers presented occlusion bodies with similar appearance and quantity (Fig. 2c). In addition, we quantified the viral production from synchronized infections in Sf9 cells. For this purpose, culture supernatants obtained at different time points were titrated by end-point dilution. The viruses replicated with the same efficiency and with no apparent differences between the kinetics of both curves (Fig. 2d).

3.5. Occlusion body analysis

By SEM, we studied and compared the polyhedra produced in cells infected with the knockout and control viruses. Indeed, both samples presented occlusion bodies with normal aspect (Fig. 3a). Nevertheless, a slight significant difference in polyhedron size was detectable between the samples when we analyzed the diameter of 1500 occlusion bodies from each sample. This record showed that *ac12*KO polyhedra were significantly smaller than those of the control virus (1.61 µm vs 1.83 µm; $p < 0.0001$; Fig. 3c). In order to discard effects produced by the alteration of other genes due to genetic manipulation, we constructed an *ac12*-repair virus (Fig. 3b) and proved that the size of *ac12*Rep polyhedra did not show significant differences compared with those from AcPHGFP virus (1.81 µm vs 1.83 µm; $p = 0.3939$; Fig. 3c).

TEM images of cells infected with *ac12*KO and control viruses demonstrated that the lack of *ac12* did not produce any alteration in the typical infection signs. Moreover, a virogenic stroma and newly formed nucleocapsids getting their envelope were evident in nuclei. Normally shaped polyhedra with virions inside were also present (Fig. 4a). These results showed that *ac12* was not responsible for any of these processes and that in its absence the different stages of viral infection proceed normally in these cells.

Bioassays were performed using third instar *Rachiplusia nu* larvae to assess the oral infectivity of the *ac12*KO polyhedra. Tissue liquefaction, typical of most baculovirus infections, occurred in diseased specimens inoculated with both viruses and occlusion bodies were present in the dead larvae, as evidenced by light microscopic examinations (data not shown). The mortality curves of larvae fed with the different inocula were similar (Fig. 4b). This suggests that *ac12* is not necessary for the primary infection of the midgut cells or for the viral infection spread throughout the insect. The reduced size of the *ac12*KO occlusion bodies seemed not to alter their infectivity in the assayed conditions.

3.6. Effect of ac12 deletion on viral genes transcription

To characterize whether the transcription of some viral genes is affected by *ac12* deletion, we performed RT-qPCR. The transcripts selected as representatives of genes expressed in different stages of viral infection (*ie1*, *gp64* and *polyhedrin*) in Sf9 cells were normalized to the cellular 28S rRNA transcript. The data obtained evidenced significant differences between Ac12KO and AcPHGFP. The absence of *ac12* caused a deregulation of these genes, with a downregulation at early time points and an upregulation at late time points after infection (Fig. 5a). The expression of the studied genes was similar between Ac12Rep and AcPHGFP (Fig. 5b). These results suggest that *ac12* is involved in the regulation of transcription of viral genes in cultured cells.

3.7. Analysis of ac12 homologous sequences

We analyzed nucleotide and amino-acid *ac12* sequences and identified homologous genes not only in PlxyMNPV, RoMNPV and LdMNPV (Rohrmann, 2013), but also in other six nucleopolyhedroviruses: Bombyx mori nucleopolyhedrovirus (BmNPV), Thysanoplusia

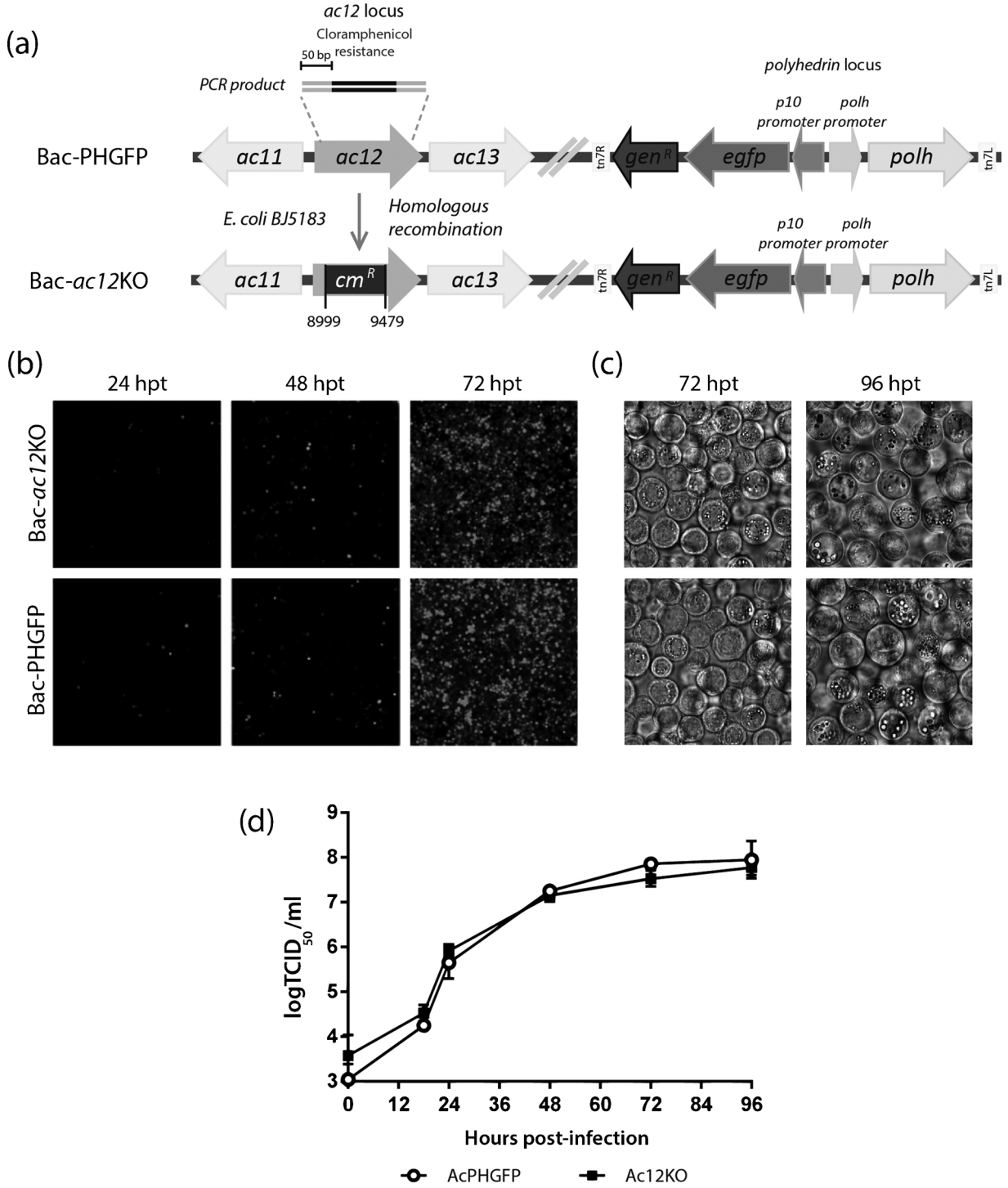

Fig. 2. Deletion of *ac12* does not affect AcMNPV replication in Sf9 cells. (a) Schematic diagrams of the *ac12*KO and PHGFP bacmid constructs. (b) Transfection of either Bac-PHGFP or Bac-*ac12*KO in Sf9 cells. Virus replication and dispersion can be observed by the intracellular fluorescence of eGFP. (c) Capacity of polyhedra production as seen in bright field microscopy. (d) One-step curves obtained by infection with AcPHGFP and Ac12KO at high MOI in Sf9 cells.

orichalcea nucleopolyhedrovirus (ThorNPV), Dendrolimus kikuchii nucleopolyhedrovirus (DekiNPV), Cyclophragma undans nucleopolyhedrovirus (CyunNPV), Dasychira pudibunda nucleopolyhedrovirus (DapuNPV), Lymantria xylina multiple nucleopolyhedrovirus (LyxyMNPV) and in one granulovirus, Erinnyis ello granulovirus (ErelGV). Except for BmNPV sequence, which lacks an internal fragment of 364 nt, the nucleotide alignment showed identities from 50 to 99%. The comparison of the predicted amino acid sequences showed identities ranging from 30%, with distant viruses such as ErelGV, DapuNPV, LdMNPV and LyxyMNPV, to 40%–50%, with ThorNPV, DekiNPV and CyunNPV, and higher than 90%, with the more related baculoviruses PlxyMNPV and RoMNPV. BmNPV sequence was discarded from the analysis because it was not possible to find ORFs of at least 50 amino acids in the sequence. Remarkably, a highly conserved F-box domain (Kipreos and Pagano, 2000) was predicted in all sequences (Fig. 6a). The identity percentage in this region compared with AcMNPV ranged from 40 (for LdMNPV and LyxyNPV) to 100% (for PlxyMNPV). In AcMNPV, the beginning of the F-box motif coincides with the

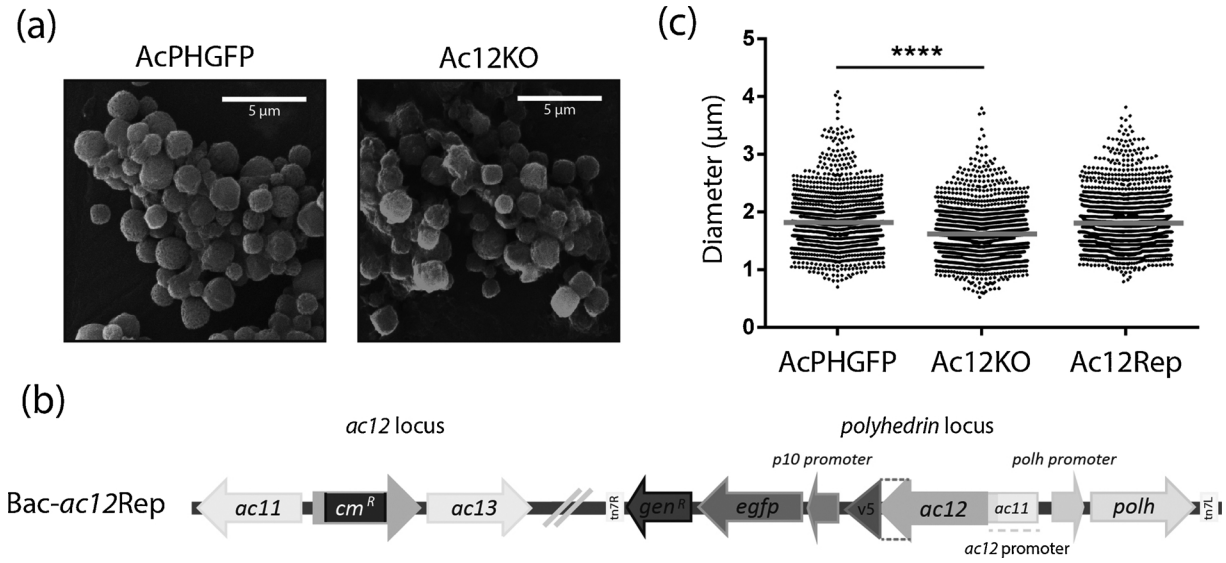

Fig. 3. Polyhedra characterization by scanning electron microscopy. (a) Polyhedra purified from infected Sf9 cells. Images taken with a scanning electron microscope. (b) Schematic diagrams of the *ac12*Rep bacmid construct. (c) Quantification of polyhedra diameters. Average value is indicated by a grey line. $p < 0.0001$.

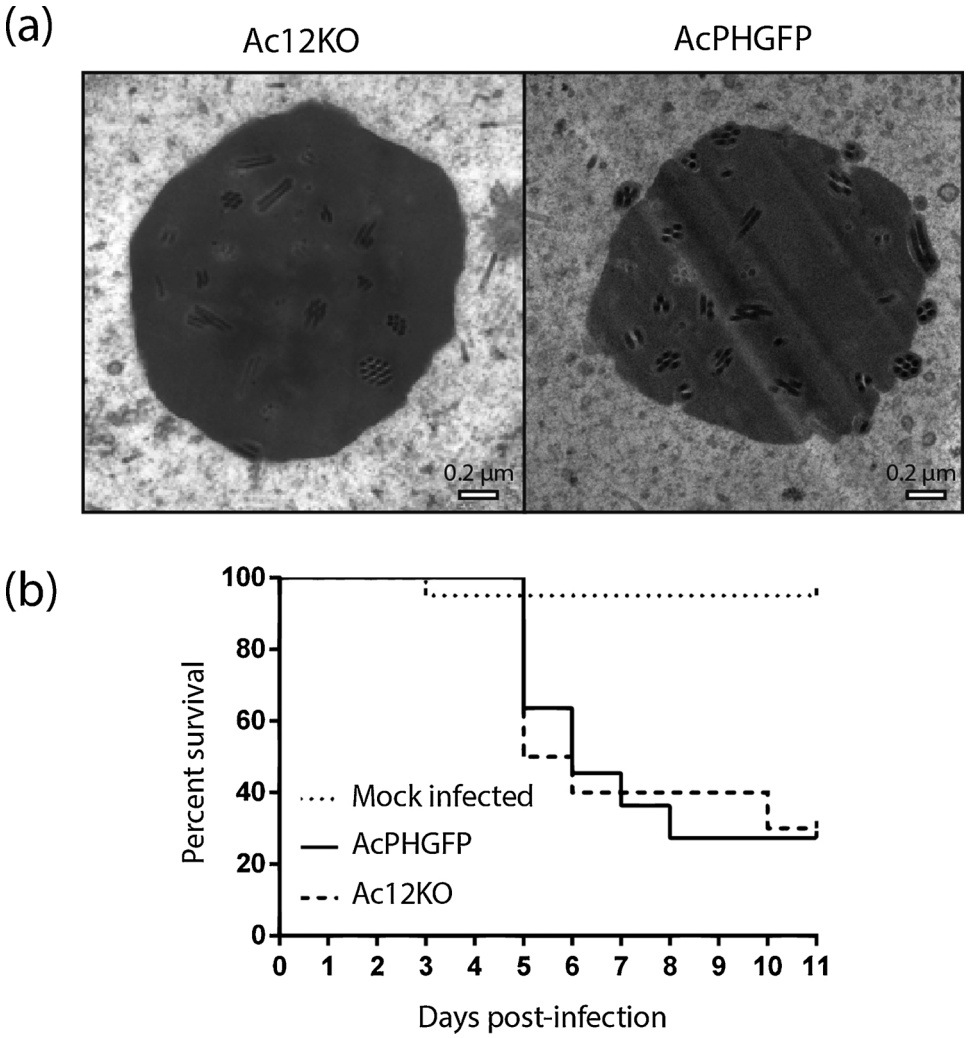

Fig. 4. Polyhedra assembly, occlusion, and oral infectivity. (a) Transmission electron microscopy of polyhedra from Sf9 cells infected with either Ac12KO or AcPHGFP viruses. (b) Survival curves of the infectivity assays performed on third-instar *Rachiplusia nu* larvae infected with occlusion bodies from Ac12KO or AcPHGFP viruses. The larvae were orally infected with 100 polyhedra each.

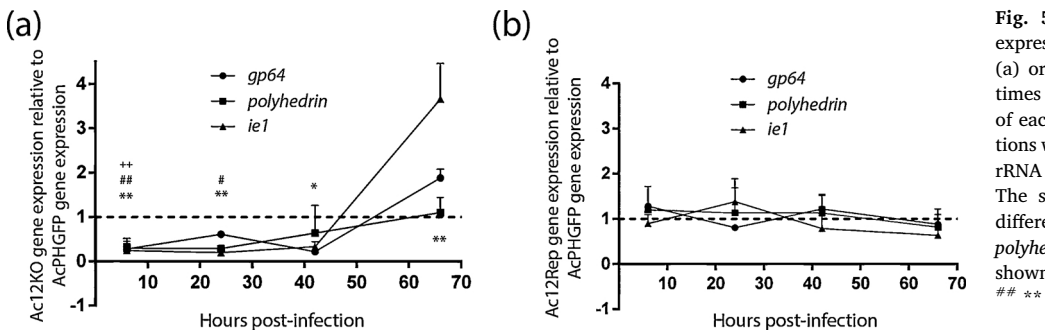

Fig. 5. Quantitative RT-PCR analysis of the expression of three viral genes in the Ac12KO (a) or Ac12Rep (b) baculoviruses at different times post-infection relative to the expression of each gene in the AcPHGFP virus. The infections were performed using a MOI of 5. The 28S rRNA sequence was used as a reference gene. The symbol * was used to show significant differences between the viruses for *ie1*, # for *polyhedrin* and the symbol + for *gp64*. Data shown are mean ± SD (n = 3). $^{\#}$ *p < 0.05, $^{\#\#}$ ** $^{++}$p < 0.01.

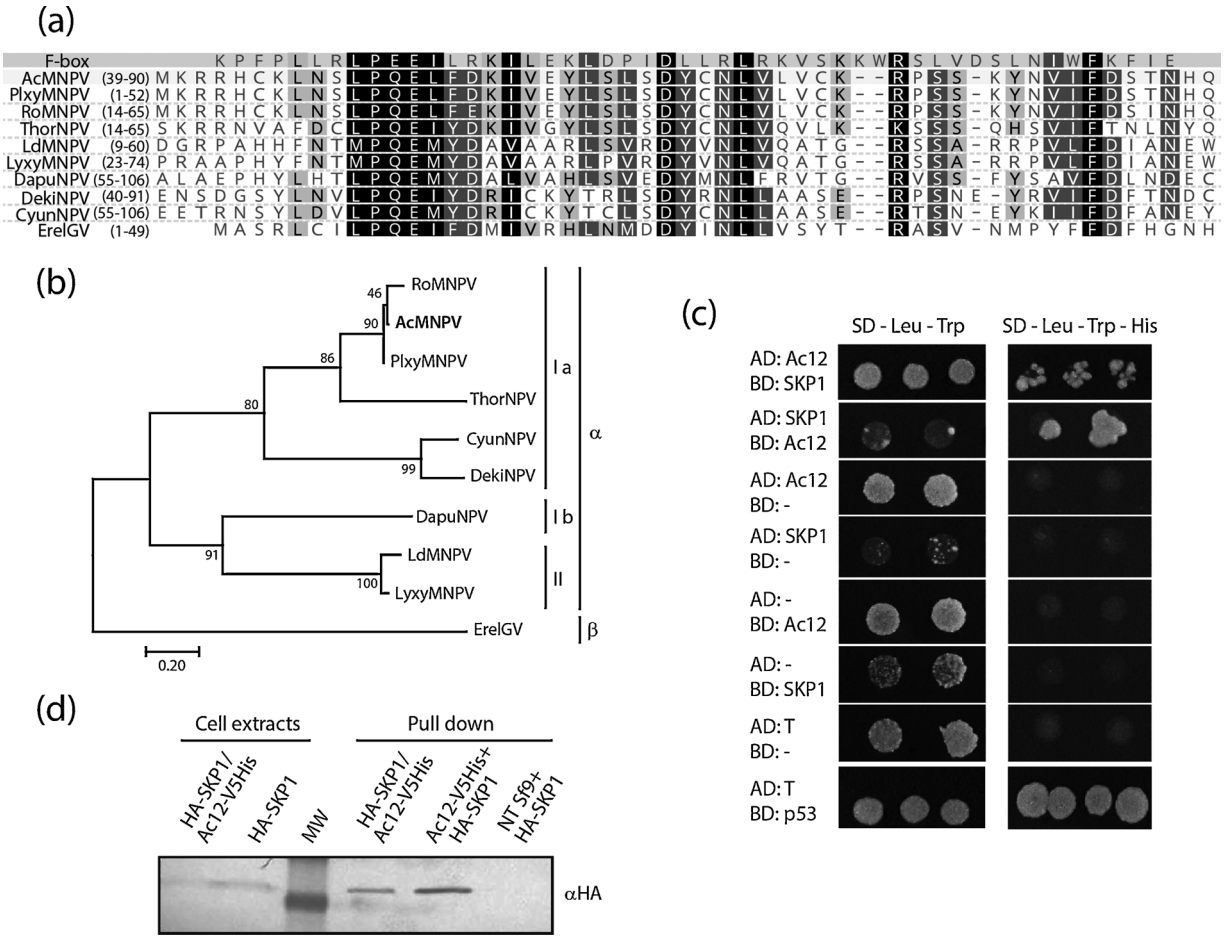

Fig. 6. Ac12 analysis. (a) Alignments of F-box residues of Ac12 homologous proteins in other baculovirus species and the F-box consensus sequence. Different tones of gray indicate degree of conservation, where black is 100% similar, dark grey is 80–100% similar, light grey is 60–80% similar and not coloured is less than 60% similar. (b) The evolutionary history of the deduced amino acid sequence of Ac12 and its homologous was inferred by using the Maximum Likelihood method. The tree with the highest log likelihood (-2460.34) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. (c) Yeast two-hybrid assay between Ac12 and SKP1 proteins. The last 6 rows are technique controls (d) Immunodetection of HA tag of in vitro and in vivo pull- down assays. Left part of the blot: extracts of Sf9 expressing HA-SKP1 or HA-SKP1/Ac12-V5-His. Right part of the blot: Ac12-V5-His were immobilized using a Ni-NTA matrix and the capture of HA-SKP1 was evaluated. HA-SKP1/Ac12-V5-His: pull down from co-expressing Sf9 cells. Ac12-V5-His + HA-SKP1: HA-SKP1 pull down with previously immobilized Ac12-V5-His. NT Sf9 + HA-SKP1: HA-SKP1 pull down with extract from non-transfected Sf9. The band of the marker corresponds to 17 kDa.

translation start site of the detected putatively functional protein. A maximum likelihood tree based on the homologous nucleotide sequences of *ac12* (Fig. 6b) allowed us to infer an evolutionary history based on this gene.

3.8. Interaction of Ac12 with Sf9-SKP1

In host cells, the SKP1-cullin 1-F-box protein (SCF) type of E3 ubiquitin ligase complex binds and transfers ubiquitin to its substrates, thus leading to degradation by the proteasome. The SCF complex consists of a scaffold protein, an Rbx1 protein that recruits the E2 enzyme, and SKP1 as the adaptor protein to bridge F-box proteins. F-box proteins target certain regulatory proteins such as ubiquitin substrates that are critically involved in cell cycle regulation (Hershko and Ciechanover, 1998; Hoeller and Dikic, 2009; Komander and Rape, 2012). In order to establish if Ac12 F-box domain can interact with the cellular ubiquitination complex through SKP1 protein, we first performed yeast two-hybrid (Y2H) assays. Ac12-SKP1 interaction was

clearly evidenced by yeast growing in SD-Leu-Trp without-histidine (Fig. 6c).

Furthermore, in vitro and in vivo polyhistidine pull-down assays were performed to confirm the interaction. Sf9 cells expressing either, Ac12-V5-His, HA-SKP1 or co-expressing Ac12-V5His and HA-SKP1 were lysated. In vitro pull-down results showed the capturing of HA-SKP1 by immobilized Ac12-V5-His using a Ni-NTA resin. In the in vivo pull down the interacting proteins were bound to Ni-NTA resin. A specific band corresponding to HA-SKP1 was detected in the elution fraction only when Ac12-V5-His was present (Fig. 6d).

Altogether, the results strongly support the existence of a functional F-box motif in Ac12 protein that interacts with cellular SKP1.

4. Discussion

In this work we studied *ac12* for the first time. The existence of a gene cluster (ORF1-ORF12) that could be transcriptionally co-regulated with synchronized expression has been previously suggested (Jiang et al., 2006). Nevertheless, transcriptomic studies of infected *Trichoplusia ni* cells have demonstrated differential temporal expression of the genes included in this part of the genome (Chen et al., 2013). We were able to detect *ac12* transcription in early steps of the infection, from 6 hpi, and through all the viral cycle. Chen et al. (2013) classified *ac12* as an early gene because they did not find canonical late or early motifs upstream of its sequence. In that study, however, they also mentioned that its transcription profile is similar to that of the very late gene *polyhedrin*. Our analysis of the *ac12* sequence evidenced an early promoter element upstream of the putative ORF start and nearby the TATA box sequences. This finding supports the observed kinetics of the transcription and suggests that RNA polymerase II participates in this process. Through 5' RACE analysis, we also studied its transcription start site but did not find a single start nucleotide position. Our results are in agreement with those of Chen et al. (2013), who found more than one TSS by using different approaches. One TSS, beginning upstream of the putative ATG codon, was detectable in a very low amount, whereas the other one, more abundant, did not encompass that start codon. By contrast, a single nucleotide position was identified as the 3' end of this mRNA, 3 nucleotides downstream of the polyadenylation site obtained for the transcriptome of AcMNPV (Chen et al., 2013). Thus, the smaller transcript would result in a protein 38 amino acids shorter and in frame with the first one. Even though a variety of modifications were made in the 5'RACE protocol to avoid technical artifacts owing to stable secondary structures of the RNA, methodological impediments cannot be ruled out. Alternative starts in baculoviral genes have been already described. In some cases there are late promoters with nested early promoters that are switched on or off depending on the state of the infection cycle (Chang and Blissard, 1997; Chisholm and Henner, 1988). For example, the *p34* gene in OpMNPV is a viral transactivator and its amino-terminal truncated version acts as an inhibitor of the complete form (Wu et al., 1993). Besides, a protein isoform without a functional domain could allow a negative regulation mechanism. In the case of *ac12*, our results suggest the co-existence of transcripts with differential beginnings at 48 hpi. Further studies are needed to elucidate the participation of both transcripts to define their biologic roles and their representation into the course of the infection.

The sequence analyses showed that ORF12 is conserved in seven other alphabaculoviruses of group I (PlxyMNPV, RoMNPV, BmNPV, ThorNPV, DekiNPV, CyunNPV and DapuNPV), in two of group II (LdMNPV, LyxyMNPV) and in one betabaculovirus (ErelGV). Indeed, according to these analyses, LdMNPV, LyxyMNPV and DekiNPV contain *ac12* homologues at different genome localization and in opposite orientations. Except for the BmNPV sequence, in which the central region of the gene is missing, the amino acid alignment of Ac12 and its homologous showed different start sites of the predicted ORFs and a highly conserved F-box motif. Phylogeny studies of the complete genomes have classified *ac12* as an independently gained gene in AcMNPV and LdMNPV linages (Herniou et al., 2003); which suggests that the gene could be beneficial for the development of some viral functions that we have not covered in this study.

The study of baculoviral genes by using a knockout bacmid obtained in a bacterial system is a widely used strategy. In this work we replaced *ac12* with a resistance bacterial cassette by homologous recombination and compared its performance vs a control bacmid. Transfection of the recombinant genome in insect cells (Sf9) evidenced that *ac12* is a non-essential gene. The replication curve of the *ac12*-deleted virus suggests that this gene is not involved in the dispersion ability and in the amount of budded viruses produced. Indeed, there are no differences in the ability and temporality of the production of occlusion bodies. The appearance of infected cells presenting a virogenic stroma with rod shaped nucleocapsids and virions acquiring nuclear envelopes allowed us to conclude that the *ac12* deletion does not affect cellular modifications through infection. Many examples in the literature have reported deletion of certain baculoviral genes without an evident effect in cell cultures, some of them involved in regulatory processes or in vivo infectivity. For example, the deletion of *ac130* does not affect viral production, viral DNA replication or viral gene transcription but causes a delayed mortality in *Spodoptera exigua* larvae (Yang et al., 2014). Liang et al. (2015) and Li et al. (2008) observed similar effects with *an ac124* deletion and an *ac68* deletion, respectively, in *Trichoplusia ni*. Also, Liu et al. (2016) detected more genes (i.e. *ac110*) whose deletions did not alter the infection ability in larvae by the haemocoelic route but affected it by the oral route. The TEM and SEM observations of this study showed that the *ac12*KO viruses are able to produce occlusion bodies, with enveloped virions inside them, morphologically similar to those obtained with the control virus. These results show that *ac12* is not involved in the most relevant process of ODV morphogenesis and their subsequent inclusion in the polyhedrin matrix. Nevertheless, through SEM observations of the purified polyhedra, we could detect a minor but significant decrease in size of the *ac12*KO compared with control polyhedra. These results suggest that *ac12* is involved in regulatory pathways of polyhedrin production or in its assemble into polyhedra, with no consequences in viral occlusion. Polyhedrin point mutations that alter OB size have been described (Lin et al., 2000; López et al., 2011; Ribeiro et al., 2009). Except for *ac43*, whose deletion results in significantly larger OBs (Tao et al., 2013), to our knowledge, however, no other baculoviral gene influencing the polyhedron size has been reported yet. In our work, bioassays performed by infecting *R. nu* larvae by the oral route demonstrated that the effect of *ac12* deletion is restricted to a mild morphogenesis alteration but does not alter the lethality of polyhedra in the larval species under the conditions tested.

Some baculoviral genes have no obvious roles in the infectious cycle or have regulatory functions with slight viral replication effects. *Bm59* from BmNPV is nonessential for viral replication and dispensable for occlusion body morphogenesis in culture cells (Hu et al., 2016). An *ac43* deletion resulted in an altered polyhedrin expression, which yielded lower polyhedron production without affecting the in vivo mortality curves (Tao et al., 2013). On the other hand, a *pp31* knockout derived in small differences in viral replication and a slight decrease in transcription of many early and late genes (Yamagishi et al., 2007). Our quantitative RT-PCR results evidenced regulatory effects of *ac12* on, at least, some representative genes of immediate early, early, late and very late stages of the viral cycle that seemed not to affect budded virus production in the studied cells. This was evidenced by a viral growth curves that remained unaltered. The observed delay in reaching the required levels of *polyhedrin* transcription may produce the alteration of the polyhedron size.

Until now, no Ac12 protein product has been detected. In infected cells, we detected a tagged Ac12 peptide regulated from its own promoter. In accordance with proteomic results previously obtained in both viral phenotypes, we were unable to detect Ac12 in an immunoblot assay of purified budded- and occlusion- derived Ac12V5 viruses. This result suggests Ac12 is not a virion structural protein

(Braunagel et al., 2003; Wang et al., 2010). The molecular weight of the observed fusion protein in infected cells coincided with a transcript with the downstream TSS detected by 5'RACE. Noticeably, this alternative initiation codon would give rise to a protein with the same open reading frame as the peptide postulated from the genome analysis (Ayres et al., 1994) and beginning in the predicted F-box domain. The latter belongs to the F-box family of proteins that act as mediators of protein-protein interactions in some biologic processes as ubiquitination, transcriptional elongation and translation repression. F-box proteins bind SKP1, which is a component of SCF complexes that mediate ubiquitination of target proteins that are degraded via the ubiquitin proteasome system (UPS), and provide specificity to the E3 ligase complex since they interact with the substrate to be ubiquitinated. F-box domains are also present in viral proteins from vertebrate viruses and their presence is a common strategy in the interference of interferon pathways (Correa et al., 2013). Polerovirus and enamovirus, viruses that infect plants, have P0 F-box like protein acting as an RNA silencing suppressor through its direct or indirect interaction with AGO1 protein from RISC complexes (Fusaro et al., 2012; Pazhouhandeh et al., 2006). In view of the existence of this conserved sequence in Ac12 protein, we hypothesized that Ac12 could interact with cellular protein SKP1. F-box proteins act binding SKP1 and the substrate that will be ubiquitinated. Yeast two-hybrid, in vitro and in vivo pull down results revealed this positive interaction. Thus, Ac12 may act as a regulator factor for cellular responses to viral infection. AcMNPV could also hijack the UPS machinery to degrade viral proteins when they are not necessary or even interfere with infection. In this way, Ac12 could interact with cellular or viral proteins to be ubiquitinated and degraded. In AcMNPV, two genes codify F-box like proteins: *ac12* and *lef-7*. The protein product from *lef-7* also interacts with SKP1 from insect cells and regulates the DNA damage response thus diminishing its antiviral effects (Mitchell et al., 2013). This effect might be achieved by inhibiting histone H2AX phosphorylation (Mitchell et al., 2013). Lef-7 showed differential infection phenotypes, depending on the cell system used. In Tn368, viral replication of Lef7KO virus remains unaltered, whereas in Sf21 and Se1c, viral titers were reduced by 90% (Chen and Thiem, 1997). The authors concluded that *lef-7* is a regulatory gene with a host dependent function. Even though *ac12* does not play a relevant role in *Spodoptera frugiperda* cells, the study of its function in other cell systems could provide evidence of other roles. Alternatively, the *ac12* function could be rescued by *lef-7*; which would imply that both proteins play a complementary role.

In sum, this work suggests that *ac12* codifies a polypeptide, with a conserved F-box motif, that is implicated in the regulation of the viral cycle, possibly through the interaction with cellular SKP1, which is a component of UPS machinery. Ac12 is a non-essential protein with a slight effect on the morphogenesis of the occlusion bodies. Further studies could help us to elucidate the precise role of *ac12* in the viral cycle.

Conflict of interest

Authors declare no conflict of interest.

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References

Alfonso, V., Maroniche, G.A., Reca, S.R., Lopez, M.G., del Vas, M., Taboga, O., López, M.G., del Vas, M., Taboga, O., 2012. AcMNPV core gene ac109 is required for budded virion transport to the nucleus and for occlusion of viral progeny. PLoS One 7, e46146. https://doi.org/10.1371/journal.pone.0046146.

Ayres, M.D., Howard, S.C., Kuzio, J., Lopez-Ferber, M., Possee, R.D., 1994. The complete DNA sequence of Autographa californica nuclear polyhedrosis virus. Virology 202, 586–605. https://doi.org/10.1006/viro.1994.1380.

Beas-Catena, A., Sánchez-Mirón, A., García-Camacho, F., Contreras-Gómez, A., Molina-Grima, E., 2014. Baculovirus biopesticides: an overview. J. Anim. Plant Sci. 24, 362–373.

Braunagel, S.C., Russell, W.K., Rosas-Acosta, G., Russell, D.H., Summers, M.D., 2003. Determination of the protein composition of the occlusion-derived virus of Autographa californica nucleopolyhedrovirus. Proc. Natl. Acad. Sci. U. S. A. 100, 9797–9802. https://doi.org/10.1073/pnas.1733972100.

Braunagel, S.C., Summers, M.D., 1994. Autographa californica nuclear polyhedrosis virus, PDV, and ECV viral envelopes and nucleocapsids: structural proteins, antigens, lipid and fatty acid profiles. Virology 202, 315–328. https://doi.org/10.1006/viro.1994.1348.

Chang, M.J., Blissard, G.W., 1997. Baculovirus gp64 gene expression: negative regulation by a minicistron. J. Virol. 71, 7448–7460.

Chen, C.J., Thiem, S.M., 1997. Differential infectivity of two Autographa californica nucleopolyhedrovirus mutants on three permissive cell lines is the result of lef-7 deletion. Virology 227, 88–95. https://doi.org/10.1006/viro.1996.8341.

Chen, Y.R., Zhong, S., Fei, Z., Hashimoto, Y., Xiang, J.Z., Zhang, S., Blissard, G.W., 2013. The transcriptome of the baculovirus Autographa californica multiple nucleopolyhedrovirus in Trichoplusia ni cells. J. Virol. 87, 6391–6405. https://doi.org/10.1128/JVI.00194-13.

Chisholm, G.E., Henner, D.J., 1988. Multiple early transcripts and splicing of the Autographa californica nuclear polyhedrosis virus IE-1 gene. J. Virol. 62, 3193–3200.

Correa, R.L., Bruckner, F.P., de Souza Cascardo, R., Alfenas-Zerbini, P., 2013. The role of F-Box proteins during viral infection. Int. J. Mol. Sci. 14, 4030–4049. https://doi.org/10.3390/ijms14024030.

Di Rienzo, J., Robledo, W., Casanoves, F., Balzarini, M., Facultad de, C.A.N., 2000. FG Statistics, Infostat, Software estadístico.

Friesen, P.D., Miller, L.K., 1986. The regulation of baculovirus gene expression. Curr. Top. Microbiol. Immunol. 131, 31–49.

Fusaro, A.F., Correa, R.L., Nakasugi, K., Jackson, C., Kawchuk, L., Vaslin, M.F.S., Waterhouse, P.M., 2012. The Enamovirus P0 protein is a silencing suppressor which inhibits local and systemic RNA silencing through AGO1 degradation. Virology 426, 178–187. https://doi.org/10.1016/j.virol.2012.01.026.

Garavaglia, M.J., Miele, S.A., Iserte, J.A., Belaich, M.N., Ghiringhelli, P.D., 2012. The ac53, ac78, ac101, and ac103 genes are newly discovered core genes in the family Baculoviridae. J. Virol. 86, 12069–12079. https://doi.org/10.1128/JVI.01873-12.

Herniou, E.a, Olszewski, J.a, Cory, J.S., O'Reilly, D.R., 2003. The genome sequence and evolution of baculoviruses. Annu. Rev. Entomol. 48, 211–234. https://doi.org/10.1146/annurev.ento.48.091801.112756.

Hershko, A., Ciechanover, A., 1998. The ubiquitin system. Annu. Rev. Biochem. 67, 425–479. https://doi.org/10.1146/annurev.biochem.67.1.425.

Hoeller, D., Dikic, I., 2009. Targeting the ubiquitin system in cancer therapy. Nature 458, 438.

Hu, X., Shen, Y., Zheng, Q., Wang, G., Wu, X., Gong, C., 2016. Bm59 is an early gene, but is unessential for the propagation and assembly of Bombyx mori nucleopolyhedrovirus. Mol. Genet. Genomics 291, 145–154. https://doi.org/10.1007/s00438-015-1094-7.

Javed, M.A., Biswas, S., Willis, L.G., Harris, S., Pritchard, C., van Oers, M.M., Donly, B.C., Erlandson, M.A., Hegedus, D.D., Theilmann, D.A., 2017. Autographa californica Multiple Nucleopolyhedrovirus AC83 is a Per Os Infectivity Factor (PIF) Protein Required for Occlusion-Derived Virus (ODV) and Budded Virus Nucleocapsid Assembly as well as Assembly of the PIF Complex in ODV Envelopes. J. Virol. 91, e02115–16. https://doi.org/10.1128/JVI.02115-16.

Jiang, S.S., Chang, I.-S., Huang, L.-W., Chen, P.-C., Wen, C.-C., Liu, S.-C., Chien, L.-C., Lin, C.-Y., Hsiung, C.A., Juang, J.-L., 2006. Temporal transcription program of recombinant Autographa californica multiple nucleopolyhedrosis virus. J. Virol. 80, 8989–8999. https://doi.org/10.1128/JVI.01158-06.

Jones, D.T., Taylor, W.R., Thornton, J.M., 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199.

Kipreos, E.T., Pagano, M., 2000. The F-box Protein Family. Genome Biol. 1, reviews3002.1. https://doi.org/10.1186/gb-2000-1-5-reviews3002.

Komander, D., Rape, M., 2012. The ubiquitin code. Annu. Rev. Biochem. 81, 203–229. https://doi.org/10.1146/annurev-biochem-060310-170328.

Kost, T.A., Condreay, J.P., Jarvis, D.L., 2005. Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat. Biotechnol. 23, 567–575. https://doi.org/10.1038/nbt1095.

Kumar, S., Stecher, G., Tamura, K., 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol. Biol. Evol. 33, 1870–1874. https://doi.org/10.1093/molbev/msw054.

Li, G., Wang, J., Deng, R., Wang, X., 2008. Characterization of AcMNPV with a deletion of ac68 gene. Virus Genes 37, 119–127. https://doi.org/10.1007/s11262-008-0238-9.

Liang, C., Lan, D., Zhao, S., Liu, L., Xue, Y., Zhang, Y., Wang, Y., Chen, X., 2015. The Ac124 protein is not essential for the propagation of Autographa californica multiple nucleopolyhedrovirus, but it is a viral pathogenicity factor. Arch. Virol. 160, 275–284. https://doi.org/10.1007/s00705-014-2277-y.

Lin, G.Y., Zhong, J., Wang, X.Z., 2000. Abnormal formation of polyhedra resulting from a single mutation in the polyhedrin gene of Autographa californica multicapsid nucleopolyhedrovirus. J. Invertebr. Pathol. 76, 13–19. https://doi.org/10.1006/jipa.2000.4934.

Liu, J., Zhu, L., Zhang, S., Deng, Z., Huang, Z., Yuan, M., Wu, W., Yang, K., 2016. The Autographa californica multiple nucleopolyhedrovirus ac110 gene encodes a new per os infectivity factor. Virus Res. 221, 30–37. https://doi.org/10.1016/j.virusres.2016.05.017.

López, M.G., Alfonso, V., Carrillo, E., Taboga, O., 2011. Description of a novel single mutation in the AcMNPV polyhedrin gene that results in abnormally large cubic polyhedra. Arch. Virol. 156, 695–699. https://doi.org/10.1007/s00705-010-0885-8.

López, M.G., Diez, M., Alfonso, V., Taboga, O., 2018. Biotechnological applications of occlusion bodies of Baculoviruses. Appl. Microbiol. Biotechnol. 102, 6765–6774. https://doi.org/10.1007/s00253-018-9130-2.

Lykhova, A.A., Kudryavets, Y.I., Strokovska, L.I., Bezdenezhnykh, N.A., Semesiuk, N.I., Adamenko, I.N., Anopriyenko, O.V., Vorontsova, A.L., 2015. Suppression of proliferation, tumorigenicity and metastasis of lung cancer cells after their transduction by interferon-beta gene in baculovirus vector. Cytokine 71, 318–326. https://doi.org/10.1016/j.cyto.2014.10.029.

Mitchell, J.K., Byers, N.M., Friesen, P.D., 2013. Baculovirus F-box protein LEF-7 modifies the host DNA damage response to enhance virus multiplication. J. Virol. 87, 12592–12599. https://doi.org/10.1128/JVI.02501-13.

O'Reilly, D.R., Miller, L., Luckow, V.A., 1994. Baculovirus Expression Vectors: a Laboratory Manual. Oxford University Press.

Pazhouhandeh, M., Dieterle, M., Marrocco, K., Lechner, E., Berry, B., Brault, V., Hemmer, O., Kretsch, T., Richards, K.E., Genschik, P., Ziegler-Graff, V., 2006. F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function. Proc. Natl. Acad. Sci. U. S. A. 103, 1994–1999. https://doi.org/10.1073/pnas.0510784103.

Ramakers, C., Ruijter, J.M., Deprez, R.H.L., Moorman, A.F.M., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66. https://doi.org/10.1016/S0304-3940(02)01423-4.

Ribeiro, B.M., Generino, A.P.M., Acacio, C.N.L., Kalapothakis, E., Báo, S.N., 2009. Characterization of a new Autographa californica multiple nucleopolyhedrovirus (AcMNPV) polyhedra mutant. Virus Res. 140, 1–7. https://doi.org/10.1016/j.virusres.2008.10.010.

Rohrmann, G.F., 2013. Baculovirus Molecular Biology.

Salem, T.Z., Allam, W.R., Thiem, S.M., 2014. Verifying the stability of selected genes for normalization in q PCR experiments of Spodoptera frugiperda cells during AcMNPV infection. PLoS One 9, e108516.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671–675. https://doi.org/10.1038/nmeth.2089.

Shoji, I., Aizaki, H., Tani, H., Ishii, K., Chiba, T., Saito, I., Miyamura, T., Matsuura, Y., 1997. Efficient gene transfer into various mammalian cells, including non-hepatic cells, by baculovirus vectors. J. Gen. Virol. 78 (Pt 10), 2657–2664. https://doi.org/10.1099/0022-1317-78-10-2657.

Tao, X.Y., Choi, J.Y., Wang, Y., Roh, J.Y., Lee, J.H., Liu, Q., Park, J., Bin Kim, J.S., Kim, W., Je, Y.H., 2013. Functional characterization of Autographa californica multiple nucleopolyhedrovirus ORF43 and phenotypic changes of ORF43-knockout mutant. J. Microbiol. 51, 515–521. https://doi.org/10.1007/s12275-013-3058-0.

van Oers, M.M., Vlak, J.M., 2007. Baculovirus genomics. Curr. Drug Targets 8, 1051–1068.

Vanarsdall, A.L., Okano, K., Rohrmann, G.F., 2006. Characterization of the role of very late expression factor 1 in baculovirus capsid structure and DNA processing. J. Virol. 80, 1724–1733. https://doi.org/10.1128/JVI.80.4.1724-1733.2006.

Wang, R.R., Deng, F., Hou, D.H., Zhao, Y., Guo, L., Wang, H.L., Hu, Z.H., 2010. Proteomics of the Autographa californica nucleopolyhedrovirus budded virions. J. Virol. 84, 7233–7242. https://doi.org/10.1128/jvi.00040-10.

Wu, Q., Yu, F., Xu, J., Li, Y., Chen, H., Xiao, S., Fu, Z.F., Fang, L., 2014. Rabies-virus-glycoprotein-pseudotyped recombinant baculovirus vaccine confers complete protection against lethal rabies virus challenge in a mouse model. Vet. Microbiol. 171, 93–101. https://doi.org/10.1016/j.vetmic.2014.03.037.

Wu, X., Stewart, S., Theilmann, D.A., 1993. Alternative transcriptional initiation as a novel mechanism for regulating expression of a baculovirus trans activator. J. Virol. 67, 5833–5842.

Yamagishi, J., Burnett, E.D., Harwood, S.H., Blissard, G.W., 2007. The AcMNPV pp31 gene is not essential for productive AcMNPV replication or late gene transcription but appears to increase levels of most viral transcripts. Virology 365, 34–47. https://doi.org/10.1016/j.virol.2007.02.034.

Yang, M., Huang, C., Qian, D.D., Li, L.L., 2014. Functional characterization of Autographa californica multiple nucleopolyhedrovirus gp16 (ac130). Virology 464–465, 341–352. https://doi.org/10.1016/j.virol.2014.07.030.