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BRIEF REPORT

Effects of deletion of the ac109 gene of *Autographa californica* nucleopolyhedrovirus on interactions with mammalian cells

Victoria Alfonso[1] · Sabrina Amalfi[2] · María Gabriela López[1] · Oscar Taboga[1]

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Abstract Baculoviruses are able to enter into mammalian cells, where they can express a transgene that is placed under an appropriate promoter, without producing infectious progeny. ORF109 encodes an essential baculovirus protein that participates in the interaction of the baculovirus with mammalian cells. To date, the mechanisms underlying this interaction are not yet known. We demonstrated that although a Ac109 knock out virus maintained its ability to enter into BHK-21 cells, there was a marked reduction in the expression efficiency of the nuclear transgene. Moreover, the amount of free cytoplasmic viral DNA, which was detected by transcription of a reporter gene, was severely diminished. These results suggest Ac109 could be involved in maintaining the integrity of the viral nucleic acid.

Keywords Baculovirus · ac109 · mammalian cells

Autographa californica multiple nucleopolyhedrovirus (AcMNPV) is the prototype member of the *Baculovirade* family which comprises a variety of large DNA viruses that infect arthropods [9]. AcMNPV has important biotechnology applications as a biological control agent, recombinant protein producer and vaccine vector [17, 21, 25, 28]. Indeed, in the last years, AcMNPV has been proposed as vector for gene therapy in mammals because it can enter these cells, reach the nucleus and transcribe genes from mammalian promoters, without completing their full infectious cycle [11, 12]. More recently, some research groups have described the immunomodulation and adjuvant properties of baculoviruses in mammals and poultry [1, 20, 22]. Nevertheless, the interaction of AcMNPV with mammalian cells is not yet well understood.

In infected insect cells, baculovirus genomes are packaged in a proteinaceous capsid, and then enveloped in a lipid membrane. Two phenotypes can be found in the infectious cycle: budded viruses (BVs) and occlusion derived viruses (ODVs) [30]. In insects and mammals, BVs enter the cell by receptor-mediated endocytosis and, after a pH-dependent fusion of the envelopes, nucleocapsids escape from the endosomes and access the cytoplasm [4, 8, 14, 18, 24]. About four hours post infection the nucleocapsids reach the nuclear boundaries and enter into the nucleus through nuclear pores [13, 24]. Once in the nucleus, the viral genome is released from the capsid. The efficiency of this transduction process is low and seems to depend on the mammalian cell type; the proportion of cells expressing a transgene is therefore quite variable [4, 5]. The use of baculoviruses in gene therapy is favored by higher amounts of genomes reaching the nucleus. Furthermore, some authors have suggested that the presence of baculoviruses in the cytoplasm of mammalian cells would trigger an antiviral response that could be exploited [2, 10]. Thus, knowing in depth the proteins involved in the first events of the baculovirus infection, i.e. from when the virions enter cells until they reach the nucleus, could help to improve and expand the uses of baculoviruses in biotechnology.

ORF109 belongs to the group of baculoviral core genes that are present in all baculoviral sequenced genomes [27],

✉ Oscar Taboga
taboga.oscaralberto@inta.gob.ar

[1] INTA, CONICET, Instituto de Biotecnología, CICVyA, Nicolás Repetto y de los Reseros S/N, Hurlingham, CP 1686, Buenos Aires, Argentina

[2] INTA, Instituto de Biotecnología, CICVyA, Nicolás Repetto y de los Reseros S/N, Hurlingham, CP 1686, Buenos Aires, Argentina

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and codes for an essential structural protein, Ac109, which is localized in ODVs and in the nucleocapsids of BVs [3]. The deletion of *ac109* from the AcMNPV genome allows the replication of the transfected viral DNA in insect cells [3, 6, 15, 16]; and most of these reports showed that the nucleocapsids are able to assemble and that the virions bud from the cytoplasmic membrane [3, 6, 15]. Although indistinguishable from wild type budded viruses, these BVs are not infectious and therefore the infection cannot proceed beyond the initially infected cell. These BVs, thus, give rise to a single-cell phenotype mutant [3, 6, 15]. The Ac109 knock out (Ac109KO) mutants enter normally into insect cells but no nucleocapsid is detected inside the nucleus. This result indicates a failure, either in the escape of the Ac109KO virions from the endosome, or in the import of these virions into the nucleus, probably due to an alteration in the architecture of the virions [3].

In an attempt to understand the non-productive infectious process between baculovirus and mammalian cells, we studied the role of Ac109 in this interaction. In these type of cells only a low proportion of nucleocapsids reach the nuclei; unfortunately, the viral molecular factors that are implicated in this process are not well defined. Therefore, we assessed the entrance of Ac109KO virions into BHK-21 cells, a fibroblastic cell line derived from baby hamster kidney, as well as the presence of accessible viral genomes for transcription in the cytoplasm and in the nucleus.

For this purpose, we designed the recombinant baculovirus Ac109KO$_{T7}$Ch$_{CAG}$GFP (Ac109KO). In this construct, the synthetic CAG promoter [19], which is active in mammalian nuclei, drives the expression of the *egfp* gene, whereas the T7 promoter regulates the cytoplasmic transcription of *mCherry* gene when the T7 RNA pol is provided *in trans*. Both genes were placed into the polyhedrin locus by using Bac-to-Bac Baculovirus Expression System (Invitrogen, Life Technologies) technology. The recombinant baculovirus was obtained by using a previously constructed *ac109*KO bacmid [3]. The final bacmid which was obtained by transposition with a transference vector was purified with the Large Construct Kit (QIAGEN). Figure 1a shows the details of the construct. Ac$_{T7}$Ch$_{CAG}$GFP virus (AcC) was used as a control virus in all the experiments.

The Ac109KO non-infectious BVs were harvested and purified from culture supernatants of transfected Sf9 insect cells. The AcC BVs were obtained by infecting Sf9 cells and then quantified by end point assays. In order to employ the same amounts of AcC and Ac109KO viral particles in all the subsequent assays, we assessed the BVs yields by performing a quantitative PCR (qPCR) reaction. The BVs were concentrated by ultracentrifugation and viral DNA was purified as previously described [23]. The DNA was resuspended in distilled water and used as the template to quantify the *vp39* copies. Transfection of 5 x 10^6 Sf9 cells with 3 μg of Bac-ac109KO in three independent assays yielded 5.99 x 10^7 ± 5.47 x 10^6 viral genomes and 5 x 10^6 TCID$_{50}$ of AcC yielded 7.90 x 10^9 ± 7.25 x 10^8 viral genomes. Additionally, viral particles were visualized by immumodetection of GP64 membrane glycoprotein. In agreement with qPCR results, the viral load obtained from transfection of 5 x 10^6 Sf9 cells with 3 μg Bac-ac109K0 showed a western blot signal more intense than 2 x 10^4 TCID$_{50}$ of AcC and less intense than 1 x 10^5 TCID$_{50}$ of AcC.

By immunofluorescence, using a confocal microscope, we first evaluated the entrance and permanence of virions lacking the Ac109 protein in BHK-21 cells. For this purpose, AcC and Ac109KO BVs were adsorbed onto mammalian cells at 4 °C and then the cells were incubated for 3 or 24 h at 37 °C. Next, the cells were fixed and VP39, which is the major BV nucleocapsid protein, was detected using an anti-VP39 monoclonal antibody [29]. As shown in figures 2a and b, at 3 h post infection (hpi), the infected cells contained similar quantities of VP39 (56.13 ± 2.27 green spots/field for AcC and 57.47 ± 4.45 green spots/field for Ac109KO, from three independent experiments),

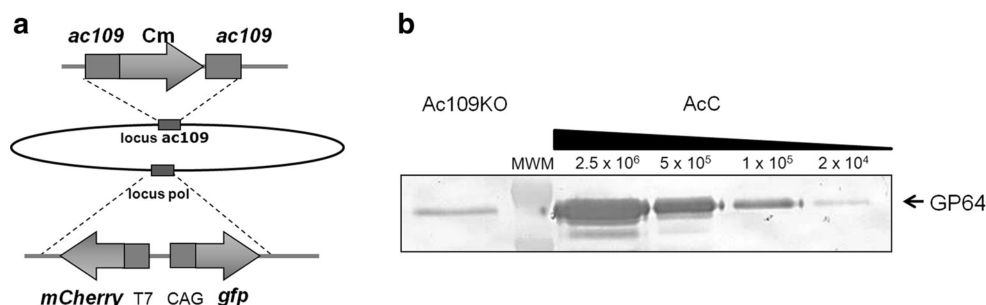

Fig. 1 Construction of Bac-*ac109*KO and quantification assays. (a) Schematic representation of the *ac109*KO bacmid. The *ac109* locus was previously interrupted by a Cm cassette and T7-mCherry and CAG-egfp constructs were inserted in the polh locus using the Bac-to-Bac system. (b) Western blot of Ac109KO and AcC BVs. Budded viruses from 5 x 10^6 Sf9 cells transfected with 3 μg of Bac-*ac109*KO, and 2.5 x 10^6, 5 x 10^5, 1 x 10^5 and 2 x 10^4 TCID$_{50}$ of AcC BVs were concentrated through a 25% w/v sucrose cushion and analyzed using a 12% acrylamide gel. GP64 protein (arrow) was detected with AcV5 antibody [11]. MWM: molecular weight marker

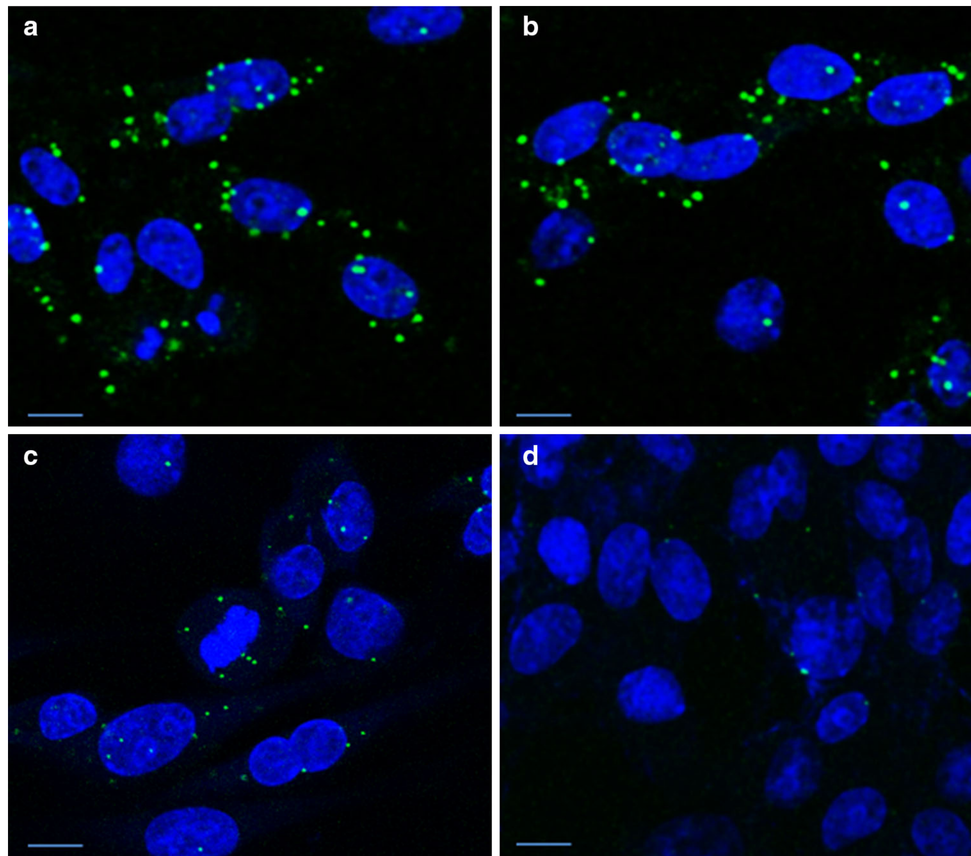

Fig. 2 Entry of Ac109KO BVs into mammalian cells. Immunofluorescence of BHK-21 infected with AcC or with Ac109KO BVs. 2.5 x 10^5 cells were incubated with BVs equivalent to 6 x 10^7 viral genomes of control (a and c) or Ac109KO (b and d) for 45 min at 4 °C and then rinsed twice with PBS. The cells were then incubated for 3 h (a and b) or 24 h (c and d) at 37 °C in DMEM supplemented with 2.5% FCS in a 5% CO_2 atmosphere. The fixed cells were treated with an anti-VP39 monoclonal antibody and an Alexa Fluor® 488 conjugated anti-mouse antibody. Nuclei were visualized by staining with TO-PRO®-3 (Invitrogen). Scale bars: 10 µm

either with Ac109KO or AcC BVs. No significant differences were found (Student's t-test). This finding strongly suggests that Ac109 is not essential for adsorbing onto and entering into BHK-21 cells.

These results are consistent with previous reports that state that the uptake of BVs in mammalian cells is driven by the same molecules and mechanisms as in insect cells [26]. This process requires an intact viral envelope exposing GP64 in the peplomers, a structure which is present in Ac109-defective particles. In baculovirus infections, the efficiency of reaching the nuclear pores in mammalian cells is much lower than in insect cells; thus at 24 hpi 55% of AcC virions remained detectable in BHK-21 cells. Nevertheless, only 11% of Ac109KO BVs were detectable at the same time point (the mean of spots were 30.83 ± 0.29 for AcC and 6.38 ± 0.21 for Ac109KO; $p < 0.001$ by Student's t-test). Figure 2 shows representative fields of BHK-21 cells for each condition. These results suggest that the lack of Ac109 in viral particles accelerates nucleocapsid disassembly of the virions that remain in the cytoplasm. An alternative explanation is that the lack of this protein improves the entrance and/or the disassembly of these virions into the nucleus.

Subsequently, we evaluated the efficiency of these recombinant baculoviruses to transcribe the nuclear transgene. For this purpose, we infected BHK-21 cells with equal amounts of AcC or Ac109KO BVs and recorded the number of eGFP positive cells 48 hpi. Three independent assays showed that upon infection the number of BHK-21 cells displaying eGFP fluorescence was higher in AcC baculovirus infected cells (0.0397% ± 0.0035%) than in Ac109KO infected cells (0.0004% ± 0.0004%) ($p < 0.01$). These data were obtained using BVs equivalent to 6 x 10^7 genomes of Ac109KO (or the equivalent AcC) in 2.5 x 10^5 cells. Figure 3a displays representative fields of view from both viral infections. These results indicate that the efficiency of reporter transcription, achieved in the nuclei of this type of mammalian cell, decreases up to 100-fold without Ac109. Thus, although the transcription of the *egfp* gene was markedly reduced, it was not completely eliminated. This result contrasts with the results obtained for insect cell infection [3, 6]. The decrease in

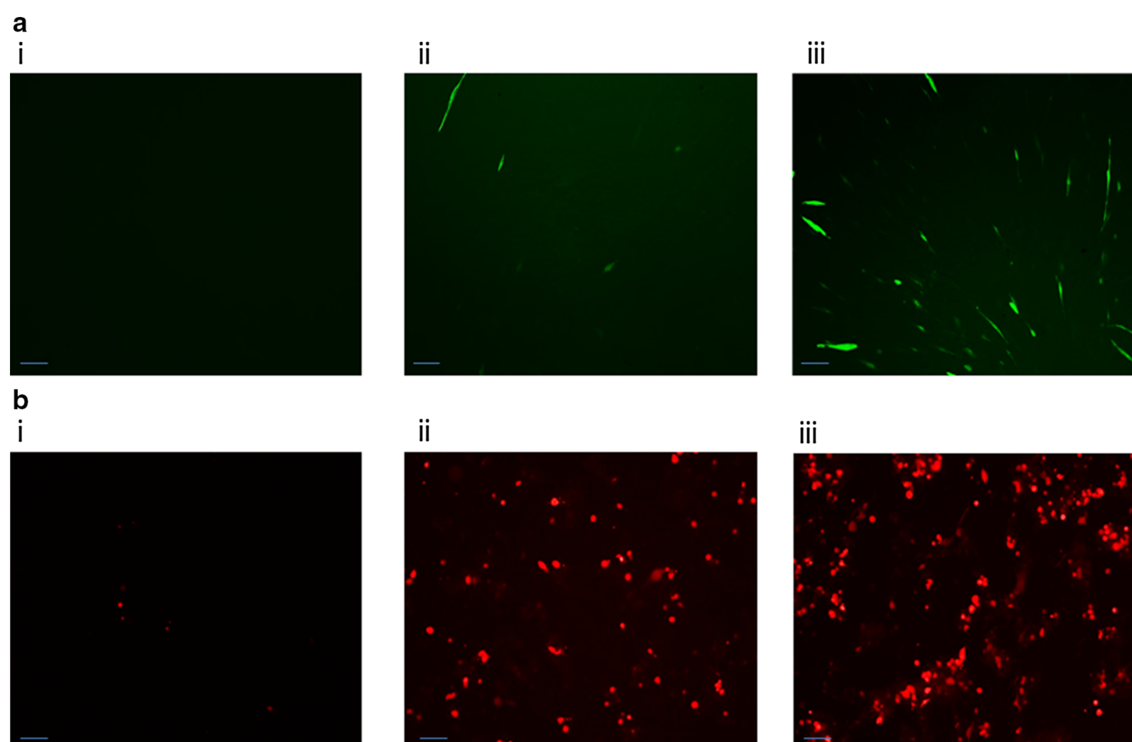

Fig. 3 Detection of viral genomes in the nuclei and cytoplasm of mammalian cells. (a) Nuclear transcription of viral DNA. BHK-21 cells were incubated with BVs equivalent to 6 x 10^7 viral genomes of Ac109KO (i), BVs equivalent to 6 x 10^7 viral genomes (ii) or 6 x 10^8 viral genomes (iii) of AcC for 4 h at 27 °C, and then for 48 h at 37 °C in DMEM supplemented with 2.5% FCS in a 5% CO_2 atmosphere. Next, the cells were examined for eGFP fluorescence and the positive cells were quantified. (b) Cytoplasmic transcription of viral DNA. BHK-21 cells were infected with VTF7-3 at a multiplicity of infection of 1 and incubated for 1 h at 37°C. Then, the viral inoculum was removed and BVs equivalent to 6 x 10^7 viral genomes of Ac109KO (i), BVs equivalent to 6 x 10^6 viral genomes (ii) or 6 x 10^7 viral genomes of AcC (iii) were added. The cells were incubated for 4 h at 27 °C and then for 24 h at 37 °C in DMEM supplemented with 2.5% FCS in a 5% CO_2 atmosphere. Next, the cells were examined for mCherry fluorescence and the positive cells were quantified. Scale bars: 50 µm

transgene expression indicates that there were fewer Ac109KO genomes available for transcription than in the control infection. The Ac109KO nucleocapsids did not accumulate in the cytoplasm (Figure 2). Altogether, these results suggest a role for Ac109 in maintaining the integrity of viral particles or viral genomes.

Next, we analyzed the presence of viral DNA in the cytoplasm for both virion types. For this purpose, we studied viral DNA transcription after infection in BHK-21 cells by assessing *mCherry* expression. Expression of this reporter gene is governed by a T7 RNA pol promoter, which is recognized by the T7 RNA polymerase. This polymerase is active in the cytoplasm and was provided by infection with the recombinant vaccinia virus VTF7-3 [7]. Figure 3b shows representative fields of view of viral infections performed with Ac109KO BVs, or with different amounts of AcC BVs. The analysis from three independent assays indicated that the percentage of fluorescent cells in a monolayer of 2.5 x 10^5 cells, upon infection with BVs equivalent to 6 x 10^6 genomes of AcC, was 0.58% ± 0.05%, whereas in the infection with Ac109KO (6 x 10^7 viral genomes) it was 0.024% ± 0.003% ($p < 0.01$). Thus, when the viral genome was assembled in Ac109KO virions, the availability of the viral DNA for cytoplasmic transcription decreased 242-fold. As the nuclear transcription was also affected in Ac109KO, the last results indicate a drop in the capability of the viral DNA to express a transgene, both in the nucleus and in the cytoplasm. These reduced capacities suggest an accelerated degradation of the nucleic acid in the absence of Ac109 protein, probably in association with a diminished stability of the viral particles. Faster capsid degradation cannot explain all of the decrease in genome transcription, as a 5-fold reduction in vp39 is detected at 24 hpi. This reduction could correspond with an improper viral particle conformation that promotes DNA degradation. In agreement with previous reports, the viral genomes in the control infection were detected at much higher levels (100-fold) in the cytoplasm than in the nucleus; suggesting that most viral particles are retained in the cytoplasm [13, 24, 26]. As mentioned before, the presence of the viral genomes was measured on the basis of accessibility for

gene transcription. In insect cells, Ac109 is necessary for the nucleocapsid to enter into the nucleus.

Here we demonstrated, using BHK-21, a mammalian fibroblast cell line, that Ac109 is required for efficient transgene expression in the nucleus and availability of the viral genome in the cytoplasm. This in turn could allow triggering of the innate immune response.

Altogether, this study shows that Ac109 could be important for baculovirus infection in mammals, where this viral vector has several biotechnological applications. Other proteins must also play important roles in the interaction of BV with mammalian cells. For this reason, future work should focus on studying the effects of different proteins on this interaction, by means of knocking out relevant baculoviral genes. This knowledge could, in turn, help us design improved gene therapy vectors and vaccines as well as to provide innovative antiviral strategies.

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Compliance with ethical standards

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Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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