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Trans-complementation of polyhedrin by a stably transformed Sf9 insect cell line allows occ− baculovirus occlusion and larval per os infectivity

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ABSTRACT

Oral infection of insect larvae with baculovirus is an advantageous methodology for producing high levels of recombinant proteins and for achieving plague control. However, many recombinant baculoviruses express a foreign protein in lieu of the polyhedrin and hence do not form occlusion bodies (occ−), resulting in extremely reduced *per os* infectivity in larvae.

To overcome this limitation, stably transformed insect cell lines expressing polyhedrin capable of occluding occ− recombinant baculovirus by trans-complementation were developed to obtain oral inoculum for insect larvae infection.

First, the optimum regulatory region of polyhedrin promoter was determined utilizing chloramphenicol acetyl transferase (CAT) as the reporter gene. After infection with occ− baculovirus, the higher expression levels of CAT were achieved when a region of 2735 bp that contained sequences known to have transcriptional enhancer functions were present upstream the polyhedrin coding sequence. This regulatory region was selected to drive polyhedrin expression in insect cell lines.

Transfection of Sf9 cells with plasmid carrying polyhedrin gene and stable cell lines established by selection with blasticidin showed polyhedrin expression and, moreover, crystalline polyhedron-like structures were visualized by optic microscopy. Oral infectivity was demonstrated by fluorescence detection in *Rachiplusia nu* larvae infected with occluded AcGFPpolh− baculovirus obtained using the system presented here.

1. Introduction

The baculovirus-insect cells system is one of the most powerful eukaryotic expression systems. Recombinant baculoviruses are widely used for successful expression of foreign genes from many different sources (Demain and Vaishnav, 2009). Although hundreds of foreign genes have been expressed in infected insect cells, the main disadvantage for mass production of foreign gene products using this system is its very high cost. Therefore, an *in vivo* system such as recombinant baculovirus-insect larvae results a very attractive alternative for the expression of large amounts of foreign proteins in an inexpensive way (Kost et al., 2005).

In nature, wild type baculoviruses are protected from proteolysis and other environmental hazards by occlusion bodies (OB) or polyhedra: a crystalline matrix of a protein coded by the viral polyhedrin gene (polh). When a susceptible insect ingests polyhedra, the alkaline conditions of its midgut dissolve the polyhedrin matrix, releasing the virions designated occlusion derived virus (ODV). Then, the ODVs cross the peritrophic membrane of the gut and infect epithelial cells. The viral DNA is transcribed and replicated, and new nucleocapsids (NC) are assembled in the nucleus. The NCs pass out of the nucleus and bud through the plasma membrane; this viral phenotype is called budded virus (BV). Later in infection, NCs that remain in the nucleus are enveloped by newly synthesized nucleus membranes. The resulting ODVs are then encased in newly made polyhedra (Theilmann et al., 2005). The BVs spread the infection from cell to cell within the insect and in cell cultures but will not infect an insect orally (Wood et al., 1993). In fact, injection of BVs in the haemolymph is the methodology of choice for infecting insect larvae.

Since the construction of most baculovirus expression vectors has been based on the replacement of the polyhedrin coding region with a foreign sequence under the transcriptional control of the polh promoter (Sun et al., 1997; Sriram and Gopinathan, 1998), the resulting recombinant baculoviruses are non-occluded (occ−). In the absence of a functional polyhedrin gene, the newly made virions cannot be assembled into OB, loosing their oral infectivity (Hamblin et al., 1990).

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The aim of the work presented here was to trans-complement occ− baculoviruses with polyhedrin, and to make them infectious to insects by the oral route.

The first step for the development of an insect cell line expressing polyhedrin was the design of a vector carrying the polh gene downstream regulatory regions known to bind cellular and viral factors needed for the correct expression of polyhedrin at very late stages of infection.

An Sf9 cell line expressing polyhedrin after infection was developed and used to produce occluded viruses genotipically occ−. These polyhedra were able to infect *Rachiplusia nu* larvae by the oral route.

This approach brings out a new methodology that can be used either to produce recombinant proteins or baculovirus as biopesticides (Moscardi, 1999).

2. Materials and methods

2.1. Cells and viruses

Spodoptera frugiperda Sf9 cells obtained from American Type Culture Collection (ATCC) were cultured at 27 °C as a monolayer in serum-free Sf900 II insect medium (Invitrogen) with antibiotic-antimycotic solution (GIBCO). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) wild type (wt) was obtained from Pharmingen. Wt and recombinant baculoviruses were propagated in Sf9 cells.

2.2. Construction of recombinant baculoviruses

Recombinant baculovirus AcGFPpolh− (occ−) was generated using the Bac-to-Bac methodology according to the supplier's suggestions (Invitrogen). The 716 bp eGFP coding sequence was amplified using oligonucleotides *GFP-for* (5′ TTCTAGAGTATGGTGAG CAAGGGC 3′) and *GFP-rev* (5′ TTCTAGACTTGTACAGCTCGTC 3′) both with underlined XbaI restriction enzyme site. eGFP gene was cloned into pGEMT-easy vector (Promega) and subcloned into pFastBac-1 plasmid (Invitrogen) using the XbaI site to obtain pFast-eGFP transfer vector. The recombinant bacmid DNA obtained was utilized to transfect Sf9 cells using Cellfectin® reagent (Invitrogen). Cells were incubated for 5 h at 27 °C after which the transfection medium was replaced by 5 ml of Sf900 II medium. After 5 days, the transfection supernatant was collected and after two passages in cells, the viral stocks were titered by end point dilution (O'Reilly et al., 1994).

For the construction of the recombinant occ+ baculovirus, eGFP coding sequence was amplified by PCR and cloned into pGEMT-easy vector. PGEM-eGFP was digested with BamHI and pBacPA8K vector (BD Biosciences) was digested with BglII. Both vector and insert were ligated to obtain plasmid pBacPAK-GFP. Recombinant AcGFPpolh+ baculovirus was obtained by cotransfection of Sf9 cells with pBacPAK-GFP and BacGOZA bacmid as previously described (Je et al., 2003).

2.3. Construction of CAT transfer vectors

Vector p_LCAT was obtained by substitution of the early promoter OpIE2 of plasmid pIB/V5-His/CAT (Invitrogen) with the polh promoter flanked by a NaeI site, 222 bp upstream the minimal polh promoter region of 95 bp. Vector p_SCAT was originated from p_LCAT by digestion with NaeI and EcoRV and religation, maintaining only the minimal polh promoter region upstream CAT.

A 700 bp region upstream the NaeI site of polh promoter (AcMNPV nucleotides 3497–4196), designated XL, was amplified by PCR using oligonucleotides *XL-for* (5′ AATCATGATTGCAAACGTGGTT TCGTGTGC 3′) and *XXL-rev* (5′ AATCATGAAACATATTGTACAAAACCG ACGA 3′) (BspHI sites are underlined) from plasmid pVL1393 (Pharmingen). Region XL was subcloned in pCR 2.1-TOPO vector (Invitrogen), and TOPO-XL was digested with BspHI to recover XL which was ligated to p_LCAT, digested with the same restriction enzyme. The resulting vector was named p_{XL}CAT.

Homologous region 1 (hr1) fragment (AcMNPV nucleotides 133696–682) was amplified by PCR from total AcNPV genomic DNA with oligonucleotides *hr1-FOR* (5′ ATTGATCATCATGAATCGAT GTTGACCCCAACAAAA 3′) (BclI and BspHI sites are underlined) and *hr1-REV* (5′ ATTGATCAATCGATTATTGCTCCA ATACTAG 3′) (BclI site is underlined). The 880 bp fragment was cloned in pCR 2.1-TOPO vector and then recovered by digestion with BclI. Hr1 was then introduced into pVL1393 plasmid digested with BclI to originate vector pVLhr1, used as a template for a PCR using oligonucleotides *hr1-FOR* and *XXL-rev* (5′ AATCATGAAACATATTGTACAAAACCGACGA 3′) (BspHI site is underlined). The amplification product of 2418 bp was designated XXL and was inserted upstream basic promoter into p_LCAT vector, to originate plasmid p_{XXL}CAT.

All transfer vectors were sequenced to confirm their identity. A simplified scheme of CAT-based constructions is presented in Fig. 1.

2.4. Transient transfections and CAT assay

1.5×10^5 Sf9 cells were seeded in each well of 24-well plates, transfected with 0.8 μg of vectors p_SCAT, p_LCAT, p_{XL}CAT and p_{XXL}CAT using Cellfectin® reagent and incubated at 27 °C. Four hours post-transfection (hpt) some of the transfected cells were infected with recombinant baculovirus AcGFPpolh− at a multiplicity of infection (moi) of 1. The remaining transfected cells were left uninfected. Sf9 cells, mock transfected cells, mock transfected and infected cells, and infected cells were included as controls. Each condition was assayed in triplicates. After treatments, cells were incubated at 27 °C in Sf900 II medium and harvested at 1, 2, 3, 4, 5, 6 and 7 days post-transfection (dpt) in reporter lysis buffer 1× (Promega). CAT expression and activity was quantitatively determined using 14C-chloramphenicol and n-butiryl CoA (Promega) as substrates.

The reaction product (n-butiryl-chloramphenicol) was separated from free chloramphenicol by differential solubility in organic solvents. Cell extracts were analyzed in a liquid scintillation counter EG&G/Wallac 1414-001 WinSpectral using a ^{14}C window. The results of CAT measurement in counts per minute (cpm) were averaged for the triplicates of each sample and standard deviation was obtained to plot the total cpm values of each samples.

2.5. Construction of p_{XXL}POL transfer vector and transient transfections

Viral polyhedrin gene (AcMNPV nucleotides 4515–5261) was amplified by PCR from total AcMNPV genomic DNA, using oligonucleotides *POLATG* (5′ TAAATATGCCGGATTATTCA 3′) and *POLSTOP* (5′ TGTTTTAATACGCCGGACCAG 3′) and was cloned in pCR 2.1-TOPO vector.

P_{XXL}CAT was digested with SpeI and XhoI to release the reporter gene, which was replaced by the polyhedrin coding sequence obtained by digestion of vector TOPO-POL with EcoRI. Ligation was performed after treatment of the vector and the insert with Klenow fragment, to generate p_{XXL}POL.

Transient transfections were carried out by seeding 7×10^5 cells per well in 6-well plates and transfecting them with 2.5 μg of plasmid and 10 μl of Cellfectin® per well. After 4 hpt, one well of each of the ones transfected with the plasmid was infected with AcGFPpolh− at a moi of 1, while the other was left uninfected. Cells were monitored every day using an inverted light microscope (Olympus LH50A) to observe polyhedra formation. Six dpt, cells

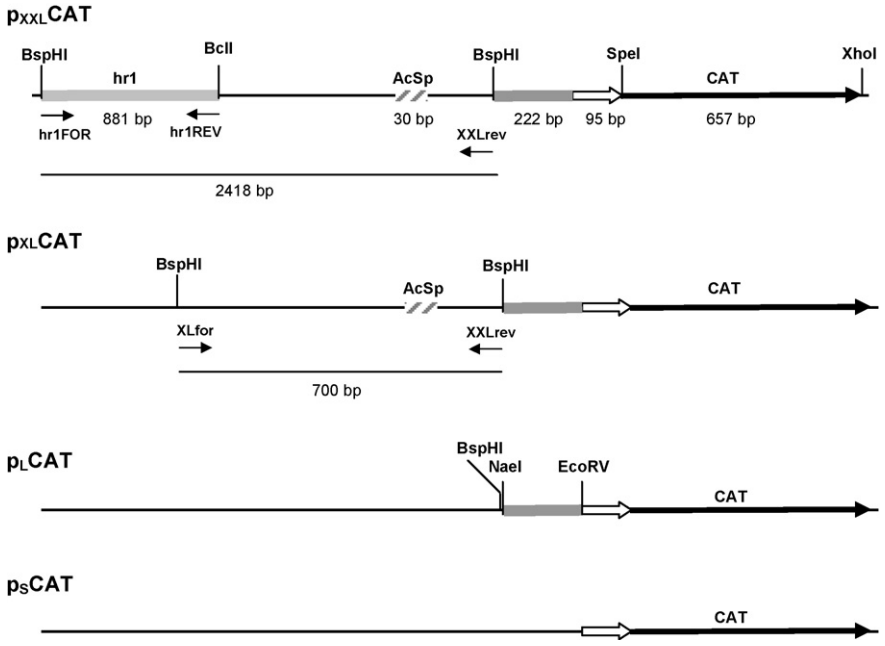

Fig. 1. Schematic drawing of plasmid constructs with different polyhedrin regulatory regions upstream reporter gene CAT. Open arrow corresponds to minimal polh promoter sequence of approximately 95 base pairs (bp). Small black arrows stand for designed oligonucleotides, previously detailed in Section 2.

were harvested in 1× PBS pH 6.2 and prepared for protein analysis in SDS-PAGE electrophoresis.

2.6. Selection of a stably transformed cell line

For the establishment of the polyhedrin cell line (designated Sf9polh line), 2×10^6 cells in 60 mm^2 culture dishes were transfected with 6 μg of plasmid p_{XXL}POL using 10 μl of Cellfectin® reagent for each reaction. Experimental controls consisted of: transfected cells without Cellfectin, mock transfected cells, and untreated cells. In parallel, Sf9 cells were transfected with pIB/V5-His-GFP plasmid construct to monitor the efficiency of transfection.

Recombinant cellular clones were selected by blasticidin resistance, starting from a concentration of 60 μg/ml blasticidin previously determined in a cell death curve (López, personal communication). Confluent monolayers were subsequently subcultured in larger plates, while blasticidin concentration was reduced up to 10 μg/ml, which was the concentration used to maintain cell lines.

2.7. Polyhedra samples

Polyhedra from Sf9polh line were prepared by infecting 3×10^7 cells grown in T150 flasks with AcGFPpolh− at a moi of 1. As a control, 3×10^7 Sf9 cells were infected with AcNPV wt. Five days post-infection (dpi), polyhedra were purified according to O'Reilly et al. (1994). Briefly, cells were sedimented at 5000 × *g* for 5 min and resuspended in 1 ml of 0.5% SDS. The lysate was centrifuged at 5000 × *g* for 10 min and resuspended in the same volume of 0.5 M NaCl. Polyhedra were sedimented at 5000 × *g* for 5 min, resuspended in 200 μl of ddH_2O and counted in a Neubauer chamber. For higher purity, polyhedra were loaded onto a linear 40–65% sucrose gradient and centrifuged at 96,000 × *g* at room temperature for 3 h.

In order to investigate the presence of occluded virus inside the polyhedra, approximately 1×10^8 wt or AcGFPpolh−/Sf9polh polyhedra were sedimented and resuspended in cracking buffer 1× (120 mM Tris–HCl pH 6.8, 4% SDS, 0.02% bromophenol blue, 1.4 M 2-β-mercaptoethanol, 20% glycerol). The samples were heated at 65 °C for 10 min to eliminate baculovirus that could be adsorbed on the polyhedra. After centrifugation at 9000 × *g*, the supernatant (S) was set aside and pellets were treated with 0.5 N Na_2CO_3 (P). Supernatant and pellet treatments were separated in polyacrilamide gels using Laemmli's discontinuous buffer system under denaturing conditions and gels were western blotted. VP39 was detected using a specific monoclonal antibody (1:2000) (Whitt and Manning, 1988) followed by an alkaline phosphatase-conjugated anti-mouse antibody (1:15000) (SIGMA).

2.8. Detection of polyhedrin expression

To analyze the expression of polyhedrin, transfected and infected Sf9 cells were collected at 6 dpi, washed with chilled PBS pH 6.2 and resuspended in 500 μl of cracking buffer. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes.

Polyhedrin was detected using a chicken-made polyclonal antibody (GenTex®) in a dilution of 1:1000 and a second alkaline phosphatase-conjugated anti-chicken antibody diluted 1:30000 (AXELL).

2.9. Insect larvae infection

R. nu larvae were kindly provided by technicians from INTA Saenz Peña, Chaco, who have a laboratory colony maintained on a high wheat germ semisynthetic diet (Ignoffo, 1963). Third-instar larvae were randomly selected and used in groups of twelve. After overnight starving, each group was orally infected with 20 μl of a suspension of 5×10^4 polyhedra absorbed on the diet. The same volume of PBS was used in negative controls. Infected larvae were monitored every day at UV light microscope (Olympus LH50A) using 10× magnification.

3. Results

3.1. Determination of the optimum regulatory region to be used in the stable Sf9 polyhedrin line: transient transfections using CAT reporter gene

To determine the regulatory regions upstream polh promoter needed for a correct polyhedrin transcription in stable lines, different constructions were obtained using pIB/V5-His/CAT vector as a backbone. Modifications consisted in the replacement of OplE2 promoter with polyhedrin regulatory regions of variable length. The resulting vectors were designated: p_SCAT, p_LCAT, p_{XL}CAT y p_{XXL}CAT, as the complexity of the regulatory region increased. The plasmid p_{XXL}CAT included the hr1 sequence, known to have transcriptional enhancer functions.

These constructions were individually transfected in Sf9 cells, and replicates were infected with baculovirus as described in Section 2. Transient expression of CAT was measured from the first to the seventh dpt.

The results showed that in the absence of baculovirus infection, none of the vectors carrying inducible promoters presented reporter activity. On the other hand, when transfected cells were also infected with baculovirus, all the constructions bearing polyhedrin regulatory regions induced CAT expression. Among the experimental constructions, the highest CAT activity was obtained when using vector p_{XXL}CAT. The high expression level observed was maintained until day 7 post-transfection. The other vectors, containing shorter inducible promoters, did not reach such levels of CAT expression, showing, most of them, a CAT activity peak at 6 dpt (Fig. 2).

These results demonstrated that the presence of additional regulatory regions upstream polh promoter play a positive role in the enhancement of CAT expression, differing from the other regulatory regions in intensity and time course expression achieved.

3.2. Polyhedrin expression in transiently transfected Sf9 cells infected with occ− baculovirus

Once the optimum regulatory region was determined, a definitive vector for polyhedrin expression in Sf9 cells was constructed as described in Section 2. Cells were transiently transfected with plasmid p_{XXL}POL in 6-well plates and infected with AcGFPpolh− baculovirus at a moi of 1. Transfected/infected cells were daily observed at light microscope and under UV light. In those cultures, green fluorescent cells were visualized from the second dpi. At 3 dpi, visible polyhedron-shaped structures were observed in p_{XXL}POL transfected/infected cells (Fig. 3A). No such structures were visible inside only transfected or infected cells (data not shown).

To determine if those intracellular structures consisted of polyhedrin protein, cells were collected and resolved in SDS-PAGE. Comparison of polyhedrin protein contents between p_{XXL}POL transfected cells with and without infection with AcGFPpolh− was made using a Western blot assay. AcNPV wt infected cells were used as positive control. The results are shown in Fig. 3B. A neat band of the electrophoresis mobility expected for polyhedrin appeared in p_{XXL}POL transfected/infected cells, similar to the one obtained by infection of Sf9 cells with wt baculovirus (Fig. 3B, lanes 2 and 4).

3.3. Stable transformation of Sf9 cells with polyhedrin allowed trans-complementation of occ− baculovirus and polyhedra formation

To establish a polyhedrin line, transfections with selected plasmid p_{XXL}POL were performed as described. Stable cell lines was selected using blasticidin.

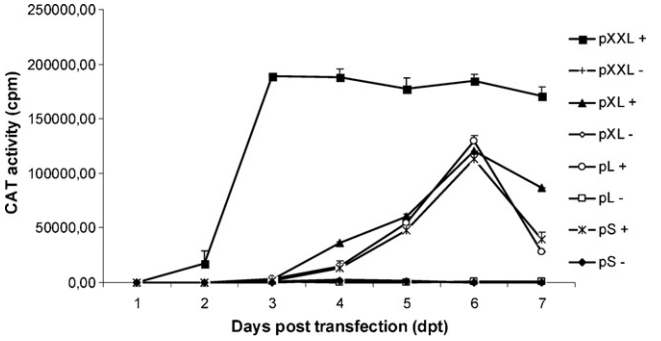

Fig. 2. Expression levels reached by different polh promoter constructions regulating CAT. The constructions depicted in Fig. 1 were used to transiently transfect Sf9 cells. CAT activity was measured for every construction assayed alone and followed by occ− baculovirus infection. Cell extracts were collected from 1 to 7 dpt. Signs + and − stand for infected or uninfected, respectively.

After infection with AcGFPpolh− baculovirus, the stably transformed Sf9polh line was visualized at light microscope every day. Sf9polh line showed polyhedrical and intracellular structures from the second to third day, similarly to transiently transfected/infected Sf9 cells (Fig. 3C). Detection of polyhedrin expression by Western blot in stably transfected cells confirmed the identity of those occlusion bodies (data not shown).

3.4. Polyhedra obtained by infection of the stably transformed cell lines occluded occ− baculovirus

To determine if polyhedra obtained from infected Sf9polh line occluded recombinant baculovirus, Western blot assays to detect the major protein of the capsid of AcNPV, VP39, were performed as described in Section 2.

Wt and Sf9polh/AcGFPpolh− infected polyhedra samples were treated with cracking buffer and heated at 65 °C and centrifugated to eliminate possible contaminating baculovirus from the surface of polyhedra. Supernatants (S) of this treatment were analyzed by SDS-PAGE and pelleted polyhedra (P) were dissolved in order to analyze its content by SDS-PAGE. The result is shown in Fig. 3D, where a clear band corresponding to VP39 appeared in the sample P obtained from infected Sf9polh line, as well as in the P control sample containing wt polyhedra. This result suggests baculovirus occlusion into polyhedra in the Sf9polh line.

3.5. Determination of the infectivity of polyhedra obtained using Sf9polh line in R. nu larvae

R. nu larvae starved overnight were fed with polyhedra of different origins: wt polyhedra, polyhedra obtained by infection of Sf9 cells with AcGFPpolh+ baculovirus and polyhedra obtained by infection of Sf9polh line with AcGFPpolh− baculovirus. A group of larvae were left uninfected as a control. Larvae were daily monitored by optic microscope visualization of fluorescence to follow infection progress. Fig. 4 shows one larva of each group as a representative of fluorescence detection under UV light. All larvae infected *per os* with polyhedra derived from the infected Sf9polh line as well as those infected with AcGFPpolh+, showed high GFP expression, indicating that AcGFPpolh− was successfully occluded and recovered its oral infectivity.

4. Discussion

The most widely used methodology to obtain recombinant baculoviruses is based on the allelic replacement of polyhedrin gene by the gene of interest in the viral genome. This methodology gener-

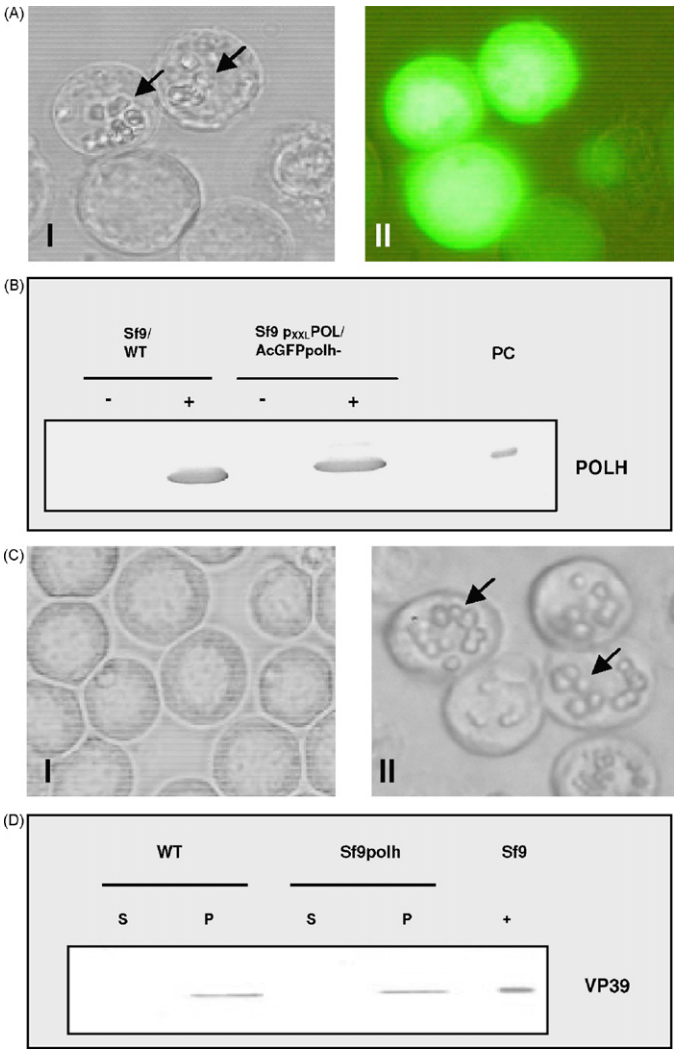

Fig. 3. Polyhedrical structures appeared inside Sf9 cells transfected with p_{XXL}POL construction and infected with occ− baculovirus. (A) Optical microscopies at 100× augment of Sf9 cells transiently transfected with p_{XXL}POL and infected with AcGFPpolh− baculovirus. Pictures were taken at 4 dpt. Arrows show refringent polyhedra inside transfected and infected cells; (I) under white light and (II) under UV light. (B) Polh expression detected in cell extracts. From left to right, lanes 1 and 2 correspond to a control of Sf9 cells uninfected (−) or infected with AcMNPV wt (+); lanes 3 and 4 correspond to Sf9 transfected with p_{XXL}POL plasmid, uninfected (−) or infected with AcGFPpolh− (+). PC at the right lane is a positive control of soluble polyhedrin protein. (C) (I) Sf9polh line, uninfected and (II) Sf9polh line infected with AcGFPpolh− baculovirus. Arrows indicate visible refringent polyhedra at 4 dpi. (D) VP39 detection in samples from the surface of Sf9polh-derived polyhedra (S) and from its content (P). Sf9 (+) is an extract of wt infected cells as a positive control. See Section 2 for detailed protocols.

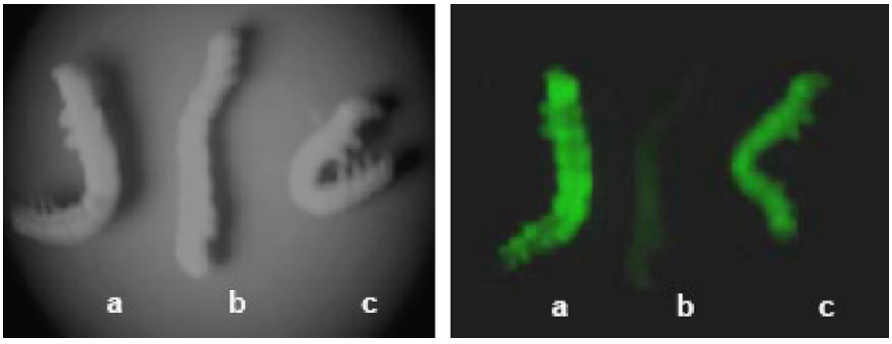

Fig. 4. Occ− baculovirus were infective for larvae by the oral route. Groups of twelve *R. nu* larvae were infected orally with polyhedra of different composition. After 4 dpi, infected larvae were visualized under UV light. (a) Larvae fed with polyhedra containing AcGFPpolh+. (b) Larvae fed with wt polyhedra. (c) Larvae fed with Sf9polh line-derived polyhedra containing AcGFPpolh−.

ates baculoviruses occ− which are unable to infect insect larvae by the oral route. Although recombinant baculoviruses preserving polh gene (occ+) are often used to overcome this problem (Luckow et al., 1993), the co-expression of polh gene may be disadvantageous. For heterologous recombinant protein production, it would be desirable to avoid competition between foreign mRNA and polh mRNA for the cell translational machinery (Chaabihi et al., 1993). On the other hand, when using recombinant bac-

uloviruses as biological *pesticides*, an occ+ virus could be not safe, as the subsequent generations would be able to propagate in nature.

Chen et al. (2009) were able to occlude *Bombyx mori* nucleopolyhedrovirus (BmNPV) by infection of a BmN cell line stably transformed by using a transposon-based methodology with the polyhedrin gene under the regulation of a constitutive promoter. However, the generation of a Sf9 cell line expressing polyhedrin only after AcNPV infection could be advantageous over polyhedrin constitutive expression due to the possible side-effects in cellular growth and viability. The expression of polyhedrin in a transformed Sf21 cell line after infection with a baculovirus has been previously proposed but not achieved by Nagamine et al. (1995). They informed absence of polyhedra in the infected cell line due to a lack or a very low expression of polyhedrin in the cellular genome context, even though it was regulated by its own promoter. Nevertheless, the inclusion of regulatory regions acting as enhancer elements could overcome this problem. Upstream polyhedrin (pu) sequences were identified as novel enhancer-like elements in AcNPV. It has been demonstrated that their presence allows the achievement of higher expression levels of foreign genes in transient expression assays (Wu et al., 2008), and this activity is due only to viral factors (Lo et al., 2002). In contrast to pu sequences, the known baculoviral enhancers hrs, which locate over 3 kbp upstream the polh gene in the AcNPV genome, can function independently of viral factors, indicating that they can respond to certain host factors (Guarino and Summers, 1986). It has been shown through plasmid-based transient expression assays that hr1 augments transcription from polh promoter in a position and orientation independent manner (Habib et al., 1996). This enhancement also occurs in non-insect systems in which baculoviral promoters are functional (Viswanathan et al., 2003). Another regulatory sequence that binds host cellular factors of the family of Sp proteins is believed to play an important role in transcription from polh promoter. This region, that is designated AcSp and contains the binding consensus of Sp1 protein, has been shown to enhance *in vivo* expression of a polh promoter-driven luciferase gene (Ramachandran et al., 2001).

In view of this evidence, we decided to develop a stably transformed Sf9 cell line expressing polyhedrin including baculoviral enhancer regions in the constructions. Our first step was to determine the upstream sequences that are sufficient to regulate polyhedrin expression. For that purpose, different regulatory regions were first analyzed in transient expression assays using CAT as reporter gene. The presence of a regulatory region that included sequences that bind viral and cellular factors (like AcSp and hr1) upstream polh promoter resulted in an increased transcription of CAT. Our result is in accordance to the ones reported by Habib et al. (1996), which demonstrated that hr1 sequence, carrying five 28-bp core palindromes, enhances expression from the very late polyhedrin promoter in a classical enhancer manner in transient expression assays.

After 4 dpi with an occ− baculovirus, Sf9polh line cells showed structures compatible with polyhedra, indicating that polyhedrin was able to trans-complement the polyhedrin-defective baculovirus and polyhedra morphogenesis was possible in the cell line. The presence of baculovirus inside these occlusion bodies was suggested by VP39 detection in Western blot assays and confirmed in bioassays performed with isolated polyhedra from the Sf9polh cell line infected with AcGFPpolh− baculovirus. Fluorescence of *per os* infected larvae showed that the inoculum obtained by this newly described technology resulted infectious *per os* to *R. nu* larvae.

The approach described here can be applied not only in the generation of oral inoculum for insect larvae for the production of high levels of recombinant proteins but also in the development of viral pesticides. Occluded occ− baculovirus cannot generate polyhedra by themselves. Thus, polyhedrin provided *in trans* by a stable insect cell line is also a powerful tool with broad potential uses in entomology biotechnologies.

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