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Characterization of a new *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) polyhedra mutant

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ABSTRACT

In the very late phase of baculovirus infection, virions are occluded in a crystalline matrix called polyhedra, which is mainly composed of polyhedrin. This protein is highly conserved among baculoviruses and changes in its amino acid sequence may lead to mutant polyhedra. During the purification of an AcMNPV recombinant virus, a mutant virus was isolated. Structural and ultrastrutural analysis by light and transmission electron microscopy (TEM) of insect cells infected with this mutant virus did not show polyhedra formation and differed from the wild-type infection by the presence of a proteinaceous mass dispersed in the cytoplasm and nucleus of the infected cells, which was confirmed by immunogold labelling to be polyhedrin. The polyhedrin gene was amplified by PCR and sequenced. The only change observed was the substitution of a G to a T at the nucleotide +352, which resulted in a Val to Phe change. A recombinant virus was constructed by transferring the mutant gene into a polyhedrin negative virus. The phenotype of this recombinant virus was the same as the mutant one, confirming that this single mutation alone was responsible for the mutant phenotype.

1. Introduction

Baculoviruses are insect viruses used as biological control agents (Moscardi, 1999) and vectors for the expression of heterologous proteins in insect cells in culture and insect larvae (Jarvis, 1997). These viruses have large dsDNA genomes, and enveloped rod-shaped nucleocapsids. The *Baculoviridae* family is divided into two genera: *Nucleopolyhedrovirus* (NPV) and *Granulovirus* (GV) (Theilmann et al., 2005). The *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV) is the type species of the NPV genus and is the most studied baculovirus to date.

Baculoviruses produce, during their replication cycle, two phenotypes: the extracellular or budded virus (ECV or BV), responsible for the spread of infection within the insect larvae and the occluded derived virus (ODV), responsible for the infection from insect to insect. The BV is produced early in infection and the ODV is produced late in infection, inside the nucleus of infected cells (Harrap, 1972). The ODVs are found occluded in a protein matrix made of a protein called polyhedrin forming occlusion bodies, also called polyhedra (Maruniak, 1986) which are responsible for protecting the virions in the environment before a new susceptible host is infected (Steinhaus, 1960). The polyhedrin protein has a molecular mass of around 29 kDa and it is highly expressed late in infection of insect cells.

Polyhedra formation seems to depend on interactions between polyhedrin and other proteins present on the virions envelope (Woo et al., 1998). However, the polyhedra morphology seems to take into account not only polyhedrin interactions with other viral and or host cell proteins (Woo et al., 1998), but its own amino acid sequence (Carstens et al., 1992; Cheng et al., 1998; Hu et al., 1999).

Polyhedrin is a highly conserved protein among baculoviruses and amino acid changes in the protein sequence could lead to the formation of mutant viruses with morphologically distinct polyhedra (Smith et al., 1983; Katsuma et al., 1999). A domain required for supramolecular assembly of the polyhedra has been proposed for AcMNPV's polyhedrin (Jarvis et al., 1991).

Some AcMNPV polyhedra mutants have been isolated and characterized (Brown et al., 1980; Carstens, 1982; Duncan and Faulkner, 1982; Duncan et al., 1983), with most of them containing point mutations in the polyhedrin gene sequence responsible for the altered phenotype of the polyhedra (Carstens et al., 1986, 1987, 1992; Lin et al., 2000).

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In this work, we have isolated and characterized a new polyhedra mutant AcMNPV virus (vSynlitx1B12P) isolated during the construction of a recombinant AcMNPV virus.

2. Materials and methods

2.1. Cells and viruses

Trichoplusia ni (BTI-Tn-5B1–4 or Tn-5B) cells (Granados et al., 1994) were grown in TC-100 medium (Gibco-BRL) supplemented with 10% fetal calf serum at 27 °C. This cell line served as host for the *in vitro* propagation of AcMNPV, the polyhedra mutant virus and the recombinant virus constructed in this work.

2.2. Isolation of an AcMNPV polyhedra mutant

During the isolation of a recombinant AcMNPV virus containing a toxin gene from the spider *Loxosceles intermedia* (not shown), we isolated a polyhedra mutant virus (vSynlitx1B12P). In brief, the transfer vector pSynXIVVI+X3 (Wang et al., 1991) was used to clone a cDNA from the toxin gene (LiTxX1) from *L. intermedia* (De Castro et al., 2004). The recombinant plasmid pSynLitx1 was used in a co-transfection with DNA from the recombinant virus vSynVI$^-$gal DNA (Wang et al., 1991), previously linearized with the restriction enzyme *Bsu*36I (New England Biolabs), into a monolayer of Tn-5B cells (10^6 cells) in a 60-mm plate, using liposomes (Cellfectin®, Invitrogen). The plate was incubated 1 week at 27 °C until the appearance of polyhedra, when the supernatant was collected and used to purify the recombinant virus through end-point dilution in 96-well plates (O'Reilly et al., 1992). The single *Bsu*36I site of vSynVI$^-$gal is located inside the β-galactosidase gene, and linearization makes it non-infective (Kitts et al., 1990), facilitating recombinant virus purification. Furthermore, the pSynLitx1 plasmid possesses, besides the toxin gene, the polyhedrin gene (disrupted in vSynVI$^-$gal). Upon homologous recombination of plasmid and viral DNA during co-transfection, vSynVI$^-$gal regains expression of polyhedrin, which is made evident by the formation of occlusion bodies by the recombinant virus. During the first round of isolation, using the protocol described by O'Reilly et al. (1992), we noted a well in a 96-well plate containing different cells with few polyhedra and dark patches inside the cytoplasm. The supernatant of this well was collected and used in three rounds of serial dilution for the isolation of the possible mutant virus which was designated vSynlitx1B12P.

2.3. Isolation, cloning and sequencing of the polyhedrin gene from the mutant virus

The isolated mutant virus (vSynlitx1B12P) was amplified in Tn-5B cells (5×10^6 cells) in a 100-mm plate (TPP), purified by ultracentrifugation and its DNA extracted following the methodology described by O'Reilly et al. (1992). The vSynlitx1B12P DNA was then used in a PCR reaction with specific oligonucleotides (ORF603 5′-CAGCCATTGTAATGAGACGC-3′ and AcpolR 5′-CAACAACGCACAGAATCTAG-3′) for the amplification of the polyhedrin gene. The ORF603 primer anneals at 162 nucleotides upstream from the start codon of polyhedrin gene and the AcpolR primer anneal at 37 nucleotides downstream of the polyhedrin stop codon. The amplified PCR fragment was cloned into the pGEM-T vector following the manufacturer's instructions (Promega), completely sequenced (ABI-PRISM) and the sequences analyzed using the open reading frame finder (ORF finder) and BLAST programs (Altschul et al., 1990) at the NCBI home page (http://www.ncbi.nlm.nih.gov).

2.4. Construction of a recombinant AcMNPV with the mutated polyhedrin gene

The plasmid containing the polyhedrin gene from vSynlitx1B12P (pGEMPOL) and the transfer vector pSynXIVVI+X3 were digested with the KpnI and BamHI restriction enzymes, separated by electrophoresis in a 0.8% agarose gel (Sambrook et al., 1989). A 460 and 5000 bp fragments from pGEMPOL and pSynXIVVI+X3, respectively, were purified from the gel using the GFX band prep kit following the manufacturers' instructions (GE healthcare), ligated and used to transform *Escherichia coli* DH5-α cells using standard protocols (Sambrook et al., 1989). The recombinant plasmid pSynpolmut was isolated, mixed with vSynVI$^-$gal DNA and transfected into Tn-5B cells as described above. The recombinant virus containing the mutant polyhedrin gene (vSynpolmut) was then isolated by end point dilution as previously described.

2.5. SDS-PAGE and Western blot

Tn-5B cells were seeded at 1×10^6 cells per well in six-well plates, mock infected and infected with wild type, vSynVI$^-$gal, vSynlitx1, vSynlitx1B12P and vSynpolmut viruses. Cells were harvested at 72 h.p.i. and centrifuged at $750 \times g$ for 7 min. The pellet was washed in PBS and boiled for 5 min in SDS sample buffer (ESB: 0.0625 M–Tris HCl, pH 6.8, 1% SDS, 2% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue). Protein samples were subjected to two 12% SDS polyacrylamide gels using a Mini-Protean apparatus and following the manufacturer's instructions (Bio-Rad). After electrophoresis, one gel was stained in a mixture of acetic acid:methanol:water (10:40:50) containing 0.1% Coomassie brilliant blue and destained in the same solution without Coomassie brilliant blue. The proteins present in the other gel were blotted onto a nitrocellulose membrane (GE Healthcare) using the Trans-Blot SD device, following the manufacturer's instructions (Bio-Rad). The polyhedrin protein was detected using a rabbit polyclonal antibody raised against the AcMNPV polyhedrin using standard methodology (Alves Júnior et al., 2008). In brief, the membranes were incubated for 1 h in 1% non-fat powdery milk in PBS-T [1× PBS (10 mM): 0.23 g NaH_2PO_4 (anhydrous; 1.9 mM phosphate), 1.15 g Na_2HPO_4 (anhydrous; 8.1 mM phosphate), 8.75 g NaCl (150 mM Na^+), pH 7.2 in 1 l of water] plus 0.5% Tween 20, and then 1 h in anti-polyhedrin (1:2000) in 0.5× PBS. After washing the membranes three times in 5-min steps with 0.5× PBS, they were incubated with 1:10,000 (v/v) goat anti-rabbit IgG alkaline phosphatase conjugate in 0.5× PBS for 1 h. The washing steps were repeated and NBT/BCIP (Sigma) substrate (33 ml of a 5% solution in DMF of NBT and 22 ml of a 5% solution in 70% DMF) in 7.5 ml of 0.1 M Tris buffer, pH 9.5, containing 0.1 M NaCl and 5 mM $MgCl_2$, was added to develop the reaction (Sambrook et al., 1989).

2.6. Structural, ultrastructural and immunogold labelling analysis of infected cells

Sixty millimeter-plates were seeded with Tn-5B cells (1×10^6) and infected with wild-type, mutant and recombinant viruses (10 pfu/cell). At 72 h.p.i., insect cells were observed and photographed in a light Axiophot microscope (Zeiss) and processed for electron microscopy. In brief, samples were fixed for 30 min (2% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer pH 7.4 with 5% sucrose), centrifuged at $750 \times g$ for 5 min, the pellet washed in the same buffer, post-fixed (1% osmium tetroxide, 0.8% potassium ferricyanide in the same buffer), contrasted *in block* with 0.5% uranyl acetate, dehydrated in acetone, and embedded in Spurr's resin. The ultrathin sections were contrasted with uranyl acetate/lead citrate and observed in a TEM JEOL 100C and