CHARACTERIZATION OF AUTOGRAPHA CALIFORNICA MULTIPLE NUCLEOPOLYHEDROVIRUS ORF17

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Summary. – The expression of Autographa californica multiple nucleopolyhedrovirus (AcMNPV) ORF17 was examined. The respective transcript of 492 nts could be detected by RT-PCR at 3–72 hrs p.i., while the corresponding protein could be assessed by Western blot analysis at 6–72 hrs p.i. Its size was determined at about 19 K in agreement with the predicted value of 18.5 K, suggesting that no major posttranslational modification of primary gene product. The ORF17 protein was primarily located in the cytoplasm. All these results together with earlier data on early AcMNPV promoter motifs suggest that ORF17 as an early gene encoding a protein located in the cytoplasm of infected cells.

Key words: Autographa californica; Autographa californica multiple nucleopolyhedrovirus; baculovirus; gene expression; ORF17; transcription; translation; subcellular localization

Introduction

The family *Baculoviruses* includes a large group of viruses that infect invertebrate species belonging mainly to the order *Lepidoptera*. Baculoviruses have enveloped rod-shaped particles containing a circular supercoiled double-stranded DNA genome ranging in size from 81.7 kbp to 178.7 kbp (Zhang *et al.*, 2005). Based on their occlusion body morphology, baculoviruses occupy two genera, *Nucleopolyhedrovirus* and *Granulovirus* (Fauquet *et al*., 2005). AcMNPV is the type species of the *Nucleopolyhedrovirus* genus. Baculoviruses are used as insect pest control agents and vectors for expression of heterologous genes in insect-derived cells as well as in host caterpillars (Ignoffo, 1973; Cunningham, 1998; Luckow and Summers, 1988; Fraser, 1992; King and Possee, 1992).

AcMNPV is the most studied baculovirus and most frequent tool for the expression of foreign genes. The complete nucleotide sequence of its genome is known (Acc. No. L22858). It contains 133,894 bp and 154 potential methionine-initiated ORFs of more than 150 nts (Ayres *et al*., 1994). In addition, complete genome sequences of another 26 baculoviruses have been reported (Zhang *et al.*, 2005). Analysis of these genomes has revealed many interesting features of baculoviruses (Herniou *et al*., 2003). Sixty-three genes, inclusive of AcMNPV ORF17 and its homologs, are shared by all the sequenced Lepidopteran nucleopolyhedroviruses (NPVs) and are regarded as core genes (Zanotto *et al*., 1993; Herniou *et al*., 2001). AcMNPV ORF17-like genes play an essential role in viral replication in Lepidopteran hosts. AcMNPV ORF17 (nt 13,738–14, 232) encodes a putative protein of 164 aa with a predicted M_r of 18.5 K. It is transcribed in the same orientation as the polyhedrin gene. Nevertheless, it is still uncertain whether this gene is really functional.

Baculovirus gene expression is believed to be regulated in a cascade fashion (Blissard and Rohrmann, 1990). Regarding the time, the transcription can be divided into three phases, early, late and very late (Friesen, 1997). Early

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Abbreviations: AcMNPV = Autographa californica multiple nucleopolyhedrovirus; GST = glutathione S-transferase; EGFP = enhanced green fluorescent protein; GV = granulovirus; MNPV = multiple nucleopolyhedrovirus; NPV = nucleopolyhedrovirus; SeNPV ORF116 = Spodoptera exigua NPV ORF 116; SeNPV ORF117 = Spodoptera exigua NPV ORF117

transcription is mediated by host RNA polymerase and is independent of viral DNA replication. After virion uncoating, early transcription takes place in the cell nucleus at 0–4 hrs p.i. (Rice and Miller, 1986). Many baculovirus early promoters contain a conserved sequence CAGT or CATT at or near the transcription start site and a functional TATA box (IJkel *et al*., 2002;Theilmann and Stewart, 1991). Two *cis*-regulatory elements, TATA and CAGT or CATT are essential for start site selection and initiation efficiency of early genes, respectively, and work in a cooperative manner (Blissard *et al*., 1989, 1992). In contrast, late and very late transcriptions require viral RNA polymerase and their promoters contain a TAAG motif.

In this study, we examined the transcription and translation of AcMNPV ORF17 and location of the respective protein product in Tn5B-1-4 cells.

Materials and Methods

Virus and cell line. *Trichoplusia ni* Tn5B-1-4 cells (High Five) (Invitrogen, USA), were cultured in the TNM-FH insect medium (Sigma, USA) with 10% fetal bovine serum. Wild AcMNPV (C6 clone) was propagated in the Tn5B-1-4 cells.

Computer-assisted sequence analysis. The AcMNPV ORF17 sequence (Acc. No. AAA66647) was analyzed using softwares of the ExPASy server (www.expasy.ch) for predicting domains, motifs, signal sequences and post-translational modifications (Appel *et al*., 1994). The AcMNPV ORF17 protein sequence was compared with those of homologous proteins obtained from BLASTP search in the updated GenBank/EMBL and SWISS-PROT databases. Multiple sequence alignments were carried out using ClustalW (version 1.8) (http://www.ebi.ac.uk/clustalw/) and Genedoc softwares.

RT-PCR. Total RNA was isolated from 1 x 10^6 Tn5B-1-4 cells infected with wild AcMNPV at a multiplicity of 10 $TCID_{50}$ per cell using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentration was determined spectrophotometrically at 260 nm. For cDNA synthesis, total RNA treated with DNase, an oligo(dT)-primer and Superscript II reverse transcriptase (LifeTechnologies) were used. The cDNA was amplified by PCR using the primers P1 (5'-AGGATCCATGA ATCT CAAAGTGATATT-3', nt 13,738–13,757, a *Bam*HI site underlined) and P2 (5'-ACTCGAGTTATTTTACTA AAACA GATT-3', nt 14,232–14,213, a *Xho*I site underlined), designed on the basis of the AcMNPV ORF17 sequence (Acc. No NC_001623). The reaction consisted of initial denaturation at 94°C/3 mins, 35 cycles of 94°C/1 min, 58°C/1min and 72°C/2 mins, and final extension at 72°C/10 mins. The PCR product was electrophoresed in 1% agarose gel. To check possible contamination of the PCR with undigested AcMNPV DNA, total RNA extracted from virus-infected cells was used as negative control.

Antiserum to AcMNPV ORF17 protein. The PCR-amplified AcMNPV ORF17 was first cloned in pGEM-T easy vector (Promega, USA) and then subcloned in pGEX-4T-2 expression vector (Pharmacia, USA), thus producing pGEX-4T-2-Ac17 vector. In this vector, the AcMNPV ORF17 insert was fused with a glutathione S-transferase (GST) tag. After confirmation by sequencing, this vector was used for transformation of *Escherichia coli* BL21 (DE3). In the transformed bacteria, the fusion protein designated as GST-Ac17 was expressed by induction with IPTG. After confirmation by Western blot analysis using an anti-GST antibody (Pharmacia, USA), GST-Ac17 was purified by means of a GST-binding column (Pharmacia, USA) and used for raising a polyclonal antibody in rabbits. Each New Zealand rabbit was injected four times with 100 µg of the protein; the first hypodermical dose was followed by 3 intramuscular doses after 3, 5 and 6 weeks, respectively. To collect the serum, the rabbits were bled 1 week after the last dose.

Western blot analysis. Monolayers of Tn5B-1-4 cells were infected with AcMNPV at a multiplicity of 10 $TCID_{50}$ per cell. Cells were harvested, resuspended in PBS and lysed in a SDS-PAGE loading buffer (50 mmol/l Tris pH 7.5, 500 mmol/l NaCl, 1mmol/l EDTA, 10% glycerol, 10 mmol/l ß-mercaptoethanol and 1%Triton-100) by boiling for 10 mins. The lysates were subjected to SDS-PAGE (10% gels) and the gels were electro-blotted to Immobilon-P nitrocellulose membranes (Millipore, USA) (Ausubel *et al*., 1994). The blots were incubated overnight in 3% skimmed milk in PBST (PBS with 0.2% Tween 20) at 4°C and then allowed to react with the GST-Ac17 antiserum diluted 1:5,000 for 1 hr at room temperature. After washing in PBST for 3 x 15 mins, the blots were incubated for 1 hr at room temperature with a goat anti-rabbit IgG-HRP (Southern Biotech, USA) diluted 1:5,000 in PBST. After washing in PBST for 3 x 15 mins, the blots were stained with DAB substrate (IJkel *et al*., 2000).

Subcellular localization. The AcMNPV ORF17 was expressed using the Bac-to-Bac system (Invitrogen, USA) in Tn5B-1-4 cells. The target fragment was retrieved by digestion with *Bam*HI and *Xho*I from pGEX-4T-2-Ac17 and subcloned into the *Bam*HI-*Xho*I sites of pFastBacHTe vector. The pFastBacHTe vector was reconstructed from pFastBacHTb vector (Invitrogen, USA) by inserting the enhanced green fluorescent protein gene (EGFP). The target gene was fused in frame with the EGFP in the vector. The plasmid was designated as pEGFP-Ac17. DH10 cells were transformed with pEGFP-Ac17 and a recombinant Bacmid, designated as vpEGFP-Ac17, was transfected into Tn5B-1-4 cells. The latter were examined by confocal microscopy using a Zeiss LSM510 scanning confocal microscope. A recombinant Bacmid with EGFP gene (vpEGFP) was used as a control.

Immunofluorescence analysis. Monolayers of Tn5B-1-4 cells were infected with wild-type AcMNPV. At 36 hrs p.i., the cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 10 mins at room temperature. The cells were washed three times with PBS, treated with 0.2% Triton X-100 in PBS for 10 mins and incubated with the antiserum to Ac17 diluted 1:5,000 for 1 hr at room temperature. The antiserum was removed by washing three times with PBS and the cells were allowed to react for 1 hr with protein G fused to EGFP (G-EGFP protein). After washing three times with PBS, the cells were examined by confocal microscopy for fluorescence detection. The AcMNPV-infected Tn5B-1-4 cells, which were reacted with G-EGFP protein only, were used as a negative control.

Results

Sequence analysis of AcMNPV ORF17

AcMNPV ORF17 consisting of 492 nts encodes a protein of 164 aa with a predicted M_r of 18 K. It is transcribed in the same orientation as the polyhedrin gene. A baculovirus consensus early promoter motif CATT and a TATA box were found 10 and 56 nts upstream of the initiation codon (ATG), respectively, suggesting that AcMNPV ORF17 might be an early gene. Two polyadenylation signal sequences, AATAAA, were localized 41 nt and 132 nts downstream of the stop codon, TAA.

To identify any similarities to potential biologically significant domains or motifs in existing protein families, respective search was carried out. It revealed a casein kinase II phosphorylation site (aa 69–72), two N-glycosylation sites (aa 78–81and 158–161) and a 1N-myristylation site (aa 151–156), but no domain.

The search for homologs revealed that all the available sequences of lepidopteran NPV genomes contained a homolog of AcMNPV ORF17: Bombyx mori NPV ORF9 (Gomi *et al*., 1999), Helicoverpa armigera SNPV ORF128 (Zhang *et al*., 2005), Helicoverpa zea SNPV ORF132 (Chen *et al*., 2002); Spodoptera litura NPV ORF119 (Pang *et al*., 2001), Adoxophyes honmai NPV ORF120 (Nakai *et al*., 2003), Lymantria dispar NPV (ORF128 (Kuzio *et al*., 1999), Spodoptera exigua NPV ORF29 (IJkel *et al*., 1999), Mamestra configurata NPV ORF36 (Li *et al*., 2002), Rachiplusia ou MNPV ORF15 (Harrison and Bonning, 2003), defective Choristoneura fumiferana NPV ORF14 (Acc. No. AY327402), Choristoneura fumiferana NPV ORF16 (Acc. No. NC_004778), Orgyia pseudotsugata NPV ORF16 (Ahrens *et al*., 1997), Epiphyas postvittana NPV ORF14 (Hyink *et al*., 2002), Spodoptera frugiperda NPV ORF-B, SfB (Acc. No. AY250076), and Amsacta albistriga NPV ACORF17 homolog gene (Acc. No. AF204882). However, no homolog was identified in all seven so far completely sequenced genomes of granuloviruses and baculoviruses isolated from non-lepidopteran invertebrates. These results indicated that the AcMNPV ORF17 homologs were thus gene-specific for Lepidopteran NPVs. Sequence alignment of AcMNPV ORF17 with its homologs from other NPVs revealed 6 completely conserved amino acid residues (Fig. 1), which likely play an important role in the functioning of these ORFs and/or their protein products.

Expression of AcMNPV ORF17 in Escherichia coli

The AcMNPV ORF17 protein fused to GST was expressed from the pGEX-4T-2-Ac17 vector in *E. coli* cells. SDS-PAGE revealed the production of a 42 K protein (Fig. 3A) and Western blot analysis confirmed that this protein was the fusion protein (Fig. 3B).

Analysis of AcMNPV ORF17 expression in Tn5B-1-4 cells

Analysis of AcMNPV ORF17 transcription in Tn5B-1-4 cells by RT-PCR revealed a band with an expected size of 492 bp at 3–72 hrs p.i. (Fig. 2). An appropriate control excluded contamination of the reaction by genomic AcMNPV DNA. This result suggested that AcMNPV ORF17 might be an early gene.

Western blot analysis revealed at 6–72 hrs p.i. a specific band of approximately 19 K in agreement with the predicted 18.5 K value, suggesting that no major posttranslational modification of the respective transcript occurred (Fig. 4).

Subcellular localization of AcMNPV ORF17 protein

Confocal microscopy localized the Ac17-EGFP fusion protein mainly in the cytoplasm along the nuclear membrane but not in a network of granular material, known as virogenic stroma (Fig. 5). The non-fused protein was primarily localized in the cytoplasm (Fig. 6).

Discussion

In this study, the analysis of transcription of AcMNPV ORF17 in Tn5B-1-4 cells by RT-PCR showed that, as it started at 3 hrs p.i. it might be an early gene using an early promoter CATT motif.

AcMNPV ORF17 protein, compared to its mRNA, was first detected later, at 6 hrs p.i. This could be due to the fact that the Western blot analysis was not sensitive enough to detect low levels of the protein at early time and/or the affinity of the antiserum to the protein was too low. The size of translational product of the AcMNPV ORF17 gene in Tn5B-1-4 cells was about 19 K in accord with the predicted 18.5 K value. The same protein expressed in the same cells using the Bac-to-Bac system had a similar M_r (~19 K). This suggests that, despite the presence of several potential posttranslational modification motifs in the sequence, no major changes of this kind occurred in the respective primary protein product.

The subcellular localization of the AcMNPV ORF17 protein yielded different results depending on the method used. The Ac17-EGFP fusion proteins were primarily located in the cytoplasm along nuclear membrane, while the non-fused protein occurred mainly in the cytoplasm. A larger size of the Ac17-EGFP fusion protein compared to the non-fused protein might be responsible for their different localization. The non-fused protein did not appear in the

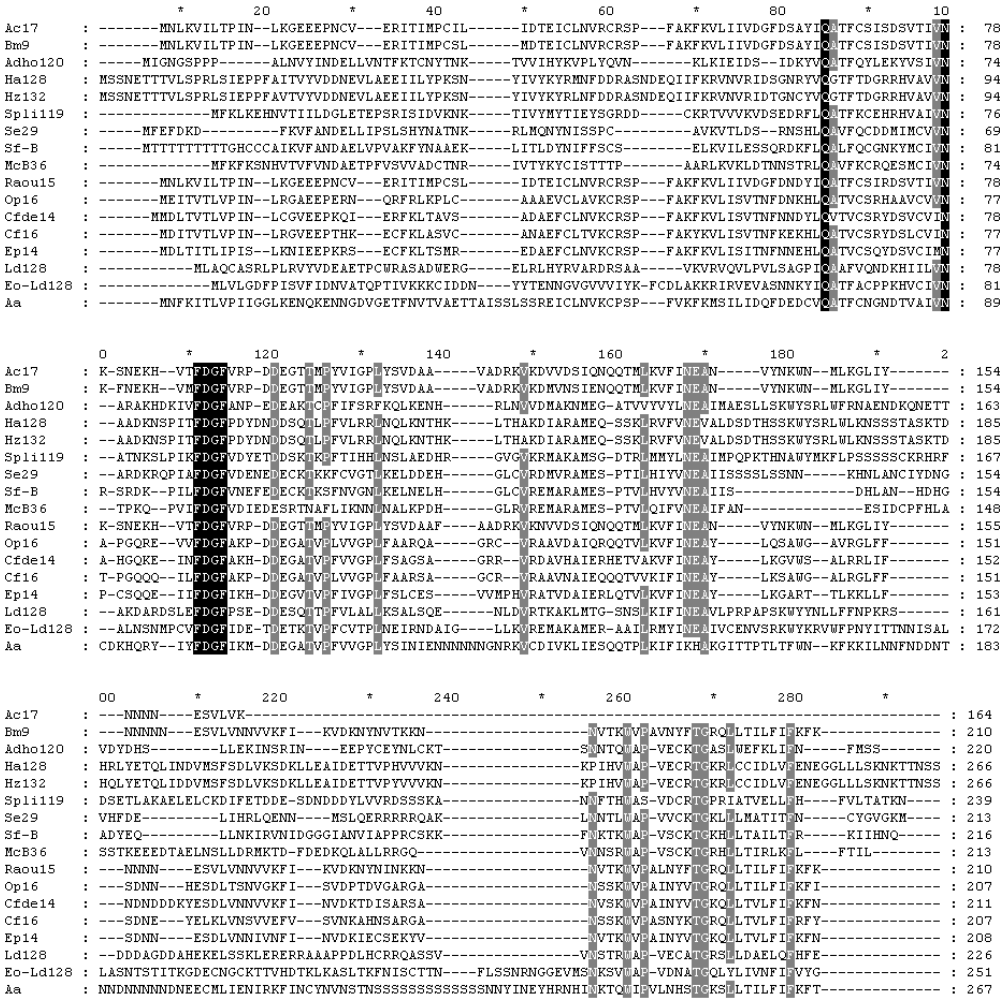

Fig. 1

Multiple sequence alignment of AcMNPV ORF17 and its homologs

Residues with complete identity are shaded in black while residues with similar character are shaded in grey.

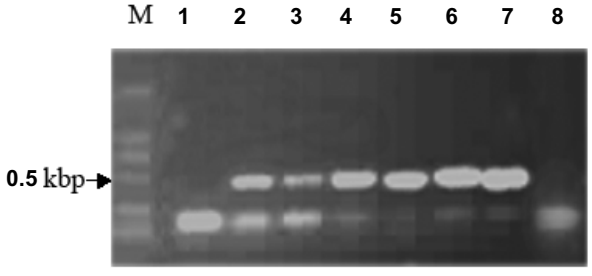

Fig. 2

RT-PCR analysis of AcMNPV ORF17 transcripts

The analysis performed on total RNA extracted from AcMNPV-infected Tn5B-1-4 cells at 0, 3, 6, 12, 48 and 72 hrs p.i. (lanes 1–7). Size markers (lane M), negative control (lane 8).

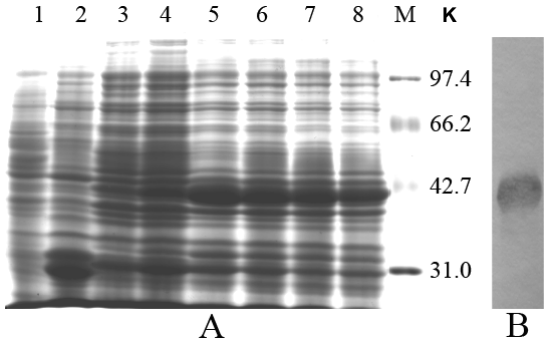

Fig. 3

SDS-PAGE (A) and Western blot analysis (B) of expression the GST-Ac17 fusion protein in *E. coli*

A. Non-transformed cells (lane 1). Cells transformed with pGEX-4T-2 (lane 2). Cells transformed with pGEX-4-2-GST-Ac17 and induced using IPTG in the concentrations of 6.7, 13.4, 26.8, 53.6, 80.4 and 134 ng/ml, respectively (lanes 3–8). Size markers (M). B. Western blot analysis.

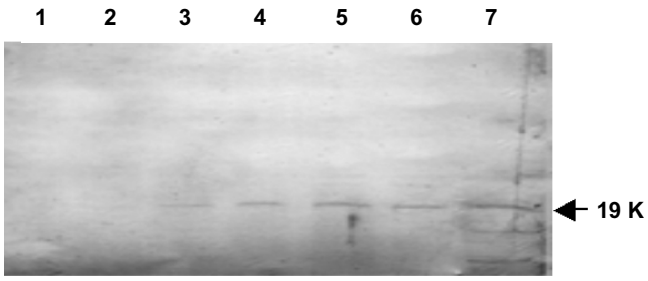

Fig. 4

Western blot analysis of expression of AcMNPV ORF17 protein in Tn5B-1-4 cells

Lysates were prepared from AcMNPV-infected cells at 0, 3, 6, 12, 24, 48 and 72 hrs p.i. (lanes 1–7) and assayed for AcMNPV ORF17 protein by Western blot analysis.

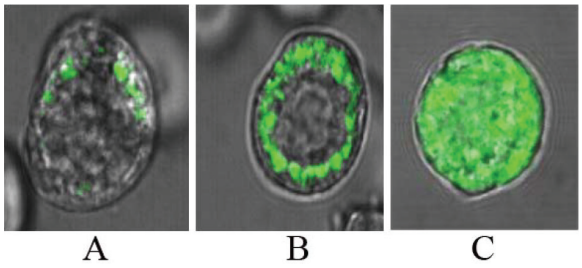

Fig. 5

Localization of EGFP-Ac17 fusion protein in Tn5B-1-4 cells by confocal microscopy

The Tn5B-1-4 cells were transfected with vpEGFP-Ac17 and subjected to confocal microscopy at 24 hrs p.i. (A) The cells transfected with vpEGFP, 72 hrs p.i. (B). The cells transfected with vpEGFP (control) (C).

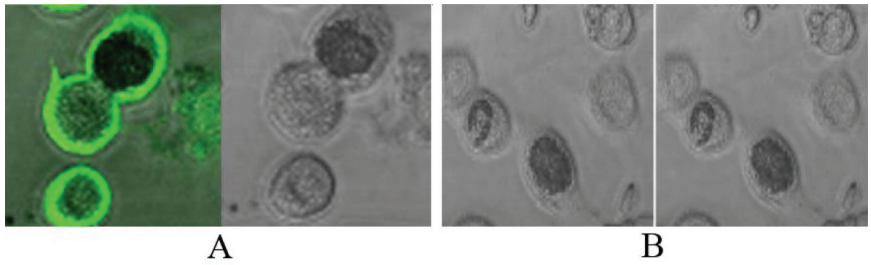

Fig. 6

Localization of AcMNPV ORF17 protein in Tn5B-1-4 cells by confocal microscopy

The Tn5B-1-4 cells infected with AcMNPV were fixed at 36 hrs p.i., incubated with the anti-Ac17 serum and protein-G-EGFP and subjected to confocal microscopy (A). AcMNPV-infected cells reacted only with protein-G-EGFP (negative control) (B).

region of virogenic stroma in contrast to similar proteins encoded by Spodoptera exigua NPV ORFs 116 and 117 (SeNPV ORF116 and SeNPV ORF117) (Ijkel *et al*., 2001). The latter proteins fused to EGFP were primarily localized in virogenic stroma, which is believed to be the site of viral DNA replication (Fraser, 1986; Guarino *et al*., 1992). The virogenic stroma is considered a *de novo* product of baculovirus infection in which progeny virions are assembled (Williams and Faulkner, 1997). Thus the SeNPV ORF116 and SeNPV ORF117 proteins may play a specific role in virion assembly process or virogenic stroma arrangement. Summing up, the function of AcMNPV ORF17 protein on one hand and those of SeNPV ORF116 and SeNPV ORF117 on the other hand seem different. Results similar to AcMNPV ORF17 were obtained with homologous Helicoverpa armigera NPV ORF128 (An *et al.*, 2005). The location of AcMNPV ORF17 protein in the cytoplasm suggested that this protein could be associated with the regulation of late viral genes and/or the virus-host interaction.

In conclusion, our results suggest that AcMNPV ORF17 is an early gene encoding a probably functional protein, which is primarily located in the cytoplasm. However, further studies are needed to prove real function(s) of this gene and its homologs.

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