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The *p26* gene of the *Autographa californica* nucleopolyhedrovirus: Timing of transcription, and cellular localization and dimerization of product

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Abstract

p26, an early AcMNPV gene, codes for a 240-amino acid polypeptide of unknown function. Primer extension analysis showed that the *p26* transcripts, initiating at three clustered start sites, accumulated between 2 and 12 h post-infection, after which these transcripts declined in quantity. Indirect immunofluorescence studies detected the p26 protein primarily dispersed in the cytoplasm of infected cells, although some staining of the nucleus was also observed. Immunoblots of infected cell fractions also detected p26 primarily in the cytoplasm. A yeast two-hybrid assay detected p26 in association only with other p26 molecules. Biochemical analysis showed that p26 forms dimers under physiological conditions.
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Keywords: Baculoviruses; *Autographa californica*; p26; Transcription timing; Cellular location; Dimer

1. Introduction

The *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) is the most extensively studied member of the *Baculoviridae* family, which infects the larvae of lepidopterans (butterflies and moths). The genome of AcMNPV is a double-stranded, covalently closed, circular DNA, consisting of 133,894 bp (Ayres et al., 1994). There are two morphologically distinct but genetically identical phenotypes of the AcMNPV: the occluded virus (OV) and the budded virus (BV). The BVs are produced during the late phase of infection and spread the infection from cell to cell within the host. The OVs are produced during the very late phase of infection and are embedded in a virally encoded protein, polyhedrin, to form the polyhedral inclusion bodies (PIBs). These PIBs provide some resistance to heat, sunlight, and dessication, and spread the infection from insect to insect.

While mapping the transcripts of the very late gene *p10*, Rankin et al. (1985) identified and mapped two additional transcripts of 1500 and 1100 nucleotides (nt). Both these transcripts were shown to have the same 5′ terminus, and were present during the early and late phases of infection. Liu et al. (1986) sequenced the HindIII-Q genomic fragment and identified an open reading frame of 240 amino acids in both 1500 and 1100 nt transcripts. The S1 mapping studies of Rankin et al. indicated the 5′ end of the two transcripts to be at a G, 19 bp upstream of the translation start codon (ATG) of this open reading frame. A putative TATA box and a putative polyadenylation signal, ATTAAA, for the 1100 nt transcript were also identified. This open reading frame codes for a potential 26 kDa protein that was named as p26. Huh and Weaver (1990a,b) showed that *p26* transcription is α-amanitin-sensitive and thus the *p26* gene is transcribed by the host RNA polymerase II.

Bicknell et al. (1987) identified and sequenced a similar *p26* gene in the *Orgyia pseudotsugata* MNPV (OpMNPV). The location of the *p26* gene in OpMNPV is similar to the location of *p26* in AcMNPV, just upstream of the *p10* gene. Sequence analysis of the *p26* gene of the OpMNPV and its comparison to the *p26* gene of AcMNPV revealed that both the potential protein products were of similar size, 230 and 240 amino acids, respectively. The two *p26* genes showed 55% identity in their nucleotide

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sequences and 47% identity in the amino acid sequences of their potential protein products. Most of the identity was concentrated into six regions, which demonstrated 65% amino acid identity. Since then, many nucleopolyhedroviruses in both groups I and II have been found to have *p26* genes homologous to the AcMNPV gene. Some of these showed very high degrees of similarity. For example, the closely related *Rachiplusia ou* virus (RoMNPV) contains a *p26* gene that differs from the AcMNPV gene in only 9 out of 240 amino acids (Harrison and Bonning, 2003). To date, *p26* genes have not been found in granuloviruses or in nucleopolyhedroviruses that infect non-lepidopteran species.

Besides nucleopolyhedrovirus proteins, the only other protein reported to have significant sequence similarity to AcMNPV p26 is an orthopox virus protein known as v-slfn (Gubser et al., 2007). The camelpox virus version of this protein has an N-terminal domain with 22% sequence identity and approximately 41% sequence similarity to AcMNPV p26. The C-terminal domain of v-slfn is related to the conserved region of a class of murine proteins known as schlafens, but not to AcMNPV p26. v-slfn appears to limit the virulence of camelpox virus. Aside from the similarity to v-slfn, there are no motifs in the p26 sequence, including the six conserved regions, that provide hints about its function.

While analyzing the transcription of *p35*, the gene upstream of *p26*, Rodems and Friesen (1993) constructed a recombinant virus in which all but 51 of the 240 codons of *p26* were replaced by the *Escherichia coli lacZ* gene. They showed that this deletion had no effect on the production of BVs or polyhedra. Thus, *p26* is not essential for the replication of the virus in cultured cells.

Here we present the results of high resolution mapping of the start sites for the *p26* transcripts and confirm the presence of these transcripts during the early and late phases of infection. We have also localized the p26 protein primarily in the cytoplasm of infected cells by immunofluorescence and cellular fractionation. Using a yeast two-hybrid assay, we have shown that p26 associates with itself. We have determined that p26 forms a dimer by cross-linking studies. Moreover, we demonstrate by size exclusion HPLC and analytical ultracentrifugation that p26 exists predominantly as a dimer under native conditions.

2. Materials and methods

2.1. Cells and viruses

The *Spodoptera frugiperda* cell line IPLB-SF21 (SF-21) was propagated in TC-100 medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL), at 28 °C. The *A. californica* nuclear polyhedrosis virus (AcMNPV) L-1 strain was used as the wild-type virus.

2.2. Transcription analysis of the p26 gene

Total cellular RNA was isolated from mock-infected and wild-type AcMNPV-infected SF-21 cells as described by Ausubel et al. (1989). 1×10^7 cells were infected (m.o.i. of 10) in 100 mm plates. RNA was isolated from cells at various times post-infection. Cells were harvested by lysis with guanidinium isothiocyanate solution and the lysate was centrifuged on a CsCl cushion to pellet the RNA. The RNA pellet was resuspended and reprecipitated with ethanol.

Primer extension analysis was performed using 30 μg of total RNA isolated from each time point. Each reaction was carried out in 20 μl containing 4 μl of 5× reverse transcription buffer; 20 units of RNasin (Promega); 2.5 μCi of [α-^{32}P]dATP; 250 nM of dCTP, dGTP, and dTTP; 20 pmol of an 18 nt antisense primer with the sequence 5′-CACATTGTCGACTTGCTC-3′ (3′ end of primer located 54 nt downstream of the translation start codon of the *p26* ORF) and 6 units of AMV (avian myeloblastosis virus) reverse transcriptase (Promega). Initially, the reaction was incubated at room temperature for 20 min. The elongation reaction was then carried out at 43 °C for 1.5 h after increasing the dNTP concentration to 50 μM. The reaction was stopped using formamide stop solution. The primer extension samples were electrophoresed on a denaturing sequencing gel along with sequencing reactions performed on the pUC12–HindIII-Q plasmid using the same primer.

2.3. Over-expression of p26 in E. coli and generation of anti-p26 antibody

2.3.1. Cloning of the p26 ORF in the bacterial expression vector

The *p26* ORF was obtained by PCR using as template a pUC12 plasmid with the HindIII-Q fragment inserted into the HindIII site and the following p26-specific primers: forward primer: 5′-ATATGCTGCAGATGGAATTGTATAATA-TAA-3′; reverse primer: 5′-ATATGCTGCAGTTAAGTATA-ATATATTGTG-3′. The PCR reaction was performed in a volume of 50 μl containing: 100 ng of template DNA, 10 μM of each of the primers, 5 μl of Taq polymerase 10× reaction buffer, 5 μg of bovine serum albumin (BSA), 1.5 mM $MgCl_2$, 200 μM dNTPs and 5 units of Taq DNA polymerase. The PCR was performed at 55 °C for annealing, 72 °C for elongation, and 95 °C for denaturation. The PCR product was purified using a Wizard PCR Preps DNA Purification System (Promega) and cleaved with PstI. It was then ligated with PstI-cut pTrcHis-C expression vector (Invitrogen) that had been treated with calf intestine phosphatase (CIP). $CaCl_2$-treated *E. coli* DH5-α cells were transformed with the ligated DNA, and positive clones were identified by restriction enzyme digestion. Clones with the *p26* insert in the correct orientation (pTrcHis/p26) were selected. All cloning procedures were carried out according to Sambrook et al. (1989).

2.3.2. Purification of recombinant p26 protein

One hundred milliliters of fresh pre-warmed LB media supplemented with 100 μg/ml of ampicillin were inoculated with 1 ml of an overnight culture of pTrcHis/p26 and grown to mid-log phase (OD_{600} = 0.4–0.5). The cells were then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 2.5 mM and grown for an additional 3 h. The cells were then pelleted by centrifugation and resuspended in 20 mM phosphate buffer (pH 7.8) containing 500 mM NaCl. The cells were lysed with lysozyme (final concentration 100 μg/ml) followed

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