The p6.5 gene region of a nuclear polyhedrosis virus of *Orgyia pseudotsugata*: DNA sequence and transcriptional analysis of four late genes

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The gene encoding the basic DNA-binding protein (p6-5) of the multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* (OpMNPV) was localized by Southern blot analysis using a cDNA probe containing the *Autographa californica* virus (AcMNPV) p6-9 gene. The OpMNPV p6-5 gene was mapped to the *Hind III* G fragment at map unit 67. Nucleotide sequence and transcriptional analysis of a 3.26 kb region encompassing this area revealed four open reading frames (ORFs 1 to 4) oriented in the same direction. ORF 1 demonstrated a seven codon overlap with ORF 2. Messenger RNAs initiated upstream of each of the four ORFs late in infection and were coterminal at a single site downstream of the fourth ORF. The conserved late gene promoter/mRNA start site sequence (ATAAG) was present upstream of all the ORFs, but did not appear to be the major site of mRNA initiation for the third ORF, as determined by primer extension analysis. The fourth ORF in this series encoded a predicted peptide of 51 amino acids (6-5K), which was 80% similar to the p6-9 basic DNA-binding protein of AcMNPV.

Introduction

The multicapsid nuclear polyhedrosis virus of *Orgyia pseudotsugata* (OpMNPV) has a double-stranded, supercoiled DNA genome of approximately 130 kb packaged in a rod-shaped enveloped nucleocapsid and has the potential of encoding well over 100 average size (30K) proteins. Baculoviruses produce two virion phenotypes, the polyhedra-derived virus (PDV), which are occluded in polyhedral inclusion bodies, stable in the environment and spread the infection between insects, and the budded virion phenotype (BV) which spreads the virus infection from cell to cell within the insect. Although the structural organization of the virions is unclear some proteins are present in both phenotypes, whereas others are unique to a specific phenotype (Volkman, 1983). Recently a gene encoding an envelope glycoprotein (gp64) specific to the BV phenotype was characterized in both *Autographa californica* virus (AcMNPV) and OpMNPV (Whitford et al., 1989; Blissard & Rohrmann, 1989). An antiserum produced against a synthetic peptide composed of part of the p6.9 sequence reacted with an acid-soluble, chromatin-associated protein of 6.9K (Wilson, 1988). Evidence for a protein kinase associated with virions has been reported for the granulosis virus of *Plodia interpunctella* (Wilson & Consigli, 1985). An antiserum produced against a synthetic peptide composed of part of the p6-9 sequence reacted with an acid-soluble, chromatin-associated protein of 6-9K (Wilson, 1988).

In order to determine whether a gene related to the AcMNPV p6-9 exists in OpMNPV and then to examine its expression in OpMNPV, we used an AcMNPV p6-9 cDNA clone to screen Southern blots of the OpMNPV genome. The homologous gene was located on the *Hind III* G-fragment of the OpMNPV genome. In this report we describe the sequence and transcriptional analysis of 3.26 kb of DNA located on the left end of the
HindIII G fragment of the OpMNPV genome. This region encodes four tandemly organized late-expressed ORFs oriented in the same direction with coterminal transcripts. The fourth ORF encodes the OpMNPV homologue of the AcMNPV p6.9 gene.

Methods

Insect cell lines and virus. The Lymantria dispar cell line and the production of OpMNPV were described previously (Quant-Russell et al., 1987). Cell monolayers were infected with second passage OpMNPV at an m.o.i. of ≥5 for isolation of viral mRNA.

Southern blot analysis. Probes for Southern blot analysis were radioactively labelled with [α-32P]dATP (New England Nuclear) by hexamer labelling (Feinberg & Vogelstein, 1984) of insert DNA from a cDNA clone derived from mRNA of the AcMNPV p6.9 gene (Wilson et al., 1987). Non-stringent hybridization conditions were utilized (30% formamide) according to the methods of Maniatis et al. (1982).

Cloning and DNA sequencing. The vector pBS(−) (Stratagene Cloning Systems) was used, modified to contain a BglII site in the polylinker. This modified vector is 3216 nucleotides (nt) in length. Sequencing was carried out using DNA from clones of appropriate restriction enzyme fragments and exonuclease III deletion clones produced according to the method of Henikoff (1987). Plasmid sequencing was performed by the chain termination method of Sanger et al. (1980) using [α-35S]dATP (New England Nuclear) and Sequenase (United States Biochemical). M13 universal and reverse primers were used for sequencing as well as two 18-mer oligonucleotides that were synthesized (nt 499 to 516 forward and 1089 to 1106 reverse, relative to Fig. 2). The DNA sequence was determined in both directions.

RNA isolation. Isolation of total RNA from OpMNPV-infected L. dispar cells for Northern hybridization, primer extension and S1 nuclease analysis was described previously (Blissard et al., 1989).

Northern hybridization analysis. Northern blotting of viral mRNAs, production of cRNA transcripts, prehybridization and hybridization of the blots were essentially as described by Blissard et al. (1989). For the Northern hybridization probe a deletion clone in pBS(−) containing a 155 nt insert (nt 2755 to 2910; Fig. 1 and 2), which contained all but the first 20 nucleotides of open reading frame (ORF) 4 was cut with PstI and the fragment containing the insert DNA was gel-purified. A labelled cRNA transcript was synthesized from this PstI fragment using T7 RNA polymerase (Boehringer) and [32P]UTP (Blissard et al., 1989). RNAs were electrophoresed in 1.2%, agarose-6% formaldehyde gels.

Primer extension, RNA sequencing and S1 nuclease analysis. To identify transcription start sites for the four ORFs, oligonucleotides complementary to the mRNA for each ORF were synthesized and used in primer extension analyses, as described by Blissard et al. (1989). The location of each primer is indicated in Fig. 2. The primers were annealed at 51, 58 and 41°C and extended at 49, 55 and 41°C for ORFs 2, 3 and 4, respectively. Because the primer for ORF 1 failed to produce a primer extension product, the location of the mRNA start site was determined by S1 nuclease analysis (Favaloro et al., 1980). DNA was end-labelled with [γ-32P]ATP and polynucleotide kinase. RNA sequencing was done as described in Blissard et al. (1989). Primer extension products, RNA sequencing ladders and S1-protected fragments were sized on 8% polyacrylamide–7 M-urea gels.

In order to identify the 3' termini of the transcripts, S1 nuclease protection experiments were performed using probes specific for transcripts running through ORFs 1, 3 and 4 (Fig. 4d) using methods described by Favaloro et al. (1980). All probes were 3' end-labelled with [γ-32P]ATP using the Klenow fragment of Escherichia coli DNA polymerase (Maniatis et al., 1982). For the ORF 1 probe, a pBS(−) recombinant containing the 3007 nt EcoRI/HindIII fragment was cleaved at the EcoRI site (Fig. 2 and 4d). The approximately 6200 nt (3007 nt insert + 3200 nt vector) fragment was gel-purified and 3' end-labelled. The ORF 3 probe was generated by digesting the pBS–EcoRI/HindIII clone used for the ORF 1 probe with EcoRI and SalI, and the approximately 4500 nt fragment (3200 nt vector + 1307 nt SalI/HindIII insert, Fig. 4d) was gel-purified. This fragment was 3' end-labelled and again gel-purified. For the ORF 4 probe a 1890 nt (40 nt vector + 1850

Fig. 1. Location of the OpMNPV p6.5 gene region. (a) HindIII restriction enzyme map of the OpMNPV genome indicating the location of polyhedrin (PH), DNA polymerase (DNA pol), capsid protein (p39) (Blissard et al., 1989), BV envelope glycoprotein (gp64) (Blissard & Rohrmann, 1989), polyhedron envelope protein (PE) (Gombart et al., 1989) and p10 (Leisy et al., 1986) genes. The location of the OpMNPV DNA polymerase gene was determined by hybridization of the AcMNPV Smal/PstI fragment (map units 40-3 to 42; Tomalski et al., 1988) to OpMNPV and by subcloning and then sequencing portions of the OpMNPV fragment to which hybridization occurred. (b) Restriction map of the left-hand HindIII G region, indicating the location of ORFs 1 to 4. Abbreviations: A, Asp718; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; P, PstI; S, SalI; Ss, SstI.
bp insert) Asp718 fragment derived from a PstI clone (extending from the PstI site at nt 3006 in Fig. 2 to 2.45 kb downstream) and which contains the 3' end of p6.5 (Fig. 4d) was 3' end-labelled and gel-purified.

Results and Discussion

Location of the OpMNPV p6.5 gene

In order to determine the location of the gene homologous to the AcMNPV p6.9 gene, labelled insert DNA from the AcMNPV p6.9 cDNA clone (Wilson et al., 1987) was hybridized under non-stringent conditions to blots of OpMNPV cosmid DNA (data not shown). This probe hybridized to the 5-2 kb HindIII fragment of cosmid 39 (Fig. 1). Subsequent blots of EcoRI, PstI and SstI digests of cosmid 39 further localized the hybridization to a 600 bp PstI/SstI fragment at the left end of HindIII G, at approximately map unit 67 (Fig. 1). The genomes of OpMNPV and AcMNPV have been shown to be collinear (Leisy et al., 1984) and these results confirmed that the gene was located in a similar position in both genomes.

Nucleotide sequence

Restriction fragments cloned into pBS(−) and exonuclease III deletion mutants generated from these clones were sequenced (Fig. 2). From the sequence analysis we identified four major ORFs (ORFs 1 to 4) (Fig. 1 and 2) oriented in the same direction. The ORFs varied in size from 153 to 1233 nt, encoding predicted proteins of 6-5K to 48K (Table 1). ORF 1 overlapped ORF 2 by seven codons. To rule out the possibility of a sequencing error causing ORFs 1 and 2 to appear to overlap, a HincII fragment (nt 655 to 1239) was cloned into HincII-cut pUC18. All five recombinants examined that had the insert cloned in the 5' to 3' orientation in the pUC18 lacZ gene produced blue colonies, indicating that the deduced ORF 1 reading frame from nt 655 to 1239 (Fig. 2) is open. The 5' flanking region of each of the four ORFs contains the ATAAG consensus sequence at various distances from the translation start (ATG) (Fig. 2 and 3). This sequence has been implicated as necessary for transcription of baculovirus late genes (Rohrmann, 1986; Possee & Howard, 1987; Rankin et al., 1988). The nucleotide at the −3 position, relative to the translation start codon of all the ORFs, contains a purine base (Fig. 3). Similarly, the nucleotide at the +4 position of all the ORFs, except ORF 1, contains a purine. This indicates that, in general, these reading frames conform to Kozak's rules (Pu-NATGpu) for efficient eukaryotic translation initiation (Kozak, 1986). A single 3' processing/polyadenylation signal (AATAAA) was found downstream of the ORFs at nt 3057, approximately 175 nt downstream of the termination codon of ORF 4 (Fig. 2).

Mapping the 3' termini of the transcripts

To determine transcriptional organization of these ORFs, the site of termination of mRNAs from this region was examined. S1 mapping at the 3' end was accomplished using three DNA fragments that had been 3' end-labelled at restriction sites within each of three ORFs from this region. These included ORF 1 (EcoRI at nt 259), ORF 3 (SalI at nt 1599) and ORF 4 (Asp718 at nt 2869) (Fig. 2 and 4d). Protected fragments of approximately 2800 and 1100 nt for ORFs 1 and 3, respectively, were identified (Fig. 4a, b). 3' S1 mapping of ORF 4 allowed more accurate sizing of protected fragments and two fragments of 214 and 215 nt were evident (Fig. 4c), which mapped to nucleotides 3082 and 3083 (Fig. 2). These results indicate that mRNA from ORFs 1, 3 and 4 are overlapping and coterminate downstream of ORF 4 (Fig. 4d). Such patterns of overlapping and coterminating mRNA are similar to that observed in other regions of the genomes of both OpMNPV and AcMNPV (Oellig et al., 1987; Gombart et al., 1989a, b). A single 3' processing/polyadenylation consensus sequence (AA-TAAA) is located downstream of the four ORFs at nt 3057, about 20 nt upstream of the 3' mRNA termination site.

Mapping transcription initiation sites

The transcriptional start sites for ORFs 2, 3 and 4 mRNAs were determined by primer extension analysis of total RNA from infected L. dispar cells using DNA oligonucleotides complementary to sequences near the 5' ends of each ORF (Fig. 2). RNA from uninfected L. dispar cells was used as a control. Because the primer complementary to ORF 1 mRNA failed to produce primer extension products, the transcriptional start site for ORF 1 was determined by S1 nuclease protection

<table>
<thead>
<tr>
<th>ORF</th>
<th>Size (nt)</th>
<th>Size (Amino acids)</th>
<th>Size (Mr)</th>
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<tbody>
<tr>
<td>1</td>
<td>1233</td>
<td>411</td>
<td>47.89K</td>
</tr>
<tr>
<td>2</td>
<td>336</td>
<td>112</td>
<td>12.16K</td>
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<tr>
<td>3</td>
<td>1062</td>
<td>354</td>
<td>39.84K</td>
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<tr>
<td>4</td>
<td>153</td>
<td>51</td>
<td>6.51K</td>
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amino acid sequence of each ORF is given below the nucleotide sequence. Major restriction enzyme sites and the location of the primers polyadenylation signal (AATAAA) (nt 3057) and the mRNA termination site (nt 3082 to 3083) are underlined. The mRNA start sites are indicated by overlines above the relevant nt and the translational start (ATG) sites for ORFs 1 to 4 are indicated. The predicted

Fig. 2. Nucleotide sequence of the OpMNPV p6.5 gene region. The putative promoter element (ATAAG) upstream of ORFs 1 to 4, the polyadenylation signal (AATAAA) (nt 3057) and the mRNA termination site (nt 3082 to 3083) are underlined. The mRNA start sites are indicated by overlines above the relevant nt and the translational start (ATG) sites for ORFs 1 to 4 are indicated. The predicted amino acid sequence of each ORF is given below the nucleotide sequence. Major restriction enzyme sites and the location of the primers used in the extension analysis are underlined.
experiments using a DNA probe (containing the EcoRI/BglII fragment), which was 5' labelled at the EcoRI site (nt 259, Fig. 2 and 5a, b). The exact location of each transcriptional start site was established by comparing the size of the primer extension or S1-protected products with M13mp18 or pBS(−) sequencing ladders (Fig. 5a). For more accurate sizing of larger primer extension products, gels were run for longer periods of time (data not shown). S1-protected fragments of 213 and 214 nt were identified for ORF 1 (Fig. 5a). These fragments correspond to mRNA start sites at nt 50 and 51 (Fig. 2), which are located at the first nt and one nt upstream of the ATAAG sequence. Primer extension from within ORF 2 resulted in a major product of 94 nt, corresponding to the ATAAG sequence. Primer extension from within ORF 2 was not the predominant product and any ATG downstream of ORF 2.

For more accurate sizing of larger primer extension products, gels were run for longer periods of time (data not shown). S1-protected fragments of 213 and 214 nt were identified for ORF 1 (Fig. 5a). These fragments correspond to mRNA start sites at nt 50 and 51 (Fig. 2), which are located at the first nt and one nt upstream of the ATAAG sequence. Primer extension from within ORF 2 resulted in a major product of 94 nt, corresponding to the first A of the ATAAG upstream of ORF 2. ORF 3 exhibited an unusual set of primer extension products, with over 16 major products that varied from 64 to over 300 nt in length (Fig. 5a and data not shown for products over 183 nt). Although the primer extension product of 127 nt corresponded to the first A of the ATAAG, it was not the predominant product and several of the other products appeared to be present at a higher concentration (Fig. 5a). Furthermore, six of the primer extension products are located within the putative ORF. Although the first ATG downstream of the ATAAG is in an optimal Kozak context (Kozak, 1986), there are several ATGs further downstream that are also in an optimal context. Thus, it is possible that the mRNAs initiating within the ORF may result in the production of ORF 3 peptides with heterogeneous amino termini. Patterns of apparent imprecise mRNA initiation have also been observed in the OpMNPV HindIII-M region (Gombart et al., 1989b). In that study two ORFs were identified which were expressed late in infection but did not show predominant initiation near the ATAAG promoter consensus sequence (Gombart et al., 1989b). The major primer extension product of ORF 4 was 88 nt and was localized to the first A of the ATAAG (Fig. 5a) at nt 2695 (Fig. 2). RNA sequence analysis using the ORF 4 primer also confirmed the location of the mRNA start site (Fig. 5a). It was observed that the flanking sequences downstream of the ATAAG in hyperexpressed genes is A + T-rich (75 to 89%) (Rohrmann, 1986) and postulated that this may influence levels of gene expression. ORFs 1, 2 and 4 have 5' flanking sequences (between the ATAAG and the ATG) of 59, 49 and 57% A + T, respectively. However, in ORF 3 ATAAG-initiated transcripts from this sequence would be 100% G + C, which may contribute to the apparent differences in the expression of this gene.

5' mapping was done using RNA isolated at 0 to 72 h p.i. and 3' S1 mapping was done using RNA from 0 to 48 h p.i. Messenger RNA was initially observed at 24 h p.i. and was present in high concentrations up to 48 h p.i., but was at low levels at 60 to 72 h p.i. (Fig. 4 and 5). This is consistent with expression of other late OpMNPV genes such as p39 (Blissard et al., 1989) and gp64 (Blissard & Rohrmann, 1989).

**Northern blot analysis**

To investigate further the transcriptional expression of these ORFs, a deletion clone containing a 155 nt insert, which included most of ORF 4 (nt 2755 to 2910, Fig. 2), was used to synthesize a labelled cRNA transcript complementary to ORF 4 mRNA. This probe was hybridized to Northern blots of total RNA isolated from OpMNPV-infected cells from 6 to 72 h p.i. (Fig. 6). Three major transcripts were identified. The transcripts of 3200, 2000 and 500 correspond to the mRNA sizes calculated from the 5' and 3' mapping of ORFs 1, 2 and 4, respectively. The lack of a transcript corresponding to an ORF 3 mRNA (approximately 1500 nt) is consistent with the primer extension data, which indicated that transcripts for this gene initiated at a broad range of sites and therefore may not be expected to yield an mRNA of defined size.

**Comparison of OpMNPV p6-5 gene region with the homologous region in AcMNPV**

In a previous study, Wilson et al. (1987) characterized a cDNA from AcMNPV that encodes an arginine-serine/threonine-rich putative DNA-binding protein termed p6-9. The homologous ORF (p6-5, ORF 4) in OpMNPV
Fig. 4. Determination of the location of the 3' ends of transcripts from ORFs 1, 3 and 4 by S1 nuclease protection assays. (a) S1 nuclease protection analysis of the 3' end of mRNAs from within ORF 1, (b) S1 nuclease protection analysis of the 3' end of mRNAs from within ORF 3 and (c) S1 nuclease protection analysis of the 3' end of mRNAs from within ORF 4. Numbers at top of gels indicate h p.i.; M indicates mock-infected cells; P indicates untreated, radioactively labelled probe. The autoradiograms for (a) and (b) were derived from a 0.7% agarose gel blotted to GeneScreen Plus. The sizes of relevant markers (1 kb ladder; BRL) are indicated between (a) and (b). The numbers to the left of (a) and to the right of (b) indicate the size of the probe (vector plus insert) used and the size of the protected fragment. Panel (c) represents an autoradiogram of an 8% polyacrylamide–7 M-urea gel. An M13mp18 sequencing ladder (order ACGT) was used to determine the size of the S1 protected fragments. The numbers to the left indicate the size of the protected fragments. (d) Strategy for mapping the 3' ends of transcripts from the HindIII G region. Solid lines under the ORFs indicate the three probes used in the 3' S1 analysis. The cross-hatched line beneath each solid probe line indicates the protected fragments. The numbers at the right indicate the size of the viral component of the probe [the total size of the probe (vector + insert) is shown in brackets] and the size of the protected fragments. The asterisks indicate the position of the 3' labelled end.
OpMNPV p6.5 gene region

(a) ORF 1

ORF 2

ORF 3

ORF 4 (p6.5)

(b)

mRNAs

ATAG

3200

2000

1500

480

1 kb

Fig. 5. Location of mRNA start sites. (a) ORF 1, S1 analysis to determine mRNA start site; ORFs 2 to 4, primer extension analysis. The numbers to the left of each autoradiogram indicate the size of the major primer extension/S1 protection products. The numbers at the top indicate the h p.i., M indicates mock-infected cells, PE indicates primer extension product (p6.5) and RNA SEQ indicates the sequence of the 5' region of the p6.5 mRNA. The samples were analysed on 8% polyacrylamide-7M-urea gels. Sequencing ladders of a modified pBS(−) or M13mp18 (order ACGT) were used for size markers. (b) Summary of transcriptional mapping data. The numbers at the left indicate the approximate size of the transcripts. The transcripts derived from ORF 3 are designated by a dashed line because of the high degree of 5' heterogeneity demonstrated by the mRNA. For abbreviation definitions see legend for Fig. 1.
At 80% sequence similarity, the p6-9 protein would be the next most highly conserved baculovirus gene.

In addition to the p6-9 gene homology the gene upstream of the OpMNPV p6-5 gene (ORF 3, p40) was 65% similar for the C-terminal 54 amino acids, predicted from the partial sequence reported by Wilson et al. (1987) (Fig. 7). There is also significant similarity to a partial ORF downstream and in the opposite orientation to p6-9 (58% for 57 predicted amino acids) (Fig. 7). To define the relatedness of this region further, an EcoRI/PstI fragment of approximately 1.3 kb (nt 259 to 1514, Fig. 2), containing most of ORFs 1 and 2, was hybridized to blots of AcMNPV DNA using non-stringent hybridization conditions. Hybridization occurred to the AcMNPV 2 kb KpnI E fragment which maps just upstream of the AcMNPV p6-9 gene (data not shown). The Northern blot analysis reported by Wilson et al. (1987) demonstrated three major transcripts (3.15, 2.0 and 0.5 kb) for AcMNPV, which is similar to that reported here for OpMNPV (Fig. 6). The AcMNPV transcripts probably correspond to mRNA from genes homologous to OpMNPV ORFs 1, 2 and 4. The data of Wilson et al. (1987) did not identify a major transcript corresponding to AcMNPV ORF 3, suggesting that it may be transcribed in a manner similar to the OpMNPV ORF 3 with no major mRNA start site. Therefore, based on sequence comparison, hybridization studies and Northern analysis there are likely to be four ORFs in the AcMNPV p6-9 gene region, which are organized and transcribed in a similar manner to the OpMNPV ORFs.

We thank Dr Marijo Wilson for the AcMNPV p6-9 cDNA clone and Dr Eric Carstens and Albert Lu for providing information on the AcMNPV p6-9 downstream region prior to its publication. We would also like to thank Dr Gary Blissard for his valuable technical advice and for critically reading this manuscript and Reg McParland of the Center for Gene Research and Biotechnology at OSU for assistance with primer synthesis. This project was supported by grants from the USDA (97-CRCR-1-2380) and the NIH (AI 21973). This is Technical Report No. 8909 from the Oregon State University Agricultural Experiment Station. The nucleotide sequence data have been assigned accession number D00514 by EMBL, GenBank and DDBJ.

References


AcMNPV p6.9 and OpMNPV p6.5

\begin{align*}
\text{AcMNPV} & : \text{MYYRRRSSTGTTYGSTRRRSSGYYRFFPRYR} \text{SRSSTGRRS-YRTRY} \\
\text{OpMNPV} & : \text{MYYRRRSRSADGTY--TRRRSSGYRFFPRYR} \text{SRSAT--RSGYRRRYY}
\end{align*}

Partial sequence upstream ORF (ORF 3)

\begin{align*}
\text{AcMNPV} & : \text{NYAVSNCKFNEIDYNIFKVMENIRKH} \text{SNKDQDELNYLGVQSNN} \text{AKKKKY} \\
\text{OpMNPV} & : \text{NYAVANCKFNEIDYNIFKGASIKKHAN} \text{TAESDELTY} \text{LGTT--AKKKII}
\end{align*}

Partial sequence of ORF downstream and in the opposite orientation of p6-9

\begin{align*}
\text{AcMNPV} & : \text{DKVIYLQNSNK} \text{K} \\
\text{OpMNPV} & : \text{DRVYLHKNKVDERTLLLHGPS} \text{GTSLAPCLHRATVER} \text{QMSFIRYCELCQMRA}
\end{align*}

Fig. 7. Comparison of predicted amino acid sequences from AcMNPV and OpMNPV, showing 80, 65 and 57% similarity for each comparison, respectively. The upstream and downstream AcMNPV hypothetical ORFs were predicted from the partial nucleic acid sequence data of Wilson et al. (1987). The upstream sequence begins at OpMNPV ORF 3, amino acid number 275.


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