

Characterization of the *Lymantria dispar* nucleopolyhedrovirus 25K FP gene

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The *Lymantria dispar* nucleopolyhedrovirus (LdMNPV) gene encoding the 25K FP protein has been cloned and sequenced. The 25K FP gene codes for a 217 amino acid protein with a predicted molecular mass of 24 870 Da. Expression of the 25K FP protein in a rabbit reticulocyte system generated a 27 kDa protein, in close agreement with the molecular mass predicted from the nucleotide sequence. The gene is located between 40·3 and 40·8 map units on the viral genome. It is transcribed in a counterclockwise direction with

respect to the circular map at late times during the infection cycle from a consensus baculovirus late promoter. The LdMNPV and *Autographa californica* nucleopolyhedrovirus (AcMNPV) 25K FP proteins exhibit 52% amino acid identity with several regions showing greater than 75% identity. Homologues to the AcMNPV *orf59* and *orf60* were also identified upstream (with respect to the genome) of the 25K FP gene in LdMNPV and exhibit 52% and 45% amino acid identity, respectively.

Introduction

Nucleopolyhedroviruses (NPV) are members of the *Baculoviridae* which produce two morphological distinct forms, a budded virus form and a virus form that is occluded into a protein structure termed a polyhedron. Insects are infected by NPVs after ingestion of the polyhedron and release of the occluded virions by dissolution of the polyhedron in the alkaline environment of the insect midgut. Early after infection the budded virus (BV) form is produced, which buds through the plasma membrane and then infects other cells. Later during the infection cycle the occluded form of the virus is generated (reviewed by Blissard & Rohrmann, 1990).

During serial passage of NPVs in cell culture, a class of virus termed few polyhedra (FP) mutants arises at a high frequency and becomes predominant. These mutants have the characteristics of altered plaque morphology, production of few polyhedra that contain very few viral nucleocapsids, increased release of BV, defective envelopment of viral nucleocapsids within the nucleus, and in *Lymantria dispar* NPV (LdMNPV) a decrease in the percentage of cells that produce polyhedra in comparison to the wild-type (many polyhedra, MP) virus (Hink & Vail, 1973; MacKinnon *et al.*, 1974;

Ramoska & Hink, 1974; Hink & Strauss, 1976; Potter *et al.*, 1976; Fraser & Hink, 1982; Slavicek *et al.*, 1992; Harrison & Summers, 1995; Slavicek *et al.*, 1995).

Specific mutations in FP mutants of *Autographa californica* NPV (AcMNPV) and *Galleria mellonella* NPV (GmMNPV) have been identified and characterized. The appearance of the FP phenotype during virus passage in cell culture correlates with the presence of DNA insertions/deletions (Fraser *et al.*, 1983; Kumar & Miller, 1987; Cary *et al.*, 1989) and with the absence of a specific 25 kDa protein (Fraser *et al.*, 1983). These insertions/deletions range from approximately 0·4–2·8 kbp and occur predominantly within a specific region [36–37 map units (m.u.)] on the AcMNPV and GmMNPV genomes. Analysis of this genomic region has identified a gene coding for a 25 kDa protein that is necessary for the MP phenotype (Beames & Summers, 1988). The 25K FP gene is essential for polyhedron formation and virion occlusion since deletion of this gene is sufficient to eliminate these processes (Beames & Summers, 1989). In addition, different mutations in the 25K FP gene generate all phenotypical characteristics of FP mutants (Harrison & Summers, 1995).

In contrast to the AcMNPV and GmMNPV FP mutants, DNA insertions and/or deletions do not correlate with the appearance of FP mutants in LdMNPV, as determined by DNA restriction endonuclease analysis (Slavicek *et al.*, 1995). This suggests that LdMNPV FP mutants may arise through a different mechanism than the formation of FP mutants in AcMNPV. Alternatively, the mechanism of FP mutant for-

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The GenBank accession number of the nucleotide sequence reported in this paper is U58676.

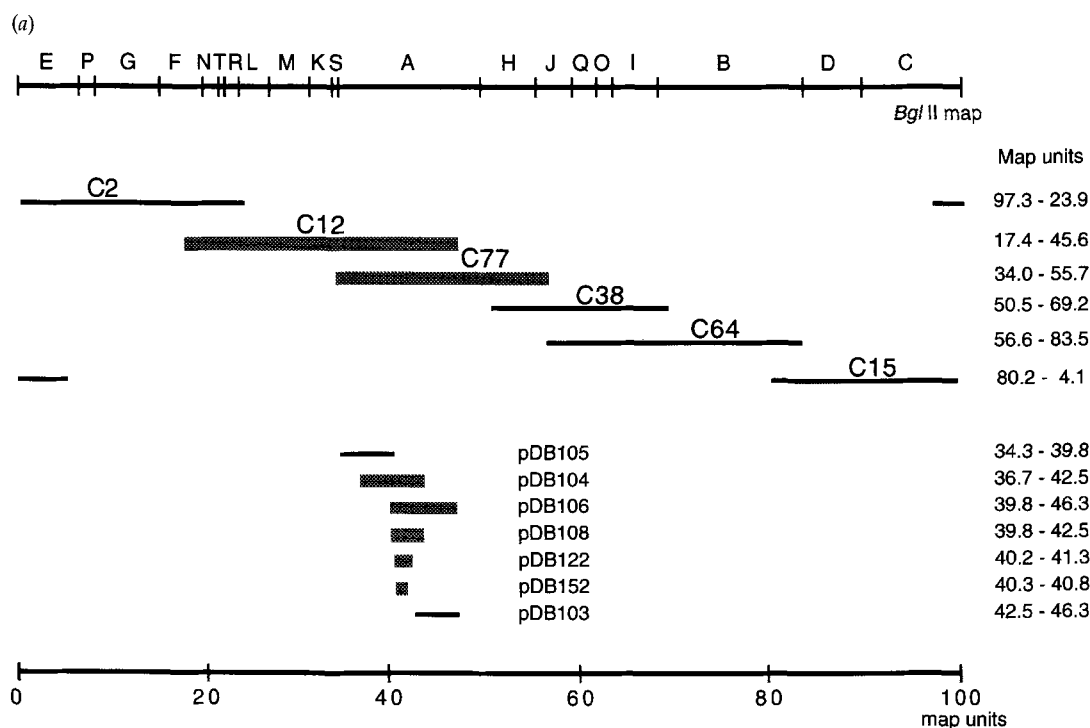


Fig. 1. For legend see facing page.

mation in these viruses may be similar; however, mutation(s) within the LdMNPV 25K FP gene may not be readily detectable. As a prerequisite to the molecular analysis of the 25K FP gene in LdMNPV FP mutants, we have identified, cloned and characterized the LdMNPV 25K FP gene.

Methods

■ **Cells and virus.** *Lymantria dispar* 652Y (*Ld*652Y) cells were grown as monolayers in Goodwin's IPL-52B (GI) medium supplemented with 6.25 mM-glutamine and 10% fetal bovine serum. Cell cultures were inoculated with either LdMNPV isolate A21-MPV, which produces wild-type polyhedra (Slavicek *et al.*, 1996), or the FP mutant A21-2 (Slavicek *et al.*, 1995).

■ **Viral DNA isolation.** Non-occluded virus from plaque purified LdMNPV A21-MPV or A21-2 was isolated from infected *Ld*652Y cells as described previously (Bischoff & Slavicek, 1994), and used as a source of genomic DNA for cosmid library construction and marker rescue experiments. Viral DNA was digested with restriction endonucleases and fractionated on 0.8% agarose-TBE gels.

■ **Cosmid construction and subclones.** A cosmid library of LdMNPV A21-MPV viral DNA was constructed using the SuperCos 1 cosmid vector (Statagene) following protocols provided with the vector. Viral DNA was partially digested with *Sau*3AI and cloned into the *Bam*HI site of SuperCos 1 to generate cosmids with LdMNPV inserts that were approximately 40–45 kbp in length. Cosmids containing overlapping viral DNA were identified by restriction endonuclease analysis. Subclones of the cosmids were constructed in pUC18 using standard techniques.

■ **Marker rescue mapping of the A21-2 mutation.** P6 wells (Corning) were plated with *Ld*652Y cells (4.5×10^5 cells per well) and the

cells allowed to attach to the bottom for 1 h. The media was removed, the cells were washed twice with 3 ml of GI medium (without fetal bovine serum) and then covered with 1.5 ml of medium. Cosmid or plasmid DNA (2.5 µg) and mutant viral DNA (2.5 µg) were mixed in a polystyrene tube, diluted to 50 µl with water, and then heated to 65 °C for 15 min. An equal volume (50 µl) of Lipofectin reagent (BRL) was added to the DNA mixture and incubated at room temperature for 15 min. The Lipofectin-DNA complex (100 µl) was added dropwise around the well. After incubation at room temperature for 2 h, the medium was replaced with fresh GI medium supplemented with 6.25 mM-glutamine and 10% fetal bovine serum. The cells were incubated at 27 °C for 2 h. Gentamicin (BRL) was added to a final concentration of 10 µg/ml, and then further incubated at 27 °C for up to 14 days. Cells were viewed with a Nikon Diaphot-TMD inverted microscope at 100× magnification.

To further localize the A21-2 mutation to the 25K FP gene, a 934 bp region containing the 25K FP gene was amplified from the A21-MPV viral genome by PCR. Primers were designed from the wild-type 25K FP gene sequence (see below) from isolate A21-MPV: 5' primer, GAGC-ACATGACCGTTTCG (complementary to positions 1627–1644 in Fig. 3); 3' primer, GGTAATCGAGCACGCTC (positions 711–728 in Fig. 3). PCR reactions (100 µl) contained buffer (50 mM-KCl, 10 mM-Tris-HCl at pH 9.0, 0.1% Triton X-100), 2.0 mM-MgCl₂, 200 µM-deoxy-nucleotides, 0.5 µM-primers, 20 ng viral DNA and 2.25 units of *Taq* DNA polymerase. Thermal cycling was carried out in a Perkin Elmer Cetus thermocycler. After denaturation at 94 °C for 3 min, 35 cycles were performed at 94 °C for 1 min, 48 °C for 1 min, and 70 °C for 1 min. The entire reaction was run-out on a 1% agarose-TBE gel, the 934 bp fragment was excised, and the DNA purified using the GeneClean II kit (Bio101). The fragment was cloned into the TA-cloning vector (Invitrogen) to create pDB152 and used for marker rescue.

Budded virus was recovered from the transfections which rescued the mutant phenotype and plaque purified as previously described (Slavicek *et al.*, 1995). *Ld*652Y cells (5×10^5 cells per T25 flask) were infected in

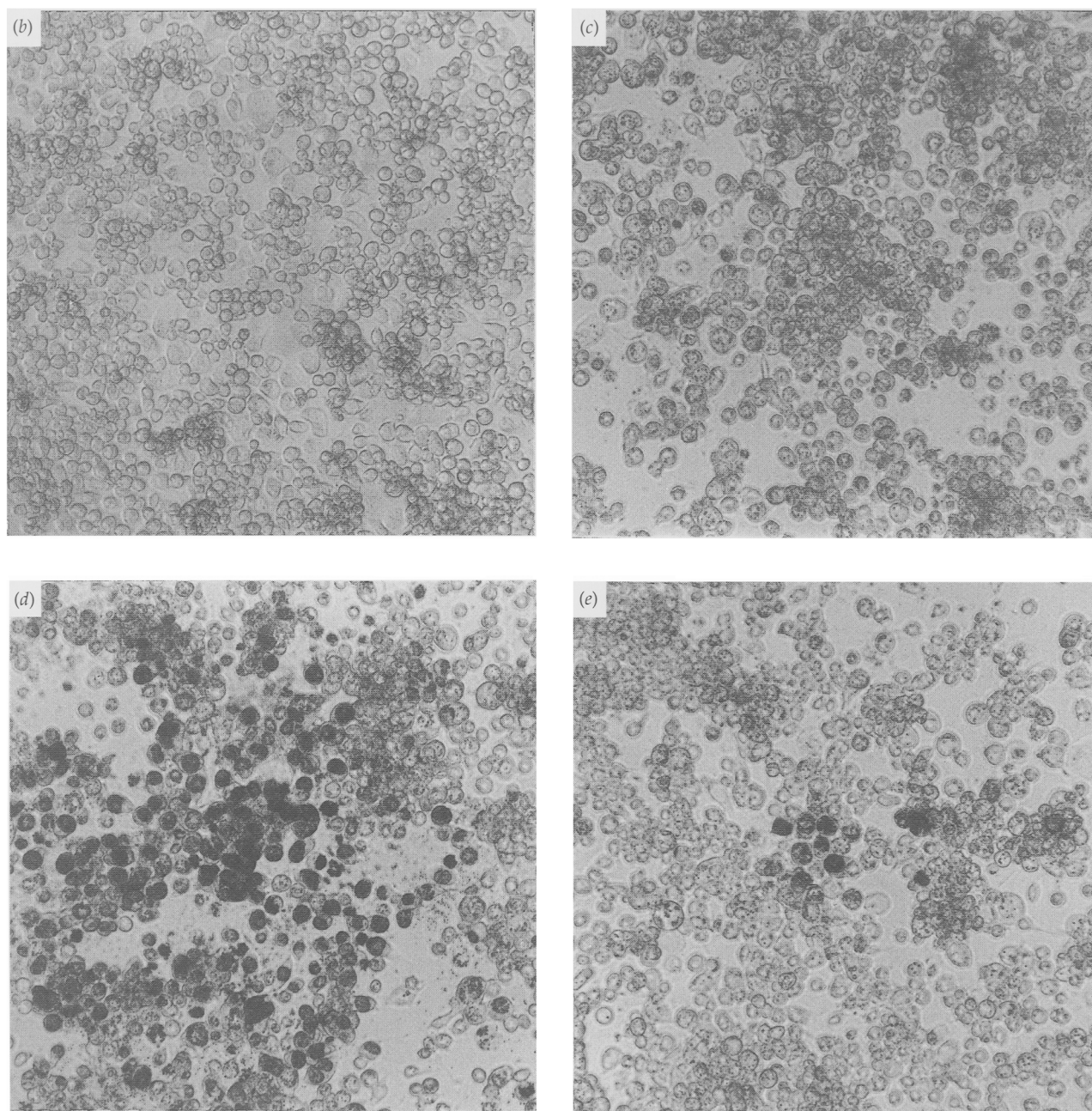


Fig. 1. Marker rescue mapping of A21-2 mutation. (a) Location of clones used to map the A21-2 mutation are shown below the LdMNPV *Bgl*II restriction map. Cosmid and plasmid inserts are shown as lines. Inserts that could repair the mutation are shaded. (b–e) Appearance of Ld652Y cells viewed by light microscopy after cotransfection with A21-2 viral DNA and one of the cosmids or plasmids in the A21-MPV genomic library. Cells were viewed with a Nikon Diaphot-TMD inverted microscope at 100 × magnification. A21-2 DNA alone (b); A21-2 with C38 (c); A21-2 with C12 (d); A21-2 with pDB152 (e).

quadruplicate with 5 TCID₅₀ units per cell of LdMNPV isolates A21-MPV, A21-2 or C12-2-1, an MP plaque purified from the C12 transfection. The number of polyhedra present per flask was determined at 6 days post-infection (p.i.) as previously described (Slavicek *et al.*, 1995).

■ **Sequencing.** The sequence of the 25K *FP* gene region from A21-MPV was determined on both strands of pDB108 using the dideoxynucleotide sequencing method. Plasmid and single-strand M13 DNA

templates were sequenced with the Sequenase version 2.0 DNA sequencing kit (USB) or the *fmo*I DNA sequencing system (Promega) using protocols supplied. [α -³⁵S]dATP was supplied by NEN. Sequence analysis was done using the MacVector program (IBI).

■ **RNA isolation and Northern blot analysis.** A21-MPV infected Ld652Y cells were harvested at various times (p.i.). Cytoplasmic RNA was isolated as described by Friesen & Miller (1985). Total RNA was

separated on 1.2% agarose gels containing formaldehyde and transferred to nitrocellulose. Northern blot analysis was performed as described by Mahmoudi & Lin (1989). A 33 base oligonucleotide (positions 1087–1119 in Fig. 3). was end-labelled with [γ - 32 P]ATP (NEN) and used as a strand-specific probe to detect the 25K FP transcripts.

■ Primer extension mapping of transcripts. Primer extension reactions were performed using the method of Crawford & Miller (1988). Total RNA was isolated at 48 h p.i. from Ld652Y cells infected with LdMNPV A21-MPV. An 18 base oligonucleotide (positions 1489–1506 in Fig. 3) was used in the reactions after being end-labelled with [γ - 32 P]ATP (NEN). The primer was extended using Moloney murine leukaemia virus (M-MLV) reverse transcriptase. Primer extension products were fractionated on 6% polyacrylamide–8 M-urea gels and visualized by autoradiography.

■ In vitro transcription and translation of the 25K FP gene. A 1.8 kbp *Sst*II fragment containing the 25K FP gene was subcloned into pBluescript SK(+) to generate pDB122. The 25K FP protein was expressed from pDB122 with the TnT coupled reticulocyte lysate system and T7 RNA polymerase (Promega) using directions provided with the kit. The expressed protein was labelled by the addition of [35 S]methionine (NEN). Reaction products were analysed by SDS-PAGE and autoradiography.

Results and Discussion

Cosmid construction

A cosmid library of isolate A21-MPV was constructed in the SuperCos 1 vector after partial digestion of LdMNPV

A21-MPV viral DNA with *Sau*3AI under conditions that generated fragments approximately 40–45 kbp in length. Cosmid inserts were analysed by restriction endonuclease analysis and comparison with the restriction pattern of LdMNPV clonal isolate 5-6 (Riegel *et al.*, 1994). Six cosmids were isolated that contained overlapping viral DNA and spanned the entire length of the viral genome (Fig. 1a).

Marker rescue of the A21-2 mutation

Isolate A21-2 exhibits the characteristics of a typical FP mutant (Slavicek *et al.*, 1995). In order to identify the genomic location of the gene mutated in this isolate, marker rescue/repair experiments were done in which Ld652Y cells were transfected with A21-2 viral DNA and DNA from each of the cosmids. Focal areas of cells appearing opaque as a consequence of the presence of a large number of polyhedra were only found in transfections with C12 and C77 (Fig. 1a, d). Transfection with the other cosmids generated cells with the A21-2 polyhedron formation phenotype (Fig. 1b, c). Since C12 and C77 are overlapping cosmid clones, the mutated gene mapped to the 20 kbp of overlapping DNA (32.7 to 45.1 m.u. on the viral genome) contained within these cosmids.

The 20 kbp of overlapping DNA contained within C12 and C77 was subcloned and the mutation further mapped in marker

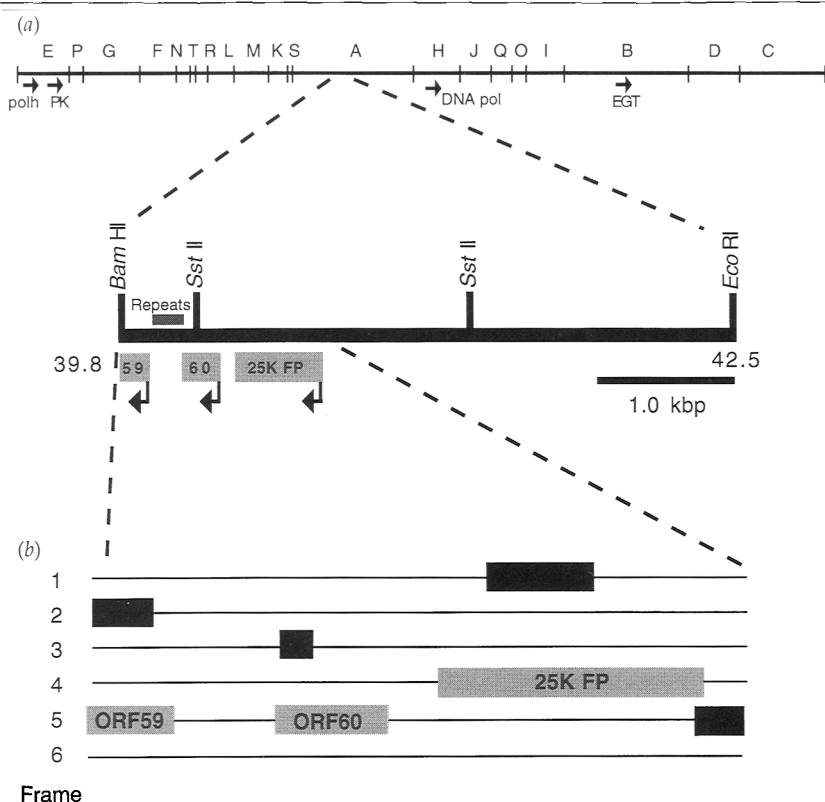


Fig. 2. Genomic location of LdMNPV *orf59*, *orf60* and 25K FP genes. (a) *Bgl*II restriction map of the LdMNPV viral genome. The locations of other LdMNPV genes are indicated: *polh* (polyhedrin; Smith *et al.*, 1988), *EGT* (ecdysteroid UDP-glucosyltransferase; Riegel *et al.*, 1994), *DNA pol* (DNA polymerase; Bjornson *et al.*, 1992) and *PK* (viral protein kinase; Bischoff & Slavicek, 1994). The enlarged map shows the 4.3 kbp *Bam*HI–*Eco*RI fragment at 39.8–42.5 m.u. on the viral genome. The position of the genes within this fragment and the direction of transcription are indicated. Restriction sites used in subcloning and expression of the gene are shown. (b) ORF analysis of this fragment in all six reading frames. The black boxes indicate ORFs that are at least 25 amino acids in length and which begin with an ATG start codon. The shaded boxes indicate the ORFs corresponding to LdMNPV *orf59*, *orf60* and 25K FP genes.

Fig. 3. Nucleotide sequence of LdMNPV *orf59* (partial), *orf60* and 25K FP genes with predicted amino acid sequences. The consensus late promoter sequence (ATAAG) located prior to the 25K FP gene coding region is shaded with the late transcriptional start sites indicated with arrows. A potential polyadenylation signal sequence (AATAAA) after the 25K FP gene coding region is underlined as are oligonucleotides used in transcriptional mapping and primer extension (see text).

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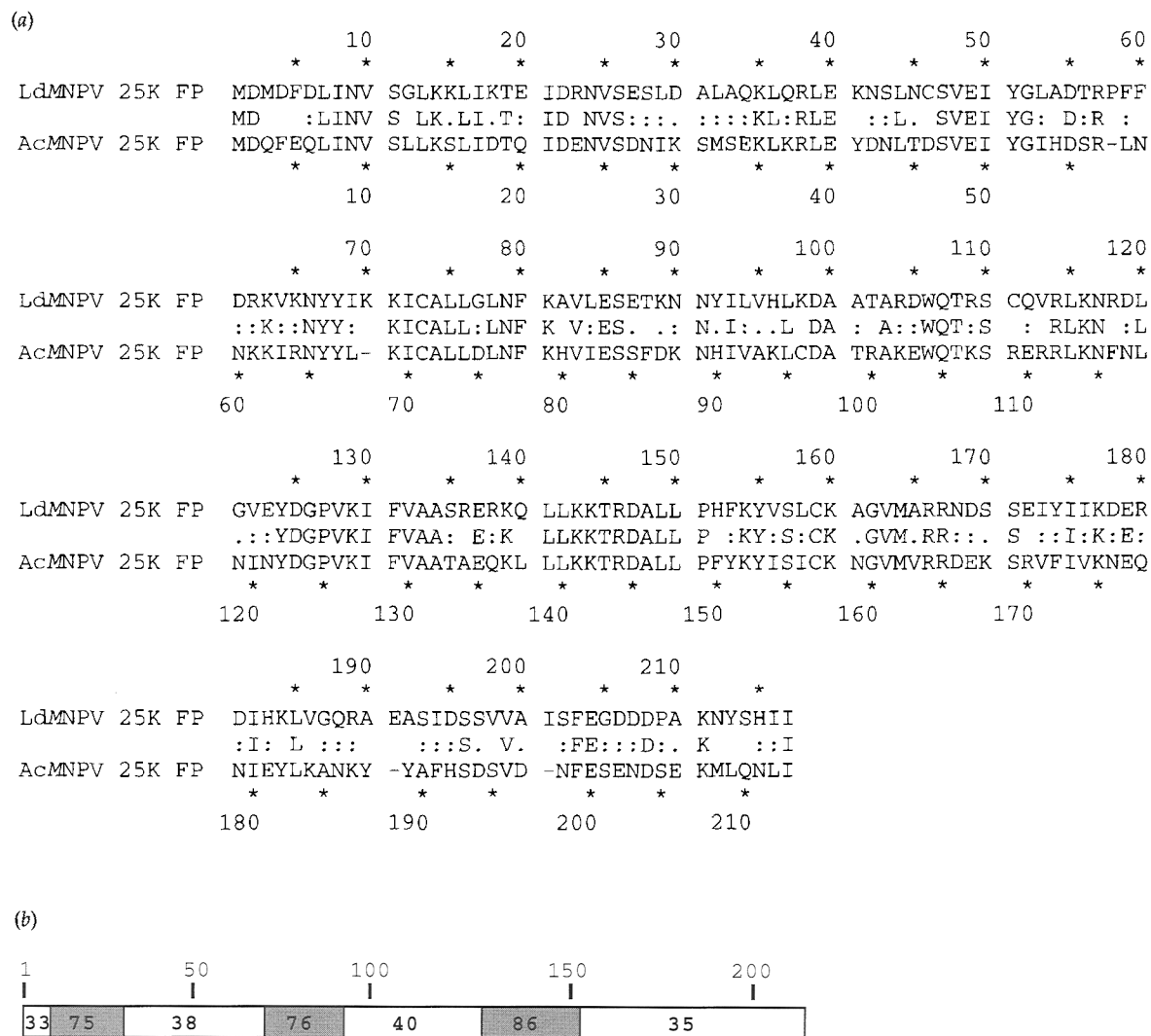
1  GGAT CCA TCT GTT GTT CAA TTT GAC GTA GCG CCG CTC GAC CGC CTT GAA
   I   W   R   N   N   L   K   V   Y   R   R   E   V   A   K   F
50  GGC CAA GTG CAA AGC TAT ATT TTC GTC GCC GCC GTC CAG CTT GTG GTA
   A   L   H   L   A   I   N   E   D   G   G   D   L   K   H   Y
98  CTT GCG AAA AGT CTC TAT GAA CAG TCG CCG CGC CCG GGC CGG CAT CTC
   K   R   F   T   E   I   F   L   R   R   A   R   A   P   M   E
146 TTC CTT TAA CAA AAC CTC GTT CAA ATA AAA CAT GAC AAT AGC CTT ATT
   E   K   L   L   V   E   N   L   Y   F   M   V   I   A   K   N
194 ATA TTG AAA TCG CTC GCG CAT ATCGTT AAAGTTTATA TATATGTTA ATTGCGT
   Y   Q   F   R   E   R   M   (orf59)
247 TATATTAAAATAGTTTGTG
266 TATTAAA TAGTTTG
280 TATATTAAAATAGTTTG
297 TATATTAAA TAGTTTG
313 TATATTAAA TAGTTTGTG
331 TATTAAA TAGTTTGTG
347 TATTAA TAGTTTGTA
362 TATTAAAATAGTTTGAC
379 TGTTAAAATAGTT GAGC
396 TATTAAAATAGTTTGGC
413 TATTAGAATAGTTTG
428 TATTAAA TTGTTTA
442 TACATTAAAA TTA TAT GTC ATC GTC GGA ATT TGT
   *** I D D D S N T
476 TTC ATT ATC AGT GGT GGT GGT GGT GGA AGA GTC CTC CTC CGT GGT
   E N D T T T T S S D E E E T T
524 GGT GGT GTC GTA ATC GTT GGC GTC CGC GAA CGG CAG CCA CTC GCC GCG
   T T D Y D N A D A F P L W E G R
572 GTC GCA ATA GTA TTT ACG CTT GAC GGC GGT CCA CGC GAT GGC GTC GGC
   D C Y Y K R K V A T W A I A D A
620 CGT CGT CGG CGA GTT GCG CAT TTT GAT GCT TTT ATT GTA AAA TTT TAA
   T T P S N R M K I S K N Y F K L
668 ATA AAT GCG CTT GCC GTT GTA GGG CAA GGC TCG CGT CGA AGT GGG TAA
   Y I R K G N Y P L A R T S T P L
716 ATC GAG CAC GCT CGT AAA TAA CAT ATTCAAATACAATAATCACAACCAATTCAGT
   D L V S T F L M (orf60)
771 CTTTATTTCGATCCTTAACGCTATTGAGATTCAACTTGAAAGGGTCCGCGTAGAATAATTAAA

834 GCGAGGT CGTCCGTCCG AGGGTTCGCG CCTCGCTCA AAT AAT GTG GGA GTA ATT
   *** I I H S Y N
888 TTT GGC CGG GTC GTC GTC GCC TTC GAA ACT GAT CGC GAC GAC GCT GCT
   K A P D D D G E F S I A V V S S
936 GTC GAT GCT GGC CTC CGC GCG CTG TCC GAC CAA TTT GTG AAT GTC TCG
   D I S A E A R Q G V L K H I D R
984 CTC GTC CTT AAT TAT GTA TAT TTC GCT GCT GTC GTT GCG GCG CGC CAT
   E D K I I Y I E S S D N R R A M
1032 GAC GCC GGC CTT GCA CAA CGA CAC GTA CTT GAA GTG CGG CAG CAG GGC
   V G A K C L S V Y K F H P L L A
1080 GTC GCG CGT CTT CTT CAA CAG CTG TTT GCG CTC GCG ACT GGC GGC CAC
   D R T K K L L Q K R E R S A A V
1128 GAA AAT CTT GAC CGG CCC GTC GTA CTC GAC GCC GAG GTC GCG ATT CTT
   F I K V P G D Y E V G L D R N K
1176 GAG GCG CAC CTG GCA CGA GCG CGT TTG CCA GTC GCG CGC CGT GGC CGC
   L R V Q C S R T Q W D R A T A A
1224 GTC CTT CAA GTG CAC GAG AAT ATA ATT GTT CTT GGT TTC GGA CTC GAG
   D K L H V L I Y N N K T E S E L
1272 CAC CGC CTT GAA GTT GAG GCC GAG CAG CGC GCA AAT TTT TTT GAT GTA
   V A K F N L G L A C I K K I Y
1320 ATA GTT TTT CAC TTT GCG GTC GAA GAA GGG CCG CGT GTC GGC GAG CCC
   Y N K V K R D F F P R T D A L G
1368 GTA AAT CTC CAC GCT GCA GTT GAG CGA GTT CTT TTC GAG CCG CTG CAG
   Y I E V S C N L S N K E L R Q L
1416 CTT TTG CGC GAG CGC GTC CAG ACT CTC GCT CAC GTT GCG GTC GAT TTC
   K Q A L A D L S E S V N R D I E
1464 GGT TTT GAT CAA TTT TTT CAA ACC CGA CAC GTT GAT GAG ATC GAA ATC
   T K I L K K L G S V N I L D F D
1512 CAT GTC CAT GA CCG CGC GGG CGT GTG TGT GTG TGT GTG CGA GGT TTG
   M D M (25K FP)
1559 AATATG TAA ATT AAT TAC TCC TAG TGA GCC GCG AGG CGC GCAG

1608 TCGATCGGAAACCTCGCCCCGAAACGGTCATGTGCTC

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Fig. 3. For legend see facing page.



52% overall amino acid identity

Fig. 4. Comparison of the LdMNPV and AcMNPV 25K FP proteins. (a) Amino acids that are positively related are shown with a colon and those with a zero value relationship are shown with a period. Amino acids with a negative relationship are shown with a blank. Identical amino acids are shown. Alignment was initially performed using the MacVector program (IBI). (b) Map indicating percentage amino acid identity (boxed numbers) within regions of the LdMNPV and AcMNPV 25K FP protein. Numbers above show the location of these regions with respect to the LdMNPV 25K FP protein. Regions exhibiting greater than 75% identity are shaded.

rescue/repair experiments (Fig. 1a). Plasmids pDB103 and pDB105 could not repair the mutation, whereas pDB104, pDB106 and pDB108 could, indicating that the gene was present on the 4.3 kbp *Bam*HI-*Eco*RI fragment (39.8–42.5 m.u. on the viral genome) contained within pDB108. Partial sequencing of this fragment revealed the presence of the LdMNPV 25K FP gene (see below). The 1.8 kbp *S*ssII fragment containing the 25K FP gene was subcloned (pDB122) and also could repair the mutation. PCR primers were designed from the sequence and used to amplify and subclone a 934 bp fragment (pDB152; 40.3–40.8 m.u.) containing only the wild-type 25K FP allele from A21-MPV. When tested for marker rescue this fragment could restore the MP phenotype (Fig. 1e).

The amount of polyhedra produced by rescued A21-2 was quantified to confirm the MP polyhedra synthesis phenotype. The BV generated during the C12 and C77 transfections was plaque purified and screened for the presence of plaques with a MP phenotype. MP plaques were recovered from both of the transfections. One of the MP plaques recovered from the C12 transfection was further analysed to determine the number of polyhedra produced by this isolate. LdMNPV isolate C12-2-1 produced significantly (ANOVA, Fisher's PLSD, $P < 0.05$) more polyhedra ($1.1 \times 10^8 \pm 9.8 \times 10^6$ polyhedra per flask) than the FP mutant A21-2 ($3.4 \times 10^6 \pm 6.2 \times 10^5$ polyhedra per flask) and a similar amount of polyhedra compared to isolate A21-MPV ($1.1 \times 10^8 \pm 3.6 \times 10^7$ polyhedra per flask).

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          10      20      30      40      50      60
      *   *   *   *   *   *   *   *   *   *
LdMNPV ORF59 MRERFQYNKA IVMFYLNELV LKEEMPARAR RLFIETFRKY HKLDGGDENI ALHLAFKAVE
              M: : :L :E.MP:RA: :LF:ETF KY HK::GGDE:I A:H A KA:E
AcMNPV ORF59      MYQIPDML YNEKMPPRAK KLFVETFTKY HKMNGGDEDI AMHKARKALE
              *   *   *   *   *   *   *   *
          10      20      30      40
          70
      *   *
LdMNPV ORF59 RRYVKLNNRW I>>
              :YVK:..
AcMNPV ORF59 EKYYVKIDTLQ NLGFRAKPPT R
              *   *   *   *
          50      60

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52% amino acid identity

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          10      20      30      40      50      60
      *   *   *   *   *   *   *   *
LdMNPV ORF60 MLFTSVLDLP TSTRALPYNG KRIYLFYNG SIKMRNSPTT ADAIAWTAVK RKYVCDRGEW
              LP . :.LPYNG KRI: KFY:: S:: :S. . A. :A AV: :KY G.W
AcMNPV ORF60      MLP KYQNLPLYNG KRIFEKFYDR SLQKYKSTQI ATKLACCAVR KKYMLIDGkW
              *   *   *   *   *   *   *   *
          10      20      30      40      50
          70      80      90
      *   *   *   *   *   *   *
LdMNPV ORF60 LPFADANDYD TTTTTEEDSS TTTTDTNETN SDDDI
              P :DAN: D TTT ..EDS: T.T.T: .T. .D:
AcMNPV ORF60 QPRPDANNSD TTT-STDST TDTETEYSTT EDELA
              *   *   *   *   *
          60      70      80

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45% amino acid identity

Fig. 5. Comparison of the LdMNPV and AcMNPV *orf59* (partial) and *orf60* homologues. Amino acids that are positively related are shown with a colon and those with a zero value relationship are shown with a period. Amino acids with a negative relationship are shown with a blank. Identical amino acids are shown.

Identification of the 25K FP gene

The restriction map of the 4.3 kbp *Bam*HI–*Eco*RI fragment is shown in Fig. 2(a). Computer analysis of a 1.6 kbp region within this fragment revealed several open reading frames (ORFs) that may encode proteins (Fig. 2b). The predicted amino acid sequence of these ORFs was compared with other proteins in GenBank at the National Center for Biotechnology Information (NCBI) using the BLAST network service (Altschul *et al.*, 1990). This search revealed that three of the ORFs showed homology to genes in AcMNPV. The largest ORF (651 bp in frame 4) is homologous to the AcMNPV 25K FP gene (Beames & Summers, 1989). The LdMNPV 25K FP gene is located at 40.3–40.8 m.u. on the viral genome and is

transcribed counterclockwise with respect to the circular viral genome. The other two ORFs upstream (with respect to gene location) of the LdMNPV 25K FP gene (213 and 285 bp in frame 5) exhibited homology to AcMNPV *orf59* and *orf60*, respectively, located immediately upstream (with respect to gene location) of the 25K FP gene in AcMNPV (Ayres *et al.*, 1994). Previous reports have indicated that the genes present in the genomes of AcMNPV and LdMNPV are organized in a similar array within short segments; however, overall the organization is not collinear (Bischoff & Slavicek, 1994; Bjornson *et al.*, 1992; Riegel *et al.*, 1994). The 25K FP, *orf59* and the *orf60* genes in LdMNPV and AcMNPV are at approximately the same location within the viral genomes in terms of

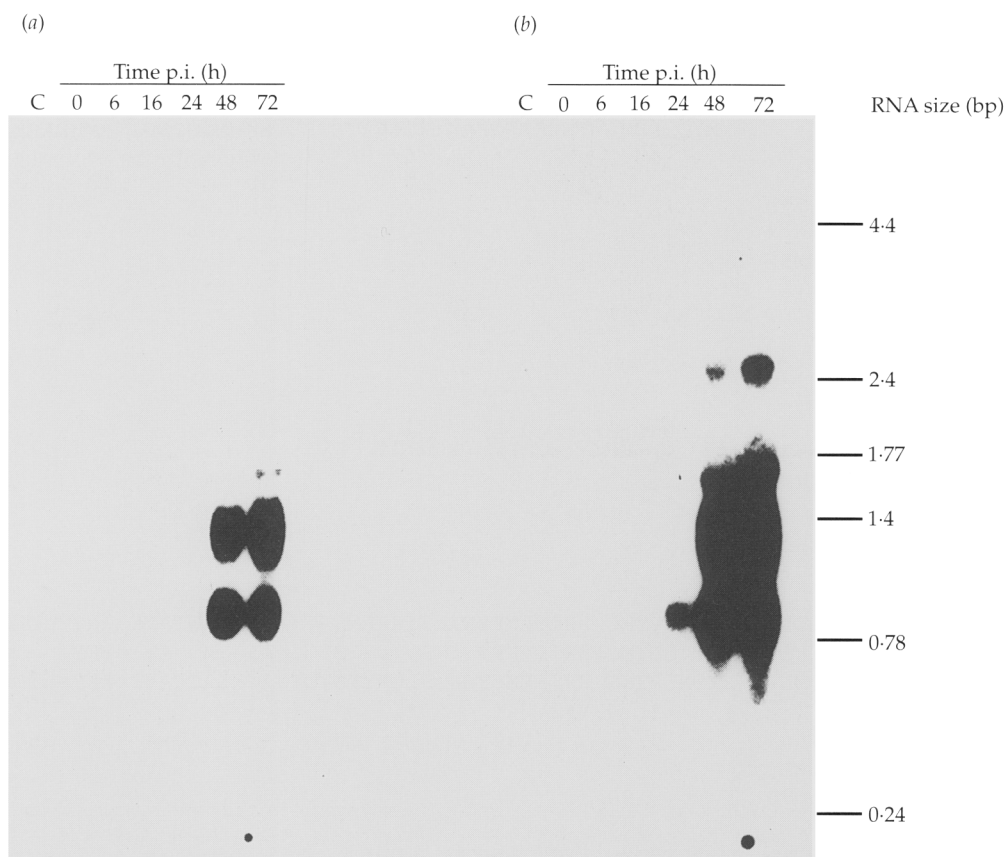


Fig. 6. Expression analysis of the LdMNPV 25K FP gene. (a) *L. dispar* 652Y cells were infected with 10 TCID₅₀ units per cell of LdMNPV A21-MPV. At the times indicated the cells were harvested, and cytoplasmic RNA was isolated. Total RNA (40 µg) was separated by formaldehyde-agarose gel electrophoresis, blotted, and probed with a 25K FP strand-specific ³²P-labelled oligonucleotide complementary to the nucleotide sequence at positions 1087–1119 (Fig. 3). RNA from uninfected cells was used as a control (lane C). The same Northern blot is shown in (a) shorter exposure and (b) longer exposure. RNA size standards are indicated.

map units (AcMNPV 36.4–37.4 m.u.) and are transcribed in the same direction.

Characteristics of the nucleotide sequence

The nucleotide sequence of the 1644 bp fragment encoding the 25K FP gene, *orf59* and *orf60* is presented in Fig. 3 with the predicted amino acid (aa) sequences of the proteins encoded by these genes. Only the N-terminal sequence up to the *Bam*HI site of *orf59* has been determined.

The 25K FP ORF begins at position 1520 and ends at position 869. This gene could encode a 217 aa protein with a predicted molecular mass of 24 870 Da. Late transcriptional start sites are at positions 1582 and 1583 as determined by primer extension mapping (see below). These start sites are within the consensus baculovirus late promoter sequence ATAAG (Rohrmann, 1986). A potential polyadenylation signal (AATAAA), beginning at position 778, was identified after the 25K FP gene coding sequences. The size of the transcript predicted from the DNA sequence from the late

promoter to the polyadenylation signal would be approximately 805 or 806 nt [not including a poly(A) tail].

orf60 is located immediately upstream (with respect to gene location) of the 25K FP gene in both LdMNPV and AcMNPV. The ORF could encode a 95 aa protein with a predicted molecular mass of 10 880 Da. The gene begins at position 739 and ends at position 454 (Fig. 3). There is a potential late promoter sequence, TTAAG (positions 784–788), starting 49 bp 5' to the ATG start codon, and a potential alternate polyadenylation signal (AATTAA), beginning at position 242. *orf59* begins at position 214. The region of the ORF sequenced could encode 71 aa. There is a TATA box sequence located at positions 191–196 and a potential late promoter sequence ATAAG beginning at position 192. These promoter elements are located within the ORF but are just before an internal methionine residue from which the homology alignment between the LdMNPV and AcMNPV proteins begin (see below).

Upstream of *orf60* is a region (positions 247–452) with 13 imperfect direct repeats of about 15 bp with the consensus core

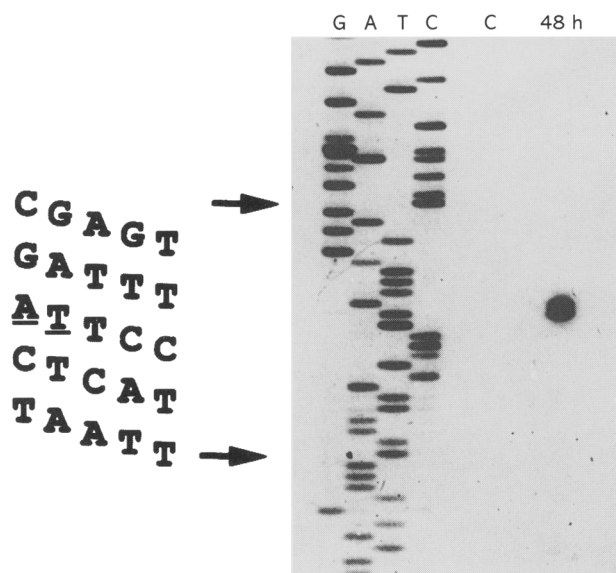


Fig. 7.

Fig. 7. Determination of the 5' end of the 25K FP transcripts. Primer extension analysis of 25K FP RNAs isolated at 48 h p.i. Total RNA (40 µg) was incubated with an end-labelled 18 base primer complementary to the 25K FP nucleotide sequence at positions 1489–1506 (Fig. 3). After extension of the primer using M-MLV reverse transcriptase, the extension products were fractionated by PAGE and visualized by autoradiography. RNA from uninfected cells was used as a control (lane C). The sequence ladder was generated using the same primer.

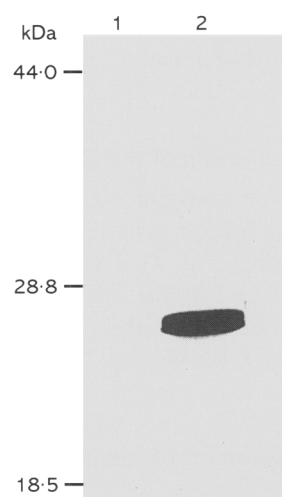


Fig. 8.

Fig. 8. SDS-PAGE analysis after expression of the 25K FP gene under control of the T7 promoter. Autoradiograph of the 25K FP protein expressed in a rabbit reticulocyte transcription and translation system and labelled with [35 S]methionine. Lane 1, pBluescript control plasmid; lane 2, pDB122 expressing the 25K FP protein. Molecular mass standards are indicated.

sequence TATTAATAAGTTTG. This repeat region is approximately 84% AT-rich. This region does not show any significant homology to the homologous regions (*hrs*) of AcMNPV (Ayres *et al.*, 1994) or LdMNPV, nor to the AT-rich Domain II of the LdMNPV *hr4* (Pearson & Rohrmann, 1995). These repeats are not present in AcMNPV. There is only 11 bp between the stop codon of *orf60* and the start codon of *orf59* (Ayres *et al.*, 1994) in AcMNPV compared with the 240 bp present in LdMNPV.

Characteristics of the protein sequences

The LdMNPV 25K FP protein shows 52% aa identity to the AcMNPV protein (Fig. 4a) over the entire length of the proteins. Regions within the proteins that exhibited the highest identity (Fig. 4b) were from residues 7–26 (75% aa identity), 66–86 (76%) and 124–151 (86%). The LdMNPV and AcMNPV proteins exhibit 78% similarity when conservative residue changes are taken into consideration.

LdMNPV *orf60* and *orf59* (first 58 aa) exhibit 52% and 45% aa identity (62% and 81% similarity), respectively, to the AcMNPV proteins (Fig. 5). The 25K FP, *orf59* and *orf60* proteins show no strong homology to any other known protein, nor were any known sequence motifs identified within the predicted aa sequences.

Transcriptional expression analysis of the LdMNPV 25K FP gene

A 33 base oligonucleotide was designed from the nucleotide sequence (positions 1087–1119 in Fig. 3) and used as a strand-specific probe to characterize the temporal expression of the 25K FP transcript(s). The 25K FP gene is expressed as two late transcripts, of approximately 0.95 and 1.3 kb in length, at high levels, and as a 2.5 kb transcript at lower levels (Fig. 6a, b). The smaller 0.95 kb transcript can be detected as early as 24 h p.i. upon overexposure of the blot (Fig. 6b). The AcMNPV 25K FP gene is expressed as two late transcripts (0.8 and 1.6 kb).

A strand-specific probe that hybridizes 50 bp 5' to the 25K FP late promoter was used to determine if the 1.3 and 2.5 kb transcripts contained sequences upstream or downstream of the 25K FP gene coding sequence. This probe did not hybridize to the 1.3 or 2.5 kb transcripts (data not shown), indicating that these transcripts contain sequences 3' to the 25K FP gene. This is consistent with primer extension data in which transcription was found to initiate at only two adjacent residues within the late promoter sequence (see below). The 1.3 kb transcript, therefore, contains the 25K FP gene and at least part (if not all) of *orf60*. This transcript may initiate at the 25K FP gene late promoter sequence and terminate at the

potential polyadenylation signal 3' to the *orf60* coding sequence (position 242 in Fig. 3).

Mapping of the 5' ends of the 25K FP transcripts

Primer extension reactions were carried out using total RNA isolated from *L. dispar* cells at 48 h p.i. to map the 5' end of 25K FP transcripts (Fig. 7). Transcription initiates at two residues 62 and 63 bp 5' to the 25K FP start codon (see Fig. 3). These start sites are within the consensus baculovirus late promoter sequence ATAAG.

In vitro transcription and translation of the 25K FP gene

The 25K FP gene was expressed from plasmid pDB122 (containing the 1.8 kbp *Sst*II fragment at 40.2–41.3 m.u.) in a rabbit reticulocyte *in vitro* transcription and translation system. A protein doublet migrating at approximately 27 kDa was seen after SDS-PAGE and autoradiography (Fig. 8). The observed doublet may be due to translational initiation at another methionine located at the third residue position of the protein (see Fig. 3). The size of the 25K FP protein is predicted to be 25 kDa from the nucleotide sequence. No radiolabelled band was detected from the parent plasmid.

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