Journal of General Virology (1997), **78**, 2119–2128. Printed in Great Britain

The p10 gene of *Spodoptera littoralis* nucleopolyhedrovirus: nucleotide sequence, transcriptional analysis and unique gene organization in the p10 locus

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The p10 gene of the *Spodoptera littoralis* (Spli) multicapsid nucleopolyhedrovirus (MNPV) was identified. With a coding sequence of 315 nucleotides (nt), corresponding to a protein of 104 amino acids, the SpliMNPV p10 gene is the longest p10 gene known. This gene codes for a putative protein with an M_r of 11 130 and was found to be most closely related to the *Spodoptera exigua* (Se) MNPV p10 (49·4% amino acid identity) and most distant from the *Autographa californica* (Ac) MNPV p10 (20·0% amino acid identity). Characterization of the protein's secondary structure and a comparison with other p10 protein species suggested that this p10 has an extended alpha-helical domain with high probability of forming a large coiled-coil structure. The p10 mRNA was about 1500 nt long, as determined by Northern blot analysis. Primer extension assay mapped three transcription start sites to a conserved baculovirus late promoter motif, TAAG. In the SpliMNPV genome, the p10 gene is not flanked by genes similar to p26 and p74, as found in SeMNPV, AcMNPV, *Choristoneura fumiferana* MNPV and *Orgyia pseudotsugata* MNPV. Instead, an open reading frame (ORF) of 945 bp is located downstream from the p10 gene and is followed by another ORF in opposite orientation, encoding the p74 protein. Upstream of the p10 sequences, an ORF of 552 bp was identified that potentially encodes a 184 amino acid protein of M_r 20925, which showed 52·2% identity with the encoded product of the SeMNPV xb187 gene.

Introduction

The nucleopolyhedroviruses (NPVs), a subgroup of the *Baculoviridae* (Murphy *et al*., 1995), have enveloped, rod-shaped virions carrying a circular, double-stranded DNA genome. Many NPV species infect lepidopteran insects and some are of potential use as biological insecticides (for review, see Wood, 1991). During the baculovirus replication cycle, two morphologically distinct forms of progeny virus are generated. Budded viruses are released from the infected cell and are responsible for virus spread in tissue culture and among tissues of the infected insect. At a late stage in the infection cycle, virions form into clusters and occlude within a paracrystalline protein matrix or occlusion body. Occlusion bodies are released from dead insects, remain in the environment and mediate the spread of infection through the insect population (Blissard & Rohrmann, 1991).

The late-infection stage of insect cells by NPVs is characterized by the synthesis of massive amounts of polyhedrin, the major component of the occlusion bodies (Rohrmann, 1986). A second protein, p10, is produced at high levels and is associated with the formation of large fibrillar structures in both the nuclei and cytoplasm of infected cells (van der Wilk *et al*., 1987; Quant-Russell *et al*., 1987; Vlak *et al*., 1988). Studies with p10 deletion mutants of *Autographa californic*a (Ac) MNPV have revealed that intact p10 is also involved in nuclear disintegration and in polyhedron morphogenesis (Williams *et al*., 1989; van Oers *et al*., 1993). However, p10 was not essential for virus replication and infection of insect cells with a p10-deleted virus could produce occlusion bodies (Williams *et al*., 1989). These features suggest the p10 promoter as a suitable site for cloning and the effective production of large quantities of heterologous proteins in insect cells, as demonstrated by expression of the cauliflower mosaic virus gene I (Vlak *et al*., 1990), and as described by Miller (1993) and O'Reilly *et al*. (1994). Although p10 was found to be nonessential for virus replication, its presence in several baculoviruses was found to be conserved. The p10 gene has been identified and studied in detail in AcMNPV

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The nucleotide sequence of the ORF552–p10–ORF945 cluster has been assigned accession number X99377 by EMBL and GenBank.

(Kuzio *et al.*, 1984) and *Orgyia pseudotsugata* (Op) MNPV (Leisy *et al.*, 1986), and identified in *Bombyx mori* (Bm) NPV (Yaozhou, 1992; Hu *et al.*, 1994), *Perina nuda* (Penu) MNPV (GenBank/EMBL accession no. U50411), *Spodoptera exigua* (Se) MNPV (Zuidema *et al.*, 1993) and *Choristoneura fumiferana* (Cf) MNPV (Wilson *et al.*, 1995). Comparative sequence analysis of the different p10 genes shows considerable divergence in the primary protein sequences, whereas higher similarity exists in the secondary protein structure and within specific domains (van Oers *et al.*, 1994; Wilson *et al.*, 1995). This similarity suggests a similar role for p10 in different baculoviruses although specificity for some of the p10 functions was detected between the p10 of SeMNPV and that of AcMNPV (van Oers *et al.*, 1994).

Spodoptera littoralis (Spli) MNPV is a pathogen of several *Spodoptera* species, and may be useful as a pest-control agent (Jones *et al.*, 1994). Distinct SpliMNPV genotypes have been identified in field isolates (Kislev & Edelman, 1982; Mialhe *et al.*, 1984; Cherry & Summers, 1985; Maeda *et al.*, 1990) and found to be genetically distant from the well-characterized AcMNPV (Kislev & Edelman, 1982; Maeda *et al.*, 1990). To increase our knowledge of the genomic organization and speciation of SpliMNPV, and to enable the design of a genetically improved virus, the p10 gene of this virus was identified. Here, we report the nucleotide sequence of the SpliMNPV p10 (the longest known baculoviral p10 gene), compare the secondary structure of its encoded product to other p10 proteins, and report the transcriptional analysis and the unique gene organization in the p10 locus relative to other baculoviruses, for which the respective genes have been identified.

Methods

■ **Virus, cells and insects.** SpliMNPV-B isolate E15 was plaque-purified from field-infected *S. littoralis* larvae as previously described (Faktor *et al.*, 1995). SL-2 cells permissive for SpliMNPV (Chejanovsky & Gershburg, 1995) were maintained in TNM-FH medium (Sigma), supplemented with 10% heat-inactivated foetal calf serum, at 27 °C (Mialhe *et al.*, 1984). The SpliMNPV-B isolate E15 was propagated in *S. littoralis* larvae. Haemolymph from virus-infected larvae was used as a source of extracellular virus for infection of cultured cells. SpliMNPV DNA was extracted from virions liberated by alkali treatment of polyhedral inclusion bodies, followed by sucrose-gradient centrifugation as described by O'Reilly *et al.* (1994).

■ **Southern blot hybridization, cloning, sequencing and data analysis.** To locate a viral DNA fragment containing the p74 gene, SpliMNPV DNA was digested with several restriction endonucleases, fractionated in Tris–borate–EDTA–0·8% agarose gels and transferred to Hybond-N membranes (Amersham). These membranes were used in a Southern hybridization with a 700 nucleotide (nt) ^{32}P-labelled *Eco*RI–*Hin*dIII DNA fragment of the AcMNPV *Hin*dIII-P fragment (Kool & Vlak, 1993) as a DNA probe, prepared using a commercial kit (Promega) according to the procedure described by Sambrook *et al.* (1989). This AcMNPV DNA fragment contained the 3′ end of the p74 gene sequence (Kuzio *et al.*, 1989). SpliMNPV DNA fragments that were found to hybridize with the AcMNPV probe were isolated from agarose gels by the freeze–squeeze method (Tautz & Renz, 1983) and cloned into the appropriately digested pUC19 vector (New England Biolabs).

The viral DNA fragments were sequenced with the Sequenase kit (US Biochemical) and [α-^{35}S]dATP (Amersham) using either commercial or specific primers. Sequence data were analysed using the Wisconsin UWGCG computer software package, version 8.0 and updated GenBank/EMBL databases.

■ **RNA isolation and transcription analysis.** Total RNA was isolated from virus-infected SL-2 cells at 5, 24, 48, 72, 96 and 120 h post-infection (p.i.), essentially as described by Chomczynski (1993), using commercial Tri Reagent (Molecular Research Center). RNA preparation from infected larvae, including grinding the larvae in liquid nitrogen prior to extraction with Tri reagent, was as described previously (Toister-Achituv & Faktor, 1997). For Northern blot analysis, total RNA was denatured, separated by electrophoresis on formaldehyde-containing gels (Sambrook *et al.*, 1989), and blotted onto Hybond N membranes. To identify specific transcripts, blots were hybridized for 20 h at 42 °C with a single-strand, antisense, ^{32}P-labelled probe in hybridization buffer containing 50% formamide (Sambrook *et al.*, 1989). After hybridization, blots were washed for 30 min at room temperature in 2 × SSC, 1% SDS, and 30 min at 60 °C in 0·1 × SSC, 0·1% SDS. Membranes were exposed to Fuji X-ray film using intensifying screens. Primer extension analysis was performed essentially as previously described (Walker *et al.*, 1983), using 5 μg of total RNA isolated from mock-infected or virus-infected cells at 24, 48 and 120 h p.i. A ^{32}P-labelled 19 base oligonucleotide (5′ GATATCCTGACGGATTACC 3′), complementary to the SpliMNPV p10 mRNA at nucleotide (nt) position 21–39 relative to the translation start codon, was annealed to the RNA and elongated by AMV reverse transcription reaction (Promega) at 42 °C for 1 h. The reaction was stopped by adding 5 μl of a solution containing 95% formamide, 0·01% xylene cyanol and 0·01% bromphenol blue, and loaded onto a 6% polyacrylamide sequencing gel. To determine the size of the extended products, a sequencing reaction was run alongside the reverse transcription reactions. The sequencing reaction was generated with a plasmid containing the p10 gene and the described 19 base oligonucleotide using a Sequenase kit. Gels were dried and exposed to Fuji X-ray film using intensifying screens.

Results

Identification of the 3′ end of the SpliMNPV p74 gene

Available information on the nucleotide sequences of different p10 genes indicated a relatively low percentage of nucleotide sequence identity (van Oers *et al.*, 1994). This prevented the use of a known p10 gene as a DNA probe for the direct identification of the corresponding gene in the SpliMNPV genome. Based on the relatively higher sequence conservation between p74 gene species, and the clustering of p26, p10 and p74 in several viruses (Zuidema *et al.*, 1993), we identified part of a SpliMNPV p74 gene homologue with the aim of using it as a reference point for subsequent identification of the p10 gene. To identify the p74 gene, SpliMNPV DNA was digested with various restriction enzymes. The resultant DNA fragments were separated by gel electrophoresis and analysed by Southern hybridization. An AcMNPV-derived

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   1 AGGAGGAGGA GGCGGCGGCG GCGTCCGAGG CAATGTCGGT GTCCAAAATA TAAGGTGATG ATTGAATAGG ACATGGAATT   80
                                                                                     Ⓜ  E  F >
  81 TGACAAAGAT ATCGCGCGGT TGACCGCTGC CATCGGGCAA AATGTCGATG TACAATCCGC GTCGCCGTCT CCAAAGCTGG  160
      D  K  D   I  A  R  L   T  A  A   I  G  Q   N  V  D  V   Q  S  A   S  P  S   P  K  L  G >
 161 GCGATGTTTT GCAGCAACTC GGACGCAAAA GGAACGGTCT GCTGTCGAGA AAGAAAGACG AAAACTTTGA CATTGCCGAA  240
        D  V  L   Q  Q  L   G  R  K  R   N  G  L   L  S  R   K  K  D  E   N  F  D   I  A  E  >
 241 ACAATCGAGC TGTCCGACGC GACAAAAGAC TATTTGAACG TGTTGCAATT GGAAAAGTTG TACGCGTGCA GGGCGTGCTA  320
     T  I  E  L   S  D  A   T  K  D   Y  L  N  V   L  Q  L   E  K  L   Y  A  C  R   A  C  Y  >
 321 CGAACGCGAC GACTCTCGCC GCTGTTGGTT TCACAAAAAG TACATATTCA CCAAAAACAT GAAGACGCAC TACGACGAGT  400
      E  R  D   D  S  R  R   C  W  F   H  K  K   Y  I  F  T   K  N  M   K  T  H   Y  D  E  Y >
 401 ACGTGCAATT TTTAAACAGT CAAATGGGCA TCGTGTCGTA CGTAGAGCTA TATTACACGT ATCTGGCGGC GCCGCCGTTT  480
        V  Q  F   L  N  S   Q  M  G  I   V  S  Y   V  E  L   Y  Y  T  Y   L  A  A   P  P  F  >
 481 TGGAATGCTA CCGCCAAGAT CATGCTAGAC GAACTGACCG GACATTCGTC GATCGCGTCT CTGCTAAAGC ATCACGGACA  560
     W  N  A  T   A  K  I   M  L  D   E  L  T  G   H  S  S   I  A  S   L  L  K  H   H  G  H  >
 561 CGAAACCTCG ATCGACGCCG ACGAACCTGC CGCCGTCGCG ATGGACACGT GTGAGGAAAA ATAAGAATTC ATATCGATTT  640
      E  T  S   I  D  A  D   E  P  A   A  V  A   M  D  T  C   E  E  K   *  ORF552 end        >
 641 TGTGTGTATA TTATAAAAAA AAGTATATAA TAAAAATATG AGTCAAAACA TTTTATTGGT AATCCGTCAG GATATCAGTA  720
                                             Ⓜ   S  Q  N  I   L  L  V   I  R  Q   D  I  S  N >
 721 ACCTCAGCGA TCAGGTCACG GCTCTACAGG GCGCCGTCGA CGATGTGCGC GCAAATCTGC CGGATGTCAC CGAGATAAAT  800
        L  S  D   Q  V  T   A  L  Q  G   A  V  D   D  V  R   A  N  L  P   D  V  T   E  I  N  >
 801 GATAAACTCG ATGCGCAGAA CGCTCAGTTA GTGAGTCTGG AGGCGTCGAA CGAAGCGATC AGTACACTCG TTCAGTCCCT  880
     D  K  L  D   A  Q  N   A  Q  L   V  S  L  E   A  S  N   E  A  I   S  T  L  V   Q  S  L  >
 881 GTCCGAAGCG GTACAGAACA TTACGGACAT TTTGACGCCC GAGATACCAG ATTTGCCCAT ACCCAATCCT CCTTTAGGCA  960
      S  E  A   V  Q  N  I   T  D  I   L  T  P   E  I  P  D   L  P  I   P  N  P   P  L  G  K >
 961 AAAAGAACAA TGGAGGAATC AATAAAAAGT AACGAAAAGA TTCTAGACAT GTTGGCGTTT TACGTGGACC AAGACGGCGG 1040
       K  N  N   G  G  I   N  K  K  *  p10 end                                                >
               Ⓜ  E  E  S   I  K  S   N  E  K  I   L  D  M   L  A  F   Y  V  D  Q   D  G  G  >
1041 CAACTCGCCA GCCATAGTAC GAGTTGTAAA CATTTTAAAT TATATAAATT CAAATTTTGT TAGAGTTAAA CTGTTGGACG 1120
      N  S  P   A  I  V  R   V  V  N   I  L  N   Y  I  N  S   N  F  V   R  V  K   L  L  D  D >
1121 ACAATCTGGG CATAGACGTG ATGGACGCAT CGTCGCCCGT CTCGATATCC GAGGGACGTA TGAGTTGTGA CCTACAAATG 1200
        N  L  G   I  D  V   M  D  A  S   S  P  V   S  I  S   E  G  R  M   S  C  D   L  Q  M  >
1201 TCCATGTTCA ACGTTGACTT TGATTGGTAC ACCGACAACG TTGTCAAGAA CGTTACGCAT CGTAAAGAGC CGGCCGACAG 1280
     S  M  F  N   V  D  F   D  W  Y   T  D  N  V   V  K  N   V  T  H   R  K  E  P   A  D  S  >
1281 CGAGCTTAGA ATAATCGATT GCGACAGACA CTGGCCTCTC ACCTACAATA TTAGAAACTT TACATTGTTT CGAGAGAAGA 1360
      E  L  R   I  I  D  C   D  R  H   W  P  L   T  Y  N  I   R  N  F   T  L  F   R  E  K  S >
1361 GTGCGGCGAC GACGACGACG ACGACGACAA GTCTTTCGCC CACGGCCGCT TTAATTTTCA AAGTTTAGA ATGCCACTAT 1440
        A  A  T   T  T  T   T  T  T  S   L  S  P   T  A  A   L  I  F  K   V  L  E   C  H  Y  >
1441 ACTAAATGTG TTCACGTCGA TCGCGGCGCC GATAACAACG ACACTTGTGT GTCATGCGAC GATTACCGAT TCGATTTTGA 1520
     T  K  C  V   H  V  D   R  G  A   D  N  N  D   T  C  V   S  C  D   D  Y  R  F   D  F  E  >
1521 AGACTGCAAT CATTTTCATA AATCATCATC ATCGTCAAGC CAACAAGATT ACAGACTCGA CGAGATCAGG CAAGCTATTC 1600
      D  C  N   H  F  H  K   S  S  S   S  S  S   Q  Q  D  Y   R  L  D   E  I  R   Q  A  I  R >
1601 GTAAATCCAA AAACCACATG TACGTCGAGT TTGAGAGGCA CGAGCTAGTG CCCGAACTGA TGGTGGTTAG AAGAGACGAG 1680
        K  S  K   N  H  M   Y  V  E  F   E  R  H   E  L  V   P  E  L  M   V  V  R   R  D  E  >
1681 CGAGACCGGC GGTGTCATTT CTTTTACTCG CATCCCAGAT CGACTTTGCC GTACGCGGTC TTGTTCACGT TGCCCGACAA 1760
     R  D  R  R   C  H  F   F  Y  S   H  P  R  S   T  L  P   Y  A  V   L  F  T  L   P  D  K  >
1761 ACTTGCCAAC ATCGATTGTC CGGGCGGCGG CAAAGACAAA ATAAATTTAT ACCAACACTG CACATTCAAA CGGGGAGCGG 1840
      L  A  N   I  D  C  P   G  G  G   K  D  K   I  N  L  Y   Q  H  C   T  F  K   R  G  A  V >
1841 TCATGCTCGC CGCCGACGCC GACGTGATCA CGTACCGAAA CGCGATCGAC CATAATGTCG TTGTGCCCGA TTTTGTACAA 1920
        M  L  A   A  D  A   D  V  I  T   Y  R  N   A  I  D   H  N  V  V   V  P  D   F  V  Q  >
1921 AAATTTTTAT TTGCTACCGA ATAAACTCTT TTGAAAAAAA AATTGTTTTT TTTATAATCA GTAATTTATG TACCAAAGCG 2000
      K  F  L  F   A  T  E   *  ORF945 end                 p74 end   *   Y  N  I  Y  W  L    <
2001 GATCGCCGGC GGCGAACCGT TGCATTCCAA TAAAATACAT GAACGGAGCG TTGATTAGTT TAAAAACTAT CATGAGTATA 2080
     P  D  G  A   A  F  R   Q  M  G   I  F  Y  M   N  G  A   L  I  S   L  K  T  I   M  S  I  <
2081 AAGATTATAA CGAAGACTGG ACATGACGGG GCCGCCGGCG ATCCACGCGA TTCCGGCGCA CGCAAAAGCA GAAACGTTAG 2160
        K  I  I   T  K  T   G  F  P  A   N  I  L   K  F  V   I  M  L  I   F  I  I   V  F  V  <
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Fig. 1. Nucleotide sequence of the p10 locus in the SpliMNPV genome. The nucleotide sequence of 2160 nt from the 3·8 kbp *Bam*HI SpliMNPV DNA fragment containing ORF552, the p10 gene, ORF945 and the 3′ end of the p74 gene is presented with the predicted amino acid sequences of each ORF in the one-letter code. Presumed initiation methionines are circled and the direction of translation is shown with arrows at the right end of each line. A putative TATA box and the late promoter initiation motif (TAAG) are underlined and the functional TAAG site is double underlined. Putative transcription poly(A) signals (AATAAA) are underlined, or overlined when present on the complementary DNA strand.

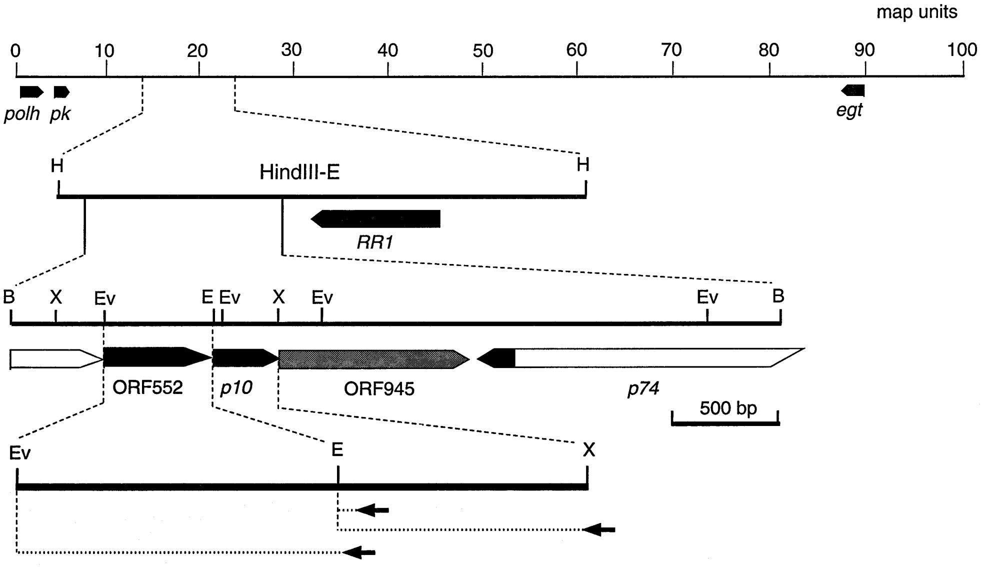


Fig. 2. Location of the ORF552–p10–ORF945–p74 cluster on the SpliMNPV genomic map. Schematic representation of the SpliMNPV genome in map units showing the position of the polyhedrin gene (*polh*), protein kinase gene (*pk*) and ecdysteroid UDP-glucosyltransferase gene (*egt*). The restriction fragment *Hin*dIII-E containing the ORF552–p10–ORF945–p74 cluster and a ribonucleotide reductase subunit 1 gene (*RR1*) is shown under the genomic map. The line below represents the 3·8 kbp *Bam*HI DNA fragment with the restriction sites *Bam*HI (B), *Eco*RI (E), *Xba*I (X) and *Eco*RV (Ev). Large arrow-boxes indicate the size and position of the identified genes. ORFs for which only a partial sequence is available appear as white boxes. Dotted lines and arrows show the direction of the primer extension reaction (a) and the direction of single-stranded DNA probe synthesis for the p10 gene (b) and ORF552 (c).

DNA probe, a 700 nt *Eco*RI–*Hin*dIII DNA fragment of the *Hin*dIII-P fragment which contains the 3′ portion of the p74 gene (Kool & Vlak, 1993), was used for the Southern hybridization, under non-stringent conditions. According to the results of this hybridization (not shown) the p74-like gene is located on the *Hin*dIII-E fragment (Croizier *et al.*, 1989; Faktor *et al.*, 1995), and a 3·8 kbp *Bam*HI fragment included within the *Hin*dIII-E fragment was cloned into a commercial plasmid (pUC19). A physical map of the location of restriction enzyme sites was generated for this fragment and the exact location of the 3′ portion of the p74 gene was determined using the same 700 bp AcMNPV DNA probe. Sequence determination of 300 nt of this fragment and comparison with the sequence of the AcMNPV p74 gene (Kuzio *et al.*, 1989) confirmed that the 3′ part of the SpliMNPV p74-like gene had been identified.

The SpliMNPV p10 gene is separated from the p74 gene by a 945 bp open reading frame

Based on the positioning of p10 genes in the immediate 3′ flanking sequences of the p74 gene in AcMNPV, OpMNPV, CfMNPV and SeMNPV, we searched for a p10 open reading frame (ORF) in the SpliMNPV genome by determining the nucleotide sequence of 2·0 kbp of the region following the p74 termination codon. Sequence analysis of this region revealed that instead of a p10 gene, the p74-like gene is followed by an ORF of 945 bp (ORF945), in opposite orientation relative to the p74 gene (Fig. 1). No TATA box or late transcription initiation motif (TAAG) were found in the 5′ flanking sequences of ORF945. A canonical poly(A) signal (AATAAA) was identified at the ORF translation stop codon TAA (Fig. 1). A comparison of the ORF945 sequence with available databases revealed it to be unique. Within ORF945, seven GAC triplets (Fig. 1, nt 1368–1389) were observed. Similar repeats were observed in other locations in the SpliMNPV genome, as well as in the SeMNPV genome in the 5′ non-translated region of the p10 gene (Zuidema *et al.*, 1993) and in the intergenic region between the ORF *v-ubi* and xb187 (van Strien *et al.*, 1996). The function of these repeats, if any, is unknown.

Further analysis of sequences upstream of ORF945 revealed another ORF of 315 bp (Fig. 1). This ORF was found, by a search of the databases, to resemble the p10 gene and was therefore designated as the SpliMNPV p10 gene. The presumed p10 of SpliMNPV has the potential to encode a 104 amino acid protein of M_r 11130. The gene is preceded by a late promoter motif, TAAG, and a poly(A) signal was identified nine nucleotides before the ORF termination codon.

Localization of the p10–ORF945–p74 cluster in the SpliMNPV genome

To localize the p10 gene in the SpliMNPV genome and to determine its position relative to other, previously identified, genes such as polyhedrin (*polh*) (Croizier & Croizier, 1994), protein kinase (*pk*) (Faktor *et al.*, 1996), ribonucleotide reductase subunit 1 gene (*RR*1) (O. Faktor, unpublished results) and

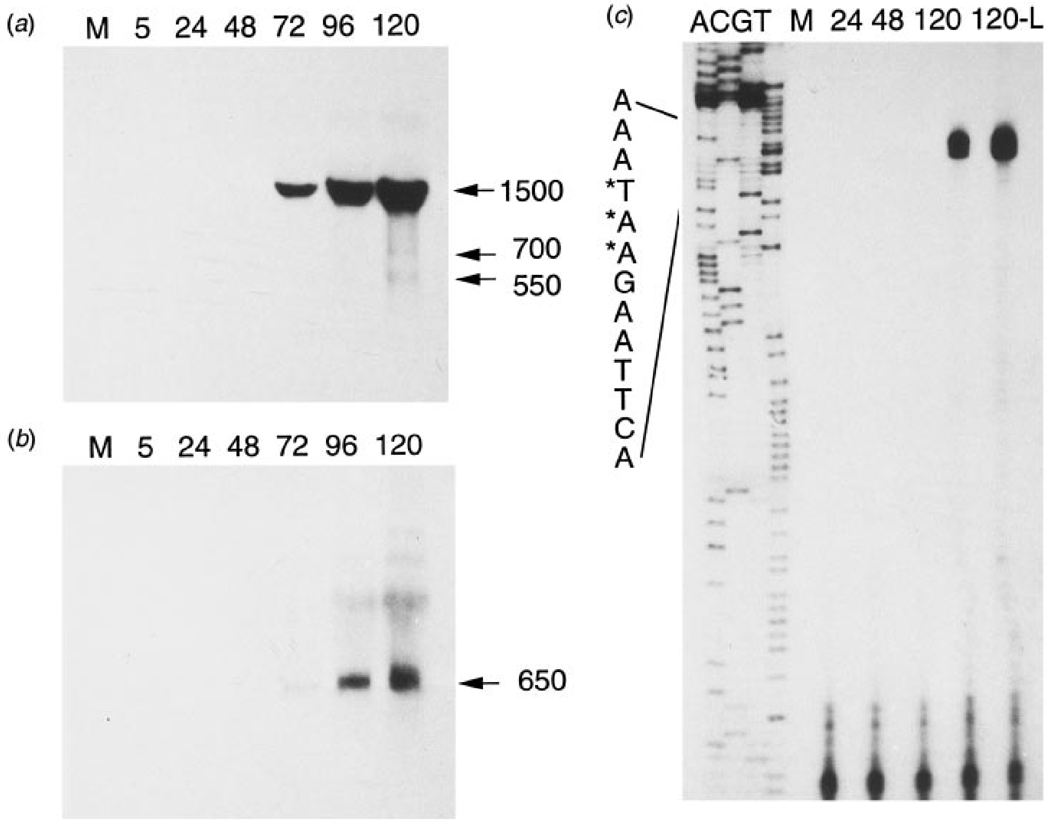


Fig. 3. Transcriptional analysis of the SpliMNPV p10 gene and ORF552. (*a*) Northern blot analysis of total RNA extracted from mock-infected cells (M) and SpliMNPV-infected cells at 5, 24, 48, 72, 96 and 120 h p.i. The p10 transcripts were detected with a single-strand ^{32}P-labelled DNA probe complementary to the p10 transcript. The p10 transcripts are indicated by arrows with sizes estimated according to RNA size markers. (*b*) The ORF552 transcripts were detected on a similar Northern blot membrane with a single-strand ^{32}P-labelled DNA probe complementary to the ORF552 transcript. (*c*) Primer extension analysis of the SpliMNPV p10 gene transcripts was performed with a p10-specific, ^{32}P-end-labelled, 19 base oligonucleotide complementary to the p10 mRNA. The oligonucleotide was annealed to total RNA (5 μg) from mock-infected cells (M), SpliMNPV-infected cells at 24, 48 and 120 h p.i., and RNA prepared from infected larvae at 120 h p.i. (120-L), elongated by reverse transcription. The size of the extension products was determined by comparison with a sequence ladder (lanes A, C, G, T) obtained by sequencing a p10-gene-containing plasmid using the same 19 base oligonucleotide as a sequencing primer. The sequence of the promoter region (mRNA strand) is indicated by the lettering at left and the specific start sites are indicated by asterisks.

ecdysteroid UDP-glucosyltransferase (*egt*) (Faktor *et al.*, 1995), we performed Southern hybridization analyses with viral DNA digested with several restriction enzymes, as described earlier. A p10 gene-containing DNA fragment was used as a probe for the hybridization. The results shown in Fig. 2 revealed that the p10 gene is located within the *Hin*dIII-E fragment of the virus. The region between the p10 gene and the polyhedrin gene was mapped using an *Eco*RI plasmid library representing the complete viral genome. According to this physical map, the distance from the p10 gene to the 3′ end of the polyhedrin gene is about 17·5 kbp. The p10 gene was found to be in the same orientation as the polyhedrin gene (Fig. 2).

Transcriptional analysis of the p10 gene

Transcriptional activity of the SpliMNPV p10 gene was determined by Northern blot analysis of RNA isolated at various times p.i. by using a strand-specific probe of the p10 gene (Fig. 2). A major transcript of approximately 1500 nt was detected at a late stage of infection (Fig. 3*a*). It was first detected 48 h p.i. (observed following a longer exposure of the blot shown in Fig. 3*a*) and its expression level increased with time. In addition, less intense bands of 550 and 750 nt were observed from 96 h p.i. Since the predicted size of the p10 transcript(s) was about 360 nt plus a poly(A) tail, we further characterized its 5′ end by primer extension analysis. A reverse transcription assay was carried out with an oligonucleotide complementary to sequences from nt 21 to 34 with respect to the translation start site (Fig. 2). Primer extension products were absent in RNA prepared from mock-infected or virus-infected cells 48 h p.i., but were observed in samples prepared 120 h p.i., as well as in total RNA prepared from infected larvae 120 p.i. Three adjacent stops at nt A, A and T located −54, −55, and −56 nt from the translation start site were detected

	SpliMNPV nt identity	SpliMNPV aa identity
SeMNPV	55.4%	49.4%
AcMNPV	36.5%	20.0%
BmNPV	38.9%	21.7%
OpMNPV	46.0%	28.5%
PnMNPV	46.4%	27.4%
CfMNPV	41.1%	28.0%

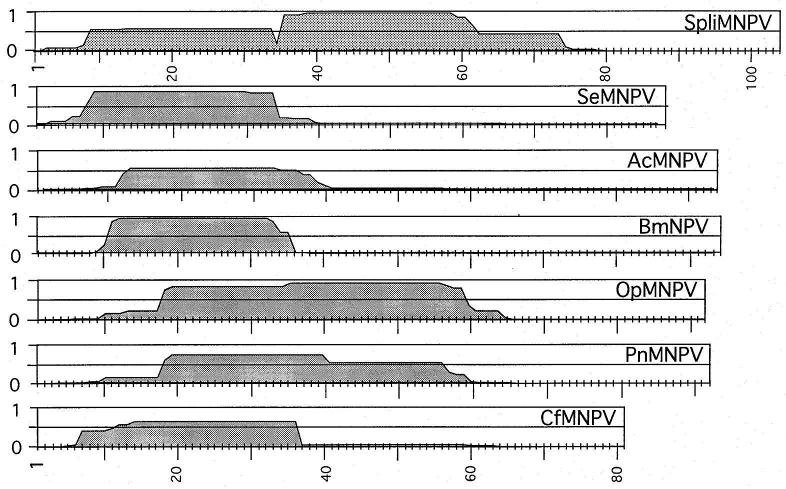
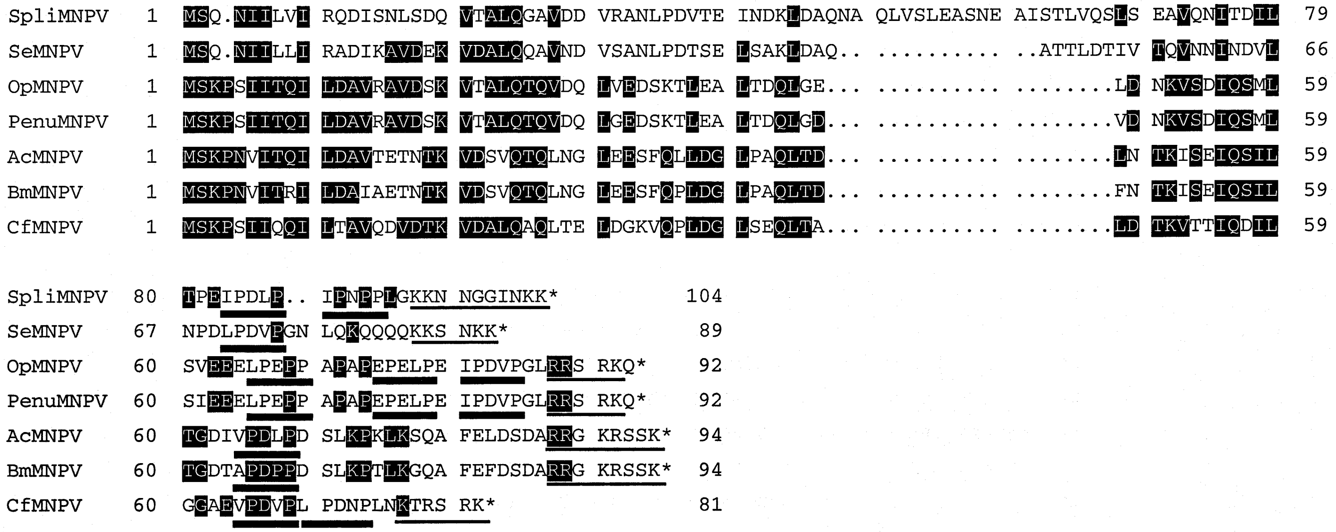


Fig. 4. Sequence comparison between the SpliMNPV p10 gene and other p10 species and the predicted probability of coiled-coil-structure formation in p10 proteins. Sequence comparison is shown on left. Numbers indicate the percentage nucleotide (nt) and amino acid (aa) sequence identities. A graphical representation of the predicted probability of coiled-coil-structure formation is shown on the right. The vertical scale denotes the prediction for coiled-coil probability between 0 and 1. The horizontal scale shows the amino acid number. Predictions were made using the COILS program and were based on a 21 residue window as described by Lupas *et al.* (1991). The virus acronym is indicated within each scheme.



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SpliMNPV   1  MSQ.NIILVI RQDISNLSDQ VTALQGAVDD VRANLPDVTE INDKLDAQNA QLVSLEASNE AISTLVQSLS EAVQNITDIL  79
SeMNPV     1  MSQ.NIILLI RADIKAVDEK VDALQQAVND VSANLPDTSE LSAKLDAQ.. .......... ..ATTLDTIV TQVNNINDVL  66
OpMNPV     1  MSKPSIITQI LDAVRAVDSK VTALQTQVDQ LVEDSKTLEA LTDQLGE... .......... ........LD NKVSDIQSML  59
PenuMNPV   1  MSKPSIITQI LDAVRAVDSK VTALQTQVDQ LGEDSKTLEA LTDQLGD... .......... ........VD NKVSDIQSML  59
AcMNPV     1  MSKPNVITQI LDAVTETNTK VDSVQTQLNG LEESFQLLDG LPAQLTD... .......... ........LN TKISEIQSIL  59
BmMNPV     1  MSKPNVITRI LDAIAETNTK VDSVQTQLNG LEESFQPLDG LPAQLTD... .......... ........FN TKISEIQSIL  59
CfMNPV     1  MSKPSIIQQI LTAVQDVDTK VDALQAQLTE LDGKVQPLDG LSEQLTA... .......... ........LD TKVTTIQDIL  59

SpliMNPV  80  TPEIPDLP.. IPNPPLGKKN NGGINKK*            104
SeMNPV    67  NPDLPDVPGN LQKQQQQKKS NKK*                 89
OpMNPV    60  SVEEELPEPP APAPEPELPE IPDVPGLRRS RKQ*      92
PenuMNPV  60  SIEEELPEPP APAPEPELPE IPDVPGLRRS RKQ*      92
AcMNPV    60  TGDIVPDLPD SLKPKLKSQA FELDSDARRG KRSSK*    94
BmMNPV    60  TGDTAPDPPD SLKPTLKGQA FEFDSDARRG KRSSK*    94
CfMNPV    60  GGAEVPDVPL PDNPLNKTRS RK*                  81
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Fig. 5. Multiple alignment of p10 amino acid sequences as deduced from the DNA sequence of AcMNPV (Kuzio *et al.*, 1984), *Bombyx mori* (Bm) NPV (Yaozhou, 1992), *Choristoneura fumiferana* (Cf) MNPV (Wilson *et al.*, 1995), *Orgyia pseudotsugata* (Op) MNPV (Leisy *et al.*, 1986), *Perina nuda* (Penu) MNPV (GenBank/EMBL accession no. U50411), *Spodoptera exigua* (Se) MNPV (Zuidema *et al.*, 1993), and SpliMNPV generated by the PILEUP program from the UWGCG computer software package. Dots denote gaps introduced by the computer program to maximize sequence alignment. Identical amino acid residues are in dark boxes. Basic amino acid residues are underlined, and conserved proline-rich domains with the consensus (E/A/V/L/I)P(D/E/N)(V/L/I/P)P are underlined with a thick line.

in this assay (Fig. 3*c*). These initiation sites are part of the baculovirus canonical late promoter motif, TAAG.

The initiation of p10 transcripts within the TAAG sequences and the size of 1500 nt indicated that the poly(A) signals located nine nucleotides upstream of the stop codon of the p10 gene (Fig. 1) and further downstream within ORF945, 1168 nt from the TAAG motif of the p10 gene, are not used. Two additional poly(A) signals at the end of ORF945, 1308 and 1387 nt from the TAAG motif, could be used, if we assume a poly(A) of about 200 nt.

Multiple sequence alignment of p10 species and structural features of the presumed SpliMNPV p10 protein

The nucleotide and amino acid sequences of p10 were compared to those of other p10 species (Fig. 4). The p10 gene

Table 1. Sequence comparison between SpliMNPV ORF552 and similar baculovirus genes

	SpliMNPV	SeMNPV*	OpMNPV	BmNPV	AcMNPV
AcMNPV †	**46·4‡**	**48·3**	**57·6**	**93·5**	–
BmNPV	**47·3**	**50·3**	**57·7**	–	97·2/**94·9**§
OpMNPV	**48·5**	**47·9**	–	71·3/**52·4**	71·8/**51·9**
SeMNPV	**58·5**	–	50·8/**31·3**	59·7/**32·8**	60·7/**34·4**
SpliMNPV	–	65·7/**52·2**	51·4/**35·8**	59·7/**32·8**	50·0/**30·3**

* The sources of the different sequences are described in the legend to Fig. 6.

† ORFs corresponding to ORF552 are described in Fig. 6.

‡ Numbers in bold in the top left of the Table are percentage nucleotide sequence identities generated using the GAP program from the UWGCG.

§ Numbers in the bottom right of the Table are percentage amino acid sequence similarities and, in bold, percentage amino acid sequence identities.

of SeMNPV, showing 55·4% nucleotide sequence identity and 49·4% amino acid sequence identity, was most closely related to SpliMNPV p10, whereas the AcMNPV p10 gene was the most distant, showing only 36·5% nucleotide sequence identity and 20% amino acid sequence identity.

Compared with other p10 proteins with sizes ranging between 81 and 94 amino acids, the putative SpliMNPV p10 protein is the longest (104 amino acids). Multiple alignment of these proteins using the GAP program generated a gap of 14 amino acids in the SeMNPV p10 protein starting at amino acid 48, and a gap of 21 amino acids in other p10 proteins starting at amino acid 48 (Fig. 5). Thus, the alignment suggests that the additional amino acid residues of SpliMNPV p10 protein reside within a defined region, rather than being scattered throughout. This region has been suggested in other p10 species to form an alpha-helical structure which was shown to have a high probability of forming a coiled-coil structure. To evaluate this probability, we employed the COILS program using a window of 21 amino acids as described by Lupas *et al.* (1991). According to the protein alignment (Fig. 4) and the prediction of coiled-coil formation (Fig. 5), the SpliMNPV p10 protein was predicted to generate a longer coiled-coil region relative to other p10 species. This relatively long region of coiled-coil is disrupted after four heptad repeats by the presence of a proline residue at position 35 and a shift of three amino acids in the next heptad repeat. Following the alpha-helical region, the SpliMNPV p10 contained a variable region rich in proline residues which fits the consensus (E/A/V/L/I)P(D/E/N)(V/L/I/P)P (Fig. 5), followed by a basic carboxy-terminal end, as described for the p10 of CfMNPV by Wilson *et al.* (1995), and similar to the structure of other p10 proteins.

A 552 bp ORF resides upstream of the p10 gene

Unlike a number of baculoviruses such as SeMNPV, AcMNPV, CfMNPV and OpMNPV, which contain a p26–p10–p74 gene cluster, analysis of the sequence upstream of the p10 gene in SpliMNPV revealed an ORF of 552 bp (ORF552). ORF552 has the potential to encode a 183 amino acid protein of M_r 20925. It is in the same orientation as the p10 gene and is preceded by a TAAG motif and a TATA box in the same location. The termination codon (TAA) is located within the TAAG motif of the p10 gene, and 45 bp downstream from this TAA codon there is a poly(A) site (Fig. 1).

A comparison of the ORF552 nucleotide sequence with the GenBank and EMBL databases (Table 1) revealed 58·5% identity to the xb187 gene of SeMNPV (van Strien *et al.*, 1996) and equal or less than 48·5% identity to similar ORFs from OpMNPV (ORF26; Ahrens *et al.*, 1997), BmNPV (ORF2; GenBank/EMBL accession no. L33180) and AcMNPV (ORF34; Ayres *et al.*, 1993). The presumed encoded protein of ORF552 was most closely related to SeMNPV xb187 (52·2% identity) and showed less than 36% identity with the other ORFs. A search of updated GenBank and EMBL databases did not show significant homology between these genes and others.

Northern blot analysis of ORF552 was performed on RNA prepared from infected cells as described for the p10 gene, using a single-strand probe (Fig. 2). A 650 nt transcript was detected at late stages of infection (Fig. 3*b*). Longer exposure of the Northern blot revealed that ORF552 transcript starts to be expressed as early as 48 h p.i. The transcript size corresponded to the expected size of ORF552 with a presumed poly(A) tail of about 100 nt.

Discussion

Identification of SpliMNPV p74 using the heterologous AcMNPV p74 gene as a probe facilitated the subsequent identification of the SpliMNPV p10 gene. A unique feature of the presumed p10 protein is its size, 11130 kDa, resulting from an extended coiled-coil structure with ten heptad repeats. This

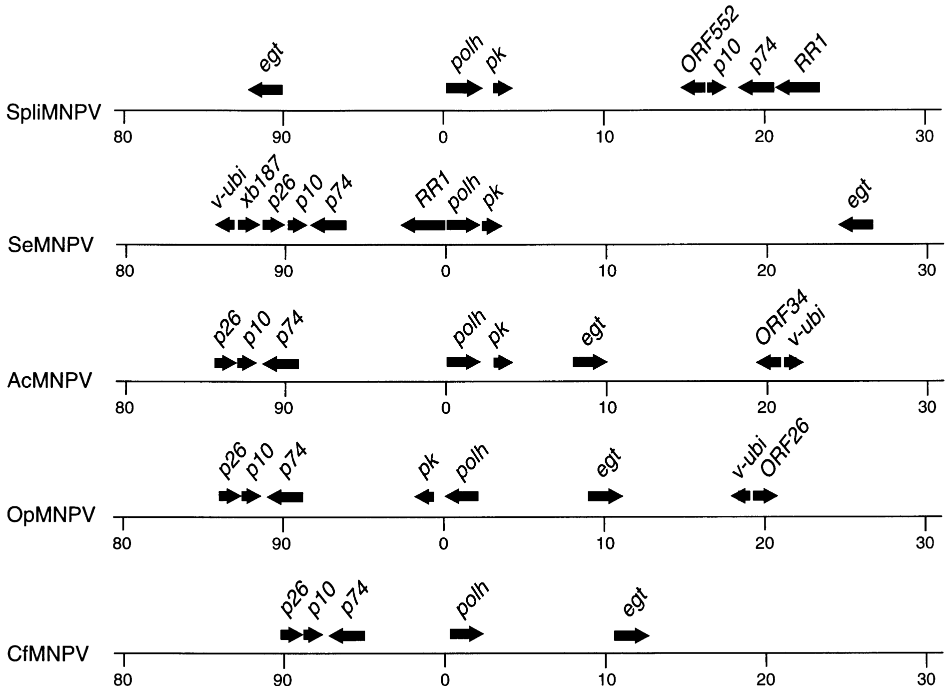

Fig. 6. Schematic alignment of the genomes of SpliMNPV, SeMNPV, OpMNPV and AcMNPV. The genomic scale is given in map units. Black arrows indicate the location of the polyhedrin gene (*polh*) in SpliMNPV (Croizier & Croizier, 1994) and CfMNPV (Qiu *et al.*, 1996) and as described previously (van Strien *et al.*, 1996); p10 gene (*p10*) as described in Fig. 4; ORF552 and its similar ORFs in SeMNPV (xb187), AcMNPV (ORF34; Ayres *et al.*, 1994) and OpMNPV (ORF26) as described previously (Ahrens *et al.*, 1997); ubiquitin gene (*v-ubi*) as described previously (van Strien *et al.*, 1996); ecdysteroid UDP-glucosyltransferase gene (*egt*) in SpliMNPV (Faktor *et al.*, 1995), CfMNPV (Barrett *et al.*, 1995) and as descried previously (van Strien *et al.*, 1996); protein kinase gene (*pk*) in SpliMNPV (Faktor *et al.*, 1996), SeMNPV (E. A. van Strien & J. G. M. Heldens, personal communication), AcMNPV (Ayres *et al.*, 1994) and OpMNPV (Ahrens *et al.*, 1997); ribonucleotide reductase gene (*RR1*) in SeMNPV (E. A. van Strien, personal communication) and SpliMNPV (O. Faktor, unpublished results); p26 and p74 genes in SeMNPV (Zuidema *et al.*, 1993), AcMNPV (Ayres *et al.*, 1994), OpMNPV (Leisy *et al.*, 1986) and CfMNPV (Wilson *et al.*, 1995).

region is suggested to play a role in the aggregation of p10 proteins (Wilson *et al.*, 1995), and we expect that variability in the size of the coiled-coil regions or in the number of heptad repeats exerts a comparable function. This region is followed by a proline-rich domain, a variable region, and a basic carboxy terminus lacking the serine or arginine residues found in the other proteins (Fig. 4). Variability within the presumed p10 protein domains may determine specificity for some functions of this protein, as was demonstrated by loss of the nuclear disintegration function when an AcMNPV p10 gene was exchanged with that of SeMNPV (van Oers *et al.*, 1993).

Transcription analysis of the SpliMNPV p10 gene revealed a major transcript of about 1500 nt and less abundant transcripts of 700 nt and 550 nt. Poly(A) signals were found for the 550 nt and 1500 nt transcripts. Alternative uses of the poly(A) signals resulting in overlapping transcripts with sizes longer than the p10 gene itself have been detected in AcMNPV (Rankin *et al.*, 1986). In OpMNPV (Leisy *et al.*, 1986) and SeMNPV (Zuidema *et al.*, 1993), the p10 major transcript sizes were 650 and 450 nt, respectively. The three adjacent transcripts of the SpliMNPV p10 gene for which the 5′ ends were mapped to the very late motif TAAG are suggested to be the major 1500 nt transcript. Moreover, Northern blotting analysis of ORF552, which is located upstream of the p10 gene, revealed a single, late 650 nt transcript that is suggested to be polyadenylated at the end of the ORF. The size of this transcript supports the suggestion that the 1500 nt major transcript of p10 initiates within the TAAG motif of p10, and not in upstream sequences, and is polyadenylated at the end of ORF945. The late expression of the SpliMNPV p10 gene (48–120 h) reflects the slow replication of this virus in SL-2 cells. We used Southern hybridization of DNA prepared from infected-SL-2 cells, at various time-points p.i. to estimate that viral DNA replication initiates at 30 h p.i. A very late expression similar to that of the p10 gene has also been found for the polyhedrin gene of SpliMNPV (Faktor *et al.*, 1996).

This study reports the identification of the SpliMNPV p10 gene using AcMNPV p74 as the DNA probe. However, gene organization in the p10 locus of SpliMNPV deviates significantly from the p26–p10–p74 gene cluster found in AcMNPV, BmNPV, OpMNPV, CfMNPV and SeMNPV (Fig. 6). In the 1·5 kbp of sequence upstream of p10, a p26-like gene is not found and instead ORF552 and a partial ORF resembling the OpMNPV ODVP6E gene (Theilmann *et al.*, 1996) are found

(Fig. 2). Downstream of the p10 gene, an ORF (ORF945) with no known function or similarity to other sequences in the databases appears to separate the p10 gene from a p74-like ORF.

The unique clustering of the p10 gene with other genes in the genome of SpliMNPV is concurrent with a different orientation of genes relative to the polyhedrin gene. In the baculoviruses shown in Fig. 6, the p26–p10–p74 gene cluster is oriented a such a way that the p74 gene is closer to the polyhedrin gene than the p10 gene, except for SpliMNPV in which the opposite localization occurs. The position of the *v-ubi*–xb187 cluster (van Strien *et al.*, 1996) also differs in the SpliMNPV genome relative to its position in other baculoviruses. In SpliMNPV, ORF552, which resembles xb187, is located upstream of the p10 gene and no *v-ubi* is found, either next to ORF552 or in the 1000 nt upstream sequences. A unique gene organization has also been observed for the polyhedrin–*pk* gene cluster and *egt* gene of SpliMNPV (Fig. 6). The observed differences in gene positioning and clustering indicate several rearrangement events in the SpliMNPV genome, in accordance with a previous suggestion that the genome organization of baculoviruses has been rearranged during their evolutionary history (Gombart *et al.*, 1989).

In addition to the unique gene organization in the SpliMNPV genome, phylogenetic analyses based on the polyhedrin and *egt* genes (Cowan *et al.*, 1994; Clark *et al.*, 1996) have suggested that SpliMNPV belongs to a distinct baculoviral group. Further information on SpliMNPV genes and their genomic localization would be useful in analysing phylogenetic relationships among baculoviruses and would contribute to an understanding of the mechanisms involved in their evolution and genetic diversity.

We thank Dr N. Chejanovsky for providing us with the SL-2 cells. This research was supported by grant no. 93/20 from DIARP, the Dutch–Israeli Agricultural Research Program.

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Received 8 January 1997; Accepted 14 April 1997