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The *Melolontha melolontha* Entomopoxvirus (*Mm*EPV) Fusolin Is Related to the Fusolins of Lepidopteran EPVs and to the 37K Baculovirus Glycoprotein[1]

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We have cloned and sequenced a 1.7-kbp DNA fragment of the *Mm*EPV genome encompassing the major polypeptide of the spindle-shaped inclusions gene termed fusolin. The sequence contained a single open reading frame of 1203 nt capable of coding for a polypeptide of 45.8 kDa. The 13 N-terminal amino acid (aa) residues were hydrophobic and could act as a signal peptide. The aa sequence also contained 13 cysteine residues very likely involved in paracrystal formation. This sequence showed significant homologies with the fusolins of two lepidopteran EPVs, the *Choristoneura biennis* EPV (*Cb*EPV) and the *Heliothis armigera* EPV, and also with the 37K glycoproteins of *Autographa californica* and *Orgyia pseudotsugata* baculoviruses. No homology was found between the *Mm*EPV fusolin and the 100K *Mm*EPV spherulin, nor with the 110K polypeptide of the *Cb*EPV and *Amsacta moorei* EPV spheroidins. These data were confirmed by Western blot analysis. Transfection of vaccinia-infected mammalian cells with a plasmid encompassing the fusolin sequence plus the upstream regulatory region resulted in transient expression of the gene. This indicated that the vaccinia transcription machinery is able to transcribe the fusolin gene. The fusolin was also expressed in insect cells via a recombinant baculovirus.

INTRODUCTION

In 1963, Hurpin and Vago described a new disease of the common cockshafer *Melolontha melolontha* L. under the name "maladie à inclusions cytoplasmiques fusiformes". Owing to its striking morphological features, the causative agent was identified as belonging to the poxvirus group (Vago, 1963) and termed entomopoxvirus (EPV) (Bergoin *et al.,* 1969a). Several EPVs were subsequently isolated from other insects including Coleoptera, Lepidoptera, Diptera, Orthoptera, and Hymenoptera (for recent review see Arif and Kurstak, 1991).

The disease caused by the *M. melolontha* EPV (*Mm*EPV) is characterized by the presence in heavily infected adipose cells of two types of cytoplasmic inclusions: spindle-shaped inclusions of 1 to 15 μm in length and larger ovoid inclusions of 10 to 20 μm, originally termed fuseaux and spherules, respectively (Amargier *et al.,* 1964). Ultrastructural studies clearly identified the spherules as electron-dense bodies containing viruses occluded in a paracrystalline lattice (Bergoin *et al.,* 1968). These inclusions can therefore be assimilated into other virus-containing inclusions such as nuclear and cytoplasmic polyhedra typical of baculovirus and cypovirus infections, respectively.

In contrast, spindle-shaped inclusions are devoid of virions and consist of a pure paracrystal (Bergoin *et al.,* 1969b). These inclusions, which lack infectious and toxic properties, are detected several days before the spherules during the infectious cycle, and their significance is still enigmatic. On thin sections, they always appear surrounded by a bilamellar membrane of smooth endoplasmic reticulum origin (Bergoin *et al.,* 1976).

Biochemical analysis of purified spindle and spherule suspensions revealed their proteinaceous nature but their distinctive amino acid (aa) composition (Bergoin *et al.,* 1970). Furthermore, serological comparisons showed that spindles and spherules are antigenically unrelated (Croizier and Veyrunes, 1971).

While the spindles are always present in EPV infections of Coleoptera, they are less frequently observed in EPVs infecting Lepidoptera and absent from EPV infections of Orthoptera and Diptera (Arif and Kurstak, 1991). In some EPVs infecting Lepidoptera, the spindles are occluded along with the virions in the matrix of spherules (Bird, 1974). Curiously, the EPV associated with bumblebees lack both types of inclusion (Clark, 1982). Although the spindles appear as a distinguishing feature of EPV infections, there are a few cases reported of spindle-shaped inclusions associated with nuclear polyhedrosis

The nucleotide sequence data reported in this article have been deposited with the EMBL Database under Accession No. X77616.

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virus (NPV) infections (Huger and Krieg, 1968; Amargier and Smirnoff, 1974).

Only a few EPV genes have been cloned and sequenced so far, including the sequence of the major occlusion body protein of *Amsacta moorei* EPV (*Am*EPV) (Hall and Moyer, 1991; Banville *et al.*, 1992), *Choristoneura biennis* EPV (*Cb*EPV) (Hall and Moyer, 1993), and *Mm*EPV (Sanz *et al.*, 1994), and also the major 50K polypeptide of *Cb*EPV (Yuen *et al.*, 1990) and *Heliothis armigera* EPV (*Ha*EPV) spindles (Dall *et al.*, 1993). To get further insight into the function of the spindles in the biology of EPV, we have cloned the fusolin gene of *Mm*EPV and we report its sequence in this communication. Comparison of the fusolin sequence with data banks revealed significant homologies with the 50K polypeptide of both *Cb*EPV and *Ha*EPV, and also with the gp37 glycoproteins of *Autographa californica* (*Ac*MNPV) and *Orgyia pseudotsugata* (*Op*MNPV) baculoviruses (Wu and Miller, 1989; Vialard *et al.*, 1990; Gross *et al.*, 1993).

MATERIALS AND METHODS

Viruses and cells

*Mm*EPV and *Am*EPV were propagated in third instar larvae of *M. melolontha* L. and *Lymantria dispar* L., respectively. *Cb*EPV spherules were a gift from Dr. J. C. Cunningham, Sault Ste. Marie, Canada. The Copenhague strain of vaccinia virus (*wt* VV) was propagated on monolayer cultures of baby green monkey kidney cells (BGM) in Dulbecco's modified MEM medium supplemented with 10% fetal calf serum at 37°. The virus was titrated according to standard assays (Mackett *et al.*, 1985). *Ac*MNPV baculovirus was amplified on *Spodoptera frugiperda* (Sf9) cells according to the procedures previously described (Summers and Smith, 1987).

Purification of spindles and spherules

Spindles and spherules from *Mm*EPV were extracted from heavily infected adipose tissues and separated on potassium iodide gradient as previously reported (Bergoin *et al.*, 1970). Spherules from *Am*EPV and *Cb*EPV were purified from infected larvae according to the same protocol.

Purification and partial peptide sequencing of fusolin

A spindle suspension virtually free of cell contaminants and spherules was dissolved in an alkaline reducing buffer (0.1 *M* Na_2CO_3 and 0.3 *M* sodium thioglycolate, pH 10.5) and subjected to SDS–PAGE analysis (Laemmli, 1970). The major 50-kDa band corresponding to fusolin was detected by Coomassie brillant blue staining, electroeluted from acrylamide gel, and purified on HPLC column chromatography.

Since the NH_2-terminal region of fusolin was inaccessible to Edman degradation, the polypeptide was cleaved by an endoprotease treatment. An aqueous solution of fusolin (50 μg/ml) was subjected to a mild treatment at 37° with trypsin at 5 μg/ml final concentration in a 0.1*M* *N*-ethyl morpholin buffer. After 4 hr of incubation at 37°, 5 μg/ml of trypsin was added and the incubation proceeded 2 hr more. The peptides resulting from this digestion were separated by HPLC on two successive microbore columns (C8 and C18) and sequenced using an automatic sequencing apparatus (Applied Biosystem Model 470).

Preparation of a DNA probe

A mixture of 16 17-mer oligonucleotides was 3′ labeled by addition of digoxigenin 11–dUTP using terminal transferase according to the protocol recommended by the supplier (Boehringer Mannheim). Two hundred nanograms of oligonucleotidic DNA was incubated with 2.5 nmol of digoxigenin-11–dUTP and 25 U of terminal transferase in tailing buffer (140 m*M* Na cacodylate, 30 m*M* Tris, pH 7.2, 1 m*M* $CoCl_2$, 0.1 m*M* DTT) at 37° for 1 hr. The probe was hybridized to Southern blots and revealed by using an antiserum coupled to alkaline phosphatase.

Purification of viral DNA and analysis of restriction fragments

*Mm*EPV DNA extraction: A sample of spherules was dissolved in an alkaline reducing buffer (0.1 *M* Na_2CO_3, 0.3 *M* sodium thioglycolate, pH 10.5, and 2% sarcosyl) to liberate the virions and the viral DNA was purified by phenol/chloroform extractions as previously described (Joklik, 1962). Five micrograms of *Mm*EPV DNA was digested by various restriction endonucleases and the resulting DNA fragments were separated on 25 × 25-cm 1% agarose gels. The DNA fragments were then transferred onto a nylon membrane (Southern, 1975) and probed with the mixture of labeled oligonucleotides. *Ac*MNPV DNA was extracted from *Sf*9 infected culture supernatants (Summers and Smith, 1987).

Cloning of restriction fragments

*Mm*EPV DNA fragments were cloned into pEMBL 18 and recombinant plasmids were used to transform *Escherichia coli* DH5α F′Q cells using standard methods (Sambrook *et al.*, 1982). The viral inserts were analyzed by Southern blots using the labeled oligonucleotide.

Sequencing of fusolin gene

The *Mm*EPV DNA fragment containing the fusolin sequence was subcloned using appropriate restriction endonucleases and sequenced by the chain termination method, (Sanger *et al.*, 1977) using single-stranded DNA templates and the sequencing kit USB-Sequenase version 2. Sequence data were analyzed using DNA STRIDER (Marck, 1989) or MULTIALIGN software (Cor-

pet, 1989) and submitted to comparisons with DNA and protein libraries (EMBL).

Transfection procedure

Confluent monolayer BGM cell cultures in 25-cm^2 flasks were first inoculated with *wt* vaccinia virus at a multiplicity of infection of 10. Two hours postinfection at 37°, the viral inoculum was removed, the monolayer was washed twice with phosphate-buffered saline, and 2 ml of MEM medium containing 40 μl of DOTAP reagent (Boehringer) and 15 μg of purified plasmid DNA was added. After 4 hr, this solution was replaced by 4 ml of MEM medium supplemented with 5% fetal calf serum. Twenty-four hours later the cells were harvested and lysed in Laemmli buffer prior to SDS–PAGE analysis.

Construction of a fusolin-recombinant baculovirus

The 21-mer oligonucleotide TTTAATAAAGATCTTTGGTAA surrounding the fusolin initiating ATG codon was used to create a *Bgl*II site by site-directed mutagenesis. The fusolin gene, deprived of its ATG translational start, was inserted in frame 4 codons downstream from the polyhedrin gene in the transfer vector pGmAc168 (from Dr. M. Cerutti) which is a pUC8 vector containing the *Eco*RI-I fragment of *Ac*MNPV genome deleted in the polyhedrin gene from position +6 to +679 relative to the polyhedrin ATG initiation codon. A *Bgl*II linker is inserted in the deleted region to facilitate the insertion of foreign genes. The resulting plasmid was used to create a recombinant *Ac*MNPV baculovirus.

A semiconfluent 25-cm^2 Sf9 monolayer cell culture was cotransfected with 15 μg of recombinant plasmid DNA, 5 μg of wild-type *Ac*MNPV DNA, and 40 μl of DOTAP reagent (Boehringer). The cells, dispersed in a serum-free medium, were plated for 1 hr at room temperature. Then the medium was poured off and replaced by 3 ml of the transfecting solution. Four hours later, a fresh serum-supplemented medium was added and the cells were incubated at 28° for 1 week.

Four OB$^-$ recombinant baculoviruses were plaque purified (Summers and Smith, 1987). The expression of fusolin was monitored by infecting a 25-cm^2 Sf9 semiconfluent monolayer cell culture with 10^8 PFU of recombinant baculovirus. Three days postinfection, the cells were suspended and centrifuged at 1000 rpm for 5 min. Pellet and supernatant were collected and treated for Western blot analysis (Ausubel *et al.*, 1987). For electron microscopy studies, a pellet of Sf9 cells infected for 2 days was fixed in a 2% glutaraldehyde solution, postfixed in a 2% osmium tetraoxide solution, and dehydrated in successive ethanol washings (respectively 30, 50, 70, and 100%). The cell pellet was then embedded in Epon and processed for thin sectioning.

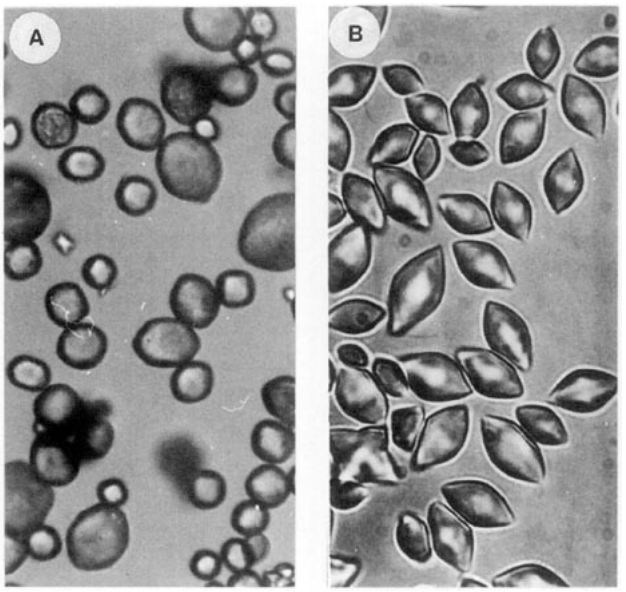

FIG. 1. Purified *Mm*EPV spherules (A) and spindle-shaped inclusions (B) observed under light microscopy (A, ×800; B, ×1800).

Antiserum production

A highly purified suspension of spindles was dissolved in denaturing buffer (0.3 *M* sodium thioglycolate, 0.3 *M* sodium carbonate, pH 10.5) and centrifuged briefly to remove undissolved materials. The protein solution was dialyzed against water for 48 hr and adjusted to 1 mg fusolin/ml.

Three inoculations of 400 μl fusolin solution were injected intraperitoneally into a mouse at 10-day intervals. The first two contained an equal volume of complete Freund's adjuvant, whereas the last was mixed with ascitic fluid (TG 180 strain from Institut Pasteur, France). Fourteen days later, the ascitic fluid was punctured and stored at −20° after centrifugation. The antiserum diluted 1/200 was used for fusolin immunodetection on Western blots. The same protocol was used to obtain antisera against spherules of *Mm*EPV, *Cb*EPV, and *Am*EPV. All the antisera were titrated by ELISA before use.

RESULTS

Characterization of the fusolin polypeptide

A suspension containing a mixture of spindles and spherules was subjected to a potassium iodide gradient centrifugation to separate both types of inclusions. The band at 1.37 density, examined under light microscopy, consisted of a pure suspension of spindles virtually free from spherules (Fig. 1B). The 1.34-density band contained essentially spherules, with less than 1% contamination by small spindles (Fig. 1A). Both preparations of inclusions were dissolved in an alkaline reducing buffer

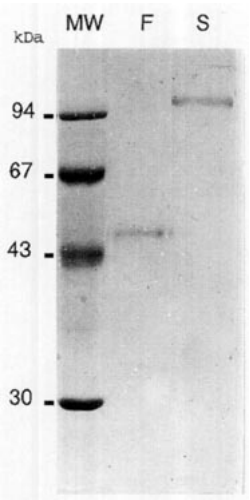

FIG. 2. SDS–PAGE analysis of polypeptides from purified *Mm*EPV spindle-shaped inclusions (F) and *Mm*EPV spherules (S). MW, molecular weight markers (Pharmacia) from top to bottom, phosphorylase b (94,000 Da), bovine serum albumin (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da).

and analyzed by SDS–PAGE (Fig. 2). Two polypeptides with clearly distinct sizes were revealed. The solution of spindles showed a major 50-kDa band, whereas the major component of spherules appeared as a polypeptide slightly above 100 kDa. These polypeptides were termed fusolin and spherulin, respectively.

Cloning of the fusolin gene

The fusolin gene was identified by probing Southern blots of restricted *Mm*EPV DNA with a degenerated probe deduced from the aa microsequencing of fusolin. Toward this end, the major 50K fusolin polypeptide was separated by polyacrylamide gel electrophoresis, electroeluted, purified by HPLC, and subjected to Edman degradation. Attempts to obtain the N-terminal aa sequence of fusolin repeatedly proved unsuccessful. Therefore, the fusolin was digested with trypsin, and the tryptic peptides were purified as described under Materials and Methods and sequenced. Among the random oligopeptides obtained, one contained a stretch of seven aa residues with the less degenerated sequence MFQQDNE that served to synthesize the 16-fold degenerated 17-mer oligonucleotide TACAA (G/A) GT (T/C) GT (T/C) CT (A/G) TT.

After 3′ labeling, the probe was used to detect the fusolin gene by Southern blot analysis. The probe hybridized to a unique band for each restriction profile. Thus, *Eco*RI, *Hin*dII, and *Xba*I restriction fragments of 3.5, 2.3, and 0.8 kbp, respectively, were detected by the probe (data not shown). A partial genome library was obtained by cloning the *Hin*dII-restricted *Mm*EPV DNA into the pEMBL 18 cloning vector and the 2.3-kbp fragment containing the putative fusolin gene was identified by hybridization to the probe. Two plasmids, designated pHF51 and pHF52, containing the *Hin*dII fragment in both orientations, were purified and used for sequencing.

Sequence and analysis of the fusolin gene

A partial restriction map of the 2.3-kbp *Hin*dII fragment along with the strategy used for sequencing are shown in Fig. 3. The 1686-nt sequence corresponding to the *Sna*BI–*Hin*dII fragment is presented in Fig. 4. This sequence contained a 1202-nt ORF, from nt 217 to nt 1419. The first in-frame ATG was 42 nucleotides downstream from the beginning of the ORF. Thus, the coding sequence corresponds to 1161 nt or 387 codons. The size of the corresponding polypeptide determined by computer analysis was 45.8 kDa, a value in good agreement with the size of 50 kDa estimated from SDS–PAGE analysis.

Computer analysis for hydrophilicity and hydrophobicity of the molecule revealed different regions within the fusolin polypeptide (see Fig. 6). The first 13 aa residues were highly hydrophobic, forming a potential secretory signal peptide. In contrast, the C-terminal moiety of the polypeptide contained a high percentage of hydrophilic aa residues with four alternating acidic and basic regions. Curiously, this region presented a stretch of 37 aa consisting essentially of Y, E, and N residues. The 13 cysteine residues randomly distributed along the fusolin sequence were potentially able to form multiple disulfide bonds. No potential N-glycosylation site (Asn–X–Tyr or Ser) was observed in this sequence.

Sequence comparisons

Comparison of the aa sequence of *Mm*EPV fusolin with the NBRF data library revealed 51% homology with both the *Cb*EPV and the *Ha*EPV fusolin polypeptides, as well as 41 and 37% with the *Ac*MNPV and the *Op*MNPV gp37 polypeptides, respectively. These homologies were scattered almost regularly within the 2/3 of the sequence for the four polypeptides. This region contained stretches with very high homology, including a 23-aa stretch (aa 226 to 248) with 20 aa common to all five polypeptides

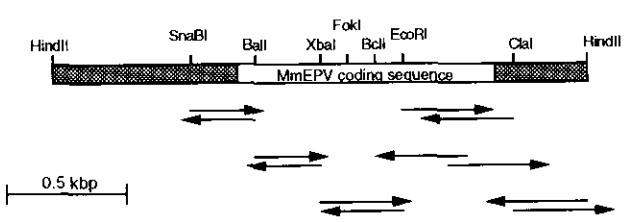

FIG. 3. Strategy for the sequencing of the *Mm*EPV fusolin gene. The 2.3-kbp *Hin*dII fragment of *Mm*EPV DNA presumed to contain the fusolin gene was cloned at the *Hin*dII polylinker site of the pEMBL 18 plasmid. A restriction map of this fragment was established and appropriate sites were used for subcloning and sequencing. As shown by the arrows, both strands of the *Sna*BI–*Hin*dII fragment were sequenced.

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SnabI
GTA.CGT.ATA.TTA.ATC.ATG.ATT.GGA.TAA.ATA.TAT.TTG.AAA.AAA.TTG.AAT.TAT.TAA.AAA.ATA
ATA.AAA.AAA.ATT.ATA.TTG.ATG.ATT.ATT.GGA.TCA.AAC.AAT.CTT.CTT.TAG.ATG.ATT.TAT.TAT
TAT.ATA.TAT.CAA.ATA.AAG.AAA.CTA.ATG.TAT.AAT.ATA.TTT.TTA.TTA.TAT.AGA.TAT.TTT.TAT
AAA.TAA.TAA.ATT.TAT.TGA.ATG.AAA.AAT.AAG.TAT.TAG.AAA.TTA.AAA.TAC.TCA.CAA.ATT.CAG
TTC.CTA.TTA.TTA.CCA.AAG.ATG.TTT.ATT.AAA.ATA.TTA.CCT.ATT.TTA.ATA.TTA.TTT.TTA.GAT  +42 (14)
                        Met Phe Ile Lys Ile Leu Pro Ile Leu Ile Leu Phe Leu Asp

TAT.GTT.AGT.GGA.CAT.GGT.TAT.ATA.ACT.TTT.CCT.ATA.GCT.AGA.CAA.AGA.AGA.TGT.AAT.GTT  +102 (34)
Tyr Val Ser Gly His Gly Tyr Ile Thr Phe Pro Ile Ala Arg Gln Arg Arg Cys Asn Val
                          BalI
CAA.GGT.GGA.TTC.TGG.TGG.CCA.CCA.GGT.GGT.TCT.GGA.ATA.CCT.GAT.CCT.ATG.TGT.AGA.GCA  +162 (54)
Gln Gly Gly Phe Trp Trp Pro Pro Gly Gly Ser Gly Ile Pro Asp Pro Met Cys Arg Ala

GCT.TAT.CAG.AAT.GTA.TAT.AAT.AAA.GTT.TTA.CAA.CAA.GGA.GGT.ACA.ATA.GAT.CAG.GCA.GCA  +222 (74)
Ala Tyr Gln Asn Val Tyr Asn Lys Val Leu Gln Gln Gly Gly Thr Ile Asp Gln Ala Ala

AGT.GCA.GCT.CAA.TAC.ATG.TTC.CAA.CAA.GAT.AAT.GAA.TAT.GCA.GCT.TTA.GCT.GGA.CCT.AAT  +282 (94)
Ser Ala Ala Gln Tyr Met Phe Gln Gln Asp Asn Glu Tyr Ala Ala Leu Ala Gly Pro Asn
      XbaI
TAT.CTA.GAT.CAA.AAT.CAT.ATA.AGA.AAT.AAT.GTA.GTT.CCT.AAT.TAT.TTA.TGT.GCA.GCT.CAT  +342 (114)
Tyr Leu Asp Gln Asn His Ile Arg Asn Asn Val Val Pro Asn Tyr Leu Cys Ala Ala His

GCT.ACT.ACT.TGG.AGA.ATT.AGA.CCA.TTT.GGA.GAT.AAA.ACT.GGT.ATG.GAT.GTA.TCA.GGA.AGT  +402 (134)
Ala Thr Thr Trp Arg Ile Arg Pro Phe Gly Asp Lys Thr Gly Met Asp Val Ser Gly Ser

TGG.ACA.CCA.ACT.GTA.ATT.CCT.CTC.CAG.GAT.AAT.ACT.GTA.AGT.ACA.GTA.CCT.ATT.GAA.TTT  +462 (154)
Trp Thr Pro Thr Val Ile Pro Leu Glu Asp Asn Thr Val Ser Thr Val Pro Ile Glu Phe

GAA.TTT.TGT.CCA.ACT.GCT.ATT.CAT.GAA.CCT.AGT.TTC.TTT.GAA.ATT.TAT.ATA.ACA.GTT.CCT  +522 (174)
Glu Phe Cys Pro Thr Ala Ile His Glu Pro Ser Phe Phe Glu Ile Tyr Ile Thr Val Pro
                          BclI
AGT.TTC.AAT.GTA.TAT.ACT.GAT.CAA.GTT.ACT.TGG.CAG.CAA.TTA.ATA.AAT.ATA.TTT.ACT.GGT  +582 (194)
Ser Phe Asn Val Tyr Thr Asp Glu Val Thr Trp Gln Gln Leu Ile Asn Ile Phe Thr Gly

CCA.ATA.CCT.TTA.GTC.CAG.AGA.AGA.CCT.GAT.TCA.CAA.TGT.AAT.GCT.CAT.AAT.CTT.GTT.TAT  +642 (214)
Pro Ile Pro Leu Val Gln Arg Arg Pro Asp Ser Gln Cys Asn Ala His Asn Leu Val Tyr
                     EcoRI
AGA.ACA.ACT.GTT.GGA.ATT.CCA.GTT.AGA.CAA.ACA.CAA.TTT.GTT.CTA.TAT.GTT.AGA.TGG.CAA  +702 (234)
Arg Thr Thr Val Gly Ile Pro Val Arg Gln Thr Gln Phe Val Leu Tyr Val Arg Trp Gln

CGT.AAT.GAT.CCA.GTT.GGA.GAA.GGA.TTT.TAT.AAT.TGT.GCT.GAT.GTT.ATA.TTT.GCA.CAT.AGA  +762 (254)
Arg Asn Asp Pro Val Gly Glu Gly Phe Tyr Asn Cys Ala Asp Val Ile Phe Ala His Arg

TTA.GGT.ATT.AAT.GAA.GAA.GAT.AAA.ATA.CGT.CCT.CCA.AAA.ATG.AAA.TGT.AAA.GGA.AAT.GAT  +822 (274)
Leu Gly Ile Asn Glu Glu Asp Lys Ile Arg Pro Pro Lys Met Lys Cys Lys Gly Asn Asp

AAA.GAT.TGT.TAT.AAA.CAT.CAT.CAT.AGA.CAT.AAT.AGA.TAT.GAA.AAT.GAT.TAT.GAA.AAT.AAT  +882 (294)
Lys Asp Cys Tyr Lys His His His Arg His Asn Arg Tyr Glu Asn Asp Tyr Glu Asn Asn

TAT.GAA.AAT.TAT.GAA.AAT.TAT.GAA.AAT.AAT.TAT.GAA.AAT.AAT.TAT.GAA.AAT.AAT.TAT.GAA  +942 (314)
Tyr Glu Asn Tyr Glu Asn Tyr Glu Asn Asn Tyr Glu Asn Asn Tyr Glu Asn Asn Tyr Glu

AAT.AAT.TAT.GAA.TAT.GAG.TAT.GAA.TAT.GAT.CGT.AAT.AAT.CGT.GAA.CAT.TAT.CAT.AAA.TGT  +1002 (334)
Asn Asn Tyr Glu Tyr Glu Tyr Glu Tyr Asp Arg Asn Asn Arg Glu His Tyr His Lys Cys

AAA.CAC.CAC.TCA.TGT.ATG.CAA.CAT.AAT.TAC.TAT.GAA.AGA.CAA.TAT.AAT.ACC.AAA.GAT.TTT  +1062 (354)
Lys His His Ser Cys Met Gln His Asn Tyr Tyr Glu Arg Gln Tyr Asn Thr Lys Asp Phe
      SnaBI
AAT.TAC.GTA.GAA.TGG.AAT.GAT.GAT.TAT.TCT.GAT.TAT.ATT.GAG.ATC.ATA.CAG.GAT.AAT.AGA  +1122 (374)
Asn Tyr Val Glu Trp Asn Asp Asp Tyr Ser Asp Tyr Ile Glu Ile Ile Gln Asp Asn Arg

GAT.ATG.TGT.GAT.TCA.ACT.ACT.AAA.TGT.TGT.TAT.AAA.AAA.TAA.AAT.ATT.TTA.AAA.TAT.TAT  +1161 (387)
Asp Met Cys Asp Ser Thr Thr Lys Cys Cys Tyr Lys Lys STOP
                                                                    ClaI
ATT.TTA.AAA.AAT.ATT.TTA.ATA.TGT.CAG.TCA.AAA.TAT.ATT.TTA.ATT.AAT.TTT.TAT.TAT.CGA
TTT.ATA.AAT.ATT.ATT.GAG.CAT.TTC.AAA.AAT.ACA.TAA.AAA.TAT.TTA.TAT.ATT.ATC.ACA.ATT
ATC.ATT.TTG.GAA.TAT.TTT.TAC.AAT.ATA.GTA.TCA.TTT.TTT.TTT.TAA.AAA.ATA.TTA.ACA.TGT
TTA.AAG.ACA.TTA.GTG.TTA.TCA.TCA.TTG.ATA.AAA.TAA.TAT.CTA.TAG.ACA.TCA.TAT.TCA.TAA
TCT.GTT
    HindII
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FIG. 4. Nucleotide sequence of the *Sna*BI–*Hin*dII *Mm*EPV DNA fragment containing the 387 codons of the fusolin protein. The first 13 hydrophobic aa residues presumed to function as a signal peptide are printed in italic. The amino acid sequence of the protein obtained by microsequencing is underlined. The probe used to identify the fusolin gene was derived from the first 6 codons of this sequence. Nucleotides are numbered from the first ATG to the last lysine codon. Amino acids are numbered in parentheses. The 13 cysteine residues are in bold characters.

(Fig. 5). Beyond aa 265, no significant homology was detected in the C-terminal moiety of the polypeptides.

Parallel to these aa homologies, computer-generated hydropathy plot comparisons with the Kyte and Doolittle (1982) algorithm showed great similarities in the organization of the five molecules: a short N-terminal hydrophobic domain, a central region with alternate hydrophilic and hydrophobic peaks, and a clearly hydrophilic C-terminal domain (Fig. 6).

No further homologies were present between the *Mm*EPV fusolin and the EMBL sequence data bank, including EPV occlusion body proteins. The AT-rich 5′ noncoding sequence upstream of the initiation ATG codon is consistent with transcriptional regulatory signals of vertebrate poxviruses (Moss, 1990). However, no significant homology with early or late vaccinia promoter consensus sequences (Davison *et al.*, 1989a,b) could be detected. In particular, the putative promoter of the fu-

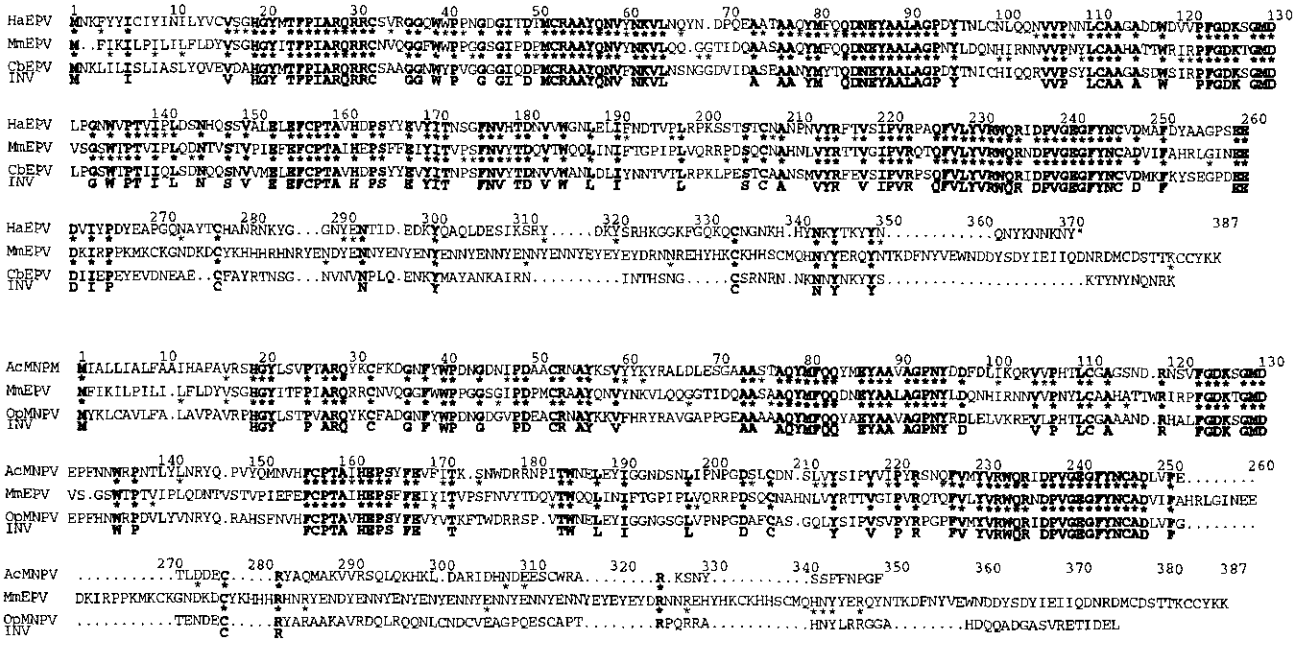

FIG. 5. Amino acid sequence comparisons between *Mm*EPV fusolin, *Cb*EPV 50K polypeptide (Yuen *et al.*, 1990), *Ha*EPV fusolin (Dall *et al.*, 1993), and gp37 *Ac*MNPV (Wu and Miller, 1989) and gp37 *Op*MNPV (Gross *et al.*, 1993) glycoproteins. Invariants (INV) are in bold characters.

solin lacks the TAAATG consensus sequence typical of poxvirus late promoters, including those of the *Am*EPV spheroidin (Hall and Moyer, 1991; Banville *et al.*, 1992), the *Cb*EPV spheroidin (Hall and Moyer, 1993), and the *Mm*EPV *spherulin* (Sanz *et al.*, 1994).

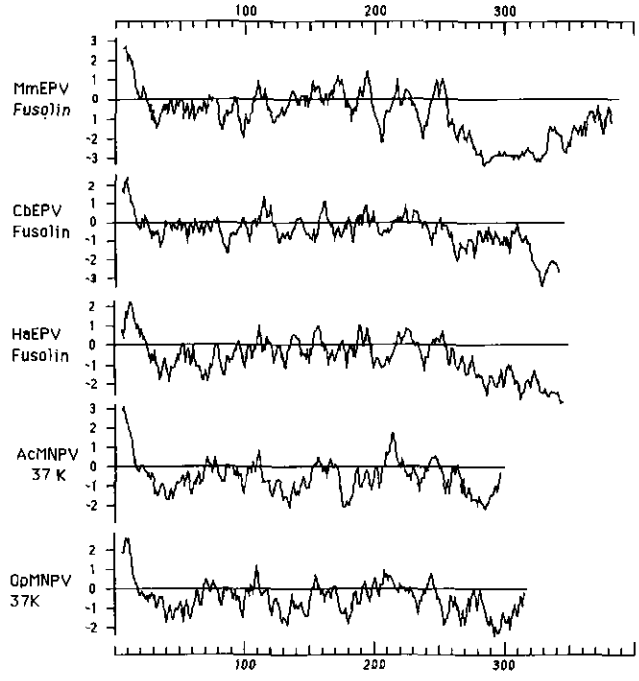

FIG. 6. Computer-generated hydropathy plots of the *Mm*EPV, *Cb*EPV, and *Ha*EPV fusolins and of the *Ac*MNPV and *Op*MNPV 37K glycoproteins. The Kyte and Doolittle (1982) algorithm was used with a window of 11 amino acid residues.

Transient expression of fusolin in mammalian cells

BGM cells infected 2 hr earlier with *wt* VV were transfected with the plasmid pHF51 encompassing the entire fusolin gene plus upstream and downstream regulatory sequences. Twenty-four hours postinfection, the cells were collected and analyzed by PAGE followed by immunoblotting. A polypeptide comigrating with fusolin was specifically detected using a polyclonal anti-fusolin antiserum (Fig. 7, lanes 1 and 3). No fusolin expression was seen in control *wt* VV-infected cells (Fig. 7, lane 2), uninfected cells, or cells transfected with pHF51 DNA alone (data not shown).

Fusolin gene expression in recombinant *Ac*MNPV

A recombinant *Ac*MNPV expressing the fusolin gene was constructed according to the steps illustrated in Fig. 8. Briefly, following the modification of the ATG initiation codon of the fusolin by site-directed mutagenesis, the fusolin sequence was inserted in frame with the polyhedrin coding sequence in a baculovirus transfer vector. The resulting vector named pGmAcF12 was used to cotransfect Sf9 cells with *wt Ac*MNPV DNA.

Among the four occlusion body-minus recombinant baculoviruses purified, one named A2264 was used to infect Sf9 cells. Three days later, the cells were pelleted and treated for SDS–PAGE analysis. As shown on Fig. 9, lane 3, a high level of fusolin expression was detected by Western blot. Despite sequence homologies between *Mm*EPV fusolin and *Ac*MNPV gp37 polypeptide, anti-fusolin antiserum failed to detect any polypeptide in cells infected with *wt Ac*MNPV used as control (Fig. 9, lane 2).

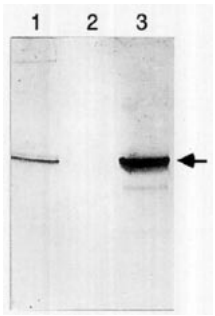

FIG. 7. Western blot analysis of polypeptides synthesized in vaccinia-infected BGM cells transfected with pHF51 plasmid DNA containing the fusolin gene. $3 \cdot 10^6$ cells were infected with *wt* vaccinia virions at 10 PFU/cell. Two hours postinfection, the cells were transfected with 15 μg of pHF51 DNA and 40 μl of DOTAP (Boehringer) reagent. Cells were harvested 24 hr later and polypeptides were separated on 12% acrylamide gel, blotted on nitrocellulose film, and revealed with a polyclonal anti-fusolin immune serum. Lane 1, infected and transfected BGM cells. Infected BGM cells (lane 2) and purified fusolin (lane 3) served as negative and positive controls, respectively.

Attempts to detect spindle-shaped inclusions in infected cells by light and electron microscopy were unsuccessful (data not shown).

Serological comparisons between fusolin and EPV occlusion body proteins

*Mm*EPV, *Cb*EPV, and *Am*EPV spherules and *Mm*EPV spindles were dissolved in reducing conditions prior to SDS–PAGE. The same amount of each sample was submitted to electrophoresis. Gels were transferred onto nitrocellulose membranes and challenged with polyclonal antisera prepared against each type of inclusion and previously titrated.

As shown in Fig. 10 B, *Mm*EPV fusolin antibodies detected not only the homologous fusolin protein but also a 50K protein in dissolved *Mm*EPV and *Cb*EPV spherules. No polypeptide was detected in *Am*EPV spherules. *Cb*EPV antibodies (Fig. 10C) detected not only the 100K spherulin proteins of *Cb*EPV and *Am*EPV but also the 50K polypeptide of *Cb*EPV and a lower molecular weight protein of about 35K in *Am*EPV dissolved spherules. This antiserum also weakly detected the 50K *Mm*EPV fusolin. *Am*EPV antibodies detected *Am*EPV 100K spherulin and, to a lesser extent, the 100K *Cb*EPV spherulin and the 50K fusolin polypeptide (Fig. 10D). This antiserum failed to detect the *Am*EPV 35K revealed by *Cb*EPV antibodies (Fig. 10C).

DISCUSSION

The assignation of the 1203-nt-long ORF of the *Mm*EPV genome to the viral gene encoding the major component of the spindles, a 50K polypeptide designated fusolin, is supported by several pieces of data: (i) The spindle suspensions were virtually free of spherules and cellular or viral contaminants, as revealed by light microscopy examination (Fig. 1B). These suspensions were used to raise specific anti-fusolin antibodies and to define a strategy for identifying the gene. (ii) PAGE analysis of purified spindles showed a single major 50K polypeptide specifically revealed by immunoblot assays. (iii) The predicted molecular weight of 45.8 kDa of the protein encoded by this gene was in good agreement with the data obtained from PAGE. (iv) Fusolin-specific antibodies failed to detect the 110K band expected to represent the *Mm*EPV spherulin, the major component of spherules. (v) In order to confirm that this gene coded for the fusolin, it was transiently expressed in vaccinia virus-infected mamma-

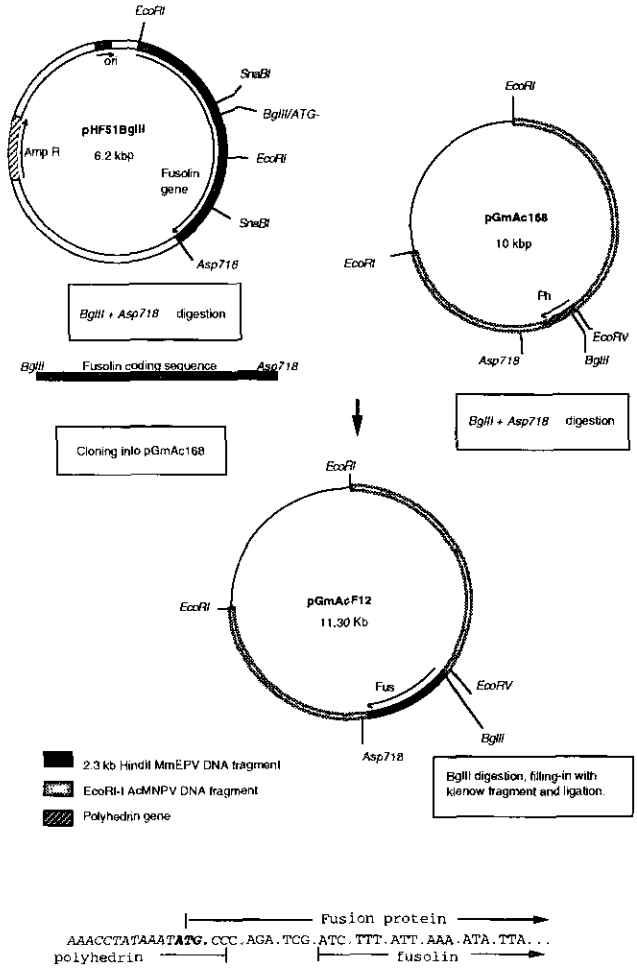

FIG. 8. Cloning of the *Mm*EPV fusolin coding sequence into a baculovirus transfer vector under the control of the *Ac*MNPV polyhedrin promoter. The plasmid pHF51–*Bgl*II contains the *Hin*dII 2.3-kbp fragment of *Mm*EPV DNA encompassing the fusolin gene (see Fig. 3), in which the ATG initiation codon has been mutated to an ATC codon so as to create a unique *Bgl*II restriction site. The 1.4-kbp *Bgl*II–*Asp*718 fragment containing the fusolin gene deprived of its promoter was excised from this plasmid and inserted at the *Bgl*II–*Asp*718 unique sites of pGmAc168 transfer vector (see Materials and Methods). The *Bgl*II site was then blunt-ended and religated in order to position the fusolin gene in frame with the polyhedrin gene, 4 codons downstream from the polyhedrin ATG initiation codon (plasmid pGmAcF12). Detail of the fusion between the polyhedrin (italic) and the fusolin sequences is presented below.

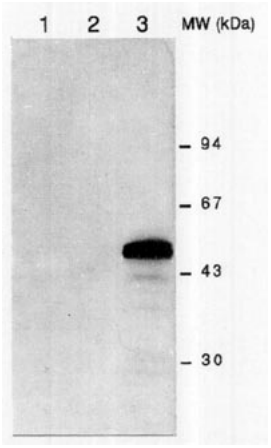

FIG. 9. Western blot analysis of polypeptides synthesized in Sf9 cells infected with a recombinant *Ac*MNPV baculovirus expressing the fusolin gene under control of the polyhedrin promoter. 10^6 cells were infected at a m.o.i. of 10 with the recombinant baculovirus and collected 72 hr later. Lane 1, mock-infected Sf9 cells. Lane 2, Sf9 cells infected with *wt Ac*MNPV baculovirus. Lane 3, Sf9 cells infected with the recombinant baculovirus expressing the fusolin polypeptide. The positions of the molecular weight markers (MW) are indicated on the right: from *top to bottom, phosphorylase b (94,000 Da), bovine serum albumin* (67,000 Da), ovalbumin (43,000 Da), carbonic anhydrase (30,000 Da).

lian cells under the control of its own promoter, or in insect cells by a recombinant baculovirus under the control of the polyhedrin promoter. In both cases, a polypeptide with the expected size of fusolin was clearly identified as such, by Western blotting.

The fact that no spindle could be detected by electron microscopy on thin sections of Sf9 cells infected by the recombinant baculovirus may be due to unfavorable conditions for crystallization of the fusolin protein. Transient expression of fusolin in VV-infected BGM cells clearly demonstrated the ability of the vertebrate poxvirus machinery to transcribe an invertebrate poxvirus gene. This result is consistent with a previous report of Pearson *et al.* (1991), showing that the 5′ noncoding sequence of the *Cb*EPV 50K gene can function as a late promoter in a vaccinia expression system.

The sequence of the fusolin gene presents some striking features. It contains a characteristic N-terminal sequence of 13 highly hydrophobic amino acids which could act as a signal peptide. This sequence is very likely involved in the targeting of the fusolin to the endoplasmic reticulum, thus providing an explanation for earlier electron microscopy observations showing that these inclusions are always surrounded by a membrane of endoplasmic reticulum (Bergoin *et al.*, 1976). Another characteristic of the fusolin gene is the numerous cysteine residues scattered along the molecule. These residues are probably responsible for intra- as well as interchain disulfide bonds and are very likely involved in crystal formation as suggested by the need for strong reducing conditions to dissolve these inclusions (Bergoin *et al.*, 1970).

An unexpected result of sequence comparisons using protein data libraries was the surprising high level of homology (about 56% at the aa level) between the *Mm*EPV fusolin and the *Cb*EPV 50K polypeptide, especially in a stretch of 229 aa (aa 19 to 248 for fusolin and aa 19 to 251 for *Cb*EPV 50K), showing 84% homology between the two polypeptides. These homologies are not fortuitous but very likely correspond to two related proteins. The antigenic commonalities detected by Western blot analysis between the *Mm*EPV fusolin and the 50K *Cb*EPV polypeptide are consistent with such hypothesis. *Cb*EPV is known to generate spindle-shaped inclusions, and electron microscopy observations have clearly demonstrated the presence of spindles occluded along with the virions in the paracrystalline lattice of *Cb*EPV inclusions (Bird, 1974). Similar observations were later reported for other EPVs of Lepidoptera (Arif and Kurstak, 1991). It is thus logical to assimilate the *Cb*EPV 50K poly-

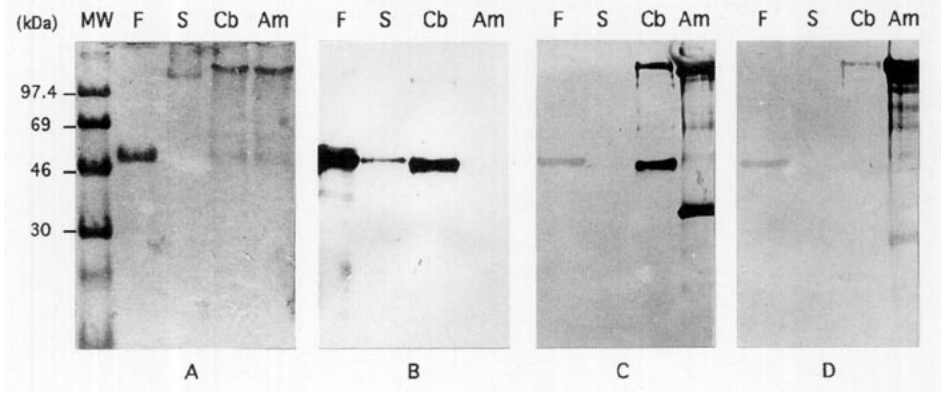

FIG. 10. Western blot comparisons between *Mm*EPV fusolin and *Mm*EPV, *Cb*EPV, and *Am*EPV occlusion body polypeptides. (A) SDS–PAGE analysis of *Mm*EPV fusolin (F), *Mm*EPV spherulin (S), *Cb*EPV spherulin (Cb), and *Am*EPV spherulin (Am). Molecular weight markers (MW; Rainbow Marker, Pharmacia) from top to bottom, phosphorylase b (97,400 Da), bovine serum albumin (69,000 Da), ovalbumin (46,000 Da), carbonic anhydrase (30,000 Da). (B, C, and D) Western blot analysis of polypeptides shown on A revealed with polyclonal immune sera prepared against purified fusolin (B), *Cb*EPV spherules (C), and *Am*EPV spherules (D).

peptide originally identified as the spheroidin (Yuen *et al.*, 1990) protein to the *Cb*EPV fusolin. The recent cloning and sequencing of a *Cb*EPV gene encoding a 100K polypeptide (Hall and Moyer, 1993) homologous to the *Am*EPV spherulin (Hall and Moyer, 1991; Banville *et al.*, 1992) is consistent with this conclusion. The lack of homology at both the nucleotide and aa levels between the fusolin on one side and the *Mm*EPV, *Cb*EPV, and *Am*EPV 100K spheroidins on the other side clearly indicate that they belong to two unrelated families of genes. These assertions are supported by the lack of detection of *Mm*EPV, *Cb*EPV, and *Am*EPV spherulins by the anti-fusolin antibodies (Fig. 5) and by our previous data showing that the anti-*Mm*EPV spherulin antibodies failed to detect the *Mm*EPV fusolin (Sanz *et al.*, 1994).

Attempts to detect a "fusolin-like polypeptide" in *Am*EPV inclusion bodies using anti-fusolin antibodies failed. This is consistent with the absence of spindle in these inclusions and, more generally, in *Am*EPV-infected cells (Roberts and Granados, 1968). However, previous reports indicated that the expression of the fusolin gene might be host-dependent, since spindle-shaped inclusions were observed in *Galleria mellonella* larvae infected by the *Am*EPV (Bergoin, unpublished data). Therefore, the presence of a cryptic fusolin gene in the *Am*EPV genome cannot be ruled out. The detection of *Mm*EPV but not *Cb*EPV fusolin by *Am*EPV antibodies was difficult to interpret. It might result from a nonspecific interaction between anti-*Am*EPV immunoglobulins and an excess of *Mm*EPV fusolin. Similarly, the strong detection by *Cb*EPV antibodies of a 35-kDa polypeptide of *Am*EPV spherules undetected by homologous antibodies was enigmatic.

The significant homologies between the *Mm*EPV, *Cb*EPV, and *Ha*EPV fusolin sequences indicated a high level of conservation of this gene among EPV genera A and B. More intriguing was the homology observed between the EPV fusolins and the gp37 of *A. californica* and *O. pseudotsugata* baculoviruses. While previous works showed spindle-shaped inclusions in baculovirus-infected cells (Huger and Krieg, 1968; Amargier and Smirnoff, 1974), no clear role could be attributed to the baculovirus gp37 glycoproteins (Gross *et al.*, 1993). However, the failure to obtain *Ac*MNPV mutants deleted of this sequence strongly suggested that this polypeptide is essential for the viral cycle (Wu and Miller, 1989). Similarly, the biological function of EPV fusolins is still unknown. Nevertheless, the high homologies found in some regions of both EPV fusolins and baculovirus gp37 glycoproteins strongly support the idea that these conserved sequences might correspond to a still unknown but possibly essential biological function common to these two groups of DNA insect viruses. Recently, Xu and Hukuhara (1992) demonstrated that a component of solubilized spheroids of *Pseudaletia separata* EPV (*Ps*EPV) significantly enhanced the infectivity of the nuclear polyhedrosis virus of *P. unipuncta* for *P. separata* larvae. The enhancing factor was identified as a 38-kDa fraction of spheroid proteins. According to the protocol used by the authors to purify spheroids, it is clear that the suspension was contaminated with spindle-shaped inclusions produced in *Ps*EPV infection (Hukuhara *et al.*, 1990). Conceivably, the 38K enhancing factor could be the *Ps*EPV fusolin itself.

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