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Cloning and Sequencing of the Spherulin Gene, the Occlusion Body Major Polypeptide of the *Melolontha melolontha* Entomopoxvirus (*Mm*EPV)

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In the late stage of infection, virions of the *Melolontha melolontha* entomopoxvirus (*Mm*EPV) are occluded into cytoplasmic paracrystalline proteinaceous occlusion bodies designated spherules (A. Amargier, C. Vago, G. Meynadier, 1964, *Mikroskopie* 19, 309–315). We have cloned and sequenced a 4-kpb DNA fragment of the *Mm*EPV genome encompassing the spherule major protein gene named spherulin. The spherulin gene contains an open reading frame able to code for a 942-amino-acid (aa) polypeptide (MW 109 kDa), consistent with a size above 100 kDa determined by SDS–PAGE for purified spherulin. The *Mm*EPV spherulin showed more than 40% aa homology with the *Amsacta moorei* EPV (*Am*EPV) spheroidin and shared homologies with the partially sequenced *Choristoneura biennis* EPV (*Cb*EPV) spheroidin, indicating that this biologically important polypeptide is well conserved among EPVs infecting phylogenetically as distant groups of insects as lepidoptera and coleoptera. Western blot analyses confirmed the relationships between the three polypeptides. In contrast, no homology was detected between the *Mm*EPV spherulin and EPV fusolins or vertebrate poxvirus A-type inclusion proteins. The 45 bases upstream from the ATG initiation codon of spherulin shared 60% homology with the vaccinia virus late promoters including the highly conserved TAAATG consensus sequence. Furthermore, the 5′ extremity of the spherulin mRNA consisted of a poly(A) tract of about 20 nucleotides just upstream from the AUG translational initiation codon. These are characteristic features of vertebrate poxvirus late mRNAs suggesting similar modalities of gene expression for vertebrate and insect poxvirus genomes.

INTRODUCTION

The *Melolontha melolontha* entomopoxvirus (*Mm*EPV) was the first poxvirus to be described as associated with an insect disease (Vago, 1963). Since then, several entomopoxviruses (EPVs) have been found infecting coleoptera, lepidoptera, diptera, hymenoptera, and orthoptera (for recent reviews see Arif, 1991; Goodwin *et al.*, 1991). In the late stage of EPV infections, the virions are generally occluded into a paracrystalline proteinaceous matrix giving rise to large oval-shaped or paraspherical occlusion bodies originally designated as "spherules" in *Mm*EPV infection (Amargier *et al.*, 1964; Bergoin *et al.*, 1967, 1968a). Once released from cadavers, the spherules ensure the survival of the virions during the extracellular phase of their life cycle, thus allowing the natural propagation of the disease. In this respect, these occlusion bodies fulfil the same functions as the polyhedra or granules of baculoviruses (Bilimoria, 1991) or the polyhedra of cypoviruses (Hukuhara and Bonami, 1991). The A-type inclusions of some vertebrate poxviruses such as cowpox (Ichihashi *et al.*, 1971) can be considered as biologically equivalent to spherules, although they lack a rigid paracrystalline structure.

A salient feature of several EPV infections, including those of coleoptera and of some lepidoptera, is the presence of a second type of cytoplasmic spindle-shaped inclusions originally used to designate the *Mm*EPV-induced disease as the so-called "maladie á inclusions fusiformes" (Hurpin and Vago, 1963). These cytoplasmic paracrystalline inclusions were named "fuseaux" (Amargier *et al.*, 1964). Unlike the spherules, they are devoid of virions and their biological significance is still enigmatic (Bergoin *et al.*, 1971; Gauthier *et al.*, submitted).

We have previously reported the ultrastructure (Bergoin *et al.*, 1968b; 1971), the chemical properties (Bergoin *et al.*, 1967) and the amino acid (aa) composition (Bergoin *et al.*, 1970) of *Mm*EPV spherules. We report here the cloning and sequencing of the *Mm*EPV gene encoding the major polypeptide of spherules designated spherulin. The sequence of this polypeptide contains 942 aa residues with as much as 46 cystein residues. Comparison of the *Mm*EPV spherulin aa sequence with sequences of *Amsacta moorei* EPV and *Choristoneura biennis* EPV spheroidins revealed significant homologies, thus indicating that these occlusion body polypeptides belong to the same family of genes. In contrast, no homology was detected between *Mm*EPV spherulin and the 50-kDa EPV

The nucleotide sequence data reported in this paper have been deposited with the EMBL Data Library under Accession No. X77052.

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fusolin polypeptides. Furthermore, several elements of this gene showed that it shared the main features of vertebrate poxvirus late genes. This strongly suggests that the genes of vertebrate and invertebrate poxviruses are expressed and very likely regulated by similar processes.

MATERIALS AND METHODS

For the restriction, the cloning in bacterial plasmids and the sequencing of the *Mm*EPV DNA, classical methods of molecular biology were used as described in *Molecular Cloning: A Laboratory Manual* (Maniatis *et al.*, 1989) and in *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994).

Viruses and larvae

*Mm*EPV was propagated in *M. melolontha* third instar larvae reared at 20° as previously reported (Bergoin *et al.*, 1970). *Am*EPV was propagated in *Lymantria dispar* third instar larvae collected in the field and reared at 28° on oak tree leaves. *Cb*EPV spherules were a gift of Dr. J. Cunningham (Forest Pest Management Institute, Sault Ste. Marie, Ontario, Canada).

Purification of spherules

A mixture of spherules and spindles was purified from heavily infected *M. melolontha* larval adipose tissue and spherules were separated from spindles by potassium iodide density gradient (Bergoin *et al.*, 1970). Spherules from *Am*EPV and *Cb*EPV were purified according to the same protocol.

Purification and sequencing of MmEPV spherulin

Spherules were dissolved in a reducing alkaline buffer (0.15 *M* sodium thioglycolate, 0.15 *M* Na_2CO_3, pH 10.5) as previously reported (Bergoin *et al.*, 1967). Spherulin was purified by HPLC on a Beckman C3 column, and was then submitted to partial digestion by mild treatment of 70 μg of spherulin in 100 μl of 0.2 *M* carbonate buffer, pH 11.0, containing 0.1% SDS and 0.04 unit of Lys-C endopeptidase (Boehringer) for 30 min at 37°. The digestion products were purified by HPLC on a Microbore C8 column. Microsequencing of oligopeptides was performed by the recurrent method of Edman (Edman and Begg, 1967) using an automatic sequencing apparatus (Applied Biosystem Model 470).

Viral DNA extraction and construction of *Mm*EPV genomic libraries

Spherules were treated by the reducing alkaline solution above mentioned and viral DNA was phenol extracted. Following precipitation with cold absolute ethanol, DNA was dissolved in TE buffer (10 m*M* Tris, 1 m*M* EDTA, pH 8.0). DNA concentration was estimated by spectrophotometry. *Eco*RV- and *Xba*I-restricted *Mm*EPV DNA was cloned at the *Sma*I and *Xba*I unique sites of the pEMBL19$^+$ (Dente *et al.*, 1983) polylinker, respectively, in order to produce partial libraries. Recombinant plasmids were amplified in *Escherichia coli* strain DH5αF'1Q.

Detection and sequencing of the spherulin gene

*Mm*EPV DNA libraries were screened with a 3' digoxigenin (Boehringer)-labeled degenerated oligonucleotide probe. The *Eco*RV and *Xba*I clones hybridizing with the probe were submitted to nested exonuclease III mung bean nuclease (Stratagene) deletions (Henikoff, 1984) in both orientations and DNA sequencing was done by the dideoxy chain termination method (Sanger *et al.*, 1977) with [α-^{35}S]dATP (Amersham) and Sequenase Version 2.0 DNA sequencing kit (U.S. Biochemical).

Isolation of mRNAs and sequence of the 5' end of spherulin mRNA

One month postinfection, *M. melolontha* larvae were dissected and the adipose tissue mixed with GIT buffer (4 *M* guanidine isothiocyanate, 25 m*M* sodium acetate, pH 6.0, and 0.1 *M* β-mercaptoethanol) was homogenized through a syringe. Following clarification at 16,000 *g* for 10 min, the supernatant was centrifuged through a 5.7 *M* CsCl cushion (160,000 *g* for 12 hr at 20°). The pellet was dissolved in distilled H_2O and submitted to a phenol–chloroform extraction. Poly(A)$^+$ RNAs were purified through an oligo(dT) cellulose column (Boehringer) and mRNA concentration was estimated by spectrophotometry.

The 5' terminal sequence of spherulin mRNA was performed according to a protocol derived from primer extension (Ausubel *et al.*, 1994), using the pool of poly(A)$^+$ mRNAs as template. A 35-mer oligodeoxynucleotide complementary to the spherulin mRNA and hybridizing between nt +100 and nt +66 relative to nt +1 for the A of the AUG translation initiation codon was used as primer. Sequence was performed by the dideoxy termination chain method (Sanger *et al.*, 1977).

Preparation of antibodies

500 μl of dissolved purified *Am*EPV, *Cb*EPV and *Mm*EPV spherules at a concentration of 1 mg/ml were injected intraperitonealy into mice with an equal volume of Freund's incomplete adjuvant. At 10-day intervals, two similar injections were performed, and a third where the Freund's adjuvant was replaced by a suspension of ascitic cells was also performed (TG 180 strain from Institut Pasteur, Paris). Ten days later, the ascitic fluid was collected from mice, then centrifuged 10 min at 4000 *g* to pellet the ascitic cells. The resulting polyclonal antisera were used for immunodetection of spherulins on Western blots. In order to prepare highly specific anti-*Mm*EPV spherulin polyclonal antibodies, the 100-kDa band corre-

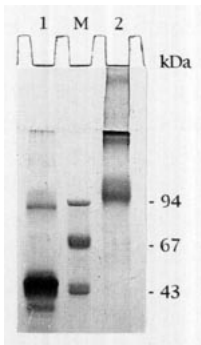

FIG. 1. SDS–PAGE analysis of *Mm*EPV spherulin and fusolin. Purified suspensions of spherules and fuseaux were dissolved in a reducing alkaline buffer (see Materials and Methods), subjected to a 12% SDS–PAGE and stained with Coomassie blue. Lane 1, fuseaux; lane 3, spherules; lane M, molecular weight marker.

sponding to spherulin was extracted from a polyacrylamide gel and used to immunize mice according to the same protocol as above.

Western blot analysis

Proteins separated by SDS–PAGE using 12% polyacrylamide gels (Laemmli, 1970) were electrophoretically transferred using a semidry transfer unit (ABCOS) onto nitrocellulose membranes (Schleicher & Schuell). The membrane was preincubated in 50 m*M* Tris–HCl, 200 m*M* NaCl, 5% milk powder, and 0.05% Tween-20, pH 7.4, before incubation for 2 hr at 25° with 1:300 dilution of spherulin antisera. After several washes, the membrane was incubated for 2 hr at 25° with a 1:400 dilution of rabbit anti-mouse IgG conjugated with horseradish peroxydase (Institut Pasteur). After washing, revelation was done with 3-amino-9-ethylcarbazole (Sigma) and H_2O_2 in a 0.05 *M* acetate buffer, pH 5.0.

Analysis of the DNA sequence

Computer analyses of the DNA and the deduced amino acid sequence of the *Mm*EPV spherulin gene were performed using the Bisance package and DNA STRIDER, version 1.0 (Marck, 1989).

For DNA and Protein homologies, BLAST and FAST programs were used. Data libraries examined were GenBank Release 80 (12/1993) and EMBL Release 36 (12/1993).

RESULTS

PAGE and Western blot analysis of spherulin

A mixture of *Mm*EPV spherules and spindles extracted from heavily infected grubs were separated and purified as previously reported (Bergoin *et al.*, 1970). Similarly, suspensions of *Am*EPV and *Cb*EPV spherules were purified (see Materials and Methods). Each suspension was solubilized in an alkaline reducing buffer (0.1 *M* sodium thioglycolate, pH 10.6) and allowed to migrate in a denaturing polyacrylamide gel. As shown on Fig. 1, the *Mm*EPV spherules consisted of a major polypeptide of about 100–110 kDa named spherulin whereas the major constituent of *Mm*EPV spindles was a 50-kDa polypeptide designated fusolin (Gauthier *et al.*, submitted). The *Am*EPV and *Cb*EPV spherules showed the same pattern as the *Mm*EPV spherules with a major polypeptide of about 100 kDa (data not shown).

On Western blots, antibodies directed against purified *Mm*EPV spherulin strongly revealed the 100-kDa homologous spherulin polypeptide but failed to reveal the 50-kDa fusolin (Fig. 2). In contrast, antisera raised against *Mm*EPV spherules clearly revealed both the *Cb*EPV and *Am*EPV above 100-kDa major polypeptides (Fig. 2), a result strongly suggesting that there was a significant homology between the occlusion body proteins from different EPVs. Western blot analyses using antibodies raised against dissolved *Am*EPV or *Cb*EPV spherules gave similar results (data not shown).

Identification of the *Mm*EPV spherulin gene

In order to identify the *Mm*EPV spherulin gene, we needed an appropriate nucleotide (nt) probe. Since no EPV spherulin sequence was available when we started this investigation, we had to prepare an oligonucleotide probe deduced from a microsequencing of spherulin. We first tried to obtain the N-terminal sequence of the protein. Several attempts in this direction were unsuccessfull, very likely because this terminus was blocked (Derancourt and Caponi, C.R.B.M. Montpellier, personal communication). The spherulin was then submitted to a partial digestion using the Lys-C endopeptidase (see Materials and Methods) and the products of digestion were purified by HPLC. Among the few oligopeptides obtained, one was sequenced. Its sequence, SFEELP-MEENYEKYL, contained a less degenerated hexapeptide from which the mix of 16 oligonucleotides 5′ TCG/ATAG/ATTT/CTCT/CTCCAT 3′ were synthetized and used to probe the viral DNA.

Cloning and sequencing of the spherulin gene

*Mm*EPV DNA was digested by *Bam*HI, *Eco*RI, *Hin*dIII, *Eco*RV, *Xba*I, and *Bgl*II restriction enzymes and the re-

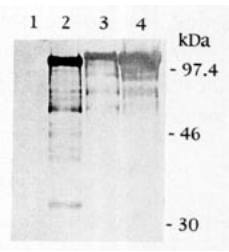

FIG. 2. Western blot analysis of *Mm*EPV-purified and -dissolved fuseaux (lane 1), *Mm*EPV-purified and -dissolved spherules (lane 2), *Cb*EPV-dissolved spherules (lane 3), *Am*EPV-dissolved spherules (lane 4) revealed with a polyclonal mouse antiserum raised against purified *Mm*EPV spherulin. Molecular weight values for protein standards are indicated.

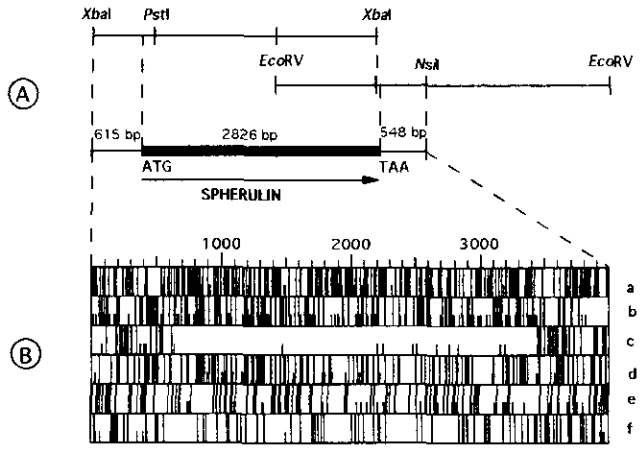

FIG. 3. Schematic diagram showing the location of the 3989 bp sequenced encompassing the spherulin gene within the overlapping 3.4-kbp *Xba*I and 4-kbp *Eco*RV fragments of the *Mm*EPV genome (A). Open reading frames on both strands of the 3989-bp sequence of the *Mm*EPV genome encompassing the spherulin gene (B). Complete bars indicate termination codons, incomplete bars indicate ATG codons (DNA STRIDER 1.0 software, Marck, 1989).

sulting fragments were separated by electrophoresis on 0.8% agarose gels (data not shown). By summing up the lengths of the fragments generated for each enzyme, a size of about 200 kbp was estimated for the viral DNA. Southern blots of DNA fragments were then hybridized to the digoxigenin-labeled oligoprobe. Two fragments in the range of 3–4 kbp were revealed, one in *Eco*RV and the other in *Xba*I profiles (data not shown). Therefore, *Eco*RV and *Xba*I *Mm*EPV partial DNA libraries were constructed in the bacterial vector pEMBL19$^+$. Among the recombinant plasmids analyzed, two reacted positively to the probe: one contained a 3.4-kbp *Xba*I fragment, the other a 4.0-kbp *Eco*RV fragment.

Subcloning and total sequencing of the 3.4-kbp *Xba*I fragment showed that it contained a long incomplete open reading frame (ORF) of 2747 nt downstream from a 5′-terminal noncoding sequence of 615 nt. Partial sequencing of the 4.0-kbp *Eco*RV fragment up to a *Nsi*I restriction site revealed a sequence of 1785 nt starting with a 1237-nt ORF overlaping on 1158 nt with the sequence of the *Xba*I-cloned fragment, followed by a 548-nt noncoding sequence. We concluded from these data that the sequence coding for spherulin consisted of a 2826-nt ORF and that the spherulin gene was located in a viral sequence overlapping the cloned *Xba*I and *Eco*RV fragments (Fig. 3A).

Sequence analysis and comparisons

The 3989-bp sequence corresponding to the *Xba*I–*Nsi*I fragment of *Mm*EPV DNA is shown on Fig. 4. This very (A + T)-rich sequence (79.4%) contains a single ORF of 942 codons in frame c (Fig. 3B) corresponding to a 109-kDa protein. The potential coding capacity of this ORF is in good agreement with a size above 100 kDa estimated by SDS–PAGE for the major spherule polypeptide (see Fig. 1). The presence in the translation product of this ORF of a 15-aa stretch (aa 733 to aa 747, see Fig. 4) identical to the oligopeptide sequence obtained by microsequencing, clearly identified it as the sequence encoding the spherulin. The sequence contains 46 cystein residues regularly distributed along the molecule except for a gap of 142 aa between cystein residues at positions 716 and 858 in the C-terminal region. Moreover, 5 putative sites for N-glycosylation (Asn–X–Thr or Asn–X–Ser) are present at aa positions 123, 522, 556, 753, and 871 (Fig. 4).

Comparison of the DNA and aa sequences of *Mm*EPV spherulin with data libraries failed to reveal any significant homology with sequences of vertebrate poxviruses, including the cowpoxvirus A-type inclusion gene (Funahashi *et al.*, 1988) or its vaccinia virus (VV) homolog (De Carlos and Paez, 1991). Among entomopoxvirus sequences, no homology was found with the *C. biennis* 50-kDa gene (Yuen *et al.*, 1990) nor with the 50-kDa *Heliothis* EPV (HaEPV) (Dall *et al.*, 1993) or *Mm*EPV fusolins (Gauthier *et al.*, submitted). In contrast, the 3989-nt *Mm*EPV sequence showed 56.8% homology with the *A. moorei* EPV DNA sequence encompassing the spheroidin gene (Hall and Moyer, 1991). This homology spanned the whole sequence, including the 5′ and the 3′ noncoding regions. At the aa level, the two ORFs shared 41.9% identity and 15.8% similarity (Fig. 5). The homologies appeared to be regularly distributed along the two sequences with three more conserved internal regions: from aa 119 to aa 325 with 53.3% identity and 10.1% similarity, from aa 466 to aa 595 with 53% identity and 14.6% similarity, and from aa 606 to aa 778 with 59.9% identity and 15.8% similarity (see Fig. 5).

The 77 aa residues corresponding to the partial sequence of the *Cb*EPV spheroidin (Hall and Moyer, 1993) could be easily aligned with the *Mm*EPV spherulin between aa 324 and aa 401. Along this stretch, the *Mm*EPV spherulin showed the same homologies with the *Am*EPV and the *Cb*EPV spheroidins (Fig. 5). In the C-terminal moiety, homologies were more scattered.

The sequence upstream from the ATG initiation codon of spherulin was very (A + T)-rich with only 6 (C + G) out of 98 bases. The 45 first ones showed 60% homology with the *Am*EPV spheroidin gene promoter (Hall and Moyer, 1991; Banville *et al.*, 1992) and the VV late promoter consensus sequence (Davison and Moss, 1989) (Fig. 6).

5′-Terminal sequence of the spherulin mRNA

In order to determine the transcription start of the spherulin gene, total poly(A)$^+$ mRNAs were isolated from infected larval adipose tissue (see Materials and Methods). A 35-mer oligonucleotide with a sequence comple-

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TCTAGAATATATGAATATTGGAATCATAATGAAGACATTATATGGGGATATATTATAGGATTATTTTTTAGCATAATGTCTTTTAATGTTTTGAAGAACG   100
ATATTGAATAAATAATTATAAATGTTATTTTTAAAAATGCCAATCCTTTATTCATTTTTTATATATATTCTTATGAGATTTAAATTAATATTTAATTAAA   200
AAGTTATTTTTTTTTTAAAATAATTTTTTTGGATTGTATAGATAAATATTTGTGTAGTAAATTTAGAATAGATAATTATATAATTATGTTTTTATTAAATAT   300
ATATAATAATTTAATAAAAGTAGAAAAATATCAAAAATAATAAAAATTTGATATATTAACATATTATCAAAATATATGAGGAATATTACGTCTATAAACA   400
TTATGTTCTTATGTATTAATATGTTAGATTATAAATTTTTATATATAATAATATAAAATATAATAAATATTTTAATTTTATTGTTATCTATAATAAAAATCTA   500
ATTAATTTTAACTTATATTTTTTATATATAAAATAATGATAAAAAATAATGTATAAATTTATATTATAAAAATATATACTGTTCTTTTAAATTTTTACTA   600

AATAATTTTTTATAAATGGATAACGTTCCTATGACAATTGAGGAGATAATTAAAATTAATGATGGTAAATATGAAGTTATTTTTTGTCTAAAAGATAAAC   700
    Spherulin >  M  D  N  V  P  M  T  I  E  E  I  I  K  I  N  D  G  K  Y  E  V  I  F  C  L  K  D  K   (28)

AAAGAATATTTGATAGAAAAGAAATAGAATTATTAGTACCATTATCTGATGGAGATGATCCATATAGTGCTGTCACTGTTGAATCTGCAGATGATAAAAC   800
Q  R  I  F  D  R  K  E  I  E  L  L  V  P  L  S  D  G  D  D  P  Y  S  A  V  T  V  E  S  A  D  D  K  T   (62)

TGAAGTATTAGAATTAGATAAAACACATTATAGATTAATATTAAAATGTACTTTAGAGGATAAATTAAGATTTTATTTTGTATTTTCTCATTGTAAATGT   900
   E  V  L  E  L  D  K  T  H  Y  R  L  I  L  K  C  T  L  E  D  K  L  R  F  Y  F  V  F  S  H  C  K  C   (95)

TTAGAAGGTGTATGGAAATATATTGGAGATTTATTACTTGCTAATTTAAAAATATGTAAAAAGATTATAAAATTATGTAATTATACAGTAAATCTTAATG  1000
L  E  G  V  W  K  Y  I  G  D  L  L  L  A  N  L  K  I  C  K  K  I  I  K  L  C  N  Y  T  V  N  L  N   (128)

AAAAACATATACCTCTTTGTGAATTACATCCTAGATTATTTATAGGATTTTATGATGATAATGGATATTATGGATTAATTACTAGACATAATATTGAAAC  1100
E  K  H  I  P  L  C  E  L  H  P  R  L  F  I  G  F  Y  D  D  N  G  Y  Y  G  L  I  T  R  H  N  I  E  T   (162)

TGGAACATTAGTAGTTAGTAAAACTGCAAATTATATTGAAATATTTCCACAACATATTTATTGTGAACATGGTAGAGATATATATTTAAATCATAAATCA  1200
   G  T  L  V  V  S  K  T  A  N  Y  I  E  I  F  P  Q  H  I  Y  C  E  H  G  R  D  I  Y  L  N  H  K  S   (195)

GTTATAGATGTATGTCCTGAAGTATGTAATGCAGTGTTAGATTTAAGAAAATCTGTTAATATTTGTGTATCATTTACATCAATAAATTATGAAGAATGTG  1300
V  I  D  V  C  P  E  V  C  N  A  V  L  D  L  R  K  S  V  N  I  C  V  S  F  T  S  I  N  Y  E  E  C   (228)

ATTCAGTCCAATTAGCATTATTAAAATCATTAGTAAATGATTATGGTGTATTTGATGTATATAATGCAGATACAGGTTTAGTATATGCTAAAGGTCTCAG  1400
D  S  V  Q  L  A  L  L  K  S  L  V  N  D  Y  G  V  F  D  V  Y  N  A  D  T  G  L  V  Y  A  K  G  L  R   (262)

AATTAATAGTAAATCATTAGTTATACAAGTTGATAAAATACCTGTTAGATTAAAGGTTAAAGCATATATGAAAGGATTAGAGGGAGAAAGATTATGTTTC  1500
   I  N  S  K  S  L  V  I  Q  V  D  K  I  P  V  R  L  K  V  K  A  Y  M  K  G  L  E  G  E  R  L  C  F   (295)

ATAAGAATTACATCAAGTACAGTAACTAATCCAGAATATGTTGCAAGTCATACAGCTACTCTTGGTTGTTTAACAGTATATAAGAAATTCAAAAAAGCTA  1600
I  R  I  T  S  S  T  V  T  N  P  E  Y  V  A  S  H  T  A  T  L  G  C  L  T  V  Y  K  K  F  K  K  A   (328)

TAGTTGATTTACTTATTCATGATTTACACACAAAAACAGTAATACCTGGAGGAAATGTAGTACTAAAATTAGTTGATTGTAATGAATATGTTAAAAAAGT  1700
I  V  D  L  L  I  H  D  L  H  T  K  T  V  I  P  G  G  N  V  V  L  K  L  V  D  C  N  E  Y  V  K  K  V   (362)

TAGTTATGGTTCTCATCTAAATGTTGGAGTTTATAAAATTGATAAAATTTATTTGAAAAATAATTTTGATAATATTCATCTTAAAACATTAGAAACTCAT  1800
   S  Y  G  S  H  L  N  V  G  V  Y  K  I  D  K  I  Y  L  K  N  N  F  D  N  I  H  L  K  T  L  E  T  H   (395)

TTTGAATGTGATAAGAAAATATTTAAGGAATATTCAACTTTATCAAGACATGATTGTACAAGAGATAAATGTAAAAAATATGGATGTAATAGATATGATG  1900
F  E  C  D  K  K  I  F  K  E  Y  S  T  L  S  R  H  D  C  T  R  D  K  C  K  K  Y  G  C  N  R  Y  D   (428)

ATGGTTGGTACACAACTGATAATAAAATATGTATTGTAGCAGGTGCTCCAAGAATACATATTAATATATGGGCAAAAATTAAAAATCTAGGATATAGGAA  2000
D  G  W  Y  T  T  D  Y  D  A  S  R  Y  I  K  L  H  P  D  G  S  L  D  L  D  L  C  Y  K  K  C  T  D  D   (462)

ACCAATATATAATCTTCATTTATGGGGATGGATATTTGATTATGATGCATCACGGTATATAAAACTTCATCCTGATGGTTCATTAGATTTAGATTTATGT  2100
   L  T  L  Y  E  A  A  R  K  K  Y  I  N  N  K  I  C  I  V  A  G  A  P  R  I  H  I  N  I  W  A  K  I   (495)

TATAAAAAATGTACAGATGATTTAACATTATATGAAGCAGCAAGAAAAAAATATATTAATGATATAATTTTAGAAAATGCTAGTTGTTATAAAAATGGTA  2200
K  N  L  G  Y  R  K  P  I  Y  N  L  H  L  W  G  W  I  F  D  D  I  I  L  E  N  A  S  C  Y  K  N  G   (528)

TGATATCATTAGGAAATCATAAATATCAAAATATATTTGAAATGGATAAATGTAGAGCATCAATAAATACTTATACTAATTTTACAAAAGAAAGACAAGA  2300
M  I  S  L  G  N  H  K  Y  Q  N  I  F  E  M  D  K  C  R  A  S  I  N  T  Y  T  N  F  T  K  E  R  Q  D   (562)

TCTCAACAATTTCGGATGTGTATTAGGAATAAATATTGGAAAACAGGTTAGTATACAAGAATTACCAGGTTGGTTAACTTGTGATGAAATTGAAATTCTT  2400
   L  N  N  F  G  C  V  L  G  I  N  I  G  K  Q  V  S  I  Q  E  L  P  G  W  L  T  C  D  E  I  E  I  L   (595)

GCTTGTGCTCCAATTGATGAAATAAAATGTTTCTGTGAGAAATTCTGTAAAATTACTAATCCAAGATTTGTACAAATGGCAACAGATATAATAAGTTTAT  2500
A  C  A  P  I  D  E  I  K  C  F  C  E  K  F  C  K  I  T  N  P  R  F  V  Q  M  A  T  D  I  I  S  L   (628)

TATTTATGTGTAATTATGTAAATATAGAAATAGATGAAGCATTAATTGATTATCCTGGATATATAGTTTTATTTGCTAGAGCAGTTAAAGTAATAAATGA  2600
L  F  M  C  N  Y  V  N  I  E  I  D  E  A  L  I  D  Y  P  G  Y  I  V  L  F  A  R  A  V  K  V  I  N  D   (662)

TTTATTATTAACTAATGGTATTTGTAATTTATGTGGTTATTCAATATCTATTCCAGTTATGTGTGGTTGTTTCGGTAAAACATTACCACACTTTGATAAT  2700
   L  L  L  T  N  G  I  C  N  L  C  G  Y  S  I  S  I  P  V  M  C  G  C  F  G  K  T  L  P  H  F  D  N   (695)

GGTGGAGTAGAAAAAAGATTCAAAGAAAAATTCTTAAAATTAAATCTTAGAGAATTAATGTGTGATGAGGAATTTGTAGAAACACCATTATATGTATCTA  2800
G  G  V  E  K  R  F  K  E  K  F  L  K  L  N  L  R  E  L  M  C  D  E  E  F  V  E  T  P  L  Y  V  S   (728)

CATATTTTAAATCATTTGAAGAATTACCTATGGAAGAAAATTATGAAAAATATTTAATAGAATATGCTAATCAATCTCAGGATTTATTACAAGGTTTATT  2900
T  Y  F  K  S  F  E  E  L  P  M  E  E  N  Y  E  K  Y  L  I  E  Y  A  N  Q  S  Q  D  L  L  Q  G  L  L   (762)

AAATACTTATACTGTAGAAGATACAAATGCTAGAGTAATATCATCTGTATATGCATTTACTTATAGAGATAAATATTTTAATGATAAATATAATACTAAA  3000
   N  T  Y  T  V  E  D  T  N  A  R  V  I  S  S  V  Y  A  F  T  Y  R  D  K  Y  F  N  D  K  Y  N  T  K   (795)

GAATATGAAGCAGGAGTTCCAAGAGAAGGAGTATTTTATAGAGATGGAGACTATAAAGAAAGAAATTATTATGATTATAATAGAAGACATAAATATTTAA  3100
E  Y  E  A  G  V  P  R  E  G  V  F  Y  R  D  G  D  Y  K  E  R  N  Y  Y  D  Y  N  R  R  H  K  Y  L   (828)

ATCCAGAAGCATTGAGTAAACTTGTCAAAGTAATAACAGAATCTGGTAATATGGGAATGATAAATAAATTACAAGAAGATTATACATGTATGCCATCATG  3200
N  P  E  A  L  S  K  L  V  K  V  I  T  E  S  G  N  M  G  M  I  N  K  L  Q  E  D  Y  T  C  M  P  S  C   (862)

TGATGAAGGATATTCAACTATAAATGTTAGCAAATCTTATTGTGATTGGTCATGTGAACCAAATAATAATTATGAATTAATTTGTAAATATGGATATAAA  3300
   D  E  G  Y  S  T  I  N  V  S  K  S  Y  C  D  W  S  C  E  P  N  N  N  Y  E  L  I  C  K  Y  G  Y  K   (895)

TTGATAGATTTAGAAAGAATACATCAATTATTAAAAGTTGCATGTTCAATACCTGATCTAGAATGTATTTATGAAAATGAAGAATGTAATAAATGTTGTG  3400
L  I  D  L  E  R  I  H  Q  L  L  K  V  A  C  S  I  P  D  L  E  C  I  Y  E  N  E  E  C  N  K  C  C   (928)

ATGTAGTAAAGGGATATGAACCGATTTATCAAACACATGTTTTAAAAATAATTGTTATAAATAATTATATATTATTTTTTGTATAAAAATATAATAATATA  3500
D  V  V  K  G  Y  E  P  I  Y  Q  T  H  V  *                                                            (942)

AATATTGTTTCAAATATATTATTAATTTATTTACATATCATATTGAATATTTATATAAAAATAACATTTTCATAATATATAATTTGTACTATATTAATAA  3600
ATTATTTTAATAGATAATAATTTATATTATATATAATTATAATTAATAATAAGAATATAATATAATAAAGAATCATGTTCAATGTATTAAAATATTAACT  3700
ATATAGATTATAATTTATAAAAAATTAATAAATATTTTATTAAAATTTCATATAGTGTTTTGAAAAAAAAATGTAATTAATATGATACCTTATCATATTA  3800
TTATAATAATGATTTCGATTTTTATAATAATATTGATATTGATTGTAAAATCAAATCAAAATTATATTATTCAAAATGATAATTTAATTATCTCAAATAA  3900
AAATTTAACAAATATAAATCATTATACATTTTATTCAAAATTTAAAAGTATTGATTTATCATATAATTATTTAAATACAATAGATGCAT              3989
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FIG. 4. Nucleotide sequence of the 3989-bp *Xba*I–*Nsi*I fragment of *Mm*EPV DNA encompassing the complete spherulin gene, plus upstream and downstream flanking regions. The deduced amino acid sequence of spherulin is shown with the first methionin codon, the cystein residues and the TAA stop codon in bold characters. The TAAATG consensus sequence typical of vertebrate Poxvirus late promoters, the stretch of 15 amino acids (aa 733 to aa 747) corresponding to the sequence obtained by microsequencing and the putative N-glycosylation sites (Asn–X–Thr or Asn–X–Ser) are underlined.

FIG. 5. Amino acid alignment of *Mm*EPV spherulin, *Am*EPV spheroidin (Hall and Moyer, 1991), and partial sequence of *Cb*EPV spheroidin (Hall and Moyer, 1993). The sequences were aligned using FASTA. The double bars correspond to identical aa residues, the single bars to conserved aa changes.

mentary to nt +100 to nt +66 downstream from the first AUG codon of the spherulin mRNA was used as primer. The sequence was established by primer extension using the AMV reverse transcriptase. As shown on Fig. 7, the mRNA had a sequence complementary to its DNA matrix up to the A of the AUG translational start codon. Upstream from this base, the DNA and RNA sequences differed and the spherulin mRNA ended by a poly(A) track of about 20 nt in length.

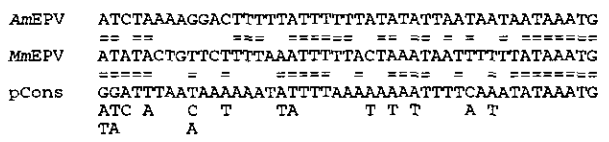

FIG. 6. Nucleotide sequence alignment of the 45 bases upstream and including the ATG initiation codon (last three characters in each line) of *Mm*EPV spherulin gene, *Am*EPV spheroidin gene (Hall and Moyer, 1991), and vaccinia virus late promoter consensus sequence (pCons) (Davison and Moss, 1989).

DISCUSSION

In this paper, we present evidence that the 3989-nt restriction fragment of the *Mm*EPV genome that we have cloned and sequenced encompasses an ORF encoding the occlusion body major polypeptide gene, designated spherulin: (i) Its potential coding capacity of 942 codons is compatible with a size of 100 kDa estimated by SDS–PAGE for the spherulin. (ii) It contains in its C-terminal moiety a stretch of 15 residues identical to the aa sequence obtained by microsequencing of an oligopeptide

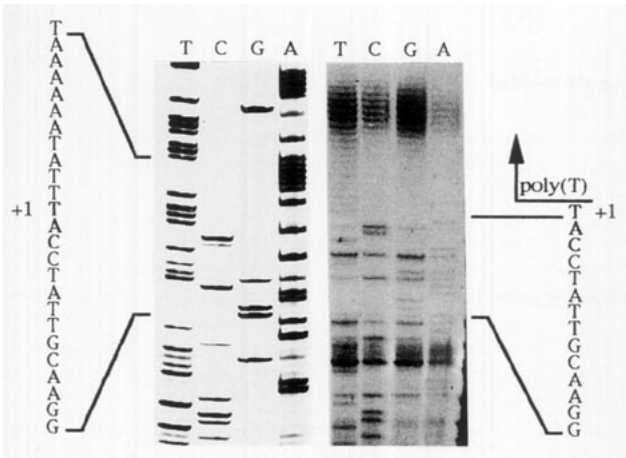

FIG. 7. 5′ End sequence of the *Mm*EPV spherulin mRNA. The sequence was determined by using a 35-mer oligonucleotide primer complementary to nt +100 to +66 relative to the AUG initiation codon of the spherulin mRNA and AMV reverse transcriptase. The same primer served to determine the viral DNA sequence of this region with Sequenase Version 2.0 DNA sequencing kit (see Materials and Methods). (Left) DNA sequence of the viral DNA strand complementary to that transcribed. (Right) DNA sequence complementary to the spherulin mRNA.

resulting from partial digestion of spherulin. (iii) The aa and nt sequences of the spherulin show significant homologies with the major occlusion body polypeptides of other EPVs.

The very high cystein content of the spherulin (46 residues) and their regular distribution along the molecule strongly suggest that these residues are able to form intra- and (or) interchain disulfide bonds. Although crystallographic studies are necessary to elucidate the three-dimensional structure of this protein, it is very likely that the interchain disulfide bonds are responsible for the supramolecular assembly of *Mm*EPV spherulin into a paracrystal. This in turn explains the difficulties encountered in dissolving *in vitro* these inclusions and the need, in addition to alkaline buffer, for reducing agents such as thioglycolate or β-mercaptoethanol in order to liberate virions (Bergoin *et al.*, 1967). It is likely that similar conditions somehow prevail in the gut lumen of *Melolontha* larvae where the occluded virions are released from spherules in order to initiate infection.

As previously suggested (Bergoin *et al.*, 1969), the abundance of virions at the periphery of spherules in the process of being occluded in the paracrystalline lattice of spherulin implies that strong interactions exist between this polypeptide and a polypeptide located at the surface of the viral envelope. Recently, McKelvey and coworkers (McKelvey *et al.*, 1992) demonstrated that the occlusion of VV virions in cowpox A-type inclusions is under the control of the 58-kDa virion protein, the major component of viral surface tubule elements (Stern and Dales, 1976). We assume that a similar process involving specific interactions between spherulin and the polypeptide(s) forming globular subunits at the surface of mature virions (Bergoin *et al.*, 1971) are responsible for the occlusion process.

The lack of homology between the *Mm*EPV spherulin and fusolin sequences (Gauthier *et al.*, submitted) clearly demonstrated that the two types of cytoplasmic inclusions observed in infected larval adipose cells of the host, the spherules and the fuseaux (Amargier *et al.*, 1964), are the products of two distinct genes. These data explain in turn the lack of antigenic community between the two polypeptides as observed either by Western blot analysis (this paper and Gauthier *et al.*, submitted) or immunoprecipitation tests (Croizier and Veyrunes, 1971).

In contrast, both sequence and antigenic comparisons between the *Mm*EPV spherulin and the 100-kDa *Cb*EPV and *Am*EPV occlusion body polypeptides proved that the three polypeptides are phylogenetically related. Thus, the gene encoding the major occlusion body polypeptide appears to be highly conserved among EPVs infecting insects phylogenetically so distant as coleoptera and lepidoptera. Considering the important biological function of this polypeptide in the packaging of mature virus particles into a paracrystalline structure so as to protect them during the extracellular phase of their life cycle, this conservation is not surprising. The high efficiency of this protection is worth mentioning since we could demonstrate that lyophilized spherules kept on the bench for more than 20 years still retained their infectivity (our unpublished results).

Although having a slightly longer sequence, the *Am*EPV spheroidin shares many properties with the *Mm*EPV spherulin including a very similar distribution of hydrophobic regions (data not shown) and cystein residues (Fig. 5) along the molecule. However, the cystein content of *Am*EPV spheroidin is significantly lower than that of *Mm*EPV spherulin (38 residues versus 46). This difference, along with their smaller size, may explain the more rapid solubilization of *Am*EPV spherules under milder conditions compared to those of *Mm*EPV (Roberts and Bergoin, 1970).

Two ORFs immediately adjacent to the *Am*EPV spheroidin ORF (G5R ORF, according to the *Am*EPV *Hin*dIII map) have been described, one upstream (G4R) of unknown function, the other downstream (G6L), corresponding very likely to NTPase I gene (Hall and Moyer, 1991, 1993). Unlike this situation, no significant ORF was detected in the 615 bp upstream or in the 548 bp downstream of *Mm*EPV spherulin ORF, except perhaps a potential ORF starting at nt 3764 of the sequence (Fig. 3B). This suggests that homologous genes are differently positioned on *Mm*EPV and *Am*EPV genomes.

The significant level of homologies of fusolin polypeptides (Gauthier *et al.*, submitted) is also a stricking feature of EPVs, although the biological function(s) of these polypeptides still need to be elucidated. The same type of conservation is observed among the polyhedrins and granulins of baculoviruses (Rohrmann, 1986; De A. Za-

notto, 1993), whereas the few polyhedrins of cypoviruses so far sequenced appear more heterogenous (Fossiez *et al.*, 1989).

The lack of homology between the 3989-bp sequence of *Mm*EPV DNA and the sequence of the VV genome (Goebel *et al.*, 1990) or other sequences of vertebrate poxviruses including the A-type inclusion protein gene sequence of cowpoxvirus (Funahashi *et al.*, 1988) clearly demonstrates the long-term divergent evolution of vertebrate and insect poxviruses.

With 88.9% of A and T, a TAAATG box including the ATG translation initiation codon, and 60% homology with VV late promoter consensus sequence (Davison and Moss, 1989), the 45 bases upstream of the spherulin coding sequence fulfil the criteria for vertebrate poxvirus late promoters. The presence at the 5′ terminus of the spherulin mRNA of a poly(A) tract approximately 20 nt in length just upstream of the AUG translation initiation codon is a characteristic feature of vertebrate poxvirus late mRNAs (Bertholet *et al.*, 1987; Patel and Pickup, 1987; Schwer *et al.*, 1987; Ahn and Moss, 1989). Being abundantly expressed (several milligrams of spherules are produced per infected larva), the spherulin gene can thus be regarded as under the control of a strong late promoter which could be a good candidate for the development of entomopox-derived expression vectors.

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