Protein requirements for assembly of virus-like particles of Junonia coenia densovirus in insect cells

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The coding sequences of four overlapping polypeptides starting at four different in-frame AUG codons and co-terminating at the stop codon of the cap gene of Junonia coenia densovirus (JcDNV) were inserted under the control of the p10 promoter of Autographa californica nucleopolyhedrovirus (AcMNPV) to generate AcMNPV-VP1 (four polypeptides), AcMNPV-VP2 (three polypeptides), AcMNPV-VP3 (two polypeptides), and AcMNPV-VP4 (one polypeptide) recombinant viruses. In all cases, infection of Spodoptera frugiperda cells (Sf9) by each of the four recombinant viruses resulted in the production of virus-like particles (VLPs) 22–25 nm in diameter. The VLPs produced by the three recombinants AcMNPV-VP2, AcMNPV-VP3 and AcMNPV-VP4 were abundant and contained three, two and one polypeptide, respectively. VP4, the shortest polypeptide, thus appears to be sufficient for assembly of VLPs morphologically similar to those formed with two to four polypeptides. The ratio of VPs did not appear to be critical for assembly of the particles. The polypeptide starting at the first AUG immediately downstream from the p10 promoter was always the most abundantly expressed in infected cells, regardless of the construct. In contrast, plaque-purified AcMNPV-VP1 recombinants were unstable and produced less than one-twentieth of the VLPs produced by the others. All VP transcripts started at the TAAG late motif of the p10 promoter and had a poly(A) tail 14 nt downstream of a poly(A) addition signal located 98 nucleotides downstream of the common stop codon. No significant transcription initiation inside the cap sequence of AcMNPV-VP2, AcMNPV-VP3 and AcMNPV-VP4 was observed.

Introduction

Parvoviruses are animal viruses with linear single-stranded DNA genomes packaged into icosahedral capsids 18–22 nm in diameter. The genomic organization, the structure of the ends of the DNA molecule and the modalities of gene expression differ among the members of the six genera of the Paroviridae family. Densovirus is one of the three genera of invertebrate paroviruses (subfamily Densovirinae). Densovirus is unusual in that structural (cap) gene and non-structural (NS) genes are segregated on different DNA strands. In this genus both strands are individually packaged in equal proportions (for a review of the Densovirinae see Bergoin & Tijssen, 1998). The number of polypeptides forming the capsids of paroviruses varies from two to four. Capsid proteins VP1, VP2, VP3 and VP4 of Junonia coenia densovirus (JcDNV) are all encoded by cap. Multiple translational initiation events occur at the in-frame AUG codons AUG1, AUG2, AUG3, and AUG4 or AUG5, the latter two being adjacent, along a unique 2·6 kb transcript driven by the p9 promoter. The mechanism of initiation at four positions on a unique mRNA remains to be established at the molecular level. All four peptides are present in JcDNV particles. Expression of the VP1, VP2, VP3 and VP4 proteins was obtained by in vitro translation of mRNAs produced from plasmids containing the cap gene driven by either the SP6 or T7 prokaryotic promoters. Using AUG1, AUG2, AUG3 and AUG4 initiation codons, proteins were simultaneously translated from constructs presenting one to four AUGs on unique unspliced transcripts (Y. Li & M. Bergoin, unpublished data). However, the level of in vitro translation products was too low to detect the assembly of virus-like particles (VLPs).

VLPs of various icosahedral viruses, belonging to different families, have been produced recently using classical baculovirus expression systems (Kinnbauer et al., 1993; Le Gall-
Reculé et al., 1996; Pawliita et al., 1996). The production of JcDNV capsid polypeptides by an Autographa californica nucleopolyhedrovirus (AcMNPV) expression vector under the control of a strong promoter may be sufficient to permit self-assembly of VLPs and to determine which of the different VP proteins are required to produce these structures. Classical baculovirus expression systems (O’Reilly et al., 1992; López-Ferber et al., 1995) require the insertion of the desired coding sequence in a transfer plasmid prior to the production of recombinants. For quicker insertion of the foreign sequences in the recombinant baculovirus genome, we took advantage of a recent technique of in vitro insertion of the foreign gene in an AcMNPV genome (Lu & Miller, 1996; Grabherr and the assembly of VPs in insect cells in the absence of non-coding fragment was substituted for the pentanucleotide sequence separating the two sites in pAcSeBsu to give plasmid pAcBS1020. The transplacement plasmid pAcBS1020 was then transfected into Sf9 cells (O’Reilly et al., 1992) together with AcMNPV-β-Gal, containing the β-gal gene in lieu of the polyhedrin gene. Recombinants presenting a white, occlusion” phenotype (occ') were purified by three rounds of plaque purification in the presence of X-Gal and then analysed for the presence of 1020 nt spacer sequence inserted at position 4000 nt relative to the AcMNPV C0 strain (Ayres et al., 1994).

Construction of the recombinant viruses. PCR products with Bsu36I and Se8387I sites near their 5’ and 3’ extremities respectively were prepared using either 5’-oligonucleotide primers VP1, VP2, VP3 or VP4 and the common 3’-primer VP1–4. Plasmid pBRJ (Jourdan et al., 1990) was used as a template for the synthesis of the sequence to be transfected (Dumas et al., 1992). Bold letters in the following oligo-nucleotide primers indicate the restriction sites. Initiation codons and stop codon complementary sequence are in italics and the annealing part of the primer is underlined.

PCR products were ligated into BsmI and SceI digested recombinant baculovirus DNA previously used for VLP assembly. In addition, the influence of the VP ratio expressed in cells on the peptide composition of VLPs was examined. Transcription analysis was also undertaken to establish whether the multiple production of VPs was associated with multiple internal starts of transcription in the heterologous baculovirus system.

Methods

Cells and virus. Spodoptera frugiperda Sf9 cells (ATCC CRL 1711), a derivative of Sf21AL, were used in all experiments. The cells were maintained at 27 °C in TC100 medium enriched with 1.0 g/l yeastolate, 3.33 g/l lactalbumin hydrolysate and supplemented with 10% heat-inactivated foetal calf serum (FCS). AcMNPV-C6.2 is a plaque-purified virus with a natural deletion of ORF6 (Durantel et al., 1998). AcMNPV-β-Gal, possessing a β-galactosidase (β-gal) gene in place of the polyhedrin gene of AcMNPV-C6.2, was generously provided by M. Cernuti (CNRS, Saint Christol-les-Alès).

Construction of the baculovirus vector. An Sce8387I site (CTCAGGAG) was inserted into the unique EcoR-I fragment of the AcMNPV EcoRI-I fragment to give pAcSe (Crozier et al., 1988). A 99 nt dsDNA containing a short p10 promoter and a Bsu36I site (CCTAGG) was obtained by annealing oligoA (5’-GTATGAATTAAGAGAGAATTATCATCTTGTATATTAAATACATATGCAGCAG-3’) and oligoB (5’-GCGGCATCTGAGGCTATGATTTAAATACATATGCAGCAG-3’). This promoter was introduced into the Sce8387I site of pAcSe to give pAcSeBsu (Fig. 1 A). Out of the two possible orientations of the insert, the positive orientation was chosen and the constructs verified by sequencing. Two cloning sites, Bsu36I (shown in bold type in oligos A and B) and Se8387I (shown in italic type in oligos A and B), followed the p10 promoter. To facilitate the subsequent double digestion of the recombinant baculovirus DNA at the two unique sites of the expression vector, a 1020 nt Sce8387I and Bsu36I non-coding fragment was substituted for the pentanucleotide sequence separating the two sites in pAcSeBsu to give plasmid pAcBS1020. The transplacement plasmid pAcBS1020 was then transfected into Sf9 cells (O’Reilly et al., 1992) together with AcMNPV-β-Gal, containing the β-gal gene in lieu of the polyhedrin gene. Recombinants presenting a white, occlusion” phenotype (occ’) were purified by three rounds of plaque purification in the presence of X-Gal and then analysed for the presence of 1020 nt spacer sequence inserted at position 4000 nt relative to the AcMNPV C0 strain (Ayres et al., 1994).

Selection of four different recombinant viruses potentially expressing between one and four of the VPs of the wild-type virus particle permitted determination of the minimum VPs required for VLP assembly. In addition, the influence of the VP expression vector containing various parts of this gene. PCR products of cap sequences beginning at each functional AUG were inserted in vitro between two unique Bsu36I and Se8387I sites in our engineered AcMNPV vector. To investigate further the translation of the unique cap gene and the assembly of VPs in insect cells in the absence of non-structural genes and genomic DNA, we constructed a baculovirus expression vector containing various parts of this gene. PCR products of cap sequences beginning at each functional AUG were inserted in vitro between two unique Bsu36I and Se8387I sites in our engineered AcMNPV vector. The selection of four different recombinant viruses potentially expressing between one and four of the VPs of the wild-type virus particle permitted determination of the minimum VPs required for VLP assembly. In addition, the influence of the VP ratio expressed in cells on the peptide composition of VLPs was examined. Transcription analysis was also undertaken to establish whether the multiple production of VPs was associated with multiple internal starts of transcription in the heterologous baculovirus system.

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Selection of AcMNPV-VP recombinants after immunofluorescence assays. To search for recombinants with a VP-sequence insertion, supernatants obtained from individual plaques were used to infect Sf9 cells in 24-well plates (30 µl per well). After 48 h incubation at 27 °C cell monolayers were prepared for immunofluorescence assay as described by Li et al. (1996). Briefly, cells were fixed in PBS (1:36 M NaCl, 0.026 M KCl, 0.03 M NaHPO₄, 0.014 M KH₂PO₄, pH 7.4) with 3% glutaraldehyde and then treated with 3% Triton X-100. Fixed cells were incubated in a mouse anti-JcDNV ascitic fluid (diluted 1/1000). Primary antigen–antibody complex was stained with a rabbit anti-mouse β-globulin coupled to fluorescein (diluted 1/200) (Diagnóstico Pasteur) in the presence of Evans’ blue (1/100000 final concentration). Cells were examined under an epifluorescence microscope. Recombinant baculoviruses from two plaques each of AcMNPV-VP1, -VP2, -VP3 or -VP4 were selected to ensure the occurrence of the sequence to be transfected (Dumas et al., 1992). Bold letters in the following oligo-nucleotide primers indicate the restriction sites. Initiation codons and stop codon complementary sequence are in italics and the annealing part of the primer is underlined.
were purified by three additional rounds of plaquing and selected for further analyses.

■ Genomic analysis of the recombinant virus. Viral DNA was purified from occ' viruses purified from Sf9 cells by established techniques (O’Reilly et al., 1992) and then subjected to RFLP and Southern blotting probed with a digoxigenin (DIG)-labelled dsVP4 probe and revealed by chemiluminescent detection with an anti-digoxigenin–AP Fab fragment and CSPD (DIG-High prime random priming and detection kit, Boehringer).

■ VLP purification and electron microscopy (EM). Sf9 cells were infected with each of the four recombinant viruses AcMNPV-VP1 to -VP4 with 4 day post-infection (p.i.) medium from Sf9 cells infected with the corresponding virus. Four days p.i., 1.25 × 10^8 cells infected respectively with AcMNPV-VP2, AcMNPV-VP3 and AcMNPV-VP4 and 2.5 × 10^8 cells infected with AcMNPV-VP1 were harvested. Each batch of cells was centrifuged at 1600 g for 10 min. The pellets were dispersed in 2 ml PBS and disrupted with a Dounce homogenizer. The resulting suspension was clarified at 10000 g for 10 min. The supernatants
were layered onto a 20–76 % Radioselectan (Schering Laboratories) density gradient. After 15 h centrifugation at 115,000 g, visible virus bands were individually recovered and then dialysed against PBS for 48 h. Dialysed virus particles layered on carbon-coated grids were negatively stained with 2 % phosphotungstic acid, pH 7.0 (Brenner & Horne, 1959), and examined using a Zeiss EM 10C/CR transmission electron microscope at 80 kV.

**Western blotting.** AcMNPV-VP1, -VP2, -VP3, VP4 recombinant baculoviruses were propagated on Sf9 cells in 6-well plates. Three days p.i., cells were harvested, centrifuged at 3000 × g for 5 min, and suspended in 40 μl of PBS buffer. One-fifth of the cell suspension (8 μl) was placed in SDS–PAGE buffer (125 mM Tris pH 6.8, 2 % SDS, 5 % β-mercaptoethanol, 50 % glycerol, 0.01 % bromophenol blue), denatured at 100 °C for 5 min, and proteins were separated on a 10 % SDS–polyacrylamide gel (Laemmli, 1970). Proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane (Schleicher and Schuell) for 5 min, and proteins were separated on a 10 % SDS–polyacrylamide gel (Laemmli, 1970). Proteins were either stained with Coomassie brilliant blue or transferred to a nitrocellulose membrane (Schleicher and Schuell) by semi-dry blotting. VPs were detected with a rabbit primary anti-VP4 antibody (received as a gift from Franc L. Croizier and others) conjugated with peroxidase as second antibody (Diagnostics Pasteur). The VP antibody (prepared by injecting a rabbit with crushed polyacrylamide band containing VP4 isolated from an SDS–PAGE gel of purified JcDNV particles) was stained by the 3-amino-9-ethylcarbazol chromogenic reaction to peroxidase (Sigma).

**Transcription analysis.** RNAs were extracted from the fat body of fifth instar *Galleria mellonella* larvae infected with AcMNPV-VP1, -VP2, -VP3 and VP4 by using a Promega SV Total RNA Isolation System kit. Analysis of the 3′ ends of the four RNAs via RT–PCR was performed using the Promega Access RT–PCR System kit. The same primers were used for all constructs, corresponding to the sequence nt 2523–2547 of the JcDNV DNA sequence (Dumas et al., 1992) and oligo(dT). The sequence of each product was determined by automatic fluorescent sequencing, using an internal primer corresponding to nt 2839–2858 of the JcDNV DNA sequence.

Primer extension was performed on different RNA samples using a combination of primers that allowed the scanning of sequences between the p10 promoter and the region downstream of ATG4 (Fig. 1B). Oligonucleotides PE-VP1 (592–568), PE-VP2 (755–735) and PE-VP4 (1717–1686) were labelled with [γ-32P]ATP and primer extension was done with the Promega Primer Extension System kit. The products were resolved on a 5 % polyacrylamide sequencing gel. HindIII-restricted φX174 was labelled with [γ-32P]ATP. M13mp18 single-stranded DNA was sequenced using a Pharmacia T7 Sequencing kit, labelled with [α-32P]dATP. Both φX174 HindIII restriction fragments and M13mp18 sequence ladder were used as molecular mass markers.

**Results**

**Construction of a baculovirus vector with two unique restriction sites for direct in vitro insertion of the JcDNV cap gene**

The construction of the baculovirus vector is described in Methods. This vector, AcMNPV-BS1020, possesses a p10 promoter followed by a *Bsu36I* cloning site. The unique *Sse8387I* site located 1020 nt downstream on the positive strand allowed the directed cloning of a foreign gene with 5′- terminal *Bsu36I* and 3′-terminal *Sse8387I* sites (Fig. 1A). The promoter is composed of the 81 nt preceding the initiation codon of the wild-type p10 gene (Fig. 1C). Transcripts of the expressed foreign genes and the polyhedrin transcripts are synthesized on the positive strand (Fig. 1A).

**Construction and isolation of AcMNPV-VP1, AcMNPV-VP2, AcMNPV-VP3 and AcMNPV-VP4 recombinants expressing JcDNV cap gene products**

VP1, VP2, VP3 and VP4 cap gene sequences were PCR-amplified, ligated into the AcMNPV-BS1020 vector and transfected into Sf9 cells. Immunofluorescence assays using an anti-VP4 antibody showed that 25, 62.4, 85.7 and 70.8 % of the plaques isolated from the supernatants of Sf9 transfected cells were infected by AcMNPV-VP1, AcMNPV-VP2, AcMNPV-VP3 and AcMNPV-VP4 respectively. Interestingly, the plaque phenotype for the recombinants AcMNPV-VP2, -VP3, and -VP4 was typical of the wild-type baculovirus, with numerous polyhedra per cell, whereas plaques generated by AcMNPV-VP1 recombinants showed substantially fewer polyhedra. Immunofluorescence assays with plaque-purified AcMNPV-VP2, -VP3 and -VP4 showed that almost all Sf9 cells were positive with bright nuclear fluorescent spots whereas less than half of the cells infected with the AcMNPV-VP1 recombinant were positive, showing diffuse immunofluorescence reactions (data not shown).

**Genomic analysis of the AcMNPV-VP recombinants**

To demonstrate the fidelity of the AcMNPV-VP constructs, two AcMNPV-VP1 plaques (VP1-13 and VP1-18) and a representative plaque of each of the AcMNPV-VP2, -VP3, and -VP4 recombinants were cloned by three rounds of plaque purification and then amplified in 105 Sf9 cells. The viral DNAs were purified and digested with *PstI*. As expected, the *PstI*/*Sse8387I* profiles of AcMNPV-VP2, -VP3, and -VP4 were identical to the vector AcMNPV-BS1020 *PstI*/Sse8387I profile except for the possession of the different version of the *cap* sequences or the 1020 nt fragment identified by the *PstI* site upstream of the p10 promoter and by the *Sse8387I* cloning site (Fig. 2A). In two independent assays, extraction of AcMNPV-VP1-13 and VP1-18 genomes yielded less DNA than the other recombinants (data not shown). AcMNPV-VP1-13 DNA showed the expected *PstI* VP1 fragment as revealed by Southern blot using a VP DNA probe (Fig. 2B). *PstI* digestion of AcMNPV-VP1-18 DNA failed to reveal the expected *PstI*/Sse8387I VP1 fragment (data not shown). Further analysis of AcMNPV-VP1-13 and -18 DNA revealed genomic rearrangements in the *Bsu36I*/Sse8387I region (data not shown) but no attempt was made to study further the instability of the AcMNPV-VP1 recombinants during replication in Sf9 cells.

**VLP formation by the four AcMNPV recombinant viruses**

EM of negatively stained Sf9 cell homogenates infected with AcMNPV-VP2, -VP3 or -VP4 repeatedly revealed abundant production of VLPs. In contrast, few VLPs could be
observed in homogenates of cells that had been infected with AcMNPV-VP1 (data not shown). The cell homogenates from cultures infected with each of the four recombinants were subjected to density-gradient centrifugation. Two discrete bands were observed in the lower part of the gradient at an identical position regardless of the virus inoculated. The upper band contained densovirus VLPs (Fig. 3). The lower band comprised empty baculovirus capsids (data not shown). VLPs produced by the four AcMNPV recombinant viruses were of similar size (22–25 nm in diameter). The appearance of VLPs varied according to the intensity of the negative staining in the EM field observed. In high-contrast fields (Fig. 3A, B) phosphotungstic acid penetration was partial or total and the majority of VLPs appeared as distinct circles, whereas in low-contrast fields most of the VLPs were not penetrated (Fig. 3C, D). No significant qualitative differences were observed between the parvovirus pseudoparticles from any of the four recombinant viruses. Nevertheless, as previously mentioned, the level of VLP production in Sf9 cells infected by AcMNPV-VP1 was very low.

JcDNV cap gene products generated by AcMNPV-VP recombinants in Sf9 cells and VLP composition

In each construct the first initiation codon of the VP1, VP2, VP3 and VP4 overlapping sequences was separated from the p10 promoter by 7 nt. The 5′ C of the Bsu36I site at position –8 overlapped the 3′ C of the p10 promoter (Fig. 1C). The Bsu36I site was followed by a T. For each of the four inserted ORFs, this resulted in a translation context close to that of the initiation codon AUG2 of the JcDNV cap gene.

PAGE analysis of protein extracts from Sf9 cells infected with AcMNPV-VP2, -VP3 and -VP4 recombinant viruses revealed the presence of VP2, VP3 and VP4 peptides,
respectively (Fig. 4A). Western blot analysis using anti-VP4 antibodies confirmed the presence of one, two and three specific VP peptides in Sf9 cells infected with AcMNPV-VP4, -VP3 and -VP2 respectively. In contrast, the same antiserum failed to reveal any viral polypeptide in cell extracts from Sf9 cells infected with the AcMNPV-VP1 recombinant (Fig. 4A'). The relative amount of each VP differed from the proportion observed in the wild-type virion (Fig. 4). The peptide translated from the first AUG codon, i.e. that immediately downstream of the p10 promoter, was always highly expressed whereas the peptides resulting from the postulated reinitiation sites of translation were consistently less abundant.

The polypeptides of purified VLPs were analysed by Western blotting and then compared to the homologous VP polypeptides present in infected Sf9 cells (Fig. 4A', B'). The ratios of the different polypeptides in the particles were

Fig. 4. Western blots of capsid proteins expressed by AcMNPV-VP1 to -VP4 recombinant baculoviruses in Sf9 cells. (A) Cell extracts subjected to 10% SDS–PAGE and stained with Coomassie brilliant blue. (A') Protein blotting to nitrocellulose membrane from gel in (A). (B) VLPs purified from Sf9 cells infected with AcMNPV-VP2 to -VP4 subjected to 10% SDS–PAGE and stained with Coomassie brilliant blue. (B') Protein blotting to nitrocellulose membrane from gel in (B).
Fig. 5. Primer extension of the cap gene transcripts in different constructs. A single extension is formed with the PEVP4 primer on AcMNPV-VP4 (lane 8), while no similar extension is observed with the same primer on viruses AcMNPV-VP2 and -VP3. The 270 extension (lane 7) and the 410 extension (lane 6) correspond to the genuine starts of VP3 and VP2 respectively. This VP2 start-point is detected at position 130 by primer PEVP2 in lane 5. An accidental stop of extension (lanes 6, 7 co-ordinate 148) attributed to a hairpin is illustrated on the right-hand side of the figure.

Mapping the 5’ and 3’ ends of the cap gene transcripts for AcMNPV-VP2, -VP3 and -VP4

Sequencing of the RT–PCR products of the 3’ untranslated moieties of the cap messenger RNA indicated the existence of a poly(A) tail starting 14 residues downstream from the third AATAAA motif after the cap stop codon, regardless of the construct (Fig. 1D). Primer extension experiments with RNAs extracted from Galleria mellonella fat body tissue infected with each of the three AcMNPV-VP recombinants indicated a unique transcription start at the ATAAG motif of the p10 late promoter (Fig. 5). No other significant transcription starts were observed between the primer and the ATAAG motif. An extension of 148 nt was observed in AcMNPV-VP3 and AcMNPV-VP2 with the PEVP4 oligonucleotide. This corresponded to a stop of the reverse transcriptase, most likely due to a hairpin structure in the RNA (Fig. 5).

Discussion

Recombinant AcMNPVs expressing different JcDNV structural polypeptides were obtained by direct insertion of a series of PCR-amplified cap gene DNA sequences. About 25–85% of the budded virus present in the supernatant of SF9 cells 48 h p.i. was recombinant. Among randomly picked plaques, the percentage of those positive by immunofluorescence assays was sufficient to select plaques with recombinant viruses. Thus, in the absence of negative selection due to the gene expressed, no more than ten plaques picked at random were sufficient to isolate at least one or several recombinants. Furthermore, titres of budded virus in the supernatant of transfected SF9 cells usually exceed 10^9 p.f.u./ml. This type of vector appears particularly appropriate for the production of JcDNV capsid proteins with deletions or extensions either at their N- or C-terminal portions to check the translation efficiency of the different VPs or to determine the amino acid sequences involved in self-assembly.

The expression of the structural cap gene of JcDNV in AcMNPV recombinants did not abolish the translational reinitiation of their mRNA nor the self assembly of VPs into capsid-like structures. The nonstructural gene products NS1, NS2 and NS3 are not required for either process to occur. The reinitiation of translation, regardless of the mechanisms involved, was demonstrated with the AcMNPV-VP2 and AcMNPV-VP3 recombinants. Polycistronic transcripts are common features in the transcription of wild-type AcMNPV (Brown et al., 1996). However, no experiment has been estimated by densitometric analysis. These ratios are not similar for each VLP, but roughly reflect the ratio in the cells they were obtained from (data not shown). Owing to the very low level of production, the peptide composition of VLPs extracted from AcMNPV-VP1-infected cells could not be determined.
undertaken to establish whether or not the second AUG is used for initiation of translation. For the first published baculovirus expression vector, in which the human interferon-β gene was inserted out of frame at the N-terminal moiety of the polyhedrin gene, translation of the interferon coding sequence always started at the interferon initiation codon (Smith et al., 1983). More recently, translation from different AUGs present in or out of frame in baculovirus vectors loaded with DNA sequences presenting overlapping ORFs has been described (Lamb et al., 1996; Suzuki et al., 1996). Wild-type JcDNV multiple products of the cap gene are hypothesized to result from a leaky scanning process (Bergoin & Tijssen, 1998). In the present work, a VP2-like context of translation initiation, corresponding to nucleotides from position −4 to +5 of the AUG codon, was chosen. Production of VP3 and VP4 with AcMNPV-VP2 and of VP4 with AcMNPV-VP3 was demonstrated.

A very high level of expression of cap gene products was obtained with the AcMNPV recombinant under control of the AcMNPV p10 late promoter. VLPs were produced in Sf9 cells infected with each of the four (AcMNPV-VP1–4) recombinants in the absence of JcDNV nonstructural genes or the complete parovirus genome. Remarkably, the protein composition of purified VLPs mirrored the protein composition observed in the cellular extract. VP4 alone was sufficient to form pseudocapsids and mixtures of VP2, VP3 and VP4 in ratios different from that present in wild-type nucleocapsids could aggregate to form pseudocapsids indistinguishable by microscopy from VP4-built pseudocapsids. These observations indicate a central role for the JcDNV VP4 peptide in assembly of the VLPs. In the particle of Galleria mellonella (GmDNV), a densovirus closely related to JcDNV, 60 copies of the common C-terminal domain of VP1, VP2, VP3 and VP4 constitute the ordered part of the icosahedral capsid, indicating the importance of the C-terminal moiety of the densovirus VPs for particle assembly (Simpson et al., 1998). In fact, JcDNV and GmDNV VP4 correspond to co-migrating VPs initiating at a MAM sequence (Bergoin & Tijssen, 1998). AcMNPV-VP4 produces the shortest VP4 version starting at the second methionine of the MAM sequence. In the present work, the ability of peptides with a common C-terminal moiety and N-moieties of different sizes to assemble into capsids was observed. The capacity to form stable VLPs from mixtures of VPs in different ratios is consistent with the model proposed for the GmDNV particle, since 60 copies of the VP4 domain are sufficient to constitute a capsid, regardless of the N-terminal domains of VP1, VP2 or VP3. The mode of assembly of VLPs of JcDNV in connection with the use of our baculovirus vector system opens various technical means to add sequences encoding foreign epitopes into the PCR products to be inserted into the baculovirus vector, leading to an epitope–VLP–carrier immunization system. Whether the stoichiometry of JcDNV VLPs is crucial for packaging of the genome, as reported for adeno-associated virus, is unknown (Steinbach et al., 1997).

The correct insertion of VP1 sequence in the AcMNPV vector and its expression were ascertained by the observation of a few VLPs in the early steps of plaque-purification of the AcMNPV-VP1 recombinant. However, these constructs proved to be unstable since genomic rearrangements occurred in the insert region during the very early passages. As a consequence, AcMNPV-VP1-infected Sf9 cells did not yield enough transcripts to be analysed. A phospholipase A2 (PLA2) activity encoded by the N-terminal moiety of VP1 sequence was recently observed in GmDNV (Zadori et al., 1999). It is tempting to speculate a possible negative selection for AcMNPV-VP1 possessing the PLA2 sequence.

Numerous nested transcripts are common for different regions of baculovirus genomes (O’Reilly et al., 1992). Different transcripts run through the polyhedrin gene area of rec-AcMNPV (Gonzalez et al., 1989). In the present study, the analysis of transcription was purposely focused within the area of the cap gene insertion in order to screen for any internal transcription start sequences. In this area, no transcript other than the expected transcript starting at the ATAGA late promoter motif and finishing about 100 nt after the cap stop codon was detected, although three proteins are produced. This transcription analysis clearly indicates that no cryptic promoter is present in the cap sequences and the proposed leaky scanning process is not dependant on nonstructural densovirus proteins.

References


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**JcDNV virus-like particle assembly**