Molecular cloning and characterization of Antheraea mylitta cytoplasmic polyhedrosis virus genome segment 9

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Genome segment 9 of the 11-segment RNA genomes of three cytoplasmic polyhedrosis virus (CPV) isolates from Antheraea mylitta (AmCPV), Antheraea assamensis (AaCPV) and Antheraea proylei (ApCPV) were converted to cDNA, cloned and sequenced. In each case, this genome segment consists of 1473 nucleotides with one long ORF of 1035 bp and encodes a protein of 345 amino acids, termed NSP38, with a molecular mass of 38 kDa. Secondary structure prediction showed the presence of nine α-helices in the central and terminal domains with localized similarity to RNA-binding motifs of bluetongue virus and infectious bursal disease virus RNA polymerases. Nucleotide sequences were 99.6% identical between these three strains of CPVs, but no similarity was found to any other nucleotide or protein sequence in public databases. The ORF from AmCPV cDNA was expressed as a His-tagged fusion protein in E. coli and polyclonal antibody was raised against the purified protein. Immunoblot as well as immunofluorescence analysis with anti-NSP38 antibody showed that the protein was not present in polyhedra or uninfected cells but was present in AmCPV-infected host midgut cells. NSP38 was expressed in insect cells as soluble protein via a baculovirus expression vector and shown to possess the ability to bind poly(rI)–(rC) agarose, which was competitively removed by AmCPV viral RNA. These results indicate that NSP38 is expressed in virus-infected cells as a non-structural protein. By binding to viral RNA, it may play a role in the regulation of genomic RNA function and packaging.

Introduction

Cytoplasmic polyhedrosis viruses (CPVs) belong to the genus Cypovirus in the family Reoviridae (Payne & Mertens, 1983; van Regenmortel et al., 2000). These viruses infect midgut cells of a wide range of insects belonging to the orders Diptera, Hymenoptera and Lepidoptera (Belloncik, 1989; Belloncik & Mori, 1998; Fouillaud & Morel, 1994; Hukuhara & Bonami, 1991; Payne, 1981). Virus infection is characterized by the production of large numbers of occlusion bodies called polyhedra in the cytoplasm of infected cells. These polyhedra are formed by the crystallization of a virus-encoded protein, polyhedrin, synthesized late during infection. Many virus particles are embedded within each polyhedron.

Polyhedra function to protect virions from physico-chemical inactivation in the environment and to ensure the accurate delivery of virus particles to midgut cells, where virions are released from these protective occlusion bodies in the alkaline pH of the insect gut (Belloncik, 1989; Belloncik & Mori, 1998).

The genomes of CPVs, like those of other members of the Reoviridae, are usually composed of 10 double-stranded (ds) RNA segments (S1–S10) (Payne & Mertens, 1983) although, in some cases, a small, eleventh segment (S11) has been reported (Arella et al., 1988). Each dsRNA segment is composed of a plus-strand mRNA and its complementary minus strand in an end-to-end base-paired configuration except for a protruding 5' cap on the plus strand. On the basis of electrophoretic migration patterns of the dsRNA segments in agarose or acrylamide gels, they have been classified into 14 different types (Belloncik et al., 1996; Fouillaud & Morel, 1994; Payne & Rivers, 1976; Payne & Mertens, 1983).

Among the family Reoviridae, complete nucleotide sequences of dsRNA genomes have been reported for members of the genera Orthoreovirus, Rotavirus, Orbivirus and Phytoreo-
virus and putative members of Fijivirus and Cypovirus (Duncan, 1999; Estes & Cohen, 1989; Roy et al., 1990; Suzuki, 1995; Nakashima et al., 1996). From the cypoviruses, segment 10, encoding the polyhedrin gene, has been cloned and sequenced from Bombyx mori CPV (BmCPV), Euxoa scandens CPV, Orgyia pseudotsugata CPV, Heliothis armigera CPV and Choristoneura fumiferana CPV (Arella et al., 1988; Echeverry et al., 1997; Fossiez et al., 1989; Galinski et al., 1994; Mori et al., 1989). However, no sequence similarities were found. In the case of BmCPV, segments 9, 8 and 5, encoding non-structural proteins NS5, p44 and p101 (Hagiwara et al., 1998a, b, 2001), and segments 4, 6 and 7, encoding structural proteins VP3, VP4 and VP5 (Hagiwara & Matsumoto, 2000; Ikeda et al., 2001), have also been cloned and sequenced. Recently, nucleotide sequences of segments 1, 2 and 3 of BmCPV and complete sequences of Lymantria dispar CPV, Trichoplusia ni CPV and Choristoneura fumiferana CPV have also been deposited in GenBank.

The Indian non-mulberry Saturniidae silkworms Antheraea mylitta, Antheraea assamensis and Antheraea proylei are wild in nature and produce exotic varieties of silk called Tasar and Muga silk (Jolly et al., 1974). A. mylitta is a tropical variety whereas A. assamensis and A. proylei are semi-temperate and temperate varieties. Each year, CPV infection destroys a major crop of tasar silk (Jolly et al., 1974).

We have previously characterized the structure of CPVs from these three silkworm species by electron microscopy and their genomes by electrophoresis and found that the genomes are similar to that of a type IV CPV that infects Actias selene and consist of 11 dsRNA molecules. The molecular sizes of the different RNA segments of Antheraea mylitta CPV (AmCPV), Antheraea assamensis CPV (AaCPV) and Antheraea proylei CPV (ApCPV) isolates are similar (3–9, 3–8, 3–6, 3–3, 2–15, 1–9, 1–8, 1–7, 1–45, 1–4 and 0–35 kb) but are quite different from those of BmCPV, which is a type 1 CPV (Qanungo et al., 2000).

In order to compare the AmCPV, AaCPV and ApCPV isolates further with each other and with other known members of the genus Cypovirus such as BmCPV, we describe the molecular cloning and sequencing of RNA segment 9 from these three isolates and the characterization of their encoded proteins by expression in E. coli and insect cells. We show that a new CPV infects all three Indian Saturniidae silkworms and its segment 9 encodes a novel protein. By immunoblot and immunofluorescence analysis, we also show that the product of segment 9 is expressed in infected cells as a non-structural protein and binds viral RNA. Thus, it appears that it may play a role in the regulation of genomic RNA function and packaging.

Methods

Silkworms, virus and cell line. CPV-infected Indian non-mulberry silkworms A. mylitta, A. assamensis and A. proylei were respectively collected from West Bengal, Assam and Manipur states of India (Qanungo et al., 2000). The Spodoptera frugiperda cell line S69 was obtained from Invitrogen and maintained using TC-100 (Sigma) or TNM-FH (Grace’s basic media, supplemented with yeast and lactalbumin hydrolysate (Life Technologies) media with 10% foetal bovine serum (Hyclone).

Purification of polyhedral bodies, isolation of total genomic RNA and extraction of segment 9 RNA. Polyhedra from infected larvae were purified by sucrose-density-gradient centrifugation according to a modification (Qanungo et al., 2000) of the method of Hayashi & Bird (1970). The purity of the polyhedral preparations as checked by light and scanning electron microscopy was more than 95%. Genomic RNA was extracted from purified polyhedra by a standard guanidium isothiocyanate method (Ausubel et al., 1995). RNA was then fractionated in 10% polyacrylamide gels and the segment 9 RNA was excised from ethidium bromide-stained gels and eluted by the crush and soak method (Sambrook et al., 1989).

Molecular cloning and sequencing of segments 9 of the AmCPV, AaCPV and ApCPV genomes. cDNA of the segment 9 genomic RNA of AmCPV was prepared as described previously for the cloning of the polyhedrin gene of BmCPV (Arella et al., 1988). In brief, approximately 200 ng segment 9 RNA (1–5 kb) was denatured by heating at 95 °C in the presence of 95% DMSO and then polyadenylated with ATP and poly(A) polymerase (Life Technologies).

First-strand cDNAs of both RNA strands were synthesized using the primer 5′-AACGAGTGGTAACACAGGCTACTGYN3′ (N=A, C, G or T; V=A, G or C) (Clontech) and Superscript II AMV reverse transcriptase (Gibco) according to the manufacturer’s protocol. RNA was then removed with RNase H (Roche) at 37 °C for 20 min. The resulting cDNAs of the two strands were annealed at 65 °C for 60 min, ends were repaired by Taq DNA polymerase (Bioline) and the cDNAs were cloned into pCR2.1 TOPO (Invitrogen) to create recombinant plasmid pCR2.1 TOPO/AmCPV-9. After transforming E. coli TOP10 cells (Invitrogen), plasmids were isolated and characterized by EcoRI digestion. A recombinant plasmid containing the full-length insert (~1·5 kb) was then sequenced by using an ABI 377 automated DNA sequencer with M13 forward and reverse primers as well as internal primers designed from deduced sequences. Homology searches were done using BLAST (Altschul et al., 1997) and the secondary structures of segment 9-encoded proteins were predicted using Swissprot (Rost & Sande, 1994).

For cloning segment 9 RNA from ApCPV and AaCPV, primers AG8 (5′-GAATCGAGTGCTGCTTTGGAC-3′; forward primer) and AG9 (5′-TCATACGACGCAAGTCTCTCAT-3′; reverse primer) were synthesized on the basis of terminal RNA sequences from AmCPV segment 9. Purified segment 9 dsRNA (200 ng) from AaCPV and ApCPV was denatured at 100 °C for 5 min, chilled rapidly on ice and then converted to cDNA by incubating at 50 °C for 60 min in a reaction mixture containing 20 pmol forward and reverse primers, 20 U Rnase inhibitor, 40 U Thermoscript reverse transcriptase (Gibco), 10 mM DTT and 2 mM of each dNTP. The reaction was stopped by heating at 95 °C for 10 min and, after cooling, RNA was removed with RNase H at 37 °C for 20 min. The resulting cDNAs of the two strands were then annealed, ends were repaired with Taq DNA polymerase and cDNAs were cloned into pCR2.1 TOPO as described above.

Northern hybridization. In order to verify cloning of the segment 9 cDNA from the corresponding RNA of AmCPV, all of the genomic dsRNA segments were separated in a 10% polyacrylamide gel and observed by staining with ethidium bromide. The RNA segments in the gel were then denatured by brief treatment with 0·1 M NaOH, neutralized in TAE buffer and electroblotted onto nitrocellulose membrane. The membrane was then hybridized with 32P-labelled cloned...
segment 9 cDNA from AmCPV, washed and autoradiographed (Bittner et al., 1980; Feinberg & Vogelstein, 1983; Qanungo et al., 2000).

**Expression of AmCPV segment 9 in E. coli.** The entire 345 amino acid protein-coding region of AmCPV segment 9 cDNA, from nucleotide 31 to 1068, was amplified by PCR from plasmid pCR2.1 TOPO/AmCPV-9 by using Advantage Taq DNA polymerase (Clontech) and two synthetic primers, AGQ1 (5’ GTAGGTTTGTAGGGAGCTA-GATGTC 3’; forward primer) and AGQ2 (5’ CGCGCATTGTAACCTGTT-GAAATTACTTTG 3’; reverse primer), complementary to bases 8–33 and bases 1001–1036, respectively, and containing BamHI (in the forward primer) and HindIII (in the reverse primer) restriction enzyme sites (underlined). The amplified PCR product (1–kb) was digested with BamHI and HindIII, separated on a 1% agarose gel and purified from the gel by using a Qiagen gel extraction kit (Qiagen). The purified DNA was ligated to BamHI/HindIII-digested pQE-30 vector (Qiagen) in-frame with a sequence encoding six histidine residues at the N terminus. The resulting recombinant plasmid, pQE-30/NSP38, was then transformed into E. coli M15 and colonies were screened following BamHI and HindIII digestion.

For protein expression, recombinant bacteria were grown in 5 ml LB medium containing ampicillin (60 µg/ml) for 4 h at 37 °C and then induced with 1·5 mM IPTG for an additional 5 h at the same temperature. Bacteria was harvested by centrifugation, lysed by boiling with sample loading buffer (60 mM Tris–HCL, pH 6·8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol and 1 µg/ml bromophenol blue) for 3 min and then loaded onto a 3·5% stacking gel cast above a 10% resolving SDS–polyacrylamide gel (Laemmli, 1970). After electrophoresis, the protein bands in the gel were stained with Coomassie brilliant blue (Ausubel et al., 1995). The molecular mass of the recombinant NSP38 was determined by comparison to standard protein molecular mass markers and by using Quantity One software in Gel-Doc 2000 (Bio-Rad).

**Purification of His-tagged protein.** Recombinant bacteria containing pQE-30/NSP38 were grown in 1·1 LB medium and induced with IPTG as described above. The insoluble 6 × His-tagged NSP38 fusion protein was first prepared to 60% purity from inclusion bodies (Caligan et al., 1995). After solubilizing the inclusion bodies in 8 M guanidine hydrochloride, further purification of protein was carried out by using a Ni–NTA agarose kit (Qiagen) according to the manufacturer’s protocol and then by PPLC using a Superdex 75 column (Pharmacia) in the presence of 8 M urea. The total amount of purified NSP38 was determined by the method of Bradford (1976) using BSA as the standard and purity was checked by SDS–10% PAGE (Laemmli, 1970).

**Rabbit immunization and production of polyclonal antibodies.** One rabbit was immunized with bacterially expressed, purified, recombinant His-tagged NSP38 protein by standard methods (Harlow & Lane, 1988). In brief, purified protein (625 µg) was mixed with Freund’s complete adjuvant and injected intramuscularly at multiple sites. Three booster doses with Freund’s incomplete adjuvant and the same amount of protein were administered via the same route at 4-week intervals. Twelve days after the final booster, blood was collected, serum was prepared and the antibody titre was determined by ELISA (Harlow & Lane, 1988). Specific antibody was purified by antigen (NSP38–SEPHAROSE) affinity chromatography (Harlow & Lane, 1988; Sambrook et al., 1989).

**Transient expression of NSP38 in insect cells.** The NSP38 ORF was amplified from pCR2.1 TOPO/AmCPV-9 by PCR by using the primers AG10S (5’ GGATCCCTAGGATCCCTTATGCAAAGC 3’; forward primer) and AG12S (5’ TGGTATTACCTCGGGATGGATG 3’; reverse primer) and PWO high-fidelity Taq polymerase (Roche). KpnI and SacII sites were introduced in the forward and reverse primers, respectively (underlined). The PCR-amplified product (~ 1·1 kb) was digested with KpnI and SacII, gel-purified (Qiagen) and cloned into pITZ/V-5His vector (Invitrogen) to make the recombinant plasmid pITZ/V-5His-NSP38.

Sf9 cells (2 × 10⁶) were transfected with purified pITZ/V-5His-NSP38 plasmid (1 µg) by Bacfectin (Clontech) and cells were checked 48 h after transfection for the expression of green fluorescent protein (GFP) in the transfected cells by fluorescence microscopy. Three days post-transfection, the cells were harvested and a cytoplasmic extract was prepared by the method of Behrens et al. (1996). In brief, cells were resuspended (7·5 × 10⁶ cells/ml) in buffer A (10 mM Tris–HCl, pH 8·0, 1·5 mM MgCl₂, 10 mM NaCl, 1 mM DTT, 1 mM PMSF) and allowed to swell on ice for 30 min. After vortexing vigorously, glycerol (10%, v/v), NP-40 (1%, v/v) and CHAPS (0·5%, v/v) were added and the mixture was incubated on ice for an additional 1 h with occasional shaking. Cell debris was removed by centrifugation at 8000 g for 10 min and the supernatant was assayed for NSP38 expression by immunoblotting and polyclonal (rC)–agarose binding (Hagiyama et al., 1998a).

**Construction of recombinant baculovirus and expression of NSP38 in Sf9 cells.** The protein-encoding region of NSP38 was excised from pQE-30/NSP38 by BamHI and HindIII digestion and cloned into the baculovirus transfer vector pBlueAc-His2A (Invitrogen) at the 3’ end of baculovirus polyhedrin promoter. The resulting recombinant baculovirus transfer vector and Boul-digested, linearized Autographa californica nuclear polyhedrosis virus DNA (Invitrogen) were co-transfected into Sf9 cells by using Insectin Plus according to the manufacturer’s protocol (Invitrogen). Four days post-transfection, the culture medium was collected and recombinant baculovirus showing cytopathic effects but not the production of polyhedral occlusion bodies was isolated by plaque purification (O’Reilly et al., 1992). For the expression of NSP38, Sf9 cells (2 × 10⁶ cells in a 1 l spinner flask) were inoculated with the recombinant baculovirus at an m.o.i. of 5. The cells were harvested 6 days post-infection by centrifugation and a cytosolic extract was prepared by the method of Behrens et al. (1996). Baculovirus-expressed, His-tagged NSP38 was then purified by Ni–NTA affinity chromatography (Invitrogen).

**SDS-PAGE and immunoblotting.** In order to localize NSP38 expression, guts were dissected from virus (AmCPV)-infected and uninfected fifth instar larvae and homogenized with PBS and supernatants were collected after centrifugation at 10 000 g for 10 min. Recombinant baculovirus-infected Sf9 cells, Sf9 cells transiently transfected with pITZ/V-5His-NSP38 plasmid and uninfected Sf9 cells (2 × 10⁶) were homogenized and lysates prepared in the same way. Each protein sample was boiled in SDS–PAGE sample buffer and separated by SDS–10% PAGE under reducing conditions. After electrophoresis, proteins were transferred to Duralose membrane (Strategene) using a transblot cell (Pharmacia) according to Towbin et al. (1979). The membrane was blocked for 1 h at room temperature with 0·02% casein in TTBS (100 mM Tris–HCl, pH 7·5, 0·9% NaCl, 0·1% Tween 20), washed with TTBS and then incubated with 10 000-fold diluted, affinity-purified anti-NSP38 polyclonal antibody for 1 h at room temperature. After washing with TTBS as above, the membrane was incubated with Protein A-conjugated horseradish peroxidase at a dilution of 1:200 for 1 h and then washed and colour development was done by using the HPO colour development kit (Bio-Rad).

**Binding assay of NSP38 using poly(rI),poly(rC)–agarose.** Poly(rI),poly(rC)–agarose (Pharmacia) (100 µl) was washed three times with wash buffer (20 mM HEPES–NaOH, pH 7·5, 150 mM KCl, 10%
glycerol, 5 mM magnesium acetate, 1 mM DTT, 1 mM benzamidine hydrochloride and 0%–5% NP-40) and then incubated with crude cytosolic extracts of Sf9 cells or baculovirus-expressed, purified, soluble, Histagged NSP38 for 60 min at 4°C with occasional gentle mixing. Poly(rI):poly(rC)–agarose resin was then pelleted by centrifugation at 1000 g for 30 s and then incubated three times with wash buffer. Bound protein was recovered from the resin by boiling in sample loading buffer and then analysed on SDS–10% PAGE. For competition assays, purified NSP38 was preincubated with 5 or 50 µg total AmCPV dsRNA as well as heat-denatured AmCPV ssRNA for 60 min before being mixed with poly(rI):poly(rC)–agarose (Hagiwara et al., 1998a).

Immunofluorescence assay. Cells were either grown on a slide or a tissue smear was prepared, washed twice with PBS, fixed and permeabilized with ice-cold acetone for 2 min at −20°C. Slides were dried in air, rehydrated in PBS and blocked with 0.2% casein in PBS for 60 min at room temperature. Slides were then incubated with affinity-purified anti-NSP38 polyclonal antibody (1:100) (Sigma), washed, mounted with glycerol and observed by fluorescence microscopy (Harlow & Lane, 1988).

Results

Genetic analysis of segment 9 RNA

Segment 9 RNA was isolated from AmCPV, AaCPV and ApCPV, converted to cDNA and cloned into pCR2.1 TOPO and the nucleotide sequences were determined in both the forward and reverse directions. The segment 9 cDNA of AmCPV consisted of 1473 nucleotides and contained a single long ORF of 345 amino acids starting with the ATG codon (at base 31) and ending with a TAA stop codon (at base 1068) (Fig. 1a). Thirty nucleotides upstream of the initiation codon and 407 nucleotides downstream of the termination codon were present as 5′ and 3′ untranslated sequences. The deduced molecular mass of the encoded protein was 38.28 kDa and the protein was termed NSP38. No sequence similarity was found by searching nucleic acid and protein sequence databases using BLAST. A comparison of the deduced amino acid sequences of the segment 9 ORFs of AmCPV, AaCPV and ApCPV isolates revealed that the nucleotide sequences were identical in all three CPV isolates except at two positions in ApCPV. At position 989, A was replaced with a G residue, changing asparagine to serine, and, at position 1400 (3′ untranslated region), there was a deletion of a G residue in ApCPV. The deduced amino acid composition resulted in an isoelectric point of 7.96 and showed that the protein is rich in alanine, leucine and serine (8–70% each) as well as asparagine (7.54%) and threonine (6.09%) residues. Two potential N-linked glycosylation sites (at positions 205–207 and 337–339) (Fig. 1a) and several phosphorylation sites (not marked) were found. Secondary structure analysis (Rost & Sander, 1994) showed that 44.35% of the amino acids would form random coils, 38.55% would form α-helices and 17.10% would form extended sheets (Fig. 1b). Some sequences in the central as well as terminal regions showed localized similarity to the conserved RNA-binding motifs of infectious bursal disease virus (IBDV) and bluetongue virus (BTV) RNA-dependent RNA polymerases (see Fig. 2) are underlined. The asparagine residue that is replaced by serine in ApCPV is boxed.

Northern analysis

Of the 11 dsRNA segments found in AmCPV, the cDNA of segment 9 hybridized specifically with segment 9 genomic
Antheraea mylitta CPV segment 9

Fig. 2. Alignment of AmCPV NSP38 with four conserved RNA-binding motifs of RNA-dependent RNA polymerase of IBDV and BTV. Numbers following the virus abbreviations indicate the position of the first amino acid of each motif according to the NBRF database. Identical residues are indicated by colons (:); dashes indicate gaps in the alignment.

Fig. 3. Northern blot analysis of AmCPV genome RNA. AmCPV genome segments were resolved by PAGE as discrete bands (lane 1) (segment 11 was not visible as it ran off the gel under these running conditions) and hybridized with \(^{32}P\)-labelled cloned segment 9 cDNA (lane 2). Positions of molecular size markers are indicated in kb.

RNA, confirming the cloning of segment 9 genomic RNA from AmCPV, AaCPV and ApCPV isolates (Fig. 3).

Analysis of recombinant NSP38 protein

The ORF of segment 9 was cloned, expressed and purified from E. coli as a 6 × His-tag–NSP38 fusion protein (Fig. 4, lanes 2 and 3). A polyclonal antibody was raised in a rabbit, affinity-purified, titrated (10\(^{-5}\) by ELISA) and used for immunoblotting and immunofluorescence analysis. Since NSP38 was produced as insoluble inclusion bodies in E. coli, the recombinant NSP38 was transiently expressed in Sf9 cells via pITZ/V-5-His vector. Transfection was monitored by the expression of GFP (present as part of the expression vector) through fluorescence microscopy (data not shown) and the production of recombinant NSP38 was monitored by immunoblotting (Fig. 4, lane 5).

Localization of NSP38 in infected gut cells

In order to localize the expression of NSP38 in cells naturally infected with AmCPV, uninfected and infected gut cells of A. mylitta larvae were analysed by Western blot with anti-NSP38 antibody. A protein band of 38 kDa was detected in infected midgut cells (Fig. 4, lane 8), but no immunoreactive proteins were detected in uninfected cells or in polyhedral bodies containing thousands of virions (Fig. 4, lanes 7 and 9). Immunofluorescence studies corroborated these results, showing the localization of NSP38 in the cytoplasm of infected columnar epithelial cells of the A. mylitta larval midgut (Fig. 5d) but not in the gut cells of uninfected larvae (Fig. 5b).

Expression of NSP38 by baculovirus and binding assay to viral RNA

In order to produce large amounts of functional, soluble recombinant NSP38, the protein was expressed via baculovirus expression and monitored by immunoblotting and immunofluorescence assay using anti-NSP38 antibody. As shown in
Fig. 5. Localization of NSP38 in midgut columnar epithelial cells of *A. mylitta* larvae by immunofluorescence. Midgut cells from uninfected (a, b) and CPV-infected (c, d) *A. mylitta* larvae were collected and stained with rabbit anti-NSP38 antibody and FITC-conjugated anti-rabbit IgG. The cells were observed by phase contrast (a, c) and fluorescence (b, d) microscopy.

Fig. 6. Phase contrast (a, c) and fluorescence (b, d) micrographs of Sf9 cells. Uninfected Sf9 cells (a, b) and Sf9 cells infected with recombinant baculovirus expressing NSP38 (c, d) were stained with rabbit anti-NSP38 antibody and FITC-conjugated anti-rabbit IgG.

Fig. 6(d), recombinant baculovirus-infected cells showed green fluorescence after staining with rabbit anti-NSP38 antibody and FITC-conjugated anti-rabbit IgG. In the immunoblot, an immunoreactive 38 kDa protein was also found (Fig. 4, lane 6). The baculovirus-expressed, recombinant His-tagged NSP38 was purified from insect cells by Ni–NTA agarose and the purified protein appeared as a single band in SDS–PAGE (Fig. 7, lane 2).

Since the NSP38 sequence showed some similarities to RNA-binding motifs of BTV and IBDV RNA polymerases, the RNA-binding ability of soluble NSP38 produced in baculovirus-infected insect cells was examined by poly(rI).poly(rC)–agarose binding. We found that NSP38 binds to poly(rI):poly(rC):agarose (Fig. 7, lane 3), and that the binding was reduced by pre-incubation with 5 µg AmCPV dsRNA (lane 4) or ssRNA (lane 6) and abolished by pre-incubation with 50 µg AmCPV dsRNA (lane 5) or ssRNA (lane 7). Similar results were observed for soluble NSP38 produced after transient transfection of Sf9 cells with pITZ/V-5His/NSP38 (data not shown).

**Discussion**

We report the cloning and complete nucleotide sequence of segment 9 dsRNA, one of the 11 discrete RNA segments of the three CPV isolates AmCPV, AaCPV and ApCPV. A cDNA from AmCPV encoding a protein of 345 amino acids with a deduced molecular mass of 38 kDa was expressed in both *E. coli* and insect cells. The predicted molecular mass of the encoded protein was in agreement with that estimated from the electrophoretic mobility of the expressed protein in SDS–PAGE. Two potential N-linked glycosylation sites are
found in the sequence. However, it is unlikely that these sites are used for glycosylation, because the non-glycosylated NSP38 synthesized in E. coli cells co-migrated in an SDS–polyacrylamide gel with transiently expressed NSP38 from insect cells, recombinant baculovirus-expressed NSP38 and native NSP38 from virus-infected midgut cells. Moreover, the anti-NSP38 antibody recognized both potentially glycosylated and non-glycosylated NSP38.

The AmCPV and AaCPV segment 9 cDNAs showed 100% nucleotide sequence identity, whereas the ApCPV sequence showed two nucleotide changes. One of these changed an asparagine to serine and the other was found in the 3′ untranslated region. These data suggest that all three CPV isolates are the same and infect different Antheraea species in different geographical regions. However, whether other genome segments are the same is not yet known.

When the nucleotide and deduced amino acid sequences of AmCPV segment 9 were compared with sequences in the GenBank and EMBL/GenPept databases using BLAST of FASTA programs (Altschul et al., 1997), no significant similarity was found to any gene or protein sequence. This indicates that segment 9 encodes a novel protein conserved among a new type of CPV that infects these three Saturniidae silkworms. Amino acid sequence comparisons showed three regions of similarity in NSP38 to the RNA-binding motifs of IBDV and BTV RNA-dependent RNA polymerases (Poch et al., 1989; Roy et al., 1990; Yamaguchi et al., 1997). However, NSP38 had no GDD sequence motif for the NTP-binding site found in the polymerases of single-stranded and double-stranded RNA viruses (Bruenn, 1991; Dolja & Carrington, 1992), although a YDD sequence found at amino acids 324–326, an LDD sequence at amino acids 29–31 and an FDD sequence at amino acids 167–169 of NSP38 may be important in binding to CPV RNA.

Immunoblotting and immunofluorescence analysis using anti-NSP38 antibody failed to detect NSP38 in virions or uninfected cells, but the protein was present in virus-infected cells. This suggests that NSP38 is not a structural protein, but may play a role in the regulation of AmCPV genome replication or function.

NSP38 was expressed in E. coli as insoluble inclusion bodies but in soluble form in transiently transfected insect cells or baculovirus-infected insect cells. Transiently produced or baculovirus-expressed and purified NSP38 bound poly-(rI).poly(rC)–agarose and this binding was abolished competitively by AmCPV dsRNA and ssRNA, indicating that NSP38 produced in insect cells possessed the ability to bind viral dsRNA and ssRNA. In BmCPV, the segment 9-encoded non-structural protein NS5 is expressed in virus-infected cells and binds viral dsRNA (Hagiwara et al., 1998a). In mammalian orthoreovirus, the non-structural protein eNS binds preferentially to ssRNA, like a single-stranded DNA-binding protein, better than dsRNA and regulates the replication of the reovirus RNA genome (Gillian et al., 2000). Specific binding of rotavirus non-structural proteins NSP1 and NSP3 to viral RNA has also been reported (Hua et al., 1994; Poncet et al., 1993), but their functions in assortment and replication of RNA are not understood. Rotavirus non-structural protein NSP2 has been reported to be expressed at high levels in infected cells, accumulates in the viroplasm in multimeric forms and binds strongly to viral ssRNA (Petrie et al., 1984; Taraporewala et al., 1999). It also possesses nucleoside triphosphatase activity, which may provide the energy necessary for the protein to function as a molecular motor that directs the packaging of viral mRNA (Petrie et al., 1984; Taraporewala et al., 1999). Another rotavirus protein, NSP5 (a glycosylated phosphoprotein), has been found to be autophosphorylated and to form a complex with NSP2 and viral polymerase that then participates in virus replication and assembly (Blackhall et al., 1997; Fabbretti et al., 1999). It is also rich in serine and
threonine residues. Since NSP38, encoded by AmCPV segment 9, also contains a large proportion of serine (87%) and threonine (61%) residues and binds viral RNA, we hypothesize that NSP38 may undergo autophosphorylation during its expression in virus-infected cells and that phosphorylated NSP38 binds viral RNA to play a role in the regulation of viral mRNA function or replication and packaging of the viral RNA genome.

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