Expression of plant virus genes in animal cells: high-level synthesis of cowpea mosaic virus B-RNA-encoded proteins with baculovirus expression vectors

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The baculovirus expression system has been used to produce non-structural proteins encoded by bottom-component RNA (B-RNA) of cowpea mosaic virus (CPMV). For this, cDNAs containing the 60K, 87K, 110K and 170K protein coding sequences were each provided with an ATG start codon and the cDNA containing the 60K coding sequence with a TAA stop codon immediately downstream of the coding sequence. Recombinant baculoviruses were retrieved which harboured the modified B-cDNA sequences under the control of the polyhedrin promoter of Autographa californica nuclear polyhedrosis virus (AcNPV). Upon infection of Spodoptera frugiperda cells with these recombinant baculoviruses, proteins were produced which were indistinguishable from the viral proteins found in CPMV-infected plants as judged by their migration in polyacrylamide gels and their reactivity with CPMV-specific antisera. Specific processing of CPMV polyproteins in cells infected with the 110K- and 170K-encoding baculovirus recombinants proved that the CPMV-encoded 24K protease activity contained in these polyproteins is active in these cells. Approximately 10% of the 110K protein was processed into 87K and 24K proteins and the 170K protein almost completely into the 110K, 87K, 84K, 60K and 24K polypeptides. In S. frugiperda cells infected by recombinant AcNPVs harbouring the 87K or 110K coding sequences, the CPMV-specific proteins amounted to 10 to 20% of the total cellular protein content, whereas in cells infected by recombinants encoding the 60K and 170K polypeptides the amounts of CPMV-specific proteins synthesized were much lower. Northern blot analysis indicated that the low-level synthesis of the 60K and 170K polypeptides was not due to inferior transcription of the cloned genes but was probably the result of inefficient translation of the RNAs derived from these constructs. It is concluded that plant virus genes can be efficiently expressed in an animal cell expression system to yield proteins that are structurally and, in at least one case (24K protein), functionally identical to the authentic plant virus proteins.

Introduction

Cowpea mosaic virus (CPMV), the type member of the comoviruses, possesses a bipartite, plus-sense RNA genome. Both RNAs (denoted B- and M-RNA) carry a small protein VPg covalently linked to their 5' terminus, a poly(A) tail at their 3' terminus and encode a large polyprotein from which the functional proteins are generated through proteolytic processing by a viral protease (for reviews see Goldbach & van Kammen, 1985; Eggen & van Kammen, 1988). M-RNA encodes the two capsid proteins and protein(s) required for cell-to-cell transport (van Wezenbeek et al., 1983; Wellink & van Kammen, 1989), whereas B-RNA encodes all viral functions necessary for viral RNA replication (Goldbach et al., 1980; Eggen & van Kammen, 1988). Knowledge of the precise function of each of the B-RNA-encoded proteins in CPMV replication is, however, still very limited and partly based on amino acid homology of some CPMV proteins with those of the picornaviruses (Franssen et al., 1984a; Argos et al., 1984; Goldbach, 1986, 1987).

Purified replication complexes from CPMV-infected cowpea leaves capable of elongating viral plus-sense RNA, the synthesis of which has already been initiated in vivo, contain the B-RNA-encoded 110K polypeptide (Dorssers et al., 1984). This polypeptide is believed to be the active viral RNA replicase and is composed of the 24K protease and 87K core polymerase (Fig. 1). So far it has not been possible to obtain a template-dependent
CPMV RNA replicase activity from CPMV-infected cowpea plants (Dorssers et al., 1983, 1984) or Chenopodium amaranticolor (Eggen et al., 1988). The study of viral RNA replication is further hampered by interfering host plant activities, such as a 130K RNA-dependent RNA polymerase (Dorssers et al., 1983; van der Meer et al., 1984) and a terminal uridylyl transferase (Zabel et al., 1981). As an alternative approach to the study of the role of viral proteins in RNA replication, the B-RNA-encoded 87K and 110K proteins were produced in an Escherichia coli expression system (Richards et al., 1989). Neither the 87K nor the 110K protein showed RNA synthesizing activity under conditions where poliovirus polymerase (protein 3D) similarly produced in E. coli was highly active (Richards et al., 1989). Reasons for the lack of polymerase activity for the CPMV proteins may be (i) the low level of expression in E. coli, (ii) incorrect protein folding or lack of post-translational modifications in a prokaryotic system, (iii) the low stability of the proteins in E. coli, (iv) requirement for additional proteins such as the viral 60K protein and (v) the need for a polyprotein from which the polymerase is simultaneously cleaved and incorporated into an active replication complex.

In order to investigate whether a eukaryotic system may alleviate the potential shortcomings of a prokaryotic system, and to produce larger amounts of the proteins encoded by CPMV B-RNA, an animal cell expression system was employed. It has been reported that baculovirus expression vectors produce high amounts of biologically active proteins in insect cells which are post-translationally modified in a fashion similar to that of the authentic proteins (for reviews see Luckow & Summers, 1988a; Miller, 1988). The high level of expression is based on the exploitation of the strong polyhedrin promoter by the allelic replacement of the baculovirus polyhedrin gene by a heterologous sequence. So far the gene of phaseolin has been the only plant gene expressed with this system (Bustos et al., 1987); the expression of plant virus genes has not yet been reported. In this paper we report the expression of the 60K, 87K, 110K and 170K coding regions of CPMV B-RNA in insect cells by Autographa californica nuclear polyhedrosis virus (AcNPV) recombinants.

Fig. 1. Diagram of the genetic organization of CPMV B-RNA and its translation products. B-RNA contains a single open reading frame represented by an open bar on which the positions of the start and stop codons are indicated. VPg is indicated by a black square, other proteins by a single line. The initial polyprotein is processed into smaller functional proteins by specific proteolytic cleavages at the indicated sites. :, Gin-Met; ·, Gin-Gly; ¶, Gin-Ser.

Methods

Viruses, plasmids and cells. AcNPV, strain E2 (Summers & Smith, 1987) and recombinant viruses were grown in Spodoptera frugiperda IPLB-SF-21 cells (Vaughn et al., 1977) in TNM-FH medium (Hink, 1970) supplemented with 10% foetal bovine serum as described by Summers & Smith (1987). Baculovirus transfer vectors and other plasmids were propagated in the E. coli strains DH5α(F') and JM109. Single-stranded DNA containing UMP residues was isolated from the E. coli strain RZ/032.

Plasmid pTB1G (Eggen et al., 1989) is a full-length cDNA clone of the CPMV B-RNA sequence (Lomonosoff & Shanks, 1983) downstream of the bacteriophage T7 promoter from which infectious RNA transcripts can be generated. The baculovirus transfer vector pAcRP23 (Posse & Howard, 1987) was constructed by cloning the EcoRI I fragment of AcNPV, containing the polyhedrin gene, into pUC8.

DNA manipulations. Standard recombinant DNA techniques were used for the isolation and ligation of DNA fragments and transformation of DNA in competent E. coli cells (Maniatis et al., 1982). Enzymes were purchased from Gibco-BRL or from Boehringer-Mannheim and used as described by the manufacturer. Oligodeoxynucleotides were synthesized with a Cycloone DNA synthesizer (Biosearch). Site-directed mutagenesis with these oligodeoxynucleotides was performed as described by Kunkel (1985).

Construction of the baculovirus transfer vector pAcHB60. An XbaI–SstI fragment [nucleotides (nt) 899 to 2301] from pTB1G was inserted in M13mp18 and a start codon was introduced at positions 1185 to 1187 using the oligonucleotide 5'-GGACAATGCATAT-GAGTCCGTTAATCTTCC-3', which, at the same time, created an Ndel site (CATATG). The mutagenized XbaI–SstI fragment was then reintroduced into pTB1G resulting in the plasmid pTBHM60 (HM refers to the histidine–methionine residues encoded by the Ndel recognition site). A TAA stop codon was added at the end of the 60K coding region by inserting a SstI–Spfl fragment (nt 2301 to 3156) of pTB1G in M13mp19 followed by mutagenesis with the oligonucleotide 5'-GGCCAGAGCCAAATTCTTGGAT-3'. The newly created stop codon was subsequently inserted in pTBHM60 by exchanging the homologous SstI–KpnI fragments (nt 2301–3134) of pTB1G in M13mp19 followed by mutagenesis with the oligonucleotide 5'-GGGCAGAGCACAATTCTTGGAT-3'. The resulting plasmid was then digested with Ndel, blunted with Klenow polymerase, partially digested with KpnI and the 1970 bp Ndel–KpnI fragment was isolated. The baculovirus transfer vector pAcBP23 was digested with BamHI, the protruding ends were filled in with Klenow polymerase and digested with KpnI. Ligation with the 1970 bp Ndel–KpnI fragment from pTBHM60STOP resulted in the 11250 bp expression vector pAcHB60 containing the 60K coding region.

Construction of transfer vector pAcHB87. An SstI–BamHI fragment (nt 2301 to 3857 in the B-RNA) from pTB1G was inserted in M13mp18 and subjected to mutagenesis using the oligonucleotide
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5'-ATTCTTCAGCCATATGCCGCTTG-3'. This resulted in an NdeI recognition site and an ATG start codon at the beginning of the 87K coding region. The mutation was transferred to pTB1G, resulting in plasmid pTBHM87, which was subsequently cleaved with NdeI (partially) and with Clal (nt 3671 to 5935). The baculovirus transfer vector pACRP23 was digested with BamHI and Kpnl and the 9.3 kb fragment was isolated, blunted with Klenow polymerase and dephosphorylated with calf intestinal phosphatase. Linkage of this fragment and the blunted NdeI–ClaI fragment, containing the 87K coding region, produced vector pACRB87. Confirmation of the proper orientation of the CPMV insert downstream of the polyhedrin promoter was established by restriction enzyme analysis and dideoxynucleotide sequencing (Sanger et al., 1977; Korneluk et al., 1985).

**Construction of transfer vector pAcHB110.** The 1556 bp Srl–BamHI fragment of pTB1G was inserted in M13mp19 and an NdeI site was introduced at positions 3045 to 3050 using the oligonucleotide 5'-GGCACGCGCATATGGCTTATTTAG-3'. This mutation was transferred to pTB1G resulting in pTBHM110, which was subsequently digested with NdeI (partially) and Clal (nt 3046 to 5935). The isolated NdeI–Clal fragment, containing the 110K coding region, was blunted with Klenow polymerase and ligated to the 9.3 kb linearized and blunt-ended Kpnl and the isolated 8–2 kb fragment was ligated with the blunted fragment of pTB1G (nt 735 to 5319) and the 0.5 kb NdeI–Clal fragment, containing the 110K coding region downstream of the polyhedrin promoter was confirmed by restriction enzyme analysis and sequencing.

**Construction of transfer vector pAcHB170.** pAcHB60 was digested with Kpnl, blunted with Klenow polymerase and digested with Srl1: the 10.4 kb fragment was isolated. pTB1G was digested with BamHI, filled in with Klenow polymerase and digested with Srl1. The 1556 bp fragment (nt 2301 to 3857 of the B-cDNA) was isolated and ligated to the 10.4 kb pAcHB60 fragment so that the BamHI site was repaired. The resulting plasmid pAcHBam was digested with BamHI and BstEI and a 5.7 kb fragment was isolated. pAcHB110 was also digested with BamHI and BstEI and the isolated 8.2 kb fragment was ligated with the 5.7 kb fragment to create pAcHB170.

**Selection of recombinant viruses.** Monolayers of S. frugiperda cells (1 x 10⁶ cells in 35 mm tissue culture dishes) were cotransfected with wild-type (wt) AcNPV DNA and transfer vector DNA using the calcium phosphate precipitation procedure of Summers & Smith (1987). After incubation of cells for 5 days at 27°C the supernatant was collected and putative recombinants, recognized by their polyhedron-negative phenotype, were picked and plaque-purified three times to reach genetic homogeneity.

**Analysis of proteins synthesized in infected cells.** S. frugiperda cells (1 x 10⁶ cells per 35 mm Petri dish) were infected with AcNPV recombinants containing CPMV B-cDNA inserts at a m.o.i. of 10 and incubated at 27°C for 48 h. The cells were pelleted by low-speed centrifugation (300 g for 2 min), washed twice in cold phosphate-buffered saline (10 mm-Na₂HPO₄, 100 mm-NaCl pH 7.5) and disrupted in sample buffer (10 mm-Tris–HCl pH 8.0, 1 mm-EDTA, 10% v/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol and 0.001% w/v bromophenol blue). An equal amount of S. frugiperda cells infected with wt AcNPV and mock-infected cells served as controls. Samples were boiled for 3 min and aliquots corresponding to 10³ to 50 x 10³ cells were electrophoresed on 10% SDS-polyacrylamide gels (Laemmli, 1970). These gels were either stained with Coomassie brilliant blue (CBB) or analysed by immunoblotting using either rabbit anti-24K (Wellink et al., 1987), anti-170K (Franssen et al., 1984a) or anti-VPg serum (Eggen et al., 1988) with anti-rabbit IgG-alkaline phosphatase conjugate (Promega Biotech) as a second antibody (Blake et al., 1984).

**Northern blot analysis.** Total RNA was isolated from uninfected and infected S. frugiperda cells at 24 h post-infection (p.i.) essentially as described by de Vries et al, (1988). Briefly 1 x 10⁶ infected cells were mixed at 53°C with a 1:1 mixture of RNA extraction buffer (100 mm-Tris–NaOH pH 9.0, 100 mm-LiCl, 10 mm-EDTA and 1% w/v SDS) and phenol and vortexed for 5 min. After the addition of 0.5 vol. of chloroform the cells were vortexed for another 10 min followed by 30 min centrifugation at 10000 g. The aqueous layer was removed and again extracted with chloroform. The RNA was precipitated in 2 M-ammonium acetate, 100 mm-EDTA, 10% w/v polyethylene glycol 6000 and ethanol and collected and putative recombinants, recognized by their polyhedron-negative phenotype, were picked and plaque-purified three times to reach genetic homogeneity.

Blotting was on GeneScreen (NEN Research Products) as recommended by the manufacturer. Nick translations of the 4.6 kb Accl fragment from pTB1G (nt 735 to 5319) and the 0.5 kb EcoRV–Kpnl fragment (nt 93 to 634 of the polyhedrin gene) fragment from pAcRP23 were used as radioactive probes.

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**Fig. 2. Schematic diagram of the construction of the transfer vectors.** Vector construction is described in Methods. (a) The full-length cDNA clone of CPMV B-RNA is represented by an open bar on which the restriction sites that were used in the different cloning steps are indicated. The newly created NdeI sites and TAA stop codon are indicated with an asterisk. The regions that were cloned downstream of the polyhedrin promoter of transfer vector pACRP23 are represented by single lines. (b) The BamHI–Kpnl fragment of the polyhedrin coding region (black bar) of pAcRP23 was exchanged with the CPMV coding sequences, resulting in the pAcHB transfer vectors.
Results

Construction of recombinant baculoviruses

For the cloning and expression of the CPMV B-RNA sequences encoding the 87K (core polymerase), 110K (protease and polymerase), 60K (58K and VPg) and 170K (60K and 110K) polypeptides, start and stop codons and restriction enzyme cleavage sites were introduced in the B-cDNA. This was achieved by site-directed mutagenesis (Kunkel, 1985) in such a way that the first codon of a sequence to be expressed was changed into an ATG start codon which was also part of an NdeI recognition site (CATATG). Using the newly created restriction sites the modified B-cDNA sequences were then inserted in the baculovirus transfer vector pAcRP23 (Possee & Howard, 1987) behind the intact polyhedrin promoter, resulting in the transfer vectors pAcHB87, pAcHB110, pAcHB60 and pAcHB170 (Fig. 2). In these constructs almost the entire polyhedrin coding region (nt +1 to +633) has been deleted and replaced by a CPMV coding sequence in such a way that the open reading frame is under the control of the polyhedrin promoter and that translation initiation will be at the ATG start codon provided by the CPMV sequence. This start codon was either naturally present (pAcHB110) or introduced by site-directed mutagenesis. In the latter case the

Fig. 3. Transcripts expected to be generated from the polyhedrin promoter for the different recombinant baculoviruses. (a) The mRNAs are represented by bars on which the positions of the start and stop codons and the polyadenylation signal (P.S.) are shown. The lines indicate the length of segments of the transcripts as the number of nucleotides (nt) and the virus from which these segments originated. (b) Comparison of the leader sequences of the CPMV genes inserted in the transfer vector to that of the AcNPV polyhedrin gene (Hooft van Iddekinge et al., 1983). The sequences were verified by sequence analysis.
methionine codon substitutes a serine codon (pAcHB60 and pAcHB170) or a glycine codon (pAcHB87) of the authentic CPMV proteins. For the expression of the 60K coding region a TAA stop codon was introduced immediately behind the 60K coding region, at the position of the first amino acid of the 24K protease (Fig. 1; Wellink et al., 1986). In all other constructions the natural stop codon (TAG) of the CPMV B-cDNA is present.

Each of the transfer vectors were used with wt AcNPV DNA for cotransfection of S. frugiperda cells. In approximately 0.5% of the infections recombination occurred between the sequences flanking the CPMV inserts and the homologous sequences of the wt AcNPV DNA. The recombinant AcNPV DNAs, denoted AcHB87, AcHB110, AcHB60 and AcHB170, were characterized by their occlusion body-negative phenotype, as they lack the polyhedrin gene. The CPMV-specific mRNAs expected to be generated for each construct are depicted in Fig. 3a. Sequencing of the transfer vectors revealed that, as expected, the 5' untranslated regions were identical for all constructs (Fig. 3b).

Identification of CPMV polypeptides in insect cells

Plaque-purified recombinant viruses were used at a m.o.i. of 10 to infect S. frugiperda cells and, after incubation for 48 h at 27 °C, proteins from these cells were analysed on SDS-polyacrylamide gels. Following electrophoresis the gels were either stained with CBB (Fig. 4) or used for immunoblotting (Fig. 5, 6). The CBB-stained gel revealed that a 30K protein representing polyhedrin was present in wt AcNPV-infected cells (Fig. 4, lane 2) but not in cells infected with each of the recombinants. Instead, with the recombinants AcHB87 and AcHB110 extra bands of proteins of 87K and 110K were clearly visible which made up about 20% of the total stainable protein content (Fig. 4, lanes 4 and 5). Furthermore, the 110K protein seemed to be partially processed (approximately 10%) into the 87K protein. No additional proteins could be detected by CBB staining with recombinants AcHB60 and AcHB170 as compared to wt AcNPV-infected cells.

The expression products from the recombinants were further analysed on immunoblots by treatment with three different CPMV-specific antisera. The analysis showed that the 87K and 110K proteins, which were detected with CBB, migrated with the same electrophoretic mobility as the 87K and 110K proteins present in plant fractions of CPMV-infected cowpea plants and were immunoreactive with anti-170K serum (Fig. 5, lanes 3, 4, 9 and 10). With anti-24K serum a 24K protein could also be detected in cells infected with AcHB110

![Fig. 4. Expression of CPMV sequences by recombinant baculoviruses.](image)

![Fig. 5. Immunoblot analysis of proteins synthesized in S. frugiperda cells infected with wt AcNPV or recombinant viruses AcHB87 and AcHB110. Detection was with anti-170K or anti-24K serum as a primary antibody and anti-rabbit alkaline phosphatase conjugate as the detection antibody. Cells were collected and fractionated by PAGE as in Fig. 4. Lanes 1 and 5, uninfected Sf21 cells (control); lanes 2 and 6, AcNPV; lane 3, AcHB60; lane 4, AcHB87; lane 5, AcHB110; lane 6, AcHB170; lane 7, M, markers. The AcNPV polyhedrin protein and the 87K and 110K proteins of CPMV are indicated by pointers.](image)
indicating, that the 110K protein was processed into 24K and 87K proteins. In S. frugiperda cells infected with recombinant \( \text{AcHB60} \), a 60K protein reactive with anti-VPg serum could be shown (Fig. 6, lane 3). This protein was also present in cells infected with \( \text{AcHB170} \) (Fig. 6, lane 5) as were proteins of sizes of 170K, 110K, 84K, 87K and 24K, which were immunoreactive with anti-24K serum (Fig. 6, lane 7) or anti-170K serum (Fig. 6, lane 9). All of the immunoreactive species correspond to authentic viral proteins present in fractions of CPMV-infected cowpea plants and were absent in uninfected or AcNPV-infected \( S. \text{frugiperda} \) cells. It is thus concluded that the proteins detected for the AcNPV recombinants could be attributed to expression of CPMV sequences. Furthermore, the results demonstrate that whenever the 24K protein was synthesized, processing of approximately 10% of the 110K protein and almost complete processing of the 170K protein into products of the proper sizes occurred (see also Fig. 1). In addition to the specific products of proteolytic processing, additional cleavage products could also be detected with anti-24K and anti-170K serum.

**Differences in expression levels for the various recombinants**

Significant differences in the expression levels of the various recombinant baculoviruses was observed (Fig. 4). For the detection of CPMV-specific proteins on immunoblots, a 20-fold greater number of cells was used in the case of recombinants \( \text{AcHB60} \) and \( \text{AcHB170} \) as compared to \( \text{AcHB87} \) and \( \text{AcHB110} \). The AcNPV DNA recombinants with low expression levels both have the 60K coding region in common, whereas recombinants with high expression levels do not harbour this sequence. \( S. \text{frugiperda} \) cells producing the 60K protein exhibited abnormal cytopathic effects and showed lysis within 48 h p.i. Furthermore, using electron microscopy, it appeared that these cells contained large numbers of vacuoles in the cytoplasm (results not shown). Therefore, the proteins produced by recombinants \( \text{AcHB60} \) and \( \text{AcHB170} \), notably the 60K protein, may have a deleterious effect on cells. \( S. \text{frugiperda} \) cells were infected with a mixture of two recombinants at an m.o.i. of 15 for each recombinant in the following combinations: \( \text{AcHB60 + AcHB87 + AcHB110 + AcHB170 + AcHB60 + AcHB87 + AcHB110} \). At 48 h p.i. the cells were harvested and assayed for the presence of the 110K protein (Fig. 7). Similar amounts of the 110K protein were found for each set of recombinants (Fig. 7, lanes 1 to 3), demonstrating that the proteins produced in \( \text{AcHB60} \) or \( \text{AcHB170} \)-infected cells were not detrimental to the expression of other genes. To determine whether the low expression of the 60K and 170K proteins by recombinants \( \text{AcHB60} \)
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The results described in this paper demonstrate that the baculovirus expression vector system is very useful for the high-level expression of plant virus genes. The CPMV-specific 87K and 110K proteins produced by AcHB87 and AcHB110 amounted to up to 20% of the total CBB-stainable protein content of infected insect cells (Fig. 4). On the other hand, the CPMV-specific 60K and 170K proteins produced by AcHB60 and AcHB170 were detectable only by immunoblotting. Similar differences in the amounts of CPMV-specific proteins were found upon expression in E. coli cells (Richards et al., 1989; S. Peters, unpublished results) but overall the amounts of protein produced in the baculovirus expression vector system were larger than those in E. coli. Moreover, the polypeptides synthesized in S. frugiperda cells were more stable than those produced in E. coli, as very little non-specific proteolytic cleavage occurred. Specific proteolytic processing of the CPMV polyproteins was observed in cells infected with AcHB110 or AcHB170, which both produced the 24K protease. About 10% of the 110K protein was processed into 87K and 24K proteins (Fig. 5) whereas the 170K protein was almost completely processed to give 110K, 87K, 84K, 60K and 24K polypeptides (Fig. 6). The 110K protein could be precipitated by low-speed centrifugation from cell extracts and therefore appears to be aggregated into large insoluble complexes. The low degree of processing of the 110K protein as compared to the 170K protein may be attributed to this aggregation of the 110K protein. The polyproteins and products of processing synthesized in insect cells corresponded to the CPMV proteins produced in infected cowpea plants as judged by their electrophoretic mobility and their reaction with CPMV-specific antisera. The occurrence of the specific cleavage products in insect cells indicates that the 24K protease is biologically active and acts at the proper glutamine-methionine cleavage site between the 60K and 110K proteins and the glutamine-glycine cleavage site between the 87K and 84K proteins.

We wondered whether the low amounts of 60K and 170K polypeptides produced in cells infected with AcHB60 and AcHB170 might be due to a deleterious effect of these proteins on the S. frugiperda cells. It is

and AcHB170 was due to a reduced transcription level, RNA was isolated at 24 h p.i. from cells infected with AcNPV recombinants. The amount and quality of the isolated RNA was analysed on an ethidium bromide-stained agarose gel. Similar quantities of RNA were used for electrophoresis under denaturing conditions and for Northern blotting. It was verified that comparable amounts of viral RNA were present by treating the blot with a probe specific for the gene of the abundant envelope surface glycoprotein (gp67; Whitford et al., 1989) of AcNPV as an internal standard (results not shown). Using probes specific for CPMV B-RNA and polyhedrin coding sequences, no significant differences in mRNA levels transcribed from the polyhedrin promoter were detected for the recombinant with low expression level (AcHB170) and the recombinants with high expression levels (AcHB87 and AcHB110; Fig. 8). For the other recombinant with the low expression level (AcHB60) there seemed to be less mRNA present but this was probably due to masking of the signal by rRNA present at the same position on the gel. Cells infected with a mixture of two recombinants contained comparable amounts of mRNA detectable on Northern blots. Hence, it may be deduced that the low expression of the 60K and 170K sequences was not the result of an impeded transcription level in these cells. The origin of the high Mr bands in Fig. 8 (lanes 2 to 8) is unknown. The same bands appeared with the same intensity with the gp67 probe and are thus not specific for CPMV sequences. The sizes of the mRNAs detected on the Northern blots also demonstrate that transcription does not stop at the poly(A) sequence provided by the CPMV cDNA but proceeds to the polyhedrin transcription termination site.

Discussion

The results described in this paper demonstrate that the baculovirus expression vector system is very useful for the high-level expression of plant virus genes. The CPMV-specific 87K and 110K proteins produced by AcHB87 and AcHB110 amounted to up to 20% of the total CBB-stainable protein content of infected insect cells (Fig. 4). On the other hand, the CPMV-specific 60K and 170K proteins produced by AcHB60 and AcHB170 were detectable only by immunoblotting. Similar differences in the amounts of CPMV-specific proteins were found upon expression in E. coli cells (Richards et al., 1989; S. Peters, unpublished results) but overall the amounts of protein produced in the baculovirus expression vector system were larger than those in E. coli. Moreover, the polypeptides synthesized in S. frugiperda cells were more stable than those produced in E. coli, as very little non-specific proteolytic cleavage occurred. Specific proteolytic processing of the CPMV polyproteins was observed in cells infected with AcHB110 or AcHB170, which both produced the 24K protease. About 10% of the 110K protein was processed into 87K and 24K proteins (Fig. 5) whereas the 170K protein was almost completely processed to give 110K, 87K, 84K, 60K and 24K polypeptides (Fig. 6). The 110K protein could be precipitated by low-speed centrifugation from cell extracts and therefore appears to be aggregated into large insoluble complexes. The low degree of processing of the 110K protein as compared to the 170K protein may be attributed to this aggregation of the 110K protein. The polyproteins and products of processing synthesized in insect cells corresponded to the CPMV proteins produced in infected cowpea plants as judged by their electrophoretic mobility and their reaction with CPMV-specific antisera. The occurrence of the specific cleavage products in insect cells indicates that the 24K protease is biologically active and acts at the proper glutamine-methionine cleavage site between the 60K and 110K proteins and the glutamine-glycine cleavage site between the 87K and 84K proteins.

We wondered whether the low amounts of 60K and 170K polypeptides produced in cells infected with AcHB60 and AcHB170 might be due to a deleterious effect of these proteins on the S. frugiperda cells. It is
rather striking that these cells showed a c.p.e. characterized by large arrays of vacuoles and lysis of the cells within 48 h p.i. whereas such effects were not found in cells infected with AcHB87 and AcHB110. Production of vacuoles in cells infected with recombinant viruses has been reported before (Matsuura et al., 1987; Luckow & Summers, 1988b) but in these cases there was high expression of the foreign gene. Moreover, insect cells infected both by AcHB110 and AcHB60 showed the characteristic c.p.e. whereas the amounts of 110K and 60K proteins produced were not affected. It is therefore not very likely that the c.p.e. is the major cause of the low production of the 60K and 170K proteins. Neither it seems are the low amounts of 60K and 170K polypeptide found in insect cells the result of more rapid turnover of these proteins as compared to the 87K and 110K proteins. In cells infected with AcHB170 low quantities of 170K protein are found, as are similarly low amounts of its cleavage products, 110K, 87K, 84K, 60K and 24K proteins (Fig. 6). If the different CPMV-specific proteins varied in stability, accumulation of the 110K and 87K polypeptides would be expected, as occurs in cells infected with AcHB110 and AcHB87. Northern blot analysis revealed no significant difference in the amounts of mRNA produced from the polyhedrin promoter of the recombinant with the low expression level (AcHB170) as compared to the recombinants with high expression levels (AcHB87 and AcHB110; Fig. 8) but apparently less 60K mRNA was present in AcHB60-infected cells. However, it is not very plausible that this is caused by a transcriptional defect because the promoter region of the transfer vector pAcHB170 originated from pAcHB60. Furthermore, it is unlikely that transcription signals were affected during cloning or recombination because, for each recombinant AcNPV DNA, at least five independent clones were isolated. It is concluded that recombinants AcHB170 and AcHB60 produce similar amounts of mRNA from the polyhedrin promoter and that the hybridization signal for AcHB60 is weakened by the presence of rRNA. Therefore, the relatively low expression level of the 60K and 170K coding sequences as compared to the 87K and 110K coding sequences does not appear to be the result of a transcriptional defect. The only possibility left is that the low production of 60K and 170K proteins in AcHB60- and AcHB170-infected cells is caused by inefficient translation of the corresponding mRNAs. On the other hand it is difficult to understand what the structural basis of the difference in efficiency of translation of the mRNAs is, since the 5' non-coding regions were identical in all constructs. Also, the sequence around the AUG start codon in the different mRNAs does not give a plausible explanation for the different translation efficiencies. The mRNAs for the 87K, 110K, 170K and 60K proteins all have T at the −3 position. It has been reported that when a pyrimidine at position −3 is replaced by a purine, translation becomes more sensitive to changes at position +4 (Kozak, 1986). The mRNAs generated from the polyhedrin promoter of AcHB87, AcHB110 and AcHB60/170 have a G, a T and an A residue, respectively, at the +4 position (Fig. 3b). Consequently one would expect that these mRNAs would be equally translated (Rohrmann, 1986; Luckow & Summers, 1988b). This, however, is not the case. The low translation level seems to be an intrinsic property of the mRNA of the 60K coding region that also occurs at the 5' end of the mRNA of the 170K coding region. A similar suggestion has been made for the expression of the human tissue type plasminogen activator in insect cells (Luckow & Summers, 1988b). Deletion of various domains of the 60K coding sequence may answer the question of which region is responsible for the poor translation.

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References


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