Protoplasts transiently expressing the 200K coding sequence of cowpea mosaic virus B-RNA support replication of M-RNA

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In order to identify the viral polymerase involved in cowpea mosaic virus (CPMV) RNA replication the 87K, 110K and 170K proteins as well as the complete 200K polyprotein of CPMV B-RNA have been produced in cowpea protoplasts, using expression vectors based on the 35S promoter of cauliflower mosaic virus. CPMV-specific proteins were obtained that were indistinguishable from proteins found in CPMV-infected protoplasts. Proteolytic processing of precursor proteins synthesized from the expression vectors proved that the 24K protease contained within these proteins is active. Moreover, it was established that protoplasts transfected with the expression vector containing the entire 200K coding sequence, but not those transfected with vectors containing the 170K, 110K or 87K coding sequences, were able to support replication of co-inoculated M-RNA. Despite the ability to support replication of M-RNA for protoplasts transiently expressing the 200K coding region, CPMV-specific RNA polymerase activity dependent on exogenous added template RNA could not be detected in extracts of these protoplasts in assays using poly(A)·oligo(U) or other template/primer combinations. In contrast, extracts of protoplasts in which poliovirus polymerase was produced exhibited RNA polymerase activity in such assays. These results indicate that the CPMV polymerase, unlike the poliovirus polymerase, is not able to use oligo(U) as a primer or cannot function on exogenous template and primer RNA.

Introduction

The genetic information of the comovirus cowpea mosaic virus (CPMV) is located on two positive-sense RNA molecules, designated B- and M-RNA. Upon translation of both RNA molecules large polyproteins are produced from which functional proteins are generated by a number of defined proteolytic cleavages executed by a B-RNA-encoded protease (Wellink et al., 1986; Verver et al., 1987). The viral functions that are required for viral RNA replication are all supplied by B-RNA (Goldbach et al., 1980), whereas the proteins encoded by M-RNA are indispensable for virus cell-to-cell movement (Wellink & van Kammen, 1989).

Characteristic cytopathic structures arise in the cytoplasm of cowpea cells at early stages of infection with CPMV (de Zoeten et al., 1974). These structures consist of numerous vesicular membranes surrounded by electron-dense material in which the bulk of the non-structural proteins encoded by B-RNA are found (Wellink et al., 1988). Replication of CPMV RNA is associated with these membranous vesicles (de Zoeten et al., 1974) and, accordingly, viral RNA-dependent RNA polymerase activity capable of elongating in vivo initiated positive-sense RNA has been detected in the crude membrane fraction of CPMV-infected leaves (Dorssers et al., 1983). Extensive purification of the RNA polymerase activity from the membrane fraction resulted in a preparation in which the B-RNA-encoded 110K protein was the sole detectable viral protein and this protein was therefore assigned to be the viral RNA polymerase (Dorssers et al., 1984). In addition to the 110K protein, the active preparation contained two major host proteins of 57K and 68K, but it is not known whether these polypeptides also fulfil a role in viral RNA replication. On the other hand, the inhibition of RNA replication in cowpea protoplasts by actinomycin D early in infection with CPMV suggests that one or more host proteins are needed for CPMV RNA replication (Rottier et al., 1979; de Varennes et al., 1985).

The 87K protein of CPMV, which together with the 24K protease constitutes the 110K protein, has significant sequence homology with poliovirus polymerase 3D<sup>″</sup> and also shares conserved sequence motifs with the putative RNA-dependent RNA polymerases of other viruses (Kamer & Argos, 1984; Franssen et al., 1984a; Poch et al., 1989). However, unlike poliovirus (Flanagan...
polymerase and digested with manipulations were carried out by standard recombinant DNA (position 3672; Lomonossoff & Shanks, 1983), filled in with Klenow virus 3CD coding region (van Bokhoven techniques (Sambrook et al., 1989; 1992). Although the proteins thus produced were very similar to the corresponding viral proteins found in plant cells, it was not possible to demonstrate oligo(U)-primed RNA polymerase activity in cell extracts containing CPMV-specific proteins. Even insect cells in which the complete 200K polyprotein encoded by B-RNA was synthesized did not contain any virus-specific RNA-dependent RNA polymerase activity (van Bokhoven et al., 1992). It was concluded that CPMV polymerase, in contrast to poliovirus 3Dpol, is not able to make use of exogenous added template and primer RNA or requires an additional plant component to function as a polymerase. In this paper we have exploited a plant expression vector containing the 35S promoter of cauliflower mosaic virus (CaMV) to produce the 87K, 110K, 170K and 200K proteins encoded by CPMV B-RNA in cowpea protoplasts. Transfected protoplasts were subsequently assayed for CPMV-specific RNA polymerase activity.

Methods

Construction of plasmids. E. coli strain DH5α was used for growth and maintenance of all plasmids. Plant expression vector pMON999 was kindly provided by Dr C. Hemenway of the Monsanto Company. Plasmid pBI121, containing the gene for β-glucuronidase (GUS) has been described by Jefferson et al. (1987). Plasmids pTB1G and pTM1G contain full-length cDNA copies of CPMV B- and M-RNA respectively, from which infectious RNA transcripts can be generated using T7 RNA polymerase (Egen et al., 1989). Plasmids pTBHM60, pTBHM87, pTBHM110 and pTB200N are derivatives of pTB1G with an engineered Ndel recognition site just at the 5’ end of the coding sequence for the protein each denotes (van Bokhoven et al., 1990, 1992). Baculovirus transfer vector pACHB3CDA contains the poliovirus 3CD coding region (van Bokhoven et al., 1991). All DNA manipulations were carried out by standard recombinant DNA techniques (Sambrook et al., 1989).

Plasmid pMGUS was constructed by ligating the XbaI–SstI fragment from pBI121 containing the GUS gene into the corresponding sites of pMON999.

To create pMB87, plasmid pTBHM87 was digested with Ndel (position 3672; Lomonossoff & Shanks, 1983), filled in with Klenow polymerase and digested with CiaI just downstream of the poly(A) sequence at position 5935. The 2·3 kb Ndel–CiaI fragment was ligated into pMON999 cleaved with BglII (filled in) and CiaI, thus restoring the BglII site. Plasmid pMB110 was constructed by digestion of pTBHM110 with Ndel (position 3048; filled in) and XhoI (position 3978) and insertion of the isolated 0.9 kb fragment into pMB87 digested with BglII (filled in) and XhoI.

For construction of pMB170, an Ndel (filled in)–BamHI fragment (positions 1185 to 3857) from pTBHM60 was first subcloned in pUC19 digested with Asp718 (filled in) and BamHI. The 60K coding region was retrieved from this plasmid by digestion with SstI and BamHI and subsequently ligated into the corresponding sites of pMON999 to give pMB60. Expression vector pMB170 was then created in a three-fragement ligation using DNA fragments from pTB1G (Ndel–XhoI; positions 2080 to 3978), pMB60 (SstI–Ndel) and pMB87 (PstI–XhoI).

To create expression vector pMB200, subclone pMBA32 was constructed by insertion of a 0.7 kb Ndel (position 207, filled in)–XhoI (position 899) fragment from pTB200N into pMON999 digested with BglII (filled in) and XhoI. DNA fragments from pTB1G (XhoI–XhoI; positions 899 to 3978), pMBA32 (XhoI–BamHI) and pMB170 (XhoI–BamHI) were used in a three-fragement ligation to give expression vector pMB200.

Plasmid pMP3CD was constructed by insertion of the poliovirus 3CD coding region, contained in a BamHI–KpnI fragment from pACHB3CDA, in the BglII–KpnI sites of pMON999.

For the generation of negative-sense transcripts of CPMV B-RNA a SnaBI site was introduced at positions –4 to +2 of the B-cDNA sequence. For this, the SalI–SstI (positions –40 to 2301) fragment of pTB1G was inserted in M13mp19 and subjected to mutagenesis (Kunkel, 1985) using the oligonucleotide 5’ GATTTTTAATACGTTATAGTGAAGC 3’, resulting in an insertion of a C nucleotide in the complementary sequence (represented by the bold G) and thereby the generation of a SnaBI recognition site (underlined). This clone was digested with SalI and Ndel (position 2080) and the 2·1 kb fragment carrying the mutation as well as the T7 promoter was isolated and exchanged with the homologous sequence of pTB1G to give pTBSnA. The T7 promoter of plasmid Bluescript (SK−) was removed by deletion of the PvuII–SalI fragment. Plasmid pSnaB was then created by insertion of the SalI–CiaI fragment of pTBSnA, containing the T7 promoter and the entire B-cDNA sequence, into this modified BlueScript clone. Upon linearization of pSnaB with SnaBI and in vitro transcription with T3 polymerase, B-RNA transcripts of negative-sense polarity were obtained that carried only one additional C at the 3’ end as compared to negative-sense B-RNA found in CPMV-infected plants.

Transfection of cowpea protoplasts. Cowpea (Vigna unguiculata L.) mesophyll protoplasts were prepared essentially as described previously (Reizenman et al., 1989), except that 10 mM-CaCl2 was included in the enzyme solution. CsCl-purified vector DNA (10 μg in 25 μl ice-cold water) was added to 1 x 106 protoplasts in 100 μl ice-cold 0·6 M-mannitol containing 10 mM-CaCl2, immediately followed by addition of a 0·5 ml solution of 40 % polyethylene glycol (PEG; M, 6000), 0·5 M-mannitol and 0·1 mM-Ca(NO3)2 (Negruțiu et al., 1987). The protoplast suspension was gently mixed for 10 to 15 s, diluted with 4·5 ml 0·6 M-mannitol, 15 mM-MgCl2, and 0·1% MES pH 5·6 and kept at room temperature for 20 min. The protoplasts were then washed with 0·6 M-mannitol and 10 mM-CaCl2 and incubated for 16 to 66 h as described previously (Rottier et al., 1979).

Determination of GUS activity. GUS activity in extracts of protoplasts was determined essentially as described by Jefferson (1987). One unit of GUS activity is defined as the amount of enzyme required to produce 1 pmol of methylumbelliferone in 1 min at 37 °C using 1 mM-4-methylumbelliferyl glucuronide (X-Glu) as a substrate. GUS activity in individual protoplasts was assayed at 42 h post-transfection (p.t.). For this, the protoplast culture medium was replaced...
with a solution of 100 mM-sodium phosphate pH 7.0, 10 mM-EDTA, 0.5 mM-potassium ferricyanide, 0.5 mM-potassium ferrocyanide and 2 mM-X-Gluc substrate. Reactions were carried out at 37 °C until an indigo precipitate was visible (usually after a few hours). After staining, protoplasts were rinsed in 70% ethanol and examined by light microscopy.

Analysis of proteins synthesized in cowpea protoplasts. At 16 to 42 h.p.t. protoplasts were harvested to determine expression of sequences under control of the CaMV 35S promoter. Transfected protoplasts were stained by the indirect fluorescent antibody technique (Hibi et al., 1975; Maule et al., 1980), using one of the following antisera: anti-24K (Wellink et al., 1987), anti-VPG (Eggen et al., 1988), anti-32K (Franssen et al., 1984b), anti-110K (van Bokhoven et al., 1992) or anti-poliovirus 3D (kindly provided by Dr O. C. Richards, University of Utah Medical Center). Upon treatment with goat anti-rabbit antibody conjugated to fluorescein isothiocyanate (FITC; Nordic) the protoplasts were examined by fluorescence microscopy. For immunoblot analysis of the proteins synthesized by the expression vectors, protoplasts were collected by centrifugation at 100 g for 2 min, washed and resuspended in 50 mM-Tris-acetate pH 8.0, 25% glycerol, 50 mM-potassium acetate, 1 mM-EDTA, 5 mM-DTT and 0.5 mM-PMSE at 1 × 10^5 protoplasts per ml. Cells were disrupted by centrifugation at 30000 g for 30 min. Aliquots, corresponding to 2 × 10^5 protoplasts, of the resulting pellet (P30) and supernatant (S30) fraction were electrophoresed in 10% SDS-polyacrylamide gels as described by Laemmli (1970). Immunoblots of these gels were prepared as described previously (van Bokhoven et al., 1990) using the antisera mentioned above.

Detection of RNA polymerase activity. At 60 h.p.t. protoplasts, co-transfected with plasmid DNA and M-RNA transcript derived from pTM1G, were collected by centrifugation and pellets were frozen at -80 °C. Total RNA was extracted from the protoplasts as described by van Bokhoven et al. (1988) and electrophoresed in a 1% denaturing agarose gel (McMaster & Carmichael, 1977). The separated RNAs were transferred to GeneScreen (NEN Research Products) and hybridized with 32P-labelled probe prepared by random-primer labelling (Feinberg & Vogelstein, 1984) of a HindIII fragment (positions 482 to 2231) of pTM1G for detection of M-RNA.

RNA polymerase assays were performed on crude extracts and S30 supernatant fractions, prepared as described above. Determination of RNA polymerase activity in these extracts was as described previously (Flanagan & Baltimore, 1977; van Bokhoven et al., 1991).

**Results**

**Optimal conditions for transfection of cowpea protoplasts**

An expression system based on the CaMV 35S promoter was developed for use in cowpea protoplasts. All expression vectors used in this study are derived from plasmid pMON999 by insertion of sequences of interest in the multiple cloning site in this vector that is located between an enhanced CaMV 35S promoter and a nopaline synthase terminator (Fig. 1). Plasmid pMGUS, containing the bacterial reporter gene coding for GUS (Jefferson, 1987), was used to determine the optimal conditions for transfection of cowpea protoplasts. The protoplasts were transfected with plasmid DNA using electroporation (Hibi et al., 1986), liposomes (Malone et al., 1989) or PEG (Negrutiu et al., 1987). The highest GUS activity, as determined by fluorometry, was found if protoplasts were transfected in a medium with 40% PEG, 0.5 M-mannitol and 0.1 M-Ca(NO₃)₂. Under such conditions, using 10 µg DNA per 1 × 10^6 protoplasts, approximately 70% of the protoplasts survived and

Fig. 1. Schematic diagram of the expression vectors used in this study. (a) Expression vector pMON999. Indicated are the restriction sites and the relevant sequences contained within the plasmid: P-e35S, enhanced CaMV 35S promoter; NOS 3', nopaline synthase terminator; Clal(d), dam-methylated Clal site. (b) Top, genetic organization of CPMV B-RNA. VPg at the 5' end of the RNA is drawn as a black square. The single open reading frame codes for a 200K polyprotein and is represented by an open bar in which final cleavage products (the 32K, 58K, 24K and 87K proteins and VPg) are indicated. The other protein species mentioned in the text are composed of these final cleavage products as follows: 170K, 58K–VPg-24K–87K; 60K, 58K–VPg; 84K, 58K–VPg-24K; 112K, VPg-24K-87K; 110K, 24K-87K. The positions of the restriction sites in the B-cDNA sequence used for the construction of the expression vectors are indicated. The open circles represent the engineered Ndel sites. Bottom, expression vectors containing CPMV B-cDNA sequences. The sequences represented by solid lines were cloned in the polylinker of pMON999. (c) Genetic organization of poliovirus RNA and relevant position of the cDNA sequence inserted in pMON999 to create expression vector pMP3CD. For details of vector constructions see Methods.
protoplasts vectors resided in the supernatant fraction (data not shown). The specific proteins that could be detected on polyacrylamide gels stained with Coomassie brilliant blue. The pMB110 and pMB87 contained amounts of CPMV-specific proteins that could be detected on polyacrylamide gels stained with Coomassie brilliant blue. The expression from pMB170 was less but still sufficient for detection of proteolytic processing products of the 170K protein on immunoblots (Fig. 2).

The 87K protein produced by pMB87 showed an electrophoretic mobility and immunoreactivity with anti-110K serum identical to those of the authentic 87K protein found in CPMV-infected protoplasts (Fig. 2, lanes 5 and 7). Expression of the 110K coding sequence by pMB110 resulted in the synthesis of proteins co-migrating with the authentic 110K protein from CPMV-infected protoplasts. Proteolytic cleavage of the 110K protein occurred very inefficiently because hardly any 87K protein was visible on immunoblots of proteins extracted from protoplasts at 16 h.p.t. At later times (> 42 h.p.t.) only about 5% of the 110K protein encoded by pMB110 was processed into proteins of 24K and 87K (data not shown). These results are in agreement with in vitro translation studies that have revealed that efficient cleavage at the 24K and 87K junction requires sequences upstream of the 24K protease (Dessens & Lomonossoff, 1992).

The processing patterns of the 200K polyproteins synthesized in protoplasts transfected with either pMB200 or CPMV B-RNA were very similar (Fig. 2, lanes 2 and 7; see Fig. 1 for the composition of the different protein species). In both cases proteolytic processing of the 200K polyprotein into proteins 170K and 32K appeared very efficient as the 170K protein was the only CPMV-specific protein that could be detected on Coomassie brilliant blue-stained polyacrylamide gels (data not shown). Further processing of the 170K protein into 112K, 110K, 87K, 84K and 60K proteins occurred to only a limited extent (Fig. 2, lanes 2 and 7, and data not shown). The 60K protein seems to be under-represented in pMB200-transfected protoplasts. We have no explanation for this discrepancy. In protoplasts transfected with pMB170 the B-RNA-specific proteins that could be identified were the 112K, 110K and 87K C-terminal cleavage products (Fig. 2, lane 3, and data not shown), indicating that the 170K protein was processed very efficiently. Remarkably, the 60K and 84K proteins were not detected in protoplasts transfected with pMB170.

Expression vector pMP3CD was created for the synthesis of poliovirus protein 3D in cowpea protoplasts. On an immunoblot of protoplasts transfected with pMP3CD a protein of approximately 53K was visible that was immunoreactive with anti-3D serum and co-migrated with poliovirus protein 3D found in infected HeLa cells (data not shown). This protein band was not present in extracts of protoplasts transfected with pMGUS (data not shown), indicating that indeed poliovirus protein 3D had been produced by pMP3CD. The presence of 3D protein in protoplasts strongly expressed the GUS gene as seen with X-Gluc staining. In a typical experiment the GUS activity was 1500 units/100000 protoplasts at 16 h.p.t. and up to 2000 units/100000 protoplasts at 42 and 66 h.p.t.

The transfection conditions for introducing DNA into protoplasts were also very efficient for inoculation of cowpea protoplasts with viral RNA. As little as 0.1 μg CPMV RNA sufficed to infect 80% of 1 x 10⁶ protoplasts (data not shown). This protein band was not visible that was immunoreactive with anti-3D serum and co-migrated with poliovirus protein 3D found in infected HeLa cells (data not shown). The 60K protein seems to be under-represented in pMB200-transfected protoplasts. On an immunoblot of protoplasts transfected with either pMB200 or CPMV B-RNA were very similar (Fig. 2, lanes 2 and 7; see Fig. 1 for the composition of the different protein species). In both cases proteolytic processing of the 200K polyprotein into proteins 170K and 32K appeared very efficient as the 170K protein was the only CPMV-specific protein that could be detected on Coomassie brilliant blue-stained polyacrylamide gels (data not shown). Further processing of the 170K protein into 112K, 110K, 87K, 84K and 60K proteins occurred to only a limited extent (Fig. 2, lanes 2 and 7, and data not shown). The 60K protein seems to be under-represented in pMB200-transfected protoplasts. We have no explanation for this discrepancy. In protoplasts transfected with pMB170 the B-RNA-specific proteins that could be identified were the 112K, 110K and 87K C-terminal cleavage products (Fig. 2, lane 3, and data not shown), indicating that the 170K protein was processed very efficiently. Remarkably, the 60K and 84K proteins were not detected in protoplasts transfected with pMB170.

Expression of CPMV and picornaviral sequences in protoplasts

The multiple cloning site of pMON999 was used to insert the coding sequences for the 87K, 110K, 170K and the complete 200K polyprotein of CPMV B-RNA, resulting in expression vectors pMB87, pMB110, pMB170 and pMB200 respectively (Fig. 1). Cowpea protoplasts were transfected with these plasmids or inoculated with CPMV RNA under the conditions mentioned above. At 16 h.p.t. the protoplasts were harvested, fractionated by centrifugation and proteins from the supernatant (S30) fraction were fractionated in 10% polyacrylamide gels. Detection of CPMV-specific proteins was with anti-110K and anti-VPg sera. * Cowpea proteins that cross-react with these antisera. Lane 1, mock-inoculated protoplasts; lanes 2 to 6, protoplasts transfected with expression vectors: 2, pMB200; 3, pMB170; 4, pMB110; 5, pMB87; 6, pMGUS; lane 7, protoplasts inoculated with CPMV RNA.

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Protoplasts transfected with plasmids pMB200, pMB110 and pMB87 contained amounts of CPMV-specific proteins that could be detected on polyacrylamide gels stained with Coomassie brilliant blue. The presence of 3D protein in protoplasts strongly
Table 1. Immunofluorescent staining of cowpea protoplasts

<table>
<thead>
<tr>
<th>Protoplasts*</th>
<th>Anti-110K</th>
<th>Anti-CPMV</th>
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<tbody>
<tr>
<td>B-RNA</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>pMB200</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>pMB170</td>
<td>20</td>
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</tr>
<tr>
<td>pMB110</td>
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<tr>
<td>pMB87</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>pMGUS</td>
<td>0</td>
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* Cowpea protoplasts were transfected with B-RNA transcripts or expression vector DNA in the presence of M-RNA transcripts.
† Immunofluorescent staining was with the indicated antisera. Numbers in the table represent the percentage of fluorescing protoplasts as obtained in a typical experiment.

![Fig. 3. Immunofluorescent staining of cowpea protoplasts inoculated with CPMV RNA (a) or transfected with pMB200 (b, c) or pMB170 (d). Detection of CPMV-specific proteins was with anti-VPg (a, b) or anti-110K serum (c, d) followed by treatment with goat anti-rabbit IgG conjugated with FITC. Note that the immunofluorescent staining in protoplasts transfected with CPMV RNA or pMB200 is concentrated in a few restricted areas.](image)

suggests that proteolytic processing of precursor protein 3CD had occurred.

Localization of CPMV proteins by immunofluorescence

By immunofluorescence staining with anti-110K serum and goat anti-rabbit IgG conjugated with FITC, it was possible to quantify the number of protoplasts in which considerable amounts of CPMV-specific protein were produced (Table 1). For each of the expression vectors and RNAs used for transfection, the percentage of protoplasts showing fluorescence was in good agreement with the amount of protein detected on immunoblots (Table 1).

Immunofluorescent staining of transfected protoplasts was also used to locate B-RNA-specific proteins within the protoplasts. A remarkable difference in the localization of the fluorescent label was observed between, on the one hand, protoplasts transfected with CPMV RNA or pMB200 and, on the other hand, protoplasts transfected with pMB87, pMB110 or pMB170. Typical examples of these localization studies are shown in Fig. 3. In protoplasts transfected with CPMV RNA (Fig. 3a) or pMB200 (Fig. 3b) the fluorescent signal after treatment with anti-VPg serum was concentrated in one or a few restricted areas in the cytoplasm of the protoplast. Use of anti-32K, anti-24K and anti-110K antisera resulted in a similar localized immunofluorescence, except that the anti-110K serum always gave additional fluorescence in other parts of the protoplast (Fig. 3c). These results indicate that the majority of the different viral proteins had accumulated at restricted areas in these protoplasts. In contrast, such accumulation of CPMV-specific proteins was not observed in protoplasts transfected with pMB87, pMB110 or pMB170 (Fig. 3, and data not shown). In these cases the fluorescence after treatment with anti-110K serum was dispersed over the entire cell (Fig. 3d).

Expression of cDNA from the 200K coding region supports replication of co-inoculated M-RNA

Having established that the proteins synthesized by the expression vectors are similar to the authentic viral proteins in immunogenic properties, electrophoretic mobility and, at least in one case, activity (24K protease), it was of interest whether the B-RNA-specific proteins produced by the expression vectors were capable of replicating viral RNA. For that purpose protoplasts were transfected with the different expression vectors together with M-RNA. As a control, protoplasts were transfected with B- and M-RNA transcripts.

Immunofluorescent staining (Table 1) with anti-CPMV serum at 16 h.p.t. detected the presence of the capsid proteins encoded by M-RNA in 20% of the protoplasts transfected with B- and M-RNA transcripts, indicative of the occurrence of replication of M-RNA in these protoplasts. Replication of M-RNA was also observed in approximately 10% of the protoplasts transfected with pMB200 together with M-RNA, as determined by immunofluorescent staining. No fluorescent staining with anti-CPMV serum was observed in protoplasts transfected with pMB170, pMB110 or pMB87 in the presence of M-RNA, whereas immunofluorescent staining with anti-110K serum verified that
Fig. 4. Determination of M-RNA replication in cowpea protoplasts. Total RNA extracted from protoplasts at 16 h p.t. was separated in a 1% denaturing agarose gel followed by transfer of the RNAs onto GeneScreen. The blot was hybridized with a probe specific for M-RNA and exposure was for 16 h (with a screen) to a Kodak XAR film. The position of M-RNA is indicated by an arrowhead. Lane 1 contains RNA from protoplasts inoculated with M-RNA only (mock). Lanes 2 to 6 contain RNA from protoplasts transfected with expression vectors as in Fig. 2 together with M-RNA. Lane 7, RNA from protoplasts inoculated with B- and M-RNA.

Table 2. Poly(A)-oligo(U) polymerase activity in cell extracts

<table>
<thead>
<tr>
<th>Extract*</th>
<th>Poly(A)-oligo(U)</th>
<th>No template/primer</th>
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<tr>
<td>AcHB3CD</td>
<td>126000†</td>
<td>160</td>
</tr>
<tr>
<td>HeLa</td>
<td>7200</td>
<td>150</td>
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<tr>
<td>pMP3CD</td>
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<td>pMB87</td>
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<td>130</td>
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<tr>
<td>pMGUS</td>
<td>150</td>
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* Extracts of protoplasts were prepared as described in the text. AcHB3CD is a crude extract of insect cells containing poliovirus 3D pol and HeLa is a partially purified preparation of 3D pol from poliovirus-infected HeLa cells.

† Polymerase activity is expressed as the amount of [3H]UTP label incorporated (c.p.m.) after 30 min incubation.

expression of the B-RNA coding sequences had indeed occurred in these protoplasts. Thus it appeared that the proteins expressed from pMB200, but not those from pMB170, pMB110 and pMB87, are able to support replication of M-RNA. This was confirmed by analysis of the RNA content of the protoplasts. At 42 h p.t. total RNA was extracted from protoplasts and equal amounts of RNA were analysed on a Northern blot hybridized with an M-RNA-specific probe. Fig. 4 shows that RNA from protoplasts transfected with pMB200 gave a hybridization signal on the blot at the same migrational position as M-RNA produced in protoplasts inoculated with B- and M-RNA. In none of the other lanes was a hybridization signal detected even after prolonged autoradiography.

Polymerase assays

Cowpea protoplasts isolated 16 h after transfection with the different expression vectors were used to prepare fractions to test for RNA-dependent RNA polymerase activity using poly(A) as a template and oligo(U) as a primer (Table 2). Partially purified poliovirus 3D polymerase obtained from poliovirus-infected HeLa cells and a crude extract of insect cells in which 3D pol was produced by a baculovirus expression vector (van Bokhoven et al., 1991) were used as controls for the assays and exhibited template- and primer-dependent polymerase activity. An S30 fraction of pMP3CD-transfected protoplasts contained a similar RNA-synthesizing activity dependent on addition of both template and primer, indicating that biochemically active poliovirus polymerase (3D pol) was synthesized in cowpea cells. In striking contrast, no polymerase activity was detected in any of the extracts (S30, P30 or crude extracts) containing CPMV proteins (Table 2). The assays were repeated with other template/primer combinations to investigate whether the absence of polymerase activity might be due to specificity of CPMV polymerase for its homologous RNA. But none of the other substrates used, i.e. CPMV RNA with or without oligo(U) and negative-sense B transcripts with or without plus-sense B primer, resulted in detectable CPMV polymerase activity, whereas poliovirus polymerase was active with these templates (data not shown). No stimulatory effect on CPMV polymerase activity was obtained by varying the assay conditions, including conditions which have been used to demonstrate RNA-elongating activity in crude membrane fractions of CPMV-infected cowpea plants (Dorssers et al., 1984).

Discussion

We have shown that expression vector pMON999 lends itself very well to the synthesis of CPMV-specific proteins in cowpea protoplasts. The expression levels obtained with this system were remarkably high, especially for the 87K, 110K and 200K coding regions of CPMV B-RNA, even though the amount of these proteins was lower than obtained for these proteins with the baculovirus expression system (van Bokhoven et al., 1990, 1992). Expression in cowpea protoplasts has an advantage over the baculovirus expression system in that it provides a tool for examining the characteristics and behaviour of individual CPMV proteins and mutants thereof in plant cells. Hitherto, such experiments have
not been possible since the production of viral proteins in cowpea cells was dependent on the occurrence of viral RNA replication. Furthermore, the system proves useful for the production of proteins of animal virus origin, as is illustrated by the synthesis of functional 3Dpol of poliovirus.

The proteins produced by pMB200 were active in supporting replication of M-RNA transcripts in cowpea protoplasts. No replication of M-RNA was observed in protoplasts transfected with either pMB170, pMB110 or pMB87, suggesting that expression of the entire 200K coding region is required for replication. Since the 32K protein is the only additional viral protein produced by pMB200 in comparison to pMB170, the 32K protein seems to serve a crucial role in achieving viral RNA replication. Recently, it was demonstrated that the 32K protein has a regulatory role in the proteolytic processing of the 170K protein and it was proposed that this regulation may be important in building an active replication complex (Peters et al., 1992a). The present results show that in cowpea protoplasts the rate of proteolytic processing is also strongly affected by the presence of the 32K protein. In extracts of protoplasts transfected with pMB200 or with CPMV RNA, the 170K protein was the most abundant viral protein and processing of this primary cleavage product occurred to a limited extent only. In contrast, no 170K protein could be detected in pMB170-transfected protoplasts in which the 32K protein was not produced. Rather strikingly, only the 112K, 110K and 87K cleavage products from the C-terminal part of the 170K protein, and not the 60K and 84K proteins from the N-terminal region, were found in cells transfected with pMB170 (see Fig. 1 for the composition of these proteins). It could be argued that VPg was removed from the 60K and 84K proteins, preventing their detection with anti-VPg serum. However, in in vitro proteolytic processing studies the 60K and 84K proteins are clearly detectable (Peters et al., 1992b). Therefore it seems likely that the 60K and 84K proteins are not stable in protoplasts transfected with pMB170. This may point to a role of the 32K protein in keeping the proteins with a function in viral RNA replication together and/or in guiding them in forming an active replication complex. As long as the instability of the 60K and 84K proteins in pMB170-transfected cells is not understood we should be cautious in concluding that pMB170 will not be capable of replicating co-inoculated M-RNA.

Upon infection of cowpea cells with CPMV, characteristic cytopathic structures appear in the cytoplasm, consisting of numerous membranous vesicles and amorphous electron-dense material (Assink et al., 1973; de Zoeten et al., 1974). It has been shown previously that in protoplasts inoculated with CPMV RNA most of the B-RNA-encoded proteins accumulate in these electron-dense structures (Wellink et al., 1988). Immunofluorescent staining of protoplasts transfected with pMB200 or inoculated with CPMV RNA revealed that the non-structural proteins encoded by B-RNA were aggregated in one or a few areas in the cytoplasm of the protoplast. In protoplasts transfected with either pMB87, pMB110 or pMB170 viral proteins were found dispersed over the entire cell. Thus it seems that the 32K protein, which is the only unique domain in the 200K polypeptide in comparison with the other B proteins that have been synthesized, is somehow involved in keeping together B-RNA-encoded proteins in defined areas of the cell. In this context it is remarkable that the 32K protein is known to interact with the 170K, 84K, 60K and 58K proteins encoded by B-RNA (Franssen et al., 1984b; Peters et al., 1992a). The immunofluorescent localization studies are in agreement with the results on the expression of B-RNA-encoded proteins in insect cells by means of the baculovirus expression system (van Bokhoven et al., 1992). Insect cells that produce the 200K polypeptide of B-RNA developed electron-dense structures, whereas the independent synthesis of the 170K, 110K, 87K and 60K proteins did not result in the induction of electron-dense structures in such cells (van Bokhoven et al., 1992). Therefore, the localized areas of fluorescent staining probably represent the occurrence of electron-dense structures. The biological significance of the electron-dense structures in CPMV-infected cells remains obscure. They may be the storage sites where B-RNA-encoded proteins are maintained in an active conformation for RNA replication or other processes of the viral life cycle or, alternatively, may be waste-baskets for abundant viral proteins.

Although pMB200-transfected protoplasts supported replication of co-inoculated M-RNA, it was not possible to demonstrate in vitro RNA-dependent RNA polymerase activity in extracts of these protoplasts, nor in extracts of protoplasts transfected with pMB170, pMB110 or pMB87. In contrast, poliovirus polymerase 3Dpol produced in cowpea protoplasts by pMP3CD exhibited RNA polymerase activity in vitro, providing evidence that the polymerase assay functions in extracts of protoplasts. Why then is the CPMV polymerase not able to function under these conditions? Although we cannot exclude the possibility that an essential host factor could have been dissociated from the CPMV proteins prior to the in vitro polymerase assays we consider it more likely that the CPMV polymerase cannot use oligo(U) as a primer nor function on any exogenous template and/or primer. Recently, we have obtained evidence that replication of both B- and M-RNA is tightly linked to translation of the RNAs (H. van Bokhoven, O. Le Gall, D. Kasteel, J. Verver, J. Wellink
& A. van Kammen, unpublished). Therefore, it may be anticipated that in vitro CPMV replicase activity using exogenous template RNA will only be measurable under conditions that also facilitate translation of the added template RNA molecules. Poliovirus 3Dpol, on the other hand, is capable of in vitro RNA synthesis in a template- and primer-dependent fashion (Flanagan & Baltimore, 1977). Perhaps the ability of the poliovirus polymerase to use poly(A)-oligo(U) (or any other template/primer combination) may be a fortuitous property not shared by the CPMV polymerase, which may be more fastidious in accepting any other than the natural primer. Indeed, the oligo(U)-primed polymerase activity of 3Dpol is not at all specific for the poliovirus RNA template and furthermore does not reflect the in vivo initiation of replication events in which VPg is likely to be involved (Kuhn & Wimmer, 1987). Thus far, true replication in vitro of poliovirus RNA, i.e. synthesis on a genomic RNA template of a negative-sense RNA which is subsequently used as a template for the synthesis of the complementary plus strand, has been accomplished only in extracts of uninfected HeLa cells that support translation of the exogenous RNA molecules (Molla et al., 1991).

Notwithstanding the difficulties of studying CPMV RNA replication in vitro, the expression system described in this paper offers new opportunities for the examination of the viral RNA replication process in vivo. One interesting question to be answered is whether mRNAs generated by expression vector pMB200 can be used as templates for the synthesis of complementary negative-strand RNA. Although the mRNAs produced by pMB200 have additional nucleotides at their 3' termini compared to viral B-RNA, this should not interfere with minus strand RNA synthesis because addition of about 400 non-viral nucleotides at the 3' end of B-RNA did not have any effect on virus infectivity (Eggen et al., 1989). The pMB200 mRNAs further differ from B-RNA in that they lack the viral 5' non-coding region. It will be of interest to create expression vectors that contain the 5' end of the B-cDNA and to examine whether the mRNAs that will be generated are replicated by the proteins they encode. Another question now amenable to further investigations is whether a functional replication complex can only be obtained with viral proteins obtained by proteolytic processing of a 200K polypeptide or whether an active replication complex can also be constructed from 32K and 170K proteins (or other combinations) produced by separate expression in one cell.

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