Evidence for dissimilar properties of comoviral and picornaviral RNA polymerases

Hans van Bokhoven,1* Mick Mulders,1,2 Joan Wellink,1 Just M. Vlak,2 Rob Goldbach2 and Ab van Kammen1

1Department of Molecular Biology, Agricultural University, Dreyenlaan 3, 6703 HA Wageningen and 2Department of Virology, Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands

The poliovirus RNA polymerase has been synthesized in Spodoptera frugiperda cells by using the baculovirus expression system. Crude sonicates of these cells exhibited an RNA-elongating activity of a synthetic oligo(U) primer with poly(A) or cowpea mosaic virus (CPMV) RNA as a template. A similar polymerase activity was found in extracts of insect cells in which foot-and-mouth disease virus (FMDV) proteins, including the putative polymerase, were produced. The analogous CPMV 87K protein and several of its precursors, synthesized in S. frugiperda cells, did not show any detectable polymerase activity in the same assay under a variety of conditions. The results indicate that, in contrast to the picornaviral polymerases, the CPMV polymerase is unable to function in an oligo(U)-primed polymerase assay.

Introduction

The organization and expression of the genome of cowpea mosaic virus (CPMV), type member of the plant comoviruses, is strikingly similar to that of the animal picornaviruses (Franssen et al., 1984; Argos et al., 1984; Goldbach, 1987). The major difference is that the genome of CPMV consists of two separately encapsidated RNA molecules and that of picornaviruses only one (Fig. 1). The single-stranded positive-sense RNA molecules of both the comovirus group and the picornaviruses contain a small protein, VPg, covalently linked to their 5' ends and a poly(A) tail at their 3' termini. Translation of the genomic RNAs results in the synthesis of large polypeptides, which are processed into functional proteins by virus-encoded proteases. Significant sequence identity exists between the 60K, 24K and 87K non-structural proteins encoded on CPMV B-RNA and the picornavirus non-structural proteins 2C, 3C and 3D, respectively, which are also similarly arranged on the genome (Fig. 1; Franssen et al., 1984). Protein 3D has been assigned as the primer- and template-dependent RNA polymerase in poliovirus RNA replication (Lundquist et al., 1974; Flanegan & Baltimore, 1977; Van Dyke & Flanegan, 1980). In contrast, neither the 87K nor the 110K proteins of CPMV exhibited such an activity when synthesized in E. coli and assayed under the same conditions (Richards et al., 1989). Previously we have described the expression of CPMV B-RNA coding sequences in insect cells using the baculovirus vector expression system (van Bokhoven et al., 1990). Here we report assays for RNA polymerase activity of the CPMV proteins as well as the poliovirus and FMDV polymerase, which were also produced in these cells.

Methods

Vaccines, plasmids and cells. Autographa californica nuclear polyhedrosis virus (AcNPV, strain E2; Smith & Summers, 1978) and recombinant AcNPVs 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. Baculovirus transfer vectors and other plasmids were propagated in E. coli strain DH5α. Expression vector pEXC-3D contains the poliovirus 3CD coding sequence under the control of the TrpE promoter and produced an active 3D polymerase in E. coli (Richards et al., 1987). Baculovirus transfer vector pAcRP23 has been constructed by cloning the EcoRI I fragment of AcNPV containing the polyhedrin promoter in pUC8 (Possee & Howard, 1987). The construction and expression of recombinant baculoviruses containing CPMV sequences (AcHB60, AcHB87, AcHB110 and AcHB170) or FMDV sequences (AcJR4/MRI4) have been described by van Bokhoven et al. (1990) and Roosien et al. (1990), respectively.

Construction of transfer vectors pAcHB3CD and pAcHB3CDA. Plasmid pEXC-3D was partially digested with HindIII and with Saff to completion and the poliovirus fragment encoding 3CD (about 2 kb) was isolated from an agarose gel. This fragment was inserted into the multiple cloning site of plasmid Bluescript KS+ (Stratagene), to isolate the 3CD coding sequence as a transfer vector pAcRP23 (Possee & Howard, 1987) was also digested with BamHI and KpnI and ligated with the 3CD fragment to create pAcHB3CD. The number of nucleotides between the polyhedrin promoter and the start codon provided by the 3CD sequence was reduced by deleting a Smal–EcoRV fragment originating from the Bluescript plasmid, resulting in pAcHB3CDA. Sequence analysis confirmed that the transfer vectors pAcHB3CD and pAcHB3CDA contain 38 and 20 additional nucleotides, respectively, upstream of the ATG start codon compared to the polyhedrin gene.

Selection of recombinant baculoviruses. Monolayers of S. frugiperda cells (1 × 10⁶ cells per 35 mm Petri dish) were cotransfected with wild-type (wt) AcNPV DNA and transfer vector DNA using the calcium phosphate precipitation procedure of Summers & Smith (1987). After 5 days incubation at 27 °C the supernatant was collected and used for infection of S. frugiperda cells under agarose. Putative recombinants recognized by their polyhedron-negative phenotype were picked and plaque-purified three times to reach genetic homogeneity.

Analysis of proteins synthesized in S. frugiperda cells. S. frugiperda cells (1 × 10⁶ cells per 35 mm Petri dish) were infected with AcNPV recombinants at an m.o.i. of 10 and incubated for 3 days at 27 °C. The cells were pelleted by low-speed centrifugation (300 g for 2 min), washed twice in cold phosphate-buffered saline (PBS; 10 mM-sodium phosphate pH 7.5, 100 mM-NaCl) and lysed by boiling for 3 min 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). Aliquots corresponding to 5 × 10⁴ cells were electrophoresed on 10% SDS–polyacrylamide gels (Laemmli, 1970), which were either stained with Coomassie brilliant blue or analysed by immunoblotting using antiseraum against a TrpE/poliovirus polymerase fusion protein (gift of Dr O. C. Richards) and with antirabbit IgG–alkaline phosphatase conjugate (Promega). Equal amounts of S. frugiperda cells infected with wt AcNPV and mock-infected cells served as controls.

Polymerase assays. S. frugiperda cells were infected with the AcNPV recombinants at an m.o.i. of 10. At 3 days post-infection the cells were harvested and sedimented by low-speed centrifugation (300 g for 2 min). The cells were washed twice in cold PBS and finally resuspended in TGKEDP buffer (50 mM-Tris–acetate pH 8.0, 25% v/v glycerol, 50 mM-potassium acetate, 1 mM-EDTA, 5 mM-DTT and 0.5 mM-PMSF) at 4 × 10⁶ cells per ml. Cell suspensions on ice were sonicated with a Vibra Cell sonicator in two cycles of 10 pulses separated by a 2 min interval for cooling.

Poly(A)-oligo(U) polymerase assays were performed essentially as described by Flanagan & Baltimore (1977). Reaction mixtures contained 50 mM-HEPES pH 8.0, 0.5 mM-ATP, -GTP and -CTP, 10 μM-UTP, 3 mM-magnesium acetate, 60 μM-ZnCl₂, 3 μg/ml actinomycin D, 1 μg poly(A) or 2.5 μg of CPMV RNA, 0.34 μg oligo(U) and 2.5 μCi [³H]UTP (Amersham) and 5 to 10 μl of crude sonicat in a total volume of 50 μl. Samples (20 μl) taken at time zero and after 30 min incubation at 30 °C were precipitated in 5% Na₂PO₄ on Whatman DE81 filter discs, and counted in Insta-Fluor (Packard) scintillation fluid. Each extract was also assayed in the absence of primer and/or template.

For an analysis of the products of the polymerase assays the reactions were performed with 0.5 μCi of [³²P]UTP (Amersham) instead of [³H]UTP. A sample of assay mixture (5 μl) was spotted onto a filter and used for scintillation counting. The remaining 45 μl of the reaction mixture was, after a phenol extraction and ethanol precipitation, used for electrophoresis on a denaturing 1% agarose gel (McMaster & Carmichael, 1977).

Results and Discussion

Expression of picornavirus and CPMV sequences in insect cells

The construction and expression of four recombinant AcNPVs containing CPMV B-RNA coding sequences under the control of the polyhedrin promoter has been described (van Bokhoven et al., 1990). These recombinants, AcHB60, AcHB87, AcHB110 and AcHB170, produce the proteins they denote (60 K etc.) and also the proteolytic cleavage products derived from them (Fig. 1; van Bokhoven et al., 1990). In AcHB110-infected cells about 10% of the 110K protein was processed into the 87K and 24K proteins and approximately 80% of the 170K protein produced by AcHB170 was processed into the 110K, 87K, 84K, 60K and 24K polypeptides. Recombinant AcJR4/MRI4 has also been described and produces, amongst others, protein 3D of FMDV (Fig. 1; Roosien et al., 1990).

For the synthesis of the poliovirus polymerase in insect cells two transfer vectors, pAcHB3CD and pAcHB3CDA, were constructed which contain the poliovirus 3CD coding region under the control of the polyhedrin promoter separated by 38 and 20 additional nucleotides, respectively, directly upstream of the ATG start codon as compared to the polyhedrin gene. Cotransfection of S. frugiperda cells with these two transfer vectors together with wt AcNPV DNA generated the recombinant baculoviruses AcHB3CD and AcHB3CDA. These recombinants were plaque-purified and used at an m.o.i. of 10 to infect S. frugiperda cells. After incubation for 3 days at 27 °C proteins from these cells were analysed by electrophoresis on 10% SDS–polyacrylamide gels. Polioviral proteins could not be detected by staining with Coomassie brilliant blue (results not shown), but on an immunoblot treated with antiseraum raised against a TrpE/poliovirus polymerase fusion protein, two bands were clearly detectable at positions corresponding to the bands of poliovirus proteins 3CD and 3D found in infected HeLa cells (Fig.
Comoviral and picornaviral RNA polymerases

Fig. 1. Diagram showing the genetic organization of the monopartite picornavirus RNAs of FMDV and poliovirus and the bipartite comoviral RNA of CPMV. Each RNA molecule contains a single open reading frame represented by a bar on which the positions of the final proteolytic cleavage products are indicated. Regions of amino acid sequence similarity are shaded similarly. Also indicated are: Tra, putative transport protein(s); CP, coat proteins; MEM, membrane protein; pro, protease; pol, core RNA-dependent RNA polymerase. The dashed lines in protein 3B of FMDV represent three tandemly arranged VPgs. The coding regions that were cloned downstream of the polyhedrin promoter of a baculovirus transfer vector are indicated by single solid lines drawn underneath the genomic RNA.

2, lanes 3 to 5). These bands were not present in uninfected or AcNPV-infected S. frugiiperda cells (Fig. 2, lanes 1 and 2). The amounts of 3CD and 3D proteins produced in the AcHB3CD- and AcHB3CDA-infected cells were similar. Furthermore, the immunoblot demonstrates that partial processing of the 3CD protein has occurred resulting in the release of approximately 75% of the 3D protein from its precursor 3CD.

The amount of 3CD and 3D protein produced in AcHB3CD- and AcHB3CDA-infected S. frugiiperda cells is lower than is usually found with the baculovirus expression system. In comparison with the polyhedrin gene, AcHB3CD has 38 and AcHB3CDA has 20 additional nucleotides upstream of the ATG start codon and it is possible that these extra nucleotides prevent the high expression level that is possible with this system. On the other hand, it is clear that the amount of protein produced in the baculovirus expression system is also influenced by the nature of the protein or the efficiency with which the corresponding messenger RNAs are translated. For example, the CPMV 87K and 110K proteins synthesized by AcHB87 and AcHB110 in S. frugiiperda cells amounted to 20% of the total cellular...
Table 1. Oligo(U)-dependent polymerase activity of crude sonicates of wt and recombinant AcNPV-infected S. frugiperda cells

<table>
<thead>
<tr>
<th>Extract</th>
<th>Poly(A) template</th>
<th>CPMV RNA template</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcNPV</td>
<td>70*</td>
<td>70</td>
</tr>
<tr>
<td>AcHB3CD</td>
<td>109000</td>
<td>31000</td>
</tr>
<tr>
<td>AcJR4/MR41</td>
<td>1100</td>
<td>ND†</td>
</tr>
<tr>
<td>AcHB80</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>AcHB87</td>
<td>60</td>
<td>40</td>
</tr>
<tr>
<td>AcHB110</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>AcHB170</td>
<td>70</td>
<td>50</td>
</tr>
</tbody>
</table>

* The amount of [3H]UTP label incorporated in the absence of primer and/or template and at time zero varied between 10 and 100 c.p.m.
† ND, Not determined.

protein content, whereas the CPMV 60K and 170K coding sequences in constructs with the same 5' non-coding region produced much lower amounts of protein (van Bokhoven et al., 1990), which were comparable to the amounts of 3CD and 3D in AcHB3CD(A)-infected cells. It has previously been shown that the FMDV-specific proteins 3CD, 3C and 3D synthesized in S. frugiperda cells infected with AcJR4/MR41 together with AL-1AB, 1AB, 1CD, 1C, 1D and P1 are produced in amounts that are detectable only by immunoprecipitation (Roosien et al., 1990). Therefore it was suggested that the low level of production may be due to the toxicity of the 3C protease to the baculovirus system. If so, the low amounts of poliovirus proteins might likewise be explained by a toxic effect of the poliovirus 3C protease on the insect cells.

Polymerase activity in crude sonicates of infected S. frugiperda cells

S. frugiperda cells were infected at an m.o.i. of 10 with each of the AcNPV recombinants described and, after 3 days incubation at 27 °C, crude extracts of these cells were tested for polymerase activity using poly(A) as a template and oligo(U) as a primer (Table 1). A substantial RNA synthesizing activity was detected in cells infected with AcHB3CD which was not present in the absence of primer and/or template (results not shown), indicating that poliovirus protein 3D synthesized in S. frugiperda cells exhibits a primer- and template-dependent RNA polymerase activity. Polymerase activity was also detected in cells producing FMDV-specific proteins, but this activity was much lower than the activity present in AcHB3CD-infected cells (Table 1). This is not surprising as the FMDV-specific proteins are produced in much smaller amounts than the poliovirus proteins (Roosien et al., 1990). This result shows that one of the FMDV proteins synthesized in insect cells, most likely 3D (Lowe & Brown, 1981), exhibits an oligo(U)-primed RNA-synthesizing activity similar to that of 3D polymerase of poliovirus. We were unable to retrieve recombinants expressing only the FMDV 3CD sequence, which might be due to the toxic effect of protease 3C for the host cell (Falk et al., 1990).

In striking contrast to the extracts with picornavirus polymerase proteins, the extracts containing CPMV proteins did not show [3H]UTP-incorporating activity above background levels in these assays either with poly(A) or with CPMV RNA as a template (Table 1). Remarkably, when crude extracts containing the poliovirus proteins were assayed with 2.5 μg of CPMV RNA as a template, the amount of incorporated label was only three- to fourfold lower than that with 1 μg poly(A) as a template.

The polymerase assays with AcHB3CD extracts were repeated with [32P]UTP to perform a product analysis. A range of products, migrating around 0.2 kb, was found when poly(A) was used as a template (Fig. 3). On the other hand, when CPMV RNA was used as a template the products were almost exclusively present in two distinct bands at the B- and M-RNA positions (Fig. 3). The M-RNA band was reproducibly found to be more intense than the B-RNA band. This can be explained by the longer average length of the poly(A) tail of M-RNA (20 to 400 nucleotides) as compared to B-RNA (10 to 170 nucleotides; Ahlquist & Kaesberg, 1979), but it may also
be caused by a higher affinity of poliovirus 3D for the M-RNA template. It is surprising that CPMV RNA seems to be such an efficient template and that even full-length copies were synthesized with crude insect cell extracts as the source of the polymerase.

The polymerase assay with CPMV RNA as a template was repeated in the absence of unlabelled nucleotides, except that CTP (0.5 mM) was maintained in the reaction mixture as it was found that the presence of at least one of the nucleotides gave a more than 10-fold stimulation of the RNA-synthesizing activity of poliovirus 3D polymerase in a poly(A)-oligo(U) polymerase assay (results not shown). A high incorporation of label was found, due to the absence of competing unlabelled nucleotides, but only short products were formed (Fig. 3), indicating that oligo(U) was elongated to a limited extent. This shows that the B- and M-RNA bands were the result of genuine polymerase activity and not of a terminal transferase.

**Differences between the comoviral and picornaviral RNA polymerases**

It is remarkable that the poliovirus 3D polymerase and also the presumed FMDV 3D polymerase display such an easily detectable RNA-elongating activity whereas the analogous CPMV proteins do not. Mixing of extracts from cells infected with AcHB3CD with any of the extracts in which CPMV proteins were present revealed that there was no activity inhibiting RNA elongation in extracts containing CPMV proteins (results not shown). The assays for CPMV polymerase activity were performed under a large number of different conditions (pH, temperature, monovalent and divalent ions) including conditions optimal for RNA elongation in crude replication extracts from CPMV-infected cowpea plants (Dorssers et al., 1984). Since no stimulation of incorporation of [3H]UTP was observed, it seems unlikely that the conditions in the assay system are responsible for the lack of polymerase activity of CPMV proteins. The observed proteolytic processing activities of CPMV proteins which contained the protease domain suggest that precursor proteins are folded properly in insect cells. Thus, the absence of RNA polymerase activity is probably not due to incorrect protein folding. It remains possible that some form of post-translational modification, that does not occur in *S. frugiperda* cells, is required for CPMV polymerase activity but not for the proteolytic activity or functioning of picornaviral polymerases.

However, it is known that the 87K and 110K proteins found in CPMV-infected plants are not glycosylated (H. van Bokhoven & J. Wellink, unpublished results).

It is also conceivable that the CPMV polymerase is not able to function in an oligo(U)-primed assay. In the case of poliovirus at least two host factors have been described that can replace oligo(U) as a primer to allow 3D polymerase to initiate RNA synthesis (Dasgupta et al., 1980; Morrow et al., 1985; Andrews & Baltimore, 1986), although the relevance of these proteins in the replication of poliovirus RNA has been questioned (Hey et al., 1987). In studies with crude replication complexes from poliovirus-infected HeLa cells the presence of another activity has been demonstrated that catalyses the formation of VPG–pUpU (Takegami et al., 1983), which may serve as a primer for RNA synthesis (Takeda et al., 1986). Similar activities have not been demonstrated in CPMV-infected cowpea leaves, but it is known that CPMV RNA replication is inhibited by actinomycin D during early stages of infection, suggesting the requirement for a host factor (Rottier et al., 1979; de Varennes et al., 1985). Mixing of insect cell extracts containing CPMV proteins with plant extracts from CPMV-infected or non-infected cowpea plants did not result in any detectable RNA-synthesizing activity (results not shown). However, it is possible that during the formation of a viral replication complex a host factor is needed to obtain functional proteins. Obviously, expression of the CPMV polymerase genes in a homologous system is required to demonstrate such a mechanism. Finally, another viral protein may be required for polymerase activity. It is known that the B-RNA of CPMV encodes all viral functions necessary for viral RNA replication (Goldbach et al., 1980). The only B-RNA-encoded proteins which were not tested in our polymerase assays were the 32K protein and the entire 200K polyprotein. Vos et al. (1988b) have shown that a mutation of the 32K protein renders the B-RNA non-infectious. Recent experiments suggest a function for the 32K protein in the regulation of polyprotein processing (J. Wellink, J. Wery & R. Milek, unpublished results). Assuming that processing and the formation of an active replication complex are tightly linked, the 32K protein may have a crucial role in the establishment of such a complex.

The authors wish to thank Dr. J. Roosien for AcJR4/MR41 and Dr O. C. Richards for TrpE/poliovirus polymerase antiserum and valuable suggestions during the course of this work. Magda Usmany for technical assistance and Piet Madern for artwork. This work was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for Scientific Research (NWO).

**References**


(Rceived 21 September 1990; Accepted 13 November 1990)