Synthesis of foot-and-mouth disease virus capsid proteins in insect cells using baculovirus expression vectors

Jan Roosien,1* Graham J. Belsham,2 Martin D. Ryan,2 Andrew M. Q. King2 and Just M. Vlak1

1Department of Virology, Agricultural University Wageningen, P.O. Box 8045, 6700 EM Wageningen, the Netherlands, and 2AFRC Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking GU24 0NF, U.K.

Foot-and-mouth disease virus (FMDV) cDNA casettes containing sequences encoding the capsid precursor P1-2A with and without those encoding the proteases L and 3C were introduced into Autographa california nuclear polyhedrosis virus (AcMNPV) expression vectors. Procapsid proteins 1AB, 1C and 1D were produced in cells infected with recombinant baculoviruses, when L and 3C were present in the constructs, indicating that these FMDV proteases were active in insect cells. Unlike P1 processing in poliovirus, which has been shown to be catalysed mainly by the 3CD gene product, the 3C protease of FMDV was able to process P1 independently of 3D. Cytotoxicity of the L protease for insect cells prevented the use of the optimized transfer vector, pAcRP23, for inserting L-containing cassettes into AcMNPV. By contrast, viable AcMNPV-FMDV recombinants could be made without restriction on choice of the transfer vector when the L gene was either not expressed or inactivated by an in-frame deletion. In the latter case, normal cleavage at the L–P1 junction no longer occurred in cis, and a new processing event, probably catalysed by 3C, was observed within the C-terminal region of the residual L protein. Analysis of baculovirus-expressed products in sucrose gradients showed that a fraction of the capsid proteins is present in an aggregated form, migrating at 70S and possibly resembling FMDV empty capsid particles.

Introduction

Foot-and-mouth disease virus (FMDV) is an important disease of farm animals. The virus is endemic in large parts of the world and the disease results in considerable economic losses. Control of the disease is mainly achieved using vaccines based on inactivated virus. However, in areas of low disease incidence, e.g. Europe, occasional outbreaks of the disease are often associated with the manufacture and use of such vaccines. Therefore, a synthetic or recombinant vaccine lacking infectious RNA is an attractive alternative.

FMDV (family Picornaviridae, genus aphthovirus; Matthews, 1982) has a genome of positive-stranded RNA, 8500 nucleotides long (Forss et al., 1984) with a protein (VPg) at the 5' end and a 3' poly(A) tail. The RNA molecule is encapsidated in an icosahedral virus particle, consisting of 60 molecules each of proteins 1A (VP4), 1B (VP2), 1C (VP3) and 1D (VP1). FMDV proteins are translated as a polypeptide precursor, which is cleaved by virus-encoded proteases into mature proteins. Unlike that of most picornaviruses, the N-terminal part of the FMDV polypeptide is not the capsid precursor (P1), but a protease (L) which cleaves itself away from P1 (Strebel & Beck, 1986). Cleavage at the C terminus of the capsid precursor occurs initially at the 2A–2B junction in a rapid reaction which is believed to be catalysed by the 16 residue 2A protein (Vakharia et al., 1987; Clarke & Sangar, 1988; Ryan et al., 1989). The protease 3C ultimately cleaves the capsid precursor P1 in trans into the mature capsid proteins 1D, 1C and 1AB, the latter being cleaved presumably autocatalytically into 1A and 1B upon entry of the viral RNA into the procapsid.

The antigenic sites on the FMDV capsid involved in virus neutralization have been mapped by selection and sequence analysis of neutralizing monoclonal antibody-resistant mutants (Thomas et al., 1988; McCahon et al., 1989). In serotype O, two regions of 1D and areas of 1B and 1C have been found to contribute to four distinct antigenic sites (McCahon et al., 1989; J. D. A. Kitson, D. McCahon & G. J. Belsham, unpublished results). Serotype A has also been shown to possess multiple antigenic sites at similar locations on the virion surface (Thomas et al., 1988). These studies establish that all three major capsid proteins, 1B, 1C and 1D, contribute to the antigenicity of FMDV. It has been shown that empty capsid particles of FMDV retain the antigenicity
(Grubman et al., 1985) and immunogenicity (Rowlands et al., 1975) of infectious virus particles and hence may represent an efficient and safe alternative vaccine.

The aim of this study was to exploit the baculovirus expression vector system (Luckow & Summers, 1988a; Miller, 1988; Maeda, 1989) for the production of empty capsid particles by expression of FMDV cDNA clones. Based upon the allelic replacement of the polyhedrin gene of Autographa californica nuclear polyhedrosis virus (AcMNPV), recombinants can be obtained which express the foreign gene under the control of the polyhedrin promoter. Transfer vectors have been designed for the insertion of foreign genes, while maintaining the signals required for optimal transcription (Summers & Smith, 1987; Matsuura et al., 1987). This paper describes the construction of recombinant baculoviruses containing FMDV capsid protein genes together with genes for proteolytic processing enzymes, and the use of these recombinants for studying the synthesis, processing and assembly of FMDV capsid proteins in insect cells.

Methods

Virus and cells. AcMNPV, strain E2 (Smith & Summers, 1978), was propagated at 27°C in Spodoptera frugiperda (SF) cells (IPLB-SI21; Vaughn et al., 1977) using TNM-FH medium (Hink, 1970) supplemented with 10% foetal bovine serum (FBS).

Isolation and analysis of DNA. Viral DNA was isolated from non-occluded virus preparations or from infected SF cells as described (Summers & Smith, 1987). Plasmid DNA isolation, restriction endonuclease digestions and Southern blotting were carried out using methods described by Maniatis et al. (1982).

DNA sequencing. Transfer vector DNA was sequenced directly from the plasmid as described (Chen & Seeburg, 1985), using a 17-mer oligonucleotide primer, dAATGATAACCATCTCGC, which spans the sequence from nucleotide -76 to -60 upstream of the ATG start codon.

Viral DNA was isolated from non-occluded virus preparations and from infected SF cells as described (Summers & Smith, 1987). Plasmid DNA isolation, restriction endonuclease digestions and Southern blotting were carried out using methods described by Maniatis et al. (1982).

AcMNPV plasmid DNA was sequenced directly from the plasmid as described (Chen & Seeburg, 1985), using a 17-mer oligonucleotide primer, dAATGATAACCATCTCGC, which spans the sequence from nucleotide -76 to -60 upstream of the ATG start signal of the polyhedrin gene.

AcMNPV transfer vectors. The AcMNPV transfer vectors pAc610, pAc611, pAc360, pAcVL941 (Summers & Smith, 1987; Luckow & Summers, 1988a, b, 1989), pAcRP23 (Possee & Howard, 1987) and pAcYM1 (Matsuura et al., 1987) were a gift from Dr M. D. Summers (Texas A & M University, College Station, Tx, U.S.A.) and Dr R. D. Possee (NERC Institute of Virology & Environmental Microbiology, Oxford, U.K.), respectively. The various transfer vectors including the derivatives described below are depicted in Fig. 1.

Transfer vector pAcJR4 consists of the 1.9 kb Sphl-BamHI fragment of pAcVL941 (Luckow & Summers, 1989) ligated to the 6.7 kb BamHI-Sphl fragment of pAc611, providing this vector with the complete polyhedrin promoter sequence, additional 5’-terminal polyhedrin-coding sequences, and unique BamHI, Smal, SstI and EcoRI cloning sites. The additional polyhedrin-coding sequences should give enhanced expression (Luckow & Summers, 1989). Transfer vector pAcJR5 was constructed by ligation of the 1.9 kb Sphl-BamHI fragment of pAcVL941 with the 15 kb BamHI-SalI fragment of pAcYM1 and the 5.2 kb SalI-Sphl fragment of pAc610. In pAcJR5 a unique BamHI cloning site is present along with a larger deletion of the polyhedrin-coding sequence present in pAcYM1 (Matsuura et al., 1987). In both pAcJR4 and pAcJR5 the 5’-terminal sequence up to nucleotide 35 is present. Since the original ATG of the polyhedrin gene in pAcVL941 is converted into an ATG, the expression of a foreign gene is dependent upon its own translational start (Fig. 1).

Vector pAcJR10 was constructed by ligation of the 1.9 kb Sphl-BamHI fragment of pAc630 with the 6.7 kb BamHI-Sphl fragment of pAc610 (Summers & Smith, 1987). To obtain pAcJR11, the unique BamHI site was converted into a unique BglII site by insertion of the oligonucleotide GATCAGATCT. This vector and pAcJR10 allow expression of foreign gene products as fusions with the 11 N-terminal amino acids of polyhedrin. Unique BglII, Xbal and PstI cloning sites are present (Fig. 1).

FMDV cDNA cassettes. cDNAs from type O1 KauKauburgen (O1K) (Fors et al., 1984) and type A10 (Carroll et al., 1984) FMDV strains were used to construct the cassettes including in-frame fusions between L, P1 and 3C. In addition, as the A10 cDNA was incomplete with respect to the 5’ end of the coding region, chimeras had to be made from O1K and A10 cDNA. In Fig. 2 the origin and extent of the FMDV sequences present in these cassettes are indicated. The cassettes were inserted into pSP64 (Melton et al., 1984). Details of the construction of the pMR1, pMR2 and pMR4 cassettes and their introduction into pSP64 are described elsewhere (Ryan et al., 1989; Belsham et al., 1990).

To obtain pMR32, pMR1 DNA was digested with NruI and PvuI (site in pSP64). A 5.9 kb fragment was ligated to the 1.3 kb PvuI-Smal fragment obtained after double digestion of pSP64 with these enzymes. In this way BamHI sites were provided at both ends of the FMDV cassette. To construct pMR35, A10 cDNA was partially digested with NruI and to completion with XhoI. The 2.8 kb NruI (position 357)–XhoI (position 3142) fragment was cloned along with the 2.7 kb XhoI–PvuI (in pSP64) fragment of pMR2 and the 1.3 kb PvuI–Smal fragment of pSP64. As with pMR32 this cloning strategy provided the FMDV cassette with BamHI sites at both ends. These cassettes lack the 5’ end of the L gene and initiate translation at AUG codons seven and 14 triplets downstream of the NruI site. pMR41 is based on cassette CA103 (Belsham et al., 1990) modified by the addition of a 1.7 kb PstI fragment of O1K cDNA (Fig. 2), which spans the PstI site in 3C (position 6297) and the PstI site in the original cDNA vector.

AcMNPV–FMDV transfer vectors. FMDV cassettes from pMR1 and pMR2 were cloned into two types of transfer vectors, pAc610 and -611, and pAcRP23, which differed in promoter structure (Fig. 1). pMR1 was digested with NaeI and partially with SstI. The 4.2 kb fragment extending from the Nael site to the SstI site in the multi-linker region of pSP64 was then ligated into SmaI- and SstI-digested pAc611 to give transfer vector pAc611/MR1. To introduce the MR1 cassette into pAcRP23, which has only one BamHI cloning site, the 4.2 kb Nael–SstI fragment was cloned first into SmaI- and SstI-digested pUC19. The complete MR1 cassette was then obtained after partial BamHI digestion from this intermediate construct and cloned into the BamHI site of pAcRP23 to give pAcRP23/MR1.

After HindIII digestion of pMR2 and pMR4, the HindIII sites were made blunt with the Klenow fragment. The DNA was then cleaved with BamHI and 3.8 kb and 2.9 kb fragments were cloned into Smal- and BamHI-digested pAc610 to give pAc610/MR2 and pAc610/MR4, respectively. To insert the MR2 cassette into pAcRP23, pMR2 was digested with HindIII, blunt-ended with the Klenow fragment and partially digested with SstI. The 3.8 kb fragment was cloned into Smal- and SstI-digested pUC19, thereby providing the cassette with BamHI sites at both ends. The 3.8 kb BamHI fragment was cloned into pAcRP23, resulting in the transfer vector pAcRP23/MR2.

The construction of the transfer vectors pAcYM1/MR35 and pAcJR5/MR35 was achieved by introducing the 3.9 kb BamHI fragment of pMR35 into the unique BamHI cloning sites of pAcYM1 and pAcJR5, respectively. Expression of FMDV proteins using these
Expression of FMDV proteins in insect cells

Fig. 1. AcMNPV transfer vectors used in these studies. pAc610, pAc611 and pAc360 have been described in detail in Summers & Smith (1987); pAcYM1 and pAcRP23 are described in Matsuura et al. (1987) and pAcVL941 in Luckow & Summers (1989). The construction of the derivatives pAcJR4, pAcJR5 and pAcJR11 is described in Methods.

The FMDV cassette of pMR41 was obtained by first digesting the DNA with HindIII and blunting the ends with the Klenow fragment. After digestion with EcoRI, a 0.4 kb HindIII–EcoRI fragment was cloned into Smal- and EcoRI-digested pAcJR4. This intermediate plasmid was then digested with EcoRI. A 5.3 kb EcoRI fragment from pMR41, which extends from the EcoRI site in the L deletion up to the EcoRI site in the pTL/RI 'termination' cassette, was introduced into this intermediate vector to give pAcJR4/MR41.

All the plasmids were checked by restriction analysis for proper orientation and size of the inserts. The relationships among the various FMDV cDNA constructs, AcMNPV transfer vectors and AcMNPV recombinants are indicated in Table 1.

The junctions between the AcMNPV leader and FMDV inserts were sequenced. The 5' end of the cassettes in pMR2- and pMR4-based vectors revealed the presence of an inadvertant stop codon, eight triplets after the first initiation codon. The next ATGs available for (re)initiation are found 27 and 34 triplets into 1A.

Transfection, plaque assay and infection. A mixture of 950 μl, consisting of 1 μg AcMNPV DNA and 15 μg CsCl-purified transfer vector DNA in HEPES-buffered saline (21 mM-HEPES, 137 mM-NaCl, 5 mM-KCl, 1.4 mM-Na2HPO4, 12 mM-glucose, pH 7.1) and 50 μl of 2.5 mM-CaCl2, was left for 30 min at room temperature to form a precipitate. This was added to 1.5 x 106 Sf cells in a 35 mm Petri dish. After 1 h incubation at 27 °C, the transfection mixture was replaced with 2 ml Hink's medium plus 10% FBS. The culture fluid was harvested after 4 to 6 days and used at different dilutions in a plaque assay (Brown & Faulkner, 1977). Plaques without polyhedra were selected using inverted phase-contrast microscopy, purified in three consecutive plaque assays, and propagated into high titre stocks. Infections of Sf cells were carried out at 27 °C using an m.o.i. of 5 to 10 TCID50 units per cell.

Analysis of proteins from infected cells. At appropriate times after inoculation, cells were harvested, washed three times in phosphate-buffered saline (PBS) and boiled in disruption buffer (40% glycerol, 8% SDS, 4 mM-EDTA, 22% 2-mercaptoethanol, 40 mM-Tris–HCl, 0.02% bromophenol blue, pH 8.0). For radiolabelling of Sf cells, 106 cells in 35 mm dishes were starved for 2 h in Grace's medium without methionine (Summers & Smith, 1987), and then incubated at 27 °C for 3 h with [35S]methionine in the same medium. Cells were lysed in 300 μl TEN (50 mM-Tris–HCl, 5 mM-EDTA, 150 mM-NaCl, pH 8.0), 0.5%
Table 1. Relationship between cDNA constructs, transfer vectors, recombinants and FMDV products

<table>
<thead>
<tr>
<th>cDNA construct</th>
<th>Transfer vector</th>
<th>Recombinant</th>
<th>Products</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A10 serotype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMR2</td>
<td>pAc610</td>
<td>Ac610/MR2</td>
<td>ΔP1, 1CD, Δ1AB, 1C, 1D</td>
</tr>
<tr>
<td>pMR2</td>
<td>pAcRP23</td>
<td>AcRP23/MR2</td>
<td>ΔP1, 1CD, Δ1AB, 1C, 1D</td>
</tr>
<tr>
<td>pMR4</td>
<td>pAc610</td>
<td>Ac610/MR4</td>
<td>ΔP1</td>
</tr>
<tr>
<td>pMR35</td>
<td>pAcYM1</td>
<td>AcYM1/MR35</td>
<td>ΔL-P1, 1CD, ΔL-1AB, 1C, 1D</td>
</tr>
<tr>
<td>pMR35</td>
<td>pAcJR5</td>
<td>AcJR5/MR35</td>
<td>ΔL-P1, 1CD, ΔL-1AB, 1C, 1D</td>
</tr>
<tr>
<td><strong>O1K serotype</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMR1</td>
<td>pAc611</td>
<td>Ac611/MR1</td>
<td>P1, 1CD, 1AB, 1C, 1D</td>
</tr>
<tr>
<td>pMR1</td>
<td>pAcRP23</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>pMR32</td>
<td>pAcJR11</td>
<td>AcJR11/MR32</td>
<td>ΔL-P1, (ΔL-P1*), 1CD, ΔL-1AB, ΔL-1AB*, 1C, 1D</td>
</tr>
<tr>
<td>pMR41</td>
<td>pAcJR4</td>
<td>AcJR4/MR41</td>
<td>ΔLb-P1, ΔLb-P1, (ΔLb-P1*), (ΔLb-P1*), 3D, 1CD, ΔLb-1AB, ΔLb-1AB, ΔLb-1AB*, 1C, 1D</td>
</tr>
</tbody>
</table>

NP40, and debris was removed by centrifugation (10 min at 12000 g). To monitor total protein synthesis, 5 μl of the supernatant fraction was boiled in disruption buffer prior to loading onto gels. In immunoprecipitations, 75 μl of the supernatant fraction was mixed with 75 μl IPPT buffer (500 mM-NaCl, 20 mM-Tris–HCl, 2 mM-EDTA, 0.2% NP40, pH 7.5) and 5 μl hyperimmune guinea-pig anti-FMDV serum (serotype O1K or A10). After 1 h at room temperature 20 μl of Pansorbin (Calbiochem), previously washed three times in IPPT buffer, was added and incubation was continued for 20 min. After centrifugation (1 min at 12000 g) the pellet was washed twice with RIPA buffer (150 mM-NaCl, 50 mM-Tris–HCl, 10 mM-EDTA, 0.2% NP40, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, pH 7.4) and once with TBS buffer (20 mM-Tris–HCl, 140 mM-NaCl, pH 7.5) by cycles of vortexing and centrifugation. The residual pellet was boiled in disruption buffer for 3 min, and after centrifugation the solution was electrophoresed in 10% SDS-PAGE gels (Laemmli, 1970). Gels were then dried and autoradiographed.

Sucrose gradient centrifugation. Gradients of 10 to 30% sucrose in TEN buffer, 0.2% NP40, pH 7.5, were centrifuged for 3 h at 38000 r.p.m. in a Beckman SW41 rotor after loading of [35S]methionine-labelled infected cells, prepared as above.

Results

**FMDV cDNA cassettes**

Various FMDV cDNA constructs, derived from serotypes O1K and A10, and possessing different regions of the FMDV genome, were made in pSP64. To ensure expression of the mature FMDV procapsid proteins, cDNA cassettes of FMDV, containing the capsid precursor region P1-2A and the sequence for the proteases required for correct processing (L and 3C), were constructed (Fig. 2). The presence of XhoI sites in 2B and the beginning of 3B enabled the construction of in-frame fusions, thereby deleting 2C and 3A and truncating 2B and 3B. As a control for cellular proteolytic activity or adverse effects of the 3C protease on Sf cells, a construct was included (pMR4), which lacked the sequence for this protease (Fig. 2). The integrity of the A10 and O1K constructs was tested in pSP64 by *in vitro* transcription and translation. In all cases the expected products were observed (see for pMR1, Ryan *et al.*, 1989; and for pMR2 and pMR4, Belsham *et al.*, 1989, 1990), which indicates that the cassettes were assembled correctly and could give rise to mature FMDV proteins.

**Isolation of baculovirus recombinants**

The FMDV cDNA cassettes were subcloned into a number of AcMNPV transfer vectors (Fig. 2; Table 1) and used to obtain AcMNPV recombinants. Analysis of the recombinants was performed using restriction endonucleases and Southern blotting followed by hybridization to confirm the proper insertion of the FMDV cassettes into the AcMNPV genome.

With the cassette from pMR1 which contains the complete L protease sequence, no recombinants could be retrieved when pAcRP23 was used as transfer vector. Of the 16 polyhedron-negative plaques, none contained the FMDV insert, whereas when pAc611 was used three out of four polyhedron-negative plaques contained the correct FMDV sequences. It might be that when the FMDV insert is expressed using a more efficient vector (pAcRP23), the L protease surpasses a critical toxic level, rendering recombinants non-viable. The yield of virus from cells infected with viable recombinants was usually 10-fold lower than for wt AcMNPV.

**Characterization of FMDV proteins expressed in insect cells**

When Sf cells infected with recombinants were analysed for their protein composition at 48 h post-infection (p.i.), no evidence for the expression of FMDV proteins was
observed in SDS–polyacrylamide gels stained with Coomassie blue (data not shown). Also when [35S]-
methionine-labelled cell extracts were analysed, no
prominent bands indicative of the synthesis of FMDV
products were evident. No increased level of protein
expression was observed at other times after infection.
These results indicated that there was little expression of
FMDV proteins in insect cells. Only in cells infected with
the recombinant Ac610/MR4 did we observe an addi-
tional protein that migrated near the position of the P1
precursor (not shown).

(i) A10 cassettes

FMDV products isolated from recombinant AcMNPV-
infected cells 48 h p.i. were detected by immunoprecipita-
tion using guinea-pig anti-FMDV sera followed by
SDS–PAGE and autoradiography (Fig. 3). Using A10
cassettes, a protein was observed in cells infected with
Ac610/MR4 (lane 3) which had an approximate size
expected for the precursor protein P1. This indicated
that in Sf cells no processing of P1 occurred as a
consequence of insect cell proteolytic activity.

When recombinants that also contained protease 3C
(AcRP23/MR2 and Ac610/MR2) were used, P1 was
processed (Fig. 3, lanes 6 and 7); some residual P1 was
found, but most of the protein was cleaved into 1C, 1D
and a very heterogeneous set of products, probably
related to 1AB (lanes 7 and 6). This heterogeneity, also
observed for P1 in Ac610/MR4-infected cells (Fig. 3,
lane 3), is probably due to initiation occurring at
different AUG codons close to the 5' end of P1. Due to
the occurrence of a stop codon eight triplets after the first
initiation codon in the MR2 and MR4 cassettes, the
copolyprotein precursor P1 probably started from an AUG
inside 1A. In fact recombinants containing MR2 and
MR4 cassettes gave rise to P1 and 1AB products that
were somewhat smaller than the authentic products (Fig.
4, lanes 3, 6 and 7). Therefore, a cassette containing
AUG start sites in L (pMR35; Fig. 2) was devised. It was
hoped that the truncated L protein would be removed
from the P1-2A precursor by the 3C protease as happens

---

Fig. 2. FMDV cDNA cassettes derived from O1K and A10 serotypes, present in the vectors used in this study. The segments
originating from A10 are indicated by the dashed areas. Restriction sites are indicated: A, ApaI; B, BamHI; H, HindIII; K, KpnI; Na,
NaeI; Nr, NruI; P, PstI; R, EcoRI; S, SalI; X, Xhol.
Fig. 3. Analysis of FMDV proteins expressed by various baculovirus expression vectors. Autoradiograph of a SDS-polyacrylamide gel loaded with immunoprecipitates of [35S]methionine-labelled extracts of Sf cells infected with wt AcMNPV (lanes 2 and 9), Ac611/MR1 (lane 8), Ac610/MR4 (lane 3), AcYM1/MR35 (lane 4), AcJR5/MR35 (lane 5), AcRP23/MR2 (lane 6), Ac610/MR2 (lane 7), AcJR11/MR32 (lane 10), AcJR4/MR41 (lane 11) and of mock-infected Sf cells (lanes 1 and 12). PI, 3D, 1CD, AL-1AB, lAB*, lAB, 1D and 1C mark the positions of the respective FMDV proteins; PH denotes polyhedrin. The A10 proteins 1C and 1D nearly comigrate.

with cardiovirus polyproteins (Parks et al., 1986). The recombinants containing the MR35 cassette also produced correctly processed 1D and 1C and a modified 1AB product due to the fusion of the C-terminal portion of the L protein (Fig. 3, lanes 4 and 5). Thus the truncated L was not cleaved from the P1 precursor.

It is noteworthy that no products larger than the expected precursor P1 were observed from the MR2 or MR35 recombinants. This suggested that the cleavage between 2A and 2B occurred at the correct site in this insect cell system.

In mock-infected cell extracts (Fig. 3, lanes 1 and 12) two proteins of low Mr were non-specifically precipitated and in the wt AcMNPV-infected cells some of the highly expressed polyhedrin protein was observed in the immunoprecipitates (lanes 2 and 9). No apparent qualitative or quantitative differences in expression level were observed between recombinants made using the transfer vectors pAc610/611 and those made using pAcYM1, pAcRP23 and pAcJR5.

(ii) O1K cassettes

Three types of recombinants, containing cDNA from the O1K strain of FMDV, were isolated (Fig. 2). As indicated above, the MR1 cassette, containing L, P1 and 3C, could be introduced only into a recombinant virus using the vector pAc611, and not in the more efficient transfer vectors, pAcJR4 and pAcJR11. However, the MR32 and MR41 cassettes which contained truncated L sequences were inserted in the more efficient pAcJR11 and pAcJR4 vectors, respectively (Fig. 2; Table 1), and subsequently used to isolate viable recombinants. The MR32 cassette is essentially an O1K analogue of the A10-derived MR35 cassette (Fig. 2), whereas in the MR41 cassette an in-frame deletion was introduced within the L gene. This cassette also contained 3D, since in poliovirus the 3CD product was found to process P1 much more efficiently than 3C protein alone (Jore et al., 1988; Ypma-Wong et al., 1988). In the case of FMDV only a small change in processing efficiency was observed in vitro (Ryan et al., 1989).

The expression of these chimeric cassettes was determined after immunoprecipitation of [35S]-methionine-labelled cell extracts (Fig. 3, lanes 8 to 12). In Ac611/MR1-infected cells (Fig. 3, lane 8), a small amount of P1-2A and the processing intermediate 1CD was observed, but most of the material was in the form of fully processed procapsid proteins 1AB, 1C and 1D. Hence, both L and 3C proteases were functional in this system.

Extracts from cells infected with the recombinant AcJR11/MR32 (Fig. 3, lane 10) contained correctly processed 1D and 1C. The 1AB product was slightly larger as it represents the polyhedrin-ΔL-1AB fusion product. However, an additional FMDV product, which migrated only slightly slower than 1AB, was also observed. This may be the result of an aberrant cleavage within the C-terminal region of L, mediated by 3C (also reflected in the presence of two forms of ΔL-P1). The AcJR4/MR41 recombinants produced two ΔL-1AB products (lane 11). This may be due to the unusual possession by FMDV of two efficient alternative initiation codons for L (Clarke et al., 1985). Protein 3D and a small amount of the 3CD precursor were also observed. Processing of P1 with 3C alone occurred as efficiently as with 3CD.

As already observed for the A10 constructs, O1K chimeras also did not give rise to higher expression of proteins, even using fusion vectors pAc360 and pAcJR11 which contained all of the polyhedrin gene regulating sequences.

Detection of particles

Only recombinant Ac611/MR1 contained an actively expressed L gene and was thus able to produce the correctly processed 1AB, 1C and 1D in insect cells. These are the components of empty capsid particles and
Expression of FMDV proteins in insect cells

Discussion

Baculovirus recombinants were isolated with the objective of obtaining expression of the FMDV capsid proteins 1AB, 1C and 1D. These recombinants contained subgenomic FMDV cDNA cassettes containing sequences encoding L (or parts of L) and P1 fused to the genes required for cleavage (3C) of this precursor into procapsid proteins (Fig. 1). Correct cleavage of the FMDV polyprotein occurred in infected insect cells at the L-1A (L-mediated) and at the 1B-1C, 1C-1D and 1D-2A (3C-mediated) junctions. Inclusion of 3CD instead of 3C alone in a recombinant did not affect the efficiency of protein processing significantly, in contrast to the requirement for 3CD in processing poliovirus P1 in vitro (Ypma-Wong et al., 1988). Processing in Sf cells was more efficient than in reticulocyte lysates (Ryan et al., 1989) and similar in efficiency to that observed in mammalian cells using vaccinia virus recombinants (Belsham et al., 1990). It should be noted that insect cells are maintained at 27 °C rather than the temperature evidence was sought for the assembly of these proteins into empty capsid structures. Extracts were centrifuged in a sucrose gradient and fractions were analysed by immunoprecipitation and SDS–PAGE (Fig. 4). Most of the FMDV products stayed at the top of the gradient (see lanes 2 and 3) and no FMDV proteins were detectable in lanes 5 to 8. In lanes 9 to 12 the pattern of 1AB, 1C and 1D typical of procapsid proteins was observed. Empty capsid particles obtained from a natural FMDV infection of BHK cells sedimented to the same part of the gradient (data not shown).

Fig. 4. Sedimentation analysis of FMDV capsid proteins. Autoradiograph of an SDS–polyacrylamide gel loaded with immunoprecipitates of a [35S]methionine-labelled extract of Ac611/MRL-infected Sf cells, fractionated on a 10 to 30% sucrose gradient. The material applied onto the gradient (lane 1) was fractionated after centrifugation into 22 aliquots. Lane 2 contains a sample from the top of the gradient, whereas lanes 3 to 12 represent alternate fractions. Empty capsids of FMDV-infected BHK cells sediment into fraction 17 (lane 10).
(37 °C) usually used for mammalian cell culture. It is also of interest that the cleavage at the 2A–2B junction, which is very rapid in in vitro translation reactions (Ryan et al., 1989), is also efficient in the insect cell system in vivo. The results described here confirm those obtained in other systems (Clarke & Sangar, 1988; Ryan et al., 1989) that this cleavage reaction occurs independently of L and 3C activity, and supports the idea that it may be mediated by 2A itself, even though this protein is only 16 residues long in FMDV.

Since the expression of L may have a negative effect on the generation of recombinants, truncated versions of L were also used (Fig. 2). The presence of a product 1AB*, migrating heterogeneously and slightly slower than 1AB with recombinants in which L had been truncated, might indicate that 3C is able to cleave within the C-terminal region of L. It is interesting to note that in L, 13 residues before the C terminus, there is a L/N sequence which is the same as that at the 3C-mediated cleavage site between 1D and 2A. The finding that under certain conditions a truncated form can be found (Sangar et al., 1988) may be related to this phenomenon.

The expression levels of FMDV proteins were low with all recombinants. We have observed some variation in the levels of transcription with the various recombinants and this is a subject for further study. Reduced levels of expression may have been caused by the presence of 27 or more nucleotides, dependent on the recombinant, upstream from the ATG start codon. In some cases the nucleotide at position −3 with respect to the first initiation codon of L was not a purine, and therefore does not conform to the Kozak (1984) consensus for optimal initiation of translation. However, the use of transfer vectors modified for optimal expression, including polyhedrin fusions such as pAcJR11/MR32 (Fig. 2; Table 1) did not markedly increase this level as determined by immune precipitation (Fig. 3). Recently, poliovirus proteins have been expressed in Sf cells (Urakawa et al., 1989). In that study expression levels were also low, even though very efficient vectors were used and although the initiation codon was in a Kozak consensus.

Some other features of the FMDV inserts might also have impaired efficient expression. The codon usage in the FMDV open reading frame is considerably different from that observed for the major late AcmNPV genes, polyhedrin and p10. However, similar differences in codon usage have not been found to inhibit the efficient expression of proteins of similar size e.g. β-galactosidase (Summers & Smith, 1987). The consensus sequence ATAAAG, essential for transcription of baculovirus late genes (Leisy et al., 1986), is not present in either strand of the FMDV inserts. This makes it unlikely that interference occurs at the level of transcription by illegitimate initiation or as a result of the synthesis of antisense RNA. The influence of these and other factors such as protein location within the cell, protein stability and RNA stability on expression levels will be the subject of future examination.

Using the MR1 cassette containing the complete L-P1-3C sequence we were unable to isolate recombinants using transfer vectors modified for optimal expression (pAcRP23, pAcYM1, pAcVL941) (Fig. 1). This may suggest that the L protein, when cleaved off P1, is toxic to the cells, thus causing a negative selection for good FMDV expressors. This may also explain the retrieval of only a single recombinant, Ac611/MR1, expressing L. Using the same FMDV cassette from pMR1 no recombinants could be obtained in the vaccinia virus system (Belsham et al., 1990). Moreover, it has been suggested that L plays a role in the shut-down of host cell protein synthesis by inducing the cleavage of the capsid-binding protein p220 (Devaney et al., 1988). Hence, inefficient expression of picornavirus capsid proteins might be a general feature of this insect virus–cell system and proteins other than L may also impair protein synthesis. It was shown recently that expression of poliovirus proteins by vaccinia virus recombinants was also impaired by the action of the 2A protease (Jewell et al., 1990).

Since L is required for the correct (lysine–glycine) cleavage between itself and 1A, truncated versions of L will be unable to carry out the correct cleavage to provide a N-terminal glycine. This glycine is the substrate for myristylation of 1A (Chow et al., 1987), which may be important for FMDV capsid formation. Therefore, constructs lacking L and starting with 1A create a situation similar to that of poliovirus, the polypeptide of which starts naturally with 1A.

Some of the capsid protein expressed from the MR1 cassette sedimented in sucrose gradients in an aggregated form in what may be empty procapsids, since they migrate at a rate characteristic of FMDV empty capsid particles from infected BHK cells. The efficiency of assembly was low, possibly due to the low concentration of protein subunits within the insect cells. We were not able to detect capsid particles in the electron microscope although poliovirus particles produced by a baculovirus expression vector were detected with this technique (Urakawa et al., 1989).

We thank J. K. Brangwyn and M. Usmany for their skilful technical assistance and C. C. Abrams for the construction of the L deletion. We appreciated the continuous interest of Professor Rob W. Goldbach and his critical reading of the manuscript. This investigation was carried out under research contracts BAP-0118-NL and BAP-0119-UK of the Biomolecular Action Programme of the Commission of the European Communities.
Expression of FMDV proteins in insect cells

References


(Received 4 December 1989; Accepted 19 March 1990)