Assembly of foot-and-mouth disease virus empty capsids synthesized by a vaccinia virus expression system

Charles C. Abrams, Andrew M. Q. King and Graham J. Belsham*

BBSRC Institute for Animal Health, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, UK

cDNA cassettes encoding the foot-and-mouth disease virus (FMDV) structural protein precursor (P1-2A) together with the 3C protease, which cleaves this molecule to 1AB, 1C and 1D, were constructed. These cassettes were introduced into vaccinia virus (VV) transfer vectors. Attempts to isolate recombinant VVs constitutively expressing these cassettes were unsuccessful. However, when the P1-2A-3C cassette was placed under the control of the bacteriophage T7 promoter, stable VV/FMDV recombinants were isolated. Co-infection with recombinant VV vTF7-3 (which expresses T7 RNA polymerase) led to the production of correctly processed FMDV capsid proteins. Analysis by sucrose gradient centrifugation showed that material which co-sedimented with natural empty capsid particles (70S) was formed. Electron microscopy revealed empty capsid-like particles with diameters of about 30 nm. Studies using monoclonal antibodies specific for conformational epitopes indicated that the antigenicity of the synthetic particles was similar to whole virions and natural empty capsid particles. Surprisingly, merely the modification of a single amino acid residue within the myristoylation consensus sequence at the N terminus of P1-2A allowed the isolation of a recombinant VV which constitutively expressed the correctly processed proteins. However, the capsid proteins expressed from this mutant cassette failed to assemble into 70S empty particles.

Introduction

Foot-and-mouth disease virus (FMDV) is the sole member of the aphthovirus genus within the picornavirus family. Seven distinct serotypes of FMDV have been identified, namely O, A, C, South African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1, each of which can be divided into a number of different subtypes. The disease caused by the virus continues to be of major economic importance across the world. The virion contains a single copy of the positive-sense single-stranded RNA genome which encodes a single long open reading frame. Following infection of cells, the genome is released from the virion and is translated to produce a polyprotein which is proteolytically processed by three virus encoded proteases. The Leader (L) protease cleaves itself in cis or in trans at its C terminus from the P1-2A capsid precursor. The 2A protease cleaves itself at its C terminus to release P1-2A from P2. Processing of P1-2A is effected by the 3C protease to produce the capsid proteins 1AB (VP0), 1C (VP3) and 1D (VP1) (for review see Belsham, 1993). In the virion, cleavage of 1AB occurs to produce 1A (VP4) and 1B (VP2). All other processing events within the polyprotein require the 3C protease.

Many details of the assembly of picornaviruses are not known. However, several intermediates have been isolated from infected cell extracts and from in vitro translation experiments. These are: (i) the 5S protomer which contains a single copy of 1AB, 1C and 1D, (ii) the 12S pentamer consisting of five copies of the 5S protomer and (iii) the 70S empty capsid containing 12 copies of the 12S pentamer, but no RNA genome (Rueckert, 1990). Natural FMDV empty particles have been shown to bind to susceptible cells (Rowlands et al., 1975) and to be indistinguishable from whole virus when assayed with a panel of monoclonal antibodies (MAbs) (Grubman et al., 1985). Furthermore, such empty particles have been shown to stimulate the same level of neutralizing antibodies as full particles (Rowlands et al., 1975; Rweyemamu et al., 1979). The proportion of full to empty particles isolated from infected cells varies for different virus strains. In cells infected with A Pando and A24 Cruzeiro strains greater than 50% of the virus sized particles consisted of 70S empty capsids, whereas for other virus types such as O1 Kaufbeuren (O1K) less than 5% were empty capsids (Rweyemamu et al., 1979).

The N-terminal glycine residue of the picornavirus capsid precursors (P1 or P1-2A) has been shown to be modified by the addition of a myristate moiety (Chow et al., 1987). This modification is essential for the infectivity of poliovirus (PV) (Marc et al., 1989, 1990; Krausslich et
al., 1990), another member of the picornavirus family, and is required for efficient assembly of the PV capsid (Marc et al., 1990). It does not appear to be essential for proteolytic processing of the PV or FMDV capsid precursors (Marc et al., 1990; Ansardi et al., 1992; Belsham et al., 1991). Few studies on the role of myristoylation have been performed on picornaviruses other than PV but some observations suggest that other members of this family may differ from PV in this respect. Studies by Lewis et al. (1991) showed a low level of assembly of FMDV capsid proteins expressed in *Escherichia coli*, in the absence of any myristoylation. Evidence indicates that myristoylation of hepatitis A virus (HAV) proteins is not required for virus assembly or infectivity (Tesar et al., 1993).

Previous reports have indicated that picornavirus empty capsid-like particles can be synthesized using the vaccinia virus (VV) expression system. Winokur et al. (1991) and Zhu et al. (1994) each constructed VV–HAV recombinants encoding the complete open reading frame of HAV. Both groups showed, using electron microscopy, that the recombinant viruses synthesized HAV-empty capsids. However, attempts to isolate recombinant VV including the FMDV L protein (Belsham et al., 1990) or the PV 2A protease (Turner et al., 1989; Jewell et al., 1990) were unsuccessful. Both of these enzymes are involved in the processing of the viral polyprotein. However they also direct the cleavage of the 220(?) subunit of the cap-binding complex (eIF-4F) (Krausslich et al., 1987; Devaney et al., 1988) which results in the inhibition of cap-dependent protein synthesis. Therefore, strategies have been developed to obtain the expression of just the regions of the viral polyprotein required for the correct expression and processing of the structural protein precursor. Ansardi et al. (1991) reported the synthesis of poliovirus empty capsids using the co-infection of two VV–PV recombinants. One virus encoded the P1 region and the second virus encoded the 3CD region of PV. The assembly of these particles was dependent on myristoylation of the capsid precursor (Ansardi et al., 1992). We have shown that following precise deletion of the L protein coding sequence from the O1K type FMDV cDNA correct processing and myristoylation of the capsid proteins could be achieved in transient expression studies using a P1-2A-3C cassette (Belsham et al., 1991).

In this report we describe the isolation of recombinant VVs containing P1-2A-3C cDNA cassettes (derived by precise deletion of the L coding sequence), encoding the capsid precursor from three different FMDV subtypes representing two different serotypes. Surprisingly, recombinant VVs could still only be isolated when the cassettes were placed under the control of the T7 promoter and were not isolated when using a constitutive promoter. Co-expression of the T7 RNA polymerase resulted in the production of self processing capsid precursors which self assemble to form empty capsid-like structures. These particles have an antigenic structure similar to whole virus particles. The assembly process is blocked by modification of the myristoylation site.

**Methods**

**Construction of FMDV cDNA cassettes encoding P1-2A-3C.** The structures of the cDNA cassettes used in these studies are indicated in Fig. 1. The CAK cDNA cassette containing the P1-2A region of strain O1K was described previously (Belsham et al., 1991). In order to construct the CA2 cassette, the mutagenesis technique of Venkitaraman (1989) was used to specifically delete the L sequence from plasmid pMR8 (Ryan et al., 1989) which includes the P1-2A region of FMDV strain A061. A HindIII–KpnI fragment of plasmid pMR8 containing the L coding sequence was cloned into M13mp18. The deletion was achieved with the oligonucleotide dACCACTGAACACATGGGT-GCTGGACCAGTCC (the initiation codon is underlined) and the enzyme T7 DNA polymerase Sequenase version 2.0 to create M13CA2. The mutated fragment from the replicative form of this DNA was substituted back into the parental plasmid pMR8 to create pCA2. The CA24IRES cDNA cassette was constructed containing both the 5'NCR (downstream from the poly(C) tract) and the complete P1-2A capsid coding region of FMDV strain A24 Cruzeiro. Originally two cDNA clones, SK2.0 and CT13 (kindly provided by C. Tahourdin), were prepared using FMDV A24 vRNA and included the 5'NCR and L-1P-1.2A regions of strain A24. The L coding sequence of clone SK2.0 was deleted in a similar fashion using the oligonucleotide dATACA-TGGATCGATGGGGGCCGGGCAA to produce SK2.0ALT. This clone was combined with clone CT13 into the plasmid pMR8, replacing the P1-2A region of strain A061 with the 5'NCR-P1-2A region of strain A24 to produce the cassette CA24IRES. The presence of an ATG start codon immediately upstream of the P1-2A-3C cassette for both pCA2 and pCA24IRES was confirmed by DNA sequencing (data not shown).

**Construction of myristoylation negative cassette pCA2M1.** A 700 bp fragment was generated by PCR using the M13CA2 ssDNA as template and the mutagenic oligonucleotide MYRA10 (dAAGCTTAAATGGGTGCTGGACCAGGCC) encoding the change from Ser to Ala (within the consensus sequence) and the M13 reverse primer (dAACAGCTATGACCATG). The fragment was digested with HindIII and KpnI, ligated back into pCA2 that had been digested with HindIII and KpnI to create pCA2M1 and the sequence was verified.

**Construction of VV transfer vectors.** Initially the plasmids pCAK, pCA2 and pCA24IRES were digested with HindIII and DraI and the P1-2A-3C cassettes cloned into HindIII–Smal cut pH3IV vector (the VV transfer vector pH3IV, derived from pGS20 (Mackett et al., 1984), was described previously (Belsham et al., 1990)) to place the FMDV cDNA cassette under the control of the VV p7.5K early/late promoter in plasmids pVCAK, pVCA2 and pVCA24IRES respectively. Additionally, the same parental plasmids were digested with HindIII and DraI, blunt ended with Klenow enzyme and the P1-2A-3C cassette cloned into BamHI cut and blunt ended pBG200 plasmid under the control of the T7 promoter to produce vectors pT7CAK, pT7CA2 and pT7CA24IRES. Vector pBG200 was constructed by cloning a 200 bp BgII–BgII fragment from pAR2529 (Fuerst et al., 1986), which contains the T7 promoter and terminator sequence separated by a unique BamHI site, into the vector pBGH3IV (a derivative of pH3IV in which the p7.5K promoter was removed and replaced with a BgII

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**References**


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Fig. 1. Representation of the structure of the FMDV cDNA cassettes and VV transfer vectors used in this study. The P1-2A coding regions derived from three different strains of FMDV are differentially shaded to indicate this. Each of the cassettes used the 3C protease from FMDV Aog61. The myristoylation consensus sequence mutation is indicated by an asterisk (*) in cassette M1 and CA2M1. The transfer vector pBG200 contains the bacteriophage T7 promoter and transcription terminator (T7φ) sequences, inserted into the VV TK sequence. The HindIII restriction sites are indicated by H3.

linker) (see Fig. 1). To create the vector p7.5CA2M1, the myristoylation site mutant plasmid pCA2M1 was digested with HindIII and DraI and the insert ligated into HindIII–SmaI digested pH3IV as above.

Isolation and characterization of recombinant VVs. The procedure used for isolating recombinant VVs was essentially that described by Mackett et al. (1984). Briefly, BSC40 cells were infected with VV (WR strain) and transfected with 5 μg of VV transfer vectors (pT7CAK, pT7CA2, pT7CA24IRES and p7.5CA2M1) containing the FMDV cassettes. Viruses were harvested after 48 h and those lacking thymidine kinase activity were selected in the presence of 5-bromo-2'-deoxyuridine (BUdR) (25 μg/ml). Recombinant viruses (vT7CAK, vT7CA2, vT7CA24IRES and v7.5CA2M1) containing the FMDV cassettes were harvested after 48 h and those lacking thymidine kinase activity were selected in the presence of 5-bromo-2'-deoxyuridine (BUdR) (25 μg/ml). Recombinant viruses (vT7CAK, vT7CA2, vT7CA24IRES and v7.5CA2M1) containing the FMDV cassettes were plated on human 143TK− cells in the presence of BUdR until all plaques were positive as screened by hybridization to 32P-labelled FMDV cDNA as described previously (Belsham et al., 1990). Virus stocks were then grown in the TK− cells in the presence of BUdR.

SDS-PAGE analysis of FMDV proteins and particles. Twenty hours post infection with VV derivatives, BSC40 or TK− 143 cells were incubated for 2 h with [35S]methionine. Cell extracts were prepared in 0.15 M-NaCl, 10 mM-Tris-HCl pH 8.0, 1 mM-EDTA, 0.5% NP40 and immunoprecipitated using hyperimmune guinea-pig anti-FMDV sera and Pansorbin (Calbiochem) as previously described (Belsham et al., 1990). For sucrose gradient centrifugation analysis FMDV infected BHK-21 cells or VV infected BSC40 cells were radiolabelled with [35S]methionine as described above. Cell extracts were loaded onto a 10–30% (w/v) sucrose–TNE gradient and centrifuged at 35000 r.p.m. (Beckman type 41 rotor) for 2.5 h at 4°C. Gradients were fractionated and proteins immunoprecipitated with anti-FMDV sera (as above) before SDS-PAGE (Laemmli, 1970) and autoradiography.

Detection of FMDV antigens by ELISA. Microtitre plates were coated with anti-FMDV type A rabbit sera at a dilution of 1:5000 in carbonate coating buffer overnight at 4°C. All subsequent incubations,
using 50 μl of reagent added to each well, were performed at 37 °C for 1 h. Purified FMDV AstrId virions (2.5 μg/ml) or empty capsid particles (2.5 μg/ml) or cell extracts from BSC40 cells (35 mm dish samples were harvested in 100 μl) infected with recombinant VVs were added to the wells. Plates were then incubated with MAb 3.7 or MAb 3.9 (1:500 dilution) (Thomas et al., 1988), which recognize conformation dependent epitopes on the type A virion (as judged by screening against whole and denatured virions, see below) followed by peroxidase linked guinea-pig anti-mouse IgG conjugate (Dako) (diluted 1:1000). The reaction was developed with a solution containing o-phenylenediamine (1 mg/ml) and hydrogen peroxide (0.012%) and the colour development was stopped after 15 min with 1 M-H₂SO₄ and quantified using an ELISA reader.

Results

Constitutive expression of the P1-2A-3C cassettes is not tolerated by VV

In previous studies (Belsham et al., 1990) we showed that recombinant VVs containing a cDNA of the structure L-P1-2A-3C could not be isolated by screening for TK⁻ recombinant viruses. However, when an in-frame deletion of the L protein coding sequence was introduced into the cassette then recombinant VVs containing this cassette could be readily isolated. Since it was shown that the L protein initiated the cleavage of the cap-binding complex (Devaney et al., 1988) this apparent toxicity was readily explicable. We reasoned that the coding sequence for L could be precisely deleted to produce an arrangement similar to that found in the enteroviruses (e.g. PV) in which the first component of the polyprotein is the capsid precursor. This modification was performed, as described previously for the type O cassette (CAK; Belsham et al., 1991), on the type A cassettes resulting in loss of L sequences and the positioning of an initiation codon adjacent to the N terminus of the P1-2A coding sequence (Fig. 1). The resulting cassettes of the form P1-2A-3C, containing type O and A cDNA, were transferred into VV transfer vectors containing the p7⁻⁵K promoter to produce pVCAK, pVCA2 and pVCA24IRES. The latter plasmid includes a portion of the 5'NCR from this virus including the complete FMDV internal ribosome entry site (IRES) (Belsham & Brangwyn, 1990). Each of the plasmids was assayed for their ability to produce the expected products. In a transient expression experiment BSC40 cells were infected with wild-type VV (WR strain) and transfected with each of these three vectors. Analysis of the proteins produced from each FMDV cassette showed correct initiation of translation and also processing of the capsid proteins (data not shown). Homologous recombination experiments were carried out with wild-type TK⁺ (WR) VV and each of the three VV transfer vectors. TK⁻ VV were selected on human 143 TK⁻ cells in the presence of BUdR. In total, some 600 TK⁻ plaques were screened using a radioactive FMDV cDNA probe. However, none of the TK⁻ plaques was found to contain FMDV sequences (this contrasts with about 50% of TK⁻ plaques carrying the appropriate inserts in parallel experiments). The reason why recombinant VVs containing the P1-2A-3C cassette under the control of the VV p7⁻⁵K promoter are not readily isolated is not known.

Isolation and characterization of recombinant VVs containing the P1-2A-3C cassette under the control of the T7 promoter

In an attempt to overcome the apparent toxicity of constitutive expression of these cassettes the P1-2A-3C cassette from each of the three vectors pCAK, pCA2 and pCA24IRES was cloned into the VV transfer vector pBG200 (Fig. 1), which contains the T7 promoter and terminator sequences. The three transfer vectors were then used in a homologous recombination experiment with VV (WR) to produce the VV/FMDV recombinants
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Fig. 3. Assembly of FMDV empty capsid particles. Extracts from BHK-21 cells infected with FMDV A1061 or HTK-143 cells infected with vTF7-3 and vT7CA24IRES and metabolically labelled with [35S]methionine were analysed on a 10–30% sucrose gradient. Fractions from the top of the gradient are shown on the right. FMDV proteins in each fraction were immunoprecipitated with guinea-pig anti-FMDV sera and analysed by SDS-PAGE. The locations of FMDV natural empty capsids and mature virions present within FMDV infected cells are indicated. Note that the presence of capsid protein 1B in FMDV A1061 natural empty capsid particles has been observed previously (Curry et al., 1995).

vT7CAK, vT7CA2 and vT7CA24IRES respectively. All three recombinant viruses were obtained at a frequency of between 40–60% of selected TK- plaques in contrast to the results described above.

The ability of these recombinant viruses to express the correct FMDV proteins was determined by co-infecing cells with vTF7-3 and either vT7CA2, vT7CAK or vT7CA24IRES. Twenty hours after infection the cells were labelled with [35S]methionine for 2 h. The cells were then lysed and the FMDV proteins immunoprecipitated with a hyperimmune guinea-pig anti-FMDV antisera before analysis by SDS-PAGE (Fig. 2). In the presence of vTF7-3, the VV/FMDV recombinants vT7CA2, vT7CAK and vT7CA24IRES (lanes 4, 6 and 8) each expressed the correctly processed empty capsid components 1AB, 1C and 1D (note that capsid proteins from the different strains run at different positions). No expression was observed in the absence of vTF7-3 (lanes 3, 5 and 7).

Assembly of FMDV capsid proteins into empty particles

To demonstrate the ability of the expressed FMDV capsid proteins to assemble into empty particles, the viruses vT7CA24IRES and vTF7-3 were used to co-infect BSC40 cells. In parallel, BHK-21 cells were infected with FMDV strain A1061. After [35S]methionine labelling of the cells, extracts were prepared and analysed by sedimentation on a 10–30% sucrose gradient. Fractions were collected and FMDV proteins were immunoprecipitated and analysed by SDS-PAGE. The results (Fig. 3a) showed that a major fraction of the FMDV protein isolated from vT7CA24IRES and vTF7-3 co-infected cells sedimented at the same rate (70S) as empty capsid particles isolated from FMDV A1061 infected cells (Fig. 3b).

Note that the FMDV infected cells also contained a peak of material sedimenting at 140S corresponding to complete virus particles. Densitometric scanning of FMDV proteins expressed in vT7CA24IRES and vTF7-3 co-infected cells showed that approximately 50% of the expressed proteins assembled into empty particles. Similar sucrose gradient analysis has been performed on extracts from cells infected with vT7CAK (type O1K FMDV) and vTF7-3. Again, a peak corresponding to 70S particles was observed but in this case only about 10% of the FMDV proteins synthesized by vT7CAK assembled, the vast bulk of the capsid proteins remaining unassembled (data not shown). Using a recombinant virus expressing the O1K strain cDNA CA103 cassette (ΔL-P1-2A-3C) (Belsham et al., 1990), which produces an inactive portion of the L protein fused to the N
terminus of 1AB together with 1C and 1D, no assembly was observed.

We wanted to confirm that the relatively low level of formation of material sedimenting at 70S from the vT7CAK virus did represent true assembly of particles. Cells were co-infected with vT7CAK and vTF7-3, and after 20 h these cells were scraped off the dish and stained with 2% phosphotungstic acid. Analysis of samples by electron microscopy revealed empty capsid-like particles of approximately 30 nm in diameter, similar to that of natural empty particles (Fig. 4). No such particles were detected in cells infected with the VV vTF7-3 alone (not shown).

Role of myristoylation in the assembly of FMDV empty capsids

The requirement for myristoylation of the capsid precursor in the assembly process was investigated following mutagenesis of the myristoylation consensus sequence (MGXXXS/T; Towler et al., 1988). Myristoylation of the wild-type molecule (which contains a Ser residue at position 6 of this motif) occurs on the Gly residue which becomes the amino terminus of P1-2A following removal of the terminal Met residue. A single amino acid substitution (Ser to Ala) was introduced into P1-2A-3C to produce the MYR-P1-2A-3C cassette, termed CA2M1 (Fig. 1). The N-terminal Gly residue was unaltered. This mutant cassette was introduced into the pH3IV transfer vector, containing the constitutive p7.5K promoter, as above. In contrast to our experience with the wild-type cassette, recombinant VVs that constitutively express the MYR-P1-2A-3C cassette were readily isolated. Hence, a single amino acid substitution in the myristoylation consensus sequence overcame the inhibitory effect of this cassette on VV. Metabolic labelling of cells infected with these recombinant viruses indicated that the correct pattern of proteins, i.e. 1AB, 1C and 1D, was produced (Fig. 5b), although it was observed that processing of the MYR-P1-A precursor to these products was less efficient than in the wild-type counterpart (Fig. 5a). Furthermore, sedimentation analysis of such extracts on sucrose gradients indicated that the expressed products failed to assemble into stable empty capsids (Fig. 5b) since all the FMDV proteins remained near the top of the gradient. In contrast, the proteins from the wild-type vT7CA2 VV assayed in parallel (Fig. 5a) efficiently assembled and the sedimentation profile closely resembled that of the proteins expressed from vT7CA24IRES, shown in Fig. 3 (a).

Antigenicity of expressed FMDV proteins

In order to determine the antigenicity of FMDV proteins expressed in cells co-infected with vT7CA2 and vTF7-3 these were analysed by capture ELISA experiments using MAbs raised against FMDV strain A10 Holland (Thomas et al., 1988). This panel of MAbs was screened by ELISA with native and denatured viruses and the two antibodies, MAb 3.7 and MAb 3.9, were selected since they efficiently
Fig. 5. Non-assembly of FMDV capsid proteins expressed from a myristoylation consensus site mutant cassette. Cells were infected with vTF7-3 and vT7CA2 (a) or v7.5CA2M1 (b). After 20 h cells were labelled with [35S]methionine, extracts were prepared and analysed on a 10–30% sucrose gradient. FMDV proteins were immunoprecipitated from fractions with anti-FMDV antisera and analysed by SDS-PAGE. Two truncated versions of 1AB (indicated by an asterisk) are more apparent in the proteins expressed from the CA2M1 cassette, perhaps indicative of a change in context of the initiation site.

recognized intact virus but not the denatured products (C. C. Abrams, A. M. Q. King & G. J. Belsham, unpublished). Similar binding curves were obtained for MAb 3.7 with extracts from vT7CA2 and vTF7-3 co-infected cells as with purified FMDV A1061 virions and empty capsid particles (Fig. 6, compare a, b and c). Similar results were also obtained with MAb 3.9 (data not shown). No specific interaction of these antibodies was observed with extracts from cells infected with vTF7-3 alone (Fig. 6d). Furthermore, by comparison of the level of binding found in the capture ELISA test (within the linear range) to the vT7CA2 expressed empty particles and to the purified virus particles it can be calculated that the yield of recombinant empty capsids was of a similar magnitude to that of natural FMDV empty capsids isolated from the same number of cells.

Discussion

We have reported before (Belsham et al., 1990), that it is not possible to isolate VV/FMDV recombinants containing an intact L gene. A similar problem has been observed with the 2A protease gene of poliovirus (Turner et al., 1989; Jewell et al., 1990). The L protease of FMDV and the 2A protease of PV each direct the cleavage of the p220 subunit of the cap-binding complex. This cleavage correlates with the inhibition of cap-dependent translation and presumably accounts for this toxic effect. No functional analogue of these proteins has been identified in HAV which presumably explains why recombinant
Although a consensus sequence for myristoylation is present, mutagenesis of this site does not compromise HAV viability.

VVs expressing the complete polyprotein of this virus have been obtained (Winokur et al., 1991; Zhu et al., 1994).

The precise deletion of the FMDV L coding sequence to create a P1-2A-3C cassette was expected to overcome this problem. However, no VV/FMDV recombinants could be isolated containing this cassette under the control of the constitutive VV p7.5K promoter. In contrast, recombinant viruses could be isolated at a high frequency when the P1-2A3-C cassette was placed under the control of the T7 promoter. FMDV proteins are only expressed by these recombinant viruses upon co-infection with a second recombinant VV vTF7-3. The requirement for an inducible promoter suggests that the capsid expressing cassettes retain a degree of toxicity for VV even in the absence of the L coding sequence. [It should be noted that cassettes containing an intact L coding sequence cannot be recombined into VV even under the control of the T7 promoter (C. C. Abrams, A. M. Q. King & G. J. Belsham, unpublished.)] The cause of the residual toxicity is not clear. It is not due to the activity of 3C since previously the AL-P1-2A-3C cassette was successfully used to create recombinant VVs which constitutively expressed a fusion protein AL-1AB, 1C and 1D (Belsham et al., 1990). No myristoylation of the N terminus of the modified VP0 was observed (Belsham et al., 1991). Since we have shown here that a mutant cassette in which the myristoylation consensus sequence is modified can also be constitutively expressed within VV it could be that the myristoylation of the FMDV capsid precursor is inhibitory. However, recently a VV/FMDV recombinant which contains just the P1-2A region under the control of the constitutive p7.5K promoter has been isolated (C. C. Abrams, A. M. Q. King & G. J. Belsham, unpublished) which argues against this explanation. One characteristic common to all the VV/FMDV recombinants which could be isolated is that their products do not assemble into empty capsids. Thus the problem in isolating recombinant viruses which constitutively express the P1-2A-3C cassette appears to lie either in the production of myristoylated 1AB (rather than myristoylated P1-2A) or in the assembly of FMDV empty capsids. It is unlikely that free 1AB exists in significant amounts since it is usually associated with 1C and 1D (and hence this does not seem significantly different from uncleaved P1-2A). However, since HAV empty capsids can be expressed constitutively it is not entirely clear what is the basis of this incompatibility. It is noteworthy in this context that no evidence for the myristoylation of HAV capsid proteins has been obtained (Tesar et al., 1993). Although a consensus sequence for myristoylation is present, mutagenesis of this site does not compromise HAV viability.

It was apparent that the non-myristoylated P1-2A precursor was less well processed by 3C than the wild-type P1-2A (Fig. 5). The inefficient processing is consistent with initial observations made by Marc et al. (1989) and Krausslich et al. (1990) who showed that modification of the N-terminal Gly residue of the PV P1 precursor blocked processing in vitro, but that this block was not absolute within cells (Marc et al., 1990). Previous studies on FMDV cassettes have also shown that processing can occur in the absence of an appropriate myristoylation site (Belsham et al., 1990, 1991). However, the efficiency of the process compared to that of the wild-type cassettes has not been determined quantitatively. It does appear that the block on myristoylation affects not only capsid assembly or stability but also the formation of the processed products.

Ansardi et al. (1991, 1992) have reported results with the PV capsid proteins which are closely analogous to those reported here. The PV capsid precursor is the first component of the PV encoded polyprotein and this is a significant difference between this virus and FMDV which possesses a leader protein. The mutagenesis we have used to remove the FMDV L protein coding sequence therefore produced a structure similar to that which occurs in PV. Ansardi et al. (1991) employed two separate VV/PV recombinants to synthesize the empty capsids. One recombinant virus contained the PV P1 cassette and the second recombinant contained the 3CD region necessary for the processing of the P1 precursor. The results presented here and those of Ansardi et al. (1992) show that there is a strong requirement for myristoylation in empty capsid assembly. It is difficult from the experiments described here to rule out any assembly occurring in the absence of myristoylation and a low level efficiency may be compatible with the findings of Lewis et al. (1991) who observed limited assembly of FMDV capsid proteins expressed in E. coli (neither the system nor the cassette used permitted myristoylation).

The level of expression of the different FMDV cassettes was quite similar in each case and was not appreciably affected by the presence of the FMDV IRES (see Fig. 2). Studies by Elroy-Stein et al. (1989) showed that the translation of T7 derived mRNA transcripts encoding chloramphenicol acetyltransferase could be improved in cells by the insertion of the IRES of encephalomyocarditis virus (EMCV) immediately downstream of the T7 promoter. Our results suggest that for these FMDV specific mRNAs the VV capping system is probably capable of modifying most of the transcripts and that intrinsically a capped transcript, lacking an extensive or highly structured 5'NCR, is as efficient as a monocistronic mRNA containing an IRES element (as observed previously in transient expression assays, Belsham & Brangwyn, 1990). The correct initiation of
translation in the presence of a highly structured 5'NCR of over 700 nt containing several AUG codons indicates the FMDV IRES in this construct is functional.

It is noteworthy that the yield of empty capsids from the expressed FMDV proteins was much greater with the serotype A cDNA cassettes than from the type O cDNA. This closely mirrors findings from the analysis of empty capsid formation from FMDV infections of cells (Rweyemamu et al., 1979). This indicates that some structural difference in the capsid particles exists between these two serotypes which affects empty capsid assembly or stability rather than there being a difference in the efficiency of RNA replication or packaging. Knowledge of the three-dimensional structure of FMDV of both serotypes (Acharya et al., 1989; Curry et al., 1995; S. Curry, D.I. Stuart & A.M.Q. King, unpublished) should help in the identification of the key features underlying this difference. In this context, the observation that serotype A empty capsid particles, either produced in the VV system or in an infection by FMDV, are more stable at low pH than virions, is of interest (Curry et al., 1995). Perhaps, differences in pantemeric interactions underlie both effects.

The potential utility of these recombinant, non-infectious, FMDV empty capsids for diagnostic and vaccine purposes is apparent.

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