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DISTINCTIVE FEATURES OF FOOT-AND-MOUTH DISEASE VIRUS, A MEMBER OF THE PICORNAVIRUS FAMILY; ASPECTS OF VIRUS PROTEIN SYNTHESIS, PROTEIN PROCESSING AND STRUCTURE

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I. INTRODUCTION

Foot-and-mouth disease virus (FMDV) is responsible for one of the most economically important diseases of cloven-footed animals (this includes cattle, pigs and sheep). The disease is present in many parts of the world although it has been successfully eliminated or excluded from Western Europe, North America and Australasia. The control of the disease is achieved by vaccination with chemically inactivated preparations of virus. Seven distinct serotypes of the virus have been defined and many different subtypes. Some, as indicated by their names, have distinct geographical distributions, i.e. Asia 1 and the South African Territories (SAT) types 1, 2 and 3. Serotypes A, O and C are present across large areas of the world.

The FMD viruses constitute the genus aphthovirus of the picornavirus family. Other members of this family include poliovirus (PV) and other enteroviruses, the rhinoviruses (which cause the common cold) and encephalomyocarditis virus (EMCV, a cardiovirus). In common with other picornaviruses FMDV has a single-stranded RNA genome of positive sense containing a poly A tail at the 3′ terminus and a small virus encoded protein, VPg, is covalently attached to the 5′ terminus. The FMDV genome is approximately 8400 bases in length, slightly larger than all other picornaviruses, for example the PV genome is about 7400 bases long. The virus particle (25–30 nm in diameter) consists of a single copy of the genome encapsidated by 60 copies of four different virus encoded proteins. The three-dimensional structure of the virus has recently been determined by X-ray crystallography to atomic resolution (Acharya *et al.*, 1989). Complete genome sequences are available for type O, A

and C FMDVs and partial sequences are also available for the other serotypes and many strains within particular serotypes.

In order to replicate a virus particle has to enter a cell, uncoat and deliver its genome intact to the cellular translation machinery where it behaves as a mRNA and protein is produced. A complex pathway of proteolytic processing follows to produce the mature virus proteins. The genome also has to act as the template for replication by the virus encoded RNA polymerase so that both viral RNA and virus proteins are produced. New virus particles assemble and are released on cell lysis. FMDV is able to complete this cycle of events in about 4 hr in tissue culture and in the process may produce 100,000 particles from a single cell.

Although it is often considered that PV is the prototype virus within the picornavirus family it has become clear in the last few years that the molecular biology of PV and other enteroviruses differs significantly from that of FMDV and the cardioviruses in certain respects. Discussion of the molecular features of FMDV which distinguish it from other picornaviruses will be the major emphasis of this review. Other reviews (Rueckert, 1990; Stanway, 1990) covering picornaviruses discuss the general aspects of these viruses.

II. STRUCTURE OF THE FMDV GENOME

The structure of the genome of FMDV and the proteins it encodes are depicted in Fig. 1. The complete nucleotide (nt) sequence (8400 nt) of the O1Kaufbeuren (O1K) strain of FMDV has been determined (Forss *et al.*, 1984; Zibert *et al.*, 1990). The 5′ non-coding region (NCR) of FMDV is exceptionally long, it contains about 1300 nt; this may be compared with PV containing about 740 nt and typical cellular mRNAs which have 5′NCRs of 50–100 nt. As will be discussed below this region of the FMDV genome has functions distinct from those of a cellular mRNA, however the reason for the exceptional size of the 5′NCR of FMDV compared to other picornaviruses is not clear.

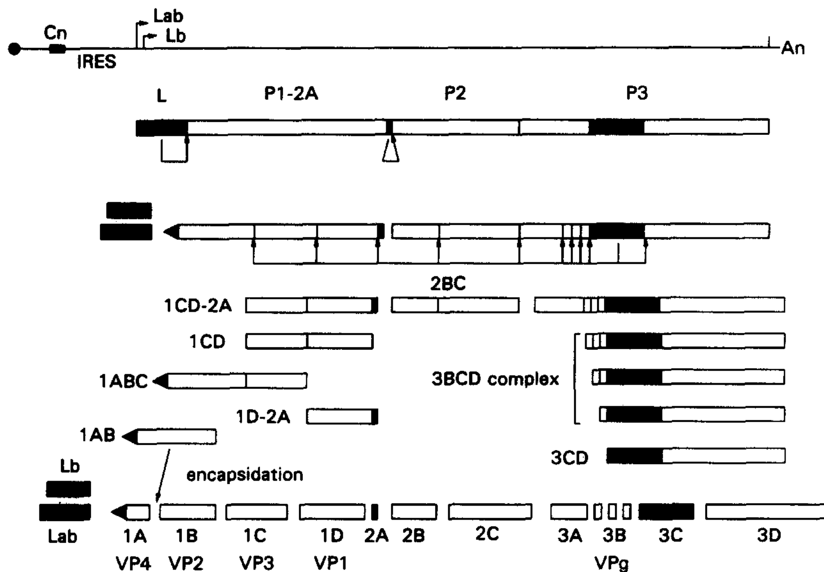

FIG. 1. The genome organization of FMDV and the proteins it encodes. The sequences involved in proteolytic processing are indicated by the solid shading. Various possible cleavage intermediates are not indicated largely for the sake of clarity. All the intermediates indicated have been observed (see Ryan *et al.*, 1989). The 5′ terminus of the genome is blocked by the virus protein 3B (VPg) and is indicated by a filled circle. The modification of the amino-terminal glycine residue of 1A (also in precursors) by myristate is indicated by a filled triangle. All of the cleavages requiring 3C are indicated as being mediated just by this protein but they may (also) be mediated by precursors of this protease.

The 5′ terminus of the genomic RNA is blocked by the small virus encoded protein 3B, frequently also still called VPg. This protein occurs in three different forms uniquely in FMDV. The 3B1 species is 23 amino acids long while 3B2 and 3B3 have 24 residues. All three species have been identified attached to viral RNA (King *et al.*, 1980) in virions.

The sequence of the first region (termed the S fragment) of the RNA from the 5′ terminus, about 400 bases in length, has been determined for a number of strains. The sequence has been predicted to form a large hairpin structure (Clarke *et al.*, 1987; Escarmis *et al.*, 1992) but no biochemical studies on the structure of this region of the genome have been reported. The characterization of a cloverleaf type structure at the 5′ terminus of PV (Andino *et al.*, 1990), comprising about 100 bases, involved in RNA replication suggests that an analogous structure may be found within other picornaviruses possibly including FMDV. Thus a reassessment of the secondary structure prediction may be required. No specific functions have been attributed to any part of this region of FMDV so far.

The second region of the genome is an extended, essentially homopolymeric, tract of cytidyl residues, termed the poly C tract. A few uridine residues are also present within these tracts. Only the cardioviruses and FMDVs have such a poly C tract which contains about 150–250 bases in many strains of FMDV. Its function is also unknown but recent studies suggest that this tract behaves differently in FMDV than in cardioviruses such as mengovirus. These studies are referred to in the following section.

The third part of the genome contains a further stretch of 5′ non-coding region (about 720 bases) which includes, close to the poly C tract, 3–4 (dependent on the strain examined) imperfect repeats which are predicted to form pseudo-knots (Clarke *et al.*, 1987). These structures were initially identified at the 3′ end of various plant virus RNAs (see review by Pleij, 1990). They have also been implicated in the process of ribosome frame shifting when located within the open reading frame of certain viral mRNAs (reviewed by ten Dam *et al.*, 1990). The function of the pseudo-knots in FMDV is totally unknown. In cardioviruses it has been suggested that pseudo-knots are also present within the 5′NCR but on the 5′ side of the poly C tract in contrast to their position in FMDV (Duke *et al.*, 1992).

Also within this region is the structure termed the internal ribosome entry sequence (IRES) comprising some 435 bases immediately upstream of the first AUG initiation codon (Belsham and Brangwyn, 1990; Kuhn *et al.*, 1990). This element is also predicted to have an extensive secondary structure and will be discussed in some detail later.

The major portion of the virus genome is a single very large open reading frame of 6996 nucleotides encoding a polyprotein of 2332 amino acids (type O, Forss *et al.*, 1984). The polyprotein may be considered as 4 different components, termed L, P1-2A, P2 and P3. In contrast to most picornaviruses two distinct initiation sites for protein synthesis, separated by 84 bases, are used in FMDV. The two AUG codons are in the same reading frame but initiation at the second start site produces a truncated version of the first component, the leader (L) protein. The complete polyprotein is never observed within infected cells since a complex series of proteolyic processing events occurs to produce the mature products indicated in Fig. 1. Some of these processing events occur extremely rapidly, probably while the translation process is occurring. The processing events are discussed further below. The P1-2A product is the precursor of the capsid proteins while the P2 and P3 precursors are processed to non-structural proteins involved in virus RNA replication and protein processing. The roles of some of these proteins are discussed further within this review, however the functions of several proteins, namely 2B, 2C and 3A have still not been identified. Protein 3C is the major protease (see Fig. 1) and 3D is the RNA-dependent RNA polymerase. It is possible that other functions remain to be identified either for some of the precursor proteins or for the mature products in addition to those already defined.

A short 3′NCR of 89 bases precedes a poly A tract. No role has so far been attributed to this region of the genome but it is of interest that a mutation within the 3′NCR of PV produced a temperature-sensitive virus (Sarnow *et al.*, 1986); the significance of this result remains to be established.

1. *Modifications to the Structure of the Genome*

Manipulation of picornavirus genomes has been readily accomplished since the construction of full-length cDNA clones which were capable of producing infectious virus when transfected into cells (Racaniello and Baltimore, 1981). The process has been greatly improved by the use of vectors containing promoter sites for RNA polymerases so that full-

length RNA transcripts can be produced. The infectivity of these transcripts is much higher than the cDNA clones themselves. In general the specific infectivity of the RNA transcripts is still rather less than the virus RNA, probably because of the presence of some vector sequences within the transcripts which have to be deleted during the replication process.

The presence of the poly C tract within FMDV inhibited progress towards the production of infectious cDNA for FMDV. However an infectious cDNA copy of FMDV has now been constructed using the O1K strain (Zibert *et al.*, 1990); this infectious clone contains just 32 C residues in the poly C tract. This number was something of a compromise between the number that could be stably maintained within a bacterial plasmid and that apparently required for viability. In the recovered viruses the poly C length was found to contain 60 or more residues. Very recently a second infectious cDNA based on the A12 FMDV cDNA has been assembled and a range of constructs containing poly C tracts from 2–35 C residues has been isolated. RNA transcripts prepared from each of these plasmids were infectious. Analysis of the genomes of the viruses recovered from the RNAs containing 6 or more C residues indicated that the poly C tracts had grown to 75–140 bases in length in agreement with the work on the type O infectious cDNA. In contrast the genomes of the viruses recovered from the transcripts containing just 2 C residues at the position of the poly C tract maintained this small tract. These viruses exhibited a microplaque phenotype and grew more slowly than the wild-type virus in BHK cells (E. Rieder and P. Mason, personal communication). Field isolates of FMDV generally have a poly C tract of about 150–200 bases. Multiple passage and attenuation of FMDV in tissue culture has been accompanied by reduced length (about 100 residues) of poly C tract (Harris and Brown, 1977). In contrast a type C FMDV (VR100) isolated after 100 passages of persistently infected cells had a poly C tract of 420 bases (the longest yet described) while that of its parental virus is only about 275 bases long. This change is the greatest difference between these two viruses (Escarmis *et al.*, 1992). The VR100 virus is greatly attenuated in cattle and mice (Diez *et al.*, 1991). However this virus is described as hypervirulent in tissue culture (de la Torre *et al.*, 1988) so it has two apparently opposite characteristics. It should be realized that a number of other substitutions have occurred between the VR100 virus and its parental CS8 strain (about 1% of nucleotides are different) and the contribution of each change to the virus phenotype is not yet clear. Interestingly recent studies on the cardioviruses have shown that deliberate reduction of the length of the poly C tract in mengovirus (another cardiovirus closely related to EMCV) (from 60 Cs down to about 10) considerably reduced the neurovirulence of the viruses in mice without inhibiting their ability to induce an immune response (Duke *et al.*, 1990). The poly C tract remained short in these viruses during propagation in both tissue culture and in mice. Such viruses may prove useful as prototype vaccines. The difference in stability of the introduced short poly C tracts within mengovirus compared to that within FMDV is unexplained.

The first application of the infectious copy of FMDV has been an analysis of the role of the three 3B proteins. It was shown that the virus is capable of replicating with only a single copy of 3B, however the full complement increases the fitness of the virus (Falk *et al.*, 1992). Each one of the 3B proteins can apparently act in FMDV replication but a reduced yield of RNA synthesis compared to the wild-type virus was observed.

III. PICORNAVIRUS PROTEIN SYNTHESIS

The RNAs from FMDV and from EMCV have long been recognized as very efficient mRNAs when assayed by translation in rabbit reticulocyte lysates. In contrast PV RNA is rather poorly and inaccurately translated in this system unless the lysate is supplemented with a HeLa cell extract. Within host cells all of the RNAs clearly must translate efficiently. Although these RNAs behave like mRNAs and in some respects resemble cellular mRNAs, e.g. the presence of the poly A tail at the 3′ terminus, in other respects picornavirus mRNAs differ significantly from cellular mRNAs and have to break the rules, identified by Kozak (see review by Kozak, 1989) for initiation of protein synthesis (Jackson *et al.*, 1990). The infection of cells by these viruses is also accompanied by inhibition of cellular protein synthesis and it is

now apparent that the picornavirus RNAs employ a distinct mechanism for the initiation of protein synthesis.

To appreciate the differences between the initiation of protein synthesis on picornavirus RNAs and that which occurs on cellular mRNAs I will briefly describe the relevant aspects of the process that is believed to occur normally within cells (for detailed reviews of this process see Kozak, 1989; Hershey, 1991). Cellular mRNAs contain a cap-structure at their 5′ termini which consists of 7-methyl G linked by a 5′–5′ linkage to the terminal base of the RNA. This cap structure is important for recognition of the mRNA (see Fig. 2). The cap-binding complex (eIF-4F) consists of 3 polypeptides. These are the alpha (25 kDa) (eIF-4E) (which has cap-binding activity), beta (eIF-4A) (which has ATP dependent helicase activity and gamma (called p220, with no known function) subunits. The complex is involved in the assembly of the initiation complex which includes the 40S small ribosome subunit which is then believed to scan along the mRNA until an AUG codon is encountered (usually within 50–100 bases from the 5′ terminus) when protein synthesis initiates. The context of the AUG is also important, a consensus sequence (A/G)XXAUG(A/G) for efficient initiation has been derived by analysis of numerous mRNA sequences and also by site directed mutagenesis (Kozak, 1989). Extensive secondary structure within the 5′NCR inhibits the translational efficiency of a mRNA presumably by slowing the scanning process.

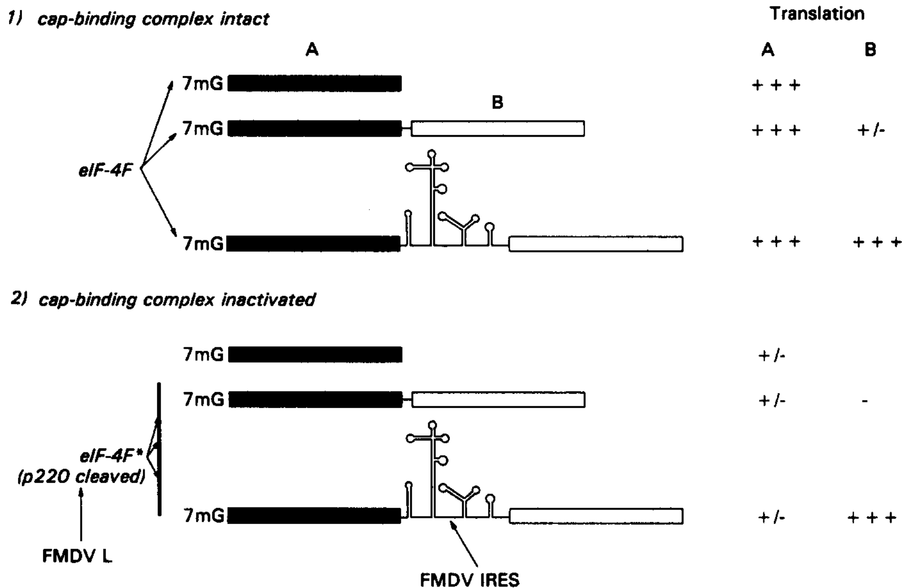

FIG. 2. Representation of the activity of the FMDV IRES in directing cap-independent initiation of protein synthesis. Panel (1) indicates the normal pattern of protein synthesis while panel (2) indicates the effect of the expression of the FMDV L protein. An analogous figure could have been drawn with the PV IRES and the effect of the PV 2A protein. The p220 component of the cap-binding complex eIF-4F is cleaved in the presence of either the FMDV L or the PV 2A proteins. The modified form of the cap-binding complex is indicated as eIF-4F* which no longer recognizes the cap structure.

Several features of the 5′NCR of picornaviruses preclude this standard mechanism of protein synthesis initiation. As mentioned above the genomes of picornaviruses have a virus protein attached to the 5′ terminus and no cap structure. Furthermore initiation of protein synthesis occurs several hundred nucleotides downstream from the 5′ terminus. This region is predicted to contain extensive secondary structure (Fig. 3). In FMDV the context of the first initiation codon (AUG 9 in type O) is quite poor and the other AUG codons present within the 5′NCR are apparently ignored. Many picornaviruses also suppress the initiation of translation of cellular mRNAs by inducing the cleavage of the p220 component of the cap-binding complex (Etchison *et al.*, 1982; Krausslich *et al.*, 1987; Devaney *et al.*, 1988). All of

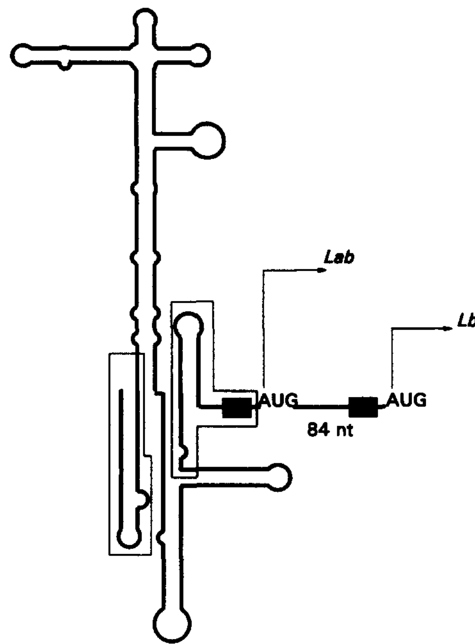

Fig. 3. Secondary structure prediction for the IRES of FMDV. The position of the two polyprimidine tracts upstream from the two initiation sites (producing the Lab and Lb forms of the first component of the polyprotein) are indicated by filled rectangles. An almost identical structure prediction can be made for the IRES of the cardioviruses (see Pilipenko *et al.*, 1989). The regions of the IRES identified as interacting with p57 are boxed.

this information has been known for several years and it was widely accepted that the translation of picornaviruses occurred by a distinct mechanism.

1. *Identification of the Internal Ribosome Entry Site*

The major step forward was achieved by Pelletier and Sonenberg (1988) and Jang *et al.* (1988) who demonstrated for PV and EMCV respectively that introduction of the 5′NCR from these viruses as the intergenic spacer in artificial bicistronic mRNAs directed efficient translation of the second open reading frame. These results led to the concept of internal initiation of protein synthesis becoming widely accepted. This activity has now been demonstrated for a member of each genus of the picornavirus family including FMDV (Belsham and Brangwyn, 1990; Kuhn *et al.*, 1990; Brown *et al.*, 1991). The translation of both open reading frames from bicistronic mRNAs has become the favoured assay for internal initiation and allows the ready analysis of both cap-dependent and cap-independent translation directed by the 5′NCR of the picornaviruses at the same time. It has been demonstrated that the translation directed by the picornavirus 5′NCRs occurs totally independently from the translation of the first open reading frames. The element within the 5′NCR which confers this activity is now generally called the "internal ribosome entry site" (IRES) but it has also been termed the "ribosome landing pad" in PV. A scheme illustrating the ability of an IRES to direct a novel mechanism of initiation of protein synthesis is shown in Fig. 2.

The IRES elements in FMDV and the cardioviruses are predicted to form very similar secondary structures (Pilipenko *et al.*, 1989) and they probably operate by a very similar mechanism, although the nucleotide similarity is only about 50%. The element in PV although functionally analogous has no apparent sequence similarity to that of FMDV and EMCV and the secondary structure predictions are also unrelated (Jackson, 1990; Jang *et al.*, 1990). It is also apparent that the 3′ ends of the IRES of FMDV and EMCV are close to

the initiation codon whereas in PV over 100 nt are present between the 3′ end of the structure and the start site. In rhinoviruses much of this sequence is deleted.

Studies on the 5′NCR of FMDV using artificial bicistronic mRNAs have shown that a region of 435 bases immediately upstream from the first initiation site of FMDV is necessary and sufficient to direct internal initiation of protein synthesis within cells (Belsham and Brangwyn, 1990). Furthermore when cap-dependent protein synthesis was inhibited by the co-expression of the FMDV L protein (which induces the cleavage of the p220 cap-binding complex component) the expression of the first open reading frame was almost abolished while the expression of the open reading frame following the FMDV IRES continued. In other studies deletion analysis of the FMDV 5′NCR suggested similar limits for the FMDV IRES using *in vitro* translation assays (Kuhn *et al.*, 1990). The only disparity in the data between the two studies on FMDV lies in the requirement for sequences at the 5′ end of the IRES. The *in vitro* translation data suggested that the sequence on the 5′ side of an XbaI site some 400 bases upstream of the initation codon could be deleted with only a modest drop in activity (50%). However Belsham and Brangwyn (1990) showed that a construct expressing a bicistronic mRNA in which the IRES lacked the sequences on the 5′ side of this XbaI site failed to express the product from the second open reading frame within cells. Re-examination of this question using sensitive reporter genes (rather than metabolic labelling and immunoprecipitation) has demonstrated a low level of internal initiation directed by the element lacking the 35 bases upstream of the XbaI site (J. Drew and G. J. Belsham, unpublished data) within cells. Studies on the EMCV IRES, using *in vitro* translation assays of monocistronic transcripts, showed that removal of sequences in an analogous position caused a large drop in activity. However further loss of sequence which removed the residual sequences from the particular stem loop structure resulted in partial restoration of activity (Duke *et al.*, 1992). Other studies conflict with these results. Both Evstafieva *et al.* (1990) and Jang and Wimmer (1990) found that the 5′ terminal stem removed by Duke *et al.* (1992) is required for activity. Clearly further work is required to clarify this issue. These inconsistencies may indicate that partial residual stem loop structures, in close proximity to the IRES, may interfere with its function. Slightly different deletions may give conflicting results depending on the nature of the interference. No evidence has been obtained for the FMDV/cardiovirus IRES that any of the internal stem-loop elements are dispensible for IRES activity. However in PV deletion of one internal stem-loop structure seems not to greatly affect IRES activity (Nicholson *et al.*, 1991; Percy *et al.*, 1992). This particular structure is not present within the 5′NCR of a related enterovirus, namely bovine enterovirus (Earle *et al.*, 1988). There is a need for studies on the physical structure of the IRES elements including identification of regions within the predicted secondary structures which interact.

One feature which is conserved between the IRES element of PV and that found in EMCV and FMDV is the presence of a polypyrimidine tract. This feature is about 20 bases upstream from the initiation codon in FMDV and the cardioviruses and a similar distance from a conserved AUG codon in PV, however in this case the AUG codon is not the initiation site. The significance of this tract is unclear, some studies in PV indicate that the introduction of G residues into the tract are deleterious (Nicholson *et al.*, 1991). However other studies converting all the residues of the polypyrimidine tract to A residues had only a modest effect on the efficiency of the EMCV directed translation using both *in vitro* and *in vivo* expression systems (A. Kaminski, G. J. Belsham and R. J. Jackson, unpublished observations).

2. *Mechanism of Internal Initiation*

So how does the IRES work? It is clear that no virus proteins are required for its function since it will operate independently from any virus coding sequences. Studies have therefore focused on cellular proteins which interact with the IRES. Luz and Beck (1990, 1991) have identified two locations within the FMDV IRES which interact with a p57/p58 protein using UV cross-linking to specific RNA species. It is probable that this protein corresponds to the protein found by similar studies to interact with the EMCV IRES (Jang and Wimmer, 1990) although in this case only a single site of binding has been detected (corresponding to the 5′ proximal site identified in the FMDV IRES). This protein seems to be unrelated to

previously identified protein synthesis initiation factors. Recent studies (R. Jackson, personal communication) have identified this protein as the polypyrimidine tract binding protein (Garciablanco *et al.*, 1989). This protein has been identified as having a role within the nucleus in the process of RNA splicing. In PV, evidence for interaction of the IRES with a protein termed p52 has been obtained (Meerovitch *et al.*, 1989). Interaction with this protein appears both to enhance translation and to improve the extent of correct initiation of protein synthesis in rabbit reticulocyte lysate. This protein has been very recently identified as La (N. Sonenberg, personal communication). This protein has been implicated in the maturation of RNA polymerase III transcripts within the nucleus. Hence the two proteins identified as interacting with the IRES elements of the picornavirus RNAs located within the cytoplasm have both been previously assigned functions within the nucleus. Further studies are required to determine the function of these proteins within the cytoplasm and to assess their role in the activity of the IRES elements.

Although it has become apparent that a complex RNA structure is required for the IRES to function, an unexpected phenomenon has been observed in studies on the PV IRES. Deletion of certain domains within this element completely abolished its ability to direct the translation of the second ORF (CAT) from a bicistronic construct when assayed alone, however when the construct was cotransfected with a full-length PV cDNA, the expression of CAT activity was again observed (Percy *et al.*, 1992). The expression of the full-length PV cDNA clone was accompanied by the inhibition of cap-dependent translation. The enhanced CAT expression was interpreted as suggesting that some of the sequences within the IRES were dispensible within cells in which cap-dependent translation was abolished (Percy *et al.*, 1992). It was presumed that additional initiation factors that would otherwise be utilized by capped transcripts became available. Recent studies have extended these observations and demonstrated that co-transfection with a plasmid containing just the PV 5′NCR unlinked to any protein coding sequence also enhances the translation directed by the defective PV IRES (D. M. Stone, J. W. Almond, J. K. Brangwyn and G. J. Belsham, unpublished data). Thus the restoration of IRES activity is achieved in the absence of any virus protein or any inhibition of host cell protein synthesis. We currently interpret these data as indicating complementation at the level of the RNA between the wt IRES and the defective IRES within the bicistronic mRNA. Analogous data have been generated using the FMDV IRES, which as mentioned above has an IRES very different in structure and sequence from that of PV (J. Drew and G. J. Belsham, unpublished results). Control experiments and the efficiency of the process (typically at least 20% of wild-type IRES activity) appear to rule out any recombination event as the basis for these observations.

Since cells appear to be equipped to interact with viral IRES elements, an important question was whether cells make use of such motifs themselves and hence whether viruses have merely exploited an existing cellular function. The first evidence for the presence of a cellular IRES was presented by Macejak and Sarnow (1991) who showed that within the 5′NCR of the mRNA encoding BiP (heavy chain binding protein, or the 78 kDa glucose regulated protein, grp78) an element was present which displayed IRES activity. This was consistent with the continued expression of this protein in poliovirus infected cells when the expression of most cellular proteins is abolished (Sarnow, 1989). Cap-dependent translation is abolished during heat shock and also during normal cell division (mitosis); one mechanism for continuing the synthesis of specific proteins during this period of the cell cycle would be the presence of an IRES element within the 5′NCR of their mRNAs. Hence there is considerable interest in identifying other cellular mRNAs which contain such an element.

3. *Selection of Two Distinct Initiation Sites*

All seven serotypes of FMDV have conserved the feature of utilizing two distinct initiation sites so that two forms of the L protein are generated (Sangar *et al.*, 1987). The ratio of synthesis does differ between different serotypes. The studies of Kaminski *et al.* (1990) on EMCV showed that a very precise initiation site selection system is mediated by this IRES. In constructs containing the EMCV IRES, ribosomes fail to recognize AUG10 which is just 8 bases upstream of the usual initiation site AUG11. In the absence of this IRES, efficient

utilization of the AUG10 codon could be observed. In both cases downstream AUG codons were poorly utilized. In the light of the presumed similarity between the mechanism of action of the EMCV IRES and that of FMDV it was of interest to understand how the two different initiation sites in FMDV were recognized. In FMDV type O the two initiation sites are separated by 84 bases and both sites are used with similar efficiency. Experiments were performed to examine the selection of the different initiation sites in FMDV under a variety of conditions (Belsham, 1992). It was shown that initiation of protein synthesis occurred at both sites on RNAs containing either just 60 bases upstream of the first initiation site or the complete IRES element. The presence of the IRES within the RNAs slightly biased the selection of the AUGs towards the second start site. Secondly the inhibition of host cell protein synthesis, achieved by the co-expression of the intact L protein, did not modify the site selection on RNAs containing the IRES. In the absence of the IRES expression of the L protein abolished synthesis from both start sites as expected. In type C FMDV an upstream AUG just 8 bases upstream of the first initiation codon is present, analogous to the situation in EMCV. However in this case, the open reading frame following this upstream AUG is very short, only three amino acids would be joined. Surprisingly the presence of the upstream mini-ORF in the type C constructs did not affect the site selection either in the presence or absence of the IRES, probably as a result of the very short ORF following this upstream AUG (Belsham, 1992).

An additional experiment was aimed at determining the mechanism by which ribosomes reached the second start site. Two possibilities were considered (Fig. 4). Firstly ribosomes could start recognizing the RNA immediately upstream of the first initiation site, as in EMCV, but only a portion of them may recognize the first site so that a proportion of the ribosomes would scan through the region to the next start site (Fig. 4, panel b). A second possibility was that a second site of ribosome recognition is present in FMDV (Fig. 4, panel c). To distinguish between these possibilities two additional AUG codons were introduced, in frame, between the two start sites. Analysis of this mutant clearly demonstrated the utilization of these two additional start sites (as in Fig. 4, panel b) indicating that ribosomes do scan through this region (Belsham, 1992). In EMCV the context of the AUG11 matches well to the Kozak consensus sequence for efficient initiation whereas the context of the first initiation codon of type O FMDV is poor. Recently Davies and Kaufman (1992) have shown that modification of the good context of AUG11 in EMCV promoted read through to a downstream AUG codon. Thus it appears that ribosomes start recognizing both the FMDV RNA and the EMCV RNA just to the 5′ side of the start site and then scan as for normal mRNAs. In EMCV the scanning distance to AUG11 is extremely short. In PV and other enteroviruses within the picornavirus group it appears that ribosomes start recognizing the RNA over 100 nucleotides upstream from the initiation codon. The mechanism by which the ribosomes reach the initiation codon is not fully established in this instance since only fairly drastic modifications to the structure of the intervening sequence have been made. However the inclusion of an AUG codon into a 72 base insertion was deleterious to PV translation whereas the 72 base insertion lacking an AUG had little effect (Kuge *et al.*, 1989). Hence a classical scanning mechanism would be consistent with these observations.

4. *Inhibition of Host Cell Protein Synthesis*

As mentioned above, infection of cells by PV induces the cleavage of the p220 cap-binding complex component and this inactivates the complex. This process is mediated by the PV 2A protease, however the p220 cleavage activity is apparently separable from 2A, hence it appears that the process is indirect (Lloyd *et al.*, 1986). A further feature of this process is a recently identified requirement for eIF3 when using purified preparations of PV 2A and p220. The initiation factor eIF3 is a multi-subunit complex and it is not yet clear how this protein is involved in this process. No modification of eIF3 by 2A has been observed (Wyckoff *et al.*, 1992).

The cleavage of p220 has not been observed in cardiovirus infected cells (Mosenkis *et al.*, 1985) and inhibition of host cell protein synthesis appears to be dependent on the cell line

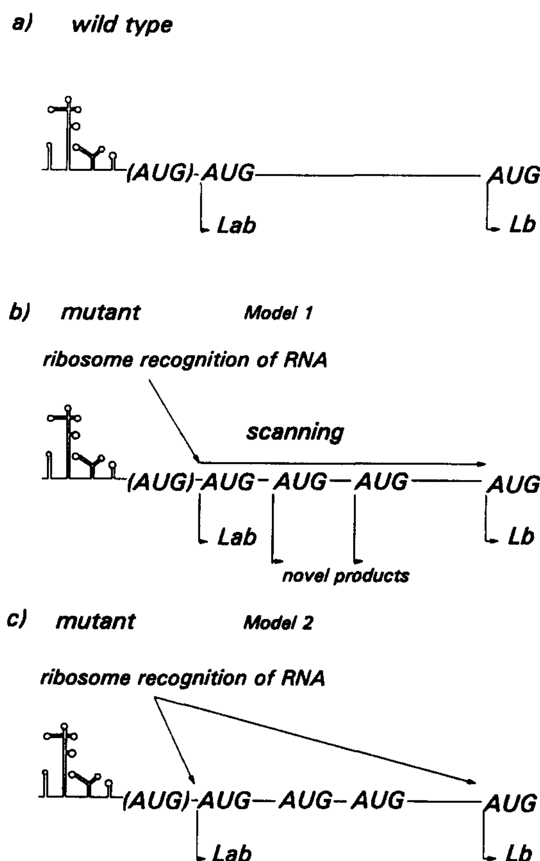

FIG. 4. Models for the recognition of the two initiation sites in FMDV. Panel (a) indicates the utilization of the two initation sites on the wild type virus RNA. Panels (b) and (c) indicate the two different predictions made for the translation of a mutant RNA in which two additional AUG codons were introduced between the two authentic initiation codons. The results from these analyses (Belsham, 1992) were that the extra AUG codons were efficiently utilized indicating that the model shown in panel (b) is correct.

(Jen and Thach, 1982). Modification of the ionic conditions within cells may be involved in the inhibition of protein synthesis achieved by EMCV (Alonso and Carrasco, 1981).

In contrast the inhibition of host translation is observed with FMDV infection and this is accompanied by cleavage of p220 (Devaney *et al.*, 1988). It has been shown that this effect is induced by the L protein as indicated above (Vakharia *et al.*, 1987; Belsham and Brangwyn, 1990; Medina *et al.*, 1993). It has been reported that the p220 cleavage products induced by FMDV infection differ in mobility from those induced by PV infection (Lloyd *et al.*, 1988; Kleina and Grubman, 1992). This could suggest that the mechanisms of p220 inactivation used by PV and FMDV are totally distinct but probably reflects differences in the species of cell used for the preparation of the different extracts. Indeed recent studies transiently expressing the PV 2A protein and the FMDV L protein within the same cell type, from plasmid cDNA, produced p220 cleavage products with the same mobilities (Medina *et al.*, 1993). No apparent homology exists between PV 2A and the FMDV L protein. Both proteins also display cleavage activity to release themselves from the structural protein precursor (see below). It has to be established whether the cleavage of p220 by FMDV is also indirect and also whether eIF3 is required. Mutants of PV have been constructed with amino acid substitutions in 2A which are deficient in p220 cleavage activity but are still viable, this implies at least a partial independence of the proteolyic *cis* cleavage from the p220ase induction. It has to be determined whether the p220 cleavage induction by the FMDV L protein is separable from the L–P1 cleavage activity.

The Lb protein formed by initiation of protein synthesis at the second start site is sufficient

to induce p220 cleavage (Medina *et al.*, 1993) and the inhibition of host cell protein synthesis. Modification of the Lb initiation codon in constructs encoding Lab and Lb generated plasmids capable of only expressing a mutant Lab protein. The mutant Lab products also efficiently cleaved the L/P1 junction and inhibited cap-dependent translation. Thus no difference in function has been detected between the two forms of L and hence the significance of the two start sites which are conserved in all seven serotypes of FMDV remains unknown. It is of interest that it has recently been reported that the PV 2A protein has a transactivation effect on the PV IRES which is independent from its ability to inhibit cap-dependent translation (Hambidge and Sarnow, 1992). It may be that additional roles for FMDV Lab and Lb remain to be identified which may differ between these two proteins.

IV. POLYPROTEIN PROCESSING

1. *Proteolysis*

At least four different types of protein processing are involved in the conversion of the polyprotein precursor to the mature polypeptides. Figure 1 depicts 15 mature polypeptides but other partially digested intermediates may be stable and functional, hence the total number of different species generated is rather greater than this. The different cleavages are those mediated by L, 3C, 2A and that which occurs on encapsidation of the RNA when cleaving the capsid protein precursor 1AB to 1A and 1B. A major difference between the processing pathways of the entero-/rhinoviruses and the cardio-/aphthoviruses is the position of the 3C-independent cleavage around 2A. In PV it has been shown that the cleavage mediated by 2A occurs at the P1/2A junction. In contrast, in EMCV and FMDV a 2A mediated cleavage occurs at the 2A/2B junction and the P1/2A junction is cleaved by 3C (see reviews on polyprotein processing by Jackson, 1989; Palmenberg, 1990).

(a) *The L/P1 cleavage*

For most picornavirueses the cleavage around 2A is the primary cleavage (Jackson, 1989; Palmenberg, 1990), however in FMDV the first processing event is uniquely the L/P1 cleavage. Amongst the picornaviruses only the cardioviruses and FMDV have an L protein and only in FMDV is the L protein a protease (Strebel and Beck, 1986). This cleavage occurs in *trans* and almost certainly also in *cis*, although this has not been rigorously proved. Little of the P1 precursor needs to be synthesized for the cleavage to occur (Ryan *et al.*, 1989). In cardioviruses the L/P1 junction is cleaved by 3C. Little characterization of the FMDV L protease has been performed. No strong similarity with other proteases has been identified but a thiol protease inhibitor has been shown recently to inhibit the function of L (Kleina and Grubman, 1992). Gorbalenya *et al.* (1991) have indicated a very limited relationship of L to cysteine proteases. As mentioned above both the smaller L protein (Lb) and the larger Lab species are fully competent to cleave the L/P1 junction in *trans* (Medina *et al.*, 1993).

(b) *The 2A/2B cleavage*

The second very rapid cleavage event within the FMDV polyprotein occurs at the 2A/2B junction. Within the context of native FMDV proteins this junction always appears completely cleaved even using *in vitro* translation assays (Ryan *et al.*, 1989, 1991). It has been shown that this cleavage is independent of both L and 3C. In EMCV the 2A/2B cleavage is attributed to the 2A protein (143 amino acids), however in FMDV only a 16 amino acid peptide is present between the C-terminus of 1D and the N-terminus of 2B. It is noteworthy that this peptide is closely related to the C-terminus of the cardiovirus 2A proteins and deletion of the amino-terminal two-thirds of the EMCV 2A did not block its function (Palmenberg *et al.*, 1992). Recent studies have shown that introduction of a 19 amino acid segment, including the FMDV 2A sequence, into a totally foreign protein (influenza virus haemagglutinin) induces efficient cleavage of the novel protein at the 2A/2B junction (G. P. Thomas and M. D. Ryan, unpublished observations). It has not yet been proven whether the 2A/2B junction is very efficiently recognized by host proteases in a wide variety of host cells or whether the 16 amino acids truly represent a very small *cis*-acting protease.

Palmenberg *et al.* (1992) state that the tetrapeptide NPGP (corresponding to the conserved cleavage site in both FMDV and EMCV) spontaneously cleaves at pH 8.5. This suggested that this rare sequence might be unstable, however this cleavage produces NP and GP. In contrast the cleavage in the polyprotein occurs at the NPG/P junction, suggesting a different mode of cleavage.

(c) *Cleavage of 1AB on RNA encapsidation*

The cleavage of the capsid protein precursor 1AB only occurs on encapsidation of the virion RNA to produce virus particles. The mechanism of this process is so far unknown. Following the determination of the crystal structure of poliovirus (and rhinovirus) it was suggested that a serine residue (ser 10) within 1B could act as a nucleophile for the reaction. However when the structure of FMDV was solved no appropriate residue was present in a suitable position (Acharya *et al.*, 1989). Furthermore substitution of ser 10 in 1B of poliovirus had no effect on poliovirus viability clearly proving that this residue was not essential for this process (Harber *et al.*, 1991). Hence the mechanism of 1AB cleavage remains unresolved.

(d) *3C mediated processing*

Apart from the processes described above, all other proteolytic processing events within the picornavirus polyprotein require the 3C protease. In PV it is clear that at least some of the cleavages (those within the capsid precursor) require the precursor 3CD (Jore *et al.*, 1988; Ypma-Wong *et al.*, 1988), the 3C protein alone is insufficient. However in FMDV all the capsid protein processing events to produce 1AB, 1C and 1D can be mediated by 3C alone (Vakharia *et al.*, 1987; Ryan *et al.*, 1989; Belsham *et al.*, 1990). The details of P2 and P3 processing have not yet been fully elucidated in FMDV or other picornaviruses. There may be mechanisms which control the series of potential processing pathways if different intermediates are required for different functions either simultaneously or at different times in the life cycle of the virus. Evidence of strong specificity in the order of some cleavages within the P2 and P3 regions of PV has recently been presented (Lawson and Semler, 1992).

2. *Myristoylation*

A second form of protein processing that occurs on FMDV (and other picornavirus) proteins is the post-translational modification of the capsid precursor P1-2A so that the N-terminal glycine residue is blocked by a myristate moiety (Chow *et al.*, 1987). Processing of the P1-2A yields the myristate attached to N-terminus of 1AB and subsequently on encapsidation to 1A. Modification of the glycine to other residues, incapable of being modified by this reaction, is lethal to PV (Marc *et al.*, 1989; Krausslich *et al.*, 1990). The initial experiments on PV using *in vitro* translation studies suggested that the lack of myristoylation inhibited proteolytic processing of the P1 precursor. However these results are at variance with more recent studies performed in cells with both PV and FMDV where apparently normal capsid protein processing is observed in the absence of this modification (Marc *et al.*, 1990; Belsham *et al.*, 1990, 1991; Lewis *et al.*, 1991). PV RNA transfection experiments followed by sucrose gradient analyses showed that within a 12S fraction PV 1B was formed from the myristoylation negative RNA transcripts, indicating that 1AB cleavage (concomitant with RNA packaging) had occurred (Marc *et al.*, 1990). This suggests that assembly to the capsid structure and packaging of the RNA are not dependent on myristoylation but that the stability of the capsid is adversely affected in the absence of this modification. Ansardi *et al.* (1992) recently demonstrated that the production of PV empty capsid particles from expressed P1 and 3CD is also dependent on myristoylation. This result may also reflect instability of the non-myristoylated empty capsid or else it is not clear how the PV 1B protein is generated in the experiments of Marc *et al.* (1990). Such direct studies have not been performed on other picornaviruses but some studies suggest that the results may differ in other systems. Indeed using FMDV cDNA Lewis *et al.* (1991) expressed a P1-2A + 3C cassette (with an additional foreign four amino-acids fused to the amino-terminus of

1A) in *E. coli* and observed a low level of assembly into particles sedimenting at 70S (indicative of empty capsids). These particles contained 1C, 1D and a modified form of 1AB. Due to the presence of the additional residues at the amino-terminus no myristoylation would be possible, furthermore the bacterial host used does not perform this modification.

Hepatitis A, another member of the picornavirus family, has two potential initiation sites separated by just 6 bases. It has recently been demonstrated that each of these sites can be used by the virus. Surprisingly modification of the amino-terminus of the polyprotein resulted in a modified version of the capsid protein precursor 1AB being expressed within cells infected with the mutant viruses. The consequence of this result is that the putative myristoylation signal within the capsid precursor is not used on protein 1AB since this would require removal of some of the terminal sequences (a presumed leader protein). If myristoylation of the 1A protein does occur, for the assembly of the virions, then it must occur when encapsidation and 1AB cleavage occurs (Tesar *et al.*, 1992). However no evidence for myristoylation of Hepatitis A virus 1A protein has been presented. Thus within the picornavirus family there are different requirements for myristoylation and further studies are needed on FMDV and the cardioviruses to establish the importance of its role in these systems.

V. VIRUS STRUCTURE

The 3D structure of FMDV has been determined at atomic resolution (Acharya *et al.*, 1989) (see Fig. 5). The basic organization of the particle follows that of the other picornaviruses published previously (Rossmann *et al.*, 1985; Hogle *et al.*, 1985). An ironic twist to these studies was that one of the most intensively studied regions of FMDV, the region of 1D (VP1) between residues 135–158 (the βG–βH loop) was not resolved (Fig. 5). This region has been shown to represent one of the antigenic sites of the virus (see below) and synthetic peptides including this region are capable of inducing protection against the virus in animals (DiMarchi *et al.*, 1986). The lack of resolution of this region of the virus is due to some disorder (or flexibility) in the structure of this region.

Recently it has been possible to resolve the structure of this βG–βH loop by addition of the reducing agent dithiothreitol to crystals (Logan *et al.*, 1993) (see Fig. 5, panel d). The reduction of the disulphide bridge between cys 134 of 1D and cys 130 of 1B allows the loop to adopt a more stable conformation in the crystal. It seems probable that the loop will still have flexibility in the particle itself. A second disulphide bridge between cys 7 residues in 1C located around the 5-fold axis of symmetry is also present in the oxidized virus. Since the virus is assembled under reducing conditions, within cells, it is probable that the reduced form of the virus is the native state and that which is normally responsible for infecting cells.

Another feature within the 135–158 region of 1D is an amino acid triplet RGD (arginine–glycine–aspartate) which is a motif found in a number of cell attachment proteins and recognized by some members of the integrin family. Most, but not all, strains of FMDV have conserved this motif. It has been suggested that this region represents the cell attachment site for FMDV and indeed this motif appears exposed in the reduced form of the virus when the βG–βH loop is resolved (see Fig. 5, panel d). Recently the cellular receptor for echovirus 1 (another picornavirus) has been identified as VLA-2, which is a member of the integrin family (Bergelson *et al.*, 1992). Fox *et al.* (1989) showed that certain synthetic peptides containing the RGD sequence inhibited FMDV binding to cells. However quite high concentrations (>10 mM) were required and it is not certain that the virus binding assay used was measuring binding of the virus to the cellular receptor involved in the internalization of the particle. Other members of the picornavirus family have a "canyon" in PV and rhinovirus (Rossmann *et al.*, 1985) or a "pit" in mengovirus (Luo *et al.*, 1987) on the surface of the virion. It is believed that the cellular receptors for the virus interact with residues at the side and base of this feature. The "canyon hypothesis" which was formulated in the light of these findings suggests that this arrangement allows for change on the surface of the virion (under antibody selection pressure) without affecting the antibody inaccessible site on the virus which has to interact with the cell. If the cellular receptor for FMDV recognizes the RGD motif, which is an exposed feature, then this hypothesis does not apply to this

virus. It has been suggested that the high variability of residues flanking the RGD motifs permits conservation of the RGD motif while allowing antigenic change (Fry *et al.*, 1990).

Both the major group rhinovirus receptor and the poliovirus receptor have been identified. Both proteins are members of the immunoglobulin superfamily. The rhinovirus receptor is ICAM-1 (Greve *et al.*, 1989) while the PV receptor has not been characterized previously (Mendelsohn *et al.*, 1989).

Further work is required to establish the identity of the cellular receptor for FMDV and to ascertain the nature of the interaction of the virus particle with it.

A characteristic of FMDV is its extreme lability when exposed to even mildly acidic conditions; dramatic loss of infectivity occurs on exposure to pH 6.5 or below. It has been suggested that His residues at the protomer interfaces may mediate this instability (Acharya *et al.*, 1989) but no studies have been reported to verify this. The isolation and characterization of acid-resistant mutants may help to identify the determinants of low pH instability.

1. *Antigenic Structure*

In recent years the analysis of antigenic sites of picornaviruses has been performed using panels of neutralizing monoclonal antibodies (Mabs) to select and screen Mab-resistant mutants. Sequence analysis of such mutants has identified distinct clusters of residues within each antigenic site which are considered to lie within the contact area of the antibodies on the virus. Work on PV (reviewed in Minor, 1990), HRV14 (Sherry *et al.*, 1986) and FMDV (Xie *et al.*, 1987; Thomas *et al.*, 1988; Kitson *et al.*, 1990) have identified 3–4 distinct antigenic sites in each virus. The location of the residues identified by sequence analysis of Mab escape mutants of type O1K FMDV (Xie *et al.*, 1987; Kitson *et al.*, 1990) have been mapped onto the 3D structure of the virus as determined by X-ray crystallography and they are shown in Fig. 5 (panel b). Two sites are located on 1D (VP1). Site 1 includes residues within the 140–160 (βG–βH loop) region (not visualized in the initial X-ray structure determination) and residue 208 close to the carboxy-terminus (residue 213). The carboxy-terminus of each 1D molecule lies across its neighbouring protomer and hence the interaction between these two parts of 1D actually represents interaction between two different molecules of this protein (see Fig. 5, panel c). The involvement of both the βG–βH loop and the carboxy-terminus in a single antigenic site presumably underlies the improved effectiveness of the 140–160 peptide as a vaccine when linked to the 200–213 region (DiMarchi *et al.*, 1986). Antigenic site 3 is on the βB–βC loop of 1D involving sequences between residues 42–47. Site 2 was identified as involving the βB–βC loop of 1B (VP2) (residues 72–77) and the adjacent βE–αB loop (residue 131). The antigenic site 4 includes residues 56–58 of 1C (VP3) within an insertion within the βB of this protein. Parallel studies on the antigenic sites of A10 FMDV provided evidence for a similar distribution of antigenic sites on this virus too (Thomas *et al.*, 1988).

Parry *et al.* (1990) isolated Mab-resistant mutants of FMDV strain O1BFS and found mutations within the βB–βC loop of VP1 analogous to those defined by Kitson *et al.* (1990) as being within site 3. However, using synthetic peptides, Parry *et al.* (1990) had mapped the binding sites of the Mabs used to isolate these mutants to the 140–160 loop of VP1. They therefore proposed that mutations within one region of this protein were perturbing the antigenicity of the virus by altering the conformation of a separate region of the protein (so-called "action at a distance"). Crystallographic analyses of these mutants also showed that some residues within the 140–160 region which were not resolved in the wild-type virus structure were visible in the mutant structures which was interpreted as support for this effect. No good evidence for "action at a distance" has been obtained using other picornavirus systems (Minor, 1990) and other studies have produced results which conflict with the conclusions of Parry *et al.* (1990). One approach has been to analyse the properties of the regions identified as antigenic sites of FMDV in isolation from other FMDV sequences. The construction of PV/FMDV chimaeras has been successful in this area. The insertion of the βG–βH loop of FMDV into the βB–βC loop of PV VP1 produced a chimaeric virus which could be neutralized by anti-FMDV antisera (Kitson *et al.*, 1991). It

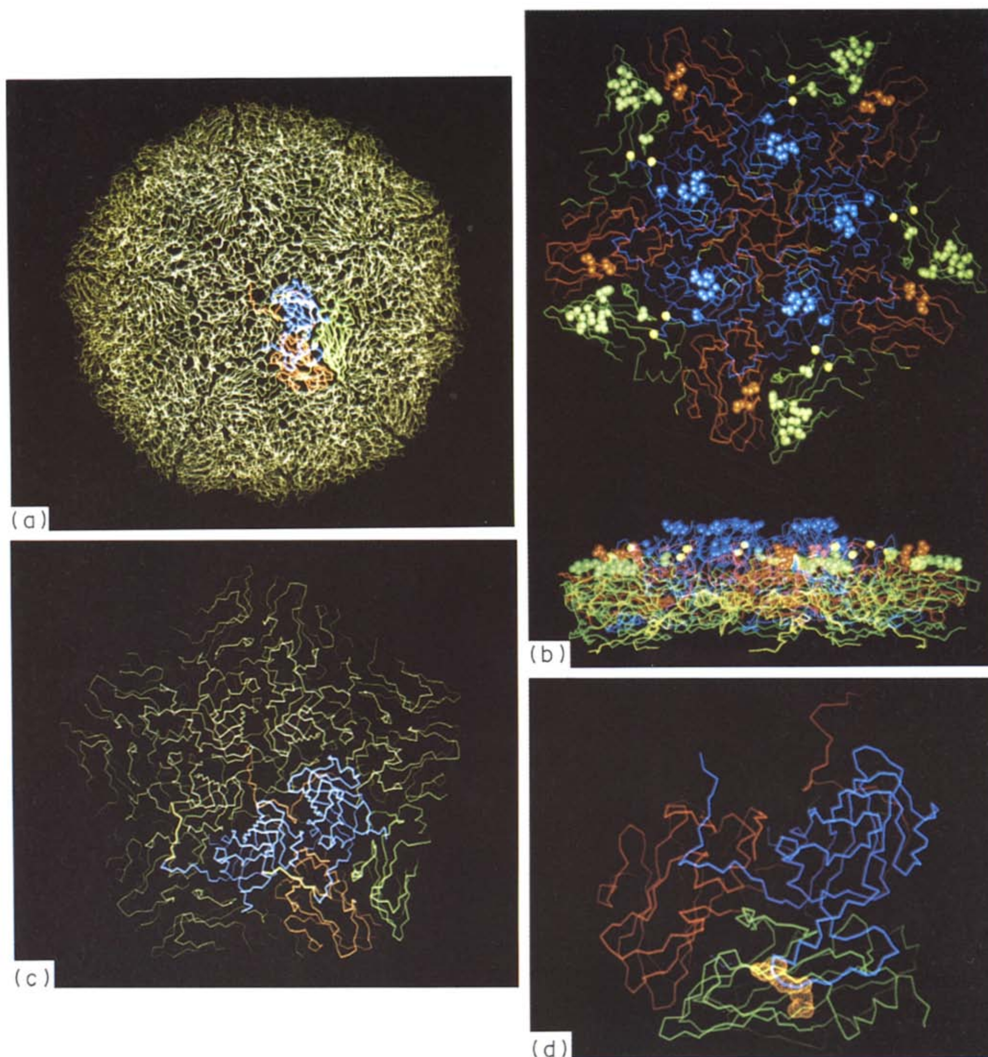

FIG. 5. Panel (a) shows the 3D structure of FMDV. A single protomer is highlighted derived from a single P1-2A precursor. The 1D (VP1) sequences are indicated in blue, the 1C (VP3) sequences are in red and the 1B (VP2) residues are shown in green. Sixty protomers constitute the capsid. Panel (b) shows the location of residues associated with specific antigenic sites on type O FMDV. Data presented by Kitson *et al.* (1990) are indicated on a pentamer (composed of five protomers), plan and side views are shown. Each of the four antigenic sites is composed of a distinct cluster of residues exposed at the surface of the capsid. In this representation the residues 144, 148, 154 of 1D identified within site 1 are not shown since the βG–βH loop was not visualized in the initial determination of the structure. The termini of the available information at residues 134 and 157 within protein 1D are indicated. Panel (c) shows the interaction between the carboxy terminus of 1D from one protomer with the 1D sequence of the adjacent protomer. A single pentamer is shown. The carboxy terminal residues (200–208) of one molecule of protein 1D are highlighted in yellow while the rest of the 1D molecule and its neighbour are depicted in blue as above. It should be noted that the sequence of 1D extends to 213 but the extreme terminus is not visualized in the structure. Panel (d) shows the structure of the βG–βH loop of 1D identified in the reduced form of the virus. The panel shows the structure of a single protomer. The RGD motif (residues 145–147) within the βG–βH loop is indicated by the highlighted residues. The residues 144 and 148 identified as key residues for the recognition of the virus by site 1 specific neutralizing monoclonal antibodies flank this motif.

was therefore possible to select antigenic mutants of this virus which showed a sequence modification corresponding to the FMDV residue 148. This residue has previously been identified as a key residue within this antigenic site (Kitson *et al.*, 1990). Hence the PV/FMDV chimaeras are capable of presenting epitopes in a functional form which closely reflects their behaviour within FMDV itself. These studies also provided independent evidence for the antigenic activity of the FMDV βB–βC loop of VP1 (site 3), in isolation from other regions of this protein. In this case a chimaeric poliovirus was produced which contained residues 40–49 of FMDV VP1 (Kitson *et al.*, 1991). This chimaera also induced site specific anti-FMDV neutralizing antibodies in guinea pigs (Kitson *et al.*, 1991). This indicates that this region of FMDV does indeed represent an independent antigenic site and antigenic changes in this site are not necessarily mediated through the βG–βH loop.

Recently crystallographic studies have been performed on a mutant of type O FMDV selected for resistance at all four antigenic sites. When examined in the presence of a reducing agent no alterations in the structure of the βG–βH loop apart from the residues actually substituted were observed. This further indicates that the changes in antigenicity at the other sites are not mediated through perturbation of the βG–βH loop (A. M. Q. King, personal communication).

VI. CONCLUSIONS

FMDV has features which distinguish it from all other picornaviruses at a variety of different levels. Many areas of the molecular biology of this virus remain to be studied and the availability of infectious cDNA clones will assist in some areas. However these clones will not answer all questions; this is apparent since infectious cDNA clones of PV have been available for over 10 years.

Other areas of future interest are common to all picornaviruses, however it will not be a surprise if different members of the picornavirus family use different mechanisms to reach the same end. The study of the interaction between the virus and the cell is still at a very early stage for all picornaviruses. Although the cellular receptors for certain viruses have been identified, this interaction is only the first of many. The mechanism by which the virus RNA is delivered to the cell cytoplasm is far from clear. Studies are still at an early stage in determining the proteins which interact with the IRES element to permit the efficient translation of the RNA. The involvement of cellular proteins in the replication of the viral RNA and the formation of virus particles is also poorly understood, but it is clear that a major change in the structure of the cell and the intracellular membranes occurs on virus infection. Indeed it has been shown recently that the drug brefeldin A, which disrupts the golgi apparatus, completely inhibits PV replication (Maynell *et al.*, 1992). The study of some of these processes will no doubt lead to and depend on increased understanding of the molecular biology of the cell.

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