FOOT AND MOUTH DISEASE VIRUS
RNA REPLICATION

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ABSTRACT

Infection of susceptible cells with foot and mouth disease virus (FMDV) results in multiplication of the RNA genome and assembly of mature virions. The entire process of genome replication is completed in a few hours and encompasses many intracellular events. Like other picornaviruses, FMDV uses a peptide primed RNA replication mechanism. The factors that are required to uridylylate each of the three FMDV VPg peptides and the role of the FMDV cis-acting replication element (cre or 3B Uridylylation Site (bus) in VPg uridylylation have been determined. The native N-terminus of the FMDV 3Dpol enzyme is a pre-requisite for VPg uridylylation in vitro and the effects of mutations in the RNA template are consistent with a slide-back mechanism. The role of the poly(A) tail in uridylylating VPg was insignificant using full-length FMDV RNA transcripts suggesting the possibility of an alternative mechanism of VPg incorporation into negative strand RNA. The optimal RNA sequences required for VPg uridylylation were found to be within the 5' non-coding region (NCR). Furthermore, the results also showed evidence for RNA-RNA interactions between distinct structures from within the 5' NCR that influence VPg uridylylation. The polymerase precursor 3CDpro is also a prerequisite for uridylylation of each of the FMDV VPg peptides. However 3Cpro alone can substitute for 3CD, but is much less efficient. It also appeared that the overall charge of the VPg peptides determines their recognition by the FMDV 3Dpol. The RNA binding activity of the 3C was found to be required for its stimulatory effects on VPg uridylylation. Unlike the poliovirus cloverleaf, the FMDV S-fragment (at the 5' end of the genome) does not interact with the FMDV 3CD precursor protein; however it binds specifically to a cellular factor p48. The crude replication complexes (CRCs) isolated from FMDV-infected cells were found to synthesize viral RNA very efficiently and an in vitro RNA replication system developed using these CRCs can be used to study the complete RNA replication events of FMDV.
ACKNOWLEDGEMENTS

It is with a great sense of enthusiasm and pride that I write about the people who are involved in this work and also for the people who made it possible. I would like to convey my sincere regards and gratitude to my supervisor Dr Graham Belsham whose expert guidance and constant support made this work possible. From my personal point of view it is his continuous encouragement and faith in my ability to deliver results that was my source of inspiration throughout my studies. I would also like to thank my supervisor and the late Dr Nat Bumstead for arranging the IAH studentship for my final year. I am also very thankful to my university supervisor Dr Lisa Roberts for her help, patience and kindness in fulfilling the requirements of the University of Surrey. I am grateful to the Commonwealth Scholarship Commission (CSC), UK and Human Resource Development (HRD), Government of INDIA for funding my initial two years of study.

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### 2.2.13.11 pSP64polyA/S-fragment
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<td>A</td>
<td>Alanine</td>
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<tr>
<td>aa</td>
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<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAV</td>
<td>Equine rhinitis A virus</td>
</tr>
<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>EV I I</td>
<td>Enterovirus 11</td>
</tr>
<tr>
<td>FA</td>
<td>Formaldehyde Agarose</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>FD</td>
<td>Faraday</td>
</tr>
<tr>
<td>FMDV</td>
<td>Foot-and-mouth disease virus</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G</td>
<td>Glycine</td>
</tr>
<tr>
<td>g</td>
<td>Acceleration due to gravity</td>
</tr>
<tr>
<td>GDD</td>
<td>Glycine-aspartate-aspartate</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>Gu-HCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>HAV</td>
<td>Hepatitis A virus</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>HCV</td>
<td>Hepatitis C virus</td>
</tr>
<tr>
<td>HDV</td>
<td>Hepatitis delta (δ) virus</td>
</tr>
<tr>
<td>HeLa cells</td>
<td>Henrietta Lack's cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl) piperazine-N'- (2-ethane) sulfonic acid</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HpeV 1</td>
<td>Human parechovirus 1</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous nuclear ribonucleoproteins</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HRV</td>
<td>Human rhinovirus</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
</tr>
<tr>
<td>I</td>
<td>Isoleucine</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosome entry site</td>
</tr>
<tr>
<td>ITAF 45</td>
<td>IRES trans-activating factor 45</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>La</td>
<td>Lupus autoantigen</td>
</tr>
<tr>
<td>LB</td>
<td>Luria bertani</td>
</tr>
<tr>
<td>LMP</td>
<td>Low melting point</td>
</tr>
<tr>
<td>L protein</td>
<td>Leader protein</td>
</tr>
<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>mA</td>
<td>Milliamps</td>
</tr>
<tr>
<td>Mab</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>ml</td>
<td>Millilitre</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mol</td>
<td>Mole</td>
</tr>
<tr>
<td>MTOC</td>
<td>Microtubule organising centre</td>
</tr>
<tr>
<td>mQ</td>
<td>milli Q</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MT</td>
<td>Microtubule</td>
</tr>
<tr>
<td>mut</td>
<td>Mutant</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
</tr>
<tr>
<td>NaOAc</td>
<td>Sodium acetate</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NCR</td>
<td>Non-coding region</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>nM</td>
<td>Nanomolar</td>
</tr>
<tr>
<td>NP40</td>
<td>Nonidet P40</td>
</tr>
<tr>
<td>nt</td>
<td>Nucleotide</td>
</tr>
<tr>
<td>NTP</td>
<td>Nucleotide tri-phosphate</td>
</tr>
<tr>
<td>N-terminus</td>
<td>NH₂ terminus</td>
</tr>
<tr>
<td>O1K</td>
<td>O1Kaufbeuren</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>Ori</td>
<td>Origin of replication</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly (A) binding protein</td>
</tr>
<tr>
<td>PAP</td>
<td>Poly(A) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PCBp</td>
<td>Poly (C) binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>Pfu</td>
<td><em>Pyrococcus furiosus</em></td>
</tr>
<tr>
<td>PIRC</td>
<td>Pre-initiation replication complexes</td>
</tr>
<tr>
<td>PK</td>
<td>Pseudoknot</td>
</tr>
<tr>
<td>pmole</td>
<td>Picomole</td>
</tr>
<tr>
<td>PNK</td>
<td>Polynucleotide kinase</td>
</tr>
<tr>
<td>pol</td>
<td>Polymerase</td>
</tr>
<tr>
<td>pro</td>
<td>Protease</td>
</tr>
<tr>
<td>Pro-Pol</td>
<td>Precursor form of the polymerase</td>
</tr>
<tr>
<td>PTB</td>
<td>Polypyrimidine tract binding protein</td>
</tr>
<tr>
<td>PV</td>
<td>Poliovirus</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>RCs</td>
<td>Replication complexes</td>
</tr>
<tr>
<td>RdRp</td>
<td>RNA dependant RNA polymerase</td>
</tr>
<tr>
<td>rER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RF</td>
<td>Replicative form</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-glycine-aspartate amino acids</td>
</tr>
<tr>
<td>RHDV</td>
<td>Rabbit hemorrhagic disease virus</td>
</tr>
<tr>
<td>RI</td>
<td>Replicative intermediates</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RRM</td>
<td>RNA recognition motif</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcriptase</td>
</tr>
<tr>
<td>RVF</td>
<td>RNA virus fold</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>S</td>
<td>Serine</td>
</tr>
<tr>
<td>SAP</td>
<td>Shrimp Alkaline Phosphatase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Serpin</td>
<td>Serine protease inhibitor</td>
</tr>
<tr>
<td>SL</td>
<td>Stem-loop</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>T7</td>
<td>T7 phase</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFB</td>
<td>Transforming buffer</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer chromatography</td>
</tr>
<tr>
<td>TMEV</td>
<td>Theiler's murine encephalitis virus</td>
</tr>
<tr>
<td>TPB</td>
<td>Tryptose phosphate broth</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>ts</td>
<td>Temperature sensitive</td>
</tr>
<tr>
<td>U</td>
<td>Units</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitin</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>VPg</td>
<td>Viral protein, genome linked</td>
</tr>
<tr>
<td>VTC</td>
<td>Vesicular tubular cluster</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside</td>
</tr>
</tbody>
</table>
CHAPTER ONE

INTRODUCTION

1.1 Picornaviruses

Picornaviruses constitute one of the most important groups of animal and human pathogens. They are among the smallest of viruses measuring 22 to 30 nm in size. Picornaviruses infect a broad range of hosts and produce a variety of disease symptoms. Foot-and-mouth disease virus (FMDV), the first animal virus to be discovered (Loeffler and Frosch, 1897) and Poliovirus (PV), one of the most important human pathogens, both belong to the family Picornaviridae. Based upon the physical properties and nucleotide sequence, the Picornaviridae family has been subdivided into nine genera: Enterovirus, Rhinovirus, Cardiovirus, Aphthovirus, Hepatovirus, Parechovirus, Erbovirus, Teschovirus and Kobuvirus (Fauquet et al., 2005). The genomes of picornaviruses are positive sense single-stranded RNAs of about 7500 nucleotides and are infectious (Baltimore 1969; Belsham and Bostock 1988). They have distinct but similar genome organisations and follow similar replication strategies. Picornaviruses replicate in the cytoplasm of infected cells and the genome directly acts as a messenger RNA to produce the viral proteins required for RNA replication and virion assembly.

1.2 Foot and mouth disease virus (FMDV)

Foot-and-mouth disease virus (FMDV) causes one of the most important diseases of farm animals. It is the prototype aphthovirus and infects a broad range of cloven footed domestic and wild animals including cattle, sheep, goat, pig, deer etc. Cattle and pigs infected with the virus shows severe clinical manifestation of the disease
whereas symptoms are often sub-clinical in sheep and goats. The replication strategy of the virus resembles that of other picornaviruses. However certain distinct features like its broad host range and presence of three distinct copies of the genome linked protein VPg makes FMDV unique among the picornaviruses. It is interesting to note that even equine rhinitis A virus (ERAV), the other member of the genus aphthovirus only possesses a single copy of VPg. The recent outbreak of foot and mouth disease in the UK (2001) caused tremendous economic losses of about £8 billion due to slaughter of all those infected animals and the severe trade restriction imposed on countries with the disease. At present some of the factors like poor understanding of the replication mechanism of the virus, broad host range, high infectivity, diverse antigenicity and limited efficacy of the currently available vaccine make it imperative for continued research and understanding of the molecular biology of the virus.

1.2.1 Structure of foot-and-mouth disease virus

Foot-and-mouth disease virus is non-enveloped and has a capsid with an icosahedral symmetry. The capsid is composed of 60 copies of each of the viral capsid proteins VP1-VP4. VP1-VP3 constitute the outer surface; in contrast VP4 is completely internal. VP1-VP3 assume a fold that is conserved among RNA viruses and called the RNA virus fold (RVF). One of the important features of the FMDV capsid is that, it is devoid of pits and the canyon found in certain other picornaviruses that acts as the anchoring point for receptor attachment (Luo et al., 1987; Hogle et al., 1985; Rossman et al., 1985; Acharya et al., 1989). In FMDV, the G-H loop (spanning residues 140-160 of VP1) is highly accessible on the surface and is the site of receptor binding on host cells (Leippert et al., 1997). A triplet of amino acid residues
motif the Arg-gly-asp (RGD) within this loop interacts with the integrin receptor found on susceptible cells (Baxt and Becker., 1990). Mutation within the RGD motif prevents virus binding to cells and infectivity (Mason et al., 1994). Furthermore viruses with a deletion in the RGD motif are attenuated in cattle and pigs (McKenna et al., 1995). The FMDV capsid possesses the highest buoyant density in CsCl amongst the picornaviruses (Acharya et al., 1989). The capsid is unstable in an acidic environment, pH below 6.8 causes its dissociation into 12S pentameric subunits. In the case of PV and human rhinovirus (HRV) the β-barrel of VP1 contains a hydrophobic pocket that accumulates a fatty-acid molecule called pocket factor. From a biological point of view, release of this pocket factor following virus binding to receptors on host cells leads to an altered structure called the activated particle (A-particle) in which the VP4 is externalised and the viral RNA is released to the cytoplasm. The entire process is called uncoating. However FMDV does not assume any such intermediate structure like PV and HRV for uncoating (Fry et al., 1999; Rueckert, 1996).

1.2.2 FMDV receptors

Foot and mouth disease virus preferentially infects epithelial cells of the host. This epitheliotropic nature is due to the affinity of the virus for integrin receptors found on the surface of these cells. Integrins are a family of integral membrane anchored proteins which are heterodimers consisting of α and β subunits. They have important cellular functions including cell-cell and cell-matrix interactions in addition to transmitting signals in and out of the cells. Out of the different integrin complexes αβ3, αβ6, αβ1 have been shown to act as FMDV receptors by binding to the RGD motif within VP1 (Berinstein et al., 1995; Neff et al., 1998; Jackson et al.,
2000, 2002). Recent findings have shown that αvβ3 is predominantly found on endothelial cells rather than epithelial cells leading to the belief that αvβ6 is the true or primary receptor for FMDV rather than αvβ3 (Monaghan et al., 2005).

Besides using an integrin as a primary receptor for infection, certain tissue culture adapted viruses possess the affinity to bind heparan sulphate (HS) as an alternative receptor (Jackson et al., 1996; Sa-Carvalho et al., 1997). This change in receptor specificity has been attributed to a net gain in positive charge on the outer capsid surface in tissue culture (Fry et al., 1999). However viruses having higher affinity for heparan sulphate are attenuated in cattle (Sa-Carvalho et al., 1997).

1.2.3 FMDV entry into cells

Having bound to the receptor on the cell surface, internalisation of the FMDV results from endocytosis into early endosomes. The FMDV capsid is highly labile in the acidic environment of the endosome where protonation of a cluster of histidine residues at the pentamer boundary leads to disassembly of the 12S pentameric subunits (van Vlijmen et al., 1998) resulting in uncoating of the virus and by an unknown mechanism the release of the viral RNA to the cytoplasm. Pre-treatment of the cells with certain lysomotropic ionophores like monensin and concanamycin-A increases the endosomal pH and prevents virus infection (Baxt 1987; Miller et al., 2001).

1.2.4 Genome organisation of FMDV

Foot and mouth disease virus RNA is about 8.4 kilo bases in length and directly acts as a messenger RNA for protein synthesis. Unlike cellular messenger RNA, the 5' end is devoid of a modified nucleotide m7GpppN or the cap structure. Instead the 5'
end is attached to a viral encoded short peptide 3B/VPg. FMDV is different from other picornaviruses having three distinct copies of VPg which contain 23 or 24 amino acids (Forss and Schaller 1982). The presence of tyrosine residue at position three of the VPg is absolutely conserved and is required for its linkage to the 5' end of the genome. Both positive sense viral RNA and the antisense negative strand RNA are linked to one copy of VPg at the 5' end suggesting a role of VPg in the RNA priming process during viral RNA replication. It is known that each VPg peptide can be linked to the viral RNA (King et al., 1980).

The foot and mouth disease virus genome begins with the 5' untranslated region (UTR), and is separated from the 3' untranslated region by a single large open reading frame (FIG. 1.1). The FMDV 5' UTR is about 1300nt in length and is the longest among the picornaviruses (Forss et al., 1984). It begins with a single long stem loop structure (about 370nt) called the short fragment or S-fragment, which is followed by a 150-250 nt RNase T1-resistant tract that contains about 90% C residues called the poly(C) tract (Brown et al., 1974; Newton et al., 1985). To date no specific function of the S fragment has been investigated; however deletion of the S fragment makes the RNA transcript non-infectious suggesting that this structure will have a role in virus RNA replication. The S-fragment is believed to be the functional counterpart of the cloverleaf RNA in PV and the highly base-paired nature of the S fragment could prevent cellular exonuclease digestion of the viral RNA. Since VPg is cleaved soon after release of the viral RNA into the cytoplasm, there might be a role for the S-fragment in providing stability to the FMDV RNA. The poly(C) tract has been implicated with virulence and pathogenesis of the virus (Harris and Brown 1977). However the presence of the polyC tract is not absolutely
FIG. 1.1. Genome organisation of foot-and-mouth disease virus. Foot and mouth disease virus RNA is about 8.3 kb and contains three distinct regions. The 5' non-coding region includes secondary structures namely S-fragment, poly(C) tract, pseudoknots (PKs), cis-acting replication element (cre/bus) and internal ribosome entry site (IRES). The coding region is translated into a single polypeptide that undergoes co-translational and post-translational cleavage to produce 4 structural and 10 non-structural proteins. FMDV is unique in encoding three distinct copies of VPg. The 3' non-coding region also contains regions of secondary structure including a 100nt heteropolymeric tract and a poly(A) tail.
required for virus propagation. Virus having only a 2'C' tract (C2) can be stably propagated in cell culture although the viruses have a higher particle:infectivity ratio compared to virus having longer polyC tracts (Rieder et al., 1993; Rieder and Mason unpublished data). It is also interesting to observe that virus having a short polyC tract can rapidly acquire a longer polyC tract during propagation in cell culture indicating a selective advantage for maintaining a longer polyC tract in the genome for stable propagation. Though the exact role of the polyC tract is not yet known, it has been hypothesised that the polyC binding protein (PCBP) a host cell protein that interacts with the clover leaf of the PV will also interact with the polyC tract of FMDV and might have a role in genome circularization as suggested for PV (Herold and Andino 2001). The FMDV polyC tract is followed by three to four pseudoknots (PKs) of unknown function. Recently a simple stem loop structure has been identified just downstream of the PKs called a cis-acting replication element or cre (Mason et al., 2002) which has been implicated in genome replication. Unlike FMDV, other picornaviruses possess a cre structure in the polyprotein coding region instead of the 5' untranslated region (this will be described in more detailed later). The 3' end of the 5' untranslated region contains a well characterised structure (~450 nt) called the internal ribosome entry site (IRES) that directs the initiation of cap-independent translation of viral protein synthesis (Belsham and Brangwyn 1990).

The FMDV RNA contains a single open reading frame that encodes a polypeptide of approximately 260 kDa and separates the 5' UTR from the 3' UTR. The 3' UTR resembles that of cellular messenger RNA, which consists of a short heteropolymeric tract of about 100 nt that fold into secondary structures followed by a polyA tail.
1.2.5 FMDV RNA translation

Following virus entry into a host cell, the viral RNA is released to the cytoplasm by means of capsid dissociation. Subsequently the VPg protein at the 5' end of the RNA is cleaved by a eukaryotic cellular enzyme called VPg unlinkase (Gulevich et al., 2002). This leaves the 5' terminal of the viral RNA beginning with two uridine residues (UU) an important feature required for picornavirus RNA replication. Any change at the 5' terminal UU can abrogate viral RNA replication in a cell free system (Herold and Andino 2000). Though it seems beneficial for the virus to have a free 5' end for viral RNA replication, at the same time translation needs to be initiated for synthesis of viral proteins required for RNA replication and virion assembly. Translation of most cellular mRNA requires the m^7G cap structure found attached to the 5' end of the RNA. However, the absence of any such structure at the 5' end of the picornaviral RNA necessitates the use of an alternate mechanism for the initiation of viral protein synthesis. In FMDV, the 3' portion of the 5' untranslated region contains a specialised region of secondary structure of about 450 nt where the ribosome lands for internal initiation of protein synthesis. This is called an internal ribosome entry site (Belsham and Brangwyn 1990; Belsham and Jackson 2000). It has been demonstrated that FMDV uses two AUG initiation codons (84 nt apart) for RNA translation thereby producing two forms of Leader protease Lab and Lb respectively (Belsham 1992). It has been demonstrated that Lb start site is required for virus infectivity while the Lab site can be removed (Cao et al., 1995; Piccone et al., 1995a). The FMDV IRES along with the encephalomyocarditis virus (EMCV) IRES comprise the group II of IRES element which function efficiently in rabbit reticulocyte lysates in vitro (Belsham and Jackson 2000). At the early stage of infection the leader protease brings about the cleavage of the eukaryotic translation
initiation factor eIF4G a scaffold component of the cap binding complex (eIF4F) and thereby inhibits cap-dependent protein synthesis (Devaney et al., 1988; Medina et al., 1993; Belsham et al., 2000). Except for eIF4E, the FMDV IRES essentially requires all the canonical translation initiation factors (including the C-terminal cleavage fragment of the eIF4G) for its activity (Pilipenko et al., 2000). In addition picornavirus IRES elements also require tissue specific cellular factors for efficient IRES dependent translation (Belsham and Sonenberg 2000). Cellular factors like polypyrimidine tract binding protein (PTB), La protein and poly(rC) binding protein (PCBP2) are required for efficient PV IRES dependant translation (Gosert et al., 2000; Craig et al., 1997; Blyn et al., 1997). It has been demonstrated that FMDV requires PTB and ITAF45 for its activity in vitro (Luz and Beck 1991; Niepmann et al., 1997; Pilipenko et al., 2000).

1.2.6 Polyprotein processing and mature virus production

During a picornavirus infection in cells, the factors required for successful infection and progeny virus production are produced from the polyprotein. Under normal conditions the polyprotein corresponding to the entire open reading frame is never observed in the cells. As the polyprotein is synthesized it is cotranslationally and post-translationally cleaved by viral encoded proteinases to produce mature and precursor proteins to serve specific functions in the virus life cycle (FIG. 1.2). The polyprotein has been characterised to contain three distinct domains based on functionality. The P1 region codes for viral proteins VP4, VP2, VP3 and VP1 which are required for capsid assembly and hence is called the structural domain. The remainder of the polyprotein is divided into P2 and P3 regions which are known as replicative domains for their role in RNA replication.
In poliovirus the entire structural domain is efficiently cleaved by the action of the 3CDpro with the help of a cellular factor/chaperone (Blair et al., 1993). Though 3Cpro can carry out this function, 3CDpro has been characterised to be 100-1000 times more efficient than the 3Cpro (Parsley et al., 1999). Initial processing of the P1 region leads to appearance of VP0(VP4+VP2), VP3 and VP1. Subsequently VP0, VP3 and VP1 assemble to form 5S protomers. Five individual protomers combine to form 14S pentamers. Assembly of the 150S provirion can take place by two different pathway; a) in the absence of poliovirus RNA 12 pentamers can assemble to form an 80S procapsid into which a single RNA is finally threaded to form 150S provirions, or b) in the presence of virion RNA individual pentamers assemble themselves with the viral RNA to form the 150S provirion. This 150S provirion is short lived and undergoes a final maturational cleavage by an unknown mechanism in which VP0 is cleaved to VP4 and VP2 to form the mature virion (Arnold et al., 1987).

Within the family Picornaviridae, both aphthoviruses and cardioviruses encode leader proteins (L) at the N-terminus of the polyprotein. In FMDV the leader protein is a protease and cleaves itself from the polyprotein (at the L/P1 junction) as the primary cleavage event in polyprotein processing. In addition in FMDV, the L protease plays an important role in host cell protein synthesis shut-off (Strebel and Beck 1986; Devaney et al., 1988; Medina et al., 1993; Belsham et al., 2000). In cardioviruses L protein does not possesses any detectable protease activity and is cleaved from the P1 precursor by the action of the 3Cpro (Parks et al., 1986). In contrast, in enteroviruses and rhinoviruses cleavage at the P1/P2 junction (which takes place in cis at the N-terminus of the 2A) is the primary cleavage event and is carried out by the 2A protease. In aphthovirus and cardiovirus 2A mediated cleavage
takes place at the 2A/2B junction that leads to the formation of P1-2A and L-P1-2A precursors respectively instead of P1 precursor alone (Grubman and Baxt 1982). Subsequently 2A is separated from the P1 by the action of 3Cpro in FMDV (Ryan et al., 1989). In all picornaviruses the rest of the polyprotein processing is carried out by the 3Cpro and its precursor 3CDpro. In poliovirus 3CDpro is the major protease that carries out the majority of the polyprotein processing, in contrast 3Cpro in FMDV is sufficient to carry out almost all the polyprotein processing (Farsley et al., 1999; Bablanian and Grubman, 1993; Grubman and Baxt 2004). The P2 non-structural domain in enteroviruses and rhinoviruses is processed into three mature proteins 2A, 2B and 2C whereas the P3 non-structural domain is processed into 3A, 3B, 3C and 3D.

1.2.7 Switching from translation to replication

In picornavirus infected cells viral protein synthesis occurs on rough endoplasmic reticulum (rER) derived membranes on polyribosomes (Caliguiiri and Tamm 1969). This leads to the formation of multiple copies of the viral polyprotein and post-translational cleavage by viral proteases 2A, 3C and 3CD to produce the structural and non-structural proteins along with various precursor polypeptides (Kitamura et al., 1981; Nicklin et al., 1987; Palmberg 1990). At some stage of the picornavirus life cycle the translation of the viral RNA needs to be switched off so that the RNA can act as a template for negative strand synthesis. It has been reported that the viral proteins are synthesized in PV infected cells for several hours after RNA replication has started (Levintow 1974). Thus it appears that the entire translating apparatus on viral RNA need not be switched off for RNA replication as there is interdependence of viral RNA translation and replication. The switch from translation to replication to
synthesize negative strand could occur on a single RNA molecule and may then be
followed by multiple rounds of positive strand RNA synthesis. It is interesting to
note that in PV and may be in some other picornaviruses; proteins involved in RNA
replication are required in cis (Novak and Kirkegaard 1994) thereby providing a
partial check on genome functionality. In principle there is a potential conflict
between the RNA translation and RNA replication machinery. During RNA
replication the polymerase enzyme moves from 3' to 5' end whereas the ribosome
during translation proceeds in a 5' to 3' direction. Evidence has been presented that
the translating ribosome inhibits negative strand synthesis (Gamarnik and Andino
1998; Barton et al., 1999). Drugs like cyclohexamide (CHI) inhibit polypeptide
chain elongation and freeze the ribosome on the viral RNA template and prevent
normal clearance of the ribosome. Such blocked RNA templates do not support
negative strand synthesis (Gamarnik and Andino 1998; Barton et al., 1999)
suggesting the polymerase of the poliovirus can not dislodge the translating
ribosome from viral RNA template. This necessitates the blockage of translation to
allow switching of the viral RNA from translation to replication. Therefore there
must be critical interplay between the translation and replication machinery on the
same RNA molecule so that they do not collide with each other. One of the
intriguing features of picornaviral RNA replication is the RNA dependent RNA
polymerase (3Dpol) should be delivered on the same RNA molecule from where it
has been synthesized (Novak and Kirkegaard 1994). This suggests there must be
some specific cis-signal associated with the genomic RNA, excluding the polyA
sequence at the 3' end that allows the viral polymerase to recognise cis-RNA rather
than other viral RNA and cellular mRNA.
Switching from translation of viral RNA to RNA replication necessitates the production of all proteins required for replication in sufficient quantities. It has been proposed that the concentration of the viral protein 3CD, the precursor of RNA polymerase is critical for this switching (Gamarnik and Andino 1998). Once the level of 3CD reaches a critical level it may bind to the stem-loop D of the cloverleaf RNA and repress viral RNA translation. That process could promote negative strand RNA synthesis, indeed it has been observed that supplying an excess of 3CD in trans specifically inhibits viral translation. However, subsequent work suggested that this mechanism may not be totally true. It has been found that binding of the 3CD to the cloverleaf RNA increases the cloverleaf’s affinity for the cellular transacting factor PCBP-2 (Walter et al., 2002). PCBP-2 is functionally required for poliovirus translation and binds to stem loop IV of the IRES during this process. Thus, it was suggested that upon binding of 3CD to the 5’ cloverleaf structure, PCBP-2 might preferentially bind at the cloverleaf structure and dissociate from the IRES (Gamarnik and Andino 1998). Dissociation of PCBP-2 from the IRES would have the effect of inhibiting translation, thereby allowing the synthesis of negative strand to proceed. The reverse could be achieved when the 3CD protein has been cleaved into its components, 3C and 3D. These proteins have a very low affinity for the cloverleaf structure, thus freeing PCBP-2 to bind stem loop IV of the poliovirus IRES (Andino et al., 1993). However it is now believed that the amount of PCBP-2 available within the cell may be sufficient to bind to both sites on the poliovirus genome. Thus, the model suggested earlier, which relies on PCBP-2 dissociation from the IRES is unlikely to be the mechanism by which viruses switch between translation and replication of their genomes (Gamarnik and Andino 2000). However, the concentration of PCBP-2 in the area of the cell where viral translation and
replication takes place has not yet been determined. Demonstration that the local concentration of PCBP-2 is sufficient to bind to the IRES and the 5' cloverleaf structure simultaneously is required before this model can be totally disregarded (Gamarnik and Andino 2000). Recently polypyrimidine tract binding protein (PTB) has also been implicated to be involved in molecular switching from translation to RNA replication. PTB or PTB1 has been reported to occur in three different isoforms (PTB1, PTB2 and PTB4) (Michael et al., 1995). Drugs like actinomycin D that inhibit cellular transcription lead to partial redistribution of PTB from the nucleus to the cytoplasm (Back et al., 2002). Though PTB predominantly remains inside the nucleus (Perez et al., 1997; Romanelli et al., 1997) during PV infection similar distribution takes place between the nucleus and cytoplasm (Back et al., 2002). PTB has four RNA recognition motifs (RRM), out of which the first two form the N-terminal segment that are required for interaction with other cellular proteins like PCBP2, hnRNP K, hnRNP L also to PTB itself (Hahm et al., 1998; Kim et al., 2000). The C-terminal domain that includes the other two RRM's interacts with the IRES during translation. PV 3Cpro and/or 3CDpro cleaves all three isoforms of PTB which are required for IRES dependent translation (Gosert et al., 2000). At the early stage of PV infection both PTB and PCBP2 facilitate the translation of PV RNA. PTB has been shown to interact with PCBP2 via RRM1 and RRM2 (Kim et al 2000) and with the PV IRES by RRM3 and RRM4 (Oh et al., 1998; Hellen et al., 1993) and it has been suggested that PTB stabilises the binding of PCBP2 to the IRES. Cleavage of PTB during the late stage of infection with 3Cpro and/or 3CDpro leads to localisation of the N-terminal segment in the cytoplasm and the C-terminal segment of the PTB remains attached to the IRES (Back et al., 2002). This results in a decrease of intact PTB attached to IRES and thereby causes the dissociation of
PCBP2 from the IRES as the binding domain of the PTB for PCBP2 is not present in the C-terminal fragment. In addition the presence of the C-terminal fragment of PTB on the IRES may produce a dominant negative effect by preventing the binding of intact PTB and thus reducing the affinity of the PCBP2 for the IRES. Disconnection of the PTB and PCBP2 from the IRES might facilitate the association of the PCBP2 with the RNA-protein ternary complex formed at the cloverleaf (CL) along with the 3CDpro of the PV and cause repression of translation and molecular switching to negative strand synthesis. The N-terminal segment of PTB might also be involved in repressing translation by sequestering other RNA binding protein like PCBP2, hnRNP K, hnRNP L which are known to interact with it (Back et al., 2002).

1.3 Picornavirus RNA replication mechanism

All picornavirus RNA replication follows a similar strategy, the viral genome is first transcribed into a complementary RNA (negative strand), which in turn is used as a template for multiple rounds of synthesis of new positive sense progeny genomes (FIG. 1.3 and FIG. 1.4A). For both these processes the RNA dependent RNA polymerase (3D\(^{pol}\)) plays a central role. The 3' terminus of the positive-sense RNA (the poly(A) tail) and the negative-sense RNA template (antisense cloverleaf or S-fragment) are very different in sequence. Thus, the nature of the recognition process by the RNA polymerase must be complex but is not currently defined. However for this process to occur the polymerase enzyme needs a protein primer. The viral protein 3B (VPg) serves this role, it must firstly be uridylylated to form VPgpUpU followed by template dependent elongation by the polymerase enzyme (FIG. 1.3). This reaction can be reproduced in-vitro by using polyA or the cis-acting replication element (cre),
FIG. 1.3. VPg uridylylation during positive strand synthesis involves a slide-back mechanism. In poliovirus it has been suggested that 3Dpol catalyses the linkage of the first ‘U’ residue to VPg to form VPgpU using the first ‘A’ residue of the conserved AAACA motif as a template. In the subsequent step there is a slide back of the VPgpU to hydrogen bond with second ‘A’ residue. In the next step addition of the second ‘U’ residue takes place to form VPgpUpU using the first ‘A’ as template nucleotide.
a small stem-loop structure as template (Paul et al., 1998). The role of the cre will be discussed in detail further below. Genetic studies have revealed the requirement of several non-structural proteins for the process of RNA synthesis (Kirkegaard 1992; Li and Baltimore 1988; Giachetti and Semler 1991; Burns et al., 1989). Furthermore for poliovirus RNA synthesis to occur the formation of a ternary ribonucleoprotein (RNP) complex is necessary in which viral proteins as well as host proteins are involved (Andino et al., 1993; Andino et al., 1990; Harris et al., 1994; Herold and Andino 2001).

1.3.1 Genome circularization model for poliovirus RNA replication

An open question concerning the replication of RNA viruses is how the same viral replication machinery can initiate RNA synthesis from both positive and negative strands, considering they carry very different cis-acting elements. It is also intriguing that specific binding of the viral polymerase precursor (3CD) to the 5'-end of the RNA genome is required for the negative-strand RNA synthesis (Barton et al., 2001; Gamarnik and Andino 1998; Herold and Andino 2001), given that initiation of negative-strand synthesis takes place at the opposite end of the genomic RNA. Clearly, successful initiation of negative-strand RNA synthesis depends on the specific recognition of the viral RNA as a template as well as the point of initiation for replication. The negative-strand synthesis of poliovirus is initiated within the poly(A)-tail of the genomic RNA (Herold and Andino 2001). Thus, the poliovirus poly(A)-tail is an important cis-acting element for RNA replication and removal or shortening of the poly(A)-tail results in impaired RNA replication (Barton et al., 1996; Sarnow 1989; Spector et al., 1975). This observation may reflect modification of the stability of the RNA or possibly a requirement for interactions between the 3'
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Positive sense  

\[ \text{AAAA(n)} \]

Negative sense  

\[ \text{UUUU} \]

FIG. 1.4A. Linear solid bar depiction of replication mechanism in poliovirus
The polyA tail at the 3' end of the poliovirus genome is considered to be an important cis acting RNA element required for the negative strand synthesis. For initiation of negative strand synthesis, the 3Dpol enzyme uridylylates VPg to form VPgpUpU possibly using polyA tail as a template. Subsequently the negative strand acts as a template for multiple round of positive strand synthesis. This process is highly asymmetric since the replication machinery favours an overwhelming proportion of positive strand synthesis against the negative strands. As a consequence during the virus life cycle more than 95% of the RNA that accumulates in the cytoplasm of the infected cells constitutes the positive strands. For initiation of positive strand synthesis 3Dpol uridylylates VPg by a slide-back mechanism using the cre/bus as a template.

FIG. 1.4B. Genome circularization model for poliovirus RNA replication. In poliovirus RNA replication, negative strand RNA synthesis initiates with the formation of a ternary ribo-nucleo protein complex at the 5' end of the genome. The cloverleaf binds to viral proteinase 3CD and cellular polyC binding protein (PCBP). PolyA binding protein (PABP) forms a RNA-protein complex with the polyA tail at the 3' end of the genome. PABP and PCBP forms a protein-protein complex, as a result genome circularization takes place via a RNA-protein-protein-RNA bridge.
and 5' termini of the RNA potentially involving the poly(A) binding protein (PABP) which binds optimally to a sequence of at least 25 'A' residues. However, the poly(A)-tail cannot be the only cis-acting element that specifically ensures replication of the viral RNA, as all cellular mRNAs contain poly(A) tails. Cis-acting elements at the 5' end of the genome are also required for initiation of negative strand synthesis (Barton et al., 2001, Herold and Andino 2001). Furthermore, it appears that the opposite ends of the viral genome interact with each other through a protein-protein bridge (Herold and Andino 2001). The viral 3CD binds to the 5'-end of the genomic RNA and reaches its site of action within the poly(A)-tail of the genome via circularization of the genomic RNA using an RNA-protein-protein-RNA-bridge that involves at least two cellular factors, PCBP and PABP1 (FIG. 1.4.B). These results explain the requirement for a long poly(A)-tail in RNA replication and reveal a direct role for the 3CD binding to the cloverleaf in negative-strand RNA synthesis. Currently, no protein interactions with the FMDV S-fragment have been identified. However, if the S-fragment does exist in a stable stem-loop structure, as predicted, then the 5' end of the RNA would be close to the poly(C) tract which is likely to be bound to the poly(rC) binding protein, potentially achieving the same effect as proposed for the PV cloverleaf (Belsham and Martinez-Salas 2004). The formation of a circular genomic structure by interaction of the 5'- and 3'-ends may be a general mechanism by which positive-stranded RNA viruses initiate their replication.
1.4. Role of the non-structural proteins in RNA replication

The non-structural proteins of picornaviruses carry out the necessary host cell modifications required for viral RNA synthesis and ultimately progeny virus production. Proteins of the P2 non-structural domain are responsible for morphological and biochemical changes associated with virus replication, that include inhibition of host cell transcription and translation (for enteroviruses and rhinoviruses), active remodelling of the cellular cytoskeleton and vesicular membrane structure, inhibition of protein secretion and also nucleocytolasmic transport. Proteins from the P3 non-structural domains along with some precursors are physically involved in RNA replication. The roles of these non-structural proteins in the virus life cycle are summarized in Table-1.
<table>
<thead>
<tr>
<th>Non-structural proteins</th>
<th>Functions</th>
</tr>
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</table>
| **2Apro**               | **Poliovirus**  
|                         | Host cell protein synthesis shut off (Etchison et al., 1982)  
|                         | Polyprotein processing  
|                         | Apoptotic cell death (Goldstaub et al., 2000)  
| **FMDV**                | Part of the P1-2A precursor (Ryan et al., 1999) |
| **2B**                  | **Poliovirus**  
|                         | Blocks protein secretion (Doedens et al., 1997)  
|                         | Cell permeabilisation (van Kuppeveld et al., 1997)  
| **FMDV**                | Membrane association  
|                         | Does not block protein secretion (Moffat et al., 2005) |
| **2C**                  | **Poliovirus**  
|                         | ATPase and GTPase activity (Rodríguez and Carrasco 1993)  
|                         | RNA binding activity (Banerjee et al., 1997)  
|                         | Negative strand synthesis (Blenz et al., 1980)  
|                         | Increases intracellular Ca$^{2+}$  
|                         | Guanidine resistance  
|                         | Regulatory effects on proteolytic activities of 2A and 3C (Banerjee et al., 2004)  
| **FMDV**                | Guanidine resistance (Saunders and King 1982) |
| **2BC**                 | **Poliovirus**  
|                         | Membrane alteration  
|                         | ATPase activity (Pfister and Wimmer 1999)  
| **FMDV**                | Blocks protein secretion (Moffat et al., 2005) |
| **3A**                  | **Poliovirus**  
|                         | RNA replication  
|                         | Membrane association  
|                         | Blocks protein secretion (Doedens et al., 1997)  
|                         | Recruits 3Dpol to replication complex (Datta and Dasgupta 1994)  
|                         | Brefeldin resistance (Crotty et al., 2004)  
| **FMDV**                | Membrane association (Moffat et al., 2005)  
|                         | Pathogenesis (Pacheco et al., 2003)  
|                         | Host range and virulence (Nunez et al., 2001) |
| 3AB | **Poliovirus**  
Membrane association (Datta and Dasgupta 1994)  
RNA replication  
Binds to cloverleaf (Harris et al., 1994)  
Binds to 3'UTR  
Stimulates 3Dpol, 3CDpro activity in vitro (Lama et al., 1994) | 3CD | **Poliovirus**  
Major protease for polyprotein processing (Ypma Wong et al., 1988)  
Strong affinity for the cloverleaf (Andino et al., 1993)  
Stimulates VPg uridylylation  
Stimulates virus production in cell-free system  
Interacts with 3' UTR together with 3AB (Paul et al., 1994) | 3C | **Poliovirus**  
Binds to cloverleaf (Andino et al., 1990)  
Stimulates VPg uridylylation (Yin et al., 2003)  
**FMDV**  
Oligomerises in non-reducing condition |  | **FMDV**  
Major protease for polyprotein processing (Grubman and Baxt 2004)  
Cleaves host cell histone protein (Tesar and Marquardt 1990) and eIF4A (Belsham et al., 2000) |  | **FMDV** (Not known) |
1.4.1 Role of P2 non-structural proteins in RNA replication

1.4.1.1 2A viral protease

In foot-and-mouth disease virus the 2A oligopeptide is about 18 amino acid long and mediates its own cleavage at its C-terminal ends to release it from the 2B region (Grubman and Baxt 1982). That is why the 2A peptide remains with the P1 structural proteins precursor as P1-2A following initial cleavage of the polyprotein. Later on, the viral proteinase 3C brings about the cleavage of the 2A from the P1-2A precursor. Although the 2A protein does not have any direct involvement in RNA replication in PV, the 2A protein mediates cleavage of the eukaryotic initiation factor 4G (eIF4G) and thus has been implicated in the host cell protein synthesis shut-off mechanism (Etchison et al., 1982). It also induces apoptotic cell death in infected cells by a mechanism that differs from caspase mediated physiological cell death (Goldstaub et al., 2000).

The mechanism of cleavage mediated by the FMDV 2A at -NPG|P- junction has been studied in detail. Modelling of the 2A indicates the presence of an amphipathic helix and the conformation of the 2A sequence produces strain on the peptidyltransferase centre of the ribosome (Ryan et al, 1999). That results in repositioning of the peptidyl (2A)-tRNA ester linkage. The steric effect that is generated by this process prevents the nucleophilic attack by the incoming (prolyl)-tRNA which is the first amino acid of the 2B protein thereby causing the release of the N-terminal product. It has been suggested that a proportion of the ribosomes stop translation while the rest carry out the translation of the downstream products. Further studies have suggested that protein synthesized upstream of the 2A sequence were always present in greater molar excess than that of the protein downstream of
the 2A sequence (Donnelly et al., 2001b). This leads to the idea that 2A-2B cleavage is not a proteolytic cleavage, rather an alternation of the translation machinery by the 2A sequence or peptide which allows the release of the P1-2A from the ribosome while permitting the synthesis of the downstream proteins (Donnelly et al., 2001a, b).

1.4.1.2 Picornavirus 2B protein as a viroporin

Among different FMDV serotypes, the 2B region is found to be most conserved. In poliovirus, 2B protein has been shown to increase the permeability of cell membranes and it also blocks the protein secretory pathways (Doedens and Kirkegard 1995). Both in FMDV and PV, 2B along with 2C have been found to localise in the membranous vesicles which are the site of virus replication (Gosert et al., 2000; Suhy et al., 2000; Moffat et al., 2005). There is very little literature on the FMDV 2B function, however information derived from PV and recent studies in FMDV indicates that picornavirus 2B has got an important role in cell permeabilization during virus infection. The non-structural protein 2B, its precursor 2BC and 3A in poliovirus have the ability to alter the cell membrane permeability when expressed individually in cells (Doedens and Kirkegard 1995; Barco and Carassco 1995; van Kuppeveld et al., 1997). However 2BC protein is the most active in terms of membrane alteration and shows some properties that differ from 2B and 2C alone (Barco and Carassco 1995). In addition 2B, 2BC and 3A (3A being the most potent) also impair glycoprotein trafficking through the secretory transport system (Doedens and Kirkegard 1995; Doedens et al., 1997). 2B protein alone has been shown to localise to the Golgi complex when expressed alone (De Jong et al., 2003). FMDV 2B is found to be located in structures closely associated with the ER,
and at high levels of expression, the 2B protein caused rearrangement of the ER into a honeycomb of membranes close to the nucleus (Moffat et al., 2005). Unlike poliovirus, even though FMDV 3A, 2B, and 2C were located in membranes of the early secretory pathway, they did not individually block protein transport. However the precursor 2BC is able to block the secretory pathway (Moffat et al., 2005).

Picornavirus 2B proteins are about 100 amino acids in length and contain two hydrophobic stretches in their sequences. In the Coxsackievirus 2B protein mutations that alter the first domain’s amphipathicity (residues 37-54) or the second domain’s hydrophobicity (residues 63-80) have an effect on the ability of the 2B protein to increase membrane permeability and viral RNA replication (van Kuppeveld et al., 1995; 1996). The yeast two-hybrid approach has shown that 2B and 2BC alone can mediate homomultimerization on their own (De Jong et al., 2002) and the domain that is involved in this reaction is located in the hydrophobic domain of the 2B protein. This might have important implications in the virus life cycle by creating a microenvironment required for virus replication as a result of membrane permeabilization. The ability of the 2B protein to permeabilise the membrane has been assessed using large unilamellar vesicles which support the idea that 2B acts as a viroporin that creates transmembrane pores which allow free diffusion of small solutes (Agirre et al., 2002). The mechanism currently proposed is that an oligomer form of the 2B, most likely a tetramer, forms a permeating unit or pore on the membrane surface (Nir and Nieva 2000; Nieva et al., 2003). As a result of this, compounds with molecular weight less than 1000 Da are able to enter and leave the cytoplasm during the mid phase of infection which leads to alteration of the
monovalent ion concentration and hence membrane depolarisation, this finally leads to cell lysis.

1.4.1.3 2C protein has both RNA binding and NTPase activities

2C protein is the most conserved protein among the picornaviruses and has multiple functions (Argos et al., 1984). In poliovirus, the N-terminal region of 2C encompassing residues 21-54 contains a putative amphipathic helix supposed to play an important role in membrane binding (Argos et al., 1984). It has also been reported that PV 2C has specific binding affinity for the 3' end of the negative strand RNA but not to the complementary sequence of the positive sense RNA (Banerjee et al., 1997). In addition Echovirus-9 2C protein can bind to both positive and negative sense viral RNA non-specifically (Klein et al., 2000) indicating a conserved RNA binding activity of picornavirus 2C protein. The membrane binding and RNA binding properties suggests that PV 2C might have an important function in viral RNA replication by keeping the 3' end of the negative sense RNA immobilised on the replication complex where the necessary viral and cellular factors can assemble to initiate positive sense RNA synthesis.

PV 2C has been reported to contain three conserved motifs. Motif A and motif B constitute the NTP-binding site whereas motif C has been found to be present in members of the helicase superfamily III. The function of the motif C is not known and no helicase activity of the 2C has been reported yet. Motif A and motif B are involved in ATPase and GTPase activity which are required for RNA replication (Rodriguez and Carrasco 1993). Point mutations resulting in changes in conserved amino acid residues in the NTP-binding motif have been shown to yield RNA
transcripts that are non-infectious and act by interfering with RNA replication (Mirzayan and Wimmer 1994; Teterina et al., 1992). Multiple studies on PV 2C have revealed two distinct functions of this protein in viral RNA synthesis: a cis-acting, guanidine-sensitive RNA synthesis initiation event and a trans-acting elongation process. Low concentrations of guanidine-hydrochloride (up to 2-10 mM) inhibit picornavirus replication and are found to inhibit negative strand synthesis by interfering with the function of 2C (Bienz et al., 1980). In polioviruses and foot-and-mouth disease viruses resistance to guanidine has been associated with mutations that have been mapped to be within the 2C coding region (Saunders and King 1982; Pincus and Wimmer 1986; Pariente et al., 2003). Evidence has been presented that many mutations in the 2C region can be lethal. In addition some of the non-lethal 2C mutants have been shown to display abnormalities in RNA replication such as a temperature-sensitive phenotype, or guanidine hydrochloride resistance or dependence phenotype (Wimmer et al., 1993). In addition to the role of 2C in viral RNA replication it has been found to be involved in virus encapsidation (Vance et al., 1997). Very recently it has been reported that serine protease inhibitor or ‘serpin’ motifs are found to be present throughout the 2C sequence which are capable of inhibiting the activity of viral protease 3C(pro) and 2A(pro) in-vitro (Banerjee et al., 2004). These workers also suggested that serpins in the 2C might interacts with the 3C active site, inhibiting its proteolytic activity and may thereby play a key role in viral replication and pathogenesis.
1.4.2 Role of P3 non-structural proteins in RNA replication

1.4.2.1 3A protein

Foot-and-mouth disease virus 3A protein is about 153 amino acids in length and differs in size from other picornavirus 3A protein. It is about 50% larger than the PV 3A protein which is only 87 amino acids (Kitamura et al., 1981). In all the picornaviruses, the 3A protein contains a 15-20 amino acid hydrophobic domain located 20-23 amino acids downstream of the N-terminus. In FMDV the region downstream of the hydrophobic region has been implicated in species specificity. Earlier studies have indicated that the length of the 3A protein has important implications for producing disease in different hosts. Live attenuated strains of FMDV that have been passaged through chicken embryo cells displayed an apparent reduction in 3A size that has been suggested to be required for an attenuated phenotype in bovines. Subsequently these changes were mapped to a deletion of about 20 amino acids in the C-terminal third of the 3A protein (Giraudo et al., 1990). Subsequent evidence of a role of 3A length on species specificity became clearer after the outbreak of FMDV in Taiwan in pigs which had a similar deletion in 3A; this virus did not affect the cattle population (Beard and Mason 2000). It has also been shown that deletions within the 3A sequence (amino acids 93-102 and amino acids 93-144) resulted in reduced RNA synthesis in both bovine and porcine kidney cells indicating impaired RNA replication in both types of cells. Such virus strains showed an attenuated phenotype using bovine cells in culture. However such deletions within 3A had no detectable effect on growth in porcine kidney cells (Pacheco et al., 2003; O’Donnell et al., 2001; Sagedahl et al., 1987 Giraudo et al., 1987). Recently the ability of FMDV isolate to produce disease in pigs has been mapped to a point mutation in 3A (changes from glutamine (Q) to an arginine (R) at
residue 44) suggesting a role for 3A in FMDV virulence and its broad host-range (Nunez et al., 2001).

In addition to the role of 3A in pathogenesis, it also plays a major role in the biochemical and morphological changes that are associated with picornavirus replication. In PV infected cells, the 3A protein blocks endoplasmic reticulum (ER) to Golgi traffic causing accumulation of secretory components and swelling of the ER lumen (Doedens et al., 1997). Removal of the N-terminal ten amino acids of the 3A protein significantly reduced the ability of the protein to inhibit protein secretion suggesting a role for the N-terminus either in membrane interaction or proper folding of the protein required for 3A protein secretory inhibitory function. It has been suggested that PV 3A inhibits ER-to-Golgi transport by blocking the formation or budding of the COP II-coated vesicles known to mediate anterograde transport from ER-to-Golgi (Doedens et al., 1997). Recently a brefeldin resistant phenotype in polioviruses has been mapped to either a single point mutation in the 2C (G→A at nucleotide 4361) or in the 3A (C→U at nucleotide 5190) coding sequence suggesting a role of 3A for interaction with cellular proteins like ARFl and other homologues that play an important role in the cellular vesicular trafficking system (Crotty et al., 2004). Recent studies on FMDV 3A protein suggest a different role of 3A in RNA replication. The FMDV 3A protein is found to be located in the membrane structure within the ER, but does not block protein trafficking in the cells. However 2B precursor protein 2BC has been assigned a role in blocking protein transport from the ER to Golgi (Moffat et al., 2005).
1.4.2.2 3B/VPg

Unlike any other picornaviruses, FMDV encodes and uses three distinct copies of VPg during RNA replication. Even the other sole member of the aphthovirus genus equine rhinitis A virus (ERAV) only possesses a single copy of the VPg. Each copy of VPg can be linked to the 5' end of the genomic RNA in approximately equimolar ratio (King et al., 1980; Forss and Schaller, 1982). To date no natural isolates of FMDV have been discovered that contain less than three copies of the VPg, indicating a selective pressure in maintaining apparently redundant copies of the VPg. Recently it has been shown that FMDV lacking VPg1 and VPg2 sequence in its genome could replicate well in BHK cells and porcine kidney cells, albeit with lower efficiency in fetal bovine kidney cells suggesting a role of VPg copy number in broad host-range and pathogenicity (Pacheco et al., 2003). VPg copy number in FMDV is also known to influence RNA synthesis and production of infectious virus particles in cell culture (Falk et al., 1992). Studies with single-VPg viruses have shown that they are >1000 fold less pathogenic than the three-VPg viruses based on their ability to produce vesicular lesions at the site of inoculation (Pacheco et al., 2003). But all three single-VPg viruses were capable of systemic infection albeit with symptoms milder compared to the three-VPg containing viruses. It was also reported that single-VPg copy viruses are attenuated in both cattle and pigs which explains the maintenance of the three copies of VPg found in all natural isolates of FMDV.

Among the three copies of the VPgs, VPg3 is critical for virus replication in cultured cells. However such a defect was attributed to improper proteolytic processing associated with 3B/3C cleavage junction (Falk et al., 1992). However, the possibility that this phenotype was due to replication defect as well cannot be fully excluded. To
date amongst all isolates examined, VPg3 is the most conserved whereas VPg1 and VPg2 are more variable and undergo selection (VPg1 aa 4, 11 and VPg2 aa 17, 18 and 19) suggesting a role for VPg1 and VPg2 in FMDV virulence and host range (Pacheco et al., 2003).

The tyrosine residue at the position three is conserved among all picornaviruses VPgs and is required to form a covalent bond with the 5' terminal uridine residue of the picornavirus genome (Ambros and Baltimore 1978; Rothberg et al., 1978). The importance of this residue can be easily assessed from the fact that mutation in this residue in VPg abolishes RNA replication. The linkage of the VPg in nascent positive strands, negative strands and replicative intermediates indicates a role for the VPg in the RNA priming reaction during RNA replication (Wimmer 1982). The function of VPg in the RNA priming reaction was made evident from the finding that VPg acts as a substrate in vitro to form VPgpUpU (the functional peptide primer for RNA replication) in the presence of 3D and 3CD using PV 2C RNA transcripts as a template (Paul et al., 1998; Goodfellow et al., 2000; Paul et al., 2003a,b).

1.4.2.3 3C protease

Structural studies on the picornavirus 3C proteases from poliovirus (PV), hepatitis A virus (HAV), human rhinovirus (HRV) and recently FMDV have provided insight into a unique class of cysteine proteases with three dimensional structures similar to chymotrypsin-like serine proteases. All of them possess a Cys-His-Asp/Glu catalytic triad at the active site (Allaire et al., 1994; Matthews et al., 1994; Mosimann et al., 1997; Birtley et al., 2005). In FMDV, the 3C protease has an important role in viral protein expression strategy by carrying out most of the post-translational cleavages
of the polyprotein (10 out of 13) (Grubman and Baxt 2004). The crystal structure of the FMDV 3C protease has shown that the overall fold of the enzyme is similar to that of chymotrypsin containing two β-barrel domains, each having a pair of four-stranded anti-parallel β-sheets that pack together to form a shallow peptide binding cleft. Unusually FMDV 3C structure lacks the extended β-ribbon that folds over the peptide binding/substrate binding cleft found in other picornavirus 3C proteases. That makes FMDV 3C substrate binding cleft more solvent exposed and may account for the more relaxed substrate specificity (Birtley et al., 2005). In PV, 3C mediated cleavage is specific to sites between Gln-Gly (Palmenberg, 1990); whereas FMDV 3C shows cleavage specificity for a range of dipeptides sequence including Gln-Gly, Glu-Gly, Gln-Leu and Glu-Ser (Palmenberg, 1990; Robertson et al., 1985).

Studies on PV indicates that the precursor 3CD is the major enzyme involved in structural protein processing (Ypma-Wong et al., 1988; Parsley et al., 1999) whereas in FMDV, 3C alone is sufficient to carry out most of the proteolytic processing (Ryan et al., 1989; Bablanian and Grubman, 1993). In addition to the role of FMDV 3C in polyprotein processing, it also cleaves the cellular protein eIF4A (an RNA helicase associated with the cap-binding complex) and eIF4G (in BHK cells and some rodent cells) during late stage of infection (Belsham et al., 2000; Strong and Belsham 2004). PV 3C is also involved in the cleavage of specific transcription factors including TATA-binding factors involved in RNA polymerase II mediated transcription (Clark et al., 1991; Yalamanchili et al., 1997; Kundu et al., 2005). The viral function of this inhibition could be to block host defences that require new RNA synthesis, to increase the intracellular concentration of ribonucleotides, or to disassemble nuclear complexes. FMDV 3C also brings about the cleavage of the N-terminal 20 amino acid of the histone H3 (Tesar and Marquardt 1990) suggesting...
FMDV might have an alternate mechanism of host-cell transcription shut-off compared to other picornaviruses.

The picornavirus 3C protein and its precursor 3CD have been reported to possess RNA binding properties that are required for viral RNA replication (Andino et al., 1990; 1993). In PV the RNA binding site has been mapped to the highly conserved amino acid sequence motif KFRDI, which is located on the face opposite to the active site of the enzyme. Within this motif R84 and I86 have been reported to be involved in RNA binding. The importance of the RNA binding activity of the 3C in VPg uridylylation can be easily assessed from the fact that the mutation R86S blocks the ability of this protein to support PV VPg uridylylation (Paul et al, 2000). Subsequently it was found that 3C or its precursor protein 3CD stimulates VPg uridylylation in vitro by forming a RNA-protein complex with the cre (Yin et al., 2003). Previous studies have reported the binding of the 3C protein to the clover-leaf (CL) found at the 5' terminus of many picornaviruses RNAs (Andino et al., 1990, 1993; Kusov et al., 1997; Walker et al., 1995). However in some other members of the picornaviruses including aphthoviruses and cardioviruses the CL structure is replaced by an S-fragment which has been predicted to form a single large stem-loop structure (Clarke et al., 1987).

1.4.2.4 3D is an RNA dependent RNA polymerase

The 3Dpol or RNA dependent RNA polymerase is the key non-structural protein that initiates RNA replication with the aid of other viral proteins and possibly with some cellular factors. In PV infected cells 3Dpol was found to be associated with a membranous replication complex (Bienz et al., 1983; Schlegel et al., 1996, 1997)
and recruitment of the 3Dpol to the replication complex is believed to be carried out by the 3B precursor protein 3AB (Datta and Dasgupta. 1994).

X-ray crystallographic structure of the PV polymerase, the first RNA dependent RNA polymerase to be studied, provided a classic example for understanding the structure, function and evolution of the rest of the picornavirus polymerases (Hansen et al., 1997). Foot and mouth disease polymerase and poliovirus polymerase belong to the group 1 polymerases (Koonin 1991). The overall topology resembles other classes of nucleic acid polymerase, which is like a cupped right hand with finger, palm and thumb domains (Hansen et al., 1997). The palm domain contains four structural motifs (A-D) which are found in all classes of polymerase. It also has a fifth motif (E) found only in enzymes such as RNA dependent DNA polymerases which utilise RNA templates.

Across all species of positive strand viruses of eukaryotes, only six residues within the polymerase are completely conserved. All picornavirus polymerases possess a conserved GDD sequence which is located in the motif C region of the poliovirus polymerase. The first aspartic acid is supposed to be critical for enzyme function and postulated to be involved in the metal ion co-ordination at or near the catalytic active site of the enzyme (Delarue et al., 1990). Mutational analyses have shown that any changes at the first aspartic acid results in an enzyme that is inactive in-vitro as well as in-vivo, however mutation at the second aspartic acid resulted in an enzyme with a metal ion requirement for Mn$^{2+}$ for activity both in vitro as well as in vivo (Jablonski and Morrow 1995). In addition, in the poliovirus polymerase, the residue lys-61 is implicated in the catalytic activity in the polymerization reaction and
regarded as the nucleotide tri-phosphate (NTP) binding site. Residues Val391, Phe377, Arg379 and Glu382 are all supposed to be involved in the 3AB-protein binding that recruits the polymerase to the membrane bound replication complex and mutation of these residues affects the process of VPg uridylylation (Lyle et al., 2002). In vitro studies have shown that the PV 3Dpol exhibits co-operativity in its polymerase activity (Pata et al., 1995) and it has been suggested that PV 3Dpol functions as a multimer in vivo.

In addition to PV 3Dpol, the three dimensional crystal structure of polymerases of many other positive strand RNA viruses have been solved to date including FMDV and HRV (picornaviridae family) (Hansen et al., 1997; Appleby et al., 2005), hepatitis C virus and bovine viral diarrhea virus (Flaviviridae family) (Bressanelli et al., 1999; Lesburg et al., 1999), rabbit hemorrhagic virus and Norwalk virus (Caliciviridae family) (Ng et al., 2002; Ng et al., 2004) and the double-stranded RNA bacteriophage Φ6 (Butcher et al., 2001). One of the common important features of this class of polymerase is the presence of a fully encircled active site.

Foot and mouth disease virus polymerase shares about 29% sequence identity to PV polymerase. The recent three dimensional crystal structure of the FMDV 3Dpol provides further understanding of picornavirus polymerases (Orta et al., 2004). The N-terminal segment comprising residues 1-96, called the finger tips, bridges the fingers and thumb sub-domains which encircle the active site of the enzyme similar to PV and HRV. In addition, comparison of the FMDV 3D in its bound and free form (template-primer RNA decanucleotide) showed no movement of sub-domains to accommodate the template-primer unlike the Human immunodeficiency virus
(HIV) Reverse transcriptase (RT). For HIV RT, movement of the region following motif E is required as a pivot point for the movement of the thumb sub-domain to accommodate the template-primer RNA complex. Unlike the PV polymerase, the crystal structure of FMDV polymerase did not show any evidence of higher order structure, like the head-to-tail fibres described for PV.

However, differences exist among viruses with respect to the mechanism of initiation of RNA synthesis by the polymerase. HCV and bacteriophage Φ6 polymerase follows a de-novo RNA synthesis mechanism without the aid of any primer. Both HCV and Φ6 polymerase posses a large and extensive thumb sub-domain with a β-hairpin extension. It has been proposed that the β-hairpin and the C-terminal region of the enzyme form a scaffold upon which the 3' terminal initiation complex assembles (Hong et al., 2001; O'Farrell et al., 2003). PV, HRV and RHDV use a protein primed initiation mechanism for RNA synthesis where a small virus encoded peptide VPg/3B, is first uridylylated to form VPgpU(pU) which then acts as a functional primer for RNA synthesis (Paul et al., 2003; Machin et al., 2001). In the case of PV, 3Dpol is inactive in the context of its precursor 3CDpro (Thompson and Peersen 2004). Proteolytic cleavage of 3Dpol is required for activation of the enzyme for its biological activity. In all picornaviruses, glycine is the first amino acid of the polymerase. In PV, deletion of the first glycine residue or addition of a single amino acid residue at the N-terminus results in complete loss of polymerase activity (Thompson and Peersen 2004). Based upon the above observation, it was contemplated that the N-terminus could be an integral part of the active site. Recent studies with the PV polymerase suggested that the proteolytic processing of the 3CDpro leads to polymerase activation following a structural change in which the N-
terminal glycine residue is buried in a pocket at the base of the finger domain. The buried N-terminus stabilises the structure and allows the direct positioning of the aspartate 238 for binding to the 2' OH group of the incoming nucleoside in the active site (Thompson and Peersen 2004). In RNA polymerase, this aspartate hydrogen bond to the 2'OH of the incoming rNTP allows the selection of rNTPs over dNTPs (Huang et al., 1997; Gohara et al., 2004). In addition, the interaction between the finger and thumb completely encircles the active site and creates an NTP entry tunnel at the back of the polymerase (Thompson and Peersen 2004). The unique extensive interactions between the thumb and finger domains associated with all RdRp results in a closed globular conformation (Thompson and Peersen 2004; Appleby et al., 2005), whereas a U shaped conformation is observed in the case of the DNA polymerases, DNA-dependent RNA polymerases, and the HIV RT.

The 3Dpol from PV and HRV catalyse two different types of phosphoryl transfer reaction. The enzyme can nucleotidylate the virus encoded peptide, 3B/VPg to generate the functional primer for initiation of RNA synthesis a process called VPg uridylylation. The second function includes the ability of the enzyme to elongate the nascent chain of nucleotides during the RNA elongation reaction (Paul et al., 1998; Gerber et al., 2001b). Both the reactions require Mg$^{2+}$ or Mn$^{2+}$ as cofactors. The requirements and specificities of both type of reaction are considered to be different and are not known. For VPg uridylylation 3Dpol uses a ‘slide-back’ mechanism for nucleotidylation of the VPg peptide to form VPgpUpU in the presence of the cre as a template (Paul et al., 2003a,b). The nucleotide elongation activity of the enzyme has been well studied and the detailed mechanism of action determined. In addition 3D$^{30}$ has both terminal transferase activity (Neufeld et al., 1994) and strand displacement
activities (Cho et al., 1993). The enzyme is efficient in template switching and is capable of catalysing primer-independent RNA synthesis (Arnold and Cameron 1999).

All classes of nucleic acid polymerase (e.g. DNA dependent DNA polymerase (e.g. Klenow Fragment) and RNA dependent DNA polymerase (e.g. HIV RT) can use two divalent cations as the cofactor for the phosphoryl transfer (Brautigam and Steitz 1998). Most of the polymerases known use Mg$^{2+}$ as a cofactor which resembles the conditions in vivo (Kornberg and Baker 1991). Mn$^{2+}$ has been shown to be an effective cofactor for a variety of polymerase systems (Huang et al., 1997). However Mn$^{2+}$ usually alters the biochemical properties of the polymerase, decreasing the stringency of the substrate selection and incorporation fidelity permitting increased use of DNA template, nucleotides with incorrect bases or nucleotides with incorrect sugar configurations (e.g. 2'-dNTPs, 3'-dNTPS and 2',3'-ddNTPs) (Arnold et al., 1999).

In the presence of Mn$^{2+}$, the ground state binding of the correct nucleotide is more stable than in the presence of Mg$^{2+}$ suggesting Mn$^{2+}$ has additional adventitious interactions with the enzyme. Being a transition metal it has been suggested that during the course of interaction with the enzyme, Mn$^{2+}$ has the additional ability to interact with the backbone or nitrogen and sulphur containing residue ligands near the binding site of the metal (Bock et al., 1999).

Though RNA dependent RNA polymerases are associated with an extraordinarily high mutation rate (Domingo et al., 1996), recent data suggested that the intrinsic
fidelity of PV 3Dpol is similar to that of the T7 DNA polymerases (Wong et al., 1991). The important aspects of the kinetic data suggested that once 3Dpol has initiated RNA synthesis it will not dissociate from the nascent RNA until it reaches the end of the genome (Arnold and Cameron 2004).

1.4.3 Role of precursor proteins in RNA replication

Positive strand RNA viruses including picornaviruses have limited genetic information due to their relatively small genomes. During the virus life cycle many positive strand RNA viruses follow different strategies to subvert the cellular machinery for their favour that allows efficient replication and new progeny virus production. One of the important strategies is to utilise cellular proteins for recruitment into their translation and replication machinery. They also produce proteins that have multiple functions. In addition, during polyprotein processing they generate many intermediate precursor proteins; these can have completely different functions compared to their mature cleavage counterparts (Wimmer et al., 1993).

Viral proteins 2C and its precursor 2BC are known to possess ATPase activity (Pfister and Wimmer 1999) and are capable of membrane rearrangements in the infected cell. Protein 2B and its precursor 2BC increase the intracellular calcium level, due to disruption of the endoplasmic reticulum and alter the permeability of the plasma membrane (Aldabe et al., 1996; 1997; Irurzu et al., 1995; Vance et al., 1997). However, striking difference in the activities of 3CDPro and 3C polypeptide has been observed in picornaviruses. 3C has been shown to possess RNA binding determinants and the motif required for this could be separated from those required for proteolytic activity. Though the proteinase and the RNA binding sequences reside within 3C, 3CD complexs differs from 3C with respect to these activities. In
poliovirus both the 3C and 3CD recognize the RNA cloverleaf element at the 5’ end of positive strand genome, in the presence of cellular protein PCBP2, to form a ternary complex (Parsley et al., 1997). However 3CD has an enhanced ability to form this ternary complex compared to 3C, and for this reason the 3CD/PCBP2/RNA complex is thought to be the biologically relevant one (Andino et al., 1990). In addition, as proteinases, 3CD has a dramatically enhanced ability to cleave the structural (P1) protein precursor compared to 3C (Ypma-wong et al., 1988). No specific role for the FMDV 3CD protein has yet been determined and FMDV 3C is sufficient for all FMDV polyprotein processing.

In addition to 3C, poliovirus 3D is the other cleavage counterpart of 3CD and mediates uridylylation of VPg to form VPgpUpU that acts as the primer to initiate RNA replication (Paul et al., 1998). The 3CDpro from PV does not possess any elongation activities in vitro. However evidence suggests that the precursor (Pro-pol) form of the polymerase in caliciviruses is the most active and biologically relevant form of the enzyme (Wei et al., 2001). Recently a more defined role of the 3CDpro in RNA replication has been established. It has been shown that 3CD stimulates the VPg uridylylation in vitro up to 100 fold by forming a stable complex with the PV cis-acting replication element (cre) (Yin et al., 2003). In addition supplementation with mRNA encoding 3CDpro or purified 3CDpro stimulates virus production in a cell-free system up to 100 fold, but has no effect on protein translation (Franco et al., 2005). This stimulatory effect is only observed when 3CDpro is added in the reaction within 2-4 hrs of the initiation of cell-free reaction indicating the time of maximum protein translation and replication complex assembly. It should be noted that both 3CDpro and 3Cpro inhibits virus production when added at the beginning
of the reaction. Furthermore 3CDpro has no effect on pre-initiation replication complexes isolated after treatment with guanidine. This indicates that 3CDpro produces its stimulatory effects only when it is added to the system at the time of replication complex assembly but loses its activity once replication complexes are formed. Since 3C or 3D alone do not stimulate this reaction it suggests that besides 3C, the 3D domain is also involved in the stimulation process. Mutations in the 3D domain in the context of 3CDpro in interface I that disrupt oligomerization of 3Dpol also results in loss of the stimulatory activity suggesting that either interaction between 3CDpro molecules or interaction between 3CD and 3D is required for this stimulation (Franco et al., 2005). In addition, mutations in the 3C domain within the context of 3CD that disrupts the RNA binding properties of 3C (R84S/I86A) also destroys the stimulatory activity (Franco et al., 2005). However 3Cpro alone, with intact RNA binding activity does not have any stimulatory effect on virus yield at any stage of in vitro virus synthesis. It is well known that along with 3CD, 3C alone can stimulate cre template mediated VPg uridylylation, thus it was suggested that the 3CD stimulation of virus yield is mediated by a CI-3CDpro mediated interaction. In other words, it was suggested that this stimulatory effect of the 3CD on virus yield in cell-free system is cre independent (Franco et al., 2005).

The 3CD protein has also been shown to interact with the 3’NCR of PV, which may provide a source for a polymerase molecule proximal to the 3’ end of the RNA to initiate negative strand RNA synthesis (Harris et al., 1994). This 3’ NCR interaction may be stabilised further by 3D domain mediated contacts with the viral polypeptide 3AB. Evidence for a 3D-3AB protein-protein interaction has been generated previously using the yeast two hybrid system (Hope et al., 1997; Xiang et al., 1998).
The viral protein 3AB, the precursor of VPg, has been suggested to be an important component of the replication complex because of its ability to interact both with membranes and the RNA polymerase (Datta and Dasgupta, 1994). The yeast two-hybrid system has shown a strong interaction between the 3AB or 3B with the polymerase and the contact point between the two proteins has been mapped to be in the 3B domain of the 3AB. In addition, the polymerase activity of purified 3D polymerase (Lama et al., 1994; Paul et al., 1994; Plotch and Palant, 1995) and proteolytic activity of the 3CD<sup>pro</sup> (Lama et al., 1994) are both stimulated by addition of the detergent solubilized 3AB but not by 3A in vitro. However, detergent solubilized 3AB is not a substrate for 3CD cleavage, whereas membrane-associated 3AB is (Molla et al., 1994). 3AB has also been implicated to have non-specific RNA binding activities as well as a specific affinity for the CL structure at the 5' end of the genome (Harris et al., 1994; Paul et al., 1994). These in vitro properties are consistent with a function for 3AB in the replication of the viral genome, and interpretation of viral defects stemming from mutations in 3A has frequently presumed that the mutations were disrupting 3AB function. Both 3A and 3AB are membrane associated in infected cells (Semler et al., 1982) and this association is thought to be mediated by a 22 amino acid hydrophobic domain near the C-terminus of the 3A protein. It has also been suggested that in addition to 3B/VPg protein, other precursor proteins like 3AB, 3BC could also act as substrate for VPg uridylylation.
1.4.4 Cis-acting RNA elements involved in picornavirus RNA replication

Studies carried out using poliovirus as a model organism have shed light on the requirements of proteins and RNA structures required for viral RNA replication. These RNA structures are located in the 5’ non-coding region that is the cloverleaf (OriL), the 3’ non-coding region (including the heteropolymeric tract and the polyA tail (OriR) and 2C(cre)(OriC) have been well documented (Andino et al., 1990; 1993, Filipenko 1992, 1996, Wang et al., 1999; Melcher et al., 2000; Goodfellow et al., 2000). However very little work has been pursued in this area for foot-and-mouth disease virus. The structure and location of these cis-acting RNA elements involved in FMDV replication vary from those of the poliovirus, however a similar pattern of function in FMDV RNA replication could be expected.

1.4.4.1 The cloverleaf of PV participates in the formation of a ternary ribonucleoprotein complex

In poliovirus the first 90 nt has been predicted to form a cloverleaf-like secondary structure that is necessary for viral RNA replication (Riviera et al., 1988; Skinner et al., 1989). Detailed structural analysis of the CL revealed the presence of 4 domains namely stem a, stem-loop b (SLB), stem-loop c (SLC) and stem-loop d (SLD). SLD is again divided into sub-domains d1, d2, d3 and d4 (Andino et al., 1990;1993). SLB and the sub-domain d3 play important roles in negative strand synthesis by participating in the formation of a ternary ribonucleoprotein complex around the 5’ end of the poliovirus RNA by interacting with cellular protein PCBP2 and the viral proteinase 3CD respectively (Andino et al., 1990; Garmarnik and Andino 1997;
Parsley et al., 1997; Barton et al., 2001). The importance of this interaction between CL and 3CD was made clearer following the observation that an insertion of 4 nts in the CL resulted in compensating mutations in the 3C domain of the 3CD that suppressed the replication defects (Andino et al., 1990). The CL has been reported to be involved in negative strand synthesis but not in the positive strand synthesis (Andino et al., 1990). Recently poliovirus 5' terminal CL has also been shown to be required in cis for VPg uridylylation and negative strand synthesis (Rieder et al., 2003). In addition to the role of CL in RNA replication recent evidence suggests a role for the CL in viral RNA stability (Murray et al., 2001).

If the S-fragment in FMDV has to function as a CL substitute in RNA replication, as a matter of speculation it may be expected to interact with viral protein 3C or 3CD alone or in combination with other viral and cellular proteins to form a ribonucleoprotein complex at the 5' end of the RNA genome. To date no such interactions between the S-fragment and any viral or cellular proteins have been established.

1.4.4.2 Role of 3' non-coding region in viral RNA replication

In picornaviruses the 3' non-coding region has been known to fold into higher order structural elements that interact with both viral and cellular proteins for RNA replication (Harris et al., 1994; Pilipenko et al., 1996; Mirmomeni et al., 1997; Melchers et al., 1997, 2000; Wang et al., 1999; Waggoner and Sarnow 1998; Meredith et al., 1999). The poliovirus 3' UTR RNA contains two hairpins, designated domain X and Y and a third domain designated the K (kissing) domain which involves interactions between the X and Y domains. Experimental data have shown the importance of the 3' non-coding region in RNA replication as it can not
be replaced with non-viral sequences (Rohll et al., 1995). It is reasonable to assume that the 3' non-coding region plays an important role in poliovirus RNA replication as it contains the binding site for the poliovirus protein 3AB and 3CD (Harris et al., 1994). The 3' UTR of FMDV RNA consists of two components, a region of about 100 nt of heterogeneous sequence and a poly(A) tail. Deletion of the unique (heterogeneous) 3'UTR sequence blocks infectivity of FMDV RNA (Saiz et al, 2001). This may not be true for other picornavirus like PV and HRV, where viable viruses lacking the unique 3'UTR sequence can be propagated albeit less efficiently (Todd et al., 1997).

It has been also reported for HRV 14 that the 3'UTR forms a complex with a 34 to 38 kDa host protein which is induced during viral replication (Todd et al., 1995). In contrast to the PV 3'UTR, the rhinovirus 3'UTR forms only a single stem-loop, which, at the level of secondary structure is highly conserved amongst rhinoviruses. Mutations in the loop and in the stem that altered the stability of the stem have been observed to reduce rhinovirus replication efficiency (Rohll et al., 1995). A mutation in the 3' NCR of poliovirus has been reported that resulted in a temperature-sensitive defect in RNA replication (Sarnow et al., 1986). Furthermore, it has been reported in encephalomyocarditis virus (EMCV) that the poly(A) tract is required for the recognition of the viral genomic 3' end sequence by purified EMCV 3D<sup>10</sup> (Cui et al., 1993). Studies on the 3' UTR of cardiovirus RNA have shown evidence for 3 stem-loop structures, one of which is essential for virus viability (Duque and Pahnenberg 2001). It is possible that sequences within the viral coding sequence (but perhaps near the 3' end of the genome) are required to provide the specificity of viral RNA recognition that must be achieved by the replication machinery, especially for
the PV mutant lacking the usual 3’ UTR sequences (Todd et al., 1997). The length of the poly(A) tail determines the infectivity of PV RNA (Spector & Baltimore 1974), but the mechanism involved in this is not elucidated. In addition the polyA tail has been proposed to be involved in negative strand synthesis by acting as a template for VPg uridylylation (Paul et al., 1998).

Another crucial feature of the poliovirus RNA is the binding affinity of the polyA tail to a cellular protein polyA binding protein (PABP) (Herold & Andino 2001). The viral 3CD binds to the 5’-end of the genomic RNA and reaches its site of action within the poly(A)-tail of the genome via circularization of the genomic RNA using an RNA-protein-protein-RNA-bridge that involves two cellular factors, PCBP and PABP (Herold & Andino 2001).

1.4.4.3 Cis-acting replication element (cre)

Evidence has accumulated for the presence of cis-acting RNA elements located internally within picornavirus genomes that are required for RNA replication. Initially it was found that certain sequences within the P1-coding sequence of HRV-14 were required to achieve efficient replication of replicons based on this virus (McKnight and Lemon 1996). This contrasted with previous studies on PV that had demonstrated that the entire capsid coding sequence could be deleted without affecting RNA replication (Kaplan and Racaniello 1988). Further work (McKnight and Lemon, 1998) identified the critical RNA element as a specific stem-loop structure, termed a cis-acting replication element (cre), and located within the 1D (VP1) coding region of the HRV-14 genome. Subsequently, similar cre motifs have been identified in other picornavirus genomes (Gerber et al., 2001; Goodfellow et
al., 2000; Lobert et al., 1999). Each of the cre’s includes a conserved sequence motif of AAACA located within a loop at the end of a stable stem structure. These elements (about 50-60nt in length) occur in different places within the picornavirus genomes; the cre’s from HRV-14, HRV-2, cardioviruses and PV are within the coding regions for 1D, 2A, 1B and 2C respectively (Gerber et al., 2001; Goodfellow et al., 2000; Lobert et al., 1999; McKnight and Lemon 1996). Furthermore the cre’s can be moved without blocking function (Goodfellow et al., 2000). It has been shown that the PV and HRV-2 cre structures act as the template for the uridylylation of VPg (3B) (to produce VPgpU and VPgpUpU) by the 3D RNA polymerase in vitro (Gerber et al., 2001; Paul et al., 2000). Evidence has been presented that this process involves a “slide-back” mechanism on the AAACA motif (Paul et al 2003). The first A residues in the AAACA sequence acts as template for the addition of two uridine residue to the VPg peptide to form VPgpUp(U). The VPgpUpU has been thought to acts as a primer for both positive and negative strand synthesis (Paul et al., 2000; Goodfellow et al., 2003). However it is noteworthy that, within cell-free replication systems, the PV cre is only required for the synthesis of +ve sense RNA strands (Morasco et al., 2003; Murray and Barton 2003; Goodfellow et al., 2003). In addition it has been found that there is a direct correlation between the ability of the cre to support uridylylation in vitro and replication in vivo (Yang et al., 2002).

Recently, Mason et al., 2002 presented evidence for a cre within the genome of FMDV. This element is about 54nt in length and includes a conserved AAACA sequence but in contrast to all other picornavirus cre structures it is located within the 5’UTR of the viral genome, immediately upstream of the IRES. This FMDV cre could be moved to the 3’ UTR whilst retaining activity. This feature corroborates the
assumption that the sequence of the cre and its secondary structure rather than the coding sequence is important for RNA replication. A role for this FMDV sequence in the uridylylation of FMDV VPg (3B) has not been reported but is assumed. The identification of a cre within the 5' UTR of FMDV explained the phenotype of a temperature-sensitive (ts) mutant (ts303) of FMDV which is RNA replication defective under the non-perrmissive conditions (Tiley et al., 2003). Unexpectedly, the ts lesion in this virus was located within the 5' UTR and this mutation is now known to reduce the stability of the stem of the FMDV cre (Tiley et al., 2003). A revertant of the ts mutant had a second mutation that restored the stability of this structure. A remarkable feature of this mutant is that its defect in replication could be complemented in trans, thus it appears that the FMDV cre can function in trans and it was suggested (Tiley et al., 2003) that a better name for this element may be a 3B-uridylylation site (bus). It is interesting to note that the role of the PV cre in the in vitro uridylylation of the PV VPg can also be performed in trans (Goodfellow et al., 2003).

One of the important features of the cre is that the structural stability of the cre plays an important role in VPg uridylylation since the stem must present the unpaired terminal loop in a proper spatial configuration. Though the whole viral genome contains a number of AAACA motifs, not all of these sequences are capable of supporting VPg uridylylation. The poliovirus CL possesses such a motif that is incapable of supporting VPg uridylylation. It appears that the flanking nucleotide sequence and local structure adopted by the AAACA motif in the CL promotes complete base pairing (Andino et al., 1990, 1993). Further evidence came into the picture by ribonuclease mapping of the PV cre with ssRNA-specific T2 nuclease that
indicated an unpaired nature for the terminal loop of the cre (Goodfellow et al., 2003). In PV the entire stem of the cre can be replaced with an artificial stem of similar stability without compromising its function (Goodfellow et al., 2003). This suggests that the stability of the stem, not its sequence, is critical for cre function. Structural stability of the cre is also temperature dependent and the optimum temperature to support uridylylation is 30-34°C in vitro. Interestingly in a ts mutant of FMDV where the replication is significantly suppressed at non-permissive temperature, the mutation was mapped to the stem of the cre (transition of the C-U at the 47 position) making it less stable (Tiley et al., 2003). Revertant mutants with compensating mutations that stabilised the stem enabled the virus to replicate again at the elevated temperature making it reasonable to assume that the temperature dependence of the cre function has a role in uridylylation in vitro and also during in-vivo replication. Despite the difference in the location of the cre and limited sequence similarities it is probable that all picornavirus members may have evolved a conserved mechanism for RNA replication. Furthermore the presence of multiple functional cre elements in the genome does not have any effect on the replication process (Yin et al., 2003). Recently the solution structure of the minimum functional cre sequence (33 nt) of HRV has been studied by NMR (Thiviyathan et al., 2004). One of the important observations that were made is the orientation of the conserved adenosines towards the inside of the loop that participate in a 'slide-back' mechanism of VPgpUpU synthesis. However it was suggested that during VPg uridylylation the adenosine residues are available for base templating (Thiviyathan et al., 2004).
1.4.5 Replication complex assembly

One of the important features of positive strand RNA viruses is that they assemble specialised replication complex machinery in infected cells. It leads to extensive structural reorganisation of the cellular organelles that leads to cytopathology and ultimately cell death. The replication complex provides a platform for accumulation of all the replication factors (both cellular and viral proteins) required for RNA synthesis. It has been proposed that translation of PV RNA into proteins, generation of vesicles and their association into functional replication complex is a coupled process (Egger et al., 2000). In other words, for vesicle assembly and RNA replication all the proteins and RNA must be provided in cis. PV utilises membrane structures from the host cell for vesicle assembly. The exact nature and source of these membranes from where they are derived is not clear. In PV it has been suggested that the early vesicles are derived from the ER (Rust et al. 2001) whereas the late vesicles arise from other cellular organelles like lysosomes and the Golgi and co-localize with Golgi protein markers (Rust et al., 2001; Bolten et al., 1998; Schlegel 1996). Infection by PV, EV11 and EMCV results in vesicles similar in morphology which are heterogeneous in size and arranged as tightly packed clusters. However another picornavirus, human parecho virus 1 (HpeV-1), forms homogenously sized vesicles which are less numerous and do not form clusters (Gazina et al., 2002). In the case of PV, the replication complex appear like rosettes in infected cells, which are covered by double layer membrane structures that surround and protects the input and newly synthesized viral RNA rendering majority of them resistant to the action of RNaseA (Fogg et al., 2003). Such a structure is functionally important for the virus survival as it sequesters the replication process from other potentially competing events such as translation inside the cells. In
addition, it also safeguards the virus against any double stranded RNA induced host defence response such as RNA interference or interferon induced responses (Ahlquist, 2002).

Poliovirus assembles its replication complex using membrane structures derived from the components of the cellular anterograde membrane traffic pathway (Rust et al., 2001; Suhy et al., 2000). Such replication complexes are associated with both viral structural and non-structural proteins. In mammalian cells cellular secretion between ER and Golgi is dependent upon the function of two coat protein complex, COP I and COP II. The secretory pathway follows a route from the endoplasmic reticulum to the microtubule organising centre (MTOC) harbouring the Golgi complex. In the first step ER protein cargo is transported through COP II coated vesicles to a vesicular tubular cluster (VTC). In the next step the COP I coated VTC, without any trace of COP II, travels to the Golgi complex (Stephen at al., 2000). In both steps, the transport is aided by microtubule infrastructure present in the cell (Rios and Bornens., 2003). Different picornaviruses show different degrees of sensitivity to the action of drug brefeldin A. Replication of PV, EV11 and BEV are sensitive to the action of this drug. However both FMDV and EMCV are resistant (Gazina et al., 2002; O'Donnell et al., 2001) and HpeV-1 is partially resistant (Gazina et al., 2002). The possible explanation for insensitivity to the action of BFA in case of FMDV and EMCV might be due to involvement of different cellular factors for vesicle assembly or they might have a different site of replication. Recent work has suggested that PV resistance to BFA action can replicate efficiently in cells where the secretory function has been completely blocked and determinants of such BFA resistant have been mapped within 2C and 3A. These data suggest that BFA
inhibits a specific step in PV replication that may involve direct interaction of 2C and 3A with the cellular factor ARF1 (Crotty et al., 2004)

Infection of susceptible cells with FMDV results in the accumulation of the majority of the cellular organelles to a distinct region located to one side of the nucleus termed the virus replication site (Monaghan et al., 2004). The morphological changes that occur during this process are different from those seen with other picornaviruses. Changes in the ER appear first, these are followed by structural changes in the Golgi. The number of vesicles grows in number as the infection progresses towards the late stage of infection. FMDV forms considerably fewer vesicles than PV and does not form clusters or rosettes, these observation are similar to those seen with HpeV-1 (Gazina et al., 2002). Though both single and double layered vesicles appears in FMDV infected cells, the latter structure is rare compared to PV and appears only during the mid and late phase of virus replication (Monaghan et al., 2004).

It has been demonstrated that the site of capsid dissociation after virus entry and site of RNA replication are different in location inside PV infected HeLa cells. The former takes place towards the peripheral region, whereas the latter localises to the perinuclear position (Egger and Bienz, 2002) suggesting a pattern of migration of the components of the PV replication machinery. Once the viral RNA is released in the cytoplasm it migrates to the endoplasmic reticulum in a microtubule (MT) independent manner where the translation of the viral RNA starts to synthesize the proteins required for RNA replication. This nascent replication complex then migrates towards the perinuclear location in a MT dependent manner and is thus
sensitive to the treatment of Nocodazole (Egger and Bienz, 2005). Nocodazole treatment results in the appearance of viral RNA dispersed throughout the cytoplasm but does not block replication indicating that the migration step is not absolutely required for RNA replication. When the Replication complex (RC) reaches the perinuclear location the number of individual RCs decreases as they coalesce. It has been suggested earlier that such coalescence would increase the chance of mixed RCs inside the cell infected with two different virus strains (Egger and Bienz, 2002). This process might have an important selective advantage in evolution of new strains of virus by means of recombination.

1.4.6 Cell free replication systems for picornaviruses

In picornaviruses the infection cycle begins with the synthesis of all viral proteins and these are required for initial negative strand and subsequent positive strand synthesis. The whole process of initial translation and subsequent replication is interdependent. That makes it difficult to dissect individual steps in a synchronous manner during virus infection in tissue culture. Subsequently this difficulty has been overcome by the development of cell-free replication system in poliovirus (Molla et al., 1991) and more recently in EMCV (Svitkin and Sonenberg 2003). Essentially cell-free replication reactions constitute the assembly of an in-vitro reaction using HeLa S10 extract programmed with saturating amounts of viral RNA transcripts that does not necessitate the generation of new viral mRNA for protein translation (Molla et al., 1991; Barton and Flanagan 1993; Barton et al., 1995). Such a system allows the dissection of individual steps including viral protein synthesis, RNA replication and infectious virion particle production in synchrony that can be detected by $^{35}$S and $^{32}$P pulse labelling. However addition of VPg linked RNA isolated from virion itself
support RNA synthesis more efficiently compared to RNA transcripts made in-vitro (Elizabeth Rieder 2005, Europic meeting, The Netherlands; Paul et al., 2005 Europic meeting). In addition, the stimulatory effects of 3CD protein on RNA synthesis in vitro also requires VPg linked RNA (Franco et al., 2005). This in-vitro system also allows the isolation of pre-initiation replication complexes (PIRC) by using guanidine at low concentration (2mM) (Barton and Flanagan 1997). Guanidine at low concentrations is non-toxic to the cells and acts as a reversible inhibitor of initiation of negative strand synthesis, but does not have any effects on the elongation of negative strands, initiation of positive strands or elongation of positive strand synthesis. Such PIRC can be made replication competent by removing the guanidine which then allows the synthesis of negative strands and asymmetric positive strand synthesis. To summarize the utility of this system, it allows the complete replication cycle in vitro in a step by step manner that includes translation of viral RNA, assembly of membrane associated replication complexes, VPgpUpU formation, VPg linked negative strand RNA synthesis, VPg linked positive strand RNA synthesis and complete infectious virion particle production (Morasco et al., 2003; Murray et al., 2003). However it should be noted that programming of the HeLa S10 extracts with T7 RNA polymerase derived transcripts allows the detection of negative strand synthesis but undetectable levels of positive strand synthesis (Barton and Flanagan 1997; Herold and Andino 2000). In vitro derived T7 RNA transcripts generate RNA with two ‘G’ residues at the 5’ end that are copied into two ‘C’ residues in the 3’ end of the negative sense RNA which then blocks VPgpUpU mediated positive strand synthesis. However T7 transcripts with two ‘G’ residues are corrected in tissue culture and there is generally a lag phase of a few hours to correct the non-viral ends. It is still not clear why two non-viral ‘G’ residues can not be
corrected in cell-free replication system. However this problem can be overcome in cell-free system by using either virion RNA or T7 RNA polymerase derived RNA transcript with a hammer-head ribozyme at the 5' end. Ribozymes are antisense RNA molecules that are enzymatically active (Scott 1997). They function by binding to the target moiety through Watson-Crick base pairing and act by cleaving the phosphodiester backbone at a specific target site. The name of hammerhead ribozyme is given due to the similarity between its secondary structure and the shape of a hammerhead (Birikh et al., 1997). They are the best understood subcategory of all ribozymes. Insertion of a hammerhead ribozyme sequence at the 5' end of the viral cDNA produces RNA transcripts in vitro with an authentic 5'end that replicate efficiently in the cell-free system (Herold and Andino 2000).
1.5 Research objectives

1.5.1 Development of a VPg uridylylation assay for foot-and-mouth disease virus

Previous studies with poliovirus (PV) and human rhinovirus (HRV) have shown the requirement of VPg peptide for RNA replication. The 3Dpol enzyme first uridylylate VPg peptide to form VPgpUpU that acts as the functional primer for both positive and negative strand synthesis. Synthesis of VPgpUpU can be reproduced in vitro by using either cre or polyA as template ((Paul et al., 1998; Goodfellow et al., 2000). Unlike other picornaviruses foot and mouth disease virus (FMDV) contains genetic information that encodes three distinct copies of the VPg and the cre function can be complemented in trans (Tiley et al., 2003). On the premise of information available for other picornaviruses, a role of VPg in FMDV RNA replication has been assumed. To this end we are interested in developing a VPg uridylylation assay for FMDV. In addition, defined roles for cre/bus and 3Dpol precursor protein 3CDpro in this assay will be determined. The functional significance of the three distinct copies of the VPg and their efficiency in FMDV uridylylation system also need to be determined.

Poliovirus 3Dpol is able to utilize as a substrate, in addition to its own VPg, the VPg of HRV14 when either poly(A) or cre(2C) RNA is used as a template. In contrast to HRV14 VPg, the VPgs of HRV2 and HRV89 have barely detectable substrate activities on both templates (Paul et al., 2003). Based on those observations, using the poliovirus polymerase, PV VPg as well as the PV cre, the compatibility of these components with the FMDV uridylylation assay will be determined.
1.5.2 Functional analysis of proteins and RNA elements required for RNA replication

It has been suggested for PV that some of the precursor proteins like 3AB, 3BC, 3BCD (precursors of the VPg) could be uridylylated in vitro; the objective will be to determine the role of the precursor proteins in the FMDV uridylylation system. In addition, analysis of the minimum cre sequence required for VPg uridylylation will be performed.

The function of the 3CDpro can be substituted with the 3C protein in PV uridylylation system (Pathak et al., 2002). FMDV 3C differs from PV 3C with respect to polyprotein processing and the RNA binding activity of the FMDV 3C protein has not been established. The nature of the RNA binding activity of the FMDV 3C protein and its role in RNA replication will be determined.

1.5.3 Development of an in-vitro RNA replication assay for FMDV

In FMDV infected cells RNA replication takes place on specialised membranous vesicles (Monaghan et al., 2004). In PV it has been shown that such replication complexes are capable of supporting RNA synthesis in vitro (Takeda et al., 1986). In this context, the ability of isolated replication complexes, derived from FMDV infected BHK cells will be used to analyse FMDV RNA synthesis in vitro.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Bacterial cells, cell lines and viruses

2.1.1 Bacterial cells

E.coli cells DH5α (Invitrogen), SCS 110 (Stratagene), M15 (Qiagen), BL21DE3 (Novagen), BL21DE3pCG1 (Modified BL21DE3), ER2925 (NEB) and BL21DE3pLysS (Novagen) were streaked onto LB (Luria-bertani) agar plates supplemented with antibiotics as required and incubated at 37°C overnight. A single colony was picked, grown overnight at 37°C in 5ml LB medium. 50μl of the overnight culture was added to 5ml LB medium and grown at 37°C until cells reached mid log phase OD$_{600}$: 0.2-0.8. 400μl of this culture was then added to 600μl of a 20% LB-glycerol solution (Appendix II). The cell suspension was vortexed gently for uniform mixing and frozen at −70°C until required.

2.1.2 Cells and viruses

Baby Hamster Kidney (BHK) cells were cultured in Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 5% foetal calf serum (FCS), 2 mM glutamine, penicillin (100 SI Units/ml) and streptomycin (100 μg/ml). The virus used in this study was the FMDV strain O1BFS. This virus has been passaged through BHK cell lines multiple times and uses heparan sulphate (HS) as its receptor in the absence of functional integrins (Jackson et al., 1996).
2.2 DNA techniques

2.2.1 PCR

Polymerase Chain Reactions (PCR) (gradient PCR, overlapping PCR) were performed using the oligonucleotides listed in Appendix III.

25-50 ng of template DNA was added to 10 pmol of each oligonucleotide (forward and reverse), 1 μl of dNTP mix (10 mM of each dNTP, Promega), 5 μl of 10X Pyrococcus furiosus (Pfu)/Thermus aquaticus (Taq) enzyme buffer and 0.5 μl of Pfu/Taq enzyme. The reaction volume was made up to 50 μl with mQ H₂O. The reactions were subjected to the following amplification cycle:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Step 1: Initial denaturation</td>
<td>95°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Step 2: Denaturation</td>
<td>95°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Step 3: Primer annealing</td>
<td>55°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>Step 4: Elongation</td>
<td>72°C</td>
<td>2-5 minutes</td>
</tr>
<tr>
<td>Step 5: Repeat steps 2-4 29 times</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Step 6: Final elongation</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
</tbody>
</table>

The PCR samples were stored at -20°C until required.

2.2.2 Restriction digest

For analytical digests, 5 μl of the miniprep DNA, or 0.25 μg of the midiprep DNA were treated with appropriate restriction enzyme, 2 μl of the 10X restriction digestion buffer and made up to 20 μl with mQ H₂O. Digests were incubated for 2 hours at
37°C. Samples were loaded into the wells of the agarose gels following addition of
4μl of blue/orange 6x loading dye (Promega) and visualized under UV.

For preparative digests, 2.5μg of the DNA was added to 5 units of restriction
enzyme, 2μl of 10X restriction digestion buffer and made up to 20μl with mQ H₂O.
Digests were incubated for 2 hours at 37°C water bath and 0.5-1μl of the digest was
analyzed on a 1% agarose gel.

2.2.3 Alkaline Phosphatase treatment
Linearised plasmid vectors used for cloning and expression purposes were treated
with Calf Intestinal Alkaline Phosphatase (CIAP) or Shrimp Alkaline Phosphatase
(SAP) to prevent recircularization and unwanted backgrounds during the cloning
procedure.

2.2.3.1 CIAP treatment
The digested DNA was incubated for 45 minutes at 37°C water bath using 1μl CIAP
enzyme/μg of DNA (1U/μl) and 5μl 10x dephosphorylation buffer (Promega) in a
50μl reaction volume. For recessed ends a second aliquot of the fresh enzyme was
added and incubated for 45 min at 55°C. After the incubation, the enzyme was
inactivated by heat treatment at 75°C for 10 minutes and the fragments were gel
purified.
2.2.3.2 SAP treatment

For SAP treatment, digested vectors were treated with SAP enzyme (1U/µg of vector DNA) and 10x dephosphorylation buffer (Promega) for 30 min at 37°C and inactivated at 65°C for 15 min.

2.2.4 Agarose Gel Electrophoresis

Agarose gels (1%) were made using electrophoresis grade agarose powder (Invitrogen) and 1x Tris-acetate-EDTA (TAE) buffer (Appendix II). Ethidium bromide was added to the molten agarose at a final concentration of 0.5µg/ml and the gel was allowed to set for at least 30 min at room temperature. Gels were run in a 1x TAE buffer containing 0.5µg/ml ethidium bromide. Bands were visualized on GelDoc (BIO-RAD).

2.2.5 Low Melting Point Gel Electrophoresis

Low melting point (LMP) agarose gels were used for purification of DNA. LMP agarose gels (1%) were made using 1x TAE buffer using electrophoresis grade LMP agarose powder (Invitrogen). Ethidium bromide was added to the molten agarose at a final concentration of 0.5µg/ml and the gel was then cast. Gels were run in 1x TAE buffer containing 0.5µg/ml ethidium bromide. The DNA bands were visualized under low intensity UV light and the appropriate bands were excised from the gel using a scalpel. The DNA was eluted and purified from the agarose using Amersham GFX™ PCR DNA and Gel Band purification kit, according to the manufacturer’s protocol. The DNA was eluted in 50µl of mQ H2O and the purified DNA was quantified by comparison with λIII DNA marker on a 1% agarose gel.
2.2.6 Ligation

2.2.6.1 Directional cloning

Ligations were carried out using 25-100 ng of vector and insert DNA at a molar ratio of 1:1 or 1:3. To this, 1 μl of 10x T4 DNA ligase buffer (Promega) and 0.5 μl of T4 DNA ligase enzyme (Promega) was added and the reactions made up to 10 μl with nuclease-free H₂O. The ligations mixtures were incubated at 16°C overnight and then transformed into suitable competent cells.

2.2.6.2 Blunt end ligation of PCR products

PCR fragments amplified using Pfu enzyme were ligated into 25 ng of the pT7-blue blunt ended linearised vector (Novagen). Inserts were made up to a final volume of 8.7 μl with nuclease-free H₂O and incubated with 1 μl T4 DNA ligase buffer (Promega) and 0.3 μl PolyNucleotide Kinase (PNK) (Promega) for 30 minutes at 37°C and then for a further 15 minutes at 70°C for the heat denaturation of the enzyme. The pT7-blue vector was then added to each reaction with 0.3 μl of T4 DNA ligase. The reactions were incubated at room temperature overnight and transformed into NovaBlue (Novagen) or DH5α cells.

PCR fragments amplified using *Thermus aquaticus* (Taq) polymerase were first treated with end conversion mix (Novagen) to polish the ends for blunt end ligation.

2.2.6.3 Oligonucleotide ligation

Complementary 5’ phosphorylated oligonucleotides were mixed in a single PCR tube (1 μl each of the 100 pmole/μl forward and reverse primer stock), denatured for
2 min at 100°C and allowed to cool down gradually at room temperature for 1 hr. A small aliquot of the reaction (0.1 μl - 0.2 μl) was added to the restriction digested vector for ligation at 16°C overnight. 5 μl of the ligation reaction was used for transformation.

2.2.7 Preparation of competent *E. coli* cells

*E. coli* cells from the glycerol stocks were streaked on an LB-agar plate and left at 37°C overnight. Single colonies were picked and grown overnight at 37°C in LB broth containing 20mM MgSO₄. 1 ml of the overnight culture was added to 10 ml fresh LB broth containing 20mM MgSO₄ and incubated at 37°C until the cells had reached mid log (OD₆₀₀ = 0.2-0.8). These cells were then transferred to a 1 liter flask containing 50 ml LB broth and 20mM MgSO₄ and grown at 37°C until cells reached OD₆₀₀ 0.9. 200 ml LB broth containing 20mM MgSO₄ was then added to the cells and they were incubated at 37°C until cells reached OD₆₀₀ 0.6. The cells were then centrifuged at 4°C, 4,200 rpm for 15 minutes. The pellet of cells was resuspended in 50 ml fresh transforming buffer 1 (TFB1) (Appendix II) and incubated on ice for 5 minutes, before being spun at 4°C, 4,200 rpm for 15 minutes. The resulting pellet was resuspended in 10 ml of ice cold transforming buffer 2 (TFB2) (Appendix II) and the cell suspension aliquotted into 200μl sample. These were snap frozen and stored at -70°C until required.

2.2.8 Transformation of the competent cells

10 μl of the ligation reactions were added to 190 μl of TCM (Appendix II) and kept for 5 minutes on ice. Competent cells were thawed on ice and 100 μl added to the
mixture for one hour. The samples were then heat shocked at 42°C for 30 seconds and again kept on ice for 5 minutes. Then 700µl of room temperature SOC (Appendix II) was added and incubated for a further 60 minutes at 37°C with constant shaking. The cultures were centrifuged at 10,000 rpm for one minute and most of supernatant removed. The pellet was resuspended in the remaining media and the transformed bacterial cells were then plated out onto LB-agar plates containing the appropriate antibiotics. For midi plasmid preparations, the cells were added to 50ml LB media containing the appropriate antibiotic.

2.2.9 Mini preparation of plasmid DNA

Colonies were picked from the agar plates and grown up overnight at 37°C in 3ml LB media containing appropriate antibiotics. 1ml of the overnight culture was centrifuged for 2 minutes at 14,000 rpm and the supernatant removed. The plasmid DNA was obtained from the pelleted cells using the Wizard Plus Miniprep DNA purification system (Promega) as described by the manufacturer. DNA was eluted in 50µl mQ H2O and 5µl of the sample was analyzed by restriction analysis and agarose gel electrophoresis.

2.2.10 Plasmid midi preps

Midi-prep DNA was obtained using the Qiagen Hi-Speed Plasmid Midi Prep Kit and the DNA eluted in 1ml mQ H2O. The DNA was then precipitated using 2.5 volumes 100% ethanol and 1/10th volume salt (3M NaOAc). The DNA was quantitated using the spectrophotometer.
2.2.11 QuikChange site directed mutagenesis

Quik-change site directed mutagenesis reactions were performed using mutagenic oligonucleotides with desired nucleotide changes according to the manufacturer's instructions (Stratagene). Briefly target DNA was grown in *E. coli* cells with dam\(^+\) genotype. QuikChange site directed mutagenesis was performed by using *PfuTurbo* (Stratagene) enzyme in a thermal cycler for 12-18 cycles depending upon the number of nucleotide changes. Following temperature cycling the amplified product was treated with 1 \(\mu\)l of *DpnI* restriction enzyme (which only cuts methylated DNA) for 2hr at 37\(^\circ\)C, transformed into DH5\(\alpha\) cells and colonies selected on LB plates containing suitable antibiotics. Five colonies were picked and mini prep DNA was isolated. Sequencing of the plasmids DNA was carried out to confirm the presence of the desired mutation.

2.2.12 Sequencing of DNA

2.2.12.1 DNA Preparation

0.25-0.50 \(\mu\)g of plasmid DNA was dissolved in \(\text{dH}_2\text{O}\) to a final volume of 8.8\(\mu\)l and the mixture was heated for 2 minutes at 96\(^\circ\)C. The DNA sample was immediately placed on ice for 2 min and 3.2pmol of the sequencing primer was added to the DNA. 8\(\mu\)l of Quickstart Reaction Mix (Beckman Coulter) was added to each reaction and the reactions were then incubated in the mastercycler PCR machine (Eppendorf) using the following amplification cycle:-

Step 1: 96\(^\circ\)C 20secs
Step 2: 50\(^\circ\)C 20secs
Step 3: 60\(^\circ\)C 4mins
Step 4: Repeat steps 1-3 30 times
2.2.12.2 Ethanol Clean-Up

5µl stop solution was added to each reaction (Appendix II). The mixture was vortexed and 60µl ice cold 95% ethanol added to each tube. The reactions were then centrifuged for 10 mins at 14,000 rpm, at 4°C and the resulting supernatant removed. 200µl ice cold 70% ethanol was added to the pellet and the reaction was centrifuged at 14,000 rpm for 5 minutes to wash the pellet. This step was repeated and then all the supernatant was removed and the pellet allowed to air dry. Once dry, each pellet was resuspended in 40µl sample loading solution, loaded onto the sequencing plates and covered with mineral oil.

2.2.12.3 Sequencing Reaction

The plates were loaded into the CEQ 8000 Sequencer (Beckman Coulter) and the reactions were run.

2.2.12.4 Analysis of Results

The sequencing data was exported into the DSgene sequencing software package and analysed.

2.2.13 Plasmid construction

The full-length FMDV infectious cDNA clone (pT7S3) of the O1Kaufbeuren strain (Ellard et al., 1999) was used as the template for PCR amplification of specific FMDV coding sequences.
2.2.13.1 pQE30/3Dpol

The coding sequence of the FMDV 3Dpol was amplified in a PCR with primers 3DFORBGL and 3DREVSPH, which contain BgiII and SphI restriction sites respectively (Appendix III), using Pfu DNA polymerase. The product was ligated into the pT7blue vector (Novagen) to produce pT7/3Dpol, from this plasmid the BgII-SphI fragment was excised and ligated into the bacterial expression vector pQE30 (Qiagen) previously digested with BamHI and SphI to produce pQE30/3Dpol. The vector adds an N-terminal hexa-His tag to the 3Dpol coding sequence.

2.2.13.2 pQE30/3CDprov(C163G)

The coding sequence of 3CDpro was amplified by PCR using oligonucleotides 3CFORBGL and 3DREVSPH with BgiII and SphI restriction sites respectively (Appendix III). The fragment was cloned in pT7 blue vector (Novagen) by blunt end cloning. pT7/3CDprov containing the wild type sequence of FMDV 3CDpro was used as a template for modification of the codon (using primers C163FOR and C163REV, Appendix III) corresponding to cysteine 163 using the Quikchange site directed mutagenesis to a glycine codon as described previously (Grubman et al., 1995). The resulting plasmid was digested with BgII and SphI and the modified fragment was ligated between the BamHI and SphI sites of the pQE30 vector.

2.2.13.3 pQE30/3Cpre(C163G)

The coding sequence of O1K 3Cpro was amplified by PCR using oligonucleotides O1K/3CFOR and O1K/3CREV with BamHI and HindIII restriction sites respectively (Appendix III) using pQE30/3Cprov(C163G) as a template. The fragment was cloned in pT7 blue vector (Novagen) by blunt end cloning. The
resulting plasmid was digested with BamHI and HindIII and the released 3C insert was ligated between the BamHI and HindIII sites of the pQE30 vector.

2.2.13.4 pET11-M3C(C163A,C95K)R92S/R95S/R97S

pET11M-3C(C163G, C95K) plasmid was a gift from Dr. Stephen Curry (Imperial College, London). The plasmid, under appropriate conditions expresses FMDV type A10 3C protein (Birtley et al., 2005). This plasmid was used as a template for modification of each of the arginine (R) residues at position 92, 95, 97 to serine residues by QuikChange site directed PCR mutagenesis using suitable primers (Appendix III).

2.2.13.5 pUb-3Dpol

For unmodified FMDV 3Dpol expression, a forward primer (Ub3DpolSacII) was designed with a SacII site, the coding sequence for the carboxy terminus of ubiquitin followed by the first twenty one nucleotides of the 3Dpol sequence as described previously (Gohara et al., 1999). The reverse primer (Ub3DpolBglII) was designed such that it contained a BglII site. The fragment was amplified and cloned into the blunt cloning site of the pT7 blue vector (Novagen). Modification of the internal SacII site within the FMDV 3Dpol sequence was carried out by using site directed mutagenesis kit using SacIImutFOR and SacIImutREV primers (Appendix III). Removal of the internal SacII site was confirmed by sequencing as well as by restriction digestion. The modified 3Dpol (O1K strain) sequence was ligated between the SacII and BamHI site of the pET26b-Ub vector. The newly constructed plasmid was named as Ub-3Dpol.
2.2.13.6 Ub-3B\textsubscript{3}3C, Ub-3B\textsubscript{123}3C

For expressing FMDV non-structural precursor proteins 3B\textsubscript{3}3C 3B\textsubscript{123}3C in the ubiquitin system, the respective DNA fragments were amplified by PCR using suitable primers (Appendix III) and cloned into pT7-blue blunt linearised vector. Mutations within the 3C protease at the Cysteine (Cys) residue to a Glycine (Gly) residue in pT7-3B\textsubscript{3}3C, pT7-3B\textsubscript{123}3C constructs were carried out using Quik-Change site directed mutagenesis protocol as described previously. The respective fragments were excised from pT7 blue clones using SacII and BamHI and cloned into similarly digested pET26b-Ub vector. All the desired mutations in the expression constructs were confirmed by sequencing.

2.2.13.7 pGC-cre (wt and mut forms)

The plasmid pGC was a gift from Dr Graeme Conn (University of Manchester), and contains the Hepatitis D virus (HDV) \delta ribozyme cDNA (Walker et al., 2003). Oligonucleotides T7creFOR and T7creREV (Appendix III) corresponding to the FMDV OVI cre/bus (Tiley et al., 2003) and preceded by a T7 promoter were annealed and ligated into this vector upstream of the ribozyme between EcoRI and Nhel sites to produce pGC-cre (see results). This plasmid can be used to produce, from a T7 promoter, RNA transcripts that correspond to the FMDV cre/bus. Shorter versions of the artificial cre/bus DNA constructs encompassing 37 and 31 nucleotides respectively were generated using similar methods. The plasmid was modified using mutagenic primers (Appendix III) and a QuikChange mutagenesis kit (Stratagene) to construct plasmids which can be transcribed to produce mutant transcripts with A to C substitutions at positions 1-3 within the conserved AAACA
motif and also mutation in the stem (C47U) within the cre. All the desired mutations were verified by sequencing.

2.2.13.8 Generation of full-length infectious pT7S3 clones with cre/bus point mutations

Point mutations within the cre/bus in the first 3 ‘A’ residues was carried out using the Quik-change site directed mutagenesis protocol. Briefly, the pT7S3 infectious clone cDNA was digested with SpeI and NotI to release a 3068 bp fragment which was ligated into similarly digested pGEM-SZ vector (Promega) to produce pGEM-SZ/SN. The newly generated clone was used as a template for modification of each of the first 3 ‘A’ nucleotide of the AAACA motif within the cre/bus using QuikChange site mutagenesis using O1Kcremut primers listed in Appendix III. The modified fragments containing the required mutations were released from pGEM-SZ/SN derivatives and reconstructed back into the pT7S3 backbone. The respective mutations in the full-length cDNA were verified by sequencing.

To achieve a deletion of the AAA sequence from the AAACA motif, an overlap PCR mutagenesis protocol was performed using the O1KcreDel primers (Appendix III) with pT7S3 as the initial template. The external primers were designed to generate an amplified product (about 1250nt) that contained the naturally occurring SpeI and XbaI sites of the pT7S3 infectious clone. The final product was digested with SpeI and XbaI to generate a smaller 743 bp fragment which was ligated into the similarly digested pT7S3 backbone to produce pT7S3creDel. The presence of the required mutation in the cre/bus region in this plasmid was verified by sequencing.
2.2.13.9 Generation of infectious pT7S3 clones with hammerhead ribozyme

Hammerhead ribozyme constructs were made using forward primer (pT7S3-Ribo-FOR) containing a SpeI restriction site, T7 promoter, hammer-head ribozyme core sequence followed by the 5' 17 nucleotides of the FMDV genome (FIG. 2.1). The reverse primer (pT7S3-Ribo-REV) was designed to include the natural Nhel site just upstream of the polyC tract (Appendix III). The product was PCR amplified using Taq DNA polymerase (Invitrogen) and cloned into pT7-blue blunt vector (Novagen). The resultant clone was digested with SpeI and Nhel to release a fragment (469 bp) and ligated into the similarly digested pT7S3 infectious clone. Presence of the hammer-head ribozyme in the final constructs was verified by sequencing and also by in-vitro transcription.

2.2.13.10 Construction of FMDV infectious cDNA clone with single amino acid substitution (arginine to serine at position 92, 95 and 97) in the 3C protease

A PCR fragment (1750 bp) encompassing the FMDV 3C coding sequence along with the natural BclI and BtpI restriction sites was amplified using primers O1KBclI and O1KBtpI (Appendix III) and ligated into the pT7-blue blunt end cloning vector. The newly constructed clone (pT7-BB) was used as a template for site directed mutagenesis using QuikChange mutagenesis kit (Stratagene) to introduce mutations in the codon for the amino acid arginine at position 92, 95 and 97 to encode serines (for R92S, R95S, R97S mutagenic primers, see Appendix III). Positive clones were grown for midi-prep plasmid isolation using dam' and den' E. coli strains (ER2925 from NEB) and digested with BclI and BtpI to release an insert of 1729 bp and introduced into similarly digested FMDV infectious c-DNA clone (with or without ribozyme).
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FMDV Ribozyme forward oligo

\[
\begin{array}{c}
\text{Spe I} & \text{T7 promoter} & \text{Base pair with FMDV 5' terminus} \\
\text{TGGACTAGT} & \text{TAATACGACTCATATAGGG CTTTCAA} \\
\end{array}
\]

Ribozyme core

\[
\begin{array}{c}
\text{CTGATGAGGCCGAAAGGCCGAAAACCCCGTATCGGGTC} & \star \\
\text{TTGA} \\
\end{array}
\]

17 nt at FMDV 5' terminus

AAGGGGGGCATTAG

FIG. 2.1. Designing FMDV ribozyme construct. Panel A. Shows the forward primer designed for FMDV ribozyme construct that contains the FMDV stuffer sequence (with SpeI restriction site), T7 promoter sequence, and hammer-head ribozyme core sequence. Panel B. Secondary structure prediction of the above mentioned nucleotide sequence using M.Zucker RNA folding server.
2.2.13.11 pSP64polyA/S-fragment. The nucleotide sequence of the FMDV S-fragment was amplified using oligonucleotides S-frag-FOR and S-frag-REV (Appendix III) with PstI and XbaI restriction sites respectively. The resulting fragment was cloned into pT7 blue vector by blunt end cloning and subcloned into the corresponding sites of pSP64polyA vector (Promega). RNA transcripts corresponding to the S-fragment were produced by in-vitro transcription using XbaI linearised pSP64polyA/S-fragment clone.

2.2.13.12 pT7-(PK+cre+IRES). The nucleotide sequence of the FMDV 5' UTR encompassing the pseudoknots (PKs), cre/bus and IRES was amplified by using oligonucleotides poly(C)-down-FOR and IRES/REV (listed in the Appendix III). The amplified PCR product was cloned in pT7-blue blunt end cloning vector as described previously.

2.3 RNA techniques

2.3.1 In-vitro transcription

Plasmids meant for in-vitro transcription were linearized with suitable restriction enzymes. The DNA was purified by phenol extraction and ethanol precipitation and then used as template for transcription reaction as follows using MEGAscript high yield transcription kit according to manufacturer instructions (Ambion)

\[
\begin{align*}
2 \text{ µl} & \quad \text{ATP solution} \\
2 \text{ µl} & \quad \text{CTP solution} \\
2 \text{ µl} & \quad \text{GTP solution} \\
2 \text{ µl} & \quad \text{UTP solution}
\end{align*}
\]
For shorter RNA transcripts (cre/bus) the reaction mixtures were incubated overnight at 37°C whereas for longer transcripts, reaction mixtures were incubated for 2-4 hrs (shorter duration) at 37°C. RNA transcripts were purified by phenol extraction and ethanol precipitation. The cre/bus RNA transcripts were analyzed by denaturing urea-PAGE and the longer transcripts were visualized by native TBE gel/denaturing formaldehyde-agarose gels. The yield of the RNA transcripts was determined by spectrophotometry.

2.3.2 Urea-PAGE electrophoresis

Polyacrylamide urea gels (8%) were used to analyze the in vitro transcribed cre/bus RNA transcripts.

The following recipe were used for 10ml of gel solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% acrylamide</td>
<td>1.9ml</td>
</tr>
<tr>
<td>bis-acrylamide</td>
<td></td>
</tr>
<tr>
<td>10M urea</td>
<td>7.0ml</td>
</tr>
<tr>
<td>10X TBE</td>
<td>1.0ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.1ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>5.0 μl</td>
</tr>
</tbody>
</table>

All the component were mixed quickly, poured into the gel apparatus and allowed to polymerize for at least 1hr. Gel wells were rinsed with TBE buffer prior to electrophoresis. The gel was run at 150-200v in 1X TBE running buffer and stained
with ethidium bromide at 0.5 μg/ml for 10-20 minutes and photographed under UV illumination (305nm).

### 2.3.3 Native agarose gel electrophoresis of RNA

To assess the overall integrity and quality of the larger RNA transcripts native agarose gel electrophoresis were carried out using 1% agarose gel in TBE. Usually 1μg of the RNA transcripts were loaded per well along with RNA molecular weight markers (Novagen) and visualized by ethidium-bromide (EtBr) staining.

### 2.3.4 Denaturing formaldehyde-agarose gel electrophoresis of RNA

For denaturing gel electrophoresis appropriate amounts of agarose, 10X FA gel buffer (Appendix II) and RNAse free water were mixed and boiled in a microwave. The molten agar then cooled to 65-70°C in a hot water bath. After cooling, for 100ml gel, 1.8 ml of 37% (12.3 M) formaldehyde and 5 μl of a 10mg/ml ethidium bromide were added in a fume hood. The gel was poured on to a gel tray to a thickness of 3-5 mm. Combs were inserted immediately into the gel and allowed to set for at least 30 min. Electrophoresis tanks were treated with detergent solution (0.5 % SDS), rinsed with RNase-free water, and then rinsed with ethanol and allowed to dry. The gel was allowed to equilibrate with the FA running buffer (Appendix II) for 30 min prior to sample loading. The RNA samples were treated with denaturing sample buffer II (Appendix II) and heated for 10 min at 75°C. The denatured samples were snap cooled in ice for 10 min and loaded into the gel which was allowed to run for 2hr at 80V and RNA bands were visualized under UV.
2.3.5 UV shadowing

Non-radioactive cre/bus RNA transcripts were mixed with 2X gel loading buffer II (Appendix II), heat denatured at 75°C for 2 min and snap cooled on ice for 5 min. Samples were loaded directly to freshly rinsed well of 8% urea-PAGE gel and run at 170V till the bromophenol blue (BPS) dye front reached the bottom of the gel. The glass plate was carefully removed and the gel was covered with a thin saran wrap on both sides. The wrapped gel was placed over the top of a fluorescent thin-layer chromatography (TLC) plate (the white side of the TLC plate faced the gel). RNA transcripts corresponding to the cre/bus were visualized by a hand held UV light source (254 nm) as a purple band and marked with a marker pen over the saran wrap. The marked band was cut with a razor blade and put in RNA elution buffer (10mM Tris, 0.1% SDS, 1mM EDTA) overnight. The elution buffer was collected and the RNA was extracted with phenol: chloroform: isoamyl alcohol and subsequently precipitated with absolute ethanol.

2.3.6 Preparation of [³²P]-labeled RNA

Radioactive probes for UV-crosslinking were prepared by using Ambion's maxi­
script kit. To generate probes with the highest specific activity [³²P]α-UTP were used as the limiting nucleotide without any cold UTP. The in-vitro transcription reactions were set up using linearised template DNA as follows.

<table>
<thead>
<tr>
<th>Volume (μl)</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10mM ATP solution</td>
</tr>
<tr>
<td>1</td>
<td>10mM CTP solution</td>
</tr>
<tr>
<td>1</td>
<td>10mM GTP solution</td>
</tr>
<tr>
<td>5</td>
<td>[³²P]-UTP solution</td>
</tr>
<tr>
<td>2</td>
<td>10 x reaction buffers</td>
</tr>
</tbody>
</table>
The reaction mixture was incubated for 1-2 hr at 37°C and stopped by adding 1μl of 0.5M EDTA. Residual DNA template from the reaction was removed by adding 1μl DNase I and incubated for 30 min at 37°C. An equal volume of 2X gel loading buffer was added, heated for 2 min at 75°C and loaded into the well of freshly rinsed 8% urea-PAGE gel. Electrophoresis was stopped once the bromophenol blue dye reached the bottom of the gel. The gel was covered with a saran wrap and an autoradiograph was developed by exposing the film for 30 sec-2 min. A dark band on the autoradiograph was marked with a marker pen. The marked area on the autoradiograph was excised using a sharp scalpel and again placed over the gel. The gel corresponding to the cut region was carefully removed and put in RNA elution buffer (10mM Tris, 1mM EDTA, 0.2% SDS) overnight. The elution buffer was collected and treated with ammonium acetate and glycogen. The probe was recovered by phenol extraction and ethanol precipitation.

2.3.7 UV-cross linking

Approximately 50 μg of cell extracts (BHK S10 extracts (uninfected or infected), HeLa S10 extracts) or 0.5 μg of purified recombinant proteins (3CD, PCBP1, PCBP2) were incubated with uniformly radiolabeled probe (2x10^5 cpm) in 20 μl of 10 mM HEPES-KOH (pH 7.9), 25 mM KCl, 2mM MgCl2, 10% glycerol, 0.05% NP-40, 0.5 mM DTT, 10 μg t-RNA. After 30 min incubation on ice, the reaction mixtures were UV cross-linked for 30 min on ice keeping the sample 10 cm from the UV source. The un-protected probes in UV-irradiated extracts were digested with 2
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µl RNase cocktail (Ambion) for 30 min at 37°C. The RNA-cross linked proteins were resolved in 10% SDS-polyacrylamide gels. After gel electrophoresis, the gels were stained in coomassie blue, destained, dried and 32P-labelled complexes were visualized by autoradiography.

2.3.8 Electrophoretic mobility shift assay (EMSA)

Gel purified in vitro transcribed cre/bus RNA (50 µM) and FMDV A10 3C proteins [(wt 3C or mutant 3C (R92S, R95S, R97S)] (20-250 µM) were incubated in a 20 µl reaction mixture in buffer conditions similar to the in vitro uridylylation assay (50 mM HEPES, 8% glycerol, 2 mM MgCl2) for 30 min at 30°C to allow complex formation. Samples were run on a 5% native polyacrylamide gel in 0.5X TBE buffer at room temperature for 2 hr. After electrophoresis the gel was stained in EtBr and visualised under UV. To visualise the 3C protein in the RNA-protein complex the gel was stained with coomassie brilliant blue.

2.3.9 Trizol extraction of RNA from tissue culture cells

Membrane fractions from FMDV infected BHK cells were treated with Trizol (500 µl Trizol/ 50 µl of membrane fraction) and incubated for 5 min at room temperature. Subsequently 100 µl of chloroform was added, mixed well by vortexing and kept for another 3 min at room temperature. The samples were centrifuged at 12,000 rpm for 15 min at 4°C. Phenol and chloroform formed a lower organic red phase that contained all the protein and DNA. The upper clear aqueous phase containing RNA was collected carefully and this was precipitated by using 2µg glycogen and 250 µl of isopropanol at room temperature for 10 min. The samples were centrifuged at 12,000 rpm for 10 min at 4°C. The pellet obtained was washed for 5 min at 12,000 rpm
by using 70% ethanol to remove any salt. RNA samples obtained were dissolved in RNA storage solution (Ambion) and stored at -70°C.

2.3.10 Electroporation of RNA into BHK cells

Fresh BHK cells were passaged the day before the electroporation to obtain 60%-70% confluency. Cells were split by trypsinisation and resuspended in 10 ml of electroporation buffer (Appendix II). Viable cell counts were carried out and total cells were collected by centrifugation at 200g for 3 min at 4°C. The cell pellet was resuspended at 2x10⁶/ml of electroporation buffer and 0.8 ml of the cells was transferred into an ice-cold electroporation cuvette. Approximately 4 µg of the FMDV RNA was added to the cuvette and shocked (0.75kV and 25µFD) using an electroporator (Bio-Rad). The cuvette was placed at room temperature for 10 min and cells were transferred to a 25cm² culture flask containing 5ml of virus growth media. The flask was incubated overnight at 37°C in a CO₂ incubator.

2.3.11 Plaque assay

Cells were seeded into 35 mm dishes, 16 hours prior to infection. Monolayers (~80% confluent) were washed with phosphate buffer saline (PBS) (pH 7.5 containing 2 mM CaCl₂, 1mM MgCl₂). Virus dilutions (200µl in PBS) were added to the cells for 15 min at 37°C. The cell monolayers were then overlayed with 4 ml of molten Eagles overlay (Eagles medium supplemented with 0.6% indubiose, 5% tryptone phosphate broth, 1% foetal calf serum, 100 SI Units/ml penicillin and 100 μg/ml streptomycin). Cells were then incubated at 37°C, 5% CO₂ for 40-48 hours. Plaques were visualised by staining cell monolayers with methylene blue, 4% formaldehyde in PBS.
2.3.12 Isolation of replication complexes from FMDV infected BHK-21 cells

BHK-21 cells were grown overnight up to 100% confluent in 175-cm$^2$ flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS. The excess media was decanted leaving little residual media without making the monolayer dry. FMDV O1BFS virus was adsorbed onto the monolayer with or without the presence of guanidine hydrochloride (10mM) and kept on a rocking platform at 37°C for 15 min followed by 15 min at 37°C incubator. An additional 25 ml of DMEM media with 10% FCS was then added. The virus was allowed to grow till the CPE was observed in the infected cells. A mock infected BHK-21 monolayer was kept as a negative control. At about 3-4 hrs when the cells started rounding up (but had not detached from the plastic surface) the media was removed and the cells were washed with cold PBS. The cells were resuspended in 15 ml of fresh PBS and then pelleted at 500xg for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml cold hypotonic TN buffer (Appendix II) and kept for 15 min in ice. Cells were lysed with 30-40 strokes in a cold 15 ml glass Dounce homogenizer. The lysate was transferred to 1.5 ml Eppendorf tubes and centrifuged for 5 min at 900xg at 4°C to remove any unlysed cells and nuclei. The supernatant was transferred to a fresh Eppendorf tube and centrifuged for 20 min at 20,800xg at 4°C. The resultant pellet (FMDV replication complexes (RCs) or mock RCs) was resuspended in 120 µl TN buffer with 15% glycerol and stored at -70°C.

2.3.13 In-vitro RNA replication assay

RNA replication assays were performed (as described by Green et al., 2003) using reaction mixtures containing 18 µl of FMDV RCs or mock RCs, 50mM HEPES (pH 8.0), 10mM DTT, 3mM MgCl$_2$, 0.25 mM GTP, 0.25 mM CTP, 1.0 mM ATP, 0.04
mM [α-35P]UTP and unlabelled UTP, 10 μg/ml Actinomycin D, 25 μg/ml Creatine phosphokinase (CPK), 5mM Creatine phosphate, 800 U of RNasin/ml, 50 mM potassium acetate. Gu-HCl was added when required (at a final concentration 10mM) in the reaction mixture. Assays were carried out for 1 hr at 30°C. Reactions were stopped by adding 150 µl of TENSK at 37°C (Appendix II). RNA extraction was carried out using phenol: chloroform: isoamylalcohol (25:24:1). An aliquot of the extracted RNA (usually 1/5 to 1/10 of the sample) was run in a native agarose gel at 100 volt for 2 hrs. The bottom of the gel was cut to remove any unincorporated nucleotide and gel dried under vacuum for 30 min without heat followed by drying at 60°C for 30 min. The dried gel was exposed overnight for autoradiography.

2.3.14 RNaseA protection assay

In vitro RNA replication assays was performed as described above. The whole aliquot of the reaction was centrifuged at 12,000 rpm for 5 min to pellet the entire membrane fraction. The supernatant was discarded that contained the unincorporated radiolabelled UTP. The membranous pellet was treated with Triton-X 100 in RNase A buffer to a final concentration of 0.1%. RNase A was added at the rate of 1mg of RNase A/ml of RNase A buffer containing 300mM NaCl and 10mM Tris (pH 7.8). A duplicate reaction was performed without RNaseA as control. The reaction mixture was incubated for 30min at 37°C. The RNA from the replication complex was isolated by Trizol (Invitrogen) as per the manufacturer’s instruction and run in a 1% TBE agarose gel. The gel was dried and exposed.
2.3.15 RNaseA sensitivity Assay

RNA replication assays were performed as described above and RNA was extracted by Phenol:chloroform:isoamylalcohol. An aliquot (1/5\textsuperscript{th}) of the total RNA was dissolved in RNaseA digestion buffer with 300mM salt. The residual RNA was treated with RNaseA at the rate of 0.2 µg of RNaseA/ml of RNaseA buffer. The whole reaction mixture was incubated at 23°C for 15 min. Both RNaseA treated and untreated samples were extracted with phenol:chloroform:isoamylalcohol and samples were analysed on a 1% TBE gel.

2.4 Protein techniques

2.4.1 Expression and purification of FMDV native 3Dpol

Protein expression and purification was carried out using the protocol described by Gohara et al., 1999. The plasmid Ub-3Dpol was transformed into BL21(DE3)pCG1 cells for induction and expression of the protein. Briefly BL21(DE3)pCG1 cells containing the Ub-3Dpol plasmid were grown overnight in 100 mL of NZCYM media supplemented with Kanamycin (25µg/mL), Chloramphenicol (20µg/mL), and 0.4% glucose at 30°C. The overnight culture was used to inoculate 1L of fresh NZCYM media supplemented with Kanamycin and chloramphenicol, and grown to OD\textsubscript{600} 1.0 at 37°C. The cells were cooled down to 28°C, induced with IPTG (final concentration of 500 µM) and grown for an additional 4 h at 28°C. The cells were harvested using a Sorval GS-3 rotor at 6000 rpm for 15 min, washed once in 200 mL of TE (10mM Tris, 1mM EDTA). The cell paste obtained was weighed and stored at -70°C for future use.
For protein purification, the frozen cells were thawed in ice and suspended in lysis buffer (50mM potassium phosphate (pH 8.0), 500mM sodium chloride, 20% glycerol, 10mM mercaptoethanol, protease inhibitor cocktail set III, 0.1% NP40) using 5mL/g of cell paste. The suspended bacterial cells were frozen in liquid N\textsubscript{2} and thawed in ice-cold water for 30 min. The thawed samples were lysed by sonication on ice. Protease inhibitor cocktail set III (Calbiochem) was added to a final concentration of 1mM to inhibit any protease mediated protein degradation after lysis. Precipitation of the nucleic acid was carried out by using polyethylenimine (PEI) to 0.25% (v/v). PEI was added drop by drop at 4\textdegree\text{C} for 30 min with constant stirring. The extract was centrifuged for 30 min at 15,000 rpm at 4\textdegree\text{C} to pellet the cellular debris and nucleic acid. The PEI supernatant was collected and solid powdered ammonium sulphate was slowly added up to 30%-40% saturation. The ammonium sulphate-precipitated material was then pelleted by centrifugation for 20 min at 9,000 rpm at 4\textdegree\text{C}. The supernatant was decanted and the pellet was suspended in buffer A (50mM Tris, pH 8.0, 20% glycerol, 50mM sodium chloride, 10mM mercaptoethanol and 0.1% NP40) and dialysed overnight.

Subsequent purification of the native FMDV 3Dpol was carried out using an Akta-Prime protein purification system from Amersham Biosciences. The dialysed sample was loaded onto a 15mL phosphocellulose column (P11, Whatman) equilibrated with buffer A at a flow rate of 0.5 mL/min. The column was washed to baseline with buffer A containing 50mM NaCl and protein was eluted using a linear gradient (5 column volume) from 50mM to 1M NaCl in buffer A. 1.5 mL fractions were collected and protein purity was analysed by Coomassie staining of the SDS-PAGE gel. Peak fractions with 3Dpol protein were pooled and dialysed against buffer A.
with 50mM NaCl. The dialysed sample was loaded onto a buffer A equilibrated 1 mL hi-trap Q-sepharose column at 0.2 mL/min. The column was washed with buffer A with 50 mM salt and eluted with linear gradient (5 column volumes) from 50mM to 2.0M NaCl in buffer A. Pure 3Dpol fractions were pooled and dialysed for 3 h against buffer A containing 50mM salt and further concentrated by loading onto a buffer A equilibrated 1 mL Heparin column and eluted using 2 column volumes of buffer A in a linear gradient from 50mM to 2.0M salt. The concentrated fraction was dialysed against buffer A (50mM salt) for 6hrs and stored at -70°C.

2.4.2 Expression and purification of histidine-tagged 3Dpol, 3Cpro and 3CDpro

To express His-tagged FMDV O1K 3Dpol, 3Cpro and 3CDpro, the appropriate plasmids were transformed into *Escherichia coli* strain M15. Growth and induction of the cultures were done essentially as described for the native 3Dpol. For 3Cpro/3CDpro, IPTG induction was carried out with 100 μM final concentration of IPTG. His-tagged 3Dpol expression and purification was performed essentially as described above except the phosphocellulose column purified sample were directly loaded onto a 1-ml hi-trap His-column (Amersham Bioscience) and eluted by step elution using 500 mM imidazole. For 3Cpro/3CDpro purification 500 mM NaCl concentration was kept constant throughout lysis, binding and for elution. The eluted 3Dpol and 3Cpro/3CDpro samples were dialysed against Buffer A containing 50mM and 500 mM NaCl respectively.

2.4.3 Purifications of FMDV VPg precursor protein 3BC

Expression and purification of the native 3BC proteins (3B13C 3B123C) were performed essentially as described for native 3Dpol. The phosphocellulose column
purified samples were directly loaded onto a 1-ml hi-trap SP sepharose column (Amersham Bioscience) and eluted by gradient elution using buffer A with 500-750 mM salt.

2.4.4 Protein Dialysis

Purified protein samples were dialysed overnight using a 'Slide-a-Lyser' cassette (Pierce) with 7 kilo Dalton molecular weight cut off (7 kD MWCO) against 1 litre of suitable buffers. The samples were collected, aliquotted and stored at -70°C.

2.4.5 Estimation of protein concentration

Protein concentration was determined by using the following molar extinction coefficients: 1280 M⁻¹ cm⁻¹ (VPg1/VPg2/VPg3), 51850 M⁻¹ cm⁻¹ (3Dpol), 61170 M⁻¹ cm⁻¹ (3CDpro), 9320 M⁻¹ cm⁻¹ (3Cpro), 10600 M⁻¹ cm⁻¹ (3B13C) and 13160 M⁻¹ cm⁻¹ (3B1233C). These values were determined by using protein parameters tool (ProtParam) on the ExPASy site (www.expasy.org). The absorbance values were measured at 280 nm in 6 M guanidine HCl (Gu-HCl), pH 6.5.

2.4.6 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were analyzed by SDS-PAGE. A 10% separating gel and 5% stacking gel containing 0.1% SDS was prepared (Sambrook and Russell 2001 3rd edition). The electrophoresis was carried out in Tris-Glycine buffer on a vertical slab gel electrophoresis system (Bio-Rad) using constant current of 40mA. Prior to loading, the samples were heated at 95°C for 5 minutes in 3X sample buffer (Bio-Rad). Gels were stained using Coomassie Brilliant Blue. Standard Pre-stained
molecular weight markers (NEB) were used to determine the size of the protein samples.

2.4.7 Tris-tricine polyacrylamide gel electrophoresis

For separation and visualization of small VPg peptides (~2kD), the Tris-tricine gel electrophoresis system was used. To analyse the results of the in-vitro uridylylation assay, 14% Tris-tricine gels were prepared using tricine gel buffer (Appendix II), 40% acrylamide:bisacrylamide (29:1) and glycerol as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>Resolving gel</th>
<th>Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricine gel buffer</td>
<td>5 ml</td>
<td>1.5 ml</td>
</tr>
<tr>
<td>40% acrylamide:bisacrylamide</td>
<td>5.3 ml</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>2 g</td>
<td>-</td>
</tr>
<tr>
<td>mQ water</td>
<td>up to 15 ml</td>
<td>6.0 ml</td>
</tr>
<tr>
<td></td>
<td>APS 100 µl</td>
<td>TEMED 15 µl</td>
</tr>
</tbody>
</table>

The gel was allowed to set for at least 1 hr. Samples were prepared by heating at 95°C for 2 min in 3X tricine sample buffer (Appendix II) and loaded on to a freshly rinsed well of the gel. Electrophoresis was carried out by using 1X cathode buffer (Appendix II) at 150v till the dye front run off the gel.

2.4.8 Western blot

Cell lysates or purified proteins (approximately 100 ng) were resolved by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) using Tris-glycine running buffer as described above. Protein samples were transferred
electrophoretically to nitrocellulose membrane (Immobilion) in a wet transfer tank (Bio-Rad). The membrane was then blocked overnight at 4°C with 5% blocking solution (skimmed milk powder in TBS-T) (TBS + 1% Tween, see Appendix II). The membrane was then incubated at room temperature with primary antibody (using a suitable dilution in blocking solution) for 1 hr. The membrane was washed extensively three times 5 min each using TBS-T. Subsequently the membrane was incubated with HRP conjugated secondary antibody (Amersham biosciences) diluted in blocking solution for 1 hr at room temperature. The membrane was then washed extensively as above and detection was achieved using the ECL plus system (Amersham Biosciences).

2.4.9 PolyU polymerase activity assays

PolyU polymerase assays were performed (as described by Gohara et al., 1999) in a total volume of 20 µl containing 50 mM HEPES buffer (pH 7.5), 10 mM 2-mercaptoethanol, 5 mM MgCl₂/MnCl₂, 60 µM ZnCl₂, 500 µM UTP, 0.2 µCi/µL [α-³²P]UTP, 1.8 µM dT₁₅/0.15 µM poly(rA)₉₀₀ primer/template and 1 µM 3Dpol. Reactions were carried out at 30°C for 5 min followed by quenching with 0.5 M EDTA to final concentration of 100 mM. 10 µl of the quenched reaction was spotted onto DE81 filter paper discs and dried completely. The discs were washed three times for 5 min in 5% dibasic sodium phosphate and rinsed in absolute ethanol. Bound radioactivity was quantified by liquid scintillation counting in 5 mL of Escoscint scintillation fluid and the number of pmoles of UTP incorporated per µmol of 3Dpol/min was measured.
2.4.10 In vitro uridylylation assay

The synthesis of VPgp(U)pU was measured by using an assay similar to that described before (Paul et al., 1998 and Goodfellow et al., 2003). The reaction mixture in total volume of 20 µL contained 50 mM HEPES (pH 7.5), 8% glycerol, 2 mM MgCl₂, 12.5 µM VPg (any deviation in the VPg concentration is listed in appropriate figure legends), 1 µM cre/bus RNA transcripts, 0.75 µCi [α-³²P]UTP (100 µCi/µL, Amersham Bioscience), 10 µM UTP, 1 µM 3CDpro [(C163G) with amino terminal His tag] and 1 µM 3Dpol were mixed together and added to the reaction. The final NaCl concentration in the reaction was kept at 6.25 mM. Reactions were carried out for 30 min at 30°C and stopped by addition of 10 µl of Tris-tricine loading dye. The samples were analyzed by Tris-tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 14 % polyacrylamide. Gels were dried without fixing and autoradiographed. The reaction products were quantitated with a PhosphorImager (Molecular Imager FX, BIO-RAD) by measuring the amount of [³²P] UMP incorporated. VPgpU(pU) refers to the sum of VPgpU and VPgpUpU.

To determine the differential efficiency of uridylylation of each VPg(1-3) peptides, standard reactions were assembled as described above with varied concentrations of VPg1-3 ranging from 1 µM to 10 µM for 10 minutes. The samples were analysed by using a bigger Tris-tricine gel. The purpose is to separate three radiolabeled band that is end-labeled cre/bus RNA products, VPgpU(pU) and the unincorporated hot UTP. The gel was carefully removed and covered with a saran wrap. The radioactivity of each band was quantified without drying the gel by PhosphorImager and the percentage of [³²P] UMP incorporated into each VPg was determined.
CHAPTER THREE

Development of a foot and mouth disease virus VPg uridylylation assay

Recent studies on the mechanisms of replication initiation of PV and HRV RNA have established the requirements for \textit{in vitro} uridylylation of VPg to be: VPg, 3D\textsuperscript{pol}, 3CD, RNA transcripts containing the \textit{cre} plus UTP and divalent metal ions, either Mn\textsuperscript{2+} or Mg\textsuperscript{2+} (Gerber et al., 2001a, b; Paul et al., 2000, 2003a,b). To establish a similar assay system for the FMDV it was necessary to generate the equivalent reagents.

3.1 Recombinant protein production

3.1.1 Expression and purification of histidine tagged FMDV 3D\textsuperscript{pol}

The coding region for FMDV 3D\textsuperscript{pol} was amplified using a PCR and inserted into the bacterial expression vector pQE30 (Qiagen). An N-terminal His-tagged form of 3D\textsuperscript{pol} was efficiently expressed from this vector in \textit{E. coli} M15 (FIG. 3.1, Lane 2) and purified using Hi-Trap His-column (Amersham Biosciences) to near homogeneity as judged by coomassie blue staining and immunoblotting using anti-3D antibodies (FIG. 3.2, Panel A and Panel B).

3.1.2 Expression and purification of histidine tagged FMDV 3CD\textsuperscript{pro}

cDNA encoding the FMDV 3CD precursor protein was amplified by PCR. Within the FMDC 3C, His-46, Cys-163, Asp-84 are part of the catalytic triad and His-181 is part of the binding pocket (Grubman et al., 1995). The catalytic activity of the 3C protease was blocked by modification of the cysteine codon (TGC) at residue 163 to a glycine (GGC) codon (FIG. 3.3, Panel A). This modification was verified by DNA
FIG. 3.1 SDS-PAGE analysis of uninduced and induced M15 cells harbouring pQE30-3Dpol and pQE30-3CDpro plasmids. Expression of 3Dpol and 3CD proteinase (proteolytically inactive) was achieved as described in the materials and methods. 0.5 ml of the sample was pelleted and suspended in 90 µl of PBS. 30µl of 3X loading dye (Bio-Rad) was added, heated for 5 min at 95°C and loaded onto an 10% SDS-PAGE gel. The gel was stained with Coomassie brilliant blue R-250 and subsequently destained. Lane 1, uninduced M15 cells harbouring pQE30-3Dpol, lane 2, M15 cells harbouring pQE30-3Dpol and lane 3, M15 cells harbouring pQE30-3CD after 4-h induction.
FIG. 3.2. Gel analysis of FMDV 3Dpol, His-3D, 3CD, and VPg1 to 3. Purified recombinant His-tagged 3D (termed His-3Dpol), His-tagged 3CD(C163G) (termed His-3CD), and the untagged 3D (3Dpol) were analyzed by SDS-PAGE and either stained directly with Coomassie blue (A) or transferred to an Immobilon membrane and detected using an anti-3D monoclonal antibody (kindly provided by E. Brocchi) with peroxidase-labeled goat anti-mouse immunoglobulin and chemiluminescence reagents (B). (C) Synthetic peptides (10 nmol [lanes a], 20 nmol [lanes b], or 30 nmol [lanes c]) corresponding to 3B1, 3B2, and 3B3 were analyzed on Tris-Tricine gels (16.5% acrylamide) and stained with Coomassie blue. Lane M contains a molecular weight marker (175,000 to 6,000; New England Biolabs).
sequencing and the mutant 3CD coding sequence was inserted into pQE30 as above.

The His-tagged 3CD(C163G) was also efficiently expressed in *E. coli* M15 (FIG. 3.1, Lane 3) and purified to near homogeneity as detected by coomassie blue staining and immunoblotting (FIG 3.2, Panels A and Panel B). As expected, the 3CD migrated more slowly than the 3Dpol confirming that the modification of the 3C sequence blocked its proteolytic activity.

### 3.1.3 Expression and purification of native FMDV 3Dpol

To express the FMDV 3Dpol without any additional sequences, the 3Dpol cDNA was amplified using a PCR, modified to remove an internal *SacII* site, and inserted into the vector pET26-Ub as described (Gohara et al., 1999, FIG. 3.3, Panel B) to generate pUb3Dpol. This plasmid efficiently expressed a ubiquitin-tagged FMDV 3Dpol in *E. coli* and when co-expressed with the ubiquitin-specific protease Ubp2, by co-transformation of *E. coli* BL21 (DE3) with pUb3Dpol and pCG1, cleavage occurred to liberate the untagged FMDV 3Dpol (FIG. 3.4, Panel A). It was observed that even after 4 hrs of co-expression the processing of the ubiquitin-tagged 3Dpol to native 3Dpol was incomplete (FIG. 3.4, Panel A). However during the course of the purification, the ubiquitin-tagged 3Dpol was completely processed (FIG. 3.4, Panel B) during the ammonium sulphate precipitation step (Materials and Methods). The native FMDV 3Dpol was then purified to near homogeneity using phosphocellulose, anion exchange and heparin chromatography (FIG. 3.5, Panel A, Panel B and Panel C respectively). This untagged FMDV 3Dpol migrated slightly faster on SDS-PAGE than the His-tagged 3Dpol (FIG. 3.2, Panels A).
FIG. 3. Strategy for production of proteolytically inactive 3CD and authentic 3Dpol. Panel A. To generate intact 3Dpol precursor protein 3CD, QuikChange site directed mutagenesis protocol was followed to mutate the amino acid codon for Cysteine 163 to a Glycine within the 3C coding region (shown with star in panel A). This mutation was intended to completely block the protease activity of the 3C to produce uncleaved 3CD in the bacterial expression system. Panel B. To produce unmodified FMDV 3Dpol the internal Sac II site was modified by QuikChange site directed mutagenesis protocol without changing the arginine amino acid (shown with delete mark) and the Bam HI site was made irrelevant by introducing a Bgl II restriction site in the reverse primer. Panel B depicts the diagrammatic representation of the overall strategy followed for native FMDV 3Dpol expression using the system as described in Gohara et al., 1999. The cleavage junction between ubiquitin and FMDV 3Dpol cleaved by the ubiquitin protease is indicated.
CHAPTER THREE FMD VpG URIDYLYLATION ASSAY

FIG. 3. SDS-PAGE analysis of uninduced and induced BL21DE3(pCG1) cells harbouring Ub-3Dpol plasmid. Unmodified 3Dpol was expressed as described in the Materials and Methods. Lane 1 represents the uninduced BL21DE3(pCG1) harbouring Ub-3Dpol plasmid. Lane 2, lane 3, lane 4 and lane 5 corresponds to BL21DE3(pCG1) harbouring Ub-3Dpol plasmid induced with IPTG (final concentration 0.5 mM) for 1-hr, 2-hr, 3-hr and 4-hr respectively.

FIG. 3. SDS-PAGE analysis of Ubiquitin-tagged and fully cleaved native 3Dpol protein. Lane 1, represents the induced BL21DE3 cells harbouring the Ub-3Dpol plasmid. Lane 2 represents ammonium sulphate precipitated fraction of the native 3Dpol as mentioned in the Materials and Methods section. As expected ubiquitin-tagged 3Dpol migrated slower compared to the fully cleaved 3Dpol.
### CHAPTER THREE

**FMDV VPg URIDYLATION ASSAY**

**Phosphocellulose (P11) column purification**

Ammonium sulphate precipitation fraction of the FMDV 3Dpol (FIG. 3.4, Panel B) was loaded on to a phosphocellulose column to remove any nuclease contamination (Panel A). Subsequently 3Dpol sample was purified by Q column anion exchange chromatography (panel B) and heparin column chromatography (panel C). Fraction 25, 26 and 27 were pooled together and dialysed against buffer A containing 50 mM salt (see Materials and Methods section).

![Phosphocellulose (P11) column purification](image)

![HiTrap™ Q Sepharose XL column purification](image)

![HiTrap™ Heparin HP column purification](image)

**FIG.3.5. Ion exchange chromatography purification of FMDV 3Dpol enzyme.**
3.2 Synthesis of synthetic peptides corresponding to VPg1, VPg2, and VPg3.
Synthetic peptides corresponding to the individual FMDV VPg1, VPg2, and VPg3 peptides and a modified version containing a single-amino-acid substitution (Y to F) were produced by Lawrence Hunt (Institute for Animal Health, Compton Lab) and purified by high-performance liquid chromatography. Aliquots were analysed in Tris-tricine gel (14%) and stained with coomassie brilliant blue (FIG. 3.2, Panel C).

3.3 Production of wild-type and mutant FMDV cre/bus transcripts.
The plasmid pGC-cre (wt) and the various mutants were used as templates for production of RNA transcripts (FIG. 3.6 and FIG. 3.7). Plasmid DNAs were linearized with Dra I and purified by phenol extraction and ethanol precipitation, and RNA was produced using a MEGAscript high-yield transcription kit according to the manufacturer's instructions (Ambion). The products were treated with DNase I and purified using urea-polyacrylamide (8%) gel electrophoresis to recover the cre/bus transcripts only. The transcripts were detected by UV shadowing, eluted in EDTA (1 mM) and sodium dodecyl sulfate (SDS, 0.5%), and isolated by phenol extraction and ethanol precipitation. The purified RNA transcripts were analysed using 8% polyacrylamide-urea gels and were stored at -70°C.

3.4 PolyU polymerase activity assays
The functional activity of the three purified proteins (His-3Dpol, native 3Dpol and His-3CD) were tested using an in vitro RNA polymerase assay (Gohara et al., 1999) using poly(A) as template. The His-tagged 3Dpol and the His-tagged 3CD(C163G) exhibited little or no RNA polymerase activity in the presence of Mg^{2+} but both proteins displayed significant activity in the presence of Mn^{2+} (FIG. 3.8 Panel A and
FIG. 3.6. Production of FMDV cre/bus transcripts. Panel A, represents the cloning strategy of the FMDV cre/bus to produce the pGC-cre plasmid. Panel B represents the in vitro transcription of the linearized pGC-cre plasmid (cut with Dra I) to generate cre/bus transcripts and the self-cleaved ribozyme.
FIG. 3. Diagrammatic representation of the wild type and mutant cre. Mutations were introduced within the AAACCA motif of the loop region of the cre/bus by QuikChange site directed mutagenesis as per the manufacturer's instructions.
Panel B). This result should be compared with the complete lack of RNA polymerase activity exhibited by the PV 3CD (Thompson et al., 2004). Indeed the complete inactivity of the PV 3CD was confirmed in our assays (data not shown). There is conflicting evidence about the ability of different picornavirus 3D<sup>pol</sup> molecules with some form of N-terminal extension to function as an RNA polymerase. The PV 3D<sup>pol</sup> poorly tolerates even a single extra amino acid (Gohara et al., 1999) whereas the 3D<sup>pol</sup> from HRV2 within a GST-3D<sup>pol</sup> fusion protein exhibited similar RNA polymerase activity to the untagged 3D<sup>pol</sup> (Gerber et al., 2001a, b). The native FMDV 3D<sup>pol</sup> exhibited high RNA polymerase activity using poly(A) as a template (FIG. 3.8, Panel A). This activity was also markedly greater in the presence of Mn<sup>2+</sup> compared to Mg<sup>2+</sup> (compare FIG. 3.8, Panel A). It is noteworthy that the incorporation of UMP by the unmodified 3D<sup>pol</sup> in the presence of Mg<sup>2+</sup> is comparable to that achieved by the His-tagged 3D<sup>pol</sup> and the His-3CD in the presence of Mn<sup>2+</sup>. The specific activity of the unmodified FMDV 3D<sup>pol</sup> in the presence of Mn<sup>2+</sup> was 247 pmol/min/µg whereas in the presence of Mg<sup>2+</sup> it was 15 pmol/min/µg.

3.5 In vitro uridylylation assay

Standard in vitro uridylylation assays were performed (Materials and Methods) using RNA transcripts corresponding to the FMDV cre/bus with the FMDV VPg peptides, (FIG. 3.8, panel C and Panel D). No uridylylation of the peptides was observed using the His-tagged 3D<sup>pol</sup> in the presence of Mg<sup>2+</sup> (FIG. 3.8, panel C). Reactions were then performed to analyze the ability of the untagged FMDV 3D<sup>pol</sup> to uridylylate the FMDV VPg1 peptide. Under these conditions efficient VPg1 uridylylation was observed (FIG. 3.8, Panel C and Panel D). It was found that the
FIG. 3.8. In vitro RNA synthesis and VPg1 uridylylation by FMDV 3Dpol. (A and B) In vitro RNA synthesis assays using a poly(rA)500 (0.15 μM) template and dT15 (1.8 μM) primer were performed with the His-tagged 3Dpol (1 μM), His-tagged 3CD (1 μM), and untagged 3Dpol (1 μM) as indicated in the presence of Mn<sup>2+</sup> (5 mM) (A) or Mg<sup>2+</sup> (5 mM) (B). The incorporation of [32P]UMP (from UTP) was measured at 5 min when the assays were within a linear range, and the reactions for panels A and B were performed in parallel. (C) In vitro 3B uridylylation assays were performed using the 3B1 peptide (12.5 μM) with [α-32P]UTP in the presence of Mg<sup>2+</sup> (2 mM) with His-tagged 3CD (1 μM), the cre/bus RNA transcripts (1 μM), and either the untagged 3Dpol or the His-tagged 3Dpol, as indicated. The uridylylated 3B1/VPg1 product, termed VPgpU(pU), is indicated. (D) In vitro 3B uridylylation assays were performed as described for panel C, and the individual components of the uridylylation reaction were separately omitted, as indicated. +, present; −, absent.
production of [\(^{32}\)P]-uridylylated VPg required the presence of the 3CD(C163G) precursor and an RNA transcript (56nt) corresponding to the FMDV cre/bus. Omission of this short RNA transcript, or His-3CD(C163G), or 3D \(^{2}\) totally abrogated uridylylation of VPg (FIG. 3.8, Panel D). To achieve the standard VPg uridylylation assay optimisation of each and individual components were carried out as summarized below.

### 3.6 Optimization of the in-vitro uridylylation assay

#### 3.6.1 Stoichiometry of 3CD

To find out the stoichiometry of the 3CD concentration for optimal VPg uridylylation, in vitro uridylylation assays were carried out using 1\(\mu\)M 3Dpol, 1\(\mu\)M cre/bus RNA transcripts, 10 \(\mu\)M VPg and varied concentrations of 3CD protein. It was observed that 1\(\mu\)M of purified 3CD is optimal for the reaction and any concentration above or below is suboptimal in this conditions (FIG. 3.9). Henceforth all the future experiments were carried out using 1\(\mu\)M 3CD.

#### 3.6.2 Optimum VPg concentration for the uridylylation assay

To titrate the optimum VPg concentration, uridylylation assay was carried out using 1\(\mu\)M 3Dpol, 1\(\mu\)M cre/bus RNA transcripts, 1\(\mu\)M 3CD and different concentration of VPg peptides (VPg1-3) ranging from 1-100 \(\mu\)M. Incorporation of UMP residues in the VPg to form VPgpUpU was measured by phosphorimaging and the values obtained were plotted against the different VPg concentrations. The three VPgs were uridylylated with slightly different efficiencies. The optimal reaction was observed at 6.0 or 12.5 \(\mu\)M VPg but higher concentrations inhibited the reaction (FIG. 3.10). All the future experiments were performed at 12.5 \(\mu\)M VPg concentration.
FIG. 3.9. Uridylylation of VPg1 in vitro using cre/bus as a template with varied concentration of 3CD. The production of VPg1pUpU was measured as described in the Materials and Methods using 1 μM 3Dpol, 1 μM cre/bus, 10 μM VPg1 and different concentration of 3CD as indicated in the Panel A. The optimal concentration of 3CD required for the in vitro uridylylation assay was determined from the graph depicted in Panel B.
FIG. 3.10. In vitro uridylylation assays using varied concentrations of VPg. Optimal concentration of VPg required for the uridylylation of VPg1-3 were determined using 1μM 3DPol, 1μM cre/bus, 1μM 3CD and VPg1-3 peptide ranging from 1μM-100μM for 20 min at 30°C (Panel A). The synthesis of VPgpU(pU) for each peptide was quantified using a phosphorimageter (Bio-Rad). The range of VPg concentrations that supports the linear range of VPgpU(pU) synthesis in the in vitro reaction was determined from the graph depicted in Panel B.
3.6.3 Titration of divalent cations/cofactors

The effects of divalent cations Mg\(^{2+}\) and Mn\(^{2+}\) on uridylylation activity of the polymerase enzyme were assessed by using 1\(\mu\)M 3Dpol, 1\(\mu\)M cre/bus RNA transcripts, 1\(\mu\)M 3CD and 12.5 \(\mu\)M VPg1 with varied ion concentrations as indicated (FIG. 3.11 and FIG. 3.12). Production of VPgpUpU was quantified by phosphorimaging. For both magnesium chloride (MgCl\(_2\)) and manganese acetate (MnOAc), the optimum concentrations were found to be 2mM.

3.6.4 Determination of optimum temperature

To determine the optimum temperature for VPg uridylylation, uridylylation reactions were assembled as above using 2mM Mg\(^{2+}\) and incubated at different temperatures for 1 hr as indicated (FIG. 3.13). The optimum temperature was found to be 30\(^\circ\)C-37\(^\circ\)C. At 42\(^\circ\)C, the reaction was greatly inhibited.

3.6.5 Optimization of time of incubation

VPg uridylylation assays were assembled and carried out as above for different durations as indicated (FIG. 3.14). The linearity of the reaction was determined from the plotted values and 30 min incubation time was found to be optimum for the reaction.

3.7 The role of the cre/bus in the uridylylation of the FMDV VPg peptides

To study the effects of mutations in the loop region of the FMDV cre/bus in the VPg uridylylation assay, mutant cre/bus plasmids were generated. The first A residue in the conserved A\(^1\)A\(^2\)A\(^3\)CA motif from the PV cre acts as the template for the addition of two U residues to the VPg peptide primer, the second and third A residues
FIG. 3.11. Effects of magnesium ion (MgCl₂) on VPg uridylylation. Panel A. Standard VPg uridylylation assays were assembled as described in the Materials and Methods except for the varied magnesium ion concentration as indicated. Panel B. The production of VPg1pUpU(pU) was measured and plotted as a function of Mg²⁺ concentration. Optimal quantity of Mg²⁺ required for uridylylation of VPg1 was determined.
FIG. 3.12. Effects of Manganese ion (MnOAc) on VPg uridylylation. Panel A. Standard VPg uridylylation assays were assembled as described in the Materials and Methods except for the varied manganese ion concentration as indicated in the legend. Panel B. The production of VPg1pUpU(pU) was measured and plotted against the Mn$^{2+}$ concentration. Optimal quantity of Mn$^{2+}$ required for uridylylation of VPg1 was determined.
FIG. 3.13. Effects of temperature on VPgpU(pU) synthesis. Standard assays were carried out as indicated in the Materials and Methods except for the varied temperature ranging from 23°C to 42°C. The rate of VPgpU(pU) formation by the FMDV 3Dpol (Panel A) at different temperatures at the indicated time points was measured and plotted on a graph (Panel B). The optimal incubation temperature corresponds to the maximum VPgpU(pU) formation.
FIG. 3.14. Uridylylation assay time course experiments in the presence of 2 mM MgCl₂. Panel A. Standard uridylylation assays were carried out using 1 μM 3Dpol, 1 μM cre/bus, 1 μM 3CD and 12.5 μM VPg using Mg²⁺ (2 mM) as a cofactor at 30°C for varied times as indicated. Panel B. The incorporation of UMP into the VPg to form VPgpU(pU) was plotted against Y-axis as function of time of incubation. The linearity of the reaction was determined from the graph.
modulate this process (Paul et al., 2003). Mutations (A to C) were introduced in the FMDV cre/bus at the A₁, A₂, A₃ positions individually and a fourth mutant was made with mutations at both the A₁ and A₂ positions (FIG. 3.7). RNA transcripts were prepared from each of these plasmids and their ability to support the uridylylation of FMDV VPg₁ was tested in the presence of Mn²⁺ or Mg²⁺ ions (FIG 3.15, Panels A and Panel B). In the presence of each of the divalent metal ions, the wt cre/bus transcript supported very efficient uridylylation of VPg₁. However, substitution of A * alone or A₁ and A₂ together in the cre/bus transcripts completely blocked the reaction. In the presence of Mg²⁺ ions (FIG. 3.15, Panel A and Panel B), the A₂ and A₃ point mutants were also inactive but, in the presence of Mn²⁺ ions, low level uridylylation of the VPg₁ peptide was also observed using the A₂ and A₃ mutant transcripts as template (FIG. 3.15, Panel A).

3.8 Uridylylation of the three different FMDV VPg peptides

Uniquely, FMDV encodes and uses three distinct forms of VPg (3B) which differ in size and sequence (FIG. 3.16, Panel A). Each of the different forms of FMDV VPg has been found to be associated with genomic RNA and hence all must be functional (King et al., 1980). The activity of the three different peptides in the in vitro uridylylation assay was compared (FIG. 3.16). It was apparent that each of the VPg peptides could be uridylylated in vitro by the untagged 3Dpol in the presence of the cre/bus transcripts and His-3CD. However, VPg3 was a better substrate in this assay than VPg2, which in turn was also better than VPg1. It was also apparent that the uridylylation reaction occurred with similar efficiency in the presence of Mn²⁺ or Mg²⁺ (FIG. 3.16) and the relative efficiency of uridylylation of the different VPg peptides was the same with each divalent metal ion. Each of the VPg peptides
FIG 3.15. Critical role of the AAACA motif within the FMDV cre/bus for in vitro uridylylation of FMDV 3B1. In vitro uridylylation assays using the FMDV 3B1 peptide were performed using wt or mutant FMDV cre/bus RNA transcripts as indicated in the presence of Mn^{2+} (A) or Mg^{2+} (B) with FMDV 3Dpol and His-3CD. Reaction mixtures were incubated at 30°C for 30 min. +, present.
FIG. 3.16. In vitro uridylylation of all three FMDV 3B peptides. Panel A. Comparison of VPg sequences from FMDV(01 Kaufbeuren) and PV (type 3). The conserved Tyr (Y) (residue 3) is the site of uridylylation. Panel B. In vitro uridylylation assays were performed in the presence of purified FMDV components 3Dpol (1 μM), His-3CD (1 μM), cre/bus RNA transcripts (1 μM), and either Mn<sup>2+</sup> (2 mM) or Mg<sup>2+</sup> (2 mM), as indicated, using each of the FMDV 3B peptides (12.5 μM). Products were analyzed on Tris-Tricine gels. Panel C. Incorporation of [α-<sup>32</sup>P]UMP into VPgpU(pU) was quantitated for each peptide using a phosphorimager. Panel D. The time course of uridylylation of VPg1, VPg2, and VPg3 was determined in parallel. Reactions were performed as described for Panel B with Mg<sup>2+</sup> (2 mM), and each VPg peptide was used at 1 μM. The time points were 30 s (A), 1 min (B), 2 min (C), 5 min (D), and 10 min (E). Panel E. The incorporation of UMP in the reactions shown in panel D was quantified using a phosphorimager.
migrate slightly differently on the Tris-tricine gels (FIG. 3.16, Panel B). Thus, although VPg1 is one amino acid shorter than VPg2 and VPg3 (FIG. 3.16, Panel A), it migrates at an intermediate position and VPg3 migrates the fastest (FIG. 3.16, Panel B). To confirm the differences in efficiency of uridylylation, we determined the rate of UMP incorporation in a time course assay for each VPg in parallel (FIG. 3.16, Panel D and Panel E). Consistent with the results shown in FIG. 3.16, Panel B, Panel C and Panel D the rate of VPg3 (3B3) uridylylation was the fastest, while that for VPg1 (3B1) was the slowest and VPg2 (3B2) was intermediate (FIG. 3.16, Panel E).

3.9 Differential uridylylation efficiency of VPg1, VPg2 and VPg3.

To determine more accurately the differential ability of each copies of the VPg to undergo uridylylation, the incorporation of \([^{32}\text{P}-\alpha]UTP\) into VPg-pUpU was monitored by a Tris-Tricine gel and buffer system. The uridylylation of each VPg was carried out with different concentrations for a fixed time period of 10 min. Both VPg-pU(pU) and the unincorporated labeled UTP were separated, and the band intensity quantified by phosphorimager (FIG. 3.17). The uridylylation efficiency of each peptide was determined from the % incorporation of UTP into VPgpU(pU) (FIG. 3.18). Consistent with earlier results % incorporation of UTP into VPg3 was highest (~4%) followed by VPg2 (~3%) and VPg1 (~2%).

3.10 Specificity of VPg recognition in the uridylylation assay. The linkage of VPg (3B) to RNA occurs through the hydroxyl group on a Tyr residue. To confirm that the uridylylation reaction observed with the FMDV components was dependent on the presence of the Tyr residue, a variant of the VPg3 peptide was synthesized in
FIG. 3.17. Differential uridylylation efficiency of VPg1. Standard uridylylation assay reactions were set up with a range of VPg concentrations as indicated in the figure legend. The incubation temperature was set for 10 min to measure the incorporation of \(^{32}\)P UTP when the reaction is within the linear range. Band intensities were quantified for VPg1pU(pU) and unincorporated UTP for each concentration of VPg in each lane and the incorporation percentage of UTP was calculated. Similar reactions were set up for VPg2 and VPg3 and the relative efficiency of uridylylation of each VPg peptide was determined.
### CHAPTER THREE

**FMDV VPg URIDYLATION ASSAY**

#### FIG. 3.18
Tabular presentation of the differential uridylation efficiency of the FMDV VPg peptides

<table>
<thead>
<tr>
<th>[VPg] (µM)</th>
<th>VPglpU(pU) Unincorporated UTP</th>
<th>VPglpU(pU)+ unincorporated UTP</th>
<th>% incorporation of UTP into VPglpU(pU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>12762</td>
<td>810477</td>
<td>1.55</td>
</tr>
<tr>
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which the Tyr was replaced by a Phe residue (which lacks the hydroxyl group). This peptide was inactive in the uridylylation reaction, as anticipated (FIG. 3.19, Panel A).

3.11 Compatibility between FMDV and PV uridylylation assay system

Poliovirus 3Dpol is able to utilize as a substrate, in addition to its own VPg, the VPg of HRV14 when either poly(A) or cre(2C) RNA is used as a template. In contrast to HRV14 VPg, the VPgs of HRV2 and HRV89 have hardly detectable substrate activities on both templates (Paul et al., 2003). Based on those observations we analysed the activity of poliovirus polymerase, PV VPg and PV cre within the FMDV uridylylation assay system and vice versa. PV VPg is significantly different in sequence from each of the FMDV VPgs (FIG. 3.16, Panel A). Surprisingly, the PV VPg was very efficiently uridylylated using the FMDV components (FIG. 3.19, Panel B). However none of the rest of the PV components was recognised in the FMDV uridylylation assay system using Mg2+ as a cofactor. In contrast, use of the PV uridylylation assay system showed high specificity for the PV components and none of the FMDV components were able replace the PV components (FIG. 3.19, Panel C).
FIG. 3.19. Specificity of the VPg uridylylation reaction. (A) In vitro uridylylation assays were performed with [α-32P]UTP using the FMDV cre/bus RNA transcript in the presence of Mg^{2+} with FMDV 3Dpol and His-3CD with either the FMDV VPg3 peptide or the VPg3(Y3F) peptide, as indicated. The latter peptide has the Tyr (Y) residue replaced by a Phe (F) residue. (B) In vitro uridylylation assays were performed using the FMDV assay system where each of the FMDV components was separately replaced with PV components as indicated. (C) In vitro uridylylation reactions were performed using PV assay system where each and single component was replaced separately with FMDV components as indicated.
3.12 Discussion

The studies that have been performed here have shown for the first time the mechanism by which the FMDV 3Dpol selects its viral RNA structure as a template for uridylylation of the genome linked short peptides VPg to form VPgpU(pU), a primer for viral RNA synthesis. This has been demonstrated in vitro by using defined purified components. In picornavirus VPgs, the tyrosine residue at position 3 is found to be conserved. The importance of this residue in RNA replication has been well known. In recent times the exact function of this residue has become clearer when it was established that this residue participates in a nucleophilic attack on the α-phosphate of UTP to form VPgpU(pU) in vitro (Paul et al., 1998, 2003). In our study we have shown that in addition to UTP (with divalent metal ions) and a FMDV VPg peptide, this uridylylation reaction required RNA transcripts containing the cre/bus, the untagged 3Dpol, and the 3CD precursor. Each of the three distinct FMDV VPg peptides is an efficient substrate in this reaction, although the VPg3 showed the highest activity. Within the RNA, the AAACA motif within the cre/bus was shown to be the sole or primary motif for activity.

3.12.1 An authentic N-terminus of the FMDV 3Dpol is a pre-requisite for efficient RNA polymerisation and VPg uridylylation activity

The synthetic activity of PV and HRV 3Dpol in vitro has been well studied (Paul et al., 2000; Gerber et al., 2001). The transcription activity of the enzyme is required for complementary strand synthesis. The nucleotidylation activity is required for formation of a covalent linkage between a nucleotide(U) and the hydroxyl group of tyrosine. Both these activities are required for the synthesis of a protein primer and subsequent positive and negative strand synthesis. Both types of synthetic activities
are quite similar to the activities of some of the double stranded linear DNA viruses with a genome linked protein including phage 29, PRD1 (a lipid-containing double stranded DNA bacteriophage), and adenoviruses (see Introduction). Within the picornaviruses the first amino acid glycine in 3Dpol is absolutely conserved. Recent studies have suggested that PV 3Dpol undergoes an elegant proteolytic processing-dependent allosteric switch for polymerase activation that involves burying the N-terminal glycine residue in a pocket at the base of the fingers domain. The buried N-terminus is also involved in positioning Asp238 in the active site (Thompson and Peersen 2004). The replacement of the glycine with an alanine significantly reduces the polymerization activity of the enzyme (~54% of the wt) whereas replacement with a serine almost completely destroyed the enzymatic activity (1.6% activity of the wt) (Thompson and Peersen 2004). The presence of an extra –CH$_3$ group in the alanine pushes the N-terminus out of its binding pocket by ~0.9 Å compared to the native Gly1 residue. These studies indicate that a clear correlation exists between enzymatic activity and the positioning of Asp238 for the interaction with the 2' OH of the incoming NTP.

The importance of the native N-terminus in polymerase enzyme activity has also been reported for other viruses like Sindbis virus, a prototype alpha-virus, in which an authentic amino terminus is essential for minus strand synthesis (Lemm et al., 1994). Similarly, the coxsackie virus A21 is only active in an untagged form (Gerber et al., 2001), whereas in contrast, the 3D$^{pol}$ from HRV-2 displayed similar activity in both oligo-dT primed polyU synthesis and uridylylating Vpg in an untagged form or as part of a glutathione S-transferase fusion protein. In studies shown here the activity of FMDV 3D$^{pol}$ is clearly severely affected by the presence of the N-terminal
His tag or by the presence of 3C sequences and has little or no activity in the presence of Mg$^{2+}$ in the standard RNA polymerase assay. However, these proteins do display significant activity in the same assay with Mn$^{2+}$. The structure of the native FMDV 3D$^\text{pol}$ has recently been published (Ferrer-Orta et al., 2004). It was found that the N-terminal region of the protein encircles the enzyme active site; hence, it is perhaps not very surprising that the presence of an N-terminal His tag perturbs the activity. It is clearly possible, however, for the presence of the tag to differentially affect the RNA polymerase activity compared to the uridylylation reaction.

3.12.2 FMDV 3Dpol uridylylates VPg efficiently in presence of either Mg$^{2+}$ or Mn$^{2+}$

All known DNA and RNA polymerases require divalent cation cofactors for optimal activity. Because of relatively higher concentration of magnesium (Mg$^{2+}$) in vivo in comparison to Mn$^{2+}$ it is considered that Mg$^{2+}$ is the true physiological cofactor inside the cell. Evidence has been presented that besides Mg$^{2+}$ and Mn$^{2+}$ other divalent cations like Co$^{2+}$ and Zn$^{2+}$ also stimulate the PV VPg uridylylation reaction (Paul et al., 2003). However other divalent cations like Ca$^{2+}$, Fe$^{2+}$ and Ni$^{2+}$ are inactive in VPg uridylylation. The activity of the 3D$^\text{pol}$ from PV and HRV-2 have both been shown to be much (10- to 100-fold) higher in the presence of Mn$^{2+}$ than in the presence of Mg$^{2+}$ (Arnold et al., 1999; Gerber et al., 2001). The FMDV 3D$^\text{pol}$ was also much more active (over 10-fold) with Mn$^{2+}$ in the RNA polymerase assay. Within cells, the enzymes presumably function with Mg$^{2+}$. Studies suggest that the specificity of these RNA polymerases is relaxed in the presence of Mn$^{2+}$ compared to Mg$^{2+}$, and this can lead to a higher level of misincorporation of nucleotides (Arnold et al., 1999). The ability to use mutant forms of the short FMDV crelbus transcripts...
in the uridylylation assay was also enhanced in the presence of Mn$^{2+}$, but there was little difference in the activity observed with each of these divalent metal ions when the wt crelbus was used as a template with each of the VPg peptides (FIG. 3.16). These results closely match observations made with the PV 3Dpol (Paul et al., 2003; Dr. Ian Goodfellow, personal communication).

3.12.3 Loss of virus viability due to VPg3 deletion could be due to loss of primer function

It is noteworthy that the uridylylation ability of the FMDV 3Dpol reduces significantly at concentrations of VPg above 10 μM (FIG. 3.10). However this is not true for PV 3Dpol. PV 3Dpol can uridylylate PV VPg very efficiently well above 100 μM (Pathak et al., 2002). It is also interesting that the FMDV VPg3 peptide was the most efficient substrate for uridylylation by the FMDV 3Dpol (FIG. 3.16). This was further confirmed by quantification analysis of the UTP incorporated into each of the VPgs during a fixed period of time and at a fixed concentration (FIG. 3.17 and FIG. 3.18). So the ability of the FMDV 3Dpol to recognise and uridylylate FMDV VPg3 more efficiently might suggest that FMDV 3Dpol forms a more stable complex with VPg3. It is also possible that since VPg3 is closer to the 3D and 3C in the polyprotein, the availability of VPg3 being selected for uridylylation is greater than VPg2 and VPg1. In addition, 3C or 3CD is also a prerequisite for the VPg uridylylation, this leads to the possibility that VPg/3B along with the 3C as precursor protein (3BC) might also serve as a substrate for uridylylation (see Chapter 4). Deletion of the individual VPg sequences from within FMDV RNA transcripts had shown that only the VPg3 sequence was essential for viability (Falk et al., 1992). However, this was attributed to a defect in proteolytic processing that was observed.
using in vitro translation reactions of the mutant transcripts. Recently it has been reported that amongst the three different copies of the VPg, the amino acid sequence of the VPg3 is most conserved among all FMDV isolates known to date compared to VPg2 and VPg1 (Carrillo et al., 2005). VPgl and VPg2 have been implicated in the broad host range of the virus (Pacheco et al., 2003) unlike that of the VPg3. It can be suggested that VPg3 might have a conserved function in all hosts that is solely in RNA replication. In addition, since VPg3 also seems to be the most efficient substrate for in vitro uridylylation, it may be that the loss of this sequence may also have a deleterious effect on RNA replication, especially at the early stages of infection when the VPg peptides will be present at low levels. On the other hand, there was apparently little difference in the efficiency of virus rescue from the three different mutant transcripts in which two of the three 3B tyrosine (Y) residues were changed to phenylalanine (F). However, no sequence analysis of the rescued viruses was reported and each rescue was about 100-fold-less efficient than that of the wt (Falk et al., 1992).

3.12.4 FMDV 3Dpol uses a mechanism of VPg uridylylation that is consistent with a 'Slide-Back' mechanism proposed for poliovirus 3Dpol

Mutagenesis of the AAACA motif within the FMDV crelbus showed that the A\(^1\) nucleotide is critical for the uridylylation reaction (FIG. 3.15). This result is consistent with studies using a FMDV replicon containing modifications in this motif (Mason et al., 2002). Modification of the A\(^2\) and A\(^3\) nucleotides within this motif also greatly depressed viral RNA replication, and it is now shown that the in vitro uridylylation of VPg is also severely inhibited by these mutations in the crelbus. Thus, the results on the requirements for the crelbus in a FMDV replicon (Mason et
al., 2002) closely match the in vitro studies on the uridylylation of VPg presented here. Furthermore, they are in accord with previous results on the role of the AAACA motif obtained in previous studies on the PV and HRV core structures (Paul et al., 2003a; Yang et al., 2002). These results lead to the hypothesis that the first A nucleotide within this motif acts as the template for the addition of both the first and second U to 3B/VPg using a "slide-back" mechanism (Paul et al., 2003b) as has been suggested for nucleotidylation reaction observed in adenoviruses and phage 29. Our results are also consistent with a 'slide-back' back mechanism of VPg uridylylation by FMDV 3Dpol.

3.12.5 Amino acid composition of the VPg peptides might be responsible for 3Dpol recognition

The ability of the PV VPg to act as a substrate in the FMDV uridylylation assay was quite surprising considering the fact that PV VPg peptide is significantly different in sequence from each of the FMDV VPgs (FIG. 3.16). In contrast, FMDV VPg could not replace PV VPg in the PV uridylylation assay (FIG. 3.19). Similar studies using HRV2 has shown that the 3Dpol can efficiently uridylylate PV1 VPg, HRV14 VPg and HRV89 VPg in the HRV2 uridylylation assay (Gerber et al., 2001). Conversely PV 3Dpol could not utilise HRV2 and HRV 89 VPg in the PV uridylylation assay (Paul et al., 2003a). Both HRV2 and HRV89 are closely related and contain two negatively charged glutamic acid residues which are absent in polioviruses. It is believed that the negatively charged amino acids in the HRV2 and HRV89 VPgs make them incompatible substrates in the PV uridylylation assay. Similarly all the three FMDV VPgs also contain two negatively charged amino acids, this may explain why they are unsuitable substrates for the PV uridylylation assay.
In the VPg of many enteroviruses, Tyrosine (position 3), Glycine (position 5) and Arginine (position 17) are found to be conserved. The tyrosine residue is absolutely critical for the replication of the virus. A mutant PV VPg peptide with mutation at position 3 (T3Y4) where tyrosine at position 3 was replaced with threonine (an amino acid residue that contains free -OH group like tyrosine) and the amino acid at the fourth position was replaced with a tyrosine has been found to be completely inactive in the uridylylation reaction suggesting the high degree of specificity of the PV 3Dpol enzyme for the identity and location of the tyrosine in the VPg peptide (Gerber et al., 2001). As expected, mutation at the position 3 (Y3F) of the FMDV VPg completely abolished the uridylylation of the VPg. In addition in PV, replacement of the positively charged arginine (residue 17) with a negatively charged glutamic acid, produced a mutant peptide that was completely inactive in the uridylylation assay (Paul et al., 1998; Paul et al., 2000). Thus it is not too surprising that all the FMDV VPg peptides with two negatively charged glutamic acid in them are completely inactive as a substrate in the PV system. Furthermore in the compatibility experiments between FMDV and PV, none of the cre transcripts could replace the function played in the homologous system. However it is noteworthy that HRV14 cre is a very good template in the PV uridylylation assay system and was stimulated by the addition of PV 3CD (Paul et al., 2000). From this point of view it is quite intriguing, how 3C of PV could complement the function of HRV 3C in the PV uridylylation system. So it seems that the specificity of the VPg uridylylation reaction provided by the 3C/3CD in the in-vitro uridylylation assay is due to non-specific structure based RNA binding activity rather than any sequence specific recognition of the 3C/3CD protein. This argument could be further substantiated, for example a PV chimeric cre structure in which the entire sequence of the stem was
replaced with an artificial sequence of similar stability supported 3CD dependant VPg uridylylation very efficiently (Goodfellow et al., 2003). In the FMDV uridylylation assay PV 3C could not complement the function of the FMDV 3C or vice versa. Recently it has been proposed that the base of the loop and upper stem in the cre determines the 3C binding site (Yin et al., 2003). It is interesting, however, that the FMDV cre/bus does not contain the consensus sequence derived from the PV and HRV cre sequences, 1GXXXAAAXXXXXXA14, or even the more relaxed version, RXXXAAAXXXXXXR. In the FMDV sequences studied, nt 14 is a C (see references Mason et al., 2002; Paul et al., 2003b; and Tiley et al., 2003).

3.12.6 FMDV 3CD protein provides specificity to VPg uridylylation assay

The uridylylation ability of the FMDV 3Dpol was stimulated up to 80-100 times in the presence of 3CD (FIG. 3.8, Panel D). This suggests that 3CD provides a high degree of specificity to this reaction when the cre/bus is used as a template either by recruiting the 3Dpol or 3Dpol/VPg complex on the cre/bus template. Another possibility is, 3CD might provides a great degree of stability to the cre/bus RNA structure because of its RNA binding activity providing an optimum orientation of the AAACA motif in the cre/bus for it to act as a template. In PV it has been shown that the RNA binding activity of the 3CD/3C is required for the stimulation of the uridylylation activity of the 3Dpol (Gerber et al., 2001). To date the 3C recognition motif in the cre/bus has not been identified. In addition, the structure-function analysis of the cre-3C complex and its relevance in VPg uridylylation assay has not been established. Recently the structure of the HRV cre has been determined by NMR (Thiviyanathan et al., 2004) which suggests that the adenosine residues (AAACA) in the cre loop are oriented towards the inside of the loop. But it was
suggested that the loop was twisted in a spiral fashion such that sufficient space was provided for two uridine residues to base-pair with the loop without significant disruption of the structure. However the stimulation of VPg uridylylation by addition of the 3C might be because the RNA binding activity of the 3C produces some conformational changes in the overall configuration of the structure that could present the loop in such a manner that the adenosine residues are more exposed and accessible to stimulate VPg uridylylation.

Studies on the PV system have found that the PV 3C alone can replace 3CD (Pathak et al., 2002). There are significant differences between the properties of the PV 3C and 3CD. Both proteins are active proteases, but the PV 3CD is required for processing of the capsid precursor P1 (Leong et al., 2002), whereas in contrast, the FMDV 3C is able to achieve all capsid processing by itself (Ryan et al., 1999). Hence, it will be interesting to determine whether the role of the FMDV 3CD in VPg uridylylation can also be replaced by 3C.
CHAPTER FOUR

Characterisation of RNA elements and proteins required for FMDV replication

All picornavirus RNA replication follows a similar strategy, where the viral genome is first transcribed into a negative strand, which in turn is used as a template for synthesis of positive sense progeny genomes. We have shown in the previous chapter that the viral protein 3B (VPg) serves as a source of primer for replication to be initiated. Considering the fact that during polyprotein synthesis VPg/3B is synthesized within precursor proteins the exact source of the VPg to acts as a primer is still not known. Furthermore for poliovirus RNA synthesis to occur the formation of a ternary ribonucleoprotein (RNP) complex is necessary in which both the viral proteins as well as host proteins participate (Andino et al., 1990, 1993; Herold and Andino 2001). The negative-strand synthesis of poliovirus is initiated within the poly(A)-tail of the genomic RNA (Herold and Andino 2001). The viral protein 3CD binds to the 5’-end of the genomic RNA and reaches its site of action within the poly(A)-tail of the genome via circularization of the genomic RNA using an RNA-protein-protein-RNA-bridge that involves two cellular factors, PCBP and PABP1 (Herold and Andino 2001). This type of RNA-protein interactions necessary for PV RNA replication have not yet been shown for any other picornaviruses including FMDV. In addition it is important to mention that the RNA structures that are involved in PV replication within the 5’ UTR are different from the RNA structures present in the FMDV genome. Therefore we have attempted to look at the requirements of both RNA structures within the FMDV genome and non-structural proteins that play important roles in FMDV RNA replication.
4.1. Determination of RNA sequence required for FMDV VPg uridylylation

4.1.1 A functional AAACA motif of the cre/bus within the FMDV full-length RNA is required for efficient VPg uridylylation

In the previous chapter, a small RNA transcript including the FMDV cre/bus sequence was used as the template in the uridylylation assay. Clearly, this element normally has to function in the context of a full-length (FL) viral RNA and secondly it is possible that uridylylation of VPg may also occur on the poly(A) tail at the 3' terminus of the viral RNA. To investigate this, plasmid pT7S3 (containing a full-length infectious c-DNA of FMDV) and its derivatives with point mutations in the A1, A2 and A3 positions within the AAACA motif of the cre/bus or with complete deletion of the A1A2A3 residues were linearized downstream of the poly(A) tail at a unique HpaI site and used for the production of full-length RNA transcripts as described in the Materials and Methods. The transcripts were purified by phenol extraction and ethanol precipitation and quantified using a spectrophotometer. Truncated transcripts lacking the poly(A) tail or the entire coding sequence were generated in the same way but using templates that had been linearized with EcoNI or XbaI, respectively, as indicated in the figure legends.

Uridylylation reactions were performed using 3Dpol, His-3CD, VPg3, and RNA transcripts corresponding to the wt FL RNA. Efficient uridylylation was observed in the presence of all of these components but omission of any one of them blocked the reaction (FIG.4.1, panel A). A very low level of product was observed in the absence of His-3CD but this was over 100-fold less abundant. When mutant FL transcripts were used in which the cre/bus sequence was modified or deleted, it was found that
modification of the A\textsuperscript{1} position within the AAACA motif of the cre/bus or deletion of A\textsuperscript{1}, A\textsuperscript{2} and A\textsuperscript{3} (as in the creDel transcript) completely blocked activity but a weak signal (about 1% of wt) was observed in the presence of Mg\textsuperscript{2+}, when A\textsuperscript{2} or A\textsuperscript{3} alone were modified (FIG. 4.1, Panel B). The overall pattern of these results closely matches those observed with the short cre/bus transcripts (Chapter 3) but it is apparent that the FL transcripts with A\textsuperscript{2} or A\textsuperscript{3} modified were more active than the short cre/bus transcripts containing these modifications. In the absence of a functional AAACA motif within the FL RNA no uridylylation of the VPg3 occurred indicating that the poly(A) tail was not functional in this assay (FIG. 4.1, Panel B).

RNA transcripts corresponding to the FMDV 5'UTR down to an XbaI site (about 40nt downstream from the cre/bus within the FMDV IRES) were also very efficient as templates for the uridylylation of VPg3. Indeed, since within this assay the various RNA transcripts were used at the same molar concentration, it is apparent that the full-length RNA and the truncated 5'UTR were significantly better templates than the cre/bus element alone (FIG. 4.1, Panel B). In previous studies (Paul et al 2000), it has been shown that the PV 3D\textsuperscript{pol} can generate VPgpU(pU) using a poly(A) template in the presence of Mn\textsuperscript{2+} and this occurs most efficiently in the absence of 3CD. These results were reproduced using PV 3D\textsuperscript{pol} (FIG. 4.1, Panel C). However, in contrast, no significant uridylylation of FMDV VPg3 was observed using FMDV 3D\textsuperscript{pol} with a poly(A) template in the presence of Mg\textsuperscript{2+}, but a very weak signal was observed, on long exposure, in the presence of Mn\textsuperscript{2+} (FIG. 4.1, Panel C).

4.1.2 Context of the RNA template determines uridylylation efficiency.

The above results suggested that 5' UTR RNA transcripts lacking most of the IRES element is about as good a template as the full-length FMDV RNA for VPg.
FIG. 4.1. The cre/bus is required for FMDV 3B3 uridylylation and the context of this structure influences the efficiency of the reaction. Panel A. *In vitro* uridylylation assays were performed with [α-^32^P]-UTP in the presence of Mg^{2+} using the purified components VPg3 (12.5 μM), 3Dpol (1 μM), His-3CD (1 μM) with full-length (FL) wt FMDV RNA transcripts (25 nM) as indicated. Panel B. Uridylylation assays were performed with [α-^32^P]-UTP in the presence of Mg^{2+} using the purified components VPg3 (12.5 μM), 3Dpol (1 μM), His-3CD (1 μM) using the different RNA transcripts indicated. Each transcript was used at the same concentration (25 nM). The full-length (FL) RNA transcripts (wt or mutant as indicated) were derived from HpaI linearized plasmid DNA. Alternatively, truncated transcripts were produced following linearization with EcoNI which removes the 3' UTR plus the coding sequence for the C-terminus of 3Dpol or linearization with XbaI which removes all of the coding sequence plus most of the IRES but the transcript still includes the S-fragment and the cre/bus. The uridylylated VPg (VPgpU(pU)) product is indicated. Panel C. Uridylylation reactions were performed using FMDV or PV components (as indicated, in the absence of 3CD) with poly(A) as the RNA template. Reactions were performed in the presence of Mg^{2+} or Mn^{2+} for 30 min (a) or 60 min (b) as shown.
uridylylation and both were much better template than the cre/bus transcript alone. This interesting observation raised the possibility that larger RNA transcripts improved the uridylylation efficiency. However evidence of a lack of functionality of the polyA tail in the uridylylation assay suggested that the sequences required for optimal VPg uridylylation reside solely in the 5' UTR. In addition, the significant improvement in the uridylylation efficiency using the 5' UTR transcripts lacking the IRES compared to the cre/bus transcripts indicated that RNA sequences within the 5' UTR sequence other than the IRES element are also involved in modulating the uridylylation efficiency (FIG. 4.1, Panel B). In order to investigate the possibility that distinct structures within the FMDV 5' UTR (FIG. 4.2, Panel A) might be required for determining the optimum template efficiency for VPg uridylylation, a number of different RNA transcripts were made as described in the figure legends (FIG. 4.2, Panel B). VPg uridylylation assays using such transcripts showed that the complete FMDV 5' UTR transcripts is better than the FMDV 5' UTR lacking most of the IRES. However an RNA transcript that contains PK+cre+IRES is the best template in the assay and was much better than RNA transcripts that contain only the PK+cre RNA sequence. This result suggests that IRES RNA structure also influences this reaction by enhancing the production of VPgpUpU in vitro. It has been demonstrated earlier that distinct structural domains of the FMDV IRES undergoes long-range RNA interaction in optimum conditions of magnesium and potassium (Ramos and Salas 1999). To investigate the possibility of similar RNA-RNA interactions within the FMDV 5' UTR that might influence FMDV VPg uridylylation, trans stimulation experiments were carried out using two separate RNA transcripts in the same uridylylation assay each at 35 nM concentration. It is very interesting to observe that addition of IRES transcripts in trans stimulated the
VPgpUpU production about ten fold in the presence of PK+cre RNA transcripts (FIG. 4.3, Panel A). However in similar experiments the IRES RNA transcripts could not produce any significant trans stimulation in the presence of the FMDV 5' UTR RNA transcripts lacking most of the IRES RNA sequence except for the first 40 nt (FIG. 4.3, Panel B, Panel C). This finding suggests that either some signals located in the S-fragment or polyC tract or both interfere with RNA-RNA interaction seen between PK+cre and IRES transcripts or the RNA sequence required for interaction between the PK+cre and IRES is determined by the first 40 nt of the IRES element. It was also observed that the IRES could not stimulate significantly in trans when cre/bus alone is used as a template (FIG. 4.3, Panel C). This result showed that the RNA sequence present within the PK+cre+IRES contain almost all the signals required for most optimum FMDV VPg uridylation (FIG. 4.2). Since the IRES has been well characterised to be involved in RNA translation we also suggests that there might be some overlapping sequence present within the IRES that interacts with cre/bus when in the context PK+cre. In addition since the PK+cre is a better template when compared to cre/bus transcripts alone, it indicates that the RNA sequence present immediately upstream (PK) and downstream (IRES) of the cre/bus are involved in stabilisation of the shorter cre/bus transcripts enabling it to act as a better template compared to cre/bus or PK+cre transcripts alone.

4.1.3 The cre/bus derived from a temperature sensitive mutant of FMDV (ts303) is a less efficient template in VPg uridylylation

Previous studies (Tiley et al., 2003) have suggested that a temperature sensitive mutant of FMDV (ts303) could not grow at the non-permissive temperature (42°C), however it has a wild type growth phenotype at 37°C. The lesion that resulted in
FIG. 4.2. The minimal and optimal sequences required for FMDV VPg uridylylation is located within the 5' UTR. Panel A. Secondary and tertiary folding of the FMDV 5' UTR region. Panel B. In vitro uridylylation assays were carried out using VPg3 as a substrate and RNA transcripts of different length from the 5' UTR as depicted in the figure legend. The reactions were carried out for 30 min in presence of Mg^{2+} and the production of VPgpUpU was measured. All the reactions were carried out using the same concentration (35 nM) of RNA including that of the cre/bus transcripts.
FIG. 4.3. RNA sequences within the FMDV 5' UTR complement in trans in VPg uridylylation.

To detect any specific RNA-RNA interaction between FMDV IRES and other secondary structures within the FMDV 5' UTR, that might contribute to the increased efficiency of VPg uridylylation, standard in vitro uridylylation assays were carried out using separate transcripts in equimolar ratio in the same in vitro reaction as shown in the figure legend. The production of VPgpUpU was measured by phosphoroimaging and plotted as bar diagrams.
such a phenotype has been attributed to a single point mutation (C47U) in the stem of the cre/bus. It was suggested that this mutation reduced the stability of the cre/bus compared to the wild type. To test the hypothesis that such a cre/bus template is also weaker in the VPg uridylylation reaction, the same mutation was introduced into the stem of the cre/bus by QuikChange site directed mutagenesis (FIG. 4.4, Panel C) and RNA transcripts were made using the Megascript kit. Uridylylation assays using such mutated transcripts resulted in reduced production of VPgpUpU compared to the wild type cre/bus transcripts at different temperature points under similar in vitro conditions (FIG. 4.4, Panel A and Panel B). The difference in VPg uridylylation is more marked at temperatures above 34°C but the reaction was inhibited for both wild type and mutant cre/bus transcripts at 42°C.

4.1.4 Shorter cre/bus transcripts do not support VPg uridylylation

In human rhinovirus 14 (HRV 14) although the full length cre is about 66 nt, the minimum optimal cre sequence required for VPg uridylylation is 34 nt (Thiviyanathan et al., 2004). To determine whether smaller cre/bus sequence would function in the FMDV VPg uridylylation assay shorter cre/bus (cre37 and cre31) constructs were made by oligonucleotide ligations (see Materials and Methods). The deletions were introduced into the stem of the cre/bus with a few nucleotide changes (C44U in cre/bus37 and U42C, U43C in cre/bus31) as shown in the FIG. 4.5, Panel B and panel C. These changes were designed to stabilize the stem of the cre/bus structure. VPg uridylylation assays using such modified transcripts resulted in very limited production of VPgpUpU in the reaction when compared to the wild type cre/bus (cre 54) (FIG. 4.5, Panel D).
FIG. 4.4. The cre/bus stability determines the efficiency of FMDV VPg3 uridylylation. Panel A. Standard in vitro VPg uridylylation assays were performed in the presence of Mg²⁺ using wild type (wt) cre/bus or mutant (mt)(ts303) cre/bus transcripts at temperatures ranging from 30°C to 42°C. The production of VPgpU₅₅ in the reactions was measured at each temperature point and plotted as a graph. Panel B. Measurement of VPg3 uridylylation using wt and mt cre/bus transcripts at a narrower range of temperature starting from 35.1°C to 42.3°C.
CHAPTER FOUR

ANALYSIS OF FMDV RNA AND PROTEINS

FIG. 4.5. Deletions in cre/bus stem has deleterious effects on VPg uridylylation. Uridylylation assays were carried out using the wild type and modified cre/bus transcripts (cre37/cre31) under similar conditions. The synthesis of VPgpUpU in the reactions were monitored by tris-tricine gel electrophoresis and autoradiography. The precise yield of the VPgpUpU in all the three reactions could not be compared due to the large difference in their uridylylation efficiency (over 100 fold).
4.2 Determination of protein requirements for FMDV VPg uridylylation

As discussed earlier, for PV replication to occur, a complex web of interactions must be established that includes interaction of viral RNA along with both viral and cellular proteins. The importance of the viral protein 3CD in PV RNA replication is well known. However, the properties and function of the PV 3CD and FMDV 3CD are not similar. In FMDV, the 3C protease can carry out all the polyprotein processing and the role of the 3CD in FMDV replication or polyprotein processing is not clear. Therefore, it is interesting to see whether FMDV 3CD and 3C possess similar properties to that of the PV in the uridylylation assay.

To observe the suitability of 3C in FMDV VPg uridylylation instead of 3CD, it was necessary to express the relevant recombinant proteins in the E. coli system. In addition, mutations were introduced within the 3C protein in the predicted RNA binding domain to see the effects on VPg uridylylation and RNA replication.

4.2.1 Expression and purification of FMDV O1K 3C protein

The coding region for FMDV O1K 3C was amplified using a PCR using pQE-3CD(C163G) (described in chapter 3) as a template and inserted into the bacterial expression vector pQE30 (Qiagen). An N-terminal His-tagged form of 3C(C163G) was efficiently expressed from this vector in E. coli M15 and purified using Hi-Trap His-column (Amersham Biosciences) to near homogeneity as judged by coomassie blue staining (FIG. 4.6)
4.2.2 FMDV A10 3C proteins and its derivatives

The plasmid pETm11-3C, that encodes the 3C(C163A) protease from FMDV A1061, was a generous gift from Dr Stephen Curry (Imperial college, London). This plasmid was used as a template to generate modified 3C plasmids with amino acid changes from arginines (R) to serines (S within the purported RNA binding motif of 3C by QuickChange site directed mutagenesis. C-terminal His-tagged form of A1061 3C(C163A) and its derivatives that contain mutations in each of the arginine residues to serines at 92, 95 and 97 positions respectively were efficiently expressed in *E. coli* BL21DE3pLysS as described in Birtley et al., 2005. Purification of the proteins were carried out using Hi-Trap His-column (Amersham Biosciences) to near homogeneity as judged by coomassie blue staining (FIG. 4.6).

4.2.3 FMDV 3C can substitute for FMDV 3CD for the uridylylation of FMDV VPg

In Chapter 3, it was shown that the uridylylation of FMDV VPgs required UTP plus Mg$^{2+}$ (or Mn$^{2+}$) and was also dependent on the presence of each of the following FMDV components: 3Dpol and 3CD plus an RNA template including the *cre/bus*. Recently Pathak et al., 2002 have demonstrated that the role of PV 3CD in this reaction can be achieved by 3C alone. It was decided to determine whether the FMDV 3C protein could replace 3CD in the FMDV VPg uridylylation assay. The results obtained using the *cre/bus* transcript as template showed that the 3C protein derived from both serotype O (O1K) and A (A1061) can each substitute for 3CD in this assay (FIG. 4.7). However the reaction is significantly less efficient than achieved with 3CD (FIG. 4.8). The production of VPgpUpU was steadily increased with increased concentration of 3C protein, albeit at 10μM 3C, the level of VPg.
FIG. 4.6. Purification of the His-tagged 3C proteases by Ni-NTA affinity chromatography.
Proteolitically inactive A10 3C(C163A) and mutant versions of this protein were grown, induced and expressed in BL21 DE3pLysS cells as described by Birtley et al., 2005. O1K version 3C(C163G) protein was expressed in M15 cells. All the proteins were purified using a similar protocol as described for His-tagged 3CD protein in Chapter 3 using Hi-Trap His-tagged column (Amersham biosciences). The purity of the proteins were judged to be more than 90% from the coomassie blue staining.
FIG. 4.7. The factor that contributes to the stimulatory activity of the 3CD in VPg uridylylation resides in 3C. Standard VPg uridylylation assays were carried out (see Materials and Methods) except that the 3CD protein was replaced with 3C protein. To observe if there is any significant differences in the ability to stimulate VPg uridylylation in vitro, 3C(C163A) or 3C(C163G) proteases of both type A10 and type O1K were expressed and purified using similar protocols and equal amount of proteins were used in the assay (1μM-2.5 μM) under similar conditions. Formation of VPgpUpU in vitro was measured by phosphorimaging as shown in the graph above.
FIG. 4.8. 3CD mediated VPg uridylylation in vitro is much more efficient than 3C protein alone. In vitro VPg uridylylation assays were carried out using both 3CD and 3C at different concentrations as depicted in the figure legends either using cre/bus (1 μM) (Panel A) or 5’ UTR RNA transcripts (25 nM) (Panel B) as templates. Reactions were carried out for 30 min at 30°C and production of VPgpUpU was quantified and depicted as bar graphs.
uridylylation was only about 25% of that achieved with 1µM 3CD (FIG. 4.8, Panel A). In contrast when the cre/bus transcripts was replaced by the FMDV 5' UTR as a template the reaction was inhibited with 3C concentration above 1-2 µM (FIG. 4.8, Panel B).

4.2.4 Identification of a putative RNA binding site on the surface of FMDV 3C protein

In certain picornaviruses the ability of the 3C or its precursor 3CD to bind cloverleaf RNA at the 5' end of the genome is well established (Andino et al., 1990, 1993; Kusov et al., 1997; Walker et al., 1995). In PV 3C, the individual residues that contribute to this RNA-protein interaction have been identified (Andino et al, 1993). A consensus motif that includes the amino acid sequence KFR^\_^\_^\_DI^\_\_\_\_^\_ is found to be conserved among enteroviruses, rhinoviruses and hepatovirus (Andino et al., 1993; Mosimann et al., 1997; Bergmann et al., 1997). For the PV 3C protein, residues R84 and I86 within this motif were found to be critical and modification of R84 blocked the ability of the PV 3CD to support VPg uridylylation (Paul et al., 2000). In FMDV the analogous motif is K\_\_\_\_VRDI (A10) or R\_\_\_\_VRDI (O1K and Type C) (FIG. 4.9). In addition among the picornaviruses mentioned excluding the HAV other viruses also showed the presence of a conserved amino acid arginine corresponding to residue 92 of FMDV 3C (R79 in PV) (FIG. 4.10). It should be noted that the arginine residue at the 84 position (R84) in PV corresponds to the arginine residue at position 97(R97) in FMDV. Since the structure of the FMDV 3C protein has been determined recently (Birtley et al., 2005) it is possible to locate this motif and residue R97, in particular, on the surface of 3C. Consistent with observations on the PV and HAV 3C proteins, this motif on the FMDV protein is located on the opposite face of the
FIG.4.9. Sequence alignment of the 3C proteases from three FMDV serotypes.

Multiple sequence alignment of the 3C protein derived from three different serotypes of the FMDV (A, O, C) using DS gene software. The high degree of consensus among the three different serotypes is presented in the figure legend. The solid-line box represents the absolute conserved nature of the predicted RNA binding site of the three different FMDV serotypes.
FIG. 4.10. Sequence alignment of the 3C proteases from multiple picornaviruses. Amino acid sequences of the 3C protease derived from three different FMDV serotypes (O, A, C) and other picornaviruses including enteroviruses (PV), rhinoviruses (HRV 14) and hepatovirus (HAV) were aligned using the programme BIOEDIT. The residues marked with red star were the position where mutations were introduced in the A10 3C expression constructs by QuikChange site-directed mutagenesis (see Materials and Methods). In all cases the positively charged arginine residues were mutated to a neutral amino acid serine.
FIG. 4.11. Three dimensional x-ray crystallographic structure of 3C protease depicting the basic surface opposite to the protease active site. Panel A. Surface representation of the 3C protease coloured by electrostatic potential. The positively charged residues (blue) are indicated with single amino acid letter code. Panel B. Secondary structure showing the positions of the mutated arginine residues to serines on the 3C surface. Panel C. The 90° vertical rotation of the 3C structure in Panel B showing the catalytic triad of the 3C protease just opposite to the predicted RNA binding site. The asterisk C163* represents the mutation in the cysteine to an alanine within the active site of the A10 3C protease.
protein from the protease active site (FIG. 4.11, Panel C). It is apparent that other positively charged residues are close to R97 on the surface of the FMDV 3C, thus residues R92 and R95 together with R97 form a cluster of positively charged residues that may bind to RNA. In addition since the amino acid sequence alignment of three different serotypes of the FMDV showed a high degree of similarity among them (FIG. 4.9) and the A10 3C structure has been solved (FIG. 4.11) we have used the A10 3C protein for further analysis of this protein.

4.2.5 Analysis of putative RNA binding residues in FMDV 3C.

In order to test the role of these basic residues for the uridylylation function, mutations were introduced into the 3C sequence individually to change residues R92, R95 and R97 to an uncharged serine (S) residue in each case. The mutant Histagged 3C proteins were each expressed in *E. coli* and purified to near homogeneity. Equal amounts of each of these proteins were then tested in uridylylation assays in conjunction with 3DPol and either the cre/bus transcript (FIG. 4.12, Panel A) or with the entire FMDV 5'UTR (FIG. 4.12, Panel B). Very similar results were obtained with each template. The R97S mutant was almost completely inactive in these assays, similarly the R92S mutant was also severely defective but a low level of VPg uridylylation was observed. In contrast, the R95S mutant displayed about 25% of wt activity. Competition experiment using such mutant proteins demonstrated that these proteins do not compete with wt 3C protein for VPg uridylylation (FIG. 4.13).

4.2.6 Electrophoretic mobility shift assay (EMSA)

To confirm that the stimulatory properties of the FMDV 3C protein in the VPg uridylylation assay is due to the RNA binding activity, gel shift assays were
FIG. 4.12. Modification of the positively charged arginine residues to serines in 3C protein influences VPg uridylylation. In vitro uridylylation assays were carried out using VPg3 as a substrate in the presence of either 3C(C163A) or 3C(C163A) with mutations in arginines to serines at position 92, 95 and 97 respectively either using cre/bus (Panel A) or 5’ UTR RNA (Panel B) as templates. Equal amount of the proteins were used in each reaction those were detected by western blotting (Panel A). The amount of VPgpUpU synthesized in Panel A and Panel B were quantified and depicted in bar graph.
FIG. 4.13. Mutant 3C proteins do not compete with wt AlO 3C(C163A) protein for VPg uridylylation. To test the ability of the mutant 3C protein (R92S, R95S and R97S) to inhibit wt 3C(C163A), VPg uridylylation assays were carried out using 2.5 μM VPg3 and 2.5 μM 3C(C163A) in all reactions with further addition of 2.5 μM 3C (wt/mut) as indicated in the figure legend. Production of VPgpUpU was measured and showed as a bar diagram.
FIG. 4.14. Electrophoresis mobility shift assay of recombinant 3C(C163A) with FMDV cre/bus.

Panel A. FMDV cre/bus RNA transcripts (100 µM) were incubated with 3C(C163A) (20µM-250µM) at 30°C in reaction conditions similar to that of the in vitro VPg uridylylation assay (see Materials and Methods) for 20 min and run in a 8% TBE-polyacrylamide gel. RNA was stained with ethidium-bromide and visualized using UV light. Arrow on the left represents the free RNA and on the right present the RNA-protein complex. Panel B. Coomassie brilliant blue staining of the ethidium bromide stained polyacrylamide gel to visualize the position of the 3C(C163A) in the gel.
performed to determine the ability of the 3C protein to form complexes with cre/bus transcripts. Essentially reaction conditions of the binding buffer were set similar to that of VPg uridylylation assays containing the cre/bus RNA with 3C(C163A) protein. By Et-Br staining it was found that in the presence of 3C, the mobility of the cre/bus transcript was greatly impaired, consistent with the formation of an RNA/protein complex (FIG. 4.14, Panel A).

4.2.7 Generation of FMDV full-length RNA transcripts with authentic 5' end
To establish whether there is any connection between the ability of the 3C to support VPg uridylylation in vitro and its importance in RNA replication in vivo we introduced the same mutations into the FMDV infectious clone (Materials and Methods). Such mutations were introduced into the infectious cDNA clone pT7S3 and pT7S3-Ribo (contains a hammer head ribozyme upstream of the 5' end of the virion cDNA). Wild type RNA transcripts generated from these clones were electroporated into BHK 21 cells and plaque assay was carried out. A direct comparison of the infectivity of both type of transcripts showed that wild type FMDV RNA transcripts without ribozyme is slightly better than that of the wild type transcripts with ribozyme (FIG. 4.15).

4.2.8 FMDV S-fragment interacts with a cellular protein p48
To detect any interactions between the S-fragment and viral and cellular proteins UV cross linking experiments were carried out (Materials and Methods) using 32P-labelled S-fragment RNA. It was observed that neither the recombinant viral protein (3CD) nor cellular proteins (PCBP1 and PCBP2) showed any evidence of RNP
FIG. 4.15. Plaque assay using pT7S3(wt) and pT7S3Ribo(wt) RNA transcripts in BHK 21 cells. Wt RNA transcripts (with or without ribozyme) were electroporated with BHK 21 cells. After 8 hr incubation the virus was harvested and 200 µl of the neat or diluted virus was added to fresh BHK 21 cells (60%-70% confluent) in soft agar. Staining of the petridishes were carried out after 48 hr incubation at 37°C. The number of plaques at each dilution were counted as above and compared.
FIG. 4.16. Interaction of the S-fragment with viral and cellular proteins. Panel A. UV cross-linking experiments were carried out using radiolabeled S-fragment probe in the presence of either purified recombinant proteins (3CD, PCB1, PCB2) or cell extract (BHK S10) following protocol as described in the Materials and Methods. Reaction samples were mixed with 3X sample loading buffer (BioRad), boiled for 5 min at 75°C and run in a 10% SDS-PAGE gels. The gel was stained with coomassie brilliant blue for 15 min, destained for 30 min and dried for 1 hr at 68°C and gel was exposed overnight for autoradiography. Panel B. The specificity of the UV-cross linking experiment was checked by titrating the competitor t-RNA in the reaction. Panel C, demonstrates the interaction of the radiolabeled S-fragment and PK+cre+IRES probes using cell extracts derived from uninfected (BHK S10, HeLa S10) and FMDV infected BHK extracts.
complex formation (FIG 4.16, Panel A). However FMDV S-fragments specifically interacted with a 48 kDa cellular protein (p48) both in BHK cell extracts and HeLa cell extracts (FIG 4.16, Panel A, Panel B). However a similar interaction was not detected with radiolabelled probe comprising the FMDV pseudoknots, cre/bus and IRES together (PK+cre/bus+IRES) indicating the specificity of this interaction (FIG 4.16. Panel B, Panel C).

4.2.9 3B precursor proteins can be a substrate in the FMDV uridylylation assay
For PV it has been suggested that VPg containing precursor proteins like 3AB, 3ABC, 3ABCD and 3BC could be substrates in the uridylylation assay. To investigate this possibility for FMDV, native protein expression and purifications of the non-structural proteins 3BC123C and 3B3C were carried out using ubiquitin vector system (FIG. 4.17, Panel A) as described in the Chapter 3. Purifications of the untagged version of the 3BC123C and 3B3C were carried out by ion exchange chromatography (Materials and Methods) (FIG. 4.17, Panel B). In vitro uridylylation assays using these purified proteins resulted in efficient uridylylation of these precursor proteins in vitro. It was found that that both the precursor proteins can be uridylylated in vitro in the absence of 3CD (since they each contain 3C), but the reaction was stimulated in its presence (FIG. 4.18, Panel A and Panel B). However it should be noted that uridylylation of the 3BC123C resulted in the formation of some radiolabelled breakdown product that was again enhanced by the presence of the 3CD protein (FIG. 4.18, Panel B). Treatment of the 3BC123C uridylylation assay products with RNase A and Proteinase K resulted in complete disappearance of the breakdown products indicating their protein nature (FIG.4.18, Panel C). At the present time the explanation of this phenomenon is not known.
FIG. 4.17. Expression and purifications of FMDV non-structural precursor proteins. Panel A. Coomassie brilliant blue staining of the untagged recombinant proteins expressed in *E. coli* cells (total cell extracts). Panel B. Purification of the native VPg precursor protein 3B₁₂₃C and 3B₃C. Expression of the native VPg precursor proteins 3B₁₂₃C and 3B₃C were carried out as described for of the native 3Dpol protein in Chapter 3. Purification of the proteins were carried out by ion-exchange chromatography using P11 (phosphocellulose) and High-Trap SP sepharose column. Purity of the proteins were judged by coomassie brilliant blue staining.
FIG. 4.18. Precursor protein 3BC can be a substrate in FMDV uridylylation assay. In vitro uridylylation assays were carried out using VPg precursor proteins 3B\(_{123}3C\) and 3B\(_3\)3C as substrates using \textit{coccid} as a template. Panel A. FMDV uridylylation assays were carried out using varied concentrations of 3B\(_3\)3C with or without 3CD. Panel B. 3B\(_{123}3C\) uridylylation assay in presence or absence of 3CD. Panel C. Depicts the effects of RNase A (lane b) and proteinase K (lane c) in separate in vitro reactions at 37°C on the 2.5 \(\mu\)M 3B\(_{123}3C\) uridylylation products.
4.3 Discussion

4.3.1 The FMDV cre/bus could be the primary template for VPg uridylylation

In the previous chapter the requirements of the FMDV uridylylation assay in vitro were described in which cre/bus acts as a template. However an important question remaining to be answered is how the cre/bus functions in vivo when it is in the context of the full-length FMDV RNA. Within the 5' UTR of the FMDV genome, besides the cre/bus there are other important structures which are present including the S-fragment, internal ribosome entry site (IRES), pseudo knots (PKs) and the polyC tract (FIG. 4.2, Panel A). Therefore at this juncture it could not be ascertained whether the cre/bus alone or any other RNA structure within the 5'UTR participates in RNA replication. Previously it has been suggested for PV that the polyA tail at the 3' end of the genome primes VPg uridylylation internally (Paul et al., 1998). To investigate these possibilities for FMDV, in vitro uridylylation assays were carried out using RNA transcripts with an intact polyA tail but with point mutations in each of the first 3 'A' residues within the AAACA motif of the cre/bus. To our surprise any mutations that debilitated the VPg uridylylation using cre/bus as a template (as in Chapter 3) also showed similar results with the same mutations within the cre/bus in the context of the full-length FMDV RNA. This indicates that the polyA tail is an inefficient template for VPg uridylylation in vitro in the presence of magnesium. This is in agreement with a recent report on poliovirus where mutation in the first 'A' residue within the AAACA motif blocked the production of VPgpUpU using a cell-free reaction (Murray et al., 2003). Therefore we suggest that at least for FMDV, cre/bus is the primary template for VPg uridylylation in vitro and it might be possible that cre/bus dependent VPg uridylylation primes both positive and negative strand synthesis. It was also found that wild type full-length FMDV RNA is a
significantly better template for VPg uridylylation in comparison to cre/bus alone (FIG. 4.1, Panel B).

4.3.2. RNA-RNA interactions in the FMDV 5' non-coding region influence VPg uridylylation

It is noteworthy that the stability of the PV cre as a template also determines the uridylylation efficiency in vitro (Goodfellow et al., 2003). We assume that when the FMDV cre/bus is within the full-length FMDV RNA the overall stability and tertiary folding is significantly influenced by the neighbouring structures either by direct contact with nearby structure or some type of long distance kissing interactions. Since we know that the polyA tail is an insignificant template in VPg uridylylation, the enhanced ability of the FL-FMDV RNA to support VPg uridylylation is most likely determined by the 5' UTR structures. To test this hypothesis a series of truncated FMDV 5' UTR RNA transcripts were used which showed that RNA transcripts encompassing the PK+cre+IRES are the most efficient template in the reaction (FIG. 4.2). This result suggested that neither the S-fragment nor the polyC tract is involved in any sort of stabilization of the cre/bus structure. However it was evident from the RNA trans complementation experiments that RNA-RNA interactions exists among the secondary and tertiary structures of the PK, cre/bus and IRES which supports VPg uridylylation as a single template most efficiently than any other structures present in the 5' UTR (FIG. 4.2, FIG. 4.3). In addition a comparison of the uridylylation efficiency of the cre/bus alone and that of the full-length FMDV RNA and 5' UTR FMDV RNA (FIG. 4.1 and FIG. 4.2) showed that the ability of the FMDV 5' UTR and FMDV full-length RNA to support VPg
uridylylation is very similar suggesting that the optimal sequences for uridylylation reside solely in the 5' UTR.

4.3.3 The FMDV cre/bus derived from a temperature sensitive mutant is defective in VPg uridylylation

In foot and mouth disease virus temperature sensitive mutants have been identified in which the virus could not grow at the non-permissive temperature (42°C). For one mutant (ts303) this defect was attributed to a lesion in the stem of the cre/bus (Tiley et al., 2003). Based on this information we generated the cre/bus transcripts with a mutation at position 47 of the stem from a cytosine residue to a uracil residue (FIG. 4.4). The ability of this transcript to support VPg uridylylation was measured in vitro. It was found that this mutated cre/bus transcript could not support VPg uridylylation to similar extent as that of the wild type cre/bus transcripts under similar conditions of temperature and time. Analysis of the secondary structure of the wild type and mutant FMDV cre/bus RNA sequence using mfold RNA prediction server (http://bioinfo.math.rpi.edu/~mfold) showed that the stability of this mutated cre/bus RNA is reduced because of the mutation (ΔG value -15.2 to -13.5). In addition the effect of this mutation on virus growth was only observed at elevated temperature in vivo but not at the permissive temperature of 37°C. Our in vitro data suggested that the defects in VPg uridylylation in vitro using mutated cre/bus as a transcript was observed throughout the temperature range of 30°C to 42°C (FIG. 4.4, Panel B). The difference in sensitivities between the in vivo data and in vitro data could be attributed to the relative stability of the cre/bus transcripts alone in vitro compared to that of the cre/bus element in the context of full-length FMDV RNA. These results suggested that the temperature dependence of the cre/bus
in VPg uridylylation corresponds to the stability of the cre/bus structure. It is also being reported that the stem of the PV cre provides the stability to the loop for VPg uridylylation (Goodfellow et al., 2003). Recently it has been shown that although HRV14 cre is about 66nt in length the minimal functional sequence required for VPg uridylylation in vitro is 34 nt (Thiviyanathan et al., 2004). To explore the minimal sequence for FMDV cre/bus we generated two shorter version of the cre/bus with 31 and 37 nt respectively. In vitro uridylylation assay using these shorter cre/bus transcripts resulted in very little VPg uridylylation compared to the wild type cre/bus (54 nt) (FIG. 4.5). This suggests that the minimal optimal sequence for VPg uridylylation in FMDV is more than 37 nucleotides.

4.3.4 The FMDV 3C protein can replace 3CD in VPg uridylylation assay

We have shown in the previous chapter that 3CD is a pre-requisite for the VPg uridylylation in vitro. Earlier studies with PV have shown that the RNA binding activity of the 3C domain within the 3CD is responsible for the stimulation of the VPg uridylylation (Pathak et al., 2002). To explore the possibility that FMDV 3C also possesses similar properties, we replaced the FMDV O1K 3CD with 3C proteins of A10 and O1K serotypes in the uridylylation assay. We found that 3C alone can replace the 3CD in the uridylylation assay (FIG. 4.7) but it is less efficient than 3CD (FIG. 4.8). In addition we also found that the O1K and A10 3C protein possess similar activity in the uridylylation assay. A titration experiment showed that even at 10 μM 3C concentration, efficiency of VPg uridylylation was only about 25% of the level observed in the presence of 1 μM 3CD (FIG. 4.8, Panel A). This suggests that 3CD is more specific for the uridylylation of the VPg than the 3C alone. However there was a sharp contrast in the extent of stimulation of VPg uridylylation using the
3C protein with respect to RNA template. There was steady stimulation of VPg uridylylation by 3C in the presence of cre/bus as template, whereas the reaction was inhibited beyond 1 μM 3C in the presence of the 5' UTR (FIG. 4.8, Panel B). This phenomenon could be explained on the basis that there was a great difference in the concentration of RNA in the two systems. 5' UTR transcripts were used 40 times lower compared to the cre/bus transcripts in similar assays. It is possible that the excess 3C protein to template ratio could block the accessibility of the template thereby causing non-specific inhibition of the reaction.

4.3.5 The RNA binding activity of the FMDV 3C protease could be responsible for stimulatory effects in VPg uridylylation

Recently the crystal structure of the FMDV (A10) 3C protease has been solved (Birtley et al., 2005) which showed a region of the 3C that is rich in positively charged amino acids on the opposite face from the protease active site (FIG. 4.11). This region was hypothesized to be the RNA binding region of the FMDV 3C protein. Based upon the sequence alignment with other picornaviruses (FIG. 4.10) we predicted that arginine residues at position 92(R92), 95(R95) and 97(R97) might be involved in RNA binding properties of the FMDV 3C protein. We mutated the positively charged arginine residues at positions 92, 95 and 97 to serine residues. Replacement of the wild type 3C protein with mutant proteins resulted in varied degrees of VPg uridylylation. Substitution of the arginines at 97 and 92 almost completely inhibited the reaction; whereas mutation in the arginine 95 residue resulted in 75% reduction in VPg uridylylation (FIG. 4.12). Since it has been suggested that the RNA binding activity of the 3C contributes to the stimulation of uridylylation, we explored this possibility by electrophoretic mobility shift assay
(EMSA). Initial experiments suggest that wild type 3C protein binds to the cre/bus in a dose dependent manner (FIG. 4.14). The binding activity of the mutant 3C proteins is the focus of current investigation. In addition, studies on PV have shown that modifications of the 3C protein at the arginine residue at position 84 which corresponds to the FMDV arginine 97 inhibits RNA replication by disrupting the ternary complex formation between clover leaf and 3CD protein (Andino et al., 1990, 1993). To date no such studies has been carried out to find out the role of 3C or 3CD in FMDV RNA replication. Furthermore a defined role of the S-fragment in FMDV RNA replication has not yet been established.

The formation of a ribonucleoprotein complex between 3CD and cloverleaf RNA of poliovirus and its significance in RNA replication is long established (Andino et al., 1990). In addition there is strong evidence of linkage between the ability of the cre to support VPg uridylylation in vitro and its effects on RNA replication (Yang et al., 2002). At this juncture we have little information regarding either the biological relevance of the RNA binding activity of the FMDV 3C/3CD in RNA replication or formation of any ribonucleoprotein complex formation between the FMDV 3C/3CD to any RNA structure at the 5' UTR. Based upon the band-shift assay (FIG. 4.14) we assume that the RNA binding activity of the 3C is also required for FMDV VPg uridylylation. However we do know that mutations in the arginine residues in 3C at 92, 95 and 97 positions affect FMDV VPg uridylylation, thus it may be expected that such mutations will affect RNA replication. Hence it makes sense to carry out further experiments to elucidate the role of 3C/3CD in FMDV RNA replication by introducing these mutations into the full-length FMDV infectious clone and analysing their effects on RNA replication by introducing such full-length modified
RNA into BHK cells. It is well established that transcripts generated by in vitro transcription reaction using T7 RNA polymerase adds two non-viral GG residues at the beginning of the transcripts that delays the onset of RNA replication in transfected cells until the ends are corrected (Herold and Andino 2001). To avoid such problems we introduced sequence encoding a ribozyme into the infectious cDNA clone of the FMDV and a direct comparison was carried out using transcripts with or without ribozyme. Unexpectedly we found the transcripts generated in vitro without ribozyme was slightly better when compared to transcripts with the ribozyme with respect to the number of plaques formed on BHK cells under identical condition (FIG. 4.15). Similar observations were made during the course of development of a cell-free replication system for FMDV using BHK 21 extracts (Data not shown). This discrepancy might be attributed to the fact that the in vitro condition at present for RNA transcription may not be ideal for the optimal cleavage of the ribozyme from the 5’ end of the FMDV transcripts.

4.3.6 The FMDV S-fragment interacts with a cellular protein (p48)

We have suggested earlier (see Introduction) that if PV and FMDV follow a similar replication strategy then the FMDV 3C/3CD may be expected to interact with the S-fragment, which is considered to be the counterpart of the cloverleaf RNA to form a ternary ribonucleo-protein complex. To investigate this hypothesis we carried out the UV-cross linking experiment using $^{32}$P-labelled S-fragment probe in the presence of both cellular and purified recombinant proteins including FMDV 3CD, PCBP1, PCBP2 and BHK and HeLa cell extracts. It was found that S-fragment does not participate in the formation of RNP complex either with viral protein 3CD, or with the cellular proteins PCBP1 and PCBP2 (FIG. 4.16, Panel A). These findings
suggested that FMDV S-fragment did not interact with any of those proteins stated above individually. It might be possible that 3C or 3CD together with other viral or cellular proteins interacts with the FMDV S-fragment. Since FMDV replicates in BHK 21 cells and all positive strand RNA viruses utilizes host components for RNA replication it was quite obvious to try out the BHK cell extracts to detect any RNA-protein interactions. It was observed that the S-fragment specifically interacts with a cellular protein p48 (ca.48 kDa) even in the presence of competitor RNA (FIG. 4.16, Panel B). To exclude the possibility that this interaction might be due to a RNA binding protein La (an autoantigen required for translation of many positive strand RNA viruses including PV and HCV which has a general RNA binding activity) we used another radiolabelled probe that contains the FMDV PK+cre+ IRES sequence. UV cross linking with such a probe resulted in interaction with a much larger protein (~ 100 kDa) but not the p48 (FIG. 4.16, Panel C). Similar observations were also made using HeLa cell extracts and infected BHK 21 cells extracts (FIG. 4.16, Panel C). The identity of this protein (p48) remains to be elucidated.

4.3.7 Non-structural precursor protein 3BC is a good substrate in FMDV uridylylation assay

In this chapter we have also explored the possibility of using non-structural precursor proteins as a substrate for FMDV uridylylation assay. It has long been believed that either 3AB or 3A containing precursors are the source of VPg for uridylylation. Since 3A and 3AB both remain attached to the membrane (Towner et al., 1996) it is likely that 3AB undergoes cleavage in the presence of 3CD to produce VPg molecules for primer formation required for both negative and positive strand synthesis. It is also possible that the uncleaved form of the 3B and 3C (3BC) might
also acts as a substrate for VPg uridylylation in the presence of 3D. It was found that both forms of the 3BC precursor proteins either \( 3B_{123}3C \) or \( 3B_{3}3C \) are good substrates for VPg uridylylation with or without the presence of 3CD (FIG. 4.18).
Characterisation of foot and mouth disease virus replication complexes (RCs) in vitro

All positive strand RNA viruses replicate inside the cells in association with cytoplasmic membranes (Salonen et al., 2005). Previous studies on poliovirus (Takeda et al., 1986) and recent studies on other positive strand RNA viruses like hepatitis C virus (HCV) (Ali et al., 2003; El-Hage and Luo 2003) and feline calicivirus (FCV) (Green et al., 2002) have also shown a requirement for membrane associated replication complexes (RCs) in RNA replication. To date no such studies have been undertaken to characterise the replication complexes in FMDV infected cells in vitro. In addition, a cell-free system capable of replicating FMDV RNA in vitro has not been developed so far. Such a system could greatly contribute to our understanding of the RNA replication mechanism of FMDV. It would be possible to analyse individual steps in RNA replication and clarify some of the other individual processes like the requirement for replication factors and also the effects of inhibitors of RNA replication like guanidine in vitro. In the present studies we have been able to develop an in vitro RNA replication assay system using a membrane fraction from FMDV infected BHK 21 cells that faithfully synthesizes full length FMDV RNA molecules along with intermediate RNA species.

5.1 Features of the FMDV in vitro replication assay system

Based upon the observations with some positive strand RNA viruses that a membrane fraction obtained from cells infected with these viruses is fully capable of synthesizing virion RNA in vitro (Takeda et al., 1986; Green et al., 2002; Ali et al., 2003) we envisaged developing a homologous system for FMDV. Like other
positive strand RNA viruses, FMDV assembles replication complexes using membranes derived from the vesicle structures inside the cytoplasm (Monaghan et al., 2004). In this context we have prepared a post nuclear, crude membrane fraction from FMDV infected BHK21 cells (see Materials and Methods) which was supplemented with an ATP regenerating system using creatine phosphate as a substrate and creatine phosphokinase (CPK from rabbit muscle) as an enzyme. Addition of all the 4 rNTPs along with $^{32}$P-aUTP into the system resulted in synthesis of FMDV RNA that included single stranded RNA (ss RNA), double stranded RNA replicative form (dsRNA RF) and partially double stranded replicative intermediate (dsRNA RI) molecules (FIG. 5.1, Panel B). To observe the effects of guanidine on RNA synthesis, BHK21 cells were treated with this inhibitor (10mM concentration) prior to virus infection and crude membrane fractions obtained from these cells were incapable of synthesizing RNA (FIG. 5.1, Panel B). Ethidium-bromide staining of RNA isolated from the in vitro RNA replication assay showed that RNA synthesis was only observed when the BHK cells were infected with virus in the absence of guanidine (FIG. 5.1, Panel A). In all cases the membrane fraction was found to be associated with similar levels of 18S and 28S ribosomal RNA.

5.2 Association of FMDV specific structural and non-structural polypeptides with the crude replication complexes

In poliovirus infected cells, RNA translation, vesicle assembly and RNA replication are coupled processes (Egger et al., 2000). Similarly we may expect that all the non-structural proteins required for FMDV RNA replication and the structural proteins required for virus capsid assembly will also be present in the FMDV crude replication complexes (CRCs). In order to investigate this possibility we analysed for
FIG. 5.1. RNA synthesis from membrane fraction of FMDV infected BHK cells. In vitro RNA replication assays were carried out using RC isolated from mock, guanidine treated and FMDV infected BHK cells using $^{32}$P-αUTP at 30°C. Panel A. RNA synthesized in this assay was extracted using phenol:chloroform:isoamyl alcohol and analysed by ethidium-bromide staining using a 1% native agarose gel. The first lane contains in vitro transcribed full-length (FL) viral RNA as a marker. Second and third lanes contain RNA synthesized from mock and FMDV infected BHK cells with prior treatment with guanidine hydrochloride (10 mM) respectively. Lane four shows the synthesis of FL FMDV RNA within the RC. 18S and 28S r-RNA appeared in all lanes as indicated. However presence of the FL FMDV RNA was only observed in the fourth lane. Panel B. Ethidium-bromide stained gel was dried under vacuum without heat for 30 min followed by drying at 60°C for an hour on the gel dryer. The dried gel was exposed to x-ray film for 1hr and image was developed using a Compact X4 X-Ograph.
the presence of both non-structural and structural proteins in these replication complexes isolated at different time points post infection. Western blot analysis of these membrane fractions using anti-3C, anti-3D monoclonal antibody revealed that non-structural precursor polypeptides appeared at around 2hr post infection (FIG. 5.2, Panel A and Panel B) and by 3hr most of the non-structural proteins including both precursor and mature proteins were present (FIG. 5.2, Panel A and Panel B). As expected the membrane preparations from mock infected and guanidine treated FMDV infected cells did not show any detectable protein synthesis in western blot (FIG. 5.2, Panel A and Panel B). Similar experiments using polyclonal serum raised against the capsid protein of O1K virus also revealed the appearance of the structural proteins in the CRCs isolated from FMDV infected cells (FIG. 5.3).

5.3 Synthesis of peptide primer VPgpUpU within the CRCs

In picornavirus RNA replication, synthesis of the peptide primer VPgpUpU is the primary step in both positive and negative strand synthesis. To analyse whether true RNA replication initiation takes place within these replication complexes, in vitro replication assay was carried out as described in the Materials and Methods. VPgpUpU synthesis within these replication complexes was analysed using methods described by Vartapetian et al., (1984) (see Materials and Methods). Earlier work using poliovirus has suggested that VPg bound to the short oligonucleotides partitioned to the organic phenol phase, whereas the virion RNA is sequestered to the aqueous phase (Ambrose and Baltimore 1978; Vartapetian et al., 1982). Our preliminary experiments using FMDV CRCs in the RNA replication assay showed similar results with two $^{32}$P-$\alpha$UTP labelled products were being present in the phenol phase (FIG. 5.4) which are interpreted as VPgpU(pU).
FIG. 5.2. Analysis of non-structural proteins associated with RC isolated from mock and FMDV infected BHK cells with anti-3C Mab. Panel A. Monoclonal antibody raised against the 3C protein was used to detect the RC proteins during the course of FMDV infection. The appearance of 3C along with its precursors 3BC, 3CD and larger precursors are marked by arrows. A non-specific band of approximately 50 kDa appears in every lane irrespective of virus infection. The values in the left are molecular sizes in kilodalton. Panel B. The same sample were analysed using a monoclonal antibody raised against the 3D polymerase. The appearance of 3D and its precursor 3CD and larger 3D containing precursors are indicated.
FIG. 5.3. Analysis of structural proteins associated with the RC from mock and FMDV infected BHK cells. Proteins synthesized during the course of in vitro assays were transferred to a nitrocellulose membrane and analysed using guinea pig serum raised against FMDV. Appearance of the VP3, VP2/VP1 and VP0 was indicated by an arrow.
FIG. 5.4. De novo synthesis of VPgpUpU in the FMDV crude replication complexes (CRCs). Standard in vitro RNA replication assays were performed as described previously. The samples were solubilised with SDS (final concentration of 1%) and extracted with chloroform/methanol (2:1, v/v). The aqueous phase was again extracted with phenol/chloroform:isoamyl alcohol (25:24:1). Products in the organic phenol phase were precipitated with 4 volume of acetone and production in the aqueous phase were precipitated with 2.5 volume of ethanol. The samples were dissolved in tris-tricine dye and analysed using 14% tris-tricine gel electrophoresis.
5.4 FMDV RNA synthesis in crude replication complexes (CRCs)

The ability of the FMDV CRCs to synthesize RNA was analysed by isolating the membrane fractions from the FMDV infected BHK21 cells at different times post infection starting from 1hr to 3.5 hrs. In vitro RNA replication assays were performed (see Materials and Methods) to detect the synthesis of the viral RNA. It was found that CRCs isolated as early as 2hr post infection (FIG. 5.5) were sufficiently active enzymatically to synthesize full-length virion RNA, which closely matched the results of the western blot analysis with appearance of the replication proteins like 3D polymerase (FIG. 5.2). However CRCs isolated at 3 hr post infection were most active in synthesizing virion RNA (FIG. 5.5) when there is greatest abundance of the mature and precursor forms of the non-structural proteins (FIG. 5.2). The 3.5 hr CRCs were also active in synthesizing virion RNA though less efficiently compared to the 3hr CRCs.

5.5 Time course of RNA synthesis in CRCs

The accumulation of FMDV RNA in the replication complexes was analysed by using the 3hr post infection membrane fractions in an in vitro RNA replication assay. A master mix reaction was assembled and aliquoted in to 7 tubes for 7 different time points. At each time point the reaction was stopped by adding the TENSK solution (see Appendix II). Analysis of the RNA extracted from these replication complexes showed that viral RNA synthesis could be detected as early as 5 min (FIG. 5.6) and gradually increased until it reaches maximum at 1hr time point (FIG. 5.6).
FIG. 5.5. Analysis of RNA synthesis in RC isolated from FMDV infected BHK cells at different time points. BHK cells were infected with O1 BFS virus at high MOI to observe more than 95% of cell infection. Replication complexes (RCs) present in the membrane fraction were isolated at 1hr, 2hr, 3hr and 3.5 hr post-infection. The ability of these RC to synthesise full length (FL) virion RNA were assessed by in vitro RNA replication assay. Newly synthesized RNA were extracted using phenol: chloroform: isoamyl alcohol followed by ethanol precipitation. Subsequently the RNA pellets were dissolved in RNA storage solution (Ambion). 1/10th of the aliquot was treated with non-denaturing orange dye and run on a 1% native agarose gel at 80 volts for 3hrs. The gel was dried for 30 min under vacuum without heat followed by drying at 60°C for another hour. The dried gel was exposed to Biorad X-ray film for approximately 1hr and image was developed using Compact X4 X-Ograph.
FIG. 5.6. Time course of RNA synthesis in vitro using 3hr replication complexes. RNA replication assays were carried out in seven individual tubes at 30°C for the indicated times. RNA was extracted from each tube and run on a 1% native agarose gel. The gel was dried and exposed to film.
5.6 Effect of guanidine on RNA synthesis in vitro

To determine the effects of the guanidine in vitro, this inhibitor (10mM) was added to the reaction mixture at the beginning of the RNA synthesis. The idea was to detect the action of the guanidine at the very early stage of the initiation of viral RNA synthesis within these replication complexes to observe the effects on negative strand synthesis. For this, CRCs isolated from BHK 21 cell infected with FMDV in the absence of guanidine at different time point were used in the reaction either with or without guanidine. Earlier it has been reported using poliovirus that guanidine inhibits the synthesis of negative strands in a reaction using pre-initiation replication complexes (PIRC) isolated from a cell-free in vitro translation-replication reaction. However guanidine did not have any effects on positive strand synthesis (Barton and Flanagan 1997). In FMDV the reaction mixtures containing guanidine were partially inhibited in their ability to support RNA synthesis (approximately 50%) compared to CRCs without guanidine (FIG. 5.7). This suggested that within this system true initiation of negative strands occurred.

5.7 Analysis of RNA species synthesized within the FMDV CRCs

To determine the nature of the RNA species synthesized within the FMDV CRCs, total RNA was isolated from the in vitro replication assay by phenol extraction and ethanol precipitation and subsequently treated with RNase A under high salt buffer conditions. High salt buffer conditions are supposed to keep the double stranded RNA intact (and hence resistant to RNase A) compared to the low salt buffer condition that allows the relaxation of the double stranded RNA interaction. Analysis of the RNA sample in a native agarose gel after the digestion showed that the single stranded RNA species is completely degraded by RNase A, whereas the
FIG. 5. Effects of guanidine hydrochloride on RNA synthesis in vitro. To observe the effect of guanidine hydrochloride on RNA synthesis, in vitro RNA replication assays were carried out using RC isolated from FMDV-infected BHK cells at different time points. In all reactions the final concentration of the Gu-HCl was 10mM. Reactions were carried out for a period of 1hr at 30°C with or without Gu-HCl as indicated and stopped by adding TENS buffer and the RNA was extracted. 1/10<sup>th</sup> of the total aliquot of the RNA was run on a 1% native gel. Subsequently the gel was dried and exposed to film.
FIG. 5.8. Double stranded RNA species are synthesized in the RC. RNA replication assays were performed, the product RNA were extracted with phenol-chloroform-isooamyl alcohol and precipitated with ethanol. One quarter of the isolated RNA was kept untreated and the rest of the sample were digested with 0.2 μg of RNase A per ml in 0.3 M NaCl for 15 min at 23°C. The RNA samples were separated by gel electrophoresis using a native 1% TBE-agarose gel.
double stranded RNA (RF) along with some of the replicative intermediate (RI) RNA species are resistant (FIG. 5.8).

5.8 All RNA species synthesized within the FMDV CRCs are protected from RNase A

To determine whether FMDV RCs remained inside or outside of the vesicle membrane structure, membrane fractions containing CRCs were treated with RNase A either with or without detergent treatment. As indicated in FIG. 5.9, in the presence of detergent TritonX-100, the RNA was completely accessible to RNase A thus this treatment resulted in complete digestion of the bound RNA within the replication complexes. However in the absence of TritonX-100 nearly all RNA was protected indicating that the RNA synthesized within the replication complex was tightly bound inside the membrane vesicles (FIG. 5.9).
FIG. 5.9. Effects of Triton X-100 on RNA synthesis in vitro. In vitro RNA replication assays were carried out for one hour as described for FIG. 5.8. The total reaction mixture was spun down for 3 min at maximum speed. The supernatant containing un-incorporated nucleotide was discarded. The remaining membranous pellet was resuspended in 40 µl of RNase A digestion buffer by gentle pipetting. The resuspended pellet was treated with or without Triton X-100 as indicated and RNase A to a final concentration of 0.1% and 1 mg/ml respectively. The reaction mixtures were incubated at 37°C for 30 min. RNA was extracted by Trizol method. 1/10th aliquot of the total RNA isolated was run in a native agarose gel, dried and exposed to a x-ray film.
5.10 Discussion

The studies that have been performed here show for the first time the complete RNA replication events in vitro within the crude replication complexes (CRCs) isolated from FMDV infected BHK21 cells. These events include the formation of the functional peptide primer VPgpUpU and subsequent positive and negative strand synthesis. In the absence of an authentic cell free replication system for FMDV, this system mimics the replication events inside the cells and allows the dissection of individual steps in RNA replication. Usually an authentic cell free system is supplied with a saturating amount of virion RNA to initiate the complete replication cycle in vitro. However the current system described in this chapter utilises both de novo peptide primer (VPgpUpU) and endogenous virion RNA as a source of template RNA to initiate the replication cycle.

5.10.1 The production of VPgpUpU in vitro suggests true initiation of RNA synthesis

It has been demonstrated that FMDV is capable of utilising all the three copies of VPg/3B peptides as primer for RNA replication. This was achieved in vitro using defined purified components as described in Chapter 3. As a matter of fact, the CRCs contain all the replication protein required for RNA replication including VPg, 3Dpol, and 3CD. The presence within these CRCs of these non-structural proteins was established by western blot analysis of the membrane fraction using specific monoclonal antibodies directed against 3C and 3D protein (FIG. 5.2). So it should be able to synthesize the VPgpUpU using endogenous RNA template and VPg/3B peptide without any exogenous substrate. Indeed it was found that supplementation of these CRCs with an ATP generating system resulted in true synthesis of
VPgpUpU in vitro though in limited quantities (FIG. 5.4). However due to unavailability of specific antibodies against FMDV VPgl, VPg2 and VPg3 true identification of the copies of VPg that were uridylylated could not be ascertained.

5.10.2 The FMDV in vitro RNA replication assay support positive and negative strand synthesis

In the case of a complete RNA replication cycle within the cells negative strand synthesis follows VPgpUpU synthesis and subsequently the negative strand acts as template for the synthesis of positive strands. Previous studies have shown that within this system the majority of the RNA strands synthesized are of positive polarity (Takegami et al., 1983). We also suggest that most of the RNA synthesized in the FMDV in vitro replication assay could be of run-off transcripts but de novo initiation of positive and negative strand synthesis could not be ruled out. In the present system the experiment we have conducted is not capable of determining the polarity of RNA synthesis within this system. However evidence that supported the true initiation of negative strand synthesis within this system was obtained and these could have been used as template for positive strand synthesis. The use of guanidine, a reversible inhibitor of picornavirus RNA replication, in this system resulted in approximately 50% reduction in RNA synthesis suggesting that there are true events of initiation of negative strand synthesis. The fact that guanidine does not have any effects either on positive strand synthesis or elongation of the already initiated nascent negative strand synthesis support our conclusion that de novo initiation of negative and positive strand synthesis does occur in this system. Time course experiments of the 3 hr CRCs showed that RNA synthesis could be detected as early as 5 min with the appearance of partially double stranded RNA molecules and
mature single stranded RNA molecules (FIG. 5.6). The RNA synthesis reached a maximum at about 1 hr. We assume that within this time period there is initiation of negative strand synthesis that lead to appearance of RI and RF and mature positive strand synthesis. In fact we validated the presence of RI and RF by treating the total RNA isolated from these replication complexes with RNase A. As expected all the single stranded RNA molecules were completely degraded whereas all the double stranded (RI) and partially double stranded was resistant (FIG. 5.8).

5.10.3 The FMDV CRCs contain both structural and non-structural proteins

Analysis of the protein components of the CRCs showed the presence of non-structural proteins, both mature and precursor polypeptides, and also the presence of all the capsid proteins. This suggests that assembly of provirions and mature virions also takes place within the replication complexes once the positive sense progeny virion RNA molecules are formed. One of the interesting observations is the detection of 3BC protein in the membrane preparation with 3C monoclonal antibody which has been found earlier to be a precursor protein that undergoes uridylylation to form 3BCpUpU (Chapter 4). At this stage we cannot say whether this uridylylated precursor is a cleavage product of the 3ABC that is subsequently auto-processed to generate VPgpUpU, the true primer for RNA replication. Another important observation is, by 3.5 hrs of infection in the BHK21 cells, the structural capsid protein synthesis is at maximum (FIG. 5.3), this may explain the reduced RNA synthesis from the 3.5 hr CRCs preparation (FIG. 5.5) compared to the 3hr replication complex (FIG. 5.5). It appears that at three hours post infection the RNA replication is at a peak but by 3.5 hr the RNA production has decreased.
5.10.4 The synthesis of FMDV RNA takes place inside of the replication complexes

Studies conducted on poliovirus have shown that replication-complex associated membrane structures closely protect the replication intermediates (RI) rendering them resistant to the action of RNase A. However, the single stranded mature RNA molecules are located outside of the replication complex and are susceptible to the action of RNase A (Bienz et al., 1992). In contrast, similar studies on hepatitis C virus (HCV) have suggested that the majority of the RNA remained protected against the action of the RNase A in the absence of detergent within the replication complexes (El-Hage and Luo., 2003). Addition of detergent resulted in solubilization of the replication complexes both with PV and HCV thereby rendering them susceptible to the action of RNase A (Bienz et al., 1992; El-Hage and Luo 2003). Our present work using FMDV CRCs showed that the single stranded, partially double stranded (RI) and double stranded RNA (RF) species synthesized within this system are completely resistant to the action of the RNase A in the absence of detergent TritonX 100 (FIG 5.9). High resolution autoradiography and in situ localisation studies have suggested that PV RNA synthesis proceeds on the outer surface of the vesicles and both positive and negative strand RNA are found to be attached to the vesicular membrane (Bienz et al., 1987; Bolten et al., 1998). To date no such studies in FMDV have been carried out. Such studies might help in understanding the location of the RNA synthesis within the vesicular structures in the FMDV infected cells and may shed light into the completely resistant nature of the FMDV RNA.
In summary, the ability of this system to support de novo synthesis of VPgpUpU followed by both positive and negative strands synthesis is promising with respect to studying FMDV replication in vitro. This information should contribute to the development of a FMDV cell free system using RNA from infectious cDNA clones to recover infectious virions which will help in dissecting individual steps in FMDV RNA replication in vitro.
CHAPTER SIX

FINAL DISCUSSION

Foot and mouth disease is probably the most contagious disease of cloven footed livestock and wild mammals. The disease has the potential to spread very quickly with severe economic consequences. The replication of the virus is remarkably efficient and one of the most important aspects of FMDV is its ability to infect a wide range of hosts. In the recent past a great deal of importance has been given to looking at the epidemiology, pathogenesis and vaccine development. Very little attention has been paid to study the replication mechanism of the virus. In this project the emphasis has been to look specifically at the mechanism of replication at the molecular level.

In the picornavirus lifecycle during the course of RNA replication both positive and negative strands are linked to the viral encoded peptide VPg (Paul 2002). In addition, in poliovirus infected cells, uridine linked VPg (VPgpUpU) is found in the cytoplasm (Ambrose and Baltimore 1978). This observation led to the impression that VPg peptide acts as a primer for both positive and negative strand synthesis. However only recently this became clearer when Paul et al (1998) showed, for the first time, that VPg peptide can be linked to uridine residues (a process called uridylylation) in vitro by the PV polymerase in the presence of poly(A) template. Subsequently it was shown that the cis-acting replication element (cre) acts as a better template for VPg uridylylation which provided further validation for the role of the cre in RNA replication in vivo. In 2002, Mason et al demonstrated that for FMDV the cre is located in the 5' non-coding region. No further work has been pursued since to elucidate the role of cre in priming the replication mechanism. In
this chapter the replication mechanism of the FMDV RNA will be discussed including the identification of non-structural proteins and RNA elements that play important roles in FMDV replication along with the characterisation of the structural and functional aspects of the replication complexes (RCs) that provide a platform for replication to occur.

6.1 VPg uridylylation and RNA polymerisation are two distinct functions of the FMDV polymerase enzyme (3Dpol)

Previous work with FMDV has shown that all the three copies of the FMDV VPgs, which are non-identical to each other, can be linked to the 5' end of the RNA at almost equal frequency (King et al., 1980). Therefore it appears that all the three copies of VPg must be able to form phosphodiester bond with the terminal uridine residue of the viral RNA. It also generates the question whether all the three copies of VPg can be uridylylated in vitro with equal efficiency. To address these questions we developed an in vitro assay that measures the production of uridylylated form of the VPg peptide (VPgpUpU) using an un-tagged FMDV polymerase enzyme. It is interesting to mention that picornavirus polymerases constitute a class of polymerase in which the N-terminal segment forms an important component of the active site of the enzyme. It is now known that in the unmodified polymerase enzyme the N-terminus is buried in a pocket that encircles the active site (Hobson et al., 2001; Ferrer-orta et al., 2004). This interaction of the N-terminus with the active site determines the polymerisation activity. This is very much consistent with our observation that the N-terminal His-tagged version of the FMDV polymerase lacks the ability to uridylylate VPg in vitro. Whereas using an unmodified polymerase
enzyme uridylylated VPg was produced very efficiently under identical conditions. It is noteworthy that although the N-terminal His-tagged polymerase enzyme is devoid of the uridylylation activity it still retains some RNA elongation activity. The native polymerase enzyme differed hugely in polymerization activity in response to the presence of divalent metal ions (Mg$^{2+}$/Mn$^{2+}$) in the polyU polymerisation reaction. The same enzyme showed no significance difference in the uridylylation ability in the presence of these different cations. These findings suggest that the RNA polymerisation activity and uridylylation activity are two distinct functions of the enzyme perhaps contributed by different domains of the polymerase enzyme. The uridylylation activity is more likely to be dependent on additional interactions with other cofactors (eg. 3C) along with the catalytic activity of the enzyme.

6.2 Importance of VPg in RNA replication

In the FMDV uridylylation assay it was established that all the three copies of VPg can be uridylylated efficiently, though VPg3 was found to be the best substrate. Surprisingly, although PV VPg has little similarity with the three FMDV VPgs, it was still efficiently recognised as a substrate in the FMDV uridylylation system. In contrast, using the PV uridylylation system it was not possible to replace the PV VPg with FMDV VPg. This phenomenon was explained on the basis of the presence of negatively charged glutamic acids in the FMDV VPgs. Therefore it can be suggested that the overall charge of the VPg is also crucial for polymerase recognition and VPg-polymerase complex formation. It also makes sense to argue that amongst the VPgs, VPg3 is the best recognised substrate with formation of most stable complex with FMDV polymerase. This might be linked with the genetic evolution of the virus.
in which VPG3 is the closest in proximity to 3C and 3D the two essential viral proteins for VPg uridylylation and viral RNA replication. Therefore the earlier report of the loss of viability (Falk et al., 1992) of the virus with the deletion of the VPG3 in the viral genome which was explained as a consequence of a defect in polyprotein processing may also have resulted from the lack of primer function.

6.3 Precursor proteins could be a source of VPg for RNA replication

In all picornaviruses, polyprotein processing generates mature and precursor polypeptides that serve distinct functions in the virus life cycle (Leong et al., 2002). One of the important questions that arises is whether VPg needs to be cleaved from its precursor for uridylylation or whether the precursor form can first undergo uridylylation and then subsequent cleavage to liberate the uridylylated form of the VPg. It has long been suggested that 3AB might serve this function by acting as a source of VPg (Towner et al., 1996). 3CD brought about the cleavage of the 3AB thereby releasing 3B for uridylylation. We have shown for the first time that both precursor forms of the VPg (3B3C and 3B_{123}3C) and VPg alone can be uridylylated in vitro. Therefore we hypothesize that precursor proteins like 3AB, 3B3C and 3B_{123}3C, 3ABC, 3BCD might be source of VPg for uridylylation instead of 3AB alone.
6.4 The cre/bus supported VPg uridylylation could be the source of VPgpUpU for both positive and negative strand synthesis

At this juncture it is hard to explain how the entire process of VPg uridylylation occurs inside the cells. It appears that the poly(A) tail is not the primary template for FMDV VPg uridylylation. The poly(A) segment might be involved in stabilization of the viral RNA inside the cell against the action of exonucleases by forming a complex with polyA-binding protein (PABP). It is also possible that polyA-PABP RNP complex is involved in genome circularisation of the FMDV RNA by interacting with the 5' end. Unlike other picornaviruses, the cre/bus is located in the 5' UTR and the accessibility of this template for VPg uridylylation is not defined. One hypothesis could be that once the VPg-3Dpol complex forms, it translocates to the 5' UTR for uridylylation. Another possibility is that, by long distance interactions, either by RNA-RNA or RNA-protein bridges, the cre/bus template is brought into close proximity with the VPg-polymerase complex. It can also be suggested that once a pool of VPgpUpU forms inside the cell this may be used for both positive and negative strand synthesis (summarized in FIG.6.1). This could explain the ability of the FMDV cre/bus to act in trans (Tiley et al., 2003). It was also determined that the sequences required for optimal FMDV VPg uridylylation reside solely in the 5' UTR region.

6.5 FMDV S-fragment forms a binary complex with a cellular protein p48

In the FMDV uridylylation system the efficiency of the reaction was enhanced by 100-fold when 3CD was added to the reaction. Similar observations have been
FIG. 6.1. Proposed model for FMDV RNA replication. The FMDV RNA once released into the cytoplasm the 5'end terminal VPg is cleaved by the cellular VPg unlinkage enzyme. Translation of the viral RNA produces the proteins that are required for RNA replication and capsid assembly. The FMDV cre/bus is functional in positive sense polarity and the anti-sense cre/bus is non-functional for priming VPg for uridylylation. The cre/bus in positive sense primes VPg uridylylation that generates a pool of VPgpUpU inside the cells. This VPgpUpU translocates to the 3' end of the viral RNA to form hydrogen bonds with the proximal 3' end of the poly(A) tail to initiate nascent negative strand synthesis. The VPgpUpU from the cellular pool also primes the 3' end of the negative sense RNA by forming hydrogen bond with the terminal AA- residues to synthesize the nascent positive strand.
reported in other picornaviruses including PV and HRV. The role of 3CD as an efficient cofactor in this reaction has been attributed to its RNA binding activity. It also seems that in FMDV system the RNA binding activity of the 3CD/3C contributes to this effect. Mutations of the arginine residues that are predicted to form the RNA binding motif of the FMDV 3C severely debilitated the uridylylation reactions. In PV, 3CD also forms a component of the biologically relevant ternary ribonucleoprotein (RNP) complex with the cloverleaf RNA that participates in PV RNA replication. The striking difference between FMDV and PV is the lack of a cloverleaf RNA in FMDV; instead a larger S-fragment is present at the 5' end of the RNA. A definitive function for the S-fragment in FMDV RNA replication has not been established. It has been hypothesized that the S-fragment at the 5' end may prevent the degradation of the viral RNA through the action of exonucleases; a similar function has been assigned to the PV cloverleaf (Murray et al., 2001). However no supportive evidence has been presented in this regard for FMDV S-fragment. Our experiments suggest that FMDV S-fragment does not interact with FMDV 3CD or the polyC binding protein (PCBP1/PCBP2), these are the two factors that bind to the PV cloverleaf for negative strand synthesis (Herold and Andino 2001). However it is interesting to mention that FMDV S-fragment interacts with a cellular factor of molecular weight approximately 48 kilo Dalton (p48). Further identification and characterisation of this factor needs to be done.

6.6 FMDV replication complexes (RCs) are enzymatically active

Like other picornaviruses FMDV replicates inside the cell using membrane fractions derived from vesicular structures like the ER and Golgi (Moffat et al., 2005). A post-
nuclear membrane fraction containing crude replication complexes (CRCs) derived from BHK 21 infected cells efficiently synthesized both positive and negative sense RNAs including replicative form (RF) and replicative intermediates (RI). The production of the functional peptide primer VPgpUpU was also detected in this system. These findings suggested that all the replication events can be mimicked using these replication complexes in vitro. It should be noted that unlike with PV (Bienz et al., 1992), FMDV crude replication complexes (CRCs) efficiently protect the newly synthesized virion RNA from the action of RNase A. This in vitro RNA replication system can be a useful tool to dissect the individual steps in FMDV RNA replication in the absence of a true cell-free system for virus production as described for PV and EMCV (Molla et al., 1991; Svitkin and Sonenberg 2003).

6.7 Future directions

At present the above findings contributed to further understanding of the molecular biology of FMDV and more specifically of the RNA replication mechanism at the molecular level. At the same time still there are lot questions remaining to be answered.

6.7.1 Functional dissection of cre/bus RNA and poly(A) tail in FMDV RNA replication

It is interesting to mention that for FMDV, the poly(A) tail is an inefficient template for VPg uridylylation in the presence of Mg$^{2+}$ in vitro. Since Mg$^{2+}$ is the true physiological cofactor it could be the same phenomenon in vivo. Other findings like
the replication of mutant PV lacking poly(A) tail, and the inability to detect polymeric VPg species (VPgpUpUpUpUpUpU ... ) in the cytoplasm of the picornavirus infected cells support the hypothesis that the poly(A) tail might not be the template for VPg uridylylation. On the contrary internal priming on the poly(A) tail could be the case where abortive VPg uridylylation takes place but terminating once two uridine residues added to the VPg (VPgpUpU) thereby allowing for the accumulation of VPgpUpU inside the cell. This might be an intrinsic property of the VPg-3Dpol complex to allow addition of only two uridine residues. If this is the case then there might be other cellular mechanisms involved in this process as well. For instance if internal priming of the VPg takes place on a poly(A) template then there is every possibility of having shorter poly(U) tail in the negative strand and consequently shorter poly(A) tail in the mature positive strand. However a definite length of the poly(A) tail is maintained in a particular virus during a particular virus life cycle. Hence the role of the poly(A) polymerase (PAP) a cellular enzyme come in to picture. These hypotheses could be further clarified by using a cell-free replication system for FMDV. The role of the cre/bus in positive and negative strand synthesis can be studied by using FL FMDV RNA with mutations or complete deletion of the cre/bus (A1A2A3) and further detecting the effects on positive and negative strand synthesis. If cre/bus is required for both positive and negative strand synthesis such mutations in the cre/bus should block the formation of VPgpUpU in a cell-free system. In addition the role of the poly(A) polymerase (PAP) in maintaining the length of the FMDV poly(A) tail and in RNA replication could be determined by depleting the PAP in a cell-free system with specific antibodies or siRNA approach.
6.7.2 Defining the role of S-fragment in FMDV RNA replication

The FMDV S-fragment that forms the 5' terminal 371 nt of the FMDV RNA is predicted to fold into a single hairpin structure. The equivalent region in PV and HRV form the cloverleaf that folds into 4 domains and each domain has an important role in RNA replication including interactions with cellular protein (PCBP) and viral protein (3CD). For FMDV, the relevance of the specific interaction of a 48 kDa cellular protein (p48) to the S-fragment in RNA replication needs to be investigated. For this identification of the protein (p48) need to be carried out. Once the identification is done it will be possible to clone and express the protein. Specific antibody could be raised against the protein to deplete the cell-free system and which could then be programmed with FMDV RNA to see the effects. It will also be interesting to see if any specific interaction exists between FMDV 3' UTR with any cellular protein in BHK S10 extracts. The identification of such RNA-protein interactions at the 5'end and 3'end of the viral RNA will further enhance the understanding of the role of cellular proteins in FMDV RNA replication and might also shed information regarding the interaction between 5'end and 3'end of the FMDV RNA a mechanism that has been proposed for replication of PV.
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APPENDIX I

PUBLICATIONS

Arabinda Nayak, Ian G. Goodfellow, and Graham J. Belsham. 2005. Factors Required for the Uridylylation of the Foot-and-Mouth Disease Virus 3B1, 3B2, and 3B3 Peptides by the RNA-Dependent RNA Polymerase (3D\textsuperscript{pol}) In Vitro. J.Virol. 79: 7698-7706


Yasmin Chaudhry, Arabinda Nayak, Marie-Eve Bordeleau, Junichi Tanaka, Jerry Pelletier, Graham J. Belsham, Lisa O. Roberts and Ian G. Goodfellow. 2006. Caliciviruses differ in their functional requirements for eIF4F components. (Submitted)

Arabinda Nayak, Ian G. Goodfellow, Kathryn E. Woolaway, James Birtley, Stephen Curry and Graham J. Belsham. 2006. Identification of a critical region on the surface of foot-and-mouth disease virus 3C protein required for the uridylylation of VPg and virus replication. (Submitted)
APPENDIX II
BUFFERS, SOLUTIONS AND REAGENTS

II.1 Solutions for DNA Preparations and Analysis

LB Broth
Bacto-tryptone 10g
Bacto-yeast extracts 5g
NaCl 10g
dH₂O 950ml
Adjust to pH 7 with 5M NaOH
Adjust volume to 1L

20% LB Glycerol
80ml LB
20ml Glycerol
Autoclave for 1 hour

TAE (50X) Buffer
242g Tris
57ml Glacial Acetic Acid
100ml 0.5M EDTA pH 8.0
Make up to 1L with dH₂O

Agarose Gel (1%)
1g Agarose (Gibco)
2ml TAE (50X) buffer
98ml ddH₂O
10µl EtBr

LMP Agarose gel (0.5%)
0.5g LMP Agarose (Gibco)
48ml ddH₂O
1ml TAE (50X) buffer
5µl EtBr
APPENDIX II
BUFFERS, SOLUTIONS AND REAGENTS

TFB1 for making competent cells
30 mM KOAc
50 mM MnCl₂
100 mM KCl
10 mM CaCl₂
15% Glycerol
H₂O to a volume of 50 ml

TFB2 for making competent cells
10 mM Na-MOPS
75 mM CaCl₂
10 mM KCl
15% Glycerol
H₂O to a volume of 50 ml

TCM (1.1X)
1 M Tris (pH 7.0) 11 µl
1 M MgCl₂ 11 µl
1 M CaCl₂ 11 µl
dH₂O 967 µl

SOB medium
20 g Bacto-tryptone
5 g Bacto-yeast extract
0.5 g NaCl
Dissolve in 1 litre of water adding KCl to 2.5 mM and adjust pH to 7.0 with NaOH, autoclave to sterilize and add sterile MgSO₄.

SOC medium
SOB
20 mM Glucose

Ampicillin Stock (100 mg/ml)
2 g AMP (GmBH)
10 ml H₂O
10 ml 100% EtOH
Use at a concentration of 100 µg/ml

X-Gal stock (80 mg/ml)
1 g X-GAL (Roche)
12.5 ml Dimethylformamide
Use at a concentration of 40 µg/ml
APPENDIX II BUFFERS, SOLUTIONS AND REAGENTS

Sequencing Stop Solution
100mM EDTA (pH 7.0) 20μl
3M NaOAc 20μl
Glycogen (20mg/ml) 10μl

λIII Marker
λIII marker (500μg/ml) 2.5μl
6x load dye 10μl
dH2O 46.5μl
Run 5μl

II.2 Solutions for RNA Analysis

10X TBE
121g Tris (Trizma base)
62g Boric acid
18.6g EDTA
Make volume upto 1 litre with deionised water

10M urea
Dissolve 60.06 grams of urea in 100 ml of dH2O

HEPES (1M)
Dissolve 1.191 grams in 5ml dH2O, adjust the pH to 7.5

Gel Loading Buffer II
95% Formamide
0.025% Xylene cyanol,
0.025% Bromophenol blue
18Mm EDTA
0.025% SDS

FA gel buffer (10X)
41.9 g MOPS
6.8 g Na-acetate.3H2O
20 ml 0.5 M EDTA
Adjust pH to 7.0 with NaOH
FA gel running buffer (1L)
100 ml 10X FA gel buffer
20 ml 37% (12.3M) formaldehyde
880 ml RNase-free water

Electroporation buffer
21 mM HEPES, pH 7.0
137 mM NaCl
5.0 mM KCl
0.7 mM Na₂HPO₄
6.0 mM Glucose
Solution was prepared in RNase free water and filter sterilised

Probe/RNA elution buffer
0.2% SDS
10 mM Tris
1 mM EDTA

TN hypotonic buffer
10 mM Tris, pH 6.8
10 mM NaCl

TENSK
100 mM NaCl
10 mM Tris pH 8.0
0.1 mM EDTA
0.5% SDS
10 µg/ml Proteinase K

Low salt RNase A digestion buffer
10 mM Tris, pH 7.8
5 mM EDTA
100 mM NaCl

High salt RNase A digestion buffer
10 mM Tris, pH 7.8
5 mM EDTA
300 mM NaCl
APPENDIX II  
BUFFERS, SOLUTIONS AND REAGENTS

5% TBE-PAGE gel
1.7 ml 30% acrylamide:bisacrylamide
500 μl 10X TBE
1 ml Glycerol
6.8 ml Deionised water
100 μl 10% APS (Ammonium per sulphate)
5 μl TEMED

3X UV-cross linking buffer (1 ml)
30 μl 1M HEPES
105 μl 1M KCl
6 μl 1M MgCl₂
300 μl Glycerol
15 μl 10% NP-40
1.2 μl 1.25M DTT
543 μl RNase free water

EMSA binding buffer (1 ml)
50 μl 1M HEPES
2 μl 1M MgCl₂
80 μl Glycerol
868 μl RNase free water

II.3 Media and solutions for Protein expression, purification and Analysis

NZCYM broth
Tryptone 10g
Yeast extract 5g
Casamino acid 1g
NaCl 5g
MgSO₄ 1g
Dissolve 22.0 grams per litre of dH₂O and autoclave

Lysis Buffer (for protein purification)
500 mM NaCl
50 mM Potassium phosphate, pH 8.0
10 mM mercaptoethanol
20% glycerol
40μl of Protease inhibitor cocktail (Calbiochem)
0.1% NP40
Buffer A (for protein purification)
50 mM Tris
20% Glycerol
10 mM BME
0.1% NP40

10% running gel (20ml)
6.7 ml Acrylamide (Sigma)
5.0 ml 1.5M Tris pH 8.8
7.9 ml ddH2O
200μl 10% SDS
200μl 10% APS
10μl TEMED

5% stacking gel (5ml)
0.83 ml Acrylamide (Sigma)
0.63 ml 1M Tris pH 6.8
3.4 ml ddH2O
50μl 10% SDS
50μl 10% APS
10μl TEMED

SDS-PAGE running buffer
28.8 g Glycine
6 g Tris
2 g SDS
Make final volume up to 2 L with ddH2O

Coomassie Brilliant Blue
Brilliant Blue G 5 g
Destain 15 ml

Coomassie Brilliant Blue Destain Solution
Methanol 400 ml
Glacial acetic acid 150 ml
ddH2O 1450 ml

IPTG Stock (100 mM)
IPTG 1.2 g
ddH2O 50 ml
Tricine gel buffer 0.5 litre (3M tris, 0.3% SDS, pH 8.45)
181.65 g Tris
1.5 g SDS (adjust pH to 8.45 using HCl)
mQ H₂O up to 500 ml

Tricine cathode buffer 0.5 litre (10X) (1M tris, 1M tricine, 1% SDS)
60.55 g Tris
89.60 g Tricine
5.0 g SDS/ 50 ml 10% SDS
mQ H₂O up to 500 ml

Tricine sample buffer
200 mM Tris-HCl (pH 6.8)
2% SDS
40% Glycerol
0.04% Coomassie Brilliant Blue G-250

Western Blot Running Buffer
Glycine 28.8g
Tris base 6g
SDS 2g
dH₂O 2L

Western Blot Transfer Buffer
Glycine 28.8g
Tris base 6g
dH₂O 1600ml
Methanol 400ml

10x TBS-0.1%Tween
Tris base 48.4g
NaCl 160g
dH₂O 1800ml
Adjust to pH to 7.6 with HCl
Tween-20 20ml
Make up to 2L with dH₂O
Dilute to 1x before use

Blocking Buffer
Marvel Milk 5g
1x TBS-Tween 100ml
### APPENDIX III

**OLIGONUCLEOTIDES**

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