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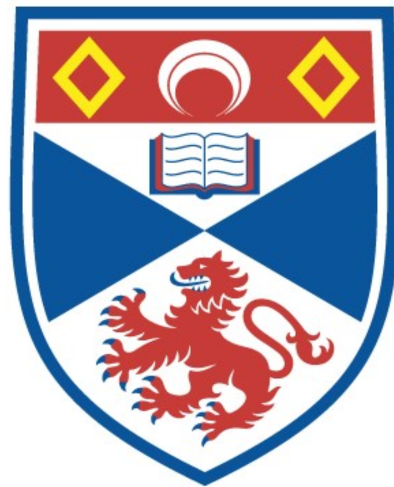

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Cloning, Expression and Functional Analysis of Picornavirus Protein 2C.

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A thesis submitted in partial fulfilment

of the requirements for the

degree of Master of Philosophy

December 2002

Th E426

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Abstract

Picornavirus-infected cells are characterised by the appearance of smooth vesicles and the disappearance of the Golgi apparatus. Replication of the viral RNA takes place on these virus-induced vesicles. Expression of the viral protein 2BC, and to some extent 2C, has similar effects on cells. Protein 2C has been proposed to hold these replication complexes together and is known to bind to the membranes, however the mechanism of this binding is unknown. Previous work by Knox *et al* showed an interaction between FMDV protein 2C and the protein kinase A anchoring protein 10 (AKAP 10). The 2C gene from the CA9, ECHO 22, FMDV, HAV HM175, HAV wt1, HeCV, HRV 85 and TME picornaviruses were cloned into both translation and expression vectors (the FMDV and HRV 85 were donated by C. Knox and N. Moir respectively). Translation products were then used in a binding assay and interaction with AKAP 10 was shown for ECHO 22, FMDV, HAV wt1, HeCV, HRV 85 and TME. These results indicate AKAP 10 binding as a general function of 2C in all picornaviruses. An expression system was optimised for HRV 85 2ABC, 2BC and 2C proteins, and was shown to produce the other picornavirus 2C proteins at similar levels. Cellular expression of protein 2BC is known to bring about membrane rearrangements and the binding of 2C to AKAP 10 may help explain this aspect of the cytopathic effect and the targeting of virus replication to membranes.

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Abbreviations.

AKAP 10	protein kinase A anchoring protein 10
APS	ammonium persulphate
ARF1	ADP-ribosylation factor 1
beads	sepharose beads
BSA	bovine serum albumin
CA9	coxsackievirus A9
CBPC	cap-binding protein complex
CPE	cytopathic effect
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
dsRNA	double stranded ribonucleic acid
DTT	dithiothreitol
ECHO 22	enteric cytopathic human orphan virus type 22
E. coli	*Eschericia coli*
EDTA	ethylenediamine tetra-acetic acid
ER	endoplasmic reticulum
FMDV	foot and mouth disease virus
GEB	glutathione elution buffer
GST	glutathione S-transferase
HAV	hepatitis A virus
HBB	2-(α-hydroxybenzyl)-benzimidazole
HCl	hydrochloric acid
HeCV	human enteric calicivirus
HRV 85	human rhinovirus 85

IAP3	clone encoding the C-terminal 55aa of AKAP 10 fused to GST.
IRES	internal ribosome entry site
IPTG	β-D-isopropyl-thiogalactopyranoside
Kb	kilobase
LB	Luria-Bertani
NaAc	sodium acetate
NP40	nonidet P40
ntr	nontranslating region
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PKA	protein kinase A
PMSF	phenylmethylsulfonylflouride
RGS	regulator of G protein signalling
RNA	ribonucleic acid
RE	restriction enzyme
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNARE	soluble NSF attachment protein receptor
TAE	Tris/acetic acid/EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TGN	trans Golgi network
TME	Theiler's murine encephalitis virus
Tris	tris(hydroxymethyl)aminomethane
vRNA	viral RNA
wt	wild type
X-Gal	5-bromo-4-chloro-3-indolyl-β-galacto-pyranoside

1 Introduction.

1.1 Picornaviruses.

The foot and mouth disease virus (FMDV) is an important pathogen in livestock, and can have devastating effects on a country; the outbreak in Great Britain caused financial stress on most farms in England, Wales and Southern Scotland. This is just one member in a family of economically and medically important viruses called picornaviruses. The picornaviruses were the first viruses to be recognised (FMDV in 1898 by Loeffler and Frosch) and to be propagated in cultured cells (poliovirus). Many important developments in virology were made using poliovirus, including purification and crystallisation methods that led to structural analysis, and assays for infectivity (reviewed in 76).

The *picornaviridae* has six genera, each containing viruses that have medical importance worldwide. These genera are: rhinoviruses; the most important etiologic agents in the common cold, enteroviruses; (including poliovirus and coxsackieviruses) widely used to study picornaviruses in general, aphthoviruses; containing the FMDV, cardioviruses; animal viruses affecting the heart, parechoviruses; including ECHO 22 and finally, hepatovirus; containing the human and simian hepatitis A viruses (HAV) (38, 70, 76).

Picornaviruses have a small protein capsid, with a diameter of around 30nm, enclosing a single stranded, positive sense RNA. There is no lipid envelope: the virus is therefore unaffected by organic solvents. The pH stability of the capsid varies between the genera: enteroviruses, cardioviruses and hepatitis A viruses

are acid stable (able to survive pH 3 or lower), the rest are acid labile (labile at pH 7-6) (65).

Picornaviruses are a family of RNA based viruses who's RNA contains various structures important in replication and life cycle. The single stranded RNA varies only slightly between genera, ranging from roughly 7,200 to 8,400 bases. The RNA has either 11 or 12 genes, the extra gene encoding the leader (L) protein, which is found only in cardioviruses and aphthoviruses (76). The RNA has the viral protein 3B (VPg) covalently bound to its' 5' terminus (FMDV has three 3Bs bound) (44). The 3' end of the RNA is polyadenylated, ranging from 35 to 100 Adenine residues. The function of the poly(A) tail is unknown, however shortening results in a decrease in infectivity (77) .

The coding section is flanked by two nontranslating regions (ntr). The 5' ntr is unusually long, 620-1,200 bases (76), and encompasses two distinct, independently acting components. In poliovirus and HRV 14 the first 100 nucleotides form a cloverleaf configuration, which is required for replication, whilst in HAV they form three stem-loop structures (4 and reviewed in 76). The remaining nucleotides form the internal ribosomal entry site (IRES) (68). The folds of the IRES are thought to bind different initiating factors, and therefore may have a role in determining host range and neurovirulence (80).

The 3' ntr is much shorter (47-126 bases) and the sequences and structures thought to be involved in replication are less clear (30). Replacing the 3' ntr of poliovirus with analogous regions from other picornavirus has no effect on

viability despite little homology between viruses in this region (75). Conversely changes in 3' structures, such as a pseudoknot in poliovirus, or the terminal loop in HRV 14, causes adverse effects on replication (58,35). This suggests that 3' ntr has a role in replication, however as this region can be deleted without completely preventing replication, it is not essential (83, 30).

1.1.1 Picornavirus Life Cycle.

The picornavirus infectious cycle last 5 to 10 hours and replication occurs only in the cytoplasm. The initial step, uncoating, is a four-step process commencing with attachment to specific receptors in the plasma membrane (Step 1, Figure 1.1.1 [51]). The receptors vary between virus genera and partly define the cell tropisms (76, 19), however most are involved in the immune response. More receptors are recruited, drawing the virus into a receptor-coated pit (Step 2). The eclipse is the irreversible conformational change caused by the loss of 1A (Step 3 [51]), which results in the extrusion of N-terminus of the viral protein1B, and capsid swelling. The viral RNA (vRNA) is then delivered across the membrane into the cytosol (Step 4).

Once in the cytosol, the host cells' translation machinery is sequestered to produce the polyprotein (Step 5). The machinery required by the virus is not fully known, however it includes ribosomes, tRNA and enzymes (76), nevertheless it does not involve the cap-binding protein complex (CBPC) (68). Since the 40s ribosome can attach directly to the IRES (5), the eukaryotic cap-dependent ribosomal process can be targeted for inhibition of cellular protein

production. The polyprotein is proteolytically processed both co- and post-translationally, initially producing three precursor proteins - P1, P2 and P3 -, which are further processed yielding the mature proteins (17, 66). Up to 80 ribosomes may translate the vRNA simultaneously.

In vRNA synthesis a complementary minus (-ve) strand of the genome is produced (Step 6), from which the positive (+ve) strands are quickly made (Step 7). Positive strand vRNA synthesis is performed in replication centres on virus-induced vesicles. In early stages these newly synthesised +ve strands are recruited for translation, and for transcription to make more –ve strands, which form new replication centres (Step 8). These vesicles probably separate the viral polymerase from cytoplasmic RNA to allow efficient replication of the viral RNA (10), since the poliovirus polymerase copies RNA non-specifically (85). As the concentration of +ve strands – and capsid proteins - increases they are packaged into virions. The +ve strand RNA synthesis is exclusive to replication centres on virus-induced vesicles (10 and 17), and are thought to be responsible for the efficient synthesis of +ve strand RNA, which is 20-50 times faster than –ve strand replication (81, 5).

The formation of virions begins with cleavage of P1 by the virus protein 3C (the function of viral proteins are discussed in sections 1.2-1.4) to produce the immature protomer (Step 9). P1 is cleaved into 1AB, 1C and 1D, however these proteins remain bound to each other (29). The protomers binds to form pentamers (Step 10) 12 of which assemble with the genome to produce the provirion (Step 11). It is not known if the genome is threaded into the capsid, or

if the proteins form around the RNA, however infected cells often contain empty shells that are able to dissociate into pentamers (reviewed in 76). Finally 1AB must be cleaved into 1A and 1B (29) in a process known as the 'maturation cleavage' (Step 12), before the virus particles are released (Step 13) by infection mediated host cell disintegration. Some viruses, such as hepatitis A, can cause nonlytic infections that persist indefinitely (41). It is conceivable that in these cells the viruses may be released by budding. Figure 1.1.1 shows the typical picornavirus life cycle.

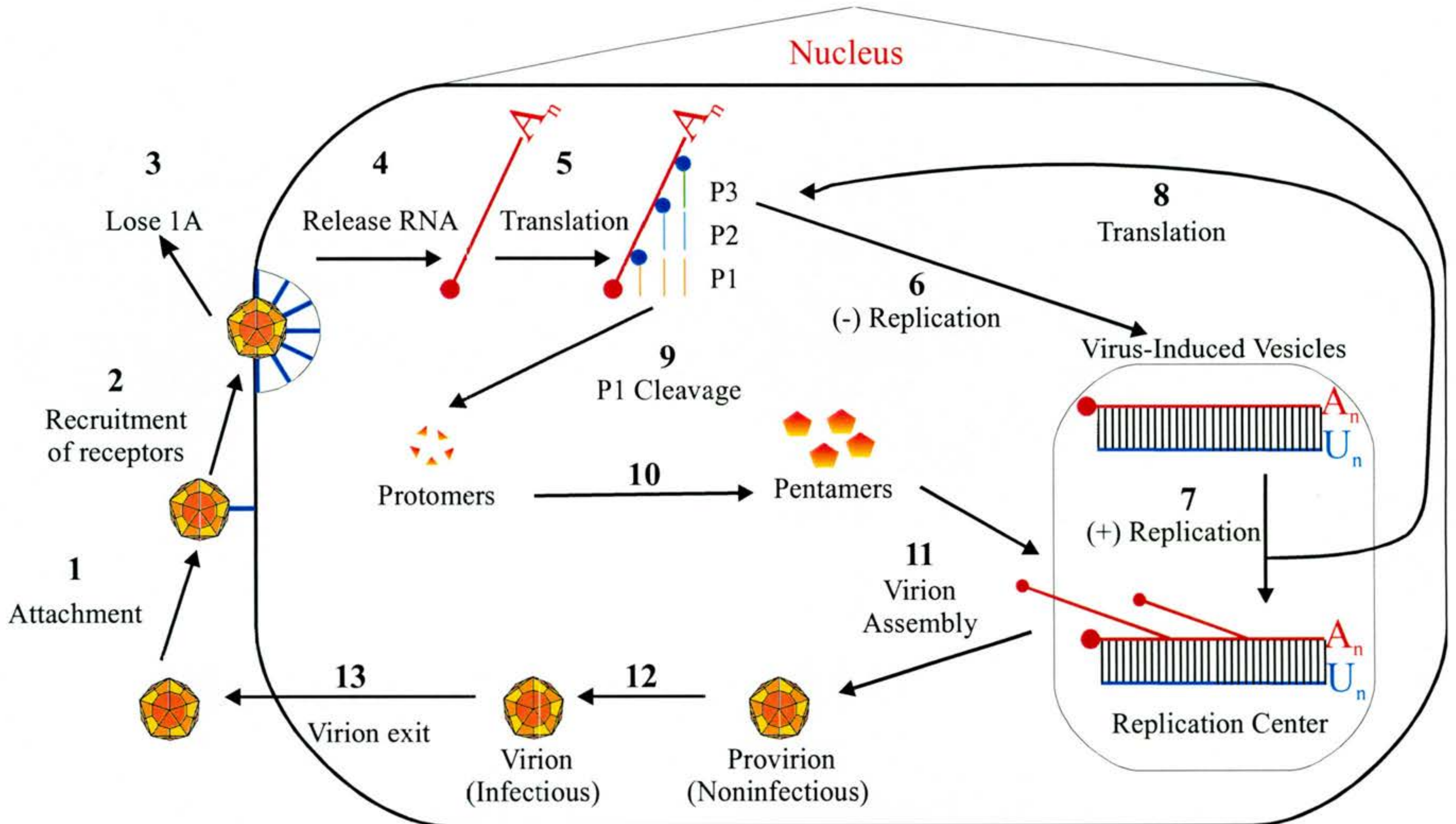

Figure 1.1.1. Typical infection cycle for members of the picornavirus family. The virus enters the cell (steps 1-4), the viral proteins are produced (5), the RNA is replicated (6-8) and the virus assembled (9-12).

1.1.2 Cytopathic Effect of Picornaviruses on the Host Cell.

Picornavirus-infected cells are easily identified by the appearance of smooth vesicles (150-400nm in diameter, [9, 17]) often with a double lipid bi-layer and the disappearance of the Golgi apparatus. These virus-induced vesicles are a prerequisite for vRNA synthesis (11). This increase in vesicles and rearrangement of membranes has been proposed as the underlying cause of the cytopathic effect (CPE) (10) and is not seen in non-cytopathic infections, such as with HAV (81). The vesicles form on membranous protrusions at the rough endoplasmic reticulum (rER) (9), however contain protein markers from the whole secretary apparatus (79). Inhibition of retrograde traffic from Golgi apparatus to the ER by the drug brefeldin A inhibits poliovirus replication (56, 17), suggesting that either traffic between the membranes, or integrity of the membranes is vital for replication.

As these vesicles have double lipid bi-layers, a simple budding system cannot account for their formation, however in normal cells similar vesicles form when nitrogen or amino acid starved. These vesicles, called autophagic vacuoles, are initially produced when ER membranes wrap around a pool of cytoplasm. Suhy *et al* drew similarities between these vacuoles and picornavirus-induced vesicles and suggested this as a mechanism by which they may form (79).

Information about the CPE is based on research on polioviruses, and less is known about it in other picornaviruses. One of the first effects, in as little as 30 minutes, is the inhibition of cellular protein synthesis. This steadily decreases to a minimum at around 2 hours, and then peaks at approximately 3 hours, before

declining once more, after which cell death occurs (reviewed in 76). Within one hour of infection there is margination of the chromatin (48). Membranous vesicles appear in the cytoplasm between 2 ½ and 3 hours, radiating out from the nuclear envelope (21). The appearance of the vesicles is associated with changes in plasma membrane permeability, increase in intracellular calcium concentration (39), modification of lipid turnover and phospholipase activities (40), and leakage of cellular contents (13). There is also a decrease in the rate of cellular RNA synthesis as the viral RNA synthesis increases, and inhibition of cell division (76). The underlying cause of the CPE is unknown, however the non-structural proteins are all thought to have a role.

Inhibition of cellular protein synthesis is thought to occur due to many factors. The increase in double stranded RNA, the presence of viral protein coat, an increase in sodium and decrease in potassium cytoplasmic concentrations, competition for machinery and the inactivation of initiating factors have all been implicated in the inhibition. However, it seems that the latter two are the main factors. Viral protein synthesis does not require the cap-binding complex as the IRES allows internal binding of ribosomes. The virus can therefore cleave p220 in the CBPC, without affecting its own synthesis, using either the 2A protease (most picornaviruses) or the leader protein (aphthoviruses). In cardioviruses there is no cleavage of the CBPC and these rely on an exceptionally active IRES, which out-competes with the host mRNAs (reviewed in 76).

The decrease in cellular RNA synthesis is due to virus 3C protein modifying transcription factor complexes, for example cleaving the TATA-binding protein

subunit of transcription factor IID. 3C mainly effects polymerase II, but does, however, degrade both polymerase I and III to a degree (reviewed in 76).

1.1.3 Polyprotein Processing.

Picornavirus positive strand RNA has one open reading frame from which all proteins are translated. The full-length translation product is never detected in host cells since the large polyprotein (2100-2400 amino acids) is co-translationally cleaved. In aphthoviruses the leader protein cleaves itself from the polyprotein, however in cardioviruses this initially remains attached to the polyprotein (76). The first fragment, P1, is cleaved during translation by the protein 2A, at tyr-gly pairs (24), at its N-terminus, however in certain viruses, for example FMDV, 2A cleaves at its C-terminus (76). If P1 is not removed from the nascent chain translation slows noticeably. P2P3 is cleaved by 3C at gln-gly pairs (73). Further cleavage is achieved by 3C in a concentration-dependent manner *in trans*.

P1 is cleaved to produce the structural proteins, and has four products: 1A, 1B, 1C and 1D, however 1AB is not cleaved until the virion is made and maturation can occur (29). P2 and P3 are processed into non-structural proteins, and produce 3 and 4 products respectively (2A, 2B and 2C; 3A, 3B, 3C and 3D). These regions also produce functionally active proteins from partially cleaved polypeptides, such as 2BC, 3AB and 3CD. The genomic structure can be seen in figure 1.1.2.

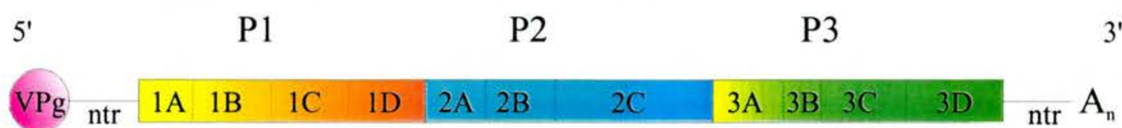

Figure 1.1.2 Diagrammatical representation of the general Picornavirus genome.

Unless stated otherwise all the above cleavages are completed by 3C. 2A can also cleave 3CD, although this is not essential for virus viability and cleaves at a point within 3D (66). In addition the P3 segment can be cut in a concentration independent manner - an *in cis* intramolecular cleavage by 3C. The mature proteins from the P3 region are therefore produced more rapidly. This is important since the P3 proteins are required for both cleavage and vRNA synthesis.

1.2 Structural Proteins.

The P1 region proteins form the dodecahedral protein capsid, which is spherical and has a diameter of 24-30ηm. The capsid forms the only protection for the genome and recognises specific receptors in the host cell membrane. The P1 region is initially cleaved into three segments, 1AB, 1C and 1D (29). These non-identical proteins assemble forming the protomers, which are identical four segmented subunits. Five protomers bind to form pentamers, using a urea sensitive bond. This construction initiates assembly of 12 pentamers, possibly forming around the RNA genome, producing the immature virion. The pentamers are held together with an acid sensitive bond. In order for the virion to become infective the 1AB protein must be cleaved to form 1A and 1B, which

occurs before the release of the virus from the host cell. Both the cleavage site and the protein 1A are buried deep within the virion, and therefore possibly occurs autolytically. The diagram below (figure 1.1.3) depicts the protein capsid structure (reviewed in 76).

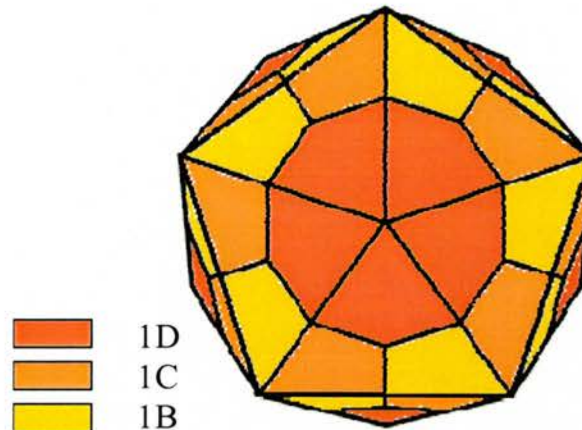

Figure 1.1.3. Diagrammatic representation of the dodecahedron structure of the protein capsid.

1.3 Non-Structural Proteins.

1.3.1 P3 Proteins.

The proteins in the P2 and P3 regions are mainly involved with RNA replication. Protein 3A has been suggested to sequester viral proteins for formation and function of viral replication complexes (79). When expressed in isolation it inhibits traffic from the ER to the golgi (23), and radically alters the ER structure (not like the modification seen in virus infected cells, [79]). Protein 3A binds to ER membranes and requires the integrity of 22 amino acids in a hydrophobic region near the C-terminus (79, 24) to anchor 3A and 3AB to membranes. Membrane associated 3AB binds directly to the polymerase (3CD), therefore stimulating the activity of the protease to cleave the 3CD, leaving 3D anchored to

the membrane (79). 3AB associates with the 3' ntr in the absence of other proteins (6) and has been proposed to bind 2C and 2BC onto the replication complex (60).

3B is the protein that attaches to the 5' end of the genome (VPg) and is important in RNA synthesis initiation (8). It has also been implicated in other roles, for example, when uridylated it acts as a primer in both +ve and –ve strand RNA replication (24 and 30). It also may act as a primer when in it is partially cleaved form 3AB (79). Mutation studies imply that it could act in later functions such as encapsidation (24).

Proteins 3C and 3CD are proteinases that cleave the viral polyprotein into mature proteins. Both 3C and 3CD bind to viral RNA (24) and 3CD is implicated in cleavage of 3AB during initiation of +ve strand RNA synthesis (3). Protein 3CD forms a ribonucleoprotein complex in association with other cellular proteins, with the first 100 nucleotides of the viral RNA. This is required for +ve, but not –ve strand RNA synthesis (82). The ribonucleoprotein complex also contains p36, which is thought to be a cleavage product of EF-1α (5, 6). Others have indicated that either 3AB or cellular poly-C binding protein type 2 (PCBP2) binds to the cloverleaf structure with 3CD (30). The ribonucleoprotein complex may be involved in suppression of viral translation (30). 3CD has also been shown to bind to 3' ntr in the absence of other proteins (6).

The protein 3D is a primer-dependent, RNA-dependent RNA polymerase (82). This has also been shown to possess an RNA duplex unwinding activity (16),

important in separating double stranded RNA (dsRNA) during RNA replication. During *in vitro* elongation of viral RNA the only protein required is 3D. It also has a role in uridylylating the protein primer VPg (70, 82). The poliovirus 3D copies non-specifically several different virus and cellular RNA (85).

1.3.2 P2 Proteins.

The proteins from the P2 region are not as well understood. In entero- and rhinoviruses 2A is a viral protease, cleaving at a Tyr-Gly amino acid pair at its N-terminus - the P1/P2 junction (24). Some evidence suggests 2A plays a role in viral replication (24), and in shutting down the host protein synthesis through cleavage of cellular factor eIF-4G (29).

2B is thought to be involved in host range determination, and in RNA synthesis as mutations in the 2B region lead to noncomplementable defects in RNA replication (42). This implies that either 2B or 2BC is needed *in cis* for RNA replication or that mutations in the 2B region disrupt the RNA structure causing a defect in RNA synthesis (24). Early studies showed that 2BC was involved in the induction of membrane proliferation within the host cell (10). Insertion of IRES between 2B and 2C was lethal; therefore showing that 2BC is necessary for poliovirus replication (90)

2C is the most conserved gene in the picornavirus family, especially within the central third of the protein (51, 67). Mutational studies have suggested that it is involved in RNA synthesis. As 2C possesses an NTPase activity, it has been

proposed that it may have a helicase activity utilising the energy from the NTPase for dsRNA unwinding (76). Both 2C and 2B are found exclusively within the replication complex and on vesicular membranes in infected cells (11).

1.4 Picornavirus 2C Protein.

2C is the most conserved picornavirus gene, both for its antigenicity and its amino acid sequence (51, 67). Mutations within this sequence inhibit virus replication; therefore it is important to understand its function within picornavirus proliferation. Most 2C studies have been performed on poliovirus, however due to its highly conserved nature, inferences may be made as to the function of 2C for the whole family. 2C proteins are approximately 330 amino acids long with a molecular mass of around 37kDa (67, 24, 71). Unless stated otherwise all sequence nomenclature is based on the poliovirus sequence.

The growth of picornaviruses can be selectively inhibited by guanidine hydrochloride (0.1 to 2.0 mM [72, 46]), primarily through the blockage of vRNA synthesis, with no effect on the host cell. Pincus *et al* (1986) produced two viruses, one resistant to and the other dependent on guanidine. Both mutants had a change in 2C at amino acid 179, whilst the dependent mutant also had a change in 2C at position 227. As these were the only mutations within the genomes, guanidine specifically inhibits RNA synthesis initiation through an action on 2C (72).

The antiviral drug 2-(α-Hydroxybenzyl)-Benzimidazole (HBB) specifically inhibits enterovirus RNA synthesis (46, 31) at non-toxic concentrations. Cells infected with a HBB dependent virus will stop producing infectious particles 15min after HBB is removed. In one virus the mutation was at position 229 on the 2C protein near the C motif for nucleotide binding (NTP-B) (figure 1.4.2), in the other it was at position 291 in the C-terminal amphipathic helix. This suggests that there is a link between 2C and virion assembly, requiring both NTPase and the C-terminus. Position 229 is also a hot spot for guanidine mutations, therefore both drugs may inhibit 2C in a similar manner, despite their different antiviral spectrums, and furthermore there may be a link between vRNA synthesis and virion assembly (31).

Li *et al* (1988) isolated 2C mutants that were temperature-sensitive for vRNA synthesis, however cleavage of the polyprotein remained unaffected. They showed 2C is continuously required for vRNA synthesis. They also showed the mutation can be complemented *in trans* and that no excess of dsRNA is seen, contradicting guanidine mutants, 2C may therefore have multiple functions within vRNA synthesis (50).

A temperature sensitive revertant from this mutant was defective in virion uncoating at 32°C (51). Two secondary point mutations were found down stream of original insert and could be complemented *in trans*. 2C has not been detected within virions; therefore the defect must be in the virus's capsid structure and cannot be due to a direct effect of 2C. Only newly made RNA is incorporated into virions (12), therefore replication and formation of virions may

be coupled in membranes. Further evidence supporting this theory comes from facilitation of *in vitro* capsid formation by 2C-containing membrane fractions (69). Picornavirus encapsidation is inhibited by 5-(3,4-dichlorophenyl)methyl-hydantoin, and in resistant viruses the mutations are situated in the 2C protein (86). 2C, 2B, 2BC, 3AB and capsid proteins form a detergent-resistant complex (26), further supporting the notion that 2C has a role in virion formation.

A large proportion of 2BC remains uncleaved in poliovirus-infected cells. 2B and 2BC enhance membrane permeability and block the exocytic pathway (7, 41), whilst expression of 2C and 2BC promotes membrane proliferation in the cytoplasm (81). Poliovirus 2BC causes many cytopathic effects, including growth inhibition, intracellular membrane proliferation, enhanced membrane permeability and blockage of exocytic pathway (7). The area specifically required for membrane proliferation is located in 2C, whilst membrane permeability, and inhibition of the exocytic pathway is located in 2B. Areas in both 2B and 2C are required for 2BC-induced cytotoxicity (7).

Poliovirus infected cells undergo an increase in cytoplasmic calcium concentration from 4 hours post-infection (2). Protein 2BC enhances cytoplasmic calcium concentrations in a manner similar to poliovirus-infected cells. Calcium concentrations are key in the regulation of many cell functions, and modification of intracellular Ca^{2+} concentration is important in the life cycle of many viruses. Although 2B can modify membrane permeability, it is unable to affect intracellular Ca^{2+} concentration (2). A release of Ca^{2+} from internal stores could stimulate Ca^{2+} entry across the plasma membrane, or increased Ca^{2+}

concentrations could be due to activation of plasma membrane Ca^{2+} ion channels. Equally activation of phospholipase C and subsequent increase in inositol-1,4,5-triphosphate in poliovirus infected cells could trigger an increase in cytosolic Ca^{2+}. The role of increasing intracellular Ca^{2+} in picornaviruses is not known (2). Similar results were found for HAV proteins (41), however coxsackievirus 2B protein is sufficient to increase intracellular Ca^{2+} concentrations (2BC was not tested [87]).

1.4.1 Interaction of 2C and Related Proteins with Host Cell Membranes.

The disappearance of the golgi apparatus and the appearance of replication complexes, with associated 2C (10), is characteristic of picornavirus-infected cells (17). These rosette-like structures contain a number of vesicles (200-400nm in diameter) surrounding an elongated replication complex. The presence of guanidine *in vivo* destroys this formation, indicating 2C maintains the integrity (11). However guanidine does not affect 2C membrane binding in the absence of other viral proteins (24). UV photo-cross-linking during the peak of vRNA synthesis induced covalent bonds between vRNA and 2C/2BC. Thus 2C (and 2BC) attaches vRNA to vesicular membranes, providing the correct spatial organisation for the replication complex (11).

2C lacks domains normally found in membrane bound proteins, thus has been suggested to bind to membranes via 3AB (60, 24). However 2C and 2BC can associate with cytoplasmic membranes without other viral proteins and are not modified by glycoslyation or signal peptidases (81, 24, 41). A region of the N-

terminus of 2C (amino acids 6-35, [24, 70]) has been predicted to be a class 'A' amphipathic helix (67). These can be further differentiated; "type 1" is found in enteroviruses and rhinoviruses whilst "type 2" is in aphthoviruses, parechoviruses and cardioviruses. The HAV helix is thought to be significantly different from all other picornaviruses. Mutations within this region demonstrate that the helix is required for normal polyprotein processing and perhaps for vRNA synthesis and virus viability. A weaker amphipathic helix at the C-terminus (amino acids 290-308) has also been predicted (67, 60).

Deletions of 21, 54 and 88 N-terminal amino acids from 2C have an increasing effect on membrane binding, from none, through significant reduction to abolishment, respectively. Thus amino acids 21-54, and therefore the N-terminal helix, are important in membrane binding (24). Protein 2C membrane binding does not require the B, C and D NTPase motifs and the C-terminus amphipathic helix (24). These results support the idea of the class 'A' amphipathic helix being the site of membrane binding in 2C. In fact an N-terminal fragment of 2C is capable of targeting a soluble protein to cellular membranes (unpublished data, Banerjee *et al*)

Poliovirus 2C and 2BC expression produces vesicles (150-350nm diameter) similar to those found in poliovirus-infected cells, even with mutations in the NTP-binding motifs (1, 4, 79 and 17). Protein 2C alone also forms extensive myelin-like tubular structures to which 2C is associated. Only when 2BC and 3A are expressed together is vesicle formation consistent with poliovirus-infected cells (79). Guanidine HCl does not influence the patterns of vesicles;

nevertheless it abolishes 2C-mediated formation of tubular structures, as does mutations in NTPase binding domain (17). GTPase activity is required for eukaryotic vesicle formation and traffic, however as this is not required in 2C then the virus may use another mechanism for vesicle formation, or may utilize the normal cellular process.

Membrane arrangements found in poliovirus-infected cells have not been seen in HAV infected cells (81, Egger and Bienz, unpublished observations), yet expression of HAV 2C and 2BC in cells shows efficient membrane association and reorganisation (81). Despite the common gene positioning in HAV, there is limited nucleotide or amino acid sequences similarity with other picornaviruses and wild type HAV is unable to replicate in cultured cells. Mutations in 2B/2C and the 5' ntr are necessary for cell culture adaptation. 2C N-terminal mutations in culture adapted HAV may indicate a role for the amphipathic helix in growth efficiency, however 2C and 2BC from wild type, cell culture adapted and cytopathic HAV varies little in membrane association and reorganisation (81). Thus the inefficient replication property of HAV is not a consequence of the inherent inability of 2C/2BC to interact with membranes (81) and is more likely a result of the small amount of 2C and 2BC in infected cells (41). This is further substantiated by the relatively high similarity between 2C from HAV and other picornaviruses (81).

1.4.2 NTP-Binding Motifs and RNA Binding Domains in Picornavirus 2C.

2C contains nucleotide binding (NTP-B) motifs present in NTPases: the A site (GXXXXGKS) containing a flexible Gly-rich loop involved in binding purine nucleotides; and the B site (DD preceded by hydrophobic amino acids), which chelates Mg^{2+} of the complex Mg-NTP (73). Another is present at position 217-223, motif C (a region of hydrophobic residues, preceding an invariant N), which is found in members of the helicase superfamily III (71, 84). All NTP motifs in 2C can be seen in figure 1.4.2. Mutations within these regions not only abolish NTP-binding/hydrolyses, but also produce non-infectious virus, unable to replicate vRNA (60, 82, 59, 71). Deletion mutants show that amino acids 160-255, and therefore the B site, are necessary for NTPase activity (73). One study showed that guanidine did not inhibit ATPase activity (60), whilst another showed the opposite (71). Considering that guanidine mutations map to the vicinity of the B motif it seams likely that the latter is true (72). Guanidine-induced 2C mutants show normal NTPase activity (73, 60).

Helicase activity is often associated with NTPase activity, however 2C does not contain all the necessary motifs and has never shown helicase activity (71, 73). NTPases, however, also act in membrane trafficking, protein folding and nucleic acid replication. 2C could equally have a role in one or more of these, rather than acting as helicase, which is further substantiated by the fact $3D^{pol}$ shows strand displacement (16).

2C has been shown to bind RNA both *in vivo* (11) and *in vitro* (73). Mutational studies showed that 2C and 2BC fusion proteins bound RNA, however cleavage

mutants did not, suggesting the RNA binding domain lay beyond amino acid position 255 of 2C (73). Paul *et al* (1994) reported that dsRNA binding consensus sequences for proteins with N-terminal amphipathic helices had extensive homology with 2C, and that some 2C mutations are found in this proposed RNA binding site (50, 67). Further studies showed 2 regions of 2C involved in RNA binding: positions 21-45, overlapping the N-terminus amphipathic helix, and an Arg rich region (45) at positions 312-319. Deletion of either site abolishes RNA binding; however removal of a much larger C-terminal region, including the NTP-binding motif, does not affect RNA binding (74). As this occurs in other RNA binding proteins, it is not an experimental anomaly and shows that NTPase activity is not required for RNA binding.

Poliovirus 2C binds specifically to the 3'end cloverleaf of -ve RNA, but does not bind to the 5'end cloverleaf of the positive strand (6). A series of mutations showed that the highly conserved (in enteroviruses and rhinoviruses) sequence UGUUUU in stem A of the negative strand 3'cloverleaf was critical for 2C binding and that when this was double-stranded 2C's binding was enhanced. Therefore it is likely that 2C anchors –ve strand RNA to membranes, facilitating the transfer of proteins from 5' positive strand on to the 3' negative strand. 2C from HAV and HRV 14 also bind specifically the 3' end of negative strand RNA, and shows little interaction with the 5' end of positive RNA (5). It must be mentioned, however, that echovirus 2C has been shown to bind equally to 3' positive and negative single stranded RNA, but not to 5' or double stranded RNA (45).

There is also a cysteine-rich motif ($CX_{2-6}cX_{6-8}CX_{3-4}C$) in enterovirus and rhinovirus 2C between the B and C NTPase motif (amino acids 269-286). These cysteines are not involved with disulphide bonds and are likely to be engaged in zinc binding (70). This suggested zinc finger could be implicated in either protein-protein or protein-nucleic acid interaction, therefore could have a role in RNA binding, or 2C oligomerisation (20, 84).

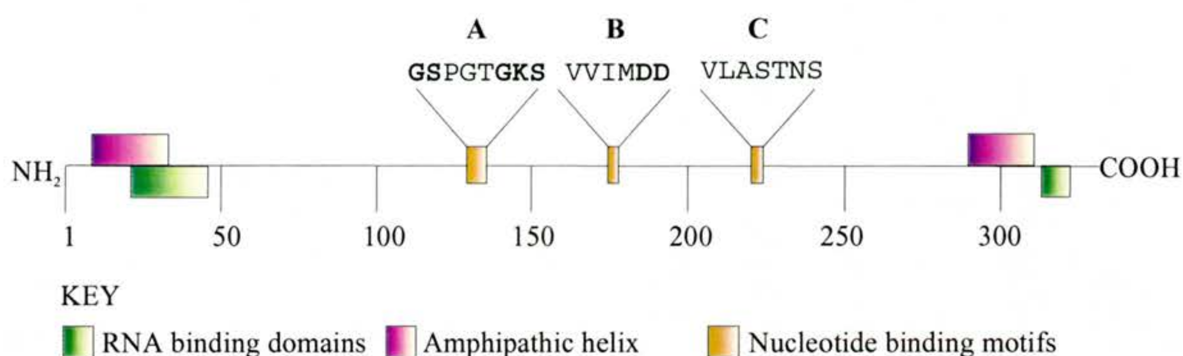

Figure 1.4.2. Positioning of RNA binding domains, nucleotide binding motifs and amphipathic helices on the 2C gene.

Work by Wileman and Ryan laboratory showed FMDV 2C produces rosette-like structures similar to those described earlier, and that were absent when a single amino acid within the NTP-binding domain of 2C (amino acid 116) was altered. They also showed that 2C could not bind to trypsinised membrane preparations, which puts doubt over the role of the amphipathic helices.

Recently Knox *et al* found that FMDV 2C bound to membranes through interaction with the C-terminal region of a protein kinase A (PKA) anchoring protein 10 (AKAP 10), which is a membrane bound protein. This was seen initially in a yeast two-hybrid screen then confirmed by biochemical binding assays using a GST-fusion of AKAP 10 C-terminal and radiolabelled 2C

proteins. 2C binds to the C-terminal region of AKAP 10 (termed IAP3) at the same site as the regulatory subunit (RI or RII) of PKA. A 20 amino acid fragment of the N-terminus of 2C is sufficient for the interaction with AKAP 10.

1.5 Protein Kinase A Anchoring Proteins.

Activation of G proteins by transmembrane receptors causes an increase in cAMP, which results in activation of cAMP dependant protein kinase (PKA), one of 1000's of kinases in human cells. As an inactive holoenzyme, PKA consists of 2 regulatory (R) and 2 catalytic (C) subunits (34, 18). The binding of 2 cAMPs to each regulatory subunit stimulates dissociation, therefore releasing free active catalytic subunits. There are three types of catalytic ($C\alpha$, $C\beta$ and $C\gamma$) and four regulatory ($R_I\alpha$, $R_{II}\alpha$, $R_I\beta$ and $R_{II}\beta$ [49, 43]) subunits, and PKA is divided into type I (containing either $R_I\alpha$ or $R_I\beta$) and type II (containing either $R_{II}\alpha$ or $R_{II}\beta$). PKA type I is mainly soluble, whereas type II is generally found in association with cell structures (43, 18). The binding affinity of the different regulatory subunits to cAMP varies; $R_I\alpha$ has a relatively higher affinity than $R_{II}\alpha$, which in turn has a greater affinity than $R_{II}\beta$ (28). Therefore PKA I and PKA II decode cAMP signals depending on their duration and intensity.

PKA has many roles within a cell: phosphorylation of cAMP response element binding (CREB) causing expression of specific genes (37); control of transport of protein through the Golgi (61); modulation of Ca^+ channels (27); activation of glycogen break down via glycogen phosphorylase (37); and, prevention of cell apoptosis by inactivating BAD (32). These few examples of PKA's many roles

reveals that tight regulation is required to ensure specific signals produce relevant responses. This complex control is poorly understood, however it is known that most type II PKA, and some type 1 PKA, can be localised within the cell via interaction with different AKAPs (49, 43). AKAPs can bind to a variety of cellular organelle including structural proteins and organelles of the secretory pathway, therefore compartmentalising PKA close to relevant substrates and ensuring specificity of cellular phosphorylation events (reviewed in 78).

AKAPs bind the dimerised RII (64, 78) targeting the inactive form of PKA. RII N-terminal amino acids 6-30 are required for both dimerisation and AKAP binding, whilst amino acids 3 and 5 are only necessary for the former (49). RII dimerisation creates an X-type four-helix bundle motif with extended hydrophobic face (64) onto which the hydrophobic face of the amphipathic helix in AKAPs (residues 14-18) can bind (18, 25, 63). Most AKAPs have been shown to bind RII, however some bind only RI and others bind both RI and RII – so called 'duel-specificity' AKAPs (D-AKAPs) (34, 33). Mutational studies indicate consensus positions 2 and 10 on the amphipathic helix are necessary for PKA binding, whilst position 6 determines isoform specific binding. The size of the side chains at these residues is important, at position 2 they are generally small, at 10 they are normally large (and hydrophobic) and at position 6 they are either large (type II PKA) or small (type I PKA) hydrophobic side chains. These residues, whilst necessary, are not sufficient for PKA binding (57).

AKAP 10, or D-AKAP 2 as it was originally designated, was first identified as a duel specificity AKAP (35). It binds $R_I\alpha$, $R_{II}\alpha$, and $R_{II}\beta$ but not $R_I\beta$, and has

two putative regulator of G protein signalling (RGS) domains (93). Full-length AKAP 10 from different tissues have dissimilar molecular weights (88), and it has a complicated mRNA expression pattern (35), therefore AKAP 10 may have alternative splicing patterns, as is seen with other AKAPs, such as D-AKAP 1 (36). Full length human AKAP 10 is located mainly on mitochondria, however is seen dispersed around the cell. If other isoforms exist they may be targeted to other locations within the cell.

1.6 Aims and Objectives.

This project aims to identify whether other picornavirus 2C proteins interact with AKAP 10, thereby indicating if this is a general function of 2C in all picornaviruses or a specific function of FMDV 2C. If indeed this function is found to be universal amongst *picornaviridae* then further theories and investigations can be made to explain why this occurs. Any further work will require a large amount of soluble 2C protein; therefore a general expression system for producing pure concentrated protein will be investigated.

2 Material and Methods.

Unless stated otherwise all chemicals were from Sigma and all other materials were obtained from Promega.

2.1 Plasmids.

Plasmids pGEM-T, pGEM-7ZF, and pBLUESCRIPT II SK were used for cloning and for programming *in vitro* translation reactions. These plasmids contain an ampicillin-resistance gene, both the T7 and SP6 promoters and the α peptide of the LacZ gene (also containing the multiple-cloning site). Successfully ligated vectors were selected for using the blue-white screen when grown in the presence of X-Gal (final concentration 40μg/ml) and ampicillin (final concentration of 100μg/ml). Once cloned into these vectors the gene was transferred to a bacterial expression vector, either pGEX 4T-1 or pGEX 4T-2 (Pharmacia Biotech). These vectors also contained the ampicillin-resistance gene.

2.2 Bacterial Strains.

E.coli JM109 strain was used for plasmid proliferation by growth in LB broth with ampicillin (100μg /ml) when required. The *E.coli* strain B834 was used for protein expression and was also grown in LB broth with ampicillin (final concentration of 100μg /ml).

2.3 Agar Plates.

For cloning into the pGEM and pBLUESCRIPT translation vectors special agar plates were made. L-agar (500ml) was melted in a microwave (low power) for approximately 40 minutes, whilst constantly mixing contents. The bottle was kept in a water bath at 55°C until hand cool, at which point ampicillin (100μl, 100mg/ml), X-Gal (80μl, 50mg/ml) and IPTG (100μl, 0.1M) were added. Plates were poured to a ½cm depth and left to set and dry next to a flame. The plates were stored at 4°C for a maximum of a month. Plates for all other purposes were made as above, excluding the X-Gal and IPTG.

2.4 Agarose Gel Electrophoresis.

DNA separation was achieved by horizontal agarose gel electrophoresis. The 1% gels consisted of 3g SeaKem LE agarose (FMC Bioproducts) prepared in 300ml 1×TAE (Tris-acetate; EDTA) solution. This was heated in the microwave for 5 minutes (full power) to melt the agar and then cooled to hand touch. Ethidium bromide (15μl, 5mg/ml) was added before pouring into the casting platter. The running buffer was 1700ml 1×TAE with ethidium bromide (85μl, 5mg/ml). Usually 8μl of 1Kb DNA marker was loaded alongside the samples, which were loaded in 6× Blue/Orange loading dye (15% Ficoll 400, 0.03% bromophenol blue, 0.03% xylene cyanol FF, 0.4% orange G, 10mM Tris-HCl (pH 7.5), 50mM EDTA). Gels were typically set at 180V for 45min and were then visualised via an ultraviolet transilluminator.

2.5 Oligonucleotide Primers.

Primers were obtained commercially from Interactiva. The primers were designed to include the full-length 2C sequences specific for the individual picornavirus. The forward primers contained a start codon (ATG) and the reverse contained the stop codon (TGA) required for the *in vitro* translation reactions. The forward and reverse primers were designed to contain restriction enzymes sites, chosen so the PCR product could be cloned into p-GEM 7ZF then the insert moved into either p-GEX 4T-1 or 4T-2. The restriction enzymes used were *Bam HI*, *Eco RI* and *Sma I* and the specific sites used in the primer depended on the presence of these within the 2C sequences (table 2.5.1). To clone CA9 2BC the same reverse primer was used; however the forward primer was made to interact with the beginning of the beginning of the 2B protein. Once again both 2B and 2C sequences were checked to ensure they did not contain the restriction enzyme sites used in the primers.

Primer	Sequence	Restriction sites
CA9 forward	GAA TTC ATG AAT GAT AGC TGG CTC AAG AAA	*EcoRI*
CA9 2BC forward	GAA TTC ATG ACG CTA TGG AGC AGG GTG TGA	*EcoRI*
CA9 2C reverse	CCC GGG TCA CTG GAA TAA TGC CTC AAG GGA	*SmaI*
ECHO 22 forward	GAA TTC ATG GGA CCT TTT AAA GGA TTC AAT	*EcoRI*
ECHO 22 reverse	CCC GGG TCA CTG ATT TTC CAA TTG TTG TTT	*SmaI*
HAVHM175 forward	CCC GGG ATG AGT TTT TCC AAC TGG TTA AGA	*SmaI*
HAVHM175 reverse	GGA TCC TCA CTG AGA CCA CAA CTC CAT GAA	*BamHI*
HeCVSoton forward	GAA TTC ATG GGA CCT GAA GAC TTG GCA CGA	*EcoRI*
HeCVSoton reverse	GGA TCC TCA CTG TAG CTG GAA CTC ATC CTG	*BamHI*
TME forward	CCC GGG ATG GGG CCT CTA CGC GAG GCC AAT	*SmaI*
TME reverse	GGA TCC TCA CTG GGC AAC CAA GCT GTT CAT	*BamHI*

Table 2.5.1 List of primers used to clone Picornavirus 2C proteins

2.6 Polymerase Chain Reaction.

PCR reactions (100μl) contained; PCR buffer (10μl), dNTPs (1μl, 10mM stock), oligonucleotide primers (0.5μl each; 100ρmol/μl stock) and DNA template (1μl), water then being added to a final reaction volume of 100μl. After mixing, paraffin (100μl) was placed over the top of the reaction mixture and the tubes were positioned in a programmable thermocycler. The initial parameters were 94°C for 5 minutes, then 85°C for 1 minute during which DNA *Taq*-polymerase (1μl) was added. The PCR then entered the cycling phase with 25 cycles: 94°C for 1 minute (denatures the DNA), 55°C for 1 minute (primers anneal to the DNA template) and 72°C for 2 minutes (extension of the primers). The reaction was completed with a final extension phase of 5 minutes at 72°C. The reaction mixture was then pipetted from below the paraffin into a new Eppendorf tube, and stored at -20°C.

2.7 DNA Purification from Agarose Gels.

In general cDNA was separated by agarose gel electrophoresis, then purified using QIAquick Gel Extraction Kit (QIAGEN, Hybaid Ltd.). The DNA extraction was carried out according to the manufactures instructions, completing all optional steps. Briefly, the DNA band of interest was excised from the agarose gel, removing all excess gel. To the weighed sample 3 volumes buffer QG to 1 volume gel (100mg ~ 100μl) was added. This was incubated at 50°C for 10 minutes to dissolve the gel, before addition of one gel volume isopropanol. The sample was then placed in a QIAquick column and centrifuged for 1 minute (13,000rpm). The column was washed with 0.5ml QG buffer (13,000rpm for 1

minute), then with 0.75ml PE buffer (13,000rpm for 1 minute). The column was centrifuged for an additional minute to remove residual ethanol, and the DNA eluted from the column with RNase-free water (50μl), re-centrifuged (13,000rpm for 1 minute) and finally stored at -20°C.

2.8 Phenol/Chloroform Extraction.

Phenol/chloroform extraction was used to further purify DNA for translation reactions. One volume phenol/chloroform was added to one volume DNA, mixed until milky white, and then centrifuged for five minutes at 13,000rpm. The top layer was transferred to a clean tube. This was repeated twice, after which ethanol (2.5 volumes, 100%) and Na acetate (0.05 volumes, 2M) were added. This was incubated for 30 minutes at -70°C, and then centrifuged (13,000rpm at 4°C) for 30 minutes. The supernatant was removed and the pellet washed with ethanol (500μl, 70% v/v). After centrifuging for 10 minutes at 4°C (13,000rpm) the ethanol was removed and the pellet air-dried. The pellet was then resuspended in water (20μl) and stored at -20°C.

2.9 DNA Digestion.

DNA digestion was generally used for cloning, cutting both the vector and the insert with the two restriction enzymes as determined by the primers used in the insert. A normal 20μl reaction contained; 10× R.E. buffer concentrate (2μl, specific for each enzyme, as indicated by the manufacturer), acetylated BSA (0.2μl), DNA (4μl) and enzyme (1μl of each) in water (11.8μl). The reaction

mixture was gently mixed, then incubated at 37°C for three hours. When *Sma I* was used the reaction was performed in two stages. The reaction mixture was made as above, excluding *Sma I*, and incubated at 37°C for three hours. Then *Sma I* was added and the reaction mixture incubated at 25°C for three hours. In situations where different buffers were required, the reaction was also performed in two steps. The reaction mixture was made using the buffer with the lowest salt concentration, the appropriate enzyme added and incubated for three hours at the appropriate temperature. The second buffer and enzyme were then added to the reaction, which was incubated for a further three hours at the correct temperature. The samples were separated on an agarose gel to verify digestion, and the digested product purified by DNA gel extraction.

2.10 Ligation.

When possible, ligation steps were prepared with the plasmid:insert ratio between 3:1 and 1:3, preferably 1:1. To estimate the concentration of the insert, 1µl of the sample was compared to 0.5µl and 1µl of the plasmid pGEM-3ZF on an agarose gel (0.1µg and 0.2µg respectively). The ligation reaction mixture for the plasmid pGEM-T was as follows: 2X ligase buffer (5µl), pGEM-T vector (1µl), PCR product (2µl), T4 DNA ligase enzyme (1µl) and water (1µl). These were incubated for 2 hours at 16°C, overnight at 4°C or over the weekend at 4°C. Two control reactions were carried out; one with a control insert in place of the PCR product, the other with both the insert and PCR product replaced with water.

For the other vectors (pGEM-7ZF, pBLUESRIPT II SK, pGEX 4T-1 and pGEX 4T-2) the following amounts were used: 10X ligase buffer (2µl), pGEM-T vector (2µl), PCR product (4µl), T4 DNA ligase enzyme (1µl) and water (11µl). Two control reactions were carried out; in the first the insert was replaced by water, in the second the insert and PCR product were substituted for water. These reactions were generally incubated overnight at 16°C.

2.11 Transfection.

Transfections were used to amplify successfully ligated DNA. Competent JM109 cells were defrosted on ice. pGEM-T ligation reactions (2µl) or pGEX ligation reactions (10µl) were placed in 1.5ml eppendorfs on ice. To each of these competent *E. coli* cells (50µl) were added and the tubes gently flicked. These were incubated on ice for 20 minutes, heated to 42°C for 1 minute and then placed on ice for at least 2 minutes. SOC medium (950µl) was added to each reaction and, which were then incubated at 37°C for one hour. The mixture (100µl) was then spread onto agar plates. The remaining medium was centrifuged at 13,000rpm for two minutes. The supernatant (800µl) was removed; the cells were resuspended in the remaining medium (100µl) and spread onto agar plates. These were incubated overnight at 37°C. Ampicillin plates were used for expression vectors, and the ampicillin, X-GAL and IPTG plates for the translation vectors.

Transfections were also performed when vectors needed to be amplified from QIAGEN Miniprep samples. Since this technique used fully ligated vectors, it had a greater success rate and therefore required less cells and DNA. The experiments were performed as before, however only 2μl DNA and 20μl cells were used in each reaction. When expression vectors were transfected into *E. coli* strain B834 cells the protocol was slightly altered again. As before the DNA used was from QIAGEN Miniprep kits, therefore less was required. *E. coli* B834 cells were made competent in the laboratory, rather than the competent *E. coli* JM109 cells which were obtained commercially. *E. coli* B834 cells were less competent than the *E. coli* JM109 cells and, therefore, more *E. coli* B834 cells were used routinely. The experiments were performed as described above, however using, DNA (2μl) and *E. coli* B834 cells (50μl).

2.12 Small Scale DNA Preparation.

Once cloned plasmid DNA was purified from JM109 bacterial cells using QIAprep Miniprep kit (QIAGEN, Hybaid Ltd.), following the manufacturers instructions. A sample (3ml) was taken from an overnight culture (5ml), and centrifuged for 2 minutes at 13,000rpm. The pelleted *E. coli* cells were resuspended in Buffer P1 (250μl) and then buffer P2 (250μl) was added. After gently mixing, buffer N3 (350μl) was added and again mixed by inverting. These were centrifuged for 10 minutes, at 13,000rpm, to remove cellular debris. The supernatant containing the DNA was poured onto a column that was centrifuged for 1 minute at 13,000rpm. The column was washed with buffer PB (0.5ml) and then buffer PE (0.75ml) by centrifugation at 13,000rpm for 1 minute

after each addition. This was centrifuged for an additional minute at 13,000rpm, after which the DNA was eluted in water (50μl) collected by re-centrifugation (13,000rpm, 1min). The DNA was stored at -20°C.

2.13 Large Scale DNA Preparation.

When a large quantity of DNA was required it was replicated using QIAGEN Plasmid Maxi Kit (QIAGEN, Hybaid Ltd.) following manufacturers instructions. Cells containing plasmid grown overnight in LB (100ml) were harvested by centrifuging at 4,500rpm for 15 minutes at 4°C. The resulting pellet was then resuspended in buffer P1 (10ml), mixing until no lumps remained. To the lysed cells buffer P2 (10ml) was added. This was mixed gently and incubated at room temperature for 5 minutes. Chilled buffer P3 (10ml) was added before the mixture was placed in the cartridge, where it was incubated for 10 minutes at room temperature. At the same time the QIAGEN-tip 500 column was equilibrated with buffer QBT (10ml). The lysate was then applied to the column by plunging the cartridge. After the column emptied by gravity, it was washed twice with buffer QC (30ml) and the DNA eluted with buffer QF (15ml). The DNA was precipitated by addition of isopropanol (10.5ml), which was mixed and centrifuged for 30 minutes (4°C at 10,500rpm). The pellet was then washed with 70% ethanol (5ml) and centrifuged (10 minutes at 10,500rpm). The resulting DNA pellet was air dried for 10 minutes and then resuspended in water (500μl). The DNA was stored at -20°C

2.14 Plasmid Screening,

To test whether plasmids contained the insert of interest, each DNA sample was digested using the restriction enzymes corresponding with the sites in the primers as earlier described. With pGEM-T vectors restriction enzymes were chosen to determine whether cloning had been successful, and also to demonstrate the orientation of the insert (one restriction enzyme site in the MCS the other in the insert).

In general a 10µl reaction was used in which 1µl 10× buffer, 0.1µl BSA acetylated, 1µl DNA and 0.5µl of each enzyme was added to 6.9µl water. The reaction mixture was gently mixed and then incubated at 37°C for at least an hour. When *Sma 1* was used this was added separately after an hour, then the reaction was incubated at 25°C for at least an hour. If the enzymes required different buffers the reaction mixture was made using the buffer with the lowest salt concentration, the appropriate enzyme added and incubated for one hour. The second buffer and enzyme were then added to the reaction, which was incubated for a further hour. The samples were then loaded onto an agarose gel to show the size and purity of each insert.

2.15 Nucleotide Sequencing.

Plasmids containing the insert were sequenced at the University of St Andrews DNA sequencing unit using ABI PRISM™ 377 DNA sequencer. Computer-assisted alignments and comparisons of sequences were carried out using the computer programme DNA-MAN. For each reaction, DNA (5µl),

oligonucleotide primer (0.5µl, of 10µM stock) and water (6.5µl) was used. These were initially sequenced using the T7 promoter primer. Any discrepancies within the sequencing were investigated by further sequencing reactions with other primers.

2.16 Glycerol Stock.

Once cloned, *E. coli* JM109 cells containing the plasmid were made into glycerol stocks for future use. Freshly grown bacterial culture (0.85ml) and sterile glycerol (0.15ml) were placed in a screw cap storage tube. This was then vortexed and placed on dry ice for 10 minutes to ensure the sample was properly frozen. The samples were then transferred to -70°C where they were stored. To recover cells, the surface of the frozen stock was scraped and streaked across an LB agar ampicillin plate. This was incubated overnight at 37°C.

2.17 *In Vitro* Translation Reactions.

In vitro translation reactions were performed using the TNT Quick coupled transcription/translation system. In general for each reaction the following amounts were used; master mix (20µl), ^{35}S methionine (1.5µl) and DNA (1µl). These were gently mixed and then incubated at 30°C for 90 minutes in Eppendorf tubes. The reaction was centrifuged for 30 minutes at 4°C, 14,000rpm to remove debris. The supernatant was transferred to new tubes and stored at -20°C. Generally, the reaction mixture (2µl) was denatured in sample

buffer (2μl; glycerol, SDS 0.2M Tris pH 6.8 and 4% β-mercaptoethanol) at 100°C for two minutes for analysis by SDS-PAGE.

2.18 SDS PAGE.

The SDS-PAGE consisted of two parts: the running gel and the stacking gel. The running gel was prepared with acrylamide (1.25ml, 40% w/v), bisacrylamide (0.863ml, 2% w/v), water (0.92ml), Tris-HCl pH8.8 (1.875ml, 1M), APS (30μl, 10% w/v), SDS (50μl, 10% w/v) and TEMED (8.3μl). After all these were mixed, they were immediately loaded onto the gel rig and overlaid with water. Once set, the water was removed and the running gel completely dried. The stacking gel was prepared using acrylamide (0.2ml, 40% w/v), bisacrylamide (0.33ml, 2% w/v), water (1.17ml), Tris-HCl pH6.8 (0.25ml, 1M), APS (20μl, 10% w/v), SDS (20μl, 10% w/v) and TEMED (4μl). This was mixed, then immediately loaded on top of the running gel, the combs inserted and left to set.

The samples (in sample buffer, previously denatured) were loaded onto the gels. The proteins were separated with a current of 40mA. Once run the gels were stained in Coomassie Brilliant blue R250 (0.2%, w/v), methanol (50%, v/v) and acetic acid (10%, v/v), then destained in methanol (25%, v/v) and acetic acid (10%, v/v): throughout both step the gels were gently agitated. Where applicable, the distribution of radiolabelling was determined by phosphorimaging.

2.19 Preparation of Sepharose Beads.

Cells containing either GST or GST-IAP3 pGEX-2T plasmids were grown overnight. An inoculum of each culture (50μl) was added to LB ampicillin (2ml) and grown at 37°C until the absorbance (600nm) reached between an O.D. of 0.4-0.6. IPTG (0.5ml, 0.1M) was then added to the cultures to induce protein expression. After 2 hours the cells were pelleted by centrifugation (2 minutes, 13,000rpm), the supernatant removed and the cells resuspended in cold PBS (500ml) containing PMSF (1mM) and benzamidine (2mM). The cells were sonicated twice for 20 seconds and centrifuged for 2 minutes (13,000rpm). Each supernatant was added to sepharose beads (100μl) and incubated for 10 minutes at room temperature on a carousel, to produce either GST bound sepharose beads (GST beads) or IAP3-GST bound sepharose beads (IAP3 beads). The beads were washed eight times in PBS (1ml; supplemented with PMSF and benzamidine), and then two times in PBS. They were stored in a 50% slurry with PBS (NaN_3) at 4°C until required. Before use 1ml PBS (10mg/ml BSA) was added to the required bead volume, and this was incubated for 30 minutes at room temperature. This was repeated and then they were resuspended in PBS/BSA as a 50% slurry.

2.20 Binding Assay.

Two polypropylene tubes were set up for each TNT reaction, with binding buffer (1ml; 20mM Tris pH 7.5), potassium acetate (100mM), DTT (1mM), BSA (10mg/ml), NP40 (0.05%, v/v), and 10μl from the TNT *in vitro* translation reaction. GST beads (10μl) were added to one-tube and IAP3 beads (20μl) to the

other. The disparity in the amount of beads is attributable to the larger amount of GST compared with IAP3 on the beads. These reactions were placed on a carousel for 30 minutes at 4°C. The beads were then washed 5-6 times in 1ml ice cold binding buffer without the BSA. Sample buffer was added and the proteins denatured for 2 minutes at 100°C.

2.21 Preparation of Competent B834 Cells.

LB medium (2.5ml) was inoculated with a single colony from an LB plate of *E. coli* B834 cells, and grown overnight at 37°C. LB (75ml) containing $MgSO_4$ (20mM) was then inoculated with 0.75ml of the overnight culture. The cells were grown at 37°C until the absorbance at 600nm reached 0.4-0.6, and then pelleted by centrifugation (4,500rpm at 4°C) for 5 minutes. All the following steps were carried out at 4°C using chilled pipettes, tubes and flasks with minimal handling to protect the cells. The cells were gently resuspended in ice-cold, filter sterilised, TFB1 (30ml; 30mM potassium acetate, 10mM calcium chloride, 50mM manganese chloride, 100mM rubidium chloride and 15% glycerol, pH 5.8 - using acetic acid), and incubated on ice for 5 minutes. After pelleting the cells by centrifugation (4,500rpm, 4°C) for 5 minutes, they were gently resuspended in ice-cold TFB2 (3ml; 10mM MOPS pH 6.5 -using potassium hydroxide-, 75mM calcium chloride, 10mM rubidium chloride and 15% glycerol) and incubated on ice for 5 minutes. The cells where then placed in screw cap tubes in 100µl and 200µl aliquots and quick frozen on dry ice. They were stored at -70°C.

2.22 2C Protein Expression.

Expression of 2C initially followed the following protocol, however this was adapted to obtain the best expression, as detailed in the results section. An agar plate was streaked with freshly transfected *E. coli* B834 cells containing the expression vector and incubated overnight at 37°C. An LB culture (5ml) was inoculated with a single colony and grown overnight at 37°C. The next day LB was inoculated with 100th volume of the overnight culture and grown at 37°C until the absorbance at 600nm reached 0.6-0.8. IPTG was added to a concentration of 0.1mM and the cells incubated at 37°C for a further three hours. The cells were then centrifuged for 5 minutes at 14,000rpm, 4°C. The supernatant was removed and the pelleted cells frozen at -20°C. This prevents the protein from degrading and helps lyse the cells for protein extraction.

2.23 2C Protein Extraction.

The frozen *E. coli* B834 cells expressing the protein were resuspended in cold PBS (supplemented with 1mM PMSF and 2mM benzamidine). The cells were then sonicated using a cell disrupter for 2 x 30 seconds with cooling on ice in between. The sonicate was centrifuged for 30 minutes at 18,000rpm at 4°C leaving two fractions; the supernatant containing the soluble proteins, and the pellet containing cellular debris and insoluble proteins. The supernatant was transferred into new tubes and glutathione sepharose beads were added (1ml 50% slurry with PBS per 100ml culture). This was incubated on a carousel at 4°C for 30 minutes to allow the GST fusion protein to bind to the beads. The beads were then washed 8-10 times in PBS (supplemented with 1mM PMSF and 2mM

benzamidine) and, if necessary, stored in a 50% slurry with PBS (supplemented with 1mM PMSF and 2mM benzamidine) at 4°C. Before being used the beads were washed twice in PBS.

2.24 2C Protein Elution.

Sepharose beads containing GST fusion 2C protein were washed 3 times with PBS. One volume Glutathione Elution Buffer (GEB) was added per 1 bed volume of beads. This was gently mixed and incubated at room temperature for 10 minutes. After centrifugation for 5 minutes (14,000rpm) the supernatant was collected into a new tube. These steps were repeated another two times, pooling the supernatants. The supernatants were then dialysed three times in 500ml PBS for 1 hour and stored at 4°C.

2.25 Removal of GST from Protein 2C.

Cleavage of GST from 2C was achieved using thrombin. When cleaving directly from the sepharose beads, 50 units of thrombin per 1ml of beads (bed volume) and one bed volume of PBS were placed in a tube with the beads, which was then incubated on a carousel for 16 hours at room temperature. The beads were centrifuged and the supernatant transferred to a new tube. This should contain the cleaved protein of interest and was stored at 4°C until required. When cleaving protein eluted from the beads, 15 units of thrombin per 1ml eluate was used. This was incubated on a carousel for 16 hours at room temperature. The

solution was stored at 4°C until required, and contained both GST and the cleaved protein of interest.

3 Results.

3.1 Cloning.

The initial aim was to clone the 2C gene from the following picornaviruses into both translation and bacterial expression vectors; CA9, ECHO 22, FMDV, HAV HM175, HAV wt1, HAV wt2, HeCV, HRV 85 and TME. The FMDV 2C (gift of Vanessa Cowton) and the HRV 85 2C, 2BC and 2ABC (gift of Nicolas Moir) had previously been cloned in the laboratory into both translation (pGEM) and expression (pGEX) vectors.

3.1.1 Polymerase Chain Reaction Amplification of cDNAs Encoding Picornavirus 2C Proteins.

The polymerase chain reaction amplification of the 2C gene produced bands of 1KB, as expected, for all the different picornaviruses (CA9, ECHO 22, HAV HM175, HAV wt1, HeCV, and TME), except HAV wt2. The HAV PCR was completed using primers designed for the cell culture adapted HAV since the sequences for either wild type HAV were not available. These primers were not specifically designed for wild type HAV, thus explaining why the PCR for HAV wt2 did not work. The CA9 2BC PCR produced a band of 1.3KB as expected. Figure 3.1.1 demonstrates the bands produced by these PCR reactions.

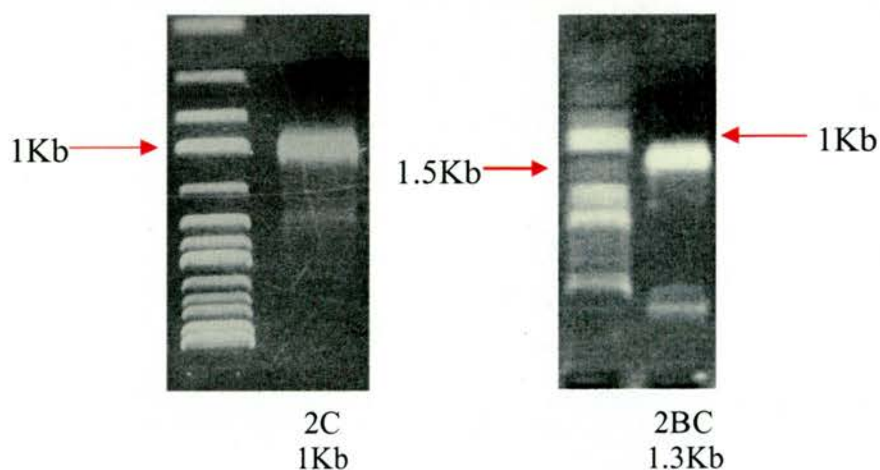

Figure 3.1.1 Examples of two agarose gels showing the 1Kb and 1.3Kb products from the 2C and 2BC PCR reactions respectively.

3.1.2 Translation vectors.

The primers used for the PCR step were designed so the inserts were suitable for ligation into both pGEM-7ZF and bacterial expression vectors. The intention was to cut the PCR products with appropriate restriction enzymes and ligate them into the pGEM-7ZF vector (figure 3.1.2), then to transfer them to the bacterial expression vector system.

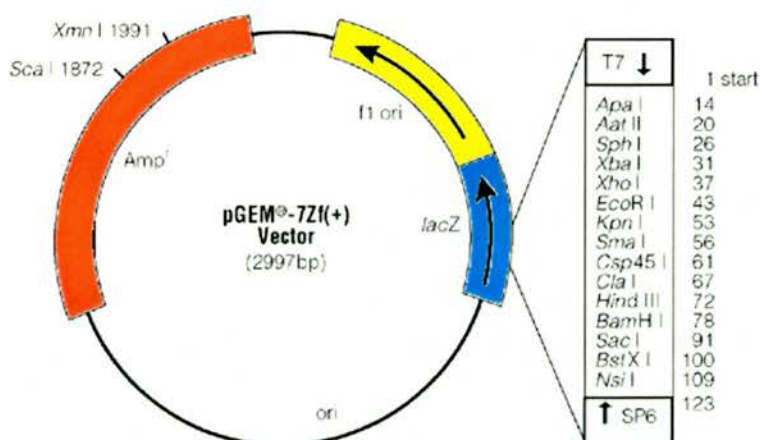

Figure 3.1.2 The vector map of the plasmid pGEM-7ZF (Promega)

After numerous attempts only TME was successfully cloned. The difference between restricted and non-restricted PCR product is not detectable by gel electrophoresis and, therefore, the cloning strategy was altered. The pGEM-T system (figure 3.1.3) requires no digestion of the PCR product. Ligation can be achieved directly from the PCR product using the adenosine 3′-overhangs left by TAQ polymerase, since the pGEM-T vector is pre-cut and supplied with thiamine 5′-overhangs. Hence no digestion step is required and the PCR product is directly ligated into the plasmid. Using this method cDNA encoding all of the 2C proteins, with the exception of the HAV products, were successfully cloned.

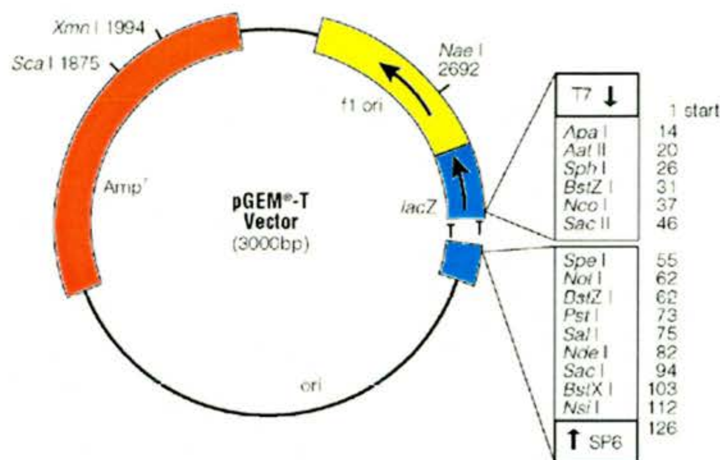

Figure 3.1.3 The vector map of the plasmid pGEM-T (Promega).

PCR products may be inserted into the pGEM-T system in either orientation. The vector contains, however, a T7 promoter for one direction, and a SP6 for the other. In order to determine in which orientation inserts had been ligated, digestion mapping was performed. The results (not shown here) were used to select clones that were oriented in the positive (mRNA) sense with regards the T7 transcription promoter.

Despite numerous attempts, with various parameters altered, cloning cDNA encoding the HAV 2C into the pGEM-T system remained highly problematic. The initial system was returned to and this was eventually successful. This time the pGEM-3ZF, rather than the pGEM-7ZF vector, was used to reduce the number of steps during transfer into an expression vector. This vector differs from pGEM-7ZF only in the restriction enzyme sites in the MCS. CA9 2BC was cloned directly into the pGEM-T system without any complications. All translation vector clones were inspected using DNA sequencing and DNA alignment. The vectors contained exact copies of the respective gene.

3.1.3 Bacterial Expression vectors

Bacterial expression vectors allow the expression of the desired gene in bacterial cells to give a potentially high concentration of the required protein. The system used in these experiments was that of the glutathione S-transferase (GST) fusion vectors. These vectors allow insertion of a gene on the C-terminus of the GST gene. This gene contains an ATG and ribosome-binding site and is under control of the *Lac*-promoter, which is inducible by IPTG. The protein can then be separated from the cellular material in a simple purification step. GST binds to glutathione beads, therefore incubating the cellular lysate with these beads will bind GST, or GST-fusion proteins, and the protein remaining can be washed off in a 'pull-down' assay. The desired protein can then be cleaved from the GST (and beads) using thrombin.

Once cloned into the translation vectors, the genes were then transferred to either the pGEX 4-T1 or pGEX 4-T2 vector system. Both vectors were required since the gene had to be inserted in the correct orientation and in the correct reading frame for expression to occur. The method by which these were transferred was dependent on the restriction enzyme sites both within the gene, the plasmid and those artificially added to either end during PCR.

When cloning the CA9 2BC and 2C and the ECHO 22 2C inserts into an expression vector the following system was used (figure 3.1.4). Using the restriction enzyme sites added by the primers, the required gene was excised from the pGEM-T vector. This was then ligated into a pGEX 4T-1 vector that had previously been digested with the same enzymes (*Eco RI* and *Sma I*). A restriction digestion was used to confirm if they contained the correct insert.

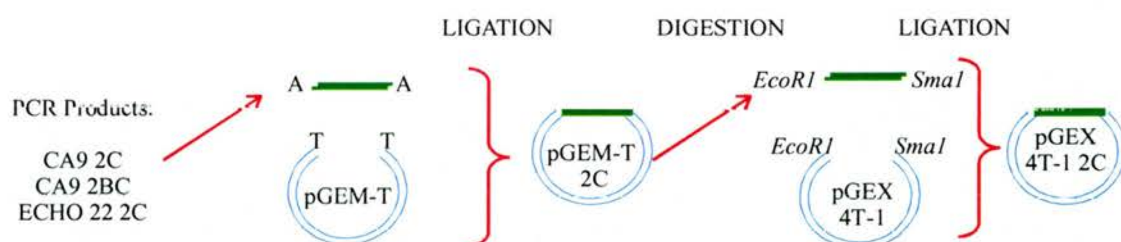

Figure 3.1.4 Diagrammatic representation of the cloning strategy for CA9 2C, 2BC and ECHO 22 2C

For the cloning of HeCV 2C into an expression vector the following method was used (figure 3.1.5). The 2C was excised from the pGEM-T vector using one restriction enzyme built in during PCR (*Eco RI*) and one in the pGEM-T vector (*Not I*). This was ligated into a pGEX 4T-1 vector digested with the same enzymes. Restriction digestion was used to show cloning was successful.

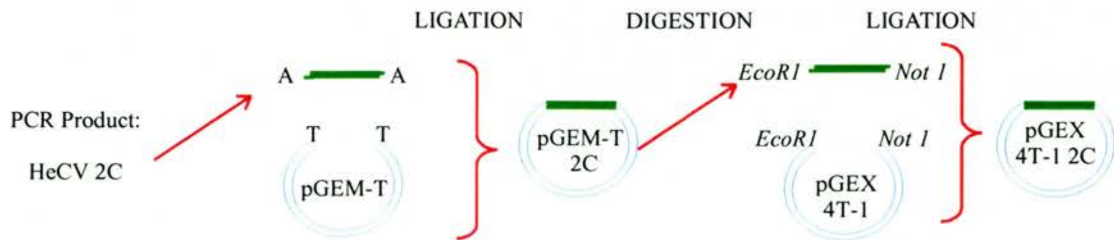

Figure 3.1.5 Diagrammatic representation of the cloning strategy for HeCV 2C

Cloning HAV HM175 2C, HAV wt1 2C and TME 2C into an expression vector was conducted as follows (figure 3.1.6). The TME 2C was excised from the pGEM 7ZF vector using the restriction enzymes built in during PCR (*Sma I* and *Bam HI*). This was ligated into pGEM 3ZF. The HAV's were directly cloned into this vector. All 2C's were cut from this vector using *Sma I* (from primer) and *Sal I* (from MCS of pGEM 3ZF) and ligated into pGEX 4T-2 also cut with these enzymes. Restriction digestion was used to show cloning was successful.

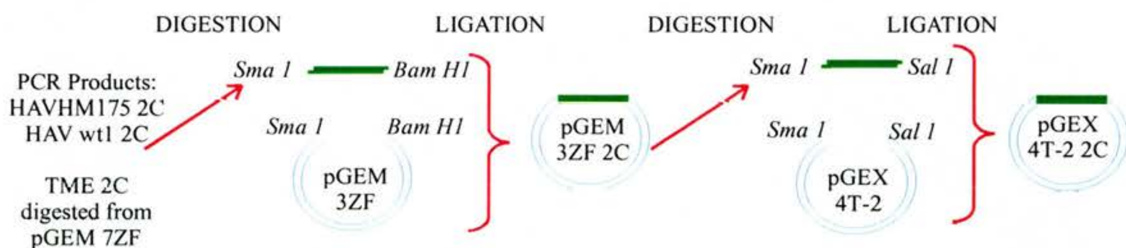

Figure 3.1.6 Diagrammatic representation of the cloning strategy for HAVHM175 2C, HAV wt1 2C and TME 2C.

3.2 2C:AKAP 10 Binding Assay.

Previous studies using yeast two-hybrid screen then confirmed by biochemical binding assays proteins have shown that FMDV 2C binds to the C-terminal portion of AKAP 10 (D-AKAP 2) at the same site as the regulatory subunit (RI or RII) of PKA. For the purpose of these experiments this peptide, IAP3, was

used to test the interaction of other picornavirus 2C proteins with AKAP 10 in a binding assay, which allows for the detection of small amounts of 2C binding to this oligopeptide tract. The GST-IAP3 fusion protein was to be used in all of the binding assays and was, therefore, produced on a large-scale using bacterial expression. GST-IAP3 bound to glutathione beads could then be incubated with radiolabelled 2C (produced by translation *in vitro*), and the washed beads assayed for the presence of 2C by gel electrophoresis – a GST 'pull-down' assay (see section 3.1.3 for a summary of 'pull-down' assays). The presence of 2C bound to IAP3 beads, but not bound to (the control) GST beads would indicate that the 2C from that virus bound specifically to AKAP 10.

3.2.1 *In vitro* Translation of 2C Plasmid Constructs.

In vitro translation systems permits the production of a protein from a defined template, thus greatly reducing the background resulting from endogenous RNA that occurs with *in vivo* systems. The system can also be used to specifically label products; therefore allowing subsequent monitoring of the protein in future experiments. There are many systems available, however the one used in these experiments was a eukaryotic coupled-transcription/translation system, thereby allowing *in vitro* translation directly from DNA samples. The translation reaction mixture contained all required components barring methionine, therefore the protein could be radio-labelled by addition of [^{35}S]methionine.

Once the translation vectors were cloned they were tested for ability to translate the 2C protein. The translation products of all the clones are shown in figure

3.2.1. Some are much stronger translations than others and some are more specific than others (due primarily to internal initiation of translation). The specificity of the translation product should not be too important, since if the binding is specific then only the 2C product will appear in the binding assay. The efficiency of the translation reaction can depend on many factors, such as purity of DNA sample (for example ethanol), the amount of DNA used and the sequence comprising the translation initiation signal.

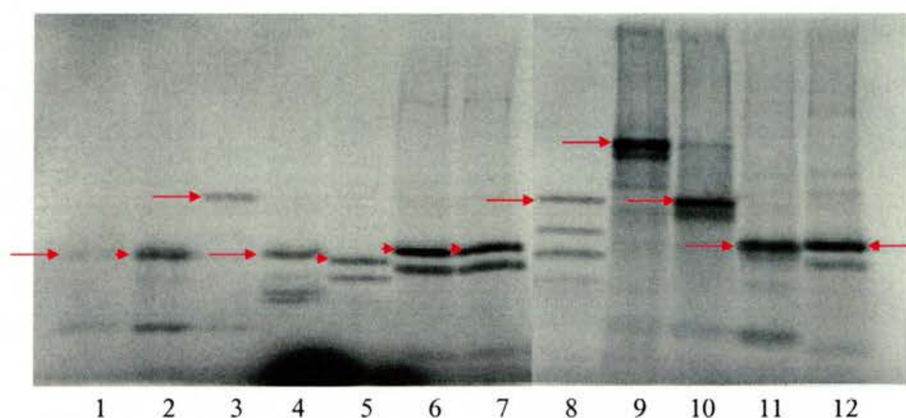

Figure 3.2.1 Translation products from all translation vectors: Ca9 (lane 1), Ca9 pBluescript (lane 2), Ca9 2BC (lane 3), Echo 22 (lane 4), FMDV (lane 5), HAV M175 (lane 6), HAV wt1 (lane 7), HeCV (lane 8), Hrv85 ABC (lane 9), Hrv85 BC (lane 10), Hrv85 C (lane 11), TME (lane 12). The translation products are indicated by the red arrows.

As can be seen the yield of the full-length translation product of CA9 2C is very poor, and as shown more clearly in figure 3.2.2, there is an apparent internal initiation. To try and improve the translation the CA9 2C was transferred directly into pBluescript. The difference in the sequence before the start codon was hoped to improve initiation of the full-length protein. Whilst the translation in general has improved there is still a much stronger translation from the internal initiation site (figure 3.2.1, lane 2 and figure 3.2.2a). In all other cases the top strongest band is the translation product required.

Figure 3.2.2a clearly shows that a smaller product of CA9 2C is produced preferentially over the full-length product. Analysis of the CA9 2C sequence shows that there are 3 start codons (AUG) in the first 55 bases of the sequence, two of which are in the correct reading frame. Either of these may be responsible for the secondary initiation that produced the smaller product.

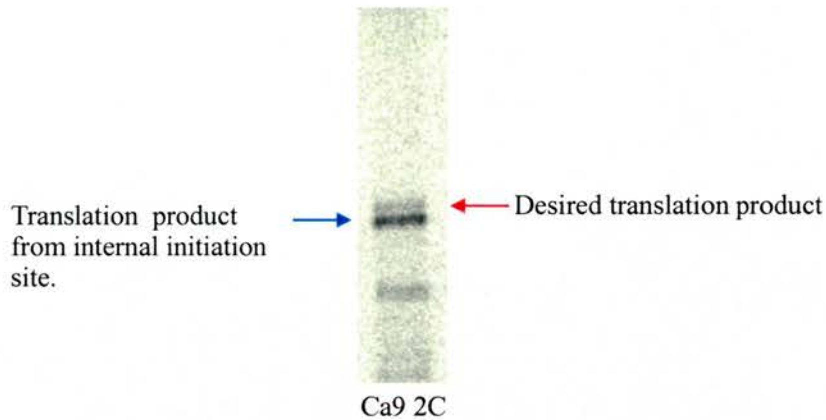

Figure 3.2.2a An example of CA9 2C translation products clearly showing two bands. The upper band is the full-length product, and the lower is the product from the internal initiation.

3.2.2 Initial Binding Assay.

Initially the original parameters as prescribed by prior 2C IAP3 binding work were successful. Those 2C translation vectors that were already cloned were tested, and all of these except CA9 2C worked (figure 3.2.2b). Some, such as FMDV, HRV 85 A and TME bound better than others, whilst CA9 2C did not bind, but this was expected, as the translation of the full-length product was too weak for the assay. The other HRV 85 2C proteins (2BC and 2ABC) were already successfully tested in this system (Nick Moir).

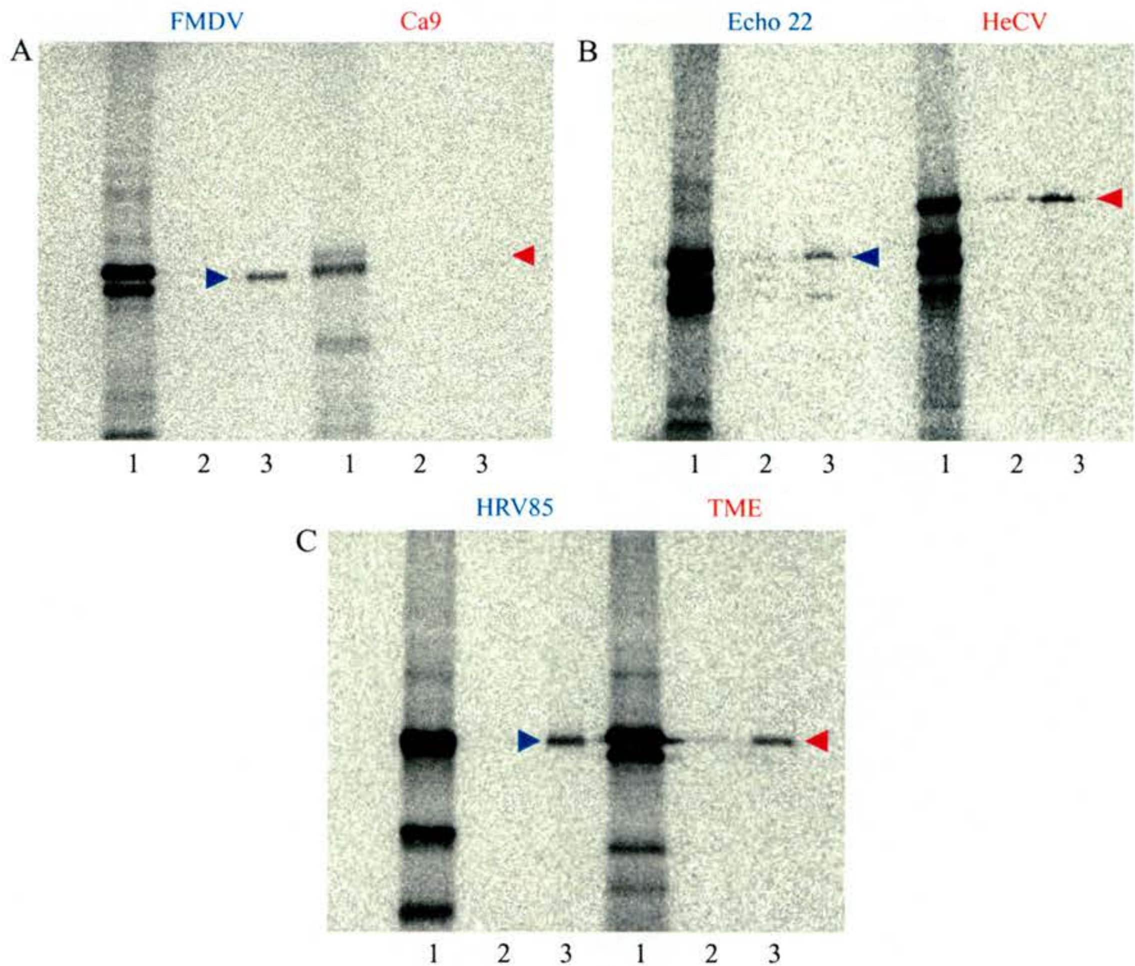

Figure 3.2.2b SDS-PAGE showing results for the binding assay for the viruses CA9, ECHO 22, HeCV, HRV 85 and TME. FMDV is used as a control. In lane one the translation product is shown. Lane 2 shows the translation reaction bound to GST beads and in lane 3 the translation reaction bound to IAP3 beads.

3.2.3 Revised Binding Assay.

Once the other 2C translation vectors were cloned they too were tested in this binding assay, however during the period in which they were cloned better conditions had been established (Binding buffer: 20mM Tris (pH 8), 500mM magnesium acetate, 1mM DTT, 10mg/ml BSA and 0.075% NP40, Wash buffer: the same as binding buffer but without BSA). These conditions were tested, however even those previously shown to work would no longer bind (Figure

3.2.3). The experiment resulted in non-specific binding to both GST beads and IAP3 beads. The previous methodology was used, but this too was unsuccessful for all translation products. Due to the time delay between the experiments it was hypothesised that the solutions may have become contaminated, or destabilised. New solutions were used, but again these were ineffective and the problem with non-specific binding remained.

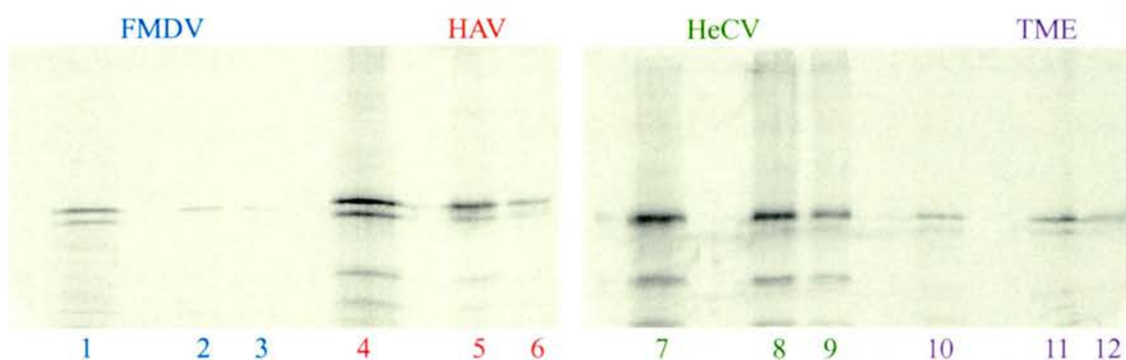

Figure 3.2.3. Non-specific binding that was typical for the failed binding assay. The example shown includes the viruses TME, HAV and HeCV, with FMDV as a control. The translation product is shown in lanes 1, 4, 7 and 10. The translation reaction bound to GST beads is in lanes 2, 5, 8 and 11, whilst lanes 3, 6, 9 and 12 show the translation reaction bound to IAP3 beads.

The binding assay had many parameters that may have been the cause of failure in 2C's binding. The trouble seamed to be with non-specific binding as all the proteins produced in the TNT reactions bound to both bead preparations. Therefore all parameters that may affect non-specific binding were tested. Firstly the number of washes was increased to at least eight vigorous washes, however this made little difference to the binding. Since the purity of the DNA can seriously affect the translation reaction, it is possible that this may affect GST binding. Thus the binding assay was repeated, this time using translation products from DNA cleaned by phenol/chloroform extraction, but no improvement was detected.

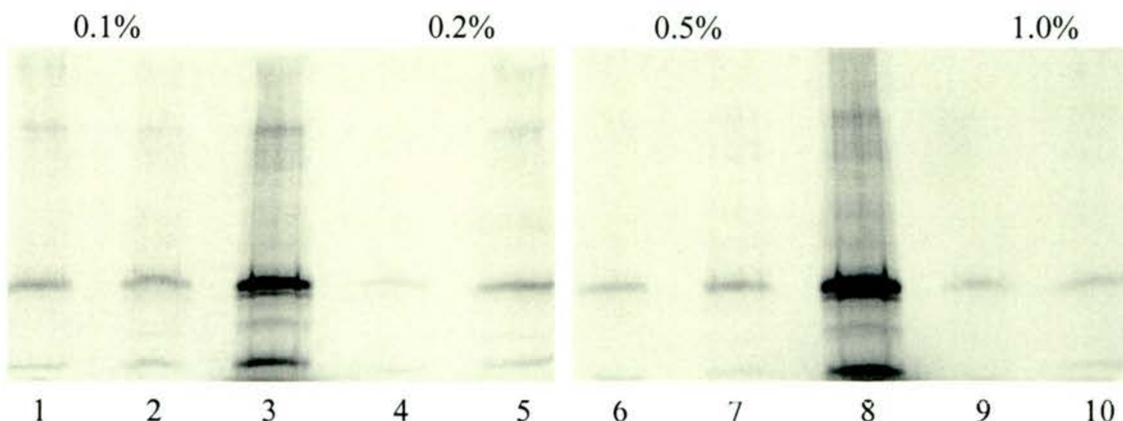

Figure 3.2.4. Differing concentrations of NP40 in the binding assay. The translation product is shown in lanes 3 and 8. The translation reaction bound to GST beads is in lanes 1, 4, 6 and 9, whilst lanes 2, 5, 7 and 10 show the translation reaction bound to IAP3 beads.

Differing concentrations of NP40 were tested using the latest protocol (figure 3.2.4), and there appeared to be a decrease in the non-specific binding. The ideal concentration found was 0.2% NP40, however this still did not completely remove the non-specific binding. Another theory that was tested was that the longer the incubation of 2C with beads the greater the risk of BSA being displaced by the translation products. Thus the incubation period was reduced to 20 minutes using the revised binding assay conditions, with 0.2% NP40. This time the assay was successful, however only for one 2C protein. As can be seen in figure 3.2.5 the HAV wt1 2C protein binds with the IAP3 beads, but not the GST beads, therefore indicating specific binding between HAV wt1 2C and IAP3. A low level of 'background' non-specific protein binding can be seen in the GST lane, although much less than the IAP3 binding.

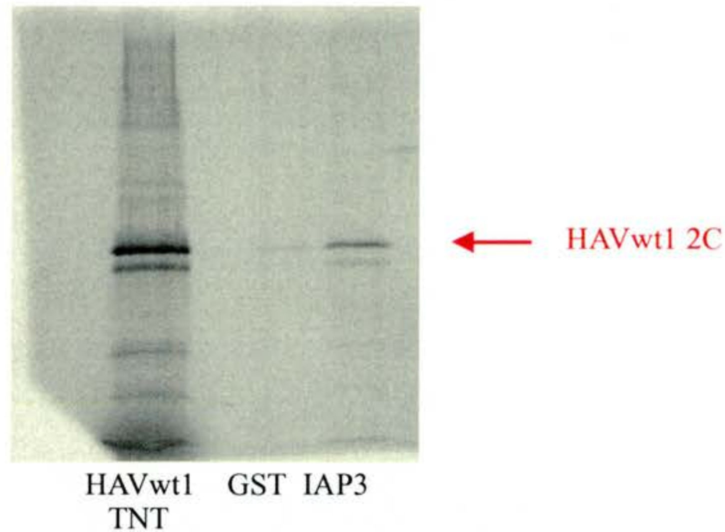

Figure 3.2.5 SDS PAGE depicting the translation product of HAV wt1 2C and the binding of this protein to both GST beads and IAP3 beads.

Whilst these conditions were successful for HAV wt1 2C, there was no improvement seen in any other binding assay, and so a final alteration was tested. The washing buffer contains no BSA, however the TNT reaction mixture is not fully removed in the first wash, indeed a large percentage still remains. Thus it is possible that the wash buffer is removing non-specific bound BSA, before the majority of unbound protein is removed. The binding assay was repeated using two wash buffers. The first, the same as the binding buffer, was used for the majority of washes, the second, containing no BSA, was used for the last few washes. However this showed no improvement in the non-specific binding.

3.3 Expression of Protein 2C.

Once the genes were transferred into the expression vectors, investigation into the best conditions for protein expression started. The initial conditions used were general expression protocol for proteins that are known to be insoluble; as

previous work had indicated the 2C protein was insoluble (Mike Flint, PhD Thesis, University of Reading). Step by step the protocol was optimised for 2C proteins, using HRV 85 2ABC, 2BC and 2C proteins as models.

3.3.1 Growth Temperature.

The first parameter to be optimised was temperature. The optimum temperature for growth with the bacteria used was 37°C. Insoluble proteins can become more soluble when expressed at 25°C, therefore both 37°C and 25°C were tested. Once the bacteria had reached the log growth stage the cultures were grown in new conditions for a further three hours. They were either left at 37°C, or transferred to grow at 25°C. At both temperatures there were two conditions, in one culture the bacterium were left, in the other IPTG was added (as in 2.19) to induce the desired protein production.

As can be seen in figure 3.3.1 there is an increase in protein production when the cultures are induced, however there appeared to be little difference between the cultures induced at 25°C or 37°C. The normal bacterial protein production was greater in the culture grown at 37°C compared to the culture grown at 25°C. As some of the normal bacterial protein is of a similar size to the viral proteins, there is an impression of similarity that may be false. Further tests isolating the induced proteins showed a greater production of 2C protein when induced at 25°C (results not shown).

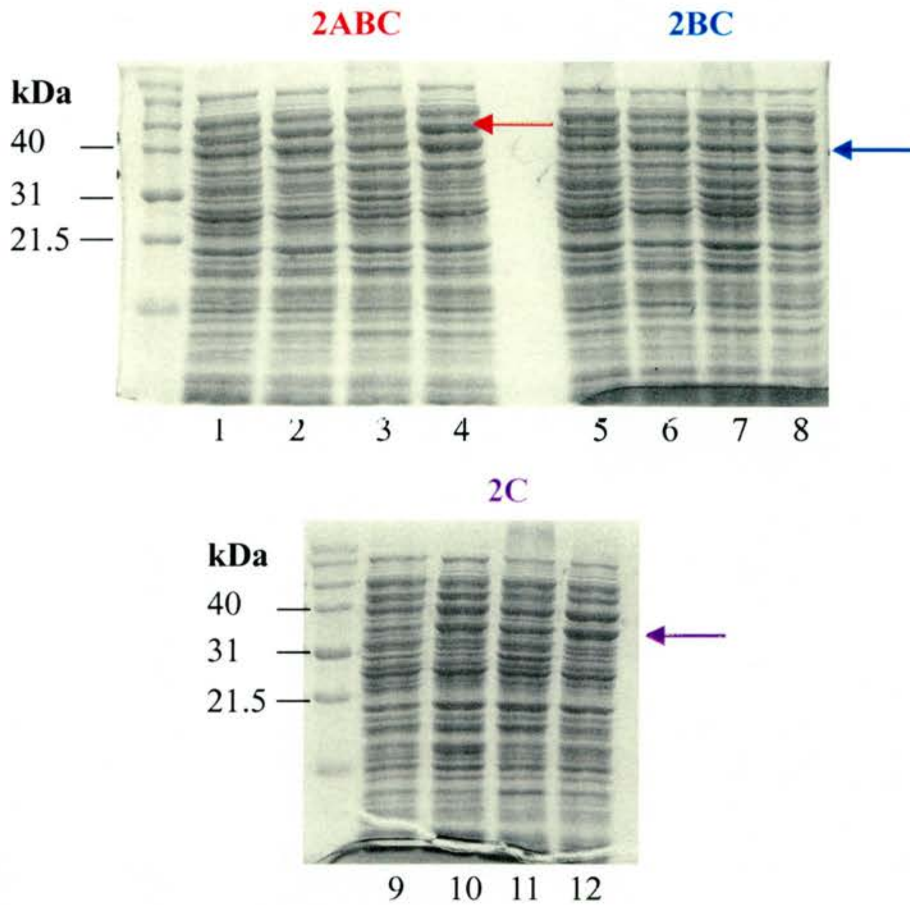

Figure 3.3.1 Diagram showing total soluble protein from bacterium lysate in the expression of HRV 85 2ABC, 2BC and 2C proteins under four conditions: uninduced 25°C (lane 1), induced 25°C (lane 2), uninduced 37°C (lane 3) and induced 37°C (lane 4).

3.3.2 Heat Shock of Cultures.

Recent findings had shown that heat shocking bacteria just before induction can cause bacteria to produce heat shock proteins. These chaperonins assist with correct folding, therefore making proteins more soluble. This method may have proved useful in the production of soluble viral proteins (personal communication) and so the protocol was tested. As before bacteria containing the appropriate vector were grown to log phase. IPTG added to one sample, which was then transferred to 25°C. The other was heat shocked at 42°C for 2 minutes before IPTG was added and then the culture transferred to 25°C. Both

were induced for three hours. Heat shocking the bacteria appeared to reduce the amount of both bacterial and viral protein produced (figure 3.3.2). There appeared to be no increase in viral proteins, and further testing by purification showed that heat shocking the bacteria was shown to reduce the amount of soluble viral protein.

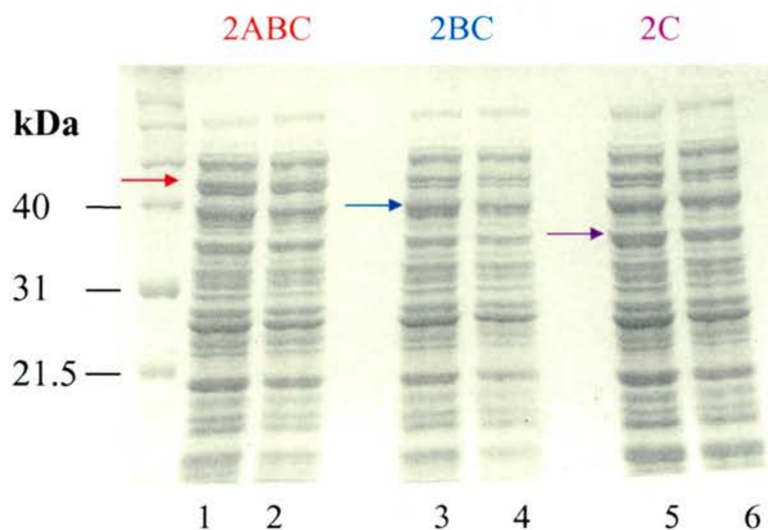

Figure 3.3.2 Total soluble proteins in bacterial expression of HRV 85 2ABC, 2BC and 2C proteins. In lanes 1, 3 and 5 the culture was grown and induced as normal (25°C). In lanes 2, 4 and 6 the culture was grown as normal, and then heat shocked for 2 minutes before being induced as normal.

3.3.3 Duration of Expression Following Induction.

The time in which expression lasts can be crucial so that the maximum amount of desired protein is produced with the minimal amount of bacterial protein. Therefore cells containing HRV 85 vectors were grown to log phase, IPTG was added and the cultures were transferred to 25°C. They were grown for 0, 1, 2, 3 and 16 hours and compared for expressed protein. The results clearly show an increase in the expressed protein with longer time, however an increase in cellular protein can also be noted (figure 3.3.3). The figure does not clearly

show the much larger amount of protein in the culture after 16 hours, as less than half was loaded onto the SDS PAGE. The amounts loaded in the first four samples were equal. Whilst there is a lot more viral protein after 16hours, the ratio between viral and bacterial protein decreases with time. Therefore the ideal time frame appears to be a three-hour incubation. At three hours there is a large amount of viral protein production, however the bacteria protein is not much greater than at 1 or 2 hours.

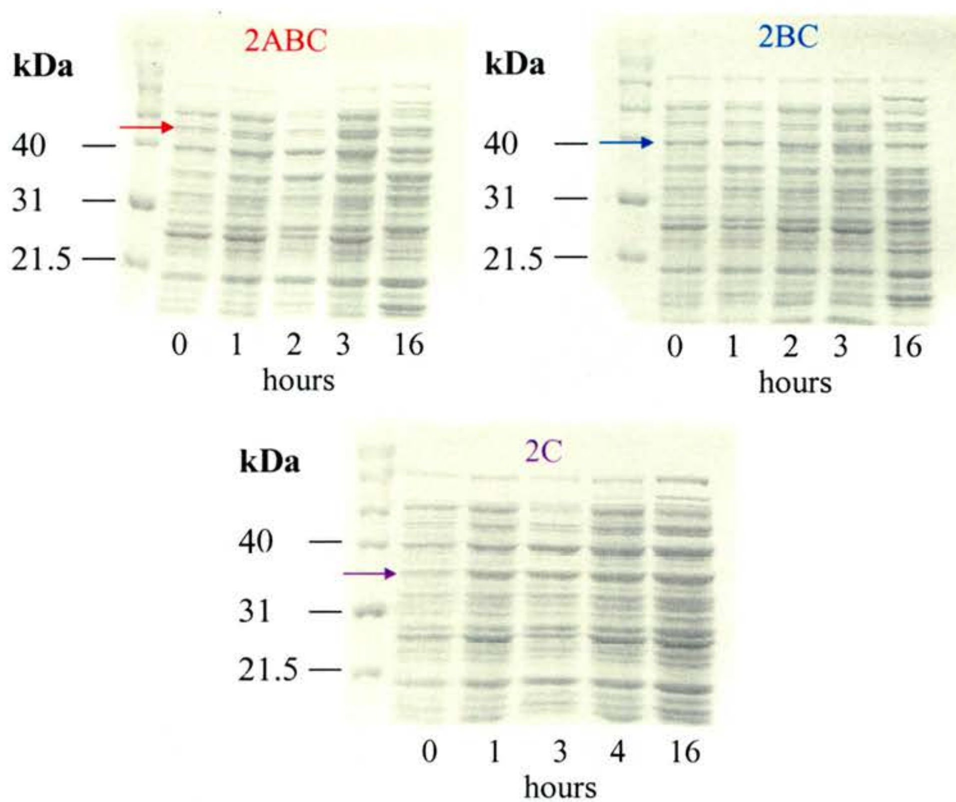

Figure 3.3.3 Expression of HRV 85 2ABC, 2BC and 2C proteins 0, 1, 2, 3 and 16 hours after being induced. The loading is equal apart from the sample in the last lane of each gel (16 hours), which has 3/8th the volume.

3.3.4 Solubility.

Partially soluble proteins can be made more soluble with the use of detergents in the lysis buffer. However detergents can interfere in experiments that the

expressed proteins may be required for, for example in x-ray crystallography, ionic detergents can cause problems. Therefore the isolation of the HRV 85 proteins from bacterial lysate was tested using PBS, PBS with NP40 (0.05% and 0.1%) and PBS with N-Octyl β-D glucopyranoside (0.05% and 0.1%). The addition of these detergents does not appear to affect the amount of HRV 85 2ABC protein in the soluble portion of the bacterial lysate (Figure 3.3.4). There is a slight increase in HRV 85 2BC and 2C proteins with the detergents, however there is little difference between the two detergents, or the concentrations used.

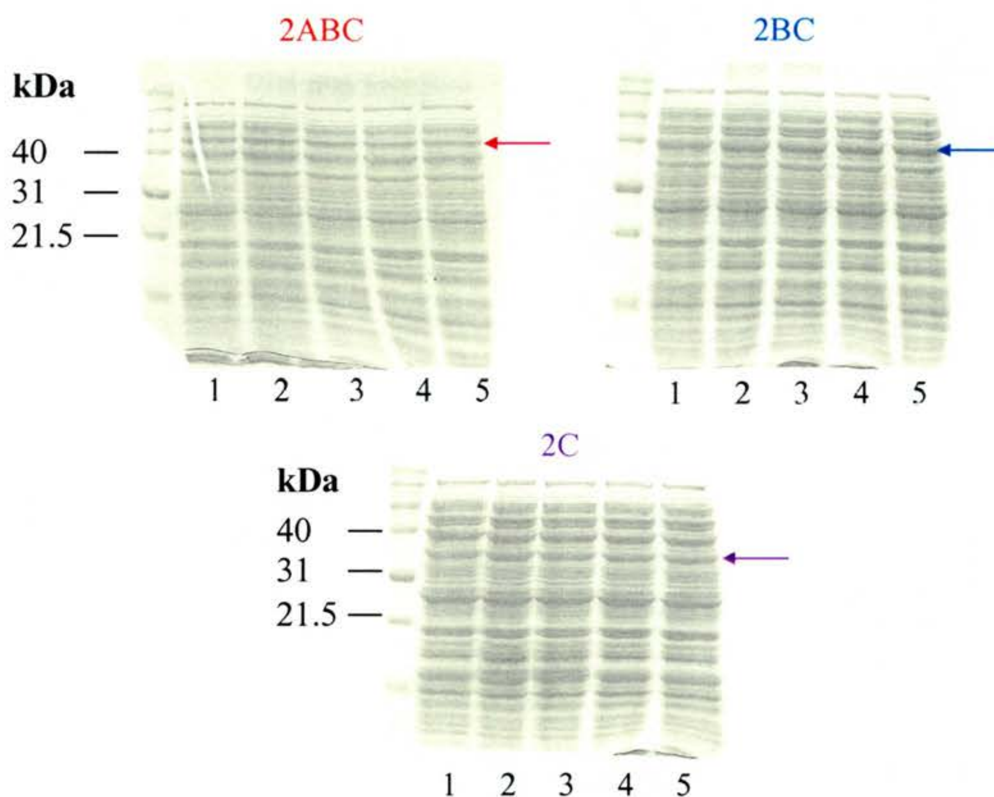

Figure 3.3.4 Soluble fraction of lysate from bacteria expressing HRV 85 2ABC, 2BC and 2C proteins. Bacteria lysed in PBS (lane 1), PBS with 0.05% and 0.1% NP40 (lanes 2 and 3 respectively) and PBS with 0.05% and 0.1%N-Octyl β-D glucopyranoside (lanes 4 and5 respectively).

3.3.5 Use of Detergents.

The use of detergents in the lysate buffer was inconclusive, with very little, if any, increase in soluble viral proteins. The separation of viral from bacterial

protein would show more decisively if there were any difference in the five preparations. Therefore the samples were incubated with beads for 20 minutes (4°C), washed with PBS and protease inhibitors (plus the respective detergent concentrations) and then with PBS (plus the respective detergent concentrations). There was a slight improvement in the binding of HRV 85 2ABC to the beads when N-Octyl β-D glucopyranoside was used in the lysis buffer, however no increase was seen using N-Octyl β-D glucopyranoside with the other proteins (figure 3.3.5). The results clearly show that viral protein extracted using NP40, especially 0.1%, gave greater binding to beads. This suggests that there was more soluble protein available for binding when lysed with this detergent.

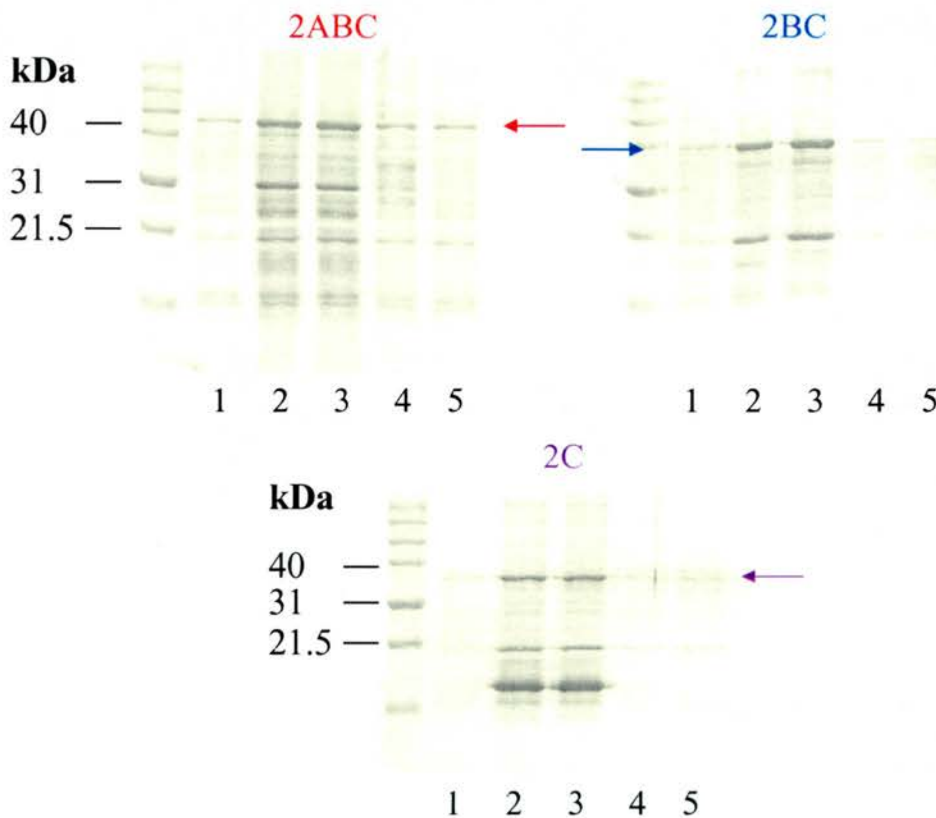

Figure 3.3.5 Binding of HRV 85 2ABC, 2BC and 2C proteins to beads when lysed in PBS (lane 1), PBS with 0.05% and 0.1% NP40 (lanes 2 and 3 respectively) and PBS with 0.05% and 0.1%N-Octyl β-D glucopyranoside (lanes 4 and 5 respectively).

3.3.6 Washing of Beads.

When purifying the proteins it is best to use the cleanest sample possible, therefore it is important that as few bacterial proteins bind to the beads as possible. To improve the purity of beads the washes were considered. As the beads are incubated with BSA most non-specific binding sites should be blocked, however it is possible impurities due to non-specific hydrophobic interactions occurred. To test this theory the washes were completed using 0.1, 0.5 and 1.0% NP40 in the normal wash buffers. The use of higher concentration of detergent did not seem to improve the problem with non-specific interactions (figure 3.3.6). There is no reduction seen in any of the bacterial protein bands. Although the viral proteins were beginning to degrade there was still enough viral protein for the experiment and no change was seen in any other banding, so it is unlikely that this affected the results.

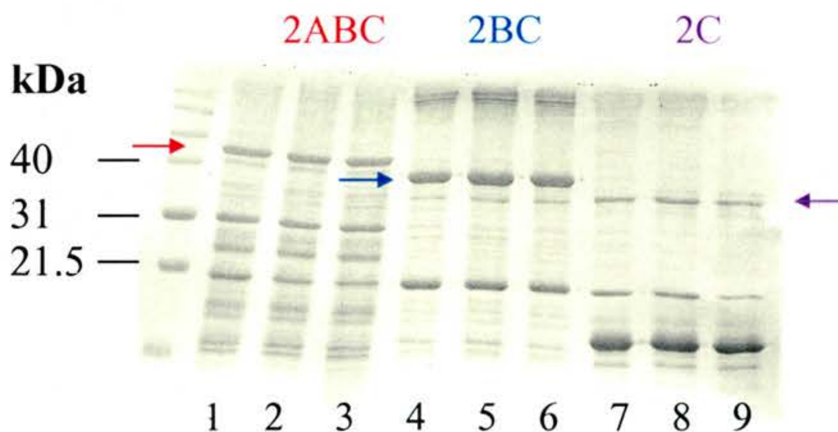

Figure 3.3.6 Investigation into the use of NP40 in the wash buffer for HRV 85 2ABC, 2BC and 2C proteins. Using bacteria lysate (0.1% NP40) bound to beads, which were washed with 0.1% (lanes 1, 4 and 7), 0.5% (lanes 2, 5 and 8) or 1.0% (lanes 3, 6 and 9) NP40 in the wash buffer.

Since no improvement was observed using NP40, the stronger detergent N-Octyl β-D glucopyranoside was tested in the last few washes (figure 3.3.7). The extract from the experiment in section 3.3.4 was bound to beads, which were

washed as normal using PBS with protease inhibitors and 0.1% NP40. The final two washed were completed using PBS with either 0.1% NP40 or 0.1% N-Octyl β-D glucopyranoside. The use of the stronger ionic detergent in the final wash had no effect on non-specific binding (figure 3.3.7).

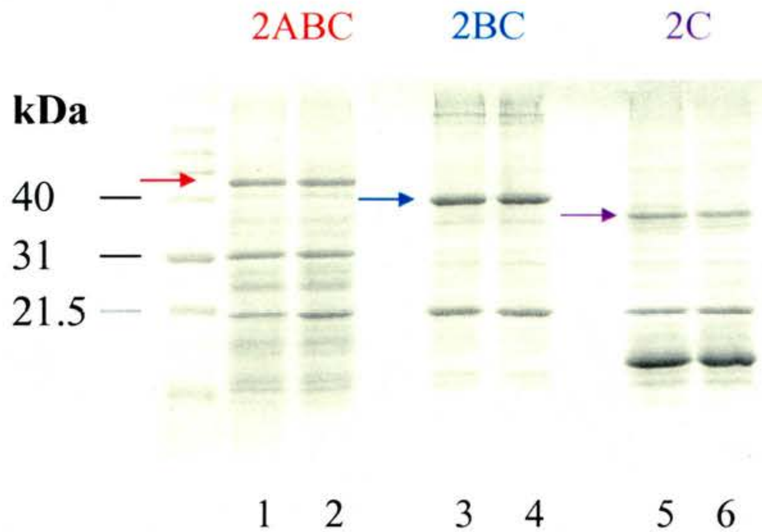

Figure 3.3.7 Expressed HRV 85 2ABC, 2BC and 2C proteins bound to beads. Last two washes using either PBS with 0.1% NP40 (lanes 1, 3 and 5) or PBS with 0.1% N-Octyl β-D glucopyranoside (lanes 2, 4 and 6).

3.3.7 Purification of Protein 2C.

The proteins must be able to be removed from the GST in order to be used in future experiments. The ideal way to achieve this would be to cleave the fusion protein whilst still attached to the beads as this would result in a relatively pure sample of the desired protein, whilst the GST and other non-specific bound proteins would remain on the beads. The other option would be to elute the protein from the beads, and then cleave it in solution. The resulting solution would contain the desired protein and GST, and so would require further purification. Thus the viral proteins were cleaved with thrombin directly from

the beads. Initial experiments using this method were unsuccessful and little, if any, protein was cleaved (results not shown).

After a few tests the experiment was changed and the protein was eluted from the beads, dialysed, and then cleaved. The protein used was newly expressed and whilst expression of HRV 85 2ABC was poor, 2BC was reasonable and 2C was very good. The elution does not model this and very similar quantities of each were eluted (Figure 3.3.8).

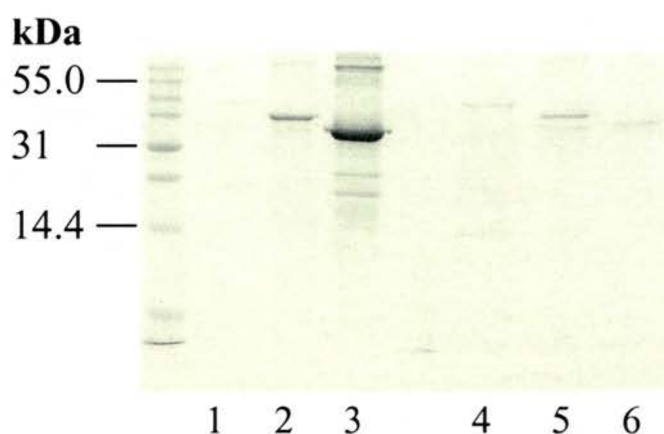

Figure 3.3.8. Expression (lanes 1-3) and elution (lanes 4-6) of HRV 85 2ABC (lane1 and 4), 2BC (lane 2 and 5) and 2C (lane 3 and 6) proteins.

The eluted protein was then cleaved using thrombin to produce the HRV 85 proteins and GST. The cleavage was only partial, with a lot of the protein remaining as a fusion protein (Figure 3.3.9). The amount of cleaved protein was very small, and in the case of HRV 85 2C there was no visible product, and very little GST. This may have been caused by the use of a shorter cleavage step (14 hours). Whilst so little protein was made, it was still superior to cleaving directly from the beads.

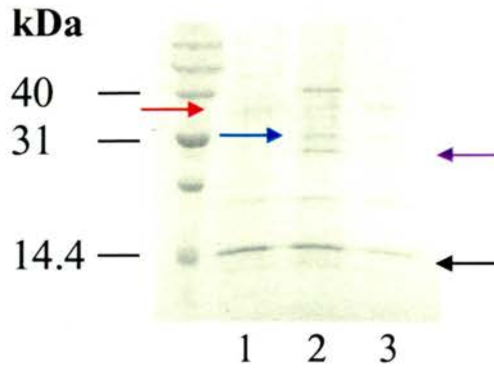

Figure 3.3.9 Thrombin cleavage of eluted HRV 85 2ABC, 2BC and 2C proteins. Cleaved GST protein is indicated by the black arrow, 2ABC by the red arrow (lane 1), 2BC by the blue arrow (lane 2) and cleaved 2C by the purple arrow (lane 3).

The elution and cleavage of HRV 85 proteins was repeated using the same bead preparation. This time elution was modified so that the incubation with GEB lasted 20 minutes and the dialysis step was removed. This step had not appeared to purify the eluted protein and may have lost some of the desired cleaved product. The cleavage with thrombin was repeated with more thrombin (1.5 times the amount used before) for the full 16 hours. The longer elution step resulted in much better elution (figure 3.3.10). Cleavage of the protein was also improved with the fusion protein completely cleaved to produce the desired product and GST.

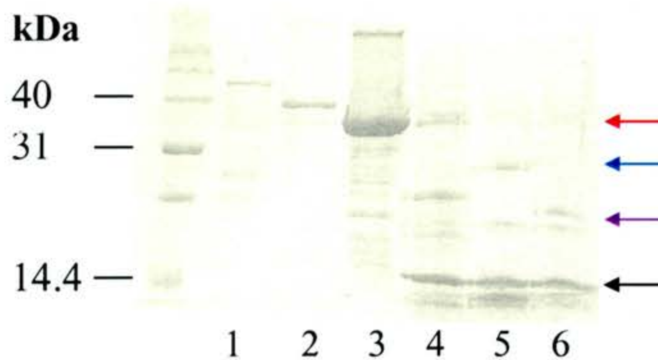

Figure 3.3.10 Elution (lanes 1-3) and cleavage (lanes 4-6) of HRV 85 proteins 2ABC, 2BC and 2C. Cleaved GST protein is indicated by the black arrow, 2ABC by the red arrow (lane 4), 2BC by the blue arrow (lane 5) and cleaved 2C by the purple arrow (lane 6).

3.3.8 Application of Modified Technique to Other 2C Proteins.

Once the best conditions for expression and binding of HRV 85 proteins were determined they were tested on all the other picornavirus 2C proteins cloned. The same conditions for expression were used and the results were very promising. It appeared that almost all those tested expressed at the same levels as, or better than HRV 85 proteins, and so this would appear to be the best conditions for these proteins (figure 3.3.11). Others like ECHO 22 and HeCV may require further testing to improve their expression to the same levels.

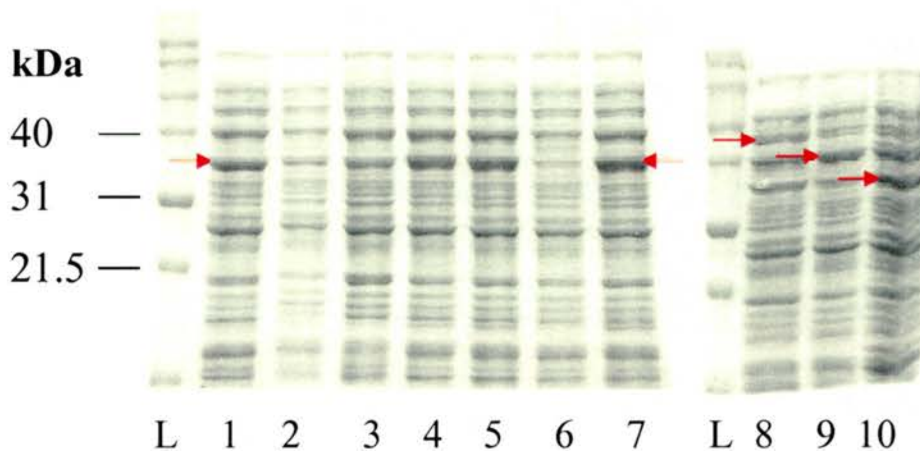

Figure 3.3.11 Expression of Picornavirus 2C proteins using condition optimised with HRV 85 2C run next to protein ladder (L). These are from Picornavirus CA9 (lane 1 2C), ECHO 22 (lane 2), FMDV (lane 3), HAV HM175 (lane 4), HAV wt1 (lane 5), HeCV (lane 6), TME (lane 7). The controls in lane 8-10 are HRV 85 2ABC, 2BC and 2C proteins.

These expressed proteins were then used to test the conditions for bead binding optimised with HRV 85 proteins. As figure 3.3.12 demonstrates few of the proteins bound as well as the HRV 85 2C protein, with the exception of TME and CA9. The other proteins, however, did bind fairly successfully. ECHO 22 and FMDV bound better than HRV 85 2ABC protein, and the HAV proteins bound nearly as well as HRV 85 2C protein. Only HeCV was very

disappointing, with very little binding, this obviously needs other conditions for better binding.

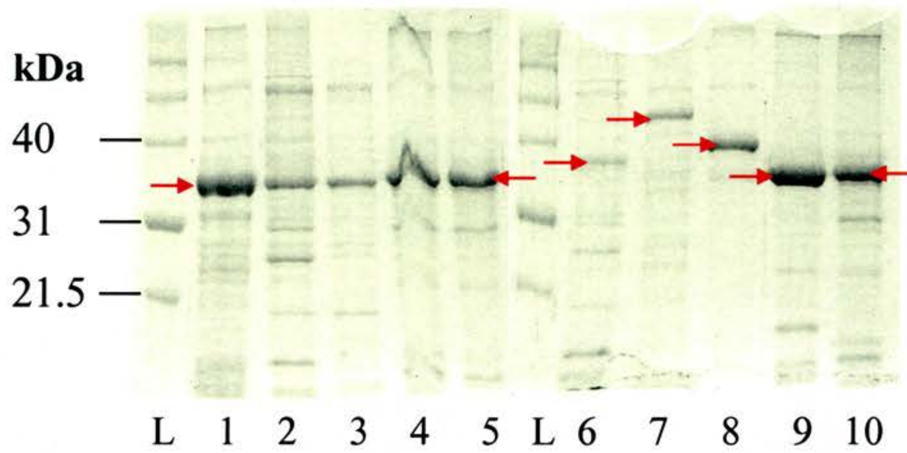

Figure 3.3.12 Picornavirus 2C proteins bound to beads using conditions optimised with HRV 85 proteins. These are from Picornavirus CA9 (lane 1), ECHO 22 (lane 2), FMDV (lane 3), HAV HM175 (lane 4), HAV wt1 (lane 5), HeCV (lane 6), TME (lane 10). The controls in lane 7-9 are HRV 85 2ABC, 2BC and 2C proteins.

4 Discussion.

Before investigations into picornavirus 2C proteins could begin the genes were cloned into expression and translation vectors. The restriction digestion sites and vectors used were kept as similar as the differences in the genes would allow, enabling direct comparisons between the genes in future experimentation. Whilst there were initial unknown problems during cloning, especially with the HAVs and ECHO 22, through meticulous trial and error all, barring HAV wt2, were successfully cloned.

Cloning was attempted with three different HAVs: cell cultured (cc) HAV (HAV HM175), HAV wt1 and HAV wt2, two strains of wild type (wt) HAV, taken from HAV infected patients. Since the gene sequence was only available for cc HAV the primers were designed using this sequence, and cloning was attempted with the same primers for all three HAV 2Cs. There were no problems during PCR for cc HAV, or HAV wt1, however HAV wt2 could not be obtained even after lowering the annealing temperature to allow for any differences in the primer sequences. It is well documented that 2C is a major site for mutation when HAV is adapted to grow in cell culture (92). The apparent difference in sequence between these HAVs could explain the failure of HAV wt 2 PCR. It should also be noted that use of these primers for HAV wt 1 might have introduced changes to the original sequence. In order to gain comprehensive insight into the differences between wt and cc, the wt HAV sequences should be found, and primers designed accordingly.

Once cloned into the translation vector the different 2Cs were investigated for IAP3 binding in a tried and tested assay. Whilst this initially gained positive results for many of the different genera, there were problems repeating these experiments. Difficulties in cloning some of the genes meant there was a long delay between the first set of tests and the next group, by which point Caroline Knox had improved the binding assay, and new conditions were recommended (personal communication). These proved to be unsuccessful, thus the old conditions were returned to, however numerous attempts produced unspecific binding to both IAP3 and GST. Investigations into the unsuccessful binding assay did not reveal the reason for the failure. There are many factors involved in the binding assay and time limitations prevented a comprehensive investigation.

The first binding assays were successful for all 2Cs tested apart from CA9 2C, which appeared to give a double band at translation. The larger protein was the right size for 2C, suggesting that the other was produced from an internal initiation site within the 2C gene. The second, smaller band was produced to a greater extent than full-length 2C (figure 3.2.2). Knox has shown that the first 20 amino acids are responsible for binding of 2C to IAP3, however if internal initiation were the cause of this second band most, if not all, of this binding site would be missing. If this is true then the majority of the translation product is missing this domain, therefore a negative result does not indicate CA9 2C's inability to bind IAP3.

It was thought that by optimising initiation of the full-length CA9 2C translation product would increase the proportion of full-length product, and therefore enable testing of IAP3 binding. The sequence preceding the AUG codon was thought to be inhibiting translation, and therefore a later start codon was being preferentially translated. To test this theory the gene was transferred into another plasmid, however similar translation results were observed. Since FMDV 2BC and HRV 85 2BC were also shown to bind IAP3 (Knox and Ryan unpublished results), CA9 2BC was cloned in the hope that full-length 2BC translation product could be obtained. Full-length 2BC was effectively translated, however by this point the binding assay was not showing specific binding even for the control. Obviously the assay needs to be revised, however once it has been perfected, CA9 2BC can be tested and all the 2C-binding assays can be repeated to validate the results.

Two key elements were considered during investigation of 2C expression; the need for large quantities of the protein - for other assays - and, more importantly, the need for pure protein, intended for x-ray crystallography. The basic protein expression protocol was systematically examined and optimised for HRV 85 2C, 2BC and 2ABC proteins. Initial appearance revealed little difference between expression at 25 °C or 37 °C, whether or not heat shock was used. Binding to glutathione beads highlighted these differences, and indicated that soluble expression was best achieved at 25°C without heat shock.

When investigating the length of expression three hours was selected, however this was not the optimum time for 2C expression. Slightly more 2C was

produced if left longer, yet after three hours the increase in 2C concentration was less significant than the rise in bacterial protein. Increased concentrations of bacterial protein may require more purification steps, thereby increasing the amount of 2C lost. Therefore three hours should give maximum concentration of 2C with minimum purification.

Whilst studying solubility and bead washing two types of detergent were investigated, a strong ionic and a weaker non-ionic detergent. Detergents need to be kept to a minimum when purifying protein if it is required for x-ray crystallography, since they cover hydrophobic areas of a protein, preventing stacking required for crystal formation. Experiments showed that 0.1% NP40 was as efficient as the same concentration of a stronger ionic detergent at extracting soluble 2C and at washing the beads. Further investigation is required to test whether the last couple of washes of the beads can be completed without any detergent, as this would allow simple removal of detergent from the protein.

Elution and cleavage of the protein from GST was only briefly investigated, and optimisation was not, therefore, performed. Nevertheless eluting the fusion protein before cleaving produced more of the desired product than cleaving directly from the beads. This method has the added problem of GST contamination, however this should be easily removed by a brief incubation with glutathione beads. Much of the protein was lost with either method, probably remaining bound to the beads, therefore further investigation is required if the protein is to be used in any other form than bound to glutathione beads.

The conditions for expression and bead binding optimised for the HRV 85 proteins were tested for the other cloned picornavirus 2C proteins. The results were encouraging, as almost all showed expression levels in the range of HRV 85 proteins. The low expression level of HeCV was disappointing, and individual optimisation of conditions may be required to obtain comparable quantities. It is possible that the expression of other 2C proteins could also be improved, as CA9, HRV 85 2C and TME expressed better than others. Nevertheless the conserved nature of the protein and the similarity of their expression suggest that they should have analogous requirements. The high level of HAV wt1 expression supports the idea that there is no substantial difference between HAV and other picornavirus 2C proteins, despite the lack of CPE in HAV-infected cells (81). Very little non-structural proteins are seen in HAV-infected cells (41), however as 2C expresses normally it is unlikely that the 2C sequence is causing this low level of expression, thus there must be another controlling factor involved.

The results that FMDV (an aphthovirus), HRV 85 (a rhinovirus), wt HAV (a hepatovirus), HeCV (a calicivirus), ECHO 22 (a parechovirus) and TME (a cardiovirus) all bind IAP3 indicate a conserved role for 2C in all picornaviruses. A 20 amino acid N-terminal fragment is sufficient for the interaction of 2C and AKAP 10, therefore the ATPase action of 2C is not necessary for AKAP 10 binding (Knox and Ryan, unpublished results). It is well documented that 2C binds to cellular membranes and vRNA, and has been suggested to hold the RNA replication complexes together (11). AKAP binding could be the mechanism by which this occurs. Membrane associated 2C is completely and rapidly degraded by proteases (24), AKAP binding regions in 2C map to the same region as

membrane binding, and 2C is unable to locate to trypsonised cells (Knox and Ryan, unpublished results), thus AKAP binding and membrane binding maybe one and the same.

AKAPs are important in maintaining the compartmentalisation of PKA that is thought to be vital to the control its multi-functionality. If the interaction of 2C with AKAP-10 upsets this balance by displacing PKA, it could have a knock-on effect throughout the cell, perhaps causing many changes that occur in picornavirus-infected cells. The low presence of 2C in HAV infected cells (41) would suggests that whatever the effect of displacing PKA may not be seen in HAV infected cells. There are two main differences between cells infected with HAV or other picornaviruses. The method of replication is by persistent infection, therefore there is no CPE (81) and there is no alteration in membrane structures normally seen in the host cell, although this has also been linked with CPE (10).

Whilst AKAP 10 is mitochondria-associated, it is also seen dispersed around the cell (88), and may have isoforms located in other membranous regions in a similar fashion to D-AKAP 1 (36, 35). 2C has been co-fractionated with ER membranes and virus-induced vesicles (79), which form the replication centres and are thought to originate from ER (79). This suggests that AKAP 10, or an isoform, may be located in ER membranes. The location of AKAP 10 is important as it determines the effect of specific PKA displacement by 2C.

Isoforms of protein kinase A are known to localise in the ER and Golgi complex (43) and protein phosphorylation is part of the regulation of cellular transport pathways. There are many PKA-regulated events, including transfer of protein from the ER to the Golgi (22), budding and movement of vesicles within the Golgi apparatus and to the plasma membrane (62), exo-, endo- and transcytosis and endosome fusion (52 and 91). It is likely that disruption of PKA within this tightly controlled pathway may effect the structural organisation of the Golgi stack. Indeed the changes caused by infection with picornaviruses are similar to those seen in mitosis. Free vesicles, clusters and tubular structures are seen to replace the Golgi apparatus in mammalian cells during mitosis (reviewed in 53). Accumulation of vesicles has been linked to the inhibition of both the export of proteins from the ER (14) and the fusion of vesicles, through phosphorylation of proteins involved in tethering and docking (53). Similarly, osmotic stress in vertebrate cells causes inhibition of traffic between the ER and the Golgi, and the Golgi is seen to disappear into the ER (47).

The docking of vesicles is controlled by the binding of t-SNAREs, which are found in target membranes, to v-SNAREs, which are located in vesicles. The Ypt/Rab family of small GTP-binding proteins promote docking as they catalyse the removal of inhibitory t-SNARE binding proteins. All of these proteins may be regulated in mitosis, however two, Rab1 and Rab4, are phosphorylated during mitosis. Rab1, which is involved in transport between the ER and Golgi complex, has enhanced membrane binding when phosphorylated, whereas Rab4, found on putative recycling endosomes, has decreased binding, thus the effect of phosphorylation is confusing (reviewed in 53). Vesicles are thought to tether to

the membrane before fusing. This occurs by the binding of p115, found on COP1 vesicles, and GM130, found on the target membrane (reviewed in 89). Phosphorylation of GM130 in mitosis by cyclin-dependent kinase 2 inhibits this tethering (54) and may account for the accumulation of COP1 vesicles in mitotic cells. In neurones synaptic transmission is up regulated by phosphorylation of Snapin by PKA. This increases its affinity for SNAP-25, therefore enhancing fusion of synaptic vesicles with SNARE complexes (15). Clearly phosphorylation plays an important regulatory role in vesicle fusion and could explain vesicle accumulation in picornavirus-infected cells.

Transport from the TGN (trans Golgi network) is dependent on PKA, whilst transport from ER to *cis*/middle Golgi is sensitive to PKA. The same study also suggested that PKA was required to maintain the Golgi organisation (61). Further studies indicated that PKA was required for the budding of vesicles at the TGN, and to some extent at ER and *cis* Golgi (62), possibly through the recruitment of coat proteins. Recruitment of COP1 and adaptor coat proteins is controlled by ARF1 (ADP-ribosylation factor 1). Activated (GTP bound) ARF1 binds to intracellular membranes, whereas inactive (GDP bound) ARF1 is cytosolic. Dephosphorylated Golgi membranes have a reduced ability to recruit ARF1 and redistribution of ARF1 to Golgi membranes is seen with increased PKA activity (55). Interestingly the drug brefeldin A, which inhibits ARF1 activation and membrane association (55), also inhibits poliovirus replication and retrograde traffic from Golgi apparatus to the ER (56, 17), suggesting a common link.

AKAP 10 is known to be located on the mitochondria (88); therefore it is possible that 2C effects PKA activity at this location. A significant mitochondria-bound PKA role is the control of apoptosis via phosphorylation of BAD. Disruption of the association of PKA with AKAPs causes inhibition of BAD phosphorylation, and therefore promotes cell death (32). If 2C displaces PKA at the mitochondria in a similar fashion, then the CPE could be explained by activation of BAD, and therefore promotion of cell death. The limited concentration of 2C and the lack of CPE in HAV infected cells supports this hypothesis (81 and 41). In such low concentrations of protein 2C there is unlikely to be much AKAP binding, thus displacement of PKA would be minimal and BAD would remain under PKA control.

Other examples of factors that PKA control include induction of gene expression through CREB and activation of L-type Ca^+ channels. Activated PKA can enter the nucleus and phosphorylate CREB (cAMP response element-binding protein), which then induces specific gene expression (reviewed in 37). Cellular protein expression decreases dramatically in picornavirus-infected cells, however it increases again towards then end of the virus life cycle (76). PKA anchoring is necessary for modulation of L-type Ca^+ channels (reviewed in 49). Another cytopathic effect seen in picornavirus-infected cells is an increase in intracellular calcium concentration (39). Displacement of PKA by 2C may be contributing to one or both of these changes, however other factors cannot be ruled out.

PKA is a multifunctional protein, and very few of its cellular actions have been mentioned. It is believed that compartmentalisation via AKAPs controls PKAs specificity, therefore displacing PKA from its anchor would alter this tightly

regulated system, and could result in a variety of cellular changes. 2C binds specifically to AKAP 10, known to localise to mitochondria (88), yet *in vivo* 2C is located in ER and virus induced vesicles (79). Further work is required to show why 2C is located in ER and ER-originated vesicles, when it binds to a mitochondrial AKAP. Since 2C can localise to ER membranes without other virus proteins, it is possible that it binds to another AKAP, or another isoform of AKAP 10.

This, and other work by Ryan *et al*, indicates that 2C specifically interact with membranes through AKAP 10, however the possibility that 2C binds to other isoforms, and indeed different AKAPs need to be excluded. It is possible that this is not the mechanism by which 2C attaches to membranes *in vivo*, since others have reported significant membrane binding in 18KDa protein from secondary initiation site, located after the proposed AKAP binding site in 2C (observations by Echeverri *et al*). However if 2C does localise to membranes via an interaction with AKAPs then the consequences of this binding should be further examined. 2C and RIIα share an identical binding domain on AKAP 10, therefore redistribution of PKA induced by the binding of 2C to AKAP 10 could explain the effects on membrane trafficking observed in infected cells. Although it is feasible that this binding could account for part of the picornavirus-induced CPE this cannot be the only contributing factor as 3A has also been linked to the CPE (81).

5 Conclusion.

All genera of the picornavirus were successfully cloned into both expression and translation vectors. These were then used to successfully translate the 2C

proteins, which were used in the IAP3 binding assay. This initially showed binding between AKAP 10 and 2C proteins from all six picornavirus genus indicating that AKAP 10 binding is a function of 2C common to even the more distantly related picornaviruses. Despite this initial success further binding could not be displayed due to problems with the binding assay. Therefore the assay needs to be revised before it can be used to test the remaining picornaviruses for the AKAP 10 binding. Expression was optimised for HRV 85 2ABC, 2BC and 2C proteins and the protocol was shown to produce high levels of many other picornavirus 2C proteins. Further investigations are required to gain optimum purification. Finally the potential of PKA displacement by 2C in picornavirus-infected cells was considered and comparisons between these infected cells and normal cellular processes were used to outline a number of possible consequences. Competition studies between PKA and 2C should be performed to test for displacement, and if applicable the effect of this displacement *in vivo* should be examined. Lastly the numerous explanations for the disparity between *in vivo* location of AKAP 10 and membrane-bound 2C should be investigated.

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