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ENTEROVIRUS TYPE 70:  
RECEPTOR INTERACTIONS AND  
CELL ENTRY

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A Thesis presented for the degree of Doctor of Philosophy  
in the Faculty of Biomedical and Life Sciences at the  
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Division of Infection and Immunity  
Institute of Biomedical and Life Sciences

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## **Abstract**

Enterovirus type 70 (EV 70) is the causative agent of a highly infectious haemorrhagic conjunctivitis that can rarely be complicated by a polio-like flaccid paralysis, and is one of only two enteroviruses (out of over 90) that infect the eye. EV70 has previously been reported to bind to sialic acid and decay accelerating factor (DAF), however the mechanism by which EV70 enters the cell is unknown. Using a minimally passaged isolate of the J670/71 type strain of EV70 the receptor interactions and cell entry processes of this virus have been investigated. As part of this study, an infectious cDNA and a subgenomic replicon of EV70 were generated.

In the work presented here multiple different experimental approaches demonstrated that sialic acid is the primary determinant of EV70 binding and infection and that DAF does not play a significant role. Sialic acid is also responsible for EV70-mediated haemagglutination and binding to a range of non-primate mammalian cells as diverse as *Xenopus* in origin. On erythrocytes further characterisation showed the receptor to be an  $\alpha$ 2-3 linked sialated GPI-anchored glycoprotein; although binding of EV70 to MRC5 cells was unaffected by the removal of  $\alpha$ 2-3 linked sialic acid from the cell surface, suggesting potential variation in receptor usage. Further studies have demonstrated that EV70 uses clathrin-mediated endocytosis as its initial route of entry to MRC5 cells, and that tyrosine kinases and the actin cytoskeleton are important. The evidence is also suggestive of a role for low pH in exit of the viral genome from late endosomes.

The availability of infectious cDNAs for enteroviruses has allowed reverse genetic approaches to be used during analysis of virus structure and function. Prior to this study such reagents did not exist for EV70. Long range PCR was used during the generation of an infectious cDNA for EV70 that, in turn, was used to derive a subgenomic replicon encoding a luciferase reporter gene. The specific infectivity of *in vitro* generated RNA, and growth characteristics of recovered virus, indicated that the cDNA was truly representative of the EV70 J670/71 stock. These reagents represent invaluable tools for further research into all aspects of the EV70 life cycle.

## Declaration

This work was completed at the University of Glasgow between January 2003 and April 2007 and has not been submitted for another degree. The work is original and unless otherwise stated in the text, has been completed by the author.

Signed

Date 19-04-07

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## Abbreviations

ATCC	American Tissue Culture Collection
bp	base pairs
BSA	bovine serum albumen
CAR	coxsackie-adenovirus receptor
CPE	cytopathic effect
cpm	counts per minute
CVA	coxsackie A virus
CVB	coxsackie B virus
DAF	decay accelerating factor
dH <sub>2</sub> O	deionised water
DMEM	Dulbecco's minimum essential medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
EMEM	minimum essential medium with Earle's salts
EV68	enterovirus type 68
EV70	enterovirus type 70 (from ATCC)
EV70 <sup>FLC</sup>	enterovirus type 70 (derived from infectious full-length EV70 DNA)
EV94	enterovirus type 94
FcDAF	human DAF-Immunoglobulin fusion protein
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GPI	glycosylphosphatidylinisitol
HA	haemagglutination
HCV	hepatitis C virus
HEV-A to D	human enterovirus groups A to D
HIV	human immunodeficiency virus
ICAM-1	intracellular adhesion molecule 1
IF	immunofluorescence
IRES	internal ribosome entry site

kbp	kilo base pairs
m.o.i.	multiplicity of infection
NH <sub>4</sub> Cl	ammonium chloride
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PV3	poliovirus type 3
pfu	plaque forming units
PLC	phospholipase C
PIPLC	phosphatidylinositol specific phospholipase C
PVR	poliovirus receptor
RBC	red blood cell, erythrocyte
RCA	regulator of complement activity
RNA	ribonucleic acid
rNTPs	ribonucleotide triphosphates
RTPCR	reverse transcription and polymerase chain reaction
SCR	short consensus repeat
SDS	sodium dodecyl sulphate
TCID <sub>50</sub>	50% tissue culture infectious dose
UTR	untranslated region
VOPBA	virus overlay protein blot assay
VP1 to 4	viral capsid proteins 1 to 4

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## CHAPTER 1

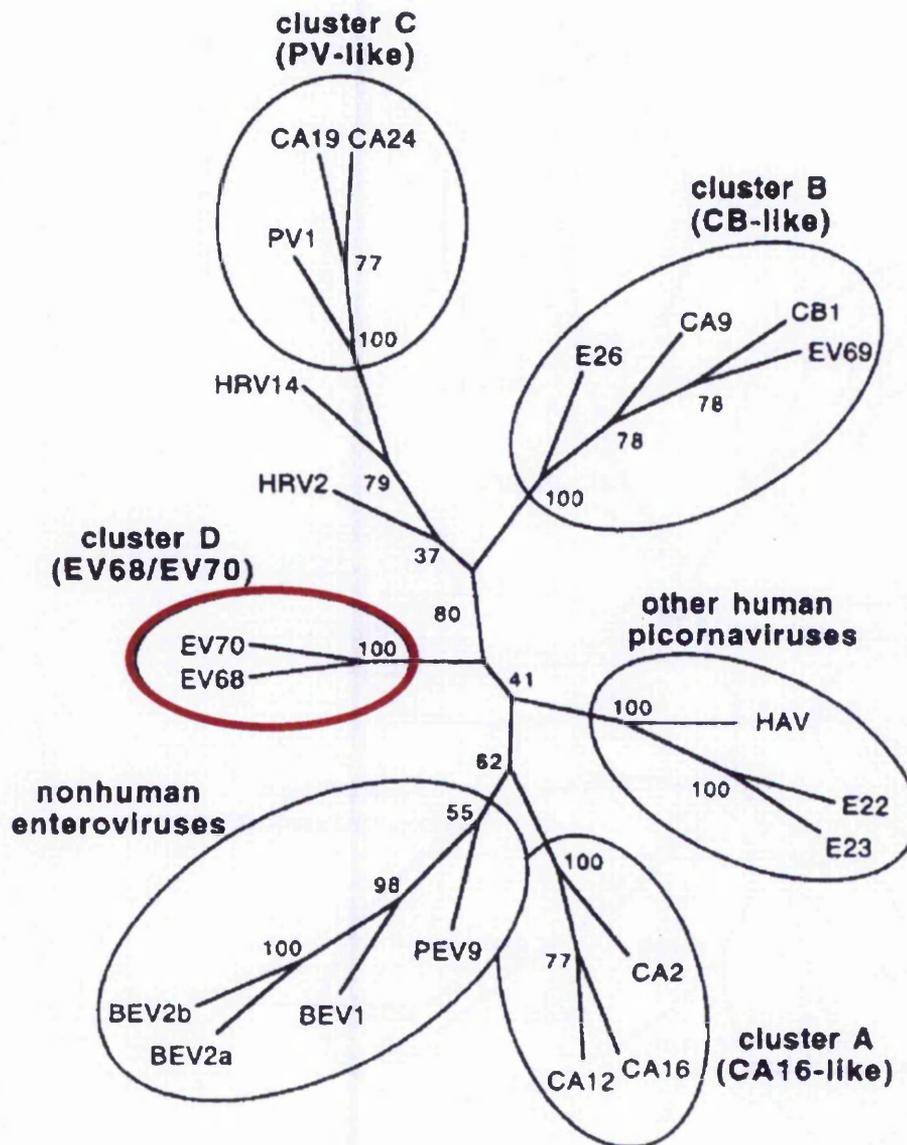
### Introduction

Enterovirus type 70 (EV70) is a member of the enterovirus genus of the *Picornaviridae* family. It is the cause of epidemics of acute haemorrhagic conjunctivitis and cases of acute flaccid paralysis.

EV70 is an important subject of study, both as a significant human pathogen and in relationship to a number of key features which differ from those seen in other human enteroviruses, of which over 90 are now described (Oberste et al., 2005). In particular the virus is unusual in terms of its tissue and host tropism. It is one of only two human enteroviruses that primarily infect the eye, and is reported to have a much greater host range, at least in tissue culture, than other members of the genus. Study of the early stages in the viral life cycle, specifically interaction of the virus with its cellular receptors, will be crucial in uncovering the mechanisms behind this unusual tropism.

#### 1.1 Classification

Taxonomy within the *Picornaviridae* is constantly evolving. Among other genera it includes: cardioviruses (murine viruses); aphthoviruses (including the causative agent of foot and mouth disease in cattle); hepatovirus (hepatitis A virus); rhinoviruses (cause of over half of cases of the common cold) and enteroviruses (Fields and Knipe, 2004; Minor, 1999). Classically enteroviruses and rhinoviruses are distinguished by their stability to acidic environments, with enteroviruses being acid-stable and rhinoviruses acid-labile. Molecular phylogenetic analysis, however, suggests that enteroviruses and rhinoviruses cannot be regarded as distinct, and proposals are underway to merge them as a single genus (Hyypia et al., 1997; *Picornaviridae* Study Group Proposals, 2005). There have also been changes in the organisation of viruses within the enterovirus genus (Stanway et al., 2005); originally the viruses were grouped into polioviruses, coxsackie A viruses (CVA), coxsackie B viruses (CVB) and echoviruses on the basis of differing pathogenesis in newborn mice. Classification difficulties resulted in more recently identified viruses being assigned a number; hence enterovirus type 70. The enteroviruses are now arranged on the basis of sequence analysis into four phylogenetic groupings (figure 1.1), human enterovirus



**Figure 1.1 Consensus phylogenetic tree for human enteroviruses.**

Taken from Oberste et al (1999). Shows the phylogenetic relationship of EV70 in cluster D (HEV-D) to other human enteroviruses based on VP1 sequence. Note also the recent identification of EV94, which is the third virus to be assigned to HEV-D (Smura et al, 2007). BEV, bovine enterovirus; CA, coxsackie A virus; CB, coxsackie B virus; E, echovirus; HAV, hepatitis A virus; HRV, human rhinovirus, PEV, parechovirus; PV, poliovirus; EV, enterovirus. Figures at branch points are bootstrap values, being the percentage of times random computer sampling of subsets of the viral sequences used to construct the tree gives a branch point at that location (Page and Holmes, 1998).

(HEV)-A, HEV-B, HEV-C and HEV-D (Oberste et al., 1999). EV70 is a member of the smallest group HEV-D, with the only other two members being enterovirus type 68 (EV68) and the recently identified enterovirus type 94 (EV94). EV68 has subsequently been shown to be an isolate of rhinovirus type 87, and indeed shares biological features with the rhinoviruses, again emphasising the overlap between the two genera (Blomqvist et al., 2002; Ishiko et al., 2002; Oberste et al., 2004; Savolainen et al., 2002). EV94 was isolated from environmental samples in Egypt and two cases of acute flaccid paralysis in The Democratic Republic of Congo (Smura et al., 2007). Initial studies have suggested that it shares the broad tissue range seen for EV70.

## **1.2 Epidemiology and disease**

### **1.2.1 Epidemiology**

Some picornaviruses seem to have been present in the human population for a long time; indeed there is evidence for poliovirus in Egypt as far back as 1300BC. Disease attributable to EV70 is a much more recent phenomenon. The probable first reported outbreak of acute haemorrhagic conjunctivitis due to EV70 was in 1969 in Ghana, although the virus was not isolated until 1971 (Kono et al., 1972). Although most enteroviruses have a worldwide distribution, to date EV70 has been observed primarily in Southeast Asia and Africa. A massive epidemic occurred in 1970/1971 affecting large areas of Southeast Asia, in particular India and Japan. The EV70 reference strain, J670/71 dates from this epidemic. It is estimated that over one million cases occurred in Calcutta alone within a three month period in 1971, and that overall 15 to 20 million were affected between 1969 and 1971 (Higgins, 1982). Smaller outbreaks and epidemics have continued to occur at intervals throughout the following decades, mainly in Southeast Asia, but also involving South and Central America, the Caribbean and the Southern states of the USA (Hatch et al., 1981; Maitreyi et al., 1999).

It is not clear why outbreaks of enteroviral conjunctivitis were not known prior to 1969. EV70 antibodies, though not the virus, have been reported in a variety of animals (Kono et al., 1981) and the virus has been reported to infect a range of non-primate mammalian cell lines (Yoshii et al., 1977). This is not the case for other human enteroviruses, which are restricted to human or primate hosts, with the possible exception of the newly identified EV94. These observations raise the possibility that

EV70 may have evolved from an animal enterovirus which adapted to infect humans. It has been proposed that human EV70 strains diverged from a single common ancestor around 1967 (Takeda et al., 1994). This was based on sequencing of the gene encoding the major viral capsid protein (VP1) in eighteen isolates from 1971 to 1981, and estimating the time required to accumulate the nucleic acid substitutions observed. This estimate is only two years prior to the first recorded acute haemorrhagic conjunctivitis cases, and contrasts dramatically with the time believed to have elapsed before human immunodeficiency virus (HIV) was first detected in the human population after its transmission from the chimpanzee, which is believed to have occurred in the 1930s (Hillis, 2000). This difference is readily explained as EV70 is highly infectious with a very short incubation period and causes a readily identifiable disease; in contrast, HIV exhibits much lower transmissibility, with years required to symptomatic disease, which then presents as susceptibility to other illnesses and was therefore much harder to differentiate as a distinct disease.

It is of interest to note that the only other cause of infective haemorrhagic conjunctivitis is a variant strain of the enterovirus CVA24, and that it too emerged around 1970 (Higgins, 1982).

### **1.2.2 Route of spread**

Enteroviruses are characteristically spread by the faecal-oral route (Minor, 1999), however EV70 has only rarely been isolated from faeces, despite its reported acid stability (Kono et al., 1972), and thus a faecal-oral or faecal-eye route seems unlikely (Higgins, 1982). It is postulated that spread is by direct contact from eye to eye or via fomites, analogous to the transmission of adenoviral epidemic keratoconjunctivitis. As with faecal-oral spread, this route is particularly relevant in areas of socio-economic deprivation, correlating with many of the larger outbreaks.

### **1.2.3 Disease associations**

A distinctive feature of the enteroviruses is the number of different pathologies with which they are associated (Minor, 1999), reflecting variation in tissue tropism. While poliovirus targets the anterior horn cells of the spinal cord and causes acute flaccid paralysis, other viruses target a range of different organs and systems, with different viruses causing illnesses such as aseptic meningitis, encephalitis, myocarditis,

vesicular lesions, as in hand, foot and mouth disease and herpangina, and more non-specific febrile illnesses and severe systemic disease.

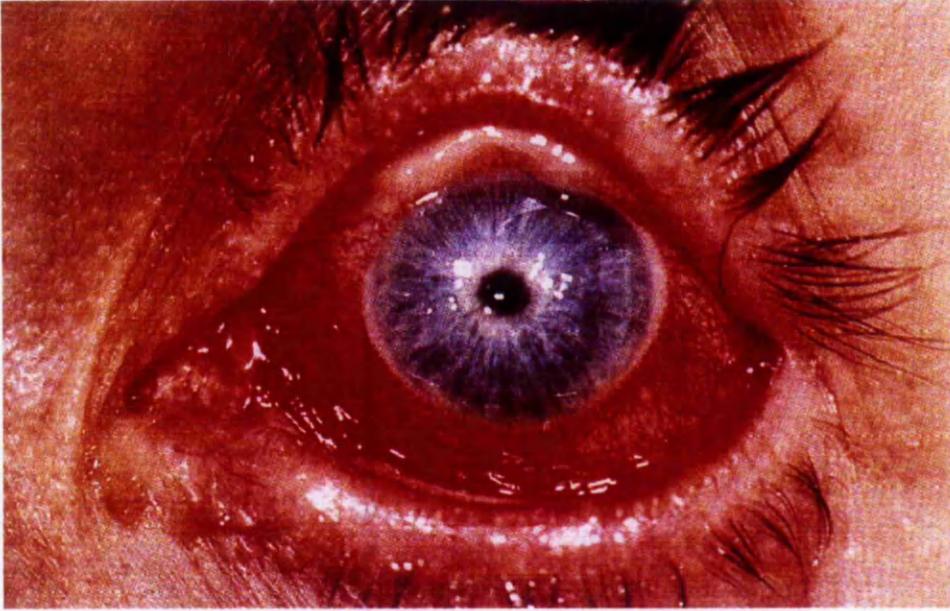
The primary manifestation of infection with EV70 (Seal et al., 1998; Wright et al., 1992) is acute haemorrhagic conjunctivitis (figure 1.2). This arises rapidly with an incubation period of 24 hours. Symptoms are usually bilateral and are severe, with conjunctival haemorrhage, excessive lacrimation and pain. The pain usually decreases within two to three days, with full resolution within ten days. There are no long-term ocular consequences.

Acute flaccid paralysis was first noted during the 1971 epidemic in India (Bharucha et al., 1972). Symptoms are indistinguishable from poliomyelitis and occur with a frequency of one in ten thousand cases of conjunctivitis. Although relatively rare, this is nonetheless highly significant given the size of epidemics. Symptoms develop from two to eight weeks after the onset of conjunctivitis with a prodrome of fever and malaise. In about fifty percent of cases there is some degree of permanent disability. Paralysis has been shown in cynomolgous monkeys after intraspinal injection of the virus (Kono et al., 1973). Cranial nerve palsies have also been reported (Katiyar et al., 1981; Wadia et al., 1983).

### **1.3 Viral structure and life cycle**

#### **1.3.1 Viral morphology**

Picornaviruses consist of a non-enveloped capsid with icosahedral symmetry, which contains a single-stranded RNA genome of messenger sense. The atomic structures of rhinovirus type 14 and poliovirus type 1 were first published in 1985 (Hogle et al., 1985; Rossmann et al., 1985), and picornaviruses conform to a basic structural pattern. The capsid consists of 60 protomers each comprising one molecule of the four virion proteins: VP1, VP2, VP3, and VP4. Each of the 20 faces of the icosahedron is made up of three protomers, with VP4 being entirely internal. Five copies of VP1 form each of the twelve pentameric apices, which in enteroviruses and rhinoviruses are encircled by a cleft known as the 'canyon'. This has been shown to be a receptor binding site for many viruses such as poliovirus type 1, rhinovirus type 16 and CVA21 (He et al., 2000; Olson et al., 1993; Xiao et al., 2001), although this appears not to be the case for receptor binding of other picornaviruses, such as echovirus types 7



**Figure 1.2 Acute Haemorrhagic Conjunctivitis.** From Seal et al., 1998.

and 11, and human rhinovirus type 2 (He et al., 2002; Hewat et al., 2000; Pettigrew et al., 2005). The canyon is not a universal feature of picornaviruses, however, and is absent in foot and mouth disease virus which possesses a relatively smooth capsid (Acharya et al., 1989). In addition to the capsid proteins, the N terminus of VP4 is covalently attached to a myristic acid residue (Chow et al., 1987). This contributes to the surface of the virion apices and may have a role in receptor binding and entry.

### 1.3.2 Genome structure

The full genome sequence of the EV70 reference strain (J670/71) was published in 1990 (Ryan et al., 1990). It consists of a single stranded messenger-sense RNA of 7390 nucleotides (figure 1.3) and shares the general organisation of enteroviral genomes (Minor, 1999). The 5' untranslated region (UTR) is 726 bases in length and is highly structured. The first ninety bases consist of a cloverleaf structure which interacts with both host proteins and the viral polymerase during replication of the genome, and this is followed by the internal ribosome entry site (IRES), a much larger element of RNA secondary structure, which functions to recruit ribosomes to the viral RNA for translation of the single open reading frame (ORF) which follows. The EV70 open reading frame is 6582 bases in length and thus codes for a polyprotein of 2194 amino acids, it is divided into three regions based upon primary proteolytic cleavage events. The P1 region, located at the 5' end, encodes the structural proteins in the order VP4, VP2, VP3 and VP1 (genome segments 1A, 1B, 1C and 1D respectively). The P2 and P3 regions, which follow P1, encode the non-structural proteins (sections 2A, 2B and 2C, and 3A, 3B, 3C and 3D respectively). Not all the functions of the non-structural proteins are known, however they include the virally encoded proteases, 2A and 3C, and the viral 3D polymerase. Following the ORF is the 3'UTR, which is 79 bases in length and thus much smaller than the 5'UTR. Like the 5'UTR, it contains elements of secondary structure and has a role in replication. The genome terminates in a sequence of 40-100 adenosine residues that are not virally encoded. In addition to the RNA, the 5' end of picornaviruses genomes is known to be covalently attached to a small protein of 22 amino acids, known as VPg (encoded by 3B), which is involved in the initiation of replication.

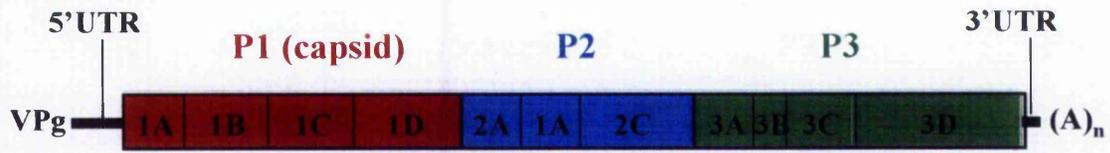


Figure 1.3 Organisation of enteroviral genomes. See text for details.

### **1.3.3 Overview of the enterovirus life cycle**

Enteroviruses share a common life cycle (Fields and Knipe, 2004; Minor, 1999). Individual viruses bind to their target cell via specific cell membrane proteins or receptors, they are then internalised by a variety of mechanisms. These early events are the subject of the investigations presented in this thesis and are discussed in sections 1.4 and 1.5 below.

Once in the cytoplasm translation of the viral RNA is initiated after binding of a ribosome to the IRES. A single viral polyprotein is produced, which is co-translationally cleaved by the viral 2A protease into the three primary products (P1, P2 and P3). Subsequent cleavages are carried out by the viral 3C protease. VP4 and VP2 remain uncleaved, as the precursor VP0, until late in infection and the mechanism by which they are cleaved is unclear. Soon after the start of viral protein synthesis, the viral 2A protease catalyses the cleavage of the cellular initiation factor eIF-4G, which is required for cap-dependant translation, and thus host cell protein synthesis is shut off.

Viral replication requires a number of factors including the viral proteins 3D polymerase and VPg and a number of *cis*-acting replication elements, such as the cloverleaf within the 5'UTR and a recently identified element within the poliovirus 3CD region (Goodfellow et al., 2000). A negative sense genome intermediate is made from which further positive strands are produced, both for more translation and for packaging into virions.

Capsids are initially assembled as pentamers of VP0, VP1 and VP2. The exact mechanism of RNA insertion is unclear, but appears to occur in association with VP0 cleavage. Virions are generally released by cell lysis, but other mechanisms may also be important.

## **1.4 Viral receptors**

Productive infection of a cell requires that the virus first attach to the cell, generally by binding to a specific receptor, and then delivers its genome to the site of viral replication. The presence or absence of a specific receptor on the cell surface is thus an important determinant of whether a particular virus can infect a cell and hence of its tissue tropism (Evans and Almond, 1998).

A diverse range of cell surface molecules have been reported as receptors for different picornaviruses. For example, the first identified and most well characterised is the poliovirus receptor (PVR), an immunoglobulin-like transmembrane protein (Mendelsohn et al., 1989). Other identified immunoglobulin family receptors are the intracellular adhesion molecule one (ICAM-1), receptor for several coxsackie A viruses and the major group rhinoviruses (Greve et al., 1989; Shafren et al., 1997a); and the coxsackie-adenovirus receptor (CAR), used by the coxsackie B viruses (Bergelson et al., 1997). Foot and mouth disease virus requires an integrin for binding (Berinstein et al., 1995), as does echovirus type 1 (Bergelson et al., 1992). Decay Accelerating Factor (DAF) is a commonly used enterovirus receptor. It has been shown to be required for the cellular binding of several coxsackieviruses and echoviruses (Shafren et al., 1995; Shafren et al., 1997b; Ward et al., 1994).

EV70 was reported in the early 1980s (Utagawa et al., 1982a) to require sialic acid for binding to red blood cells (RBC), but prior to the current studies had also been reported to bind to DAF on HeLa cells (Karnauchow et al., 1998; Karnauchow et al., 1996). Both sialic acid and DAF are known to be expressed on the surface of the conjunctiva of the human eye (Kawano et al., 1984; Medof et al., 1987).

#### **1.4.1 The poliovirus model for receptor binding and particle uncoating.**

The interaction of poliovirus with its receptor PVR has been studied in detail, and binding is known to occur within the depression on the viral surface at the five-fold axis of symmetry known as the canyon (He et al., 2000). The canyon has also been shown to be the site of interaction for other picornaviruses and their receptors, such as rhinovirus type 14 with ICAM-1 (Olson et al., 1993) and CVB3 with CAR (Xiao et al., 2001). Binding of poliovirus to PVR-expressing cells triggers the process of viral uncoating. This is required for the release of free RNA into the cytoplasm. Uncoating can be tracked by changes in the particle sedimentation coefficient. The 160S native infectious virus loses one of the capsid proteins (VP4), forming 135S 'A' particles. As VP4 is an internal capsid protein and not present on the surface of the 160S particles, this marks a major conformational change, during which the N-terminus of VP1 is also externalised. Subsequently the viral nucleic acid is released with the formation of empty (80S) particles. Both 135S and 80S particles are poorly infectious (Curry et al., 1996) and can be detected both intracellularly and extracellularly during productive virus infection, indeed binding of poliovirus to its soluble receptor alone is sufficient to

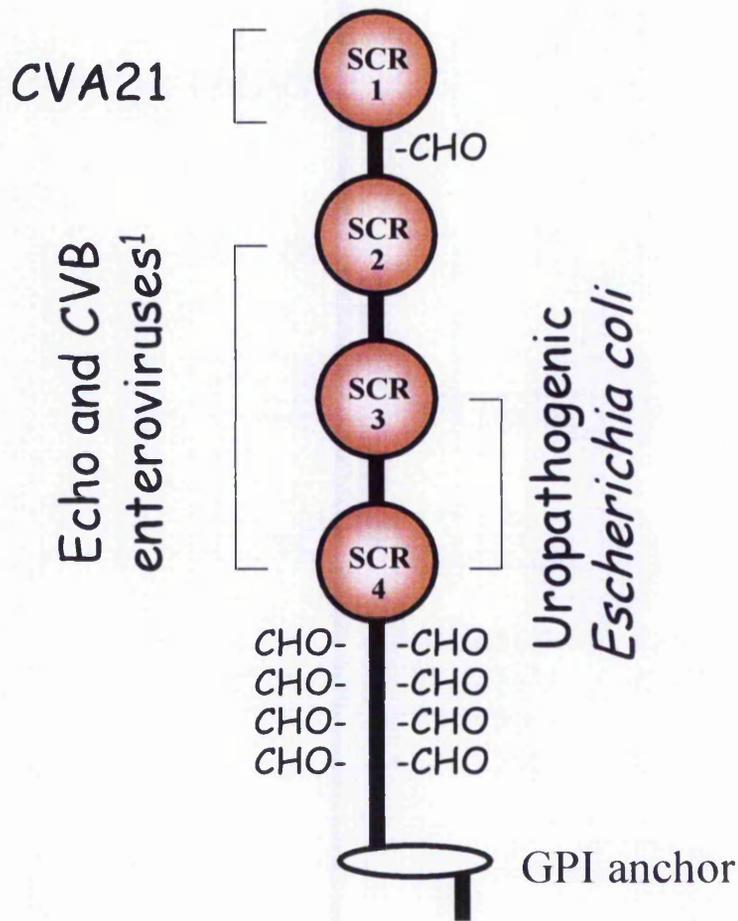
trigger the full uncoating process (Kaplan et al., 1990). It should be noted, however, that it is not yet clear whether 'A' particle formation itself is a necessary step for the uncoating of poliovirus or other enteroviruses.

#### 1.4.2 DAF as a viral receptor

DAF is a widely expressed 70kD cell surface protein which acts to prevent cell damage from the complement cascade by accelerating the breakdown of the C3 and C5 convertases (Campbell et al., 1988; Nicholson-Weller and Wang, 1994). It is a member of the RCA (regulator of complement activity) group of proteins, other notable members being CD46, which is used as a receptor by the Edmonston strain of measles virus (Dorig et al., 1993), and CR2, the Epstein Barr virus receptor on B lymphocytes (Fingerth et al., 1984). RCA proteins are characterised by 60 amino acid short consensus repeat domains (SCRs). DAF is made up of four such domains connected to the cell membrane by a lipid glycosylphosphatidylinositol (GPI) anchor rather than a transmembrane domain. SCR4 is separated from the GPI anchor by a serine/threonine rich region that is heavily O-glycosylated. In addition there is an N-glycosylation site between SCR 1 (the most membrane distal domain) and SCR 2 (figure 1.4).

DAF has been implicated as an important receptor for a considerable number of enteroviruses (figure 1.4), in addition it is also bound by the fimbriae of uropathogenic *Escherichia coli* (Nowicki et al., 1993). DAF's viral receptor role was first identified for echovirus 7 (Ward et al., 1994). A novel technique was used in which non-permissive cells were transfected with cDNA clones in high efficiency expression vectors, cells rendered permissive for echovirus 7 infection were identified using a viral antigen-specific mouse monoclonal antibody and an anti-mouse IgG antibody conjugated to  $\beta$ -galactosidase, leading to blue colouration on the addition of substrate. Plasmid DNA was extracted directly from individual positive cells, transformed into *Escherichia coli* and the DAF-encoding gene identified by sequencing. Subsequently many other enteroviruses have been demonstrated to bind DAF, including numerous echoviruses, some of the coxsackie B viruses and CVA21 (Powell et al., 1998; Powell et al., 1999).

The majority of DAF binding enteroviruses require SCR 3 interactions (Powell et al., 1999). However CVA21 has been shown to interact with SCR 1 (Shafren et al., 1997b). The observation that different enteroviruses bind to different domains of DAF has led to the hypothesis that DAF-binding has evolved convergently, and that there is some advantage to the viruses in using this protein as a receptor. Members of the RCA



**Figure 1.4 DAF structure and sites of pathogen binding.** Diagrammatic representation of the DAF molecule, showing the GPI anchor and the four SCR domains, and illustrating the domains to which the binding sites of various pathogens have been mapped. CHO indicates glycosylation sites. Adapted from Powell et al, 1999. <sup>1</sup>Echoviruses types 3, 6, 7, 11-13, 20, 21, 29 and 33; Coxsackievirus B viruses types 1, 3 and 5.

family act as receptors for not only measles virus and Epstein-Barr virus, but also for a wide range of pathogenic bacteria (Lindahl et al., 2000), and it may be that a consequence of binding to these molecules is to allow pathogens to evade the complement response. A further possible advantage might be the location of DAF on the cell surface. GPI-anchored proteins, including DAF, are known to be concentrated in areas of the cell membrane known as lipid rafts (Brown and London, 1998). These are detergent-resistant, sphingolipid and cholesterol-rich microdomains. They act as functional platforms for many cell signalling and endocytotic processes, and may facilitate signalling processes required for subsequent viral entry and uncoating (Coyne and Bergelson, 2006; Greber, 2002; Simons and Toomre, 2000).

Evidence to date suggests that the interaction of DAF with enteroviruses does not involve docking of the receptor into the canyon, but rather binding occurs at the two-fold axis of symmetry (He et al., 2002; Pettigrew et al., 2005). Further work has looked at the kinetics of the interaction of soluble DAF and echovirus type 11 using surface plasmon resonance (Lea et al., 1998). It was shown that the interaction is of a low affinity with a very fast dissociation rate constant, similar to the kinetics seen with cell to cell recognition molecules (Lin et al., 2001; van der Merwe and Barclay, 1994). In contrast interaction of ICAM-1 with the rhinovirus canyon has a low dissociation rate constant, consistent with binding at a more inaccessible site (Casasnovas and Springer, 1995).

DAF further differs from canyon binding receptors, in that in soluble form it does not induce uncoating of DAF binding viruses as is the case for poliovirus and PVR, although uncoating does occur when virus binds to DAF on the cell surface (Powell et al., 1997). The fact that soluble DAF does not cause viral uncoating suggests that either multiple interactions between the virion and DAF molecules are required for uncoating, or that some other cellular factor is required. Indeed coxsackie B viruses require both DAF and ICAM-1. In this case it has been shown that binding of virus to DAF acts as an initial attachment event, activating pathways required to relocate the virus to the relatively inaccessible tight junction where ICAM-1 is located and uncoating occurs (Coyne and Bergelson, 2006). Alternatively for some viruses, such as the major group rhinoviruses, uncoating requires receptor binding in combination with exposure to low pH in the cellular late endosome (Nurani et al., 2003).

EV70 has been reported to bind DAF on HeLa cells, following the observation that a DAF-specific mouse monoclonal antibody could block virus binding

(Karnauchow et al., 1996). Using chimeric molecules containing domains from both DAF and CD46 it was shown that, as is the case for CVA21, this interaction occurred with SCR 1, the most membrane-distal domain of DAF (Karnauchow et al., 1998).

### 1.4.3 Sialic acid as a viral receptor

Sialic acids are negatively charged monosaccharides, which are commonly found as the terminal components of carbohydrate (glycan) chains in glycoproteins and glycolipids (Flint et al., 2000; Schauer, 2004; Varki, 1999). They are found in all higher animals and some microorganisms, but there is ongoing debate regarding their presence in invertebrates. Sialic acids are structurally diverse, with the potential for a number of substitutions at the amino and hydroxyl groups. The most common form on human cells is N-acetylneuraminic acid (Neu5Ac), shown in figure 1.5, which has an N-acetyl group at the 5-carbon position. Further diversity is generated by variation in the linkage from the 2-carbon to the underlying sugar, usually galactose. Most commonly this takes the form of an  $\alpha$ 2-3 or  $\alpha$ 2-6 linkage. This can vary in a species- and tissue-specific fashion, such that, for example,  $\alpha$ 2-3 predominates in the avian intestine and  $\alpha$ 2-6 predominates in the majority of the human respiratory tract, with  $\alpha$ 2-3 found in human lung alveolar cells (Shinya et al., 2006). Glycosylation sites can be present on lipids or proteins; in the latter case, attachment to the underlying protein is via either a serine or threonine residue (O-glycosylation) or an asparagine residue (N-glycosylation). The same molecule can have a different glycosylation pattern in different tissues, and indeed glycosylation is believed to play an important role in cellular differentiation (Schauer, 2004; Varki, 1999).

Influenza virus was the first virus to be shown to use sialic acid as its cellular receptor (Klenk and Stoffel, 1956; Skehel and Wiley, 2000), and since then it has been shown to be an important receptor component for multiple other viruses, including enveloped viruses such as parainfluenza, mumps, Newcastle Disease virus and some coronaviruses; and non-enveloped viruses such as the human polyomaviruses, adenovirus type 37, and some rotaviruses (Arnberg et al., 2000; Gee et al., 2006; Isa et al., 2006; Schwegmann-Wessels et al., 2003; Villar and Barroso, 2006). While all these viruses bind sialic acid there is variation in the form of sialic acid, the requirement for specific downstream sugars and the linkage to them. In addition specificity may be determined by components of the underlying lipid or protein. For example human influenza virus appears to bind to all  $\alpha$ 2-6 sialated glycolipids and glycoproteins,



although a requirement for cell surface N-glycoproteins for endocytosis post attachment has been reported (Chu and Whittaker, 2004). In contrast polyomaviruses seem to require a specific glycolipid (Tsai et al., 2003). For some sialic acid binding viruses, such as reoviruses and polyomaviruses, there is known to be a requirement for an additional receptor distinct from sialic acid (Barton et al., 2001; Caruso et al., 2003).

The interaction of influenza virus haemagglutinins with sialic acid has been studied in great detail (Skehel and Wiley, 2000). Species tropism is largely dependant on the linkage of the sialic acid to galactose, with avian viruses, such as the H5N1 currently circulating in poultry, binding to the  $\alpha$ 2-3 form present in avian intestine and human viruses binding to the  $\alpha$ 2-6 form in the human respiratory tract. Linkage preference therefore not only determines species tropism, but also the primary site of viral infection. Studies of H5N1 influenza suggest that several separate adaptive changes would be required to change its receptor specificity to  $\alpha$ 2-6 linked sialic acid, and this is postulated to be part of the reason for the lack of human to human spread observed in the sporadic human cases associated with the outbreaks (Russell et al., 2006).

One striking feature of many viruses predominantly infecting the human conjunctiva is the proportion which utilise sialic acid for binding (Olofsson et al., 2005). These include adenovirus 37 (Arnberg et al., 2000), one of the primary causes of viral keratoconjunctivitis, and the avian viruses influenza virus H7N7 (Fouchier et al., 2004; Koopmans et al., 2004) and Newcastle Disease Virus (Ferreira et al., 2004), which both cause conjunctivitis in humans. EV70 also fits into this group as another virus that infects the eye and has a requirement for sialic acid. EV70 infection was first reported to potentially involve sialic acid in 1982 when loss of viral-induced haemagglutination (HA) was observed on removal of sialic acid from the RBC membrane using neuraminidase (Utagawa et al., 1982b). Subsequently it has been shown that the DAF binding phenotype is distinct from the sialic acid requirement, as removal of the DAF N- and O-glycosylation sites, by site-directed mutagenesis and the use of chimeric proteins respectively, did not affect DAF binding by the virus (Alexander and Dimock, 2002). Interestingly EV68, the other HEV-D group virus, has also been shown to require sialic acid for infection (Uncapher et al., 1991). No other human enterovirus has been reported to use sialic acid as a receptor

#### 1.4.4 Species Tropism

EV70 is unusual among human enteroviruses in that it has been shown to replicate in a range of non-primate mammalian cell lines, including rodent, rabbit, porcine and bovine (Yoshii et al., 1977), and antibodies to EV70 have been reported in a number of animals (Kono et al., 1981). These observations support the hypothesis that the human virus may have emerged from an animal reservoir, although EV70 has only been isolated from humans.

DAF homologues have been identified in several species, including the mouse (Fukuoka et al., 1996). The divergence between human and murine DAF is 63% at the nucleotide level, which is sufficient to prevent other DAF binding viruses from binding and infecting murine cells. Sialic acid is found on all vertebrate cells, although the relative amounts of the different side chain substituted forms, and tissue-specific patterns of expression, may vary (Varki, 1999). The recently identified enterovirus HEV-D member, EV94, has also been reported to have wide tropism in tissue culture (Smura et al., 2007). Its receptor is unknown, although preliminary studies suggest it does not bind DAF. EV68, the other HEV-D member has been reported to require sialic acid (Uncapher et al., 1991), however it has not been observed to have a wide tissue tropism.

#### 1.4.5 Tissue culture adaptation and receptor usage

Viruses adapt to growth in tissue culture, and this is especially true for RNA viruses due to the high error rate of RNA polymerases. This is not a new concept as serial passage of virus *in vitro* has long been used to generate attenuated strains of viruses for use in vaccination. One commonly observed culture adaptation is a change in viral receptor usage due to changes in the viral capsid. For example, foot and mouth disease virus adapts to use heparin sulphate as a receptor on culture in CHO cells (Sa-Carvalho et al., 1997), and cultured measles virus adapts to use CD46 as a receptor (Ono et al., 2001).

The majority of research into viral life cycle and pathogenicity has been carried out using reference strains, which are highly passaged. Tissue culture induced changes are thus a very important consideration. Particularly misleading results could occur in the development of antiviral drugs where inhibition of a passaged virus *in vitro* may not reflect activity against circulating strains. Antiviral candidates with activity against

enteroviruses, such as pleconaril, have mainly targeted the viral capsid, which appears particularly susceptible to adaptive change (Zhang et al., 2004).

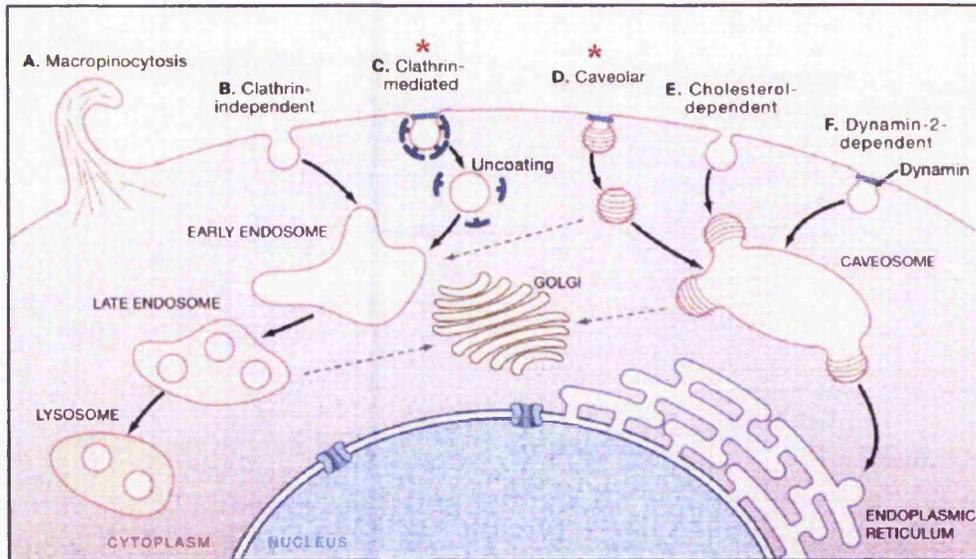
All the studies to date on EV70 have used the reference strain, J670/71, obtained during the 1970/71 epidemic in Southeast Asia. As with all reference strains, it has undergone extensive passage in tissue culture. Different laboratories have used a variety of cell lines to culture this virus (Karnauchow et al., 1996; Ryan et al., 1990; Yoshii et al., 1977). It is therefore important to interpret results using reference strains with caution and where possible to attempt to verify the observations made, using clinical isolates.

### **1.5 Viral entry**

Following attachment, a virus (or its genome) requires a mechanism to enter the cytoplasm of the cell. A virus may either penetrate the plasma membrane directly, or utilise one of the existing endocytotic pathways. The latter have the potential advantage of specifically delivering the virus to its site of replication, however a mechanism is still required for the virus to exit the endocytic vesicle and release its nucleic acid into the cytoplasm. The identity of the viral receptor plays an important role in viral entry, by virtue of its location (for example within lipid rafts), and also potentially by triggering signalling events required for internalisation.

Cells utilise a large number of different endocytic pathways (Marsh and Helenius, 2006; Pelkmans and Helenius, 2003; Sieczkarski and Whittaker, 2002a), most of which can be exploited by viruses (figure 1.6). These include the clathrin-mediated pathway, best studied as the route of influenza virus entry, and the caveolar pathway, best studied as the route of entry of the polyomavirus SV40, and a number of less well-defined non-clathrin, non-caveolin-dependant pathways. Some viruses have been reported to enter the cell via multiple routes. For example, influenza virus can still enter the cell when the clathrin route is blocked (Sieczkarski and Whittaker, 2002b), suggesting that it can utilise alternative entry pathways.

Different enteroviruses appear to use different mechanisms as their primary route of entry into the cell, partially reflecting the variety of receptors used. These include direct entry via the plasma membrane, as suggested for poliovirus (Hogle, 2002), and endocytic entry via the clathrin or caveolar pathways. The entry route of EV70 is unknown.



**Figure 1.6 Diagram of cellular endocytotic pathways.** From Marsh and Helenius, 2006. \* The clathrin and caveolar pathways are discussed in the text. The other pathways shown have also been implicated in viral entry, but are less well defined (Marsh and Helenius, 2006).

### 1.5.1 The poliovirus model for cell entry

Despite poliovirus being arguably one of the best-studied viruses, and certainly the most studied picornavirus, the mechanism of its entry into the cell remains the subject of considerable uncertainty and debate. A general consensus now seems to be emerging (Hogle, 2002; Rossmann et al., 2000), aided by recent studies on the structure of the 135s 'A' particle (Bubeck et al., 2005) and the use of liposome-based systems to allow the analysis of entry in a controlled, cell free system (Tuthill et al., 2006).

Growing evidence suggests that poliovirus RNA does not enter the cell by an endocytic pathway, but rather does so directly via the formation of a pore within the plasma membrane. This process appears to be associated with the formation of the 135S 'A' particle following viral binding to PVR on the cell surface. As mentioned in section 1.4.1, this structural change results in externalisation of the VP4 capsid protein and the N-terminus of VP1. The latter has a sequence compatible with an amphipathic helix and has been shown to allow tethering of viral particles to liposomes, independently of receptor. Evidence suggests that both the N-terminus of VP1 and VP4 may be involved in the formation of a membrane pore, through which viral RNA can be extruded directly into the cytoplasm.

Little work has been done examining the potential for this model to be applied to other enteroviruses and rhinoviruses. Although all have a predicted amphipathic helix at the N-terminus of VP1, not all the viruses can attach to liposomes in their 135S form (Airaksinen et al., 2001). One virus that appears not to is EV68, another group D enterovirus. Although not all viral receptors bind in the canyon and can induce 135S formation directly, 135S particles are still observed during productive infection. The additional cellular or physical factors that might be required for this are unclear.

In contrast to poliovirus, endocytosis does appear to have a critical role in the entry of many other enteroviruses. It remains possible, however, that similar mechanisms to those used by poliovirus may play a role in the exit from endocytotic vesicles following internalisation. Additional factors, such as pH, may be required to trigger further changes in the virion within the endocytotic vesicle, which could potentially be associated with pore formation within the vesicle membrane.

### 1.5.2 The clathrin-mediated endocytic pathway as a route of viral entry

The clathrin-mediated route was the first pathway of endocytosis described and is the most studied and best understood mechanism (Brodsky et al., 2001). In brief, receptor binding triggers the recruitment of clathrin to the cytoplasmic side of the membrane (figure 1.6). Subsequent invagination results in the formation of a clathrin coated vesicle within the cytoplasm. Clathrin then uncoats from the vesicle, which fuses to early endosomes, with progression through late endosome and lysosome stages. The pH of these compartments progressively drops to 6.0 in the early endosome and 5.5 in the late endosome. These cellular compartments also contain a number of acid-dependant proteases.

Increasing research is making apparent the complexities involved in these processes. Many additional proteins are required, such as adapter proteins for the recruitment of clathrin and for subsequent vesicle formation. Elements of the cytoskeleton, such as actin and dynamin, also seem to have a role, which as yet remains poorly defined. The pathway is also capable of targeting ligands or viruses to distinct locations, as endosomes are not all identical in terms of function and location (Marsh and Helenius, 2006). One major requirement for clathrin endocytosis is the activation of cell signalling pathways. Recent work suggests that over 90 kinases may be involved in the clathrin-mediated entry of vesicular stomatitis virus (Pelkmans et al., 2005). Membrane lipid composition is believed to play an important part in vesicle formation and lipid rafts can have a role in clathrin-mediated endocytosis. Although these are classically the site of internalisation via the caveolar route, clathrin entry can also occur at these sites, and raft-based cell signalling is known to have important roles in the clathrin-mediated endocytosis of receptors such as the epidermal growth factor receptor, the B cell receptor and the HIV receptor CCR5 (Puri et al., 2005; Signoret et al., 2005; Stoddart et al., 2002).

Influenza is perhaps the best studied virus to use the clathrin pathway (Skehel and Wiley, 2000). After entering late endosomes, the low pH induces a conformational change in the viral haemagglutinin, which results in fusion of the viral and endosomal membranes with release of viral RNA into the cytoplasm. Some rhinoviruses are also reported to require low pH for entry (Nurani et al., 2003). In addition to direct pH effects on viral proteins, viruses such as coronaviruses and rotavirus, require the action of proteases present within late endosomes for release of their nucleic acid (Ebert et al., 2002; Simmons et al., 2005).

Picornaviruses believed to enter cells via a clathrin route include human rhinoviruses type 2 and 14 (DeTulleo and Kirchhausen, 1998), parechovirus 1 (Joki-Korpela et al., 2001) and foot and mouth disease virus (Berryman et al., 2005). Many of these are reported to be dependant on late endosomal pH for entry, however there is ongoing debate regarding the exact role of pH and the exact method by which the viral RNA is released. This could involve pore formation comparable to that suggested for poliovirus or disruption of the endosome itself.

### **1.5.3 The caveolar endocytotic pathway as a route of viral entry**

A subset of lipid rafts contain caveolin and can be observed by electron microscopy to form pits or caveolae in the plasma membrane. Caveolar endocytosis is somewhat slower than that mediated by clathrin. Following internalisation ligands or viruses are taken up into pH-neutral vesicles known as caveosomes and hence to the endoplasmic reticulum (Anderson, 1998). Low pH endosomes and lysosomes do not appear to be involved (figure 1.6). The caveolar pathway has been best studied as the route of entry of the simian polyomavirus, SV40 (Pelkmans et al., 2001). As with clathrin pathways, caveolar entry pathways are complex involving several protein components, as well as signalling pathways, the cytoskeleton and lipids. The cellular uptake of SV40 may be associated with changes in the activity of 80 different kinases, largely distinct from those affecting clathrin-mediated endocytosis (Pelkmans et al., 2005). SV40 penetration into the cytosol is believed to occur via the ER and an ER protein has been shown to be capable of causing a structural change in the SV40 capsid resulting in exposure of a hydrophobic domain (Magnuson et al., 2005), perhaps in a manner similar to poliovirus uncoating and pore formation.

Picornaviruses believed to enter cells via the caveolar route include echovirus type 1 (Pietiainen et al., 2004) and the coxsackie B viruses (Coyne and Bergelson, 2006). There is currently no information on how these viruses might release their RNA from caveosome or ER compartments.

## **1.6 Use of full-length clones and replicons**

The RNA alone of picornaviruses is infectious on introduction into cells (Evans, 1999; Racaniello and Baltimore, 1981). As the positive sense RNA genome acts as its own messenger RNA, and its translation is dependant on host rather than viral proteins,

the viral polyprotein can be produced from the RNA. Subsequently polyprotein cleavage and viral replication occur with production of viable viral particles. Using full-length DNA clones of the viral genome, with subsequent *in vitro* transcription and RNA transfection, viable virus can be efficiently recovered from transfected cells.

Complimenting the use of full-length clones are subgenomic replicons. These contain reporter genes, usually in place of capsid-encoding sequences, allowing viral replication to be quantified and studied (Percy et al., 1992).

The first demonstration of an infectious enterovirus clone was of poliovirus (Racaniello and Baltimore, 1981). Viral sequences were cloned directly from multiple cDNAs derived from portions of the poliovirus genome. More recently PCR (polymerase chain reaction) -based cloning strategies have been used, and direct cloning of entire enterovirus genomes using long distance reverse transcription and polymerase chain reaction (RT-PCR) has been reported (Lindberg et al., 1997). The construction of a poliovirus subgenomic replicon was first reported in 1985 and included a chloramphenicol acetyltransferase reporter gene (Percy et al., 1992). More recently firefly luciferase has been used as a reporter gene, allowing more rapid automated quantification (van Kuppeveld et al., 1996).

Enteroviral clones have been used extensively for reverse genetic research approaches, where specific mutations are introduced into the viral cDNA, allowing virus containing these mutations to be generated (Evans, 1999). Full-length clones also allow virus isolates that have not undergone passage in tissue culture to be used in research. It is not practical to generate full-length clones for all clinical isolates, as it remains a difficult and time consuming procedure. An alternative strategy of subcloning portions of the viral genome of interest into a generic backbone, such as that generated from a reference strain, is more feasible, and could enable the study of larger numbers of clinical isolates. Subgenomic replicons are particularly useful for the investigation of viral translation and replication, and have also been used to investigate viral packaging by co-transfecting helper virus RNA to supply capsid proteins *in trans*.

Subgenomic replicons and infectious viral clones have also been used in the study of other viruses. Until recently there has been no cell culture model for hepatitis C virus (HCV) infection, however research has been greatly facilitated by the availability of subgenomic HCV replicons (Lohmann et al., 1999). These replicate in a hepatoma cell line and have been used to investigate HCV translation and replication. More recently a full-length HCV clone has been derived from a patient with fulminant

hepatitis which enables productive HCV infection of hepatoma cells and is currently revolutionising research into HCV infection (Zhong et al., 2005).

Both full-length clones and subgenomic replicons are proving invaluable tools in many areas of picornavirus research, however, before now, these have not been available for EV70. Such clones would be extremely valuable in future studies of EV70, in particular by enabling the use of virus derived directly from circulating strains.

## **1.7 Aims**

As can be seen from the preceding introduction, much work has been done, and is ongoing, regarding the interaction of enteroviruses with the cell via specific receptors and the subsequent pathways of cellular entry. In addition the importance of cell signalling pathways and the cytoskeleton to virus infection is becoming increasingly apparent. Although this research has encompassed members of HEV-A, B and C relatively little work has been done on the HEV-D viruses, in particular EV70.

EV70 is an important human pathogen in terms of morbidity and the potential economic impact of epidemics. From a virological point of view it is of interest as an unusual enterovirus in terms of its tropism for the conjunctiva, reported broad species range and use of sialic acid as a receptor.

Although it has been reported to bind both sialic acid and DAF, the relative importance of these is unknown, as is their importance in determining the tropism of the virus. In addition little is known regarding the sialic acid component of the receptor, for example whether a specific sialated protein or lipid is required, or if there is specificity for a particular linkage to the underlying sugar. Furthermore the route used by EV70 to enter the cell is completely unknown.

The overall aim of this study was to determine the molecular basis of the early events in the EV70 life cycle.

### **Specific aims**

- To develop tools to facilitate research into the EV70 life cycle, and enable the use of viruses derived directly from uncultured clinical isolates. Specifically the construction of a full-length infectious EV70 clone and subgenomic replicon.

- To determine the receptor interactions required by EV70 to enable binding and infection of the cell.
  
- To investigate the route of entry of EV70 into the cell, including the potential involvement of cellular signalling pathways and the cytoskeleton.
  
- To investigate the determinants of EV70 tissue tropism.

## CHAPTER 2

### Materials and Methods

#### 2.1 Reagents

##### 2.1.1 Antibodies and conjugates

Antibodies were diluted in phosphate buffered saline (PBS) with 0.5% bovine serum albumin (BSA) for flow cytometry or immunofluorescence (IF) and in blocking buffer for western blots (WB). The supplier and dilution used for each technique is indicated below.

Anti-actin rabbit polyclonal (Sigma) – 1:40 (IF)

Anti-alpha tubulin mouse monoclonal (Abcam) – 1:50 (IF)

Anti-Caveolin1 rabbit polyclonal (Santa Cruz) – 1:2 000 (WB)

Anti-DAF rabbit polyclonal antiserum (raised against *Pichia* expressed human DAF in-house, Prof. DJ Evans laboratory) – Working concentrations detailed in relevant sections

Anti-enterovirus 70 mouse monoclonal 854 (Chemicon) – 1:667 (flow cytometry),  
1:250 (IF)

Anti-enterovirus mouse monoclonal 5-D8/1 (Dako) – 1:400 (flow cytometry, IF)

Anti-mouse IgG Fluorescein isothiocyanate (FITC) conjugate produced in goat (ImmunologicalsDirect) – 1:400 (flow cytometry, IF)

Anti-rabbit IgG FITC conjugate produced in goat (Sigma) – 1:320 (IF)

Anti-rabbit IgG peroxidase conjugate produced in goat (Sigma) – 1:3 000 (WB)

Anti-*src* rabbit monoclonal (Cell signaling) – 1:1 000 (WB)

Transferrin from human serum, fluorescein conjugated (Invitrogen) – 5mg/ml in PBS

##### 2.1.2 Growth Media and supplements

Dulbecco's minimum essential medium (DMEM) (Invitrogen)

F12 Ham (Invitrogen)

Foetal Calf serum (FCS) (Invitrogen)

Glutamine 200mM (Invitrogen)

Leibovitz's L-15 medium (Invitrogen)

Minimum essential medium with Earle's salts (EMEM) (Invitrogen)

Methionine/cysteine free DMEM (Sigma)

Non-essential amino acids 10mM (Invitrogen)

Opti-MEM – reduced serum medium (Invitrogen)

Penicillin/Streptomycin 100x, 10 000IU/10 000µg per ml (Invitrogen)

Sodium Bicarbonate 7.5% (Invitrogen)

Sodium Pyruvate 100mM (Invitrogen)

Trypsin, porcine, 25g/litre (Invitrogen)

Versene – 0.2g/litre ethylenediaminetetraacetic acid (EDTA) in PBS (Invitrogen)

### 2.1.3 Buffers and Solutions

Brij-58 (Sigma) – 1% in 2mM EDTA in PBS

Crystal Violet solution – 0.5g crystal violet dissolved in 20ml ethanol, made up to 1 litre  
in a solution of 4% v/v formaldehyde, with 0.9g NaCl.

Paraformaldehyde – 4% v/v in PBS, stored at –20°C

PBS/BSA - 0.5% v/v BSA in PBS, stored at 4°C

PBS/Tween (PBST)– PBS with 0.1% v/v Tween 20

Plaque assay overlay medium- 1 x EMEM (from 10 x EMEM), 2% v/v FCS, 2mM  
glutamine, 1x penicillin/streptomycin, 3ml 7.5% sodium bicarbonate, 20mM MgCl<sub>2</sub>  
and 0.6% low melting point agarose (made up as 2% stock in deionised water  
(dH<sub>2</sub>O), melted and added just prior to use)

TAE (50x) – 242g Tris base, 57.1ml glacial acetic acid, 0.05M EDTA pH8.0, made up  
to 1 litre

TM buffer – 10mM TrisHCl pH 7.5, 1mM MgCl<sub>2</sub> (1 protease inhibitor tablet/10ml).

TNE buffer – 10mM TrisHCl pH7.5, 150mM NaCl, 1mM EDTA

Tris-glycine electrophoresis buffer (10x) – 250mM Tris base, 2.5M glycine, 1% sodium  
dodecyl sulphate (SDS)

Triton X100 - 0.1% v/v in PBS

Western Blot Blocking Buffer – PBST with 4% v/v skimmed milk powder

Western Blot Transfer Buffer – 25mM Tris, 0.2M glycine, 20% v/v methanol, 10% SDS

### 2.1.4 Chemicals, inhibitors and enzymes

Solvent and concentration of initial stock solution are given; working concentrations are detailed in the relevant results sections. All other unlisted basic chemicals were obtained from Sigma, unless noted elsewhere in this section.

$\alpha$ 2-3 specific neuraminidase from *Streptococcus pneumoniae* (Sigma) – 1U/ml in PBS

Ammonium Chloride (NH<sub>4</sub>Cl) (Sigma) – 3M in dH<sub>2</sub>O

Ampicillin (Sigma) – 100mg/ml in dH<sub>2</sub>O

Bafilomycin (Sigma) - 40 $\mu$ M in dimethyl sulphoxide (DMSO)

Bisindoylmaleimide (Sigma) – 2mM in DMSO

Brefeldin A (Sigma) – 36mM in ethanol

Chlorpromazine (Sigma) – 78mM in dH<sub>2</sub>O

Chymotrypsin (Sigma) – 100mg/ml in H<sub>2</sub>O

Cytochalasin B (Sigma) – 20mM in DMSO

Genistein (Sigma) – 18mM in DMSO

Guanidine Hydrochloride (Sigma) – 200mM in dH<sub>2</sub>O

Kanamycin (Sigma) - 50mg/ml in dH<sub>2</sub>O

Methyl- $\beta$ -cyclodextrin (Sigma) - 38mM in dH<sub>2</sub>O

N-Acetylneuraminic Acid (Sialic Acid, Sigma) – 100mg/ml in dH<sub>2</sub>O

Neuraminidase from *Clostridium perfringens* (Sigma) – 1U/ml in PBS

Nocodazole (Sigma) – 33mM in DMSO

Nystatin (Sigma) – 52mM in dH<sub>2</sub>O

Phospholipase C (PLC, Sigma) – 5U/ml in PBS

Phosphatidylinositol specific phospholipase C (PIPLC, sigma) – 5U/ml in PBS

PP2 (Calbiochem) – 10mM in DMSO

Progesterone (Sigma) – 32mM in ethanol

Protease Inhibitor Tablets (Roche) – 1Tablet/10ml solution (added just prior to use)

Proteinase K (Sigma) – 1mg/ml in dH<sub>2</sub>O

Trypsin (Sigma) – 100mg/ml in dH<sub>2</sub>O

### 2.1.5 Molecular biology enzymes, reagents and kits

Plasmids are listed in table 2.1

Agarose (Roche) Used as a 1% solution in 1x TAE

Table 2.1 Description of plasmids

Plasmid	Source	Description
<b>p5'70Rep3</b>	This project (section 3.3.1 / figure 3.10)	Chimeric PV3 luciferase-encoding subgenomic replicon, with EV70 5'UTR. Contains ribozyme and T7 promoter. Ampicillin resistant.
<b>pBluescript II SK-</b>	Stratagene	Cloning vector with polylinker and <i>LacZα</i> blue-white selection. Ampicillin resistant.
<b>pFLC70</b>	This project (section 3.1.5 / figure 3.4)	EV70 full length infectious clone with ribozyme and T7 promoter. Ampicillin resistant.
<b>pFLC70β</b>	This project (section 3.1.4 / figure 3.2)	EV70 full length infectious clone with ribozyme and T7 promoter. Contains changes within 5'UTR (table 3.3). Ampicillin resistant.
<b>pLuc70</b>	This project (section 3.3.1 / figure 3.10)	EV70 luciferase-encoding subgenomic replicon with ribozyme and T7 promoter. Ampicillin resistant.
<b>pLuc70intermediate</b>	This project (section 3.3.1 / figure 3.10)	Intermediate in the construction of pLuc70. Contains EV70 5'UTR, partial P1, P2, P3 and 3'UTR. Also encodes luciferase and a small portion of the PV3 P1/2 junction. Ampicillin resistant.
<b>pPCR-BluntII-TOPO</b>	Supplied with Zero Blunt TOPO PCR Cloning Kit (Invitrogen)	TOPO cloning vector with <i>ccdB</i> lethal gene selection, T7 and SP6 promoters and M13 forward and reverse primer binding sites. Supplied linearised, with <i>Vaccinia</i> virus DNA topoisomerase I covalently linked to 3' end of each strand. Kanamycin resistant.
<b>pT7rep3-L+R</b>	Prof. DJ Evans, in house (Goodfellow et al., 2000)	PV3 luciferase-encoding subgenomic replicon with ribozyme and T7 promoter. Ampicillin resistant.
<b>pTOPOFLC70</b>	This project (section 3.1.2 / figure 3.2)	EV70 full length infectious clone with T7 promoter. Contains changes within 5'UTR (table 3.3). No ribozyme. Kanamycin resistant.

BCA Assay Kit (Pierce)

Deep Vent DNA Polymerase (Invitrogen). Supplied with 100mM MgSO<sub>4</sub> and 10x thermopol reaction buffer

Deoxyribonucleotide triphosphates (dNTPs): dATP, dUTP, dCTP, dGTP (Invitrogen) supplied separately at 100mM each and diluted to a single stock solution in dH<sub>2</sub>O containing 10mM each

Ethidium Bromide Solution 10mg/ml (BioChemika)

KOD Hot Start DNA polymerase (Novagen). Supplied with 25mM MgSO<sub>4</sub>, dNTP solution containing dATP, dTTP, dCTP and dGTP at 2mM each and 10x PCR buffer

Lipofectamine 2000 (Invitrogen)

Low Melting Point Agarose (Roche) Used as a 1% solution in TAE

Luciferase Assay System (Promega)

Megascript T7 kit (Ambion)

Platinum Pfx DNA Polymerase (Invitrogen). Supplied with 50mM MgSO<sub>4</sub> and 10x PCR buffer

Primers (VHbio) - diluted on receipt to a stock of 100 picomolar in dH<sub>2</sub>O

QIAfilter Plasmid Midi Kit (Qiagen)

QIAquick Gel Extraction Kit (Qiagen)

QIAquick PCR purification Kit (Qiagen)

QIAamp Viral RNA Mini Kit (Qiagen)

RNaseOUT (Invitrogen)

RNeasy Mini Kit (Qiagen)

Ribonucleotide triphosphates (rNTPs): rATP, rUTP, rCTP, rGTP (Promega) supplied separately at 100mM each and diluted to a single stock solution in dH<sub>2</sub>O containing 10mM each

RQ1 RNase-Free DNase (Promega)

Restriction endonucleases (New England Biolabs)

SP6 RNA polymerase (Invitrogen). Supplied with 5x buffer and 0.1M DTT

Superscript II Reverse Transcriptase (Invitrogen)

T4 DNA Ligase (New England Biolabs). Supplied with 10x buffer

T7 RNA Polymerase (Invitrogen). Supplied with 5x buffer and 0.1M DTT

Zero Blunt TOPO PCR Cloning Kit (Invitrogen)

## **2.2 Cell lines and tissue culture**

All cells were grown at 37°C in 5% carbon dioxide unless otherwise stated. Cells were washed with PBS and detached in versene supplemented with 2.5g/litre trypsin.

MRC5 are primary human embryonic lung fibroblast cells and were obtained from the European Collection of Cell Cultures and LGC Promochem. The cells were grown in EMEM, supplemented with 0.1mM non-essential amino acids, 2mM glutamine, 1x penicillin/streptomycin and 10% FCS. Cells were recovered on receipt (16 population doublings) and frozen at approximately 18 population doublings. Following recovery they were split 1:6 and used for assays up to 36 total population doublings.

RD are human embryo rhabdomyosarcoma cells. They were grown in DMEM, supplemented with 2mM glutamine, 1x penicillin/streptomycin and 10% FCS and were split 1:5 to 1:20.

RD-ICAM are RD cells engineered to express human ICAM-1, and were a generous gift from Dr Darren Shafren, University of Newcastle, Australia. The cells were grown under the same conditions as RD cells.

HeLa are human cervix epitheloid carcinoma cells. They were grown in DMEM supplemented with 2mM glutamine, 1x penicillin/streptomycin and 10% FCS and were split 1:5 to 1:10.

RK13 are rabbit kidney cells, obtained from LGC Promochem. They were grown in EMEM supplemented with 0.1mM non-essential amino acids, 1mM sodium pyruvate, 2mM glutamine, 1x penicillin/streptomycin and 10% FCS and were split 1:5 to 1:20.

CHO are chinese hamster ovary cells. They were grown in F12 Ham medium, supplemented with 2mM glutamine, 1x penicillin/streptomycin and 10% FCS and were split 1:5 to 1:20.

CHODAF are CHO cells engineered to express human DAF. Cells were transfected with a DAF expression vector based on the plasmid pDR2 $\Delta$ EF1 $\alpha$  and stable transfectants selected (in-house, Prof. DJ Evans laboratory). The cells were grown under the same conditions as CHO cells.

XTC-2 are *Xenopus laevis*, African clawed toad cells and were generously provided by Prof. R Elliott, University of Glasgow. They were grown at 28°C in Leibovitz's L-15 medium, supplemented with 2mM glutamine, 1x penicillin/streptomycin and 10% FCS and were split 1:5 to 1:10.

XTC-2DAF are XTC-2 cells engineered to express human DAF. Cells were transfected with a DAF expression vector based on the plasmid pDR2ΔEF1α and stable transfectants selected (in-house, Prof. DJ Evans laboratory). The cells were grown under the same conditions as XTC-2 cells.

C6/36 are *Aedes albopictus* larvae cells and were generously provided by Prof. R Elliott, University of Glasgow. They were grown in EMEM supplemented with 0.1mM non-essential amino acids, 1mM sodium pyruvate, 2mM glutamine, 1x penicillin/streptomycin and 10% FCS and were split 1:5 to 1:10.

## **2.3 Viruses, passage and quantification**

### **2.3.1 Viruses**

The viruses used are listed in table 2.2, together with details of where the viruses were obtained and the growth conditions used for cultivation of viral stocks.

### **2.3.2 Viral Passage**

Cells were grown to 80-90% confluency, washed with PBS and inoculated with virus at a multiplicity of infection (m.o.i.) of at least three in a volume of under 1ml. After 45min absorption at room temperature, the inoculum was removed and cells washed with PBS before adding 10mls of serum-free growth medium. Inoculated cells were incubated in 5% carbon dioxide at the temperatures detailed in table 2.2. After the appearance of full cytopathic effect (CPE), virus was harvested at 16-24hr by two freeze-thaw cycles followed by centrifugation for 5min at 4 000rpm. The clarified virus-containing supernatant was then stored in 1ml aliquots at -70°C (EV70) or -20°C (other viruses).

**Table 2.2 List of viruses used with details of source and culture conditions**

<b>Virus</b>	<b>Source</b>	<b>Cell line used for cultivation</b>	<b>Growth temperature</b>
EV70 (also referred to as wild type EV70)	A fresh aliquot of EV70 (strain J670/71) from ATCC was passaged in MRC5 cells and all work carried out using virus obtained from the fifth passage	MRC5	33°C
EV70 <sup>FLC</sup>	Derived from pFLC70 full length EV70 infectious clone (chapter 3)	MRC5 (RNA transfection)	33°C
Echovirus type 7	Dr D Williams (University of Glasgow), originally derived from an infectious clone generously provided by Prof. M. Lindberg (University of Kalmar, Sweden)	RD	37°C
Echovirus type 11 (strain 207)	Generously provided by Dr T. D. K. Brown (University of Cambridge), Stuart et al., 2002	RD	37°C
CVA21	Generously provided by Prof. Glyn Stanway, (University of Essex)	RD-ICAM	37°C
Influenza B virus	Generously provided by Dr Wendy Barclay, (University of Reading)	Egg, allantoic fluid	37°C

### **2.3.3 Plaque Assay**

A ten-fold dilution series of virus in serum-free EMEM was made, and 200µl of each added to wells of a 6-well tissue culture dish containing 90% confluent MRC5 cells. Following 45min adsorption, 4ml plaque assay overlay medium was added per well without washing. Once set, plates were inverted and incubated for 72hr at 37°C or 33°C as appropriate (table 2.2). Plates were stained with Crystal Violet solution overnight prior to the removal of overlay, and plaques counted.

### **2.3.4 50% Tissue Culture Infectious Dose (TCID<sub>50</sub>) Assay**

Four replicates of a ten-fold dilution series of virus in serum-free EMEM were made in 50µl volumes in a 96-well tissue culture plate. MRC5 cells were detached in trypsin and made up to a concentration of  $1 \times 10^5$  cells/ml in growth medium and 100µl added to each well. A further volume of 50µl serum-free EMEM was added to make a final volume of 200µl/well. Cells were incubated for 96hr at 37°C or 33°C as appropriate (table 2.2). The proportion of wells at each dilution showing CPE was scored and the TCID<sub>50</sub> calculated. Where required, the effect of potential inhibitors of viral infection was determined, by treating cells prior to their addition to the viral dilution series.

### **2.3.5 Growth Curves**

90% confluent cells in 35mm dishes were infected with virus at an m.o.i. of three and dishes incubated at 33°C. Dishes were frozen at -20°C at time points detailed in the relevant sections. After two freeze-thaw cycles virus was harvested and titrated by plaque assay or TCID<sub>50</sub>.

## **2.4 Molecular Biology Protocols**

A list of plasmids used is provided in table 2.1.

### **2.4.1 DNA Purification**

Small scale plasmid DNA preparation was carried out by alkaline lysis using published protocols (Sambrook and Russel, 2000). Larger scale plasmid DNA preparation was carried out using the QIAfilter Plasmid Midi Kit (Qiagen), according to the manufacturer's protocol.

Purification of PCR products was carried out using the QIAquick PCR purification Kit (Qiagen) or, following electrophoresis, with the QIAquick Gel Extraction Kit (Qiagen), both according to the manufacturer's protocol with a final elution volume of 30µl dH<sub>2</sub>O.

Phenol/chloroform extraction was carried out by adding an equal volume of phenol: chloroform: isoamyl ethanol (25:24:1) to the solution. The sample was then vortexed and centrifuged for 5min at 13 000rpm. The upper aqueous, DNA containing layer was then transferred to a fresh sample tube.

Ethanol precipitation of DNA was carried out by the addition of 1/10 volume 3M sodium acetate pH5.3 and 2½ volumes 100% ethanol. Samples were then centrifuged at 13 000rpm at 4°C for 30min, washed with 70% ethanol, centrifuged for a further 10min at 13 000rpm and resuspended in dH<sub>2</sub>O.

#### **2.4.2 DNA, RNA and protein quantification**

DNA and RNA were quantified using an Eppendorf BioPhotometer. Protein quantification was carried out using a BCA assay kit (Pierce), following the manufacturer's protocol and using an MRX Microplate Reader (Dynex Technologies).

#### **2.4.3 DNA Electrophoresis**

Gel electrophoresis of DNA was carried out at 5V/cm in 1% agarose supplemented with 0.1µg/ml Ethidium Bromide Solution. The running buffer was 1x TAE. Where purification of DNA fragments from the gel was required low melting point agarose was used.

#### **2.4.4 Viral RNA extraction and reverse transcription**

Viral RNA was extracted from viral tissue culture supernatant using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's protocol. 140µl of clarified viral tissue culture supernatant was used per spin column.

Reverse transcription was carried out using 10µl of extracted viral RNA, primer E70rt (Table 3.1) and superscript II reverse transcriptase (Invitrogen) in a reaction volume of 20µl. The enzyme supplier's protocol was used with the modification of a longer extension incubation of 130min at 46°C. Following reverse transcription, RNA was

hydrolysed by the addition of 1M sodium hydroxide to a final concentration of 0.1M, and incubation at 37°C for 20min. The cDNA was precipitated with ethanol and resuspended in 20µl dH<sub>2</sub>O. Integrity of the full-length RTPCR product was then assessed using PCR targeted at the viral 5'UTR (section 2.4.5, table 2.3).

#### **2.4.5 PCR**

PCR was carried out using PTC 200 DNA engine Thermal Cycler (MJ Research). Conditions were optimised at a range of magnesium concentrations and annealing temperatures using a gradient PCR block. The optimised conditions used are detailed in table 2.3. Reactions were analysed by electrophoresis, using 2-5µl of the reaction mixture.

#### **2.4.6 Restriction endonuclease digestion**

Reactions were carried out with a minimum of 5 units of enzyme per digest for 2hr using the recommended temperature and buffer.

#### **2.4.7 Cloning Protocols**

Detailed descriptions of the cloning strategies used to make the EV70 full-length infectious clone and subgenomic replicon are contained in chapter 3. Where necessary plasmids were digested with the restriction endonucleases detailed in the relevant sections, and insert and vector fragments separated by agarose gel electrophoresis prior to purification using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's protocol. Ligation, transformation and the selection of insert containing clones are described in sections 2.4.8 and 2.4.9. For cloning using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen), the manufacturer's protocol was followed using a vector to insert ratio of 1:3.

#### **2.4.8 Ligation**

Ligations were carried out in a 10µl reaction volume containing 50-100ng of vector, insert (in a vector to insert ratio of 1:3), 400 units T4 DNA ligase and 1x ligase buffer. Reactions were incubated overnight at 16°C, DNA was precipitated with ethanol and 5µl used per transformation.

Table 2.3 PCR reaction contents and conditions

PCR*	Polymerase	Primers†		Initial denaturation	Cycles (30)			Final extension
		Sense	Anti-sense		Denaturation	Annealing	Elongation	
<b>Full length EV70</b>	1unit KOD Hot Start DNA Polymerase (Novagen)	EV70s5p	EV70a3p	2min 94°C	15s 94°C	30s 58.5°C	390s 72°C	20min 72°C
<b>EV70 capsid</b>	2.5 units Pfx DNA polymerase (Invitrogen)	EV70sJUP1	EV70aJP12	2min 94°C	15s 94°C	30s 46°C	120s 72°C	20min 72°C
<b>EV70 5'UTR</b>	1unit KOD Hot Start DNA Polymerase (Novagen)	EV70s5p	EV70aJUP1	2min 94°C	30s 94°C	30s 57°C	50s 72°C	20min 72°C
<b>EV70 P1/2 junction</b>	1unit KOD Hot Start DNA Polymerase (Novagen)	EV70s3121	EV70a3596	2min 94°C	15s 94°C	30s 57°C	30s 72°C	20min 72°C

\*Reactions were carried out in a 50µl volume containing the polymerase and 0.4µl of the primers detailed, and in the appropriate polymerase buffer with 4µl cDNA, 2mM MgSO<sub>4</sub> and 0.2mM dNTPs. † Primer sequences are detailed in table 3.1.

#### 2.4.9 Bacterial transformation and selection of positive clones.

Transformations were carried out using either commercially available chemically competent TOP10 *E.coli* (Invitrogen) according to the supplier's protocol, or alternatively, using in-house electrocompetent *E.coli* DH5 $\alpha$  (see below). For TOPO cloning One Shot<sup>®</sup> Electrocompetent *E.coli* (Invitrogen) were used following the manufacturer's protocol.

Electrocompetent *E.coli* DH5 $\alpha$  were made as described in Sambrook and Russel, 2000. Plasmid or ligated DNA was added to 45 $\mu$ l of electrocompetent cells. Electroporation was then carried out using an ECM 630 electroporator (BTX) at settings of 1600V, 400 $\Omega$  and 25 $\mu$ F. After addition of 1ml of L-Broth, cells were incubated at 37°C for 60min and plated on selective agar.

For all transformations agar were supplemented with either kanamycin at 50 $\mu$ g/ml or ampicillin at 100 $\mu$ g/ml as appropriate. Plates were incubated inverted overnight at 37°C.

#### 2.4.10 Sequencing

Sequencing was carried out by The Sequencing Service, School of Life Sciences, University of Dundee, Scotland ([www.dnaseq.co.uk](http://www.dnaseq.co.uk)), and analysed using Bioedit (Tom Hall, Ibis Therapeutics) and Clustal X software (Thompson et al., 1997). Plasmid templates were purified using the QIAfilter plasmid midi kit (Qiagen) and PCR templates purified using the QIAquick PCR purification Kit (Qiagen), according to the manufacturer's protocol. Sequencing primers used are listed in table 2.4. In addition M13 forward and reverse primers supplied with the TOPO cloning kit (Invitrogen) were used to sequence the ends of cloned inserts.

#### 2.4.11 RNA transcription

5 $\mu$ g of Plasmid DNA, from the viral full-length clone or subgenomic replicon, was linearised in a 50 $\mu$ l volume using the restriction endonuclease *Sal* I, extracted with phenol/chloroform, ethanol precipitated and resuspended in 20 $\mu$ l dH<sub>2</sub>O. The transcription reaction was carried out for 2hr at 37°C in a 50 $\mu$ l volume containing 10 $\mu$ l linearised DNA, 100U T7 or SP6 RNA polymerase as appropriate, 120U RNaseOUT, 1x polymerase buffer and 1mM rNTPs. Transcribed RNA was treated with RQ1 DNase

**Table 2.4 Primers used for sequencing of EV70 genome**

<b>Primer*</b>	<b>Sequence (5' to 3')</b>
<b>497F</b>	ATCCTGTGAGTGGTTTGTCG
<b>518R</b>	TACGACAAACCACTCACTGG
<b>1057F</b>	CTACCTGATAATGAGGCAG
<b>1442F</b>	CTCTTGTATAACCCTCACCAG
<b>1620R</b>	TGGCACATAGGAGTTTGAG
<b>2620F</b>	TCTGCACTTGTGTGTATGCG
<b>2801R</b>	CTAAGGGTGGGTACAATAG
<b>3200F</b>	TGCCCTATAACCAACATCCTC
<b>3552F</b>	CCCTGGTATTCAGTGGATC
<b>4107F</b>	ATTGAGAGGCCTGGATTGG
<b>4690F</b>	ATTGAGAGGCCTGGATTGG
<b>4877F</b>	CAGAAAAGTGCAGACCAACC
<b>4882R</b>	TTTCTGGGTGCACAACCTCC
<b>5246F</b>	CGCTACAGGCAATAACCAC
<b>6419R</b>	GCCCTTCTCTACTTTCTCTC
<b>6432F</b>	GAAGGGCAAGTCTAGTGTC
<b>6992F</b>	CAGCAGACAAGAGTAGTGG
<b>7092F</b>	CCCAGTTATGCCAATGAAGG

\* In the primer names, numbers refer to the position of the 5' base in the published EV70 sequence (Ryan et al, 1990) and 'F' and 'R' represent sense and antisense orientations with respect to the EV70 genome.

(Promega) according to the supplier's protocol and purified using the RNeasy mini kit (Qiagen), according to the manufacturer's protocol, with a final elution volume of 50 $\mu$ l. RNA integrity was checked by gel electrophoresis (as for DNA electrophoresis above, using ribonuclease free solutions and buffers) and RNA quantified by spectrophotometry. Where a large amount of viral RNA transcript was required a Megascript T7 kit (Ambion) was used, according to the manufacturer's protocol.

#### **2.4.12 Eukaryotic Cell transfection**

For electroporation cells were detached with trypsin, washed and resuspended at  $2 \times 10^6$  cells/ml. 2 $\mu$ g of RNA was added to 450 $\mu$ l of cells and electroporation carried out using a BioRad GenePulserXCell at exponential wave settings of 250V, 250mF,  $\infty\Omega$ . Cells were then added to 4ml of growth medium, transferred to a 6-well tissue culture dish, and incubated at 33°C.

Lipofection was carried out in 6-well dishes using Lipofectamine 2000 (Invitrogen) and following the manufacturer's protocol. In brief 2 $\mu$ g of RNA in 100 $\mu$ l Opti-MEM (Invitrogen) was added to 4 $\mu$ l lipofectamine 2000 in 100 $\mu$ l Opti-MEM, mixed, and incubated at room temperature for 20mins. Growth medium was removed from a monolayer of cells in one well of a 6-well dish, replaced with 800 $\mu$ l of Opti-MEM and the 200 $\mu$ l solution of RNA/lipofectamine added. Cells were incubated at 33°C and the culture medium replaced after 1-2 hours.

## **2.5 Experimental Protocols**

### **2.5.1 Chemical treatments of cells**

All reagents were made up in serum-free EMEM from stock solutions (section 2.1.3) at the concentrations detailed in the relevant chapters. Controls were treated with serum-free EMEM alone. Cells were used at 90% confluency. To prevent detachment, plates to be used for cytochalasin B treated cells, were pre-treated with 0.01% poly-L-lysine (Sigma), according to the supplier's protocol. All cells were washed in PBS and treated with reagents in a volume of 200 $\mu$ l per coverslip, 500 $\mu$ l per well of a 6-well plate or 6ml per 150cm<sup>2</sup> flask. For binding assays and the TCID<sub>50</sub>-based infectivity assay, cells were first detached, washed with PBS and resuspended in 500 $\mu$ l reagent per 10<sup>6</sup> cells.

Incubations were for 1hr at 37°C for non-specific and  $\alpha$ 2-3 specific neuraminidase, 1hr at 4°C for anti-DAF rabbit polyclonal and 30min at 37°C for NH<sub>4</sub>Cl, bafilomycin, bisindoylmaleimide, brefeldin A, chlorpromazine, cytochalasin B, genistein, methyl- $\beta$ -cyclodextrin, nocodazole, nystatin & progesterone and PP2. The agents were also maintained in the culture medium during virus adsorption and incubation stages.

### 2.5.2 Toxicity Assay

MRC5 cells were treated with agents for 6hr at 37°C. All cell treatment solutions were tested on Whatman pH 6-8 indicator paper to ensure maintenance of neutral pH. Subsequently cells were detached with trypsin and stained with trypan blue (Sigma), to indicate viability. Only intact, unstained (viable) cells were counted. Three wells were counted per reagent concentration and compared to the numbers of intact cells in three control wells. Concentrations resulting in more than a 0.5 log<sub>10</sub> reduction in viable cells were considered toxic.

### 2.5.3 pH stability assay

A series of solutions were made up between pH 1 and pH 7 by dilution of 1M HCl with 1M NaOH using a PH211 Microprocessor pH Meter (Hanna Instruments). These were used to dilute viral stock from tissue culture supernatant to 1 in 100. In parallel, supernatant from a mock-infected flask incubated with the viral cultures was also diluted 1:100 in the pH solutions, and the pH measured to obtain an accurate pH at the time of the assay. Virus containing solutions were incubated for 20min at 37°C, adjusted to pH 7 with 7.5% sodium bicarbonate using Whatman® pH 6-8 indicator paper and assayed by TCID<sub>50</sub>, using the neat solution (1:100) as the starting dilution.

### 2.5.4 Flow cytometry -based Infectivity Assay

MRC5 cells were treated with enzymes, inhibitors or antibody in 6-well plates as detailed in section 2.5.1. Cells were washed with PBS and infected with virus at an m.o.i. of three. Assays were carried out in duplicate with three wells being used per replicate. After 6hr incubation, cells were detached with trypsin, washed with PBS and transferred to a V-bottom 96-well plate. Subsequent steps were carried out at room temperature. Washes were carried out by centrifugation for 3min at 2 000rpm, followed by resuspension of the cell pellet in PBS/BSA. Cells were fixed in 4% paraformaldehyde for 20min, washed once and permeabilised with 0.1% triton X100 for

10min. After a further wash, cells were treated with the primary antibody for 1hr, washed twice and treated with the secondary antibody (goat anti-mouse IgG FITC) for 1hr. After a further three washes stained cells were analysed using a FACSCalibur Flow Cytometer (Becton Dickinson). A minimum of 10 000 events were counted per sample.

### **2.5.5 Transferrin Uptake Assay**

Cells were pre-treated with enzymes or inhibitors in 6-well plates as detailed in section 2.5.1, with all assays being conducted in duplicate. Plates were transferred to 4°C and washed with PBS. Fluorescein-conjugated transferrin was made up from stock to 0.2mg/ml in serum-free EMEM and 500µl added per well. Plates were incubated for 20min at 4°C and then either processed immediately or transferred to 37°C for 15min. For analysis, cells were detached with trypsin and washed once with 0.1M glycine-0.1M NaCl (pH3) to remove non-internalised conjugate, and washed again with PBS. Cells were then analysed using a FACSCalibur Flow Cytometer (Becton Dickinson), with a minimum of 5 000 events counted per sample.

### **2.5.6 Phospho *Src* specific ELISA**

Cells were treated with enzymes or inhibitors as detailed in section 2.5.1. The assay was carried out in duplicate using one 150cm<sup>2</sup> flask of MRC5 cells per replicate. Cells were detached with versene and lysed in a volume of 100µl of cell extraction buffer. Lysates were tested neat and at a dilution of 1:2 in cell extraction buffer. Tyr<sup>418</sup> phosphorylated *src* was assayed using the PhosphoDetect *Src* (pTyr<sup>418</sup>) ELISA Kit (Calbiochem), following the manufacturer's protocol.

### **2.5.7 Indirect Fluorescence**

Cells were grown on coverslips and either infected with virus at an m.o.i. of three, and incubated for 6hr at 37°C (echovirus type 7) or 33°C (EV70), or treated with cytochalasin B or nocodazole as described in section 2.5.1. All washes were carried out with PBS/BSA and all incubations were at room temperature. Cells were washed, fixed with 4% paraformaldehyde for 20min and permeabilised with 0.1% triton X100 for 10min. Coverslips were treated with primary antibody for 1hr, washed twice, treated with secondary antibody (goat anti-mouse or goat anti-rabbit IgG FITC) for 1hr and washed a further three times before mounting on microscopy slides. Cells were viewed and photographed using a LSM 510 Meta Confocal Microscope (Zeiss).

### 2.5.8 Haemagglutination and haemagglutination inhibition assays

Human blood of type O positive was obtained from the blood transfusion service at the Western Infirmary, Glasgow. The units used were generally within the week after becoming out-of-date for transfusion purposes. RBC were washed at 4°C by 5 min centrifugation at 2 000rpm, with resuspension in PBS until the supernatant was clear (3-4 washes) and the packed cells were retained. Assays were done on ice, unless otherwise stated. EV70 and CVA21 were first concentrated from infected tissue culture supernatant by sucrose cushion ultracentrifugation (section 2.5.9). Echovirus type 7 was used directly from tissue culture supernatant at a titre of at least  $1 \times 10^7$ /ml and influenza virus used in allantoic fluid.

HA assay - Packed RBC were resuspended in PBS to give a 0.5% v/v solution. A two-fold dilution series of virus was made in duplicate in 50µl PBS in a U-bottom 96-well plate. A starting dilution of 1 in 10 was used and PBS only controls included. 50µl of 0.5% v/v solution RBC in PBS was added to each well and the plate incubated on ice until a clear RBC pellet was seen in control wells (approximately 2-3hr). HA was assessed visually and the HA titre scored as the highest dilution displaying HA in both duplicate wells (1 HA unit).

HA Inhibition assay (RBC treatment) – 4HA units of virus in 50µl of PBS were added to wells in a U-bottom plate. 10% v/v RBC were incubated for 1hr at 37°C in enzyme solutions made up in PBS from stock solutions, at the concentrations detailed in the relevant chapters. Treated RBC were washed with PBS, made up to 0.5% RBC v/v with PBS and 50µl added to wells containing virus or controls. Plates were incubated on ice and HA titre scored as above.

HA Inhibition assay (virus treatment). A two-fold dilution series of soluble F<sub>C</sub> DAF (Harris et al., 2005) or N-acetylneuraminic acid (sialic acid) was made in 25µl PBS in a U-bottom plate at the concentrations detailed in the relevant chapters. 4HA units of virus in 50µl of PBS were added to each well and plates incubated on ice for 1hr. 25µl of a 1% v/v solution of RBC was added to each well. Plates were incubated on ice and HA titre scored as above.

### 2.5.9 Preparation of $^{35}\text{S}$ labelled virus

90% confluent cells in a 75cm<sup>2</sup> flask were washed and incubated in methionine/cysteine free DMEM (Sigma) for 1hr and infected with virus at an m.o.i. of between five and ten. Following 45min adsorption, cells were washed and 10ml methionine/cysteine free DMEM added. Flasks were then incubated at 37°C or 33°C as appropriate. 200µCi Pro-mix -[ $^{35}\text{S}$ ] methionine/cysteine (Amersham) was added after 2hr (EV70, echovirus type 7) or 4hr (CVA21) incubation. After overnight incubation and the development of full CPE, labelled virus was harvested by addition of 100µl NP40 (Sigma) and centrifugation for 5min at 4 000rpm. Virus-containing supernatant was concentrated and partially purified by pelleting through a 2.5ml 30% sucrose cushion at 40 000rpm for 6hr in a Sorvall Ultra Pro 80 centrifuge using a Sorvall TH641 rotor. The pellet was resuspended in 300µl of PBS. To obtain 160S infectious particles, the virus was further centrifuged through a 12ml 10-25% linear sucrose gradient (prepared using a Biocomp Gradient Master) for 90min at 40 000rpm in a Sorvall Ultra Pro 80 centrifuge using a TH641 rotor. The gradient was harvested in 500µl fractions using a Biocomp Gradient Fractionator and 5µl of each fraction added to 3ml of scintillation fluid (Ecoscint A, National Diagnostics) prior to counting in a Beckman LS5000CE liquid scintillation counter. The two fractions containing peak activity (typically the 5<sup>th</sup> and 6<sup>th</sup> fraction from the bottom of the gradient) were further centrifuged at 40 000rpm for 6hr in a Beckman TL-100 benchtop ultracentrifuge using a Beckman TLS55 rotor. Pelleted, labelled virus was resuspended in 200µl PBS and stored in aliquots at -70°C.

### 2.5.10 Binding Assays

Cells were detached with versene. If required they were then treated with antibody, enzymes or inhibitors as detailed in section 2.5.1. Treated or untreated cells were washed in PBS/BSA and 10<sup>6</sup> cells incubated on ice with 10 000-20 000 counts per minute (cpm) of  $^{35}\text{S}$  labelled virus in a volume of 50µl for 1hr. Cells were washed three times in 4°C PBS/BSA, transferred to scintillation vials and counted as described in section 2.5.9. All assays were conducted in duplicate.

### 2.5.11 Raft fractionation

Cells were detached with versene and 10<sup>8</sup> used per fractionation. After two washes in serum free EMEM cells were incubated for 1hr on ice with 200 000cpm of  $^{35}\text{S}$  labelled

160S viral particles. Cells were washed twice with TM buffer to remove unbound virus, made up to 5ml with TM buffer and broken up using 80 strokes in a Dounce homogeniser. Unbroken cells were removed by centrifugation at 2 000rpm for 5min at 4°C. Supernatant was then centrifuged at 33 000rpm for 15min at 4°C in a Beckman TL-100 benchtop ultracentrifuge using a beckman TLA100.3 rotor. This was repeated a total of three times, the pellet being resuspended in TM buffer each time. The final membrane pellet was resuspended in 1ml 1% Brij-58 solution, and incubated on ice for 2hr. An equal volume of 80% sucrose in TNE buffer was added to give a 40% solution and overlaid stepwise in a 5ml ultracentrifuge tube with 2ml of 30% sucrose and 1ml of 4% sucrose (both in TNE buffer). Gradients were centrifuged at 40 000rpm for 16hr at 4°C in a Sorvall Ultra Pro 80 ultracentrifuge using a Sorvall AH650 rotor. 500µl fractions were collected using a Biocomp Gradient Fractionator. 250µl of each fraction was transferred to a scintillation vial and counted as described in section 2.5.9. A portion of each fraction was used for western blot analysis.

### **2.5.12 Western Blot**

Cell lysates or membrane fractions were denatured at 100°C for 2min and subjected to SDS polyacrylamide gel electrophoresis on a 12.5% polyacrylamide gel using the BioRad Miniprotean 3 system at 30mA in 1x tris-glycine electrophoresis buffer. Samples were subsequently transferred to nitrocellulose membrane using western blot transfer buffer and a BioRad TransBlot SD for 1hr at 200V. The membrane was then blocked using western blot blocking buffer for a minimum of 1hr at room temperature and incubated with primary antibody for either 1hr at room temperature (anti-caveolin 1) or overnight at 4°C (anti-*src*). Membranes were washed three times in PBST and incubated for 1hr with anti-rabbit IgG peroxidase conjugate. After a further three washes with PBST the protein was detected using ECL Western Blot Detection Reagents (Amersham), and the blot exposed for 1-5min on Kodak film. Films were developed using a Compact X2 Automatic Film Processor (X-ograph Ltd.).

### **2.5.13 Luciferase assay**

RNA was transcribed from subgenomic replicons and 2µg transfected into cells by electroporation or lipofection as described in section 2.4.12 (if required cells were first treated with inhibitors or enzymes as described in section 2.5.1). Cells were then added to 4ml of growth medium and 2ml transferred to two wells of a 6-well tissue culture

dish. One of the duplicate wells was supplemented with 4mM guanidine hydrochloride. Cells were incubated at 33°C. All samples were tested in duplicate (4 wells per sample). The Promega Luciferase Assay System was used. Cells were harvested at 6hr (electroporation) or 10hr (lipofection) by scraping of cells, which were then washed with PBS and lysed with 100µl of lysis buffer for 5min at room temperature. Following centrifugation the protein content of the lysate was quantified using the BCA assay. 100µl of luciferase substrate was added to 20µl of lysate (normalised for protein), and assayed using a TD-20/20 luminometer (Turner BioSystems).

#### **2.5.14 Specific Infectivity Assay**

RNA was transcribed from full-length clones and quantified. Six serial ten-fold dilutions were made in a volume of 250µl and transfected into cells by lipofection as described in section 2.4.12. After 3hr lipofectamine containing medium was removed, cells washed in PBS and 4 ml plaque assay overlay medium added. Subsequent processing was as for standard plaque assays. Results were expressed as plaque forming units (pfu) per µg of input RNA.

## CHAPTER 3

### Construction and Characterisation of an Enterovirus type 70

#### Full-Length Infectious Clone and Subgenomic Replicon

The study of picornaviruses has been greatly facilitated by the availability of both full-length and subgenomic clones of viral genomes. RNA transcribed *in vitro* from full-length clones can be transfected into cells to generate viable virus particles, while RNA from subgenomic replicons can be transfected into cells for the study of viral translation and replication processes. To date there have been no reports of an EV70 full-length infectious clone or subgenomic replicon.

Infectious viral clones enable reverse genetic approaches in research and where available, have become extremely valuable research tools. They are used not only in picornavirus research, but are also useful tools in other fields of virology. One very important recent development is the report of a full-length HCV clone which is capable of generating infectious virus in a hepatoma cell line (Zhong et al., 2005). As there has not previously been an *in vitro* model of HCV infection, this now makes possible infectivity studies, which previously could only be done in humans and chimpanzees.

A full-length infectious clone of the EV70 reference strain J670/71, in which all studies have been carried out to date, would be extremely valuable tool in the study of all aspects of the viral life cycle. In particular, in the study of the interaction between EV70 and its receptor/s, it would facilitate reverse genetic approaches aimed at identifying the specific capsid residues involved in binding and potentially in determining cellular and species tropism. In addition it would allow one of the major concerns with viral receptor studies, including those in EV70, to be addressed – that is, that in highly passaged viral reference strains, receptor usage may have diverged from that of wild type virus. It is not practical to generate full-length clones for all clinical isolates as it remains a difficult and time-consuming procedure, however, an alternative strategy of subcloning portions of the viral genome, specifically the capsid sequences, into a generic backbone is more feasible, and could enable the study of larger numbers of clinical isolates.

Infectious clones have been made using several strategies. The first poliovirus clone was made directly from multiple viral cDNAs spanning the whole genome (Racaniello and Baltimore, 1981). Subsequently a CVB3 infectious clone was made

from the cloning of a single full-length viral cDNA (Kandolf and Hofschneider, 1985). More recently clones have been constructed using RTPCR of viral genome segments. The successful construction of full-length enteroviral clones using a single long distance RTPCR has now also been reported (Lindberg et al., 1997). The drawbacks of this latter method include difficulties with optimisation of long distance RTPCRs and the potential for introduction of reverse transcriptase and PCR generated sequence errors. However, this is a potentially timesaving method, requiring fewer cloning steps than construction of the clone from smaller segments of the genome. For these reasons, long distance RTPCR was chosen here for the construction of a full-length EV70 clone, based on the approach previously described by Lindberg et al (1997).

An important consideration in the making of an enteroviral infectious clone is the requirement that the *in vitro* transcribed RNA have both an accurate 5' and 3' end. These are necessary for efficient poliovirus replication (Herold and Andino, 2000; Sarnow, 1989). At the 3' end a polyadenosine sequence is required, followed by a restriction site to allow removal of additional sequences prior to transcription. An accurate 5' end can be achieved with the use of the hammerhead ribozyme, derived from a plant viroid, (Birikh et al., 1997). When positioned immediately upstream of the viral sequence within an infectious clone and transcribed into RNA, it catalyses cleavage of the phosphodiester bond immediately upstream the first base, thereby generating the correct genomic 5' end.

In addition to full-length clones, subgenomic viral replicons have also proven valuable tools in picornavirus and HCV research (Lohmann et al., 1999; Percy et al., 1992). These contain a reporter gene, such as chloramphenicol acetyltransferase or firefly luciferase. The reporter gene sequence replaces the capsid-encoding section of the genome, which is not required, as viral binding and entry are by-passed by direct RNA transfection of the cell. On transfection of RNA into the cell, the viral polyprotein, including reporter sequences, is translated and cleaved into its constituent proteins. Subsequent viral replication results in a proportionate increase in reporter gene product, which can then be measured. For HCV, the availability of subgenomic replicons (Lohmann et al., 1999) has allowed research into HCV translation and replication to progress prior to the availability of an *in vitro* culture system.

In addition to the requirement for a ribozyme (as for full-length clones), there are a number of further considerations in the construction of a subgenomic replicon. The reporter gene sequence replaces the capsid-encoding section of the virus and must be

cloned in-frame with the rest of the viral open reading frame, both 5', to ensure the reporter gene is correctly translated, and 3', to allow correct translation of the P2 and P3 portions of the polyprotein required for proteolytic processing and viral replication. It is also important to include an intact P1/P2 junction to ensure correct processing by the viral 2A protease (Skern et al., 1991). It was decided to construct the EV70 subgenomic replicon with a luciferase reporter gene. This allows highly sensitive and rapid quantification of replication.

### **Chapter Outline**

This chapter details the construction of a full-length infectious EV70 clone and functional luciferase-encoding subgenomic replicon. Characterisation of these clones is described, including sequence data and the specific infectivity and growth characteristics of RNA and virus derived from the full-length clone.

### **3.1 Construction and Sequence Analysis of a Full-Length EV70 Clone using Long Distance Enterovirus RTPCR**

To construct an EV70 infectious clone using long distance RTPCR of the viral genome, the strategy previously described by Lindberg et al. was used (Lindberg et al., 1997).

#### **3.1.1 Reverse Transcription and PCR**

The primer used for reverse transcription, E70rt (table 3.1), included a *Sal* I restriction site to facilitate future cloning and 27 thymidine residues for annealing to the viral poly A tail. The reaction was carried out using viral RNA extracted from EV70 J670/71 tissue culture supernatant. In view of the large size of the target (7390 bases) an extension step of 130 minutes at 46°C was used (Lindberg et al., 1997).

Following the reverse transcription step, the entire 7390 base EV70 sequence was amplified by PCR. The sense primer, EV70s5p, included a *Sma* I restriction site for future cloning and the initial 27 bases of the EV70 genome. The antisense primer, E70a3p, was identical to that used for reverse transcription with the addition of a further 24 bases complimentary to the 3' end of the EV70 genome. Problems were encountered with the efficiency and fidelity of several different proofreading polymerases during the

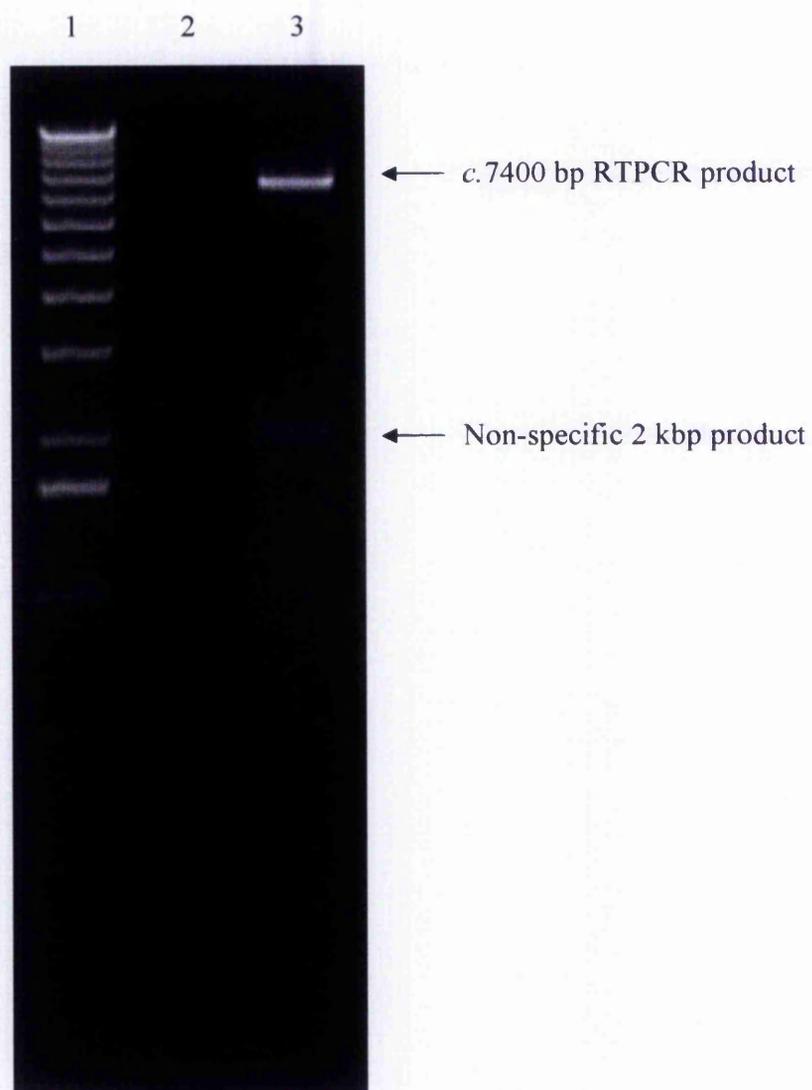


optimisation of the PCR reaction, including platinum pfx DNA polymerase (Invitrogen) and Deep Vent DNA polymerase (New England Biolabs). Despite extensive attempts at optimisation of the PCR reactions using these enzymes, the yield of full-length product remained poor, with even very minor changes in the reaction conditions resulting in failure to obtain detectable product. Attempts to amplify a smaller 3.5kb segment of the genome using pfx polymerase resulted in improved yield, but sequencing revealed multiple errors in the products. In contrast, KOD polymerase (Takagi et al., 1997), from Novagen, proved to be much more reliable in terms of both yield and fidelity, it gave consistently good yield of the full-length product and was much less sensitive to changes in magnesium concentration or annealing temperature. KOD polymerase was therefore used for this and all subsequent PCR-based cloning. 30 PCR cycles, with extension times of 390 seconds, was identified as the minimum required to reliably yield sufficient product. The expected product of 7451 base pairs (bp) was produced, with a further non-specific product of approximately 2 kilobase pairs (kbp) (figure 3.1).

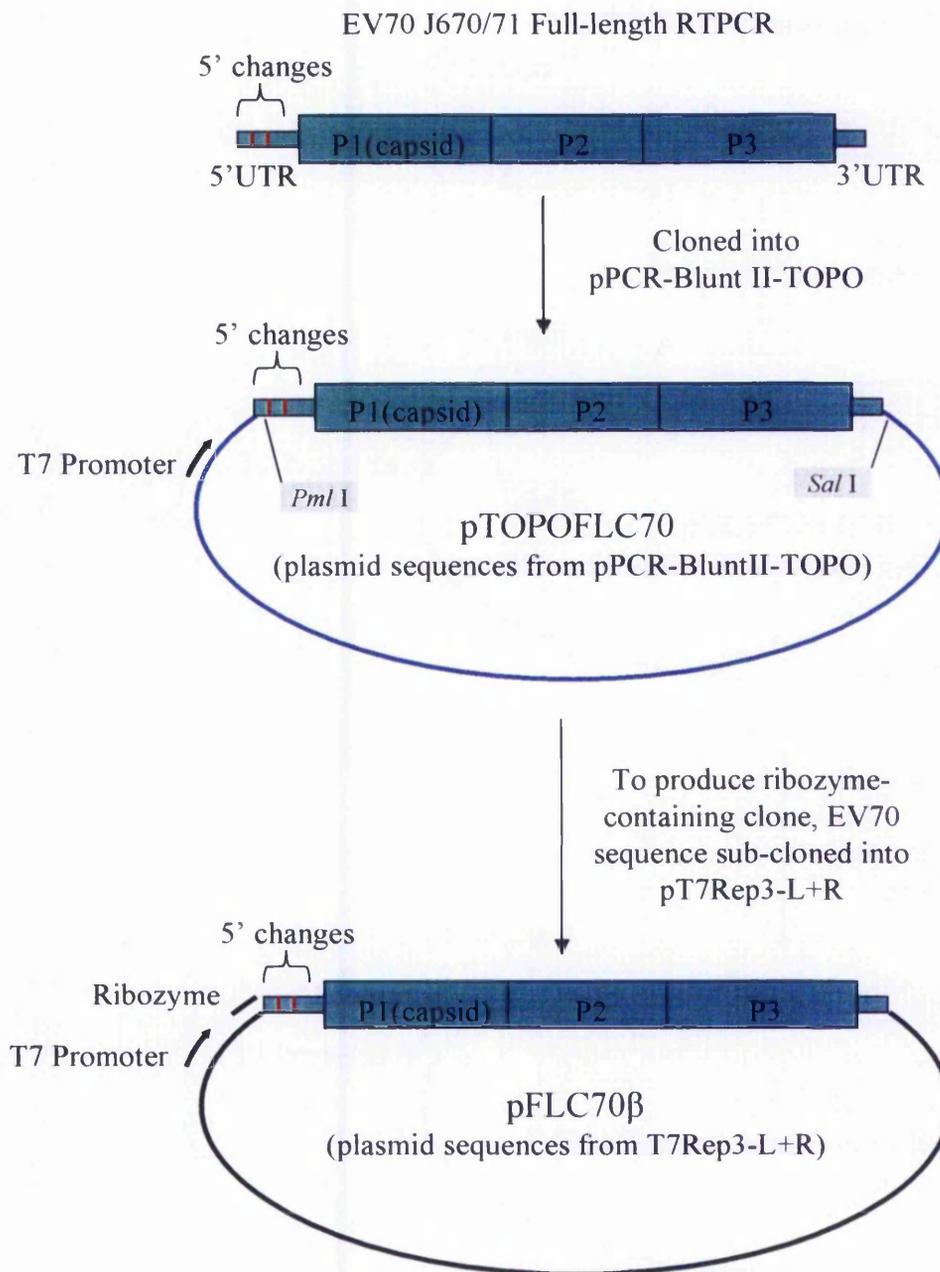
### 3.1.2 Cloning of full-length RTPCR product

A diagrammatic representation of the initial cloning steps is provided in figure 3.2. The full-length RTPCR product was first purified from an agarose gel, to remove the non-specific product and any other smaller products that might interfere with cloning. It was initially cloned into the pPCR-Blunt II-TOPO cloning vector using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). The vector was supplied linearised, with *Vaccinia* virus topoisomerase I covalently linked to the 3' end of each strand, which acts as a ligase, and facilitates efficient cloning of blunt-ended PCR products. This vector also includes T7 and SP6 RNA polymerase promoters at either end of the insert site.

Sixteen clones were identified as containing inserts of the expected size and restriction pattern. Fourteen contained inserts orientated for transcription using the T7 promoter, and two for transcription using the SP6 promoter. To test whether these clones were capable of generating infectious EV70 virus, the DNA was linearised at the 3' end of the poly A tail using the *Sal*I restriction site and RNA transcripts obtained using T7 or SP6 RNA polymerase as appropriate. 2µg of transcribed RNA was transfected by electroporation into MRC5 cells and incubated at 33°C. Cells transfected with twelve of the clones demonstrated CPE, seven by 48 hours and a further five by 96 hours. RNA from the remaining four clones showed no CPE even after seven days



**Figure 3.1 Gel electrophoresis of full-length EV70 RTPCR product.** Ethidium bromide stained agarose gel showing the full-length EV70 RTPCR product of 7451bp at the expected size. There is also a further non-specific band of just over 2 kbp. Lane 1, DNA size marker; lane 2, No template control; lane 3, EV70 full-length RTPCR.



**Figure 3.2 Construction of pFLC70 $\beta$ , a full-length EV70 clone with a ribozyme, but with 5'UTR sequence changes.** The full-length EV70 RTPCR product (containing two 5' changes) was cloned into pPCR-Blunt II-TOPO which contains a T7 promoter but no ribozyme using topoisomerase (full description in section 3.1.2). To obtain a ribozyme-containing clone, the full EV70 sequence, excluding the first 42 bp of the EV70 5'UTR, was sub-cloned into pT7Rep3-L+R, a luciferase-encoding PV3 replicon (see figure 3.10), using the restriction endonucleases *Pml* I and *Sal* I. All PV3 and luciferase-encoding sequences were removed from the latter during cloning, except for the first 42 bases of the PV3 5'UTR which are identical to the EV70 sequence (full description in section 3.1.4). ■ EV70 sequence.

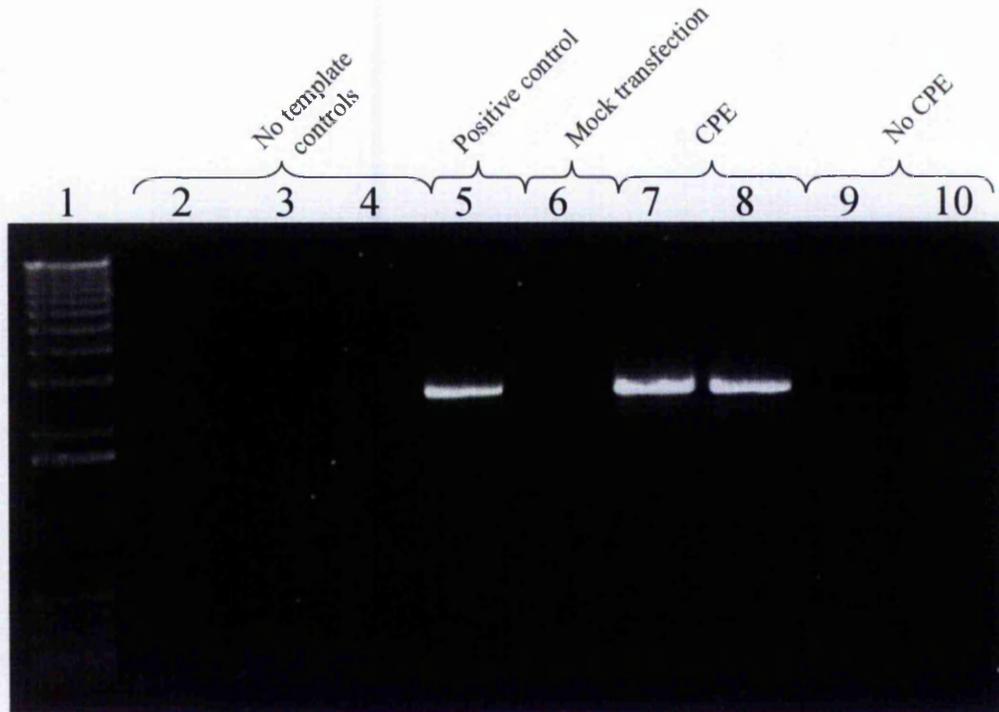
incubation. Supernatant from the transfections demonstrating CPE was passaged onto fresh cells following clarification by centrifugation and filtration. All twelve showed CPE at 24 hours, excluding the possibility that the initial observations were due to toxicity following electroporation. To confirm that the CPE was due to EV70, RTPCR of the viral capsid sequence was carried out on the passaged supernatant using EV70-specific primers. The expected product of 2435 bp was observed for all CPE-exhibiting clones, but not for those without CPE (figure 3.3).

### 3.1.3 Sequence analysis of full-length clones

The two clones showing the most marked CPE 48 hours after transfection were fully sequenced and compared with the published J670/71 EV70 sequence (Ryan et al., 1990). The clone with fewer differences from the published sequence (21 bases) was designated pTOPOFLC70 (tables 3.2 and 3.3). To establish whether the sequence differences were present in the original virus stock or were introduced during RTPCR, the RTPCR product and seven additional clones were sequenced.

Fifteen of the differences were present in the RTPCR product and in all clones (table 3.2). These are assumed to be true differences between the J670/71 strain used here and that originally sequenced. Of these fifteen differences, one was a G nucleotide insertion in the 5' UTR, eight were synonymous and three resulted in conservative changes to related amino acids (alanine to valine, aspartate to glutamate and threonine to serine in VP1, 3A and 3D respectively). The remaining three differences resulted in changes of amino acid of arginine to glutamine and aspartate to asparagine, both in VP1, and alanine to threonine in 2C.

The remaining six differences were not found in the RTPCR sequence (table 3.3). Two resulted in conservative changes (leucine to isoleucine in VP2 and isoleucine to valine in 3A). These were both found in a proportion of the other clones sequenced, suggesting they may form part of the viral quasispecies. The remaining four were found only in single clones, and although these could be minor components of the quasispecies, they may also be due to errors during RTPCR. Two (in VP3 and VP1 respectively) are synonymous and so of less concern, the remaining two lie within the 5'UTR, and could therefore impact on either the structure or function of this region, which is important in viral translation and replication. All six changes were maintained after a further three passages in MRC5 cells, suggesting that there was no strong selection for reversion to wild type or consensus sequence at these sites. MFOLD



**Figure 3.3 Gel electrophoresis of EV70 capsid RTPCR on cells transfected with RNA from pTOPOFLC70 clones.** PCR primers and conditions are detailed in tables 3.1 and 2.3 respectively. The expected product size of 2435 bp is observed only for cells showing CPE. Lane 1, DNA size marker; lanes 2, 3 and 4, no template - extraction, RT and PCR controls respectively; lane 5, EV70 wild type culture supernatant; lane 6, mock transfection; lanes 7 and 8, two clones showing CPE; lanes 9 and 10 two clones showing no CPE.

**Table 3.2 Differences from published EV70 sequence present in EV70 RTPCR product and all sequenced clones.**

<b>Location</b>	<b>Base Position*</b>	<b>Base Change</b>	<b>Amino Acid Change</b>
<b>5'UTR</b>	<b>352</b>	<b>G insertion</b>	-
<b>1D</b>	3051	C to T	No change
	<b>3233</b>	<b>C to T</b>	<b>Ala to Val</b>
	<b>3239</b>	<b>G to A</b>	<b>Arg to Gln</b>
	3252	C to T	No change
	<b>3277</b>	<b>G to A</b>	<b>Asp to Asn</b>
<b>2C</b>	<b>4780</b>	<b>G to A</b>	<b>Ala to Thr</b>
	4959	G to A	No change
<b>3A</b>	5202	C to T	No change
	<b>5220</b>	<b>C to G</b>	<b>Asp to Glu</b>
<b>3C</b>	5721	C to T	No change
<b>3D</b>	5940	T to G	No change
	<b>5963</b>	<b>C to G</b>	<b>Thr to Ser</b>
	5970	G to C	No change
	5976	A to C	No change

5'UTR and coding changes are marked in bold.

\* Numbered relative to published sequence (Ryan et al, 1990).

**Table 3.3 Differences from published EV70 sequence present in one or more clones, but not seen in EV70 RTPCR product.**

	<b>Location</b>	<b>Base Position*</b>	<b>Base Change</b>	<b>Amino Acid Change</b>	<b>Frequency of changes†</b>
<b>1</b>	<b>5'UTR</b>	<b>267</b>	<b>T to C</b>	-	<b>1 of 9</b>
<b>2</b>	<b>5'UTR</b>	<b>443</b>	<b>G to A</b>	-	<b>1 of 9</b>
<b>3</b>	<b>1A (VP4)</b>	<b>735</b>	<b>A to T</b>	No change	Introduced during cloning
<b>4</b>	<b>1B (VP2)</b>	<b>1558</b>	<b>T to A</b>	<b>Leu to Ile</b>	<b>4 of 9</b>
<b>5</b>	<b>1C (VP3)</b>	<b>2241</b>	<b>T to C</b>	No change	1 of 9
<b>6</b>	<b>1D (VP1)</b>	<b>3027</b>	<b>T to C</b>	No change	1 of 9
<b>7</b>	<b>3A</b>	<b>5275</b>	<b>A to G</b>	<b>Ile to Val</b>	<b>3 of 9</b>

All changes are present in pTOPOFLC70 and pFLC70 $\beta$  with the exception of change 3. pFLC70 contains changes 3, 4 and 7 only. pLuc70 contains change 7.

\* Numbered relative to published sequence (Ryan et al, 1990)

† Refers to full-length clones sequenced (section 3.1.3)

5'UTR and coding changes are marked in bold.

prediction (Zuker, 2003), and comparison to the proposed secondary structure of poliovirus (Stewart and Semler, 1997), suggests that both 5' changes occur in stem-loop IV of the IRES structure, with the change at base 267 likely to be present in an unpaired loop, and the change at base 443 disrupting the base pair at the base of the stem.

### 3.1.4 Construction of a ribozyme-containing full-length EV70 clone (pFLC70 $\beta$ ).

Transcripts generated from pTOPOFLC70 using T7 RNA polymerase will not have an accurate viral 5' end, which is important for efficient replication in poliovirus clones (Herold and Andino, 2000). The EV70 viral sequence was therefore subcloned into pT7Rep3-L+R (figure 3.2). This vector encodes a poliovirus type 3 (PV3) subgenomic luciferase-encoding replicon (Goodfellow et al., 2000), and was used here as it contains the hammerhead ribozyme sequence and also suitable restriction sites and a T7 polymerase promoter. To generate insert and vector fragments for ligation, both pTOPOFLC and pT7Rep3-L+R were digested with the restriction enzymes *Pml* I, which cleaved within the 5'UTR 42 bases from the end of the genome, and *Sal* I, which cleaved at the end of the poly A tail. This resulted in the removal of the PV3 replicon sequences, with the exception of the initial 42 bases of the PV3 5'UTR which are identical to those of EV70. The resultant clone was designated pFLC70 $\beta$  and contains all the sequence differences present in pTOPOFLC70 (table 3.2 and 3.3). Transfection of RNA from this clone into MRC5 cells reproducibly resulted in CPE within 24 hours.

### 3.1.5 Construction of a ribozyme-containing full-length EV70 clone lacking 5'UTR changes (pFLC70).

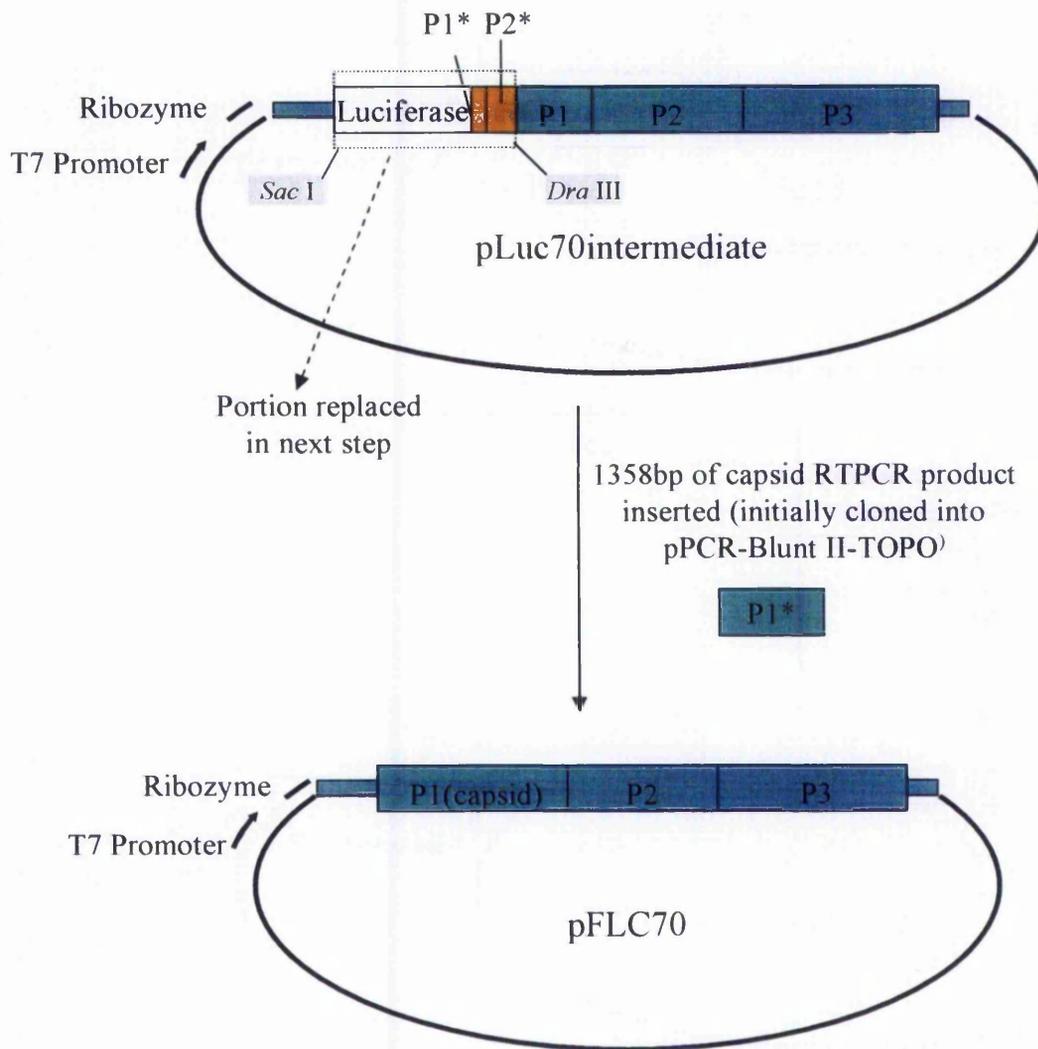
As shown in table 3.3, and discussed in section 3.1.3, the infectious EV70 clones constructed directly from full-length RTPCR product, pTOPOFLC70 and pFLC70 $\beta$ , contain six sequence changes in comparison to the consensus sequence of the parent virus. Two of these result in conservative amino acid changes and are present in other clones, suggesting they form part of the viral quasispecies, however the other four are not seen in other clones, and may represent true RTPCR errors. The two non-coding changes in VP3 and VP1 are unlikely to be detrimental, as enteroviral capsid sequences are not thought to have significant secondary structure or function at the RNA level. The 5'UTR changes, however, are of concern as they occur in an area rich in secondary structure required for translation and replication. In addition these changes are not present in the subgenomic replicon, pLuc70 (see section 3.3), making difficult full

comparisons of infectivity and replication, based on the use of these clones. The cloning strategy described here resulted in a full-length EV70 clone lacking these 5' changes and also the two non-coding capsid changes.

The clone was constructed using pLuc70intermediate (figure 3.4). This is a plasmid generated from pTOPOFLC70 as an intermediate during the construction of a luciferase-containing EV70 replicon (section 3.3.1). pLuc70intermediate contains the wild type EV70 5'UTR without errors and the full P2, P3 and 3'UTR regions and partial P1 region from pFLC70 $\beta$ . It also contains sequence encoding luciferase and a small stretch of poliovirus sequence.

RTPCR was carried out of the EV70 capsid coding region using viral RNA extracted from EV70 J670/71 tissue culture supernatant. The sense PCR primer, EV70sJUP1, contained a single non-coding base change from the published sequence to introduce a *Sac* I restriction site for cloning (table 3.1) within P1, nine bases downstream of the translational start site. The antisense primer, EV70aJP12, contained three non-coding base changes, and introduced a *Mlu* I restriction site (although this was not required for the cloning strategy used here). The 2434 bp product was initially cloned into pPCR-Blunt II-TOPO, and this was digested with the restriction enzymes *Sac* I and *Dra* III to generate a 1358 bp fragment from the 5' end of the capsid sequence. This was subcloned into pLuc70intermediate in place of the luciferase gene and PV3 sequences forming a ribozyme-containing infectious EV70 clone without 5' changes, designated pFLC70. This clone contains all the sequence changes from the published sequence which are also seen in the RTPCR product (table 3.2), however it contains only three further changes not seen in the RTPCR (table 3.3). These are the presumed viral polymorphism in the 3A region (derived from pFLC70 $\beta$ ), the non-coding change required to introduce the *Sac* I restriction site in the 1A region and the presumed viral polymorphism in the 1B region (also present in the capsid RTPCR product cloned here).

Following linearisation of pFLC70 at the 3' end and T7 RNA transcription a single predominant transcript was obtained (figure 3.5). On transfection into MRC5 cells, CPE was observed within 24 hours. Virus recovered from cells transfected with RNA transcribed from pFLC70 will be referred to as EV70<sup>FLC</sup> for the remainder of this thesis.



**Figure 3.4 Construction of pFLC70, a full-length EV70 clone with a ribozyme, and wild type 5'UTR sequence.** To obtain a full-length EV70 viral clone without 5' changes, the EV70 capsid RT-PCR product was initially cloned into pPCR-Blunt II-TOPO (not shown) and then a fragment containing the 5' 1358 bp was sub-cloned into pLuc70intermediate in place of the PV3 and luciferase sequences using the restriction endonucleases *Sac* I and *Dra* III (full description in section 3.1.5).

\*Partial P1 or P2 sequence ■ EV70 sequence ■ PV3 sequence



**Figure 3.5 Gel electrophoresis of pFLC70 and pLuc70 RNA transcripts.** RNA was transcribed using T7 polymerase and run on a 1% agarose gel. The RNA transcript from pFLC70, of expected size 7417 bases, runs slightly higher than the RNA transcript from pLuc70 of expected size 6844 bases. Lane 1, DNA size marker as point of reference only; lane 2, T7 transcript from pFLC70; lane 3, T7 transcript from pLuc70.

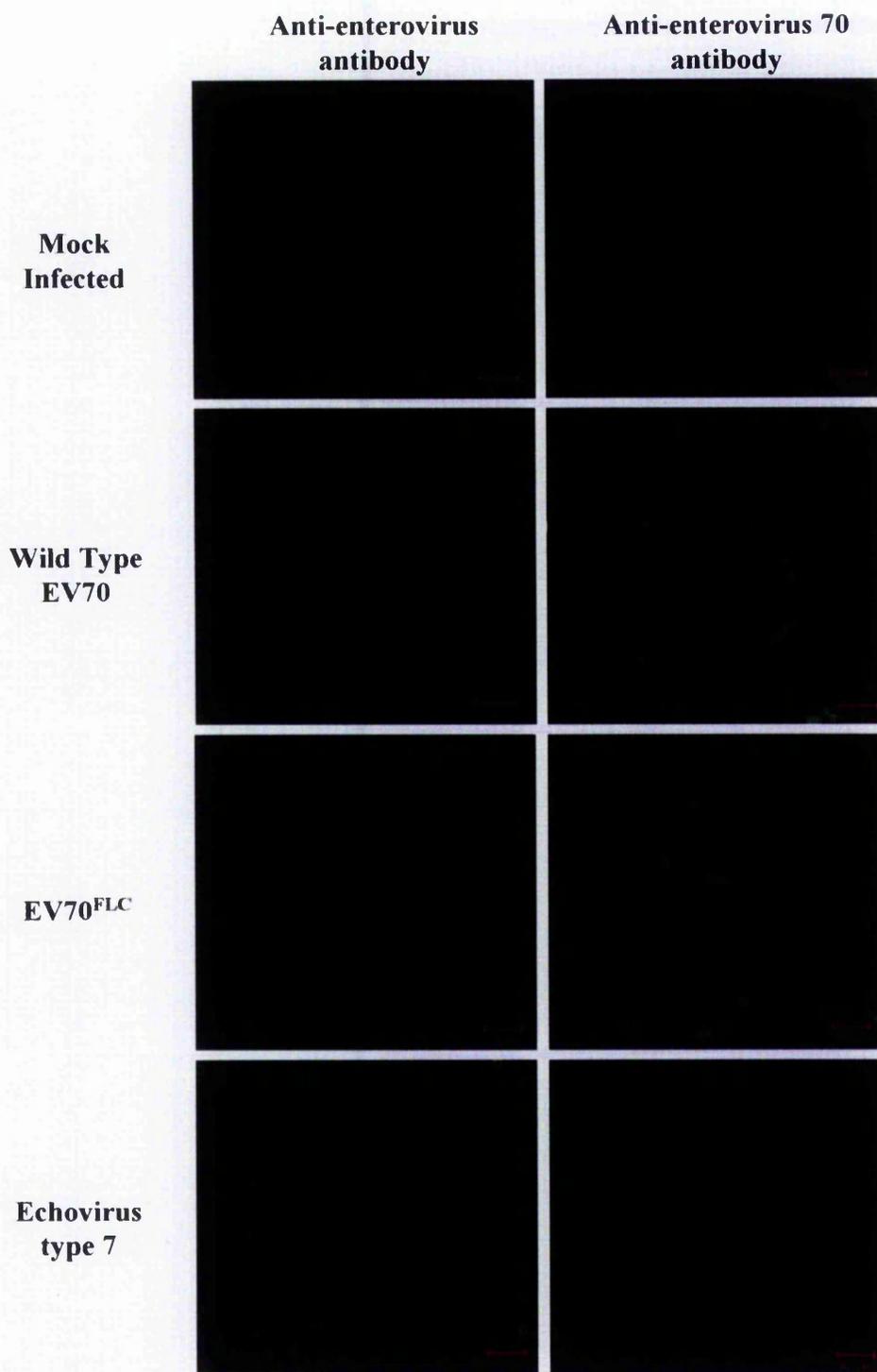
### **3.2 Characterisation of pFLC70 and other full-length EV70 clones**

During the cloning procedures outlined in the preceding sections three related but distinct EV70 infectious clones were derived: pTOPOFLC70 lacking a ribozyme and containing the potentially deleterious 5'UTR changes; pFLC70 $\beta$  with a ribozyme, but still containing the 5'UTR changes; and pFLC70 with a ribozyme and without the 5'UTR changes.

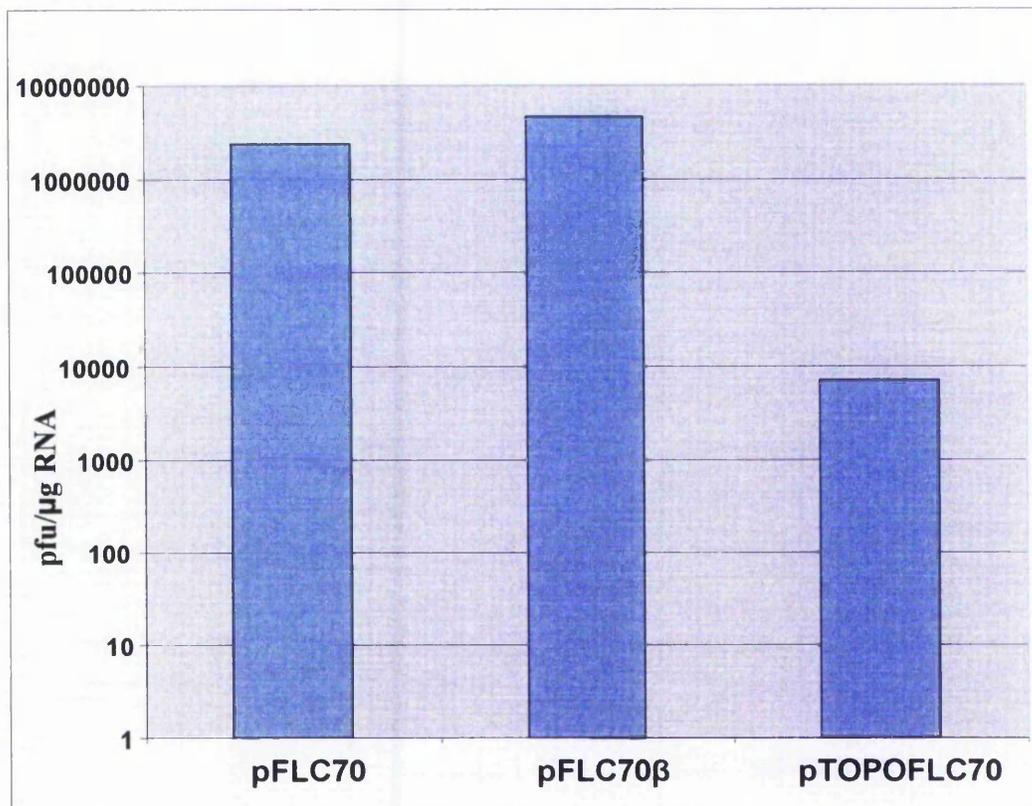
Virus recovered from cells transfected with RNA from all three clones was passaged successfully in MRC-5 cells and confirmed to be EV70 by indirect immunofluorescence of the infected cells using an EV70-specific mouse monoclonal antibody (data from EV70<sup>FLC</sup> is shown in figure 3.6). The cells exhibited only very pale staining with a broadly reactive Dako enteroviral antibody, as is also observed for the wild type virus. An echovirus type 7 control failed to stain with the EV70 antibody, but did stain with the enterovirus antibody. The three clones were further characterised with respect to growth characteristics and kinetics.

Specific infectivity of transcribed viral RNA was assessed in MRC5 cells (figure 3.7). Naked poliovirus RNA is reported to have a specific infectivity in HeLa cells of around  $10^6$  pfu/ $\mu$ g RNA (Crotty et al., 2001; Koch, 1973). The specific infectivity of RNA transcribed from pFLC70 was above  $10^6$  pfu/ $\mu$ g RNA. This was comparable to that of RNA transcribed from pFLC70 $\beta$ , suggesting that the sequence differences between these two clones (consisting of the two 5'UTR changes and two non-coding changes in the capsid-encoding sequence) did not significantly affect specific infectivity. RNA transcribed from pTOPOFLC70 had a specific infectivity over two  $\log_{10}$  lower than that of the other EV70 clones. Its sequence is identical to pFLC70 $\beta$  and the difference must therefore be ascribed to the lack of a ribozyme and accurate 5' end, as is known to be important for the specific infectivity of poliovirus RNA (Herold and Andino, 2000).

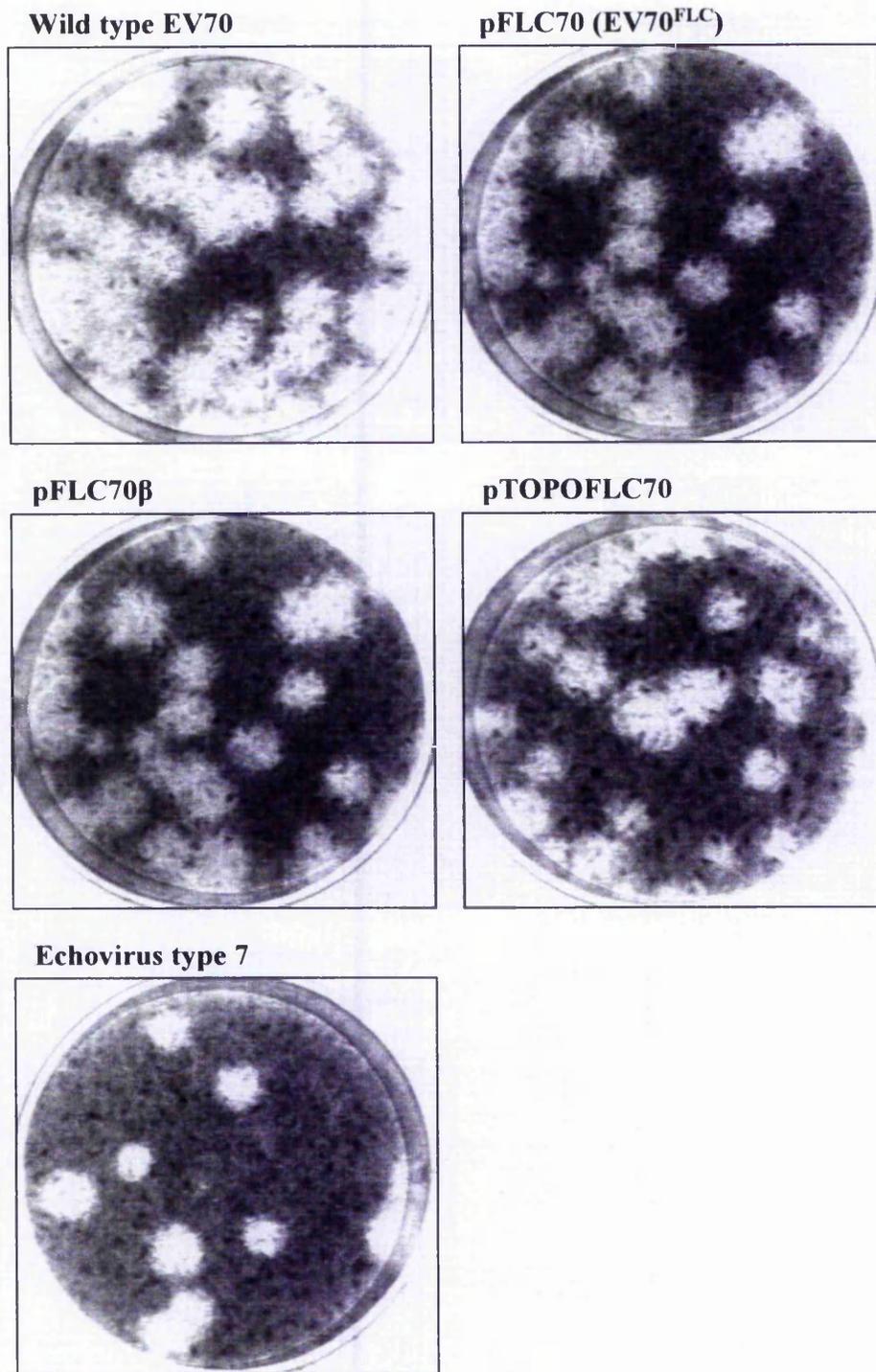
Virus recovered from all three clones showed very similar plaque phenotype on MRC5 cells, in terms of both size and plaque margins. The latter were more irregular than those of another enterovirus, echovirus type 7, which exhibited much smoother plaque margins in these cells (figure 3.8).



**Figure 3.6 Indirect Immunofluorescence of clone-derived EV70 in MRC5 cells.** Cells were infected with virus at an m.o.i. of three and stained after 6hrs incubation with either the broadly reactive Dako anti-enterovirus antibody 5-D8/1 or with EV70 monoclonal 854. Very pale staining was observed for wild type EV70 and EV70<sup>FLC</sup> using the enterovirus antibody at the time of microscopy, but is less clear on reproduction. Identical staining patterns to wild type EV70 and EV70<sup>FLC</sup> were obtained from virus recovered from cells transfected with RNA transcribed from the clones pFLC70 $\beta$  and pFLCTOPO70 (data not shown).



**Figure 3.7 Specific Infectivity of RNA transcripts derived from infectious clones.** Serial 10-fold dilutions of RNA transcribed from the infectious EV70 clones were transfected into MRC5 cells using lipofectamine 2000 (Invitrogen) and overlaid with plaque assay overlay medium. After 72hrs incubation plaques were counted and specific infectivity expressed as pfu/ $\mu$ g transfected RNA.



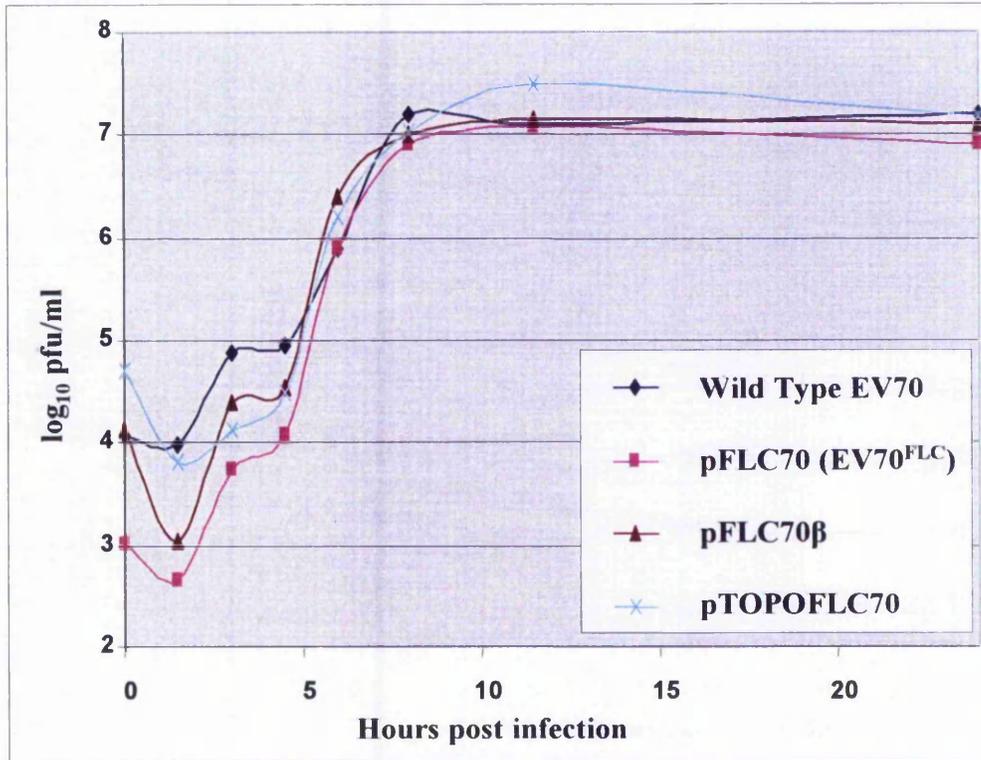
**Figure 3.8 Plaque Phenotypes of clone-derived EV70 in MRC5 cells.** MRC5 cells were infected with a 10-fold dilution series of virus and overlaid with plaque assay overlay medium. After 72hrs incubation plaques were viewed and photographed. Where appropriate virus was first recovered from MRC5 cells transfected with RNA transcribed from the infectious clones.

One-step growth curves in MRC5 cells were derived (figure 3.9). Despite irregularities during the initial phases, which may have been due to variation in the amount of virus in the initial inocula, virus recovered from all three infectious clones exhibited very similar growth kinetics, comparable to wild type EV70. These consisted of an exponential growth phase at between four and six hours, and a final plateau at a viral titre of  $10^7$  pfu/ml. Despite the lack of a ribozyme and the lower specific infectivity of RNA derived from pTOPOFLC70, the wild type growth curve is not unexpected for virus recovered from this clone. After transfection and an initial low efficiency round of replication, a correct 5' end would be generated, resulting in recovered virus identical to that obtained from pFLC70 $\beta$ .

In summary virus recovered from all three infectious EV70 clones had phenotypic and growth characteristics comparable to wild type virus. The primary difference between the clones is the lower specific infectivity of pTOPOFLC70 derived RNA due to the lack of a ribozyme and therefore of an accurate 5' end. The sequence differences between pFLC70 and pFLC70 $\beta$  do not appear to affect viral growth or infectivity.

### **3.3 Construction and Characterisation of an EV70 Subgenomic Viral Replicon**

The EV70 genome does not contain suitable sites to allow the direct cloning of the luciferase reporter gene into the full-length infectious clone. It was therefore decided to construct the EV70 subgenomic replicon by taking advantage of the restriction sites in the plasmid pT7Rep3-L+R, the poliovirus subgenomic replicon also used to construct pFLC70 $\beta$ . In addition to the firefly luciferase gene, this vector also contains a T7 RNA polymerase promoter sequence required for in vitro RNA transcription and a hammerhead ribozyme sequence to generate the accurate 5' end required for efficient replication of the replicon RNA.



**Figure 3.9 One step growth curves of clone-derived EV70 in MRC5 cells.** Cells were infected with virus at an m.o.i. of 3 and titres determined by plaque assay on virus harvested by freeze-thawing after 0, 1.5, 3, 4.5, 6, 8, 12 and 24hrs incubation. Where appropriate virus was first recovered from MRC5 cells transfected with RNA transcribed from the infectious clones.

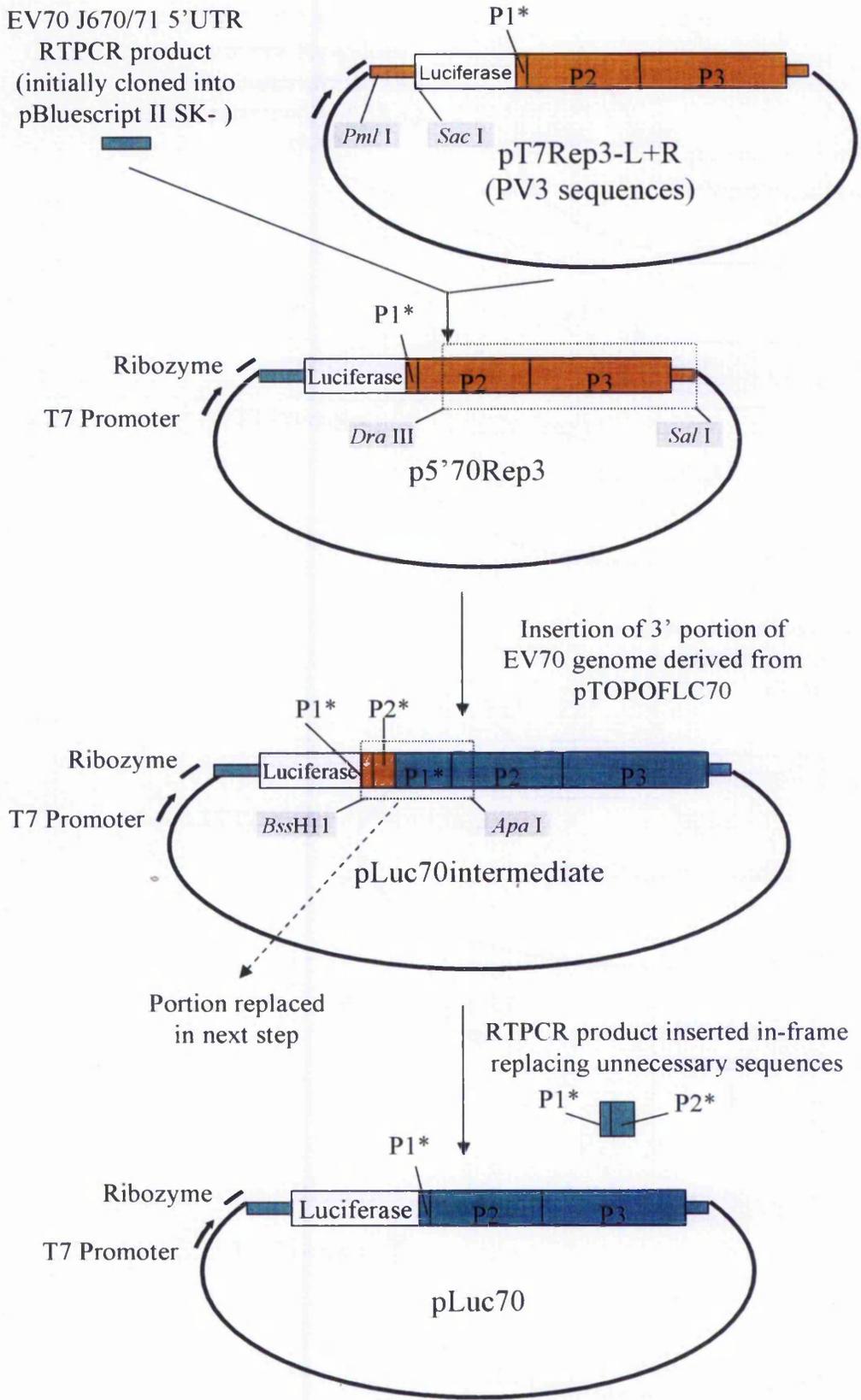
### 3.3.1 Construction of a ribozyme-containing EV70 subgenomic replicon with luciferase reporter gene (pLuc70).

Cloning was carried out in three steps: insertion of the EV70 5'UTR upstream of the luciferase gene, insertion of the 3' end of the EV70 genome downstream of the luciferase gene, and a final step to remove unnecessary sequences (figure 3.10).

In the first step the EV70 5'UTR was amplified by RTPCR using viral RNA extracted from EV70 J670/71 tissue culture supernatant. The sense *Sma* I-containing primer E70s5p, used in the full-length PCR, was used, with the antisense primer E70aJUP1, which contained a single non-coding base change from the published sequence to introduce a *Sac* I restriction site for cloning (table 3.1). This change was within P1, nine bases downstream of the translational start site. The 766 bp product was initially cloned into pBluescript II SK- using the restriction endonucleases *Sma* I and *Sac* I and then subcloned into pT7Rep3-L+R in place of the PV3 5'UTR. Both insert and vector fragments for this ligation reaction were generated by digestion with the restriction enzymes *Pml* I and *Sac* I. *Pml* I cleaved within the 5'UTR 42 bases from the end of the genome, ensuring maintenance of the intact ribozyme sequence within the vector. The initial 42 bases of the PV3 5'UTR are identical to those of EV70. Sequencing showed the insert contained no changes from the published EV70 5'UTR sequence. The resultant plasmid was designated p5'70Rep3, it contains the luciferase gene and preserves the open reading frame. It encodes a chimeric replicon with the EV70 5'UTR in a PV3 backbone.

In the second step the 3' end of the EV70 genome, derived from the pTOPOFLC70 full-length clone, was cloned into p5'70Rep3. Fragments for ligation were generated from both plasmids using the enzyme *Dra* III, which cleaves within the P1 region of EV70 and the P2 region of PV3, and *Sal* I, which cleaves at the end of the poly A tail. The resultant clone included the entire EV70 P2, P3 and 3'UTR and also 1247 bp of the P1 region. This clone was designated pLuc70intermediate and still contained some PV3 sequence immediately downstream of the luciferase gene.

In the final step a 475 bp RTPCR product from the EV70 P1/P2 junction was inserted, removing all remaining PV3 sequences and leaving only 198 bp of the EV70 P1. The sense primer, EV70s3121 contained four mismatches from the EV70 sequence to introduce a *Bss*HIII restriction site for cloning downstream of the luciferase gene (table 3.1), however these occurred in the redundant residual P1 section. Fragments for



**Figure 3.10 Construction of pLuc70, an EV70 subgenomic replicon.** (See legend on following page)

**Figure 3.10** (previous page) **Construction of pLuc70, an EV70 subgenomic replicon.** The EV70 5'UTR RTPCR product was initially cloned into pBluescript II SK- vector (not shown) and then sub-cloned (excluding the first 42bp which are identical to the PV3 5'UTR) using the restriction endonucleases *Pml* I and *Sac* I, into pT7Rep3-L+R, in place of the PV3 5'UTR, forming p5'70Rep3. In the second step the 3' portion of the EV70 genome derived from pTOPOFLC70 and extending 1247bp into P1, was subcloned using the restriction endonucleases *Dra* III and *Sal* I, to form pLuc70intermediate which retains 81bp and 319bp of the PV3 P1 and P2 respectively. In the final step a 475 bp EV70 RTPCR product comprising 198 bp of P1 and 214 bp of P2 was inserted in-frame, using the restriction endonucleases *Bss*HIII and *Apa* I, and removing the remaining PV3 sequences. Full description in section 3.3.1.

\*Partial P1 or P2 sequence  EV70 sequence  PV3 sequence

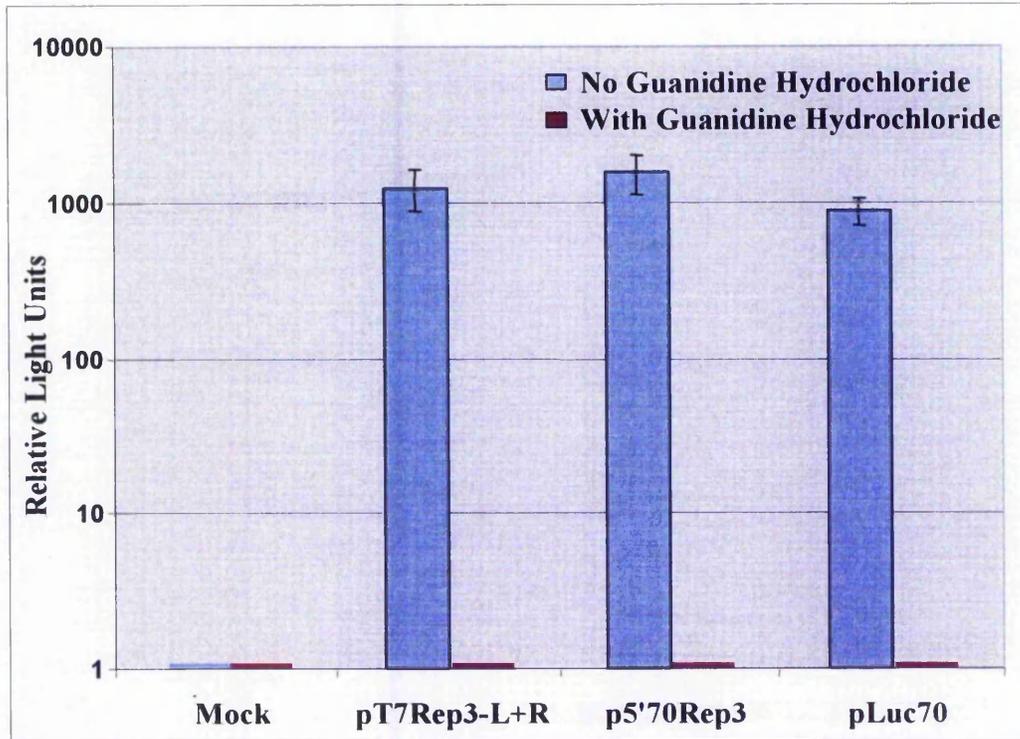
cloning were generated using the restriction endonucleases *Bss*HIII and *Apa* I, which cleaves 214 bases from the 5' end of the 2A sequence. This final clone contained the luciferase gene in frame with the viral polyprotein and was designated pLuc70. It contains the EV70 5'UTR upstream of luciferase with the EV70 P2, P3 and 3'UTR downstream. It also retains 214bases of the EV70 P1, so maintaining the P1/P2 junction sequences necessary for cleavage of the polyprotein by the viral 2A protease. This replicon contains all the sequence changes outwith the P1 region, listed in tables 3.2 and 3.3, with the exception of the two base changes in the 5'UTR.

### 3.3.2 Testing of pLuc70 subgenomic replicon

Following linearisation of the replicon with the restriction endonuclease *Sal* I, an RNA transcript was generated from pLuc70 utilising T7 RNA polymerase. A single band could clearly be seen on agarose gel (figure 3.5), indicating the presence of a single predominant transcript. The expected size of this transcript was 6844 bases, and the band seen was marginally smaller than that for the transcript generated from the full-length clone, pFLC70 (expected size 7417 bases). Luciferase was assayed in MRC5 cells transfected with 2µg of RNA transcripts from pT7Rep3-L+R (a well-characterised PV3 replicon, Goodfellow et al., 2000), pLuc70 and p5'70Rep3 (the chimeric PV3 replicon with the 5'UTR of EV70). All three replicons gave strong luciferase readings within 0.3 of a log<sub>10</sub>, suggesting comparable replication efficiencies (figure 3.11). Guanidine hydrochloride is a potent inhibitor of the replication of polioviruses (Baltimore et al., 1963) and other enteroviruses, and the failure to detect luciferase activity in its presence in cells transfected with pLuc70, confirms that the signals seen are due to replication of the replicon RNA.

## **3.4 DISCUSSION**

A full-length infectious clone of the EV70 reference strain (J670/71) has been constructed, with subsequent characterisation demonstrating that virus derived from the clone is comparable to the wild type virus on which it was based. In addition a luciferase-encoding subgenomic replicon of EV70 (J670/71) has been constructed, with replication demonstrated by the production of luciferase on transfection of RNA. These clones will prove extremely valuable resources for future investigation of all aspects of the EV70 life cycle.



**Figure 3.11 Luciferase assay comparing replication of subgenomic replicons.** Luciferase was assayed 6 hours after MRC5 cells were transfected with 2 $\mu$ g of RNA transcribed from luciferase-encoding subgenomic replicons, in the presence and absence of the replication inhibitor guanidine hydrochloride. Error bars show the range of results over duplicate assays. Replication of the EV70 subgenomic replicon pLuc70 is comparable to that of the PV3 subgenomic replicon (pT7Rep3-L+R), and a chimeric PV3 replicon with EV70 5'UTR sequence (p5'70Rep3).

The virus used in the current studies contains fifteen differences from the published EV70 J670/71 sequence (Ryan et al., 1990). These are present in both the RTPCR product and all clones sequenced. As both sequences are derived from the same reference strain it is likely that the differences have occurred during passage in tissue culture, either by progressive sequence drift or active selection by growth in different cell types. The virus sequenced by Ryan et al was grown in Hep-2c cells, while here MRC5 cells have been used. The earlier passage history of both viruses is unknown. One difference, the G nucleotide insertion at position 352 of the 5'UTR is also present in other EV70 5'UTR sequences in genbank and may represent an error in the original sequence. Of the other changes, within the open reading frame of the virus, the majority are non-coding or represent conservative amino acid changes. Of note is that four changes occur within a 37 base region of the viral polymerase sequence, although as three are non-coding and one highly conservative it is not possible to suggest a common selective pressure for these at the protein level. Of more potential import to receptor studies is a cluster of four changes within a 45 base region of 1D, of which three are coding changes, two being non-conservative. This region encodes the major viral capsid protein VP1 and the differences may be the result of selective pressure, potentially at the level of receptor binding.

As mentioned in the text, problems were encountered attempting to amplify the 7451 bp RTPCR product using a variety of proofreading DNA polymerases. These related to low and variable yield and multiple RTPCR errors. The polymerase eventually used was KOD polymerase (Takagi et al., 1997). This is derived from the archaeon *Pyrococcus* sp. strain KOD1. It contains polymerase and 3'-5' exonuclease activity. Fidelity is claimed to be comparable to that of other proofreading polymerases in the same assay, however the manufacturers also claim that the enzyme has ten to fifteen times greater processivity than similar polymerases. The improved reliability and yield observed in the full-length EV70 PCR with KOD polymerase seem likely to be the result of this improved processivity. Low fidelity observed in the EV70 RTPCR with other enzymes may be a function of poor processivity on a long target with the resultant inability to accurately optimise PCR conditions. These findings emphasise the importance of the choice of polymerase when carrying out RTPCR on long targets and suggests the advisability of assessing a panel of different proofreading enzymes.

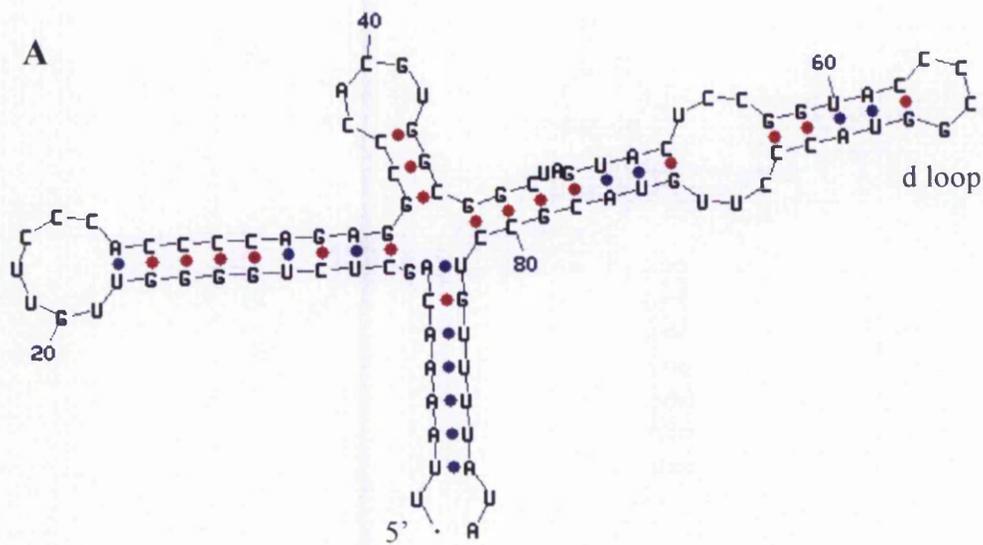
CPE was evident by 48 hours in seven of the sixteen full-length EV70 clones obtained. Five clones required a further 48 hour incubation before CPE was observed.

Although this may have been due to variation in transfection efficiency, it seems likely that some contained RTPCR errors resulting in defective viral sequence. The remaining four clones showed no CPE after prolonged incubation of transfected cells, suggesting fatal errors during RTPCR. The final clone used from the full-length RTPCR cloning, contained six differences from the published sequence of EV70 J670/71, which were also not found in the RTPCR sequence. Two result in conservative changes in amino acid; leucine to isoleucine in the VP2 sequence and isoleucine to valine in 3A. Both were present in other clones sequenced, suggesting that they form part of the viral quasispecies. Of the remaining four differences two are non-coding changes within the capsid and two occur in the 5'UTR. Although these changes were not apparent on sequencing of the EV70 RTPCR product or found in the additional clones sequenced, it is possible that some or all of them may be represented at low frequency within the viral quasispecies, alternatively they may represent true PCR errors. KOD polymerase is reported to have an error rate equivalent to other proofreading DNA polymerases of approximately 1 in  $10^6$  nucleotides (Cline et al., 1996; Takagi et al., 1997), in contrast the viral RNA dependant RNA polymerase has no proofreading capacity and has a much higher error rate, estimated at approximately 1 in  $10^4$  for the poliovirus polymerase (Ward and Flanagan, 1992). Therefore the viral polymerase is not an unlikely source of the sequence differences seen. The capsid sequences of enteroviruses are not believed to have significant RNA secondary structure, in view of which the two non-coding capsid changes seem unlikely to be of significance. The 5'UTR changes occur in the EV70 IRES, an area rich in functional secondary structure and are therefore potentially significant. However, the changes are predicted to be in an unpaired loop and at the base of a stem-loop structure, therefore neither would be expected to cause major structural disruption, although binding of factors essential to translation might still be compromised.

Three EV70 full-length infectious clones were constructed: pTOPOFLC70, made by direct cloning of a full-length RTPCR product, which lacks a ribozyme; pFLC70 $\beta$  which is identical to pTOPOFLC70 but has been subcloned into a vector containing a ribozyme; and pFLC70 which encodes a ribozyme but does not contain the two 5'UTR changes and two non-coding changes in P1 present in the other two clones. All three viruses show similar profiles on a single-step growth curve to wild type EV70 and indistinguishable plaque phenotypes. This, together with the absence of reversion of the changes on passage of recovered virus, suggests that they do not confer a significant

growth disadvantage on the virus in tissue culture. The potential for an *in vivo* effect still remains. The major difference noted between the clones relates to specific infectivity. Both ribozyme-encoding clones generate over  $10^6$  pfu/ $\mu$ g RNA, a result comparable to that seen for poliovirus virion RNA in HeLa cells (Crotty et al., 2001; Koch, 1973). However, RNA transcribed from pTOPOFLC70, which lacks a ribozyme, has a specific infectivity over two  $\log_{10}$  lower. The presence of an upstream ribozyme results in transcribed RNA being cleaved to generate an accurate 5' end to the viral genome. It has been shown that an accurate 5' end is necessary for the efficient replication of poliovirus RNA (Herold and Andino, 2000). This result illustrates that an accurate 5' end is also required for efficient EV70 infection. Following transfection, however, the additional nucleotides are removed (Herold and Andino, 2000) and replicated viral RNA and progeny virions will have the correct 5' end, as evidenced by the growth curve for virus recovered from pTOPOFLC70 which is comparable to that of the ribozyme-encoding clones and wild type virus.

The construction of a luciferase-containing EV70 subgenomic replicon has also been described. A luciferase replication assay has shown that this replicon is functional and replicates to comparable levels as the equivalent well-characterised PV3 subgenomic replicon. In addition, an intermediate from the first step of construction of the replicon, which consists of a chimera with the 5'UTR of EV70 in the backbone of the PV3 replicon, also replicates to comparable levels. Although not directly related to the subject of this study this is an interesting observation and will be briefly discussed here. It is known that the initial 100 bases of the 5'UTR forms a cloverleaf structure which interacts with the viral 3CD polymerase (Andino et al., 1993; Andino et al., 1990). Substitution of the cloverleaf of CVB4 or human rhinovirus type 2 into a poliovirus replicon result in replication competent chimeras, whereas substitution with the human rhinovirus type 14 sequence does not (Rohll et al., 1994). It has further been shown (Rieder et al., 2003) that 3CD polymerase binding and replication can be restored in the rhinovirus type 14/PV3 chimera by both the insertion of a G nucleotide within the loop of stem-loop d of the cloverleaf to form a tetra loop and an additional mutation to a C nucleotide at the third position of the loop (figure 3.12B). MFOLD analysis (Zuker, 2003) reveals that the EV70 cloverleaf d-loop has a similar structure to that of PV3 and has a tetra loop with a C nucleotide at the third position (figure 3.12), predicting that there is likely to be a functional interaction with the PV3 3CD



**B**

	d loop	Replication competent chimera with PV3
PV3	CACG	Yes
EV70	-C--	Yes
CVB4	UC--	Yes
Human Rhinovirus type 2	U---	Yes
Human Rhinovirus type 14	U-U.	No
M2-R14	U---	Yes

**Figure 3.12 Sequences important for poliovirus 3CD polymerase binding.** **A** MFOLD prediction of EV70 cloverleaf bases 1-90 (Zuker, 2003). **B** Table based on Rohll et al, 1994 and Rieder et al, 2003, showing the d-loop sequence for several viruses and their compatibility with poliovirus polymerase, including a mutant of human rhinovirus type 14 (M2-R14, Rieder et al, 2003) which confers replication competence on a human rhinovirus type 14/PV3 chimera. The EV70 d loop is included, which forms a replication competent chimera with PV3 (figure 3.11 and section 3.3.2).

polymerase. This is consistent with the observed efficient replication of the EV70 5'UTR/PV3 chimera in the luciferase assay.

The availability of a functional subgenomic replicon and infectious clone for EV70 will provide valuable tools for further research into all areas of the EV70 life cycle, including investigations into viral receptors and cell entry. In the current study the replicon has been utilised to assess the relative importance of replication, as opposed to viral binding and entry, in the species' tropism of EV70, and to exclude non-specific effects on viral replication when using inhibitors to investigate the EV70 entry pathway.

The EV70 infectious clone has also been utilised in the investigation of species tropism. It has not yet been possible, however, to construct a clone with the capsid from an unpassaged EV70 isolate, due to difficulty obtaining a clinical EV70 isolate. This is planned as future work, as is the use of the clone in reverse genetic strategies to identify the capsid sequences important for receptor binding and cell entry.

## CHAPTER 4

### Characterisation of the Enterovirus type 70 Receptor/s

Virus attachment to the cell requires interaction with a specific molecule on the cell surface. It follows therefore that the presence of the viral receptor is the primary determinant that defines a cell's susceptibility to virus infection and therefore the tissue and species tropism of the virus. EV70 is unusual as an enterovirus, both in terms of its tropism for the human conjunctiva and reported ability to infect non-primate mammalian cells in culture (Yoshii et al., 1977), and in terms of its receptor usage. Both sialic acid and DAF have been implicated as EV70 receptors (Karnauchow et al., 1996; Utagawa et al., 1982b).

Sialic acid was first implicated as a receptor for EV70 when it was reported that HA by EV70 was sensitive to the action of the sialic acid-cleaving enzyme neuraminidase (Utagawa et al., 1982b). Although sialic acid is used by a variety of different viruses, it is unusual as a receptor within the *picornaviridae*, having only been implicated for human EV68 and equine rhinitis virus (Stevenson et al., 2004; Uncapher et al., 1991).

In 1996 DAF was implicated as the HeLa cell receptor for EV70 (Karnauchow et al., 1996). Although DAF is the receptor for a number of echoviruses and coxsackieviruses, EV70 was again unusual as the interaction with DAF was shown to occur at SCR domain 1, the most membrane distal of the four SCR domains in the DAF structure (Karnauchow et al., 1998). In contrast, SCR domain 3 of DAF is required for the binding of the majority of DAF-binding enteroviruses, with only CVA21 having been shown to bind SCR1 (Shafren et al., 1997b).

Prior to the start of this project it was also reported that EV70 bound to DAF even after removal of its N- and O-glycosylation sites, by site-directed mutagenesis and the use of chimeric proteins respectively (Alexander and Dimock, 2002). This suggests that the sialic acid requirement is distinct from any requirement for DAF. In addition, using metabolic inhibitors of O-linked and N-linked glycosylation, it was reported that an O-linked glycoprotein was involved in EV70 binding to HeLa cells (Alexander and Dimock, 2002).

The relative roles and importance of sialic acid and DAF in cellular binding and infection by EV70, therefore, remain unclear. In addition the nature of the sialic acid

element of the receptor is unknown: whether sialic acid itself is sufficient for binding; whether the linkage to the underlying sugar is important, as for influenza virus; whether there is a requirement for specific downstream sugars or glycan structure; or indeed whether a specific glycoprotein or glycolipid is required as appears to be the case for polyomaviruses (Tsai et al., 2003).

One major concern in receptor studies is the potential for adaptation of receptor specificity during passage in tissue culture. This is particularly the case for RNA viruses where error-prone RNA polymerases and the consequent existence of the virus as a quasispecies result in the potential for the rapid emergence of viral variants. For example, foot and mouth disease virus (a picornavirus) adapts to use heparin sulphate, in preference to integrins, on culture in CHO cells (Sa-Carvalho et al., 1997), and laboratory adapted vaccine strains of measles virus adapt to use CD46 as a receptor (Ono et al., 2001). The EV70 reference strain J670/71 was isolated in Japan from conjunctival scrapings during the pandemic of 1970/1971. It is held by the American Tissue Culture Collection (ATCC), who unfortunately have no information regarding the early passage of the virus. Recent passage at ATCC has been in WI-38 cells, a primary embryonic fibroblast cell line. When using a reference strain the virus has, almost by definition, been extensively passaged in culture. Efforts can still be made, however, to standardise the passage history of the virus used during studies, and minimise the risks of any further adaptation. Results from such studies can then be used as the basis for future studies using unpassaged clinical isolates, to confirm viral receptor usage *in vivo*.

### **Chapter Outline**

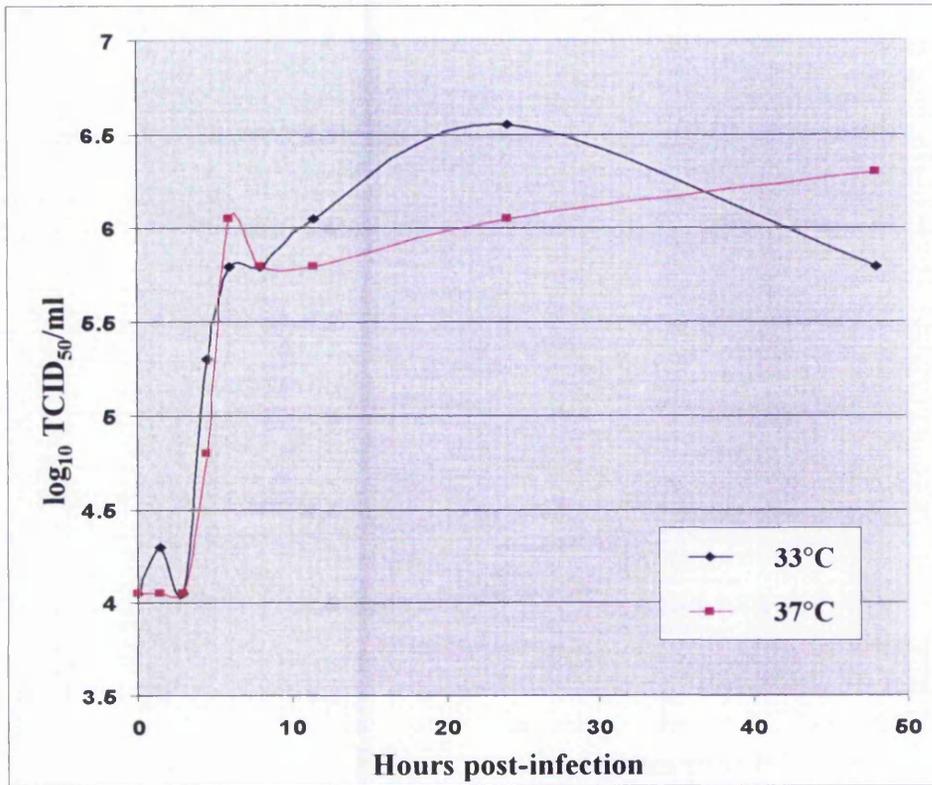
This chapter describes the derivation and passage of the EV70 J670/71 reference strain used in these studies and outlines a series of experiments to investigate the requirements of this virus for binding to cells. This involves studies of the importance of sialic acid and DAF to EV70-mediated HA, and to viral binding and infection of MRC5 cells and a variety of non-mammalian cell lines. It includes further characterisation of the nature of the sialic acid requirement and investigation of the site of viral binding on the cell surface.

### **4.1 Derivation and passage of EV70**

A fresh aliquot of EV70 J670/71 was obtained from the ATCC. As mentioned above this had a recent passage history in WI-38 cells, a human fibroblast cell line. Viral growth was assessed in several human cell lines including rhabdomyosarcoma (RD), HeLa, human embryonic kidney, WI-38 and MRC5 (primary embryonic lung fibroblast). Only in WI-38 and MRC5 was a CPE observed. These are both primary embryonic lung fibroblast cell lines. MRC5 cells were chosen for future viral passage, as the viral titres obtained were marginally better than those in WI-38 cells. In MRC5 cells EV70 grows to approximately  $10^7$  pfu per ml. MRC5 cells have the advantages of being human derived, and thus from the natural host of EV70, and of not having been transformed or immortalised. However, as a primary cell line, MRC5 cells will only undergo 40 to 50 population doublings before the onset of senescence. Therefore to ensure a constant stock of cells for these studies, cells were frozen in aliquots at eighteen population doublings and used from recovery until not more than 36 doublings. EV70 yield was not significantly different when grown in MRC5 cells of 20 and 36 population doublings.

Although ATCC recommend that EV70 is grown at 37°C, previous studies have used a temperature of 33°C (Karnauchow et al., 1996; Utagawa et al., 1982b). Empirically this is more in keeping with the peripheral location of the human conjunctiva. To determine the optimum temperature for viral growth, one step growth curves were examined in MRC5 cells infected with EV70 and incubated at either 37°C or 33°C (figure 4.1). It can be seen that both curves are similar, although maximum growth at 24 hours was seen at 33°C.

To standardise the passage history of the virus used in the current studies and to minimise the risk of any further viral adaptation, EV70 stock from ATCC was passaged a standard five times in MRC5 cells at 33°C, and this virus was then used for all subsequent work.



**Figure 4.1 Effect of incubation temperature on EV70 one step growth curves in MRC5 cells.** MRC5 cells were infected with EV70 at an m.o.i. of 3, and incubated at either 33°C or 37°C. Virus was harvested by freeze-thawing at 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours and titres determined by TCID<sub>50</sub>.

## **4.2 The haemagglutination phenotype of EV70 is due to viral binding to sialic acid and not to DAF**

Viral HA can be defined as the agglutination or clumping of RBC by virus, forming a diffuse film in the well of a microtitre plate. This is in contrast to the defined pellet seen in non-haemagglutinating controls. Viruses which have receptors expressed on RBC may exhibit this phenomenon. As both sialic acid and DAF are expressed at significant levels on RBC, both sialic acid binding viruses such as influenza virus (Ito et al., 1997), and DAF binding enteroviruses (Powell et al., 1998), can cause HA. HA and HA inhibition assays provide a straightforward and rapid method for investigating the interaction of viruses with their receptors and it was therefore decided to use this as an initial strategy to investigate the receptor specificity of EV70.

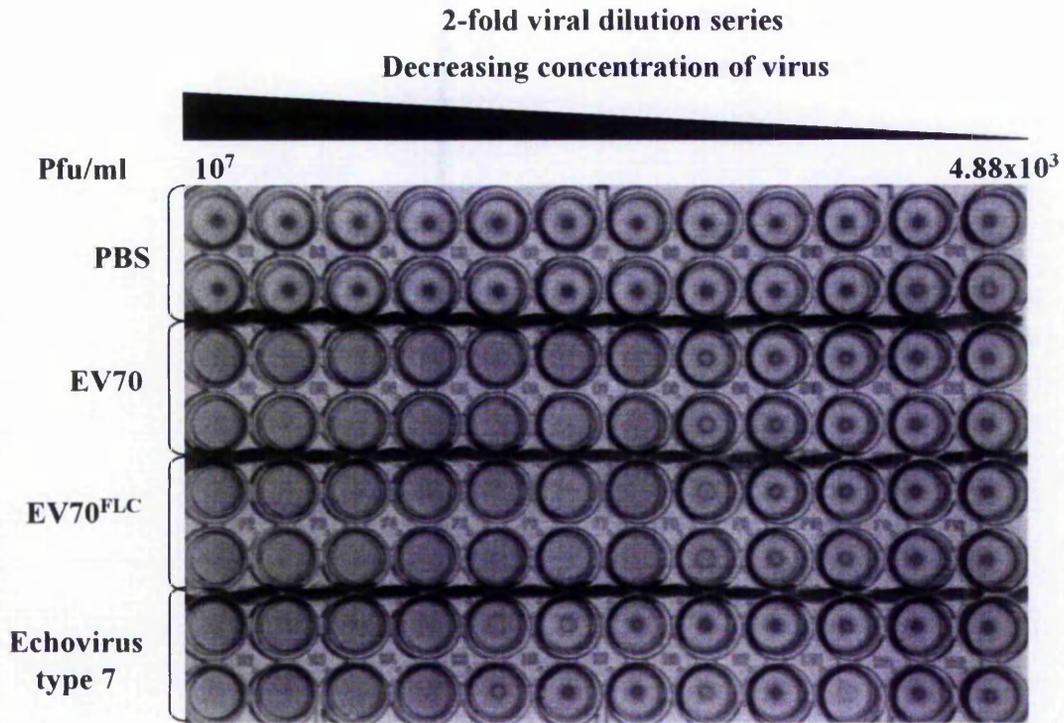
### **4.2.1 EV70 causes haemagglutination.**

Although EV70 culture supernatant does not haemagglutinate RBC, if the virus is first purified by ultracentrifugation through a sucrose cushion, HA is readily observed (figure 4.2). This dependence on the use of purified virus has also been observed for CVA21 (unpublished observation, Prof. DJ Evans laboratory).

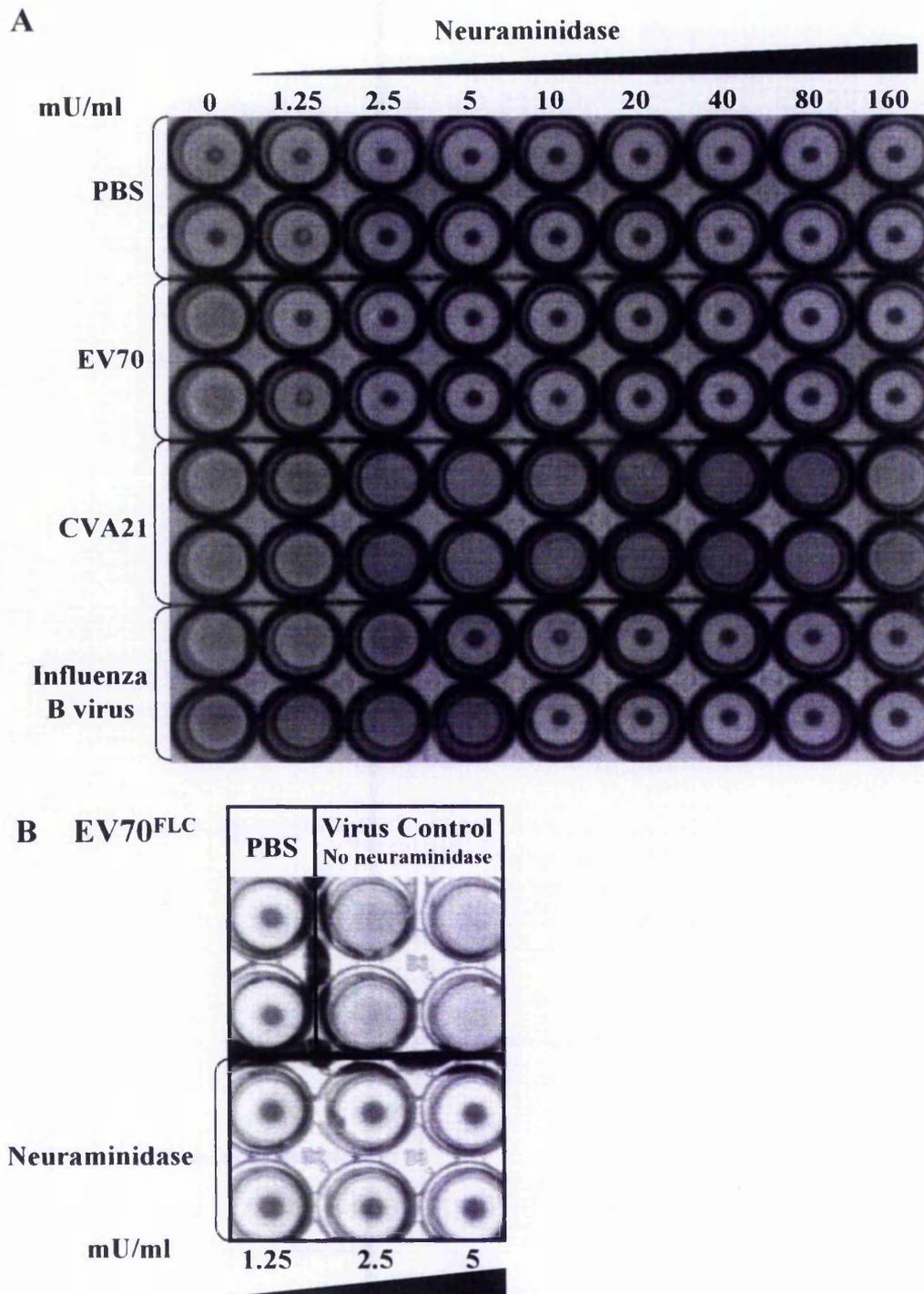
In figure 4.2 the DAF binding echovirus type 7 is used as a positive HA control and PBS as a negative control. The initial viral dilution used for all three viruses was  $10^7$  pfu/ml, and HA by a two-fold dilution series of virus was recorded. Both EV70 from ATCC derived stock and EV70<sup>FLC</sup> from the infectious clone, clearly demonstrate HA. As purified EV70 shows HA up to an eight-fold higher dilution than echovirus type 7 culture supernatant, the requirement for initial purification of EV70 is not accounted for purely by a concentration effect, and is most likely due to the removal of an inhibitory contaminant in the culture medium.

### **4.2.2 EV70 haemagglutination is inhibited by neuraminidase**

To determine the contribution of sialic acid binding to the EV70 HA phenotype, an HA inhibition assay was carried out using the enzyme neuraminidase to remove sialic acid from the RBC surface (figure 4.3A). The controls in this assay were influenza B virus, which haemagglutinates via sialic acid binding, and CVA21, which



**Figure 4.2 Haemagglutination by EV70.** Two-fold dilutions of virus, from a starting concentration of  $10^7$  pfu/ml (lowest concentration  $4.88 \times 10^3$ ), were mixed with RBC and HA recorded visually. EV70 and EV70<sup>FLC</sup> were first purified by ultracentrifugation through a sucrose cushion, while echovirus type 7 was used directly from clarified tissue culture supernatant. Adjacent rows are duplicates. EV70, EV70<sup>FLC</sup> and echovirus type 7 all demonstrate HA.



**Figure 4.3 Haemagglutination inhibition with neuraminidase.** RBC, pre-treated with neuraminidase at the concentrations shown, were mixed with a constant 4HA units of virus and HA recorded visually. EV70, EV70<sup>FLC</sup> and CVA21 were first purified by ultracentrifugation through a sucrose cushion, while influenza B virus was tested in allantoic fluid. Adjacent rows are duplicates. **A.** Neuraminidase inhibits HA by EV70 and influenza B virus, but not by CVA21. **B.** Neuraminidase inhibits HA by EV70<sup>FLC</sup>.

haemagglutinates via DAF binding. The latter was used in these assays as it is known to bind the same domain of DAF (SCR1) as has been reported for EV70 (Shafren et al., 1997b). A standard amount of four HA units of each virus was added to RBC treated with neuraminidase at various concentrations. The neuraminidase used from *Clostridium perfringens* is reported to cleave  $\alpha$ 2-3,  $\alpha$ 2-6 and  $\alpha$ 2-8 linked sialic acid.

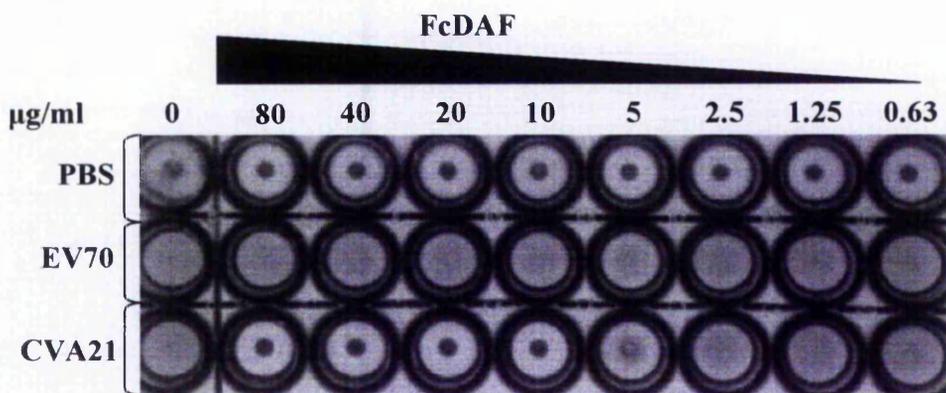
As expected, neuraminidase had no effect on HA by CVA21, but did inhibit influenza B virus HA down to a neuraminidase concentration of 10mU/ml. EV70 HA was also inhibited by neuraminidase, down to the lowest concentration tested of 1.25mU/ml. This was equally the case for EV70<sup>FLC</sup> (figure 4.3B). The inhibition of EV70 HA was observed at a significantly lower neuraminidase concentration than that required for inhibition of influenza B virus HA. This could be due to the differences in the affinity of the viruses for sialic acid or the number of receptor binding sites involved on the EV70 capsid, as compared to the influenza B virus membrane.

These results confirm the early observation of sialic acid involvement in HA by EV70 (Utagawa et al., 1982b), and further demonstrate that molecularly cloned virus exhibits similar binding characteristics to those of the virus obtained from the ATCC from which it is derived.

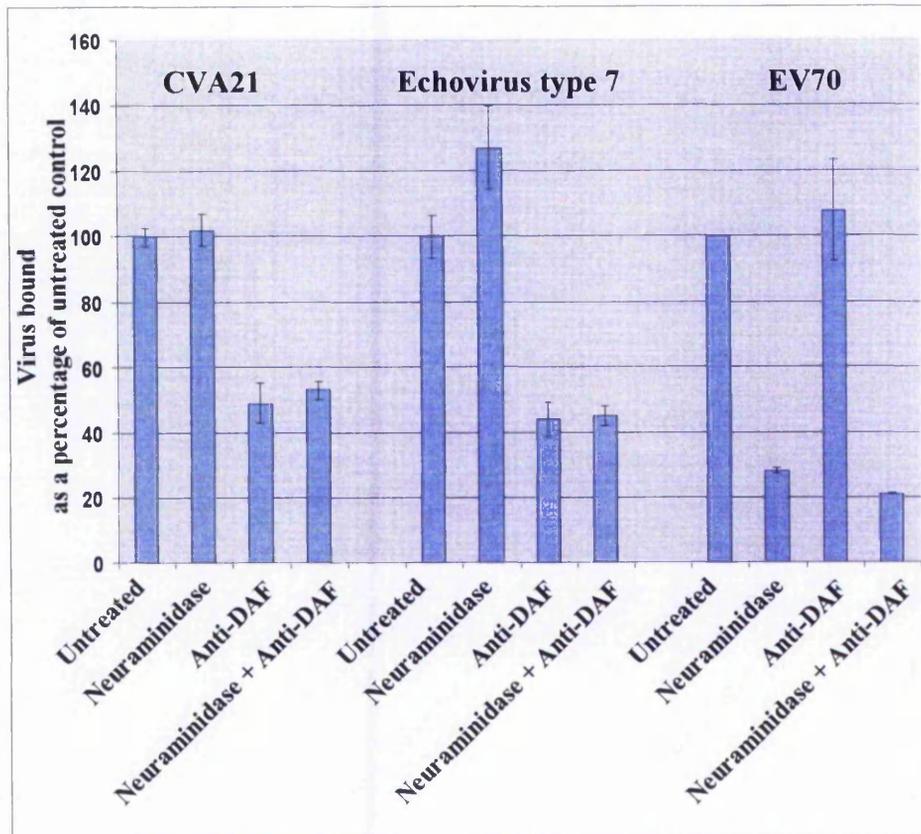
#### 4.2.3 EV70 haemagglutination is not inhibited by soluble DAF

Although the above studies using neuraminidase demonstrate an absolute requirement for sialic acid in the HA phenotype of EV70, they do not rule out a role for DAF in addition to sialic acid. Therefore, to determine the contribution of DAF binding to the EV70 HA phenotype, an HA inhibition assay was carried out using a soluble form of DAF expressed as a dimer, FcDAF (figure 4.4). FcDAF is a dimeric human DAF-Ig fusion protein comprising two DAF molecules (SCR domains 1-4) fused to the amino terminus of human IgG1 Fc in place of the antibody Fab arms (Harris et al., 2002). CVA21 was again used as a positive control. A standard amount of four HA units of each virus was used and pre-incubated with a range of concentrations of FcDAF. As expected, CVA21 HA was inhibited by FcDAF, down to a concentration of 10 $\mu$ g/ml. In contrast no inhibition was seen for EV70, even up to concentrations of 80 $\mu$ g/ml FcDAF.

These results demonstrate that DAF binding is not a significant determinant of the EV70 HA phenotype and that DAF does not represent a major binding site for EV70 on RBC.



**Figure 4.4 Haemagglutination inhibition with soluble FcDAF.** A constant 4HA units of virus, pre-incubated with FcDAF at the concentrations shown, was mixed with RBC and HA recorded visually. EV70 and CVA21 were first purified by ultracentrifugation through a sucrose cushion. Adjacent rows are duplicates. FcDAF inhibits HA by CVA21, but not by EV70.



**Figure 4.5 Viral binding to MRC5 cells after treatment with neuraminidase and polyclonal DAF antibody.** MRC5 cells were pre-treated with 25mU/ml neuraminidase and/or polyclonal antibody raised against DAF at a dilution of 1 in 250, and incubated with 20 000cpm of  $^{35}\text{S}$  labelled CVA21, echovirus type 7 or EV70. After 1hr cells were washed and binding assessed using a scintillation counter. Binding is shown as a percentage of binding to untreated MRC5 cells. Error bars show the range of results over duplicate assays.

### **4.3 EV70 binding and infectivity in MRC5 cells is dependant on sialic acid, but not DAF**

Having shown that EV70 binding to RBC is mediated primarily by sialic acid, rather than DAF, the roles of sialic acid and DAF were investigated in the binding to, and infection of, nucleated cells that the virus productively infects. The MRC5 cell line, used to cultivate EV70 viral stocks, was used as the host cell line for these studies.

#### **4.3.1 Binding of EV70 to MRC5 cells is dependant on sialic acid, but not DAF**

To determine the contributions of sialic acid and DAF to the binding of EV70 to MRC5 cells, a binding assay was carried out using <sup>35</sup>S radiolabelled purified 160S virus particles (figure 4.5). MRC5 cells were pre-treated with either 25mU/ml neuraminidase to remove sialic acid, or with a 1 in 250 dilution of rabbit polyclonal DAF antibody to block potential binding sites on DAF, or with both. Control viruses were CVA21, which binds SCR1 of DAF, and echovirus type 7, which binds predominantly to SCR3.

As can be seen from figure 4.5, the DAF antibody reduced the binding of both CVA21 and echovirus type 7, as expected, by approximately 50%, but had no effect on EV70 binding. In contrast neuraminidase reduced the binding of EV70 by over 70%, but resulted in no reduction in binding of the other viruses tested, indeed there was an increase in the binding of echovirus type 7 after neuraminidase treatment. Pre-treating cells with both DAF antibody and neuraminidase had no additional effect on the binding of any of the viruses. The increased binding by echovirus type 7 after removal of sialic acid could be due to improved accessibility of the virus to DAF on the cell surface, either due to a steric effect or due to a change in cell surface charge (sialic acid being highly negatively charged).

As previously shown for RBC binding, these results demonstrate that binding of EV70 to MRC5 cells is mediated by sialic acid, and additionally, that DAF is not an important determinant of viral binding to these cells.

#### **4.3.2 Infection of MRC5 cells by EV70 is dependant on sialic acid, but not DAF**

Although DAF does not appear to be required for binding of EV70 to MRC5 cells, it may still have an important role in the infection process; for example in virus

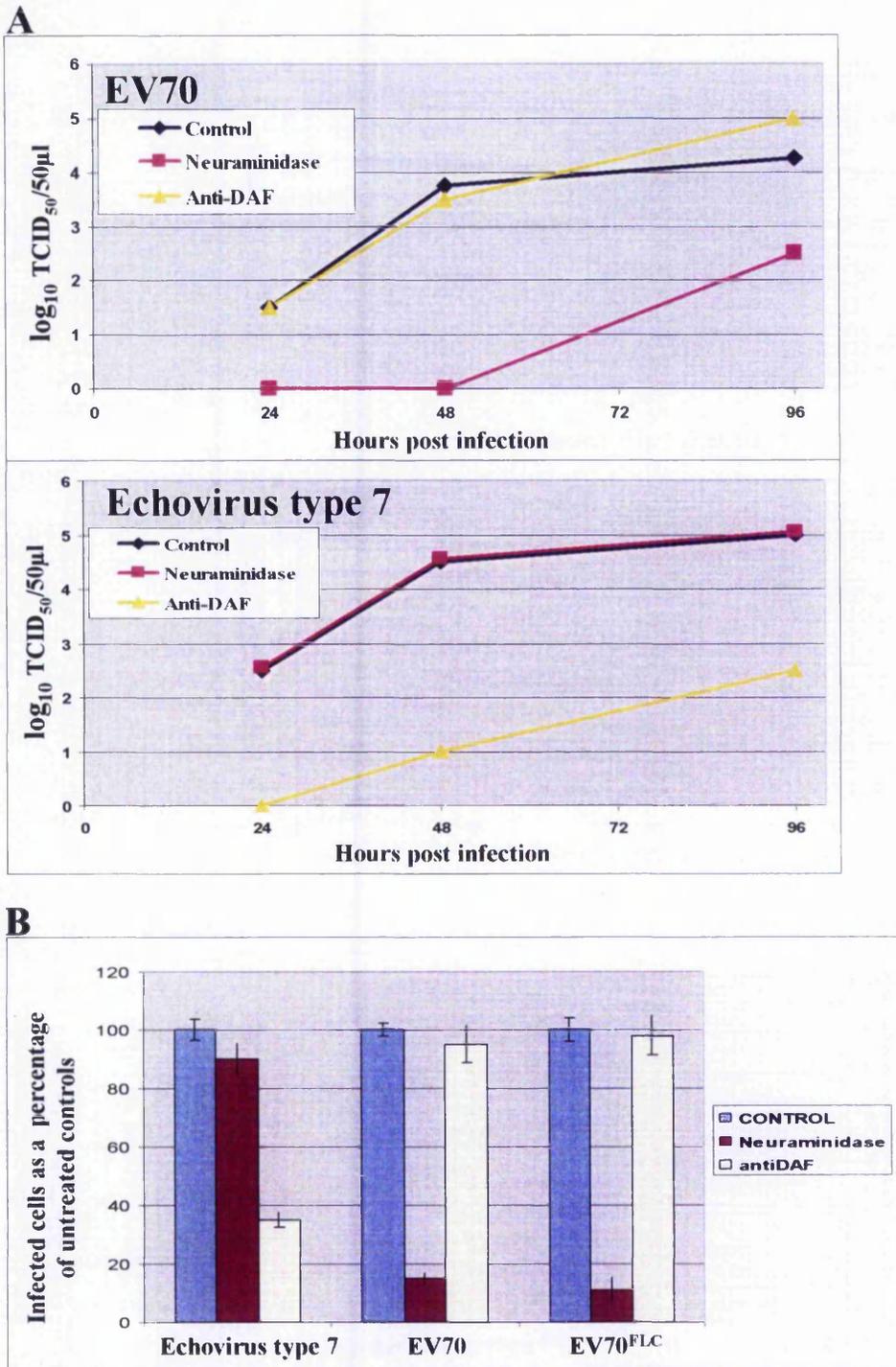


entry or downstream signalling events. To determine the contributions of sialic acid and DAF to the infectivity of EV70 in MRC5 cells, two different assays were performed. A TCID<sub>50</sub>-based assay, where virus was titrated on MRC5 cells pre-treated with neuraminidase or polyclonal DAF antibody (figure 4.6A), and a flow cytometry-based assay using pre-treated cells infected with virus and scored for the numbers of infected cells at 6 hours (figure 4.6B).

In the TCID<sub>50</sub> assay neuraminidase concentrations of 5, 15 and 25mU/ml and rabbit polyclonal DAF antibody dilutions of 1 in 1000, 1 in 500 and 1 in 100 were used. For clarity of presentation only the results for 5mU/ml neuraminidase and the 1 in 100 antibody dilution are presented (figure 4.6A). As can be seen the DAF antibody reduced echovirus type 7 infection of MRC5 cells by over two log<sub>10</sub> at all time points, but had no effect on the titres obtained for EV70. In contrast, EV70 infection of MRC5 cells is blocked completely by neuraminidase up to 48 hours, whereas similar treatment of cells did not affect the yield of echovirus type 7. The growth of EV70 seen at 96 hours is likely to be a consequence of regeneration of the sialated receptor in the treated MRC5 cells. Similar results were obtained in the TCID<sub>50</sub> assay at the other concentrations used, with DAF antibody dilutions up to 1 in 1000 still capable of blocking echovirus type 7 infection by several orders of magnitude, but with no effect on EV70; and higher concentrations of neuraminidase having no effect on echovirus type 7 and having a greater effect on the 96 hour EV70 titres than for 5mU/ml.

The TCID<sub>50</sub> assay results were confirmed using the flow cytometry-based assay, assessing the percentage of infected cells (figure 4.6B). In addition EV70<sup>FLC</sup> was included to confirm that the molecularly cloned virus had the same characteristics as the virus from which it was derived. The polyclonal DAF antibody at a dilution of 1 in 250 reduced the number of cells infected with echovirus type 7 by over 60%, but had no effect on EV70 or EV70<sup>FLC</sup>. In contrast, neuraminidase at 25mU/ml reduced the number of EV70 and EV70<sup>FLC</sup> infected cells by over 80% with no effect on echovirus type 7.

These results demonstrate that sialic acid is a key determinant, not only of viral binding, but also for the productive infection of MRC5 cells by EV70. There is no evidence of a role for DAF in either binding or infection by EV70 of these cells routinely used to cultivate the virus.



**Figure 4.6 Viral infectivity in MRC5 cells after treatment with neuraminidase and polyclonal DAF antibody.** MRC5 cells, pre-treated with 5mU/ml neuraminidase and/or polyclonal antibody raised against DAF at 1 in 100, were infected with EV70, EV70<sup>FLC</sup> or echovirus type 7. **A** TCID<sub>50</sub>-based infectivity assay. A 10-fold dilution series of virus was added to pre-treated cells. Titres were read visually after incubation for 24, 48 and 96 hrs. **B** Flow cytometry-based infectivity assay. Pre-treated MRC5 cells were infected with virus at an m.o.i. of 3 and the number of infected cells assessed by flow cytometry 6hrs post infection. Error bars show the range of results over duplicate assays.

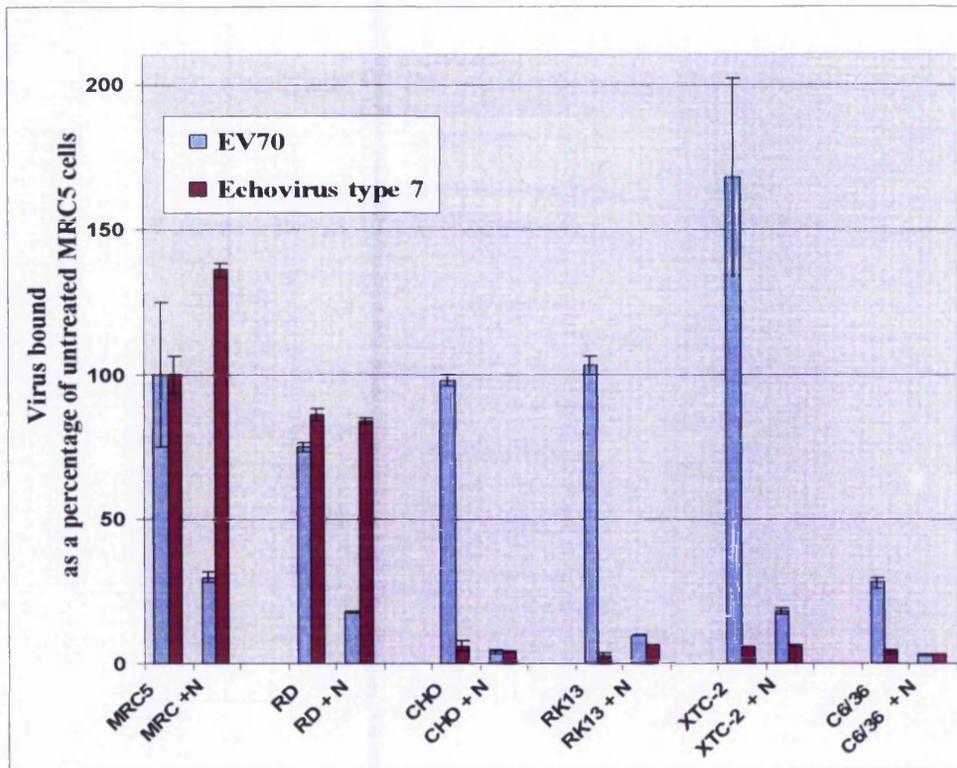
#### **4.4 The role of sialic acid and DAF in EV70 binding and infection in a variety of cell lines**

EV70 has been reported to bind and infect several non-primate mammalian cell lines (Yoshii et al., 1977). Both sialic acid and homologues of DAF are present on mammalian cells and could be contributing to binding. It was decided to investigate binding and infection of EV70 in a range of cell lines. The human cell lines used were MRC5 and RD (rhabdomyosarcoma). The non-primate mammalian cell lines used were CHO (Chinese hamster ovary) and RK13 (rabbit kidney), the latter having been reported by Yoshii et al. to support EV70 infection to titres equivalent to primate cells. In addition two non-mammalian cell lines were also used; XTC-2 (*Xenopus laevis*, South African clawed toad) and C6/36 (*Aedes albopictus*, mosquito larva). Sialic acid is known to be present on *Xenopus* cells. Generally sialic acids are not found in invertebrates, however the situation is controversial and there are exceptions such as *Drosophila* embryos (Varki, 1999).

##### **4.4.1 EV70 binds to non-human cell lines in a sialic acid-dependant manner**

The binding of <sup>35</sup>S radiolabelled purified 160S virus particles to the cell lines under investigation is shown in figure 4.7. Echovirus type 7 bound only to the human cell lines, confirming that there is insufficient homology between human DAF and any homologues on these cells to preserve DAF binding. Although a DAF homologue has not yet been identified on hamster or rabbit cells, the murine DAF homologue exhibits only 63% homology to human DAF (Fukuoka et al., 1996). In contrast EV70 bound to all the cell lines to levels similar or greater than that seen for MRC5 cells. The exception was the C6/36 (mosquito) cell line; however even for these cells binding was detected at levels 28% of that seen in untreated MRC5 cells. In all cases, over 70 percent reduction in binding was seen after pre-treatment of the cells with neuraminidase. EV70 therefore binds to non-primate cell lines, as diverse as *Xenopus*, in a sialic acid-dependant manner.

The presence or absence of sialic acid on invertebrate cells is controversial, as mentioned above (Varki, 1999). Although the level of EV70 binding is low on C6/36 cells, the reduction with neuraminidase strongly suggests that on these cells binding of



**Figure 4.7 EV70 binding to a variety of cell lines, with and without neuraminidase treatment.**  $10^6$  cells, untreated or treated with 25mU/ml neuraminidase (+ N), were incubated with 20 000cpm of  $^{35}\text{S}$  labelled EV70 or echovirus type 7. After 1hr cells were washed and binding assessed using a scintillation counter. Binding is shown as a percentage of binding to untreated MRC5 cells. Error bars show the range of results over duplicate assays.

the virus was mediated by sialic acid. The low level of binding observed might be due to a low level of sialic acid on the surface of these cells or to differences between sialic acid on human and mosquito cells.

#### **4.4.2 Expression of human DAF on non-primate cell lines does not affect EV70 binding**

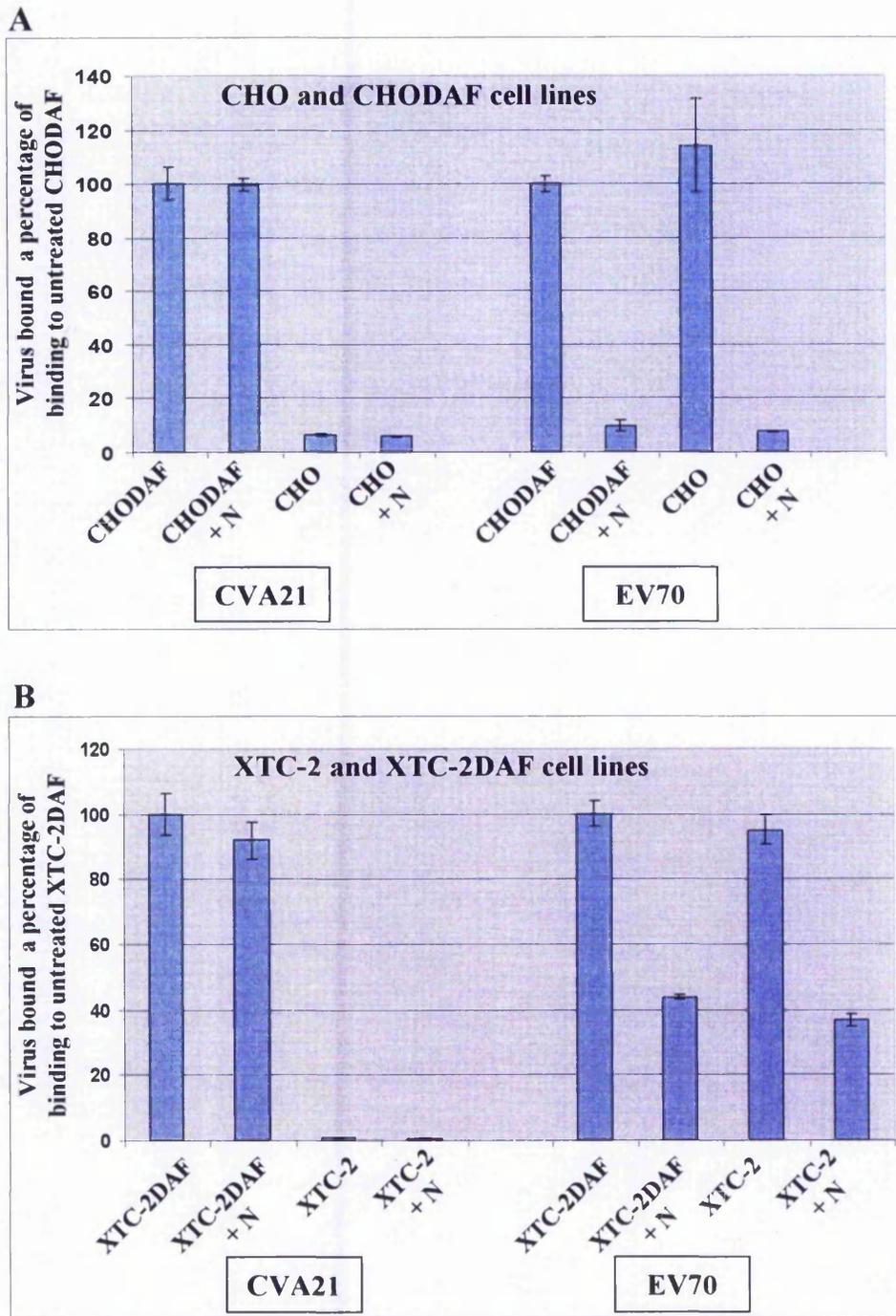
The ability of EV70 to bind to DAF was further assessed using CHO (hamster) and XTC-2 (Xenopus) cell lines engineered to express human DAF (figure 4.8). CVA21 was used as a control, and only bound the cell lines expressing human DAF. As seen in figure 4.7, EV70 efficiently bound both CHO and XTC-2 cells. Binding was not significantly increased when human DAF was expressed on these cells. Both the DAF expressing cell lines and their non-engineered parent lines showed significant and equivalent reductions in binding after removal of sialic acid with neuraminidase. Indeed, in DAF expressing CHO cells binding was reduced by over 90 percent. These results again indicate that DAF is not involved in the binding of EV70 to cells.

In contrast to CHO cells, in this assay neuraminidase treatment of XTC-2 cells reduced EV70 binding by only 60%, the residual binding could be due to binding to a non-sialated protein, or a failure of neuraminidase to remove all forms of sialic acid on these cells, however, a decrease in binding of almost 90% was seen in previous binding assays in XTC-2 cells treated with neuraminidase (figure 4.7), suggesting that this observation is likely to be due to interassay variability, such as differences in the quality of different batches of neuraminidase.

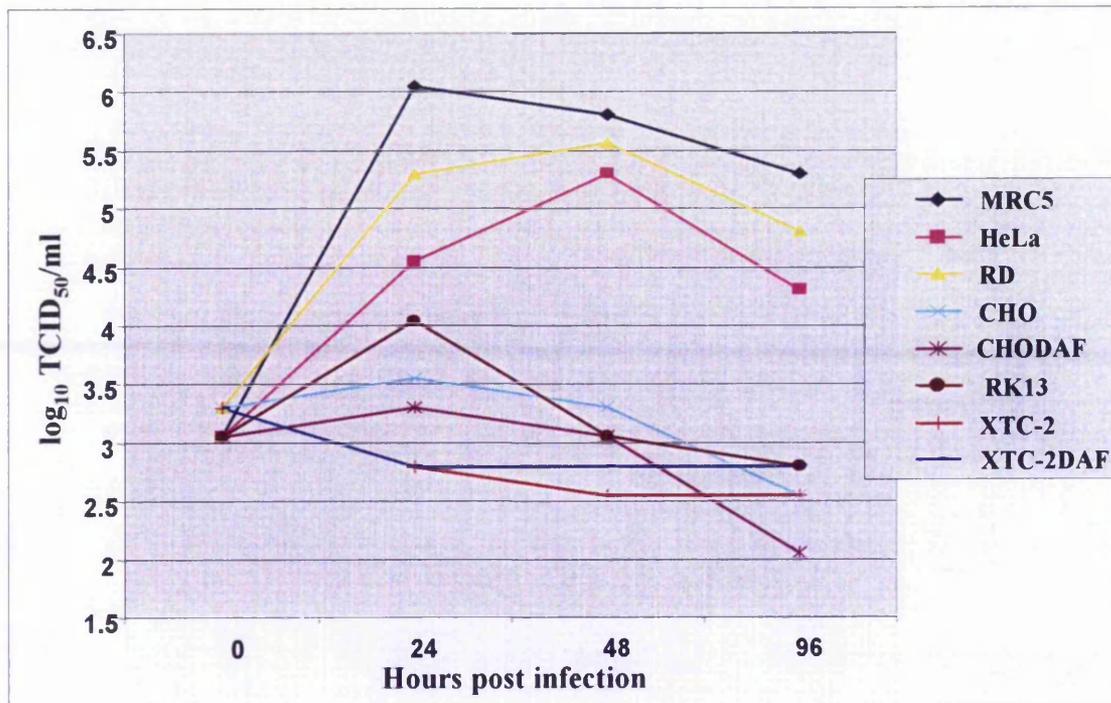
#### **4.4.3 EV70 does not significantly infect non-primate cell lines, even when expressing human DAF**

The infectivity of EV70 in multiple cell lines was investigated using multi-step growth curves over 96 hours (figure 4.9). The virus showed good growth in the three human cell lines used, MRC5, RD and HeLa. Of note was that despite being permissive for viral infection, neither RD nor HeLa cells exhibited CPE. This remained the case after virus was further passaged in these cell lines five and three times respectively (data not shown).

There was a one log<sub>10</sub> increase in viral titres in RK13 (rabbit) cells at 24 hours, however this rapidly reduced, suggesting that these cells are not fully permissive for EV70 infection. This contrasts with the good viral yield from these cells observed by



**Figure 4.8** Viral binding to CHO and XTC-2 cell lines expressing human DAF, with and without neuraminidase treatment.  $10^6$  cells, untreated or treated with 25mU/ml neuraminidase (+ N), were incubated with 20 000cpm of  $^{35}\text{S}$  labelled EV70 or CVA21. After 1hr cells were washed and binding assessed using a scintillation counter. Binding is shown as a percentage of binding to untreated CHODAF or XTC-2DAF cells. Error bars show the range of results over duplicate assays. **A** CHO and CHODAF cell lines. **B** XTC-2 and XTC-2DAF cell lines.



**Figure 4.9 EV70 multi-step growth curves for EV70 in a variety of cell lines.** Cells were infected with EV70 at an m.o.i. of 3 and titres determined by TCID<sub>50</sub> on virus harvested by freeze-thawing after 0, 24, 48 and 96 hours.

Yoshii et al, 1977 and may be due to differences in the passage history of the virus or of the cells themselves. Significant infection by EV70 is not seen in CHO and XTC-2 cells and this remained the case in the cell lines engineered to express human DAF, in keeping with the lack of binding to DAF described above.

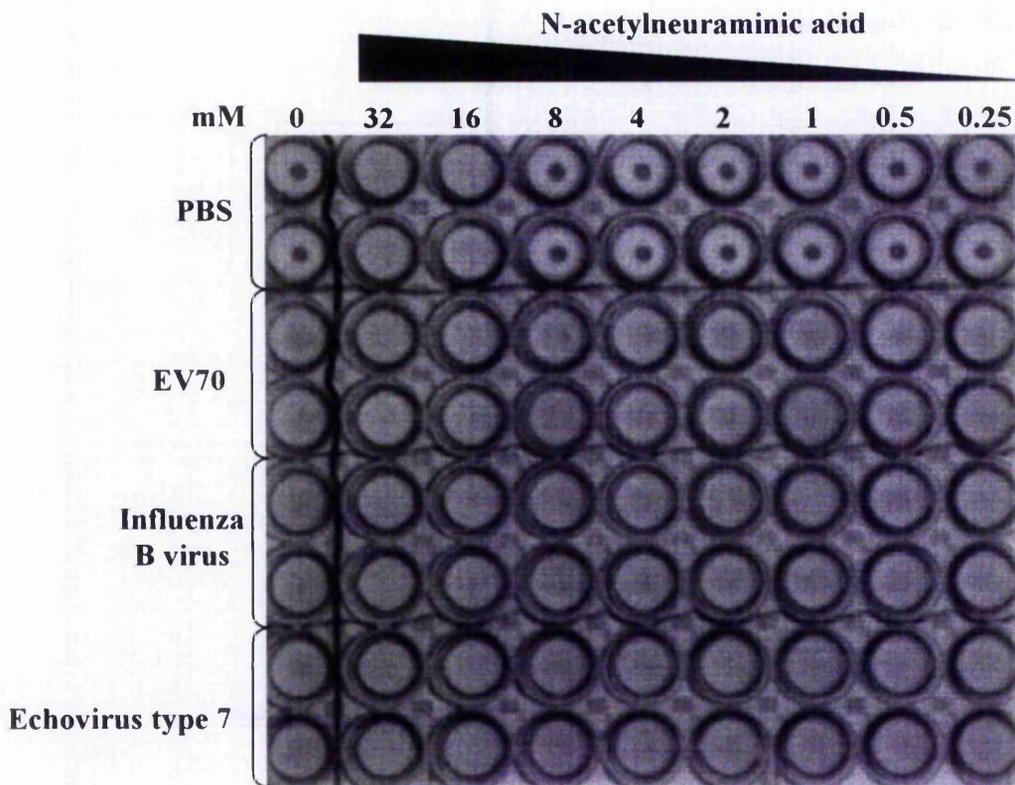
Sialic acid-mediated binding to non-primate cell lines is therefore not sufficient for productive infection by EV70. This may be due to additional requirements for viral entry to the cell or incompatibility with the cellular replication machinery. These possibilities are further investigated in chapter five.

#### **4.5 The EV70 receptor on red blood cells is a GPI-anchored, $\alpha$ 2-3 linked sialated glycoprotein**

Having ascertained that sialic acid is the major determinate of EV70 cellular binding, the nature of the sialic acid required was further investigated. As discussed in section 4.2, HA assays provide a convenient and rapid method for studying viral binding to cells and were again utilised here. Three aspects of the sialic acid requirement were explored: whether sialic acid itself is sufficient for binding, regardless of its molecular attachments; the specificity for sugar linkage; and the requirement, if any, for location on a specific glycolipid or glycoprotein.

##### **4.5.1 EV70 haemagglutination is not inhibited by N-acetylneuraminic acid**

To ascertain whether EV70 can bind to any sialic acid on the surface of RBC regardless of its molecular attachments, an HA inhibition assay was carried out using free sialic acid in the form N-acetylneuraminic acid (figure 4.10). The controls used were the DAF binding echovirus type 7, and the sialic acid binding influenza B virus. A standard amount of four HA units of each virus was used and pre-incubated with a range of concentrations of N-acetylneuraminic acid. No inhibition of HA was seen for any of the viruses. Although influenza virus is believed to bind sialic acid present on any glycolipid or glycoprotein, it does have specificity for downstream linkages, and free sialic acid has been found to be a poor inhibitor of influenza virus binding (Pritchett and Paulson, 1989). The reason for this is believed to be at least partly due to a need for polyvalent co-operative associations between the virus and multiple sialic acid binding sites. Indeed synthetic polymeric molecules with multiple sialic acid residues have



**Figure 4.10 Haemagglutination inhibition with N-acetylneuraminic acid (sialic acid).** A constant 4HA units of virus, pre-incubated with N-acetylneuraminic acid at the concentrations shown, was mixed with RBC and HA recorded visually. EV70 was first purified by ultracentrifugation through a sucrose cushion, while influenza B virus and echovirus type 7 were tested directly from allantoic fluid and tissue culture supernatant respectively. Adjacent rows are duplicates. N-acetylneuraminic acid does not inhibit HA by any of the viruses. Note that at 32mM and 16mM concentrations N-acetylneuraminic acid alone causes HA, as shown by the lack of a pellet in wells treated solely with PBS.

proven to be potent inhibitors of influenza virus binding (Lees et al., 1994; Reuter et al., 1999). Pritchett and Paulson (1989) did observe inhibition of influenza virus binding to RBC at a concentration of 10mM N-acetylneuraminic acid. In this study the highest concentration giving analysable data was 8mM, with no inhibition. Concentrations of 16 and 32mM resulted in RBC lysis, possibly due to the high negative charge on sialic acid.

Although it has not been possible to demonstrate inhibition of EV70 HA with free sialic acid, this does not conclusively prove that the virus is incapable of binding to sialic acid alone, but may be due to limitations of the assay method, such as cell lysis at high concentrations of sialic acid, and/or a requirement for sialic acid presented in multivalent form.

#### **4.5.2 EV70 haemagglutination is inhibited by $\alpha$ 2-3 specific neuraminidase**

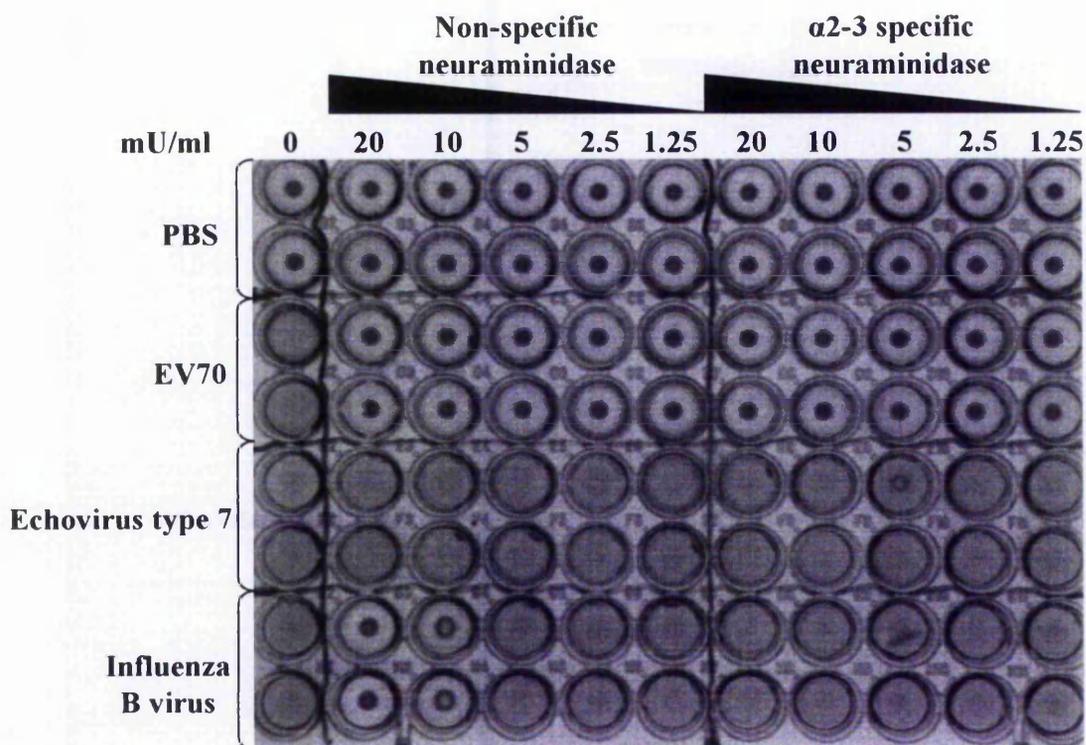
To determine whether EV70 binding is dependant upon a particular linkage of sialic acid to the underlying sugar, an HA inhibition assay was carried out using an  $\alpha$ 2-3 specific form of neuraminidase derived from *Streptococcus pneumoniae* (figure 4.11). The controls in this assay were the DAF-binding echovirus type 7 and influenza B virus. Human influenza B virus has a specificity for  $\alpha$ 2-6 linked sialic acid (Suzuki et al., 1992). A standard amount of four HA units of each virus was used and RBC were treated with a range of concentrations of both the non-specific and  $\alpha$ 2-3 specific forms of neuraminidase.

As expected neither neuraminidase had an effect on HA by echovirus type 7. Also as expected, the  $\alpha$ 2-3 specific form of neuraminidase had no effect on HA by influenza B virus, while the non-specific form of the enzyme inhibited HA at concentrations down to 10mU/ml, comparable to those seen in figure 4.3A. In contrast both the non-specific and  $\alpha$ 2-3 specific forms of neuraminidase inhibited EV70 HA and this was seen down to the lowest concentration used (1.25mU/ml).

All observable HA by EV70 was blocked by removal of  $\alpha$ 2-3 linked sialic acid from RBC, demonstrating that EV70 binds to  $\alpha$ 2-3 linked sialic acid on RBC and that this interaction is required for the HA phenotype of the virus.

#### **4.5.3 EV70 haemagglutination is inhibited by PLC, PIPLC and Proteinase K.**

To determine whether EV70 has a binding specificity for sialic acid on a specific glycolipid or glycoprotein on the RBC surface, HA inhibition assays were carried out



**Figure 4.11 Haemagglutination inhibition with  $\alpha$ 2-3 specific neuraminidase.** RBC, pre-treated with a non-specific neuraminidase or  $\alpha$ 2-3 specific neuraminidase at the concentrations shown, were mixed with a constant 4HA units of virus and HA recorded visually. EV70 was first purified by ultracentrifugation through a sucrose cushion, while influenza B virus and echovirus type 7 were tested directly from allantoic fluid and tissue culture supernatant respectively. Adjacent rows are duplicates. The non-specific neuraminidase inhibits HA by EV70 and influenza B virus, but not by echovirus type 7, while the  $\alpha$ 2-3 specific neuraminidase inhibits only HA by EV70.

using a range of lipases and proteases. Again echovirus type 7 and influenza B virus were used as controls.

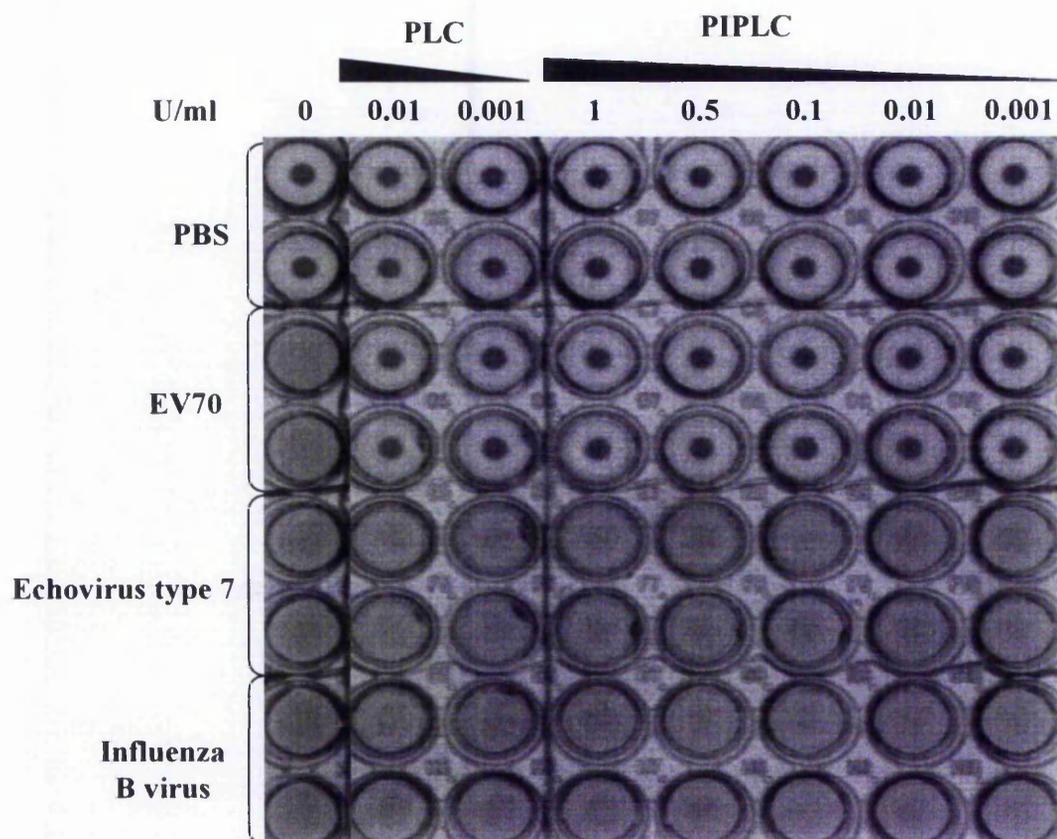
To determine whether the sialated receptor contained a lipid component the lipases PLC and PIPLC were used (figure 4.12). PLC hydrolyses the phosphate bond on glycerophospholipids, including the GPI anchor of GPI-anchored proteins. PIPLC specifically cleaves GPI anchors. The concentrations used ranged from 0.001-1 U/ml, however concentrations of PLC of 0.1 U/ml and over resulted in RBC lysis and so no data is available at these concentrations. As expected HA by influenza B virus, which can bind sialic acid on both lipids and proteins, was not affected by either lipase. HA by echovirus type 7 was also unaffected by these enzymes. Although echovirus type 7 binds DAF, a GPI- anchored protein, it has previously been shown that the GPI anchor of DAF in RBC contains a fatty acid substitution which renders it resistant to cleavage by these enzymes (Walter et al., 1990). In contrast EV70-mediated HA was inhibited by both PLC and PIPLC, the latter down to low concentrations of at least 0.001 U/ml. These results suggest that EV70 HA of RBC is due to the interaction of the virus with a protein attached to the cell surface via a GPI anchor.

To confirm that the virus was indeed binding to a sialated protein, an HA inhibition assay was carried out using the proteases proteinase K and chymotrypsin (figure 4.13). As expected neither protease had an effect on influenza B virus HA. Inhibition was seen at 6 to 8mg/ml of both proteases for echovirus type 7 and down to at least 2mg/ml Proteinase K for EV70. Chymotrypsin had no effect on EV70 HA. These results confirm that EV70 is binding to a protein on the RBC surface. Further, the protease cleavage profile differs from that of DAF bound by echovirus type 7.

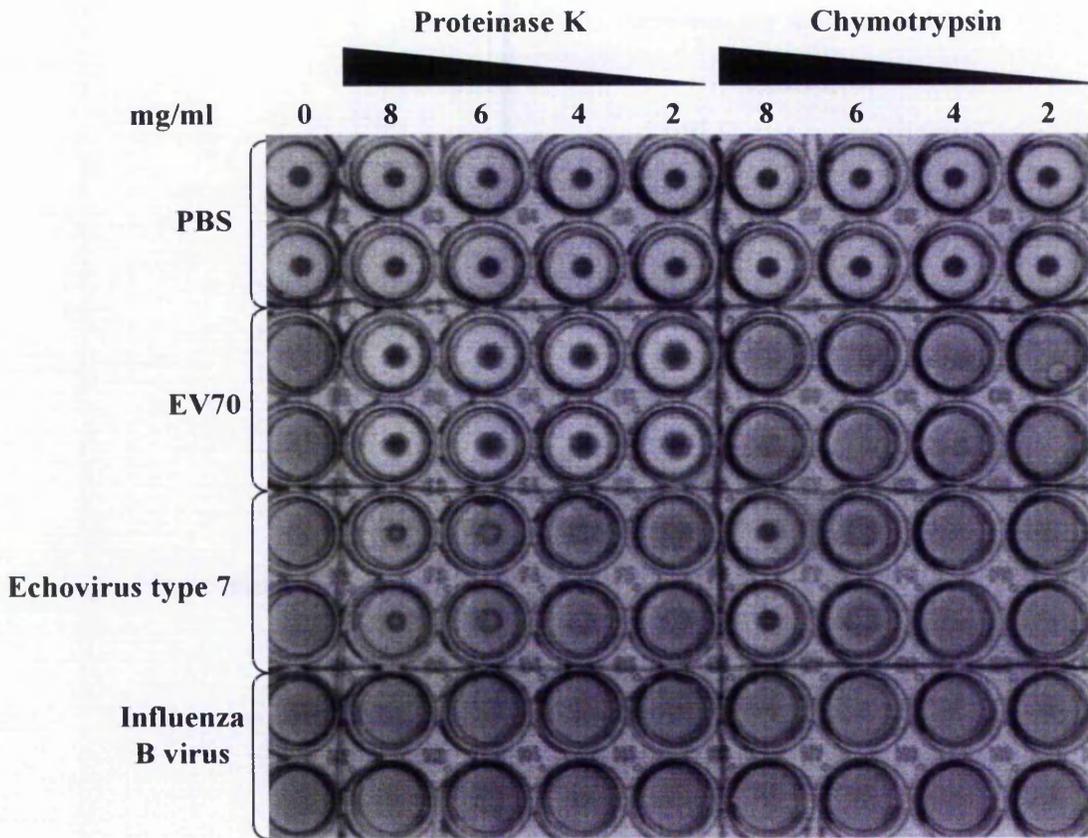
In summary, the above results suggest that the HA of RBC seen with EV70 is due to binding of the virus to sialic acid on a specific molecule, or possibly group of molecules, on the RBC surface and that this receptor molecule is a GPI-anchored, sialated,  $\alpha$ 2-3 linked glycoprotein.

#### **4.6 The EV70 receptor on MRC5 cells is not $\alpha$ 2-3 linked**

Having shown that EV70 binding to RBC is potentially mediated by a GPI-anchored, sialated,  $\alpha$ 2-3 linked glycoprotein, further work was aimed to investigate whether this was also the case for binding to MRC5 cells. Unfortunately lipases and



**Figure 4.12 Haemagglutination inhibition with PLC and PIPLC.** RBC were pre-treated with either PLC or PIPLC at the concentrations shown, and the assay conducted as in figure 4.11. Both PLC and PIPLC inhibit HA by EV70, but not by influenza B virus or CVA21. Concentrations of PLC of 0.1U/ml and above, resulted in RBC lysis and are not shown.



**Figure 4.13 Haemagglutination inhibition with proteinase K and chymotrypsin.** RBC were pre-treated with either proteinase K or chymotrypsin at the concentrations shown, and the assay conducted as in figure 4.11. Proteinase K inhibits HA by EV70 at all the concentrations tested, but HA by echovirus type 7 only at the higher concentrations. Chymotrypsin inhibits HA by echovirus type 7 at 8mg/ml, but has no effect on HA by EV70. HA by influenza B virus was not affected by either protease.

proteases were found to be highly toxic for MRC5 cells and it has therefore not been possible to assay the effect of these enzymes on EV70 binding to these cells.

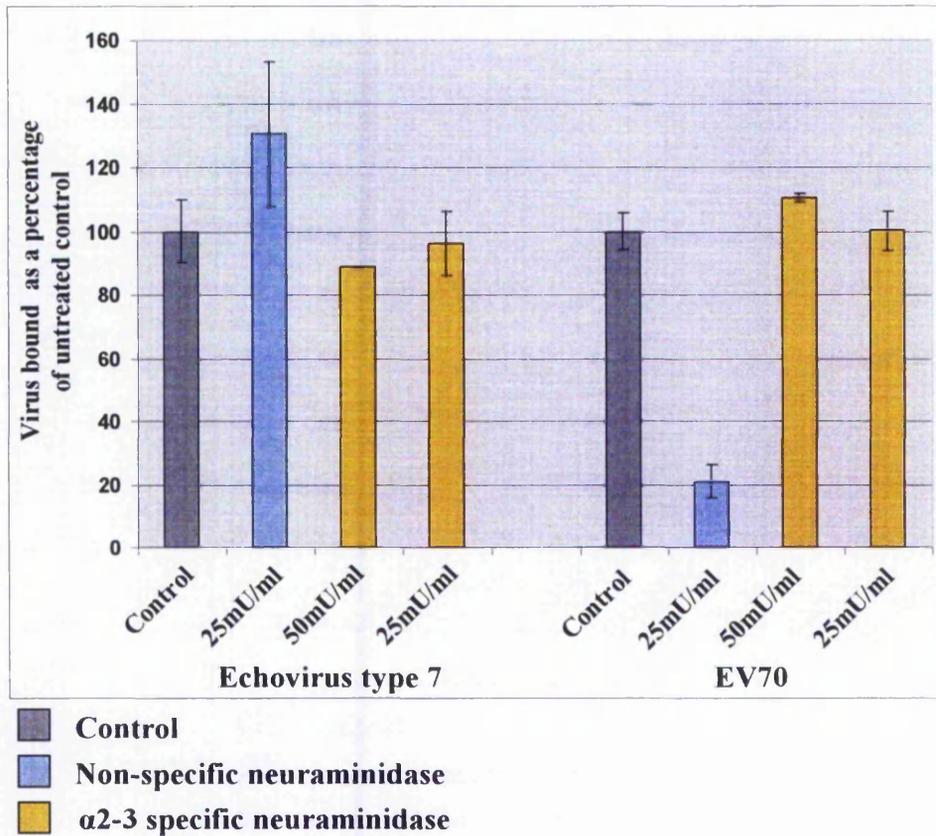
To determine whether EV70 has a binding specificity for  $\alpha$ 2-3 linked sialic acid on MRC5 cells, a binding assay was carried out on MRC5 cells pre-treated with either the non-specific or  $\alpha$ 2-3 specific forms of neuraminidase (figure 4.14). The concentrations used were 25mU/ml for the non-specific neuraminidase and 50 and 25mU/ml for the  $\alpha$ 2-3 specific neuraminidase.

Echovirus type 7 used as a control showed no reduction in binding with either neuraminidase, as expected. EV70, although showing the previously noted reduction in binding to MRC5 cells with the non-specific neuraminidase, did not show any reduction with the  $\alpha$ 2-3 specific form. It must be noted that further work requires to be done to establish whether the  $\alpha$ 2-3 specific neuraminidase is efficiently removing  $\alpha$ 2-3 specific sialic acid from the cell surface under these conditions. If confirmed, however, this result suggests a difference in the EV70 binding determinants on RBC and MRC5 cells. Several possibilities exist to explain this: it may be that in both cases the same GPI-anchored glycoprotein is being bound, but that the molecule is differently glycosylated in terms of sialic acid linkage in the two cell types; it may be that the virus is using an entirely different receptor on MRC5 cells from that bound on RBC; alternatively these results may be artefacts of the different assay formats used (HA inhibition and  $^{35}\text{S}$  virus binding). These assays are likely to vary in sensitivity and the lack of availability of other linkage-specific neuraminidases means that binding to  $\alpha$ 2-6 could not be directly assessed.

#### **4.7 EV70 binds to lipid raft domains on MRC5 cells**

Lipid rafts are detergent-resistant microdomains on cellular membranes that have important functions in endocytosis and cell signalling. They are also sites rich in certain lipids and proteins, and in particular are known to be enriched with GPI-anchored proteins (Brown and London, 1998). As a GPI-anchored protein has been implicated in the binding of EV70 to RBC (section 4.5), the association of the virus with lipid raft domains was investigated.

A membrane preparation was made from MRC5 cells pre-bound with either  $^{35}\text{S}$ -labelled EV70 or echovirus type 7, and floated on a step-wise sucrose gradient by ultracentrifugation. Using this method the raft fractions float to the interface of a four



**Figure 4.14 Viral binding to MRC5 cells after treatment with  $\alpha$ 2-3 specific neuraminidase.** MRC5 cells were pre-treated with non-specific neuraminidase or  $\alpha$ 2-3 specific neuraminidase at the concentrations shown and incubated with 20 000cpm of  $^{35}\text{S}$  labelled echovirus type 7 or EV70. After 1hr cells were washed and binding assessed using a scintillation counter. Binding is shown as a percentage of binding to untreated MRC5 cells. Error bars show the range of results over duplicate assays.

percent and twenty percent sucrose layer. Equal volume fractions were collected from the top of the centrifuge tube and analysed for viral content by scintillation counting and for the protein caveolin-1 by western blot. Caveolin-1 is known to be almost exclusively present in lipid raft domains of the cell membrane and therefore can be used as a marker for raft-containing fractions (Quest et al., 2004). Figure 4.15B shows western blots for fractions collected from membrane flotation gradients of MRC5 cells pre-bound with EV70 and echovirus type 7. In both gradients caveolin-1 was present in fractions two and three near the top of the gradient, as expected for raft fractions. Both echovirus type 7 and EV70 were found predominantly in the raft fractions (figure 4.15A). Echovirus type 7 binds DAF, a GPI-anchored, raft associated protein, and it is therefore unsurprising that the virus was largely located in the raft fractions. Likewise, the association of EV70 with raft domains is consistent with binding to a GPI-anchored protein, as suggested by the HA inhibition assays.

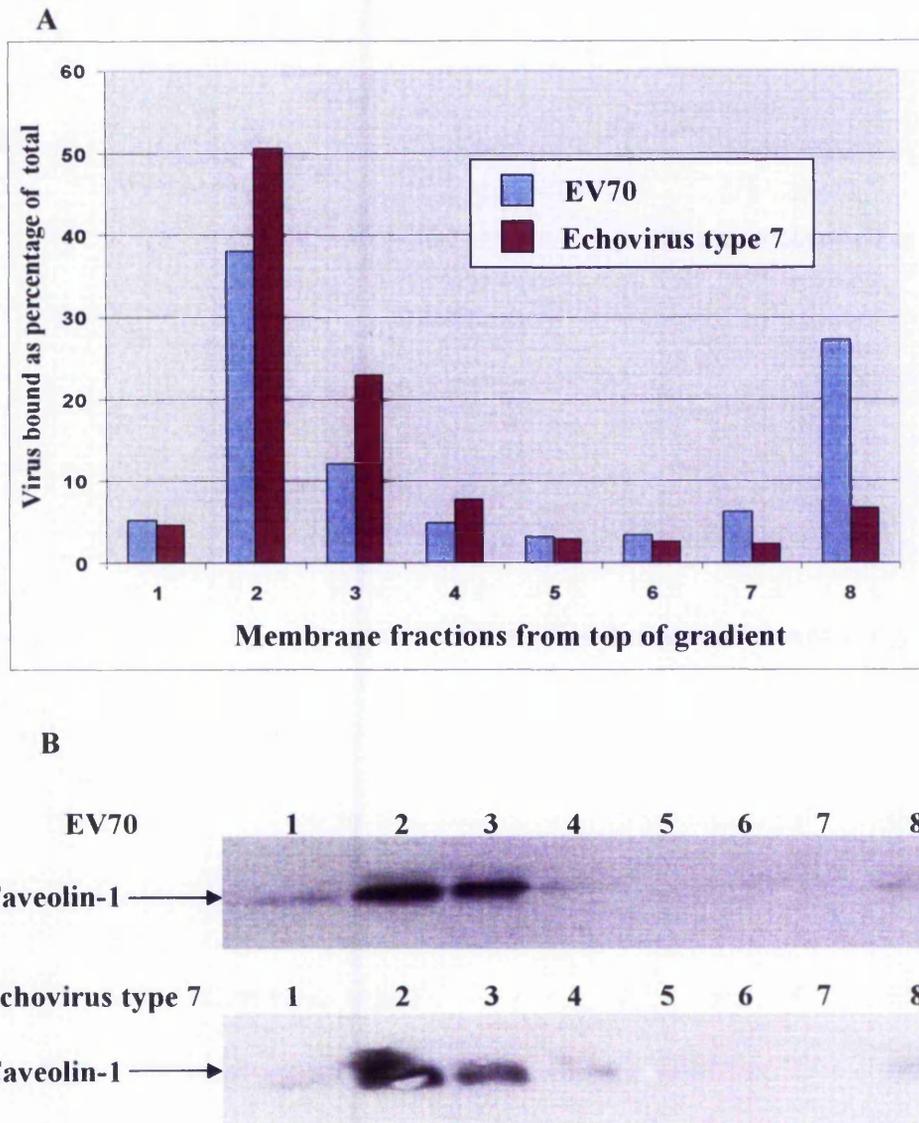
## **4.8 DISCUSSION**

The studies described in this chapter confirm the role of sialic acid in EV70 binding to RBC and binding to and infection of MRC5 cells. In contrast, DAF does not appear to be required for these processes. The studies further show that on the RBC surface EV70 binds to an  $\alpha$ 2-3 linked sialated GPI-anchored glycoprotein. Although the requirement for  $\alpha$ 2-3 linked sialic acid has not been demonstrated in viral binding to MRC5 cells, it has been shown that EV70 binds primarily to a protein associated with lipid rafts. In addition, it has been demonstrated that EV70 binds to a range of non-human cell lines in a sialic acid-dependant manner, but that this does not result in fully productive infection.

### **4.8.1 The role of sialic acid in EV70 binding and infection**

The importance of sialic acid in EV70 binding, first reported by Utagawa et al (1982), has been confirmed by the current studies. It has been clearly demonstrated that sialic acid is important for the HA of RBC, and binding and infection of MRC5 cells. Furthermore binding to HeLa and RD cells is also dependant on sialic acid.

Binding of EV70 to non-primate mammalian cell lines (CHO and RK13) and XTC-2 (Xenopus) cells has been demonstrated, although it does not cause significant infection of these cells. The level of binding is reduced by neuraminidase, confirming



**Figure 4.15 Raft fractionation and viral binding assay.** MRC5 cells were incubated with 200 000cpm of  $^{35}\text{S}$  labelled EV70 or echovirus type 7. After 1hr cells were washed, membrane components purified and raft flotation carried out on a stepwise sucrose gradient as detailed in section 2.5.11. Fractions were collected and binding assessed using a scintillation counter, and the fractions containing the raft marker caveolin-1 determined by western blot **A**. EV70 and echovirus type 7 present in each membrane fraction as a percentage of the total virus detected across each gradient. Both viruses are mainly contained in fraction 2, and to a lesser extent fraction 3. **B**. Western blots of EV70 and echovirus type 7 containing gradients, probed with antibody to caveolin-1. Numbers above the lanes refer to fractions counted from the top of the gradient. Caveolin-1 is primarily detected in fractions 2 and 3.

that attachment to these cells is sialic acid-dependant. It is unusual for picornaviruses to bind to cells that are not closely related to the viral host; human picornaviruses generally interact only with primate cells, perhaps reflecting the fact that most bind protein receptors. The broad range of cell types bound by EV70 can be explained by the ubiquitous nature of glycosylation and in particular sialidation. Sialic acid can be found on all vertebrate species and some invertebrates (e.g. echinoderms). There is debate regarding its presence in other invertebrates, but there are reports of its presence particularly in non-adult cells such as drosophila larvae; this would explain the binding seen on C6/36 cells (mosquito larvae cell line). The observed binding of EV70 to sialic acid on the non-human cell lines does not result in significant yield of virus, however this could be due to blockage at several points in the viral life cycle, including translation and replication. The use of a ubiquitous receptor such as sialic acid could certainly have facilitated a cross-species jump, such as that postulated for the emergence of EV70 as a human pathogen.

#### **4.8.2 The role of DAF in EV70 binding and infection**

These studies have demonstrated that DAF has no significant role in cellular binding and infection by EV70. This has been investigated using a variety of approaches and reagents. Firstly, soluble DAF does not prevent the HA of RBC by EV70. This contains all four domains of DAF, including the most membrane distal (SCR 1), to which EV70 has been reported to bind, and effectively inhibits HA by CVA21, the other SCR 1 DAF binding enterovirus. Secondly, a polyclonal antibody against DAF has no effect on the ability of EV70 to bind to or infect MRC5 cells. Although there is no formal data to show that this antibody binds to SCR 1 of DAF, indirect evidence comes from its ability to block binding by CVA21. Thirdly, using XTC-2 and CHO cell lines engineered to express human DAF, it has been shown that the ability of EV70 to bind or infect these cells is not affected by the presence or absence of human DAF on the cell surface. Indeed treatment of CHODAF cells with neuraminidase consistently reduces EV70 binding by greater than 90%, with no evidence of significant residual binding to human DAF.

The initial studies showing DAF binding were carried out in the laboratory of Dimock and colleagues using HeLa cells (Karnauchow et al., 1996). Binding was blocked by monoclonal antibodies to DAF. In addition they reported that viral binding to NIH3T3 cells (mouse fibroblasts) was increased 2-3 fold upon expression of human

DAF, and the DAF expressing NIH3T3 cells were found to support EV70 growth at low level, while the parent cells were non-permissive. However, more recently the same group have published data showing that DAF is not required for the attachment to, nor infection of, a number of human leukocyte cell lines (Haddad et al., 2004). For example neither EV70 binding nor infection of U-937 cells (derived from a human histiocytic lymphoma) were reduced by monoclonal DAF antibodies known to block viral binding to HeLa cells. This was the case, despite the fact that U-937 cells were shown to express comparable levels of DAF to HeLa cells. The authors hypothesised that the amount or nature of the sialic acid receptor on these cell lines was such that the interaction of EV70 with DAF was circumvented. However the authors also stated that neuraminidase treatment of these leukocyte cell lines completely abolished viral binding, indicating that DAF is not only not required for EV70 binding, but, that even in the absence of sialic acid, EV70 is not capable of binding to DAF on these cells. The group have previously shown that sialidation of DAF is not necessary for binding (Alexander and Dimock, 2002).

Therefore, although initially, the previously published data on DAF involvement and the current studies appear contradictory, this is not the case. The work presented here taken together with the previous studies actually indicates that the primary component of EV70 binding and infection is sialic acid-dependant and that DAF, if involved, is relevant only in a limited number of cell types. Indeed HeLa cells are the only human cell line in which DAF binding has been demonstrated and even here binding is still sialic acid-dependant (Alexander and Dimock, 2002). It is also of importance to consider the relevance of DAF binding to EV70 infection of the human conjunctiva. Although DAF is known to be expressed on the human conjunctiva it is also present in large amounts in a soluble form in the tear film (Medof et al., 1987). If the virus bound DAF it might be expected that significant levels of soluble DAF might block binding of the virus to cell surface expressed DAF.

#### **4.8.3 Further characterisation of the EV70 receptor**

It has been shown using HA inhibition studies that the RBC receptor of EV70 is a sialated GPI-anchored glycoprotein. This protein shows different chemical properties to DAF, another GPI-anchored glycoprotein. HA by echovirus type 7, a known DAF binding virus, is inhibited by chymotrypsin, presumably due to proteolytic cleavage of the receptor. In contrast, chymotrypsin has no effect on EV70-mediated HA, and the

EV70 receptor is also more sensitive to Proteinase K. Additionally DAF on RBC is known to be poorly cleaved by PIPLC due to a fatty acid substitution in its GPI anchor and therefore echovirus type 7 HA is not inhibited by this enzyme, whereas EV70 HA is strongly inhibited by PIPLC.

These results are interesting as GPI-anchored proteins are predominantly found concentrated at specific cell membrane locations, lipid rafts, and have important roles in both cell signalling and endocytic pathways (this is discussed more fully in chapter five). Thus the variation in DAF requirement by EV70 may reflect conservation during culture adaptation of the association with lipid rafts and specific internalisation and cell signalling pathways, rather than of the specific receptor.

Although the HA inhibition results show the RBC receptor to be a sialated GPI-anchored glycoprotein, differentiated RBC are enucleate cells that the virus cannot infect. Therefore these results need to be corroborated in eukaryotic cells that are permissive for viral infection. The proteases and lipases used in the RBC assays have proven toxic for MRC5 cells, and so it has not yet been possible to fully compare the HA results to binding and infection of MRC5 cells. However, it has been shown that EV70 binding in MRC5 cells is localised to lipid rafts, a result consistent with binding to a GPI-anchored protein.

The HA inhibition assays also suggested that RBC binding was mediated via sialic acid with an  $\alpha$ 2-3 linkage. This used  $\alpha$ 2-3 specific neuraminidase that inhibited HA. However, no other linkage specific neuraminidases are available to assess whether  $\alpha$ 2-3 is the only sialic acid linkage involved in HA. HA assays are designed to detect HA inhibition by as little as four-fold and so additional binding to other forms of sialic acid such as  $\alpha$ 2-6 linked sialic acid cannot be ruled out. Dimock and colleagues (Nokhbeh et al., 2005) have recently also reported that EV70 recognises  $\alpha$ 2-3 linked sialic acid on human corneal epithelial cells and U-937 cells (a histiocytic lymphoma cell line). The group used  $\alpha$ 2-3 specific neuraminidase, but also blocked virus binding to cells with specific lectins known to bind either  $\alpha$ 2-3 or  $\alpha$ 2-6 linked sialic acid.

Use of an  $\alpha$ 2-3 linked sialated receptor would be consistent with other viruses known to primarily infect the conjunctiva e.g. H7N7 influenza virus and adenovirus (Arnberg et al., 2000; Olofsson et al., 2005; Skehel and Wiley, 2000), and suggests the interesting hypothesis that  $\alpha$ 2-3 linked sialic acid is responsible for the conjunctival tropism of these viruses (Olofsson et al., 2005). One function of the conjunctiva is the secretion of heavily glycosylated proteins known as mucins, which are also found as

membrane bound proteins and form an important part of the tear film (Varki, 1999). Unfortunately very little information is available on sialic acid linkages in ocular mucins or elsewhere on the conjunctiva. Studies looking at lacrimal duct epithelium have detected both  $\alpha$ 2-3 and  $\alpha$ 2-6 linked sialic acid (Paulsen et al., 1998; Thale et al., 2001). The lacrimal duct epithelium is continuous with the conjunctival epithelium, but differs functionally. Both  $\alpha$ 2-3 and  $\alpha$ 2-6 linked sialic acid have also been detected in separate studies of ocular secreted mucins (Argueso et al., 1998; Aristoteli and Willcox, 2006).

Surprisingly, given these results, the use of an  $\alpha$ 2-3 specific neuraminidase did not affect the binding of EV70 to MRC5 cells, even at high concentration. To confirm this result it will be important to establish that the  $\alpha$ 2-3 specific enzyme is efficiently removing  $\alpha$ 2-3 linked sialic acid from the surface of MRC5 cells. There are a number of explanations for these results if they are confirmed. It is possible that the virus binds to an entirely different molecule on MRC5 cells than on RBC, or the same molecule with a different glycosylation pattern. However, it is also possible that these results could be a consequence of the different assay formats used on RBC (HA inhibition) and MRC5 cells ( $^{35}$ S viral binding assay), and that in fact the virus is capable of recognising both linkage forms. The recent work from Nokhbeb et al (2005) provides evidence that there may be major differences in the EV70 receptor requirement between cell types. Their results indicate that on a human corneal cell line the receptor is an  $\alpha$ 2-3 linked O-glycosylated, non-GPI-anchored glycoprotein, while on U-937 cells an  $\alpha$ 2-3 linked glycolipid was implicated (Nokhbeb et al., 2005), while on HeLa cells the same group's previous work has shown involvement of a GPI-anchored glycoprotein (DAF) (Karnauchow et al., 1996). It is possible that the virus has specificity for a particular glycosylation pattern, which might be present on different proteins or lipids in different cell types. As the glycosylation patterns seen in different species are much more conserved than protein sequence (Varki, 1999), this would tie in well with the observation that EV70 binds well to non-human and non-mammalian cell lines. If the MRC5 result is confirmed, and the virus is not primarily binding to  $\alpha$ 2-3 linked sialic acid in these cells, this might then suggest that although sialic acid is important to viral binding its orientation may be unimportant or may vary in different cell types depending on the underlying molecule. Indeed the influenza virus H1 haemagglutinin can bind to both  $\alpha$ 2-3 and  $\alpha$ 2-6 linked sialic acid, due to flexibility in the position of a specific residue (Glutamine 226) in the viral haemagglutinin (Skehel and Wiley, 2000).

Similarly human influenza virus can effectively replicate in transgenic mice lacking  $\alpha$ 2-6 linked sialic acid (Glaser et al., 2007).

Further studies will be needed to unravel this complex picture. Centering future research on the interaction of the EV70 and the conjunctiva could circumvent the problems engendered by the variation of receptor usage between cell lines. There is currently no commercially available conjunctival epithelial cell line. Although Nokhbeh et al (2005) have looked at infection of a human corneal cell line; this is likely to have different properties to conjunctival cells, particularly in relationship to glycosylation patterns and mucin expression. Several groups are currently attempting to create conjunctival cell lines maintaining the features of conjunctival differentiation (De Saint Jean et al., 2004; Diebold et al., 2003; Gipson et al., 2003), such a cell line would be extremely valuable in research on agents causing conjunctivitis, such as EV70.

#### **4.8.4 Tissue culture adaptation of the viruses used in studies of EV70 receptors**

Research on the EV70 receptor has to date been primarily carried out in the laboratory of Professor K Dimock, and they have reported a variable DAF requirement depending on tissue-type using the reference EV70 strain, J670/71 (Haddad et al., 2004). The work in this study, also using J670/71, has shown no DAF involvement for EV70 binding to RBC or MRC5 cells, and no evidence of the virus binding to human DAF on CHO and XTC-2 engineered to express it. It is possible that further studies using additional cell lines with the virus used in the current work might demonstrate a role for DAF, consistent with tissue-specific variation in receptor usage. This cannot be the entire explanation, however, as there are several areas of direct conflict between the previously reported studies by Dimock and colleagues (Alexander and Dimock, 2002; Haddad et al., 2004; Karnauchow et al., 1996) and those presented here. Firstly the virus used by Dimock and colleagues is cytopathic in HeLa cells, which is not the case for the virus used here (although this could be explained by differences in HeLa cell lineages), secondly the virus used here binds non-human cell lines to levels equivalent to MRC5 cells. In particular the virus binds well to CHO cells and NIH3T3 cells (data not shown), while the previous reports suggested very poor binding to these cell lines except when engineered to express human DAF. A further difference exists between the current data and data from Yoshii et al (1977). This early paper provided evidence suggesting that EV70 J670/71 growth was comparable between HeLa cells and RK13 (rabbit) cells, while here only very limited growth was observed in RK13 cells

(although again this could be explained by differences in cellular lineages rather than the viruses). These apparent data conflicts may be explained by differences in passage history of the J6/70 EV70 reference strain used here and that used in the other studies. Yoshii et al., used virus passaged in primary cynomolgous monkey kidney cells and that used by Dimock and colleagues was passaged in LLC-MK<sub>2</sub> rhesus macaque kidney cells, a continuous cell line. No information is given in these studies on the earlier passage history of the viruses, nor whether the passage history of virus used was standardised. In the current studies the virus was grown in MRC5 primary human embryonic lung fibroblast cells, and used after a standard five passages in MRC5 cells. Therefore although all studies have used the reference strain of EV70, J670/71, there are differences in the more recent passage history which may well have been responsible for adaptation of the viral receptor usage and could explain differences in the data obtained. Indeed, as reported in chapter three, there are sequence differences in the capsid-encoding VP1 sequence of the virus between that used in the current study and the previously published sequence for EV70 J670/71, which might be the molecular basis of such adaptation.

Studies along the lines of those discussed above could eventually provide an exact molecular profile of what the EV70 reference strain is binding to on a particular cell line. However, given the possibility of confounding results due to the adaptation of the virus in culture, an approach using virus that has not been extensively cultured, would present a much more clinically relevant picture. This could be done using the strategy outlined in chapter three of inserting capsid sequence from a clinically isolated virus directly into an infectious EV70 clone.

## CHAPTER 5

### Investigation of Enterovirus type 70 Entry into the Cell

For productive infection to occur, the viral genome must enter the cell. Attachment of the virus to its cellular receptor is only the first step in this process. Following attachment the virus must have a mechanism to allow the virus or its genome to reach the site of viral replication. For picornaviruses the genome must be delivered to the cytoplasm, either by crossing the plasma membrane or the membranes of internal cellular structures. Although the term 'viral entry' will be used here, it is currently unclear for EV70 and many other viruses, whether the intact virion, or the viral nucleic acid alone, enters the cell.

Entry to the cytoplasm may occur directly via the formation of a pore within the plasma membrane, as seems to be the case for poliovirus (Hogle, 2002; Rossmann et al., 2000), or may occur via the utilisation of the host cell's endogenous endocytic processes, such as the clathrin-mediated or caveolar pathways. Both of the latter two pathways have been implicated as the entry routes for different picornaviruses; CVB3, human rhinoviruses type 2 and 14, parechovirus type 1 and foot and mouth disease virus are believed to use clathrin pathways (Berryman et al., 2005; Chung et al., 2005; DeTulleo and Kirchhausen, 1998; Joki-Korpela et al., 2001; Snyers et al., 2003), while echovirus type 1 and coxsackie B viruses are believed to enter via caveolae (Coyne and Bergelson, 2006; Pietiainen et al., 2004). However, both clathrin and caveolar routes of cell entry result in the virus being contained within a cytoplasmic vesicle (endosome and caveosome respectively), which the virus must exit to enable translation and replication to occur.

Some viruses require a pH-dependant step for entry to the cytoplasm, particularly in viruses entering via the clathrin route, where they pass through the acidic environment (pH 5.5-6) of the late endosome. Such a pH dependence has been best characterised for influenza virus where low pH triggers conformational changes in the viral haemagglutinin resulting in fusion of the viral and late endosomal membranes. Non-enveloped viruses have also been shown to be dependant on endosomal acidification, such as some rhinoviruses and CVB3 (Chung et al., 2005; Nurani et al., 2003). In these cases exit from the endosome may involve lysis of the endosome or the formation of a membrane pore analogous to that of poliovirus.

Early understanding of endocytic processes was relatively simplistic, in comparison to the increasing complexities being revealed by current research. Multiple routes of cell entry exist. In addition to the well characterised clathrin and caveolar routes, there are many other more poorly understood pathways (Marsh and Helenius, 2006; Pelkmans and Helenius, 2003; Sieczkarski and Whittaker, 2002a), and even within clathrin and caveolar pathways there is variation, for example cargo entering cells via the same general route, can be found in distinct cytoplasmic structures or locations (Marsh and Helenius, 2006). One important feature of cell entry pathways which is now emerging, is the crucial involvement of multiple signal transduction pathways (Coyne and Bergelson, 2006; Pelkmans et al., 2005). Many viruses enter cells using proteins which cluster in lipid raft domains, which are now known to play key roles in both cellular internalisation pathways and cell signalling (Brown and London, 1998). These domains, once believed to be primarily linked to caveolar entry, are now believed to have a more general role in entry pathways, including clathrin-mediated endocytosis (Anderson, 1998; Stoddart et al., 2002). Additionally viral entry pathways involve elements of the cellular cytoskeleton such as actin and tubulin, although the precise role of these is often unclear (Apodaca, 2001; Coyne and Bergelson, 2006; Lakadamyali et al., 2003; Stuart et al., 2002).

There is not a single common route for enterovirus entry. It may be that this reflects the variety of different receptor and co-receptor molecules used by the different viruses within the genus. Binding to a particular receptor, in a particular site might dictate not only the route of viral entry, but also trigger specific down-stream signalling events. As one of only two human enteroviruses with a sialic acid component to its receptor, EV70 is of particular interest to study in this respect. The other enterovirus where sialic acid has been implicated is the less well studied EV68, now known to be a strain of rhinovirus type 87 (Blomqvist et al., 2002; Uncapher et al., 1991).

To date there has been no published work regarding the route by which EV70 gains entry to the cell following binding, nor any information relating to the importance of pH, signalling cascades, or the cytoskeleton during this process. EV70 has been classified as an enterovirus partially on the basis of acid stability (Kono et al., 1972). Virus from the initial EV70 epidemics and the reference strain J670/71 were found to be stable at pH3 (Kono et al., 1972). EV70 is unusual, however, in that it is only very rarely isolated from faeces in infected patients (Higgins, 1982); in contrast other enteroviruses can be easily isolated from the faeces after surviving passage through the

acidic environment of the stomach. Interestingly, a more recent isolate was found to be acid sensitive at pH3 (Oberste et al., 2004), suggesting that the presumed acid stability of this serotype may not be universal.

One striking feature of EV70 is its ability to bind to multiple non-primate cell lines (see section 4.4), even though these do not support fully productive infection. Although these cells may be non-permissive for viral replication, the blockage might occur at the stage of viral entry. If the latter, these cell lines may provide a useful resource for determining the precise molecular requirements for the entry of EV70.

### **Chapter Outline**

This chapter describes a series of experiments to determine the route by which EV70 enters the cell. This involved the use of a range of potential inhibitory agents to investigate the entry pathway used by the virus and the importance of pH, cellular signalling and the cytoskeleton in the process. In addition the stage at which EV70 infection in non-primate cells is blocked was investigated, using the EV70 subgenomic replicon and full-length infectious clone.

## **5.1 Clathrin-mediated endocytosis is important for EV70 cellular entry**

A number of chemicals exist which can disrupt entry pathways (Sieczkarski and Whittaker, 2002a). Although often not completely specific for a particular entry route, taken together these represent a good method for gaining an initial indication of the route of entry used by a particular virus (Pietiainen et al., 2004; Stuart et al., 2002). Results from such analyses can identify pathways for more targeted investigation, using dominant negative mutant proteins, which specifically inhibit steps in clathrin or caveolar entry pathways, or direct observation, perhaps using confocal microscopy approaches.

A panel of four treatments were chosen; chlorpromazine, brefeldin A, methyl- $\beta$ -cyclodextrin and the combination of nystatin & progesterone. Chlorpromazine is an inhibitor of clathrin-mediated endocytosis, it acts by causing the accumulation of clathrin lattices on endosomal membranes and preventing clathrin pit assembly on the

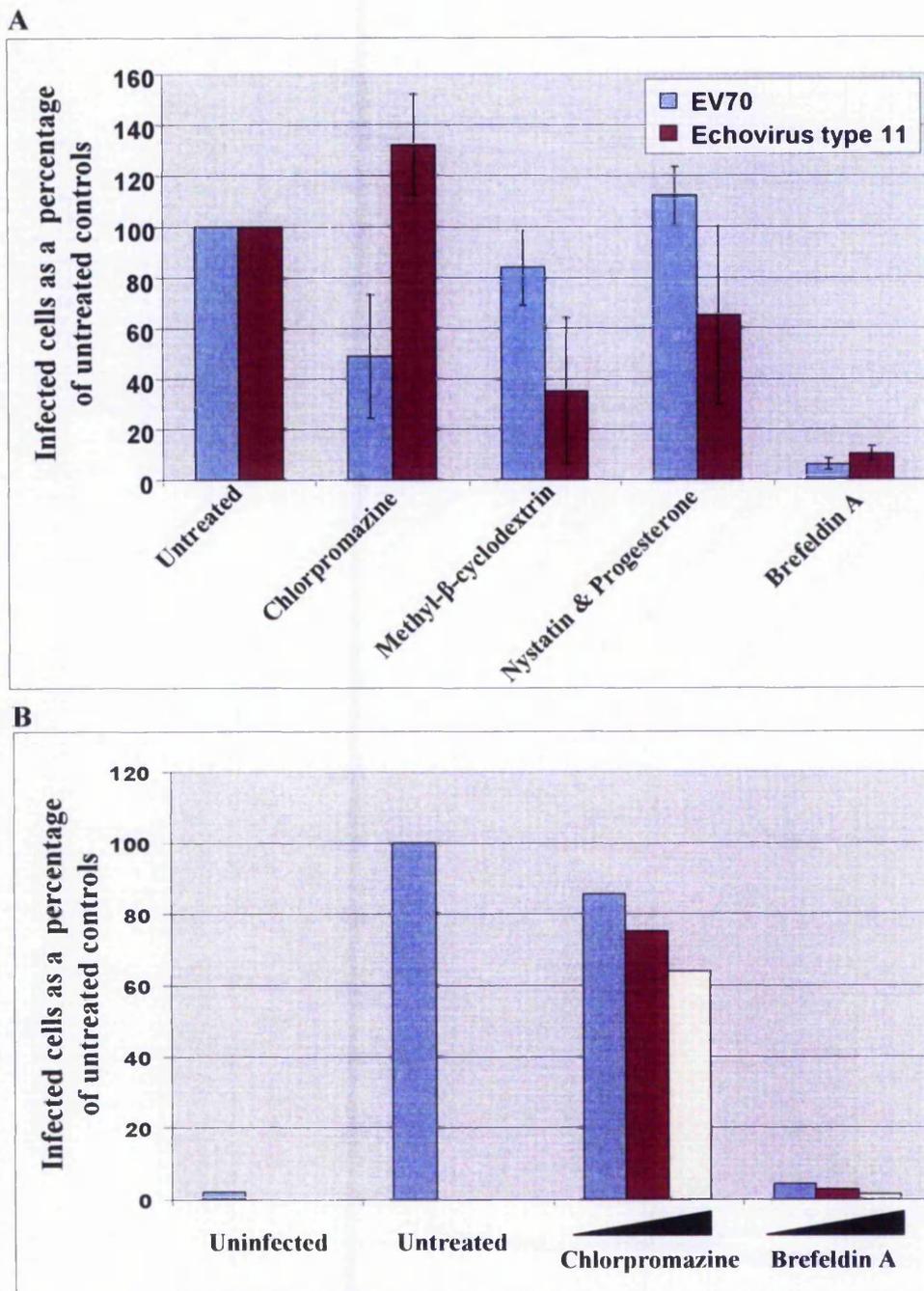
cell surface (Wang et al., 1993). Brefeldin A has been used as an inhibitor of caveolar endocytosis (Dinter and Berger, 1998; Richards et al., 2002), and is also known to cause disruption of the golgi (Dinter and Berger, 1998). Methyl- $\beta$ -cyclodextrin and the combination of nystatin & progesterone are commonly used methods for disrupting lipid rafts. Methyl- $\beta$ -cyclodextrin causes cholesterol redistribution (Hailstones et al., 1998), while nystatin and progesterone cause the sequestration of cholesterol and inhibition of cholesterol synthesis respectively (Simons and Toomre, 2000). Previously disruption of lipid rafts has been used as a method for preventing caveolar entry, however it is now established that lipid rafts can also be important for clathrin-mediated endocytosis (Puri et al., 2005; Signoret et al., 2005; Stoddart et al., 2002). EV70 has been shown to associate with lipid rafts on receptor binding (section 4.7), however this does not prove a functional raft requirement.

### **5.1.1 Chlorpromazine and brefeldin A reduce EV70 infectivity**

The effect on EV70 entry of the pre-treatment of MRC5 cells with chlorpromazine, methyl- $\beta$ -cyclodextrin, brefeldin A and the combination of nystatin & progesterone was investigated. Toxicity of these agents in MRC5 cells was first assessed using a range of concentrations, based on those shown to be effective in previous studies (Pietiainen et al., 2004; Stuart et al., 2002). This was done by visual inspection of the treated cells and by assessing the number of viable cells using trypan blue exclusion. Both methyl- $\beta$ -cyclodextrin and nystatin showed significant toxicity in MRC5 cells at the concentrations used in the previous studies (10mM and 25mM respectively), and so were retested at lower concentrations. In contrast, chlorpromazine and brefeldin A showed no toxicity at the doses used in previous studies (6 $\mu$ M and 8 $\mu$ M respectively), and were tested again at higher concentrations. These differences are likely to be due to the relative sensitivities of MRC5 cells in comparison to the cell lines used in other studies. The highest concentration of each agent for which no toxicity was observed on repeat testing, were 14 $\mu$ M chlorpromazine, 3mM methyl- $\beta$ -cyclodextrin, and 13 $\mu$ M nystatin with 32 $\mu$ M progesterone, and these concentrations were used in subsequent assays of viral entry. Initial assays suggested a profound effect of brefeldin A on viral infection, even at very low concentrations, and so in this case a lower working concentration of 0.4 $\mu$ M was used. Cell culture medium was tested to ensure that pH was not affected by the addition of these compounds.

Monolayers of MRC5 cells were treated in duplicate with agents at the above concentrations, prior to infection with EV70 or echovirus type 11 (strain 207). This strain of echovirus type 11 has previously been investigated using similar methodology and is believed to enter via caveolae (Stuart et al., 2002). The adsorption and incubation steps of infection were carried out in the continued presence of the chemicals. At six hours post-infection cells were stained and a flow cytometry-based infectivity assay used to determine the proportion of infected cells. This had the advantage over viral titration-methods of excluding confounding effects on viral egress. An example of flow cytometry data from one representative assay is provided in appendices 1 and 2 (histogram plots). The results from individual assays are detailed in appendix 3. Figure 5.1A shows the mean results with standard deviations, based on a minimum of three assays carried out on separate occasions. Results are expressed as a percentage of the infected cells in the untreated controls within individual assays. There was significant variation in some of the results between separate assays, which may have been partly due to the fact that MRC5 cells are a primary cell line and small differences in confluence, or in the number of passages undergone, might have affected the responsiveness of the MRC5 cells to the agents used. Attempts were made to minimise this effect by using cells well before senescence and at a consistent confluency. Despite this variation, a number of clear results could be observed.

Treatment of MRC5 cells with the clathrin pathway inhibitor chlorpromazine reduced EV70 infection by an average of 50% (figure 5.1A). The use of a range of chlorpromazine concentrations confirmed this to be a dose related reduction (figure 5.1B), and suggested a role for clathrin-mediated endocytosis in EV70 entry. In contrast chlorpromazine caused an increase in echovirus type 11 infection, consistent with previous observations that this virus does not use the clathrin route (Stuart et al., 2002). Increased activity of caveolar entry pathways may be a consequence of the blockage of other entry routes, potentially explaining the increased infection seen with echovirus type 11. Brefeldin A is used to inhibit caveolar entry pathways, and did cause a reduction in infection by echovirus type 11 of over 90% (figure 5.1A), however a similar dramatic reduction was seen for EV70 infection, with a dose response assay showed the effect of brefeldin A on EV70 over a range of concentrations (figure 5.1B). This result is not consistent with the reduction seen in EV70 infection using chlorpromazine. However, brefeldin A is also known to cause disruption of the golgi and may be



**Figure 5.1 Viral infection in MRC5 cells treated with inhibitors of cell entry.** **A** Flow cytometry-based infectivity assay showing cumulative data from several experiments with standard deviations (see appendix 5). MRC5 cells were pre-treated with 14 $\mu$ M chlorpromazine, 3mM methyl- $\beta$ -cyclodextrin, 13 $\mu$ M nystatin with 32 $\mu$ M progesterone or 0.4 $\mu$ M brefeldin A. Cells were infected with virus at an m.o.i. of 3, incubated for 6 hours in the presence of the chemical and the proportion of infected cells assessed by flow cytometry. **B** Flow cytometry-based infectivity assay showing dose response of EV70 infection to increasing concentrations of chlorpromazine (at 1 $\mu$ M, 7 $\mu$ M and 14 $\mu$ M) and brefeldin A (at 0.1 $\mu$ M, 0.4 $\mu$ M and 2 $\mu$ M).

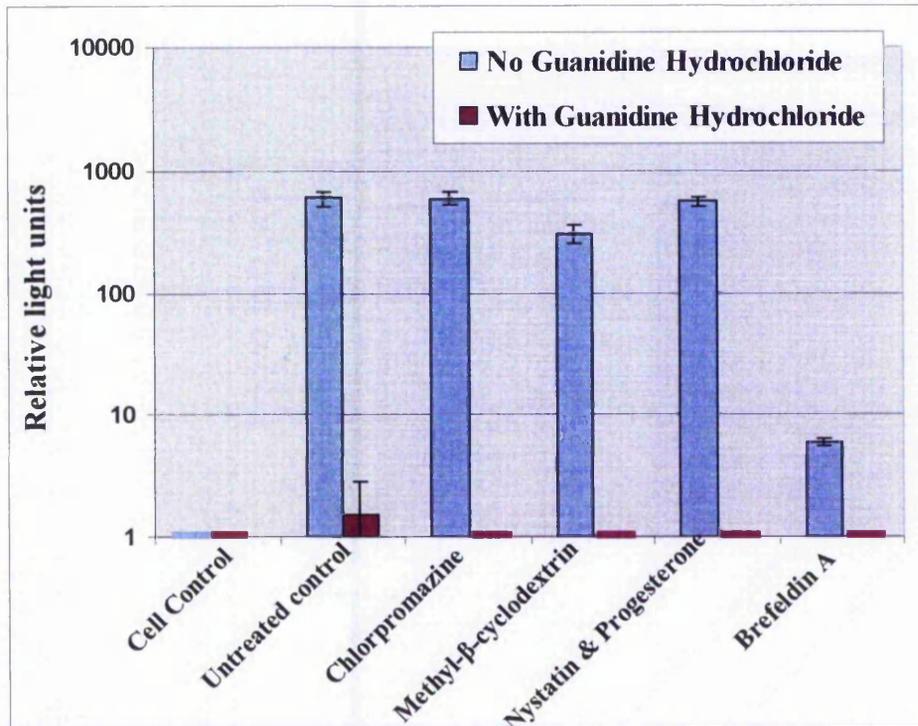
affecting viral replication rather than viral entry; this was assessed further in section 5.1.2.

Echovirus type 11 infection was reduced in cells treated with both methyl- $\beta$ -cyclodextrin and the nystatin & progesterone combination (figure 5.1A), although for nystatin & progesterone the error bar reaches the level of the untreated control. In contrast there was no effect of nystatin & progesterone on EV70 infection, and a small reduction, if any, with methyl- $\beta$ -cyclodextrin. However, due to toxicity, both treatments were carried out using lower concentrations than those used in previous studies, and indeed in those studies greater effects on echovirus type 11 infection were observed. Therefore further work is required to assess the extent to which these concentrations are disrupting lipid rafts in MRC5 cells. Most methods to assess disruption of lipid rafts rely on the secondary effect of the disruption of caveolae and the caveolar entry pathway. One method which has been used, is to assess the effect of treatments on the entry of cholera toxin B, which is known to primarily enter via caveolae and lipid rafts (Pietinen et al., 2004). Although the results of the studies presented in this section are inconclusive regarding the functional role of EV70 binding to lipid rafts, the previous results showing that EV70 does associate with lipid rafts (section 4.7) suggest that they may well be important.

### **5.1.2 Brefeldin A reduces EV70 infection by inhibition of viral replication, while chlorpromazine acts at the level of cell entry.**

The reduction in EV70 infection seen with both chlorpromazine and brefeldin A could be due to effects of these agents on aspects of the viral life cycle other than entry, in particular effects on viral replication or binding.

The subgenomic replicon, pLuc70, was used to assess the effect of chlorpromazine, methyl- $\beta$ -cyclodextrin, nystatin & progesterone, and brefeldin A on EV70 replication. RNA was transcribed from the replicon and transfected into MRC5 cells pre-treated at the concentrations used in the previous assays. After 6 hours incubation in the continued presence of the agents, luciferase activity was assayed (figure 5.2). Chlorpromazine, methyl- $\beta$ -cyclodextrin, and nystatin & progesterone treated cells exhibited luciferase levels comparable to untreated cells, demonstrating that these agents do not significantly effect EV70 replication. However, replication was reduced by two  $\log_{10}$  in brefeldin A treated cells. Therefore, although an effect of



**Figure 5.2 EV70 replication in MRC5 cells treated with potential inhibitors of cell entry.** MRC5 cells were pre-treated with inhibitors at the concentrations detailed in figure 5.1A, and transfected with 2 $\mu$ g of RNA transcribed from pLuc70. Luciferase was assayed after 6 hours incubation in the continued presence of the chemicals and in the presence and absence of the replication inhibitor guanidine hydrochloride. Error bars show the range of results over duplicate assays.

brefeldin A on EV70 entry cannot be excluded, the reduction in infection seen can be fully explained by an effect on viral replication.

A binding assay using  $^{35}\text{S}$  radiolabelled EV70 was used to assess EV70 binding to cells treated with chlorpromazine (figure 5.3). Levels of binding were very similar for both untreated and chlorpromazine treated MRC5 cells, suggesting that this treatment did not affect the level of EV70 receptor expression or viral binding.

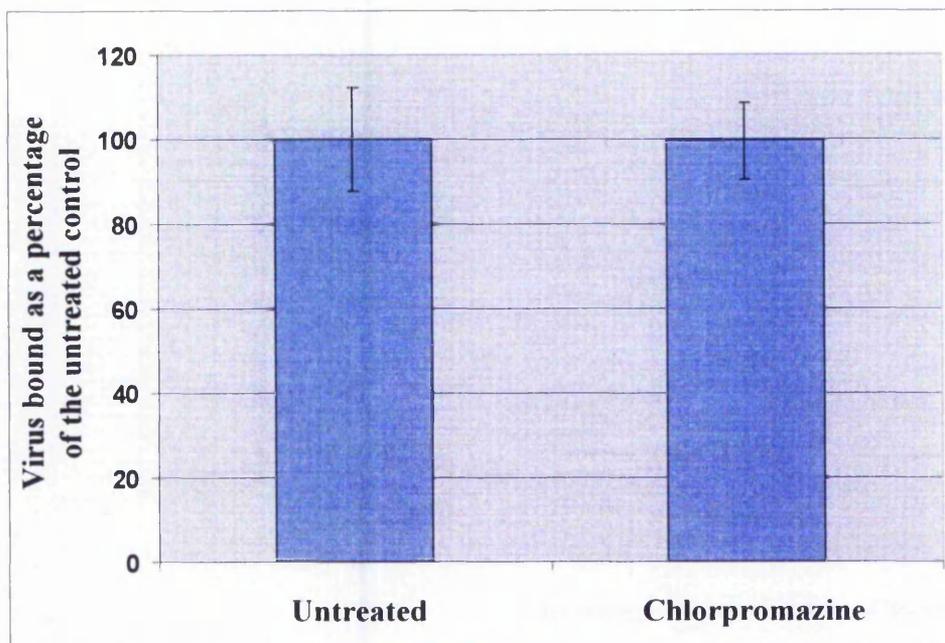
Chlorpromazine is therefore not affecting either EV70 binding or replication. The flow cytometry-based assay used does not depend on post-replication events for the detection of viral infection and therefore the reduction in EV70 infection caused by chlorpromazine must be occurring at the level of viral entry to the cell.

### **5.1.3 Chlorpromazine is inhibiting clathrin-mediated endocytosis in MRC5 cells at the concentration used.**

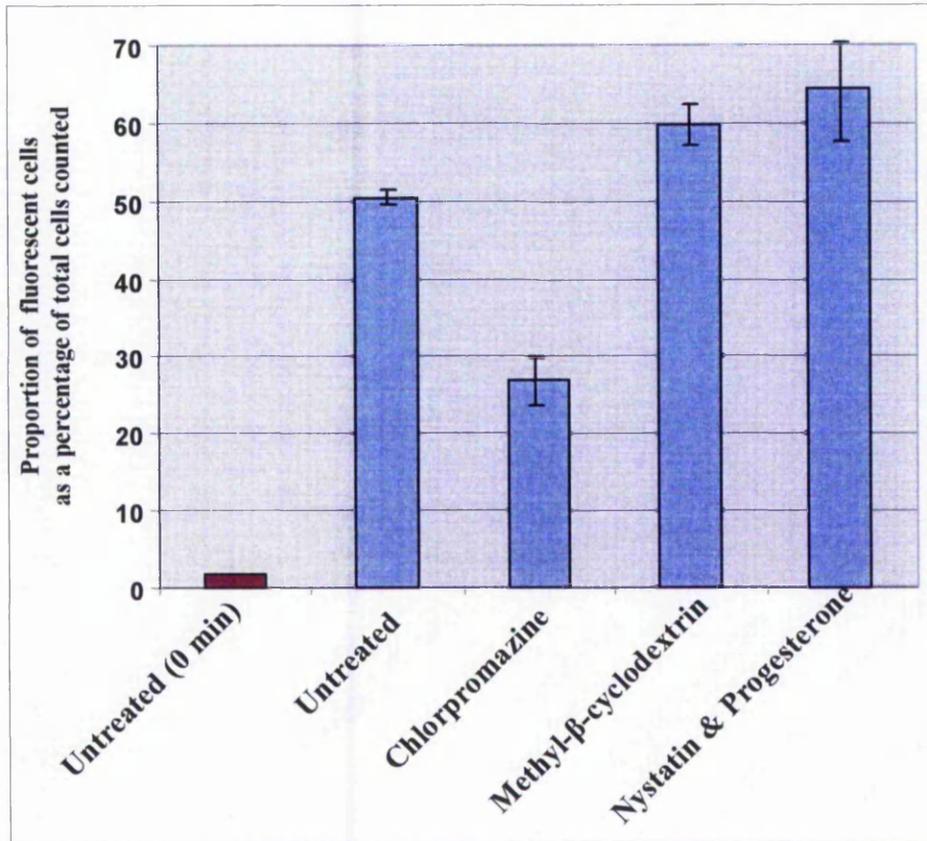
It was concluded in the preceding section that the reduction in EV70 infection of MRC5 cells treated with chlorpromazine was due to inhibition of viral entry. To confirm that chlorpromazine was disrupting clathrin-mediated endocytosis in MRC5 cells as expected, a transferrin uptake assay was used, as transferrin is known to be endocytosed via the clathrin route. Fluorescein conjugated transferrin was added to untreated cells, or cells treated with chlorpromazine, methyl- $\beta$ -cyclodextrin, or nystatin & progesterone. After fifteen minutes incubation at 37°C, non-internalised transferrin was removed from the cell surface using a glycine buffer at pH3, and the proportion of cells containing transferrin determined by flow cytometry (figure 5.4).

Less than 2% of untreated cells were positive for transferrin uptake prior to the 37°C incubation, showing that non-internalised transferrin had been efficiently removed. After 37°C incubation over 50% of untreated cells, and cells treated with methyl- $\beta$ -cyclodextrin, or nystatin & progesterone, showed detectable transferrin uptake. This was reduced to 27% in MRC5 cells treated with 14 $\mu\text{M}$  chlorpromazine, a 50% reduction in comparison to uptake into untreated cells. This was directly comparable to the 50% average reduction in the number of EV70 infected cells seen after chlorpromazine treatment, and demonstrated that under these conditions chlorpromazine is inhibiting clathrin-mediated endocytosis.

In conclusion the reduction in EV70 infection seen in chlorpromazine treated cells is likely to be due to inhibition of clathrin-mediated endocytosis, suggesting that this is the route used by the virus to gain entry to the cell.



**Figure 5.3 Binding of EV70 to MRC5 cells treated with chlorpromazine.** MRC5 cells were pre-treated with  $14\mu\text{M}$  chlorpromazine and incubated with 20 000cpm of  $^{35}\text{S}$  labelled EV70. After 1hr cells were washed and binding assessed using a scintillation counter. Binding is shown as a percentage of binding to untreated MRC5 cells. Error bars show the range of results over duplicate assays. Chlorpromazine treatment of MRC5 cells does not reduce EV70 binding.



**Figure 5.4 Transferrin uptake in MRC5 cells treated with inhibitors of cell entry.** MRC5 cells were pre-treated with inhibitors at the concentrations detailed in figure 5.1A, and fluorescein conjugated transferrin added. Prior to incubation (untreated cells), or after 15 minutes incubation at 37°C (untreated and treated cells), cells were washed with a pH3 glycine buffer to remove non-internalised transferrin and analysed by flow cytometry, counting 5 000 events per sample. Error bars show the range of results over duplicate assays. Only chlorpromazine reduces the uptake of transferrin.

## **5.2 pH has a role in the EV70 life cycle**

After entry via the clathrin-mediated route, viruses pass through late endosomal compartments where they experience an acidic pH. To determine the potential significance of this in EV70 entry into the cytoplasm, the effect of pH on the EV70 life cycle was assessed, firstly by assessing the stability of the virus to a range of pH conditions outside the cell, and secondly, by assessing the effect on viral infectivity of agents which raise the pH of late endosomes.

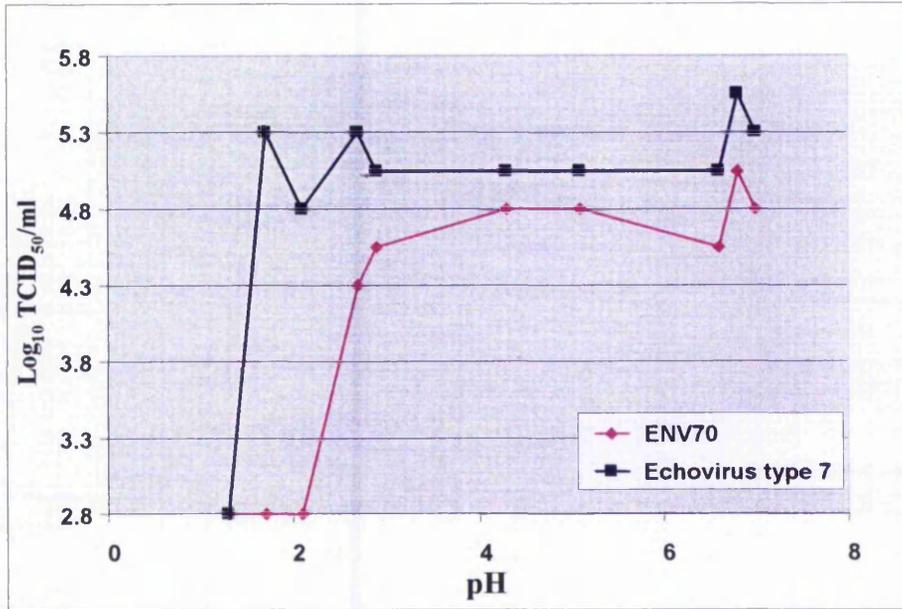
### **5.2.1 The EV70 particle is stable at late endosomal pH, but not at gastric pH**

In order to assess the acid stability of EV70, sensitivity of the virus was tested to a range of pH from one to seven. EV70 and the echovirus type 7 were incubated in solutions of known pH for 20 minutes at 37°C, before being assayed for infectivity in MRC5 cells by TCID<sub>50</sub> (figure 5.5). Echovirus type 7 is a classically acid-stable virus found in the faeces of infected individuals.

There were broad similarities in the pH sensitivities of these viruses, with both showing no, or insignificant, loss in infectivity down to a particular pH at which there was a dramatic reduction. However whereas echovirus type 7 was stable at pH 1.7 and above, EV70 was only stable above pH 2.7. This difference was confirmed on repeat testing. Although a small difference, it is significant in terms of gastric pH. Gastric pH ranges from one to four and the increased sensitivity of EV70 to this pH range may explain its absence from human faeces in infected individuals. However, at the pH of late endosomes (pH 5.5-6) EV70 remained stable.

### **5.2.2 The effect of inhibitors of endosomal acidification on EV70 infection**

Although isolated EV70 virions were found to be stable at late endosomal pH, it is still possible that low pH is an important factor in EV70 entry. Low pH may induce a conformational change in the virus which facilitates exit from the endosome, but which does not affect the infectivity of the EV70 virion. Alternatively, factors additional to pH may be required, such as attachment to the viral receptor, or the action of endosomal proteases. The role of late endosomal pH in EV70 infection was therefore further assessed.

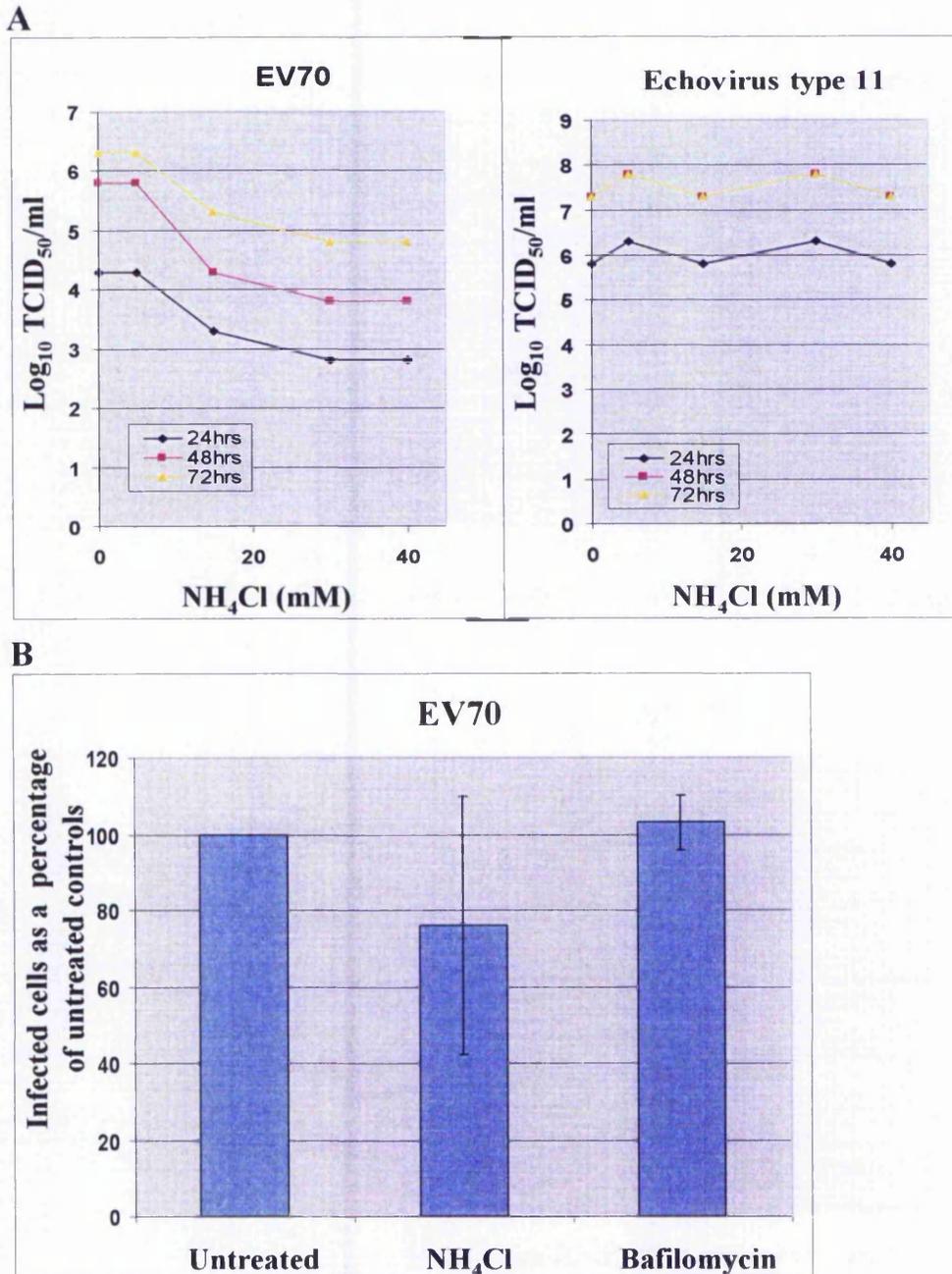


**Figure 5.5 Effect of pH on viral infectivity in MRC5 cells.** Viral tissue culture supernatant was diluted 1:100 in solutions at a range of pH. Parallel dilution of supernatant from mock infected cells, and determination of the pH, gave an accurate indication of the pH of virus-containing solutions of 1.3, 1.7, 2.1, 2.7, 2.9, 4.3, 5.1, 6.6, 6.8, and 7. Virus containing solutions were incubated at 37°C for 20 minutes, before the pH was adjusted to 7 with sodium bicarbonate and viral titres determined by TCID<sub>50</sub> in MRC5 cells.

$\text{NH}_4\text{Cl}$  is a weak base causing elevation of late endosomal pH (Eliassen et al., 2000; Glomb-Reinmund and Kielian, 1998). MRC5 cells were pre-treated with a range of concentrations of  $\text{NH}_4\text{Cl}$  from 5mM to 40mM. These doses were not found to be toxic in MRC5 cells and did not significantly affect the pH of the culture medium. A TCID<sub>50</sub>-based infectivity assay was carried out using these cells in the continued presence of  $\text{NH}_4\text{Cl}$ . EV70 titres were assessed at 24, 48 and 72 hours (figure 5.6A). In this assay a dose-related reduction in EV70 titre of up to 1.5-2 log<sub>10</sub> was clearly seen at all time points. In contrast  $\text{NH}_4\text{Cl}$  had no effect on echovirus type 11, a virus that is believed to enter via the caveolar route and therefore not to pass through late endosomes.

To further confirm the reduction in EV70 titres seen here, a flow cytometry-based infectivity assay was carried out using MRC5 cells treated with either 30mM  $\text{NH}_4\text{Cl}$ , a dose resulting in maximum reduction of EV70 titres in the TCID<sub>50</sub> assay, or bafilomycin. Bafilomycin is a vacuolar ATPase inhibitor and acts to prevent acidification of late endosomes. Previous publications have used a wide range of bafilomycin concentrations, and so the highest concentrations found to be non-toxic in MRC5 cells, 0.3 $\mu\text{M}$ , was used. MRC5 cells were pre-treated with  $\text{NH}_4\text{Cl}$  or bafilomycin, and then infected with EV70 at an m.o.i. of 3, with the agents present throughout a six-hour incubation. The results from individual assays are detailed in appendix 3, with the cumulative results from these assays presented in figure 5.6B. Although wide variation was seen in the results from individual assays, an overall reduction in EV70 infection was seen after  $\text{NH}_4\text{Cl}$  treatment in the flow cytometry-based assay, although much less marked than in the TCID<sub>50</sub>-based assay. Bafilomycin at 0.3 $\mu\text{M}$  did not have a demonstrable effect on EV70 entry in the flow cytometry-based assay. The difference in  $\text{NH}_4\text{Cl}$  results in the two assays and lack of a bafilomycin effect in the flow cytometry-based assay may be due to the different assay formats used, an additional factor may be the extent to which bafilomycin is affecting endosomal pH.

Therefore, although the  $\text{NH}_4\text{Cl}$  results overall suggested a role for late endosomal pH in EV70 infection, it will be important to assess the extent to which bafilomycin in particular is affecting endosomal pH in MRC5 cells. One potential way to do this would be the use of pH sensitive fluorescent probes which accumulate in acidic cellular compartments, and either fail to accumulate or alter their emission spectrum when endosomal pH is raised (e.g. lysosensor probes available from invitrogen).



**Figure 5.6 EV70 and Echovirus type 11 infection in MRC5 cells treated with NH<sub>4</sub>Cl and Bafilomycin. A.** TCID<sub>50</sub>-based infectivity assay. Cells were pre-treated with a range of concentrations of NH<sub>4</sub>Cl as shown, and used to directly determine EV70 or echovirus type 11 titres in a TCID<sub>50</sub> format. Titres were determined visually at 24, 48 and 72 hours. **B.** Flow cytometry-based infectivity assay using EV70 showing cumulative data from several experiments with standard deviations (see appendix 5). MRC5 cells were pre-treated with 30mM NH<sub>4</sub>Cl or 0.3µM bafilomycin. Cells were infected with EV70 at an m.o.i. of 3, incubated for 6 hours in the presence of the chemical and the proportion of infected cells assessed by flow cytometry.

### **5.3 Cell signalling pathways and the cytoskeleton are important for EV70 cell entry**

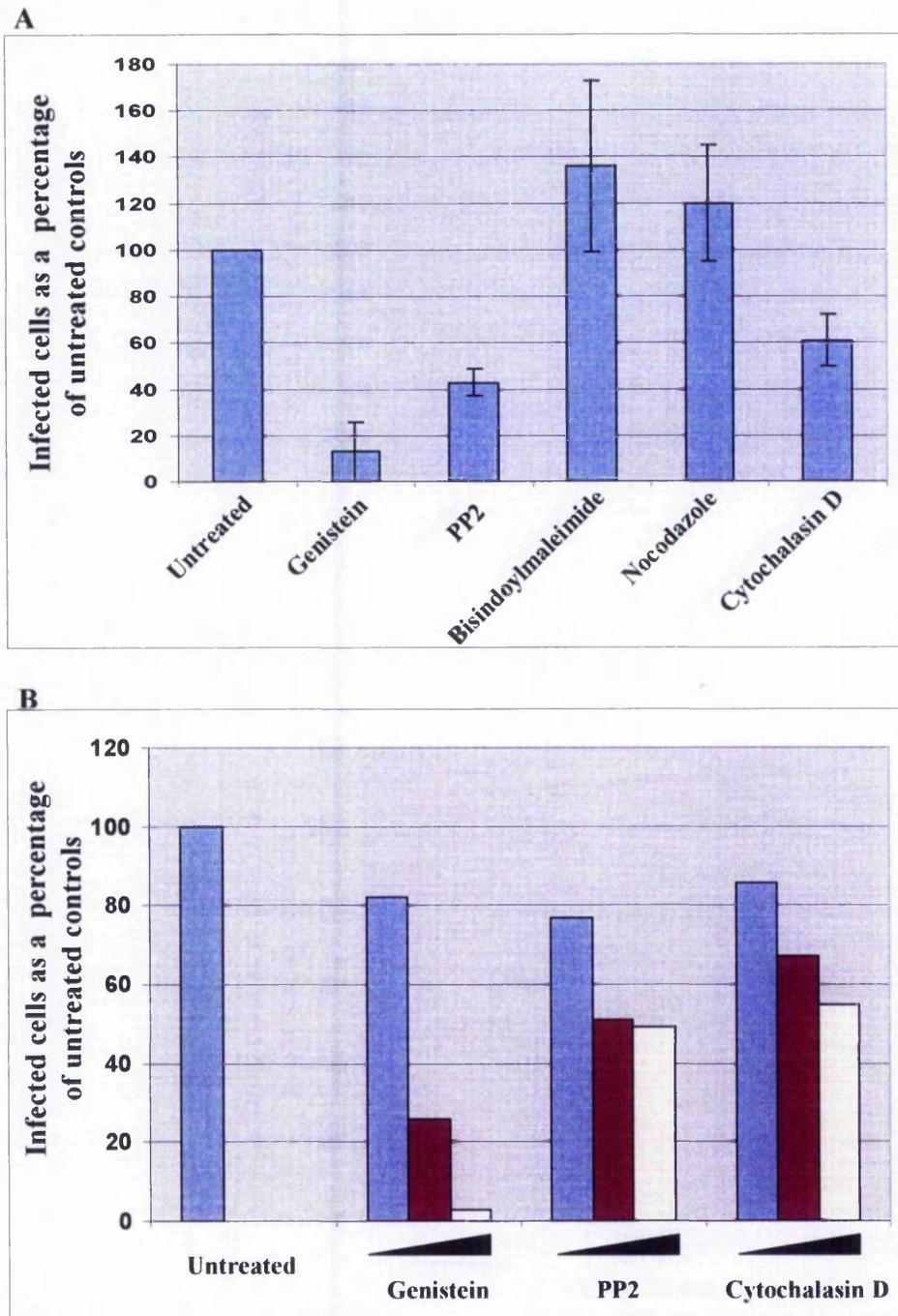
To obtain an initial indication of the importance of cell signalling and the cellular cytoskeleton in EV70 infection, infectivity was assessed in cells treated with a number of agents known to target specific signalling events or cytoskeletal elements. Genistein is a competitive inhibitor of tyrosine kinases, including both *abl* and *src*-like kinases, the phosphatase PP2 is a potent selective inhibitor of *src*-like kinases, and bisindoylmaleimide is a potent inhibitor of serine/threonine kinases, and protein kinase C in particular. In addition the effects of cytochalasin D and nocodazole were assessed. Cytochalasin D is an inhibitor of actin polymerisation whereas nocodazole causes the destabilisation of microtubules.

#### **5.3.1 Inhibition of tyrosine kinases and disruption of actin reduce EV70 infectivity**

Toxicity in MRC5 cells was first assessed using a range of concentrations, based on those shown to be effective in previous studies (Pietiainen et al., 2004; Stuart et al., 2002). The highest concentration of each agent for which no toxicity was observed on repeat testing, were 20 $\mu$ M genistein, 60 $\mu$ M PP2, 13nM bisindoylmaleimide, 2 $\mu$ M cytochalasin D and 6.6 $\mu$ M nocodazole, and these concentrations were used in all subsequent assays.

MRC5 cells were treated in duplicate with chemicals at the above concentrations prior to infection with EV70. In the case of cytochalasin D and nocodazole, culture dishes were pre-treated with poly-L-lysine to prevent cellular detachment. The virus adsorption and incubation steps of infection were carried out in the continued presence of the chemicals. At six hours post-infection cells were stained and a flow cytometry-based infectivity assay used to determine the number of infected cells. The results from individual assays are detailed in appendix 3, with the cumulative results from these assays presented in figure 5.7.

Genistein, PP2 and cytochalasin D caused clear reductions in the number of EV70 infected cells, by an average of 87%, 57% and 40% respectively, while neither bisindoylmaleimide, the protein kinase C inhibitor, nor the nocodazole-mediated disruption of microtubules, had a demonstrable effect. Use of a range of concentrations



**Figure 5.7 EV70 infection in MRC5 cells treated with kinase inhibitors and agents that disrupt the cytoskeleton. A** Flow cytometry-based infectivity assay showing cumulative data from several experiments with standard deviations (see appendix 5). MRC5 cells were pre-treated with 20  $\mu$ M genistein, 13 nM bisindoylmaleimide, 60  $\mu$ M PP2, 6.6  $\mu$ M nocodazole and 2  $\mu$ M cytochalasin D. Cells were infected with virus at an m.o.i. of 3, incubated for 6 hours in the presence of the chemical and the proportion of infected cells assessed by flow cytometry. **B** Flow cytometry-based infectivity assay showing dose response of EV70 infection to increasing concentrations of genistein (at 5  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M), PP2 (at 40  $\mu$ M, 50  $\mu$ M and 60  $\mu$ M), and cytochalasin D (at 0.2  $\mu$ M, 1  $\mu$ M and 2  $\mu$ M).

confirmed the reductions by genistein and cytochalasin D to be dose-dependant (figure 5.7B). A dose response for PP2 was less clear at the concentrations used, with little difference in the percentage of infected cells between 50 $\mu$ M and 60 $\mu$ M.

### 5.3.2 Genistein, PP2 and cytochalasin D do not affect EV70 replication

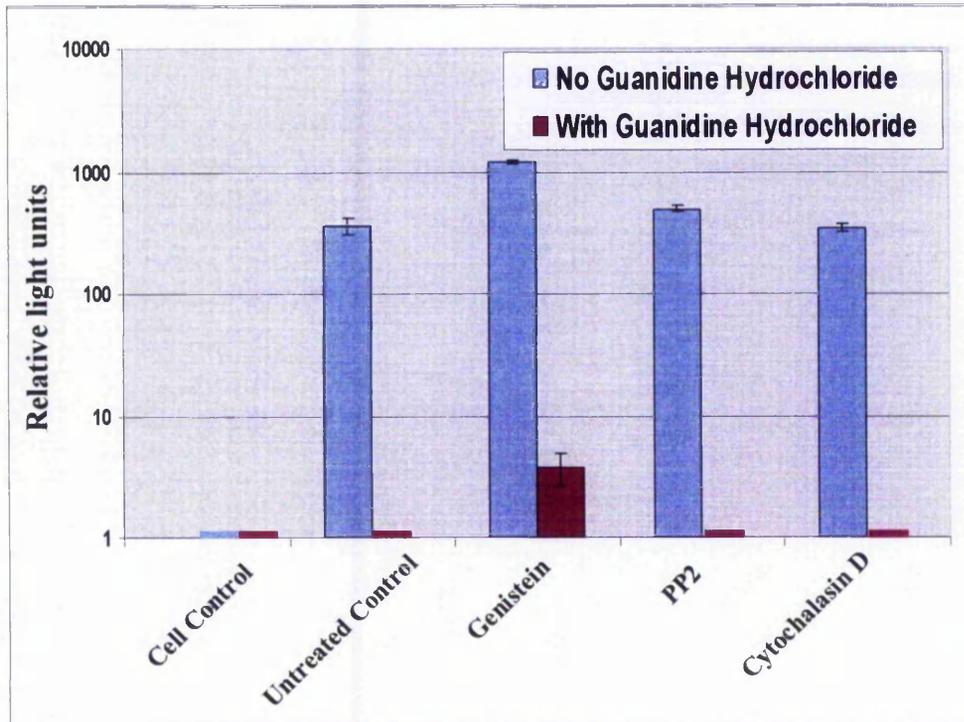
To confirm that the reductions in EV70 infection seen were not due to inhibition of EV70 replication, the subgenomic replicon, pLuc70, was used to assess the effect on EV70 replication. RNA was transcribed from the replicon and transfected into MRC5 cells pre-treated at the concentrations used in the previous assays. After 6 hours incubation in the continued presence of the agents, luciferase activity was assayed (figure 5.8).

The level of EV70 replication observed in all treated cells was comparable to that seen in untreated cells, demonstrating that genistein, PP2 and cytochalasin D do not affect EV70 replication, but are acting to reduce EV70 infection earlier in the viral life cycle.

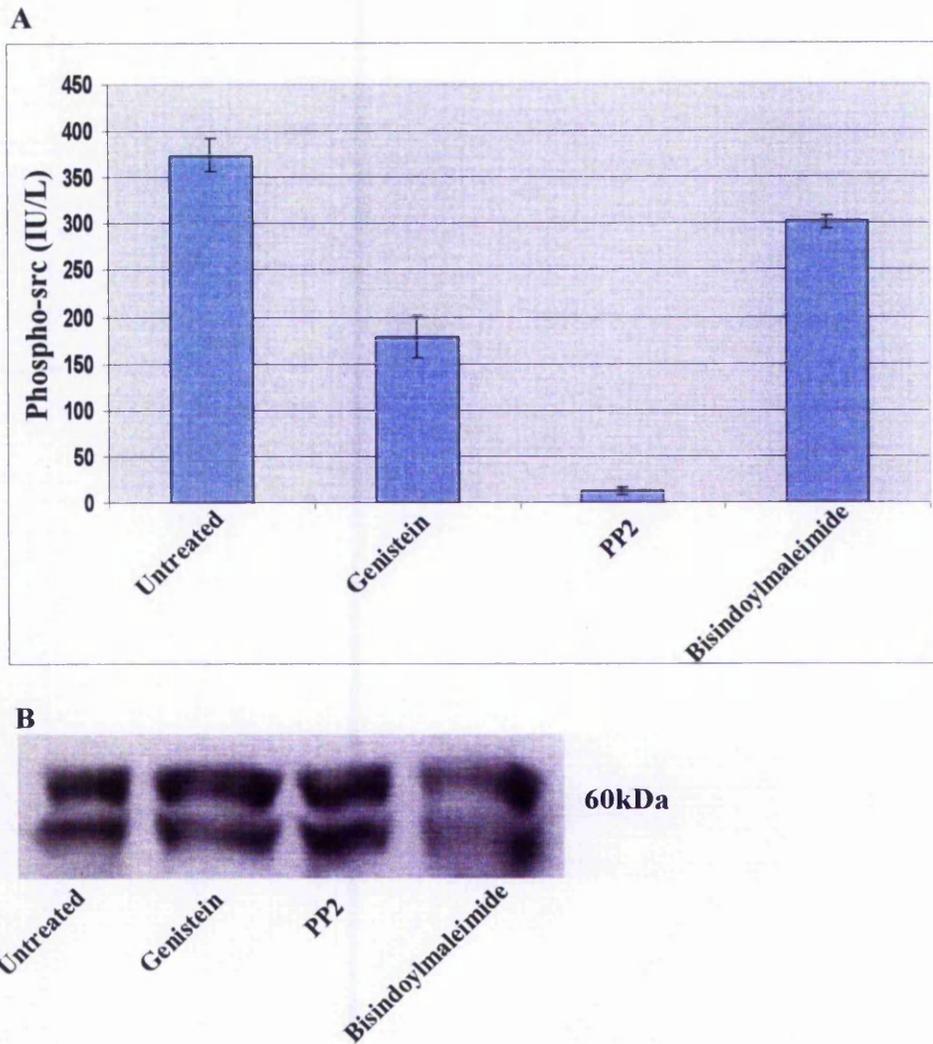
### 5.3.3 Genistein, PP2, cytochalasin D and nocodazole have the expected effects in MRC5 cells.

Further assays were conducted to confirm that the concentrations of the agents used in the above assays were having the expected effects in MRC5 cells. This was assessed by determining the levels of phosphorylated *src* in the kinase treated cells, and by immunofluorescence in cells treated with cytochalasin D and nocodazole.

*Src* is a tyrosine kinase that is autophosphorylated at tyrosine residue 418. The level of *src* phosphorylated at tyrosine 418 was assayed in cells treated with the kinase inhibitors (figure 5.9). Using an ELISA-based assay (figure 5.9A), it can be seen that both tyrosine kinase inhibitors reduce phosphorylation of this protein, genistein by over 50% and PP2 by over 90%. Bisindoylmaleimide, the protein kinase C inhibitor, also appears to cause a small reduction in *src* phosphorylation, but to a much lesser degree. That the observed reductions are due to changes in the levels of *src* phosphorylation, rather than changes in the amount of *src* protein in the cell, was confirmed by a western blot for *src* on the cell lysates used in the ELISA assay. Using a non-phosphospecific *src* antibody a duplex band was seen at the expected size of 60kDa, with broadly similar intensity in all samples. This antibody is reported to identify a single band; the duplex band seen here may be due to the presence of alternately glycosylated or modified forms



**Figure 5.8 EV70 replication in MRC5 cells treated with kinase inhibitors and agents that disrupt the cytoskeleton.** MRC5 cells were pre-treated with inhibitors at the concentrations detailed in figure 5.7A, and transfected with 2 $\mu$ g of RNA transcribed from pLuc70. Luciferase was assayed after 6 hours incubation in the continued presence of the chemicals and in the presence and absence of the replication inhibitor guanidine hydrochloride. Error bars show the range of results over duplicate assays.



**Figure 5.9 Levels of tyrosine 418 phosphorylated *src* in MRC5 cells treated with kinase inhibitors . A** Phospho-*src* quantification in MRC5 cells following treatment with various cell-signalling inhibitors. Inhibitor concentrations were as detailed in figure 5.7A. Error bars show the range of results over duplicate assays **B** Western blot of cell lysates used for figure 5.9A, probed with antibody against *src* (not phosphospecific). Similar band intensities show that the differences seen in figure 5.9A are not due to variation in protein levels.

of *src* in MRC5 cells. PP2 is a much stronger inhibitor of *src* kinases than genistein, nevertheless genistein was observed to cause the greater reduction in EV70 infection. This is likely to reflect the broader spectrum of tyrosine kinases which genistein inhibits and therefore suggests that other tyrosine kinase families, such as *abl*, may be involved in EV70 infection.

Cytochalasin D and nocodazole treatments were confirmed to be disrupting the cell cytoskeleton by indirect immunofluorescence for alpha-tubulin and actin respectively (figure 5.10). Clear disruption of the cell sheet is seen in cytochalasin D treated cells stained for actin and in nocodazole treated cells stained for alpha-tubulin.

In conclusion the results in this section show that the actions of tyrosine kinases, and *src*-like kinases in particular, are important in signalling pathways involved in EV70 cellular entry, and in addition that an intact actin cytoskeleton is necessary for efficient infection.

#### **5.4 The block in EV70 infection in non-primate mammalian cell lines occurs at cell entry**

EV70 has been shown to bind to hamster (CHO), rabbit (RK13) and xenopus (XTC-2) cells (figure 4.7), but does not result in fully productive infection in these cells (figure 4.9). It is not known at what step in the viral life cycle this block in infection is occurring. If there is a block at the level of viral entry, future studies using these cells have the potential to provide insights into this step in the EV70 life cycle.

The ability to introduce viral RNA from a full-length clone directly into the cell, makes possible the assessment of the viral life cycle in isolation from the early events of binding and entry. In addition a subgenomic replicon allows viral translation and replication to be specifically assessed. These clones are therefore useful tools in investigating the block to fully productive EV70 infection observed in non-primate cells.

**Anti-actin antibody**



**Untreated**



**Cytochalasin D**

**Anti-tubulin antibody**



**Untreated**



**Nocodazole**

**Figure 5.10 Immunofluorescence for actin and tubulin in MRC5 cells treated with agents that disrupt the cytoskeleton.** Cells pre-treated with cytochalasin D or nocodazole, were fixed, permeabilised and actin or alpha tubulin detected using an anti-alpha tubulin mouse monoclonal or an anti-actin rabbit polyclonal antibody respectively. Anti-mouse or rabbit IgG FITC conjugates were used as the secondary antibodies as appropriate.

#### **5.4.1 In non-primate mammalian cells EV70 infection is blocked at the level of cell entry.**

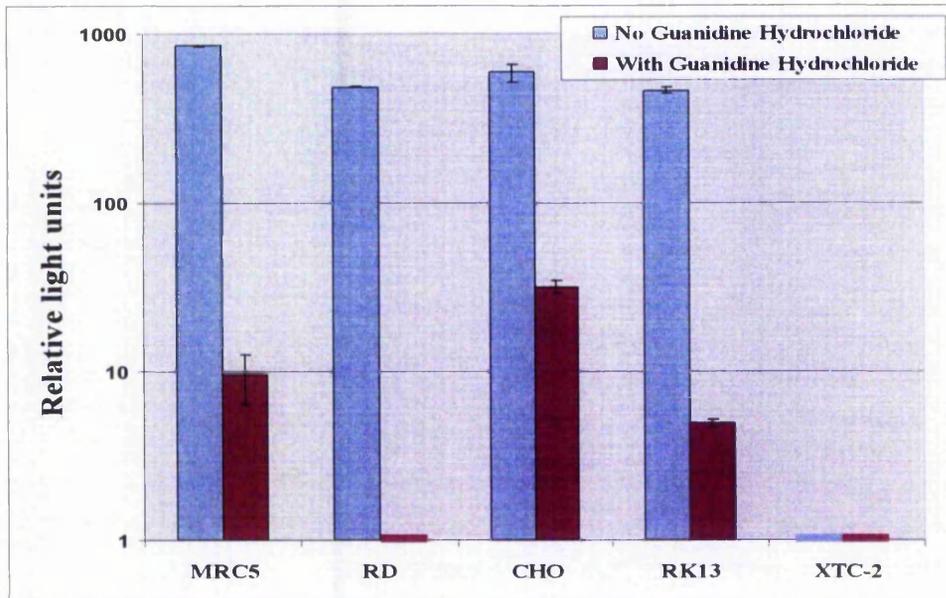
To assess EV70 replication, RNA from the luciferase-encoding EV70 subgenomic replicon, pLuc70, was transfected using lipofectamine, into three non-permissive cell lines; CHO (hamster), RK13 (rabbit), and XTC-2 (xenopus) and two permissive cell lines (MRC5 and RD). Lipofectamine was used, as electroporation did not result in efficient transfection in all the cell lines. As can be seen in figure 5.11, luciferase levels at 6hrs were comparable between the two rodent cell lines and the permissive cell lines, showing that EV70 replication is not blocked in these cells. Perhaps not unexpectedly there was no evidence of replication in XTC-2 cells which, being of amphibian origin, are much more divergent from human cells than those of other mammals.

Further evidence that CHO and RK13 cells fully support EV70 replication was obtained from transfection of full-length EV70 RNA transcribed from the full-length clone, pFLC70 (figure 5.12). EV70 titres were measured in both culture supernatant and in cellular lysates 6hrs post transfection. CHO and RK13 cells demonstrated EV70 titres comparable to MRC5 and RD cells. This shows that in addition to translating and replicating viral RNA, these cell lines also allow the formation of correctly assembled viral progeny. The observation that EV70 titres are comparable between culture supernatant and cell lysate shows that the virus is also capable of being released from these cells. This is an important observation as EV70 is not cytopathic in CHO, RK13 or RD cells, and so suggests an active process of viral release, rather than cell lysis. In keeping with the lack of replication seen in the replicon assay, infectious virus was not recovered from XTC-2 Xenopus cells.

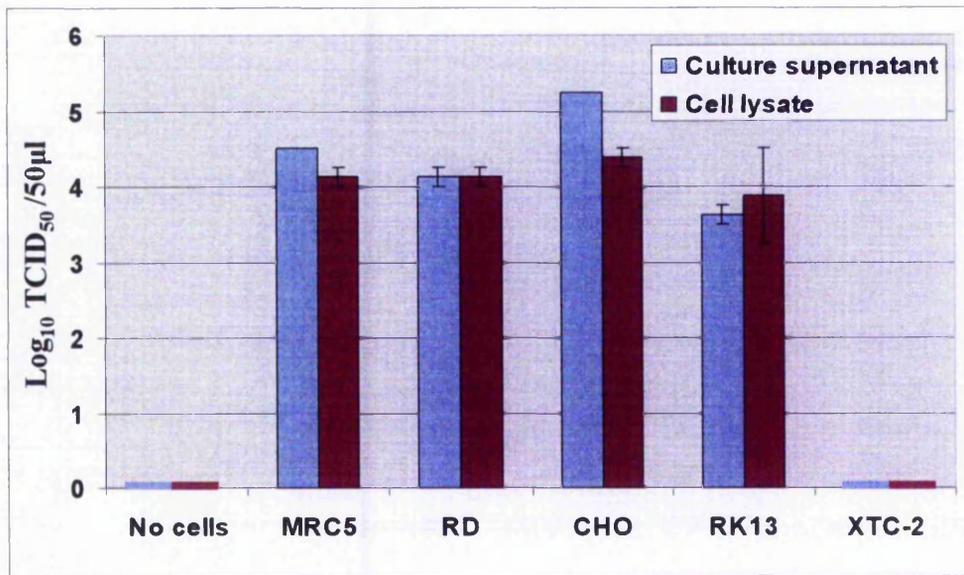
These results show that the two non-primate mammalian cell lines tested from rodents are fully permissive for EV70 replication, assembly and release. As it has been shown that the virus efficiently binds to these cells, this implies that the block to full infection is most likely to be occurring at the level of viral entry.

### **5.5 DISCUSSION**

The results presented above provide preliminary evidence that EV70 enters cells via clathrin-mediated endocytosis, that the acid environment of late endosomes may be



**Figure 5.11 EV70 replication in a variety of cell lines.** 2 $\mu$ g of RNA transcribed from pLuc70 was transfected into cells by lipofection and luciferase assayed at 6 hours. The assay was carried out in the presence and absence of the replication inhibitor guanidine hydrochloride. Error bars show the range of results over duplicate assays.



**Figure 5.12 EV70 titres after transfection of EV70 RNA into a variety of cell lines.** 2 $\mu\text{g}$  of RNA transcribed from pFLC70 was transfected into cells by lipofection and viral titres were measured by  $\text{TCID}_{50}$  after six hours. Error bars show the range of results over duplicate assays.

important and that *src* family tyrosine kinases and actin filaments are involved during early, pre-replication, stages in the viral life cycle. In addition the virus has also been shown to bind to lipid raft domains of the plasma membrane (chapter 4).

### 5.5.1 The dependence of EV70 on clathrin-mediated endocytosis.

The inhibitor of clathrin endocytosis, chlorpromazine, has been used here to demonstrate that EV70 entry into MRC5 cells is via clathrin-mediated endocytosis. Although non-specific effects on other entry pathways cannot be entirely excluded, chlorpromazine had no effect on echovirus type 11, a virus believed to enter via caveolae. It has been shown that this is a specific inhibition of the EV70 entry process, as viral binding and replication are unaffected. Further, using transferrin as a marker, it has been shown that chlorpromazine is blocking the clathrin pathway in MRC5 cells under the assay conditions used.

The clathrin route was the first described and is the most widely documented method of viral entry to the cell (Marsh and Helenius, 2006). It is a rapid process, and results in viral passage through the increasingly acidic early and late endosomes, where penetration occurs with the virus entering the cytoplasm. Influenza virus is perhaps the best characterised of the clathrin using viruses (Lakadamyali et al., 2003; Skehel and Wiley, 2000). Other viruses using this entry route include adenovirus types 2 and 5 (Lakadamyali et al., 2003; Meier and Greber, 2004), semliki forest virus (Marsh et al., 1984; Meier and Greber, 2004) and a range of picornaviruses, such as foot and mouth disease virus, human parechovirus type 1, and human rhinovirus types 2 and 14 (Berryman et al., 2005; DeTulleo and Kirchhausen, 1998; Joki-Korpela et al., 2001; Snyers et al., 2003). However, not all picornaviruses use this route, as both coxsackie B viruses and echovirus type 1 are believed to use the caveolar method of entry (Coyne and Bergelson, 2006; Pietiainen et al., 2004).

The use of an established endocytic pathway allows viruses to rapidly traverse the highly structured submembranous region of the cell. While precise information on the EV70 life cycle is limited, there are a number of features of the clathrin pathway that may be significant in facilitating viral entry. One feature is the exposure of the virus to acidic conditions in late endosomes, which could then be exploited by the virus to allow entry from the vesicle into the cytoplasm. The reduction in infection seen by raising endosomal pH with  $\text{NH}_4\text{Cl}$  suggests that this may be important for EV70. Clathrin-mediated entry may also allow specific delivery of the virus to its site of

transcription and translation, as it becoming apparent that the clathrin pathway allows internalised cargo to be delivered to functionally and geographically distinct endosomes within the cell (Marsh and Helenius, 2006). Entry route is undoubtedly influenced by the receptor/s used by the virus; this may not only associate the virus with a specific internalisation site on the cell membrane, such as lipid rafts, but viral binding can initiate signalling events via the receptor to facilitate entry as discussed below.

An important point is that viruses may use more than one route of entry to the cell, either simultaneously or when the preferred pathway is blocked. For example, influenza virus, though well characterised as using the clathrin route, can also enter the cell by a non-clathrin, non-caveolar route (Rust et al., 2004; Sieczkarski and Whittaker, 2002b). It is also possible that the entry pathway may vary depending on the cell type.

Further work will be needed to confirm the chlorpromazine-based results and use of the clathrin-mediated endocytic pathway for EV70 entry. One more specific way of determining whether a virus enters via the clathrin or caveolar routes is the use of dominant negative proteins. These have been successfully used to investigate the route of entry of a number of viruses (Sieczkarski and Whittaker, 2002b). Various dominant negative mutants have been created; in particular mutants of the Eps 15 protein, which is associated with clathrin coated pits (Benmerah et al., 1999), and a dominant negative form of caveolin (Pelkmans et al., 2001). Attempts were made during this project to confirm the EV70 entry route by expressing these mutant proteins in MRC5 cells, however, it was found that the transfection process used in the assay greatly reduced the ability of MRC5 cells to be infected by EV70 (data not shown). Further possibilities are to use these proteins to investigate EV70 in a different cell line or the use of confocal fluorescent microscopy to examine co-localisation of the virus and cellular components involved in entry processes, as has been done for a number of viruses (Pietiainen et al., 2004; Rust et al., 2004).

### **5.5.2 Viral Penetration and requirement for a low pH environment**

EV70 has been classified as an enterovirus partially on the basis of acid stability at pH3 (Kono et al., 1972), although the expected reclassification of enteroviruses and rhinoviruses indicates that acid stability may not necessarily be a distinguishing feature of this genus. It is unusual, however, in that it can only very rarely be isolated from faeces in infected patients (Higgins, 1982), in contrast other enteroviruses can be easily

isolated from the faeces after surviving passage through the acidic environment of the stomach.

In this chapter EV70 has been shown to be acid-labile below pH 2.7. This is an important observation in relation to viral pathogenesis, in that virus will not be expected to survive the gastric environment, and explains the failure to isolate virus from the faeces of infected patients. A more recent isolate strain of EV70 has been found to be acid labile at pH 3 (Oberste et al., 2004), suggesting that relatively few changes may be required to render the EV70 capsid sensitive to a wider range of pH. In contrast, the virus is clearly stable at the pH of late endosomes, suggesting that the virus should be stable in these compartments. However this does not rule out a possible role for endosomal pH in viral penetration. Additional factors, present in endosomes, may be important in regulating the viral response to low pH, such as continued attachment to the viral receptor or exposure to endosomal proteases. Preliminary work, showing a dose-dependant reduction in EV70 infection in MRC5 cells treated with  $\text{NH}_4\text{Cl}$ , suggests that pH may well be important in EV70 entry. This result clearly requires further confirmation, especially as bafilomycin had no apparent effect on viral entry in MRC5 cells (although this may have been due to toxicity preventing effective treatment concentrations from being used).

Many viruses entering via the clathrin route have been shown to require a low pH step during infection, most obviously influenza virus where acid pH causes a conformational change in the viral haemagglutinin resulting in membrane fusion (Skehel and Wiley, 2000), but also for non-enveloped viruses, such as adenoviruses. Adenoviruses exit via endosomal lysis following a conformational change in the penton base of the capsid induced by low pH (Medina-Kauwe, 2003) and low pH steps have also been implicated for foot and mouth disease virus, CVB3 and rhinoviruses (Berryman et al., 2005; Chung et al., 2005; Nurani et al., 2003), although here the significance remains uncertain. Some viruses, such as reoviruses and coronaviruses, require the additional action of endosomal proteases (Ebert et al., 2002; Simmons et al., 2005).

If EV70 infection is confirmed to be reliant on low endosomal pH, there are a variety of ways in which this could be acting. Low pH may trigger conformational change within the EV70 capsid, potentially in combination with viral receptor attachment or endosomal proteases. Such capsid changes might trigger the uncoating of the virus, potentially forming a pore within the endosomal membrane through which the

virus can penetrate, analogous to the membrane pore formed by poliovirus (Hogle, 2002; Rossmann et al., 2000), or endosomal lysis might result, analogous to the adenoviruses (Medina-Kauwe, 2003).

### 5.5.3 Lipid rafts and cellular signalling in EV70 entry

EV70 has been shown to associate with lipid rafts upon binding to MRC5 cells via sialic acid. As yet the evidence is insufficient to determine whether this interaction is absolutely necessary for EV70 infection. However, as lipid rafts are dynamic regions of the cell surface with multiple functions in endocytic and signalling pathways (Brown and London, 1998), it is likely that this association has a strong functional basis.

Although traditionally associated with caveolar entry pathways (Quest et al., 2004), rafts have been implicated as a site of endocytosis via other pathways; clathrin-mediated endocytosis (Puri et al., 2005; Signoret et al., 2005; Stoddart et al., 2002) and non-clathrin, non-caveolin routes (Zeng et al., 2003). This perhaps reflects the importance of cell signalling in all cell entry processes, as rafts function as cell signalling platforms within the cell membrane (Simons and Toomre, 2000). External stimuli, such as viral binding, can result in dramatic changes in the protein composition of lipid rafts, with the recruitment of further proteins. Particular proteins are preferentially associated with rafts; these include GPI-anchored proteins, *src*-family kinases and certain transmembrane proteins and G proteins. Simons and Toomre (2000), suggest that rafts act as 'concentrating platforms for individual receptors, activated by ligand binding'. Rafts can thus recruit proteins to an environment where they are exposed to the actions of a variety of kinases and phosphatases and act as the starting point for signal transduction pathways. The potential importance of this in viral infection is highlighted by the finding that binding of coxsackie B viruses to DAF present in lipid rafts, results in concentration and cross-linking of DAF, with the clustering of proteins including activated *src* kinases and *abl* (Coyne and Bergelson, 2006).

The sensitivity of EV70 infection to the inhibitors genistein and PP2 has demonstrated the dependence of early stages in the EV70 life cycle on the activity of tyrosine kinases, and of *src*-family kinases in particular. No effect was observed in cells treated with the protein kinase C inhibitor, bisindoylmaleimide, however it is important to note that inhibition of protein kinase C activity has not yet been confirmed under the assay condition used. Tyrosine kinases play key roles in signal transduction pathways,

and *src* family kinases, in particular, are known to associate with lipid rafts (Simons and Toomre, 2000).

The importance of signal transduction in viral entry, including clathrin pathways, is now well established (Brodsky et al., 2001; Greber, 2002). The entry of vesicular stomatitis virus via the clathrin route has been shown to be associated with alterations in the activities of over 90 distinct kinases (Pelkmans et al., 2005). Over 95% of the clathrin pits with which influenza virus associates are formed *de novo* after viral binding (Rust et al., 2004), presumably through virus-induced signalling events. Viruses may directly activate signalling pathways by binding to a receptor with integral signalling functions, as is the case for the low-density lipoprotein receptor of minor group rhinoviruses (Hofer et al., 1994). Alternatively viral binding may cause interactions between the receptor and other molecules involved in signalling pathways. This is the method by which proteins with a GPI anchor initiate signalling; as they lack a cytoplasmic domain, they exert their effects via interactions with the external or transmembrane domains of other signalling proteins. This may be the situation for EV70 if the receptor is indeed GPI-anchored, as suggested by HA studies (chapter 4). Signalling may not only facilitate viral entry by direct stimulation of entry pathways, but also by recruiting necessary co-receptors to the lipid raft (Simons and Toomre, 2000), or by triggering translocation of the virus to the site of a second receptor. The latter has been shown for coxsackie B viruses (Coyne and Bergelson, 2006), where binding to DAF triggers *abl* kinase activity, resulting in movement of the virus to the epithelial tight junction, where it can bind to CAR, an otherwise inaccessible receptor.

#### 5.5.4 The role of the cytoskeleton in EV70 entry

Cytochalasin D reduces EV70 infectivity in MRC5 cells, but has no effect on replication. As this agent blocks actin polymerisation, this suggests a role for the actin cytoskeleton in EV70 entry. In contrast, the disruption of microtubules using nocodazole had no effect on EV70 infection.

Both microtubules and actin form part of the cellular cytoskeleton and have important roles in endocytic processes (Apodaca, 2001). Microtubules are involved in the localisation of organelles within the cell and transport between organelles. They are one of the primary mechanisms by which viruses have been found to traverse the cytoplasm (Greber and Way, 2006). The precise role of microtubules in endocytic pathways however, is not clear, as depolymerisation with nocodazole has variable

effects depending on the cell type and end-point being assessed (Apodaca, 2001). That EV70 endocytosis itself does not require intact microtubules in MRC5 cells might therefore not be an unexpected finding, however the lack of effect of nocodazole on EV70 infection further suggests that EV70 uses a non-microtubule-based route, after release from the endosome, to reach its site of replication.

Actin is abundant in the submembranous region forming the cortical cytoskeleton of the cell. It has been strongly linked to endocytic processes, although the need for actin varies depending on cell type (Fujimoto et al., 2000), growth conditions and specific membrane subdomains (Apodaca, 2001). For example, actin appears to be required for the uptake of influenza virus and vesicular stomatitis virus from the apical, but not basolateral membranes of polarised epithelial cells (Gottlieb et al., 1993). The precise role of actin in endocytosis is unknown, it may be acting as a scaffold for assembly of the endocytic machinery or to regulate the entry of receptors into vesicles (Apodaca, 2001). The requirement for an intact actin cytoskeleton in EV70 infection might therefore be associated with its requirement for endocytosis of the virus into MRC5 cells. Another explanation is that actin is required for movement of the virus, either via translocation to a separate region of the cell membrane prior to entry, or within the cytoplasm following exit from the endosome. Coyne and Bergelson (2006), showed that localised actin rearrangements, induced by tyrosine kinases were responsible for the translocation of DAF bound coxsackievirus to the tight junction for interaction with CAR. In the cytoplasm, tyrosine kinase-mediated signalling induced by SV40 and vaccinia viruses, results in the formation of comet-like actin tails, believed to propel the viruses through the cytoplasm (Frischknecht et al., 1999; Pelkmans et al., 2002). If actin tails are involved in EV70 movement within the cytoplasm, this might explain the lack of effect of the disruption of microtubules on EV70 infection.

#### **5.5.5 Species restriction in EV70 infection**

It has been shown that the block in EV70 infection in CHO (hamster) and RK13 (rabbit) cells occurs after viral binding but before replication. This suggests that the block is occurring during viral entry and penetration of the cytoplasm. In contrast EV70 was not found to be capable of replicating in XTC-2 cells (*Xenopus*).

The finding that EV70 RNA is not replicated after transfection into cells of *Xenopus* origin is perhaps not surprising given the evolutionary distance between humans and amphibians. Similarly, poliovirus RNA is not replicated after injection into

Xenopus oocytes. Interestingly, however, the addition of only two factors from HeLa cell extracts allows efficient translation and replication to occur (Gamarnik and Andino, 2006), and this may therefore also be a useful model for future investigations into the cellular requirements for EV70 replication

The block to EV70 entry in CHO and RK13 cells has a number of potential explanations. One possibility is that the entry pathway in these cells is not compatible with that required by EV70. Although clathrin-mediated endocytosis is observed in all mammalian cells, there are likely to be differences in either the components of the pathway or the signalling pathways associated with it. However, poliovirus can productively infect mouse L cells engineered to express the poliovirus receptor (Mendelsohn et al., 1989; Wood and Hull, 1999) and CVA21 can infect L cells expressing its receptor ICAM-1 (Shafren et al., 1997a), suggesting that at least some potential routes of enteroviral entry are sufficiently conserved. Another possible reason for the block in infection observed with EV70 is lack of an effective receptor or receptors. Although these cells express a sialated receptor to which EV70 can bind, the nature of the underlying molecule may differ between non-permissive and permissive cells, such that entry does not follow binding. Alternatively there may be a lack of an essential co-receptor. Many viruses have been shown to require more than one receptor. Coxsackie B viruses are recruited to the cell via the ubiquitously expressed DAF, but require interaction with CAR for uncoating and release of viral RNA (Coyne and Bergelson, 2006), and it is hypothesized that rotaviruses require as many as three separate receptor interactions (Arias et al., 2002). Thus the sialated receptor may be sufficient to recruit the virus to the cell, but another receptor/s may be needed for EV70 entry.

CHO or RK13 cells are therefore potentially valuable tools in future EV70 research. By transfecting in cDNA libraries, clones could be found which result in the fully productive infection of these cells by EV70. These might then identify the functional receptor and/or co-receptors required for EV70 entry and infection of cells.

## CHAPTER 6

### Final Discussion and Future Work

#### 6.1 Future directions in EV70 receptor and entry studies

##### 6.1.1 Current understanding and future research goals.

Although improving, the current understanding of how enteroviruses gain entry to the cell for replication remains limited. Only for poliovirus are the steps required relatively clear and, even for this well understood virus, there is some debate and uncertainty. The evidence suggests differences in the routes and mechanisms used for different members of the genus. Therefore, as the best characterised group D enterovirus, and one of only two human enteroviruses with a requirement for cell surface sialic acid, further understanding of EV70 entry may help give a fuller appreciation of the overall picture of how enteroviral genomes gain entry to the cellular cytoplasm.

The work presented in this thesis has demonstrated the essential role of sialic acid as a receptor in the binding and infection of MRC5 cells by EV70. Additionally sialic acid has been shown to be the primary determinant of virus-mediated HA and binding to non-human cell lines. The central role of sialic acid is corroborated by other studies using cell lines productively infected by EV70 (Alexander and Dimock, 2002; Haddad et al., 2004; Nokhbeh et al., 2005). On RBC the virus has been shown to bind a GPI-anchored glycoprotein with  $\alpha$ 2-3 linked sialic acid. However this was not the case in MRC5 cells, where binding was unaffected by the removal of  $\alpha$ 2-3 linked sialic acid, and other work has suggested the involvement of a variety of different categories of sialated molecules on different cell types (Nokhbeh et al., 2005). Potential reasons for the apparent variation in the identity of the sialated receptor have been discussed in chapter 4, such as specificity for a specific glycosylation pattern (rather than a specific glycoprotein or lipid) or adaptation of the virus to growth in different cell lines.

The sialic acid-mediated binding of EV70 to rodent cell lines is not sufficient to permit entry of the virus into the cell. As discussed in chapter 5 this could be due to the absence of a specific coreceptor on these cells, or to the virus binding to a sialated molecule which cannot support internalisation or uncoating.

A number of features of EV70 entry into MRC5 cells have been determined. The current study suggests that internalisation occurs via the clathrin-mediated endocytic pathway and that this occurs in association with lipid raft domains in the cell membrane, although a functional role for lipid rafts in EV70 entry has not yet been demonstrated. Additionally there has been shown to be involvement of cellular signalling pathways (*src* tyrosine kinases in particular) and the actin cytoskeleton. Furthermore, the acidic pH in late endosomes is likely to be important for EV70 penetration, although further work is required to confirm this, and determine its specific role.

The aims of future research will be to identify the GPI-anchored protein or proteins to which the virus is binding on RBC, and to determine the nature and identity of the receptor on MRC5 cells if this is different. It will be important to determine whether the virus interacts with a specific individual glycosylated molecule, or to a variety of related molecules, and if the latter, to establish the common carbohydrate structures and protein or lipid components required for viral binding. In addition further investigation is required to ascertain whether these interactions are sufficient to enable subsequent viral entry into the cell, or whether a further coreceptor may be necessary.

In addition to the identification and characterisation of the EV70 receptor/coreceptors, elucidation of the interaction between the receptor and viral capsid is important. In particular whether the interaction of the virus and receptor occurs within the viral canyon, as with poliovirus, human rhinovirus type 14 and CVB3 (He et al., 2000; Olson et al., 1993; Xiao et al., 2001), or elsewhere on the capsid as for DAF binding viruses (He et al., 2002; Pettigrew et al., 2005). The direct consequences of the binding of EV70 to its receptor/s also need to be established; particularly whether, and in what circumstances, the receptor interaction results in viral uncoating and the nature, and importance for productive infection, of any down-stream signalling events that might be directly triggered by viral binding.

The preliminary findings relating to the entry pathway of EV70 need to be confirmed, with the aim of future studies being to establish an integrated picture of the precise molecular and cellular steps involved in delivery of the viral genome to the cytoplasm.

### 6.1.2 Future research strategies and approaches

There are a number of approaches that can be used to identify viral receptor candidates. These include methods directly assessing viral binding, such as virus overlay protein blot assays (VOPBA) and recent glycan microarray technology, as well as screening using panels of antibodies and cDNA libraries.

VOPBA would be a particularly useful technique in the case of EV70, both to further investigate the nature of viral binding, and to specifically identify potential candidate receptors. The assay involves binding of labelled virus to membrane proteins extracted from the cells of interest and separated on a native gel. VOPBA has been used to identify several viral receptors including a 55kDa protein which is the receptor for adenovirus type 37, a cause of keratoconjunctivitis (Wu et al., 2001). The assay could potentially provide information on whether EV70 is binding to a single GPI-anchored protein on RBC, or a variety of proteins, by observation of binding to single or multiple protein bands respectively. Overlay assays using RBC could be compared with those using MRC5 cells or even non-primate cell lines. Analysis of binding patterns in cells treated with neuraminidase would give further information, in particular on whether an additional non-sialated coreceptor exists. There are however, several drawbacks to such analyses; if the virus binds to a significantly large numbers of different sialated proteins it may not be possible to differentiate specific binding from background, and in addition, binding to glycolipids would not be identified. However, should discreet bands be seen, mass spectrometry could then be used to identify potential receptors.

The non-permissive cell lines, CHO and RK13, could be particularly useful in the screening of cDNA libraries, looking for clones capable of rendering these cell lines fully permissive for viral infection. This approach would identify sialated or non-sialated proteins that are specifically required for EV70 entry, rather than just binding. Cells rendered permissive for EV70 infection could be detected using a  $\beta$ -galactosidase-linked antibody and the cDNA clone present identified by sequencing. Again, however, this approach is limited by its failure to allow identification of glycolipid receptors, and also as the defect in entry may not be receptor-related. Another potential problem is that the glycosylation of human proteins may not be conserved when expressed in non-human cell lines.

An alternative approach, that has the advantage of being able to identify both glycoprotein and glycolipid receptors, is the use of pools of monoclonal antibodies raised against membrane components. These can be screened for the ability to block

viral infection. Subsequently the specificity of blocking antibodies can be established. ICAM-1 was initially identified as a receptor by identifying a 95 kDa protein recognised by an antibody capable of blocking rhinovirus infection (Greve et al., 1989).

Although the above approaches may identify candidate receptors for EV70, they will not directly provide information on the requirement for specific glycosylation patterns or glycan sequences, nor will they yield useful results if the binding specificity is purely for a specific glycan moiety. If a receptor protein is identified its glycosylation can be determined, but this is not a straightforward task and requires first that the glycoprotein or glycolipid be purified and then the glycan groups detached and analysed using a combination of techniques including chromatography and mass spectrometry (Varki, 1999). As many proteins contain multiple glycan groups, and the exact structure of these can vary between individual molecules at the same site, interpretation is often complex. One particularly attractive technique for directly identifying the glycan structures to which a virus can bind, is the use of recently developed glycan microarrays (Blixt et al., 2004). These include over 200 glycan sequences, representing the major glycan structures found on glycoproteins and glycolipids, to which virus binding can be assessed. This would allow direct assessment of the glycan structures to which EV70 can bind; however, useful results would only be obtained if the virus is capable of binding to these in the absence of an underlying protein or lipid.

The availability of a full-length infectious clone now makes possible reverse genetic approaches to examine the effect of mutations at sites of interest within the EV70 genome. However, studies to identify the site of receptor binding on the EV70 capsid are limited at present, as the exact receptor is not known, and also due the lack of information on the precise structure of the EV70 virion that would enable accurate identification of the position of residues on the capsid surface. Residues potentially involved in binding in the canyon or at the axes of symmetry could be determined by comparison to known enterovirus structures; however there is likely to be too much sequence variation to make this a reliable strategy. One potentially informative approach would be to investigate the significance of the differences in capsid sequence between the virus used in these studies, and the published EV70 sequence; in particular the relevance to viral binding of the cluster of four base changes within a short 45 base region of VP1. Once a receptor is identified, it may be possible to examine crystal structures of the virus-receptor complex.

Viral uncoating of EV70 has not yet been studied, however this is an important step in release of the viral genome into the cytoplasm. Uncoating assays involve looking for evidence of a change in the viral sedimentation coefficient from the native 160S particle to the 135S or 'A' particle (Curry et al., 1996). Although there is still some doubt regarding whether the 135S particle is a true intermediate in the process, it has been used as a convenient method for monitoring uncoating. Questions that could be addressed include whether EV70 uncoats upon binding to the surface of MRC5 cells, or whether there are further requirements, such as exposure to low pH, as is the case with some rhinoviruses (Nurani et al., 2003). Uncoating could also be assessed on non-primate cell lines, giving information on the functionality of the receptor on these cells.

The use of dominant-negative proteins to investigate the effect on viral entry of inhibiting specific endocytic pathways has been discussed (section 5.5.1). However, another potential approach, which could also allow wider aspects of the entry pathway to be assessed, is the use of fluorescent microscopy. This would require either the availability of an antibody raised against EV70 that binds the 160S particle, or the production of fluorescently labelled virus. Indeed, previous studies have shown that enterovirus capsids can be successfully labelled without affecting receptor interactions (Kremser et al., 2004a; Pietiainen et al., 2004). Confocal microscopy could then be used at various time points following binding to examine the co-localisation of virus with potential receptors, markers of intracellular structures or elements of the cellular cytoskeleton. In addition the associations of virus capsids and viral RNA could be differentiated by taking advantage of the dynamic nature of the capsid, which allows viral RNA to be labelled inside intact particles (Kremser et al., 2004b). Studies can be enhanced by following the movement of individual virus particles and the use of real-time fluorescent microscopy, as has been done for influenza virus (Lakadamyali et al., 2003). However, such studies may be more challenging where there is a high particle to infectious unit ratio as is the case for enteroviruses.

### **6.1.3 Source of virus for future studies**

Virus can be derived either from cultured virus or from infectious clones. The use of virus recovered from infectious clones enables a ready supply of virus, which can be recovered unchanged over time, and which can easily be used by different laboratories to allow collaboration and ready comparison of research results. However, such virus will be uniform, and not representative of the variety of sequences present

within the viral quasispecies. The quasispecies may well be of clinical relevance, for example in terms of pathogenesis (Domingo et al., 2006). One possibility is that the rare occurrences of acute flaccid paralysis due to EV70 are due to a minor component of the quasispecies that allows the virus to access the central nervous system. If this were the case, studies using cloned virus might not be relevant to this aspect of the viral pathogenesis.

The potential problems of the use of highly passaged virus and reference strains have been discussed, and indeed observations during this study have suggested that there may be adaptation of EV70 to growth in different cell lines; for example changes from the published sequence and variation in findings between different laboratories. One way to circumvent this problem would be the use of clinical isolates; in particular to engineer sequences from these into the infectious EV70 clone, avoiding the need for further culture. It might also be possible to compare virus isolated from the conjunctiva and the central nervous system of patients with paralysis and compare binding characteristics and potential differences in tropism. One problem that has been encountered in this study is the difficulty of obtaining clinical isolates. The last outbreak of EV70 reported in the literature was in 1996 in Delhi, India (Maitreyi et al., 1999). Despite contact with a number of laboratories across Asia, it was not possible to obtain any samples from EV70 epidemics. It is likely that a more extensive search would locate some such samples, additionally closer contacts with diagnostic laboratories within South East Asia would allow early collaboration should a further outbreak be detected.

#### **6.1.4 Cell lines and models for use in future studies**

The studies here have primarily centred on EV70 infection of MRC5 cells. There were a number of advantages to use of this cell line; the virus grows cytopathically in it and to good titre, it is derived from the natural host of the virus (human), and it is a primary cell line that is not transformed and transformation is known to have wide-ranging effect on the expression of cell surface molecules and their glycosylation (Varki, 1999). Additionally recent passage of the virus at ATCC has been in WI-38 cells, another embryonic lung fibroblast cell line. However there were a number of disadvantages to the use of MRC5 cells, including a number uncovered during this study. As a primary cell line, it is possible that increasing passage results in changes within the cells. Cells were used over a span of 18 population doublings well

before senescence, and this was shown not to affect viral yield, however other more subtle effects could not be excluded. Certain approaches were not possible using MRC5 cells, due to problems of sensitivity to treatment with proteases and lipases and reduced EV70 infection in cells that had undergone transfection. Although human, these cells are fibroblasts, rather than epithelial like the cells of the conjunctiva. Epithelial cells are characterised by polarity, having distinct apical and basal membranes, and this may affect receptor expression patterns or endocytic pathways.

To overcome this last point, it would be desirable to use a conjunctival cell line. As yet no good conjunctival cell lines are readily available. One cell line derived from human conjunctiva (Chang), has been shown to be less differentiated and differ in several key characteristics from primary conjunctival cells (De Saint Jean et al., 2004). Other candidate immortalised conjunctival cell lines, which show at least the same pattern of mucin expression to native conjunctiva are potential future candidates (Diebold et al., 2003; Gipson et al., 2003). The use of neuronally derived cell lines would also be useful, particularly where neuronal tropism and pathogenesis is being investigated.

Of course cell lines do not accurately represent the *in vivo* situation, and one hope for the future would be the availability of an animal model of EV70 infection. Intraspinal injection of the virus caused paralysis in chimpanzees, however EV70 (at least in this study) does not appear to efficiently infect rodent cell lines. It may be possible by serial passage of virus in murine cell lines to derive a virus that can efficiently infect them, which might then also permit infection of the host species, but with the risk of alteration in the viral receptor usage. Another solution would be the generation of transgenic animals, perhaps expressing a candidate human receptor protein. For example poliovirus research has been greatly helped by the availability of a transgenic mouse model that expresses PVR (Ren et al., 1990). However, the expression of a receptor molecule may not always result in the expected pathology; for example CVA21 causes paralysis in mice transgenic for ICAM-1, as opposed to the respiratory symptoms associated with human infection. Further cell culture based research will be important in elucidating the requirements for the generation of an animal model for EV70. Such a model would be particularly important for future studies investigating EV70 tropism and pathogenesis.

## **6.2 Central themes in viral attachment and entry**

### **6.2.1 Receptor usage and delivery of the viral genome into the cell**

A successful virus must be capable of transferring its genome between cells and between organisms. To do this it must be capable of associating with a cell that can support its life-cycle, and be able to deliver its genetic material, and any viral proteins required for replication and translation, to the interior of that cell.

The choice of receptor is obviously an important factor in this process. As will be discussed in the next section, the receptor largely dictates which cell types and species a virus can infect. The cell type infected must be able to efficiently support the viral life-cycle, not only intracellularly, but also in terms of the organism as a whole and the mechanism of transmission of the virus. EBV, for example, is believed to have dual tropism for its primary site of replication and latency, the B cell, and for the epithelial cells of the oral mucosa which permit efficient transmission between individuals in saliva (Faulkner et al., 2000). Viruses that cause acute illnesses, with relatively short periods of viral excretion, must be able to efficiently infect tissues to which they have ready access and exit. Infection of the respiratory tract, which is seen for a very wide range of viruses including influenza viruses, rhinoviruses and adenoviruses, is not only facilitated by inhalation, but is also an easy site for exit and transmission between individuals by sneezing and coughing (often a consequence of viral infection). Viruses can enter the digestive tract easily by ingestion, and exit easily in the faeces where transmission may be further facilitated by diarrhoea; the ease by which such enteric infections can be transmitted by faecal oral contact and fomites is attested to by the frequency and extent of rotavirus outbreaks in children, and intractable hospital outbreaks of norovirus (Bishop, 1996; Chadwick et al., 2000). The conjunctiva also represents an easily accessible target for EV70, particularly in situations of poor hygiene, where rubbing of eyes (which will be increased due to the irritation of inflammation), result in contamination of hands and the environment. In the case of viruses which cause chronic or latent infection poor accessibility of tissues may be overcome by the length of time for which virus can be excreted. For example HCV infects the liver, which is accessed primarily via the blood stream, either iatrogenically or via needle-sharing. However, in the 80% of infected individuals where chronic HCV

infection is established, the continued presence of the virus in the blood stream for the life-time of the patient ensures opportunities for transmission (Shepard et al., 2005).

The receptor is not only important in associating the virus with the correct target cell, but also in facilitating entry and the viral life-cycle within the cell. In chapter 5, the importance of cellular binding in terms of entry was discussed, with important factors being the location of the receptor on the cell surface and the triggering of endocytic pathways. However, there may also be wider effects. Signal transduction effects may not only assist internalisation, but may also render the cell receptive for later stages of the viral life-cycle; for example by upregulating the expression of proteins required for viral translation and replication. One of the major challenges a virus has to overcome is the host immune system. Many viruses, such as adenoviruses and herpes viruses, encode a number of proteins with cytokine and immune functions. Viruses with smaller genomes, such as picornaviruses, may achieve similar results by using as receptors proteins with immune functions. For example, it has been suggested that binding to members of the RCA family of proteins by Epstein-Barr virus, measles virus and many enteroviruses, may be selected for, in some way allowing the viruses to avoid complement related cell damage (Lindahl et al., 2000). However, DAF may be favoured as a receptor for other reasons: for example via its association with cell signalling pathways, such as those involved in the lipopolysaccharide receptor complex, of which DAF is a part (Heine et al., 2003). In addition DAF has been observed to be removed from the cell surface both by endocytic mechanisms, potentially favouring viral entry, and also by shedding from the cell surface, which might be a mechanism for promoting eventual viral release from the cell.

Conceptually viral internalisation (as opposed to actual crossing of cellular membranes) is not a difficult challenge for a virus, as many cell surface molecules are naturally endocytosed following ligand binding. Indeed viruses seem capable of using a variety of routes either between families, within families (such as the *Picornaviridae*) and even for individual viruses, as influenza virus can use both clathrin-mediated and clathrin-independent methods of endocytosis (Marsh and Helenius, 2006; Sieczkarski and Whittaker, 2002b). It may be postulated that a specific route of entry is required to ensure delivery of the virus to the correct site of viral replication in the cell. This is potentially important for some viruses, however for others it seems an unlikely constraint on the virus; for example RNA derived from cloned enteroviruses is efficiently replicated, even when delivered non-specifically to the cytoplasm by

electroporation. Perhaps the important challenge for the virus is the requirement to deliver its genome, and any necessary proteins, to the internal cellular environment. This requires that the viral nucleic acid cross cell membranes, either the plasma membrane or the membrane of intracellular vesicles, and also that the virus is released from the viral particle itself (uncoating), and these processes are often tightly linked.

Virus particles can be described as existing in a metastable state (Marsh and Helenius, 2006; Steven et al., 2005). They require a structure that is sufficiently stable in the extracellular environment to allow efficient transfer from one infected cell to the next and from one infected organism to the next. However, this structure must also be capable of being readily destabilised to allow uncoating, and release of the viral nucleic acid into the cell. Perhaps the primary requirement of a specific route of endocytosis, and partly for a specific receptor, is in fact to ensure exposure of the virus to a set of factors that trigger this process. Poliovirus, for example, requires only contact with the viral receptor on the cell membrane to trigger uncoating, with the formation of a membrane pore allowing penetration, while other picornaviruses also require receptor interaction, but with the additional factor of low pH in late endosomes (Nurani et al., 2003), and this may also be the case for EV70. Many enveloped viruses, such as influenza virus, also require low pH to induce conformational changes in viral proteins that trigger membrane fusion and nucleic acid release (Marsh and Helenius, 2006; Skehel and Wiley, 2000). Other viruses may require specific cellular proteases or reducing environments as triggers of uncoating and penetration (Ebert et al., 2002; Greber et al., 1996; Li et al., 1998; Simmons et al., 2005).

### 6.2.2 Tropism and pathogenesis

It is well established that the viral receptor is the primary determinant of species tropism. The engineering of transgenic mice to express specific viral receptors, has been used to create animal models for a number of viral infections, including poliovirus, measles and the SARS coronavirus (Patterson et al., 2001; Ren et al., 1990; Tseng et al., 2007). In the case of influenza viruses the receptor specificity largely dictates the host species, with  $\alpha$ 2-3 linked sialic acid specific for avian strains and  $\alpha$ 2-6 for human strains. In avian viruses a change in specificity, from  $\alpha$ 2-3 to  $\alpha$ 2-6 linked sialic acid, is associated with an increase in the ability of the virus to infect and be transmitted among humans. Changes in receptor specificity may therefore be an important mechanism by

which animal viruses might emerge as new human pathogens, and may well have been a contributory factor in the emergence of EV70.

It is clear that receptor usage is also of primary importance in viral tissue tropism; if the receptor is not expressed on a particular tissue the virus will not infect it. Some viral receptors are indeed expressed on a very limited range of cell types; for example CD4, the HIV receptor is found primarily on T-lymphocytes, and the RBC P antigen is the receptor for human parvovirus B19, which specifically infects RBC progenitors (Heegaard and Brown, 2002). However, many viral receptors are expressed on a much wider range of tissues than are infected *in vivo*. In transgenic mice, PVR, the poliovirus receptor, is expressed in a wide range of tissues, including the kidneys and lung, which is similar to PVR expression in humans (Ren and Racaniello, 1992); despite this, in both humans and mice, poliovirus replication is largely restricted to the intestine and neuronal tissues. Human influenza virus predominantly infects the respiratory tract, despite the presence of  $\alpha$ 2-6 linked sialic acid on a wide range of tissues, and although avian H7N7 influenza virus (with a specificity for  $\alpha$ 2-3 linked sialic acid) can infect the conjunctiva, other avian influenza viruses with the same specificity do not. Therefore other factors must exist that influence tissue tropism *in vivo*. Perhaps one of the most obvious is that the virus must have physical access to a particular tissue to infect it. Thus major group rhinoviruses may not infect the gut epithelium, not because these cells lack ICAM-1, but because viral infectivity is destroyed by gastric acid before the virus reaches the intestine. Poliovirus may not infect the kidney as they do not have ready access to this organ, and this possibility is to some extent corroborated by the fact that renal cell cultures derived from PVR transgenic mice do support poliovirus infection (Ren and Racaniello, 1992). Other determinants of tissue tropism might relate to viral requirements for specific entry routes, activation of cell signalling pathways or the presence of essential coreceptors. In addition to these early events in the viral life-cycle, there may be a requirement for tissue-specific cellular factors in viral replication, transcription and translation.

Pathogenesis is usually a result of the primary tissue tropism of a virus; for example HIV infects T-lymphocytes and causes immunodeficiency, HCV infects hepatocytes and causes hepatitis, human parvovirus B19 infects RBC precursors and causes anaemia and EV70 infects the conjunctival epithelium and causes conjunctivitis. However, where a particular infection causes less common pathology, the picture is less clear. EV70 causes acute flaccid paralysis rarely, in one in ten thousand cases, and this

is also a rare result of infection with a number of other non-polio enteroviruses. This may be due to the inability of these viruses to replicate in neuronal tissue unless there exists within the quasispecies a virus with altered tropism, or to a chance event that allows the virus to cross the blood brain barrier and access the central nervous system. Alternatively central nervous system infection or symptoms may be determined by host factors, such as the ability to mount an effective immune response against the virus.

### **6.3 EV70 as a human pathogen**

EV70 infection has been characterised by a dramatic emergence in the human population. Assuming estimates of the date of a common, presumably animal-based, EV70 ancestor are correct (Takeda et al., 1994), within four years of its emergence, EV70 had spread to several countries and infected over 20 million people. This viral success story was presumably due to its emergence as a highly infectious virus that was easily spread between individuals, particularly in areas of crowding or poor hygiene. However, now forty years on, EV70 outbreaks are observed in much smaller clusters and have been separated by up to ten years. There is also a suggestion of changes in the clinical presentation of the infection.

Following the initial Southeast Asian pandemic of 1970/1971 numerous smaller outbreaks were reported in Africa, India and Japan until the early 1980s (Higgins, 1982). During a ten-year period, from the mid-1980s, EV70 infection was rarely detected (Saitoh-Inagawa et al., 1999); however, in the mid-1990s several epidemics (affecting thousands, rather than millions) occurred in India and Japan (Maitreyi et al., 1999; Uchio et al., 1999). The last outbreak of EV70 infection reported in the literature was in Delhi in 1996. These later outbreaks were less well reported than the initial pandemic, however a number of interesting observations were made. Firstly the virus proved more difficult to culture than previously, and secondly, outbreaks were smaller and symptoms milder, with fewer haemorrhagic features and potentially fewer neurological side effects (although with the smaller numbers affected this cannot clearly be determined), there also seemed to be an increase in the proportion of infections occurring in children (Bern et al., 1992; Maitreyi et al., 1999; Uchio et al., 1999).

The decrease in the incidence and size of EV70 outbreaks may have been due to high levels of immunity in the population following the 1970/1971 pandemic, and this may not have supported further outbreaks in these regions. However, infection has been

observed in areas and individuals previously infected, suggesting that immunity is not complete (Bern et al., 1992), and additionally with the birth of a new largely susceptible generation the frequency of infection would have been expected to increase after an interval, and this is not the pattern seen. Perhaps a more likely explanation, given the potential changes in symptoms, epidemiology and viral characteristics, is that the virus is adapting to its new human host. Given the initial success of the virus this is most likely to have been due to immune pressures, with the severity of the initial symptoms suggesting a vigorous immune response in the conjunctiva. Further adaptation may have reduced the immune stimulus, allowing prolonged viral shedding and a less long-lasting immunity. An alternative explanation, which cannot be discounted, is that there has been more than one introduction of the virus into the human population following changes in the virus within its natural host that rendered it more transmissible to humans.

EV70 remains a potentially significant human pathogen, however, and despite its recent reduction in incidence and potentially severity, it is still capable of causing large epidemics with the potential for severe neurological consequences in the individual. Due to its capacity for rapid spread it can incapacitate large proportions of the working population in a small geographical area within a short period of time, with resultant economic chaos, and in a world where paralysis due to poliovirus is becoming increasingly rare due to the effectiveness of the eradication campaign (Kew et al., 2005), other viral causes of acute flaccid paralysis are gaining in significance. A further risk is, that as an RNA virus capable of considerable adaptation, and also as a relatively new human pathogen still adapting to the human host, a strain may emerge in the future with increased neurotropism or the potential to cause long-lasting ocular damage. It is also relevant that EV70 was being considered as one of only three viruses in Iraq's Bioweapons program (Stone, 2002).

The changes and adaptability of the virus present unique problems in the diagnosis and management of EV70 infection. With fewer cases and milder symptoms the clinical diagnosis may be less obvious, with symptoms being overlooked or misdiagnosed as adenoviral conjunctivitis. Traditionally enteroviral infections have been diagnosed by tissue culture that may fail to isolate more recent fastidious strains, indeed even the reference strain does not result in observable CPE in many standard cell lines. Where CPE is observed, enterovirus identification requires further typing by neutralisation, which is now more difficult due to the withdrawal of the World Health

Organisation's serotype-specific antibody neutralisation pools. Currently many diagnostic laboratories have switched to PCR as a method for identifying viral infections, including enteroviruses. The identification of EV70 would then require that the primers used for generic enterovirus PCRs and typing by sequencing (usually VP1 specific) are capable of amplifying EV70. Given that there are currently only three known group D enteroviruses, the data set for designing such primers is very small. Even where specific EV70 primers are used, changes in viral sequence may prevent detection of more recent isolates.

There are currently no effective antivirals in use against picornaviruses. The most promising compounds are the so-called WIN compounds (originally developed at Sterling Winthrop in the USA), which act by occupying the base of the viral canyon and stabilising the capsid, thus preventing uncoating. Pleconaril is one such compound that showed promise in early trials in enterovirus infections and was used clinically on a compassionate basis from the late 1990s (Webster, 2005). Pleconaril was shown to have a broad activity against rhinoviruses and enteroviruses, although was never formally tested against EV70. However in 2003, following failure to obtain a licence for the treatment of the common cold in the USA, pleconaril production was discontinued. Another treatment which might prove useful against EV70 infection is polyvalent sialic acid compounds, which have been shown to be effective against influenza virus (Matrosovich and Klenk, 2003).

EV70 is a highly adaptable virus, which remains a threat to the human population. Further research into early steps in the viral life-cycle, and in particular the interaction of the viral capsid with its receptor and the cell, is essential to our understanding of viral pathogenesis and of the action of potential antiviral compounds. Of particular importance is that these studies are not limited to use of the reference strain, but that clinically relevant viral strains or cloned viral sequences are used to test the efficacy of potential treatments and to compliment future research.

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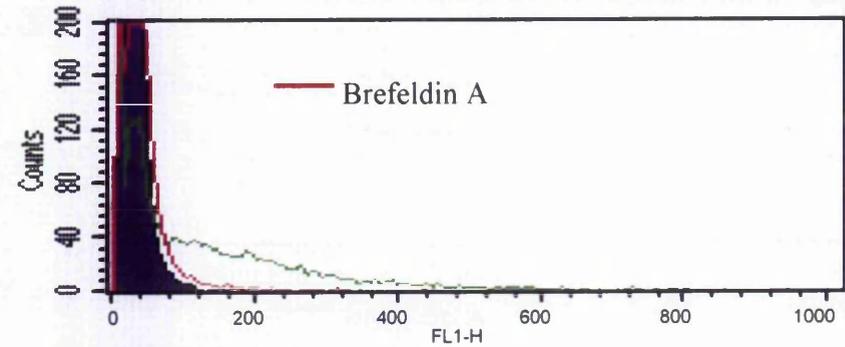
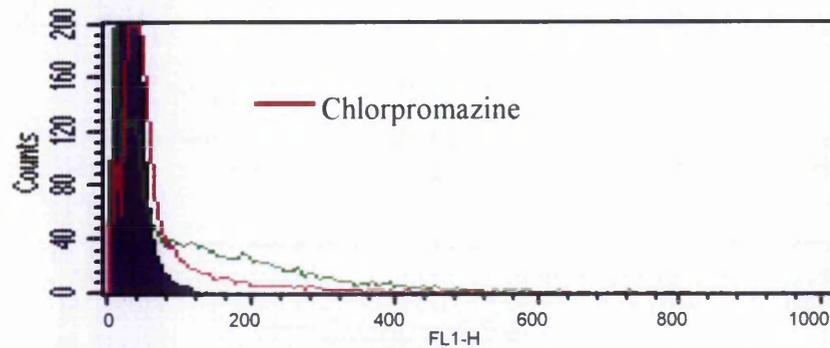
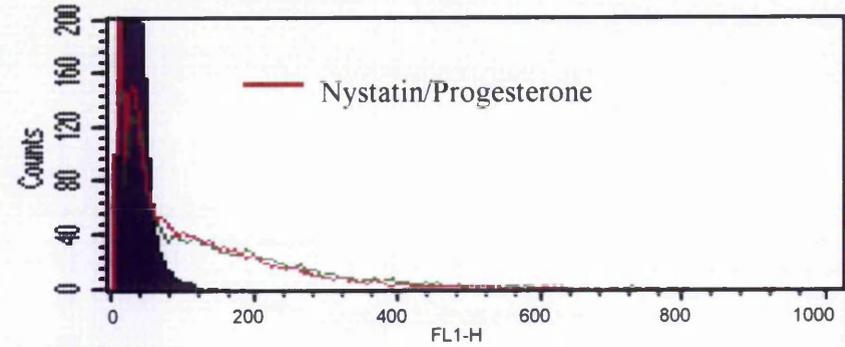
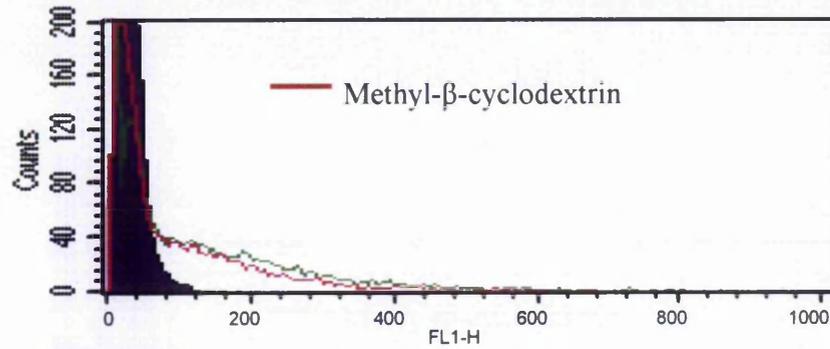
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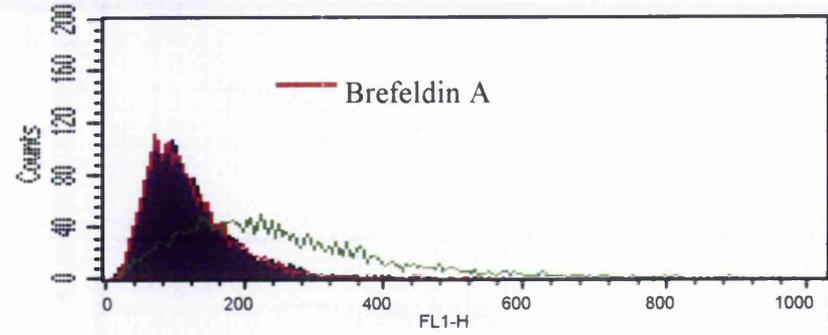
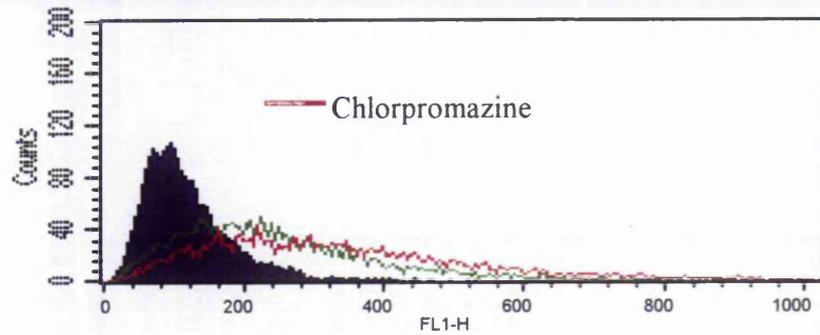
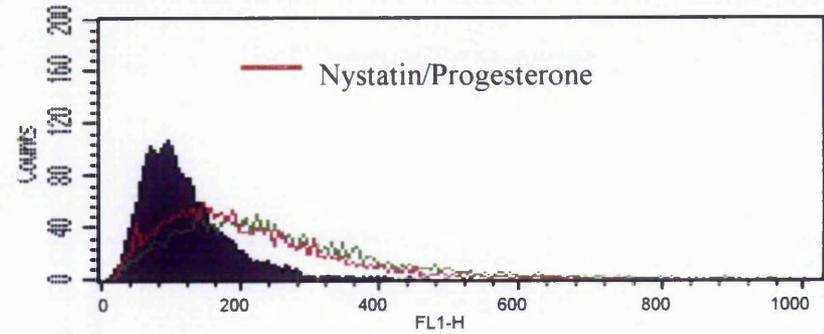
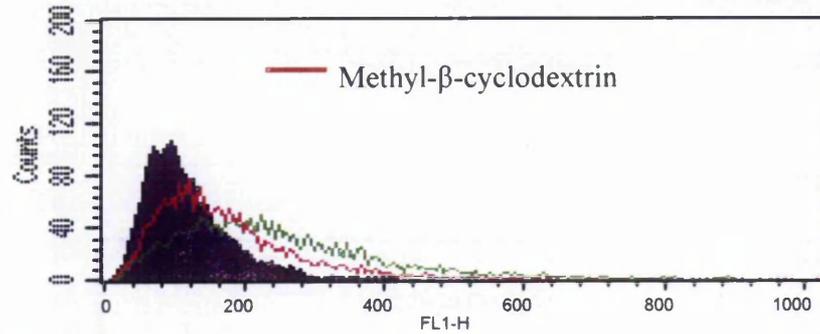
## EV70



**Appendix 1 Example of histogram plots from one flow cytometry-based entry assay using EV70.** MRC5 cells were treated with the agents indicated, at the concentrations detailed in section 5.1.1 and figure 1.5A, and infected with EV70 at an m.o.i. of 3. After 6 hours incubation in the continued presence of the inhibitors, cells were washed, fixed, permeabilised and EV70 detected using the primary antibody, anti-enterovirus 70 mouse monoclonal 854, and an anti-mouse IgG FITC conjugate as the secondary. Cells were analysed by flow cytometry. A plot of forward versus side scatter for the untreated cells was used to gate the population of intact cells. 10 000 gated events were counted per sample, and the number of counts plotted against FL1-H (FITC staining).

■ Control, uninfected cells. — Control, EV70 infected untreated cells. — EV70 infected treated cells.

## Echovirus type 11



**Appendix 2 Example of histogram plots from one flow cytometry-based entry assay using Echovirus type 11.** MRC5 cells were treated with the agents indicated, at the concentrations detailed in section 5.1.1 and figure 1.5A, and infected with echovirus type 11 at an m.o.i. of 3. After 6 hours incubation in the continued presence of the inhibitors, cells were washed, fixed, permeabilised and echovirus type 11 detected using the primary antibody, anti-enterovirus mouse monoclonal 5-D8/1, and an anti-mouse IgG FITC conjugate as the secondary. Cells were analysed by flow cytometry. A plot of forward versus side scatter for the untreated cells was used to gate the population of intact cells. 10 000 gated events were counted per sample, and the number of counts plotted against FL1-H (FITC staining).

Control, uninfected cells.
  Control, echovirus type 11 infected untreated cells.
  echovirus type 11 infected treated cells.

**Proportion of infected cells, as a percentage of the untreated, infected controls for each assay.**

<b>EV70</b>	<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>	<b>F</b>	<b>G</b>	<b>H</b>	<b>I</b>	<b>J</b>	<b>K</b>	<b>Mean</b>	<b>SD</b>
Chlorpromazine			65	39	92			29	35	36		<b>49</b>	24.37
MethylBcyclodextrin	68	83	102		100			68	93	71		<b>84</b>	14.95
Nystatin / Progesterone		110	122		117			112		91	121	<b>112</b>	11.41
Brefeldin A	7		4		7			5		10	4	<b>6</b>	2.32
Genistein						28	3		9			<b>13</b>	13.05
PP2									40	39	49	<b>43</b>	5.51
Bisindoylmaleimide						157	93		157			<b>136</b>	36.95
NH4Cl						48	85		95			<b>76</b>	33.86
Bafilomycin						109	104		95			<b>103</b>	7.09
Nocodazole						148	100		112			<b>120</b>	24.98
Cytochalasin D						55			74	55		<b>61</b>	10.97

<b>Echo 11</b>								<b>H</b>	<b>I</b>	<b>J</b>	<b>K</b>	<b>Mean</b>	<b>SD</b>
Chlorpromazine								150	134	111		<b>132</b>	19.6
MethylBcyclodextrin								35	6	64		<b>35</b>	29
Nystatin / Progesterone								60		102	32	<b>74</b>	35.23
Brefeldin A								9		9	13	<b>10</b>	2.31

**Appendix 3 Results from flow cytometry-based entry assays used to construct figures 5.1A and 5.7A.** Columns A to K represent assays done on separate occasions. Monolayers of MRC5 cells were pre-treated with chemicals at the concentrations detailed in the relevant sections, and infected with either EV70 or echovirus type 11 at an m.o.i. of 3. Cells were then incubated for 6 hours in the presence of the chemical and the proportion of infected cells assessed by flow cytometry, counting 10 000 events per sample. The proportion of infected cells is given as a percentage of the untreated infected controls in each assay. The results presented in columns A to K represent the mean results from duplicate assays done in parallel, while the final two columns give the mean results, with standard deviation, for assays done on separate occasions. Inter-assay variation was noted to be far greater than intra-assay variation (data not shown).