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University of Glasgow

**Enteroviral evolution: interspecies
recombination and implications for
picornavirus research**

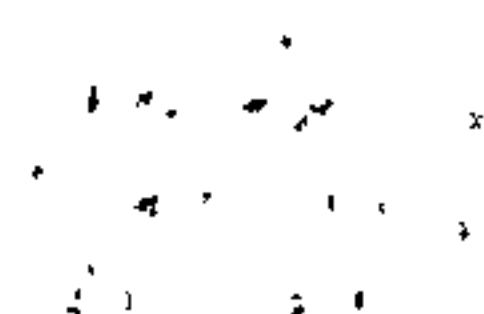
By

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A thesis submitted for the degree of Doctor of Philosophy at the University of Glasgow

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Statement

**The research reported within this thesis is my own work, except where otherwise stated,
and has not been submitted for any other degree**

Claire Blanchard

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Abstract

Poliomyelitis is a seriously debilitating human disease, caused by poliovirus. Since the initiation of the World Health Organisation (WHO) and the Pan American Health Organisation (PAHO) poliomyelitis eradication program, mass immunisation of the worldwide population and better monitoring of acute flaccid paralysis (AFP) cases enabled the reduction in the number of poliomyelitis cases worldwide and the eradication of circulating wild-type polioviruses in a large number of countries. The oral live-attenuated poliovirus vaccine (OPV) and inactivated poliovirus vaccine (IPV) have been used extensively as a tool for achieving poliomyelitis eradication. Since the introduction of OPV, neurovirulent revertant poliovirus vaccine strains have been reported to cause rare cases of paralysis in vaccinees and/or contacts of vaccinees. However, recent reports of vaccine-associated acute paralysis (VAAP) outbreaks, caused by newly emerging circulating vaccine-derived polioviruses (cVDPVs), in Hispaniola and Madagascar have raised the existing concerns about the potential of poliovirus vaccine strains to recombine with human enteroviruses (HEV). The original aim of the project was to determine whether a non-poliovirus HEV could evolve to use the poliovirus receptor (PVR). A variety of methods were used to exploit aspects of the evolutionary capacity of viruses to achieve this goal. Although the aim was not attained, the investigation of recombinants between different HEVs yielded interesting results, which were pursued further. Two approaches were developed: *in vitro* generation of recombinant viruses and phenotypic analysis of such chimeras and the selection for recombinant viruses *in vivo*.

In vitro generation of reciprocal recombinants between the structural and the non-structural coding region of coxsackievirus A21 (CVA21) and poliovirus type 3 (PV3) was initiated. Transfected and passaged chimeras did not produce infectious virions.

Immunofluorescence analysis of VP1 protein expression suggested that the recombinants were not acytopathic. A series of assays were then carried out to investigate the nature of the defect. HeLa S10 translation/transcription reactions of the *in vitro* generated recombinants expressed the correct protein-processing pattern suggesting efficient processing occurred *in vitro*. Replication assays demonstrated that the chimeras were replication competent. Trans-encapsidation experiments were then carried out and preliminary results strongly suggested that the defect could lie at the packaging level. Additional studies are required.

Selection of recombinants *in vivo*, without predetermining the crossover sites, was also conducted. Under the conditions used, recombination between CVA21 and PV3 impaired genomes and echovirus 7 (EV7) and PV3 impaired genomes proved to be unsuccessful. This could have been due to both genomes localising to separate replication compartments rendering replication-dependent recombination impossible. This needs to be verified. Characterisation of the impaired parental genomes used for the experiment needs to be carried out. However, recent reports of recombinants between Sabin polioviruses and HEV-C confirm the possibility of such a recombination event occurring and emphasize concerns regarding the success of the polio eradication program.

RNA viruses are also subjected to polymerase error rate, another evolutionary mechanism. Ribavirin, a random mutagen, was used to increase the error rate of CVA21, a HEV-C, to investigate whether this particular HEV-C could evolve to use an alternative receptor for attachment and entry. However, no novel tropic viruses were recovered under the experimental conditions applied.

Abbreviations

AFP	Acute flaccid paralysis
ATP	Adenosine tri-phosphate
bp	base pair
BEV	Bovine enterovirus
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
CNS	Central nervous system
CO ₂	Carbon dioxide
CPE	Cytopathic effect
cpm	counts per minute
CRE	<i>Cis</i> -acting replication element
cDNA	DNA complementary to RNA
CVA21	Coxsackievirus A21
DAF	Decay accelerating factor
dH ₂ O	Distilled water
DI	Defective interfering
DMEM	Dulbecco's modified Eagle's medium
DNase	Deoxyribonuclease
DTT	Dithiothreitol
dNTP	Deoxyribonucleoside 5' triphosphate
DNA	Deoxyribosenucleic acid
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediamine tetra acetic acid
eIF	Eukaryotic initiation factor
EMCV	Encephalomyocarditis virus
ER	Endoplasmic reticulum
ERAV	Equine rhinitis virus
EV7	Echovirus 7
FITC	Fluorescein isothiocyanate
FLC	Full length clone
FMDV	Foot and mouth disease virus
g	Gram(s)
GPI	Glycosylphosphatidylinositol
GTP	Guanosine tri-phosphate
GuHCl	Guanidine hydrochloride
HAV	Hepatitis A virus
HCV	Hepatitis C virus
HEPES	N-((2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid))
HEV	Human enterovirus
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
HRVA	Major rhinovirus group
HRVB	Minor rhinovirus group
HRV14	Human rhinovirus 14
ICAM-1	Intracellular adhesion molecule-1
IPV	Inactivated poliovirus vaccine
IRES	Internal ribosome entry site
kDa	Kilodaltons

kb	Kilobases
mg	Milligram(s)
MHC	Major histocompatibility complex
ml	Millilitre(s)
mM	Millimolar
M.O.I.	Multiplicity of infection
mRNA	Messenger RNA
NIDS	National immunisation days
nPTB	Neuronal form of PTB
nt	Nucleotides
NTR	Non-translated region
OPV	Oral poliovirus vaccine
ORF	Open reading frame
PABP	Poly (r) A binding protein
PAGE	Polyacrylamide gel electrophoresis
PAHO	Pan American Health Organisation
PBS	Phosphate buffered saline
PCBP	Poly (r) C binding protein
PCR	Polymerase chain reaction
PEV	Porcine enterovirus
pmol	Picomole(s)
PTB	Polypyridimine tract binding protein
PV	Poliovirus
PV3	Poliovirus type-3
PVR	Poliovirus receptor
RD	Rhadosarcoma cells
RD-ICAM	Rhadosarcoma cells expressing ICAM-1
RdRp	RNA dependent RNA polymerase
RF	Replication form
RFLP	Restriction fragment length polymorphism
RI	Replication intermediate (picornavirus)
RNA	Ribonucleic acid
RNA-i	RNA-mediated interference
rpm	Rotations per minutes
RTP	Ribavirin triphosphate
siRNA	Small interfering RNA
SNIDS	Sub-national immunisation days
SCR	Short consensus repeat
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
TNF	Tumour necrosis factor
TMEV	Theiler's murine encephalomyocarditis virus
tRNA	Transfer RNA
Tris	Tris (hydroxymethyl) methylamine
U	Units
UTR	Untranslated region
UV	Ultra violet
VAPP	Vaccine-associated paralytic poliomyelitis
VDPV	Vaccine-derived poliovirus
v/v	Volume by volume

WHO	World Health Organisation
w/v	Weight by volume
X-Gal	5-bromo-4-chloro-3-indolyl-beta-galactopyranoside
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
°C	Degrees Celsius

Discrepancies observed in CVA21 related plasmid names were due to a recent change in the nomenclature and conservation of the original plasmid names in the laboratory database (see table of plasmids and appendix 2).

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1 Introduction

1.1 Picornaviruses

Picornaviruses are some of the smallest RNA viruses: pico (very small unit of measurement 10^{-12}) na (RNA) viruses. The *Picornaviridae* family comprises some of the oldest known viruses such as the foot and mouth disease virus (FMDV), one of the first viruses to be recognised (Loeffler & Frosch, 1898), and poliomyelitis, which has been prevalent since at least the 18th Egyptian dynasty (1550-1333 BC) as is shown in an illustration of a doorkeeper on a funeral stone (Melnick, 1982).

1.2 Classification of the *Picornaviridae* family

The *Picornaviridae* family is composed of over 200 serotypes. The original taxonomy was based on physical properties (particle density and pH-sensitivity) and serological relatedness. However, picornavirus classification was recently revised based on nucleotide sequences and divided into 9 genera: Aphthoviruses, Cardioviruses, Enteroviruses, Erboviruses, Hepatoviruses, Kobuviruses, Parechoviruses, Rhinoviruses and Teschoviruses (see table 1.1). The nine genera include pathogens that cause a wide range of mild to acute diseases.

Genus	Species	Serotypes
<i>Aphthovirus</i>	<i>Foot and Mouth Disease Virus</i> <i>Equine Rhinitis A virus</i>	FMDV-O, A, C, Asia 1, SAT1-3 ERAV
<i>Cardiovirus</i>	<i>Encephalomyocarditis Virus</i> <i>Theileovirus</i>	EMCV TMEV, VHEV
<i>Enterovirus</i>	<i>Poliovirus</i> <i>Human Enterovirus A</i> <i>Human Enterovirus B</i> <i>Human Enterovirus C</i> <i>Human Enterovirus D</i> <i>Bovine Enterovirus</i> <i>Porcine Enterovirus A</i> <i>Porcine Enterovirus B</i> <i>Simian Enterovirus A</i>	PV 1-3 CVA 2-8, 10, 12, 14, 16, EV71 CVB 1-6, CVA9, E1-33, EV69-73 CVA 1, 11, 13, 15, 17-24 EV-68, EV- 70 BEV-1, BEV-2 PEV-8 PEV-9, PEV10 SEV-A
<i>Erbovirus</i>	<i>Equine Rhinitis virus</i>	ERBV 1 and 2
<i>Hepatovirus</i>	<i>Hepatitis A virus</i> <i>Avian Encephalomyelitis Virus</i>	HAV AEV
<i>Kobuvirus</i>	<i>Aichi virus</i> <i>Bovine Kobuvirus</i>	AiV BKV
<i>Parechovirus</i>	<i>Human Parechovirus</i> <i>Ljungan Virus</i>	HpeV-1 and 2 LV
<i>Rhinovirus</i>	<i>Human Rhinovirus A</i> <i>Human Rhinovirus B</i>	HRV 1, 2, 7, 8, 9 –13 etc HRV 3-6, 14, 17, 26 etc
<i>Teschovirus</i>	<i>Porcine Teschovirus</i>	PTV 1-11

Table 1.1: Classification of members of the *Picornaviridae*.

1.3 Pathogenesis within a host

Pathogenesis is defined by the broad spectrum of symptoms triggered by viral infection and the host range or tissues susceptible to infection by specific viruses. Because of the nature of this project, emphasis and detailed analysis of the human enterovirus (HEV) pathogenesis and life cycle, and not that of other genera of the *Picornaviridae* family, is contained in this chapter. The majority of HEV infections cause asymptomatic or mild syndromes (see table 1.2). However, HEVs have also been involved in cardiac and central nervous system (CNS) syndromes. Invasion of the CNS is characteristic of poliovirus, the prototype picornavirus, resulting in acute flaccid paralysis (AFP). However, Coxsackieviruses A have also been shown to cause AFP. In fact recent studies carried out in ICAM-1 transgenic mice have shown that CVA21 could cause identical symptoms to those seen in poliovirus infected PVR-transgenic mice (Dufresne et al., 2004).

	Clinical diseases associated with Infection
<i>Enteroviruses</i>	
<i>Poliovirus type 1, 2 and 3</i>	Paralytic poliomyelitis and mild febrile illness
<i>Coxsackievirus A</i>	Aseptic meningitis Hand, foot and mouth disease Common cold
<i>Coxsackievirus B</i>	Aseptic meningitis Myocarditis, pleurodynia, severe systemic disease in infants Upper respiratory tract illness, pneumonia and post-viral fatigue syndrome
<i>Echoviruses</i>	Aseptic meningitis Paralysis Respiratory disease Myocarditis Epidemic myalgia Hepatic disturbances
<i>Enterovirus 68</i>	Pneumonia
<i>Enterovirus 70</i>	Acute haemorrhagic conjunctivitis
<i>Enterovirus 71</i>	Hand, foot and mouth disease
<i>Rhinoviruses</i>	
<i>Major receptor group rhinoviruses</i>	Common cold
<i>Minor receptor group rhinoviruses</i>	Common cold
<i>Parechoviruses</i>	
<i>Echoviruses types 22 and 23</i>	Respiratory disease Encephalitis
<i>Hepatoviruses</i>	Hepatitis
<i>Aphthoviruses</i>	Foot-and-mouth disease
<i>Cardioviruses</i>	
<i>Encephalomyocarditis virus and Mengovirus</i>	Encephalitis and myocarditis
<i>Theiler's murine encephalomyocarditis virus</i>	Encephalomyelitis (in mice)

Table 1.2: Clinical diseases associated with picornavirus infection and exacerbation.

1.3.1 Poliovirus pathogenesis and life cycle within a human host

Poliovirus is the prototype HEV and has been extensively studied since it was first identified as the etiologic agent of poliomyelitis in 1909 (Landsteiner & Popper, 1909). Infection with poliovirus causes mild or asymptomatic syndromes in 90% of cases and infection only progresses to poliomyelitic paralysis in 1% of cases (Melnick et al., 1996 a). Poliovirus infects cells of the digestive tract (oropharyngeal cells and intestinal cells), multiplies locally at the initial sites of virus ingestion (tonsils, Peyer's patch) and virions are shed into the environment through faeces (see figure 1.3). The translocation of poliovirus particles across M-like cells, which are found in the epithelial sheet of the Peyers patches (Ouzilou et al., 2002) enables the virus to come in contact with cells from the immune system, which are then thought to favour spread to cervical and mesenteric lymph nodes. These events lead to a primary viraemia during which poliovirus can replicate in peripheral blood mononuclear cells (Freidstadt & Eberle, 1996). Once inside the bloodstream the infectious virus particles spread to other susceptible tissues such as other lymph nodes, brown fat, muscle, and the central nervous system (CNS). Poliovirus has been shown to cross the blood-brain barrier in mice (Yang et al., 1997). Invasion of the CNS is primarily thought to occur by means of peripheral or cranial nerve retrograde axonal transport, stimulated by skeletal muscle injury (Gromeier & Wimmer 1998), and leads to the development of poliomyelitis. The fast retrograde axonal transport pathway of poliovirus from the axon to the neuronal cell body occurs in a PVR-dependent manner by the PVR interaction with the dynein-motor complex component Tctex-1 (Mueller et al., 2002). Poliovirus is then able to attach, enter, infect and destroy motor neurons, in particular neurons of the anterior horn of the spinal cord leading to paralytic poliomyelitis

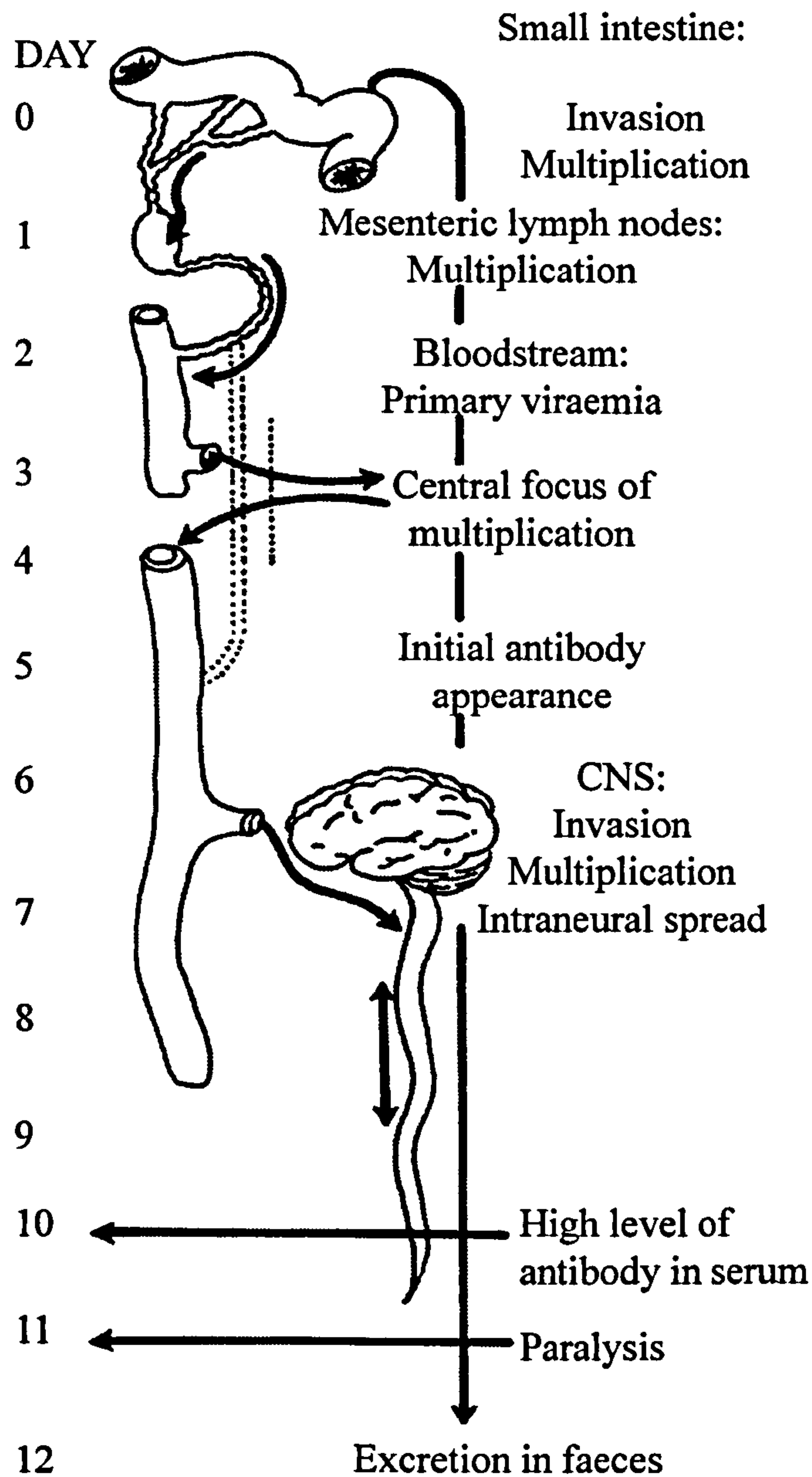


Figure 1.3: Poliovirus pathogenesis

This schematic illustration represents a longstanding view of the pathogenesis of poliovirus. Note that additional data emphasize the importance of retrograde axonal flow of virus into the central nervous system. Virus enters by way of the alimentary tract and multiplies locally at the initial sites of virus implantation (tonsils, Peyer's patch) or the lymph nodes that drain these tissues, and virus begins to appear in the throat and in the feces—leading to virus shedding into the environment. At this time, it is possible that virus may spread to the CNS by peripheral or cranial nerve axonal flow. Secondary virus spread occurs by way of the bloodstream to other susceptible tissues—namely, other lymph nodes, brown fat, muscle, and the CNS. Virus can then also spread into the CNS at this time by means of peripheral or cranial nerve retrograde axonal flow—for example, from muscle. If a high level of virus replication occurs within the CNS, motor neurons die and paralysis ensues. (Diagram and legends taken from Melnick et al., 1996a)

in humans, primates and PVR transgenic mice (Ren et al., 1990). The following description of the virus life cycle focuses on poliovirus unless otherwise specified.

1.3.2 Host cell infection and poliovirus life cycle

1.3.2.1 Overview (see figure 1.4)

The complete life cycle of poliovirus within a host cell occurs in approximately 5 to 10 hours and is initiated by the attachment of the virus to its receptor: PVR (CD155) (Hogle et al., 1985). Conformational changes induced by receptor attachment lead to the uncoating and release of the viral genomic RNA into the host cell cytoplasm (Belnap et al., 2000), in which the entirety of the reproductive life cycle of poliovirus occurs. Once the genomic RNA has entered the cell, the 5' covalently-linked VPg is cleaved off by a cellular enzyme, releasing the RNA for translation into a single polyprotein (Pelletier et al., 1988). The translated polyprotein undergoes a cascade of autocatalytic events known as proteolytic processing, generating functional viral proteins (Schechter et al., 1967; Ypma-Wong et al., 1988). Once the non-structural proteins are generated, the host cell endoplasmic reticulum membranes are altered and re-arranged to form replication complexes where initiation of the negative-sense strand synthesis occurs (Bienz et al., 1987; Beinz et al., 1990). The negative-sense strands are then used as a template for the positive-sense strand synthesis. The newly synthesised positive-sense strand progeny RNA molecules are translated, enhancing the number of replication complexes formed and amplification of the viral genome (Jarvis et al., 1992). The VPg-linked progeny RNA genomes are then packaged into the virus capsid proteins VP1, VP3 and VP0, resulting from the polyprotein processing (Arnold et al., 1987). Encapsidation leads to the final maturation of the immature virions into infectious icosahedral particles, which are then released into the intercellular environment (Hellen et al., 1992). Poliovirus infection can be

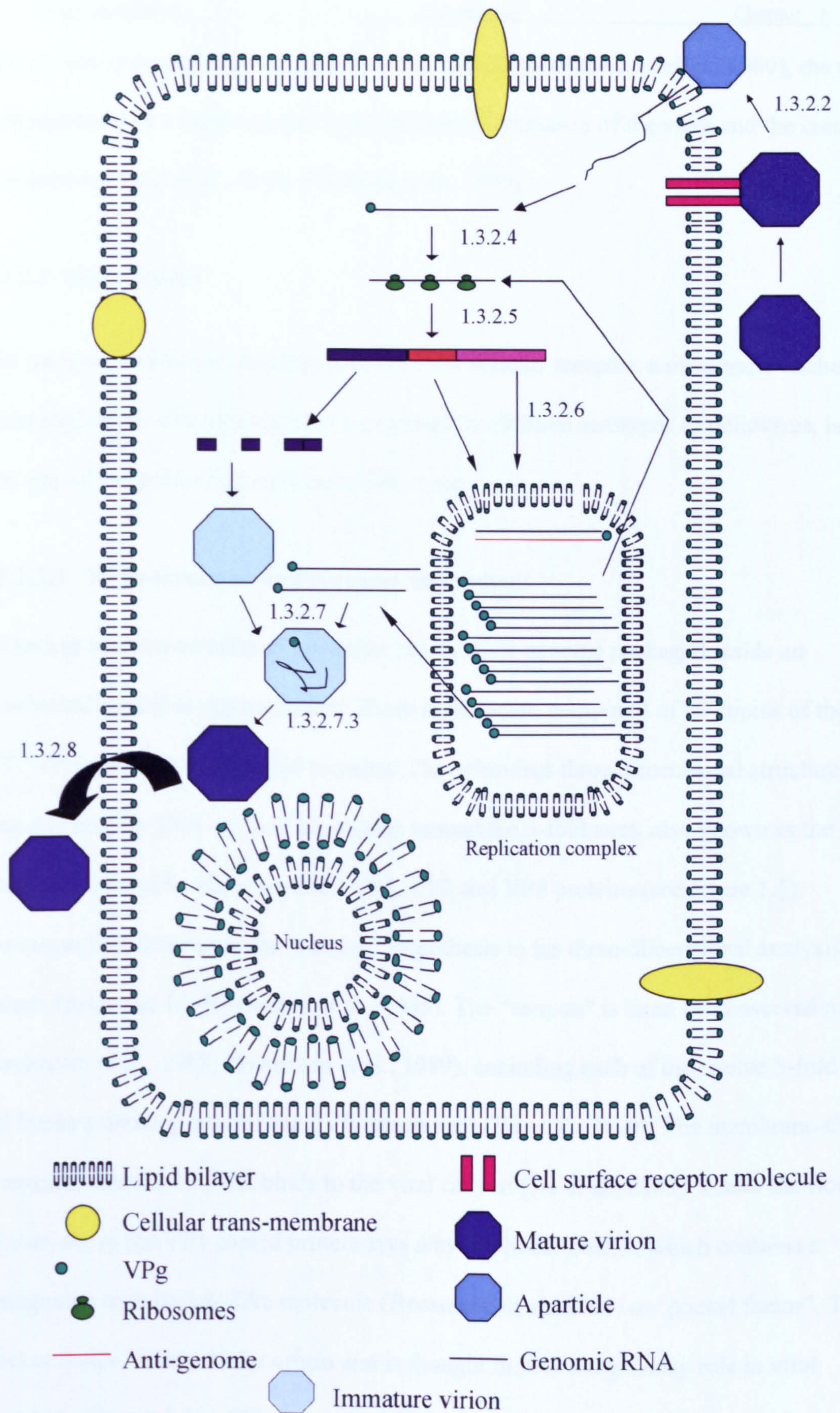


Figure 1.4: Poliovirus life cycle during host cell infection

Numbering corresponds to sections in the thesis describing the particular part of the life cycle.

lytic or non-lytic depending on the host cell type (Lopez-Guerrero et al., 2000), the state of differentiation of a particular cell type, the genetic make-up of the virus and the conditions of infection (Agol et al., 1998, Tolskaya et al., 1995).

1.3.2.2 Attachment

The recognition and the attachment to the PVR cellular receptor, a membrane-anchored immunoglobulin-like glycoprotein recognised by all three serotypes of poliovirus, is the first step of the poliovirus replication life cycle.

1.3.2.2.1 Virus structure and receptor attachment

Poliovirus is a non-enveloped virus that has an RNA genome packaged inside an icosahedral capsid of approximately 25nm in diameter, composed of 60 copies of the VP1, VP2, VP3 and VP4 viral capsid proteins. The poliovirus three-dimensional structure has a deep and narrow 20 Å-surface depression around the 5-fold axes, also known as the “canyon”, formed by the assembled VP1, VP2 and VP3 proteins (see figure 1.5).

Rossmann first developed the “canyon” hypothesis in his three-dimensional analysis of the human rhinovirus 14 (Rossmann et al., 1985). The “canyon” is lined by conserved residues (Rossmann et al., 1988; Rossmann et al., 1989), encircling each of the twelve 5-fold axes, and forms a docking site for the PVR attachment (He et al., 2000). The membrane-distal N-terminal domain of PVR binds to the viral canyon (He et al., 2003). Under the floor of the canyon, in the VP1 capsid protein lays a hydrophobic pocket, which contains a sphingosine or palmitate-like molecule (Rossmann et al., 1994) or “pocket factor”. The “pocket factor” is of cellular origin and is thought to play a regulatory role in viral assembly and uncoating (Filman et al., 1989) as well as stabilising the virions during the transit phase from infected cells to non-infected cells. Binding of the PVR to the canyon depression of the poliovirion destabilises the “pocket factor”, which triggers a

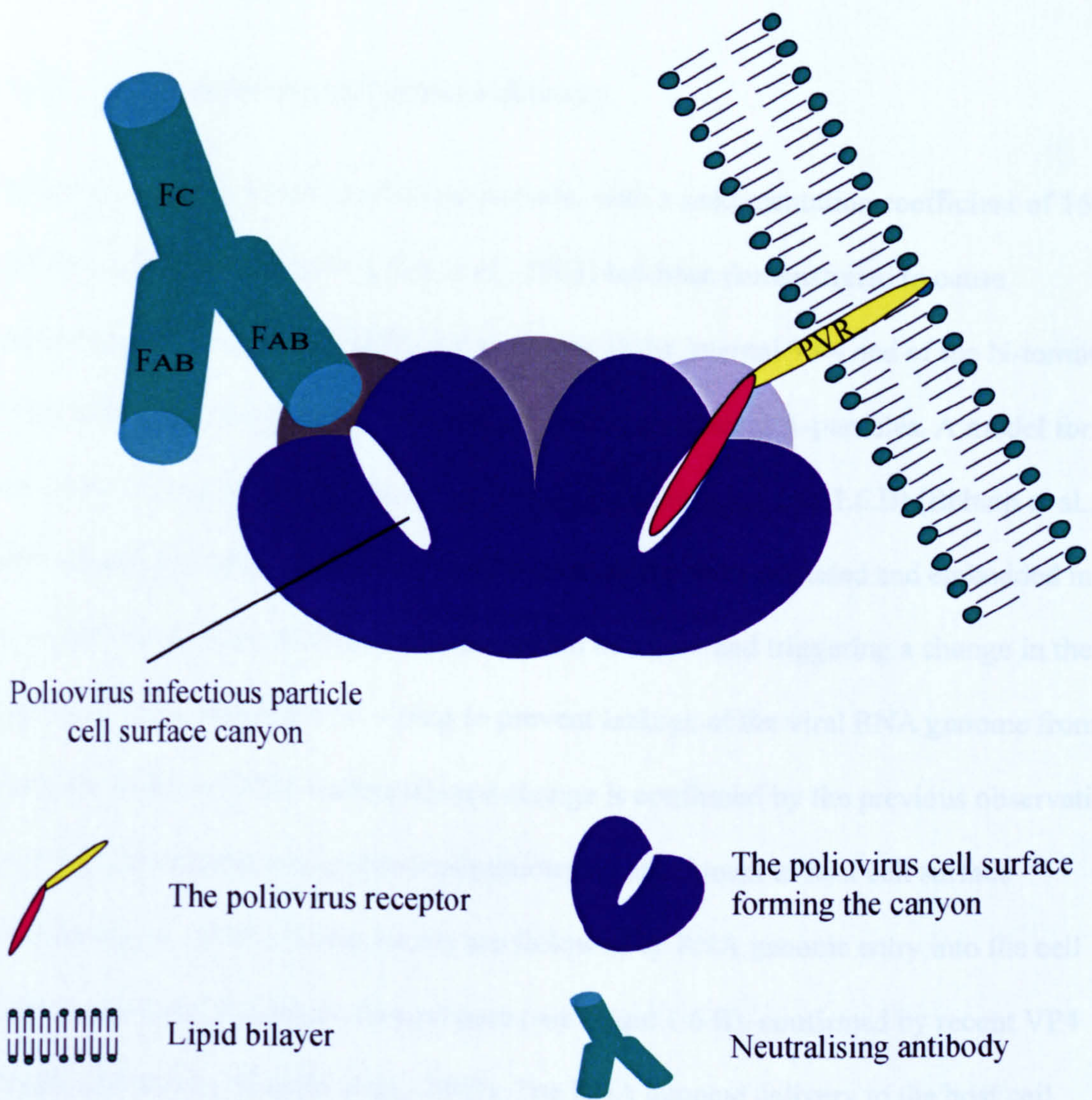


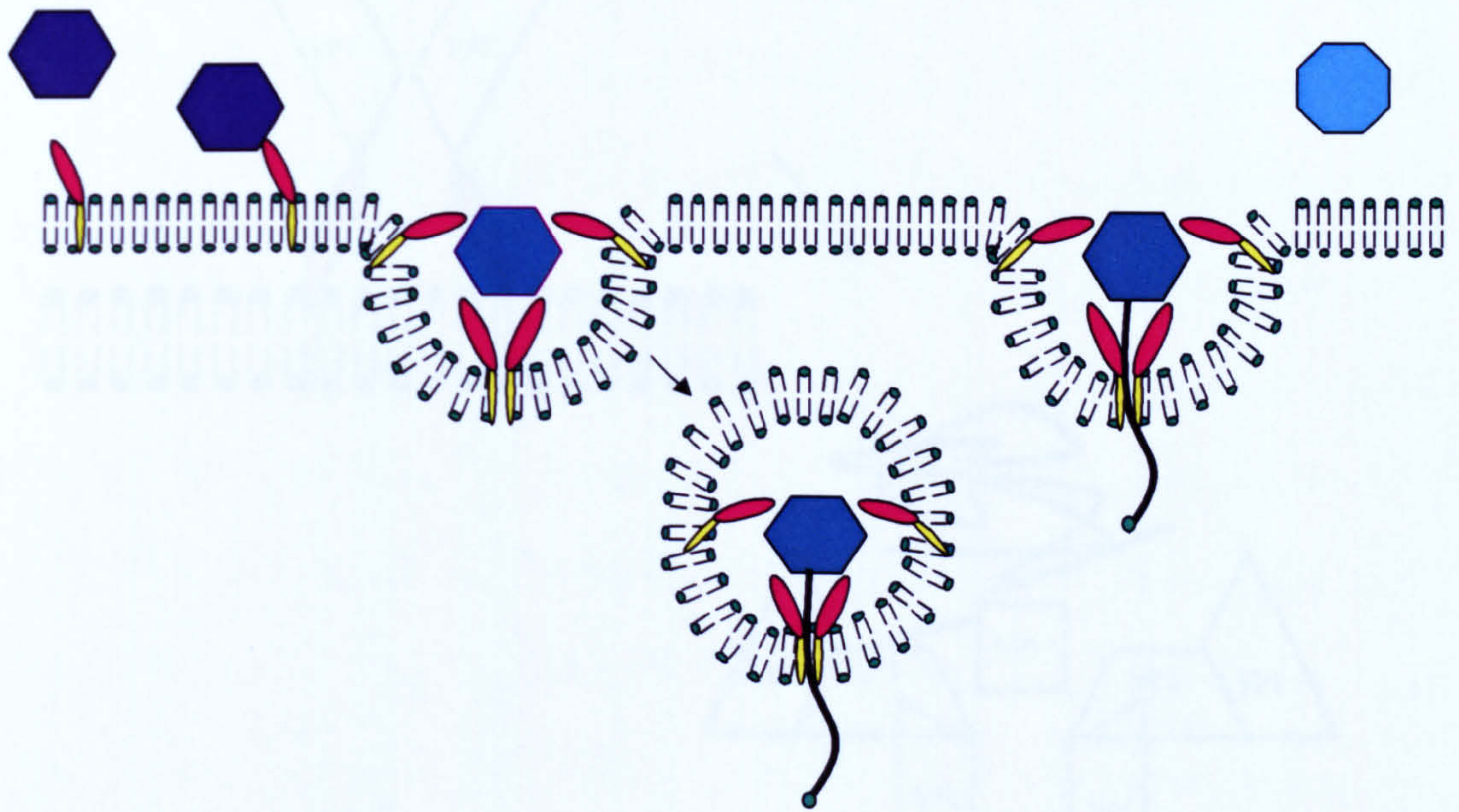
Figure 1.5: Canyon hypothesis.

Diagram showing the binding of the PVR to the deep narrow depression, known as the canyon, of poliovirus. Diagram adapted from Rossmann (1985).

conformational change in the virus particle leading to the uncoating and release of the viral genomic RNA into the host cell cytoplasm (Rossmann et al., 1994).

1.3.2.3 Viral uncoating and genome delivery

The interaction of a mature poliovirus particle, with a sedimentation coefficient of 160S, with the cognate receptor PVR (He et al., 2003) has been demonstrated to cause conformational changes including the extrusion of the internal VP4 and of the N-terminus of VP1 (Fricks & Hogle, 1990; Hogle et al., 2002) generating A-particles. A model for virus entry was proposed by Belnap in 2000 (see figure 1.6.A and 1.6.B) (Belnap et al., 2000) whereby myristylated VP4 and VP1 N-terminus were extruded and embedded in the cell plasma membrane leading to the formation of a pore and triggering a change in the location of VP3, which acts as a plug to prevent leakage of the viral RNA genome from infectious particles. This conformational change is confirmed by the previous observations of a 10% size expansion of poliovirus particles on attachment to host cell surface (Incardona et al., 1964). These events are followed by RNA genome entry into the cell cytoplasm through the newly formed pore (see figure 1.6 B), confirmed by recent VP4 mutagenesis studies (Danthi et al., 2003). The RNA genome delivery to the host cell cytoplasm leads to a decrease in the extracellular particle sedimentation coefficient to 80S. Because of the unstable nature of the A-particle 135S intermediates, up to 50-90% of virus particles are eluted or unable to deliver their RNA molecule. This contributes to the very high particle to infectivity ratio of 1000 to 1 (Fenwick & Cooper, 1962). Alternative uncoating and entry pathways have been thought to exist. Indeed studies of various rhinovirus serotypes demonstrated the virus entry to be dependent on a receptor-mediated endocytosis pathway (Nurani et al., 2003; Kronenberger et al., 1998). Alternative uncoating pathways are not discussed in this thesis.









	160S infectious poliovirus particle		Poliovirus RNA genome
	The poliovirus receptor		135S A-particle
	Lipid bilayer		80S empty procapsid

Figure 1.6 A: Model of poliovirus uncoating, adapted from Belnap (2000).

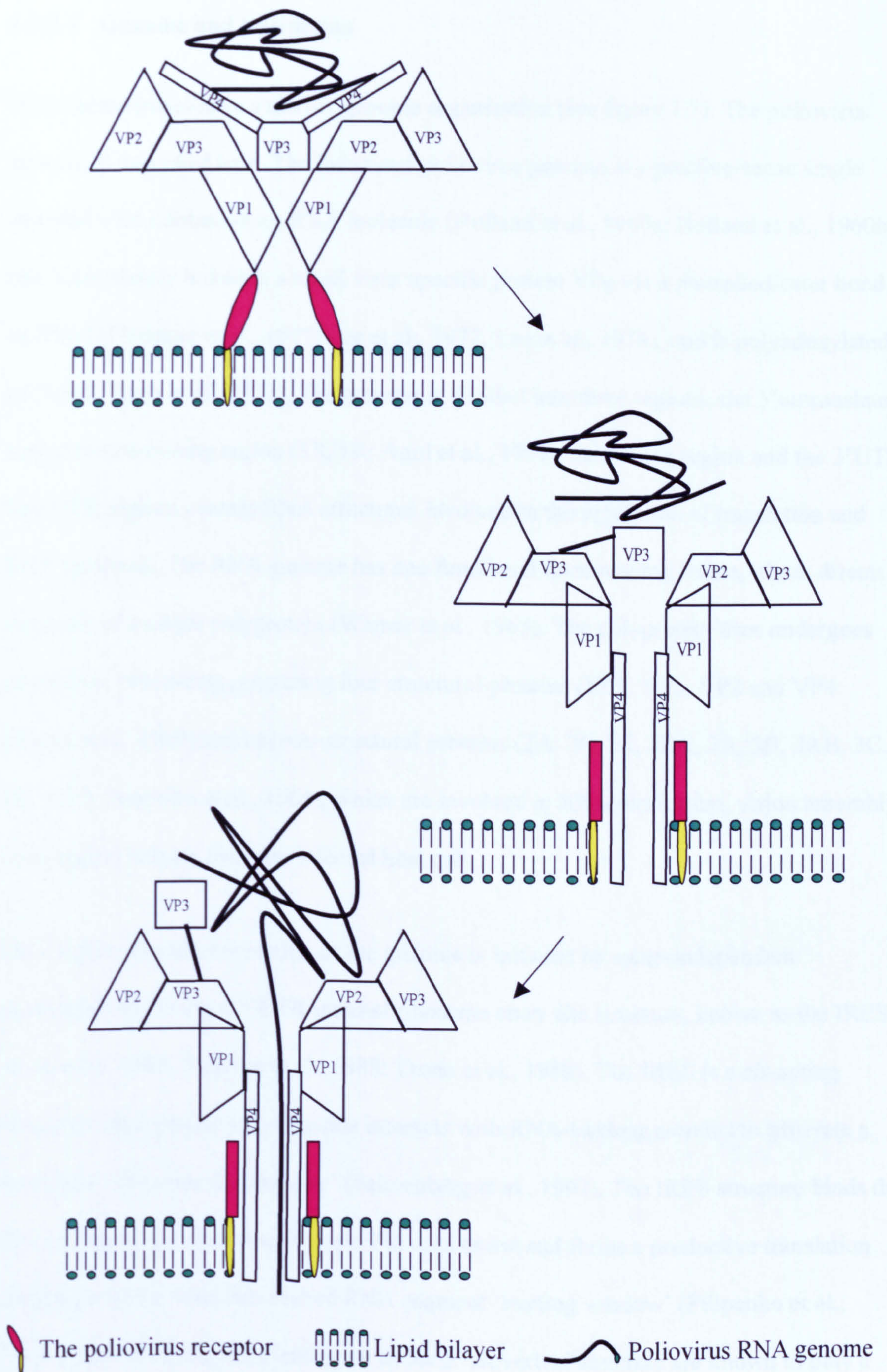


Figure 1.6 B : Model of poliovirus uncoating, adapted from Belnap et al. (2000).

1.3.2.4 Genome and translation

All picornaviruses have a similar genome organisation (see figure 1.7). The poliovirus genome is described here. The infectious poliovirus genome is a positive-sense single stranded ~7.5 kilobase-long RNA molecule (Holland et al., 1960a; Holland et al., 1960b) that is covalently linked to a small virus specific protein VPg via a phosphodiester bond at its 5' end (Flanegan et al., 1977; Lee et al., 1977, Lee et al., 1976), and is polyadenylated at its 3' end (Yogo et al., 1972). The genome is divided into three regions, the 5' untranslated region or non-coding region (5'UTR: Agol et al., 1991), the coding region and the 3'UTR. The UTR regions contain RNA structures involved in the regulation of translation and RNA synthesis. The RNA genome has one functional open reading frame, which directs synthesis of a single polyprotein (Warner et al., 1963). The polyprotein then undergoes proteolytic processing generating four structural proteins (VP1, VP3, VP2 and VP4: Maizel et al., 1963) and ten non-structural proteins (2A, 2B, 2C, 2BC, 3A, 3B, 3AB, 3C, 3D, 3CD: Summers et al., 1965), which are involved in RNA replication, virion assembly and progeny release from the infected host cell.

Once inside the cell, translation of the genome is initiated by a cap-independent mechanism involving a 5'UTR internal ribosome entry site structure, known as the IRES (Jang et al., 1988, Pelletier et al., 1988; Trono et al., 1988). The IRES is a *cis*-acting element with a tertiary structure that interacts with RNA-binding proteins to generate a functional 'ribosome-landing site' (Palmenberg et al., 1997). The IRES structure binds the 40S subunit of the ribosome in a specific orientation and forms a productive translation complex with the template limited RNA segment 'starting window' (Pilipenko et al., 1994). Other *cis*-acting elements such as the 5' cloverleaf structure are known to play a role in RNA replication and there is evidence of its role in the viral translation initiation process (Gamarnik et al., 1998; Simoes et al., 1991).

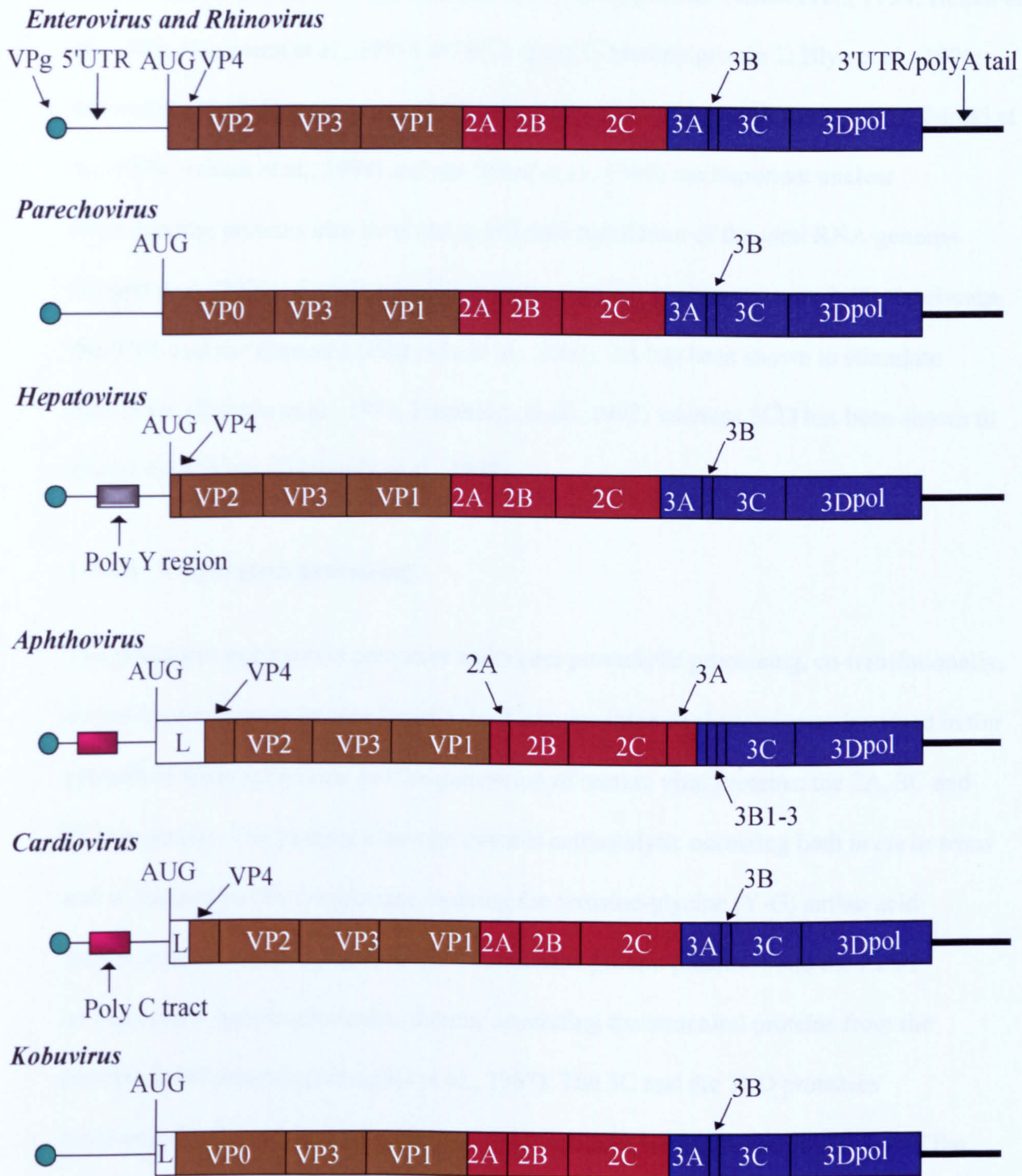


Figure 1.7: Overall picornavirus genome structure.

The overall genome structure of the different genera is similar and is summarised in this figure.

Other proteins such as PTB (pyrimidine tract binding protein: Hellen et al., 1994; Hellen et al., 1993; Niepmann et al., 1997), PCBP-2 (poly C-binding protein 2: Blyn et al., 1996; Blyn et al., 1997; Herman et al., 1998), autoantigen La (Meerovitch et al., 1993; Shiroki et al., 1999; Svitkin et al., 1994) and unr (Hunt et al., 1999) are important nuclear RNA-binding proteins also involved in efficient translation of the viral RNA genome (Gosert et al., 2000). Certain translation factors can act as chaperones or bridges between the IRES and the ribosome (Pilipenko et al., 2000). 2A has been shown to stimulate translation (Roberts et al., 1998; Hambidge et al., 1992) whereas 3CD has been shown to inhibit the process (Gamarnik et al., 1998).

1.3.2.5 Polyprotein processing

The poliovirus polyprotein precursor undergoes proteolytic processing, co-translationally, driven by viral proteases (see figure 1.8). There are three viral proteinases involved in the proteolytic cleavage events and the generation of mature viral proteins: the 2A, 3C and 3CD proteases. The primary cleavage event is autocatalytic occurring both *in cis* or *trans* and is directed by the 2A protease cleaving the tyrosine-glycine (Y-G) amino acid sequence at the junction between the P1 structural protein precursor and the P2/P3 non-structural protein precursors thereby separating the structural proteins from the non-structural proteins (Schechter et al., 1967). The 3C and the 3CD proteases preferentially cleave glutamine-glycine (Q-G) residues leading to the generation of the remainder of the viral proteins (Luke et al., 2001). In poliovirus only 9 of the 13 Q-G residues are cleaved (Hanecak et al., 1982; Kitamura et al., 1981). The lack of conservation of residues surrounding the Q-G amino acid sequences indicates that processing is not only sequence-dependent but that preferential processing must occur (Kusov et al., 1999). Studies demonstrating enhanced activity of 3CD protease over 3C protease (Parsley et al., 1999) and complete P1 processing solely achieved by 3CD protease confirms that the

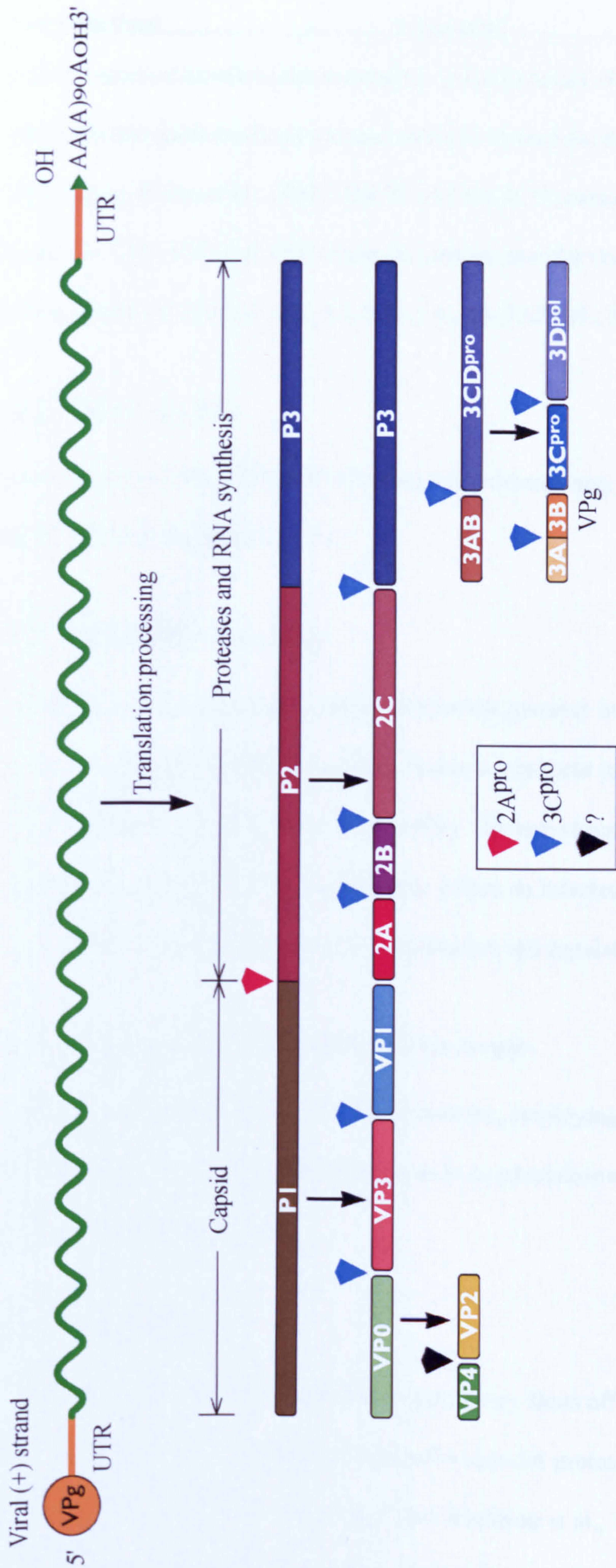


Figure 1.8: Polyprotein processing (Flint Principles of Virology). See section 1.3.2.4.

amino acid sequence cannot alone account for proteolytic activity of 3C and 3CD proteases and that structural conformations are also involved in specific protease activity (Harris et al., 1992; Ypma-Wong et al., 1988). The 3C and the 3CD proteases process the P1 precursor into VP0, VP3 and VP1 structural proteins and the non-structural protein precursors P2 and P3 into 2A, 2B, 2C, 2BC, 3A, 3B, 3AB, 3C, 3D and 3CD.

1.3.2.5.1 Capsid proteins

The capsid proteins VP0, VP3 and VP1 assemble to form empty virus capsids, a process discussed further in section 1.3.2.7.1.

1.3.2.5.2 Non-structural proteins

The P2 and P3 protein precursors contain most of the proteins important for the replication of the virus. P1 deletion mutants have been shown to replicate in tissue culture (Percy et al., 1992; Collis et al., 1992; Kaplan et al., 1988). The non-structural proteins have a variety of functions in the poliovirus life cycle within an infected host cell such as protease activity, membrane rearrangements, RNA replication and regulation of host cell functions.

1.3.2.5.3 The viral proteins and their cellular targets

Viral proteases are capable of hijacking host proteins, employing them for viral functions and shutting down the key cellular functions such as translation and transcription leading to cell cycle arrest and cell death.

1.3.2.5.3.1 Translation

Upon virus infection the host cell translation machinery shuts off. This mechanism is regulated by cellular and viral proteins. The poliovirus 2A protease exerts its proteolytic activity on host cell proteins by cleaving eIF4G (Etchison et al., 1982; Krausslich et al., 1987; Sommergruber et al., 1994), a cellular translation initiation factor, and more

specifically eIF4G-I (Haghighat et al., 1996) and eIF4G-II (Svitkin et al., 1999; Gradi et al., 1998), essential components of the host cell cap-dependent translation initiation complex. Moreover, the cleavage of the poly (A)-binding protein (PABP) by the poliovirus 2A and 3C proteases contributes to host cell translation shut off (Joachims et al., 1999). Once complete host cell protein synthesis shut off has occurred, 2 to 4 hours post infection, eIF4G is used for the viral cap-independent IRES-mediated translation (Ohlmann et al., 1996).

1.3.2.5.3.2 Transcription

Similarly, viral proteases regulate both the viral and the cellular transcription machineries. The poliovirus 3C and 3CD proteases bind viral RNA secondary structures such as the 5'cloverleaf of the positive-sense RNA molecule (Andino et al., 1993; Leong et al., 1993) and the poly(rC)-binding protein (PCBP: Parsley et al., 1997) thereby forming a complex required for poliovirus replication. 3CD has been suggested to be involved in genome circularisation as it bridges the 5'cloverleaf structure-PCBP complex with polyA-binding protein (PABP), bound to the 3' poly-A tail of the RNA molecule (Herold et al., 2001).

The 3C and 2A proteases are also involved in the regulation of the host cell transcription and apoptotic pathways upon viral infection, by exerting a proteolytic activity on a variety of host cell proteins such as TATA-binding proteins (Clark et al., 1993; Yalamanchili et al., 1997), the cAMP-responsive element (Deitz et al., 2000), the transcriptional activator Oct-1 (Yalamanchili et al., 1997), the PABP (Joachims et al., 1999; Kerekatte et al., 1999), and anti-apoptosis and PKR phosphorylation pathways (Black et al., 1993).

1.3.2.5.3.3 Membrane re-arrangement and replication compartments

1.3.2.5.3.3.1 P2 proteins

P2 proteins are involved in membrane rearrangements within a host cell, disrupting cellular functions and creating replication compartments for the generation of progeny viral RNA.

2B is thought to be involved in alteration of intracellular membrane permeability, facilitating the release of progeny virus (van Kuppeveld et al., 1997), inhibition of cellular exocytosis, dissociation of Golgi complexes and viral RNA amplification (Aldabe et al., 1996; Barco et al., 1998; Doedens et al., 1995; Sandoval et al., 1997; van Kuppeveld et al., 1995; van Kuppeveld et al., 1997; Johnson et al., 1991).

2B and 2BC proteins play a role in the formation of viral replication vesicles (Bienz et al., 1987; Bienz et al., 1983; Aldabe et al., 1995; Cho et al., 1994; Suhy et al., 2000;

Teterina et al., 1997) and results showing that 2C is involved in positive strand synthesis give further evidence of the role taken by 2C during viral replication (Barton et al., 1997; Molla et al., 1991).

1.3.2.5.3.3.2 P3 proteins

3A and 3AB proteins have also been shown to be associated with intracellular membranes (Datta et al., 1994; Semler et al., 1982; Towner et al., 1996). Indeed, 3AB, which delivers 3B, known as VPg, (Kusov et al., 1999) and the 3D polymerase (Hope et al., 1997; Xiang et al., 1998) to replication compartments, is involved in rearrangement of intracellular membranes into replication vesicles (Doedens et al., 1997; Doedens et al., 1995; Lama et al., 1992).

3D is the essential RNA-dependent RNA-polymerase that catalyses the positive-sense and the negative-sense strand synthesis RNA elongation during the replication process.

1.3.2.5.3.4 Immune evasion and immune response interference

Some poliovirus proteins have been shown to interfere with the host immune response, probably indicating that poliovirus has evolved to evade the immune response induced following viral infection. The poliovirus proteins 2BC and 3A modulate the host immune response by interfering with exocytotic pathways (Barco et al., 1995), secretion of antiviral cytokines (β -IFN, IL-6, IL-8) (Dodd et al., 2001), and antigen presentation via major histocompatibility complex class I (MHC-I) molecules (Doedens et al., 1995; Deitz et al., 2000).

1.3.2.6 RNA replication

1.3.2.6.1 Replication complex formation and membrane rearrangement

Genomic RNA replication is driven by the virally encoded RdRp and occurs in rearranged cellular membrane vesicles also known as replication complexes. The use of membranous replication compartments enables the localisation and concentration of proteins required for replication and assembly, provides protection from degradation by any cellular factors or proteins (Ahlquist et al., 2002) and could be a means of hiding the presence of double stranded RNA thereby avoiding the induction of a dsRNA activated immune response such as the protein kinase R (PKR) response and RNA silencing (Ahlquist et al., 2002).

Poliovirus replication complexes have been shown to contain viral and cellular proteins, replicative-intermediate RNA and newly synthesised progeny RNA (Bienz et al., 1994, Bienz et al., 1992; Egger et al., 1996; Troxler et al., 1992; Egger et al., 2002) and are assembled on membrane vesicles (Bienz et al., 1987; Bienz et al., 1990). In fact it has been demonstrated that HeLa S10 nuclear extracts contained cellular proteins inducing the formation of replication complexes (Jurgens et al., 2003). During poliovirus infection, COP II-coated vesicles derived from the anterograde membrane transport pathway form on

the ER but unlike other vesicles from that particular pathway, they are unable to fuse with the Golgi system or the ER-Golgi intermediate compartment (Rust et al., 2001). The involvement of the COP II-dependent pathway was supported by inhibition studies (Sandoval et al., 1997). However, replication complex formation is not solely dependent on COP II-mediated pathways and studies using Brefeldin A (BFA) demonstrated COP I-dependent poliovirus replication inhibition (Cuconati et al., 1998; Irurzun et al., 1992; Maynell et al., 1992).

1.3.2.6.2 *Cis-acting replication elements*

The viral RNA genome possesses signals, *cis-acting* elements, that allow the polymerase to differentiate between viral and cellular RNAs. The 5'UTR and 3'UTR *cis-acting* elements as well as elements within the genome-coding region have been identified to be involved in replication. The 5'UTR IRES ensures the formation of a pre-initiation template/ribosome complex. The 5'UTR cloverleaf stemloop d has been shown to interact with the 3CD^{pro} and PCBP has been shown to interact with the stem loop b of the cloverleaf thereby forming a ternary ribonucleoprotein complex (Andino et al., 1990a; Andino et al., 1990b). The cloverleaf structure has also been involved in the switch between translation and replication, as the two processes are mutually incompatible on a single template (Barton et al., 1999).

The CRE, a secondary RNA structure, initially identified in the P1-coding region of HRV14 (McKnight et al., 1998), is involved in replication. Subsequent studies identified the presence of similar structures in the P1-coding region of cardioviruses (Lobert et al., 1999) and in the 2C-coding region of poliovirus (Goodfellow et al., 2000). These structures contain a conserved GXXXAAAXXXXXXA motif (Yang et al., 2002), where the first two A residues of the AAA triplet template addition of pUpU onto VPg. The uridylylated VPg

(VPg-pUpU) (Paul et al., 2000; Rieder et al., 2000) is then used as a primer for the initiation of the positive-sense strand synthesis but not negative-sense strand synthesis (Goodfellow et al., 2003; Morasco et al., 2003; Murray et al., 2003).

As opposed to the 5'UTR, the 3'UTR has not been studied extensively and the precise role of the latter in replication of picornaviruses is still unclear. The 3'UTR is not essential for viability of picornaviruses and mutagenesis and deletion of the 3'UTR did not prevent viability, though a reduction in viral fitness was observed (Meredith et al., 1999; Todd et al., 1997). The 3'UTR of enteroviruses has been identified to have two or three stem loops (Pilipenko et al., 1992), supported by thermodynamic studies (Jacobson et al., 1993; Melchers et al., 1997) and phylogenetic studies (Pilipenko et al., 1992). The 3'UTR can be exchanged, between PV3 and HRV14 (Rohll et al., 1995) and a defect in replication can be seen in viruses containing an altered 3'UTR (Melchers et al., 1997, Mirmomeni et al., 1997, Pilipenko et al., 1996). The 3'UTR of poliovirus has also been shown to interact with cellular factors such as p105, p68 and p45 (Waggoner et al., 1998). Further studies expressing these cellular proteins and investigating the nature and functions of the interactions need to be conducted in order to understand the precise role of the 3'UTR *cis*-acting element. The poly-A tail, attached to the 3'end of the genome, is essential for poliovirus infectivity (Rohll et al., 1995; Sarnow et al., 1989), however other functions of the poliovirus poly-A tail still remain to be investigated.

1.3.2.6.3 Model of poliovirus replication

The model of the poliovirus replication has been proposed to occur in two phases: the synthesis of negative-sense strands followed by the production of positive-sense strands templated from the negative-sense strands. The first step to occur is that of genome circularisation (Herold et al., 2001) by protein-protein interaction that enable the formation

of a bridge between two molecules of $3CD^{pro}$. The uridylylated VPg is then thought to serve as a primer for negative strand synthesis and elongation, catalysed by the RdRp $3D^{pol}$ (Goodfellow et al., 2003; Morasco et al., 2003; Murray et al., 2003). Following the termination of the negative-sense strand synthesis, the viral RdRp uses VPg-pUpU complexed with the 3' termini of double stranded negative-sense strands, known as the replicative form or RF, as a primer for initiation of positive strand synthesis (see figure 1.9). Binding of the $3D^{pol}$ -VPg-pUpU complex to the RF is responsible for unwinding and disrupting the double-stranded helices thereby generating partially double-stranded negative strands or replication intermediates (RI). This event leads to the exposure of more residues free for interaction with numerous polymerase/primer complexes. The CRE-generated VPg-pUpU primer is then released from the template (Goodfellow et al., 2003). The large number of released VPg-pUpU primers, during negative-sense strand synthesis, enables the polymerase to initiate multiple rounds of positive strand synthesis from a single RF template. This asymmetric replication gives rise to high ratios of readily packageable positive-sense progeny RNA molecules to negative-sense template strands (Jarvis et al., 1992).

1.3.2.7 Encapsidation

1.3.2.7.1 Viral particle assembly and RNA encapsidation

Viral particle assembly and encapsidation of the newly synthesised viral genome are essential steps of the poliovirus life cycle. These processes have been hypothesised to occur via two alternate pathways.

Copies of the structural proteins VP0, VP1 and VP3 assemble into 60 protomers to form poliovirus capsids. A late VP0 cleavage event during the maturation of the virus particles gives rise to VP4 which is located inside the mature virus particle and VP2. The carboxyl

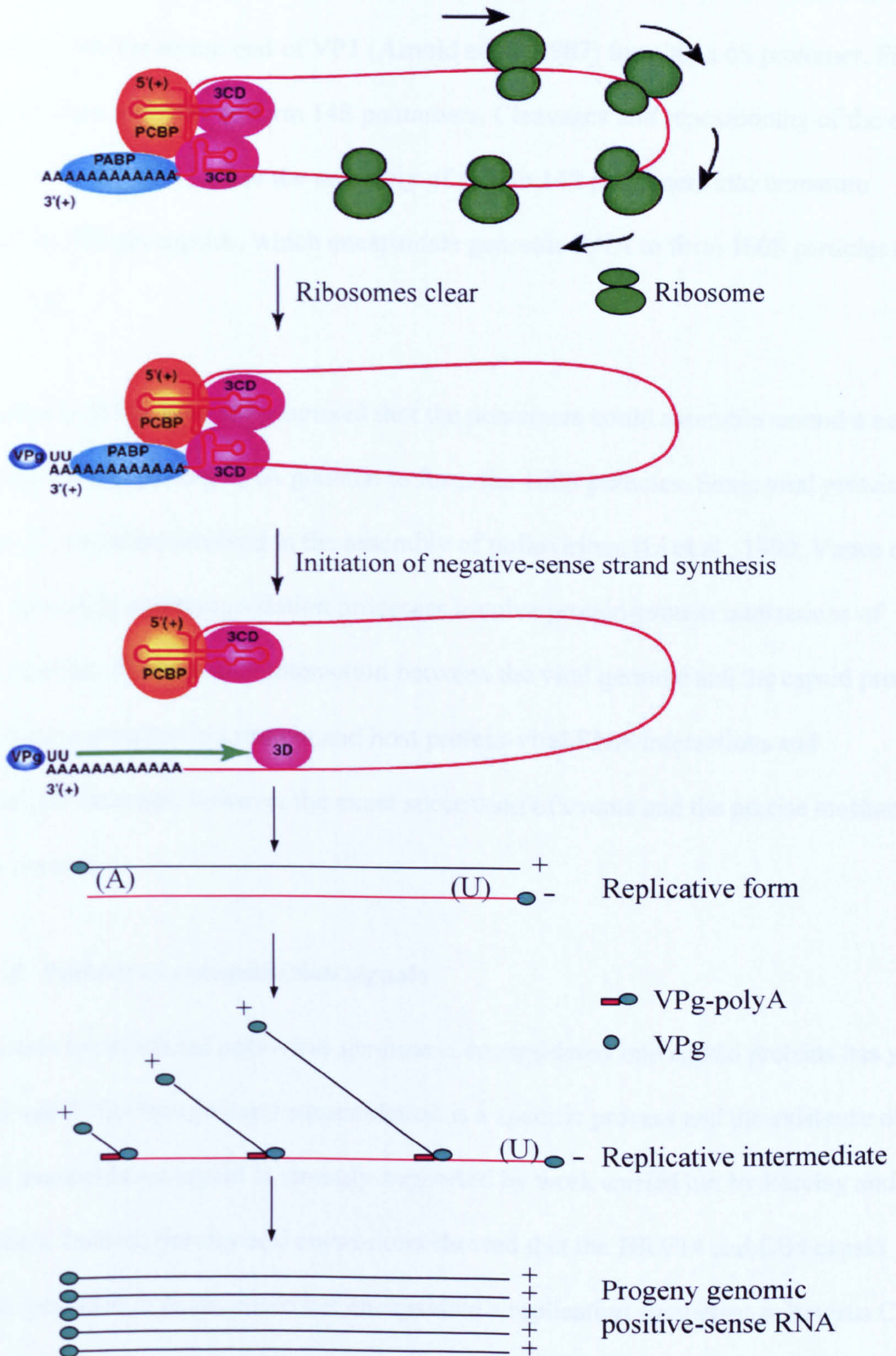


Figure 1.9: Poliovirus translation, poliovirus genome circularisation and genome replication.

end of VP0 is associated with the amino end of VP3 and the carboxyl end of VP3 is associated with the amino end of VP1 (Arnold et al., 1987) forming a 6S protomer. Five 6S protomers then assemble to form 14S pentamers. Cleavages and repositioning of the ends of VP1, VP3 and VP0 lead to the assembly of twelve 14S pentamers into immature icosahedral 80S procapsids, which encapsidate genomic RNA to form 160S particles (see figure 1.10).

Alternatively, it has been hypothesised that the pentamers could assemble around a newly synthesised progeny viral RNA genome to form the 160S particles. Some viral proteins, such as 2C, could be involved in the assembly of poliovirions (Li et al., 1990; Vance et al., 1997). Assembly and encapsidation processes involve protein-protein interactions of capsid proteins, RNA-protein interaction between the viral genome and the capsid proteins, potential host protein-viral protein and host protein-viral RNA interactions and autocatalytic cleavage, however the exact succession of events and the precise mechanisms remain unclear.

1.3.2.7.2 Poliovirus encapsidation signals

The process by which the poliovirus genome is encapsidated into capsid proteins has yet to be resolved. Poliovirus genome encapsidation is a specific process and the existence of a specific encapsidation signal is strongly supported by work carried out by Barclay and co-workers. Indeed, Barclay and co-workers showed that the HRV14 and CB4 capsid proteins, provided *in trans*, could not encapsidate a replication competent poliovirus CAT (Chloramphenicol acetyl transferase) replicon, lacking most of the P1 coding region. However, poliovirus type 1, poliovirus type 2 and poliovirus type 3 capsid proteins were able to specifically encapsidate the poliovirus CAT replicon (Barclay et al., 1998).

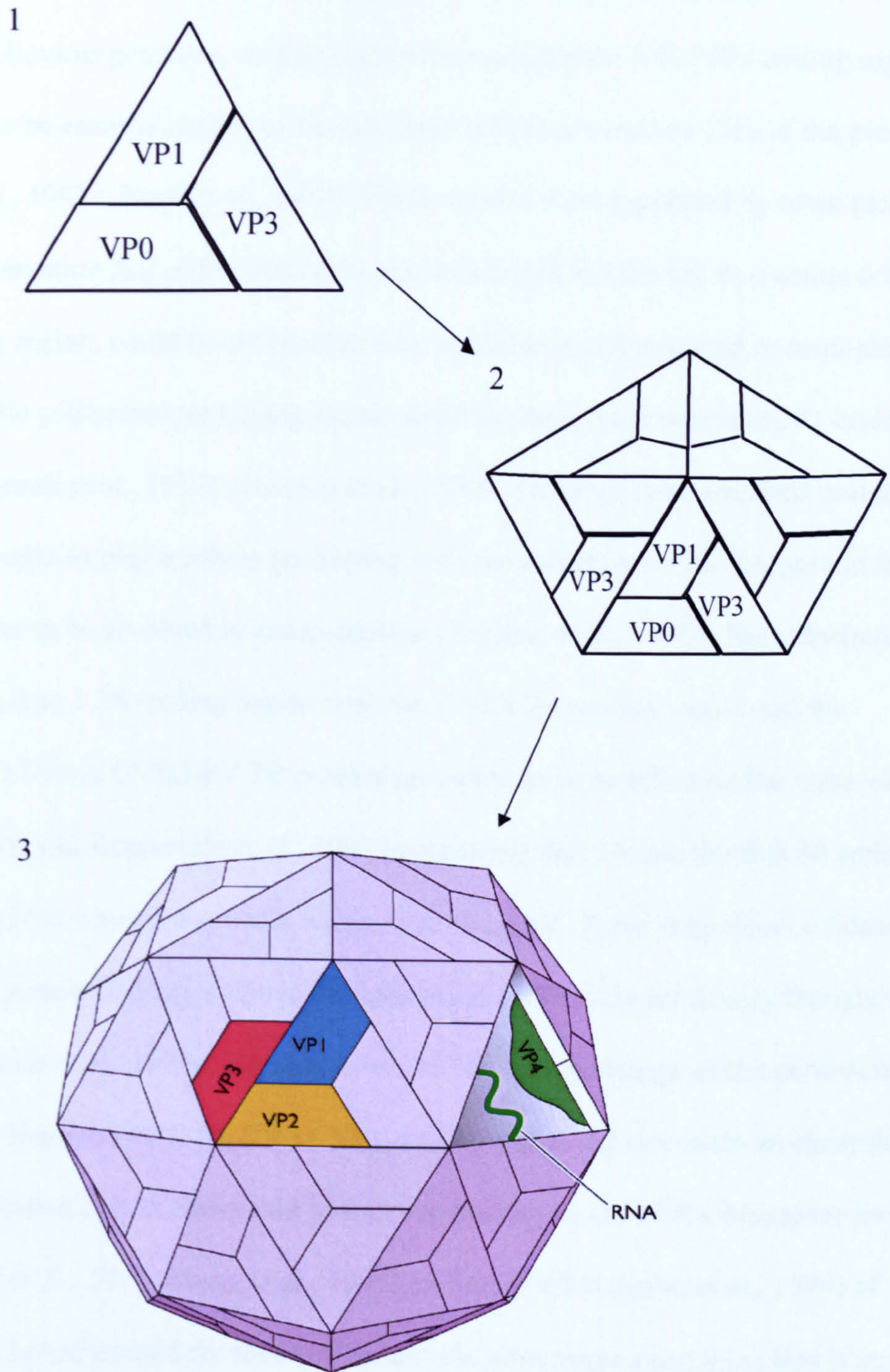


Figure 1.10: Model of poliovirus particle assembly

1- VP0, VP1 and VP3 assembly into 6S protomer; 2- assembly of five protomers into pentamers; 3- assembly of 12 pentamers into empty 80S procapsids and encapsidation of newly synthesised RNA genome leading to maturation cleavage and generation of mature icosahedral progeny virion.

The exact localisation of the specific encapsidation signal remains unidentified. Defective interfering poliovirus genomes, containing deletions within the VP2/VP3-coding regions, were shown to be encapsidated even though these lacked as much as 13% of the genome (Ansardi et al., 1993a, Kuge et al., 1986). These studies were supported by more recent data. The observation that poliovirus replicons, lacking all but the last two amino acids of the P1-coding region, could be encapsidated by capsid proteins provided *in trans* showed that the specific poliovirus-packaging signal could not be located within the P1-coding sequence (Ansardi et al., 1993b; Barclay et al., 1998). Other genome-encoded proteins have been thought to play a role in packaging with the exception of the 2A protein that does not appear to be involved in encapsidation (Ansardi et al., 1995). The substitution of the poliovirus type 1 2A-coding region with the CVB4 2A-coding region and the generation of a hybrid CVB3/PV 2B protein proved to have no effect on the virus viability (Lu et al., 1995; van Kuppeveld et al., 1997) suggesting that 2A and the first 30 amino acids of 2B did not contain a specific encapsidation signal. There is no direct evidence of the VPg viral protein playing a role in encapsidation, which was previously thought to be the case (Nomoto et al., 1977a; Nomoto et al., 1977b). An exchange of the poliovirus type 3 VPg-coding sequence with the CVB4 VPg-coding region did not cause an encapsidation defect (unpublished data of Percy and Moon; see Barclay et al., 1998). Moreover the 5'UTR (Rohll et al., 1994; Xiang et al., 1995) and the 3'UTR (Rohll et al., 1994) of poliovirus can be exchanged for those of certain picornaviruses suggesting that if an encapsidation signal exists it is most likely to reside in the region of the genome encoding 2B to 3D (Barclay et al., 1998) or to be discontinuous.

However, the previous results do not exclude 2A, part of 2B, capsid proteins and RNA structures from playing a role in the packaging of the poliovirus genome. Indeed the stimulation of encapsidation by unidentified signals of the 5'UTR strongly supports this

statement (Johansen et al., 2000). Moreover the specificity of the encapsidation process is not absolute as some poliovirus RNA appears to be packageable by coxsackievirus (Holland et al., 1964; Soloviev et al., 1968; Barclay et al., 1998), HRV and mengovirus particles (Jia et al., 1998). The overall encapsidation efficiency of the poliovirus genome by heterologous capsid proteins provided *in trans* is however significantly lower than encapsidation efficiency of the poliovirus genome by homologous capsid proteins. The specificity of the interspecies trans-encapsidation process could be due to the existence of specific replication compartments and thus inefficient mixing (Ansardi et al., 1993a), or to a requirement for specific interactions between genomic RNA sequences or structures and the capsid proteins (Barclay et al., 1998).

1.3.2.7.3 Maturation cleavage

Juxtaposition of catalytically active amino acid residues in the immature virion (Hindiyeh et al., 1999) and encapsidation of the RNA genome (Arnold et al., 1987) trigger the final stages of assembly by “maturation” cleavage of the VP0 precursor protein between the C-terminal asparagines (N) of VP4 and the N-terminal serine (S) of VP2 (Basavappa et al., 1994; reviewed in Hellen et al., 1992). This autocatalytic event provides the concluding stage to the production of mature infectious progeny virions (Ansardi et al., 1996; Rueckert et al., 1996) and release into the extracellular environment.

1.3.2.8 Release

Lytic release of newly synthesised virions, seen in poliovirus infection, can occur via virus-induced cytopathic effect or host-cell apoptosis defense mechanism, triggered by 2A (Barco et al., 2000b; Goldstaub et al., 2000) and 3C (Barco et al., 2000a) protease damage. Apoptosis can be interrupted by the activation of anti-apoptotic pathways, which have been shown to be inhibited by interaction of VP2 of CVB3 with the *in vivo* host apoptotic

regulatory protein (Henke et al., 2000). Recent studies have provided evidence that the enterovirus 2B protein was able to form pores in the ER and Golgi membranes thereby reducing Ca^{2+} levels and interfering with the ER-mitochondrial Ca^{2+} signalling. This prevented premature apoptosis and allowed completion of the virus reproductive cycle (van Kuppeveld et al., 2005). Most poliovirus apoptotic and anti-apoptotic pathways have been reviewed by Blondel (Blondel et al., 2004).

Several poliovirus proteins have been involved in host cell translation/transcription shut off. PV 2A mutagenesis studies demonstrated that 2A was involved in specific host cell shut off, a process different from that induced by the use of non-viral host inhibitors (Bernstein et al., 1985). PV 3A is a protein involved in the rearrangement of host cell membrane structures and was also shown to have a cytopathic effect on poliovirus-infected cells (Lama et al., 1998). Furthermore, accumulation of progeny virions and increase in size of the infected host cell causes cell burst and release of progeny virions into the intercellular environment, where these are then able to infect the neighbouring cells.

1.4 Receptors: pathogenesis and tropism

1.4.1 Picornavirus receptors and diseases

The pathogenesis of picornaviruses or range of clinical symptoms observed as a result of virus infection varies depending on a number of factors such as the receptor and tissue tropism, the dose required for infectivity and the cellular immune response to a particular viral infection. Picornaviruses are pathogens that cause a wide range of diseases such as respiratory diseases, encephalitis, hepatitis, foot-and-mouth disease, myocarditis, non-specific febrile symptoms, aseptic meningitis, congenital and neonatal infections, encephalomyelitis, the common cold and poliomyelitis (see table 1.11). Picornaviruses

have evolved to use a variety of cell surface molecules as receptors for attachment and entry of picornaviruses into the cell. This section discusses enterovirus receptors and tropism in more detail.

<u>Virus genus/species</u>	<u>Receptors</u>	<u>Accessory factors</u>	<u>Pathogenesis</u>
Enteroviruses			
<i>Poliovirus type 1, 2 and 3</i>	PVR (Ig-like)	N/A	Paralytic poliomyelitis and mild febrile illness
<i>Coxsackievirus A13, 18, 21</i>	ICAM-1 (Ig-like)	N/A	Aseptic meningitis Hand, foot and mouth disease Common cold
<i>Coxsackievirus A9</i>	$\alpha_v\beta_3$ Vitronectin receptor (integrin)	N/A	
<i>Coxsackievirus A21</i>	DAF (SCR-like)	ICAM-1 (Ig-like)	
<i>Coxsackievirus B1, 3, 5</i>	DAF (SCR-like)	$\alpha_v\beta_6$ (integrin)	Aseptic meningitis Myocarditis, pleurodynia, severe systemic disease in infants Upper respiratory tract illness, pneumonia and post-viral fatigue syndrome
<i>Coxsackievirus B1, 6</i>	CAR (SCR-like)	N/A	
<i>Echoviruses 1</i>	$\alpha_2\beta_1$ (integrin)	β_2m	Aseptic meningitis Paralysis Respiratory disease Myocarditis Epidemic myalgia Hepatic disturbances
<i>Echoviruses 3, 6, 7, 11-13, 20, 21, 24, 29, 33</i>	DAF (SCR-like)	β_2m	
<i>Echoviruses 22</i>	$\alpha_v\beta_3$ Vitronectin receptor (integrin)	N/A	
<i>Enterovirus 70</i>	Sialic acid	N/A	Acute haemorrhaging conjunctivitis
Rhinoviruses			
<i>Major receptor group rhinoviruses</i>	ICAM-1 (Ig-like)	N/A	Common cold
<i>Minor receptor group rhinoviruses</i>	LDLR (low density lipoprotein receptor)	N/A	
<i>Rhinovirus 87</i>	Sialic acid (carbohydrate)	N/A	
Hepatoviruses			
<i>HAV</i>	HAVcr-1 (Ig-like and mucin-like)	N/A	Hepatitis

Aphthoviruses			
FMDV	$\alpha_3\beta_5$ Vitronectin receptor, $\alpha_v\beta_6$ (Jackson et al., 2000), $\alpha_v\beta_1$ (Jackson et al., 2003)- integrins	Heparan sulphate proteoglycan	Foot-and-mouth disease
Cardioviruses			
Encephalomyocarditis virus	VCAM-1 (Ig-like) or Sialylated glycoporphin A (carbohydrate)	N/A	Encephalitis and myocarditis

Table 1.11: Picornavirus receptors and accessory molecules involved in cell infection.

Table was adapted from Evans & Almond (1998). A list of clinical diseases associated with picornavirus infection and exacerbation was added to provide a complete picture of receptors and role in pathogenesis. Abbreviations used: β_2m , β_2 -microglobulin; CAR, coxsackievirus and adenovirus receptor; DAF, decay-accelerating factor; HAVcr-1, hepatitis cellular receptor type 1; N/A, not applicable (no accessory molecules are currently implicated in virus infection); PVR, poliovirus receptor; SCR, short consensus repeat (s); VCAM-1, vascular adhesion molecule type 1.

1.4.2 Identified enterovirus receptor

1.4.2.1 Receptors

Human enteroviruses exhibit great diversity in the range of molecules used as cell surface molecules for attachment. Enteroviruses have evolved to use cell surface receptors that have a defined role in cell recruitment, attachment, infiltration or other aspects of the immune system. Certain enteroviruses, such as some coxsackieviruses and poliovirus, appear to have evolved to attach immunoglobulin-like molecules. It is possible either that the viruses have evolved to use immunological molecules or that the receptor bound by these viruses are immunoglobulin-like as the structural fold of these molecules is stable. The majority of enterovirus receptors are immunoglobulin-like molecules, other proteins such as integrins or SCR-like molecules, glycosaminoglycans (GAGs), sialic acid and other molecules such as heparan sulphate, which are all involved in a broad number of cellular functions (see figure 1.12).

The intracellular adhesion molecule type 1 (ICAM-1), the poliovirus receptor PVR, CD155 and the Coxsackievirus and adenovirus receptor (CAR) belong to the immunoglobulin-like family of cell surface molecules.

ICAM-1, a molecule involved in the adhesion between leukocytes and endothelial cells, has been identified as the receptor for human enterovirus species C coxsackieviruses A (Colonno et al., 1986; Shafren et al., 1997a) and the major receptor group human rhinoviruses (HRV) (Greve et al., 1989; Staunton et al., 1989).

CD155 is an 80kDa cell surface molecule, expressed as four splice variants of which α and δ act as a receptor for poliovirus attachment and entry (PVR) (Mendelsohn et al., 1989). Subsequent studies demonstrated that infection of non-transgenic mice with the mouse-adapted strain of poliovirus type 2 (Lansing) induced fatal poliomyelitis in mice (Murray et al., 1988), as seen in infection of PVR-transgenic mice with poliovirus type 1 (Ren et al., 1990). These two pathogens were shown to infect different cell types by histopathological analysis. The study of the PVR-transgenic mice infected with poliovirus type 1 Mahoney strain implies that the use of PVR is involved in causing poliomyelitis (Ren et al., 1990) and defines a virus as a poliovirus. However the precise role of the PVR in the host still remains to be identified even though it is involved in embryonic CNS development and cell to matrix contacts by specific binding to vitronectin (Lange et al., 2001). The PVR was recently shown to be a ligand for the natural killer cell (NK) triggering receptor DNAM-1 (CD226). Upon binding of CD155 to DNAM-1, activation of lysis of PVR-expressing cells by NK occurred. This was inhibited by the use of anti-DNAM-1 monoclonal antibody (Bottino et al., 2003). Until the identification of a CD155 mouse homolog (Morrison et al., 1992), poliovirus was believed to only infect primates as no non-primate CD155 homologs were thought to exist (Koike et al., 1992). Transgenic mice expressing the human PVR (Koike et al., 1991; Ren et al., 1990) have proved to be a very useful animal model for the study of poliovirus pathogenesis in a laboratory environment. Studies investigating the expression of CD155 have suggested that the protein seems to be regulated on a developmental level. CD155 expression in embryonic anatomical structures giving rise to spinal cord anterior horn motor neurons may contribute to the restricted host cell tropism of poliovirus for these compartments of the CNS (Gromeier et al., 2000). The PVR has also been suggested to have a part in intracellular transport (Mueller et al., 2002) and maybe retrograde axonal transport.

The CAR molecule, the identified receptor for CVBs and adenoviruses type 2 and type 5 (Bergelson et al., 1997), is expressed during the embryonic development in the central and peripheral nervous systems (Tomko et al., 2000) as well as in many other human tissues. CAR has cell adhesion functions (Honda et al., 2000).

The enteroviral receptor DAF, a GPI-anchored complement regulatory cell surface molecule protecting host cells from autologous lysis (Lublin et al., 1989) has four short consensus repeats (SCRs), which mediate binding with human enteroviruses species B, C and D. DAF was demonstrated to be the human receptor for enterovirus 70 (Karnauchow et al., 1996). Haemagglutinating echovirus strains (Ward et al., 1994; Powell et al., 1997; Williams et al., 2004), echovirus 7 (Powell et al., 1997), CVA21 (Shafren et al., 1997a; Newcombe et al., 1994; Johansson et al., 2004) and CBV1, 3 and 5 (Bergelson et al., 1995; Shieh et al., 2002) have since been shown to interact with DAF. DAF-inhibition studies have shown that EV6, EV7, EV12 and EV21 infections were DAF-dependent (Powell et al., 1998; reviewed in He et al., 2002). Many picornaviruses have been shown to bind DAF indicating that adaptation to use DAF as a receptor is likely to result from evolutionary selective pressure. DAF may therefore offer a selective advantage over other cell surface molecules. DAF has been thought to facilitate internalisation of virus receptor or recruitment of naïve cells and activation of the apoptotic pathway and therefore virus release and spread (Evans et al., 1998). DAF is also a molecule involved in controlling the host immune response and adaptation of viruses to use DAF could enable masking and inhibition of the immune system.

Heparan sulphate is a ubiquitous cell surface glycosaminoglycan (GAG) that interacts with a wide range of virus families, such as α herpesviruses (Shieh et al., 1992) and picornaviruses (Jackson et al., 1996). A number of human enteroviruses have been reported to use heparan sulphate as a receptor for attachment and entry. Coxsackievirus B3

uses heparan sulphate as well as CAR for virus entry (Zautner et al., 2003) and echovirus 6 clinical isolates use heparan sulphate as an alternative receptor to DAF for virus attachment and entry (Goodfellow et al., 2001).

Sialic acid, a family of unique 9-carbon monosaccharides, plays an essential role in enterovirus 70-attachment (Alexander et al., 2002). Indeed EV70 is able to bind neuraminidase-treated cells, in which DAF was neutralised, as the molecule is sensitive to neuraminidase. This is further supported by Haddad's work demonstrating that DAF-binding is not required for EV70 infection of a human leukocyte cell line (Haddad et al., 2004). It is thought that EV70 primarily binds sialic acid. EV70 is likely to have evolved to use DAF as a receptor by passage in cell culture (Karnauchow et al., 1996).

Increasing evidence indicates that dual tropism (simultaneous or sequential binding of a virus to two cell surface molecules) can be used for attachment and entry of enteroviruses into cells. The CVB3-Nancy strain uses the CAR receptor for attachment and entry and is unable to infect RD cells, as they do not express the CAR receptor. CVB3 serially passaged in RD cells (CVB3-RD) was able to productively infect these cells by DAF entry (Bergelson et al., 1995; Schmidtke et al., 2000) whilst retaining its ability to bind CAR (Schmidtke et al., 2000). CVB3-RD was however unable to infect CHO-DAF cells suggesting that accessory factors, molecules involved in subsequent stages of entry and uncoating, present in RD cells but not in CHO-DAF cells might play a role in infection with CVB3-RD adapted strain. Recent studies demonstrated that CVB3 was also able to use heparan sulphate as well as CAR for virus entry (Zautner et al., 2003). CVA9 is another enterovirus that is able to use more than one cell surface molecule for attachment. CVA9 uses the vitronectin receptor as a molecule for attachment to host cell (Roivainen et al., 1991). The inactivation of an RGD motif of the virus, by trypsin cleavage, does not prevent infection of African green monkey cells thereby indicating that there is an

alternative cell surface molecule used by CVA9 for attachment and entry (Roivainen et al., 1994). Echoviruses use DAF as a receptor (Bergelson et al., 1994; Powell et al., 1998; Ward et al., 1994) however recent studies suggested the potential use of DAF and heparan sulphate, a second receptor, by these viruses (Goodfellow et al., 2001)

Dual tropism could be due to capsid surfaces having evolved to contain two receptor-binding sites. However, distinguishing between molecules only involved in binding and molecules required for subsequent stages of infection such as entry and uncoating (known as accessory factors) can be difficult.

1.4.2.2 Accessory factors

As we understand more about viruses it is clear that accessory factors are important for infection. A number of accessory factors have been identified for different families of viruses and are thought to be important determinants of tissue specific pathogenesis and tropism. Accessory factors still need to be established for picornaviruses, however studies strongly suggest the involvement of such factors in the infection process (see table 1.11). Moreover, several entities have been reported to enhance infectivity of enteroviruses; in fact DAF has been demonstrated to enhance efficiency of CVA21 uptake, an ICAM-1 using virus (Shafren et al., 1997a; Johansson et al., 2004). MAP70, an MHC class I protein, has been suggested to be an accessory factor in infection with CVA9 (Triantafilou et al., 2000). CD59, a complement control protein, appears to be important in echovirus infection, in particular infection with echovirus 7 (Goodfellow et al., 2001). $\alpha_v\beta_6$ is thought to enhance CVB lytic infection of human colon cancer cells (Agrez et al., 1997). β_2 -microglobulin (β_2 -m), a component of the MHC class I has been indirectly implicated in the infection of RD cells with echoviruses, since treatment with β_2 -m antibody was able to block infection but not binding in RD cells (Ward et al., 1998). A similar event was

observed in CVA9 infection of CHO cells, where CVA9 was able to bind $\alpha_v\beta_3$ but no infection was observed after β_2 -m antibody treatment (Triantafilou et al., 1999)(see table 1.11). In all these cases, the role of these accessory factors remains ill defined.

1.4.3 Tropism and its determinants

It is clear that several factors are involved in the determination of pathogenesis and tropism, the affinity of picornaviruses for a population of host cells or a specific host tissue composed of susceptible cells (Shafren et al., 1997a; Johansson et al., 2004; Triantafilou et al., 2000; Goodfellow et al., 2001; Agrez et al., 1997; Triantafilou et al., 1999). The tropism of a virus is often defined by the receptor usage. Within each genus exist sub-divisions of viruses using the same receptor for attachment and entry. Some genera are directly correlated to receptor usage such as aphthoviruses that use the vitronectin receptor, and rhinoviruses and Coxsackievirus A21, both causing respiratory tract infections, that use either the intracellular adhesion molecule type 1 (ICAM-1: major receptor group) or the low-density lipoprotein receptor (LDLR: minor receptor group). However there is little or no direct correlation between the genetic similarity and receptor use for the remaining picornaviruses, for which the receptor has been identified, in particular for human enteroviruses as they exhibit the most diversity in receptor usage.

Pathogenesis is also variable. Different viruses can cause the same clinical symptoms (Melnick, 1996). Indeed Coxsackievirus B3 and Coxsackievirus B4 (CVB3 and CVB4) strains can vary in their ability to cause particular syndromes such as cardiovirulence and diabetes (Cao et al., 1991; Dunn et al., 2000). The two different viruses, closely related in their non-structural coding region of the genome, can infect the same cell type through attachment to the same receptors DAF or different receptors: DAF and CAR (Bergelson et al., 1995; Shafren et al., 1997b). This and the recent recombination data (described in

Chapter 3) suggest that the predominant – if not the primary – determinant of virus pathogenesis is the receptor to which it attaches.

Similarly, the same virus can infect different cell types, expressing the same cellular receptors, situated at distinct locations in the host, causing different symptoms. Poliovirus is a very good example as it can infect cells of the intestinal epithelium causing mild enteric syndromes whereas infection of the CNS motor neurons, expressing the same PVR cell surface receptor molecule, causes AFP.

The choice of receptor or cell surface molecule, determining the cell type infected, is a major factor contributing to symptoms observed in a viral infection. Receptors are therefore very important pathogenesis and tropism determinants. However, it is unclear whether these cell surface molecules are the sole determinants of tropism or whether other factors are involved. Factors such as the immune status of a particular host and different environmental conditions may be involved in the determination of tropism and pathogenesis. Whilst poliovirus appears not to require additional factors for cellular infection to occur, this is not the case for all enteroviruses.

Non-poliovirus enteroviruses, like CVA7, CVA9 (Grist et al., 1970), EV70 (Wadia et al., 1983), EV71 (Chumakov et al., 1979) and recently CVA21 (Shaffren et al., 1997) are nonetheless able to cause poliomyelitis-like syndromes. Despite acquiring particular receptor affinity, infection by some of these viruses can be blocked by antibodies to their original receptors. EV70 and EV71 have been shown to cause acute haemorrhagic conjunctivitis and hand-foot-and-mouth disease (Wang et al., 1999; Yin-Murphy et al., 1984). It has been thought by many that non-poliovirus enteroviruses could potentially acquire affinity for CD155 through adaptive changes in the P1 structural proteins coding

region (Gromeier et al., 1999). The hypothesis still needs to be tested and the following project was an attempt to determine whether such an evolutionary event could occur.

1.5 Evolution

RNA virus evolution occurs under selective pressure, provided by physical, selective, functional, developmental and/or genetic constraints, and is driven by two mechanisms enabling the viruses to adapt, survive and multiply in a new cellular environment: polymerase error rate leading to the introduction of mutations within progeny genomes and RNA recombination. The evolution of picornaviruses is driven by accumulation of point mutations, short insertions/deletions and genomic changes due to the exchange of genetic material, with related or non-related sequences, that lead to the generation of divergent RNA and protein sequences, with conservation of essential functions and viability of the virus. The evolution of picornaviruses occurs via both these mechanisms.

1.5.1 Evolution of picornaviruses

1.5.1.1 Mechanisms of evolution

1.5.1.1.1 RdRp mutation rate and error polymerase rate

Replication of all positive-sense strand RNA viruses is driven by RNA-dependent RNA-polymerases (RdRp), apart from retroviruses that replicate using RNA-dependent DNA-polymerases. RdRps lack a proofreading mechanism (Ishihama et al., 1986) and are therefore unable to correct mutations or mismatches introduced during viral replication. The polymerase error rate of picornaviruses has been shown to be as high as 2.1×10^{-4} to 5.9×10^{-4} substitutions per nucleotide (Crotty et al., 2001; Sierra et al., 2000). As a result positive-sense strand RNA viruses exist as quasispecies, or an ensemble of related but genetically different viral genomes (Eigen et al., 1988), and exhibit high evolution rates,

discussed further in chapter 2. Existing as a quasispecies is an evolutionary advantage as the mutant spectrum contains viral sequences that may be efficient at evading the immune response, having increased resistance to antiviral agents, escaping specific antibodies and cytotoxic T cells and altering their induction of interferon capacity (Ruiz-Jarabo et al., 2000).

1.5.1.1.2 Recombination

1.5.1.1.2.1 Definition and description

The second process that drives positive-sense single strand RNA virus evolution is that of recombination between viral RNA genomes and was first described in poliovirus in the 1960's (Hirst et al., 1962; Ledinko et al., 1963). It is a widespread mechanism known to occur in animal, plant and human viruses (Chetverin et al., 1997; Lai et al., 1992b; Slobodskaya et al., 1996) and it enables the generation of fitter viruses, able to survive in an altered environment, or the preservation of wild-type viral sequences. Recombination is the generation of a new combination of genes that are more advantageous for the survival of a specific viral population. Recombination between two pieces of RNA can be either homologous, and occur between two identical or similar molecules recombining in a precise (junction sites occupy different positions in recombining molecules) or imprecise (producing molecules with duplications and deletions) manner, or non-homologous, and occur between two different RNA molecules.

1.5.1.1.2.2 Different processes of recombination

Recombination of positive-sense single strand non-segmented RNA genomes can occur via two distinct mechanisms: a replication-dependent copy-choice mechanism where the viral replicase-nascent strand complex switches from one template to another during replication (Coffin et al., 1979; Kirkegaard et al., 1986; Figleworicz et al., 1997; Figleworicz et al.,

1998) and a replication-independent mechanism of breakage and rejoining of pieces of genetic material (Kirkegaard et al., 1986). Until recently, very few studies had investigated the second process, however recent studies have provided definitive evidence of recombination occurring via a replication-independent mechanism of template breakage and rejoining (Chetverin et al., 1997; Gallei et al., 2004; Gmyl et al., 2003).

1.5.1.1.2.2.1 RdRp copy choice mechanism

Copy-choice recombination is the result of the RdRp template switching during replication (Figlerowicz et al., 1998; Jarvis et al., 1992; Kirkegaard et al., 1986; Lai et al., 1992a; Nagy et al., 1997; Nagy, 1998; Pilipenko, 1995), a process described in studies of pseudorevertant genomes in Theiler's murine encephalomyelitis virus (TMEV) and poliovirus (Pilipenko et al., 1995). Pausing of the nascent strand due to misincorporation by the RdRp, dissociation of the RNA polymerase from the nascent strand, re-annealing of nascent and template strands and resumption of the synthesis are the various stages of the copy-choice mechanism (reviewed Nagy et al., 1997). Studies in the 1960's demonstrated that poliovirus recombinants could arise as a result of mixed infection of a cell with two different serotypes of polioviruses (Hirst et al., 1962; Ledinko et al., 1963). This was later shown to occur inside specific compartments used for RNA replication (Bienz et al., 1990).

1.5.1.1.2.2.2 Replication-independent recombination

Unlike template switching, non-replicative recombination has not been studied extensively. Chetverin carried out experiments on Q β replication demonstrating non-replicative recombination of RNA fragments (Gmyl et al., 1999; Chetverin et al., 1997). These *in vitro* studies, however, did not exclude the possible low levels of replication due to the presence of the Q β replicase required for amplification of the recombinant molecules.

Recombination could still be accounted for by a copy-choice mechanism. Even if we assume that Chetverin's studies demonstrate non-replicative recombination *in vitro* it has

not been demonstrated in an *in vivo* system until recently. Experiments studying the co-transfection of incomplete poliovirus RNA sequences demonstrated the generation of viable viruses and gave strong but not definitive evidence of a replication-independent recombination mechanism (Gmyl et al., 2003). However, the definitive evidence of such an event occurring was provided by the recent studies carried out in BVDV, where viable recombinants would arise from co-transfection of segments of RNA, each containing deletions in the RdRp-coding genes thereby completely ruling out the possible involvement of replication in the process (Gallei et al., 2004).

1.5.1.2 Evidence of emergence of recombinants in nature

The emergence of viruses as a result of recombination represents an important threat to the world as they have the potential to cause major outbreaks and pandemics. Indeed virus recombination, leading to the emergence of completely new viruses, has been a concern for many years. The recent outbreak of SARS, a coronavirus that is thought to have recently evolved from its natural reservoir to infect humans, illustrates the impact such emerging viruses can have both economically and demographically. Another example of recombination occurring in nature is that of bovine viral diarrhea viruses (BVDVs). Recent phylogenetic studies of BVDVs suggested that recombination between different virus strains had occurred in animal hosts (Nagai et al., 2004). HIV is an RNA human retrovirus that has had a huge impact on the world. Recombination has been reported to be a frequent event contributing to the generation of genetically diverse strains of HIV with different pathogenesis characteristics (Robertson et al., 1995).

1.5.1.3 Evolution amongst enteroviruses

Recombination plays a major role in enterovirus evolution. In fact, numerous recombination events have been reported in both a natural host environment and a

laboratory environment. Certain human enteroviruses are thought to have arisen from ancient recombination events and this was suggested to have occurred for CVA16 and bovine enteroviruses as sequence comparison studies of these viruses indicated strong phylogenetic relationships (Hyypiä et al., 1997). Genetic recombination between different human enteroviruses type B echovirus serotypes has been reported to occur when multiple epidemic strains were circulating simultaneously (Oprisan et al., 2002). This was confirmed by recent work studying the phylogenetic relationships between various enteroviruses species B (Oberste et al., 2004a). Further phylogenetic analysis of enteroviruses species C (Brown et al., 2003) and enteroviruses species A (Oberste et al., 2005) included strong evidence that recombination amongst enteroviruses is a common occurrence. CVA21, currently defined as an enterovirus expressing the same tissue tropism as HRV and causing respiratory infections, was suggested to be a recombinant between a rhinovirus and a poliovirus in studies carried out in the mid 1980's (Stanway et al., 1984).

1.5.1.4 VDPV and vaccine revertants

Live-attenuated strains of poliovirus, containing mutations rendering the strains unable to cause poliomyelitis but still able to infect cells; have proved to be an essential tool for vaccination and eradication of poliovirus. Sabin was one of the first generated live-attenuated poliovirus vaccine strains (Krugman et al., 1961), and these are now used in many countries as means of immunisation through vaccination with OPV, a live-attenuated vaccine containing three serotypes of live-attenuated poliovirus wild-type strains. Since the first use of live-attenuated poliovirus strains, the secretion of neurovirulent revertants have been reported (reviewed Dowdle et al., 2003) and have been shown to cause infection in immunodeficient patients and the non-immune population in close contact with the excreting vaccinee. However, recent data from retrospective studies have shown the presence of recombinant VDPVs (vaccine-derived poliovirus) circulating in the wild.

VDPDs isolates were circulating in Egypt for over 10 years, between 1983 and 1993, and circulation ceased with rising coverage of OPV vaccination (Yang et al., 2003). These studies not only suggest that OPV vaccine strains can recombine and cause acute flaccid paralysis (AFP) but that VDPVs can circulate for extended periods of time in an environment where low vaccination coverage is prevalent. Further retrospective studies of the poliovirus Sabin type 3-associated outbreak in Poland in 1968 (Martin et al., 2000) demonstrated that VDPV-related outbreaks were not restricted to tropical developing countries such as Hispaniola (Kew et al., 2002) and that VDPVs were able to cause AFP in developed countries with temperate climates and moderate population densities if the population immunity to one of the vaccine strains of poliovirus is low. Moreover studies of 1963-1966 VDPV isolates in Belarus (Korotkova et al., 2003) reinforced the point that maintenance of sufficient levels of OPV vaccination coverage is essential to avoid circulating VDPVs from occurring.

1.5.1.5 Recent outbreaks and recombinants between poliovirus and human enterovirus C

The outbreak of recombinant vaccine strains in Hispaniola in 2000-2001 (Kew et al., 2002) caused an immediate public health concern, as the country had been free of wild-type poliovirus since 1991. Isolates were collected, sequenced and had Sabin poliovirus type 1 5'UTR and P1/2A-coding sequences and unidentified human enterovirus C 3'UTR, 2B/2C/P3-coding sequences, thereby giving evidence for a recombination event. This recombination event is the first report of human enteroviruses C recombining with a Sabin poliovirus vaccine strain with a crossover point within the 2A/2B-coding region (see figure 3.38). Other circulating VDPVs were also identified in the Philippines in 2001 (MMWR 2001). More recent studies carried out by Delpyroux's group isolated strains from outbreaks of AFP in Madagascar (Rousset et al., 2003) and identified a number of human

enterovirus C strains that were circulating at the time of vaccination (Rousset et al., 2004). Studies of both Haitian and Madagascan cVDPVs suggested that live-attenuated poliovirus vaccine strains were able to recombine with circulating enterovirus C causing a poliomyelitis-like disease. In fact, further studies carried out by Delpeyroux's group identified two unspecified recombination crossover points within the 2A-coding region and a definitive recombination between vaccine strains Sabin type 2 and enteroviruses species C (Rousset et al., 2004). These studies not only highlighted the risk of recombinant viruses emerging and causing outbreaks, as a result of low vaccination coverage, but the fact that enteroviruses C were able to recombine just confirmed the difficult obstacles the WHO would have to overcome in order to succeed in eradicating poliovirus from the world. The emergence of such recombinant viruses may lead to a persistence of circulating polioviruses in the wild. So far the circulating recombinant VDPVs isolated and analysed from outbreak cases had structural proteins of a vaccine strain and the remainder of the virus genome had sequences of unidentified viruses within species of HEV-C (Stanway et al., 2000). An urgent understanding of such recombinants is essential for the monitoring and control of transmission or re-emergence of poliomyelitis post-eradication. Failure to investigate concerns regarding the polio eradication programme could lead to the re-emergence of poliovirus and actually reverse the efforts and progress already achieved by the WHO.

1.5.1.6 Vaccines and revertants

Vaccine-derived polioviruses have been responsible for many reported acute flaccid paralysis (AFP) cases. Stability of live-attenuated vaccines has been a concern since the first evidence of the appearance of vaccine-associated paralytic poliomyelitis VAPP soon after the OPV vaccine was licensed for commercial use. With a large part of the world being polio-free OPV has always been considered as a very efficient and fairly safe

vaccine. However, recent retrospective studies and outbreaks of paralytic poliomyelitis associated with circulating vaccine-derived polioviruses (cVDPVs) have highlighted pre-existing concerns.

1.5.2 WHO eradication program and rising concerns

In the 1950's poliomyelitis was an important disease prevalent in both the developed and the developing world. Salk vaccine, a poliovirus inactivated vaccine (IPV), was initially used as a prevention method and is still used in many countries. Since the 1960's Sabin's OPV (trivalent live-attenuated oral poliovirus vaccine) has been used, in most parts of the world, as the preferred prevention method. IPV and OPV are still being used but the low cost of OPV production makes its administration in developing countries more manageable and realistic. This pattern is likely to change as eradication is achieved and every means possible will be made to exclude the potential reintroduction of circulating paralytic poliovirus.

The 1979 smallpox eradication prompted the Pan American Health Organisation (PAHO) and World Health Organisation (WHO) to initiate a poliovirus eradication program, in 1985, aiming to eliminate poliomyelitis worldwide by 1990 using the OPV as the means to the end. Mass immunisation campaigns were initiated with national immunisation days (NIDS), in which millions of children in a country are immunised in a single day, and sub-national immunisation days (SNIDS), in which smaller areas are immunised in one day. These have shown to abruptly reduce the number of susceptible individuals thereby interrupting transmission of the virus. However, widespread vaccination can prove to be challenging and some parts of tropical and developing countries have very low immunisation coverage. This leads to the emergence of vaccine-associated paralytic poliomyelitis (VAPP) caused by circulating vaccine-derived poliovirus strains (cVDPVs).

The WHO polio eradication program has achieved the eradication of circulating wild-type poliovirus in many parts of the world (figure 1.13), however the transmission of wild-type poliovirus still occurs in India, Pakistan, Afghanistan, Egypt, Niger and Nigeria and the emergence of more VAPP cases by cVDPVs in Haiti (Kew et al., 2002), Egypt (Yang et al., 1997), Philippines (MMWR 2001) and Madagascar (Rousset et al., 2003) have raised issues about the successful eradication of poliomyelitis. Better monitoring has enabled the detection of emerging recombinant viruses between vaccine strains and human enterovirus species C (Kew et al., 2002; Rousset et al., 2003), able to cause outbreaks, highlighting the importance of vaccine-derived polioviruses (VDPV) and the need for a better understanding of recombinants both *in vitro* and *in vivo*.

The re-emergence of poliomyelitis due to undetected circulating wild-type strains or recombinant vaccine strains is a major concern. Monitoring and control methods need to be carried out and extended to the post-eradication era as poliovirus could escape or be released from laboratories and potentially cause a pandemic in a non-immune susceptible population. Vaccine strains need to be kept in case such an event was to happen and it is likely that vaccination in the developed world will switch to IPV in the end stages of the eradication program. Non-poliovirus enteroviruses are known to be capable of causing a poliomyelitis-like disease in humans. The eradication of poliovirus does not remove the possibility of another enterovirus evolving to use the poliovirus ecological niche left vacant post-eradication and cause poliomyelitis.

1.6 Aims of the project

As the world gets closer to poliomyelitis eradication, progress in vaccination distribution, detection methods and laboratory containment are becoming more important. The emerging VDPVs causing outbreaks of AFP are a major source of concern and various

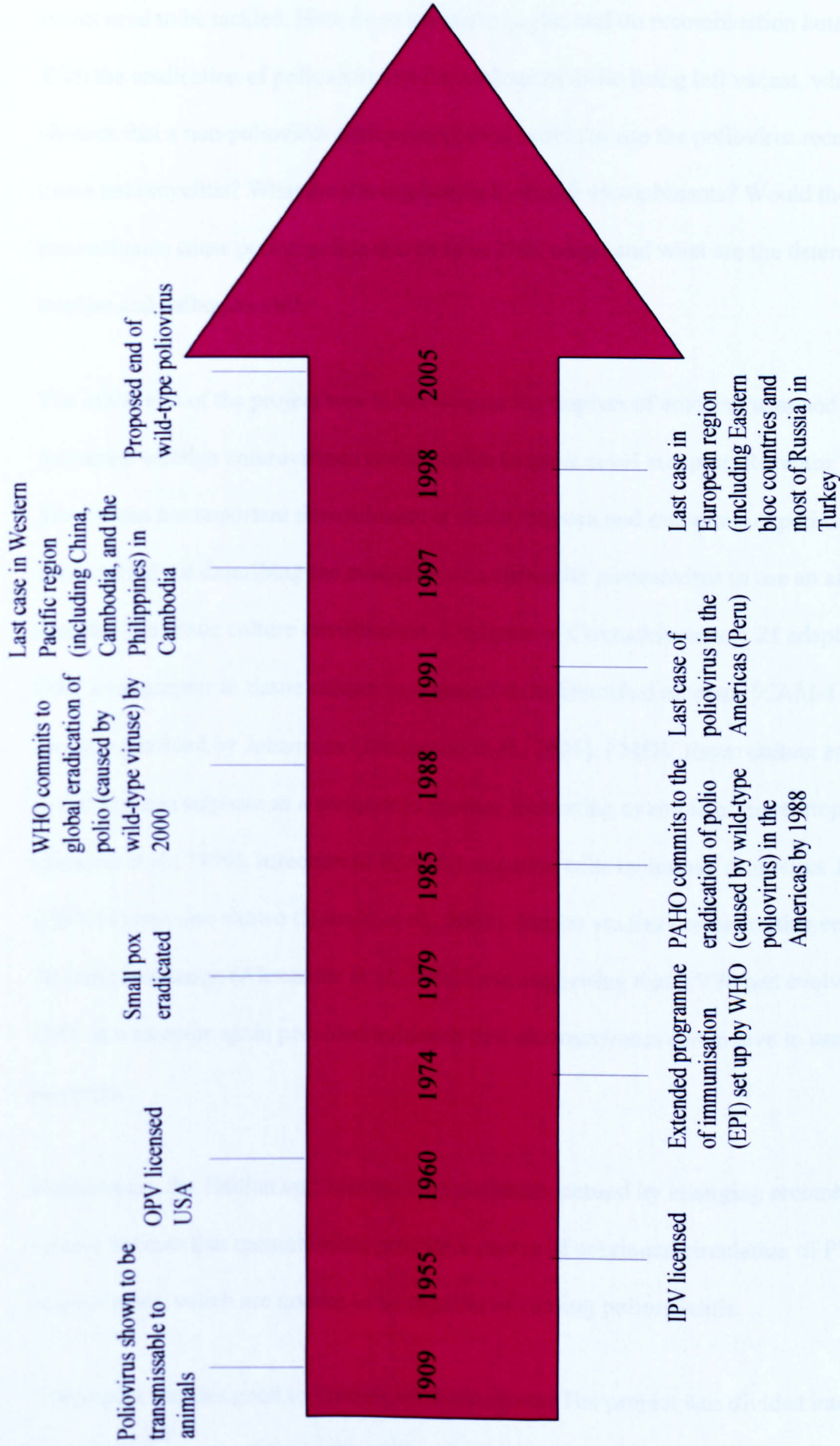


Figure 1.13: Important events in poliovirus eradication (figure taken from "Polio eradication, cessation of vaccination and re-emergence of disease" P. Minor, 2004, Nature Reviews).

issues need to be tackled. How do recombinants arise and do recombination hotspots exist? With the eradication of poliovirus and the ecological niche being left vacant, what are the chances that a non-poliovirus enterovirus could evolve to use the poliovirus receptor and cause poliomyelitis? What are the implications of such recombinants? Would these recombinants cause poliomyelitis due to their PVR usage and what are the determinants of tropism and pathogenesis?

The initial aim of the project was to investigate the tropism of enteroviruses and to determine whether enteroviruses could evolve to use a novel receptor. Receptor interactions are important determinants of tissue tropism and numerous experiments have been carried out describing the evolution of a particular picornavirus to use an alternate receptor in a tissue culture environment. Evidence of Coxsackievirus A 21 adapting to use DAF as a receptor in tissue culture as opposed to its identified receptor ICAM-1 was recently provided by Johansson (Johansson et al., 2004). FMDV tissue culture adaptation to use heparan sulphate as a receptor is another interesting example of novel tropism (Jackson et al., 1996). Infection of ICAM-1 negative cells by human rhinovirus 14 (HRV14) was also shown (Reischl et al., 2001). Recent studies demonstrating enterovirus 70 sialic acid usage (Alexander et al., 2002) and suggesting that EV70 had evolved to use DAF as a receptor again provided evidence that picornaviruses can evolve to use alternate receptors.

Furthermore, the Haitian and Madagascan outbreaks caused by emerging recombinant viruses indicate that recombinants provide a source of continued circulation of PVR-using enteroviruses, which are known to be capable of causing poliomyelitis.

The project was designed to investigate these issues. The project was divided into three major sets of experiments: mutagenesis selection and adaptation studies, *in vitro* generation

and characterisation of recombinant viruses and *in vivo* selection for novel tropic recombinant viruses.

Mutagenesis studies to select for novel tropic viruses using an antiviral mutagen were carried out. These were designed to enable the generation of divergent mutant viruses using a process reproducing evolution.

In vitro generation of recombinants with pre-determination of the crossover sites was initiated to investigate heterologous processing requirements of enteroviruses. The aim was to generate recombinant viruses that had the capsid-coding region of a non-poliovirus enterovirus and the remainder of the genome of a poliovirus. Reciprocal recombinants were also constructed in attempt to provide a better understanding of the VDPVs recombined with enterovirus species C (Kew et al., 2002; Rousset et al., 2003). The circulating VDPVs, isolated from outbreak cases in Haiti and Madagascar, were demonstrated to be recombinants between the structural protein-coding region of vaccine strains, Sabin poliovirus type 1 (Haiti) and Sabin poliovirus type 2 (Madagascar), and the non-structural protein-coding region of human enteroviruses species C. The viability of these isolates indicated that the 3CD of an enterovirus type C could efficiently process the P1 of a poliovirus vaccine strain.

In vivo selection for novel tropic recombinant viruses by co-transfection of two defective viral RNA sequences constituted the final part of the project. Previous evidence of successful generation of recombinants by co-transfection of incomplete viral RNA transcripts, reports of emergence of recombinants between human enteroviruses C and Sabin poliovirus vaccine strains and the implication of such recombinants in view of poliomyelitis eradication formed the basis of the next set of experiments. Successful selection of capsid recombinants in tissue culture without predetermining crossover

points/exact recombination sites and characterisation of such recombinants could provide valuable information on properties, stages of life cycle affected, tropism determinants, potential location of recombination hotspots of enteroviruses and the potential emergence of a virus with a novel sequence to occupy the ecological niche that would be left vacant following poliovirus eradication. Generation of such recombinants could also potentially be a tool for enabling identification of compatible domains of the genome involved in packaging and give us insights into protein-protein, protein-RNA and potentially RNA-RNA interactions involved in replication.

2 Selection of novel tropic viruses using a mutagen

2.1 Introduction

All RNA viruses, except for retroviruses, use RNA-dependent RNA-polymerases (RdRps) as a catalytic enzyme for RNA synthesis. RdRps lack proofreading activity thereby introducing errors to the newly synthesised progeny genomes. RNA viruses can therefore exhibit a high mutation rate. These viruses exist as quasispecies or large population of related but divergent viral sequences, which may be an evolutionary advantage. The frequency at which errors occur in a viral genome is known as error frequency, which is the measure of genetic drift or gradual adaptation to new environments due to external stresses also known as selective pressure.

However, the evolution rate of RNA viruses is not solely determined by the fixation rate of mutations as it is also dependent on environmental factors. Measles virus exhibits a high mutation rate but has evolved slowly due to a low fixation rate of mutations (Schrag et al., 1999) whereas HIV shows a higher evolution rate even though its mutation rate is similar to that of measles virus. The evolution rate of HIV has been extensively documented and has been shown to be dependent on mutation rate as well as on the host immune system (Wolinsky et al., 1996) and other factors, which select for viruses that exhibit particular phenotypes. Indeed a virus could evolve faster in a new host but keep the same polymerase error rate or fidelity, due to a variability in the fixation rate of mutations. Recent studies carried out by Andino (Crotty et al., 2001), Domingo (Ruiz-Jarabo et al., 2000; Sierra et al., 2000) and co-workers confirmed the mutation rate of picornaviruses to be between 2.1×10^{-4} and 5.9×10^{-4} substitutions per nucleotide. These figures were obtained by direct sequencing analysis of many viral genomes and by using the frequency at which genetic markers are isolated from a given virus population. These studies confirmed the initial

poliovirus mutation rate calculation (Ward et al., 1988). More investigations have quantified the poliovirus mutation rate in a long-term immunodeficient excretor to be 1% per base per annum (Martin et al., 2000; Minor, 1996), again supporting initial calculations (Ward et al., 1988). Recent investigations demonstrated that the VP1-coding region of Sabin type 1 VDPV, isolated from the gut of a healthy child, exhibited a sequence drift of over 2% per base per annum. This figure proves that certain VDPVs exhibit higher mutation rates than that initially observed for poliovirus, and that these viruses are able to replicate in the gut of healthy individuals for long periods of time (Martin et al., 2004).

Selective pressure occurs during processes such as replication, cell lysis and virion release into the intercellular environment, where these come into contact with cells from the immune system. The combination of variation and large replication yield during infection is considered to be advantageous to virus populations. If the host specific immune response results in the near elimination of wild-type viruses, quasispecies may contain escape mutants that do not express the specific epitopes recognised by cytotoxic T cells, antibodies or innate responses. These escape mutants will then quickly replicate and give rise to a new population of quasispecies, allowing the survival of the viral population as was shown for Hepatitis C virus (HCV) (Erickson et al., 2001) and HIV (Pircher et al., 1990). However, errors can lead to the generation of non-viable viruses and the extinction of a particular virus population in the event of excessive number of mutations being introduced, a consequence known as error catastrophe (see figure 2.4 and 2.5).

RNA virus recombination can be beneficial to mutant viral sequences and can allow recovery of fitness-impaired sequences (Chao et al., 1997). However, recombination can also lead to the generation of non-viable viruses due to incompatibility between genomes, RNA structures and/or proteins.

In an environment where the mutation rates are high, a significant proportion of mutations are deleterious. This leads to the generation of non-viable or otherwise disadvantaged genomes. Müller's ratchet is the selection by serial passages of virus populations through a narrow genetic bottleneck, an evolutionary process whereby a significant percentage of a virus population is killed or prevented from reproducing, which leads to the reduction in the population and survival of a subset of viruses (transmitted from host to host) (Duarte et al., 1992; Chao et al., 1990; Domingo et al., 1985). In this context, wild-type viruses are defined as viruses that have sufficient fitness to survive and be transmitted from host to host. Müller's ratchet could have significant implications for variability of disease severity during virus outbreaks.

Evolution of picomaviruses, and more specifically poliovirus, occurs via two mechanisms: the introduction of mutations due to the error rate of RdRp and intra-/inter-typic recombination. The fixation rate and phenotypic selection may result in viruses with altered tropism at an enhanced level of mutation. The use of a mutagen *in vitro* may enable the selection for novel tropic mutant progeny viruses.

2.1.1 Ribavirin: a mutagen and its effect on virus life cycle

Ribavirin, 1- β -D-ribofuranosyl-1,-2,4-triazole-3-carboxamide (see figure 2.1), is a synthetic broad-spectrum non-specific ribonucleoside analog with antiviral activity.

Ribavirin is currently used for the treatment of a variety of RNA virus infections. In humans, the drug is used as monotherapy for Lassa fever virus infections and severe respiratory syncytial virus infections, and in combination with interferon- α as treatment for Hepatitis C virus (HCV) infections (Crotty et al., 2002a).

Despite the extensive use of ribavirin, the detailed understanding of the activity of the drug is still unclear thereby preventing further development of enhanced derivatives with improved properties for clinical use. Recent studies have shown that ribavirin is an RNA virus mutagen (Crotty et al., 2000) and lethal mutagenesis has been proposed to be its mechanism of action (Crotty et al., 2001).

Once inside the cell ribavirin is phosphorylated by adenosine kinase to generate ribavirin monophosphate (RMP). Sequential phosphorylation events then give rise to ribavirin triphosphate (RTP). RMP is an inosine monophosphate dehydrogenase (IMPDH) inhibitor (Muller et al., 1977; Streeter et al., 1973). IMPDH is a cellular enzyme used for *de novo* synthesis of guanosine triphosphate (GTP), which is itself required for translation, transcription and replication of all viruses. IMPDH inhibition triggers a decrease of intracellular levels of GTP, which could account for antiviral activity.

RTP is a substrate for the poliovirus RNA-dependent RNA polymerase and acts as a purine nucleoside analog by presenting two distinct hydrogen-bonding conformations to complementary bases. Low intracellular GTP levels favour the incorporation of RTP into

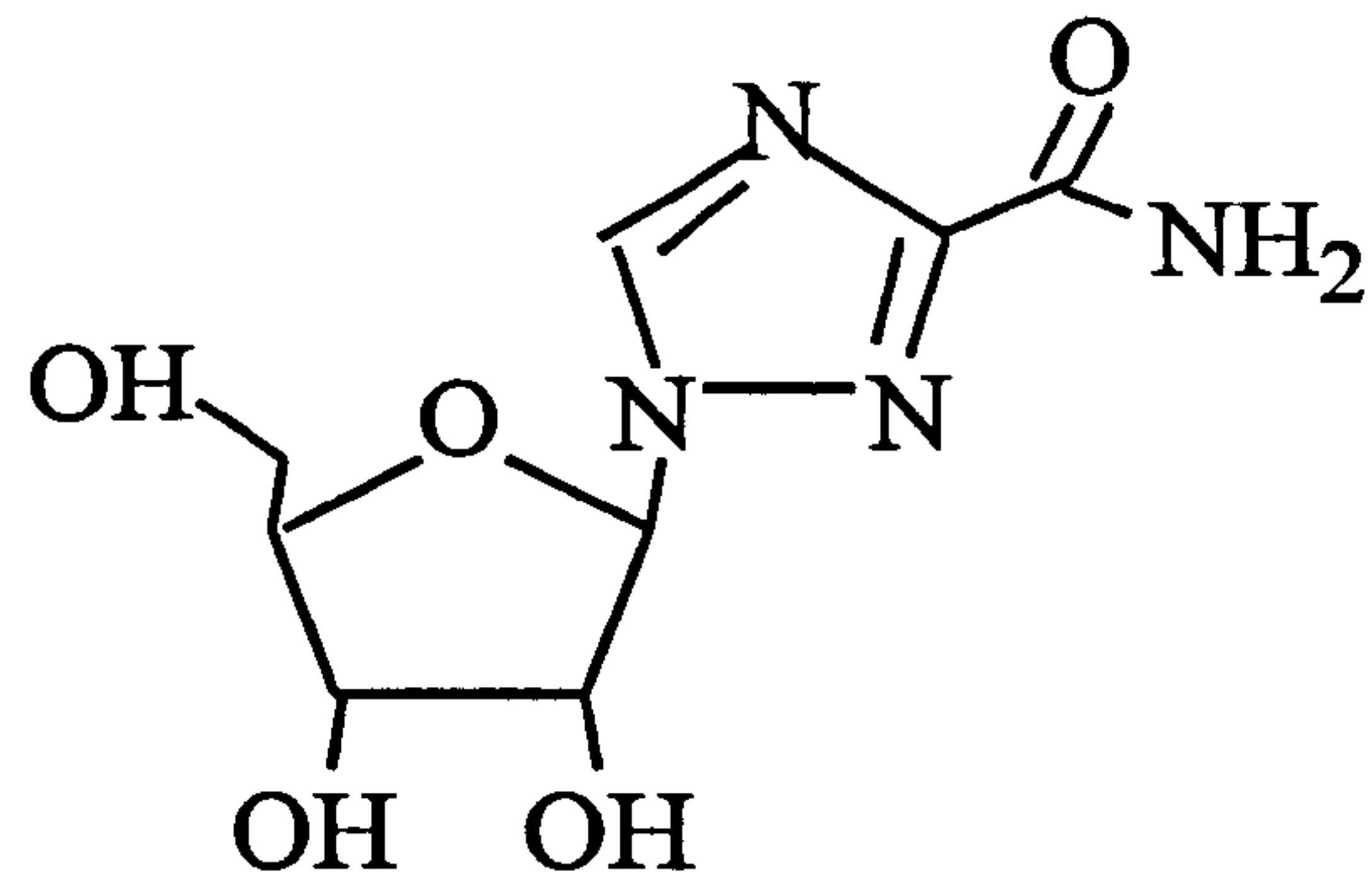


Figure 2.1: Chemical structure of ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide).

Figure taken from Crotty et al. (2002 b).

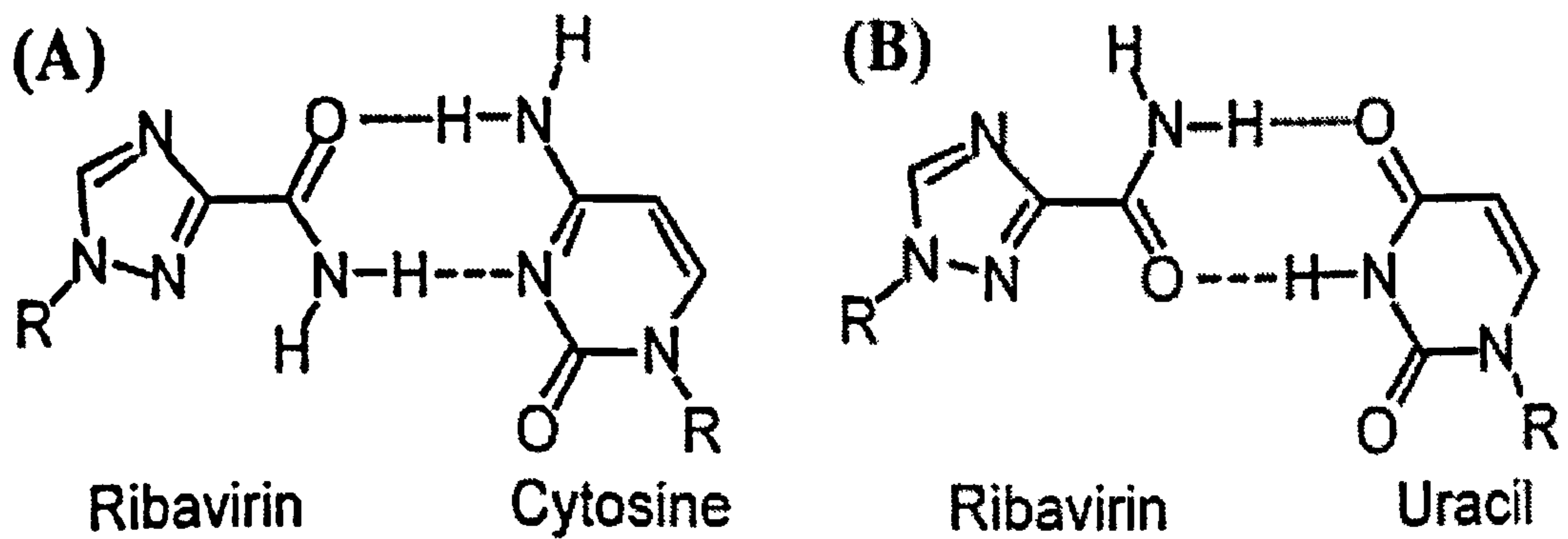


Figure 2.2: Hydrogen-bonding conformations of ribavirin, a purine analog, with cytosine and uracil, pyrimidine bases.

Figure taken from Graci et al. (2002).

the genome, promoting G to A and A to G transitions due to its ability to base pair with C and U (see figure 2.2) and disrupting RNA structures, which can be critical for virus replication (Crotty et al., 2000).

Ribavirin concentration	Number of mutations observed in the sequenced window		Total mutation frequency (/10 ⁴ nucleotides)	Total mutation frequency (/genome)
	G→A	C→T		
0 μM	0.5	1.2	2.1	1.5
100 μM	0	1.3	2.5	1.8
400 μM	4.4	5	9.3	6.9
1,000 μM	6.8	12	20.8	15.6

Table 2.3: Mutation frequency in ribavirin-treated RNA poliovirus populations. Data was taken from Crotty et al. (2001).

2.1.2 Effect of ribavirin on poliovirus infection life cycle

Andino and co workers (Crotty et al., 2000) established a relationship between ribavirin concentration and the mutation rate of poliovirus. In their study they also showed the effect ribavirin exerted on viral translation and production. The increase of the mutation frequency caused by ribavirin, shown in table 2.3 and the effect of ribavirin on translation and production of poliovirus were demonstrated by Andino and co-workers (Crotty et al., 2000). Poliovirus replication and translation, determined using a subgenomic replicon, is reduced only very slightly in the presence of up to 1mM ribavirin. Under similar conditions virus yield is reduced by up to 3 log₁₀. The normal mutation rate of poliovirus, 1.5 mutations per genome (Crotty et al., 2001), was increased to approximately 6.9 mutations per genome in the presence of 0.4 mM ribavirin. This was shown to cause a 95%

decrease in the fitness of the viral population compared to that of wild type (Crotty et al., 2001). Because the normal poliovirus population exists on the threshold of error catastrophe, accumulation of too many mutations can lead to lethal mutagenesis of poliovirus leading to a decrease in virus titres to near background levels (see figure 2.4: Crotty et al., 2001). RNA viruses are unstable and a small increase in mutation levels can result in a reduction of virus viability. This has been shown for poliovirus, vesicular stomatitis virus (Holland et al., 1990), and HIV (Loeb et al., 2000). Moreover, serial passages in the presence of a mutagen were shown to be sufficient for viral extinction of HIV populations (Loeb et al., 2000). The relationship between virus survival and error catastrophe is fragile and the application of random mutagenesis experiments might cause a shift in the equilibrium relationship towards error catastrophe (see figure 2.5).

2.2 Aims

Will the poliovirus niche remain vacant after the eradication of poliomyelitis or could viruses evolve to occupy the empty ecological niche? Enteroviruses exhibit a high mutation rate in nature. Over time, mutations cause changes in the capsid-coding region that could lead to a change in receptor usage. Moreover, it has been extensively reported that picornaviruses can adapt to use alternative receptors in tissue culture. Various examples provide evidence that picornaviruses can use more than one cell surface molecules for cell attachment. Indeed, FMDV can adapt through a single mutation to use heparan-sulphate as a receptor in tissue culture as opposed to the identified receptor $\alpha_v\beta_3$, (Jackson et al., 1996). Human rhinovirus 14 has been shown to be able to infect ICAM-1 negative cells (Reischl et al., 2001). Coxsackieviruses have adapted to attach DAF and infect RD cells (Reagan et al., 1984). A DAF-binding echovirus 6 has been demonstrated to have the ability to use heparan sulphate as a receptor (data not published David Williams). An echovirus 11 variant has also adapted to not use DAF as a receptor for

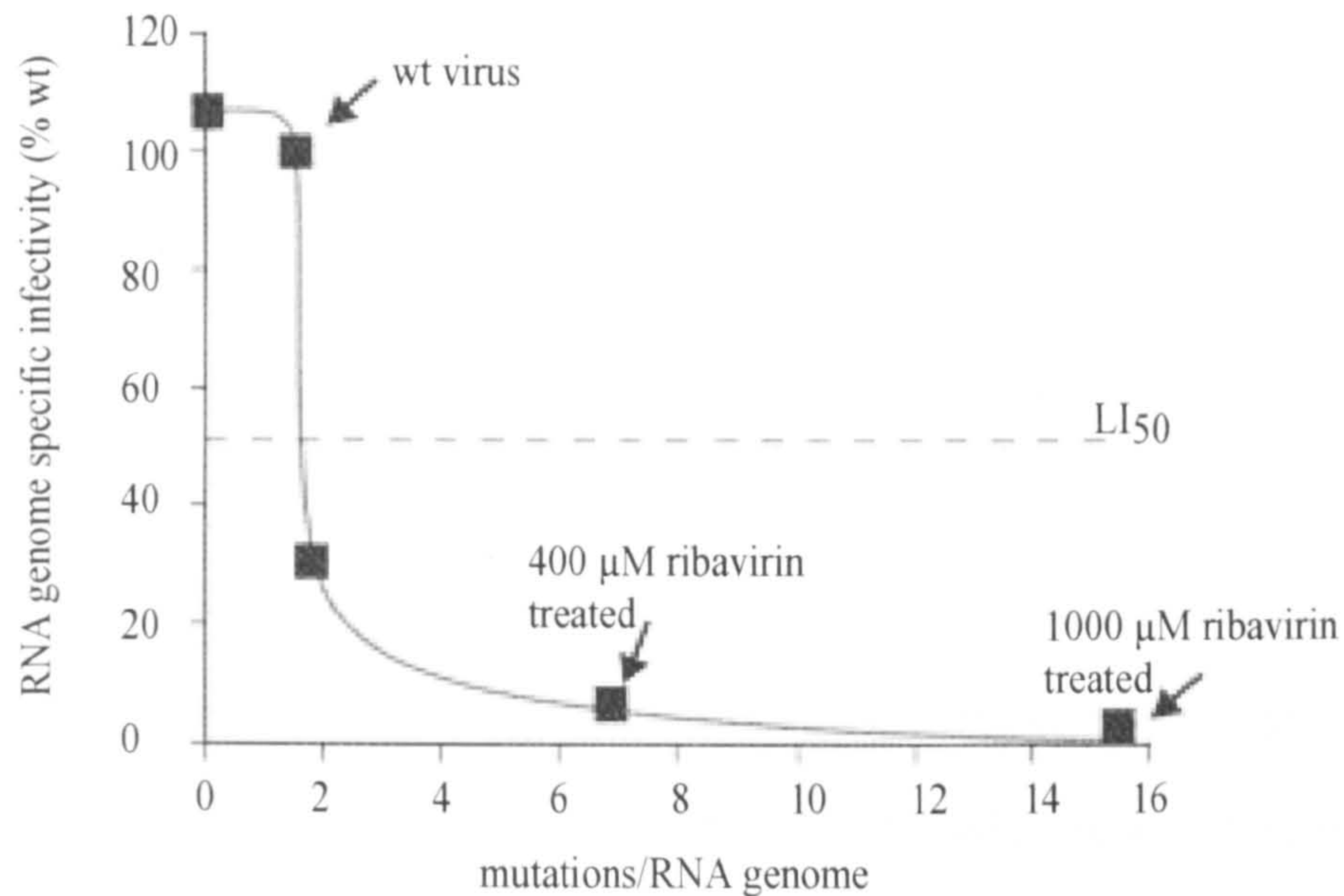


Figure 2.4: Error catastrophe

Relationship of mutation frequency to genomic RNA infectivity (Crotty et al., 2002 b). Specific infectivity of normal poliovirus RNA was set to 100%. The graph shows that poliovirus populations exist near the edge of error catastrophe, as there is a rapid decline in RNA genome infectivity at levels of mutagenesis only slightly higher than normal. The LI50 (50% loss of specific infectivity) is defined as the mutation frequency at which 50% of the viral genomes are lethally mutated, indicated by the dashed line. Wild-type poliovirus genomes contain an average ~1.5 mutations/genome. Poliovirus genomes from cells treated with 400 μM and 1000 μM ribavirin respectively contain an average ~6.9 mutations/genome and ~15.5 mutations/genome. Taken from Crotty et al. (2001).

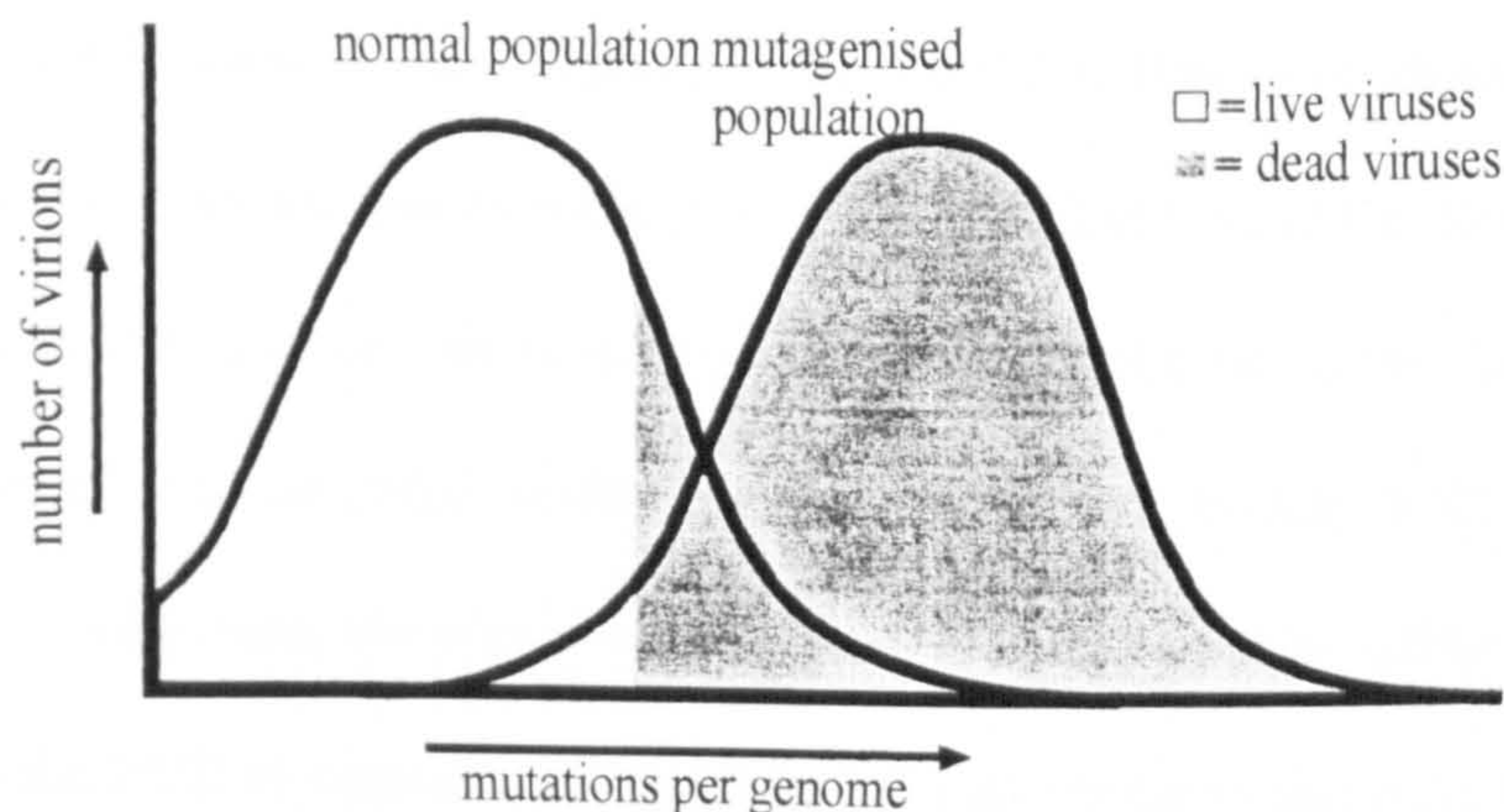


Figure 2.5: Model of error catastrophe

The majority of viruses in a normal picornavirus population are viable. But a small increase in mutation frequency is predicted to push the virus population into error catastrophe (the mutagenised population, Right), where the number of errors per viral genome is sufficiently high to lethally mutate a majority of the virus population. White indicates live virus, gray indicates dead virus. Figure and legend taken from Crotty et al. (2001).

infection (Stuart et al., 2001). Finally, CVA21 DAF-binding has been demonstrated and confirmed (Shafren et al., 1997; Johansson et al., 2004).

The aim of the experiment was to investigate whether accelerating evolution *in vitro*, by combining the use of a non-specific mutagen with the natural variability exhibited by enteroviruses, would generate key mutations resulting in a virus receptor-usage shift.

Selection of mutagen-treated virus populations, by conducting serial passages on a variety of cell lines, could enable the isolation of specific tissue-tropic viruses. The potential outcome of such experiments could be the selection of a population of enteroviruses that have evolved to use an alternative receptor or the selection of a population of non-poliovirus enteroviruses that use the PVR.

To investigate this, a closely related human enterovirus species C to poliovirus was used as a HEV representative for this experiment. CVA21 is a HEV-C closely related to PV3 with an amino acid sequence identity of 84.2%. CVA21 and PV3 have evolved from a common ancestor to use different virus receptors. The aim of this experiment was to try and select for mutated CVA21 virus populations that bind the PVR. However, divergence from the common ancestor is an ancient event and it is possible that CVA21 is too genetically distant from poliovirus to be able to evolve to use the PVR (see figure 2.6). However, there are similarities in virus-receptor interaction of both viruses, as they both interact with Ig-like receptor molecules via a canyon interface. It is therefore possible that CVA21 could evolve to use the PVR as opposed to the ICAM-1 cell surface molecule. Can CVA21, a well-characterised HEV-C, undergo a shift in receptor usage in the presence of ribavirin? Can CVA21 evolve to use PVR? Such findings would be crucial information for the WHO poliomyelitis eradication program and would contribute to our understanding of virus evolution and the evolution of receptor tropism.

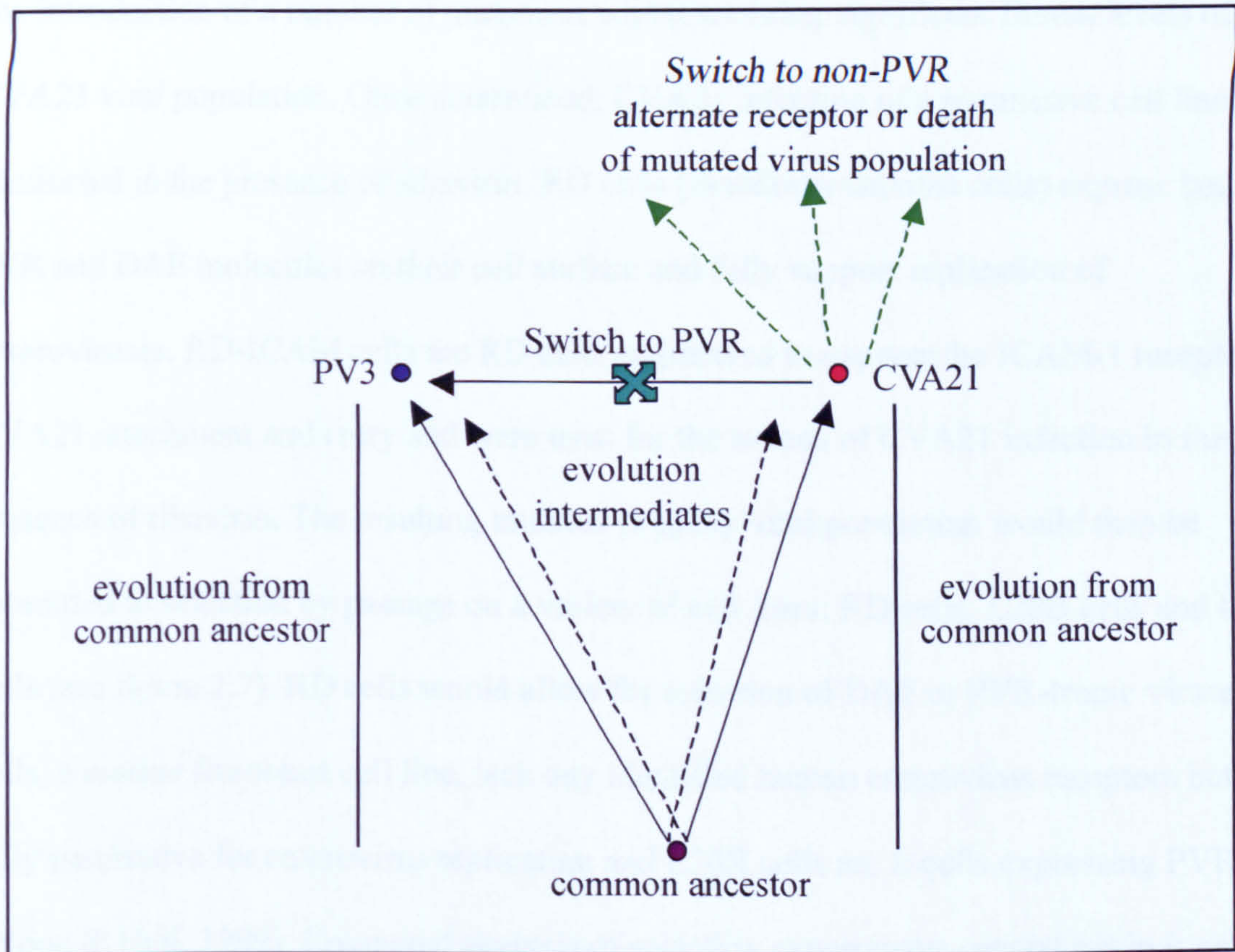


Figure 2.6: Compared evolution of CVA21 and PV3

Figure illustrating different possible outcome of the designed strategy: switch from ICAM-1 usage to PVR-usage, switch to non-PVR alternate receptor-usage and death of mutated virus population due to introduction of fatal errors or mutations that do not allow alternate receptor usage. The switch from ICAM-1 usage to PVR-usage could occur, however it is also possible that the shift in receptor requires too great a genetic shift. Discussed section 2.2, chapter 2.

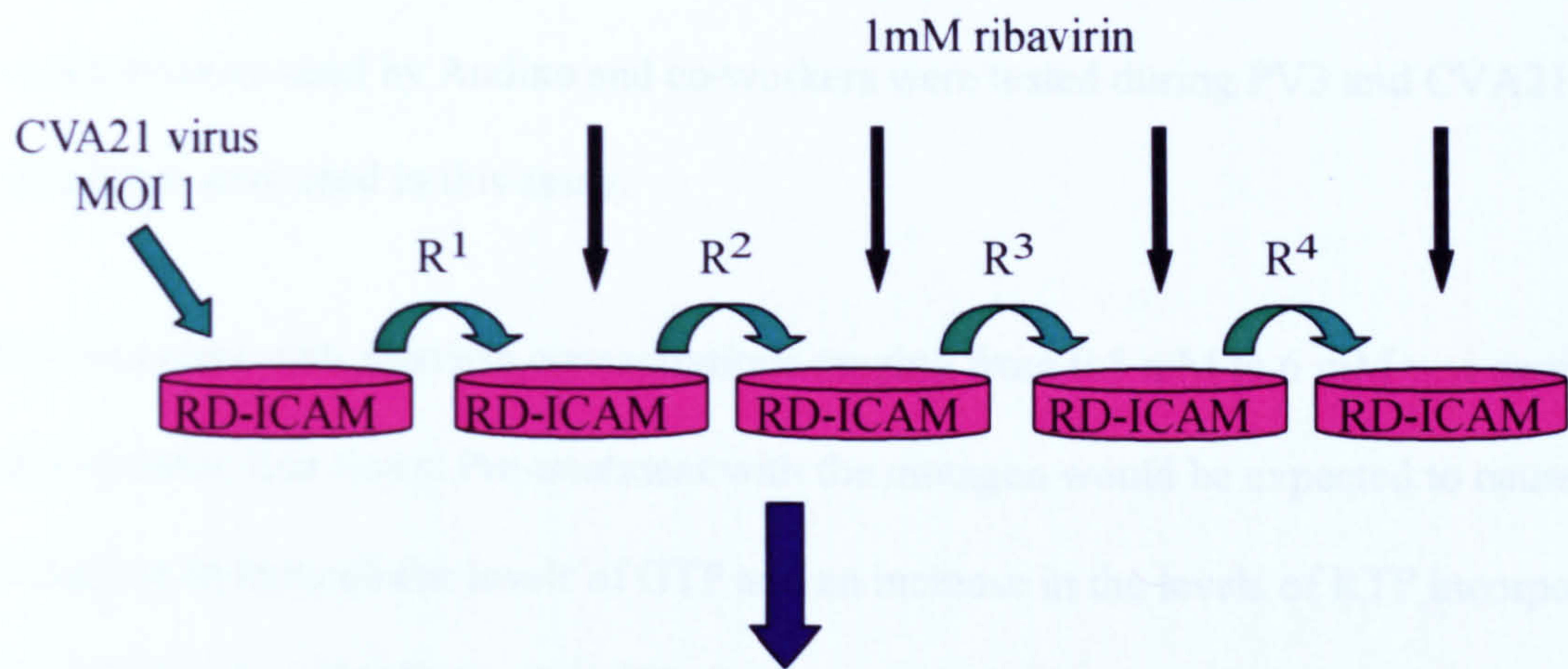
2.2.1 Experimental model

Initial titration of ribavirin was carried out to determine a concentration that would lead to the introduction of a number of mutations whilst retaining significant fitness levels of a CVA21 viral population. Once determined, CVA21 infection of a permissive cell line was conducted in the presence of ribavirin. RD cells (rhabdomyosarcoma cells) express both PVR and DAF molecules on their cell surface and fully support replication of enteroviruses. RD-ICAM cells are RD cells engineered to express the ICAM-1 receptor for CVA21 attachment and entry and were used for the rounds of CVA21 infection in the presence of ribavirin. The resulting mutated progeny viral population would then be submitted to selection by passage on a variety of cell lines: RD cells, L20B cells and L cells (see figure 2.7). RD cells would allow for selection of DAF or PVR-tropic viruses. L cells, a murine fibroblast cell line, lack any identified human enterovirus receptors but are fully permissive for enterovirus replication and L20B cells are L cells expressing PVR (Wood & Hull, 1999). Compared analysis of selection experiments carried out in L cells and L20B cells would be the most restrictive strategy. Mutated enterovirus populations growing on L20B cells but not L cells would be PVR-tropic. This could lead to the first *in vitro* report of an enterovirus that had evolved to use PVR.

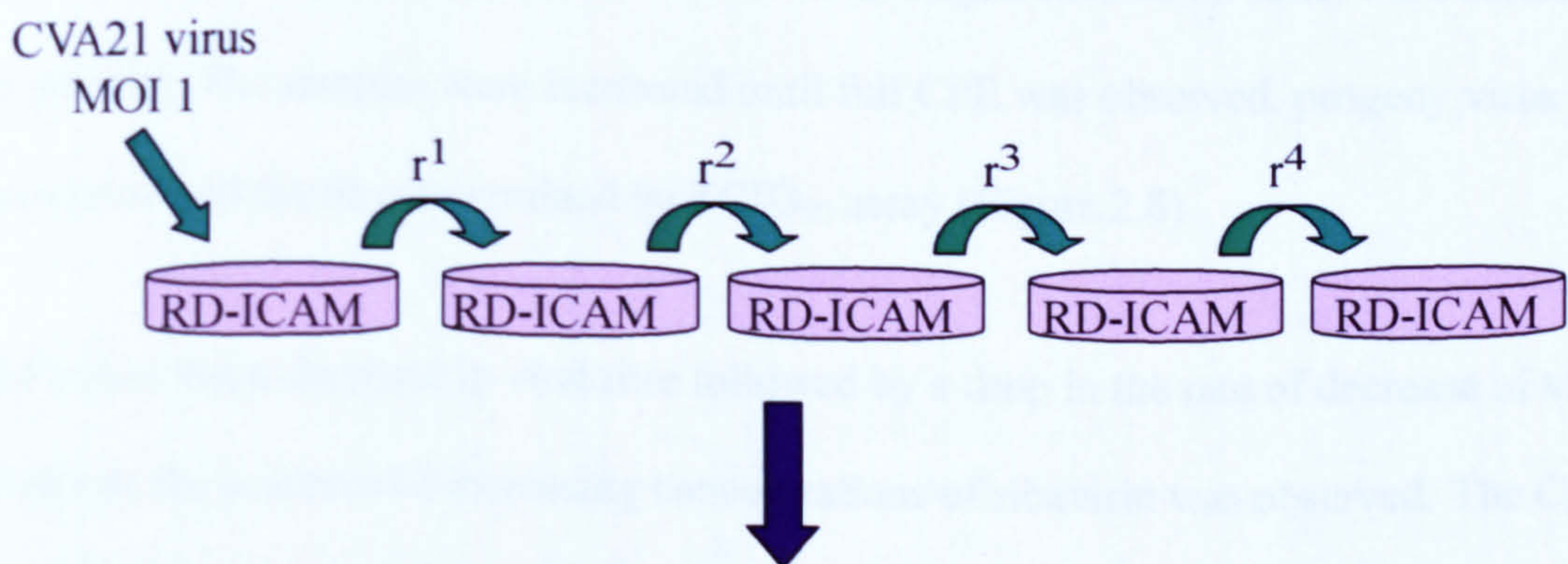
2.3 Results and Discussion

2.3.1 Effect of ribavirin on CVA21 yields and determination of concentration to use for experimental system

The introduction of 6-9 mutations per poliovirus genome resulted in a 95% reduction in fitness of the viral populations (Crotty et al., 2001). The effect of a range of ribavirin concentrations on CVA21 was not expected to differ greatly from those observed with poliovirus, due to the similarity of the virus, the replication cycle and the similarity of



Testing on RD cells, L cells and L20B cells in the absence of ribavirin



Testing on RD cells, L cells and L20B cells in the absence of ribavirin

Figure 2.7: Selection of novel tropic viruses: infection of CVA21 in the presence of ribavirin (a random mutagen).

Flow chart representing a diagram of the experimental procedures. Rⁿ indicates passage in the presence of ribavirin, where n is the passage number, and rⁿ indicates passage in the absence of ribavirin, where n is the passage number.

growth of the viruses *in vitro*. Only concentrations spanning the upper limits of ribavirin concentrations used by Andino and co-workers were tested during PV3 and CVA21 viral infections were used in this assay.

Pre-treatment with ribavirin concentrations ranging from 0.5 mM to 6 mM was carried out and repeated four times. Pre-treatment with the mutagen would be expected to cause a reduction in intracellular levels of GTP and an increase in the levels of RTP incorporated in viral genomes (Muller et al., 1977; Streeter et al., 1973), as a GTP analog. Cells were then infected with CVA21 or PV3 viruses at an MOI of 1. CVA21 and PV3 infections of untreated cells in the presence and absence of a virus replication inhibitor GuHCl were conducted to check that the observed CPE were virally induced. Mock infections in the presence of ribavirin, to monitor the effect the mutagen exerted on cells, were carried out in parallel. The samples were incubated until full CPE was observed, progeny virus was harvested and the titre determined by TCID₅₀ assay (Figure 2.8).

An initial sharp decrease in viral titre followed by a drop in the rate of decrease of viral yields in the presence of increasing concentrations of ribavirin was observed. The CPE exhibited by PV3 infection of ribavirin pre-treated RD-ICAM cells and the effect of ribavirin on virus titres was similar to those previously observed (Crotty et al., 2000).

Unlike PV3, CVA21 did not show as strong a decrease in titre at lower concentrations of ribavirin. A 1-log titre reduction of CVA21 was observed in the presence of 1mM ribavirin, a 2-log decrease was observed when CVA21 was treated with 3mM ribavirin and a 4-log decrease was seen in the presence of 6mM of the mutagen. With such a significant decrease the overall fitness of the mutated viral populations was expected to be too low due to an excessive number of mutations being introduced and the volume handled in the assay would need to be too high. 1mM of ribavirin was the chosen concentration for the following assays, as a sufficient decrease in CVA21 virus titres (1-log) was achieved and a

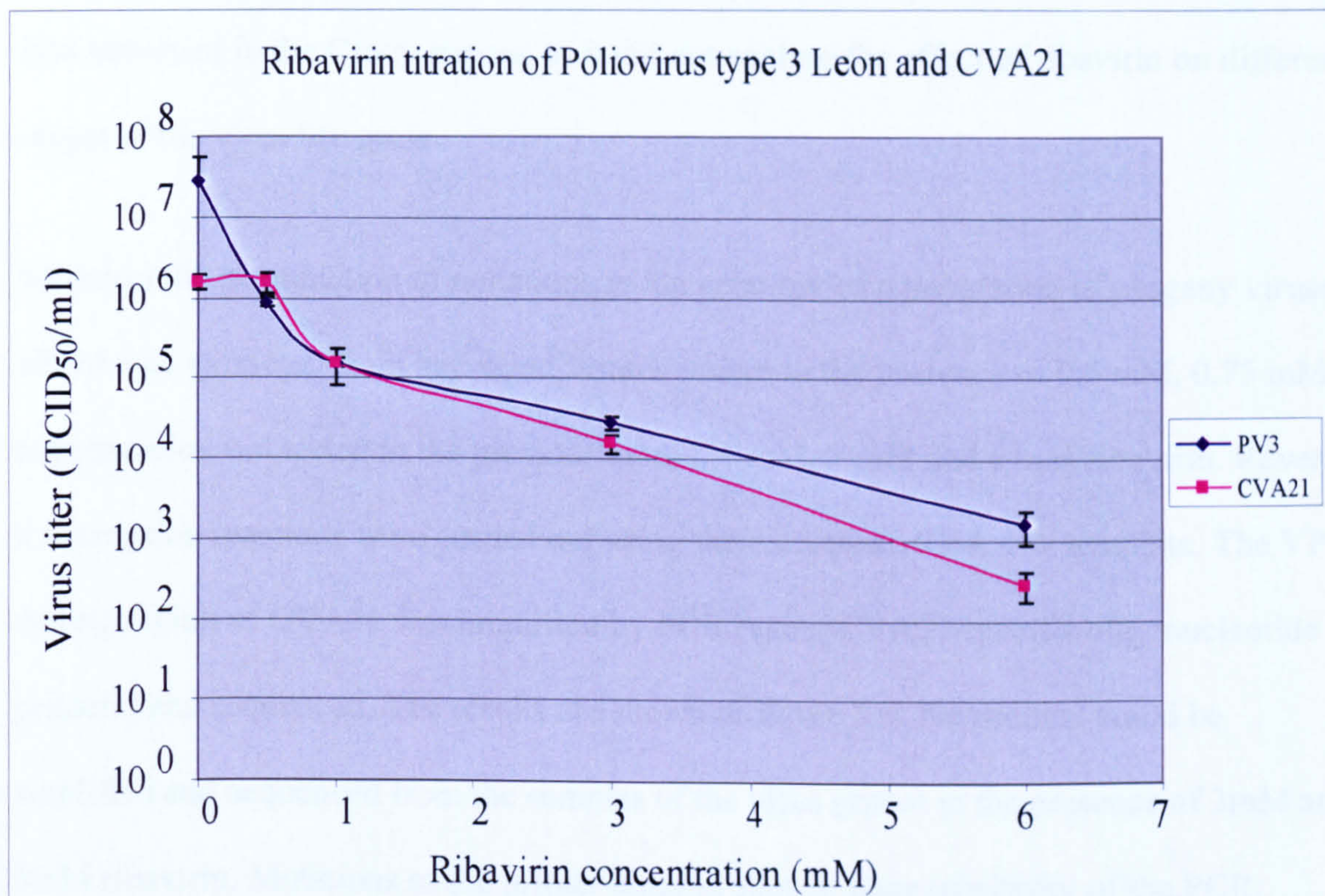
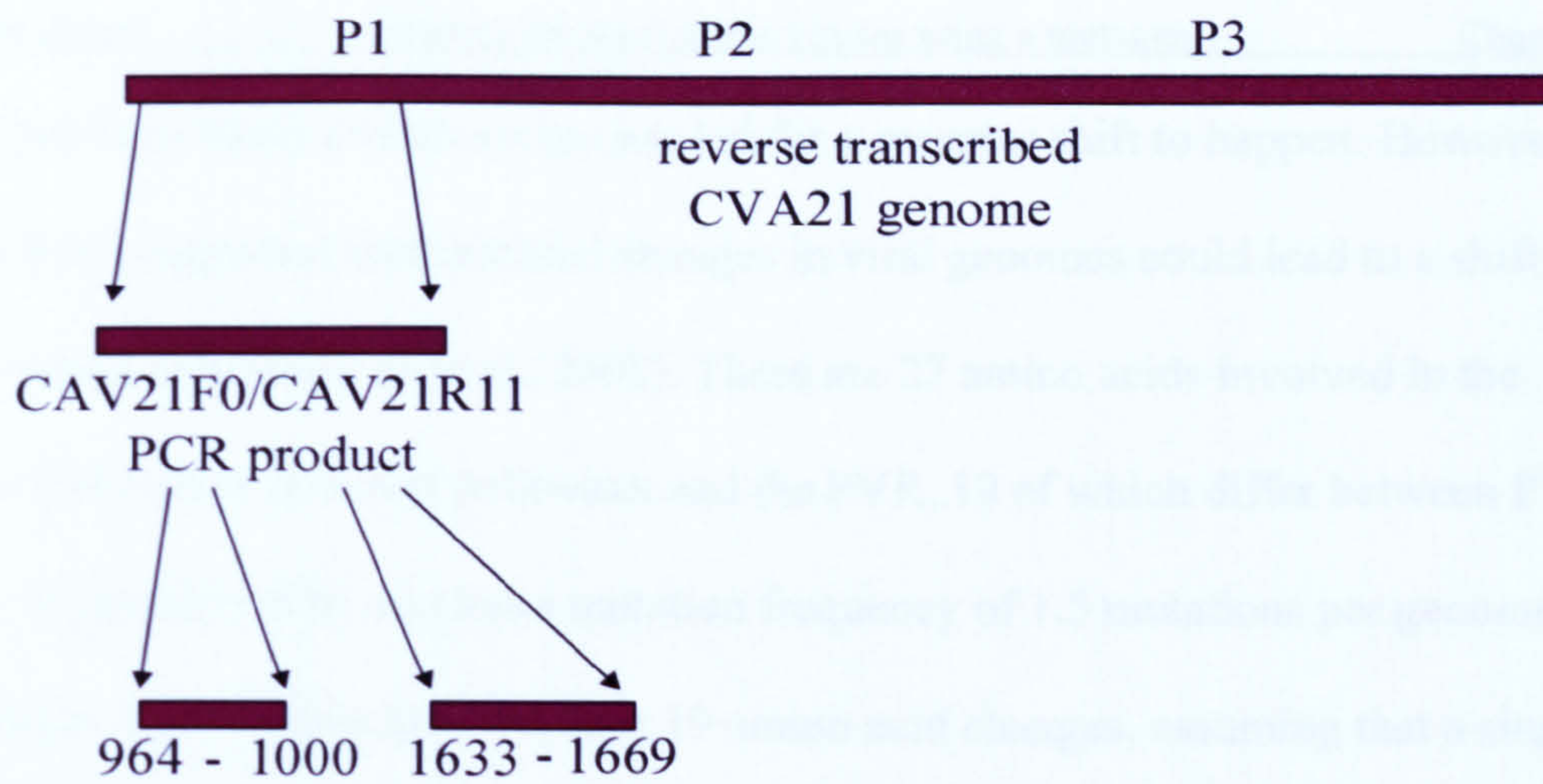


Figure 2.8: Titration of CVA21 and PV3 in the presence of increasing concentrations of ribavirin, a nucleoside analog.

TCID₅₀ assays of PV3 and CVA21 virus grown in the presence of a range of ribavirin concentrations were carried out four times. Plates were fixed and stained. Titres were calculated and plotted using a log scale. The 4 repeats of the assay exhibited similar results. Standard deviation values were calculated and are represented as error bars on the graph. 10mM ribavirin concentrations exerted a cytotoxic effect on mock infected cells and was therefore not used in the experiment.

significant number of mutations were expected to have been introduced. None of the ribavirin concentrations used (with the exception of the 10mM samples), had a cytotoxic effect on the cells. This basic titration assay did not enable any direct comparison with the data observed in the Crotty papers, as it did not analyse the effect of ribavirin on different stages of the virus life cycle.

To detect the introduction of mutations in the genomes of a population of progeny viruses, vRNA was extracted from harvested viruses grown in the presence of 0.5 mM, 0.75 mM (a concentration not tested in the previous assay), 1 mM, 3 mM and 6 mM ribavirin. Reverse transcription reactions were carried out using the extracted vRNA as a template. The VP1 coding region of CVA21 was amplified by PCR (using CVA21 specific oligonucleotide primers) and sequenced. The results are shown in figure 2.9. No product could be amplified and sequenced from the samples of the virus grown in the presence of 3mM and 6mM ribavirin. Mutations of the primer binding sites or poor sensitivity of the PCR reaction could both be responsible for this phenomenon. A window of the CVA21 VP1-coding region of the virus samples grown in the presence of 0.5 mM, 0.75 mM and 1 mM ribavirin was amplified by PCR and sequenced. The results are shown in figure 2.9. The sequencing results show that within a 1778-bp window two C to T coding changes, reflecting C to U substitutions in the original viral sequence, that were absent from the original CVA21 viral sequence, were introduced when the virus was grown in the presence of 1 mM ribavirin and no third-base changes were seen. No changes were detected in the sequences of the 0.5mM and 0.75mM ribavirin samples. Amplification reactions were carried out from a population of DNA molecules since the fragments were not cloned therefore the mutations seen are likely to have been fixed at early stages of the replication cycle and calculation of mutation rates would therefore be inaccurate.



	964											1000
CVA21	I	T	L	G	N	S	T	I	T	T	Q	E
	ATC	ACT	TTA	GGT	AAC	TCG	ACC	ATT	ACC	ACA	CAG	GAA
CVA21+0.5mM	I	T	L	G	N	S	T	I	T	T	Q	E

CVA21+0.75mM	I	T	L	G	N	S	T	I	T	T	Q	E

CVA21+1.0mM	I	T	L	G	N	S	I	I	T	T	Q	E
T.

	1633											1669
CVA21	F	A	A	T	S	S	P	Q	V	P	I	T
	TTT	GCC	GCA	ACA	TCG	TCA	CCA	CAG	GTG	CCT	ATT	ACA
CVA21+0.5mM	F	A	A	T	S	S	P	Q	V	P	I	T

CVA21+0.75mM	F	A	A	T	S	S	P	Q	V	P	I	T

CVA21+1.0mM	F	A	A	T	S	S	S	Q	V	P	I	T
	T.

Figure 2.9: Sequencing analysis of input CVA21 virus and CVA21 virus sequences passaged in the presence of 0.5mM, 0.75mM and 1.0mM ribavirin.

The vRNA of the various samples was extracted using a QIAamp® Viral RNA mini kit, vRNA was reverse transcribed and newly synthesised cDNA was amplified by PCR using CVA21 P1 specific oligonucleotide primers (CVA21-F0, CVA21-R11). The PCR products were purified and sent for sequencing. Here the two nucleotide changes, both from C to T, due to the presence of ribavirin are illustrated and cause two coding amino acid changes.

It is unclear how many mutations are needed for a receptor shift to happen. However, it has recently been suggested that minimal changes in viral genomes could lead to a shift in receptor usage (Baranowski et al., 2001). There are 27 amino acids involved in the interface interaction between poliovirus and the PVR, 19 of which differ between PV3 and CVA21. A normal poliovirus has a mutation frequency of 1.5 mutations per genome or 1 mutation per 5000 nucleotides. To have 19 amino acid changes, assuming that a single nucleotide change is sufficient to lead to an amino acid change, every virus would have a 1 in $(5 \times 10^3)^{19}$ or 2×10^{70} chance to mutate the correct 19 nucleotides. Even with a mutation rate of 1 mutation per 600 nucleotides, increase induced by treatment with a mutagen such as ribavirin, every virus would have a 1 in $(6 \times 10^2)^{19}$ or 6×10^{52} chance to mutate the correct 19 nucleotides and therefore amino acids for a shift in receptor usage. However, it is likely that more than one nucleotide change is required for an amino acid change and the chance of getting all 19 amino acid mutations would therefore be expected to be even lower. This describes the worst-case scenario whereby all 19 amino acids involved in the interface that differ between CVA21 and PV3 would need to be changed for a switch in the receptor usage of CVA21 to occur.

However, due to the nature of the interaction of the canyon with the virus receptor, the importance of all amino acids involved in the interaction between a virus particle and a receptor molecule is not equal. For example, only 3 amino acids out of the 30 residue-interface contribute 80% of the total energy of the interaction between EV7 and DAF (Bhella 2004). Similarly, Akio Nomoto's group suggested that only 3 amino acids are key to the interaction between PV1 and the PVR (Arita et al., 1998). A single amino acid change has been shown to alter Echovirus type-6 receptor usage (unpublished data by David Williams). So it is very likely that significantly fewer amino acids than all 19 of those that differ between PV3 and CVA21, involved in the interaction, would need to be

changed for the virus to use a different cell surface molecule as a receptor for attachment and entry. If three amino acids need to be changed for the receptor swap to occur at a mutation rate of 1 in 5×10^3 then a population of $(5 \times 10^3)^3$ or 1.25×10^{11} would have to be screened, a more reasonable and manageable number. In fact in a hypothetical case where the mutation rate was increased to 1 mutation in 600 nucleotides the minimum population screened would have to be $(6 \times 10^2)^3$ or 2.1×10^8 viruses. A smaller number of mutations than that required for CVA21 adaptation to use the PVR could be required for CVA21 to adapt to use DAF, heparan sulphate or other enterovirus receptor molecules. The use of a random mutagen to accelerate evolution and select for a human enterovirus type C that has evolved to use an alternate receptor, and more specifically the PVR, is a feasible experiment, due to the small number of mutations actually required for a receptor shift to occur and due to the possible handling of large population sizes in small volumes.

2.3.2 Determination of optimal concentration of ribavirin

The assays carried out in this chapter do not enable the determination of the optimal ribavirin concentration to attain the correct equilibrium of introducing enough mutations and maintaining sufficient fitness of the mutated viral populations. Sequencing of independent clones from PCR reactions at each concentration and overlaying graphs plotting the number of mutations introduced versus ribavirin concentrations and the viral titre drop versus ribavirin concentrations (see figure 2.10) could be carried out. There is likely to be a balance between the number of mutations introduced and the decrease in viral titres. It would therefore be possible to determine the optimal ribavirin concentration needed to introduce a maximal number of mutations in a virus population without shifting to error catastrophe.

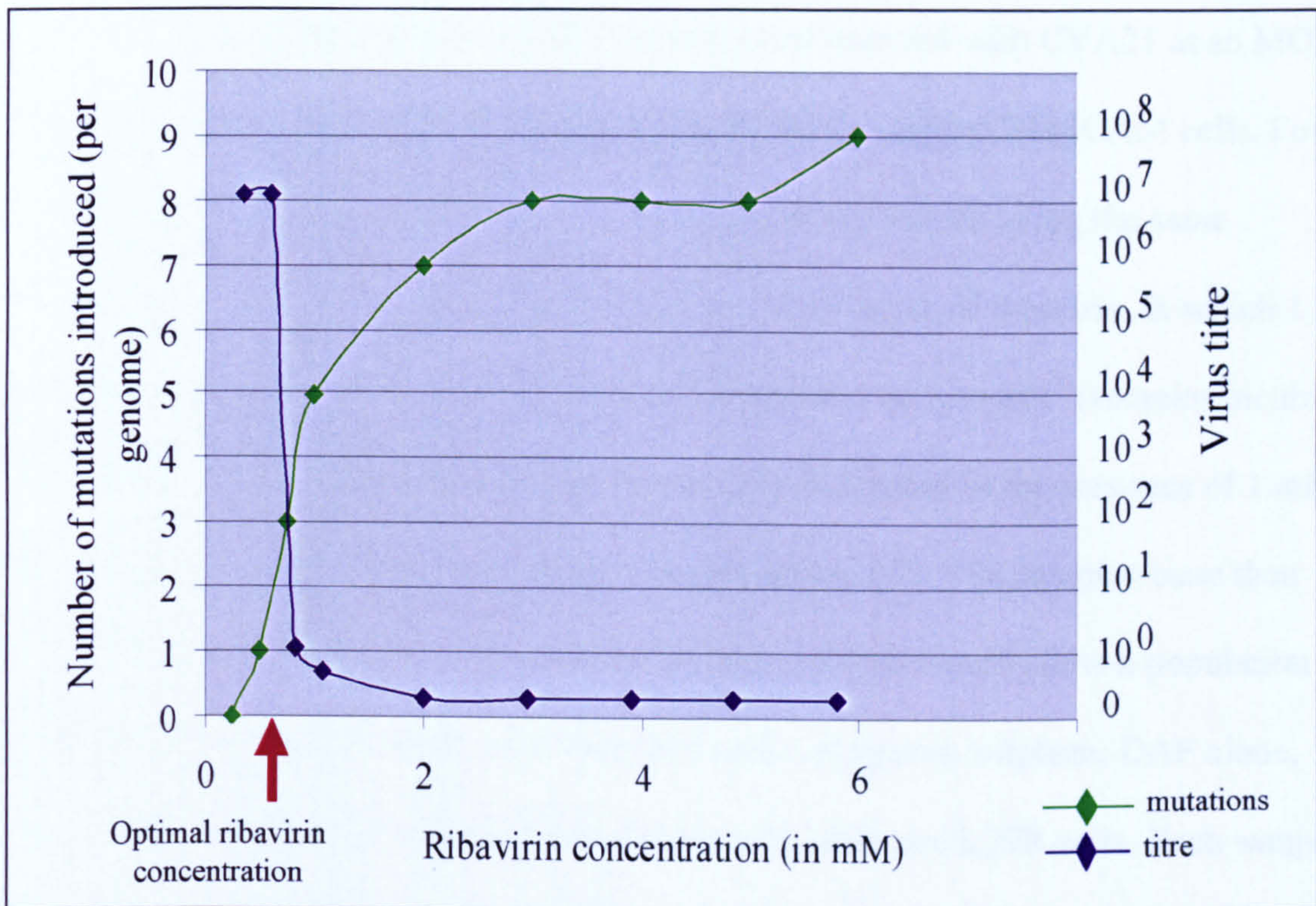


Figure 2.10: Graph of a hypothetical determination of optimal ribavirin concentration to use for the selection of novel tropic mutant viruses experiment.

The optimal concentration is determined based on the amount of ribavirin needed to cause a significant number of mutations whilst retaining the ability to replicate and produce progeny viruses. In this hypothetical situation the optimal concentration to use, shown by the red arrow, would be that causing a 2-log reduction in titre whilst introducing 2.5 mutations per genome. Such an assay needs to be carried out and the experiment needs to be repeated using the determined optimal ribavirin concentration.

2.3.3 Results and discussion of the selection experiment

RD-ICAM cells, pre-treated with 1mM ribavirin, were infected with CVA21 at an MOI of 1. Each sample was harvested and passaged onto fresh pre-seeded RD-ICAM cells. Four subsequent serial passages or rounds of selection were carried out using the same pre-incubation, infection and incubation stages in the presence of ribavirin, in which 1 ml out of 5mls were transferred. The first round of selection was named r^1 (samples incubated in the absence of the mutagen) or R^1 (for the samples incubated in the presence of 1 mM ribavirin), the second round r^2 or R^2 and so on (see figure 2.7). The samples were then screened on various cell lines. The selection strategy applied would allow a population of mutated viruses to evolve to use other receptors such as heparan sulphate, DAF alone, murine DAF or PVR, by comparison of infection of L cells and L20B cells. Each sample from each round of selection was tested on each cell line and serially passaged on the respective cell lines in order to enable potential infection and amplification of less fit novel tropic mutant CVA21 populations. The results are shown in table 2.11. The assay was repeated four times. On no occasion were novel tropic mutant viruses detected and isolated from the reactions.

Test samples	Cell line infected			
	RD-ICAM	RD	L	L20B
CVA21 r ¹	■	■	□	□
CVA21 R ¹	■	■	□	□
Cells R ¹	□	□	□	□
CVA21 r ²	■	■	□	□
CVA21 R ²	■	■	□	□
cells R ²	□	□	□	□
CVA21 r ³	■	■	□	□
CVA21 R ³	■	■	□	□
cells + R ³	□	□	□	□
CVA21 r ⁴	■	■	□	□
CVA21 R ⁴	■	■	□	□
cells R ⁴	□	□	□	□
CVA21 r ⁵	■	■	□	□
CVA21 R ⁵	■	■	□	□
cells R ⁵	□	□	□	□

Table 2.11: Results of screening of samples of CVA21 serially selected in the presence of ribavirin. The read-out of the experiment was the presence (■) or absence (□) of CPE observed in the transfected cell sheet. ■ represent very low levels of CPE observed on the infected cell sheet (~20% CPE).

Ribavirin did not have a cytotoxic effect on any of the cell lines and serial passaging in the presence of the mutagen did not affect the readout of the experiment. No CPE was observed in L cells or L20B cells implying that CVA21 had not evolved to use murine fibroblast cell surface molecules or the PVR. As expected RD-ICAM cells always exhibited CPE in the passaged virus samples tested, but not in the absence of the virus. The ribavirin-independent result in RD cells was however intriguing. CVA21 virus alone and CVA21 virus incubated in the presence of 1 mM ribavirin caused low levels of CPE in RD cells. This outcome could be due to a positive selection for a subpopulation of virus that is able to grow. However the CPE levels were not amplified after several passages in the cell line indicating that low CPE due to a positive selection is unlikely. Possible contaminations of either the CVA21 virus stocks with RD-tropic virus or the RD cells with low levels of RD-ICAM or another cell line permissive for CVA21 growth were checked and different virus stocks were tested on different batches of RD cells. No contamination was detected. A likely explanation for the limited CPE observed in RD cells could be that CVA21 is able to use DAF as a cell surface receptor (Shafren et al., 1997; Johansson et al., 2004).

Although the strain of CVA21 used for the experiment was not adapted to grow in RD cells, the limited CPE observed in the assay could be due to the fact that in the absence of ICAM-1 cell surface molecules CVA21 is able to attach DAF to some extent and actually cause some CPE.

Under the conditions tested, the selection for novel tropic viruses was not successful, however the use of different parameters could potentially allow for the selection of such viruses. Several factors, such as the ribavirin concentration, the incubation time, the cell type chosen and the pre-incubation stage, might have biased the experiment. Reducing the pre-incubation time could mean that the intracellular levels of GTP were still significant.

Intracellular GTP could therefore have been used during virus replication as opposed to RTP, leading to the generation of wild-type virus sequences and the absence of mutations. It would be interesting to repeat the experiment with a longer pre-incubation period so that a better environment for the introduction of mutations may be created.

A ribavirin concentration of 1mM caused a 1-log reduction in CVA21 viral titres which, combined with the long incubation stage after infection of RD-ICAM cells, could have affected the outcome of the experiment. Indeed the risk of carrying out a long incubation is that of allowing fitter viruses, like wild-type viruses, to outgrow novel tropic mutants of lesser fitness. High mutation rates could lead to the introduction of lethal mutations affecting essential protein coding sequences and the overall RNA structure. Using random mutagenesis as a selection method combined with high mutation rates increases the risk of lethal mutagenesis. If random mutations are introduced at a high rate the probability of affecting a key sequence is increased and the mutated virus is less likely to be able to survive and outgrow wild-type viruses during the first round of infection in RD-ICAM cells in the presence of ribavirin. Multiple nucleotide changes may disrupt the overall RNA structure and therefore seriously impair downstream events that are dependent on such a structure. By attempting to speed up evolution there is a risk of reaching extinction without going through the various transitional stages. RNA viruses such as CVA21 and PV3 already exist on the brink of error catastrophe and random mutagenesis might have destroyed a fine equilibrium and might have accelerated extinction. However, the experiment provides no evidence for error catastrophe in the studied virus populations, as virus titres were still significant (3.6×10^4 TCID₅₀/ml, data not shown) after multiple passages of CVA21 in the presence of 1 mM ribavirin.

2.3.4 Future work

Some variables of the described experiment could be altered. Indeed, repeating the experiment using high multiplicity of infection coupled with rapid passages could potentially alter the outcome. Furthermore, determination of the optimal ribavirin concentration to use for the introduction of a sufficient number of mutations is essential. Moreover, a variety of alternative assays with a more stringent selection applied to a variety of reference viruses, could be designed.

The selection for novel PVR-tropic viruses would potentially mean the loss of ICAM-1 affinity by the emerging virus populations. One way to select for recombinants *in vivo* is to select against parental viruses using ICAM-1, by repeating the assay in the presence of both ribavirin and soluble ICAM-1. The samples would then be washed to get rid of ICAM-1 tropic viruses allowing for recovery and growth of non-ICAM-1 binding novel tropic recombinant viruses. Should novel tropic mutant viruses be selected for, characterisation of such viruses by assays such as antigenicity testing, specific infectivity assessment and nucleotide sequence analysis would be carried out.

Due to time restrictions and progress on the generation of recombinants *in vitro* project it was not possible to carry out the described assays.

3 Chapter 3: *In vitro* recombination

3.1 Introduction

Human enterovirus (HEV) evolution is a consequence of selective pressure, error rate and recombination. Recombination plays a major role in the emergence of viruses with novel sequences. Emerging recombinant enteroviruses have been a major concern for the WHO poliomyelitis eradication program and have been reported to occur both in the laboratory environment and in nature. The exact circumstances, processes and factors contributing to the emergence of such recombinant viruses are unclear.

So far the circulating vaccine-derived polioviruses (cVDPVs), isolated from outbreak cases, were found to have a structural protein-coding region derived from a vaccine strain and the remainder of the genome from an unidentified virus strain from the same species as poliovirus (Stanway et al., 2000). This was the case of the cVDPVs isolated in Egypt (Yang et al., 2003) and the Philippines (WMMR, 2001). Recently, cVDPVs isolated from Haiti, Egypt, the Philippines and Madagascar seemed to have arisen from a recombination event between Sabin poliovirus strains and enteroviruses circulating in the gut of vaccinees at the time of vaccination (Kew et al., 2002; Rousset et al., 2003). These emerging cVDPVs (reviewed in Kew et al., 2004) highlight the importance recombination plays in enterovirus evolution.

The initial aim of the project was to generate and study viruses with mosaic non-poliovirus HEV-C P1-coding sequence resulting from shuffling that may use the PVR, thereby mimicking evolution. The generation of viable viruses would require *cis*-processing of the polyprotein. However, the exact requirements for the processing of the P1-coding region by a heterologous 3CD protease to occur *in trans* were unclear. The *cis*-processing

requirements were therefore investigated prior to shuffling by constructing and analysing recombinants with a precise exchange of the P1-coding region. These *in vitro* generated recombinants exhibited interesting phenotypes and further characterisation was conducted.

3.1.1 Aims

To attempt to provide a greater understanding of the different factors involved in the generation and infection of recombinant enteroviruses, chimeras between a poliovirus and a human enterovirus type C (HEV-C) were generated *in vitro*. The studies described in this chapter were designed to broaden the understanding of key factors and mechanisms and enable a better knowledge of processing requirements.

It was proposed to generate recombinants with a precise exchange of the P1-coding region between a poliovirus and a non-poliovirus HEV-C, using basic molecular cloning techniques. The source of the genomes used for the experiments were selected on the availability of infectious clones. Poliovirus type 3 Leon (PV3), a neurovirulent poliovirus, was chosen as one of the wild-type viral sequences. Sabin poliovirus type 1 (Sabin 1), a vaccine poliovirus strain, was an additional parental genome used for the experiments (kindly provided by Dr A. Macadam, National Institute for Biological Standards and Control).

As the non-poliovirus HEV-C, a genome closely related to that of poliovirus type 3 Leon was chosen. CVA21 exhibits 84.2% identity of the polyprotein with PV3 (Brown et al., 2003). The amino acid identity deduced from nucleotide sequence relationships of a range of HEV-C viral proteins is illustrated in appendices 3, 4 and 5 of this thesis. Other HEV-Cs are more closely related to PV3; for example CVA20 has 90.2% amino acid identity to PV3. However, CVA21 was the closest relative to PV3 for which an infectious clone was

available. Recombinants between Sabin 1 P1-coding region and the remainder of the CVA21 genome and PV3 P1 coding region and the remainder of the CVA21 genome were generated for subsequent investigation. Reciprocal recombinants between the structural coding region of CVA21 and the remainder of the PV3 genome were also constructed.

3.2 *In vitro* generation of recombinants

3.2.1 Construction of recombinant plasmid DNA

The recombinant sequences were generated so as to have reciprocal precise exchanges of heterologous P1-coding regions without modifying the backbone sequences. The phenylalanine/glycine (F/G in the case of CVA21) and tyrosine/glycine (Y/G in the case of PV3 and Sabin 1) amino acid sequences at the VP1/2A junctions, essential for correct processing to occur, were retained (Luke et al., 2001). This would therefore create a perfect exchange of the P1-coding region.

3.2.1.1 Generation of backbone cassette vectors

CVA21 and PV3 backbone vectors lacking the P1-coding regions were constructed. The first 14 amino acids of VP4 are identical in human enteroviruses. Synonymous codon variation was used to maintain, in the case of PV3, or introduce, in the case of CVA21, a *Sac* I cloning site at the start of the P1-coding region for the construction of backbone vectors.

Synonymous codons were also used to maintain the native amino acid sequence at the start of the 2A-coding region of the backbone vectors. PV3 and CVA21 2A-coding regions start with respective GGC and GGG codons encoding a glycine (G). A *Sma* I site was created

by PCR in the PV3 backbone vector, where the second half of the site (GGG) replaced the original GGC sequence thereby maintaining the amino acid at the start of the PV3 2A.

Similarly a *Nae* I site was introduced by PCR in the CVA21 backbone vector. The second half of the *Nae* I site has a GGC sequence coding for a glycine and again enabled conservation of the glycine present at the start of the native CVA21 2A.

3.2.1.1.1 PV3 backbone vector (PV3BK)

The PV3 backbone vector was derived from pT7FLC/REP3 (Barclay et al., 1998), using GEN-3'BK'F and GEN-3'BK-R oligonucleotide primers and the *Nsi* I restriction endonuclease. The PV3 backbone vector cloning strategy is illustrated figure 3.1.

3.2.1.1.2 CVA21 backbone vector (CVA21BK)

The CVA21 backbone vector was derived from pRibo-CAV21-NaeIDel (David Williams). A 5' fragment of the 5'UTR and start of the coding-region was amplified by PCR using T7-F forward primer and CA21-5'-BK-R *Sac* I-containing reverse primer. The 5' PCR fragment was ligated to a *Sac* I digested 3' fragment, amplified by PCR, using GEN-3'BK-F-Nae (a *Sac* I and *Nae* I – containing primer) and GEN-3'BK-R (reverse primer annealing the 3C coding-region). A 3.9kb ligated fragment was re-amplified by PCR using T7-F and GEN-3'BK-R. The *Mlu* I and *Bgl* II double-digested PCR fragment was then ligated to a *Mlu* I and *Bgl* II double-digested pRibo-CAV21-NaeIDel. The CVA21 backbone vector cloning strategy is illustrated figure 3.2. The clones were checked by RFLP analysis and sequencing of the ligation junctions.

Once the backbone vectors were constructed, P1-coding regions were introduced to generate recombinants. The complete P1 sequences were generated by PCR using forward oligonucleotide primers, containing a *Sac* I site, and virus-specific blunt ended reverse oligonucleotide primers.

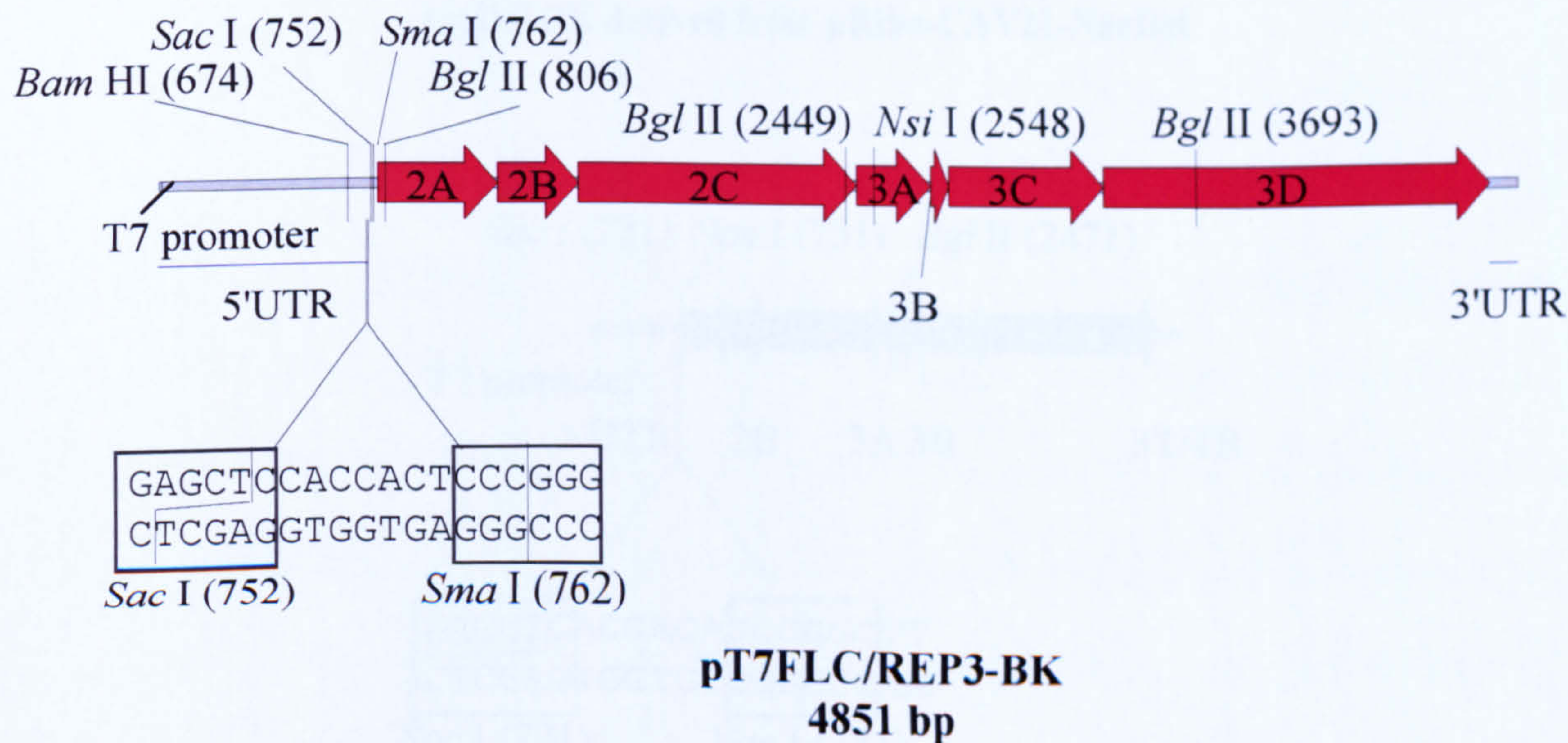
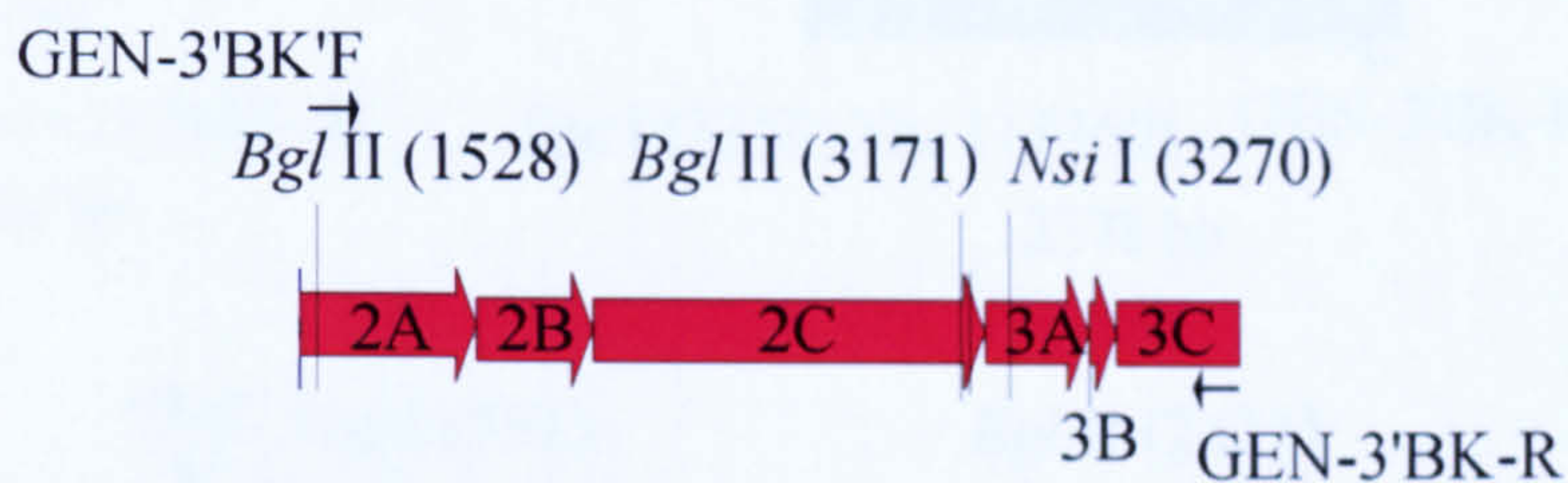
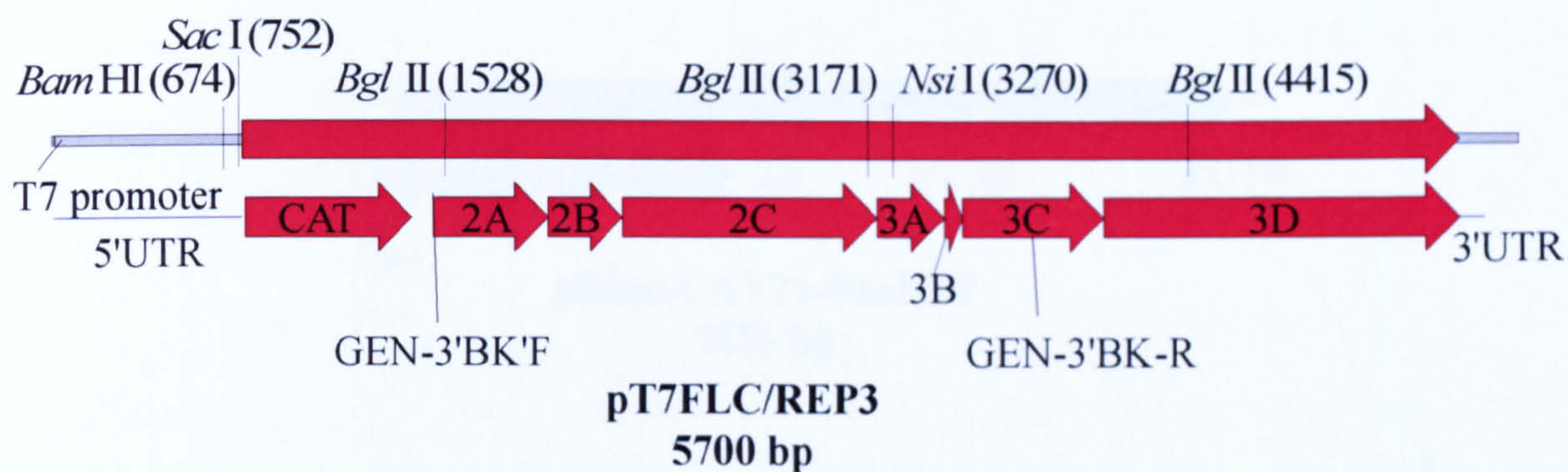
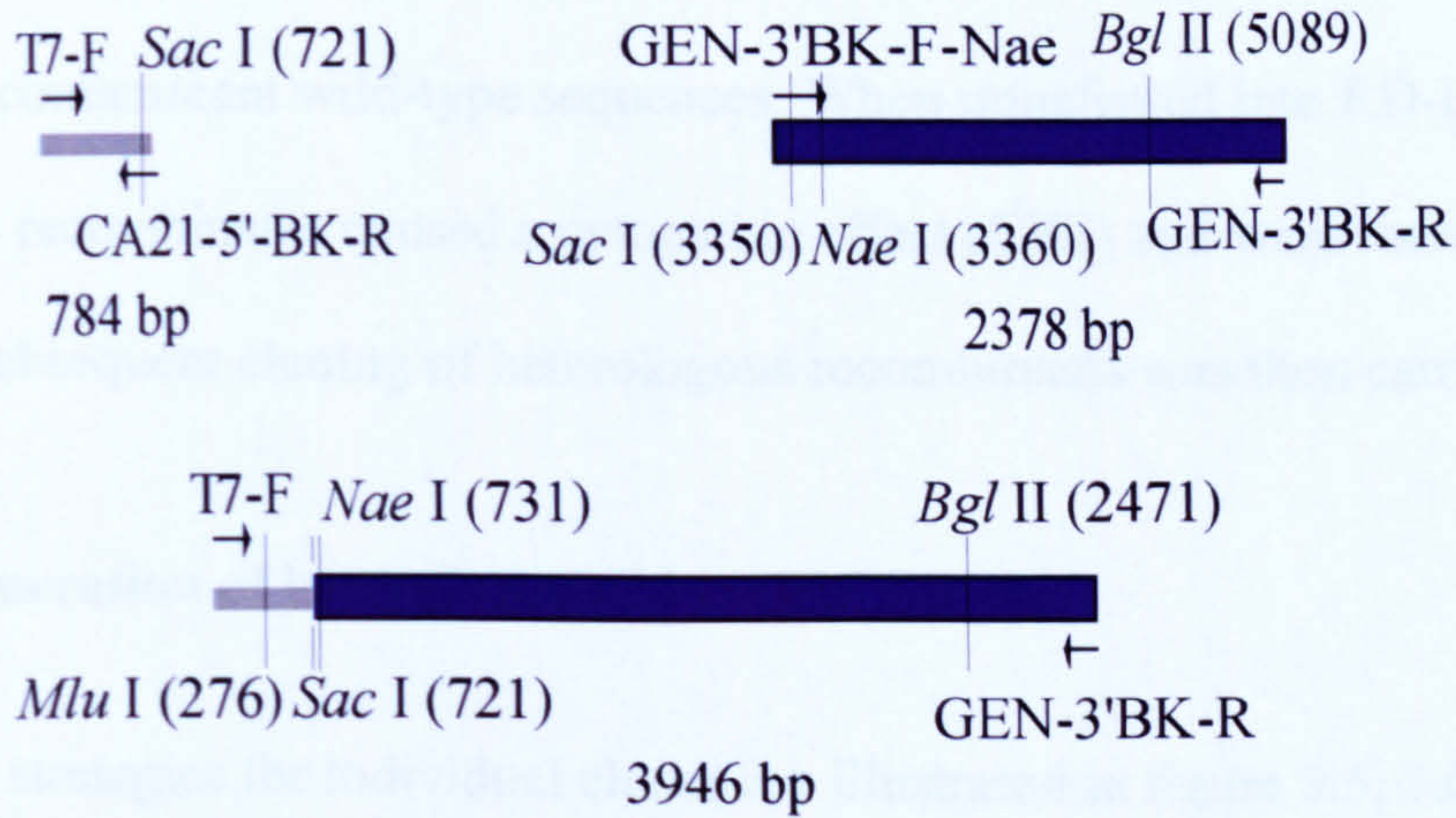
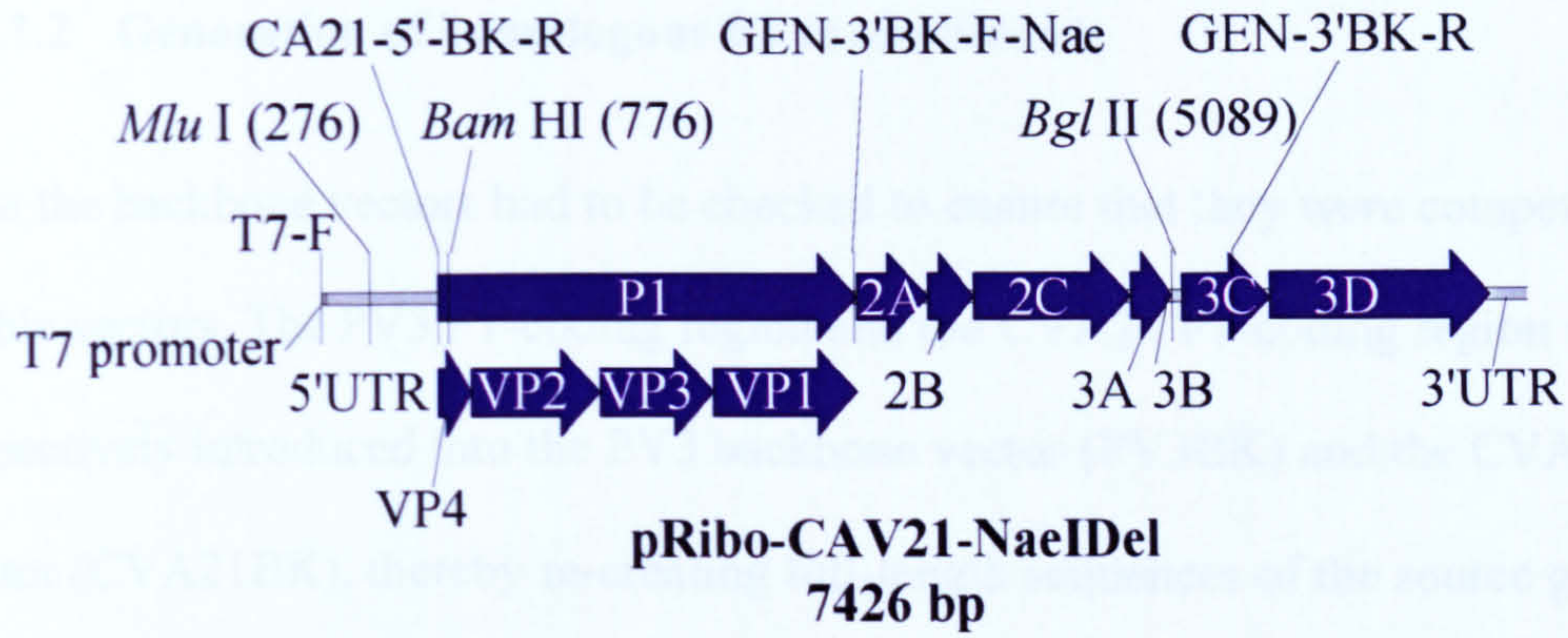


Figure 3.1: The construction of pT7FLC/REP3-BK (henceforth referred to as PV3BK).



PCR products generated for construction of pRibo-CAV21-NaeDel-BK derived from pRibo-CAV21-NaeDel

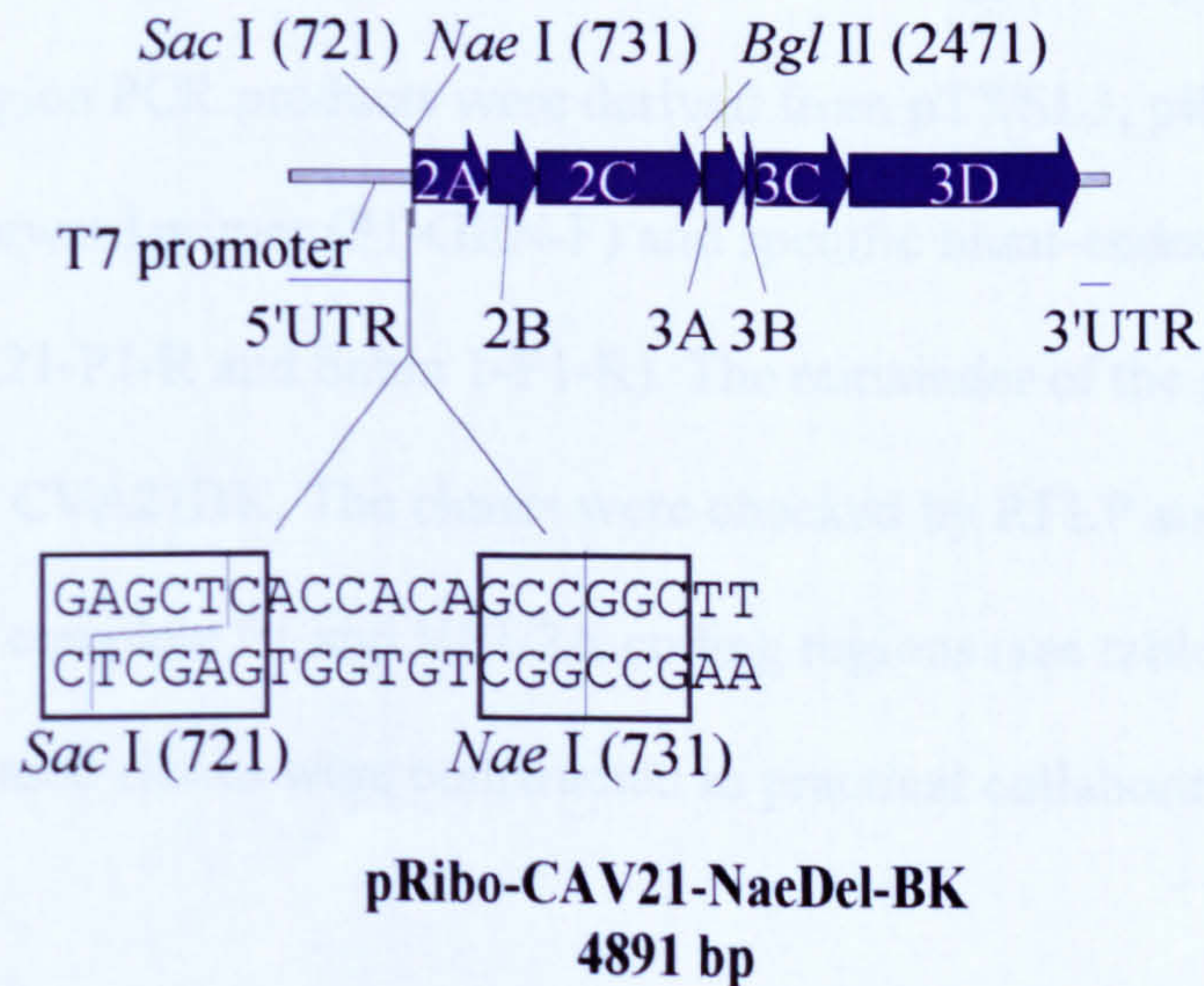


Figure 3.2: The construction of pRibo-CAV21-NaeDel-BK (henceforth referred to as CVA21BK).

3.2.1.2 Generation of homologous P1 recombinants

First the backbone vectors had to be checked to ensure that they were competent to make viable vectors. The PV3 P1-coding region and the CVA21 P1-coding region were respectively introduced into the PV3 backbone vector (PV3BK) and the CVA21 backbone vector (CVA21BK), thereby re-creating full-length sequences of the source genomes (figures 3.3 and 3.4). The clones were sequenced at the key junctions. The expected non-coding changes, introduced by PCR, were present thereby excluding the possible presence of contaminant wild-type sequences. When transfected into RD-ICAM cells, the homologous recombinants caused a cytopathic effect (CPE) and were therefore assumed to be viable. Subsequent cloning of heterologous recombinants was then carried out.

3.2.1.3 Generation of heterologous P1 recombinants

The cloning strategies for individual clones are illustrated in figure 3.5, 3.6 and 3.7. *Sac* I-digested blunt-ended P1-coding regions amplified by PCR were introduced to respective *Sac* I and *Sma* I double-digested PV3 backbone vector and *Sac* I and *Nae* I double-digested CVA21 backbone vector. The same strategy was applied to all the clones. The P1-coding region PCR products were derived from pT7/SL3, pRiboCAV21 and pS1F using a generic forward primer (P1-GEN-F) and specific blunt-ended reverse primers (PV3-P1-R, CVA21-P1-R and Sabin 1-P1-R). The remainder of the genome was derived from PV3BK and CVA21BK. The clones were checked by RFLP analysis and sequencing of the 5'UTR/P1, complete P1 and VP1/2A coding regions (see table 3.8.2 and figure 3.9). The CVA21BK based clones were constructed in practical collaboration with David Williams.

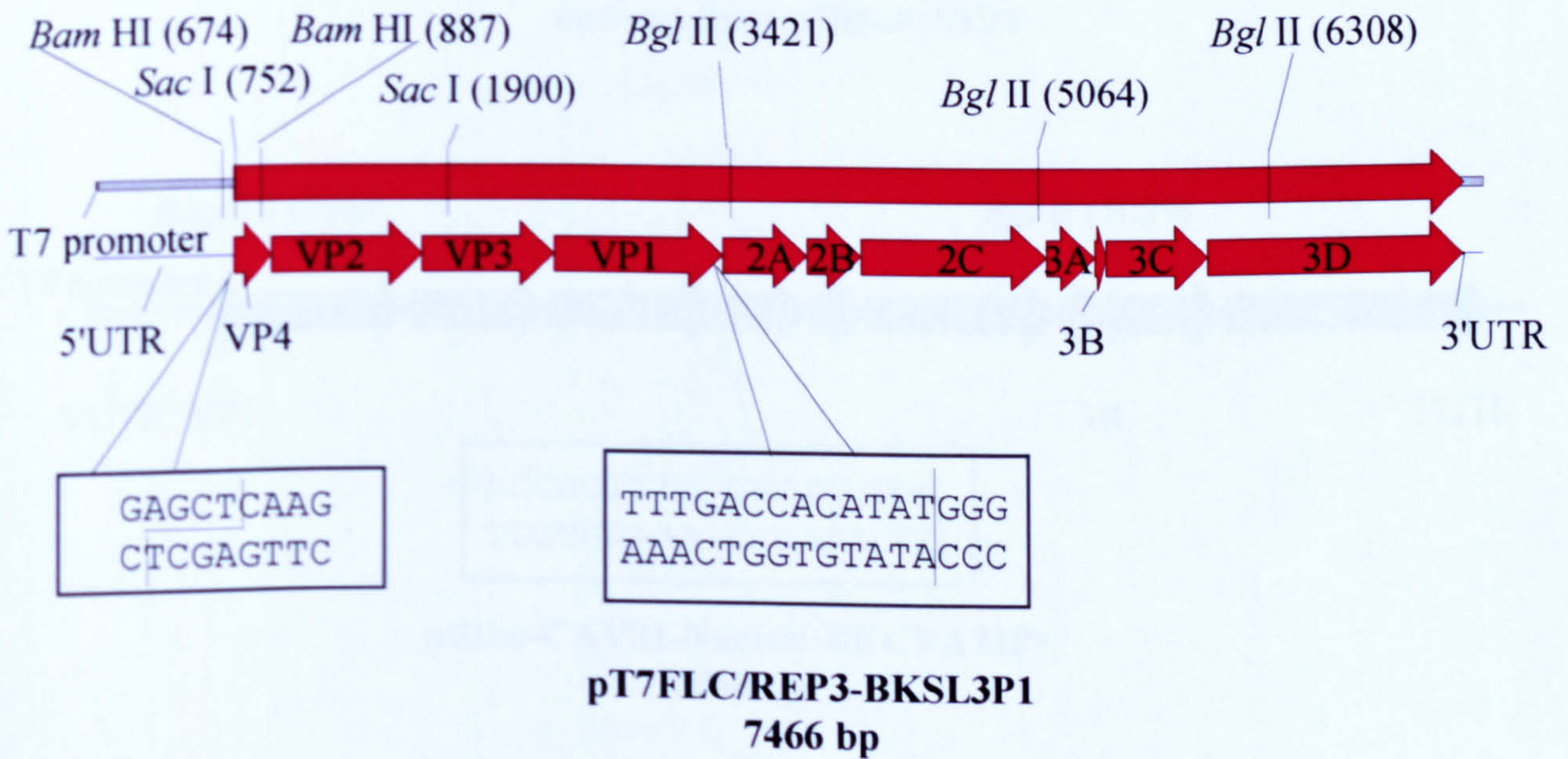
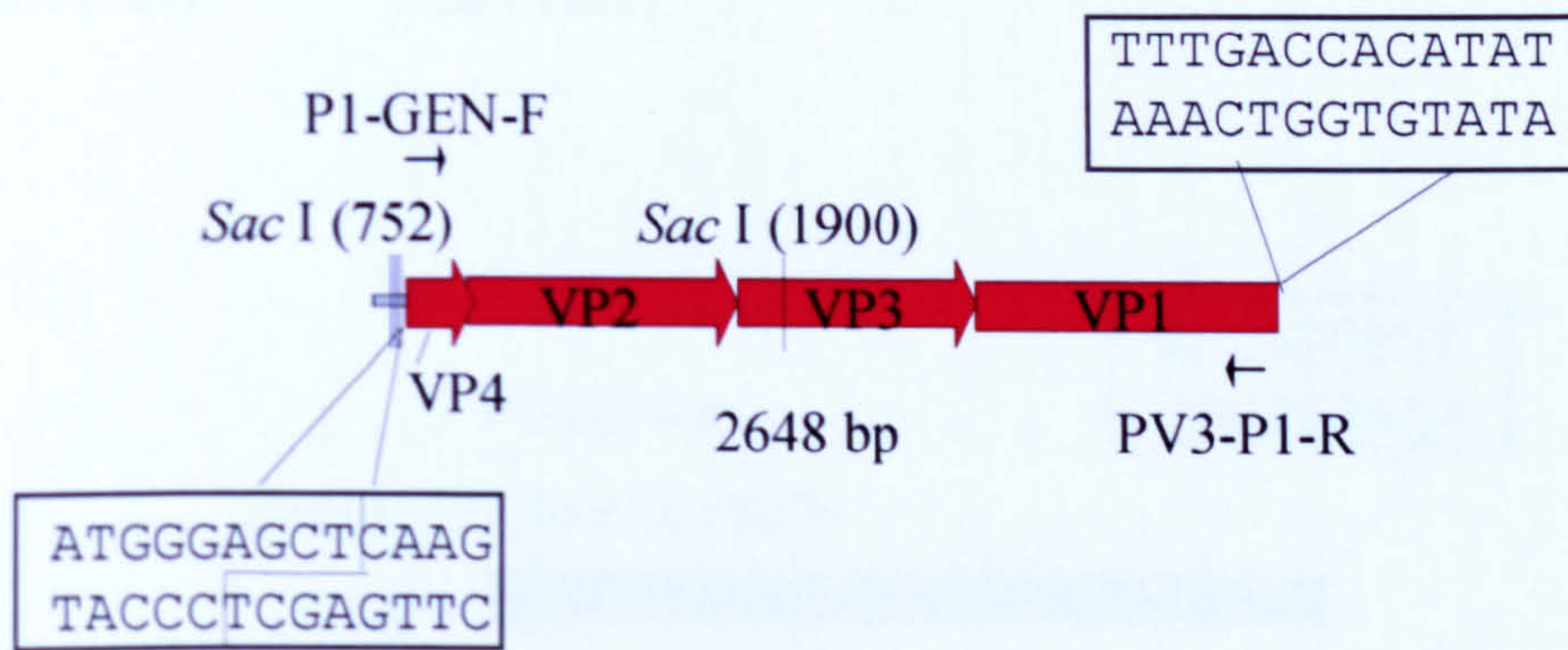
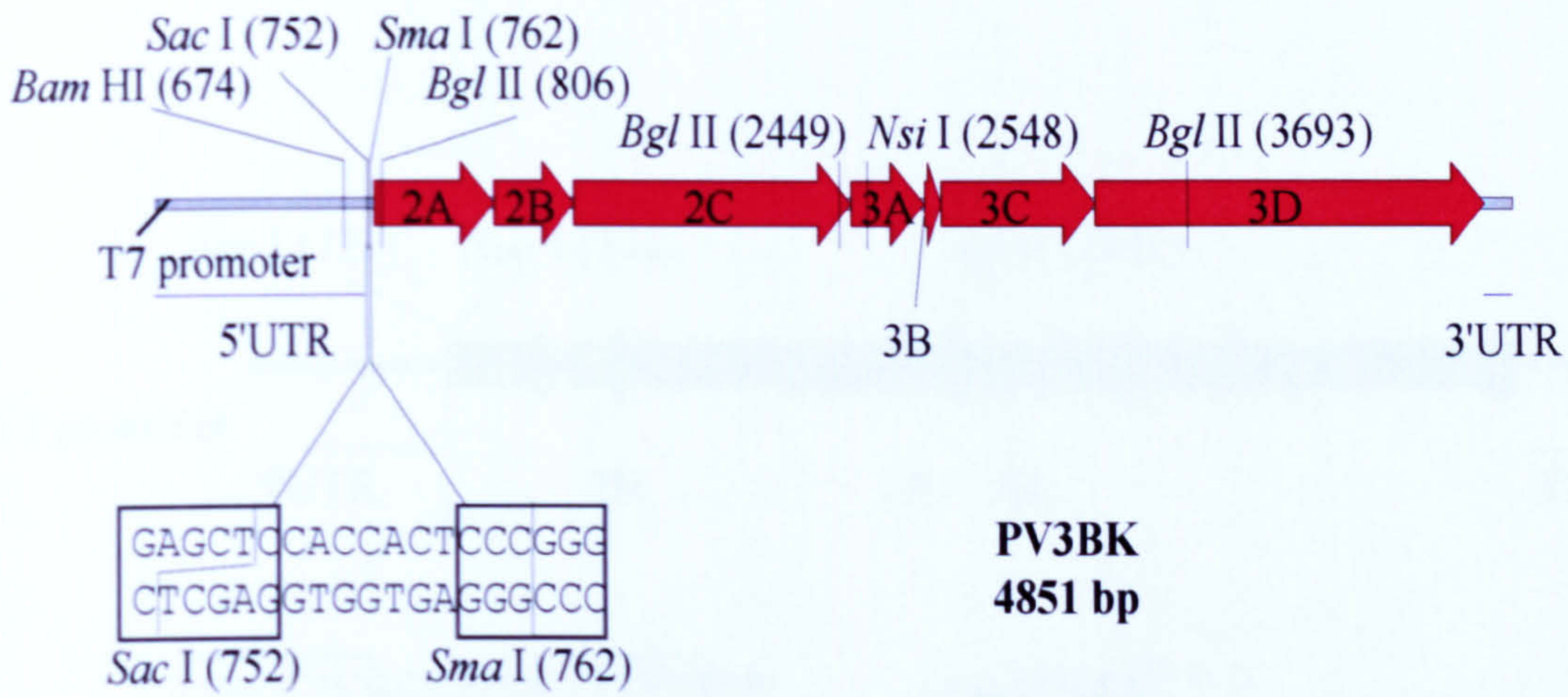
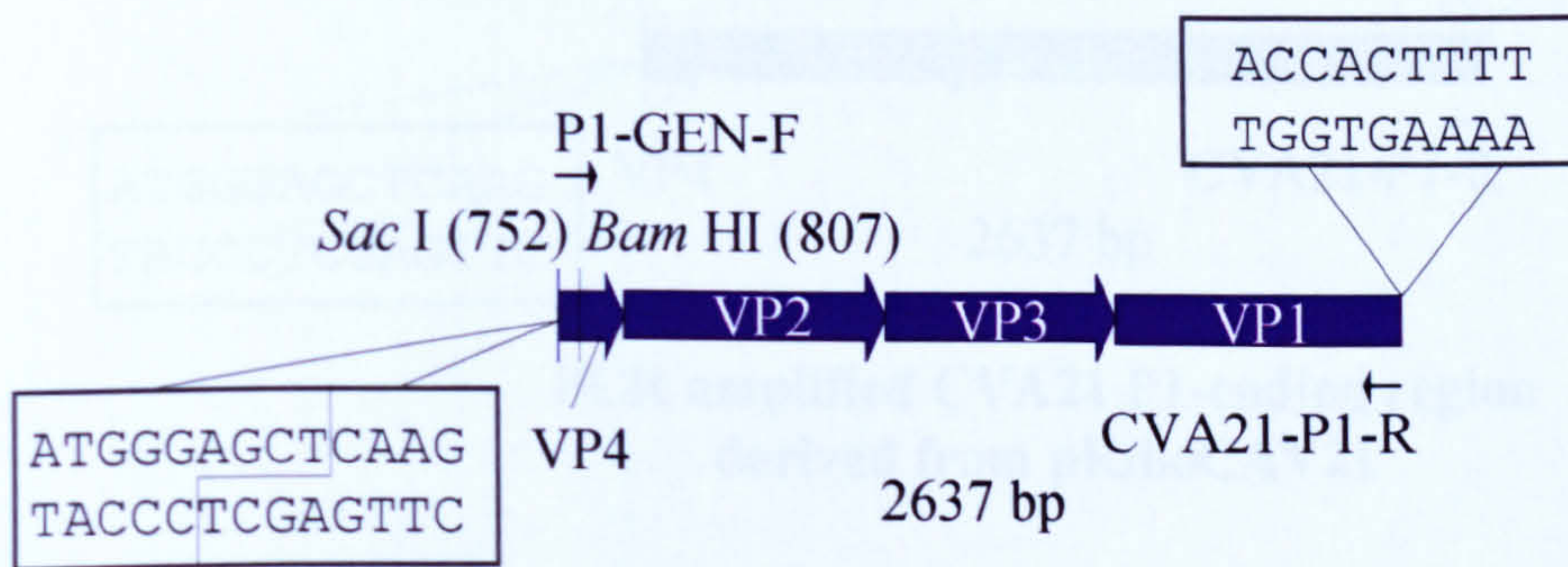
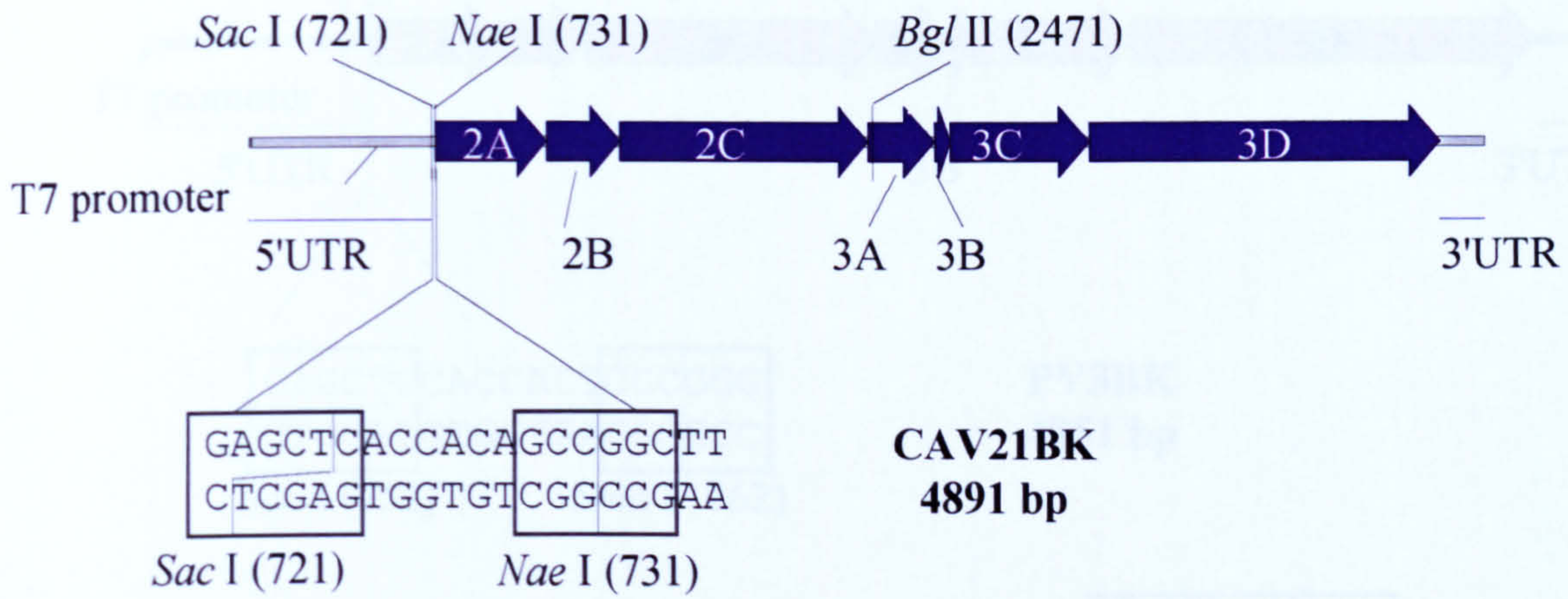


Figure 3.3: The construction of pT7FLC/REP3-BKSL3P1 (henceforth referred to as PV3BKPV3P1).



**PCR amplified CVA21 P1-coding region
derived from pRiboCAV21**

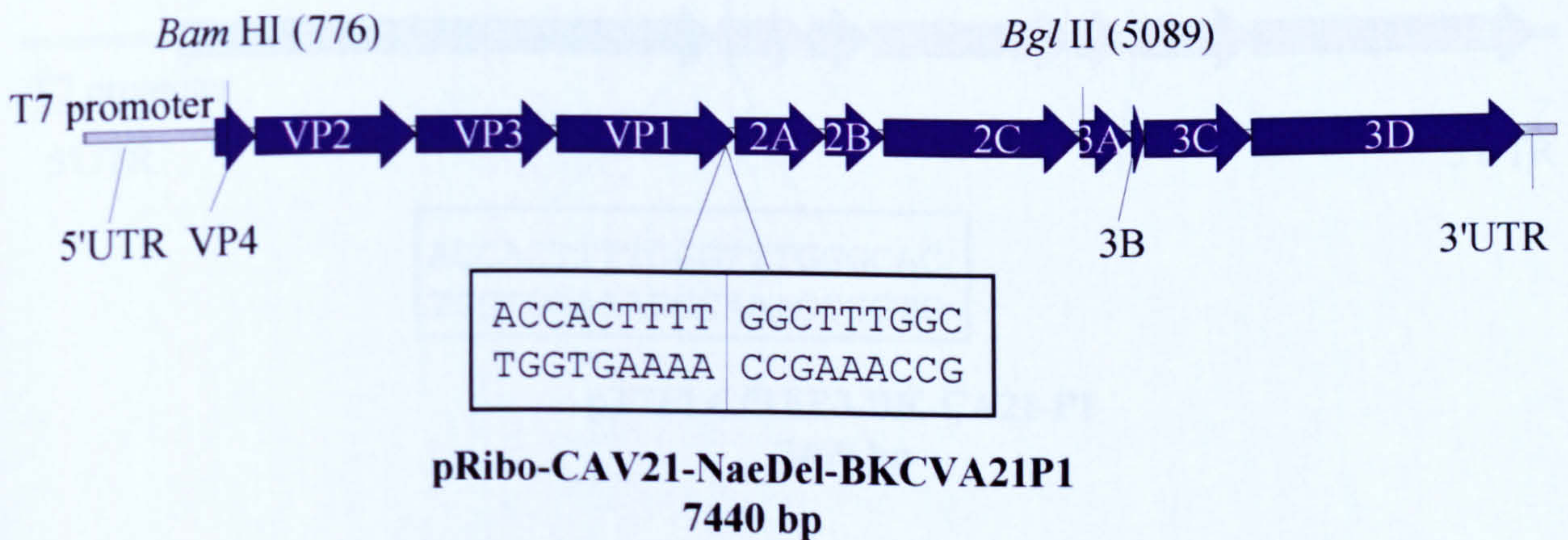


Figure 3.4: The construction of pRibo-CAV21-NaeDel-BKCVA21P1 (henceforth referred to as CVA21BKCVA21P1).

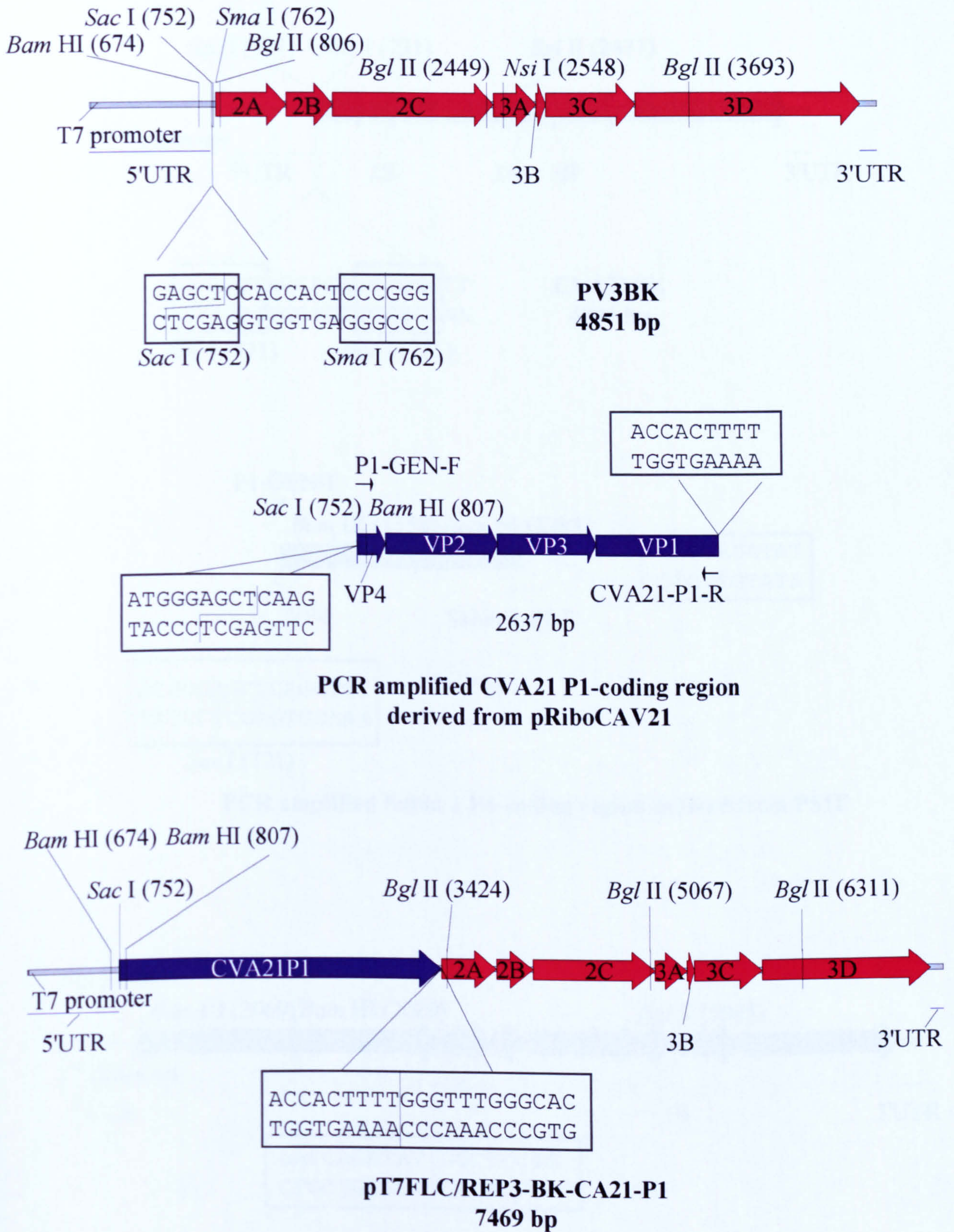
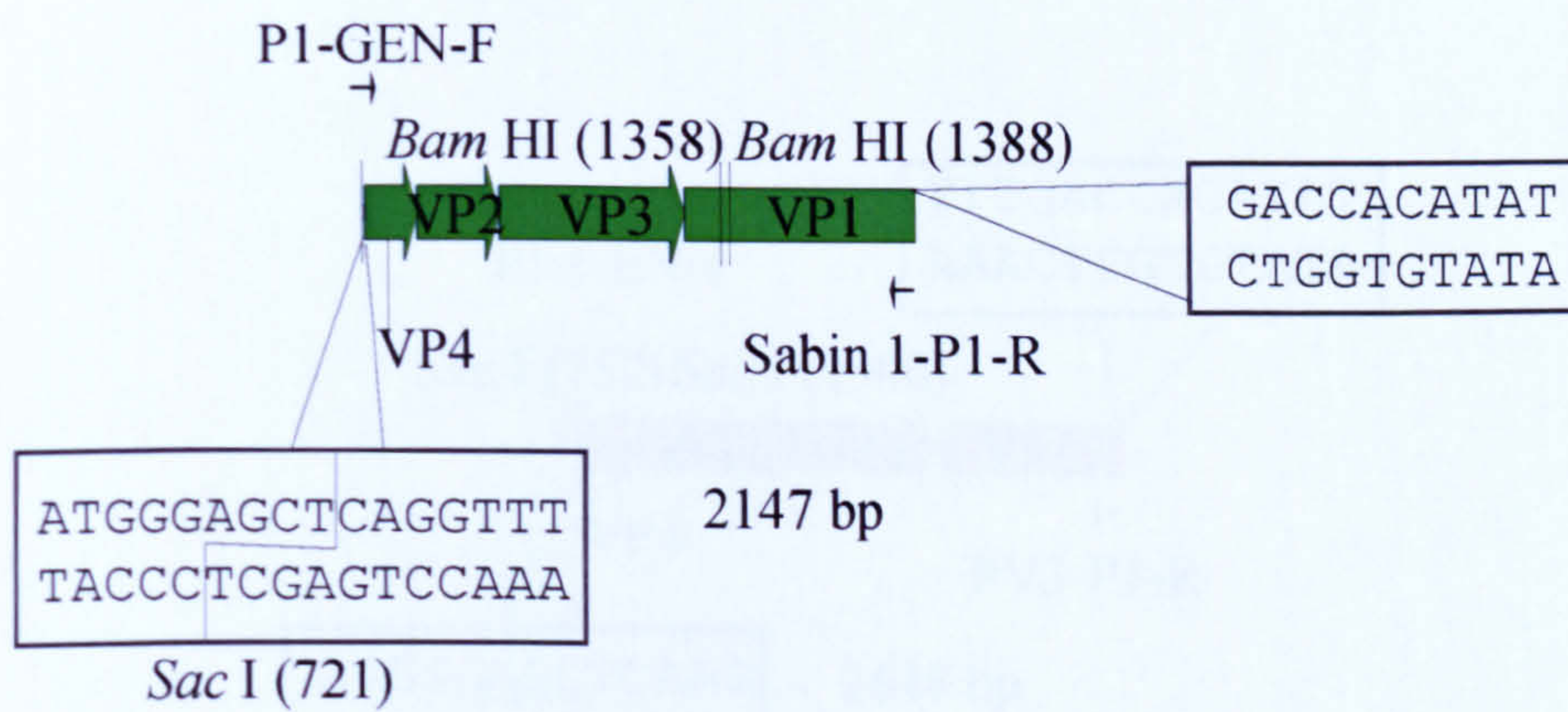
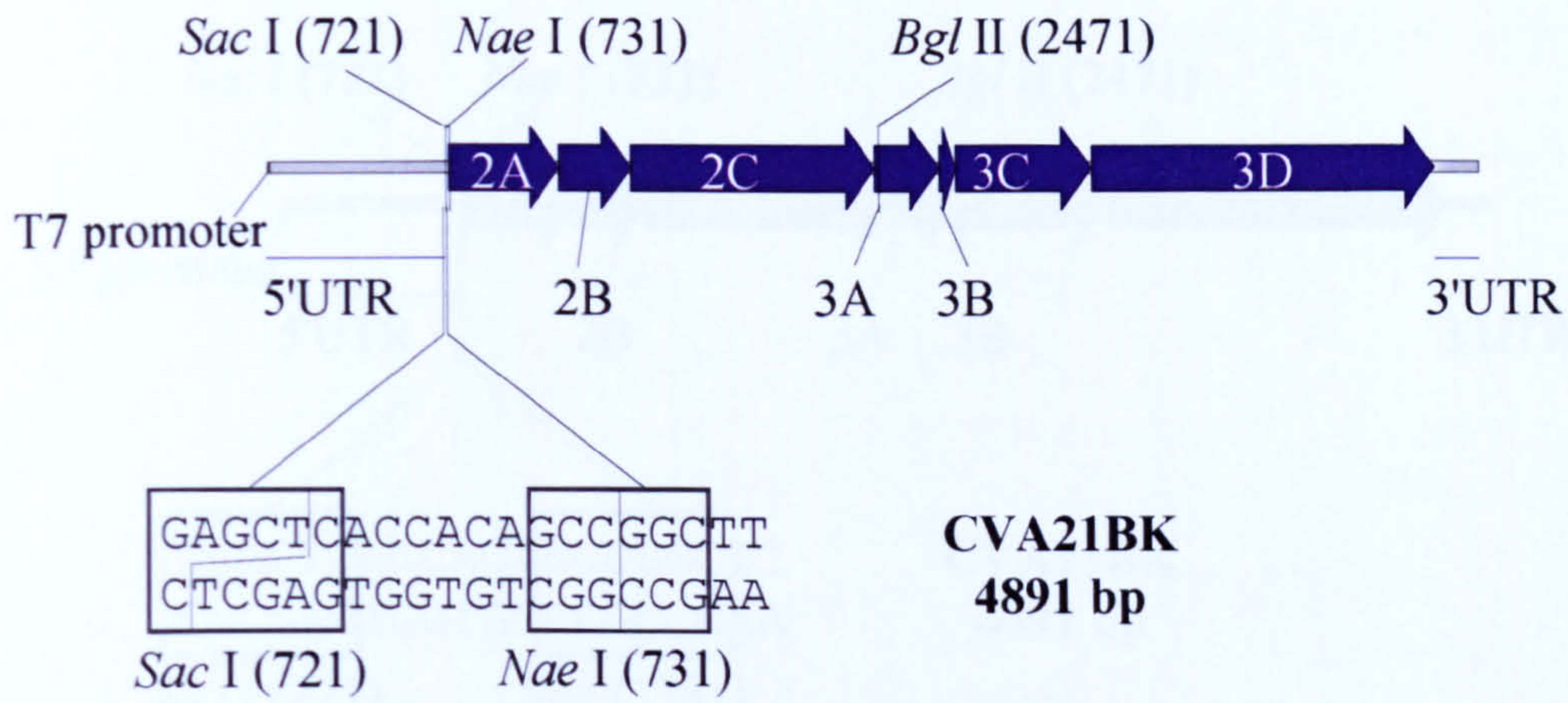


Figure 3.5: The construction of pT7FLC/REP3-BK-CA21-P1 (henceforth referred to as PV3BKCVA21P1).



PCR amplified Sabin 1 P1-coding region derived from PS1F

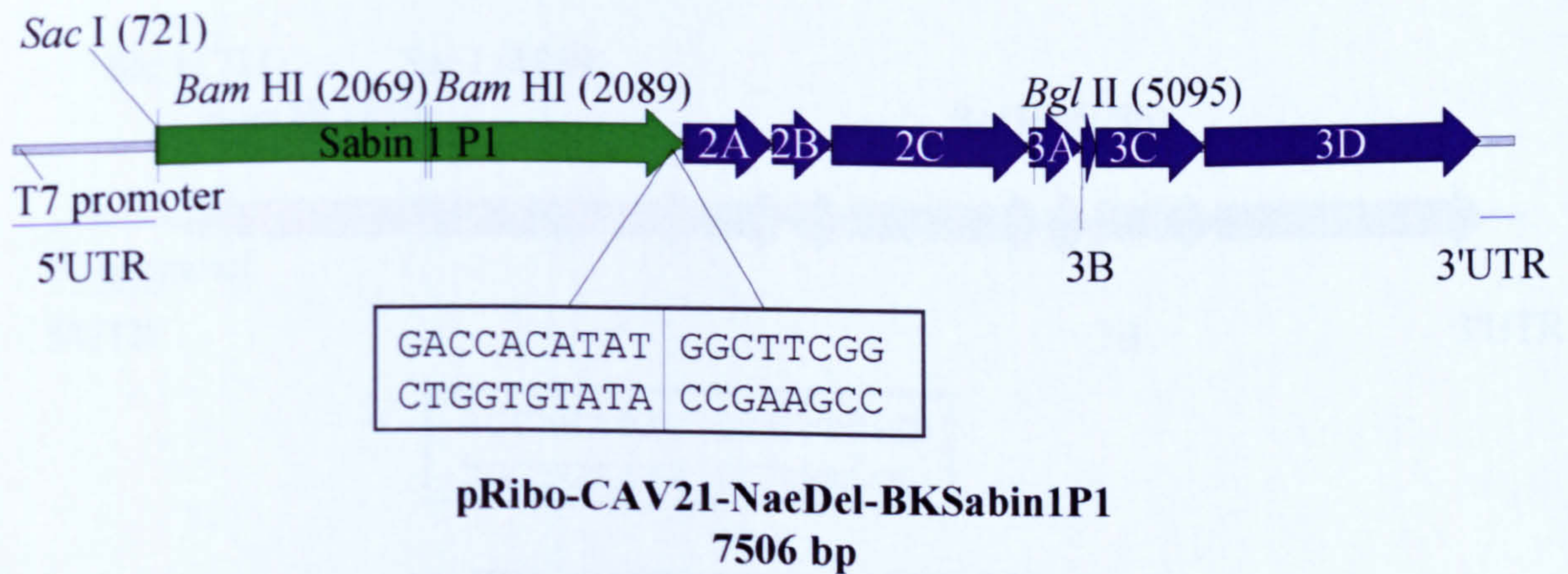
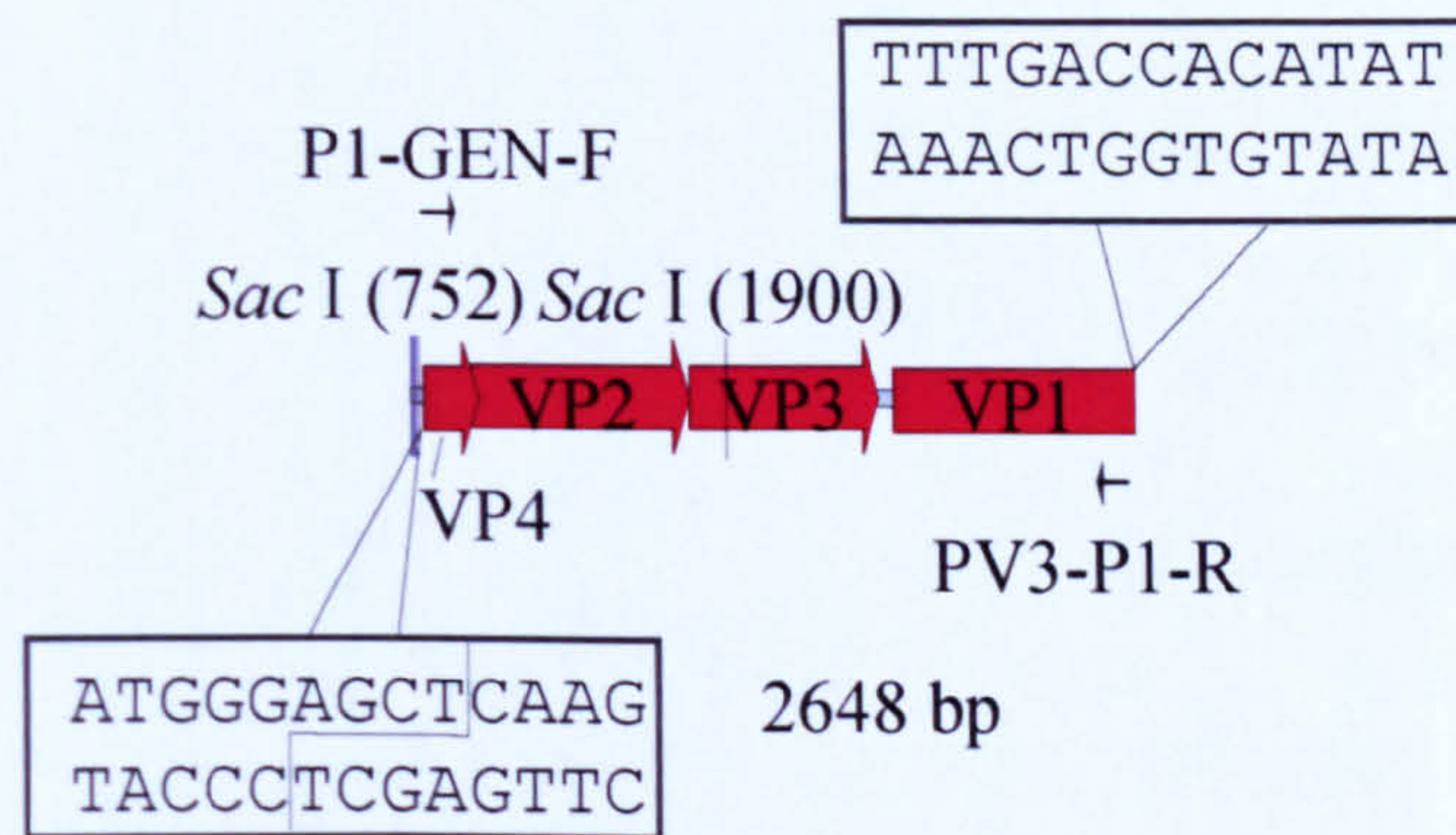
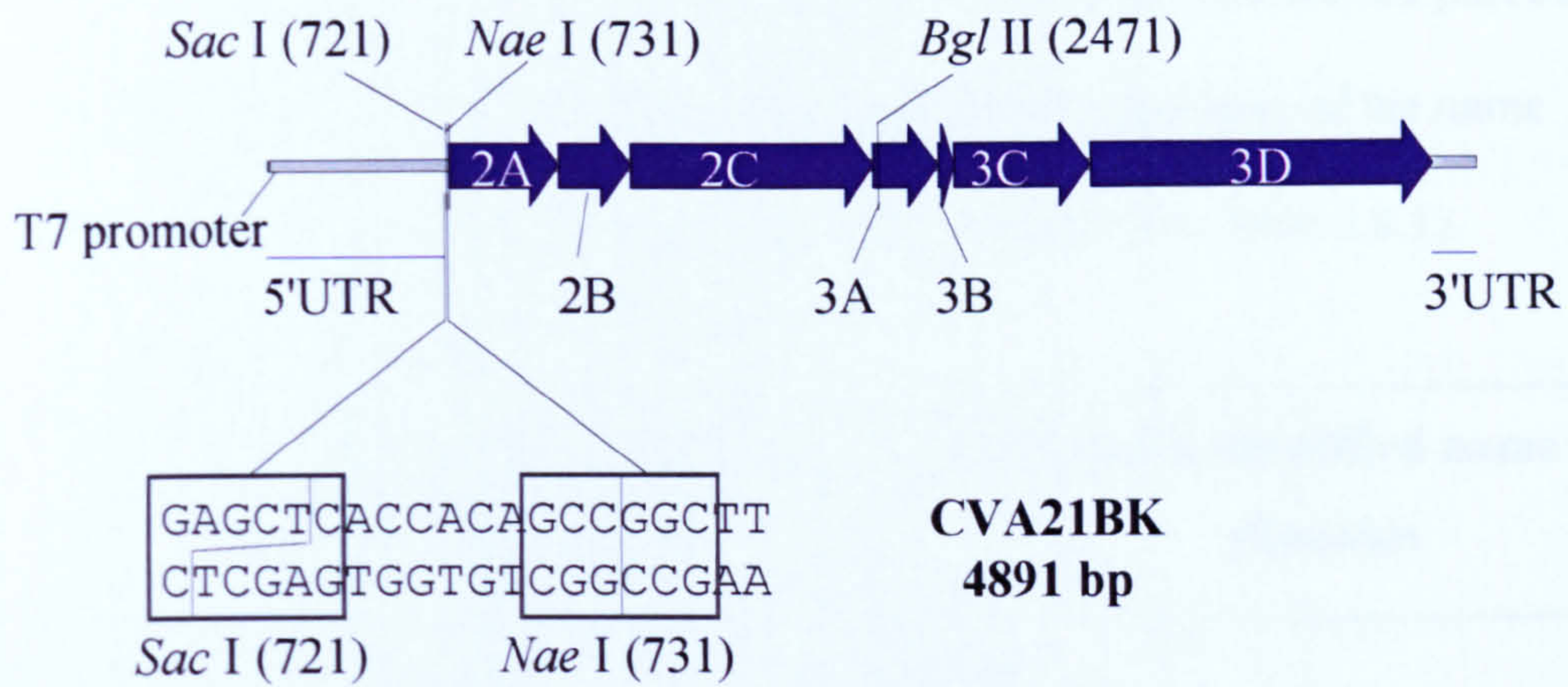


Figure 3.6: The construction of pRibo-CAV21-NaeDel-BKSabin1P1 (henceforth referred to as CVA21BKSabin1P1).



PCR amplification of the pT7/SL3 P1 coding region

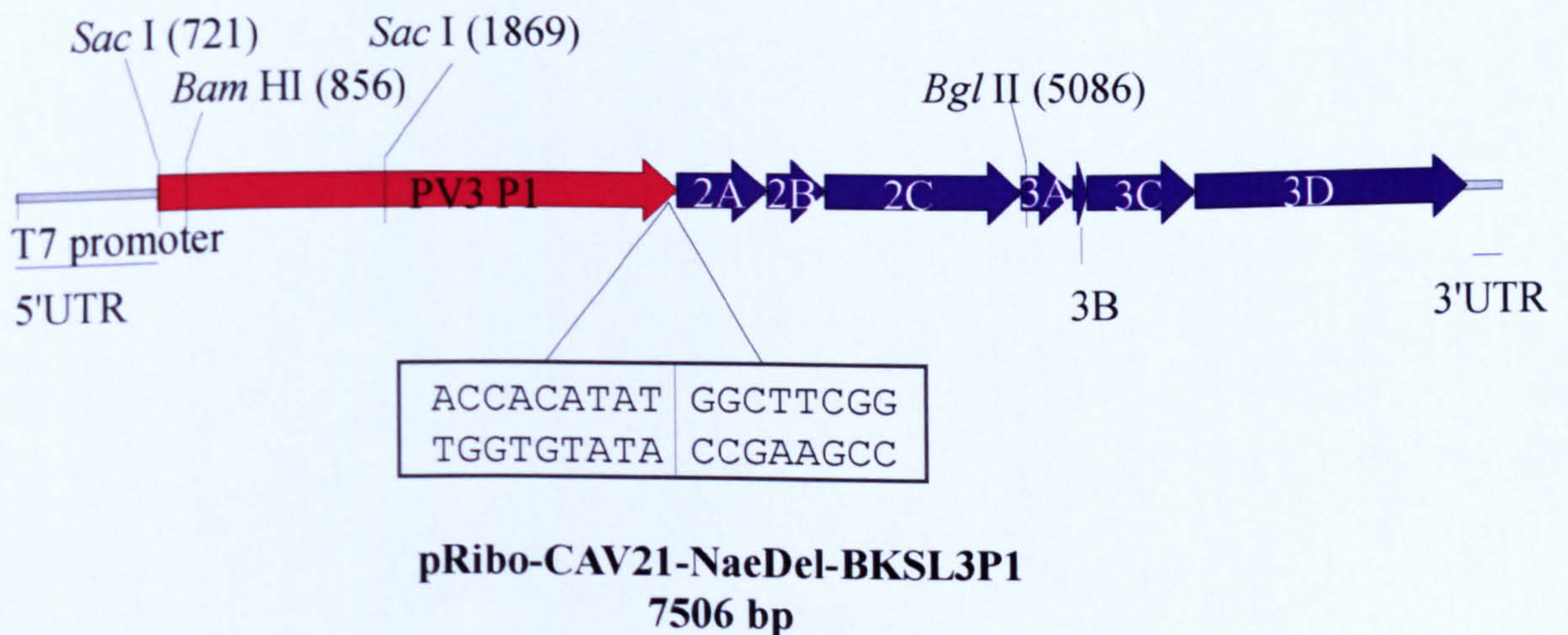


Figure 3.7: The construction of pRibo-CAV21-NaeDel-BKSL3P1 (henceforth referred to as CVA21BKPV3P1).

The *in vitro* generated recombinants were named in two parts. The second part of the name corresponded to the origin of the P1-coding region and the first part of the name corresponded to the source of the remainder of the genome (see table 3.8.1).

Name of the plasmid as indicated in the laboratory database	Henceforth simplified name of the plasmids
pT7FLC/REP3-BK	PV3BK
pRiboCAV21-NaeDel-BK	CVA21BK
pT7FLC/REP3-BKSL3P1	PV3BKPV3P1
pRibo-CAV21-NaeDel-BKCVA21P1	CVA21BKCVA21P1
pT7FLC/REP3-BK-CA21-P1	PV3BKCVA21P1
pRibo-CAV21-NaeDel-BKSabin1P1	CVA21BKSabin1P1
pRibo-CAV21-NaeDel-BKSL3P1	CVA21BKPV3P1

Table 3.8.1: Names of constructed plasmids

Digested plasmid DNA	<i>Bam</i> HI (sizes in bp)	<i>Bgl</i> II (sizes in bp)
pT7FLC/PV3	10996 133	8241 1643 1244
PV3BK	8513	5626 1643 1244
PV3BKCVA21P1	10998 133	8244 1643 1244
pRiboCAV21	10429	10429
CVA21BKCVA21P1	10429	10429
CVA21BKPV3P1	10426	10426
CVA21BKSabin1P1	10405 30	10435
pS1F	5721 2471 1879 30	10101

Table 3.8.2: Predicted RFLP analysis of the *in vitro* generated P1 recombinants. The fragments expected from the restriction digests reactions are in base pairs (see figure 3.9 for actual result).

The RFLP analysis data indicated that the clones constructed were correct. The key junctions 5'UTR/P1-coding region and the VP1/2A-coding regions of the constructed clones were checked by sequencing. The cDNA sequence of the entire P1-coding region of the CVA21BKSabin1P1 61-9, CVA21BKPV3P1 c6 and PV3BKCVA21P1 3 clones were also determined and shown to be correct (data not shown). The clones were amplified, purified from bacterial cultures and stored in the laboratory communal stocks. Subsequent analyses were then initiated.

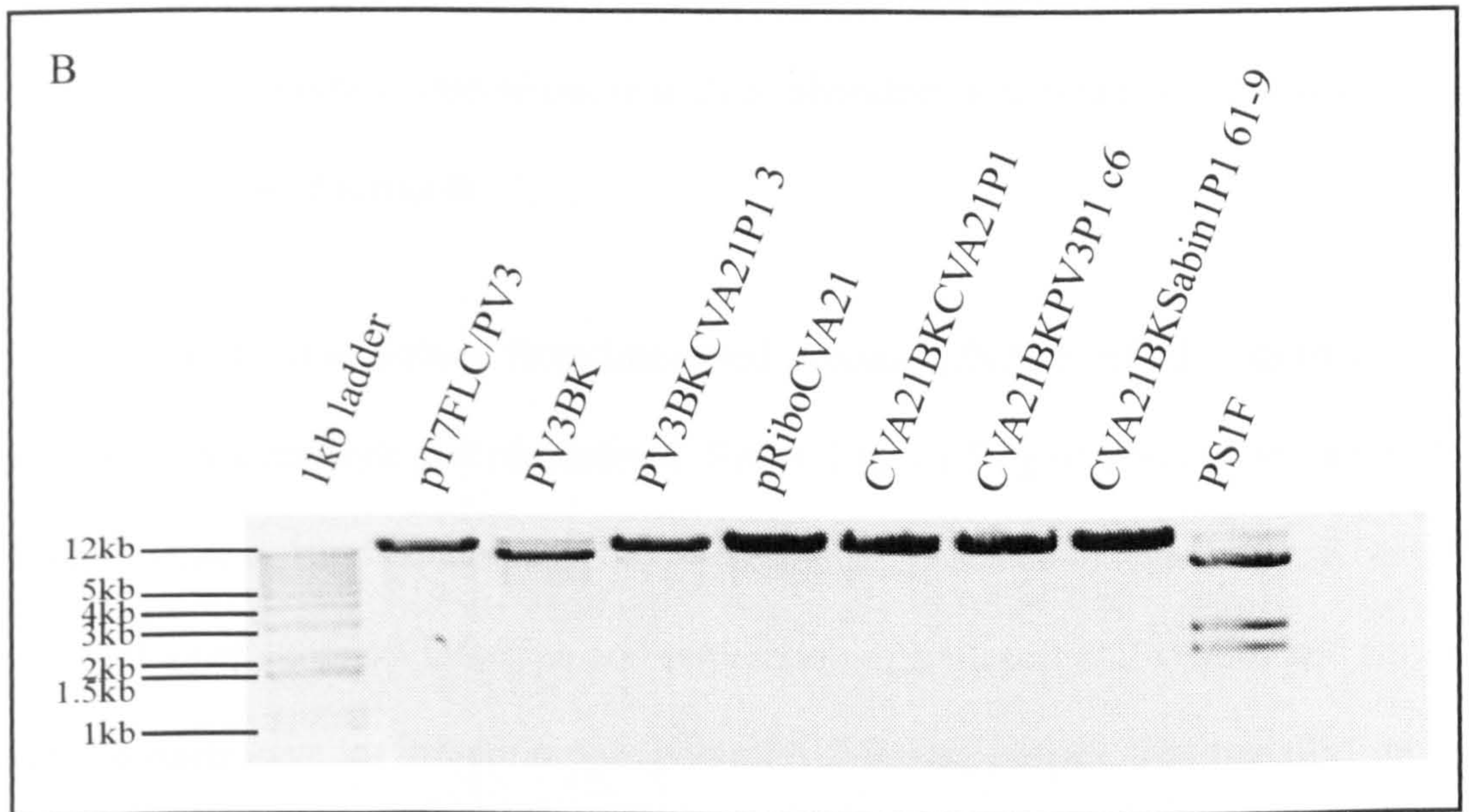
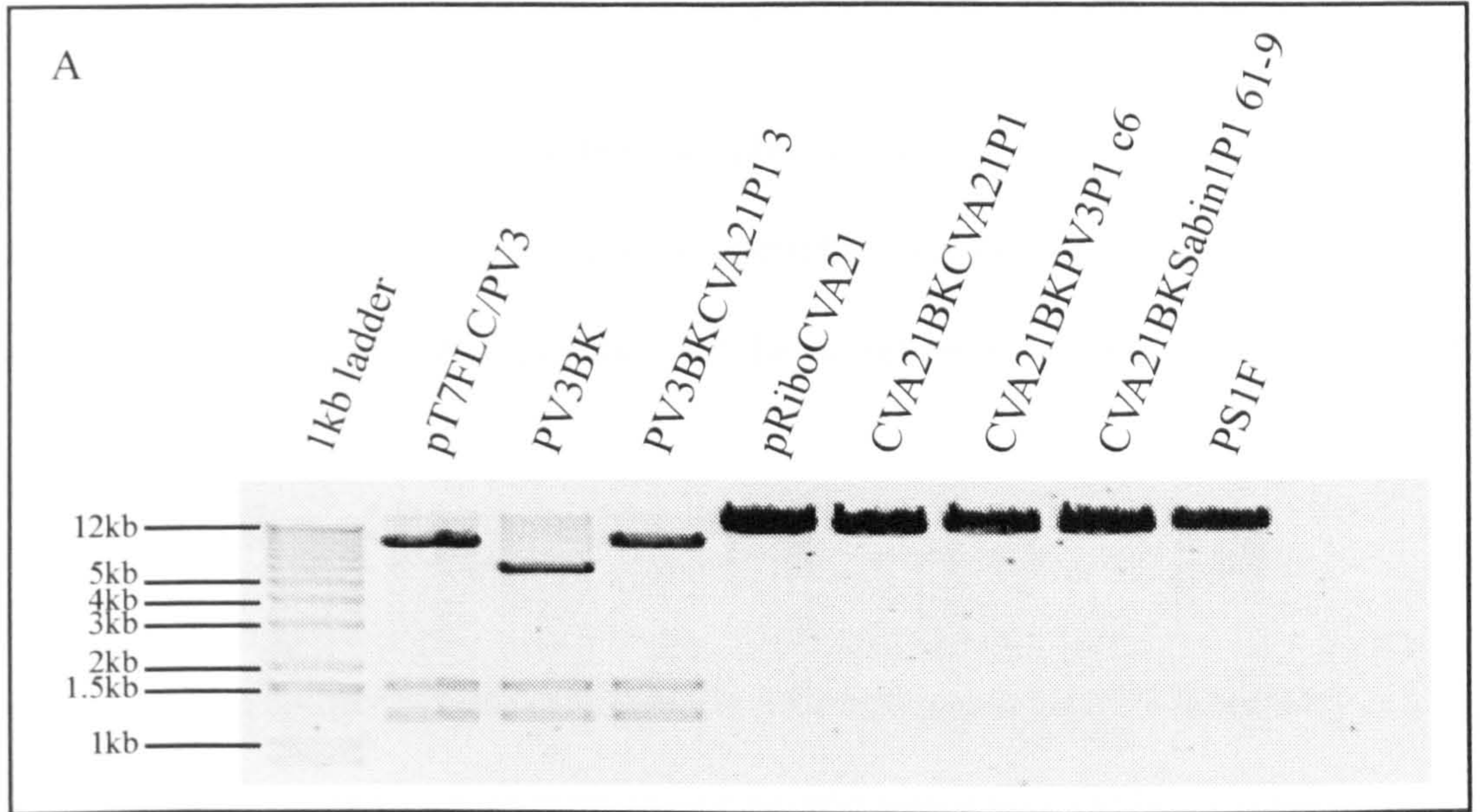


Figure 3.9: Restriction length polymorphism analysis of in vitro generated P1 recombinants and controls.

Recombinants and controls plasmid DNA was digested with *Bgl* II (A) and *Bam* HI (B) and run on a 1% agarose gel.

3.3 Phenotypic analysis of *in vitro* generated recombinants

Phenotypic analysis of *in vitro* generated recombinants was carried out by transfection of T7 RNA transcripts derived from linearised plasmid DNAs in both RD-ICAM cells and HeLa cells, and the samples were screened for the presence or absence of cytopathic effect.

3.3.1 Transfection of P1 recombinants

Transfection of the chimeras was carried out in a Containment Level 3 laboratory (CL3). Recombinants with enhanced pathogenicity or altered tropism could be generated and higher safety levels should be applied when handling the potential viruses resulting from the reaction. The CL3 rules prevented transfected cells from being imported. All transfections were therefore carried out in a CL3 laboratory and were conducted so as to avoid the production of aerosols.

T7 RNA transcripts were derived from linearised plasmid DNA, purified, quantified by spectrophotometric analysis and normalised. Either 1 µg or 5 µg of RNA were transfected, by DEAE-dextran or Lipofectamine 2000, into RD-ICAM cells and HeLa cells. RD-ICAM cells and HeLa cells support infection and replication of both parental viruses and should therefore be permissive for infection with potential chimeric viruses. The transfection samples were incubated at 37°C with 5% CO₂ for 3-5 days to allow for any recombinant viral genomes of lesser fitness to recover and be amplified by repeated replication/infection cycles. Cells were examined by light microscopy to observe a cytopathic effect. The results are shown in table 3.10. No photographic evidence was provided due to the equipment restrictions and difficulty of removing material from the CL3 environment.

Input T7 RNA transcripts	Cytopathic effect monitoring	
	RD-ICAM cells	HeLa cells
CVA21	■	■
PV3	■	■
pS1F (Sabin 1)	■	■
PV3BK	□	□
PV3BKPV3P1	■	■
PV3BKCVA21P1 1	□	□
PV3BKCVA21P1 3	□	□
PV3BKCVA21P1 4	□	□
CVA21BK	□	□
CVA21BKCVA21P1	■	■
CVA21BKPV3P1 c6	□	□
CVA21BKSabin1P1 61-5	□	□
CVA21BKSabin1P1 61-9	□	□
CVA21BKSabin1P1 66-6	□	□
CVA21BKSabin1P1 66-8	□	□
CVA21BKSabin1P1 69-1	□	□
CVA21BKSabin1P1 69-3	□	□
PV3 linearised in 3D	□	□
Mock transfection	□	□
Cells alone	□	□

Table 3.10: Cytopathic readout of transfection of chimeric T7 RNA transcripts into RD-ICAM cells and HeLa cells. The read-out of the experiment was the presence (■) or absence (□) of cytopathic effect observed in the transfected cell sheet. The numbers after each plasmid name represent individual clone numbers resulting from the cloning reaction.

As expected, control transfections of PV3, CVA21 and Sabin 1 RNAs caused CPE in cells after both transfection and serial passage. Similarly CPE was observed in the cells transfected with PV3BKPV3P1 and CVA21BKCVA21P1 RNAs. It was concluded that the experimental system allowed virus recovery following transfection and that the vectors could be used as a basis to create viable recombinants. These were consistent with the results previously seen.

A number of heterologous P1 recombinant clones were prepared and analysed. These were based on CVA21BK and PV3BK, suffixed with clone numbers, and were expected to behave in a similar manner. RD-ICAM cells transfected with each of the heterologous P1 recombinant clones RNAs exhibited very low levels of CPE. However, serial passage of the samples did not appear to increase CPE and it appeared as though viruses could not be amplified. The initial low levels of CPE observed were likely to have been induced by the production and activity of 2A protease, a cytotoxic viral protein. The assay was repeated on several occasions and the same results were obtained. In addition, the same observations were made from transfections carried out in HeLa cells indicating that the observed results were not cell specific and were reproducible in other cell lines. The transfected chimeric T7 RNA transcripts did not cause CPE in HeLa cells. The results were consistent and identical to those observed in RD-ICAM cells (see table 3.10). It is possible that the transfection of recombinants did not give rise to viable viruses or that acytopathic viruses were produced, explaining the lack of CPE.

The CVA21BKCVA21P1 and PV3BKPV3P1 clones were viable thus indicating that there were no defects in the backbone vectors. It appeared unlikely that the recombinants had errors in the sequences as all the clones were derived from the CVA21BK and PV3BK

vectors. Furthermore, RFLP analysis and sequencing of key junctions of all the clones and complete P1 coding region sequencing of the CVA21BKSabin1P1 61-9, CVA21BKPV3P1 c6 and PV3BKCVA21P1 3 chimeras confirmed there were no errors in the constructed clones. It therefore seemed likely that the inability to produce viable virus was due to a defect in the replication cycle, rather than the chimeric viral genome. Further characterisation of the *in vitro* generated recombinants was therefore initiated. The possible acytopathogenicity of the *in vitro* generated P1 recombinants was investigated by an immunofluorescence assay to detect virus antigen.

3.3.2 Immunofluorescence study of viral protein expression and determination of the acytopathogenic phenotype of chimeras

Immunofluorescence analysis was carried out to detect VP1 protein production, using a specific anti-enterovirus VP1 mouse monoclonal antibody (DAKO). The specificity of the antibody for the P1 capsid precursor protein was unclear. Subsequent *in vitro* translation studies appeared to indicate that the antibody was specific to VP1 as no extra bands were observed in western blot analysis (see figure 3.17).

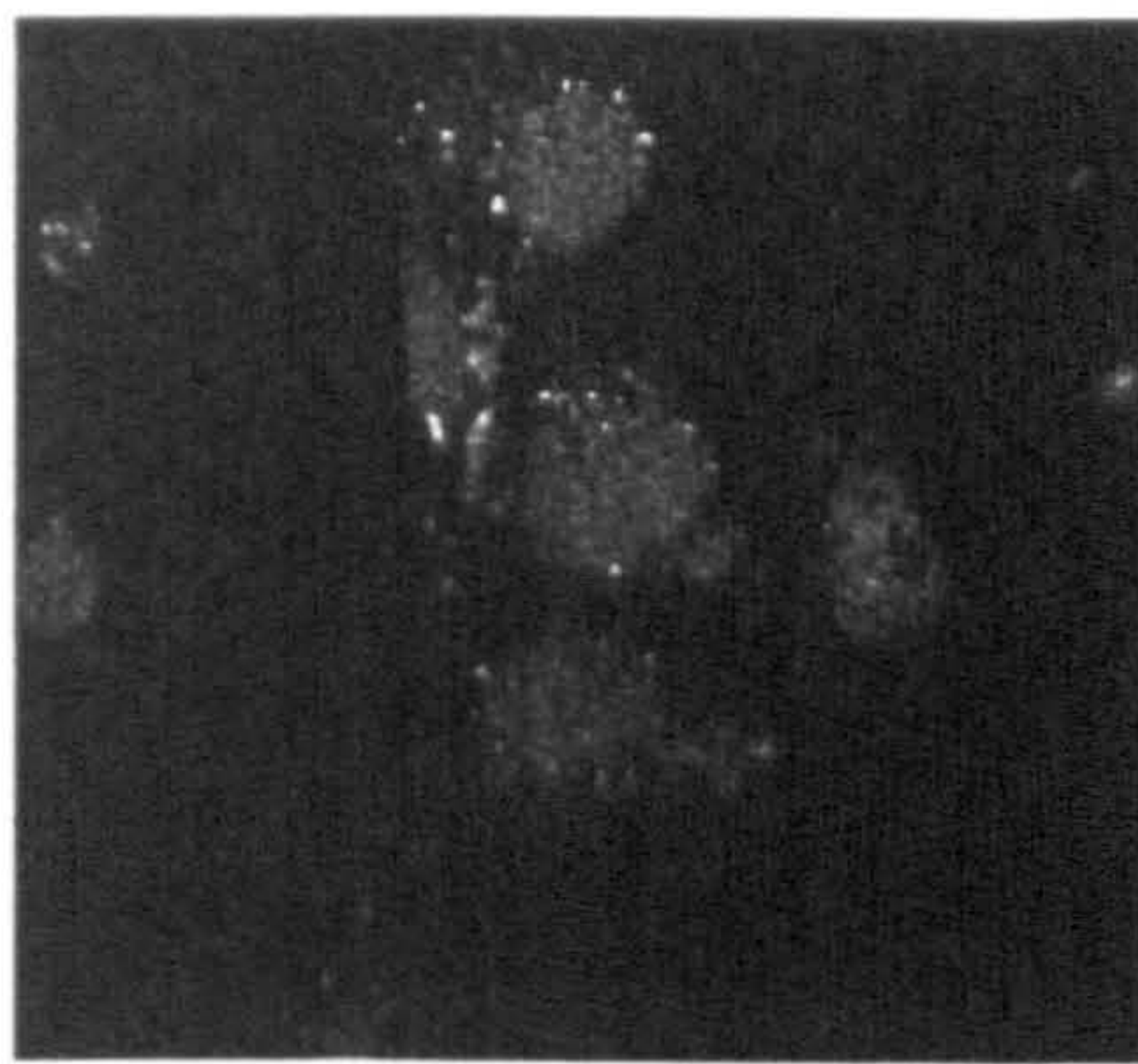
The VP1 capsid protein production would be expected to occur post-transfection. Viable chimeric viruses would be expected to produce VP1 and protein expression would be detected after passage of RNase treated transfected samples. The detection of VP1 expression in both transfected RD-ICAM cells and RD-ICAM cells in which harvested RNase treated samples had been passaged would suggest that the chimeric viruses were acytopathic.

Duplicates of the transfected cells were lysed to release any intracellular virus particles, samples were harvested and RNase treated to prevent RNA carry over onto a fresh cell

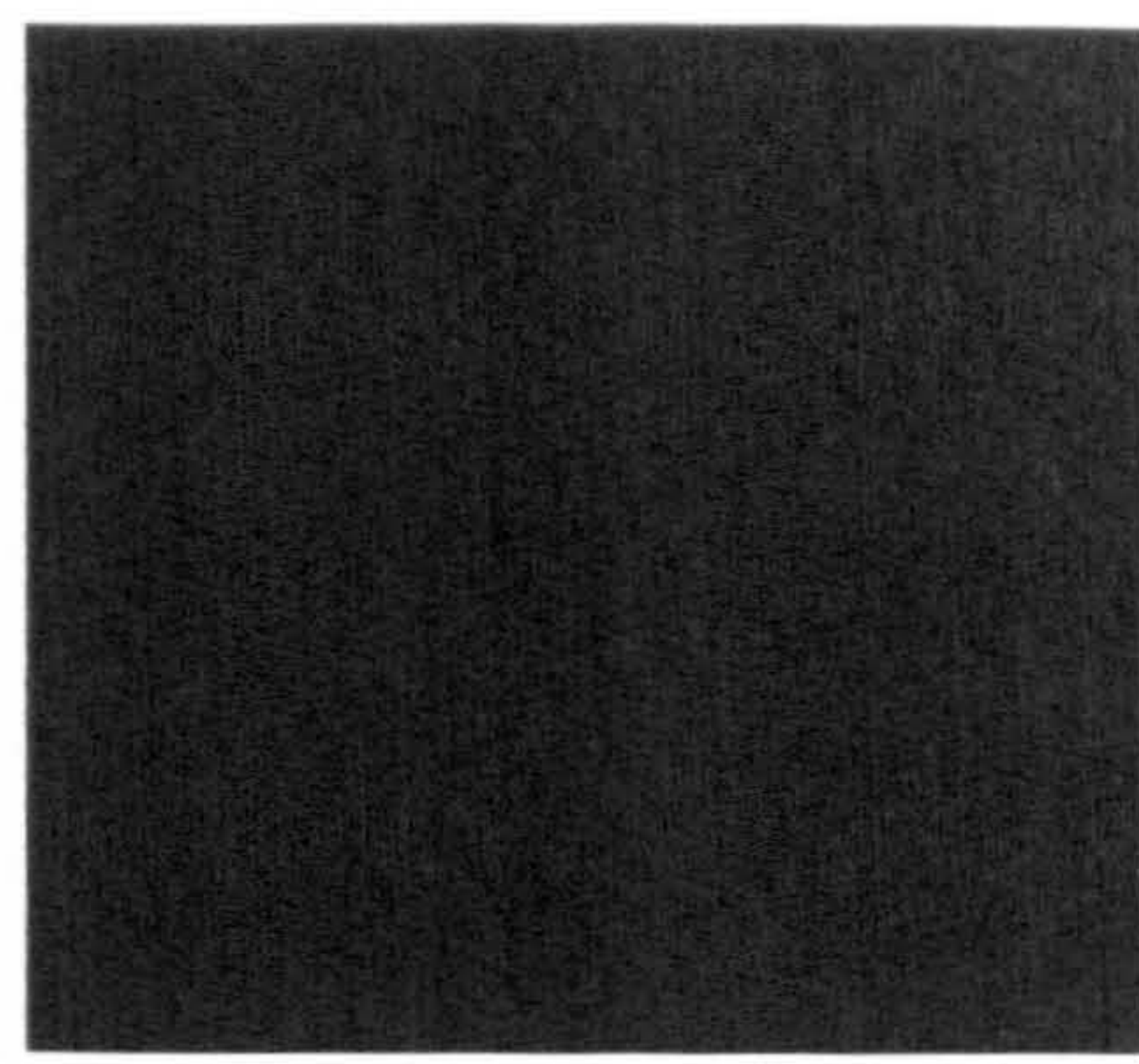
sheet. The harvested samples were passaged onto fresh cell sheets and VP1 immunolabelling was conducted. Cells were treated with a primary anti-VP1 DAKO mouse monoclonal antibody followed by treatment with a secondary anti-mouse FITC-conjugated polyclonal antibody (see appendix 1). Sample-coverslips were mounted on slides and visualised using a confocal microscope. All the confocal imaging was carried out at the same time. The results are illustrated in figure 3.11, 3.12 and 3.13 using a range of magnifications.

VP1 expression was detected in cells transfected and passaged with control samples PV3, Sabin 1 and CVA21. These control samples are known to produce cytopathic viruses. These results suggested the assay had been successful. Mock transfection and passage controls were carried out in parallel as negative controls for non-FITC labelled cells and did not exhibit expression of VP1 after transfection or passage. A PV3-infected control singly labelled with the secondary FITC-conjugated antibody was used as a control for monitoring background levels of non-specific detection of FITC fluorescence.

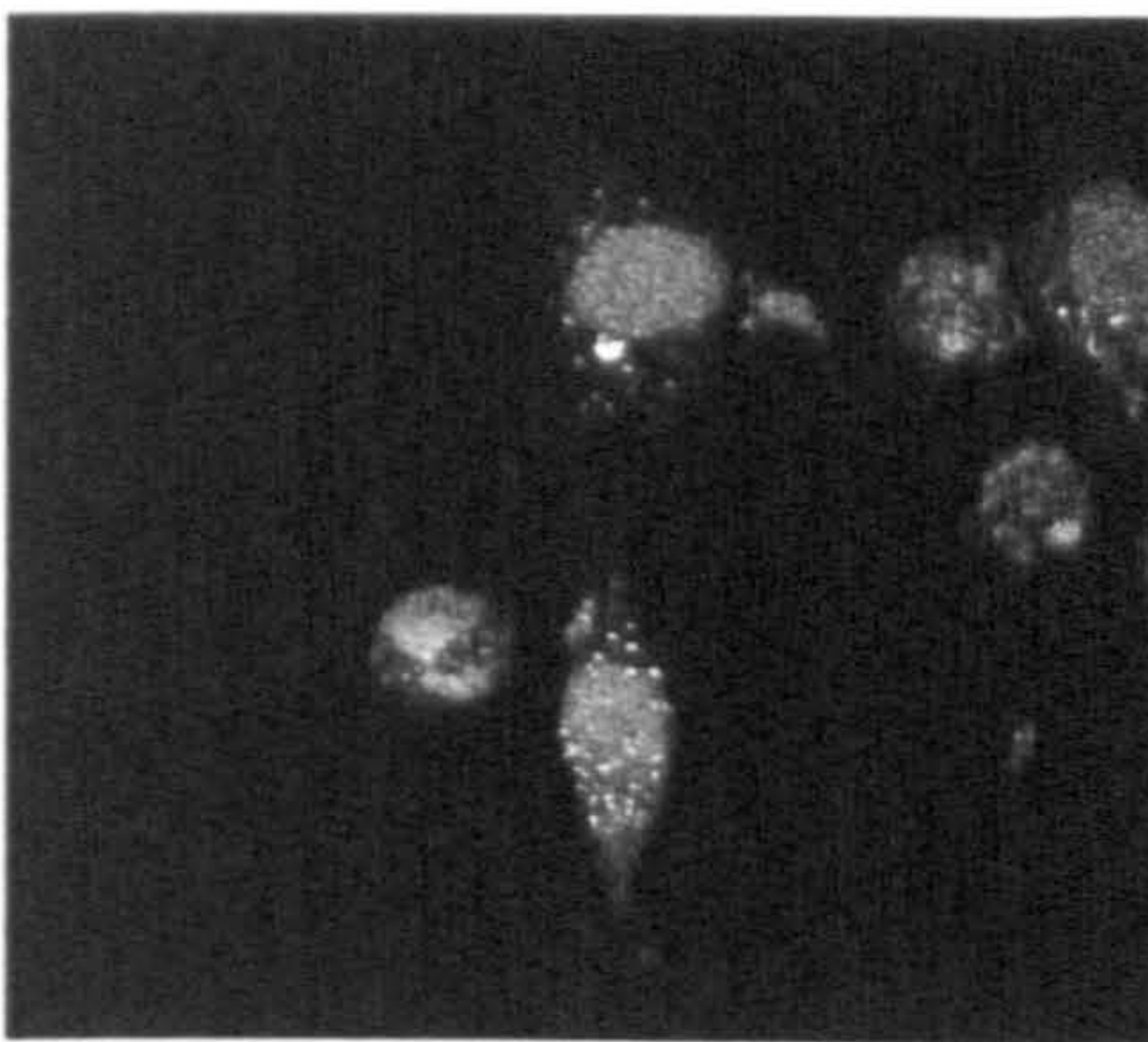
VP1 expression was detected in all cell sheets transfected with RNA derived from recombinant cDNAs but not in other cells. A weak signal was detected in the cells passaged with the CVA21BKSabin1P1 RNA transfected cell harvest solutions. The detection of VP1 expression in the transfected cells indicated that translation had occurred. The lack of VP1 expression in cells passaged with samples harvested from the chimera transfections would suggest that the recombinants were not acytopathic. If the recombinants were acytopathic the levels of VP1 expression in the passage samples were below the level of detection by immunofluorescence. These results suggested that the defect had to be at some stage post-transfection, such as processing, replication, packaging, assembly, and release or host cell entry. Further experiments were conducted to investigate



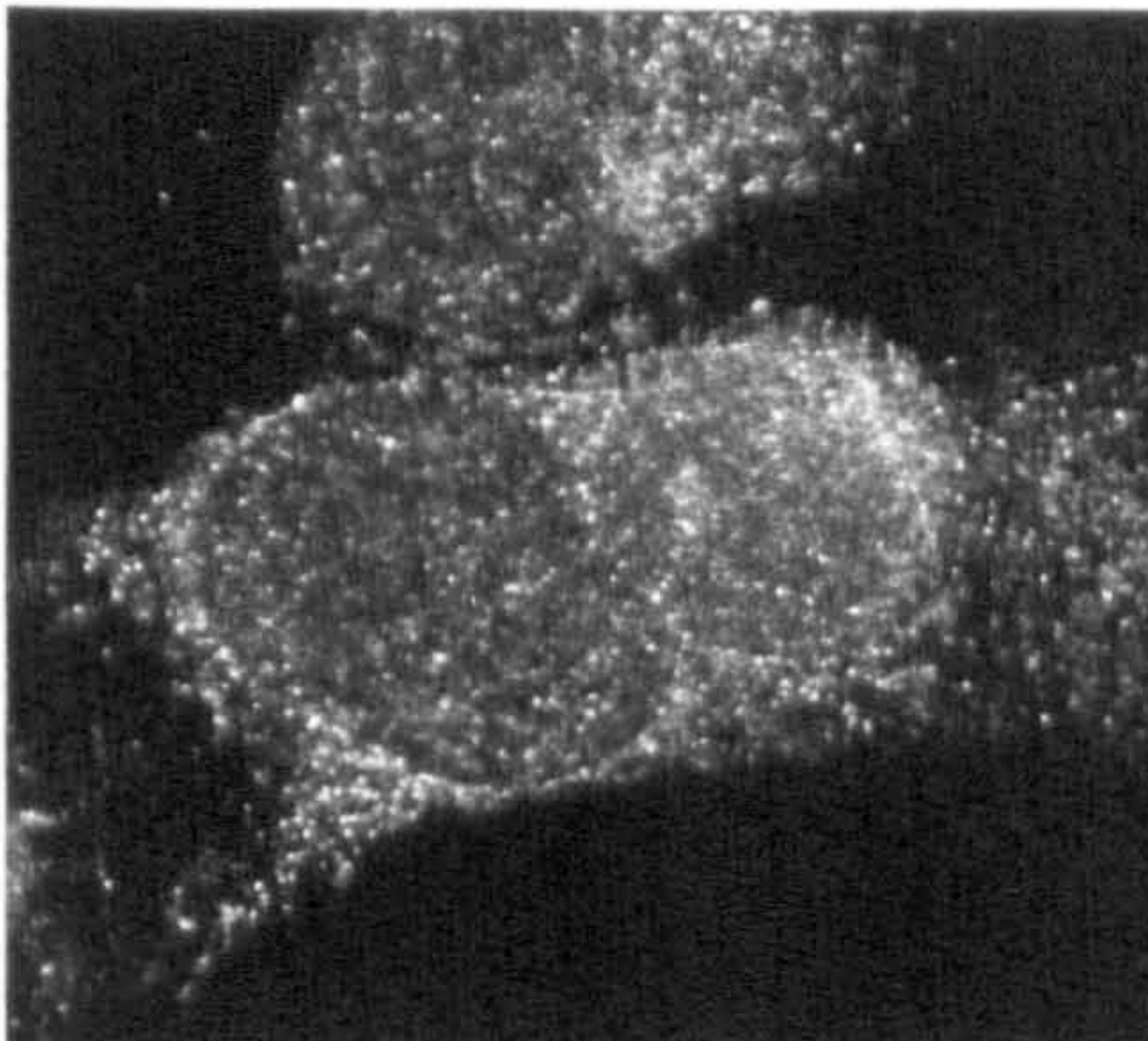
PV3BKCVA21P1 3



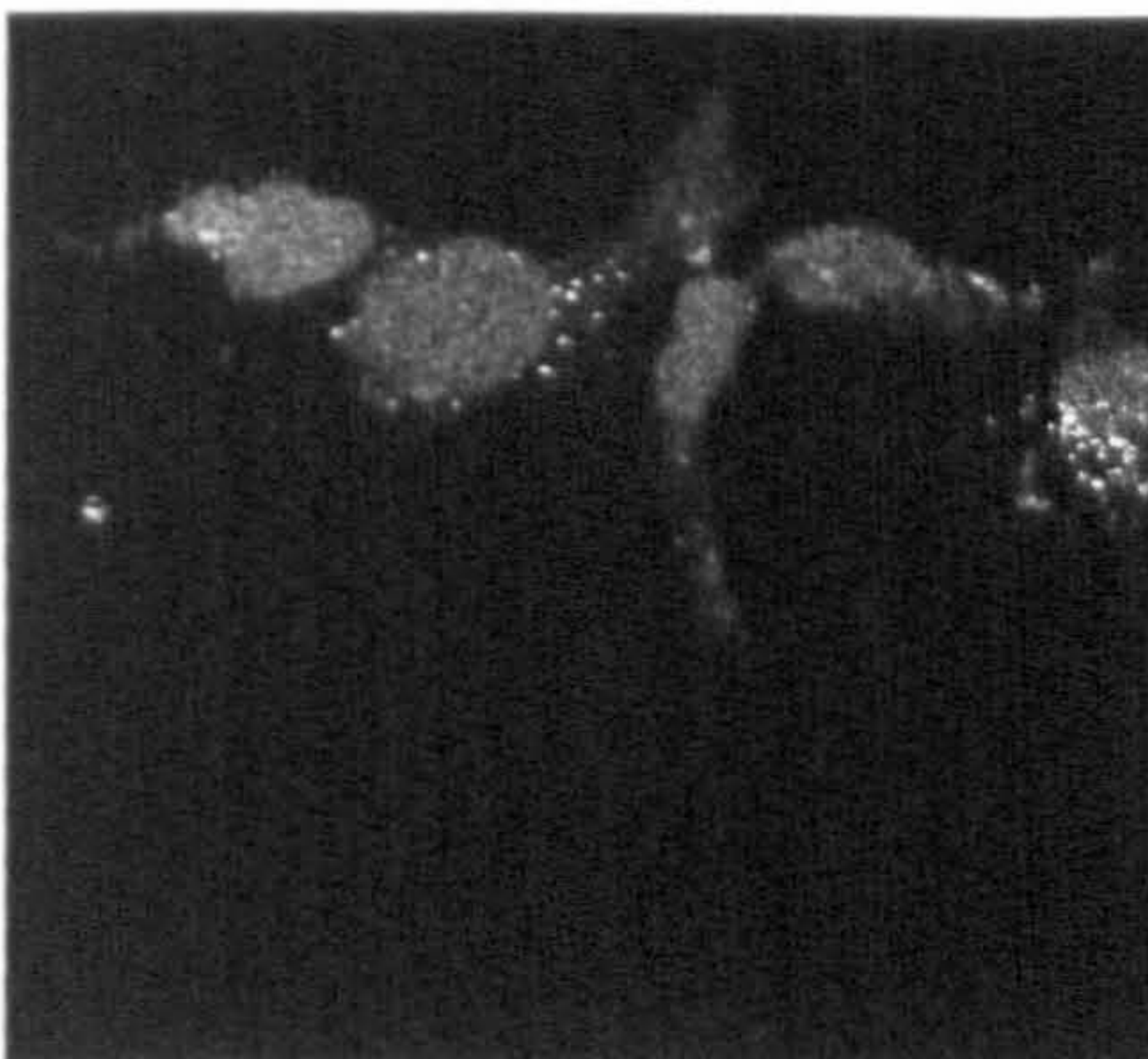
PV3BKCVA21P1 3



CVA21



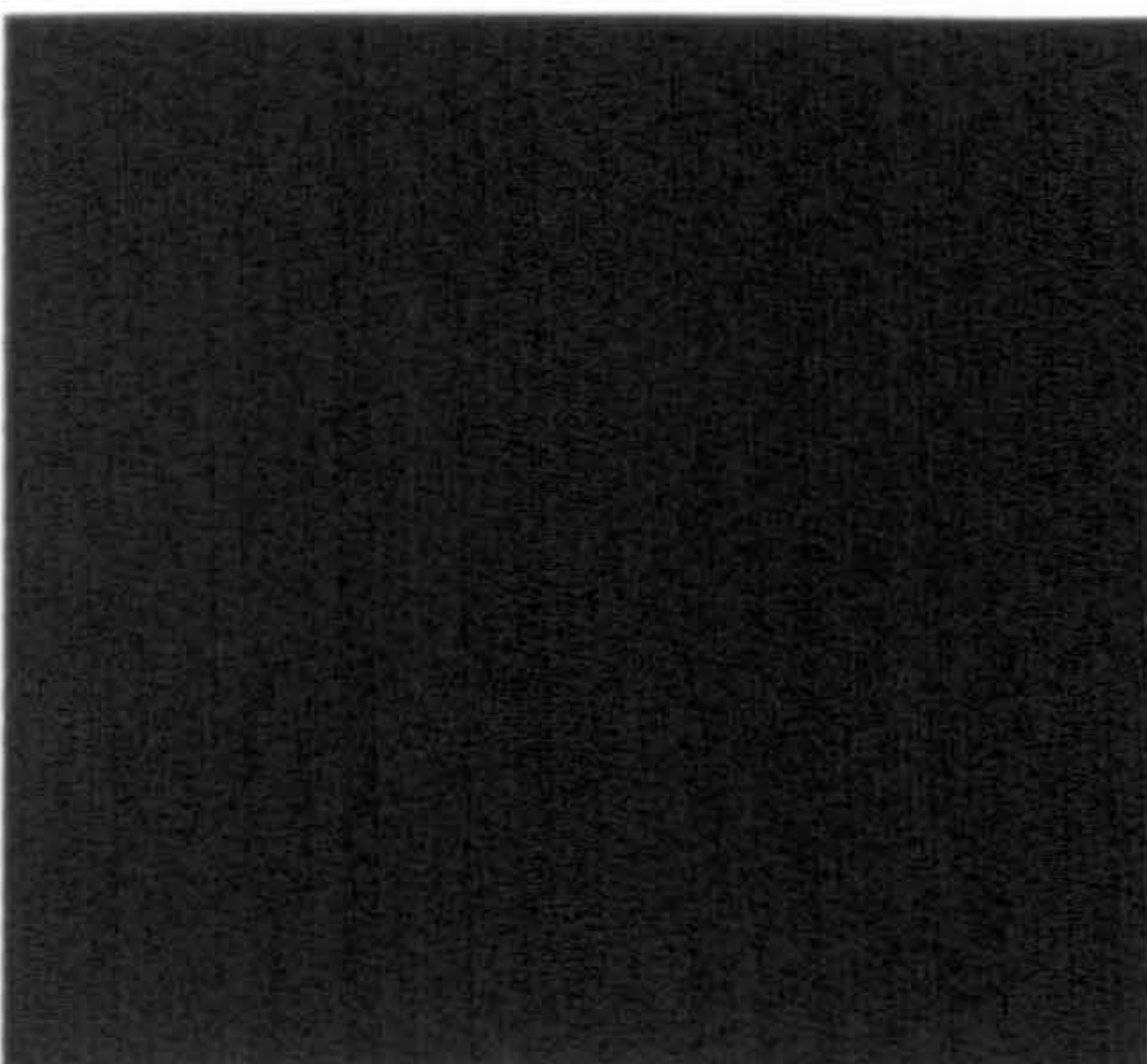
CVA21



PV3



PV3



Cells alone



Cells alone

Figure 3.11: Detection of PV3BKCVA21P1 3 VP1 expression

The left hand column represent the pictures of transfected cells and the right hand column are pictures of the supernatant passaged onto fresh cell sheets. RNA transcripts from the indicated plasmids were transfected into RD-ICAM cells. The presence of VP1 virus antigen was determined by immunofluorescence analysis using as primary antibody the anti-HEV VP1 (DAKO) MAb at 10 hours post-transfection or 10 hours after passaging of RNase-treated culture supernatant to fresh cells. Mock transfected cells are also shown.

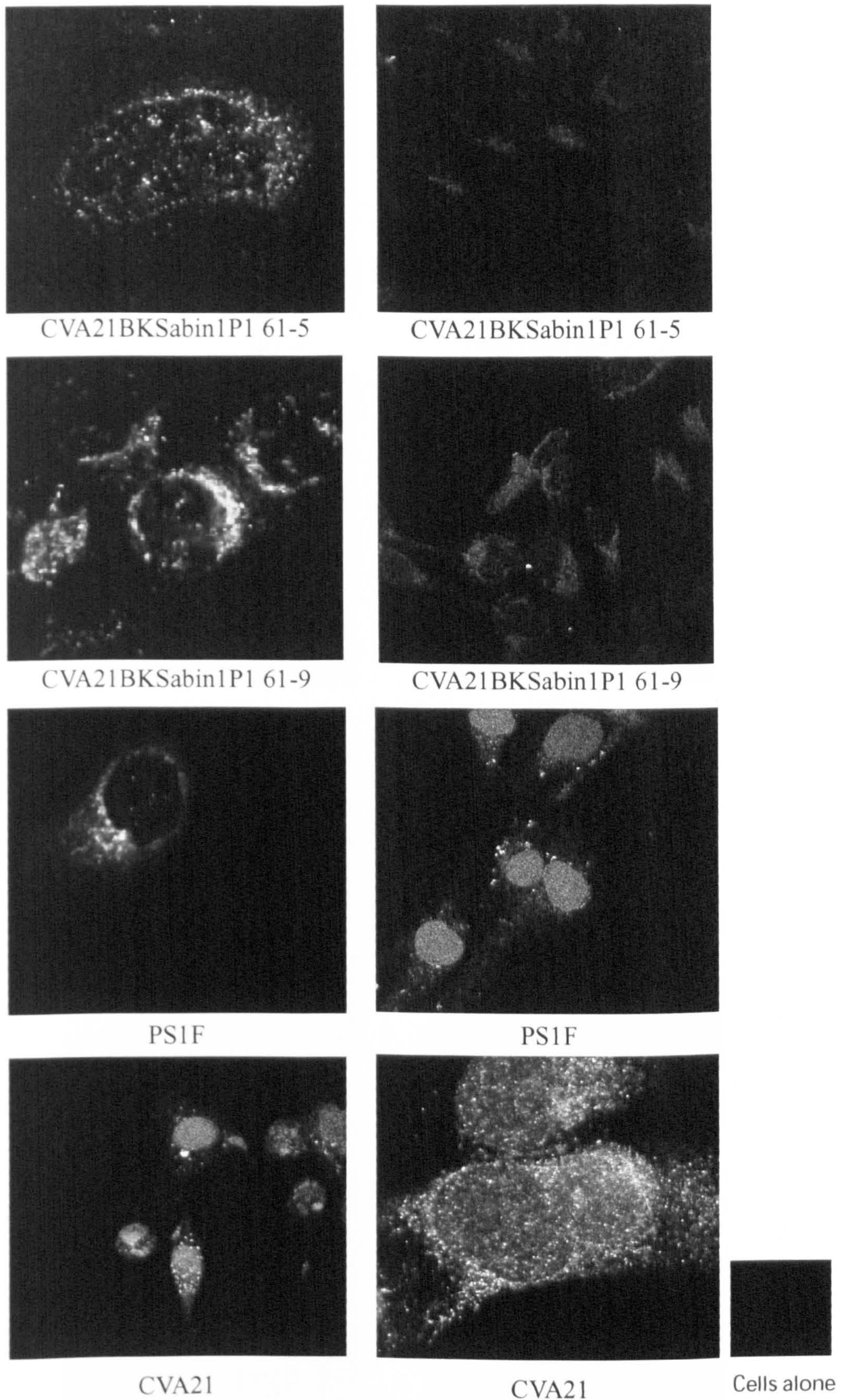
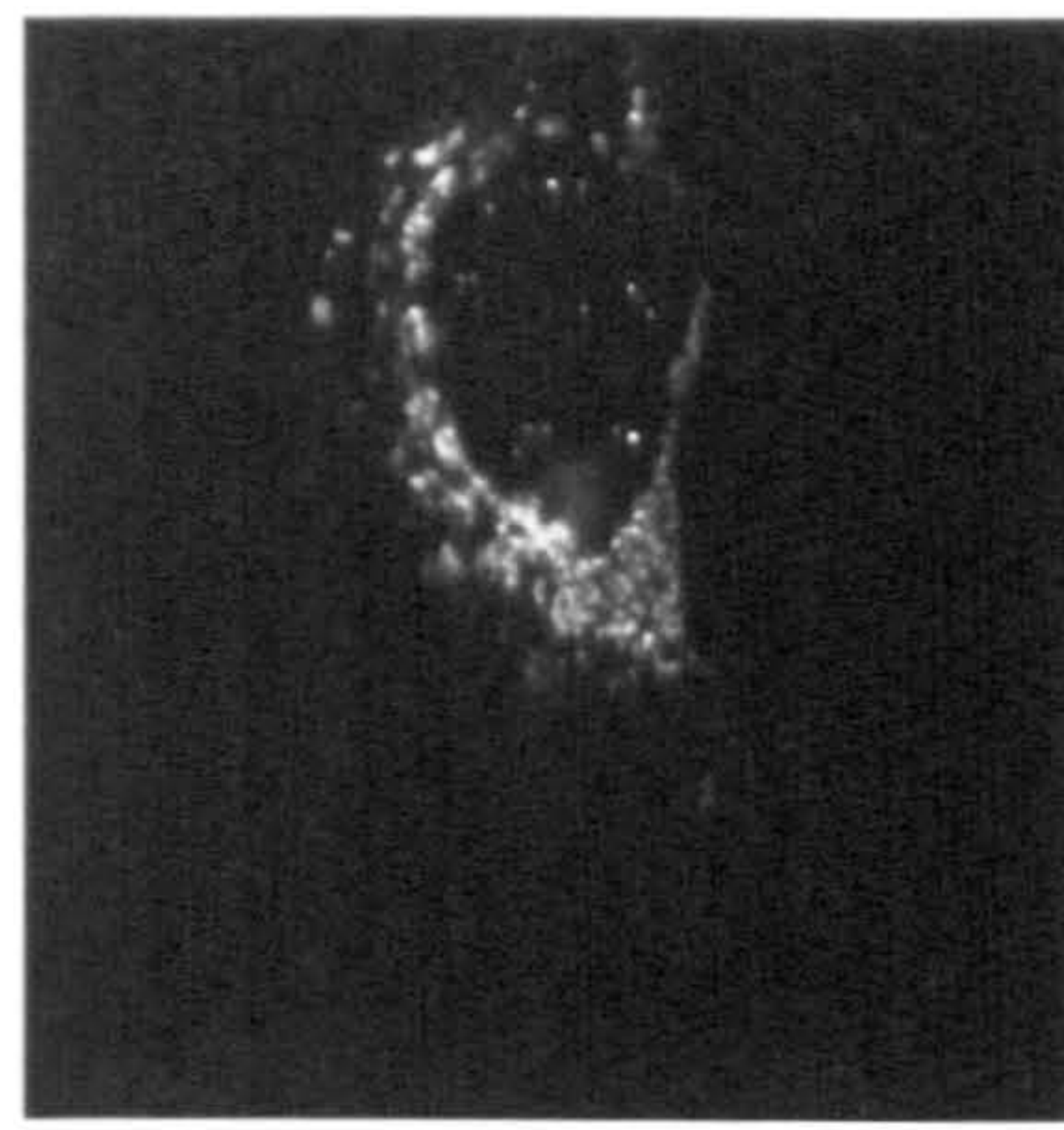
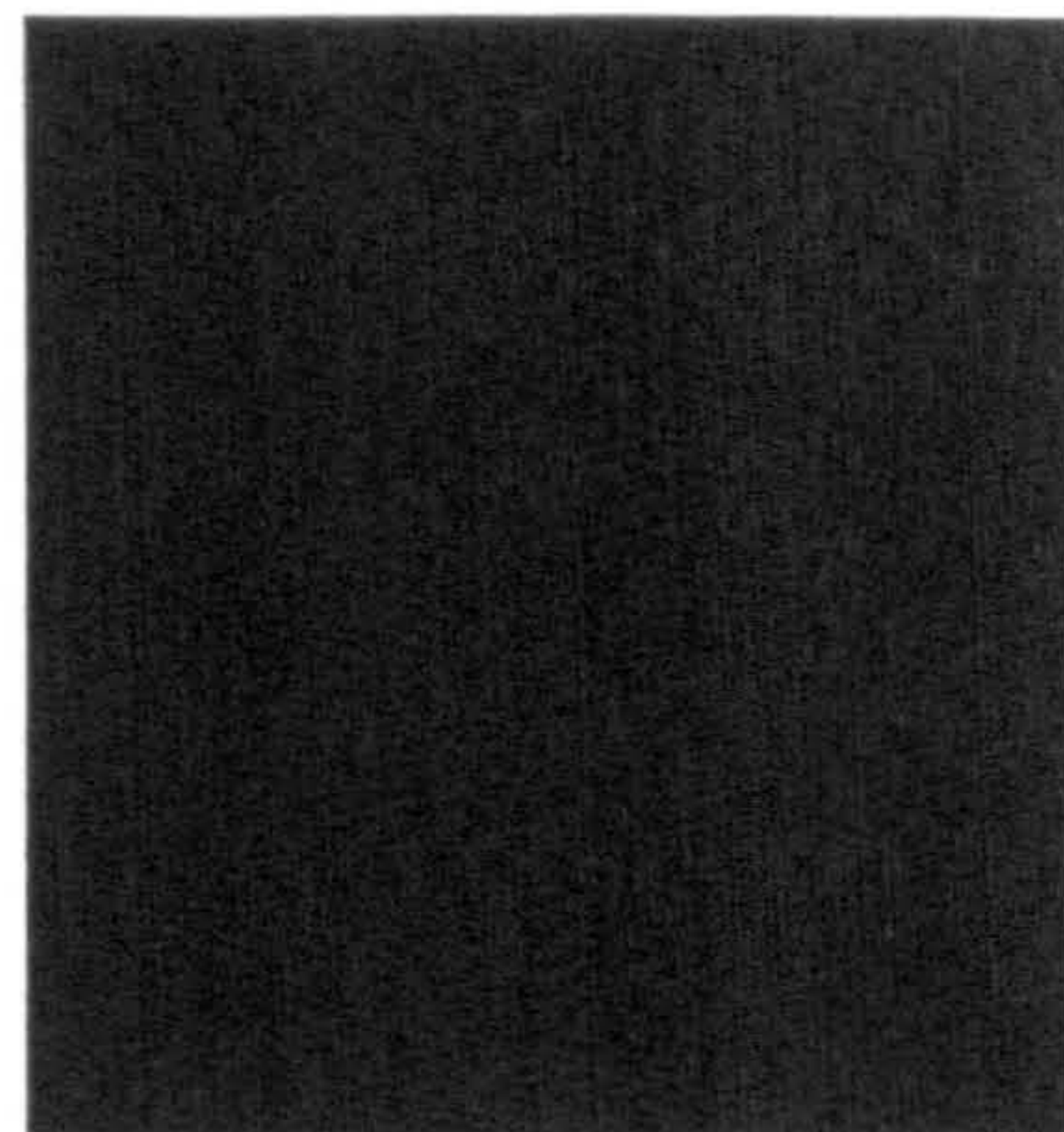


Figure 3.12: Detection of CVA21BKSabin1P1 61-5 and 61-9 VP1 expression

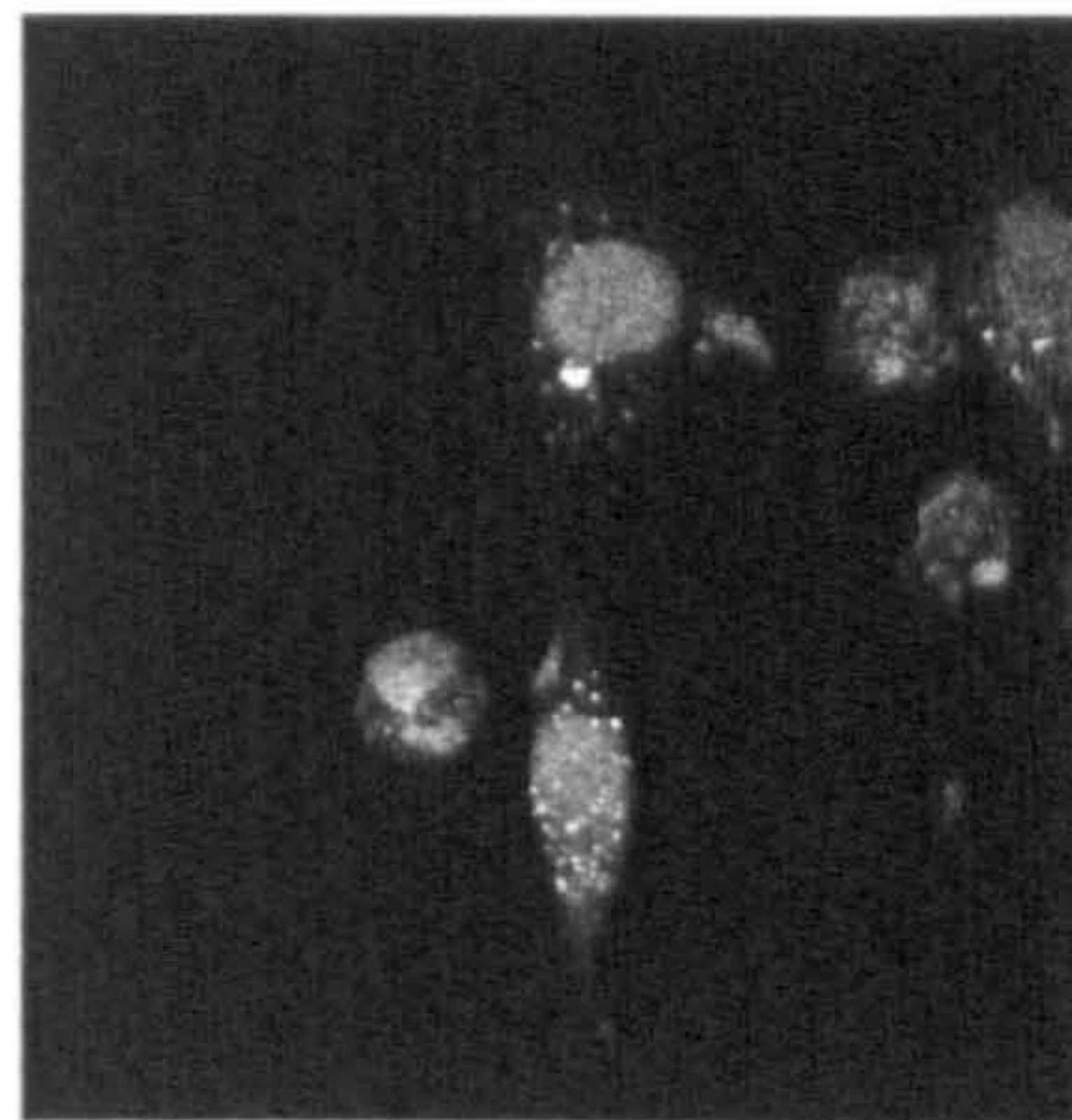
The left hand column represent the pictures of transfected cells and the right hand column are pictures of the supernatant passaged onto fresh cell sheets. RNA transcripts from the indicated plasmids were transfected into RD-ICAM cells. The presence of VP1 virus antigen was determined by immunofluorescence analysis using as primary antibody the anti-HEV VP1 (DAKO) MAb at 10 hours post-transfection or 10 hours after passaging of RNase-treated culture supernatant to fresh cells. Mock transfected cells are also shown.



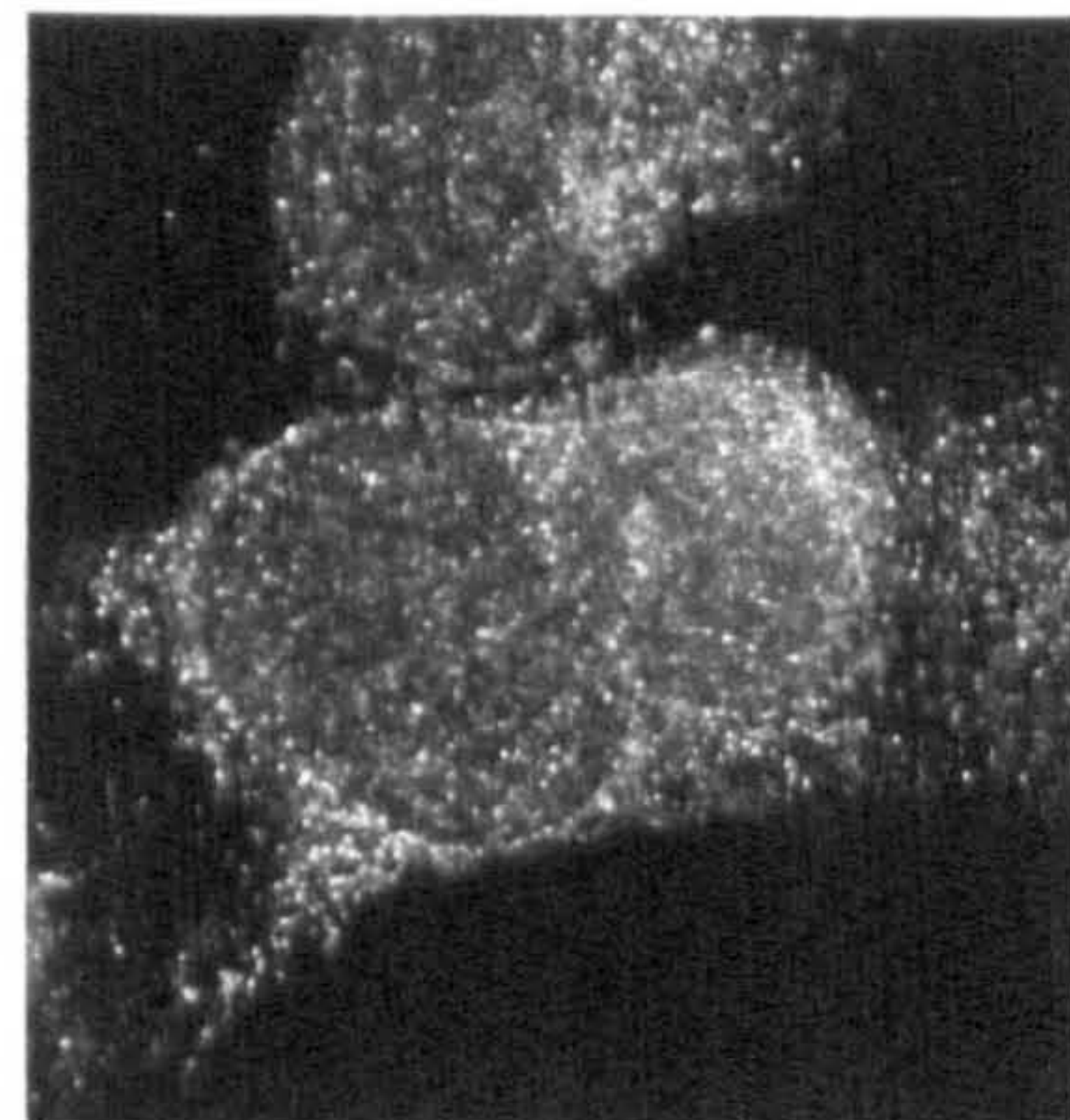
CVA21BKPV3P1 c6



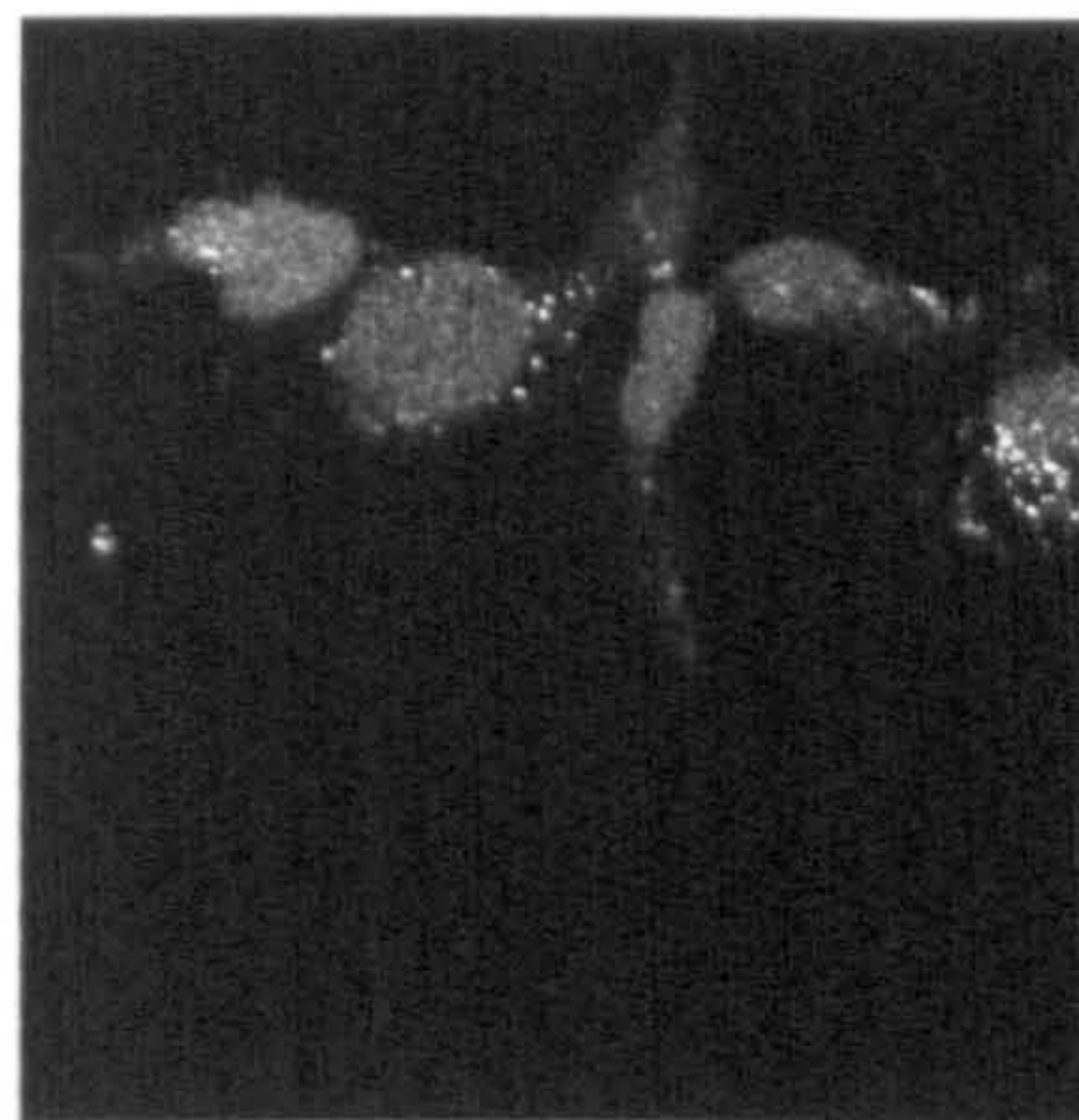
CVA21BKPV3P1 c6



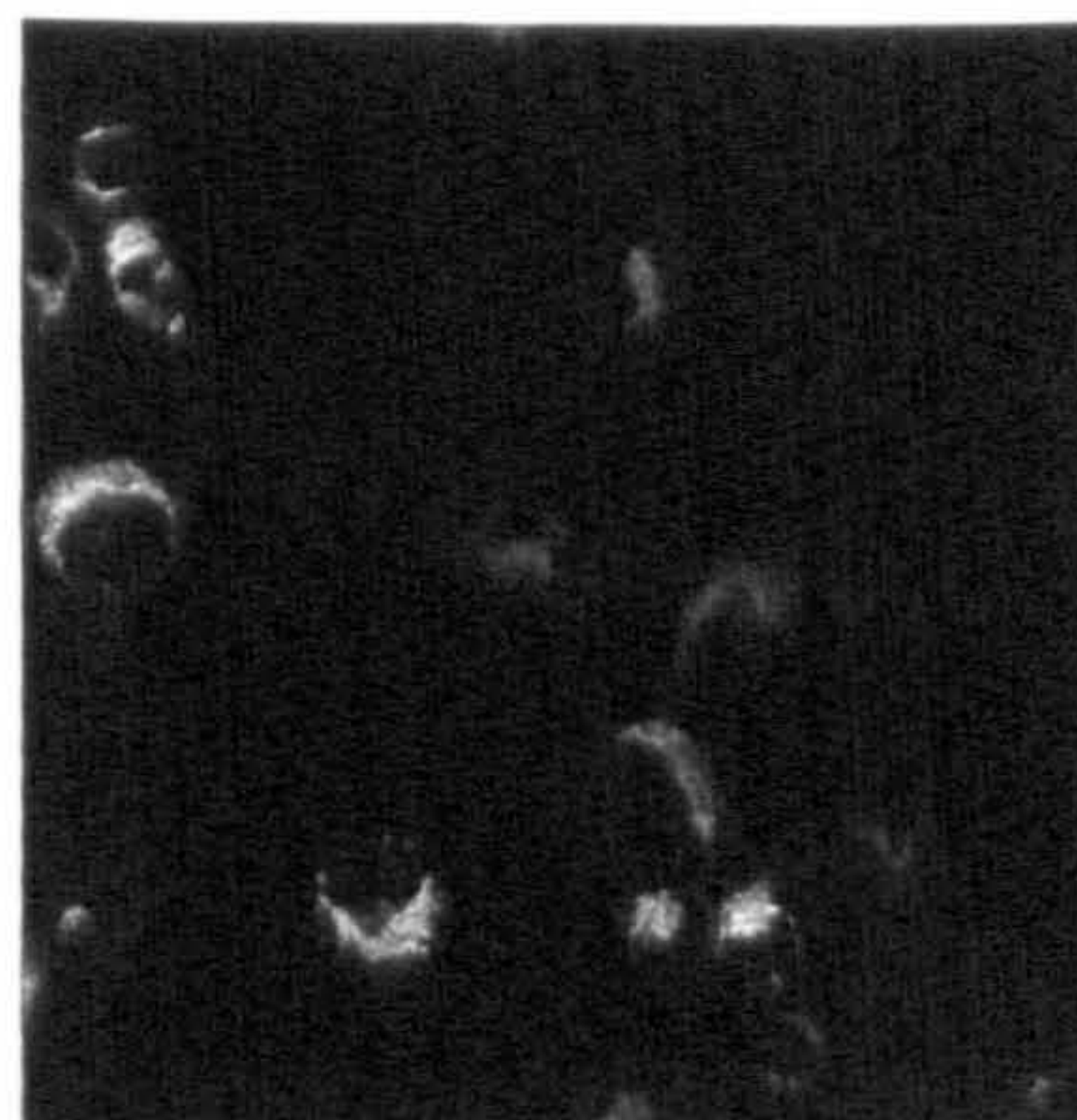
CVA21



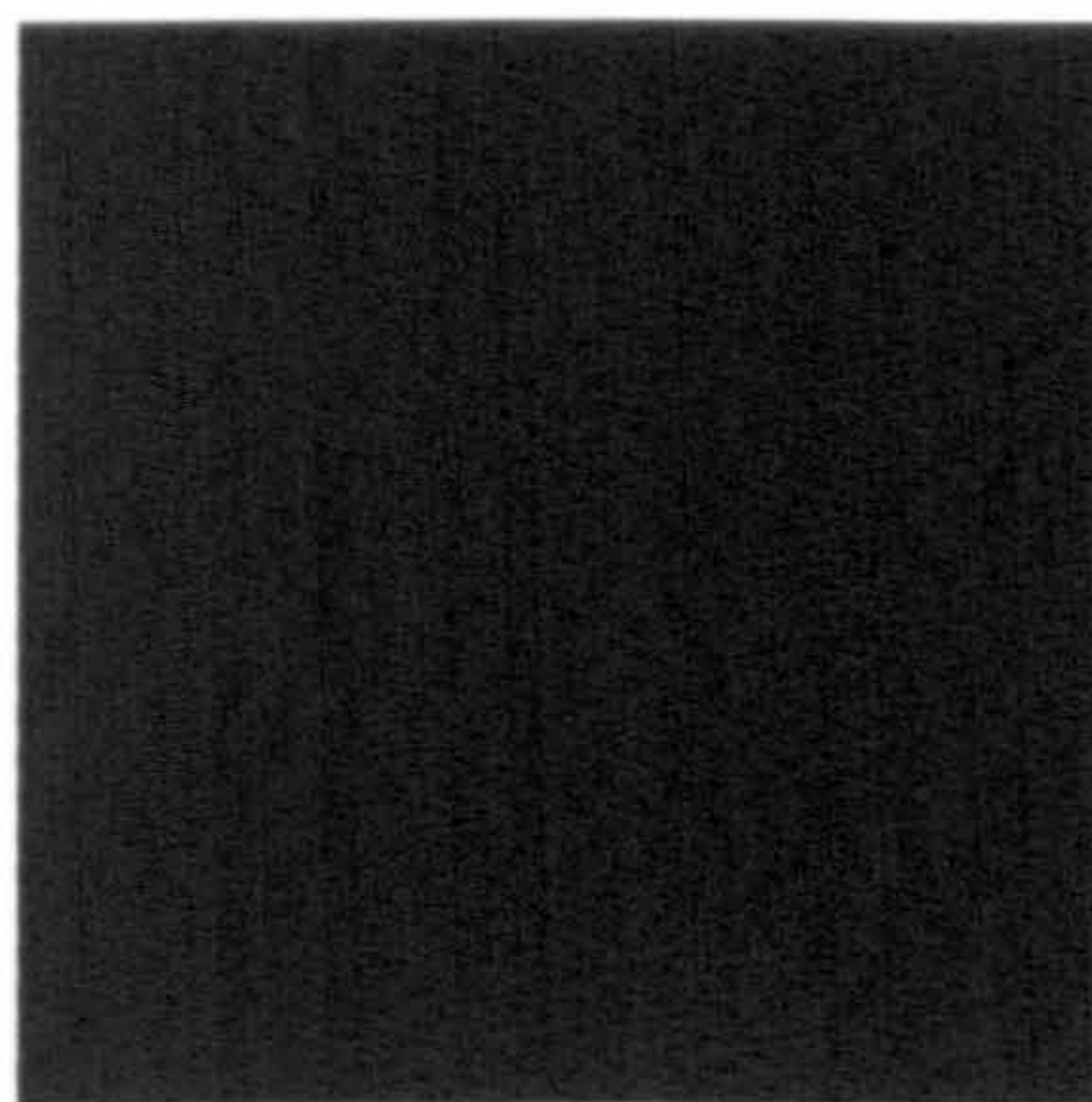
CVA21



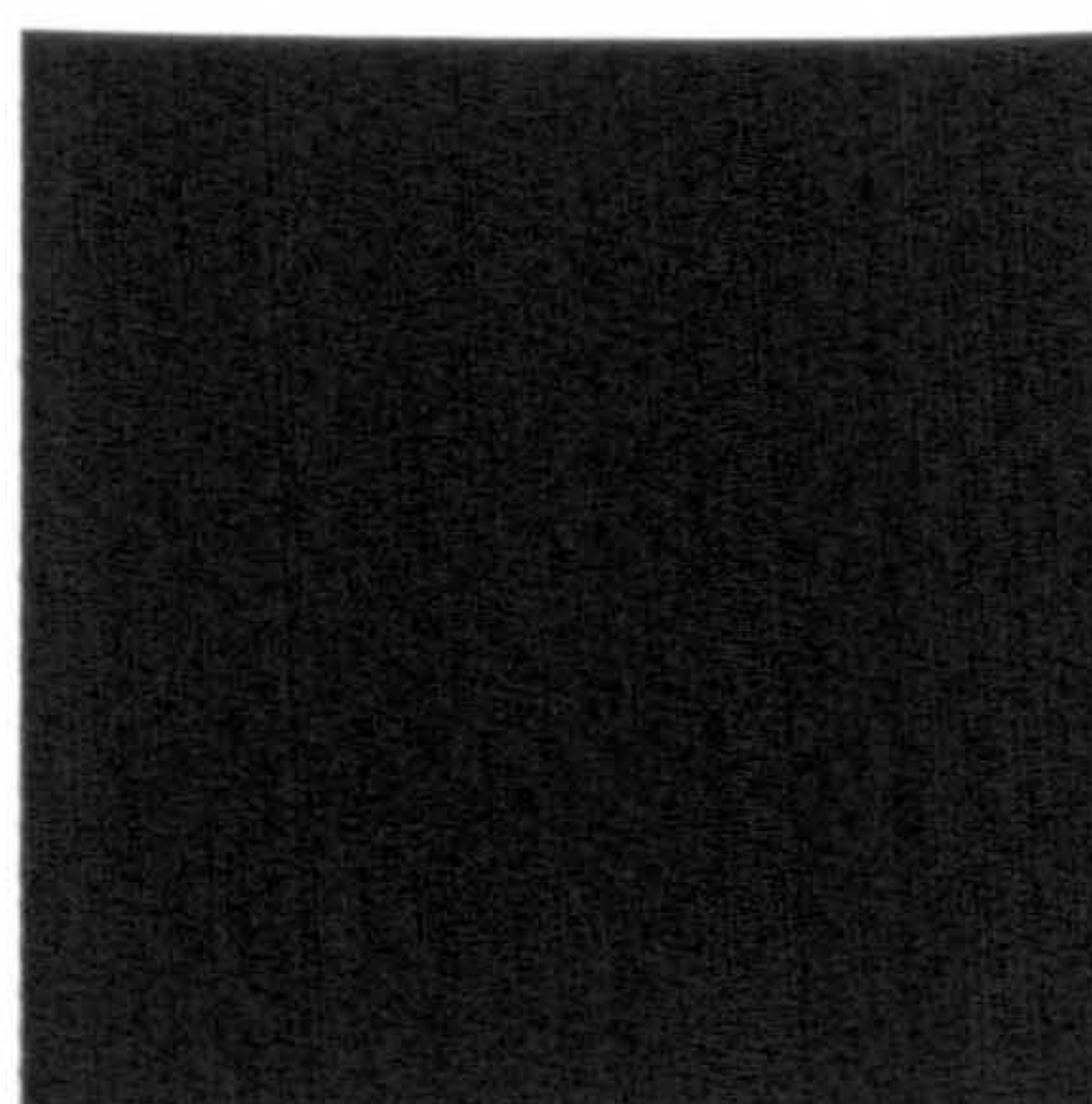
PV3



PV3



Cells alone



Cells alone

Figure 3.13: Detection of CVA21BKPV3P1 c6 VP1 expression

The left hand column represent the pictures of transfected cells and the right hand column are pictures of the supernatant passed onto fresh cell sheets. RNA transcripts from the indicated plasmids were transfected into RD-ICAM cells. The presence of VP1 virus antigen was determined by immunofluorescence analysis using as primary antibody the anti-HEV VP1 (DAKO) MAb at 10 hours post-transfection or 10 hours after passaging of RNase-treated culture supernatant to fresh cells. Mock transfected cells are also shown.

the defect. As the results suggested that translation had occurred, *in vitro* translation assays were undertaken to confirm the initial observations.

3.3.3 *In vitro* translation

The processing of the P1 capsid precursor and its cleavage from the non-structural proteins are mediated by two viral proteases, 3C and 2A. The proteases and the capsid proteins, in the *in vitro* generated recombinants, are derived from separate viral sources and processing could be absent, incomplete or inefficient due to an incompatibility of the proteases with the P1 capsid precursors. Therefore the investigation of the protein processing properties of the *in vitro* generated chimeras, by *in vitro* translation assays, was essential. Initial characterisation of wild-type viral proteins and processing pattern was therefore conducted.

3.3.3.1 Wild-type controls characterisation

Radiolabelled infected cell lysates of control viruses CVA21, PV3 and Sabin 1 were prepared as an *in vivo* source of large amounts of correct processing products (figure 3.14). These were later used for comparison with the *in vitro* processing products, obtained using HeLa S10 extracts. Control radiolabelled infectious virus particles, with a sedimentation coefficient of 160S, were also prepared in parallel, to identify the capsid proteins. VP1, VP3 and sometimes VP2 of the 160S particles were observed on the gels and comparison with the migration patterns exhibited by the infected cell lysates samples enabled the identification of capsid proteins (see appendix 1). These are illustrated in figure 3.14. CVA21 160S particles and Sabin 1 infected cell lysates consistently labelled poorly. CVA21 VP1 and VP3 labelled protein bands are indicated with dots in figure 3.14. CVA21 VP2 detection was not apparent due to the poor labelling of the virus.

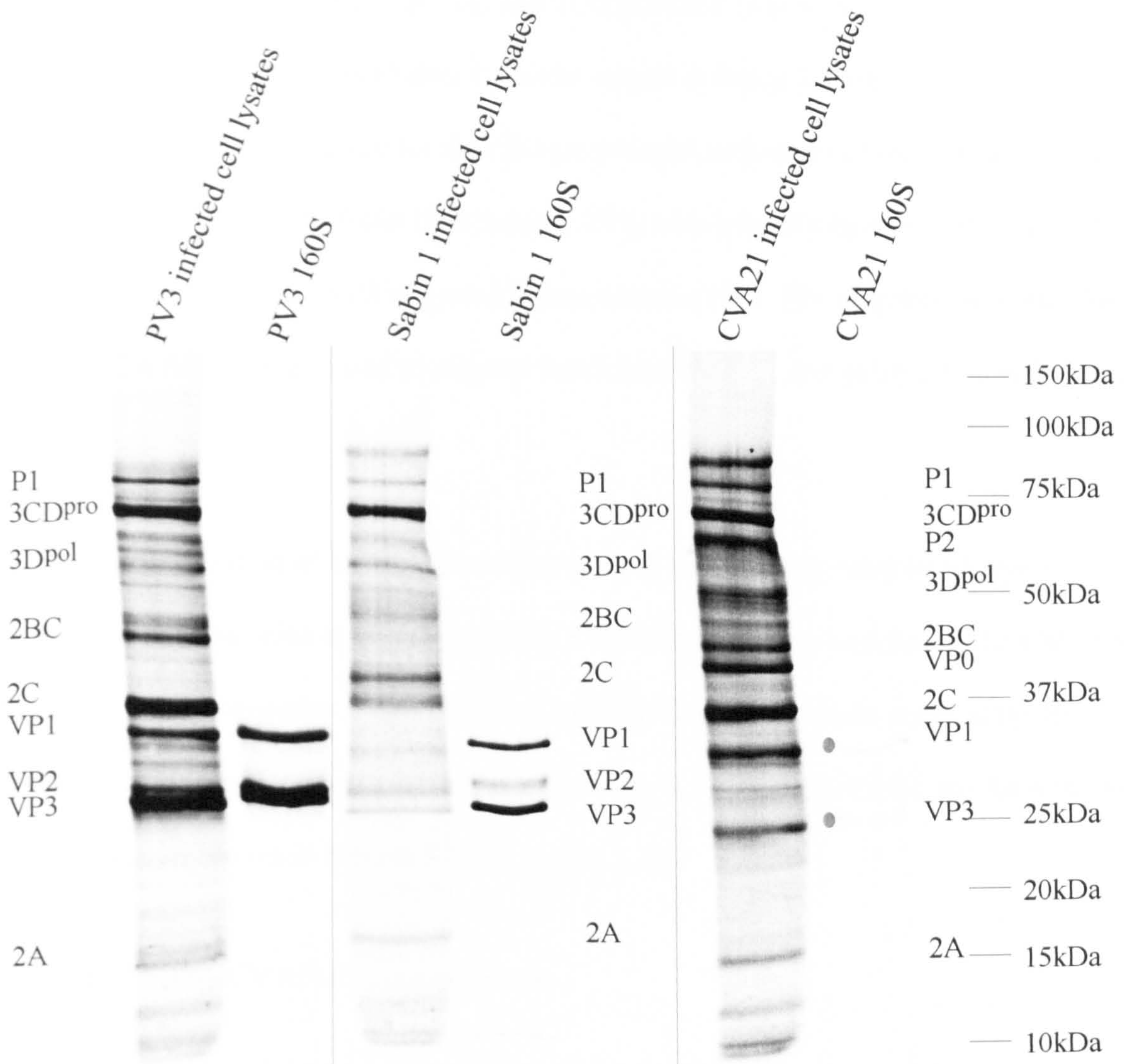


Figure 3.14: Wild type characterisation of PV3, CVA21 and Sabin 1 capsid proteins using 160S virus particles and infected cell lysates.

12.5% SDS-PAGE gel of the PV3, CVA21 and Sabin 1 processing pattern and identification of capsid proteins. CVA21 160S particles consistently labelled poorly and the labelled VP1 and VP3 bands are highlighted with dots. Overexposure of the gel showed the presence of an extra band migrating slightly slower than VP3 in the radiolabelled CVA21 160S sample lane.

The SDS-PAGE gel (figure 3.14) shows that CVA21 and PV3 exhibited a similar migration pattern for the P1, 2BC, 2C and 3CD proteins. However, VP1, VP3 and 2A proteins of PV3, CVA21 and Sabin 1 control viruses differed in their migration patterns and were therefore diagnostic for the relevant parental sequences of each chimera. CVA21 VP1 appeared to migrate faster than Sabin 1 VP1, which itself migrated faster than PV3 VP1. Similarly, CVA21 VP3 migrated fastest whereas PV3 VP3 migrated slowest. The CVA21 2A protease appeared to migrate faster than PV3 2A and pS1F 2A, which migrated the slowest.

Once characterisation of control viruses processing patterns was completed, *in vitro* transcription/translation assays using HeLa S10 nuclear extracts supplemented with flexi rabbit reticulocyte lysates were conducted to determine the products encoded by the chimeras. The reactions were denatured and run on a 12.5% SDS-PAGE gel for analysis. The results are shown in figures 3.15, 3.16 and 3.18.

3.3.3.2 PV3BKCVA21P1 recombinants

The *in vivo* infected cell lysates samples patterns and the *in vitro* patterns obtained using HeLa S10 nuclear extracts of control viruses were compared on the PV3BKCVA21P1 processing gel (figure 3.15). These patterns were similar, apart from few additional cellular proteins present in the *in vivo* infected cell lysates. The CVA21 and PV3 infected cell lysates samples consistently migrated slightly faster than other lanes. This was taken into consideration for recombinant sample analysis.

The PV3BKCVA21P1 recombinants appeared to exhibit the correct processing pattern (see figure 3.15). PV3BKCVA21P1 recombinants should express a PV3 non-structural protein pattern and a CVA21 capsid protein pattern. 3CD, P1, 2BC and 2C could not be used for comparison purposes since the migration pattern of these proteins did not differ amongst

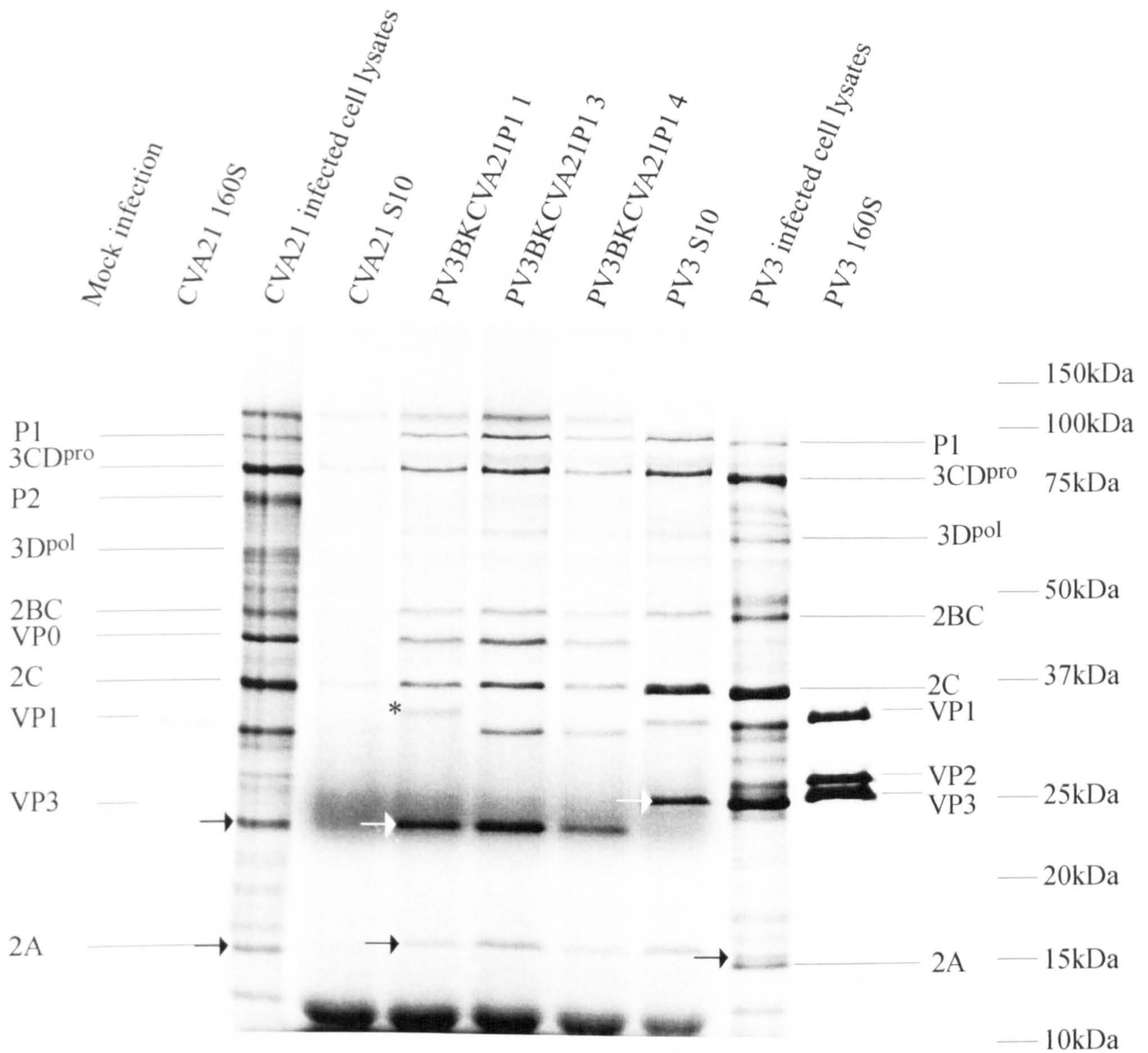


Figure 3.15: PV3BKCVA21P1 recombinants in vitro translation reaction (using HeLaS10 extracts supplemented with rabbit flexi reticulocyte lysates).

The samples were run alongside the 2 control viruses. PV3BKCVA21P1 1, PV3BKCVA21P1 3, PV3BKCVA21P1 4 represent individual clones isolated from the cloning experiment. The arrows highlight the bands used for characterisation and analysis and the asterisk represent an unidentified band in one of the recombinant samples. PV3 and CVA21 infected cell lysates appeared to migrate faster than PV3 and CVA21 HeLa S10 samples.

various samples. PV3BKCVA21P1 2A had the same migration phenotype as that of PV3 2A and migrated slightly slower than the CVA21 2A. However, the CVA21 S10 sample consistently labelled poorly and 2A was hardly detectable on the protein gel. These results indicated that the non-structural proteins of the PV3BKCVA21P1 recombinants appeared to be PV3-like.

VP1 of CVA21 and PV3 had similar migration patterns and could not be used as identification proteins for the PV3BKCVA21P1 recombinants analysis. However, VP3 of PV3 migrated significantly slower than CVA21 VP3 and the gel demonstrated that PV3BKCVA21P1 VP3 was CVA21 VP3-like. The results suggested that PV3BKCVA21P1 3 and PV3BKCVA21P1 4 proteins were correct and that the processing pattern *in vitro* was also correct. Therefore the P1 of CVA21 was processed *in trans* by the 3CD of poliovirus.

VP1 of the PV3BKCVA21P1 1 recombinant migrated slower than that of other recombinants and control viruses (indicated by an asterisk in figure 3.15).

PV3BKCVA21P1 1 VP1 was aberrant in size and appeared to be approximately 35 kDa as opposed to native VP1, which is approximately 33 kDa. The 2A protease being correct, inefficient 2A-mediated cleavage event could not have caused the observed result. The 2-kDa difference in size would correspond to an 18 amino acid difference, or 54 nucleotides. This difference in size would not have been apparent during the RFLP analysis of the clone. Moreover, initial sequencing analysis of the constructed clones was restricted to the 5'UTR/VP4 and VP1/2A junctions. Complete sequencing of the P1-coding region was only carried out on the clones that appeared to express the correct protein-processing pattern but not on the PV3BKCVA21P1 1 clone. Either PCR error or aberrant processing at the VP3/VP1 junction could account for the observed result.

3.3.3.3 CVA21BKSabin 1P1 and CVA21BKPV3P1 recombinants

Comparison of the infected cell lysates with the HeLa S10 samples of CVA21, PV3 and Sabin 1 control viruses demonstrated similar processing patterns. The *in vivo* samples consistently migrated slightly faster than the *in vitro* samples. The *in vivo* samples are not shown in the CVA21BKSabin1P1 and CVA21BKPV3P1 SDS-PAGE gels due to poor gel quality.

The results of the CVA21BKSabin1P1 SDS-PAGE gel are illustrated in figure 3.16. VP3 and 2A were the two proteins used for the identification and analysis of the CVA21BKSabin1P1 recombinants *in vitro* processing. The gel shows that VP3 of the CVA21BKSabin1P1 recombinants migrated as Sabin 1 VP3 and 2A migrated as CVA21 2A (see figure 3.16). The presence of VP1, as opposed to the uncleaved VP1/2A product, was detected by DAKO anti-VP1 labelling western blot. VP1 was present in all the samples, apart from the mock, and was estimated to be the correct size (see figure 3.17). No other bands were present on the western blot, confirming the specificity of the antibody, the correct cleavage of the P1 capsid protein precursor from the non-structural proteins and efficient processing into VP1.

The CVA21BKPV3P1 c6 recombinant processing pattern was then analysed (figure 3.18). CVA21BKPV3P1 c6 appeared to express a PV3 VP3 migration pattern and a CVA21 2A migration pattern suggesting that the recombinant processed efficiently *in vitro*. It is important to note that the PV3 P1 chimera consistently labelled poorly, explaining the poor quality of the SDS-PAGE analytical gel.

However *in vitro* translation did not enable the study of the kinetics of the processing cascade and *in vivo* translation should be investigated. Pulse-chase labelling type assays

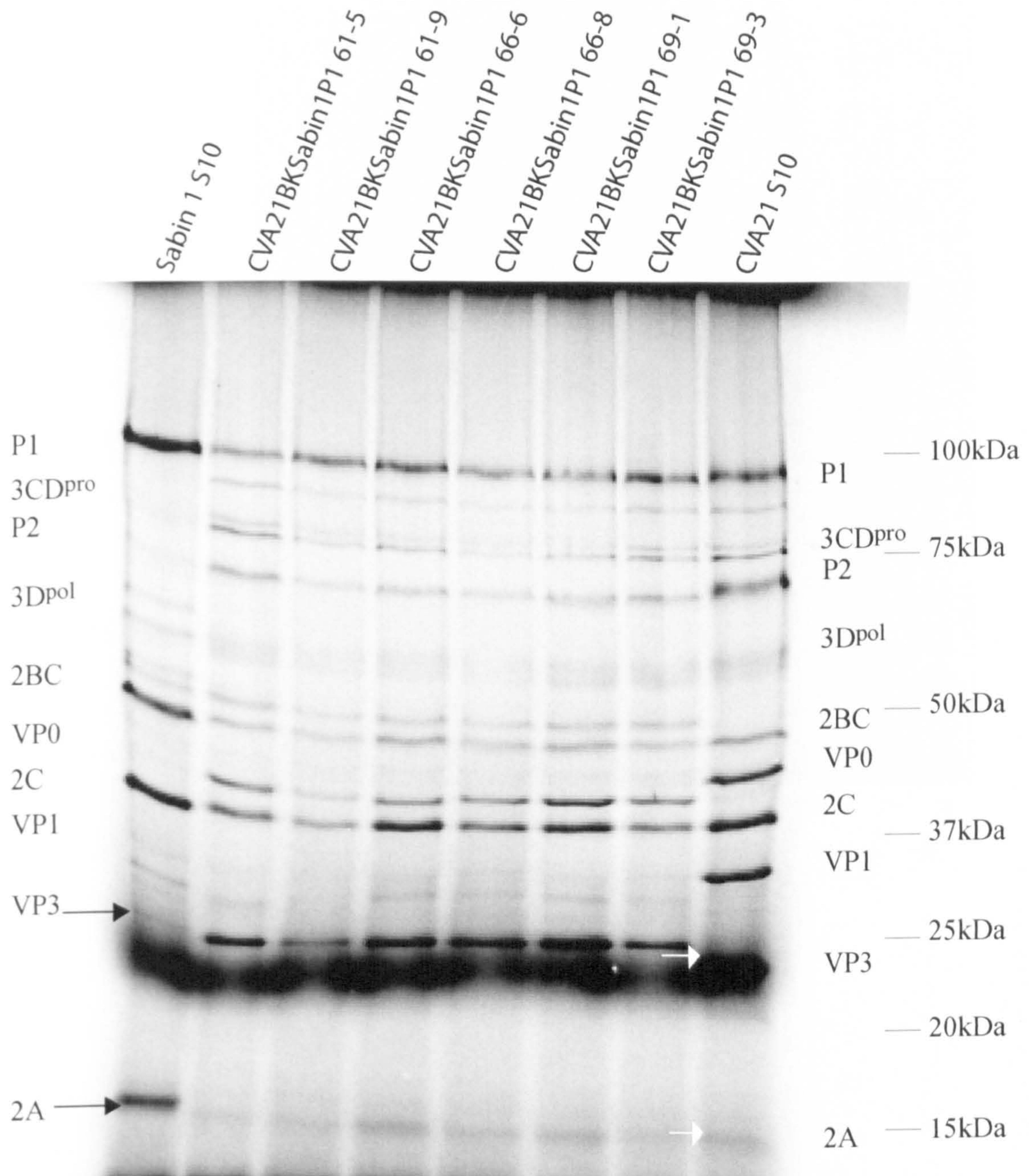


Figure 3.16: CVA21BKSabin1P1 recombinants in vitro translation reaction (using HeLaS10 extracts supplemented with rabbit flexi reticulocyte lysates).

The sample was run alongside the 2 control viruses. CVA21BKSabin1P1 61-5, 61-9, 66-6, 66-8, 69-1, 69-3 represent individual clones isolated from the cloning experiment. The arrows highlight the bands used for identification and characterisation.

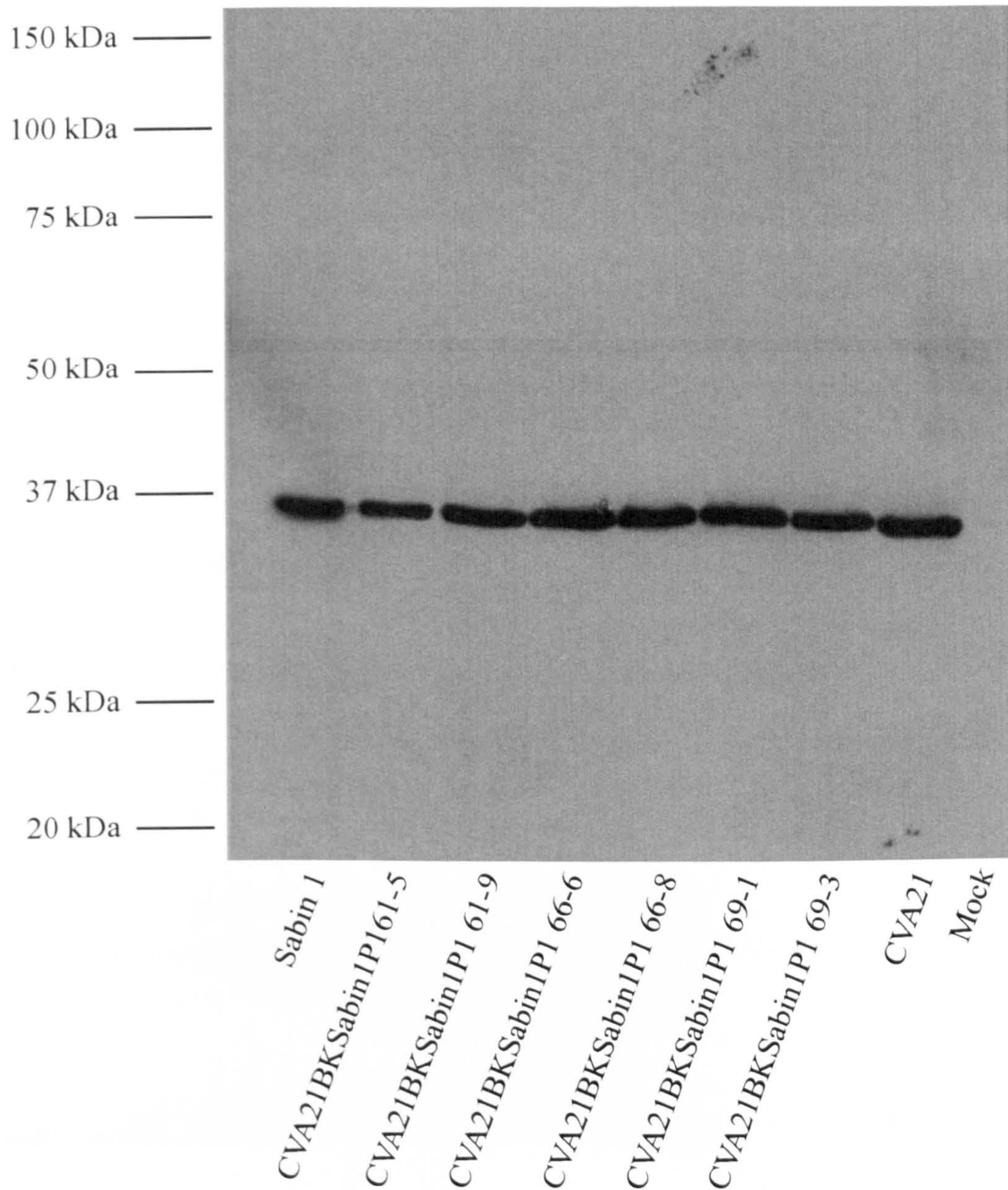


Figure 3.17: Western Blot probing for HEV VP1

VP1 western blotting using a HEV-specific DAKO anti-VP1 mouse monoclonal antibody (1:1000). Samples, generated during the *in vitro* S10 translation/processing assay, were run on an SDS-PAGE gel, transferred onto a membrane for protein analysis and probed for VP1. A secondary anti-mouse HRPO-conjugated secondary antibody was then used at a dilution of 1/5000 in PBS-A. The western blot was developed with ECL western blot developing reagents for a couple of minutes and exposed on an X-Ray film. The film was developed and the bands represent VP1 proteins.

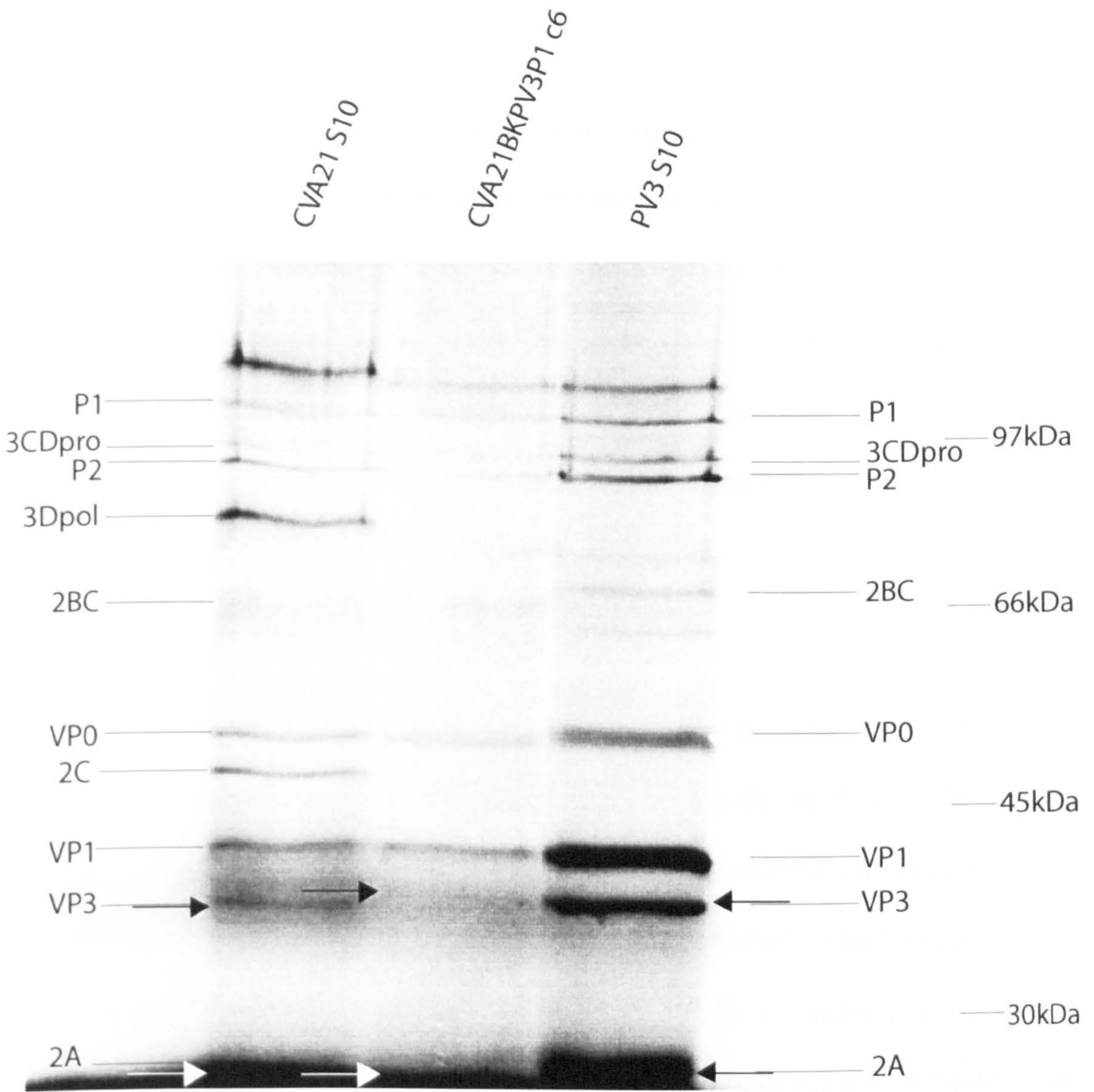


Figure 3.18: CVA21BKPV3P1 c6 recombinant in vitro translation reaction (using HeLaS10 extracts supplemented with rabbit flexi reticulocyte lysates).

The samples were run alongside the two control viruses. The arrows highlight the bands used for identification and characterisation of the recombinant processing pattern. Poor labelling of the CVA21BKPV3P1 c6 sample was consistently observed.

based on transfection would not be practical. It is therefore possible that the recombinants may have temporal defects in translation.

In vitro translation assays suggested that the correct proteins were produced and the processing pattern appeared to be authentic. The recombinant proteins were consistently processed, the P1 and the VP1 proteins released from the non-structural proteins and the proteins involved in replication expressed. The chimeras would therefore look like sub-genomic replicons and would be expected to replicate. Replication of the virus genomes was therefore analysed by dot blot hybridisation assays.

3.3.4 Replication assay

Replication studies were carried out as described in Chapter 6: Materials and methods. RNA samples, normalised to 1 µg, were transfected individually into RD-ICAM cells. The transfected cells were harvested at different time points post-transfection and viral RNA extraction was carried out. The RNA samples were then transferred and fixed onto a membrane and hybridised to a generic [³²P] probe. The probe was expected to bind the 3' polymerase-coding region of all viral RNAs used in the reaction and would therefore be expected to enable quantification of RNA production. The membrane was exposed to a phosphoimager screen. Figure 3.19 shows an example dot blot membrane. Radioactivity was then quantified and plotted onto graphs. The results are expressed in percentage fold-increase of viral RNA levels and are illustrated in figures 3.20 and 3.21 and summarised in table 3.22.

Samples	Replication phenotype
CVA21BKSabin1P1 61-5	+++
CVA21BKSabin1P1 61-9	+
CVA21BKPV3P1 c6	+++
PV3BKCVA21P1 1	+
PV3BKCVA21P1 3	+
pRiboCVA21	++/+++
pS1F	++
PV3	+++
PV3 + 4mM GuHCl	-
Cells alone / mock transfection	-

Table 3.22: Summary of replication phenotype determination assay of *in vitro* P1 recombinants. The replication phenotype was determined from graphs obtained, figure 3.20 and 3.21, based on [³²P] signal detection and quantification. Replication competent (+) and replication incompetent (-) samples are illustrated. Number of + signs indicate different levels of replication (see graphs 3.20 and 3.21).

Graphs summarising the results of the time course/dot blot assay are illustrated figures 3.20 and 3.21. The control samples behaved as expected. The control CVA21 and PV3 parental genomes exhibited an increased signal over time indicating that these were replicating.

PV3 transfected in the presence of 4mM GuHCl, an inhibitor of viral replication (Molla et al., 1993), did not replicate.

However, the Sabin 1 genome control sample, derived from the pS1F infectious clone, showed apparent lower replication levels than the PV3 and CVA21 controls. This was surprising considering the *in vitro* protein-processing pattern of Sabin 1 appeared to be correct. However, throughout the project the pS1F plasmid caused problems and exhibited

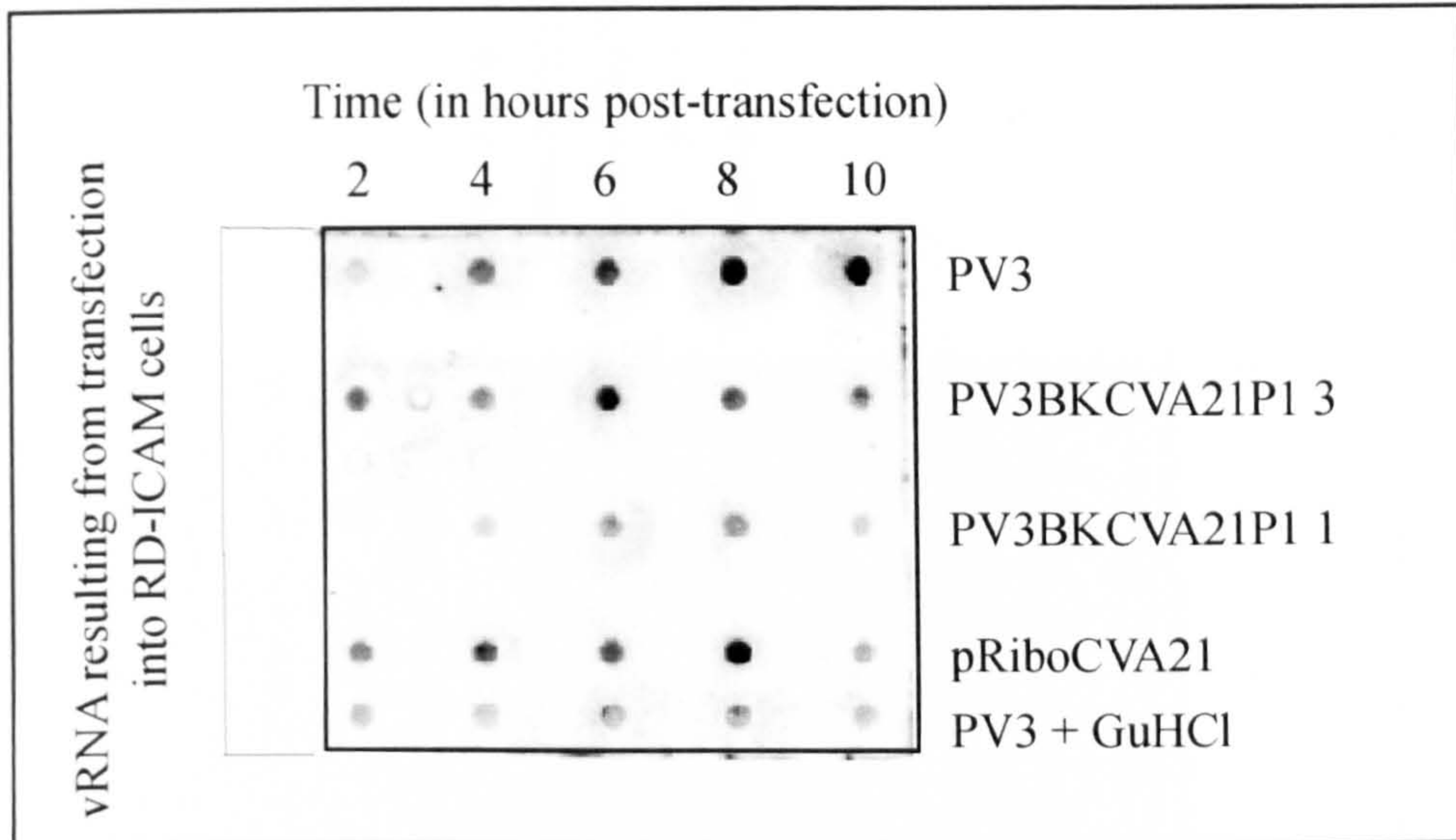


Figure 3.19: Sample dot blot.

This figure is an example dot blot similar to those used for the replication curves. Several dot blots were carried out. The primary data was then used for quantification and plotted onto graphs.

PV3BKCVA2IP1 recombinants replication assay (in RD-ICAM cells)

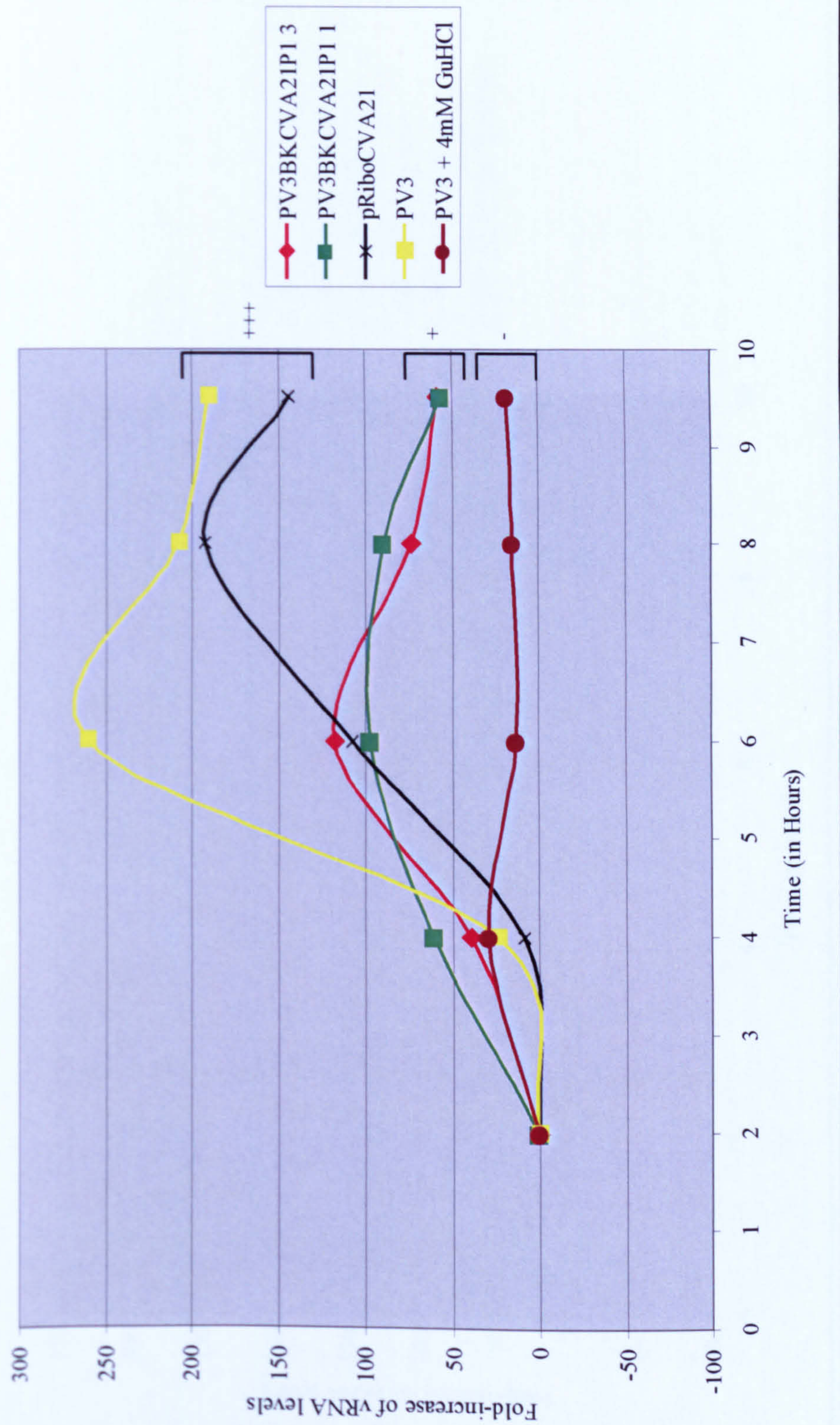


Figure 3.20: Replication assay by dot-blot probing analysis of the PV3BKCVA2IP1 1 and PV3BKCVA2IP1 3 recombinants.

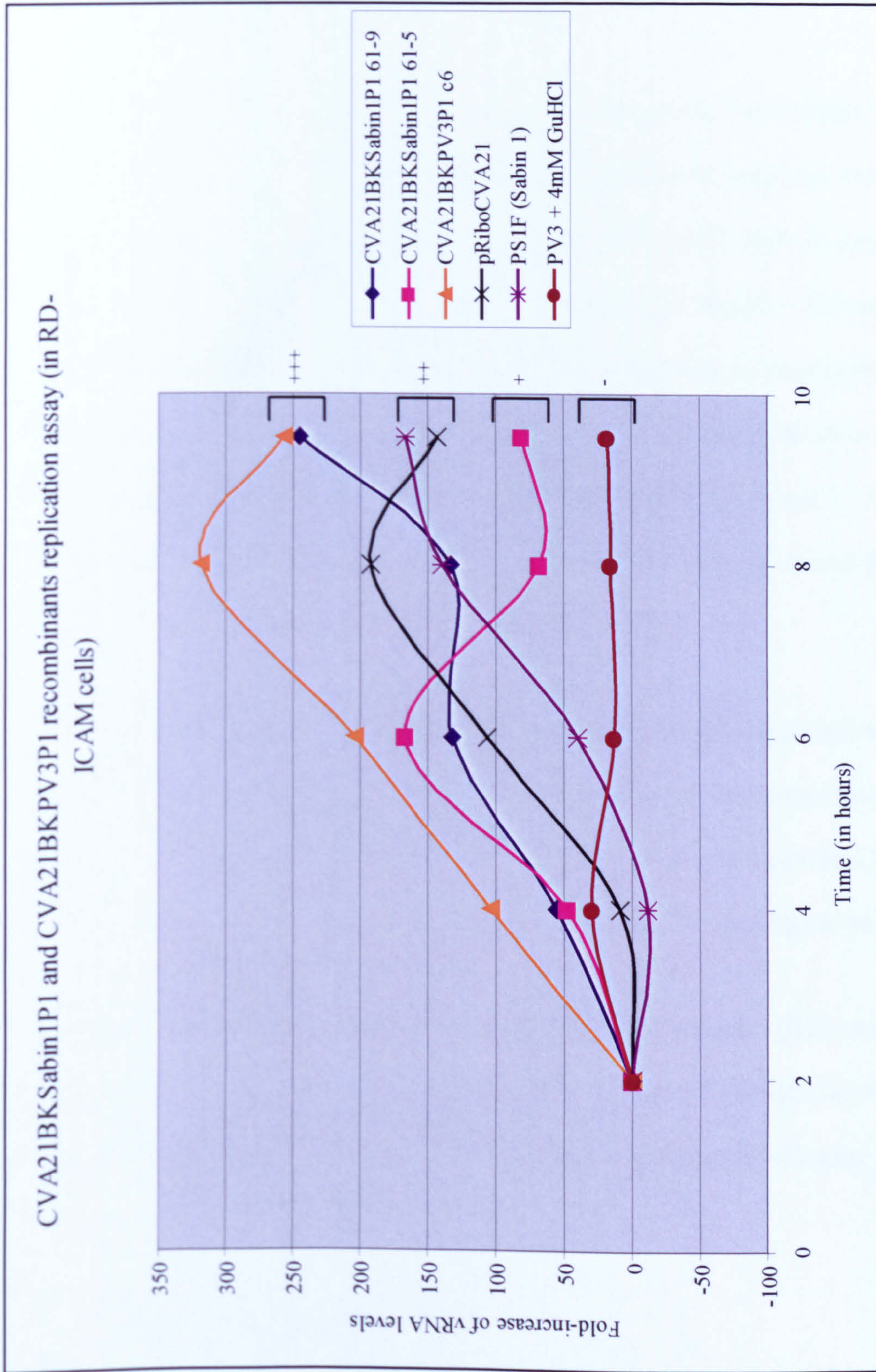


Figure 3.21: Replication assay by dot-blot probing analysis of the CVA21BKSabin1P1 61-5, CVA21BKSabin1P1 61-9 and CVA21BKPV3P1 c6 recombinants.

a few unexplained abnormalities, such as slow virus production and genome replication. It is not clear why this was the case.

All the recombinants exhibited different levels of replication. The PV3BKCVA21P1 1 and PV3BKCVA21P1 3 recombinants appeared to be replication-competent. However, levels of replication were not as high as those observed in the positive control samples (see figure 3.20). The CVA21BKPV3P1 c6 recombinant appeared to be highly efficient at replicating. The CVA21BKSabin1P1 61-9 recombinant replication seemed to be at least as efficient as the control virus replication. CVA21BKSabin1P1 61-5 was also replication-competent but to lower levels than those observed in the other test samples (see figure 3.21). These results confirmed the *in vitro* translation assay initial findings whereby the correct proteins had been synthesised and could catalyse the replication process.

Under the conditions used, the levels of replication at different time points were reproducible. Each individual clone analysed is characteristic and consistent with previous assays. The assay needs to be repeated for the data to be statistically relevant. Repeating the assay in L cells may enable cleaner and more statistically relevant data to be generated.

The defect, which prevented the chimeras from causing cytopathic effect must therefore be due to a late event such as maturation, assembly, packaging or infection of new cells.

Because of the availability of relevant expertise and reagents, the packaging phenotype of the recombinants was analysed.

3.3.5 Trans-encapsidation assays

The trans-encapsidation assays were conducted to determine whether the P1 recombinant viral genomes had a packaging defect.

3.3.5.1 Trans-encapsidation experiment

3.3.5.1.1 Method

The trans-encapsidation assay was conducted to monitor whether the T7 RNA transcripts of the P1 recombinants and progeny RNA, synthesised after transfection of the transcripts into RD-ICAM cells, could be encapsidated by CVA21 or PV3 helper viruses (protocol described in figure 3.23). Both CVA21 and PV3 are infectious and generate viral capsid proteins that assemble and package progeny genomes leading to the production of mature virions. Chimeric genomes being packaged into the helper virus empty capsids would demonstrate that the recombinants are competent for packaging.

The recombinants are effectively replicon systems, as they are able to translate, process and replicate. However, these lack a reporter gene or other readily assayable marker and encapsidation was therefore screened for by PCR. An RFLP type analysis was then carried out to differentiate between chimeric genomes packaged into helper virus capsids and helper viruses, taking advantage of the differences at the 5'UTR/P1-coding regions.

T7 RNA transcripts derived from the recombinants and controls were transfected into RD-ICAM cells. Six hours post-transfection the samples were infected with the helper viruses and incubated for 8-10 hours. Viral RNA was extracted from the various samples and reverse transcribed to generate cDNA. A 900-1000 bp window of the cDNA 5'UTR/P1-coding regions were amplified by PCR, using generic oligonucleotide primers (CVA21-CAPF and ENT-GEN-2R), digested with *Bam* HI endonuclease and run on a 2% agarose gel for analysis. Predicted RFLP patterns were generated using Vector NTI and are illustrated in figures 3.24 and 3.25. The ability of Vector NTI to predict priming of oligonucleotide to sequences does not take into consideration factors such as PCR

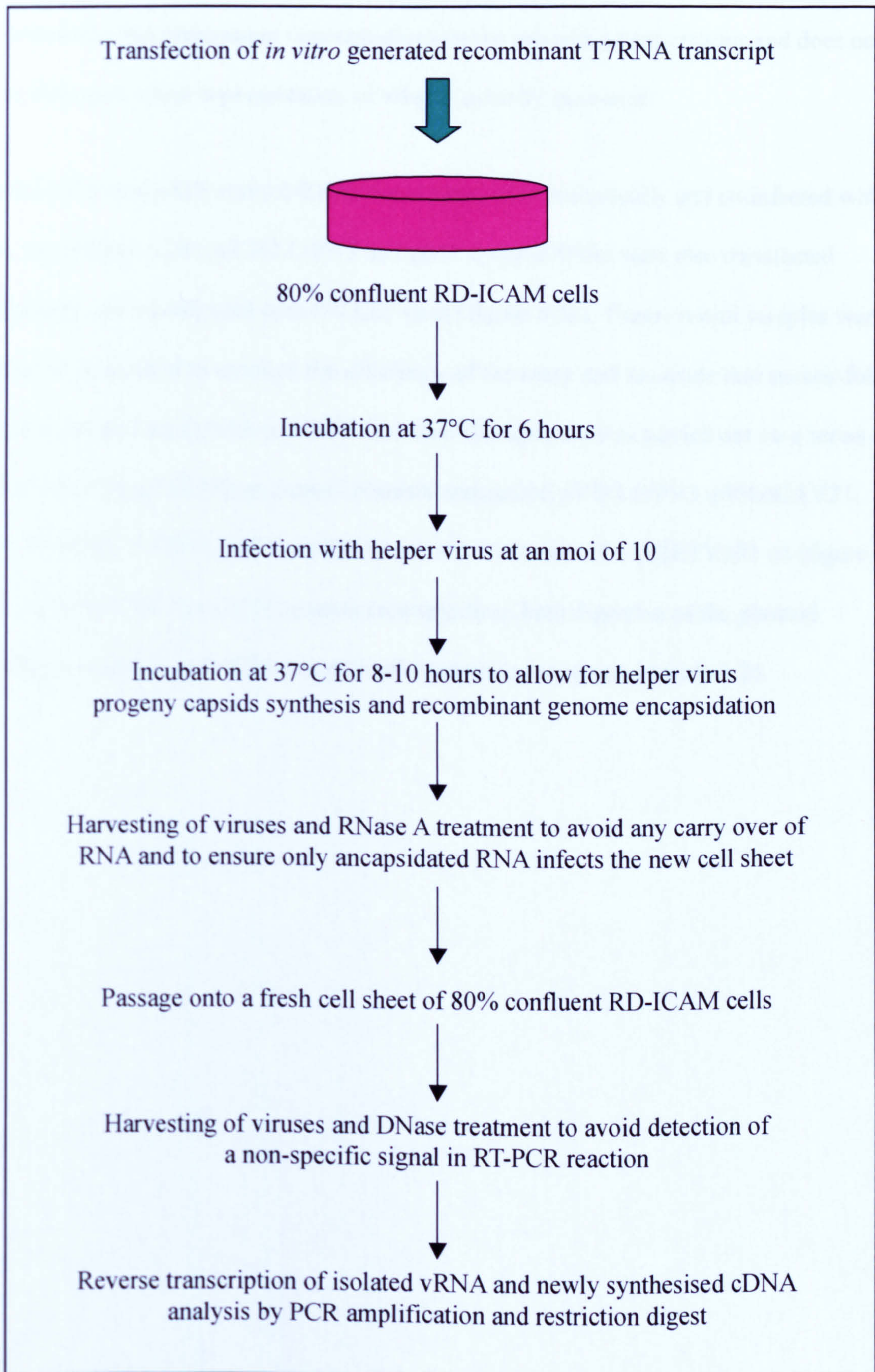


Figure 3.23: Flow chart summarising the method and analysis of the trans-encapsulation of recombinant genomes by helper virus assay.

conditions like the magnesium concentrations or the annealing temperatures and does not necessarily give a true representation of what is actually generated.

pRiboCAV21 and pS1F control RNAs were transfected individually and co-infected with PV3 virus (figure 3.28). pT7FLC/PV3 and pS1F control RNAs were also transfected individually and co-infected with CVA21 virus (figure 3.28). These control samples were carried out in parallel to monitor the efficiency of the assay and to ensure that successful heterologous packaging had occurred. The *Bam* HI digestion was carried out on a series of PCR products amplified from control plasmid sequences: pT7FLC/PV3, pRiboCAV21, pS1F, PV3BKCVA21P1 3, CVA21BKSabin 1P1 61-9 and CVA21BKPV3P1 c6 (figure 3.27). The expected sizes of fragments resulting from both digestion of the plasmid amplified sequences and of the encapsidation samples are shown in table 3.26.

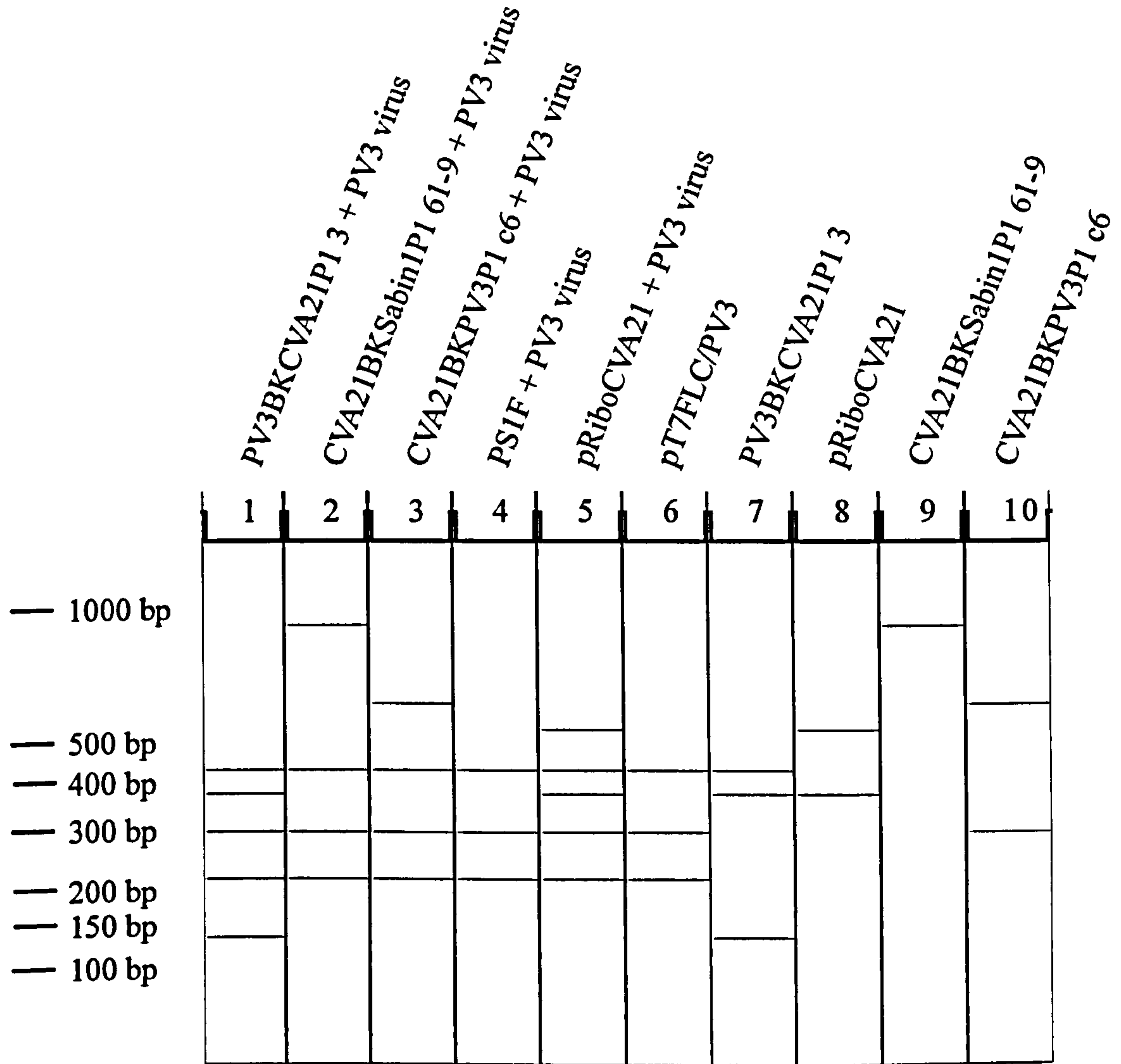


Figure 3.24: Predicted RT-PCR-*Bam* HI RFLP analysis of the trans-encapsidation experiment with PV3 helper virus.

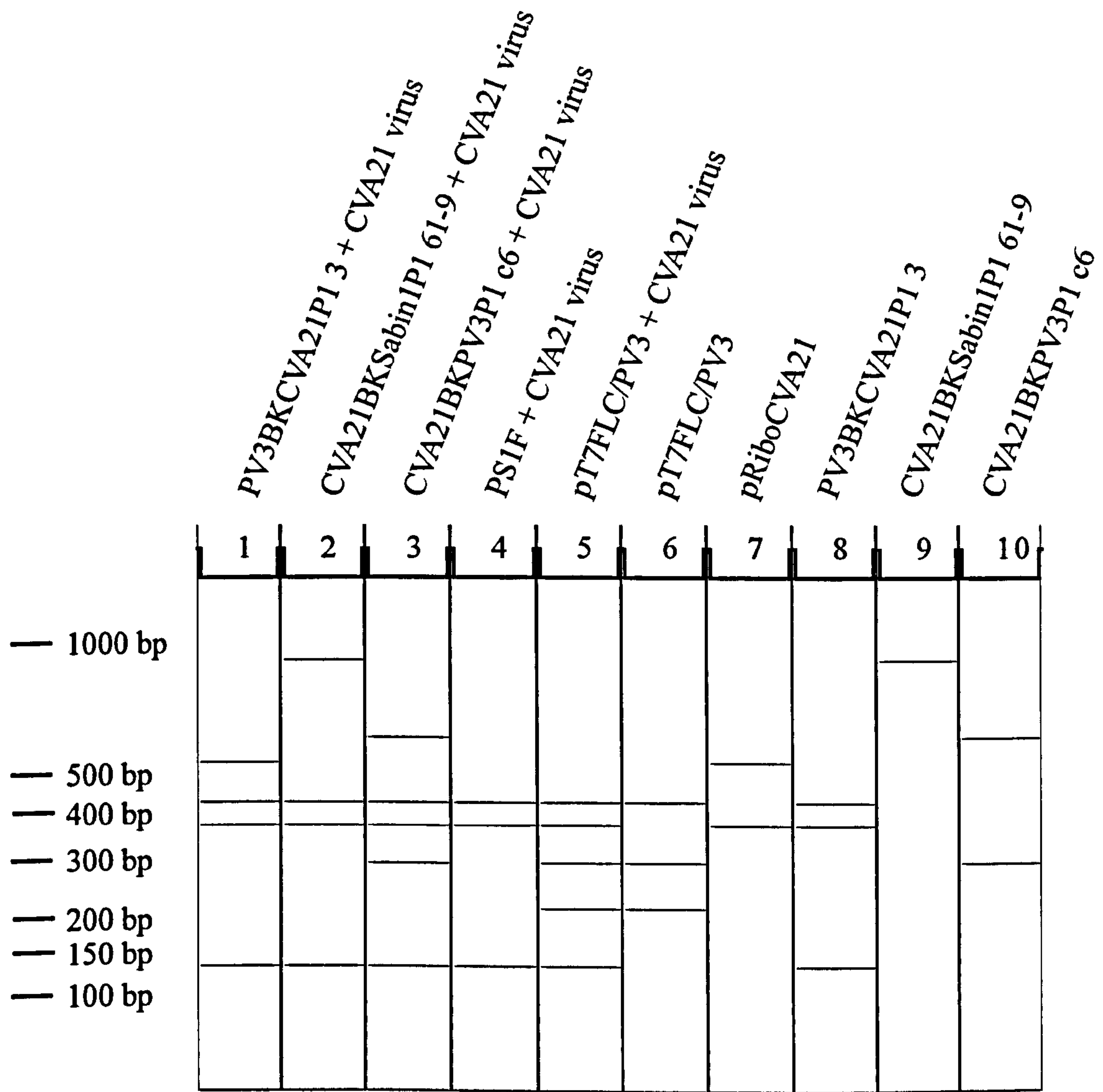


Figure 3.25: Predicted RT-PCR-*Bam* HI RFLP analysis of the trans-encapsidation experiment with CVA21 helper virus.

Trans-encapsidation samples : RNA + virus	<i>Bam</i> HI digests (in kb)
PV3BKCVA21P1 3 + PV3 virus	0.433, 0.430, 0.393, 0.313, 0.213, 0.133
CVA21Sabin1P1 61-9 + PV3 virus	0.9, 0.433, 0.313, 0.213
CVA21BKPV3P1 c6 + PV3 virus	0.615, 0.433, 0.344, 0.313, 0.213
pS1F + PV3 virus	0.9, 0.433, 0.313, 0.213
pRiboCVA21 + PV3 virus	0.535, 0.454, 0.433, 0.313, 0.213
PV3BKCVA21P1 3 + CVA21 virus	0.535, 0.454, 0.430, 0.393, 0.133
CVA21Sabin1P1 61-9 + CVA21 virus	0.9, 0.535, 0.454
CVA21BKPV3P1 c6 + CVA21 virus	0.615, 0.535, 0.454, 0.344
pS1F + CVA21 virus	0.9, 0.535, 0.454
pT7FLC/PV3 + CVA21 virus	0.535, 0.454, 0.433, 0.313, 0.213
pT7FLC/PV3	0.433, 0.313, 0.213
pRiboCVA21	0.535, 0.454
pS1F	No fragment generated, CAV21-CAPF does not anneal to pS1F.
PV3BKCVA21P1 3	0.430, 0.393, 0.133
CVA21BKSabin1P1 61-9	0.9
CVA21BKPV3P1 c6	0.615, 0.344

Table 3.26: RFLP analysis of alternative trans-encapsidation assay sample testing for packaging capacity of *in vitro* generated P1 recombinant DNAs. Fragments expected from the *Bam* HI restriction digests.

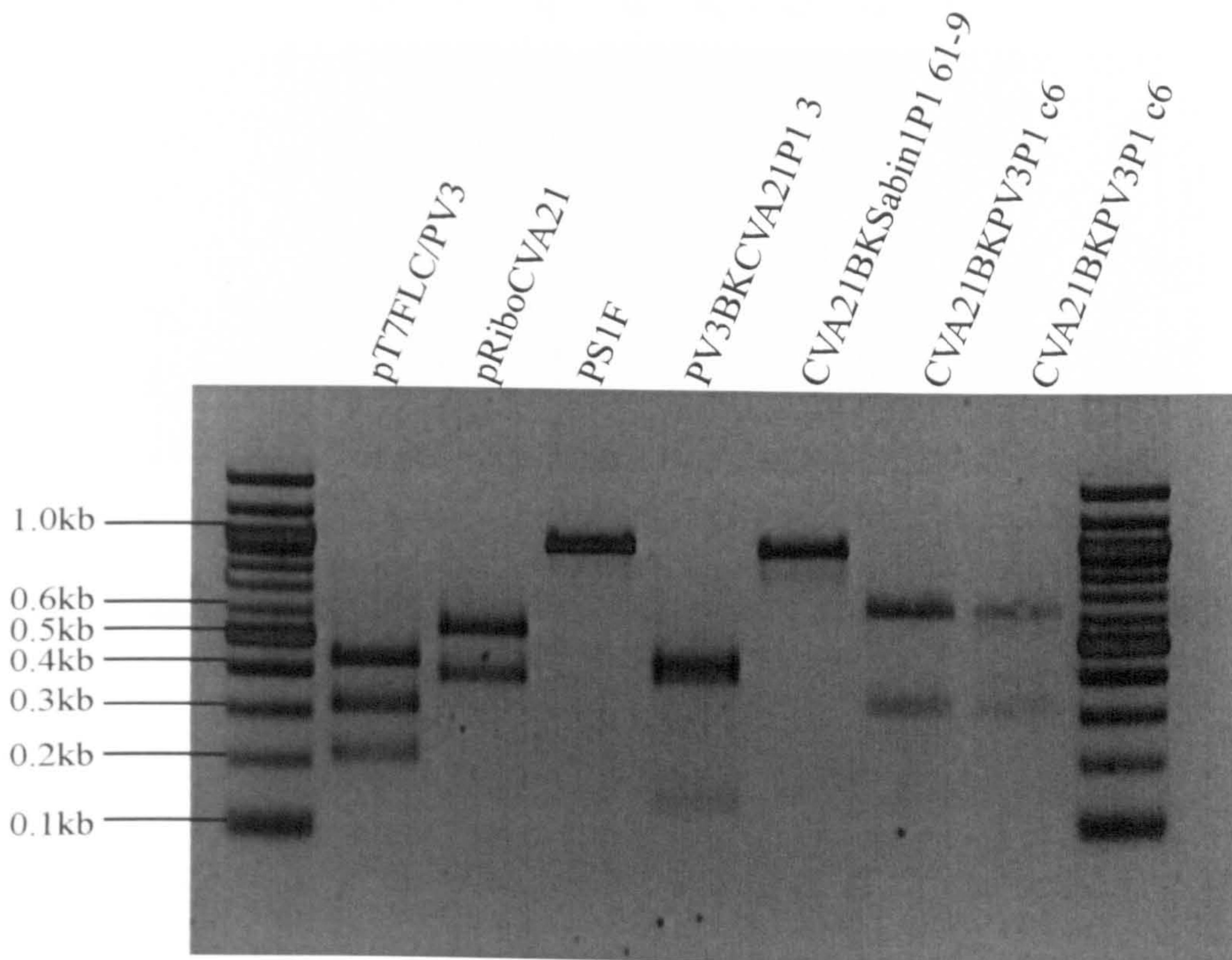


Figure 3.27: Analysis of control plasmids by PCR and *Bam* HI RFLP analysis.

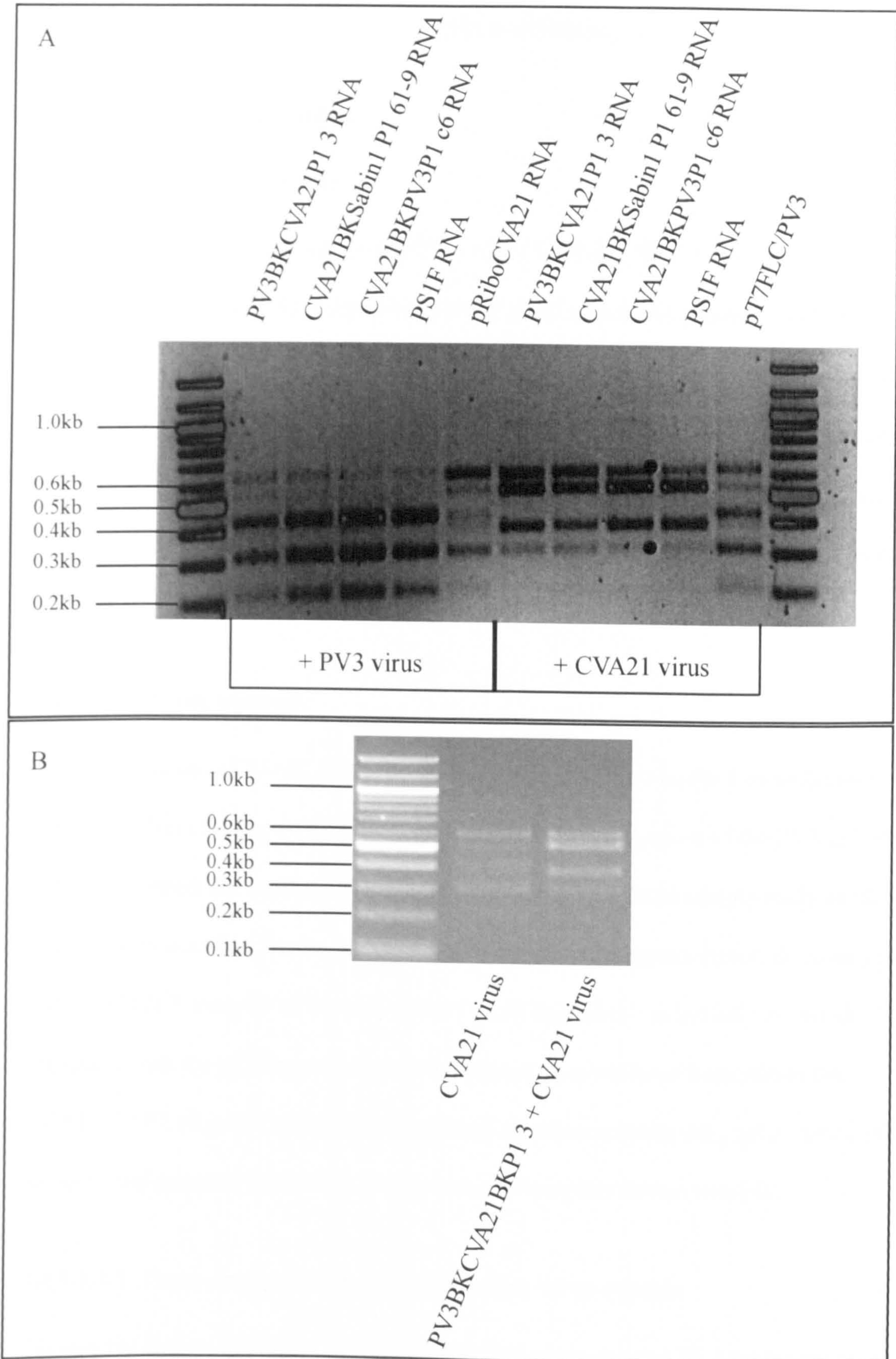


Figure 3.28: Analysis of trans-encapsidation assay by PCR and *Bam* HI RFLP analysis.

The dots in figure A indicate artefacts seen in the vRNA/RT-PCR/digests of CVA21 virus shown in figure B. These were therefore not taken into consideration during the analysis of results.

3.3.5.1.2 Results and analysis

3.3.5.1.2.1 Plasmid controls

PCR and *Bam* HI RFLP analysis of pT7FLC/PV3, pRiboCVA21, pS1F, PV3BKCVA21P1 3, CVA21BKSabin1P1 61-9 and CVA21BKPV3P1 c6 control plasmid DNAs are illustrated figure 3.27. The fragment sizes obtained were as expected and were used as controls for sample analysis. The generation of an unexpected ~0.9 kb PCR product resulting from amplification of pS1F was surprising. The observed result could have been due to the CVA21-CAPF oligonucleotide predicted to bind pS1F 0.927 bp upstream of the ENT-GEN-2R pS1F-binding site with 45% homology.

3.3.5.1.2.2 Virus controls

Control infections of RD-ICAM cells with CVA21, PV3 and Sabin 1 virus samples were also undertaken (see figure 3.28.B). The PCR/RFLP investigation of the PV3 infected sample generated the expected fragments. The CVA21 infected sample study exhibited two additional unidentified bands, not detected in the plasmid digests (result discussed later). The PCR/RFLP analysis of Sabin 1 did not yield fragments, as initially expected. This conflicted with the pS1F plasmid control analysis. It could have been due to the CVA21-CAPF oligonucleotide having primed a vector-specific site, not present in the Sabin 1 viral genome. The Sabin 1 data were not used for further analysis.

3.3.5.1.2.3 Trans-encapsidation by PV3 helper virus capsids

The control sample of pRiboCAV21 RNA + PV3 virus showed PV3 fragments as well as CVA21 fragments. It is important to emphasize that the detection of both CVA21 and PV3 bands could have been due to both PV3 helper virus and CVA21 virus production from pRiboCVA21 RNA transfection. However, the presence of both CVA21 and PV3 bands

could indicate that pRiboCAV21 derived genomic RNA had been encapsidated in PV3 capsids.

The screening method used in this assay did not enable the detection of Sabin 1-specific bands, as one of the oligonucleotide primers used for amplification was unable to anneal to the Sabin 1 genome. The pS1F RNA + PV3 virus and pS1F RNA + CVA21 virus samples were therefore not used for the analysis.

The test samples co-infected with PV3 virus presented a PV3-like RFLP pattern. No other bands were observed suggesting that no recombinant genomes could be detected.

3.3.5.1.2.4 Trans-encapsidation by CVA21 helper virus capsids

As expected, the control sample of pT7FLC/PV3 RNA + CVA21 virus exhibited PV3-like bands as well as CVA21 virus-like bands. This result suggested that either the PV3-like bands were derived from PV3 virus resulting from the transfection of pT7FLC/PV3 RNA or that a heterologous packaging event had occurred. The two possibilities could not be distinguished.

Two unidentified bands, absent from the *Bam* HI digested pRiboCAV21-derived PCR product, were present in all the samples. The bands were observed in the *Bam* HI digestion of the CVA21 virus alone control and were thought to have been artefacts resulting from the vRNA extraction and/or reverse transcription reactions. These were excluded from the characterisation studies. All the samples co-infected with CVA21 virus, only had a CVA21-like RFLP pattern.

The trans-encapsidation assay results indicated that no packaged recombinant genomes could be detected in the reactions. However, it is not possible to differentiate between lack of encapsidation and poor sensitivity or screening of the experiment. The former would not

be surprising; as previous studies have demonstrated that poliovirus subgenomic replicons could be encapsidated by poliovirus capsids and to some levels by CVA21 capsids but no conclusive evidence was given for the encapsidation of a poliovirus subgenomic replicon by CB4 or HRV14 heterologous capsids (Barclay et al., 1998). Contradictory studies reporting that successful encapsidation of a poliovirus subgenomic replicon by CVB4 and HRV14 heterologous capsid proteins had occurred (Ansardi et al., 1996). The *in vitro* generated recombinants could lack a specific functional packaging signal. The design and use of highly specific primers, amplifying the 5'UTR/P1 region, may enable a more sensitive analysis of the recombinant genome trans-encapsidation experiment.

Can capsid proteins, generated from transcription/translation of the recombinant RNA genomes, assemble and encapsidate a known packageable genome? To investigate this, a reciprocal trans-encapsidation experiment was carried out.

3.3.5.2 Luciferase encapsidation by recombinant capsids

3.3.5.2.1 Method

To detect whether the P1 recombinant genomes were capable of providing capsids *in trans* a luciferase-encoding subgenomic replicon was provided in the same cells (see figure 3.29). To confirm the packaging assay behaved correctly, a poliovirus type 3 packageable luciferase subgenomic replicon (pT7REP3-L) was co-infected with PV3, CVA21 and Sabin 1 control viruses into RD-ICAM cells (figure 3.30).

Co-transfection of pT7REP3-L RNA with chimeric RNA was then carried out. The co-transfected RD-ICAM cells were incubated at 37°C overnight to allow generation of progeny virus. The samples were harvested, RNase treated, passaged onto a fresh RD-ICAM cell sheet and incubated for 6-8 hours at 37°C to allow expression of the

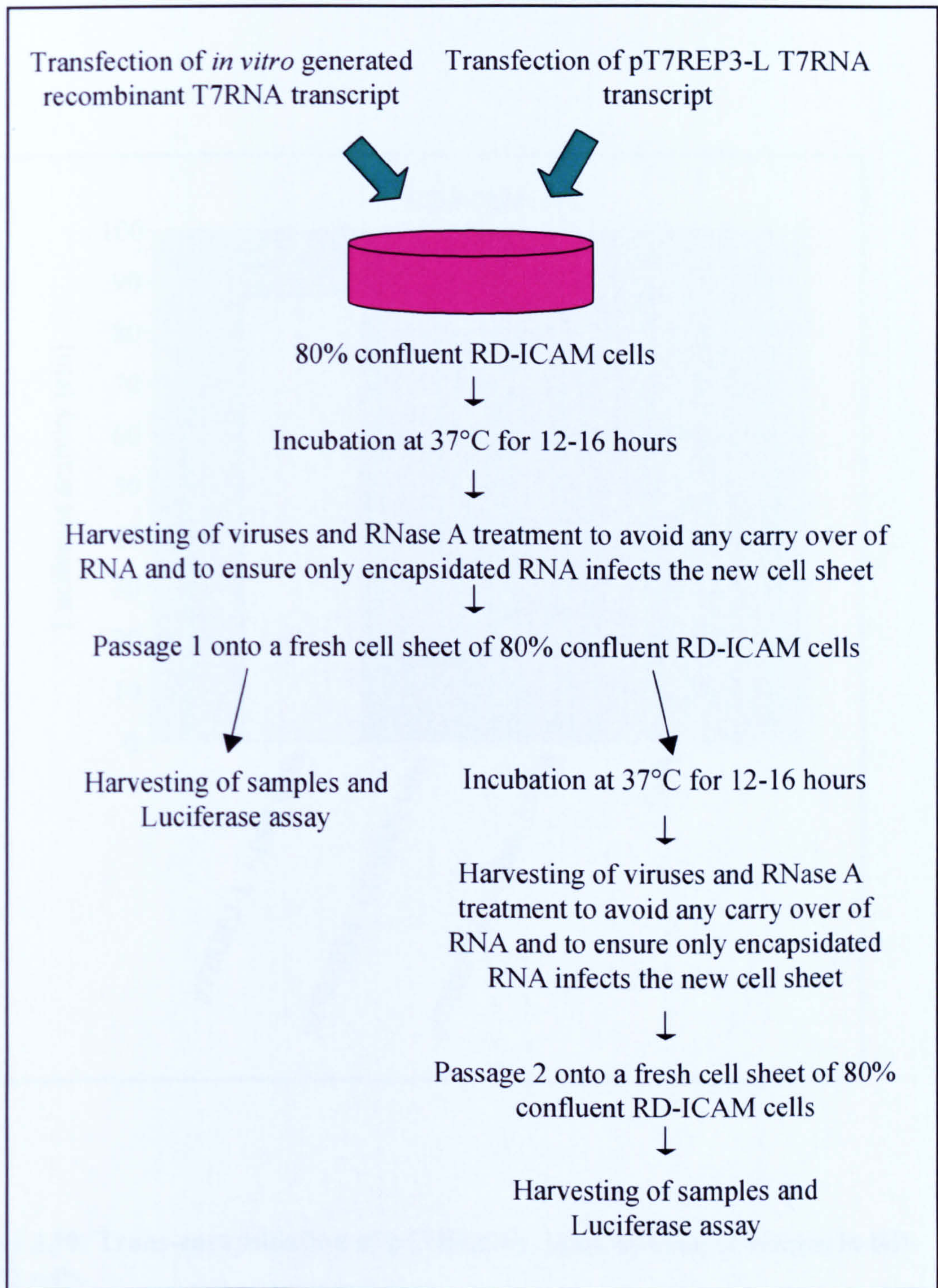


Figure 3.29: Flow chart summarising the method and analysis of the trans-encapsulation assay by co-transfection

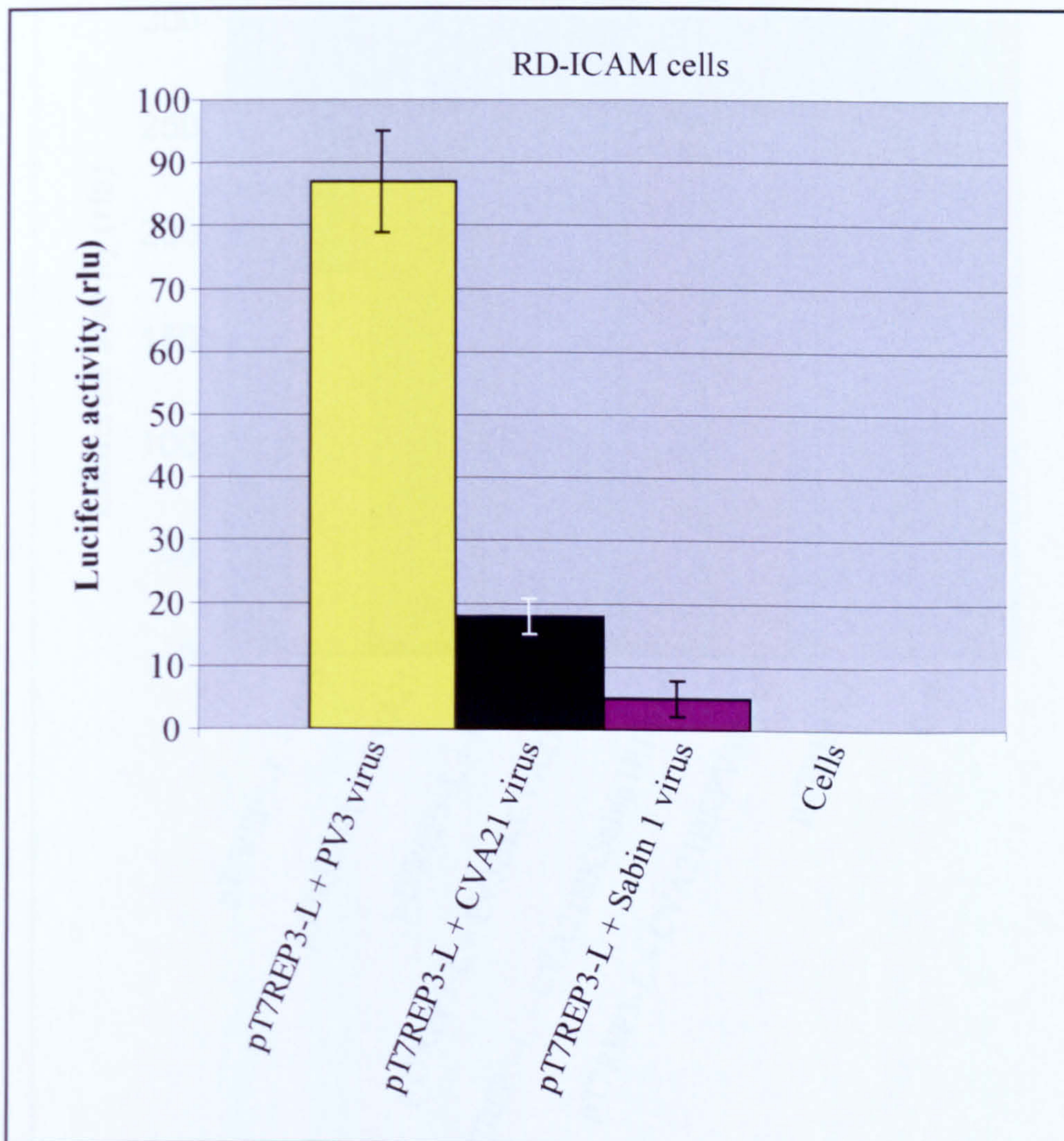


Figure 3.30: Trans-encapsidation of pT7REP3-L RNA by control viruses in RD-ICAM cells.

The assay was carried out five times, standard deviation values were calculated and are illustrated as error bars in the graph.

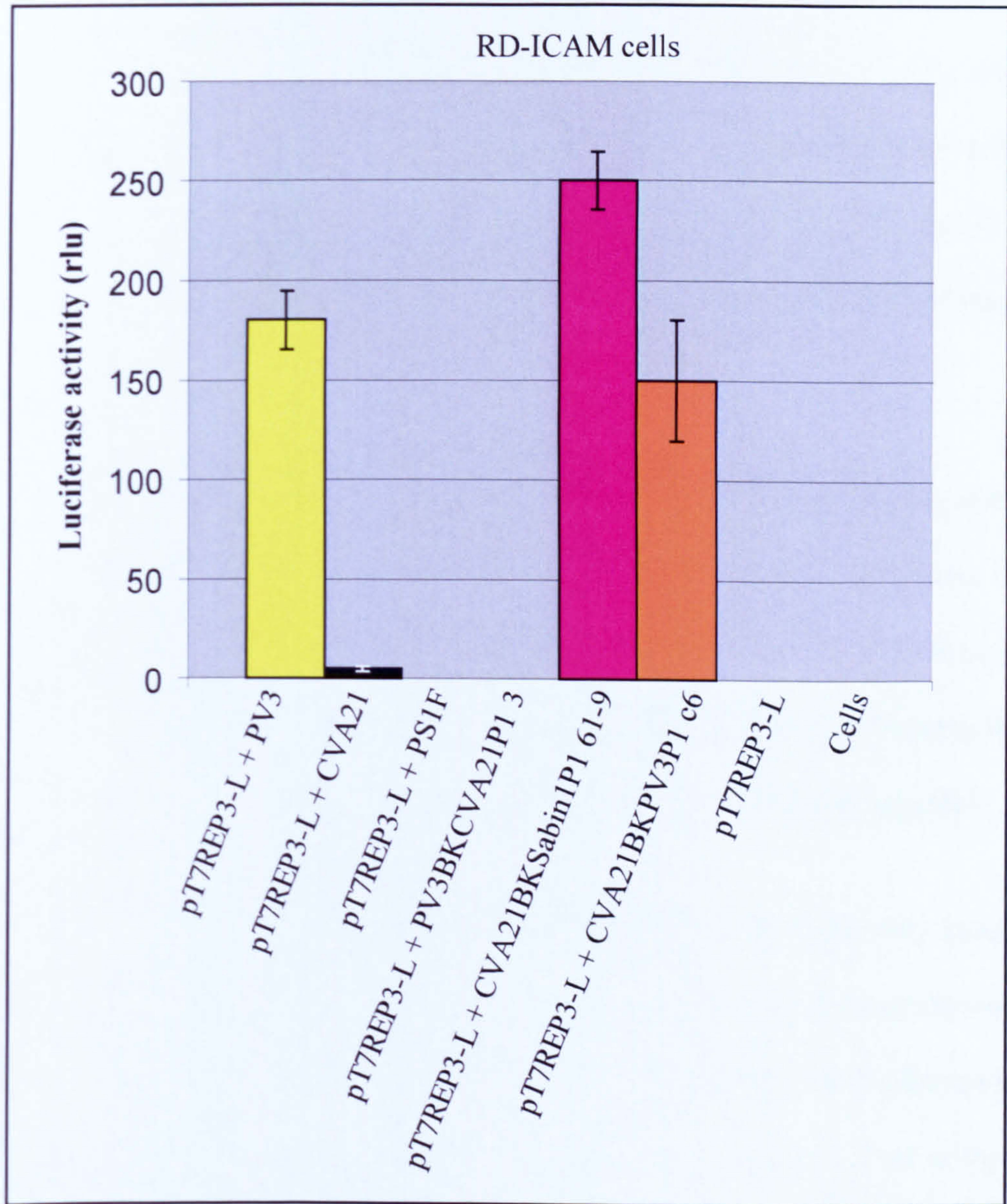


Figure 3.31: Trans-encapsidation of pT7REP3-L RNA by recombinants: first passage into RD-ICAM cells post-co-transfection of T7 RNA transcripts.

The assay was carried out five times, standard deviation values were calculated and are illustrated as error bars in the graph.

luciferase gene. A luciferase assay was carried out and results were plotted as histograms (figure 3.31). Viable capsid proteins cannot be produced as a result of transfection with the poliovirus type-3 luciferase subgenomic replicon, as most of the P1-coding region is substituted for the luciferase reporter gene. The recombinant genomes are unable to produce progeny viruses. Any transfer of the luciferase signal onto a fresh cell sheet, after RNase treatment, would be expected to be due to a luciferase genome packaged into capsids derived from the chimeras.

The assay was also conducted on L20B cells (figure 3.33) and L cells (data not shown), cell lines, which could only be used to test PV3- containing capsid sequences. The PV3BKCVA21P1 3 + pT7REP3-L sample would therefore not be expected to yield a luciferase signal in L20B cells and was used as a negative control monitoring for the detection of non-specific luciferase activity. These were first passage samples.

Samples passaged for the first time onto a fresh RD-ICAM cell sheet were incubated for 12-16 hours at 37°C, harvested, RNase treated and passaged for the second time onto a third RD-ICAM cell sheet. Should a luciferase signal be detected in the first passage of the test recombinant samples, transfer onto a third cell sheet should not occur as the recombinants are unable to make viable viruses that would enable the signal transfer. The assay was repeated five times.

3.3.5.2.2 Results and analysis

3.3.5.2.2.1 Control samples of transfected pT7REP3-L RNA co-infected with helper virus

As expected, the control sample co-infected with PV3 virus exhibited a significant luciferase activity. The control sample co-infected with CVA21 virus exhibited a detectable but low luciferase signal indicating poor encapsidation of the poliovirus replicon

by heterologous capsids. The sample co-infected with Sabin 1 virus exhibited very low luciferase activity indicating poor if not non-existent trans-encapsidation. The consistent abnormal pS1F and Sabin 1 virus behaviour could not be explained.

The control assay demonstrated efficiency of the method and was applied to the recombinant samples.

3.3.5.2.2.2 Co-transfection of pT7REP3-L RNA with Sabin 1, CVA21 and PV3 control RNAs

Genomic RNA derived from PV3, CVA21 and pS1F co-transfected with pT7REP3-L RNA exhibited the expected luciferase activities in all the cell lines tested and confirmed the success of the trans-encapsidation assay. As expected, no luciferase signal was detected in any cell line tested after passage of the transfected pT7REP3-L alone sample and mock transfections.

3.3.5.2.2.3 Co-transfection of pT7REP3-L RNA with PV3BKCVA21P1 3 RNA

No luciferase signal was detected in the co-transfection of luciferase type 3-replicon RNA with PV3BKCVA21P1 3 RNA (pT7REP3-L + PV3BKCVA21P1 3) samples in RD-ICAM cells (see figure 3.31). This could be due to inability of PV3BKCVA21P1 3 capsid proteins (CVA21-like) to package the luciferase genome. In fact previous reports showed that CVA21 capsids appeared to reproducibly package poliovirus subgenomic replicons at very low levels (Barclay et al., 1998).

3.3.5.2.2.4 Co-transfection of pT7REP3-L RNA with CVA21BKSabin1P1 61-9 RNA

Significant luciferase signals were detected in RD-ICAM cells, from the co-transfected luciferase type 3-replicon RNA and CVA21BKSabin1P1 61-9 RNA (pT7REP3-L + CVA21BKSabin1P1 61-9). The CVA21BKSabin1P1 61-9 recombinant

should synthesise Sabin 1-like capsids. The luciferase signal was also detected in L20B cells (figure 3.33), but not L cells (data not shown), implying that virus entry was PVR-dependent. Only very low amounts of viral RNA could be extracted and reverse transcribed. PCR amplification of a range of windows of the 5'UTR/P1, P1/P2 and 3D-coding regions, RFLP and sequencing analysis of the samples were inconclusive. When the samples were passaged for the second time onto a fresh RD-ICAM cell sheet, a very low luciferase signal, close to background levels and considered as negative, was detected in the pT7REP3-L + CVA21BKSabin1P1 61-9 sample (figure 3.32). These results suggested that CVA21BKSabin1P1 61-9 was capable of encapsidating the luciferase type 3-replicon genome.

3.3.5.2.2.5 Co-transfection of pT7REP3-L RNA with CVA21BKPV3P1 c6 RNA

The samples co-transfected with T7 RNA transcripts of the luciferase type 3-replicon and CVA21BKPV3P1 c6 exhibited a high luciferase signal in both RD-ICAM cells and L20B cells (figures 3.31 and 3.33) but not L cells (data not shown), again implying a PVR-dependent signal. The CVA21BKPV3P1 c6 recombinant seemed to be able to generate capsid proteins that were then able to assemble and encapsidate the luciferase type 3-replicon progeny RNA. The output luciferase signal of the CVA21BKPV3P1 c6 + pT7REP3-L sample passaged onto a second cell sheet was strikingly high (figure 3.32). This was confirmed by repeating the entire trans-encapsidation assay several times.

A possible contamination event with an infectious virus could explain this result. However, a contamination with poliovirus seemed unlikely as all the test samples were put in an incubator prior to handling the control samples, samples that would generated a positive signal. Moreover, ART-filter tips were used throughout the experiments to avoid aerosol contamination of the pipettes.

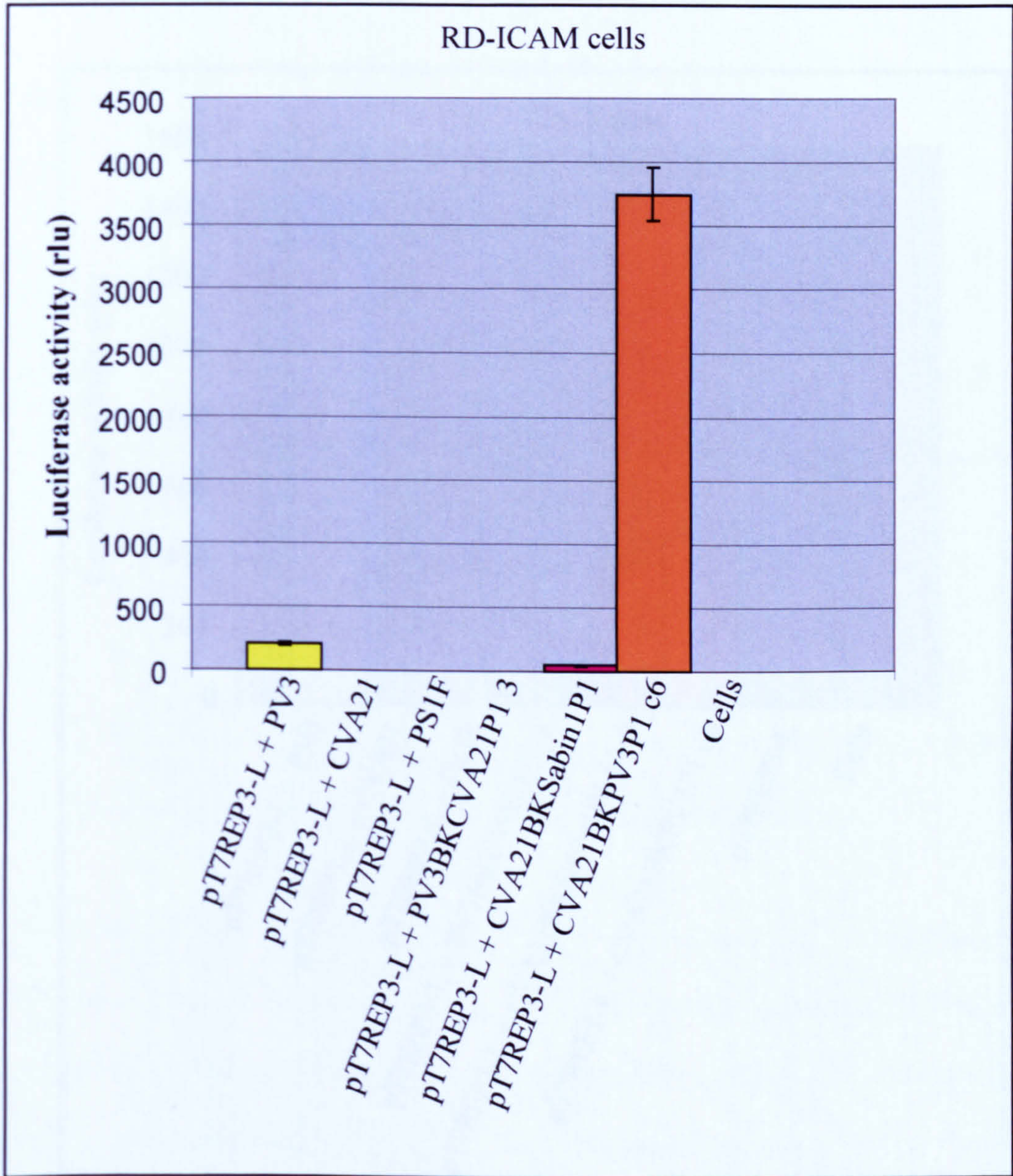


Figure 3.32: Trans-encapsidation of pT7REP3-L RNA by recombinants: second passage into RD-ICAM cells post-co-transfection of T7 RNA transcripts.

The assay was carried out five times, standard deviation values were calculated and are illustrated as error bars in the graph.

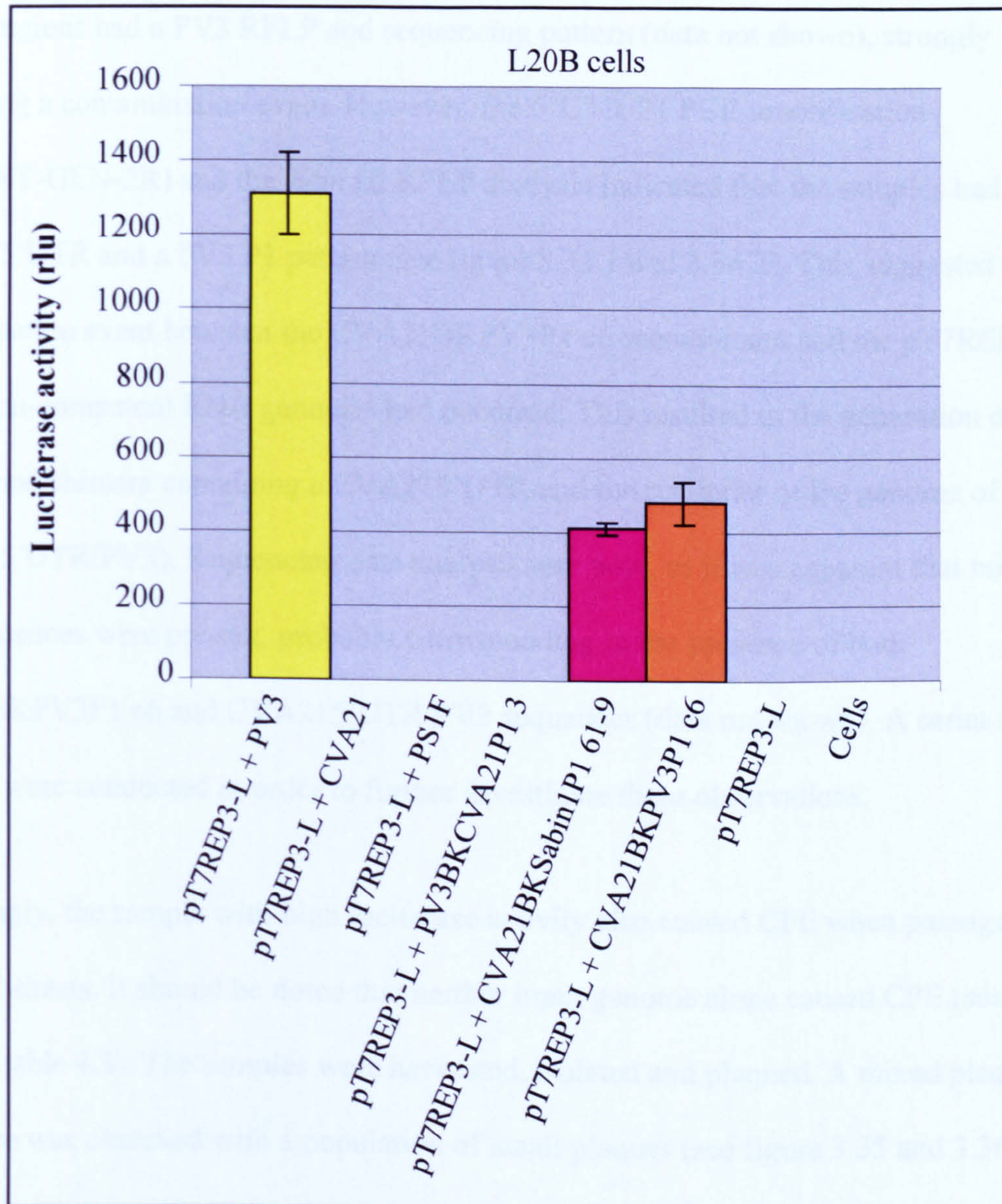


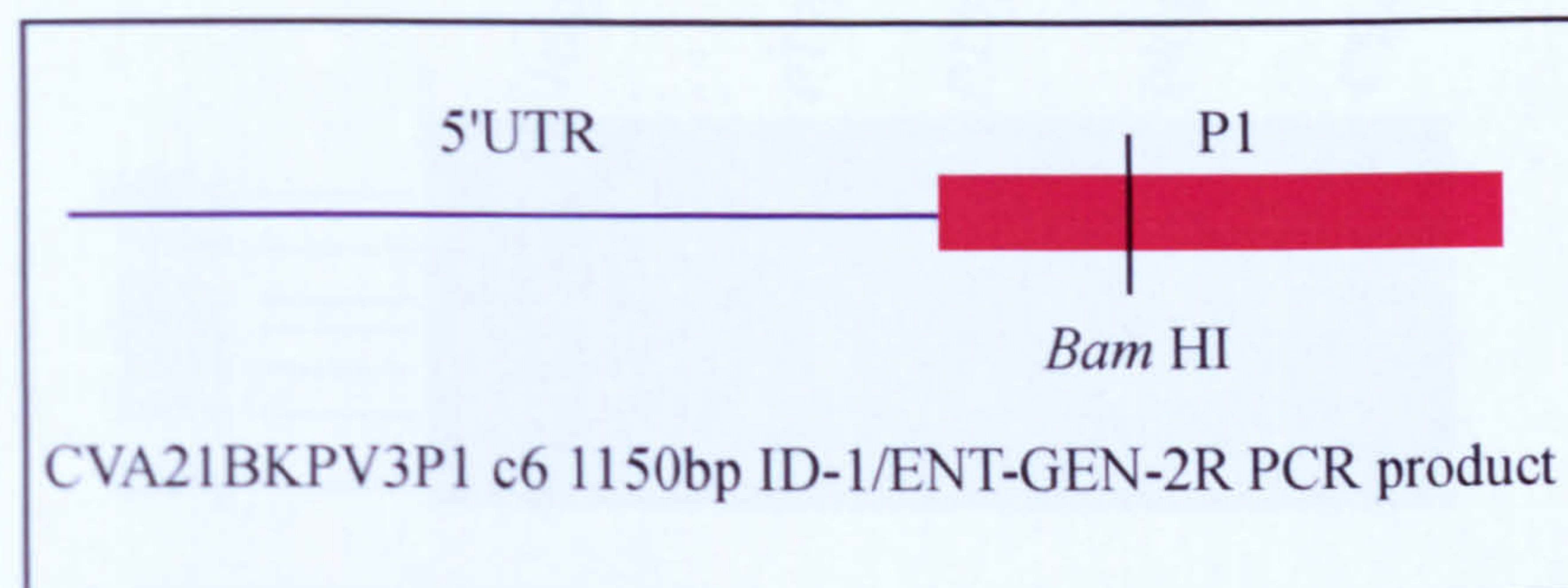
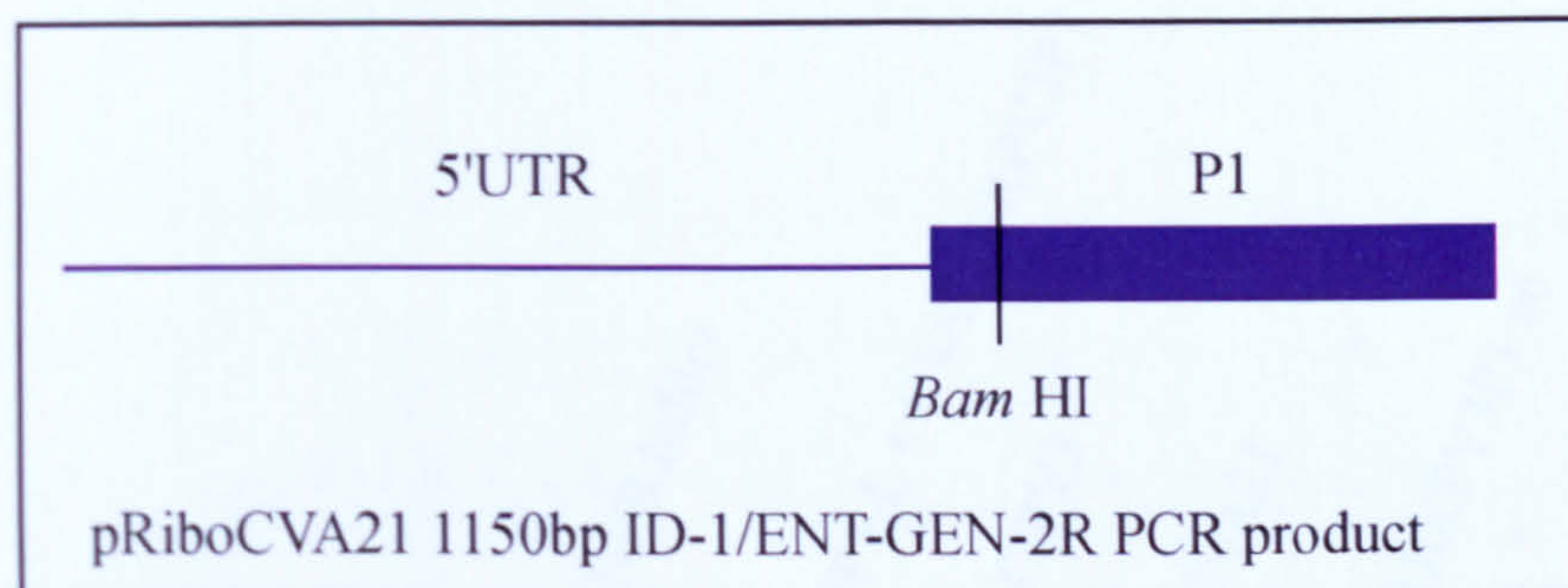
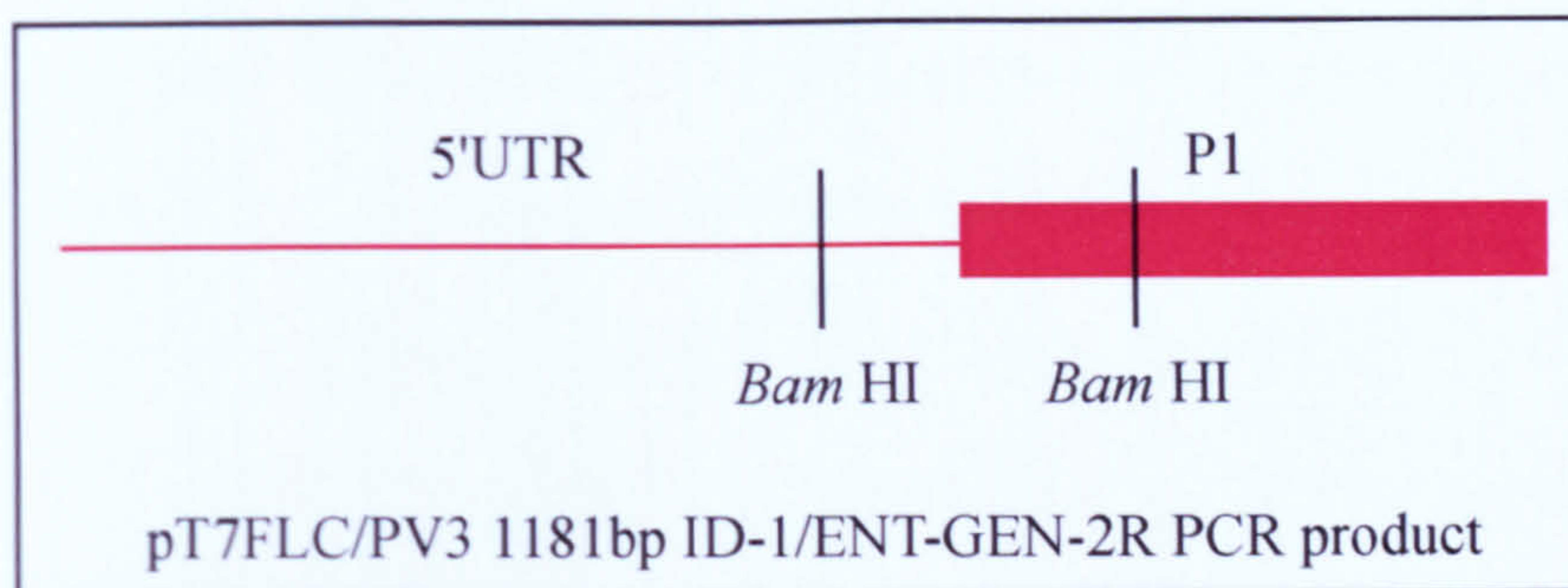
Figure 3.33: Trans-encapsidation of pT7REP3-L RNA by recombinants: first passage into L20B cells post-co-transfection of T7 RNA transcripts.

The assay was carried out five times, standard deviation values were calculated and are illustrated as error bars in the graph.

Further investigation of this surprising result was conducted. Viral RNA extraction, reverse transcription, amplification of the 5'UTR/P1-coding region, P1/P2 and 3D-coding regions by PCR, RFLP and sequencing analysis of the sample were carried out. The P1/P2 and 3D coding-regions had a PV3 RFLP and sequencing pattern (data not shown), strongly suggesting a contamination event. However, the 5'UTR/P1 PCR amplification (ID-1/ENT-GEN-2R) and the *Bam* HI RFLP analysis indicated that the samples had a CVA21 5'UTR and a PV3 P1 pattern (see figure 3.34.1 and 3.34.2). This suggested that a recombination event between the CVA21BKPV3P1 c6 recombinant and the pT7REP3-L replication-competent RNA genomes had occurred. This resulted in the generation of a recombined chimera containing a CVA215'UTR and the remainder of the genome of PV3 (CVA215'UTR/PV3). Sequencing data analysis was poor, as it was apparent that mixed viral sequences were present, probably corresponding to the presence of both CVA21BKPV3P1 c6 and CVA215'UTR/PV3 sequences (data not shown). A series of analyses were conducted in order to further investigate these observations.

Interestingly, the sample with high luciferase activity also caused CPE when passaged onto fresh cell sheets. It should be noted that neither input genome alone caused CPE (see table 3.10 and table 4.3). The samples were harvested, isolated and plaqued. A mixed plaque phenotype was observed with a population of small plaques (see figure 3.35 and 3.36) that were consistently smaller than those seen in a typical PV3 infection (data not shown).

Previous studies demonstrated that, when 220 nucleotides of the 5'UTR of CVB3 were changed for PV1, the viability of the virus was maintained. These viruses, however, had smaller plaques than that of PV3 wild-type plaques (Zell et al., 1995). Replacement of the complete PV3 5'UTR with the CVB4 5'UTR was demonstrated not to affect sub-genomic replicon replication (Rohll et al., 1994). Furthermore, chimeric CVB4/PV3 5'UTR did not appear to affect levels of virus production (Rohll et al., 1994).



Fragments generated from the *Bam* HI digestion of the ID-1/ENT-GEN-2R amplified PCR products (in kb).

pT7FLC/PV3	0.673, 0.296, 0.213
pRiboCVA21	0.775, 0.376
CVA21BKPV3P1 c6	0.855, 0.296

Figure 3.34.1: *Bam* HI digestion of ID-1/ENT-GEN-2R PCR products of pT7FLC/PV3, pRiboCVA21 and CVA21BKPV3P1 c6.

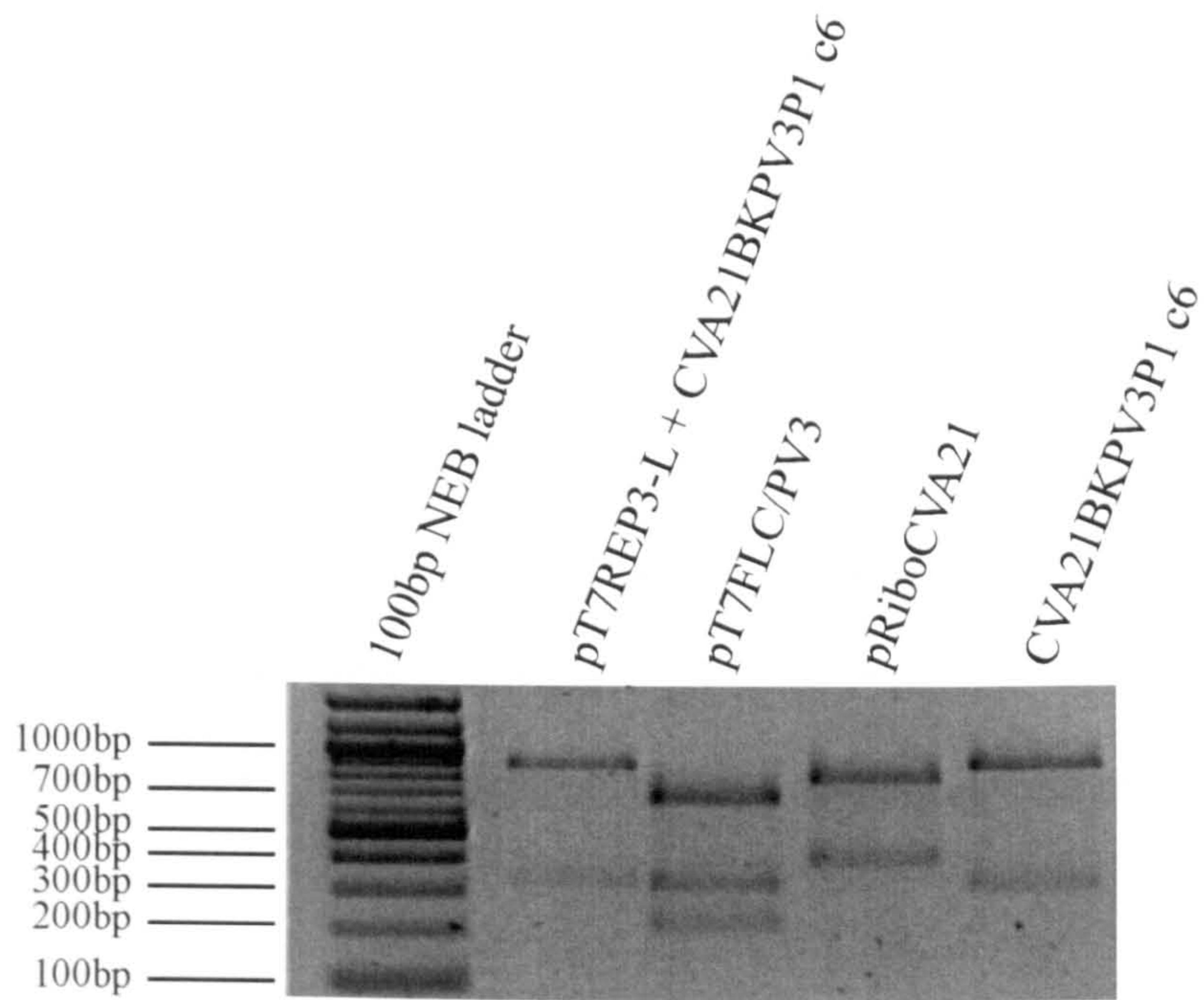


Figure 3.34.2: 5'UTR/P1 RT-PCR RFLP analysis of the trans-encapsulation samples

Samples, resulting from the trans-encapsulation transfection experiments, were reverse transcribe and the 5'UTR/VP4-coding region was amplified by PCR using generic primers (ID-1 and ENT-GEN-2R). The amplified fragments were then digested with *Bam* HI and run on a 2% agarose gel.

Infectious recombined chimeric viruses would have been able to infect the third cell sheet and provide capsids *in trans* as they appear to cause CPE. Furthermore it is likely that the luciferase subgenomic replicon was packaged into CVA215'UTR/PV3 capsids during the first passage. The particles were then able to attach, enter and release the replicon into fresh cells. This could explain the transfer of a luciferase signal onto a third cell sheet.

Another possible explanation is that a recombination event occurred generating a large genome containing both the luciferase-coding gene and the complete genome coding sequence. This is improbable, as a larger genome is less likely to be packaged into the empty virus particles, as it is not an evolutionary advantage. The plaque purified isolated viruses were passaged onto a fresh cell sheet and luciferase activity detection assay was carried out. No luciferase signal was detected thereby suggesting the absence of a large super-recombinant genome.

The elevated luciferase signal seen after the first passage could also be due to the CVA21BKPV3P1 c6 recombinant capsid proteins assembling to form a virus particle packaging the luciferase replicon genome (see figure 3.30). This could also have been the case, as similar results were observed in the case of CVA21BKSabin1P1 61-9 (see figure 3.30). It is likely that the two events together- trans-encapsidation and subsequent transfer of the luciferase genome and recombination to form a viable CVA215'UTR/PV3 genome-were responsible for the present observations. The latter would amplify the former. However, it is still unclear why such high readings were observed.

3.4 Discussion

In vitro generated recombinants between polioviruses and a HEV-C, with a perfect exchange of the complete P1-coding region, were unable to cause cytopathic effect when

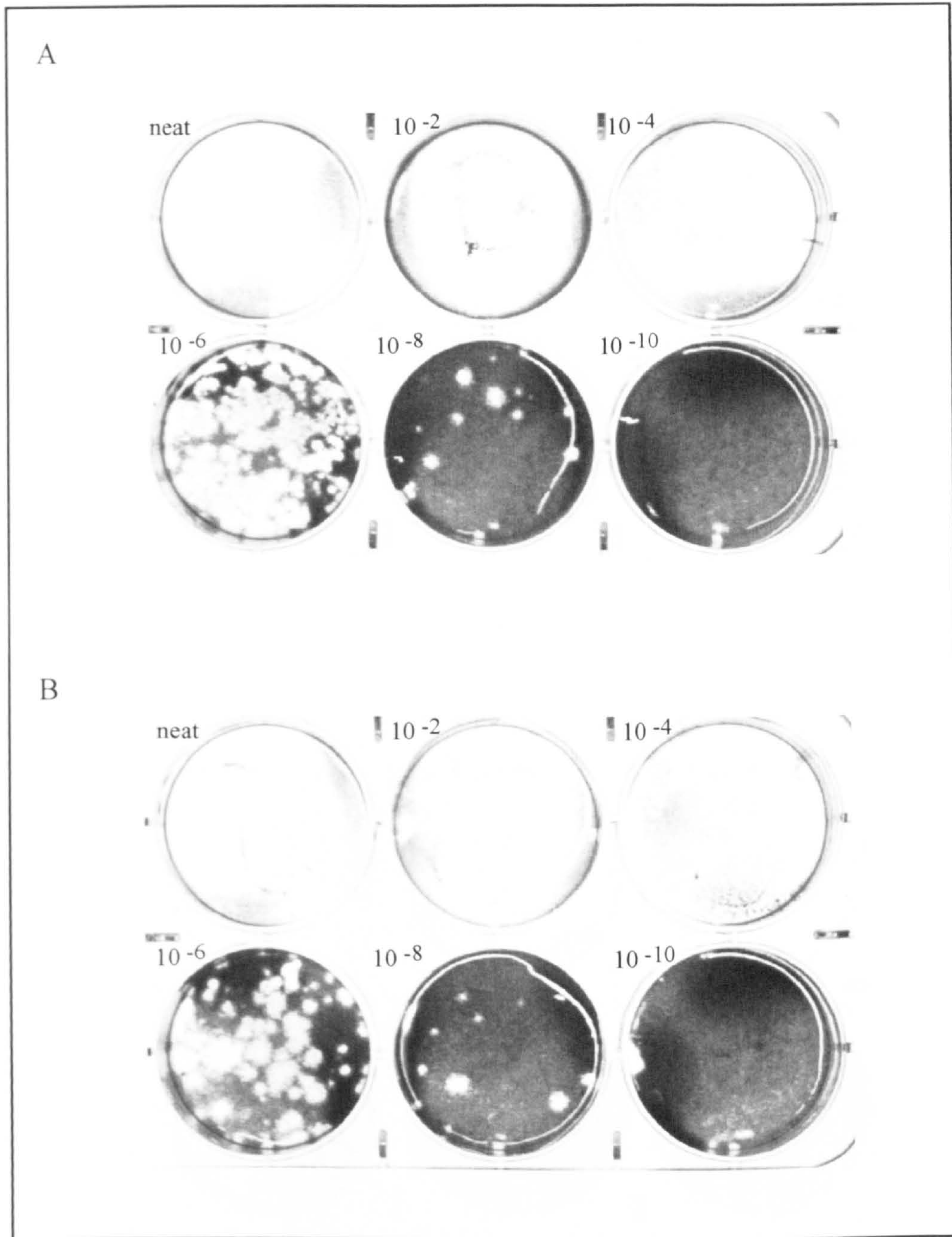


Figure 3.35: Plaque phenotype determination

in RD cells of plaque purified virus isolated after the second passage of CVA21BKPV3P1 c6 + pT7REP3-L samples and plaque purified twice:

A- pT7REP3-L + CVA21BKPV3P1 c6 4,

B- pT7REP3-L + CVA21BKPV3P1 c6 8

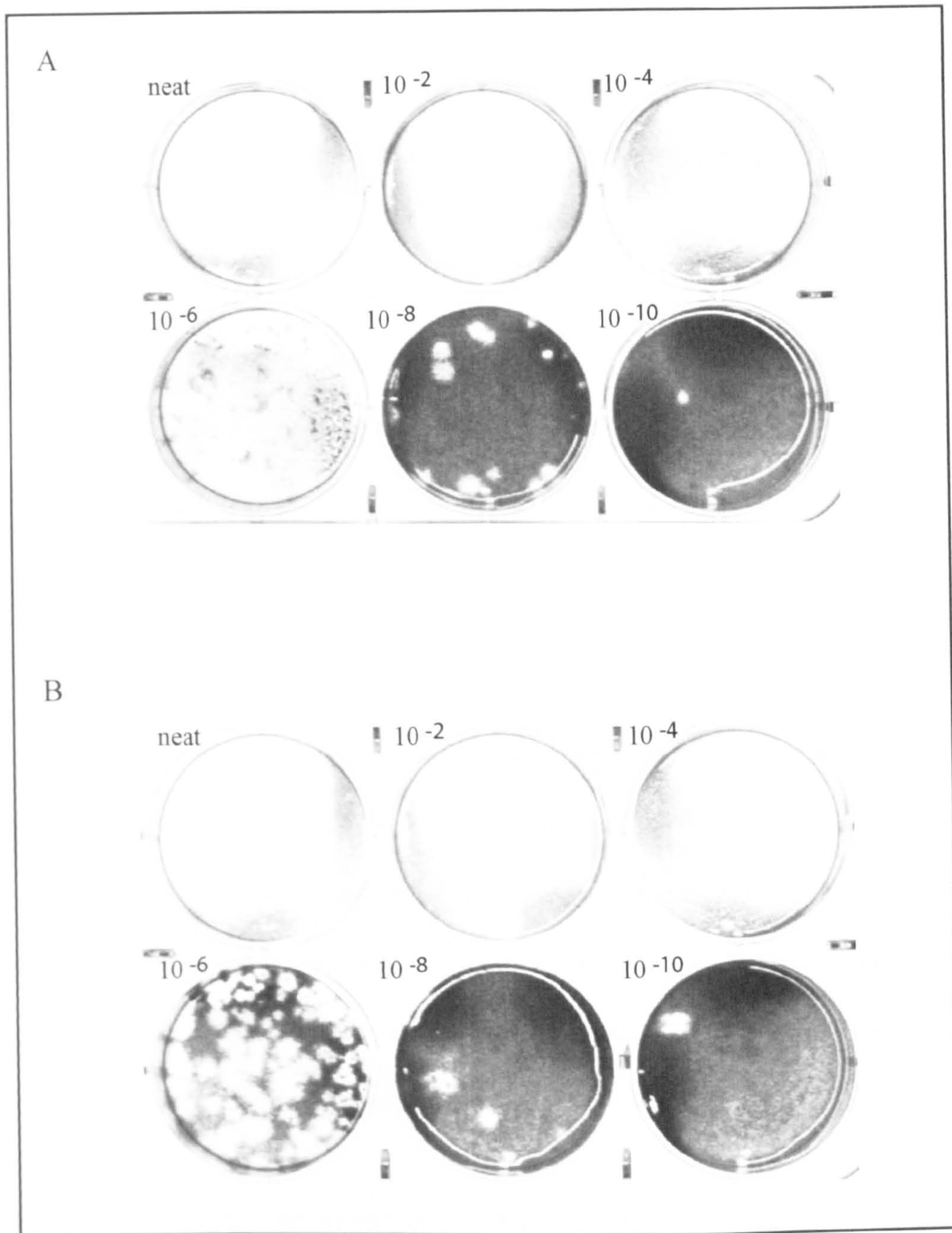


Figure 3.36: Plaque phenotype determination (continued)

in RD cells of plaque purified virus isolated after the second passage of CVA21BKPV3P1 c6 + pT7REP3-L samples and plaque purified twice:

A- pT7REP3-L + CVA21BKPV3P1 c6 11,

B- pT7REP3-L + CVA21BKPV3P1 c6 12

transfected into permissive cell lines. The generated chimeras were initially thought to be acytopathic or replication-defective due to a combination of protein coding regions leading to the generation of non-viable viruses. Immunofluorescence studies suggested that the chimeras were not acytopathic but that VP1 was generated and that translation had occurred. Therefore a defect must have affected a post-translation event of the life cycle such as processing, replication, assembly, encapsidation, maturation or entry of progeny virions into uninfected cells. A series of assays were undertaken to determine the nature and location of the defect affecting the recombinants.

In vitro translation assays were conducted to confirm the immunofluorescence study results. Results indicated that the recombinants appeared to translate efficiently and exhibit the correct *in vitro* protein-processing pattern. *In vivo* control samples appeared to have identical protein processing patterns as the *in vitro* translation control samples. The *in vitro* translation assay data suggested that the correct viral proteins had been made thereby implying that proteins derived from chimeras were likely to be able to drive genome replication. However, the translation assay was carried out *in vitro* and it is possible that in a particular cellular environment the polyprotein processing would not occur as efficiently or would be unable to generate functional proteins.

Recombinant genome replication was then checked by dot blot hybridisation. The experiment confirmed that the *in vitro* generated recombinants were replication-competent. Some replicated to wild-type genome levels, whilst others were shown to be less efficient at replicating. These observations indicated that the defect was either located at maturation, assembly, and packaging processes or affected the infection of new cells.

The level of expertise in the laboratory enabled trans-encapsidation assays to be conducted. Two assays, testing for the presence or absence of a packaging signal in the recombinant

genomes and for the capacity to encapsidate a replicon genome by the capsid proteins synthesised from the chimeric genomic RNA, were carried out. Trans-encapsidation of recombinant genomes by infectious helper viruses was investigated first. No packaged recombinant genomes were detected from the RT-PCR and RFLP screening. However, the sensitivity of the technique was not evident, as control samples did not enable a definite conclusion. Subsequent co-transfection of a luciferase type-3 poliovirus replicon with the recombinant RNA genomes into RD-ICAM cells, sample harvesting, RNase treatment and passage onto a fresh cell sheets constituted the second trans-encapsidation assay. The results obtained from this assay suggested that both a recombination event and the P1 recombinants generating capsids were likely to account for encapsidation of the luciferase subgenomic replicon. This could be explained by the recombinant genomes lacking a packaging signal or having an incompatibility essential for key interactions involved in encapsidation. This defect would have prevented the formation of viable virions.

Studies investigating poliovirus packaging have not yet provided a detailed understanding of this mechanism and the proteins involved in such processes have yet to be identified. The poliovirus packaging signal was shown not to be located in the 2A-coding region or in the P1 minus the last few amino acids of VP1-coding region and was suggested to be located elsewhere in the remainder of the genome (Kuge et al., 1986; Barclay et al., 1998). However, packaging may be more complex and dependent on a number of factors.

Infectious cVDPVs, isolated from the Haiti outbreaks, were shown to be recombinants between Sabin poliovirus type 1 and HEV-Cs, with a crossover site located in the 2A/2B-coding regions (Kew et al., 2002 see figure 3.37). *In vitro* generated recombinants and the Haiti isolates differed in the location of their crossover sites. Similar recombination events were observed in Madagascar cVDPVs isolates that proved to be Sabin poliovirus

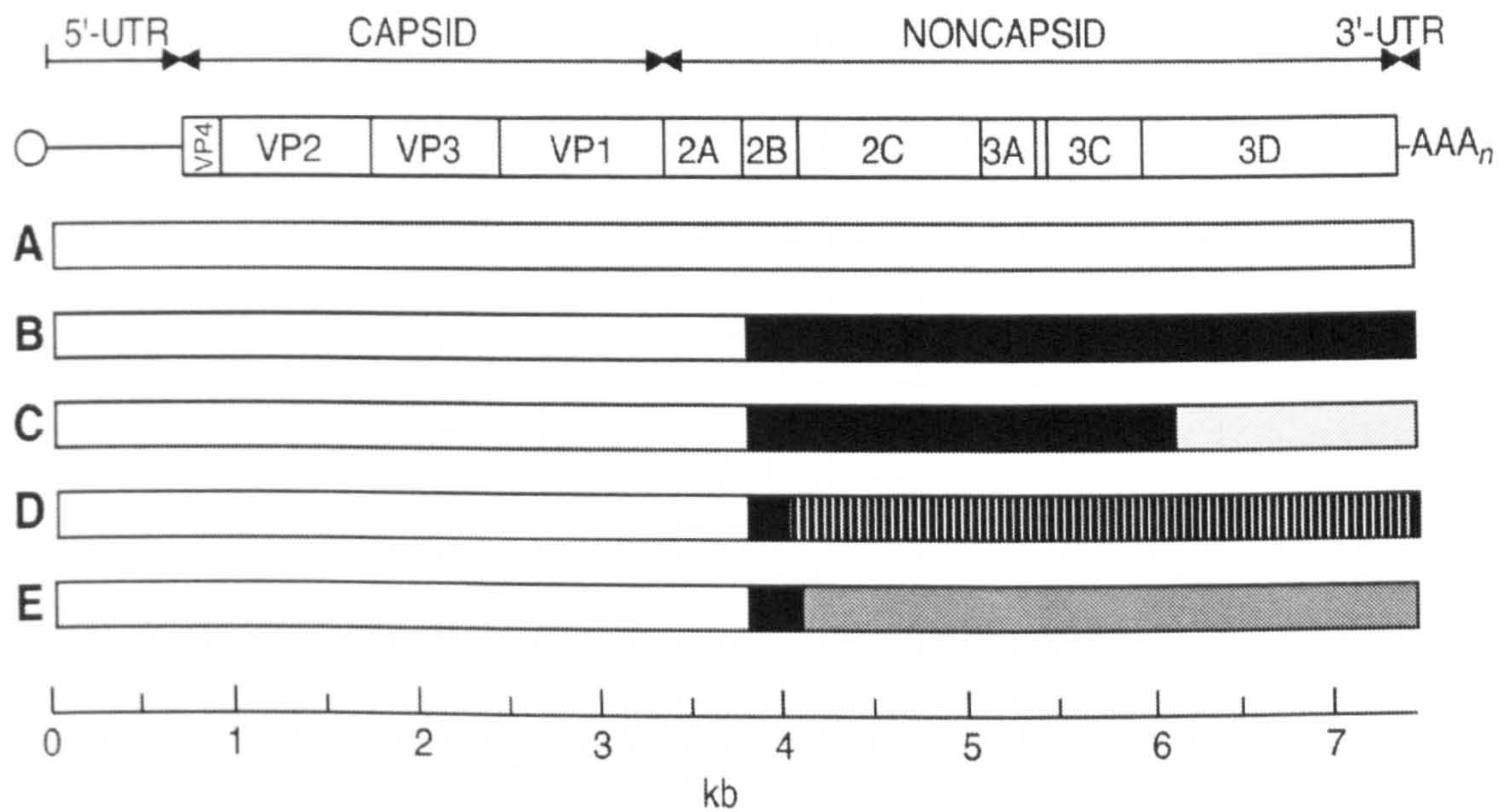


Figure 3.37: Location of recombination breakpoints of the four different recombinant classes found among Hispaniola VDPV isolates.

In the schematic of the poliovirus genome, the single open reading frame is indicated by a rectangle, flanked by the 5'- and 3'-untranslated regions (UTR). In rectangles A to E, Sabin 1-derived sequences are indicated by white fill, and sequences derived from enteroviruses other than the Sabin OPV strains are indicated by shaded or hatched fills. The rectangles symbolize sequences of A, Sabin 1; B, DOR00-013; C, DOR00-041c1; D, HAI00-003; and E, HAI01-007. Figure and legend taken from Kew et al. (2002).

type 2/HEV-Cs chimeras, with a non-specified crossover site within the 2A-coding region (Rousset et al., 2004).

The studied viable cVDPVs had P1, 2A and/or part of the 2B-coding regions derived from the same source genomes. The non-viable *in vitro* generated P1 recombinants differed from the cVDPVs as the P1 and 2A/2B-coding regions were derived from separate source genomes. In light of these results, packaging may be more complex than initially thought. A packaging signal may depend on localisation of genomes and proteins and/or on compatibility required for specific interactions to occur. It is possible that the genome and the capsid proteins of a particular virus localise in specific intracellular areas. These intracellular areas may differ between viruses, which may prevent specific interactions, and thus packaging from occurring. Compatibility between the P1 with some of the 2A/2B-coding regions may be necessary for efficient packaging to occur, whether the compatibility is based on RNA structures, protein-RNA or protein-protein interactions. Unassembled or partially assembled capsid proteins could exhibit high specificity for a particular RNA structure, RNA sequence or protein present within homologous 2A/2B-coding regions. This would explain the discrepancy seen between the results presented in this thesis and those observed in Haiti and Madagascar (Kew et al., 2002; Rousset et al, 2004).

A repeat of the assay by sequential transfection as opposed to simultaneous co-transfection may prevent the occurrence of recombination events between the chimeric genomes and the luciferase genomes and ensure a better data interpretation.

Further investigations need to be carried out in order to determine the exact nature of the defect and to enable a better understanding of this particular stage of enterovirus life cycle. Electron microscopy (EM) studies should enable the detection of mature virus particles.

The absence of mature virions would support the existence of a packaging defect. If such virions were to be detected in the supernatant of transfected cells, then the defect would have to be at the attachment and entry level of the next cell sheet, since they cannot infect fresh cell sheets. If no virions were to be detected in the supernatant but were present in the lysed cell suspension, this would imply that the mature virions could not be released into the extracellular environment or that the production or release of such virus particles would be much slower. Due to equipment breakdown and restrictions of EM, such experiments were not completed. Should microscopy studies confirm the absence of mature virus particles, the detection of assembly intermediates would be undertaken.

The detection of assembly intermediates by sedimentation coefficient assay should enable the determination of the particular stage of the life cycle affected by the defect and potentially the assembly step at which packaging of the genomic RNA occurs. The determination of the presence of assembly precursors by density gradients would also establish whether there was a defect in the maturation process or whether the lack of maturation was a consequence of a packaging defect. The method used would be designed based on early experiments (Moscufo et al., 1991) whereby radiolabelled HeLa cell suspension cultures, infected with a high MOI of control viruses or transfected with high concentrations of recombinant RNA, would be harvested and lysed. Alternatively, microinjection of high concentrations of sample RNA into oocytes, supplemented with HeLa nuclear extracts necessary for initiation of translation and RNA synthesis (Gamarnik et al., 1996), may be conducted to ensure a more efficient delivery of the samples. Samples would then be centrifugated to eliminate cell debris and the supernatants would then be applied to 6-25% and 10-30% sucrose gradients. Centrifugation to allow separation of different assembly intermediates with different sedimentation coefficients would then be conducted. The 6-25% gradient would enable isolation of the 6S protomers and 14S

pentamers and the 10-30% gradient would allow isolation of 80S empty capsids and 160S mature virions. The detection of 160S particle production would suggest that assembly and packaging had occurred. However, the detection of assembly intermediates may give an insight into what stage of assembly was affected by the defect. Results could be interesting for the understanding of the picornavirus packaging and assembly.

In the present assays, the cells were lysed by freeze thawing, thereby releasing any intracellular viable and infectious progeny virions. An incapacity of recombinant viruses to attach and enter cells was not likely, as the entire P1-coding sequence of the *in vitro* generated recombinants, checked in its entirety by sequencing, remained unchanged. The capsids, being identical to source capsids, would be expected to have wild-type infectious parent virion conformation and to attach and enter cells.

A number of experiments need to be conducted in order to get a better understanding of the defect seen in the *in vitro* generated P1 recombinants and the requirements necessary for packaging to occur. One way to investigate this would be the generation of P1/2A recombinants where P1 and 2A-coding regions would be swapped between CVA21 and PV3 or Sabin 1. The *in vitro* generation of such recombinants might give us an insight into the localisation of an encapsidation signal and the compatibility requirements for efficient encapsidation of the genomes to occur. This would also represent a scenario closer to that described in nature (Kew et al., 2002; Rousset et al., 2003; Rousset et al., 2004).

The selection of novel tropic recombinant viruses *in vivo*, enabling the generation of recombinant viruses without predetermining crossover sites, and the *in vitro* generation of P1/2A chimeras, may enable a better understanding of the results described in this chapter and of the encapsidation and assembly processes.

4 Results 3: *In vivo* recombination

4.1 Introduction

Since the introduction of the OPV, vaccine-derived polioviruses (VDPVs) have been reported to cause vaccine-associated paralytic poliomyelitis (VAPP) in both immunocompromised patients and healthy individuals (see chapter 1: Introduction). The recent outbreaks of VDPVs in Hispaniola (Kew et al., 2002), Madagascar (Rousset et al., 2003), the Philippines (MMWR, 2001) and Egypt (Yang et al., 2003) have raised concerns about the WHO eradication program and have highlighted the threat VDPVs cause and the need for a better understanding of such viruses.

A variety of reports (see chapter 1: Introduction) have shown that human enteroviruses can recombine in the natural host. In fact, intraspecies recombination is a common occurrence in nature, and has been reported in human enterovirus species A (Oberste et al., 2004b), human enterovirus species B (Oprisan et al., 2002; Oberste et al., 2004a) and human enterovirus species C (Brown et al., 2003). Moreover recent studies provided evidence that intertypic recombination could occur between a HEV-C and a poliovirus. In fact, the poliomyelitis-causing VDPVs isolated in Haiti and Madagascar were shown to be recombinants between poliovirus Sabin type 1/poliovirus Sabin type 2 and human enteroviruses species C (Kew et al., 2002, Rousset et al., 2004).

When recombinants between a poliovirus and a HEV-C with a precise exchange of the P1-coding region were generated *in vitro*, these proved to be non-viable. Can viable recombinants between poliovirus and non-poliovirus human enteroviruses occur in a tissue culture environment? Can recombination occur in a particular cellular environment such as RD-ICAM cells or does recombination only occur in the M cells of Peyer's patches? *In*

vivo recombination studies could provide valuable information and determine whether recombination can occur in RD-ICAM cells or whether these cells lack specific requirements, only found in the gut environment, necessary for a recombination event to occur. What viruses are most likely to recombine? The experiments described in this chapter were designed to select for viable recombinant viruses in a tissue culture environment by co-transfecting two impaired genomes in the same cellular environment.

Two selectable defective genomes unable to make viable virus progeny would be introduced to the same cellular environment, passaged and growing viruses isolated. This would bias the assay and recombination, where the crossover sites have not been pre-determined, would therefore be more likely to occur in the particular cellular environment. The choice of input genome was critical. Various defects such as in-frame deletion within the capsid-coding region, mutations preventing a complete replication cycle or sensitivity to a particular drug, such as GuHCl, were considered for these experiments. Should recombinant viruses arise from the experiment, these would be isolated, analysed, characterised, the exact location of the crossover points identified, and the existence of recombination hot spots and the relevance of these in virus evolution would be identified. In this chapter, assays carried out in a tissue culture environment are defined as *in vivo*.

The successful selection and characterisation of capsid recombinants in tissue culture without predetermining crossover points and exact recombination sites should provide very valuable information on properties of interspecies recombinant viruses, tropism, viability and fitness determinants. Previous findings, described in chapter 3, showed that recombinants engineered *in vitro* to have a precise exchange of the P1-coding region were non-viable. These were suggested to have a late replication defect such as packaging. *In vivo* studies may allow a better understanding of the defect affecting the *in vitro* generated

recombinants and may provide valuable information on sequences and interactions involved in processes such as encapsidation.

4.2 Experimental model

In the model used here, two impaired viral genomes were co-transfected in an 80%-confluent cell monolayer. The co-transfected cell sheets were incubated for 3 to 5 days at 37°C to allow for recombination, recovery and multiplication of novel chimeras to occur. Any virus recovered would be isolated, analysed and characterised.

The model was designed based on a number of studies. Evidence of the generation of recombinants resulting from the co-transfection of two impaired viral DNA sequences, with an efficiency of 80-100%, has been provided in baculoviruses (Chaabihi et al., 1997) and adenoviruses (Elahi et al., 2002). The study of poliovirus 5' and 3' overlapping RNA fragments (Gmyl et al., 2003) conclusively demonstrated the production of recombinants resulting from a co-transfection assay. Furthermore, recent experiments describe the generation of cytopathic recombinant viruses from *in vivo* co-transfection of non-replicating subgenomic transcripts of the pestivirus BVDV (bovine viral diarrhea virus) (Gallei et al., 2004). The described method can therefore lead to cell death due to the emergence of chimeric viruses. This suggested that the proposed model offered a suitable strategy to select for recombinants *in vivo*.

4.2.1 Strategy and experimental evidence

In order to test the viability of the experimental system two impaired poliovirus type 3 Leon genomes were chosen, based on availability of infectious cDNAs, subgenomic replicons and replication-incompetent clones in the laboratory. A PV3 subgenomic replicon (REP3-L) was selected as one of the parental genomes. This replicon lacks all but 82 nucleotides encoding the P1 region, but replicates well upon transfection of permissive cell lines. The second chosen parental impaired viral genome was a replication-incompetent full-length poliovirus type 3 genome (SL3), derived from the plasmid pT7/SL3. pT7/SL3 has eight mutations in the 2C CRE region preventing the formation of the stem loop structure thereby inhibiting the formation of the VPg-pUpU primer and thus the initiation of the positive-sense strands synthesis. However, the SL3 genome is still capable of generating negative-sense strands, a process required for replication-dependent recombination (Goodfellow et al., 2003), and the remainder of the genome is PV3 sequences. Interestingly, SL3 revertants to replication-competency could not be recovered (Goodfellow et al., 2000).

4.2.2 Transfection efficiency calculation

The transfection efficiency of poliovirus type 3 in RD-ICAM cells was calculated by blue cell assay, to determine favourable experimental conditions. Transfection by CaPO₄ precipitation (Elahi et al., 2002), DOTAP (Chaabihi et al., 1996), electroporation (Gallei et al., 2004) and DEAE-dextran (Gmyl et al., 2003) have all been shown to be successful at generating recombinants. The use of Lipofectamine 2000 is another very efficient RNA transfection method. To maximise the chances of observing a recombination event a variety of transfection methods, post-transfection incubation times, cell densities and RNA

concentrations were tested. Transfection method and RNA concentrations test results are illustrated in table 4.2.

Transfection method	[PV3 T7 RNA] transfected into RD-ICAM cells			
	0 μ g	1 μ g	5 μ g	10 μ g
DEAE-dextran	1%	10-20%	30-40%	30-40%
Lipofectamine 2000	5%	50%	70-90%	70-90%
Electroporation (250V, 400 Ω , 250 μ F)	5%	60%	70-90%	70-90%

Table 4.2: Summary of *in situ* β -galactosidase detection of a bound ligand assay to determine the conditions for efficient transfection of PV3 T7 RNA transcript in RD-ICAM cells. The values represent the percentage of blue cells in each sample.

The negative controls for transfection surprisingly exhibited reasonably high background level for the samples transfected by electroporation and Lipofectamine 2000. This was thought to be due to non-specific staining of unattached cells. Transfection by electroporation often results in a significant number of dead or unattached cells that never recover. Similarly, large numbers of Lipofectamine 2000 liposomes fuse with the cell membranes, which can disrupt these structures resulting in significant levels of cell death. The background levels of blue cell staining observed in DEAE-dextran transfected cells was significantly lower than that seen post-electroporation and Lipofectamine 2000 treatment. This is because dextran permeabilises the cell membrane allowing entry of RNA and results in very little cell death. However, the transfection of poliovirus type 3 into RD-ICAM cells by electroporation and Lipofectamine 2000 (70-90%) was significantly

more efficient than transfection by DEAE-dextran (30-40%). The amounts of RNA that yielded greater transfection efficiency were that of 5 μ g and 10 μ g.

However, these figures were calculated based on transfection of one parent and, due to time restrictions, co-transfection efficiency was not determined. Assuming the transfection efficiency was equally high for other genomes the theoretical co-transfection efficiency was calculated. Around 49% (70% of 70%) of the transfected cells would be expected to contain copies of both parental genomes. This figure was thought to be sufficiently high since recombination is a frequent event. The parameters (electroporation of a total of 10 μ g of RNA into 10⁶-10⁷ cells/ml) were standardised and applied to the experimental model.

4.2.3 Co-transfection of SL3 and REP3-L genomes in L20B cells

The experimental model is described in figure 4.1.A. RNA synthesised *in vitro* from pT7/SL3 and pT7REP3-L were co-transfected by electroporation into L20B cells (kindly provided by Dr A. Macadam, National Institute for Biological Standards and Control), a murine cell line expressing the PVR. To ensure that the differences in transfection efficiencies would not bias the assay, different ratios of normalised concentrations of RNA transcripts derived from the two parental genomes were used. RNAs synthesised from individual control plasmids pT7/SL3 and pT7REP3-L were transfected in parallel and a mock transfection was carried out to monitor the effect of the transfection conditions on L20B cells. A parallel control transfection of the pT7FLC/PV3 RNA transcript positive control was conducted under the same experimental conditions at a different time to ensure no carryover. The results are listed in table 4.3.

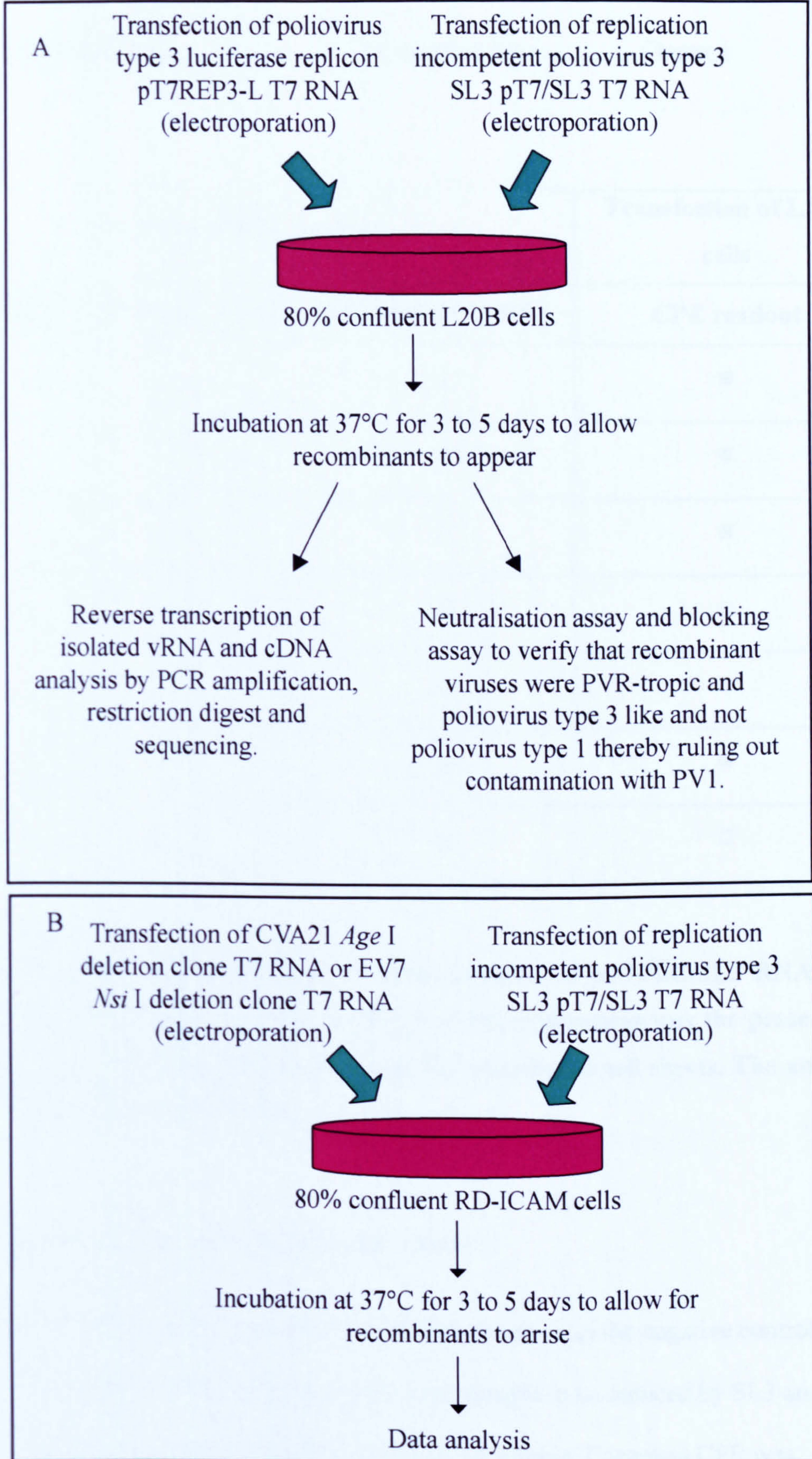


Figure 4.1: Experimental model

Experimental procedure of co-transfection experiments for the selection of recombinant viruses. A- Co-transfection method of the pT7REP3-L T7 RNA transcript and pT7/SL3 T7 RNA transcript. B- Co-transfection method of the CVA21 *Age* I deletion clone T7 RNA transcript or EV7 *Nsi* I deletion clone T7 RNA transcript and pT7/SL3 T7 RNA transcript.

Amounts of RNA (in μg)			Transfection of L20B cells
pT7/SL3 RNA	pT7REP3-L RNA	PT7FLC/PV3 RNA	CPE readout
5	5	0	■
2	8	0	■
8	2	0	■
10	0	0	□
0	10	0	□
0	0	10	■
0	0	0	□

Table 4.3: Results of co-transfection by electroporation of pT7FLC/SL3 RNA with pT7REP3-L RNA in L20B cells. The read-out of the experiment was the presence ■ or absence □ of cytopathic effect observed in the transfected cell sheets. The amount of RNA transfected is expressed in μg .

4.2.3.1 Analysis of recovered recombinant viruses

As expected, no CPE or very low levels of CPE were observed in the negative control samples. The very low levels of CPE observed were thought to be induced by SL3 and REP3-L production of the cytotoxic functional viral 2A protein. Complete CPE was observed in cells transfected with the positive control full-length wild-type PV3. Finally, L20B cells co-transfected with both defective genomes exhibited high levels of CPE,

comparable to wild-type levels. The viruses were isolated from the samples, analysed and characterised.

The co-transfection of a replication-impaired SL3 genome with a REP3-L luciferase replicon *in vivo* led to the successful generation of viable recombinant viruses (SL3-L) with PV3-like sequences and properties. The expectation that two impaired PV3 viral genomes can recombine *in vivo* was confirmed and the viable recombinant viruses were analysed by receptor blocking, virus neutralisation, plaque phenotype, RFLP and sequencing studies.

4.2.3.2 Receptor blocking assay

Infections with the viruses resulting from the co-transfection of pT7/SL3 RNA and pT7REP3-L RNA were blocked by treatment with an anti-PVR monoclonal antibody (see table 6.1) indicating that the isolated viruses were PVR-binding viruses (see figure 4.4). In the absence of an anti-PVR polyclonal antibody PV3, PV1 and the isolated recombinant viruses (SL3-L) were able to exert a cytotoxic effect on L20B cells. In the presence of the anti-PVR polyclonal antibody infections by PV3, PV1 and the SL3-L viruses were blocked and no cytolysis was observed. This result confirmed that SL3-L viruses were PVR-tropic.

4.2.3.3 Neutralisation assay

SL3-L recombinant viruses had a PV3-like antigenicity as neutralisation of virus infection in L20B cells was observed in the presence of an anti-PV3 antibody but not in the presence of an anti-PV1 antibody (see figure 4.4). PV1 infection was neutralised by anti-PV1 antibody, PV3 infection was neutralised by anti-PV3 antibody and no antibody cross-reactivity was observed.

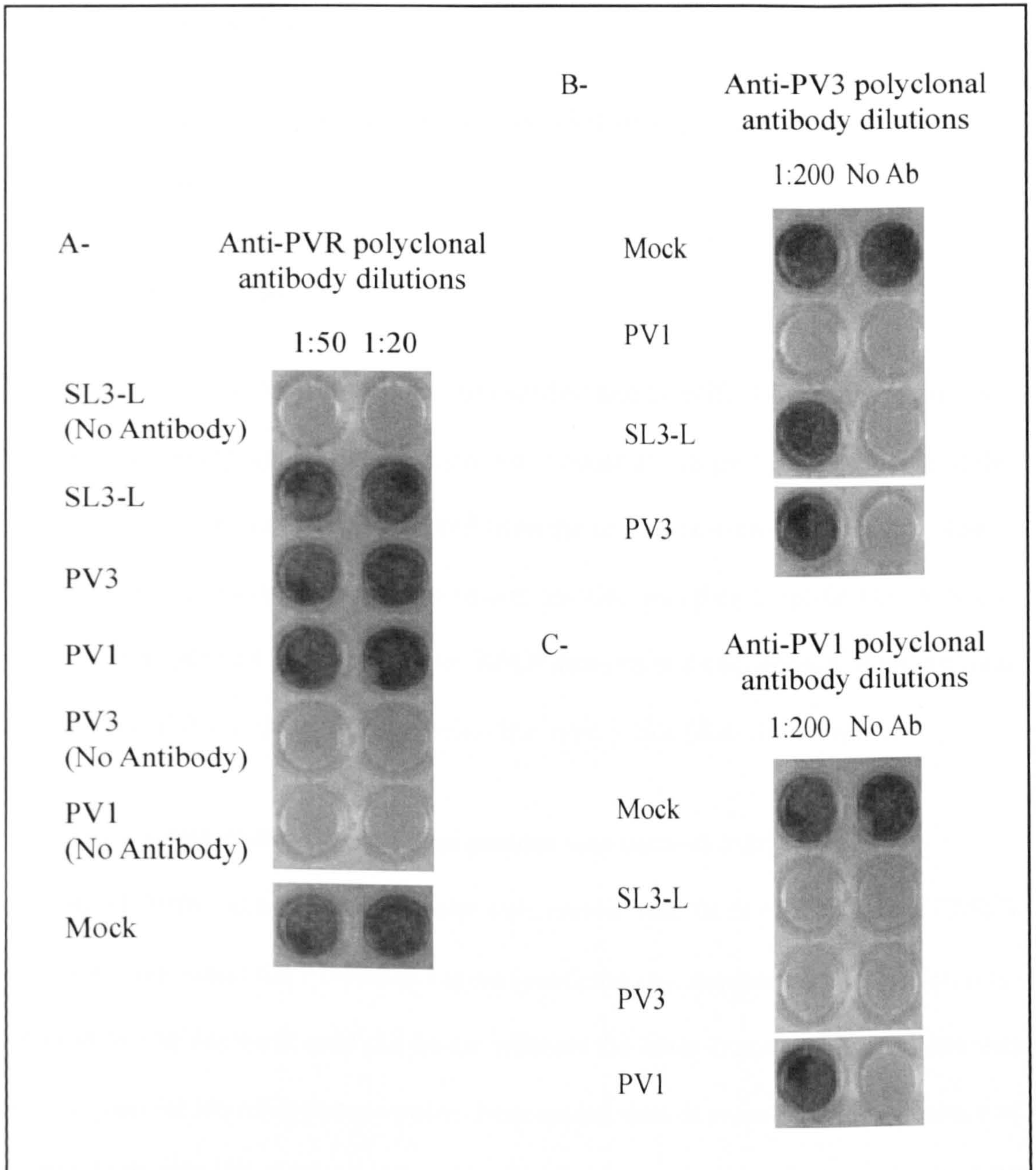


Figure 4.4: Phenotypic analysis of viruses generated by co-transfection of pT7/SL3 T7 RNA with pT7REP3-L T7 RNA.

Neutralisation and blocking assay of plaque purified viruses generated by co-transfection of pT7/SL3 T7 RNA transcript with pT7REP3-L T7 RNA transcript. A- Blocking assay using anti-PVR polyclonal antibody. B- Neutralisation assay using an anti-PV3 polyclonal antibody. C- Neutralisation assay using an anti-PV1 polyclonal antibody.

4.2.3.4 Plaque phenotype

The plaque phenotype of SL3-L viruses was indistinguishable from that of PV3 viruses (see figure 4.5 and 4.6).

4.2.3.5 Genetic analysis

SL3-L viral RNA was extracted, reverse transcribed and amplified for further analysis. RFLP and sequencing studies were carried out in order to determine the genotype of the SL3-L viable recombinant viruses isolated from the *in vivo* co-transfection assay. The cDNA generated from the reverse transcription reaction was then amplified by PCR and the product was purified and submitted to RFLP analysis and sequencing. Both analyses confirmed the SL3-L sequences were poliovirus type 3-like (data not shown).

To check the capsid-coding region of the genome was derived from the SL3 replication-defective genome *Sac* I digests were carried out. Both pT7FLC and pT7/SL3 have a *Sac* I site within the P1-coding region (confirmed by sequencing). However, it is unclear as to why but the former can be cut whereas the latter cannot. This *Sac* I site was therefore used for identification purposes. Sequencing data demonstrated the presence of a *Sac* I site within the P1-coding region of the SL3-L cDNA. This site was not digested by *Sac* I confirming that the SL3-L viruses isolated had SL3 P1-coding region sequences.

The SL3 clone has a *Swa* I site, situated in the mutated 2C-coding region. Screening for the absence of that site in the SL3-L virus sequences verified that the 2C CRE region was PV3-like and not SL3-like. Indeed the PV3 cDNA and SL3-L cDNA sequences were not digested by the *Swa* I restriction endonuclease whereas SL3 control sequences were.

Partial genome sequencing data further supported the result; the *Swa* I site was absent from the SL3-L cDNA. Contamination with wild-type poliovirus type 3 could not be excluded in

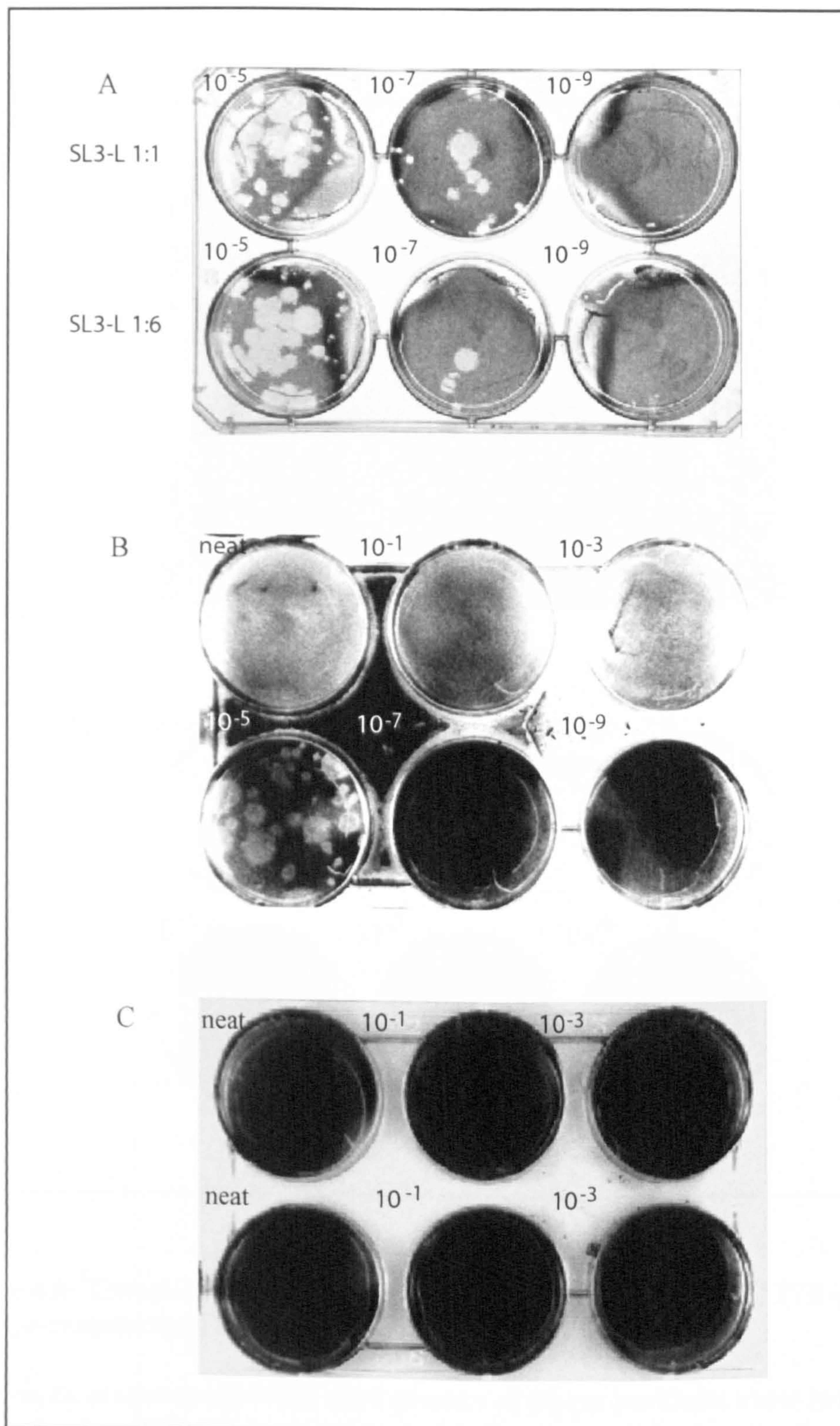


Figure 4.5: Plaque phenotype of recombinant viruses generated by co-transfection of RNA transcripts derived from pT7/SL3 with pT7REP3-L.

Two of the plaque purified viruses are illustrated here. The plaque phenotypes of control viruses PV3 and SL3 are also illustrated here. The plaque assays were carried out on L20B cells. A- Plaque assay of two of the plaque purified SL3-L viruses generated from co-transfection of RNA transcripts derived from pT7/SL3 with pT7REP3-L. 1:1 and 1:6 represent initial ratios of RNA used for transfection. B- Plaque assay of pT7FLC/PV3 T7 RNA transcript transfection. C- Plaque assay of pT7/SL3 T7 RNA transcript transfection.

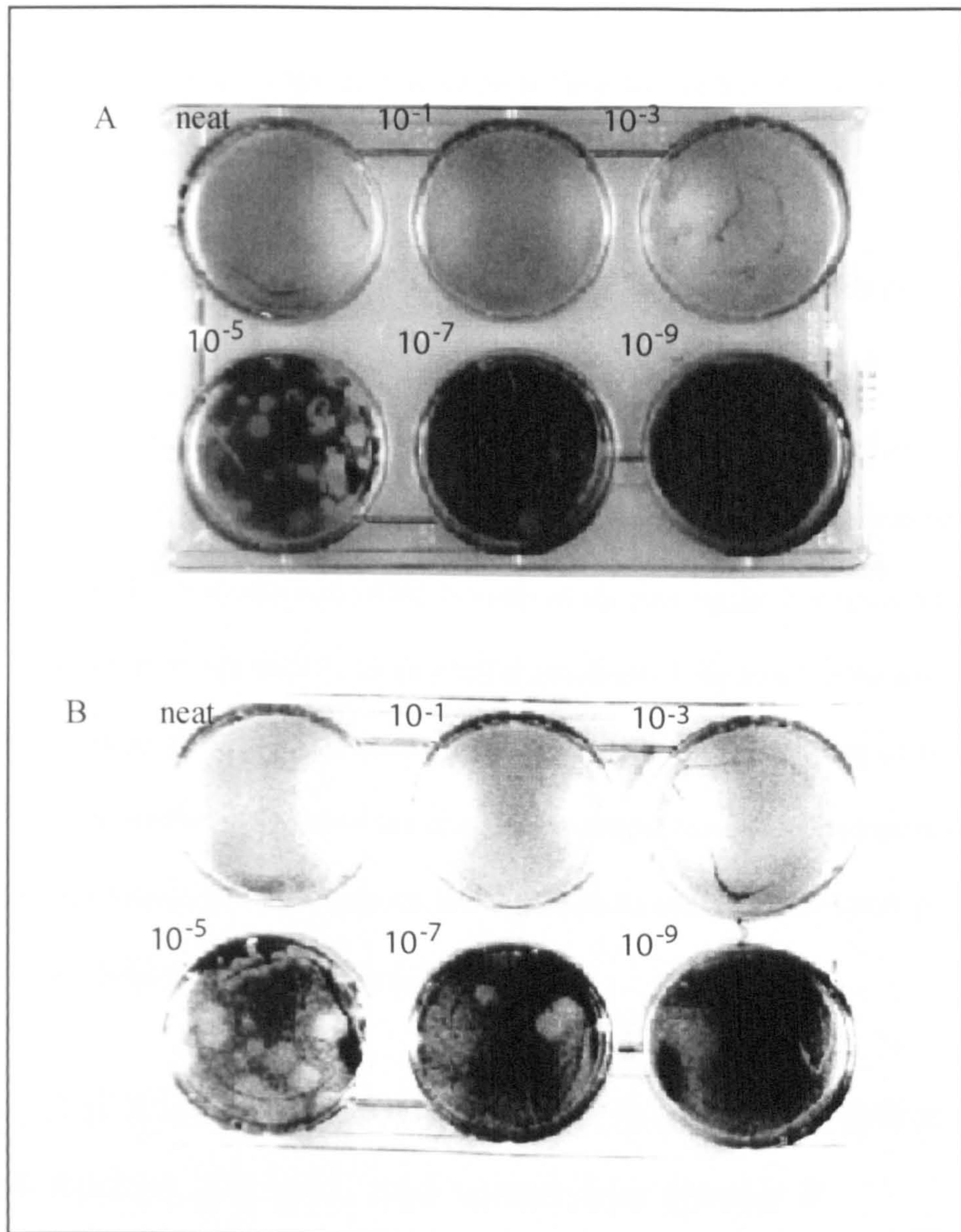


Figure 4.6: Titration of virus resulting from pT7/SL3 RNA and pT7REP3-L RNA co-transfection.

Titration by plaque assay of the third passage of plaque purified viruses generated by transfection of T7 RNA transcripts in L20B cells. A- Plaque assay of one of the passaged (p3) plaque purified viruses generated by co-transfection of pT7/SL3 T7 RNA transcript with pT7REP3-L T7 RNA transcript on L20B cells: 1.25×10^7 pfu/ml. B- Plaque assay of the passaged (p3) plaque purified virus generated by transfection of pT7FLC/PV3 T7 RNA transcript on L20B cells: 1.25×10^7 pfu/ml

these experiments but was very unlikely due to the precautions taken whilst carrying out the experiment and control transfections. Further analysis of possible crossover sites was impractical as both parental impaired genomes had type 3 sequences.

Repeating the experiment with defective genomes derived from different poliovirus serotypes could enable a more detailed analysis. Indeed, the successful recovery of viable recombinant viruses from co-transfection of a poliovirus-type 1 impaired parent with a poliovirus-type 3 impaired parent, could lead to the identification of recombination crossover points and the potential identification of recombination hot spots due to the sequence differences exhibited by the parental genomes. Time constraints and lack of availability of defective type 1-genomes prevented this assay from being carried out. This additional assay would add little to the aims of the study. However, the present result indicated that successful recombination between two homologous defective poliovirus type-3 RNAs could occur *in vivo*, in L20B cells.

4.3 *In vivo* selection for recombinants between poliovirus and enterovirus species C and enterovirus species B

Can a non-poliovirus enterovirus recombine with poliovirus in tissue culture? The *in vivo* selection for viable recombinant viruses between a poliovirus and a non-poliovirus enterovirus was therefore carried out using the experimental system as previously described and tested. Before initiating the assays the appropriate and available impaired parental genomes were carefully chosen and designed. The aim was to choose a human enterovirus C, closely related to poliovirus. These would be expected to recombine *in vivo*, as HEV-C are known to recombine with poliovirus vaccine strains in nature (Kew 2002, Rousset 2004). A human enterovirus B reference genome, more distantly related to

poliovirus, was also chosen for the assay, as it would be equally interesting in the selection for recombinants *in vivo*.

The choice of available impaired parental genomes was limited as there are very few or no current replicon or SL3 versions of HEV-Cs or HEV-Bs. One of the parent genomes was required to be poliovirus capsid-encoding as the aim was to select for PVR-tropic viruses. The poliovirus SL3 replication incompetent clone was chosen as one of the impaired parents. HEV representatives were chosen based on availability of infectious clones in our laboratory and homology levels to PV3. CVA21, a HEV-C closely related to polioviruses, has an 84.2% polyprotein amino acid identity with PV3, and EV7, a HEV-B more distantly related to polioviruses, only has a 64% amino acid identity with PV1. CVA21 and EV7 were the chosen as HEV representatives and best available parents for the experiment.

CVA21 and EV7 cDNAs were genetically engineered to create an in-frame deletion within the capsid-coding regions so that infectious wild-type virus could not be generated due to the absence of functional capsid proteins. Due to time constraints the replication phenotype of the P1-deletion clones could not be tested. Their competence to replicate was assured from knowledge of the poliovirus and poliovirus sub-genomic replicon system and from the sequencing to verify an in-frame deletion was present. The cloning strategies are illustrated in figure 4.7 and 4.8 and described in section 4.3.1.1 and 4.3.1.2.

4.3.1 Design of defective genomes

4.3.1.1 Cloning strategy for the generation of in-frame deletion within the P1-capsid coding region of CVA21 (enterovirus C)

A pT7CVA21 Δ P1 deletion clone was derived from the pCAV21 plasmid, which contains an infectious CVA21 cDNA. pCAV21 has three *Age* I sites within the VP2 and VP4

coding regions of the genome. Following *Age* I digestion of pCAV21, T4 DNA polymerase treatment was carried out to fill in 5' overhangs to form blunt ends, which were ligated together. The ligation reaction was then transformed, amplified in ER2738 bacterial cells and the DNA was purified. The clones were screened by standard digestion with *Nco* I, *Bam* HI and *Eag* I, a site created upon ligation (data not shown), and sequence analysis of PCR amplified VP4/VP3 coding regions (see figure 4.7). A window of the 5'UTR/P1-coding region spanning the in-frame deletion was amplified by PCR (using forward and reverse oligonucleotide primers CAV21-CAPF and ENT-GEN-2R) and sequenced. The analysis confirmed that the constructed clones were correct. The 5'UTR/P1 junction coding region of pT7CVA21 Δ P1 clones 6 and 7 sequences contained the *Eag* I site and were as predicted. The PCR fragments, were approximately 600bp-long suggesting that clones 6 and 7 did contain a deletion, compared to the wild-type pCAV21 PCR product, which was approximately 900bp-long.

4.3.1.2 Cloning strategy for the generation of an in-frame deletion within the P1 capsid coding region of EV7 (enterovirus B)

A pT7EV7 Δ P1 deletion clone was derived from the pT7EV7 plasmid, containing the complete infectious EV7 cDNA. pT7EV7 was digested with *Nsi* I, purified and self-ligated. The ligation reaction was digested with *Sal* I preventing religated vector from being recovered. The reaction was then transformed and amplified in ER2738 bacterial cells and the DNA was purified. The clones were screened by standard restriction digest reactions with *Bam* HI, *Nco* I and *Sal* I (data not shown) and sequenced. The sequencing analysis of a PCR amplified window of the 5'UTR/P1 coding region (using specific forward and reverse oligonucleotide primers CB3-1F and 591-5R: see figure 4.8) confirmed that the recovered clones had in-frame deletions and that the sequences were correct. Sequencing of PCR products generated with primers specific for sequences coding

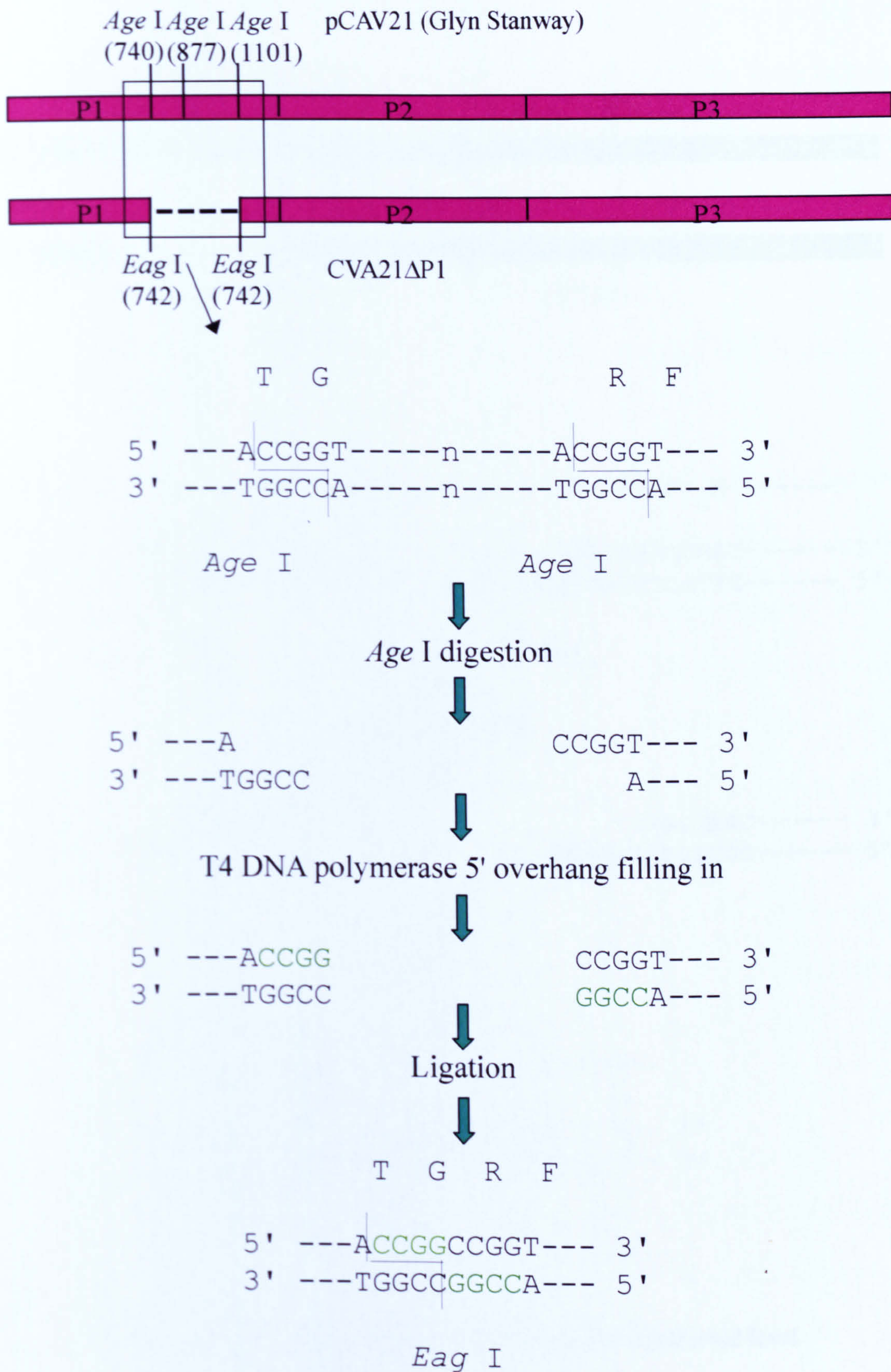
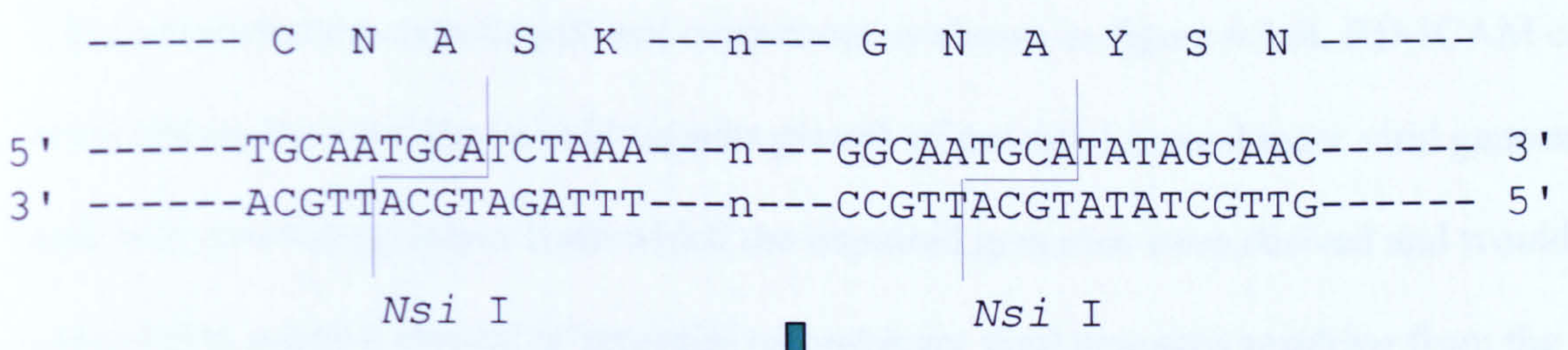
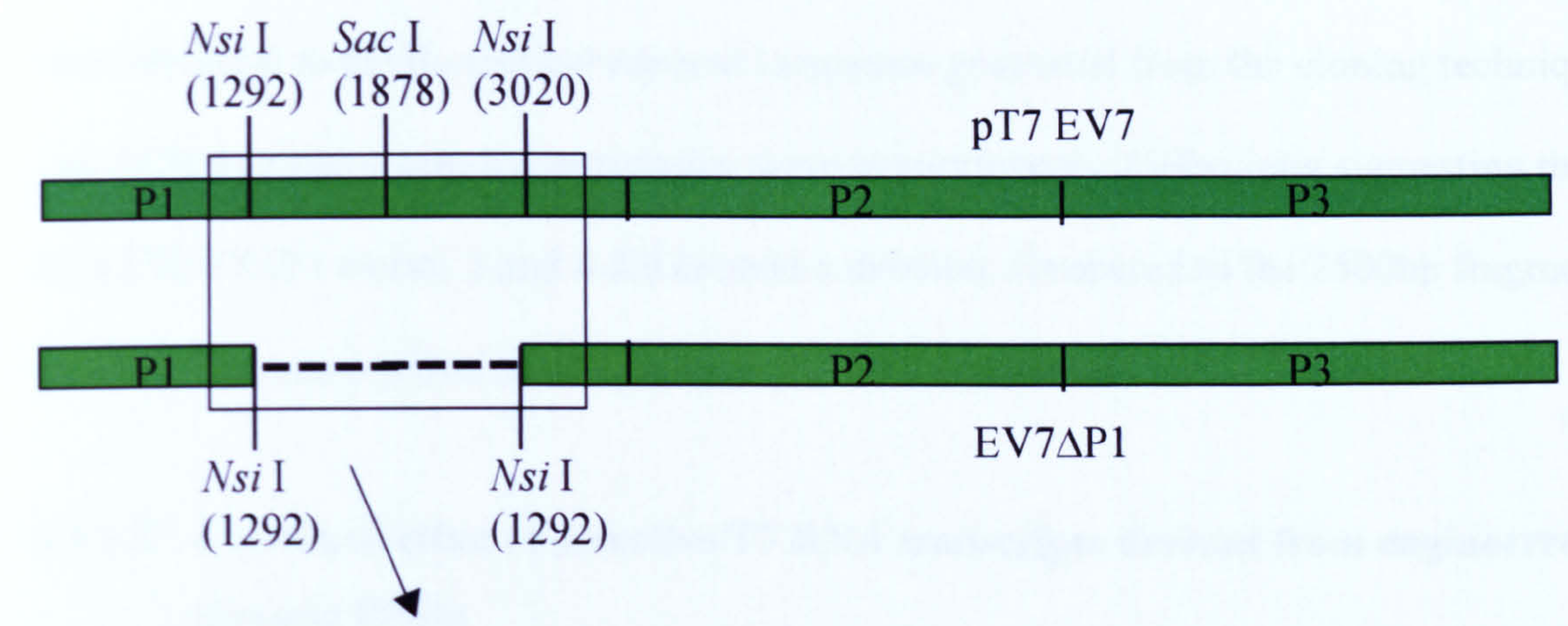


Figure 4.7: Cloning strategy of CVA21ΔP1 at the nucleotide level



↓
***Nsi* I digestion**



↓
Ligation

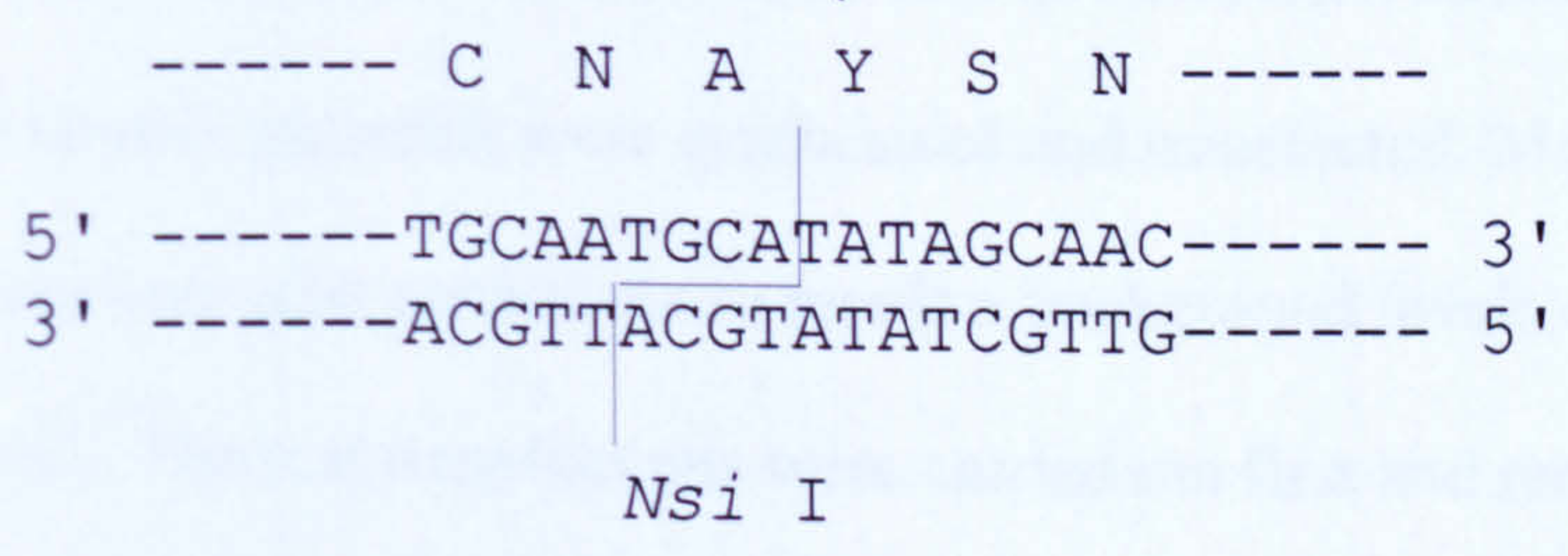


Figure 4.8: Cloning strategy of EV7ΔP1 at the nucleotide level

for the 5'UTR/P1 junction of pT7EV7 Δ P1 clones 3 and 4 was carried out. The sequences were identical to the theoretical expected sequence generated from the cloning technique. The PCR fragments sent for sequencing were approximately 750bp-long suggesting that the pT7EV7 Δ P1 clones 3 and 4 did contain a deletion, compared to the 2500bp fragment obtained from amplification of wild-type pT7EV7 sequences.

4.3.1.3 Co-transfection of defective T7 RNA transcripts derived from engineered plasmid DNAs

The co-transfection experiment was carried out as shown in figure 4.1.B. RD-ICAM cells were chosen because they would support growth of potential recombinant viral genomes and both parental genomes from which the impaired genomes were derived and would be expected to support growth of potential recombinant viral genomes resulting from the reaction. RD-ICAM cells also have a great ability to recover post-electroporation. The laboratory HeLa cell line, another permissive cell line, exhibited higher levels of cell death post-electroporation and was therefore not used for the assay. RNA synthesised *in vitro* from pT7CVA21 Δ P1-6 and -7, pT7EV7 Δ P1-3 and -4, pT7/SL3 were DNase treated, purified, measured using a spectrophotometer and co-transfected into RD-ICAM cells. The RNA derived from each individual clone as well as RNA derived from pCAV21, pT7EV7, pT7FLC/PV3 control plasmids were synthesised and transfected. Mock transfections of RD-ICAM cells were also carried out to monitor background levels of cytolysis post-transfection. The test transfections were carried out first and removed from working environment to a 37°C incubator. The controls were then transfected under the same conditions. The results are shown in table 4.9 and 4.10.

Amounts of RNA transfected into RD-ICAM cells (in μg)					
<i>pT7CVA21ΔP16</i>	<i>pT7CVA21ΔP17</i>	<i>pT7/SL3</i>	<i>pCVA21</i>	<i>pT7FLC/PV3</i>	CPE readout
5	0	5	0	0	□
2	0	8	0	0	□
8	0	2	0	0	□
0	5	5	0	0	□
0	2	8	0	0	□
0	8	2	0	0	□
10	0	0	0	0	□
0	10	0	0	0	□
0	0	10	0	0	□
0	0	0	10	0	■
0	0	0	0	10	■
0	0	0	0	0	□

Table 4.9: Results of co-transfection of the CVA21 Δ P1 RNA with the SL3 RNA in RD-ICAM cells. The read-out of the experiment was the presence ■ or absence □ of cytopathic effect observed in the transfected cell sheet. The amounts of RNA transfected are expressed in μg .

Recovery of recombinant viruses generated *in vivo* between a replication-incompetent PV3 and CVA21 with an in-frame deletion within the P1-coding region was unsuccessful. The CPE status of control samples was as expected, however no CPE was observed in the dually transfected samples.

Amounts of RNA transfected into RD-ICAM cells (in μg)					
<i>pT7EV7ΔP1-3</i>	<i>pT7EV7ΔP1-4</i>	<i>pT7/SL3</i>	<i>pT7EV7</i>	<i>pT7FLC/PV3</i>	CPE readout
5	0	5	0	0	□
2	0	8	0	0	□
8	0	2	0	0	□
0	5	5	0	0	□
0	2	8	0	0	□
0	8	2	0	0	□
10	0	0	0	0	□
0	10	0	0	0	□
0	0	10	0	0	□
0	0	0	10	0	■
0	0	0	0	10	■
0	0	0	0	0	□

Table 4.10: Results of co-transfection of the EV7 Δ P1 RNA with the SL3 RNA in RD-ICAM cells. The read-out of the experiment was the presence ■ or absence □ of cytopathic effect observed in the transfected cell sheet. The amounts of RNA transfected are expressed in μg .

As expected, the negative controls did not induce CPE in the transfected cells whereas transfection with RNA transcripts derived from the complete cDNAs of control viruses caused full CPE of the transfected cells. The absence of CPE observed in the test samples suggested that, under the conditions used, no viable recombinant viruses could be recovered between a replication-incompetent PV3 and EV7 with an in-frame deletion within the P1-coding region.

4.4 Discussion

Viable recombinant viruses were successfully recovered from *in vivo* selection experiments using two impaired PV3 viral RNA genomes. The experimental model was then applied to human enterovirus species C and species B defective genomes. Co-transfection of CVA21 Δ P1 and SL3 defective genomes and EV7 Δ P1 and SL3 defective genomes in RD-ICAM cells failed to generate viable recombinant viruses under the conditions used.

Previous studies have shown the successful generation of recombinants resulting from co-transfection of two impaired viral genomes *in vivo* (Chaabihi et al., 1997; Elahi et al., 2002; Gmyl et al., 2003; Gallei et al., 2004). Furthermore, the Hispaniola (Kew et al., 2002) and Madagascar (Rousset et al., 2004) outbreaks were demonstrated to have been caused by viable circulating VDPV strains that had emerged as a result of recombination between poliovirus vaccine strains and human enterovirus species C strains. It was therefore surprising that co-transfection of CVA21 and PV3 did not give rise to a recombination event leading to the generation of viable viruses.

4.4.1 Further investigations of the experimental conditions

Further control experiments are required to investigate whether the results seen were due to the defective clones being unable to generate functional proteins and replicate or whether recombination between CVA21 and PV3 is not possible under the conditions used. Study of CVA21 Δ P1 and EV7 Δ P1 translation needs to be carried out by *in vitro* translation assays using HeLa S10 nuclear extracts and rabbit flexi-reticulocyte lysates. The replication competence of the generated defective RNAs also need to be determined by dot-blot assay or replication assay using HeLa S10 nuclear extracts, as the unsuccessful generation of viable recombinant viruses from the *in vivo* selection experiment could have been due to the P1-deletion genomes having a defect at the level of replication. Such a defect could have prevented replicative recombination from occurring.

The precise co-transfection efficiency needs to be determined to ensure that the conditions used are optimal. Introduction of a tag-epitope during the *in vitro* generation of the P1 deletion defective CVA21 (CVA21-tag) and co-transfection of SL3 RNA with the CVA21-tag RNA into RD-ICAM cells or L20B cells would constitute the basis of a specific probing assay. Eight hours post-transfection the cells would be fixed and permeabilised. Transfected cells would then be treated with both anti-VP1 DAKO mouse monoclonal antibody (detecting the SL3 VP1 proteins), followed by treatment with a secondary anti-mouse antibody conjugated with FITC for example, and an anti-tag polyclonal antibody (probing for the CVA21-tag genome), followed by treatment with a secondary anti-polyclonal antibody conjugated with a different fluorochrome like TRITC. Confocal microscopy analysis would then be carried out to detect the percentage of dually transfected cells detected by co-localisation of the two different fluorochromes. The use of a fluorescence activated cell sorter (FACS) would enable detection and a more accurate quantification of dually-transfected cells. Once the co-transfection efficiency has been

determined, an improved design of the transfection technique will be possible thereby increasing the efficiency at which recombinants are generated.

Recent studies, have provided definitive evidence that recombination could occur via a replication-independent pathway (Gallei et al., 2004), probably involving cellular processes, suggesting that even in the event of the P1 deletion genomes being replication-incompetent, recombination could have occurred in a replication-independent manner. This hypothesis is further supported by the fact that defective RNA molecules can exert a complementing effect in a cellular environment and lead to cytopathic cell death (Garcia-Arrazia et al., 2004). The cellular environment used could have affected the results seen. The use of caco-2 cells, lining the intestinal epithelium, or pharyngeal cell lines may provide a more favourable environment for recombination between enteroviruses to occur. Moreover using such cell lines may better reflect a natural environment as it is in the sites of infection that recombination between two enteroviruses is more likely to occur.

Co-transfection of defective EV7 RNA with SL3 replication-incompetent RNA did not generate viable viral genomes. This could be due to the distant genetic relationship between EV7, a human enterovirus type B, and PV3. As we were unable to reproduce the situation observed in nature for human enterovirus species C *in vivo* or *in vitro* such a conclusion could not be made. However, it is possible that human enterovirus species B will never be able to recombine with human enterovirus species C. Because different species use different cell surface receptors for infection, it may be impossible for both genomes to be present in the same cell type at any one time. If the genomes are not in the same cellular environment they may never be able to recombine and could be why there has been no reported evidence of such an event happening in nature.

4.4.2 Speculations

There exist differences between SL3 and PV3 P1-coding regions. For example, the *Sac* I site present in the middle of the P1-coding region is not digested in the SL3, as opposed to the PV3 genome. It is known that these do not affect the production of viable viruses (Goodfellow et al., 2003; table 3.10; table 4.3).

It is possible that the lack of pre-determined crossover sites and the randomness of recombination sites could have affected RNA structures preventing viable viruses from being generated.

It is also possible that acytopathic recombinant viral genomes were generated in these experiments. The CVA21 2C CRE may have been unable to interact with the rest of the SL3 genome and therefore unable to take part in key interactions necessary for the generation of viable viruses. The primary screening method used in the described experiment did not enable the detection of acytopathic recombinant viruses and testing to determine whether the samples contained acytopathic viruses was not carried out due to time restrictions and prioritisation of other parts of the project.

Recombination during mixed infection with various strains of poliovirus has been known to be a common occurrence in vaccinees. Recombination has also been shown to occur during mixed infection with various strains of poliovirus, where both genomes replicated in the same compartments (Egger et al., 2002). However, no study so far has provided proof of different enterovirus species replicating in the same compartments and it could be that strains belonging to either the same species (CVA21 and PV3) or different species (EV7 and PV3) replicate in separate replication compartments thereby making replication-dependent recombination impossible.

4.4.3 Alternative reference genomes

It is expected that a genome exhibiting high homology with that of poliovirus would be more likely to recombine. Recent sequence analysis studies in which mass sequencing of complete genomes of the human enterovirus species A (Oberste et al., 2004b), human enterovirus species B (Oberste et al., 2004a) and human enterovirus species C (Brown et al., 2003) was carried out and inter- and intra-species amino acid percentage identity deduced from nucleotide sequences were calculated. These studies provided a better insight on evolutionary stages of human enteroviruses and a very useful report of homology levels that exist between enteroviruses. Some HEV-Cs, other than CVA21, have been shown to exhibit greater homology to PV3. CVA20, for example, would have been expected to be a better member to choose for the experiment, as it is 90.2% identical, based on complete amino acid sequence, to PV3. A more detailed comparison between CVA21, CVA20 and PV3 is described in table 4.11, based on the published data (Brown et al., 2003). The way the assay was conducted did not enable us to use CVA20, as no infectious cDNA was available.

Amino acid identity with PV3 (in %)	CVA21	CVA20
5'UTR	79	81
3'UTR	97	99
P1	70	78
P2	79	96
P3	96	97
Complete polyprotein	84.2	90.2

Table 4.11: Amino acid sequence relationships (percent identity) between two members of the human enterovirus species C (CVA21 and CVA20) and PV3 (data adapted from Brown et al., 2003).

An interesting alternative assay would be the transfection of SL3 RNA and subsequent co-infection with CVA20 virus. The progeny viruses would be harvested and passaged onto L20B cells, assuming CVA20 does not infect L20B cells, thereby only selecting PVR-binding viruses. It is unlikely that CVA20 would infect L20B cells as these are L cells expressing the PVR on their cell surface and that L cells are non-permissive for infection with any human enteroviruses. This would enable the selection of infectious recombinant viruses that have acquired an SL3 P1 coding region. This alternative assay may allow for recombination to occur. The characterisation of viable *in vivo* recombinants would then allow the mapping of recombination crossover sites and potential determination of recombination hotspots.

4.4.4 Suppression of synonymous sequence variation (SSSV)

Suppression of synonymous sequence variation (SSSV) is a program designed by David Evans. The program differentiates equal distribution of the codons encoding a single amino acid from bias towards the use of a preferred codon encoding for an amino acid in a particular area of the genome. If there is equal distribution of codons encoding an amino acid, no bias towards use of a particular codon exists and therefore no suppression of synonymous sequence variation is observed. This is represented by high synonymous variation values. However, a trough would indicate a region where synonymous sequence variation was suppressed and a bias towards the use of a particular codon was observed. A trough is a region where there is less variation in the sequence than the amino acid sequence would allow. These troughs are thought to represent conserved regions that may play key roles in the virus life cycle. This has been confirmed to be the case of the first trough observed on the graphs (figure 4.12). The trough corresponds to the 2C CRE stemloop structure, a structure essential for priming and initiating genome replication. It is possible that other regions of synonymous sequence variation are structured regions.

HEV-Bs and HEV-Cs sequences have been submitted to the SSSV analysis and the results are plotted on graphs illustrated figure 4.12. HEV-Bs and HEV-Cs exhibit different SSSV patterns in the non-structural coding region. The graph of HEV-Bs exhibits one trough, whereas the graph of HEV-Cs exhibits an additional two areas of strong suppression of synonymous variation. The trough present in both HEV-Bs and HEV-Cs graphs represents the region corresponding to the 2C CRE. The functions fulfilled by the two additional regions where troughs were observed are unknown, however sequence and maybe structure conservation suggests a functional role. If the HEV-Bs have a similar life cycle to that of HEV-Cs, some reactions are likely to be carried out in different ways, explaining the lack

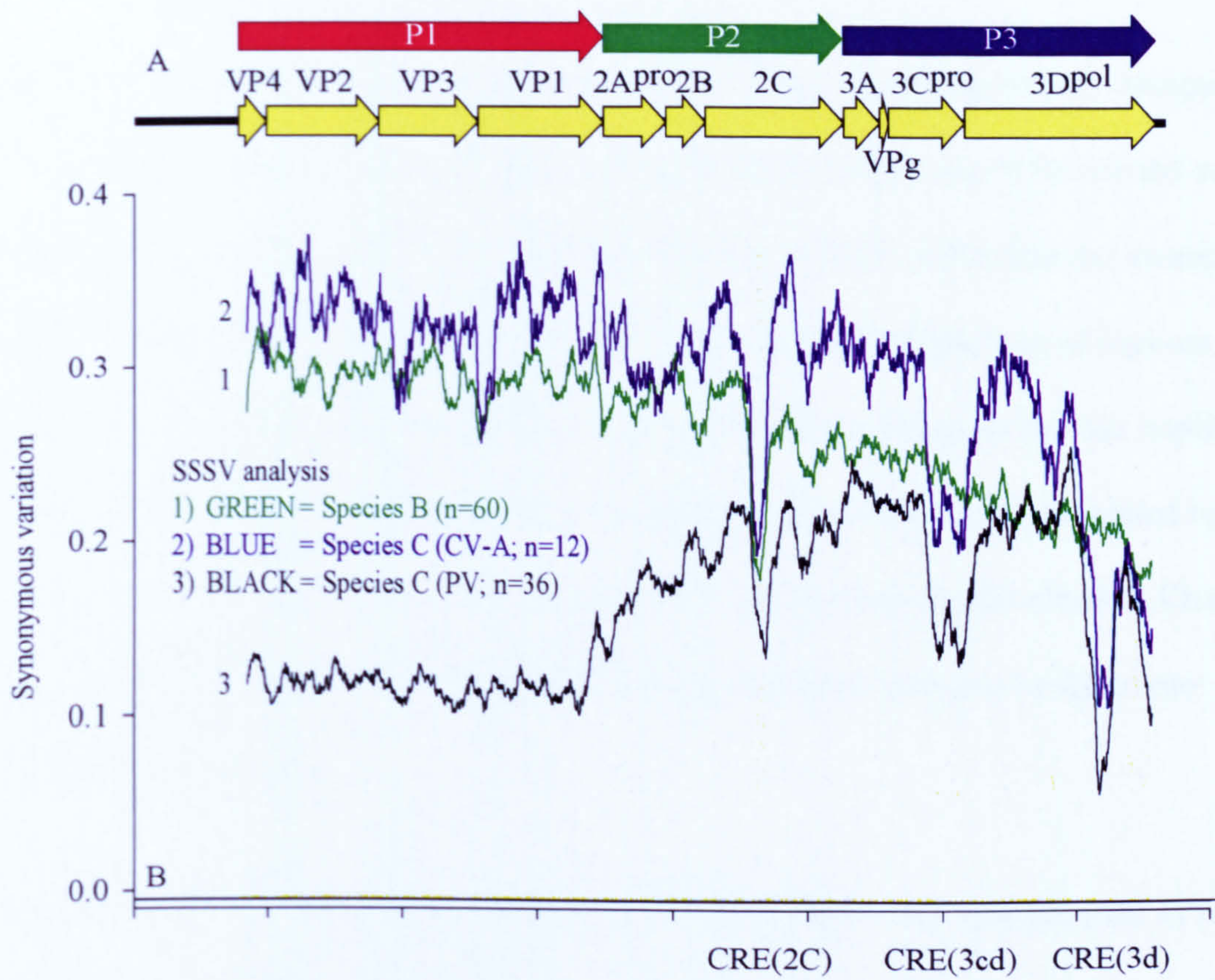


Figure 4.12: Suppression of synonymous sequence variation analysis, comparison of human enterovirus genera (figure generated by David Evans).

of troughs. Should one of these functions involve interaction with homologous capsid proteins, this would explain the absence of recombinants between HEV-Bs and HEV-Cs.

4.4.5 Future experiments

Should recombinant viruses be generated *in vivo* using a more accurately designed method or alternative assays, a series of characterisation experiments would be carried out. A number of sequencing studies would be initiated in order to determine the location and nature of the recombination crossover sites. Analysis and comparison of various recombinants may enable determination of recombination hotspots and the implications of such hotspots in enteroviral evolution. Comparison of viable *in vivo* generated recombinant viruses with the non-viable *in vitro* generated P1 recombinants, described in Chapter 3, may enable identification of the defect affecting the latter and give insights into picornaviral research.

An infectious centre type assay could also be undertaken to try and generate *in vivo* recombinants. Co-transfection of L cells, which have been shown to support replication of human enteroviruses post-transfection, with the two impaired parental genomes, could be carried out. 4-6 hours post-transfection, time point at which the cells would have recovered following the transfection but no newly generated virus would have been released, the co-transfected L cells would be lifted from the transfection flasks and transferred onto an 80%-confluent monolayer of L20B cells, cell line supporting poliovirus recombination, RD-ICAM cells or RD cells. Overlay media would be added and the samples would then be incubated at 37°C for three to five days to allow plaque formation of any *in vivo* generated recombinants. These would then be isolated from plaques, purified, amplified and characterised. This method is an alternative to the technique described in this chapter

and may enable isolation of *in vivo* generated recombinant viruses as opposed to populations of mixed recombinants.

The advantage of generating recombinants *in vivo* is that a scenario closer to that seen in nature is reproduced in a tissue culture environment. As opposed to *in vitro* generation of recombinants the crossover sites are not pre-determined and are more likely to mimic a natural situation. However, the use of novel techniques such as small interfering RNAs (siRNAs) and DNA shuffling to select for recombinants between human enterovirus species in a laboratory environment are interesting alternatives to the method used in this chapter (see chapter 5: General discussion).

5 General discussion

In view of the poliomyelitis eradication program, several issues have become major concerns. What are the chances of a non-poliovirus enterovirus evolving to use the poliovirus receptor? Would such a virus cause poliomyelitis? What are the implications of such recombinants? The project aimed to investigate some of these issues.

It was initially proposed to use DNA shuffling to create chimeric mosaic capsids that may interact with different cell surface receptors. DNA or gene shuffling is an elegant process that enables the production of *in vitro* recombinants with mosaic sequences of gene families and diverse libraries of progeny genes. This technique is very ambitious, as it requires very technically demanding DNA manipulations, and a number of different stages could become problematic. Firstly, stop codons, truncation of the sequences and incorrect open reading frames leading to inefficient translation could arise from DNA shuffling experiments. Secondly, full-length nonsense sequences encoding the capsid proteins could have been generated rendering any resulting viruses non-viable. Finally, full-length in-frame generated sequences of the capsid protein-coding regions could be processed inefficiently by heterologous 3C and 3CD proteases. It was therefore important to study processing requirements closely. To do so, recombinants with a precise exchange of the P1-coding region between poliovirus type 3 and CVA21, a HEV-C, were generated *in vitro*. The experiment developed into a very interesting analysis of chimeras. The *in vitro* generated recombinants were unable to cause cytopathic effects upon transfection and passage into cell lines permissive for infection and replication of both parental genomes. However, the chimeras appeared to produce the correct proteins and to exhibit the correct protein-processing pattern. Further assays indicated that the recombinants were replication-competent and suggested that these may have had a late replication defect such

as a packaging defect. These results indicated that relatively divergent capsids could be processed by viral proteases suggesting that the heterologous mosaic capsids created by DNA shuffling may also be processed by 3CD protease.

The DNA shuffling technique has been applied to create recombinants of single genes such as cytokine (Chang et al., 1999), thymidine kinase (Christians et al., 1999), green fluorescence protein (Crameri et al., 1996), subtilisin (Ness et al., 1999) and a fucosidase (Zhang et al., 1997). However, until recently DNA shuffling had not been applied to viral genes. In 2000, studies described the use of the method to alter receptor tropism of a retrovirus by creating a recombinant mosaic of the envelope glycoprotein gene (Soong et al., 2000). Since then, a variety of studies have used DNA shuffling for recombinant generation, functional gene analysis and other purposes. RACHITT (RANdom CHimeragenesis on Transient Templates) is one of the best available DNA shuffling techniques (Pelletier et al., 2001; Coco et al., 2001; Coco et al., 2003) and it was planned to use this approach for the project.

RACHITT, a templated assembly of mosaic sequences, could be used to generate HEV-C/PV recombinants with a mosaic P1-coding region, by shuffling several HEV-Cs or polioviruses P1 genes. Although ambitious, the potential recovery and characterisation of a PVR-tropic recombinant HEV-C virus would be very valuable.

A range of relatively closely related input polioviruses and non-poliovirus HEV-Cs would be used for the experiment. The entire capsid-coding gene or a range of individual capsid genes of various polioviruses would be shuffled and introduced into a non-poliovirus HEV-C backbone vector containing the remainder of the coding and non-coding sequences. The experimental method is illustrated figure 5.1. First, top strands of the P1-coding regions of heterologous polioviruses would be partially digested with DNase I.

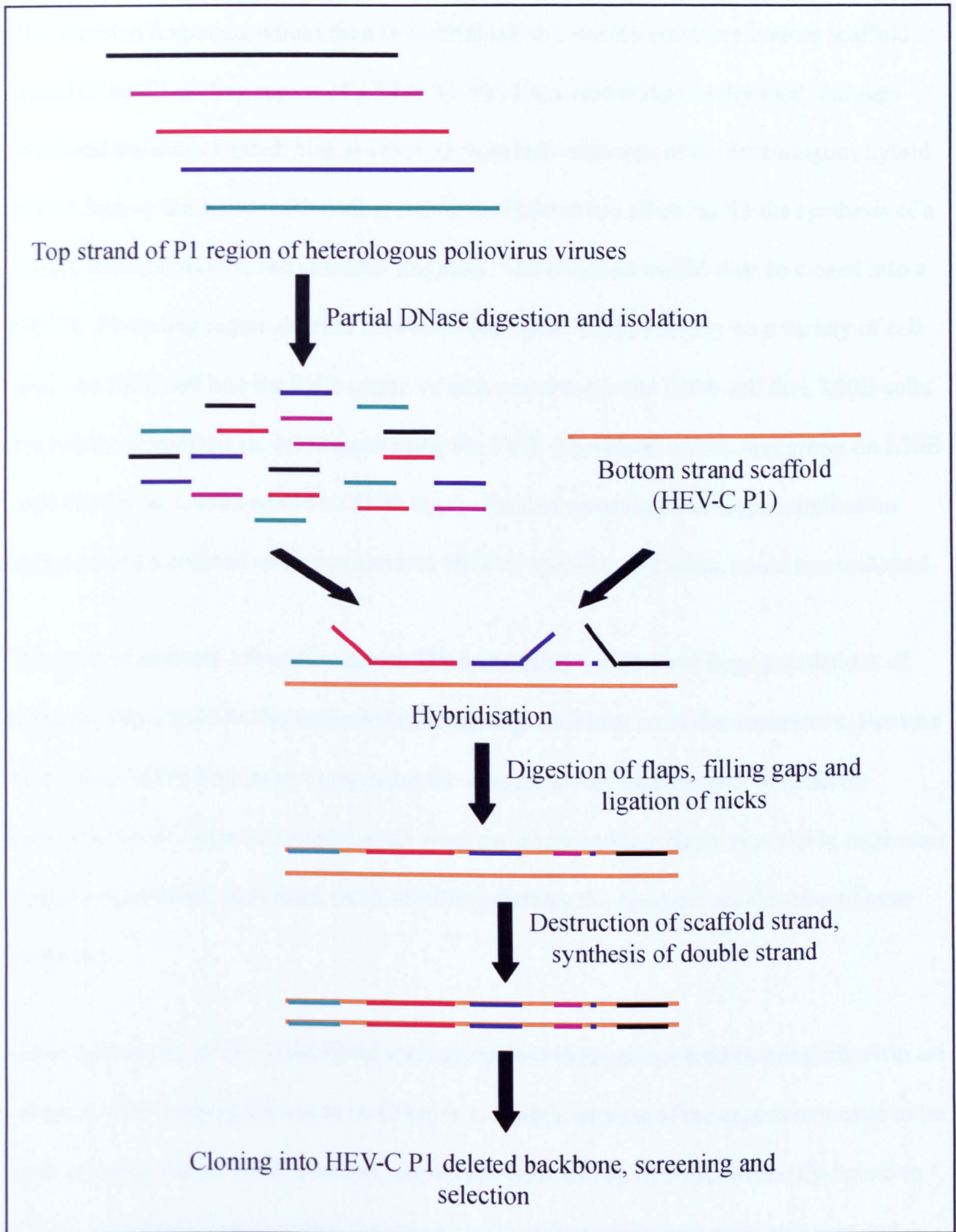


Figure 5.1: DNA shuffling

DNA shuffling using RACHITT: Random chimeragenesis on transient templates, method first used by Stemmer *et al.* (1994) for retroviruses. Adapted for accelerated HEV-C evolution.

The digested fragments would then be hybridised to a uracil-containing bottom scaffold strand of the P1-coding region of a HEV-C. The flaps would then be digested, the gaps filled and the nicks ligated. Uracil-DNA-glycosylase treatment of the heterologous hybrid would destroy the HEV-C P1-coding region scaffold strand allowing for the synthesis of a double stranded mosaic recombinant fragment. The fragment would then be cloned into a HEV-C P1-coding region deleted backbone and screened for viability on a variety of cell lines. An ideal cell line for PVR-tropic viruses screening is the L20B cell line. L20B cells are murine fibroblasts (L cells) expressing the PVR. Therefore, a virus that grows on L20B cells but not on L cells would be PVR-tropic. Further screening utilising neutralisation assays, using a cocktail of non-poliovirus HEV-C specific antibodies, could be conducted.

The method presents a few drawbacks. DNA shuffling would yield large populations of chimeras that would not be subjected to screening until later on in the experiment. Because of the sizes of the fragments composing the mosaic, a very high number of different combinations of sequences could result from the assay and inevitably non-viable sequences would be generated. However, DNA shuffling enables the dramatic acceleration of gene evolution.

Other approaches to DNA shuffling such as random mutagenesis studies using ribavirin are possible. This assay is discussed in Chapter 2. Certain aspects of the experiment need to be more formally tested. Such a method combined with the use of PVR covalently-bound to affinity chromatography column would make the screening process more stringent and could increase the chances of selecting for novel tropic viruses. However, upon binding to PVR viral uncoating occurs and the chromatography isolation of mutated virus populations binding the PVR with different degrees of affinity would need to be carried out at 0-4°C. Elution of low-affinity PVR-bound viruses using high salt concentrations and RNA extraction for subsequent transfection would then be carried out. Following transfection the

samples would be submitted to several rounds of replication thereby enhancing the affinity for PVR of the isolated viruses. This method may enable isolation and study of novel tropic mutated viruses.

Although the project diverged from the initial aim, the results obtained from the *in vitro* generated recombinant studies were very interesting and could be very valuable for poliovirus research. Further investigations could lead to the identification of a, yet to be determined, specific encapsidation signal and/or interactions involved in packaging of the viral genome. In order to investigate this result further, forced recombination in a tissue culture environment without predetermining the crossover sites should to be carried out. *In vivo* recombination experiments did not yield any significant data. However, there are alternate ways of applying selective pressure and forcing recombination between two viruses in tissue culture, such as RNA interference.

RNA interference (Fire et al., 1998) is a very useful tool for functional gene analysis, gene silencing and therapeutic use (Cheng et al., 2003). Small double stranded fragments of RNA (small interfering RNAs: siRNAs) have been shown to induce sequence specific degradation of RNA. In fact, binding of siRNAs to single genes leads to suppression of gene expression. RNA interference has been identified in many organisms such as plants, trypanosomes and fungi and is thought to be a means of protecting an organism against viral infection (reviewed Saleh et al., 2004). Recent studies indicated that 19-23 nucleotide-long synthetic siRNA fragments could trigger strong and specific gene silencing (Elbashir et al., 2001).

The use of siRNAs, in a tissue culture environment, may enable the generation of recombinant viruses with altered tropism, and more specifically may allow recovery of a PVR-tropic non-poliovirus HEV-C. An experimental assay was designed to investigate this

and is illustrated in figure 5.2. Recent studies have demonstrated the existence of siRNA escape mutants that are resistant to specific RNA degradation necessitating the use of multiple siRNAs for efficient silencing of the input genome (Gitlin et al., 2005). Hence, several siRNAs would be synthesised to bind a number of genes: CVA21 VP1, VP2, VP3 and VP4-coding regions binding siRNAs and PV3 2C CRE and 3C-specific binding siRNA fragments. Due to the highly specific nature of RNA interference, any input sequence could be used and a range of genomes could be tested. Initial silencing would be carried out on both CVA21 and PV3 genomes in a tissue culture environment, thereby disabling the genomes to yield viable viruses. These two partners, when present in the same cellular environment, should be subjected to sufficient selective pressure to generate chimeras. The assay could then be extended to a wider range of different partners.

The emergence of siRNA escape mutants, the need for high numbers of siRNAs and the off-target effects exerted by siRNAs on host cells should not be ignored (Couzin et al., 2004). However, the method would allow a forced recombination event between different junctions points. Analysis, identification of crossover points, characterisation of the biology of potentially viable *in vivo* generated recombinants and comparison with the *in vitro* generated precise exchange of the P1-coding region recombinants may give an insight into different areas of picornavirus research such as genome cellular localisation, *cis*-acting replication and encapsidation of progeny viral genomes into newly synthesised capsids.

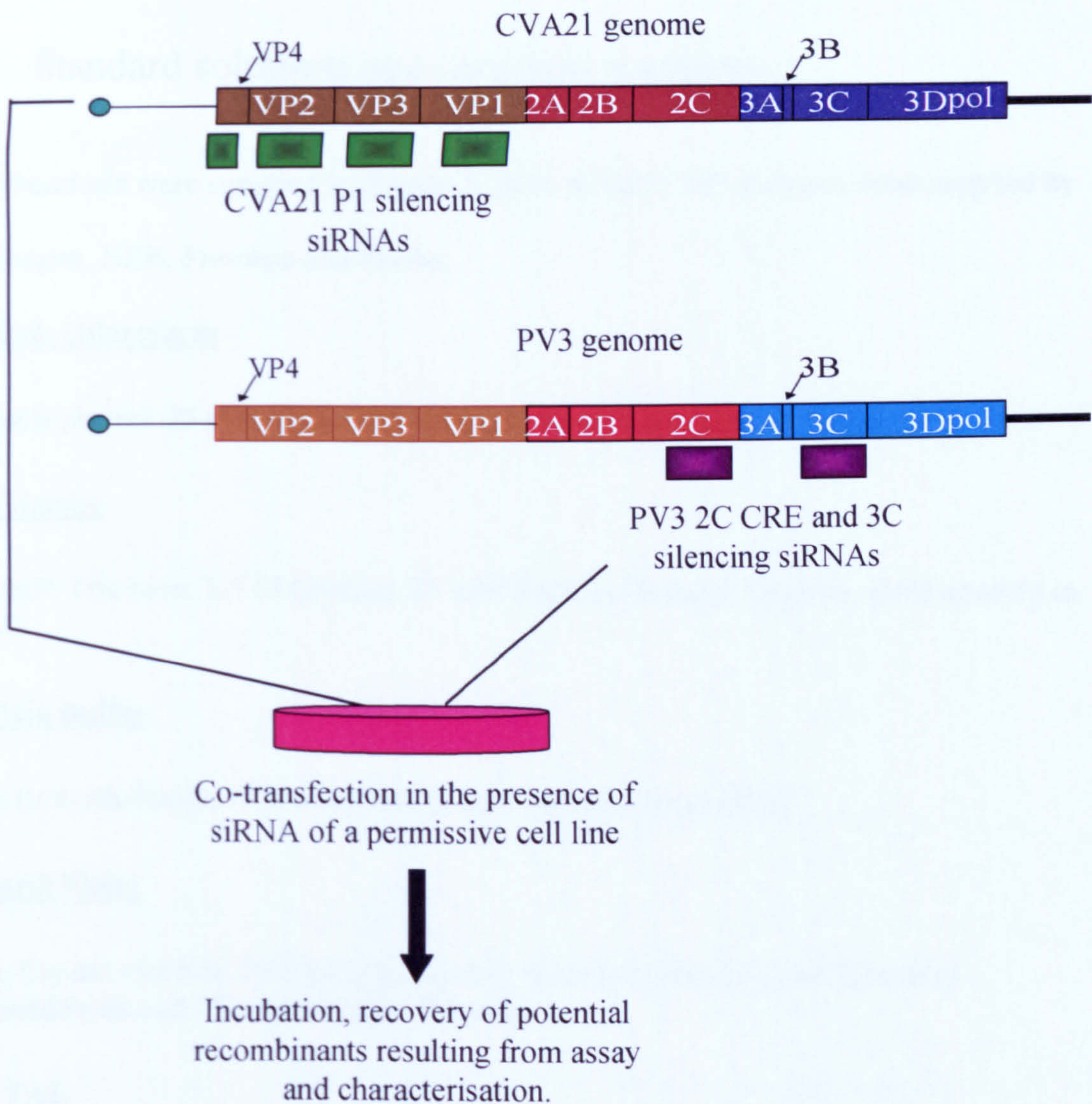


Figure 5.2: siRNA gene silencing recombination in tissue culture

Diagram illustrating the use of siRNAs in tissue culture as means of obtaining recombinants without predetermining crossover sites.

6 Materials and Methods

6.1 Standard solutions and chemical suppliers

All chemicals were supplied by Sigma-Aldrich or BDH. All enzymes were supplied by Invitrogen, NEB, Promega and Roche.

Phenol: chloroform

25 parts phenol: 25 parts chloroform: 1 part isoamyl alcohol

10x Gringo

250 mM Tris-base, 2.5 M Glycine, 35 mM Sodium Dodecyl Sulphate made up to 5 l in dH₂O

Destain buffer

10% (v/v) Methanol, 10% (v/v) Acetic acid and 80% (v/v) dH₂O

Crystal Violet

0.5 g Crystal violet in 20ml of Ethanol, 880 ml dH₂O, 10% (v/v) of 37%(w/v) Formaldehyde and 15 mM Sodium Chloride.

50x TAE

2 M Tris-base, 57.1 ml Glacial Acetic Acid, 10 mM EDTA (pH 8.0) made up to 1 l in dH₂O

Mini-prep solutions

Solubilisation: solution 1 (4°C)

50 mM Glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0)

Cell lysis: Solution 2 (room temperature)

200 mM Sodium Hydroxide and 1% SDS

Neutralisation: Solution 3 (4°C)

3 M Potassium Acetate, 11.5 ml Glacial Acetic Acid and 28.5 ml dH₂O

RNase A

2 g RNase A (SIGMA, kept at -20°C) in 200 mls 10 mM Tris (pH 7.8) and 15 mM Sodium Chloride. Boiled for 15 minutes to destroy DNase and allow to cool slowly. Store at -20°C

DEPC-treated water

0.1% DEPC (SIGMA) to dH₂O. Left at 37°C overnight and autoclaved twice.

2x protein reducing gel loading buffer

2 ml Glycerol, 1.5 ml 10% Sodium Dodecyl Sulphate, 0.625 ml 1M Tris (pH 6.8), 0.375 ml dH₂O, a spatula's worth of Bromophenol Blue and 50 µl Mercaptoethanol was added to every 1 ml of solution.

Guanidine Hydrochloride (GuHCl)(SIGMA)

100 mM GuHCl in dH₂O

6x DNA gel loading buffer

7 ml dH₂O, 3 ml Glycerol and a spatula's worth of Bromophenol Blue or Orange G.

10% (w/v) Ammonium Persulfate**20xSSC**

3 M Sodium Chloride, 300 mM Sodium Citrate made up to 500 mls in dH₂O.

Hybridisation buffer

Rapid-hyb buffer supplied by Amersham Biosciences.

RNA denaturation buffer

660 µl Formamide, 210 µl 37%(w/v) Formaldehyde and 130 µl 10x MOPS electrophoresis buffer.

10x MOPS

200 mM MOPS, 80 mM Sodium Acetate, 1 mM EDTA and 100 ml of dH₂O.

Western Blot Transfer buffer

1% (w/v) Sodium Dodecyl Sulphate, 570 mM Glycine, 50 mM Tris-base, 20% (v/v) Methanol and 160 ml dH₂O.

Western Blot Blocking buffer

5% (w/v) dried skimmed milk, 0.1% (v/v) Tween 20 in Phosphate Buffered Saline (PBS).

Cell lysis buffer

50 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0) and 100 mM Sodium Chloride.

“Spiller” radiolabelling buffer

1 M Sodium Chloride, 20 mM Tris-HCl (pH 7.5) and 0.1% BSA in dH₂O.

Blue cell assay developing solution

9.560 ml PBS, 2.500 ml 0.5M Potassium Ferrocyanide, 2.500 ml 0.5M Potassium Ferricyanide, 0.500 ml 1M Magnesium Chloride and 6.250 ml X-gal (1,4-O-β-D-galactopyranosyl-D-glucose) (20 mg/ml).

Plaque overlay media

10% (v/v) 10x EMEM: Eagle's minimum essential medium (GIBCO Life technologies), 50% (v/v) dH₂O, 1% (v/v) L- glutamine (GIBCO Life technologies), 2% (v/v) FCS (Foetal calf serum), 1% (v/v) Penicillin/ Streptomycin (GIBCO Life technologies), 3% (v/v) of 7.5% Sodium bicarbonate (GIBCO Life technologies) and 30% (v/v) of 2% Bactoagar.

6.2 List of antibodies

Antibodies	Database reference	Dilution	Usage
Anti-PVR 280	A-AB-5H	1 in 50/ 1 in 200	Monoclonal antibody to PVR
Anti-PV3	A-AA-1E	1 in 200	Rabbit polyclonal antibody to poliovirus type 3
Anti-Polio type 1	A-AB-1D	1 in 200	Sheep 16- sheep polyclonal to poliovirus type 1
Anti-VP1 DAKO		Western blot: 1 in 1000 Immunofluorescence: 1 in 400	DAKO mouse monoclonal antibody against enterovirus VP1
Anti-mouse FITC		1 in 200	Anti-mouse FITC conjugated polyclonal antibody
Anti-mouse HRPO		1 in 5000	Anti-mouse HRPO conjugated polyclonal antibody

Table 6.1: Table of Antibodies

6.3 List of oligonucleotide primers

Name of oligonucleotide	Database reference	Nucleotide sequence
ID6	O-AB-9I	GGTGTAGAGAAAATTGGTTTTGG
T7R3D	O-AD-9C	AGGGATATCACTCAGCATAATAATGAGTCAAGCCAAC
GEN-3'BK'F	O-AA-2F	ATATGAGCTCACCACWCCCGGGTTTGGSCAYCAG
GEN-3'BK-R	O-AA-7E	CCYACATACATGTTGGGGTACTTACTAGTGTTTC
T7-F	O-AA-7H	ATACGACTCACTATAGGGCG
CA21-5'-BK-R	O-AA-1F	ACTTGCGCTCCCATTTCAC
GEN-3'BK-F-Nae	O-AB-7C	RASGAGCTCACCACWGCCGGCTTYGGMCACCARAAC
GEN-3'BK-R	O-AA-7E	CCYACATACATGTTGGGGTACTTACTAGTGTTTC
P1-GEN-F	O-AA-8E	ATGGGAGCTCARGTDC
PV3-P1-R	O-AD-6D	ATATGTGGTCAAACCTTTCTC
CA21-P1-R	O-AC-3B	AAAAGTGGTAATTGAGTCTAC
PV3/CA21-P1-R	O-AA-3F	TGSCCAAATACGTAWGTGG
Sabin1-P1-R	O-AC-4B	ATATGTGGTCAGATCCTTG
ENT GEN 2F	O-AA-9C	GGAACCGACTACTTTGGG
ENT GEN 2R	O-AA-1D	TCNNGNARYTTCCACCAC
ENT PCD 6R	O-AA-5E	TGGTARTTRCWRYTYTTG
CAV21 F0	O-AA-6G	CGGTGCACACGAGAATCAAA
CAV21 R11	O-AA-5G	CGACTGGAAGTCATCAGATG
CVA21P1delDJE1	O-AE-8E	CCGGGATCTCGAGG
CVA21P1delDJE2	O-AE-9E	CTAGCCTCGAGATC
Polio-End	O-AA-7B	ATAAGTCGACTTTTTTTTTTTTTTTTTTTTTTTTCTCCT CCGAATTAAAGAAAATTACC
ENT PCD 6F	O-AA-6E	TMACHGCHGTBGARACWGG
IDI	O-AB-2I	TTAAAACAGCTCTGGGGT
LUCF	O-AB-9I	CATCTTCGACGCGGGCGT
PV3-VP2-F	O-AD-8D	GCCATTCTGCCCTTATCACCG
PV3-VP3-R	O-AD-9C	CATTACTAATCCACGGCAC
PV33D-T7R	O-AD-9D	AGGGATATCACTCAGCATAATAATGAGTCAAGCCAAC
PV3-VP3-3F	O-AD-7D	GCCAATGACCAGATTGGTG
CAV21 CAPF	O-AA-1H	CTTCGAGAAGCCTAGTATCG
CB3-1F	O-AB-8A	ATCCGGTGACCAATAGAGC
591-5R	O-AD-5C	TGTCTGAAGTACAGCTTGCC

Table 6.2: List of oligonucleotides

6.4 List of plasmids

Plasmid name	Description	Simplified plasmid name
pS1F	Full length poliovirus Sabin type 1 infectious clone	pS1F
CAV21	CVA21 full-length infectious clone	CAV21
pT7FLC	Full length poliovirus type 3 Leon P3/Leon/37 infectious clone	pT7FLC
pT7FLC/REP3	Poliovirus type 3 Leon P3/Leon/37 CAT replicon derived from pT7FLC	pT7FLC/REP3
pT7FLC/REP3-BK	Backbone vector lacking the complete P1-coding gene derived from pT7FLC/REP3	PV3BK
pT7REP3-BK-SL3-P1	Backbone vector PV3BK with insertion of the complete PV3 P1-coding region introduction derived from pT7/SL3	PV3BKPV3P1
pT7REP3-BK-CA21-P1	Backbone vector PV3BK with insertion of the complete CVA21 P1-coding region introduction derived from CAV21	PV3BKCVA21P1
pT7/SL3	Full length poliovirus type 3 Leon P3/Leon/37 with mutations in the 2C CRE derived from pT7FLC	pT7/SL3
pT7REP3-L	Poliovirus type 3 Leon P3/Leon/37 luciferase replicon derived from pT7FLC/REP3	pT7REP3-L
pRibo1	Vector containing a ribozyme hammerhead	pRibo1
pRibo-CAV21	CVA21 full-length infectious clone introduced in a ribozyme hammerhead-containing vector derived from CAV21 and pRibo1	pRibo-CAV21
pRibo-CAV21-Nae1Del	CVA21 full-length infectious clone with a deleted Nae I site, derived from pRibo-CAV21	pRibo-CAV21-Nae1Del
pRibo-CAV21-Nae1Del-backbone	CVA21 backbone vector lacking the complete P1-coding gene, derived from pRibo-CAV21-Nae1Del	CVA21BK

Plasmid name	Description	Simplified plasmid name
pRibo-CAV21-BK/CA21 P1	CVA21 backbone vector with insertion of the complete CVA21 P1-coding region, derived from pRibo-CAV21-Nae1Del-backbone and CAV21	CVA21BKCVA21P1
pRibo-CAV21-BK/PV3-P1	CVA21 backbone vector with insertion of the complete PV3 P1-coding region, derived from pRibo-CAV21-Nae1Del-backbone and pT7/SL3	CVA21BKPV3P1
pRibo-CAV21-BK/Sabin1 P1	CVA21 backbone vector with insertion of the complete Sabin1 P1-coding region, derived from pRibo-CAV21-Nae1Del-backbone and pS1F	CVA21BKSabin1P1
pT7EV7	Full length echovirus 7 infectious clone	pT7EV7
EV7 Δ P1	EV7 genome with an in-frame Nsi I deletion in the P1-coding region, derived from pT7EV7	EV7 Δ P1
CVA21 Δ P1	CVA21 genome with an in-frame Age I deletion in the P1-coding region, derived from CAV21	CVA21 Δ P1

Table 6.3: List of plasmids

6.5 Cell culture and virological methods

6.5.1 Digestion of DNA with restriction endonucleases

Restriction digest reactions were carried out in the 1x buffer recommended by the manufacturers in the presence of 1 unit (U) of restriction enzyme per μg of DNA. The reactions were made up to final volumes of 20 μl in the case of analytical digests or 50 μl in the case of preparative digests and were carried out at the temperatures recommended by the manufacturers for 1 to 4 hours.

6.5.2 Plasmid DNA preparation for *in vitro* transcription

5 μg of plasmid DNA, containing a T7 promoter, was linearised with the appropriate restriction endonuclease, phenol-chloroform extracted and ethanol precipitated. After washing in 70% ethanol, the DNA was pelleted, dried and resuspended in 20 μl clean dH_2O .

6.5.3 *In vitro* transcription using T7 RNA polymerase

1 μg of resuspended DNA was incubated in 1x transcription buffer (NEB), 1mM rNTPs, 0.5 μl of RNase OUT (Invitrogen), 1 μl of T7 DNA polymerase (NEB) made up to a final reaction volume of 25 μl with clean dH_2O . The reaction was incubated at 37°C for 2 hours. 10% of the reaction volume was checked on an agarose gel and quantified on a spectrophotometer.

6.5.4 *In vitro* transcription using Ribomax

1 µg of linearised DNA was incubated in 6 µl of 5x T7 buffer, 2.3 µl rATP, 2.3 µl rGTP, 2.3 µl rCTP, 2.3 µl rUTP, 3 µl of T7 enzyme mix and clean dH₂O (30 µl reaction mix) provided in the RiboMAX™ Large scale RNA Production Systems-T7, for 2 hours at 37°C. The newly transcribed RNA reactions were then RQ1Dnase treated (1 U/µg of DNA transcribed) for 30 minutes at 37°C and heat inactivated at 65°C for 15 minutes. Samples were checked on an agarose gel and quantified in a spectrophotometer.

6.5.5 RNA transfection of mammalian cell lines

6.5.5.1 DEAE-dextran

1-10 µg of T7 RNA transcripts was incubated in 1xHepes-buffered saline, pH 7.1 in clean dH₂O with 200 µg/ml DEAE-dextran and 10 µl 10x filtered glucose solution on ice for 30 minutes. Prior to cell transfection, the pre-seeded 80% confluent 6-well dish cell monolayers (RD-ICAM cells, Hela cells, RD cells) were washed with Serum Free DMEM (SF-DMEM). To each well, 100 µl of the reaction mix was added and incubated at room temperature for 30 minutes ensuring the cell monolayers did not dry out. The transfection mix was then removed and 4 ml of DMEM supplemented with 10% FBS (Foetal Bovine Serum) were added to each well and the dishes were incubated at 37°C in a humidified 5% CO₂ incubator.

6.5.5.2 Electroporation

Cells of a confluent T175 flask were resuspended using PBS-125mM EGTA and centrifuged at 2000 rpm for 5 minutes (Multifuge 3 S-R Heraeus). The cell pellet was resuspended in 500 µl PBS per reaction and transferred with 1-10 µg of T7 RNA to 4 mm

electroporation cuvettes for mammalian cells (CLP). The settings used for transfection were: 250 V, 400 Ω , 250 μ F. The cells were then added to a T25 tissue culture flask containing 4.5 ml of 10% DMEM and incubated at 37°C in a humidified 5% CO₂ incubator.

6.5.5.3 Lipofectamine 2000

10-15 μ g of RNA in solution was made up to 100 μ l in OPTIMEM medium (GIBCO) and mixed and vortexed with a solution of 10-15 μ l of LipofectamineTM 2000 (Invitrogen) and 90-85 μ l of OPTIMEM. The mixture was incubated at room temperature for 10-15 minutes and a further 800 μ l of OPTIMEM was added to the mix. Before transfection the 80%-confluent cell sheet was washed with OPTIMEM. 1 ml of the vortexed solution was added to each well of a 6-well dish and incubated at 37°C for 4-5 hours. The mix was removed and 4 ml of 10% DMEM was added to each well and incubated at 37°C.

6.5.6 Infection of cell monolayers with virus

80% confluent cell monolayers were pre-washed with SF-DMEM, virus was added to an MOI (multiplicity of infection) of 1-10 and incubated at 37°C in a humidified 5% CO₂ incubator for 30 minutes. The virus supernatant was discarded and DMEM supplemented with 10% FBS was added to the infected cell sheet.

6.5.7 Purification of viral RNA

6.5.7.1 Using Trizol[®] Reagent

Flasks of virus-infected cells were frozen and thawed twice. 750 μ l Trizol[®] Reagent (Invitrogen) was added to virus-infected cells, vortexed and left at room temperature for 10 minutes to ensure homogenisation and degradation of DNA. 200 μ l of chloroform was

added and centrifuged for 15 minutes at 13,000 rpm (in a Biofuge *pico* Heraeus instruments) at 4°C. The upper phase was added to isopropanol and left at room temperature for 10 minutes before precipitation by centrifugation at 13,000 rpm for a further 10 minutes at 4°C (in a Biofuge *pico* Heraeus instruments). The RNA pellet was washed with 75% ethanol and then the pellet was left to air-dry. The air-dried pellet was resuspended in 50 µl clean dH₂O and incubated at 55°C for 10 minutes to ensure correct solubilisation of the extracted viral RNA.

6.5.7.2 Using QIAamp® Viral RNA mini kit

vRNA extraction was carried out using a QIAamp® Viral RNA mini kit. A volume of 140 µl of harvested virus was added to 560 µl of prepared Buffer AVL containing carrier RNA in a 1.5 ml clean tube. The mixture was mixed by pulse-vortexing for 15 seconds and incubated at room temperature for 10 minutes. The samples were centrifuged briefly to remove drops from the inside of the lid and 560 µl of 100% ethanol were added to each tube. The mixture was mixed by pulse-vortexing for 15 seconds and centrifuged briefly. 630 µl of the mix were carefully applied to a QIAamp spin column, which were then centrifuged at 8000 rpm for 1 minute (in a Biofuge *pico* Heraeus instruments). This step was repeated with the remainder of the sample and using a clean collection tube. Clean collection tubes were used for individual steps. 500 µl of Buffer AW1 were then added to each column and these were centrifuged at 8000 rpm for 1 minute (in a Biofuge *pico* Heraeus instruments). 500 µl of Buffer AW2 were added to each column and these were centrifuged at 13000 rpm for 3 minutes and then at 13000 rpm for 1 minute (in a Biofuge *pico* Heraeus instruments). 60 µl of Buffer AVE or elution buffer were added to each column in clean collection tubes, the columns were incubated at room temperature for

1 minute and centrifuged at 8000 rpm for 1 minutes (in a Biofuge *pico* Heraeus instruments).

6.5.8 TCID₅₀

Infectious titre of virus was routinely calculated by TCID₅₀ (Tissue culture infectious dose 50). One tenth of a confluent T75 tissue culture flask of cells was evenly distributed in 100 µl volumes across a 96-well plate containing quadruplicates of 50 µl set of serial 10-fold dilutions of virus. Three-to four days post infection crystal violet stain, supplemented with formaldehyde, was added to the monolayers for two to three hours. The non-infected cell sheets were stained and the cells, killed by infection, did not retain staining. The virus titre was expressed as the dilution of the virus required to infect 50% of the cultures (Reed & Muench, 1938). If CPE was observed in half of the wells at a dilution of 10⁻⁷, the titre was 10⁷ TCID₅₀ units in 50 µl. If all wells show CPE at 10⁻⁷ and none at 10⁻⁸, the titre is 10^{7.5} TCID₅₀ units in 50 µl. One well corresponds to approximately 10^{0.25} TCID₅₀ units. Titres were expressed as TCID₅₀/ml.

6.5.9 Plaque Assay

RD, Hela, L20B or RD-ICAM cells as appropriate were seeded into 6-well plates and grown to 80% confluent monolayers. Serial 10- to 100-fold dilutions of the virus stocks were made in serum-free media. The cell sheets were then washed a couple of times in serum free media and inoculated with 200 µl of the diluted virus. The virus was allowed to absorb for 30–45 minutes at room temperature and the plaque overlay media was added to the infected monolayers. The inverted dishes were incubated for three to four days at 37°C in a humidified 5% CO₂ incubator. Crystal violet solution was added to the dishes for two hours, the plaque overlays were then removed and further staining was carried out for two

to three hours. The plaques were counted and expressed as plaque forming units per ml (pfu/ml).

6.5.10 Virus neutralisation assay

Viruses were pre-incubated with two-fold serial dilutions (50 μ l) of neutralising antibody in serum free DMEM starting at 1:50 for one hour at 37°C. 100 μ l of cells were added to the wells and infection was allowed to proceed for 24-48 hours prior to crystal violet staining.

6.5.11 Virus blocking assay

Monolayers of the appropriate cell line in a 96-well plate were incubated with two-fold serial dilutions (50 μ l) of neutralising antibody in serum free DMEM starting at 1:50 for one hour at 37°C. 10^4 TCID₅₀ (100 μ l) of virus was added to the wells and infection was allowed to proceed for 24-48 hours prior to crystal violet staining.

6.5.12 Synthesis of radiolabelled virus

RD, RD-ICAM or Hela cells were pre-treated with Methionine-free Minimum Essential Medium Eagle (MEM) (Sigma) for one hour at 37°C in a humidified 5% CO₂ incubator. The 80% confluent cell sheet of a T75 tissue-culture flask was infected with virus at an MOI of 10. The virus was adsorbed at room temperature for 30 minutes and the cells were washed and incubated with 10ml Methionine-free MEM for 2 hours. 10 ml Methionine-free MEM supplemented with 200 μ Ci [³⁵S] methionine was added to the infected cells and this was incubated overnight at 37°C in a humidified 5% CO₂ incubator. The virus was then harvested after 2 freeze-thaws and filtered through a 0.20 μ m filter. The radiolabelled virus was partially purified by pelleting through a 12 ml linear 30% sucrose

gradient at 40,000 rpm (Sorvall Ultra *Pro* 80 centrifuge) using a TH641 rotor at 4°C for 6 hours. The CVA21 pellet was resuspended in 300 µl Spiller buffer. All other viruses were resuspended in 300 µl of PBS-BSA.

The virus was further purified by centrifugation through a 12 ml linear 10-25% sucrose gradient at 40,000 rpm (Sorvall Ultra *Pro* 80 centrifuge) using a TH641 rotor at 4°C for 1 hour and 20 minutes to obtain infectious 160S particles. The gradient was harvested from the bottom in 500 µl aliquots. 5 µl of each fraction was counted in 3 ml of scintillant (Ecosint A scintillation fluid by National Diagnostics) by scintillation counting (Beckman LS5000CE machine). 160S particles, detected by a peak reading, were typically found in fractions 5 and 6. The sucrose was removed by washing with PBS-BSA or Spiller buffer and loaded into 1.5 ml Beckman Ultracentrifuge tubes in equal portions and centrifuged overnight at 40,000 rpm (Beckman TL-100 Ultracentrifuge) using a TLS55 rotor at 4°C. The 160S-pelleted particles were carefully resuspended in 100 µl of PBS-BSA and stored at -20°C for up to 4 weeks.

6.5.13 Synthesis of infected cell lysates

An 80%-confluent cell sheet was infected at an MOI of 10 for 30 minutes at room temperature and washed with SF-DMEM. 1 ml of 10% DMEM was added to the infected cells and incubated for 2 hours at 37°C in a humidified 5% CO₂ incubator. Timing enabled viral proteases-mediated host cell shut off and insured that the proteins labelled were of virus origin. The washed cells were supplemented with 1 ml of Methionine-free MEM for 30 minutes at 37°C in a humidified 5% CO₂ incubator. The medium was removed and 1 ml of Methionine-free MEM supplemented with 100 µCi [³⁵S] methionine was added and incubated at 37°C for 30 minutes to 1 hour. The cells were washed and resuspended in 1 ml PBS using cell scrapers. Cells were pelleted by centrifugation in a Biofuge *pico* microfuge

(Heraeus instruments) at 5,000 rpm for 5 minutes and resuspended in 100 μ l of 1x Cell Lysis Solution (CLS) and incubated at room temperature for 5 minutes. Cell debris were removed by centrifugation and the supernatant added to 100 μ l 2x reducing SDS-PAGE loading buffer.

6.5.14 Immunostaining assays

6.5.14.1 “Blue cell” assay

“Blue cell” assay is a rapid and sensitive method, known as CELICS (cloning by enzyme-linked immunocolourimetric screening (Evans et al., 1998)). 10 to 12 hours post RNA-transfection, the transfected cells were fixed in Methanol:Acetone (1:1) for 10 minutes at room temperature and washed with PBS. The cells were then incubated overnight in PBS-BSA (0.5%) at room temperature. The cells were washed and incubated with DAKO mouse anti-enterovirus VP1 antibody (diluted to 1:400 in PBS-BSA) for 2 hours at room temperature. The cells were washed 3 times in PBS-BSA and incubated for a further 2 hours at room temperature in the presence of anti-mouse β -galactosidase antibody (diluted to 1:800 in PBS-BSA). The cells were washed and incubated for 30 minutes to 1 hour at 37°C in colour developing solution.

6.5.14.2 Immunofluorescence assay

6.5.14.2.1 Cell preparation

13 mm coverslips (VWR international) were flame sterilised and put into a 24-well tissue culture plate. RD-ICAM cells were then seeded so as to reach 50% confluency on the day of infection and were incubated in 10% FCS DMEM supplemented with Penicillin: streptomycin at 37°C in a humidified 5% CO₂ incubator.

6.5.14.2.2 Fixation and immunofluorescence labelling

10 hours post RNA-transfection the transfected cells were fixed in 1 ml per well of Methanol:Acetone (1:1) mix for 30 minutes at -20°C and rehydrated with 1.5 ml of PBS for 10 minutes. The transfected cells were then blocked in PBS + 2% FCS for 10 minutes on a rotating shaker. The cells were incubated with DAKO mouse anti-enterovirus VP1 antibody (diluted to 1:400 in PBS + 2% FCS) for 2 hours at room temperature on a rotating shaker. The cells were washed 3 times in PBS + 2% FCS and incubated for a further 2 hours at room temperature in the presence of anti-mouse FITC conjugate antibody (diluted to 1:400 in PBS + 2% FCS). The 24-well dish was wrapped in tin foil, as FITC is light sensitive. The cells were washed three times in PBS + 2% FCS and once with PBS.

6.5.14.2.3 Analysis on the confocal microscope

The coverslips were rinsed in dH_2O and mounted onto a drop of PBS-glycerol on a glass slide, cells facing down. The coverslips were fixed by applying nail varnish around the edges of the coverslips. Slides were labelled and allowed to dry in a dark environment for 15-30 minutes. The slides were then analysed using a confocal microscope set to detect FITC wavelength. The samples were photographed and analysed using LSM Meta 510. The figures were prepared with Adobe Illustrator 10.

6.5.15 Packaging assays

The trans-encapsidation assays were adapted from methods described in (Barclay et al., 1998; Percy et al., 1992). T7 RNA transcripts were transfected into permissive cell lines and either co-transfected with helper T7 RNA transcript or co-infected 6 hours post-transfection with a helper virus. The samples were then incubated in a 37°C humidified 5% CO_2 incubator until CPE was seen. The samples were freeze-thawed and

clarified. Each sample was then digested with 400 µg/ml of RNase A for 30 minutes at 37°C. The RNase digested samples were passaged onto a fresh cell sheet of RD-ICAM cells. The infected cells were incubated in a 37°C humidified 5% CO₂ incubator for 8-10 hours. The samples were harvested and screened by RT-PCR and RFLP analysis or by Luciferase assay.

6.5.15.1 Luciferase assay

The supernatants of the passaged samples were discarded and the cells were scraped off and resuspended in 1 ml of PBS. The cells were pelleted by centrifugation at 6000 rpm for 5 minutes (in a Biofuge *pico* Heraeus instruments). The supernatant was discarded and the cells were resuspended in 1x cell lysis buffer (Promega®). Luciferase activity was detected from 10 µl of the lysed samples, using a TD20/20 luminometer (Promega), after the addition of 100 µl of room temperature luciferase assay substrate + luciferase assay buffer solution (Luciferase assay system, Promega®). The Luciferase activity was measured using a luminometer and the data was expressed in relative light units.

6.6 Molecular techniques

6.6.1 Isolation of plasmid DNA

10 ml and 100 ml overnight cultures of transformed *E.coli* in Luria broth with the appropriate antibiotic selection medium were used to perform small or medium-scale isolations of plasmid DNA. These were carried out following the alkaline lysis protocol described by Sambrook (Sambrook et al., 1989).

6.6.2 Restriction endonuclease digestion

Restriction digests were carried out using 1 unit of the appropriate enzyme per μg of DNA in the buffer and at the temperature recommended by the manufacturer for one to three hours. Samples were visualised by electrophoresis on a 1% agarose gel in TAE buffer (40 mM Tris-acetate, 2 mM EDTA) containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide. The DNA was visualised on a UV transilluminator. The size of the DNA fragments was estimated using commercially available size markers, (GIBCO 1 kb ladder).

6.6.3 Isolation of DNA fragments from an agarose gel

DNA fragments were isolated from agarose gel using QIAquick[®] PCR and gel purification kit (QIAGEN). To an agarose slice containing the required DNA fragment in an eppendorf tube three volumes of Buffer QG were added and incubated at 50°C for 10 minutes to dissociate the agarose. The solubilised sample was then applied through a purification column, washed with buffer PE and eluted in 30 μl of buffer EB. A fraction of the purified DNA sample was then checked by visualisation on an agarose gel.

6.6.4 Ligation of DNA fragments

All ligation reactions were performed in 10 μl volumes at a molar ratio of insert to vector of 3:1 in 1x ligation buffer (10 mM MgCl_2 , 200 mM Tris-HCl pH 7.6, 50 mM DTT, 1 mM ATP) with 1 unit of T4 DNA ligase. Reactions were incubated at 16°C for 2 hours or at 4°C overnight. Ligation reactions were always purified by ethanol precipitation and resuspended in 5 μl clean dH_2O for transformation.

6.6.5 Preparation of electro-competent *Escherichia coli*

A single colony of *Escherichia coli* ER2738 or DH5 α was picked and inoculated in 10 ml of LB, containing the appropriate antibiotic selection medium, overnight at 37°C on an orbital shaker. The 10ml was added to 1 l of LB with antibiotic and incubated at 37°C on an orbital shaker until the solution reached an OD₆₀₀ of 0.5-0.7 before being pelleted for 10 minutes at 3000 rpm in a Beckman bench-top centrifuge at 4°C. The bacteria were resuspended in 100 ml of ice-cold dH₂O and centrifuged again at 3000 rpm for 10 minutes at 4°C (Multifuge 3 S-R Heraeus). The cells were then resuspended in 100 ml of ice-cold autoclaved 10% Glycerol solution and centrifuged at 2000 rpm for 20 minutes at 4°C (Multifuge 3 S-R Heraeus). The pellet was resuspended in 2 ml 10% Glycerol solution. Aliquots were snap-frozen in liquid N₂ and stored at -70°C for up to three months. The cells were tested for their efficiency the following day by transforming 10 pg of pUC19 and plating it onto an LB agar plate containing ampicillin. Electro-competent cells routinely produced 10⁷-10⁸ colony forming units per μ g of DNA transformed.

6.6.6 Transformation of plasmid DNA in electrocompetent *E.coli*

45 μ l of thawed electro-competent *E.coli* cells were added to each 5 μ l purified ligation reaction and electroporated in a 1 mm electroporation cuvette (CLP) at 1600 V, 400 Ω , 25 μ F with a time constant of 7-10ms. The reaction were transferred to 500 μ L of LB and incubated at 37°C for 45 minutes before plating on to LB agar plates containing 50 μ g/ml ampicillin (or other appropriate antibiotic selection) and incubating at 37°C overnight.

6.6.7 Amplification of DNA by PCR (Polymerase Chain Reaction)

3 μ g of template DNA was added to a reaction mix containing 1x Thermopol buffer (10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄,

0.1% Triton X-100), 2.5 mM dNTPs, 20 pmoles of the relevant forward and reverse primers and 1 unit of Vent_R® proof-reading DNA Polymerase (New England BioLabs NEB) in a 50 µl reaction volume. The DNA was preheated at 95°C for 3 minutes prior to adding the polymerase and amplified by 6-15 (for further cloning)-25 (analysis and sequencing) cycles at 95°C for 45s, 45-55°C for 45s, 73°C for 2-3 minutes (extension step was extended for longer products) followed by a final extension step at 73°C for 10 minutes in a PTC-200 Peltier Thermal Cycler. The amplified DNA was analysed by gel electrophoresis in a 1% TAE agarose gel and purified following the isolation of DNA fragments from an agarose gel protocol.

6.6.8 DNA sequencing

200-300 ng of purified PCR products or double stranded midi plasmid DNA along with 3.2 µM forward and reverse oligonucleotides were sent to the University of Dundee Sequencing Service for sequencing. Data was analysed using Bio Edit and aligned with the appropriate DNA sequence, from the Vector NTI database.

6.6.9 Western immunoblotting

6.6.9.1 Preparation of samples

Appropriate samples were mixed in a 2x reducing SDS-PAGE gel-loading buffer. The samples were then heated at 99°C for a few minutes to denature proteins.

6.6.9.2 Western blotting

Protein samples, normalised for total amount of protein, were subjected to SDS-PAGE on a 10% or 12.5% polyacrylamide gel and transferred to a nitrocellulose membrane for ECL (Amersham Pharmacia) using a Bio-Rad Transfer Blot at 200 V for an hour. The

membrane was then soaked in blocking buffer at room temperature for about one hour, and washed three times in PBS-0.1% Tween 20. Incubation with a primary antibody diluted in blocking buffer to obtain a 1:1000 dilution was carried out at room temperature for two hours. The membrane was washed three times in PBS-0.1% Tween 20 and incubated with a secondary antibody HRP, diluted to 1:5000 in blocking buffer, at room temperature for two hours. A final three washes with PBS-0.1% Tween 20 were carried out prior to detection using an Amersham Biosciences ECLTM Western Blotting Detection Kit. The blots were exposed to a Kodak X-omat UV film and developed.

6.6.10 Dot blot replication assay

6.6.10.1 Preparation of DNA probe

The sequences for the probe were obtained from the pT7FLC/PV3 by amplifying the 3D polymerase using primers T7R3D and ID6. To 3 µg DNA was added 1x Thermopol buffer (10 mM KCl, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM (NH₄)₂SO₄, 2 mM MgSO₄, 0.1% Triton X-100), 2.5 mM dNTPs, 20 pmoles of the relevant forward and reverse primers and 1 unit of Vent_R® proof-reading DNA Polymerase (New England BioLabs NEB) in a 50 µl reaction volume. The PCR conditions were as follows: 95°C-3mins, 25 cycles of 95°C-45secs, 45°C-45secs, 73°C-2mins and a single final extension step at 73°C for 10 minutes. The PCR fragment was gel purified using a QIAquick[®] PCR and gel purification kit (QIAGEN) according to manufacturers instructions.

25 ng of template DNA were mixed in dH₂O to a final volume of 11 µl. The DNA was denatured by heating in boiling water for 10 minutes and quickly chilling on ice. 4 µl of High Prime, supplied by Roche, were mixed thoroughly and added to the denatured DNA reaction. The reaction tube was then supplemented with 5 µl (50 µCi) EasyTidesCytosine

5' Triphosphate (NEN [^{32}P] α -rCTP). The solution was then mixed, briefly centrifuged and incubated at 37°C for 10 minutes. The reaction was stopped by heating at 65°C for 10 minutes. The DNA probe was then purified through a Sephadex G-50 column to remove unbound nucleotides.

6.6.10.2 Northern type hybridisation

10 μl of RNA samples was added to 30 μl of RNA denaturation buffer, incubated at 65°C for 5 minutes and quenched on ice. A HybondTM-N Nylon membrane optimised for nucleic acid transfer (Amersham Pharmacia) was presoaked with dH₂O before being placed on the BioRad Bio-DotTM microfiltration apparatus. The membrane was washed with 10xSSC buffer and the denatured RNA samples were added and dotted onto it by vacuum. Further washes with 10x SSC buffer were carried out and the membrane was dried and samples were fixed by UV cross-linking at 1200 $\mu\text{Joules} \times 100$ for 30 seconds in a UVStratalinker 1800 (Stratagene). The membrane was transferred to a hybridisation chamber containing 10 ml of Rapid-Hyb buffer (Amersham Pharmacia) and incubated at 65°C for 2 hours.

The DNA probe mixture was added to the membrane in the hybridisation chamber and incubated at 65°C for 12-18 hours. The membrane was then washed with 1xSSC-0.1%SDS three times and with 0.5xSSC-0.1%SDS twice. The membrane was covered in cling film and exposed to phosphoimager screen for 30 minutes to 1 hour before visualising with BioRad Quantity One software.

6.6.11 *In-vitro* translation assay

A mix of 30 μl of Hela S10 extracts, 5 μl of Flexi®Rabbit Reticulocyte Lysate System (Promega), 5 μl of 10xbuffer (10 mM rATP, 2.5 mM rCTP, 2.5 mM rGTP, 600 mM KOAc, 300 mM Creatine phosphate, 15 mM Hepes pH 7.0 made to 1 ml in dH₂O)

supplemented with Phosphocreatine kinase (Sigma), 1 μ l of 100 mM GuHCl, 3 μ l of dH₂O, 50 μ Ci of [³⁵S] Methionine and 1 μ g of Ribomax prepared RNA diluted in 1 μ l of dH₂O was incubated at 30°C for 5 hours. 60 μ l of 2x SDS reducing protein gel loading buffer was added to the reaction mix post-incubation and samples were denatured by incubation at 100°C for a few minutes. Samples were then run on an SDS-PAGE gel and visualised using Quantity One software after exposure to a phosphoimager screen for 10 to 12 hours.

7 Bibliography

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Appendix 1

Viral protein sizes (in kDa)			
Control virus	Poliovirus Leon type 3	Sabin poliovirus type 1	Coxsackievirus A21
Viral proteins			
<i>P1</i>	95.9	97.3	97
<i>VP0</i>	37.4	37.6	37.5
<i>VP1</i>	32.3	33.4	33
<i>VP3</i>	26.2	26.3	26.5
<i>VP2</i>	29.7	30.1	29.9
<i>VP4</i>	7.4	7.5	7.4
<i>VP1-2A</i>	48.6	49.9	49.4
<i>P2</i>	63.4	63.7	63.3
<i>2A</i>	16.4	16.5	16.4
<i>2B</i>	10.6	10.8	10.6
<i>2C</i>	36.4	36.4	36.3
<i>2BC</i>	47	47.2	46.9
<i>P3</i>	82.8	83.5	82.8
<i>3A</i>	9.5	9.7	9.5
<i>3B</i>	2.3	2.4	2.3
<i>3C</i>	20	20.2	20
<i>3D</i>	51	51.	51
<i>3CD</i>	71	71.4	71
<i>3AB</i>	11.8	12.1	11.8

Table listing the viral protein sizes of PV3, Sabin 1 and CVA21

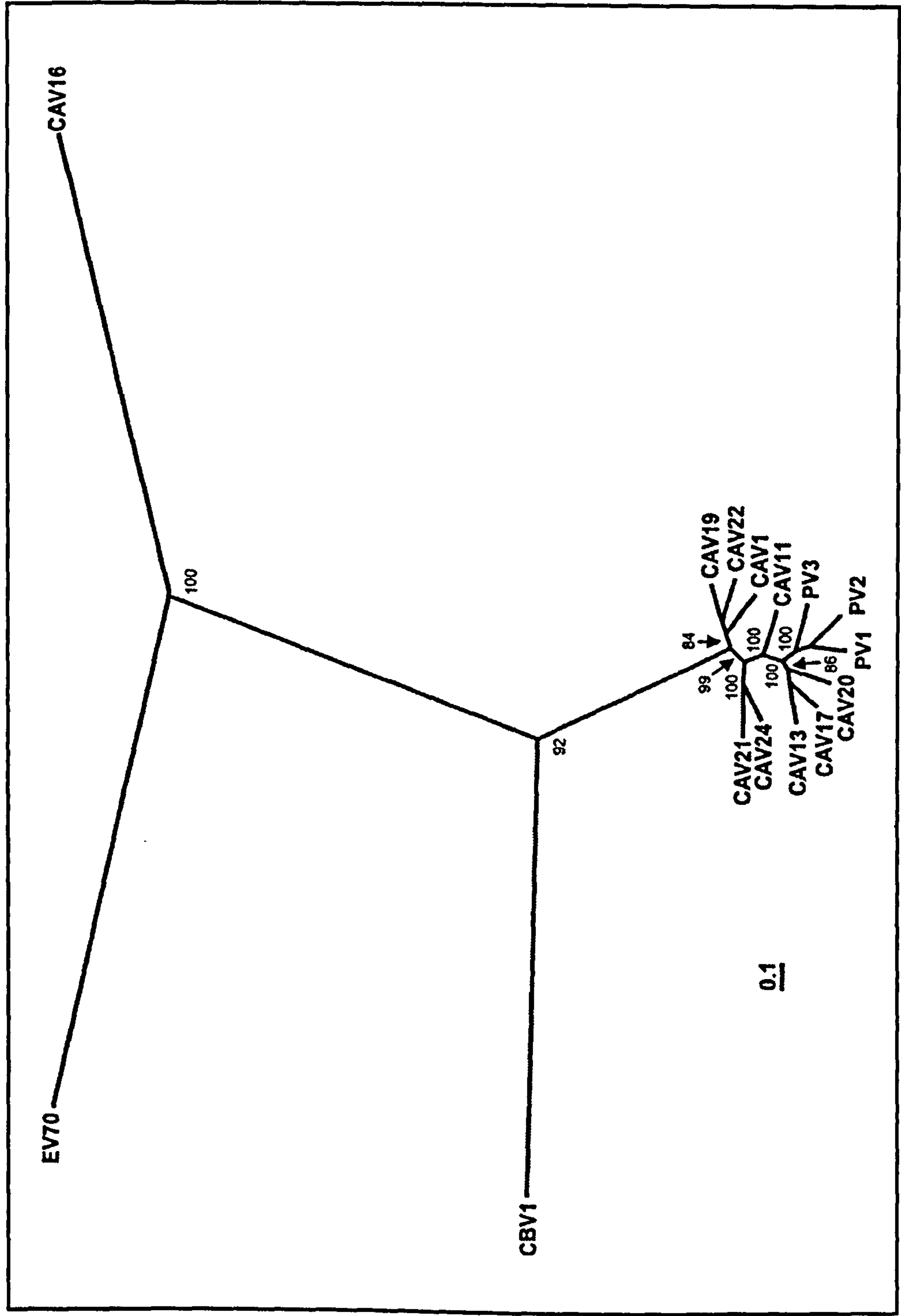
Appendix 2

Plasmid name	Description	Published
pS1F	Full length poliovirus Sabin type 1 infectious clone	Provided by A. Macadam NIBSC, London
CAV21	CVA21 full-length infectious clone	Hughes et al., 1989
pT7FLC	Full length poliovirus type 3 Leon P3/Leon/37 infectious clone	Goodfellow et al., 2000
pT7FLC/REP3	Poliovirus type 3 Leon P3/Leon/37 CAT replicon derived from pT7FLC	Barclay et al., 1998
pT7FLC/REP3-BK	Backbone vector lacking the complete P1-coding gene derived from pT7FLC/REP3	
pT7REP3-BK-SL3-P1	Backbone vector PV3BK with insertion of the complete PV3 P1-coding region introduction derived from pT7/SL3	
pT7REP3-BK-CA21-P1	Backbone vector PV3BK with insertion of the complete CVA21 P1-coding region introduction derived from CAV21	
pT7/SL3	Full length poliovirus type 3 Leon P3/Leon/37 with mutations in the 2C CRE derived from pT7FLC	Goodfellow et al., 2000
pT7REP3-L	Poliovirus type 3 Leon P3/Leon/37 luciferase replicon derived from pT7FLC/REP3	Goodfellow et al., 2003
pRibol	Vector containing a ribozyme hammerhead	Constructed by Simmonds 2001
pRibo-CAV21	CVA21 full-length infectious clone introduced in a ribozyme hammerhead-containing vector derived from CAV21 and pRibol	
pRibo-CAV21-Nae1Del	CVA21 full-length infectious clone with a deleted Nae I site, derived from pRibo-CAV21	
pRibo-CAV21-	CVA21 backbone vector	

Nae1Del-backbone	lacking the complete P1-coding gene, derived from pRibo-CAV21-Nae1Del	
pRibo-CAV21-BK/CA21 P1	CVA21 backbone vector with insertion of the complete CVA21 P1-coding region, derived from pRibo-CAV21-Nae1Del-backbone and CAV21	
pRibo-CAV21-BK/PV3-P1	CVA21 backbone vector with insertion of the complete PV3 P1-coding region, derived from pRibo-CAV21-Nae1Del-backbone and pT7/SL3	
pRibo-CAV21-BK/Sabin1 P1	CVA21 backbone vector with insertion of the complete Sabin1 P1-coding region, derived from pRibo-CAV21-Nae1Del-backbone and pS1F	
pT7EV7	Full length echovirus 7 infectious clone	Lindberg et al.,1997
EV7 Δ P1	EV7 genome with an in-frame Nsi I deletion in the P1-coding region, derived from pT7EV7	
CVA21 Δ P1	CVA21 genome with an in-frame Age I deletion in the P1-coding region, derived from CAV21	

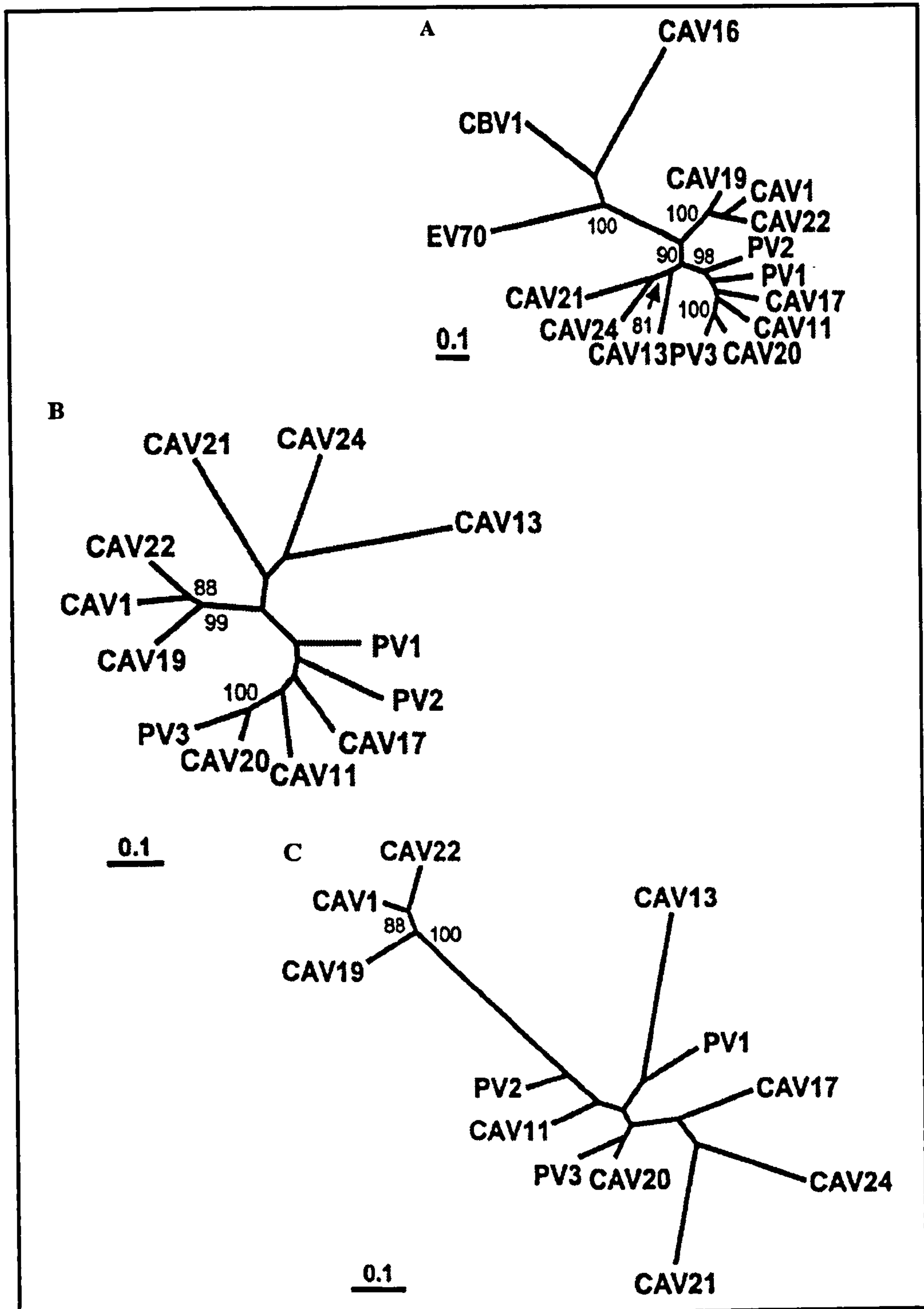
Table listing plasmids used for this project

Appendix 3: Phylogenetic relationship based on P1 coding sequence comparison studies



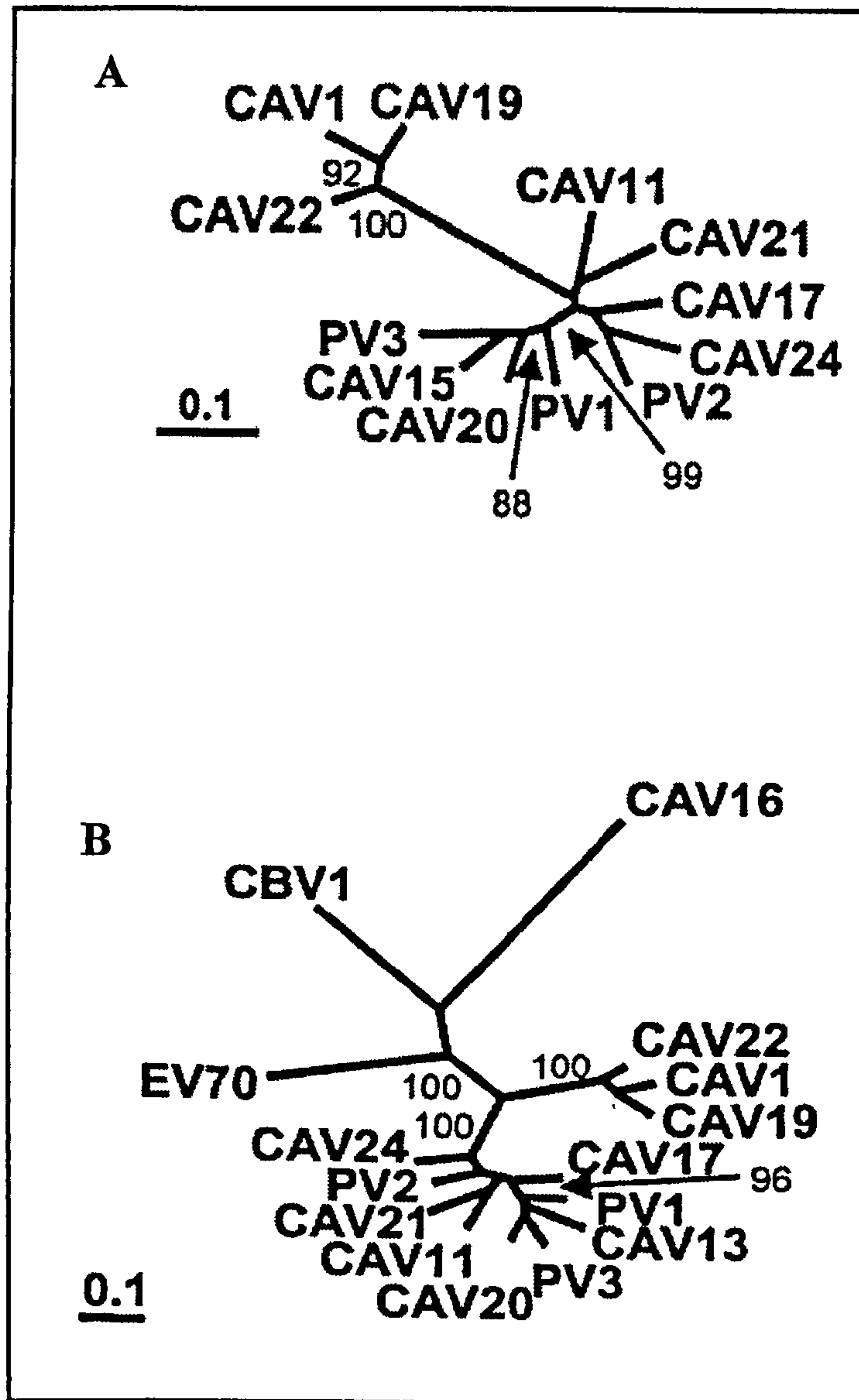
Phylogenetic relationship of HEV-C based on P1 coding region sequence analysis comparison from Brown et al. (2003).

Appendix 4: Phylogenetic relationship based on P2, 2A and 2B coding sequence comparison studies.



Phylogenetic relationship of HEV-C based on A: P2 coding region B: 2A coding region and C: 2B coding region sequence analysis comparison from Brown et al. (2003).

Appendix 5: Phylogenetic relationship based on P3 and 3D coding sequence comparison studies.



Phylogenetic relationship of HEV-C based on A: 3D coding region and B: complete P3 coding region sequence analysis comparison from Brown et al. (2003).

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