MODIFICATION OF THE E1-PIX REGION OF THE ADENOVIRUS 5 GENOME FOR USE IN CANCER GENE THERAPY

Susanna Kallioinen

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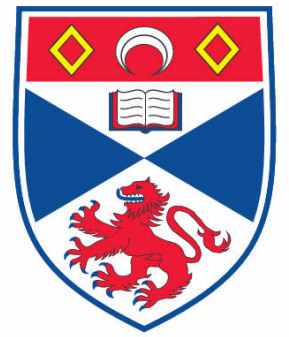

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4th October 2007

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Abstract

Currently the use of adenoviruses in cancer gene therapy is limited by efficient delivery of the virus into the tumour cells, detargeting of the virus from the liver, and the efficient spread of the virus within the tumour. Rapid and easy modification of adenoviruses enables expression of different genes from the genome of an oncolytic virus. I developed a system where the E1-pIX region of the adenovirus 5 genome could be mutated via recombination of a recipient virus with the deleted E1-pIX region flanked by a *loxP* and an attB-site and an "addback" plasmid with the mutated E1-pIX region flanked by a *loxP* and an attP-site. The recipient virus was found not to be producible even on a pIX-complementing cell line. The pIX was further modified by fusing GFP, FCU1 and MMP7 to the C-terminus with a 2A sequence that enables the ribosome to skip one specific peptide bond enabling the expression of genes flanking this sequence. Two different 2A sequences were used: FMDV 2A (F2A) and PTV-1 2A (P2A). The pIX-P2A-GFP expressing virus was found to have similar heat stability, CPE, burst size and plaque size characteristics as the parental virus, whereas the pIX-F2A-GFP expressing virus was found to have reduced heat stability, CPE, burst size and smaller plaque size. The viruses expressing FCU1 and MMP7 were found only to be producible on a pIX-complementing cell line due to the low expression of pIX from these constructs. I concluded that 2A sequences can be used in the context of adenoviruses but optimisation of the sequence may be needed depending on the fusion partners.

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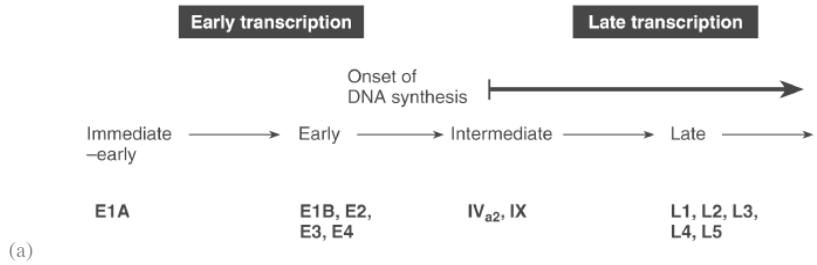
List of Abbreviations

4EBP1	4E binding protein 1
Ad	Adenovirus serotype
ADP	Adenovirus death protein
BAP	Biotin acceptor protein
CAR	Coxsackie adenovirus receptor
CMV	Cytomegalovirus
CPE	Cytopathic effect
cre	Cyclization recombination
DBP	DNA binding protein
DNA PK	DNA protein kinase
E1, E2, E3, E4	Early unit 1, 2, 3, 4
E2F	E2 factor
EGF	Epithelial growth factor
EGFP	Enhanced green fluorescent protein
EYFP	Enhanced yellow-green fluorescent protein
FMDV	Foot-and-mouth disease virus
GFP	Green fluorescent protein
HA	hemagglutinin
HD	Helper-dependent
HSV-1	Herpes simplex virus 1
iPCR	Inverted PCR
IRES	Internal ribosomal entry site
ITR	Inverted terminal repeat
L1 to L5	Late unit 1 to 5
MHC	Major histocompatibility complex
MLP	Major late promoter
MLTU	Major late transcription unit
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
ND10	Nuclear domain 10
NF-κB	Nuclear Factor κB

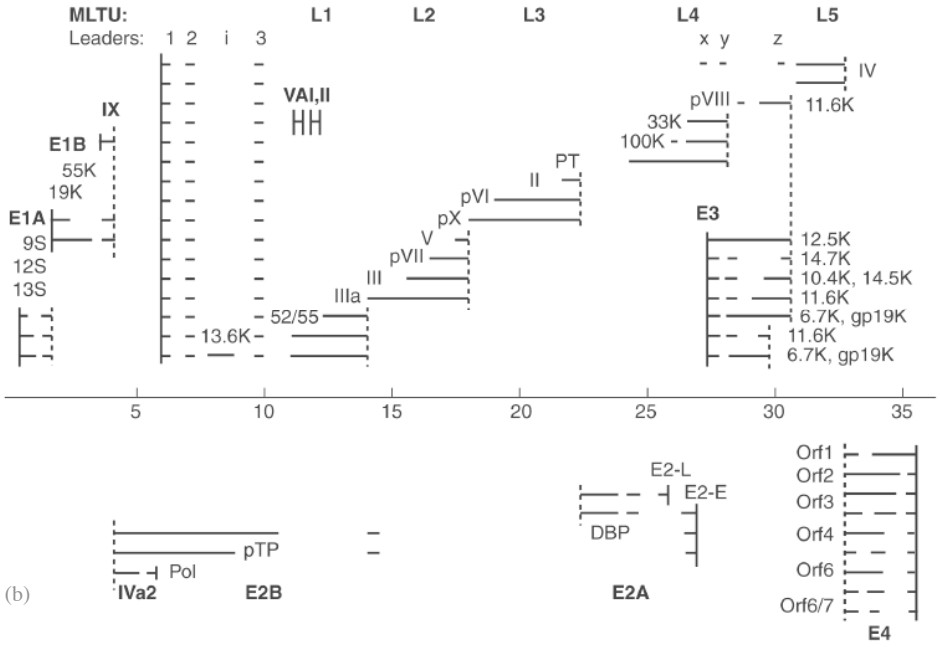
PFU	Plaque forming unit
PI3K	Phophatidylinositol 3 kinase
Pol	Polymerase
RGD	Arginine-Glycine-Aspartic acid
PCR	Polymerase chain reaction
PML	Promyelocytic leukaemia protein
POD	PML oncogenic domain
PP2A	Protein phosphatase 2A
pRb	Retinoblastoma protein
(p)TP	(pre) Terminal protein
TCF/LEF	T cell factor/Lymphoid enhancer binding factor
TNFα	Tumour necrosis factor α
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
VA RNA	Virus associated RNA
Wt	wild type

1. Introduction

Currently the use of adenoviruses in cancer gene therapy is limited by different factors. These include the lack of efficient delivery of the virus into the tumour cells, detargeting of the virus from the liver, and the efficient spread of the virus within the tumour (for a recent review see Alemany, 2007). This project aims to improve the spread of the virus within the tumour by developing a system for rapid modification of the E1-pIX region of the virus to enable the expression of anti-tumoural genes from the adenoviral genome. The E1-pIX region of the adenovirus is located near the ITR at the left end of the viral genome (Figure 1b, page 2). It is approximately three kilobases in size. The E1 region is divided into two primary transcripts, E1A and E1B. Both transcripts are regulated by their own promoters. Protein IX (pIX) is located at the end of the E1B region sharing its poly(A) signal with E1B but being regulated by its own promoter.



(a)



(b)

Figure 1: (a) Schematic diagram showing the adenoviral life cycle. (b) Schematic diagram showing the organisation of adenovirus 5 genome. The sizes are in kilobases. (Dimmock et al., 2001)

1.1 The Adenovirus 5 Genome

The adenovirus 5 (Ad5) genome is 36 kb in length and encodes its own early and late promoters as well as its own replication proteins (Figure 1a, p. 2). Deletion of certain regions is possible without hindering the viral function in cancerous cells.

1.1.1 Early proteins

The E1 region consists of two early transcripts, E1A and E1B. The E1A genes drive the cells to enter the S phase of the cell cycle. E1A inhibits the action of pRB that would otherwise prevent E2F transcription factors from activating genes that drive the cells through S phase (Chellappan et al., 1991; Nevins et al., 1988). The E1A promoter is constitutively active (Hearing and Shenk, 1983) and can be transactivated by pIX (Lutz et al., 1997). The E1B region also consists of two early transcripts that code for E1B19K and E1B55K. The E1B proteins prevent E1A-induced apoptosis by preventing procapsase-9 activation (E1B19K) (Chiou et al., 1994) and by binding to p53 (E1B55K) (Yew et al., 1994).

The adenovirus 5 genome also encodes three other early transcription units, E2, E3 and E4. Similarly to the E1 region, E2 region can be divided into two groups of transcripts, E2A and E2B. E2A encodes for the single-stranded DNA binding protein (DBP) that binds to the DNA chain stabilising it during the replication of the adenoviral genome (Friefeld et al., 1983). DBP has also been shown to activate a variety of viral promoters (Chang and Shenk, 1990), although other reports show DBP having inhibitory effects on early viral promoters (Carter and Blanton, 1978). E2B codes for the adenoviral polymerase (Ad Pol) and the precursor of the terminal protein (pTP). pTP is covalently attached to the termini of the adenoviral genome, and mediates the attachment of the adenoviral DNA with nuclear matrix proteins. pTP also serves as a primer for initiation of the adenoviral DNA replication, and in a later stage during packaging pTP gets cleaved to terminal protein (TP) to enable the release of the DNA from the nuclear matrix and facilitate its packaging into virions (Fredman and Engler, 1993).

The E3 region codes for seven proteins involved in the immune modulation of the host (Horwitz, 2001). E3-gp19k is a transmembrane protein that prevents antigen presentation on the cell surface by binding to the MHC class I heavy chain and retaining it in the endoplasmic reticulum (Burgert and Kvist, 1985). E3-10.4K and E3-14.5K (RIDα&β) form heterotrimers that inhibit TNFα and Fas ligand-mediated apoptosis via internalization of their receptors and promotion of their degradation in lysosomes (Wold et al., 1999). E3-14.7K also inhibits TNFα-induced cell death by enhancing IKKγ phosphorylation of IKγ, thus stabilising NF-κB (Horwitz, 2001). E3-6.7K functions in the specific modulation of the two death receptors for tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). The E3-6.7K protein is expressed on the cell surface and forms a complex with the 10.4K and 14.5K proteins. This complex is sufficient to induce down-modulation of TRAIL receptor-1 and -2 from the cell surface, and to reverse the sensitivity of infected cells to TRAIL-mediated apoptosis (Benedict et al., 2001). The E3-11.6K protein, also called adenoviral death protein, or ADP, is a glycosylated transmembrane protein that accelerates cell lysis in late stages of infection and induces virus release from the host cell. ADP was described as an early protein but is actually expressed late during infection from the MLP and is therefore primarily considered as to be a late protein (Tollefson et al., 1996). Finally, the function of the remaining protein, E3-12.5K, is unknown.

The E4 region codes for six different polypeptides named E4orf1 to E4orf6/7 after their arrangement of open reading frames inside the region. They are mainly involved in facilitating viral mRNA metabolism and protecting the viral DNA ends. They also promote cell growth, proliferation and survival via interactions with the PI3K pathway. E4orf3 and E4orf6 are associated with E1B55K. E4orf3 redistributes components of nuclear bodies or PML oncogenic domains (PODs) from spherical nuclear bodies into fibrous structures, while E4orf6 plays a role, with E1B55K, in the preferential cytoplasmic accumulation of viral late mRNAs and in the degradation of p53 (Carvalho et al., 1995; Harada et al., 2002; Querido et al., 2001). Both E4orf3 and E4orf6 also enhance viral replication and target the double strand break repair machinery to prevent concatenation of viral genomes. E4orf3 mislocalizes the double-stranded break repair complex Mre11-Rad50-NBS1 and E4orf6 together with

E1B55K target it for degradation (Stracker et al., 2002). E4orf3 also protects the ends of the viral DNA from non-homologous end joining by binding to the DNA-dependent protein kinase (DNA PK) and inhibiting the DNA PK-dependent double stranded break repair from occurring (Boyer et al., 1999). E4orf6/7 interacts with E2F that dimerises and transactivates the E2 promoter (Obert et al., 1994) via binding of the two inverted E2F-binding sites in the Ad5 E2 early promoter (Hardy and Shenk, 1989) and in the cellular E2F-1 promoter (Schaley et al., 2000). E4orf6/7 is able to activate E2 expression by displacing pRb and the related protein p107 from E2F complexes (O'Connor and Hearing, 2000). E4orf1 and E4orf6 have partial transforming activities as they are able to stimulate E1 transformation. E4orf1 interacts with PDZ domain-containing cellular proteins (Glaunsinger et al., 2000), which leads to PDZ protein-dependent activation of PI3K. This adds to the transforming potential of E4orf1 (Frese et al., 2003). E4orf4 interacts with protein phosphatase 2A (PP2A) (Kleinberger and Shenk, 1993), and hypophosphorylates c-Fos and E1A, which leads to downregulation of AP-1 (Muller et al., 1992). E4orf4 is also thought to dephosphorylate SR proteins which leads to inactivation of SR-mediated splicing in L1 (Estmer Nilsson et al., 2001). E4orf4 has also been shown to induce growth arrest in yeast and mammalian cells at the G2/M phase of the cell cycle (Kornitzer et al., 2001). Finally, it is thought that E4orf4 interacts with the PI3K pathway through activation of the mammalian target of rapamycin (mTOR). E4orf4 deleted mutant adenovirus failed to activate mTOR or phosphorylate the mTOR target 4EBP1. E4orf4 was also shown to activate the mTOR effector $p70^{S6K}$ upstream of mTOR (O'Shea et al., 2005).

1.1.2 Intermediate proteins

The IVa2 protein is a minor component of the adenoviral capsid that has been found to have DNA binding properties (Russell and Precious, 1982). Later, it was found to act as an enhancer for initiation of transcription from the adenovirus major-late promoter (MLP) (Tribouley et al., 1994). IVa2 was also found to be able to bind to the packaging signal and be involved in the encapsidation of the viral genome (Tyler et al., 2007). IVa2 was shown to be crucial in adenoviral capsid formation, as IVa2

deleted viruses are unable to form capsids (Zhang and Imperiale, 2003). IVa2 is transcribed in an intermediate state of the viral life cycle.

Another intermediate protein is the protein IX (pIX). It is a cement protein found between hexons in the capsid of the virus. Besides its role as a structural protein, it was shown to have roles as a transcriptional activator and reorganising nuclear proteins to provide a more replication-friendly environment for the virus (reviewed by Parks, 2005). The functions of pIX will be discussed in detail later.

1.1.3 Late proteins

The expression of late proteins commences at the start of the viral DNA replication. The late transcripts are grouped into five different families, L1 to L5, that as a whole form the adenovirus major late transcription unit (MLTU). The MLTU is differentially spliced and polyadenylated to yield the different families of mRNAs. The MLTU is under the control of the major late promoter (MLP) that is activated by IVa2 (Lutz and Kedinger, 1996), pIX (Lutz et al., 1997) and via DNA replication (Toth et al., 1992). Three functionally different types of genes are encoded by the MLTU. Capsid proteins fibre (IV), penton (III), and hexon (II), as well as cement proteins (pVIII, IIIa, VI) located in the capsid of the virus. Non-structural proteins are also encoded by the MLTU. They have various functions in the assembly of the viral capsid and encapsidation of the viral genome, such as L1 52/55 K protein that jointly with IVa2 and pVII binds to the packaging sequence enables the encapsidation of the viral genome (Perez-Romero et al., 2006; Zhang and Arcos, 2005).

1.1.4 Other regions of the adenovirus 5 genome

The packaging signal (Ψ) directs the encapsidation of the adenoviral DNA into the precursor capsid of the virus. It is a 192-nucleotide long sequence located near the left inverted terminal repeat (ITR) of the adenovirus 5 genome within the E1A enhancer region. It consists of seven adenosine/thymidine-rich sequence motifs, A repeats (A1-A7) (Tyler et al., 2007). The packaging signal can be moved next to the right ITR in

an inverted orientation without losing function. It can also be moved several hundred nucleotides away from the ITR without affecting viral yield. The ITRs (103 bp) are also thought to be involved in the encapsidation process (Grable and Hearing, 1990, 1992), as well as in the replication of the adenoviral genome, where it serves as a docking site for nuclear factor I and III that recruit the Ad Pol-pTP complex for initiation of replication (Lichy et al., 1982).

1.2 Protein IX

Protein IX (pIX) is the smallest of the minor capsid proteins. It is 140 amino acids in length and 14.3 kDa in size. It is situated at the end of the E1B region sharing its poly(A) signal with E1B. E1B transcription inhibits the expression of pIX by inhibiting the function of the pIX promoter. pIX expression occurs after DNA replication when an E1B product activates the IX gene (Vales and Darnell, 1989).

1.2.1 Role as a capsid protein

pIX is not produced from the adenovirus major late promoter (MLP) like other structural proteins but rather is expressed from its own promoter (Boulanger et al., 1979). pIX is located between hexons in the groups-of-nine (GONs) in the capsid of the virus (Figure 2, p. 10) (Boulanger et al., 1979; Everitt et al., 1973; Maizel et al., 1968). It is thought to be a cement protein stabilising molecular interactions between hexons in the capsid (Colby and Shenk, 1981; Furcinitti et al., 1989; Ghosh-Choudhury et al., 1987; Maizel et al., 1968). It is present in 240 copies in the 20 facets of the capsid. pIX is thought to form four trimers inside one GON (Fabry et al., 2005; Furcinitti et al., 1989; Saban et al., 2005; Saban et al., 2006; Stewart et al., 1991; Stewart et al., 1993) with the N-terminus buried inside the capsid while the C-terminus points towards the outside of the capsid (Akalu et al., 1999). The N-terminus has been shown to be important for the incorporation of pIX into the capsid where as the C-terminus has other roles in the life cycle of the virus (Akalu et al., 1999; Rosa-Calatrava et al., 2001; Vellinga et al., 2005a). Despite being widely accepted there is some contradictory evidence in the location of pIX in the capsid. Instead of forming four trimers inside a GON, it has been proposed that pIX is located near the peripentonal hexons, which in the classical model is occupied by protein IIIa (Campos et al., 2004b; Vellinga et al., 2005b). In this position it is difficult to see how pIX could affect the stability of the GONs (Vellinga et al., 2005b).

pIX deletion results in reduced heat stability of the virus (Colby and Shenk, 1981). pIX was also shown to be essential for packaging of full-length viral genomes from

wild type size to 105% of wild type. This left room for insertion of approximately 2 kb of extra sequence (Bett et al., 1993; Caravokyri and Leppard, 1995; Ghosh-Choudhury et al., 1987; Sargent et al., 2004b). Viruses with deleted pIX grown on a 293pIX complementing cell line could accommodate up to 37.3 kb of viral DNA but these virions could not infect non-complementing cell lines (Sargent et al., 2004b). Viruses with a genome size of 105% of that of a wild type virus were slightly less stable than wild type viruses (Bett et al., 1993). Although pIX has been shown to be dispensable for the virus (Boulanger et al., 1979; Colby and Shenk, 1981; Sargent et al., 2004a) many helper cell lines have been created in order to complement for pIX deficiencies. These are used for generation of helper-dependent (HD) adenoviruses (Caravokyri and Leppard, 1995; Krougliak and Graham, 1995; Vellinga et al., 2006). pIX is one of the adenoviral genes present in 293 cells (Graham et al., 1977; Louis et al., 1997) but it does not get expressed in detectable amounts and cannot complement for pIX deficiency. However, recombination between the viral pIX and the pIX sequence present in 293 cells is possible (reviewed by Parks, 2005).

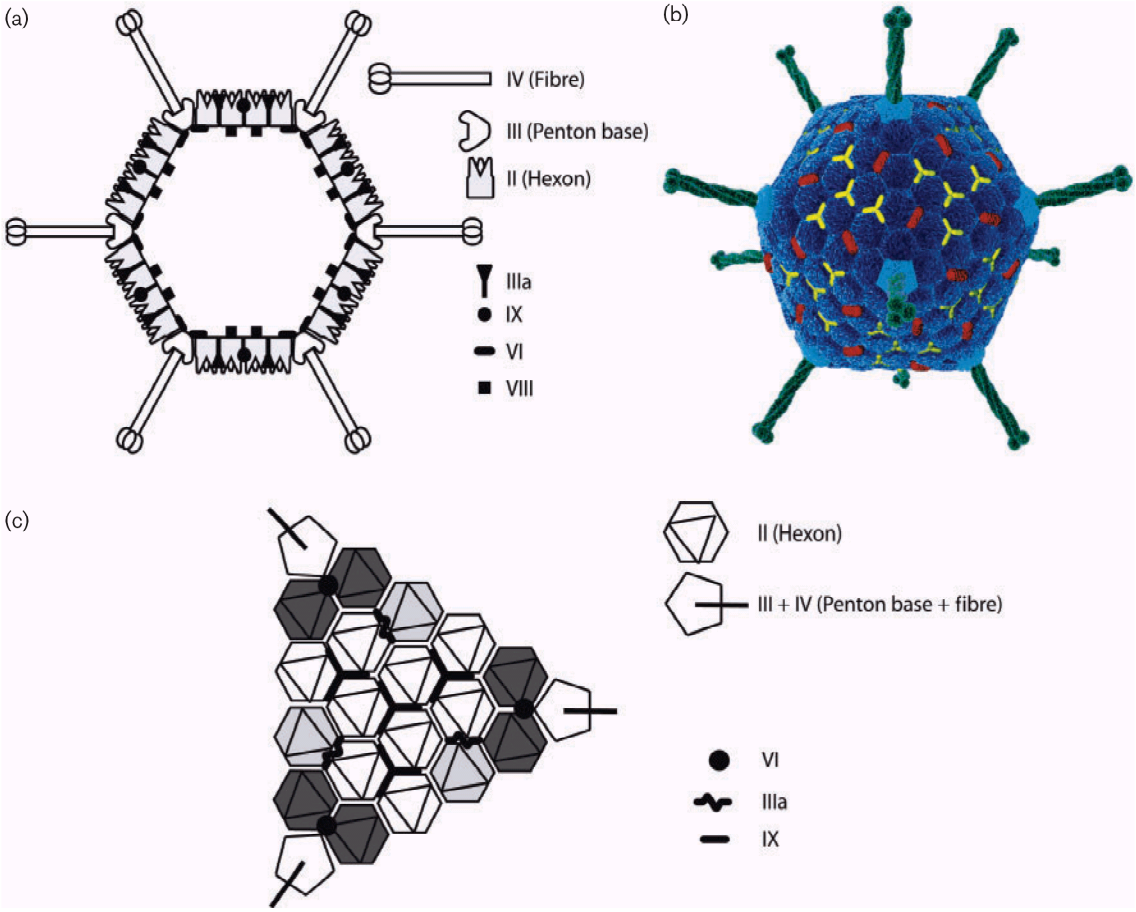


Figure 2: Location of pIX on the capsid of the virus. (a) pIX are represented by black circles that reside in the capsid. (b) Three-dimensional representation of the icosahedral virion. pIX is depicted in yellow. (c) Diagram representing one of the adenoviral capsid facets. pIX is shown to form four trimers in between the hexons that form the GONs. Hexons are depicted in white. (Vellinga et al., 2005b)

1.2.2 Role as a transcriptional activator

Besides being a structural protein pIX also has a function as a transcriptional activator and it stimulates TATA-containing promoters of both viral and cellular origin (Lutz et al., 1997). However, it does not show direct DNA-binding properties (Rosa-Calatrava et al., 2001). It has been shown that the C-terminal leucine putative coiled-coil domain is critical for the transactivating function (Lutz et al., 1997; Rosa-Calatrava et al., 2001). Although no pIX formation was detected early in infection, it was thought that pIX released from the capsid of the infecting virion, as a result of decapsidation, could accumulate in the nucleus 45 minutes post-infection and stimulate viral transcription programme via activation of the E1A promoter (Lutz et al., 1997; Rosa-Calatrava et al., 2001). Although it was shown that pIX could activate the E1A promoter, this did not happen during the normal viral replication (Sargent et al., 2004a).

The leucine repeat in the C-terminus of pIX also enables self-assembly that results in the formation of specific nuclear structures after pIX accumulates in the nucleus (Rosa-Calatrava et al., 2001). The pIX inclusions were shown to co-localise with the promyelocytic leukaemia (PML) protein (Rosa-Calatrava et al., 2003). Production of a virus containing a mutant pIX that lacked the ability to form nuclear inclusions was significantly reduced when compared with a wild type adenovirus (Rosa-Calatrava et al., 2003). PML protein is the major reorganiser of PML bodies (PODs), also called nuclear domain 10 (ND10) (reviewed by Maul et al., 2000). PODs seem to have a role in antiviral response (Chee et al., 2003; Maul, 1998), and many viruses disrupt the formation of PML bodies, as does E4orf3 in adenovirus (Carvalho et al., 1995). Hence, it is possible that pIX acts as a back-up for disrupting the formation of PML bodies (Parks, 2005).

1.2.3 pIX-fusions

As antibodies specific for the C-terminus of pIX were discovered to bind to intact virions, it was concluded that the C-terminus of pIX was pointing toward the outer

surface of the viral capsid (Akalu et al., 1999). It was shown that it is possible to fuse peptides into the C-terminus of pIX. This made it possible to alter the tropism of the virus by inserting targeting ligands into pIX (Dmitriev et al., 2002). Several different proteins have been fused to pIX (reviewed by Campos and Barry, 2006; Hedley et al., 2006).

Green fluorescent protein (GFP) has been fused to pIX in order to enhance vector detection in targeting schemes (Le et al., 2004), to track canine adenovirus 2 (CAV2) infection in tissues and the nuclear localisation of pIX (Le et al., 2005), and to see if large protein tags fused to pIX would interfere with the functioning of the virus (Meulenbroek et al., 2004). Le *et al.* (2004) found that DNA packaging and thermostability of the virus were marginally hampered but DNA replication, cytopathic effect (CPE) and CAR-dependent binding were not affected. Meulenbroek *et al.* (2004) found that fusing a large ligand to the C-terminus of pIX did not interfere with its incorporation into the capsid of the virus nor did it affect the virus viability or infectivity.

Enhanced yellow-green fluorescent protein (EYFP) has also been fused to the C-terminus of pIX to visualise the corporation of pIX into the capsid (Zakhartchouk et al., 2004). EYFP was found to be located on the surface of pIX indicating that the C-terminus of pIX is pointing towards the outside of the capsid (Zakhartchouk et al., 2004). A FLAG tag (DYKDDDDK) was also added to the end of pIX to see if it can be displayed on the surface of the virus. This was shown to be the case (Kurachi et al., 2006).

Red fluorescent proteins mRFP1 and tdimer2(12) were added to the end of pIX in order to monitor adenovirus replication both *in vitro* and *in vivo* (Le et al., 2006). The fluorescent labels were observed to have little effect on viral DNA replication, encapsidation, CPE, thermostability, and CAR-binding (Le et al., 2006).

The herpes simplex virus 1 (HSV-1) thymidine kinase (TK) was fused to the C-terminus of pIX to see if a large protein of this type could retain functionality in this context (Li et al., 2005). It was found that HSV-1 TK could retain functionality, while

DNA packaging and CPE of the virus were not affected. CAR-dependent binding of the virus to the host cell was marginally affected (Li et al., 2005).

An RGD motif (CDCRGDCFC) has also been added to the end of pIX to see if this enhanced the infectivity of integrin-positive cells (Kurachi et al., 2006; Vellinga et al., 2004; Zakhartchouk et al., 2004) and CAR-negative cells (Kurachi et al., 2006; Vellinga et al., 2004). Zakhartchouk *et al.* (2004) demonstrated that the addition of RGD to the C-terminus of pIX in the bovine adenovirus 3 enhanced the infection efficiency. Kurachi et al. (2006) showed that the RGD modification with or without a 78 Å spacer did not affect the transduction efficiency of the adenovirus vector, where as Vellinga et al. (2004) claimed that the RGD modification in the end of pIX with or without a 78 Å spacer increased transduction efficiency of the virus.

A biotin acceptor peptide (BAP) was fused to the C-terminus of pIX so biotinylated ligands may be displayed on the surface of the virus (Campos and Barry, 2006; Campos et al., 2004a). These ligands include antibodies, transferrin, EGF, and cholera toxin. However, the ligands failed to mediate robust vector targeting when displayed using pIX-BAP (Campos and Barry, 2006; Campos et al., 2004a).

A common epitope of the hemagglutinin (HA) protein of the influenza A virus was added to the end of pIX to see if it is possible to induce antigen-specific immunity in mice (Krause et al., 2006). No significant anti-HA titres were detected when mice were immunized with the adenovirus containing a pIX fused to the HA epitope (Krause et al., 2006).

Single-chain antibody fragments (scFv) 13R4 against β-galactosidase were fused to the C-terminus of pIX with a MYC-HIS tag by using a spacer to lift the ligand out of the proposed cleft in which pIX resides in the capsid of the virus (Vellinga et al., 2007). Single-chain antibodies are thought to have the potential to achieve cell type specificity and changed adenoviral tropism. Vellinga et al. (2007) chose to incorporate the 13R4 scFv to the end of pIX as it has the capacity to fold correctly in a reducing environment. The 13R4 scFv was shown to be functional in this context as it was able to bind its ligand β-galactosidase (Vellinga et al., 2007). In a comparison of

different spacer lengths, the accessibility of the ligands was increased with increasing spacer length. The most efficient spacer for displaying ligands on the surface of the virus appeared to be the 113 amino acids (78 Å) long spacer (Vellinga et al., 2004). However, the virus with the 78 Å long spacer fused to pIX showed reduced heat stability suggesting that large fusions to pIX might hinder the stability of the capsid of the virus (Vellinga et al., 2007; Vellinga et al., 2006).

1.3 Expression of Genes

1.3.1 Regulation of gene expression

Viruses have developed several ways to exploit the host cell machinery to control the transcription and translation of their own genomes. Due to their small genome size RNA viruses are especially abundant in using methods for packaging their genomes in smaller space. Many RNA viruses use polyproteins, where all the proteins coded by the viral genome are expressed from one long open reading frame (orf) (Donnelly and Luke, 2001). The have also developed control mechanisms to manipulate the transcriptional and translational machinery of the host cell, which include leaky scanning of start codons, ribosomal shunting, reinitiation of translation, ribosomal frameshifting and suppression of termination.

In leaky scanning the ribosome does not necessarily initiate translation at the first start codon (AUG) but can translate many proteins from overlapping orfs. Start codons may be overlooked due to the context of the surrounding sequence in which they lie (Williams and Lamb, 1989).

Ribosomal shunting is another way of manipulating the translation of mRNAs. In this case the ribosome scanning machinery enters via the 5' end of the viral mRNA but is shunted to an acceptor site downstream bypassing intervening segments by a *cis*-acting element within the 5' UTR of the viral mRNA (Futterer et al., 1993).

Yet another mechanism used by RNA viruses to manipulate the host cell machinery is reinitiation of translation. Here tandem orfs are closely linked, and the ribosome reinitiates translation at another start codon in quite close proximity either up- or downstream of the termination site of the first orf (Ahmadian et al., 2000).

In ribosomal frameshifting the ribosome starts to translate one orf but before reaching the stop codon frameshifts to another orf. This results in a fusion of the two proteins coded by these two orfs. This mechanism seems to require a secondary structure motif to allow the close proximity of the sites where the frameshifting occurs. These structure motifs may need to contain a "slippery site" and the ability to form a

pseudoknot. In several cases frameshifting has been reported to occur less than 30% of the time (Dinman et al., 1991; Somogyi et al., 1993).

As opposed to leaky scanning where start codons are overlooked, in suppression of termination a stop codon is overlooked. This results in the generation of a long fusion protein that is cleaved in subsequent steps by proteinases. This mechanism seems to need a particular context for the stop codon. In Sindbis virus a cytidine residue is needed immediately downstream of the UGA termination codon for efficient read-through to occur (Li and Rice, 1993; Strauss et al., 1983). In Murine leukaemia virus a complex bipartite signal is needed to achieve read-through at the UAG termination codon. This signal includes a purine-rich sequence of eight nucleotides and an RNA pseudoknot structure downstream of the UAG termination codon (Feng et al., 1992; Yoshinaka et al., 1985).

Initiation of translation by internal ribosomal entry sites (IRESs) is a commonly used method in biotechnology. In this case, as with the mechanisms described above, the secondary structure of the mRNA is important. The IRESs form hairpins that allow the ribosome to initiate translation in the middle of an mRNA (reviewed by de Felipe, 2002). In addition to these methods another sequence, called 2A, was discovered in picornaviruses. This sequence causes the ribosome to skip one specific peptide bond.

1.3.2 2A sequences

2A was originally discovered in Foot-and-Mouth Disease Virus (FMDV), which is a member of the Picornavirus family. 2A allows co-expression of genes flanking the 2A sequence. It is a short peptide sequence that causes the ribosome to skip one specific peptide bond resulting in the generation of two peptides upstream and downstream of 2A. In FMDV 2A is found at the junction between the transcription regions for the replicative and the capsid proteins (Ryan and Drew, 1994). The proposed cleavage mechanism involves the last three peptides of the consensus sequence (-DxExNPG) and the first proline (P') of the following protein 2B. The upstream sequence of 2A is thought to form an α-helix that interacts with the exit tunnel of the ribosome (Donnelly et al., 2001). This causes the last three peptides of the consensus sequence

to form a tight turn that prevents the formation of the final peptide-bond between the last glycine (G) of the 2A sequence and the P' (Ryan et al., 1999). 2A and 2A-like sequences can be found for many different viruses, and they have previously been utilised in gene therapy (de Felipe, 2004) and biotechnology (de Felipe et al., 2006) applications.

1.4 Recombination Methods

In order to modify adenoviral vectors, different recombination methods may be exploited. These methods include Cre-mediated homologous recombination and ϕC31-mediated integration.

1.4.1 Cre recombinase

Cre is a recombinase of the Int family (Argos et al., 1986), a product of the *cre* gene of the bacteriophage P1 (Sternberg et al., 1986). It is 38 kDa in size (Sternberg et al., 1986) and mediates a bidirectional recombination between identical *loxP*-sites either intra- or intermolecularly (Hoess and Abremski, 1990). The *loxP*-sites are 34 bp in length comprising an 8 bp region between two inverted repeats of 13 bp in length. The inverted repeats have been found to act as recognition sites for two Cre enzymes that appear to have strong cooperation between each other (Hoess and Abremski, 1990).

The Cre-*lox* system was first discovered by Sternberg in the 1970's (Sternberg, 1979), and since then it has been used widely to manipulate both prokaryotic and eukaryotic genomes. The first use of the Cre-*lox* recombination in a eukaryotic genome was with the excision of a floxed (flanked with *loxP* sites) LEU2 gene from the chromosome of *Saccharomyces cervisiae* (Sauer, 1987). The functionality of the system was also shown in mammalian cells both extrachromosomally (Sauer and Henderson, 1988) and when the *loxP*-sites were located in the mouse genome (Sauer and Henderson, 1989). Since the first trials of the Cre-*lox* system in mammalian cells, the methods have been widely established as a technique to precisely genetically modify the mouse genome (Sauer, 1998; reviewed by Feil, 2007).

The Cre-*lox* recombination system has also been widely used in the generation and modification of adenoviral vectors (Hardy et al., 1997; Mitani et al., 1995; Ng et al., 1999; Parks et al., 1996; Sandig et al., 2000; Tashiro et al., 1999). The most utilised application for the Cre-*lox* mediated recombination in the adenovirus field is the generation of helper-dependent adenoviruses, or so called gutless adenoviral vectors.

These vectors are devoid of all coding viral regions only containing the essential *cis*-acting elements, for example the packaging signal, that are required for packaging and replication of the virus. The helper-dependent adenoviruses rely on a complementing virus, or helper virus, to provide the necessary proteins *in trans* for packaging of the virus. These viruses were developed to ease the cloning of viruses, as well as to reduce the immunogenicity of the adenoviruses (reviewed by Alba et al., 2005).

Since the discovery of the Cre-*lox* recombination system, other recombination systems have also been described and utilised in generation of helper-dependent adenoviruses. These include the FLP (Ng et al., 2001) and FLPe (*in vitro* evolved FLP) (Buchholz et al., 1998; Umana et al., 2001) mediated recombination. FLP is a yeast site-specific recombinase that catalyzes recombination between frt-sites (Som et al., 1988). Another recombinase that may be used is the ϕC31 integrase.

1.4.2 ΦC31 integrase

ϕC31 integrase is a member of the resolvase/integrase family of recombinases (Stark et al., 1992; reviewed by Groth and Calos, 2004). ϕC31 integrase mediates unidirectional recombination between two non-identical att-sites, attB and attP, that results in the generation of other two non-identical att-sites, attL and attR (Table 1, p. 20). The integration is strictly controlled by the non-identical attB and attP recombination sites (Kuhstoss and Rao, 1991; Rausch and Lehmann, 1991). ϕC31 does not seem to show strong preference for position or orientation of the recombination sites, nor does the reaction depend on supercoiling of the DNA as linear substrates have been shown to recombine *in vitro* (Thorpe and Smith, 1998; Thorpe et al., 2000). It has also been shown that the ϕC31 integrase does not need any other proteins for the reaction to occur (Thorpe and Smith, 1998).

ϕC31 was shown to be active in human cells (Groth et al., 2000). ϕC31-mediated integration has been used previously in gene therapy applications (reviewed by Calos, 2006), as well as in combination with the Cre-*lox* system in the manipulation of vertebrate chromosomal DNA (Dafhnis-Calas et al., 2005). The ϕC31-system has also

been used in the generation of helper-dependent adenoviruses, for flanking the packaging signal. The attB-site inserted left from the packaging signal impaired the packaging of the virus regardless of the presence of the ϕC31 enzyme, and delayed the viral life cycle resulting in lower yields at early time points when compared with a wild type virus (Alba et al., 2007).

Part of this project was to combine the Cre-*lox* and the ϕC31 systems to enable the rapid modification of the E1-pIX region of the adenovirus 5 genome. This was done by deleting the E1-pIX region from the viral genome and flanking the deleted site with a *loxP*- and an attB-site. The modification would involve a series of recombinations with an addback plasmid containing a mutated E1-pIX region flanked with a *loxP*- and an attP-site. This would result in the generation of a mutant E1-pIX adenovirus containing a single *loxP*-site and an attR-site (Figure 3, p. 29).

Name	**Sequence**
attB	CCGCGGTGCGGGTGCCAGGGCGTGCCC**TTG**GGCTCCCCGGGCGCGTACTCC
attP	AGTAGTGCCCCAACTGGGGTAACCT**TTG**AGTTCTCTCAGTTGGGGGCGTA
attL	CCGCGGTGCGGGTGCCAGGGCGTGCCC**TTG**AGTTCTCTCAGTTGGGGGCGTA
attR	AGTAGTGCCCCAACTGGGGTAACCT**TTG**GGCTCCCCGGGCGCGTACTCC

Table 1: Sequences of the att-sites. The consensus triplet (TTG) is marked in black. The attB- and attP-sites recombine to give the attL- and attR-sites. (Thorpe et al., 2000)

2. Methods and Materials

Cell lines. 293T and SW480 cells were supplied by American Type Culture Collection (ATCC), Manassas, VA. C7 cells (Amalfitano and Chamberlain, 1997) were provided by Dr J. Chamberlain. HER911 cells (Fallaux et al., 1996) were supplied by Dr P. Beard (Swiss Institute for Experimental Cancer Research (ISREC), Epalinges, Switzerland). All cells were cultured in DMEM with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA).

Lentiviruses. 293T cells were transfected with lentiviruses containing pIX (pLV-CMV-pIX-IRES-eGFP and pLV-CMV-pIX-IRES-NPTII) and pIX with a 75 Å spacer (pLV-CMV-pIX.flag.75.MYC-IRES-NPTII) (Vellinga et al., 2006) (provided by Dr. R. Hoeben) together with pSD11 (gag-pol) and pSD16 (packaging plasmid). The supernatant was harvested 24 hours later, complemented with 8 μg/ml Polybrene (Sigma, Saint Louis, MI), filtered through 0.22 μm filter, and stored at -70°C.

cSK cell lines. HER911, 293T and C7 cells were infected with the lentiviruses LV-CMV-pIX-IRES-eGFP, LV-CMV-pIX-IRES-NPTII, and LV-CMV-pIX.flag.75.MYC -IRES-NPTII in medium complemented with 8 μg/ml Polybrene (Sigma). 24 hours after the cells were washed with PBS, and fresh medium was added. The medium was complemented with 8 μg/ml Polybrene (Sigma) and 5 μg/ml G418 (Invitrogen) for the lentiviruses containing a neomycin marker and the cells passaged after the cells in a control well had died. See Table 2 for details of cell lines (cSK1-9). The pIX-expression was tested by immunoblotting.

Antibodies. Rabbit anti-pIX antibody (Caravokyri and Leppard, 1995) was provided by Dr K. Leppard. Rabbit antibody against the last amino acids of the PTV-1 2A sequence (Holst et al., 2006) was provided by Dr D. Vignali. Rabbit anti-Ad5 hexon and anti-pIX antibodies were provided by Prof. W. Russell, School of Biology, University of St Andrews, UK. Monoclonal mouse anti-V5 tag (Southern et al., 1991) was provided by Dr. R. Randall. Polyclonal rat anti-FCU1 was supplied by Transgene S.A, Strasbourg, France. Monoclonal mouse anti-α-Tubulin antibody (clone B-5-1-1) was supplied by Sigma. Mouse anti-GFP antibody was supplied by Roche, Basle,

Switzerland. Mouse anti-E1A antibody (M58) was supplied by BD Biosciences, San Diego, CA. Peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L; 115-035-003) and anti-rabbit IgG (H+L; 111-035-003) were supplied by Jackson ImmunoResearch, West Grove, PA. ECL Anti-rat IgG, Horseradish Peroxidase-Linked Species-Specific whole antibody from goat was supplied by GE Healthcare, Little Chalfont, United Kingdom.

Cloning. All the restriction enzymes and polymerases used were supplied by New England Biolabs, Ipswich, MA.

vpSK1. A fragment containing the floxed mutated packaging signal together with pIX was obtained by PCR from H14 virus (Sandig et al., 2000) with primers G76 (CGGAAT TCAAGC TTAATT AACATC ATCAAT AATATA CC) and oPF3 (GCCGCC GCTAGC ATGAGC ACCAAC TCGTTT GA), using Taq polymerase. TOPO TA cloning (Invitrogen) was used to ligate this insert into the pCRII-TOPO vector (Invitrogen) giving pSK1. An attB site was inserted after the floxed packaging signal by inverse PCR using primers oSK1 (TGGGCT CCCCGG GCGCGT ACTCCA GCAAGT GTCTTG CTGTCT TTATTT AGGGGT TTTGC) and oSK2 (AGGGCA CGCCCT GGCACC CGCACC GCGGCT AGAGGA TCCGAA TTCAGT), simultaneously deleting the pIX gene from the vector to yield pSK2. The right end of the adenovirus 5 genome was cloned from pCF6 (unpublished, derived from pLS77 (Brunori et al., 2001)) in a SalI-blunt fragment into pBSK- vector (Stratagene, La Jolla, CA) giving pSK3. pSK3 was used as the acceptor vector for a SpeI-EcoRV fragment from pSK2, yielding pSK5. A PacI fragment was cloned from pSK5 into pSK13 to give pSK6. pSK13 was obtained by self-ligation of PacI digested pPZ6, a YAC/BAC derived from pNKBAC39 (Gagnebin et al., 1999). pSK6 was cut with PacI and gap repaired in yeast (Gagnebin et al., 1999) with Ad5 genomic DNA (ATCC VR5) to give vpSK1 (Figure 4, p. 31).

The "addback" plasmid. pSK14 contains the left ITR and the packaging signal. It was obtained via BglII-ClaI digestion of pCF4 (derivative of pLS77 containing the left end of the Ad5 genome up to 952 nt). A wild type lox site was inserted between the left ITR and the packaging signal by inverse PCR using primers oSK3 (TTCAAT

AGAATT CTAAGG ATCCAA TGAGCT CACTTT TGCCAC ATCCGT) and oSK4 (GCATAT CGTATG TAATAT GCTTCA ATAGAC GTTTTT GGTGTG CG). A SacI-SacII fragment was cloned from the resulting pSK7 into a SacI-SacII digested pBSK- plasmid (Stratagene) to yield pSK8. pSK15 contains the pIX-IVa2 region of the Ad5 genome. pSK15 was obtained via SphI-BglII digestion of pPF2 (see above; Figure 9, p. 38) to reduce the size of the plasmid. The attP-site would then be inserted into pSK15 by inverse PCR with primers oSK5 (GAGAAC TCAAAG GTTACC CCAGTT GGGGCA CTACTT GATCCA AATCCA AAC) and oSK6 (TCAGTT GGGGGC GTAAAG GTACCA GCAAGT GTCTTG CTGTCT TTATT) and reamplified with oSK9 (TACTTG ATCCAA ATCCAA AC) and oSK10 (TCAGTT GGGGGC GTAAAG GT) to amplify truncated PCR products that may have arisen from truncated primers, due to the great length of the primers. From the resulting pSK9 a SacII-KpnI fragment would be cloned into SacII-KpnI digested pSK8 to give pSK10. An EcoRI-BamHI fragment from pLVTHM (Wiznerowicz and Trono, 2003) containing a tet-operator would be cloned into pSK10 to give pSK11. A SacII fragment from wild type Ad5 YAC/BAC, pMB20 (Brunori et al., 2001) containing the middle part of the E1-pIX region would be cloned into SacII digested pSK11 to give pSK12 (Figure 7, p. 35).

Viruses: vKM11 and vKM31. The pIX-2A-GFP viruses are called vKM11 (FMDV 2A, "F2A") and vKM31 (PTV-1 2A, "P2A"). The 2A sequences were inserted into the genome of vKH6 (Homicsko et al., 2005) by two-step gene replacement in yeast (Gagnebin et al., 1999). pPDF2 (unpublished) is a pcDNA3.1(+) (Invitrogen) derived vector with a hybrid CMV/T7 promoter and deletion of the *neo* cassette. pRS406 is a yeast integrating vector (Sikorski and Hieter, 1989). Overlapping pIX-IVa2 fragments of Ad5 genomic DNA (ATCC VR5) were amplified by PCR and cloned into pPDF2 [pIX region: primers oPF3 (GCCGCC GCTAGC ATGAGC ACCAAC TCGTTT GA) and oPF4 (GGTACC CCATCA TTATGG ACGAAT GCATGG AAA)] and pRS406 [IVa2 region: primers oPF7 (ATGCAT GGATCC ATAATG ATGGCA ATGGGC C) and oPF8 (GCCACG GGTACC AGGGGC TGGACT ATGACA C)] to give pPF2 and pPF3, respectively. The PTV-1 2A site was inserted into pPF2 by inverse PCR with primers oPF5 (CCACGT CTCCTG CTTGCT TTAACA GAGAGA AGTTCG TGGCTC CGGACC CTCTAG AAACCG CATTGG GAGGGG

AGGAAG CC) and oPF6 (TCCGTC GACGCG GCCGCG AATTCC AATGCG GTTTAA AACATA AATA). pPDF16 is an unpublished derivative of pL-P-Δ1D2A-G from which a run of nine thymidines between FMDV 2A and GFP was deleted (De Felipe and Izquierdo, 2000; Felipe and Izquierdo, 2003). An FMDV 2A-GFP cassette was cloned from pPDF16 into pPF4 on an XbaI-NotI fragment to give pPF5. The remaining steps in the construction of the gene replacement vectors (pPF13 for F2A, pPF14 for P2A) are shown in Figure 9, p. 38. pPF13 and pPF14 were linearised with SacII for insertion into vpKH6 (Homicsko et al., 2005). The resulting plasmids containing the modified, full length viral genomes are called vpKM1 (F2A) and vpKM3 (P2A). They were cut with PacI to liberate the viral DNA then transfected into C7 cells to produce virus. After plaque purification on SW480 cells the viruses were called vKM11 (F2A) and vKM31 (P2A). They were expanded on SW480 cells, purified by two rounds of $CsCl_2$ equilibrium gradient centrifugation, buffer exchanged using HR400 columns (GE Healthcare) into 1 M NaCl, 100 mM Tris-HCl pH 8.0, and stored at -70°C. The plaque forming unit (pfu) titre was determined on HER911 cells.

Viruses: vPZ2 and vPZ7. The FCU1 gene was obtained by PCR with primers oPF12 (CTTTCA GGGCCC ATGGTG ACAGGG GGAATG GCAAG) and oPF13 (CGCGTG GCGGCC GCTTAA ACACAG TAGTAT CTGTCA) from pTG13046 (Transgene S.A). The PCR product was cut with ApaI and NotI, and inserted into ApaI-NotI digested pPF5 (F2A) and pPF6 (P2A) (see above; Figure 9, p. 38) vectors to yield pPF9 (F2A) and pPF10 (P2A). A SacI-BstXI fragment containing the pIX-2A-FCU1 cassette was cloned from both pPF9 and pPF10 into SacI-BstXI cut pPF3 to yield the gene replacement vectors pPF15 (for F2A) and pPF16 (for P2A) (Figure 15, p. 48). pPF15 and pPF16 were linearised with SacII for insertion into vpKH6 (Homicsko et al., 2005). The resulting plasmids containing the modified, full-length viral genomes are called vpPZ2 (F2A) and vpPZ7 (P2A). They were cut with PacI to liberate the viral DNA then transfected into cSK8 cells to produce virus. They were expanded on cSK2 cells.

Viruses: vPZ4 and vPZ6. The polylinker between the MMP7 gene and the V5 tag in the pMMP7 plasmid (provided by Dr. I. Stamenkovic, Department of Experimental Pathology, University of Lausanne, Switzerland) was deleted by digesting the plasmid

with NotI and Sac II and blunting the overhangs with T4 polymerase. This yielded pPF7. The size of the V5 tag was reduced by PCR with primers oPF14 (GAGCTC GGGCCC ATGCGA CTCACC GTGCTG TGTGCT) and oPF15 (TGATGA GCGGCC GCTTAC GTAGAA TCGAGA CCGAGG AGAG). The PCR fragment was digested with ApaI and NotI and cloned into ApaI-NotI digested pPF5 and pPF6 vectors (see above; Figure 9, p. 38) to give pPF11 (F2A) and pPF12 (P2A). These were digested with NheI, blunted with T4 polymerase and partially digested with BstXI to give a fragment containing the pIX-2A-MMP7 cassette that was cloned into NheI digested, T4 polymerase blunted, and BstXI digested pPF3 vector (see above; Figure 9, p. 38) to yield the gene replacement vectors pPF17 (F2A) and pPF18 (P2A) (Figure 16, p. 49). pPF17 and pPF18 were linearised with SacII for insertion into vpKH6 (Homicsko et al., 2005). The resulting plasmids containing the modified, full-length viral genomes are called vpPZ6 (F2A) and vpPZ4 (P2A). They were cut with PacI to liberate the viral DNA then transfected into cSK8 cells to produce virus. They were expanded on cSK2 cells.

Influence of MMP7 on pIX-P2A expression. pPF12 (plasmid containing the CMV-driven pIX-P2A-MMP7 expression cassette) was cut with ApaI, blunted with T4 DNA polymerase and self-ligated to generate a stop codon downstream of P2A. This plasmid, called pGF3, was transfected into 293T cells, and the cell lysate was immunoblotted for pIX.

Coupled transcription/translation *in vitro*. Coupled transcription-translation reactions were performed in rabbit reticulocyte lysates (TnT T7 system, Promega, Madison, WI). Translation reactions performed in a total volume of 7 µl were programmed with 0.1 µg plasmid DNA and incubated in the presence of ^{35}S-methionine (5µCi, GE Healthcare) at 30ºC for 90 minutes. Reactions were analysed by autoradiography of SDS-PAGE gels.

Immunoblotting. To test the pIX expression cassette, 293T cells were transfected with pPF2, pPF5, pPF6, pPF9, pPF10, pPF11, and pPF12, and harvested 24 hours later. To test protein expression from viruses, SW480 cells were infected with vKH6, vKM11 and vKM31 at a multiplicity of infection (MOI) of 0.5 pfu/cell, the medium

was changed after four hours and the cells were harvested 12 hours later. To test expression of pIX from the cSK cell lines, cells were harvested after at least five passages, washed with PBS and suspended in 2x sample buffer. Nitrocellulose membranes (GE Healthcare) were probed with primary antibodies against E1A, hexon, pIX, 2A, GFP, FCU1, V5 tag and tubulin, followed by secondary antibodies coupled to HRP and developed using chemiluminescence (ECL, GE Healthcare).

Protein IX half-life. To test pIX half-life HER911 cells were infected with vKM11 and vKM31 at MOI 0.5 of pfu/cell. The medium was replaced at 24 hours post-infection with medium containing 20 μg/ml of cycloheximide (Sigma). Samples were harvested 0, 2, 4, 8 and 16 hours after changing the medium. The samples were immunoblotted for pIX.

Pictures of viral infection. Photographs of viral infections were taken with a Nikon Coolpix 990 camera, using GFP filters (49002, Chroma, Rockingham, VT) on an Olympus CKX41 microscope at four times magnification.

Measurement of plaque size. HER911 cells were infected at an MOI of 20 pfu/well in six well plates and covered with agar. The wells were stained with propidium iodide and pictures were taken 10 days after infection. The size of the plaques was measured using ImageJ (NIH, Bethesda, MD). A minimum of 22 plaques were measured per virus. Error bars are SEM. Photographs of individual plaques were taken with a Nikon Coolpix 990 camera, using mCherry filters (49008, Chroma) for propidium-stained cells and GFP filters (49002, Chroma) for viral GFP-expressing cells, on an Olympus CKX41 microscope.

Burst assay. SW480 cells were infected at an MOI of 0.1 pfu/cell, and harvested after 48 hours. Virus was released from the cells by three rounds of freeze-thawing and titred by pfu assay on HER911 cells. The burst size is expressed as output pfu per input pfu.

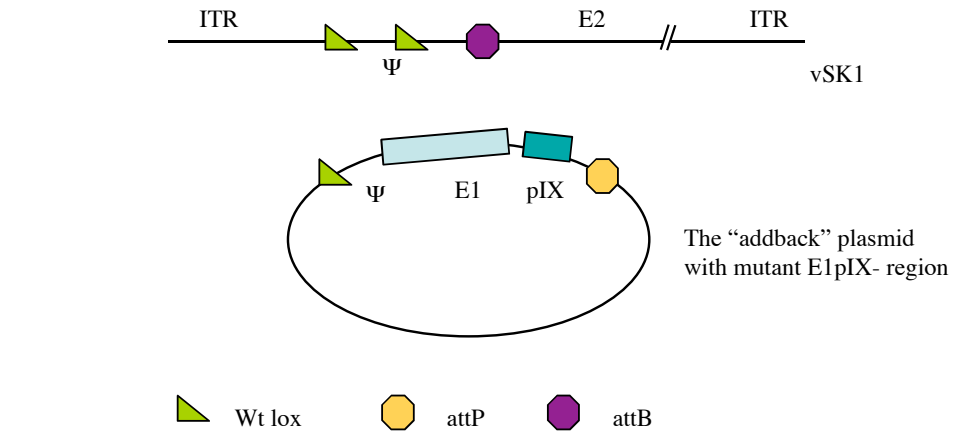
Cytopathic effect assay. SW480 cells were infected at an MOI ranging from 0.0002 to 0.2 pfu/cell. The medium was changed four hours after infection, and cells were stained after seven days with crystal violet.

Heat stability assay. The viruses were heated to 45°C for 0, 4, 8 or 12 minutes in the storage buffer. The titre of infectious virus was then measured by plaque assay on HER911 cells. The wells were stained with propidium iodide and the plaques counted.

3. Results

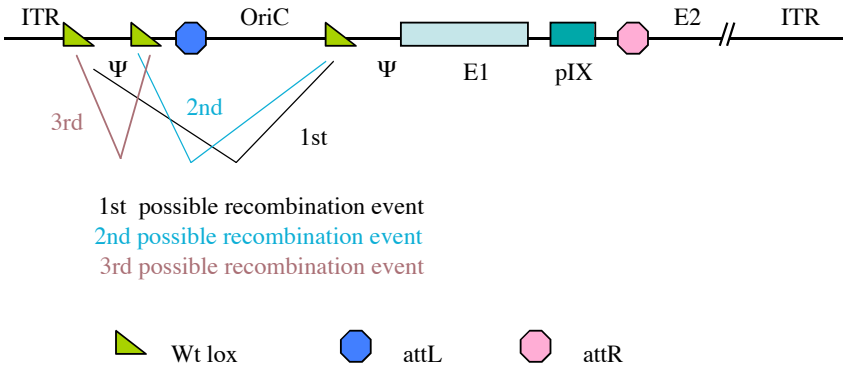
3.1 E1pIX-Philoxera System

To enable rapid modification of the E1-pIX region of adenovirus 5 an E1-pIX deleted virus with *loxP* and attB sites flanking the deleted region was constructed. Together with an "addback" plasmid containing *loxP* and attP sites flanking a mutated E1-pIX region, the E1-pIX region may be modified via two recombination events that take place, consecutively, after transfections with Cre and ΦC31 recombinases (Figure 3, p. 29). First, the attB and attP sites recombine to produce a fused viral genome with the whole of the addback plasmid inserted between the recombined attL and attR sites (see Table 1 for att-sequences, p. 20). After Cre mediated recombination the plasmid pops out leaving the mutated E1-pIX region in the newly created virus. There are four possible outcomes of the recombination events: two *lox* sites left in the pop-out plasmid; two *lox*-sites left in the recombined virus with a packaging signal in the middle; two *lox* sites left in the recombined virus with attL site in the middle; or recombined virus and two plasmids all containing one *lox*-site. The fourth option is the most likely as the other three may still undergo intramolecular recombination to give two plasmids and a recombined virus all containing one *lox*-site each. In all cases the recombination events result in the formation of a replication competent adenovirus with a mutant E1-pIX region.



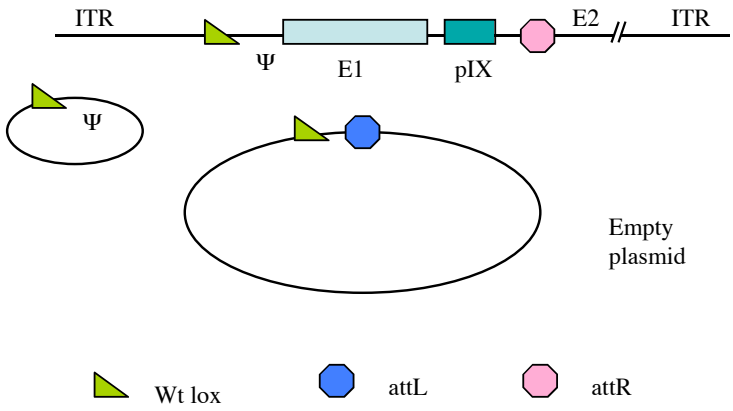
(a)

The pIX-Philoxera System



(b)

After transfection with ΦC31



(c)

After transfection with Cre

Figure 3: The pIX-Philoxera system. (a) The recombination events take place between the philoxeraΔE1ΔpIX-virus (vSK1) and the "addback" plasmid. (b) After transfection with ΦC31 the virus has recombined with the plasmid from the att-sites. The Cre-enzyme can recombine the *loxP*-sites in three different ways. (c) After transfection with Cre, if all three possible recombination events have occurred, a recombinant virus with a mutant E1pIX-region is generated along with two plasmids.

3.1.1 Construction of vpSK1

The construction of vpSK1 is shown in detail in Figure 4, p. 31. A fragment containing the floxed mutated packaging signal together with pIX was obtained by PCR from H14 virus (Sandig et al., 2000) with primers G76 (CGGAAT TCAAGC TTAATT AACATC ATCAAT AATATA CC) and oPF3 (GCCGCC GCTAGC ATGAGC ACCAAC TCGTTT GA), using Taq polymerase. TOPO TA cloning (Invitrogen) was used to ligate this insert into the pCRII-TOPO vector (Invitrogen) giving pSK1. An attB site was inserted after the floxed packaging signal by inverse PCR using primers oSK1 (TGGGCT CCCCGG GCGCGT ACTCCA GCAAGT GTCTTG CTGTCT TTATTT AGGGGT TTTGC) and oSK2 (AGGGCA CGCCCT GGCACC CGCACC GCGGCT AGAGGA TCCGAA TTCAGT), simultaneously deleting the pIX gene from the vector to yield pSK2. The right end of the adenovirus 5 genome was cloned from pCF6 [unpublished, derived from pLS77 (Brunori et al., 2001)] in a SalI-blunt fragment into pBSK- vector (Stratagene, La Jolla, CA) giving pSK3. pSK3 was used as the acceptor vector for a SpeI-EcoRV fragment from pSK2, yielding pSK5. A PacI fragment was cloned from pSK5 into pSK13 to give pSK6. pSK13 was obtained by self-ligation of PacI digested pPZ6, a YAC/BAC derived from pNKBAC39 (Gagnebin et al., 1999). pSK6 was cut with PacI and gap repaired in yeast (Gagnebin et al., 1999) with Ad5 genomic DNA (ATCC VR5) to give vpSK1.

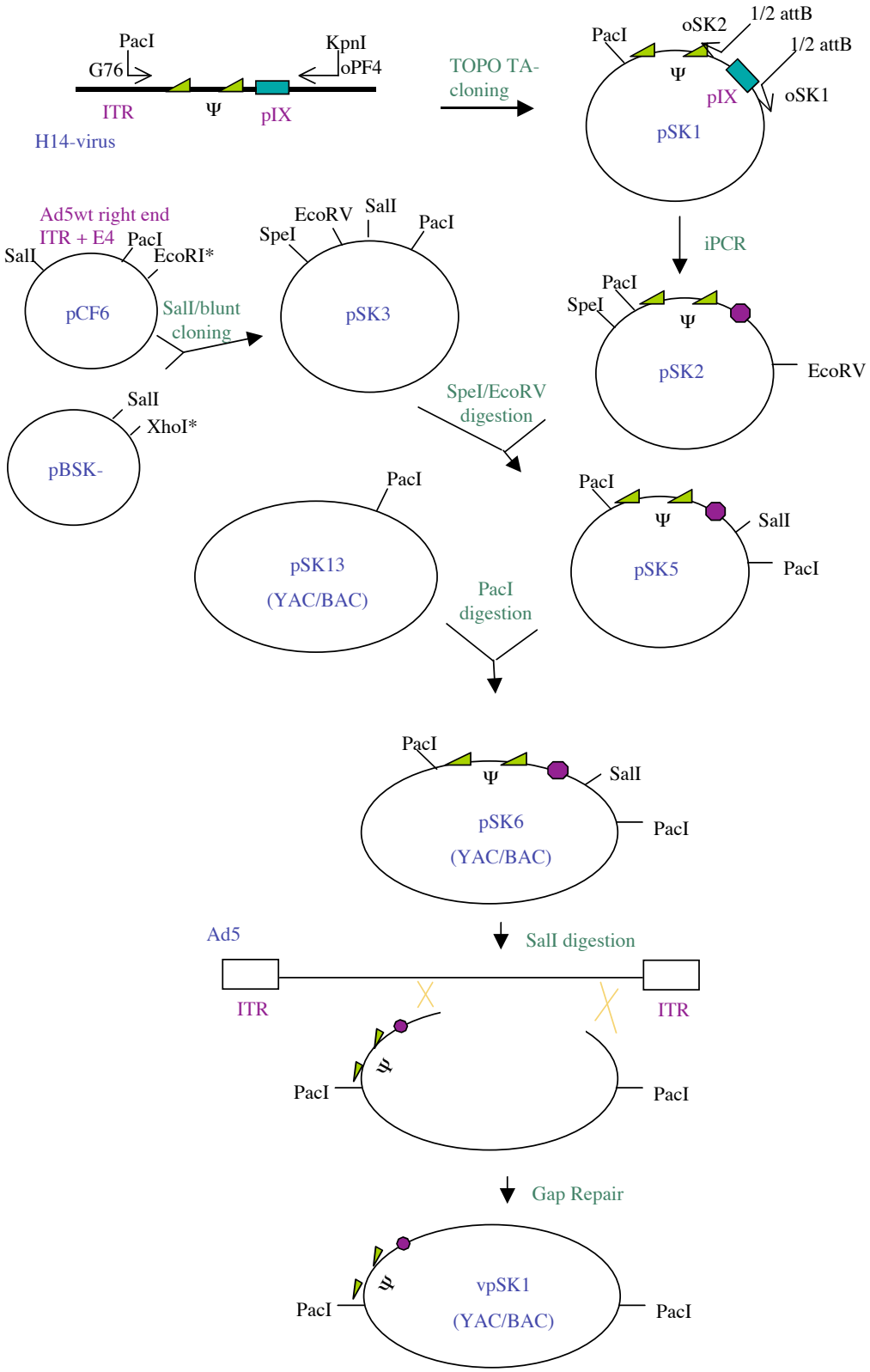


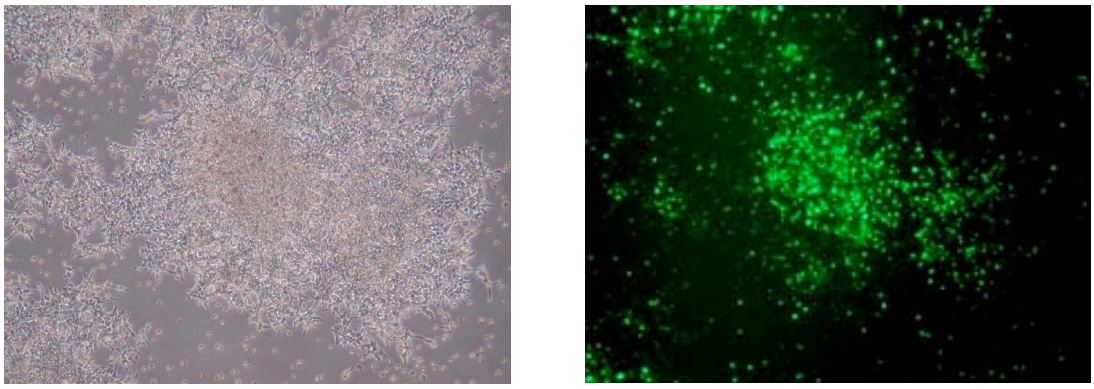
Figure 4: Schematic diagram showing the cloning strategy for the vpSK1 YAC/BAC that contains the wt Ad5 genome with E1pIX deletion, and *loxP*- and attB-sites.

3.1.2 Production of vSK1

The initial production of vSK1 was carried out by transfecting 293T cells with PacI digested viral DNA from vpSK1. The production of the virus was found to be difficult as the virus would not give any cytopathic effect after first passage. The attenuation was thought to be due to the lack of pIX in the capsid of the virus. To test this hypothesis PacI digested vpSK1 was transfected into 293T and C7 cells that had been previously transfected with pPF5 to complement for the pIX deficiency in the virus. pPF5 is a plasmid containing the pIX-F2A-GFP cassette (see Section 3.2). The virus vSK1 appeared to prefer the cells containing pPF5, as the green cells displayed more cytopathic effect (Figure 5, p. 33). This is not a pIX-induced phenomenon, as the control wells with only pPF5 transfected into them did not show any cytopathic effect. The cells were lysed more efficiently when pIX was present than absent but the conversion of plasmid DNA to virus was inefficient. Therefore, nine different pIX-helper-cell lines were produced by transfecting HER911, 293T and C7 cell lines with lentiviruses containing pIX (pLV-CMV-pIX-IRES-eGFP and pLV-CMV-pIX-IRES-NPTII) and pIX with a 75 Å spacer (pLV-CMV-pIX.flag.75.MYC-IRES-NPTII) (Vellinga et al., 2006) (see Table 2 for details on cell lines, p. 32). The pIX-complementation was tested with western blots and all of the cell lines express pIX (Figure 6, p. 33). PacI digested vpSK1 was transfected into cSK8 (pIX-complemented C7 cell line). This demonstrated cytopathic effect. The lysate was used to infect cSK2 cells (a pIX-complemented HER911 cell line) but no cytopathic effect could be detected.

Name	Origin	Lentiviral plasmid used	Marker	Complementation
cSK1	HER911	pLV-CMV-pIX-IRES-eGFP	eGFP	pIX
cSK2	HER911	pLV-CMV-pIX-IRES-NPTII	NPTII	pIX
cSK3	HER911	pLV-CMV-pIX.flag.75.MYC-IRES-NPTII	NPTII	pIX.flag.75.MYC
cSK4	293T	pLV-CMV-pIX-IRES-eGFP	eGFP	pIX
cSK5	293T	pLV-CMV-pIX-IRES-NPTII	NPTII	pIX
cSK6	293T	pLV-CMV-pIX.flag.75.MYC-IRES-NPTII	NPTII	pIX.flag.75.MYC
cSK7	C7	pLV-CMV-pIX-IRES-eGFP	eGFP	pIX
cSK8	C7	pLV-CMV-pIX-IRES-NPTII	NPTII	pIX
cSK9	C7	pLV-CMV-pIX.flag.75.MYC-IRES-NPTII	NPTII	pIX.flag.75.MYC

Table 2: The cSK-cell lines created to complement for pIX. The lentiviral plasmids (Vellinga et al., 2006) were provided by Dr. R. Hoeben.



White light GFP

Figure 5: Photographs taken of vSK1 infections on pPF5 (pIX-F2A-GFP) transfected 293T cells. The pictures show that the virus prefers to lyse green cells. These cells are expressing pIX-F2A, pIX-F2A-GFP and GFP.

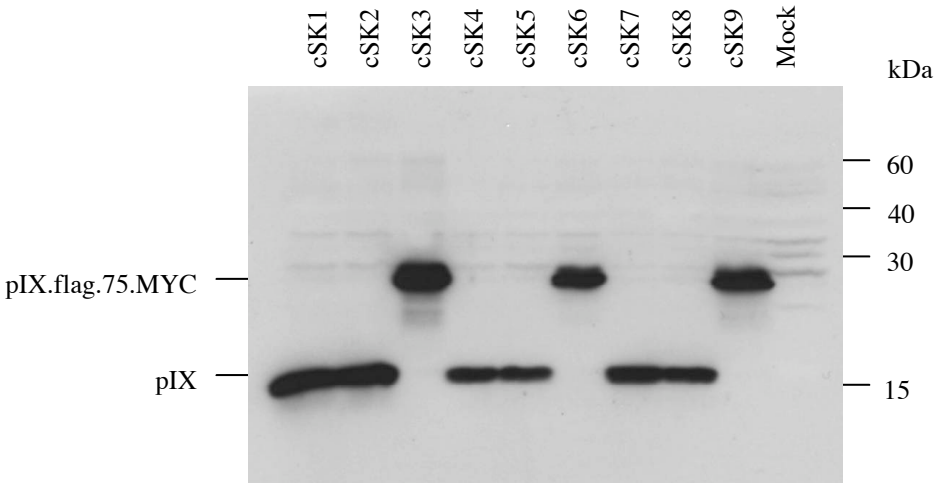


Figure 6: Expression of pIX from the cSK cell lines. Cells were harvested after at least five passages, washed with PBS and suspended in 2x sample buffer. The western blot was probed with anti-pIX.

3.1.3 Construction of the "addback" plasmid

In order to modify the E1-pIX region using the Philoxera system an "addback" plasmid with the potential to be easily modified to include mutant E1-pIX regions has to be generated. The cloning plan for this plasmid is shown in Figure 7, p. 35. pSK14 contains the left ITR and the packaging signal. It was obtained via BglII-ClaI digestion of pCF4 (derivative of pLS77 containing the left end of the Ad5 genome up to 952 nt). A wild type lox site was inserted between the left ITR and the packaging signal by inverse PCR using primers oSK3 (TTCAAT AGAATT CTAAGG ATCCAA TGAGCT CACTTT TGCCAC ATCCGT) and oSK4 (GCATAT CGTATG TAATAT GCTTCA ATAGAC GTTTTT GGTGTG CG). A SacI-SacII fragment was cloned from the resulting pSK7 into a SacI-SacII digested pBSK- plasmid (Stratagene) to yield pSK8. pSK15 contains the pIX-IVa2 region of the Ad5 genome. pSK15 was obtained via SphI-BglII digestion of pPF2 (see below; Figure 9, p. 38) to reduce the size of the plasmid. Due to difficulties with the insertion of the attP-site by inverse PCR into pSK15, to obtain pSK9 (Figure 7, p. 35), the cloning of the "addback" plasmid has not been completed. The attP-site would be inserted into pSK15 by inverse PCR with primers oSK5 (GAGAAC TCAAAG GTTACC CCAGTT GGGGCA CTACTT GATCCA AATCCA AAC) and oSK6 (TCAGTT GGGGGC GTAAAG GTACCA GCAAGT GTCTTG CTGTCT TTATT) and reamplified with oSK9 (TACTTG ATCCAA ATCCAA AC) and oSK10 (TCAGTT GGGGGC GTAAAG GT) to amplify truncated PCR products that may have arisen from truncated primers, due to the great length of the primers. From the resulting pSK9 a SacII-KpnI fragment would be cloned into SacII-KpnI digested pSK8 to give pSK10. An EcoRI-BamHI fragment from pLVTHM (Wiznerowicz and Trono, 2003) containing a tet-operator would be cloned into pSK10 to give pSK11. A SacII fragment from wild type Ad5 YAC/BAC, pMB20 (Brunori et al., 2001) containing the middle part of the E1-pIX region would be cloned into SacII digested pSK11. This would give pSK12, which would be the first addback plasmid that could be used to recombine with vSK1 to initially yield a wild type adenovirus 5 with lox and att-sites in the E1-pIX region (Figure 7, p. 35). pSK12 could be further modified to yield mutated E1-pIX regions, with modifications either in the E1A and E1B promoters, or in the pIX region.

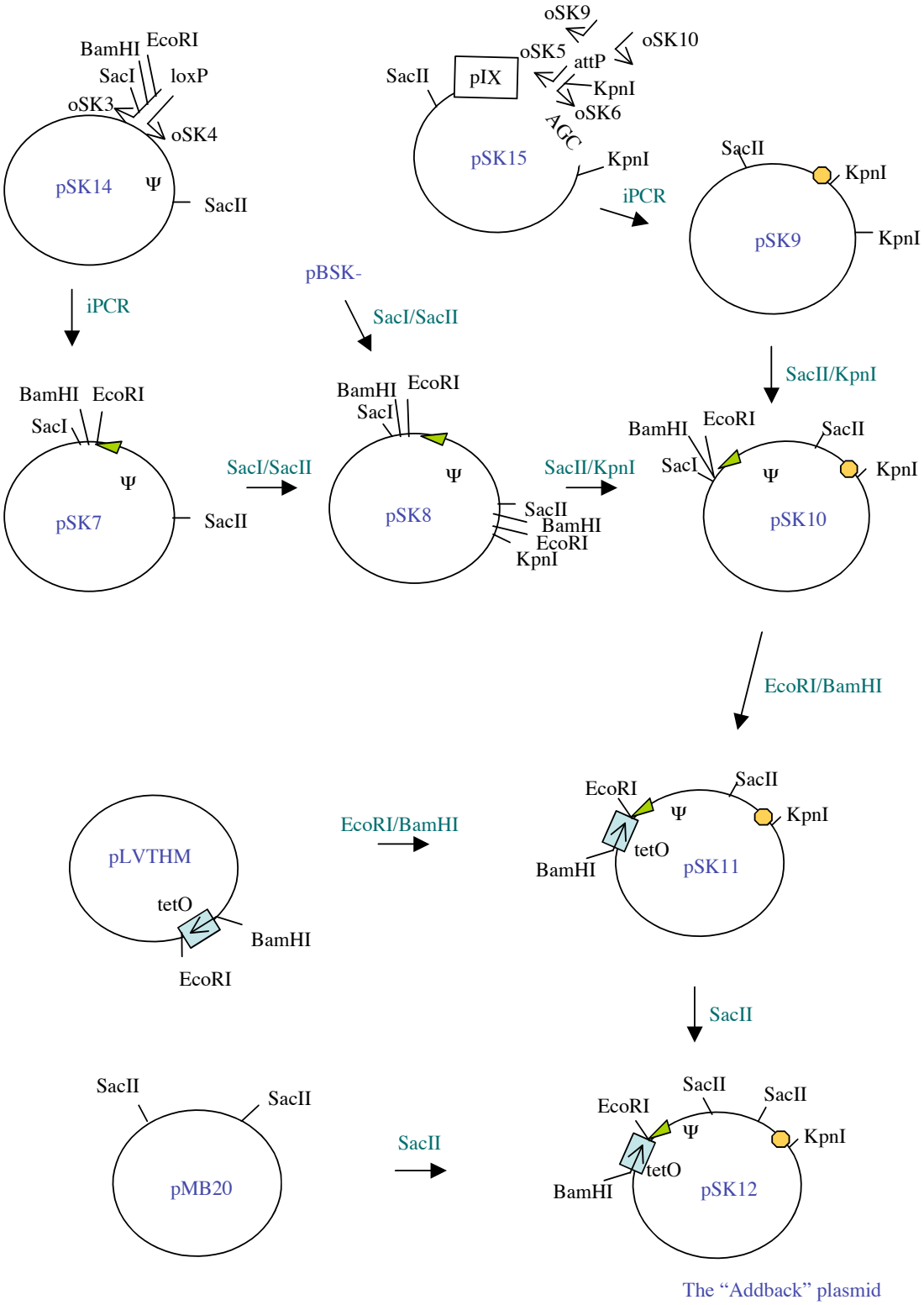


Figure 7: Schematic diagram showing the cloning strategy for the “addback” plasmid.

3.2 Expression of Transgenes After pIX

To test whether picornaviral 2A sequences can be used to express foreign genes in adenoviruses, 2A skipping sites were inserted after the pIX gene in an oncolytic virus that targets colon cancer cells. A schematic diagram of the constructed viruses is shown in Figure 8, p. 36. In addition to the modifications to pIX, the viruses also contain Tcf sites in the ITRs and E1B promoter, as well as an RGD modification in the fibre region as described earlier (Homicsko et al., 2005). In this study two different 2A sequences of varying lengths were tested: a 58 amino acid sequence that includes the FMDV 2A sequence itself (F2A) preceded by 39 amino acids from the FMDV 1D protein (Donnelly et al., 2001); and a 22 amino acid sequence that includes the PTV-1 2A-like sequence (P2A) preceded by a 3 amino acid spacer that improves cleavage (Holst et al., 2006; Szymczak et al., 2004). The sequences are shown in Figure 8, p. 36.

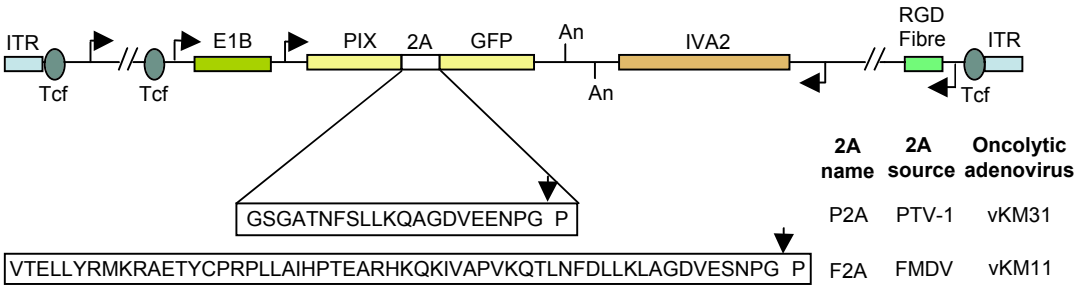


Figure 8: Schematic diagram showing the genome organisation of the pIX-F2A-GFP and pIX-P2A-GFP viruses (vKM11 and vKM31). The viruses contain Tcf sites in the ITRs and E1B promoter. There is an RGD motif in the fibre HI loop. The pIX region has been mutated to contain pIX-2A-GFP fusion genes with the indicated 2A sequences. The 2A skipping site is marked with an arrow.

3.2.1 Expression of green fluorescent protein

In order to determine whether 2A can be used to express genes after pIX, two different viruses with varying lengths of 2A-sequences attached to green fluorescent protein (GFP) were constructed and tested *in vivo*. GFP was used as it permits easy visualisation of infected cells. Before the construction of the viruses, pIX-2A-GFP expression cassettes were cloned to test the skipping of the 2A sequence *in vitro*. A diagram illustrating the construction of the expression cassettes is shown in Figure 9, p. 38. pPDF2 (unpublished) is a pcDNA3.1(+) (Invitrogen) derived vector with a hybrid CMV/T7 promoter and deletion of the *neo* cassette. pRS406 is a yeast integrating vector (Sikorski and Hieter, 1989). Overlapping pIX-IVa2 fragments of Ad5 genomic DNA (ATCC VR5) were amplified by PCR and cloned into pPDF2 [pIX region: primers oPF3 (GCCGCC GCTAGC ATGAGC ACCAAC TCGTTT GA) and oPF4 (GGTACC CCATCA TTATGG ACGAAT GCATGG AAA)] and pRS406 [IVa2 region: primers oPF7 (ATGCAT GGATCC ATAATG ATGGCA ATGGGC C) and oPF8 (GCCACG GGTACC AGGGGC TGGACTA TGACAC)] to give pPF2 and pPF3, respectively. The PTV-1 2A site was inserted into pPF2 by inverse PCR with primers oPF5 (CCACGT CTCCTG CTTGCT TTAACA GAGAGA AGTTCG TGGCTC CGGACC CTCTAG AAACCG CATTGG GAGGGG AGGAAG CC) and oPF6 (TCCGTC GACGCG GCCGCG AATTCC AATGCG GTTTAA AACATA AATA). pPDF16 is an unpublished derivative of pL-P-Δ1D2A-G from which a run of nine thymidines between FMDV 2A and GFP was deleted (De Felipe and Izquierdo, 2000; Felipe and Izquierdo, 2003). An FMDV 2A-GFP cassette was cloned from pPDF16 into pPF4 on an XbaI-NotI fragment to give pPF5. A SacI-BstXI fragment containing the pIX-2A-GFP cassette was cloned from both pPF5 and pPF6 into SacI-BstXI cut pPF3 to yield the gene replacement vectors pPF13 (for F2A) and pPF14 (for P2A). pPF13 and pPF14 were linearised with SacII for insertion into vpKH6 by two-step gene replacement in yeast (Gagnebin et al., 1999). vpKH6 is the YAC/BAC containing the genome for the parental virus, vKH6, that has an RGD peptide in the HI loop of the fibre protein and Tcf sites in the E1A, E1B and E4 promoters (Homicsko et al., 2005). The resulting plasmids containing the modified, full-length viral genomes are called vpKM1 (F2A) and vpKM3 (P2A).

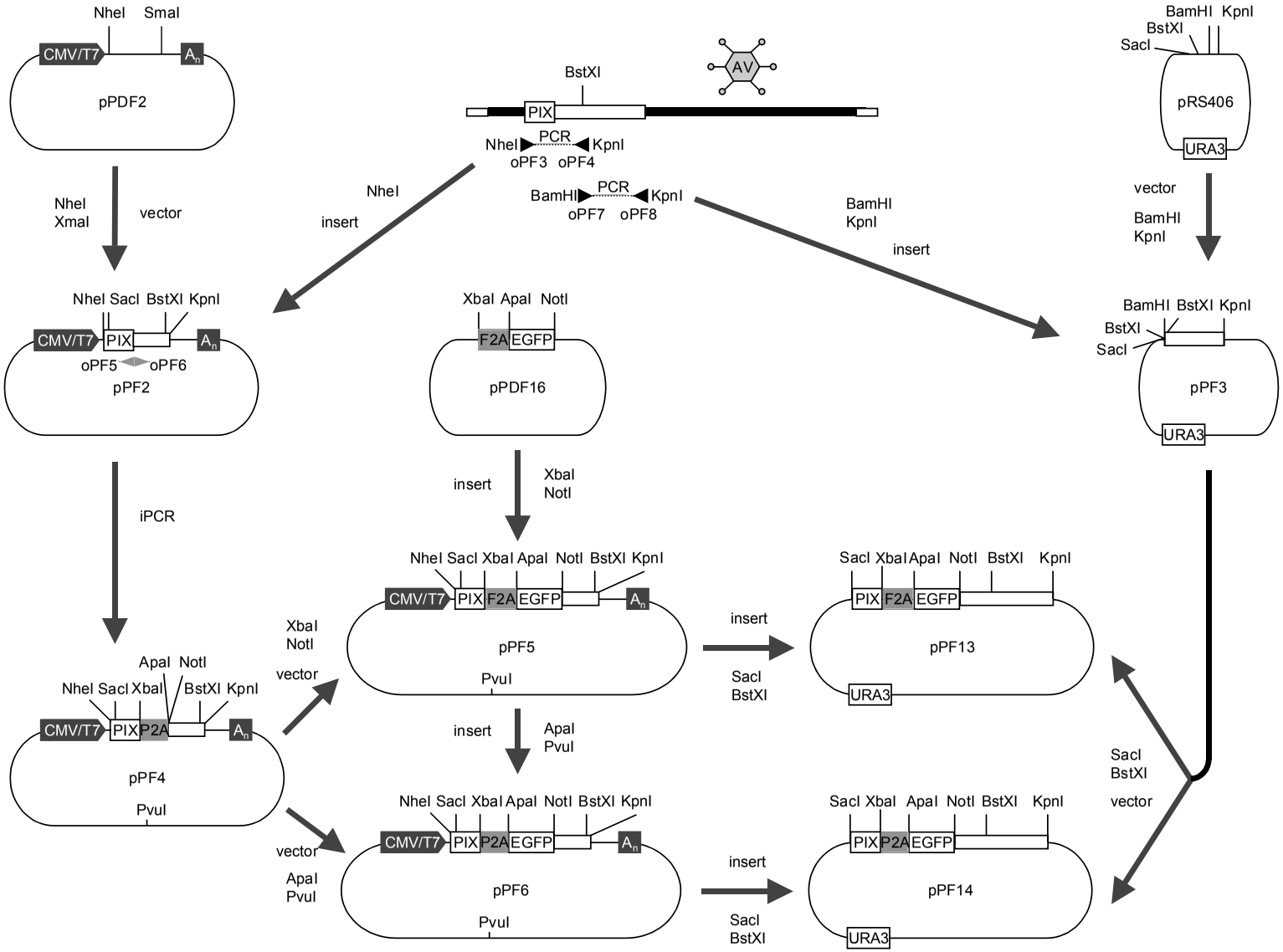


Figure 9: Schematic diagram showing the cloning strategy for the pIX-2A-GFP cassettes. The final plasmids shown, pPF13 and pPF14, are yeast integrating vectors that were used to insert the pIX-2A-GFP cassettes into plasmids containing the entire viral genome (YAC/BACs, Gagnebin et al., 1999).

To test the pIX-2A-GFP cassettes *in vitro* before construction of recombinant viruses, they were cloned downstream of a hybrid CMV/T7 promoter in a plasmid expression vector (pPF5&6, Figure 9, p. 38). Skipping at the arrows in Figure 8 (p. 36) should lead to the formation of pIX proteins with the 2A consensus site at the carboxy-terminus and GFP proteins with a single additional proline at the amino-terminus. *In vitro* transcription/translation (TnT) in the presence of ^{35}S-labelled methionine was used to study *in vitro* skipping at the 2A sites. Both 2A constructs showed efficient skipping to yield GFP and pIX proteins of the expected size (Figure 10a, p. 40; the wild type pIX, pIX-P2A and pIX-F2A proteins are predicted to be 14, 17.5 and 21 kDa, respectively). Figure 10a (p. 40) also shows pIX expressed from a construct containing just pIX-P2A without a downstream open reading frame (pPF4, Figure 9, p. 38). The pIX-P2A protein expressed from this construct is the same size as the pIX-P2A protein expressed from the pIX-P2A-GFP construct. Small amounts of pIX-2A-GFP fusion proteins can also be seen (Figure 10a, p. 40). It can be concluded that ribosomal skipping occurs at the 2A site in the absence of exogenous viral proteins yielding proteins of the expected size, as previously described (Donnelly et al., 2001; Holst et al., 2006).

To test whether skipping occurs *in vivo*, 293T cells were transfected with the fusion constructs, and cell lysates were immunoblotted for pIX, 2A and GFP. Immunoblotting for 2A confirmed the presence of the 2A sequence in the pIX-2A proteins (Figure 10b, p. 40). Since the antibody against 2A was made against the PTV-1 2A sequence, the relative abundance of the two forms cannot be assessed from this blot as the antibody appears to prefer the P2A epitope. Skipping at the P2A site was less efficient than at the F2A site, as shown by the presence of more pIX-2A-GFP fusion protein, but in both cases the quantity of GFP was far greater than that of the fusion proteins (Figure 10b, p. 40). I conclude that the pIX-2A expression strategy gives good expression of GFP.

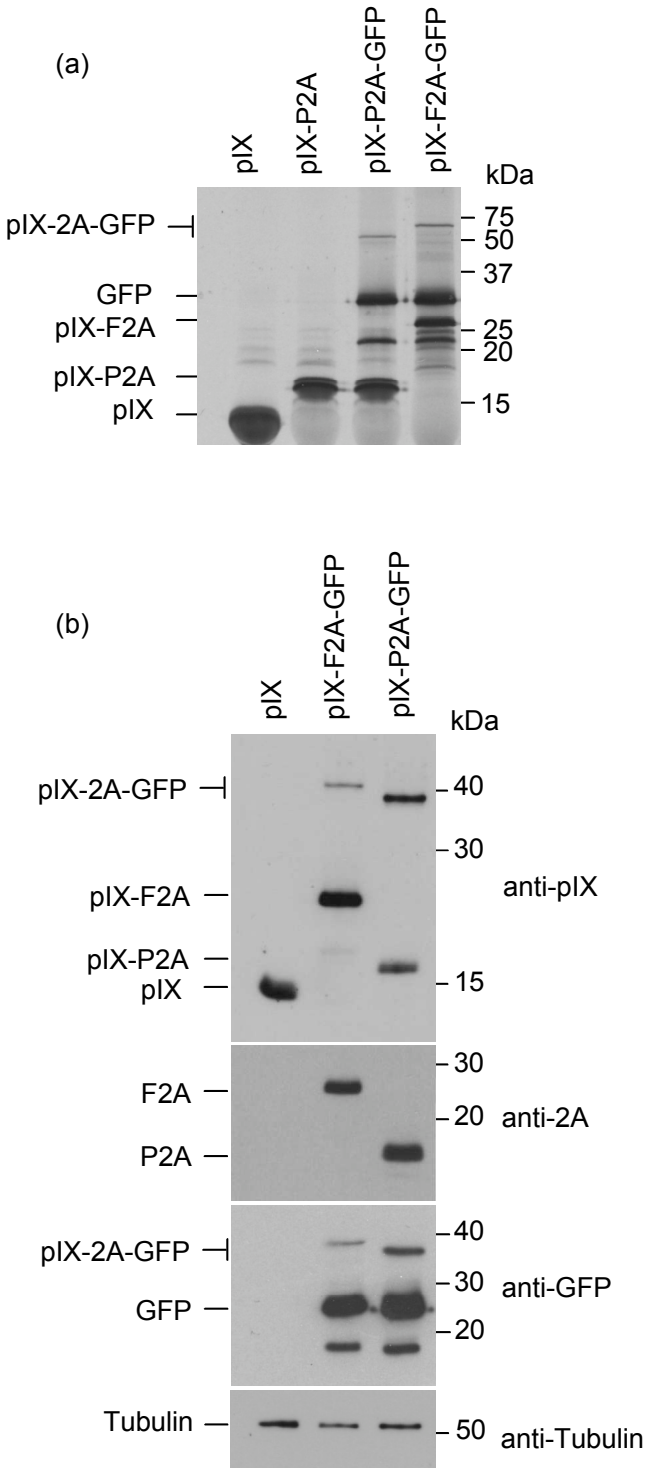


Figure 10: (a) In vitro transcription/translation of pIX, pIX-P2A, pIX-P2A-GFP and pIX-F2A-GFP proteins. Transcription/translation reactions were programmed with plasmids pPF5 (F2A) and pPF6 (P2A, see Figure 9, p. 38). (b) Expression of pIX, pIX-P2A-GFP and pIX-F2A-GFP genes after transfection of 293T cells with plasmids expressing pPF2 (pIX), pPF5 (pIX-P2A-GFP) and pPF6 (pIX-F2A-GFP) from the CMV promoter. Western blots were probed with anti-pIX, anti-2A and anti–GFP antibodies as indicated. Tubulin was used as a loading control. Note that the anti-2A antibody has a higher affinity for the P2A than the F2A epitope.

To test skipping in the context of the virus, the pIX-2A-GFP cassettes were cloned into an oncolytic viral genome by two-step gene replacement in yeast (Gagnebin et al., 1999). The resulting YAC/BACs vpKM1 and vpKM3 were cut with PacI to liberate the viral DNA then transfected into C7 cells to produce the viruses vKM11 (pIX-F2A-GFP) and vKM31 (pIX-P2A-GFP). The viruses were plaque purified on SW480 colon cancer cells to reduce the risk of unwanted recombination events, expanded on SW480 cells and purified by two rounds of $CsCl_2$ equilibrium density gradient centrifugation. Immunoblotting of the purified virions for 2A (Figure 11a, p. 42) confirmed the presence of the 2A epitope in the pIX-F2A and pIX-P2A proteins of the vKM11 and vKM31 viruses, respectively; the weaker F2A signal is expected given the higher affinity of the antibody for the P2A epitope. The ratio of pIX to hexon was similar for the parental virus, vKH6, and the two progeny viruses, vKM11 (pIX-F2A-GFP) and vKM31 (pIX-P2A-GFP). The pIX-F2A protein in the virion showed signs of degradation, with the appearance of lower bands (Figure 11a, p. 42, asterisk). Only trace amounts of the pIX-2A-GFP proteins were present, suggesting that the viruses may preferentially incorporate pIX-2A protein into the capsid.

The ability of the viruses to express the pIX-2A and GFP proteins correctly was tested by infecting SW480 cells at a multiplicity of infection (MOI) of 0.5 pfu/cell. Cells were harvested 16 hours after infection to avoid losing cells that detached from the plate. Immunoblotting for E1A showed that the early steps of infection were not affected by the presence of the pIX-2A proteins in the capsid (Figure 11b, p. 42). There was more pIX-2A-GFP protein present after infection with the P2A virus, again suggesting that skipping is less efficient with the P2A sequence. The level of the pIX-F2A protein was substantially higher than that of either the wild type or P2A proteins (Figure 11b, p. 42). The same trend was visible after transfection with the plasmid constructs (Figure 10b, p. 40). In both cases the ratio of GFP to pIX-P2A-GFP was much higher than the ratio of pIX to pIX-P2A-GFP, which was close to one. The reason is unclear, since all three proteins initiate from the same ATG. Despite these differences, both 2A viruses expressed GFP in equal amounts. I conclude that, consistent with findings in other biological systems, 2A sequences allow efficient expression of transgenes in adenoviruses.

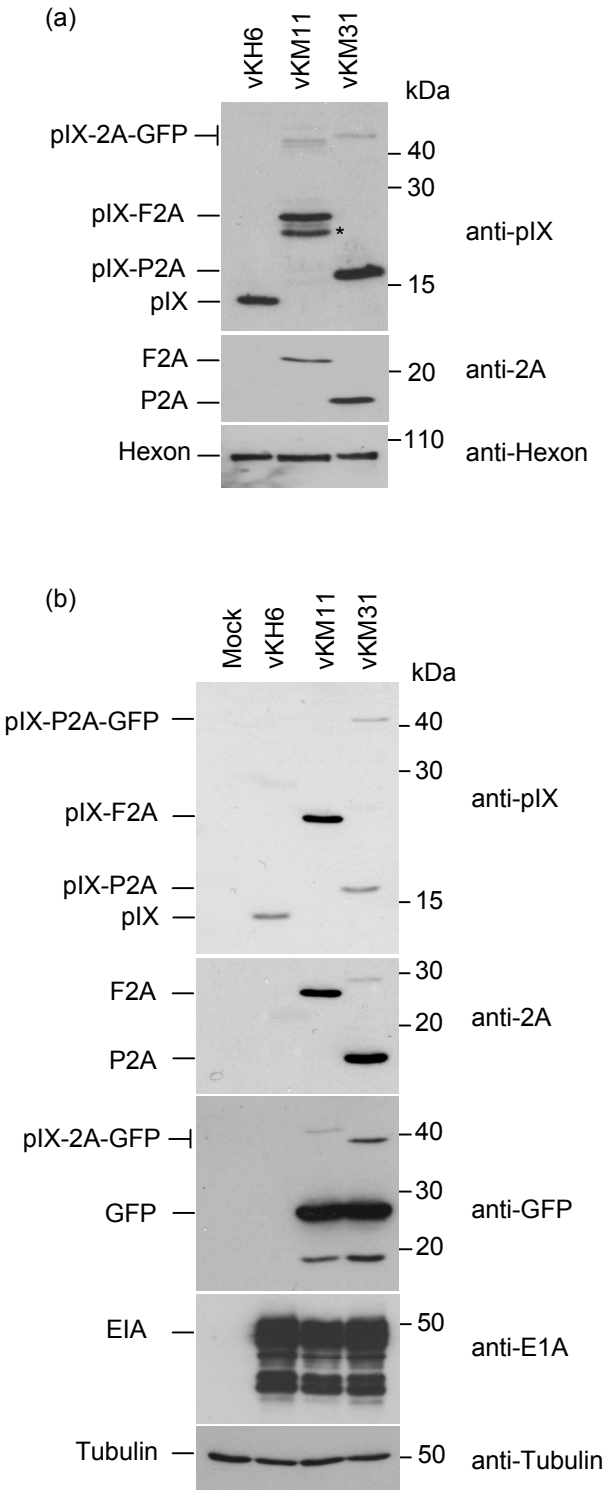


Figure 11: (a) Western blots showing the presence of pIX-2A proteins in virions. Blots of purified virus were probed with anti-pIX and anti-2A antibodies. Ad5 hexon was used as a loading control. pIX-F2A degradation products are marked with an asterisk. (b) Expression of pIX and GFP after infection of SW480 cells with vKM11 (pIX-F2A-GFP), vKM31 (pIX-P2A-GFP) and vKH6 (parental virus). Cells were harvested 16 hours after infection. Western blots were probed with anti-pIX, anti-2A, anti–GFP and anti-E1A antibodies. Tubulin was used as a loading control.

To test whether the difference in the pIX-2A expression is due to differences in pIX-2A half-lives HER911 cells were infected with vKM11 and vKM31 at MOI of 0.5 pfu/cell. Medium was changed to contain 20 μg/ml of cycloheximide after 24 hours, and samples were harvested at certain time points after changing the medium. Immunoblotting for pIX showed that both pIX-F2A and pIX-P2A have long half-lives (Figure 12, p. 43). Unexpectedly the half-life of pIX-F2A seems to be shorter than that of pIX-P2A. The reason is unclear. However, the half-life of pIX does not seem to be the reason for the differential expression patterns of pIX-F2A and pIX-P2A.

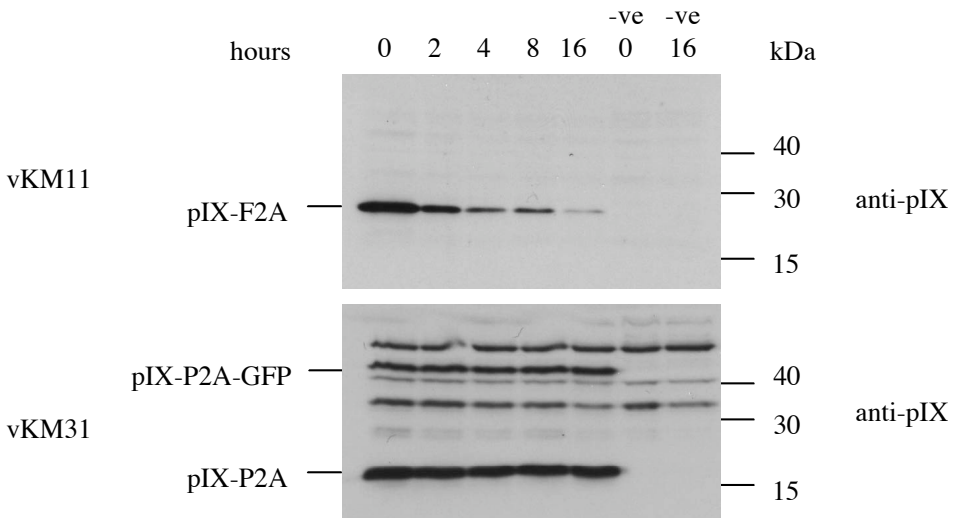


Figure 12: Protein IX half-life. HER911 cells were infected with vKM11 and vKM31, the medium was changed 24 hours post-infection to contain cycloheximide, and samples harvested at 0, 2, 4, 8 and 16 hours after changing the medium to contain cycloheximide. Samples were immunoblotted for pIX.

The pIX-F2A virus was more difficult to produce than the pIX-P2A virus or the parental virus. This suggests that the pIX-F2A protein may be partially defective. Visual inspection showed that pIX-P2A plaques were generally larger than pIX-F2A plaques (Figure 13a, p. 45). To pursue this plaque size was measured by staining dead cells with propidium iodide and measuring the surface area of plaques photographed through mCherry filters (Figure 13b, p. 45). This showed that the pIX-F2A virus forms significantly smaller plaques than the other viruses (Figure 13b, p. 45). To test whether the pIX-F2A virus is less cytopathic than the other viruses, SW480 cells were infected with log dilutions of virus. SW480 cells are highly permissive for the parental virus, vKH6, because they have high Tcf activity, leading to strong activation of Tcf-regulated promoters. The cytopathic effect of the pIX-P2A virus (vKM31) was similar to that of the parental virus, whereas that of the pIX-F2A virus (vKM11) was reduced 10-fold (Figure 13c, p. 45). To identify the reason for the reduction in activity of the pIX-F2A virus, a burst assay was performed. SW480 cells were infected at a multiplicity of infection of 0.1 pfu/cell and virus was harvested after 48 hours. The burst size of the pIX-P2A virus was two-fold lower than that of the parental virus, whereas that of the pIX-F2A virus was reduced 90-fold (Figure 13d, p. 45). The CPE assay was performed in conditions where several cycles of infection were required, so the difference in burst size is a potential explanation for the reduction in CPE. Viruses defective in pIX function rapidly lose activity at mildly elevated temperatures. To test whether this might have contributed to the reduction in CPE of the pIX-F2A virus, aliquots of virus were heated to 45°C and plaque assays were performed to measure the decline in titre. This showed a marginal difference in stability of the pIX-P2A virus compared to the parental virus, and a large reduction in stability of the pIX-F2A virus (Figure 14, p. 46). I conclude that addition of the FMDV 2A sequence to the carboxy-terminus of the pIX protein interferes with pIX function and this leads to a reduction in CPE, burst size and stability of the capsid.

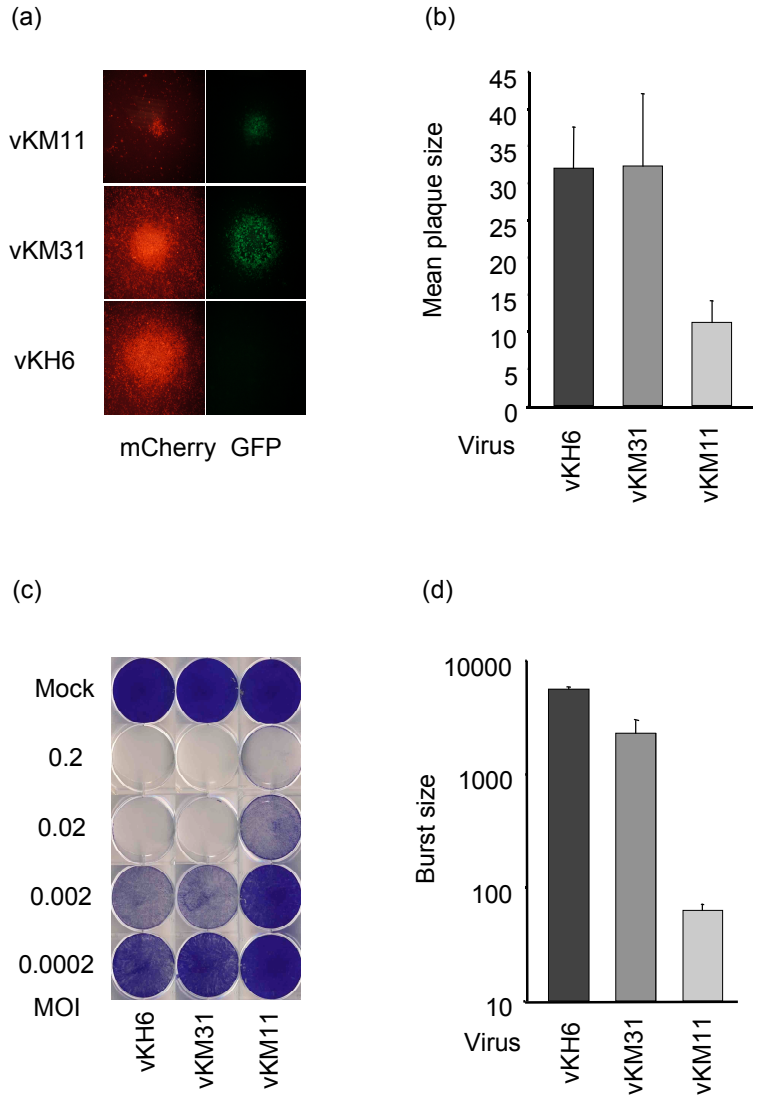


Figure 13: (a) Measurement of plaque size. Photographs of plaques 10 days after infection of HER911 cells. Left panels, mCherry filters were used to detect propidium iodide staining of dead cells. Right panels, GFP filters were used to detect GFP expression by vKM11 and vKM31. (b) Area of propidium-stained plaques. At least 22 plaques were counted for each virus. Error bars represent SEM. p values: vKH6 vs vKM11, 0.002; vKH6 vs vKM31, 0.98;vKM11 vs vKM31, 0.05. (c) Cytopathic effect assay. SW480 cells were infected at the indicated multiplicities of infection (MOI, pfu/cell) and stained with crystal violet after 7 days. (d) Viral burst assay. SW480 cells were infected at an MOI of 0.1 pfu/cell and harvested 48 hours later. The burst size is expressed as pfu per input pfu. Error bars represent SEM.

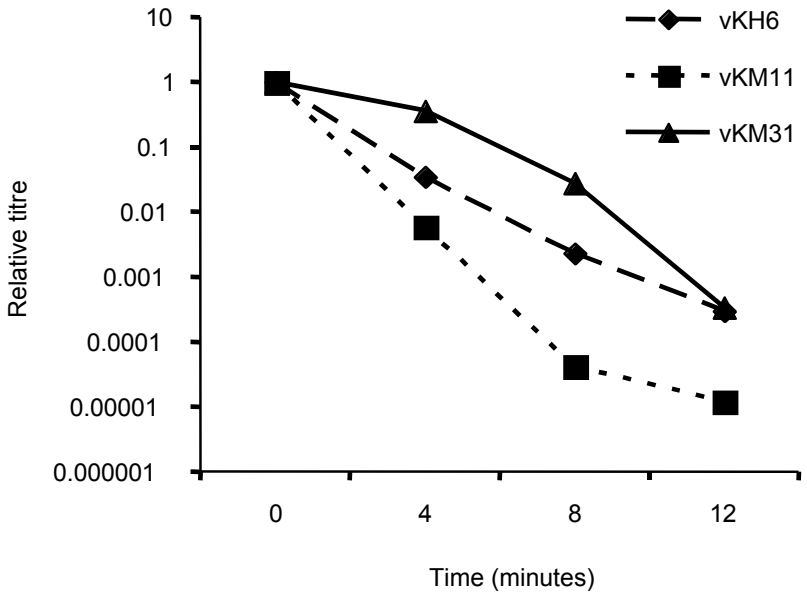


Figure 14: Heat stability assay. Aliquot of pure virus was heated to 45°C for the indicated times then titred on HER911 cells.

3.2.2 Expression of FCU1 and MMP7

In addition to GFP, two other transgenes were inserted after pIX-F2A and pIX–P2A. These genes were MMP7, a matrix metalloprotease, and FCU1, a suicide gene that converts a non-toxic antifungal agent, 5-FC, into the toxic metabolites 5-fluorouracil and 5-flurouridine-5'-monophosphate (Erbs et al., 2000).

Similar to the pIX-2A-GFP constructs, the pIX-2A-FCU1 and pIX-2A-MMP7 cassettes were also cloned downstream of a hybrid CMV/T7 promoter in a plasmid expression vector (Figures 15, p. 48, and 16, p. 49) to test the expression of pIX-2A and the transgenes *in vivo*. The FCU1 gene was obtained by PCR with primers oPF12 (CTTTCA GGGCCC ATGGTG ACAGGG GGAATG GCAAG) and oPF13 (CGCGTG GCGGCC GCTTAA ACACAG TAGTAT CTGTCA) from pTG13046 (Transgene) (Figure 15, p. 48). The PCR product was cut with ApaI and NotI, and inserted into ApaI-NotI digested pPF5 (F2A) and pPF6 (P2A) (see above; Figure 9, p. 38) vectors to yield pPF9 (F2A) and pPF10 (P2A). A SacI-BstXI fragment containing

the pIX-2A-FCU1 cassette was cloned from both pPF9 and pPF10 into SacI-BstXI cut pPF3 to yield the gene replacement vectors pPF15 (for F2A) and pPF16 (for P2A). pPF15 and pPF16 were linearised with SacII for insertion into vpKH6 (Homicsko et al., 2005). The resulting plasmids containing the modified, full-length viral genomes are called vpPZ2 (F2A) and vpPZ7 (P2A).

The construction of the pIX-2A-MMP7 cassettes is shown in Figure 16, p. 49. The polylinker between the MMP7 gene and the V5 tag in the pMMP7 plasmid (provided by Dr. I. Stamenkovic, Department of Experimental Pathology, University of Lausanne, Switzerland) was deleted by digesting the plasmid with NotI and Sac II and blunting the overhangs with T4 polymerase. This yielded pPF7. The size of the V5 tag was reduced by PCR with primers oPF14 (GAGCTC GGGCCC ATGCGA CTCACC GTGCTG TGTGCT) and oPF15 (TGATGA GCGGCC GCTTAC GTAGAA TCGAGA CCGAGG AGAG). The PCR fragment was digested with ApaI and NotI and cloned into ApaI-NotI digested pPF5 and pPF6 vectors (see above; Figure 9, p. 38) to give pPF11 (F2A) and pPF12 (P2A). These were digested with NheI, blunted with T4 polymerase and partially digested with BstXI to give a fragment containing the pIX-2A-MMP7 cassette that was cloned into NheI digested, T4 polymerase blunted, and BstXI digested pPF3 vector (see above; Figure 9, p. 38) to yield the gene replacement vectors pPF17 (F2A) and pPF18 (P2A). pPF17 and pPF18 were linearised with SacII for insertion into vpKH6 (Homicsko et al., 2005). The resulting plasmids containing the modified, full-length viral genomes are called vpPZ6 (F2A) and vpPZ4 (P2A).

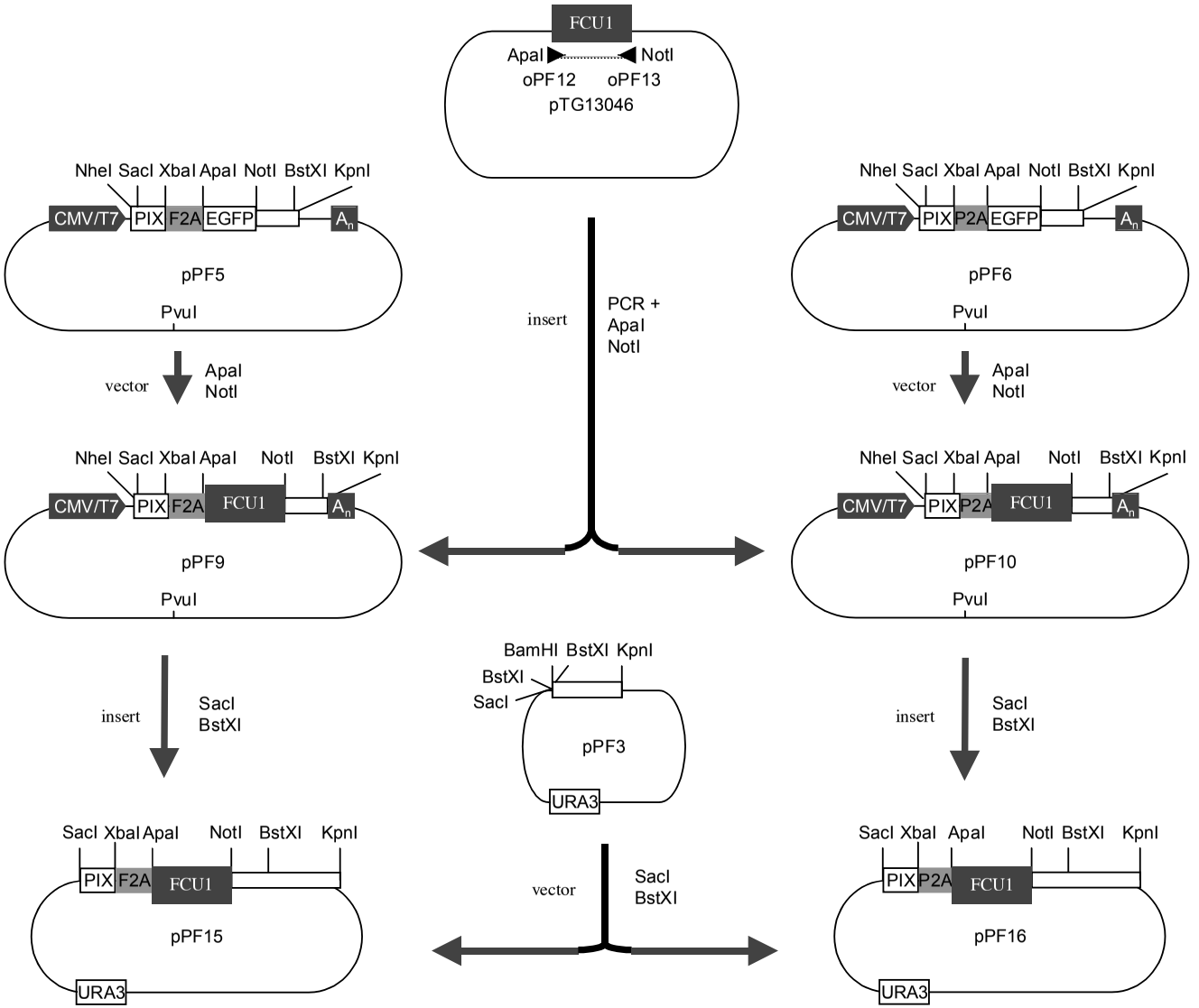


Figure 15: Schematic diagram showing the cloning strategy for the pIX-2A-FCU1 cassettes. The final plasmids shown, pPF13 and pPF14, are yeast integrating vectors that were used to insert the pIX-2A-GFP cassettes into plasmids containing the entire viral genome (YAC/BACs, Gagnebin et al., 1999).

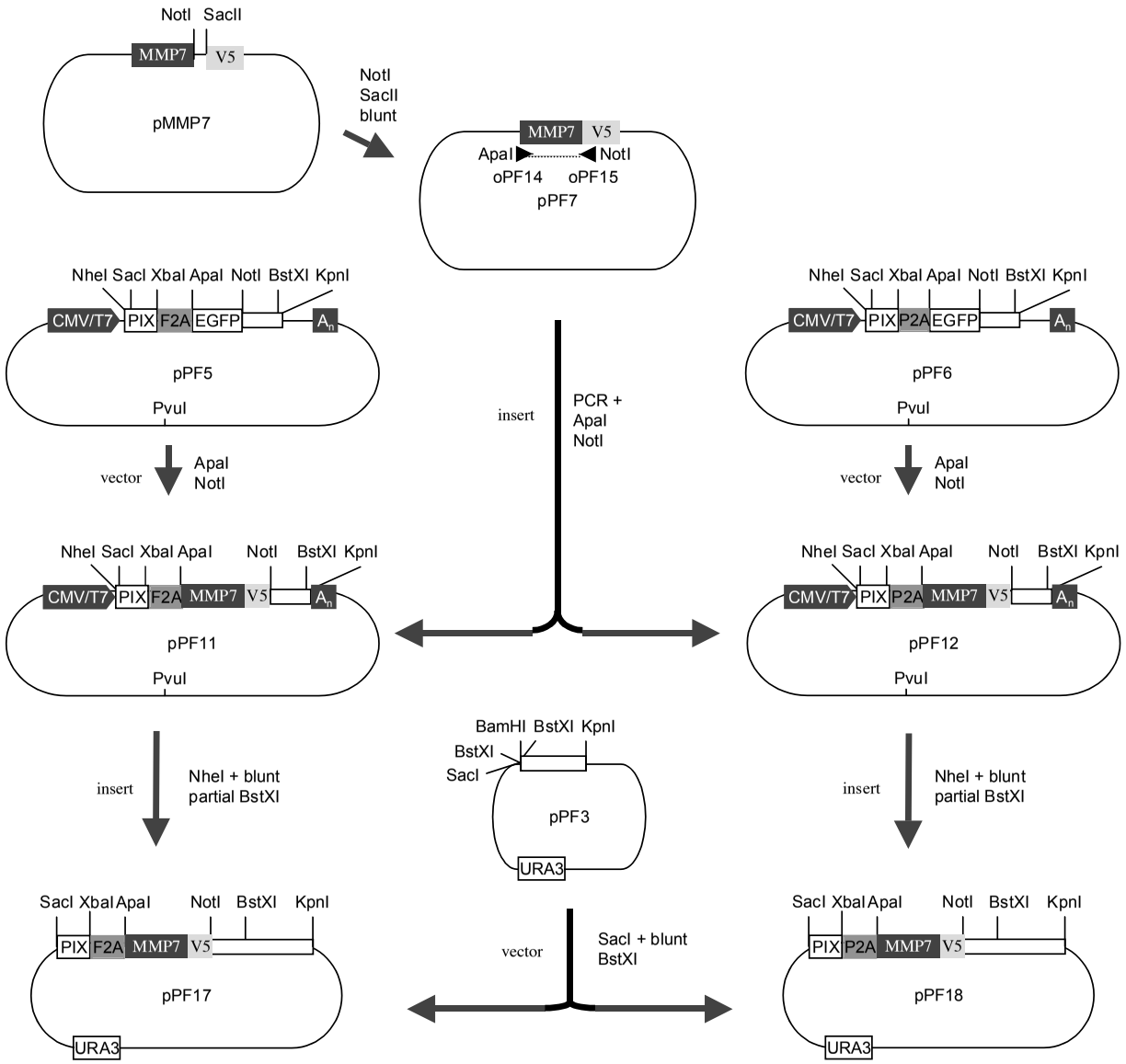


Figure 16: Schematic diagram showing the cloning strategy for the pIX-2A-MMP7 cassettes. The final plasmids shown, pPF13 and pPF14, are yeast integrating vectors that were used to insert the pIX-2A-GFP cassettes into plasmids containing the entire viral genome (YAC/BACs, Gagnebin et al., 1999).

To test if the skipping of the 2A sequence also occurs with the FCU1 and the MMP7 cassettes, the constructs were transfected into 293T cells, and cell lysates were immunoblotted for pIX, 2A and either FCU1 or the V5 tag depending which gene was cloned after pIX-2A (Figure 17, p. 51). The blot for 2A confirmed the presence of the 2A epitope in the construct but nothing can be said about the amounts expressed as the 2A antibody prefers the P2A epitope over the F2A epitope as established above (Figure 17, p. 51). Immunoblotting for pIX showed the presence of pIX-2A proteins but the quantities are significantly reduced compared to the levels detected with the pIX-2A-GFP cassettes. The pIX-2A-FCU1 fusion protein can be detected in small amounts but the pIX-2A-MMP7 fusion proteins cannot be detected (Figure 17, p. 51). Immunoblotting for FCU1 showed the presence of the protein expressed in equal amounts with both F2A and P2A constructs (Figure 18a, p. 52). No pIX-2A-FCU1 fusion protein could be detected from the blot for FCU1. Immunoblotting for V5 tag present in the pIX-2A-MMP7 constructs showed the equal expression of MMP7 from both F2A and P2A cassettes (Figure 18b, p. 52). The pIX-2A-MMP7 fusion proteins were barely visible. With both proteins FCU1 and MMP7 the levels of the transgene expression appear to be much higher than the expression of pIX-2A or the pIX-2A-trangene fusion protein. The reason is unclear but may be due to destabilising effects of the transgenes over the pIX-2A protein.

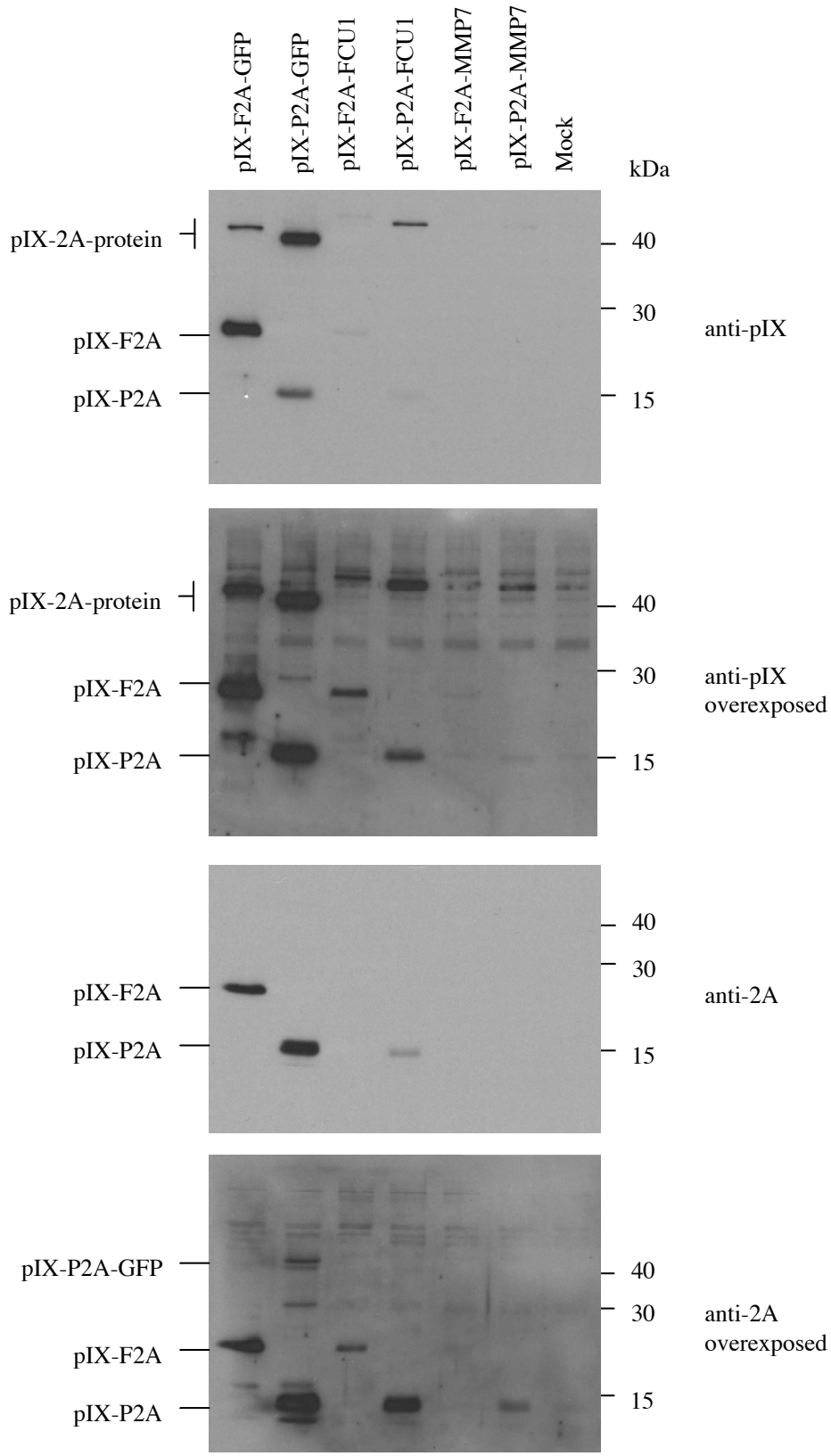


Figure 17: Expression of pIX and pIX-2A-protein after transfection of 293T cells with plasmids expressing pPF2 (pIX), pPF5 (pIX-F2A-GFP), pPF6 (pIX-P2A-GFP), pPF9 (pIX-F2A-FCU1), pPF10 (pIX-P2A-FCU1), pPF11 (pIX-F2A-MMP7), and pPF12 (pIX-P2A-MMP7) from the CMV promoter. Western blots were probed with anti-pIX and anti-2A antibodies as indicated. Note that the anti-2A antibody has a higher affinity for the P2A than the F2A epitope.

To produce the viruses containing the pIX-2A-FCU1 and pIX-2A-MMP7 cassettes, vpPZ2, vpPZ7, vpPZ6, and vpPZ4 were cut with PacI to liberate the viral DNA and then transfected into C7 cells to produce the viruses vPZ2, vPZ7, vPZ6, and vPZ4 respectively. Conversion of plasmid DNA into virus using conventional packaging cells was not possible. To circumvent this problem, packaging cells expressing pIX (cSK8, Table 2, p. 32) were used. The viruses were producible on pIX-complementing cell lines. After the initial production of cSK8 cells, the viruses were expanded on cSK2 cells (Table 2, p. 32).

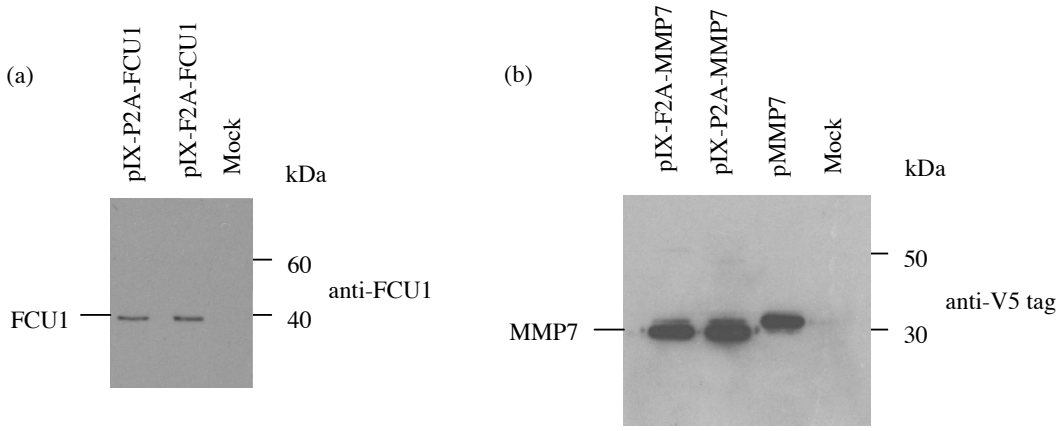


Figure 18: (a) Expression of FCU1 after transfection of 293T cells with plasmids expressing pPF9 (pIX-F2A-FCU1) and pPF10 (pIX-P2A-FCU1) from the CMV promoter. The western blot was probed with anti-FCU1. (b) Expression of MMP7 after transfection of 293T cells with plasmids expressing pPF11 (pIX-F2A-MMP7) and pPF12 (pIX-P2A-MMP7) from the CMV promoter. The western blot was probed with anti-V5 tag that is fused to the C-terminus of MMP7.

To test whether the differences in the expression of pIX-P2A and pIX-F2A and between the different transgene constructs were due to antibody affinities, plasmids containing the pIX-2A-transgene CMV-driven expression cassettes were transfected into 293T cells, and immunoblotted with another anti-pIX antibody provided by Prof. W. Russell (Figure 19, p. 53). The expression patterns are the same as with the blots immunoblotted with the other anti-pIX antibody (Figure 10, p. 40, Figure 11, p. 42, and Figure 17, p. 51). I conclude that antibody affinity cannot explain the differences in the pIX-F2A and pIX-P2A expression patterns.

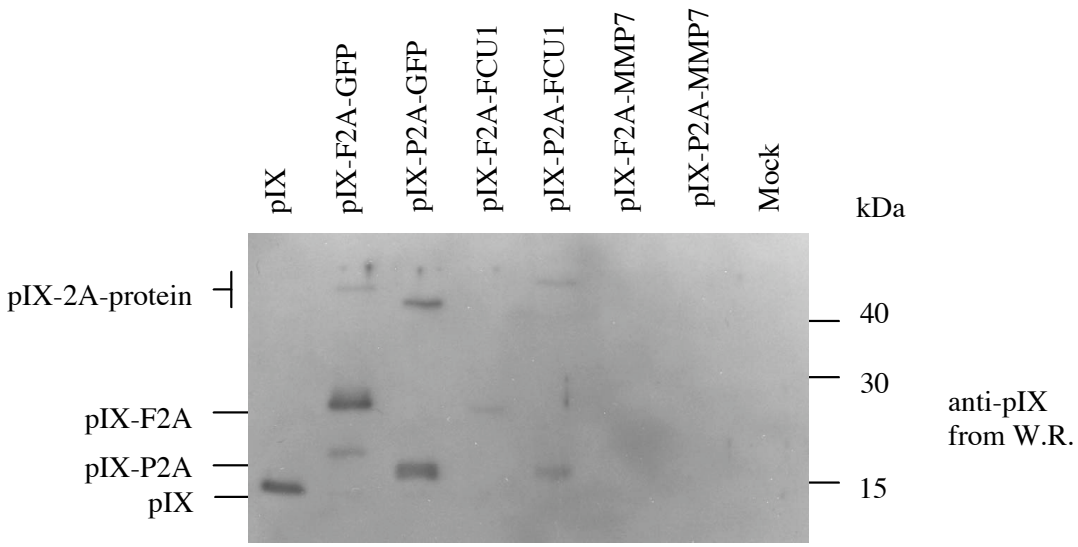


Figure 19: Expression of pIX and pIX-2A-protein after transfection of 293T cells with plasmids expressing pPF2 (pIX), pPF5 (pIX-F2A-GFP), pPF6 (pIX-P2A-GFP), pPF9 (pIX-F2A-FCU1), pPF10 (pIX-P2A-FCU1), pPF11 (pIX-F2A-MMP7), and pPF12 (pIX-P2A-MMP7) from the CMV promoter. Western blots were probed with an alternative anti-pIX antibody provided by Prof. W. Russell, School of Biology, University of St Andrews, UK.

The expression of pIX-P2A from the pIX-P2A-MMP7 construct was only barely visible in an overexposed immunoblot for pIX when compared with wild type pIX (Figure 17, p. 51). To investigate this further pPF12, the plasmid containing the CMV-driven pIX-P2A-MMP7 expression cassette (see above, Figure 16, p. 49) was cut with ApaI, blunted with T4 DNA polymerase and self-ligated to generate a stop codon downstream from P2A. The resulting plasmid, called pGF3, was transfected into 293T cells, and the cell lysate was immunoblotted for pIX. The expression of pIX-2A is clearly visible (Figure 20, p. 54). I conclude that the lower expression of pIX-P2A seen in Figure 17 (p. 51) is due to the MMP7 protein.

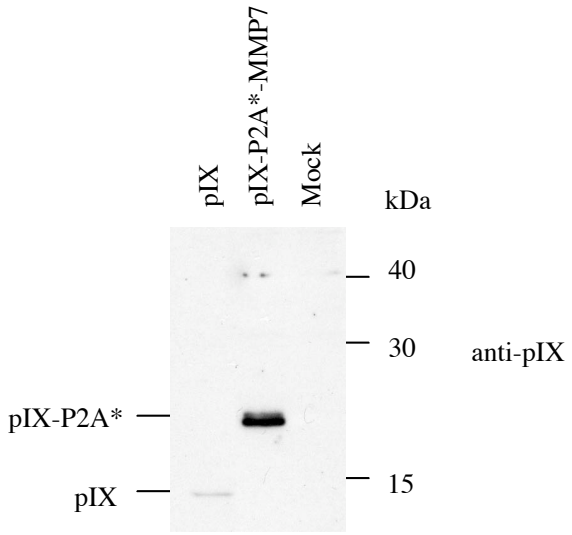


Figure 20: Expression of pIX and pIX-2A* after transfection of 293T cells with plasmids expressing pPF2 (pIX), and pGF3 (pIX-P2A*-MMP7) from the CMV promoter. Asterisk (*) denotes a stop codon. The western blot was probed with anti-pIX antibody.

4. Discussion

This project aimed to improve the spread of an oncolytic adenovirus within a tumour by developing a system for rapid modification of the E1-pIX region of the virus to enable the expression of anti-tumoral genes from the adenoviral genome.

4.1 The Philoxera System

To be able to rapidly modify the adenoviral genome in the E1-pIX region, a system was created in which a recipient virus devoid of the E1-pIX region would recombine with an "addback" plasmid that contains the mutated E1-pIX region. However, the recipient virus, vSK1, was not producible. This was initially thought to be a result of the lack of pIX in the virus. As the production of the virus failed on a pIX-complementing cell line, the reason for the crippling of the virus must be due to other factors. Alba et al. (2007) showed that the addition of an attB-site left of the packaging signal in the adenovirus genome resulted in impaired packaging of the genome, lengthening the viral life cycle up to 60 hours. It seems that excision of the packaging signal is not needed, as the delay occurs in the absence of ΦC31. Thus, other mechanisms must be involved. In vSK1 the attB-site was inserted right from the packaging signal that was already flanked by *loxP*-sites. If the presence of an attB-site near the packaging signal attenuates the virus this would explain why the production of vSK1 was not successful. A possible method to overcome this may be to insert an attP-site into the virus and an attB-site into the "addback" plasmid. There is a potential problem with this approach in that pseudo attP-sites can be found in mammalian genomes (Thyagarajan et al., 2001), therefore it is possible that the "addback" with the attB-site could recombine with the genome of the transfected cell. Of course, it is possible that this is happening with vpSK1 as well. According to Alba et al. (2007) the addition of an attP-site right of the packaging signal did not result in the attenuation of the packaging of the genome and the virus behaved in the same way as the control one. Based on these results the addition of an attP-site instead of an attB-site might indeed result in the generation of a producible recipient virus. Another possibility is that IVa2 is not functioning properly as the attB-site was inserted in the

junction of IX and IVa2 genes. Complementing for IVa2 may also overcome difficulties in producing the recipient virus.

4.1.1 Adenovirus expression library

A possibility with the philoxera-system is the creation of an expression library. Hillgenberg et al. (2006) constructed an adenovirus-based cDNA expression library using the Cre-*lox* system. The transgene was inserted into a plasmid containing the viral 5' ITR, the full-length packaging signal and a single *loxP* site. The plasmid was transfected into a Cre-expressing cell line previously infected with a donor virus containing a partially deleted packaging signal flanked by *loxP* sites. This system is bidirectional whereas the system using the ΦC31-mediated integration together with the Cre-*lox* system is unidirectional. In creating an "addback" plasmid that is easy to modify, it would be possible to create a library with different E1-pIX mutations. To select for desirable traits, the generated E1-pIX-mutant adenoviral bank could be injected into mice with tumour xenografts. To see which virus preferentially targets tumour cells, virus could be extracted from the xenografts and tested for the presence of different viruses (Figure 21, p. 57). For this approach to succeed the recombination even should be quite efficient with low yields of the recipient virus left in the bank. As the ΦC31-mediated integration is unidirectional it is more likely to achieve high recombination rate with this rather than with the Cre-mediated recombination system.

Adenovirus pIX library

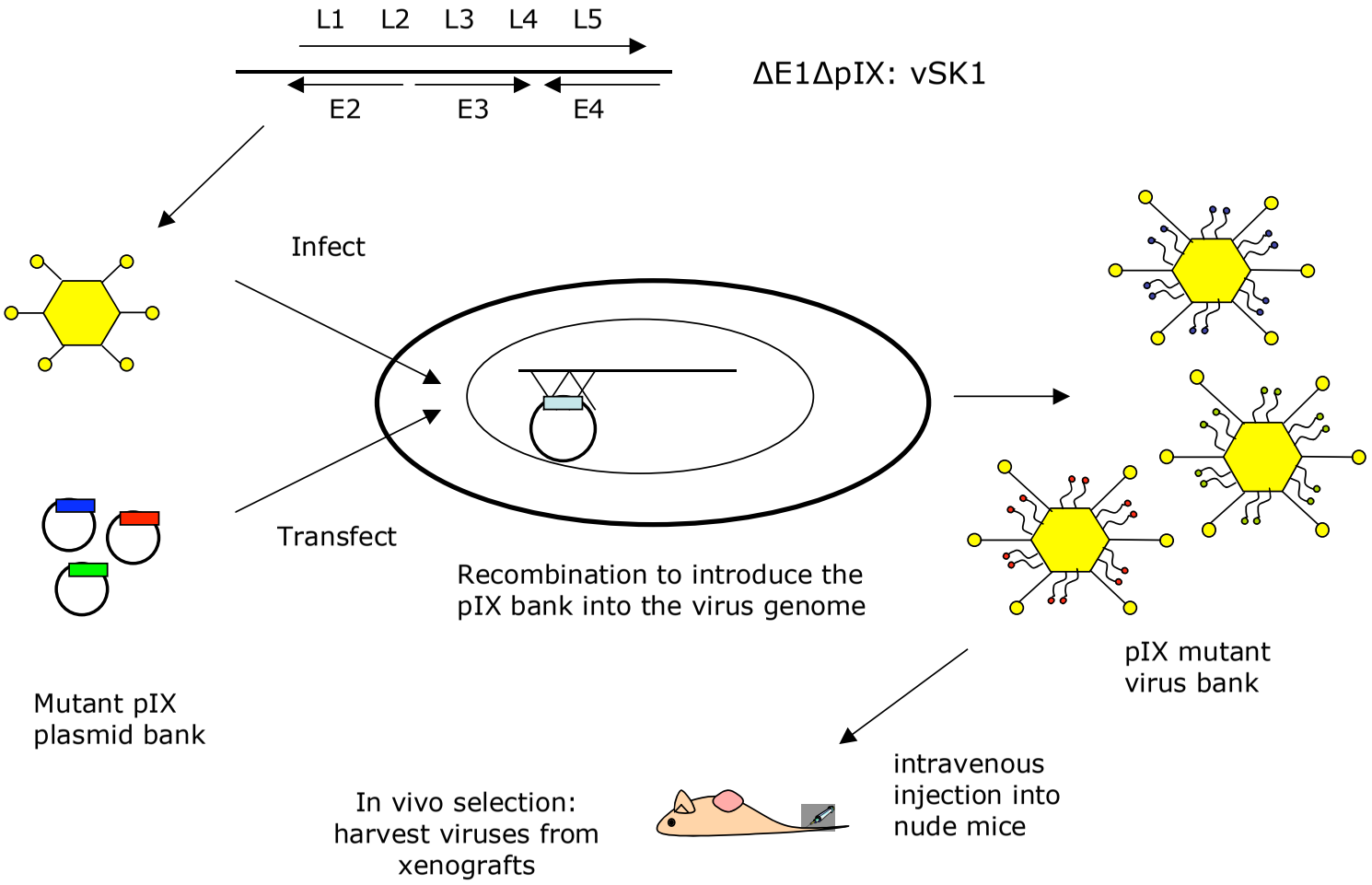


Figure 21: The adenovirus pIX library. The mutant viruses will be generated via recombination of the recipient virus vSK1 and the "addback" plasmid containing a mutant E1pIX-region. After the infection with vSK1 and transfection with the "addback" plasmid the viral bank would be screened for mutant viruses with desirable traits by injecting the viral bank into nude mice and harvesting mutant virus from the xenografts.

4.1.2 Future work

The rapid modification of the E1-pIX region of the adenovirus 5 genome would be a highly efficient way to express transgenes or achieve preferential replication in tumour cells by modifying the E1-promoters. Other regions of the adenovirus 5 genome could also be flanked with att-sites to generate similar systems. Efficient utilisation of the systems requires a recipient virus that can be easily propagated to a high yield, and a highly efficient recombination system. As it was not possible to produce the recipient virus with the attB-site inserted 3' from the packaging signal, either the insertion of an attP-site to the same site or the utilisation of another recombination system could generate a high-yielding recombination system. As gap repair in yeast (Gagnebin et al., 1999) is a highly efficient way of modifying YAC/BACs, the viral recombination system has to be very efficient to yield higher numbers of recombinant viruses than is possible with gap repair in the same amount of time. The advantage of the viral recombination system over gap repair is that there is no need to convert plasmid DNA to viral DNA via transfection of PacI digested plasmid DNA. Often this is the most time-consuming step in the generation of mutant viruses. A high-yielding recombinant system is also a prerequisite for the generation of mutant adenovirus libraries. If the generation of the recipient virus may be overcome, then indeed it could be possible to rapidly modify the desired region of adenovirus and generate expression libraries.

4.2 The pIX-2A-Fusions

Another part of the project involved the expression of transgenes after pIX using 2A sequences. It was shown that 2A sequences do work in the context of oncolytic adenoviruses, and they can be used to express transgenes from the genome of an adenovirus. Two 2A sequences were used of different lengths: the FMDV 2A sequence (F2A) of 58 amino acids in length and the PTV-1 2A sequence (P2A) that is 22 amino acids in length.

4.2.1 The pIX-2A-GFP viruses

The F2A turned out to attenuate the function of the virus, although the skipping activity of the 2A sequence was better than of the P2A. These results were confirmed by many different experiments. The pIX-F2A-GFP virus (vKM11) showed smaller plaque size, reduced burst size and CPE activity, and loss of heat stability. Due to the loss of heat stability, it can be deduced that the impairment is a result of destabilising effects on the capsid. Another possible explanation is a defect in the transactivation capability of pIX but this is not thought to play a major role in normal viral replication (Sargent et al., 2004a). As the pIX-F2A virus showed reduced heat stability, it is possible that the incubation of the infected cells at 37°C for long periods of time in the course of the experiments lowered the efficiency of the virus that could be seen in the burst size, CPE and plaque assays, as well as in the initial production of the pure virus. The deletion of pIX has been previously shown to result in reduced stability of the virus (Colby and Shenk, 1981; Sargent et al., 2004b). This is consistent with the result that the pIX-F2A virus is deficient in pIX function. As the F2A sequence is longer than the P2A, the extension of the F2A sequence might interfere with the capsid structure. Besides having the 2A sequence from the FMDV, the F2A sequence also contains a part of the 1D structural protein coded upstream of 2A in the genome of the FMDV. Thus, it might be possible that this sequence from 1D contains a domain that imposes a tertiary or a quaternary structure on pIX that hinders its normal functioning. The C-terminus of pIX contains a leucine coiled coil domain that enables self-association of pIX (Rosa-Calatrava et al., 2001). This, however, has been shown

not to be essential for incorporation of pIX into the capsid nor the heat stability of the virus (Vellinga et al., 2005a).

Although the F2A seems to hinder the normal viral function, the skipping activity of the sequence was higher than that of the P2A sequence as the ratio of pIX-F2A to pIX-F2A-GFP is higher than that of pIX-P2A to pIX-P2A-GFP (Figures 10b, p. 40, and 11b, p. 41). As the F2A virus seems to be attenuated, the skipping activity does not seem to play a major role, as the expression of GFP seems to be equal between the viruses. This causes a problem as the expression of GFP is equal between the viruses, the expression of pIX-2A would also be expected to be the same (Figures 10, p. 40, and 11, p. 42). This seems not to be the case. As the ratio of the pIX-F2A-GFP and pIX-P2A-GFP is the same in the anti-pIX as it is the anti-GFP blots (Figures 10b, p. 40, and 11b, p. 42) the greater expression of pIX-F2A when compared to pIX-P2A cannot be explained by differences in antibody affinity. When the level of expression of pIX-P2A and pIX-F2A is compared with the parental virus, vKH6, pIX expression it seems that pIX-F2A expression is too abundant (Figure 11b, p. 42). The difference does not seem to be due to differences in half-life of the proteins (Figure 12, p. 43), nor the affinity of the anti-pIX antibody, since the same difference was seen with a different anti-pIX antibody serum (Figure 19, p. 53). However, the difference might be due to premature activation of the pIX promoter in the pIX-F2A virus, or possibly congregation of pIX-P2A in a form that does not show up in Western blotting.

4.2.2 Expression of FCU1 and MMP7

In addition to GFP, FCU1 and MMP7 were also fused to the end of pIX with the two different 2A sequences. The expression ratios seemed to be the same when compared to the pIX-2A-GFP constructs except that the levels of pIX-2A expressed were much lower with FCU1 and almost undetectable with MMP7 (Figure 17, p. 51). However, the expression of FCU1 and MMP7 were clearly detectable, and equal between the constructs (Figure 18, p. 52). As the differences in the pIX expression were not due to antibody affinities (Figure 19, p. 53), a stop codon was generated downstream of P2A in the pIX-P2A-MMP7 construct. Immunoblotting revealed that the MMP7 indeed does influence the stability of pIX-P2A as the construct with the stop codon clearly

expressed pIX-P2A. This also means that the stability arises at the protein level. It is not clear why MMP7 would have such an effect on pIX expression. As MMP7 is a protease, it may be possible that it is cleaving pIX straight after translation. The inefficiency in producing the viruses expressing FCU1 and MMP7 was clearly due to the low availability of pIX, as it was very easy to produce the viruses on a pIX-complementing cell line. This confirms the result already seen with the pIX-2A-GFP viruses that the lack of pIX attenuates the normal function of the virus. To test whether FCU1 and MMP7 get expressed from the viruses, the viruses would need to be purified, and cells infected with the purified viruses to determine the expression levels.

4.2.3 Future work

As seen from the results, it is clear that further development of pIX-2A-fusions should be based on the PTV-1 2A sequence. To enhance the activity of the skipping the P2A sequence could be optimised via mutations to better suit pIX. It would also be possible to insert a long spacer between pIX and the 2A sequence to stop any interference from the 2A sequence. However, this approach would increase the size of the adenoviral genome, and this in turn might interfere with normal functioning of the virus, if the resulting genome would be larger then the allowed packaging size. The aim of this project was also to minimally enlarge the viral genome to allow other modifications to be incorporated.

Besides pIX, other fusion partners can be utilized to express proteins using 2A sequences. It was shown that it is possible to express proteins using 2A sequences in the context of adenoviruses. Finding another fusion partner for MMP7 might be the best strategy due to the destabilising effects it has on pIX expression. If the expression of the fusion partner proves to be yet again unsatisfactory, another approach would have to be used that stops the MMP7 interfering with its fusion partner. These might include insertion of an IRES or a splice site between the fusion partner and MMP7. The same methods may be used for expressing FCU1 from the adenovirus genome. But if the expression of the fusion partner is too low and complementation is needed

for the normal functioning of the virus, it will not be possible to utilise the virus as an oncolytic virus.

5. Appendixes

5.1 Plasmid List

Name	Resistance	Backbone	Construction
pSK1	Amp	pCRII-TOPO	TOPO TA cloning with G76/oPF4 PCR on H14 as insert that contains lox+pack+lox+pIX
pSK2	Amp	pCRII-TOPO	iPCR on pSK1 with oSK1/oSK2 with lox+packaging+lox+attB
pSK3	Amp	pLS77	pCF6 -> SalI/EcoRI*blunt into pBSK- -> SalI/XhoI*blunt
pSK4	Amp	pcDNA3.1(+)	pPF5 with BglII site destroyed from position 12
pSK5	Amp	pBSK-	pSK2-> SpeI/EcorV into pSK3 -> SpeI/EcoRV, contains complete lox+mutpac+lox and attB
pSK6	Chr	pNKBAC39	pSK5 -> PacI into pSK13 -> PacI, YAC/BAC for Gap repair
pSK7	Amp	pLS77	iPCR on pSK14 with oSK3/oSK4, added loxP and EcoRI, BamHI and SacI sites
pSK8	Amp	pBSK-	pSK7 -> SacI/SacII into pBSK- -> SacI/SacII, contains loxpwt+packaging
pSK13	Chr	pNKBAC39	vpPZ4 -> PacI self-ligated, YAC/BAC backbone (1 PacI site)
pSK14	Amp	pLS77	pCF4 -> ClaI and BglII, deleted 1582 to 3359 from pCF4
pSK15	Amp	pcDNA3.1(+)	pPF2 -> NheI and BglII, blunted with Klenow, self-ligated, for reduction of the size of the plasmid
pGF3	Amp	pcDNA3.1(+)	pPF12 -> ApaI, blunted with T4 DNA polymerase, self-ligated, to insert a stop codon after P2A

5.2 Oligo List

Name	Sequence	Designed for
oSK1	5'-TGGGCTCCCCGGGCGCGTACTCCAGCAAG TGTCTTGCTGTCTTTATTTAGGGGTTTTGC-3'	Upper iPCR primer 1108 -> on pSK1 with 23 bp of attB in the 5' end, re-creates Sty I site
oSK2	5'-AGGGCACGCCCTGGCACCCGCACC GCGGCTAGAGGATCCGAATTCAGT-3'	Lower iPCR primer for pSK1 <- 501 with 28 bp of attB in the 5' end (antiparallel), re-creates Sty I site
oSK3	5'-TTCAATAgaattcTAAggatccAATgagctc ACTTTTGCCACATCCGT-3'	Lower primer for iPCR on pCF4 <- 569, has 1/3 loxpwt in the 5' end, also has EcoRI, BamHI and SacI sites
oSK4	5'-GCATATCGTATGTAATATGCTT CAATAGACGTTTTTGGTGTGCG-3'	Upper Primer for iPCR on pCF4 586 ->, has 2/3 loxpwt in the 5' end
oSK5	5'-GAGAACTCAAAGGTTACCCCAGTTG GGGCACTACTTGATCCAAATCCAAAC-3'	Lower primer for iPCR on pPF2 <- 1344 with 2/3 attP in the 5' end, recreates DdeI site
oSK6	5'-TCAGTTGGGGGCGTAAAggtaccA GCAAGTGTCTTGCTGTCTTTATT-3'	Upper primer for iPCR on pPF2 1364 -> with 1/3 attP in the 5' end, KpnI site in the middle, recreates DdeI site
oSK7	5'-ACCCATAGCAGGAGTGTGT-3'	reamplification primer for oPF22
oSK8	5'-actgctgctctgggttccag-3'	reamplification primer for oPF23
oSK9	5'-TACTTGATCCAAATCCAAAC-3'	reamplifying primer for oSK5
oSK10	5'-TCAGTTGGGGGCGTAAAggt-3'	reamplifying primer for oSK6
oSK11	5'-ATGCTATACGAAGTTATTGG-3'	primer for sequencing vpSK1 <- 468
oSK12	5'-GCTATGAGTACAACAAAATT-3'	primer for sequencing vpSK1 <- 403
oSK13	5'-GAGTATTTAGCATTAACAAC-3'	Sequencing primer for YAC/BAC 64bp before the start of the left ITR

5.3 Sequencing Data

Sequencing done on vpSK1 to see that the sequence was correct. Sequence alignment was done using Sequencher (Gene Codes Corporation, Ann Arbor, MI).

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