THE ENCAPSIDATION OF HERPES SIMPLEX VIRUS TYPE 1 DNA.

By

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Abstract.

The herpes simplex virus genome contains an approximately 400 bp direct repeat at its ends known as the 'a' sequence, which contains all of the signals necessary for the cleavage and packaging of concatemeric replication products. During replication the genome is circularised, probably by direct ligation of the termini, generating a novel junction between two tandemly repeated 'a' sequences. We have cloned such a novel junction and in agreement with previous results (Nasseri & Mocarski, 1988), have shown that it can serve as a substrate for cleavage and packaging. The novel junction contains two highly conserved regions of the 'a' sequence which have been shown to be essential components of the packaging signal (pac1 and pac2). This thesis describes the introduction of specific mutations in this region and the development of a transient packaging assay to examine the effect of these mutations. In addition the mutants were tested for their ability to be serially propagated, extending the analysis beyond cleavage and packaging. Mutations were made using the motifs previously described by Deiss *et al.* (1986). The pac1 and pac2 regions as a whole were deleted using convenient restriction enzyme sites and individual motif mutations, were introduced using a site directed mutagenesis method based on that described by Kunkel *et al.* (1991). The mutant sequences were then tested for their ability to direct cleavage and packaging in the transient packaging assay. This identified that both the pac1 and pac2 sequences as a whole represent essential components of the packaging signal. Both the location and the sequence of the pac2 T rich element were also shown to be essential in a functional cleavage and packaging signal. In addition a substitution mutation of the pac2 unconserved region reduced the efficiency of packaging directed by *wt* HSV-1 strain 17 but no effect on packaging efficiency was observed when HSV-2 strain HG52 was used. This result appears to indicate an unexpected divergence between HSV-1 and HSV-2. No function was attributable to either the conserved pac2 consensus sequence or any of the individual motifs of the pac1 sequence. However, considering the importance of the pac1 region as a whole the lack of identifiably important motifs is likely to reflect a level of redundancy within the pac1 region as opposed to a lack of biological function.

Finally the mutants were used to examine the signals necessary for the serial propagation of defective genomes. This revealed that both GC rich regions of pac1 are involved in the serial propagation of defective genomes. No sequences within pac2 were identified as important. However, this may reflect either redundancy within the region or the importance of sequences which are also involved in DNA packaging.

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Abbreviations

Amp^r	- ampicillin resistant
BHK (cells)	- baby hamster kidney (cells)
bp(s)	- base pair(s)
BSA	- bovine serum albumin
CAV	- cell associated virus
Ci	- curie
CIP	- calf intestinal phosphatase
CLB	- cell lysis buffer
cm	- centimetre
cpe	- cytopathic effect
CRV	- cell released virus
CT (DNA)	- calf thymus (DNA)
dATP	- 2'-deoxyadenosine-5'-triphosphate
dCTP	- 2'-deoxycytidine-5'-triphosphate
dGTP	- 2'-deoxyguanosine-5'-triphosphate
dNTP	- 2'-deoxyribonucleoside 5'-triphosphate
dUTP	- 2'-deoxyuridine 5'-triphosphate
DMF	- dimethyl formamide
DMSO	- dimethyl sulphoxide
DNA	- deoxyribonucleic acid
DNase	- deoxyribonuclease
DTT	- dithiothreitol
dTTP	- 2'-deoxythymidine-5'-triphosphate
EBV	- epstein barr virus
E.coli	- Escherichia coli
EDTA	- ethylenediaminetetra-acetic acid
EHV-1	- equine herpesvirus type 1
EHV-2	- equine herpesvirus type 2
EtBr	- ethidium bromide
GMEM	- Glasgow modified eagles medium
GPCMV	- guinea pig cytomegalovirus
H (mix)	- hybridisation mix
HCMV	- human cytomegalovirus
hepes	- N-[2,-hydroxyethyl] piperazine-N'-[2-ethane sulphonic acid]
HHV-6	- human herpesvirus 6

HHV-7	- human herpesvirus 7
hr	- hour
HSV-1	- herpes simplex virus type 1
HSV-2	- herpes simplex virus type 2
Kb(p)	- kilo base (pairs)
KDa	- kilo daltons
LB	- L-broth
M	- Molar
mw	- molecular weight
MCMV	- murine cytomegalovirus
MCS	- multiple cloning site
mg	- milligram
µCi	- micro curie
µg	- micro gram
µl	- micro litre
µM	- micro molar
min	- minute
ml	- millilitre
mm	- millimetre
mM	- millimolar
MOI	- multiplicity of infection
MOPS	- 3-[N-morpholino] propane sulphonic acid
NBCS	- new born calf serum
ng	- nanogram
nm	- nanometre
NP40	- nonidet P-40 detergent
NPT	- non permissive temperature
NT Buffer	- nick translation buffer
OD	- optical density
ORF	- open reading frame
p.i.	- post infection
PBS	- phosphate buffered saline
PEG	- polyethylene glycol
pfu	- plaque forming units
RNase	- ribonuclease
rpm	- revolutions per minute
SDS	- sodium dodecyl sulphate
sec	- second

ss	- single stranded
SSC	- standard saline citrate
STET	- sucrose, Tris, EDTA, triton buffer
ts	- temperature sensitive
TAE	- Tris, acetate; EDTA
TBE	- Tris, boric acid; EDTA
TE	- Tris EDTA
TEMED	- N,N,N',N'-tetra-methyl-ethylene diamine
TM buffer	- Tris, magnesium buffer
Tris	- 2-amino-2-(hydroxymethyl)-1,3-propandiol
Triton X-100	- octyl phenoxy polyethoxy ethanol
TS	- Tris buffered saline
TSB	- transformation storage buffer
UV	- ultra violet
V	- volts
v/v	- volume / volume
Vol(s)	- volume(s)
VZV	- varicella zoster virus
w/v	- weight / volume
wt	-wild type
X-Gal	- 5-bromo-4-chloro-3-indolyl-(-D-galacto pyranoside)

Chapter 1 - Introduction.

Section 1.1 - Herpesviruses - distinctive characteristics and structure.

1.1.1 - Definition of the family *Herpesviridae.*

Herpesviruses are ubiquitous eukaryotic, enveloped viruses which have linear double stranded (ds) DNA genomes.

More than 100 herpesviruses have so far been isolated from a wide variety of eukaryotic organisms. Although the bulk of known herpesviruses have been identified in vertebrate organisms (fish, amphibians, reptiles, birds and mammals), at least one herpesvirus which infects an invertebrate organism has also been identified (Comps & Cochennec, 1993).

All of the herpesviruses identified to date share four common features:

1. All specify enzymes and other factors involved in nucleic acid synthesis and metabolism e.g. DNA polymerase and dUTPase. Herpesviruses also encode at least one protease and a variable number of protein kinases.
2. The synthesis of viral DNA, capsid assembly and at least one envelopment stage all occur in the nucleus of herpesvirus infected cells.
3. Virion production invariably leads to host cell death.
4. Most herpesviruses are known to establish a latent infection in their natural host.

Latency is a central feature of herpesviruses and is defined as the persistence of virus in the absence of clinically apparent infection. After initial infection of their natural host, herpesviruses frequently establish a latent infection with reactivated virus being detected either at a constant low level or intermittently in recurrent lesions.

Natural herpesvirus infections are usually limited to a single host, where they cause limited disease. However, while inter species infections are uncommon in nature, where they do occur, they can cause serious disease. This high degree of host specificity has been proposed as evidence that herpesviruses have co-evolved with their hosts which has been supported by examining the relationships between the genome sequences of multiple herpesviruses (McGeoch *et al.*, 1995).

1.1.2 - Classification of herpesviruses.

Historically herpesviruses have been divided into three sub-families based on their biological properties, these being the *alpha herpesvirinae,* the *beta herpesvirinae,* and the *gamma herpesvirinae* (Roizman *et al.*, 1992).

Members of the *alpha herpesvirinae* sub-family are neurotropic and have a variable host range. They exhibit a relatively short reproductive life cycle, rapid spread in culture and the efficient destruction of infected cells. They also have the ability to establish latent infections in sensory ganglia. Members of this sub-family include herpes simplex virus type 1 (HSV-1) and equine herpesvirus 1 (EHV-1).

Members of the *beta herpesvirinae* sub-family have a restricted host range, they have long reproductive cycles and the spread of virus infection from cell to cell is also slow. Infected cells frequently become enlarged (cytomegalia) and latent infections may be established in a variety of tissues including kidney, and secretary glands. Members of the this sub-family include human cytomegalovirus (HCMV), and human herpesvirus 7 (HHV-7).

Members of the *gamma herpesvirinae* are lymphotropic, they also have a highly restricted host range but their reproductive cycle is variable. Latent infections are frequently established in lymphoid tissue. Members of this group include epstein-barr virus (EBV) and equine herpesvirus 2 (EHV-2).

With a few exceptions this classification corresponds to the groupings derived from the genetic relationships between the different viruses (Reviewed McGeoch *et al.*, 1993). Phylogenetic analysis of the available herpesvirus genomes has suggested a common evolutionary origin for the herpesvirus family approximately 200 million years ago. The exception to this is channel catfish virus, which appears to be even more distantly related to the herpesvirus lineage. How the recently identified oyster herpesvirus fits into the evolutionary picture is still unclear.

1.1.3 - Herpesvirus infections in humans.

Herpesviruses in general, cause a wide range of diseases in both humans and other animals. In the natural, immuno-competent, host the disease is not often fatal. In nature infections are usually restricted to a single species but, where inter species transmission does occur it can cause serious disease e.g. Pseudorabies virus infection of species other than pigs (Umene, 1998). In immunocompromised individuals herpesvirus infections can also be serious, even fatal e.g. HCMV infection of AIDS patients (Cotte *et al.*, 1993). Some examples of human herpesvirus infections are described below.

1.1.3.1 - Alphaherpesvirus infection.

The prototype alphaherpesvirus is HSV-1. Its natural host is man and primary infection usually occurs in the mucosa of the mouth or throat although HSV-1 is also capable of infecting the mucosa of the genital tract. Primary infection is usually asymptomatic but

can lead to illness which is characterised by lesions in the mouth and throat, fever and a general malaise. Latency is usually established in the trigeminal ganglia, from where reactivation may occur. Reactivation is often associated with stress, fever, exposure to UV light, tissue damage and or immuno-suppression. Reactivated virus infection usually results in lesions of the skin in the area served by the trigeminal ganglia e.g. herpes labialis (cold sores)(Umene, 1998). One of the complications which can result from an HSV-1 infection is encephalitis, although this is usually restricted to neonatal and immuno-compromised individuals. Other complications can include keratitis and a disseminated infection involving organs such as the liver and adrenal glands (Whitley, 1996).

Varicella-zoster virus, the causative agent of chicken pox, and shingles, and HSV-2 which is responsible primarily for recurrent genital infections are also alphaherpesviruses.

1.1.3.2 - Betaherpesvirus infection.

A betaherpesvirus which frequently infects man is human cytomegalovirus (HCMV). Primary infection typically occurs in morphonuclear leukocytes, monocytes or endothelial cells (Grefte *et al., 1993*; Saltzman *et al.,* 1988 & Turtinen *et al.,* 1987) and is usually asymptomatic even in immuno-compromised individuals. Where clinical disease does occur HCMV frequently causes a persistent fever and myalgia. Infrequent complications include pneumonia, hepatitis and encephalitis. The sites of viral latency remain unclear but sensitive detection methods have identified viral DNA in monocyte populations of peripheral blood (Taylor-Wiedeman *et al.,* 1991)

Human herpesviruses HHV-6 and HHV-7 also belong to the betaherpesvirus subfamily.

AIDS patients appear to be particularly vulnerable to HCMV infection and infection of the liver, lungs, and CNS are quite common. HCMV also frequently infects the gastrointestinal tract of AIDS patients where disease can vary from superficial ulceration to more severe necrosis which often leads to a fatal perforation of the gut (Cotte *et al.,* 1993).

1.1.3.3 - Gammaherpesvirus infection.

The gamma herpesvirus epstein-barr virus (EBV) infects man. Primary infection probably occurs in the mucosa of the nose and mouth, although it rapidly goes on to infect B cells which are transformed into an immortal lymphoblastoid state. Again primary infection is usually asymptomatic but EBV has been identified as the causative agent of a condition known as infectious mononucleosis in a proportion of adolescent primary infections. Symptoms range from a mild fever to months of debilitating illness including headaches, pharyngitus and general malaise. In immuno-compromised individuals the outcome of

infection can be more severe, resulting in conditions such as oral hairy leukoplakia and Burkitt's lymphoma.

Since its discovery EBV has been associated with several conditions characterised by malignant tumours, these include Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease (Magrath, 1990 & Kieff, 1995). Although EBV has been implicated in these conditions, secondary factors are also frequently involved e.g. co-infection with the malarial parasite *plasmodium falciparim* in cases of Burkitt's lymphoma (Doumbe *et al.*, 1997).

Human herpesvirus 8 (Kaposi's sarcoma) is a gamma herpesvirus which appears to be the causative agent of Kaposi's sarcoma.

1.1.4 - Herpesvirus virions.

Herpesvirus virions vary quite considerably in size but all have a similar design. The virions range in size up to 200 nm in diameter but all are approximately spherical. The virions are composed of a linear ds DNA genome, which is packaged into an icosohedral capsid, an amorphous, often asymmetrical layer known as the tegument surrounds the capsid and this in turn is surrounded by a viral protein containing lipid envelope which is derived from the host.

1.1.4.1 - Herpesvirus genomes.

The genomes of all herpesviruses are linear ds DNA. The genomes range in size from 120-240 Kbp but all are packaged into a capsid of between 100-110 nm in diameter. Although for many years the viral DNA was believed to be packaged around a protein core in a toroidal arrangement (Furlong *et al.*, 1972) more recent evidence suggests that herpesvirus DNA is packaged in a liquid crystalline arrangement, in the absence of a protein component (Booy *et al.*, 1991).

Herpesvirus genomes often contain multiple internal and terminal repeat regions. These repeats give rise to considerable variation, both in terms of differences in the size of individual genomes (due to the copy number of terminal and/or internal repeats) and in the sequence arrangements of different viruses. Based on their sequence arrangements herpesvirus genomes can be classified into 7 classes, numbered 0-6, (Roizman *et al.*, 1992; Davison & McGeoch, 1995) (figure 1.1).

Group 0 genomes consist of a single unique sequence which lacks either internal or terminal repeat regions. The genome of the tree shrew herpesvirus is characteristic of this group of genomes (Koch *et al.*, 1985).

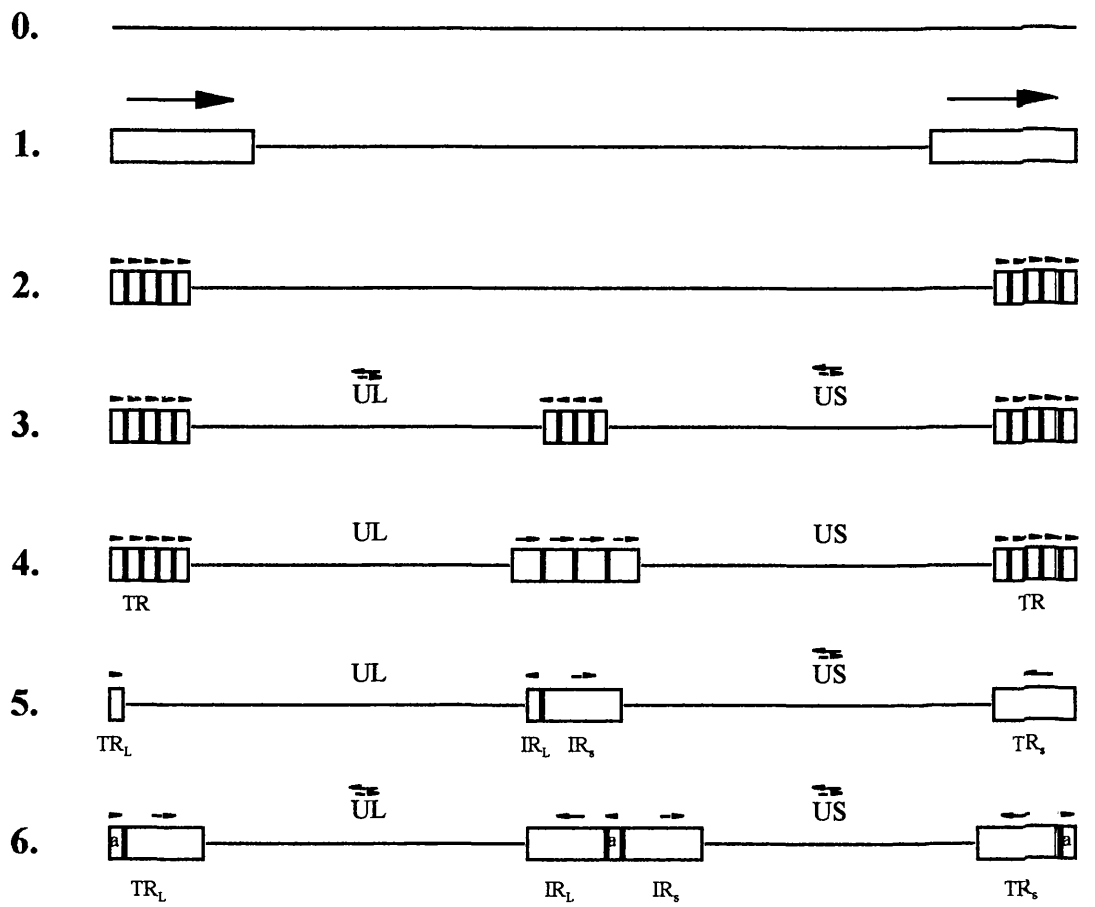


Figure 1.1 - Herpesvirus genome structures.

This figure shows a diagrammatic representation of the range of genome structures found in the *herpesviridae*. Genome structures 1-6 correspond to those defined by Davison & McGeoch (1995). Structure 0, corresponds to the F group defined by Roizman *et al.*, 1992, without large terminal repeats.

Unique and repeat regions are shown as horizontal lines and rectangles respectively. The orientations of unique and repeat sequences are shown as arrows or arrow heads.

Genomes which contain a single unique region which is flanked by direct repeats are designated as group 1 genomes. Channel cat fish virus is an example of a virus with this type of genome (Davison, 1992).

Group 2 and 3 genomes both have multiple copies of a terminal repeat sequence at their termini. However, while group 2 genomes have a single unique region, group 3 genomes have additional copies of the repeat sequence positioned internally. This gives rise to two unique regions which are flanked by inverted copies of the same repeat sequence. In addition the 2 unique regions of group 3 genomes are freely invertable which gives rise to 4 equimolar genome isomers. Group 2 genomes include the genome of herpesvirus samiri (Albrecht *et al.,* 1992), while characteristic group 3 genomes include the genome of cottontail rabbit herpesvirus (Cebrian *et al.,* 1989).

EBV has a characteristic group 4 genome having a set of internal repeats which are unrelated to the terminal repeats (Baer *et al.,* 1984).

Group 5 genomes have two unique regions which are flanked by inverted repeats. The repeats are not related and the repeat flanking the long unique region (UL) is significantly shorter than that flanking the short unique region (US). This leads to two genomic orientations of US but either completely or predominately one for UL. Varicella-zoster virus (VZV) has a characteristic group 5 genome structure (Davison & Scott, 1986).

Group 6 is similar to group 5 except that the inverted repeats flanking the UL region are larger and an additional repeat sequence known as the 'a' sequence is found at the termini and at the junction between the internal L and S segments. The presence of a larger UL flanking repeat also means that the UL region is freely invertable leading to the occurrence of 4 equimolar genomic isomers. HSV-1 and HSV-2 are examples of herpesviruses with group 6 genomes (Roizman, 1979).

1.1.4.2 - Herpesvirus capsids.

Despite the fact that very little sequence homology is apparent between the major capsid proteins of some herpesviruses, cryo-electron microscopy has shown that morphologically the capsids are very similar (Booy *et al.,* 1996). All herpesviruses encode proteins which form an approximately icosohedral nucleocapsid of more than 100 nm in diameter. Of the herpesvirus capsids examined to date, all have a similar capsid structure. Capsids having a T=16 structure and comprise 162 capsomers (150 hexons and 12 pentons). Conserved features include the heterotrimeric complexes known as a triplexes which appear to stabilise the capsid structure, a closely knit inner floor layer, the axial channel which runs through each capsomer and the chimney like protrusions seen on both hexons and pentons (Booy *et al.,* 1996).

Three capsid types have been identified in infected cells and these have been labelled A (empty), B (intermediate) and C (full) capsids. B capsids appear to be the progenitors of A and C capsids which are believed to be the products of abortive and successful packaging events respectively. B capsids contain a proteinaceous core which is composed largely of the scaffold protein around which the capsid is formed. The proteinaceous core may be large or small dependent on whether the scaffold has undergone auto proteolysis following successful capsid assembly or not. The scaffold, once cleaved by the virally encoded protease, is believed to leave the capsid at the same time as the viral genome is packaged (Rixon, 1993).

1.1.4.3 - Herpesvirus tegument.

The tegument is an amorphous structure, surrounding the capsid, which is peculiar to herpesviruses. It has been frequently reported to be asymmetrically distributed but this may be an artefact of the conditions used to prepare the samples for electron microscopy (Rixon, 1993). A variety of proteins have been identified as components of the tegument. The most notable group being the trans-activating proteins such as the HSV-1 protein UL48 (VP16, ICP25, αTIF), a transcriptional activator which is required for the efficient expression of immediate early genes expression. For HSV-1 other components of the tegument include UL41 (VHS) which acts to shut off host cell protein synthesis (Smibert *et al.*, 1992), US11 which appears to be an anti attenuation factor for transcription (Roller *et al.*, 1996) and UL36 (ICP1-2) which amongst other things has been implicated in the release of viral DNA from the capsid (Batterson *et al.*, 1983).

1.1.4.4 - The envelope.

The envelope of herpesviruses is derived from altered patches of cellular membranes (Morgan *et al.*, 1959). Herpesvirus capsids have been observed budding into the perinuclear space and this was assumed to be how the virions obtained their envelopes. There are however, other studies which suggest that the virion envelope is not derived from either the nuclear or ER membranes thus supporting the hypotheses of de-envelopment and re-envelopment stages during virion maturation (Vangenderen *et al.*, 1994; Browne *et al.*, 1996).

Under the EM the lipid envelope contains numerous protein spikes which have been identified as viral glycoproteins (Wildy & Watson, 1963). The number of glycoproteins encoded by different herpesviruses varies but HSV-1 encodes at least eleven. Some have been implicated in the early stages of infection e.g. gB, gC, gD, gH and gL (UL27, UL44, US6, UL22 and UL1 respectively). The glycoproteins may also have roles in immune

modulation (Friedman *et al.*, 1984; Johnson *et al.*, 1988) and preventing the secondary infection of cells (Campadelli-Fiume, 1988).

Although glycoproteins make up the largest proportion of virally encoded membrane proteins in HSV-1, several other non-glycosylated proteins have also been assigned to the envelope. These include UL34, UL20 and UL43, the functions of which are unknown (Campadelli-Fiume, 1994).

1.2 - Herpes simplex virus type 1.

1.2.1 - Introduction.

Herpes simplex viruses were the first human herpesviruses identified and herpes simplex virus type 1 (HSV-1) is probably the most studied herpesvirus to date. HSV-1 is the prototype herpesvirus and as such a considerable amount is known about its structure, biology and life cycle.

1.2.2 - The HSV-1 genome.

The HSV-1 genome is approximately 152 Kbp in size, with a G+C content of approximately 68%. The genome conforms to the pattern of a class 6 genome as described in figure 1.1. It contains two unique regions denoted UL and US, which are 108 and 13 Kbp, respectively, and are flanked by inverted repeats which have defined structures. The inverted repeats are composed of elements designated 'a' (250-500 bp), 'b' (8.8 Kbp) and 'c' (6.6 Kbp) sequences. The terminal L repeat (TRL) has a minimum structure of an 'a' and a 'b' sequence, there may, however, be more than one 'a' sequence present. The terminal S repeat (TRS) only ever contains a single 'a' sequence giving it the structure 'ca'. The internal repeats are inverted with respect to the terminal repeats, which means that the structure of the HSV-1 genome can be written as a_mb-UL-b'a'$_n$c'-US-ca, where m and n are variable. Figure 1.2 shows a diagrammatic representation of the HSV-1 genome (Ward & Roizman, 1994, McGeoch *et al.*, 1988, Sarisky & Weber, 1994a). The invertable nature of the two unique regions gives rise to equimolar amounts of four functionally equivalent isomers (Martin and Weber, 1996).

1.2.2.1 - HSV genes and gene expression.

The HSV-1 genome contains approximately 80 open reading frames (ORFs) which have been divided into three classes based on when they are expressed during infection. These are the α (immediate early or IE), β (early) and γ (late) genes (Jones & Roizman,

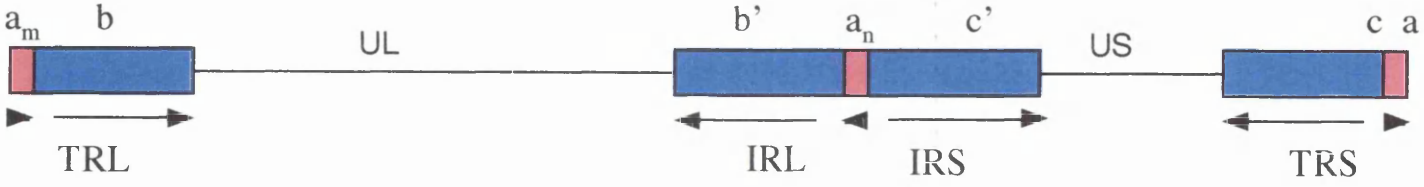


Figure 1.2 - The HSV-1 genome arrangement.

This figure shows a diagrammatic representation of the HSV-1 genome (not to scale). The genome contains two freely invertable unique sequences US and UL which are flanked by inverted repeated. The 108 Kbp UL region is flanked by an approximately 8.8 Kbp repeat sequence known as the 'b' sequence while the 13 Kbp US region is flanked by a 6.6 Kbp repeat known as the 'c' sequence. In addition the 400-500 bp 'a' sequence repeat is found at the L and S termini as well as the LS junction. Multiple copies of the 'a' sequence may be found at the L terminus and the LS junction but only a single copy is found at the S terminus. The genome can thus be described a_mb-UL-b'a_nc'US-c, where m and n are variable.

1979). Although there is some blurring of the boundaries, in general the genes are co-ordinately regulated in a cascade fashion.

The first genes to be expressed after infection are the α genes, which reach a peak of expression approximately 2 hours post infection. Four of the five IE proteins are involved in regulation of gene expression (α4 / ICP4 or Vmw175, α0 / ICP0 or Vmw110, α22 / ICP22 or Vmw68 and α27 / ICP27 or Vmw63). The fifth (α47 / ICP47 or Vmw12) plays a role in inhibiting antigen presentation by infected cells. IE genes are expressed in the absence of prior viral protein synthesis and are characterised by the presence of the sequence TAATGARAT (where R is a purine) within their promoters (Gaffney *et al.*, 1985).

β genes reach a peak of expression between 5 and 7 hours post infection and require the prior synthesis of α proteins (Honess and Roizman 1974). This class of genes has been further divided into β_1 and β_2 based on when they are first detected. The products of β^1 genes appear very quickly after infection but are differentiated from α proteins by their lack of a TAATGARAT sequence and their requirement for α proteins for efficient activation. An example of a β_1 gene is UL39 (ICP6) the large component of the viral ribonucleotide reductase. β_2 genes are exemplified by the gene for the viral DNA polymerase. The remaining six proteins required for HSV-1 DNA synthesis are also β genes.

γ genes generally reach a peak of expression after viral DNA replication has begun and again these genes have been sub-divided into two groups γ_1 and γ_2. A characteristic γ_1 gene is that encoding gD (US6) which is expressed relatively early in infection and is not dramatically affected by the inhibition of viral DNA replication. γ_2 genes, which are characterised by the gene encoding gC (UL44) are expressed late in infection and their expression is effectively inhibited in the absence of viral DNA replication (Kibler *et al.*, 1991).

1.2.2.2 - DNA replication.

The major features of herpes simplex virus DNA replication have comprehensively been reviewed by Challberg (1991) and Boehmer & Lehman (1997). Herpes simplex viruses have 3 origins of replication, which are described in sections 1.2.2.3 and 1.2.2.6, any one of these represents the essential cis-acting signal required for viral DNA synthesis. In addition HSV-1 contains seven genes that specify proteins with essential trans-acting functions in DNA synthesis. These are genes UL5 , UL8, UL9, UL29 (ICP8), UL30, UL42 and UL52. The functions of the encoded proteins have now been elucidated and their role in DNA replication characterised. UL5, UL8 and UL52 encode the three components of a trimeric helicase-primase complex. UL30 and UL42 represent the catalytic and accessory subunits of a heterodimeric DNA polymerase. UL29 encodes a single-stranded DNA

binding protein, and UL9 a homodimeric protein that binds to specific sequences within the origin regions and can also function as a DNA helicase. It is thought that the initial event in viral DNA synthesis is the binding of UL9 to the origin regions and that the other proteins are recruited by virtue of a series of specific protein-protein interactions to form a replication complex. Unwinding of the origin region then allows synthesis of RNA primers, their extension by the DNA polymerase and the establishment of replication forks. All the HSV-1 proteins except UL9 are presumed to also have essential roles at the replication forks where they allow co-ordinated synthesis of the leading and lagging strands. RNA primers are probably removed by the viral DNA polymerase, but two host proteins, a DNA ligase and a DNA topoisomerase are likely to be required for replication of the genomic DNA.

The major problem associated with the replication of linear genomes relates to the mechanism by which the ends are faithfully copied. In the case of HSV-1 there is overwhelming evidence that the virus overcomes this problem by existing in an "endless" form during most of the replicative cycle. Following infection input genomes are rapidly circularized, a process that does not require de novo viral protein synthesis. As described later circularization is most likely to occur by a direct end-to-end ligation. Double stranded circular molecules are usually replicated by either a theta form or rolling circle mechanism. In the case of HSV-1 it is proposed that, like bacteriophage lambda, an initial amplification of circular template molecules by theta form replication is followed by a phase of rolling circle DNA synthesis. The evidence for this is, however, far from conclusive. Nevertheless at late times of infection the replicated DNA clearly consists of concatemeric molecules composed of tandem repeats of the whole genome. Although this is consistent with a rolling circle mechanism other possibilities cannot be excluded, for example similar products may be formed as a result of recombination or the yeast 2 micron circle mode of replication. Whatever the mechanism, at least two process are involved in the conversion of the concatemeric molecules to mature linear genomes. Branched structure due to recombination must be removed prior to DNA packaging and it is thought that the 'alkaline exonuclease' encoded by UL12 plays a role in this process. Finally the concatemers are cleaved at specific sites, a process very tightly coupled to their packaging into the preformed viral capsids

1.2.2.3 - The UL region.

The UL region (108 Kbp) contains at least 56 genes (UL1 -UL56). A number of other genes have been proposed, some of which appear to encode genuine original products (e.g. UL26.5 and UL49.5) whilst the status of others is less clear (e.g. UL8.5, UL9.5, UL12.5, UL15.5, UL43.5). Another important function which is contained within the UL region of the HSV-1 genome is the origin of replication, Ori_L. The HSV-1 genome contains 3 origins

of replication, one copy of Ori_L in the long unique region and one copy of Ori_S in each of the TR_s and IR_s regions of the genome. The functional significance of having three origins of two types is not yet understood. It does appear however, that only one copy of either Ori_L or Ori_S is required for efficient DNA replication which implies a certain level of redundancy (Igaroashi *et al.,* 1993; Polvino-Bodnar *et al.,* 1987). Ori_L is located in the untranscribed region between the UL29 and UL30 genes. The smallest Ori_L fragment which has been shown to act as an origin of replication in a transient replication assay contains a 144 bp perfect palindromic sequence with a 20 bp region containing exclusively A and T residues at its centre (Hardwicke and Schaffer, 1995; Lockshon & Galloway, 1988; Spaete & Frenkel, 1982)

1.2.2.4 - The US region.

The US region (13 Kbp) contains at least 12 genes (US1-US12), with a thirteenth possibly represented by the more recently described US8.5 gene.

1.2.2.5 - The RL repeats.

The RL region comprises the 'a' and 'b' sequences and in HSV-1 strain 17 is 9.2 Kb in length. The 'b' sequence specifies the RL1 gene(Ackerman *et al.*, 1986; Sarisky & Weber, 1994b), the antisense open reading frame ORFP (Lagunoff & Roizman, 1995) and the RL2 gene (Everett, 1984). The RL1 gene encodes the critical neurovirulence factor ICP34.5 which appears to prevent host cell apoptosis and the associated protein synthesis shut off (Chou & Roizman, 1992). The function of the antisense transcript ORFP is unknown, but it has been proposed as an antisense regulator of RL1 expression (Randall & Roizman, 1997). The 'b' sequence also encodes the latency associated transcripts or LATs. During latency the viral genome is transcriptionally silent with the exception of the LAT region. LATs are, largely, non-polyadenylated spliced introns which accumulate in the nucleus of latently infected cells (Rock, 1993), but although a considerable amount is known about their occurrence their function remains unclear. Several suggestions have been made regarding their potential for protein expression (Perry & McGeoch, 1988; Wechsler *et al.,* 1988; Wagner *et al.,* 1988 a&b) but no LAT associated polypeptides have yet been identified. In addition the location of the LAT transcripts also overlap the RL1 gene, on the opposite strand,. This observation has lead to the hypothesis that they, like ORFP, are involved in the negative regulation of the RL1 gene (Russell *et al.,* 1987; Harris *et al.,* 1989; Clements & Stow, 1989). As described in section 1.2.2.7a both the 'b' and 'c' sequences

also play a role in the respective inversions of the UL and US regions of the genome, giving rise to the four genomic isomers.

1.2.2.6 - The RS repeats.

The RS region (6.6 Kbp) comprises the 'a' and 'c' sequences. The 'c' sequence contains the coding region of the RS1 gene. This essential α gene positively regulates most β and γ genes while negatively regulating itself and RL2 (Ward & Roizman, 1994). Each copy of the 'c' sequence also contains a copy of the Ori_S, origin of replication. HSV-1s Ori_S, has been located within an approximately 100 bp region between ICP4 and ICP22 or ICP48 genes on either side of the S region (Stow & McMonagle, 1983). The prominent feature of ori_S is a nearly perfect 45 bp palindromic sequence centred around an 18 bp AT rich sequence. Although Ori_S and Ori_L are non-identical approximately 80% sequence identity is exhibited over the region corresponding to the Ori_S palindrome and approximately 40 bp to one side of it. Both origins also contain two strong binding sites for the HSV-1 origin binding protein UL9. The activities of the origins appear to be stimulated by the presence of flanking transcription factor binding sites which are associated with the divergent US12 (or US1)/ IE175 and UL29 / UL30 genes (Hardwicke & Schaffer, 1995; Wong & Schaffer, 1991). Like the 'b' sequence, the presence of two copies of the 'c' sequence in the HSV-1 genome plays an important role in the inversion of the L and S segments.

1.2.2.7 - The 'a' sequence

The experimental work presented in this thesis is concerned with the role of the 'a' sequence in viral DNA packaging. The following section therefore reviews in greater depth our knowledge of the 'a' sequence structure and function.

The 'a' sequence of HSV-1 strain 17 is 380 bp in size and is itself composed of both quasi unique sequences and repeat elements. In addition, the 'a' sequence proper is flanked by a repeat element known as the DR1 repeat which is 20 bp long. Single 'a' sequences are flanked by separate DR1 repeats but where two 'a' sequences are tandemly repeated they share the intervening DR1 repeat. The DR1 repeat contains the site for viral DNA cleavage during the packaging of concatemeric DNA, however, the actual sequence of the DR1 repeat has been shown to be unimportant. This has lead to the hypothesis that cleavage occurs a fixed distance from a separate cleavage signals (Varmuza & Smiley, 1985).

The 'a' sequence itself contains two quasi unique regions, u_b and u_c, separated by a number of repeat elements, in the case of HSV-1 strain 17 these are known as DR2 repeats. It is the variability in the number and length of these internal repeats which gives rise to the

variability in the size of 'a' sequences from different strains of HSV-1. The u_b element is 80 bp long while the u_c element is slightly shorter at 72 bp. Separating these two elements are approximately 19 copies of the 12 bp DR2 repeat. The role of the DR2 repeats is not clear as they have been shown to be nonessential for the processes of encapsidation and genome isomerisation (Varmuza & Smiley, 1985)

A functional analysis of the 'a' sequence has identified two specific regions required for DNA cleavage and packaging which mapped to the u_b and u_c elements. These sequences were shown to correspond to regions of conserved sequence found at the termini of a range of herpesviruses and this has lead to these elements being labelled the pac1 and pac2 homologies (Deiss *et al.*, 1986; Broll *et al.*, 1999).

Based on DNA sequence comparisons, Deiss *et al.* (1986) identified four distinct regions within pac1, (1) a 40 to 50 bp GC rich region, which is followed by (2), a C or G rich region of variable length, (3) a T rich element which interestingly has a conserved location relative to the end of the genomes of a wide range of herpesviruses, (4) the final element is another G rich sequence.

Pac2 was defined as comprising (1) a short sequence with a consensus sequence CGCCGCG, (2) an unconserved region which is followed by (3) a T element which, like the pac1 T element is rich in thymidine and has a conserved start point, (4) the pac2 element finishes with a GC rich element (see figure 1.3). Subsequent investigators, using the larger number of sequences available, have been less convinced of the pac2 consensus sequence preferring to define pac2 as two GC rich regions separated by a T rich element (Broll *et al.*, 1999).

The functions of individual regions of pac1 and pac2 identified by Deiss *et al.*, (1986) have not been subsequently investigated to test their functional significance.

As mentioned above 'a' sequences are found at the L and S termini as well as at the L-S junction. This arrangement means that even in a virion containing only single 'a' sequences, a tandemly repeated 'a-a' junction, will be formed when the genome circularises, shortly after infection. This scenario conflicts however, with the observation that, single 'a' sequences predominate in concatemeric DNA (Locker & Frenkel, 1979). This seems to suggest a complex replication mechanism in which the number of 'a' sequences may first be reduced and then amplified.

i) - Genome isomerisation and recombination.

The perceived importance of the HSV-1 terminal repeats, and in particular the 'a' sequence, in genome isomerisation has varied greatly over the years.

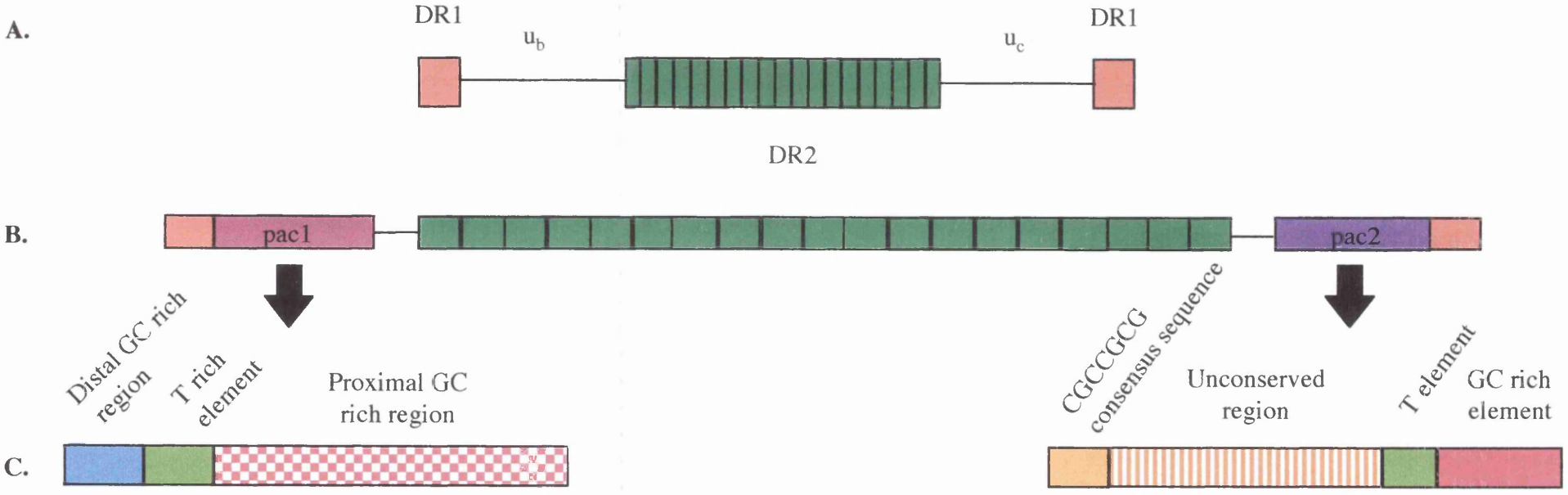


Figure 1.3 - The HSV-1 'a' sequence.

This diagrammatic expansion of the 'a' sequence shows A. the 'a' sequence as a whole, composed of two two quasi unique regions separated by an array of DR2 repeats. B. shows an expansion of the 'a' sequence including the location of the pac1 and pac2 homologies with u_b and u_c. C. shows the detailed structure of the pac1 and pac2 sequences. pac1 comprises a 12bp G/C rich sequence, an 8 bp T rich sequence and a 43 bp GC rich sequence. While pac2 comprises a 7 bp consensus sequence, a 31 bp unconserved sequence , a 6 bp T rich element and a 14 bp GC rich sequence.

Each line has been drawn to scale.

Initially in the mid 1970s it was thought that genome isomerisation was mediated by whole inverted repeats, in a process of homologous recombination (Sheldrick & Berthelot, 1974). In 1980 however, an attempt was made to examine the actual mechanism of genome isomerisation. This was achieved by taking restriction endonuclease fragments of the genome and inserting them into the UL region of the genome. Using this system it was shown that the only fragment to drive additional genome isomerisation events (i.e. produce novel genomic isomers) was a fragment which spanned the L-S junction. The fragment in question contained a complete 'a' sequence as well as portions of the 'b' and 'c' sequences. This suggested that genomic isomerisation did not require a complete terminal or internal repeat (Mocarski *et al.,* 1980). At this time the mechanism of isomerisation was believed to be one of site specific recombination. This was supported when it was shown that viral proteins were required in trans to initiate isomerisations within amplicons (an amplicon is a plasmid which contains both an origin of replication and a functional packaging signal) (Mocarski & Roizman 1982a & 1982b).

Some years later in 1988, following experiments in which duplicate L-S junctions or Tn5 transposon elements were placed into amplicons, it was shown that using only the minimum set of seven viral replication genes, isomerisation could be detected in amplicon sequences. These results indicated that recombination in HSV-1 was being mediated by the replication machinery and did not appear to be sequence specific. This made the probability of isomerisation occurring by a process of generalised recombination more likely (Weber *et al.,* 1988). This finding was supported by work published in 1992, which found that while recombination between two directly repeated 'a' sequences was detectable in the absence of replication, it was greatly increased by the process of replication. One explanation for this observation was that during replication highly recombinogenic single stranded nicks were introduced into the genome. This would also account for the close link between replication and recombination (Dutch *et al.,* 1992). In addition to the link between recombination and replication it was also observed that sections of the 'b' and 'c' sequences could lead to low levels of inversion (Longnecker & Roizman, 1986 & Vlazny & Frenkel, 1981).

All of these lines of inquiry seem to point towards a mechanism of homologous recombination for the isomerisation events observed in HSV-1. This model however fails to take into account firstly, the disproportionate recombinogenic activity of the 'a' sequence with respect to its size, and secondly the observation that sequences at each end of the 'a' sequence are particularly important in the process of recombination. Deletion of these sequences results in reduced DNA rearrangements and reduced generation of novel termini (Chou & Roizman, 1985; Smiley *et al.,* 1990). It was noticed that the recombinationally sensitive regions of the 'a' sequence corresponded to the independently isolated signals for

the cleavage and packaging of viral DNA. This, combined with the observation that inter 'a' sequence recombination could also occur between fragments which did not share any other homology, lead to the hypothesis that recombination was mediated by the free ends generated during cleavage and packaging. In early 1994 one publication gave support to the importance of this event and at the same time linked the 'a' sequence to observations supporting a mechanism of homologous recombination. The report proposed that it was double stranded breaks that were required for recombination and not specific DNA sequences. It was again confirmed that the cleavage and packaging signals were particularly important for the induction of high frequency recombination, however, by separating packaging signals from the cleavage event using a temperature sensitive mutant for packaging and introducing ds breaks into the viral genome with a non HSV-1 endonuclease, it was possible to show that it was the double stranded breaks which were responsible for the recombination. Therefore the 'a' sequence would appear to be a so called 'hot spot' simply because it coincides with a double stranded break generated by cleavage. In addition, it was noticed that the recombinogenicity of the 'a' sequence was only observed in the absence of its normal sequence environment, that is to say adjacent to the 'b' or 'c' sequences with their highly repetitious regions (Sarisky & Weber, 1994a). This suggestion is supported by work from two laboratories. Firstly the identification of a host cell endonuclease which cleaves viral DNA within DR2 repeats producing ds breaks (Wholrab *et al.,* 1991) and secondly, the discovery that HSV-1 ss DNA binding protein possess a Rec-A like ability which promotes homologous pairing and strand transfer (Bortner *et al.,* 1993).

In HSV-1 the dispensability of the 'a' sequence in the mechanism of L and S segment inversion was finally confirmed in 1996. A recombinant virus was engineered without 'a' sequences at the LS junction or the L and S termini. In order to produce a viable virus a single 'a' sequence was inserted into the thymidine kinase gene in the UL region of the genome. The resulting recombinant virus was able to invert its L and S segments at *wt* levels, producing all four genomic isomers. Interestingly only two of the genomes were packaged into virions allowing an investigation of the kinetics of recombination to be made. Infections using the virions containing only two genomic isomers resulted in regeneration of the other two isomers with a high degree of efficiency. All four genomic isomers were readily detected at a stage of infection, co-incident with the onset of DNA replication, supporting the idea that genome isomerisation is mediated by an 'a' sequence independent homologous recombination mechanism, which is enhanced by the process of DNA replication (Martin & Weber, 1996).

A number of alternative solutions to the genome isomerisation puzzle have also been proposed, although at present their validity is less well established. Firstly a mechanism for

novel rolling circle replication has been proposed (Zhang *et al.*, 1994) which could account for genomic isomers. This model proposes the existence of circular dimers of two viral genomes, which could theoretically provide all of the genomic isomers in equimolar amounts simply by using different cleavage frames. Secondly a host recombinase has been isolated from un-infected cells which promotes inter 'a' sequence recombination *in vitro*. This recombinase, however, appears to have features which are not consistent with characteristics observed for HSV-1 recombination. Its role is, for this reason probably less significant (Bruckner *et al* ., 1992; Sarisky & Weber *et al.*, 1994a).

ii) - Genome circularisation.

In 1982 the role of the terminal repeats was proposed to include the circularisation of the genome (Mocarski & Roizman, 1982b). The prevailing view at the time was that ligation of the two termini was preceded by 5'-3' exonuclease digestion of each terminal 'a' sequence, forming two cohesive ends (Wadsworth *et al.*, 1976). However, it has been shown that the L terminus terminates with 18.5 bp of a DR1 repeat, while the S terminus terminates with 1.5 bp of a DR1 repeat (in each case 0.5 bp indicates a single nucleotide 3' overhang). This means that the two might be directly ligated via their single base overhang in the process generating a complete 20 bp DR1 repeat. The free energy associated with the complementary binding of a single base pair is, however, very small making a protein interaction quite likely in this process. Although no candidate interactions have been identified to date, the fact that the each terminus contains at least one 'a' sequence suggests that signals within the 'a' sequence could prove to play an important role in this process. A mechanism of direct ligation of the termini would also appear to be important for circularization of the many herpes viruses which lack a terminally redundant element

A mechanism of genome circularisation which involves homologous recombination does however remain possible. In support of this, it has been shown that the 'a' sequence of HSV-1 can direct circularisation both by imprecise end joining and non-conservative homologous recombination (Yao *et al.*, 1997).

A subsequent study of GPCMV has directly implicated the pac2 homology in the circularisation mechanism and appears to support a mechanism of direct ligation (McVoy *et al.*, 1997). GPCMV generates three different termini which have been designated R, O and M. Viable genomes have an R terminus (containing a pac1 homology) and either an M (containing a pac2 homology) or an O terminus. Both types of genome undergo circularisation soon after infection indicating that they contain all of the sequences necessary for circularisation. The O and M termini differ in the presence (M) and absence (O) of the terminal repeat and consequently there is very little sequence homology between them. Sequence comparisons have, however, identified two regions of the O terminus which

appear to be related to the pac2 T rich element and 6 bp of the GC rich element which occur on the M termini. No region of homology corresponding to the pac2 consensus sequence was identified. Assuming that circularisation requires the specific recognition of both termini these observations appear to implicate pac2 or at least a degenerate pac2 sequence in genome circularisation. The other important aspect of this study is the finding that genome termini which lack extensive sequence similarity are efficiently joined during circularisation. This suggests that, in GPCMV at least, the principle mechanism of circularisation involves ligation of the termini rather than homologous recombination or cohesive end formation.

Finally, it also appears likely that cellular proteins are involved in the process of circularisation. The *RCC1* gene of BHK cells, which is a regulator of chromosome condensation, has been implicated as viral DNA remains linear in cells lacking RCC1 (Umene & Nishimoto, 1996).

iii) - The role of the 'a' sequence in the regulation of gene RL1.

The RL1 gene, which encodes the ICP34.5 protein, is unusual in that it lacks the usual regulatory sequences found upstream of HSV-1 genes. Surprisingly this includes the almost ubiquitous TATA box. The other striking feature about this gene, is the presence of an 'a' sequence directly upstream with its highly repetitious GC rich regions. In order to examine the regulatory mechanisms of this gene, promoter deletions were constructed. From these studies it was found that the promoter consisted of a core promoter, which had full wild type activity and two upstream silencer elements. These silencer elements mapped to the DR2 ('a' sequence) repeats and the DR6 ('b' sequence) repeats. These regions were found to posses an unwound S1 nuclease sensitive (anisomorphic) DNA structure, the structure of which was essential for repressor function. Further study, using differently super coiled DNA and an *in vivo* protein : DNA binding assay, suggest that host cell, protein binding is involved in the silencing mechanism (Sarisky & Weber, 1994b). Despite this it has since been reported that the deletion of the DR2 repeats in a recombinant virus did not significantly effect the expression of ICP34.5 (Martin & Weber, 1998)

iv)- The role of the 'a' sequence in DNA cleavage and packaging.

The role of the 'a' sequence in the cleavage and packaging of the virus genome was first identified by Stow *et al.,* (1983). Using an amplicon packaging system it was shown that a plasmid containing an origin of replication and sequences from the termini of the virus genome could be packaged into viral particles. Further investigations have shown that all of the signals required for cleavage and packaging are contained within a 179 bp sequence from

the junction between two tandemly repeated 'a' sequences (Nasseri & Mocarski, 1988). This sequence contains most of the u_c, u_b sequences separated by a single DR1 repeat. The role of the 'a' sequence in cleavage and packaging is discussed in more detail in 1.3.2.1.

1.3 Virus Genome Packaging.

Section 1.3.1 - ds DNA packaging in bacteriophage.

The study of bacterial viruses, or bacteriophages, has been underway since their discovery, as filterable agents, between 1915 and 1917. This has lead to a considerable wealth of information about their structure and biochemistry as well as their life cycle.

The DNA of ds-DNA bacteriophages is packaged in a highly condensed form, within a protein shell made of virion encoded proteins. The current view is that in most cases the DNA is packaged in a quasi-liquid crystalline arrangement which gives a packaging density which approaches the maximum possible (Cerritelli *et al.*, 1997; Booy *et al.*, 1991).

Based on the arrangement of the virion genome, ds-bacteriophages can be divided into three groups (Fujisawa & Morita, 1997): 1) Linear genomes with non-unique sequences repeated at their terminus e.g. T4. 2) Linear genomes with unique sequences repeated at each terminus e.g. λ, T3 and T7. 3) Other genome structures such as linear genomes with covalently attached protein or circular genomes. Of course considerable variation is apparent within these groups but in general they serve as a good basis for discussing the different strategies that bacteriophages employ to package their genetic material.

Despite this variation in the genome structures of ds bacteriophage, in general their replication mechanisms lead to an accumulation of concatemeric DNA, i.e. multiple copies of the phage genome joined head to tail. This high molecular weight DNA forms the substrate for the DNA packaging machinery, which produces packaged unit length genomes. Several mechanisms for DNA packaging are discussed below. However the focus is on the linear dsDNA phages such as T4 and λ as they are more likely to share similarities with herpesviruses. The mechanisms utilised by viruses such as the small circular phages P1 and P2 are not discussed.

The central elements of the DNA packaging process are thought to be similar for most ds DNA phages. DNA is packaged into a preformed protein shell known as the prohead. While the prohead may exhibit rotational symmetry the vertex through which the DNA enters the prohead is specifically known as the portal vertex. At this portal vertex the DNA enters through a non-head protein complex known as the connector. The connector is essential for DNA packaging but it is not thought to be responsible for catalysing the packaging reaction, this function is fulfilled by another non-head protein, the packaging enzyme or terminase. The energy necessary for DNA translocation is almost certainly provided by ATP hydrolysis and while it has not been exclusively shown that this is carried out by the terminase, several terminases have been shown to exhibit ATPase activity. In addition to providing the energy

required for DNA packaging, ATP has also been implicated as an allosteric effector of packaging complex formation. (Shibata *et al.*, 1987).

For a considerable number of bacteriophage the initial step in the packaging of concatemeric DNA involves the recognition of a specific sequence e.g. pac or *cos* site. This initial recognition event is followed by either a site-specific cleavage (e.g. λ, T3 and T7) or cleavage at a non-specific site(e.g. T1). This is known as the initial cleavage event. DNA is then packaged uni-directionally from the newly generated end. Once the procapsid is full a second cleavage is made. Again this cleavage may be site specific as in λ, T3 and T7 or non specific as in T1.

A variation on this type of mechanism is that employed by T4. T4 DNA packaging appears to be almost completely non site specific. The location of the initiation cleavage event is close to random, although there is some evidence of preferred initiation sites (Franklin *et al.*, 1998), and the termination cleavage is performed only when the capsid is full i.e. when 104% of a unit length has been packaged (Black, 1995; Lin & Black, 1998) This, so called headful packaging, appears to have a role in the strategy of a number of bacteriophage, even those with defined initiation and termination signals, and although the mechanisms which control it are not fully understood, it is becoming apparent that the portal proteins play a central role (Cue & Feiss, 1997).

Central to the mechanism of DNA packaging is the packaging enzyme or terminase. Where they have been identified, terminase enzymes exhibit DNA dependant ATPase activity and it is the energy released by this hydrolysis which is thought to drive the thermodynamically unfavourable packaging process. In general terminases are commonly composed of two subunits, a large and a small subunit. The large subunit is responsible for procapsid binding, DNA translocation and DNA cleavage as well as containing either the putative or demonstrated ATP binding site (Rao & Black, 1988; Babbar & Gold, 1998). It is ATP hydrolysis at these sites which is thought to drive DNA translocation. The small subunits have DNA binding activities which appear to be involved in the recognition of the DNA to be packaged (Catalano *et al.*, 1995).

1.3.1.1 - DNA packaging in bacteriophage λ.

The bacteriophage λ has been studied in some detail and for this reason the mechanism which it employs to package its DNA will be explored in a more detail.

The lambda virion is composed of an isometric (T=7) head, which contains the phage genome, a long tail, which is surrounded by a contractile sheath and a single fibre which is attached to a structure known as the base plate at the base of the tail. The genome, which is linear in the phage particle, is approximately 48.5 Kbp long and terminates in a

complementary 12 bp 5' overhang. Shortly after infection the termini are ligated together to form a covalently closed circle.

The lambda genome is replicated, initially via the theta mode of replication but at later stages rolling circle replication and recombination give rise to concatemeric DNA which forms the substrate for DNA packaging (Furth & Wickner, 1983).

a) - The packaging signal of lambda.

λ has a tri-partite cleavage and packaging signal know as the *cos* site which is found at the junction between viral genomes in the replicated concatemer. *Cos* can be divided into three components, *cos*N, *cos*B and *cos*Q (figure 1.4).

*Cos*N is 22 bp long and is the site of viral cleavage. Ten of these bases display imperfect rotational symmetry. *Cos*N is both necessary and sufficient for terminase binding as indicated by the fact that the large subunit of the terminase has nicking activity at *cos*N (Rubinchik *et al.*, 1994a). Terminase has been shown to introduce two ss nicks into *cos*N at positions N_1 and N_2 (figure 1.4b). In addition, kinetic studies of this interaction appear to suggest that it is a terminase dimer which binds to *cos*N (Tomka & Catalano 1993a).

*Cos*B is composed of 3 core elements, R1, R2 and R3. Collectively these sites are involved in the initiation of DNA packaging. Along with ATP the *cos*B site stimulates the efficiency and fidelity of *cos*N cleavage by the terminase (Cue & Feiss, 1993a; Rubinchik *et al.*, 1994a). More specifically, R1, R2 and R3 have been shown to interact directly with the small subunit of the terminase enzyme (Bear *et al.*, 1984; Shinder & Gold, 1988). Another feature of the *cos*B site is the presence of a consensus binding site (I1) for integration host factor (IHF)(Bear *et al.*, 1984). IHF is the site specific, DNA bending protein of *E. coli* and it has been shown that IHF does bind to I1 introducing a sharp bend between R3 and R2 (Kosturko *et al.*, 1989). This has been proposed to facilitate co-operative interactions between terminases bound at R3 and R2/R1, a conclusion which is supported by the observation that deletion of R2 and R1 produces a phage which is IHF insensitive (Cue & Feiss, 1992). Finally, several mutant viruses have also implicated *cos*B in the termination cleavage, it appears that *cosB* and a second site I2, which is found between *cos*N and *cos*B (Xin & Feiss, 1988; Mendelson *et al.*, 1991) act in concert to direct nicking at N_1 during termination (Cue & Feiss, 1998).

The other major element of the *cos* packaging signal is the 14 bp *cos*Q. *Cos*Q is not required for the initiation of packaging but *cos*Q⁻ mutants have been shown to be defective in the termination cleavage event (Cue & Feiss, 1993b). *Cos*Q is responsible for directing nicking at N_2 during termination, however, as mentioned above, *cos*Q alone is not the only part of *cos* which has been implicated in the termination of packaging. Thus it appears that *cos*Q constitutes one part of a larger termination signal (Cue & Feiss, 1998).

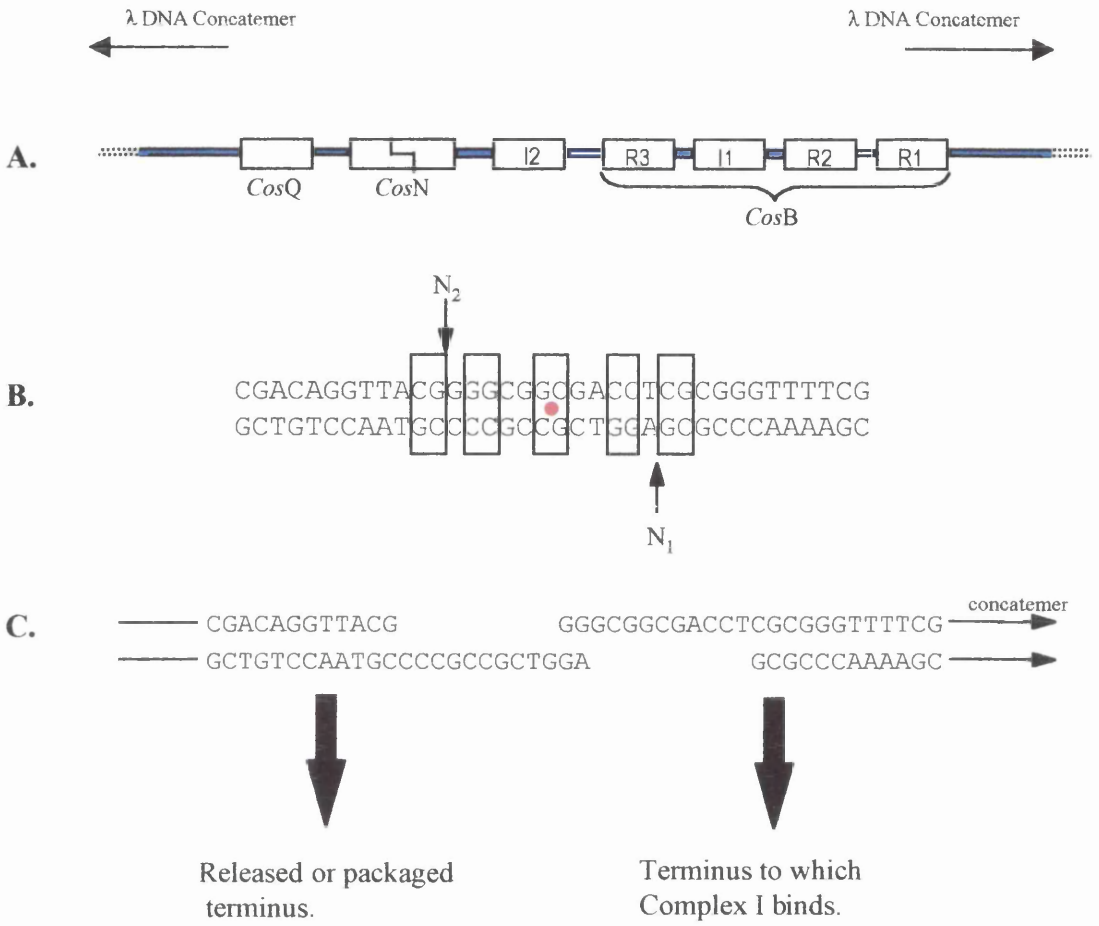


Figure 1.4 - Lambda's packaging signal.

A. shows the component features of the λ cleavage and packaging signal. The cleavage event at the initiation of packaging occurs at *cos*N in conjunction with R1-3 and I1 which are are collectively known as *cos*B. Termination cleavage involves a number of different sequences including those found at *cos*Q and I2.

B. shows the detail of the cleavage site *cos*N. Cleavage introduces two nicks which are 12 bp apart (N_1 and N_2). The cleavage site as a whole exhibits imperfect rotational symmetry about the point marked by the red dot.

C. Shows the termini generated by the cleavage event. The terminase remains specifically attached to the *cos*B containing terminus, forming complex I.

b) - λ terminase.

The lambda terminase is composed of two virally encoded subunits, gpNu1 and gpA. The combined holoenzyme is responsible for a number of functions. These include the recognition of the concatemeric lambda DNA, the initiation of DNA packaging, including the introduction of nicks at *cos*N. The translocation of the viral DNA into the procapsid, with the concomitant hydrolysis of ATP and the terminal cleavage at *cos*N, in conjunction with *cos*Q, *cos*B and I2.

gpNu1, the small subunit of the terminase, is the product of the *Nu1* gene and is 181 amino acids in size. gpA, the large subunit, is the product of gene *A* and is 641 amino acids in size. gpA has been shown to have, site specific endonuclease, DNA helicase and DNA dependant ATPase activities which closely resemble those seen for the terminase complex as a whole (Davidson & Gold, 1992; Rubinchik *et al.,* 1994a & b). In addition to these, several other motifs have been identified. These include a domain for gpA binding to the prohead, a specificity domain for gpA : gpNu1 binding (Yeo & Feiss, 1995a) and a putative leucine zipper domain which is potentially involved in terminase dimerisation (Davidson & Gold, 1992).

The same techniques used above to dissect the functional elements of gpA have also been applied to gpNu1. This identified domains for both *cos*B binding and gpNu1 : gpA interaction. The *cos*B binding domain forms a putative helix-turn-helix domain (Frackman *et al.,* 1985) which is a characteristic of many sequence-specific DNA binding proteins. In addition to these domains, and in a marked departure from the other bacteriophage which have been studied in detail, the small subunit of the lambda terminase (gpNu1) also exhibits DNA dependant ATPase activity (Becker & Gold, 1988; Tomka & Catalano 1993b; Rubinchik *et al.,* 1994b)

c) - The packaging mechanism employed by lambda.

Using what is known about *cos* and the DNA packaging proteins of lambda a model for DNA packaging can be constructed (Catalano *et al.,* 1995) (Figure 1.5).

i) - Initiation.

The initial step in the mechanism is the binding of terminase, probably as a terminase dimer, at a *cos* site on the replicated concatemeric DNA. This is followed by the introduction of two ss nicks in *cos*N (N_1 & N_2). The binding of a terminase dimer, which has been alluded to above, is based on the imperfect rotational symmetry seen at the *cos*N site and on

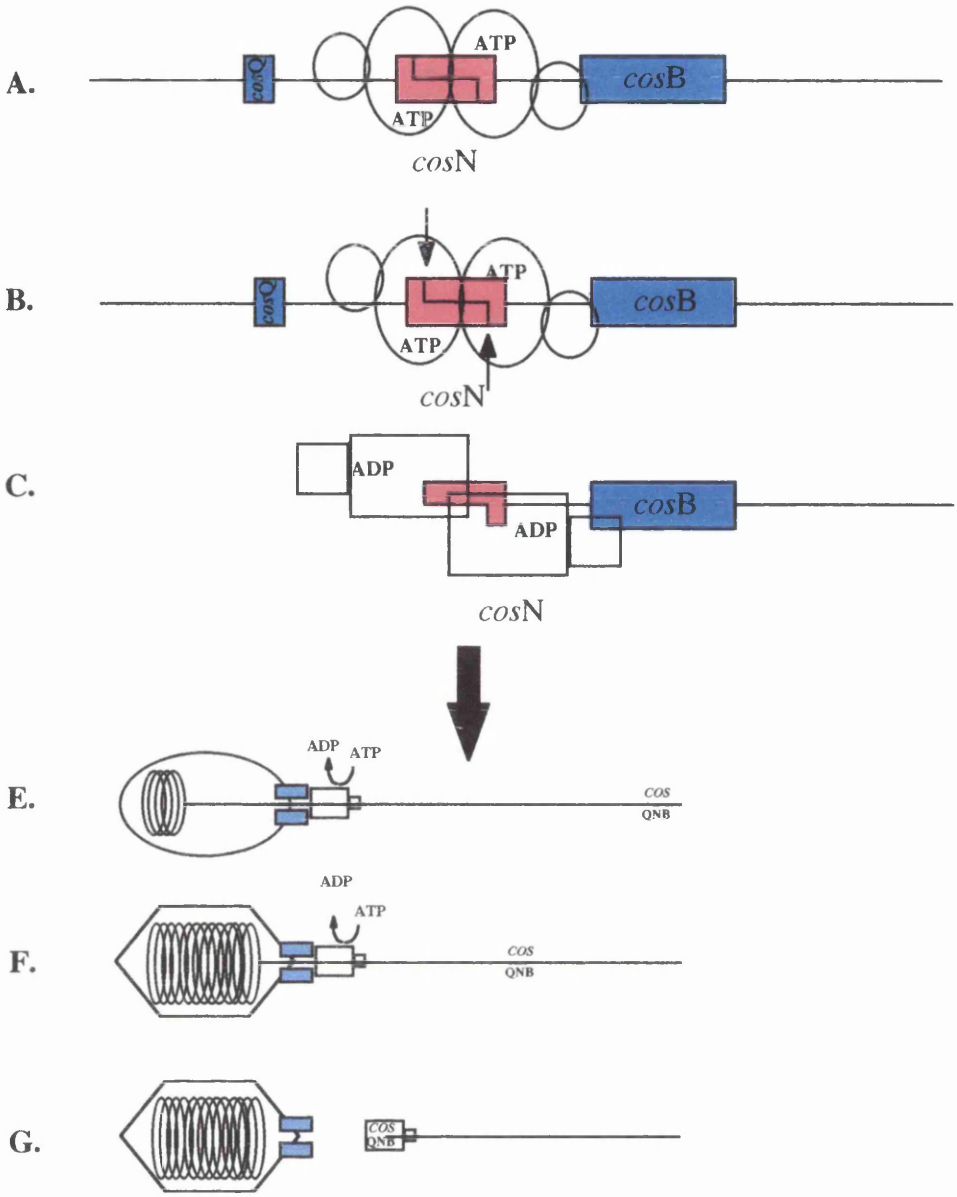


Figure 1.5 - A model for lambda DNA packaging.

This figure describes the model for λDNA packaging proposed by Catalano *et al.* (1995). A. The initial step in packaging probably involves at least one ATP associated terminase enzyme binding to *cosN and cos*B. B. Two nicks are introduced at N_1 and N_2 in a mechanism which is stimulated by ATP but does not require ATP hydrolysis. C. The cohesive strands are subsequently separated and the protein complex undergoes a structural reorganisation which results in the formation of complex I. ATP is hydrolysed during this process but it is unclear whether ATP drives the strand separation or the structural reorganisation. E. Complex I binds to the portal vertex of a prohead generating complex II, DNA packaging then commences with the concomitant hydrolysis of ATP. F. DNA packaging continues until a suitable termination signal is reached. G. On reaching the termination signal cleavage occurs and the terminase dissociates, from the now full head but remains attached to the free terminus thus regenerating complex I.

the kinetic analysis of the endonuclease activity of gpA, which predicts that two terminases are required to perform a single cleavage event (Tomka & Catalano, 1993a). In addition, the rotational symmetry of *cos*N is a common feature amongst DNA binding sites for dimeric proteins (Aggarwal *et al.,* 1988)

As well as the interactions between gpA and *cos*N, the interactions between gpNu1 and *cos*B are also important in the formation of the initial complex. The binding of IHF at I1 appears to facilitate gpNu1 : *cos*B interactions and these in turn enhance the efficiency and fidelity with which the terminase is able introduce the initial nicks at *cos*N.

In addition to the protein components of this complex, another co-factor has been identified. Where low concentrations of terminase or *cos*B mutants were used, the presence of ATP has been shown to have a marked effect on the efficiency with which terminase is able to introduce nicks at *cos*N. Importantly, ATP hydrolysis is not required as non hydrolysable nucleotide triphosphate analogues have an analogous effect (Higgins *et al.,* 1988). It has been shown that the presence of ATP does not increase the affinity of the terminase for DNA. Instead the increased efficiency of nicking at *cos*N is a consequence of an apparent increase in the endonuclease activity of gpA (Cue & Feiss, 1993a).

In summary two heterodimeric terminases, IHF and ATP are all involved in the formation of the initial complex which forms with the *cos* DNA. This complex leads to the introduction of two single stranded nicks (12 bp apart) at *cos*N.

Once the duplex has been cut by the terminase the annealed strands are separated, in a process which is performed by the gpA subunit of the terminase, and appears to require ATP hydrolysis (Higgins *et al.,* 1988). Whether ATP hydrolysis provides the energy for strand separation, in a DNA helicase type mechanism, or whether ATP hydrolysis drives the reorganisation of the protein subunits which make up the complex remains unclear. What is apparent, however, is that at the same time as strand separation significant subunit reorganisation occurs, which leads to the formation of a relatively stable complex in which the newly formed terminus is bound to the terminase (Cue & Feiss, 1992 & 1993b). This complex, first identified by Becker *et al.,* (1977), is called complex I.

It is as part of this complex that the gpA subunit(s) interact, via the connector, with the prohead. The interaction of the prohead with complex I, promoted by a viral protein gpF1 (Yeo & Feiss, 1995b; Davidson & Gold, 1987), forms complex II. This large complex contains all of the necessary components for the packaging of the viral genome. gpF1 appears to promote complex II formation by destabilising complex I, in a reaction analogous to the way in which chaperonins destabilise the gpO, gpP; DNAB protein complex which forms at the λ origin of DNA replication (Liberek *et al.,* 1988).

ii) - Translocation.

Complex II is responsible for translocating the viral genome into the prohead cavity, using the energy released by ATP hydrolysis. Very little is known about how this is achieved but a number of models have been proposed, some of which are discussed in section 1.3.1.4.

The translocation process is none-the-less quite impressive. It has been estimated that the full length lambda genome, of 48.5 Kbp, is packaged in less than 5 minutes using approximately 29,000 molecules of ATP. This rate of packaging, which equates to approximately 160 bp sec^{-1}, combined with the relatively poor ATPase activity of purified terminases has lead to the suggestion that ATP hydrolysis probably occurs at more than one site e.g. multiple terminases, multiple sites of a single terminase, or even sites within the prohead (Morita *et al.,* 1993; Shibata *et al.,* 1987). Several other properties of different DNA translocation complexes are also worthy of note at this point. For T3, the interactions between the DNA and the terminase during translocation have been shown to be weak, both in the presence and absence of ATP. The packaged DNA does not require superhelicity as both nicked and cross-linked DNA can be packaged. However, intercalating agents do inhibit packaging which suggests that the terminase recognises the exterior structure of the DNA helix (Fujisawa *et al.,* 1987; Shibata *et al.,* 1987).

iii) - Termination.

The termination of DNA packaging in lambda requires that *cos*N must be recognised and cut, the cohesive ends must be separated and the terminase must disassociate from the prohead. A feature of several viral DNA packaging mechanisms is processivity i.e. a second packaging event is initiated, directly, from the terminus generated by a termination event. For this to be possible the terminase must remain attached to the free unpackaged end.

A simple mechanism could be envisaged if *cos*N cleavage at termination were essentially similar to the *cos*N cleavage which results in the formation of complex I. However, the existence of *cos*Q suggests that termination is fundamentally different. *cos*Q, as described above, is essential for efficient DNA packaging in lambda. *Cos*Q mutants, result in virions containing greater than unit length genomes as would be expected if the termination of packaging were impaired (Catalano *et al.,* 1995). A clearer definition of *cos*Q function was provided by Cue and Feiss (1998). Their work indicated that *cos*Q alone was not a stop signal and that *cos*B and I2 also make significant contributions to the termination signal. *cos*Q appears to be involved in nicking at both N_1 and N_2, (figure 1.4). However, while *cos*Q is strictly necessary for nicking at N_1, at N_2 nicking is actually increased in the

absence of *cos*Q. *Cos*B and I2 are important for both the efficiency and fidelity of nicking at N_2.(Cue & Feiss, 1998).

Following cleavage the cohesive ends must be separated, a process which is presumed to occur via the same mechanism which generates complex I. For processivity to occur the terminase must also remain attached to the unpackaged genome terminus. This new complex probably resembles complex I and as such, is able to recruit an empty prohead to form complex II and reinitiate a second DNA packaging event (Catalano *et al.*, 1995).

1.3.1.4 - Models for DNA packaging in ds bacteriophages.

Various different models for the mechanism of DNA translocation and the structure of packaged DNA in ds bacteriophages have been proposed, however, to date none has been conclusively established.

a) - Rotating connector models for DNA translocation.

The forerunner of these models is the rotating nut model described by Hendrix (1978). This model is founded on the observation that bacteriophage heads exhibit 5 fold rotational symmetry while the tails have 6 fold rotational symmetry. Unless the connector exhibits 30 fold rotational symmetry this implies a symmetry mismatch at either the connector : head or connector : tail interface. As no protein is present in the connector at 30 copies the possibility of 30 fold rotational symmetry was thought to be unlikely.

Hendrix proposed that connectors had 6 fold rotational symmetry based on the observation that the tail : connector interaction was exclusively non-covalent but that the interaction was none the less relatively strong - the connector remaining attached to the tail during the chemical disruption of bacteriophage (Serwer, 1976). Based on these observations he also proposed that the connector was fairly weakly bound to the head, possibly being held in place by steric constraints, much in the same way that a button is held in a button hole.

The proposed symmetry mismatch between the connector and the head has implications for rotation at this interface. An examination of the kinetics of this type of interaction showed that, between a 6 subunit surface (the connector) and a 5 subunit surface (the head) the bonds are relatively weak. In addition there are no strong energy barriers to rotation.

Hendrix proposed that DNA packaging was achieved in one of two ways. For mechanism 1, ATP hydrolysis is directly linked to the passage of DNA through the connector. The movement of the DNA relative to the connector occurring in an analogous way to polymerase moving along a DNA molecule. Rotation of the connector could occur subsequently in order to accommodate any twisting strain introduced into the molecule.

Mechanism 2, proposes that ATP hydrolysis drives the rotation of the connector. This model requires that some part of the connector, which lacks 6 fold rotational symmetry, interacts directly with the DNA helix in an association analogous to the way that the threads of a nut and bold fit together. Thus, as the nut is turned the 'bolt' passes through the central hole.

Although subsequent investigators have since shown that connectors have 12 or 13 fold symmetry (Dube *et al.,* 1993) the occurrence of a symmetry mismatch holds true. The major weakness of both these models is their predicted energy requirement for packaging (approximately 1 ATP bp^{-1}), which is almost twice that measured for Ø29 packaging (0.5 ATP bp^{-1}) (Guo *et al.,* 1987)

The basic principle of a rotational motor has been subsequently adapted to form the basis of the rotating winch model (Turnquist *et al.,* 1992) and the model described by Dube *et al.* (1993). These models give rise to energy efficiencies which are more in line with the experimental data.

The rotating winch model is essentially similar to the model described by Hendrix (1978). Supercoiled DNA was found to wrap around the outside of Ø29 connectors and this was interpreted as evidence for a winch like mechanism in which connector rotation, driven by ATP hydrolysis, effectively pushes the DNA into the head (Turnquist *et al.,* 1992). This type of mechanism requires DNA which does not contain any nicks, which is at odds with the observed nicks in the packaged DNA of T4, T7 and λ (Khan *et al.,* 1995).

b) Osmotic pump models of DNA translocation.

In 1988 it was proposed that the reported ATP binding activity of the T4 major capsid protein (Rao & Black, 1985b) could be associated with an osmotic pump for DNA packaging (Serwer, 1988). This model proposes that capsid expansion on initiation of DNA packaging decreases the osmotic pressure inside the head, the portal vertex opens and DNA is driven through the pore by the osmotic gradient. In some models of this type all of the DNA is packaged in this initial event. However, in this model it was proposed that small molecules leaked into the head along with the DNA establishing the potential for a second osmotic gradient and a second packaging event. If the head were to contract after the initial expansion, a step potentially driven by ATP hydrolysis by the major capsid protein, the small molecules could be expelled through closeable pores. Subsequent re-expansion would re-establish the osmotic gradient and more DNA could be driven into the head.

This model requires that the bacteriophage capsid be capable of as much as 25% expansion, that the major capsid protein be able to contract and expand in an ATP dependent manner and that the head itself be variably porous and non porous to small molecules. These

constraints combined with the observations of Rao and Black (1985a) which suggest that T4 prohead expansion is not linked to DNA packaging appear to make this model for DNA translocation unlikely.

c) - The ratchet model of DNA translocation (Fujisawa & Morita, 1997).

This model was proposed with T3 in mind but its essential features may well apply to other viruses. The model envisages a single copy of the viral protein gp19 bound to each of the six domains of the connector. gp19 is the large subunit of the T3 terminase. In order to package DNA into the head it is proposed that an ATP associated conformation of gp19 binds to the sugar backbone of the DNA. DNA binding activates the DNA dependent ATPase activity and the hydrolysis of ATP to ADP and P_i results in a conformational change which pushes DNA into the head. When a new ATP molecule binds, gp19 dissociates from the DNA and returns to its original extended conformation. The movement of the DNA molecule allows the next gp19 to bind and the cycle is complete. When this cycle proceeds six times, six molecules of ATP will have been hydrolysed and 1 turn of the DNA helix will have been packaged. Thus this model produces a theoretical packaging efficiency of 0.59 ATP bp^{-1} which is very close to the observed value of 0.55 ATP bp^{-1} (Shibata *et al.,* 1987; Morita *et al.,* 1995)

d) - Models based on the structure of the packaged DNA.

Many different models for the structure of packaged DNA have been proposed; these include spiral folds (Black *et al.,* 1985), liquid crystals (Lepault *et al.,* 1987) twisted toroids (Hud, 1995) and coaxial spools (Cerritelli *et al.,* 1997). Although none of these models has been found to be universally applicable, several interesting features have been identified.

It appears that, for T4 at least, the DNA is packaged in such a way as to allow the first end of the DNA to be packaged to be the first end to leave the head i.e. injection is not the reverse of packaging (Black and Silverman, 1978). As part of their work these investigators also speculated on a mechanism for DNA packaging which is driven via the introduction of torsional strain into DNA molecules.

Some bacteriophages have been shown to contain a proteinacious structure which resides on the inner surface of the connector. The core has been implicated in head assembly (Roeder & Sadowski, 1977) and it may also be involved in DNA injection (Garcia & Molineux, 1996). Although its precise role has not yet been established it has been proposed that the core may influence the arrangement of DNA during packaging.

Chemical cross linking studies have shown that in a large population of virions, all parts of the genome come into close proximity to the head proteins (Widom & Baldwin,

1983; Serwer *et al.,* 1992) indicating that there is significant variation in how the DNA is packaged into an individual virion. Despite this it is also clear that on a larger scale the packaged DNA has a highly ordered structure. Several studies have proposed that packaged DNA is in a liquid crystalline or quasi liquid crystalline state (Lepault *et al.,* 1987; Cerritelli *et al.,* 1997).

Current thinking appears to favour the coaxial spool models for packaged DNA (Richards *et al.,* 1973; Earnshaw & Harrison, 1977; Earnshaw *et al.,* 1978) as this type of packaging can accommodate both the locally crystalline arrangement of packaged DNA (Harrison, 1983) and the cross linking data without the need for the energetically unfavourable kinks required for the spiral fold model. Cryoelectron microscopy of packaged T7 DNA also tends to support the co axial spool models (Cerritelli *et al.,* 1997)

Section 1.3.2 - DNA packaging in herpesviruses.

Compared to the understanding that we have of the bacteriophage systems, our understanding of herpesvirus packaging is relatively rudimentary. A variety of herpesviruses have been studied including HSV-1, HCMV, GPCMV and HHV-6. Although considerable parallels between these viruses have become apparent differences have also been identified and this has made drawing inferences from one system and applying them to another a difficult proposition.

As with the ds bacteriophage, herpesvirus replication is believed to culminate in a replicative mechanism analogous to rolling circle replication which produces high molecular weight concatemeric DNA which forms the substrate for the cleavage and packaging machinery. The concatemeric DNA is processed into unit length genomes and packaged into preformed capsids. B capsids are believed to be the progenitors of DNA containing C capsids, the scaffold proteins leaving the capsid at the same time that the DNA is packaged. The processes of cleavage and packaging have been shown to be closely linked with the majority of terminal fragments being found to be DNase resistant i.e. packaged (Deiss & Frenkel, 1986). This conclusion is also supported by studies of HSV DNA packaging mutants which fail to cleave concatemeric DNA in the absence of DNA packaging. In HSV-1 unit length genomes generated by cleavage at distinct signals are packaged although the fact that not every correctly orientated packaging signal is cleaved implies an element of consideration for headful packaging. Herpesviruses also appear to have an additional control mechanism for aberrant packaging events. Capsids which contain less than unit length amounts of DNA have been observed trapped between the inner and outer nuclear membranes suggesting that they are blocked in their subsequent maturation (Vlazny *et al.,* 1982).

1.3.2.1 - Cleavage and packaging signals.

The 'a' sequence was proposed as the sequence responsible for directing the cleavage and packaging of the herpes simplex virus genome by Mocarski & Roizman (1982b), based on the observation that both unit length DNA molecules and defective genomes contained directly repeated 'a' sequences at their termini. The 'a' sequence was directly implicated in the cleavage and packaging process by Stow *et al.,* (1983) who showed for HSV-1 that a minimal 550 bp of the L or S termini contained all of the sequences necessary for the cleavage and packaging of the viral genome. The only sequence which is common to both the L and S termini are the 400 bp 'a' sequences.

A comparison of the termini of many different herpesviruses has shown that certain sequences are particularly well conserved (Figure 3.44). These sequences are part of the quasi unique regions of the 'a' sequence but they are also found at the termini of herpesviruses which lack 'a' sequences. The implication is that these sequences are involved in the cleavage and packaging process and subsequent experimental evidence has lead to these regions being labelled the pac1 and pac2 sequences (see section 1.2.2.7). The occurrence of these sequences at the termini of herpesviruses other than the herpes simplex viruses is consistent with observations which have shown that their termini are also involved in cleavage and packaging. Sequences found at the termini of HCMV (Spaete & Mocarski, 1985), EBV (Zimmermann & Hammerschmidt, 1995), MCMV (McVoy *et al.*, 1998), HHV-6 (Deng and Dewhurst, 1998), and GPCMV (McVoy *et al.,* 1997) have all been directly implicated in the cleavage and packaging process.

Interestingly, while in general, there is considerable similarity between the pac homologies from different herpesviruses, there are some notable examples of variation from the common theme. For example, the T rich element of pac2 is particularly well conserved in almost all of the herpesviruses for which sequences are available but it is absent from the pac2 homologue of the HCMV genome (Broll *et al.,* 1999). In GPCMV there appear to be two cleavage sites, one associated with an easily identifiable pac2 homologue, the other with a region which shares only partial homology with other pac2 sequences (McVoy *et al.,* 1997).

During the 1980s several investigators sought to functionally examine cleavage and packaging signals in infected cells. One of the observations that came from this work was that the sequence at the site of cleavage, in the DR1 repeat, was non essential (Varmuza & Smiley, 1985; Deiss & Frenkel, 1986). The non-essential nature of the DR1 repeat sequence was shown in a set of experiments in which an 'a' sequence fragment, which drove cleavage and packaging but did not contain a DR1 repeat, was inserted into the thymidine kinase gene of HSV-1. Sequencing of the novel termini which were generated by cleavage at this site

revealed that the accuracy and location of cleavage with respect to the putative cleavage signals in pac1 and pac2 was unaffected by the absence of the repeat sequence.

The first study which attempted to introduce specific mutations into the packaging signal was carried out by Deiss and Frenkel (1986) and was rapidly followed by a second study (Deiss *et al.,* 1986). These studies, using HSV-1 amplicons, specifically confirmed the importance of the pac1 and pac2 sequences in the cleavage and packaging process by observing that constructs lacking these sequences were not propagated in transfection derived virus stocks.

In 1988 the central role of pac1 and pac2 was conclusively shown when it was found that all of the signals necessary for the cleavage and packaging of HSV-1 were contained within a 179 bp fragment of viral DNA which spanned the junction between two tandemly repeated 'a' sequences (Nasseri & Mocarski, 1988). The structure of the fragment is such that it contains both the pac1 and pac2 elements that are present in the u_b and u_c regions of adjacent 'a' sequences, as well as a single DR1 repeat which separates them ($[DR4]_{0.6}$-u_c-DR1-u_b-$[DR2]_1$). This arrangement is of interest for several reasons, firstly, assuming that genome circularisation occurs by ligation, it would be predicted to occur at the novel junction formed by the termini, even in a genome containing the minimum number of 'a' sequences. Taking this to be true would suggest that *in vivo* the cleavage signal is presented as the junction between two 'a' sequences. Cleavage at a single 'a' sequence could only occur after an 'a' sequence duplication event. Secondly, the novel junction formed between two tandemly repeated 'a' sequences is the only arrangement in which the pac1 and pac2 sequences are brought close together. In a single 'a' sequence these sequences are as much as 400 bp apart. Finally a striking aspect of this study, was the finding that cleavage and packaging did not require the replication of the amplicon, implying that concatemeric DNA is not the sole substrate for DNA packaging. This observation does, however, remain unconfirmed.

One of the studies using whole 'a' sequences to drive amplicon DNA cleavage and packaging which was conducted during the 1980s showed that the HCMV 'a' sequence could act as a functional cleavage and packaging signal for the HSV-1 packaging machinery (Spaete & Mocarski, 1985). This is particularly interesting as the HCMV 'a' sequence lacks the pac2 T rich element which is particularly well conserved in other herpesviruses, implying that this element is not required for cleavage and packaging. In a similar way, a more recent study of the terminal sequences of GPCMV has provided an unexpected source of additional information. As described earlier GPCMV has three potential termini designated R, M and O. The absence of repeat sequences at the O terminus or any other regions of homology between the R and O termini would appear to suggest that genome circularization occurs by

direct ligation of the termini, as opposed to homologous recombination or strand annealing (McVoy *et al.*, 1997).

Despite the information which has been gathered about the sequences which are required for cleavage and packaging, there remains uncertainty in defining the actual arrangement of signals which forms the preferred cleavage and packaging signal *in vivo*. From the work carried out by Stow *et al.* (1983) it is apparent that amplicons containing single 'a' sequences can be packaged into viral particles, despite the fact that the essential pac1 and pac2 elements are approximately 400 bp apart. Conversely a fragment of viral DNA, as small as 179 bp, which spans the junction between two tandemly repeated 'a' sequences and has pac1 and pac2 in close proximity can also function as a cleavage and packaging signal (Nasseri & Mocarski, 1988). Although single 'a' sequences were reported to predominate in the concatemeric DNA of HSV-1 (Locker & Frenkel, 1979) it is difficult to explain how the termini of HSV-1, result from cleavage at single 'a' sequences. In order to account for this observation several mechanisms have been proposed including 'a' sequence duplication events (Deiss & Frenkel, 1986), staggered cleavage events and models for DNA packaging which involve discarding a large proportion of the replicated DNA which lacks a terminal 'a' sequence (Varmuza & Smiley, 1985 & Deiss *et al.*, 1986), but none of these models seems entirely satisfactory and to date it remains unclear which arrangement forms the preferred cleavage and packaging signal *in vivo*. Overall, the complementary portions of a DR1 repeat at the extreme ends of the genome and the presence of at least one 'a' sequence at both the L and S termini suggests that cleavage is more likely to occur in the DR1 repeat between two 'a' sequences. The novel junction contained within the 179 bp sequence constitutes the minimal cleavage and packaging signal so far identified and it is of interest to note, that it is only in this arrangement that the pac1 and pac2 sequences are brought into close proximity (figure 1.6).

In addition to the direct examination of cleavage and packaging several studies have been conducted looking for protein interactions with herpesvirus packaging signals. The reasoning behind this being the site specific cleavage of viral DNA will undoubtedly involve specific protein interactions. The first study was conducted using probes constructed from the 'a' sequence of HCMV. A protein from uninfected host cells which bound a probe containing most of the pac2 element, in a sequence specific manner was identified. The protein has an apparent molecular mass of 89 KDa and was found in a variety of human cell types. The protein was named pac2 binding protein (Pac2BP) (Kemble & Mocarski, 1989). The relevance of a host cell protein that binds to the cleavage and packaging signals lies in the observation that, in bacteriophages, host cell factors can play important roles in the packaging of viral genomes (Craig, 1985).

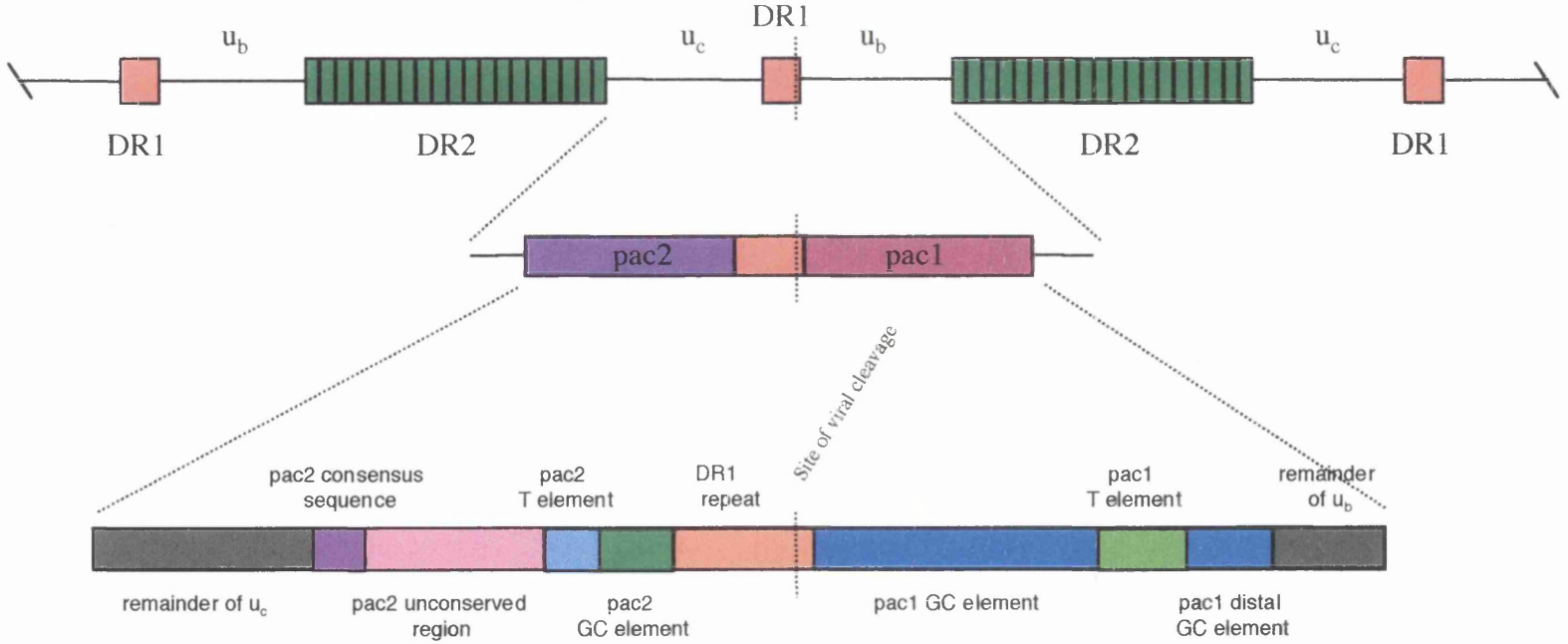


Figure 1.6 - HSV-1 strain 17 sequence arrangement at the junction between two tandemly repeated 'a' sequences.

This diagram shows a progressive expansion of the junction between two tandemly repeated 'a' sequences. This junction is the only arrangement in which the u_c and u_b sequences are brought into close proximity. The resulting sequence contains pac1 and pac2 sequences separated by a single DR1 repeat. The comparison of the termini from a variety of herpesviruses has enabled the pac sequences to be divided into a number of motifs (Deiss *et al.*, 1986). pac1 comprises a G/C rich elements of 43 and a GC rich sequence of 12 bp, separated by a T rich element of 8 bp. pac2, comprises a 7 bp consensus sequence (CGCCGCG), 31bp of unconserved sequence, a 6 bp T rich element and a 14 bp GC rich element.

Meanwhile, using HSV-1 sequences, another group isolated several sequence specific and non-specific protein complexes which formed with probes containing the pac2 element as well as parts of the DR1 and DR4 repeats. They identified two interesting complexes containing proteins from the nucleus of infected cells. The first complex contained two proteins, which were present in approximately equimolar amounts and their binding was shown to be sequence specific. One protein was identified as the virion protein ICP1 by its apparent molecular weight and by its reactivity with ICP1 specific monoclonal antibody. The second protein has an apparent molecular weight similar to that predicted for the virion protein ICP7.

The ICP1 protein, (UL36) has been implicated in the release of viral DNA from capsids. It is conceivable that this protein could be involved in escorting viral DNA in and out of the capsid (Batterson *et al.,* 1983). The identity of the second protein which remains tightly bound to ICP1 throughout the purification process remains to be confirmed (Chou & Roizman, 1989).

The second protein complex contained a single polypeptide which bound in a non-sequence specific manner. A variety of observations, including apparent molecular weight, catalytic activity and monoclonal antibody reactivity, identify this protein as the gene product of UL12. UL12 encodes the alkaline nuclease of HSV-1 and has both endonuclease and exonuclease activity. While the protein in this study was bound in a sequence non-specific manner it could theoretically cleave DNA in a sequence specific manner if it were part of a sequence specific complex (Chou & Roizman, 1989).

The HCMV homologue of HSV-1 UL28 (HCMV UL56) has also been reported to bind specifically to and cleave the HCMV 'a' sequence (Bogner *et al.,* 1998).

Interestingly, all of these reports represent single reports of sequence specific DNA binding activities but clearly reproducible data on this subject has not yet been provided.

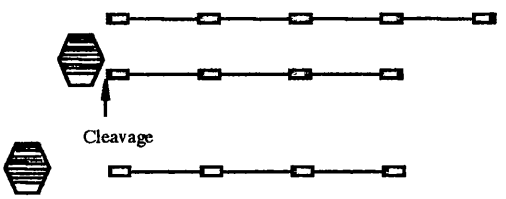
1.3.2.2 - Models for the production of unit length genomes from concatemeric DNA.

Several models for the cleavage and packaging process have been proposed in order to account for the varied observations regarding this part of the virus life cycle (Figure 1.7).

Varmuza and Smiley (1985) proposed two possible models for the cleavage of concatemeric DNA. The first, the 'staggered nick mechanism' proposed that the L and S termini arose as a product of two separate single stranded cleavages. They proposed that the cleavages are on opposite strands and at opposite ends of a single 'a' sequence. In this way each terminus would have a single-stranded 5' overhang the length of the 'a' sequence, these termini would need to be separated and then filled in by repair synthesis. For a tandem 'a'

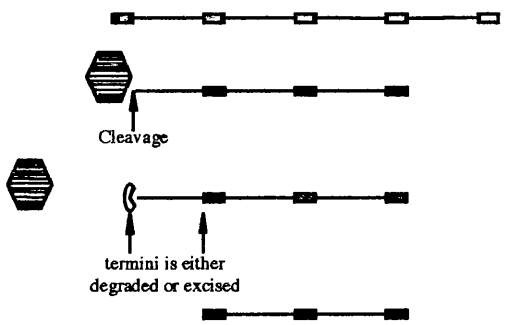
Figure 1.7 - Mechanisms for packaging herpesvirus genomes.

A.



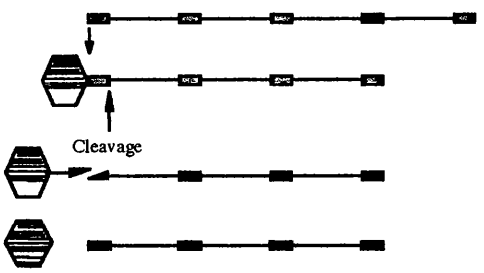
Defective genome packaging.
1. A concatemer of viral genomes is generated by rolling circle replication.
2. Packaging is initiated at an initial packaging signal and a unit length genome is packaged. The terminal cleavage is made before the terminal packaging signal giving rise to a packaged genome with only one terminal repeat.
3. The unpackaged concatemeric molecule is able to act as a substrate for subsequent cleavage and packaging events.
In herpesvirus other than GPCMV this would lead to defective virions

B.



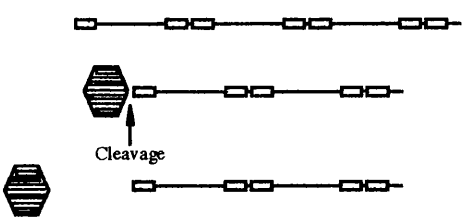
The theft model.
1. A concatemer of viral genomes is generated by rolling circle replication.
2. Packaging is initiated at an initial packaging signal and a unit length genome is packaged. The terminal cleavage is made after the terminal packaging signal.
3. A genome with two terminal repeats is packaged but the the unpackaged concatemer has an un-packageable terminus which must be either excised or degraded.

C.



'a' sequence duplication model.
1. A concatemer of viral genomes is generated by rolling circle replication.
2. Packaging is initiated at an initial packaging signal and a unit length genome is packaged. At the terminal signal, staggered nicks are introduced at either end of the repeat sequence. Repair synthesis then duplicates the terminal repeat.
3.. A genome with two terminal repeats is packaged and a packageable terminus is regenerated.

D.



'a-a' junction model.
1. A concatemer of viral genomes generated from a template containing a tandemly repeated packaging signal.
2. Packaging is initiated at an initial signal and a unit length genome is packaged. At the terminal packaging signal, a cleavage is made between the tandem repeats.
3.. A genome with two terminal repeats is packaged and a packageable terminus is regenerated.

sequence arrangement the situation would appear more simple, as a co-operative action between adjacent 'a' sequences could result in the nicks being positioned to generate a cohesive end of just a single base pair i.e. as found in virion DNA (Varmuza & Smiley, 1985). The subsequent work of Nasseri & Mocarski supports this co-operative mechanism by showing that the essential components of the packaging signal were found at the junction between two tandemly repeated 'a' sequences (Nasseri & Mocarski, 1988). The second potential mechanism proposed by Varmuza and Smiley, was the so called 'theft or sacrifice model'. This model requires that a double stranded cut be made at the L terminal 'a' sequence. This is followed by packaging until the next appropriately orientated L-S junction is found, where the S terminus is formed. This model's name is derived from the outcome of cleavage at a single 'a' sequence. If cleavage were to occur at a single 'a' sequence then that 'a' sequence would be 'stolen' from one genome at the cost of the adjacent one (Varmuza & Smiley, 1985). This model suffers from several problems, namely, that it predicts the existence of termini, which lack a terminal 'a' sequence. As these termini have not been detected they would need to have been rapidly degraded with the corresponding wastage of synthesised DNA. Neither of these two models is however, capable of explaining the observed distribution of 'a' sequences at the L (a_n) and S ($a_{n=1}$) termini.

In 1986 Deiss and Frenkel proposed an extension of the theft model, which they called the 'Modified Theft Model'. In this model a packaging complex is thought to traverse the HSV-1 DNA in either direction, starting from a random start point. They propose that cleavage occurs proximal to the first u_c element encountered. Packaging then continues until the next directly repeated junction is found. A second cleavage then occurs proximal to the u_b element (Deiss & Frenkel, 1986). This form of directional cleavage model can account for several observations, such as cleavage at directly repeated 'a' sequences and, providing that the scanning is US to UL, the observed distribution of 'a' sequences at the L (a_n) and S ($a_{n=1}$) termini.

Although there is still insufficient information on which to base a model for the translocation of herpesvirus genomes into the capsid, as with the bacteriophage systems which have been studied, the process appears to require ATP (Dasgupta & Wilson, 1999).

1.3.2.3 - Proteins implicated in the cleavage and packaging of HSV-1 DNA.

Several proteins or genes have been implicated in the cleavage and packaging process. All were identified by isolating temperature sensitive (ts) or deletion mutants, whose phenotype suggested that they had functions in this process. The proteins implicated so far are the gene products of UL6, UL12, UL15, UL17, UL25, UL28, UL32 and UL33. One other gene, UL36, may also be involved in packaging, although to date it has only been

implicated in the release of viral DNA from the nucleocapsid. In addition the structural proteins of the capsid and its scaffold are also obviously necessary for DNA to be packaged.

a) - UL6 .

UL6 is a γ' gene, indicating that it is expressed shortly after DNA replication commences. The gene encodes a protein with an apparent molecular weight of approximately 75 KDa and a study of its location revealed that it is tightly associated with the capsid proteins, most likely the hexons. The evidence suggesting that this protein is involved in cleavage and packaging comes from the study of a ts mutant tsF18. At the nonpermisive temperature (NPT) this mutant produces almost wild type levels of replicated DNA which is found as a high molecular weight concatemer. This mutant however, fails to cleave or package the DNA. B capsids also accumulate to near *wt* levels in the nucleus. The effect of this mutation can be reversed by a return to the permissive temperature (PT). There is however a requirement for *de novo* protein synthesis (Patel & McLean, 1995). This requirement for *de novo* protein synthesis may indicate that the mutant protein forms a stably inactive conformation at NPT, alternatively it may reflect a more complex situation associated with its location in the nucleocapsid. The isolation of a UL6$^-$ HSV-1 mutant has confirmed the essential nature of this protein for virus growth. This study also used a recombinant baculovirus system in which infected insect cells which expressed the seven known capsid proteins and UL6, were examined. In insect cells it was found that although UL6 was bound to the capsid, it was not necessary for capsid assembly, and in insect cells at least, UL6 binding to the capsid does not require any other viral proteins (Patel *et al.,* 1996).

b) - UL12.

The UL12 ORF of HSV-1 encodes a deoxyribonuclease which is often referred to as the alkaline nuclease because of its unusually high pH optimum (Keir & Gold, 1963). The UL12 ORF appears to encode two polypeptides designated UL12 and UL12.5 (Draper *et al.,* 1986). A deletion mutant of the UL12 locus has been shown to be capable of *wt* levels of DNA synthesis. Cleavage of the viral DNA occurs and levels of DNA packaging can be detected in the nucleus of infected cells. The capsids however, fail to egress from the nucleus suggesting that they or the DNA they contain may be aberrant in structure (Weller *et al.,* 1990; Shao *et al.,* 1993). The UL12 protein has a predicted molecular mass of 67.5 KDa, it has both endonuclease and exonuclease activity and localisation studies have shown that the nuclease locates in the nucleus, but at locations distinct from those associated with DNA replication, supporting the idea that the nuclease is not involved in DNA synthesis (Weller *et al.,* 1990 & Bronstein & Weber, 1996). The UL12.5 protein has an approximate molecular mass of 60 KDa and is transcribed, in the same frame as UL12, using a down

stream methionine start codon. UL12.5 has been shown to have similar activity, pH optimum and requirement for divalent cations as UL12 but unlike the UL12 protein it has been identified as a component of B and C capsids. Despite the fact that both proteins have similar activities UL12.5 cannot complement a UL12$^-$ virus (Bronstein *et al.*, 1997), indicating that it probably has a different biological function. It is tempting to suggest that the capsid associated UL12.5 is responsible for cleaving the viral DNA. There are however, several potential roles for the nucleases in the cleavage and packaging process. As in several other DNA viruses including bacteriophage T4, endonucleases have been shown to play a role in the resolution of intermediate structures generated by recombination prior to packaging. The UL12 protein has been implicated in the resolution of branched DNA structures which result from recombination between replicated concatemeric DNA. The importance of this role is seen by the fact that the viral yield of a UL12$^-$ virus is only 0.1-1.0% of *wt* levels (Martinez *et al.*, 1996). The fact that virus was replicated at all is important because it indicates that UL12 is not absolutely required for virus maturation, an observation which would be unexpected for a nuclease responsible for generating genomic termini. Never the less, despite the weakness of the endonuclytic activity, it remains possible that the nuclease does play a role in the cleavage between directly repeated 'a' sequences. UL12 remains a candidate for the protein which binds to ICP1 in an 'a' sequence specific complex (Chou and Roizman, 1989). Finally, several of the packaging models have a requirement for the rapid degradation of termini lacking 'a' sequences, the nuclease having both endo- and exonuclease activity may be able to perform this function (Weller *et al.*, 1990). One final point of note, regarding the alkaline nuclease, is that it is a phosphoprotein. A viral protein kinase has been identified which phosphorylates the nuclease *in vitro*. This serine threonine protein kinase, which is encoded by UL3, also has an alkaline pH optimum (Daikoku *et al.*, 1995).

In summary the UL12 gene appears more likely to have an indirect role in packaging. Its various phenotypes are likely to be the result of the inefficient removal of branched structures from DNA prior to encapsidation which results in the packaging of aberrant DNA structures.

c) - UL15.

The UL15 gene was implicated in DNA packaging through the characterisation of a ts mutant, ts66.4 (Poon & Roizman, 1993). ts66.4 accumulates high molecular weight DNA and B capsids at the NPT. The UL15 gene is highly conserved among distantly related herpesviruses and it has been shown to share limited homology, including a putative ATP binding domain, with gp17 of bacteriophage T4. gp17 is the small subunit of the T4 terminase enzyme (Weller *et al.*, 1987). UL15 is unusual in two respects, unlike most other

HSV-1 genes it is composed of two exons, which are separated by the coding region of two other genes and in addition it appears to encode multiple related polypeptides. The replacement of the two exons with a cDNA clone of the gene does not appear to affect the viability of the virus, suggesting that the splicing does not have a physiological role (Baines & Roizman, 1992). On the other hand the different polypeptides have been shown to have distinct properties.

The UL15 locus contains at least two independent ORFs. Several studies have identified two UL15 derived polypeptides of approximately 35 and 75 KDa. The 75 KDa (UL15) species is derived from the spliced 2.7 Kbp mRNA while the smaller 35 KDa species (UL15.5) is specified from the same reading frame of a 1.0 Kbp mRNA from the second exon (Baines *et al.*, 1997). UL15.5 has been shown to be independently expressed indicating that it is not a proteolytic cleavage product of the full length UL15. Localisation studies have shown that the location of the UL15 protein changes during the course of infection. At 6 hours the protein is found mainly in the perinuclear space however by 12 hours post infection the protein is found mainly in distinct nuclear structures (Baines *et al.*, 1994). A putative nuclear localisation signal is present in the protein which appears to explain its localisation. Subsequent studies have observed further forms of the UL15 protein, Proteins of 79, 80 and 83 KDa which react with UL15 specific antibodies have been found associated with B capsids. Interestingly though, in the absence of other proteins implicated in the cleavage and packaging process, only the 83 KDa form was observed associated with the capsids. This suggests that the 80 and 79 KDa forms are involved in aspects of the cleavage and packaging process which require interactions with other proteins.

Mutants which express UL15.5 but not U_L15, have also been found to accumulate high molecular weight DNA and B capsids. In several studies it was also observed that instead of accumulating in the nucleus or perinuclear space, B capsids were found in the cytoplasm (Baines *et al.*, 1997; Yu *et al.*, 1997). This observation indicates that UL15 plays a role in the mechanism which blocks aberrant capsids from further maturation (Vlazny *et al.*, 1982). Another study showed that a plasmid which encoded UL15 but not UL15.5 was able to complement a UL15/UL15.5 null virus, indicating that UL15.5 is non essential in cell culture. Another mutant containing a mutation into the putative ATP binding domain of UL15 was unable to complement a $UL15^-$ virus indicating that the putative ATP binding motif is indispensable for UL15 function (Yu & Weller, 1998a).

Finally UL15 has been found to make a functional interaction with UL28 (Koslowski *et al.*, 1999). The localisation of UL28 to the nucleus appears to be as a consequence of its interaction with UL15, as transiently expressed UL28 accumulates in the cytoplasm but fails to enter the nucleus in the absence of UL15. Conversely, the association of UL15 with B

capsids has been shown to potentially involve UL6, UL17 and / or UL28, (Yu & Weller, 1998b; Salmon & Baines 1998). The association of UL15 and UL28 with capsids has been observed for B capsids but not for mature C capsids or virions suggesting that the interaction is only a transient association as part of the cleavage and packaging process.

d) - UL17.

A UL17$^-$ virus has been identified which, as for the other packaging-impaired mutants, accumulates endless concatemeric DNA and B capsids in the nucleus (Salmon *et al.*, 1998). The mutant virus was subsequently used in a confocal immunofluorescence study which found that in the absence of UL17 the major and minor capsid proteins as well as UL6 were not targeted to the replication compartments as they were in *wt* infections. This not only suggests that UL17 is involved in the targeting of these proteins to the replication compartment but also implies that this is the site of virion assembly (Taus *et al.*, 1998). This observation conflicts with the observations of Ward *et al.* (1996) which were interpreted to indicate that replication and virion assembly occurred at discrete sites.

In a separate study UL17 was also shown to be involved, along with UL6 and UL28, in the localisation of UL15 with B capsids (Salmon and Baines, 1998).

e) - UL25.

The UL25 gene locus was implicated in the cleavage and packaging process prior to the publication of the full HSV-1 genome sequence. A ts mutant, ts1208, was isolated which had two distinguishable phenotypes. The first was that virions grown at the PT were found to be capable of strongly binding to the cell surface but were unable to penetrate at the NPT. Penetration could be facilitated by the addition of a fusogen or alternatively by a shift to the permissive temperature. The second phenotype was the accumulation of intermediate but not full capsids in the nucleus. The number of intermediate capsids was however lower than in other packaging mutants. This second phenotype suggests that the UL25 gene product is involved in the cleavage and packaging process (Addison *et al.*, 1984). More recently the UL25 protein has been expressed in *E. coli* as a GST fusion protein. The identity of the over-expressed protein was confirmed by immunoprecipitation with an antibody raised against viral UL25. The kinetics of expression were examined revealing that UL25 is expressed as a late or leaky late gene in the HSV-1 life cycle. The protein has also been identified as a virion component, probably as a tegument protein (Ali *et al.*, 1996). A UL25 mutant virus, which does not express the UL25 protein, due to the insertion of an inframe stop codon, successfully uncouples the cleavage and packaging processes. The mutant accumulates both A and B capsids in the nuclei but no C capsids. In addition pulse

field gel electrophoresis has also identified genome-sized viral DNA which is DNase sensitive, i.e. unpackaged, in infected cell extracts. These observations indicate that UL25 is required for encapsidation, or at least the retention of capsid DNA, but not for cleavage of viral concatemers (McNab *et al.,* 1998). It is thought that the packaging defect in UL25 mutants relates to their failure to retain packaged DNA within the capsid.

f) - UL28.

A role for UL28 in DNA packaging was also identified using a temperature sensitive mutant. The phenotype of the ts mutant at the NPT is similar to the other mutants, high molecular weight DNA accumulating in the nucleus along with B capsids. UL28 encodes the protein ICP18.5. Its molecular mass is predicted to be 85.5 KDa. UL28 like UL15 appears to be highly conserved between the herpesviruses (Addison *et al.,* 1990; Tengelsen *et al.,* 1993). The UL28 protein has been identified as a minor component of B capsids and its interaction with the capsid is strong, even in the absence of other packaging proteins which suggests that UL28 interacts directly with the capsid proteins. Despite this strong interaction with B capsids less UL28 protein is associated with C capsids and it is undetectable in virions. This distribution suggests that UL28 is lost during the capsid maturation (Taus & Baines, 1998; Yu & Weller, 1998b). In addition to its interaction with B capsids, as described above, the UL28 protein has also been found to have a functional interaction with UL15. This interaction is currently thought to serve as a means of localising UL28 in the nucleus but as UL28 binding to the capsid appears to be at least partly responsible for UL15 association with B capsids an extension to the role of the interaction cannot be ruled out (Yu & Weller, 1998b; Koslowski *et al.,* 1999). By analogy with the bacteriophage terminases, and considering the homology between UL15 and the large subunit of the T4 Terminase, it is possible that UL28 represents the second subunit of an HSV-1 terminase. It is also interesting to note that this interaction has been observed between the HCMV homologs of UL15 and 28, suggesting that it is conserved in distantly related viruses (Krosky *et al.,* 1998).

g) - UL32.

The UL32 locus has been implicated in DNA cleavage and packaging (Schaffer *et al.,* 1973; Sherman & Bachenheimer, 1987) and at one time it was also thought to be involved in the maturation and translocation of viral glycoproteins (Machtiger *et al.,* 1980), but this is now thought not to be the case. The product of the UL32 ORF has been identified as a 67 KDa protein which accumulates in the nucleus of infected cells. The protein has also been

shown to bind zinc *in vitro*. Based on the predicted sequence of the protein it had been suggested that UL32 was a membrane bound aspartyl protease but its accumulation in the cytoplasm and mutagenesis of the putative aspartyl protease motif has shown that this is not the case (Chang *et al.*, 1996). The function of UL32 has subsequently been more clearly defined using confocal immunofluorescence microscopy (Lamberti & Weller, 1998). This study showed that while UL32 staining was predominantly in the cytoplasm of infected cells, there was also some nuclear staining, which localised with the replication compartments. In addition it was observed that the localisation of the major capsid protein VP5 was also affected by UL32. In the presence of UL32 VP5 was also observed in the replication compartment but during infections with a UL32 mutant VP5 exhibited a more diffuse nuclear localisation. This result suggests that UL32 plays a role in the correct localisation of capsids within infected nuclei.

h) - UL33.

The importance of UL33 in DNA packaging was again identified using a ts mutant, this time ts1233. The capsids formed at the NPT were intermediate capsids which lacked dense internal structures. DNA was again observed in a high molecular weight form in the absence of genome termini. This mutant is caused by a single point mutation and, again, a downshift to the PT also requires *de novo* protein synthesis to restore virus replication (Al-Kobaisi *et al.*, 1991).

i) - UL36.

As mentioned earlier the UL36 gene has been implicated in the release of viral DNA from capsids (Batterson *et al.*, 1983). Analysis of the UL36 open reading frame confirmed that it coded for the infected cell protein 1 (ICP1). ICP1 is a 270 KDa phospho-protein which is found in the tegument of mature virions. Approximately 150 copies of the protein are found tightly associated with each nucleocapsid. The tight association is likely to explain how the protein can affect genome release after the nucleocapsid has been transported to the nucleus. Computer-assisted analysis of the predicted amino acid sequence of ICP1 has revealed several putative domains; two leucine zipper domains and two ATP binding domains. Leucine zipper domains have previously been seen in the formation of homo and hetero dimers in other DNA binding proteins e.g. c-jun. The formation of a DNA binding hetero dimer which involves ICP1 has already been described. The ATP binding sites are arranged into a pocket which could potentially provide a local environment for ATP binding and hydrolysis (McNabb & Courtney, 1992). The functional significance of these predictions has yet to been shown experimentally, however it is interesting to speculate that

these domains may be involved in the formation of a packaging complex and the hydrolysis of ATP in order to provide the necessary energy for the encapsidation of HSV-1 DNA.

This list is not exhaustive as many other proteins have indirect effects upon packaging. For example mutants of the capsid and scaffold proteins, which do not assemble viable capsids are impaired for DNA packaging (Weller *et al.,* 1987). This list does however, include those viral proteins which appear to have central roles in the cleavage and packaging process.

Chapter 2: Materials and Methods.

Section 2.1 Materials.

2.1.1 Chemicals and reagents.

Most analytical grade chemicals were purchased from Sigma Chemical Co. Ltd. The exceptions are listed below :

BDH Laboratory Supplies	- DMSO
	- DMF
Bio-Rad Laboratories	- Ammonium persulphate
	-TEMED (N,N,N',N'tetramethylethylenediamine)
SmithKline Beecham Research	- Ampicillin (Penbritin)
Melford Laboratories Ltd	- Caesium chloride
Joseph Mills (Denaturants) Ltd	- Absolute alcohol 100
Pharmacia Biotech	- dATP, dTTP, dGTP & dCTP
Fluka Chemical ltd.	- Formaldehyde
Gibco BRL	- Acrylamide
	- Acrylamide : N, N'-methylene-bis-acrylamide 19:1
Fisher Scientific UK Ltd	- Ammonia solution
Pierce	- Surfact-Amps∏ NP 40

2.1.2 Miscellaneous materials.

The suppliers of miscellaneous materials were as follows:

Amersham International Plc	- Hybond -N membrane (0.45μm)
Gibco BRL	- LipofectAce∏
Whatman International Ltd.	- Whatman 3 mm paper
Medicell International Ltd.	- Dialysis tubing
Kodak Ltd	- X-omat S film
Molecular Dynamics	- Phosphorimager screens
Pharmacia Ltd	- Sephadex G-50

1.3 Solutions.

Table 2.1 - Solutions.

Solution	Composition
β-galactosidase fix	2% formaldehyde, 0.2% glutaraldehyde in PBS
β-galactosidase stain	5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.5 mg ml^{-1} X-Gal, in PBS
blot wash	2 x SSC, 0.1% SDS
calf thymus DNA (denatured)	2 mg ml^{-1} in TE pre-incubated @ 100°C / 30 minutes.
calf thymus DNA (transfections)	3 mg ml^{-1} in TE
chloroform : isoamyl alcohol	chloroform : isoamyl alcohol 24:1 v/v
CLB (2x)	10 mM Tris.HCl (pH 7.5), 1 mM EDTA, 0.6% w/v SDS
Denhardt's solution (50x)	1% ficoll 400, 1% polyvinylpyrolidone, 1% BSA
DNase dilution buffer	50% glycerol, 50 mM NaCl
gel soak I	0.6 M NaCl, 0.2 M NaOH
gel soak II	0.6 M NaCl, 1.0 M Tris.HCl (pH 8.0)
H-mix	6 x SSC, 5 x Denhardt's solution, 0.05% SDS, 50 μg ml^{-1} denatured CT DNA, 20 mM Tris.HCl (pH 7.5), 1 mM EDTA
HeBS	137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM D-glucose, 21 mM Hepes, pH 6.95-7.15 with NaOH
Loening's buffer	40 mM Na_2PO_4, 36 mM Tris.HCl, 1 mM EDTA
loading buffer dyes	50% w/v Sucrose, 0.25% bromophenol blue, in 5x running buffer
methyl cellulose	3% carboxymethylcellulose sodium salt in H_2O
nick translation (NT) buffer (10x)	0.5 M Tris.HCl (pH 7.5), 0.1 M $MgCl_2$, 10 mM DTT, 0.5 mg ml^{-1} BSA
oligonucleotide elution buffer	0.1% w/v SDS, 0.5 M ammonium acetate, 10 mM magnesium acetate

PBS ABC (complete)	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 6.8 mM $CaCl_2$, 4.9 mM $MgCl_2$
PEG precipitation buffer	20% PEG, 2.5 M NaCl
Pol/Lig buffer (5x)	50 mM $MgCl_2$, 100 mM Tris.HCl (pH 8.0), 10 mM DTT, 2.5 mM dNTPs, 5 mM ATP
pre-hybridisation buffer	6 X SSC, 5 x denhardts solution, 0.1 % SDS, 20 $\mu g\ ml^{-1}$ denatured CT DNA
RFI	100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% v/v glycerol, pH 5.8
RFII	10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, pH 6.8
RSB	10 mM Tris.HCl (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$
SOB	2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$
SOC	20 mM D-glucose in SOB
SSC (20x)	3 M NaCl, 0.3 M tri-sodium citrate
STET buffer	8% w/v sucrose, 0.5% v/v Triton X-100, 50 mM EDTA, 10 mM Tris base
sucrose reagent	0.25M sucrose, 2 mM $MgCl_2$, 50 mM Tris.HCl (pH 8.0)
TE	10 mM Tris.HCl (pH 7.5 or 8.0) 1 mM EDTA
TAE (1x)	40 mM Tris.acetate, 1 mM EDTA
TBE (1x)	90 mM Tris base, 89 mM boric acid, 1 mM EDTA
TM buffer	200 mM Tris.HCl (pH 8.0), 20 mM $MgCl_2$
Triton reagent	0.5% Triton X-100, 62.5 mM EDTA, 50 mM Tris.HCl (pH 8.0)
trypsin	0.25% trypsin in Tris saline
TS	137 mM NaCl, 0.7 mM Na_2HPO_4, 5 mM KCl, 1 mg ml^{-1} dextrose, 100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin
TSB	10 mM $MgCl_2$, 10 mM $MgSO_4$, 10% w/v PEG 4000, 5% v/v DMSO, in L-broth

TSB / glucose	20 mM D-glucose in TSB
versene	0.6 μM EDTA, 0.02% phenol red in PBS

2.1.4 Enzymes.

Unless otherwise stated restriction enzymes were supplied by **New England Biolabs (NEB)** or **Boehringer Mannheim**. Other enzymes are listed below :

Sigma Chemical Co. Ltd.	- Protease XIV (pronase from *S.griseus*)
	- Lysozyme (from chicken egg whites)
	- RNase T_I (grade IV)
	- RNase A (type A1)
	- DNase I
New England Biolabs	- DNA polymerase I
	- T7 DNA polymerase
	- T4 DNA Ligase
Boehringer Mannheim	- Calf intestinal phosphatase (alkaline phosphatase)

2.1.5 Radiochemicals.

All radiochemicals were supplied by Amersham International plc.

5' [α-^{32}P] deoxy ribonucleoside triphosphates @ 3000 Ci mmol^{-1} (10 μCi μl^{-1})

2.1.6 Cells and culture media.

Baby hamster kidney 21 clone 13 (BHK-21 C13) cells (Macpherson & Stoker, 1962) were used for almost all of the experimental work including the preparation of virus stocks. BHK-21 C13 stocks were obtained from the Institute cytology department and stored at -190°C.

Vero cells (Rhim & Schell, 1967) were also obtained from the Institute cytology department stocks.

The following reagents were used in the growth, maintenance and storage of cells :

Glasgow modified Eagle's Medium (GMEM)	- Supplied by **Gibco BRL**
wash	- GMEM plus 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin
EC5	- wash plus 5% NBCS
ETC10 (Vero)	- wash plus 10% FCS and 7% tryptose phosphate broth

ETC10 (BHK)	- wash plus 10% NBCS and 7% tryptose phosphate broth
BHK storage media	- wash plus 20% NBCS and 10% glycerol
methyl cellulose overlay	- 1.5% methyl cellulose, 0.9x wash supplemented with 5% NBCS, sodium bicarbonate and L-glutamine
Optimem	- Supplied by **Gibco BRL**

2.1.7 Viruses.

Two herpes viruses were used in the course of the experimental work :
Herpes simplex virus type 1 (HSV-1) strain 17+ (McGeoch *et al.,* 1985; McGeoch *et al.,* 1988).
Herpes simplex virus type 2 (HSV-2) strain HG52 (Dolan *et al.,* 1998)
Both of these viruses were obtained from the Institute of Virology stocks.
Bacteriophage R408 (Russel *et al.* 1986) was used during the mutagenesis.

2.1.8 Bacterial strains and culture media.

Plasmids were manipulated and propagated in *E.coli* strain DH5α (Hanahan, 1985). As part of the site directed mutagenesis protocol two different strains of *E. coli* were used. Strain XL1-BLUE was used to propagate the R408 bacteriophage and strain CJ236 was used to prepare the uridine rich ssDNA. The CJ236 bacteria have the genotype *F' cat(=pCJ105;M13^{s}Cm^{r}/dut ung^{-1} thi-1 relA1 spoT1 mcrA)* which means that their DNA contains an increased frequency of incorporated uridine in place of thymidine residues.

For high efficiency transformations, Library Efficiency∏ DH5 competent cells were used. (Gibco BRL).

All bacteria were grown in L-broth, supplemented with 50 μg ml^{-1} ampicillin and 0.1 mg ml^{-1} chloramphenicol when appropriate.

Bacteria containing plasmids were stored at -70°C in growth media supplemented with 7% DMSO.

2.1.9 Plasmids.

The parental plasmid vector used in this work was pAT153 (Twigg and Sheratt, 1980). pAT153 is a high copy number plasmid derived from pBR322. It contains ampicillin and tetracycline resistance loci. The *Eco*RI, *Hin*dIII and *Bam*HI sites, were used in the construction of the plasmids. pAT153 is the parent of all the following plasmids except pE*LacZ* and the pTZ based plasmids.

The following plasmids were provided by Dr N.D.Stow.

Table 2.2 - Parent plasmids.

Plasmid Name	Description
pS1	pAT153 with a 500 bp HSV-1 Ori_S fragment cloned into the *Bam*HI site (Stow and McMonagle, 1983).
pY1	pS1 with a 1762 bp HSV-1 fragment (125644-127405)containing a single 'a' sequence (Stow *et al.*, 1983). The insert was cloned between the *Eco*RI and *Hin*dIII sites.
pZ1	pS1 with a 2161 bp HSV-1 fragment (125644-127405) containing two tandemly repeated 'a' sequences (Stow *et al.*, 1983). The insert was cloned between the *Eco*RI and *Hin*dIII sites.
pSA1	pS1 with a novel 200 bp HSV-1 sequence containing the junction between two tandemly repeated 'a' sequences (u_c-DR1-u_b fragment) (see figure 3.31). The insert was cloned between the *Eco*RI and *Hin*dIII sites.
pE*LacZ*	pCMV10 containing the *LacZ* gene under the regulation of the CMV major immediate early promoter.
pTZ18U	An f1 origin containing plasmid based on pUC19. pUC19 contains ampicillin resistance loci and an MCS, as well as universal and reverse sequencing primer sites.

The numbers shown in brackets for pY1 and pZ1 indicate the location of the *Hin*fI restriction enzyme sites used to clone these fragments (McGeoch *et al.*, 1988)

u_c-DR1-u_b mutants were prepared and sequenced in pTZ18U derived plasmids (pTZ series of plasmids). The *Eco*RI / *Hin*dIII inserts were transferred to a pS1 backbone to derive mutant amplicons (pPH series of plasmids). Both the pTZ derived plasmid and the mutant amplicon produced are listed together in the table below. Further details are provided in the results section.

Table 2.3 - Mutant Plasmids

Mutation, pTZ and pPH number	Description
2	*wt* HSV-1 stain 17 u_c-DR1-u_b fragment
3	pac2 deletion
4	pac1 deletion
6	DR1 substitution
7	pac1 T element substitution
8	pac1 T element deletion
9	pac2 T element substitution
10	pac2 T element deletion
11	pac1 GC distal element deletion
12	pac1 GC distal element substitution
15.1	pac2 GC element deletion
15.2	pac2 GC element deletion + other pac2 deletion
16	pac2 GC element substitution
17	pac2 consensus deletion
18	pac2 consensus substitution
19	pac2 unconserved deletion
20	pac2 unconserved substitution
21	pac1 proximal GC element deletion
22	pac1 proximal GC element substitution

2.1.9 Oligonucleotides.

The sequences of the mutagenic oligonucleotides used in the site directed mutagenesis are shown below :

Table 2.4 - Oligonucleotides.

Mutation number	Mutation	Oligonucleotide sequences
6	DR1 substitution	CGCGCCCGCGGGGGGCAATAGTACTCCGCCGCCACCGCTT
7	pac1 T element substitution	GCGACCCCCGGGGGGGCTCGAGGGGGGGGGGCCCGTTT
8	pac1 T element deletion	GCGACCCCCGGGGGGGGGGGGGCCCGTTT
9	pac2 T element substitution	GGACCGCCGCCCGCCCTCGAGGCGCGCGCGCGCC
10	pac2 T element deletion	GGACCGCCGCCCGCCGCGCGCGCGCC
11	pac1 GC distal element deletion	CGGGGGGTGTGTTTTTTTTCGGCGTCTGGC
12	pac1 GC distal element substitution	CGGGGGGTGTGTTTTCCCAGTACTGCCTTTTCGGCGTCTGGC
15	pac2 GC element deletion	CCGCCCGCCTTTTTTCCGCGGGGGGCCCGG
16	pac2 GC element substitution	CCGCCCGCCTTTTTTCGAGTACTCACGTACCGCGGGGGGCCCGG
17	pac2 consensus deletion	CCCGCCCCCCACGCCCGCGCGCACGCCGCC
18	pac2 consensus substitution	CCCGCCCCCCACGCCGAGTACTCGCGCGCACGCCGCC
19	pac2 unconserved deletion	CCCCACGCCCGCCGCGTTTTTTGCGCGCGCGC
20	pac2 unconserved substitution	CCCCACGCCCGCCGCGGACGTAACAAGCACATCTCGAGGTGTGAGGGTTTTTTGCGCGCGCGC
21	pac1 proximal GC element deletion	GGGGGGCCCGGGCTGCTGTGTTTTGGGGGGGGC
22	pac1 proximal GC element substitution	GGGGGGCCCGGGCTGCTTGTAGGGGATGAGGTCTCCCCTGGCGCACAGCTCGAGTACATTGTGTTTTGGGGGGGGC

For a comparison of the mutation-containing oligonucleotides and the viral sequence see Table 3.6 in the results section.

Section 2.2 METHODS.

Unless otherwise stated BHK and Vero cells were incubated throughout at 37°C in an atmosphere supplemented with 5% CO_2.

2.2.1 - Tissue culture and preparation of virus stocks.

2.2.1.1 - Serial passage of cells.

BHK and Vero cells were passaged in ETC10(BHK) and ETC10(Vero) respectively. 175 cm^2 tissue culture flasks were inoculated with not less than $1.5x10^6$ cells. Confluent monolayers were washed with versene (20 ml) and then with versene : trypsin (1:1 v/v) before being resuspended in 10 ml of the appropriate ETC10. Cells were occasionally stored o/n at 4°C, more usually however, an aliquot of resuspended cells was used immediately to seed another 175 cm^2 flask. Cells were discarded after approximately 30 passages.

2.2.1.2 - Long term storage of BHK cells.

The cells from an 80% confluent 175 cm^2 flask were resuspended in 9 ml of BHK storage medium. The resuspended cells were then divided into 1 ml aliquots, cooled slowly to -70°C before being transferred to liquid nitrogen storage (-190°C)

The cells were recovered from long term storage by thawing, pelleting the cells (1000 rpm, Beckman GPR centrifuge for 5 minutes) and resuspending the pellet in fresh ETC10 prior to inoculating a fresh 175 cm^2 flask.

2.2.1.3 - Preparation of *wt* stocks of HSV-1 and HSV-2.

BHK monolayers (approximately 80% confluent) in 175 cm^2 culture flasks were infected with approximately $4x10^6$ pfu virus in 4 ml of EC5. The virus was allowed to adsorb for 1 hour at 37°C before 40 ml of EC5 was then added. The flasks were then returned to 37°C for between 3 and 5 days. Once extensive cpe was evident the cells were harvested using sterile glass beads. The cells were spun down at 2000 rpm (Beckman GPR centrifuge) for 2 minutes and the supernatant decanted. In the preparation of HSV-1 the supernatant was spun at 4°C at 12,000 rpm for 240 minutes (Sorvall GSA rotor). The pellet was resuspended in 10 ml EC5, sonicated and stored at -70°C as cell released virus (CRV) stock. The HSV-2 supernatant was discarded.

The pelleted cells from the first spin were resuspended in 5 ml EC5 and sonicated extensively. The cell debris was pelleted at 2000 rpm for 5 minutes (Beckman GPR

centrifuge) and the supernatant was decanted and stored at -70°C as cell associated virus (CAV) stock.

2.2.1.4 - Titration of HSV-1 and HSV-2.

Virus titrations were carried out on 90-95% confluent BHK cell monolayers in 35 mm dishes. The plates were inoculated with 0.1 ml of virus stock dilution of 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}. The virus was allowed to adsorb for 1 hour at 37°C before being overlaid with methyl cellulose overlay to prevent secondary plaque formation. Incubation was continued at 37°C for 3 days. The monolayers were fixed and stained with giemsa stain for 24 hours at 4°C. The stain was removed with running water and the plaques counted using a dissecting microscope.

2.2.1.5 - Sterility checks.

Sterility checks were carried out by streaking BHK or virus stocks onto blood agar plates. The plates were then incubated at 37°C for up to 5 days.

2.2.1.6 - Preparation of R408 bacteriophage.

A single R408 plaque on an *E. coli* (XL1-Blue) lawn was picked into 1.5 ml of L-broth containing 20 µl of *E. coli* (XL1-Blue) overnight culture. The phage culture was incubated in an orbital shaker overnight at 37°C. After approximately 16 hours 2 ml of fresh *E. coli* (XL1-Blue) culture and the phage overnight culture were added to 100 ml of L-broth and incubated at 37°C in an orbital shaker. After 24 hours the remaining bacteria in the culture were heat inactivated at 55°C for 1 hour and the culture was centrifuged at 5000 rpm for 10 minutes (Sorvall GSA rotor). The supernatant was then decanted and re-spun as described above. The supernatant from this final spin was divided into aliquots and stored at 4°C as bacteriophage stock.

2.2.1.7 - Titration of R408 bacteriophage.

Serial dilutions of bacteriophage stock were made (10^{-5}-10^{-7}). 100 µl of each dilution was plated out onto L-broth agar plate. After being allowed to adsorb the phage was overlaid with 3 ml of top agar containing 200 µl of *E.coli* (XL1-Blue) overnight culture.

Plates were incubated overnight at 37°C and plaques counted the following day.

2.2.2 - Preparation of plasmid DNA.

2.2.2.1 - Large scale plasmid preparation (CsCl banding).

5 ml overnight cultures containing appropriate antibiotics were inoculated with a single colony picked from an LB-agar plate or 10 µl of bacterial stock (stored in 7% DMSO at -70°C). Cultures were shaken overnight at 37°C and then used to inoculate 400 ml LB cultures in 2 litre conical flasks, again containing appropriate antibiotics. 400 ml cultures were grown to an A_{600} of 0.7 and 1 ml chloramphenicol (34 mg ml^{-1}) was added. Incubation was continued overnight at 37°C. After approximately 16 hours the bacteria were pelleted at 8000 rpm for 10 minutes at 4°C (Sorvall GS3 rotor). The bacteria were washed with 8 ml TE (pH 7.5) and re-pelleted at 5000 rpm for 5 minutes 4°C (Sorvall GS3 rotor). The pellet was resuspended in 2 ml sucrose reagent, 400 µl of fresh lyzozyme (20 mg ml^{-1}) was added and the reaction was incubated at 4°C for 30 minutes. 800 µl EDTA (0.25M) and 3.2 ml triton reagent was then added and the lysis reaction was returned to 4°C for a further 15 minutes.

The lysed bacteria were spun at 35,000 rpm for 30 minutes, 4°C (Sorvall T865). The supernatant was decanted and its volume adjusted to 7.5 ml using 200 µl EtBr (10 mg ml^{-1}) and distilled water. 7.5 g of CsCl was then added to give a final density of 1.55-1.60 g ml^{-1}. The mixture was transferred to cellulose nitrate tubes and capped with paraffin oil. Tubes were centrifuged at 44,000 rpm 15°C for 36 hours (Sorvall T1270 rotor). The supercoiled DNA band in the gradient was extracted using a hypodermic needle and a 2 ml syringe. The ethidium bromide was removed by repeated extraction with 1.5 ml of isoamyl alcohol and the CsCl removed by dialysing the DNA preparation against 2 x 2 litres of TE (pH 7.5). Plasmid DNA preparations were stored at -20°C.

The concentration of the DNA was established by measuring UV absorbance at 260 nm (an absorbance of 1.0 corresponds to 50 µg ml^{-1} dsDNA). The quality of the DNA was examined by measuring its absorbance at 280 nm and by running 0.5 µg of linearised DNA on a 0.8% agarose minigel. Generally the A260 : A280 ratios were close to 2.0.

2.2.2.2 - Qiagen preparation.

DNA for ds plasmid sequencing was prepared using the Qiagen plasmid midi kit (**Qiagen**).

5 ml LB cultures containing the appropriate antibiotic were inoculated with either a single colony picked from an LB-agar plate or 10 µl of bacterial stock (stored in 7% DMSO at -70°C) and incubated overnight. 1 ml of overnight culture was added to 100 ml of LB media containing the appropriate antibiotic. The culture was grown to an A_{600} of

approximately 1.0 and 0.3 ml chloramphenicol (34 mg ml^{-1}) was added. The culture was incubated at 37°C for a further 16 hours, the cells were pelleted at 9000 rpm (Sorvall GSA rotor) for 15 minutes at 4°C and resuspended in 4 ml buffer P1. 4 ml buffer P2 was then added. After 5 minutes incubation at room temperature 4 ml of buffer P3 was added and the incubation continued for 15 minutes on ice. The bacterial lysate was centrifuged at 10,000 rpm for 30 minutes at 4°C (Sorvall SS34 rotor). The supernatant was decanted and re-spun at 10,000 rpm for 15 minutes, 4°C. Meanwhile a Qiagen Tip-100 was equilibrated with buffer QBT. The supernatant from the bacterial lysate was applied to the tip which was then washed with 2 x 10 ml buffer QC. The plasmid was eluted from the column with 5 ml of buffer QF and the DNA precipitated with 3.5 ml of isopropanol. The precipitate was spun at 10,000 rpm for 30 minutes at 4°C (Sorvall SM24 rotor). The pelleted DNA was washed twice with 70% ethanol and resuspended in dH_2O. The DNA yield and quality were determined as described in section 2.2.2.1

2.2.2.3 - Small scale plasmid preparation (miniprep) (Sambrook *et al.*, 1989).

5 ml LB cultures, containing the appropriate antibiotic, were inoculated with either a single bacterial colony picked from an LB agar plate or 10µl of bacterial stock (stored in 7% DMSO at -70°C and shaken overnight at 37°C. 1 ml was removed and the cells pelleted at 13,000 rpm for 1 min (MSE microfuge). The supernatant was discarded and the bacterial pellet was resuspended in 100 µl STET buffer. 16 µl of fresh lysozyme (10 mg ml^{-1}) was added for 30 seconds and the samples were boiled for 1 min. After boiling the lysed bacteria were immediately centrifuged at 13,000 rpm for 10 minutes (MSE microfuge). The soft lysis pellet was removed and the DNA precipitated with 100 µl of isopropanol for 5 minutes at -20°C. The DNA was pelleted at 13,000 rpm for 10 minutes (MSE microfuge) and resuspended in 100 µl TE (pH 8.0) containing 1x RNase mix.

Miniprep DNA was stored at -20°C and 30 µl aliquots were used for restriction enzyme digests.

2.2.3 - Transfection of cells with plasmid DNA.

2.2.3.1 - Calcium phosphate transfection of BHK cells.

1 ml of HeBS (optimised pH) was mixed with 0.25-6 µg of plasmid DNA, an optimised amount of calf thymus DNA usually 12-16 µg and 70 µl, 2M $CaCl_2$. The mixture was vortexed and allowed to precipitate at room temperature for 5 minutes. Confluent BHK

cell monolayers in 35 mm dishes (set up the previous day) were washed and drained prior to adding 0.4 ml of precipitate. The monolayers were incubated at 37°C for 40 minutes, overlaid with 3 ml EC5 and returned to 37°C. 4 hours post transfection the cells were washed with 2 ml of wash and treated with 2 ml 25% DMSO in HeBS for 4 minutes. The monolayers were then washed to remove the DMSO and overlaid with 2 ml EC5 before being returned to the incubator at 37°C/5% CO^2.

2.2.3.2 - Lipofection of BHK cells.

Approximately $1.5x10^5$ BHK cells were used to seed 35 mm plates 24 hours prior to lipofection yielding approximately 25% confluent monolayers.

0.25-6 µg of plasmid DNA was mixed with 0.5 ml Optimem. 15 µl of LipofectACE∏ was mixed with 0.5 ml Optimem in a separate tube. The DNA and liposomes were mixed and allowed to stand at room temperature for 10 minutes. Meanwhile the monolayers were washed with Optimem and drained. The liposomes / DNA mix was then added to the monolayer and the plates incubated at 37°C. 4 hours post transfection 2 ml of EC5 was added to each plate.

2.2.4 - Staining Monolayers.

2.2.4.1 - Staining for β-galactosidase expression in transfected cells

Monolayers transfected with the plasmid pE*LacZ* were stained for expression of β-galactosidase as an indicator of transfection efficiency. 24 hours post transfection monolayers were washed in PBS/ABC then fixed with β-galactosidase fix for 5 minutes. The fix was removed by washing with PBS/ABC. 1 ml of β-galactosidase staining solution was applied to each plate and the cells were incubated at 37°C for 24 hours. Cells expressing β-galactosidase stain blue. The number of blue cells were counted and the percentage of positive cells calculated.

2.2.5 - Transient replication and packaging assay.

2.2.5.1 - Transient replication assay.

a) Transfection / superinfection protocol.

BHK-21 C13 monolayers in 35 mm dishes were transfected with 3 µg of plasmid DNA using either the calcium phosphate or lipofection procedures described in section

2.2.3. 6 hours post-transfection the cells were infected with *wt*HSV-1 strain 17, at an MOI of 10.0. The virus was allowed to adsorb for 1 hour before being overlaid with 2 ml of EC5. Monolayers were maintained at 37°C throughout the assay protocol.

b) Preparation of total cellular DNA.

16 hours post-infection the medium was removed from the transfected monolayers, 2 ml of 1 x CLB containing 0.5 mg ml^{-1} protease was added and the plates were incubated at 37°C for 6 hours. The nucleic acids were then sequentially extracted with phenol and chloroform : isoamyl alcohol, precipitated with 2.5 volumes of ethanol and resuspended in 150 µl TE pH 8.0 containing 1 x RNase mix.

30 µl of the resuspended DNA (1/5th plate) was digested with *Eco*RI and *Dpn*I. The products were resolved on a large 0.8% agarose gel in 1x Loening's buffer and the presence of replicated plasmid sequences was detected by Southern blotting and hybridisation to a ^{32}P labelled probe.

2.2.5.2 - Transient packaging assay.

a) Transfection / superinfection protocol (used in initial experiments).

BHK-21 C13 monolayers in 35 mm dishes were transfected with 3 µg of plasmid DNA using the calcium phosphate procedure described in section 2.2.3.1. At 6 hours post-transfection the cells were infected with *wt*HSV-1 strain 17 at an MOI of 10.0. The virus was allowed to adsorb for 1 hour before being overlaid with 2 ml of EC5. Monolayers were maintained at 37°C throughout the assay protocol.

b) Transfection / superinfection protocol (optimised conditions).

BHK-21 C13 or Vero monolayers in 35 mm dishes were transfected with 1 µg of plasmid DNA using the calcium phosphate procedure described in section 2.2.3.1. At 30 hours post-transfection the cells were infected with *wt*HSV-1 strain 17 or *wt*HSV-2 strain HG52 at an MOI of 1.0. The virus was allowed to adsorb for 1 hour before being overlaid with 2 ml of EC5. Monolayers were maintained at 37°C throughout the assay protocol.

c) Preparation of DNase resistant (packaged) DNA.

16 hours post-infection the medium was removed from the transfected monolayers and the cells resuspended in 2 ml RSB. The cells from each plate were divided into two aliquots.

To the first aliquot (DNase resistant DNA) 55 µl NP40 (10%) and 10 µl DNase I (10 mg ml^{-1}) was added. To the second aliquot (total cellular DNA) 55 µl NP40 (10%) was added. Both preparations were incubated at 37°C. After 2 hours 1 ml of 2xCLB containing 1 mg ml^{-1} protease was added to each preparation. This was followed by a further 2 hour incubation at 37°C. The nucleic acids were then sequentially extracted with phenol and chloroform : isoamyl alcohol, precipitated with 2.5 volumes of ethanol and resuspended in 75 µl TE pH 8.0.

30 µl of the resuspended DNA (corresponding to that recovered from 1/5th of the cells) was digested with *Eco*RI and *Dpn*I. The products were resolved on a large 0.8% agarose Loening's buffer gel and the presence of replicated plasmid sequences was detected by Southern blotting and hybridisation to a ^{32}P labelled probe

2.2.5.3 - Assay for the serial propagation of amplicons.

BHK cell monolayers in 35 mm dishes were transfected with 1 µg of plasmid DNA using the calcium phosphate procedure described in section 2.2.3.1. At 30 hours post-transfection the cells were infected with *wt*HSV-1 strain 17 at an MOI of 1.0. The virus was allowed to adsorb for 1 hour before being overlaid with 2 ml of EC5. 16 hours later the cells were resuspended in the medium and divided into two portions. Half was used to prepare total cellular DNA (pre-passage) as described in section 2.2.5.2b, except that incubation in the presence of NP40 was omitted. The remaining half was used to prepare a virus stock. The cells were sonicated in a sonicating waterbath for 5 minutes and the cell debris pelleted by centrifugation (5 minutes 2000 rpm, Beckman GPR centrifuge). The supernatant was removed and stored at -70°C as a virus stock. Half of this virus stock was subsequently used to infect a confluent BHK cell monolayer in a 35 mm dish. The virus was allowed to adsorb for 1 hour and the cells were overlaid with 2 ml of EC5. 16 hours later total cellular DNA (post-passage) was prepared as described in section 2.2.5.1b. All monolayers were maintained at 37°C throughout the assay protocol.

30 µl aliquots of both pre and post-passaged total cellular DNAs were digested with *Eco*RI and *Dpn*I. The products were resolved on a large 0.8% agarose Loening's buffer gel and the presence of plasmid sequences was detected by Southern blotting and hybridisation to a ^{32}P labelled probe as described in section 2.2.6.

2.2.6 - Southern blotting and hybridisation.

2.2.6.1 - Southern blot transfer (Southern, 1975).

The restriction enzyme products for Southern blotting were electrophoresesed through a 0.8% 0.5 cm thick slab gel in 1x Loening's buffer containing 0.5 μg ml^{-1} ethidium bromide. Typically gels were run overnight at approximately 15 volts. Prior to blotting the gel was photographed under long wave UV light to confirm that the samples were consistently recovered and digested. The DNA was denatured by gentle agitation in gel soak I for 45 minutes, and then neutralised prior to blotting in gel soak II for 45 minutes. The DNA was blotted for 6-16 hours onto Hybond-N membrane using 6 x SSC, Whatman 3 mm chromatography paper and a weighted stack of paper towels. The DNA was crosslinked to the membrane using a Stratagene UV crosslinker (1200 Jcm^2).

2.2.6.2 - Preparation of radio-labelled probe for Southern blot hybridisation

Approximately 500 ng of pAT153 was labelled with 10 μCi each of [α^{32}P] dGTP and [α^{32}P] dCTP using nick translation protocol (Rigby *et al.* 1977). The incorporation reaction (28 μl) also contained 50 μM dATP and dTTP, 1 unit of *E.coli* DNA polymerase I, 10^{-7} mg ml^{-1} DNase I and 1x nick translation buffer. The reaction was carried out for 90 minutes at 16°C. Radio-labelled probe was separated from the unincorporated nucleotides using a G50 sephadex column. Just prior to hybridisation the probe was denatured in 0.15M NaOH (1 ml) and after 10 minutes neutralised with HCl and mixed with 8.6 ml of pre-warmed hybridisation mix immediately prior to hybridisation.

2.2.6.3 - Southern blot hybridisation.

The dried Hybond-N membrane was sealed inside a hybridisation bag with 100 ml of pre-soak solution and shaken in a water bath at 68°C. After 2 hours the pre-soak solution was discarded and replaced with 10 ml of hybridisation mix containing the denatured nick translated probe. The hybridisation bag was then resealed and returned to the 68°C water bath for a further 6-16 hours.

The membrane was then removed and washed twice in 1 litre of blot wash for 1 hour. After this the blot was dried and exposed to either autoradiography film or a phosphorimager screen.

2.2.6.4 - Visualising Southern blots.

a) Autoradiography.

The Southern blot membrane was exposed to X-omat S film for 3-16 hours, using an intensifier screen. The film was developed in an automated processor.

b) Phosphorimager.

The Southern blot membrane was exposed to the phosphorimager screen for 3-16 hours and was scanned into the computer using a **Molecular Dynamics** scanner and the ImageQuant software package.

c) Quantitative analysis.

Quantitative measurements of the amount of replicated or packaged DNA were made using the ImageQuant software package (**Molecular Dynamics**). The scanned phosphorimage files were used for the analysis which measures the values of 'positive' pixels within a given area.

For each analysis the volume integration function was used, which sums the pixel values within a given object and subtracts the summed background pixel values. Identically sized rectangles were drawn around each of the objects for which intensities were to be calculated. An identically sized rectangle was also positioned somewhere on the blot, outwith the sample or marker lanes, this rectangle was used to calculate the background volume. The volumes of each of the object rectangles was then calculated, subtracting the volume of the background.

In experiments which were quantified, the intensity of each band is given as the value calculated by the volume integration function described above.

2.2.7 - DNA manipulation and propagation.

2.2.7.1 - Restriction enzyme digestion.

Restriction enzyme digestions were carried out using commercial restriction enzymes and the buffers supplied. Generally approximately 1 μg of DNA was incubated at the recommended temperature for 3 hours, with 5-10 units of enzyme. The final reaction volume was between 20-35 μl in 1 x reaction buffer.

2.2.7.2 - Purification of DNA fragments.

Where it was necessary to specifically purify an individual DNA fragment this was achieved by excising the appropriate band and purifying the DNA by electoelution and passage over an affinity column.

The restriction enzyme digest was first run on a 1 x TAE agarose gel. The DNA was visualised using long wave UV light and the required DNA band excised. The excised band was placed inside dialysis tubing with 2 ml 1 x TAE, and then electroeluted at 200 volts for approximately 2 hours. The DNA containing buffer was removed and spun at 13000 rpm (MSE microcentrifuge) for 1 minute to remove the remaining gel fragments. The DNA containing buffer was then applied to a DEAE sephacel column. The column was washed with 5 ml TE pH 8.0 containing 0.1 M NaCl, the DNA was eluted using 1 ml of TE pH 8.0 containing 1 M NaCl, was precipitated with 2.5 vols. of ethanol, washed with 70% ethanol and resuspended in a suitable volume of TE pH 8.0.

2.2.7.3 - Ligation of DNA fragments.

Prior to ligation restriction enzyme products were sequentially extracted with phenol and chloroform : isoamyl alcohol, ethanol precipitated and resuspended in TE pH 8.0. Where possible the plasmid backbone was dephosphorylated with calf intestinal phosphatase to prevent recircularisation.

During the ligation reaction an excess of insert with respect to plasmid backbone was incubated together with 1 unit of T4 DNA ligase in 1 x ligation buffer. The reaction was made up to 20 µl with dH_2O and incubated either overnight at RT or for 48 hours at 4°C. Ligation products were sequentially extracted with phenol and chloroform : isoamyl alcohol, ethanol precipitated and resuspended in TE pH 8.0 prior to transformation into *E. coli.*

2.2.7.4 - Preparation and transformation of competent *E. coli.*

a) TSB competent *E. coli.* (Chung *et al.*, 1989)

A 50 ml L-broth culture of *E. coli* was grown to an A_{600} of 0.9. 20 ml of this culture was pelleted at 2300 rpm for 10 minutes at 4°C (Sorvall SS-34). The pelleted bacteria were resuspended in 2 ml of chilled TSB / DMSO and incubated on ice for 10 minutes. 100 µl aliquots were flash frozen and stored at -70°C.

Transformation was achieved by adding ≤ 1 µg of DNA to 100 µl of bacteria. The bacteria were incubated on ice for 30 minutes, 900 µl of TSB/glucose was then added and

the incubation continued in an orbital shaker at 37°C for 1 hour. 100 µl was then plated out onto L-broth agar plates containing the appropriate antibiotic.

b) Super competent *E.coli* (Hanahan, 1985).

A 100 ml SOB culture of *E.coli* was grown to an A_{550} of 0.5. The culture was then chilled and transferred to centrifuge tubes. The cells were pelleted at 1000 rpm for 15 minutes (Sorvall SS34) and the supernatant removed. The cells were resuspended in RFI (1/3rd original volume) and incubated on ice for 60-120 minutes. The cells were then repelleted as above and resuspended in RFII (1/12.5 original volume). This suspension of bacteria was divided into 200 µl aliquots, which were flash frozen and stored at -70°C.

Transformation was achieved by adding ≤ 20 µl (≤ 50 ng) of DNA to 200 µl of cells and incubating on ice for 60 minutes. The cells were heat shocked at 42°C for 90 seconds and then immediately chilled and 800 µl of SOC added. The bacteria were then incubated in an orbital shaker at 37°C for 1 hour. Dilutions were made of this culture (typically 10^{-2}-10^{-5})and 100 µl plated out onto L-broth agar plates containing the appropriate antibiotic.

c) Commercial super-competent *E. coli* DH5α (Hanahan, 1985).

Transformation was achieved using the suppliers recommended conditions (essentially as described in section 2.2.6dii). Less than 50 ng of DNA was added to 100 µl of cells. The cells were incubated on ice for 30 minutes, heat shocked at 42°C for 45 seconds and chilled on ice for 2 minutes. 900 µl of SOC was then added and the bacteria incubated in an orbital shaker for 1 hour. Dilutions were made and 100 µl plated out onto L-broth agar plates containing the appropriate antibiotic.

2.2.8 - Site Directed Mutagenesis.

2.2.8.1 - Oligonucleotides.

a) Synthesis of mutagenic oligonucleotides.

Oligonucleotides were synthesised 'in house' using a Cruachem PS250 synthesiser.

The newly synthesised oligonucleotides were supplied still attached to their synthesis column. They were removed using 1.5 ml of concentrated ammonia solution. 0.2 ml aliquots were pushed through the column at 20 minute intervals. The oligonucleotides were deprotected at 55°C for 5 hours and lyophilised overnight. The desiccated oligonucleotides

were then resuspended in a suitable volume of distilled water and their concentrations were established by measuring their absorbance at 260 nm.

b) Phosphorylation of mutagenic oligonucleotides.

Each mutagenic oligonucleotide was 5' phosphorylated using T4 polynucleotide kinase in the presence of 1x polynucleotide kinase buffer and 1 mM ATP. The phosphorylation reaction was carried out at 37°C for 40 minutes. The polynucleotide kinase enzyme was heat inactivated at 70°C for 10 minutes and the phosphorylated oligonucleotides stored at -20°C.

2.2.8.2 - Preparation of uracil rich single stranded (ss) DNA.

Uracil rich ssDNA copies of plasmids for mutagenesis were prepared from *E. coli. CJ236 (dut, ung⁻)* transformants infected with the ssDNA bacteriophage R408.

An overnight culture of bacteria was grown up in L-broth including 50 μg ml^{-1} ampicillin and 100 μg ml^{-1} uridine. 4 ml of this overnight culture was used to inoculate a 200 ml L-broth culture , which also contained 50 μg ml^{-1} ampicillin and 100 μg ml^{-1} uridine. This large culture was shaken at 37°C for 30 minutes before 10^{11} pfu of R408 bacteriophage was added. The culture was shaken for a further 9 hours at 37°C. Phage particles containing ss DNA were recovered after first pelleting the remaining bacteria at 9000 rpm (Sorvall GSA rotor). The phage were precipitated from the supernatant, by adding 0.25 volumes (vols.) of 20 % PEG 6000 / 2.5 M NaCl incubating on ice for 30 minutes and pelleting at 12,000 rpm for 15 minutes (Sorvall GSA rotor). The pellet was resuspended in 5 ml of TE pH 8.0 and reprecipitated and pelleted using the PEG / NaCl mix. Following the second precipitation all of the supernatant was removed and the pellet was resuspended in 2 ml TE pH 8.0. The ssDNA was recovered from the phage like particles using 2 ml of equilibrated phenol. The phenol was vigorously mixed with the resuspended pellet for 1 hour before the aqueous phase was removed. This aqueous phase was repeatedly extracted (until no interphase was observed) with an equal volume of phenol : chloroform : isoamyl alcohol (24:24:1). Finally the DNA was extracted with an equal volume of chloroform : isoamyl alcohol (24:1) and ethanol precipitated in the presence of 320 mM NaCl / 4 mM EDTA. The DNA was pelleted at 7000 rpm for 20 minutes (Sorvall SM24), washed with 70% ethanol and resuspended in 100 μl of dH_2O.

2.2.8.3 - Modified Kunkel mutagenesis (Kunkel *et al.*, 1991).

a) Annealing.

Approximately 1 µg of uracil rich ss DNA was mixed with 2 µl of TM buffer and 36 ng of phosphorylated oligonucleotide in a total volume of 20 µl. The annealing reaction was then placed in a water bath containing 250-300 ml of water. The waterbath was heated to 95°C and allowed to cool slowly to RT.

b) Second strand synthesis.

To each annealing reaction, 10 µl of 5x Pol / Lig buffer, 1 unit of T7 DNA polymerase and 1 unit of T4 ligase was added. The total volume was adjusted to 50 µl and the reaction incubated at RT for 1 hour and then at 37°C for 1 hour.

The DNA from the synthesis reaction was then sequentially extracted with phenol and chloroform : isoamyl alcohol, ethanol precipitated with 2.5 volumes of ethanol and resuspended in 10-20 µl of distilled water.

3 µl of the resuspended DNA was then used to transform competent *E.coli.* DH5α (*dut*$^+$, *ung*$^+$).

2.2.8.4 - Screening for mutants.

Mutations constructed using the method described above were identified by preparing DNA from transformed colonies picked from agarose plates (see section 2.2.2.3) and screening the DNA either for engineered restriction enzyme sites or changes in the size of particular restriction fragments.

2.2.9 - Gel electrophoresis of nucleic acids.

2.2.9.1 - Non denaturing agarose gels.

Two different sizes of slab gel were used, large gels (215 x 165 x 7 mm) and minigels (80 x 70 x 9 mm). All gels were made with 0.8% agarose in 1x running buffer which contained 0.5 µg ml^{-1} ethidium bromide. Samples were loaded into the wells of the gel in 1x loading buffer.

The running buffer used for a particular gel was dependent upon the circumstances. Large gels for Southern blotting were run slowly overnight at 10-25 volts, therefore Loening's buffer was used because of its high buffering capacity. In the majority of cases

minigels were run in 1x TBE and run at 40-60 volts for 2-4 hours.. The exception to this was when bands were to be purified from the gel, in this case 1x TAE was used.

2.2.9.2 - Non denaturing polyacrylamide gels.

In order to resolve small DNA fragments, 1.5 mm thick vertical slab gels made with 8% polyacrylamide (acrylamide : bis acrylamide 59:1) were used. The acrylamide : bis acrylamide was polymerised with 0.01 volumes of 10% ammonium persulphate and 0.001 volumes of TEMED. The gels were run in 1 x TBE. DNA samples were loaded in 1x TBE loading dye and the gels were stained in 1x TBE containing 1 $\mu g\ ml^{-1}$ ethidium bromide. Polyacrylamide gels were typically run at 100 volts for 1-2 hours.

2.2.10 - Sequencing.

Plasmid DNA was sequenced directly using a **Perkin Elmer** automated sequencer. Double stranded plasmid DNA was prepared using Qiagen midi columns™ (see section 2.2.2.2). DNA samples were provided for sequencing at a concentration of 0.2 $\mu g\ \mu l^{-1}$ with 1.6 pmol primer. All of the sequencing was carried out, in the pTZ series of vectors, by Lab200 (Leslie Taylor and Aiden Dolan). The sequencing reactions utilised the -20 16mer universal priming site present in these vectors. The sequence of the universal primer used was, TGACCGGCAGCAAAATG.

Chapter 3 - Results.

Section 3.1 - Optimisation of a transient packaging assay for HSV-1.

3.1.1 - Introduction.

The data presented in this section relate to the optimisation of a transient packaging assay for HSV-1, described initially by Stow *et al.* (1983).

The transient packaging assay involves transfecting a plasmid, containing a viral origin of DNA replication and packaging signal, into a cell line permissive for HSV-1. The cells are subsequently super-infected with *wt*HSV-1, which provides the helper functions necessary for the replication and packaging of plasmid sequences. Approximately 16 hours post-infection the cells are lysed in the presence of DNase I, so that only the DNA that has been packaged into viral capsids, and therefore protected from DNase digestion, is recovered. In the experiments described here the harvested DNA is digested with *Eco*RI and *Dpn*I and the products are resolved on an agarose gel. The presence of DNase-resistant (packaged) plasmid DNA is detected by Southern blotting and hybridisation to a ^{32}P labelled probe (figure 3.1).

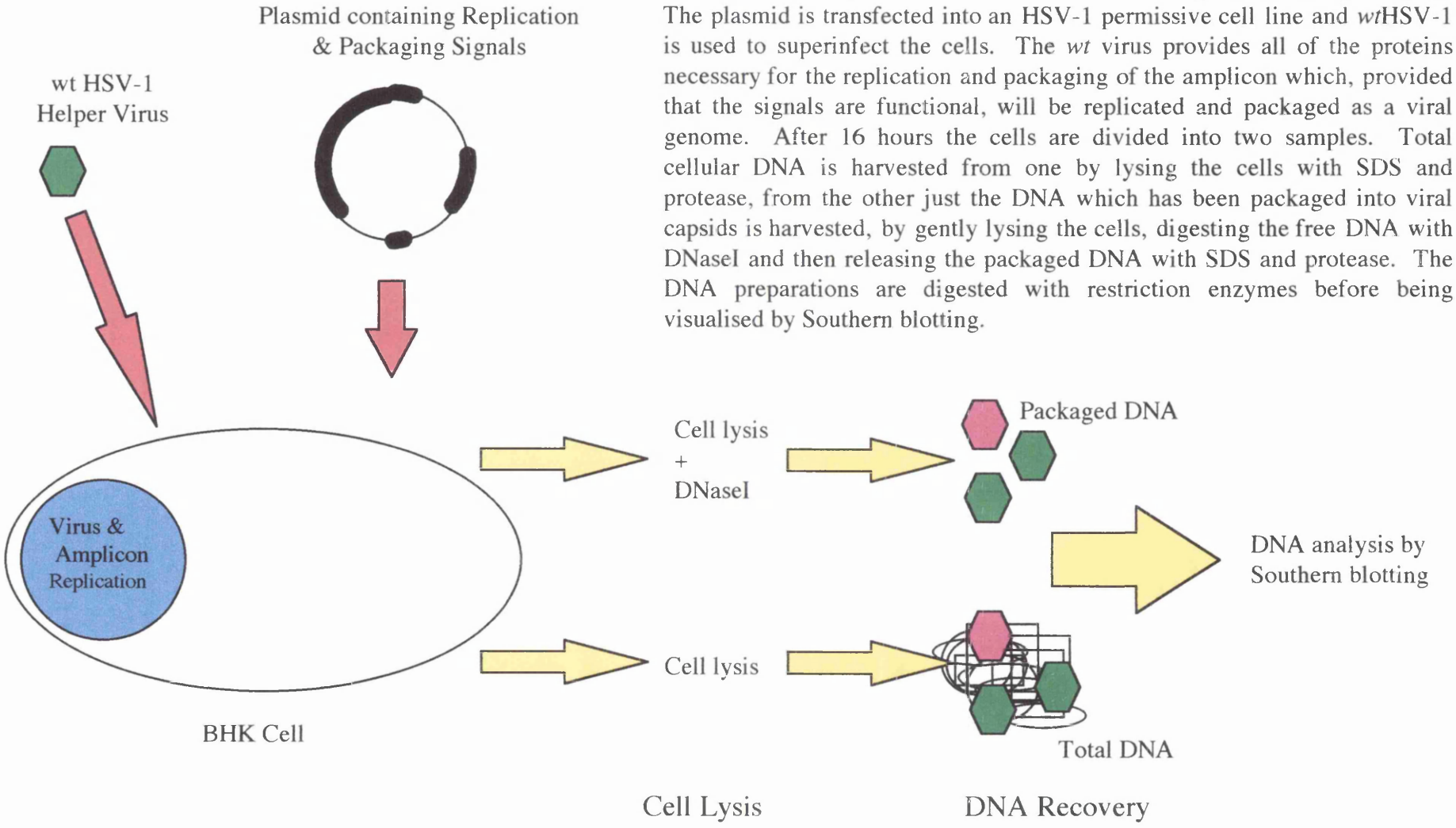
Digestion with *Eco*RI (which cuts once in every plasmid used in this study) resolves the concatemeric DNA, produced by virus-directed replication, into unit length species. *Dpn*I digestion allows a distinction to be made between the input DNA delivered by transfection and the products of virus-directed replication. This distinction is based on the requirement for *Dpn*I digestion that its recognition / cleavage site (GATC) is methylated at the A residues. The input DNA, which is prepared from *dam*$^+$ *E.coli* strains, is methylated and therefore susceptible to digestion; while the DNA which is replicated in eukaryotic cells during the course of the assay contains unmethylated GATC sequences and is, therefore, resistant to digestion.

In all the transient assay experiments described here the ethidium bromide stained gel was examined prior to blotting using a UV light box to check that recovery of DNA from all samples was consistent, and to confirm that the DNA had been properly digested.

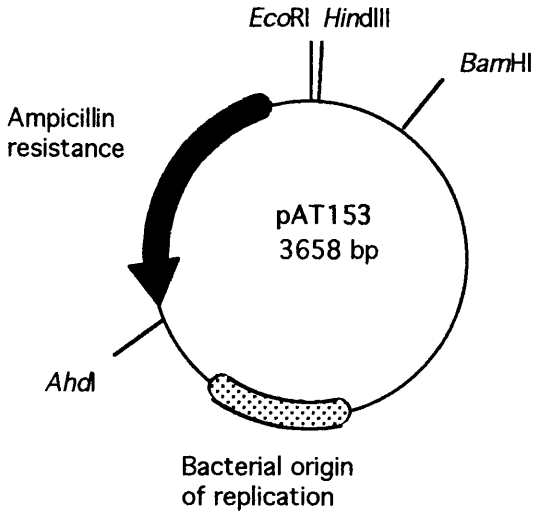
The principal parent plasmid used in these experiments was pS1 (Stow *et al*., 1983). pS1 is derived from the high copy number plasmid pAT153 (Twigg & Sherratt, 1980) (figure 3.2 A). In addition it contains a 539 bp insert containing the HSV-1 Ori_S, which is cloned into the *Bam*HI site (figure 3.2 B). In order for the replicated plasmid to be packaged during the transient packaging assay a packaging signal is also necessary. Putative packaging signals were cloned between the *Eco*RI and *Hin*dIII sites of pS1 (e.g. pSA1,

Figure 3.1 - The transient packaging assay.

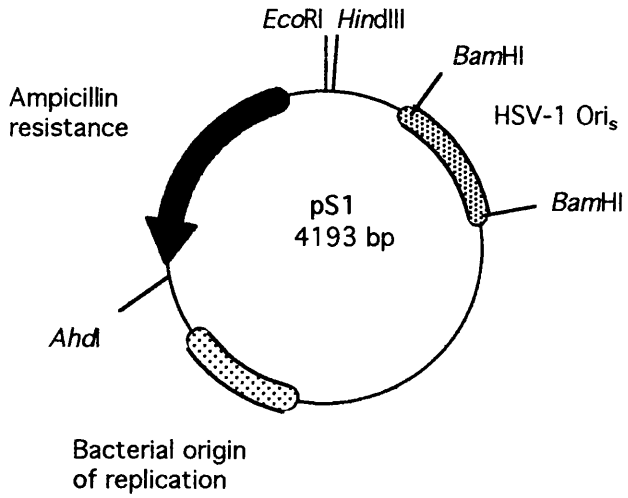
In the transient packaging assay a plasmid (known as an amplicon) is used, which contains signals for HSV-1 directed DNA replication and packaging. The plasmid is transfected into an HSV-1 permissive cell line and *wt*HSV-1 is used to superinfect the cells. The *wt* virus provides all of the proteins necessary for the replication and packaging of the amplicon which, provided that the signals are functional, will be replicated and packaged as a viral genome. After 16 hours the cells are divided into two samples. Total cellular DNA is harvested from one by lysing the cells with SDS and protease, from the other just the DNA which has been packaged into viral capsids is harvested, by gently lysing the cells, digesting the free DNA with DNaseI and then releasing the packaged DNA with SDS and protease. The DNA preparations are digested with restriction enzymes before being visualised by Southern blotting.



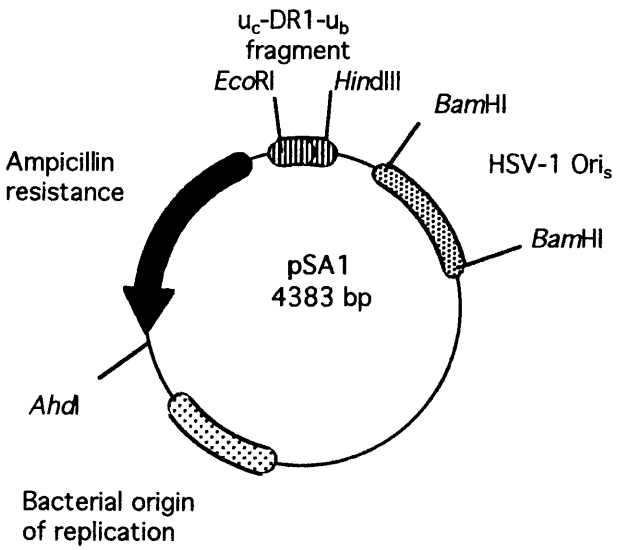
A.


EcoRI HindIII
BamHI
Ampicillin resistance
pAT153
3658 bp
AhdI
Bacterial origin of replication

B.


EcoRI HindIII
BamHI
Ampicillin resistance
HSV-1 Oris
pS1
4193 bp
BamHI
AhdI
Bacterial origin of replication

C.


uc-DR1-ub fragment
EcoRI
HindIII
BamHI
Ampicillin resistance
HSV-1 Oris
BamHI
pSA1
4383 bp
AhdI
Bacterial origin of replication

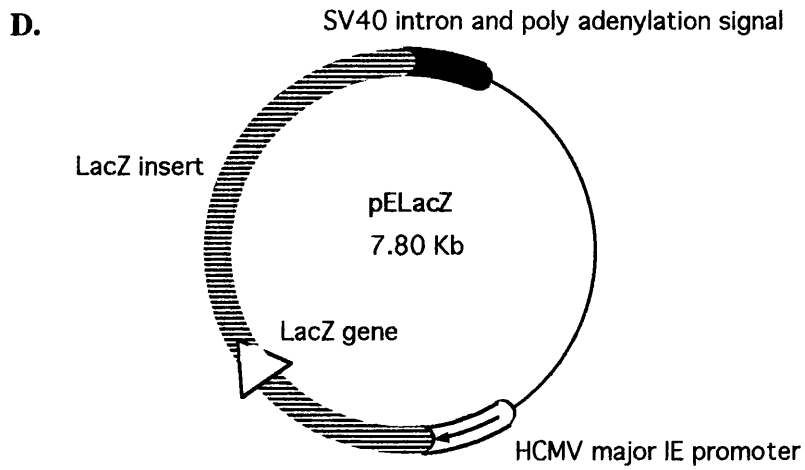


Figure 3.2 - Plasmid constructs used in the transient packaging assay.

All of the plasmids used during the transient packaging assay were based on pAT153 (A). pAT153 is derived from pBR322 (Twigg & Sherratt, 1980). The plasmid contains the β-lactamase gene for ampicillin resistance and a bacterial origin of replication.

pS1 (B) is an HSV-1 replicon. It contains 539bp of HSV-1 Ori_s cloned into the *Bam*HI site of pAT153.

pSA1 (C) is an HSV-1 amplicon. The packaging signal (in this case the 200bp u_c-DR1-u_b fragment) is cloned between the *Eco*RI and *Hin*dIII of pS1.

pE*LacZ* (D) is the plasmid used as a transfection marker, it contains the *LacZ* gene under the control of the HCMV immediate early gene promoter.

figure 3.2 C). All of these plasmids also contain a functional ß-lactamase gene which was used for the purpose of selection in bacteria.

Plasmids which contain functional HSV-1 signals that allow their replication, or replication and packaging, during the transient packaging assays are frequently referred to as replicons or amplicons respectively; where appropriate these names will be used.

3.1.2 - Linear relationship between the amount of plasmid DNA and the signal detected by the phosphorimager.

The probed membrane from each of the Southern blots, which were generated as part of this work, were exposed to a phosphorimager screen and the image captured using a specialised scanner (**Molecular Dynamics**).

In order to establish that there was a linear relationship between the amount of DNA loaded onto the gel and the value ascribed to the corresponding band by the phosphorimager software (**Molecular Dynamics**), a range of plasmid DNA amounts (0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 ng) were Southern blotted and analysed using the software.

The phosphorimage for this blot is shown in figure 3.3 panel A. The intensity of each band was analysed using the phosphorimager software (**Molecular Dynamics**), as described in the methods, to quantify the radioactivity contained in each band. This data has been plotted graphically in figure 3.3 panel B. The relationship between the amount of DNA and the corresponding band intensity is approximately linear over the range tested.

3.1.3 - Comparison of three different fragments containing HSV-1 packaging signals, in the transient packaging assay.

The aim of this section was to compare the ability of three different DNA fragments, containing HSV-1 packaging signals, to direct cleavage and packaging in a transient packaging assay.

The three different fragments were all cloned between the *Eco*RI and *Hin*dIII sites of pS1 (figure 3.2 B). The packaging signals were contained within; a 1762 bp HSV-1 DNA fragment from the junction between the L and S segments of the genome, which contains a single 'a' sequence (pY1), a 2161 bp HSV-1 DNA fragment from the same region of the genome, which contains two tandemly repeated 'a' sequences separated by a single DR1 repeat (pZ1), and a 200 bp sequence which contains sequences which span the junction between two tandemly repeated 'a' sequences (pSA1) (figure 3.2 C). The 200 bp sequence cloned into pSA1 contains the u_c and u_b regions of the 'a' sequences on either side of the junction, plus the DR1 repeat which separates them. It is the u_c and u_b elements which

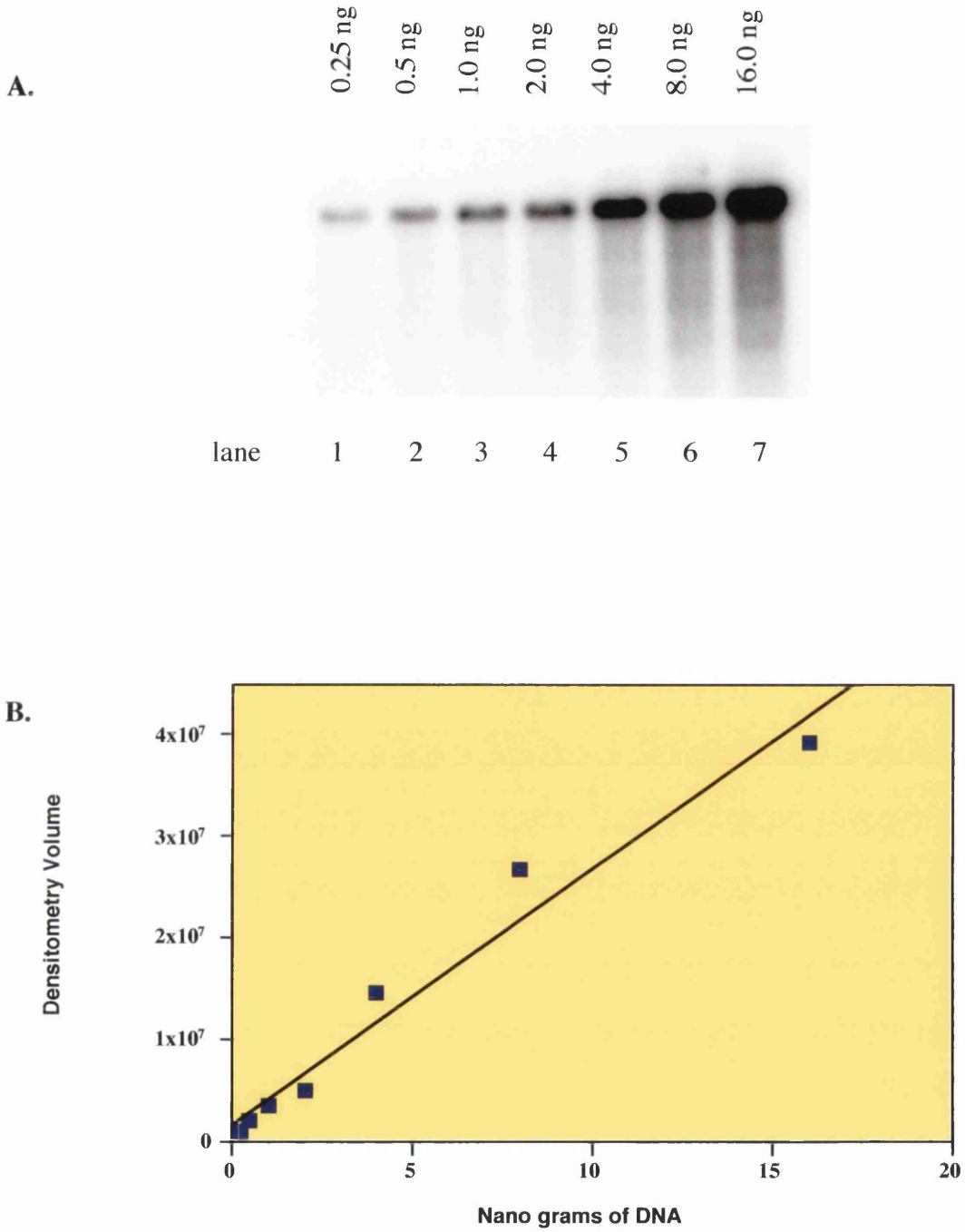


Figure 3.3 - Linear relationship between the amount of plasmid DNA and the signal detected by the phosphorimager.

Panel A, lanes 1-7 contain; 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 ng of linear pSA1 respectively. The DNA was resolved on a 0.8% agarose gel. The gel was Southern blotted and the membrane probed with ^{32}P labelled pAT153. Panel B, shows a graphic representation of the densitometry data from the phosphorimager.

contain the regions of the 'a' sequence which have been shown to be particularly well conserved at the termini of other herpesviruses. The structure of this element is referred to as u_c-DR1-u_b. The structure of each packaging signal is shown in figure 3.4.

3 μg of each plasmid were transfected into BHK-21 cells using the calcium phosphate procedure. Helper functions were provided by HSV-1 superinfection (MOI, 10.0), at 6 hours post-transfection. Total and packaged DNA were prepared 16 hours later, digested with *Eco*RI and *Dpn*I and Southern blotted as described in the methods section.

In agreement with previous results all three plasmids were replicated and packaged, indicating that both the Ori_S fragment and all three packaging signals are functional (figure 3.5 Lanes 3-8). The DNA from cells transfected with pSA1 (Lanes 3 & 4) generates a single unit length band, which comigrates with linear pSA1. Lanes 5-8, which contain DNA harvested from cells transfected with pY1 (lanes 5 & 6) and pZ1 (lanes 7 & 8), contain major bands consistent with the unit length species for each plasmid. They also, contain minor bands of a smaller size (marked *), which represent the large terminal fragments which are generated as a consequence of the restriction enzyme digestion of packaged DNA (figure 3.5). Terminal fragments are generated by two cleavage events in the terminal unit of the concatemer generated by the virus (figure 3.6). The first cleavage occurs *in vivo* as the viral proteins package the viral concatemer. The second cleavage event is carried out as the harvested DNA is digested with *Eco*RI. The *Eco*RI cleavage releases unit lengths from the middle of the replicated concatemers, but at the termini smaller fragments are released. The size of these fragments is therefore dependent upon the position of the *Eco*RI site with respect to the site of viral cleavage. In pSA1 these 2 sites are 65 bps apart, which produces two fragments of 65 bp and 4318 bp. The smaller fragment is not detected because it does not contain any vector sequences, while the larger 4318 bp fragment would comigrate with the 4383 bp monomer. For pY1 and pZ1 however, as more sequence separates the viral cleavage site from the *Eco*RI site, the large terminal fragments are significantly smaller than the monomer. This significant size difference allows the large terminal fragments to be clearly seen.

Quantification of the level of replication and packaging for each plasmid was carried out using the phosphorimager (**Molecular Dynamics**) to measure the radioactivity in the monomer bands. The data for this experiment (experiment 1) and a repeat experiment is shown in table 3.1. The amount of packaged DNA is also given as a percentage of the total DNA harvested from the cells.

The data clearly show that for each plasmid the proportion of replicated amplicon DNA packaged into viral capsids is very similar. This shows that u_C-DR1-u_b packaging signal in pSA1 functions as efficiently as the larger packaging signals in pY1 and pZ1 and is therefore

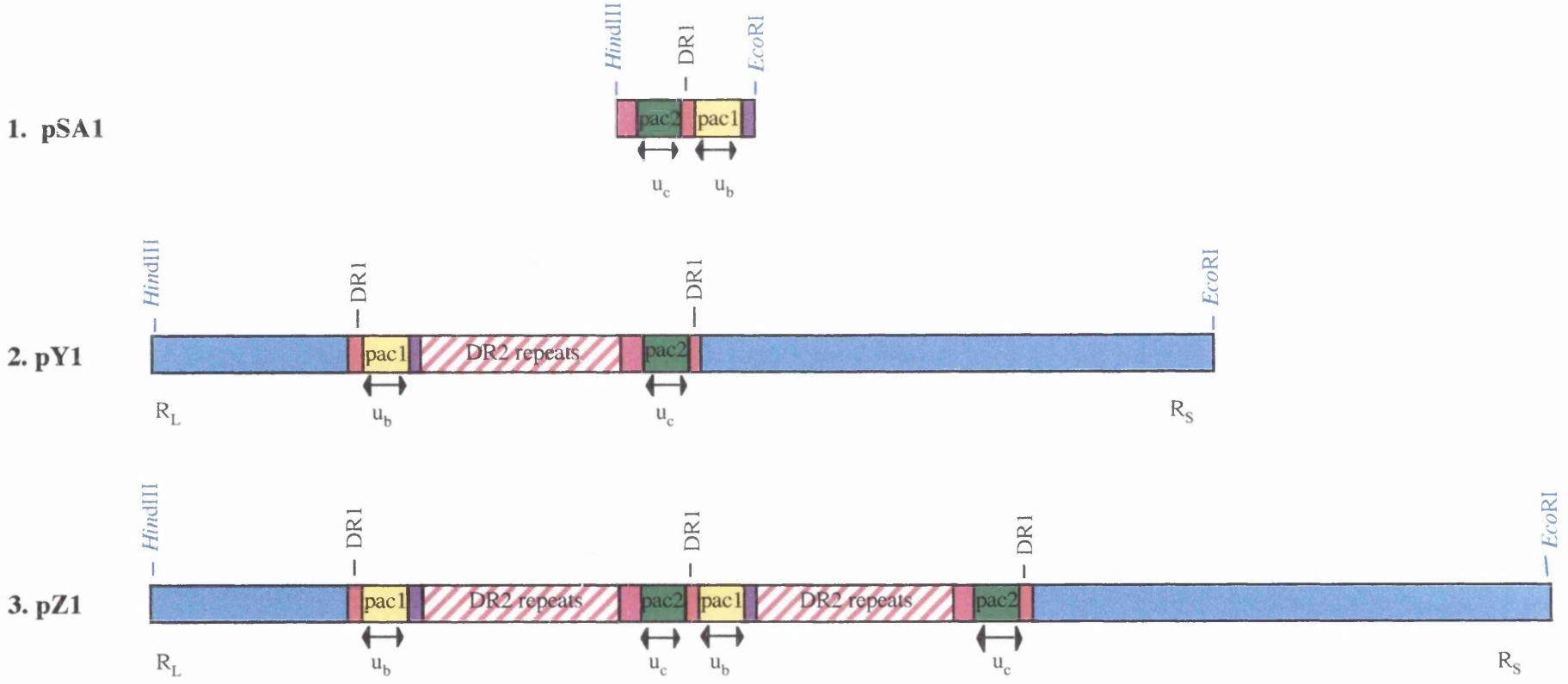


Figure 3.4 The structure of the three different packaging signals used in the transient packaging assay.

(1) pSA1 contains a 200bp u_c-DR1-u_b fragment from the HSV-1 genome. (2) pY1 contains a 1762bp fragment of the HSV-1 genome, which includes a single 'a' sequence. (3) pZ1 contains a 2161bp fragment of the HSV-1 genome, which includes two tandemly repeated 'a' sequences. The restriction enzyme sites used in the cloning of these fragments is shown in blue.

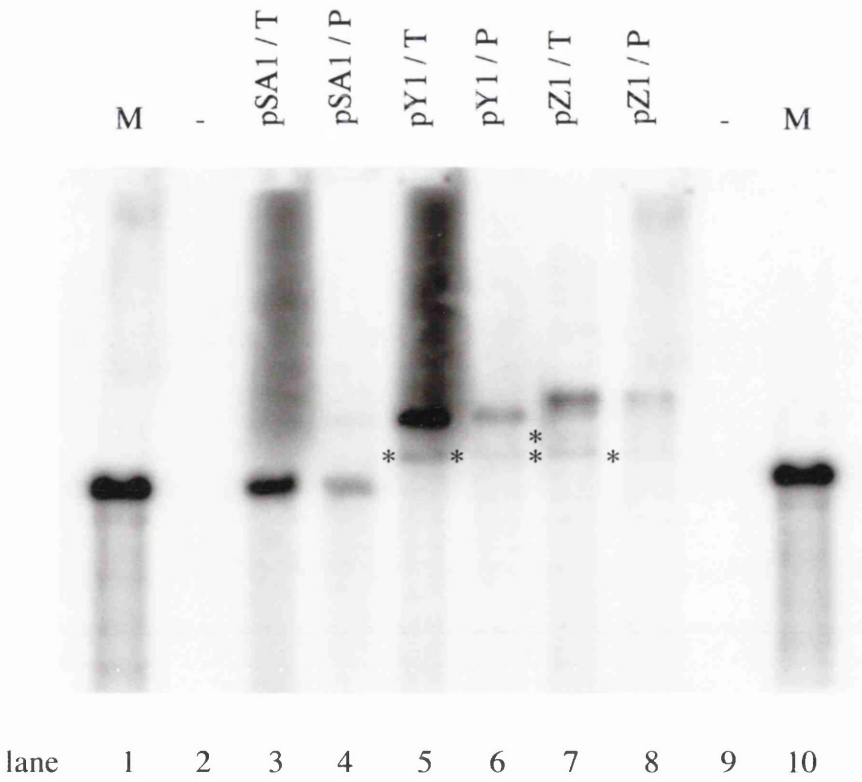


Figure 3.5 - Comparison of three different packaging signals in the transient packaging assay.

BHK 21 C13 monolayers were transfected with 3 μg of either pSA1, pY1 or pZ1 using a calcium phosphate precipitate. Monolayers were DMSO boosted at 4 hours post-transfection and super-infected with *wt*HSV-1 strain 17, (MOI, 10.0) at 6 hours post-transfection. 16 hours post-infection total and packaged DNA was harvested from the cells. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153.

The labelled blot was then exposed to a phosphorimager screen to produce the image above. Lanes 3 and 4 contain total and packaged DNA from cells transfected with pSA1. pSA1 contains a 200 bp packaging signal (u_c-DR1-u_b) which spans the junction between two tandemly repeated 'a' sequences. Lanes 5 and 6 contain total and packaged DNA from cells transfected with pY1, which contains 1.7 kbp of HSV-1 DNA within which is a single 'a' sequence. Lanes 7 and 8 contain total and packaged DNA from cells transfected with pZ1, which contains 2.1 kbp within which are two tandemly repeated 'a' sequences, separated by a single DR1 repeat.

The bands in lanes 5-8 (pY1 and pZ1 marked *), represent the large terminal fragments produced by viral cleavage and subsequent *Eco*RI cleavage. Lanes 1 and 10 (M) contain 4 ng of linear pSA1.

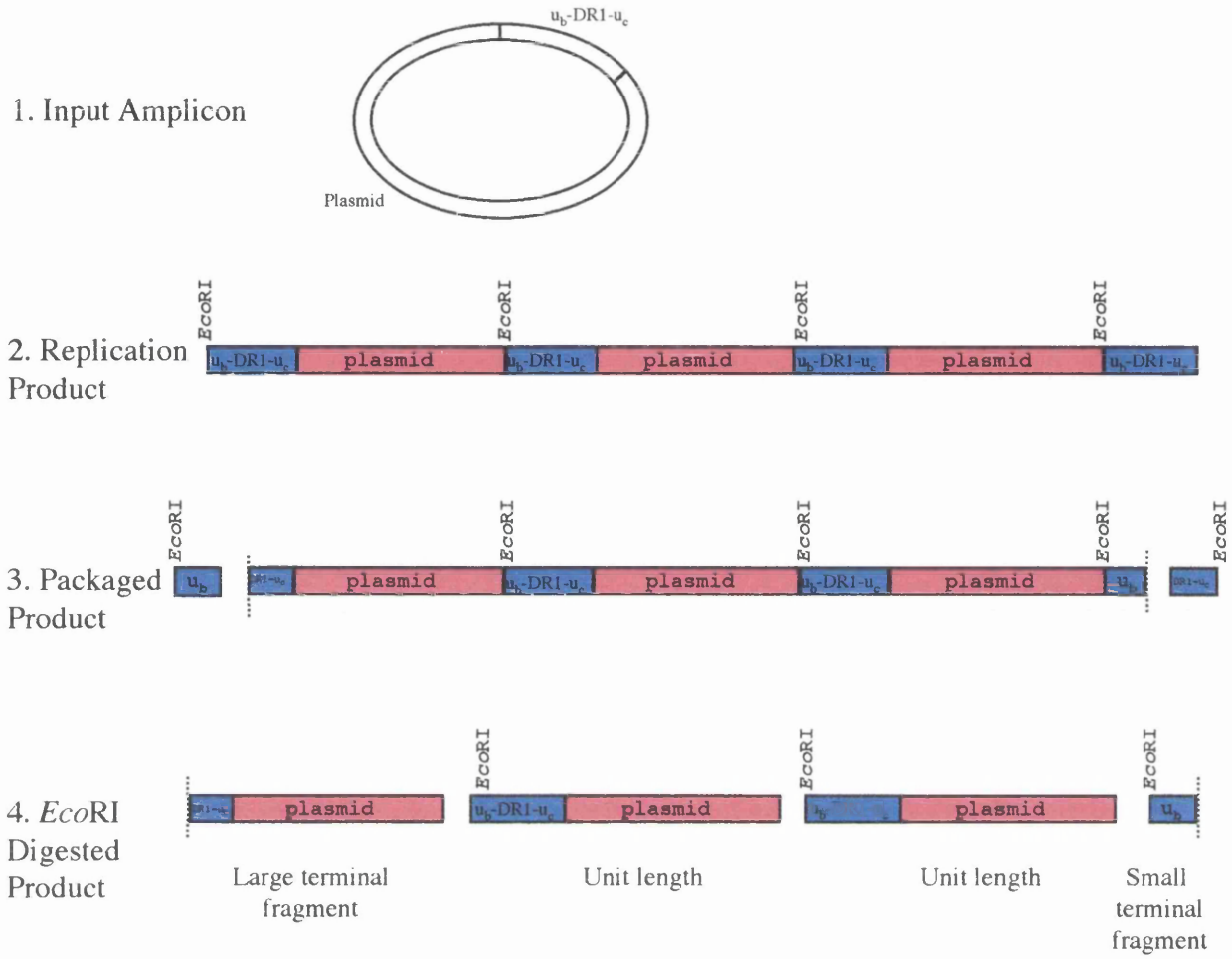


Figure 3.6 - Generation of large and small terminal fragments.
Terminal fragments are produced by two successive cleavage events in the concatameric DNA, generated by virus directed replication. The input plasmid (1) is replicated by the virus generating a concatameric product (2), which consists of head to tail repeats of the amplicon. This concatamer is the substrate for DNA packaging and, as such, the concatamer will be cleaved at a site corresponding to the virus genome termini (3). This product is subsequently harvested and digested with *Eco*RI, which releases unit lengths from the middle of the concatamer. However, from the ends terminal fragments are produced, which are smaller than unit length (4). The size of the terminal fragments is a product of the location of the *Eco*RI site with respect to the site of viral cleavage.

a suitable template for site-directed mutagenesis (see section 3.2). pSA1 was consequently used for all subsequent optimisation experiments.

Table 3.1 - Comparison of three different packaging signals in the transient packaging assay.

Plasmid	Experiment 1		Experiment 2	
	Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5	Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5
pSA1	299	69.8 (23.3%)	15.4	4.6 (30.1%)
pY1	236	58.4 (24.8%)	249	77.1 (30.9%)
pZ1	159	27.3 (17.1%)	93.3	28.5 (30.5%)

The amount of replicated and packaged DNA was quantified by 'volume' analysis using the phosphorimager (arbitrary units). The percentage of total DNA which is packaged is shown in brackets. All data is shown to 3 significant figures.

3.1.4 - Comparison of transfection methods.

The level of pSA1 replication in the transient assay was compared using two different transfection methods, calcium phosphate precipitation and lipofection. Hepes buffered saline (HeBS) was used in forming the calcium phosphate precipitate and lipofection was achieved using Lipofect^{ACE}(BRL).

0.25 µg, 0.5 µg, 1.0 µg, 2.0 µg, and 6.0 µg of pSA1 were transfected into BHK 21 C13 cells using Lipofect^{ACE}, and 0.25 µg, 0.5 µg, 1.0 µg and 2.0 µg of pSA1 were transfected into BHK 21 C13 cells using the calcium phosphate procedure. Helper functions were provided by *wt*HSV-1 super-infection (MOI, 10.0) at 6 hours post-transfection and total DNA was harvested 16 hours later. The harvested DNA was then digested with *Eco*RI and *Dpn*I and Southern blotted as described in the methods.

The phosphorimage (figure 3.7) shows that for lipofected plates (lanes 2-6) increasing the amount of input DNA results in an increased amount of replicated DNA. However, the level (even at 6 µg per plate) never reaches that obtained from plates transfected using the calcium phosphate procedure (lanes 8 - 11), which remain fairly constant across the range of input amounts. Interestingly, the smaller bands, produced by *Dpn*I cleavage of unreplicated pSA1, were more prominent in lipofected cells and increased with increasing amount of input DNA. The presence of these bands suggests that lipofected monolayers contain plasmid DNA in relatively large amounts, which may not be available for replication.

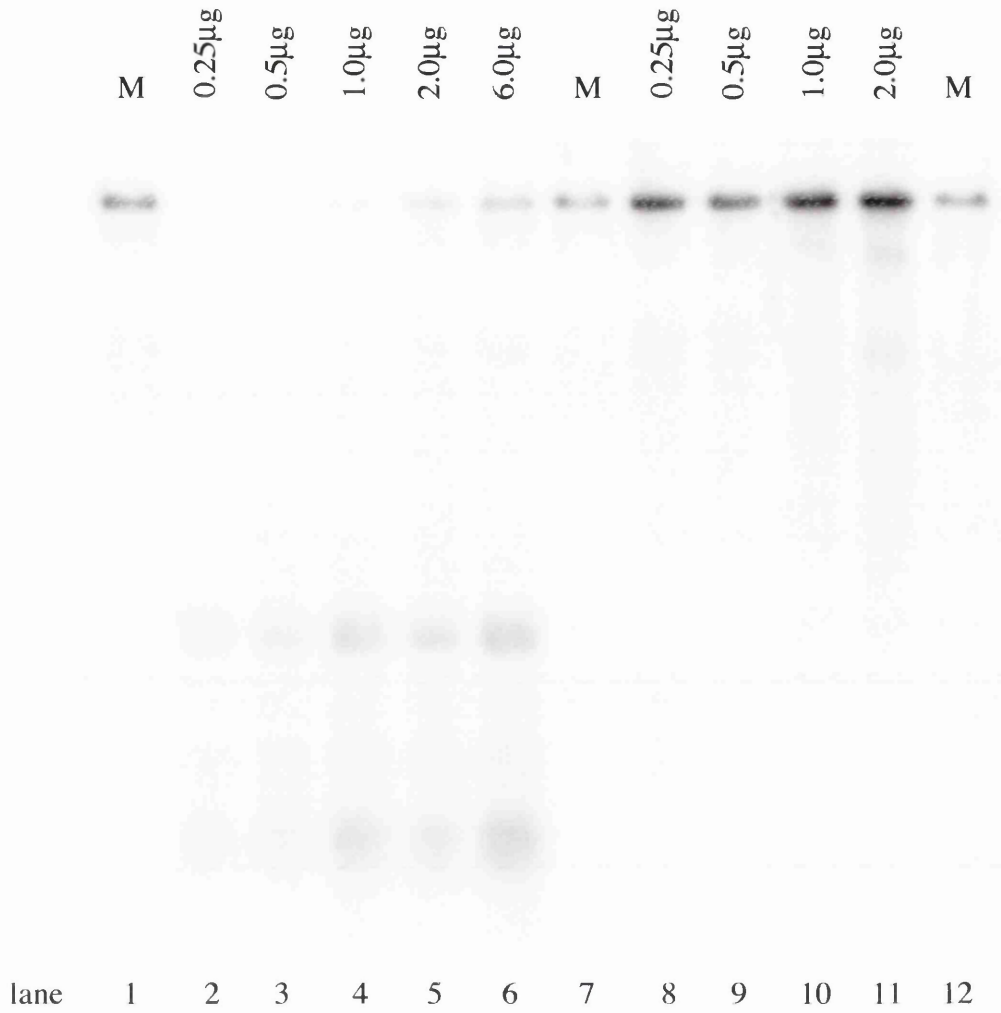


Figure 3.7 - Transient replication assay comparing calcium phosphate precipitation with lipofection.

BHK 21 C13 monolayers were transfected using either Lipofect^{ACE} (lanes 2-6) or a calcium phosphate procedure (lanes 8-11). Lipofected plates were transfected with 0.25,0.5,1.0,2.0 or 6.0 μg of pSA1 as indicated. Calcium phosphate transfected plates were transfected with 0.25,0.5,1.0 or 2.0 μg of pSA1. Monolayers were DMSO boosted at 4 hours post-transfection and super-infected with *wt*HSV-1 strain 17, (MOI, 10.0) at 6 hours post-transfection. 16 hours post-infection total DNA was harvested from the cells. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lanes 1,7 and 12 (M) contain 4 ng of linear pSA1.

Whether this DNA is bound to the cell surface or is trapped somewhere in the cell remains unclear.

Based on these observations, in subsequent experiments each monolayer was transfected with 1 μg of plasmid DNA using the calcium phosphate procedure.

3.1.5 The effect of multiplicity of infection of *wt*HSV-1.

The amount of helper virus applied to the cells was previously shown to influence the yield of packaged DNA by a group interested in HSV-1 amplicon packaging for gene therapy purposes (Wu *et al.*, 1995). Therefore, an experiment was performed using *wt*HSV-1 superinfection at a variety of different multiplicities, in order to optimise the packaging of pSA1 sequences.

BHK 21 C13 monolayers were transfected with 1 μg of pSA1 using the calcium phosphate procedure. Helper functions were supplied by *wt*HSV-1 super-infection at 6 hours post-transfection. Helper virus was applied to individual plates at one of the following MOIs; 0, 0.01, 0.05, 0.1, 1.0, 5.0, or 10.0 p.f.u cell^{-1}. Total and packaged DNA were harvested from each plate 16 hours post-infection. Harvested DNAs were then digested with *Eco*RI and *Dpn*I and Southern blotted as described in the methods section.

The phosphorimage (figure 3.8) shows that both total (lanes 2 - 8) and packaged (lanes 11 - 17) amplicon increases with increasing helper virus up to a certain point before beginning to decline. The intensity of the bands was measured using the phosphorimager software (**Molecular Dynamics**). The data for this experiment (experiment 1) and a repeat experiment are shown in table 3.2. The data from experiment 1 is also expressed graphically in figure 3.9, where the intensity of each band has been plotted against the MOI on a log scale.

Table 3.2 - Effect of multiplicity of infection of *wt*HSV-1.

MOI	Experiment 1		Experiment 2	
	Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5	Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5
0.00	0.412	0.143 (34.7%)	8.38	4.90 (58.5%)
0.01	0.585	0.126 (21.5%)	44.2	21.9 (49.5%)
0.05	2.49	0.288 (11.6%)	87.0	35.3 (40.6%)
0.10	3.27	0.997 (30.5%)	167	85.6 (51.3%)
1.0	19.6	6.86 (35.0%)	410	147 (35.9%)
5.0	29.4	5.19 (17.7%)	537	133 (24.8%)
10.0	13.0	4.22 (32.5%)	166	53.7 (32.3%)

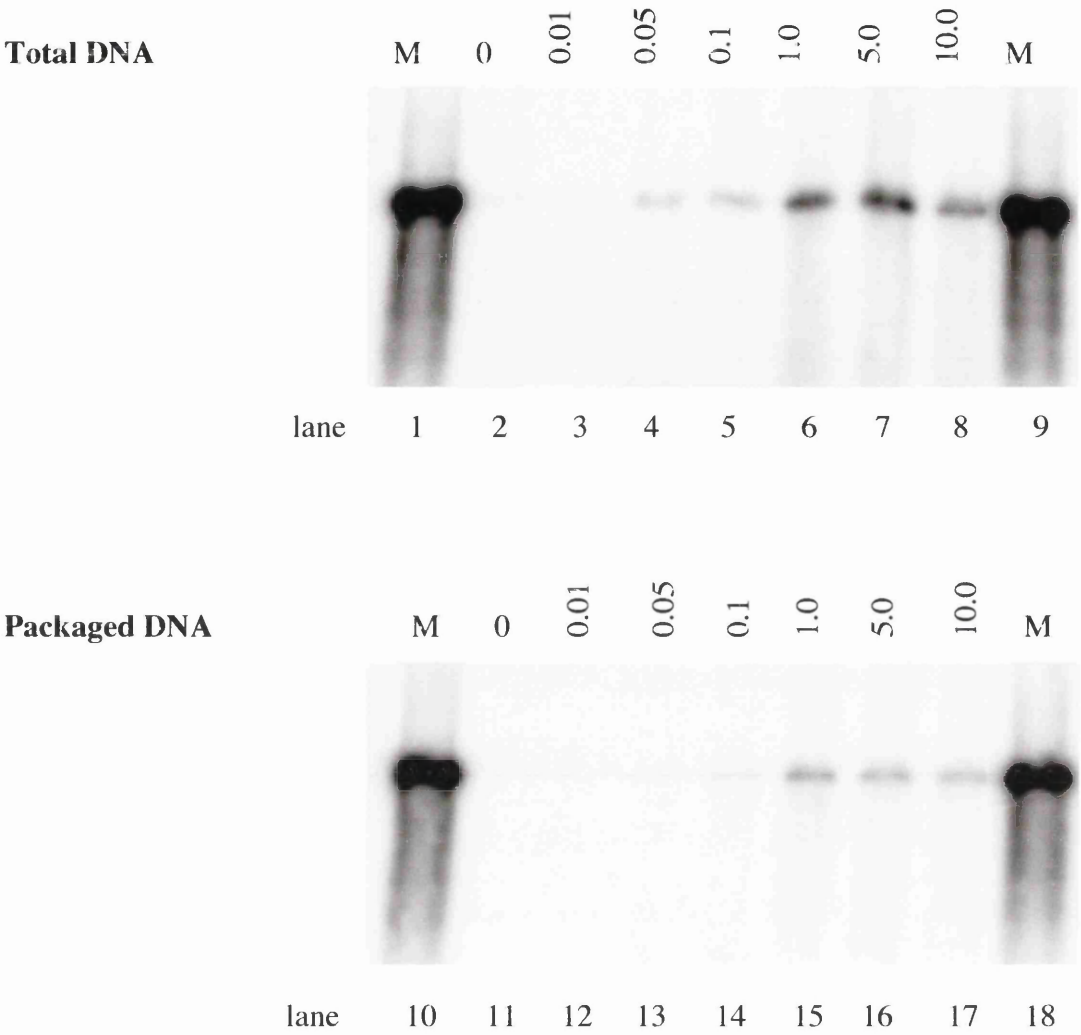


Figure 3.8 - Transient packaging assay examining the effect of multiplicity of infection (with HSV-1) on replication and packaging efficiency.

BHK 21 C13 monolayers were transfected with 1 μg of pSA1 using the calcium phosphate procedure. Monolayers were DMSO boosted at 4 hours post-transfection and super-infected with *wt*HSV-1 strain 17, (MOI of; 0, 0.01, 0.05, 0.1, 1.0, 5.0 or 10.0) at 6 hours post-transfection. 16 hours post-infection total (lanes 2-8) and packaged (lanes 11-17) DNA was harvested from the cells. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lanes 1,9,10 and 18 (M) contain 4 ng of linear pSA1.

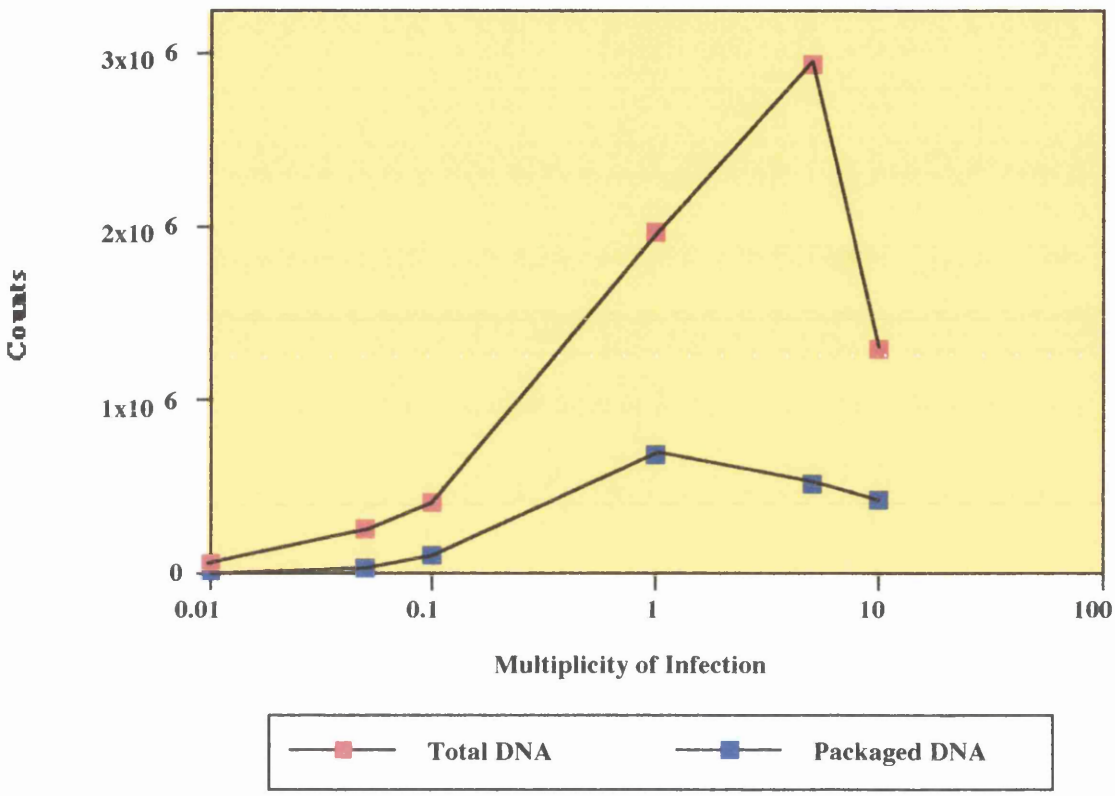


Figure 3.9 - Graphic representation of HSV-1 multiplicity of infection data.

The band intensity data from the phosphorimager was plotted against multiplicity of infection on a log scale.

All data is shown to 3 significant figures. The percentage of total DNA which is packaged is shown in brackets.

In both experiments the optimum for replication is 5, but for packaging it is 1. For this reason an MOI of 1 was used in all subsequent experiments.

3.1.6 - Delayed super-infection.

The work of Wu *et al.* (1995) also suggested that delaying super-infection with *wt* virus increased the amount of packaged amplicon that could be recovered. This was examined for the transient packaging assay using duplicate plates which were infected with *wt*HSV-1 24 hours apart.

BHK-21 C13 monolayers were transfected with 1 μg pSA1 using a calcium phosphate procedure. The plates were DMSO boosted at 4 hours post-transfection and 6 hours post-transfection one plate was super-infected with *wt*HSV-1 (MOI, 1.0). 16 hours later packaged DNA was harvested. A second plate was super-infected with *wt*HSV-1 (MOI, 1.0) at 30 hours post-transfection and again packaged DNA was harvested 16 hours later. The DNAs from both plates were then digested and Southern blotted as described in the methods section.

Figure 3.10 shows the phosphorimage from this experiment. Lane 3 contains DNA harvested from the plate which was super-infected at 6 hours post-transfection. Lane 2 contains DNA harvested from the plate transfected at 30 hours post-transfection. The intensity of the respective bands was measured using the phosphorimager software **(Molecular Dynamics).** The data demonstrated that delaying super-infection by 24 hours increases the amount of packaged DNA by approximately 1.7 fold. However, in separate experiments it has been observed that the number of cells in the 35 mm dish increased during the additional 24 hours, from 1.72×10^6 to 3.00×10^6 an increase of 1.7 fold. This suggests that, at an appropriately corrected MOI, the increase in packaged DNA may simply reflect the increased number of cells on the plate.

3.1.7 - The effect of the DMSO boost on transient packaging assay efficiency.

The treatment of a monolayer of cells with a solution of 25% DMSO (DMSO boost) has been shown to significantly increase the efficiency of calcium phosphate transfection as measured by either infectivity of viral DNA (Stow and Wilkie, 1976) or expression of a marker gene (such as *LacZ*) from a transfected plasmid (personal communication, N.Stow). The aim of this section was to establish whether the application of a DMSO boost was

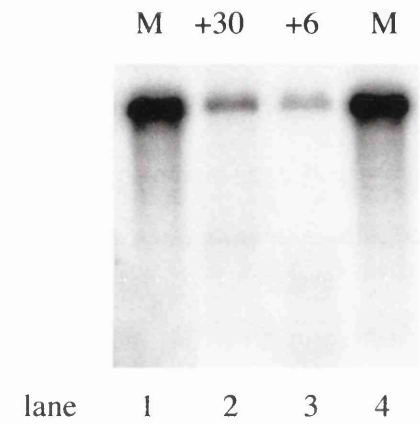


Figure 3.10 - Investigating the effect of delayed super-infection with helper virus, on packaging assay efficiency.

BHK 21 C13 monolayers were transfected with 1 μg of pSA1, using a calcium phosphate procedure. Monolayers were DMSO boosted at 4 hours post-transfection and super-infected with *wt*HSV-1 strain 17, (MOI, 1.0) at 6 (lane 3) or 30 (lane 2) hours post-transfection. Packaged DNA was harvested from the cells 16 hours post-infection. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lanes 1 and 4 (M) contain 4 ng of linear pSA1.

relevant in the context of the transient packaging assay, which does not require gene expression from the transfected DNA. The effect of a DMSO boost on *LacZ* expression from a transfected plasmid is shown in the experiment below.

A plasmid (pE*LacZ*, see figure 3.2 D) which contains the *LacZ* gene under the control of the immediate early HCMV promoter was transfected into duplicate BHK 21 C13 monolayers, using a calcium phosphate procedure. At 4 hours post-transfection one plate was boosted for 4 minutes with a 25% DMSO solution in HeBS. The other plate was mock boosted with wash. 24 hours post-transfection both plates were fixed and then stained to determine the expression of ß-galactosidase (section 2.2.4). The number of cells which stained blue were then counted using a microscope. The data is shown in table 3.3.

Table 3.3 - Effect of DMSO on β-galactosidase expression.

Observation	DMSO Boost	No DMSO Boost
	Blue cells per field of vision	Blue cells per field of vision
1	138	5
2	142	7
3	115	0
4	122	1
5	109	3
6	135	3
7	126	5
8	131	9
9	129	7
10	133	11
Average	128	5.1
Blue cells per plate	48256	1923
% efficiency	2.80±0.07%	0.11±0.02%

The number of blue cells were counted in 10 representative fields of vision and an average calculated. The area of a single field of vision is 1/377 the area of a 35 mm dish (962 mm^2) at 200 x magnification. From this the number of blue cells per plate could be calculated. Using a Heamatocytometer the number of BHK cells per plate was determined as $1.72x10^6$, (24 hours after seeding with $6x10^5$ cells). Using this figure the percentage of the cells transfected was calculated. The standard error for the calculation was calculated using the formula $se=d^{n-1}/\sqrt{n}$.

This data is expressed as a bar chart in figure 3.11 A. The DMSO boost increases the number of cells expressing ß-galactosidase more than 25 fold.

To test whether the DMSO boost also had an effect upon the efficiency of the transient packaging assay, duplicate BHK 21 C13 monolayers were transfected with 1 µg of pSA1 using a calcium phosphate procedure. One plate was DMSO boosted with 25% DMSO in HeBS at 4 hours post-transfection. The other plate was mock boosted with wash. Both sets of plates were super-infected with *wt*HSV-1 (MOI, 1.0) at 30 hours post-transfection. Packaged DNA was harvested 16 hours later. The DNAs were digested and Southern blotted as described in the methods section.

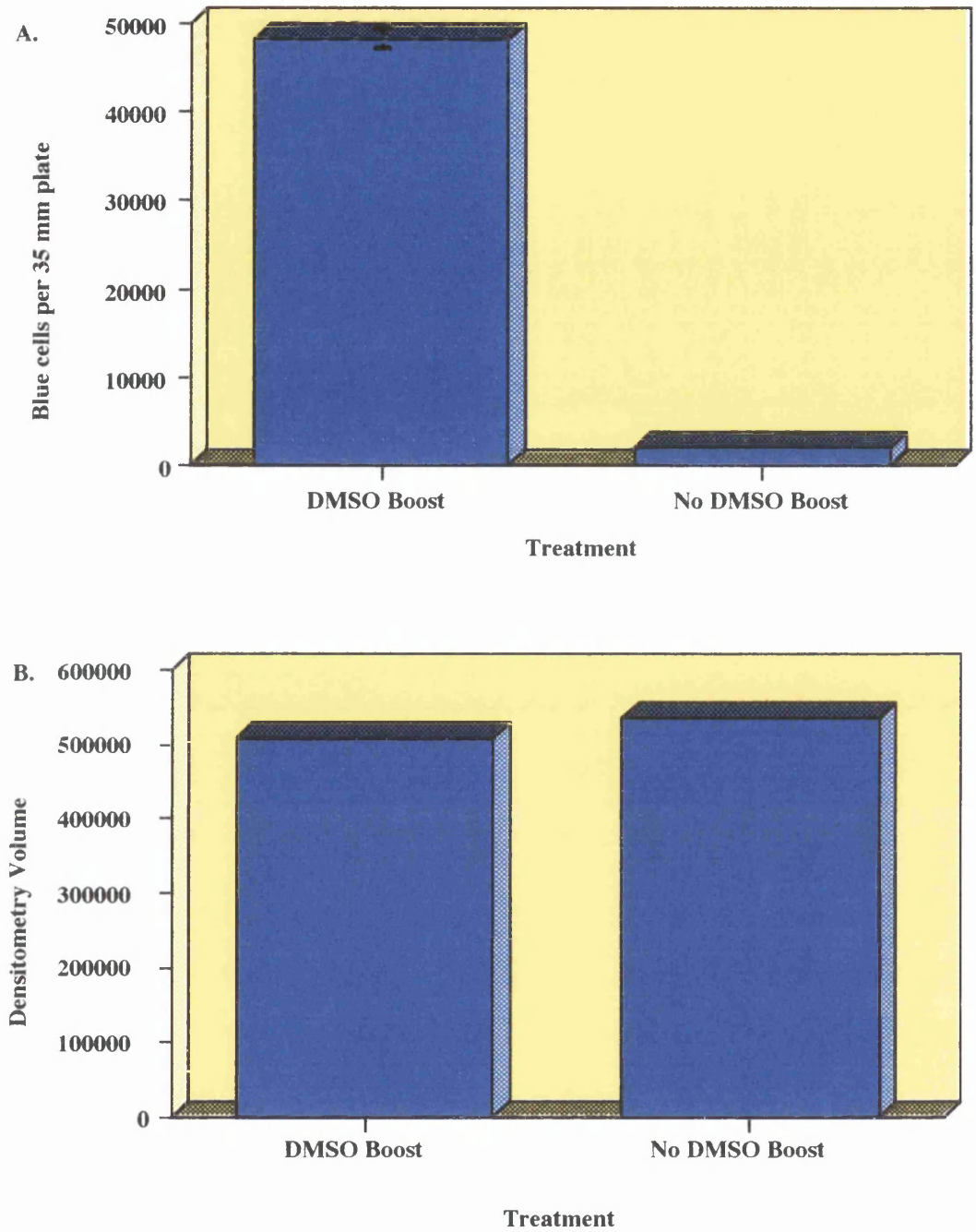


Figure 3.11 - Graphical representations of the effect of the DMSO boost.

Panel A., shows a graphical representation of the data from table 3.3. Showing the effect of a 25% DMSO boost on the number of cells expressing β-galactosidase, following calcium phosphate transfection of a plasmid containing the *LacZ* gene.

Panel B., shows shows a graphical representation of the data from figure 3.12. Showing the effect of a 25% DMSO boost on transient packaging efficiency.

The Phosphorimage from this experiment is shown in Figure 3.12. Lane 3 contains DNA from a mock boosted plate and lane 4 contains DNA from a boosted plate. The intensity of these bands was analysed using the phosphorimager software (**Molecular Dynamics**) and data is presented graphically in figure 3.11 B. This data indicates that a DMSO boost has no significant effect on the yield of packaged DNA.

A similar experiment was performed to examine the effect of DMSO on the transient packaging assay in BHK cells and a second cell line - Vero cells. Vero and BHK 21 C13 monolayers were again transfected with 1 μg of pSA1 using the calcium phosphate procedure. 4 hours post-transfection half the plates were DMSO boosted for 4 minutes with 25 % DMSO in HeBS. The remaining plates were 'mock boosted' with wash. Where appropriate the plates were mock infected or infected with *wt*HSV-1 (MOI, 1.0) at 30 hours post-transfection. Total and packaged DNA was harvested 16 hours later. The harvested DNAs were then digested and Southern blotted as described in the methods section. The phosphorimage from this experiment is shown in figure 3.13. Lanes 3-8 contain DNA harvested from Vero cells and lanes 11-18 contain DNA harvested from BHK cells. Again, in this experiment no correlation was found between the DMSO boost and assay efficiency in BHK cells. The level of replicated DNA in Vero cells appears to be slightly increased by DMSO treatment but there was little effect upon the amount of packaged DNA. In Vero cells the DMSO boost had a similar effect on *LacZ* expression from a transfected plasmid as that observed in BHK cells (data not shown). These observations combined suggest that the DMSO boost has little effect on the efficiency of the transient packaging assay in either cell line.

3.1.8 The u_c-DR1-u_b sequence serves as a cleavage and packaging signal for HSV-2.

A complicating factor in assessing the effects of mutations in the HSV-1 u_c-DR1-u_b element on packaging efficiency was considered to be the possibility of recombination between the amplicon and the helper virus to repair mutated sequences. One way to reduce recombination in the transient packaging assay might be to reduce the homology between the amplicon and the helper virus. The aim of this section was therefore to establish whether HSV-2 could replace HSV-1 in the transient packaging assay. Overall HSV-1 and HSV-2 share 83% identity at the nucleotide level, however, an alignment of the u_c-DR1-u_b sequence cloned into pSA1 with the corresponding regions of HSV-2 reveals only 54% nucleotide identity (figure 3.14). Nevertheless the 'a' sequences of HSV-1 and HSV-2 have previously been shown to be functionally interchangeable (Smiley *et al.*, 1992). Similarly, the HSV-1

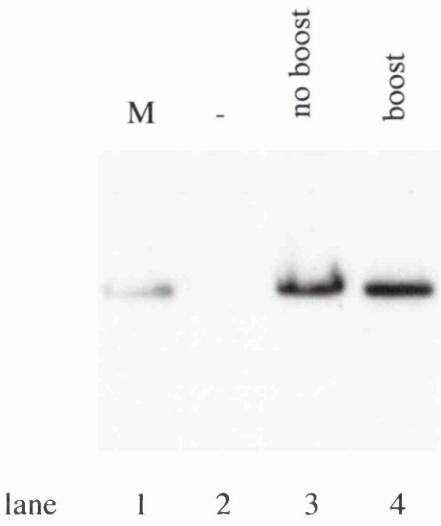


Figure 3.12 - Transient replication assay examining the effect of the DMSO boost on packaging efficiency.

BHK 21 C13 monolayers were transfected with 1 μg of pSA1, using the calcium phosphate procedure. At 4 hours post-transfection one monolayer (lane 4) was DMSO boosted with 25% DMSO in HeBS for 4 minutes. Another monolayer (lane 3) was mock boosted with wash. Each monolayer was super-infected with *wt*HSV-1 strain 17, (MOI, 1.0) at 30 hours post-transfection. Total DNA was harvested from the cells 16 hours post-infection. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lane 1 (M) contains 1 ng of linear pSA1.

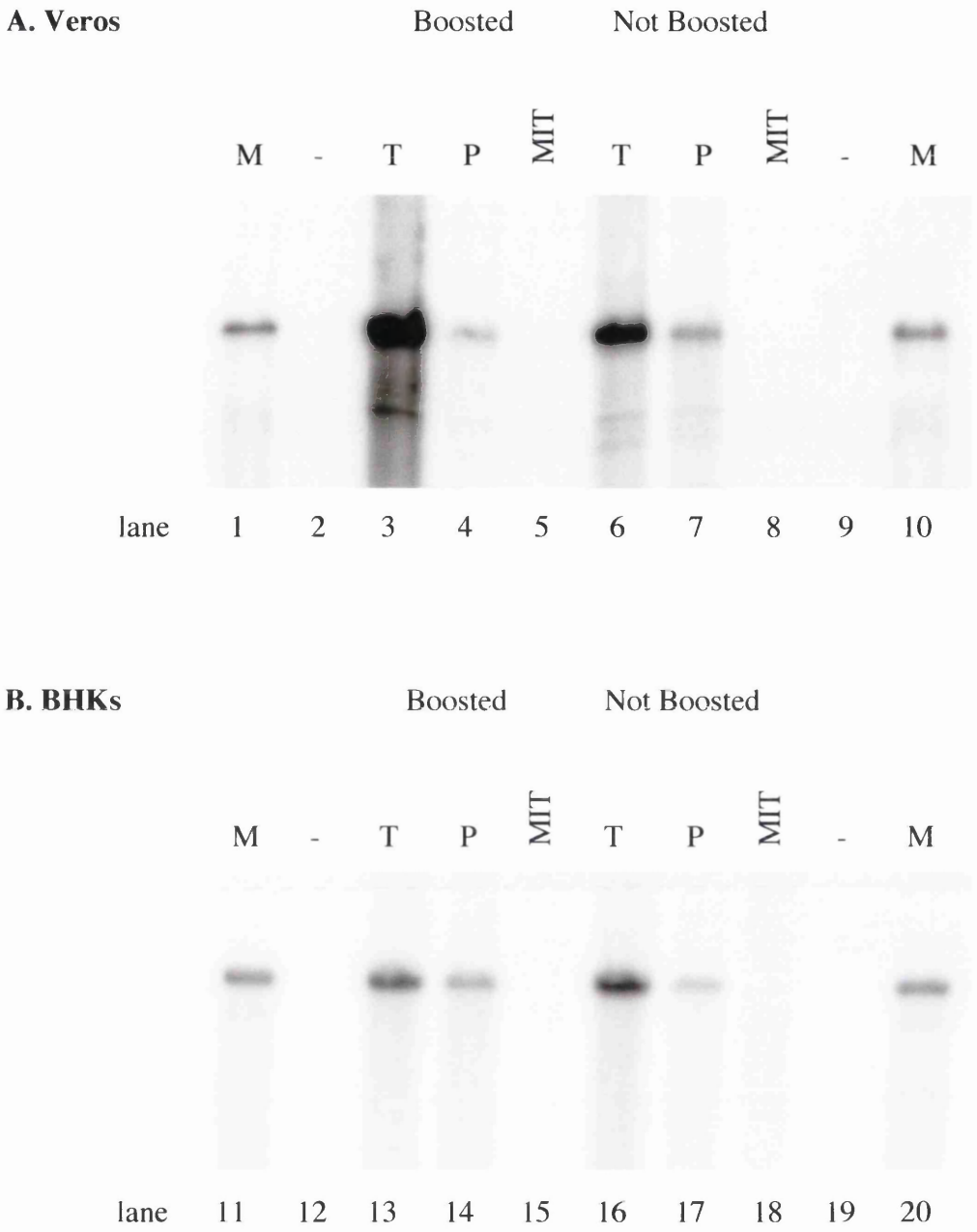


Figure 3.13 - Transient packaging assay examining the effect of the DMSO boost in vero and BHK cells.

Identical transient packaging assays were performed in either Vero (panel A) or BHK cells (panel B). Monolayers were transfected with 1 μg of pSA1, using the calcium phosphate procedure. At 4 hours post-transfection half the plates for each cell type were DMSO boosted with 25% DMSO in HeBS for 4 minutes (lanes 3-5 & 13-15). The other half (Lanes 6-8 & 16-18) were mock boosted with wash.

At 30 hours post-transfection experimental plates (3, 4, 6, 7, 13, 14, 16 and 17) were super-infected with *wt*HSV-1 strain 17, (MOI, 1.0). The control plates were mock-infected (MIT). 16 hours post-infection total (lanes 3, 5, 6, 8, 13, 15, 16 & 18) and packaged (lanes 4, 7, 14 & 17) DNA was harvested from the cells. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lanes 1,10,11 and 20 (M) contain 1 ng of linear pSA1.

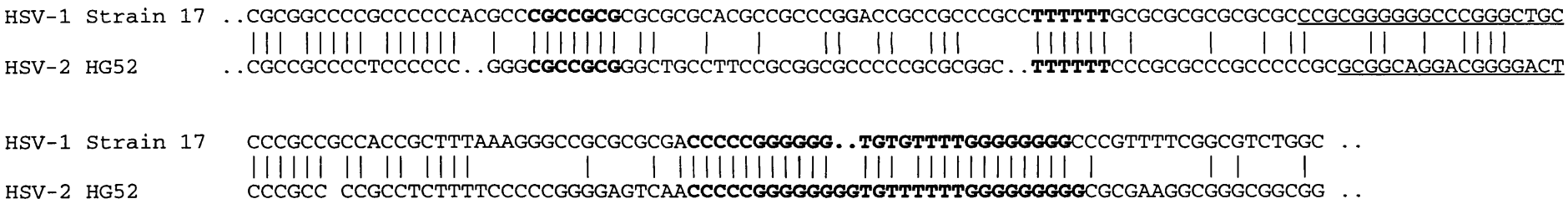


Figure 3.14 - Alignment of the HSV-1 and HSV-2 u_c-DR1-u_b sequences.

The above alignment of the HSV-1 Strain 17 and HSV-2 Strain HG52 u_c-DR1-u_b sequences indicates that there is approximately 54% nucleotide identity (the vertical bars indicate identical nucleotides), but, that the level of homology varies quite considerably across the region. The regions of homology between herpesviruses, as defined by Deiss *et al.*, (1986), are shown in bold. The DR1 repeat from HSV-1 and its equivalent in HSV-2 are underlined.

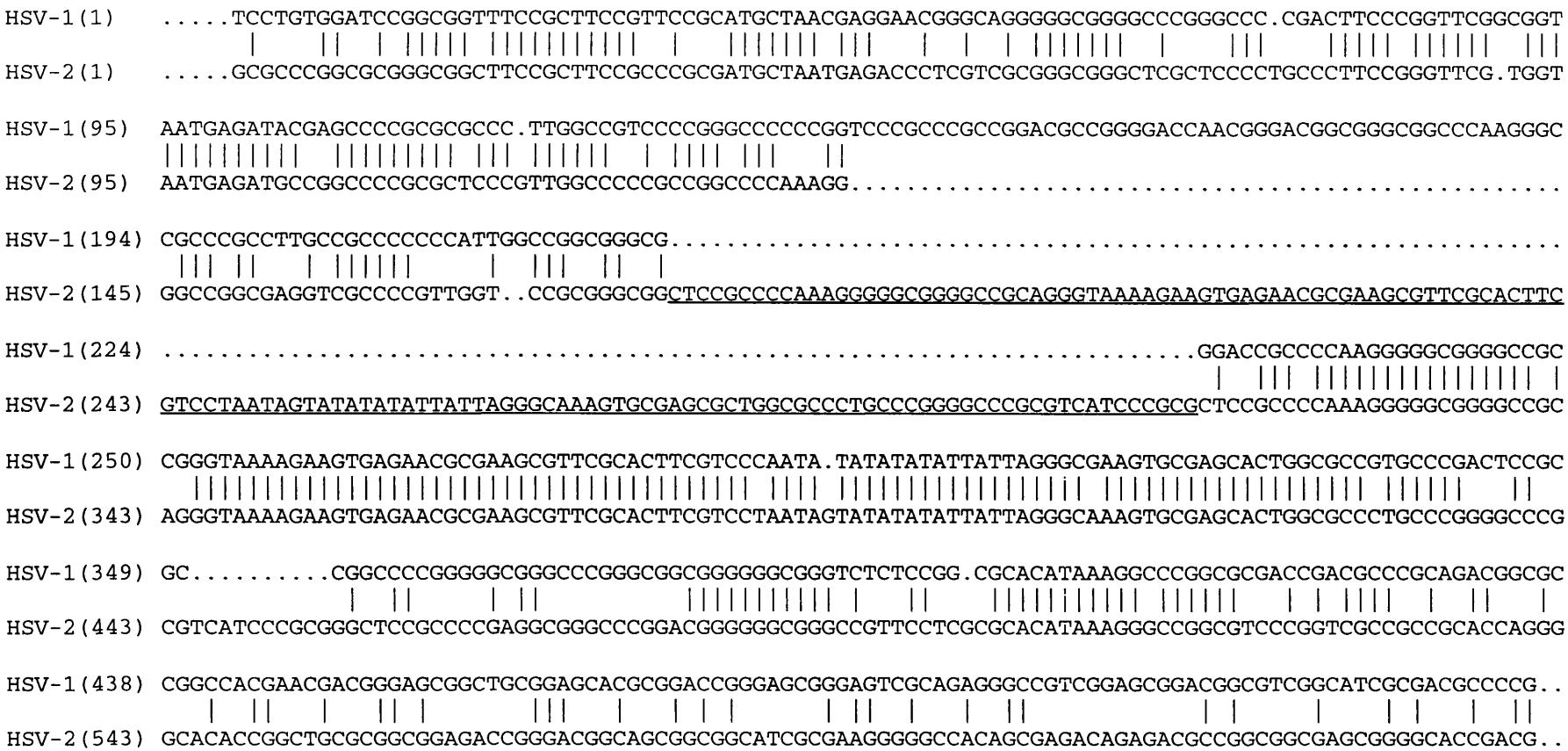


Figure 3.15 - Alignment of the HSV-1 and HSV-2 Ori$_s$ fragments.

This alignment of the HSV-1 strain 17 Ori$_s$ fragment from pSA1, with the comparable region of the HSV-2 strain HG52 genome, shows that there is approximately 56% nucleotide identity between the two sequences (305 of 536 HSV-1 residues conserved in HSV-2). The homology is however, noticeably variable. The core region of the origin, which is repeated twice in HSV-2 strain HG52 (the second repeat is underlined), is 88% identical in the two viruses while some of the flanking regions share very little homology.

Ori_s region present in pSA1 exhibits only 56% sequence identity with the corresponding region of the HSV-2 genome (Figure 3.15).

The ability of HSV-2 to utilise the HSV-1 Ori_s and u_c-DR1-u_b packaging signals in the transient packaging assay was examined in the experiment described below.

Duplicate BHK 21 C13 monolayers were transfected with 1 μg of pSA1 or pS1 using the calcium phosphate procedure. The plates were DMSO boosted at 4 hours post-transfection and helper functions were provided at 30 hours post-transfection. One plate was super-infected with *wt*HSV-1 strain 17 at an MOI of 2.0. The other plate was super-infected with *wt*HSV-2 strain HG52 at an MOI of 2.0. Total and packaged DNA was harvested 16 hours later. The harvested DNA was digested with *Eco*RI and *Dpn*I and Southern blotted as described in the methods section.

Figure 3.16 shows the phosphorimage of the blot from this experiment. Lanes 3 and 4 contain total DNA from cells transfected with pSA1 and super-infected with HSV-1 and HSV-2 respectively. Both lanes contain replicated DNA, although the total amount of replication appears slightly reduced when HSV-2 is used. Lanes 5 and 6 contain packaged DNA from cells transfected with pSA1 and super-infected with HSV-1 (lane 5), or HSV-2 (lane 6). Both viruses packaged DNA at approximately the same level. Lanes 8 - 11 contain DNA harvested from pS1 transfected cells. Again, HSV-1 replicated pS1 to a slightly higher level than HSV-2 (lanes 8 and 9). As expected, neither virus packaged any plasmid DNA (lanes 10 and 11) since pS1 lacks a packaging signal.

This experiment confirms that HSV-2 can utilise the HSV-1 Ori_s and u_c-DR1-u_b elements with similar efficiency to HSV-1. However, the reduced sequence homology between these signals and the corresponding region of HSV-2, would be expected to reduce the probability of recombination between amplicon and helper virus.

3.1.9 - Effect of HSV-2 multiplicity of infection on replication and packaging efficiency.

Having established that HSV-2 can utilise HSV-1 replication and packaging signals, the optimal MOI using HSV- 2 in the transient packaging assay was investigated.

BHK cell monolayers were transfected with 1 μg of pSA1 using the calcium phosphate procedure. The cells were DMSO boosted at 4 hours post-transfection and helper functions were provided by *wt*HSV-2 Strain HG52 at 30 hours post-transfection. Each plate was infected at one of the following MOIs; 0, 0.01, 0.05, 0.1, 1.0, 5.0, 10.0, or 20.0. Total and packaged DNA were harvested from each plate 16 hours post-infection. Harvested DNAs were digested with *Eco*RI and *Dpn*I and then Southern blotted as described in the methods section.

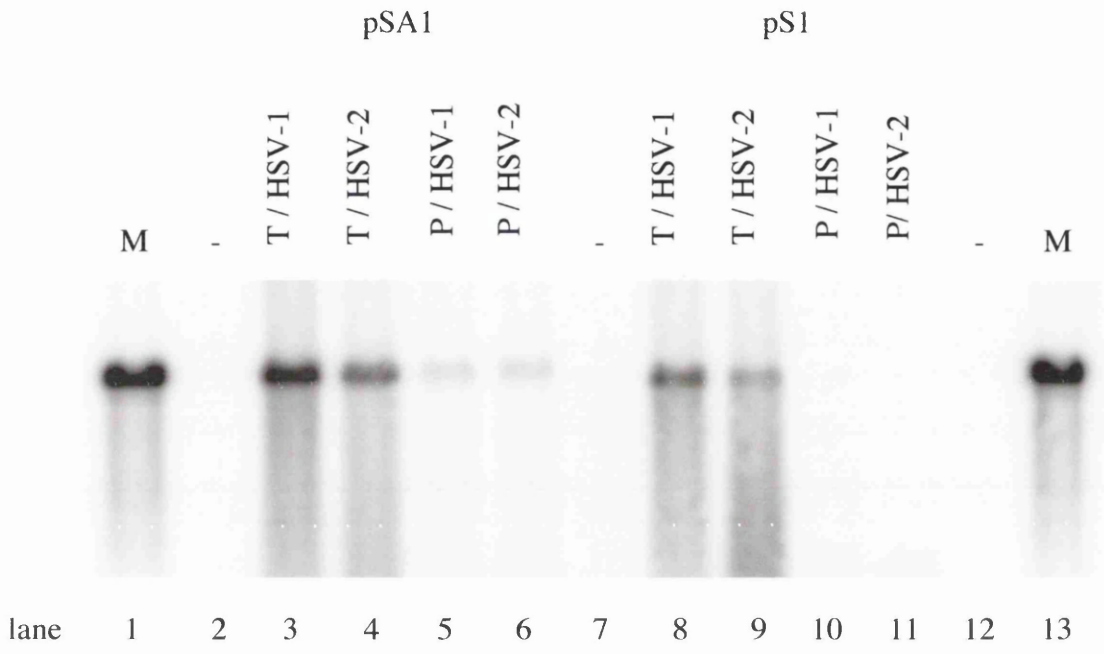


Figure 3.16 - The HSV-1 u_c-DR1-u_b sequence serves as a cleavage and packaging signal for HSV-2.

BHK 21 C13 monolayers were transfected with 1 μg of pSA1 (lanes 3-6) or pS1 (lanes 8-11), using the calcium phosphate procedure. Monolayers were DMSO boosted 4 hours post-transfection and super-infected with either *wt*HSV-1 strain 17, (lanes 3,5,8 & 10) or *wt*HSV-2 strain HG52 (lanes 4,6,9 & 11) (MOI, 2.0) at 30 hours post-transfection. 16 hours post-infection total (lanes 3,4,8 & 9) and packaged (lanes 5,6,10 & 11) DNA was harvested from the cells. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lane 1 and 13 (M) contain 4 ng of linear pSA1.

Table 3.4 - Effect of multiplicity of infection with HSV-2.

MOI	Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5
0.00	0.857	0.812 (n/a)
0.01	2.91	0.207 (7.1%)
0.05	15.2	9.88 (65.0%)
0.10	24.1	15.5 (64.5%)
1.0	42.4	12.2 (28.8%)
5.0	10.4	5.57 (53.6%)
10.0	12.8	6.48 (50.6%)
20.0	8.00	6.51 (81.4%)

All data is shown to 3 significant figures. The percentage of total DNA which is packaged is shown in brackets.

The phosphorimage (figure 3.17) shows a similar pattern to that seen with HSV-1. The amount of total (lanes 2-9) and packaged (lanes 12-19) DNA initially increased with increasing helper virus and then declined. The intensity of the bands was measured using the phosphorimager software (**Molecular Dynamics**) and the data is shown in table 3.4. and figure 3.18.

The results show that maximum efficiency for replication was obtained at 1.0 pfu $cell^{-1}$ and the maximum packaging (both in terms of absolute amount and the proportion of replicated DNA, packaged) was obtained at 0.1 pfu $cell^{-1}$.

3.1.10 - Alternative method of harvesting total and packaged DNA.

The method for harvesting total and packaged DNA from cells involves multiple steps, culminating in phenol and chloroform extractions and ethanol precipitation. The extraction of multiple samples in this way can be extremely time consuming especially when the experiment has a large number of samples. With this in mind an attempt was made to simplify the method, by replacing the phenol and chloroform extractions and ethanol precipitation with a single isopropanol precipitation. Isopropanol precipitation offers a fast and efficient method of DNA precipitation from cell lysates, e.g. precipitation of DNA from small scale DNA preparations and **Qiagen** purifications.

A BHK cell monolayer was transfected with 1 μg of pSA1 using calcium phosphate procedure. The cells were DMSO boosted at 4 hours post-transfection and helper functions

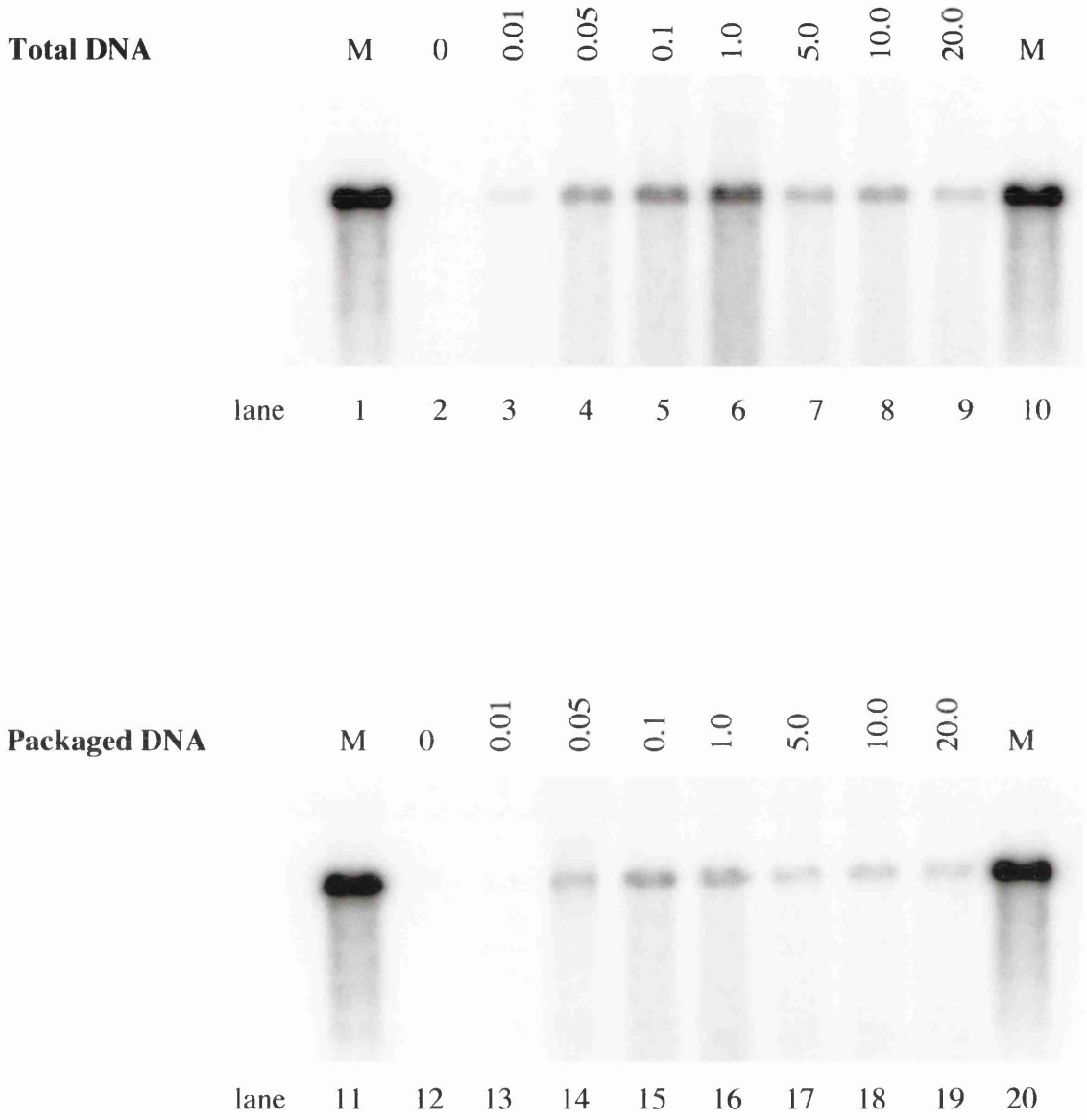


Figure 3.17 - Transient packaging assay examining the effect of multiplicity of infection (with HSV-2) on replication and packaging efficiency.

BHK 21 C13 monolayers were transfected with 1 μg of pSA1 using a calcium phosphate procedure. Monolayers were DMSO boosted at 4 hours post-transfection and super-infected with *wt*HSV-2 strain HG52, (MOI of; 0, 0.01, 0.05, 0.1, 1.0, 5.0, 10.0 or 20.0) at 30 hours post-transfection. 16 hours post-infection total (lanes 2-9) and packaged (lanes 12-19)DNA was harvested from the cells. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lanes 1, 10, 11 and 20 (M) contain 4 ng of linear pSA1

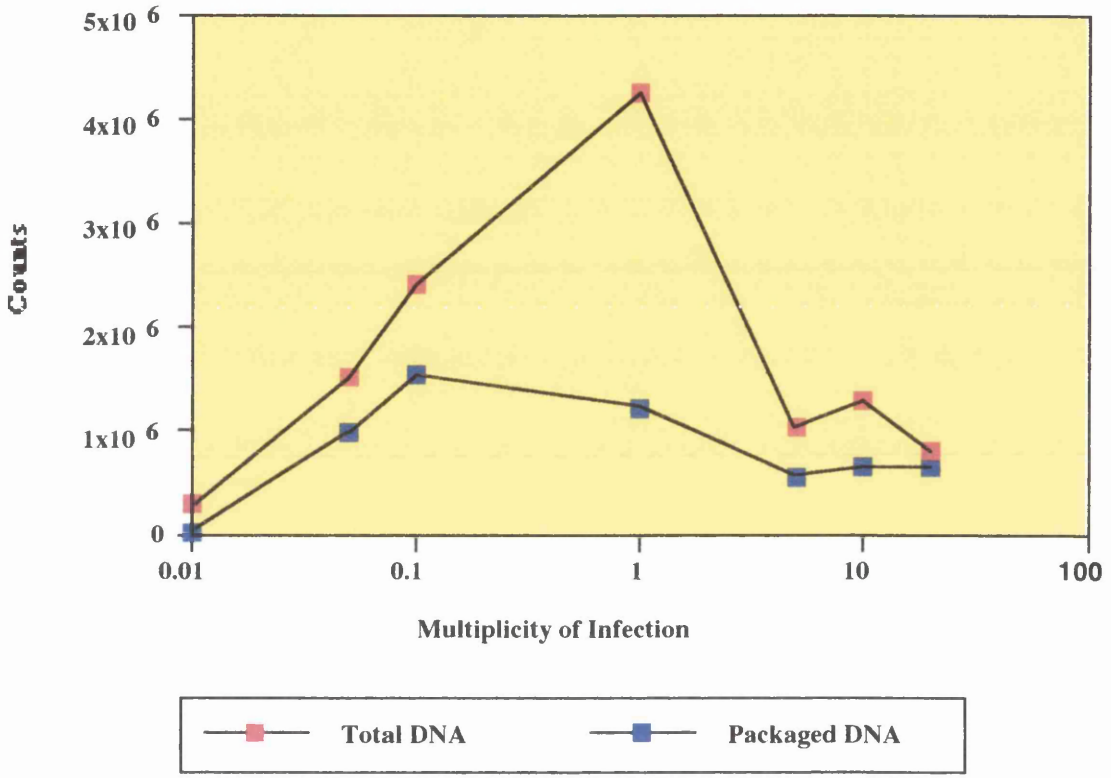


Figure 3.18 - Graphic representation of HSV-2 multiplicity of infection data.

The band intensity data from table 6 was plotted against multiplicity of infection on a log scale.

were provided by *wt*HSV-1 at 30 hours post-transfection. 16 hours later total and packaged DNA were harvested. Following incubation, with 1 x CLB and 0.5 mg ml^{-1} protease, the nucleic acids were precipitated using an equal volume of isopropanol. The harvested DNAs were then digested with *Eco*RI and *Dpn*I and Southern blotted as described in the methods section.

Figure 3.19 shows the phosphorimage from this experiment. Prior to Southern blotting, when the agarose gel was visualised using a UV lamp, the DNA appeared incompletely digested - the cellular DNA being largely near the wells as opposed to evenly distributed down the lane. This observation was confirmed when the Southern blot was inspected. Lanes 2 and 3 (which contain total and packaged DNA respectively) contain multiple high molecular weight bands which correspond to the partially cleaved concatemeric molecules produced by rolling circle replication. These intermediates would normally be resolved to unit lengths by complete *Eco*RI digestion. In this case, a possible cause of inefficient digestion of the harvested DNA was that the DNA pellet was contaminated with protease from the lysis stage.

In an attempt to circumvent this problem another transient packaging assay was performed, exactly as described above except that duplicate plates were used. The DNA from one plate was harvested as described above, and the resuspended DNA was then treated with 67 μM PMSF (PMSF is a potent protease inhibitor). The second plate was harvested as usual, with a phenol and chloroform extraction and ethanol precipitation.

The DNA from both plates was then digested with *Eco*RI and *Dpn*I and Southern blotted as described in the methods section (data not shown). Efficient digestion was observed in the presence of PMSF, however, the yields of replicated and packaged DNA was significantly reduced compared to the yields achieved with the standard protocol. This and subsequent experiments showed that, while good recovery of total DNA could be achieved using the isopropanol precipitation method, consistent recovery, especially of the small amount of packaged DNA, was a problem. For this reason the DNA in subsequent experiments was extracted with phenol and chloroform and ethanol precipitated.

3.1.11 - Comparison of original and modified protocols for the transient packaging assay.

The modified protocol, incorporating the various changes described above was compared to the original transient packaging assay in a single experiment.

One BHK cell monolayer (original protocol) was transfected with 3 μg of pSA1 using the calcium phosphate procedure. The monolayer was DMSO boosted at 4 hours post-transfection and then super-infected (at a multiplicity of 10.0) with *wt*HSV-1 at 6 hours

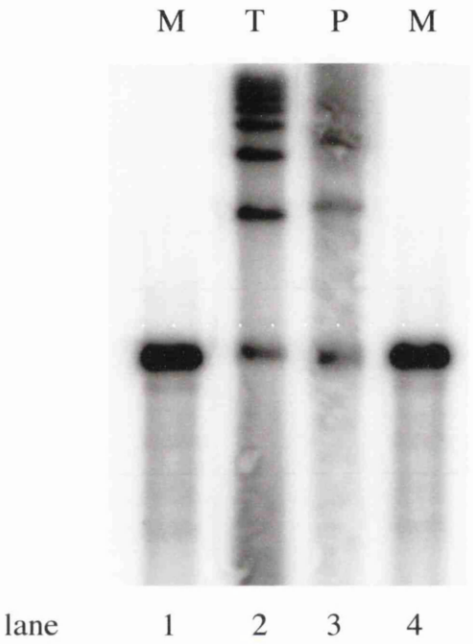


Figure 3.19 - The transient packaging assays using isopropanol precipitation of nucleic acids as opposed to phenol, chloroform and ethanol precipitation.

BHK 21 C13 monolayers were transfected with 1 μg of pSA1 using the calcium phosphate procedure. Monolayers were DMSO boosted at 4 hours post-transfection and super-infected with *wt*HSV-1 strain 17 (MOI, 2.0) at 30 hours post-transfection. Total and packaged DNA were harvested from the cells 16 hours post-infection and precipitated with an equal volume of isopropanol. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153.

Lane 2 contains total DNA and Lane 3 contains packaged DNA. The ladder of bands is indicative of incomplete digestion which is possibly caused by protease carried over from the cell lysate. Lanes 1 and 4 (M) contain 4 ng of linear pSA1.

post-transfection. Total and packaged DNA was prepared 16 hours later, phenol and chloroform extracted, ethanol precipitated and resuspended in TE pH 8.0. A duplicate monolayer (optimised conditions) was transfected with 1 μg of pSA1 using the calcium phosphate procedure. The monolayer was DMSO boosted at 4 hours post-transfection and then super-infected (at a multiplicity of 1.0) with *wt*HSV-1 at 30 hours post-transfection. Total and packaged DNA was prepared 16 hours later, phenol and chloroform extracted, ethanol precipitated and resuspended in TE pH 8.0. The harvested DNAs were digested with *Eco*RI and *Dpn*I and Southern blotted as described in the methods section.

The phosphorimage of this blot is shown in figure 3.20. Lanes 3 and 4 contain packaged and total DNA respectively, prepared using the original protocol. Lanes 6 and 7 contain packaged and total DNA respectively, prepared using the optimised protocol. The intensity of each band was measured using the phosphorimager software (**Molecular Dynamics**) and the data from this experiment (experiment 1) and a repeat experiment is shown in table 3.5. The intensity of each band is given and the percentage increase between the two protocols is also shown.

Table 3.5 - Comparison of the original and optimised protocols for the transient packaging assay.

Conditions	Experiment 1		Experiment 2	
	Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5	Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5
Original	74.9	23.1 (30.8%)	9.03	3.82 (42.3%)
Optimised	115	45.3 (39.1%)	35.0	9.09 (26.0%)
fold increase	1.53	1.96	3.87	2.38

The percentage of total DNA which is packaged is shown in brackets.

This data shows that the optimised protocol results in a significant increase in the efficiency of the assay. These conditions were therefore used in all subsequent experiments.

3.1.12 - Discussion.

3.1.12.1 - Functional packaging signals.

The observation that the HSV-1 packaging signals in pY1, pZ1 and pSA1 are all functional, while agreeing with the published data (Stow *et al.*, 1983; Nasseri & Mocarski, 1988), raises the question of which sequence represents the functional signal *in vivo*. The minimum functional packaging signal so far identified is the u_c-DR1-u_b sequence. In this arrangement the essential pac1 and pac2 homologies are brought into close proximity, the cleavage occurring between the two. It has been proposed that this element constitutes the *wt* cleavage and packaging signal, as this arrangement of sequences occurs between two tandemly repeated 'a' sequences and cleavage would generate genomic termini which

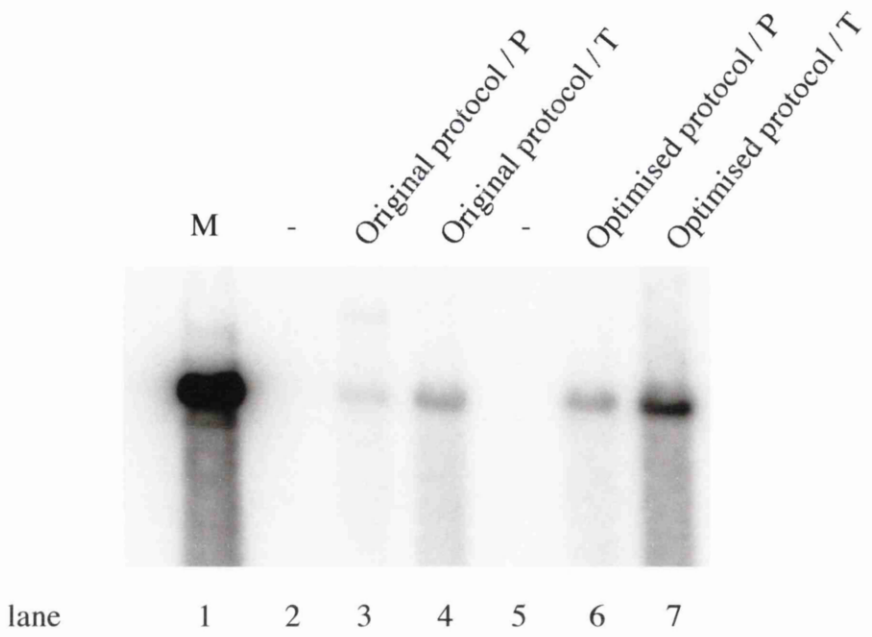


Figure 3.20 - Comparison of original and optimised protocols for the transient packaging assay.

Lanes 3 and 4 contain total (lane 3) and packaged (lane 4) DNA from a monolayer which was transfected with 3 μg of pSA1 using a calcium phosphate procedure. The monolayer was DMSO boosted at 4 hours post-transfection and super-infected with *wt*HSV-1 strain 17 (MOI, 10.0) at 6 hours post-transfection. 16 hours post-transfection the DNA was harvested using a phenol chloroform extraction and ethanol precipitation.

Lanes 6 and 7 contain total (lane 6) and packaged (lane 7) DNA from a monolayer which was transfected with 1 μg of pSA1 using a calcium phosphate precipitate. The monolayer was DMSO boosted at 4 hours post-transfection and super-infected with *wt*HSV-1 strain 17 (MOI, 1.0) at 30 hours post-transfection. 16 hours post-infection the DNA was harvested using a phenol chloroform extraction and ethanol precipitation. The harvested DNAs were *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lane 1 (M) contains 4 ng of linear pSA1.

terminate in 'a' sequences (Nasseri and Mocarski, 1988). However, while pSA1 and pZ1 contain this arrangement of sequences, pY1 (which contains a single 'a' sequence) does not. If the u_c-DR1-u_b sequence represents the functional packaging signal *in vivo* then pY1 must have gained either all, or part of, an 'a' sequence in order to generate this arrangement. Alternatively, the cleavage and packaging mechanism may not have an absolute requirement for the pac1 and pac2 homologies to be in close proximity allowing the single 'a' sequence of pY1 to direct cleavage and packaging.

Some insight into the mechanism of cleavage and packaging can be obtained by considering the terminal fragments seen in section 3.1.3. pY1 generates a single terminal band while pZ1 generates two. This suggests that pY1 contains one cleavage site and pZ1 contains two (at the time of cleavage and packaging). The size of the fragments may provide further insight. Input plasmid pY1 contains two DR1 repeats where virus directed cleavage may potentially be occurring. Cleavage at these DR1 repeats would be predicted to give rise to two large terminal fragments of 4430 and 4829 bp respectively. However, only one terminal fragment is observed and estimates of its size, based on the distance the band has migrated on the agarose gel suggests that it is 4829 bp in size. For pZ1, three putative large terminal fragments are predicted from the location of the DR1 repeats. These are 4430, 4829 and 5228 bp in size. In fact, only two are observed and their sizes are consistent with the expected locations of the two largest fragments. The estimation of fragment sizes was made by comparing the relative migration of the terminal fragments and fragments of known size such as the linearised markers. The smallest, predicted, fragments which are not observed, would be produced by cleavage in the DR1 repeat closest to the *Hin*dIII site in the plasmid producing a concatemer which lacks an 'a' sequence at its terminus. These data are compatible with the pac2 sequence in u_c being necessary for the generation of the long terminus of pY1 and pZ1 molecules, as the common feature of the long termini observed is an adjacent u_c sequence (figure 3.21). Although no experiments were done to analyse the opposite terminus of these packaged molecules, it is possible that the pac1 sequence in u_b might have a function in the generation of packaged DNA molecules containing an 'a' sequence at this terminus.

Provided that the input amplicon is the same as the packaged amplicon, then pY1, with its single 'a' sequence, will generate one terminus lacking an 'a' sequence for every terminus which possesses one. Two possible scenarios regarding DNA packaging can be derived from this. On the one hand, DNA packaging may initiate at an 'a' sequence and terminate at the next 'a' sequence, cleaving the viral concatemer in such a way as to give a packaged terminus which lacks an 'a' sequence and an unpackaged concatemer which terminates with an 'a' sequence (figure 3.22 A). Thus unpackaged concatemer would then be the substrate

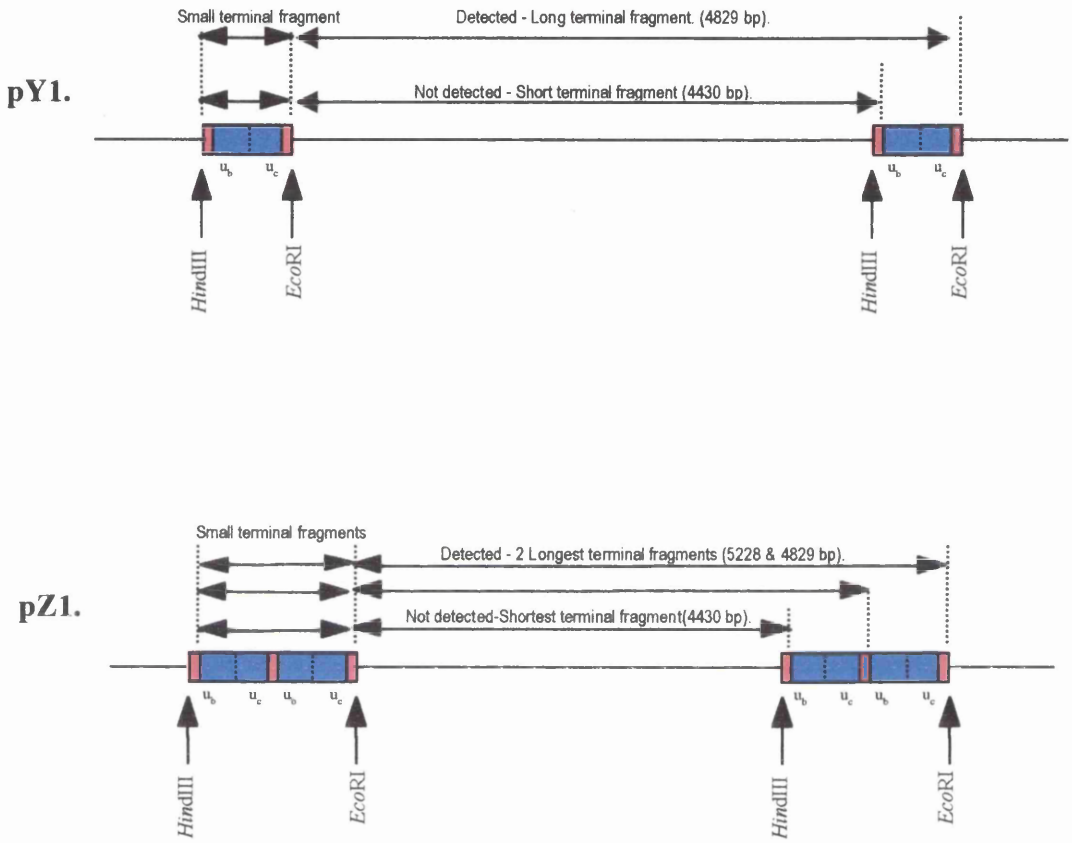


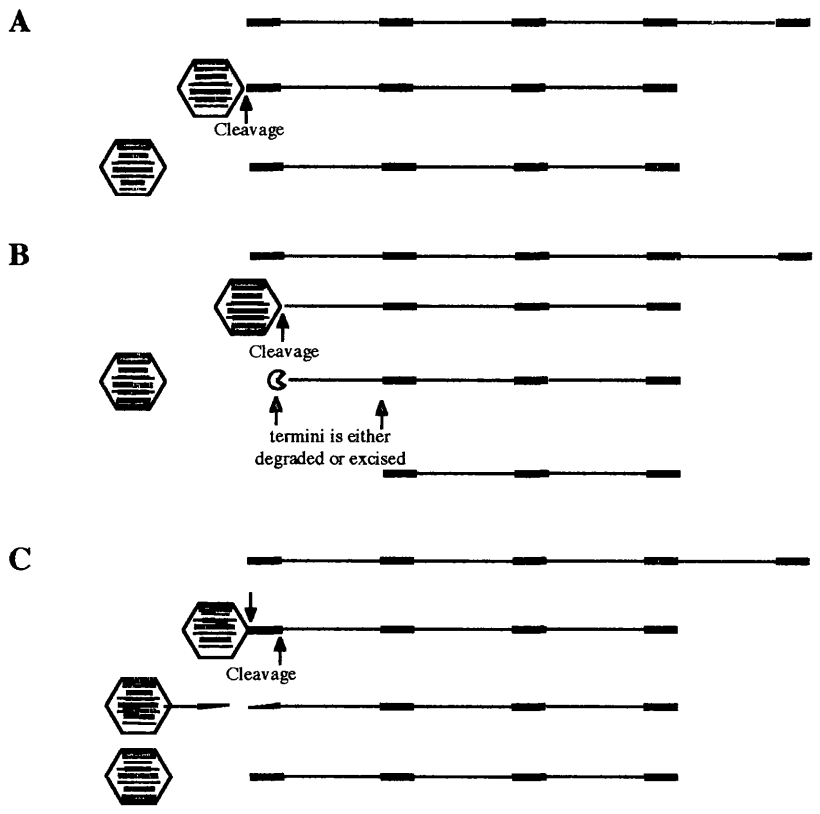
Figure 3.21 - Predicted packaged products of pY1 and pZ1 in the transient packaging assay.

The terminal fragments generated during the transient packaging assays with pY1 or pZ1 are shown. Of note is the observation that only large terminal fragments with u_c terimi were observed.

for subsequent packaging events. This mechanism possibly operates to produce R-O GPCMV genomes which lack the terminal repeat at one end (McVoy *et al.*, 1997). However, HSV-1 always has at least one 'a' sequence at each terminus so this mechanism is unlikely to operate in HSV-1. An alternative scenario is the 'theft' model of DNA packaging (Varmuza & Smiley, 1985). In this model cleavage occurs before the first 'a' sequence, at least one unit length is then packaged and a second cleavage event occurs after the next 'a' sequence. The packaged molecule thus contains an 'a' sequence at each end but the free end of the concatemer lacks an 'a' sequence (figure 3.22 B). Packaging might resume at the next 'a' sequence in the concatemer but the intervening DNA would need to be degraded or excised. This appears to represent a wasteful method of DNA packaging which the virus probably manages to avoid. This is because the virus genome contains at least one 'a' sequences at each terminus which are linked together when the viral genome circularises. The presence of tandem 'a' sequences in the replicated concatemer would allow cleavage to produce both packaged molecules and concatemer ends containing 'a' sequences. In the case of the transient packaging assay using pY1 the theft mechanism would not be as wasteful as it would be for the viral genome, which may explain why pY1 does not appear to be impaired with respect to pZ1 and pSA1. The pY1 plasmid is considerably smaller than the viral genome and concatemers containing as many as 40 copies of pY1 may be packaged into one viral capsid. After a cleavage event which produced a concatemer lacking a terminal 'a' sequence, only one unit length would be lost before a functional terminus was regenerated, (i.e. 1/40 of the amount packaged). Therefore, if pY1 were packaged via the theft of an 'a' sequence from a neighbouring molecule it would probably not appear to be noticeably impaired with respect to pZ1 and pSA1.

An alternative is that pY1 has gained all or part of an 'a' sequence in order to generate a u_c-DR1-u_b sequence arrangement. From this work the possibility that this is occurring cannot be totally eliminated, however, a number of observations suggest that it is unlikely. If high frequency recombination were occurring between the pY1 plasmid and helper virus, or even between pY1 molecules, then plasmid monomers which are greater than unit length would be observed. The addition of an entire 'a' sequence to pY1 would generate a sequence similar to that present in pZ1, and as a consequence multiple large terminal fragments would be expected to be observed. Since these effects were not observed the possibility that pY1 has gained any sequence seems unlikely.

Finally, cleavage could be occurring via the staggered nick model (Varmuza & Smiley, 1985). This mechanism requires that a nick be introduced at either end of the 'a' sequence, on alternate strands, so that either strand separation or repair synthesis could effectively generate an approximately 400 bp long cohesive end. This, however, would suggest a



1. A concatemer of viral genomes generated by rolling circle replication.
2. A viral genome with only one terminal repeat is packaged into a preformed capsid.
3. The unpackaged concatemeric molecule is able to act as a substrate for subsequent cleavage and packaging events.

1. A concatemer of viral genomes generated by rolling circle replication.
2. A viral genome with two terminal repeats is packaged into a preformed capsid.
3. The unpackaged concatemer has an un-packageable terminus which must be either excised or degraded.
4. A packageable terminus is regenerated.

1. A concatemer of viral genomes generated by rolling circle replication.
2. Packaging is initiated at an initial terminal repeat and a unit length genome is packaged. At the terminal repeat a staggered nick is introduced at either ends of the repeat.
3. The two strands of the DNA are then separated and repair synthesis copies the repeat sequence from the complementary strand. In reality these to steps would probably be performed simultaneously by repair synthesis initiating at the nicks accompanied by strand displacement.
4. A genome with two terminal repeats is packaged and a packageable terminus is regenerated.

Figure 3.22 - Mechanisms for packaging genomes with only one terminal repeat region.

Three mechanisms by which genomes (or amplicons) which only have a single 'a' sequence may be packaged are shown above. Mechanism **A** involves packaging the genomes, with only one terminal repeat. This mechanism is not seen in *wt HSV*-1 which always has two terminal repeats but it may be similar to the mechanism which generates RO genomes in GPCMV (McVoy *et al.*, 1997) and it is conceivable that it may occur in amplicon packaging systems. Mechanism **B** is the 'theft model' of DNA packaging proposed by Varmuza and Smiley (1985). In this mechanism a terminal repeat is 'stolen' from a neighbouring genome in the concatemer. The neighbour is then unpackageable and must be degraded or excised if a packageable terminus is to be regenerated. Mechanism **C** is the repair synthesis model. This mechanism duplicates a terminal repeat by separate the two strands of the repeat between two staggered repeat and regenerating the repeats by repair DNA synthesis (Varmuza and Smiley, 1985).

continued amplification of the 'a' sequence which is not observed during natural infection or the passaging of defective genomes.

Despite all of the uncertainties it is clear that the u_c-DR1-u_b sequence can act as a functional cleavage and packaging signal for HSV-1, and, as the smallest sequence available represents an ideal template with which to conduct a mutagenesis study.

3.1.12.2 - The variation in efficiency between lipofection and calcium phosphate transfection assays.

The observation that calcium phosphate precipitation results in more efficient replication and packaging than lipofection is surprising. When a *LacZ* containing plasmid was used, lipofection reproducibly resulted in far higher numbers of cells expressing ß-galactosidase than with calcium phosphate. It is possible that differences between the relative number of plasmid molecules delivered to the cells, or the nuclear sites to which they are delivered, may account for this difference. Another possible explanation may be that the process of lipofection has a detrimental effect on the subsequent expression of helper functions by super infecting virus.

3.1.12.3 - Multiplicity of infection.

The difference between the optimal MOI for replication and packaging, using HSV-1 or HSV-2, probably reflects a fine balance between providing sufficient amounts of the proteins necessary for the replication and packaging and competition for the proteins between the amplicon and viral genomes. It is worth remembering that for HSV-1 the particle to pfu ratio is between 10:1 and 50:1 (Stow & Stow, 1986). This means that for every genome which is delivered to the cell and goes on to establish a successful infection, as many as 50 genomes may also be delivered but, for some reason, fail to establish a productive infection. A proportion of this pool of viral genomes may also be able to sequester the viral proteins necessary for efficient DNA packaging.

There were two reasons for repeating the multiplicity of infection experiments with HSV-2. The origin of replication and the packaging signal in pSA1 are both from HSV-1 and although HSV-2 can functionally substitute for HSV-1 as helper virus, the interactions between these elements and the viral proteins may not be optimal. Secondly, HSV-1 helper virus was prepared as a cell released virus (CRV) preparation, i.e. virus particles released from the cell. For HSV-2, as less virus is actually released into the media, the virus was prepared as a cell associated virus (CAV) preparation. It was observed that the application of

CAV at a given MOI resulted in a far higher cpe than the same MOI applied with CRV, possibly due to the effects of intra cellular proteins.

The observed optimal multiplicities of infection with HSV-2 were lower than those observed for HSV-1, and this may have several causes. The higher cpe observed using HSV-2 probably plays a role in reducing the optimal multiplicity, because at higher MOIs increasing numbers of cells detach from the plate. It is also possible that the higher particle to pfu ratio for HSV-2 has a role. The protein expression from the larger number of non infectious genomes delivered by HSV-2 may elevate the level of viral proteins *in vivo,* with the corresponding effect of reducing the multiplicity required for maximum efficiency replication or packaging.

3.1.12.4 - Delayed super infection.

An increase in the efficiency of replication and packaging was associated with delayed super infection with wild type virus . It seems likely that the majority of the increase seen is a consequence of the increase in the number of cells available as centres for viral replication and packaging. When the cells divide after transfection the transfected pSA1 would be expected to divide randomly between the two daughter cells. This means that theoretically, provided transfected cells contain a reasonable number of plasmid molecules (e.g. 20), it is highly probable that both daughter cells will receive some plasmid DNA. As it is does not appear that the number of plasmid molecules per cell is a limiting factor in the yield of the transient assays (see section 3.1.4), spreading the same amount of DNA between a larger number of potential centres for viral replication will have the effect of increasing the overall amount of plasmid which is replicated and packaged.

3.1.12.5 - The DMSO boost.

The results presented in section 3.1.7 showed that although DMSO increased gene expression from a β-galactosidase expressing plasmid which was delivered to the cells by the calcium phosphate technique it had little effect on plasmid replication.

DMSO has a range of biological effects. It is commonly used as an antifreeze for proteins, an additive to cell storage media, and inducer of cellular differentiation (Nomura & Oishi, 1983). *In vitro* it has been shown to up-regulate transcription (Juang & Liu, 1987), as well as increasing the infectivity of viral RNAs and DNAs (Tovell & Coulter, 1969; Stow & Wilkie, 1976).

The mechanism by which DMSO, when employed in conjunction with the calcium phosphate technique, is able to increase the infectivity of viral DNA and increase the level of

expression from transfected DNA is not clearly understood. However, the failure of DMSO to increase plasmid replication in this assay provides evidence that it may act at the level of gene expression rather than directly on DNA delivery.

Section 3.2 - Site directed mutagenesis of the HSV-1 packaging signal, u_c-DR1-u_b.

3.2.1 - Introduction.

The 'a' sequence of HSV-1 is composed of a number of repeat regions and two quasi unique regions called u_b and u_c. Parts of these quasi unique regions have attracted particular attention as similar sequences have been identified at the termini of a wide variety of herpesviruses (Spaete & Mocarski, 1985; Diess *et al.*, 1986; McVoy *et al.*, 1998). The observation that these same two regions are essential for cleavage and packaging of the viral genome has directly implicated them in the cleavage and packaging process and has lead to them being labelled the pac1 and pac2 homologies (Deiss *et al.*, 1986). Comparison of the terminal sequences from many different herpesviruses, including those which lack terminally redundant sequences, has lead to a proposed breakdown of the pac1 and pac2 homologies into a number of different elements (Deiss & Frenkel, 1986). Figure 3.23 shows these different elements in the arrangement found in the 200 bp packaging signal present in amplicon pSA1. This sequence spans the junction between two tandemly repeated 'a' sequences. It is proposed that for HSV-1 pac1 comprises 3 distinct elements; 2 GC rich elements of 43 and 12 bp separated by a T rich element of 8 bp. Deiss *et al.* (1986) proposed that the pac2 homology of HSV-1 is composed of 4 distinct elements; a 7 bp consensus sequence (CGCCGCG), 31 bp of unconserved sequence, a 6 bp T rich element and a 14 bp GC rich element. The pac1-containing u_b and pac2-containing u_c sequences are separated by a single DR1 repeat region which contains the site of viral cleavage, although the actual sequence of the DR1 repeat has been shown to be non-essential for cleavage to occur (Varmuza and Smiley, 1985)

The aim of the work described in this section was to alter the u_c-DR1-u_b packaging signal by constructing specific substitution and deletion mutations. The mutated packaging signals would then be tested in the transient packaging assay described in section 3.1 to evaluate the roles of the various sequence elements.

The substitution and deletion mutations of the u_c-DR1-u_b packaging signal were largely based on the individual elements proposed by Deiss & Frenkel (1986). In addition the entire pac1 and 2 homologies were individually deleted and a substitution mutation at the site of viral cleavage in the DR1 repeat was constructed. A complete list of the designed mutations is shown in table 3.6.

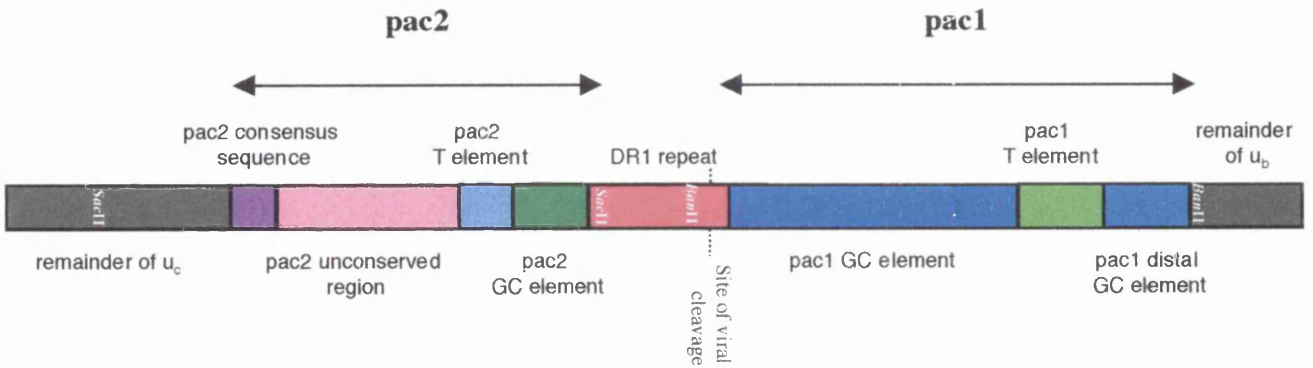


Figure 3.23 - Detailed structure of the u_c-DR1-u_b packaging signal for HSV-1.

It has been proposed that the u_c-DR1-u_b sequence of HSV-1 contains a number of distinct elements, (Deiss *et al.*, 1986) The designation of these elements is based on the comparison of terminal sequences from a wide variety of herpesviruses.

In HSV-1 the pac1 homology is composed of 2 GC rich elements of 43 and 12 bp, separated by a T rich element of 8 bp. A single DR1 repeat separates pac1 from pac2, which comprises a 7 bp consensus sequence (CGCCGCG), 31bp of unconserved sequence, a 6 bp T rich element and a 14 bp GC rich element.

The region illustrated corresponds to the 200 bp fragment of HSV-1 Strain 17 present in pSA1. The site of viral cleavage and the location of the *Ban*II and *Sac*II sites used to construct the pac1 and pac2 deletion mutants, are also shown.

Table 3.6 - Designed mutations in the u_c-DR1-u_b sequence.

Mutation	Element Size
pac1 deletion	63 bp
pac2 deletion	59 bp
DR1 substitution	20 bp
pac1 T element substitution	8 bp
pac1 T element deletion	8 bp
pac2 T element substitution	6 bp
pac2 T element deletion	6 bp
pac1 GC distal element deletion	12 bp
pac1 GC distal element substitution	12 bp
pac2 GC element deletion	14 bp
pac2 GC element substitution	14 bp
pac2 consensus deletion	7 bp
pac2 consensus substitution	7 bp
pac2 unconserved region deletion	32 bp
pac2 unconserved region substitution	32 bp
pac1 proximal GC element deletion	43 bp
pac1 proximal GC element substitution	43 bp

The deletion of the complete pac1 and pac2 regions could be achieved using restriction enzyme digestion, the remainder of the mutations were introduced into the packaging signal using a modified Kunkel mutagenesis method (Kunkel *et al.*, 1991). This method involves the synthesis of a uracil-rich single-stranded copy of the plasmid containing the region to be mutagenised. The sequence to be mutagenised is placed in a plasmid which contains the f1 origin of replication, and the plasmid is propagated in a *dut*$^-$ *ung*$^-$ strain of *E. coli*, which is unable to repair uridylate residues incorporated into the DNA in place of thymidylate. Upon subsequent infection with an f1-related bacteriophage the plasmid will be replicated and packaged (like the phage genome) as a single stranded copy. The phage particles can then be harvested and the single stranded DNA recovered. Bacteriophage R408 is used to infect the bacterial culture as it has a reduced efficiency to package its own DNA, thus reducing the amount of bacteriophage DNA in the final preparation (Russel *et al.*, 1986). The site directed mutagenesis involves synthesising the second strand of the plasmid *in vitro*. An oligonucleotide primer is used to prime DNA synthesis, the primer contains the mutation of choice flanked by approximately 15 bases which are complementary to the template. Once the second strand has been synthesised the plasmid DNA is used to transform a *dut*$^+$ *ung*$^+$

strain of *E.coli.* These bacteria are able to recognise the uridine residues incorporated in the parental strand, which they remove and repair using the mutated second strand as a template. This procedure thus enriches the proportion of bacterial colonies that contain the desired mutation. A flow diagram of this method as applied to the mutagenesis of the HSV-1 u_c-DR1-u_b sequence is shown in figures 3.24 & 3.25.

3.2.2 - Cloning the u_c-DR1-u_b sequence from pSA1 to pTZ18U.

pSA1 does not contain an f1 origin, therefore it was necessary to re-clone the u_c-DR1-u_b sequence into a suitable vector in order to produce ss plasmid DNA. The plasmid chosen for this purpose was pTZ18U (figure 3.26) as it contains all of the necessary features for *in vitro* mutagenesis and DNA sequencing.

pSA1 was digested using *Eco*RI and *Hin*dIII, which releases the u_c-DR1-u_b insert from the plasmid backbone. The digested DNA was then run on an agarose gel alongside pAT153 / *Hin*fI markers (figure 3.27 panel A). The lower band of 200 bp was excised from the gel and electroeluted. The electroeluted band was then further purified over a DEAE sephacel column, and precipitated with ethanol. The precipitated DNA was resuspended and an aliquot was run on an agarose gel (figure 3.27 panel B).

pTZ18U was digested with *Eco*RI, *Hin*dIII and CIP. After digestion the DNA was phenol chloroform extracted and ethanol precipitated. The precipitated DNA was resuspended and an aliquot run on an agarose gel, (figure 3.27 panel C).

The purified fragments were ligated together using T4 DNA ligase, in the presence of an excess of insert. The products of the ligation were sequentially extracted with phenol and chloroform and used to transform *E.coli* DH5α cells. Small scale plasmid preparations were made from ampicillin resistant colonies, the DNA was digested with *Dra*III and run on an agarose gel. *Dra*III cuts once in pTZ18U and a successful ligation of a single insert would yield a single band 200 bp larger than the linearised pTZ18U. Figure 3.28 shows the ethidium bromide stained gel of this digest and shows that lanes 2-12 contain a single band of the expected size. Lane 13 contains multiple bands which may be consistent with undigested plasmid DNA. Colony 18.7 was grown up and stored at -70°C. The plasmid was designated pTZ2. Cleavage of pTZ2 with *Eco*RI and *Hin*dIII yielded fragments which co-migrated with the 200 bp pSA1 insert and the linearised pTZ18U DNA. The identity of the insert was confirmed by DNA sequencing which revealed that the sequence was identical to that expected (see figure 3.31)

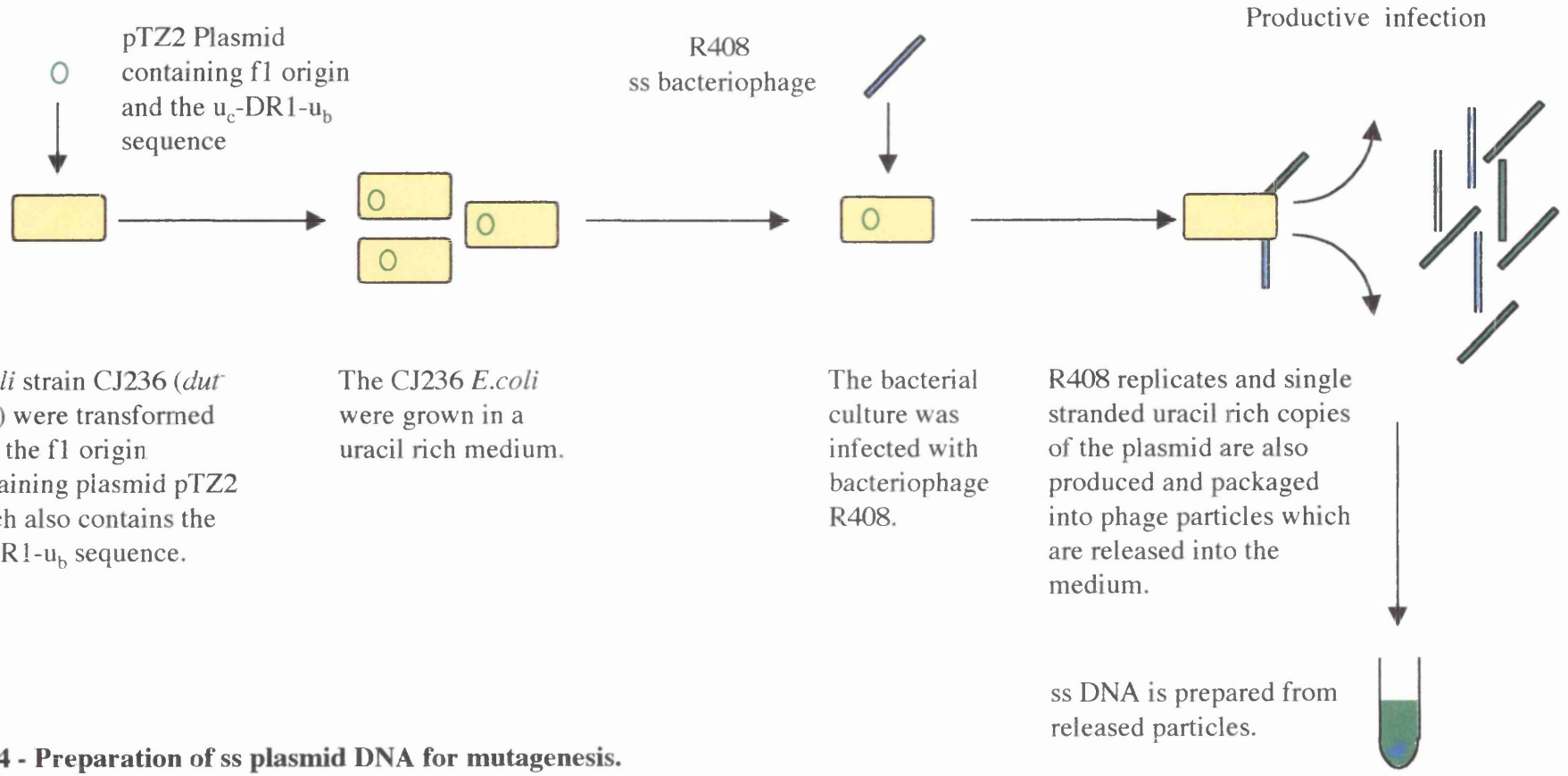


Figure 3.24 - Preparation of ss plasmid DNA for mutagenesis.

This flow diagram illustrates the procedure used to obtain uridine rich ss pTZ2 plasmid DNA, used in the modified Kunkel method of site-directed mutagenesis.

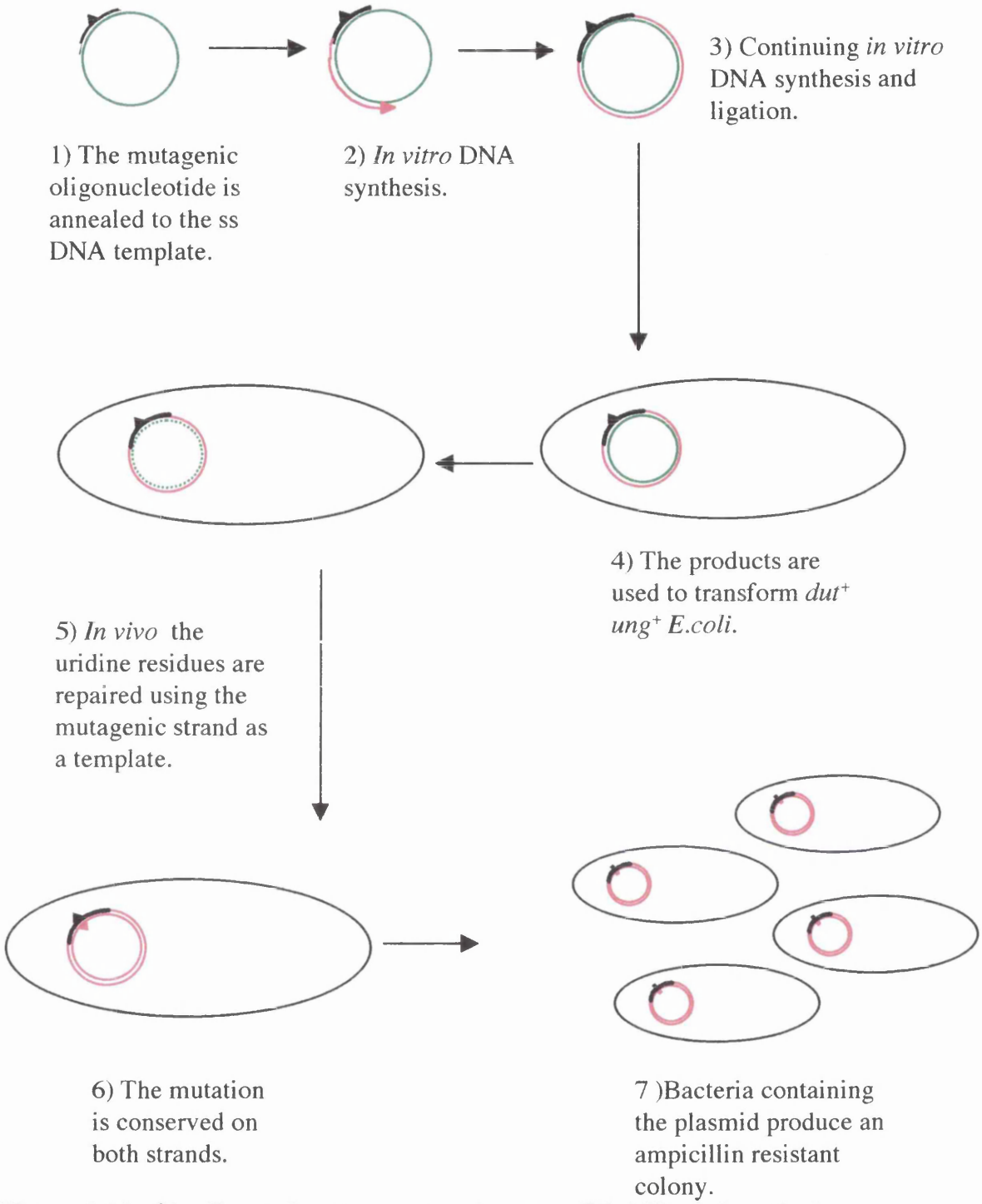


Figure 3.25 - Site directed mutagenesis using a modified Kunkel method.

This flow diagram illustrates the procedure used to make site specific mutations in the u_c-DR1-u_b sequence, using the modified Kunkel method (Kunkel *et al.*, 1991).

1) An oligonucleotide containing the mutation and 30 complementary flanking nucleotides is annealed to the single stranded template. 2) The products of the annealing reaction are used as a template for *in vitro* DNA synthesis using T7 DNA polymerase. 3) The ends of the completed new DNA strand are then ligated together by T4 DNA ligase. 4) The products of the *in vitro* DNA synthesis and ligation are used to transform *dut*$^+$ *ung*$^+$ *E.coli* DH5α, 5) The *dut*$^+$ *ung*$^+$ *E. coli* DH5α remove the uridine residues in the parental strand of the plasmid, using the mutagenic strand as a template for repair synthesis. 6) This has the effect of copying the mutation onto both strands. 7) The plasmid is then isolated from an ampicillin resistant colony.

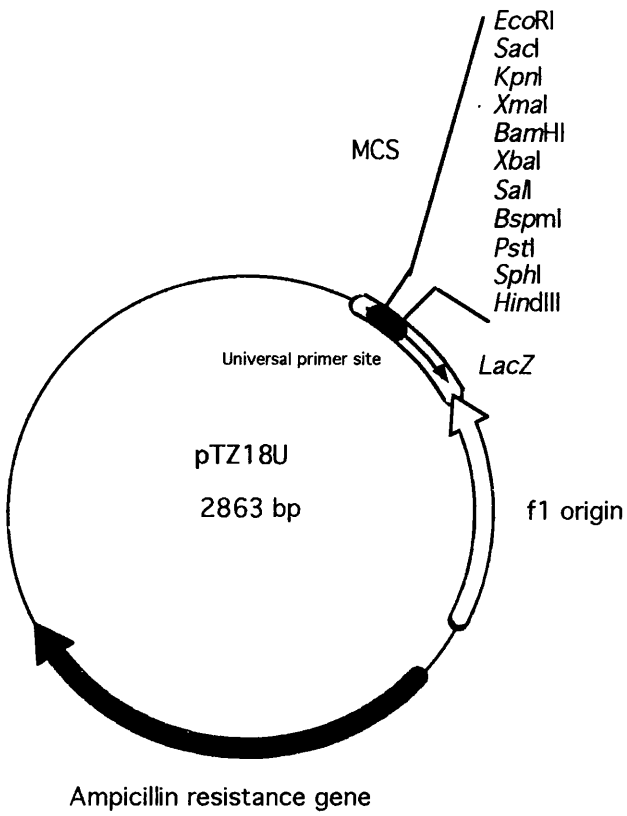


Figure 3.26 - pTZ18U, phagemid cloning vector, used for site directed mutagenesis and sequencing.

pTZ18U is a multi functional plasmid, designed to permit DNA cloning, dideoxy DNA sequencing, *in vitro* mutagenesis and *in vitro* transcription. It has the pUC18 MCS inserted into a *lacZ* gene, which is under the control of the T7 promoter. Just upstream of the T7 promoter and the MCS is a binding site for the universal sequencing primer. The plasmid also contains a *β-lactamase* gene for selection purposes.

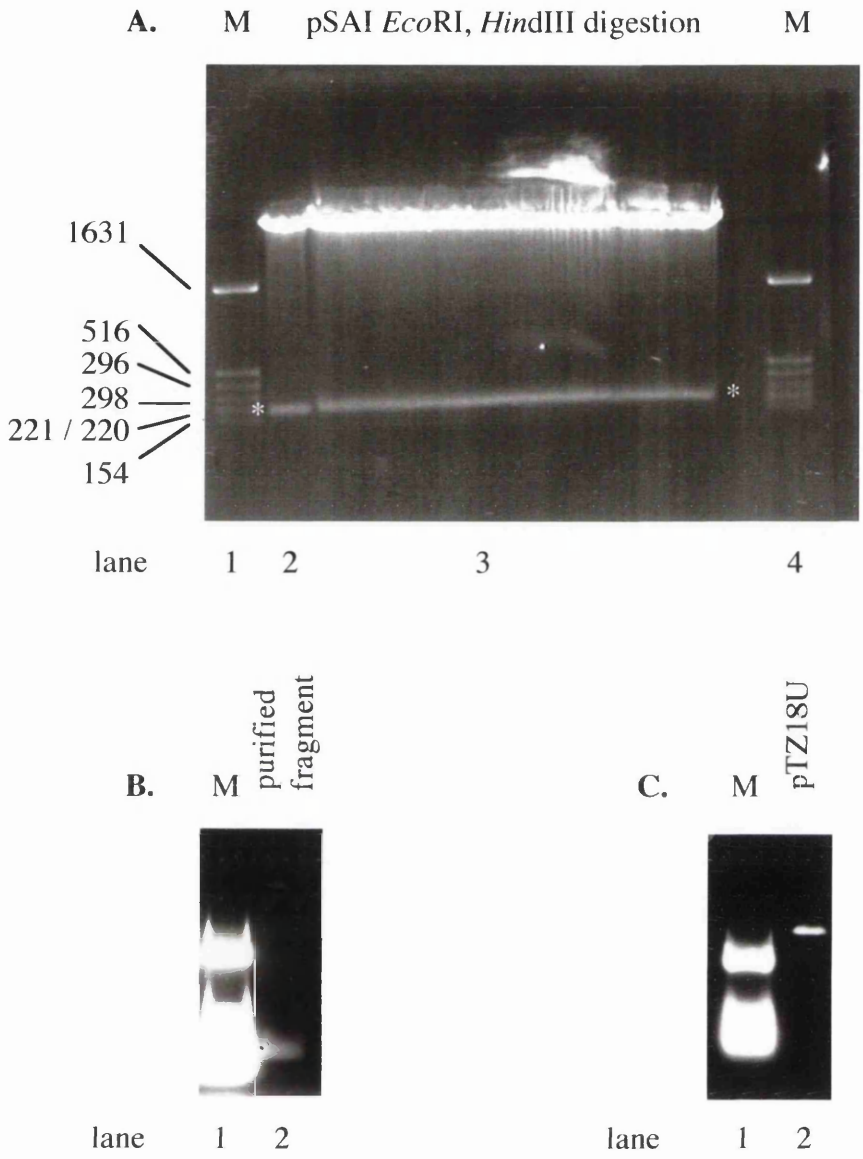


Figure 3.27 - Cloning the u_c-DR1-u_b fragment from pSA1 into pTZ18U.

Panel A lanes 2 and 3, contain 50μg of *Eco*RI, *Hin*dIII digest of pSA1. The lower band (labelled *) is the 200 bp u_c-DR1-u_b fragment. This band was excised from the gel, electroeluted and purified over a DEAE-Sephacel column. Lanes 1 and 4 contain pAT153 / *Hin*fI markers.

Panel B lane 1, contains pAT153 / *Hin*fI markers. Lane 2 contains 0.4 μg of purified u_c-DR1-u_b fragment.

Panel C lane 1, contains pAT153 / *Hin*fI markers. Lane 2 shows 0.4μg *Eco*RI, *Hind*III and CIP digested pTZ18U.

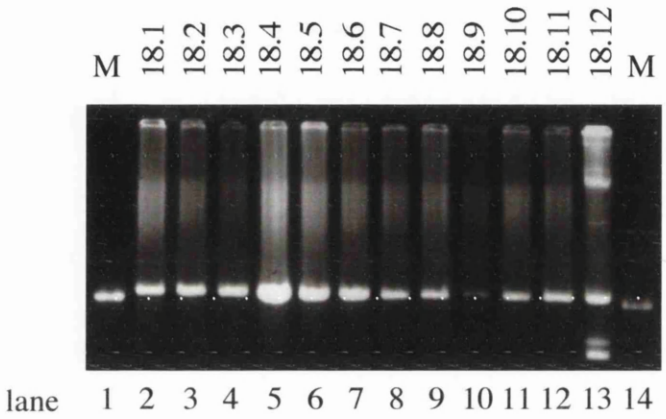


Figure 3.28 - Screening bacterial colonies transformed with pTZ18U, u_c-DR1-u_b ligation.

DNA from the colonies indicated in lanes 2-13 was digested with *Dra*III and the products resolved on a 0.8% agarose TBE gel. *Dra*III digestion will cut once in the backbone of pTZ18U based plasmids. Colonies which contain pTZ18U with the u_c-DR1-u_b sequence inserted between the *Eco*RI and *Hin*dIII sites will consequently be 200 bp larger than linearised pTZ18U.

Colonies in lanes 2-12 contain a single band which is slightly larger than the linearised pTZ18U, consistent with the insertion of the 200 bp insert. The origin of the extra bands in lane 13 is not certain but may represent partially digested plasmid molecules. Lanes 1 and 14 contain 0.4 μg of linearised pTZ18U. Clone 18.7 was selected, grown up and stored at -70°C. The plasmid was designated pTZ2.

3.2.3 - Deletion mutagenesis of the u_c-DR1-u_b sequence using restriction enzymes.

The use of restriction enzymes allowed the pac1 and pac2 elements to be individually deleted from the u_c-DR1-u_b sequence (figure 3.23).

Using *Sac*II, a fragment containing the pac2 element plus a further 18 bp of u_c and 4 bp of the DR1 repeat was excised from pSA1. Similarly, using *Ban*II, 8 bp of the DR1 repeat and all but 2 bp of pac1 was excised from pTZ2.

The plasmids were digested with the appropriate enzyme, extracted sequentially with phenol and chloroform and precipitated with ethanol. The DNA was re-ligated using T4 DNA ligase. The products of the ligation were then used to transform *E. coli* DH5α, small scale DNA preparations were made from ampicillin resistant colonies and the DNA was digested with restriction enzymes in order to identify colonies containing plasmids with the required deletions.

Figure 3.29, panel A, shows the screening of pac2 deletion colonies with *Eco*RI and *Hin*dIII. The fragment inserted between the two sites, which spans the u_c-DR1-u_b sequence, is 200 bp in pSA1 but is predicted to be 80 bp shorter in the deletion mutant. Lanes 2-11 and lane 13 contain DNA, containing the required mutation, the 120 bp fragment is marked with an arrow, lane 12 appears to contain the parental undeleted fragment (marked *). The regeneration of the *Sac*II site was also confirmed. A stock of clone 3.1 was stored at -70°C and the plasmid from this clone is subsequently referred to as pPH3. For sequencing purposes this deletion mutant of the u_c-DR1-u_b sequence was also recloned into pTZ18U. The resulting plasmid was grown up and stored at -70°C as pTZ3 stock.

Figure 3.29, panel B, shows the screening of pac1 deletion colonies. The required deletion mutant lacks one of four *Dra*I sites in the pTZ2 plasmid and screening was based on the loss of this site. *Dra*I digestion of the pTZ2 plasmid produces 4 fragments, the two largest being 1217 and 1081 bp. The loss of the *Dra*I site in the mutant means that only three fragments are produced; the two large fragments being replaced by a single one of 2218 bp. The DNA from the colonies in lanes 4,5,7,9,11,13 and 14 all show the mutant pattern. The remaining clones (Lanes 6,10,12 and 15) have the profile expected for the parent plasmid. Clone 4.1 was grown up and stored at -70°C. The plasmid from this clone is subsequently referred to as pTZ4.

The structure of both of these mutants was later confirmed by sequencing the region of the pTZ plasmid containing the u_c-DR1-u_b sequence.

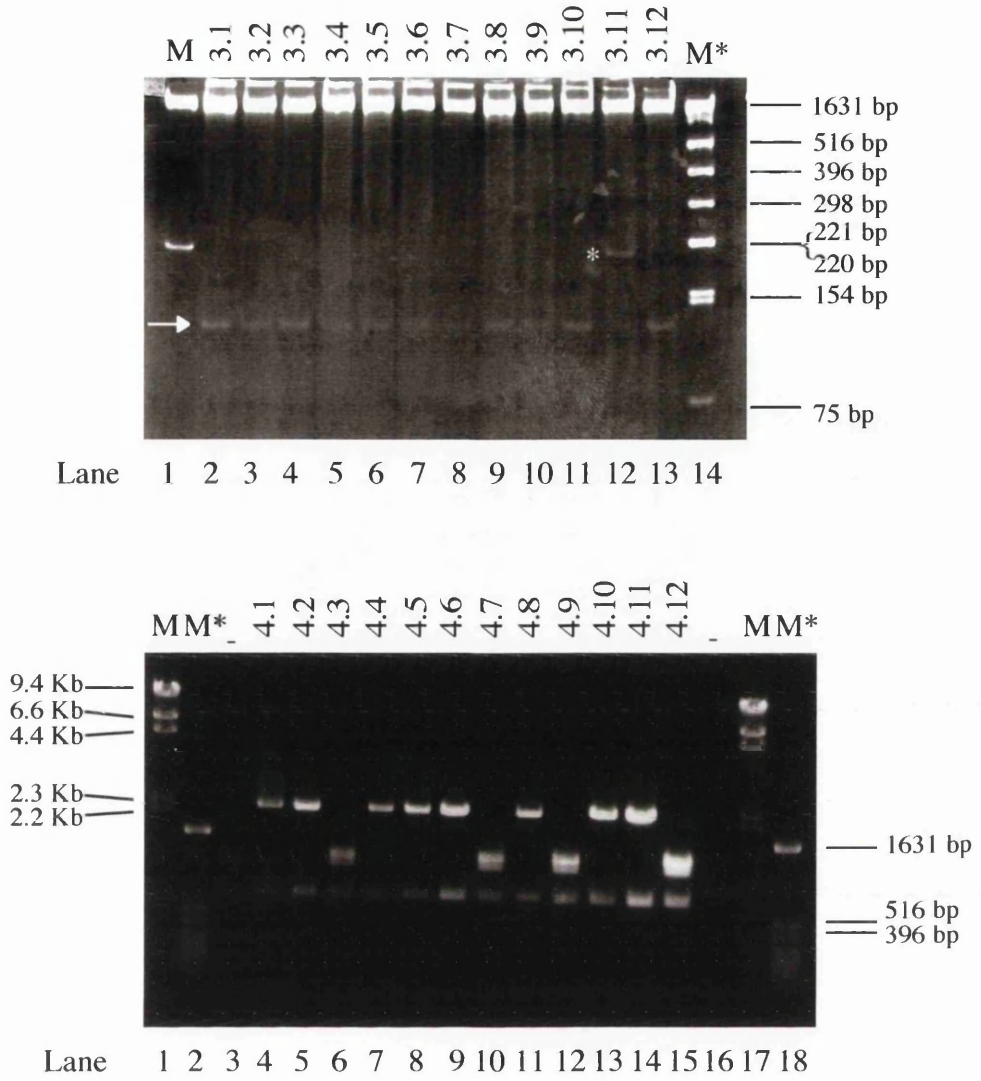


Figure 3.29 - pac1 and pac2 deletion mutagenesis of the u_c-DR1-u_b packaging signal.

Panel A shows *Eco*RI plus *Hin*dIII digested DNA, (on an 8% acrylamide TBE gel) from pac2 deletion colonies. The expected size of the insert is 120bp as opposed to the 200bp insert in the parent plasmid. The DNA in lanes 2-11 and 13 all contain an insert of the expected size (marked with an arrow). The colony labelled 3.7 (lane 8) appears not to contain an insert and the colony labelled 3.11 (lane 12) contains a 200bp insert (labelled *). Lane 1 (M) contains 0.5 μg of *Eco*RI, *Hin*dIII digested pSA1 (the parent plasmid) and lane 14, (M*) contains pAT153 / *Hin*fI marker.

Panel B shows *Dra*I digested DNA (on a 0.8% agarose TBE gel) from pac1 deletion colonies. The loss of a single *Dra*I site from the parent plasmid, caused by the deletion of pac1, results in the loss of 2 fragments (1217 & 1081bp) and the appearance of a larger fragment (2218 bp) in the mutant.

Lanes 4, 5, 7-9, 11,13 & 14 contain mutated plasmids. Lanes 6, 10, 12 and 15 show the parental pattern of bands. Lanes 1 and 17 (M) contain λ / *Hin*dIII markers and lanes 2 and 18 (M*) contain pAT153 / *Hin*fI marker.

3.2.4 - Preparation of uracil rich ss DNA from pTZ2.

In order to construct the remaining mutants it was necessary to produce uracil rich single stranded pTZ2 DNA as a template for the modified Kunkel method of site directed mutagenesis. The ss DNA was prepared in *E.coli* strain CJ236. This strain of *E.coli* has the *dut*$^-$ *ung*$^-$ genotype, which means it is deficient in the mechanism which inhibits the accumulation of uridine in the cell's DNA, and also in the repair pathway which recognises and excises the incorporated uridine. For this reason uridine residues accumulate in the DNA of cells, especially when they are grown in a uracil rich media.

The bacteria containing the pTZ2 plasmid, were grown up as an overnight culture from which an aliquot was used to inoculate a larger culture. The large culture was subsequently infected with the ss bacteriophage R408 (Russel *et al.*, 1986). The phage particles released during productive infection contain either ss bacteriophage DNA or single stranded plasmid DNA, which has been replicated and packaged as a consequence of containing the f1 origin of replication. The phage-like particles were harvested and their DNA extracted, an aliquot of which was visualised on an agarose gel.

In the experiment, shown in figure 3.30, three cultures were grown and infected with R408 in parallel. Lane 1 contains the DNA harvested from a culture of *E.coli* strain CJ236 which did not contain the pTZ2 plasmid. Lanes 2 and 3 contain DNA harvested from two parallel *E.coli* strain CJ236 cultures containing pTZ2. Lane 4 contains M13 genomic DNA, a close relative of the R408 bacteriophage. From this gel it is clear that the upper band in lanes 2 and 3 is R408 phage DNA (approximately 5900 bp) while the lower band is ss pTZ2 plasmid DNA (3063 bp). The reason for the variation in the levels of single stranded pTZ2 and R408 DNA between preparations is not clear. It is probable, however, that the variation reflects slight differences in the density of the bacterial culture at the time of infection with R408. Both DNA preparations were used equally successfully as a template for the site directed mutagenesis reactions.

3.2.5 - Site directed mutagenesis.

Specific substitution and deletion mutations in the u_c-DR1-u_b packaging signal were constructed based on the elements proposed by Deiss & Frenkel (1986). As described in the introduction to this section, the site directed mutagenesis was carried out using a modified Kunkel method (Kunkel *et al.* 1991) - using oligonucleotides which would specifically substitute or delete each of the seven elements proposed for pac1 and pac2 homologies. An additional oligonucleotide which would substitute 9 residues at the usual site for viral cleavage in the DR1 repeat was also designed.

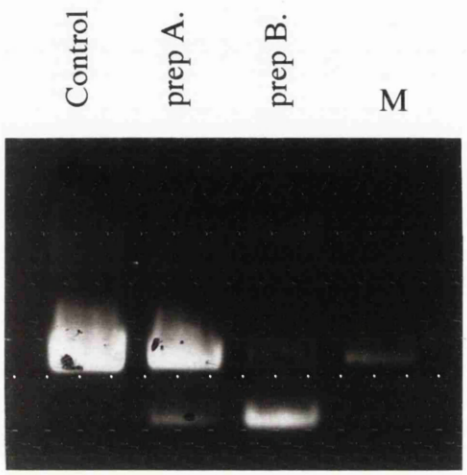


Figure 3.30 - Preparation of uracil enriched ss DNA.

Uracil enriched ss DNA was produced in *E.coli* strain CJ236 containing the pTZ2 plasmid. The bacteria were infected with the packaging-deficient ss bacteriophage R408 and the resultant phage particles harvested.

Lane 1 contains ss DNA harvested from a control culture of *E.coli* Strain CJ236 which lacked the pTZ2 plasmid. Lanes 2 and 3 contains ss DNA harvested from parallel cultures of *E.coli* strain CJ236 which contained the pTZ2 plasmid. Lane 4 contains 0.5 μg of M13 ss bacteriophage DNA.

The sequences of the mutagenic oligonucleotides and their positions with respect to the wild type sequence are shown in table 3.7 and figure 3.31. Each substitution mutation was designed to include a chosen restriction enzyme site to facilitate the identification of mutant colonies. In order to reduce nucleotide identity between the wild type and mutant sequences, both *Sca*I and *Xho*I were used. For the smaller substitutions (e.g. T rich elements) the remaining sequence was chosen to avoid identical residues occupying the same location in the wild type and mutant sequences. For the longer substitutions (e.g. pac2 unconserved region) sequences were generated using a random number generator to avoid any unintentional bias in the sequence. A series of random numbers was generated using a pocket calculator and the numbers 1-4 were ascribed to the residues G(1), A(2), T(3) and C(4). Each time a number between 1 and 4 was encountered in the series of random numbers the corresponding residue was added to the substitution sequence. This was done until the required number of bases had been generated.

In the experiment shown in figure 3.32 two mutagenesis reactions were set up using mutagenic oligonucleotide 6. The oligonucleotide was annealed to the template DNA in both, polymerase and ligase enzymes were then added to one reaction but not the other, both reactions were incubated at RT and 37°C and the products were used to transform competent *E. coli* DH5α. After the polymerisation step an aliquot from each reaction was removed and run on an agarose gel. Figure 3.31 shows this gel. Lane 2 contains the sample taken from the reaction which lacked polymerase and ligase enzymes and lane 3 contains the sample take from the reaction where the enzymes were included. The pattern of DNA was significantly altered when the annealed oligonucleotide and template were incubated in the presence of polymerase and ligase, suggesting that synthesis of the second strand had occurred. However, the profile of the DNA is not consistent with a simple shift of single stranded pTZ2 to double stranded un-supercoiled form, although a faint band corresponding to this is present comigrating with the upper band of the plasmid DNA (lane 4). The main shift in the DNA profile has been of the R408 DNA which has almost exclusively moved to a higher molecular weight (lower mobility) form. This shift may be consistent with the synthesis of R408 second strand, however, R408 second strand synthesis is initiated by a phage encoded messenger RNA which is not present in the *in vitro* reaction. It is possible that second strand synthesis has been initiated by double stranded regions of the genome

Second strand synthesis was performed using all 15 mutagenic oligonucleotides and the products were used to transform *E.coli* DH5α . Ampicillin resistant colonies were then screened for the presence of the appropriate mutation.

Table 3.7 - Sequences of mutagenic oligonucleotides compared to template sequence.

Mutation number	Mutation	Screening	Oligonucleotide sequences
6	DR1 substitution	*Sca*I	CGCGCCCGCGGGGGGCCCGGGC**T**GCCCGCCGCCACCGCTT CGCGCCCGCGGGGGGCAATAGTACTCCGCCGCCACCGCTT
7	pac1 T element substitution	*Xho*I	GCGACCCCCGGGGGGTGTGTTTTGGGGGGGGCCCGTTT GCGACCCCCGGGGGGGCTCGAGGGGGGGGGGCCCGTTT
8	pac1 T element deletion	*Eco*RI+ *Hin*dIII	GCGACCCCCGGGGGGTGTGTTTTGGGGGGGGCCCGTTT GCGACCCCCGGGGGG.....GGGGGGGGCCCGTTT
9	pac2 T element substitution	*Xho*I	GGACCGCCGCCCGCCTTTTTTGCGCGCGCGCGCGCC GGACCGCCGCCCGCCCTCGAGGCGCGCGCGCGCGCC
10	pac2 T element deletion	*Eco*RI+ *Hin*dIII	GGACCGCCGCCCGCCTTTTTTGCGCGCGCGCGCGCC GGACCGCCGCCCGCC.....GCGCGCGCGCGCGCC
11	pac1 GC distal element deletion	*Eco*RI+ *Hin*dIII	CGGGGGGTGTGTTTTGGGGGGGCCCGTTTTCGGCGTCTGGC CGGGGGGTGTGTTTT.....TTTTCGGCGTCTGGC
12	pac1 GC distal element substitution	*Sca*I	CGGGGGGTGTGTTTTGGGGGGGCCCGTTTTCGGCGTCTGGC CGGGGGGTGTGTTTTCCCAGTACTGCCTTTTCGGCGTCTGGC
15	pac2 GC element deletion	*Eco*RI+ *Hin*dIII	CCGCCCGCCTTTTTTGCGCGCGCGCGCGCCCGCGGGGGGCCCGG CCGCCCGCCTTTTTT.....CCGCGGGGGGCCCGG
16	pac2 GC element substitution	*Sca*I	CCGCCCGCCTTTTTTGCGCGCGCGCGCGCCCGCGGGGGGCCCGG CCGCCCGCCTTTTTTCGAGTACTCACGTACCGCGGGGGGCCCGG
17	pac2 consensus deletion	*Eco*RI+ *Hin*dIII	CCCGCCCCCCACGCCCGCCGCGCGCGCGCACGCCGCC CCCGCCCCCCACGCC.....CGCGCGCACGCCGCC
18	pac2 consensus substitution	*Sca*I	CCCGCCCCCCACGCCCGCCGCGCGCGCGCACGCCGCC CCCGCCCCCCACGCCGAGTACTCGCGCGCACGCCGCC
19	pac2 unconserved deletion	*Eco*RI+ *Hin*dIII	CCCCACGCCCGCCGCGCGCGCGCACGCCGCCCGGACCGCCGCCCGCCTTTTTTGCGCGCGCGC CCCCACGCCCGCCGC.....GTTTTTTGCGCGCGCGC
20	pac2 unconserved substitution	*Xho*I	CCCCACGCCCGCCGCGCGCGCGCACGCCGCCCGGACCGCCGCCCGCCTTTTTTGCGCGCGCGC CCCCACGCCCGCCGCGGACGTAACAAGCACATCTCGAGGTGTGAGGGTTTTTTGCGCGCGCGC
21	pac1 proximal GC element deletion	*Eco*RI+ *Hin*dIII	GGGGGGCCCGGGCTGCCCGCCGCCACCGCTTTAAAGGGCCGCGCGCGACCCCCGGGGGGGTGTGTTTTGGGGGGGGC GGGGGGCCCGGGCTGC.....TGTGTTTTGGGGGGGGC
22	pac1 proximal GC element substitution	*Xho*I	GGGGGGCCCGGGCTGCCCGCCGCCACCGCTTTAAAGGGCCGCGCGCGACCCCCGGGGGGGTGTGTTTTGGGGGGGGC GGGGGGCCCGGGCTGCTTGTAGGGGATGAGGTCTCCCCTGGCGCACAGCTCGAGTACATTGTGTTTTGGGGGGGGC

The template sequence is shown in grey and the oligonucleotide sequence is shown below. ... denote deleted nucleotides and the sites of mutation are underlined. For mutation 6 the residue which is at the S terminus of the viral genome is shown in bold. The mutation number corresponds to the plasmid number in both pTZ and pPH series.

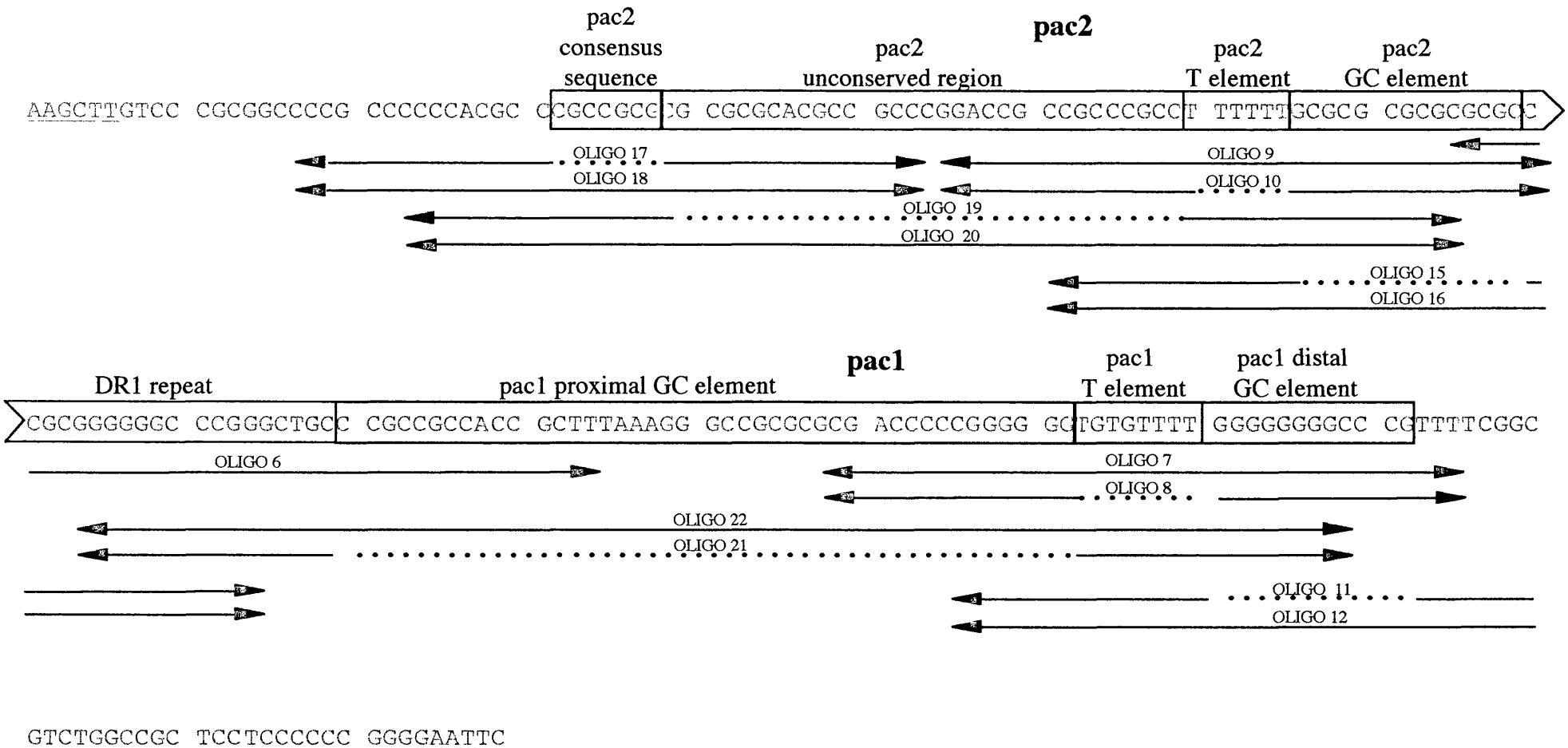


Figure 3.31 - Mutation of the u_c-DR1-u_b sequence.

This diagram show the sequence of the u_c-DR1-ub fragment, with the positions of the mutagenic oligonucletides marked. pac2 sequences are shown in blue, the DR1 repeat is shown in red and the pac1 sequences are shown in green.

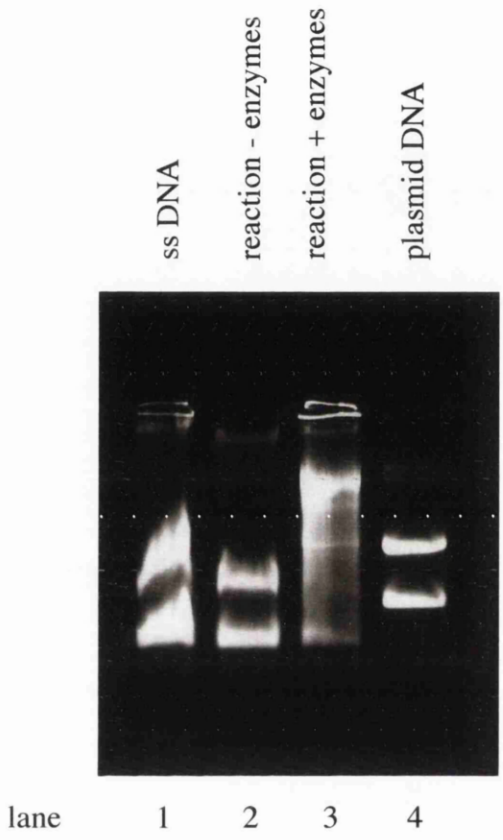


Figure 3.32 - Stages of mutagenesis.

Lane 1 contains 2 μl of the ss DNA preparation used in this mutagenesis reaction. Lane 2 contains 5 μl of mutagenesis reaction, following annealing of a mutagenic oligonucleotide and incubation with polymerisation / ligation buffer for 30 minutes at RT and 30 minutes at 37°C. Lane 3 is identical to lane 2 except that T7 DNA polymerase and T4 ligase were also added following the annealing of the mutagenic oligonucleotide. Lane 4 contains 0.5 μg of uncut ds pTZ2.

3.2.6 - Screening bacterial colonies for mutants.

Initial identification of colonies which contained mutagenised u_c-DR1-u_b sequences was achieved in one of two ways. Substitution mutants were screened on the basis of the specifically engineered restriction enzyme sites which were present in the mutagenic oligonucleotides. Deletion mutants were screened on the basis of changes in the size of the u_c-DR1-u_b fragment by digesting the plasmids with *Eco*RI and *Hin*dIII.

Small scale DNA preparations were prepared from ampicillin resistant colonies, which had been transformed with the products of the mutagenesis reactions, and the DNA was digested with appropriate enzymes. The restriction enzymes used to screen for each mutation are shown in table 3.7.

Figure 3.33 shows two representative agarose gels. Panel A contains DNA from colonies resulting from mutagenesis using oligonucleotide 6 (substitution of the cleavage site in the DR1 repeat). The DNA has been digested with *Sca*I, which cleaves once in the parent plasmid and is expected to cleave twice in the mutant. Lanes 1, 3 and 5 show the parental pattern of a single linearised band. Lanes 2 and 4 appear to contain a mixed population of parental and mutated plasmids; digestion with *Sca*I giving rise to both linear plasmid and also the 2 fragments consistent with the presence of the additional engineered site. In reality however, this was a frequently observed phenomenon when *Sca*I was used. It appears more likely that this pattern of bands reflects partial digestion of mutated plasmid DNA. Colony 6.6 (lane 6) contains the pattern of bands expected from a mutated plasmid, this colony was selected, grown up and stored at -70°C as the pTZ6 stock.

Panel B contains DNA prepared from colonies transformed with the products of a mutagenesis reaction using oligonucleotide 9 (pac2 T rich element substitution). The DNA has been digested with *Xho*I, which does not cut the parent plasmid but cuts once in plasmids carrying the required mutation. Lanes 1 and 3 contain unmutated plasmid DNA. Lanes 2, 4, 5 and 6 all contain plasmid DNA carrying the mutated sequence. Clone 9.2 (lane 2) was selected, grown up and stored at -70°C as the pTZ9 stock.

Figure 3.34 shows a representative acrylamide gel used for identifying deletion mutants. Lanes 2-9 contain small scale DNA preparations from ampicillin resistant colonies, transformed with the products of mutagenesis reactions. These colonies have been transformed with products prepared using either oligonucleotide 11 (pac1 distal GC element deletion) or oligonucleotide 15 (pac2 GC element deletion), (labelled 11.1-4 and 15.1-4 respectively). The DNA was digested with *Eco*RI and *Hin*dIII and run on a non-denaturing polyacrylamide gel. The *wt* fragment in the parental plasmid is 200 bp, mutated plasmids are predicted to contain inserts of 188 and 186 bp. The DNA in lanes 2-5 (11.1-4) show 3 inserts without deletions (lanes 2,3 and 5) and one insert containing a deletion (lane 4).

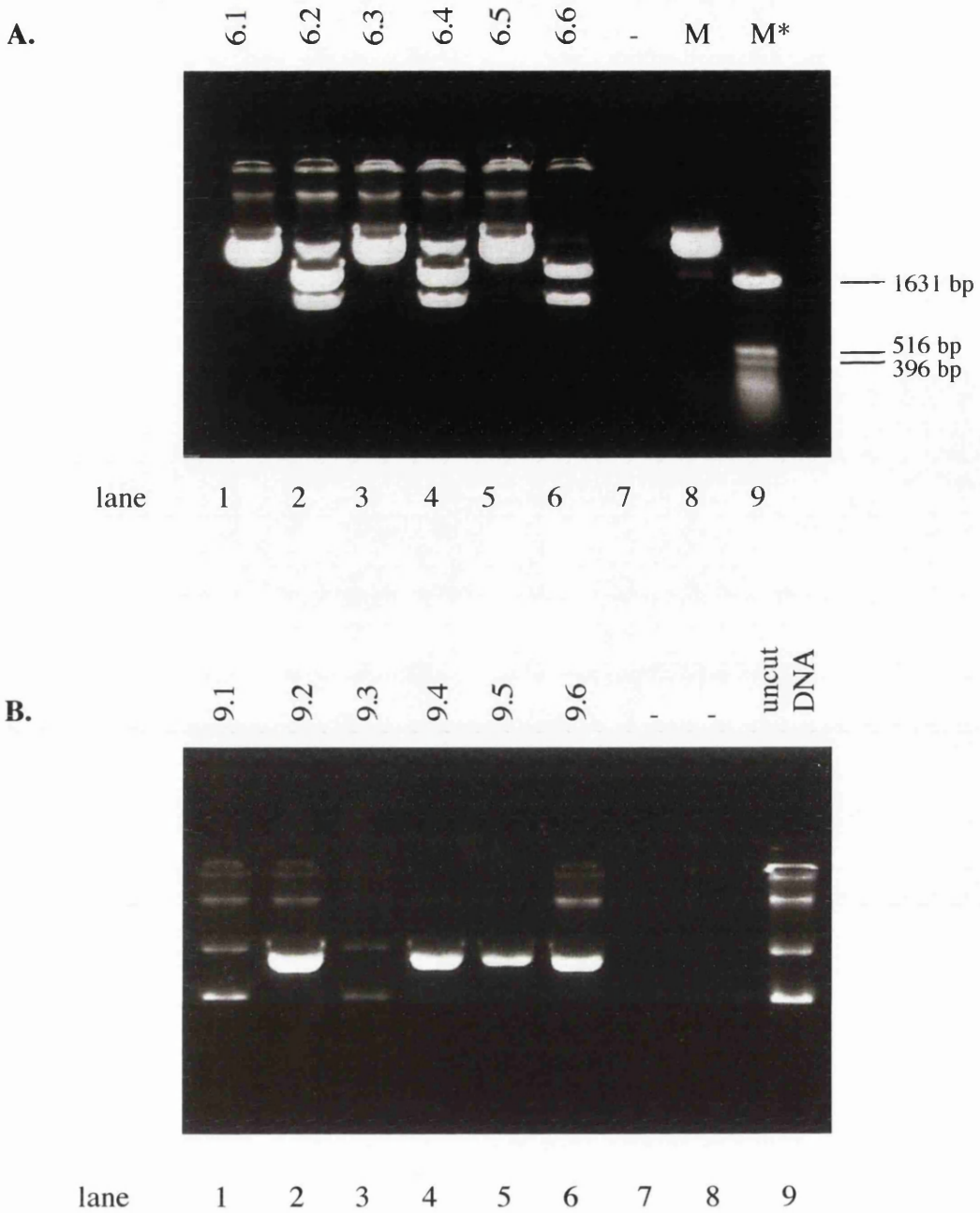


Figure 3.33 - Screening colonies, derived from mutagenesis, for the presence of engineered restriction enzyme sites.

DNA was prepared from the colonies indicated. The DNA was digested with *Sca*I (panel A) or *Xho*I (panel B) and the products resolved on a 0.8% agarose gel.

In Panel A, lanes 1-6, a profile containing 2 bands is indicative of a mutated plasmid. Lanes 1, 3 and 5 contain DNA with the parental pattern of bands. Lanes 2 and 4 appear to contain a mixed population of plasmid molecules and lane 6 contains plasmid DNA carrying the required mutation. Lanes 8 and 9 contain 0.5 μg of *Sca*I digested pTZ18U (M) and pAT153 / *Hin*fI markers (M*) respectively.

In Panel B, lanes 1-6, the presence of a single linearised plasmid band is indicative of successful mutagenesis. Lanes 1 and 3 contain DNA with the parental sequence while lanes 2 and 4-6 contain plasmid DNA with the mutated sequence. Lanes 7 and 8 are empty and lane 9 contains uncut small scale pTZ9 DNA preparation.

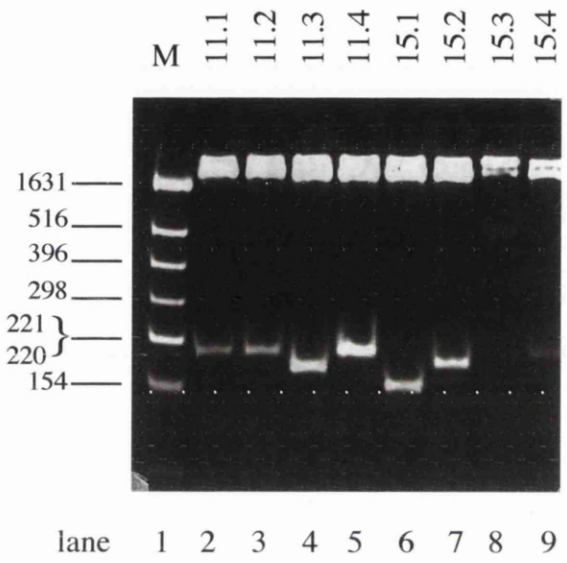


Figure 3.34 - Screening colonies, derived from mutagenesis, for the presence of deletions.

DNA was prepared from the colonies indicated in lanes 2-9 and digested with *Eco*RI and *Hin*dIII. The products were resolved on an 8% acrylamide TBE gel.

The parental fragment is 200 bp in size, deletion mutants therefore contain a smaller insert.

Lanes 2, 3, 5 and 9 contain parental 200bp inserts. Lanes 4 and 7 contain deletion mutations of approximately the expected size, lane 6 contains a mutant with a deletion significantly larger than expected. Lane 8 appears to lack either one of the restriction enzyme sites or an insert. Lane 1 contains pAT153 / *Hin*fI markers (M).

Clone 11.3 was therefore selected, grown up and stored at -70°C as pTZ11 stock. The DNA in lanes 6-9 shows a range of differently sized inserts. Lane 9 appears to contain the parental fragment (albeit faintly) and lane 8 does not appear to contain an *Eco*RI, *Hin*dIII insert. The DNA in Lanes 6 and 7 both appear to contain deletion mutations, however clone 15.1 (lane 6) appears to contain a deletion which is significantly greater than the 14 bp expected. Clone 15.2 (lane 7) contains a deletion of approximately the expected size. It was decided that in this case both clones warranted further investigation so both were grown up and stored at -70°C as pTZ15.1 and pTZ15.2 stocks.

All of the required mutants except one were obtained in this way, the exception being the deletion of the pac1 T rich element using oligo 8. Despite screening in excess of 80 colonies, gel purifying and then re-synthesising the oligonucleotide, this mutation remained elusive.

3.2.7 - Sequencing of mutations made in the u_c-DR1-u_b sequence.

In order to ensure that the mutated u_c-DR1-u_b mutants contained the specifically designed mutation and that no additional mutations were present, each mutant was sequenced using an **Perkin Elmer** automated sequencer.

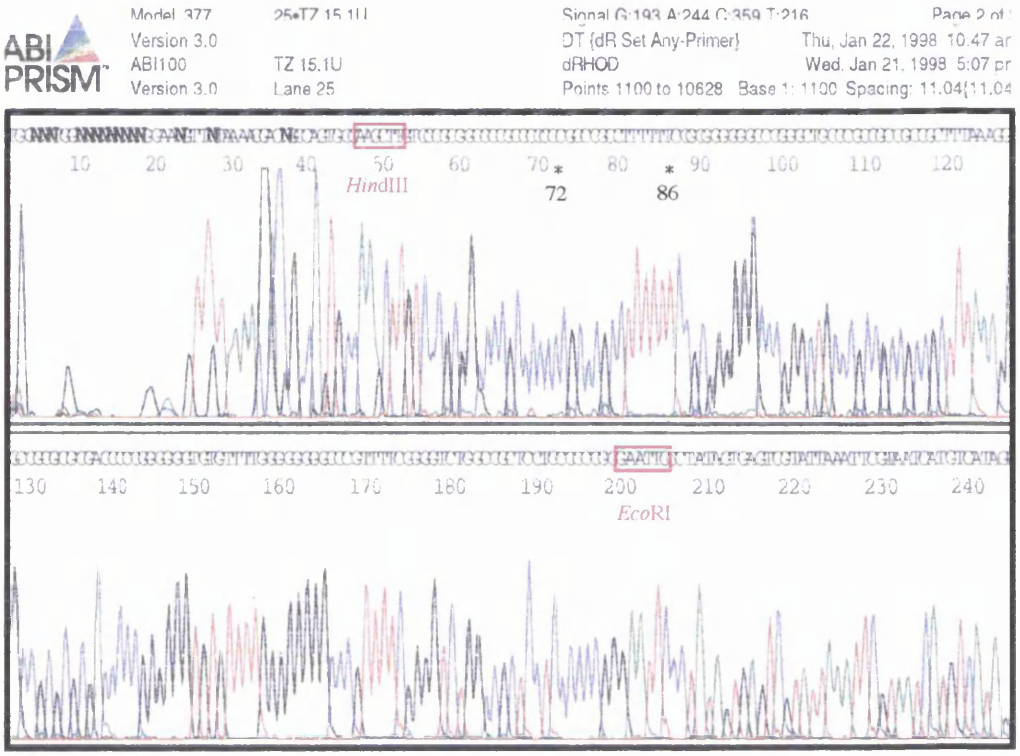
The appropriately selected plasmids were prepared using **Qiagen** midi columns™ and the double stranded DNA was resuspended at 0.2 $\mu g\ \mu l^{-1}$. An aliquot of this solution was then provided to the Institute of Virology sequencing service which sequenced the double stranded template using the M13 forward 'universal' primer.

The sequence spanning the u_c-DR1-u_b region was provided both as a printed sequence and as an electropherogram. Figure 3.35 shows the electropherogram for the sequencing of pTZ15.1. The *Eco*RI and *Hin*dIII sites which flank the u_c-DR1-u_b sequence have been marked in red and the sites of the two deletions have been marked with *. pTZ15.1 contains two deletions, the engineered deletion of 14 bp marked (*) at position 86 and a second spontaneous deletion of 35 bp also marked (*) at position 72.

The sequencing of the remaining mutants showed that each mutant contained the designed mutation with no other deviation from the wild type sequence.

3.2.8 - Cloning mutated u_c-DR1-u_b sequences into pS1.

In order to assay the mutated u_c-DR1-u_b sequences for their ability to direct cleavage and packaging in a transient packaging assay, it was first necessary to re-clone the mutated packaging signals back into the pS1 vector. This could have been achieved by individually



*72 ACGCCCCGCCGCGCGCGCACGCCGCCCGGACCGC

*86 GCGCGCGCGCGCGC

Figure 3.35 - Sequencing data from the Perkin Elmer automated sequencer.

The above electropherogram shows the results from the automated sequencing of pTZ15.1. Each coloured peak corresponds to the position of either an A(Green), T(Red), G(black) or C(Blue) residue in the sequence. The *Eco*RI and *Hin*dIII sites which flank the u_c-DR1-u_b sequence have been marked in red. The sites of the mutation have been marked (*). The spontaneous deletion at position 72 is 35 bp long and spans the pac2 consensus sequence and most of the pac2 unconserved region, the deletion at position 86 is the engineered deletion of the 14 bp pac2 GC rich element. The sequence of both deleted regions is also shown above.

gel purifying each mutated u_c-DR1-u_b fragment. However, considering the number of fragments involved an alternative strategy, avoiding fragment purification, was employed.

The mutated pTZ plasmids were digested with *Eco*RI, *Hin*dIII and *Hin*fI. The *Eco*RI and *Hin*dIII enzymes excise the u_c-DR1-u_b sequence from the plasmid backbone and *Hin*fI cuts multiple times in the pTZ plasmid, which will prevent it from re-circularising. This digestion produces a population of DNA fragments, the majority of which have *Hin*fI sticky ends. Only one fragment (containing the u_c-DR1-u_b sequence) has an *Eco*RI and a *Hin*dIII sticky end. The pS1 plasmid was prepared by digesting it with *Eco*RI, *Hin*dIII and CIP. The CIP de-phosphorylates the 5' and 3' termini, preventing the vector from circularising without a phosphorylated insert.

The restriction enzyme digests were sequentially extracted with phenol and chloroform, ethanol precipitated and the DNA resuspended. An aliquot of the digested DNA was then run on an agarose gel (figure 3.36 panel A).

Ligation reactions with T4 DNA ligase were performed using amounts of vector and pTZ DNA determined from inspection of the ethidium bromide stained gel. The products were used to transform competent *E. coli* DH5α. Small scale DNA preparations were then prepared from ampicillin resistant colonies and the DNA examined for the presence of the appropriate insert. Figure 3.36 panel B shows *Eco*RI and *Hin*dIII digested small scale DNA preparation from colonies transformed with the products from ligating pTZ19 fragments to pS1. Lanes 3 and 5-15 all contain an insert of the appropriate size(169 bp) released by *Eco*RI and *Hin*dIII digestion. Lane 4 contains plasmid DNA which does not appear to contain an insert, either as a result of vector re-circularisation in the absence of an insert or, more likely, the illegitimate insertion of a DNA fragment which did not regenerate both *Eco*RI or *Hin*dIII sites, i.e. the insert of a fragment which contains at least one *Hin*fI terminus. Colony 19.1 was grown up and stored at -70°C and the plasmid was subsequently referred to as pPH19. The lowest band in the sample lanes (3-14) is an artefact produced by the presence of 0.3% SDS in the samples loaded onto the gel. SDS was added to the loading buffers following the observation that with small scale DNA preparations the small *Eco*RI and *Hin*dIII fragments frequently exhibited a band shift effect when run on acrylamide gels. This was probably the result of bacterial DNA-binding proteins binding to the fragment and impeding its progress through the gel.

All 17 inserts from the pTZ series of plasmids were successfully transferred to pS1 to generate the corresponding pPH plasmids.

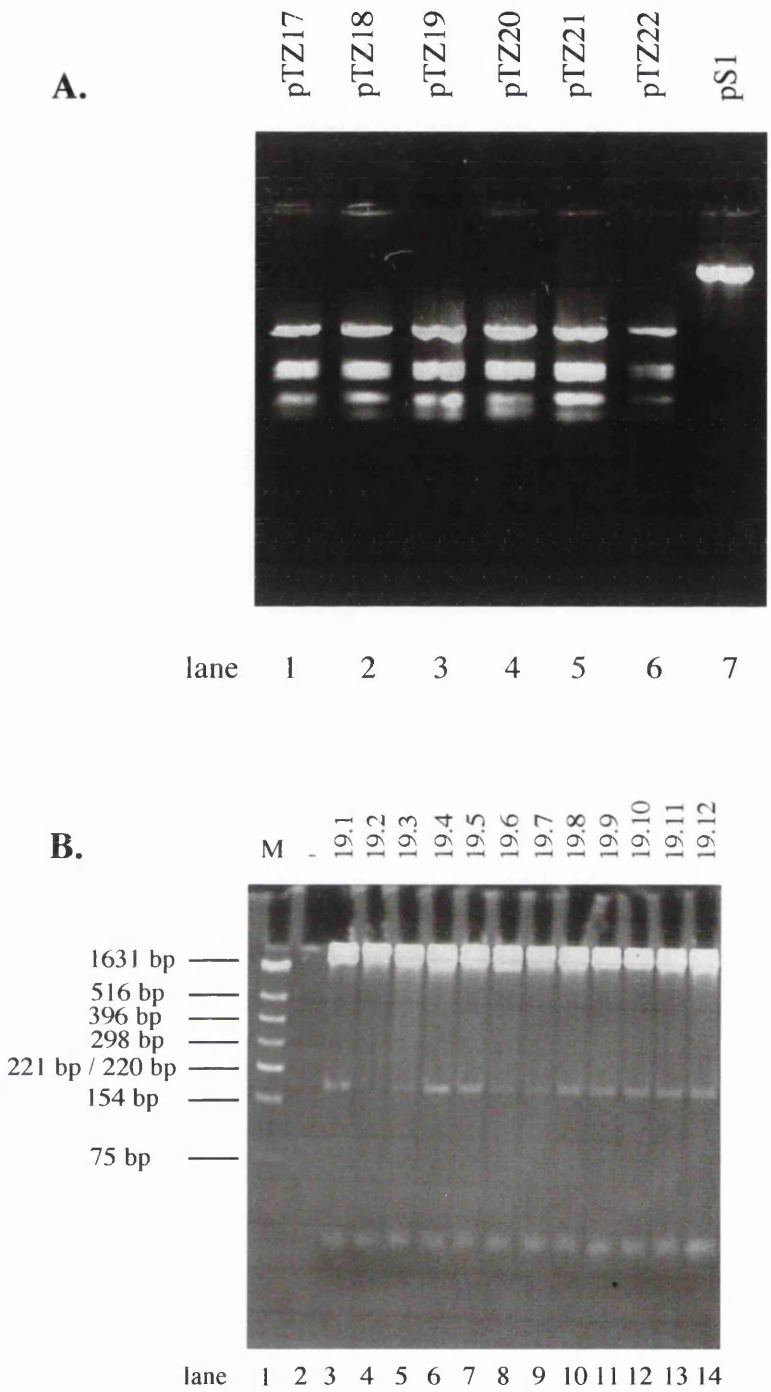


Figure 3.36 - Cloning mutated u_c-DR1-u_b fragments into pS1.

The indicated mutant plasmids were digested with *Eco*RI, *Hin*dIII and *Hin*fI (panel A lanes 1-6). pS1 was digested with *Eco*RI, *Hin*dIII and CIP (panel A lane 7). An aliquot of the products was then resolved on a 0.8 % agarose TBE gel.

Following ligation and transformation into competent bacteria, DNA was prepared from the colonies. Panel B shows 14 colonies isolated following transformation with the pTZ19 ligation products. The DNA was digested with *Eco*RI and *Hin*dIII and the products resolved on an 8 % acrylamide gel. The insert of a 200 bp fragment, in lanes 3 and 5-14 is indicative of a successful ligation. The lowest band in the sample lanes is an artefact caused by the presence of 0.3% SDS in the samples. Lane 1 contains pAT153 / *Hin*fI markers (M).

3.2.9 - Discussion.

3.2.9.1 - Isolation of mutants.

As described above 17 mutants of the u_c-DR1-u_b sequence were successfully isolated and transferred to the pS1 plasmid so that their ability to direct cleavage and packaging could be assessed. The cause of the difficulties encountered in isolating the pac1 T rich element deletion mutant were not identified during the course of this work. Examination of the u_c-DR1-u_b sequence, however, reveals that successfully generating this mutation would generate a poly G tract 14 bp long (see figure 3.31). There is no evidence whether this might interfere with the mutagenesis reaction but this single feature differentiates this mutant from the other 14 which were successfully isolated using the modified Kunkel method. The presence of the poly G tract in the mutagenic oligonucleotide may have precluded correct annealing to the single stranded DNA. An alternative is that the required product containing the poly G tract might be deleterious to or unstable in *E.coli* . It should be possible to design and test other oligonucleotides to introduce this mutation. For example, correct annealing could be investigated by performing di-deoxy sequencing reactions on the products of the annealing reaction.

3.2.9.2 - Double deletions.

The cause of the double deletions which were occasionally observed also remains unclear. They were observed for mutants prepared using oligonucleotides 15 and 21. In mutant 15.1 the additional mutation deletes 35 nucleotides spanning the consensus sequence and most of the unconserved region of pac2. In mutant 21Δ (data not shown) 84 nucleotides from 1 base into the pac2 unconserved region through to 9 bases into the pac1 proximal GC element were deleted. No correlation between the site of deletion and the oligonucleotide sequence is apparent and no direct repeats are found flanking the spontaneously deleted regions. The only common features are that both these additional deletions remove, all or most of the pac2 unconserved region and both have a single A residue at one end.

The ability of each of the mutants described above, to direct cleavage and packaging in the transient packaging assay for HSV-1 is described in the following section.

Section 3.3 - Analysis of mutated packaging signals.

3.3.1 - Introduction.

As described in section 3.2 seventeen mutants of the u_c-DR1-u_b sequence were isolated, sequenced and cloned into pS1 to generate the pPH series of plasmids. It was then possible to assay the mutated packaging signals for their ability to direct cleavage and packaging, using the optimised conditions for the transient packaging assay described in section 3.1.

In order to address the possibility that some mutants might be able to direct packaging only because the mutation had been corrected as a result of recombination with the helper virus, two investigations were carried out. The substitution mutations with an engineered *Xho*I marker were tested to see whether the packaged DNA still contained the restriction enzyme site. The continued presence of the site would indicate that the sequence at the site of mutation remains intact. In addition 16 mutants (i.e. all mutants except pPH15.1) were assayed using the optimised transient packaging assay with HSV-2 as the helper virus, in an attempt to reduce the possibilities for recombination between the plasmid and the helper virus..

The functions of the elements contained within the u_c-DR1-u_b sequence were further examined using a transient passaging assay system. This enabled the behaviour of the mutated packaging signals to be assessed in an assay which required packaged amplicon DNA to re-enter a productive round of replication i.e. to be re-circularised and act as a template for virus directed DNA replication.

The passaging assay used was described by Stow *et al,* (1983). Using this assay it is possible to identify those sequences which enable defective genomes to be serially passaged in the presence of a helper virus, i.e. not only the sequences which are necessary to direct the encapsidation of the genome but also any sequences which are required for entry into a second round of replication. The assay is similar to the transient packaging assay, except that a virus stock is prepared from the transfected and superinfected cells. This virus stock is used to infect a second monolayer of cells and, after allowing time for the replication of the virus, total cellular DNA is harvested. The presence and level of defective genome sequences in the second monolayer is indicative of the ability of the u_c-DR1-u_b variant to direct DNA packaging into virus particles and also to facilitate re-entry into a productive round of replication.

3.3.2 - Transient packaging assays using amplicons containing mutated u_c-DR1-u_b sequences.

In these experiments the mutant packaging signals described in section 3.2 were used to dissect the functional elements contained within the u_c-DR1-u_b sequence. This was achieved by testing each mutant in the transient packaging assay, using the optimised conditions described in section 3.1.

BHK-21 C13 monolayers were transfected with pSA1, pS1 or one of the pPH plasmids (except the double deletion mutant pPH15.1) using the calcium phosphate procedure. The monolayers were DMSO boosted at 4 hours post-transfection and superinfected (multiplicity of 1.0) with *wt*HSV-1 at 30 hours post-transfection. Total and packaged DNA were prepared 16 hours later. The harvested DNAs were digested with *Eco*RI and *Dpn*I before the products were resolved on an agarose gel. The ethidium bromide stained gel was then examined using long wave UV light to ensure that the samples had been recovered at a consistent level and that the restriction enzyme digestion had been completed successfully. The gel was then Southern blotted as described in the methods section. The ethidium bromide stained gels and the phosphorimages from an experiment conducted in this way are shown in figures 3.37 and 3.38.

Figure 3.37 shows the ethidium stained gels which were blotted to generate the Southern blots in figure 3.38. The ethidium bromide stained gels show that the recovery of the different samples was reasonably consistent and that the restriction enzyme digestion was successful. The ethidium bromide stained gels for other experiments in this section are not shown but those presented in figure 3.37 are a representative example.

Figure 3.38 A shows the total DNA harvested from cells transfected with pSA1, pS1 or one of the pPH plasmids. The DNA in lane 3 is from cells transfected with pSA1 and mock infected, the remaining sample lanes contain DNA harvested from cells transfected with pSA1, pS1 or one of the mutant plasmids (as labelled) and infected with *wt*HSV-1. As expected, each plasmid has been replicated at a similar efficiency enabling a good comparison of the levels of packaging. Figure 3.38 B shows the accompanying packaged samples for this experiment. Again, Lane 3 contains DNA harvested from cells transfected with pSA1 and mock infected. The remaining sample lanes contain DNA from cells transfected with pSA1, pS1 or one of the mutant plasmids (as labelled) and infected with *wt*HSV-1. pSA1 (lane 4), which is the positive control and parent plasmid, has been packaged, while pS1, which is the negative control, has been unable to direct its own packaging. Visual inspection indicates that, 6 mutants are significantly impaired for packaging, these being pPH3, 4, 9, 10, 15.2 and 19 (lanes 6, 7, 10, 11, 17 and 21 respectively). These mutants contain the pac2 deletion (pPH3), pac1 deletion (pPH4), the

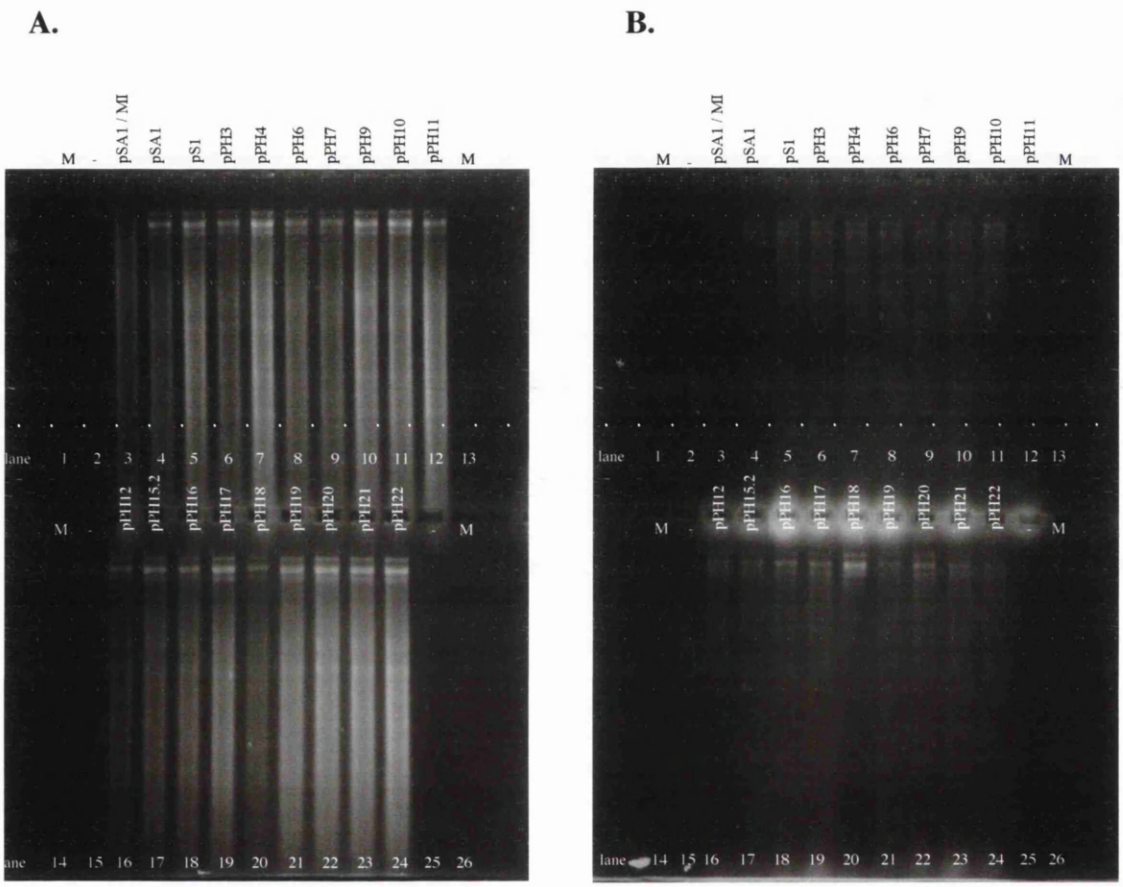


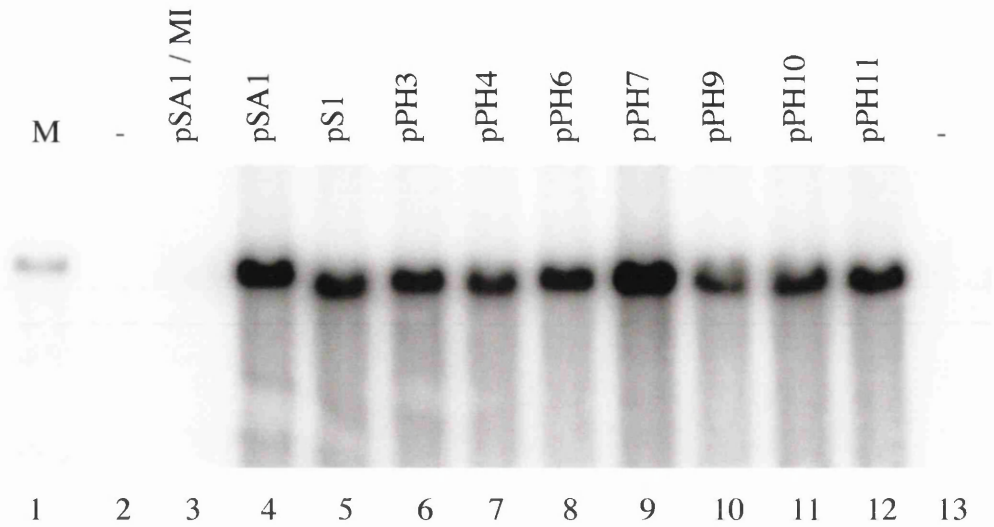
Figure 3.37 - Ethidium bromide stained gels prior to Southern blotting.

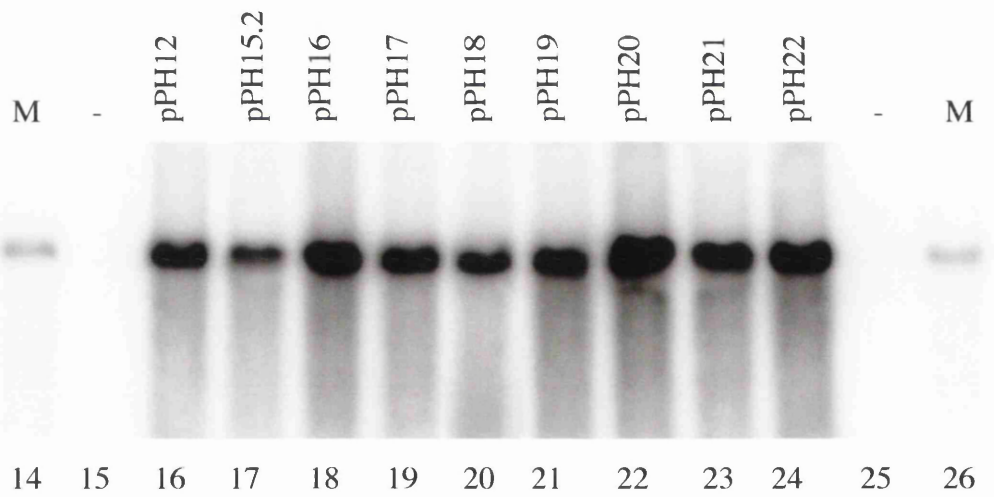
The gels above were Southern blotted to give the phosphorimages shown in figure 3.38.

Panel A corresponds to panel A of figure 3.38 and contains the total DNA samples for the experiment. The amount of DNA in each lane is reasonably uniform indicating consistent recovery.

Panel B corresponds to panel B of figure 3.38 and contains the packaged DNA samples for this experiment; again recovery is fairly consistent.

A.


M
-
pSA1 / MI
pSA1
pS1
pPH3
pPH4
pPH6
pPH7
pPH9
pPH10
pPH11
-
lane 1 2 3 4 5 6 7 8 9 10 11 12 13


M
-
pPH12
pPH15.2
pPH16
pPH17
pPH18
pPH19
pPH20
pPH21
pPH22
-
M
lane 14 15 16 17 18 19 20 21 22 23 24 25 26

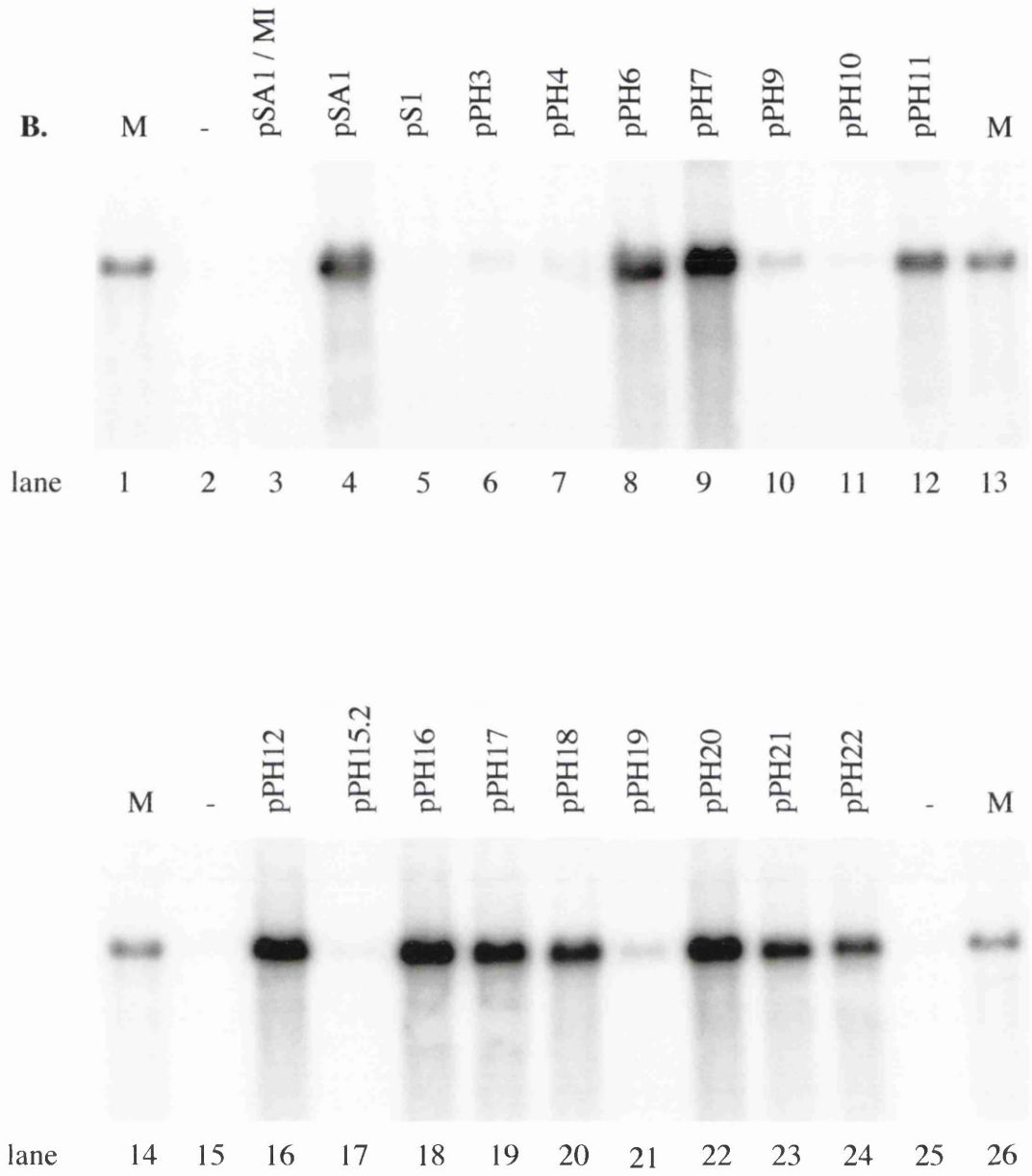


Figure 3.38 - Transient packaging assay with pPH series plasmids - I.

BHK 21 C13 monolayers were transfected with either pSA1, pS1 or one of the pPH plasmids as indicated in lanes 3-12 and 16-24 A and B. Monolayers were DMSO boosted 4 hours post transfection. All monolayers, except the control (MI) plate (lanes 3), were super-infected with *wt*HSV-1 strain 17 (multiplicity of 1.0) at 30 hours post transfection. 16 hours post infection total (A) and packaged (B) DNA was harvested. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane probed with ^{32}P labelled pAT153. Lanes 1, 14, 26 A and B, and 13 B contain 1 ng of linear pSA1.

substitution and deletion mutations of the pac2 T rich element (pPH9 and 10), the deletions of the pac2 GC rich element (pPH15.2) and the pac2 unconserved region (pPH19). The results of this experiment were further analysed by measuring the intensity of each band using the phosphorimager software (**Molecular Dynamics**), which enabled a quantitative comparison of the packaging efficiencies to be made. The data from this experiment, and a number of smaller experiments using mutant amplicons, are shown in table 3.8.

These data clearly identify the importance of the pac1 and pac2 homologies in the cleavage and packaging process, both pPH3 and pPH4 being severely impaired in their levels of packaging. It is, however, apparent that greater amounts of these plasmids are packaged than of the negative control amplicon (pS1).

The importance of the pac2 T rich element is directly indicated by two of the mutants. The deletion or substitution of this element results in a reduced or abolished ability to direct packaging (pPH10 and pPH9, respectively). pPH10 which contains the deletion mutation is reduced in its ability to direct packaging to a level comparable with that of the whole pac element deletions (pPH3 and pPH4). pPH9 which contains the substitution mutation is significantly reduced in its ability to direct packaging but, interestingly, it consistently packages slightly more DNA than the deletion mutant. There are two possible interpretations of this observation. The substitution mutation has one nucleotide which is the same as the wild type sequence and thus the substituted sequence may potentially retain some limited activity. Alternatively, and probably more likely, it is possible that the deletion of the T rich element has, in addition to deleting an important element, also disrupted some other important feature such as the spacing of other elements within the packaging signal.

Two further mutants are impaired in their ability to direct packaging, these being the deletion of the pac2 GC rich element (pPH15.2) and the deletion of the pac2 unconserved region (pPH19). These mutations do not affect DNA packaging simply through the absence of important sequences, because the substitution of these elements does not affect their ability to direct packaging. It appears instead that by deleting these elements some spatial arrangement within the packaging signal is affected, and this disrupts the ability of the mutant to direct packaging.

The pPH15.1 plasmid was also assayed using the transient packaging assay. This plasmid, which contains a u_c-DR1-u_b sequence from which the pac2 GC rich region and the majority of the pac2 unconserved region have been deleted, was seriously deficient in its ability to direct cleavage and packaging (data not shown). This is not surprising because individual deletion mutations of these regions were also shown to be deficient in their ability to direct cleavage and packaging (see table 3.8).

Table 3.8 - Transient packaging assays - I.

Plasmid Number	Plasmid Description	Experiment 1			Expt. 2	Expt. 3	Expt. 4
		Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5	Percentage Packaged	Percentage Packaged	Percentage Packaged	Percentage Packaged
1 ng Marker		20.3	20.3	**n/a**	-	-	**n/a**
pSA1 / MIT		12.4	0.398	**3.2**	-	-	-
pSA1	parental u_c-DR1-u_b sequence	326	59.4	**18.2**	**15.5**	**41.2**	**72.3**
pS1	negative control plasmid	237	1.12	**0.5**	**2.5**	-	-
pPH3	pac2 deletion	240	2.71	**1.1**	**7.0**	-	-
pPH4	pac1 deletion	203	3.95	**2.0**	**3.6**	-	-
pPH6	DR1 substitution	256	70.5	**27.6**	**16.0**	-	-
pPH7	pac1 T element substitution	491	146	**29.7**	**15.3**	-	-
pPH9	pac2 T element substitution	184	7.14	**3.9**	**5.3**	-	-
pPH10	pac2 T element deletion	228	1.86	**0.8**	**3.5**	-	-
pPH11	pac1 GC distal element deletion	250	36.5	**14.6**	-	**34.0**	-
pPH12	pac1 GC distal element substitution	284	123	**43.5**	**14.7**	-	-
pPH15.2	pac2 GC element deletion	173	4.41	**2.6**	**4.1**	-	-
pPH16	pac2 GC element substitution	462	138	**29.9**	-	**36.3**	-
pPH17	pac2 consensus deletion	320	101	**31.6**	-	**25.8**	-
pPH18	pac2 consensus substitution	355	189	**53.4**	-	**36.1**	-
pPH19	pac2 unconserved region deletion	351	10.0	**2.9**	-	-	**2.3**
pPH20	pac2 unconserved region substitution	583	150	**25.8**	-	-	**23.2**
pPH21	pac1 proximal GC element deletion	363	68.4	**18.8**	-	-	**18.2**
pPH22	pac1 proximal GC element substitution	495	50.1	**10.1**	-	-	**16.5**

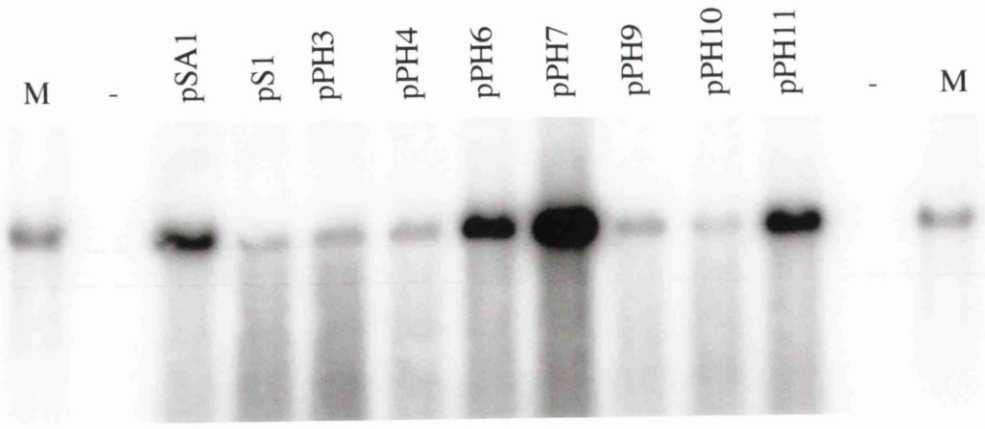
Total and packaged DNA samples were run on separate gels the data has been standardised against the intensity of the 1 ng marker. Volume counts are shown x10^{-5}.to 3 significant figures. Experiments 2,3 and 4 are smaller independant experiments, the % of replicated DNA packaged was similarly calculated. In experiment 4 the apparently high % of replicated pSA1 which is packaged, is the result of poor total DNA recovery.

3.3.3 - Apparent variation in the replicative ability of pPH plasmids.

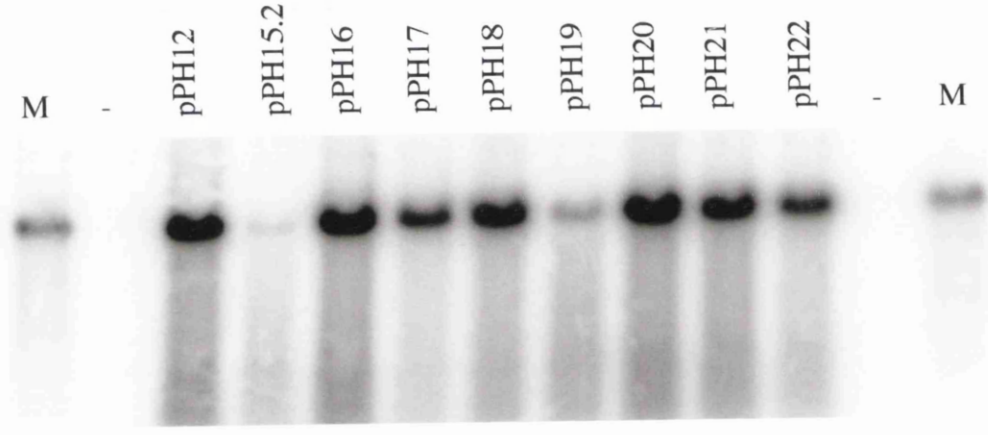
The result seen in figure 3.38, where total DNA levels were fairly constant for all of the mutants tested, contrasts with some results from other experiments which were typically similar to those shown in figure 3.39. Again, 16 mutants were assayed using the optimised conditions for the transient packaging assay described in section 3.1. However, it is immediately apparent that there is a quite noticeable variation between the apparent levels of DNA replication for individual plasmids. Lanes 3 and 4 contain DNA from cells transfected with pSA1 and pS1, the positive and negative controls respectively. pSA1 (lane 3) appears to have been replicated successfully. pS1, which is identical to pSA1 except that it does not contain the u_c-DR1-u_b sequence, appears by contrast to be noticeably impaired with respect to its replication. The correlation between apparent replication level and the presence of a functional cleavage and packaging signal continues for the mutated plasmids. The intensity of the total and packaged bands for this experiment was analysed using the phosphorimager software (**Molecular Dynamics**) and the data is shown in table 3.8. The data show that, although the absolute level of replication and packaging varies considerably between plasmids, the same plasmids as in Fig 3.38 show most efficient packaging of their replicated DNA. The one exception to this is pSA1, which in this experiment has packaged only a small proportion of its total DNA. This was explained when the photograph of the packaged DNA gel was examined: the photograph shows that the recovery of packaged DNA (as judged by the intensity of helper virus bands) was lower in this sample than it was for the others. The low level of packaged pSA1, therefore, represents an anomaly confined to this experiment.

This experiment is particularly interesting because it was performed in parallel with the experiment in figure 3.38. With one exception the experiments were performed identically, using the same cell clone, the same plasmid preparations, and the same calcium phosphate precipitate. The transfections were carried out only minutes apart and the DNA was prepared in an identical fashion. The only difference between the two experiments was in the way that the BHK 21 C13 monolayers were prepared. For the experiment shown in figure 3.38 the BHK monolayers were seeded with $6x10^5$ cells from a subconfluent 175 cm^2 flask, the monolayers were then incubated at 37°C for 24 hours prior to transfection. The BHK21 C13 monolayers used in the experiment shown in figure 3.39 were seeded with $6x10^5$ cells from a confluent 175 cm^2 flask, the monolayers were then incubated at 31°C for 48 hours prior to transfection. At the time of transfection both sets of monolayers were almost confluent and the cells appeared to be in good health.

A.



lane 1 2 3 4 5 6 7 8 9 10 11 12 13



lane 14 15 16 17 18 19 20 21 22 23 24 25 26

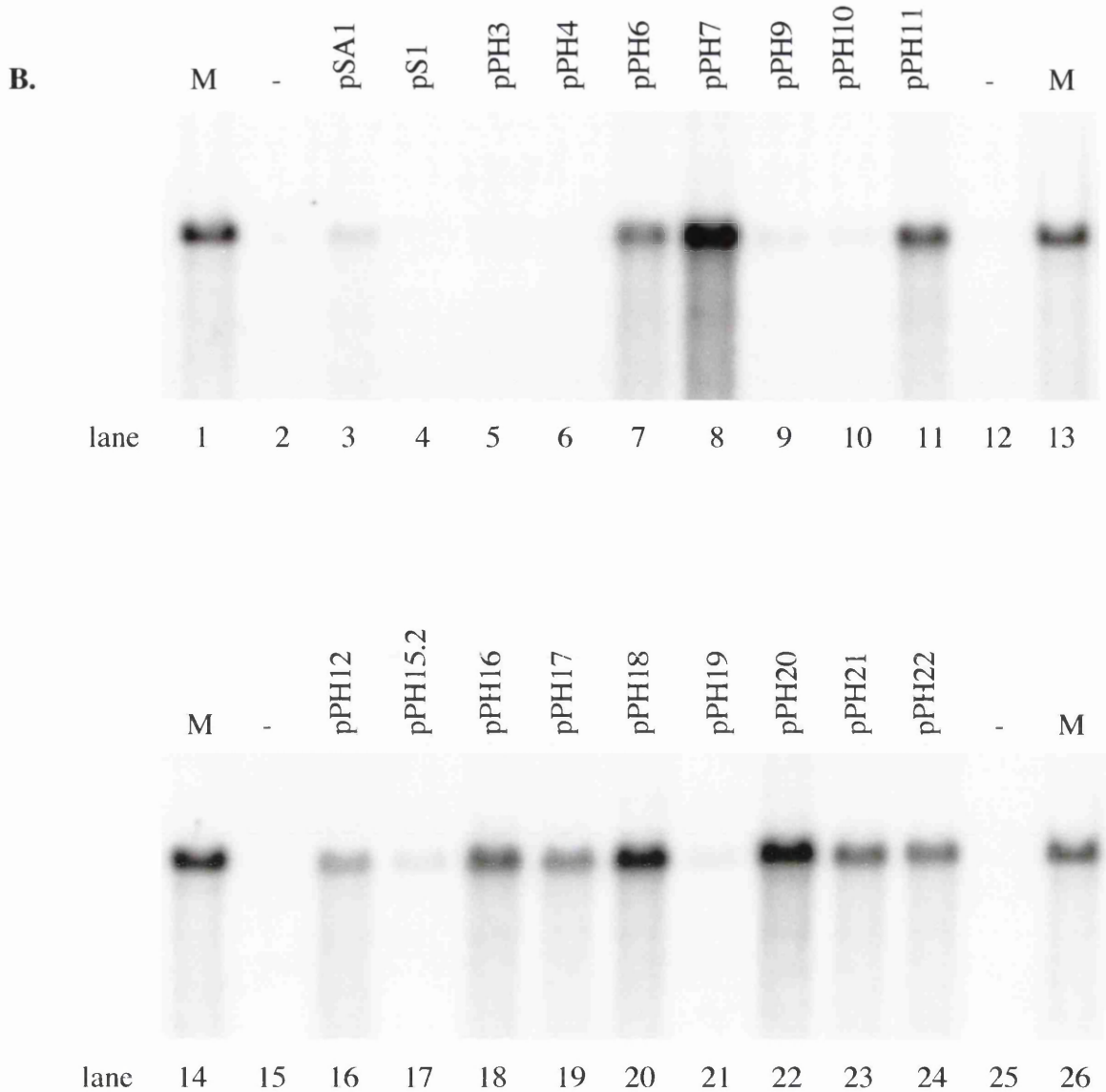


Figure 3.39 - Transient packaging assay with pPH series plasmids - II.

BHK 21 C13 monolayers were transfected with either pSA1, pS1 or one of the pPH mutated plasmids as indicated in lanes 3-11 and 16-24 A and B. Monolayers were DMSO boosted 4 hours post transfection and super-infected with *wt*HSV-1 strain 17 (multiplicity of 1.0) at 30 hours post transfection. 16 hours post infection total (A) and packaged (B) DNA was harvested. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane probed with ^{32}P labelled pAT153. Lanes 1,13,14 and 26 A and B contain 1 ng of linear pSA1.

Table 3.9 - Transient packaging assays - II.

Plasmid	Plasmid Description	Band Intensity Total DNA x10^5	Band Intensity Packaged DNA x10^5	Percentage Packaged
1 ng Marker		153	153	n/a
pSA1	parental u_C-DR1-u_B sequence	368	17.0	4.6
pS1	negative control plasmid	114	0.776	0.7
pPH3	Pac2 deletion	138	2.25	1.6
pPH4	pac1 deletion	106	5.15	4.9
pPH6	DR1 substitution	586	110	18.8
pPH7	pac1 T element substitution	1911	367	19.2
pPH9	pac2 T element substitution	187	13.9	7.4
pPH10	pac2 T element deletion	92.1	8.02	8.7
pPH11	pac1 GC distal element deletion	514	130	25.3
pPH12	pac1 GC distal element substitution	790	55.4	7.0
pPH15.2	pac2 GC element deletion	101	13.3	13.2
pPH16	pac2 GC element substitution	759	136	17.9
pPH17	pac2 consensus deletion	428	86.4	20.2
pPH18	pac2 consensus substitution	649	217	33.4
pPH19	pac2 unconserved region deletion	216	15.8	7.3
pPH20	pac2 unconserved region substitution	842	261	31.0
pPH21	pac1 proximal GC element deletion	631	120	19.0
pPH22	pac1 proximal GC element substitution	405	97.3	24.0

The total and packaged DNA samples were run on separate gels and so data has been standardised against the intensity of the 1 ng marker. All the data is shown to 3 significant figures.

The source of the variability in the amount of replicated plasmid DNA detected was considered in some detail. The photograph of the total DNA gel prior to Southern blotting was examined in order to confirm that the DNA recovery had been consistent. An attempt was also made to check that each plasmid had been transfected into the cells with approximately equal efficiency. This was achieved by digesting an aliquot of total DNA from the experiment in figure 3.39 with *Eco*RI and *Dpn*II and then Southern blotting as described in the methods section. *Dpn*II cuts DNA containing the sequence GATC, providing that the A residue is unmethylated. This leaves just the input plasmid DNA as *Eco*RI monomers. No correlation between the apparent level of replication and either the level of DNA recovery or the transfection efficiency was apparent (data not shown).

It appears likely, therefore, that the peculiar variation between these two experiments is somehow related to the 'condition' of the cells (see discussion).

3.3.4 - Possible repair of mutated sequences by recombination.

The possibility that some of the mutated packaging signals direct cleavage and packaging as a consequence of being repaired by recombination with the helper virus, was investigated in two ways. Firstly, the substitution mutants which contained an engineered *Xho*I restriction enzyme site at the position of the mutation were screened for the presence of the marker site in the packaged DNA. The presence of the marker would indicate that the site of mutation remained unaltered. Secondly, the pPH mutants (except pPH15.1) were re-assayed using the transient packaging assay with *wt*HSV-2 strain HG52 as the helper virus.

3.3.4.1 - Restriction enzyme analysis.

An aliquot of packaged plasmid DNA for each of the mutants which contained an engineered *Xho*I site was digested with *Ahd*I and *Xho*I restriction enzymes, the products were resolved on an agarose gel and Southern blotted as described in the methods section. The restriction enzyme *Ahd*I cuts once in the parental plasmid at a location approximately 1.1 Kbp from the site of mutation. The *Xho*I site is not present in the corresponding region of *wt* HSV-1 DNA, so cleavage at this second site indicates the continued presence of the substitution mutation. Digestion of mutant plasmid DNA which still contains the *Xho*I site produces two bands of approximately 3.3 Kbp and 1.1 Kbp, dependant on the position of the mutation within the u_c-DR1-u_b sequence. If the mutation has been repaired by recombination with the helper virus the *Xho*I site would be missing and only a single band, 4.4 Kbp in size, would be observed.

Plasmids pPH7, pPH9, pPH20 and pPH22 contain the engineered *Xho*I restriction enzyme site. Packaged DNA from these plasmids plus an aliquot of packaged pSA1 was digested as described above and the products were resolved on an agarose gel and Southern blotted as described in the methods section. Figure 3.40 shows the phosphorimage of this experiment. Lanes 3 & 5-8 contain the digested samples (as labelled). As the Southern blot shows, those plasmids which were packaged retained the engineered restriction enzyme site. For pPH9 (lane 6), which is significantly impaired in its ability to direct packaging, it is just possible to see the 3.3 and 1.1 Kbp bands which indicate that even the small amount which is packaged still contains the engineered mutation.

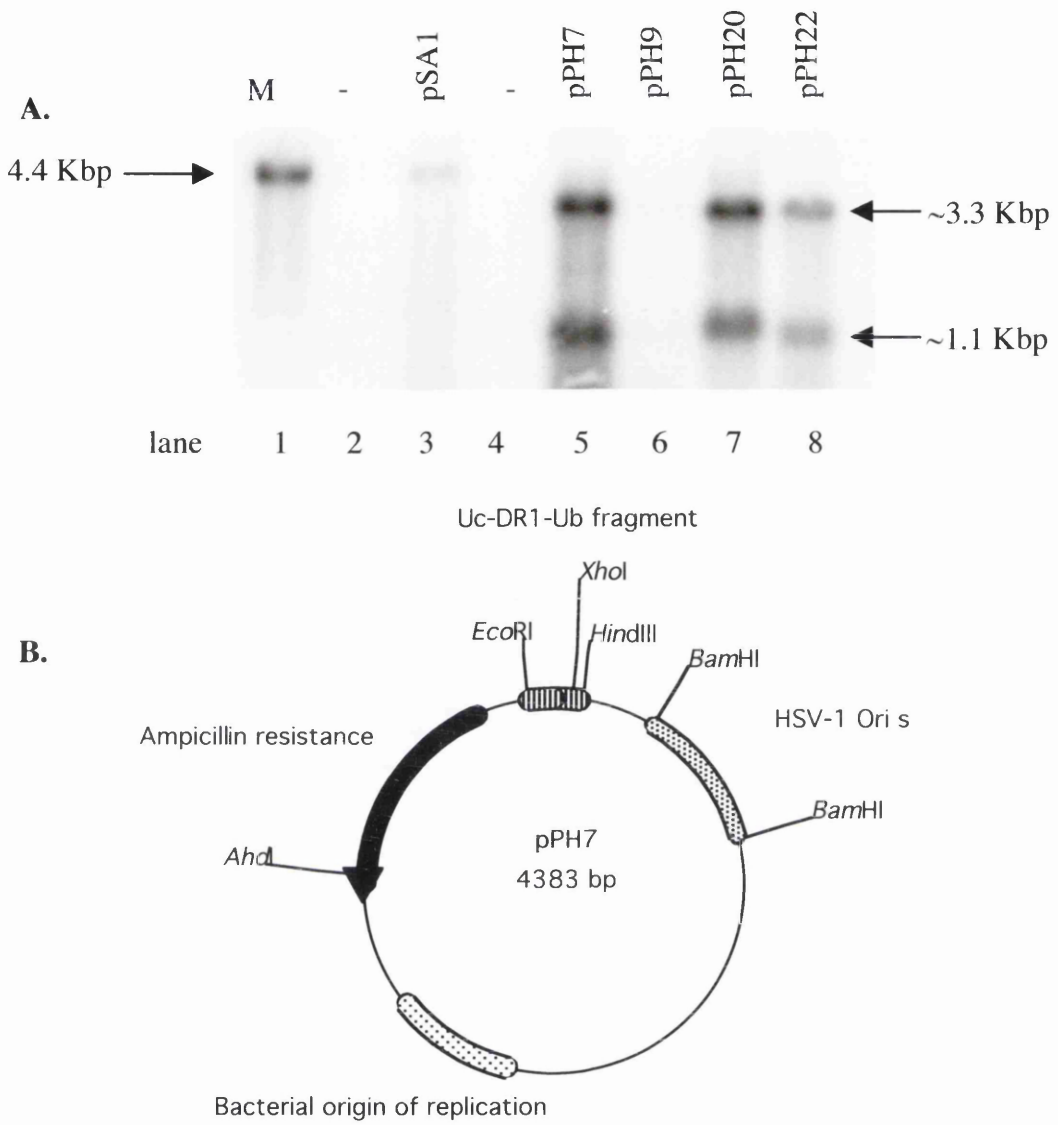


Figure 3.40 - *Ahd*I / *Xho*I digestion of packaged mutants.

Panel A shows packaged DNA from pSA1, pPH7, pPH9, pPH20 and pPH22 (lanes 3, 5, 6, 7 and 8 respectively) which was digested with *Ahd*I and *Xho*I and the products resolved on a 0.8 % agarose TBE gel. The presence of the *Xho*I site in the mutated plasmids gives rise to the profile containing two bands, as opposed to the one for the parent plasmid. Lane 1 contains 1 ng of linear pSA1 (M).

Panel B shows a diagrammatic representation of a pPH7. The engineered *Xho*I site being approximately 1.1 Kbp from the *Ahd*I site, as shown in the diagram.

This approach was also attempted to test the integrity of the substitution mutations containing the *Sca*I restriction enzyme marker. However, conclusive results using this enzyme were not obtained due to the difficulties in achieving 100% digestion with this enzyme.

3.3.4.2 - Reducing the opportunity for homologous recombination with the helper virus.

As described earlier, one way to reduce the possibility of mutations being repaired by recombination with the helper virus is to reduce the degree of homology between the mutant and the helper virus. This was achieved by testing the ability of the mutated plasmids to be packaged using *wt*HSV-2 Strain HG52 as the helper virus and the results are shown in figure 3.41.

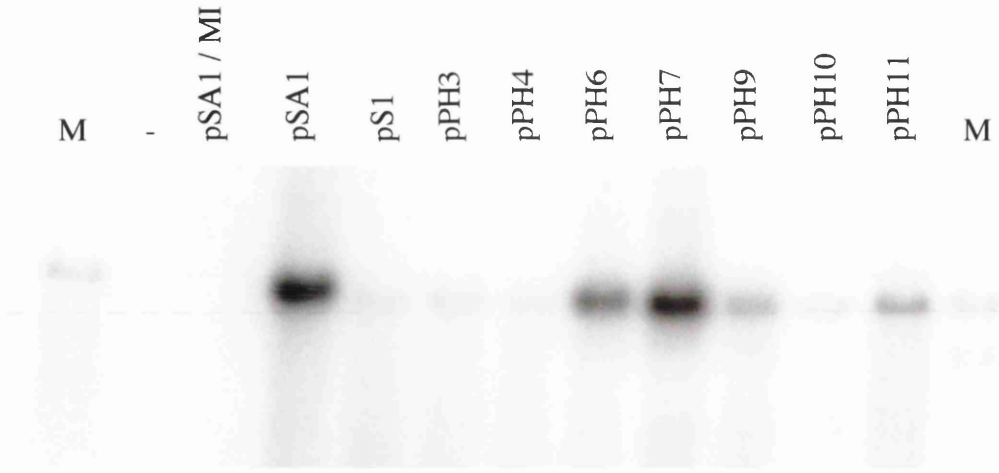
In this experiment, which unfortunately exhibits the phenomenon of reduced levels of replicated plasmid in the total DNA fraction, most of the plasmids exhibit the same behaviour with HSV-2 as the helper virus as with HSV-1. This again suggests that homologous recombination is unlikely to be occurring between plasmids and helper virus (figure 3.41B). If homologous recombination was rescuing mutant plasmids when HSV-1 was used as the helper virus it would be expected that some mutant plasmids, which had previously appeared competent for cleavage and packaging, would not be so in an HSV-2 directed assay. Interestingly, the only difference between the HSV-1 and HSV-2 directed assays was the opposite of this. pPH19, which contains a u_c-DR1-u_b sequence with the pac2 unconserved region deleted, is significantly impaired in HSV-1 directed transient packaging assays, but appears to be unimpaired when HSV-2 is used as the helper virus (figure 3.40 B lane 21).

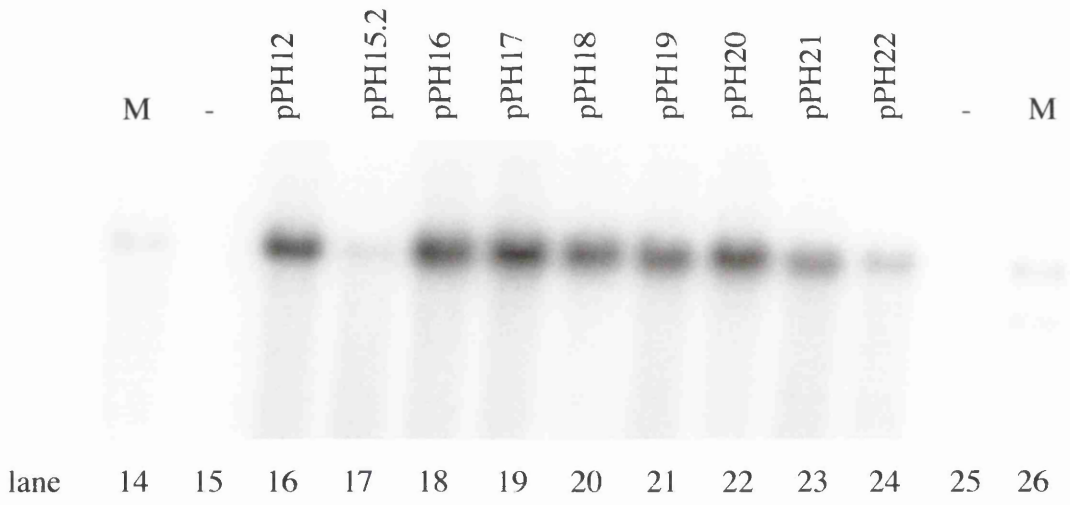
This experiment supports the evidence from the restriction enzyme analysis which indicates that mutant plasmids are not being rescued by the helper virus. The behaviour of pPH19 does, however, suggest that there may be differences between the essential components of the packaging signals of HSV-1 and HSV-2.

3.3.5 - Ability of amplicons to be serially propagated.

As described earlier (section 3.3.1) the mutant plasmids were also assayed for their ability to be serially passaged. For this to be possible the plasmids must behave as defective genomes. This means that they must be replicated, cleaved and packaged into preformed capsids, and then be released from the cells in viral particles. These particles must be able to successfully re-infect fresh cells, and the DNA must re-circularise and re-enter a second

A.


M
-
pSA1 / MI
pSA1
pS1
pPH3
pPH4
pPH6
pPH7
pPH9
pPH10
pPH11
M
lane 1 2 3 4 5 6 7 8 9 10 11 12 13


M
-
pPH12
pPH15.2
pPH16
pPH17
pPH18
pPH19
pPH20
pPH21
pPH22
-
M
lane 14 15 16 17 18 19 20 21 22 23 24 25 26

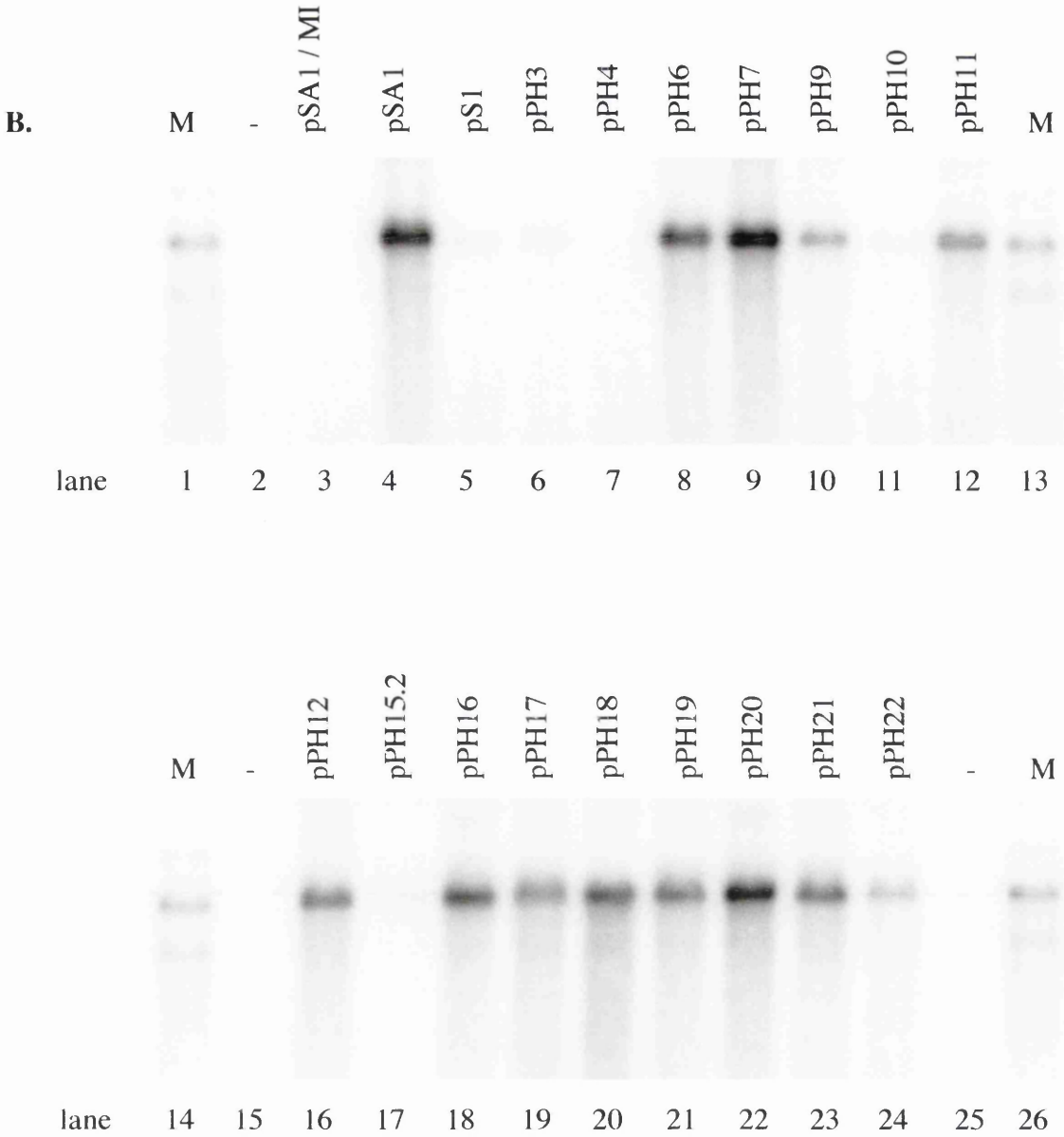


Figure 3.41 - Transient packaging assay of all mutants using *wt* HSV-2 as helper virus.

BHK 21 C13 monolayers were transfected with either pSA1, pS1 or one of the pPH plasmids as indicated in lanes 3-12 and 16-24 A and B. Monolayers were DMSO boosted 4 hours post transfection and super-infected with *wt* HSV-2 strain HG52 (multiplicity of 1.0) at 30 hours post transfection. 16 hours post infection total (A) and packaged (B) DNA was harvested. The DNA was *Eco*RI and *Dpn*I digested and the products resolved on a 0.8% agarose TBE gel. The gel was Southern blotted and the membrane probed with ^{32}P labelled pAT153. Lanes 1,13,14 and 26 A and B contain 1 ng of linear pSA1.

round of replication. The ability of HSV-1 amplicons to be serially passaged was demonstrated by Stow *et al.* (1983) using plasmids containing single and tandemly repeated 'a' sequences. The aim of this section of work was to establish whether plasmids containing the u_c-DR1-u_b sequence could be serially passaged, and whether this process was affected by any of the mutations which had been introduced into this region. The experiments would also independently assess the ability of mutated u_c-DR1-u_b sequences to direct packaging.

The serial passaging assay was based on that described by Stow *et al.* (1983) and is illustrated in Fig 3.42. Transfection and superinfection of BHK cells in 35 mm dishes was performed as in the transient packaging assay. 16 hours p.i. the cells were scraped into the medium and divided into two equal portions. Total cellular DNA was prepared from one portion and this is referred to as the 'pre passage' DNA. The second portion was sonicated to prepare a virus stock. One half of this stock was used to infect a fresh 35 mm plate of BHK cells, and 16 hours p.i. total cellular DNA was again prepared; this is referred to as the 'post-passage' DNA. Amounts the pre-passage and post-passage DNAs each representing that from one-fifth of the cells on a plate (i.e. $4x10^5$) were digested with *Eco*RI and *Dpn*I. The products then resolved on an agarose gel and analysed by Southern blotting.

Three outcomes for this experiment can be envisaged: (i) the replicated amplicon is not packaged in the original transfected cells and therefore cannot be serially passaged, (ii) the replicated amplicon is packaged but cannot enter a second round of replication when passaged (e.g. it may fail to circularise on infection), and (iii) the replicated amplicon is packaged and can enter a second round of replication when the virus stock is passaged. Since the amount of virus stock used corresponds to only 25% of the original transfected monolayer, the presence in the post-passage sample of greater than 25% of the amount of replicated plasmid DNA in the pre-passaged sample indicates that replication of the amplicon must have occurred on infection of the fresh cells.

The pPH series of plasmids (except the double mutant pPH15.1), pS1 and pSA1 were tested in this way and the results are shown in Fig. 3.43, where the 'a' and 'b' samples correspond to pre-passage and post passage DNA respectively. It can be clearly seen that in this experiment all the plasmids replicated to similar levels in the transfected cells ('a' samples. In addition this experiment clearly shows that the u_c-DR1-u_b sequence (pSA1) contains all of the sequences necessary for serial passaging, since similar intensity bands were detected in the pre and post passage DNA samples (lane 5 and 6) indicating that replication of the plasmid sequences must have occurred when the virus stock was passaged. In contrast pS1, which lacks the u_c-DR1-u_b element was detected in the pre-passage but not the post-passage DNA sample (lanes 7 and 8). The absence of pS1 DNA in the post passaged sample is probably because the DNA accumulating in the transfected cells is not

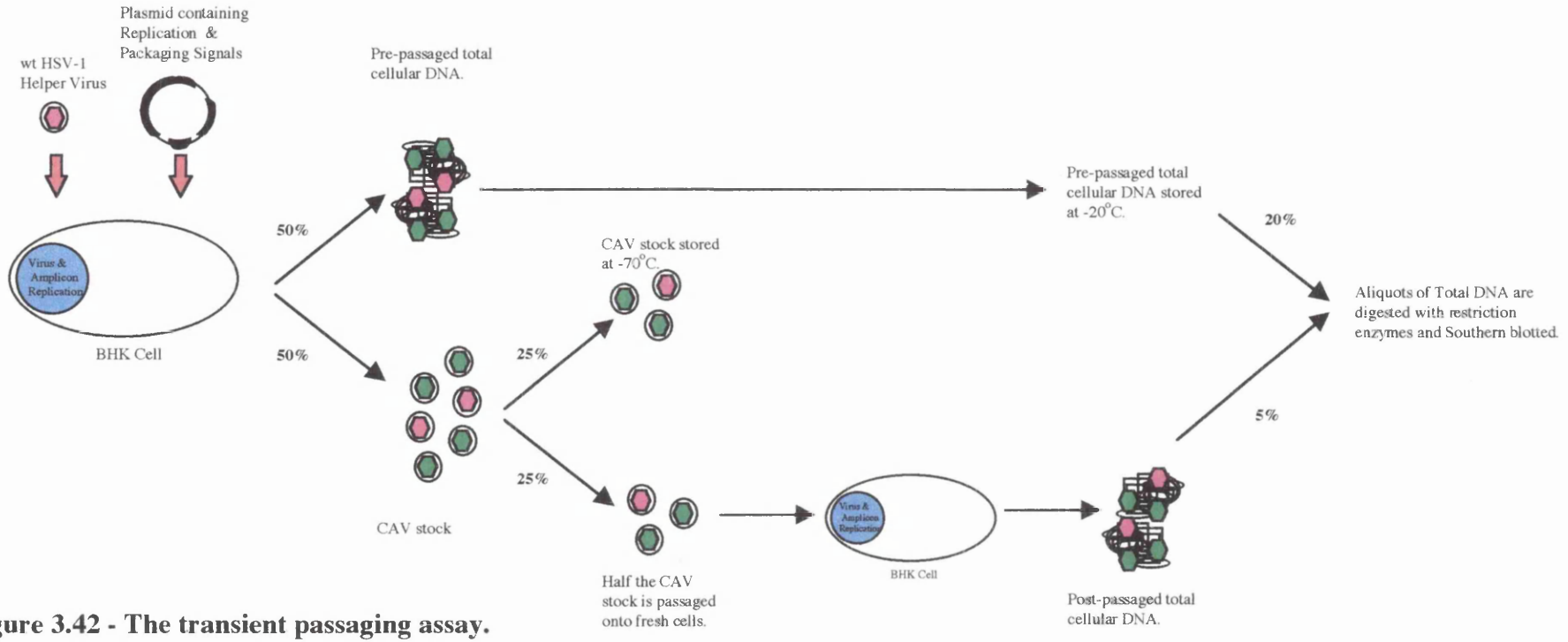
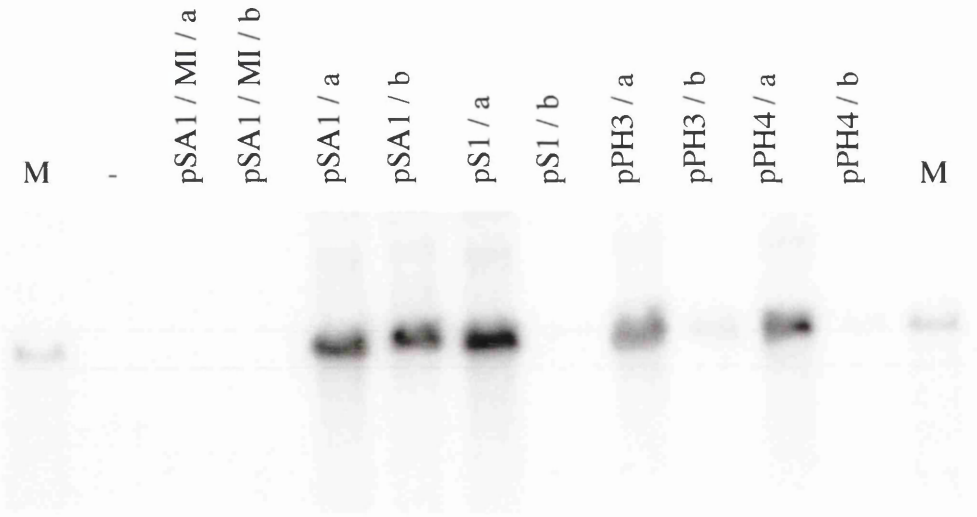


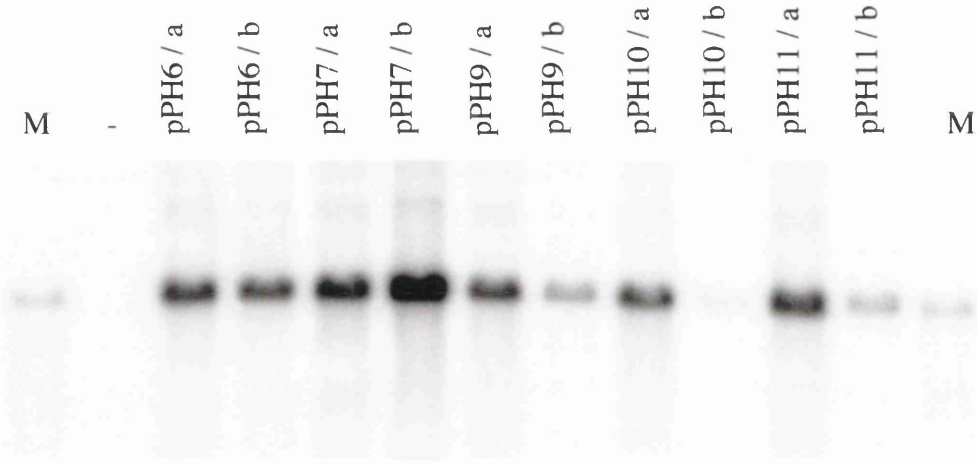
Figure 3.42 - The transient passaging assay.

The transient passaging assay used in this study was described by Stow *et al.*, (1983), it involves serially passaging an amplicon in the same way that a defective genome is serially passaged during repeated high multiplicity infections. An amplicon or putative amplicon was transfected into BHK21 C13 cells using calcium phosphate procedure, the cells were infected with *wt* HSV-1 strain 17(MOI of 1.0) and incubated at 37°C. 16 hours later the cells were divided into two portions, half were used to prepare a virus stock and half were used to prepare total cellular DNA which was stored at -20°C (pre-passaged DNA). Half the CAV stock was used to re-infect a fresh monolayer of BHK21 C13 cells from which total cellular DNA was prepared 16 hours later (post-passaged DNA). Aliquots of pre and post passaged DNA were then digested with *Eco*RI and *Dpn*I and the products analysed by Southern blotting. In each case DNA corresponding to 1/5th the DNA from the cell monolayer was digested.

The figures marked in bold denote the maximum percentage of the original plate's replicated amplicon which could be present at each step in the procedure. In reality much less amplicon DNA is passaged, because these figures suppose that all of the pre-passaged DNA is packaged into virus particles which are able to re-infect fresh cells. An equal pre and post passage band intensity would therefore reflect a minimum of four fold amplification during the serial propagation.



lane 1 2 3 4 5 6 7 8 9 10 11 12 13



lane 14 15 16 17 18 19 20 21 22 23 24 25 26

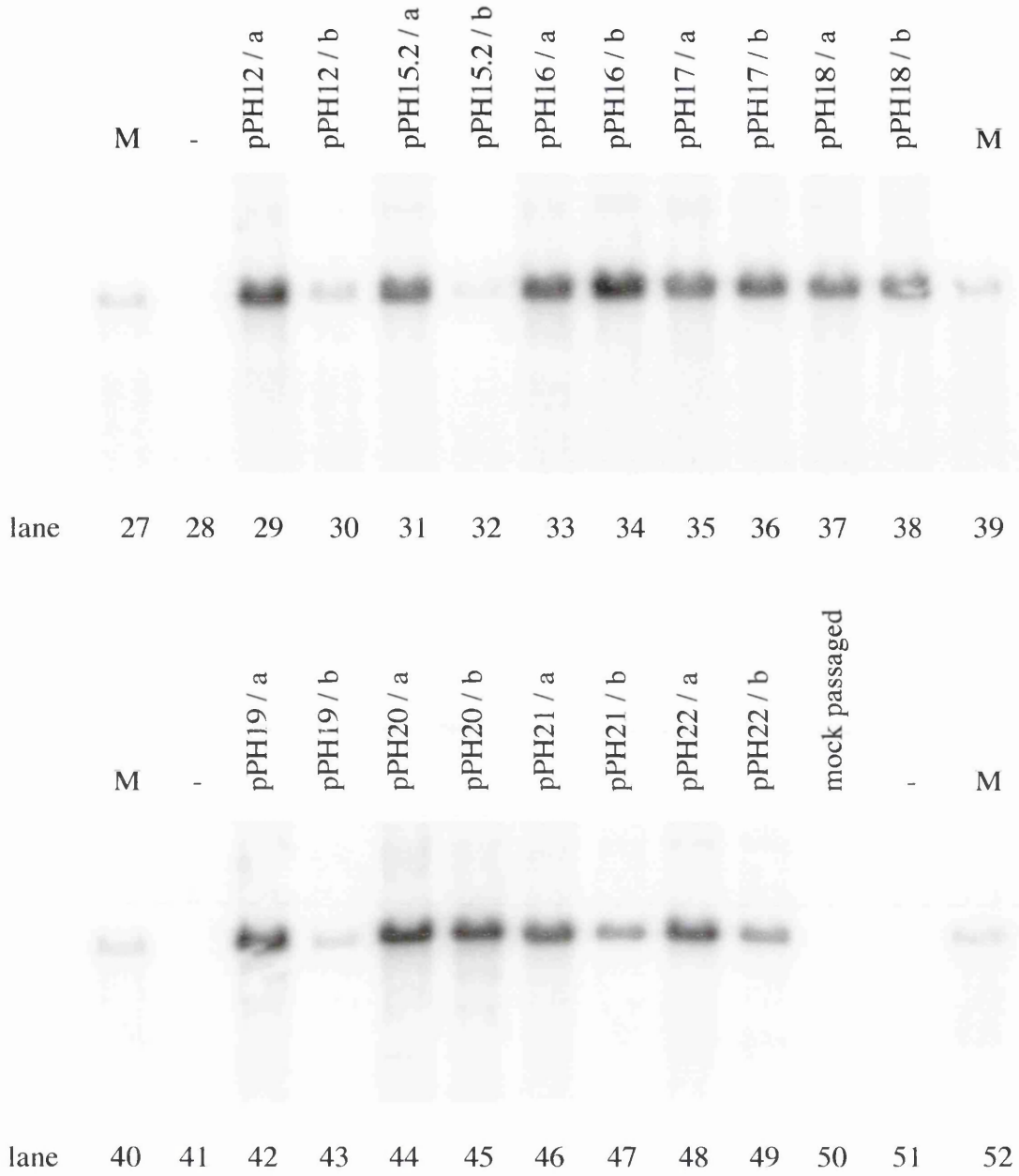


Figure 3.43 - Passaging assay with all mutants.

BHK 21 C13 monolayers were transfected with indicated plasmids (lanes 3-12, 16-25, 29-38 and 42-50). The monolayers were DMSO boosted at 4 hours post transfection and super-infected with *wt*HSV-1 strain 17 (multiplicity of 1.0) at 30 hours post transfection. 16 hours post infection the cells from each monolayer were divided into two halves. Total DNA was harvested from one half (indicated as sample 'a') The other half was used to derive a virus stock. Half of this virus stock was then used to infect a fresh BHK 21 C13 monolayer. 16 hours post re-infection total DNA was prepared (indicated as sample 'b'). DNAs 'a' and 'b' were digested with *Eco*RI and *Dpn*I and the products resolved on a 0.8 % agarose TBE gel. The gel was Southern blotted and the membrane was probed with ^{32}P labelled pAT153. Lanes 1, 13, 14, 26, 27, 39, 40 and 52 contain 1 ng linear pSA1.

packaged and is likely to be sheared into small pieces by the sonication process. These small fragments are then either not taken up or degraded by the cells in the second infection.

All the pPH plasmids which were impaired in the transient packaging assay were detected at very low levels in the post passage DNA samples (pPH3, pPH4, pPH9, pPH10, pPH15.2 and pPH19). Of the plasmids that were relatively unimpaired in the transient packaging assay several behaved similarly to pSA1 and gave post passage bands approximately equal to (or greater) in intensity to the bands in the pre-passage samples (pPH6, pPH7, pPH16, pPH17, pPH18 and pPH20). In contrast four plasmids that were relatively unimpaired in the transient packaging assay generated significantly reduced bands in the post passage samples (pPH11, pPH12, pPH21 and pPH22).

In order to examine these observations quantitatively, the intensity of each band was measured using the phosphorimager software (**Molecular Dynamics**).

Table 3.10 - Transient passaging assay.

Plasmid	Serial Passage Assay			Behaviour in transient packaging assay ✓/×	Behaviour in transient passaging assay (+- =)
	Band Intensity Total DNA x10^5 plate a	Band Intensity Total DNA x10^5 plate b	(a/b)*100		
pSA1	26.6	27.1	**101.9**	✓	n/a
pS1	38.7	1.13	**2.9**	×	n/a
pPH3	16.7	2.26	**13.5**	×	n/a
pPH4	1.90	0.684	**3.6**	×	n/a
pPH6	31.8	27.7	**86.9**	✓	=
pPH7	41.7	87.6	**210.2**	✓	+
pPH9	28.4	9.00	**31.7**	×	+
pPH10	25.2	2.00	**7.9**	×	n/a
pPH11	31.4	5.91	**18.38**	✓	-
pPH12	36.7	5.07	**13.8**	✓	-
pPH15.2	27.2	2.63	**6.7**	×	n/a
pPH16	33.7	46.9	**139.2**	✓	=
pPH17	26.0	27.8	**106.9**	✓	=
pPH18	22.6	17.8	**78.6**	✓	=
pPH19	35.6	5.05	**14.2**	×	n/a
pPH20	44.7	39.0	**87.3**	✓	=
pPH21	31.0	12.9	**41.7**	✓	-
pPH22	31.3	12.6	**40.3**	✓	-

The samples were run on 2 separate gels and so data has been standardised against the intensity of the 1 ng markers. All the data is shown x10^5 and to 3 significant figures. ✓ or × indicate plasmids that are packaged effectively or poorly, respectively, in the transient packaging assay. For the efficiently packaged plasmids the fifth column indicates whether these amplicons are passaged with similar (=), lesser (-) or Greater (+) efficient than pSA1.

The data (table 3.10) confirms that pSA1 has been serially passaged, the level of pSA1 in the passaged sample (lane 6) exceeding the maximum value of 25% carry over from the pre-passage infection. Each of the 6 mutant plasmids which were impaired for packaging (as labelled in table 3.10) are, unsurprisingly, also accumulated to low levels on passaging. One plasmid which could be considered an exception to this is pPH9. pPH9, which contains the u_c-DR1-u_b sequence with the pac2 T element substitution mutation, has a post passage band intensity of 32% the pre-passage level. This is most likely a reflection of the fact that pPH9 packages approximately 4% of its replicated DNA (cf. pPH3 which packages around 1%). This means that as much as 4 fold more DNA is available to re-establish a second round of replication. This relationship between apparent packaging efficiency and the absolute amount of plasmid seen in the post passaged sample is also apparent for the other packaging deficient plasmids.

These observations suggest that although these amplicons are packaged ineffectively, they may nevertheless be capable of being amplified when delivered to fresh cells as defective genomes within a virus stock. The quantitative analysis also confirms that pPH6, 7 , 16, 17, 18 and 20 are unimpaired in their ability to be serially passaged, whilst pPH11, 12, 21 & 22 are passaged less efficiently than pSA1. pPH11 and 12 contain the deletion and substitution mutations of the pac1 distal GC rich region while pPH21 and 22 contain the deletion and substitution mutations of the pac1 proximal GC rich region. The mutations in the distal GC rich region have resulted in pPH11 and 12 being seriously impaired for passaging, the post-passage level of plasmid being less than the 25% of the pre-passaged level. It is not possible, however, to determine whether the DNA detected in the post passage sample represents solely input DNA from the virus stock or whether limited replication of these amplicons has occurred. The mutations in the proximal GC rich region of pPH21 and 22 have a less pronounced effect, both plasmids have post passage DNA levels of approximately 40% the pre passage level. This suggests that these mutants do replicate during passaging albeit with reduced efficiency.

Another interesting observation, which is compounded in the transient passaging assay, relates to pPH7 which contains the deletion mutation of the pac1 T rich element. The data in table 3.10 shows that pPH7 has a post-passaged level of DNA which is more than twice the pre passaged level, this is also twice the level of passaged pSA1. This is consistent with the observation that pPH7 often replicates particularly well in the transient packaging assay.

The transient passaging assay has therefore shown that all of the sequences required for serially passaging a viral genome are contained within the u_c-DR1-u_b sequence, and it has highlighted the fact that the proximal and distal GC rich regions of pac1 are important in this

process. In addition, the results from this assay support the observations made using the transient packaging assay, which indicated that the GC rich region, the T rich element and the unconserved region of pac2, as well as pac1 and pac2 as a whole, are important in the encapsidation of HSV-1 DNA.

3.3.6 - Discussion.

The data presented in this section confirm that the u_c-DR1-u_b sequence contains all of the sequences necessary to act as a functional packaging signal for HSV-1 (Nasseri & Mocarski, 1988), and it extends the analysis by identifying specific regions that are important for packaging. In addition, it has been shown that the u_c-DR1-u_b sequence contains all of the sequences necessary for the HSV-1 genome to be serially passaged, highlighting two regions within the pac1 homology which appear to be important in this process. Overall, the mutagenesis has identified 5 regions of the u_c-DR1-u_b sequence which appear to have functions either in the encapsidation of the viral genome or in the ability of that genome to be serially passaged. Despite the high recombination rates reported during HSV-1 replication, no evidence of mutants being repaired by recombination with the helper virus was observed during this study.

3.3.6.1 - The importance of the pac1 and pac2 homologies.

The key nature of both pac1 and pac2 is indicated by the behaviour of pPH4, and pPH3, which are the pac1 and pac2 deletions respectively, although a low level of packaging was observed for both plasmids. This is consistent with the work of previous investigators who also found that the pac1 and pac2 homologies coincided with regions of the 'a' sequence which were important in the cleavage and packaging process (Deiss *et al.,* 1986; Nasseri & Mocarski, 1988; McVoy *et al.,* 1998. The low level of packaging could potentially be caused by recombination between the plasmids and the helper virus, integrated copies of the plasmid being packaged into capsids along with the viral genome. This possibility appears unlikely however, as no evidence of homologous recombination was observed. Another explanation may be that there is a low level of redundancy between the two pac elements. This would not be totally surprising as both elements contain a small run of T residues flanked by GC rich sequences. Alternatively, it may be that the low level of packaging observed represents packaging of DNA under unusual circumstances; for example packaging is initiated but the end of the concatemer is reached before a functional termination signal is encountered, thus circumventing the need for a termination cleavage event.

3.3.6.2 - Mutations in the pac1 homology.

Five mutations within pac1 were constructed, identifying two regions which are required for efficient serial passage of viral genomes. While no specific elements within pac1 were identified as important in the packaging process, the pac1 region as a whole is essential. It is possible that the pac1 region maintains some important contextual feature of the packaging signal, or that redundancy within the region means that loss of individual regions can be compensated for.

a) Mutations in the pac1 GC rich regions.

The transient passaging assay highlighted both the proximal and distal GC rich regions of pac1 as important for the serial passage of viral genomes. This involvement of regions of the u_c-DR1-u_b sequence in the passaging of viral genomes is not totally surprising considering that these sequences are found at the terminus of the viral genome. It is, however, surprising that the important sequences lie exclusively at one terminus. This data supports the earlier suggestion that these sequences are important, based on the observation that the proximal and distal GC rich regions of pac1 both contain quite well conserved regions across a wide range of herpes viruses (see figure 3.44). Their exact role in the passaging process cannot be established from this study, but it presumably lies in either the release of genomes from the capsid, their delivery to the replication centre or their circularisation. Circularisation would appear the most likely of these possibilities, and this could be tested by examining the fate of the amplicon DNA following passage into fresh cells.

The evidence from these single element mutations is supported by the behaviour of the pac1 and pac2 deletion mutants. The accumulation of the pac2 deletion mutant (pPH3) after passaging is consistent with the plasmid being impaired for packaging, but the small amount which is packaged being able to re-initiate infection. The lower level of the pac1 deletion mutant (pPH4) after passaging suggests that it is impaired in its ability to be packaged and also in its ability to re-initiate infection. This, therefore, implies that essential sequences for passaging lie within the pac1 homology.

b) Mutation in the pac1 T rich element.

The T rich elements of both pac1 and pac2 have been shown to have conserved locations with respect to the termini in a wide variety of herpesviruses (Deiss *et al.*, 1986). The exception to this is HCMV which appears to lack a pac2 T rich element (Broll *et al.*, 1999). This feature of conserved location has lead to at least one model for DNA cleavage

and packaging which involves cleavage events at a fixed distance from these T rich elements (Varmuza & Smiley, 1985). Despite this, the data from the transient packaging assays shows that substitution of the pac1 T rich sequence has no observable effect on the ability of the mutant to be packaged. There are a number of possible reasons which might underlie this observation. The first being that the pac1 T rich element is dispensable in a functional cleavage and packaging signal. This conclusion is supported, in part, by the fact that the T rich element of pac1 is less well conserved than the pac2 T rich element. This is clear from the alignment shown in figure 3.44. This alignment of putative pac1 and pac2 sequences from a range of herpes viruses was compiled from the sequences presented by Broll *et al.* (1999) and Deiss *et al.* (1986). There is, however, an alternative explanation. The HSV-1 pac1 T rich element consists of the sequence TGTG(T)4, but there is another (T)4 sequence 12 bases further from the terminus in the u_c region. The possibility exists that in pPH7, which has the u_c-DR1-u_b sequence with the pac1 T rich element substitution mutation, this second run of T residues is able to compensate for the loss of the principle T rich element. This scenario does not, however, account for the observation that in for example the transient passaging assay shown, pPH7 appears to replicate significantly more efficiently than pSA1. This may reflect that the pac1 T rich element acts as a negative regulator of packaging. The absence of such a signal would imply that more DNA would be packaged.

3.3.6.3 - Mutations in the pac2 homology.

Eight mutations within pac2 were constructed and these highlighted one region which was directly involved in the packaging process and a further two regions which appear to play a more indirect role in affecting either the spacing of different elements or the overall structure of the packaging signal. None of the mutations, which between them spanned the entire pac2 sequence, had any effect on the ability of the plasmids to be serially passaged (i.e. to re-initiate infection).

a) Mutation of the pac2 T rich element.

As mentioned above, the T rich elements of both pac1 and pac2 have been shown to have conserved locations with respect to the termini in a wide variety of herpes viruses (Deiss *et al.*, 1986).

The data from the transient packaging assays identifies the pac2 T element as an important component of the packaging signal. The plasmids containing the substitution (pPH9) and the deletion mutation (pPH10) of the pac2 T rich element are significantly impaired in their ability to be packaged. There is, however, a noticeable difference in the

degree to which these mutants are impaired. pPH10, which contains the deletion mutation, is impaired in packaging to a level similar to that of the mutant deleting all of pac2. pPH9, which contains the substitution mutation, is also packaged at a low level, however, it is consistently packaged more efficiently than the deletion mutation. Possible explanations for this observation are discussed in the context of several other mutations in section 3.3.6.3b.

b) Mutations in the pac2 GC rich and unconserved regions.

The observation that deletion of the pac2 GC rich region (pPH15.2) disrupts the function of the packaging signal, but that substitution (pPH16) of the same sequence has no effect, suggests that the deletion mutation is affecting the spatial arrangement of the packaging signal rather than destroying essential sequences. In the u_c-DR1-u_b sequence the pac2 GC rich region separates the pac2 T rich element from the DR1 repeat, which contains the site of cleavage, and the pac1 region. The observation that the deletion of the pac1 proximal GC rich region has no effect on packaging suggests that deleting the pac2 GC rich region is effecting the spacing between an essential pac2 element and the site of cleavage. The essential nature and the conserved location of the pac2 T rich element make it a prime candidate for this position specific element.

pPH19 contains the pac2 unconserved region deletion mutation. Again, the observation that deletion of this element disrupts the ability of the mutant to direct packaging but that the substitution mutation has no effect suggests that the mutation is affecting the spatial arrangement of the packaging signal. Further evidence supporting this hypothesis can be found by comparing the amount of DNA which is packaged by the different substitution and deletion mutants of the u_c-DR1-u_b sequence. Broadly speaking there appear to be three classes of mutation: i) Mutations which almost completely abrogate DNA cleavage and packaging, e.g. the pac2 deletion. ii) Mutations which significantly impair DNA cleavage and packaging, e.g. substitution of the pac2 T rich element. iii) Mutations which do not appear to significantly affect DNA cleavage and packaging, e.g. substitution of the pac1 T rich element. Table 3.11 shows which mutants fall into each group.

pPH15.2 (pac2 GC rich element deletion) falls into the first class, being as impaired as the complete pac2 deletion. pPH10 (pac2 T rich element deletion) also fall into this class. The substitution of the pac2 T rich element (pPH9) however, while causing significant impairment clearly enables more DNA to be packaged than pPH10, putting this mutation into the second class. pPH19 (pac2 unconserved region deletion) also falls into this class. Putting all of these observations together, it seems to suggest that there are two position-specific features within the pac2 region. The substitution of the pac2 T rich element destroys the sequence but maintains the overall spacing of the region, thus leaving the

hypothetical second element intact. The deletion of the pac2 unconserved region disrupts the spacing of the hypothetical second element but leaves the pac2 T rich element unaffected. Deletion of the pac2 T rich element or deletion of the pac2 GC rich region destroys or displaces both elements, severely impairing the ability of both these mutants to direct cleavage and packaging. The obvious importance of the pac2 T rich element indicates that this is one position-specific element, but no second element was identified during this study. The pac2 consensus sequence, as defined by Deiss *et al.* (1986), occupies a location which would be consistent with this being the position specific-element but, as discussed below, mutations in this region failed to disrupt the ability of the mutants to direct packaging.

It, therefore, remains possible that the pac2 unconserved and GC rich sequences are somehow involved in the overall structure of the packaging signal, e.g. presenting a specific DNA conformation or DNA bending within the packaging signal, as opposed to simply affecting the spacing of two position-specific elements.

Table 3.11 - The effect of different mutations on packaging efficiency.

Seriously Impaired Mutants	
Plasmid	Mutation Description
pPH3	Pac2 deletion
pPH4	pac1 deletion
pPH10	pac2 T element deletion
pPH15.2	pac2 GC element deletion

Partially Impaired Mutants	
Plasmid	Mutation Description
pPH9	pac2 T element substitution
pPH20	pac2 unconserved region substitution

Unimpaired Mutants	
Plasmid	Mutation Description
pPH6	DR1 substitution
pPH7	pac1 T element substitution
pPH11	pac1 GC distal element deletion
pPH12	pac1 GC distal element substitution
pPH16	pac2 GC element substitution
pPH17	pac2 consensus deletion
pPH18	pac2 consensus substitution
pPH19	pac2 unconserved region deletion
pPH21	pac1 proximal GC element deletion
pPH22	pac1 proximal GC element substitution

The observation that pPH19 packages at a similar level to pSA1 when HSV-2 is used as the helper virus, but is significantly impaired when HSV-1 is used, raises a puzzling issue. It was anticipated that the use of HSV-2 as the helper virus would reduce the possibility of homologous recombination occurring between the plasmids and the helper virus, thus highlighting any mutants which were being rescued as a result of recombination with HSV-1 helper virus. The observation that one mutant packages more successfully

when HSV-2 is the helper virus appears to indicate a degree of flexibility (which was not anticipated), in the HSV-2 packaging mechanism. Although surprising, similar observations have been reported else where, for example in adenovirus DNA replication. The Adenovirus 2 (Ad2) origin of replication contains three domains which are required for efficient initiation of replication. The three domains include a nine base pair region which is highly conserved in all human adenoviruses, and 2 regions which form binding sites for host proteins NFI and NFIII. NFI binding is necessary for efficient Ad2 replication *in vitro* and is absolutely essential *in vivo*. The Ad2 origin of replication can be activated by Ad4 DNA replication proteins, but it was noticed that the opposite was not the case. The reason for this is that the Ad4 virus does not require NFI binding to its origin, in fact its origin does not contain an NFI binding site. This means that Ad4 proteins can initiate replication from an Ad2 origin with the NFI binding site deleted, whilst the cognate, Ad2 proteins cannot (Hay, 1985a&b).

Obtaining further insight into this apparent flexibility within the HSV-2 packaging mechanism will probably require a more detailed understanding of the function of the pac2 unconserved region in the packaging process.

c) Mutations in the pac2 consensus sequence.

The data from the transient packaging assays shows that neither the deletion or substitution mutations of the pac2 consensus sequence have any effect on packaging. As this was difficult to reconcile with either their predicted behaviour or the behaviour of the mutants described in section b above, the presence of mutated copies of the u_c-DR1-u_b sequence was confirmed directly by re-sequencing the mutant amplicons.

The evidence therefore, suggests that the pac2 consensus sequence is not the hypothetical second position specific-element described earlier. A closer examination of the sequence of this region indicates that this might not be unreasonable. The designation of the pac2 GC rich region as a highly conserved element is based on sequence comparisons between the termini of several different herpes viruses (Deiss *et al.*, 1986). However, the GC rich sequence is itself found in a region of the genome which is highly GC rich. In fact the 14 bp deletion of the pac2 GC rich region alters the sequence, at the position of the consensus sequence, to a sequence in which five of the seven residues are identical to the *wt* sequence. Deletion of the consensus sequence itself replaces the sequence with residues which are identical in four of the seven positions. A more recent sequence comparison, using newly available sequences, has also found that the so called pac2 consensus sequence is less well conserved than had been previously thought (Broll *et al.*, 1999)(see figure 3.44)

3.3.6.4 - The apparently reduced replication of unpackageable amplicons

The reduced efficiency with which the plasmids impaired for packaging were able to replicate in some experiments was not found to be completely unprecedented. A similar connection between replication and packaging competence was reported for HHV-6 amplicons (Deng & Dewhurst, 1998). Although in this virus the packaging signal lies adjacent to the origin of replication the pac elements share extensive homology. For HHV-6 the hypothesis was presented that the activity of the packaging signal somehow up-regulated the origin of replication. In the study described here however, the effect appears to be intimately related to the cells, possibly to their 'condition' or position in the cell cycle. Subsequent investigations have revealed that the reduced level of replicated plasmid DNA in the total fraction of these experiments is probably a consequence of unpackaged DNA degradation during the preparation of the total cellular DNA (N.Stow, personal communication). This, combined with the observations made during this study (indicating that the effect is related to the cells), suggests that in older or less healthy cells nucleases are possibly produced at higher levels or are more active. These enzymes act on the unpackaged DNA following the cell lysis with NP40 and prior to SDS / protease treatment. Because the packaged DNA is protected from nuclease digestion by the viral capsid, those mutants which are deficient for packaging have a higher proportion of their total DNA which is susceptible to nuclease digestion. This leads to the apparent reduction in the levels of replication for packaging deficient plasmids.

However, these conclusions are unlikely to explain the observations made by Deng and Dewhurst (1998) described above. In that study the extra-chromosomal DNA, which is equivalent to the total DNA in this study, was prepared using the Hirt method (Hirt, 1967) which does not involve cell lysis with NP40. Despite this, it remains possible that the observed reduction in the replication of unpackageable amplicons is an artefact of the assay system used. The Hirt method of preparing extra-chromosomal DNA relies on pelleting the high molecular weight chromosomal DNA and leaving the lower molecular weight DNAs in solution. It would appear likely that at least a proportion of the high molecular weight replicated plasmid DNA, which will be in concatemeric form, may also be pelleted. As described above, those plasmids which are unpackaged will have a larger proportion of their total DNA in an unprotected concatemeric form and anything which acts to specifically reduce this unpackaged form will have the effect of apparently reducing the level of replication.

Figure 3.44 - Herpes virus pac element alignment.

Alignment of putative pac1 and pac2 sequences from 14 representative α, β and γ herpes viruses. The sequences are shown 5'-3' from left to right and the conserved motifs are marked in bold. The terminus at which pac1 and pac2 are found varies between herpes viruses e.g. pac1 is found at the left terminus of HVS and HCMV but the right terminus of HSV-1. This figure was compiled from the sequences presented by Broll *et al.* (1999) and Deiss *et al.* (1986).

pac1

	Terminus	G/C Rich Sequence	T Rich Element	G Rich Sequence	
HSV-1 17	TGCCCGCCGCCACCGCTTTAAAGGGCCGCGCGCGA	**CCCCCGGGGGG**	TGTGTTTT	**GGGGGGGG**	. .
HSV-2 HG52	CTCCCGCCCCGCCTCTTTTCCCCCGGGGAGTCAA	**CCCCCGGGGGGGG**	TGTTTTTT	**GGGGGGGGG**	. .
VZV	CCTCTCCCGGGGTCCGCCGGGCGCCCAGAAA	**CCGGGGGGGGG**	TTATTTTC	**GGGGGGGGG**	. .
PRV	CCCACCCCCCGCTCCCCGGGGGCCGCGAAAAAA	**GGGGGCGGGGC**	TTAAA	**GGGGGGGGGG**	. .
BHV-1	CCTCCGCGCCGCGCCGCGCGGGGGCCCTGCAA	**CCCCGGGGGGG**	TGTTTTT	**GGGGGGGGG**	. .
EHV-1	CCCCAGCCCGCGTCTCACCCCCGCACCCGAAT	**GGGGGGCG**	TGGTCTAAA	**GGGGGGGGG**	. .
HCMV AD169	TCCATTCCGGGCCGCGTGGTGGGTCCTCGAGGGG	**CGGGGGGG**	TGTTTTTAGC	**GGGGGGG**	. .
HHV-6	TCCTCGCGTTTCAAAAATTACTTTAAACT	**CCCCGGGGGGG**	TTAAAAAAA	**GGGGGGG**	. .
HHV-7	CCCCCCGTTTCGTATTTCAAATCCTAAATAA	**CCCCCGGGGGG**	TAAAAAAA	**GGGGGGG**	. .
MCMV	CCCCCCGGCCGTCTGAGTGCGCGCGGG	**CCCCCCGGGGGGG**	TATTTTGAT	**GGGGGGG**	. .
BHV-4	CCCCCGGCCCTCCCAGTGCCCCCAGA	**CCCCCCGGGGG**	TGGTAAAAGAT	**GGGGGGG**	. .
EBV Namalwa	CCCCCCATGCCCTCTCCAACACCCCCCGAAG	**CCCCCCGGGGG**	TCTTTCCT	**GGGGG**	. .
HHV-8	GGTCCCCGCGGCCCGCGCGCG	**CCCCCCCGGGGGGG**	TAAAACA	**GGGGGGGGGGG**	. .
HVS	CCCCTTCCCCCTCTTTGCCTACCAAGTTAT	**CCCCCGGGGGG**	AAAATCAGT	**GGGGG**	. .

pac2

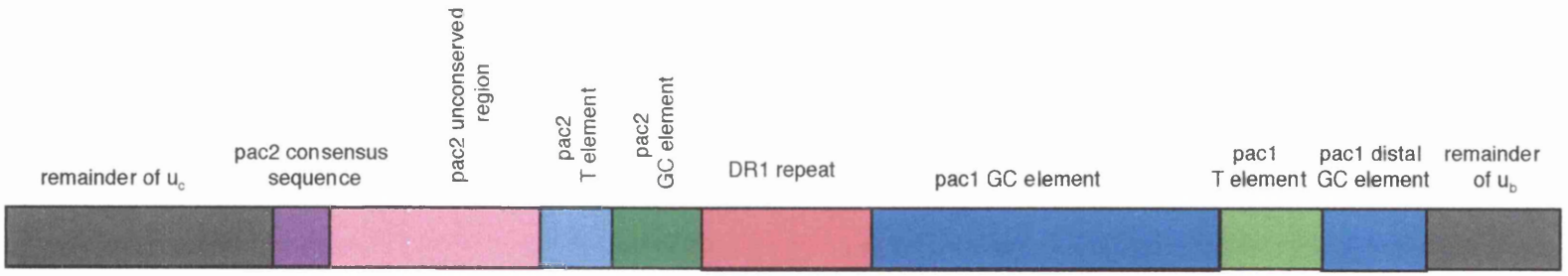
		Consensus Sequence	Unconseved Sequence	T Rich Sequence	GC Rich Sequence	Terminus
HSV-1 17	. .	CGCCGCG	CGCGCGCACGCCGCCCGGACCGCCGCCCGCC	**TTTTTT**	GCGCGCGCGCGCGCCCGCGGGGGGCCCGGGCTGCC	
HSV-2 HG52	. .	CGCCGCG	GGCTGCCTTCCGCGGCGCCCCCGCGCGGC	**TTTTTT**	CCCGCGCCCGCCCCCGCGCGGCAGGACGGGGACTCCCGCCC	
VZV	. .	CGCCCCG	CAAACGCGCGGGGAGGTGGGGTCGC	**TTTTTTTTTT**	CTCTCTCGAGGGGGCCGCGAGAGGGCTGGCCTCC	
PRV	. .	CCCCCTC	CCCATTCACCCCAATGGAAAACGCGGGGCGGGAAA	**TTTTTTTTT**	CAGAGATCCGCGCTCGGGGGAGAGCTGGGCC	
BHV-1	. .	CCCCCGC	CTGCCGCAATGCACGCGCCGCGCGCAGAGAAAA	**TTTTTTTTTT**	CTCCGCGCCCCCCCGCGCGGGGGCTGGGCCC	
EHV-1	. .	CCCCTCT	ACGATTTTCTTCGAGGCCGTCGCGAGGCCGAAAAA	**TTTTTTT**	CACGGGCCCGCGCCCGAGAGAGAGCCTAGCCC	
HCMV AD169	. .	CGCGCGG	CAGGCGCGTACCACTGGAGCGCACAGCCGCCTCCC		GGGCGCGCACCCATCTAGGTGGACGCCCGACAT	
HHV-6	. .	GCGCGCG	CGCGCGCCTCTATGGGAGGCGCCGTG	**TTTTT**	CACCACCACGCGCGCCACTGCAAGAGGCGCGTG	
HHV-7	. .	GGCAGCC	AATGTCTTGTAATGCCTTCAAGGCAC	**TTTTT**	CTGCGAGCCGCGCGCAGCACTCAGTGAAAAACA	
MCMV	. .	CGCCGCG	GCAGATACTTCTTTTTTTCATAGGGGACCTAGCTCAGTACCAC	**TTTTT**	ATCCCCCCCGGCCGTCTGAGTGCGCGCGGGCCC	
BHV-4	. .	GGCGGGC	CAGCAGTTGGTAGGCCACTCCCAC	**TTTTTTTT**	CCCCCCCGGCCCTCCCAGTGCCCCCGGCCC	
EBV Namalwa	. .	GGCCGCG	GGGGCCAGCCACGCCCCCTCCAC	**TTTTT**	CCAGGAATGCGCGGCGGCCCATGCTCCCCCCATGC	
HHV-8	. .	CCCCGCC	GGGGTACGGGGCTAGGCCACGCCTAC	**TTTTTTTTT**	CGGGCGGCCCCCCGACCCTCTCGGCCCCCC	
HVS	. .	CCCCGAG	CTCGCTCTAGCCACGCCCAGGACA	**TTTTT**	CCAGCTGCCCAGCGCCCACTGCTTGGGGCCCC	

Chapter 4 - Conclusion.

In summary, five of the eight elements within the u_c-DR1-u_b sequence have been implicated as being necessary for either DNA packaging or serial passage of the viral genome. The pac2 T rich element has been shown to be central to the packaging mechanism, both in terms of its sequence and its location. The pac2 GC rich and unconserved sequences have been shown to be required for an undefined spacing function in the packaging signal. The pac1 proximal and distal GC rich regions have been shown to be required for efficient serial propagation of amplicons, most probably in the mechanism of circularisation. The DR1 repeat which corresponds to the normal site of viral cleavage has also been confirmed as non essential. No specific function could be attributed to two regions, the pac2 consensus sequence and the pac1 T element. However, the possibility that this is due to redundancy within the packaging signal as opposed to a lack of biological function cannot be excluded. This information is summarised in figure 3.45.

Section 4.1 - Sequences involved in cleavage and packaging.

This study has directly implicated the pac2 T rich element as a central player in the cleavage and packaging process. Mutations which affect either the sequence itself or its location with respect to the site of cleavage were impaired in their ability to direct cleavage and packaging. This observation is consistent with the hypothesis presented by Varmuza and Smiley (1985) which proposed that the T rich elements of pac1 and pac2 functioned to direct separate ss cleavage events at a fixed distance. Correspondingly, the pac1 T rich element mutants might have been anticipated to behave in a similar way. There are in fact, several possible explanations for why this was not the case. As described in section 3.3.6.2b, there is a second $(T)_4$ sequence, 12 bp further away from the S terminus which may be able to compensate for the loss of the T rich element. Although if this sequence does prove to be involved it would indicate that for pac1 the importance of the location of the T rich sequence was significantly reduced. In addition to this, the observation that the pac1 T rich element is non essential in cleavage and packaging is supported by the sequence alignments presented by Broll *et al.* (1999) which showed that following analysis of a wider range of herpesviruses the pac1 T rich element is less well conserved than had been previously thought. Despite this, it is possible that the pac1 T rich element has a function in cleavage and packaging which has not been identified in this study. Firstly the termini of packaged amplicons were not directly examined as part of this study and it remains possible the pac1 T rich element has a function analogues to the role of bacteriophage λ *cos*B and I2



Substitution Mutant -pPH		**18**	**20**	**9**	**16**	**6**	**22**	**7**	**12**	
Deletion Mutant - pPH		**17**	**19**	**10**	**15.2**	n.a	**21**		**11**	
Requirement for Packaging		×	✓ SPACER	✓	✓ SPACER	×	×	×	×	
Requirement for Passaging		×	u.k	u.k	u.k	×	✓	×	✓	

Figure 3.45 - Summary.

The table above indicates the importance of each region of the U_c-DR1-U_b sequence for both DNA packaging and the passaging of the viral genome. The corresponding substitution & deletion mutant numbers are also shown. ✓ indicates that the region is required and × indicates that it is not. u.k indicates a region which is required for packaging and consequently is also required for DNA passaging. Regions which could be successfully substituted but not deleted are indicated as spacer elements.

sites in termination. During termination these sequences have been shown to have a role in increasing the fidelity of cleavage at the N_2 site (Cue & Feiss, 1998). Finally it is also possible that the pac1 T rich element, functioning as part of a termination signal, could be compensated for by a re-initiation cleavage event directed by pac2. However, this would be expected to result in at least some reduction in packaging efficiency, which was not observed.

The observation that pac1 as a whole is essential for cleavage and packaging but that no individual element proved to be so, also raises an apparent contradiction. The most probable explanation for this is that sequences within the pac1 region are essential for cleavage and packaging but that they exhibit a degree of redundancy, allowing individual elements to be deleted without effecting the function of the region as a whole. As mentioned above this may still have consequences for the fidelity of cleavage. Interestingly, parallels can be drawn between λ *cos*Q and the pac1 sequence in this regard. *cosQ* is required for the efficient termination of packaging in λ, having roles in the introduction of nicks at both N_1 and N_2. However, while total deletions of *cos*Q impair *in vivo* packaging some mutations in *cos*Q have actually been shown to increase nicking at the N_2 site (Cue & Feiss, 1998).

The other surprising result from the cleavage and packaging experiments was the un-importance of the pac2 consensus sequence. The alignment of herpesvirus termini presented by Deiss *et al.* (1986) suggested that this sequence was highly conserved and it was proposed to have a function in the cleavage and packaging signal. The present study has however, shown that this is probably not the case and more recent work tends to support this. Firstly, the observation that a degenerate pac2 homologue in GPCMV functions as a cleavage and packaging signal but does not appear to contain a sequence corresponding to the pac2 consensus sequence appears to suggest that, in GPCMV at least, this sequence is not involved in either cleavage, packaging or other events necessary for genome propagation (McVoy *et al.*, 1997). Secondly the alignment of herpesvirus termini presented by Broll *et al.*, (1999) has also cast doubt on the conservation of the consensus sequence. This however, is not the end of the story with regard to the pac2 consensus sequence. Since this element is clearly present in a range of herpesviruses including the GPCMV M terminus and this may reflect an as yet unidentified biological function.

4.2 - Sequences involved in the serial propagation of defective genomes.

The attempts to serially passage plasmids containing mutated u_c-DR1-u_b sequences identified two regions of pac1 which are important in this process but which were not identified as important for cleavage and packaging. Although both the proximal and distal

GC rich sequences of pac1 were identified as important their exact function remains unclear. It is tempting to suggest that these sequences are involved in the process of circularisation which allows the genome / plasmid to enter a second round of replication. It is however, possible that mutations in these sequences impair propagation by producing aberrant termini during cleavage or that they are involved in other aspects of serial propagation e.g. the release of viral DNA from the capsid or the targeting of released DNA to the sites of replication within the nucleus. It is interesting to note that the UL36 protein of HSV-1 has been implicated both in the release of viral DNA from the capsid and binding to parts of the 'a' sequence, although in that study the probes used were mainly from the pac2 region of the 'a' sequence (Chou & Roizman, 1989).

The lack of identifiable pac2 sequence involvement in the passaging of defective genomes may have a number of explanations. It may be that specific pac2 sequences are not required, although this would be surprising, as this would suggest that events such as circularisation only require the specific recognition of one genome terminus. Alternatively the apparent lack of involvement may reflect a level of redundancy within the pac2 sequence. However, it should also be noted that the transient passaging assay used in this study will only identify sequences involved in serial propagation where they are not also involved in cleavage and packaging. This would mean that, for example, the pac2 T rich element maybe required for efficient serial propagation but as the mutant is not packaged it is not possible to identify any subsequent functions of this sequence. In order to extend this analysis it may be possible to artificially generate linear mini genomes containing these mutations and transfect the linear DNA into BHK cells in the presence of helper virus. Subsequent replication of these plasmids would require circularisation, thus replication would be indicative of the presence of the signals necessary for circularisation.

4.3 - Future work.

Several aspects of this study warrant further investigation in order to extend or clarify the observations which have already been made.

As mentioned above, the termini of packaged mutants were not examined as part of this study but as discussed earlier mutations in the cleavage and packaging signal which appear to have little or no effect on packaging may in fact be effecting the fidelity of cleavage. Thus an examination of the precise sequences at the termini of packaged molecules may provide further insight into the functions of the packaging signal elements.

Additional mutations to delete the $(T)_4$ region with the u_b sequence and double deletion / substitution mutants within pac1 may help to more clearly identify those sequences which

are involved in the cleavage and packaging process. In addition work is already underway to insert the mutated u_c-DR1-u_b sequences generated in this study, into the *tk* locus of HSV-1 strain 17. The appearance of novel termini during infection with these recombinant viruses will indicate the presence of a functional cleavage and packaging signal and in this way test the validity of the observations made in the transient packaging assays described here, in the context of the full length viral genome.

Finally a major use for the mutants described here will be hopefully to help in the identification of protein interactions with the HSV-1 cleavage and packaging signal. The fact that the cleavage and packaging signal used in this study is approximately 200 bp in size lends itself to use in mobility shift assays for example. In this way it should be possible to identify sequence-specific DNA binding proteins responsible for the encapsidation of the viral genome, and also using the mutants to correlate the binding activity with the cleavage and packaging of DNA. It is possible that similar approaches will also be useful in dissecting the mechanism of genome circularisation.

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