CHARACTERISATION OF THE HERPES SIMPLEX VIRUS TYPE 1 MUTANT, *amb*UL12

By

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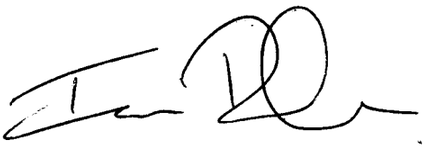

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Abstract

The herpes simplex virus type 1 (HSV-1) UL12 gene encodes an alkaline nuclease. Although the UL12 gene is not absolutely essential for replication, UL12 null mutants produce 100-1000 fold less viable virus than wt HSV-1. It has been suggested that the alkaline nuclease functions to remove branched structures from high molecular weight concatemeric DNA prior to its cleavage into monomeric genomes that are packaged into viral capsids. Failure to remove the branches results in unstable packaging of DNA into capsids which fail to egress from the nucleus.

This thesis describes detailed characterisation of the HSV-1 mutant, *amb*UL12 (Patel *et al.*, 1996) which fails to express the alkaline nuclease due to the insertion of an *amber* stop codon into the UL12 open reading frame. The ability of *amb*UL12 to replicate and package both viral genomic DNA and amplicons (plasmids containing the HSV-1 origin of replication and DNA encapsidation signal) was examined. In contrast to results obtained with other alkaline nuclease mutants, which replicate and package DNA with close to wt HSV-1 efficiency (Shao *et al.*, 1993; Martinez *et al.*, 1996b), *amb*UL12 displayed a 3-5 fold drop in replication and a 15-20 fold drop in packaging of genomic DNA. Similar reductions were observed in the replication and packaging of amplicon DNA. The replication and packaging of amplicons by *amb*UL12 in these transient assays could be partially complemented when UL12 was supplied in *trans*. Close inspection of the DNA molecules synthesised during transient assays demonstrated that amplicon replication intermediates are complex high molecular weight concatemers that undergo intermolecular recombination, analogous to viral DNA replication intermediates.

In agreement with previous results using the AN-1 *nuc* mutant (Martinez *et al.*, 1996a), *amb*UL12 high molecular weight DNA replication intermediates were found to have a more complex structure when analysed by pulsed-field gel electrophoresis (PFGE) than those of wt HSV-1. Restriction enzyme digestion of *amb*UL12 DNA resulted in a very smeared digest pattern, with larger predicted fragments (> 50 kbp) being undetectable as discrete bands. These results support the proposition that the alkaline nuclease is involved in processing the complex viral DNA replication intermediates into substrates more suitable for efficient cleavage and packaging (Martinez *et al.*, 1996a; Goldstein and Weller, 1998a).

Examination of the genomic termini present in DNase-resistant DNA revealed that *amb*UL12 exhibited reduced specificity of cleavage compared to wt HSV-1. The results suggested that aberrant cleavage may occur at either the L or S terminus. In addition, the overall efficiency of cleavage was decreased, as judged by the reduced accumulation of unit length genomes observed in PFGE studies. This differs from published data which indicated that cleavage and packaging were largely unaffected in *nuc* mutants (Shao *et al.*, 1993; Martinez *et al.*, 1996a). Further evidence of aberrant cleavage events was provided by PFGE experiments which demonstrated that the majority of encapsidated *amb*UL12 molecules migrated faster than unit length genomes (152 kbp), with estimated sizes of 130-150kbp.

Transient assays utilising various packaging signal mutants indicated that in the absence of UL12 the cleavage and packaging machinery may cleave in an abnormal way. The *amb*UL12 mutant was able to package molecules lacking a cleavage and packaging signal and these could be further propagated in the presence of wt HSV-1 helper virus.

This indicates that the alkaline nuclease may somehow confer cleavage specificity on the packaging complex. This conclusion is supported by the observation that when UL12 was supplied in *trans* the packaging of such plasmids was decreased although their level of replication was increased.

Based on the observed packaging defect of *amb*UL12, a preliminary investigation into possible interactions of UL12 with various proteins involved in packaging was also performed. The interaction of UL12 with four packaging proteins (UL6, UL15, UL28 and UL25) was examined. No convincing evidence for an interaction between UL12 and any of these proteins was obtained. However, the previously reported interaction of UL12 with the DNA replication protein UL29 (Vaughan *et al.*, 1984; Thomas *et al.*, 1988, 1992) was supported in these experiments.

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Abbreviations

µCi	- microcurie
µg	- microgram
µl	- microlitre
µM	- micromolar
*amb*UL12	- *amber* UL12
BHK (cells)	- baby hamster kidney cells
CAV	- cell associated virus
Ci	- Curie
CHO	- Chinese hamster ovary
CIP	- calf intestinal phosphate
CLB	- cell lysis buffer
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
DMSO	- dimethylsulphoxide
DNA	- deoxyribonucleic acid
DNase	- deoxyribonuclease
dNTP	- 2'-deoxyribonucleotide-5'-triphosphate
ds	- double stranded
DTT	- dithiothreitol
dTTP	- 2'-deoxythymidine-5-triphosphate
E.coli	- *Escherichia coli*
EBV	- Epstein-Barr virus
EDTA	- ethylenediaminetetra-acetic acid
EtBr	- ethidium bromide
FIGE	- field inversion gel electrophoresis
GPCMV	- guinea pig cytomegalovirus
h	- hour

h.p.i.	- hours post infection
h.p.t.	- hours post transfection
HCMV	- human cytomegalovirus
HeBS	- hepes buffered saline
hepes	- N-[2,-hydroxyethyl]piperazine-H'-[2-ethane sulphonic acid]
HHV-6	- human herpesvirus 6
HHV-7	- human herpesvirus 7
HHV-8	- human herpesvirus 8
H-mix	- hybridisation mix
hmw	- high molecular weight
HSV-1	- herpes simplex virus type 1
HSV-2	- herpes simplex virus type 2
kbp	- kilobase pairs
kDa	- kilo daltons
KSHV	- Kaposi's sarcoma associated herpesvirus (HHV-8)
LB	- L-broth
M	- Molar
m.o.i.	- multiplicity of infection
MCS	- multiple cloning site
mg	- milligram
MHC	- major histocompatability complex 1
min	- minute
ml	- millilitre
mm	- millimetre
mM	- millimolar
MW	- molecular weight
NBCS	- newborn calf serum
nm	- nanometre
NP40	- nonidet P-40 detergent
NPT	- non-permissive temperature
NT buffer	- nick translation buffer
°C	- degrees Celsius
OD	- optical density
ORF	- open reading frame

SDS-PAGE	- SDS- polyacrylamide gel electrophoresis
PAA	- phosphonoacetic acid
p.f.u.	- plaque forming unit
PBS	- phosphate buffered saline
PFGE	- pulsed field gel electrophoresis
PRV	- pseudorabies virus
RNase	- ribonuclease
rpm	- revolutions per minute
SDS	- sodium dodecyl sulphate
sec	- second
Sf	- *Spodoptera frugiperda*
ss	- single stranded
SSC	- standard saline citrate
STET (buffer)	- sucrose, Tris, EDTA, triton buffer
TEMED	- N,N,N',N'-tetra-methyl-ethylene diamine
Tris	- 2-amino-2(hydroxymethyl)-1,3-propandiol
Trition X-100	- octyl phenoxy polyethoxy ethanol
ts	- temperature sensitive
TS	- Tris buffered saline
UV	- ultra violet
V	- volts
v/v	- volume ÷ volume
VZV	- varicella-zoster virus
w/v	- weight ÷ volume
wt	- wild type
X-Gal	- 5-bromo-4-chloro-3-indolyl-(-D-galacto pyranoside)
X-junction	- Holliday junction

Amino acids

alanine	A	Ala	leucine	L	Leu
arginine	R	Arg	lysine	K	Lys
asparagine	N	Asn	methionine	M	Met
aspartate	D	Asp	phenylalanine	F	Phe
cysteine	C	Cys	proline	P	Pro
glutamate	E	Glu	serine	S	Ser
glutamine	Q	Gln	threonine	T	Thr
glycine	G	Gly	tryptophan	W	Trp
histidine	H	His	tyrosine	Y	Tyr
isoleucine	I	Ile	valine	V	Val

Chapter 1: Introduction

The work presented in this thesis is related to the role that the alkaline nuclease encoded by herpes simplex virus type 1 (HSV-1) plays in the replication and packaging of the viral genome. The introduction will begin by outlining some general properties of the herpesvirus family. This will be followed by more detailed descriptions of the processes of DNA replication and packaging in HSV-1. Finally, the literature relating to the properties and functions of the HSV-1 alkaline nuclease will be reviewed.

1.1 Herpesvirus characteristics

1.1.1 Herpesviridae

Herpesviruses are large, double stranded DNA viruses, that share a number of common morphological features including an icosahedral capsid surrounded by a tegument layer, contained within a host derived lipid envelope containing a number of virally encoded glycoproteins.

Herpesviruses infect a wide range of vertebrate hosts including mammals, birds, amphibians and fish. More recently they have also been detected in invertebrate marine bivalves. A common feature of herpesviruses is their ability to establish a persistent, latent infection within the host. The virus may then reactivate in response to stress, such as UV light or the host becoming immunocompromised. In addition, the host range of individual herpesviruses is usually limited although ostreid herpesvirus-1 has been shown to infect several species of bivalves (Arzul *et al.*, 2001). This host specificity is thought to reflect the co-evolution of the virus and host (McGeoch *et al.*, 1995).

Herpesviruses have been broadly separated into three sub-families: (i)*Alphaherpesvirinae* (including HSV-1, HSV-2, VZV and PRV). The viruses associated with this subfamily are neurotropic, they have a variable host cell range, exhibit a short life cycle and tend to spread rapidly in tissue culture. They are also able to latently infect neuronal ganglia in which they generally persist for the life of the host. (ii)*Betaherpesvirinae* (including HCMV, HHV-6 and HHV-7) have a more restrictive host cell range. Cell to cell spread of the virus is generally slower than that observed with *Alphaherpesvirinae* and the infected cells are often enlarged. Monocytes, spleen and kidney cells are sites where latent infection is known to occur. (iii)*Gammaherpesvirinae* (e.g. EBV, KSHV (HHV-8), EHV-2 and HVS) are lymphotropic, and exhibit the most restricted host cell range. Their life cycle span is variable and they generally establish latent infections in lymphocytes.

Although this classification was established largely by biological characteristics it correlates closely with genetics groupings that have emerged over the last fifteen to twenty years through DNA sequencing studies. The phylogenetic data now available suggest that herpesviruses have a common origin, the subfamilies diverging approximately 200 million years ago (McGeoch *et al.*, 1993).

1.1.2 Human herpesviruses

Eight human herpesviruses have been identified to date, with representatives of each of the *Herpesviridae* subfamilies. Infection is usually asymptomatic and non-life threatening although in neonates and in immunocompromised individuals infections are occasionally fatal.

Alphaherpesviruses:

Herpes simplex virus type 1 (HSV-1) is the most extensively studied of the herpesviruses and is the prototype virus for the family. Infection can occur at a number of body sites although the most common sites are around the lips and mouth. The virus usually remains latent in sensory neuronal ganglia, and when reactivated it generally causes cold sore lesions at, or near, the primary site of infection. HSV-2 is closely related to HSV-1 and is generally associated with genital infections.

Varicella-zoster virus (VZV) primary infections usually affect children and are classically recognised by the appearance of small puss filled vesicles that rupture and scab over, this is often associated with intense itching (chicken pox). If reactivated in adulthood the virus causes a complaint known as shingles.

Betaherpesviruses:

Human cytomegalovirus (HCMV) is the most extensively studied of the betaherpesviruses. As with most herpesviruses infection it is usually asymptomatic. However, reactivation often occurs in HIV infected individuals or organ transplant patients. In the case of HCMV reactivation is often associated with retinitis. HCMV can also cause severe disease in congenitally infected infants. HCMV is thought to be responsible for around 10% of infectious mononucleosis cases. Possible complications arising from the mononucleosis include pneumonia and hepatitis.

Two, more recently identified betaherpesviruses are HHV-6 and HHV-7. Both are members of the *Roseolovirus* genus and have very similar genomes and gene products. HHV-6 is the causative agent of exanthem subitum, an early childhood disease

characterised by a high fever followed by a rash, which lasts for one to two days. As with HCMV, reactivation of HHV-6 and HHV-7 can occur in immunocompromised individuals.

Gammaherpesviruses:

There are two known gammaherpesviruses that infect humans. Epstein-Barr virus (EBV) commonly causes infectious mononucleosis in 35 – 50% of adolescent infections. The virus has also been associated with Burkitt's lymphoma, nasopharyngeal carcinoma and Hodgkin's disease, although in these cases it is not the sole causative factor of the disease.

Kaposi's sarcoma is characterised by skin lesions and occurs in a proportion of AIDS sufferers. The most recently identified human herpesvirus is Kaposi's sarcoma associated herpesvirus (KSHV) or HHV-8 which appears to be the causative agent of these tumours.

1.1.3 Herpesvirus genomes

All herpesviruses have linear dsDNA genomes 120 – 220 kbp in length and contain 70 to 200 open reading frames (ORFs). The genomes contain various arrangements of internal and terminal repeats. To date seven genome structures have been identified in herpesviruses (Roizman *et al.*, 1992; Davison and McGeoch, 1995) and a diagrammatic representation of these can be seen in Figure I.

Group 0 genomes have a single unique coding region with no repeat units (Roizman *et al.*, 1992). Tree shrew herpesvirus is an example of this type (Koch *et al.*, 1985).

Group 1 genomes have single direct repeats at each terminus and are typified by Channel catfish virus (Davsion, 1992). Herpesvirus saimiri, with multiple repeats at each terminus but no internal repeats, is a representative of Group 2 genomes (Albrecht *et al.*, 1992). Group 3 genomes also have multiple repeats at each terminus but, in addition, have internal repeats in the opposite orientation resulting in a genome with two unique regions. Cottontail rabbit herpesvirus is an example of this group of herpesviruses, containing two unique regions of 54 and 47 kbp surrounded by a 925 bp repeat present with a variable copy number (Cebrian *et al.*, 1989). Group 4 genomes are similar to group 3 except the internal repeats share no similarity to the terminal repeats. Epstein-Barr virus (EBV) is characteristic of group 4 genomes (Baer *et al.*, 1984). With Varicella-zoster virus (VZV) genomes the inverted repeats surrounding each unique sequence (U_L and U_S) are not related. In addition, the repeats surrounding the U_S unique region are much larger than those surrounding U_L (Davison and Scott, 1986). This is a good example of a Group 5 genome. Group 6 genomes, typified by HSV-1, are very similar to group 5 genomes except the U_L repeats are larger. Group 6 genomes also contain *a* sequence repeats which are found at each terminus and in inverted orientation between the internal repeats flanking U_L and U_S (Roizman, 1979).

The presence of long inverted repeats flanking U_S in the group 5 genomes results in the U_S region being able to invert through recombination. As a result these group 5 genomes exist as a mixed population of two isomers. The increased size of the U_L repeats in group 6 genomes means that both U_L and U_S regions can invert, resulting in four genomic isomers (Hayward *et al*, 1975). The functional significance of these inversion events in the replication of herpesviruses is unknown, although HSV-1

'hich have 'fixed' orientations of U_L and U_S are unimpaired in tissue culture and Roizman, 1986; Poffenberger *et al.*, 1983).

ate the problem of replicating the terminal regions of the linear genome, there is ntial evidence that herpesviruses circularise their genome prior to replication and the replication intermediates are "endless", with DNA synthesis possibly eeding by a combination of theta form and rolling circle modes (Umene and himoto, 1996; Jacob and Roizman, 1977; Becker *et al.*, 1978).

.nalysis of the DNA sequence of the human herpesviruses predicts that between 69 .VZV) and 220 (HCMV) proteins are encoded by the various viruses (McGeoch and Davison, 1999). HSV-1 contains ORFs for at least 74 functional proteins (McGeoch *et al.*, 1988; Dolan *et al.*, 1998). Comparison with other human herpesviruses reveals that of these 74 genes, 40 are conserved throughout the mammalian and avian herpesviruses. These core genes express proteins mainly involved in virion structure, DNA replication, and cleavage and packaging of DNA (McGeoch and Davison, 1999).

1.1.5 Herpesvirus capsids

Herpesvirus capsids are constructed around a protein scaffold, which is subsequently lost. The capsid is composed of four main proteins forming an icosahedral structure composed of 150 hexamers and 12 pentamers. Groups of three capsomers are separated by a heterotrimeric complex known as the triplex. The DNA complement is densely packed and forms into a liquid crystalline configuration within the capsid. Despite the wide range in genome size all herpesvirus capsids are approximately 125 nm (HSV-1) to 130 nm (HCMV) in diameter (Schrag *et al.*, 1989; Booy *et al.*, 1991; Butcher *et al.*,

1998). In agreement with the small differences in capsid size, recent studies have shown that the HCMV genome, among the largest of herpesvirus genomes, is more densely packed than the HSV-1 genome (Bhella *et al.*, 2000).

There are three angularised capsid forms: A, B and C, which can be identified by electron microscopy and by ultracentrifugation on sucrose gradients (Gibson and Roizman, 1972). Type A capsids are empty structures, devoid of DNA or scaffolding protein and type B capsids contain mainly cleaved scaffold protein (Newcomb and Brown, 1991). These capsid types are thought to result from abortive packaging events. C type capsids contain DNA and are capable of maturing into infectious virus. A short-lived, more spherical form known as the procapsid is the precursor of A, B and C capsids (for a review see: Rixon, 1993; Homa and Brown, 1997).

1.1.6 Herpesvirus tegument

The tegument layer that separates the capsid and lipid envelope contains a matrix of viral proteins; 15 proteins have been associated with the tegument of HSV-1 (Mettenleiter, 2001). Although long considered an unstructured mass, recent studies have shown that tegument proteins associated with the capsid also have icosahedral symmetry (Zhou *et al.*, 1999).

The function of many tegument proteins remains unclear but, in HSV-1 at least, tegument protein functions include transactivation of early viral genes (UL48 gene product), the shut off of host protein synthesis (UL41 gene product), and an involvement in the cleavage and packaging of the viral genome (UL17 gene product) (Triezenberg *et al.*, 1988; Kwong *et al.*, 1988; Salmon *et al.*, 1998).

1.1.7 Herpesvirus envelope

The lipid envelope that encloses the capsid and tegument was initially thought to be derived from the nuclear membrane, with enveloped virus often being observed budding into the perinuclear space. More recent studies, however, indicate that the viral capsid is de-enveloped as it buds from the nuclear membrane, and is subsequently re-enveloped with another lipid envelope that contains the viral glycoproteins as it buds from trans-Golgi vesicles (For a review see Mettenleiter *et al.*, 2002).

The membrane embedded, virally encoded, glycoproteins are visible in electron micrographs as "spikes" emerging from the membrane of virus particles. The number of glycoproteins expressed by different herpesviruses varies but HSV-1 encodes at least eleven. The glycoproteins appear to have roles in the secondary envelopment of capsid/tegument (Brack *et al.*, 1999), attachment and entry into host cells and inhibition of secondary infections (Campadelli-Fiume *et al.*, 1988).

1.2 Herpes simplex virus type 1 lytic infection

1.2.1 Virus attachment and entry

Of the eleven identified HSV-1 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM), five are dispensable in cell culture (gC, gE, gG, gI and gM) and no single glycoprotein has been identified as essential in terms of attachment to host cells in tissue culture. In polarised cells, however, gC is needed for attachment to the apical but not the basal surface of cells (Sears *et al.*, 1991). The main attachment site on the cell surface appears to be through heparin sulphate; removal of heparin sulphate or competition by heparin reduces attachment by 85% (Shieh *et al.*, 1991; Wudunn and

Spear, 1989). Attachment could never be completely abolished in these studies suggesting that alternative attachment and entry sites exist.

By expressing human cDNAs in CHO cells, normally resistant to HSV-1 infection, at least two gene groups were identified that could act as entry receptors (Spear *et al.*, 2000): HVEM, a member of the TNF receptor family (Montgomery *et al.*, 1996), HveC and HveB, members of the nectin-1 and nectin-2 immunoglobulin family, respectively, (Geraghty *et al.*, 1998; Warner *et al.*, 1998). In addition, a modification of heparin sulphate that acts as gD mediated receptor site has also been identified (Shukla *et al.*, 1999).

Once attached the virus fuses with the cell membrane and penetration of the cell occurs. From studies of HSV-1 mutants deleted of various glycoprotein genes, it appears that glycoproteins gB, gC, gD, gH and gL are required during this penetration stage (Spear, 1993).

After penetration the tegument proteins and capsid dissociate and diffuse into the cytoplasm. Transport of the capsids to the nucleus appears to be mediated along microtubules utilising the dynein motor complex (Sodeik *et al.*, 1997). Once at the nuclear membrane the capsid attaches to the nuclear pore complex and the DNA is ejected into the nucleus (Ojala *et al.*, 2000).

1.2.2 HSV-1 gene expression

The 74 ORFs encoded by HSV-1 are transcribed in a cascade fashion and can be split into three broad groups dependent on their temporal transcription pattern: immediate

early (α), early (β) and late (γ). There are five immediate early proteins: ICP4, ICP27, ICP0, ICP22 and ICP47. The genes encoding these proteins are transcriptionally activated by a complex consisting of the tegument protein VP16 which binds to the TAATGARAT motif and host proteins Oct 1 and HCF (McKnight *et al.*, 1987; Stern *et al.*, 1989; Vogel and Kristie, 2000). The immediate early genes are transcribed in the absence of protein synthesis and their proteins are generally considered to have regulatory functions, controlling the expression of subsequent gene groups. The expression of these genes peaks 2-4 hours post infection but they remain expressed throughout an active infection. ICP47, the product of US12, is expressed with IE kinetics but rather than having a regulatory role it appears to prevent major histocompatability complex 1 (MHC1) being presented on the surface of infected cells (Jugovic *et al.*, 1998).

Immediate early proteins also exert regulatory control at the post-transcriptional level (Weir, 2001). For example, one role of the ICP27 protein is to inhibit mRNA splicing (Hardy and Sandri-Goldin, 1994). This contributes to the virus inhibiting protein synthesis in the host cell because only 4 of the 74 HSV-1 genes are spliced, and moreover, three of these four are immediate early genes expressed prior to splicing inhibition. The fourth spliced gene, UL15, is expressed later in infection but the mechanism by which it escapes the inhibitory process is not clear (Weir, 2001).

The early, or β, genes include all the genes involved in DNA replication and nucleotide metabolism. Transcription of β genes occurs prior to the onset of DNA synthesis and depends on the prior expression of immediate early proteins, especially a functional ICP4 protein.

Late genes encode mainly structural proteins, including the capsid components, tegument and glycoproteins. They can be split into two subclasses: leaky late (γ1) and true late (γ2). True late genes require the onset of DNA synthesis before transcription occurs whereas leaky late genes can be expressed prior to DNA synthesis but only reach maximal levels when DNA replication has initiated. Late genes require ICP4 and ICP27 for transcription (Preston, 1979; DeLuca and Schaffer, 1985; Godowski and Knipe, 1986; Sacks *et al.*, 1985; Rice and Knipe, 1990). It also appears that the essential DNA replication protein encoded by UL29 is also required for transactivation of late genes (Gao and Knipe, 1991; Chen and Knipe, 1996).

1.2.3 Viral DNA synthesis and encapsidation

After entry into the nucleus the viral DNA circularises probably by ligation of the genome ends although a recombination mechanism has also been proposed (Garber *et al.*, 1993; McVoy *et al.*, 1997; Yao *et al.*, 1997). The genome is then thought to replicate initially by theta form replication, followed subsequently by a rolling circle mode of replication. This produces head-to-tail concatemers which are cleaved into unit length genomes and in a linked process packaged into preformed capsids (Jacobs *et al.*, 1977; Ben-Porat *et al.*, 1977). The process of HSV-1 genome replication and packaging are described in more detail in sections 1.3 and 1.4, respectively.

1.2.4 Capsid egress and maturation

The process of nuclear egress and envelopment in herpesviruses has recently been reviewed by Mettenleiter (2002). After encapsidation of genomic DNA in the nucleus, the capsid is targeted to the nuclear membrane and through the interaction of UL31 and

UL34 (a phosphoprotein and C-terminally anchored membrane protein, respectively) it acquires its primary envelope as it buds into the perinuclear space. It is unclear whether any tegument proteins associate with the maturing virus particles at this stage.

It is now generally accepted that the capsid is de-enveloped as it leaves the outer-nuclear membrane and subsequently gains tegument and a lipid envelope containing viral glycoproteins in post-endoplasmic reticulum cytoplasmic compartments. A previous model suggested that all tegument and lipid anchored glycoproteins were gained during perinuclear envelopment and the glycoproteins were then modified *in situ*. However, recent biochemical and immunogold electron microscopy analyses have demonstrated that the lipid composition of mature virus particles differs from that of the perinuclear membrane (Van Genderen *et al.*, 1994; Skepper *et al.*, 2001). In addition, studies in pseudorabies virus have demonstrated that the homologue of the major tegument protein, UL49, is present in extracellular and intracytoplasmic enveloped virions but is absent from perinuclear enveloped virions (Klupp *et al.*, 2000).

There are at least 15 proteins associated with the tegument layer. Although this layer was previously thought to be an unstructured mixture of proteins, icosahedral symmetry has recently been observed close to the capsid, with a protein (possibly UL36) associating with the major capsid protein at the pentons (Zhou *et al.*, 1999; McNab and Courtney 1992). As well as structural proteins, the tegument also contains viral proteins that are required early in infection, such as the viral host shutoff (vhs) protein and VP16, the transactivator of immediate early genes. As well as its role as a transactivator VP16 also has an essential structural role within the tegument. In its absence virion

maturation is blocked after the primary envelopment/de-envelopment step (Mossman *et al.*, 2000).

1.3 Replication of the HSV-1 genome

1.3.1 Structure of the HSV-1 genome

The HSV-1 genome is a double stranded DNA molecule, approximately 152 kbp in length. It exhibits a group 6 genome arrangement (Figure I), containing two covalently linked segments, L and S, each of which comprises a unique region, U_L and U_S, respectively flanked by inverted repeats, TR_L/IR_L and IR_S/TR_S (Figure II). The *a* sequence represents a terminally redundant region of approximately 400 bp which is present in one or more copies at the L terminus and at the junction between the L and S segments. A single copy is present at the S terminus. The portions of R_L and R_S excluding the *a* sequence are referred to as the *b* and *c* sequences, respectively. This gives HSV-1 an arrangement of $a_m b$-U_L-$b'a'_n c'$-U_S-*ca*, where n and m are variable and ' indicates an inverted sequence. The U_L and U_S sequences are 107.9 and 13.0 kbp in length, respectively, encoding at least 71 distinct proteins. The U_L region contains 58 ORFs and the U_S region 13. Three ORFs are situated in the terminal repeats, 2 in R_L and 1 in R_S (McGeoch *et* al., 1988; Dolan *et al.*, 1998). Due to the presence of inverted repeats flanking the unique sequences, U_L and U_S can invert with respect to each other generating four equimolar arrangements for virion DNA (Figure II) (Hayward *et al.*, 1975). A recent paper by Huang *et al.* (2002) has proposed that a cellular endonuclease, endo G, may be responsible for initiating these recombination events. Endo G has a strong preference for G+C rich sequences and was demonstrated to cleave specifically within the *a* sequence, which has an 83% G+C content.

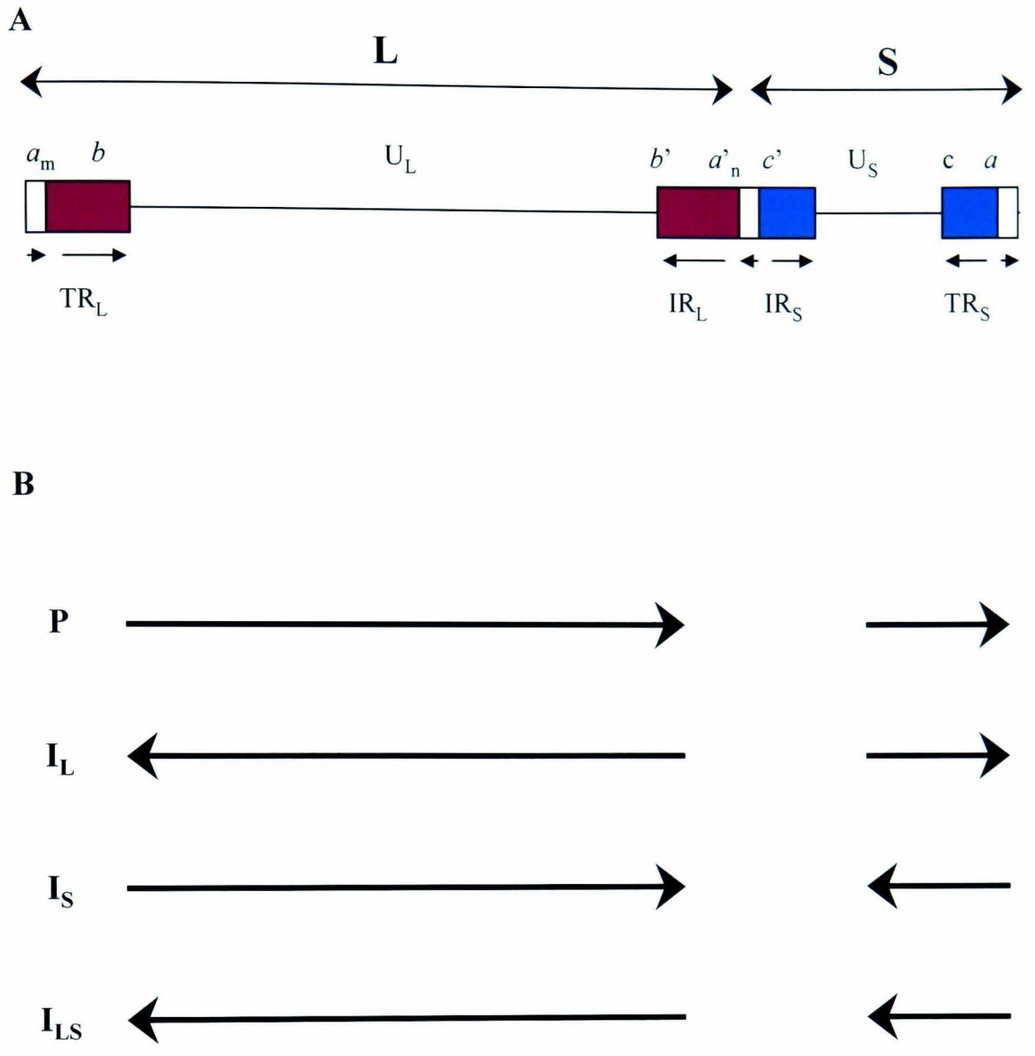


Figure II – Genome arrangement of HSV-1

(**A**) Diagram of the HSV-1 genome. The unique regions, U_L and U_S, denoted by single lines are each flanked by the R_L and R_S repeat sequences, respectively. The regions of R_L and RS which do not contain the *a* sequence are denoted *b* and *c*, respectively. The terminal (TR_L, TR_S) and internal (IR_L, IR_S) repeats are inverted with respect to each other, as indicated by the arrows. The *cis*-acting *a* sequence, required for cleavage and packaging is present at each terminus and inverted orientation separating IR_L and IR_S. (**B**) The four genomic isomers generated by the inversion of U_L and U_S regions are shown: P = prototype, I_L = inverted U_L, I_S = inverted U_S, I_{LS} = inverted U_L and U_S.

The *a* sequence contains the *cis*-acting signals required for cleavage and packaging of the viral genome and is central to a number of experiments reported in this thesis. The length of this sequence varies both within and between virus strains. In strain 17 syn^+ it is approximately 380 bp in length. The *a* sequence itself consists of a number of repeat sequences (DR1, DR2 and in some strains DR4), and two quasi-unique regions, Ub and Uc (Figure III) (Davison and Wilkie, 1981). Each complete copy of the *a* sequence is flanked by a short direct repeat (DR1). Single copies of DR1 separate tandem *a* sequences and during packaging cleavage occurs within DR1 such that the two genomic termini each contain an incomplete DR1 with a single 3' nucleotide overhang (Mocarski and Roizman, 1982). Upon circularisation of the genome a complete DR1 sequence is regenerated. The central region of the *a* sequence consists of multiple reiterations of the DR2 and/or DR4 sequences (11-24 bp). These are separated from DR1 by the Ub and Uc sequences. The Uc sequence lies closest to the L terminus whilst Ub is adjacent to the S terminus. Ub and Uc contain the *pac*1 and *pac*2 motifs, respectively, which include several conserved sequence elements found near the termini of all mammalian and avian herpesviruses (see Figure III for a schematic representation). Deletion and substitution mutations within some of these motifs had dramatic effects on the packaging and propagation of plasmids which contained the encapsidation signal, highlighting their importance in the cleavage and packaging process (Hodge and Stow, 2001). The results of this study were consistent with a model where signals for the initiation and termination of packaging were present in *pac*2 and *pac*1, respectively.

HSV-1 contains three origins of DNA synthesis. One, ori_L, is present near the centre of the U_L region between the divergent UL29 and UL30 ORFs. The other two origins (ori_S), are present as identical copies in the repeat regions flanking U_S (Spaete and

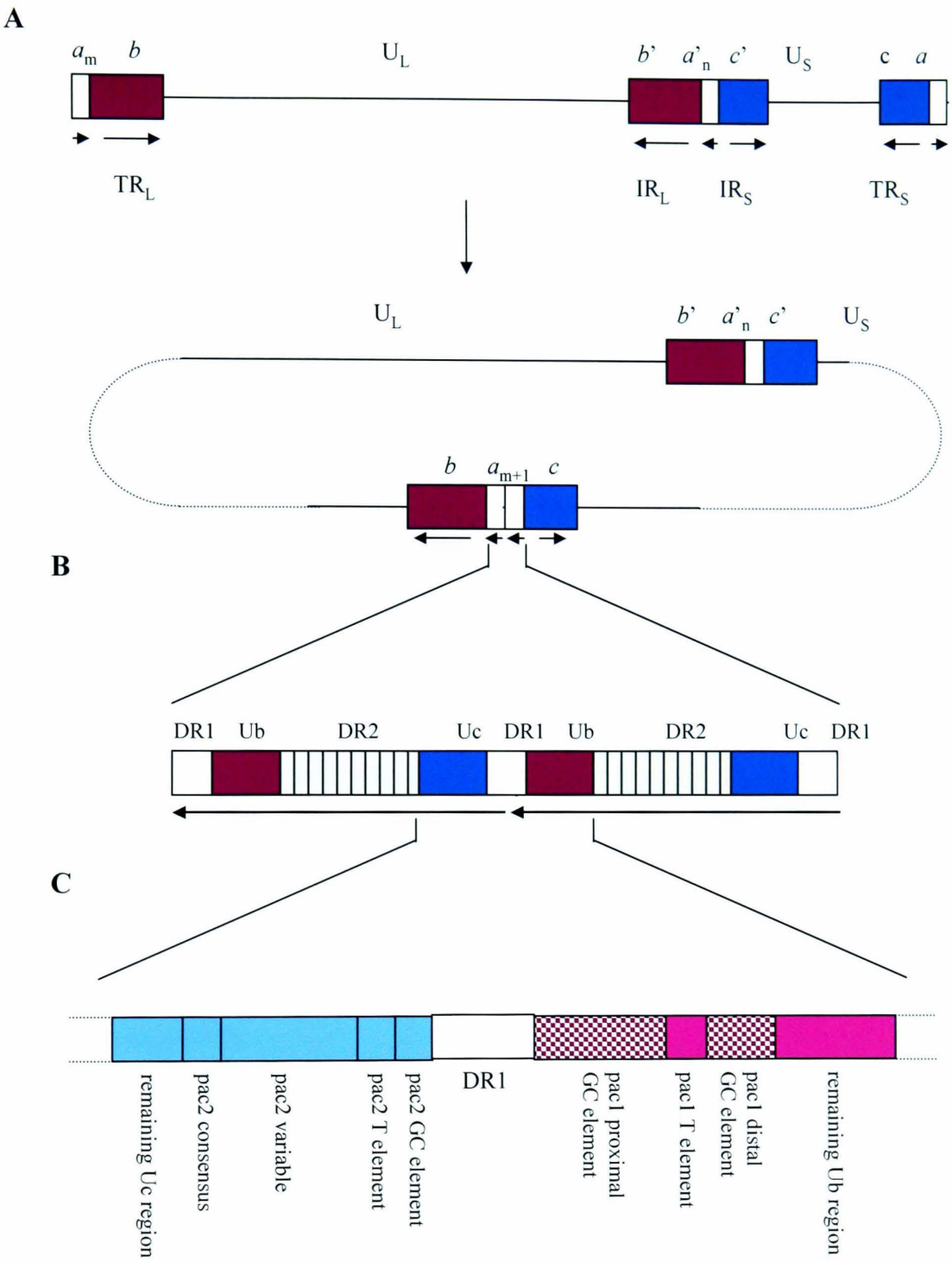


Figure III – Structure of the HSV-1 strain 17 syn$^+$ *a* sequence

(**A**) Two complete copies of the HSV-1 *a* sequence are formed upon ligation of TR_L and TR_S. (**B**) The DR1 and DR2 repeats of two consecutive *a* sequences (indicated by arrows) are shown, the quasi-unique regions Ub and Uc are also shown. (**C**) Representation of the conserved *pac*1 and *pac*2 motifs found within Ub and Uc, respectively.

Frenkel, 1982; Stow, 1982; Stow and McMonagle, 1983; Weller *et al.*, 1985). The two types of origin share considerable homology and there is a certain degree of redundancy between the two; deletion of either ori_L or ori_S does not significantly reduce the efficiency of viral replication in tissue culture (Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993). Both ori_L and ori_S contain palindromic sequences of 144 and 45 bp, respectively, and are centred around an AT rich region of approximately 20 bp. Either side of the AT rich region are sequences termed Box I, Box II and Box III. Box I and II are high affinity binding sites for the UL9 encoded origin binding protein (OBP) while Box III has a much lower binding affinity. Although the Box II and III motifs differ from Box I by only two and one base pair, respectively, they have five and 1000 times lower affinity for UL9 than Box I (Olivo *et al.*, 1988; Koff and Tegtmeyer, 1988; Elias *et al.*, 1990; Hazuda *et al.*, 1991). The ori_L sequence contains a Box I and a Box III motif either side of the AT rich region whereas ori_S contains a Box III and Box I motif on one side of the AT region and a Box II motif on the other side. The long perfect palindrome of ori_L has made the sequence difficult to clone in an intact form in *E. coli* and therefore most studies have concentrated upon ori_S. (Weller *et al.*, 1985; Hardwicke and Schaffer, 1995).

1.3.2 HSV-1 circularisation

Soon after transportation into the nucleus the HSV-1 genome circularises (Mocarski and Roizman, 1982; Poffenberger and Roizman, 1985; Garber *et al.*, 1993). The circularisation of the genome is probably completed by ligation of the incomplete 18.5 bp DR1 repeat found at the L terminus to the 1.5 bp DR1 fragment present at the S terminus. This can occur in the absence of viral gene expression suggesting that a host ligase or component of the virion is involved.

The occurrence of ligation is further supported by work on guinea pig cytomegalovirus (GPCMV) (McVoy *et al.*, 1997). This herpesvirus has a group 1 type genome, with no internal repeat. In addition it has two genome structures which are present in equimolar amounts. One genome type has single copies of the R and M repeats at the left and right end of the genome respectively. The other genome lacks the M repeat and is designated as containing an O terminus. The occurrence of two types of genome indicates the presence of two independent cleavage sites. Further analysis indicated *pac*1 homology in the R repeat and *pac*2 homology in the M repeat and at the O terminus. Through mutational analysis it was observed that R and M genomes could circularise to form a double repeat and joining of R and O to form a circular molecule with a single repeat was also feasible. In this instance ligation is the most likely mechanism for end joining since R and O termini share no significant homology.

Although the majority of experiments reported to date support a ligation mechanism for genome end-joining, another possible mechanism for circularisation that has been suggested is through non-conservative homologous recombination or an imprecise end-joining mechansim (Yao *et al.*, 1997).

1.3.3 Possible modes of HSV-1 DNA replication

It is widely held that HSV-1 genomes are initially replicated by the *theta* mode of replication followed by a rolling circle mode of replication. Although never proven conclusively there are a number of factors consistent with an initial *theta* mode of replication: (i) circularisation of the infecting HSV-1 genome (discussed above), (ii) the palindromic nature of the origins of replication suggests the ability of bi-directional

replication forks to form at the origins, and (iii) the non-linear rate of accumulation of viral DNA during replication cannot be accounted for by rolling circle replication alone. In addition, the termination of replication at early, but not late, times in infection by an inhibitor of host cell topoisomerase II suggests that decatenation of circular molecules is required (Hammarsten *et al.*, 1996). However, although the generation of circular molecules early after infection has been demonstrated, their accumulation during the early stages of DNA replication has not been observed.

The most commonly accepted evidence for the rolling circle replication mechanism is the accumulation of head-to-tail concatemers of the viral genome during replication. This was established by both sedimentation analysis, which showed replication intermediates with a higher sedimentation value than mature genomes, and restriction enzyme analysis which demonstrate that the concatemers were endless (Jacob and Roizman, 1977; Jacob *et al.*, 1979; Jongeneel and Bachenheimer, 1981). More recent experiments utilising Field Inversion Gel Electrophoresis (FIGE) and Pulsed Field Gel Electrophoresis (PFGE) techniques have determined the structure of these intermediates (Zhang *et al.*, 1994; Severini *et al.*, 1994). This work demonstrated that the high molecular weight DNA replication intermediates are composed of a complex, branched network of concatemers which would not enter pulsed field gels. In addition 2D gel electrophoresis showed that these concatemers contained many X and Y-junctions. Restriction enzyme analysis of this DNA, with an enzyme which cleaved once per genome, resulted in a high proportion of the DNA remaining in the well of the gel. Failure of this DNA to enter the gel further indicates the presence of frequent branched structures, probably the result of unresolved intra and inter-molecular recombination

events. Restriction enzyme analysis also showed that within a single concatemer both orientations of the L and S segments were present (Bataille and Epstein, 1997).

Other replication mechanisms exist that could account for the accumulation of the HSV-1 high molecular weight replicative intermediates. These include the recombination dependent replication mechanisms evident in bacteriophage T4 or the 2 micron circle mode of replication associated with *S. cerevisiae*.

1.3.4 Essential replication genes encoded by HSV-1

The identification of the viral genes involved in DNA replication was facilitated by the development of the transient DNA replication assay by Challberg (1986). Plasmids containing fragments of the HSV-1 IR_L/IR_S region (subsequently shown to contain ori_S) were identified that could be replicated if viral helper functions were supplied in *trans* (Stow, 1982). Co-transfection of cloned HSV-1 genome fragments, along with a plasmid containing ori_S, and assaying for replication of the latter plasmid identified seven viral genes: UL5, UL8, UL9, UL29, UL30, UL42 and UL52, that were both sufficient and necessary for the replication of the origin containing plasmid (Challberg, 1986; Wu *et al.*, 1988; Heilbronn and zur Hausen, 1989). Studies using both temperature sensitive (*ts*) and viral null mutants, targeted to each of these genes, confirmed their essential role in the replication of viral DNA (reviewed by Challberg, 1996; Weller, 1991). In addition, the expression of all seven genes in baculovirus infected insect cells supported the replication of an ori_S containing plasmid, indicating that any host proteins involved in HSV-1 DNA replication are sufficiently conserved between mammalian and insect cells to allow DNA synthesis (Stow, 1992). When the set of seven genes were first identified the function of many of the proteins was poorly

understood. It is now known that UL9 represents the origin binding protein, UL29 is the major single stranded DNA binding protein, UL5, UL8 and UL52 constitute a functional helicase/primase complex, and UL30 and UL42 form the viral polymerase complex. What follows is a brief description of each of these proteins and their role in the replication of the HSV-1 genome.

UL9 – Origin Binding Protein

As mentioned earlier, UL9 encodes the origin binding protein (OBP). This 851 amino acid protein exists as a homodimer with the N-terminus of each protein possessing ATP dependent helicase activity and the C-terminus containing the DNA recognition and binding domain. The N-terminus also contains residues important for dimerisation and co-operative binding of UL9 to the origins. Amino acids 535-851 are capable of sequence-specific binding to DNA but exist as monomers in solution (Elias *et al.*, 1992). Inspection of the N-terminus reveals a putative leucine-zipper domain that may facilitate the protein-protein interaction (Hazuda *et al.*, 1992).

In its role as an initiator protein, the UL9 homodimer binds the Box I and Box II motifs either side of the AT rich region of ori_S in a co-operative fashion. Hypersensitivity of the AT rich region to DNase I and micrococcal nuclease (Koff *et al.*, 1991), and observations from electron microscopy have demonstrated that this binding bends the DNA by approximately 90 degrees (Makhov *et al.*, 1996). In the presence of ATP the helicase activity of UL9 was able to unwind up to 1 kbp of DNA from the origin. This unwinding was assisted by the function of the single stranded major DNA binding protein (mDBP) encoded by UL29. However no cell free system has yet been described which will faithfully initiate DNA synthesis.

UL29 – Single stranded DNA binding protein

UL29 encodes a 130 kDa protein that binds ssDNA five times more efficiently than dsDNA, binding co-operatively and without any apparent sequence specificity (Ruyechan, 1983; Lee and Knipe, 1985). The protein acts as a helix destabilising protein, and can lower the melting temperature of duplex DNA (Boehmer and Lehman, 1993). In the presence of $MgCl_2$ the major DNA binding protein (mDBP) is also able to catalyse the reannealment of complementary strands, suggesting that it may be involved in recombination of the viral genome (Bortner *et al.*, 1993).

In conjunction with UL9, the UL29 protein appears to distort and open up the DNA around the origin. Subsequent helicase activity by UL9 and coating of the ssDNA by UL29 is presumed to allow the helicase/primase complex and DNA polymerase access to the DNA to establish a replication fork. The role of these two proteins in the initial establishment of the replication fork is further supported by several observed protein-protein interactions (Falkenberg *et al.*, 1997; Monahan *et al.*, 1998).

As well as its association with UL29, UL9 has also been shown, through immunoprecipitation experiments, to interact with the helicase/primase complex through the UL8 subunit (McLean *et al.*, 1994). UL29 has also been shown to interact with the UL8 subunit and to stimulate the helicase/primase acivity of the complex (Tanguy Le Gac *et al.*, 1996; Falkenberg *et al.*, 1997). In addition, UL29 and the UL8 subunit of the helicase/primase complex have been shown to interact (Falkenberg *et al.*, 1997). The polymerase processivity factor, encoded by UL42, has also been shown to interact with UL29 (Vaughan *et al.*, 1984). Finally, the interaction of UL9 with UL42

(Monahan *et al.*, 1998) and UL8 interacting with UL30 (Gallo *et al.*, 1988) demonstrate that all the essential replication proteins interact at some level. Taken together, this data suggests that the viral DNA replication proteins assemble to form a 'replisome' complex at the viral origins following the initial binding of the origin binding protein. In support of this, the proteins could be purified as a large multiprotein complex from *Sf* insect cells infected with baculoviruses expressing all seven replication proteins (Skaliter and Lehman, 1994).

UL5, UL8 and UL52 – The helicase/primase complex

The functional helicase/primase complex is made up of the products of the UL5, UL8 and UL52 ORFs (Crute *et al.*, 1989). The UL5 protein specifies the helicase activity and contains a number of motifs conserved throughout helicases (Gorbalenya *et al.*, 1989; Klinedinst and Challberg, 1994). The helicase can utilise either ATP or GTP (Crute *et al.*, 1988) and has been demonstrated to unwind DNA in the 5' – 3' direction (Crute *et al.*, 1988). When expressed alone in recombinant baculovirus infected cells UL5 possessed very little helicase activity, but co-expression with UL52 to form a heterodimer restored the helicase activity (Calder and Stow, 1990; Dodson and Lehman, 1991).

The UL52 protein confers the primase action of the complex. On single stranded ϕX174 DNA the helicase-primase complex produces 10-mer oligoribonucleotides, recognising the sequence 3'-AGCCCTCCCA and priming from the underlined C base (Tenney *et al.*, 1995). Like UL5, UL52 shows little or no enzymatic activity when expressed alone but when co-expressed with UL5 primase activity is restored. Although mutational studies have demonstrated that UL5 and UL52 impart helicase and

primase activities, respectively, certain mutations in UL5 can affect primase activity, similarly certain UL52 mutations affect the helicase function (Klinedinst and Challberg, 1994; Dracheva *et al.*, 1995; Zhu and Weller, 1992; Graves-Woodward and Weller, 1996).

The function of the UL8 subunit was initially unclear because it seemed to contain no intrinsic enzymatic activity and the UL5 and UL52 dimeric complex functioned as a helicase/primase in its absence (Calder and Stow, 1990; Dodson and Lehman, 1991). Subsequent studies have revealed that the addition of the UL8 subunit promotes helicase activity and stimulates primase activity by threefold (Tenney *et al.*, 1994, 1995). Although the precise action of this subunit is unclear it may promote loading of the complex onto the DNA through interactions with the mDBP. UL8 has also been shown to interact with the viral DNA polymerase and to direct the localisation of UL5 and UL52 to the nucleus (Calder *et al.*, 1992; Barnard *et al.*, 1997).

UL30 and UL42 – The DNA polymerase complex

The viral DNA polymerase exists as a heterodimer of the UL30 and UL42 gene products. The polymerase activity is associated with the 136 kDa polypeptide encoded by the UL30 gene. This protein shares extensive homology with several other polymerases including bacteriophage T4 DNA polymerase and human DNA polymerase α-primase (Digard *et al.*, 1990; Blanco *et al.*, 1991). The UL42 subunit is a 65 kDa protein that acts as an accessory factor which binds to dsDNA in a sequence independent manner increasing the processivity of the catalytic subunit (Gallo *et al.*, 1988; Gottlieb *et al.*, 1990).

The polymerase subunit has both 3'-5' exonuclease activity and RNaseH activity. In the presence of all four dNTPs the exonuclease has no activity on paired 3' residues but completely removes unpaired 3' residues (O'Donnell *et al.*, 1987). This indicates that the polymerase has an intrinsic proof reading ability. There are three conserved exonuclease motifs present in the UL30 ORF and mutagenesis of motif I greatly reduced the exonuclease activity of the protein (Hall *et al.*, 1995). The inability to produce a recombinant virus containing this mutation demonstrates the essential nature of the exonuclease activity (Hall *et al.*, 1995). The reported 5'-3' exonuclease RNaseH activity probably reflects the ability of the polymerase to remove the primers from Okazaki fragments during lagging strand synthesis (Crute and Lehman, 1989).

The UL42 protein binds to the polymerase subunit through multiple interactions (Monahan *et al.*, 1993). The role of the UL42 subunit appears to be as a processivity factor, which through its interaction with dsDNA, prevents the polymerase subunit from dissociating from the DNA template after each round of catalysis (Gottlieb *et al.*, 1990). By constructing a model primer with double and single stranded regions, Gottlieb and Challberg (1994) demonstrated in DNase I fingerprint studies that the UL30 subunit would bind at the ds/ssDNA boundary. With the heterodimeric complex of UL30 and UL42 extra protection was given to the dsDNA but not the ssDNA region. In conjunction with previous data that demonstrated UL42 bound non-specifically to dsDNA (Vaughan *et al.*, 1985; Gallo *et al.*, 1988) it has been proposed that UL42 has a role similar to "sliding clamp" processivity factors, such as PCNA. In fact, the crystal structure of UL42 has a strong similarity to PCNA (Zuccola *et al.*, 2000). However, unlike PCNA, which forms loose hexameric rings around the DNA, UL42 binds to the

DNA as a monomer but maintains a high diffusion rate of 17 to 47 bp/s (Randell and Coen, 2001).

1.3.5 Non-essential HSV-1 proteins with roles in nucleotide metabolism

Alkaline nuclease

The alkaline nuclease has 5'-3' and 3'-5' exonuclease activity as well as a low-level endonuclease activity. The main function of this protein is thought to be the removal of branched structures from DNA replication intermediates to allow efficient packaging of viral genomes. The properties and potential functions of this protein are discussed in more detail in section 1.5.

Thymidine kinase

This protein, encoded by the UL23 ORF, is able to phosphorylate various purine nucleosides in addition to thymidine. The role of the protein is unclear and UL23 deletion mutants can grow efficiently in cell culture, although they are impaired in resting cells. In animal models thymidine kinase null mutants can replicate in peripheral cells, such as in the eye but not in trigeminal ganglia. In addition, the protein does however appear to be necessary for reactivation of HSV-1 from latency (Coen *et al.*, 1989).

The main significance of this protein is in its specific phosphorylation of acyclovir (ACV), a nucleoside analogue used in the treatment of HSV. Only the viral thymidine kinase can catalyse the initial phosphorylation of this nucleoside. Host cell kinases then generate the triphosphorylated froms. Incorporation of the triphosphate into replicating DNA disables both viral and host polymerases, thus inhibiting viral replication

(Reardon and Spector, 1989). However ACV is not phosphorylated in uninfected cells and is therefore not toxic.

Uracil DNA glycosylase

Uracil glycosylase, encoded by UL2, is involved in repairing the wrongful insertion of dUTP into genomic DNA or the deamination of cytosine to uracil. If this latter lesion is not repaired the eventual mutagenesis of G-C to A-T occurs. Inactivation of the viral gene does not affect the ability of the virus to replicate in tissue culture (Mullaney *et al.*, 1989). This is probably due to the almost ubiquitous expression of uracil glycosylase in mammalian cells. As with the viral thymidine kinase, the virally encoded uracil glycosylase appears to be necessary in reactivation from latency (Pyles and Thompson, 1994).

Deoxyuridine triphosphatase (dUTPase)

This protein acts to prevent the incorporation of uracil into the viral genome and additionally generates the dTTP precursor, dUMP, by hydrolysing dUTP to dUMP and inorganic phosphate. As with thymidine kinase and uracil glycosylase mutants, dUTPase knockout mutants are unaffected in cell culture but they are less neurovirulent and reactivate poorly from latency (Pyles *et al.*, 1992).

Ribonucleotide reductase

The ribonucleotide reductase is a tetramer of two subunits encoded by each of the UL39 and UL40 genes, forming an $\alpha_2\beta_2$ complex. This complex reduces ribonucleotides to deoxyribonucleotides providing dNTPs as precursors for DNA synthesis. Although

non-essential in dividing cells, the complex appears to be necessary in neurons and non-dividing tissue culture cells (Goldstein and Weller, 1988; Idowu *et al.*, 1992).

1.4 Encapsidation of the viral genome

1.4.1 General description of capsid structure

As described in section 1.1.4, herpesviruses capsids have an icosahedral structure. In HSV-1 the capsid shell is 15 nm thick and has a diameter of 125 nm. The capsid is constructed around a central core of scaffolding proteins to form a spherical procapsid. Upon DNA packaging the scaffolding protein is cleaved and ejected. The capsid then becomes angularised and forms the mature icosahedral structure (For reviews see: Rixon, 1993; Homa and Brown, 1997).

1.4.2 Major components of the viral capsid

In HSV-1 the procapsid is constructed from seven major, virally encoded, proteins which are the products of the UL18 (VP23), UL19 (VP5), UL26 (VP21 and VP24), UL26.5 (VP22a), UL35 (VP26) and UL38 (VP19C) ORFs. As mentioned in section 1.4.1 the capsid shell comprises three distinct units, the pentamers and hexamers, collectively know as capsomers, and the triplexes. The pentamers and hexamers comprise five and six copies of VP5, respectively. There are 150 hexamers and 12 pentamers per capsid, giving a total of 960 copies of VP5 in each virion. The capsomers protrude from the capsid and flair at the base where they meet neighbouring capsomers to form the capsid floor. The triplexes consist of two copies of VP23 and one copy of VP19C. They connect groups of three capsomers, sitting above the capsid floor.

The procapsid contains an internal core of scaffolding protein, around which the capsid is constructed. The main component of the scaffold, pre-VP22a (product of the UL26.5 gene), binds to the inner face of VP5 via its C-terminus. The UL26 gene product encodes the scaffold protease. The full-length UL26 polypeptide is cleaved to form VP24, the functional protease, and VP21 (Liu and Roizman, 1993; Dilanni *et al.*, 1993;Weinheimmer *et al.*, 1993). The scaffolding protein is cleaved 24 amino acids from its C-terminus upon the onset of DNA packaging by VP24 and is ejected from the capsid by an unknown mechanism. The VP21 polypeptide is also cleaved at this time because its C-terminal 328 amino acids are identical to pre-VP22a.

There are a number of differences between the procapsid and mature capsid. The most obvious is that the procapsid is more spherical in shape than the angularised mature capsid. Secondly, the capsomers do not form a continuous floor layer in procapsids and the hexons are circular in procapsids but hexagonal in mature capsids. Thirdly, a channel which runs down the center of pentons in procapsids is closed upon capsid maturation, presumably to prevent escape of packaged DNA (Newcomb and Brown, 1994; Zhou *et al.*, 1999). Finally, the procapsid is less stable than the mature capsid (Newcomb *et al.*, 2000).

1.4.3 Proteins necessary for cleavage and packaging of concatmeric DNA

Six viral proteins: UL6, UL15, UL17, UL28, UL32 and UL33, have been identified as necessary for the cleavage and encapsidation of concatemeric DNA (Reviewed in Rixon, 1993; Homa and Brown 1997). These proteins were identified through *ts* or deletion mutants with phenotypes that resulted in the accumulation of DNase-sensitive, concatemeric DNA lacking free termini. In addition to the expected absence of C-

capsids these mutants show an accumulation of B-capsids. UL25 null mutants are unusual among HSV-1 packaging mutants in that there is also an accumulation of A-capsids (McNab *et al.*, 1998).

In contrast to our understanding of HSV-1 DNA replication, comparatively little is known about the packaging mechanism. What follows is a brief description of each of the HSV-1 proteins listed above and their possible roles in DNA packaging; largely inferred from studies involving other large, dsDNA containing viruses, such as bacteriophage T4 and bacteriophage lambda (reviewed by Catalano, 1995; Fujisawa and Morita, 1997; Catalano, 2000).

UL6 – Possible portal protein

UL6 is a 75 kDa protein that is associated with both procapsids and mature capsids. Its presence is not however necessary for assembly of procapsids (Patel and MacLean, 1995; Sheaffer *et al.*, 2001). The creation of UL6 null mutants has demonstrated the essential nature of the UL6 protein (Patel *et al.*, 1996; Lamberti and Weller, 1996). These mutants were constructed in complementing cell lines, and in non-complementing cell lines showed wt HSV-1 levels of DNA synthesis but a failure to process or package the DNA concatemers.

A recent study by Newcomb *et al.* (2001) has indicated that a dodecamer of UL6 proteins may form a portal protein at one of the procapsid vertices. In immunoelectron microscopy experiments immunogold labelling using an antibody against UL6 resulted in the deposition of particles adjacent to only one vertex of each virus particle. In addition, electron microscopy examination of UL6 protein purified from recombinant

baculovirus infected insect cells revealed heterogeneous ring structures with the major population forming a dodecameric ring with an internal diameter of 5 ± 0.7 nm.

UL15 and UL28 – A potential terminase complex

In bacteriophage such as T7 and lambda the terminase complex is responsible for the cleavage of the concatemeric genome and, through interaction with the portal protein, translocation of the genome into the capsid (for reviews see Catalano, 2000; Fujisawa and Morita, 1997). The terminase usually exists as a heterodimeric complex, containing a cleavage activity that is dependent on magnesium, and an ATP dependent DNA translocating pump. As well as its catalytic activity the terminase is also required to bind both genomic DNA and the portal protein complex.

In HSV-1 the UL15 and UL28 proteins have surfaced as potential candidates for the terminase complex. Both are essential for cleavage and packaging as initially demonstrated in *ts* mutants and confirmed using deletion mutants (Poon and Roizman, 1993; Cavalcoli *et al.*, 1993; Baines *et al.*, 1997; Yu and Weller, 1998a). Several studies have also shown that UL15 and UL28 physically interact (Koslowski *et al.*, 1997, 1999; Abbotts *et al.*, 2000). UL15 also shows limited sequence homology to gp17, the large subunit of the bacteriophage T4 terminase complex. The homology includes a consensus ATP binding motif which, in mutagenesis experiments, has been shown to be essential for the function of UL15 (Yu and Weller, 1998a). Recently the UL33 protein, also essential for cleavage and packaging, has also been observed to complex with UL15 and UL28 (Beard *et al.*, 2002).

UL15 is one of the few HSV-1 proteins that are derived from a spliced gene product. However the expression of UL15 from a cDNA copy does not appear to affect the viability of a recombinant virus (Baines and Roizman, 1992). The UL15 protein produces a number of truncated forms that appear to be the result of both proteolytic cleavage of the full length protein and expression from a truncated RNA initiated from an internal start codon that is within the second exon (UL15.5 protein) (Baines *et al.*, 1994; Baines *et al.*, 1997; Salmon and Baines, 1998). Complementation of a UL15/UL15.5 null mutant with a construct which expressed only UL15 demonstrated that, in cell culture at least, UL15.5 is non-essential (Yu and Weller, 1998a). There are three UL15 products that are associated with viral B capsids with apparent sizes of 83, 80 and 79 kDa. However in the absence of other proteins essential for the cleavage and packaging of viral DNA only the largest product associates with capsids, suggesting that an intact packaging machinery is required for the capsid association of the 80 and 79 kDa products. This observation also implies that the largest terminase product associates with capsid proteins rather than the UL6 portal protein. Interactions of HSV-1 UL6 with UL15, and UL6 with UL28 have, however, recently been observed by immunoprecipitation and immunofluoresence studies, indicative of an interaction between the HSV-1 terminase and portal proteins (C. White, personal communication).

UL28 encodes an 87 kDa protein which was identified as essential for cleavage and packaging of the viral genome through the use of *ts* mutants (Addison *et al.*, 1990; Tengelsen *et al.*, 1993). Its localisation to the nucleus appears to be dependent on the co-expression of UL15 (Koslowski *et al.*, 1999). Moreover, the pseudorabies virus UL28 homolog can also be localised to the nucleus by HSV-1 UL15, indicating the high degree of conservation of this protein between herpesviruses (Koslowski *et al.*, 1997).

Although nuclear localisation of UL28 is dependent on UL15, and both proteins have been shown to physically interact, it has been reported that UL28 can independently associate with capsids (Taus and Baines, 1998; Yu and Weller, 1998b).

Recently UL28 has been shown to interact specifically with oligonucleotides containing the *pac*1 motif of the *a* sequence (Adelman *et al.*, 2001). This interaction was dependent on the DNA forming an altered secondary structure upon denaturation and renaturation, as judged by a change in mobility of the oligonucleotide in non-denaturing gels. Introduction of previously characterised murine cytomegalovirus (MCMV) *pac*1 mutations which impaired packaging (McVoy *et al.*, 1998) into the *pac*1 oligonucleotides reduced the binding of UL28 in each case, correlating with a loss of function *in vivo*.

The physical interaction of UL15 and UL28, the capsid binding properties of both proteins, the ability of UL28 to specifically bind the *pac*1 motif and, finally, the limited homology between UL15 and the large subunit of bacteriophage T4 strongly suggest that UL15 and UL28 form the HSV-1 terminase complex. This is also supported by HCMV studies which suggest that the UL56 and UL89 proteins, homologues of HSV-1 UL28 and UL15, respectively also form a functional terminase complex. HCMV UL56, has similar biochemical properties to the HSV-1 UL28 protein (Bogner *et al.*, 1998). Also, resistance of HCMV to the drug TCRB, which inhibits DNA packaging has been linked to mutations in both UL56 and UL89 (Krosky *et al.*, 1998).

UL33

As with many of the proteins involved in the cleavage and packaging process, the UL33 gene was identified through characterisation of a *ts* mutant (al-Kobaisi *et al.*, 1991). The UL33 gene has since been identified as a late gene, in that it requires the onset of DNA synthesis to be expressed. In immunofluoresence assays the protein was predominantly associated with viral replication compartments, although a small proportion was cytoplasmic (Reynolds *et al*, 2000). As described above its interaction with UL15 and UL28 suggests that it may form a component of the putative terminase complex (Beard *et al.*, 2002).

UL17

UL17 was originally identified as a protein associated with the tegument and is essential for viral replication (Baines and Roizman, 1991). The involvement of UL17 in cleavage and packaging was discovered through characterisation of deletion mutants grown in complementing cell lines (Salmon *et al.*, 1998).

The use of UL17 null mutants revealed that UL17 is involved in targeting capsids and both major and minor capsid associated proteins to sites of viral replication. In wt HSV-1 infected cells the major capsid proteins UL19 and UL26.5, plus the cleavage/packaging protein UL6, were shown to be directed to replication compartments. However, in cells infected with the UL17 null mutant UL19 and UL26.5 formed dense aggregates separated from replication compartments, and UL6 exhibited a diffuse distribution around the nuclear periphery. In comparison, the use of UL28 and UL33 null mutants did not affect the distribution of these proteins (Taus *et al.*, 1998). Further studies demonstrated that UL17, along with UL6 and UL28, was

required for the association of various forms of UL15 with B-capsids (Salmon and Baines, 1998).

UL32

The product of the UL32 gene is a 67 kDa protein which accumulates predominantly in the cytoplasm of infected cells, although smaller amounts accumulate in the nucleus and co-localise with UL29 to replication compartments (Lamberti and Weller, 1998). As with other viral proteins involved in cleavage and packaging, UL32 null mutants accumulate endless DNA in the nucleus of infected cells with no detectable termini (Chang *et al.*, 1996). In common with UL17, UL32 appears necessary to direct capsids to viral replication compartments. Using an antibody specific for capsid hexamers, wt HSV-1 capsids were shown to localise to replication compartments, but in UL32 null mutants the capsids showed a more diffuse staining (Lamberti and Weller, 1998).

UL25

Two *ts* mutants, ts1204 and ts1208, with temperature sensitive lesions in the UL25 ORF were identified (Addison *et al.*, 1984). Electron microscopy studies demonstrated that at the non-permissive temperature both viruses produced large numbers of B-capsids and few C-capsids, suggesting a packaging defect. DNA replication was deduced to be unaffected as the viral protein profile was almost identical to wt HSV-1. The ts1208 mutant exhibited an additional defect involved in virus entry into the host cell. If absorption was performed at the non-permissive temperature the virus would bind to the cell surface but could not fuse with the plasma membrane. This defect could be overcome by subsequent incubation at the permissive temperature.

UL25 has been shown to interact with the major capsid protein, VP5 (Ogasawara *et al.*, 2001). Immunoelectron microscopy studies show that UL25 locates to both pentamers and hexamers with approximately 42 copies per B-capsid. Based upon its ability to bind DNA Ogasawara *et al.* (2001), proposed that UL25 plays a role in anchoring genomic DNA to the capsid. This work supports that of McNab *et al.* (1998) who isolated a UL25 null mutant, KUL25NS, using a complementing cell line. Characterisation of this mutant revealed that viral DNA synthesis was unimpaired and there was an accumulation of A-capsids, indicative of abortive packaging events. The most striking feature of this mutant was the presence of genome length DNA that contained free termini, indicating that the concatemeric DNA had been cleaved, which was, however, susceptible to degradation by DNase indicating that it was not encapsidated. Taken in conjunction with the accumulation of A capsids it was concluded that cleavage and packaging could occur in the absence of UL25, and that UL25 was involved in stabilising DNA containing capsids. In its absence cleaved DNA was therefore only transiently associated with the capsids.

In a subsequent study using the same mutant virus, Stow (2001) demonstrated that a small amount of genomic length DNA was stably packaged. No DNA could be detected in the cytoplasm of infected cells suggesting that there is a defect in capsid maturation and egress. Close examination of the size of packaged molecules using pulsed field gel electrophoresis demonstrated that the majority of packaged molecules were below genome length. In addition there was an over-representation of the long terminus compared to the short terminus. These results suggested that packaging was efficiently initiated but that UL25 was required for the late stage of the head-filling process to occur efficiently.

Although there are a number of contrasting features between the work of McNab *et al.* (1998) and Stow (2001), both indicate that UL25 is not absolutely required for the cleavage of genomic DNA but is involved in capsid maturation. Interestingly the phenotype of the UL25 null mutant described by Stow is not dissimilar from that of UL12 null mutants which accumulate A-capsids, package reduced amounts of DNA and exhibit a block in transportation of capsids into the nucleus (see *Results* and section 1.5).

1.5 The role of the virally encoded alkaline nuclease in the replication and packaging of HSV-1 DNA

1.5.1 Alkaline nuclease activity of HSV-1 infected cells

An increase in nuclease activity was first identified in HSV-1 infected cells in 1963 by Keir and Gold. The optimum nuclease activity was observed under very basic conditions (pH9), hence the name alkaline nuclease (Morrison and Keir, 1968). This activity was found to localise mainly to the cell nucleus and had properties distinct from known cellular nucleases, such as the ability to degrade dsDNA under alkaline conditions and lower stability than cellular nucleases at elevated temperatures (Morrison and Keir, 1968). Convincing evidence of the alkaline nuclease being virally encoded came from studies on the HSV-2 mutant, *ts*13. At the non-permissive temperature cells infected with the *ts* mutant failed to exhibit alkaline nuclease activity, whereas wt virus did (Franke *et al.*, 1978). In addition, Preston and Cordingley (1982), demonstrated that the injection of specific fragments of HSV-1 DNA (map units 0.155 to 0.185) into Xenopus oocytes resulted in production of alkaline nuclease activity.

1.5.2 Identification of the alkaline nuclease protein and open reading frame

The identification of the polypeptide and the mapping of the region of the genome that encoded the alkaline nuclease were reported in the late 1970s and early 1980s. The purification of the alkaline nuclease by Strobel-Fidler and Francke, (1980) yielded polypeptides of 90 kDa, 85 kDa and 70 kDa. Preston and Cordingley, (1982) used a reticulocyte *in vitro* translation system to express an 85 kDa protein, VI85. The VI85 mRNA co-sedimented with the mRNA which, when injected into Xenopus oocytes, displayed exonuclease activity. The alkaline nuclease also likely corresponds to the phosphoprotein Vmw85 (Marsden *et al.*, 1978) and is referred to as ICP19 by Banks *et al.* (1985).

The mRNA reported to encode exonuclease activity by Preston and Cordingley (1982) was estimated to be 3.2 kilobases in size. However subsequent studies demonstrated that the mRNA transcript for the alkaline nuclease was 2.3-kb (Costa *et al.*, 1983). Costa *et al.* (1983) also identified a 1.9-kb transcript which contained part of the nuclease reading frame, later shown to correspond to the UL12.5 ORF. The ORF specifying the alkaline nuclease was mapped and shown to correlate with the leftward orientated transcript of 2.3-kb, and to encode a 626 amino acid protein (Banks *et al.*, 1985; Draper *et al.*, 1986; McGeoch *et al.*, 1986).

Following determination of the complete DNA sequence of HSV-1 (McGeoch *et al.*, 1988) the alkaline nuclease gene was assigned as ORF UL12 (residues 26889 to 25011). The UL12 mRNA is one of five transcripts which share the polyadenylation site at position 24807. It is proceeded by a typical TATA box promoter, mapped to residue 27048 (Costa *et* al., 1983; McGeoch *et al.*, 1988) and regulated with early kinetics.

1.5.3 Biochemical properties of the alkaline nuclease

The biochemical properties of purified alkaline nuclease were examined in detail by Hoffmann and Cheng (1978). They demonstrated that the nuclease purified from both HSV-1 and HSV-2 infected cells was able to degrade dsDNA generating 5'-monophosphonucleotides. The enzyme had an absolute requirement for a divalent cation such as Mg^{2+} or Mn^{2+}. At the optimal concentration of Mg^{2+} (5mM) the nuclease was 3-5 fold more active than in the presence Mn^{2+} at its optimal concentration (0.1 – 0.2 mM). Concentrations of Mn^{2+} above 1 mM inhibited exonuclease activity. This study also demonstrated that the activity of both the HSV-1 and HSV-2 nuclease was optimal at pH 8-9. Similar properties for the alkaline nuclease were also described by Strobel-Fidler and Franke (1980), and Banks *et al.* (1983).

Although the main activity of the alkaline nuclease appeared to be as an exonuclease, acting in both 5'-3' and 3'-5' directions (Hoffmann, 1981), there was also an associated endonuclease activity (Hoffmann and Cheng, 1979; Strobel-Fidler and Francke, 1980; Banks *et al*, 1983). In comparison to the exonuclease activity the endonuclease activity was 10-fold less efficient. Moreover, the endonuclease was inhibited by lower concentrations of spermine. The endonuclease action of alkaline nuclease also appears to prefer single-stranded regions of DNA as a substrate, degrading supercoiled plasmid DNA 60 times more efficiently than relaxed, covalently closed plasmid DNA (Hoffmann and Cheng, 1979). In these experiments the endonuclease did not exhibit any sequence specificity although Henderson *et al.* (1998) demonstrated hypersensitivity sites specific to the alkaline nuclease in a fragment from plasmid

pON114, suggesting that the alkaline nuclease may contain some sequence or structure related specificity.

1.5.4 Interaction of the alkaline nuclease with other proteins

A number of antibodies have been raised against the alkaline nuclease (Banks *et al.*, 1983, 1985; Bronstein and Weber, 1996). These antibodies have facilitated studies examining the intracellular localisation of the UL12 protein during infection and the interactions of UL12 with other proteins.

Immunoabsorbant column chromatography using the monoclonal antibody Q1, specific against the HSV-1 alkaline nuclease (Banks *et al.*, 1983), resulted in co-purification of large amounts of the major DNA binding protein (mDBP) from infected cell extracts (Vaughan *et al.*, 1984). Work by Thomas *et al.* (1988) has also shown that the mDBP and the alkaline nuclease are able to form a complex with the viral DNA polymerse, although independent interaction between the polymerase and the alkaline nuclease has not been demonstrated. The interaction between the mDBP and the alkaline nuclease was confirmed using a collection of monoclonal antibodies (five specific for the alkaline nuclease and five specific for the mDBP). The interaction was mapped to the C-terminus of the mDBP, although the site of interaction in the alkaline nuclease has yet to be determined (Thomas *et al.*, 1992). In the same study it was demonstrated that both proteins co-localise to replication compartments in the nucleus of infected cells, although the alkaline nuclease was also found in the nucleoplasm. Previous studies demonstrated that the alkaline nuclease had a more diffuse staining in the nucleus, distinct from the viral DNA polymerase (Randall and Dinwoodie, 1986; Puvion-

Dutilleul *et al.*, 1986). This suggests that the alkaline nuclease may have two distinct roles in the nucleus.

The alkaline nuclease is a highly phosphorylated protein (Marsden *et al.*, 1987), suggesting a possible control mechanism through the level of phosphorylation. In HSV-2, the alkaline nuclease has been reported to be phosphorylated by the US3 gene product (Daikoku *et al.*, 1995), although similar processing has not been reported for the HSV-1 homologue. An HCMV UL97 null mutant, which does not express the HSV-1 UL13 homologue, has a very similar phenotype to UL12 null mutants (Wolf *et al.*, 2001), suggesting that the protein kinase encoded by this gene may possibly be involved in phosphorylation of the alkaline nuclease.

As will be discussed later the alkaline nuclease has a significant role in the synthesis and packaging of viral DNA. Although the nuclease has been shown to associate with proteins involved in DNA synthesis it is also possible that it interacts with the viral packaging machinery.

1.5.5 The alkaline nuclease is conserved throughout the herpesvirus family

Homologues of the HSV-1 alkaline nuclease have been reported in every herpesvirus sequenced to date and the homologues in the human herpesviruses are shown in Table I. Although there is limited overall sequence homology between alkaline nuclease ORFs, seven conserved motifs have been identified (Martinez *et al.*, 1996b). A lineup of the alkaline nuclease amino acid sequences for each of the human herpesviruses is shown in Figure IV. The HSV-1 and HSV-2, and to a lesser extent, HCMV and VZV alkaline nuclease polypeptides are unusual in that they have an extention to the N-terminus. The

function of the N-terminus is unknown but it is interesting that HSV-1 UL12 null mutants have been heterologously complemented in *trans* only by the expression of the HCMV UL98 gene indicating that the N-terminal extension may play an important role (Gao *et al.*, 1998). The HCMV protein is larger than most alkaline nucleases but still lacks 70 amino acids from its N-terminus compared to HSV-1. It is also interesting to note that no successful attempts to complement alkaline nuclease null mutants of other herpesviruses with the HSV-1 UL12 gene have been reported.

Table I - Alkaline nuclease ORFs in human herpesviruses

Herpesvirus	HSV-1	HSV-2	VZV	HCMV	EBV	HHV-6	HHV-7	KSHV
gene name	UL12	UL12	ORF48	UL98	BGLF5	U70	U70	ORF 37
protein size (a.a.)	626	620	551	584	470	488	480	486

HSV-1 - herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; VZV, varicella-zoster virus; EBV Epstein-Barr virus; HHV-6, human herpesvirus 6; HHV-7, human herpesvirus 7; KSHV, Kaposi's sarcoma associated herpes virus.

Mutational analysis of the HSV-1 alkaline nuclease has demonstrated that the N-terminal 126 amino acids can be removed without detectably altering the nuclease activity of the protein. This protein, which closely corresponds to the UL12.5 product, cannot complement a UL12 null mutant when expressed in *trans* (Henderson *et al.*, 1998). In contrast if only 49 amino acids of the C-terminus are removed nuclease activity is lost (Henderson *et al.*, 1998). Therefore, the N-terminus of the HSV-1 nuclease contains an unrecognised functional domain, although whether this domain possesses an enzymatic property or if it is required for the interaction with some, as yet unidentified, viral or cellular protein has yet to be determined.


1
sp|P04294 HSV1 MESTVGPACPPGRTVTKRPWALAEDTPRGPDSPPKRPRPNSLPLTTTFRPLPPPPQTTSA
sp|P06489 HSV2 ----MAAAATPG---AKRP----ADPARDPDSPPKRPRPNSLDLATVFGPRPAPPRPTSP
sp|P09253 VZV ----------
sp|P03217 EBV ----------
tr|P88925 HHV8 ----------
sp|P24447 HSV6 ----------
sp|P52346 HSV7 ----------
sp|P16789 HCMV ----------
61
sp|P04294 HSV1 VDPSSHSPVNPPRDQHATDTADEKPRAASPALSDAS-GPPTPDIPLSPG-GTHARDPDAD
sp|P06489 HSV2 GAPGSHWPQSPPRGQPDGGAPGEKARPASPALSEASSGPPTPDIPLSPG-GAHAIDPDCS
sp|P09253 VZV ----------MARSGLDRIDISPQPAKKIARVGGLQHPFVKTDINTINVEHHFIDTLQ
sp|P03217 EBV ----------MADVDELEDPMEEMT-SYTFARFLRSPE--TEAFVRNLD
tr|P88925 HHV8 ----------MEATPTPADLFSEDYLVDTLDGLTVDD---QQAVLASLS
sp|P24447 HSV6 ----------MDLDQISETLSSVAEEEPLTMFLLDK---LYAIREKIK
sp|P52346 HSV7 ----------MAIDYAQISCNLASIIEEDSVFLFLIDK---LNNLDISRR
sp|P16789 HCMV ----------MWGVSSLDYDDDEELTRLLAVWDDEPLSLFLMNT---FLLHQEGFR
119
sp|P04294 HSV1 PDS-PDLDSMWSASVIPNALP-SH---------ILAETFERH--LRGLLRGVRAPLAIGP
sp|P06489 HSV2 PGP-PDPDPMWSASAIPNALP-PH---------ILAETFERH--LRGLLRGVRSPLAIGP
sp|P09253 VZV KTS-PNMDCRGMTAGIFIRLSHMY---------KILTTLESP---NDVTYTTPG------
sp|P03217 EBV RPPQMPAMRFVYLYCLCKQIQ-------------EFSGETG--FCDFVSSLVQE-----
tr|P88925 HHV8 FSK-FLKHAKVRDWCAQAKIQPSMP--------ALRMAYNYF--LFSKVGEFIGS-----
sp|P24447 HSV6 QVP-FSIVRLCHVYCMLIKYN-AS---------NNNCILGRK--LIEEMQQFLCGTRV--
sp|P52346 HSV7 KIS-FNFIRLCYTYYILIKFN-SR---------FKDTFLARS--FIDYMHQNIS------
sp|P16789 HCMV NLP-FTVLRLSYAYRIFAKMLRAHGTPVAEDFMTRVAALARDEGLRDILGQRHAAEAS--
166
sp|P04294 HSV1 LWARLDYLCSLAV-VLEEAGMVDRGLGRHLWR--LTRRGPPAAADAVAPRPLMGFYEAAT
sp|P06489 HSV2 LWARLDYLCSLVV-SLEAAGMVDRGLGRHLWR--LTRRAPPSAAEAVAPRPLMGFYEAAT
sp|P09253 VZV ------STNALFF-KTSTQPQEPR----------PEELASKLTQDDIKR--ILLTIESET
sp|P03217 EBV ----NDSKDGPS------LKSIYWG----------LQEATDEQRTVLCS-----YVESMT
tr|P88925 HHV8 ----EDVCNFFVDRVFGGVRLLDV----------ASVYAACSQMNAHQRHHICCLVERAT
sp|P24447 HSV6 -DGSEDISMDLS----ELCKLYDYCP--LLCS--ALCRAPCVSVNKLFK-----IVERET
sp|P52346 HSV7 -----DFIDENV----ELSDLYSN-----IYV--RLQDASPKVVKNLFK-----ILERET
sp|P16789 HCMV ---RAEIAEALER-VAERCDDRHGGSDDYVWLSRLLDLAPNYRQVELFQ-----LLEKES
Conserved Domain 111111
223
sp|P04294 HSV1 QNQADCQLWALLRRGLTTASTLRWGPQGPCFSPQWLKHNASLR-PDVQSS------AVMF
sp|P06489 HSV2 QNQADCQLWALLRRGLTTASTLRWGAQGPCFSSQWLTHNASLR-LDAQSS------AVMF
sp|P09253 VZV RGQGDNAIWTLLRRNLITASTLKWSVSGPVIPPQWFYHHN---TTDTYGD----AAAMAF
sp|P03217 EBV RGQSENLMWDILRNGIISSSKLLSTIKNG--PTKVFEPAPIST-NHYFGG------PVAF
tr|P88925 HHV8 SSQSLNPVWDALRDGIISSSKFHWAVKQQNTSKKIFSPWPITN-NHFVAG------PLAF
sp|P24447 HSV6 RGQSENPLWHALRKYTVTATKLYDIYTTRCFL-EYKGQ-------QFFGE------AVIY
sp|P52346 HSV7 RGQSTNPLWHAMRKNCITATKIYDIYISKSFS-GIQEH-------SYLGD------AVLY
sp|P16789 HCMV RGQSRNSVWHLLRMDTVSATKFYEAFVSGCLPGAAAADGSGGGGSHYTGSRAGVSPGIQF
Conserved Domain 11111111111111111111
276
sp|P04294 HSV1 GRVNEPTARSLLFRYCVG----RADDGGEAGADTRRFIFHEP--------SDLAEENVHT
sp|P06489 HSV2 GRVNEPTARNLLFRYCVG----RADAGVNDDADAGRFVFHQP--------GDLAEENVHA
sp|P09253 VZV GKTNEPAARAIVEALFIDPADIRTPDHLTPEATTKFFNFDMLNTKSPSLLVGTPRIGTYE
sp|P03217 EBV GLRCEDTVKDIVCKLICG----------DASANRQ
tr|P88925 HHV8 GLRCEEVVKTLLATLLHP----------DETNCLD
sp|P24447 HSV6 GAKHERVIRHLVATFYVK----R----------EVKET
sp|P52346 HSV7 GIKHERIIEHLLKTFFVK----KP----------WISKT
sp|P16789 HCMV GIKHEGLVKTLVECYVMHG---RE----------PVRDG
Conserved Domain
324
sp|P04294 HSV1 CGVLMDGHTGMVGASLDILVCPRDIHGY-LAPVP-KTPLAFYEVKCRAKYAFDPMDPSDP
sp|P06489 HSV2 CGVLMDGHTGMVGASLDILVCPRDPHGY-LAPAP-QTPLAFYEVKCRAKYAFDPADPGAP
sp|P09253 VZV CGLLIDVRTGLIGASLDVLVCDRDPLTGTLNPHPAETDISFFEIKCRAKYLFDPDDKNNP
sp|P03217 EBV FGFMISPTDGIFGVSLDLCVNVESQGDFILFTDR---SCIY-EIKCRFKYLFSKSEFD-P
tr|P88925 HHV8 YGFMQSPQNGIFGVSLDFAANVKTDTEGRLQFDP--NCKVY-EIKCRFKYTFAKMECD-P
sp|P24447 HSV6 LGLLLDPSSGVFGASLDACFGISFNEDG-FLMVK-EKALIF-EIKFKYKYLRDKED----
sp|P52346 HSV7 LGLLLDPSSGVFGASIDSYYGISFNDNN-LIEVG-DKVVIF-ELKFRYKYLREKND----
sp|P16789 HCMV LGLLIDPTSGLLGASMDLCFGVLKQGSGRTLLVE-PCARVY-EIKCRYKYLRKKED----
Conserved Domain 2222222222222222 333333333333333

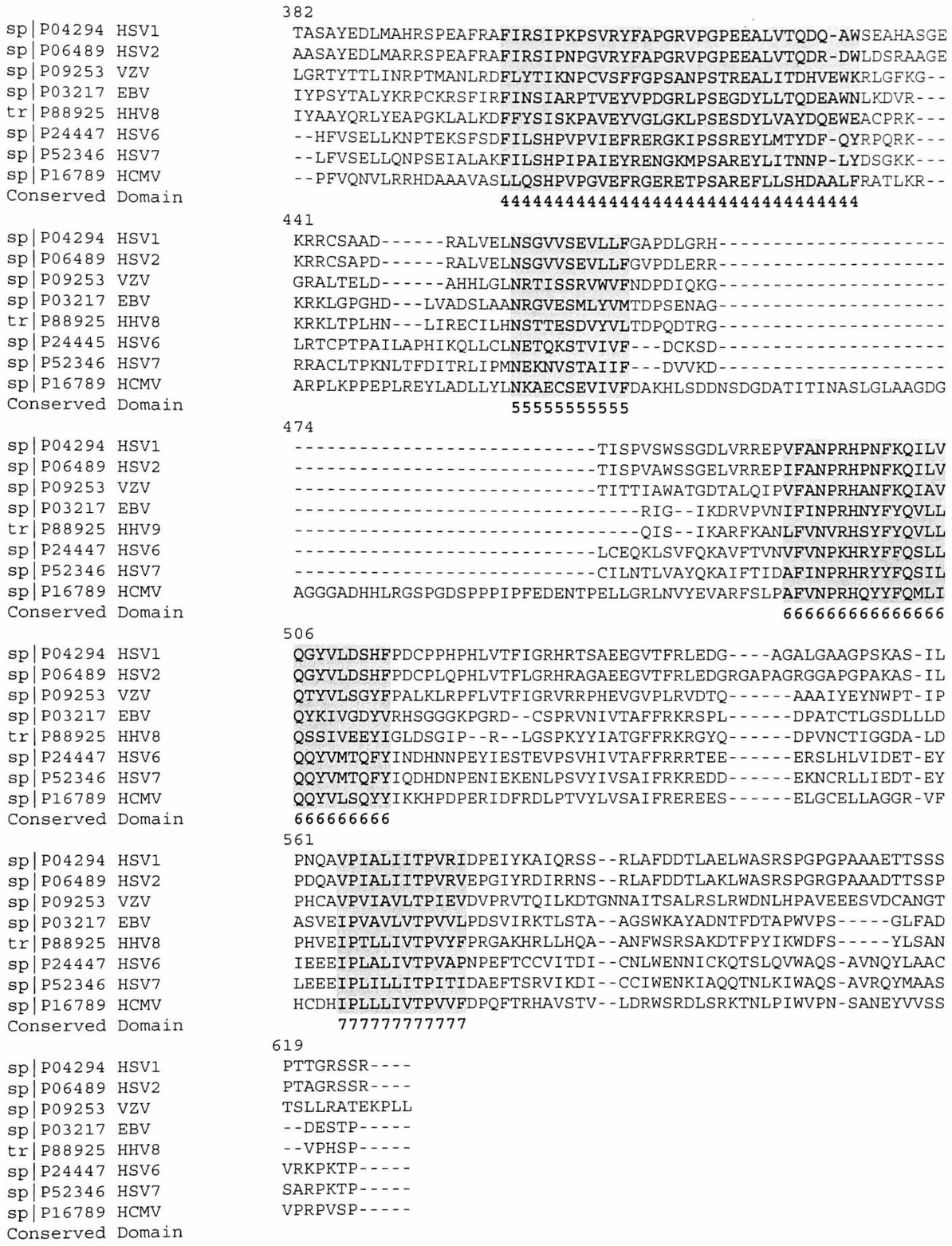


Figure IV – Alignment of the eight human herpesvirus alkaline nucleases

The eight human herpesvirus alkaline nuclease amino acid sequences are shown. Their corresponding SWISS-PROT (sp) or TrEMBL (tr) acquisition numbers are given alongside. The alignment was performed using the Quick BlastP search function and the Clustal W (multiple alignment) function of ExPASy (http://ca.expasy.org/) and related links. The highlighted regions define the conserved motifs as described by Goldstein and Weller (1998a).

1.5.6 Phenotype of UL12 null mutants

Three HSV-1, UL12 null mutants (*nuc* mutants) have been created (Weller *et al.*, 1990; Patel *et al.*, 1996; Martinez *et al.*, 1996b). In each case it was necessary to use a complementing cell line, confirming the necessity of the alkaline nuclease during viral replication. A schematic diagram indicating the lesions in each of the mutants is shown in Figure V.

Virus Yield

All *nuc* mutants produce a small amount of infectious virus in non-complementing cell lines, although the yield is reduced 100 – 1000 fold compared to wt virus. Another interesting feature of these mutants is that plaque size is reduced approximately five fold compared to wt virus. This phenotype is repaired upon growth in complementing cell lines (Weller *et al.*,1990; Patel *et al.*, 1996).

DNA replication

Initial characterisation of the AN-1 *nuc* mutant demonstrated that the virus was not impaired in its ability to replicate viral DNA (Weller *et al.*, 1990). However, subsequent studies examining the ability of this virus and other *nuc* mutants to replicate both viral and plasmid (amplicon) DNA indicate that synthesis was reduced. Shao *et al.* (1993) reported that the AN-1 mutant synthesises DNA at 50 – 63% of wt amounts, similarly the ANF-1 mutant shows DNA synthesis at 60 – 90% of wt levels (Martinez *et al.*, (1996b). Patel *et al.* (1996) also report that the *amb*UL12 *nuc* mutant is impaired in DNA synthesis, although no quantitative data was given.

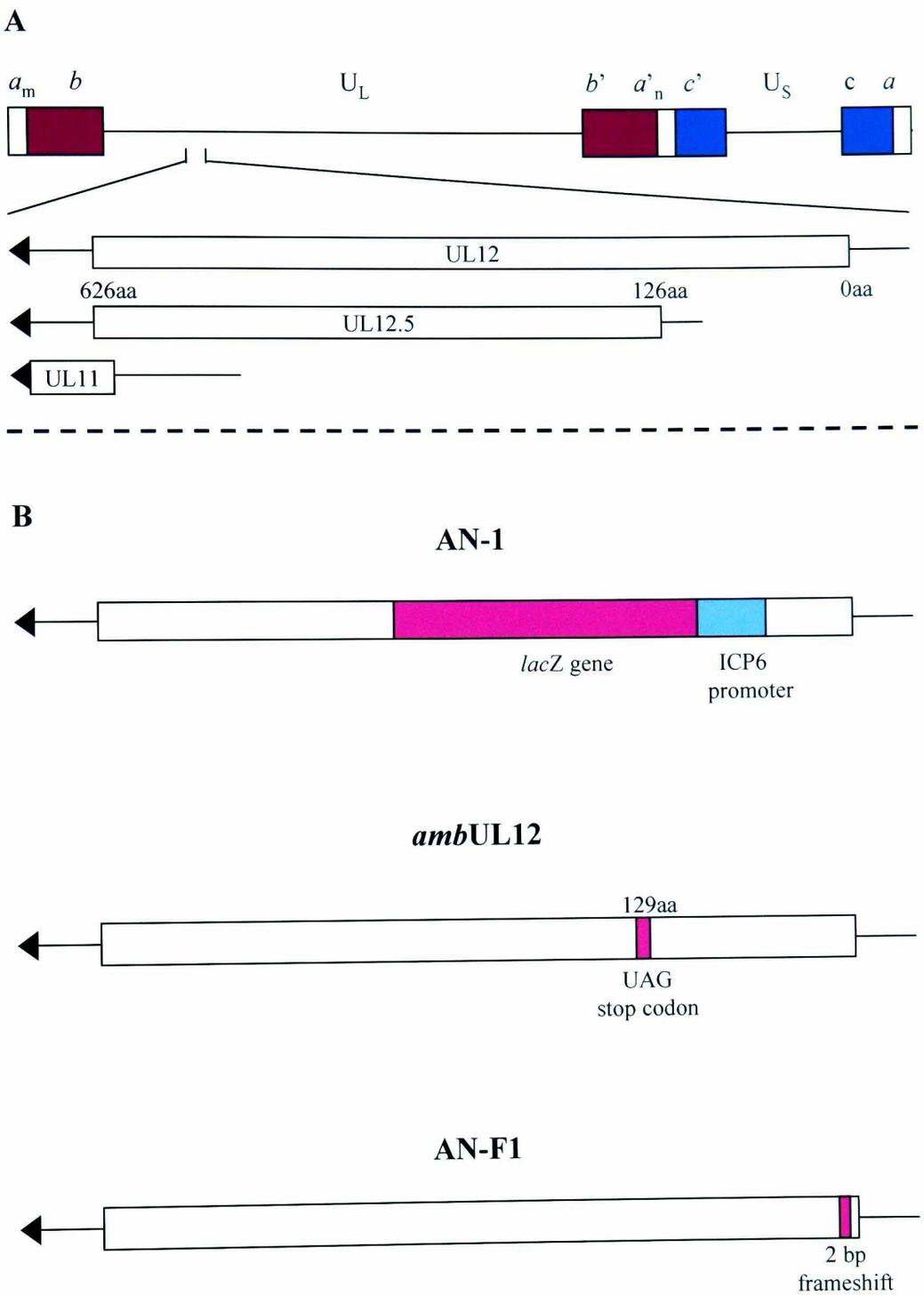


Figure V – Schematic representation of various alkaline nuclease null mutants

(**A**) The region of the genome containing the UL12 ORF is enlarged and shows the overlapping ORFs of UL12, UL12.5 and UL11. (**B**) The AN-1 mutant was generated by deleting a portion of the UL12/UL12.5 ORF and inserting the *lacZ* gene under the control of the HSV-1 ICP6 promoter (Weller *et al.*, 1990). *amb*UL12 was generated by inserting an in-frame amber stop codon into the UL12/UL12.5 ORF (Patel *et al.*, 1996). A 2bp frameshift was inserted into the UL12 ORF to generate ANF-1 (Martinez *et al.*, 1996b). The AN-1 and *amb*UL12 mutations prevent expression of functional UL12 and UL12.5 gene products whereas ANF-1 is blocked only in the expression of UL12.

Although the mechanism for impairment is not known, it has been suggested that the interaction of the alkaline nuclease with the mDBP indicates that it may have some direct, but non-essential, role in DNA synthesis. Bacteriophage T4, which has a bimodal replication mechanism, requires endonuclease VII in the second phase of replication. This endonuclease is required to initiate new replication forks through recombination dependent mechanisms (Mosig, 1998), and it is possible that the alkaline nuclease may have a similar role. Martinez *et al.* (1996a), however, demonstrated that the AN-1 mutant remains capable of intramolecular recombination. This does not completely exclude a role for the alkaline nuclease in DNA synthesis or intermolecular recombination.

Cleavage and packaging

Further characterisation of the AN-1 mutant demonstrated that packaging of viral DNA was reduced by 50% (Shao *et al.*, 1993). However the cleavage of viral DNA appeared to be almost unaffected, occurring 85 - 90% as efficiently as with wt virus. Moreover the cleavage appeared to exhibit normal specificity. Later studies, using the ANF-1 mutant, reported that cleavage activity was 54 – 68% of wt, although the sites of cleavage still appeared specific for the *a* sequence (Martinez *et al.*, 1996b).

Therefore, although slight impairments were observed in the replication and packaging of viral DNA, this did not account for the 100 – 1000 fold drop in virus yield. Electron microscopy and sucrose gradient ultracentrifugation experiments revealed that although DNA was packaged very few mature capsids were produced (Weller *et al.*, 1990; Shao *et al.*, 1993). In the nucleus only small numbers of C-capsids were present while there was an overabundance of A and B-capsids. Moreover, very few C-capsids were

detectable in the cytoplasm of infected cells. DNase and micrococcal nuclease treatment of cytoplasmic and nuclear fractions demonstrated that DNase resistant DNA could only be detected in the nucleus of AN-1 infected cells whereas protected DNA was present in both nuclear and cytoplasmic fractions of cells infected with wt virus (Shao *et al.*, 1993). From these results it was proposed that the capsids produced in *nuc* mutant infected cells were unstable, possibly due to insertion of incorrectly processed genomic DNA, resulting in DNA containing capsids that were unable to transverse the nuclear membrane and egress into the cytoplasm (Shao *et al.*, 1993). Similarly, empty A-capsids arise due to packaging events that are aborted at a relatively early stage.

Structure of DNA replication intermediates in *nuc* mutant infected cells

As described previously, replication of viral DNA produces endless, head-to-tail concatemers which are highly recombinogenic. This results in a complex DNA intermediate that contains numerous X and Y junctions. It is therefore likely that this intermediate needs to be processed into a more suitable substrate for cleavage and packaging. Failure to do so likely results in genomic DNA which contains a number of branches capable of stalling the packaging process or destabilising packaged capsids.

Evidence for the involvement of the alkaline nuclease in processing replication intermediates comes from analysis of hmw intermediates produced in wt HSV-1 and *nuc* mutant infected cells and the ability of purified alkaline nuclease to digest hmw DNA *in vitro* (Martinez *et al.*, 1996a; Goldstein and Weller, 1998b). Digestion of hmw DNA from wt HSV-1 or AN-1 infected cells with the restriction enzyme *Spe*I, which cuts only once per genome, released a small amount of genome length molecules from the wt HSV-1 but not AN-1 samples, indicating that AN-1 hmw DNA was more

complex than that of wt (Martinez *et al.*, 1996a). Similar conclusions were drawn from enzymes which cleaved more frequently although considerable cross linking is clearly present in wt HSV-1 intermediates.

From these results it was postulated that the alkaline nuclease could act as a resolvase similar to endo VII of bacteriophage T4 or endonuclease I of bacteriophage T7, which is known to linearise X and Y junctions (Severini *et al.*, 1996). Experiments using various synthetic cruciforms revealed that while endo VII of bacteriophage T4 would resolve these structures into linear molecules alkaline nuclease degraded them to completion (Goldstein and Weller, 1998b). Therefore, if the alkaline nuclease is involved in processing the HSV-1 hmw DNA intermediates it either functions in a manner not typical of other resolvases or perhaps requires the presence of an additional protein(s) to gain the appropriate specificity. Surprisingly, although the alkaline nuclease could degrade both the synthetic cruciforms and HSV-1 genomic DNA to completion it was unable to do so with hmw intermediates produced from wt HSV-1 infected cells. This degradation block did not appear to be sequence specific and the DNA released from hmw DNA ran as a heterogeneous smear on a pulsed field gel (Goldstein and Weller, 1998b). In addition, through the use of purified, mutant forms of the alkaline nuclease it was demonstrated that only the exonuclease activity of the alkaline nuclease was necessary for the release of hmw DNA. However, construction of mutant viruses which expressed these mutated forms of the alkaline nuclease proved impossible (Goldstein and Weller, 1998a).

Complementation of *nuc* mutants

Two independent, complementing cell lines have been developed for the propagation of *nuc* mutants. The 6-5 cell line (Shao *et al.*, 1993) was developed when it was realised that the S22 cell line (Carmichael *et al.*, 1988) was not fully complementing the AN-1 *nuc* mutant. However, despite producing 4-fold more alkaline nuclease activity the 6-5 cell line did not complement the *nuc* mutant significantly better than the S22 cells. This suggests the kinetics of synthesis or amount of alkaline nuclease may be critical for efficient virus replication.

The creation of plasmids expressing stable forms of alkaline nuclease which contain point mutations knocking out either exonuclease activity alone or exo- and endonuclease activity together has demonstrated that the endonuclease activity alone is not able to complement the AN-1 mutant, and that the exonuclease activity is essential (Goldstein and Weller, 1998a).

Finally, recombinant virus (ANF-1) that expresses the UL12.5 but not UL12 product was shown to be as impaired in growth as the AN-1 mutant which expresses neither protein (Martinez *et al.*, 1996a). The UL12.5 protein lacks the N-terminal 126 amino acids and is of equivalent size to alkaline nucleases produced by other herpesviruses. The nuclease produced by the ANF-1 virus also possessed both exo- and endonuclease activity. This further suggests that the N-terminus of the HSV-1 alkaline nuclease possesses some extra function or is needed for interaction with some cellular or viral protein.

1.6 Aims of the work presented in this thesis

Although the alkaline nuclease is well characterised in terms of its biochemical properties, its precise role in the replication and packaging of the viral genome has remained elusive. The UL12 null mutant, *amb*UL12, was isolated and partially characterised by Patel *et al.* (1996). The work presented in this thesis further characterises the *amb*UL12 virus, and investigates the role of the alkaline nuclease in the replication of HSV-1.

Initial work focused on the ability of *amb*UL12 to replicate and package the viral genome, paying particular attention to the structure of replicated DNA, cleavage of viral concatemers and the size of molecules encapsidated. In the course of these experiments it became evident that *amb*UL12 was severely impaired in its ability to package DNA. Therefore, preliminary experiments were performed to investigate whether UL12 might interact directly with a number of the essential packaging proteins.

The ability of *amb*UL12 to replicate and package amplicons was investigated in a transient transfection assay. The results demonstrate that the amplicons behave similarly to the *amb*UL12 genome, both in terms of the replication and packaging defects and the structure of replication intermediates. Work with the amplicon system was extended to investigate recombination and the sequence requirements for encapsidation.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Most chemicals were supplied by BDH or Sigma Chemical Co. Ltd. and were of AnalaR grade. Other sources are listed below:

Bio-Rad Laboratories	- Ammonium persulphate
	- TEMED
Fluka Chemical Ltd.	- Formaldehyde
Gibco BRL	- Acrylamide
	- Acrylamide:N,N'-methylene-bis-acrylamide 19:1
Joseph Mills (Denaturants) Ltd.	- Absolute alcohol 100
Melford Laboratories Ltd.	- Caesium chloride
Pharmacia Biotech	- dATP, dTTP, dCTP and dGTP
SmithKline Beecham Research	- Ampicillin (Penbritin)

2.1.2 Enzymes

Restriction enzymes were supplied by New England Biolabs or Roche (Boehinger Mannheim). Other enzymes used are listed below:

Sigma Chemical Co. Ltd.	- Protease XIV
	- Lysozyme
	- RNase A

	- RNase T1
	- DNase I
New England Biolabs	- DNA polymerase I
	- T4 DNA ligase
Roche	- Calf intestinal phosphatase
	- Proteinase-K

2.1.3 Stock solutions

β-galactosidase fix	-2% formaldehyde, 0.2% glutaraldehyde, in PBS
β-galactosidase stain	-5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.5 mg ml^{-1} X-Gal, in PBS.
Blot wash	- 2 x SSC, 0.1% SDS.
Boiling mix	- 6% w/v SDS, 30% v/v stacking gel buffer, 30% v/v glycerol, 0.3% w/v bromophenol blue, 210 mM β-mercaptoethanol.
Buffer E	- 100 mM Tris-HCl pH8, 100mM NaCl, 2mM EDTA, 2mM EGTA, 1% NP40, 0.5% Sodium deoxycholate.
Calf thymus DNA (denatured)	- 2 mg ml^{-1} in TE, incubated at 100°C for 30 min.
Calf thymus DNA (transfections)	- 2 mg ml^{-1} in TE.
Chloroform : isoamyl alcohol	- chloroform:isoamyl alcohol 24:1 v/v.
CLB (2x)	- 20 mM Tris-HCl pH7.5, 2 mM EDTA, 1.2% SDS.

Cell Suspesion Buffer	- 10 mM Tris-HCl pH7.2, 50 mM EDTA, 85 mM NaCl.
Denhardt's solution (50x)	- 1% ficoll 400, 1% polyvinylpyrolidone, 1% BSA.
DNase I dilution buffer	- 20 mM Tris-HCl pH7.6, 50 mM NaCl, 1 mM DTT, 50% glycerol, 0.1 mg ml^{-1} BSA.
DNase I reaction buffer	- 1 M NaAc pH5, 5 mM $MgCl_2$.
Gel soak I	- 0.6 M NaCl, 0.2 M NaOH.
Gel soak II	- 0.6 M NaCl, 1.0 M Tris-HCl pH8.0
HeBS	- 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM D-glucose, 21 mM Hepes, to pH6.95 – 7.15 with NaOH.
Hybridisation mix	- 6x SSC, 5x Denhardt's solution, 0.05% SDS, 50 μg ml^{-1} denatured CT-DNA, 20 mM Tris-HCl pH7.5, 1 mM EDTA.
Loading buffer dyes	- 50% w/v sucrose, 0.25% bromophenol blue, in 5x running buffer.
Loening's buffer	- 40 mM Na_2PO_4, 36 mM Tris-HCl, 1 mM EDTA.
Methyl cellulose	- 3% methyl cellulose sodium salt in H_2O.
Nick translation buffer	- 0.5 M Tris-HCl pH7.5, 0.1 M $MgCl_2$, 10 mM DTT, 0.5 mg ml^{-1} BSA.
PFGE cell suspension buffer	-10 mM Tri-HCl pH7.2, 20 mM NaCl, 50 mM EDTA.
PFGE WASH (1x)	- 20 mM Tris-HCl pH8.0, 50 mM EDTA.
Phosphate Buffered Saline	- 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 $MgCl_2$.

Pre-hybridisation buffer	- 6x SSC, 5x Denhardt's solution, 0.1% SDS, 20 μg ml^{-1} denatured CT-DNA.
Proteinase-K reaction buffer	- 100 mM EDTA, 0.2% sodium deoxycholate, 1% sodium lauryl sarcosine, 1 mg ml^{-1} Proteinase K.
Resolving gel buffer (4x)	- 1.5 M Tris-HCl pH 8.9, 0.4% w/v SDS.
Reticulocyte standard buffer	- 10 mM Tris-HCl pH7.5, 10 mM KCl, 1.5 mM $MgCl_2$.
Sample buffer	- 33% v/v boiling mix.
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.
Solution I	- 50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA.
Solution II	- 0.2 M NaOH, 1% SDS.
Solution III	- 5 M potassium acetate, 11.5 ml glacial acetic acid, up to 100 ml with H_2O.
SSC (20x)	- 3 M NaCl, 0.3 M tri-sodium citrate.
Stacking gel buffer (4x)	- 488 mM Tris-HCl pH 6.8, 0.4% w/v SDS.
Sucrose reagent	- 0.25 M sucrose, 2 mM $MgCl_2$, 50 mM Tris-HCl pH8.0.
Tank buffer	- 52 mM Tris, 53 mM glycine, 0.1% w/v SDS.
TBE	- 90 mM Tris, 89 mM boric acid, 1 mM EDTA.
TE	- 10 mM Tris-HCl pH7.5 or pH8.0, 1 mM EDTA.
Transfer buffer	- (25 mM Tris, 192 mM glycine, 20% v/v methanol) pH8.3.

Triton reagent	- 0.5% Triton X-100, 62.5 mM EDTA, 50 mM Tris-HCl pH8.0.
Trypsin	- 0.25% trypsin in Tris-saline.
Versene	- 0.6 μM EDTA, 0.02% phenol red in PBS.

2.1.4 Radiochemicals

α–^{32}P deoxyribonucleoside triphosphates (dNTPs) were obtained from Perkin Elmer at 3000 Ci mMole^{-1} (10 μCi μl^{-1})

2.1.5 Cells and culture media

The following cell lines were used throughout this work, from recovery to passage 30:

BHK-21 C13	- Syrian hamster kidney fibroblasts
Vero	- African green monkey kidney fibroblasts
S22	- Modified Vero cell line (Carmichael *et al.*, 1988)
FG293	- human embryonal kidney cells transformed with sheared Ad5 DNA
UL19-1	- rabbit skin cell line containing HSV-1 UL19 gene under control of the HSV-1 ribonucleotide reductase large subunit promoter (V. Preston, unpublished)
Spodoptera frugipreda (S.f.)	- strain IPLB-SF-21 (Kitts *et al.*, 1990)

The above cell lines were grown and maintained in:

EC5 (Vero and S22)	-Dulbecco's Modified Eagles Medium (DMEM), 5% Foetal Calf Serum (FCS), 1% Penicillin / Streptomycin.
ETC10 (BHK)	-BHK 21 1x medium, 10% New Born Calf Serum (NBCS), 7% tryptose phosphate broth, 1% penicillin / streptomycin.
FG293 medium	- DMEM, 10% FCS, 1% non-essential amino acids, 1% 100 x glutamine, 1% penicillin / streptomycin.
UL19-1 medium	- DMEM, 10% FCS, 2% 100 x glutamine, 1% penicillin / streptomycin.
TC100 (*Sf*)	- TC100 medium, 5% Foetal Calf Serum, 1% Penicillin / Streptomycin.
WASH	-DMEM, 1% penicillin / streptomycin.
Vero cell overlay	-200ml methyl cellulose, 140ml Eagles A, 40ml Eagles B (with phenol red), 5% FCS, 1% penicillin / streptomycin.

2.1.6 Viruses

The following herpesviruses were used during this work:

HSV-1 strain 17 syn^+ - McGeoch *et al.* (1988)

*amb*UL12 - Patel *et al.* (1996)

*amb*UL12R - Patel *et al.* (1996)

K5ΔZ - Desai *et al.* (1993)

The following baculoviruses, expressing the indicated HSV-1 gene, were used in co-immunoprecipitation studies:

AcUL6 - Patel *et al.* (1996)

AcUL12 - N.Stow (unpublished)

AcUL15-pp65 - Abbotts *et al.* (2000)

AcUL25 - Targett-Adams (2001)

AcUL28 - Abbotts *et al.* (2000)

AcUL29 - Stow (1992)

2.1.7 Bacterial Strains

Commercially available competent DH5 (Life Technologies) or SoloPack Gold (Stratagene) *E. coli* were used for the growth and maintenance of all plasmids used in this study.

2.1.8 Plasmids

The following plasmids were used during transient replication and packaging assays:

pS1 - contains a 540bp fragment from HSV-1 strain 17syn$^+$ which spans the Ori_S region inserted into the BamHI site of pAT153 (Stow and McMonagle, 1983).

pSA1 - pS1 containing the (U_c–DR1–U_b) fragment of two tandemly repeated 'a' sequences (Hodge and Stow, 2001).

pY1 - pS1 including a 1762 bp *Hin*fI fragment from the L-S junction of HSV-1 strain 17 syn+ containing a single 'a' sequence (Stow *et al.*, 1983).

pZ1 - pS1 including a 2161 bp *Hin*fI fragment from the L-S junction of HSV-1 strain 17 syn+ containing two tandemly repeated 'a' sequence (Stow *et al.*, 1983).

pPH11 - As pSA1 except for a *pac*1 GC element deletion (Hodge and Stow, 2001).

pPH12 - As pSA1 except for a *pac*1 GC element substitution (Hodge and Stow, 2001).

pPH21 - As pSA1 except for a *pac*1 proximal GC element deletion (Hodge and Stow, 2001).

pPH22 - As pSA1 except for a *pac*1 proximal GC element substitution (Hodge and Stow, 2001).

Plasmids expressing the seven essential HSV-1 encoded replication proteins were provided by N. Stow, Institute of Virology, Glasgow. The backbone for each of these vectors is the pCMV10 plasmid which contains the HCMV immediate early promoter upstream of the MCS (Stow *et al.*, 1993):

pE5 - UL5, helicase.

pE8 -UL8, helicase-primase accessory factor.

pE9 -UL9, origin binding protein.

pE29 -UL29, major DNA binding protein.

pE30 -UL30, viral DNA polymerase.

pE42 -UL42, polymerase accessory factor.

pE52 -UL52, primase.

The following plasmids were ^{32}P-labeled to produce probes for use during Southern blotting:

pAT153	- high copy number plasmid derived from pBR322 (Twigg and Sherratt, 1980)
pGX153	- pAT153 containing the HSV-1 *Bam*HI P fragment.
pBE1	- contains a fragment from the TR_L/IR_L region of HSV-1 (Stow, 2001; Figure 14).
pBN1	- contains a fragment from the TR_S/IR_S region of HSV-1 (Stow, 2001; Figure 14).
pST17	- contains a fragment from the TR_S/IR_S region of HSV-1 (Stow, 2001; Figure 14).

2.1.9 Antibodies

Q1	- monoclonal antibody against UL12 (Banks *et al.*, 1983)
BwP12	- polyclonal antibody against UL12 and UL12.5 (Bronstein and Weber, 1996)
MAb 7381	- monoclonal antibody against UL29 (unpublished, Anne Cross, Institute of Virology, Glasgow).
Anti-UL25	- monoclonal antibody against UL25 (Targett-Adams, 2001).
Anti-pp65	- monoclonal antibody against HCMV pp65 epitope tag (purchased from Capricorn Products).

YE583 - polyclonal antibody against UL6 (Patel *et al.*, 1996).

2.2 Methods

2.2.1 Tissue Culture

Cell lines were grown and passaged in 175 cm^2 tissue culture flasks containing the appropriate media. Confluent monolayers were washed in versene and trypsinised with a 1:4 dilution of trypsin:versene. Cells were then resuspended in appropriate media to a final volume of 10 ml. 175 cm^2 flasks containing fresh media were seeded with $6x10^6$ cells and incubated until confluent in a 5% CO_2 environment at 37°C.

The complementing cell lines, S22 and UL19-1, had 300 μg ml^{-1} G418 added every third passage after recovery.

S.f. cells were grown and passaged in 175 cm^2 tissue culture flasks containing supplemented TC100 media. Confluent monolayers were washed in TC100 and removed from the flask by gentle agitation. The cells were then resuspended in 10 ml supplemented TC100 and seeded into fresh flasks. Cells were grown and maintained at 28°C.

2.2.2 Preparation of Virus Stocks

Five 175cm^2 tissue culture flasks containing Vero and S22 cells at 90% confluency were infected with wt-HSV1 and *amb*UL12 respectively at 0.01 p.f.u./ml. Three days post infection the infected cells were gently tapped into the media, the supernatant

pooled and centrifuged at 2000 rpm for 5 min.

Cell released virus (CRV) was prepared by centrifuging the supernatant at 12000 rpm for 3 h. The resulting pellet was resuspended in 5 ml WASH, sonicated extensively, and stored in 1 ml aliquots at –70°C. Cell associated virus (CAV) was prepared by resuspending the pellet in 5 ml WASH, and sonicating the suspension extensively. The cellular debris pelleted by centrifugation at 2000 rpm for 5 min and the supernatant stored in 1 ml aliquots at –70°C.

2.2.3 Determination of virus stock titres

To determine the titre of virus stocks 10-fold serial dilutions of the stock were made. 35 mm Petri-dishes containing near confluent monolayers of Vero or S22 cells were inoculated with 100 µl of diluted stock virus. The plates were incubated at 37°C, 5% CO_2 for 45 min. 2 ml of Vero overlay was then added and the monolayers incubated at 37°C, 5% CO_2 for three days. The overlay was removed and 1ml of Geimsa stain added for 2 h. The Geimsa stain was then washed off and the number of plaques in each dilution counted. The virus titre was determined by the following calculation:

Titre = number of plaques on 10^{-n} dilution x 10^{n} x10 p.f.u./ml

e.g. 20 plaques on a –5 dilution plate corresponds to 20 x 10^{5} x 10 = 2x10^{7} p.f.u./ml

The average titre of at least two dilutions was used to determine the virus titre.

2.2.4 Agarose Gel Electrophoresis

LB or TBE buffer containing the desired percentage of agarose was boiled and allowed to cool. Once the agarose had cooled to approximately 60°C ethidium bromide was added to 0.5 µg ml^{-1} and the agarose poured into a mould containing a well forming

comb. Once set the gel was placed in an electrophoresis tank containing the appropriate buffer. DNA samples containing 1 x loading dye were added to individual lanes and were generally electrophoresed overnight at a constant voltage of between 10 and 50 V.

2.2.5 General Cloning Strategies

Restriction Enzyme Digests

Twenty micrograms of vector were digested with 50 U of the appropriate restriction enzyme in 100 µl of the recommended 1 x buffer. If further modification of the digested vector was required the reaction was extracted once in an equal volume of phenol, then in an equal volume of chloroform:isoamyl alcohol. The vector was then precipitated in 2.5 volumes of ethanol and resuspended in TE, pH7.5.

Preparation of Phosphatased Vector

To prevent re-circularisation during subsequent ligation reactions the vector backbone was dephosphorylated with calf intestinal phosphatase (CIP). Five micrograms of linearised vector were incubated with 10 U CIP and incubated at 37°C for 3 h. The vector was then phenol /chloroform extracted, ethanol precipitated and resuspended in TE, pH7.5.

Purification of DNA fragments

DNA was digested with the appropriate restriction enzyme to produce the desired DNA fragment, and run out on a 1% TBE agarose gel containing ethidium bromide. The DNA was visualised using long wave UV and the desired fragment excised from the gel and transferred to dialysis tubing containing 4 ml 0.5 x TBE. The DNA was then electroeluted from the agarose at 200 V for 2 h. The buffer containing the DNA was

then added to a DEAE sephacel column and the column washed with 3ml TE containing 0.1 M NaCl. The DNA was then eluted from the column with 1 ml TE containing 1 M NaCl. The DNA was precipitated in 2.5 volumes of ethanol and resuspended in 20 µl TE.

Ligation of DNA fragments

One microgram each of vector backbone and DNA fragment were mixed with 1 unit of T4 DNA ligase in 1 x ligation buffer with a total volume of 20 µl. The reaction mix was incubated overnight at 16°C, phenol/chloroform extracted and ethanol precipitated before transformation into *E. coli*.

2.2.6 Maintenance and Manipulation of Competent *E. coli*

Transformation of competent *E. coli*

Competent DH5 or SoloPack Gold cells were transformed as per the suppliers instructions. Essentially, 50 µl of competent cells were allowed to thaw slowly on ice. DNA from a ligation reaction was diluted five fold in 10 mM Tris-HCl pH7.5, 1 mM Na_2EDTA and between 1 and 10 ng of DNA was added to the cells. The cells were incubated on ice for 30 min, heat shocked at 42°C for 45 sec and then placed on ice for another 2 min. 450 µl of S.O.C. medium was then added and incubated at 225 rpm, 37°C for 1 h. This was diluted as appropriate and 100 µl was plated out on LB-agar plates containing the appropriate antibiotic and incubated overnight at 37°C.

Lysis by alkali (mini-preps) (Sambrook *et al.*, 1989)

Individual colonies from the transformation streak plate were picked and used to inoculate 4 ml universals of L-Broth. The samples were then incubated overnight at

37°C, 220 rpm. 1.5 ml of the culture was spun down in a sterile eppendorf and resuspended in 100 µl ice cold solution I. 200 µl of solution II was added and gently mixed. 150 µl of solution III was then added, the eppendorf gently vortexed in an inverted position, and incubated on ice for 5 min. The sample was then centrifuged at 13000 rpm for 5 min and the supernatant transferred to a clean tube. The sample was phenol / chloroform extracted and ethanol precipitated. The DNA was then resuspended in 100 µl TE containing 1 x RNase A mix and stored at –20°C. The plasmid contained in each transformed cell was then analysed by restriction enzyme digestion and agarose gel electrophoresis.

Storage of *E. coli*

Four millilitres L-broth, containing the appropriate antibiotic, was inoculated with transformed DH5 or SoloPack Gold *E. coli* and grown overnight at 37°C, 220 rpm. 900 µl of the overnight culture was added to 100 µl DMSO and stored at –70°C.

2.2.7 Large Scale Plasmid Preparation

Ten micro litres of bacterial stock containing the appropriate plasmid were added to 4 ml L-broth (LB) containing 50 µg ml^{-1} ampicillin and incubated overnight in a rotary shaker (37°C, 220 rpm). The overnight cultures were then added to 350 ml LB broth containing 50 µg ml^{-1} ampicillin in 2 l flasks and incubated overnight in a rotary shaker at 37°C, 220rpm. The bacteria were pelleted by centrifuging at 6000 rpm for 5 min at 4°C in a Sorvall GS3 rotor. Pellets were resuspended in 8 ml TE, pH8.0 and transferred to Oakridge tubes. Following centrifugation at 5000 rpm for 5 min at 4°C in a Beckman SS34 rotor, the pellets were resuspended in 2 ml sucrose reagent containing 400 µl 20 mg ml^{-1} lysozyme freshly dissolved in TE and incubated on ice for 30 min. 800 µl of

0.25 M EDTA and 3.2 ml Triton reagent were then added and the sample incubated on ice for a further 15 min.

Cell debris was then spun out at 35000 rpm for 30 min at 4°C in a Sorvall T865 rotor. The supernatant was decanted into a fresh tube and the volume adjusted to 7.5 ml using 200 µl 10 mg ml^{-1} ethidium bromide and distilled water. 7.5 g of caesium chloride was added to give a final density of 1.55 – 1.6 g ml^{-1}. This solution was then transferred to a T1270 tube and the remaining volume filled with paraffin oil. The tube was sealed and then centrifuged in a Sorvall T1270 rotor at 44000 rpm, 15°C for at least 36 h. The lower of the two bands in the gradient, containing the supercoiled plasmid DNA, was removed using an 18 gauge needle and 2 ml syringe and the ethidium bromide was removed by at least three extractions with equal volumes of isoamyl alcohol. The final aqueous phase was then dialysed twice, for at least 2 h, in 3 l TE, pH7.5. The DNA was stored at –20°C.

The concentration of the plasmid stock was determined by measuring the absorbance at 260 nm, assuming that an A260 reading of 1.0 corresponds to a dsDNA concentration of 50 µg ml^{-1}. The identity of the plasmid was determined by selective restriction digests.

2.2.8 Calcium phosphate transfection of mammalian cells

Preparation of Calf thymus (CT) carrier DNA

CT DNA was dissolved in TE to approximately 4 mg ml^{-1}. This was incubated with 0.5% SDS and 100 µg ml^{-1} Proteinase-K for 1 h at 55°C. 0.3 M NaCl and 5 mM EDTA

were added, the solution sequentially extracted with phenol / chloroform:isoamyl alcohol and ethanol precipitated. The DNA was resuspended in TE to a final concentration of approximately 2 mg ml^{-1}.

Calcium phosphate transfection

For every 35 mm Petri dish containing a monolayer of the appropriate cell line (2 x 10^6 cells per plate) 12 μg of calf thymus carrier DNA and 1 μg of plasmid DNA was added to 0.5 ml of HeBS (optimised pH) and precipitated with 35 μl 2M $CaCl_2$. The precipitate was added to the monolayers and incubated at 37°C, 5% CO_2 for 45 min. Two millilitres of EC5 was then added to the plate and incubated for at 37°C, 5% CO_2. After 4 h incubation the monolayers were washed and "boosted" by the addition of 1 ml 22.5% DMSO for 4 min (Stow and Wilkie, 1976). The monolayers were then washed twice and incubated with 2 ml EC5 at 37°C, 5% CO_2.

β-Galactosidase staining of pElacZ transfected cells

Monolayers were transfected with pElacZ as described above. Twenty four hours post transfection (h.p.t.) the media was removed and the monolayers washed with 2 ml of PBS. The cells were fixed by incubation with 1 ml of FIX solution for 30 min, then washed with 2 ml PBS and incubated overnight at 37°C with 1 ml β-gal staining solution. Transfection efficiency was estimated by counting the number of positive (blue) cells and averaging the percentage from five fields of view. Typically, 2 – 5% of Vero and S22 cells would be transfected while BHK cells had a transfection rate of 10 – 30%.

2.2.9 Isolation HSV-1 genomic DNA

HSV-1 infected cells were gently removed from the surface of 5 x 175 cm^2 flasks using sterile glass beads. The cells were pelleted by centrifugation at 2000 rpm for 10 min. Extracellular virions were isolated by centrifugation of the supernatant at 12000 rpm for 3 h. The supernatant was removed and the pellet resuspended in 2ml of TE. 40 µl, 0.25M EDTA; 25 µl, 20% SDS; 50 µl 20 mg ml^{-1} proteinase K was added, mixed gently and incubated at 50°C for 30 min. 100 µl 4 M NaCl / 50 mM EDTA was added and the DNA gently phenol / chloroform extracted using cut pipette tips. The aqueous phase was subsequently dialysed against 2 litres TE overnight at 4°C and again for 2 h at room temperature. The samples were then stored at –20°C.

2.2.10 Transient replication and packaging assays

Single Round Transient Transfections

Transfections were performed as described above. Six hours post transfection monolayers were superinfected with helper-virus at 3 p.f.u./cell. The monolayers were incubated at 37°C, 5% CO_2 for 45 min to allow the virus to adsorb then 2 ml of EC5 was added and the monolayers incubated at 37°C, 5% CO_2. The monolayers were generally harvested 16 h.p.i. and DNA prepared as described below.

Serial Propagation of Amplicons

Monolayers were transfected and superinfected as described above. 20 h.p.i. the supernatant was removed and extensively sonicated. Pairs of fresh monolayers were inoculated with 0.5 ml of supernatant together with 6 x 10^6 p.f.u. of wt HSV-1 and incubated for 1 h at 37°C, 5% CO_2. The inoculum was removed and residual virus was

inactivated by washing with 2 ml 0.14 M NaCl and exposing to 0.1 M glycine, 0.14 M NaCl pH 3.0. After 1 min the cells were washed once with WASH, once with EC5, then incubated for 16 h at 37°C, 5% CO_2 in 2 ml EC5 before being harvested and the DNA prepared as described below. One of each pair of monolayers had PAA added to a final concentration of 200 μg ml^{-1} to prevent replication of the propagated amplicon.

Preparation of total cellular DNA

16 h.p.i. media was removed from transfected / superinfected monolayers, the monolayers washed with 2 ml TS, scraped into 1 ml TS and centrifuged for 4 min at 3000 rpm. The cell pellets were then resuspended in 184 μl RSB / 0.5% NP40 and incubated on ice for 10 min. 184 μl of 2 x CLB, 0.5 mg ml^{-1} protease was then added and the samples incubated for 2 h at 37°C. Samples were made up to 400 μl with distilled H_2O, 32 μl of 4 M NaCl/0.5 M EDTA was added, and sequentially extracted with phenol / chloroform : isoamyl alcohol, ethanol precipitated and resuspended in 100 μl TE containing 1 x RNase A mix.

Preparation of DNase resistant DNA

Harvested cells were divided into two aliquots. One aliquot was processed for total DNA as described above. The other aliquot had 200 μg ml^{-1} DNase I added to the RSB / 0.5% NP40 mix. After incubation at 37°C for 15 min the samples were treated as for total DNA except the samples were resuspended in 50 μl TE / RNase A mix.

Nuclear and Cytoplasmic fractionation

Harvested cells were resuspended in 184 μl RSB / 0.5% NP40 and incubated on ice for 10 min. The sample were then centrifuged at 3000 rpm for 2 min to pellet the nuclei.

The supernatant, containing the cytoplasmic fraction, was transferred to a fresh eppendorf and the nuclei resuspended in a further 184 µl of RSB / 0.5% NP40. The samples were then processed for total or DNase resistant DNA as describe above.

2.2.11 Southern Blotting (Southern, 1975)

DNA samples representing the yield from 6.5 x 10^5 cells were digested with a suitable restriction enzyme in a 30 µl reaction. The reaction was stopped by the addition of 5 µl 5 x loading dye and the samples loaded on a large 0.6 - 0.8% LB agarose gel containing 0.5 mg ml^{-1} ethidium bromide and electrophoresed overnight at 15 – 30 V. A photograph of the gel was taken under long wave UV to confirm digestion had occurred and that all the lanes contained equivalent amounts of DNA.

The gel was soaked in Gel Soak I for 45 min to denature the DNA. This was followed by shaking in Gel Soak II for 45 min to neutralise the alkali. The DNA was blotted onto a nitrocellulose membrane by capillary transfer in 6 x SSC for 16 h at room temperature. Crosslinking the DNA to the membrane was achieved by exposure to 120 mJ / cm UV light. The membrane was blocked by incubation in pre-hybridisation buffer for at least 2 h at 68°C. Specific bands were detected by incubating the membrane overnight at 68°C in 10 ml hybridisation buffer containing suitably nick-translated DNA. Following 3 x 30 min washes in blotwash the membrane was exposed to a phosphorimager screen and analysed on a BioRad Personal FX FluorS MultiImager.

Preparation of radio-labelled DNA probes by nick-translation (Rigby *et al.*, 1977)

300 ng of DNA was incubated with 15 μCi each of α^{32} P dCTP and α^{32} P dGTP, 2 units of DNA polymerase I and $1x10^{-7}$ mg DNase I at 16°C for 90 min in a final volume of 30 μl of 1x NTB (containing 2 μl each of 1 mM 'cold' dATP and dTTP).

The reaction mix was made up to 100 μl with distilled H_2O and extracted once with an equal volume of phenol. The aqueous layer was then fractionated though a G50 Sephadex chromatography column to separate the radio-labelled probe from the unincorporated nucleotides. The fractions containing the probe were pooled and the volume made up to 1 ml with distilled H_2O and the DNA denatured with 0.17 M NaOH. After 10 min the solution was neutralised by the addition of 0.17 M HCl. The probe was then added to 8.6 ml hybridisation buffer and used immediately.

2.2.12 Western blotting

35 mm Petri dishes of near confluent BHK21 cells were transfected or infected as described previously. 24 h.p.t. or 16 h.p.i. the cells were harvested and resuspended in 100 μl boiling mix. The samples were then placed in a boiling water bath for 10 min and the proteins separated by SDS-PAGE. 10 μl of sample was run on an 8% running gel (39:1 acrylamide to bisacrylamide) and a 5% stacking gel (19:1 acrylamide to bisacrylamide) at 150 V using a BioRad 'Mini-protean' kit. Using a BioRad transfer kit gels were blotted onto ECL compatible nitrocellulose (Amershan Pharmacia) for 16 h at 4°C, 25 V in 1 x transfer buffer. Blotting was confirmed by the transfer of Rainbow marker onto the membrane.

The membrane was blocked in PBS, 0.05% Tween (PBST), 5% Marvel for 1 h. The primary antibody was diluted 1:2500 in PBST, 5% Marvel. After 1 h incubation the blot had 3 x 5 min washes in PBST, 5% Marvel, before being incubated in a 1:1000 dilution of the secondary antibody (HRP conjugated protein A sepharose) for 30 min. Following 3 x 5 min washes in PBST and a final wash in PBS specific bands were detected by the addition of 1 ml ECL mix, for 1 min, and exposed to auto-radiographic film.

2.2.13 Pulsed Field Gel Electrophoresis

Preparation of DNase resistant DNA for PFGE

35 mm Petri dishes of cells were scraped into 1 ml TS and centrifuged at 3000 rpm for 4 min, the pellet resuspended in 184 µl RSB, 0.5% NP40, 0.1 mg ml^{-1} DNase I and digested at 37°C for 15 min. The reaction was terminated by the addition of a one-third volume of 4 x CLB and gel loading buffer. The sample was gently mixed and a volume representing 6.5 x 10^5 cells was loaded directly onto a 1% PFGE grade agarose, 0.5 x TBE gel.

Preparation of agarose embedded cell suspensions

2 x 10^6 BHK21 or Vero cells were transiently transfected or infected as described above. 24 h.p.t. or 16 h.p.i. cells were harvested and resuspended in 50 µl cell suspension buffer and equilibrated to 50°C in a water bath. 50 µl of BioRad 2% CleanCut Agarose was then added, mixed, and the cell suspension added to BioRad plug moulds. The suspension was then placed at 4°C to allow the agarose blocks to set.

Preparation of total cellular DNA from agarose blocks

Agarose blocks were incubated overnight at 55°C in 0.5 ml Proteinase K reaction buffer containing 1 mg ml^{-1} Proteinase K. Blocks then had 4 x 30 min washes in 0.5 ml 1 x PFGE WASH and stored in 0.5 ml 1 x PFGE WASH. If the blocks were to be digested by restriction enzymes at a later stage the second wash contained 1 mM PMSF.

Preparation of DNase resistant DNA in agarose blocks

The agarose embedded cells were incubated at 37°C for 2 h in 0.5 ml RSB / 0.01% NP40 followed by a 30 min incubation in 0.5 ml DNase reaction buffer. This was then replaced with fresh DNase reaction buffer containing 0.1 mg ml^{-1} DNase I. After 2 h incubation the blocks were incubated in 0.5 ml 0.5 M EDTA for 30 min followed by 30 min in 0.5 ml Proteinase K reaction buffer. The buffer was replaced with fresh Proteinase K reaction buffer containing 1 mg ml^{-1} Proteinase K and placed on ice for 30 min before being incubated overnight at 55°C. The blocks were then washed as described in the preparation of total DNA.

Restriction enzyme digestion in agarose blocks

Agarose blocks containing total or DNase resistant DNA, prepared as described above, were washed twice in 0.1 x PFGE WASH to reduce the EDTA concentration. One third of each block was incubated in 200 µl of the appropriate 1 x restriction buffer at 37°C for 30 min. The blocks were then digested for 4 – 16 h in 200 µl fresh buffer containing 40 U of restriction enzyme. The blocks were washed twice in 0.5ml PFGE WASH before electrophoresis.

Electrophoresis and Southern blotting of PFGE samples

One gram of PFGE grade agarose was melted in 100 ml 0.5 x TBE, allowed to cool to 80°C and poured into the CHEF-DR II casting mould. One third of an agarose block was placed in each well of the gel ensuring there were no air bubbles. The wells were then sealed by filling with 1% low melting point agar. The gel was then removed from the casting stand and placed in the CHEF-DR II electrophoresis tank containing 2 l of 0.5 x TBE pre-cooled to 14°C. DNA was generally separated using a run parameter of 6 V/cm for 16 h at 14°C with a ramped switch time of 1 – 15 sec. After electrophoresis the gel was stained for 20 min in distilled water containing 0.5 mg ml^{-1} ethidium bromide before being photographed.

For Southern blotting the gel was soaked in 0.25 M HCl for 15 min, neutralised in Gel soak I for 15 min then treated as described above.

2.2.14 Co-immunoprecipitation

Each well of a 12 well plate was seeded with 6 x 10^5 *S.f.* cells in 1 ml of TC100 complete medium and incubated overnight at 28°C. The cells were then single, double or triply infected with 5 p.f.u. / cell of the appropriate recombinant baculoviruses. 24 h.p.i. the medium was replaced with 500 µl met^- TC100 containing 30 µCi ^{35}S-L-Met. After incubation for 16 h at 28°C the cells were scraped into the media and centrifuged at 6000 rpm for 1 min. The supernatant was removed and the cell pellet washed twice in ice cold, unsupplemented TC100. The pellets were resuspended in 150 µl of Buffer E with 1 µl of 0.5 M PMSF added freshly to the buffer. The sample was then incubated on ice for 20 min with occasional vortexing. Before proceeding a 15 µl sample was removed to represent the total protein fraction.

The samples were centrifuged at 35000 rpm for 20 min in a benchtop ultracentrifuge pre-chilled to 4°C. The supernatant was removed and the pellet discarded. 15 µl of the supernatant was removed to represent the soluble protein fraction. A predetermined amount of the appropriate antibody was added to the remainder of the sample and the samples incubated on an end-over shaker for 2 h at 4°C. 50 µl of a 50% (v/v) Protein A / Buffer E solution was added to the sample and incubated for a further 60 min at 4°C. The protein A and any bound protein was pelleted by centrifugation at 4°C and washed in Buffer E for 3 min, while kept on ice. This washing procedure was repeated three times. Prior to the final wash the pellet was transferred to a fresh tube.

The pellet was then resuspended in 30 µl sample buffer and incubated at 100°C for 5 min. The Protein A was pelleted and 15 µl of the sample was loaded onto an 8% SDS-PAGE gel along with the total and soluble protein fractions. The gel was subsequently dried and exposed to a phosphorimager screen and analysed using the BioRad Personal FX FluorS MultiImager.

Chapter 3: Characterisation of the HSV-1 mutant, *amb*UL12

3.1 Comparison of wt, *amb*UL12 and *amb*UL12R

3.1.1 Introduction

Several HSV-1 mutants with lesions in the UL12 gene, which fail to produce a functional alkaline nuclease, have been constructed (Weller *et al.*, 1990; Martinez *et al.*, 1996; Gao *et al.*, 1998). These UL12 null mutants (*nuc* mutants) are reported to synthesise near wt levels of viral DNA but produce mature, infectious progeny inefficiently, yielding only 0.1 – 1% as much viable virus as wt HSV-1 (Shao *et al.*, 1993).

The HSV-1 strain 17 syn^+ UL12 null mutant, *amb*UL12, which contains a stop codon after amino acid 129 (downstream of both the UL12 and UL12.5 ATG codon), and was used throughout these studies, was reported to have similar properties (Patel *et al.*, 1996). In this section the phenotypes described above were confirmed for the *amb*UL12 virus and the characterisation of the mutant extended. A phenotypically wt virus, *amb*UL12R, generated by marker rescue of *amb*UL12 with a fragment containing only the UL12 gene (Patel *et al.*, 1996) was compared to demonstrate that the amber nonsense mutation introduced into *amb*UL12 is responsible for the altered properties of the mutant virus.

3.1.2 Single step growth curves of wt HSV-1, *amb*UL12 and *amb*UL12R

Single step growth curves were used to compare the ability of *amb*UL12, the marker rescued virus, *amb*UL12R, and wt HSV-1 to produce viable virus over a single round of infection.

Stocks of *amb*UL12 were prepared and titrated on S22 cells, a Vero derived cell line containing the HSV-1 *Eco*RI D fragment (which encodes the complete ORFs of UL8 – UL14) that is permissive for the propagation of *amb*UL12 (Carmichael *et al.*, 1988; Carmichael and Weller, 1989; Patel *et al.*, 1996). Replicate 35 mm plates of S22, BHK and Vero cells were infected with 3 p.f.u. / cell of each of the viruses. At various times infected cells were scraped into the media and sonicated to lyse the cells. The samples were titrated on S22 cells and the yield of progeny virus for each sample established (Figure 1).

Throughout the time course, wt HSV-1 and *amb*UL12R produced similar titres of virus in each of the three cell lines. However, in BHK and Vero cells the growth of *amb*UL12 was clearly impaired. By 24 h.p.i. the titre was reduced approximately 700-fold in Vero cells and 300-fold in BHK cells compared to wt and *amb*UL12R. The reduced yield of the UL12 null mutant in Vero cells is similar to that previously reported (Weller *et al.*, 1990; Shao *et al.*, 1993 and Patel *et al.*, 1996). The experiment shown in Figure 1 demonstrates a comparable impairment also occurs in BHK cells. Moreover, the plaques produced in *amb*UL12 infected BHK and Vero cells were very small and easily distinguishable from the plaques produced by wt HSV-1.

The Vero derived S22 cell line partially complements the *amb*UL12 defect but the yield of *amb*UL12 in this cell line was still reduced 6 – 8 fold compared to wt HSV-1 and *amb*UL12R (Figure 1; Patel *et al.*, 1996). The failure of *amb*UL12 to grow to wt titres in S22 cells may result from the poor expression of alkaline nuclease in these cells. In S22 cells the UL12 ORF is under the control of its own promoter and should be induced

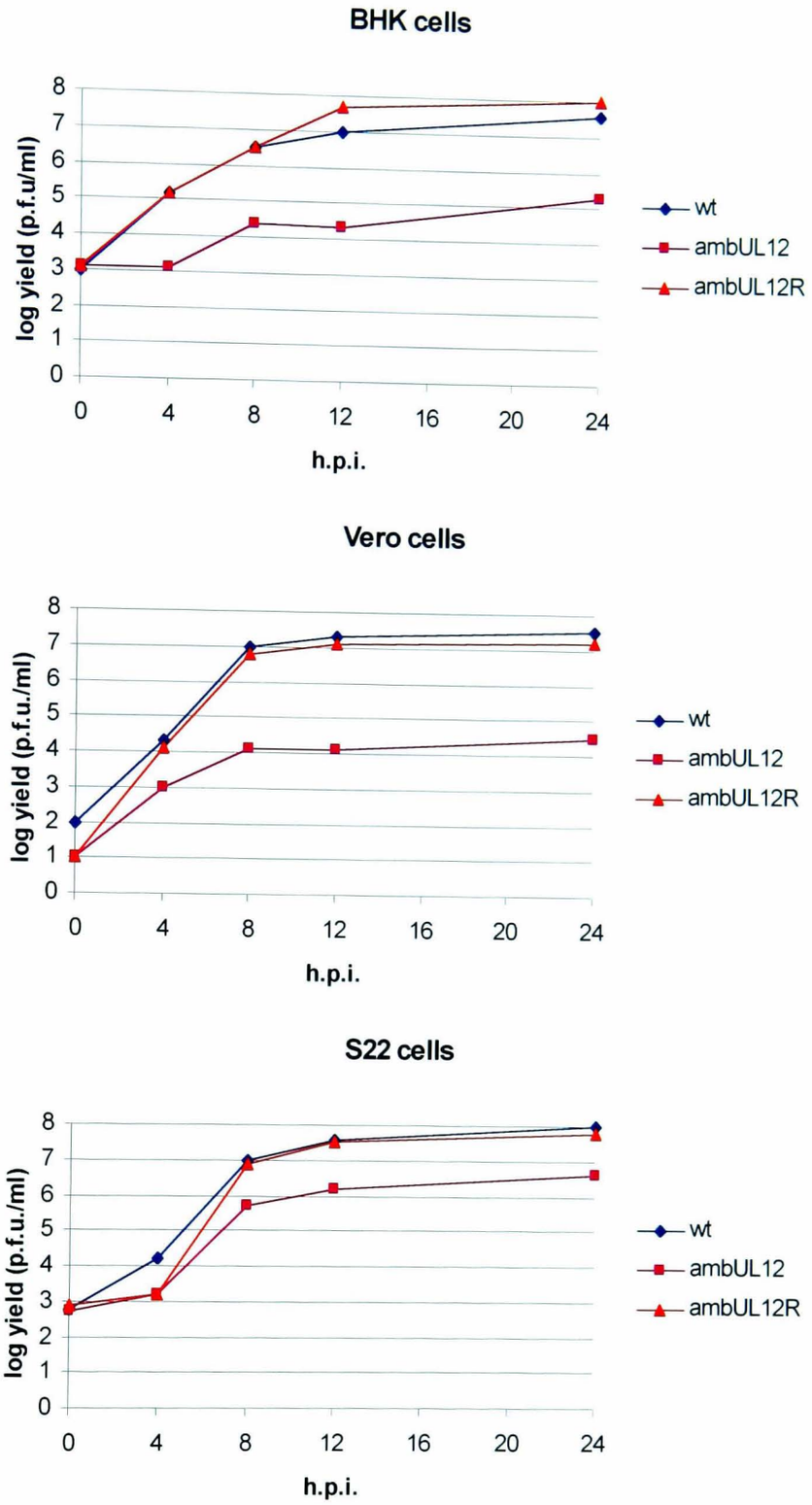


Figure 1 – One step growth curves of wt HSV-1, *amb*UL12 and *amb*UL12R.

35mm plates of BHK, Vero or S22 cells were infected at 3 p.f.u / cell. At the time points indicated the cells were scraped into the media, sonicated and subsequently titrated on 35mm plates of S22 cells. 72 h.p.i. the infected monolayers were stained with Giemsa stain and the plaques counted.

upon infection with *amb*UL12. However, western blots of *amb*UL12 infected S22 cells show that expression of UL12 is either undetectable (Weller *et al.*, 1990) or is significantly reduced compared to wt (Patel *et al.*, 1996). A second complementing cell line (6-5 cells) derived from Vero cells containing only the UL12 ORF was developed (Shao *et al.*, 1993). Upon infection with a *nuc* mutant 6-5 cells were shown to express 30-fold higher levels of the nuclease than S22 cells. Despite the increased expression of the nuclease, complementation of the *nuc* mutant was only slightly better than that observed in S22 cells. Two possible reasons were suggested for the incomplete complementation in the 6-5 cells: firstly, the nuclease requires to be expressed at some threshold level not yet reached in these cells and secondly, the temporal and spatial expression of the nuclease may be altered. Although complete complementation is not seen with either cell line they still enable sufficient virus to be obtained for experimental purposes.

3.1.3 Expression of the UL12 product in wt HSV-1, *amb*UL12 and *amb*UL12R infected cells

To confirm that *amb*UL12 failed to synthesise the alkaline nuclease protein and that synthesis had been restored in *amb*UL12R a western blot of infected BHK cells was performed. 35 mm plates of BHK cells infected with 3 p.f.u. / cell were harvested 16 h.p.i. and lysates from one fifth of a plate were separated on an 8% SDS-PAGE gel. The UL12 protein was detected on the blotted gel using monoclonal antibody Q1, which reportedly recognises an epitope within the first 126 a.a. of UL12 (Costa *et al.*, 1983; Patel *et al.*, 1996) and polyclonal antibody BwP12 (Bronstein and Weber, 1996).

As shown in Figure 2, alkaline nuclease was readily detected in both the wt HSV-1 and *amb*UL12R samples using either antibody. In addition to the 85 kDa band corresponding to the full length protein (Banks *et al.*, 1983) there are a number of other major bands, also reported by Patel *et* al. (1996) and Gao *et al.* (1998), which most likely represent breakdown products of the protein. In contrast no bands could be detected in the *amb*UL12 samples.

3.1.4 Levels of DNA replication and packaging in wt HSV-1, *amb*UL12 and *amb*UL12R infected cells

Previous studies of alkaline nuclease mutants indicated that the levels of viral DNA synthesis were similar to wt (Weller *et al.*, 1990) or slightly reduced (Shao *et al.*, 1993; Patel *et al.*, 1996) and that encapsidation of viral DNA was unaffected (Shao *et al.*, 1993). The amount of viral DNA synthesised and packaged by wt, *amb*UL12 and *amb*UL12R in BHK and Vero cells was analysed by Southern blotting.

Vero and BHK monolayers were infected with 3 p.f.u. / cell wt HSV-1, *amb*UL12 or *amb*UL12R and harvested 16 h.p.i.. Whole cell extracts (Figure 3A) or separate nuclear and cytoplasmic fractions (Figure 3B) were prepared and processed for total and DNase I resistant DNA. Samples were cleaved with *Bam*HI and Southern blots hybridised to a ^{32}P labelled pGX153 probe, which detects the HSV-1 *Bam*HI P fragment located in U_L. The membranes were exposed to a phosphorimager screen and an image captured using a BioRad Personal Molecular Imager. The radioactivity present in the bands was quantified using the band analysis function of the Quantity One software supplied by BioRad.

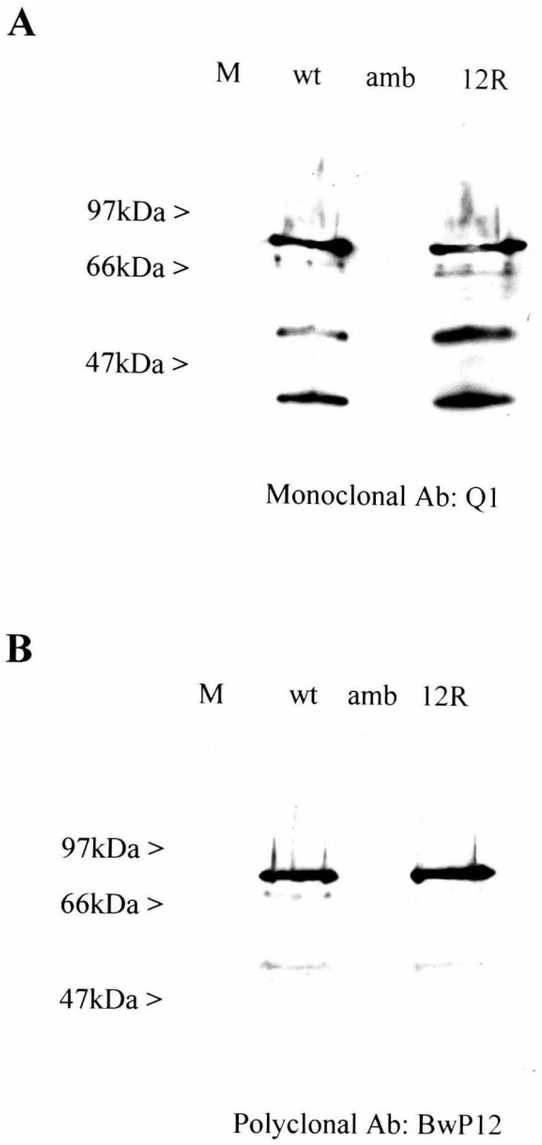


Figure 2 – Western blots of infected BHK cells.

Monolayers of BHK cells were infected with 3 p.f.u. / cell wt HSV-1(wt), *amb*UL12 (amb) and *amb*UL12R (12R) or mock infected (M). Samples were harvested 16 h.p.i. and resuspended in 50 µl of boiling mix. After incubating at 100°C for 10 min, duplicate 10 µl samples were separated on 8% SDS-PAGE gels. The gels were blotted and the membranes reacted with **(A)** monoclonal Ab: Q1 or, **(B)** polyclonal Ab: BwP12. Protein A conjugated HRP was used to detect bound antibody, and in turn was detected by ECL. The blot was exposed to autoradiographic film. The positions of molecular weight markers (Rainbow markers) are shown.

Compared to wt HSV-1 and *amb*UL12R the level of DNA synthesis in the *amb*UL12 samples was reduced in both cell lines by approximately 2 to 3-fold (Figure 3a, upper panel and Figure 3b). This result is in broad agreement with previous results that show up to a 50% reduction in DNA synthesis for the AN-1 mutant in Vero cells (Shao *et al.*, 1993). The amount of DNA packaged in *amb*UL12 infected cells was reduced approximately 7-fold and 13-fold in Vero and BHK cells, respectively, when compared to wt HSV-1 and *amb*UL12R. This contrasts with published results which suggest that packaging of viral DNA is unaffected in the *nuc* mutants and the main block is in the egress of capsids from the nucleus into the cytoplasm (Shao *et al.*,1993). Figure 3B does demonstrate that DNase-resistant *amb*UL12 DNA was detected in the cytoplasm in lower amounts than wt HSV-1 or *amb*UL12R but it cannot be concluded whether this is due to an additional defect in the nuclear egress processes, or is merely a reflection of reduced packaging in the nucleus.

3.2 Further examination of replication and packaging in wt and *amb*UL12 infected cells

3.2.1 Introduction

The above experiments established that the amber nonsense mutation in *amb*UL12 results in inefficient growth of the virus, due at least in part to a defect in the ability of the virus to package viral DNA. In addition, marker rescue can restore the virus to a wt phenotype. To quantify the effect of the mutation in greater detail replication and packaging of *amb*UL12 and wt HSV-1 were compared in repeat experiments (at least 3) in BHK and Vero cells. In addition FG293 cells were also used.

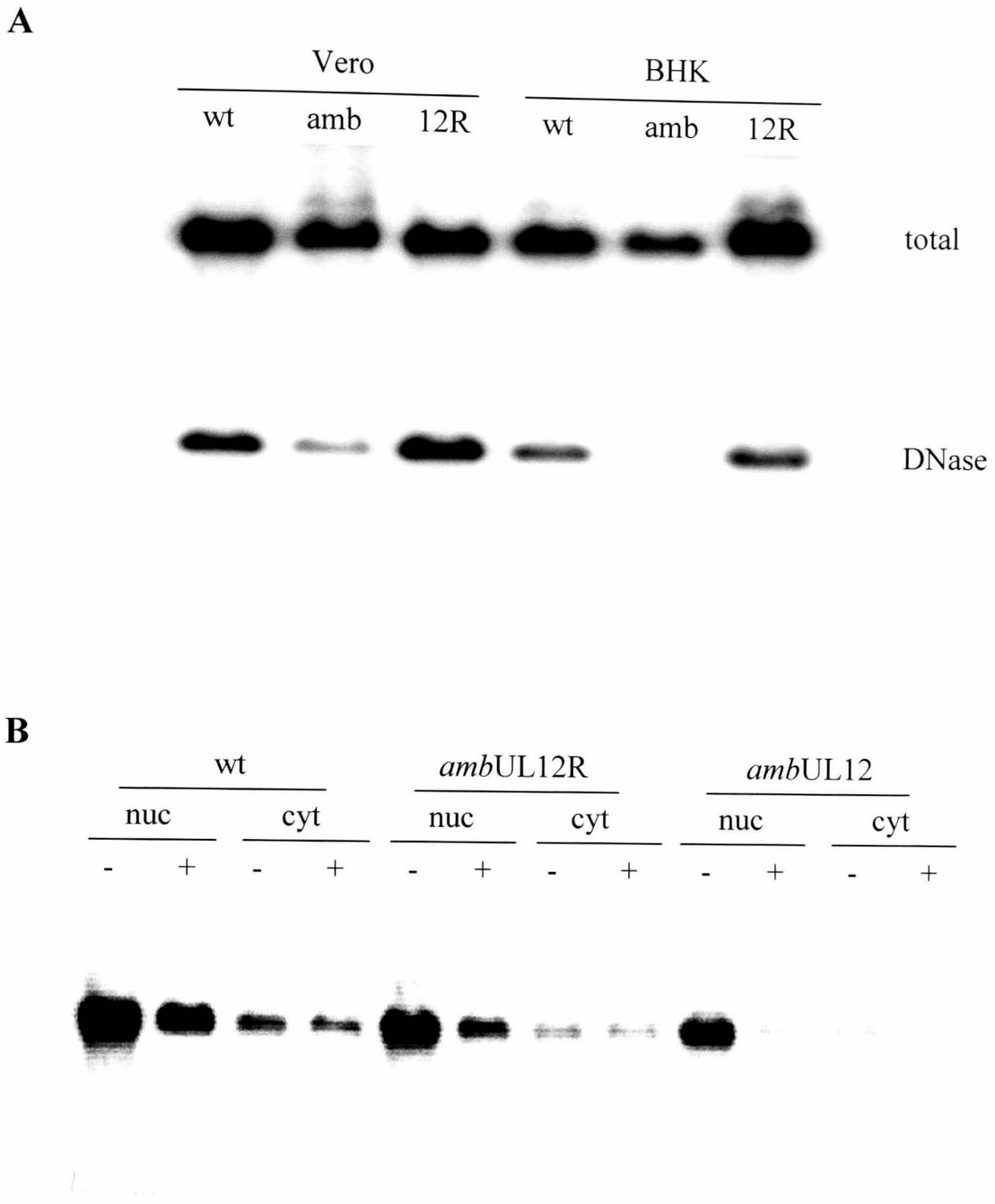


Figure 3 – Replication and packaging of wt, *amb*UL12 and *amb*UL12R.

(A) 35 mm plates of Vero and BHK cells were infected with wt, *amb*UL12 (amb) and *amb*UL12R (12R) at 3 p.f.u / cell. Cells were harvested 16 h.p.i. and processed for total DNA (upper panel) and DNase resistant DNA (lower panel). **(B)** 35 mm plates of BHK cells were infected with wt, ambUL12 and *amb*UL12R at 3 p.f.u / cell. Cells were harvested 16 h.p.i., split into nuclear (nuc) and cytoplasmic (cyt) fractions and processed for total (-) and DNase resistant DNA (+). Samples representing 1/6 of a plate (A) or 1/12 of a plate (B) were digested with *Bam*HI, separated on a 0.8% LB-agarose gel and Southern blotted. The membranes were probed with ^{32}P-labelled plasmid pGX153, exposed to a phosphorimager screen and analysed using a BioRad Personal Molecular Imager.

3.2.2 Effect of cell line on replication and packaging of *amb*UL12

S22, Vero, FG293 and BHK monolayers were infected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. Nuclear and cytoplasmic fractions were prepared 16 h.p.i. and processed for total or DNase resistant DNA. Samples representing 1/12 of a plate were digested with *Bam*HI, Southern blotted and probed with pGX153. The panels in Figure 4 represent typical results for each of the four cell lines, and quantitative data were obtained from the nuclear fraction of at least three separate experiments (Table 1). The data is presented as the counts in the wt bands divided by the counts in the corresponding *amb*UL12 bands (± SD) for total nuclear and DNase resistant nuclear DNA.

Table 1. Decrease in replication and packaging of *amb*UL12 compared to wt.

	Total nuclear DNA	DNase resistant DNA
Cell line	wt/*amb*UL12[c]	wt/*amb*UL12[c]
S22[a]	1.66 ± 0.47	6.1 ± 2.17
Vero[a]	2.83 ± 2.34	16.5 ± 8.64
BHK[b]	3.20 ± 1.92	18.85 ± 10.96
FG293[a]	1.58 ± 0.52	15.45 ± 8.76

[a] n = 4 for S22, Vero n = 3 for FG293 cells, where n = number of individual experiments.

[b] n = 11 for replicated DNA and n = 15 for packaged DNA

[c] Replication and packaging efficiency calculated as the ratio of wt counts ÷ *amb*UL12 counts ± SD.

From these results it can be seen that viral DNA synthesis was reduced to a small extent in each cell line, with a reduction of less than 2-fold in S22 and FG293 cells and approximately a 3-fold reduction in Vero and BHK cells. Clearly a more significant defect in *amb*UL12 is in the packaging of DNA. Again, the S22 cell line shows inefficient complementation with packaging being reduced by 6-fold. Vero, FG293 and BHK cells all show reductions in DNA packaging of approximately 15 to 20-fold.

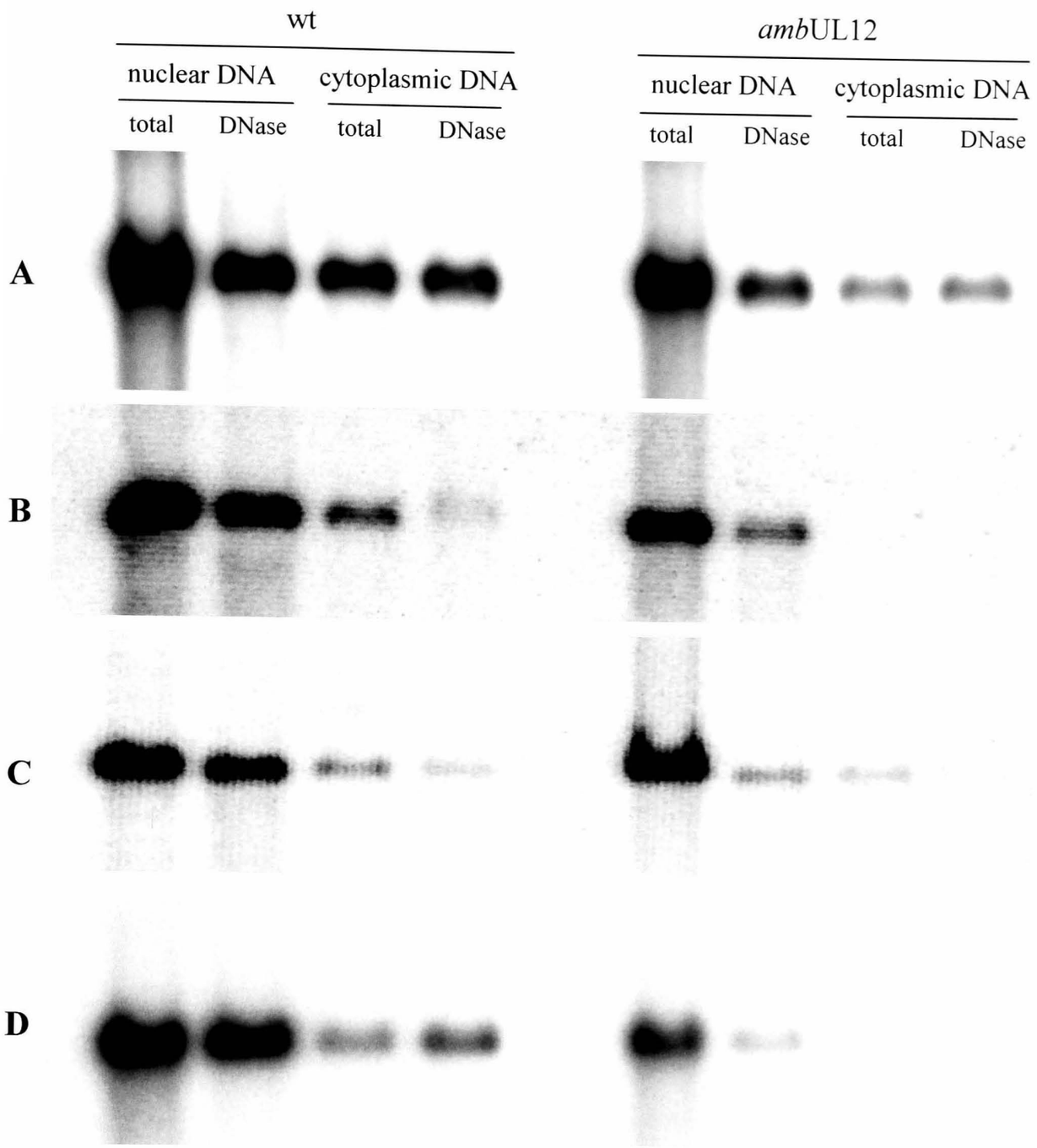


Figure 4 – Effect of cell line on replication and packaging of *amb*UL12

35mm plates of S22 (A), Vero (B), FG293 (C) and BHK (D) cells were infected at 3 p.f.u. / cell. Cells were harvested 16 h.p.i., split into nuclear and cytoplasmic fractions and processed for total and DNase resistant DNA. Samples representing 1/12 of a plate were digested with *Bam*HI, separated on a 0.8% LB-agarose gel and Southern blotted. The membrane was probed with ^{32}P-labelled plasmid pGX153.

The ability to detect viral DNA in the cytoplasm also appears to be cell type dependent (Figure 4). Vero and FG293 cells showed a similar reduction in nuclear packaging efficiency but DNase-resistant DNA could be clearly detected in the cytoplasm of only FG293 cells. Although cytoplasmic DNase-resistant DNA is not detectable in Figure 4B this was not always the case (e.g. Figure 3B). Further examination of the samples in Figure 4 show that a proportion of cytoplasmic DNA was sometimes DNase sensitive (Figure 4: Vero, wt HSV-1; FG293, *amb*UL12). The most likely explanation for this is contamination of the fraction with small amounts of nuclear DNA from lysed or damaged nuclei.

3.2.3 Time course of viral DNA synthesis and packaging

Bacteriophage T4 has two distinct phases of viral DNA replication. An initial origin-dependent replication phase is inactivated and followed by recombination-dependent replication initiation at later stages of infection (Luder and Mosig, 1982; Mosig, 1998). Recombination-deficient T4 mutants can initially replicate viral DNA with wt efficiency. However at later times in infection replication is inhibited as recombination-dependent replication initiation is implemented (Mosig *et al.*, 1995). Endonuclease VII (Endo VII), encoded by phage T4, is known to resolve Holliday and Y–Junctions. The role of Endo VII is to remove branched DNA prior to packaging, and mutations in this gene cause branched structures to remain unresolved resulting in inhibition of replication and packaging.

It is also known that HSV-1 *nuc* mutants produce branched high molecular weight (hmw) DNA intermediates that are structurally more complex than those found in wt HSV-1 infected cells (Martinez *et al.*, 1996a). In these experiments high molecular

weight DNA isolated from infected cell lysates was digested with restriction enzymes which cut only once or twice per genome. A small proportion of DNA was released into the pulsed field gel with wt HSV-1 but not the *nuc* mutant samples suggesting that the alkaline nuclease is also involved in resolving branched structures. However, the inability of purified alkaline nuclease to resolve synthetic cruciforms and Y-junctions *in vitro* suggests that the UL12 product may not function in the same way as endo VII (Goldstein and Weller, 1998b). It is, nevertheless, possible that early in infection HSV-1 *nuc* mutants replicate their DNA similarly to wt HSV-1, but as complex DNA intermediates are formed viral DNA synthesis and packaging is inhibited. To determine if this is the case a time course of viral DNA synthesis and packaging was performed.

35 mm plates of Vero cells were infected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. After 1 h incubation at 37°C the monolayers were washed with "acid glycine" to inactivate any unabsorbed virus. Cells were harvested at 0, 6, 8, 12 and 24 h.p.i., total and DNase resistant DNAs were prepared, digested with *Bam*HI and hybridised to radiolabelled pGX153 which detects the HSV-1 *Bam*HI P. The number of counts in each band of the Southern blot in Figure 5a was determined by phosphorimager and represented graphically (Figure 5b). It is apparent that from the earliest time point *amb*UL12 synthesises and packages viral DNA less efficiently than wt. The 12 and 24 h.p.i. time points also support earlier results (Table 1), with *amb*UL12 samples showing a 3-fold drop in replication and a 13 - 24-fold drop in packaging compared to wt HSV-1. From Figure 5c it can be seen that at all times during infection wt HSV-1 has encapsidated approximately the same proportion of replicated DNA. The same is true for *amb*UL12 although only 5 – 10% of the replicated DNA is packaged compared to 35 – 50% for wt HSV-1.

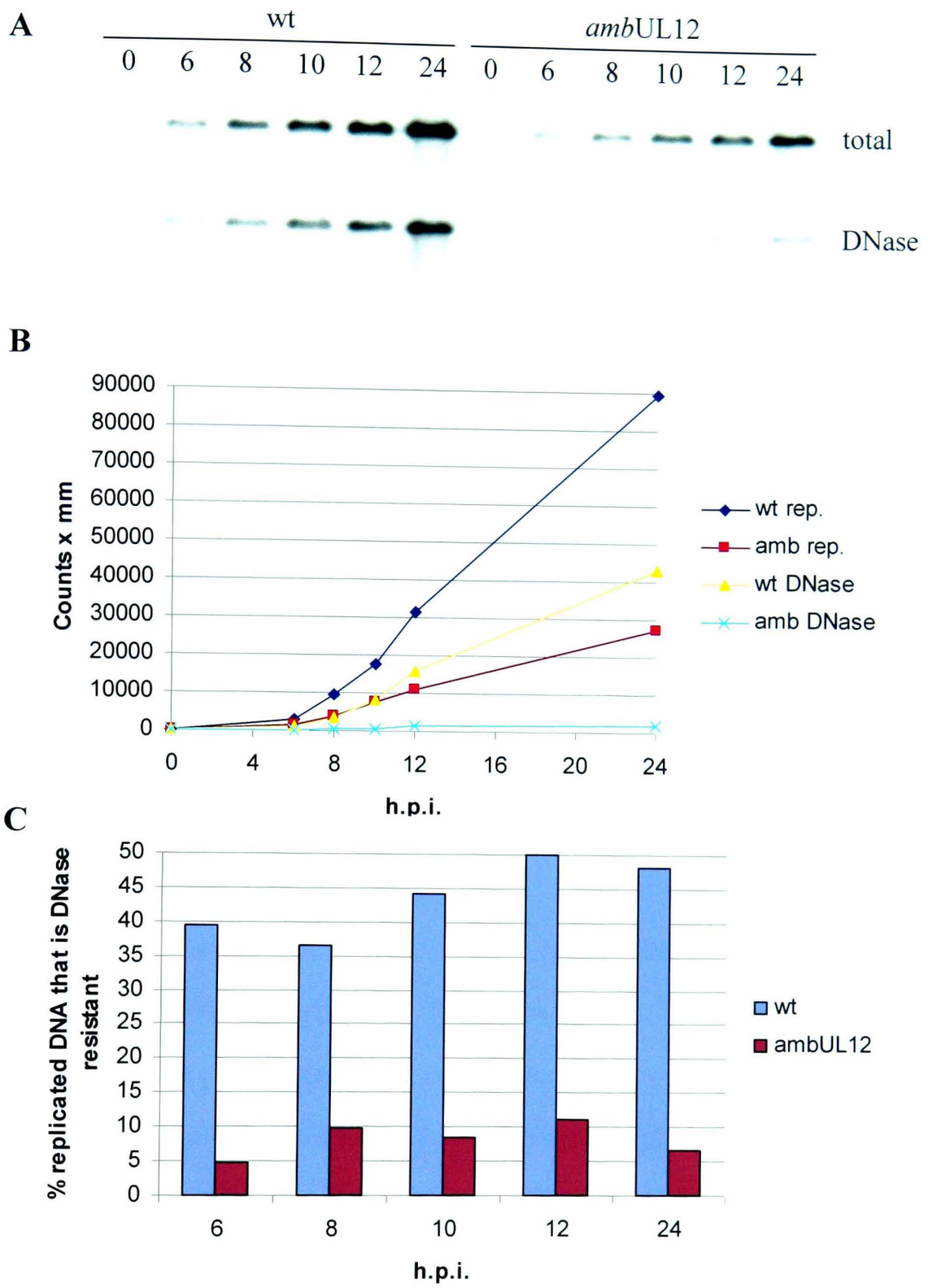


Figure 5 – Time course of viral DNA synthesis and encapsidation in wt and *amb*UL12 infected Vero cells.

(A) Vero cells were infected with 3 p.f.u. / cell wt or *amb*UL12. At the time points shown cells were harvested and whole cell extracts were processed for total and DNase resistant DNA. Samples were then treated as described in Figure 3. **(B)** The counts in each band of (A) were plotted against h.p.i. **(C)** For each time point the data is represented as the percentage of total DNA which was DNase resistant.

3.3 Pulsed Field Gel Analysis of replicated and packaged *amb*UL12 DNA

3.3.1 Introduction

Conventional agarose gel electrophoresis can routinely resolve only DNA fragments up to ~ 40 kbp in size. By cyclically changing the orientation of the electric field around a gel, Pulsed Field Gel Electrophoresis (PFGE) enables resolution of DNA fragments up to 2000 kbp in size (Schwartz and Cantor, 1984; Wagner and Lai, 1994).

A number of studies have made use of PFGE to investigate the structures of HSV-1 DNA replication intermediates (Severini *et al.*, 1994; Zhang *et al.*, 1994; Severini *et al.*, 1996) including those of *nuc* mutants (Martinez *et al.*, 1996a; Goldstein and Weller, 1998). Therefore, it was important to develop PFGE techniques in the laboratory to analyse the replication intermediates and cleavage / packaging products of *amb*UL12.

3.3.2 Establishment of PFGE run parameters

All PFGE experiments were performed on a Chef-DR II Pulsed Field Gel Electrophoresis System from BioRad. This system allows a wide degree of flexibility in the size ranges of molecules that can be separated so it was necessary to define parameters that would be useful for the present work. The following equation (Ausubel, 1999) was used to determine parameters for resolution:

Maximum well-resolved size[a] (kbp) = $0.034 \times (T + 40) \times V^{1.1} \times (3 - A)^{0.6} \times t^{0.875}$

Where T = temperature in °C; V = volts / cm; t = longest pulse time in sec; A = % agarose.

[a] Resolution is poor above this size.

For example a gel run with the following parameters: T = 14°C, V = 6 v / cm, t = 15 sec, A = 1% will resolve fragments up to 213 kbp. Increasing the switch time gradually to t during the course of the electrophoresis alters the separation of the fragments within the resolvable range.

To test the resolution, five µg of BioRad 5 kbp ladder and 5 x 5 x 1 mm agarose blocks, containing approximately 5 µg of BioRad lambda ladder (48.5 kbp ladder), were loaded into the pulsed field gel. With voltage, run time, temperature and agarose percentage kept constant at 6 v / cm, 16 h, 14°C, and 1% agarose throughout, two sets of switch times were identified as being most useful (Figure 6). A linearly ramped switch time of 1 – 50 sec allowed very large fragments, up to 800 kbp, to be resolved, suitable for analysing hmw replication intermediates. In contrast, a switch time of 1 – 15 sec could separate fragments up to only 250 kbp, more useful for investigating the mobility of encapsidated DNA molecules.

3.3.3 Preparation of agarose embedded cellular DNA

To avoid shearing of hmw DNA, infected cells were embedded in agarose and manipulated in situ, essentially using the protocols supplied by the CHEF-DR II manufacturer (BioRad) and described in detail in Methods.

3.3.4 PFGE time course of infection

It is generally accepted, although not conclusively demonstrated, that HSV-1 DNA is replicated via a rolling circle mechanism that gives rise to hmw replication intermediates. In addition branched structures are generated through homologous

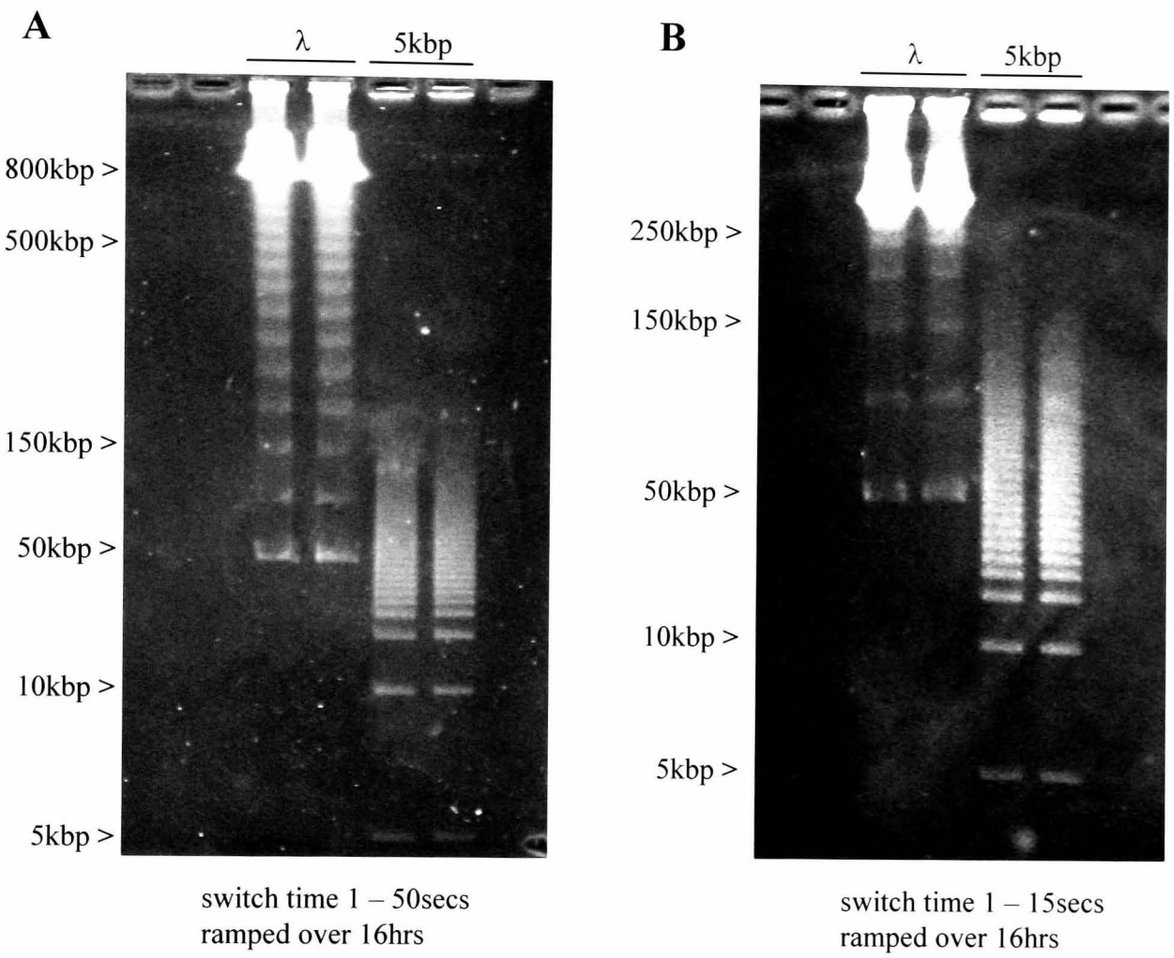


Figure 6 – Run parameters for PFGE.

1% PFGE grade agarose gels were loaded with 5µl of 5 kbp ladder (5 kbp) or 5 x 5 x1 mm agarose blocks of BioRad 48.5 kbp lambda ladder (λ), each containing approximately 5 ug of DNA. **(A)** Run parameters: 6 v / cm, 16 h, 14°C, 1% agarose, linearly ramped switch time of 1 – 50 sec. **(B)** Run parameters: 6 v / cm, 16 h, 14°C, 1% agarose, linearly ramped switch time 1 – 15 sec.

recombination events. This complex, concatemeric DNA is then cleaved into monomeric molecules which are packaged into viral capsids. The formation of hmw replication intermediates and successive appearance of unit length genomes can be monitored using PFGE (Severini *et al.*, 1994).

In order to compare wt HSV-1 and *amb*UL12 replicative intermediates, monolayers of Vero cells were infected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested at 0, 2, 4, 6, 8, 12 and 24 h.p.i. and embedded in agarose blocks as described in methods and processed for total cellular DNA. The DNA was fractionated using the conditions described by Severini *et al.* (1994): 1% PFGE grade agarose, 6 v / cm for 18 h at 14°C in 0.5 x TBE using pulse times from 2 – 70 sec, increasing linearly. The gel was then Southern blotted and probed with radiolabelled pGX153.

Figure 7 shows that in the wt HSV-1 samples monomeric units were detected at 0 h.p.i., representing the input genomes. Severini *et al.* (1994) report the disappearance of this input band until 8 h.p.i. suggesting that circularisation of the genome causes altered migration. The continued low-level presence of these bands in the 2 and 4 h.p.i. samples could be accounted for by the presence of virus that has not been uncoated. Reduction of this residual band could possibly have been achieved by acid-glycine washing of the cells after infection. In agreement with Severini *et al.* hmw DNA, which failed to enter the gel, was detected by 4 h.p.i. and steadily increased in amount as infection progressed. However, there was no significant increase in the level of monomeric DNA until 6 h.p.i., demonstrating that the viral DNA probably has to go through a hmw intermediate before cleavage into unit length genomes. In the *amb*UL12 samples hmw intermediates also increased steadily with time. However, in contrast to

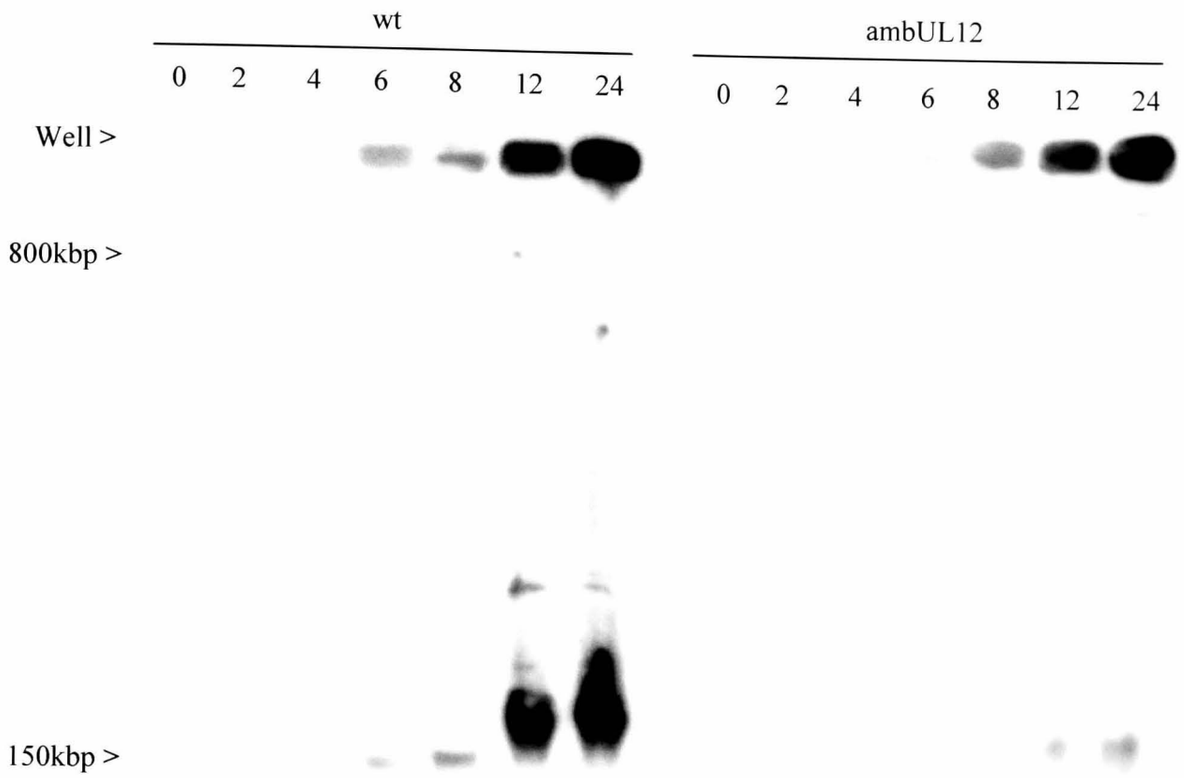


Figure 7 – Analysis of wt HSV-1 and *amb*UL12 DNA replication by PFGE.
Vero monolayers were infected at 3 p.f.u. / cell. The cells were harvested 0, 2, 4, 6, 8, 12 and 24 h.p.i., embedded in agarose blocks and total DNA was prepared in situ. 5 x 3 x 1 mm blocks, representing 1/3 of a plate, were loaded into a 1% 0.5 x TBE-agarose gel. The DNA was separated at 6 v / cm for 18 h at 14°C in 0.5 x TBE using pulse times from 2 – 70 sec, increasing linearly. The gel was pre-soaked in 0.25M HCl for 15 min, Southern blotted and probed with radiolabelled pGX153. The positions of HSV-1 genomic DNA, the highest resolved marker band (800 kbp) and the wells of the gel are indicated.

wt HSV-1, very little unit length genome was generated and at 24 h.p.i. approximately 10-fold less linear DNA had migrated into the gel.

Since cleavage and packaging are so closely linked it is reasonable to surmise that the unit length DNA represents encapsidated genomes. Therefore, this experiment suggests that packaged *amb*UL12 DNA is predominantly unit length, although further studies will be required to confirm this. In agreement with previous experiments cleavage / packaging of *amb*UL12 is clearly less efficient than wt HSV-1. Figure 7 shows that the 12 and 24 h.p.i. wt HSV-1 samples contain a large amount of cleaved DNA that migrates slower than unit length genomes. This effect was also observed by Severini *et al.* (1994) and Zhang *et al.* (1994), and is most probably a result of retarded migration caused by overloading of the gels with genome length molecules.

3.3.5 Structure of *amb*UL12 DNA

To investigate the structure of replicated *amb*UL12 DNA, restriction digest patterns of total DNA from infected cells were examined.

BHK cells were infected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i. and embedded in agarose plugs. Total cellular DNA was prepared in situ and one third of each plug, corresponding to 6.5 x 10^5 cells, was incubated with *Xba*I, *Hind*III or left undigested. The DNA was then separated in a 1% PFGE grade agarose / 0.5 x TBE gel at 6 v / cm for 16 h at 14°C in 0.5 x TBE buffer using pulse times from 1 - 15 sec, increasing linearly. The gel was then Southern blotted and hybridised to radiolabelled HSV-1 genomic DNA (Figure 8).

Once again undigested wt HSV-1 DNA produced a major band at 150 kbp corresponding to released, unit length genomes and a "well" band where hmw DNA had failed to enter the gel. *Xba*I digestion of wt HSV-1 DNA is expected to produce seven bands of approximately 55, 36, 34, 29, 28, 25 and 11 kbp. Examination of the wt HSV-1 *Xba*I sample shows distinct 11 and 55 kbp bands and an intense band which falls within the sizes of the expected 25, 28 and 34 kbp bands. There are another three predicted bands of 84, 65 and 47 kbp that corresponds to *Xba*I restriction fragments between two head-to-tail molecules within a concatemer. The 84 and 65 kbp bands are clearly visible and the 47 kbp band is probably a constituent of the intense band described above.

In contrast to wt HSV-1, the undigested *amb*UL12 sample contained no detectable full length *amb*UL12 genomes and the *Xba*I digested *amb*UL12 sample generated only a single smeared band that fell within the size range of the 24, 25 and 34 kbp bands, failing to produce discrete bands at 10, 55 and 65 kbp. The lack of detectable genome length molecules in *amb*UL12 samples was not uncommon and further emphasises the cleavage / packaging defect of the virus.

*Hind*III digestion of wt HSV-1 samples produced the expected fragments from concatemeric DNA molecules, ranging from 4 – 40 kbp whereas *amb*UL12 produced a very smeared restriction pattern with the 34, 36 and 40 kbp bands underrepresented in comparison to the other bands. In both wt HSV-1 and *amb*UL12 samples there was a substantial amount of DNA that failed to leave the wells of the gel.

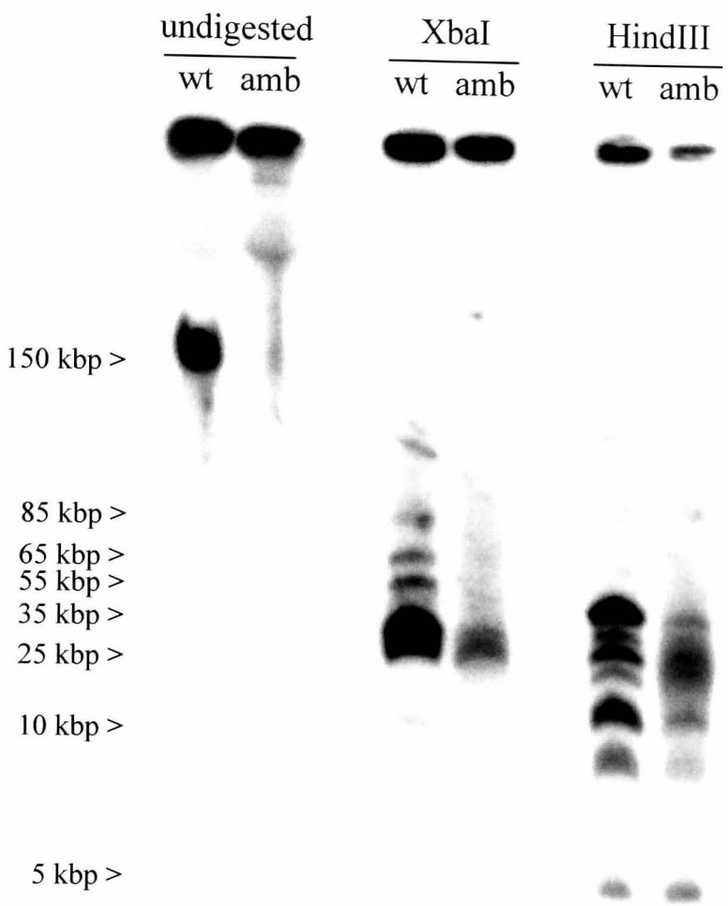


Figure 8 – Restriction enzyme digestion of agarose embedded viral DNA.

35 mm BHK plates were infected with 3 p.f.u. / cell wt HSV-1 (wt) or *amb*UL12 (amb). Cells were harvested 16 h.p.i. and embedded in agarose blocks. Total DNA was prepared *in situ*. As indicated one third of each block was digested with *Xba*I, *Hind*III or left undigested. The samples were then separated by PFGE using the following parameters: 1% PFGE grade agarose, 0.5 x TBE gel at 6 v / cm for 16 h at 14°C in 0.5 x TBE buffer using pulse times from 1 - 15 sec, increasing linearly. The gel was stained with ethidium bromide, photographed and Southern blotted. The membrane was then probed with ^{32}P labelled HSV-1 genomic DNA. The positions of molecular markers (BioRad 5 kbp ladder) are indicated.

No conditions could be established to fully remove this "well" DNA, even using the restriction enzyme *Bam*HI which cut the HSV-1 genome up to 42 times. This could suggest that a proportion of HSV-1 replication intermediates contain structures that are not accessible to restriction enzymes. However this seems unlikely because complete digestion of well DNA has been reported by other investigators (Martinez *et al.*, 1996a) and digestion of samples obtained by phenol chloroform extraction produce DNA that is able to migrate from the wells in conventional agarose gel electrophoresis. Therefore, it is more likely that Proteinase K was not completely removed from the agarose blocks or the restriction enzymes were not given adequate opportunity to fully diffuse into the agarose blocks. Nevertheless, it is clear from these results that *amb*UL12 DNA is structurally different from wt HSV-1 DNA, perhaps containing more single stranded nicks or branched structures. In addition it shows that *amb*UL12 fragments greater than ~10 kbp do not run as discrete bands and appear to be underrepresented compared to smaller bands (e.g. the 5 kbp band in lanes 5 and 6).

3.3.6 Restriction digest patterns of DNA purified from extracellular virions

A major defect in *nuc* mutants was reported to be in the egress of DNA containing capsids into the cytoplasm, possibly because of capsid instability due to the insertion of incorrectly processed DNA (Shao *et al.*, 1993). However, *nuc* mutants do produce a small amount of viable virus in non-complementing cells and it might be expected that the DNA in these virions would be fully processed and indistinguishable from that of wt HSV-1.

To investigate the structure of *amb*UL12 DNA in extracellular virions 175 cm^2 flasks of Vero and BHK cells were infected with 0.01 p.f.u. / cell wt HSV-1 or *amb*UL12. Three

days post infection the cells were suspended in the media and pelleted by centrifugation. Extracellular virions were isolated as described in Methods and DNA prepared. The DNA concentration of each of the samples was estimated from ethidium bromide stained agarose gels and the size of the isolated DNA was determined by PFGE. 0.5 μg samples of DNA were digested with either *Xba*I or *Hind*III or left undigested and subjected to PFGE. The gel was Southern blotted and the membrane hybridised to radiolabelled HSV-1 genomic DNA (Figure 9).

The undigested samples in both BHK and Vero cells produced a distinct band of 150 kbp corresponding to full size, packaged genomes. Also, in contrast to the results of 3.3.5, both wt HSV-1 and *amb*UL12 samples digested with *Xba*I or *Hind*III produced distinct restriction fragments of the expected sizes. This experiment indicates that the DNA present in released *amb*UL12 virions has a similar structure to that of wt HSV-1.

3.3.7 S1 nuclease digestion of DNA purified from extracellular virions.

Studies by Wilkie (1973) and Frenkel and Roizman (1972) showed that DNA within HSV-1 particles contained a number of nicks, although the number and distribution of the nicks was disputed. Frenkel and Roizman demonstrated that there were seven unique nicks whereas the results of Wilkie indicated that alkali labile sites were randomly distributed throughout the genome. Since the presence of single stranded nicks within the HSV-1 genome has implications for the mechanisms of replication and packaging of the genome, their frequency in the wt HSV-1 and *amb*UL12 genomes was compared by digesting genomic DNA samples with S1 nuclease, which cuts single stranded DNA.

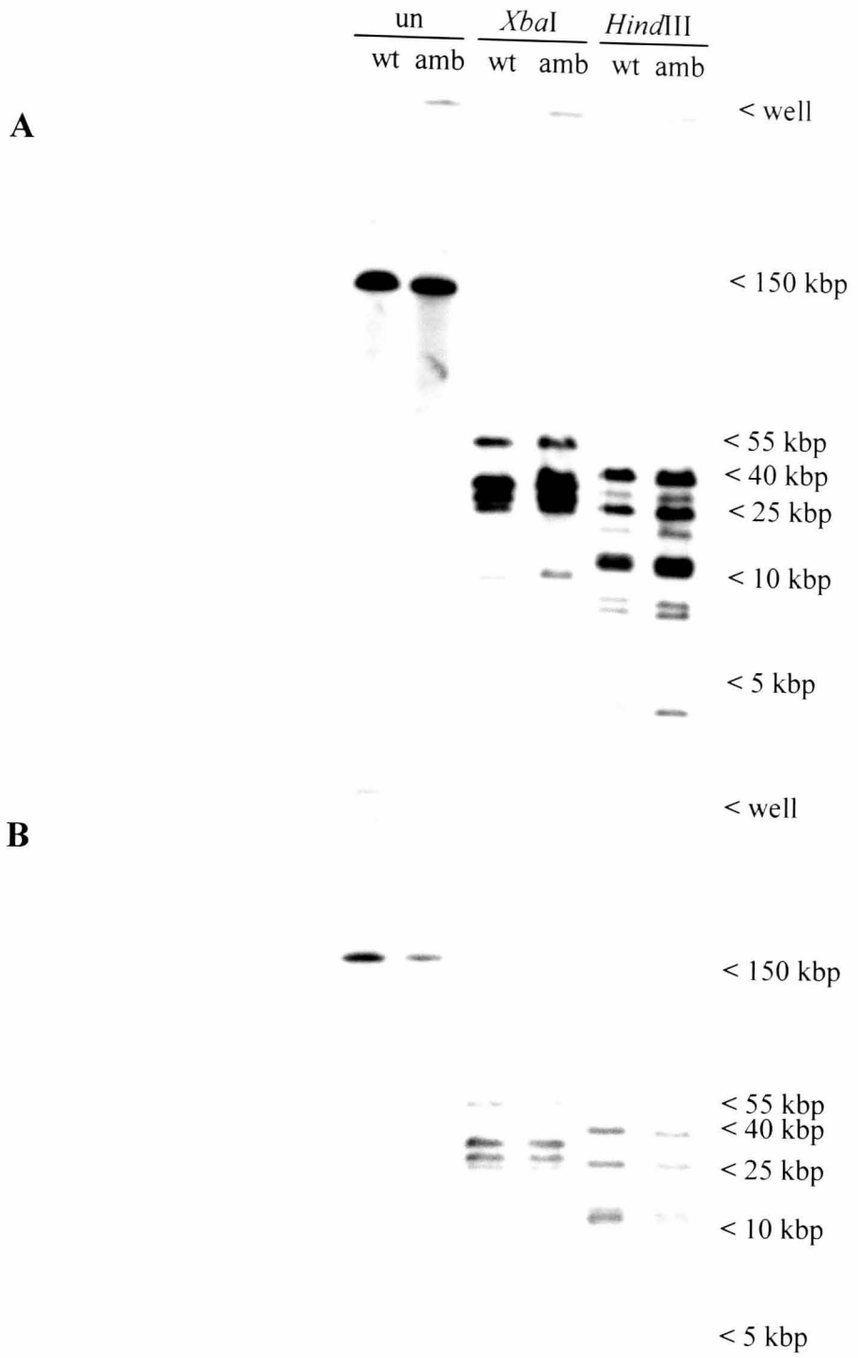


Figure 9 - Restriction digest patterns of DNA purified from extracellular virions.

175cm^2 flasks of BHK (**A**) or Vero (**B**) cells were infected with 0.01 p.f.u. / cell wt HSV-1 (wt) or *amb*UL12 (amb). DNA was extracted from extracellular virions three days post infection. As indicated 0.5 µg of each sample was digested with *Xba*I, *Hind*III or left undigested. The DNA was separated on 1% PFGE grade agarose / 0.5 x TBE gel at gel at 6 v / cm for 16 h at 14°C in 0.5 x TBE buffer using pulse times from 1 - 15 sec, increasing linearly. The gel was Southern blotted and the membrane hybridised to radiolabelled HSV-1 genomic DNA. The positions of selected bands from a 5 kbp ladder, supplied by BioRad, are indicated.

Extracellular virion DNA, obtained from Vero and BHK cells described in section 3.3.6, was used to compare the number of single stranded nicks in packaged wt HSV-1 and *amb*UL12 DNA. 0.5 μg of DNA was digested with 10 U of S1 nuclease in a final volume of 30 μl with 1 x S1 nuclease buffer (0.15 M NaAc pH 4.5, 0.5 M NaCl, 25mM $ZnSO_4$). The DNA was then separated by PFGE using the run parameters described in section 3.3.6, Southern blotted and the membrane hybridised to ^{32}P labelled HSV-1 genomic DNA.

Figure 10 ahows that S1 digestion of packaged DNA from wt HSV-1 produces fragments that are evenly distributed between the bottom of the gel (~3 kbp) and 20 kbp. The S1 fragments generated from the digestion of *amb*UL12 DNA was very similar in the BHK samples and was practically indistinguishable from wt HSV-1 in the Vero samples. This supports the results from section 3.3.6 that the DNA from extracellular *amb*UL12 virions is very similar to wt HSV-1.

Since no buffer was supplied with the enzyme, S1 nuclease buffer was made in the laboratory. To ensure the buffer did not contain any contaminating nucleases 1x S1 buffer and 0.5 μg of viral DNA were mixed and incubated at 37°C for 1 hr. As can be seen in Figure 10A (lanes 2 and 5), no degradation of genomic DNA occurred when compared with undigested samples.

3.4 Determination of the size of packaged *amb*UL12 DNA using PFGE

3.4.1 Introduction

Section 3.3.4 demonstrated that *amb*UL12 was able to cleave and presumably package unit length genomes, although at reduced levels compared to wt HSV-1. However the

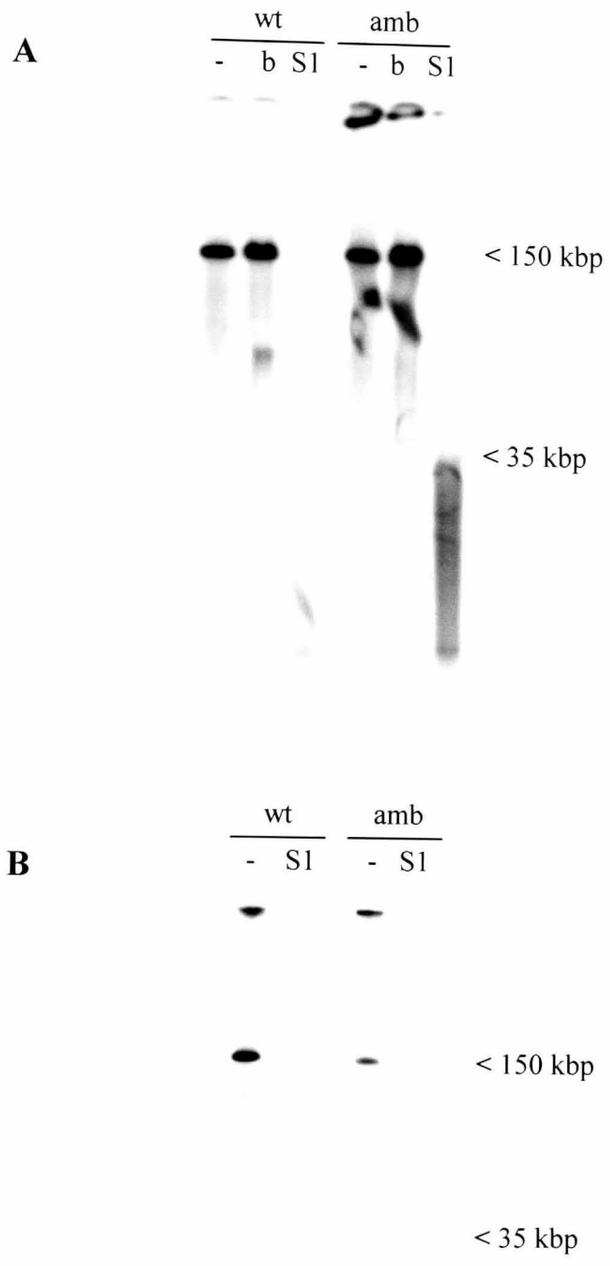


Figure 10 – S1 nuclease digestion of DNA purified from extracellular virions.

Approximately 0.5 μg samples of the DNAs described in section 3.3.6 were left undigested (-), incubated with 1 x S1 buffer alone (b) or with 10 U of S1 nuclease in 1 x S1 nuclease buffer (S1). After incubation at 37°C for 1 hr the samples were separated by PFGE. The gel was Southern blotted and the membrane hybridised to radiolabelled HSV-1 genomic DNA. The migration of selected bands from a 5 kbp ladder, supplied by BioRad, are indicated.

DNA used in that experiment was obtained from total cell extracts. To confirm that encapsidated DNA present in the nucleus of *amb*UL12 infected cells migrated with a similar mobility to wt HSV-1, PFGE analysis of DNase-resistant DNA was performed.

3.4.2 Preparation of DNase-resistant DNA by the gentle lysis method

35 mm plates of Vero cells were infected with 3 p.f.u. / cell wt HSV-1, *amb*UL12 or *amb*UL12R and harvested 16 h.p.i. The cells were harvested, nuclei prepared and incubated in 184 µl RSB, 0.5 % NP40, 0.1 mg ml^{-1} DNase for 15 min at 37°C. 184 µl 4xCLB / loading buffer (1:1) was then added. To minimise shearing of the DNA no phenol / chloroform extraction was performed and 100 µl of each sample was loaded directly onto a 1% PFGE grade agarose / 0.5 x TBE gel. Samples were separated at 6 v / cm for 16 h at 14°C in 0.5 x TBE buffer using pulse times increasing from 1 - 15 sec. The gel was Southern blotted and hybridised to ^{32}P labelled wt HSV-1 genomic DNA.

From Figure 11 it is evident that wt HSV-1 and *amb*UL12R produced considerable amounts of genome size DNA that is resistant to DNase digestion. However, *amb*UL12 produced significantly less DNase-resistant DNA and this appeared to exhibit an increased and more heterogeneous mobility (size range of 130 – 150 kbp). This experiment initially suggests that a significant proportion of packaged *amb*UL12 DNA may be slightly smaller than genome length. However, it is possible that the change in mobility of these molecules may be due to other factors, such as the presence of branched structures and not an actual change in length of the packaged molecules. It is also possible that *amb*UL12 DNA is more fragile than wt HSV-1 DNA and that portions of the genome are susceptible to mechanical shearing.

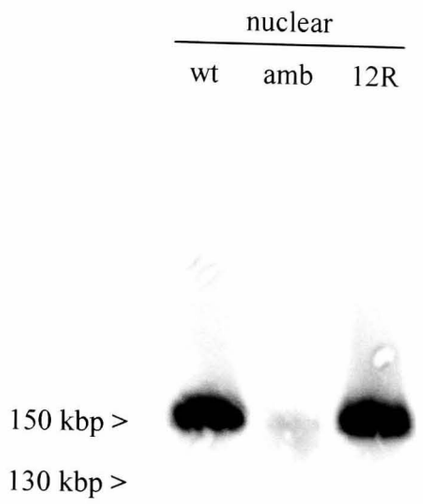


Figure 11 – Mobility of encapsidated wt HSV-1, *amb*UL12 and *amb*UL12R DNAs.

35 mm plates of Vero cells were infected with 3 p.f.u. / cell wt HSV-1 (wt), *amb*UL12 (amb) or *amb*UL12R (12R). Cells were harvested 16 h.p.i. and nuclei prepared. The samples were then incubated in 184 µl RSB, 0.5% NP40, 0.1 mg ml^{-1} DNase for 15 min at 37°C. 184 µl 4 x CLB containing loading buffer was added. 100 µl of each sample was loaded directly into a 1% PFGE grade agarose / 0.5 x TBE gel. Samples were separated at 6 v / cm for 16 h at 14°C in 0.5 x TBE buffer using pulse times from 1 - 15 sec, increasing linearly. The gel was Southern blotted and hybridised to ^{32}P labelled wt HSV-1 genomic DNA. The size markers are indicated.

3.4.3 Preparation of DNase-resistant DNA in agarose blocks

To exclude the possibility that shearing of viral DNA might be having an effect it was important to develop a technique that allowed the preparation of DNase-resistant viral DNA within agarose blocks thereby avoiding any mechanical cleavage of the DNA molecules.

Initial experiments using the approach of *in situ* DNase digestion described by Martinez *et al.* (1996a) resulted in degradation of the DNA that had been packaged. Several approaches, including the use of lower DNase I concentrations and buffers with higher EDTA concentrations were tried in an attempt to solve this problem. Eventually it was found that sensitivity of the packaged DNA to DNase I was associated with the presence of NP40. The effect of NP40 concentration in the lysis buffer was therefore examined.

35 mm plates of BHK cells were infected with 3 p.f.u. / cell wt HSV-1. 16 h.p.i. the cells were harvested and embedded in agarose blocks as described in Methods. The embedded cells were lysed by incubating the plugs at 37°C for 2 h using RSB / NP40, with NP40 content ranging from 0.05 to 0.5%. The agarose blocks were washed in 0.5 ml DNase digestion buffer. This was replaced with fresh DNase digestion buffer containing DNase I ranging in concentration from 0.04 – 0.4 mg ml^{-1} and incubated at 37°C for a further 2 h. The blocks were washed in 0.5 M EDTA and digested overnight with Proteinase K as described in Methods. The DNA from one third of each agarose block was separated by PFGE, Southern blotted and probed with radiolabelled HSV-1 genomic DNA.

Figure 12 shows that using NP40 at 0.5 % results in recovery of little or no DNase-resistant DNA (Figure 12 upper panel, lane 9 and 12 lower panel, lane 4), and even at 0.2%, the concentration routinely used by Martinez *et al.*, (1996a), very little DNA could be recovered, even when using reduced concentrations of DNase I (Figure 12 upper panel, lanes 2 – 5). It is not until NP40 is used at 0.1% that significant amounts of DNA are recovered. It was decided to use an NP40 concentration of 0.05% because this gave the most consistent recovery of DNase-resistant DNA. It can also be seen in Figure 12B that at this concentration of NP40 increasing the DNase concentration 10-fold reduced DNA recovery by less than 2-fold indicating that the capsids were stable and not permeable to DNase.

3.4.4. Mobility of packaged *amb*UL12 DNA prepared in agarose blocks

To examine the mobility of DNA molecules packaged by *amb*UL12, DNase-resistant DNA was prepared as described in Methods using the technique developed in section 3.4.2. Unfortunately no *amb*UL12 DNase-resistant DNA could be detected. Reasoning that *amb*UL12 capsids may be less stable than wt HSV-1 capsids, and therefore more sensitive to NP40 treatment, the concentration of NP40 was lowered even further. 35 mm plates of BHK cells were infected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i. and total or DNase-resistant DNA was prepared. For the preparation of total DNA an NP40 concentration of 0.5% was used whereas in the preparation of DNase-resistant DNA NP40 concentrations of 0.05, 0.01, 0.005 and 0.001% were used during the lysing of cells and DNase was used at a concentration of 0.1 mg ml^{-1}. The samples were subsequently prepared as described in Methods. One third of each sample was separated by PFGE in a 1% PFGE grade agarose / 0.5 x TBE gel at 6 v / cm for 16 h at 14°C in 0.5 x TBE buffer using pulse times from 1 - 15 sec,

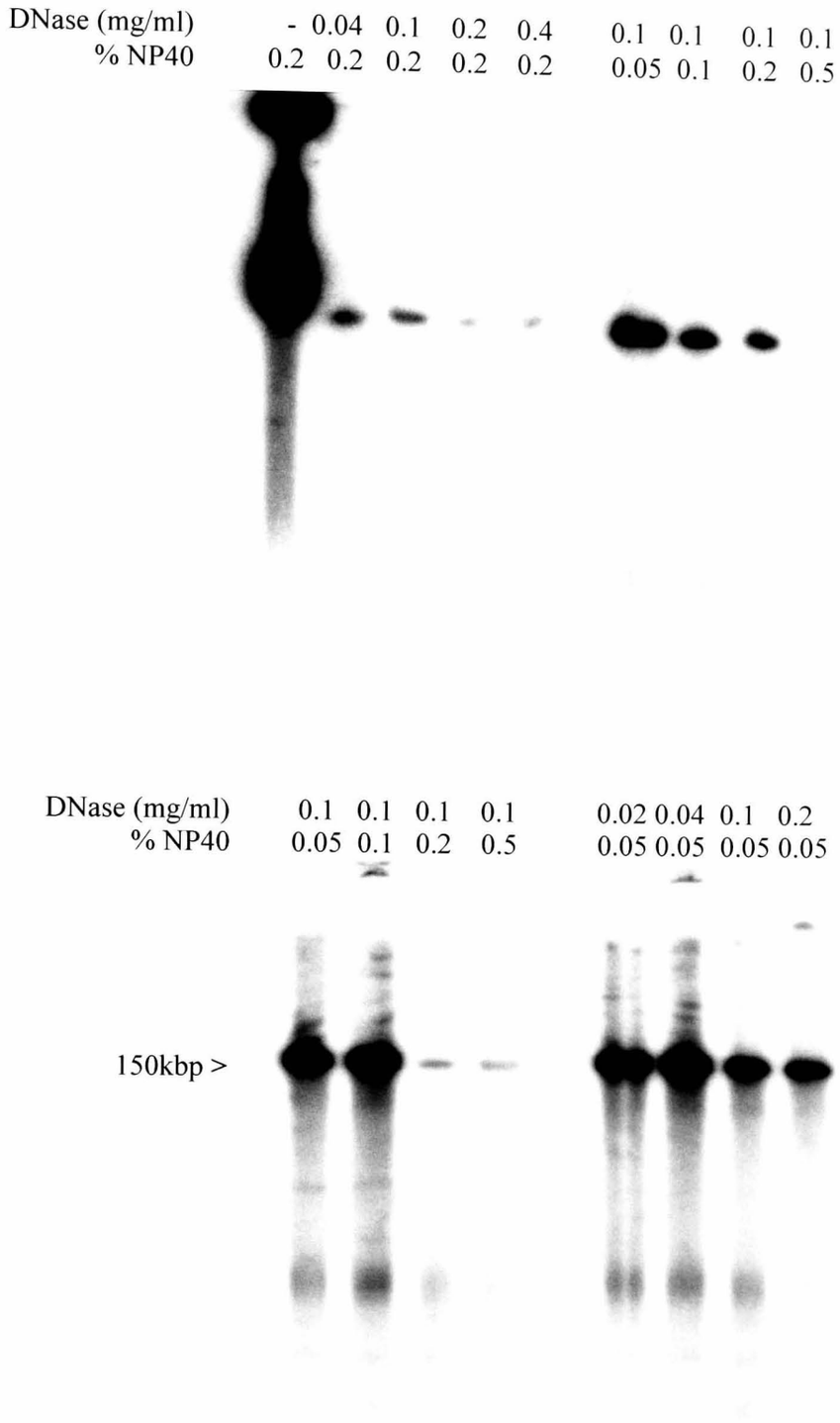


Figure 12 – Effect of NP40 and DNase concentrations in obtaining DNase resistant DNA in agarose embedded cells.

BHK cells were infected with 3 p.f.u. / cell wt HSV-1. 16 h.p.i. the cells were harvested and embedded in agarose blocks. The blocks were incubated in 0.5ml RSB containing NP40 at the percentage indicated for 2 h at 37°C. Blocks were incubated in 0.5 ml DNase reaction buffer containing DNase I at the concentrations shown for a further 2 h at 37°C. Following overnight incubation with Proteinase K one third of each block was separated by PFGE, Southern blotted and hybridised to ^{32}P labelled HSV-1 genomic DNA. The position of unit length genomic DNA is indicated.

times from 1 - 15 sec, increasing linearly. The gel was Southern blotted and hybridised to ^{32}P labelled wt HSV-1 genomic DNA.

Figure 13 shows that with NP40 below 0.05% there is no significant increase in the recovery of encapsidated wt HSV-1 DNA. It also shows that at all NP40 concentrations only a very small amount of DNase-resistant *amb*UL12 DNA can be detected. In agreement with Figure 11, the packaged *amb*UL12 DNA band is less sharp than that of wt HSV-1 forming a smear of molecules exhibiting apparent sizes in the range of 130 – 150 kbp. This experiment demonstrates that packaged *amb*UL12 molecules are close to genome length but show heterogeneity due to either being physically shorter, by up to 20 kbp, or because of some other structural defect causing altered migration.

It is unknown why capsids embedded in agarose are so sensitive to NP40. When preparing DNase-resistant DNA in the normal fashion NP40 concentrations of 0.5% and 0.2% have been routinely used in many laboratories with no adverse problems reported. It is interesting to note that even when using concentrations of NP40 as low as 0.001% (Figure 13) the amount of genomic length DNA recovered from DNase treated samples is still significantly lower than that of total DNA samples. This suggests that either the encapsidated DNA is partially sensitive to DNase using this method or the higher NP40 concentrations promote loss of DNA from the plugs during the subsequent washing stages needed to ensure removal of DNase from the agarose blocks. One way to address the latter scenario would be to treat the total DNA samples exactly as the DNase treated samples with exclusion of only the DNase.

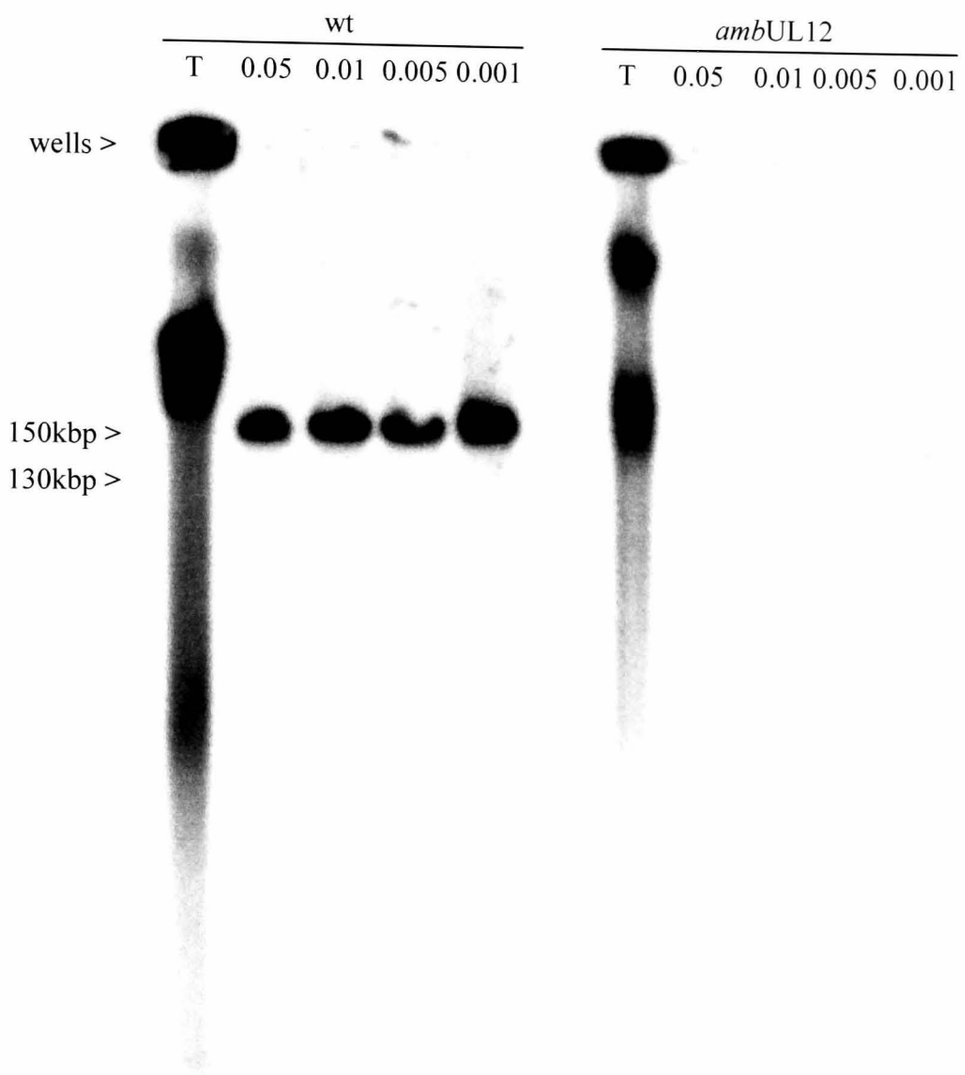


Figure 13 – Mobility of packaged *amb*UL12 DNA.

BHK monolayers were infected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i., embedded in agarose blocks and processed for total (T) or DNase-resistant DNA. Total DNA was prepared by lysing cells using RSB and 0.5% NP40. DNase-resistant samples were lysed using RSB with NP40 at the percentages indicated followed by digestion with 0.1 mg ml^{-1} DNase I. After Proteinase K treatment one third of each block was resolved by PFGE, Southern blotted and hybridised to ^{32}P labelled HSV-1 genomic DNA. The position of the wells and various markers are indicated.

3.5 Terminal fragments of packaged *amb*UL12 DNA

3.5.1 Introduction

One way to determine if the size heterogeneity seen in encapsidated *amb*UL12 molecules was due to a failure to package a complete genome length of DNA was to examine the termini contained in packaged molecules.

Previous studies by Martinez *et al.* (1996a) suggest that there is no apparent difference in the terminal fragments of *nuc* mutants when compared to wt HSV-1. However, these studies also indicated that cleavage and packaging were unimpaired with respect to wtHSV-1, which clearly differs from the results with the *amb*UL12 mutant seen in this study.

3.5.2 Terminal fragments in *amb*UL12 molecules

BHK and Vero cell monolayers were infected with wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i. and processed for total and DNase resistant DNA. Duplicate DNA samples were digested with *Bam*HI, separated on a 0.8% LB-agarose gel and Southern blotted. The membrane was divided and hybridised to radiolabelled probes that would detect the terminal repeats. pBE1 was used to detect the *Bam*HI K and S fragments, representing the joint and long terminus respectively and either pST17 or pBN1 was used to detect *Bam*HI K and Q, the joint and short terminal fragments (Figure 14).

Figures 15A and B show typical results from these experiments. Total DNA samples contain concatemeric as well as unit length viral DNA molecules. The joint fragment (*Bam*HI K) is therefore expected to be over-represented when compared to the long

terminal (TR_L) and short terminal (TR_S) fragments (*Bam*HI S and Q respectively). Figure 15A shows that this is true for the long terminus of both wt HSV-1 and *amb*UL12. A similar result was obtained with the short terminal probe (data not shown). Furthermore, when total and DNase-resistant DNA were prepared from the same sample the amount of terminal fragment found in each was similar, indicating that the majority of termini were associated with packaged molecules (results not shown).

When considering packaged molecules, it is expected that the ratio of the joint fragments to each of the terminal fragments will be one. Figure 15B shows DNase-resistant DNA samples from infected BHK or Vero cells digested with *Bam*HI and probed with pBE1 or pST17. When the counts in the joint and terminal fragments were determined for Figure 15B and the ratio of joint to each terminus was calculated it was found that the wt HSV-1 samples gave a ratio very close to one. Although not obvious by eye, the joint fragment in the *amb*UL12 samples was always over represented compared to the termini. The value of joint / TR_L and joint / TR_S was calculated for five independent experiments using BHK and Vero cells. As can be seen in Figure 15C all the wt samples gave a ratio very close to one with very little deviation from the mean. In contrast the *amb*UL12 samples show that the joint fragment is generally in excess over both termini although all the ratios showed a higher standard deviation.

There is mounting evidence that HSV-1 packages DNA molecules in the direction of U_L to U_S with encapsidation of herpesvirus genomes being initiated by cleavage and insertion of the *pac*2 sequence, which is contained within TR_L of HSV-1, into a viral capsid (McVoy and Adler, 1994; McVoy *et al.*, 2000; Stow, 2001). If packaging was initiated by cleavage at the *pac*2 site within TR_L we would expect the TR_L fragment to

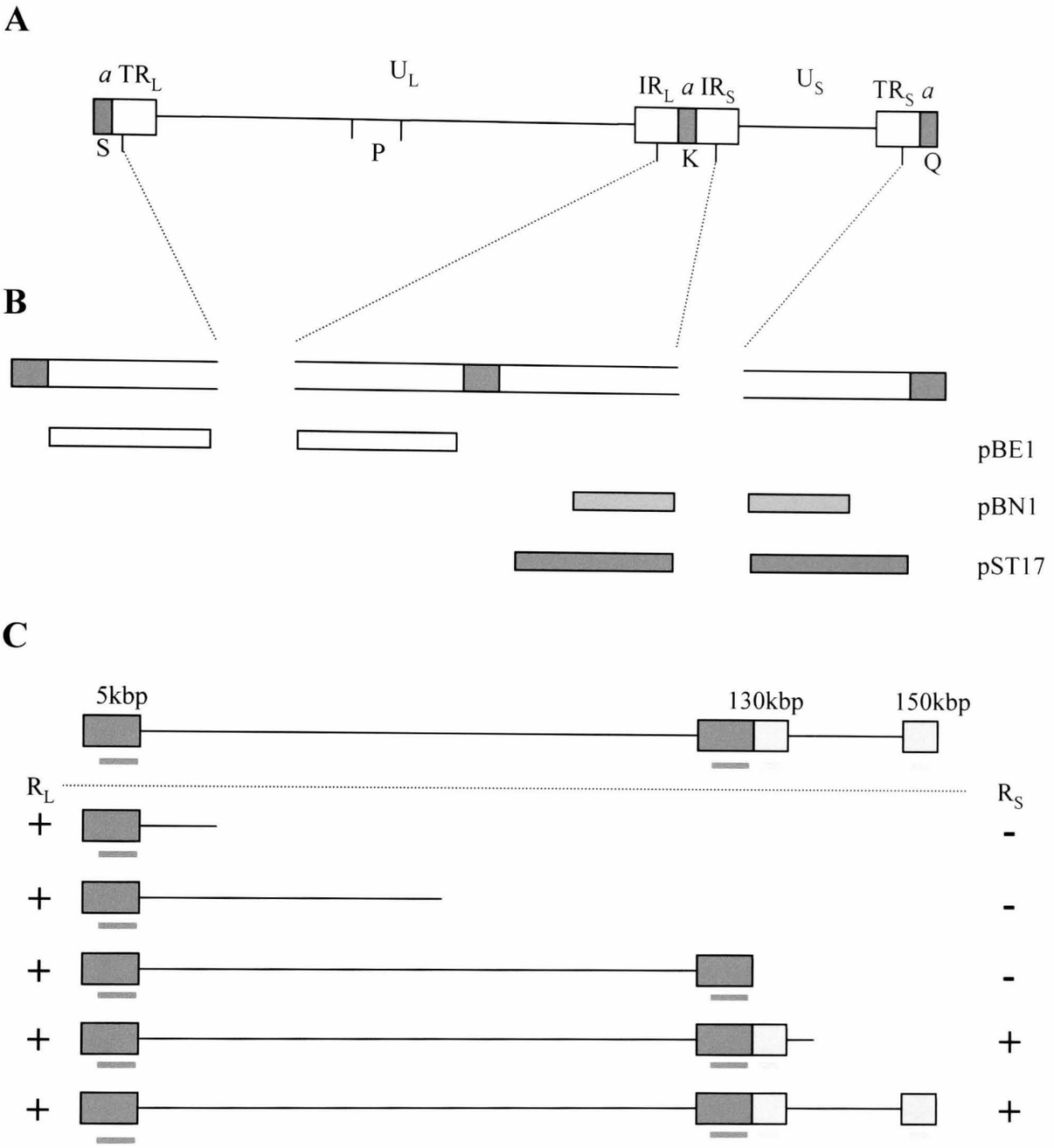
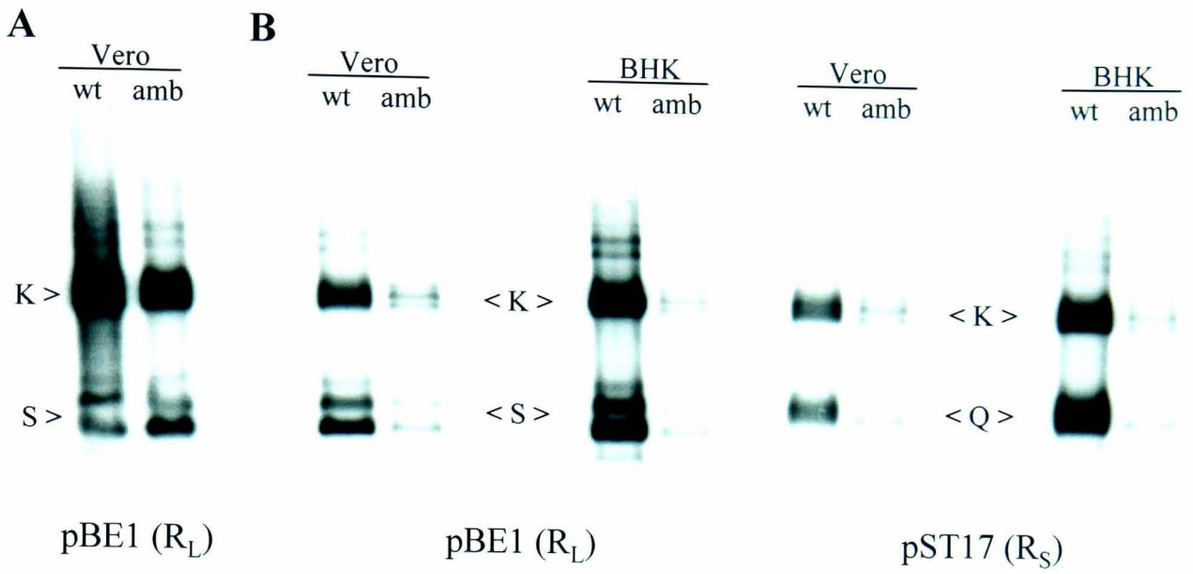


Figure 14 – Position of HSV-1 fragments used as probes for Southern blots.

(**A**) Schematic of the HSV-1 genome. The position of the unique sections U_L and U_S are shown along with the repeat sequences TR_L, IR_L, IR_S and TR_S. The shaded regions represent the position of the *a* sequences, only single copies are shown for simplicity. The location of the *Bam*HI S, P, Q and K fragments are also shown. (**B**) Expansion of the S, Q and K fragments showing the regions inserted into the plasmids pBE1, pBN1 and pST17. Aadapted from Stow, (2001).

(**C**) Diagram demonstrating the size distribution of molecules which will be detected by probes specific for R_L and R_S, assuming packaging occurs from U_L to U_S. Dark and light bars represent R_L and R_S specific probes, respectively.



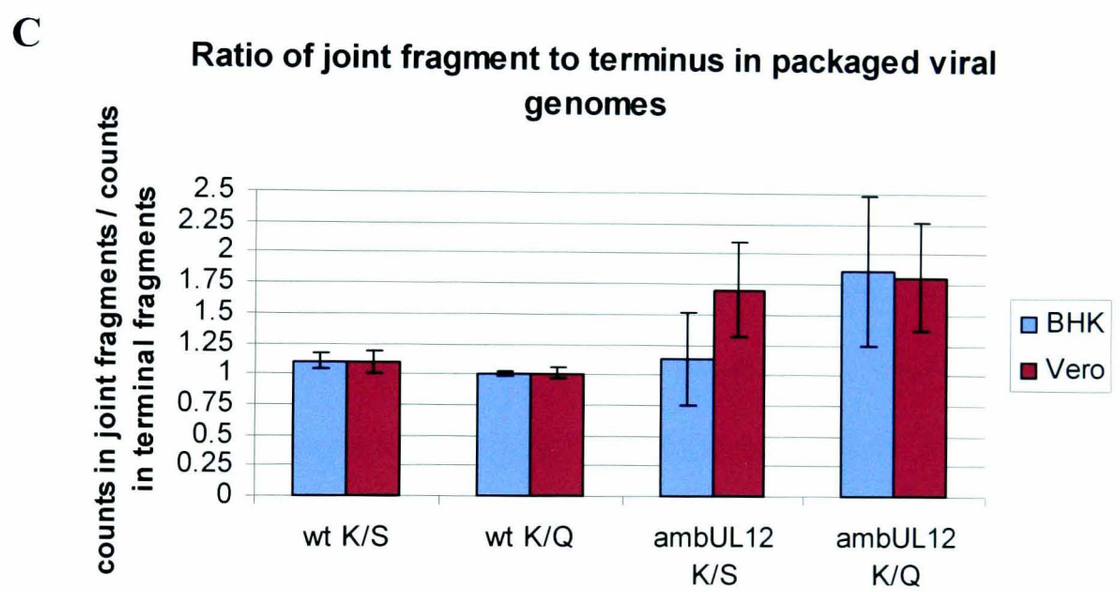


Figure 15 - Ratio of joint to termini in encapsidated wt HSV-1 and ambUL12 DNA.

BHK and Vero cell monolayers were infected with wt HSV-1 or *amb*UL12. (**A**)Total DNA was prepared 16 h.p.i. Samples were digested with *Bam*HI, separated on a 0.8% LB-agarose gel, Southern blotted and the membrane probed with ^{32}P labelled pBE1. (**B**) DNase-resistant DNA was prepared 16 h.p.i. Duplicate samples were digested with *Bam*HI and Southern blotted. The membrane was divided and probed with ^{32}P labelled pBE1 or pST17. The position of the *Bam*HI K, S and Q fragments are indicated. Note that (S) indicates the position of the fragment containing a single *a* sequence, the bands above contain 2, 3, and 4 copies of the *a* sequence. (**C**) The counts in each *Bam*HI K, S and Q fragments from five independent experiments, including those in (B), were determined and the ratio of K/S and K/Q calculated.

be in excess, or present in the same amount as the joint fragment during any incomplete packaging event and TR_S to be present in relatively lower amounts. Data for *amb*UL12 are therefore not consistent with small genomes being generated simply by failure to complete packaging. Rather, the data seems to indicate that the majority of packaged molecules have aberrant termini at either or both ends.

3.5.3 Detection of joint and terminal fragments in PFGE

The experiments described in section 3.4 demonstrated that a large proportion of the packaged *amb*UL12 genomes were below unit length. PFGE was used to determine the size distribution of molecules that contained joint and/or terminal fragments.

DNase-resistant DNA was prepared from wt HSV-1 and *amb*UL12 infected BHK monolayers as described in section 3.4.1. Replicate samples were separated at 6 v / cm for 16 h at 14°C in 0.5 x TBE buffer using pulse times from 1 - 15 sec, increasing linearly. The gel was Southern blotted, the membrane divided and each section hybridised to ^{32}P labelled wt HSV-1 genomic DNA, pBE1 (from R_L) or pST17 (from R_S). The results are shown in Figure 16.

If packaging does occur from U_L to U_S as discussed in section 3.5.2 then the pBE1 probe would detect partially encapsidated molecules containing TR_L (but not IR_L or R_S) migrating with apparent sizes of ~5 to 120 kbp. Molecules above this size would generate a signal by hybridisation to TR_L and/or IR_L. In contrast the pST17 probe would detect partially encapsidated molecules only above ~130 kbp in size by hybridisation to IR_S. Additional hybridisation to TR_S would occur only with full length molecules (Figure 14C).

With wt HSV-1 all three probes detected a band that migrated at, or slightly above 152 kbp indicating that the majority of DNase-resistant DNA is full length genomic DNA. In agreement with the above model the greatest degree of hybridisation to molecules smaller than approximately 120 kbp was detected when using the pBE1 probe and probably represents the detection of partially packaged molecules, the encapsidation of which was interrupted when the cells were harvested.

In the *amb*UL12 samples it is evident that although a small proportion of molecules migrated as full length genomes, much of the DNA migrated slightly faster, with apparent sizes of 130–150 kbp. Previously, Figure 15C demonstrated that both terminal fragments were detected in packaged *amb*UL12 DNA, and that there was a small excess of joint fragments over terminal fragments, most noticeably IR_S over TR_S. This suggests that the molecules in the size range 130-152 kbp probably correspond to the packaged molecules detected in Figure 15. The full length (152 kbp) molecules detected with both probes presumably contain both a normal TR_L and TR_S. The molecules between about 130 kbp and full length, however, are likely to contain an authentic terminal fragment from only one end suggesting non-specific cleavage may be occurring. In conjunction with the results from Figure 15C this suggests that, in BHK cells at least, the aberrant cleavage occurs more often within TR_S than TR_L. It remains possible that aberrant cleavage may take place at either or both termini within the same genome.

It should be pointed out that the data presented in Figures 15 and 16 are consistent with a model in which packaging can occur in either direction and the molecules in the size

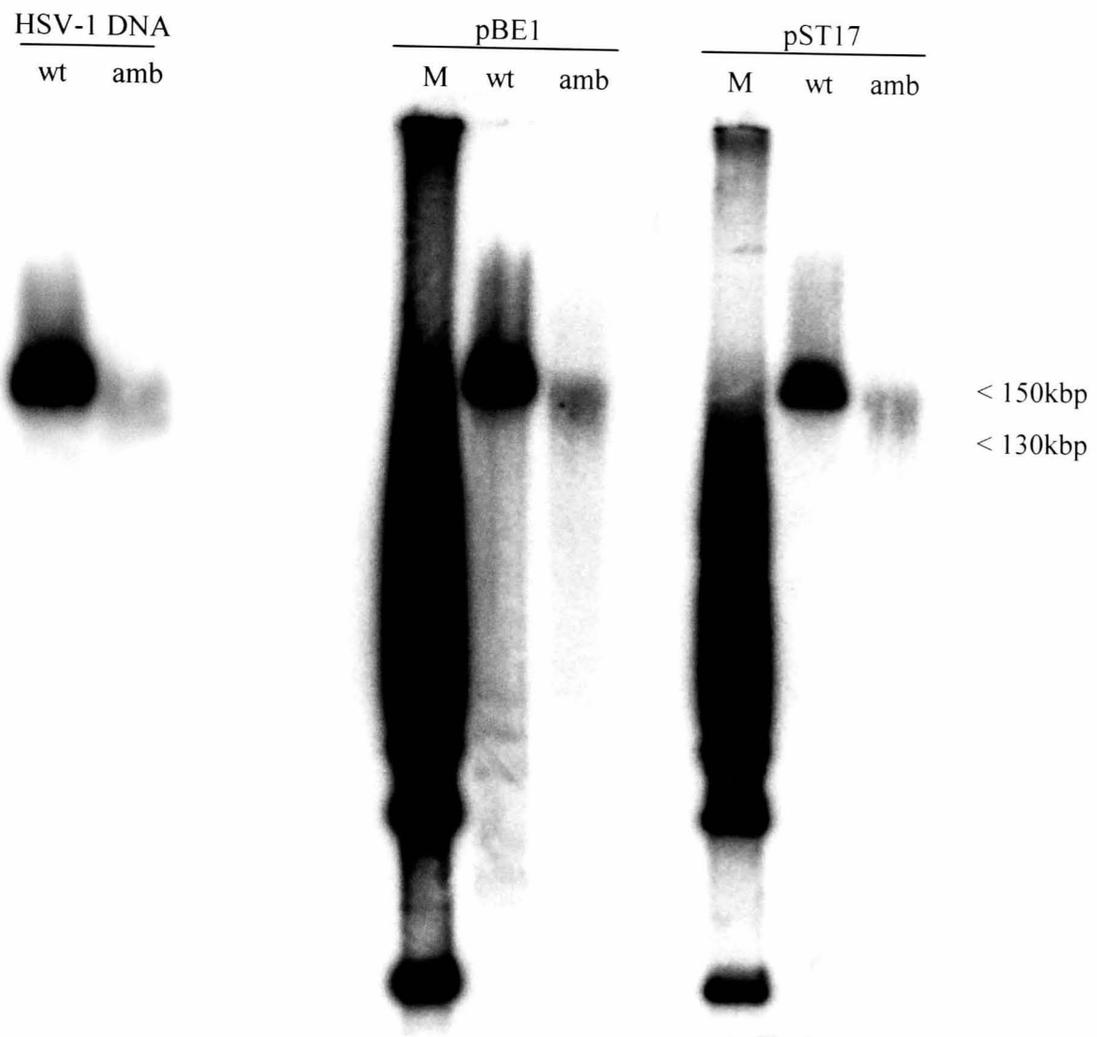


Figure 16 – Distribution of termini in packaged *amb*UL12.

BHK monolayers were infected with 3 p.f.u. / cell wt HSV-1 (wt) or *amb*UL12 (amb). 16 h.p.i. DNase-resistant DNA was gently extracted with no phenol chloroform extraction and duplicate samples representing 6.6 x 10^5 cells were immediately resolved by PFGE. The gel was blotted, divided and probed with HSV-1 genomic DNA, pBE1 or pST17. The 5 kbp marker (M) was used to determine the position of the 130 and 150 kbp fragments.

range 130-150 kbp represent species where packaging has stalled. An alternative possibility is that molecules of this size are observed because the ends of packaged full length *amb*Ul2 genomes are more susceptible to DNase than completely packaged HSV-1 molecules.

3.5.4 Investigation of the termini in extracellular *amb*UL12 virions

The packaged DNA investigated in sections 3.5.2 and 3.5.3 was derived from whole cells and therefore contains molecules originating from the nucleus and cytoplasm. Although *nuc* mutants are compromised in producing infectious virus small amounts are nevertheless generated. Egress of capsids from the nucleus into the cytoplasm appears to be linked to a head full packaging event (Homa and Brown, 1997). It was therefore decided to examine the termini of DNA molecules obtained from extracellular *amb*UL12 and wt HSV-1 virions (section 3.3.6).

Duplicate 0.5 μg DNA samples were digested with *Bam*HI and separated on a 0.8% LB-agarose gel. The gel was Southern blotted, the membrane divided and probed with ^{32}P labelled pBE1 and pST17. The blot was then analysed on a phosphorimager.

Figure 17 shows the results obtained from infected BHK cells although the results derived from infected Vero cells were essentially identical. Although only one set of samples was tested from each cell line, all samples showed ratios close to one for both junction:long terminal fragments and junction:short terminal fragment. This suggests that *amb*UL12 capsids that egress into the cytoplasm and are released from the cells are correctly processed at both termini.

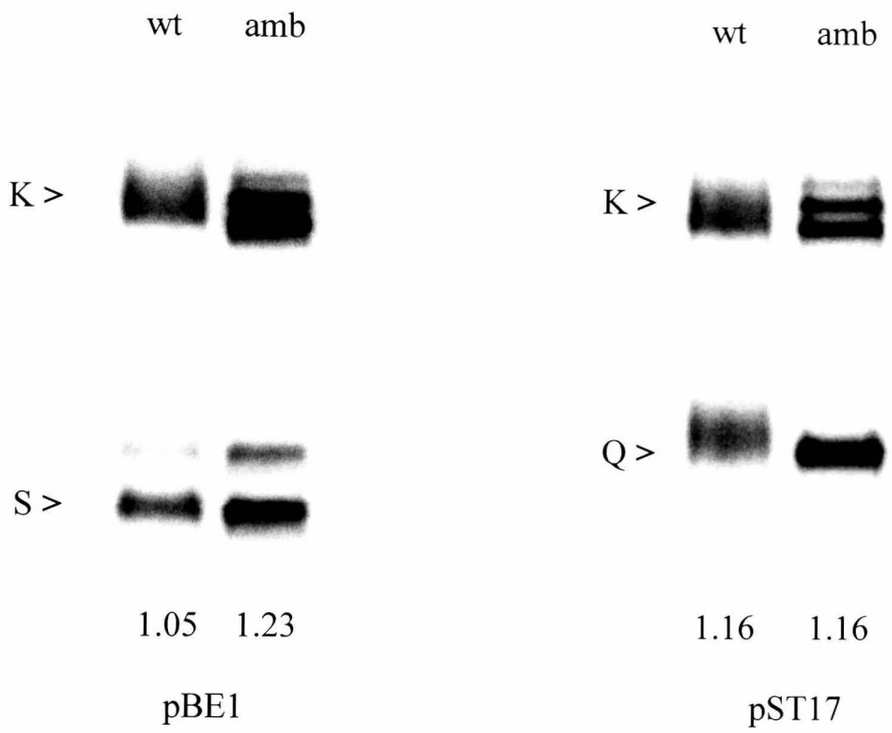


Figure 17 – Terminal fragments contained in extracellular virions.

Duplicate DNA samples from purified extracellular virions derived from infected BHK cells were digested with *Bam*HI and separated on a 0.8% LB-agarose gel. After blotting the membrane was divided and probed with pBE1 and pST17. The position of the joint fragment (K) and the terminal fragments (S or Q) are indicated. The numbers below the blot indicate the ratio of the K fragment to the total of the S or Q fragments.

3.5.5 Investigation of terminal fragments in high molecular weight DNA

A number of studies have shown that hmw HSV-1 DNA contains free long but not short termini (McVoy and Adler, 1994; Severini *et al.*, 1994; Zhang *et al.*, 1994; Martinez *et al.*, 1996; Goldstein and Weller, 1998; McVoy *et al.*, 2000). Since *amb*UL12 appears to cleave viral DNA with lower efficiency and specificity than wt HSV-1 the termini present in hmw *amb*UL12 DNA were examined.

BHK and Vero cell monolayers were infected with wt HSV-1 and *amb*UL12. 16 h.p.i. the cells were harvested, embedded in agarose blocks and processed for total cellular DNA. Each block was cut in three and subjected to PFGE (DNA was separated at 6 v / cm for 16 h at 14°C in 0.5 x TBE buffer using pulse times from 1 - 50 sec, increasing linearly). The blocks were cut from the well of the gel and the gel ethidium bromide stained to ensure that the unit length DNA had migrated into the gel. Each of the blocks were then digested with 40 U of *Bam*HI in a final volume of 200 µl at 37°C for 4 h. Following electrophoresis of duplicate samples in a 0.8% LB agarose gel the samples were Southern blotted, the membrane divided and probed with radiolabelled pBE1 or pST17.

Figure 18 shows that although there is a lot of smearing, possibly due to branched structures or partial cleavage of the DNA by the restriction enzyme, the wt HSV-1 samples generate a strong, distinct band corresponding to the joint fragment detected by both pBE1 and pST17. Bands corresponding to the long terminus containing one and two *a* sequences were also detected in the wt HSV-1 samples with the pBE1 probe. Although not very clear in Figure 18 *amb*UL12 also contained similar bands from the L terminus in hmw DNA. Surprisingly, the pST17 probe detected small amounts of

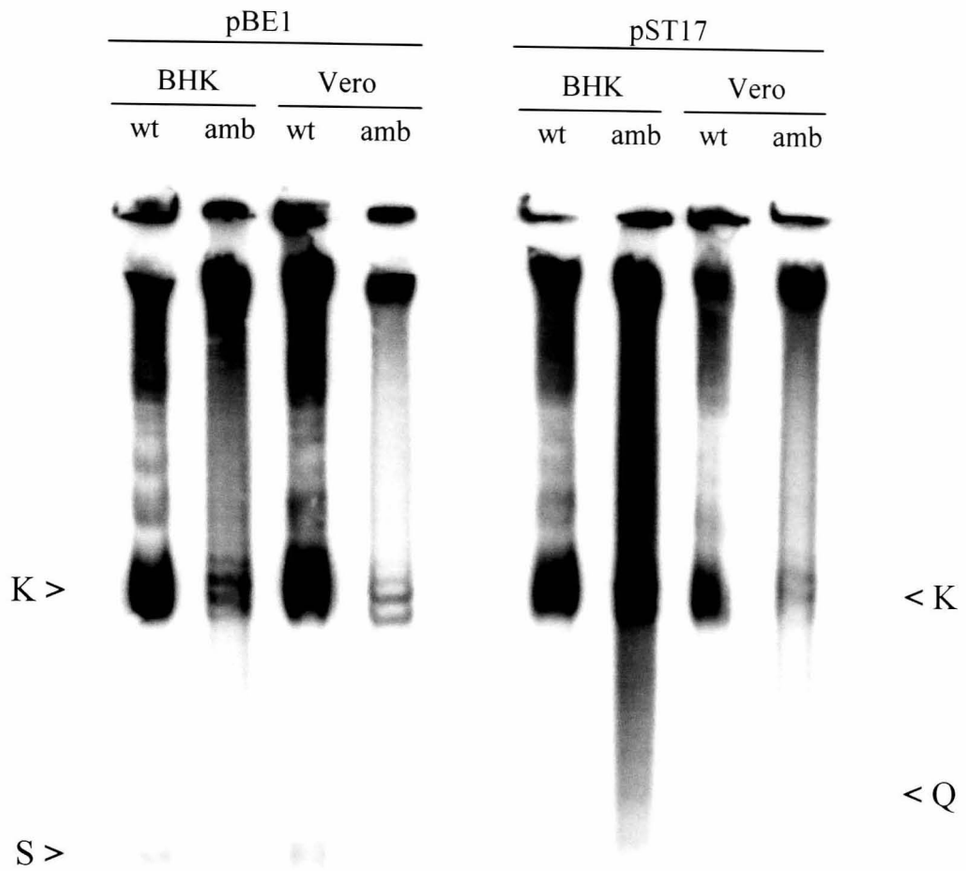


Figure 18 – Termini in high molecular weight DNA.

BHK and Vero monolayers infected with 3 p.f.u. / cell wt HSV-1 (wt) or *amb*UL12 (amb) were harvested 16 h.p.i., embedded in agarose blocks and total DNA prepared. The blocks were divided in three and the "well" DNA was separated from linear genomic DNA by PFGE. The well DNA was removed, and duplicate samples were digested with *Bam*HI and separated on a 0.8% LB-agarose gel. The gel was blotted, divided and probed with radiolabelled pBE1 or pST17. The postions of the joint (K) and terminal fragments (S and Q) are indicated.

terminal fragment corresponding to R_S. Previous studies have never detected the short terminus in hmw DNA, presuming it to be released or degraded after the initial cleavage event. Therefore its presence in the wt HSV-1 samples here cannot be easily explained. Nonetheless, the prominent smear found in the hmw *amb*UL12 DNA samples which were probed for the short terminus clearly differs from the equivalent wt HSV-1 samples. The presence of a similar smear has also been reported for other *nuc* mutants probed with S terminal DNA fragments (Martinez *et al.*, 1996a) and further demonstrates aberrant cleavage events in *nuc* mutant infected cells.

3.6 Discussion

The experiments described in this section extend the characterisation of the *amb*UL12 HSV-1 mutant. Although the general properties of *nuc* mutants were confirmed there were also a number of novel observations. (i) The *amb*UL12 mutant is deficient in both the replication and packaging of viral DNA, (ii) there appears to be an additional impairment in release of packaged DNA into the cytoplasm, (iii) the phenotype of the virus is similar in three different cell lines, (iv) cleavage of concatemeric DNA is aberrant and inefficient (v) replicated *amb*UL12 DNA is structurally distinct from wt HSV-1 DNA, (vi) the majority of intact, encapsidated *amb*UL12 DNA has a different mobility to wt HSV-1, (vii) only encapsidated genomes that are full length and possessed both termini were released from *amb*UL12 infected cells.

Previous studies on *nuc* mutants revealed that although the UL12 gene was not absolutely essential, UL12 null mutants were inefficient at producing viable virus (Weller *et al.*, 1990; Martinez *et al.*, 1996; Gao *et al.*, 1998). Studies by Patel *et al.* (1996), and confirmed here, showed that an amber mutation inserted into the UL12

ORF of *amb*UL12 generated a virus with a similar phenotype to previously described *nuc* mutants (Figure 1; Weller *et al.*, 1990; Martinez *et al.*, 1996; Gao *et al.*, 1998). Through the use of a marker rescued virus (*amb*UL12R) it was demonstrated that the amber mutation was the only lesion in *amb*UL12 contributing to the phenotype of the virus (Patel *et al.* (1996); Figure 1, 2, 3 and 11).

The major defect in *nuc* mutants has been described as the inability of capsids to egress from the nucleus into the cytoplasm (Shao *et al.*, 1996). Although there are reduced levels of viral DNA in the cytoplasm of *amb*UL12 infected cells (Figure 3 and 4) this is also due, at least in part, to defects in the viral DNA replication and packaging processes. The replication defect also appears to be cell type dependent. Infected BHK and Vero cells showed an approximately 3-fold reduction in replication compared to wt HSV-1. In contrast, FG293 and the complementing S22 cells showed only a very slight reduction in the amount of DNA synthesised. As described in section 3.2.3 there is a precedent for the involvement of virally encoded nucleases in the replication of their DNA. Endonuclease VII, encoded by gene 46 of bacteriophage T4, is required at late times of infection to initiate recombination dependent replication (Kemper and Brown, 1976; Kemper *et al.*, 1981a and 1981b; Kemper and Janz, 1976). However it is unlikely that the alkaline nuclease has a role identical to that of endonuclease VII because, as can be seen in Figure 5, *amb*UL12 synthesises less DNA than wt HSV-1 from the earliest time points. This suggests that if there is a switch from origin-dependent to recombination-dependent replication initiation then the nuclease is not involved. It has also been shown that *nuc* mutants are capable of driving intramolecular inversion of plasmids suggesting that the nuclease is not directly involved in recombination events (Martinez *et al.*, 1996a), and that the products of the seven DNA replication genes alone

are sufficient to drive inversion events (Weber *et al.*, 1988). However the association of the alkaline nuclease with the major DNA binding protein, encoded by UL29, means that a direct role in the replication of viral DNA cannot be dismissed (Littler *et al.*, 1983; Thomas *et al.*, 1992; Vaughan *et al.*, 1984).

Weller and co-workers have demonstrated in several papers, with various *nuc* mutants, that the cleavage and packaging of viral DNA is almost identical to wt HSV-1 (Weller *et al.*, 1993; Martinez *et al.*, 1996a and 1996b). However, the same conclusions cannot be drawn from the experiments presented here. Comparisons of DNase-resistant DNA obtained form wt HSV-1 and *amb*UL12 infected cells show a 15-20 fold drop in the packaging of *amb*UL12 DNA in three different cell lines (Table 1). Moreover Figure 7 shows that the cleavage of DNA is also substantially reduced, as judged by the detection of reduced amounts of unit length DNA by PFGE analysis of replicating viral DNA. This is in agreement with previous reports demonstrating close linkage of the cleavage and packaging processes (Ladin *et al.*, 1980, 1982; Sherman and Bachenheimer 1987, 1988).

Encapsidated, unit length genomes are expected to produce equimolar amounts of joint, L and S termini. As expected, examination of DNase-resistant wt HSV-1 DNA obtained from whole cell extracts showed that the ratio of joint to L terminus and joint to S terminus were both close to one (Figure 15). However in *amb*UL12 samples the joint fragment was generally in excess over both the L and S termini suggesting that cleavage at both termini was frequently aberrant. Recently the UL25 null mutant KUL25NS was examined for its ability to package DNA (McNab *et al.*, 1998; Stow, 2001). Stow observed that in KUL25NS DNase-resistant DNA there was an

overrepresentation of the L terminus and an underrepresentation of the S terminus indicating that the majority of packaging events failed to encapsidate a full length genome. These data additionally supported a model where polarity of packaging is from U_L to U_S. In contrast, it appears that *amb*UL12 has a different phenotype with both termini underrepresented compared to the joint fragment (Figure 15). Figure 16 shows PFGE separation of full length, encapsidated DNA that has been hybridised to probes for R_L (pBE1) and R_S (pST17) and demonstrates that the majority of molecules are slightly smaller than full length genomes. If packaging does occur from U_L to U_S then pBE1 would be expected to detect molecules from 3 – 150 kbp whereas pST17 would only detect molecules over 128 kbp. This is the case for both wt HSV-1 and *amb*UL12 suggesting that packaging of *amb*UL12 does occur from U_L to U_S. If the pST17 probe detected molecules shorter than 128 kbp then aberrant cleavage would have to have either occurred at both termini or packaging, alternatively, initiated from either terminus. In any case Figure 15, in conjunction with Figure 16 suggest that although many cleavage events in *amb*UL12 infected cells produce molecules with either appropriately sized R_L or R_S termini few contain both. As a result the majority of molecules are below unit length.

Figure 19 illustrates possible cleavage events that could also explain the results observed in Figure 15 and 16. In the first model the two cleavage events are identical to wt HSV-1, with the initial cleavage event directed by the *pac*2 site within Uc in the TR_L region, and the second cleavage by the *pac*1 site within Ub in the TR_S region, resulting in a genome length molecule with the correct termini being packaged. In the second model cleavage occurs as normal at the *pac*2 site but the second cleavage takes place before the *pac*1 site, and outside the region hybridising to the pST17 probe. The third

model predicts that the initial cleavage is aberrant, occurring outside the region of the pBE1 probe but the final cleavage at the *pac*1 site takes place as normal. The important aspect of models two and three is that they produce molecules that are shorter than unit length with one terminus underrepresented. It is also possible that in *amb*UL12 full length molecules are packaged (model 1) but either one or both termini are left protruding form the capsid, resulting in their susceptibility to degredation upon DNase I treatment.

Two potential explanations can be postulated for abnormal cleavage of *amb*UL12 DNA. Firstly, as *amb*UL12 DNA is more complex than wt HSV-1, in that it contains many more branched structures (Martinez *et al.*, 1996a), it is possible that the cleavage / packaging machinery cannot access the appropriate cleavage sites. The increased complexity of *amb*UL12 DNA can be clearly seen in Figure 8, in which smearing of large fragments of restriction enzyme digested hmw DNA was observed. Also, expected restriction fragments above 50 kbp could not be detected or were present at reduced levels. Martinez and co-workers have also shown that, in contrast to wt HSV-1, several *nuc* mutants were unable to release DNA from the wells of pulsed field gels when digested with enzymes that cut only a few times per genome (Martinez *et al.*, 1996). A second possibility is that the alkaline nuclease is directly involved in providing cleavage specificity. Although in vitro studies with purified alkaline nuclease show non-specific cleavage of DNA (Goldstein and Weller, 1998; Henderson *et al.*, 1998), Chou and Roizman, (1989) demonstrated that the nuclease was one of several viral proteins that could bind the *a* sequence, and although the binding was non-specific the nuclease may act in conjunction with other viral proteins which confer specificity. In the absence of UL12, sites not normally cleaved to initiate or terminate packaging

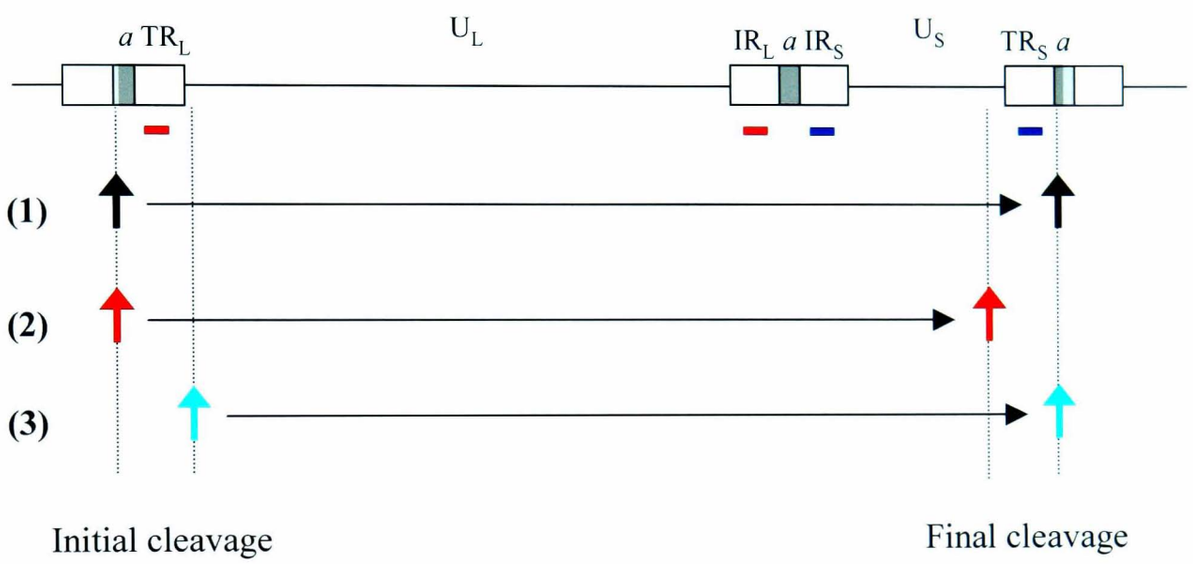


Figure 19 – Possible cleavage mechanisms in *amb*UL12.

Map of the HSV-1 genome showing where the pBE1 (▬) and pST17 (▬) probes hybridise. (**1**) Cleavage and packaging progress normally producing a full length packaged genome with correct termini. (**2**) Cleavage and packaging initiate correctly but abort at some stage within the S segment. (**3**) An initial aberrant cleavage event within the L segment is followed by termination at the correct site within TR_S. See text for details.

might become susceptible to cleavage. This can be easily envisaged at the late stages of the head filling process where more energy is required to package the DNA (Smith *et al.*, 2001) and the rate of packaging is reduced. Further evidence for reduced cleavage specificity is presented in chapter 5 of this thesis.

High molecular weight HSV-1 DNA was previously shown to contain a small amount of free long termini but no free short termini (McVoy and Adler, 1998; Severini *et al.*, 1994). In the experiment shown in Figure 18 a small amount of free long terminus was present in both hmw wt HSV-1 and *amb*UL12 DNA. It has been suggested for wt HSV-1 that after the initial cleavage event between two *a* sequences the *pac* 2 sequence within R_L is inserted into capsids while the *pac* 1 sequence is degraded (McVoy and Adler, 1994; McVoy *et al*, 2000; Stow, 2001). In contrast to previously published results similar amounts of S and L termini were present in the hmw wt HSV-1 samples. The hmw DNA found with *nuc* mutants, including *amb*UL12, also contains S termini which are detected as a heterogeneous smear (Figure 18; Martinez *et al.*, 1996a). It is not clear if this product is another example of aberrant cleavage or represents degradation of the joint fragments of concatemeric DNA. In any case, the result again suggests an impairment in the cleavage mechanism of *nuc* mutants or difference in the structure of their DNA.

In addition to the differences described above, *amb*UL12 DNA is generally of "poorer quality" than wt HSV-1. Restriction enzyme digests of *amb*UL12 DNA from whole cells are more smeared and they do not contain expected restriction fragments above 50 kbp in size (Figure 8) indicating that the replicated DNA probably contains many unresolved branched structures resulting from replication events. This has implications

for both the translocation of DNA into the capsid (the internal diameter of the portal protein is only 5 nm (Newcomb *et al.*, 2001)) and the stability of packaged capsids that may contain branched DNA products. The use of 2D agarose gel electrophoresis (Severini *et al.*, 1996) would be useful to further determine the structure of both hmw and packaged *amb*UL12 DNA.

Although there is a definite block in the egress of capsids into the cytoplasm of *amb*UL12 infected cells, a small number do mature into the cytoplasm. Analysis of DNA purified from extracellular *amb*UL12 virions reveals that: (i) The DNA has the same mobility as wt HSV-1 (Figure 9), (ii), restriction enzyme digests of extracellular DNA are identical to wt HSV-1 (Figure9), (iii) extracellular virion DNA contains the correct proportions of terminal fragments (Figure 16). These observations indicate that cleavage was more specific for these molecules, although direct investigation of the DNA ends will be necessary to conclude that cleavage occurs at an identical position to wt HSV-1. Nevertheless, these results, taken together, imply that only capsids containing correctly processed DNA molecules will egress from the nucleus. In addition S1 nuclease digestion of DNA obtained from extracellular virions revealed that wt HSV-1 and *amb*UL12 DNA contain similar amounts of single stranded nicks (Figure 10). In agreement with previous reports (Wilkie, 1973; Goldstein and Weller, 1998b) the smeared product of S1 digestion suggests that the nicks are randomly distributed.

Chapter 4: Interaction of the alkaline nuclease with other replication and packaging proteins

4.1 Introduction

There are two mutant herpesviruses; KUL25NS, an HSV-1 mutant which does not express UL25 (McNab *et al.*, 1998; Stow, 2001), and an HCMV UL97 null mutant which does not express the homologue of the HSV-1 UL13 protein (Wolf *et al.*, 2001), which have phenotypes quite similar to the *amb*UL12 mutant. Both mutants cleave concatemeric DNA at wt levels but packaging events are frequently aborted, resulting in the accumulation of A-capsids. In addition the HCMV UL97 null mutant synthesises DNA 4-6 fold less efficiently and produces 100-1000 fold less virus than wt HCMV.

The similarity in phenotype suggests that HSV-1 UL12 may interact with one or both of these proteins or have a role at the same point in the replication / packaging process. The phenotype of *nuc* mutants also suggests the possibility that the alkaline nuclease may directly interact with other components of the viral packaging machinery or be modified by other viral proteins such as the protein kinase encoded by UL13. Indeed Chou and Roizman, (1989) reported that the alkaline nuclease was one of several viral proteins that formed a complex with the *a* sequence, although it could not be confirmed if this was a direct or sequence-specific interaction. The interaction of the alkaline nuclease with at least one other protein, the major single stranded stranded DNA binding protein (mDBP) encoded by UL29, has been reported (Vaughan *et al.*, 1984; Thomas *et al.*, 1988 and 1992). Therefore, a preliminary investigation into the interaction of the alkaline nuclease with selected other viral proteins, known to be

involved in the replication and packaging processes, was performed using a panel of recombinant baculoviruses expressing various HSV-1 proteins.

4.2 Co-immunoprecipitation of UL12 and UL29

Since the interaction of UL12 and UL29 had already been reported in several publications (Vaughan *et al.*, 1984: Thomas *et al.*, 1988 and 1992) this was chosen as the first interaction to be examined.

12 well plates seeded with 1.2 x 10^6 *S.f.* cells were singly or dually infected with 5 p.f.u. / cell of the recombinant baculoviruses AcUL12 and AcUL29, expressing the alkaline nuclease and the mDBP respectively. The proteins were labelled with ^{35}S methionine as described in methods, and extracts prepared. The extracts were incubated with MAb Q1 (anti-UL12) or MAb 7381 (anti-UL29) and the complexes recovered with Protein-A sepharose beads. The immunoprecipitates were resolved on an 8% SDS-PAGE gel along with samples of total proteins and the soluble protein extracts.

UL12 and UL29 (85 and 136 kDa respectively) were readily detectable in the total and soluble protein fractions from singly and dually infected cells (Figure 20A and 20B). MAb Q1 efficiently precipitated UL12 from both AcUL12 infected samples (Figure 20A). Although a significant amount of UL29 was present in the precipitate from the co-infection when using the Q1 Ab (Figure 20A) the result is inconclusive because of the presence of a strong band of similar size in the AcUL29 single infection. Figure 20B shows that when using the anti-UL29 antibody, MAb 7381, the presence of UL29 is required for precipitation of UL12 to occur. These results, therefore, support previous reports of an interaction between UL12 and UL29 in HSV-1 infected cells,

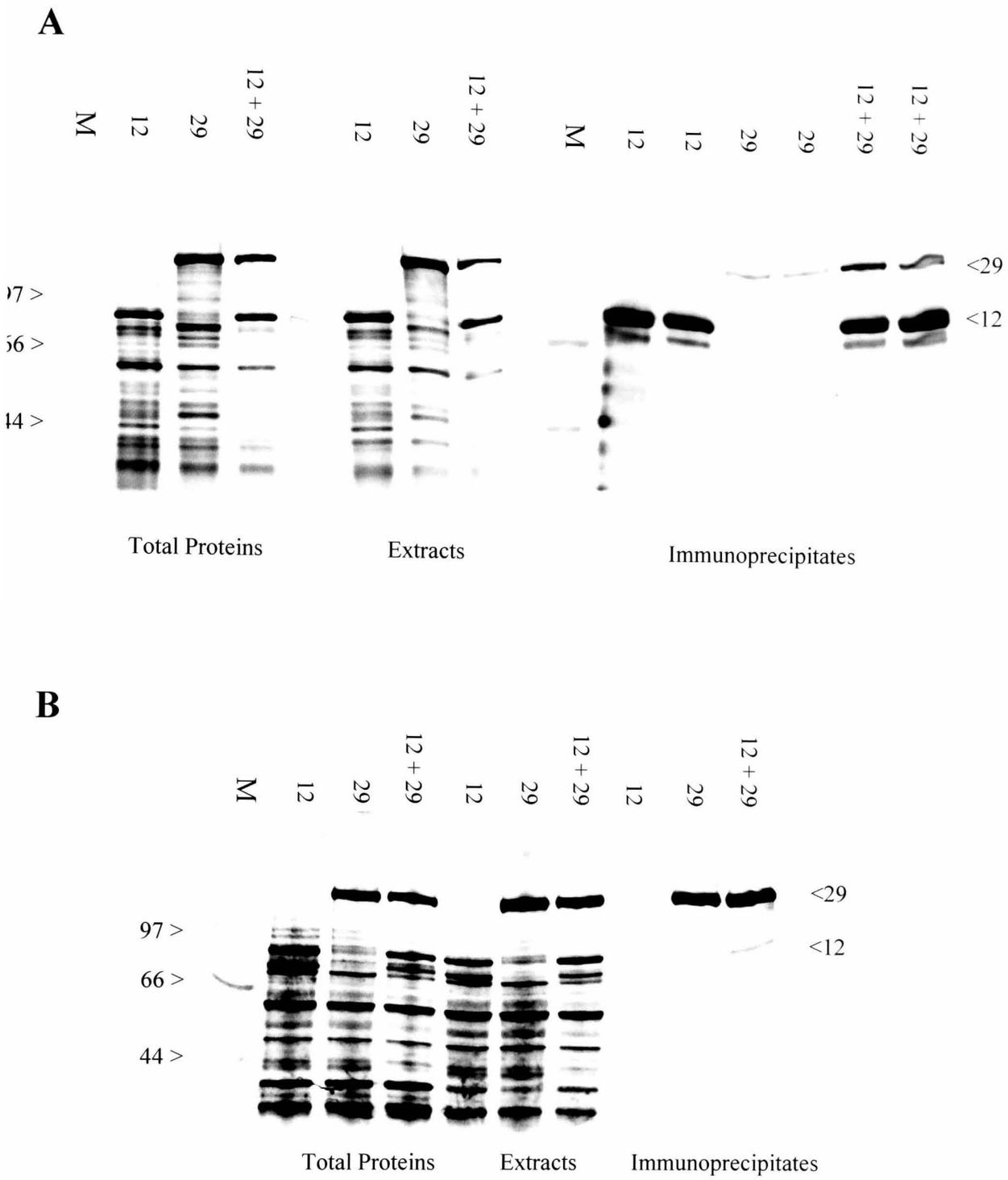


Figure 20 – Co-immunoprecipitation of UL12 and UL29 proteins.

S.f. cells were singly or dually infected with AcUL12 (12) or AcUL29 (29) and labelled with ^{35}S-methionine. The cell extracts were incubated with antibody Q1 (**A**) or antibody 7381 (**B**). The total proteins, starting extracts and immunoprecipitates were resolved on an 8% SDS-PAGE and exposed to a phosphorimager screen. The position of selected radiolabelled Rainbow Marker (M) are shown. The position of the UL12 and UL29 bands are also indicated. Note that in (**A**) the immunoprecipitations were performed in duplicate.

although more stringent washes would be required to demonstrate a definitive interaction.

4.3 Immunoprecipitation of UL12 and UL25

Because of the similarity in phenotype between UL12 and UL25 mutants, UL25 was examined for a possible interaction with UL12.

12 well plates of *S.f.* cells were infected with 5 p.f.u. / cell AcUL12 and/or AcUL25 and processed as described in section 4.2 except the antibodies used were MAb Q1 and PAb BwP12 against UL12, and MAb anti-UL25 against UL25.

Figure 21A showed that MAb Q1 precipitated UL12 from cells infected with AcUL12 alone or in conjunction with AcUL25. However, UL25 could not be detected in either sample infected with AcUL25 even though the protein was present in the soluble extract. A similar result was obtained when the polyclonal antibody BwP12, specific for UL12, was used (Figure 21B). When the MAb anti-UL25 was used, UL25 was precipitated strongly from the single and double infections using AcUL25 (Figure 21C). Although there is a weak band co-migrating with UL12 in the dual infected sample it is also present at a slightly higher amount in the singly infected AcUL12 sample indicating that precipitation of this protein is not dependent upon the presence of UL25. Taken together, this data suggests that UL12 and UL25 do not directly interact.

4.4 Immunoprecipitation of UL12, UL15 and UL28

The products of UL15 and UL28, which are essential for the packaging of viral DNA, have recently been shown to interact (Koslowski *et al.*, 1999; Abbotts *et al.*, 2000).

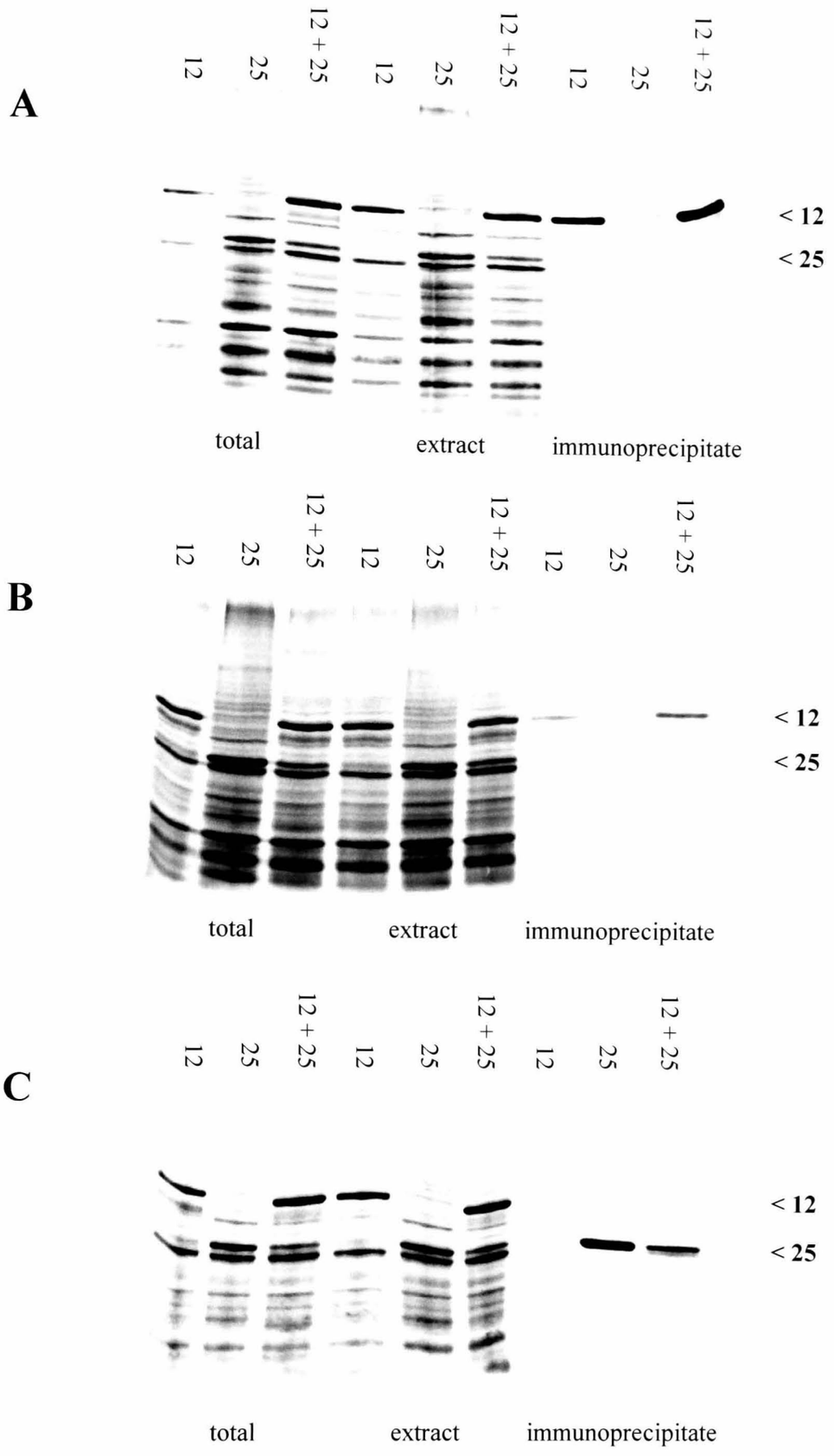


Figure 21 – Immunoprecipitation of UL12 and UL25.

S.f. cells were infected with AcUL12 (12), AcUL25 (25) or dually infected (12 + 25) and labelled with ^{35}S-methionine. Cell extracts were incubated with Q1 (**A**), BwP12 (**B**) or anti-UL25 (**C**). Total protein extracts (total), the soluble fraction (extract) and the immunoprecipitate were resolved on an 8% SDS-PAGE gel and exposed to a phosphorimager screen. The position of the UL12 and UL25 proteins are indicated.

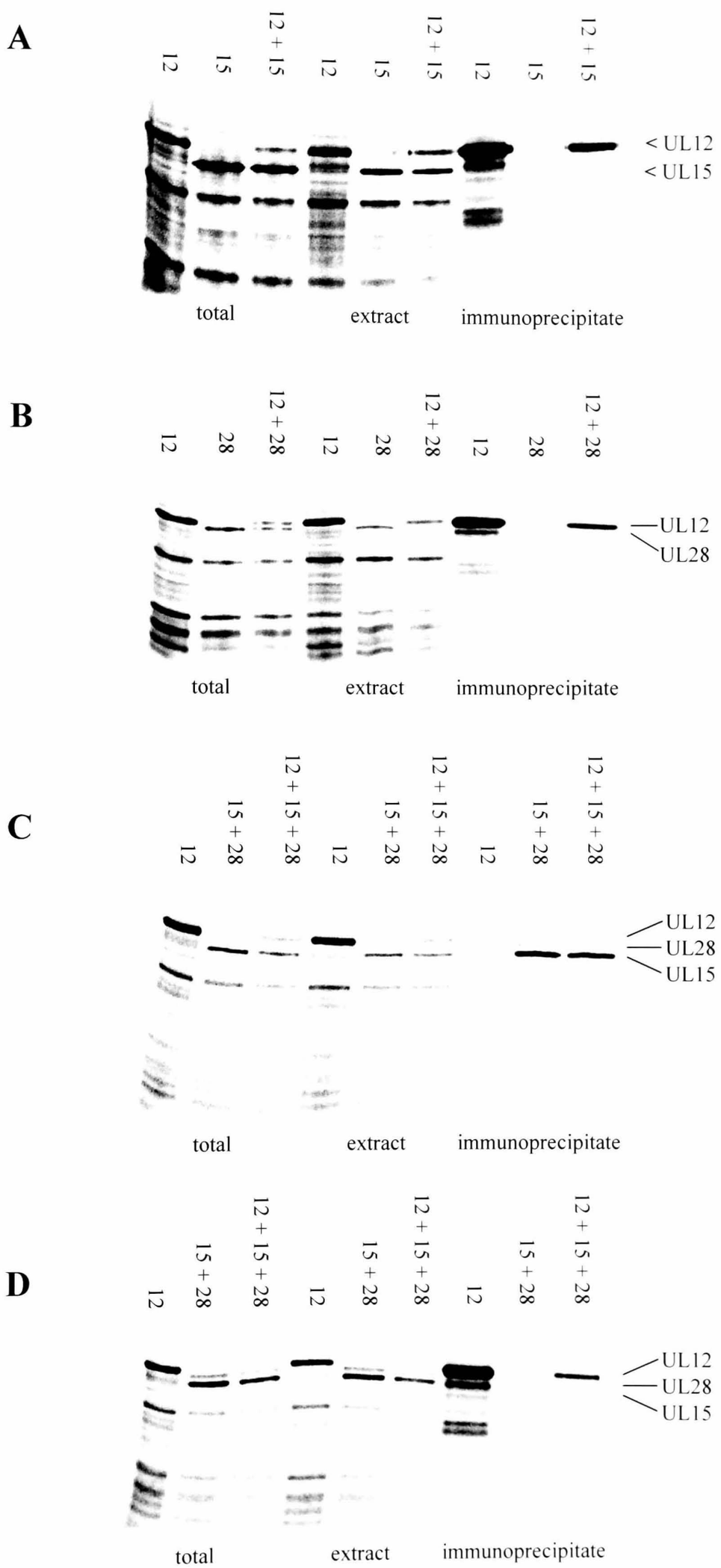
Several lines of evidence suggest they may function in a manner analogous to the terminase complexes of dsDNA bacteriophage; these include limited sequence homology (Davison, 1992) and a consensus ATP binding motif which, through mutation studies, was demonstrated to be essential for the function of UL15 (Yu and Weller, 1998). To determine if either protein, or the complex, interacts with UL12, *S.f.* cells were infected with AcUL12, AcUL15-pp65 or AcUL28 in the various combinations shown in Figure 22. The labelled soluble extracts were incubated with Mab Q1 or anti-pp65 antibody and precipitated with ProteinA beads (Figure 22).

As before MAb Q1 precipitates UL12 in the single or dual infected samples (Figure 22A and B). However, neither UL15 nor UL28 was efficiently co-precipitated with UL12 suggesting no interaction occurred between the individual proteins.

To determine whether the UL15-UL28 complex can interact with UL12, *S.f.* cells were triply infected and immunoprecipitations performed with MAb Q1, (Figure 22C) and MAb anti-pp65, (Figure 22D). In both panels all three proteins produced from the triple infection can be clearly seen in the total and soluble fractions. In Figure 22C only UL12 was precipitated. In contrast the anti-pp65 antibody co-precipitates both pp65 tagged UL15 and UL28, in agreement with previous results (Abbotts *et al.*, 2000), although UL12 was not detected. Therefore, the preliminary results presented in Figure 22 suggest that UL12 does not interact with UL15 or UL28 alone or when complexed. However, since UL15 and UL28 migrate so closely with major UL12 related bands a western blot would be necessary to exclude the possibility of weak interactions.

Figure 22 – Immunoprecipitation of UL12 and UL15 and UL28.

S.f. cells were infected with AcUL12 (12), AcUL15 (15), AcUL28 (28) in the combinations indicated and labelled with ^{35}S-methionine. Cell extracts were incubated with Q1 (**A**), (**B**) and (**D**) or anti-pp65 (**C**). Total protein extracts (total), the soluble fraction (extract) and the immunoprecipitate were resolved on an 8% SDS-PAGE gel and exposed to a phosphorimager screen. The positions of UL12, UL15 and UL28 proteins are indicated.


A
12
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UL15
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4.5 Immunoprecipitation of UL12 and UL6

The product of UL6 is one of the proteins essential for the packaging of viral DNA and has recently been shown to form the portal through which the DNA is inserted into the viral capsid (Newcomb *et al.*, 2001). To determine whether UL6 interacts with UL12 *S.f.* cells were infected with AcUL12 and/or AcUL6 and precipitated using MAb Q1 or PAb BwP12. Figures 23A and B demonstrate that with each antibody, UL12 was precipitated from both the single and dual infection samples but co-precipitation of UL6 could not be detected. Since the UL6 protein was poorly soluble the samples from the Q1 precipitated samples were re-run on an SDS-PAGE gel and western blotted using the PAb YE583, specific for UL6 (Figure 23C). It is clear that UL6 can be detected in the total and soluble fractions but not in the immunoprecipitated samples. Taken together these results indicate that UL12 and UL6 do not detectably interact.

4.6 Discussion

The aim of this section was to investigate possible interactions of the UL12 protein with other viral proteins which are involved in the replication and packaging of the viral genome.

The interaction of UL12 and UL29 (the major single stranded DNA binding protein, an essential component of the viral DNA replication machinery) had been reported previously in HSV-1 infected cells (Vaughan *et al.*, 1984: Thomas *et al.*, 1988 and 1992). The ability of baculovirus expressed UL12 to co-immunoprecipitate baculovirus expressed UL29, and vice versa (Figure 20), further suggests an interaction between these two proteins, although due to the relatively high background bands in the control lanes a definitive result cannot be reached in the absence of further experiments.

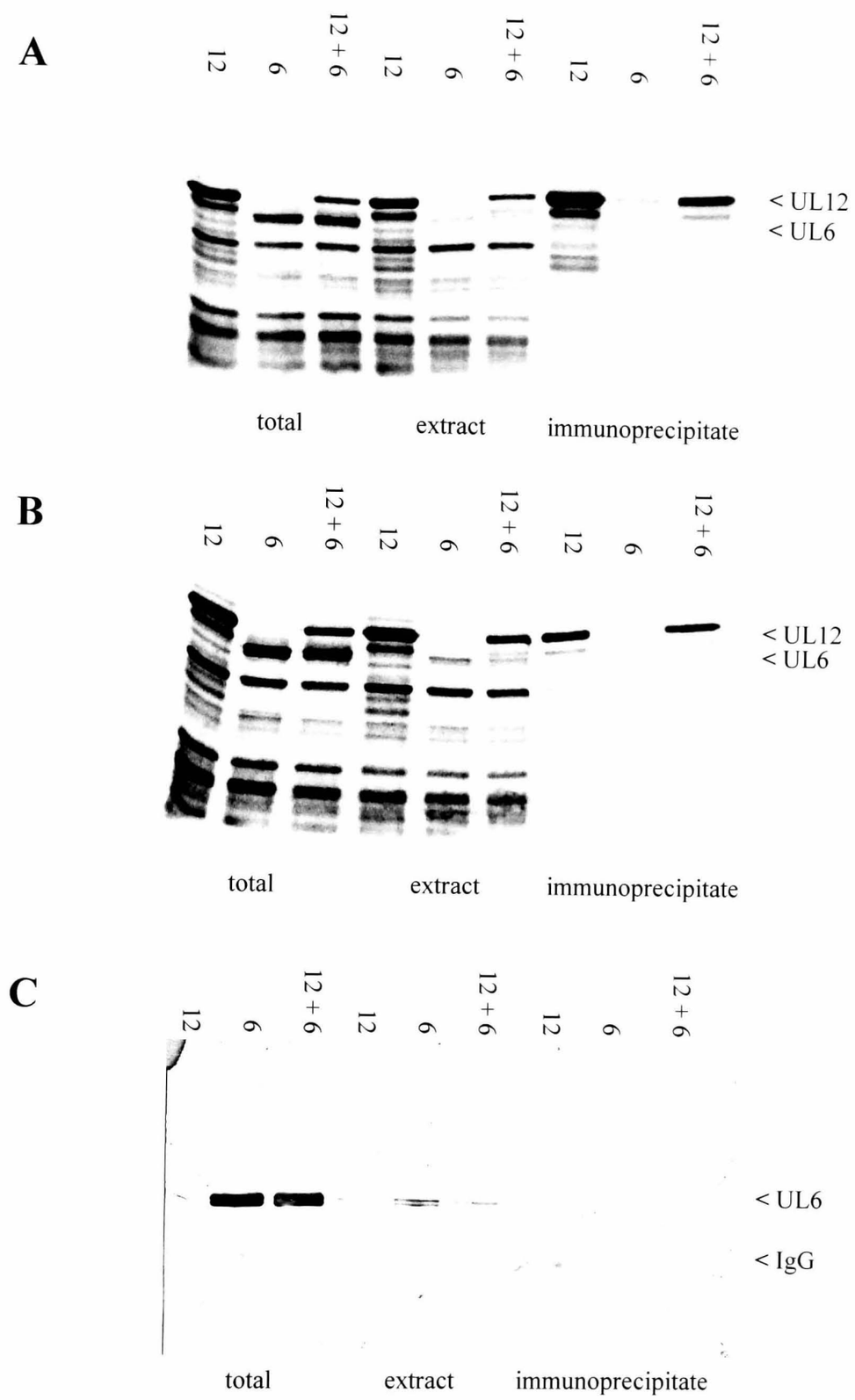


Figure 23 – Immunoprecipitation of UL12 and UL6.

S.f. cells were infected with AcUL12 (12), AcUL6 (6) or dual infected (12 + 6) and labelled with ^{35}S-methionine. Cell extracts were incubated with Q1 (**A**) or BwP12 (**B**). Total protein extracts (total), the soluble fraction (extract) and the immunoprecipitate were resolved on an 8% SDS-PAGE gel and exposed to a phosphorimager screen. The samples from (**A**) were re-run, western blotted and UL6 detected with Pab YE583 (**C**). The position of UL12, UL6 and IgG is indicated.

Nevertheless, previously published data of an interaction between UL12 and UL29, in conjunction with the results presented in in section three, which demonstrated that the *amb*UL12 null mutant was deficient in the replication of viral DNA, suggest a mechanism by which UL12 may be directly involved in the replication of viral DNA.

The UL25 null mutant, KUL25NS, shows a similar phenotype to UL12 null mutants (Shao *et al.*, 1993; McNab *et al.*, 1998; Stow, 2000), suggesting that they may have related roles in the packaging of viral DNA. However, the results presented in Figure 21 failed to detect co-immunoprecipitation of the two proteins using three separate antibodies, even though both proteins are readily detectable in the total and soluble extracts. It is therefore unlikely that these proteins interact. The possibility that the two monoclonal antibodies, Q1 and anti-UL25, both recognise epitopes that are masked in a protein complex seems unlikely since the polyclonal antibody BwP12 (specific for UL12) also did not reveal any interaction. Although the two proteins do not appear to interact this does not exclude the possibility that they may have interconnected roles in the packaging of viral DNA.

During the packaging of viral DNA any aberrant DNA structures (e.g. branches) encountered by the terminase complex could result in the termination of packaging unless the aberrant structure was resolved. Therefore, it was conceivable that UL15 and UL28, which are presumed to form the HSV-1 terminase complex (Yu and Weller,1998), might interact with UL12 thereby coupling the translocation of viral DNA into the capsid with the detection and resolution of any complex DNA structures. However, co-immunoprecipitation of UL15 or UL28, either separately or as a complex with UL12 was not be detected (Figure 22). Although there was no apparent physical

interaction between UL12 and the UL15-UL28 complex, the possibility of UL12 independently modifying non-linear DNA forms trapped at the terminase cannot be excluded.

The reason for studying a possible interaction of UL12 with UL6 uses a similar argument to that for UL15 and UL28; any non-linear DNA would be expected to either inhibit or abrogate the translocation of the viral genome through the UL6 portal complex (Newcomb *et al.*, 2001). Resolution of these structures would therefore be required for packaging to continue. The results presented in section 4.5 indicate that UL12 and UL6 do not interact.

These results, although preliminary, indicate that UL12 does not detectably interact with the packaging proteins described above. The observed interaction of UL12 with UL29 may indicate that UL12 functions to resolve aberrant structures as they are formed during replication and recombination, its actions being spatially and temporally distinct from the packaging machinery. In any case it is clear that more work is needed to define the interactions of UL12 with other viral and cellular proteins. This would help further elucidate the role of UL12 in the replication and packaging process and may give some clues as to the mechanism which must presumably control the potent nuclease activity of UL12.

Chapter 5: Replication and packaging of amplicons using *amb*UL12

5.1 Introduction

This section describes the use of a transient assay, originally described by Stow *et al.* (1983), to further investigate the role of the alkaline nuclease in the replication and packaging of HSV-1 DNA. This assay involves transfection of amplicons, plasmids that contain the HSV-1 origin of replication and packaging signals, into cells permissive for HSV-1 growth. The cells are subsequently superinfected with a helper virus, wt HSV-1 or a derivative thereof, which provides functions necessary to replicate and package the amplicon. This assay allows the *cis* and *trans*-acting factors required for the replication and packaging of viral DNA to be studied in detail (Deiss *et al.*, 1986; Stow and McMonagle, 1983; Stow *et al.*, 1998).

5.2 Cloning UL12 and UL12.5 into expression vector pCMV10

5.2.1. Introduction

To further characterise the role of UL12 and UL12.5 (the N-terminally truncated form of the protein) in the replication and packaging of viral DNA plasmids were produced which could would constitutively express the proteins when transfected into mammalian cells.

5.2.2 Cloning of UL12 and UL12.5 into pCMV10

The UL12 and UL12.5 ORFs had previously been cloned by N. Stow from HSV-1 strain 17 syn $^{+}$ into the baculovirus transfer vector pAcYM1 to generate the vectors pAc12 and pAC12.5. The ORFs were excised from the pAc12 and pAc12.5 constructs by digesting 20 ug of vector with *Bam*HI and separating the DNA on a 1% TBE agarose

gel. The bands corresponding to the ORFs were cut from the gel and purified as described in Methods. The ORFs were ligated into the MCS of plasmid pCMV10 which contains the constitutive HCMV major IE promoter and transformed into competent XL10-Gold *E. coli* (Stratagene). *Eco*RI / *Xho*I digests of plasmids isolated from the resulting colonies were performed to identify those containing inserts and determine their orientations. If inserted in the correct orientation the UL12 and UL12.5 containing plasmids would each produce a major band of 5.4 kbp and minor bands of 0.8 and 0.45 kbp respectively (Figure 24). If inserted in the opposite orientation bands of 4.6 and 1.6 kbp would be produced for UL12 and 4.25 and 1.6 kbp for UL12.5. Large scale DNA preparations were made from two colonies containing plasmids with the UL12 and UL12.5 ORFs in the correct orientation and the resulting expression constructs were named pE12 and pE12.5, respectively.

5.2.3 Expression of UL12 and UL12.5 from pE12 and pE12.5 in BHK cells

To ensure that pE12 and pE12.5 were expressing the correct products western blots were performed on lysates of transfected BHK cells.

35 mm BHK plates were transfected with 0.5 μg of pE12, pE12.5 or mock transfected using the calcium phosphate technique described in methods. Separate monolayers were also infected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. 24 hours post transfection (h.p.t.) or 16 h.p.i. cells were harvested, resuspended in 50 μl boiling mix, boiled for 10 min and 10 μl of each sample was separated on duplicate 8% SDS-PAGE gels. The gels were electroblotted and probed with MAb Q1 or PAb BwP12 at a dilution of 1:2500. HRP-conjugated Protein A sepharose was added as secondary antibody at a dilution of 1:1000 to detect bound antibody. The membranes were treated with ECL

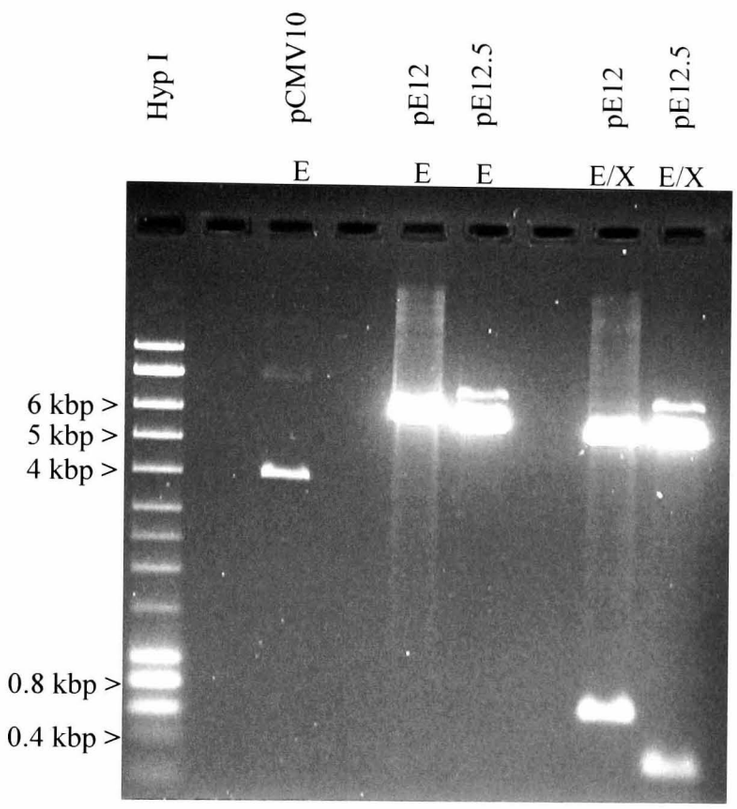


Figure 24 – Restriction enzyme digests of pE12 and pE12.5.

The ethidium bromide stained agarose gel shows *Eco*RI (E) digests of pCMV10, pE12 and pE12.5; and *Eco*RI / *Xho*I (E/X) digests of pE12 and pE12.5. Sizes of particular bands from the hyperladder I (Hyp I) are indicated at the side of the gel.

reagent and exposed to autoradiography film. To determine the transfection efficiency plates of BHK cells were also transfected with pElacZ. 24 h.p.t. the cells were treated with X-gal as described in Methods and the number of positive cells counted. The transfection efficiency for BHK cells was generally 10 – 30% positive cells.

Figure 25 shows that pE12 produced a major band of approximately 85 kDa that migrated with the corresponding major band of wt HSV-1 infected cells. Although the 85 kDa band was detected with both monoclonal or polyclonal antibodies the Q1 antibody detected many other breakdown products. Cells transfected with pE12.5 produced two products, although in reduced amounts compared to pE12, and these were detected only by the BwP12 antibody. The upper band migrated with predicted molecular weight of approximately 60 kDa, correlating with the previously reported size for UL12.5 (Costa *et al.*, 1983; Martinez *et al.*, 1996b; Bronstein *et al.*, 1997). The lower band of approximately 55 kDa has not been previously reported but probably represents a breakdown product. This experiment demonstrates that the UL12 and UL12.5 products are expressed by pE12 and pE12.5 respectively.

5.3 Transient replication and packaging assays using *amb*UL12

5.3.1 Introduction

To determine if the amplicon system yielded results similar to those in virus infected cells (see Chapter 3), the levels of replication and packaging of amplicons achieved following infection with wt HSV-1 or *amb*UL12 were compared. In addition the ability of the *amb*UL12 defect to be complemented by the expression of UL12 or UL12.5, supplied in *trans*, was assessed.

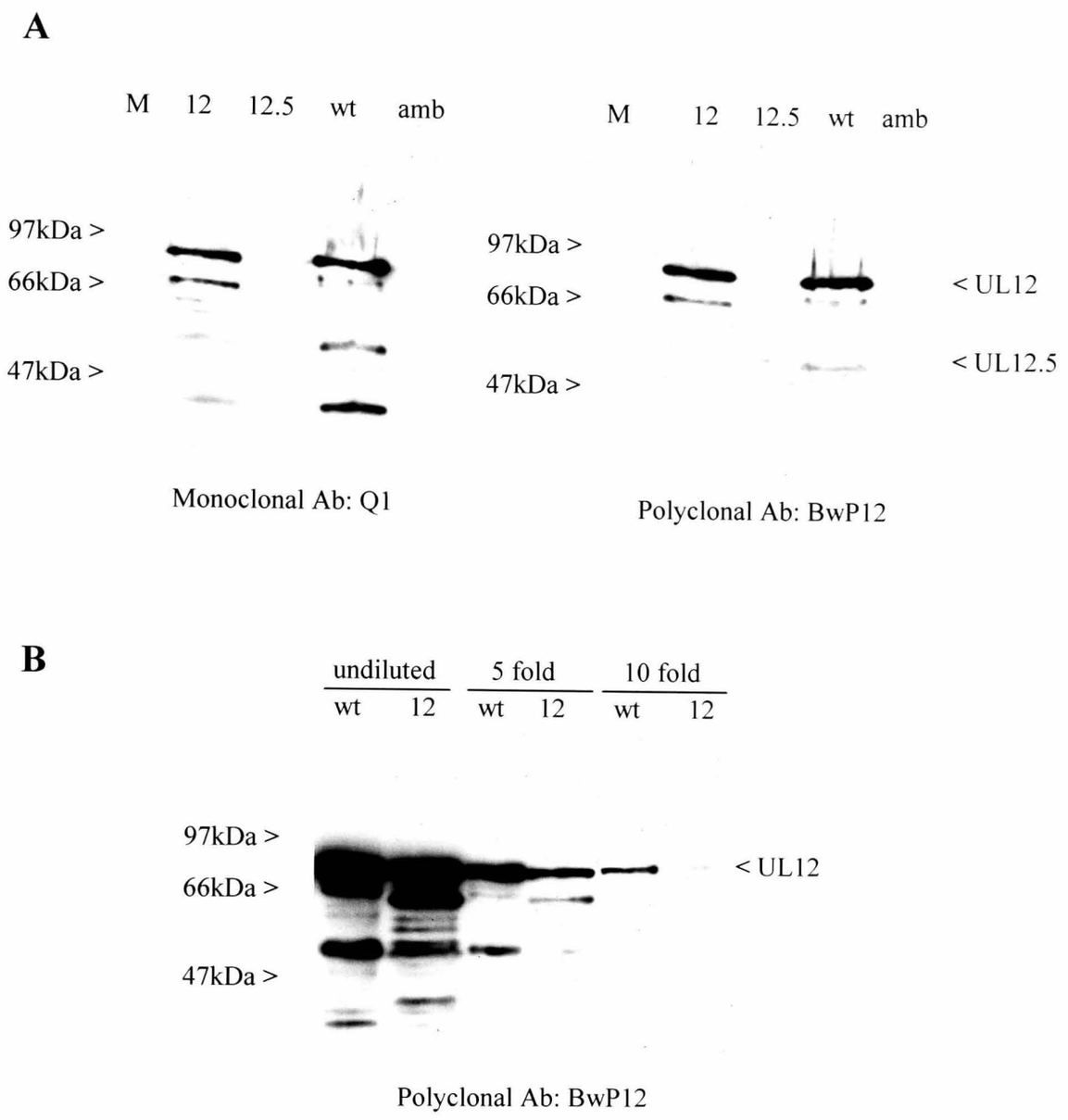


Figure 25 – Expression of UL12 and UL12.5 from pE12 and pE12.5.

(**A**) BHK monolayers were transfected with 1 μg pE12 (12), pE12.5 (12.5) or mock transfected (M). Two monolayers were also infected with 3 p.f.u. / cell wt HSV-1 (wt) or *amb*UL12 (amb). 24 h.p.t. or 16 h.p.i. cells were harvested, resuspended in 50 μl boiling mix and boiled for 10 min. Duplicate 10 μl samples were separated on a 8% SDS-PAGE gel, blotted and probed with MAb Q1 or PAb BwP12. Cells were harvested 16 h.p.i. or 24 h.p.t. and Protein A conjugated HRP was used as secondary antibody, the membrane washed with ECL reagent and exposed to autoradiographic film. (**B**) A dilution series of wt HSV-1 infected or pE12 transfected cells from A were probed with BwP12. The migration of specific bands from a Rainbow marker are indicated and the major UL12 and UL12.5 bands are indicated. Note that the (wt) and (amb) lanes in (**A**) are also shown in Figure 2.

5.3.2 Transient replication and packaging of pSA1 by *amb*UL12

The amplicon pSA1 contains the viral origin of replication, ori_S, and the minimal functional packaging signal, Uc-DR1-Ub, which represents the joint between two tandem *a* sequences (Hodge and Stow, 2001).

BHK monolayers were transfected with 1 μg pSA1 in the presence or absence of 1 μg pE12 or pE12.5 as indicated in Figure 26. The cells were DMSO boosted 4 h.p.t. as described in Methods and superinfected 6 h.p.t. with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. 16 h.p.i. the cells were harvested and processed for total and DNase-resistant DNA. Samples representing the DNA recovered from one sixth of a plate were digested with *Eco*RI and *Dpn*I (*Dpn*I digests only the input unreplicated, methylated amplicon DNA) and separated on a 0.8% LB-agarose gel. The gel was then Southern blotted and probed with ^{32}P labelled pAT153, the parental vector for pSA1.

From Figure 26 it is clear that both wt HSV-1 and *amb*UL12 are able to replicate and package pSA1. Moreover, *amb*UL12 shows a similar deficiency in replicating and packaging pSA1 as it does its own genome. Over many experiments the fold decreases in replication and packaging of pSA1, when compared to wt HSV-1, were 5.27 ± 1.83 and 15.59 ± 7.37 respectively. In an attempt to complement these defects, pE12 or pE12.5 was co-transfected along with pSA1. As can be seen in Figure 26 the co-transfection of pE12 is able to complement *amb*UL12 to a small degree but pE12.5 is not. In five separate experiments in BHK cells the fold increase in the replication and packaging of pSA1 by *amb*UL12 when pE12 or pE12.5 were co-transfected was calculated (Table 2). In support of Figure 26 there was an approximately 2-fold

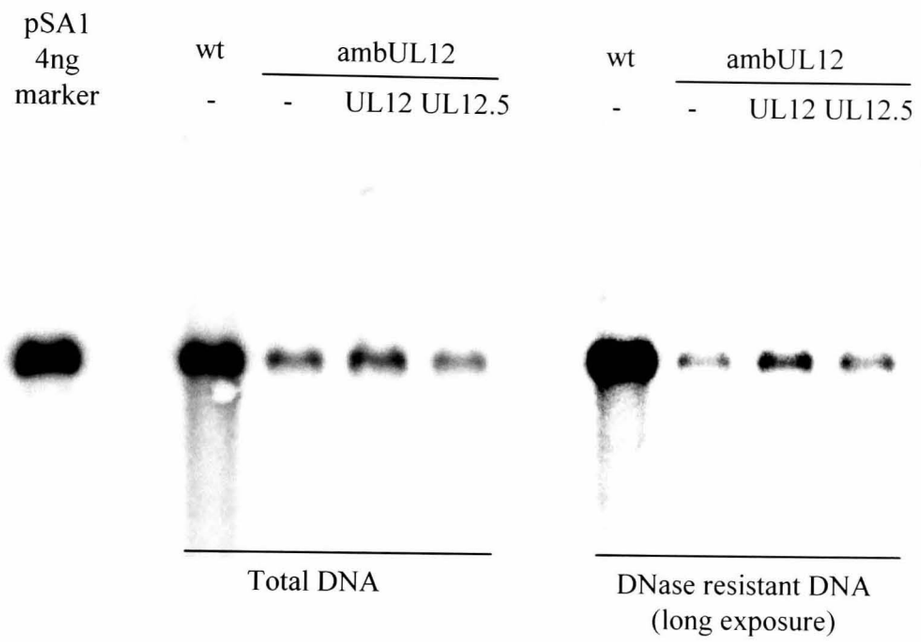


Figure 26 – Transient replication and packaging of pSA1 by *amb*UL12.

BHK monolayers were transfected with 1 μg pSA1 either alone (-), or with 1 μg pE12 (12) or pE12.5 (12.5) and superinfected with 3 p.f.u. / cell wt HSV-1 (wt) or *amb*UL12 (ambUL12). 16 h.p.i. the cells were harvested and processed for total and DNase-resistant DNA. Samples representing one sixth of a plate were digested with *Eco*RI and *Dpn*I and separated on a 0.8% LB-agarose gel. The gel was then Southern blotted and probed with ^{32}P labelled pAT153. 4 ng, *Eco*RI digested pSA1 was used as a marker. The left panel shows a 2h exposure of the total DNA samples to a phosphorimager screen. The right panel shows a 16 h exposure of the DNase-resistant samples, taken from the same membrane.

increase in the replication and packaging of pSA1 when pE12 was co-transfected with the amplicon whereas no complementation was seen with pE12.5.

Table 2. Complementation of pSA1 replication and packaging by pE12 and pE12.5

	no additional plasmid [a]	pE12[a]	pE12.5[a]
Replication	1.0	2.24 ± 0.40	0.79 ± 0.15
Packaging	1.0	2.54 ± 1.06	0.83 ± 0.32

(*a*)Replication and packaging of pSA1 by *amb*UL12 in the absence of added plasmid were assigned a value of 1.0

The average fold increase in replication and packaging upon co-transfection of pE12 or pE12.5 were calculated .± SD from 5 independent experiments.

5.3.3 Level of expression of the product expressed by pE12

One possible explanation for the low complementation by pE12 seen in the previous section was that UL12 was not expressed at a high enough level. Although the western blots in Figure 25 showed apparently similar levels of alkaline nuclease being detected in pE12 transfected cells and wt HSV-1 infected cells it is not clear whether the amounts of UL12 present were saturating the antibody. Therefore a series of dilutions were performed on cell lysates of pE12 transfected or wt HSV-1 infected BHK cells.

From the 5 and 10-fold dilution samples shown in Figure 25B it is evident that the amount of alkaline nuclease in pE12 transfected cells is reduced approximately 2 - 3-fold compared to wt HSV-1 infected cells. However, if we consider that only 10 – 30% of the transfected BHK cell population will express UL12 compared to ~100% of BHK cells infected by wt HSV-1, each pE12 expressing cell probably produces a similar

amount of alkaline nuclease to a virus infected cell. Therefore it is unlikely that the actual level of expression of UL12 from pE12 is affecting the complementation.

Interestingly Figure 25B, and to a lesser extent Figure 25A, show that the product expressed by pE12 has a slightly different pattern of breakdown products to that expressed by wt HSV-1. It has long been established that the UL12 product is extensively phosphorylated (Marsden *et al.*, 1978; Wilcox *et al.*, 1980; Banks *et al.*, 1985) and the possibility of further modifications cannot be dismissed. These observed differences suggest that viral proteins may have at least some role in the modification or breakdown of UL12. This could be investigated by comparing the UL12 related proteins in cells transfected with pE12 and either mock-infected or infected with *amb*UL12.

5.3.4 Transient replication of amplicons with plasmids expressing the seven DNA replication genes

It has been demonstrated that seven genes encoded by HSV-1 are sufficient and necessary for the replication of plasmids containing either ori_S or ori_L (Wu *et al.*, 1988). The HSV-1 genes UL5, UL8, UL9, UL29, UL30, UL42 and UL52 were cloned into pCMV10 to create a series of plasmids: pE5, pE8, pE9, pE29, pE30, pE42 and pE52 (Stow *et al.*, 1993). Transfection of these seven plasmids along with an amplicon are sufficient for amplicon replication.

To determine if the co-expression of UL12 or UL12.5 would augment the replication of amplicons in this system 1 μg of pSA1 or pS1 (the parent plasmid of pSA1 which contains ori_S but no packaging signal) was transfected into BHK cells along with the

seven replication plasmids alone, or additionally supplemented with either pE12 or pE12.5. The cells were DMSO boosted, harvested 16 h.p.t. and processed for total DNA. Following *Eco*RI and *Dpn*I digestion the samples were Southern blotted and probed with pAT153.

The fold increase in the replication of pSA1 upon co-transfection of pE12 or pE12.5 was calculated for three separate experiments (Table 3). In contrast to the results using wt HSV-1 or *amb*UL12 (section 5.3.2) there is very little increase in replication in the presence of UL12. The small increase in the replication of pSA1 samples when co-transfected with pE12.5 is difficult to explain since no increase in the replication of pS1 was observed.

Table 3. Fold increase in replication of pSA1 and pS1 by the seven HSV-1 replication genes upon co-transfection of pE12 or pE12.5.

		expt. 1	expt. 2	expt. 3	Average
pSA1	pE12	1.5	1.6	1.5	1.53
	pE12.5	3.3	2.2	2.1	2.53
pS1	pE12	1.4	1.2	1.5	1.36
	pE12.5	2.1	0.6	1.2	1.3

The failure to observe increased replication of the amplicon in the presence of UL12 could be due to the levels of expression achieved or may reflect differences between replication complexes in transfected and infected cells. As mentioned previously it is also possible that UL12 is differentially modified in transfected versus infected cells (Figure 20).

5.3.5 Terminal fragments in packaged pSA1

The termini of packaged amplicons can be detected in a similar way to those of genomic DNA. Figure 27B shows that digestion of packaged pSA1 with *Sal*I will give a major 4.3 kbp band representing the amplicon monomer and minor bands of 1.3 and 3.1 kbp signifying Uc (L) termini and Ub (S) termini, respectively. Digestion with *Pst*I will similarly generate a 3.5 kbp band representing Uc and a 0.9 kbp band representing Ub.

To investigate the effect of the UL12 gene product on the generation of amplicon terminal fragments, BHK monolayers were transfected with 1 μg pSA1 and then superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. 16 h.p.i. the cells were harvested and processed for DNase resistant DNA. Duplicate samples were then digested with either *Sal*I or *Pst*I, separated on a 0.8% LB-agarose gel and Southern blotted. The blot was then probed with radiolabelled pAT153.

It can be seen in Figure 27C that when wt HSV-1 or *amb*UL12 were used as helper virus the larger *Pst*I and *Sal*I terminal fragments, representing the equivalent of the long and short termini of viral DNA, respectively, were readily detected in both instances (The smaller fragments were more difficult to detect because they shared much less homology with the probe).

5.4 Amplicon Recombination Studies

5.4.1 Introduction

It is clear from the previous section that *amb*UL12 shows similar impairment in the replication and packaging of amplicons as it does its own genome. However, when compared to the HSV-1 genome, amplicons have a much simpler structure suggesting

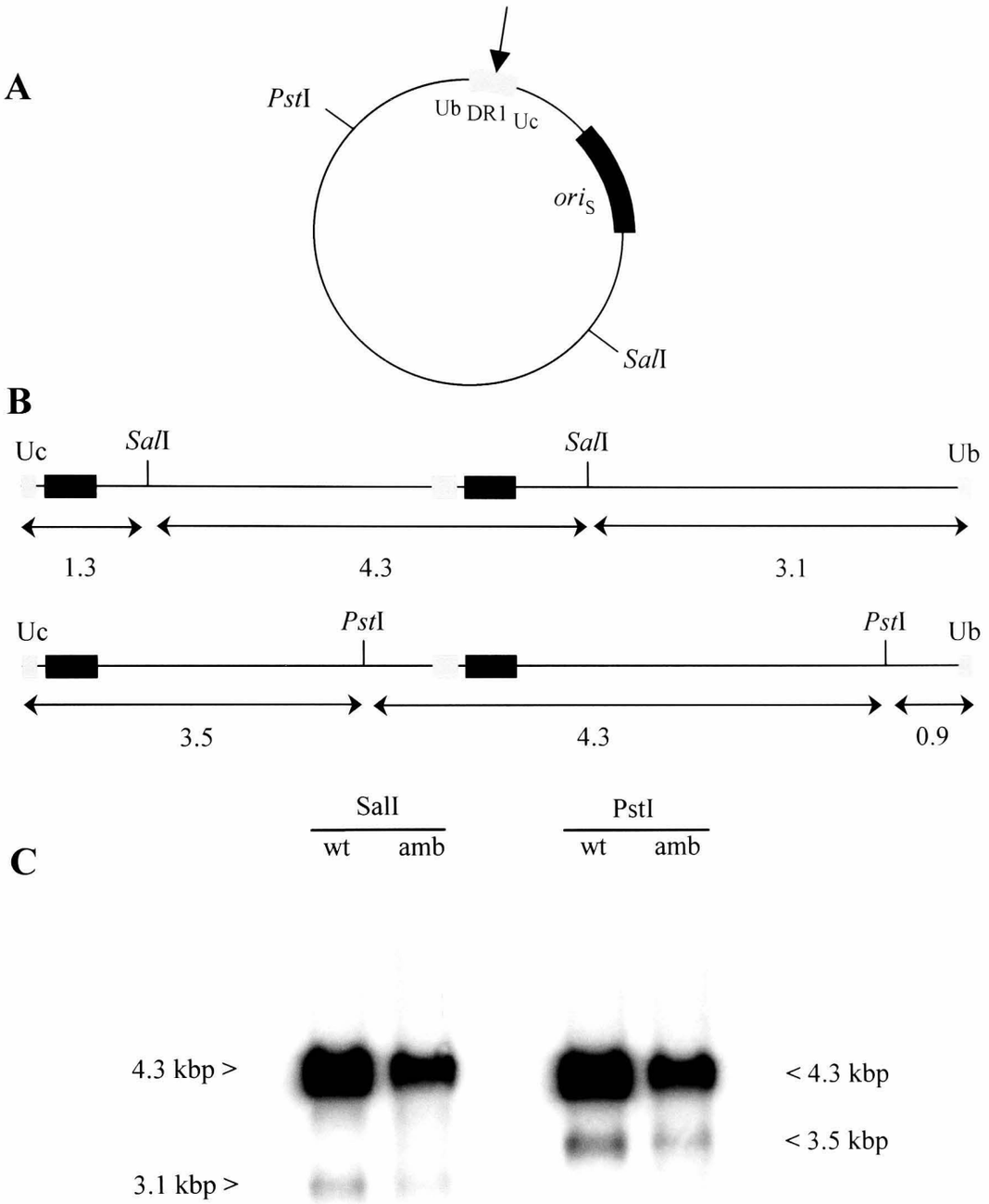


Figure 27 – Termini of packaged pSA1.

(**A**) Diagram representing pSA1. The regions containing ori_S and the *a* sequence (Ub-DR1-Uc) are indicated by thickened boxes. The cleavage site between Ub and Uc is indicated by an arrow. The *Sal*I and *Pst*I cleavage sites are indicated. (**B**) Diagram represents packaged concatemers, although only two full monomer repeats are shown. The *Sai*I and *Pst*I cleavage sites are indicated with the expected restriction fragment sizes shown below. (**C**) BHK monolayers were transfected with pSA1 and superinfected with wt HSV-1 (wt) or *amb*UL12 (amb). Cells were harvested 16 h.p.i. and processed for DNase-resistant DNA, digetsed with *Sal*I or *Pst*I, blotted and probed with radiolabelled pAT153. The 3.5 and 3.1 kbp fragments, generated by *Sal*I and *Pst*I digestion, respectively, and the 4.3 kbp monomer are indicated by arrows.

that their replication and packaging intermediates may not be as complex. Many studies have shown that amplicons recombine during replication driven by wt HSV-1 helper viruses (Dutch *et al.*, 1992; Dutch *et al.*, 1994) and also by a *nuc* mutant helper virus (Martinez *et al.*, 1996a). However these experiments examined inversion events that occur through intra-molecular recombination. Only a recent paper by Fu *et al.* (2002) has examined intermolecular recombination of plasmids replicated by HSV-1. In an effort to study the effects of intermolecular recombination when using *amb*UL12 as the helper virus, modifications were made to the pSA1 amplicon to allow detection of intermolecular recombination and to limit the extent to which branch migration can occur along amplicon concatemers.

5.4.2 Cloning of pSA1x

To be able to detect intermolecular recombination between two concatemers a derivative of pSA1 in which the unique *Eco*RI site was changed to a unique *Xba*I site was constructed. Figure 28 shows a schematic representation of the cloning strategy. An oligonucleotide was designed that would create an *Xba*I restriction site when self annealed and ligated into an *Eco*RI restriction site. 5 μg of oligonucleotide was self annealed by heating to 70°C for 10 min, followed by 2 min at 100°C and finally incubated at 37°C for 30 min. The annealed oligonucleotide was ligated into *Eco*RI digested pSA1 and the product *Eco*RI digested to relinearise any self ligated vector. The reaction was heat inactivated, phenol / chloroform extracted and the product transformed into competent DH5 *E. coli*. Colonies were picked and the plasmid DNA from transformed cells was tested for the presence of the new *Xba*I site (Figure 28). A plasmid with the required restriction site was designated pSA1x.

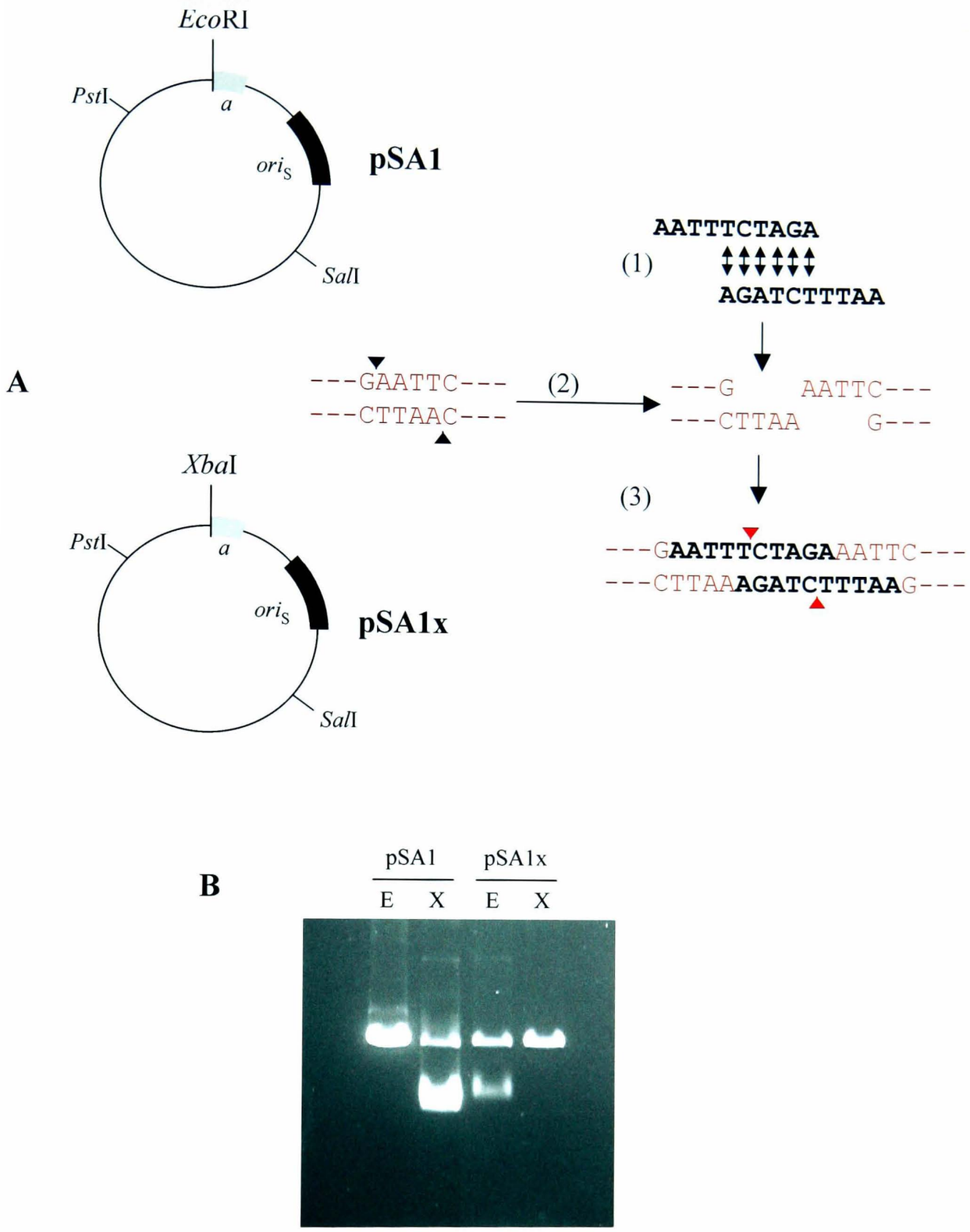


Figure 28 – Cloning of pSA1x.

(**A**) Schematic representation of cloning strategy and position of restriction sites within the plasmids: (1) The oligonucleotide was self annealled, (2) pSA1 plasmid was *Eco*RI digested, (3) the annealed oligo was ligated into the digested plasmid to create a new XbaI site and remove the *Eco*RI site. (**B**) Ethidium bromide stained agarose gel demonstrating the insertion of the XbaI restriction site. 1 µg of pSA1 and pSA1x were each digested with *Eco*RI (E) and *Xba*I (X).

5.4.3 Cloning of pSA1inv

When recombination occurs between two homologous DNA sequences spontaneous branch migration can occur in both directions. In theory branch migration along homologous plasmid concatemers can continue until another Holliday junction is met or the junction is resolved when the end of a concatemer is reached. Due to the frequent U_L and U_S segment inversions that occur within replicating HSV-1 concatemers (Slobdeman and Simmons, 1997) during replication of the HSV-1 genome any branch migration would be curtailed when segments with the opposite orientation were encountered. In order to mimic the HSV-1 sequence inversion events in replicating amplicons, the plasmid pSA1inv was constructed where the sequence around ori_S was inverted (Figure 29). If pSA1 and pSA1inv molecules recombine, branch migration would be expected to be blocked by the inverted sequence, limiting branch migration to approximately 3.5 kbp.

pSA1inv was constructed by performing a double digest of pSA1 with *Eco*RV and *Psh*A1 to produce two fragments of 3.3 and 1.0 kbp. Due to the differing optimal digestion temperatures, the *Psh*A1 digestion was performed first at 25°C. The products were phenol / chloroform extracted then digested with *Eco*RV. The fragments were re-ligated and transformed into DH5 cells and the resultant colonies picked and screened for plasmids containing both fragments but with the shorter fragment in the opposite orientation relative to pSA1. *Eco*RI / *Sal*I digestion of the derived plasmid should produce fragments of 3.8 and 0.5 kbp whereas pSA1 will produce fragments of 3.0 and 1.3 kbp. As can be seen in Figure 29C both bands of pSA1 can be clearly detected. A plasmid, pSA1inv, was identified with the insert in the opposite orientation. Figure 29C demonstrates the presence of the predicted 3.8 kbp fragment of pSA1inv.

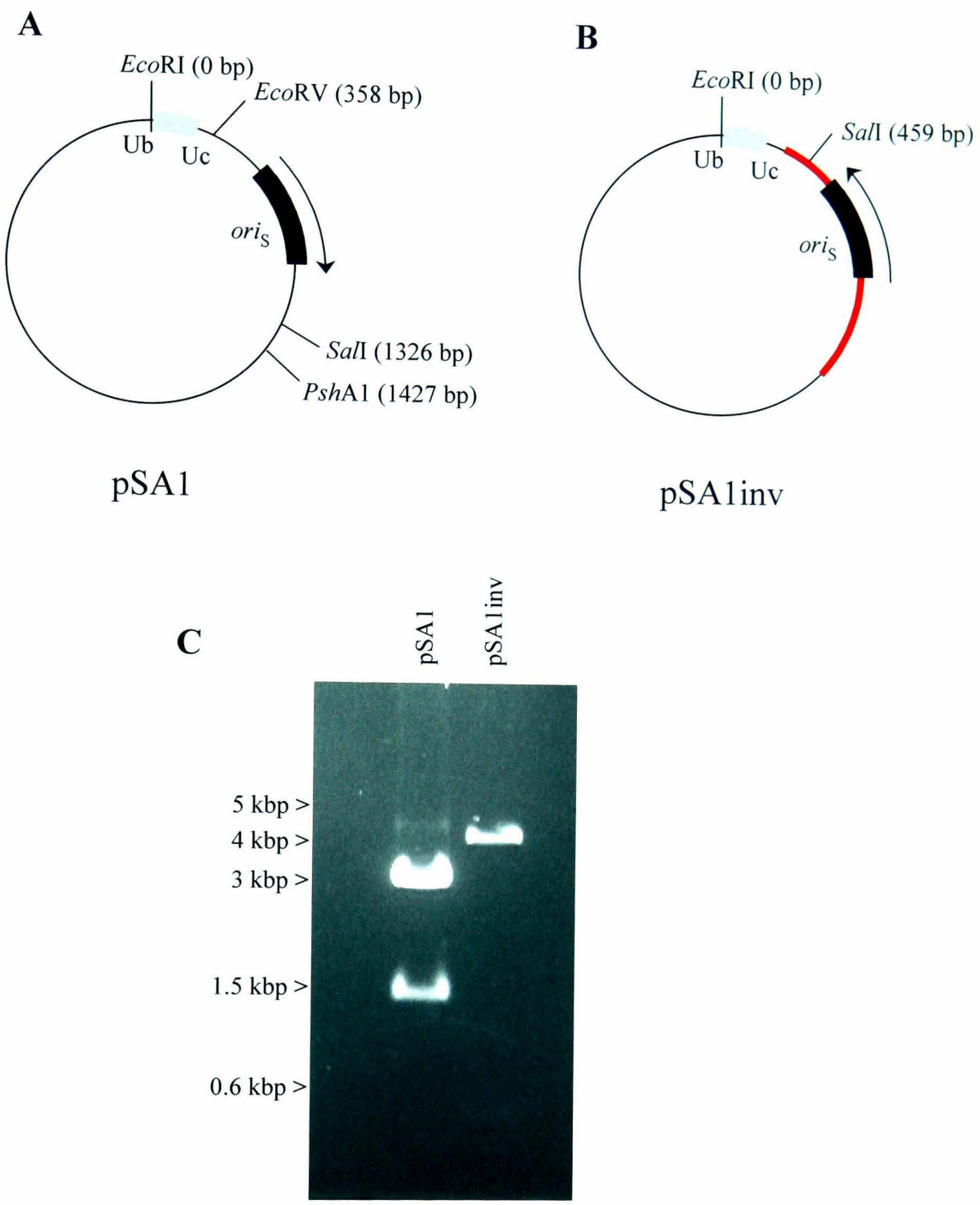


Figure 29 – Cloning of pSA1inv.

(**A**) Schematic representation of pSA1 showing the position of various restriction enzyme cleavage sites. The relative orientation of ori_S is indicated by the arrow. (**B**) Schematic representation of pSA1inv. Note the changed orientation of ori_S. The inverted region of the plasmid is marked in red. The new position of the *Sal*I restriction site is shown. The *Eco*RV and *Psh*A1 sites are absent in pSA1inv. (**C**) 1 µg of pSA1 and pSA1inv were double digested with *Eco*RI and *Sal*I and separated on a 0.8% TBE-agarose gel.

Although the smaller 0.5 kbp band cannot be detected in Figure 29C it was seen as a very faint band in other digests.

5.4.4 Recombination between pSA1, pSA1x and pSA1inv amplicons

To analyse recombination between replicating amplicons pSA1 or pSA1inv were transfected either alone or with pSA1x and superinfected with wt HSV-1 or *amb*UL12 as helper virus. Cells were then processed for total DNA 16 h.p.i., the DNA digested with *Dpn*I in combination with *Eco*RI and/or *Xba*I and separated on a 0.6% LB-agarose gel. The gel was Southern blotted and probed with ^{32}P labelled pAT153 (Figure 31).

If no recombination occurs between pSA1 and pSA1x or between pSA1x and pSA1inv concatemers digestion with either *Eco*RI or *Xba*I alone should produce two distinct bands representing the cleaved monomeric form of one amplicon and the hmw undigested concatemeric product of the other. However, if recombination does occur then digestion by either enzyme alone would be expected to produce a ladder of bands (Figure 30). As can be seen in Figure 31A digestion of replicated pSA1 or pSA1inv by *Eco*RI produced only the monomer band when using either wt HSV-1 or *amb*UL12 as the helper virus (lanes 1, 2, 11 and 12). However, *Eco*RI digests of samples co-transfected with pSA1x and either pSA1 or pSA1inv produced a ladder of bands from the monomer up to the region of non-resolution (lanes 3, 5, 7 and 9). This occurred when either wt HSV-1 or *amb*UL12 provided the helper functions, although with *amb*UL12 the ladder of bands was fainter (lanes 5 and 9). Digestion of the DNA from co-transfected samples with a combination of *Eco*RI and *Xba*I resulted in only a monomer band, which was present at approximately twice the amount of the monomer obtained from the single *Eco*RI digestion. This indicates that pSA1x was contained

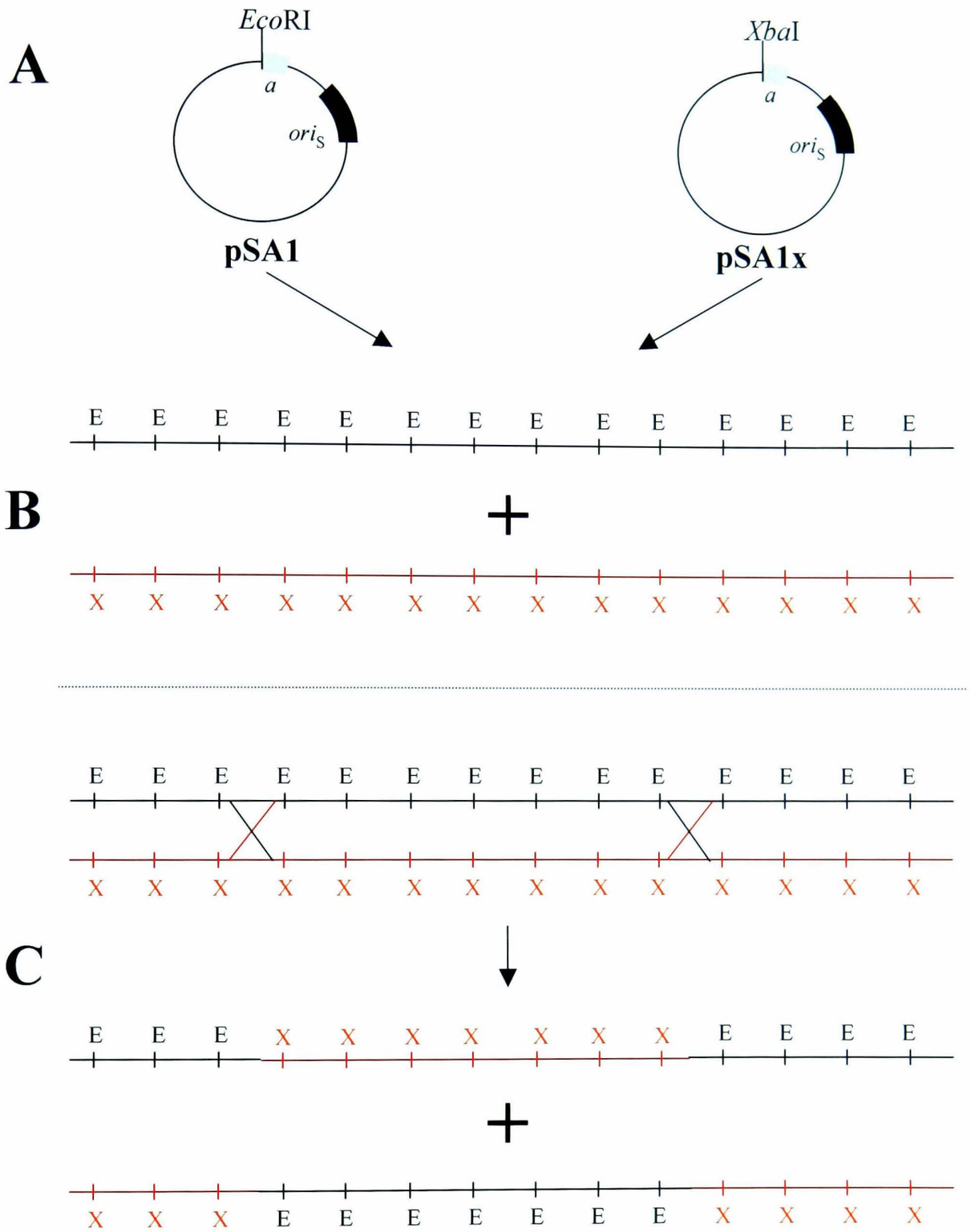


Figure 30 – Potential structures of amplicon concatemers after co-transfection and replication of pSA1 and pSA1x.

(**A**) Structure of pSA1 and pSA1x indicating the unique *Eco*RI and *Xba*I restriction sites, respectively. The position of the *a* sequence and origin or replication (ori_S) are shown. (**B**) In the absence of recombination, concatemeric replication products of the amplicons will contain only the unique *Eco*RI (E) or *Xba*I (X) site. The position of the *a* and ori_S sequences have been omitted for clarity. (**C**) In the event of intermolecular recombination events concatemeric molecules will contain both *Eco*RI (E) or *Xba*I (X) restriction sites, giving rise to ladders of bands depending on the distances between cross-over points.

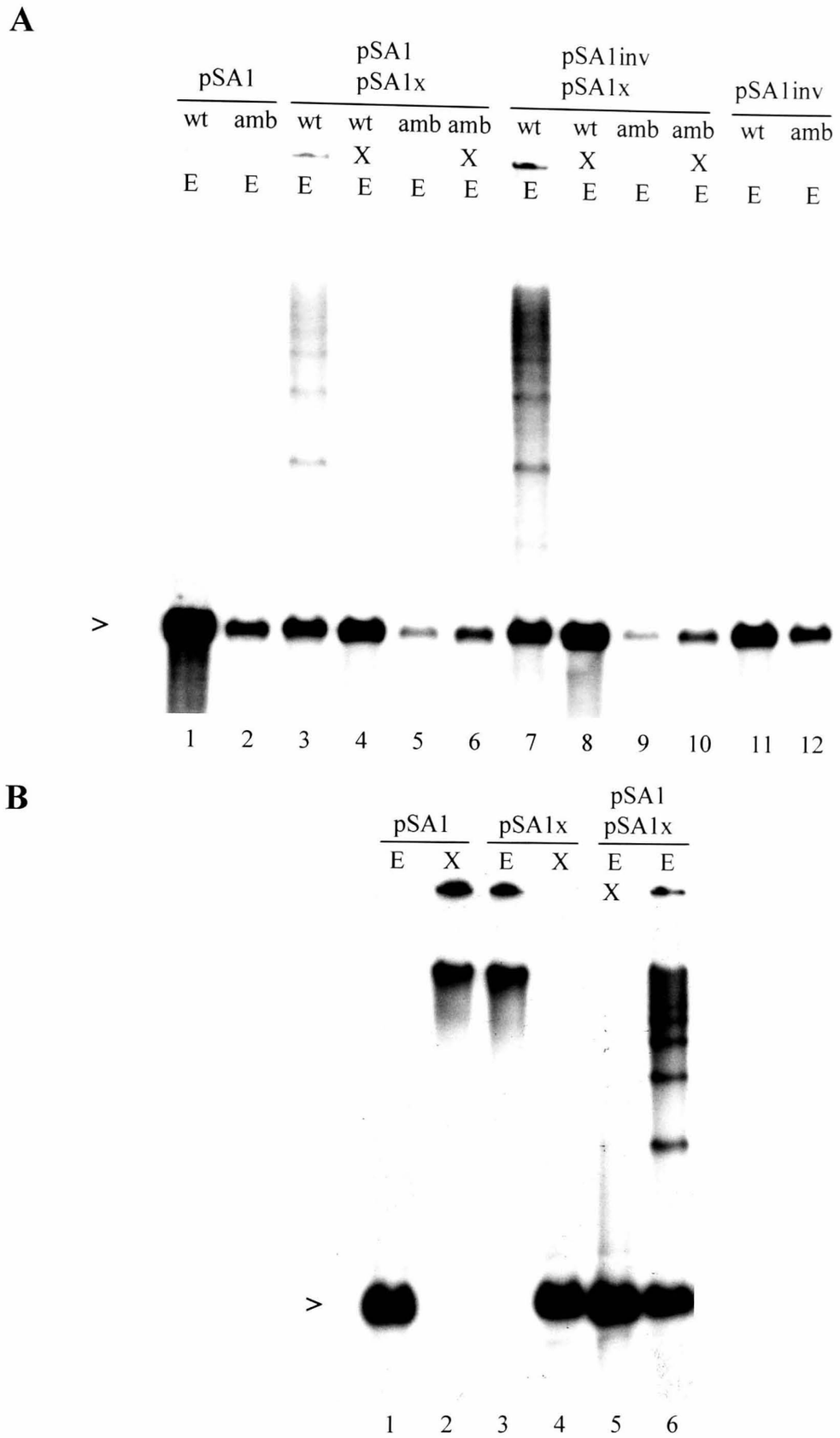


Figure 31 – Recombination of pSA1x with pSA1 and pSA1inv.

Monolayers of BHK cells were transfected with a combination of pSA1, pSA1x and pSA1inv as indicated and superinfected with (**A**) wt HSV-1 (wt) or *amb*UL12 (amb), or (**B**) *amb*UL12. 16 h.p.i. the cells were harvested and processed for total DNA. All samples were digested with *Dpn*I and *Eco*RI (E) and/or *Xba*I (X) as indicated. Samples were separated on 0.6% LB-agarose gels, Southern blotted and probed with ^{32}P labelled pAT153. The positions of amplicon monomers are indicated with an arrow.

mostly within the ladder of bands above the monomer band. In a separate experiment using *amb*UL12 as a helper virus, pSA1 and pSA1x were transfected alone or together and processed for replicated DNA. As can be seen in Figure 31B when the pSA1 or pSA1x products were digested with *Xba*I or *Eco*RI, respectively (lanes 2 and 3), no digestion occurred and the hmw concatemers migrated in the region of non-resolution. In contrast, digestion of these DNA with *Eco*RI or *Xba*I, respectively, resulted in the expected monomeric product (lanes 1 and 4). The presence of a ladder when the product of co-transfected cells were digested with *Eco*RI (lane 6), confirms that pSA1 and pSA1x recombine when using *amb*UL12 as a helper virus.

5.4.5 Replication and packaging of pSA1, pSA1x and pSA1inv

To ensure that pSA1x and pSA1inv behaved in a similar manner to pSA1 their ability to be replicated and packaged was tested. BHK monolayers were transfected with a total of 1 μg of pSA1, pSA1x, pSA1inv, pSA1 plus pSA1x or pSA1x plus pSA1inv, superinfected with wt HSV-1 or *amb*UL12 and harvested 16 h.p.i.. Total and DNase-resistant DNA was prepared from the cells and digested to a monomeric product with *Dpn*I and *Eco*RI and/or *Xba*I as appropriate. The DNA was separated on a 0.8% LB-agarose gel, Southern blotted and probed with ^{32}P labelled pAT153.

From Figure 32 it is evident that wt HSV-1 is able to replicate and package all the amplicons with similar efficiency. *Amb*UL12 also replicated and packaged all the amplicons and combinations thereof to similar levels, although at reduced efficiency compared to wt HSV-1. Although the complexity of the various amplicon DNA replication intermediates was not studied directly it was predicted that the pSA1x/pSA1inv co-transfection might produce the most complex intermediates because

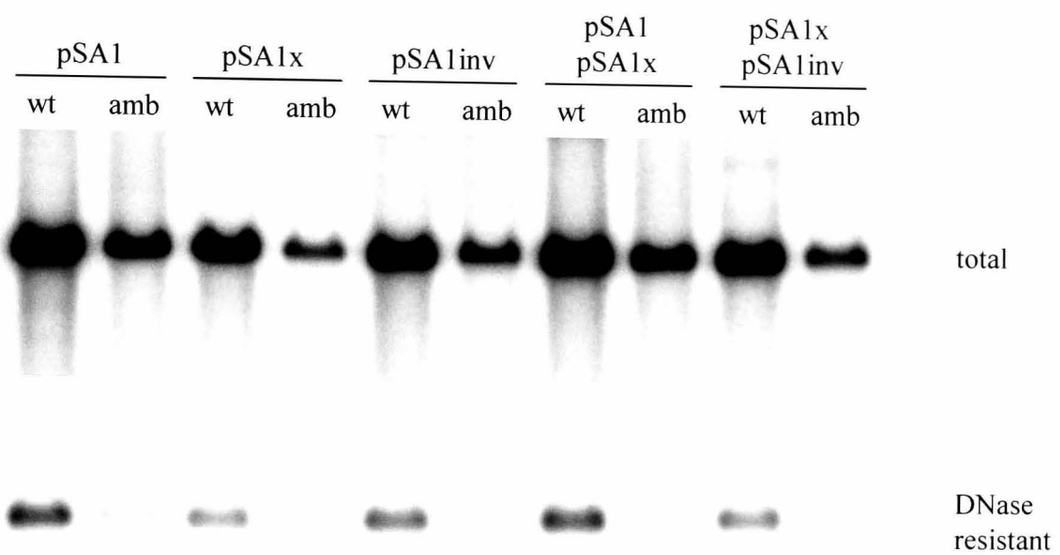


Figure 32 – Replication and packaging of pSA1, pSA1x and pSA1inv.

BHK monolayers were transfected with 1 μg pSA1, pSA1x, pSA1inv or co-transfected with 0.5 μg pSA1 and pSA1x, or pSA1x and pSA1inv. The monolayers were then superinfected with wt HSV-1 (wt) or *amb*UL12 (amb). Total and DNase-resistant DNA was prepared 16 h.p.i. The pSA1 and pSA1inv samples were digested with *Dpn*I and *Eco*RI. pSA1x was digested with *Dpn*I and *Xba*I and the pSA1/pSA1x and pSA1x/pSA1inv samples were digested with *Dpn*I, *Eco*RI and *Xba*I. The fragments were then resolved on a 0.8% LB-agarose gel, Southern blotted and probed with ^{32}P labelled pAT153.

the migration of branches might become stalled at positions where homology ceased due to the inversion. However, with this combination of amplicons there was no effect in the level of DNA packaged suggesting either that these products are not more complex, or that any increased complexity does not inhibit packaging even in the absence of the UL12 product.

5.5 Effect of the arrangement of the packaging signal on the packaging of amplicons by *amb*UL12

5.5.1 Introduction

To determine if the packaging defect found in the *amb*UL12 mutant was due to the manner in which it detected and utilised the cleavage and encapsidation signal, packaging assays were performed on amplicons containing different *a* sequence arrangements or more specific mutations within the various *a* sequence motifs.

5.5.2 Gross rearrangement of *a* sequences and effect on packaging

The amplicon pSA1 contains the minimal sequence, spanning the junction of two tandemly repeated *a* sequences, that is required to direct packaging of HSV-1 viral DNA (Hodge and Stow, 2001). To determine if other arrangements of *a* sequences would affect the packaging process, replication and packaging assays were performed with amplicons pY1 and pZ1 (Figure 33), containing a single complete, or two tandemly repeated *a* sequences, respectively.

The amplicons pSA1, pY1 and pZ1 were transfected into monolayers of BHK cells in 35 mm dishes. The cells were DMSO boosted 4 h.p.t. and superinfected with 3 p.f.u. / cell *amb*UL12 or wt HSV-1 at 6 h.p.t.. The cells were harvested 16 h.p.i. and total and

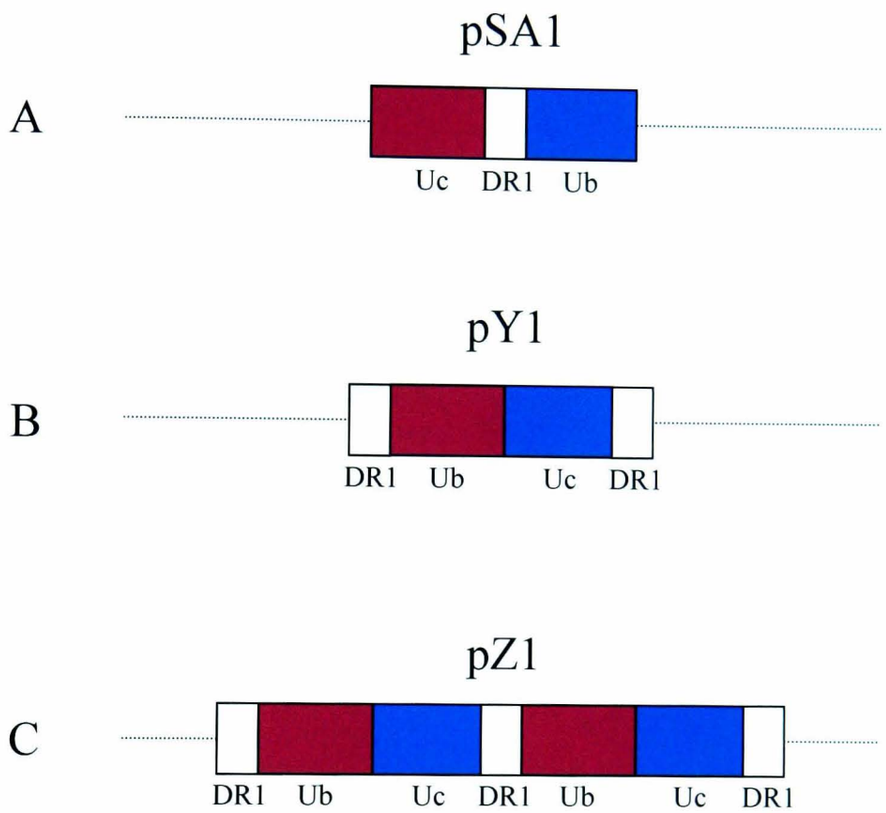


Figure 33 – Arrangement of packaging signals in pSA1, pY1 and pZ1.

Structure of the packaging signal within (**A**) pSA1, (**B**) pY1 and (**C**) pZ1. The arrangement of the DR1, Ub and Uc sequences within each of the amplicons is shown with the coloured boxes. The dotted line represents the remainder of the plasmid

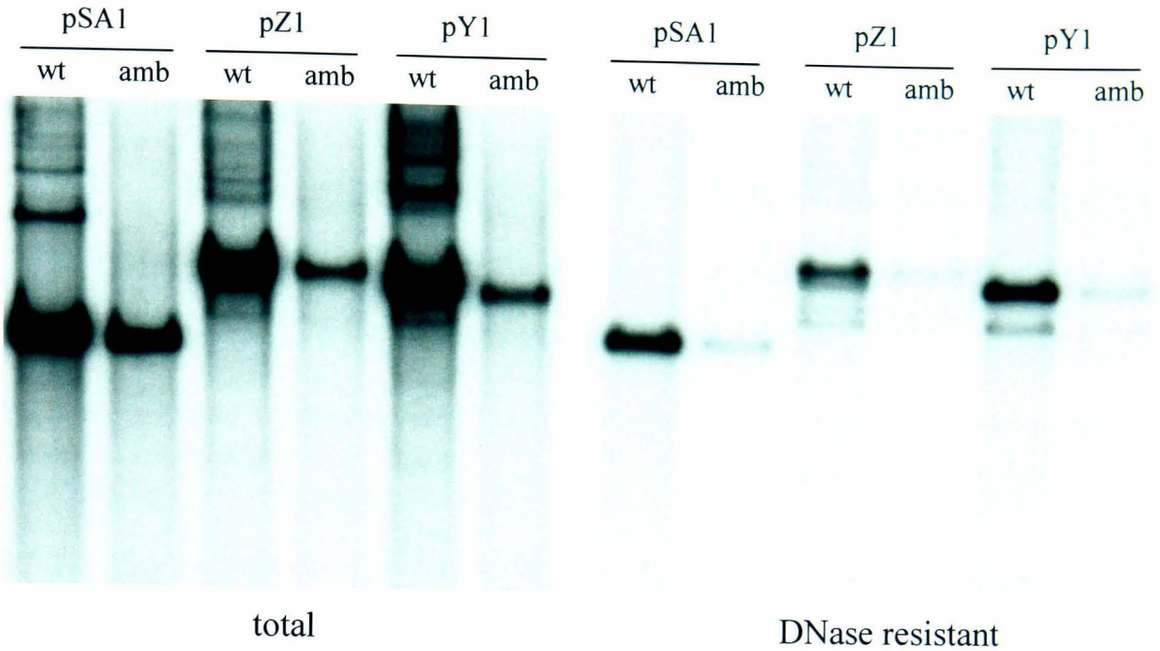
DNase-resistant DNA was prepared. After digestion with *Eco*RI and *Dpn*I the samples were separated on a 0.8% LB-agarose gel, Southern blotted and the membrane probed with radiolabelled pAT153.

Figure 34A shows a typical result from one such experiment and demonstrates that wt HSV-1 is able to replicate and pSA1, pZ1 and pY1 to similar levels. Although *amb*UL12 exhibited a reduced replication and packaging efficiency compared to wt HSV1, it was also able to package all three amplicons to similar levels. The apparent reduced replication of pY1 and pZ1 compared to pSA1 in the presence of *amb*UL12 (Figure 34A) was not reproducibly observed. Figure 34B shows that over seven repeat experiments all three plasmids exhibited a similar 5 - 8 fold decrease in replication in the presence of *amb*UL12 compared to wt HSV-1. Similarly, 15 – 25 fold reductions in packaging were observed with all three plasmids in the presence of *amb*Ul2. Because the standard deviations are relatively high it is not possible to conclude whether the relatively greater impairment of pY1 packaging in the presence of *amb*UL12 represents a real effect.

5.5.3 Ability of *amb*UL12 to package and propagate amplicons with mutations in the packaging signal

Although there was no significant difference in the ability of *amb*UL12 to replicate and package amplicons that contained different permuted forms of complete *a* sequences, a recent study by Hodge and Stow, (2001) showed that deletion and substitution mutations affecting various *a* sequence motifs within the Uc-DR1-Ub sequence of pSA1 altered the ability of the amplicon to be packaged or propagated in the presence of wt HSV-1. In particular, deletions within the *pac*1 motif allowed the modified amplicon to

A



B

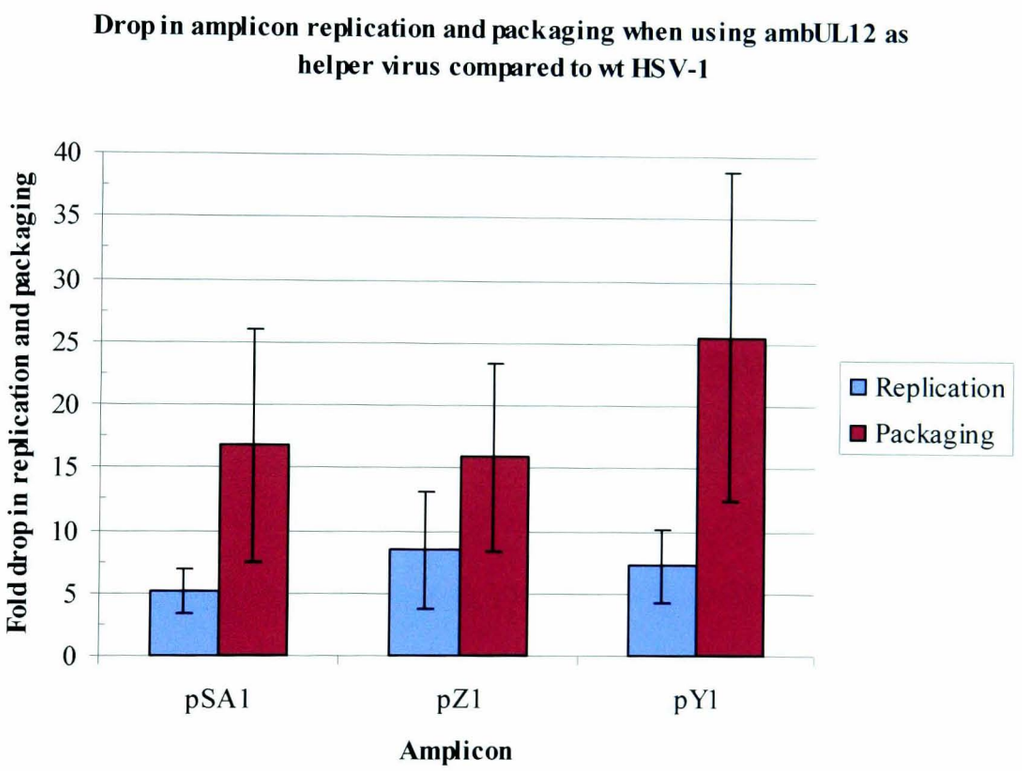


Figure 34 – Replication and packaging of pSA1, pZ1 and pY1.

(**A**) Monolayers of BHK cells were transfected with pSA1, pZ1 or pY1 as indicated and superinfected with 3 p.f.u. / cell wt HSV-1 (wt) or *amb*UL12 (amb). 16 h.p.i. the monolayers were harvested and total and DNase-resistant DNA prepared. The DNA was then digested with *Eco*RI and *Dpn*I, Southern blotted and probed with ^{32}P labelled pAT153. (**B**) Graphical representation of the fold-decrease in the replication and packaging of each of the amplicons when using *amb*UL12 as helpervirus compared with wt HSV-1. The chart shows the mean of seven separate experiments ± the S.D.

be packaged to the same level as pSA1 but caused a reduction in efficiency with which it could be serially propagated. The amplicons pPH11 and pPH12 have deletion and substitution mutations, respectively, affecting the *pac*1 distal GC element. The pPH21 and pPH22 constructs have deletion and substitution mutations which affect the *pac*1 proximal GC element. If the alkaline nuclease has no direct role in recognising or interacting with the *a* sequence then we would expect these amplicons to be packaged and propagated with the same efficiencies relative to pSA1 by either *amb*UL12 or wt HSV-1. Any differences between wt HSV-1 and *amb*UL12 may indicate that the nuclease does have a more direct interaction involving these *pac*1 motifs.

BHK monolayers were transfected with pPH11, pPH12, pPH21 or pPH22 also using pSA1 and pS1 as positive and negative controls. Following transfection the cells were superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The medium was removed from the monolayers 16 h.p.i., cell debris pelleted and the supernatant, containing released virus particles, retained. Total and DNase resistant DNA were then prepared from the cells. Fresh monolayers were inoculated with 0.5 ml samples of the media along with 3 p.f.u. / cell wt HSV-1. The addition of fresh wt HSV-1 helper was required to facilitate the replication of amplicons packaged by *amb*UL12 since the yield of infectious mutant helper virus is insufficient to enable infection at an appropriate m.o.i.. After one hour the inoculum was removed and the monolayers washed with acid glycine to inactivate any virus that had not penetrated the cells. The infected monolayers were then harvested 24 h.p.i and total DNA was prepared. All samples were then digested with *Dpn*I and *Eco*RI, Southern blotted and probed with ^{32}P labelled pAT153 (Figure 35).

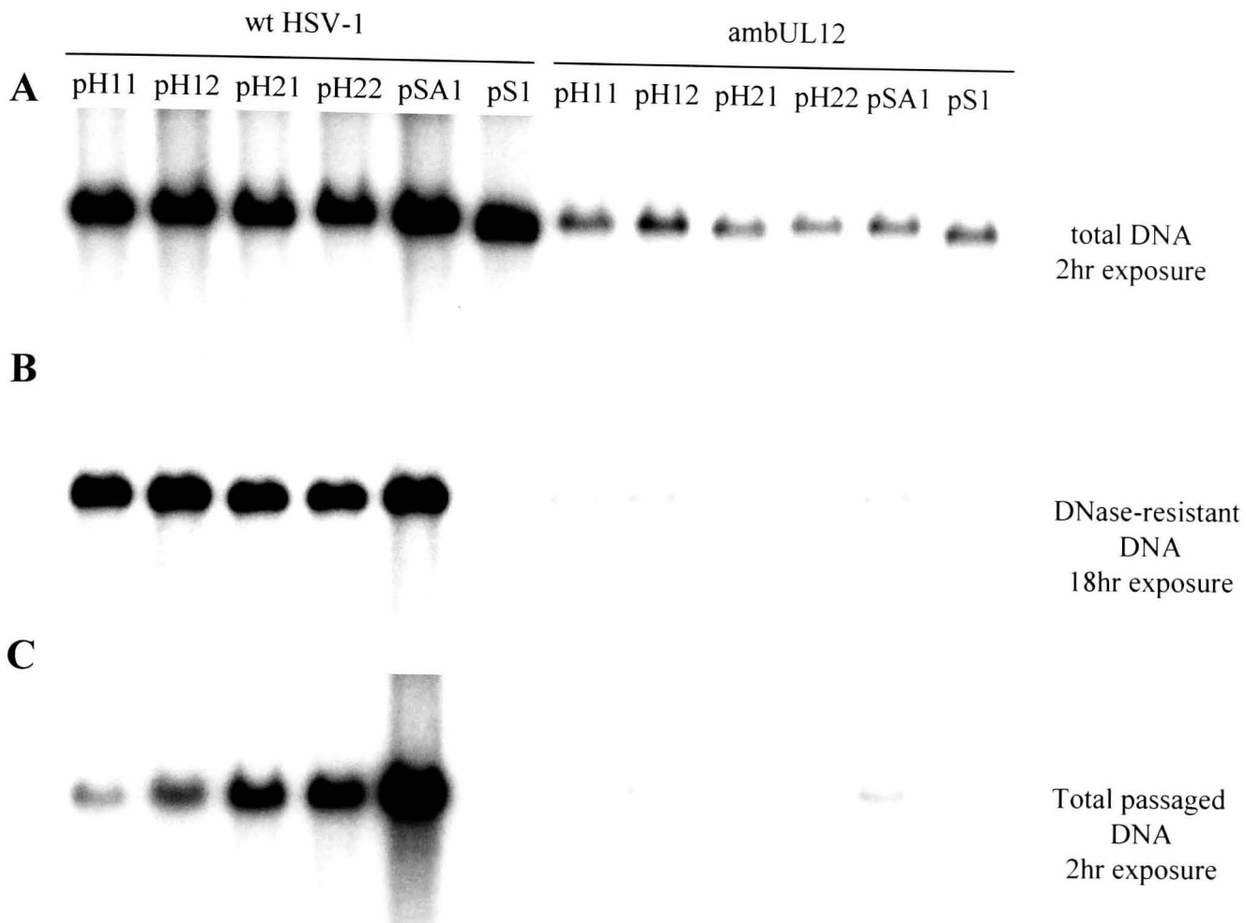


Figure 35 – Propagation of amplicons with mutations in the *pac*1 sequence.

Monolayers of BHK cells were transfected with the amplicons as indicated and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. Total (**A**) and DNase-resistant (**B**) DNA were prepared 16 h.p.i. The media removed from the monolayers were used to infect fresh monolayers along with an additional 3 p.f.u. / cell wt HSV-1. 1 hr after the addition of the inoculum the monolayers were washed with acid glycine. The monolayers were harvested 24 h.p.i. and total DNA prepared (**C**). All samples were digested with *Eco*RI and *Dpn*I, separated on an agarose gel, Southern blotted and probed with ^{32}P labelled pAT153. The gels were exposed to a phosphorimager screen for the times indicated.

Figure 35A shows that all the amplicons replicated to similar levels when using either wt HSV-1 or *amb*UL12 as helper virus, although as before more DNA accumulates in the presence of wt HSV-1 than *amb*UL12. The middle panel also shows that wt HSV-1 packages all the amplicons to a similar level apart from pS1 which lacks any *a* sequence. A very faint band of packaged pS1 was visible but represented only 0.2% of the pSA1 signal. *Amb*UL12 also packaged the amplicons containing mutated packaging signals to similar levels as pSA1. Surprisingly pS1 was packaged in greater amounts by *amb*UL12 than wt HSV-1, representing 14% of the *amb*UL12 pSA1 signal. The lower panel clearly shows that all the mutant amplicons derived using wt HSV-1 or *amb*UL12 as helper are propagated with reduced efficiency compared to pSA1. It is interesting to note that the pS1 amplicon derived from *amb*UL12 but not wt HSV-1 infected cells was able to be serially propagated.

The mean packaging and propagation efficiencies of the amplicons compared to pSA1 over three separate experiments were determined and are shown in Figure 36. It is clear from Figure 36A that the packaging efficiencies relative to pSA1 of all the amplicons except pS1 were similar whether either wt HSV-1 or *amb*UL12 was providing helper functions. The only significant difference between the two viruses was the ability of *amb*UL12 to package relatively greater amounts of pS1. In agreement with previous results the mutated amplicons were propagated less efficiently than pSA1 when using wt HSV-1 helper virus (Hodge and Stow, 2001). This was also true for the amplicons from *amb*UL12 infected cells but the patterns of impairment differed from those seen with wt HSV-1. In relative terms pPH11 and pPH12 were propagated 3-8 fold more efficiently from cells that were initially infected with *amb*UL12. In contrast pPH21 and pPH22 were propagated relatively more efficiently from cells initially infected with wt

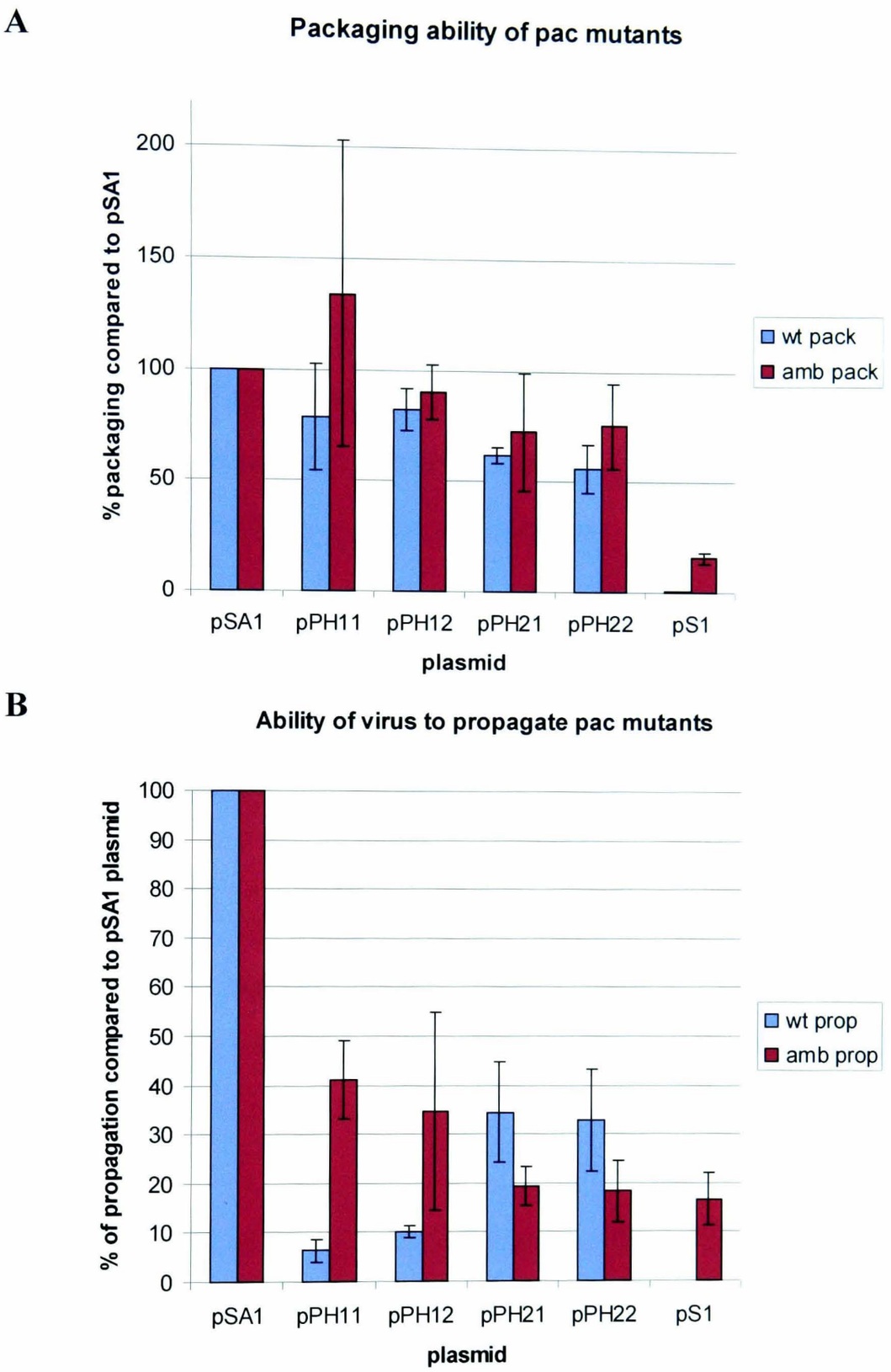


Figure 36 – Ability of amplicons with mutated Uc-DR1-Ub motifs to be packaged and propagated.

The mean and standard deviation of three independent experiments performed as described in Figure 35 were calculated and displayed graphically. The packaging (**A**) and propagation (**B**) efficiencies were calculated relative to pSA1 when using either wt HSV-1 (wt) or *amb*UL12 (amb) as original helper virus.

HSV-1. In fact the pS1 amplicon was propagated as efficiently as pPH21 and pPH22 from cells infected with *amb*UL12 even though it contains no recognised packaging signal. It should be noted that although DNase-resistant pS1 was occasionally detected at very small levels in wt HSV-1 samples no product was ever detected in passaged samples. These results indicate that there may be subtle differences between the recognition and use of the packaging signal in wt HSV-1 and *amb*UL12 infected cells giving rise to differences in the efficiencies with which packaged molecules can be propagated. Moreover, it was possible to package and propagate molecules derived from plasmids that contain no known packaging signal following infection of the transfected cells with *amb*UL12.

5.6 Packaging of the pS1 amplicon by *amb*UL12

5.6.1 Introduction

The packaging of the pS1 amplicon by *amb*UL12 suggests that the nuclease may function, in part, to control specificity of the packaging substrate. The next section aims to demonstrate that the detected pS1 molecules were truly encapsidated and not just an artefact of the assays described.

5.6.2 Packaging of pS1 by *amb*UL12 is reduced upon co-transfection of pE12

If, as suggested by the above experiments, the alkaline nuclease is involved in increasing specificity of packaging, co-transfection of the UL12 expressing plasmid pE12 should reduce the packaging levels of pS1 when *amb*UL12 is used as a helper virus.

To test this hypothesis BHK monolayers were transfected with 1μg each of pSA1, pS1, pS1 plus pE12 or pCMV10. The monolayers were superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. To confirm that the pS1 molecules were packaged into viral capsids and had not become DNase-resistant by some other means, the HSV-1 UL19 null mutant K5ΔZ (Desai *et al.*, 1993) was also used as a helper virus. UL19 encodes the major capsid protein and therefore capsids do not form in a non-complementing cell line infected with K5ΔZ. BHK or 19-1 cells (a cell line complementing UL19 null mutants: V. Preston, unpublished) were similarly transfected with pSA1, pS1 or pCMV10 and infected with 3 p.f.u. / cell K5ΔZ. All monolayers were harvested 16 h.p.i. and processed for total and DNase-resistant DNA, digested with *Dpn*I and *Eco*RI, Southern blotted and probed with ^{32}P labelled pAT153 (Figure 37).

As can be seen, wt HSV-1 replicated pSA1 and pS1 to similar levels but only pSA1 was packaged to significant levels. There is a very faint band in the DNase-resistant pS1 lane but it represents less than 0.5% of the corresponding pSA1 signal. In BHK cells K5ΔZ also replicated pSA1 and pS1 to the same level as wt HSV-1 but no DNase-resistant DNA was detected with either amplicon. However, in the complementing 19-1 cells DNase-resistant DNA was present in the pSA1 lane indicating that the resistance is a result of insertion into viral capsids. When *amb*UL12 was used as helper virus both pSA1 and pS1 were again replicated to similar levels, although reduced compared to wt HSV-1 and K5ΔZ. Nonetheless, both amplicons were evidently packaged. Although *amb*UL12 packaged pS1 slightly less efficiently than pSA1 the amount packaged was significantly greater than when wt HSV-1 was used as helper. Also, when pE12 was co-transfected with pS1 and *amb*UL12 used as helper virus, the replication of pS1 was increased but the level of DNase-resistant DNA was decreased. This confirms earlier

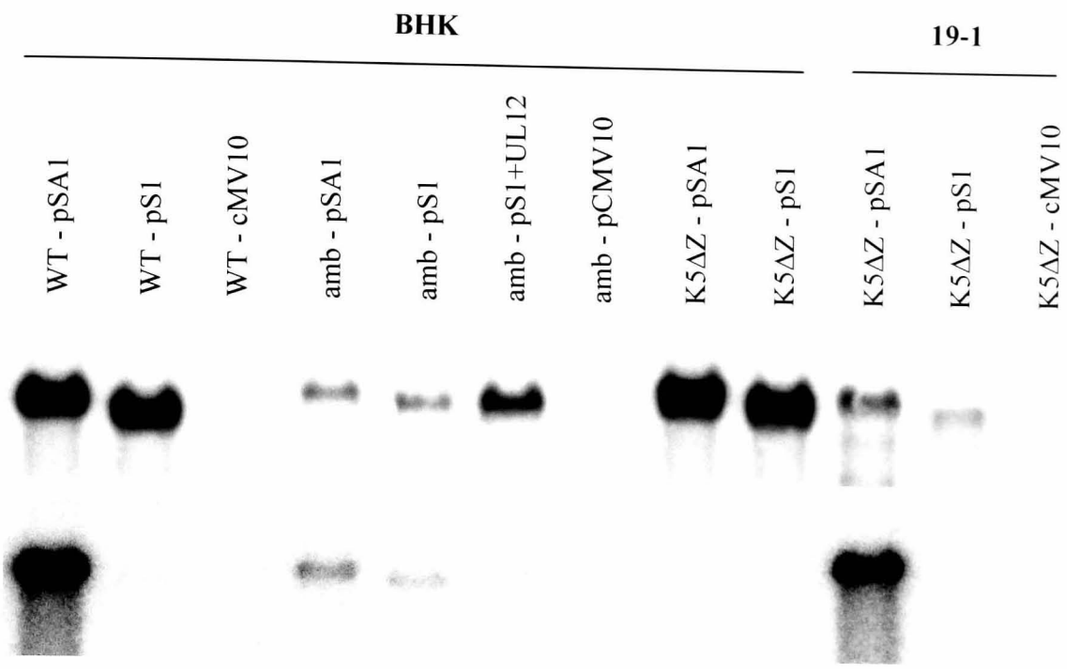


Figure 37 – Reduction in packaging of pS1 by *amb*UL12 upon co-transfection of pE12.

BHK or 19-1 monolayers were transfected as indicated and superinfected with 3 p.f.u. / cell wt HSV-1 (wt), *amb*UL12 (amb) or K5ΔZ. Cells were harvested 16 h.p.i. processed for total DNA (upper panel) and DNase-resistant DNA (lower panel). Samples were digested with *Dpn*I and *Eco*RI, separated on an 0.8% agarose gel, Southern blotted and probed with ^{32}P labelled pAT153. The lower panel shows an approximately 10-fold longer exposure to a phosphorimager screen than the upper panel.

results that the alkaline nuclease is required for maximum levels of DNA synthesis and provides further evidence that the enzyme may be involved in conferring substrate specificity during viral genome packaging.

Since a small amount of DNase-resistant DNA was present in the wt HSV-1/pS1 samples a similar experiment was performed in both BHK and Vero cells to determine whether this was a cell type specific phenomenon. The plasmids pSA1, pS1, pE12 and pE12.5 were transfected into Vero or BHK cells as indicated in Figure 38 and superinfected with wt HSV-1 or *amb*UL12. The cells were then processed for total and DNase-resistant DNA, digested with *Dpn*I and *Bam*HI, Southern blotted and probed with radiolabelled pGX153 (which detects both the viral *Bam*HI P fragment and the replicated amplicon).

Figure 38 shows that wt HSV-1 genomes were replicated and packaged to similar levels in the two cell lines; the same is true of the *amb*UL12 genomes although at lower levels. In agreement with the results of Figure 37, wt HSV-1 replicated pSA1 and pS1 to similar levels in BHK cells but only pSA1 was packaged to a significant degree (although a very small amount of DNase-resistant DNA was detected in the pS1 sample). No DNase-resistant pS1 was detectable in the wt HSV-1 Vero samples even though the plasmid had been replicated to the same extent as pSA1. In contrast, *amb*UL12 clearly packaged pS1 in both cell lines. The co-transfection of pE12 with pS1 when using *amb*UL12 as helper again increased replication and decreased packaging of the amplicon, but not the viral DNA. Although the co-transfection of pE12.5 appears to increase the level of replication of pS1 by *amb*UL12 in BHK cells this was a not reproducibly observed (see Table 2) and did not occur in the Vero cells

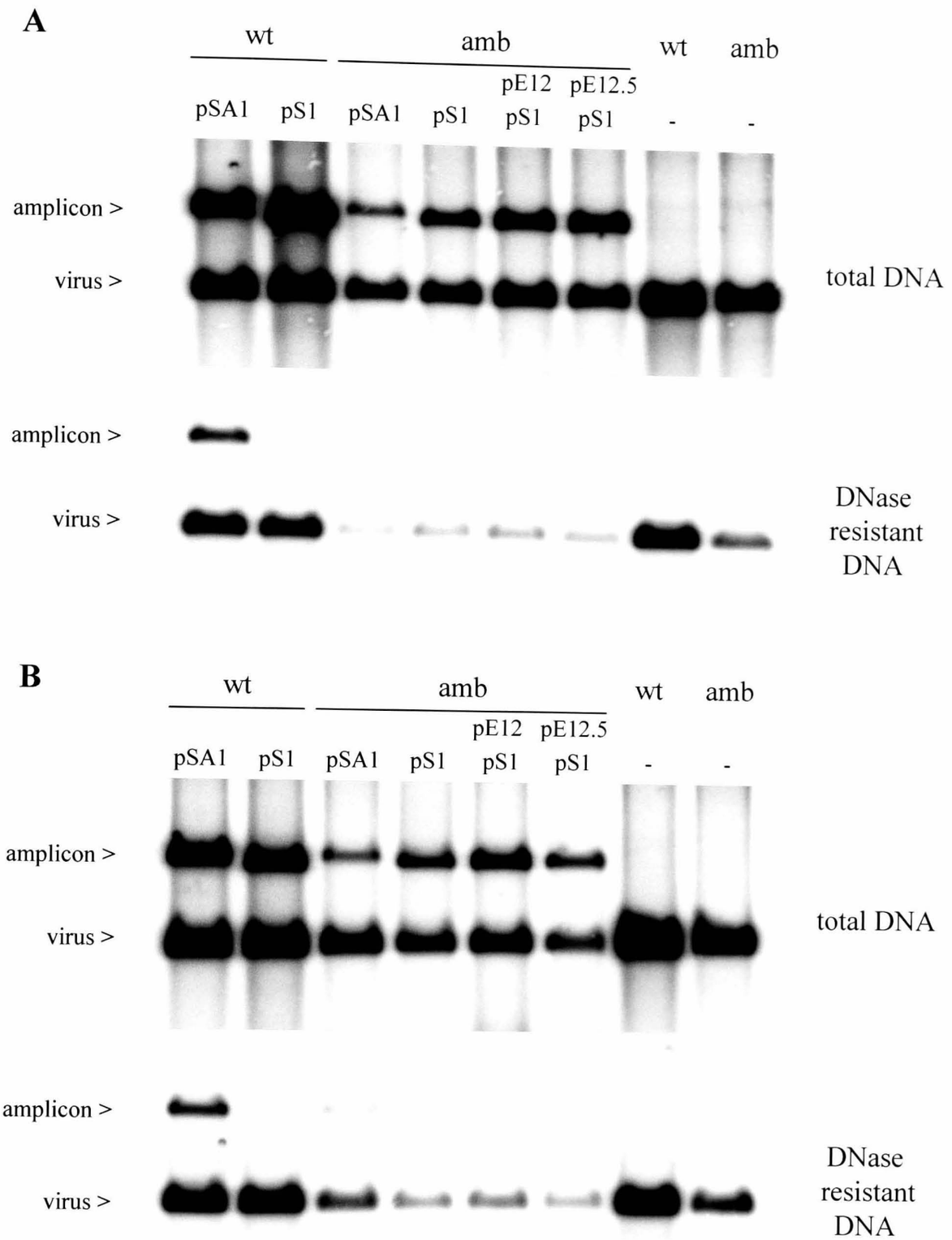


Figure 38 – Replication and packaging of viral and amplicon DNA in BHK and Vero cells.

BHK (**A**) and Vero cells (**B**) were transfected with the plasmids pSA1, pS1, pE12 and pE12.5 as indicated or mock transfected (-) and superinfected with wt HSV-1 (wt) or *amb*UL12 (amb). Total and DNase resistant DNA were prepared 16 h.p.i., digested with *Dpn*I and *Bam*HI, Southern blotted and probed with ^{32}P labelled pGX153. Total DNA panels are 2 hr exposures whereas the DNase-resistant DNA panels are 18 hr exposures. The position of the amplicon and viral DNA bands are indicated.

(Figure 38). Nevertheless, in both the BHK and Vero samples, co-transfection of pE12.5 lowered the level of packaging of pS1 to a similar extent to pE12.

5.6.3 Propagation of pSA1 and pS1 in BHK cells using wt HSV-1, *amb*UL12 and *amb*UL12R

To confirm that the observed packaging of pS1 by *amb*UL12 was a consequence of the lesion in the UL12 gene, the *amb*UL12 rescuant, *amb*UL12R, was used alongside wt HSV-1 and the null mutant in a packaging and propagation assay.

BHK monolayers were transfected with 1 μg of pSA1 or pS1 and infected with 3 p.f.u. / cell wt HSV-1, *amb*UL12 or *amb*UL12R. 16 h.p.i. the cells were harvested and total and DNase-resistant DNA prepared. The growth medium was retained from the harvested cells and sonicated extensively. Duplicate plates of fresh BHK cells were inoculated with 0.5 ml of these media supplemented with 3 p.f.u. / cell wt HSV-1. One hour after the addition of inoculum the plates were acid glycine washed and to one set of plates 200 μg ml^{-1} phosphonoacetic acid (PAA) was added to inhibit viral DNA synthesis. Total DNA was prepared 24 h.p.i.. All samples were digested with *Dpn*I and *Eco*RI, Southern blotted and probed with ^{32}P labelled pAT153.

Figure 39 shows that wt HSV-1 and *amb*UL12R replicated pSA1 and pS1 to similar levels. Only pSA1 was present in significant amounts in the DNase-resistant samples, although very faint pS1 bands were visible. Again *amb*UL12 replicated the amplicons 4 to 5-fold less efficiently than the other viruses, and DNase-resistant DNA was present in both the pSA1 and pS1 samples. Propagation of the virus from the plates originally infected with wt HSV-1 or *amb*UL12R (in the presence of additional wt HSV-1 helper)

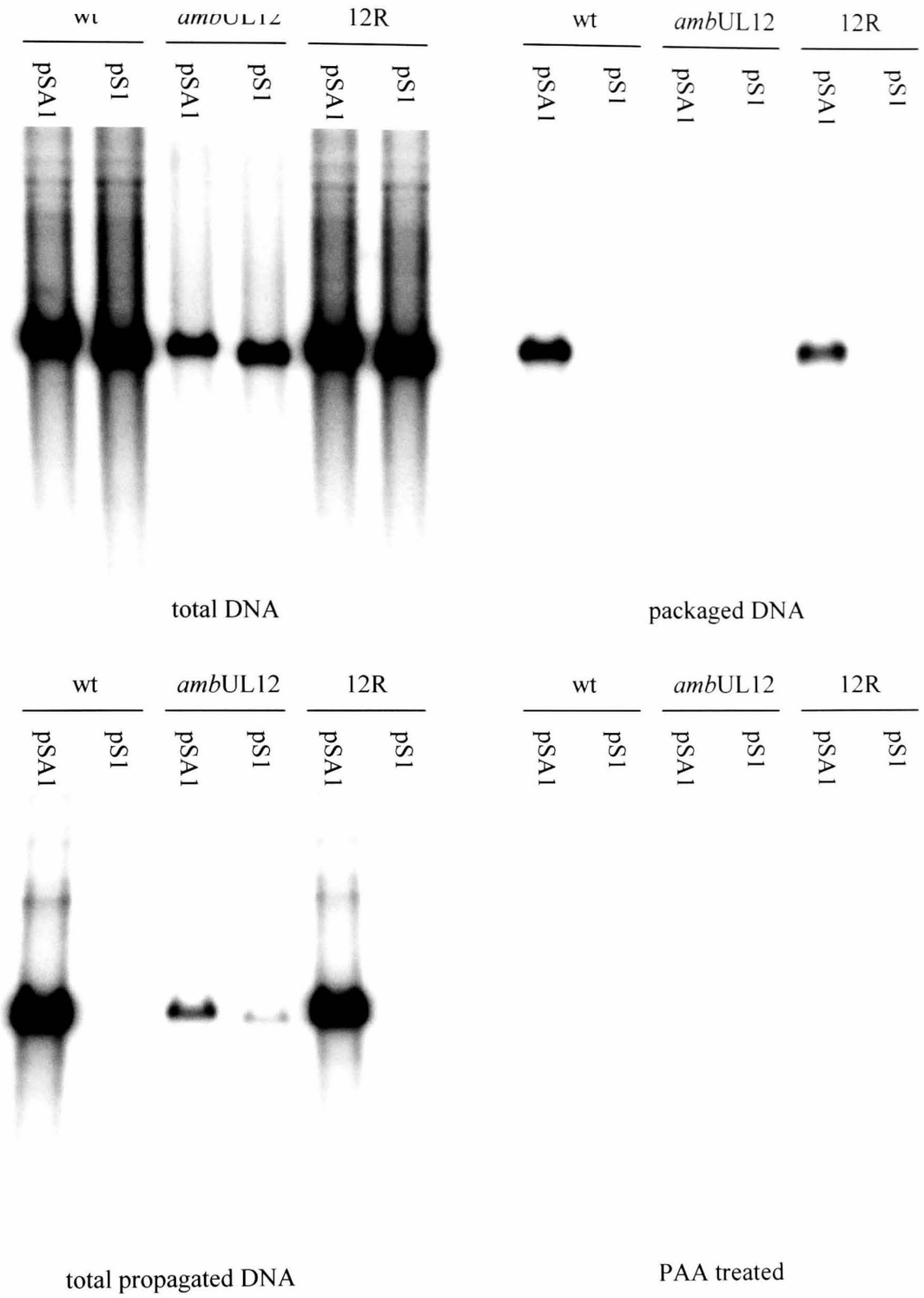


Figure 39 – Propagation of pSA1 and pS1 using wt HSV-1, *amb*UL12 and *amb*UL12R.

Monolayers of BHK cells were transfected with pSA1 or pS1 and superinfected with 3 p.f.u. / cell wt HSV-1 (wt), *amb*UL12 or *amb*UL12R (12R). Total and DNase resistant DNA was prepared 16 h.p.i. Duplicate monolayers of fresh BHK cells were each inoculated with 0.5ml of media retained from the harvested cells along with 3 p.f.u. / cell wt HSV-1. 1 hour after inoculation the plates were washed with acid glycine and medium added. 200 μg ml^{-1} PAA was added to one set of plates. 24 h.p.i. total DNA was prepared. All samples were digested with *Dpn*I and *Eco*RI, blotted and probed with ^{32}P labelled pAT153.

showed that pSA1 but not pS1 molecules could be efficiently passaged. In contrast, similar passaging of the virus from the plates originally infected with *amb*UL12 resulted in the propagation of both pSA1 and pS1. The control infections in the presence of PAA demonstrate that the signals detected following propagation result from replication and not the presence of residual input DNA. It should be noted that if additional wt HSV-1 is not added the low yield of infectious *amb*UL12 allows serial propagation to be performed at only a low m.o.i. (<1) and as a consequence packaged amplicons can not be efficiently passaged (data not shown).

5.6.4 Packaging of pS1 by wt HSV-1 at different multiplicities of infection and in different cell lines

It was possible the low level packaging of pS1 by wt HSV-1 might be a consequence of infecting the cells at a relatively high m.o.i.. To determine if this was the case monolayers of BHK cells were transfected with pS1 and then superinfected with wt HSV-1 at an MOI of 0.1, 1, 3 and 10. As a control monolayers of BHK cells were also transfected with pSA1 or pS1 and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. All monolayers were harvested 16 h.p.i. and processed for total and DNase-resistant DNA. The samples were digested with *Dpn*I and *Eco*RI, Southern blotted and probed with ^{32}P labelled pAT153.

From Figure 40 it is apparent that the m.o.i. with wt HSV-1 did not have a great effect on either the replication (upper panel) or packaging (lower panel) of pS1. Although the levels of replication of pS1 and pSA1 were very similar when using wt HSV-1 as helper virus, pS1 was packaged at less than 1% of pSA1 levels at each m.o.i. tested. In contrast, *amb*UL12 packaged pS1 at approximately 35% of the pSA1 level. In absolute

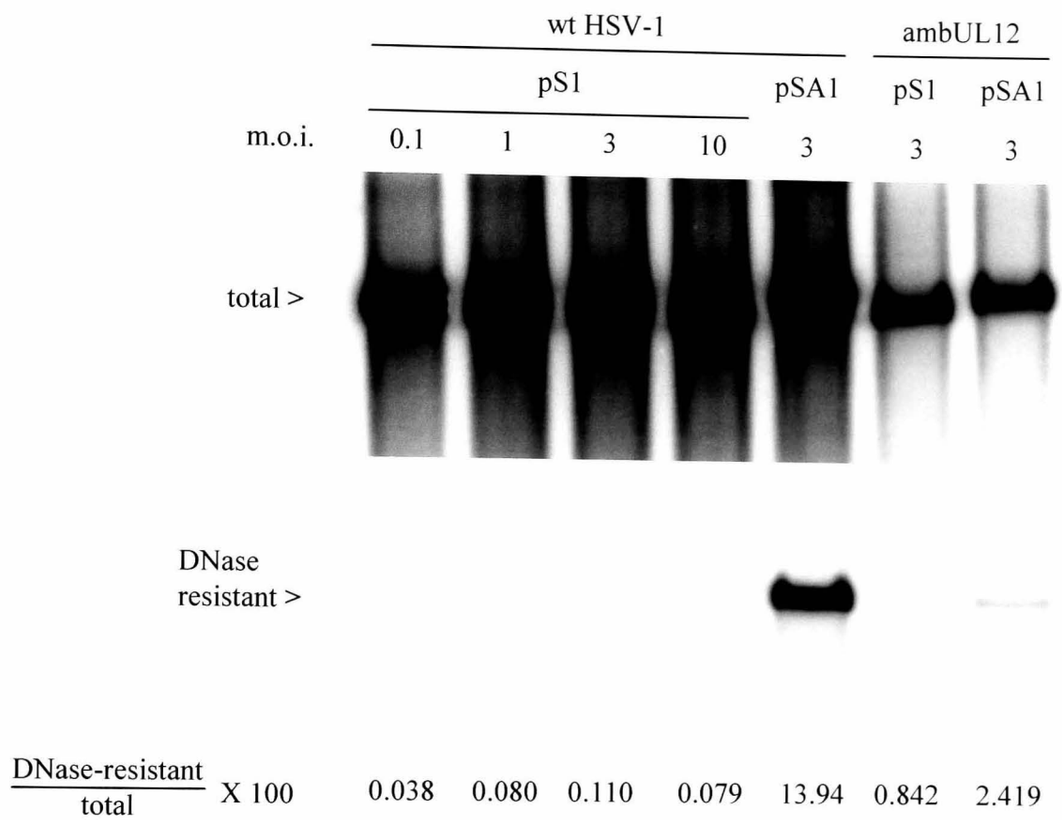


Figure 40 – Packaging of pS1 by wt HSV-1 at different MOIs.

BHK monolayers were transfected with pS1 or pSA1 and superifected with wt HSV-1 or *amb*UL12 at the m.o.i. indicated. Total (upper panel) and DNase-resistant DNA (lower panel) was prepared 16 h.p.i., digested with *Eco*RI and *Dpn*I, blotted and probed with ^{32}P labelled pAT153. The numbers below the DNase-resistant lanes indicate the percentage of total DNA that is detected in the DNase-resistant samples.

terms, *amb*UL12 packaged 2 – 10-fold more pS1 than wt HSV-1, which is also consistent with previous experiments.

The ability of wt HSV-1 to package pS1 was also examined in 293 cells. Monolayers of Vero, 293 and BHK cells were transfected with 1 μg pSA1 or pS1, superinfected with 3 p.f.u. / cell wt HSV-1 and incubated for 16 h. The cells were harvested and DNase-resistant DNA prepared. This was digested with *Eco*RI and *Dpn*I, blotted and probed with ^{32}P labelled pAT153.

The 2 h exposure of the blot shown in Figure 41B demonstrates that pSA1 was readily packaged in all three cell lines, although at varying levels. This was probably due to the varying transfection levels between the cell lines. DNase-resistant pS1 was detected only in the BHK sample even on a 24 h exposure of the blot. It should be noted that the pSA1 bands from 293 and Vero cells in the long exposure are more intense than the BHK band in the 2 h exposure in which packaging of pS1 in BHK cells was nevertheless apparent. Therefore, some property of BHK cells appears to allow pS1 to be packaged at low levels by wt HSV-1.

5.6.5 pS1 is not integrated into the viral genome

It was shown in section 4.3.3 that the pSA1 amplicon recombined at a high frequency. This suggests the possibility that replicated pS1 concatemers might be able to integrate into the viral genome DNA through ori_S, thereby becoming linked to a *cis*-acting *a* sequence and gaining the ability to be packaged.

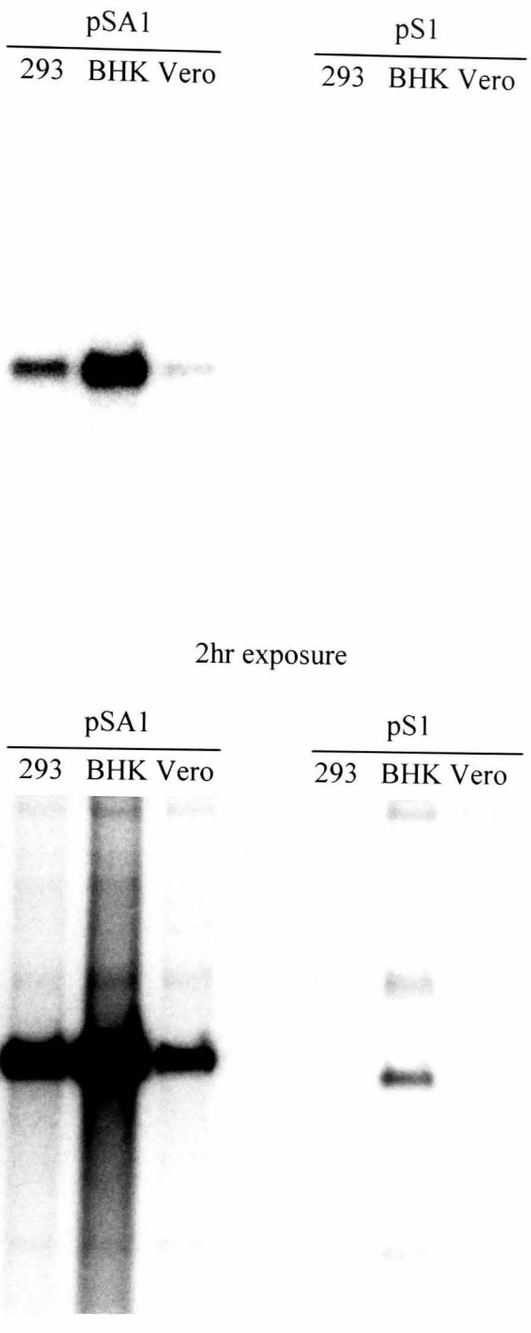


Figure 41 – Packaging of pS1 by wt HSV-1 in 293, BHK and Vero cells.

Monolayers of 293, BHK and Vero cells were transfected with pSA1 or pS1 and superinfected with 3 p.f.u. / cell wt HSV-1. 16 h.p.i. DNase-resistant DNA was prepared, digested with *Dpn*I and *Eco*RI, blotted and probed with ^{32}P labelled pAT153. The upper and lower panels show 2 and 24 h exposures of the same blot, respectively.

To determine if this was the case the structure of the packaged amplicons was examined in greater detail. BHK monolayers were transfected with pSA1 or pS1 and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12 and incubated for 16 h. The cells were harvested and DNase-resistant DNA prepared. The samples were digested with *Eco*RI/*Dpn*I, *Kpn*I/*Dpn*I or *Dpn*I alone, blotted and probed with pAT153 (Figure 42A). After exposure to a phosphorimager screen the membrane was stripped and reprobed with ^{32}P labelled HSV-1 genomic DNA (Figure 42B).

When digested with *Eco*RI (Figure 42A, lanes 1-4 and 42B, lanes 1-4) pSA1 and pS1 produce the expected monomer bands whereas the viral DNA produces a typical *Eco*RI restriction pattern. In agreement with the results described in section 3.3.5, *amb*UL12 produced a less distinct pattern that contains considerable smearing. When the samples were digested with *Kpn*I, which digests viral DNA but not amplicon DNA the viral DNA produced the expected restriction pattern (Figure 42B, lanes 4-8) while the majority of the amplicon DNA ran in the region of non-resolution (Figure 42A, lanes 4-8). The ladder of weaker bands below the region of non-resolution in Figure 42A (lanes 4-8) represent a ladder of packaged monomeric, dimeric, trimeric, etc. molecules that have been described previously (Vlazny *et al.*, 1982). Digestion of the samples with *Dpn*I will digest only unreplicated amplicon DNA. In Figure 42A the migration of the replicated amplicon DNA is unaltered when compared with the *Kpn*I digest but the viral DNA (Figure 42B) now runs in the region of non-resolution.

These results indicate that the pS1 molecule is being packaged independently from the viral DNA. Although it is difficult to exclude that a small amount of pS1 is integrated there are three strong arguments against this: (i) the pS1 amplicon behaves like pSA1

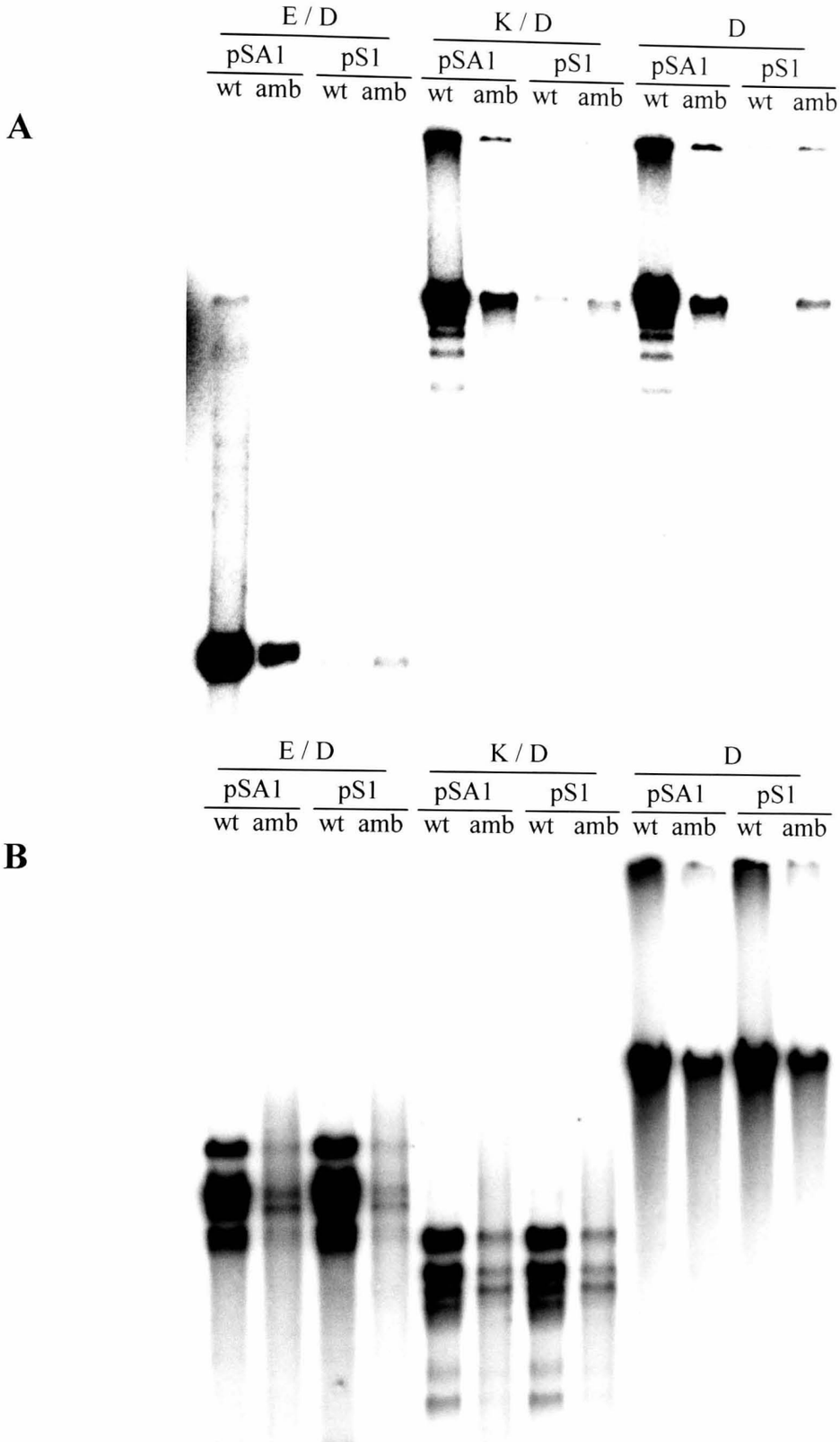


Figure 42 – Restriction patterns of DNase-resistant amplicon and viral DNA.
Monolayers of BHK cells were transfected with pSA1 or pS1 and superinfected with wt HSV-1 (wt) or *amb*UL12 (amb). DNase-resistant DNA was prepared 16 h.p.i. and digested with *Eco*RI and *Dpn*I (E/D), *Kpn*I and *Dpn*I (K/D) or with *Dpn*I alone (D). The DNA was separated on a 0.6% agarose gel, Southern blotted and probed with ^{32}P labelled pAT153 (**A**). The blot was then stripped and re-probed with ^{32}P labelled HSV-1 genomic DNA (**B**).

which is known to be independently replicated and packaged, (ii) taken together, the *Eco*RI, *Kpn*I and *Dpn*I samples indicate that the replicated pS1 DNA is concatemeric, (iii) physical packaging limits of the viral capsid would allow approximately only 12 kbp of integrated pS1 to be incorporated into the virus and the bands running in the region of non-resolution are at least 28 kbp in length.

5.7 Studies of replicated amplicons by Pulsed Field Gel Electrophoresis

5.7.1 Introduction

To further investigate the structure of amplicon DNA, PFGE was used to examine the replication products generated from pSA1 and pS1 when using wt HSV-1 and *amb*UL12 as helper-viruses.

5.7.2 Pulsed field gel analysis of replicated amplicon DNA

To determine if amplicons produced hmw intermediates analogous to viral DNA, monolayers of BHK cells were transfected with pSA1 and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i., embedded in agarose blocks and total DNA prepared *in situ*. One third of each block was then placed in a 1% pulsed field agarose gel and subjected to PFGE for 16 h at 6 v / cm, 14°C with a switch time of 1 – 15 secs. The gel was blotted and probed with ^{32}P labelled pAT153 (Figure 43A). The blot was subsequently stripped and re-probed with radiolabelled HSV-1 genomic DNA (Figure 43B).

The probe specific for the amplicon DNA shows that with both helper viruses pSA1 forms hmw concatemers that are unable to enter the pulsed field gel and are trapped in the wells of the gel (Figure 43A). In addition, a 150 kbp genome sized band is evident

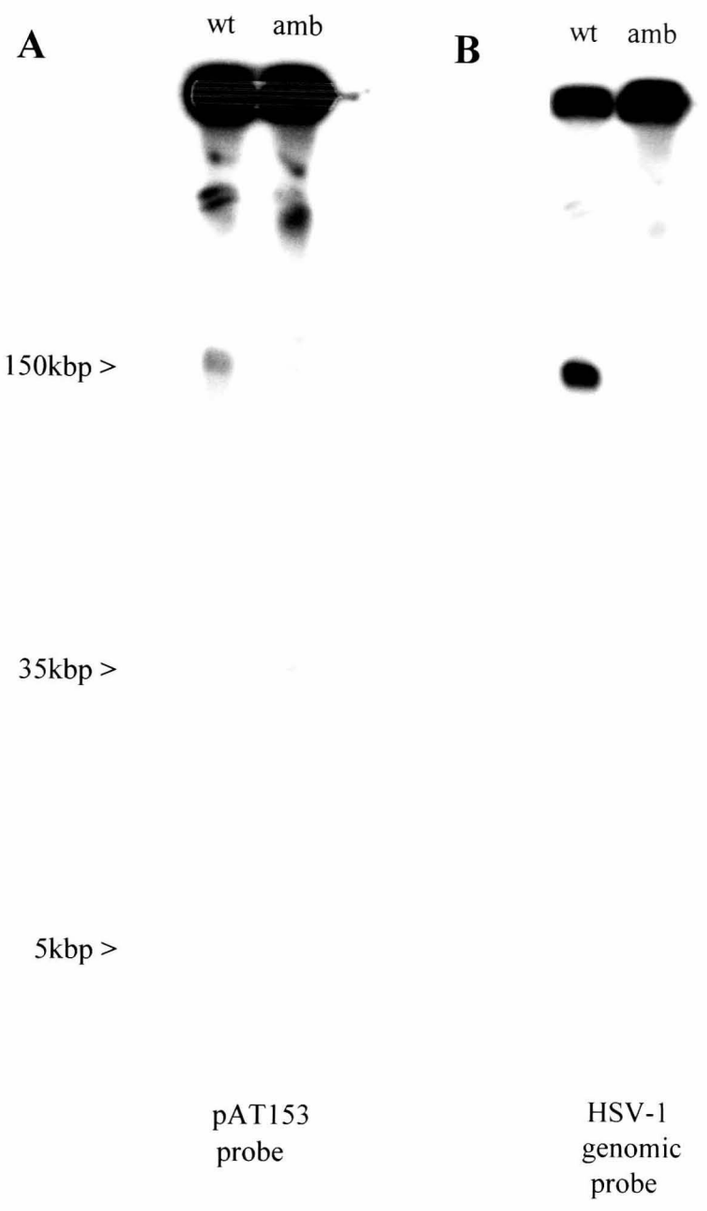


Figure 43 – PFGE analysis of replicated amplicon DNA.

Monolayers of BHK cells were transfected with pSA1 and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i. and embedded in agarose blocks. Total DNA was prepared in situ and one third of each block was subjected to PFGE. The gel was blotted and probed with radiolabelled pAT153 (**A**), stripped and re-probed with radiolabelled HSV-1 genomic DNA (**B**). The position of various bands from a size marker are indicated.

in the wt HSV-1 sample although there is considerable smearing below the band. There is a large amount of smearing in the *amb*UL12 lane which could be obscuring small amounts of a similarly sized product. When the blot was stripped and re-probed with genomic DNA the wt HSV-1 sample produced a distinct band at 150 kbp whereas *amb*UL12 yielded a smear from approximately 130 – 150 kbp. As expected, viral DNA was also detected in both "well" DNAs. Since cleavage of DNA is coupled to packaging the result suggests that, at least in the case of wt HSV-1, packaged amplicon molecules are up to genome length in size.

Interestingly two extra bands were detected with the pAT153 probe which migrated with estimated sizes of 5 and 35 kbp and were generated by both wt HSV-1 and *amb*UL12. These bands were routinely detected only with a pAT153 probe; the faint bands present in Figure 43B probably represent incomplete stripping of the pAT153 probe from the membrane.

5.7.3 Replication intermediates of pSA1 and pS1

To investigate whether the 5 and 35 kbp bands which were detected in Figure 43A were products of the packaging process, BHK cells were transfected with pSA1 or pS1 (which lacks a packaging signal) and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i. and total DNA was prepared in agarose blocks and separated on a pulsed field gel at 6 v / cm for 16 h at 14°C with switch times ranging linearly from 1 – 15 sec throughout the run. The gel was then blotted and probed with ^{32}P labelled pAT153.

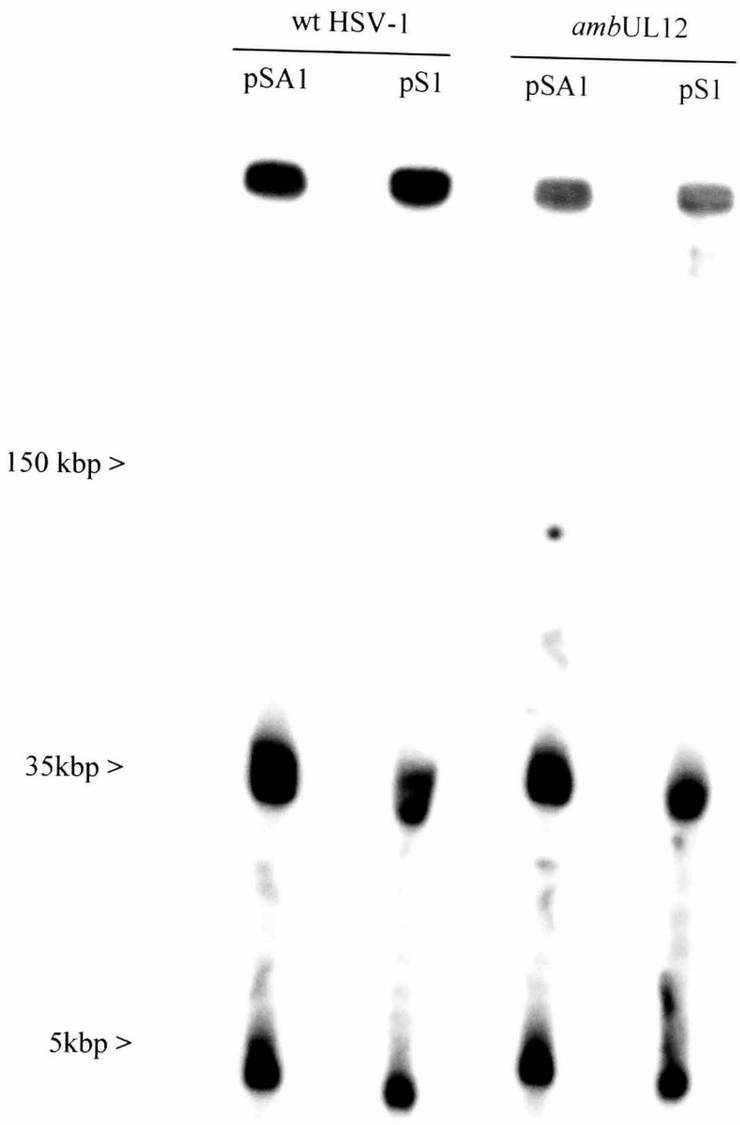


Figure 44 – PFGE analysis of pSA1 and pS1 replication intermediates.

BHK cells were transfected with pSA1 or pS1 and superinfected with wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i., embedded in agarose blocks and total DNA prepared. The DNA was separated by PFGE, blotted and probed with ^{32}P labelled pAT153. The position of various size markers are indicated.

Figure 44 shows that the 5 and 35 kbp bands appeared with a similar intensity in all the lanes irrespective of the amplicon or helper-virus used. There were also a number of intermediate bands between the two major bands. This, and the observation that the bands in the pS1 samples migrated slightly faster than those of the corresponding pSA1 samples, reflecting the different sizes of the amplicons, suggests that the bands may contain various numbers of repeats of the amplicons. Moreover, the fact that the bands also appear in the pS1 samples suggests that these bands are related to replication rather than packaging.

To confirm that these bands were replication products, one third of each plug was digested with *Dpn*I, subjected to PFGE as before, blotted and probed with radiolabelled pAT153. As can be seen in Figure 45 the upper 35 kbp bands were sensitive to *Dpn*I digestion indicating that only the lower bands are replication products. In contrast to Figure 44, the intermediate bands between 5 and 35 kbp were less evident and, in repeat experiments their appearance was inconsistent. There was also great variation in the levels of the 5 and 35 kbp bands between experiments, both in relation to each other and to the 150 kbp genome length band. In fact the genome length bands seen in Figure 43 are not apparent in Figures 44 and 45. The reason for this variation is unknown, but in the case of the 150 kbp band it could represent different overall levels of replication between experiments.

5.7.4 PFGE analysis of amplicons replicated by co-transfection of viral DNA replication genes

In order to determine if the viral DNA replication proteins alone could generate the 5 and 35 kbp bands BHK cells were co-transfected with 1 μg each of pSA1, pE5, pE8,

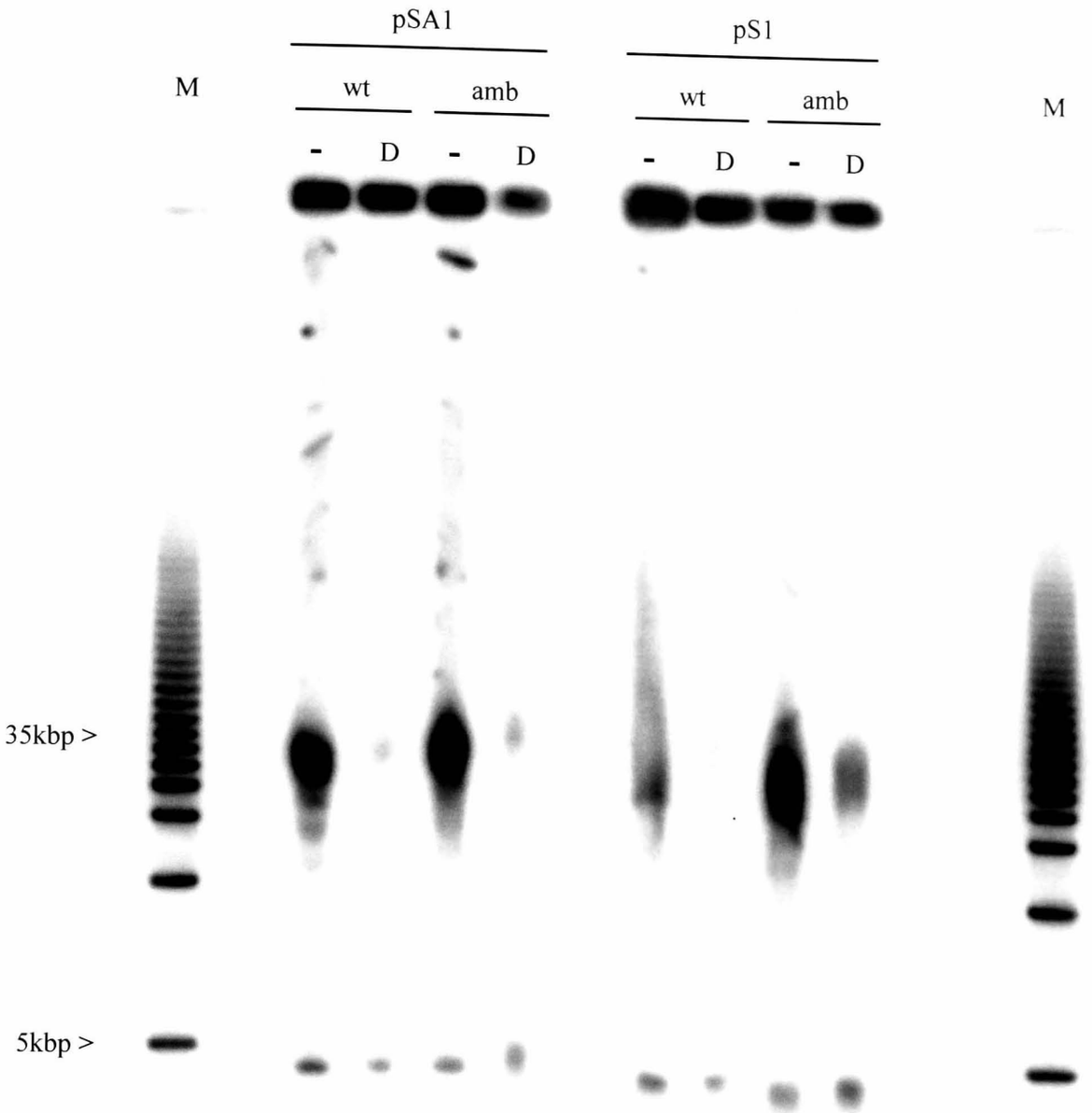


Figure 45 – PFGE analysis of *Dpn*I digested amplicon replication products.

BHK cells were transfected with pSA1 or pS1 and superinfected with wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i., embedded in agarose blocks and total DNA prepared. One third of each block was digested with *Dpn*I. The DNA in digested (D) and undigested (-) blocks was analysed by PFGE, the gel was blotted and probed with ^{32}P labelled pAT153. The sizes of two bands from the 5 kbp marker ladder (M) are indicated.

pE9, pE29, pE30, pE42 and pE52. As a control BHK cells were also transfected with pSA1 and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were then harvested 24 h.p.t. or 16 h.p.i., embedded in agarose plugs and total DNA prepared. One third of each block was subjected to PFGE using the same parameters as in section 4.7.3, the gel was blotted and probed with ^{32}P labelled pAT153.

Figure 46 demonstrates that pSA1 forms hmw concatemers when replicated in the presence of only the seven DNA replication genes. However, in contrast to amplicon replicated by helper virus no 5 or 35 kbp bands were observed, instead there was a smear ranging from approximately 40 – 90 kbp. An aliquot of the pSA1 plasmid preparation used in the transfection was included in this gel and it is interesting to note that the 5 and 35 kbp bands migrate with the presumptive supercoiled and open circular form, respectively, of the plasmid.

Taken together, the results presented in Figures 43 to 45 suggest that the 5 kbp band may represent a pSA1 replication intermediate comprising a supercoiled monomeric circle. Such a structure is compatible with initial replication occurring by a theta form mechanism.

5.7.5 PFGE analysis of DNase-resistant amplicon DNA

Studies by Vlazny *et al.* (1982) and more recently by Stow (2001) demonstrated that, in the nucleus of wt HSV-1 infected cells, packaged defective genomes and amplicons are made up of integral numbers of repeats of a single unit, from one copy up to the size of a full length HSV-1 genome. However, only the largest molecules are translocated into the cytoplasm. In an effort to determine the size of pSA1 and pS1 molecules packaged

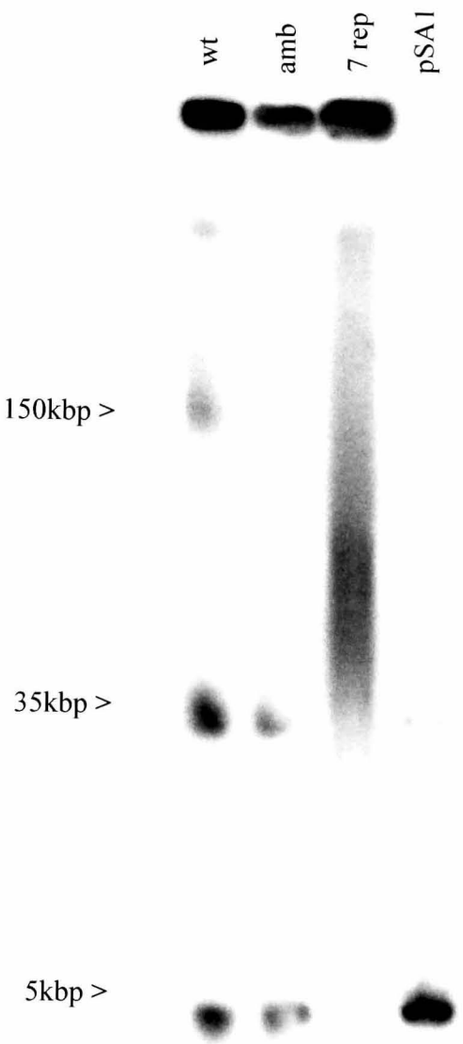


Figure 46 – PFGE analysis of pSA1 replicated by co-transfection of plasmids expressing the seven essential viral replication genes.

BHK cells were transfected with pSA1 and superinfected with wt HSV-1 (wt) or *amb*UL12 (amb), or co-transfected with plasmids expressing the seven viral replication genes (7 rep). The cells were harvested 16 h.p.i., embedded in agarose blocks and total DNA prepared, separated by PFGE, blotted and probed with ^{32}P labelled pAT153. 4 ng of the pSA1 plasmid preparation used in the transfection was also run on the gel (pSA1). The position of various size markers are indicated.

by *amb*UL12, PFGE studies were performed on DNase-resistant DNA from transfected and subsequently superinfected cells.

Plates of BHK cells were transfected with pSA1 or pS1 and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i. and processed for total and DNase-resistant DNA directly in agarose blocks. One third of each block was then loaded into a 1% pulsed field agarose gel and electrophoresed for 16 h at 6 v / cm, 14°C with a switch time ramped from 1 – 15 secs. The gel was blotted and probed with ^{32}P labelled pAT153 (Figure 47).

The total DNA samples show results consistent with Figures 43 – 46. High molecular weight 'well' DNA, a distinct genome length band in the wt HSV-1/pSA1 lane and the presence of 5 and 35 kbp bands in all the samples can be seen. In contrast, only the wt HSV-1/pSA1 sample contains detectable DNase-resistant DNA which is present as two distinct bands; one of 150 kbp and another band migrating between 130 and 140 kbp. These bands are consistent with the sizes of packaged amplicon molecules described by Stow (2001). Increased exposure of the DNase-resistant samples did not detect any genome length packaged amplicons in any of the other samples. However, small amounts of the 5 and 35 kbp bands present in the total DNA appeared to be resistant to DNase. As in section 3.4.3 the experimental conditions used for DNase I treatment resulted in the recovery of only a very small portion of the genome length (presumably packaged) molecules present in the wt HSV-1/pSA1 sample.

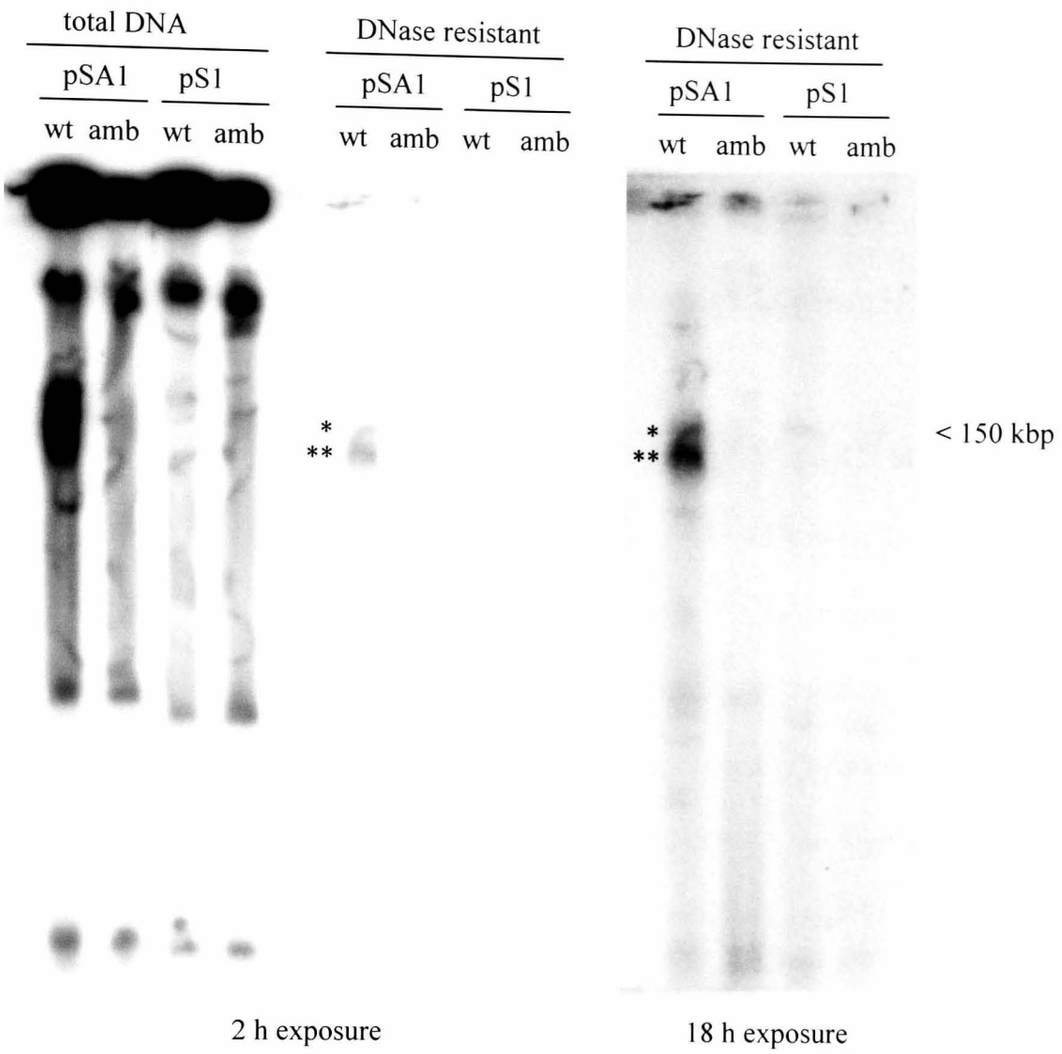


Figure 47 – PFGE analysis of total and DNase-resistant amplicon DNA prepared in agarose embedded cells.

BHK cells were transfected with pSA1 or pS1 and superinfected with wt HSV-1 (wt) or *amb*UL12 (amb). The cells were harvested 16 h.p.i., embedded in agarose blocks and total or DNase-resistant DNA was prepared. One third of each block was separated by PFGE, blotted and probed with ^{32}P labelled pAT153. * and ** show the 150 and 130-140 kbp bands discussed in the text.

5.7.6 PFGE analysis of DNase-resistant amplicons prepared by gentle lysis

Although the preparation of DNA from infected cells embedded in agarose blocks prevents shearing of hmw products, the results of section 3.4 demonstrated that if prepared gently, DNase-resistant genome length molecules could be efficiently and more reproducibly recovered by conventional techniques with minimal shearing. Therefore this method was used to further study DNase-resistant amplicon DNA.

35 mm plates of BHK cells were transfected with 1 μg of pSA1 or pS1 and superinfected with 3 p.f.u. / cell wt HSV-1 or *amb*UL12. The cells were harvested 16 h.p.i., split into nuclear and cytoplasmic fractions and DNase-resistant DNA was prepared by gentle lysis as described in section 3.4.2. Samples representing one third of a plate of cells were loaded into a 1% pulsed field agarose gel and separated at 6 v / cm for 16 h at 14°C with a switch time of 1 – 15 sec, ramping linearly throughout the run. The gel was then Southern blotted and probed with radiolabelled pAT153 (Figure 48, left panel).

The distinctive ladder of packaged molecules described by Vlanzy *et al.* (1982) can be seen in the pSA1/wt HSV-1 sample with most of the DNA concentrated within two regions ranging from approximately 130 – 140 kbp and 150 – 155 kbp. Faint bands of approximately 110 – 150 kbp are evident in both the *amb*UL12/pSA1 and *amb*UL12/pS1 lanes indicating that both amplicons are packaged to near genome length by *amb*UL12. No DNA of a similar size was detected in the wt HSV-1/pS1 lane. In general agreement with Vlazny *et al.* (1982) and Stow (2001) only the 130-140 and 150-155 kbp bands could be detected in the cytoplasmic wt HSV-1/pSA1 sample along

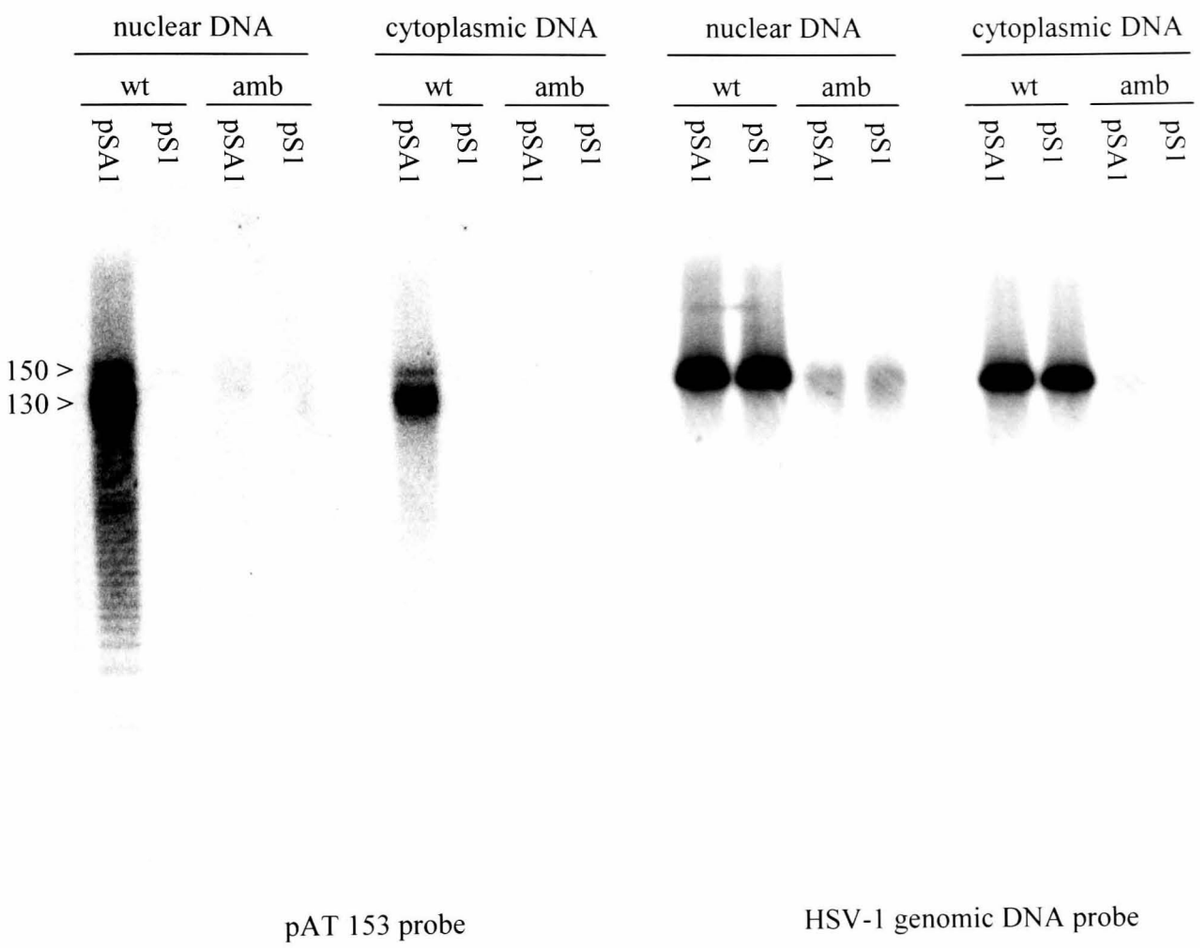


Figure 48 – PFGE analysis of total and DNase-resistant amplicon DNA prepared by the gentle lysis method.

BHK cells were transfected with pSA1 or pS1 and superinfected with wt HSV-1 (wt) or *amb*UL12 (amb). The cells were harvested 16 h.p.i., split into nuclear and cytoplasmic fractions and DNase-resistant DNA prepared. One third of each sample was separated by PFGE, blotted and probed with ^{32}P labelled pAT153 (left panel). The blot was then stripped and reprobed with ^{32}P labelled genomic HSV-1 DNA. The position of the 130 and 150 kbp marker are indicated.

with a faint smear of smaller products. However, no amplicon was detected in the *amb*UL12 cytoplasmic lanes.

To ensure that the absence of signal from certain lanes was not due to unequal loading, the blot was stripped and re-probed with radiolabelled HSV-1 genomic DNA. The right panel of Figure 48 demonstrates that near identical amounts of genomic length wt HSV-1 DNA were present in the nuclear or cytoplasmic samples from the cells which received pSA1 or pS1. Similarly the amount of genomic DNA present in the corresponding samples from *amb*UL12 infected cells was also very similar, although as previously observed packaging was reduced compared to wt HSV-1. The DNA packaged by *amb*UL12 exhibited slightly increased mobility compared to wt HSV-1, in agreement with the results of section 3.4.1. The DNase-resistant amplicon DNA detected in *amb*UL12 infected cells in earlier figures (e.g. Figures 26, 35 and 37) is presumably distributed in a weak smear down the gel.

5.8 Discussion

The results presented in this section demonstrated that the transient assay, employing amplicon plasmids, was a useful and reliable method of studying the *amb*UL12 mutant and its defects in the replication and packaging processes. Various amplicons used in this study were shown to behave in an analogous manner to DNA of the helper virus in that: (i) the replication and packaging of the amplicons were proportionally reduced by almost identical amounts to viral DNA when using *amb*UL12 as a helper virus compared to wt HSV-1, (ii) the replication and packaging defect could be partially complemented by the co-transfection of a UL12 but not a UL12.5 expressing plasmid,

(iii) the amplicons were shown to recombine at a high frequency and produce high molecular weight concatemers comparable to viral DNA and, (iv) a significant proportion of packaged pSA1 amplicon molecules were close in size to the viral genome. In addition, the amplicon system was a convenient way to study the effect of different *a* sequence arrangements and mutations on the packaging of molecules by wt HSV-1 and *amb*UL12. These experiments revealed that: (i) alternative arrangements of *a* sequence components did not affect the packaging of the amplicon by either virus, (ii) the packaging machinery in *amb*UL12 infected cells may interact with mutated *a* sequences in a manner different from wt HSV-1 and, (iii) *amb*UL12 can package and propagate an amplicon that contains no recognised packaging signal. Finally, the transient assay, in combination with pulsed field gel electrophoresis, gave preliminary evidence that amplicon replication may involve both rolling circle and theta form mechanisms. These findings are discussed in further detail below.

Because amplicons had not been used extensively in the characterisation of *nuc* mutants it was important to establish that the replication and packaging of amplicons resembled that of genomic DNA in *amb*UL12 infected cells. The initial experiments focused on the ability of *amb*UL12 to replicate and package the pSA1 amplicon, an amplicon which had been characterised previously (Hodge and Stow, 2001). The results presented in Figure 26 and Table 2 demonstrated that, relative to wt HSV-1, *amb*UL12 was as deficient at replicating and packaging amplicons as its own genome. Moreover, the cleavage of replicated pSA1 by wt HSV-1 or *amb*UL12 helper proteins was shown to occur specifically at the packaging signal, generating DNase-resistant terminal fragments of the sizes predicted for cleavage within the Uc-DR1-Ub fragment (Figure 27). The results from these experiments also demonstrated that the addition of UL12 in

trans was sufficient to at least partially complement the mutant, and indicated that the absence of UL12 was responsible for the observed defects.

The observation that co-expression of UL12 with the HSV-1 DNA replication proteins does not significantly enhance replication of an HSV-1 origin containing plasmid (Table 3) suggests that for UL12 to function in DNA synthesis it may have to be modified by some viral protein other than the seven essential viral replication proteins which were supplied in *trans*. Further evidence suggestive of differential modification of UL12 in virus-infected cells comes from the results of Figure 25 which show different UL12 breakdown products depending on whether the protein is expressed by wt HSV-1 or from the pE12 expression construct. Since UL12 is known to be phosphorylated (Wilcox *et al.*, 1980; Preston and Cordingley, 1982) this modification is most likely required for efficient complementation and may also represent a possible control mechanism for the nuclease. It is possible that the protein kinase encoded by UL13 may play a role in the phosphorylation of UL12 since an HCMV UL97 null mutant that fails to express the HCMV homologue of UL13 has a similar phenotype to HSV-1 UL12 null mutants (Wolf *et al.*, 2001).

An important feature of HSV-1 replication is the high frequency of intra and inter-molecular recombination. Recombination of HSV-1 genomes not only causes inversion of U_L and U_S relative to one another but also generates a highly branched DNA network of complex concatemeric intermediates (Severini *et al.*, 1996). Traditionally, only intra-molecular recombination has been observed during the replication of amplicons (Dutch *et al.*, 1992; Dutch *et al.*, 1994). Recently, however, intermolecular recombination of amplicons was reported in HSV-1 infected cells (Fu *et*

al., 2002). The results presented in this chapter confirm these findings (Figure 31) and demonstrate that the recombination occurs in the absence of UL12. Moreover, recombination between the parental pSA1 plasmid and the modified daughter plasmids, pSA1x and pSA1inv, does not alter the levels of replication and packaging when using either wt HSV-1 or *amb*UL12 as helper virus (Figure 32). Although recombination occurs in the absence of UL12 it cannot be determined form these studies whether the presence of UL12 influences the recombination event itself or perhaps modifies the resultant structures at a later juncture.

Another feature of HSV-1 replication is the formation of high molecular weight concatemers which are subsequently cleaved into unit length genomes. To determine if the replicated amplicon DNA also formed hmw concatemers analogous to HSV-1, pulsed field gel analysis was applied to replicated amplicon DNA. The results presented in Figure 43 demonstrate that amplicons replicated by both wt HSV-1 and *amb*UL12 generate structures that are unable to migrate from the well of the pulsed field gel and which presumably consist of complex branched hmw concatemers. Furthermore, the results shown in Figure 46 demonstrate that the seven essential replication genes are sufficient to form similar structures.

Pulsed field gel studies also confirmed the work of Vlazny *et al.* (1982) and Stow (2001) showing that, when used as helper virus, wt HSV-1 packaged pSA1 concatemers comprising integral numbers of the monomeric repeats up to approximately genome length and only the higher molecular weight species (130 – 155 kbp) were present in the cytoplasm (Figure 48). In addition it was demonstrated that *amb*UL12 was able to package small amounts of pSA1, also of approximately viral genome length. Although

no pSA1 molecules were detected in the cytoplasm of these cells, the propagation experiments described in sections 5.5.3 and 5.6.3 show that *amb*UL12 infected cells are capable of producing mature virus particles that contained pSA1 molecules, and hence packaged pSA1 molecules are presumably capable of exiting the nucleus.

The above results established that the amplicon system was a convenient way to further characterise the *amb*UL12 mutant. The amplicons behaved in a similar manner to viral DNA: they were replicated and packaged to similar relative extents as the genomes of their helper viruses, recombined at a high frequency and formed hmw replication intermediates. To determine whether the defect in *amb*UL12 packaging might be mediated by a direct or indirect interaction with specific regions of the *a* sequence the studies were extended to amplicons containing different arrangements of *a* sequences or amplicons containing mutated *a* sequences.

The pSA1 amplicon contains only the 200bp Uc-DR1-Ub fragment, the minimal sequence thus far shown to act as a packaging signal (Nasseri and Mocarski, 1998; Hodge and Stow, 2001). Studies by Hodge and Stow demonstrated that pSA1 was packaged by wt HSV-1 as efficiently as amplicons containing a single unpermuted *a* sequence or two tandem *a* sequences (Figure 33). The results presented in Figure 34 confirm those of Hodge and Stow for wt HSV-1 and also show that *amb*UL12 replicates and packages all the amplicons with a similar reduced efficiency compared to wt HSV-1. These results indicated that like wt HSV-1, *amb*UL12 utilised the minimal Uc-DR1-Ub sequence as efficiently as a single *a* sequence, in which the Uc and Ub elements are separated by approximately 300 bp, or two tandem complete *a* sequences where the Uc-DR1-Ub fragment is present.

Four mutated amplicons (pPH11, pPH12, pPH21 and pPH22), which contain lesions in the *pac*1 motif (Hodge and Stow, 2001), were used in propagation assays. The results obtained using wt HSV-1 as helper virus confirmed those of Hodge and Stow demonstrating that all four amplicons could be packaged to near pSA1 levels but were propagated with reduced efficiency, especially the pPH11 and pPH12 amplicons which contained substitution/deletion mutations affecting the distal GC element. These studies were extended by using *amb*UL12 as the helper virus. The packaging efficiencies of the four mutant plasmids were similar to pSA1 (Figure 36). Relative to pSA1, pPH11 and pPH12 exhibited a 3-8 fold greater impairment in propagation when packaged in the presence of wt HSV-1, whilst pPH21 and pPH22 were more impaired when packaged using *amb*UL12 as helper. In fact, under these conditions the propagation of pPH21 and pPH22, initially packaged by *amb*UL12, was similar to that observed with pS1 which completely lacks the Uc-DR1-Ub fragment. This suggests that the packaging machinery of *amb*UL12 may recognise or interact, either directly or indirectly, with the *a* sequence differently from that of wt HSV-1 during the cleavage and packaging processes.

Perhaps the most interesting observation from the propagation experiment using the amplicons with mutated *a* sequences was that the pS1 amplicon, which contains no known packaging sequence, was packaged to a significant level by *amb*UL12 but not wt HSV-1. In addition pS1 which had been packaged by *amb*UL12 was propagated as efficiently as the pPH21 and pPH22 amplicons even though the levels of DNase-resistant DNA detected in the cells transfected with these amplicons were 5-fold higher than for pS1 (Figure 36). This suggests that the majority of the capsids containing

pPH21 or pPH22 did not mature into infectious virions although further studies are required to determine at what stage capsid maturation was inhibited.

Upon further investigation of the ability of *amb*UL12 to package pS1 it was evident that co-transfection of a UL12 expressing plasmid increased the replication of the plasmid but inhibited its packaging (Figure 37 and 38), again suggesting a direct role for the nuclease in the packaging of viral DNA. This experiment also included the Vero cell line and demonstrated that the packaging of pS1 by *amb*UL12 was not a BHK cell line specific phenomenon. Interestingly, co-transfection of a UL12.5 expressing plasmid also reduced the level of pS1 packaging in both cell lines when *amb*UL12 was providing helper functions suggesting that this protein may have some limited functionality. In Figure 38A it also appeared to slightly increase the replication of the amplicon but this effect was not consistently observed.

To ensure that the detected DNase-resistant pS1 molecules were encapsidated and not protected from the DNase by some other means two controls were used. Firstly, the HSV-1 packaging deficient mutant K5ΔZ demonstrated that in BHK cells all replicated amplicon DNA was digested to completion but in the complementing cell line pSA1 was efficiently packaged (Figure 37). Secondly, packaged pS1 molecules from *amb*UL12 but not wt HSV-1 or *amb*UL12R infected cells could be propagated (Figure 39), indicating that this property is attributable to the lack of a functional alkaline nuclease.

It was possible that the packaging and propagation of pS1 molecules was due to integtration of the amplicon with the helper-virus genome. Analysis of packaged viral

and amplicon molecules using a restriction enzyme that cleaved only viral DNA demonstrated that digestion of the viral genome did not alter the migration of the amplicon molecules (Figure 42). In addition, the majority of pS1 signal was detected in the region of non-resolution comprising fragments over 28 kbp. Since the viral capsid can accommodate genomes only approximately 10-12 kbp longer than standard length (Gage *et al.*, 1992; Sauer *et al.*, 1987; F. Rixon, personal communication) these larger pS1 molecules must have been independently packaged.

In a number of experiments a small amount of DNase-resistant pS1 was detectable following infection with wt HSV-1. This product was also recently observed by Hodge and Stow (2001). In the experiments presented here the amount of packaged pS1 detected in wt HSV-1 infected cells represented less than 1% of that of pSA1, in agreement with the results of Hodge and Stow. Three observations, however, distinguish between pS1 encapsidated by *amb*UL12 and wt HSV-1. Firstly, although *amb*UL12 packages 15-fold less pSA1 than wt HSV-1, it packages 2-3 fold more pS1. Secondly, packaging of pS1 by wt HSV-1 appears to be a BHK cell specific effect, not seen in Vero or 293 cells (Figure 38 and Figure 41). Thirdly, only pS1 encapsidated in *amb*UL12 infected cells can be propagated in fresh monolayers (Figure 36 and Figure 39). Therefore, the mechanism of packaging of pS1 may differ in wt HSV-1 or *amb*UL12 infected BHK cells.

Finally, the pulsed field gel (PFG) analysis of replicated amplicon DNA has provided preliminary evidence that, at some point during the transient assay, amplicons may be replicated by a theta-like mechanism. In addition to the genome length and hmw products detected in PFGs of virally infected cells, the PFGs described in sections 5.7.1,

5.7.2 and 5.7.3 contained two extra amplicon bands of approximately 5 and 35 kbp. The 5kbp but not the 35 kbp product represents replicated DNA as judged by *Dpn*I digestion (Figure 45). These products are not packaged since they are present in similar amounts in pSA1 and pS1 transfected cells (Figure 44). The observation that the 5 and 35 kbp bands in the transfected cells co-migrate with the presumably supercoiled and open-circular forms, respectively, of the pSA1 plasmid used for the transfection suggests that the bands detected may represent similar topological forms. Moreover, some gels also contained a ladder of faint bands between the 5 and 35 kbp bands, possibly representing transfected plasmids with decreasing numbers of negative supercoils. The simplest way to account for the replicated 5 kbp molecules that co-migrate with the supercoiled plasmid is for a portion of the input plasmid to be replicated by a theta-like mechanism.

Although indicative of theta like replication, the results presented require a number of experiments to ensure that, a) the 5 and 35 kbp products are circular molecules (possibly by attempting to relax the product through nicking or TopoII treatment or by analysing purified DNA by restriction enzyme digestion) and, b) the 5 kbp product is replicated by the HSV-1 DNA replication machinery (e.g. the product does not appear with an ori_S^- plasmid or in the presence of PAA). Nonetheless, these results are not without precedent; work by Pfüller and Hammerschmidt, (1996) demonstrated that following induction, episomally maintained plasmids containing the Epstein-Barr Virus *oriP* and *oriLyt* sequences would initially replicate by a theta-like mechanism before producing complex, hmw products.

Chapter 6: Conclusions

My characterisation of the HSV-1 *amb*UL12 virus demonstrated that this UL12 null mutant is inefficient at replicating and packaging both viral and amplicon DNA compared to wt virus. Previous studies had indicated that the major defect in *nuc* mutants was in the egress of DNA containing capsids from the nucleus (Shao *et al.*, 1993). Although the results presented in this thesis cannot exclude an additional defect in capsid egress, it is clear that, in contrast to previous reports using the AN-1 and AN-F1 *nuc* mutants (Weller *et al.*, 1990; Shao *et al.*, 1993; Martinez *et al.*, 1996b), the HSV-1 alkaline nuclease plays a significant role in the processing and packaging of concatemeric DNA. This conclusion is supported by the observation that in transient amplicon assays *amb*UL12 packaged considerable amounts of concatemeric DNA that contained no recognised packaging signal.

The replication of both genomic and amplicon DNA by *amb*UL12 was reduced 3-5 fold compared to wt HSV-1 in Vero and BHK cells. This replication defect was detectable as early as 6 h.p.i. In transient assays for HSV-1 ori_S dependent DNA synthesis the replication defect could be partially complemented by supplying UL12 in *trans*, indicating that the reduced levels of DNA were due to the absence of the alkaline nuclease. Similarly, comparable packaging defects were observed when encapsidation of either viral or amplicon DNA was examined. In BHK and Vero cells a 15 – 20 fold reduction in packaging compared to wt HSV-1 was observed, and again this defect could be partially complemented in transient assays when UL12 was supplied.

Examination of the termini of encapsidated *amb*UL12 genomes demonstrated that

inefficient and aberrant cleavage of concatemeric DNA appears to be at least partially responsible for the packaging defect. The irregular cleavage events occurring during *amb*UL12 infection were also apparent when DNase-resistant DNA was examined by PFGE. In these experiments the majority of resistant *amb*UL12 DNA migrated faster than that of wt HSV-1, suggesting that incomplete genomes had been packaged. An alternative possibility is that these molecules represent full length genomes that, due to the absence of UL12, contain structural aberrations, which increase their mobility.

The use of amplicons containing mutations in the viral cleavage/packaging signal (*a* sequence) suggested that in the absence of the alkaline nuclease the viral cleavage/packaging machinery may interact with the *a* sequence in a different way. In addition, *amb*UL12 was able to package molecules lacking a known packaging signal. These molecules could be subsequently propagated in the presence of helper virus.

Finally, in a preliminary co-immunoprecipitation screen no convincing interactions were observed between the alkaline nuclease and four of the viral proteins (UL6, UL15, UL25 and UL28) which are necessary for the packaging of viral DNA.

Although UL12 is not essential for the replication of viral DNA the work presented in this thesis consistently demonstrated that it does have a direct or indirect role in origin-dependent DNA replication. While the interaction of UL12 with UL29 (Vaughan *et al.*, 1984; Thomas *et al.*, 1988, 1992; Section 4.2) would suggest a more direct involvement in the replication process, immunofluoresence and electron immuno-gold staining studies report that UL12 and UL29 have distinct nuclear localisation patterns. UL29 has been shown to localise to defined replication compartments

(Quinlan *et al.*, 1984; de Bruyn-Kops and Knipe, 1988) whereas UL12 has a more diffuse nuclear staining pattern (Goldstein and Weller, 1998a). Moreover, in immuno-gold electron microscopy studies UL12 has been shown to associate with electron-dense bodies while UL29 is excluded (Puvion-Dutilleul *et al.*, 1985; Puvion-Dutilleul and Prichard, 1986). These results suggest that the role of UL12 in DNA replication may be indirect, although it must also be considered that the diffuse immunofluoresence staining may reflect multi-functional properties of UL12.

It has been postulated that the role of UL12 is to process complex replication intermediates into linear substrates, more suitable for packaging (Martinez *et al.*, 1996a). It is also possible that the accumulation of these DNA intermediates inhibits replication, perhaps by causing replication forks to stall more frequently. Therefore, it is feasible that UL12 also provides, or promotes, some form of repair function by resolving these complex DNA intermediates.

Work by Bogner *et al.* (1998) identified a number of HCMV viral and cellular proteins which would bind specifically to either the HCMV *pac*1 or *pac*2 motifs. In particular, the homologue of the HSV-1 UL28 protein, UL56, was shown to bind specifically to the *pac*1 motif. A recent report has indicated that modification of the secondary structure of the *a* sequence may play an important role in its recognition by the UL28 protein (Adelman *et al.*, 2001). In these studies UL28 could bind an oligonucleotide, comprising the HSV-1 *pac*1 motif sequence, only if the oligonucleotide had been denatured and renatured to form a novel secondary structure. This binding was also primary sequence specific, and *pac*2 motif oligonucleotides were not recognised by UL28. Although these novel structures were generated *in vitro* it has also been

demonstrated that the DR2 motifs within the *a* sequence are capable of forming unusual secondary structures *in vivo* when present in supercoiled plasmid DNA (Wohlrab *et al.*, 1987), suggesting that the *pac* motifs may form similar structures *in vivo*.

Since the absence of UL12 appears to alter both packaging specificity and efficiency, it is possible that UL12 may be involved in the *in vivo* generation of the *pac* motif secondary structures. In such a model UL12 could work in both a spatially and temporally distinct location from the cleavage/packaging machinery while imparting packaging specificity. This could explain the inability to detect any interaction of UL12 with the packaging proteins tested in the immunoprecipitation assays. In support of this, UL12 has been reported to interact with the *a* sequence in an apparently sequence-independent manner (Chou and Roizman, 1989). It is, however, possible that UL12 could interact with another as yet unidentified protein that did impart sequence specificity.

6.1 Future Work

Since *amb*UL12 is defective in both replication and packaging it is difficult to determine if the alkaline nuclease is directly involved in packaging or whether the DNA replication intermediates formed in the absence of UL12 are inherently poor packaging substrates. One way to address this problem might be to use a packaging mutant with a reversible temperature sensitive defect. A possible mutant is *ts*1201, which carries a lesion in the capsid associated protease, UL26 (Preston *et al.*, 1983). The viral DNA could be replicated at the non-permissive temperature in the presence of UL12. After replication had been allowed to proceed UL12 could be inhibited by using a recently

identified inhibitor specific to the alkaline nuclease (Bronstein and Weber, 2001) and packaging allowed to proceed by shifting down to the permissive temperature. If DNA was packaged to wt levels it would suggest that UL12 is not directly involved in the packaging process.

Complex hmw DNA replication intermediates are formed during the replication of both wt HSV-1 and *amb*UL12 DNA. The branches in wt are composed of both X and Y junctions, although the Y junctions are more prevelant (Severini *et al.*, 1996). The DNA replication intermediates in *nuc* mutants are more complex (Martinez *et al.*, 1996a; section 3.3) although the nature of this complexity is not known. Examination of both packaged DNA and hmw DNA replication intermediates of *amb*UL12 using neutral agarose two-dimensional gel electrophoresis could give insights as to why cleavage is inefficient and often aberrant (e.g. do the branched structures differ from X or Y type junctions, or does the DNA merely contain an increased number of X or Y junctions compared to wt HSV-1). In addition, this approach could also determine if the abortive packaging events and instability of DNA containing capsids is due to the retention of branches or other abnormal structures in the DNA.

In the light of work which demonstrates that both ori_S and the *a* sequence are capable of forming novel conformations *in vitro* (Aslani *et al.*, 2000; Baker *et al.*, 2000; Adelman *et al.*, 2001) it would be interesting to determine whether similar structures are formed *in vivo* and also if UL12 was involved in forming these novel secondary structures.

Although the preliminary protein interaction studies produced no evidence of novel interactions it is still possible that the function of UL12 involves interactions with a

number of viral and/or cellular proteins in addition to UL29. It would therefore be useful to identify other proteins with which UL12 interacts to gain possible additional information on both functional and regulatory aspects of the protein. Further immunoprecipitation experiments with the baculovirus system described in this thesis might identify other viral proteins which interact with UL12. However, to determine if UL12 interacts with any cellular proteins it would be more useful to generate a recombinant HSV-1 virus that expressed a functional His-tagged version of UL12. This could then be used to identify and characterise viral or cellular proteins that co-precipitate with UL12. Alternatively, protein-protein interactions involving UL12 could be investigated by yeast two-hybrid screens.

It is known that UL12 is a highly phosphorylated protein. It would therefore be interesting to know what effects, if any, the levels of phosphorylation have on the activity of the protein. It has never been conclusively established whether viral or cellular proteins phosphorylate UL12 or if this phosphorylation acts as a control mechanism.

Finally, a full understanding of the role of UL12 requires definition of its *in vivo* biochemical activities and the development of appropriate enzyme assays (e.g. processing of appropriate substrates and reconstruction of functional complexes it may form with other proteins). Identification of the precise role of UL12 in replication and packaging will be greatly facilitated once cell free systems that can perform these processes have been developed.

References

Abbotts, A. P., Preston, V. G., Hughes, M., Patel, A. H. & Stow, N. D. (2000). Interaction of the herpes simplex virus type 1 packaging protein UL15 with full-length and deleted forms of the UL28 protein. *J. Gen. Virol.* **81**, 2999-3009.

Addison, C., Rixon, F. J. & Preston, V. G. (1990). Herpes simplex virus type 1 UL28 gene product is important for the formation of mature capsids. *J. Gen. Virol.* **71**, 2377-84.

Addison, C., Rixon, F. J., Palfreyman, J. W., O'Hara, M. & Preston, V. G. (1984). Characterisation of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. *Virology* **138**, 246-59.

Adelman, K., Salmon, B. & Baines, J. D. (2001). Herpes simplex virus DNA packaging sequences adopt novel structures that are specifically recognized by a component of the cleavage and packaging machinery. *Proc. Natl. Acad. Sci. U S A* **98**, 3086-91.

Albrecht, J. C., Nicholas, J., Biller, D., Cameron, K. R., Biesinger, B., Newman, C., Wittmann, S., Craxton, M. A., Coleman, H., Fleckenstein, B. & et al. (1992). Primary structure of the herpesvirus saimiri genome. *J. Virol.* **66**, 5047-58.

al-Kobaisi, M. F., Rixon, F. J., McDougall, I. & Preston, V. G. (1991). The herpes simplex virus UL33 gene product is required for the assembly of full capsids. *Virology* **180**, 380-8.

Arzul, I., Nicolas, J. L., Davison, A. J. & Renault, T. (2001). French scallops: a new host for ostreid herpesvirus-1. *Virology* **290**, 342-9.

Aslani, A., Simonsson, S. & Elias, P. (2000). A novel conformation of the herpes simplex virus origin of DNA replication recognized by the origin binding protein. *J Biol Chem* **275**, 5880-7.

Ausubel, F. M. (1999). *Current Protocols in Molecular Biology*. Lippincott, Williams & Wilkins.

Baer, R., Bankier, A. T., Biggin, M. D., Deininger, P. L., Farrell, P. J., Gibson, T. J., Hatfull, G., Hudson, G. S., Satchwell, S. C., Seguin, C. & et al. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature* **310**, 207-11.

Baines, J. D. & Roizman, B. (1991). The open reading frames UL3, UL4, UL10, and UL16 are dispensable for the replication of herpes simplex virus 1 in cell culture. *J. Virol.* **65**, 938-44.

Baines, J. D. & Roizman, B. (1992). The cDNA of UL15, a highly conserved herpes simplex virus 1 gene, effectively replaces the two exons of the wild-type virus. *J. Virol.* **66**, 5621-6.

Baines, J. D., Cunningham, C., Nalwanga, D. & Davison, A. (1997). The U(L)15 gene of herpes simplex virus type 1 contains within its second exon a novel open reading frame that is translated in frame with the U(L)15 gene product. *J. Virol.* **71**, 2666-73.

Baines, J. D., Poon, A. P., Rovnak, J. & Roizman, B. (1994). The herpes simplex virus 1 UL15 gene encodes two proteins and is required for cleavage of genomic viral DNA. *J. Virol.* **68**, 8118-24.

Baker, R. O., Murata, L. B., Dodson, M. S. & Hall, J. D. (2000). Purification and characterization of OF-1, a host factor implicated in herpes simplex replication. *J Biol Chem.* **275**, 30050-7.

Banks, L. M., Halliburton, I. W., Purifoy, D. J., Killington, R. A. & Powell, K. L. (1985). Studies on the herpes simplex virus alkaline nuclease: detection of type-common and type-specific epitopes on the enzyme. *J. Gen. Virol.* **66**, 1-14.

Banks, L., Purifoy, D. J., Hurst, P. F., Killington, R. A. & Powell, K. L. (1983). Herpes simplex virus non-structural proteins. IV. Purification of the virus-induced deoxyribonuclease and characterization of the enzyme using monoclonal antibodies. *J. Gen. Virol.* **64**, 2249-60.

Barnard, E. C., Brown, G. & Stow, N. D. (1997). Deletion mutants of the herpes simplex virus type 1 UL8 protein: effect on DNA synthesis and ability to interact with and influence the intracellular localization of the UL5 and UL52 proteins. *Virology* **237**, 97-106.

Bataille, D. & Epstein, A. L. (1997). Equimolar generation of the four possible arrangements of adjacent L components in herpes simplex virus type 1 replicative intermediates. *J. Virol.* **71**, 7736-43.

Beard, P. M., Taus, N. S. & Baines, J. D. (2002). DNA cleavage and packaging proteins encoded by genes U(L)28, U(L)15, and U(L)33 of herpes simplex virus type 1 form a complex in infected cells. *J. Virol.* **76**, 4785-91.

Becker, A. & Gold, M. (1978). Enzymatic breakage of the cohesive end site of phage lambda DNA: terminase (ter) reaction. *Proc. Natl. Acad. Sci. U S A* **75**, 4199-203.

Ben-Porat, T. & Rixon, F. J. (1979). Replication of herpesvirus DNA. IV: analysis of concatemers. *Virology* **94**, 61-70.

Bhella, D., Rixon, F. J. & Dargan, D. J. (2000). Cryomicroscopy of human cytomegalovirus virions reveals more densely packed genomic DNA than in herpes simplex virus type 1. *J. Mol. Biol.* **295**, 155-61.

Blanco, L., Bernad, A. & Salas, M. (1991). MIP1 DNA polymerase of S. cerevisiae: structural similarity with the E. coli DNA polymerase I-type enzymes. *Nucleic Acids Res* **19**, 955.

Boehmer, P. E., Dodson, M. S. & Lehman, I. R. (1993). The herpes simplex virus type-1 origin binding protein. DNA helicase activity. *J Biol Chem.* **268**, 1220-5.

Bogner, E., Radsak, K. & Stinski, M. F. (1998). The gene product of human cytomegalovirus open reading frame UL56 binds the pac motif and has specific nuclease activity. *J. Virol.* **72**, 2259-64.

Booy, F. P., Newcomb, W. W., Trus, B. L., Brown, J. C., Baker, T. S. & Steven, A. C. (1991). Liquid-crystalline, phage-like packing of encapsidated DNA in herpes simplex virus. *Cell* **64**, 1007-15.

Bortner, C., Hernandez, T. R., Lehman, I. R. & Griffith, J. (1993). Herpes simplex virus 1 single-strand DNA-binding protein (ICP8) will promote homologous pairing and strand transfer. *J. Mol. Biol.* **231**, 241-50.

Brack, A. R., Dijkstra, J. M., Granzow, H., Klupp, B. G. & Mettenleiter, T. C. (1999). Inhibition of virion maturation by simultaneous deletion of glycoproteins E, I, and M of pseudorabies virus. *J. Virol.* **73**, 5364-72.

Bronstein, J. C. & Weber, P. C. (1996). Purification and characterization of herpes simplex virus type 1 alkaline exonuclease expressed in Escherichia coli. *J. Virol.* **70**, 2008-13.

Bronstein, J. C., Weller, S. K. & Weber, P. C. (1997). The product of the UL12.5 gene of herpes simplex virus type 1 is a capsid-associated nuclease. *J. Virol.* **71**, 3039-47.

Bronstein, J. C. & Weber, P. C. (2001). A colorimetric assay for high-throughput screening of inhibitors of herpes simplex virus type 1 alkaline nuclease. *Anal BioChem.* **293**, 239-45.

Calder, J. M. & Stow, N. D. (1990). Herpes simplex virus helicase-primase: the UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities. *Nucleic Acids Res* **18**, 3573-8.

Calder, J. M., Stow, E. C. & Stow, N. D. (1992). On the cellular localization of the components of the herpes simplex virus type 1 helicase-primase complex and the viral origin-binding protein. *J. Gen. Virol.* **73**, 531-8.

Campadelli-Fiume, G., Arsenakis, M., Farabegoli, F. & Roizman, B. (1988). Entry of herpes simplex virus 1 in BJ cells that constitutively express viral glycoprotein D is by endocytosis and results in degradation of the virus. *J. Virol.* **62**, 159-67.

Carmichael, E. P. & Weller, S. K. (1989). Herpes simplex virus type 1 DNA synthesis requires the product of the UL8 gene: isolation and characterization of an ICP6::lacZ insertion mutation. *J. Virol.* **63**, 591-9.

Carmichael, E. P., Kosovsky, M. J. & Weller, S. K. (1988). Isolation and characterization of herpes simplex virus type 1 host range mutants defective in viral DNA synthesis. *J. Virol.* **62**, 91-9.

Catalano, C. E. (2000). The terminase enzyme from bacteriophage lambda: a DNA-packaging machine. *Cell. Mol. Life. Sci.* **57**, 128-48.

Catalano, C. E., Cue, D. & Feiss, M. (1995). Virus DNA packaging: the strategy used by phage lambda. *Mol. Microbiol.* **16**, 1075-86.

Cavalcoli, J. D., Baghian, A., Homa, F. L. & Kousoulas, K. G. (1993). Resolution of genotypic and phenotypic properties of herpes simplex virus type 1 temperature-sensitive mutant (KOS) tsZ47: evidence for allelic complementation in the UL28 gene. *Virology* **197**, 23-34.

Cebrian, J., Berthelot, N. & Laithier, M. (1989). Genome structure of cottontail rabbit herpesvirus. *J. Virol.* **63**, 523-31.

Challberg, M. D. (1986). A method for identifying the viral genes required for herpesvirus DNA replication. *Proc. Natl. Acad. Sci. U S A* **83**, 9094-8.

Chang, Y. E., Poon, A. P. & Roizman, B. (1996). Properties of the protein encoded by the UL32 open reading frame of herpes simplex virus 1. *J. Virol.* **70**, 3938-46.

Chen, Y. M. & Knipe, D. M. (1996). A dominant mutant form of the herpes simplex virus ICP8 protein decreases viral late gene transcription. *Virology* **221**, 281-90.

Chou, J. & Roizman, B. (1989). Characterization of DNA sequence-common and sequence-specific proteins binding to cis-acting sites for cleavage of the terminal a sequence of the herpes simplex virus 1 genome. *J. Virol.* **63**, 1059-68.

Coen, D. M., Kosz-Vnenchak, M., Jacobson, J. G., Leib, D. A., Bogard, C. L., Schaffer, P. A., Tyler, K. L. & Knipe, D. M. (1989). Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proc. Natl. Acad. Sci. U S A* **86**, 4736-40.

Costa, R. H., Draper, K. G., Banks, L., Powell, K. L., Cohen, G., Eisenberg, R. & Wagner, E. K. (1983). High-resolution characterization of herpes simplex virus type 1 transcripts encoding alkaline exonuclease and a 50,000-dalton protein tentatively identified as a capsid protein. *J. Virol.* **48**, 591-603.

Crute, J. J., Mocarski, E. S. & Lehman, I. R. (1988). A DNA helicase induced by herpes simplex virus type 1. *Nucleic. Acids. Res.* **16**, 6585-96.

Crute, J. J., Tsurumi, T., Zhu, L. A., Weller, S. K., Olivo, P. D., Challberg, M. D., Mocarski, E. S. & Lehman, I. R. (1989). Herpes simplex virus 1 helicase-primase: a complex of three herpes- encoded gene products. *Proc. Natl. Acad. Sci. U S A* **86**, 2186-9.

Daikoku, T., Yamashita, Y., Tsurumi, T. & Nishiyama, Y. (1995). The US3 protein kinase of herpes simplex virus type 2 is associated with phosphorylation of the UL12 alkaline nuclease in vitro. *Arch Virol* **140**, 1637-44.

Davison, A. J. & McGeoch, D.J. (1995) Herpesviridae. In *Molecular Basis of Viral Evolution*, pp. 290-309. Edited by A. Gibs, C. Calisher & F. Garcia-Arenal. Cambridge: Cambridge University Press.

Davison, A. J. & Scott, J. E. (1986). The complete DNA sequence of varicella-zoster virus. *J. Gen. Virol.* **67**, 1759-816.

Davison, A. J. & Wilkie, N. M. (1981). Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. *J. Gen. Virol.* **55**, 315-31.

Davison, A. J. (1992). Channel catfish virus: a new type of herpesvirus. *Virology* **186**, 9-14.

de Bruyn Kops, A., Uprichard, S. L., Chen, M. & Knipe, D. M. (1998). Comparison of the intranuclear distributions of herpes simplex virus proteins involved in various viral functions. *Virology* **252**, 162-78.

Deiss, L. P., Chou, J. & Frenkel, N. (1986). Functional domains within the a sequence involved in the cleavage- packaging of herpes simplex virus DNA. *J. Virol.* **59**, 605-18.

DeLuca, N. A., McCarthy, A. M. & Schaffer, P. A. (1985). Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J. Virol.* **56**, 558-70.

Desai, P., DeLuca, N. A., Glorioso, J. C. & Person, S. (1993). Mutations in herpes simplex virus type 1 genes encoding VP5 and VP23 abrogate capsid formation and cleavage of replicated DNA. *J. Virol.* **67**, 1357-64.

Digard, P. & Coen, D. M. (1990). A novel functional domain of an alpha-like DNA polymerase. The binding site on the herpes simplex virus polymerase for the viral UL42 protein. *J. Biol. Chem.*. **265**, 17393-6.

DiIanni, C. L., Drier, D. A., Deckman, I. C., McCann, P. J., 3rd, Liu, F., Roizman, B., Colonno, R. J. & Cordingley, M. G. (1993). Identification of the herpes simplex

virus-1 protease cleavage sites by direct sequence analysis of autoproteolytic cleavage products. *J. Biol. Chem.*. **268**, 2048-51.

Dodson, M. S. & Lehman, I. R. (1991). Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase-primase composed of the UL5 and UL52 gene products. *Proc. Natl. Acad. Sci. U S A* **88**, 1105-9.

Dolan, A., Jamieson, F. E., Cunningham, C., Barnett, B. C. & McGeoch, D. J. (1998). The genome sequence of herpes simplex virus type 2. *J. Virol.* **72**, 2010-21.

Dolan, A., Jamieson, F. E., Cunningham, C., Barnett, B. C. & McGeoch, D. J. (1998). The genome sequence of herpes simplex virus type 2. *J. Virol.* **72**, 2010-21.

Dracheva, S., Koonin, E. V. & Crute, J. J. (1995). Identification of the primase active site of the herpes simplex virus type 1 helicase-primase. *J. Biol. Chem.*. **270**, 14148-53.

Draper, K. G., Devi-Rao, G., Costa, R. H., Blair, E. D., Thompson, R. L. & Wagner, E. K. (1986). Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonucleases and overlapping proteins. *J. Virol.* **57**, 1023-36.

Dutch, R. E., Bruckner, R. C., Mocarski, E. S. & Lehman, I. R. (1992). Herpes simplex virus type 1 recombination: role of DNA replication and viral a sequences. *J. Virol.* **66**, 277-85.

Dutch, R. E., Zemelman, B. V. & Lehman, I. R. (1994). Herpes simplex virus type 1 recombination: the Uc-DR1 region is required for high-level a-sequence-mediated recombination. *J. Virol.* **68**, 3733-41.

Elias, P., Gustafsson, C. M. & Hammarsten, O. (1990). The origin binding protein of herpes simplex virus 1 binds cooperatively to the viral origin of replication oris. *J. Biol. Chem.* **265**, 17167-73.

Elias, P., Gustafsson, C. M., Hammarsten, O. & Stow, N. D. (1992). Structural elements required for the cooperative binding of the herpes simplex virus origin binding protein to oriS reside in the N-terminal part of the protein. *J. Biol. Chem.* **267**, 17424-9.

Falkenberg, M., Bushnell, D. A., Elias, P. & Lehman, I. R. (1997). The UL8 subunit of the heterotrimeric herpes simplex virus type 1 helicase-primase is required for the unwinding of single strand DNA- binding protein (ICP8)-coated DNA substrates. *J. Biol. Chem.* **272**, 22766-70.

Francke, B., Moss, H., Timbury, M. C. & Hay, J. (1978). Alkaline DNase activity in cells infected with a temperature-sensitive mutant of herpes simplex virus type 2. *J. Virol.* **26**, 209-13.

Frenkel, N. & Roizman, B. (1972). Separation of the herpesvirus deoxyribonucleic acid duplex into unique fragments and intact strand on sedimentation in alkaline gradients. *J. Virol.* **10**, 565-72.

Fu, X., Wang, H. & Zhang, X. (2002). High-Frequency Intermolecular Homologous Recombination during Herpes Simplex Virus-Mediated Plasmid DNA Replication. *J. Virol.* **76**, 5866-74.

Fujisawa, H. & Morita, M. (1997). Phage DNA packaging. *Genes Cells* **2**, 537-45.

Gage, P. J., Sauer, B., Levine, M. & Glorioso, J. C. (1992). A cell-free recombination system for site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 genome. *J. Virol.* **66**, 5509-15.

Gallo, M. L., Jackwood, D. H., Murphy, M., Marsden, H. S. & Parris, D. S. (1988). Purification of the herpes simplex virus type 1 65-kilodalton DNA- binding protein: properties of the protein and evidence of its association with the virus-encoded DNA polymerase. *J. Virol.* **62**, 2874-83.

Gao, M. & Knipe, D. M. (1991). Potential role for herpes simplex virus ICP8 DNA replication protein in stimulation of late gene expression. *J. Virol.* **65**, 2666-75.

Gao, M., Robertson, B. J., McCann, P. J., O'Boyle, D. R., Weller, S. K., Newcomb, W. W., Brown, J. C. & Weinheimer, S. P. (1998). Functional conservations of the alkaline nuclease of herpes simplex type 1 and human cytomegalovirus. *Virology* **249**, 460-70.

Garber, D. A., Beverly, S. M., Coen, D. M. (1993). Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. *Virology* **197**, 459-62.

Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J. & Spear, P. G. (1998). Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science* **280**, 1618-20.

Gibson, W. & Roizman, B. (1972). Proteins specified by herpes simplex virus. 8. Characterization and composition of multiple capsid forms of subtypes 1 and 2. *J. Virol.* **10**, 1044-52.

Godowski, P. J. & Knipe, D. M. (1986). Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. *Proc. Natl. Acad. Sci. U S A* **83**, 256-60.

Goldstein, D. J. & Weller, S. K. (1988). Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. *J. Virol.* **62**, 196-205.

Goldstein, J. N. & Weller, S. K. (1998a). The exonuclease activity of HSV-1 UL12 is required for in vivo function. *Virology* **244**, 442-57.

Goldstein, J. N. & Weller, S. K. (1998b). In vitro processing of herpes simplex virus type 1 DNA replication intermediates by the viral alkaline nuclease, UL12. *J. Virol.* **72**, 8772-81.

Gorbalenya, A. E., Koonin, E. V., Donchenko, A. P. & Blinov, V. M. (1989). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. *Nucleic. Acids. Res.* **17**, 4713-30.

Gottlieb, J. & Challberg, M. D. (1994). Interaction of herpes simplex virus type 1 DNA polymerase and the UL42 accessory protein with a model primer template. *J. Virol.* **68**, 4937-45.

Gottlieb, J., Marcy, A. I., Coen, D. M. & Challberg, M. D. (1990). The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. *J. Virol.* **64**, 5976-87.

Graves-Woodward, K. L. & Weller, S. K. (1996). Replacement of gly815 in helicase motif V alters the single-stranded DNA-dependent ATPase activity of the herpes simplex virus type 1 helicase-primase. *J. Biol. Chem.*. **271**, 13629-35.

Hall, D. L. & Darke, P. L. (1995). Activation of the herpes simplex virus type 1 protease. *J. Biol. Chem.*. **270**, 22697-700.

Hammarsten, O., Yao, X. & Elias, P. (1996). Inhibition of topoisomerase II by ICRF-193 prevents efficient replication of herpes simplex virus type 1. *J. Virol.* **70**, 4523-9.

Hardwicke, M. A. & Schaffer, P. A. (1995). Cloning and characterization of herpes simplex virus type 1 oriL: comparison of replication and protein-DNA complex formation by oriL and oriS. *J. Virol.* **69**, 1377-88.

Hardy, W. R. & Sandri-Goldin, R. M. (1994). Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect. *J. Virol.* **68**, 7790-9.

Hayward, G. S., Jacob, R. J., Wadsworth, S. C. & Roizman, B. (1975). Anatomy of herpes simplex virus DNA: evidence for four populations of molecules that differ in the relative orientations of their long and short components. *Proc. Natl. Acad. Sci. U S A* **72**, 4243-7.

Hazuda, D. J., Perry, H. C. & McClements, W. L. (1992). Cooperative interactions between replication origin-bound molecules of herpes simplex virus origin-binding protein are mediated via the amino terminus of the protein. *J. Biol. Chem.* **267**, 14309-15.

Hazuda, D. J., Perry, H. C., Naylor, A. M. & McClements, W. L. (1991). Characterization of the herpes simplex virus origin binding protein interaction with OriS. *J. Biol. Chem.* **266**, 24621-6.

Heilbronn, R. & zur Hausen, H. (1989). A subset of herpes simplex virus replication genes induces DNA amplification within the host cell genome. *J. Virol.* **63**, 3683-92.

Henderson, J. O., Ball-Goodrich, L. J. & Parris, D. S. (1998). Structure-function analysis of the herpes simplex virus type 1 UL12 gene: correlation of deoxyribonuclease activity in vitro with replication function. *Virology* **243**, 247-59.

Hodge, P. D. & Stow, N. D. (2001). Effects of mutations within the herpes simplex virus type 1 DNA encapsidation signal on packaging efficiency. *J. Virol.* **75**, 8977-86.

Hoffmann, P. J. & Cheng, Y. C. (1978). The deoxyribonuclease induced after infection of KB cells by herpes simplex virus type 1 or type 2. I. Purification and characterization of the enzyme. *J. Biol. Chem.* **253**, 3557-62.

Hoffmann, P. J. & Cheng, Y. C. (1979). DNase induced after infection of KB cells by herpes simplex virus type 1 or type 2. II. Characterization of an associated endonuclease activity. *J. Virol.* **32**, 449-57.

Hoffmann, P. J. (1981). Mechanism of degradation of duplex DNA by the DNase induced by herpes simplex virus. *J. Virol.* **38**, 1005-14.

Homa, F. L. & Brown, J. C. (1997). Capsid assembly and DNA packaging in herpes simplex virus. *Rev. Med. Virol.* **7**, 107-122.

Huang, K. J., Zemelman, B. V. & Lehman, I. R. (2002). Endonuclease g, a candidate human enzyme for the initiation of genomic inversion in herpes simplex type 1 virus. *J. Biol. Chem.* **277**, 21071-9.

Idowu, A. D., Fraser-Smith, E. B., Poffenberger, K. L. & Herman, R. C. (1992). Deletion of the herpes simplex virus type 1 ribonucleotide reductase gene alters virulence and latency in vivo. *Antiviral. Res.* **17**, 145-56.

Igarashi, K., Fawl, R., Roller, R. J. & Roizman, B. (1993). Construction and properties of a recombinant herpes simplex virus 1 lacking both S-component origins of DNA synthesis. *J. Virol.* **67**, 2123-32.

Jacob, R. J. & Roizman, B. (1977). Anatomy of herpes simplex virus DNA VIII. Properties of the replicating DNA. *J. Virol.* **23**, 394-411.

Jacob, R. J., Morse, L. S. & Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J. Virol.* **29**, 448-57.

Jenkins, F. J. & Roizman, B. (1986). Herpes simplex virus 1 recombinants with noninverting genomes frozen in different isomeric arrangements are capable of independent replication. *J. Virol.* **59**, 494-9.

Jongeneel, C. V. & Bachenheimer, S. L. (1981). Structure of replicating herpes simplex virus DNA. *J. Virol.* **39**, 656-60.

Jugovic, P., Hill, A. M., Tomazin, R., Ploegh, H. & Johnson, D. C. (1998). Inhibition of major histocompatibility complex class I antigen presentation in pig and primate cells by herpes simplex virus type 1 and 2 ICP47. *J. Virol.* **72**, 5076-84.

Keir, H. M. & Gold, E. (1963). Deoxyribonucleic acid nucleotidyltransferase and deoxyribonuclease from cultured cells infected with herpes simplex virus. *Biochimica. et Biophysica. Acta.* **72**, 263-76

Kemper, B. & Brown, D. T. (1976). Function of gene 49 of bacteriophage T4. II. Analysis of intracellular development and the structure of very fast-sedimenting DNA. *J. Virol.* **18**, 1000-15.

Kemper, B. & Garabett, M. (1981a). Studies on T4-head maturation. 1. Purification and characterization of gene-49-controlled endonuclease. *Eur. J. BioChem.* **115**, 123-31.

Kemper, B. & Janz, E. (1976). Function of gene 49 of bacteriophage T4. I. Isolation and biochemical characterization of very fast-sedimenting DNA. *J. Virol.* **18**, 992-9.

Kemper, B., Garabett, M. & Courage, U. (1981b). Studies on T4-head maturation. 2. Substrate specificity of gene-49- controlled endonuclease. *Eur J BioChem.* **115**, 133-41.

Kemper, B., Garabett, M. & Courage, U. (1981c). Studies on the function of gene 49 controlled endonuclease of phage T4 (endonuclease VII). *Prog. Clin. Biol. Res.* **64**, 151-66.

Kitts, P. A., Ayres, M. D. & Possee, R. D. (1990). Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic. Acids. Res.* **18**, 5667-72.

Klinedinst, D. K. & Challberg, M. D. (1994). Helicase-primase complex of herpes simplex virus type 1: a mutation in the UL52 subunit abolishes primase activity. *J. Virol.* **68**, 3693-701.

Klupp, B. G., Granzow, H. & Mettenleiter, T. C. (2000). Primary envelopment of pseudorabies virus at the nuclear membrane requires the UL34 gene product. *J. Virol.* **74**, 10063-73.

Koch, H. G., Delius, H., Matz, B., Flugel, R. M., Clarke, J. & Darai, G. (1985). Molecular cloning and physical mapping of the tupaia herpesvirus genome. *J. Virol.* **55**, 86-95.

Koff, A. & Tegtmeyer, P. (1988). Characterization of major recognition sequences for a herpes simplex virus type 1 origin-binding protein. *J. Virol.* **62**, 4096-103.

Koff, A., Schwedes, J. F. & Tegtmeyer, P. (1991). Herpes simplex virus origin-binding protein (UL9) loops and distorts the viral replication origin. *J. Virol.* **65**, 3284-92.

Koslowski, K. M., Shaver, P. R., Casey, J. T., 2nd, Wilson, T., Yamanaka, G., Sheaffer, A. K., Tenney, D. J. & Pederson, N. E. (1999). Physical and functional interactions between the herpes simplex virus UL15 and UL28 DNA cleavage and packaging proteins. *J. Virol.* **73**, 1704-7.

Koslowski, K. M., Shaver, P. R., Wang, X. Y., Tenney, D. J. & Pederson, N. E. (1997). The pseudorabies virus UL28 protein enters the nucleus after coexpression with the herpes simplex virus UL15 protein. *J. Virol.* **71**, 9118-23.

Krosky, P. M., Underwood, M. R., Turk, S. R., Feng, K. W., Jain, R. K., Ptak, R. G., Westerman, A. C., Biron, K. K., Townsend, L. B. & Drach, J. C. (1998). Resistance of human cytomegalovirus to benzimidazole ribonucleosides maps to two open reading frames: UL89 and UL56. *J. Virol.* **72**, 4721-8.

Kwong, A. D., Kruper, J. A. & Frenkel, N. (1988). Herpes simplex virus virion host shutoff function. *J. Virol.* **62**, 912-21.

Ladin, B. F., Blankenship, M. L. & Ben-Porat, T. (1980). Replication of herpesvirus DNA. V. Maturation of concatemeric DNA of pseudorabies virus to genome length is related to capsid formation. *J. Virol.* **33**, 1151-64.

Ladin, B. F., Ihara, S., Hampl, H. & Ben-Porat, T. (1982). Pathway of assembly of herpesvirus capsids: an analysis using DNA+ temperature-sensitive mutants of pseudorabies virus. *Virology* **116**, 544-61.

Lamberti, C. & Weller, S. K. (1996). The herpes simplex virus type 1 UL6 protein is essential for cleavage and packaging but not for genomic inversion. *Virology* **226**, 403-7.

Lee, C. K. & Knipe, D. M. (1985). An immunoassay for the study of DNA-binding activities of herpes simplex virus protein ICP8. *J. Virol.* **54**, 731-8.

Littler, E., Purifoy, D., Minson, A. & Powell, K. L. (1983). Herpes simplex virus non-structural proteins. III. Function of the major DNA-binding protein. *J. Gen. Virol.* **64**, 983-95.

Liu, F. & Roizman, B. (1993). Characterization of the protease and other products of amino-terminus- proximal cleavage of the herpes simplex virus 1 UL26 protein. *J. Virol.* **67**, 1300-9.

Luder, A. & Mosig, G. (1982). Two alternative mechanisms for initiation of DNA replication forks in bacteriophage T4: priming by RNA polymerase and by recombination. *Proc. Natl. Acad. Sci. U S A* **79**, 1101-5.

Makhov, A. M., Boehmer, P. E., Lehman, I. R. & Griffith, J. D. (1996). The herpes simplex virus type 1 origin-binding protein carries out origin specific DNA unwinding and forms stem-loop structures. *Embo. J.* **15**, 1742-50.

Marsden, H. S., Campbell, M. E., Haarr, L., Frame, M. C., Parris, D. S., Murphy, M., Hope, R. G., Muller, M. T. & Preston, C. M. (1987). The 65,000-Mr DNA-binding and virion trans-inducing proteins of herpes simplex virus type 1. *J. Virol.* **61**, 2428-37.

Marsden, H. S., Stow, N. D., Preston, V. G., Timbury, M. C. & Wilkie, N. M. (1978). Physical mapping of herpes simplex virus-induced polypeptides. *J. Virol.* **28**, 624-42.

Martinez, R., Sarisky, R. T., Weber, P. C. & Weller, S. K. (1996a). Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates. *J. Virol.* **70**, 2075-85.

Martinez, R., Shao, L., Bronstein, J. C., Weber, P. C. & Weller, S. K. (1996b). The product of a 1.9-kb mRNA which overlaps the HSV-1 alkaline nuclease gene (UL12) cannot relieve the growth defects of a null mutant. *Virology* **215**, 152-64.

McGeoch, D. J. & Davison, A. J. (1999). The molecular evolutionary history of the herpesviruses. In *Origin and Evolution of Viruses*, pp441-65. Edited by R. G. Webster, J. Holland, E. S. Domingo. Academic Press.

McGeoch, D. J., Barnett, B. & MacLean, C. (1993). Emerging functions of Alphaherpesvirus genes. *Seminars in Virology* **4**, 125-34.

McGeoch, D. J., Cook, S., Dolan, A., Jamieson, F. E. & Telford, E. A. (1995). Molecular phylogeny and evolutionary timescale for the family of mammalian herpesviruses. *J. Mol. Biol.* **247**, 443-58.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., McNab, D., Perry, L. J., Scott, J. E. & Taylor, P. (1988). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69**, 1531-74.

McGeoch, D. J., Dolan, A. & Frame, M. C. (1986). DNA sequence of the region in the genome of herpes simplex virus type 1 containing the exonuclease gene and neighbouring genes. *Nucleic. Acids. Res.* **14**, 3435-48.

McKnight, J. L., Kristie, T. M. & Roizman, B. (1987). Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its cis site requires cellular proteins. *Proc. Natl. Acad. Sci. U S A* **84**, 7061-5.

McNab, A. R., Desai, P., Person, S., Roof, L. L., Thomsen, D. R., Newcomb, W. W., Brown, J. C. & Homa, F. L. (1998). The product of the herpes simplex virus type 1

UL25 gene is required for encapsidation but not for cleavage of replicated viral DNA. *J. Virol.* **72**, 1060-70.

McNabb, D. S. & Courtney, R. J. (1992). Analysis of the UL36 open reading frame encoding the large tegument protein (ICP1/2) of herpes simplex virus type 1. *J. Virol.* **66**, 7581-4.

McVoy, M. A. & Adler, S. P. (1994). Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. *J. Virol.* **68**, 1040-51.

McVoy, M. A. & Adler, S. P. (1994). Human cytomegalovirus DNA replicates after early circularization by concatemer formation, and inversion occurs within the concatemer. *J. Virol.* **68**, 1040-51.

McVoy, M. A., Nixon, D. E. & Adler, S. P. (1997). Circularization and cleavage of guinea pig cytomegalovirus genomes. *J. Virol.* **71**, 4209-17.

McVoy, M. A., Nixon, D. E., Adler, S. P. & Mocarski, E. S. (1998). Sequences within the herpesvirus-conserved pac1 and pac2 motifs are required for cleavage and packaging of the murine cytomegalovirus genome. *J. Virol.* **72**, 48-56.

McVoy, M. A., Nixon, D. E., Hur, J. K. & Adler, S. P. (2000). The ends on herpesvirus DNA replicative concatemers contain pac2 cis cleavage/packaging elements and their formation is controlled by terminal cis sequences. *J. Virol.* **74**, 1587-92.

McVoy, M. A., Nixon, D. E., Hur, J. K. & Adler, S. P. (2000). The ends on herpesvirus DNA replicative concatemers contain pac2 cis cleavage/packaging elements and their formation is controlled by terminal cis sequences. *J. Virol.* **74**, 1587-92.

Mettenleiter, T. C. (2002). Brief overview on cellular virus receptors. *Virus Res* **82**, 3-8.

Mocarski, E. S. & Roizman, B. (1982). Structure and role of the herpes simplex virus DNA termini in inversion, circularization and generation of virion DNA. *Cell* **31**, 89-97.

Monahan, S. J., Grinstead, L. A., Olivieri, W. & Parris, D. S. (1998). Interaction between the herpes simplex virus type 1 origin-binding and DNA polymerase accessory proteins. *Virology* **241**, 122-30.

Montgomery, R. I., Warner, M. S., Lum, B. J. & Spear, P. G. (1996). Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. *Cell* **87**, 427-36.

Morita, M. & Fujisawa, H. (1997). [How do bacteriophages recognize and package their own genome DNA?]. *Tanpakushitsu Kakusan Koso* **42**, 609-18.

Morrison, J. M. & Keir, H. M. (1968). Further studies on the deoxyribonucleic acid exonuclease induced by herpes virus. *BioChem. J* **110**, 39P.

Mosig, G. & Colowick, N. (1995). DNA replication of bacteriophage T4 in vivo. *Methods Enzymol.* **262**, 587-604.

Mosig, G. (1998). Recombination and recombination-dependent DNA replication in bacteriophage T4. *Annu. Rev. Genet.* **32**, 379-413.

Mossman, K. L., Sherburne, R., Lavery, C., Duncan, J. & Smiley, J. R. (2000). Evidence that herpes simplex virus VP16 is required for viral egress downstream of the initial envelopment event. *J. Virol.* **74**, 6287-99.

Mullaney, J., Moss, H. W. & McGeoch, D. J. (1989). Gene UL2 of herpes simplex virus type 1 encodes a uracil-DNA glycosylase. *J. Gen. Virol.* **70**, 449-54.

Newcomb, W. W. & Brown, J. C. (1991). Structure of the herpes simplex virus capsid: effects of extraction with guanidine hydrochloride and partial reconstitution of extracted capsids. *J. Virol.* **65**, 613-20.

Newcomb, W. W. & Brown, J. C. (1994). Induced extrusion of DNA from the capsid of herpes simplex virus type 1. *J. Virol.* **68**, 433-40.

Newcomb, W. W., Juhas, R. M., Thomsen, D. R., Homa, F. L., Burch, A. D., Weller, S. K. & Brown, J. C. (2001). The UL6 gene product forms the portal for entry of DNA into the herpes simplex virus capsid. *J. Virol.* **75**, 10923-32.

Newcomb, W. W., Trus, B. L., Cheng, N., Steven, A. C., Sheaffer, A. K., Tenney, D. J., Weller, S. K. & Brown, J. C. (2000). Isolation of herpes simplex virus procapsids from cells infected with a protease-deficient mutant virus. *J. Virol.* **74**, 1663-73.

O'Donnell, M. E., Elias, P. & Lehman, I. R. (1987). Processive replication of single-stranded DNA templates by the herpes simplex virus-induced DNA polymerase. *J. Biol. Chem.* **262**, 4252-9.

Ogasawara, M., Suzutani, T., Yoshida, I. & Azuma, M. (2001). Role of the UL25 gene product in packaging DNA into the herpes simplex virus capsid: location of UL25 product in the capsid and demonstration that it binds DNA. *J. Virol.* **75**, 1427-36.

Ojala, P. M., Sodeik, B., Ebersold, M. W., Kutay, U. & Helenius, A. (2000). Herpes simplex virus type 1 entry into host cells: reconstitution of capsid binding and uncoating at the nuclear pore complex in vitro. *Mol. Cell. Biol.* **20**, 4922-31.

Olivo, P. D., Nelson, N. J. & Challberg, M. D. (1988). Herpes simplex virus DNA replication: the UL9 gene encodes an origin- binding protein. *Proc. Natl. Acad. Sci. U S A* **85**, 5414-8.

Patel, A. H. & MacLean, J. B. (1995). The product of the UL6 gene of herpes simplex virus type 1 is associated with virus capsids. *Virology* **206**, 465-78.

Patel, A. H., Rixon, F. J., Cunningham, C. & Davison, A. J. (1996). Isolation and characterization of herpes simplex virus type 1 mutants defective in the UL6 gene. *Virology* **217**, 111-23.

Patel, A. H., Subak-Sharpe, J. H., Stow, N. D., Marsden, H. S., Maclean, J. B. & Dargan, D. J. (1996). Suppression of amber nonsense mutations of herpes simplex virus type 1 in a tissue culture system. *J. Gen. Virol.* **77**, 199-209.

Pfuller, R. & Hammerschmidt, W. (1996). Plasmid-like replicative intermediates of the Epstein-Barr virus lytic origin of DNA replication. *J. Virol.* **70**, 3423-31.

Poffenberger, K. L. & Roizman, B. (1985). A noninverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. *J. Virol.* **53**, 587-95.

Poffenberger, K. L., Tabares, E. & Roizman, B. (1983). Characterization of a viable, noninverting herpes simplex virus 1 genome derived by insertion and deletion of sequences at the junction of components L and S. *Proc. Natl. Acad. Sci. U S A* **80**, 2690-4.

Polvino-Bodnar, M., Orberg, P. K. & Schaffer, P. A. (1987). Herpes simplex virus type 1 oriL is not required for virus replication or for the establishment and reactivation of latent infection in mice. *J. Virol.* **61**, 3528-35.

Poon, A. P. & Roizman, B. (1993). Characterization of a temperature-sensitive mutant of the UL15 open reading frame of herpes simplex virus 1. *J. Virol.* **67**, 4497-503.

Preston, V. G., Coates, J. A. & Rixon, F. J. (1983). Identification and characterization of a herpes simplex virus gene product required for encapsidation of virus DNA. *J. Virol.* **45**, 1056-64.

Preston, C. M. & Cordingley, M. G. (1982). mRNA- and DNA-directed synthesis of herpes simplex virus-coded exonuclease in Xenopus laevis oocytes. *J. Virol.* **43**, 386-94.

Preston, C. M. (1979). Abnormal properties of an immediate early polypeptide in cells infected with the herpes simplex virus type 1 mutant tsK. *J. Virol.* **32**, 357-69.

Preston, C. M. (1979). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. *J. Virol.* **29**, 275-84.

Purifoy, D. J. & Powell, K. L. (1976). DNA-binding proteins induced by herpes simplex virus type 2 in HEp-2 cells. *J. Virol.* **19**, 717-31.

Puvion-Dutilleul, F., Laithier, M. & Sheldrick, P. (1985). Ultrastructural localization of the herpes simplex virus major DNA- binding protein in the nucleus of infected cells. *J. Gen. Virol.* **66**, 15-30.

Puvion-Dutilleul, F. & Pichard, E. (1986). Viral alkaline nuclease in intranuclear dense bodies induced by herpes simplex infection. *Biol. Cell.* **58**, 15-22.

Pyles, R. B. & Thompson, R. L. (1994). Evidence that the herpes simplex virus type 1 uracil DNA glycosylase is required for efficient viral replication and latency in the murine nervous system. *J. Virol.* **68**, 4963-72.

Pyles, R. B., Sawtell, N. M. & Thompson, R. L. (1992). Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency. *J. Virol.* **66**, 6706-13.

Quinlan, M. P., Chen, L. B. & Knipe, D. M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell* **36**, 857-68.

Randall, R. E. & Dinwoodie, N. (1986). Intranuclear localization of herpes simplex virus immediate-early and delayed-early proteins: evidence that ICP 4 is associated with progeny virus DNA. *J. Gen. Virol.* **67**, 2163-77.

Randell, J. C. & Coen, D. M. (2001). Linear diffusion on DNA despite high-affinity binding by a DNA polymerase processivity factor. *Mol. Cell.* **8**, 911-20.

Reardon, J. E. & Spector, T. (1989). Herpes simplex virus type 1 DNA polymerase. Mechanism of inhibition by acyclovir triphosphate. *J. Biol. Chem.*. **264**, 7405-11.

Reardon, J. E. (1989). Herpes simplex virus type 1 and human DNA polymerase interactions with 2'-deoxyguanosine 5'-triphosphate analogues. Kinetics of incorporation into DNA and induction of inhibition. *J. Biol. Chem.* **264**, 19039-44.

Reynolds, A. E., Fan, Y. & Baines, J. D. (2000). Characterization of the U(L)33 gene product of herpes simplex virus 1. *Virology* **266**, 310-8.

Rice, S. A. & Knipe, D. M. (1990). Genetic evidence for two distinct transactivation functions of the herpes simplex virus alpha protein ICP27. *J. Virol.* **64**, 1704-15.

Rigby, P. W., Dieckmann, M., Rhodes, C. & Berg, P. (1977). Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. *J. Mol. Biol.* **113**, 237-51.

Rixon, F. (1993). Structure and assembly of herpesviruses. *Semin. Virol.* **4**, 135-44.

Roizman, B. (1979). The structure and isomerization of herpes simplex virus genomes. *Cell* **16**, 481-94.

Roizman, B., Desrosiers, R., Fleckenstein, B., Lopez, C., Minson, A. & Studdert, M. (1992). The family Herpesviridae : An Update. *Archives of Virology* **123**, 425-49.

Ruyechan, W. T. (1983). The major herpes simplex virus DNA-binding protein holds single- stranded DNA in an extended configuration. *J. Virol.* **46**, 661-6.

Sacks, S. L., Scheffer, J. R., Teh, C. Z. & Tse, A. (1985). Synthesis and antiviral activity of 11-azapentacyclo[6.2.1.0.0.0]decane. *J. Med. Chem.* **28**, 819-21.

Salmon, B. & Baines, J. D. (1998). Herpes simplex virus DNA cleavage and packaging: association of multiple forms of U(L)15-encoded proteins with B capsids requires at least the U(L)6, U(L)17, and U(L)28 genes. *J. Virol.* **72**, 3045-50.

Salmon, B., Cunningham, C., Davison, A. J., Harris, W. J. & Baines, J. D. (1998). The herpes simplex virus type 1 U(L)17 gene encodes virion tegument proteins that are required for cleavage and packaging of viral DNA. *J. Virol.* **72**, 3779-88.

Sambrook, J., Fritsch, E. & Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition : Cold Spring Harbour Laboratory Press.

Sauer, B., Whealy, M., Robbins, A. & Enquist, L. (1987). Site-specific insertion of DNA into a pseudorabies virus vector. *Proc. Natl. Acad. Sci. U S A* **84**, 9108-12.

Schrag, J. D., Prasad, B. V., Rixon, F. J. & Chiu, W. (1989). Three-dimensional structure of the HSV1 nucleocapsid. *Cell* **56**, 651-60.

Schwartz, D. C. & Cantor, C. R. (1984). Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. *Cell* **37**, 67-75.

Sears, A. E., McGwire, B. S. & Roizman, B. (1991). Infection of polarized MDCK cells with herpes simplex virus 1: two asymmetrically distributed cell receptors interact with different viral proteins. *Proc. Natl. Acad. Sci. U S A* **88**, 5087-91.

Severini, A., Morgan, A. R., Tovell, D. R. & Tyrrell, D. L. (1994). Study of the structure of replicative intermediates of HSV-1 DNA by pulsed-field gel electrophoresis. *Virology* **200**, 428-35.

Severini, A., Scraba, D. G. & Tyrrell, D. L. (1996). Branched structures in the intracellular DNA of herpes simplex virus type 1. *J. Virol.* **70**, 3169-75.

Shao, L., Rapp, L. M. & Weller, S. K. (1993). Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsids from the nucleus. *Virology* **196**, 146-62.

Sherman, G. & Bachenheimer, S. L. (1987). DNA processing in temperature-sensitive morphogenic mutants of HSV-1. *Virology* **158**, 427-30.

Sherman, G. & Bachenheimer, S. L. (1988). Characterization of intranuclear capsids made by ts morphogenic mutants of HSV-1. *Virology* **163**, 471-80.

Shieh, M. T. & Spear, P. G. (1991). Fibroblast growth factor receptor: does it have a role in the binding of herpes simplex virus? *Science* **253**, 208-10.

Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D. & Spear, P. G. (1999). A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* **99**, 13-22.

Skaliter, R. & Lehman, I. R. (1994). Rolling circle DNA replication in vitro by a complex of herpes simplex virus type 1-encoded enzymes. *Proc. Natl. Acad. Sci. U S A* **91**, 10665-9.

Skepper, J. N., Whiteley, A., Browne, H. & Minson, A. (2001). Herpes simplex virus nucleocapsids mature to progeny virions by an envelopment --> deenvelopment --> reenvelopment pathway. *J. Virol.* **75**, 5697-702.

Slobedman, B. & Simmons, A. (1997). Concatemeric intermediates of equine herpesvirus type 1 DNA replication contain frequent inversions of adjacent long segments of the viral genome. *Virology* **229**, 415-20.

Smith, D. E., Tans, S. J., Smith, S. B., Grimes, S., Anderson, D. L. & Bustamante, C. (2001). The bacteriophage straight phi29 portal motor can package DNA against a large internal force. *Nature* **413**, 748-52.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J. Cell. Biol.* **136**, 1007-21.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**, 503-17.

Spaete, R. R. & Frenkel, N. (1982). The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. *Cell* **30**, 295-304.

Spear, P. G. (1993). Entry of alphaherpesviruses into cells. *Semin. Virol.* **4**, 167-80.

Spear, P. G., Eisenberg, R. J. & Cohen, G. H. (2000). Three classes of cell surface receptors for alphaherpesvirus entry. *Virology* **275**, 1-8.

Stern, S., Tanaka, M. & Herr, W. (1989). The Oct-1 homoeodomain directs formation of a multiprotein-DNA complex with the HSV transactivator VP16. *Nature* **341**, 624-30.

Stow, N. D. & McMonagle, E. C. (1983). Characterization of the TRS/IRS origin of DNA replication of herpes simplex virus type 1. *Virology* **130**, 427-38.

Stow, N. D. (1982). Localization of an origin of DNA replication within the TRS/IRS repeated region of the herpes simplex virus type 1 genome. *Embo. J.* **1**, 863-7.

Stow, N. D. (1992). Herpes simplex virus type 1 origin-dependent DNA replication in insect cells using recombinant baculoviruses. *J. Gen. Virol.* **73**, 313-21.

Stow, N. D. (2001). Packaging of genomic and amplicon DNA by the herpes simplex virus type 1 UL25-null mutant KUL25NS. *J. Virol.* **75**, 10755-65.

Stow, N. D., Brown, G., Cross, A. M. & Abbotts, A. P. (1998). Identification of residues within the herpes simplex virus type 1 origin-binding protein that contribute to sequence-specific DNA binding. *Virology* **240**, 183-92.

Stow, N. D., Hammarsten, O., Arbuckle, M. I. & Elias, P. (1993). Inhibition of herpes simplex virus type 1 DNA replication by mutant forms of the origin-binding protein. *Virology* **196**, 413-8.

Strobel-Fidler, M. & Francke, B. (1980). Alkaline deoxyribonuclease induced by herpes simplex virus type 1: composition and properties of the purified enzyme. *Virology* **103**, 493-501.

Tanguy Le Gac, N., Villani, G., Hoffmann, J. S. & Boehmer, P. E. (1996). The UL8 subunit of the herpes simplex virus type-1 DNA helicase-primase optimizes utilization of DNA templates covered by the homologous single- strand DNA-binding protein ICP8. *J. Biol. Chem.* **271**, 21645-51.

Targett-Adams, P. (2001). Characterisation of the HSV-1 DNA packaging protein encoded by the UL25 gene. PhD Thesis, Institute of Virology, Glasgow.

Taus, N. S. & Baines, J. D. (1998). Herpes simplex virus 1 DNA cleavage/packaging: the UL28 gene encodes a minor component of B capsids. *Virology* **252**, 443-9.

Tengelsen, L. A., Pederson, N. E., Shaver, P. R., Wathen, M. W. & Homa, F. L. (1993). Herpes simplex virus type 1 DNA cleavage and encapsidation require the product of the UL28 gene: isolation and characterization of two UL28 deletion mutants. *J. Virol.* **67**, 3470-80.

Tenney, D. J., Hurlburt, W. W., Micheletti, P. A., Bifano, M. & Hamatake, R. K. (1994). The UL8 component of the herpes simplex virus helicase-primase complex stimulates primer synthesis by a subassembly of the UL5 and UL52 components. *J. Biol. Chem.* **269**, 5030-5.

Tenney, D. J., Sheaffer, A. K., Hurlburt, W. W., Bifano, M. & Hamatake, R. K. (1995). Sequence-dependent primer synthesis by the herpes simplex virus helicase-primase complex. *J. Biol. Chem.* **270**, 9129-36.

Thomas, M. S., Banks, L. M., Purifoy, D. J. & Powell, K. L. (1988). Production of antibodies of predetermined specificity against herpes simplex virus DNA polymerase and their use in characterization of the enzyme. *J. Virol.* **62**, 1550-7.

Thomas, M. S., Gao, M., Knipe, D. M. & Powell, K. L. (1992). Association between the herpes simplex virus major DNA-binding protein and alkaline nuclease. *J. Virol.* **66**, 1152-61.

Triezenberg, S. J., Kingsbury, R. C. & McKnight, S. L. (1988). Functional dissection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes. Dev.* **2**, 718-29.

Twigg, A. J. & Sherratt, D. (1980). Trans-complementable copy-number mutants of plasmid ColE1. *Nature* **283**, 216-8.

Umene, K. & Nishimoto, T. (1996). Inhibition of generation of authentic genomic termini of herpes simplex virus type 1 DNA in temperature-sensitive mutant BHK-21 cells with a mutated CCG1/TAF(II)250 gene. *J. Virol.* **70**, 9008-12.

Umene, K. & Nishimoto, T. (1996). Replication of herpes simplex virus type 1 DNA is inhibited in a temperature-sensitive mutant of BHK-21 cells lacking RCC1 (regulator of chromosome condensation) and virus DNA remains linear. *J. Gen. Virol.* **77**, 2261-70.

van Genderen, I. L., Brandimarti, R., Torrisi, M. R., Campadelli, G. & van Meer, G. (1994). The phospholipid composition of extracellular herpes simplex virions differs from that of host cell nuclei. *Virology* **200**, 831-6.

Varmuza, S. L. & Smiley, J. R. (1985). Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. *Cell* **41**, 793-802.

Vaughan, P. J., Banks, L. M., Purifoy, D. J. & Powell, K. L. (1984). Interactions between herpes simplex virus DNA-binding proteins. *J. Gen. Virol.* **65**, 2033-41.

Vaughan, P. J., Purifoy, D. J. & Powell, K. L. (1985). DNA-binding protein associated with herpes simplex virus DNA polymerase. *J. Virol.* **53**, 501-8.

Vlazny, D. A., Kwong, A. & Frenkel, N. (1982). Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length viral DNA. *Proc. Natl. Acad. Sci. U S A* **79**, 1423-7.

Vogel, J. L. & Kristie, T. M. (2000). Autocatalytic proteolysis of the transcription factor-coactivator C1 (HCF): a potential role for proteolytic regulation of coactivator function. *Proc. Natl. Acad. Sci. U S A* **97**, 9425-30.

Vogel, J. L. & Kristie, T. M. (2000). The novel coactivator C1 (HCF) coordinates multiprotein enhancer formation and mediates transcription activation by GABP. *Embo. J.* **19**, 683-90.

Wagner, L. & Lai, E. (1994). Separation of large DNA molecules with high voltage pulsed field gel electrophoresis. *Electrophoresis* **15**, 1078-83.

Warner, M. S., Geraghty, R. J., Martinez, W. M., Montgomery, R. I., Whitbeck, J. C., Xu, R., Eisenberg, R. J., Cohen, G. H. & Spear, P. G. (1998). A cell surface protein with herpesvirus entry activity (HveB) confers susceptibility to infection by mutants of herpes simplex virus type 1, herpes simplex virus type 2, and pseudorabies virus. *Virology* **246**, 179-89.

Weinheimer, S. P., McCann, P. J., 3rd, O'Boyle, D. R., 2nd, Stevens, J. T., Boyd, B. A., Drier, D. A., Yamanaka, G. A., DiIanni, C. L., Deckman, I. C. & Cordingley, M. G. (1993). Autoproteolysis of herpes simplex virus type 1 protease releases an active catalytic domain found in intermediate capsid particles. *J. Virol.* **67**, 5813-22.

Weir, J. P. (2001). Regulation of herpes simplex virus gene expression. *Gene* **271**, 117-30.

Weller, S. K., Seghatoleslami, M. R., Shao, L., Rowse, D. & Carmichael, E. P. (1990). The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant. *J. Gen. Virol.* **71**, 2941-52.

Weller, S. K., Spadaro, A., Schaffer, J. E., Murray, A. W., Maxam, A. M. & Schaffer, P. A. (1985). Cloning, sequencing, and functional analysis of oriL, a herpes simplex virus type 1 origin of DNA synthesis. *Mol. Cell. Biol.* **5**, 930-42.

Wilcox, K. W., Kohn, A., Sklyanskaya, E. & Roizman, B. (1980). Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. *J. Virol.* **33**, 167-82.

Wilkie, N. M. (1973). The synthesis and substructure of herpesvirus DNA: the distribution of alkali-labile single strand interruptions in HSV-1 DNA. *J. Gen. Virol.* **21**, 453-67.

Wohlrab, F., McLean, M. J. & Wells, R. D. (1987). The segment inversion site of herpes simplex virus type 1 adopts a novel DNA structure. *J. Biol. Chem.* **262**, 6407-16.

Wolf, D. G., Courcelle, C. T., Prichard, M. N. & Mocarski, E. S. (2001). Distinct and separate roles for herpesvirus-conserved UL97 kinase in cytomegalovirus DNA synthesis and encapsidation. *Proc. Natl. Acad. Sci. U S A* **98**, 1895-900.

Wu, C. A., Nelson, N. J., McGeoch, D. J. & Challberg, M. D. (1988). Identification of herpes simplex virus type 1 genes required for origin- dependent DNA synthesis. *J. Virol.* **62**, 435-43.

WuDunn, D. & Spear, P. G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.* **63**, 52-8.

Yao, X. D., Matecic, M. & Elias, P. (1997). Direct repeats of the herpes simplex virus a sequence promote nonconservative homologous recombination that is not dependent on XPF/ERCC4. *J. Virol.* **71**, 6842-9.

Yu, D. & Weller, S. K. (1998a). Herpes simplex virus type 1 cleavage and packaging proteins UL15 and UL28 are associated with B but not C capsids during packaging. *J. Virol.* **72**, 7428-39.

Yu, D. & Weller, S. K. (1998b). Genetic analysis of the UL 15 gene locus for the putative terminase of herpes simplex virus type 1. *Virology* **243**, 32-44.

Zhang, X., Efstathiou, S. & Simmons, A. (1994). Identification of novel herpes simplex virus replicative intermediates by field inversion gel electrophoresis: implications for viral DNA amplification strategies. *Virology* **202**, 530-9.

Zhou, Z. H., Chen, D. H., Jakana, J., Rixon, F. J. & Chiu, W. (1999). Visualization of tegument-capsid interactions and DNA in intact herpes simplex virus type 1 virions. *J. Virol.* **73**, 3210-8.

Zhu, L. A. & Weller, S. K. (1992). The six conserved helicase motifs of the UL5 gene product, a component of the herpes simplex virus type 1 helicase-primase, are essential for its function. *J. Virol.* **66**, 469-79.

Zuccola, H. J., Filman, D. J., Coen, D. M. & Hogle, J. M. (2000). The crystal structure of an unusual processivity factor, herpes simplex virus UL42, bound to the C terminus of its cognate polymerase. *Mol. Cell.* **5**, 267-78.