STUDIES ON THE US3 AND UL13 PROTEIN KINASE GENES OF HERPES SIMPLEX VIRUS TYPE 1

by

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Lesley Coulter

SUMMARY

The availability of the complete sequence of HSV-1 has permitted interpretation of HSV-1 gene function. As a result of database searches, US3 and UL13, the two HSV-1 genes studied during the course of this research, were found to possess amino acid sequence motifs characteristic of the protein kinase family. The US3 gene has indeed been shown to encode a protein kinase present in the cytoplasm of infected cells, however, protein kinase activity has not yet been assigned to the UL13 gene product, although the protein does correlate with a novel protein kinase activity in the nuclei of HSV-1 infected cells. Protein kinases constitute a very important class of enzymes which are responsible for the regulation of many cellular processes, thus, it is considered likely that these two genes play a significant role in the life cycle of HSV-1. This was investigated by constructing HSV-1 viruses which contain the US3 or UL13 genes disrupted by the insertion of the *Escherichia coli lacZ* gene. This insertional mutagenesis technique has been used successfully to investigate the functions of several other HSV-1 genes. During the course of this research three *lacZ* insertion mutants were constructed, a US3-*lacZ* insertion mutant, a UL13-*lacZ* insertion mutant and a UL13-US3 double insertion mutant. The effects of these mutations on the growth properties of HSV-1 and on the phosphoprotein profiles of infected cell extracts and virion preparations were then investigated.

The US3-*lacZ* insertion mutant used in the experiments described in this thesis was found, at a late stage of the work, to be contaminated with a low level of *wt* virus, despite 5/6 rounds of plaque purification. Nevertheless, the titres of the US3-*lacZ* virus were significantly reduced compared to *wt*, indicating that, although the gene is not essential for virus growth, its disruption does impair virus growth. This reduction in growth does not appear to be due to a decrease in the amount of DNA synthesised by the US3-*lacZ* virus but to a decrease in the infectivity of the virus particles (as indicated by the significantly higher particle:pfu ratio for the US3-*lacZ* virus). Examination of the phosphoprotein profiles of US3-*lacZ* infected cells, following *in vitro* phosphorylation, showed a reduction in the degree of phosphorylation of several proteins with estimated MWs of 200K, 80K and 30K. These proteins, which represent potential substrates of the US3 protein kinase, have not yet been identified, although the 30K protein may correspond to the UL34 gene product, which has recently been reported to be a substrate of the US3 protein kinase.

The growth of the UL13-*lacZ* insertion mutant, both *in vitro* and *in vivo*, does not differ significantly from that of *wt*, indicating that UL13 is not essential for virus growth, despite its conservation throughout the three herpesvirus families. Comparison of silver-stained gels of *wt* and UL13-*lacZ* virion preparations identified the 57K UL13 gene product as a relatively abundant component of the tegument, probably corresponding to VP18.8 - a phosphoprotein previously mapped

to this region of the genome. The phosphoprotein profiles of both UL13-*lacZ*-infected cells (nuclear extracts) and UL13-*lacZ* virions showed a reduction in the phosphorylation of several proteins, of MW 160K, 106K, 60K, 45K and 38K, indicating that the UL13 gene product does possess protein kinase activity. One of these potential substrates of UL13, the 38K phosphoprotein, has been mapped, using intertypic recombinants, to gene UL49, the product of which is known to be the virion tegument protein VP22. UL13-*lacZ* infected cell nuclear extracts were also found to contain two hyper-phosphorylated proteins with estimated MWs of 45K and 70K. The origins of these proteins are not known.

Preliminary experiments with the UL13-US3 double insertion mutant have indicated that the growth of the virus is impaired to a much greater extent than expected from the growth properties of the two single *lacZ* mutants. One possible explanation for this is that the two kinases have common substrates and can therefore substitute for each others absence in the two single mutants, however this is considered unlikely as the phosphoprotein profiles of the two single mutants have not revealed any common substrates. Alternatively, the possibility exists that the two kinases may carry out similar functions in the virus life cycle.

Thus, the research presented in this thesis provides evidence that the UL13 gene product, a relatively abundant virion protein, possesses protein kinase activity, and identifies the UL49 gene product as a major substrate for this activity.

The research has also indicated several potential substrates for the US3 protein kinase, although these have not yet been identified. Finally, preliminary experiments with the UL13-US3 double insertion mutant have revealed a considerable reduction in virus growth compared to the two single mutants.

ABBREVIATIONS

A	adenosine
Å	Angstrom
ATP	adenosine-5'-triphosphate
BHK	Baby Hamster Kidney cells
bp	base pair
BSA	bovine serum albumin
C	cytidine
cpe	cytopathic effect
cpm	counts per minute
CsCl	caesium chloride
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
EBV	Epstein-barr virus
EDTA	ethylenediamine tetra-acetic acid
EHV	equine herpes virus
EtBr	ethidium bromide
G	guanosine
HHV-6	human herpesvirus-6
HSV	herpes simplex virus

^{125}I	Iodine-125 radioisotope
ICP	infected cell protein
K	kilodalton
Kb	kilobase pair
KDa	kilodalton
LD_{50}	the dose at which 50% of animals are dead
MI	mock infected
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
mu	map unit
MW	molecular weight
N	unspecified nucleotide or amino acid
NP40	Nonidet P-40
OD	optical density
ORF	open reading frame
^{32}P	Phosphorus-32 radioisotope
PBS	phosphate buffered saline
pfu	plaque forming unit
pi	post-infection
PRV	pseudorabies virus
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
^{35}S	Sulphur-35 radioisotope
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
T	thymidine
Tris	tris(hydroxymethyl)aminoethane
u	unit

UV	ultraviolet
Vmw	apparent molecular weight of virus-induced protein
VP	virion protein
v/v	volume/volume
VZV	varicella-zoster virus
wt	wild type
w/v	weight/volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

AMINO ACIDS

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lysine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic Acid	Asp	D	Phenylalanine	Phe	F
Cysteine	Cys	C	Prolin	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

CONTENTS

CHAPTER 1: INTRODUCTION

Herpes simplex virus type 1 (HSV-1) encodes at least 70 proteins, of which, approximately one-third have no known function. The elucidation of these functions was revolutionised by the availability of the HSV-1 genomic sequence (McGeoch *et al.*, 1988a) which enabled the use of many techniques including computer based analyses, the expression of isolated genes, the construction of viruses with precise mutations and, the use of oligopeptide antisera. Indeed, it was as a result of database searches that the two HSV-1 genes studied during the course of this research, US3 and UL13, and their homologues in other herpesviruses, were found to contain short amino acid motifs characteristic of the protein kinase family (McGeoch & Davison, 1986; Smith & Smith 1989; Chee *et al.*, 1989). The presence of similar motifs within the N terminal domain of the large subunit of HSV ribonucleotide reductase (encoded by gene UL39) has led to the suggestion that this protein may also possess protein kinase activity (Chung *et al.*, 1989; Paradis *et al.*, 1991). Subsequent experiments with the US3 gene have identified the gene product as a novel protein kinase (Purves *et al.*, 1986a, 1987; Frame *et al.*, 1987) which phosphorylates the product of gene UL34 (Purves *et al.*, 1991, 1992), although the physiological significance of this phosphorylation is unknown. Less is known about the UL13 gene. The gene product is known to be a constituent of the virion and has been associated with a novel protein kinase activity present in the nuclei of infected cells (Cunningham *et al.*, 1992). As protein kinases are known to be of great importance in the

regulation of normal cellular metabolism it is anticipated that the HSV-1 encoded protein kinases play significant roles in some aspect of the virus life cycle. Thus, the objective of the research presented in this thesis was to investigate the role of the HSV-1 US3 and UL13 gene products during the lytic infection of cells, specifically, to determine if the UL13 gene product possesses protein kinase activity and to identify the substrates of the US3 and UL13 gene products. The approach chosen for this research was the construction of HSV-1 mutants with insertions in the US3 gene, the UL13 gene or both the US3 and UL13 genes.

The aims of the following introduction are; (1) to give an overview of the biology of HSV-1, concentrating on the lytic infection, to describe the structure of the HSV-1 virion, as both US3 and UL13 have been reported to be constituents of the virion, and to give brief descriptions of the pathogenicity of the virus, latency, and the role of HSV-1 in transformation and oncogenesis; (2) to summarise the use of HSV-1 mutants to elucidate HSV-1 gene function, as this was the approach taken in this research; (3) to review current knowledge on the protein kinase family, focusing on protein kinases associated with or encoded by viruses; and (4) to describe in detail both the organisation of the US3 and UL13 genes and the structure and properties of the proteins they encode.

1.1. CLASSIFICATION OF THE HERPESVIRUSES

The herpesviruses have been classified on the basis of characteristics such as the G+C content of their DNAs, genome arrangement and biological properties. According to the genome arrangement scheme the herpesviruses can be divided into six groups (A-F) depending upon the number, size and location of major repeat regions. Herpes simplex virus (HSV) falls into group E, together with human cytomegalovirus (HCMV; see Roizman, 1990). The most widely used classification, however, is based on the biological properties of the viruses and comprises three subfamilies, designated Alphaherpesvirinae, Betaherpesvirinae and Gammaherpesvirinae (reviewed in Roizman, 1990). HSV-1 and HSV-2 are members of the Alphaherpesvirinae. These viruses are characterised by their variable host range, relatively short reproductive cycle (usually less than 24 h), cytolytic infection and the ability to establish latent infection primarily in ganglia. Other examples of this subfamily include varicella-zoster virus (VZV), equine herpesvirus (EHV) and pseudorabies virus (PRV). The betaherpesviruses, such as HCMV and human herpesvirus-6 (HHV-6), have a restricted host range and a relatively long reproductive cycle. Infection often results in the formation of enlarged cells and latent infections can be established in various tissues such as secretory glands and lymphoreticular cells. Members of the Gammaherpesvirinae, such as Epstein-Barr virus (EBV), have a very limited host range with a tropism for

lymphocytes. The length of the reproductive cycle is variable and latent infections are frequently established in lymphoid tissue. Increasingly, comparison of genome sequences is being used to determine the evolutionary relationships between members of the Herpesviridae. The genomes of the alphaherpesviruses HSV-1, HSV-2, VZV, PRV and EHV-1 correspond quite closely in their content and the arrangement of homologous genes, especially in U_L (Davison & Wilkie, 1983c), while comparison of VZV and the gamma-herpesvirus EBV reveals regions of rearrangements and divergence (Davison & Taylor, 1987). In 1988 Buckmaster *et al.* used this technique to show that Marek's disease virus (MDV), previously classified as a gammaherpesvirus, is more closely related to the Alphaherpesvirinae subfamily.

1.2. PATHOGENICITY OF HSV

HSV is transmitted by direct physical contact and causes characteristic vesicular lesions on the skin and mucosal surfaces. The mouth and lips are the most common sites of HSV-1 infections (herpes labialis, "cold sores"), while HSV-2 is largely associated with genital lesions (herpes genitalis). Following a primary HSV infection, which is usually asymptomatic, a latent infection is frequently established in the neuronal cells of the sensory ganglia. The virus is periodically reactivated from the latent state by diverse stimuli such as UV light, menstruation and stress, giving rise to localised disease. Although HSV infections

are usually mild, more severe conditions can occur, such as keratoconjunctivitis and acute necrotising encephalitis. HSV-2 has also been implicated in cervical cancer, although this remains controversial. In addition, even mild HSV infections can be life-threatening in neonates and people who are immunocompromised (reviewed in Whitley, 1990).

1.3. LATENCY

A characteristic of HSV is its ability to establish latent infections in neuronal tissue (reviewed in Hill, 1985; Stevens, 1989; Feldman, 1991). Latency can be viewed in three stages: establishment, maintenance, and reactivation.

1.3.1. Establishment and Maintenance of Latency

The preferred site of HSV latency is nervous tissue although non-neuronal sites, such as the mouse footpad (Clements & Subak-Sharpe, 1988) and human corneas (Shimeld *et al.*, 1982) have also been reported. HSV enters the nervous tissue through the nerve endings that innervate the site of primary infection and is transported up the axon, by retrograde axonal transport, to the cell body where it enters the nucleus. Here, the viral DNA becomes "endless" (Rock & Fraser, 1983; Efstathiou *et al.*, 1986), implying that the DNA has integrated into the host DNA, has circularised or has concatemerised. Current evidence suggests that the latter is more likely (Mellerick & Fraser, 1987). The establishment of

latency does not require the products of the lytic genes (Russell *et al.*, 1987b; Steiner *et al.*, 1990a) and the latency associated transcripts or LATs (see section 1.3.3.) are also dispensable (Javier *et al.*, 1988; Clements & Stow, 1989; Steiner *et al.*, 1989).

It has been suggested that the host's immune response is involved in maintaining HSV in its latent state (Stevens & Cook, 1974), however, this has been disputed (Sekizawa *et al.*, 1980).

1.3.2. Reactivation from Latency

HSV can be reactivated from the latent state by a variety of stimuli including UV light, trauma, hormonal changes, stress and immunosuppression (Hill, 1985). The precise mechanism by which reactivation occurs is not known although it is likely to involve triggers to both the ganglion and the skin (Hill, 1985). Recent evidence suggests that HSV gene expression may be necessary for efficient reactivation. The immediate-early protein Vmw110 is required for reactivation in an *in vitro* latency system (Russell *et al.*, 1987b; Harris *et al.*, 1989) and the LATs have been shown to augment the reactivation process (Leib *et al.*, 1989; Steiner *et al.*, 1989; Hill *et al.*, 1990).

1.3.3. Latency Associated Transcripts (LATs)

The LATs are the only gene products detectable during HSV latency (Deatly *et al.*, 1987; Stevens *et al.*, 1987; Rock *et al.*, 1987; Wagner *et al.*, 1988a; Wechsler *et al.*, 1988; Gordon *et al.*, 1988). They are transcribed from a region within the long repeats which is known to extend 750 bp into the 3' end of Vmw110, on the opposite strands and may extend into R_S. The predominant transcripts are approximately 1.8 to 2.2 kb and 1.2 to 1.5 kb in size. These transcripts are related by splicing and are poly(A)-. The 1.8 to 2.2 kb LAT has been detected during HSV-1 replication in tissue culture cells while the smaller LATs have only been detected during latent infection (Spivack & Fraser, 1988a, 1988b).

A low abundance poly(A)+ transcript has also been detected in latently infected cells. This transcript extends from 650 bp upstream to at least 5 kb downstream of the abundant poly(A)- LATs (Stevens *et al.*, 1987; Wagner *et al.*, 1988a, 1988b; Dobson *et al.*, 1989). It has been suggested that these abundant poly(A)- LATs represent introns spliced from the larger poly(A)+ transcript. This suggestion is supported by the fact that the 5' end of the abundant poly(A)- LAT is a canonical 5' splice donor site (Wagner *et al.*, 1988a) and that the LAT promoter is situated approximately 700 bp upstream of the abundant LATs (Dobson *et al.*, 1989).

The function of LATs is not known. It was proposed that LATs

prevented a lytic infection by inhibiting Vmw110 expression through an antisense mechanism. However, it is now known that LATs are not required for the establishment of latency (Javier *et al.*, 1988; Clements & Stow, 1989; Steiner *et al.*, 1989). Another proposal is that LATs exert their effects by encoding a polypeptide. Two open reading frames have been identified in this region of the genome, the larger of which potentially encodes a 305-amino acid polypeptide (Wagner *et al.*, 1988a; Wechsler *et al.*, 1989). An antibody raised against a LAT fusion protein has identified a latency-associated antigen (LAA) of 80 KDa in latently infected neurons (Doerig *et al.*, 1991).

1.3.4. Models Used to Study Latency

The most useful animal models for studying latency are mice, guinea pigs and rabbits. In mice, latent infections are established readily following eye, footpad or ear inoculation. Reactivation does not occur spontaneously but can be induced *in vivo* by a variety of physical and chemical stimuli or *in vitro* by explantation of latently infected ganglionic tissue (Stevens & Cook, 1971).

Tissue culture latency systems have been developed in an attempt to reduce the complexities inherent in the animal models. In most of these systems latency is established by treatment of cultured cells with inhibitors of HSV replication, and reactivation is induced by chemical

manipulation, temperature change or superinfection (O'Neill *et al.*, 1972; O'Neill, 1977; Wigdahl *et al.*, 1981, 1982; Youssoufian *et al.*, 1982; Rapp, 1984; Wilcox & Johnson, 1987, 1988). Russell & Preston (1986) described a system in which HSV-2 established a latent infection in human foetal lung (HFL) fibroblasts when incubated at the supraoptimal temperature of 42°C for 6 days and subsequently downshifted to 37°C. Reactivation was achieved by superinfection with HSV or HCMV. The efficiency of this system has since been increased by using an HSV-1 Vmw65 mutant as the latent virus (Harris & Preston, 1991).

1.4. ONCOGENESIS AND TRANSFORMATION

HSV-2 has been implicated in the aetiology of human cervical cancer on the basis of seroepidemiological studies (Nahmias *et al.*, 1974) and the detection of HSV-2 DNA in cervical cancer specimens (Galloway & McDougall, 1983; Park *et al.*, 1983; Macnab *et al.*, 1985). However, a direct link between HSV-2 and cervical cancer remains controversial. It is more likely that HSV-2 contributes to the development of malignant disease by acting in synergy with another factor such as human papillomavirus (zur Hausen, 1982).

It is well established that HSV can transform cultured cells (Duff & Rapp, 1971; Galloway & McDougall, 1983; Cameron *et al.*, 1985; Jariwalla *et al.*, 1986). Three morphological transformation regions (MTRs) have been identified in the HSV

genome; one in HSV-1, designated MTRI (0.311 to 0.45 m.u., Reyes *et al.*, 1979); and two in HSV-2 designated MTRII (0.58 to 0.62 m.u., Reyes *et al.*, 1979; Galloway & McDougall, 1981; Cameron *et al.*, 1985), and MTRIII (0.54 to 0.58 m.u., Jariwalla *et al.*, 1983). In contrast to other DNA tumour viruses, such as SV40 or adenovirus, where viral DNA becomes integrated into the cellular DNA and encodes a transforming protein, the HSV MTRs do not contain a complete ORF and are not necessarily retained in the transformed cell, thus, a "hit and run" mechanism has been proposed for HSV transformation (Galloway & McDougall, 1983; reviewed in Macnab, 1987). This mechanism may involve the activation of cellular genes, either by *trans*-activation or by promoter or enhancer insertion. Indeed, a set of cellular polypeptides are induced in both transformed cells and HSV-2 infected cells (Macnab *et al.*, 1985; Hewitt *et al.*, 1991). Alternatively, the virus may introduce mutations into cellular genes; HSV infection has been shown to increase the mutation frequency of the HGPRT and supF genes (Pilon *et al.*, 1985, 1986; Clarke & Clements, 1991) and both HSV-1 and HSV-2 have been implicated in gene amplification (Lavi, 1981; Schlehofer *et al.*, 1983). Another mechanism used by many viruses to induce transformation is the expression of oncogenes. As many oncogenes encode protein kinases the products of the protein kinase genes of HSV (US3, UL13) and the N terminal domain of the UL39 product are potential candidates for a transforming protein. The regions of the genome which encode US3 and UL13 protein kinases do not

correlate with the MTRs. However, DNA fragments which span the N terminal protein kinase domain of HSV-2 UL39 have been implicated in the transformation of cultured cells (Jones *et al.*, 1986; Ali *et al.*, 1991), although there is no definite evidence that it is the protein kinase activity that is directly responsible for the transformed phenotype.

1.5. STRUCTURE OF HSV-1

1.5.1. Structure of the HSV-1 Virion

The HSV-1 virion consists of a core containing a linear, double-stranded DNA enclosed within an icosahedral capsid which is surrounded by an amorphous proteinaceous layer known as the tegument. This, in turn, is surrounded by a lipid envelope carrying surface glycoproteins (Roizman & Furlong, 1974; see Figure 1.1). The number of proteins reported to be contained in the virion ranges from 30 to 35 (Spear & Roizman, 1972; Heine *et al.*, 1974; Marsden *et al.*, 1976; Dargan, 1986).

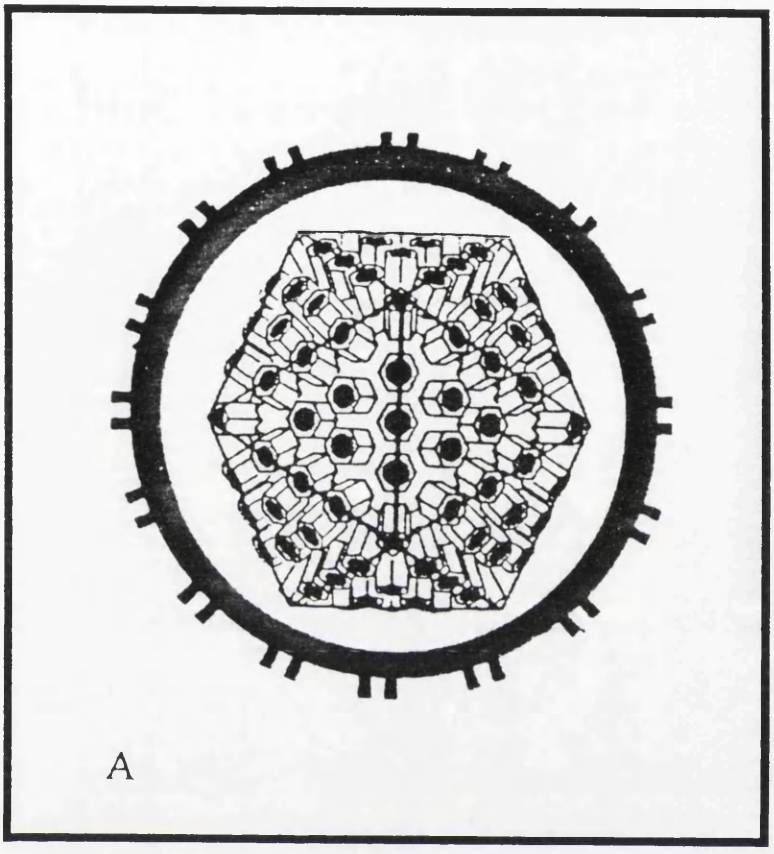
1.5.1.1. The capsid

Knowledge of the structure of the HSV-1 capsid has recently been extended using the techniques of cryoelectron microscopy and image reconstruction (Schrag *et al.*, 1989; Booy *et al.*, 1991). The outer shell of the capsid, which is 14-15nm thick, consists of 162 capsomers which lie on a T=16

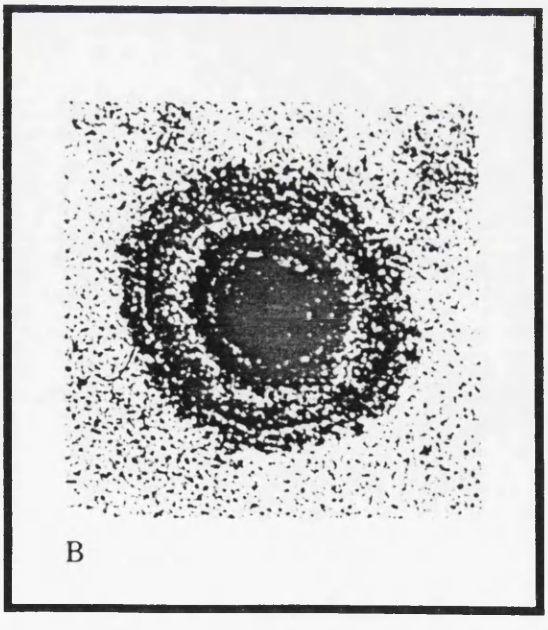
Figure 1.1 **Structure of the herpesvirus virion.**
(Reproduced from Levine, 1991)

A. Schematic diagram of a herpesvirus particle. The icosahedral inner core (capsid), shown with two-fold symmetry, is surrounded by the tegument and enclosed within an envelope from which glycoprotein spikes protrude.

B. Electron micrograph of a cross section through an HSV particle, showing the nucleocapsid core, the amorphous tegument, the outer membrane and glycoprotein spikes.



A



B

icosahedral lattice. There are 150 hexavalent capsomers (hexons) and 12 pentavalent capsomers (pentons) which are connected to each other in groups of three by trimers. A channel about 40Å in diameter runs through the long axis of each capsomer. Schrag *et al.* (1989) also reported a second, internal shell with T=4 icosahedral symmetry within which they predicted the DNA would lie. The studies of Booy *et al.* (1991) however provided no evidence for this inner shell and indicated that the viral DNA extends as far as the outer shell, appearing as a uniformly dense mass (the core). The inner shell is now presumed to have represented an artifact of image reconstruction.

The capsid is composed of the following proteins: VP5, VP19C, VP21, VP22a, VP23, VP24 (Gibson & Roizman, 1972, 1974) and a 12K protein known as VP26 (Heilman *et al.*, 1979; Cohen *et al.*, 1980; Newcomb & Brown, 1991; see Table 1.1). VP5 is essential for capsid assembly (Weller *et al.*, 1987). It is thought to be the main component of the hexons (Vernon *et al.*, 1981; Schrag *et al.*, 1989) and may also comprise the pentons (Schrag *et al.*, 1989). VP19C, also essential for capsid assembly (Pertuiset *et al.*, 1989), is linked by disulphide bonds to VP5 (Zweig *et al.*, 1979) and has been proposed to be a constituent of the pentons (Vernon *et al.*, 1981). VP21 is present only in capsids which contain DNA and has been proposed to be a constituent of the core (Gibson & Roizman, 1972). The VP22a family of proteins, which is present only in partially cored capsids (Braun *et al.*, 1984b;

Table 1.1 Capsid proteins

The seven capsid proteins are listed, together with their genes, and their alternative names.

Capsid Protein	Alternative Names of Protein	Gene Encoding the Protein	
VP5	NC-1, p155, major capsid protein	UL19	Costa *et al.*, 1984; Davison & Scott, 1986; McGeoch *et al.*, 1986a
VP19C	NC-2, p50	UL38	Braun *et al.*, 1984a; Rixon *et al.*, 1990
VP21	NC-3	UL26?	F. J. Rixon, personal communication
VP22a	NC-4, p40, ICP35	UL26.5	Liu & Roizman, 1991a
VP23	NC-5, p32	UL18	Rixon *et al.*, 1990
VP24	NC-6, p25	UL26?	F. J. Rixon, personal communication
VP26	NC-7, p12	UL35	McNabb & Courtney, 1992; F. J. Rixon, personal communication

Sherman & Bachenheimer, 1988; Rixon *et al.*, 1988) is involved in packaging viral DNA into capsids (Preston *et al.*, 1983) and has been compared to the scaffolding protein of double-stranded DNA bacteriophages (Newcomb & Brown, 1991). VP23 is located on the outer capsid surface (Braun *et al.*, 1984b) and may comprise the trimer which connects adjacent capsomers (Schrag *et al.*, 1989). Barker & Roizman (1992) have recently suggested that VP26 is encoded by a novel gene designated UL49.5 or UL49A (Barnett *et al.*, 1992). However, two concurrent studies have shown that VP26 is in fact encoded by gene UL35 (McNabb & Courtney, 1992; F. J. Rixon, personal communication).

1.5.1.2. The tegument

The tegument of HSV-1 appears as an electron-opaque amorphous region located between the capsid and the envelope. Vernon *et al.* (1982) proposed that the tegument acquired structure by attaching to the capsid at the vertices. However, on removal of the envelope from non-infectious virus-like particles (L particles) which lack capsids (Szilagyi & Cunningham, 1991), the tegument displays considerable structural integrity indicating that tegument structure is at least in part independent of the presence of either the capsid or the envelope (McLauchlan & Rixon, 1992). A major component of the tegument is the trans-acting protein Vmw65 (VP16), the product of gene UL48, which is responsible for stimulation of immediate early transcription (Post *et al.*,

1981; Batterson & Roizman, 1983; Campbell *et al.*, 1984). The tegument also contains a virion host shut off (*vhs*) protein, encoded by gene UL41 (Kwong *et al.*, 1988; McGeoch *et al.*, 1988a). The UL13 gene product, predicted to be a novel protein kinase (Smith & Smith, 1989; Chee *et al.*, 1989), has recently been identified as a constituent of the tegument (Cunningham *et al.*, 1992; Coulter *et al.*, 1993) and may account for some of the protein kinase activity which has been associated with HSV-1 virions (Rubenstein *et al.*, 1972; Lemaster & Roizman, 1980; Blue & Stobbs, 1981; Stevely *et al.*, 1985). The tegument proteins VP13/14, encoded by UL47 (McLean *et al.*, 1990; Whittaker *et al.*, 1991), together with the UL46 gene product have been shown to modulate the Vmw65-mediated transcriptional induction (McKnight *et al.*, 1987a). Other tegument proteins include VP22, a 38K protein encoded by gene UL49 (Elliott & Meredith, 1992), a 273K protein (VP1/2) which is the product of UL36 (Batterson *et al.*, 1983), a 10K protein which is encoded by US9 (Frame *et al.*, 1986) and a family of myristylated proteins encoded by UL11 (MacLean *et al.*, 1992). It is interesting to note that all of the tegument proteins described here are phosphorylated either in the purified virion or in the infected cell.

1.5.1.3. The envelope

The envelope of HSV-1 virions consists of a lipid membrane (Asher *et al.*, 1969, Ben-Porat & Kaplan, 1971) with

glycoprotein spikes of 8-10nm projecting fom its outer surface (Wildy *et al.*, 1960; Stannard *et al.*, 1987). HSV-1 encodes ten glycoproteins - gB, gC, gD, gE, gG, gH, gI (Buckmaster *et al.*, 1984; Longnecker & Roizman, 1987; Richman *et al.*, 1986; Spear, 1984), gJ (McGeoch *et al.*, 1985; Roizman & Sears, 1990), gK (Hutchinson *et al.*, 1991) and gL (Hutchinson *et al.*, 1992). These glycoproteins are encoded by genes UL27, UL44, US6, US8, US4, UL22, US7, US5, UL53 and UL1 respectively (McGeoch *et al.*, 1988a; Hutchinson *et al.*, 1991; Hutchinson *et al.*, 1992). The products of genes UL10, UL20, UL34, UL43, UL53 and UL49A may also be associated with the virion envelope as they all have hydrophobic regions which could be membrane-spanning domains (McGeoch *et al.*, 1988a; Barnett *et al.*, 1992).

1.5.2. Structure of the HSV-1 Genome

1.5.2.1. Structural properties of the HSV-1 genome

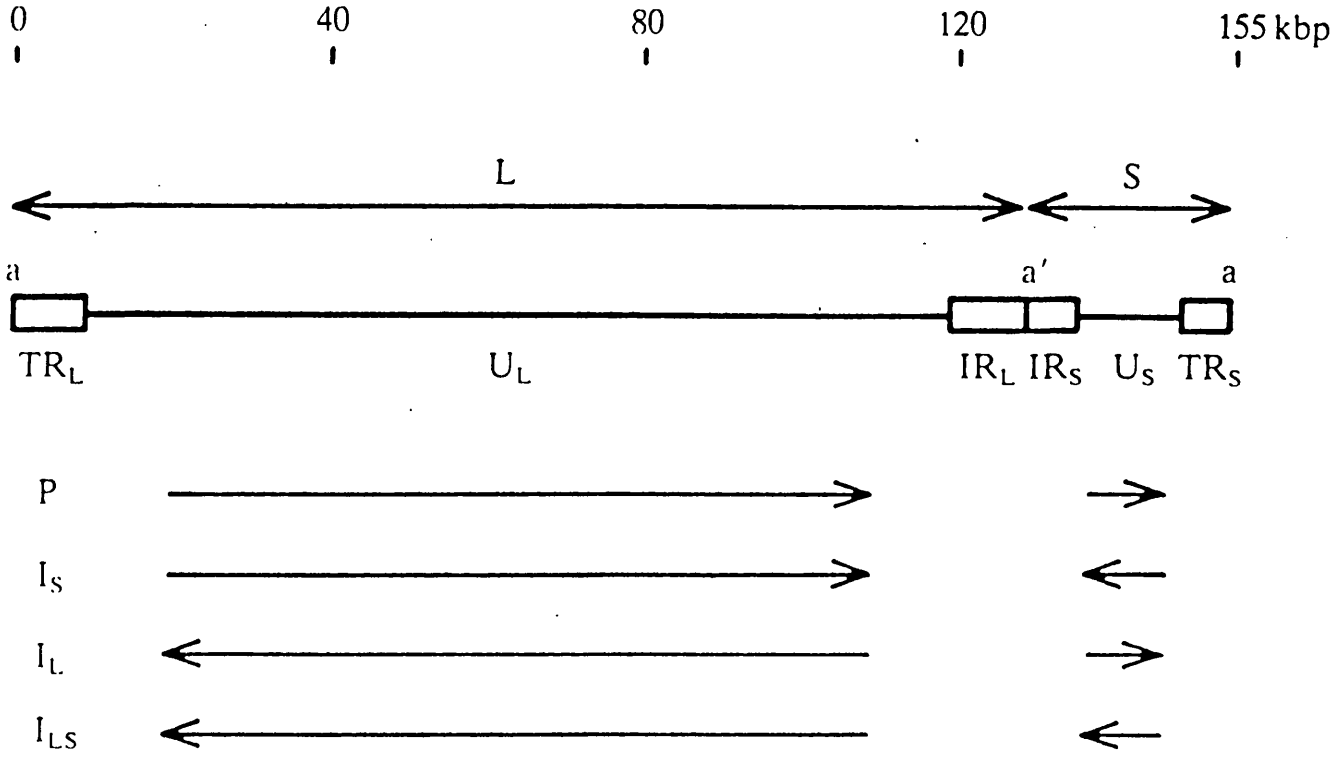
The HSV-1 genome is a linear, double-stranded DNA approximately 152 Kbp in length (McGeoch *et al.*, 1988a). It consists of two covalently joined components, L and S, which consist of unique sequences (U_L and U_S) flanked by inverted repeat sequences. The sequences flanking U_L are designated TR_L (terminal repeat) and IR_L (internal repeat), those flanking U_S are TR_S and IR_S (Sheldrick & Berthelot, 1974; Wadsworth *et al.*, 1975). The sequences of R_L and R_S are distinct. A 400bp direct repeat, known as the *a* sequence, is

present at the genome termini and, in the inverse orientation, at the L-S junction. The L terminus and the joint may possess multiple copies of the *a* sequence (Wagner & Summers, 1978). The *a* sequence of HSV-1 strain F consists of a 20bp repeat (DR1), a 64bp unique sequence (U_b), 22 repeats of a 12bp sequence (DR2), three repeats of a 37bp sequence (DR4), a 58bp unique sequence (U_c) and another copy of DR1 (Mocarski & Roizman, 1982). The number of DR elements can vary, accounting for the observed heterogeneity in the size of the *a* sequence (Davison & Wilkie, 1981; Mocarski & Roizman, 1981; Mocarski *et al.*, 1985). The *a* sequence also contains cleavage and packaging signals designated *pac1* and *pac2* which are contained within U_b and U_c, respectively (Deiss *et al.*, 1986; Varmuza & Smiley, 1985). The terminal *a* sequences possess 3' overhangs which are involved in circularization of the DNA during replication (Mocarski & Roizman, 1982). R_L and R_S also contain multiple copies of short, directly repeated sequences with unit lengths of up to 54 residues (McGeoch *et al.*, 1985; Rixon *et al.*, 1984).

The L and S components of HSV can invert relative to one another giving equimolar amounts of four isomers which appear to be functionally equivalent (Delius & Clements, 1976; Wilkie, 1976; Davison & Wilkie, 1983a, 1983b; Longnecker & Roizman, 1986). One isomer has been designated P (prototype) and the others have been designated I_L, I_S and I_{SL} (Roizman, 1979) (Figure 1.2). The function of isomerization is unclear as mutants which are frozen in one orientation are viable

Figure 1.2 **Isomerization of HSV DNA**
(Reproduced from McGeoch, 1987).

The HSV genome consists of two covalently linked segments, L and S. Each of these contains an unique sequence (U_L or U_S, solid lines) flanked by inverted repeats (TR_L and IR_L, IR_S and TR_S, open boxes). The *a* sequence is present as a direct repeat at the genome termini and, in inverted orientation, at the L-S junction. Inversion of the L and S segments occurs between inverted copies of the *a* sequence to generate four isomers found in equimolar amounts. These are designated P (prototype), I_S (inverted S), I_L (inverted L) and I_{LS} (both L and S inverted).


0
40
80
120
155 kbp
L
S
a
a'
a
TR_L
U_L
IR_L IR_S U_S TR_S
P
I_S
I_L
I_LS

(Jenkins & Roizman, 1986; Poffenberger *et al.*, 1983). It has been suggested that these inversions are due to homologous recombination between R_L and R_S. Later studies have suggested that recombinational isomerization is mediated by cis-acting sites within the *a* sequence (Mocarski *et al.*, 1980; Mocarski & Roizman, 1981; Smiley *et al.*, 1981; Chou & Roizman, 1985), however, recent work has indicated that HSV DNA fragments which lack an *a* sequence can also recombine, when duplicated in the genome (Longnecker & Roizman, 1986; Pogue-Geile *et al.*, 1985; Pogue-Geile & Spear, 1986; Varmuza & Smiley, 1985; Weber *et al.*, 1988). It has also been demonstrated that both the HSV DNA replication system and the cleavage-packaging system are inherently recombinogenic (Weber *et al.*, 1988; Smiley *et al.*, 1990). The *a* sequence, however, does appear to be more recombinogenic than other DNA fragments (Weber *et al.*, 1988; Chou & Roizman, 1985; Weber *et al.*, 1990; Deiss *et al.*, 1986; Umene, 1991).

1.5.2.2. Nucleotide sequence of HSV-1

The HSV-1 genome has been completely sequenced (McGeoch *et al.*, 1988a) giving a total genome length of 152260 residues. The genome has a high overall G+C content of 68.3% ranging from 64% in U_S to 80% in R_S. McGeoch *et al.* (1988a) identified a total of 72 genes encoding 70 distinct proteins; 56 genes in U_L, 12 genes in U_S and one gene in each copy of R_L and R_S. In addition to these genes, two novel genes have been identified in U_L; UL26.5 (Liu & Roizman, 1991a) and

UL49.5 (Barker & Roizman, 1992) or UL49A (Barnett *et al.*, 1992). The presence of the gene encoding ICP34.5 (RL1) in the long repeat region (R_L) of HSV-1 strain 17 has been confirmed (Dolan *et al.*, 1992) and a product has recently been reported for the LATs (Doerig *et al.*, 1991, see section 1.3.4.). A correction has recently been made to the published UL56 reading frame (McGeoch *et al.*, 1991; see Figure 1.3).

Approximately one third of the genes encoded by HSV-1 have no known function and, perhaps surprisingly, at least 20 have been shown to be non-essential for virus growth in tissue culture. The genes to which functions have been assigned can be categorised into (a) regulatory genes, (b) genes involved in the replication machinery, and (c) structural genes. Table 1.2 lists the HSV-1 genes and their functions, where known. (This table was compiled using unpublished information from D. J. McGeoch and P. A. Schaffer.)

Other features of the HSV-1 genome include: three origins of replications, one in U_L (ori_L) and one in each copy of R_S (ori_S); the presence of several 3'-coterminal families; and a scarcity of spliced genes.

1.6. GENETICS OF HSV-1

The isolation and characterisation of HSV-1 mutants has proven to be an effective technique in the elucidation of HSV

Figure 1.3 **Organization of genes in the HSV-1 genome** (Reproduced from McGeoch *et al.*, 1988a, with modifications).

The HSV-1 genome is shown on four successive lines, with unique regions represented by solid lines and the repeat elements as open boxes. The sizes and orientations of the proposed ORFs are shown by arrows. The genes within U_L have been labelled UL1 to UL56 (including the two novel genes, UL26.5 and UL49A). In the U_S region the genes are labelled US1 to US12. Within the repeat elements the two copies of the genes encoding ICP34.5 (RL1), IE110 (RL2) and IE175 are labelled. Locations of proposed transcription polyadenylation sites are indicated as short vertical bars. The origins of replication are marked as X. The lower scale represents kilobases and the upper scale represents fractional map units.

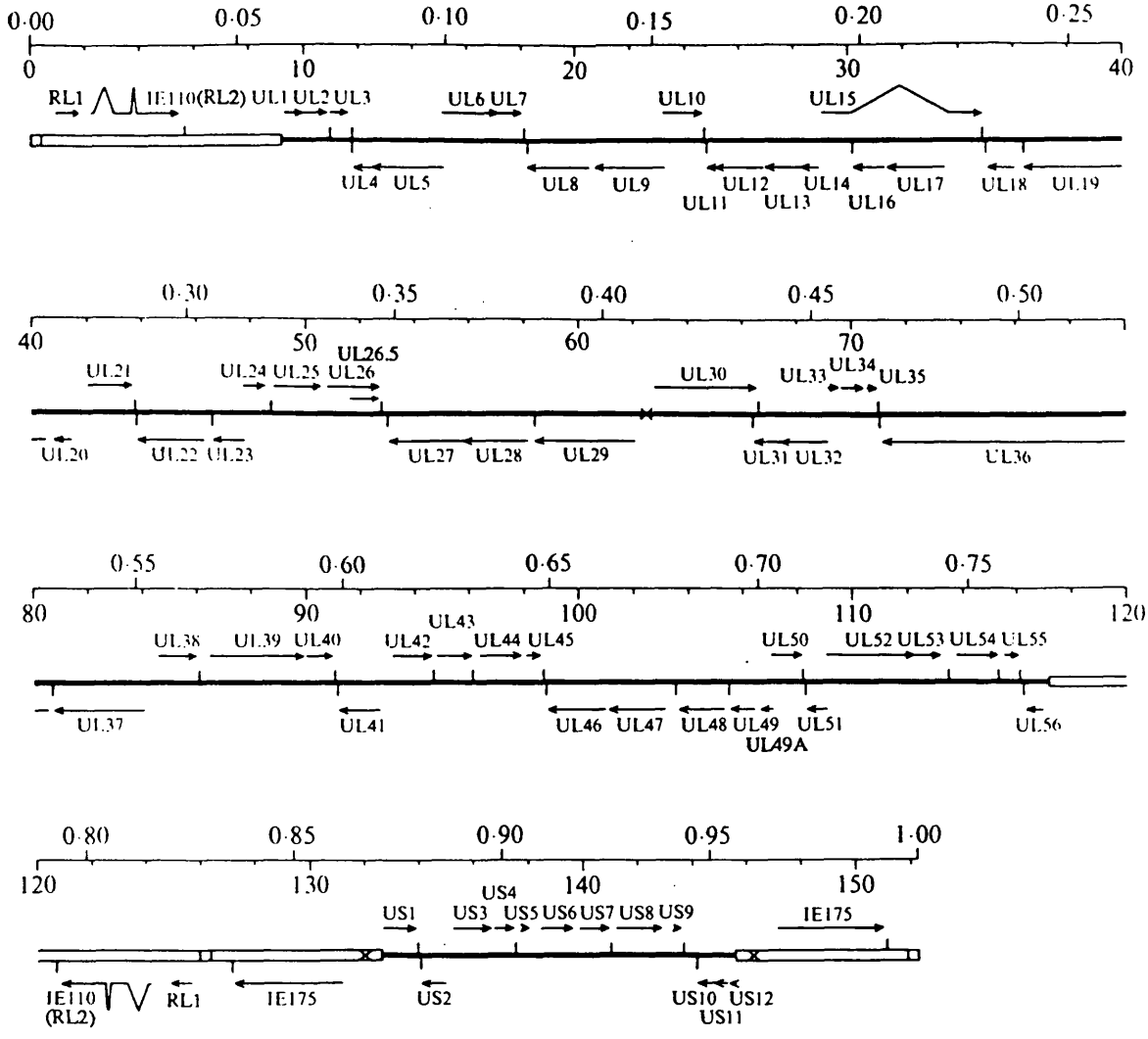

0·00
0·05
0·10
0·15
0·20
0·25
0
10
20
30
40
RL1
IE110(RL2)
UL1
UL2
UL3
UL6
UL7
UL10
UL15
UL4
UL5
UL8
UL9
UL12
UL14
UL17
UL18
UL19
UL11
UL13
UL16
0·30
0·35
0·40
0·45
0·50
40
50
60
70
UL26.5
UL21
UL24
UL25
UL26
UL30
UL33
UL34
UL35
UL20
UL22
UL23
UL27
UL28
UL29
UL31
UL32
UL36
0·55
0·60
0·65
0·70
0·75
80
90
100
110
120
UL43
UL38
UL39
UL40
UL42
UL44
UL45
UL50
UL52
UL53
UL54
UL55
UL37
UL41
UL46
UL47
UL48
UL49
UL51
UL56
UL49A
0·80
0·85
0·90
0·95
1·00
120
130
140
150
US4
US1
US3
US5
US6
US7
US8
US9
IE175
IE110
(RL2)
RL1
IE175
US2
US10
US12
US11

Table 1.2 HSV-1 genes and their functions

This table was compiled from the lists of McGeoch *et al.* (1988a); McGeoch (1989) and McGeoch & Schaffer (1993, in press).

GENE	FUNCTION	REFERENCES
RL1(γ34.5)	Neurovirulence factor (ICP34.5)	Chou *et al.*, 1990; Dolan *et al.*,1992 McGeoch & Barnett, 1991
RL2(IE1,α0)	IE protein (IE110, Vmw110, ICP0)	Everett, 1989; Stow & Stow, 1986
UL1	gL, complexes with gH	Hutchinson *et al.*, 1992
UL2	Uracil-DNA glycosylase	Mullaney *et al.*, 1989; Worrad *et al.*,1988
UL3	Unknown	Baines & Roizman, 1991
UL4	Unknown	Baines & Roizman, 1991
UL5	Component of DNA helicase-primase complex; posseses helicase motifs	Crute *et al.*, 1989; Zhu & Weller, 1992
UL6	Role in virion morphogenesis (VP11-12)	Sherman & Bachenheimer, 1988
UL7	Unknown	
UL8	Component of helicase-primase complex	Carmichael & Weller, 1989; Crute *et al.*, 1989
UL9	Ori-binding protein; DNA helicase	Olivo *et al.*, 1988; Wu *et al.*, 1988
UL10	Unknown; probable membrane protein	MacLean *et al.*, 1991
UL11	Myristylated tegument protein	MacLean *et al.*, 1989, 1992; Baines & Roizman, 1992
UL12	Deoxyribonuclease	Weller *et al.*, 1990
UL13	Protein kinase; tegument phospho-protein (VP18.8)	Cunningham *et al.*, 1992; this thesis
UL14	Unknown	
UL15	Unknown; possible NTP-binding motifs	Baines & Roizman, 1991; Dolan *et al.*, 1991 Davison, 1992
UL16	Unknown	Baines & Roizman, 1991
UL17	Unknown	Baines & Roizman, 1991
UL18	Capsid protein (VP23)	Rixon *et al.*, 1990
UL19	Major capsid protein (VP5)	Weller *et al.*, 1987
UL20	Membrane protein involved in viral egress	Baines *et al.*, 1991; MacLean *et al.*,1991
UL21	Unknown	
UL22	gH; complexes with gL; cell entry	Forrester *et al.*, 1992; Gompels *et al.*, 1991
UL23	Thymidine kinase	Darby *et al.*, 1981
UL24	Unknown; possibly cell fusion	Jacobson *et al.*, 1989a
UL25	Virion protein	Addison *et al.*, 1984
UL26	Capsid protein (VP24); proteinase	Liu & Roizman, 1991a, 1991b; Preston *et al.*, 1992
UL26.5	Internal protein of immature capsids (VP22a); processed by UL26 proteinase	Liu & Roizman, 1991a, 1991b; Preston *et al.*, 1992
UL27	gB; cell entry	Cai *et al.*, 1987; Little *et al.*, 1981
UL28	Capsid maturation	Addison *et al.*, 1990
UL29	ssDNA binding protein (ICP8)	Gao *et al.*, 1988
UL30	DNA polymerase; complexes with UL42 protein	Gottlieb *et al.*, 1990 — Gallo *et al.*, 1988
UL31	Unknown	
UL32	Unknown	Weller *et al.*, 1983
UL33	Structural, involved in DNA packaging	Al-Kobaisi *et al.*, 1991

UL34	Membrane associated protein; substrate for US3 protein kinase	Purves *et al.*, 1991, 1992
UL35	Capsid protein (VP26)	McNabb & Courtney, 1992
UL36	Large tegument protein (VP1-3)	Batterson *et al.*, 1983
UL37	DNA-binding protein	Shelton *et al.*, 1990
UL38	Capsid protein (VP19c)	Rixon *et al.*, 1990
UL39	Ribonucleotide reductase large subunit (R1, ICP6, Vmw136)	Goldstein & Weller, 1988a, 1988b
UL40	Ribonucleotide reductase small subunit (R2, Vmw38)	Preston *et al.*, 1988b
UL41	Virion protein; host shutoff factor	Fenwick & Everett, 1990
UL42	Subunit of DNA polymerase; increases processivity	Gottlieb *et al.*, 1990 Gallo *et al.*, 1988
UL43	Unknown; probable membrane protein	MacLean *et al.*, 1991
UL44	gC; cell entry	Homa *et al.*, 1986
UL45	Virion protein	Visalli & Brandt, 1991
UL46	Modulates activity of UL48 protein	Zhang *et al.*, 1991; Barker & Roizman, 1990
UL47	Tegument protein (VP13/14); modulates activity of UL48 protein	Zhang *et al.*, 1991; Barker & Roizman, 1990; McLean *et al.*, 1990
UL48	Major tegument protein; transactivates IE genes (VP16, Vmw65, α-TIF)	Ace *et al.*, 1989; Campbell *et al.*, 1984
UL49	Tegument protein (VP22)	Elliott & Meredith, 1992
UL49A	Putative membrane protein	Barker & Roizman, 1992; Barnett *et al.*, 1992
UL50	Deoxyuridine triphosphatase	McGeoch, 1990; Fisher & Preston, 1986
UL51	Unknown	Barker & Roizman, 1990
UL52	Component of helicase-primase complex	Crute *et al.*, 1989
UL53	gK	Hutchinson *et al.*, 1991; MacLean *et al.*, 1991
UL54	IE protein (ICP27, Vmw63)	Smith *et al.*, 1992; McCarthy *et al.*, 1989
UL55	Negative regulation of gene expression	Block *et al.*, 1991
UL56	Unknown	McGeoch *et al.*, 1991
LATs	Family of transcripts expressed in latency	Perry & McGeoch, 1988; Javier *et al.*,1988
IE175	IE protein (ICP4, Vmw175)	Paterson & Everett, 1990
US1	IE protein (ICP22, Vmw68)	Post & Roizman, 1981
US2	Unknown	Longnecker & Roizman, 1987
US3	Protein kinase; phosphorylates UL34 protein	Purves *et al.*, 1991, 1992
US4	gG	Longnecker & Roizman, 1987
US5	gJ	Weber *et al.*, 1987
US6	gD; cell entry	Ligas & Johnson, 1988
US7	gI	Johnson *et al.*, 1988
US8	gE	Johnson *et al.*, 1988
US9	Virion protein	Umene, 1987 Frame *et al.*, 1986
US10	Virion protein	Longnecker & Roizman, 1987
US11	Virion protein; RNA-binding protein	Roller & Roizman, 1992
US12	IE protein (ICP47, Vmw12)	Longnecker & Roizman, 1987

gene function and is the approach used in the research described in this thesis. Although spontaneous mutants do occur in *wt* stocks, most HSV-1 mutants have either been induced or engineered. The main types of mutants are described below:

1.6.1. Temperature-sensitive (*ts*) Mutants

Ts (conditional-lethal) mutants are most commonly produced by missense mutations that alter the amino acid sequence of the encoded protein. This alteration decreases the stability of the three-dimensional structure of the protein at higher temperatures, resulting in a loss of function. If the mutation is present in an essential gene, the mutant will be unable to grow at the non-permissive temperature (NPT).

Ts mutations have been induced randomly through the HSV genome using 5-bromo-2'-deoxyuridine (BUdR), nitrous acid (NA), hydroxylamine (HA), nitrosoguanidine or UV light (Schaffer *et al.*, 1970, 1973; Manservigi, 1974) or, in specific cloned HSV DNA fragments, using NA or HA (Chu *et al.*, 1979; Sandri-Goldin *et al.*, 1981). Many of these mutations have been mapped to specific genes using the marker rescue technique (Wilkie *et al.*, 1974; Stow, 1978; Preston, 1981). The nature of the screening procedure for *ts* mutants means that only mutants with defects in essential genes are isolated. Over 22 essential genes have now been identified using this technique.

Brown *et al.*, 1973; Timbury & Calder, 1976

1.6.2. Drug-resistant (*dr*) Mutants

Many HSV-1 *dr* mutants were isolated during the evaluation of compounds as potential antiviral agents. All contain mutations in the thymidine kinase (TK) gene or the DNA polymerase gene. The main types of drugs used and the *dr* mutants isolated are described below.

1.6.2.1. Nucleoside analogues

Substituted deoxyuridines such as 5-bromo-2'-deoxyuridine (BUdR) and 5-bromo-2'-deoxycytidine (BCdR) inhibit viral DNA synthesis when phosphorylated by TK. BUdR also inhibits cellular DNA replication necessitating the use of TK$^-$ cell lines for virus growth. Several BUdR and BCdR-resistant mutants have been isolated, all of which contain lesions in TK and therefore have to depend on the *de novo* pathway of thymidine synthesis to supply TTP (Kit & Dubbs, 1963; Dubbs & Kit, 1964; Jamieson *et al.*, 1974; Cooper, 1973).

Arabinose nucleosides, such as araATP (araA), inhibit HSV-1 DNA polymerase (Muller *et al.*, 1977). An araA-resistant mutant containing a lesion in the DNA polymerase has been isolated (Coen *et al.*, 1982).

The most effective antiherpetic nucleoside analogue is acycloguanosine (acyclovir, ACV) which acts as a chain

terminator during viral DNA synthesis. Many ACV-resistant mutants have been isolated and two ACV-resistant loci have been identified, the TK gene and the DNA polymerase gene (Elion *et al.*, 1977; Field *et al.*, 1980; Crumpacker *et al.*, 1980; Darby *et al.*, 1981; Darby *et al.*, 1984; Larder & Darby, 1985). Within the DNA polymerase gene, the mutations conferring ACV-resistance have been mapped to regions which are highly conserved among the human herpesviruses (Larder *et al.*, 1987).

1.6.2.2. Pyrophosphate analogues

Phosphonoacetate (PAA) inhibits viral DNA polymerase by binding to its pyrophosphate-binding site (Leinbach *et al.*, 1976). Several PAA-resistant mutants, containing lesions in the DNA polymerase gene, have been isolated (Hay & Subak-Sharpe, 1976; Purifoy & Powell, 1977).

1.6.3. Plaque Morphology Mutants

HSV-1 mutants have been isolated which cause neighbouring cells to fuse instead of undergoing lysis (Brown *et al.*, 1973). These mutants are known as syncytial (*syn*) mutants and contain lesions which have been mapped to at least seven different loci in the HSV-1 genome (reviewed in Marsden, 1987). At least five of these loci have been found to correspond to distinct genes, all of which are glycoproteins or putative membrane poteins; UL1 (gL), UL20, UL24, UL27 (gB)

and UL53 (gK) (Bzik *et al.*, 1984; Debroy *et al.*, 1985; Little & Schaffer, 1981; Pogue-Geile & Spear, 1987; Jacobson *et al.*, 1989a; Baines *et al.*, 1991). The molecular basis of the *syn* phenotype remains unclear.

1.6.4. Host-range (*hr*) Mutants

The study of HSV-1 genes essential to the virus life cycle has been aided by the construction of cell lines carrying appropriate fragments of HSV DNA. The protein expressed by the integrated HSV DNA complements the mutation in the infecting virus thereby permitting the growth of mutants containing lesions in essential genes. Conditional-lethal host-range (*hr*) mutants have been obtained in at least seven genes including genes required for DNA replication and genes encoding glycoproteins (Carmichael *et al.*, 1988; Ligas & Johnson, 1988; Weller *et al.*, 1990).

1.6.5. Monoclonal Antibody-resistant (*mar*) Mutants

Mar mutants contain altered surface antigens which are not recognized by monoclonal antibodies raised against *wt* viral proteins. These monoclonals are recognized by a second antibody to which horseradish peroxidase (HRP) has been conjugated. The HRP reacts with chloronaphthol to form a dark precipitate, thus all *wt* plaques are stained black while mutant plaques are clear. *Mar* mutants have proved useful in identifying functional regions of HSV glycoproteins (Holland

et al., 1983; Marlin *et al.*, 1986; Highlander *et al.*, 1987, 1989).

1.6.6. *In vitro* Mutagenesis

Knowledge of the HSV-1 nucleotide sequence and the development of *in vitro* mutagenesis techniques have enabled the introduction of precise mutations into the HSV-1 genome permitting detailed analysis of all aspects of the virus life cycle.

1.6.6.1. Disruption of ORFs by insertion/deletion

Post & Roizman (1981) described a method for introducing deletions into non-essential genes of HSV-1 using the TK gene as a selectable marker. HSV-1 DNA carrying a deletion in the TK gene is co-transfected with a cloned HSV-1 DNA fragment containing the gene under study into which the intact TK gene has been inserted. The insertion mutants are selected for on the basis of their TK^+ phenotype. Deletions can then be introduced by transfection of plasmid-borne HSV-1 fragments carrying the appropriate deletion. The deletion mutants are isolated by selecting for the TK^- phenotype. These procedures have been used to generate deletions in 11 of the 12 U_S genes (Longnecker & Roizman, 1987).

A second approach is the insertion of a transposable element such as miniMu (Roizman & Jenkins, 1985) or Tn5 (Weber *et*

al., 1987), into a cloned HSV-1 DNA fragment. The transposon inserts randomly into the HSV fragment, only once, disrupting any ORFs present at the site of insertion. The fragment is then transfected into the *wt* HSV-1 genome. This technique has been used to investigate the non-essential genes of U_S (Weber *et al.*, 1987).

Another approach is the disruption of an ORF by the insertion of the *lacZ* gene of *Escherichia coli* (Chakrabarti *et al.*, 1985; Panicali *et al.*, 1986). The *lacZ* gene, inserted under the control of a separate promoter such as the SV40 early promoter (Rixon & McLauchlan, 1990) or the HSV-1 UL39 promoter (Goldstein & Weller, 1988a), acts as a screenable marker with viruses containing the insert forming blue plaques in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). The insertion mutant can then be used to generate deletion mutants which produce colourless plaques in the presence of X-Gal. This approach has been successful in the investigation of several HSV-1 genes (Goldstein & Weller, 1988a; 1988b; Ligas & Johnson, 1988; Mullaney *et al.*, 1989; Carmichael & Weller, 1989; Weller *et al.*, 1990; MacLean *et al.*, 1991) and is the approach used in the research described in this thesis.

1.6.6.2. Analysis of functional domains

The insertion of small oligonucleotides, the generation of small deletions, and site-specific mutagenesis all create

local changes in proteins without causing any gross changes in their tertiary structure. These approaches have been used to study the functional domains of many HSV-1 proteins including Vmw65 (Ace *et al.*, 1988), Vmw175 (Paterson & Everett, 1988a; 1988b), gB (Cai *et al.*, 1988b; Qadri *et al.*, 1991) and TK (Inglis & Darby, 1987; Liu & Summers, 1988).

1.6.6.3. Analysis of non-coding regions

The techniques described above have also been used to analyse sequences controlling the expression of viral proteins. For example, Post *et al.* (1981) and Mavromara-Nazos & Roizman (1987) have constructed several chimeric genes, where the structural gene sequence has been fused to promoters of different genes. The promoters of UL23 (TK), UL44 (gC) and UL22 (gH) have been analysed by linker scanning (Coen *et al.*, 1986; Steffy & Weir, 1991) and Smiley *et al.* (1992) have used deletion analysis to determine the effect of Vmw175 on the gD promoter.

1.7. HSV-1 LYTIC CYCLE

1.7.1. Initial Stages of HSV-1 Infection

The initial interaction between HSV-1 and the host cell is the binding of the virion to heparan sulphate present on the cell surface (WuDunn & Spear, 1989; Shieh *et al.*, 1992). Two viral proteins have been implicated in this interaction: gC,

which is thought to be primarily responsible, and gB (Herold *et al.*, 1991; Cai *et al.*, 1988a). This initial binding of the virion to heparan sulphate is thought to promote the subsequent interaction of other virion glycoproteins, such as gD, with cellular receptors (Campadelli-Fiume *et al.*, 1988; Johnson & Spear, 1989; Johnson *et al.*, 1990). It has been reported that the basic fibroblast growth factor (bFGF) receptor acts as a receptor for HSV-1 (Kaner *et al.*, 1990; Baird *et al.*, 1990). However, subsequent studies have provided evidence that HSV-1 infection does not require the presence of this receptor (Shieh & Spear, 1991; Mirda *et al.*, 1992; Muggeridge *et al.*, 1992).

The next stage in HSV-1 infection is penetration of the cell, which is generally accepted to occur by membrane fusion rather than by endocytosis (Para *et al.*, 1980; Campadelli-Fiume *et al.*, 1988; Wittels & Spear, 1991). Penetration requires the presence of at least three glycoproteins, gB, gD and gH, and another virion protein, UL25. gB- and gD-negative viruses are unable to penetrate cells but can still bind to the cell surface, however, gH-negative virions are defective in both penetration and binding, suggesting that gH functions after gD, possibly in membrane fusion (Forrester *et al.*, 1992). The role of UL25 in penetration is unknown (Addison *et al.*, 1984).

Following penetration, the capsids are transported to the nucleus by a mechanism which probably involves the cellular

cytoskeleton (Kristensson *et al.*, 1986). The DNA is then released from the capsid by a process that requires the virion/tegument phosphoprotein UL36 (Batterson *et al.*, 1983; McGeoch *et al.*, 1988a).

1.7.2. Regulation of HSV-1 Gene Expression

The genes of HSV-1 are expressed in a cascade fashion (Marsden *et al.*, 1976; Honess & Roizman, 1974; Clements *et al.*, 1977) which is tightly controlled by both transcriptional and post-transcriptional regulatory mechanisms (Godowski & Knipe, 1986; Weinheimer & McKnight, 1987; Smith & Sandri-Goldin, 1988; Smith *et al.*, 1992). There are at least three phases of expression; immediate early (IE,α), early (E,β) and late (L,γ) (Honess & Roizman, 1974; Clements *et al.*, 1977; Jones & Roizman, 1979). The five immediate early proteins are expressed at high levels in the absence of *de novo* viral protein synthesis. Their expression has been reported to peak at the early stages of infection and decline thereafter. However, the accumulation of both IE mRNA and IE proteins has been observed during the middle and late stages of infection (Ackerman *et al.*, 1984; Harris-Hamilton & Bachenheimer, 1985; Metzler & Wilcox, 1985; Everett & Orr, 1991; Everett, 1991). The IE proteins activate the early genes which encode the proteins necessary for DNA replication. The late genes, which encode mainly structural proteins can be subdivided into leaky late (γ_1) genes which are induced shortly after

the E genes, and true late (γ_2) genes which are activated only after the onset of DNA replication.

The mechanisms by which the host RNA polymerase II differentiates between the IE, E and L genes are based on the presence of *cis*-acting DNA sequences and *trans*-acting factors.

1.7.2.1. *Cis*-acting DNA sequences

(a) Immediate early gene promoters

Transcription of the five IE genes (IE1-IE5) is mediated by both promoter and enhancer sequences. The promoter component is found within 100bp upstream of the transcription start site and contains elements, such as the TATA box, which is recognised by the transcription factor TFIID, and the GC box which is recognised by the transcriptional activator Sp1 (Jones & Tjian, 1985).

The enhancer-like elements of IE promoters are situated far upstream from the transcription start site (-140 to -340) and include the consensus sequence 5'-TAATGARAT-3' (R=purine) which is present in one or more copies in either orientation and has been shown to be essential for *trans*-induction by the virion component Vmw65 (Mackem & Roizman, 1982a, 1982b; Cordingley *et al.*, 1983; Preston *et al.*, 1984a; Kristie & Roizman, 1984; Gaffney *et al.*, 1985; Bzik & Preston, 1986;

O'Hare & Hayward, 1987). This *trans*-induction is mediated by the cellular factor OTF-I, also known as NF-AI, NFIII and OCT-1 (Staudt *et al.*, 1986; Fletcher *et al.*, 1987; Pruijan *et al.*, 1986; Stern *et al.*, 1989) which complexes with Vmw65 and binds to the TAATGARAT sequence (Gerster & Roeder, 1988; Kristie & Roizman, 1986; McKnight *et al.*, 1987a; O'Hare & Goding, 1988; O'Hare *et al.*, 1988; Preston *et al.*, 1988; Stern *et al.*, 1989). A GA-rich element, present in close proximity to at least one TAATGARAT sequence, also confers a strong, independent, response to Vmw65 (Bzik & Preston, 1986; Triezenberg *et al.*, 1988a).

The promoters of the IE genes 1, 2 and 3 also possess copies of the Vmw175 binding site (Faber & Wilcox, 1986b, 1988; Kristie & Roizman, 1986; Muller, 1987). The binding of Vmw175 to the IE1 and IE3 promoters results in repression of the promoter in transfection assays suggesting a mechanism for the observed autoregulation of IE proteins (Gelman & Silverstein, 1987a, 1987b; Resnick *et al.*, 1989; Everett & Orr, 1991; DeLuca & Schaffer, 1988; Paterson & Everett, 1988a, 1988b; Roberts *et al.*, 1988; DiDonato & Muller, 1989). However, Everett & Orr (1991) have reported that mutation of the Vmw175 binding sites in both copies of the IE1 promoter in the viral genome has no effect on IE1 expression during normal HSV-1 infection.

(b) Early gene promoters

The promoters of the thymidine kinase (TK) gene and the glycoprotein D (gD) gene have been extensively studied. The control region of the TK promoter extends 135 bp upstream of the transcription start site and contains five regulatory elements; a TATA box element, a CCAAT box, two GC box elements and an octamer binding site (Zipser *et al.*, 1981; McKnight *et al.*, 1981; McKnight & Kingsbury, 1982; Smiley *et al.*, 1983; Halpern & Smiley, 1984; Eisenberg *et al.*, 1985; Coen *et al.*, 1986; McKnight & Tjian, 1986; Parslow *et al.*, 1987; Böni & Coen, 1989). The gD promoter requires only 83bp upstream of the transcription start site for efficient induction and, like the TK promoter, contains several regulatory elements including a TATA box and two GA-rich sequences (Everett, 1983, 1984a).

The TK and gD promoters also contain Vmw175 binding sites (Kristie & Roizman, 1986; Imbalzano *et al.*, 1990; Beard *et al.*, 1986; Tedder *et al.*, 1989) although the binding of Vmw175 to the TK promoter is not necessary for transactivation (Imbalzano *et al.*, 1990; Shepard & DeLuca, 1991) and the removal of the three binding sites in the gD promoter does not affect the efficiency of gD transcription during a normal HSV-1 infection (Smiley *et al.*, 1992).

(c) Late gene promoters

The promoters of true late genes such as US11, UL44 (gC), UL38, UL49 and UL22 (gH) appear to have a simpler structure than IE or E promoters. The only consensus sequence element identified in these promoters is the TATA box. However, several studies have indicated the presence of additional *cis*-acting sequences in the late gene promoter, downstream from the TATA box (Mavromara-Nazos & Roizman, 1989; Steffy & Weir, 1991; Kibler *et al.*, 1991). These sequences do not form a recognisable consensus and the specific binding of factors to these sequences has not been observed.

Efficient expression of late promoters requires the presence of IE gene products (DeLuca *et al.*, 1984, 1985; DeLuca & Schaffer, 1985; Michael *et al.*, 1988) and functional Vmw175 binding sites have been identified in the promoters of two late genes; gene UL38 (Flanagan *et al.*, 1991) and gene UL49 (Michael & Roizman, 1989; Michael *et al.*, 1988). It is not known if these binding sites are required for activation of late gene expression.

Another *cis*-acting requirement for late gene expression is DNA replication (Mavromara-Nazos & Roizman, 1987). The onset of DNA replication may remove a transcriptional block or may provide a factor which is necessary for transcriptional activation.

There is also evidence of a negative regulatory element in late gene promoters. Costa *et al.* (1985) and Blair & Wagner (1986) have reported that the sequence between 75 and 165 bp upstream of the UL19 (VP5) cap site suppresses the activity of the promoter.

1.7.2.2. *Trans*-acting factors

(a) Vmw65

Vmw65 (VP16, α-TIF, ICP35) is an HSV-1 virion/tegument phosphoprotein (Heine *et al.*, 1974; Spear & Roizman, 1972; Lemaster & Roizman, 1980) which is encoded by gene UL48 (Campbell *et al.*, 1984; Dalrymple *et al.*, 1985; Pellett *et al.*, 1985). The protein is essential for normal virion assembly (Ace *et al.*, 1988; Weinheimer *et al.*, 1992) and specifically *trans*-activates IE gene expression (Campbell *et al.*, 1984; Post *et al.*, 1981; Batterson & Roizman, 1983). The *trans*-activating function is important for virus growth *in vivo* and also for normal virus replication at low multiplicities of infection (Ace *et al.*, 1989).

Vmw65 interacts with the *cis*-acting TAATGARAT elements present on the IE gene promoters, by forming an IE complex (IEC; also known as TRF.C and VIC) with the cellular transcription factor OFT-1 and other cellular proteins (Gerster & Roeder, 1988; Kristie *et al.*, 1989; McKnight *et al.*, 1987a; Preston *et al.*, 1988a; Stern *et al.*, 1989; Katan

et al., 1990; Xiao & Capone, 1990). OFT-1 is a member of the class of proteins known as POU proteins and contains two DNA-binding regions, the POU-homeo subdomain which is thought to interact with the TAAT sequence on the IE promoters, and the POU-specific subdomain which may interact with the sequence just upstream of this (Herr *et al.*, 1988; Sturm *et al.*, 1988; Sturm & Herr, 1988). Vmw65 may bind to the GARAT sequence (Kristie & Sharp, 1990).

The region of Vmw65 required for complex formation lies towards the amino terminus, residues 45 to 388 (Ace *et al.*, 1988; Greaves & O'Hare, 1989; 1990; Werstuck & Capone, 1989; Triezenberg *et al*, 1988b) and includes a potential phosphorylation site (serine 375), the phosphorylation of which is thought to be necessary for complex formation (Greaves & O'Hare, 1990). The domain of Vmw65 responsible for *trans*-activation is situated within the carboxy-terminal 80 amino acids and is thought to activate transcription by interacting with transcription factors or adaptors (Stringer *et al.*, 1990; Lin & Green, 1991; Berger *et al.*, 1990; Kelleher *et al.*, 1990). McKnight *et al.* (1987b) reported that this *trans*-activation can be modulated by the products of genes UL46 and UL47, however, HSV-1 mutants containing deletions in the UL46 and/or UL47 genes are viable in tissue culture (Zhang *et al.*, 1991; Barker & Roizman, 1990) and show no alteration in the synthesis or steady-state levels of Vmw65 (Zhang *et al.*, 1991).

(b) Vmw175

Vmw175 (VP4) is the product of IE gene 3 (∝4) which lies entirely within R_S and is therefore present in the genome in two copies (Rixon *et al.*, 1982). The protein, which is found in three electrophoretically distinct forms in extracts of infected cells (Pereira *et al.*, 1977), is post-translationally modified by phosphorylation and by poly-ADP-ribosylation (Preston & Notarianni, 1983; Wilcox *et al.*, 1980). The Vmw175 protein has been reported to be present in virions (Yao & Courtney, 1989) but is actually found predominantly in Light (L) particles (enveloped particles which lack capsids) (McLauchlan & Rixon, 1992). Vmw175 localises to the nucleus in a pattern which alters during infection (Randall & Dinwoodie, 1986; Knipe *et al.*, 1987).

Early studies on IE gene 3 *ts* mutants (including *ts*K) indicated that Vmw175 is continuously required for the transactivation of early and late gene expression and also for the repression of IE genes (Marsden *et al.*, 1976; Courtney *et al.*, 1976; Watson & Clements, 1978, 1980; Preston, 1979a, 1979b; Dixon & Schaffer, 1980). Recent transient transfection assays have confirmed that Vmw175 can transactivate early gene promoters (O'Hare & Hayward 1985a; Gelman & Silverstein, 1985) although Everett (1984b, 1986) suggests that this activity is the result of synergistic cooperation with Vmw110. Similar assays have demonstrated that Vmw175 can also repress transcription from IE promoters

(O'Hare & Hayward, 1985b; DeLuca & Schaffer, 1985; Gelman & Silverstein, 1986). Vmw175 is known to bind to the promoters of IE genes 1, 2 and 3 (Faber & Wilcox, 1988; Kristie & Roizman, 1986; Muller, 1987), early genes UL23 (TK) and US6 (gD) (Kristie & Roizman, 1986; Imbalzano *et al.*, 1990; Beard *et al.*, 1986; Tedder *et al.*, 1989), and late genes UL38 and UL49 (Flanagan *et al.*, 1991; Michael & Roizman, 1989; Michael *et al.*, 1988). However, several studies have shown that the binding of Vmw175 to promoter sequences is not necessary for transactivation (Everett & Orr, 1991; Imbalzano *et al.*, 1990; Shepard & DeLuca, 1991; Smiley *et al.*, 1992).

Vmw175 is proposed to consist of five domains, (McGeoch *et al.*, 1986a; DeLuca & Schaffer, 1988; Paterson & Everett, 1988a, 1988b). Domain 1 (residues 1-314), which contains a conserved potential phosphorylation site, does not appear to play a critical role in Vmw175 function. Domain 2 (315-484), however, is absolutely required for both transcription activation and repression. The region of the protein directly involved in DNA binding lies between residues 229 to 496. Transactivation is also affected by certain mutations in domain 3 (485-796), which contains the nuclear localization signal, and also in domain 4 (497-1224). Domain 5 is not thought to play a critical role in the function of Vmw175.

(c) Vmw110

Vmw110 (ICP0) is encoded by IE gene 1 (α0) which is situated entirely within the long repeat region and is therefore present in two copies (Preston *et al.*, 1978). The transcript of IE gene 1 is spliced (Perry *et al.*, 1986) and overlaps with the LATs, at the 3' end. The Vmw110 protein is a constituent of the virion tegument (Yao & Courtney, 1992). It is phosphorylated (Ackermann *et al.*, 1984) and localises to the nucleus (Pereira *et al.*, 1977, Hay & Hay, 1980).

Transient transfection assays have shown that Vmw110 can *trans*-activate transcription from IE, early, and late gene promoters, (O'Hare & Hayward, 1985a, 1985b; Mavromara-Nazos *et al.*, 1986; Shapira *et al.*, 1987; Everett, 1986), possibly in synergy with Vmw175 (Everett, 1984b), and also from heterologous promoters such as the SV40 early promoter and the HIV LTR (Everett, 1988b; Mosca *et al.*, 1987). This transactivation activity is not essential for virus growth as Vmw110 deletion mutants (Stow & Stow, 1986; Sacks & Schaffer, 1987; Everett, 1989; Cai & Schaffer, 1992; Chen & Silverstein, 1992) are viable in tissue culture. However, the mutants have high particle to pfu ratios and grow very poorly at low multiplicities of infection, suggesting that *trans*-activation may be dependent upon an interaction between Vmw110 and Vmw175, or that Vmw110 may be responsible for increasing the concentration of Vmw175 to a level required for entry into the lytic cycle (Everett *et al.*, 1991).

Mutational analyses of Vmw110 (Everett, 1987, 1988a, 1988b, 1989) have indicated that five regions of the protein, including a potential Zn finger binding domain, are important for transcriptional *trans*-activation, in synergy with Vmw175. However, only three of these regions are required for *trans*-activation in the absence of Vmw175, suggesting separate mechanisms of action.

The observation that a Vmw110 deletion virus can not reactivate latent HSV-2 from an *in vitro* latency system (Russell *et al.*, 1987b; Harris *et al.*, 1989) has led to the conclusion that Vmw110 must play a significant role in the reactivation of virus from *in vitro* latency systems.

(d) Vmw63

Vmw63 (ICP27) is encoded by IE gene 2 (α27, gene UL54) (McGeoch *et al.*, 1988a). It is modified by the addition of phosphate groups which cycle on and off during infection (Wilcox *et al.*, 1980; Pereira *et al.*, 1977; Ackermann *et al.*, 1984), and localises to the nucleus during infection (Fenwick *et al.*, 1978; Knipe & Smith, 1986).

Transfection assays (Everett, 1986; Rice & Knipe, 1988; Sekulovich *et al.*, 1988; Hardwicke *et al.*, 1989; Su & Knipe, 1989; McMahan & Schaffer, 1990) and studies with *ts* and

deletion mutants have indicated that Vmw63, which is essential for virus growth, is involved in repression of transcription from IE and some E gene promoters, and enhances transcription from L gene promoters (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice & Knipe, 1990). Vmw63 may therefore be involved in the switch from E to L gene expression.

Detailed mutational analysis of the Vmw63 polypeptide has identified two main regulatory regions; an activator region (residues 260 to 434) and a repressor region (within the C-terminal 78 amino acids) which contains a putative Zn finger binding domain (Hardwicke *et al.*, 1989; Rice *et al.*, 1989; McMahan & Schaffer, 1990; Smith *et al.*, 1991).

(e) Others

Vmw68 (ICP22), encoded by IE gene 4 (α22, US1), is phosphorylated and localises in the nucleus (Pereira *et al.*, 1977; Marsden *et al.*, 1978). A deletion mutant of Vmw68 shows a decrease in the expression of a chimeric γ_2-TK gene (Sears *et al.*, 1985) indicating that, in the absence of an as yet, unidentified host cell factor, Vmw68 is required for efficient late gene expression.

Vmw12 (ICP47) is encoded by gene IE5 (α47, US12). Unlike the other four IE polypeptides, Vmw12 is not phosphorylated and localises in the cytoplasm (Marsden *et al.*, 1982). The

role of Vmw12 during viral infection is unclear but it is known to be dispensable for virus growth (Longnecker & Roizman, 1986).

1.7.3. Post-translational Modifications of HSV-1 Proteins

1.7.3.1. Glycosylation

HSV-1 encodes at least ten glycoproteins gB, gC, gD, gE, gG, gH, gI (Buckmaster *et al.*, 1984; Longnecker & Roizman, 1987; Richman *et al*, 1986; Spear, 1984), gJ (McGeoch *et al.*, 1985; Roizman & Sears, 1990), gK (Hutchinson *et al.*, 1991) and gL (Hutchinson *et al.*, 1992) which contain both N-linked and O-linked oligosaccharides (Pizer *et al.*, 1980; Hope & Marsden, 1983; Johnson & Spear, 1983; Serafini-Cessi *et al.*, 1988). The use of glycosylation inhibitors and endoglycosidases have indicated that the N-linked oligosaccharides are important for virus infectivity (Kousoulas *et al.*, 1983; Pizer *et al.*, 1980; Svennerholm *et al.*, 1982; Spivack *et al.*, 1982; Kuhn *et al.*, 1988). However, recent oligonucleotide-directed mutagenesis studies on the three N-linked oligosaccharides of gD have shown that they are not necessary for gD function during viral infection, although the oligosaccharide at site 1 does play an important role in forming and/or maintaining the structure of the glycoprotein (Sodora *et al.*, 1991).

Three tegument proteins, VP13/14, encoded by UL47 (Whittaker *et al.*, 1991; McLean *et al.*, 1990), and VP22, encoded by UL49

(Elliott & Meredith, 1992), have been shown to contain a small quantity of O-linked oligosaccharides (Meredith *et al.*, 1991). The function of this modification is unclear.

1.7.3.2. Phosphorylation

At least eleven HSV-1 encoded proteins are post-translationally modified by the addition of phosphate (Pereira *et al.*, 1977; Marsden *et al.*, 1978). These proteins include: the transcriptional regulatory proteins Vmw175, Vmw110, Vmw68 and Vmw63; virion structural proteins including Vmw65 (Lemaster & Roizman, 1980); UL42, a subunit of the HSV-1 DNA polymerase which appears to increase the processivity of the enzyme during replication (Marsden *et al.*, 1987); and enzymes such as the large subunit of ribonucleotide reductase (RR_1, ICP6). Wilcox *et al.* (1980) demonstrated that phosphate cycles on and off several of these proteins during the course of infection. For example, two of the three electrophoretic forms of Vmw175 cycle between phosphorylated and non-phosphorylated states while the third species is stably phosphorylated throughout infection. Phosphate has also been shown to cycle on and off Vmw68, Vmw63 and RR_1. Wilcox *et al.* (1980) also demonstrated that phosphorylation affects the DNA-binding properties of several HSV-1 phosphoproteins, such as ICP6 and ICP29.

HSV-1 encodes three protein kinases, US3 (Frame *et al.*, 1987; Purves *et al.*, 1987), UL13 (Smith & Smith, 1989; Chee *et al.*,

1989; this thesis) and UL39 (Chung *et al.*, 1989; Paradis *et al.*, 1991), however, the only known substrates for these kinases are the virion protein UL34 (Purves *et al.*, 1991, 1992), Vmw 68, the product of US1 (Purves & Roizman, 1992) and the tegument protein UL49 (this thesis). It is likely that the majority of HSV-1 phosphoproteins are phosphorylated by cellular protein kinases.

The functional significance of HSV-1 protein phosphorylation is unclear, but it is possible that much of the observed phosphorylation is fortuitous and not necessary for virus growth.

1.7.3.3. Proteolytic cleavage

The ICP35 polypeptide (p40, VP22a, NCP-3) undergoes extensive post-translational processing, forming at least 20 different species (Preston *et al.*, 1983; Braun *et al.*, 1984b). The protease responsible for this processing has recently been identified as the product of gene UL26 (Liu & Roizman, 1991b; Preston *et al.*, 1992). Proteolytic cleavage is also known to be involved in the processing of HSV-1 glycoproteins. Glycoproteins gB, gC, gD, gE, gG, gH, gI, gJ and gK all have hydrophobic sequences near their N terminus and most contain cleavage sites for signal peptidase. Cleavage of these signal sequences has been demonstrated for gD, gB and gK (Matthews *et al.*, 1983; Claesson-Welsh & Spear, 1987; Ramaswamy & Holland, 1992).

1.7.3.4. Acylation

The most common fatty acid modifications are the co-translational addition of myristic acid to an N terminal glycine residue, and the addition of palmitic acid, to cysteine residue. A family of myristylated polypeptides (MW 13000 to 16000), encoded by gene UL11, has been identified in HSV-1 virions (MacLean *et al.*, 1989) and Chung *et al.* (1990) have reported that the protein kinase domain of ICP10, the HSV-2 homologue of RR_1 (ICP6), is myristylated. There is no evidence to suggest that HSV-1 RR_1 is similarly modified. To date, the only HSV-1 protein known to be palmitoylated is gE (Johnson & Spear, 1983).

1.7.3.5. ADP-ribosylation

Poly(ADP-ribosyl)ation involves the covalent attachment of ADP-ribose residues to a polypeptide using nicotinamide adenine dinucleotide (NAD) as a substrate. Preston & Notarianni (1983) identified two poly(ADP-ribosyl)ated HSV-1 polypeptides, the immediate early polypeptide Vmw175, and a 38K polypeptide which corresponds to the 38K phosphoprotein reported by Marsden *et al.* (1978) and which has subsequently been shown to be VP22, the product of gene UL49 (Elliott & Meredith, 1992).

1.7.3.6. Sulphation

At late times in infection the HSV-1 glycoproteins become sulphated by attachment of inorganic sulphate to the N-linked oligosaccharides. The major sulphated glycoprotein is gE (Hope *et al.*, 1982; Hope & Marsden, 1983).

1.7.4. HSV-1 Replication

It has been proposed that, shortly after entering the host cell, the viral DNA circularises, by a process which does not require *de novo* protein synthesis (Davison & Wilkie, 1983a; Poffenberger & Roizman, 1985; Jacob & Roizman, 1977; Jacob *et al.*, 1979). The DNA is then thought to replicate by a rolling circle mechanism which generates large concatemers of tandemly repeated viral genomes (Jacob *et al.*, 1979). These concatemers are then cleaved into unit length genomes prior to packaging into capsids.

Both *cis*- and *trans*-acting elements are necessary for replication. Evidence for *cis*-acting sequences came from studies of defective viruses generated during serial passage of HSV at high multiplicities of infection (Frenkel *et al.*, 1975, 1976). These sequences have been designated ori_S and ori_L. *Trans*-acting elements include a set of seven HSV-1 genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) which were first identified by means of a transient complementation assay (Wu *et al.*, 1988). Recently, Stow (1992) has

demonstrated that all seven of these genes expressed in recombinant baculoviruses are necessary for DNA replication from an HSV-1 ori.

1.7.4.1. *cis*-acting elements

(a) Oris

HSV-1 *oris* is located within the *c* sequence of the short repeats, between divergently transcribed genes (IE3 and IE4/IE5) and is therefore present in the genome in two copies (Stow, 1982; Stow & McMonagle, 1983). The core region of *oris* (67 to 90 bp long) contains four domains: three 11 bp UL9 binding sites -CGTTCGCACTT-(sites I to III) and an 18 bp AT-rich region. Sites I and II are inverted with respect to each other and form the arms of a 45 bp palindrome with the AT-rich region at its centre. Both of these sites have a high affinity for UL9, whereas site III, which lies just to the left of site I in inverted orientation, is a low affinity site. All four domains have been shown to be necessary for efficient replication in transient assay systems (Lockshon & Galloway, 1988; Weir & Stow, 1990; Hernandez *et al.*, 1991; Martin *et al.*, 1991). The sequences flanking the core region contain binding sites for a variety of transcriptional activators such as Vmw65 (Bzik & Preston, 1986), SpI and NF-III (apRhys *et al.*, 1989). Removal of these sequences reduces the efficiency of replication by approximately 80-fold (Wong & Schaffer, 1991). A virus lacking one copy of

ori$_S$ is viable in tissue culture (Longnecker & Roizman, 1986).

(b) Ori$_L$

Ori$_L$ is located near the middle of the long unique region of the genome between the divergently transcribed genes, UL29 and UL30 (Weller *et al.*, 1985). *Ori*$_L$ is much larger than *ori*$_S$, consisting of a 144 bp perfect palindrome. Due to the size of this palindrome, plasmids containing *ori*$_L$ are highly unstable in *E. coli* (Weller *et al.*, 1985), thus the functional domains of *ori*$_L$ have not been investigated as throughly as *ori*$_S$. A mutant virus lacking *ori*$_L$ is viable in tissue culture and *in vivo* (Polvino-Bodnar *et al.*, 1987).

1.7.4.2. Proteins directly involved in DNA replication

(a) UL30 (DNA polymerase)

HSV DNA polymerase is biochemically distinct from host DNA polymerase and is the target of many anti-viral drugs, such as acyclovir. It has been purified as a monomer of M_r 140,000 (Powell & Purifoy, 1977; O'Donnell *et al.*, 1987) and does not appear to be post-translationally modified (Thomas *et al.*, 1988). In infected cells, the polymerase associates with the UL42 gene product to form the active polymerase "holoenzyme", however, the polymerase subunit alone has catalytic activity on simple primer-templates such as

activated DNA. The subunit also possesses a 3' to 5' exonuclease activity, which probably plays a role in proof-reading (Powell & Purifoy, 1977; Knopf, 1979), and a 5' to 3' exonuclease/RNase H activity (Crute & Lehman, 1989).

The UL30 gene product contains six regions of conserved amino acids (Wong *et al.*, 1988; Larder *et al.*, 1987). Regions I to III (residues 694 to 896) contain most of the mutations which alter the drug sensitivity of the polymerase and are therefore thought to form the catalytic domain. Region IV (residues 432 to 479) is thought to be important for the 3' to 5' exonuclease activity and sequences near the N terminus have been implicated in the 5' to 3' exonuclease activity. A region of 227 amino acids near the C-terminus (residues 1008 to 1235) is required for the specific interaction with the UL42 polypeptide (Digard & Coen, 1990).

(b) UL42 (Double-stranded DNA binding protein)

The UL42 protein is a 65 KDa phosphoprotein which binds double-stranded DNA in a sequence-independent, non-cooperative manner (Vaughan *et al.*, 1985; Marsden *et al.*, 1987; Gallo *et al.*, 1988; Parris *et al.*, 1988). It is a subunit of the HSV DNA polymerase "holoenzyme" complex (Powell & Purifoy, 1977; Vaughan *et al.*, 1985; Gallo *et al.*, 1988) and appears to increase the processivity of the polymerase (Gottlieb *et al.*, 1990; Hernandez & Lehman, 1990). This increase in processivity is probably related to the DNA-

binding properties of UL42 which may be modulated by phosphorylation.

(c) UL9 (Origin binding protein)

The product of gene UL9 in an 83 KDa protein which binds to three sites in *oris* which contain the 11 bp consensus sequence CGTTCGCACTT (Elias & Lehman, 1988; Olivo *et al.*, 1988; Weir *et al.*, 1989). The DNA binding domain of UL9 has been mapped to the carboxy terminal third of the protein which contains a helix-turn-helix motif and a pseudo-leucine zipper (Weir *et al.*, 1989; Deb & Deb, 1991). UL9 possesses helicase activity and may therefore be involved in the initial unwinding of the two parental DNA strands (Bruckner *et al.*, 1991).

(d) UL5/UL8/UL52 complex

The products of genes UL5, UL8 and UL52 form a complex which exhibits both helicase and primase activities (Crute *et al.*, 1989). UL5 contains six motifs which are characteristic of helicases and which are essential for UL5 function (McGeoch *et al.*, 1988; Zhu & Weller, 1988, 1992), however, helicase activity is not observed unless UL52 is present (Dodson *et al.*, 1989; Calder & Stow, 1990). UL8 is not required for either the helicase or the primase activity of the complex (Calder & Stow, 1990; Dodson & Lehman, 1991). It may be necessary for primer utilization, possibly by stabilizing the

association of primers with the template (Challberg, 1991) or it may facilitate entry into the nucleus of the UL5 and UL52 proteins (Calder *et al.*, 1992). The UL52 gene product may possess primase activity, however this has not been demonstrated.

(e) UL29 (ICP8, major DNA binding protein)

The UL29 gene product (ICP8) is a 130 KDa DNA binding protein which localises to the nucleus where it accumulates at specific foci known as replication compartments (Quinlan *et al.*, 1984; Randall & Dinwoodie, 1986). The protein binds preferentially to single-stranded DNA in a cooperative and sequence-independent manner (Bayliss *et al.*, 1975; Powell & Courtney, 1975; Powell & Purifoy, 1976; Powell *et al.*, 1981; Ruyechan, 1983; Ruyechan & Weir, 1984) and may therefore bind to the ssDNA formed at the replication fork. UL29 may also play a role in organizing a multi-protein complex at the replication fork as it is known to interact with UL9 (Challberg, 1991), UL30 (Chiou *et al.*, 1985), UL42 and UL12 (DNase) (Vaughan *et al.*, 1984; Thomas *et al.*, 1992). The domain of UL29, responsible for binding ssDNA has been located to the carboxy-terminal portion of the protein (residues 564 to 1160; Gao & Knipe, 1989).

1.7.4.3. Proteins indirectly involved in DNA replication

(a) DNase (UL12)

The HSV-encoded nuclease exhibits both exo- and endonuclease activities and has a markedly alkaline pH optimum (Morrison & Keir, 1968; Hoffman & Cheng, 1979; Hoffman, 1981). The protein has a MW of 85 KDa (Banks *et al.*, 1983), is phosphorylated, (Banks *et al.*, 1985; Thomas *et al.*, 1988). and localises to the nucleus although it does not move into the specific replication compartments, despite its association with the major DNA binding protein (UL29) (Randall & Dinwoodie, 1986; Vaughan *et al.*, 1984; Thomas *et al.*, 1992). The nuclease is encoded by gene UL12 (Preston & Cordingley, 1982; Costa *et al.*, 1983; Wathen & Hay, 1984; Draper *et al.*, 1986; McGeoch *et al.*, 1986b, 1988b). Early studies, using *ts* mutants, came to different conclusions regarding the requirement of the DNase for viral DNA synthesis (Moss *et al.*, 1979; Francke & Garett, 1982; Moss, 1986). However, it is now apparent that the HSV-1 DNase is not essential for viral DNA replication but is required for the efficient production of infectious virus (Weller *et al.*, 1990). Possible functions of the DNase include cleavage of the viral DNA concatemers into unit length molecules (Chou & Roizman, 1989), a role in HSV DNA recombination or host chromosomal cleavage (Peat & Stanley, 1986).

(b) Thymidine kinase (TK, UL23)

HSV-1 thymidine kinase provides nucleotides for viral DNA synthesis by catalysing the phosphorylation of thymidine and other pyrimidine deoxyribonucleosides (Kit & Dubbs, 1963; Dubbs & Kitt, 1964; Jamieson & Subak-Sharpe, 1974). TK also phosphorylates nucleoside analogues such as acyclovir, converting them into the active form which results in the inhibition of HSV infection (Elion *et al.*, 1977; Fyfe *et al.*, 1978; Field *et al.*, 1980). The TK gene has been mapped to gene UL23 (Halliburton *et al.*, 1980; Reyes *et al.*, 1982; McGeoch *et al.*, 1988a), which is not essential for virus growth in dividing cells but is essential in resting cells (Dubbs & Kit, 1964; Jamieson *et al.*, 1974) and is not required for establishment of latency (Efstathiou *et al.*, 1989). The TK gene contains six regions of conserved residues including a consensus ATP binding site which is essential for TK activity (Gentry, 1985; Liu & Summers, 1988; Balasubramaniam *et al.*, 1990; Harrison *et al.*, 1991). These regions are also conserved in the cellular deoxycytidine kinase (Chottiner *et al.*, 1991) suggesting that the herpesvirus TKs may have evolved from a cellular deoxycytidine kinase (Harrison *et al.*, 1991).

(c) Ribonucleotide reductase (UL39/UL40)

Ribonucleotide reductase (RR) provides precursors for DNA synthesis by reducing ribonucleoside diphosphates (rNDPs) to their corresponding deoxyribonucleotides (dNDPs) using cellular thioredoxin as the reducing agent (Darling, 1988).

HSV RR is composed of two non-identical subunits of molecular weight 136K (R_1) and 38K (R_2) (Frame *et al.*, 1985; Cohen *et al.*, 1985; Bacchetti *et al.*, 1986) which are encoded by genes UL39 and UL40, respectively (McLauchlan & Clements, 1983). The subunits form a complex in an $\alpha_2 \beta_2$ structure (Ingemarson & Lankinen, 1987). HSV RR is essential for virus growth in "resting" cells, or at elevated temperatures (Preston *et al.*, 1984b, 1988b; Goldstein & Weller, 1988a, 1988b; Jacobson *et al.*, 1989b), and RR mutants exhibit an impaired pathogenicity in mice (Cameron *et al.*, 1988; Jacobson *et al.*, 1989b).

HSV R_1 possesses a unique N terminal domain which is not directly involved in ribonucleotide reduction (Ingemarson & Lankinen, 1987; Lankinen *et al.*, 1989; Wymer *et al.*, 1989; Conner *et al.*, 1992b). This N terminal domain contains amino acid sequences which are characteristic of serine/threonine protein kinases and has been shown to possess protein kinase activity (Chung *et al.*, 1989, 1990; Paradis *et al.*, 1991; Ali *et al.*, 1992; see section 1.9.5.).

Two peptides have been shown to inhibit RR activity. The nonapeptide YAGAVVNDL, corresponding to the C terminal of R_2, inhibits activity by interfering with subunit association (McClements *et al.*, 1988; Paradis *et al.*, 1988; Darling *et al.*, 1990) and a peptide corresponding to 15 residues of HSV-2 R_1, at the junction of the protein kinase domain and the RR

domain, disrupts a complex consisting of R_1, R_2 and a previously uncharacterised cellular 180K protein (Chung *et al.*, 1991).

(d) dUTPase (UL50)

Following infection with HSV-1, a deoxyuridine triphosphatase (dUTPase) activity is induced which catalyses the hydrolysis of dUTP to dUMP and pyrophosphate (Wohlrab & Francke, 1980; Caradonna & Cheng, 1981; Williams, 1984). This enzyme therefore provides a mechanism to minimise the incorporation of uridine into newly synthesised DNA and also maintains a supply of dUMP which can be converted into dTMP by the cellular thymidylate synthase. The dUTPase was mapped to a position consistent with UL50 (Preston & Fisher, 1984) and was found to be non-essential for virus growth (Fisher & Preston, 1986).

(e) Uracil-DNA glycosylase (UL2)

Uracil-DNA glycosylase catalyses the removal of uracil residues from DNA, thereby carrying out a DNA repair and proofreading function. HSV uracil-DNA glycosylase was first observed by Caradonna & Cheng (1981) and has been mapped to UL2 (Caradonna *et al.*, 1987; Worrad & Caradonna, 1988). Sequence analysis of UL2 has revealed a striking similarity to the uracil-DNA glycosylase of *E. coli* (Mullaney *et al.*, 1989). The uracil-DNA glycosylase is not required for virus

growth in tissue culture (Mullaney *et al.*, 1989).

(f) Others

Topoisomerase activity, which forms or removes supercoiling, has been reported in HSV-infected cells, but is likely to be of cellular origin (Leary & Francke, 1984; Pearson & Conley, 1984; Muller *et al.*, 1985; Bapat *et al.*, 1987). Topoisomerase I activity has been co-purified with HSV DNA polymerase (Biswal *et al.*, 1983).

Spadari (1976) reported an increase in ligase activity following HSV infection. However, the properties of the enzyme were similar to those of the cellular ligase.

Protein kinases are involved in the regulation of many enzyme catalysed processes and may therefore play a role in HSV replication. Indeed, several HSV proteins, directly or indirectly involved in replication, are known to be phosphorylated (e.g. UL42, RR, DNase). However, none of these proteins have yet been identified as substrates of the US3 or UL13 protein kinases (Purves *et al.*, 1991; this thesis) and must therefore be phosphorylated by cellular kinases. Whether the phosphorylation of these proteins is of functional significance or is simply fortuitous is not known.

1.7.5. Packaging of HSV-1 DNA into Capsids

HSV DNA is thought to replicate by a rolling circle mechanism generating head-to-tail concatemers which are then cleaved into unit-length genomes for packaging into preformed empty capsids (Jacob *et al.*, 1979; Vlazny & Frenkel, 1981). The processes of cleavage and packaging are linked (Ladin *et al.*, 1980; 1982; Deiss *et al.*, 1986; Deiss & Frenkel, 1986) and involve *cis*-acting sequences and *trans*-acting factors.

1.7.5.1. *cis*-acting sequences

The *cis*-acting sequences involved in cleavage and packaging are contained within the *a* sequence which consists of three direct repeats, designated DR1, DR2 and DR4 and two unique sequences, U_b and U_c (Mocarski & Roizman, 1982; see section 1.4.2.). The sequences necessary for cleavage and packaging are contained within U_b and U_c and are designated *pac*1 and *pac*2, respectively (Deiss *et al.*, 1986; Varmuza & Smiley, 1985). The site at which the DNA is cleaved is located within DR1 (Varmuza & Smiley, 1985; Mocarski *et al.*, 1985).

1.7.5.2. *Trans*-acting factors involved in cleavage and packaging

Three viral proteins with molecular weights of 250 KDa, 140 KDa and 82 KDa have been found to interact with DNA probes containing the *pac*2/DR1 sequences (Chou & Roizman, 1989).

The 250 KDa protein, which has been identified as the product of UL36 (VP1), and the 140 KDa protein, which has not yet been identified, bind to the probes in a sequence-dependent manner. The 82 KDa protein, which binds in a non-specific manner, has been identified as the HSV-encoded DNase (Chou & Roizman, 1989). This study, combined with the observation that a DNase-negative mutant is defective in the processing of viral DNA (Weller *et al.*, 1990), suggests that the DNase, in conjunction with other proteins, may be responsible for the cleavage of HSV DNA.

Studies with *ts* mutants which accumulate unprocessed DNA have led to the identification of several viral proteins involved in the *trans*-activation of encapsidation. These proteins include UL6 (Weller *et al.*, 1983, 1987), UL26 (Preston *et al.*, 1983), UL28 (Matz *et al.*, 1983), UL32 (Weller *et al.*, 1983), UL33 (Al-Kobaisi, 1990) and UL54 (Sacks & Schaffer, 1987). The roles of these proteins in encapsidation are not yet known but some, such as the IE gene UL54, are likely to play indirect roles.

1.7.5.3. Proposed packaging model

A cleavage-packaging model has been proposed (Deiss *et al.*, 1986). The concatemers produced during replication contain novel junctions formed by the fusion of the L and S termini. These junctions consist of two copies of the *a* sequence which share the intervening DR1 sequence. It is proposed that a

trans-acting protein forms an attachment between the *pac*2 sequence, of the L terminus, and an as yet unidentified structure on the capsid surface. The DNA is then looped into the empty capsid until another *pac*2 sequence, in the same orientation, is encountered (representing the S terminus). The DNA is then cleaved at the DR1 sequence adjacent to this *pac*2 sequence. This model predicts that the packaged DNA must always be the length of a unit genome, however capsids containing shorter lengths of DNA have been reported although they do not become enveloped (Vlazny *et al.*, 1982), implying that the process is more complex.

1.7.6. Envelopment of Virions and Egress From Cells

The route by which full capsids acquire a tegument and envelope has not been resolved in detail. Original reports suggested that the primary site of capsid envelopment was the inner nuclear membrane (Nii *et al.*, 1968; Schwartz & Roizman, 1969). However, evidence has also been produced suggesting that capsids become enveloped by budding into cytoplasmic vacuoles (Nii *et al.*, 1968; Smith & De Harven, 1973).

Recent reports have shown that infection with alphaherpesviruses generates enveloped particles which lack capsids and DNA. These non-infectious particles have been designated light (L) particles (Szilagyi & Cunningham, 1991; McLauchlan & Rixon, 1992). Rixon *et al.* (1992) have shown that these particles assemble independently of virion

maturation, probably by a mechanism involving the condensation of tegument proteins within the cytoplasm followed by envelopment at cytoplasmic vacuoles. It is envisaged that virions are enveloped in a similar manner, except that the tegument proteins condense around the capsids.

Irrespective of where the capsids bud, it is generally accepted that the acquired envelope contains precursor forms of the viral glycoproteins. These precursors are processed into mature glycoproteins during migration through the Golgi apparatus (Johnson & Spear, 1982; Torrisi *et al.*, 1992).

The egress of the mature virion is thought to occur by reverse phagocytosis at the plasma membrane.

1.7.7. Effect of HSV-1 Lytic Infection on the Host Cell

Lytic infection of cells with HSV-1 produces a number of changes which ultimately result in cell death.

Several alterations in the structure of the cell have been observed. For example, the nucleolus increases in size, is displaced towards the nuclear membrane and disaggregates later in infection (Roizman & Furlong, 1974). In addition, the host chromatin is seen to unwind and, at later stages, chromatid breaks and fragmentation are observed (Hampar & Ellison, 1961). This chromosomal damage does not occur

without the synthesis of IE and E proteins (Waubke *et al.*, 1968; Donner & Gönczöl, 1971; Peat & Stanley, 1986).

Another consequence of HSV-1 infection is the rapid inhibition of host macromolecular synthesis or virion host shut-off (*vhs*) (reviewed by Fenwick, 1984). This is characterised by a decrease in the transcription of both ribosomal and cellular RNA (Wagner & Roizman, 1969; Stenberg & Pizer, 1982; Mayman & Nishioka, 1985), the disaggregation of polysomes containing host mRNAs (Sydiskis & Roizman, 1967), and the destabilization and degradation of cellular mRNA (Nishioka & Silverstein, 1977, 1978; Fenwick & Walker, 1978; Schek & Bachenheimer, 1985; Strom & Frenkel, 1987). The host shut-off occurs by two independent mechanisms, an "early" shut-off, which involves a virion component (Fenwick & Walker, 1978; Fenwick & Clark, 1982; Nishioka & Silverstein, 1977; Schek & Bachenheimer, 1985) and a "delayed" shut-off, which is dependent upon *de novo* protein synthesis (Honess & Roizman, 1974; Nishioka & Silverstein, 1978). The virion component responsible for the "early" shut-off has been identified as the UL41 gene product, a non-essential, 58K tegument phosphoprotein (Read & Frenkel, 1983; Kwong *et al.*, 1988; Fenwick & Everett, 1990; Smibert *et al.*, 1992). It has been suggested that this protein interacts with a cellular factor which results in a decrease in mRNA stability (Kwong & Frenkel, 1989). The UL41 gene product also appears to be responsible for the observed degradation of viral mRNAs (Kwong & Frenkel, 1987; Orozkar & Read, 1989;

Fenwick & Owen, 1988). This regulates the accumulation of viral mRNAs allowing transition from one kinetic class to the next.

In contrast to the host shut-off described above, the transcription of some cellular proteins is increased following infection with HSV-1. A role for the IE protein Vmw175 (IE175, IE3, ICP4) in this induction has been suggested (Patel *et al.*, 1986; Latchman *et al.*, 1987), although viruses expressing an aberrant form of Vmw175 also induce cellular proteins (Notarianni & Preston, 1982; Russell *et al.*, 1987a) and Kemp *et al.* (1986) have shown that an increase in cellular transcription can occur in the absence of viral protein synthesis. The cellular proteins induced by HSV-1 infection include several heat-shock proteins (Notarianni & Preston, 1982; La Thangue *et al.*, 1984; Patel *et al.*, 1986; Latchman *et al.*, 1987) and a 90K cellular protein (Macnab *et al.*, 1985; Hewitt *et al.*, 1991).

Another effect of HSV-1 infection on the host cell is an increase in the phosphorylation of the ribosomal protein S6 (Kennedy *et al.*, 1981). This increase occurs several hours after infection and is thought to be due to an increase in the activity of a cellular protein kinase (Kennedy *et al.*, 1981; Katan *et al.*, 1986). The phosphorylation state of ribosomes appears to correlate with their affinity for mRNAs, thus this increased phosphorylation may either promote viral infection or may represent a protective response to viral

infection (Kennedy *et al.*, 1981).

1.8. PROTEIN KINASES

Phosphorylation of proteins is one of the most common forms of post-translational modification and is a major mechanism for the regulation of various cellular functions in both eukaryotes and prokaryotes. The phosphorylation state of proteins reflects the relative activities of two enzymes with antagonistic actions, protein kinases and protein phosphatases. Protein kinases catalyse the transfer of the γ-phosphate of a nucleoside triphosphate, such as ATP or GTP, to an acceptor amino acid in a substrate protein. Protein phosphatases catalyse the removal of the phosphate group (see Figure 1.4).

1.8.1. Classification of Protein Kinases

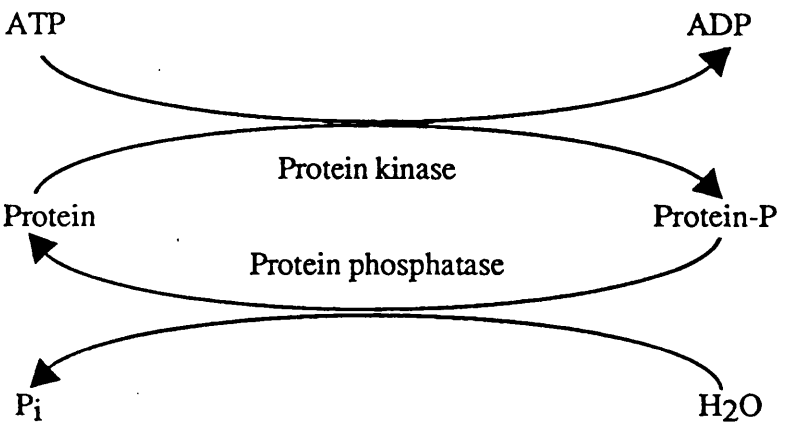
Over 100 protein kinases have been identified, initially through enzymatic studies but more recently through recognition of protein kinase sequence motifs in genes and the specific isolation of protein kinase genes from cDNA or genomic libraries. These protein kinases have been classified according to their acceptor amino acid specificity (Hunter, 1991). In cases where the amino acid specificity is not known, protein kinases have been classified indirectly by sequence comparison with protein kinases of known specificity. The five main classes of protein kinases are:

Figure 1.4

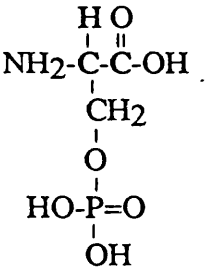
A. Phosphorylation Reactions. Proteins are phosphorylated in a reaction catalysed by protein kinases while the removal of phosphate groups is catalysed by protein phosphatases.

B. The structures of the three most commonly phosphorylated amino acid residues are shown; phosphoserine, phosphothreonine and phosphotyrosine.

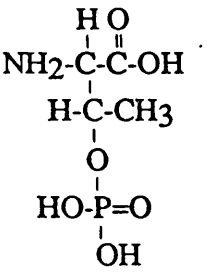
A.



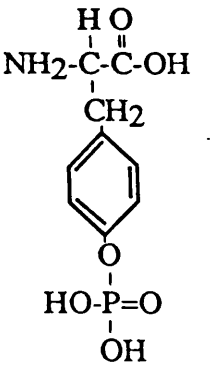
B.



Phosphoserine



Phosphothreonine



Phosphotyrosine

1. Protein-serine/threonine kinases.
2. Protein-tyrosine kinases.
3. Protein-histidine kinases, (these kinases can also use arginine and lysine as acceptors).
4. Protein-cysteine kinases.
5. Protein-aspartyl or -glutamyl kinases.

Another class of kinases may also exist, comprising protein kinases such as wee1$^+$, Clk and YPK1, which phosphorylate both serine/threonine and tyrosine amino acid residues.

Protein-serine/threonine and protein-tyrosine kinases are the most common kinases in eukaryotes and have also been detected in bacteria (Saier *et al.*, 1990). Protein-histidine kinase activity has been reported in eukaryotic cells but the enzyme(s) responsible have not been purified. Both protein-histidine and protein-cysteine kinases are involved in the phosphoenolypyruvate-dependent phosphotransferase system of prokaryotes (reviewed in Saier *et al.*, 1990). No protein-cysteine kinase activity has been detected in eukaryotic cells. Examples of protein-aspartyl kinases can be found in another prokaryotic phosphorylating system, the sensor kinase/response regulator system. This system involves the autophosphorylation of the sensor kinase on a histidyl residue with the subsequent transfer of the phosphoryl group to an aspartyl residue of the response regulator protein (reviewed in Saier *et al.*, 1990).

Within each main acceptor class the protein kinases are further divided into families. The serine/threonine kinases are very diverse and include families which are classified according to their regulators (e.g. cyclic nucleotide-dependent, diacylglycerol-dependent, and calcium/calmodulin-dependent), families classified according to their substrates (casein kinase I and II and ribosomal protein S6 kinases), families involved in cell cycle control (e.g. *CDC28* and *cdc2*$^{+}$), and a family of proto-oncogene products (raf/mos kinases). The protein-tyrosine kinases contain only two main families, kinases which are the products of oncogenes (e.g. *src* and *abl*) and kinases which function as growth factor receptors (e.g. epidermal growth factor family). Table 1.3 gives a concise list of the families within the protein-serine/threonine and protein-tyrosine kinase classes. For references and more extensive lists see Hanks *et al.* (1988) and Hunter (1991).

1.8.2. Protein Kinase Recognition Sequences

During early studies on protein kinases it was noticed that certain protein kinases, including cAMP-dependent kinase, were able to specifically phosphorylate a very wide range of substrate proteins while other kinases were able to phosphorylate only a limited number of the available phosphorylation sites on a particular substrate. It was therefore concluded that protein kinases phosphorylate their

Table 1.3 Classification of protein kinases

Protein-serine/threonine kinase family members
A. Cyclic nucleotide regulated e.g. cAPK B. Diacylglycerol regulated e.g. PKC C. Calcium/calmodulin regulated e.g. Phosphorylase kinase D. Ribosomal S6 Protein Kinases E. Casein Kinases F. Glycogen synthase 3 G. cdc2/CDC28 Kinases H. STE7 Kinases I. SNF1 Kinases J. Mos/Raf Kinases K. Others e.g. HSV1 US3
Protein-tyrosine kinase family members
A. src gene family e.g. src, yes, fgr B. abl gene family C. fps/fes gene family D. Growth factor receptors e.g. EGF-R subfamily Insulin receptor subfamily PDGF-R subfamily CSF1-R subfamily

substrates only at sites where the appropriate acceptor amino acid is surrounded by a specific local sequence. The specific phosphorylation site sequences of many protein kinases have now been characterised by studying the phosphorylation of model peptides. Extensive lists of these sequences can be found in Kemp & Pearson (1990) and Pearson & Kemp (1991). Protein kinases such as phosphorylase kinase and the myosin light-chain kinase have highly restricted specificity ranges and may only be able to phosphorylate a single substrate while others, such as the multifunctional calmodulin-dependent protein kinases, have broad specificities consistent with their ability to phosphorylate many substrates. Many protein kinases have requirements for positively or negatively charged residues surrounding the acceptor amino acid, and have been designated basotropic or acidotropic. The HSV-1 US3 protein kinase is an example of a basotropic kinase in that it requires at least three arginine residues on the N terminal side of the acceptor amino acid and cannot tolerate acidic residues on the C terminal side (Purves *et al.*, 1986b; Leader *et al.*, 1991; Purves *et al.*, 1991). Protein-tyrosine kinases tend to be acidotropic.

1.8.3. Multiple and Hiercharchical Protein Phosphorylation

Many proteins are phosphorylated at multiple sites by one, or more, protein kinases (reviewed in Roach, 1991). Examples include: phosvitin, which is one of the most densely phosphorylated proteins with over 100 phosphorylated

residues; neurofilaments; and the large subunit of eukaryotic RNA polymerases. These multiple phosphorylation sites are often concentrated in relatively short regions of the protein, usually at the N or C terminus, e.g. the C terminus of the RNA polymerases.

Multisite phosphorylation can occur by two mechanisms. The first occurs when the substrate protein contains multiple copies of specific phosphorylation sites for one or more protein kinases. For example, the neurofilaments NF-H and NF-M contain multiple copies of a -K-S-P- sequence (Lee *et al.*, 1988; Lees *et al.*, 1988). The second mechanism, known as hiercharchical phosphorylation, occurs when the introduction of one phosphate group influences the introduction of subsequent ones. The most widely known example of this mechanism is the phosphorylation of glycogen synthase by casein kinase II which provides the recognition site required for phosphorylation by the glycogen synthase kinase-3 (GSK-3) (Fiol *et al.*, 1987; reviewed in Roach, 1991).

The function of multisite phosphorylation is not known. It may be necessary to bring about a critical conformational change in the substrate or different combinations of phosphorylated sites may produce a variety of protein forms. In addition, the phosphorylation of a substrate by several protein kinases increases the complexity of the mechanisms by which the substrate may be regulated.

1.8.4. Autophosphorylation and Pseudosubstrate Sequences

Another, apparently general, characteristic of protein kinases is their ability to autophosphorylate, that is, to catalyse the transfer of phosphate groups to acceptor amino acids within the protein kinases themselves. This usually occurs via an intramolecular reaction. Evidence suggests that autophosphorylation may be involved in activation of the kinase, apparently by displacing the pseudosubstrate sequence. This element of the protein, usually located in the regulatory domain of the kinase, resembles the phosphorylation site of the protein kinase and interacts with the catalytic domain to maintain the enzyme in an inactive form. Once activated by the enzyme's regulator, e.g. cyclic nucleotides, the enzyme undergoes a conformational change which relieves this inhibitory effect and permits the autophosphorylation of the pseudosubstrate sequence which then serves to maintain the enzyme in its active form. However, it has been shown that pseudosubstrate sequences which lack a suitable acceptor amino acid also inhibit enzyme activity, in a similar manner. Pseudosubstrate sequences have been reported for the cAMP-dependent protein kinase, the skeletal and smooth muscle myosin light-chain kinases, calmodulin-dependent protein kinase II, protein kinase C and the p60$^{v\text{-}src}$ protein kinase (Kennelly *et al.*, 1987; Kemp *et al.*, 1987; Kelly *et al.*, 1988; House & Kemp, 1987; Grandori, 1989; reviewed in Hardie, 1988).

1.8.5. Protein Kinase Catalytic Domain

1.8.5.1. Sequence analysis

In recent years the sequences of many eukaryotic protein kinases have been determined. Multiple alignments of these sequences have revealed a conserved region of approximately 300 amino acids, which is considered to represent the catalytic domain of the kinases. The first comprehensive protein kinase sequence alignment was reported by Hanks *et al.* (1988) using sequences derived from 38 protein-serine/threonine kinases (including HSV-1 US3 protein kinase) and 27 protein-tyrosine kinases. This study was subsequently extended to include a total of 75 protein-serine/threonine kinases and 42 protein-tyrosine kinases (Hanks & Quinn, 1991). The catalytic domains are not uniformly conserved throughout their length but consist of a number of short characteristic conserved motifs, or subdomains, separated by regions of lower conservation which may contain large deletions or insertions. Hanks *et al.* (1988) and Hanks & Quinn (1991) identified eleven of these subdomains, designated I to XI. Table 1.4 lists the conserved sequences for both protein-serine/threonine kinases and protein-tyrosine kinases (derived from Hanks & Quinn, 1991). The conserved motifs differ slightly between the two classes of protein kinases with the protein-tyrosine kinases possessing a larger number of conserved residues. This could be

Table 1.4 Conserved subdomains of the catalytic domains of protein-serine/threonine kinases and protein-tyrosine kinases

Upper case letters indicate conserved amino acid residues. Lower case letters indicate amino acids with similar properties where:

- o = non-polar, i.e. M, L, I, V, C
- a = aromatic, i.e. F, Y, W
- b = neutral/small, i.e. A, G, S, T, P
- c = basic/polar, i.e. K, R, H

Subdomain	Consensus Sequence	
	Protein-serine/threonine kinases	Protein-tyrosine kinases
I	oG-G--b-V	oG-G-FG-V
II	aoK-o	AoK-o
III	E	E
V		G-L
VI	DoK--No	HRDL---Nooo
VII	o-DaG	Ko-DFGobR
VIII	T/S--a-bPE	P-cW-b-E
IX	D-ab-G	SDVWbaGooo-Eob-b--PY
X		G--o--P
XI	R	CW------RP-F

interpreted as an indication that the protein-tyrosine kinases are more closely related to each other than are the protein-serine/threonine kinases although this may simply be a reflection of the smaller number of protein-tyrosine kinases used in the alignment.

Similarity to protein sequences of known function, chemical modification and the use of mutagenesis techniques have given an indication of the roles these conserved motifs play in catalysis. For example, the consensus G-X-G-X-X-G of subdomain I is thought to be involved in nucleotide binding as it is present in many nucleotide binding proteins. The invariant lysine residue of subdomain II has been shown to be essential for kinase activity (Chen *et al.*, 1987; Hannick & Donoghue, 1985) and has been implicated in the phosphotransfer reaction, due to its reaction with the ATP analogue p-fluorosulphonyl 5'-benzoyl adenosine. Subdomains VI to IX form the core of the catalytic domain. Subdomain VII is thought to contribute to nucleotide binding, on account of its homology with a sequence found in a number of bacterial phospho-transferases, while the -A-P-E- consensus of subdomain VIII is thought to be located near the catalytic site and is known to be required for activity in *v-src* (Bryant & Parsons, 1983). Subdomains VI and VIII are thought to be involved in recognition of the acceptor amino acid. In subdomain VI the consensus -D-L-K-P-E-N- is an indicator of serine/threonine specificity while the protein-tyrosine consensus is either -D-L-R-A-A-N- or -D-L-A-A-R-N-. In

subdomain VIII the protein-serine/threonine kinase consensus is G-T/S-X-X-Y/F-X-A-P-E- and the protein-tyrosine kinase consensus is P-I/V-K/R-W-T/M-A-P-E-.

A greater understanding of the functions of these conserved sequences has been possible with the recent solution of the crystal structure of the catalytic subunit of the cAMP-dependent protein kinase (cAPK) (Knighton *et al.*, 1991a, 1991b; see below).

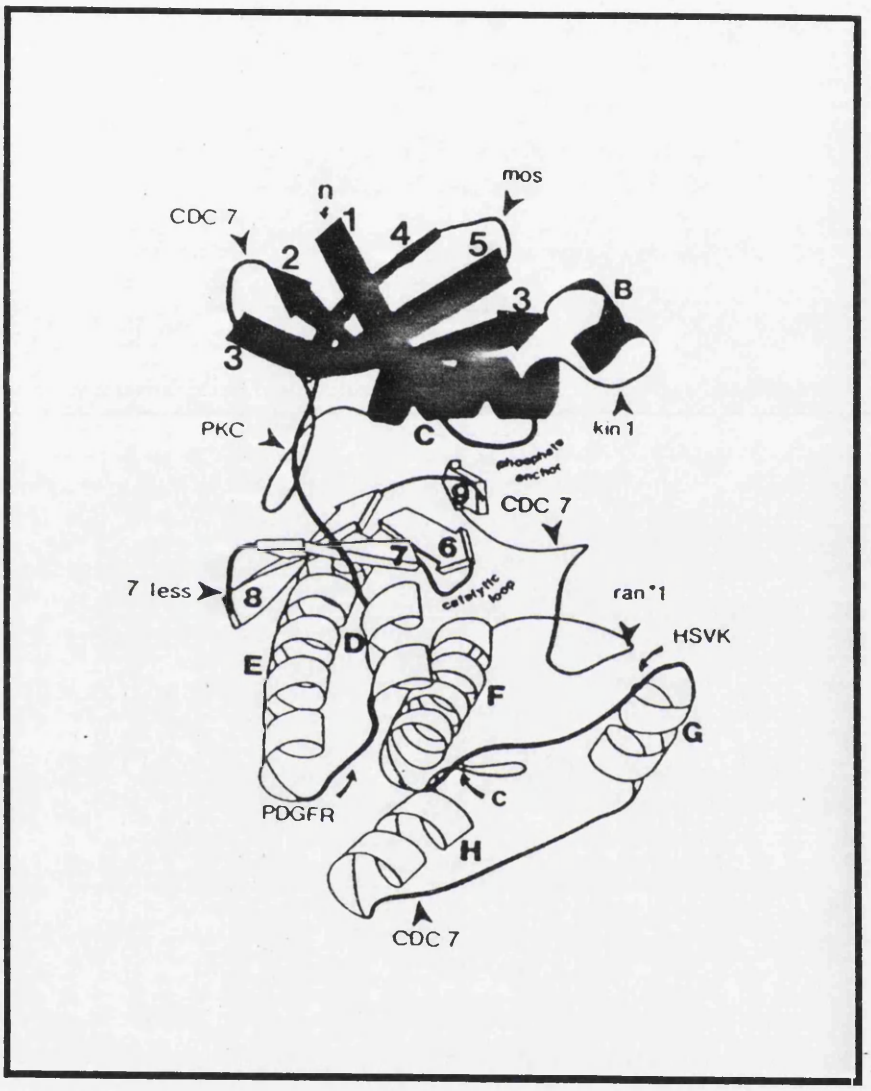
1.8.5.2. Structural analysis

The three-dimensional structure of the catalytic domain of the cAMP-dependent protein kinase has been determined by X-ray crystallography (Knighton *et al.*, 1991a, 1991b). The cAMP-dependent protein kinase was chosen for these studies because, unlike most protein kinases, the catalytic domain is located on a separate polypeptide from the regulatory domain and is relatively easy to purify. The kinase has also been well characterised (reviewed in Taylor *et al.*, 1990). The results of the X-ray crystallographic analysis indicate that the catalytic domain has a bilobal structure (Figure 1.5). The smaller lobe, consisting mostly of N terminal sequences, is characterised by a dominance of β strands and is associated with nucleotide binding. The larger lobe is predominantly alpha helical and is involved in peptide binding and catalysis. The cleft between the lobes represents the active size of the enzyme. The majority of

Figure 1.5 **Diagram of the cAMP-dependent protein kinase catalytic domain.**
(Reproduced from Knighton *et al.*, 1991a)

The small lobe (shaded) is characterised by a predominance of β strands (marked 1 to 5) while the large lobe consists of seven α helices (D to J; I and J are not marked on this diagram). The position of inserts in the protein kinase catalytic domain from the Hanks *et al.* (1988) alignment are indicated with arrows; n, amino terminal; c, carboxy terminal.

mos, cellular homologue of Moloney murine sarcoma virus oncogene; kin1, *S. cerevisiae* protein kinase; CDC7, cell division cycle 7 protein kinase; ran^{+}1, ras-related nuclear protein; HSVK, herpes virus US3 protein kinase; PDGFR, platelet-derived growth factor receptor; 7less, *sevenless* protein kinase and PKC, protein kinase


mos
n
CDC 7
1
4
5
2
3
B
3
PKC
C
kin 1
phosphate anchor
9
CDC 7
7
6
7 less
8
catalytic loop
ran*1
HSVK
D
E
F
G
PDGFR
C
H
CDC 7

the conserved subdomains identified by Hanks *et al.* (1988) are located on the surfaces that line this cleft and form three functionally important regions, the glycine-rich loop in the small lobe, the catalytic loop in the large lobe and a triad of charged residues (see Figure 1.6).

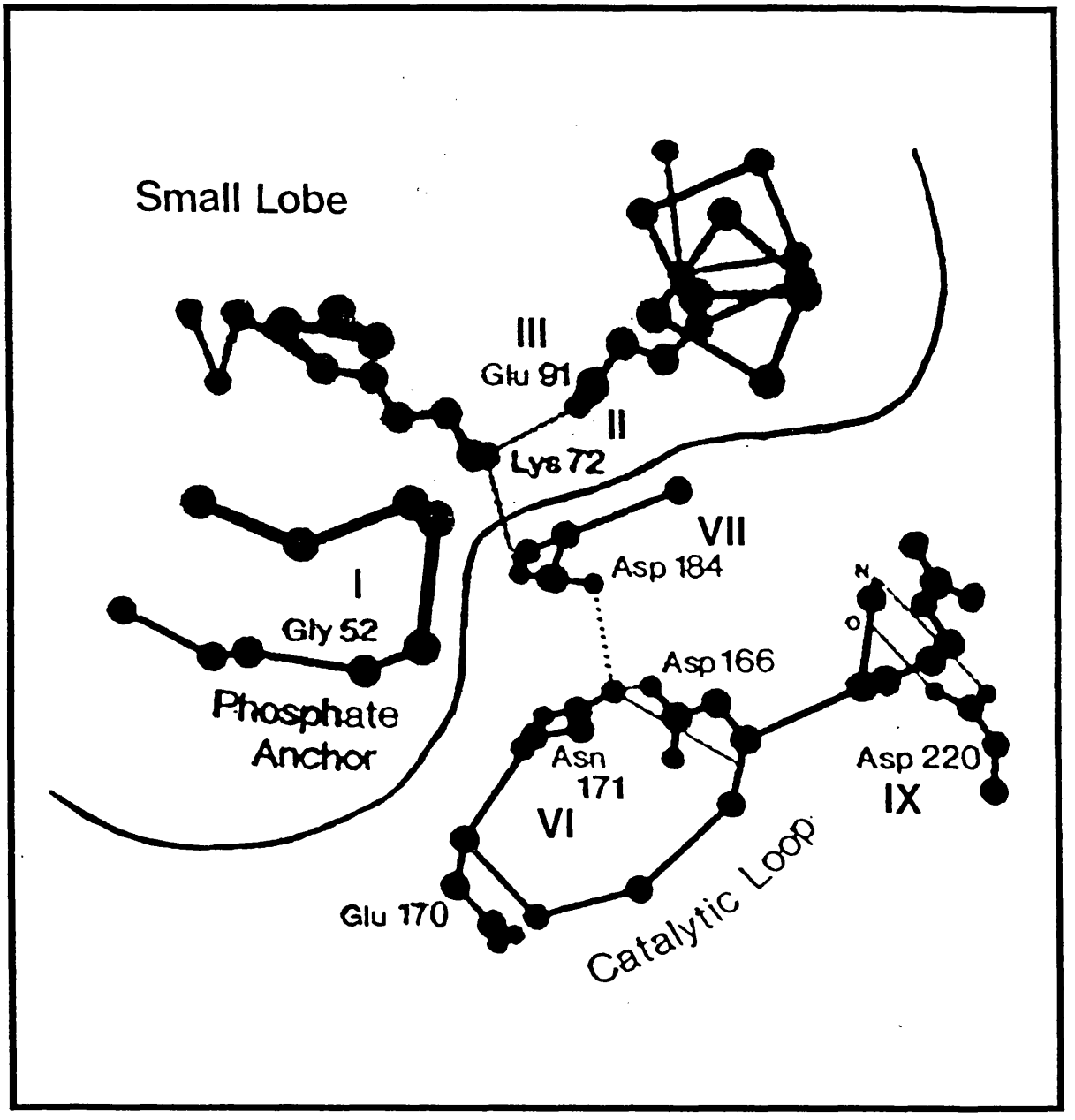
The glycine-rich loop is composed of the residues of subdomain I and forms a nucleotide binding site that is structurally distinct from both the Rossmann fold and the P loop motif which are found in many nucleotide binding sites (Saraste *et al.*, 1990). The purpose of the glycine loop is to anchor the phosphate moiety and, in particular, to hold the γ-phosphate in the correct position for transfer to occur.

The catalytic loop is composed of the conserved residues of subdomain VI. The invariant residues are important for catalysis while the less conserved residues are involved in guiding the substrate peptide into the correct orientation for catalysis to occur. The invariant aspartic acid residue of subdomain IX, Asp^{220}, (numbering refers to the cAMP-dependent protein kinase) contributes to the stability of this loop.

The Lys^{72} residue of subdomain II, the Glu^{91} residue of subdomain III and the Asp^{184} residue of subdomain VII form a triad which lies close to the γ-phosphate of MgATP. The Asp^{184} residue can also form a second triad with Asp^{166} and

Figure 1.6 **Positions of invariant amino acids at the active site of cAMP-dependent protein kinase.**
(Reproduced from Knighton *et al.*, 1991b, with modifications)

Three of the conserved residues which form the active site (Gly^{52}, Lys^{72} and Glu^{91}) are located within the small lobe. The remaining invariant residues are located in the large lobe. The subdomains in which these invariant residues are present are marked (I, II, III, VI, VII and IX). Residues close enough for hydrogen bonding or ion pairing are indicated by a dashed line while residues within 4 to 5 Å of one another are connected by a dotted line.


Small Lobe
III
Glu 91
II
Lys 72
VII
Asp 184
I
Gly 52
Phosphate
Anchor
Asp 166
N
O
Asn
171
VI
Asp 220
IX
Glu 170
Catalytic Loop

Asn^{171} of subdomain VI (the catalytic loop) and therefore has the potential to shuttle between the two conserved loops. Asp^{184} may participate in the chelation of Mg^{2+} in the MgATP complex which may alter the charge distribution in the catalytic loop. This may be necessary for efficient catalysis.

Subdomain VIII, which lies close to the catalytic loop, contains Asp^{208} which forms an ion pair with the conserved Arg^{280} of subdomain XI. This helps to maintain the tertiary structure of the domain.

The non-conserved amino acids of the catalytic domain form loops on the surface of the protein and are important for substrate recognition. These loops often contain insertions, e.g. PDGFR kinase possesses a 99 residue insertion between subdomains V and VI, and HSV US3 kinase has an extra 11 residues between subdomains IX and X (Figure 1.5).

The functions of the subdomains are summarised in Table 1.5.

1.8.6. Known Functions of Non-viral Protein Kinases

Protein phosphorylation is one of the most important mechanisms by which intracellular processes are regulated, indeed, there are few, if any, metabolic processes in eukaryotes that are not dependent upon the action of protein kinases. The following examples are some of the better

Table 1.5 Functions of the conserved subdomains.

Subdomain	Function
I	Glycine-rich loop - anchors phosphate moiety and helps position γ-phosphate for transfer
II	Recognition of phosphate groups
III	Recognition of phosphate groups
VI	Catalytic loop
VII	Conserved Asp residue shuttles between the two conserved loops - possible role in charge transfer
VIII	Forms an ion pair with subdomain XI - maintenance of tertiary structure
IX	Stabilises catalytic loop
XI	Forms an ion pair with subdomain VIII - maintenance of tertiary structure

characterised functions.

1.8.6.1. Regulation of metabolism

Protein kinases are involved in the regulation of many metabolic pathways. For example, in glycogen metabolism the shut off of glycogen synthesis and the stimulation of glycogen breakdown are both directed by the action of the cAMP-dependent protein kinase (reviewed by Cohen, 1982). Other pathways regulated by phosphorylation include fatty acid synthesis, glycolysis, gluconeogenesis and cholesterol synthesis.

1.8.6.2. Transmembrane signal transduction

Many protein kinases either span the plasma membrane or are associated with its inner face and are able to transduce signals from external stimuli, such as hormones and neurotransmitters. For example, many growth factors, such as EGF and PDGF, transmit their mitogenic effects by activating the protein-tyrosine kinase domains of their receptors (reviewed in Hunter & Cooper, 1985). These signalling pathways also play a central role in both invertebrate and vertebrate development. Examination of *Drosophila* and mouse mutants has revealed that many of the genes required for normal embryonic development contain motifs characteristic of receptor protein-tyrosine kinases or protein-serine/threonine kinases. Examples of *Drosophila* receptor protein-tyrosine

kinases include the *torso* (*tor*) gene, the gene for the *Drosophila* EGF receptor homologue (DER) and the *sevenless* (*sev*) gene. In the mouse, the *W* locus is related to the *c-kit* protoncogene and the *Patch* phenotype results from the loss of the α-type PDGF-R gene. The protein-serine/threonine kinases involved in embryonic signal transduction include the *zeste-white 3* gene, the *fused* gene, the *D-raf* gene and the *nina C* locus. *Drosophila* may also possess an eye-specific protein kinase C. It is envisaged that these serine/threonine-specific protein kinases function after the protein-tyrosine kinases, (reviewed in Pawson & Bernstein, 1990; Siegfried *et al.*, 1990).

Protein-tyrosine kinases are also involved in the signalling pathways which activate the T cell antigen receptor. This activation underlies the ability of T cells to distinguish between self and non-self and to initiate the immune response (reviewed in Klausner & Samelson, 1991). It has also been suggested that protein tyrosine phsophorylation is a pre-requisite for activation of the cytotoxic function of natural killer cells (Einspahr *et al.*, 1991).

1.8.6.3. Ion conductance

Evidence suggests that protein kinases modulate ion conductance by phosphorylating membrane proteins such as channels, pumps and ion exchange proteins. Protein kinase C is thought to regulate the extrusion of Ca^{2+} from the cell by

phosphorylating the Ca^{2+}-transport ATPase and, the cAMP-dependent protein kinase has been shown to regulate the CFTR (cystic fibrosis transmembrane conductance regulator) chloride channel (Cheng *et al.*, 1991). In addition, two transport mechanisms involved in cell volume regulation, the Na/K/2Cl system and the KCl cotransport system, are regulated by phosphorylation (reviewed in Cossins, 1991).

1.8.6.4. Neuronal function

Brain and other neuronal tissues contain high concentrations of protein kinases (reviewed by Nairn *et al.*, 1985) and phosphorylation has been implicated in the regulation of a wide range of neurophysiological processes. For example, some neurotrophic factors exert their effects through protein-tyrosine kinase receptors such as *trk B* (Klein *et al.*, 1991), protein kinases, including protein kinase C, are involved in the synthesis and release of neurotransmitters (Nairn *et al.*, 1985; Nishizuka, 1986), and casein kinase II has been implicated in microtubule protein phosphorylation which leads to neurite outgrowth (Diaz-Nido *et al.*, 1991). It is interesting to note that hyperphosphorylation of microtubule proteins is associated with Alzheimer's disease (Steiner *et al.*, 1990b).

1.8.6.5. Regulation of the cell cycle

Perhaps one of the most important functions of protein

kinases has been identified as a result of the study of cell division cycle (CDC) mutants of yeast. The products of the *CDC28* gene in *Saccharomyces cerevisiae* and *cdc2*$^{+}$, the homologous gene in *Schizosaccharomyces pombe*, have been shown to be necessary for entry into S phase and also for initiation of mitosis in late G2. Both of these genes encode proteins which possess protein-serine/threonine activity. A homologue has also been isolated from human cDNA. The mechanisms by which the p34^{cdc2} kinase regulates the cell cycle appear to be complex and have not yet been resolved. However, it is known that during the S phase and G2 the kinase associates with a protein known as cyclin A and is progressively phosphorylated. The hyperphosphorylated kinase then forms a complex with cyclin B and, at the end of G2, dephosphorylation occurs, activating the complex and initiating mitosis (reviewed in Draetta, 1990).

1.8.6.6. Regulation of protein synthesis

Several lines of evidence indicate that protein kinases play a role in the regulation of protein synthesis. For example, the phosphorylation of eukaryotic RNA polymerase II by cdc2 may be involved in transcriptional control (Cisek & Corden, 1989) and the extent of phosphorylation of ribosomal protein S6 may affect the rate at which translation occurs. The initiation of protein synthesis is controlled by the phosphorylation of eIF-2α by protein kinases such as the haem-regulated protein kinase and the double stranded

RNA-dependent protein kinase (reviewed in Edelman *et al.*, 1987).

1.8.6.7. Prokaryotic systems

The above examples have all concerned eukaryotic systems. However, it is now clear that many bacterial processes, such as intermediary metabolism, gene transcription, differentiation, virulence and photosynthetic CO_2 fixation, are also regulated by protein kinases (Saier *et al.*, 1990).

1.8.7. Phosphatases

Protein phosphatases, like protein kinases, can be classified into two main families, protein-serine/threonine phosphatases and protein-tyrosine phosphatases. Very little is known about the structure and regulation of the tyrosine-specific phosphatases, possibly due to the lack of suitable physiological substrates (Tonks *et al.*, 1988a, 1988b). Protein-serine/threonine phosphatase activity can be accounted for by four distinct enzymes which are grouped into two classes. The type 1 protein phosphatases (PP-1) selectively dephosphorylate the β subunit of phosphorylase kinase, are inhibited by inhibitor 1, inhibitor 2 (proteins purified from liver and muscle extracts; Cohen, 1989) and okadaic acid and are regulated by cAMP and Ca^{2+}. The type 2 protein phosphatases can be subclassified into three enzymes, PP-2A, PP-2B and PP-2C. All three enzymes selectively

dephosphorylate the α subunit of phosphorylase kinase and are insensitive to inhibition by inhibitors 1 and 2. They differ however, in their dependence on divalent cations, for example PP-2B and PP-2C have an absolute requirement for Ca^{2+} and Mg^{2+}, respectively, while PP-2A is active in the absence of divalent cations. In addition, PP-2A is completely inhibited by okadaic acid while PP-2B is only weakly inhibited and PP-2C is completely unaffected.

Protein phosphatase activity is required for the efficient regulation of all the processes involving protein kinases, e.g. metabolism, protein synthesis and the cell cycle (reviewed in Ingebritsen & Cohen, 1983; Cohen, 1989; Cyert & Thorner, 1989).

1.8.8. Role of Protein Kinases in Viral Transformation

It is well known that infection of cells with certain viruses, under appropriate conditions, results in abnormal cellular growth or transformation. These viruses include the acute transforming retroviruses, adenovirus, papillomavirus, polyomaviruses, and herpesviruses (reviewed in Bishop, 1985).

1.8.8.1. Acute transforming retroviruses

Acute transforming retroviruses owe their transforming capacity to the acquisition of cellular genes which are either mutated or abnormally expressed, and are designated

oncogenes. Many of these oncogenes encode protein kinases although this is not a universal attribute of a transforming gene as *v-ras*, *v-myc* and *v-sis* oncogenes do not possess any protein kinase activity.

The protein kinase-encoding oncogenes can be classified into two groups, those which resemble protein-tyrosine kinases and those which possess serine/threonine-specific characteristics. Most of the oncogenes fall into the protein-tyrosine kinase group and these can be further divided into those that span the membrane and those which associate with the membrane. The membrane-spanning oncogenes include *v-fms*, *v-erb-B* and *v-ros*. The *fms* gene was first isolated from a feline sarcoma virus and is a homologue of the cellular macrophage-colony stimulating factor (M-CSF) receptor. The *v-fms* gene product however no longer requires M-CSF to function and is constitutively activated (reviewed in Rohrschneider & Woolford, 1991). The cellular homologue of v-erb B is the epidermal growth factor receptor (Downward *et al.*, 1984). Oncogenes such as *v-src*, *v-abl*, *v-fes/fps*, *v-fgr* and *v-yes* all associate with the inner surface of the plasma membrane, usually by means of a myristyl group posttranslationally added to the N terminal glycine residue (Hunter & Cooper, 1985). *v-src*, the transforming gene of the Rous sarcoma virus, was the first oncogene to be identified and is derived from a cellular gene, *c-src*. The product of *v-src*, designated pp60$^{v\text{-}src}$, has been extensively characterised, however, its mode of action is still

unresolved (reviewed in Hunter & Cooper, 1985; Koegl & Courtneidge, 1991).

The protein-serine/threonine kinase-encoding oncogenes include *v-mil/raf* and *v-mos*. The products of these oncogenes are found in the cytosol rather than on membranes (Bunte *et al.*, 1983; Papkoff *et al.*, 1983).

1.8.8.2. Other transforming viruses

In contrast to the transforming retroviruses, the oncogenes of adenovirus (E1A and E1B), papillomavirus (E6 and E7), polyoma virus (large T and middle T antigens) and SV40 (large T antigen) do not show any extensive homology to known cellular proto-oncogenes. Investigation of the products of these oncogenes has revealed that none possess any protein kinase activity although phosphorylation does appear to play some role in T antigen-induced transformation. The middle T antigen of polyoma virus, which is partly located in the plasma membrane, forms a complex with pp60$^{c\text{-}src}$, which has protein-tyrosine kinase activity. It has been reported that middle T activates this kinase activity and it has been suggested that this forms the basis of its ability to transform cells (Bolen *et al.*, 1984; Courtneidge, 1985). In addition, studies suggest that phosphorylation of the large T antigen of SV40 modulates many of its activities including its association with p53 (Carroll & Gurney, 1982). This activity is important as large T appears to induce

transformation by binding to p53 and the retinoblastoma gene product (RB). These two proteins play an important role in suppressing the transformed phenotype and may also participate in regulation of the cell cycle. Adenovirus and papillomavirus also induce transformation by binding to p53 and RB (reviewed in Bishop, 1985; Monier, 1986; Green, 1989; Schlegel, 1990; Vousden, 1991). HSV-induced transformation is unusual in that no oncogenes have been identified (see section 1.4.).

1.8.9. Viral Phosphoproteins - Occurrence and Functions

Many viruses encode proteins which during productive infections become phosphorylated. These viruses include examples from all families of the animal viruses (see Leader & Katan, 1988). There have also been reports of a plant virus phosphoprotein (Hahn & Shepherd, 1980) and bacteriophage phosphoproteins (Robertson & Nicholson, 1990).

Within the herpesviruses, phosphoproteins have been reported in all three subfamilies. Over twelve phosphoproteins have been reported in the alphaherpesviruses, HSV type 1 and type 2 (Pereira *et al.*, 1977; Marsden *et al.*, 1978; Wilcox *et al.*, 1980). Some are present in virion preparations and may therefore play a structural role in the virus, for example, gD, UL36, UL47, UL49 and the product of UL48 (Vmw65), while others are non-structural, for example the IE *trans*-activating proteins Vmw110, Vmw175, Vmw63 and Vmw68

(Ackermann *et al.*, 1984), and the DNase (Banks *et al.*, 1985). Within the betaherpesvirus subfamily, HCMV virions are known to contain three main phosphoproteins including a 150 KDa major capsid protein (Roby & Gibson, 1986). A set of four early phosphoproteins, with common N termini, have also been reported in HCMV (Wright *et al.*, 1988). Within the recently isolated HHV-6 a phosphoprotein of MW 41 KDa has been identified (Chang & Balachandran, 1991). The phosphoproteins of EBV, a gammaherpesvirus, have not been as thoroughly investigated as the alphaherpesviruses, however certain early proteins have been shown to be phosphorylated (Epstein, 1984).

Despite the widespread occurrence of viral phosphoproteins, the relationship between their phosphorylation state and their function is largely unknown. Functions which have been assigned to certain phosphoproteins are described below.

1.8.9.1. Transcriptional activity

In vesicular stomatitis virus (VSV) a phosphoprotein (P) (previously known as NS) associates with the large protein (L) to form the RNA-dependent RNA polymerase. It has been shown that a complex of the L protein and a phosphate-free P protein is transcriptionally inactive but that transcriptional activity is restored upon phosphorylation of P protein, initially by a cellular kinase(s), and subsequently by an L-associated kinase. Thus, the

phosphorylation/dephosphorylation of P protein may represent a transcriptional control mechanism (Barik & Banerjee, 1992).

The large T antigen of SV40 plays an important role in both DNA replication and regulation of transcription. Transcriptional control is thought to depend upon the antigen's ability to bind DNA (DiMaio & Nathans, 1982) which, in turn, is modulated by the extent of phosphorylation of the polypeptide (Simmons *et al.*, 1986; Klausing *et al.*, 1988).

The E2 ORF of bovine papillomavirus type 1 (BPV-1) encodes three proteins with regulatory functions, the full length E2 gene product, which functions as a transcriptional transactivator, and two small proteins translated from the 3' end of the ORF, which inhibit the E2-mediated transactivation. All three of these proteins are phosphorylated at sites within the common C terminal domain (McBride *et al.*, 1989). It has been reported that mutagenesis of one of these phosphorylation sites results in an increase in transactivation, however it is not yet known if this effect can be attributed solely to the loss of phosphorylation at this site (McBride & Howley, 1991).

It has also been suggested that the phosphorylation of vaccinia virus core proteins may contribute to the regulation of early transcription, however, the evidence is primarily circumstantial (Moussatche & Keller, 1991).

1.8.9.2. Replication

The DNA-binding properties of the SV40 large T antigen are thought to regulate DNA replication, in addition to transcription. The specific binding of large T antigen to control sequences at the origin of replication has been reported (Tjian, 1978; Tegtmeyer *et al.*, 1983) and mutant viruses which lack some of the C terminal phosphorylation sites exhibit reduced DNA replication (Pipas *et al.*, 1983).

In BPV-1, replication requires only the products of the E1 and E2 ORFs, which form a complex (Ustav & Stenlund, 1991; Blitz & Laimins, 1991). It has recently been demonstrated that E1 only complexes with an underphosphorylated form of E2 (Lusky & Fontane, 1991), suggesting a role for phosphorylation in BPV-1 replication. This is supported by McBride & Howley (1991) who found that a mutation of the phosphorylation site within the C terminal domain of E2 resulted in an increase in the genome copy number.

1.8.9.3. Virion uncoating

Ratka *et al.* (1989) suggest that phosphorylation of poliovirus capsid proteins plays a role in uncoating of the virus. The phosphorylation is mediated by the presence of Zn^{2+} which activates one or more protein kinases and which also destabilizes the poliovirus capsid, thereby exposing the phosphorylation sites on the capsid proteins. Phosphorylation

has also been implicated in the uncoating of VSV (Witt *et al.*, 1981) and an insect granulosis virus (Wilson & Consigli, 1985).

1.8.9.4. Virion maturation

A prerequisite for virion assembly is the association of the viral nucleic acid with the structural proteins. In avian retroviruses the extent of RNA binding is decreased markedly when the phosphoprotein p12 is unphosphorylated (Sen & Todaro, 1977). Phosphorylation of the core protein of duck hepatitis B virus is also thought to play a role in virion maturation (Pugh *et al.*, 1989).

1.8.9.5. Nuclear transport

Nuclear transport of the SV40 large T antigen can be facilitated by phosphorylation of the serine residues which flank the nuclear localisation signal (Rihs & Peters, 1989). A similar role has also been suggested for the phosphorylation of the precore and core proteins of hepatitis B virus (Yeh & Ou, 1991).

1.8.10. Effect of Viral Infection on Cellular Protein Kinase Activity

Infection of cells with several different viruses activates the cellular dsRNA-dependent kinase which phosphorylates the

α subunit of the transcription factor eIF-2. The function of eIF-2 is to bind the initiator Met-tRNA$_F$ to the 40S ribosomal subunit, in a reaction in which GTP is hydrolysed and an eIF-2-GDP complex is formed. The GDP is removed from this complex by eIF-2B and active eIF-2 is released. Phosphorylation of eIF-2 by the dsRNA-dependent kinase interferes with the action of eIF-2B, thus the GDP is not displaced, the eIF-2 remains inactive and protein synthesis is inhibited. This system therefore represents an important antiviral mechanism. Indeed, during infection of cells with encephalomyocarditis virus (EMCV) the dsRNA-dependent kinase localises to the replication complexes of EMCV in an activated form (Dubois & Hovanessian, 1990), possibly representing a mechanism for the specific inhibition of viral protein synthesis.

Many viruses have developed mechanisms to nullify this protein synthesis shut-off system. For example, the adenovirus VA1 RNA and an influenza virus-encoded factor are thought to bind to the protein kinase (Gallabru *et al.*, 1989; Katze *et al.*, 1988), while proteins encoded by reovirus and vaccinia virus bind to dsRNA, the activator of the protein kinase (Imani & Jacobs, 1988; Akkaraju *et al.*, 1989). It is thought that HIV downregulates the expression of the protein kinase (Roy *et al.*, 1990) and poliovirus infection results in specific degradation of the kinase (Black *et al.*, 1989).

Following infection of cells with vaccinia virus, adenovirus,

PRV and HSV-1, phosphorylation of the ribosomal protein S6 is also stimulated (Kaerlein & Horak, 1976; Russell & Blair, 1977; Kennedy *et al.*, 1981). For the herpesviruses, it has been shown that this phosphorylation is not carried out by the virus-encoded US3 protein kinase (Katan *et al.*, 1986). Thus, it is considered likely that the increased phosphorylation of ribosomal protein S6 results from stimulation of a cellular kinase. The functional significance of this increased phosphorylation is not known.

1.8.11. Viral Protein Kinases

Protein kinases have been associated with the virions of many different viruses, including Rauscher murine leukaemia virus, avian myeloblastosis virus (Strand & August, 1971), frog virus 3 (Silberstein & August, 1976), VSV (Strand & August, 1971; Watanabe *et al.*, 1974; Imblum & Wagner, 1974), Sendai virus (Lamb, 1975; Einberger *et al.*, 1990), vaccinia virus (Kleiman & Moss, 1975; Watanabe *et al.*, 1974; Paoletti & Moss, 1972), adenovirus (Blair & Russell, 1978) and foot and mouth disease virus (Grubman, 1982). Protein kinases are also associated with many members of the herpesviruses, e.g. HSV (Rubenstein *et al.*, 1972; Lemaster & Roizman, 1980; Blue & Stobbs, 1981), PRV (Stevely *et al.*, 1985), VZV (Montalvo & Grose, 1986), EHV (Randall *et al.*, 1972), HCMV (Michelson *et al.*, 1984) and tupaia herpesvirus (Flügel & Darai, 1982). It is likely that many of these protein kinases are of cellular origin, perhaps arising from contamination during

purification procedures or acquired from the host cell membrane during budding. Indeed, Stevely *et al.* (1985) have shown that the herpesvirus virion contains enzymes which resemble cellular casein kinases I and II and protein kinase C.

Only a few protein kinases have been convincingly demonstrated to be the product of a viral gene. These are described below.

1.8.11.1. Herpesvirus protein kinases

A novel protein kinase purified from HSV-1 infected cells (Purves *et al.*, 1986a) was shown to be the product of the US3 gene (Frame *et al.*, 1987; Purves *et al.*, 1987) which possesses the sequence motifs characteristic of a protein kinase (McGeoch & Davison, 1986). This protein kinase gene has homologues in all sequenced alphaherpesviruses.

The UL13 gene of HSV-1, and its homologues in the three classes of herpesviruses, have also been identified as probable protein kinase genes, based on the presence of characteristic sequence motifs (Smith & Smith, 1989; Chee *et al.*, 1989). The work presented in this thesis confirms that the UL13 gene product has protein kinase activity.

It has recently been reported that the N terminal region of the large subunit of ribonucleotide reductase of HSV also

possesses protein kinase sequence motifs (Chung *et al.*, 1989; Paradis *et al.*, 1991).

These three protein kinase genes and their products are described fully in the next section (1.9.).

1.8.11.2. Vaccinia virus protein kinase

DNA sequence analysis revealed that vaccinia virus possesses two genes which contain conserved motifs characteristic of protein-serine/threonine kinases: B1R, which is essential for virus replication (Howard & Smith, 1989; Traktman *et al.*, 1989), and B12R (Howard & Smith, 1989). The B1R gene has sequences corresponding to all of the major conserved regions except for region VIII (according to the designation of Hanks *et al.*, 1988), where the conserved glutamate residue is replaced with an aspartate residue, and region IX where a glutamate residue substitutes for an aromatic residue (Howard & Smith, 1989). B1R had 27% amino acid identity with HSV US3 over an 85 amino acid region. Two groups have recently expressed the B1R gene product in bacteria. Lin *et al.* (1992) showed that the purified 34 KDa protein possesses protein-serine/threonine kinase activity, utilises ATP, but not GTP, as a phosphate donor, phosphorylates casein and histone H1 and is located in virions. The 34 KDa protein kinase purified by Rempel & Traktman (1992), however, possessed slightly different properties, in that it could utilise GTP as a phosphate donor, albeit inefficiently, and

did not phosphorylate histone H1. The physiological substrate(s) of the B1R kinase are not known. Mutational analysis of the B1R gene has indicated that the invariant Lys residue of subdomain II is absolutely required for kinase activity (Lin *et al.*, 1992) and that substitution of the conserved Gly residue in subdomain IX reduces activity to 3% of the *wt* level (Rempel & Traktman, 1992).

The second protein kinase-related gene of vaccinia virus, B12R, shows only weak homology to the conserved protein kinase sequences. There does not appear to be a glycine-rich nucleotide binding region (subdomain I) and invariant aspartate and glycine residues in subdomains VI and VII, respectively, are absent. Thus, it is considered unlikely that the product of this gene possesses protein kinase activity (Howard & Smith, 1989).

1.8.11.3. Bacteriophage T7 0.7 protein kinase

The 0.7 protein kinase of bacteriophage T7 has been purified from infected cells as a 37 KDa monomer (Pai *et al.*, 1975) and has recently been cloned and expressed (Michalewicz & Nicholson, 1992). The kinase activity is specific for serine and threonine residues and is cAMP-independent (Rahmsdorf *et al.*, 1974). Substrates of the kinase include the RNA processing enzyme RNase III and the β subunit of RNA polymerase (Robertson & Nicholson, 1990). The function of this protein kinase is unclear, however it is known that the

kinase activity is independent of the RNA polymerase-catalysed transcription shut-off which is also a function of the 0.7 gene product (Michalewicz & Nicholson, 1992).

The nucleotide sequence of the 0.7 gene has been determined (Dunn & Studier, 1983) and it is interesting to note that, with the exception of the nucleotide binding site of subdomain I, the 0.7 protein kinase does not exhibit any of the conserved sequence motifs seen in eukaryotic serine/threonine-specific protein kinases. In addition, the kinase does not exhibit strong homology with any other prokaryotic protein kinases. This gene therefore represents a novel class of protein kinase.

1.8.11.4. Hepatitis B virus protein kinase

The HBV genome contains four ORFs, three of which code for proteins which are essential for the viral life cycle, namely DNA polymerase-reverse transcriptase, hepatitis B surface antigen and hepatitis B core/e antigen. The fourth and smallest ORF, the hbx gene, is conserved among all mammalian hepadnaviruses and codes for a 17-KDa virion protein which is able to *trans*-activate transcription. The hbx gene has been expressed in *E. coli* and the product has been shown to possess an intrinsic serine/threonine protein kinase activity (Wu *et al.*, 1990). Thus, the hbx protein kinase may activate transcription by phosphorylating transcription factors. A recent study has indicated that the *hbx* gene is not essential

for the virus growth *in vitro*, but may be involved in the pathogenesis of the virus (Blum *et al.*, 1992).

Like the bacteriophage 0.7 protein kinase, the hbx kinase shows little homology to the sequence which characterise eukaryotic protein kinases. In addition, the protein contains only 154 amino acids, which is approximately half the size of the catalytic domain of most protein kinases. The hbx protein may therefore represent a novel protein kinase (Wu *et al.*, 1990).

1.9. HERPESVIRUS PROTEIN KINASES

1.9.1. US3

The HSV-1 US3 gene is rightward oriented with an ORF of 481 codons (residues 135222-136665), encoding a protein of MW 52831 (McGeoch *et al.*, 1985, 1988a). Mapping experiments have detected two distinct 5' termini for mRNA species, corresponding to residues 134964 to 135189. These mRNAs, designated US3a and US3b, respectively, have identical coding capacities. The 3' termini of both mRNAs correspond to residue 137508 which also represents the 3' terminus for the US4 mRNA; thus, the US3 and US4 genes form a 3' co-terminal family. The US2 gene, which lies to the left of US3 and is transcribed in the opposite direction, initiates at residue 135304 which lies 83 bp within the proposed ORF of US3 and the upstream control sequences for US3a may extend into the

US2 ORF (Rixon & McGeoch, 1985) (Figure 1.7). The US3 mRNAs are transcribed early in infection (4 h p.i.) and have declined in abundance by 7 h p.i. (Rixon & McGeoch, 1985).

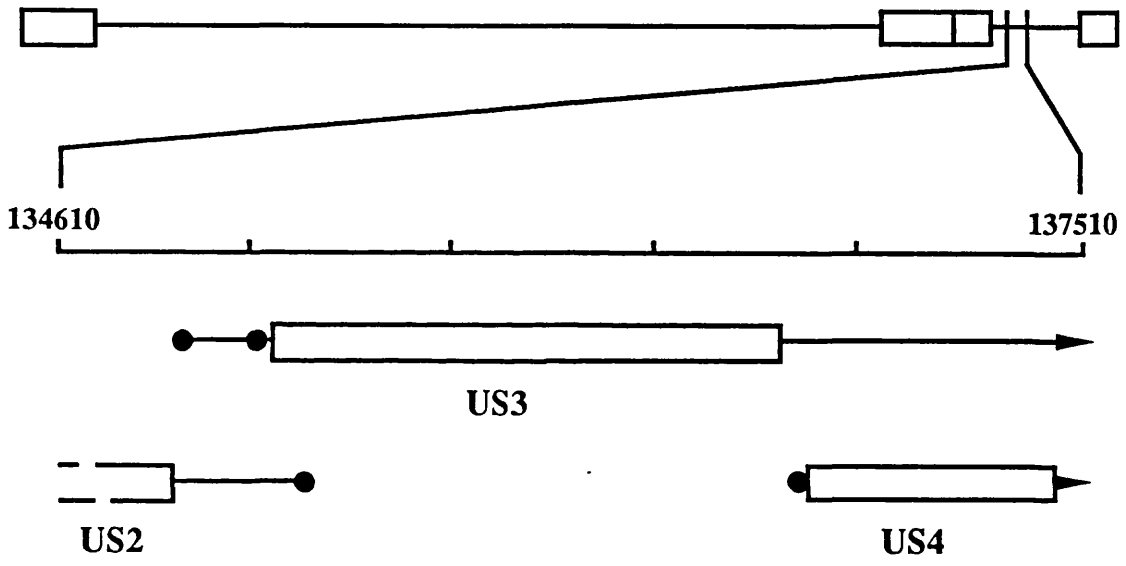
Sequence analysis of the US3 gene, and its homologues in HSV-2 and VZV, revealed homology to the protein kinase family, with greatest similarity (33%) to the yeast cell division control protein CDC28 (McGeoch & Davison, 1986).

Longnecker & Roizman (1987) described a mutant of HSV-1 strain F which contains a deletion in the US3 gene. This virus, which is TK$^-$, grows to similar titres to *wt* on Vero cells, indicating that the US3 gene is not essential for virus growth in tissue culture. *In vivo* growth studies showed that the US3$^-$ mutant (a TK$^+$ version) exhibits a significantly reduced virulence (LD_{50} = 1.8 x 10^6) compared to the *wt* (LD_{50} = 10^2). The virus was able to establish latency in the mouse eye model but with reduced efficiency (Meignier *et al.*, 1988).

A possible candidate for the US3 gene product was a novel protein kinase detected in cytoplasmic extracts of cells infected with PRV or HSV-1. The enzymatic characteristics of these alphaherpesvirus-induced kinases are indistinguishable from each other and distinct from cellular protein kinases such as protein kinase C. They utilise ATP, but not GTP, as a phosphate donor, they are active at unusually high salt concentrations (1M and above) and their activity is not

Figure 1.7 **Genomic location and organisation of the US3 gene.**

The upper line of the figure represents the HSV-1 genome with unique sequences as lines and major repeat elements as open boxes. The lower part expands the region of U_S (residues 134610 to 137510) which contains gene US3 and its neighbours, genes US2 and US4. The filled circles represent the 5' termini of the transcripts, arrowheads indicate the 3' termini of the transcripts and the open boxes indicate the locations of the predicted protein coding regions.


134610
137510
US3
US2
US4

stimulated by any known effectors. The kinases have been found to have a specificity for serine and threonine residues in basic proteins, such as protamine, but not on acidic proteins, such as casein or phosvitin. They are also capable of autophosphorylation. Both kinases are thought to exist as dimers but the subunit size differs for the two viruses; the PRV subunit has an apparent MW of 38K and the MW of the HSV-1 subunit is approximately 68K (Stevely *et al.*, 1985; Katan *et al.*, 1985; Purves *et al.*, 1986a; reviewed in Leader & Katan, 1988; Leader & Purves, 1988). Experiments by Katan *et al.* (1985) and Stevely *et al.* (1985) suggested that the alphaherpesvirus protein kinase was not a constituent of the virion. However, a more recent study has indicated that both the PRV and HSV-1 protein kinases can be immunoprecipitated from virion preparations (Zhang *et al.*, 1990).

Two lines of evidence confirmed that the novel HSV-1-induced protein kinase was the product of the US3 gene. Firstly, the kinase activity was not present in mock-infected cells or in cells infected with the US3 deletion mutant constructed by Longnecker & Roizman (1987). Kinase activity was restored when the US3 deletion was repaired (Purves *et al.*, 1987). Secondly, an antibody raised against the terminal octapeptide of the predicted US3 amino acid sequence was found to react with a protein of apparent MW 68K from an extensively purified preparation of the HSV-1 protein kinase (Frame *et al.*, 1987).

The substrate specificity of the alphaherpesvirus protein kinase has been investigated using synthetic peptides and the following consensus sequence has been defined: $R_nX(S/T)YY$, where n must be equal to or greater than 3, S/T represents the target residue, X can be R, A, V, P or S and Y can be R, A, V or S. Acidic residues and P are not tolerated at the Y position (Purves *et al.*, 1986b; Leader *et al.*, 1991; Purves *et al.*, 1991).

A physiological substrate for the HSV-1 US3 protein kinase has recently been identified. Purves *et al.* (1991) reported that in cells infected with a US3 deletion virus, a 30K phosphoprotein, which was mapped to the UL34 gene, is replaced by a higher molecular weight phosphoprotein of 33K, which was presumed to be related to UL34. Analysis of the UL34 amino acid sequence showed that the protein contains the US3 protein kinase phosphorylation consensus sequence: -RRRRTRR- (residues 191 to 197), although this is not conserved in the VZV UL34 homologue (gene 24) nor in the EHV-1 homologue (gene 26). Purves *et al.* (1991) therefore proposed that the US3 protein kinase mediates the posttranslational modification of the UL34 gene product, converting it from the 30K protein to the 33K species.

Subsequent studies (Purves *et al.*, 1992) have shown that the 30K UL34 protein is still present in cells infected with the US3$^-$ virus, but in an unphosphorylated form. These studies also indicated that the previously observed 33K phosphoprotein was one of four novel phosphoproteins with MWs

25K-35K (designated A to D) present during infection with the US3$^-$ virus. These phosphoproteins are not genetically related to the UL34 protein but do appear to associate with the unphosphorylated form of UL34 in infected cells. The UL34 protein is a constituent of virions (Marsden *et al.*, 1978; Purves *et al.*, 1992) and Purves *et al.*, 1991 have suggested that it is membrane associated, since the N terminal domain has properties similar to those of signal sequences and the C terminus is very hydrophobic (Purves *et al.*, 1991). Indeed Purves *et al.* (1992) have reported that during infection both the phosphorylated and unphosphorylated forms of the UL34 protein localise to cellular membranes. It has therefore been proposed that the UL34 protein regulates the function of infected cell kinases which associate with cellular membranes and that this regulation is dependent upon the phosphorylation state of the protein (Purves *et al.*, 1992). It is thought that the UL34 protein is essential for viral growth, although its phosphorylation by the US3 protein kinase is not (Purves *et al.*, 1991).

Homologues of the HSV-1 US3 gene have been found throughout the alphaherpesviruses: gene US3 of HSV-2, gene 66 of VZV (McGeoch & Davison, 1986), PK gene of PRV (Zhang *et al.*, 1990; van Zijl *et al.*, 1990) and gene 69 or EUS2 of EHV-1 (Telford *et al.*, 1992; Colle *et al.*, 1992). A homologue has also been detected in MDV (Ross & Binns, 1991) which, although classified as a gammaherpesvirus, is now thought to be of alphaherpesvirus lineage (Buckmaster *et al.*, 1988). An

alignment of the sequences of the catalytic domains of the six alphaherpesvirus protein kinases has been made using the PILEUP and PRETTY programs (University of Wisconsin, Genetics Computer Group) and the conserved residues compared with the motifs of Hanks *et al.* (1988) (Figure 1.8). Most of the invariant residues identified by Hanks *et al.* (1988) are conserved among the alphaherpesvirus protein kinases with the exception of the first G residue and the V residue of subdomain I, and the conserved aromatic residue (a) in the D-a-G triplet of subdomain VII. This sequence analysis has also revealed conserved residues which appear to be characteristic of this group of protein kinases, for example: a conserved E residue in subdomain I; conserved V three residues N terminal to the K of subdomain II; conserved C, P and D-L residues in subdomain V; a characteristic E-N-I-F motif in subdomain VI; two conserved A residues C terminal to the D-a-G triplet of subdomain VII; conserved V, F and E residues in subdomain XI; and an R-P-S-A motif in subdomain XI. In addition, the distance between subdomains II and III is characteristically short and all six protein kinases have an insertion between subdomains X and XI.

In contrast to the catalytic domains, the N terminal domains of these six protein kinases bear little similarity to each other. They vary considerably in size, for example, the N terminal domains of HSV-1 and HSV-2 US3 are 200 amino acids long, those of VZV, EHV-1 and MDV are approximately 100 residues long and the N terminal domain of PRV is only 55

Figure 1.8 **Sequence alignment of the catalytic domains of the US3 gene and its homologues from other alphaherpesviruses.**

The catalytic domains of HSV-1 US3, HSV-2 US3, EHV-1 gene 69, PRV PK gene, VZV gene 66 and the MDV PK gene were aligned using the PILEUP program (Genetics Computing Group (GCG), University of Wisconsin) and a consensus sequence generated using the PRETTY program (GCG, University of Wisconsin). On the consensus line the upper case letters represent conserved amino acid residues, lower case letters indicate amino acids with similar properties where:

o = non-polar, i.e. M, L, I, V, C

a = aromatic, i.e. F, Y, W

b = neutral/small, i.e. A, G, S, T, P

c = basic/polar, i.e. K, R, H

The conserved sequences of the subdomains (I to XI) identified by Hanks *et al.* (1988) are shown above the aligned sequences.

```
                        I                          II          III
                    oG-G--G-V                   aoK-o          E
HSV1   191 FTIHGALTPGSEGCVFDSSH..PDYPQRVIVKAGWYTSTS.HEARLLRRL
HSV2   191 FAIHGALIPGSEGCVFDSSH..PNYPHRVIVKAGWYASTS.HEARLLRRL
EHV1    93 YTVIKTLTPGSEGRVMVATK..DGQPEPVVLKIGQKGTTL.IEAMMLRNV
PRV     53 FEVLQPLQSGSEGRVFVARR..PGEADTVVLKVGQKPSTL.MEGMLLQRL
VZV     93 FVILKTFTPGAEGFAFACMD..SKTCEHVVIKAGQRQGTA.TEATVLRAL
MDV    102 YNIVSSLSPGSEGYIYVCTKRGDNTKRKVIVKAVTGDKTLGSEIDILKKM
Consensus a-o-----bGbEG---------------VooK------T---E---L--o

               IV                    V
HSV1   238 DHPAILPLLDLHVVSGVTCLVLPKYQADLYTYLSRRLNPLGRPQIAAVSR
HSV2   238 NHPAILPLLDLHVVSGVTCLVLPKYHCDLYTYLSKRPSPLGHLQITAVSR
EHV1   140 NHPSVIQMKDTLVSGAITCMVLPHYSSDLYTFLTKESRRIPIDQALIIEK
PRV    100 SHDNVMRMKQMLARGPATCLVLPHFRCDLYSYLTMRDGPLDMRDAGCVIR
VZV    140 THPSVVQLKGTFTYNKMTCLILPRYRTDLYCYLAAKRN.LPICDILAIQR
MDV    152 SHRSIIRLVHAYRWKSTVCMVMPKYKCDLFTYIDI.MGPLPLNQIITIER
Consensus -H--oo-o----------CoooPca--DLa-ao------o-------o-c

                              VI                 VII
                           DoK--No             o-DaG
HSV1   288 QLLSAVDYIHRQGIIHRDIKTENIFINTPEDICLGDFGAACFVQGSRSSP
HSV2   288 QLLSAIDYVHCKGIIHRDIKTENIFINTPENICLGDFGAACFVRGCRSSP
EHV1   190 QILEGLRYLHAQRIIHRDVKTENIFINSVDQVCIADFGAA...QFPVVEP
PRV    150 AVLRGLAYLHGMRIMHRDVKAENIFLEDVDTVCLGDLGAA...RCNVAAP
VZV    189 SVLRALQYLHNNSIIHRDIKSENIFINHPGDVCVGDFGAACF.PVDINAN
MDV    201 GLLGALAYIHEKGIIHRDVKTENIFLDKPENVVLGDFGAACKLDEHTDKP
Consensus -oL-bo-YoH---IoHRDoKbENIF------ooobD-GAA----------

                     VIII                  IX
                   b--a-bPE              D-ab-G
HSV1   338 FPYGIAGTIDTNAPEVLAGDPYTTTVDIWSAGLVIFETAVHNASLFSAPR
HSV2   338 FHYGIAGTIDTNAPEVLAGDPYTQVIDIWSAGLVIFETAVHTASLFSAPR
EHV1   237 ADLGLAGTVETNAPEVLARAKYNSKADIWSAGIVLFEMLAYPSTLFEDPP
PRV    197 NFYGLAGTIETNAPEVLARDRYDTKVDVWGAGVVLFETLAYPKTI.....
VZV    238 RYYGWAGTIATNSPELLARDPYGPAVDIWSAGIVLFEMATGQNSLFE...
MDV    251 KCYGWSGTLETNSPELLALDPYCTKTDIWSAGLVLFEMSVKHITFFGKQV
Consensus ---G-bGTo-TNbPEoLA---Y----DoWbAGoVoFE------------

                              X
HSV1   388 GPKRGP...CDSQITRIIRQAQVHVDEFSPHPESRLTSRYRSRAAGNNRP
HSV2   388 DPERRP...CDNQIARIIRQAQVHVDEFPTHAESRLTAHYRSRAAGNNRP
EHV1   287 STPEEYVKSCHSQLLKIISTLKINPEEFPRDPGSRLVRGY.IEYSRLERK
PRV    242 TGGDEPAINGEMHLIDLIRALGVHPEEFP..PDTRLRSEF.VRYAGTHRQ
VZV    285 RDGLDGNCDSERQIKLIIRRSGTHPNEFPINPTSNLRRQYIGLAKRSSRK
MDV    301 NG.......SGSQLRSIIRCLQVHPLEFPQNNSTNLCKHFK.QYAIQLRH
Consensus ------------o--oI--------EFb----b-L---a--------R-

                                    XI
                                 o----------R
HSV1   435 PYTRPAWTRYYKMDIDVEYLVCKALTFDGALRPSAAELLCLPLFQQK..
HSV2   435 AWTRPAWTRYYKIHTDVEYLICKALTFDAALRPSAAELLRLPLFHPK..
EHV1   336 PYTRYPCFQRVNLHIDGEFLVHKMLAFNAAMRPSAEELLSYPMFAQL..
PRV    289 PYTQYARVARLGLPETGAFLIYKMLTFDPVRRPSADEILNFGMWTV*..
VZV    335 PGSRPLWTNLYELPIDLEYLICKMLSFDARHRPSAEVLLNHSVFQTLPD
MDV    343 PYAIPQIIRKSGMTMDLEYAIAKMLTFDQEFRPSAQDILMLPLFTKEPA
Consensus b-b---------o-----a-o-K-LbFd---RPSA--oL--boa-----
```

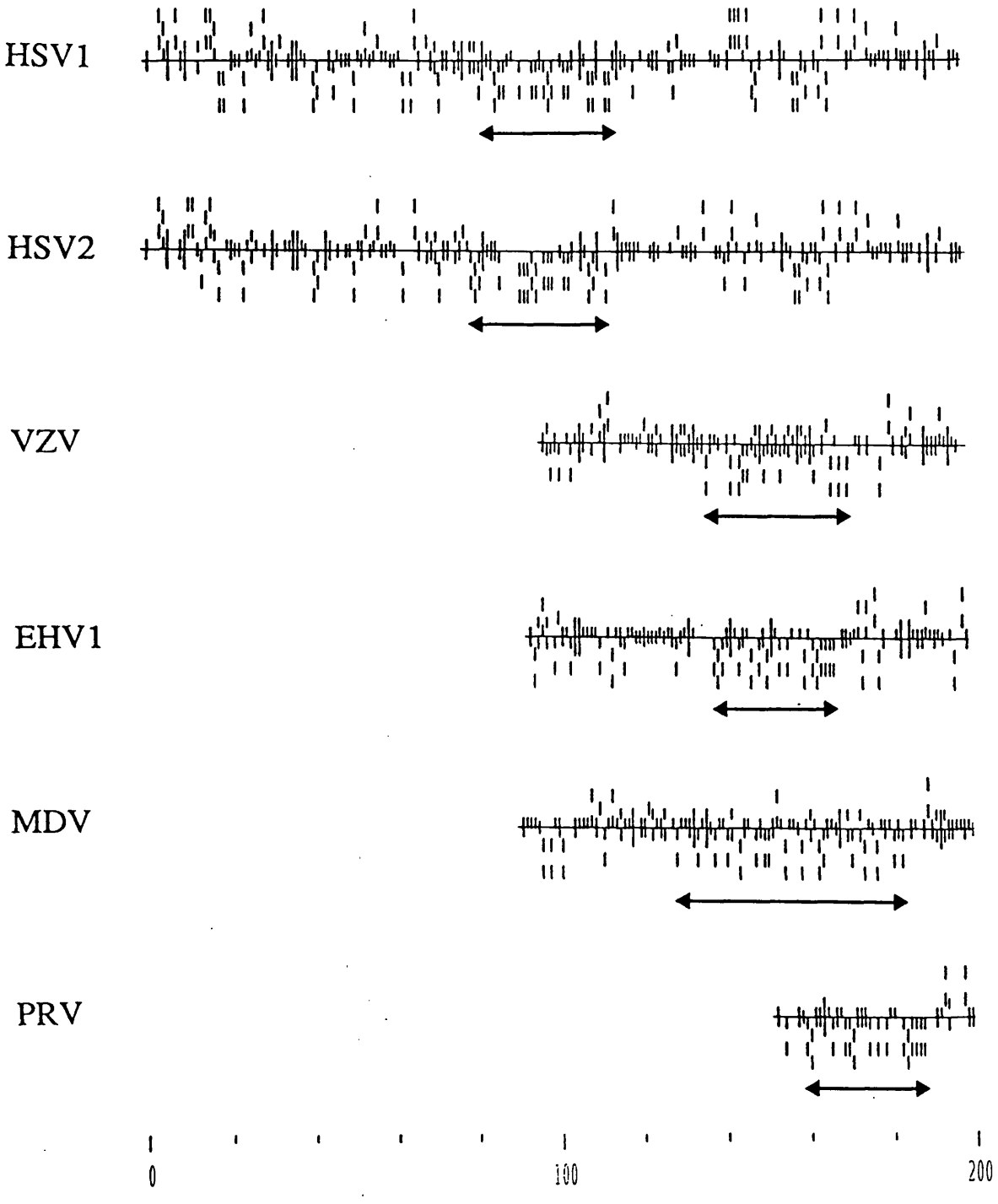
residues long. The only common feature to be detected in these N terminal domains is a cluster of acidic residues. The protein kinases can be placed into three groups according to the length of these acidic domains and their position relative to the catalytic domain (Figure 1.9). The first group consists of the HSV US3 protein kinases which have an acidic domain of between 22 and 29 residues and are situated approximately 86 residues from the first glycine of subdomain I. PRV and EHV-1 form the seond group, with acidic domains of approximately 30 residues positioned only 25 residues N terminal to subdomain I. The third group, consisting of MDV and VZV, have longer acidic clusters of 55 and 43 residues, respectively, and are situated only 17 to 18 residues from subdomain I. Three of the protein kinases (HSV-1, HSV-2 and EHV-1) also possess a proline-rich domain. In HSV-1 and HSV-2 this domain is located on the C terminal side of the acidic cluster while in EHV-1 it is located on the N terminal side. The functional significance of these N terminal subdomains is not known, however, Zhang *et al.* (1990) have proposed that the acidic clusters are involved in binding to the protein's substrate or to the catalytic domain, thereby regulating the enzyme's activity.

1.9.2. UL13

The UL13 gene of HSV-1 is leftward oriented with an ORF of 518 codons (residues 28504 to 26950), encoding a protein of MW 57193 (McGeoch *et al.*, 1986b, 1988a). Genes UL11 to UL14

Figure 1.9 Distribution of charged amino acid residues in the N-terminal domain of the US3 protein kinase and its homologues.

The amino acid sequences of HSV-1 US3 and its alphaherpesvirus homologues were analysed using the PEPPLOT program (GCG, University of Wisconsin) and aligned over their catalytic domains. Only the sequences N terminal to the start of the catalytic domains are shown.
Long dashed lines above the base-line represent basic residues whi
long dashed below the base-line represent acidic residues. Long an
short dashed lines spanning the base-line represent amides and alchols, respectively, and long and short unbroken lines spanning the base-line represent aromatic residues and aliphatic residues, respectively. The short lines on the base-line represent prolines. Alanine, glycine and cysteine are unmarked.
Clusters of acidic residues are marked with arrowed lines.


HSV1
HSV2
VZV
EHV1
MDV
PRV
0
100
200

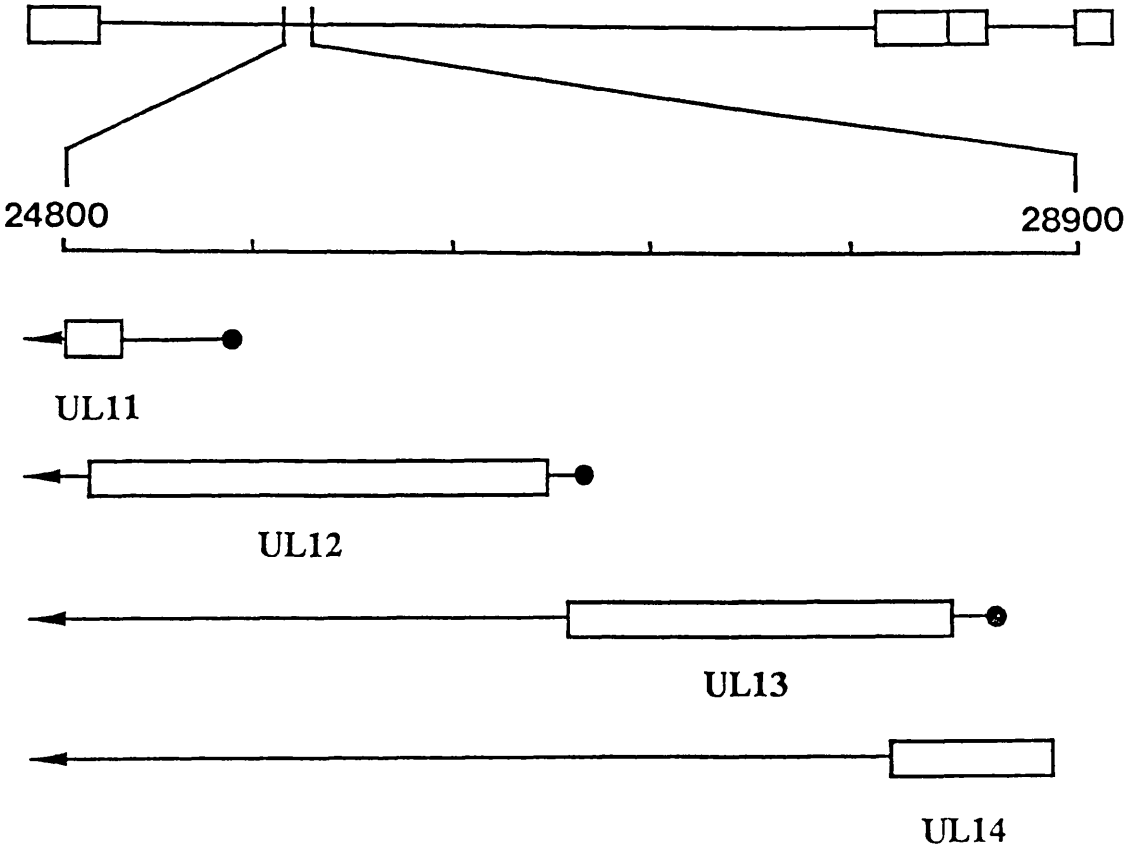
form a 3' co-terminal set of genes with the common 3' terminus of the transcripts corresponding to the AATAAA sequence at residues 24802-24807 (Costa *et al.*, 1983). The 5' terminus of the UL13 transcript has been mapped to residue 28693 (Costa *et al.*, 1983). The 3.9 kb UL13 transcript was found to be expressed late in infection and was classified as a βγ (intermediate) transcript (Costa *et al.*, 1983).

The UL13 gene overlaps extensively with its neighbours (Figure 1.10). The UL13 ORF is separated from the ORF of the downstream gene, UL12, by only 58 bp and the 5' terminus of the UL12 transcript (residue 27048) and its promoter lie within the UL13 coding region. The UL13 ORF is proposed to have an out of frame overlap of 83 codons with the coding region of its right neighbour, UL14. Thus, the 5' terminus of the UL13 transcript (residue 28693) lies within the UL14 ORF. The coding overlap does not extend into the proposed catalytic domain.

The DNA sequence of the HSV-1 (17+) UL13 gene was published by McGeoch *et al.* (1986b). Also in 1986, Draper *et al.* published sequences corresponding to the 3' terminal 101 codons of the HSV-1 (KOS) UL13 gene and its HSV-2 homologue. Homologues for UL13 have been found in all completely sequenced herpesvirus genomes (VZV gene 47, EBV BGLF4, HCMV UL97 and EHV-1 gene 49) (Smith & Smith, 1989; Chee *et al.*, 1989; Telford *et al.*, 1992) and also in HHV-6 (Chee *et al.*, 1989; Lawrence *et al.*, 1990). The gene is therefore present

Figure 1.10 **Genomic location and organisation of the UL13 gene.**

The upper line of the figure represents the HSV-1 genome with unique sequences as lines and major repeat elements as open boxes. The lower part expands the region of U_L which contains gene UL13 and its neighbours, genes UL11, UL12 and UL14 (residues 26500 to 28900). Filled circles represent the 5' termini of the transcripts while arrowheads represent the transcript stop sites. The open boxes indicate the locations of the predicted protein coding regions.


24800
28900
UL11
UL12
UL13
UL14

in all three classes of the herpesviruses.

In 1989 Smith & Smith and Chee *et al.* showed that the UL13 gene and its counterparts in other herpesviruses contained sequence motifs characteristic of the serine/threonine protein kinase family. The six sequences have been aligned using the PILEUP and PRETTY programs (University of Wisconsin, Genetics Computing Group) (Figure 1.11). The three alphaherpesvirus sequences (VZV, EHV and HSV-1) show strong homology to each other and also to the conserved subdomains identified by Hanks. The beta- and gamma-herpesviruses, however, are considerably diverged and lack certain conserved residues, which led Chee *et al.* (1989) to suggest that these proteins may represent some class of phosphotransferase other than protein kinase. All six sequences contain the glycine-rich nucleotide binding domain (subdomain I) including the first G residue and the conserved V which were absent from the US3 protein kinase family (section 1.9.1.). The conserved K of subdomain II and the E residue of subdomain III are also present, however, it should be noted that the distance between subdomains II and III in the HCMV UL97 and HHV-6 15R proteins is considerably longer than in the other UL13 homologues. Subdomain VI is well conserved in the alphaherpesvirus sequences but the invariant K, which is a determinant of serine/threonine specificity, is absent from the beta- and gamma-herpesvirus sequences. Subdomain VIII is poorly conserved with only the VZV and HSV-1 sequences possessing the invariant P-E residues. In

Figure 1.11 Sequence analysis of the catalytic domains of the UL13 gene and its homologues.

The catalytic domains of HSV-1 UL13, VZV gene 47, EHV-1 gene 49, HCMV UL97, HHV-6 15R and EBV BGLF4 were aligned using the PILEUP program (Genetics Computing Group (GCG), University of Wisconsin) and a consensus sequence generated using the PRETTY program (GCG, University of Wisconsin). On the consensus line the upper case letters represent conserved amino acid residues, lower case letters indicate amino acids with similar properties where:

o = non-polar, i.e. M, L, I, V, C
a = aromatic, i.e. F, Y, W
b = neutral/small, i.e. A, G, S, T, P
c = basic/polar, i.e. K, R, H
d = acidic/uncharged, i.e. D, E, N, Q

The conserved sequences of the subdomains (I to XI) identified by Hanks *et al.* (1988) are shown above the aligned sequences.

```
                          I              II
                       oG-G--b-V       aoK-o
VZV     129 LQIRDRPRFAGRGTYGRVHIYPSSKIAVKTMDSR.........FNRELV
EHV1    220 VALGEIPKFGGAGSYGEVQIFKQTGLAIKTASSR.........SCFEHEL
HSV1    148 LEIPGARSFGGSGGYGDVQLIREHKLAVKTIKEK.........EWFAVEL
HCMV    328 VDMSDESYRLGQGSFGEVWPLDR.YRVVKVARKH....SETVLTVWMSGL
HHV6    192 QPEKTMARVLGVGAYGKVFDLD..KVAIKTANEDESVISAFIAGVIRAKS
EBV     101 HLPITCEYLLGRGSYGAVYAHAD.NATVKLYDSV...TELYHELMVCDMI
Consensus ----------G-G--G-V---------oK--------------------

                  III
                   E
VZV     169 INAILASEGSIRAGERLGISSIVCLLGFSLQTKQLLFPAYDMDMDEYIVR
EHV1    261 AVSLLTGEDSLRAQASLGIGGIICLMAFSLPSKQMVFPAYDADLNAYGYR
HSV1    189 IATLLVGECVLRAGRTHNIRGFIAPLGFSLQQRQIVFPAYDMDLGKYIGQ
HCMV    373 IRTRAAGEQQQPPSLVGTGVHRGLLTATGCCLLHNVTVHRRFHTDMFHHD
HHV6    240 GADLLSHECVIN.........NLLISNSVCMSHKVSLSRTYDIDLHKFE
EBV     147 QIGKATAEDGQDKALVDY.........LSACTSCHALFMPQFRCSLQDYG
Consensus -------E----------------------------------------

                                                       VI
                                                    DoK--No
VZV     219 LSRRLTIPDHIDRKIAHVFLDLAQALTFLNRTCGLTHLDVKCGNIFLNVF
EHV1    311 LSRSGPPSVLVTESIERAFIGLGRALVYLNTSCGLTHLDVKGGNIFVNHF
HSV1    239 LASLRTTNPSVSTALHQCFTELARAVVFLNTTCGISHLDIKCANILVMLR
HCMV    423 QW.....KLACIDSYRRAFCTLADAIKFLNHQCRVCHFDITPMNVLIDVN
HHV6    280 DW.....DVRNVMNYYSVFCKLADAVRFLNLKCRINHFDISPMNIFLN..
EBV     188 HW.....HDGSIEPLVRGFQGLKDAVYFLNRHCGLFHSDISPSNILVDF.
Consensus ------------------F--L--Ao-aLN--C-o-H-Do---No-----

                      VII
                    o-DaG
VZV     269 NFASLEITTAVIGDYSLVTLNTYSLCTRAIFEVGNPS.HPEHVLRVPRDA
EHV1    361 HFV...ISDCVIGDLSLMTLNTNSMAMRAEFEIDTGE.EEIKTLRLPRSA
HSV1    289 S.DAVSLRRAVLADFSLVTLNSNSTIARGQFCLQEPDLKSPRMFGMPTAL
HCMV    468 PHNPSEIVRAALCDYSLSEPY.......PDYNERCVAVFQETGTARRIPN
HHV6    323 .HKKEIIFDAVLADYSLSEMH.......PNYNGTCAIAKEYDKNLQLVPI
EBV     232 TDTMWGMGRLVLTDYGTASLH.......DRNKMLDVRLKSSKGRQLYRLY
Consensus -----------o-D-b-----------------------------------

                      VIII
                    b--a-bPE
VZV     318 SQMSFRLVLSHGTNQPPEILLDYINGTGLTKYTGTLPQRVGLA......I
EHV1    407 SQMTFSFVIGHGLNQPISVIADFINNSGLAKSTGPIKHDVGLT......I
HSV1    338 TTANFHTLVGHGYNQPPELLVKYLNNERAEFTNHRLKHDVGLA......V
HCMV    511 CSHRLRECYHPAFRPMPL........QKLLICDPHARFPVAGLR.RYCMS
HHV6    365 SRNKFCDMFNPGFRPLVA........NAMILVNVCGAFDGENNPLRHCNL
EBV     275 CQREPFSIAKDTYKPLCLLSKCYILRGAGHIPDPSACGPVGAQTAL..RL
Consensus ----------------------------------------------------

                    IX
                  D-ab-G
VZV     362 DLYALGQALLEVILLGRLPGQLPISVHRTPHYHYYGHKLSPDLALDTLAY
EHV1    451 DLYALGQALLELLLVGCISPCLSVPILRTATYYYYSNKLSVDYALDLLAY
HSV1    382 DLYALGQTLLELVVSVYVAPSLGVPVTRFPGYQYFNNQLSPDFALALLAY
HCMV    552 ELSALGNVLGFCLM..RLLD...RRGLDEVRMGT..E..ALLFKHAGAAC
HHV6    407 DLCAFAQVVLSCVL..RMTD...KRGCREAQLYY..E..KRLFALANEAC
EBV     323 DLQSLGYSLLYGIM..HLADSTHKIPYPNPDMGF..DRSDPLYFLQFAAP
Consensus dL-b-b-----------------------------------------A-

                                                       XI
VZV     412 RCVLAPYILP.SDIPGDLNYNPFIHAGELNTRISRNSLRRIFQCHAVRYG
EHV1    501 RCSLYPALFP.TTPLTTIYGIPWDQVEGVFESIAGAHHREAFRAHLERYR
HSV1    432 RCVLHPALFV.NSAETNTHGLAYDVPEGIRRHLRNPKIRRAFTDRCINYQ
HCMV    593 RALENGKLTHCSDACLLILAAQMSYGACLLGEHGAALVSHTLRFVEAKMS
HHV6    448 RLNPLKYPFAYRDACCKVLAEHVVLLGLLFYRDVVEIYEKLYDFLDERGE
EBV     369 KVVLLEVLSQMWN.....LNLDMGLTSCGESPCVDVTAEHMSQFLQ.WCR
Consensus ----------------------------------------------------
```

subdomain IX the invariant D and G residues are conserved except in HCMV and HHV-6. In addition to these conserved subdomains the alphaherpesviruses also possess a conserved F-P-A-Y-D-X-D motif between subdomains III and VI. The presence of these conserved subdomains indicates that the UL13 gene and its homologues are members of the protein kinase family, however, the proteins are not closely similar to any known cellular protein kinase and also differ considerably from the US3 protein kinase family.

Like the US3 protein kinases, the UL13 homologues vary considerably in their N terminal domains. The size of the domains vary from approximately 85 residues in the BGLF4 protein of EBV, to approximately 338 residues, in the HCMV UL97 protein. There do not appear to be any conserved features among these domains.

The product of the UL13 gene has been visualised as a 50K protein following *in vitro* translation of the UL13 transcripts (Costa *et al.*, 1983). Costa *et al.* (1983) tentatively identified the 50K protein as the capsid protein VP19C. However, they had apparently confused VP19C with the phosphoprotein VP18.8 which Lemaster & Roizman (1980) had mapped to this region of the genome (see Braun *et al.*, 1984a). VP19C is now known to be the product of gene UL38 (Rixon *et al.*, 1990). More recent experiments by Cunningham *et al.*, 1992) have shown that the UL13 gene product is a heavily phosphorylated 57K virion structural protein. The

protein kinase responsible for this strong phosphorylation is distinct from known cellular protein kinases and also from the US3 protein kinase. It is present in the nuclei of HSV-1-infected cells, it phosphorylates exogenous casein but not protamine, it can use both ATP and GTP as a phosphate donor and, like the US3 kinase, it is stimulated by high salt concentrations. On the basis of the reports by Smith & Smith (1989) and Chee *et al.* (1989), Cunningham *et al.* (1992) have suggested that this novel protein kinase is, in fact, encoded by the UL13 gene and that the observed phosphorylation of the UL13 gene product represents an autophosphorylation reaction.

The fact that the UL13 protein kinase gene is conserved throughout all the herpesviruses suggests that it plays an important role in the viral cycle. Thus, the UL13 gene has been investigated with respect to its role in antiviral production and also antiviral resistance. For example, Brandt *et al.* (1991) showed that treatment of HSV-1-infected murine corneas with an oligonucleotide antisense to the UL13 gene, spanning the region encoding the F-P-A-Y-D-X-D motif (between subdomains III and VI of the catalytic domain), significantly reduced viral titres. However, viral titres were not reduced in other infected tissues and it is not known if the oligonucleotide specifically binds to the viral DNA at the UL13 locus. With regard to antiviral resistance, it was found that an HCMV virus containing a mutation in UL97 was resistant to treatment with the antiviral compound ganciclovir (Coen, 1991). It has since been reported that

the product of UL97 is directly responsible for the phosphorylation, and thus the activation, of ganciclovir (Littler *et al.*, 1992; Sullivan *et al.*, 1992).

1.9.3. UL39

The rightward oriented UL39 gene consists of 1137 codons (residues 86444-89855) and encodes a protein of MW 124,043. The 5' and 3' termini of the UL39 transcript have been mapped to residues 86217 and 90983, respectively (McLauchlan & Clements, 1983). Residue 90983 also corresponds to the 3' terminal of the UL40 transcript. The coding regions of UL39 and UL40 do not overlap.

The UL39 and UL40 genes code for the two subunits of the viral nucleotide reductase (RR), a key enzyme in DNA metabolism (see section 1.7.4.(c)); the UL39 gene encodes the 136 KDa large subunit (R1) and the UL40 gene encodes the 38K small subunit (R2). Comparison of the HSV-1 and HSV-2 R1 sequences with their homologues in other herpesviruses revealed that the HSV R1 proteins possess an additional N terminal domain of approximately 360 amino acids (Nikas *et al.*, 1986; Swain & Galloway, 1986). This domain shows only 38% intertypic homology (Nikas *et al.*, 1986), and is not required for RR activity (Ingemarson & Lankinen, 1987; Conner *et al.*, 1992b). It has therefore been suggested that this domain possesses a distinct function. This proposal correlates with the fact that fragments spanning this N

terminal domain have been implicated in the transformation of cultured cells (Jariwalla *et al.*, 1980, 1986; Jones *et al.*, 1986; Ali *et al.*, 1991), and although RR activity is a delayed-early function, the R1 promoter includes elements associated with immediate-early gene expression (Wymer *et al.*, 1989), which Chung *et al.* (1991) suggest may be associated with regulation of a distinct function.

In 1989, Chung *et al.* reported the presence of protein kinase-specific motifs within the N terminal domain of both HSV-1 and HSV-2 R1. The two R1 N terminal sequences have been aligned using the PILEUP and PRETTY programs (University of Wisconsin, Genetics Computing Group) and the location of the motifs marked (Figure 1.12). The proposed catalytic domain varies considerably from those of the known cellular protein kinases; only eight (I-VIII) of the eleven motifs identified by Hanks (1988) are present and the first G residue in subdomain I and the invariant K residue of subdomain II, reported to be essential for kinase activity, are not conserved in the HSV-1 sequence. It is therefore surprising that both proteins have been reported to auto- and trans-phosphorylate with a specificity for serine/threonine residues (Chung *et al.*, 1989; Paradis *et al.*, 1991), and that subdomains I and II have been shown to be dispensable for the protein kinase activity of the HSV-2 R1 (Luo & Aurelian, 1992). It should be noted that a recent study has shown that the activity of the *BCR* protein kinase is unaffected by the substitution of an R for the K residue of subdomain II (Maru

Figure 1.12 Sequence analysis of the catalytic domains of the HSV-1 and HSV-2 UL39 sequences.

The catalytic domains of the HSV-1 and HSV-2 UL39 genes were aligned using the PILEUP program (GCG, University of Wisconsin) and a consensus sequence generated using the PRETTY program (GCG, University of Wisconsin). The conserved sequences of the subdomains (I to VIII) identified by Hank *et al.* (1988) are shown above the aligned sequences.

```
                                 I
                              G-G--G-V
HSV1   100 SPAPFVAVTNIGAGSDGGTAVVAFGGTPRRSAGTSTGTQ.....
HSV2    91 STGAFVAISNVAAGGDGRTAVVALGGTSGPSATTSVGTQTSGEFLHGNPR
Consensus S---FVA--N--AG-DG-TAVVA-GGT---SA-TS-GTQ----------

                                                      II
                                                    aoK-o
HSV1   139 TADVPTEALGGPPPPPRFTLGGGCCSCRDTRRRSAVFGGEGDPVGPAEFV
HSV2   141 TPEPQGPQAVPPPPPPPFPWGHECCARRDAR......GGAEKDVGAAESW
Consensus T----------PPPPP-F--G--CC--RD-R------GG----VG-AE--

                                      III
                                       E
HSV1   189 SDDRSSDSDSDDSEDTD......SETLSHASSDVSGGATYDDALDSDSSS
HSV2   185 SDGPSSDSETEDSDSSDEDTGSGSETLSRSSSIWAAGATDDDDSDSDSRS
Consensus SD--SSDS---DS---D------SETLS--SS----GAT-DD--DSDS-S

                     IV
HSV1   233 DDSLQIDGPVCRPWSNDTAPL.....DVCPGTPGPGADAGGPSAVDP
HSV2   235 DDSVQPDVVVRRRWSDGPAPVAFPKPRRPGDSPGNPGLGAGTGPGSATDP
Consensus DDS-Q-D--V-R-WS---AP----------PG-PG-GA--G--SA-DP

                                                        VI
                                                     DoK--No
HSV1   275 HAPTPEAGAGLAADPAVARDDAEGLSDPRPRLGTGTAYPVPLELTPENAE
HSV2   285 RAS...ADSDSAAHAAAPQADVAPVLDSQPTVGTDPGYPVPLELTPENAE
Consensus -A----A----AA--A----D-----D--P--GT---YPVPLELTPENAE

                                                       VII
                                                     o-DaG
HSV1   325 AVARFLGDAVNREPALMLEYFCRCAREETKRVPPRTFGSPPRLTEDDFGL
HSV2   332 AVARFLGDAVDREPALMLEYFCRCAREESKRVPPRTFGSAPRLTEDDFGL
Consensus AVARFLGDAV-REPALMLEYFCRCAREE-KRVPPRTFGS-PRLTEDDFGL

                                    VIII
                                    a-bPE
HSV1   375 LNYALVEMQRLCLDVPPVPPNAYMPYYLREYVTRLVNGFKPLVSRSARLY
HSV2   382 LNYALAEMRRLCLDLPPVPPNAYTPYHLREYATRLVNGFKPLVRRSARLY
Consensus LNYAL-EM-RLCLD-PPVPPNAY-PY-LREY-TRLVNGFKPLV-RSARLY
```

& Witte, 1991). Recently, Conner *et al.* (1992a) have reported that HSV-1 R1 undergoes autophosphorylation, however, a transphosphorylating activity has not been observed.

Cheng *et al.* (1990) have reported that the HSV-2 R1 N terminal domain contains a possible transmembrane sequence and also incorporates myristic acid. On this basis they suggest that the R1 protein kinase may associate with the plasma membrane and transduce external signals in a manner similar to that of the *mos* oncogene. However, this idea is entirely speculative.

CHAPTER 2: MATERIALS

2. MATERIALS

2.1. CELLS

Baby Hamster Kidney (BHK) 21 clone 13 cells (MacPherson & Stoker, 1962) were used routinely throughout the study.

Vero cells (African Green monkey kidney fibroblasts), HFL and Flow 2002 cells (Human foetal lung fibroblasts), BSC-1 cells (African Green monkey epithelial cells) and 3T6 cells (mouse embryo fibroblasts) were obtained from stocks within the Institute or were supplied by Flow Laboratories.

2.2. TISSUE CULTURE MEDIA

All tissue culture media were obtained from Gibco-BRL.

Eagle's Medium (E)	10 x Glasgow Modified Eagle's Medium (GMEM) was diluted to 1 x GMEM with sterile distilled H_2O and supplemented with: 4mM L-Glutamine and 0.26% sodium bicarbonate.
ETC_{10}	E supplemented with: 5% tryptose phosphate broth, 100u/ml Penicillin, 100ug/ml Streptomycin and 10% Newborn calf serum (NBCS).

EC_5	E supplemented with: 100u/ml Penicillin, 100ug/ml Streptomycin and 5% NBCS.
EF_{10}	(For growth of Vero, BSC-1 and Flow 2002 cells.) E supplemented with: 10% tryptose phosphate broth, 100u/ml Penicllin, 100ug/ml Streptomycin and 10% Foetal calf serum (FCS).
DMEM	(For growth of HFL and 3T6 cells.) Dulbecco's Modified Eagle's Medium (DMEM) supplemented with: 100u/ml Penicillin, 100ug/ml Streptomycin and 10% FCS.
EMC_5	E supplemented with: 100u/ml Penicillin, 100ug/ml Streptomycin, 1.5% carboxymethylcellulose and 5% NBCS.
EMC_5/X-Gal Overlay	EMC_5 supplemented with 1mM X-Gal.
Agar/X-Gal Overlay	E (without phenol red) supplemented with: 0.64% Noble agar, 2% NBCS and 1mM X-Gal.

PIC	E (without phosphate) supplemented with 1% NBCS.
Emet/5 C_2	E containing 1/5th of normal methionine concentration supplemented with 2% NBCS.
Versene	6mM EDTA in PBS with 0.0015% (w/v) phenol red.
Trypsin	0.25% (w/v) Difco trypsin in Tris Saline (25mM Tris, 140mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 1mg/ml dextrose, 0.0015% phenol red, 100u/ml Penicillin, 100ug/ml Streptomycin, pH7.4)
Trypsin/Versene	One volume of trypsin plus 4 volumes of versene.

2.3. VIRUS

The HSV-1 wild-type virus used in these experiments was grown from a single plaque of the parental HSV-1 strain 17 syn^+ virus (Brown *et al.*, 1973).

2.4. BACTERIAL CULTURE MEDIA

L-Broth	10g/l NaCl, 10g/l Difco Bactopeptone, and 5g/l yeast extract.
L-Broth Agar	L-Broth containing 1.5% (w/v) agar.
2 x YT Broth	5g/l NaCl, 16g/l Difco Bactopeptone, 10g/l yeast extract.
Antibiotics	Ampicillin and tetracycline were added, where appropriate, to L-Broth and L-Broth Agar at concentrations of 50ug/ml and 20ug/ml, respectively.

2.5. PLASMIDS

pGX125 (Davison & Wilkie, 1983c) was provided by Dr V. G. Preston and contains the UL13 gene of HSV-1 present in the *KpnI f* fragment (residue 17789 to 28626, according to the nunbering system of McGeoch *et al.*, 1988a).

pKhx1 was provided by Dr V. G. Preston and contains the US3 gene of HSV-1 present within the *KpnI h* fragment (residue 134788 to 144893).

pGX45 (Rixon & McGeoch, 1985) was provided by Dr V. G. Preston and contains the 5' terminal portion of the US3

gene of HSV-1 in a *HindIII - BamHI* fragment (133466 to 136289).

pGX169 (Rixon & McGeoch, 1985) was provided by Dr V. G. Preston and contains the 3' terminal portion of the HSV-1 US3 gene present in a *BamHI - HindIII* fragment (136289-138344).

pFJ3 (Rixon & McLauchlan, 1990), provided by Drs F. J. Rixon and J. McLauchlan, contains the *Escherichia coli lacZ* gene under the control of the SV40 early promoter.

pJM1A was provided by Dr J. Mullaney and contains the HSV-1 *KpnI h* fragment (134788-144893) with the US3 gene disrupted by the insertion of the *lacZ* gene of *Escherichia coli*.

pAT153 (Twigg & Sherratt, 1980) was the vector used for the construction of pLC3 and pLC5.

2.6. ENZYMES

DNA polymerase I, Klenow fragment, T_4 ligase, calf intestinal phosphatase (CIP) and all restriction nucleases used were obtained from Bethesda Research Laboratories, New England Biolabs, Northumbria Biologicals Ltd or Boehringer Mannheim UK Ltd.

All other enzymes were obtained from Sigma Chemical Co Ltd.

2.7. CHEMICALS

All chemicals were of analytical grade or higher and were supplied by BDH Chemicals UK and Sigma Chemical Co Ltd, except for the following:

Acetic acid, chloroform, ether, methanol - May & Baker Ltd.
Ammonium persulphate, Gelatin, TEMED (N,N,N',N'-tetra-methylethylenediamine) - Bio-Rad Laboratories, California.
Ampicillin ("Penbritten") - Beecham Research Laboratories.
Caesium chloride - Malford Laboratories Ltd.
Ecoscint - Nuclear Medical Electronic Systems & Services Ltd, Livingstone.
En³hance - DuPont (UK) Ltd.
Ethanol - Hayman Ltd.
Ficoll 400 - Pharmacia LKB Biotechnology.
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) - Boehringer Mannheim UK Ltd.

2.8. RADIOCHEMICALS

Radiochemicals were supplied by Amersham International plc:
^{32}P-orthophosphate (carrier free), 10mCi/ml
L-[^{35}S]-Methionine, 15mCi/ml
[γ-^{32}P]-ATP, 10mCi/ml
[α-^{32}P]-dATP - 10mCi/ml
[^{125}I]-Protein A, 38mCi/mg

2.9. ANTISERA

Antisera 18377 and 18378 had been raised against the C-terminal octapeptide of the US3 ORF and were kindly provided by Dr H. S. Marsden and Mrs M. Murphy (Frame *et al.*, 1987).

The anti-UL11 antibody (MacLean *et al.*, 1989) was kindly provided by Dr C. A. MacLean.

2.10. SOLUTIONS AND BUFFERS

E Buffer	36mM Tris-HCl (pH 7.8), 30mM NaH_2PO_4, 1mM EDTA.
TE Buffer	10mM Tris-HCl (pH 7.5), 1mM EDTA.
TBE Buffer	90mM Tris base, 89mM boric acid, 2mM EDTA.
PBSA	170mM NaCl, 3.4mM KCl, 10mM NaH_2PO_4, 1.8mM KH_2PO_4 (pH 7.2).
PBS	PBSA suplemented with 6.8mM $CaCl_2$, 4.9mM $MgCl_2$.
HeBS	137mM NaCl, 5mM KCl, 0.2mM NaH_2PO_4, 9mM D-Glucose, 21mM Hepes (pH 7.05).

20 x SSC	3M NaCl, 0.3M trisodium citrate.
RSB	10mM Tris-HCl pH 7.4, 10mM KCl, 1.5mM $MgCl_2$.
TBS	20mM Tris-HCl pH 7.5, 0.5M NaCl.
WB (wash buffer)	1mM Tris-HCl pH 7.4, 0.09% NaCl, 0.01% NaN_3.
Transfer buffer	25mM Tris-HCl pH 8.3, 192mM glycine, 20% methanol.
Stet Buffer	8% sucrose, 0.5% Triton X-100, 50mM EDTA pH 8.0, 50mM Tris-HCl pH 8.0.
Detergent Lysis Buffer	0.1M Tris-HCl pH 8.0, 0.5% NP40, 0.5% sodium deoxycholate (DOC), 10% glycerol.
50 x Denhardt's Solution	1% polyvinyl pyrrolidone, 1% BSA, 1% Ficoll 400.
10 x N.T. Buffer	0.5M Tris-HCl pH 7.8, 0.05M $MgCl_2$, 10mM dithiothreitol (DTT).

Giemsa Stain	1.5% suspension of Giemsa in glycerol heated to 56°C for 90-120 min and diluted with an equal volume of methanol.
Denaturing Buffer	50mM Tris-HCl pH 6.7, 2% SDS, 700mM 2-mercaptoethanol, 10% glycerol, bromophenol blue.
DF Dye	50% (v/v) TBE, 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue.
RE Stop	5 x E buffer, 100mM EDTA, 10% Ficoll 400.

2.11. OTHER MATERIALS

"Miniprep Plus Kit" was supplied by Pharmacia LKB Biotechnology.
"Minifold II" apparatus was obtained from Schleicher & Schuell, Germany.
"GenescreenPlus" was supplied by DuPont.
"Spin-X" tubes were supplied by Costar.
Phosphorylated *XbaI* and *XhoI* linkers were obtained from New England Biolabs.

CHAPTER 3: METHODS

3. METHODS

3.1. GROWTH OF CELL AND VIRUS STOCKS

3.1.1. Tissue Culture

Baby Hamster Kidney (BHK) cells were grown in ETC_{10} in 80oz plastic roller bottles at 37°C in an atmosphere of 95% air, 5% CO_2. To harvest, the confluent monolayers were washed twice with 20ml versene followed by a trypsin/versene wash. The detached cells were resuspended in 10ml ETC_{10} and stored at 4°C for up to one week. The cells were used to seed roller bottles at a concentration of 2ml/100ml ETC_{10} and 50mm tissue culture plates at a concentration of 200ul/3-4ml ETC_{10}.

To store, detached cells were resuspended in 5ml ETC_{10} supplemented with 25% calf serum and 10% glycerol. Aliquots were stored overnight at -70°C and then transferred to liquid nitrogen (-190°C). Cells were recovered from liquid nitrogen by bringing quickly to 37°C.

All other cells used in the study were grown in large tissue flasks or roller bottles as described above, except that the growth medium ETC_{10} was replaced by EF_{10} for Vero, BSC-1 and Flow 2002 cells and by DMEM for HFL and 3T6 cells.

3.1.2. Preparation of "Resting" Cells

"Resting" cells were prepared as described by Howard *et al.* (1974). 50mm tissue culture plates were seeded with BHK cells at one fifth of the normal density, in ETC_{10}. After 24h at 37°C, the cells were then washed three times with Eagle's medium supplemented with 0.5% calf serum ($EC_{0.5}$). 2ml of $EC_{0.5}$ was added back to the cells which were then incubated at 37°C for a further 6-7 days.

3.1.3. Growth of Virus Stocks

Roller bottles of 90% confluent BHK cells were infected at a multiplicity of 1pfu per 300 cells in 40ml ETC_{10} and incubated at 31°C until total c.p.e. was obtained (about 4-5 days p.i.). The infected cells were shaken into the medium and spun at 2000 rpm for 10 min (Beckman GPR centrifuge). The cell pellet was used to prepare the cell-associated (CA) stock while the supernatant was used to prepare the Supernatant (SV) stock.

CA Stock. The cell pellet was resuspended in 5ml supernatant, sonicated, freeze-thawed, sonicated and spun at 2000 rpm for 10 min (Beckman GPR centrifuge). The supernatant (supernatant 1) was kept on ice. The cell pellet was resuspended in 5ml ETC_{10}, freeze-thawed, sonicated and spun for a further 10 min at 2000 rpm (Beckman GPR centrifuge). The supernatant was pooled with supernatant 1,

checked for sterility and stored at -70°C.

SV Stock. The supernatant was spun for 2h at 12,000 rpm (Sorvall GSA rotor). The virus pellet was resuspended in 5ml supernatant, sonicated, checked for sterility and stored at -70°C.

3.1.4. Sterility Checks

Cell and virus stocks were regularly checked for yeast and bacterial contamination using Brain Heart Infusion blood agar plates: 5.2% (w/v) Difco Bacto Brain Heart Infusion agar was autoclaved for 20 min and allowed to cool to 40°C. 10% (v/v) horse blood (Biocult) was added and the agar poured into 50mm petri dishes. The cells or virus stocks were streaked, in duplicate, on the plates which were sealed with parafilm and incubated at 37°C. If no contamination was apparent after 7 days the stocks were deemed to be sterile.

Mycoplasma contamination was checked for by Mrs J. Mitchell of Cytology. Briefly, a sub-confluent monolayer of cells was grown on a coverslip placed on the bottom of a petri dish. The cells were fixed by immersing the coverslip in a 3:1 solution of methanol:acetone for three minutes. A drop of Hoescht Stain was then placed onto the coverslip. After ten minutes the stain was washed off, the coverslip dried, mounted onto a slide and examined under the microscope at x100, full immersion. If mycoplasma was present it appeared

as small dots around the nucleus or gave the nucleus a "cotton wool" appearance. *Pseudomonas* contamination was kindly identified by the Department of Bacteriology, University of Glasgow.

3.1.5. Purification of Virions and Light Particles

Virions and light particles were purified essentially as described by Szilagyi & Cunningham (1991). 80-90% confluent monolayers of BHK cells in 80oz plastic roller bottles were infected at 0.01 pfu/cell in 40ml ETC_2. For unlabelled virions the cells were incubated at 31°C until c.p.e. was widespread (4 to 5 days). For [^{35}S]-methionine labelled virions the cells were incubated at 31°C for 2 to 3 days. The medium was replaced with 20ml Emet/$5C_2$ and 2-8h later [^{35}S]-methionine (0.5mCi/bottle) was added. Incubation was continued for a further 2-3 days.

The infected cells were shaken into the medium and pelleted by centrifugation at 2000 rpm for 30 min at 4°C (Beckman GPR centrifuge). The supernatant was then spun for a further 2h at 12,000 rpm (Sorvall GSA rotor) and the virus pellet carefully resuspended in minimal volume of Eagle's medium without phenol red. 5%-15% Ficoll 400 gradients in Eagle's medium without phenol red were prepared in 35ml cellulose nitrate centrifugation tubes. The resuspended pellet was layered onto the gradients and spun for 2h at 12,000 rpm (Sorvall AH629 rotor). The virions, which formed a well-

defined lower band, and the light particles, which formed a more diffuse upper band, were withdrawn separately from the gradient using a wide bore needle. The light particles were rebanded on another 5%-15% Ficoll 400 gradient. Both virions and light particles were pelleted overnight at 21,000 rpm (Sorvall AH629 rotor). The pellets were resuspended in Eagle's medium without phenol red and stored at -70°C. The quality of the preparations, i.e. the number of intact virus particles, was checked by Mr J. Aitken using electron microscopy.

3.1.6. Titration of Virus Stocks

Serial ten-fold dilutions of virus stocks were made using PBS containing 2% calf serum. 0.2ml of each dilution was added to plates of 90% confluent BHK cells from which the growth medium had been removed. The plates were incubated at 37°C for 1h after which the inoculum was removed and the cells were overlayed with 4ml EMC_5. After 2-3 days incubation at 37°C the monolayers were stained with Giemsa stain, the plaques counted and the titre of the virus stock calculated.

3.1.7. Particle Counts

Equal volumes of the virus stock, latex beads (at a known concentration) and stain (1% phosphotungstic acid pH 7.0) were mixed and adsorbed onto a parlodion-coated copper grid. Excess liquid was drained off and the number of virus particles and latex beads present in the field of view of the electron microscope counted. The concentration of the virus particles was calculated by comparison with the number of latex beads.

Particle counts were carried out by Mr J. Aitken.

3.1.8. Growth Curves

Growth curves were carried out at both high and low multiplicities of infection.

High moi. The medium was removed from 50mm plates of confluent BHK cells and the virus added, at a moi of 10, in a final volume of 500ul. The virus was left to absorb at 37°C for 1h after which the monolayers were washed three times with PBS. 2ml EC_5 was added and the plates were returned to 37°C. The 0h sample was harvested immediately after the washes with further samples harvested 2, 4, 6, 8, 12, 16, 20, 24 and 30h later. To harvest, the cells were scraped into the medium and subjected to ultrasonic disruption. The volume of each sample was measured. The samples were then titrated as described in 3.1.6. The virus yield at each time point was calculated as the titre x sample volume.

Low moi. This was carried out as described above except that the cells were infected at a moi of 0.001 and samples were harvested at 0, 4, 8, 12, 24, 36, 48, 60 and 72h.

3.1.9. Preparation of HSV-1 DNA Stocks

Infected BHK cells, in roller bottles, were shaken into the

medium and spun at 1000 rpm for 10 min at 4°C (Beckman GPR centrifuge). The supernatant was kept on ice while the cell pellet was resuspended in RSB containing 0.5% NP40 (5ml/bottle), incubated on ice for 10 min and spun at 3000 rpm for 10 min (Beckman GPR centrifuge). The cytoplasmic supernatant was kept and the nuclei were extracted again with RSB/0.5% NP40. The supernatants of both extractions were pooled with the original supernatant and spun at 5,000 rpm for 10 min (Sorvall GSA rotor). The pellet was discarded and the supernatant spun at 12,000 rpm for 2.5-3h (Sorvall GSA rotor). The virus pellet was resuspended in TE (2ml/bottle), sonicated and the virus particles lysed by the addition of 0.04 volumes of 0.25M EDTA and 0.025 volumes of 20% SDS. Virus DNA was extracted twice with phenol, once with phenol: chloroform (1:1) and once with chloroform. RNase A (5ug/ml) and RNase T1 (50u/ml) were added to the aqueous phase which was incubated at 37°C for 30 min. The DNA was precipitated using two volumes of ethanol, dried under vacuum and redissolved in TE.

3.2. PREPARATION AND ANALYSIS OF PLASMID DNA

3.2.1. Small Scale Plasmid Preparation

Using STET Buffer. 1.5ml of an overnight culture was spun for 10 seconds in a microcentrifuge. The supernatant was aspirated, the pellet vortexed and 100ul of STET buffer added. After vortexing, 16ul of lysozyme (10mg/ml in 0.25M

Tris pH 7.5) was added and the mixture was vortexed again. The tube was boiled for 12 min and then spun in a microcentrifuge for 15 min. The pellet was removed and 100ul isopropanol was added. The tube was left to stand at room temperature for 10 min after which it was spun for 3 min. The DNA pellet was drained, dried and dissolved in 40-50ul TE.

Using "Miniprep Plus" Kit (Pharmacia). The kit is based on a method devised by Birnboim & Doly (1979) and was used exactly as described in the instructions. Briefly, bacteria, pelleted from an overnight culture, were resuspended in one volume of a solution containing 50mM Tris-HCl pH 8.0, 50mM glucose, 10mM EDTA and 10% "Kathon". This was followed by the addition of two volumes of a 0.2M NaOH, 1% (v/v) Triton X-100 solution. Finally, sodium acetate (pH 4.8) was added to give a final concentration of 1M. The lysed bacteria were pelleted and the DNA precipitated from the supernatant using an equal volume of isopropanol. The DNA pellet was resuspended in 40-50ul TE.

3.2.2. Large Scale Plasmid Preparation

15ml L-Broth, containing the appropriate antibiotic, was inoculated with a loopful of a glycerol stock and shaken overnight at 37°C. This culture was added to 600ml of L-Broth plus antibiotic and shaken at 37°C until OD_{630} = 0.8. Chloramphenicol was then added at a concentration of 25ug/ml

and the incubation was continued overnight.

Plasmid DNA was isolated by the soft lysis method described by Katz *et al.* (1973), with modifications. The 600ml culture was centrifuged at 9000 rpm for 15 min at 4°C (Sorvall GS3 rotor). The bacterial pellets were resuspended in a total of 16ml ice-cold sucrose-Tris (25% sucrose (w/v), 50mM Tris-HCl pH 8.0) and placed on ice. Lysozyme was added to a concentration of 2.5mg/ml followed by a 5 min incubation on ice. 12ml of 250mM EDTA pH 8.0 was then added and the tube left on ice for a further 5 min. The bacterial cells were lysed by the addition of 36ml 2% Triton X-100 in 50mM Tris-HCl (pH 8.0), 62.5mM EDTA followed by 20 min incubation on ice. The lysed bacteria were then spun at 15,000 rpm for 30 min at 4°C (Sorvall SS34 rotor) to pellet the cell debris and chromosomal DNA. The lysate was extracted with phenol and the upper aqueous layer was dialysed against TE for 1.5h at 4°C with two changes. After dialysis the DNA was precipitated with 0.5 volumes of isopropanol, pelleted by centrifugation at 2000 rpm for 10 min (Beckman GPR centrifuge) and resuspended in 5ml TE.

The plasmid DNA was then purified on a CsCl gradient. To prepare the gradients 15g of CsCl plus 1ml of 10mg/ml EtBr was added to the DNA solution. Sufficient TE was then added to give a final density of 1.55g/ml. The solution was centrifuged at 40,000 rpm at 15°C for 16h (DuPont TV865B rotor). The bands were visualised using long wave

ultraviolet light and the lower band, representing the supercoiled DNA, was removed with a syringe. The EtBr was removed by successive extractions (at least 4) with CsCl-saturated isopropanol. The DNA was dialysed against TE for 1.5h at 4°C with two changes and then precipitated with 2.5 volumes of ethanol. The DNA was pelleted by centrifugation at 15,000 rpm for 15 min (Sorvall SS34 rotor), dried, resuspended in 1ml TE and stored at -20°C.

3.2.3. Glycerol Stocks of Bacteria

50ml of L-Broth, containing the appropriate antibiotic, was inoculated with a loopful of a bacterial culture and incubated overnight at 37°C in an orbital shaker. The culture was centrifuged at 9000 rpm for 15 min (Sorvall SS34 rotor) and the pellet resuspended in 3-4ml of 2% Bactopeptone plus an equal volume of 80% glycerol. The stock was aliquoted and stored at -20°C and -70°C.

3.2.4. Restriction Enzyme Digestion of Plasmid DNA

Plasmid DNA was cut with restriction enzymes, in the presence of the appropriate buffer, at a concentration of approximately 1u enzyme/1ug DNA for 3-4h at the specified temperature. For analytical use the DNA (approximately 0.5ug) was digested in a volume of 10ul all of which was loaded onto a minigel (see 3.2.5.). Preparative digests were carried out in a final volume of 30-50 ul and were either

loaded onto a minigel for separation of restriction fragments or were phenol extracted and ethanol precipitated before being used further.

3.2.5. Separation of DNA Restriction Fragments on Agarose Gels (Minigels)

50ml of a 1% agarose solution, in TBE, containing 5ug/ml EtBr, was poured into a minigel kit, a teflon comb was inserted and the gel was left to set. 5ul of DF dye was added to the samples which were then loaded into the wells and electrophoresed at 50V using TBE containing 5ug/ml EtBr as the buffer. The restriction fragments were visualised under long or short UV light.

3.3. GENERATION OF RECOMBINANT PLASMIDS

3.3.1. Extraction of DNA Restriction Fragments from Agarose Gels

DNA was cut with the appropriate enzyme (see 3.2.4.) and run on an agarose gel (see 3.2.5.). The gel was viewed under long wave UV light and the required band was excised. The DNA was extracted from the agarose by one of two ways.

Electroelution. The gel slice was placed in a piece of dialysis tubing which had been boiled and then rinsed in electroelution buffer (20mM Tris-HCl pH 7.4, 10mM sodium

acetate, 0.5mM EDTA). A minimal amount of buffer was added to the gel slice and the tubing was sealed at both ends. The gel slice was then immersed in a shallow layer of buffer and electrophoresed at 75mA for 3h. The polarity of the current was reversed for 1 min to remove DNA from the side of the tubing. The gel slice was removed and the DNA was isolated from the buffer by phenol extraction and ethanol precipitation.

Spin-X tubes. The gel slice was placed in a microcentrifuge tube and frozen for 10 min in a dry ice/isopropanol bath. The slice was then transferred to a Spin-X tube and spun for 10-15 min in a microfuge. The DNA was isolated from the buffer by phenol extraction and ethanol precipitation.

3.3.2. DNA Cloning

The vector was linearised using the appropriate restriction endonuclease in the presence of 1 unit of calf intestinal phosphatase to minimise recircularisation of the vector DNA during subsequent ligations. The DNA was phenol extracted and ethanol precipitated.

Blunt ending. For the insertion of blunt-ended fragments into sticky-ended vectors, the vector was incubated with 5 units each of T_4 polymerase and Klenow fragment in the presence of T_4 buffer (0.67M Tris-HCl pH 7.9, 67mM $MgCl_2$, 50mM DTT) and T_4 chase (2mM dATP, dCTP, dGTP, dTTP) up to a

final volume of 20ul. The incubation was carried out at room temperature for 2h. The DNA was phenol extracted and ethanol precipitated.

Ligation. The insert and vector were mixed at a ratio of 3:1 in the presence of 1-2 units of ligase and made up to 20ul with 1 x ligase buffer (Boehringer Mannheim). The mixture was incubated at room temperature or 16°C overnight.

3.3.3. Preparation of Competent *E. coli*

Calcium-shocking. JM109 *E. coli* were grown in 300ml L-Broth at 37°C in a shaking incubator until the OD_{600} was 0.3 to 0.4. The culture was spun at 9000 rpm for 15 min (Sorvall SS34 rotor) and the pellet resuspended in 0.5 volumes of 50mM $CaCl_2$/10mM Tris-HCl pH 7.5. After 20 min on ice the *E. coli* were spun as before and the pellet resuspended in 0.1 volumes of 50mM $CaCl_2$/10mM Tris-HCl pH 7.5. The competent *E. coli* were kept on ice until required.

Commercially obtained. Library Efficiency DH5α competent *E. coli* were supplied by BRL. The *E. coli* had been made competent by a modification of the procedure of Hanahan (1983).

3.3.4. Transformation of Competent *E. coli*

Calcium-shocked *E. coli*. 1ug of DNA was added to 200ul of

competent *E. coli* and incubated on ice for 40 min. The *E. coli* were then heat-shocked at 42°C for 2 min, mixed with 1.8ml of L-Broth and shaken for 1h at 37°C. Aliquots were spread on L-Broth agar plates, containing antibiotic (if appropriate), which were then incubated at 37°C overnight.

Commercially-obtained competent *E. coli*. The DH5∝ *E. coli* were transformed as described in the instructions. Briefly, 1ul of a 5-fold dilution of the ligation mixture was added to 20ul of DH5∝ cells. The mixture was incubated on ice for 30 min and then heat-shocked at 42°C for 45 sec. The *E. coli* were returned to ice and 80ul of either SOC medium (2% Bactotryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) or 2 x YT Broth was added. The mixture was shaken at 37°C for 1h and plated onto L-Broth agar plates.

3.3.5. Detection of Recombinant Plasmids Containing the *E. coli lacZ* gene

50ul of a 30mg/ml stock of X-Gal, in DMF, was spread evenly onto L-Broth agar plates and left to dry for 1-2 min. The transformed cells were then spread onto the plates which were incubated at 37°C overnight. Colonies expressing the *lacZ* gene turned blue.

3.4. GENERATION OF RECOMBINANT VIRUSES

3.4.1. Co-transfection of Viral DNA with Plasmid DNA

Co-transfection of virus and plasmid DNA into BHK cells was carried out by calcium phosphate precipitation followed by a dimethyl sulphoxide (DMSO) boost, as described by Stow & Wilkie (1976). HSV-1 DNA (0.4ug or 200 pfu) was mixed with varying amounts of linearised plasmid DNA (0.1ug to 5ug, giving an approximate 2.5-fold to 125-fold molar excess over the virus DNA) in the presence of 6ug of calf thymus as carrier. Each DNA mixture was made up to 930ul with HeBS buffer and, just before use, 70ul of 2M $CaCl_2$ was added. The tubes were inverted to gently mix the solutions. The medium was removed from the monolayers and the DNA mixtures were added dropwise, in duplicate (400ul/plate). The plates were incubated at 37°C for 40 min after which 4ml EC_5 was added to each plate. Incubation was continued for a further 4h. To boost the transfection, the monolayers were first washed with Eagle's medium without calf serum (E), then 1ml of 25% DMSO, in E, was added to each plate. After 4 min incubation at room temperature the DMSO was removed, the monolayers were washed twice with E and overlayed with 4ml EC_5. The plates were incubated at 37°C until cpe was widespread (2-4 days). The cells were then scraped into the medium, sonicated to release infectious virus, and stored at -70°C.

3.4.2. Detection and Isolation of Recombinant Viruses

The viruses harvested from the co-transfection, were titrated as described in 3.1.6. The titrations were initially overlayed with 4ml EMC_5 but when plaques were observed the EMC_5 overlay was replaced with 4ml of either EMC_5 or Agar overlay containing 1mM X-Gal. The titrations were incubated at 37°C for another day after which any plaques formed by the recombinant virus had turned blue. Individual blue plaques were picked and carried through five or six more rounds of plaque purification. Stocks were grown of 2 or 3 purified plaques which were used in subsequent experiments.

3.4.3. Restriction Enzyme Analysis of ^{32}P-labelled Viral DNA

The method used was based on that described by Lonsdale (1979). BHK cells were grown at 37°C in phosphate-free medium (PIC) in a 24 well tissue culture plate. When the cells were confluent virus was added directly to the medium at a moi of 10 and left to absorb at 37°C for 1h. The inoculum was then removed and the cells washed in 800ul PIC. 450ul of PIC was added to each well and the incubation at 37°C was continued. After 2h 50ul of PIC containing 0.1-0.2 uCi/ul of ^{32}P-orthophosphate was added to each well and the cells were incubated at 37°C for 2-3 days.

To lyse the infected cells, 0.5ml 5% SDS was added to each

well. The cells were scraped into the medium and transferred to a 10ml tube. The DNA was isolated from this lysate by extraction with an equal volume of buffer-saturated phenol followed by precipitation with 2 volumes of ethanol. The virus DNA was then pelleted by centrifugation at 2000 rpm for 10 min (Beckman GPR centrifuge). The pellet was drained, dried at 37°C for 15-20 min, and resuspended in 200ul H_2O containing 125ug/ml RNase A and 0.05ug/ml RNase T1 in a gently shaking 37°C water bath.

20ul of labelled DNA was mixed with 1-2 units of the appropriate enzyme and made up to a final volume of 30ul with the buffer supplied by the manufacturers or, in the case of *Bam*HI , with *Bam*HI buffer [6mM Tris-HCl pH 7.4, 6mM $MgCl_2$, 0.1M NaCl, 0.1mg/ml BSA (Boehringer Mannheim), 0.04% (v/v) 2-mercaptoethanol]. The samples were incubated at 37°C for at least 4h after which 10ul of RE stop was added. The restriction fragments were then separated by horizontal agarose gel electrophoresis.

To prepare agarose gels tape was placed along the 4 sides of a 265mm x 165mm glass plate which was then set in a gel former and levelled. 250ml of a 0.6% or 0.8% agarose solution in E buffer was poured into the plate and a comb was inserted. Once set, the gel was placed in a horizontal gel electrophoresis tank and E buffer was added to a level just above the gel surface. The samples were loaded using a micropipette and electrophoresed at 40V overnight. The gels

were dried and exposed to X-Omat XS-1 film either at room temperature or at -70°C against an image intensifying screen.

3.5. ANALYSIS OF HSV-1 INDUCED POLYPEPTIDES

3.5.1. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Both 9% (w/v) single concentration gels and 5%-12.5% (w/v) gradient gels were used. 30% (w/v) acrylamide stocks were prepared in water. The ratio of acrylamide to the crosslinking agent N,N'-methylene bisacrylamide was 39:1 and 19:1 for single concentration gels and gradient gels respectively. The stocks were filtered through Waterman No. 1 filter paper before use.

Gel solutions containing the appropriate percentage of acrylamide were prepared in buffer of final concentration 375mM Tris-HCl (pH 8.9) and 0.1% SDS. For gradient gels the 12.5% (w/v) solution also contained 15% glycerol to stabilise the gradient. To polymerise the gel solutions 150ul of 10% ammonium persulphate (APS) and 10ul of TEMED were added to each 24ml of gel solution except for the 12.5% solution for which only 65ul of 10% APS was added, together with 10ul TEMED.

The gel solutions were poured between 2 well-washed glass plates which were separated by 1.5mm thick spacers and sealed

with rubber tubing. The gels were left to polymerise under a thin layer of butan-2-ol. Following polymerisation a stacking gel consisting of 5% acrylamide in 122mM Tris-HCl (pH 6.7), 0.1% SDS was added. Wells were formed by a teflon comb.

Samples were boiled for 5 min in denaturing buffer with sufficient bromophenol blue to visualise the dye front. Electrophoresis was carried out in a buffer containing 52mM Tris, 53mM glycine, 0.1% SDS at 10-12mA, overnight, at room temperature.

3.5.2. Visualisation of Proteins Separated by SDS-PAGE

(a) Autoradiography: Gels were fixed for 1h in methanol: acetic acid: water (50:7:50) and then soaked in several changes of 5% methanol, 7% acetic acid. The gels were then gently shaken in 3 volumes of En^{3}hance for 45 min, followed by a wash in 10 volumes H_2O, for 10-15 min. The gels were dried, under vacuum, onto Whatman grade 182 filter paper and exposed to Kodak X-Omat XS-1 film at -70°C.

Gels were also dried prior to treatment with En^{3}hance and exposed to film at room temperature or at -70°C with an image intensifying screen.

(b) Silver staining: Gels were fixed in 30% ethanol, 10% acetic acid for at least 30 mins followed by a 30 min

incubation in a solution of 30% ethanol, 0.5M sodium acetate, 0.5% glutaraldehyde and 0.2% sodium thiosulphate. The gels were rinsed thoroughly in water, for 3 x 10 min and then soaked in 0.1% silver nitrate, 0.02% formaldehyde for 15-60 min. The protein bands were visualised by placing the gels in developer (2.5% sodium carbonate, 0.01% formaldehyde) for 5-15 min. Development was stopped by the addition of 0.05M EDTA followed by a wash in water. All water used throughout this procedure was of high purity HPLC grade.

3.5.3. Preparation of [^{35}S]Methionine-labelled Extracts

Confluent monolayers of BHK cells in 50mm plates were infected at a moi of 20. The virus was left to adsorb at 37°C for 1h after which the cells were washed twice with Eagle's medium containing one fifth the normal concentration of methionine supplemented with 2% calf serum (Emet/5 C_2). 2ml of Emet/5 C_2 was added to each plate and incubation at 37°C was continued. At the appropriate time 100uCi of [^{35}S]methionine was added to the medium and the plates were returned to 37°C.

At the required time the infected monolayers were washed twice with PBS and drained well before the addition of 500ul of detergent lysis buffer (Zweig *et al.*, 1980). After 1-2 min the lysed cells were transferred into a microcentrifuge tube, incubated on ice for 1h and spun on a microfuge, at high speed, for 10 min. The supernatant, containing the

labelled proteins, was removed and stored at -70°C.

3.5.4. Preparation of *In Vitro* ^{32}P-Labelled Virions for Immunoprecipitation

A 30ul sample of virions was mixed with 1.2ul each of 0.25M Tris-HCl pH 7.5, 0.025M $MgCl_2$, 0.025M DTT and 1.25% NP40. The mixture was incubated on ice for 1h and diluted with an equal volume of 10mM Tris-HCl pH 7.5, 1mM $MgCl_2$ and 1mM DTT.

10ul of treated virions were then mixed with 40ul of H_2O or 1.25M NaCl plus 50ul SM buffer (0.1M Tris-HCl pH 7.5, 20mM $MgCl_2$, 2mM DTT, 0.2mCi/ml [γ-^{32}P]-ATP) and incubated at 37°C. After 1h, 100ul of 2 x lysis buffer and 300ul of 1 x lysis buffer was added, the mixture was incubated on ice for 1h and then spun for 5-10 min using a microfuge. The supernatant, containing the labelled virions, was removed.

3.5.5. Immunoprecipitation

50ul aliquots of antipeptide antibody were pre-incubated wth 10ug peptide or PBS at 37°C for 30min. 100ul of [^{35}S]methionine-labelled extract (section 3.5.3.) plus 100ul of detergent lysis buffer were then added to each tube. The tubes were incubated at 4°C overnight. 60ul of Protein-A-Sepharose (50% suspension in detergent lysis buffer) was added to each tube and the incubation was continued for a further 1-2h, on ice. The Protein-A-Sepharose was pelleted

using a microfuge at low speed for 1 min and washed four times with detergent lysis buffer. The antibody-antigen complexes were eluted by boiling for 5-10 min in denaturing buffer. The samples were then spun for 1 min in a microfuge at high speed and the supernatant loaded onto SDS-polyacrylamide gels.

3.5.6. Immunoblotting

The immunoblotting procedure used was based on that described by Towbin *et al.* (1979), with modifications.

The samples used for immunoblotting were prepared by infecting BHK cells in 50mm plates or roller bottles at a moi of 10-20. (For [^{35}S]methionine-labelled samples the cells were labelled as described in section 3.5.3.) To harvest from 50mm plates, 500ul of denaturing buffer was added and the plates were then heated to 70-80°C, in an oven, for 10 min. The sample was transferred to a vial, boiled for 2 min and stored at -70°C. For roller bottles, the cells were harvested into 25ml denaturing buffer by rotating over a boiling water bath for 10 min, aliquoted and stored at -70°C.

The samples were loaded onto SDS-polyacrylamide gels in large wells (approximately 1 x 10^6 cell equivalents per cm). If the sample was unlabelled, a marker track containing [^{35}S]methionine-labelled extract was added.

Following electrophoresis the protein was transferred from the gel to nitrocellulose using a BioRad "Transblot" apparatus. The open gel holder was placed in a shallow tank of transfer buffer (cathode side down). A foam pad, presoaked in transfer buffer, was placed on top of the cathode panel. This was followed by a presoaked sheet of Whatman grade 182 filter paper and then the gel. Nitrocellulose strips (numbered and presoaked in transfer buffer) were place on top of the gel, face down, and air bubbles were excluded. The sandwich was completed with another sheet of filter paper and another foam pad. The holder was closed and placed in the "Transblot" tank containing transfer buffer. The proteins were transferred at 250mA for 3h at room temperature.

Following transfer the free binding sites on the nitrocellulose were blocked using one of the three following blocking agents: 3% gelatin in TBS; 0.05% Tween 20 in WB; or 10% FCS in TBS or WB. The strips were placed in one of these buffers and incubated at 37°C or 65°C for 1h with a change of buffer after 30 min. The strips which had been blocked with gelatin were then washed twice with 1 x WB containing 0.05% Tween 20.

The antiserum was diluted to the appropriate concentration with 1 x WB containing 0.05% Tween 20 + 1% BSA. Each strip was placed, face up, in a 5mm deep trough in a perspex block, and incubated with 3ml diluted antiserum at room temperature

overnight with gentle shaking. Unbound antiserum was removed by 4 washes in 1 x WB/0.05% Tween 20 over a period of 2h at 37°C.

To detect bound antibody the strips were transferred to clean troughs and each covered with 3ml of 1 x WB containing 3% BSA. 5 x 10^5 cpm of ^{125}I-protein-A was added to each trough and the strips were shaken gently at 37°c for 2h. The strips were then washed in 1 x WB containing 1M KI for 2h at room temperature with one change of buffer and then in PBS-A for 15 min. The strips were air-dried and stuck to card sheets which had been marked with ink containing ^{35}S and ^{125}I.

To detect which of the proteins had reacted with the ^{125}I-protein-A the strips were exposed to three films simultaneously, as described by Haarr *et al.* (1985). Film 1 was placed directly on top of the strips and was separated from the other two films by a sheet of black paper. An image intensifying screen was placed behind film 3. The [^{35}S] β emissions were detected only by film 1. The ^{125}I γ emissions passed through all three films and hit the image intensifying screen producing photons which were detected by film 3, giving an intense image and then by film 2, giving a much less intense image. Film 1 was protected from the photons by the black paper. The three films were then aligned using the radioactive ink marker.

3.6. ANALYSIS OF VIRUS DNA SYNTHESIS

3.6.1. Extraction of Viral DNA from Infected Cells

Monolayers of BHK cells in 50mm plates were mock-infected or infected at 5 pfu/cell. After 1h incubation at 37°C the monolayers were washed 3 times with PBS and overlayed with 4ml EC_5. Incubation was continued for a further 8h or 24h.

(a) Preparation of total cellular DNA. The method used was that described by Stow *et al.* (1983). The medium was removed from the infected monolayers and was replaced with 2ml lysis buffer (0.6% SDS, 10mM EDTA, 10mM Tris-HCl pH 7.5) containing 500ug pronase per ml. The plates were incubated at 37°C for 4-5h after which the lysed cells were transferred to a 10ml tube. NaCl was added to a final concentration of 200mM and followed by sequential extractions with phenol and chloroform. DNA was precipitated with two volumes of ethanol and pelleted by centrifugation at 3000 rpm for 15min (Beckman GPR centrifuge). The pellet was resuspended in water containing 10ug/ml RNase A.

(b) Preparation of encapsidated DNA. The method used was that described by Stow *et al.* (1983). The medium was removed from the infected cells, which were scraped into 1ml RSB and subjected to ultrasonic disruption. 50ug of DNase 1 was added and the cells incubated at 37°C for 2h. SDS, EDTA and pronase were added to the above concentrations and the

proteins digested for a further 2h. The encapsidated DNA was then extracted as described above for total cellular DNA.

(c) Preparation of released DNA. The medium was removed from the monolayers and spun at 3000 rpm for 10 min to pellet cell debris. An equal volume of 16% polyethylene glycol 6000, in 1M NaCl, was added to the supernatant which was then left at 4°C overnight. The virus was pelleted by centrifugation at 12,000 rpm for 30 min (Sorvall SM24 rotor). The proteins were digested and the DNA extracted as described above.

3.6.2. Transfer of DNA onto a Hybridization Membrane Using Slot Blot Apparatus

The DNA samples were denatured in 0.25M NaOH for 10 min and chilled in ice before being diluted to the desired concentration with a solution of 0.125M NaOH, 0.125xSSC. A sheet of "GenescreenPlus" membrane was cut to fit a "Minifold II" apparatus (Schleicher & Schuell) and soaked in 0.4M Tris-HCl pH 7.5 for 30 min. The apparatus was assembled according to the manufacturers' instructions and 60ul of the diluted DNA samples was loaded into each slot. After 30 min a slight suction was applied for 30 sec. The apparatus was dismantled and the membrane removed.

3.6.3. Internal Labelling of Plasmid DNA by Nick Translation

Plasmid DNA was labelled according to the method of Rigby *et al.* (1977). 1ug of DNA was mixed with 20uCi [α-^{32}P]dATP, 2 units of *E. coli* DNA polymerase I, 1ul of DNase I (10^{-4}units/ml), 10ug BSA, and 0.2mM each of dCTP, dGTP and dTTP in the presence of 1 x NT buffer in a final volume of 20 ul. The mixture was incubated at 14°C for at least 90 min and diluted to 100ul with H_2O. The labelled DNA was precipitated twice by the addition of 10ul 3M sodium acetate and 80ul isopropanol.

For use as a probe in DNA:DNA hybridisation the DNA pellet was resuspended in 100ul of 80% formamide and boiled for 5 min.

3.6.4. DNA:DNA Hybridisation

Virus DNA, transferred to a membrane by a slot blot, was visualised by hybridisation with a ^{32}P-labelled probe (section 3.6.3). The membrane was incubated in 100ml prehybridisation mix (6xSSC, 5 x Denhardt's, 0.1% SDS, 0.02mg/ml denatured DNA) for 2h at 65°C. This was discarded and replaced with 20ml of hybridisation mix (6xSSC, 2xDenhardt's, 0.5% SDS, 20mM Tris-HCl pH 7.5, 1mM EDTA, 0.05mg/ml denatured DNA) to which 455ul of 1M HCl had been added. The labelled probe was added and hybridisation was

carried out at 65°C overnight. The membrane was washed in 6xSSC, 1% SDS at room temperature for 30 min. This was followed by a 30 min wash at 65°C in 3xSSC, 1% SDS and then in 2xSSC, 1% SDS. The membrane was rinsed in water and exposed to Kodak X-Omat XS-1 film at room temperature or at -70°C with an image intensifying screen.

3.7. IN VITRO PHOSPHORYLATION REACTIONS

3.7.1. Preparation of Crude Nuclear and Cytoplasmic Extracts from Infected Cells

Extracts were prepared essentially as described by Piette *et al.* (1985). Confluent monolayers of BHK cells, in roller bottles, were infected with 5-10 pfu/cell and incubated at 37°C for 5h. The cells were scraped into 10ml PBS, pelleted by centrifugation at 2000 rpm for 10 min (Beckman GPR centrifuge) and resuspended in 10ml Buffer A (10mM Hepes pH 8.0, 50mM NaCl, 0.5M sucrose, 1mM EDTA, 0.5% Triton X-100, 1mM PMSF, 7mM 2-mercaptoethanol). The cells were then disrupted, on ice, using 20 strokes of a Dounce homogeniser and centrifuged at 3000 rpm for 10 min (Beckman GPR centrifuge). The supernatant, representing the crude cytoplasmic extract, was removed and stored at -70°C. The nuclear pellet was resuspended in 3ml Buffer A and subjected to ultrasonic disruption followed by centrifugation at 2000 rpm for 3 min (Beckman GPR centrifuge). The supernatant represents the crude nuclear extract.

3.7.2. Treatment of Virions for *In Vitro* Phosphorylation Reactions

Virions were treated with NP40 as described in the first paragraph of section 3.5.4.

3.7.3. *In Vitro* Phosphorylation of Infected Cell Extracts and Virions

5ul of extract or virion preparation was mixed with 7ul of a buffer containing 140mM Tris-HCl pH 7.5, 28.6mM $MgCl_2$, 2.86mM DTT, 1uCi [γ-^{32}P]-ATP. 8ul of an NaCl solution, at the desired concentration, was added and the mixture was incubated at 37°C for 30-45 min. The assay was analysed by SDS-PAGE.

3.8. COMPUTING

Evaluations of DNA and protein sequences were carried out on the DEC Microvax II computer using the Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.*, 1984).

CHAPTER 4: STUDIES ON THE HSV-1 US3 GENE

4. STUDIES ON THE HSV-1 US3 GENE

When this research was initiated it was known that the US3 gene of HSV-1 encodes a protein kinase with serine/threonine specificity. However, the substrates of this kinase and its role in the virus life cycle were unknown. One of the aims of this research was therefore to investigate the function of the US3 protein kinase. The approach chosen for these investigations was to construct a virus in which the US3 gene had been inactivated by the insertion of the *Escherichia coli lacZ* gene. This insertion not only disrupted the US3 ORF but also acted as a screenable marker during purification of the mutant virus. Once constructed, the growth properties of the insertion mutant were characterised in order to determine if the US3 protein kinase is required for efficient viral growth. The proteins synthesised by the US3-*lacZ* virus were also investigated and the effect of US3 disruption on viral DNA synthesis was determined. Physiological substrates of the US3 protein kinase were analysed by comparing the phosphoprotein profiles of the *wt* and mutant viruses, following *in vitro* phosphorylation assays.

4.1. CONSTRUCTION OF THE US3-*lacZ* INSERTION MUTANT

4.1.1. Construction of a Plasmid Containing a Disrupted US3 Gene (pJM1A)

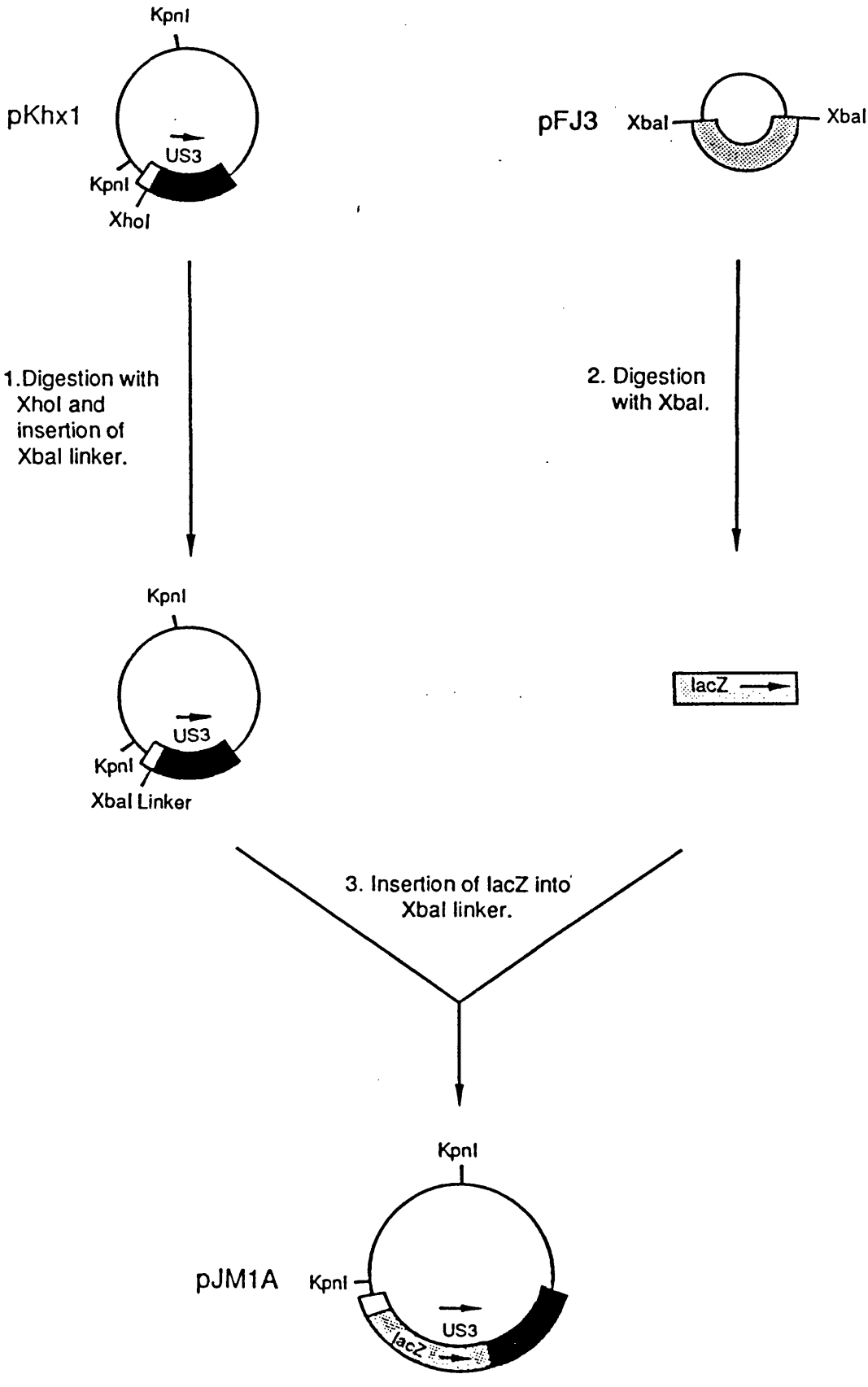
pJM1A was constructed by Dr J. Mullaney, as shown in Figure

Figure 4.1 Construction of pJM1A.

The *lacZ* gene from pFJ3 was inserted into the US3 gene at the unique *Xho*I site, via an *Xba*I linker.

The boxes represent coding sequences. The solid areas represent the predicted catalytic domain.

This construction was carried out by Dr J. Mullaney.


pKhx1
KpnI
US3
KpnI
XhoI
pFJ3
XbaI
XbaI
1.Digestion with XhoI and insertion of XbaI linker.
2. Digestion with XbaI.
KpnI
US3
KpnI
XbaI Linker
lacZ
3. Insertion of lacZ into XbaI linker.
pJM1A
KpnI
KpnI
US3
lacZ

4.1. pKhx1, which contains the *Kpn*I *h* fragment of HSV-1 cloned into pAT153, was cut at the unique *Xho*I site (residues 135727-135732) which lies within the US3 coding region (135222-136665). The linearised plasmid was blunt-ended and a phosphorylated *Xba*I linker was inserted. The *lacZ* gene, under the control of the SV40 early promoter, was excised from pFJ3 using *Xba*I, and inserted into the US3 gene via the *Xba*I linker.

The structure of the plasmid was verified by restriction enzyme digestion followed by electrophoresis on a mini-gel. This analysis also indicated that the *lacZ* gene had been inserted in the same orientation as the US3 gene, i.e. from left to right.

The *lacZ* insert (at residue 135727) is 423 bp upstream of the US2 transcript start and 1002 bp upstream of the US4 transcript start site (McGeoch *et al.*, 1988). It was therefore considered unlikely that the presence of the insert would affect transcription of the neighbouring genes.

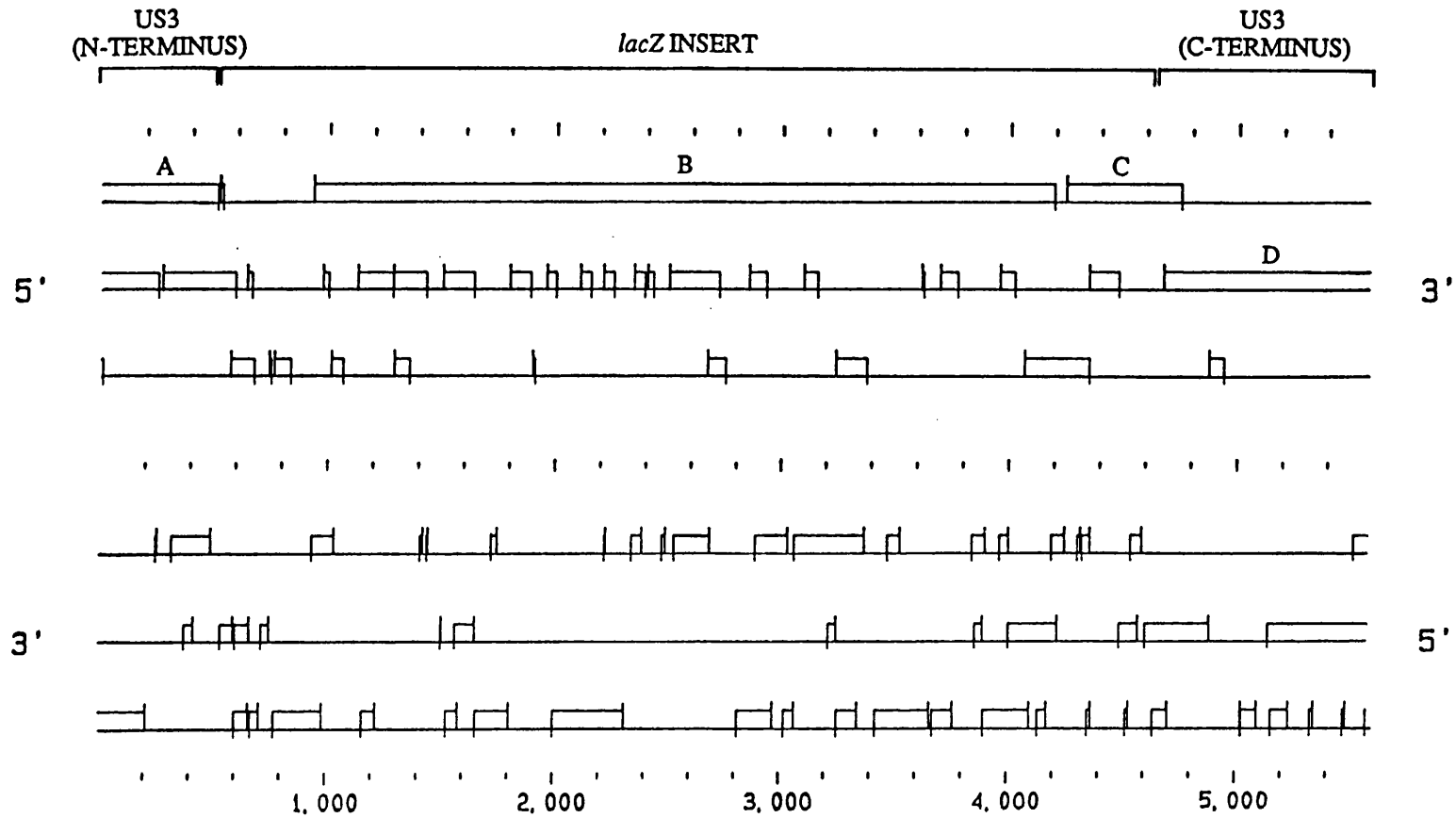
The *lacZ* insert disrupts the US3 ORF at codon 171, just upstream of the proposed catalytic domain which begins at amino acid 197 (as defined by Smith & Smith, 1989). As a result of this disruption, any translation initiated at the assigned US3 initiation codon will produce a protein consisting only of the first 171 amino acids of the US3 protein (ORFA in Figure 4.2). An initiation codon present at

Figure 4.2 **Predicted open reading frames in US3-*lacZ* construct.**

ORFs in the disrupted US3 gene, in all six translation frames, were predicted using the FRAMES program. Potential ORFs are indicated by open boxes. Potential start codons are indicated by lines extending above the reading frame box and stop codons are indicated by lines extending below the box. The four major putative ORFs are marked A-D.

A = the N-terminal 171 amino acids of the *wt* US3 protein
B = β-galactoside (approximately 120K)
C = a putative novel 17K protein
D = a putative 33K protein which includes the intact US3 catalytic domain

The scale represents nucleotides, starting with the initiation codon of the *wt* US3 protein (135222). The construct is 5579 nucleotides in length with the *lacZ* insert lying between nucleotides 512 and 4635.


US3
(N-TERMINUS)
lacZ INSERT
US3
(C-TERMINUS)
A
B
C
D
5'
3'
3'
5'
1, 000
2, 000
3, 000
4, 000
5, 000

amino acid 182 could conceivably re-initiate translation, resulting in the synthesis of a protein containing an intact catalytic domain (ORF D in Figure 4.2).

4.1.2. Co-transfection and Plaque Purification of US3-*lacZ* Virus

The US3-*lacZ* virus was generated by homologous recombination between *wt* HSV-1 DNA and pJM1A DNA during a cotransfection of BHK cells. Prior to cotransfection pJM1A was cut with *Bst*EII. There are 3 *Bst*EII sites in pJM1A situated close to each other (Figure 4.3). The digested DNA was fractionated electrophoretically on a minigel and the large restriction fragment recovered by electroelution. This DNA fragment was then cotransfected with HSV-1 DNA at the appropriate concentrations (see section 3.4.1.). The harvested cells were titrated in duplicate; one set was stained with Giemsa stain and the other was overlaid with Agar/X-Gal. The percentage of blue plaques observed was approximately 2%. For comparison, this frequency is similar to the value of 1.2% reported by Ace *et al.* (1989) for the generation of a UL48 insertion mutant, and is substantially higher than the 0.01% reported by Mullaney (1990) for the generation of a UL2-*lacZ* insertion mutant.

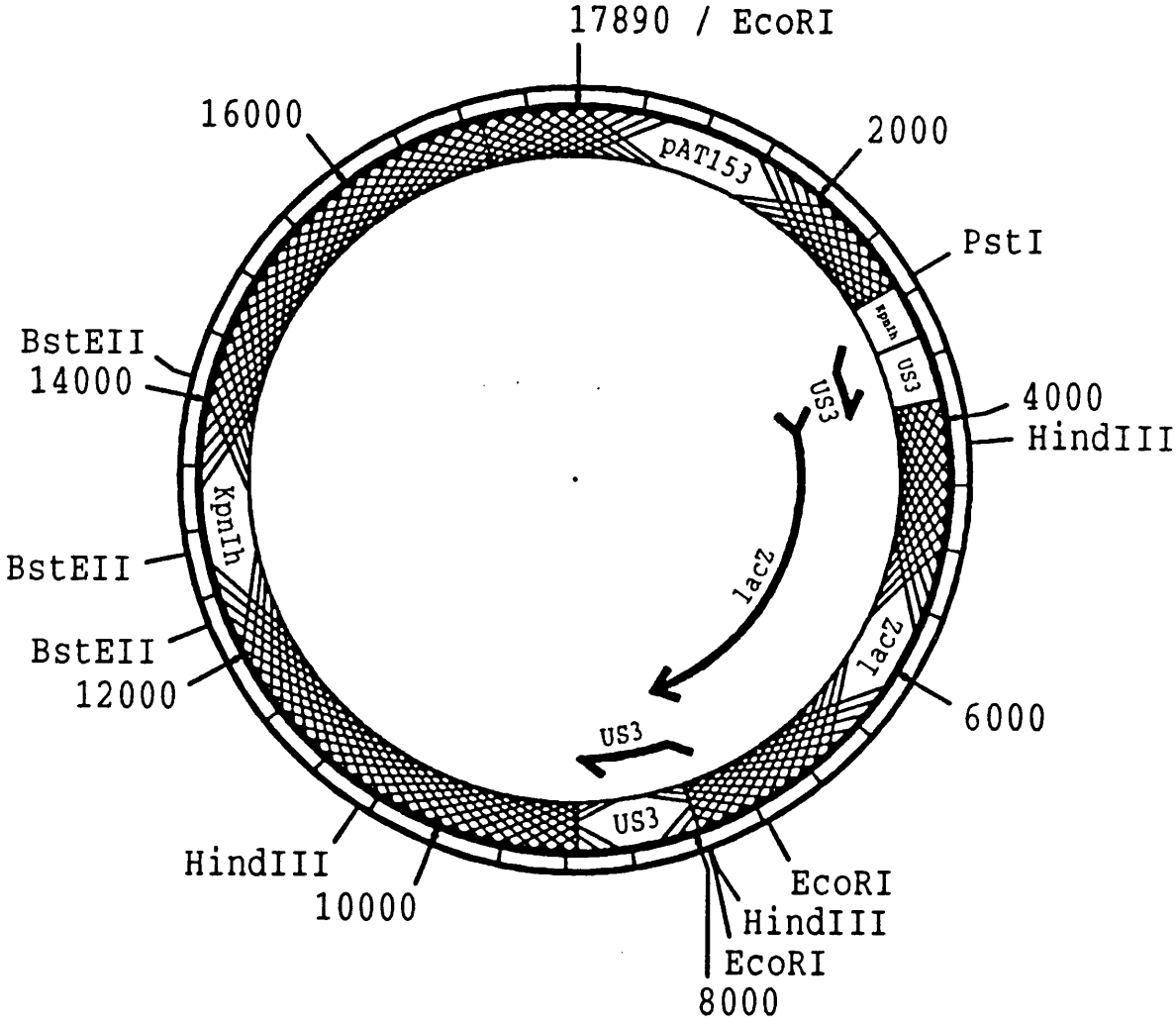
Out of the ten blue plaques which were picked, only two yielded blue progeny and these were heavily contaminated with *wt* virus. These plaques were therefore carried through a

Figure 4.3 Structure of pJM1A.

The structure of pJM1A was drawn using the PLASMIDMAP program. The scale is in nucleotides and starts at the first nucleotide of the *Eco*RI site in the vector pAT153. The plasmid is 17890 nucleotides in length.

The position and orientation of the *lacZ* insert, within the US3 gene, is shown (nucleotides 3848-7965).

The positions of several restriction enzyme sites are also indicated (*Bst*EII, *Eco*RI, *Hin*dIII and *Pst*I). The DNA fragment from the *Bst*EII site at 14149-14155 to the site at 12307-12313 was used for cotransfection.


17890 / EcoRI
2000
PstI
4000
HindIII
6000
EcoRI
HindIII
EcoRI
8000
10000
HindIII
12000
BstEII
BstEII
BstEII
14000
16000
pAT153
KpnIh
US3
US3
US3
US3
lacZ
lacZ

further 4 rounds (for the A54 and A57 isolates) or 5 rounds (for the A64 isolates) of plaque purification and stocks were grown of three purified isolates designated A54, A57 and A64. The genome structures of these viruses were checked by restriction enzyme analysis (see below).

It should be noted that these three isolates originated from the same co-transfection plate and therefore cannot be regarded as independent.

4.1.3. Restriction Enzyme Analysis of US3-*lacZ* Virus DNA

The genome arrangement of the US3-*lacZ* isolates was checked by restriction enzyme analysis of ^{32}P-labelled DNA, as described in section 3.4.3. The DNA was cut with *Eco*RI or *Hin*dIII and the fragments separated by electrophoresis on 0.8% agarose gels.

The position of *Eco*RI restriction sites in the *wt* genome and the US3-*lacZ* genome are shown in Figure 4.4. The US3 gene lies within the *Eco*RI *h* fragment. The insertion of the *lacZ* gene introduces two novel *Eco*RI sites resulting in the loss of the *h* fragment (15.2 kbp) and the generation of three novel fragments, one of 11.0 kbp (N''), which runs below the *j* fragment (12.6 kbp) on an agarose gel, one of 7.8 kbp (N'), which runs above the *k* fragment (5.6 kbp) and a small fragment of 0.5 kbp. These differences in the restriction enzyme profile of A54, A57 and A64 compared to *wt* are shown

Figure 4.4 **Location of *Eco*RI restriction sites in the *wt* and the US3-*lacZ* genomes.**

The genomes of HSV-1 *wt* (a) and the US3-*lacZ* virus (b) are shown, with the unique regions represented by solid lines and the repeat elements as open boxes. The dotted line represents the boundary between the L and the S segments.

The vertical lines mark the position of *Eco*RI restriction sites and the letters represent the names of individual restriction fragments (Davison, 1981). N' and N'' indicate the location of novel fragments in the US3-*lacZ* genome.

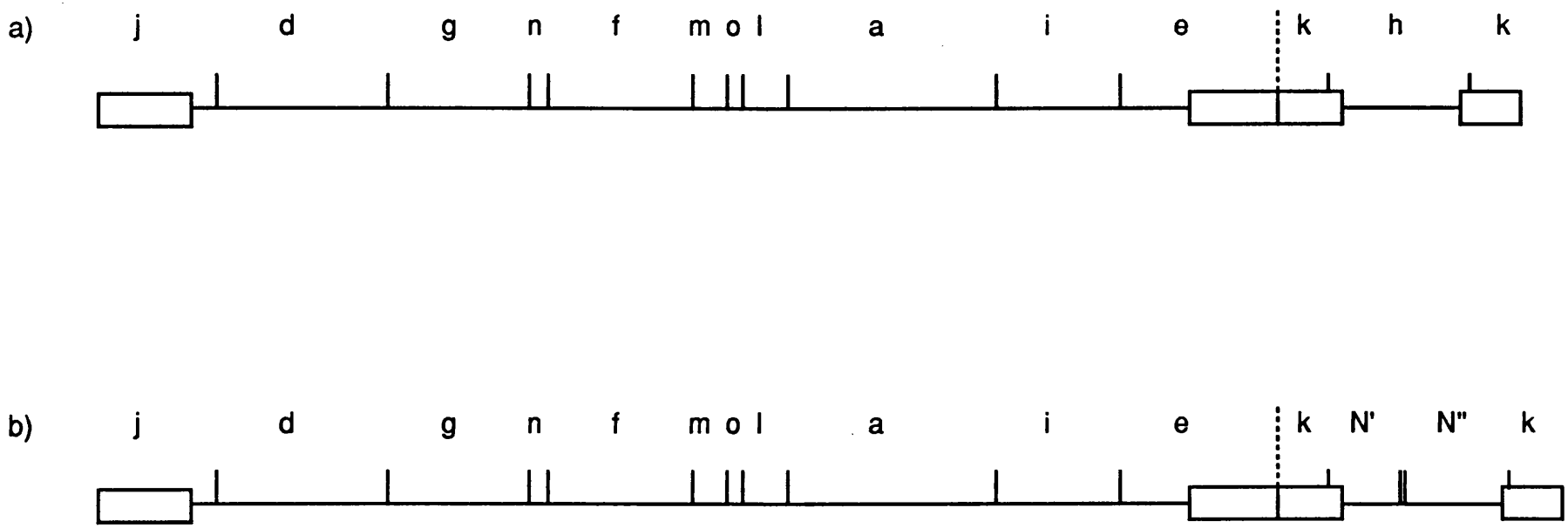
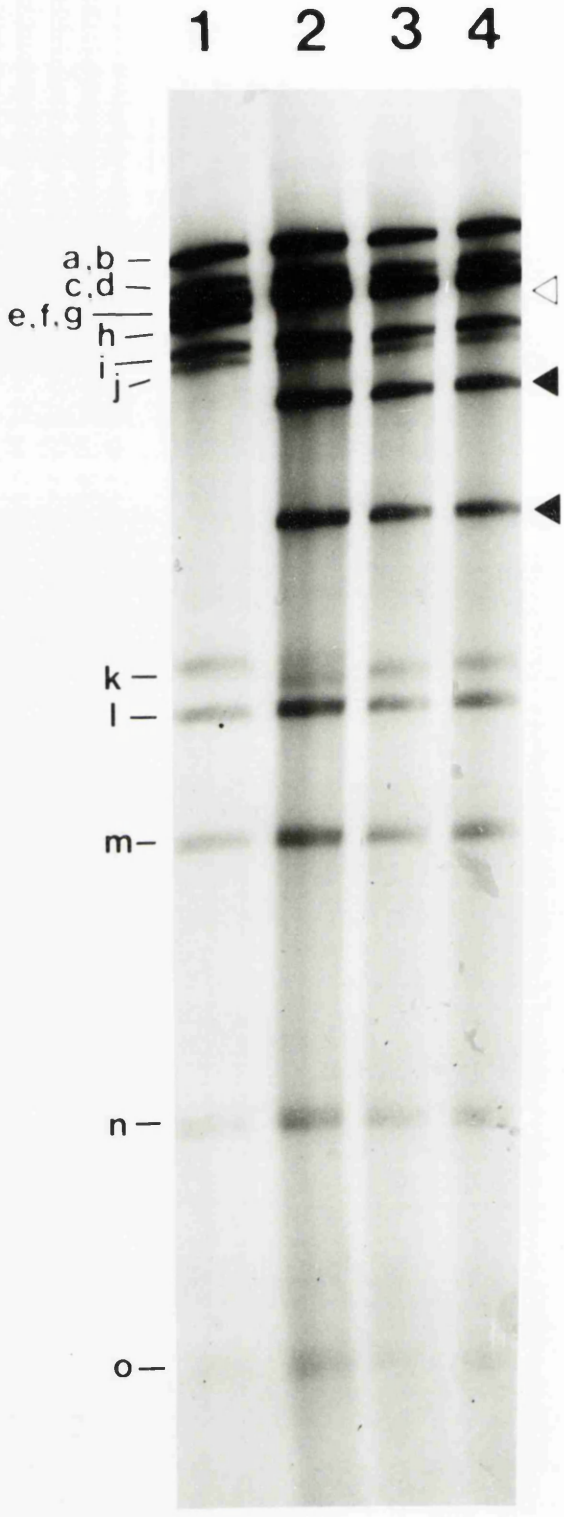

a)
j
d
g
n
f
m o l
a
i
e
k
h
k
b)
j
d
g
n
f
m o l
a
i
e
k
N'
N"
k

Figure 4.5 ***Eco*RI restriction profile of *wt* virus and the US3-*lacZ* isolates.**

^{32}P-labelled viral DNA was cut with *Eco*RI and the fragments were separated on a 0.8% agarose gel. The gel was dried and exposed to X-Omat XS-1 film. Lane 1: *wt* virus, lanes 2, 3 and 4; A54, A57 and A64, respectively. The names of the *wt* restriction fragments are to the left of the *wt* lane. Empty arrowheads (▷) indicate *wt* fragments which have been lost while filled arrowheads (▶) mark the locations of novel fragments.


1
2
3
4
a,b
c,d
e,f,g
h
i
j
k
l
m
n
o

in Figure 4.5, except for the band representing the 0.5 kbp fragment which is presumed to have run off the bottom of the gel.

The position of the *Hin*dIII restriction sites in the *wt* and the US3-*lacZ* genomes is shown in Figure 4.6. Digestion with *Hin*dIII results in the loss of the *wt n* fragment (4.9 kbp) and the generation of three novel fragments; one of 3.8 kbp (N''), which runs just below the *n* fragment on an agarose gel, and two of 2.6 kbp (N' and N'''), which run together to form a two-molar band below the *o* fragment (2.9 kbp). The loss of the *n* fragment, the appearance of a band below *n*, and the presence of a two-molar band below *o* is shown in Figure 4.7.

The observed differences in the mobility of the *k* fragment in the *Eco*RI digest and the *g* and *m* fragments in the *Hin*dIII digest are unrelated to the *lac*Z insertion. They occur because these fragments encompass the short repeat regions of the genome (IR_S and TR_S) which contain short tandemly reiterated sequences, the number of which varies between virus isolates (Davison & Wilkie, 1981; Watson *et al.*, 1981; Murchie & McGeoch, 1982; Rixon *et al.*, 1984).

In both Figures 4.5 and 4.7 it is apparent that all restriction fragments are not present in equal quantities. This is due to the isomerization of the HSV-1 genome. Restriction fragments which do not include the termini or

Figure 4.6 **Location of *Hin*dIII restriction sites in the *wt* and the US3-*lacZ* genomes.**

The genomes of HSV-1 *wt* (a) and the US3-*lacZ* virus (b) are shown, with the unique regions represented by solid lines and the repeat elements as open boxes. The dotted line represents the boundary between the L and the S segments.

The vertical lines mark the position of *Hin*dIII restriction sites and the letters represent the names of individual restriction fragments (Wilkie, 1976; Davison, 1981).

N', N'' and N''' indicate the location of novel fragments in the US3-*lacZ* genome.

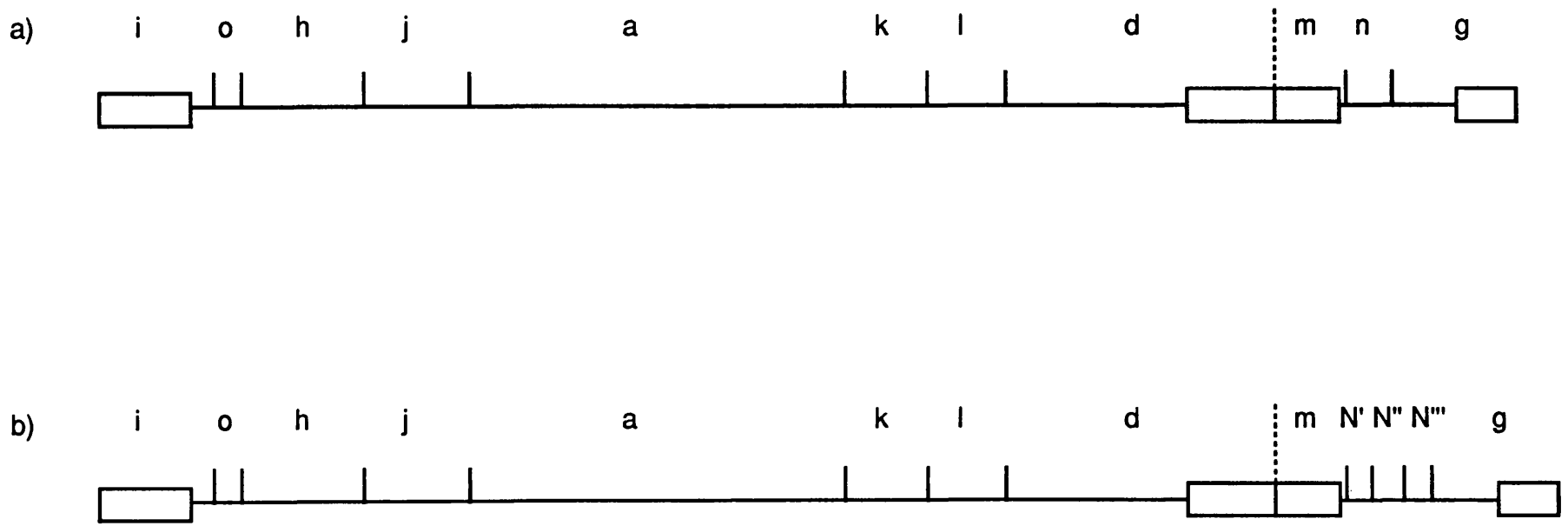
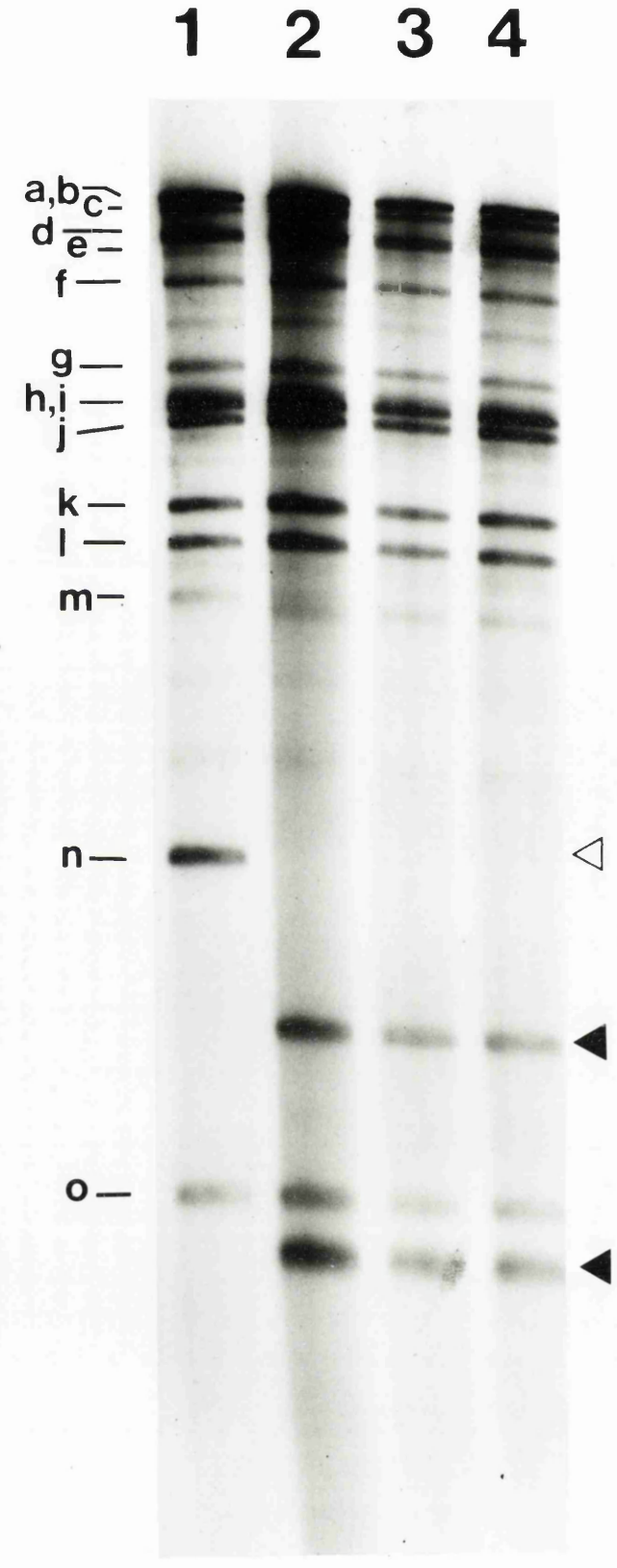

a)
i
o
h
j
a
k
l
d
m
n
g
b)
i
o
h
j
a
k
l
d
m
N'
N"
N'"
g

Figure 4.7 ***Hin*dIII restriction profile of *wt* virus and the US3-*lacZ* isolates.**

^{32}P-labelled viral DNA was cut with *Hin*dIII and the fragments were separated on a 0.8% agarose gel. The gel was dried and exposed to X-Omat XS-1 film. Lane 1: *wt* virus, lanes 2, 3 and 4; A54, A57 and A64, respectively. The names of the *wt* restriction fragments are to the left of the *wt* lane. Empty arrowheads (▷) indicate *wt* fragments which have been lost while filled arrowheads (▶) mark the location of novel fragments.


1
2
3
4
a,b
c
d
e
f
g
h,i
j
k
l
m
n
o

span the L-S junction, such as *Eco*RI *h* and *Hin*dIII *n*, are present in all four isomers and therefore appear as 1M bands. Restriction fragments which include the termini, such as *Eco*RI *k*, *e*, *j* and *Hin*dIII *i*, *d*, *m*, *g* are present in only two of the four possible isomers and therefore appear as 0.5M bands. The L-S junction fragments such as *Hin*dIII *c*, *b*, *f*, *e* form 0.25M bands as they are present in only one isomer.

The results of these digests confirm that the *lacZ* gene was inserted at the correct position and that no other major alterations of the genome have occurred.

4.2. GROWTH PROPERTIES OF THE US3-*lacZ* VIRUS

The fact that three US3-*lacZ* isolates had been plaque purified and stocks grown indicated that the US3 gene is not essential for virus growth in tissue culture. The growth characteristics of the US3-*lacZ* isolates were compared with those of *wt* HSV-1.

4.2.1. Plaque Morphology

The plaques produced by all three US3-*lacZ* isolates differed from those produced by the *wt* virus in that they were noticeably smaller.

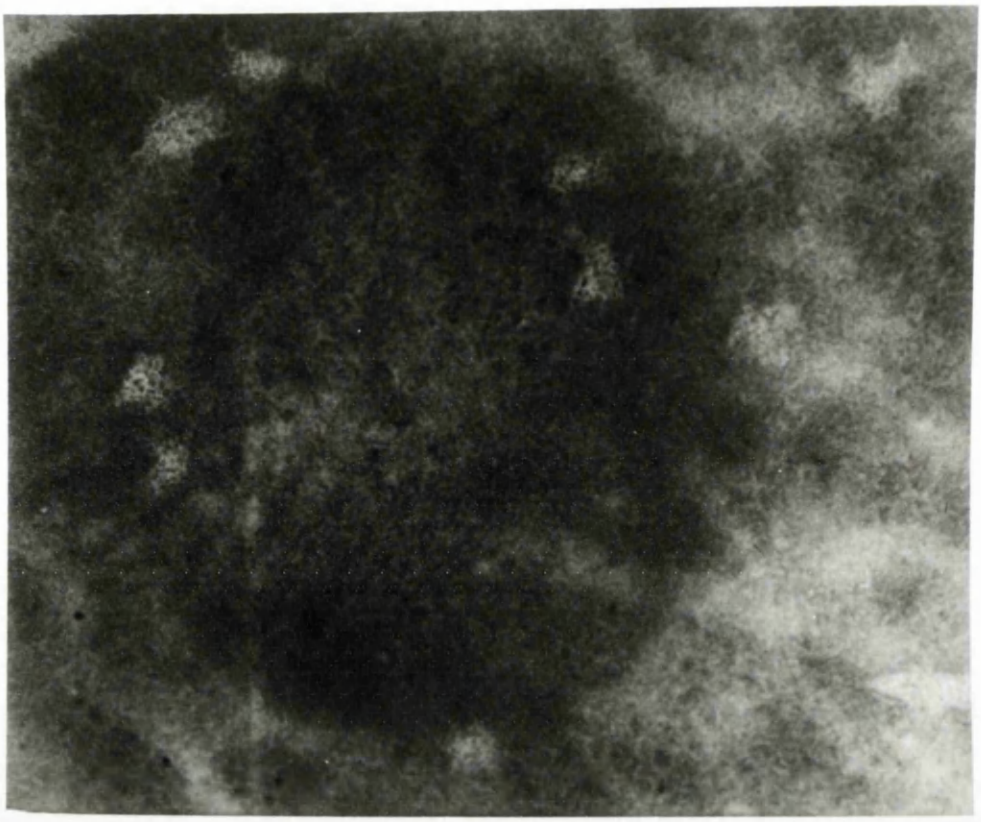
The diameters of 20 *wt* and 20 US3-*lacZ* plaques were measured and the means calculated. The US3-*lacZ* plaques were found to be approximately half the diameter of *wt* plaques. In addition, the borders of the US3-*lacZ* plaques were more distinct (Figure 4.8).

Figure 4.8 Plaque morphology of the US3-*lacZ* virus.

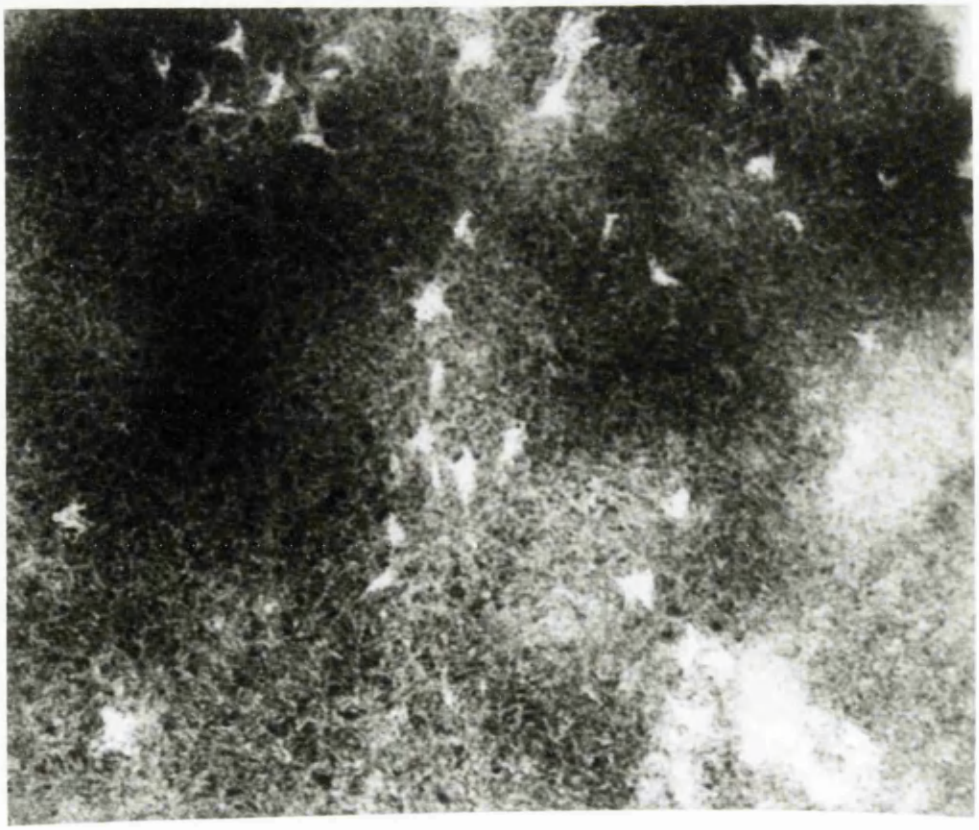
Morphology of (a) *wt* and (b) US3-*lacZ* A57 isolate plaques on BHK cells. The viruses were titrated in parallel and after two days were stained with Giemsa stain and photographed.

The bar represents approximately 2mm.

A



B



4.2.2. Growth Curve at a High moi

BHK cells were infected at 10 pfu/cell and harvested at the appropriate times, as described in section 3.1.8. The results of this one-step growth curve are shown in Figure 4.9.

The growth of the *wt* HSV-1 virus was as expected. The three US3-*lacZ* isolates, however, grew more slowly than *wt* reaching final yields which were 12-fold (A54), 8-fold (A57) or 4-fold (A64) less than that of *wt*. In order to determine if these differences between the growth of the three isolates were reproducible the experiment was repeated. The A54 isolate exhibited a 7-fold reduction in growth compared to *wt*, the A57 isolate a 6-fold reduction and the A64 isolate a 7-fold reduction (data not shown). Thus, the differences in the growth properties of the three US3-*lacZ* isolates do not appear to be significant.

It is therefore concluded that disruption of the US3 gene results in a 4- to 12-fold reduction in virus yield, following infection at a high multiplicity.

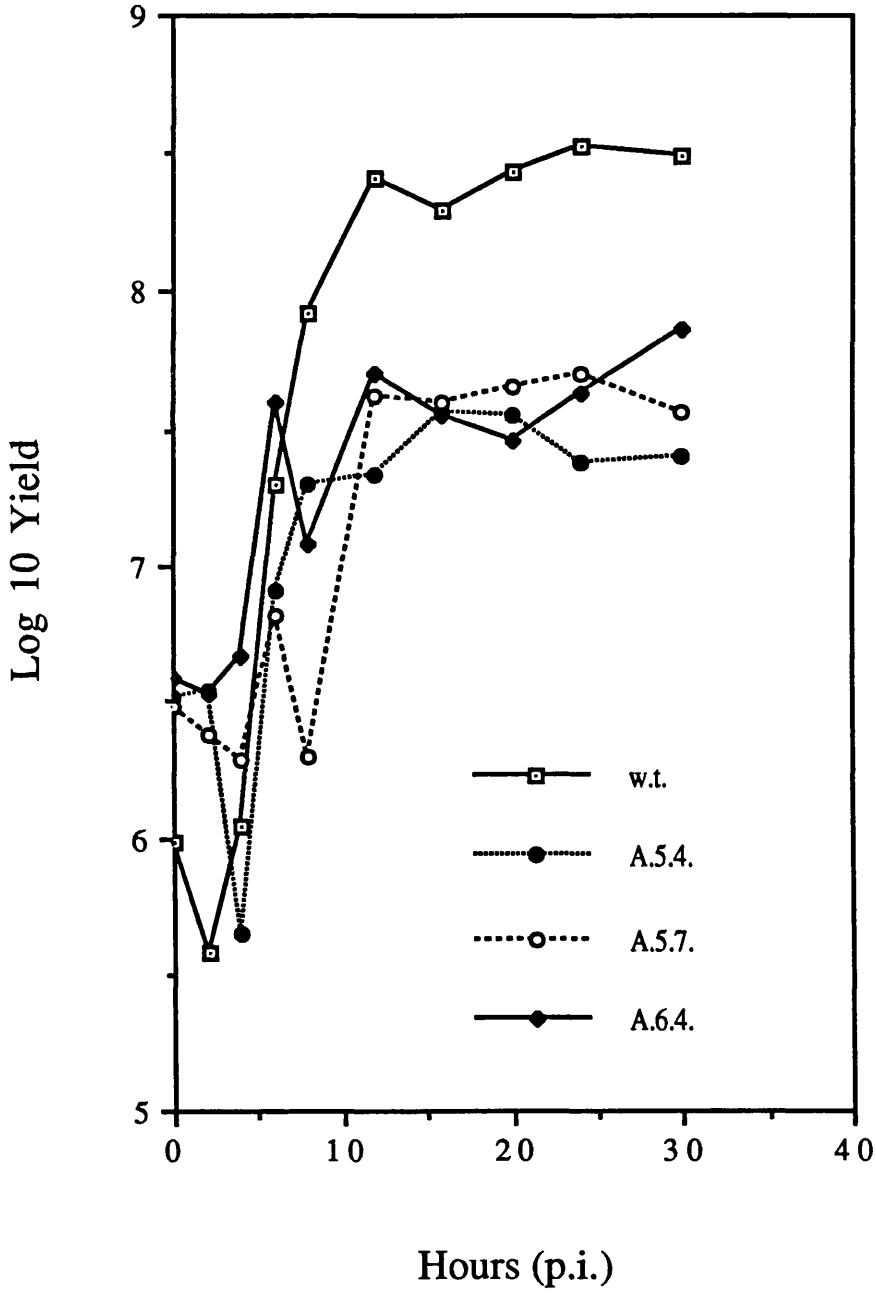
4.2.3. Growth Curve at a Low moi

Cells were infected at 0.001 pfu/cell and harvested over a period of 72h. The final yields therefore represent the result of several rounds of virus replication. The results

Figure 4.9 **Growth curve of US3-*lacZ* virus at a high moi.**

BHK cells were infected with *wt* virus or one of the three US3-*lacZ* isolates (A54, A57 or A64) at a moi of 10, incubated at 37°C and harvested over a period of 30h. The samples were subjected to ultrasonic disruption and were then titrated.

The virus yield (y axis) represents the titre (pfu/ml) x sample volume (ml).


9
8
7
6
5
0
10
20
30
40
Log 10 Yield
Hours (p.i.)
w.t.
A.5.4.
A.5.7.
A.6.4.

are shown in Figure 4.10.

The curves for the US3-*lacZ* isolates showed that their growth was impaired compared to *wt*, with decreases in the final yield of 41-fold for A54, 10-fold for A57 and 47-fold for A64. This experiment was not repeated and it is therefore not known if the higher titre obtained for the A57 isolate is reproducible. The reductions for the A54 and A64 isolates are considerably greater than those observed following infection at a high multiplicity. This is not unexpected considering that the virus, which exhibits reduced titres following one round of replication (see 4.2.2.) has undergone several more rounds of replication with less infectious virus entering each subsequent cycle.

Thus, the growth curves at both high and low moi indicate that although the US3 gene is not absolutely required for virus growth, its absence does cause a significant reduction in virus growth on BHK cells.

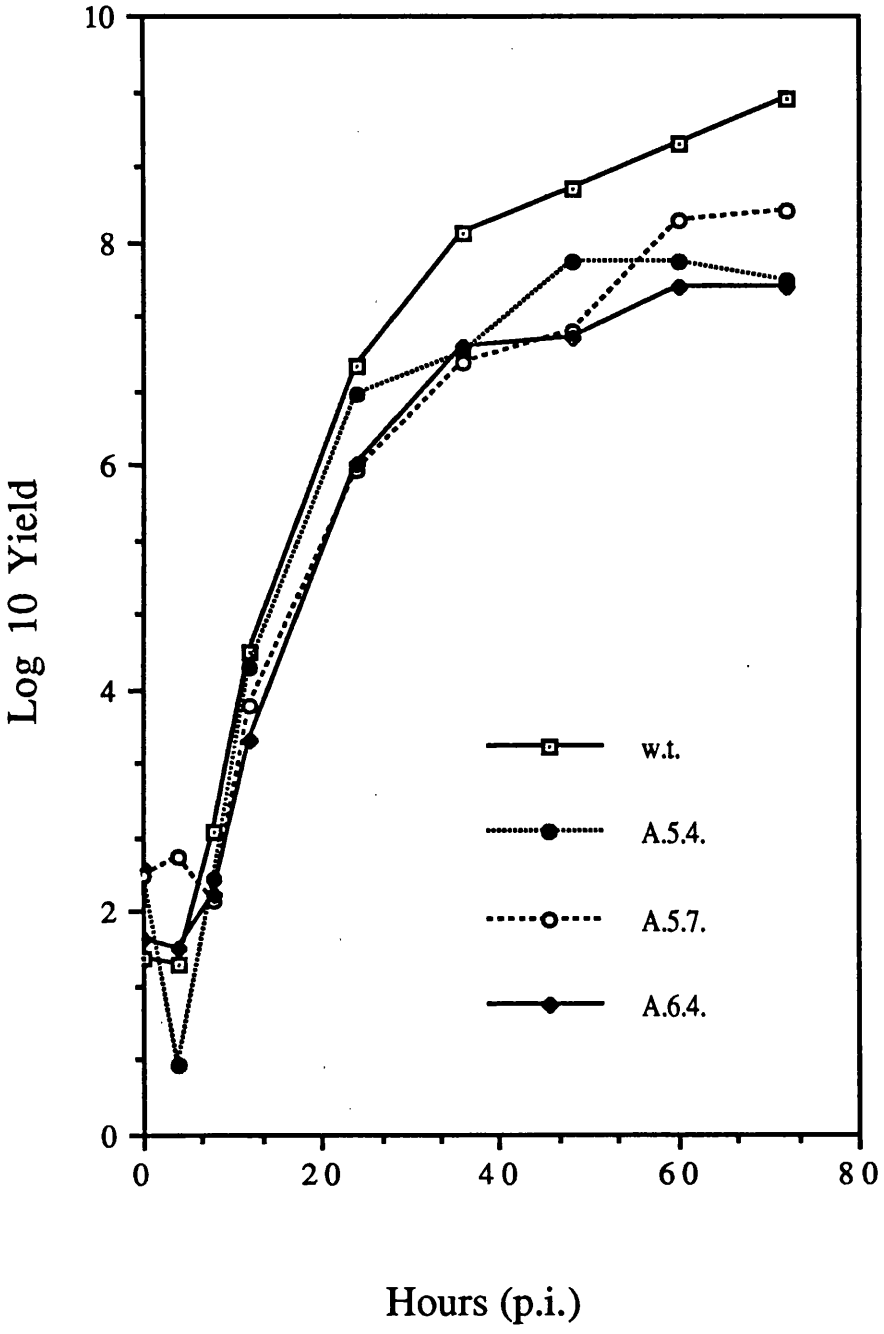
4.2.4. Investigation of US3-*lacZ* Virus Growth During the Lag (Eclipse) Phase

During the lag phase of virus growth (approximately 0-5h p.i.) the virus enters the cell, is uncoated, and is transported to the nucleus, prior to the onset of replication and transcription. The virus is not infectious during these early stages which accounts for the decrease in virus yield

Figure 4.10 Growth curve of US3-*lacZ* virus at a low moi.

BHK cells were infected with *wt* virus or one of the three US3-*lacZ* isolates (A54, A57 or A64) at a moi of 0.001, incubated at 37°C and harvested over a period of 72h. The samples were subjected to ultrasonic disruption and were then titrated.

The virus yield (y axis) represents the titre (pfu/ml) x sample volume (ml).


10
8
6
4
2
0
0
20
40
60
80
Log 10 Yield
Hours (p.i.)
w.t.
A.5.4.
A.5.7.
A.6.4.

observed at this time. However, it is apparent from figures 4.9 and 4.10 that the decrease in the yield of the US3-*lacZ* isolates during the lag phase was not as great as that observed for the *wt* virus. One possible explanation for this observation is that the US3-*lacZ* virus is slightly impaired for entry into cells. This phase of virus growth was therefore investigated more thoroughly by infecting cells at a high moi and harvesting samples every 30 min for the first 5h p.i. The results are shown in Figure 4.11.

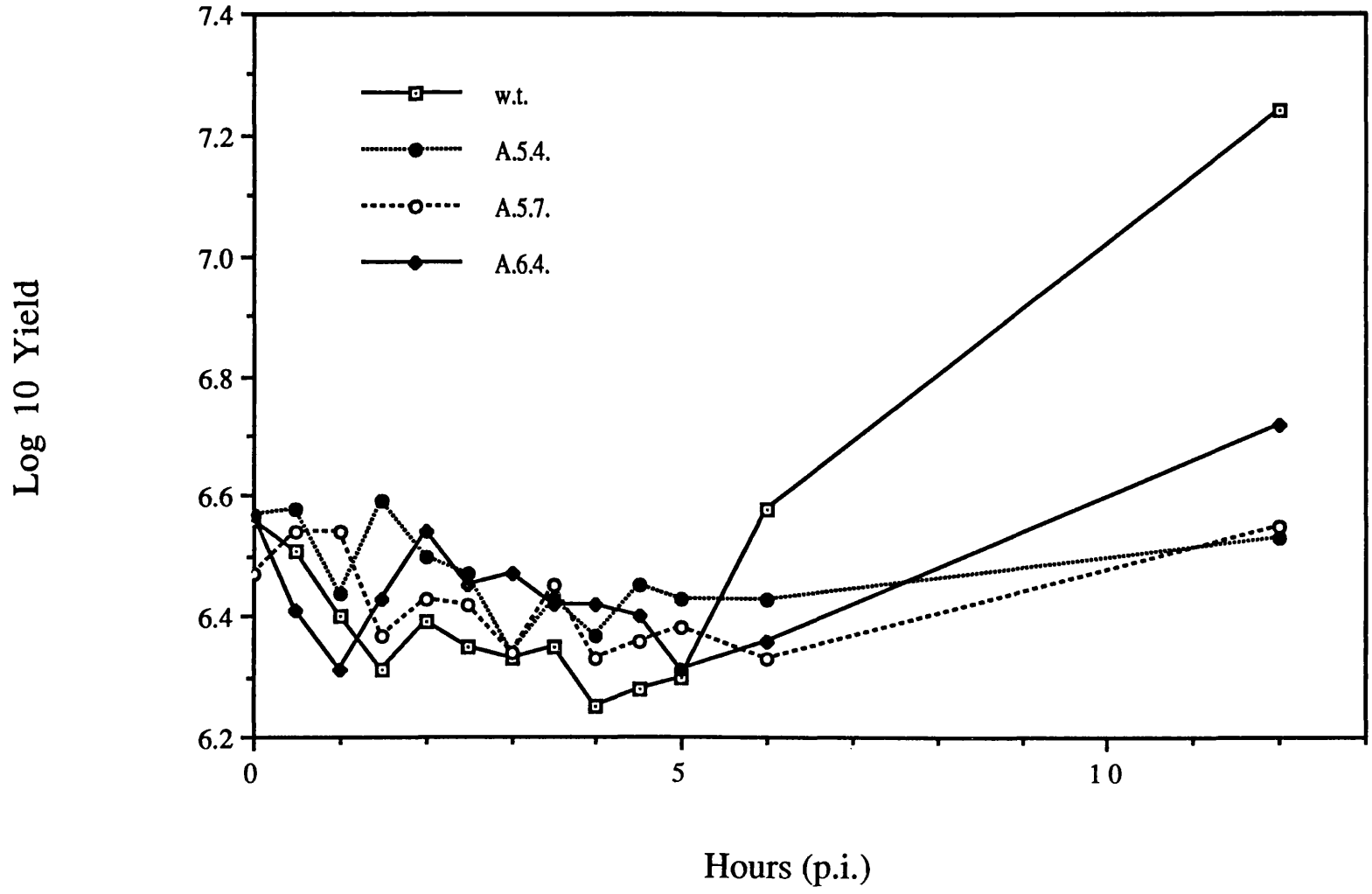
The growth curves show that at most points during the first 5h p.i. the titres obtained for the three US3-*lacZ* isolates are higher than those of *wt*, supporting the suggestion that the US3-*lacZ* virus does not enter cells as efficiently as *wt*. However, the differences observed during this experiment are not as great as those seen during the high moi growth curve (Figure 4.9). This experiment should therefore be repeated before a final conclusion is reached.

Other means of investigating virus entry into cells include using [^{3}H]-thymine-labelled virus preparations, to determine the rate of adsorption of the virus particles to the cell monolayer, and using Southern blot hybridization on nuclei preparations in order to determine the rate at which viral DNA migrates to the nucleus (Ace, 1989).

Figure 4.11 Growth of US3-*lacZ* virus during the lag (eclipse) phase.

BHK cells were infected with *wt* virus or one of the three US3-*lacZ* isolates (A54, A57 or A64) at a moi of 10, incubated at 37°C and harvested over a 12h period, with samples taken every 30 min for the first 5h p.i. The samples were subjected to ultrasonic disruption and titrated.

The virus yield represents the titre (pfu/ml) x sample volume (ml).


Log 10 Yield
7.4
7.2
7.0
6.8
6.6
6.4
6.2
w.t.
A.5.4.
A.5.7.
A.6.4.
0
5
10
Hours (p.i.)

4.2.5. Growth of US3-*lacZ* Virus at Different Temperatures

The sensitivity of the US3-*lacZ* virus to different growth temperatures was determined.

BHK cells were infected at a moi of 10 and incubated, in duplicate, at 31°C, 37°C, 38.5°C or 39.5°C overnight. The samples were harvested and titrated at 37°C. The results are shown in Table 4.1.

The growth of the *wt* virus was optimal at 37°C, with similar yields also obtained at 38.5°C. The yields at both 31°C and 39.5°C were slightly reduced.

The yields of the US3-*lacZ* isolates were consistently lower than those of *wt* at all temperatures. Optimal growth was achieved at 31°C and 37°C with the yields decreasing at the higher temperatures (38.5°C and 39.5°C).

The differences between the *wt* and the US3-*lacZ* isolate yields at each temperature, have been expressed as the average ratio of *wt* yield:isolate yield (Table 4.1). The ratios at 37°C and 38.5°C for all three isolates are similar to each other and lie within the range expected from the growth curves. The ratios at 31°C however are consistently smaller than those at 37°C or 38.5°C, reflecting the fact that while the *wt* yield has approximately halved, the US3-*lacZ* yields are unchanged. This situation is reversed at

Table 4.1 Growth of US3-*lacZ* virus at different temperatures

BHK cells were infected at a moi of 10 with *wt* or one of the three US3-*lacZ* isolates (A54, A57 and A64) and incubated overnight at 31°C, 37°C, 38.5°C or 39.5°C. The infected cells were subjected to ultrasonic disruption and titrated at 37°C.

The virus yield was calculated by multiplying the titre by the sample volume.

The growth of the US3-*lacZ* isolates at each temperature was compared with that of *wt* by calculating the ratio of *wt* yield:US3-*lacZ* yield.

Temp.	Expt. No.	Virus Yield (p.f.u./ml)				Ratio of Yields		
		wt	A54	A57	A64	wt : A54	wt : A57	wt : A64
31°C	1	6.9×10^7	1.3×10^7	1.3×10^7	8.9×10^6	4.4	5.0	6.9
	2	3.3×10^7	9.7×10^6	7.1×10^6	5.4×10^6			
37°C	1	1.3×10^8	9.6×10^6	7.9×10^6	8.5×10^6	12.0	13.6	13.4
	2	8.1×10^7	7.8×10^6	7.7×10^6	7.0×10^6			
38.5°C	1	1.1×10^8	8.4×10^6	8.1×10^6	1.1×10^7	13.6	14.7	10.1
	2	6.7×10^7	4.8×10^6	4.3×10^6	6.6×10^6			
39.5°C	1	5.5×10^7	3.2×10^6	1.1×10^6	1.9×10^6	15.7	36.9	22.8
	2	2.8×10^7	2.0×10^6	1.2×10^6	1.7×10^6			

39.5°C where the decrease in the US3-*lacZ* yields is greater than that of *wt* resulting in higher ratios.

These results suggest that while the US3-*lacZ* isolates do not display a marked *ts* phenotype they do appear to be slightly more tolerant to low temperatures and more sensitive to high temperatures than the *wt* virus.

4.2.6. Growth of US3-*lacZ* Virus on Different Cell Types

The US3-*lacZ* isolates were grown on six different cell types to see if they exhibited a host range phenotype. Cells were infected at a moi of 10, incubated overnight at 37°C, harvested and titrated. The yields are shown in Table 4.2.

The US3-*lacZ* virus, like the *wt* virus, grew on all the cell types tested. However, there was considerable variation in the yields obtained on the different cells. The *wt* virus grew well on BHK, HFL, Flow 2002 and Vero (source 2) cells but grew poorly on 3T6, BSC-1 and Vero (source 1) cells. The US3-*lacZ* isolates followed a similar pattern of growth except for the unexpectedly low yields observed on the HFL (source 2) cells.

The differences between the *wt* yields and the US3-*lacZ* yields on each cell type, expressed as the ratio of *wt* yield:US3-*lacZ* yield, are also shown in Table 4.2. The ratios for the BHK, HFL (source 1) and Flow 2002 cells all

Table 4.2 Growth of US3-*lacZ* virus on different cell types

BHK, HFL, Flow 2002, 3T6, BSC-1 and Vero cells were infected at a moi of 10 with *wt* or one of the three US3-*lacZ* isolates (A54, A57 and A64) and incubated overnight at 37°C. The infected cells were harvested and titrated on BHK cells.

All of the BHK, Flow 2002, 3T6 and BSC-1 experiments were carried out on cells which originated from the same frozen stock (within the Institute). For the HFL cells, "source 1" and "source 2" represent cells from two different frozen stocks. For Vero cells, "source 1" represents cells from a frozen stock while "source 2" represents cells obtained commercially.

Virus yield represents the titre multiplied by the sample volume.

The growth of the US3-*lacZ* isolates on each cell type was compared with that of *wt* by calculating the ratio of *wt* yield:US3-*lacZ* isolate yield.

Cell Type	Source *	Virus Yield (p.f.u./ml)				Ratio of Yields		
		wt	A54	A57	A64	wt : A54	wt : A57	wt : A64
BHK	1	6.2×10^8 7.8×10^7 4.0×10^8	5.2×10^7 7.6×10^6 4.7×10^7	5.3×10^7 3.6×10^6 5.5×10^7	6.6×10^7 8.9×10^6 5.6×10^7	10.3	13.6	8.4
HFL	1 2	2.8×10^8 1.7×10^8	2.4×10^7 3.5×10^6	2.5×10^7 2.8×10^6	1.9×10^7 3.1×10^6	11.1 47.3	10.8 59.5	12.8 52.9
Flow2002	1	1.8×10^8	1.8×10^7	1.9×10^7	2.3×10^7	10.0	9.5	7.6
3T6	1	3.3×10^6 4.6×10^6	3.7×10^6 3.1×10^6	2.3×10^6 3.2×10^6	1.5×10^6 1.9×10^6	1.2	1.4	2.3
BSC-1	1	4.4×10^7 6.2×10^7	8.6×10^6 2.2×10^7	7.2×10^6 1.2×10^7	1.1×10^7 3.8×10^7	4.0	2.9	1.0
Vero	1	2.0×10^6 1.5×10^7	5.8×10^6 5.0×10^6	2.5×10^6 5.9×10^6	8.9×10^5 6.1×10^6	1.7	1.7	2.4
	2	2.7×10^8	5.2×10^7	4.7×10^7	4.1×10^7	5.1	5.7	6.6

lie within the range expected from previous experiments with BHK cells, while the HFL (source 2) cells gave much larger ratios (47.3-59.5) due to the lower US3-*lacZ* yields. The 3T6, BSC-1 and Vero (source 1) cells all gave very low ratios. However, on the Vero (source 2) cells, which supported good *wt* growth, a reduction in the growth of the US3-*lacZ* virus compared to *wt* became apparent, with ratios ranging from 5.1 to 6.6. Thus, the low ratios obtained on the 3T6, BSC-1 and Vero cells are probably a consequence of the overall low virus yields obtained on these cells.

4.2.7. Growth of US3-*lacZ* Virus on "Resting" Cells

The previous growth experiments have demonstrated that the US3 gene product is not required for virus growth on exponentially growing BHK cells. However, it is known that exponentially growing cells can supply factors which are able to compensate for an introduced mutation. For example, Jamieson *et al.* (1974) have shown that an HSV-1 mutant which lacks any thymidine kinase activity can grow well on exponentially growing BHK cells but can not grow on serum-starved "resting" cells. This has important implications for the growth of the mutant *in vivo*, as it has been suggested that the cells infected with HSV-1 in natural infections are more likely to resemble "resting" than "non-resting" cells. Thus, the growth of the US3-*lacZ* virus on "resting" cells was investigated.

Both "resting" and exponentially growing BHK cells were infected with HSV-1 *wt* or the US3-*lacZ* isolates at a moi of 10, incubated at 37°C overnight, harvested and titrated in duplicate. The results are shown in Table 4.3.

In this experiment the *wt* yields obtained from the exponentially growing cells were much lower than expected from previous experiments. The US3-*lacZ* isolates, however, do not appear to have been similarly affected. Thus, the corresponding *wt*:US3-*lacZ* isolate ratios are considerably lower (maximum 1.4-fold reduction) than those obtained in the high moi growth curve (maximum 12-fold reduction, see section 4.2.2.).

On "resting" cells, the *wt* yields were approximately two-fold greater than those obtained on the exponentially growing cells. This difference is thought to be due more to the uncharacteristic reduced growth of *wt* on the exponentially growing cells than to a preference of *wt* for "resting" cells. Comparison of the *wt* and US3-*lacZ* isolate yields obtained on "resting" cells shows that the growth of the US3-*lacZ* virus is only slightly impaired compared to *wt* (a maximum of 4.6-fold). (It should be noted that one plate, infected with the A64 isolate, gave a much reduced yield of 4.2×10^5 pfu/ml. It is thought likely that the cells on this plate had died during the seven day incubation.) Thus, it is evident that the ability of the US3-*lacZ* virus to grow on BHK cells is not dependent upon a factor which is present only in

Table 4.3 Growth of US3-*lacZ* virus on "resting" cells

Exponentially growing and "resting" BHK cells were infected at a moi of 10 with *wt* or one of the three US3-*lacZ* isolates (A54, A57 and A64). After an overnight incubation at 37°C the infected cells were harvested and titrated.

Virus yields represent the virus titre multiplied by the sample volume.

The growth of the US3-*lacZ* isolates were compared with that of *wt* by calculating the ratio of *wt* yield:US3-*lacZ* isolate yield.

* indicates an unusually low yield which was ignored when the ratios were calculated.

Cell Type	Virus Yield				Ratio of Yields		
	wt	A54	A57	A64	wt : A54	wt : A57	wt : A64
Exponential	9.1×10^7	7.6×10^7	5.7×10^7	5.1×10^7	0.98	1.1	1.3
	6.6×10^7	8.3×10^7	9.0×10^7	6.9×10^7			
"Resting"	2.0×10^8	3.7×10^7	4.4×10^7	$^*4.2 \times 10^5$	4.6	4.5	3.8
	1.1×10^8	3.1×10^7	2.5×10^7	4.1×10^7			

exponentially growing cells. These results also suggest that the US3-*lacZ* virus will be viable *in vivo*.

4.3. PARTICLE COUNTS

The number of particles present in SV stocks of *wt* virus and the US3-*lacZ* isolates were calculated and compared with the titres to give particle:pfu ratios. The results are shown in Table 4.4. While the number of particles in the US3-*lacZ* stocks were similar to those in the *wt* stocks, the titres were less, resulting in higher particle:pfu ratios for the US3-*lacZ* isolates. In order to determine if these differences in the particle:pfu ratios are significant the results were analysed statistically.

One of the statistical methods frequently used to analyse differences between two populations is comparison of the two population means using the Student's *t*-test. The conditions of this test are that the measurements have a normal distribution and that the variances (i.e. the scatter of values around the mean) of the two groups are equal. This is tested statistically using the F-test (Wardlaw, 1985);

$$F = \frac{s_1^2}{s_2^2}$$

Table 4.4 Particle:pfu ratios of US3-*lacZ* isolates

The number of particles/ml present in three stocks of the *wt*, A54, A57 and A64 viruses were divided by the corresponding titres to give the particle:pfu ratios. The *wt* and US3-*lacZ* isolate ratios were then compared by calculating the ratio of mean isolate ratio:mean *wt* ratio ($\bar{x}$ isolate : $\bar{x}$ *wt*).

Particle counts were carried out by Mr J Aitken.

Virus	Stock	Particles (/ml)	Titre (p.f.u./ml)	Particle:pfu ratio	Mean particle: pfu ratio ($\bar{x}$)	$\bar{x}_{isolate}/\bar{x}_{wt}$
wt	1	5.9×10^{10}	3.5×10^{9}	17:1	29.33	
	2	2.6×10^{11}	7.3×10^{9}	36:1		
	3	1.1×10^{11}	3.1×10^{9}	35:1		
A54	1	2.4×10^{11}	1.1×10^{9}	215:1	325.0	11.1
	2	9.6×10^{10}	2.4×10^{8}	399:1		
	3	1.3×10^{11}	3.6×10^{8}	361:1		
A57	1	1.3×10^{11}	1.1×10^{9}	115:1	269.33	9.2
	2	1.0×10^{11}	3.9×10^{8}	266:1		
	3	1.9×10^{11}	4.4×10^{8}	427:1		
A64	1	9.0×10^{10}	2.9×10^{9}	31:1	122.67	4.2
	2	7.9×10^{10}	4.4×10^{8}	177:1		
	3	1.0×10^{11}	6.4×10^{8}	160:1		

where S^2 (variance) = $\frac{\Sigma x^2 - (\Sigma x)^2/n}{(n-1)}$

x = observations

n = no. of observations

The calculated value of F is then compared with tabulated values of F (Wardlaw, 1985) at $f_1 = n_1-1$ degrees of freedom in the numerator and $f_2 = n_2-1$ in the denominator. The data for the F-test are shown in Table 4.5(a). The F values for the three US3-*lacZ* isolates, compared to *wt* all exceed the tabulated values ($P < 1\%$) and the results are therefore significant, that is, the variances of the US3-*lacZ* isolates are not equal to that of *wt* and the Students *t*-test cannot be applied.

One of the easiest ways of processing the data from two groups with unequal variances is to use the following approximation (Bailey, 1981):

$$d = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

where $\bar{x}$ = mean.

d is then treated as being distributed approximately like Student's t and the calculated value of d is compared with the tabulated values of t (Bailey, 1981) at the appropriate

Table 4.5 Statistical analysis of the particle:pfu ratios of *wt* and the US3-*lacZ* isolates

a) F-test (to determine if the variances of the *wt* ratios and the US3-*lacZ* isolate ratios were equal). The value of F was calculated for each isolate according to the formulae given in the text. The calculated values were then compared with the tabulated values of F and the significance of the results determined.

b) Comparison of the *wt* and US3-*lacZ* isolate population means using an approximation of the Student's t-test. The formulae used to calculate *d* are given in the text. (The values of S^2 used in these calculations are as given in (a).) The significance of the results was determined by comparing the calculated values of *d* with the tabulated values of *t*, at the appropriate d.f. (Bailey, 1981).

a)

Virus	s^2	F	d.f.	P(%)	Signifi-cance
wt	114.33				
A54	9436.0	82.53	2,2	P<1	sig.
A57	24344.3	212.93	2,2	P<1	sig.
A64	6374.33	55.75	2,2	P<1	sig.

b)

Virus	$\bar{x}$	d	u	d.f.	P(%)	Signifi-cance
A54	29.33	165.21	0.012	2.05	P<0.1	highly sig.
A57	325.0	268.35	0.005	2.02	P<0.1	highly sig.
A64	122.67	134.60	0.018	2.07	P<0.1	highly sig.

degree of freedom (d.f.) which is given by:

$$d.f. = \frac{1}{\frac{u^2}{n_1 - 1} + \frac{(1-u)^2}{n_2 - 1}}$$

$$\text{where} \quad u = \frac{s_1^2/n_1}{s_1^2/n_1 + s_1^2/n_2}$$

The calculated values of d, u and d.f., for this experiment, are shown in Table 4.5(b). As can be seen from this table, the differences between the means of the US3-*lacZ* isolates and *wt* are highly significant ($P < 0.1\%$). Thus, it is concluded that the US3-*lacZ* virus particles are significantly less infectious than the *wt* particles.

The reason for the increase in non-infectious particles in the US3-*lacZ* isolates is not known.

4.4. ANALYSIS OF US3-*lacZ* VIRUS-INDUCED POLYPEPTIDES

The insertion of the *lacZ* gene into the US3 ORF is expected to produce several changes in the polypeptide profile of the virus. These changes have been analysed using the FRAMES program, which displays all of the possible ORFs in the six translation frames (Figure 4.2). Since the *wt* US3 ORF is no longer present the *wt* US3 protein, with an apparent molecular

weight of 68K (Frame *et al.*, 1987), should be absent from the US3-*lacZ* virus polypeptide profile. Other potential changes predicted by this program include the presence of four novel ORFs (marked A-D, Figure 4.2). The first of these putative ORFs (A) extends from the *wt* US3 start codon to the in-frame stop codon which lies just past the *lacZ* insert boundary. Expression from this ORF is considered likely due to the presence of the upstream *wt* US3 promoter and enhancer sequences. The expressed protein will consist of the N-terminal 171 amino acids of the *wt* US3 protein and is predicted to have a M_r of approximately 19K. The second ORF (B), which corresponds to the *lacZ* gene, will also be expressed, under the control of the upstream SV40 promoter. The protein produced, β-galactosidase, will have a MW of approximately 120K. In contrast to the above two ORFs, expression of ORF C, which spans the *lacZ* boundary, and ORF D, which includes the intact *wt* US3 catalytic domain, is purely speculative, and would be dependent upon the presence of suitable upstream promoter elements.

The polypeptide profile of the US3-*lacZ* virus was closely examined for any evidence of these changes.

4.4.1. Analysis of [^{35}S]-methionine Labelled Infected Cell Extracts

BHK cells were mock-infected or infected with *wt* or the US3-*lacZ* isolates at a moi of 20. The cells were either labelled

with ^{35}S-methionine overnight or were pulse labelled from 4h to 7h p.i. or from 7h to 10h p.i. The proteins were separated on a 5%-12.5% gradient SDS-polyacrylamide gel (Fig. 4.12) or on a 9% SDS-polyacrylamide gel (data not shown).

No change was observed in the pattern of bands around 68K in the US3-*lacZ* isolate extracts, in either gel. This is not unexpected as polypeptide profiles are complex; the US3 protein may be present only in very small quantities or may comigrate with another protein. Only one of the possible novel proteins was observed, the 120K β-galactosidase protein which appears as a novel band below the 155K major capsid protein. There were no other discernable changes in the polypeptide profiles, suggesting that the disruption of the US3 protein kinase does not adversely affect the characteristic temporal pattern of protein synthesis of the virus.

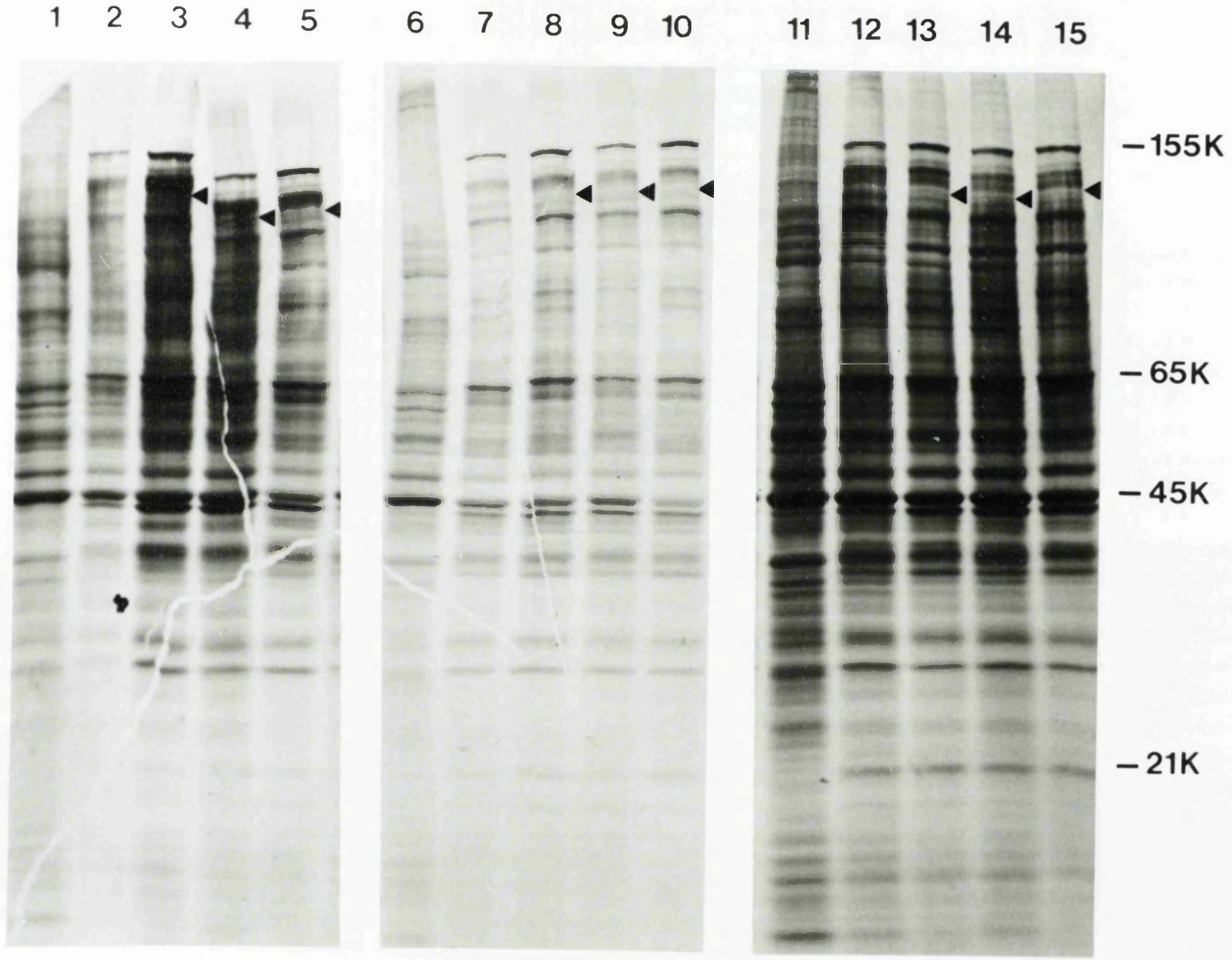
4.4.2. Analysis of Virion Peptides

Zhang *et al.* (1990) have recently reported that the US3 protein kinase is a constituent of HSV-1 virions. To investigate this further, HSV-1 *wt* and US3-*lacZ* A57 virions were prepared. Untreated samples of the virions were loaded on a 9% SDS-polyacrylamide gel, together with virions which had been treated with NP40 and phosphorylated *in vitro*. The section of the gel containing the untreated virions was silver-stained and the section containing the *in vitro*

Figure 4.12 **[^{35}S]-methionine-labelled polypeptides induced by *wt* virus and the US3-*lacZ* isolates.**

BHK cells were either mock-infected (lanes 1, 6 and 11) or infected with *wt* (lanes 2, 7 and 12), A54 (lanes 3, 8 and 13), A57 (lanes 4, 9 and 14) or A64 (lanes 5, 10 and 15) and labelled with [^{35}S]-methionine at 4-7h p.i. (lanes 1-5), 7-10h p.i. (lanes 6-10) or overnight (lanes 11-15). The cells were harvested and the proteins separated on a 5-12.5% gradient gel.

Known MWs are indicated to the right of lane 15. The novel 120K protein is indicated (◀).


1 2 3 4 5
6 7 8 9 10
11 12 13 14 15
155K
65K
45K
21K

phosphorylated virions was dried and exposed to film. There were no discernable differences in the polypeptide patterns of the *wt* and US3-*lacZ* untreated virions (data not shown) or the phosphorylated virions (Figure 4.13). Thus these results do not support the suggestion that the US3 protein kinase is a virion component. However, it is possible that the US3 protein comigrates with another virion protein which masks its absence from the US3-*lacZ* virions.

4.4.3. Analysis of Infected Cell Extracts Using Anti-US3 Antisera

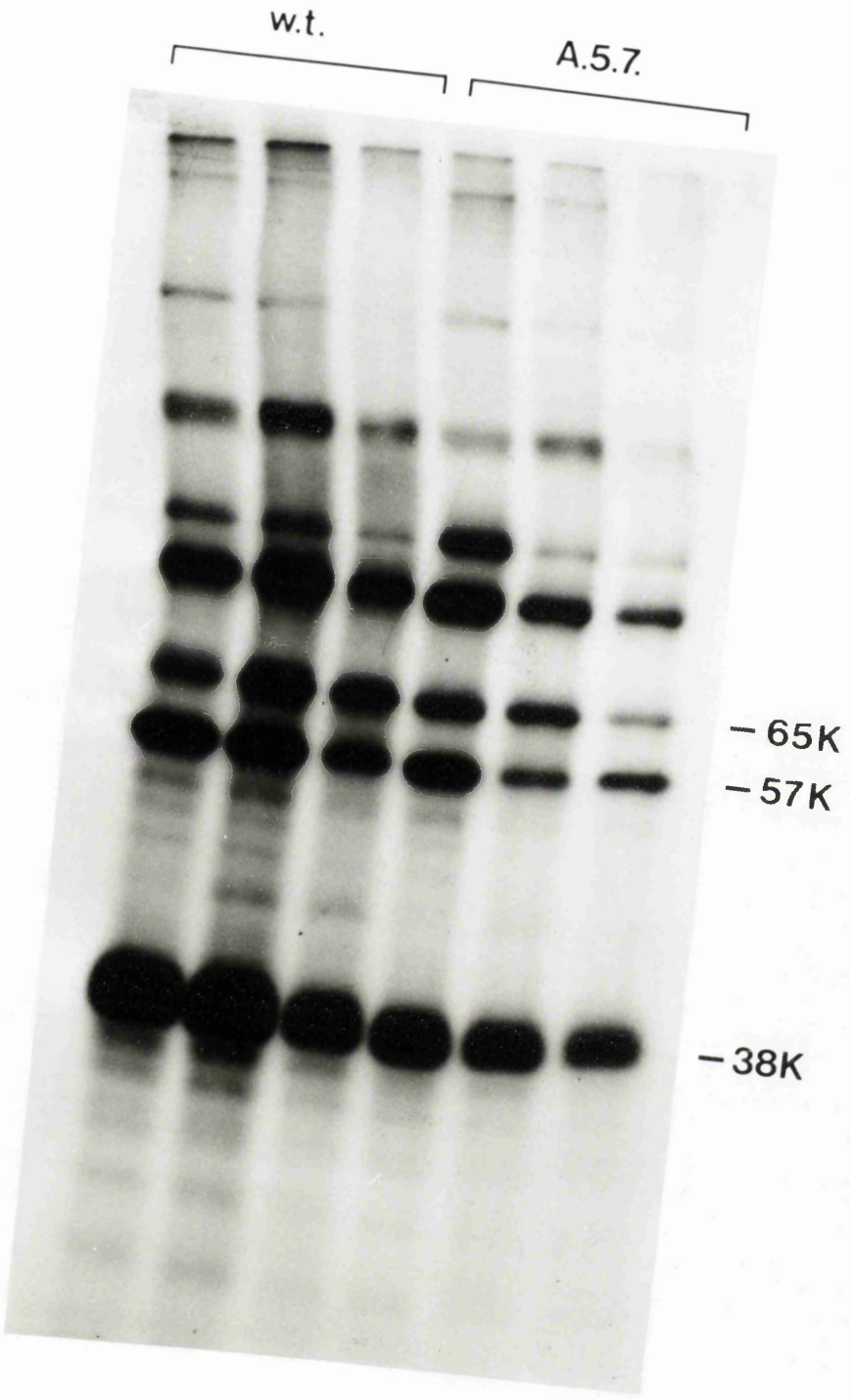
The proteins produced from the US3 locus in the US3-*lacZ* mutant were examined using two anti-peptide antibodies (18377 and 18378) which had been raised against the peptide NH_2-Tyr-Cys-Leu-Pro-Leu-Phe-Gln-Gln-Lys-COOH, which corresponds to the C-terminal octapeptide of the predicted US3 protein sequence (with a Tyr residue included for linking purposes). These antibodies had previously been used, in immunoblotting experiments, to identify the US3 protein kinase as a 68K protein present in HSV-1-infected cell extracts (Frame *et al.*, 1987). Both immunoprecipitation and immunoblotting experiments were carried out.

For the immunoprecipitations, [^{35}S]-methionine-labelled extracts of M.I. *wt* or US3-*lacZ* isolate infected cells (prepared as in section 3.5.3.) were incubated with antisera 18377 or 18378 in the presence or absence of peptide, and the

Figure 4.13 Profiles of *wt* and US3-*lacZ* virion proteins labelled with ^{32}P *in vitro*.

virions (lanes 1-3) (lanes 4-6)

wt and US3-*lacZ* A57 virions were purified, treated with NP40 and phosphorylated *in vitro*. The virion phosphoproteins were then separated on a 9% SDS-polyacrylamide gel. Known MWs are shown on the right.


w.t.
A.5.7.
65K
57K
38K

immunoprecipitated proteins were separated on 5%-12.5% SDS-polyacrylamide gels. Examination of the resulting autoradiographs (not shown) revealed that the antisera had not specifically precipitated the 68K US3 protein from the *wt* extracts indicating that the antisera do not recognise the native US3 protein.

For the immunoblotting experiments, cells were infected with *wt* and incubated at 37°C, in the presence of [^{35}S]-methionine, overnight or for five hours, as *in vitro* phosphorylation experiments have shown that the US3 protein is maximally phosphorylated at this time, which may indicate that maximal quantities of the protein are present (see 4.6.). The labelled proteins were separated on SDS-polyacrylamide gels which were then blotted onto nitrocellulose. The nitrocellulose was blocked with gelatin, Tween 20 or FCS, at a temperature of either 37°C or 65°C, and then incubated with the two anti-US3 antibodies or with normal rabbit serum, at 37°C. The resulting autoradiographs (not shown) revealed that the antisera had not recognised the 68K US3 protein.

These results were unexpected as these antisera had been successfully used by Frame *et al.* (1987) to recognise the 68K US3 protein. However, it should be noted that, in the experiments of Frame *et al.* (1987) the US3 protein had been purified prior to immunoblotting, thereby providing a concentrated source of antigen, whereas in these experiments

the US3 protein is thought to be present only as a minor protein of the infected cell extracts. In addition, the affinity of the antisera may have decreased as the antisera have aged.

4.5. ANALYSIS OF US3-*lacZ* VIRUS DNA SYNTHESIS, ENCAPSIDATION AND RELEASE

The effect of the *lacZ* insertion on the synthesis, encapsidation and release of viral DNA was investigated, as a reduction in any of these processes could explain the observed decrease in the growth of the US3-*lacZ* virus. A reduction in the amount of DNA synthesized would result in a decrease in the number of virus particles produced and consequently, a decrease in the virus titres. A defect in DNA encapsidation would also reduce the number of virus particles released, as capsids which do not contain DNA are not exported from the nucleus. Alternatively, defects in encapsidation may result in the production of particles which do not contain a complete virus genome. These particles are likely to have reduced growth properties, thereby reducing the titres of the virus stocks. A block in the release of virus particles would also result in a reduction in the titres of virus stocks. The DNA synthesis of the US3-*lacZ* virus was therefore investigated, as described below.

BHK cells were infected, at a moi of 5, with *wt* (22 plates) or with US3-*lacZ* A57 isolate (22 plates), for 8h or 24h at

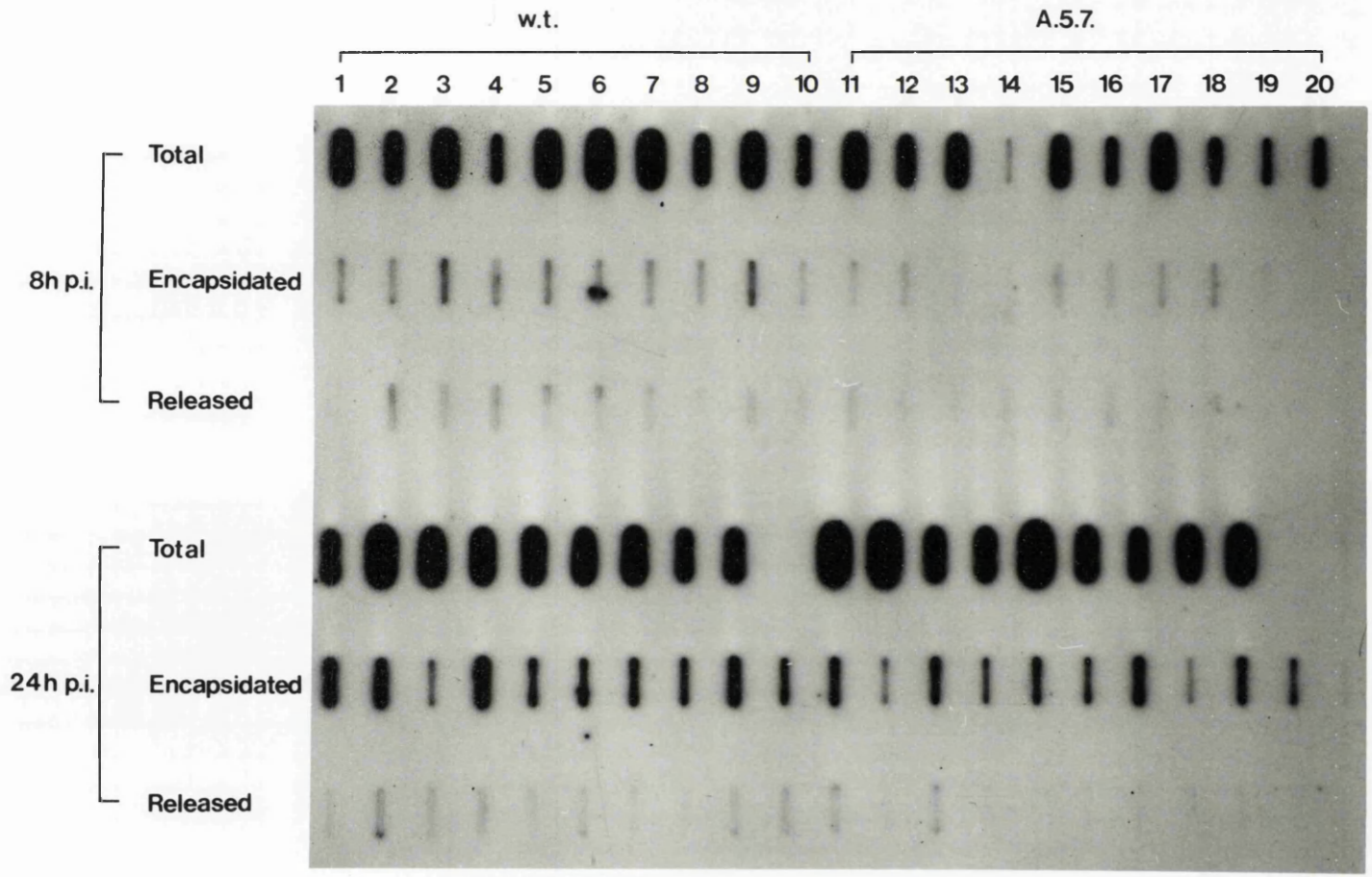
37°C. Ten of each set of virus-infected plates were then used to prepare total DNA samples and a further 10 plates were used to prepare both encapsidated DNA samples (from the monolayer) and released DNA samples (from the medium). These samples were all resuspended in 300 ul RNase solution (see 3.6.1.). The two remaining *wt* or A57 infected plates were used to determine the virus yields at the time of harvesting. Total DNA samples were also prepared from two mock-infected plates, as controls. For detection of viral DNA, 10 ul of each DNA sample, in a final volume of 60 ul, was loaded onto a slot blot and the blot was probed with the HSV-1 *KpnI f* fragment, which had been labelled with ^{32}P by nick translation. The blot was then exposed to film. Figure 4.14 shows an autoradiograph of the 30 *wt* samples and 30 A57 isolate samples harvested at 8h and 24h. As expected, the probe did not hybridise to the mock-infected samples.

Considering the weak signals from the encapsidated and released DNA samples, the experiment was repeated and these samples were resuspended in smaller volumes (150 ul or 50 ul RNase, respectively). In addition, the volume loaded onto the slot blot was increased from 10 ul to 20 ul or 30 ul, respectively, in a final volume of 60 ul (autoradiographs not shown).

Inspection, by eye, indicated that there is not a large difference in the amounts of DNA synthesised by the two viruses. To confirm this the results of these experiments

Figure 4.14 **DNA synthesis of *wt* virus and the US3-*lacZ* A57 isolate.**

Total cellular DNA, encapsidated DNA and released DNA were prepared from cells infected with *wt* (slots 1-10), or A57 (slots 11-20), at 8h or 24h p.i. The samples were loaded onto a slot blot which was probed with ^{32}P-labelled *Kpn*I *f* fragment and exposed to X-Omat XS-1 film.


w.t.
A.5.7.
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
8h p.i.
Total
Encapsidated
Released
24h p.i.
Total
Encapsidated
Released

were analysed in two ways; by densitometry (experiments 1 and 2) and by scintillation counting (experiment 1 only). Several autoradiographs of the blot, at different exposures, were analysed using a Hoefer Scientific Instruments GS300 scanning densitometer and the area under each peak calculated by the GS-360 data system using Gaussian integration analysis. The slot areas were then cut out of the blot and the radioactivity counted in the scintillation counter at 10 minutes per sample. The means of these results, together with the ratios of the means ($\bar{x}$wt/$\bar{x}$A57) are shown in Table 4.6.

The statistical significance of any differences in the means of the *wt* and A57 measurements was determined by applying the Students *t*-test.

In this statistical test the difference in the means is expressed as a single value, known as *t*, which is calculated using the values of the mean ($\bar{x}$), the standard deviation (S) and the number of measurements (n) in each of the two groups (see below). Once calculated, this value for *t* is then compared with the tabulated *t* values by consulting *t* tables (Wardlaw, 1985) at (n_1+n_2-2) degrees of freedom. If the *t* value corresponds to a P-value greater than 5% the difference is not deemed to be significant, that is, the means of the two groups are not statistically different. Values which lie between P-values of 5% and 1% are demmed to be significant and values corresponding to P-values less than 1% are highly

Table 4.6 Analysis of results from US3-*lacZ* DNA synthesis experiments

BHK cells were infected at a moi of 5 with *wt* or US3-*lacZ* A57 isolate and incubated at 37°C for 8h or 24h. Total DNA, encapsidated DNA and released DNA were prepared and loaded onto a slot blot which was then probed with ^{32}P-labelled *KpnI f* fragment. The blot was exposed to X-Omat XS-1 film and the resulting autoradiographs were analysed by densitometry. The slots were then cut from the blot and the radioactivity measured by scintillation counting.

The results of the densitometer analysis are expressed as the area under the curve, as determined by Gaussian integration analysis. The results of the scintillation counting are in cpm.

The *wt* means and A57 means for the two experiments are shown for each DNA sample. To determine if the difference in the means was significant, the Student's t-test was applied to the data. The values of *s*, *t*, the P values used and the significance of the results are shown. The ratio of *wt* mean:A57 mean for each DNA sample is also shown.

As different exposures were used for the densitometric analysis of the total, encapsidated and released DNA vertical comparisons of the densitometric means presented in this table cannot be made. The means obtained by scintillation counting can be compared vertically.

Sample	Expt. No.	Method of analysis [a]	$\bar{x}_{wt}$	n_{wt}	$\bar{x}_{A57}$	n_{A57}	s	t	P(%)	Significance	$\bar{x}_{wt} / \bar{x}_{A57}$
Total DNA 8h	1	D	1806	10	969	9	981.53	1.86	10>P>5	not sig.	1.86
	1	S	2467	10	1098	10	1324.19	2.31	5>P>2	sig.	2.25
	2	D	584	9	576	8	344.21	0.05	P>10	not sig.	1.01
Total DNA 24h	1	D	2296	9	3469	9	1828.14	1.36	P>10	not sig.	0.66
	1	S	3302	9	5813	9	3788.68	1.41	P>10	not sig.	0.57
	2	D	1742	10	1548	8	629.05	0.65	P>10	not sig.	1.13
Encapsidated DNA 8h	1	D	1001	10	361	7	396.42	3.27	1>P>0.1	highly sig.	2.77
	1	S	145	10	86	10	39.84	3.31	1>P>0.1	highly sig.	1.68
	2	D	1847	10	1240	9	1104.34	1.20	P>10	not sig.	1.49
Encapsidated DNA 24h	1	D	3622	10	2195	10	1381.31	2.31	5>P>2	sig.	1.65
	1	S	534	10	312	10	181.40	2.74	2>P>1	sig.	1.71
	2	D	1294	10	1048	10	237.74	2.31	5>P>2	sig.	1.23
Released DNA 8h	1	D	1444	9	823	8	783.83	1.63	P>10	not sig.	1.75
	1	S	80	10	59	10	17.76	2.64	2>P>1	sig.	1.36
Released DNA 24h	1	D	995	10	531	10	553.78	1.87	10>P>5	not sig.	1.87
	1	S	78	10	68	10	17.71	1.26	P>10	not sig.	1.14
	2	D	1199	10	1692	10	1144.27	0.96	P>10	not sig.	0.71

[a] D signifies that the results were analysed by densitometry.
S signifies that the results were analysed by scintillation counting.

significant, that is, the means of the two groups are statistically different.

The formula used to calculate *t* is as follows:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where $\bar{x}$ = the sample mean of the group

n = the number of measurements in the group

S = the pooled estimate of the standard deviation, which is calculated using the following formula:

$$S = \sqrt{\frac{\Sigma x_1^2 - (\Sigma x_1)^2/n_1 + \Sigma x_2^2 - (\Sigma x_2)^2/n_2}{n_1 + n_2 - 2}}$$

x represents the individual measurements within a group.

The values of $\bar{x}$, n, s, t and the significance of the results are shown in Table 4.6.

The difference in the means of the two viruses for the total DNA and released DNA samples were not significant, indicating that the two viruses synthesise and export equivalent amounts of DNA. However, the difference between the means of the encapsidated DNA samples (a maximum 2.77-fold difference, at 8h) was deemed to be statistically significant. This result is inconsistent with the conclusions reached from analysis of

the total and released DNA as any reduction in the level of encapsidated DNA should be reflected in the total DNA levels and should produce a corresponding, significant, reduction in the released DNA, neither of which was observed. In addition, the 2.77-fold reduction in encapsidated DNA does not account for the 7-fold reduction in virus growth observed in these experiments (data not shown).

Thus, although the statistical analysis is inconclusive, possibly due to insufficient data, it is considered that the observed reduction in growth of the US3-*lacZ* virus is not due to a significant reduction in DNA synthesis, encapsidation or release.

4.6. *IN VITRO* PHOSPHORYLATION OF *wt* AND US3-*lacZ*-INFECTED CELL EXTRACTS

(The following experiments were carried out in conjunction with Dr H. W. McL. Moss.)

Potential physiological substrates of the US3 protein kinase were investigated by comparing the *in vitro* phosphorylated proteins of the *wt* and US3-*lacZ* virus. US3-*lacZ* A54 and *wt* infected cells were harvested after a 5h or an overnight incubation, at 37°C. Cytoplasmic and nuclear extracts were prepared and incubated with γ-^{32}P-ATP, at a range of salt concentrations. The phosphoproteins were separated on 9% SDS-polyacrylamide gels.

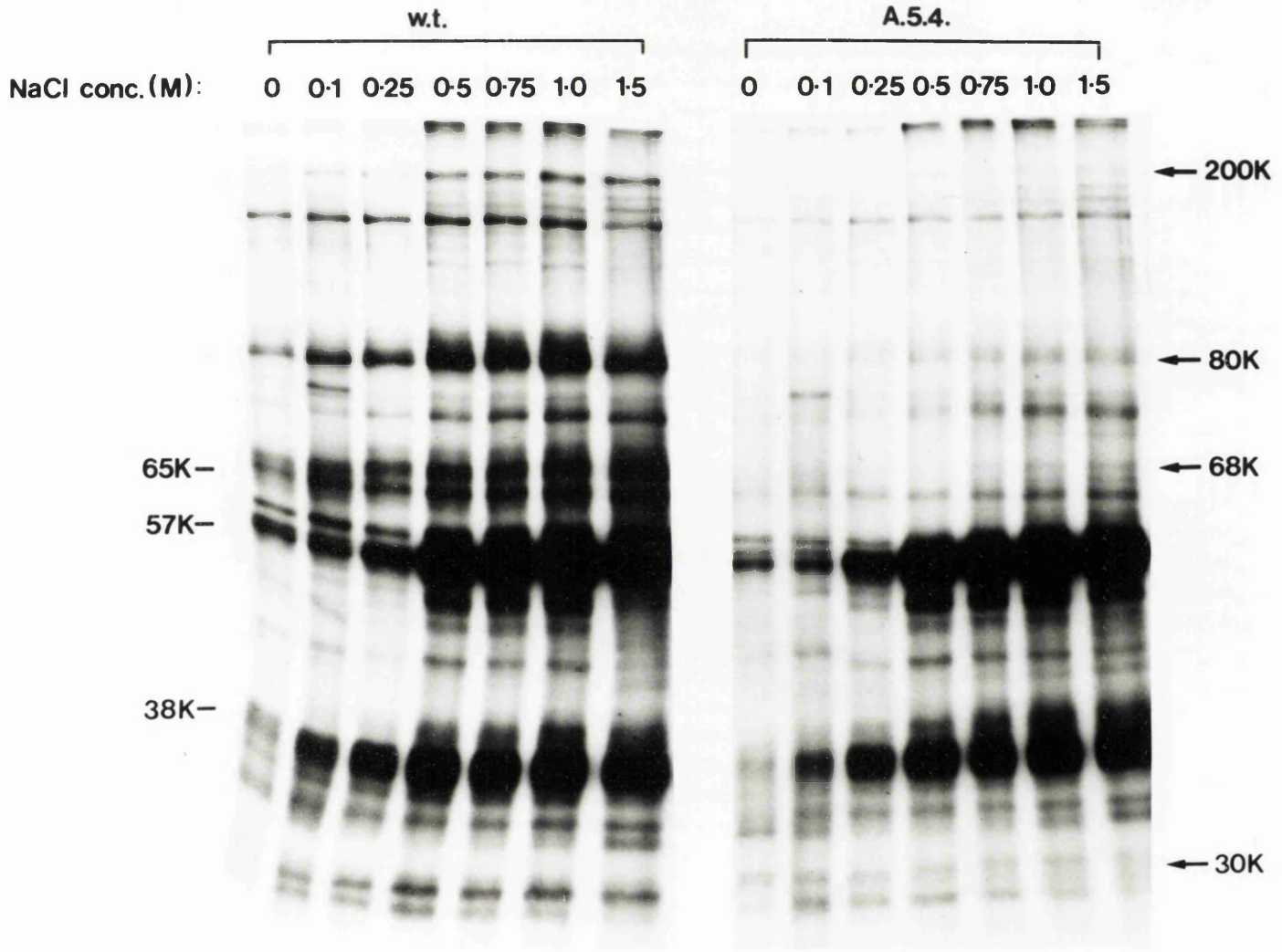
Figure 4.15 shows the phosphoproteins present in cytoplasmic extracts of cells infected with *wt* (lanes 1-7) or US3-*lacZ* A54 isolate (lanes 8-14). The *wt* extracts contain many labelled proteins, including a protein of apparent MW 68K which corresponds to the observed size of the US3 protein (Frame *et al.*, 1987). The fact that this phosphoprotein is not present in the A54 extracts (lane 8-14) supports the conclusion that this is the US3 protein. It is interesting to note that the US3 protein is strongly labelled at unusually high salt concentrations (up to 1.5M) and is not present in nuclear extracts (results not shown). The US3 protein is also absent from extracts of cells infected overnight, at 37°C (results not shown), implying that the protein has either been degraded by this stage in the virus cycle or is no longer capable of accepting phosphate groups. It is not known if the observed phosphorylation of the US3 protein is a result of autophosphorylation or the action of another kinase.

Examination of the A54 extracts (lanes 8-14) shows that the absence of the labelled 68K US3 protein is accompanied by a marked reduction in the phosphorylation of three other proteins. These proteins have estimated MWs of 200K, 80K and 30K, but they have not been identified as known virus induced species. The 200K phosphoprotein does not appear to comigrate with Vmw175 and may represent the 273K phosphoprotein (encoded by UL36), or one of its breakdown

Figure 4.15 ***In vitro*** **phosphorylation of cytoplasmic extracts, from** ***wt*** **and US3-*lacZ*-infected cells, at a range of salt concentrations.**

BHK cells were mock-infected or infected with *wt* or the A54 isolate for 5h at 37°C. Cytoplasmic extracts were prepared which were then incubated with [γ-^{32}P]-ATP in the presence of a range of salt concentrations. The phosphoproteins were separated on a 9% gel. The MWs of certain viral proteins are shown to the left of the *wt* tracks. The phosphoproteins which are absent or underphosphorylated in the US3-*lacZ* extracts are indicated, with arrows, to the right of the A54 tracks (the M.I. tracks are not shown).

This autoradiograph was prepared by Dr H. W. McL. Moss.


w.t.
A.5.4.
NaCl conc.(M):
0 0·1 0·25 0·5 0·75 1·0 1·5
0 0·1 0·25 0·5 0·75 1·0 1·5
200K
80K
68K
65K
57K
38K
30K

products. The 80K phosphoprotein migrates in the same region as the 81/82K and the 85/87K phosphoproteins and the 30K phosphoprotein may represent the UL34 gene product which has been reported to be a substrate for the US3 protein kinase (Purves *et al.*, 1991). It is interesting to note that the 57K UL13 phosphoprotein (see section 5.6.) is strongly phosphorylated in the mutant extracts, indicating that the protein is not phosphorylated by the US3 protein kinase.

In vitro phosphorylation experiments were also carried out on cytoplasmic extracts of A57 and A64 infected cells with similar results (data not shown).

These results provide further evidence that the US3 gene product is a protein kinase and indicate three possible substrates for the kinase.

4.7. US3-*lacZ* REVERTANT

When studying the properties of a mutant virus it is desirable to have a revertant virus as a control, i.e., a virus in which the mutation has been rescued using a DNA fragment containing the *wt* sequences. This ensures that the observed phenotype of the mutant virus is due only to the introduced mutation and not to a spontaneous mutation which has occurred elsewhere in the genome. The construction of the US3-*lacZ* revertant is described below.

4.7.1. Co-transfection and Plaque Purification of the US3-*lacZ* Revertant

pKhx1, which contains the *Kpn*I *h* fragment of HSV-1, was linearised with *Eco*RI and cotransfected with DNA prepared from the US3-*lacZ* A57 isolate. Recombinant progeny, lacking the *lacZ* gene, produce 'white' plaques (i.e. not stained blue) which are easily detected against the background of "blue" plaques produced by the A57 virus.

The efficiency of detection of the recombinant is reduced slightly due to the fact that "blue" viruses, in practice, often give rise to a certain percentage (usually less than 1%) of white plaques.

The percentage of "white" plaques produced by this cotransfection was higher than expected, ranging from 2.8% to a maximum of 17.5%. Out of the ten "white" plaques picked, six gave 100% white plaques when titrated. To ensure purity, these plaques were carried through several more rounds of plaque purification, their *wt* genome structures were verified by restriction enzyme analysis, and stocks were grown of three isolates.

It was at this point that the difficulties in other work to generate a US3 deletion mutant became apparent (discussed fully in section 4.8.). Briefly, it appeared that during the transfection of A57 DNA alone into BHK cells a large

percentage of *wt* virus was generated. It must be assumed that this could also have occurred during the revertant cotransfection. Since the revertant did not carry any distinguishing marker, it is uncertain that the isolated revertant actually originated from a recombination between the DNA fragment and the A57 DNA.

4.8. US3-DELETION MUTANT

An attempt was made to construct a mutant with a deletion in the US3 gene. Such a mutant is preferable to an insertion mutant as it contains no foreign DNA. The mutant should confirm the phenotype of the US3-*lacZ* insertion mutant.

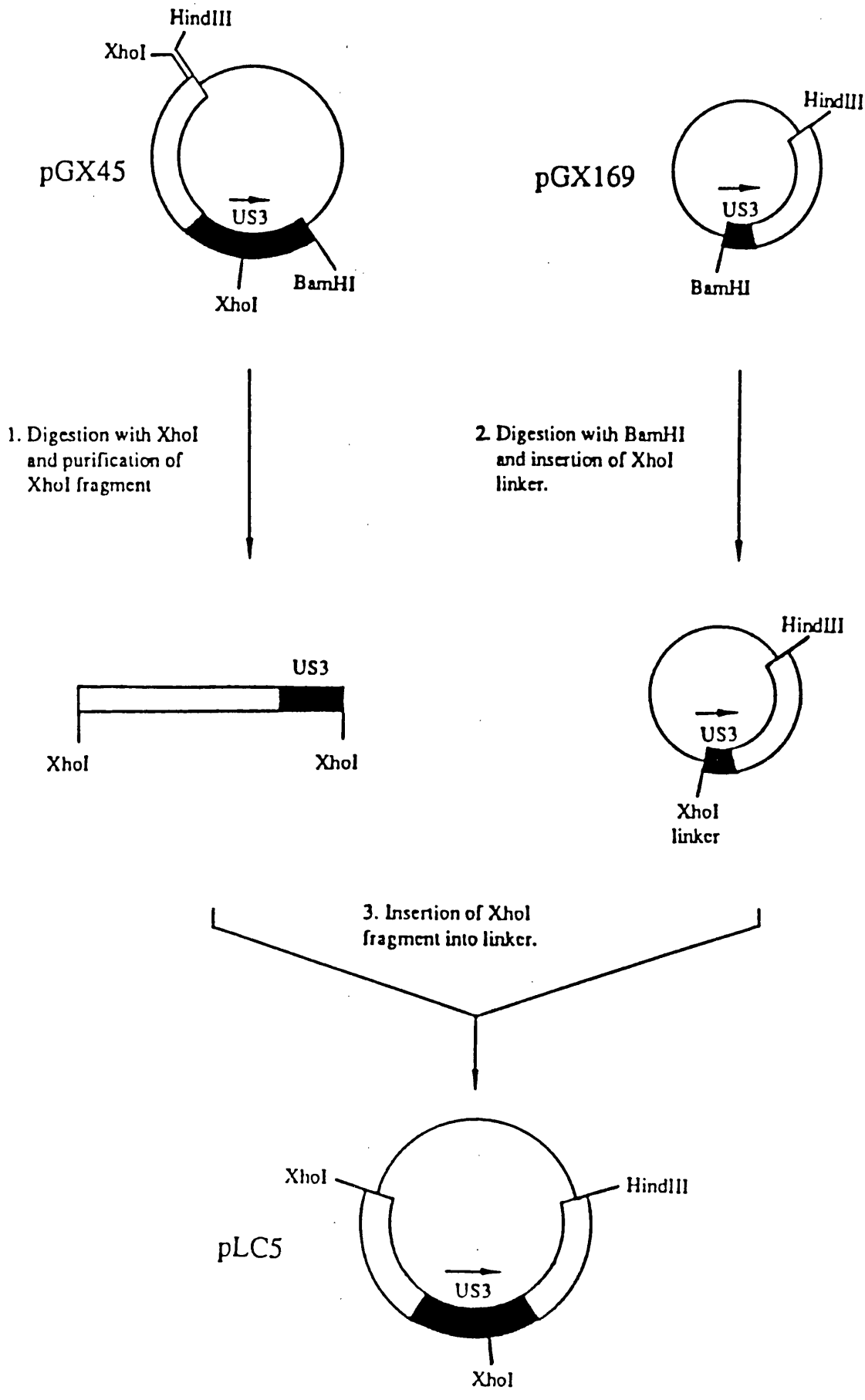
4.8.1. Construction of pLC5

pLC5, which contains a deletion in the US3 gene from the *Xho*I site (residues 135727 to 135732) to the *Bam*HI site (residues 136289 to 136294), was constructed as illustrated in Fig. 4.16. pGX45, which contains the *Hin*dIII (133466 to 133471) to *Bam*HI (136289 to 136294) subfragment of HSV-1, in pAT153, was cut with *Xho*I and the restriction fragments separated on an agarose gel. The 2.2 kbp *Xho*I fragment, containing the 5'-terminal 505 bp of the US3 gene, was purified from the gel and stored. pGX169, which contains the *Bam*HI (136289 to 136294) to *Hin*dIII (138344 to 138349) subfragment of HSV-1 in pAT153, was linearised with *Bam*HI, blunt-ended, and a phosphorylated *Xho*I linker inserted. The purified *Xho*I

Figure 4.16 Construction of pLC5.

pGX45, which contains the *Hin*dIII to *Bam*HI fragment (residues 133466 to 136289) of HSV-1 inserted into the complementary site of pAT153, was digested with *Xho*I and the resulting 2.2 kbp fragment was purified. pGX169, which contains the *Bam*HI to *Hin*dIII fragment (residues 136289 to 138344) of HSV-1 inserted into the complementary sites of pAT153, was linearised with *Bam*HI and an *Xho*I linker inserted. The *Xho*I fragment, purified from pGX45, was then inserted into this linker to give a plasmid containing a deletion in the US3 gene (pLC5).

The shaded areas represent the US3 ORF.


HindIII
XhoI
pGX45
US3
BamHI
XhoI
pGX169
HindIII
US3
BamHI
1. Digestion with XhoI and purification of XhoI fragment
2. Digestion with BamHI and insertion of XhoI linker.
US3
XhoI
XhoI
HindIII
US3
XhoI linker
3. Insertion of XhoI fragment into linker.
XhoI
HindIII
pLC5
US3
XhoI

fragment, from pGX45 was then inserted into the modified pGX169, via the *Xho*I linker to give pLC5. The structure of this plasmid was authenticated by restriction enzyme digestion followed by electrophoresis on a mini-gel. This analysis also confirmed that the insert was in the correct orientation, i.e. the two sections of the US3 gene had been ligated together to give a US3 gene with a 562 bp deletion.

The deletion represents amino acids 169 to 357 of the US3 protein which includes motifs I to VII of the catalytic domain, thus an intact catalytic domain can not be synthesised.

The deletion is not expected to interfere with transcription of the neighbouring genes.

4.8.2. Co-transfection of the US3-deletion Mutant

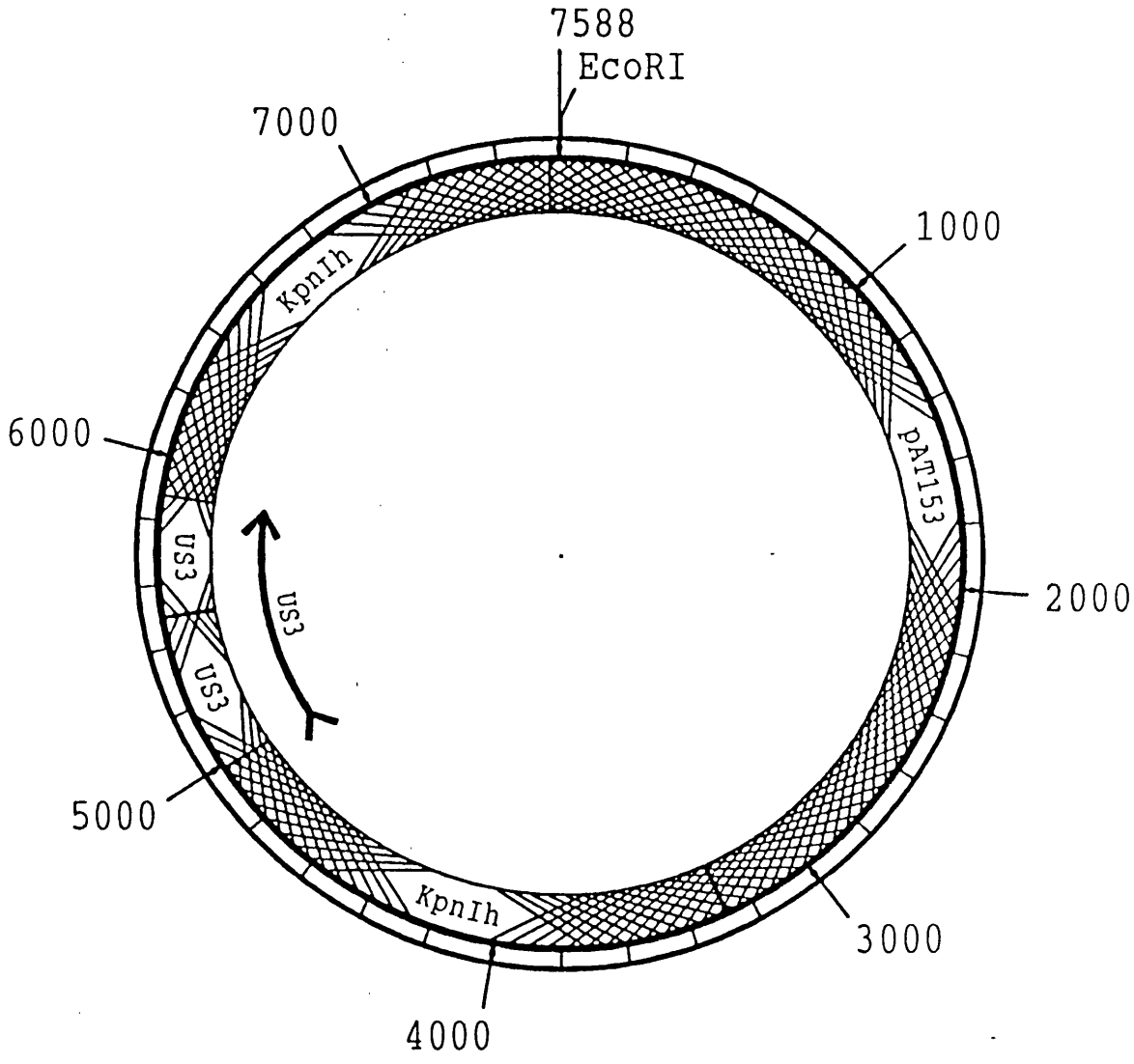
pLC5 was linearised with *Eco*RI (Figure 4.17) and cotransfected with DNA from the US3-*lacZ* A57 isolate exactly as described previously (4.1.2.). The products of the co-transfections were titrated and overlaid with EMC_5/X-Gal. As in the revertant cotransfection, recombinant progeny, lacking the *lacZ* gene should produce "white" plaques which can be easily detected against the background of "blue" plaques produced by the A57 virus.

The cotransfection resulted in very large percentages of

Figure 4.17 Structure of pLC5.

The structure of pLC5 was drawn using the PLASMIDMAP program. The scale is in nucleotides and starts at the first nucleotide of the *Eco*RI site in the vector pAT153. The plasmid is 7588 nucleotides in length.

The location and orientation of the deleted US3 gene is shown (nucleotides 4991 - 5878). The location of the *Eco*RI site, which was used to linearise the plasmid prior to cotransfection, is also indicated.


7588
EcoRI
1000
2000
3000
4000
5000
6000
7000
KpnIh
KpnIh
pAT153
US3
US3
US3

"white" plaques ranging from 27% to 100%. This, together with the fact that the plaques exhibited a *wt* morphology suggested *wt* contamination. To confirm this, the ^{32}P-labelled DNA of three plaques were analysed by digestion with *Eco*RI and *Hin*dIII. Any virus containing the deleted US3 gene is expected to show a decrease of 0.6 kbp in the size of the *Eco*RI *h* and *Hin*dIII *n* fragments. These three plaques however, gave *wt* restriction profiles.

The controls for the contransfection experiment indicated that neither the DNA solutions nor the buffers were contaminated with *wt* virus. The structure of pLC5 was checked again and found to be as expected and no *wt* virus could be detected in the A57 virus stock, from which the DNA had been made. Thus, the source of the *wt* virus was unclear.

The cotransfection was repeated using fresh solutions but, once again, large numbers of *wt* plaques were produced. The control containing A57 DNA and calf thymus DNA only was also found to contain "white" plaques (approximately 11%), suggesting that either these solutions contain a very low level of *wt* contamination, which was not detected in previous checks, or that *wt* virus is generated by the procedure of transfection itself, presumably by the loss of the *lacZ* gene from the A57 DNA.

This phenomenon was not restricted to the A57 isolate, as the A54 isolate DNA was found to produce approximately 50% white

plaques when transfected into BHK cells. (The A64 isolate gave only blue plaques.)

Another attempt was made to isolate the US3-deletion mutant, using two different batches of BHK cells. 100 plaques were picked and analysed by restriction enzyme digestion; 92 plaques were *wt*, 3 were a mixture of *wt* and A57, and 5 contained no DNA or DNA which could not be digested. It was noted that the percentage of *wt* plaques varied considerably between the two batches suggesting that the metabolic state of the cell may play a role in this phenomenon.

It was decided that isolation of the deletion mutant would be difficult while this problem was unresolved and other means of transfecting cells were therefore investigated. Initial experiments were performed with lipofectin reagent (BRL), however, this was not continued due to a lack of time.

4.9. NEUROVIRULENCE OF THE US3-*lacZ* INSERTION MUTANT

The neurovirulence of the A57 isolate of the US3 insertion mutant was compared to that of *wt*. Three-week-old BALB/c mice were inoculated in the left cerebral hemisphere with 10-fold serial dilutions (10^2 to 10^7 pfu/ml) of either the *wt* or A57 virus. Deaths from encephalitis were scored up to 21 days post-inoculation and LD_{50} values calculated. (This work was kindly carried out by Dr Lesley Robertson.) The results, shown in Table 4.7 indicate that the A57 virus, with an LD_{50}

of 1 x 10^4 pfu/animal, is at least 300-fold less neurovirulent than *wt* ($LD_{50} \leqslant 10^{1.5}$).

The virus present within the brains of the A57-infected mice which had died was analysed by homogenising the brain tissue, isolating single plaques and digesting the DNA using an appropriate restriction enzyme. (This work was kindly carried out by Dr Christine MacLean.) The results (not shown) indicated that the A57 virus was contaminated with significant amounts of *wt* virus. Thus, the LD_{50} value calculated for A57 in this experiment is incorrect.

The A57 working stocks were re-checked for *wt* contamination. The working stock was used to set up a low moi infection (0.001 pfu/cell) which was incubated at 37°C for 72h. Upon titration of this infection, approximately 10% white plaques were observed. This experiment was repeated using the A54 and A64 working stocks which were found to have 8% and 3% "white" plaques, respectively. The DNA isolated from these "white" plaques were analysed by restriction enzyme digestion. The profiles were found to be consistent with those of *wt* DNA (Dr C. A. MacLean).

As a result of these findings the A57 isolate elite stock was subjected to four more rounds of plaque purification to give isolate A91. This isolate will be characterised and used in any subsequent experiments.

These results obviously have important implications for all the work previously carried out using the US3 insertion mutant. For example, it is anticipated that the yields obtained during all the growth experiments, especially those carried out at a low moi, will have been increased by the presence of 8% (A54), 10% (A57) or 3% (A64) *wt* contamination. Reductions in the synthesis, encapsidation or release of the US3-*lacZ* virus may not have been detected due to the presence of *wt* DNA and, in the *in vitro* phosphorylation experiments the activity of *wt* US3 protein kinase may have obscured other potential substrates of the kinase. Thus, all of these experiments should be repeated with the plaque purified A91 isolate before any conclusions can be reached.

The *wt* contamination also explains the difficulties encountered when trying to construct a US3 deletion mutant and a revertant.

CHAPTER 5: STUDIES ON THE HSV-1 UL13 GENE

5. STUDIES ON THE HSV-1 UL13 GENE

At the start of this project, little was known about the function of the UL13 gene of HSV-1. The predicted sequence of the UL13 protein had been reported to contain amino acid motifs that are characteristic of known protein kinases or phosphotransferases (Smith & Smith, 1989; Chee *et al.*, 1989), and the gene is known to be conserved throughout the three sub-families of the herpesviruses. Thus, it was considered likely that the UL13 gene product may play an important role in the virus life cycle. This role was investigated by constructing a virus which contains the UL13 gene of HSV-1 disrupted by the insertion of the *E. coli lacZ* gene. The effect of this disruption on the growth properties of the virus were investigated and *in vitro* phosphorylation experiments were carried out to determine if the UL13 gene product possesses protein kinase activity.

5.1. CONSTRUCTION OF THE UL13-*lacZ* INSERTION MUTANT

5.1.1. Construction of pLC3

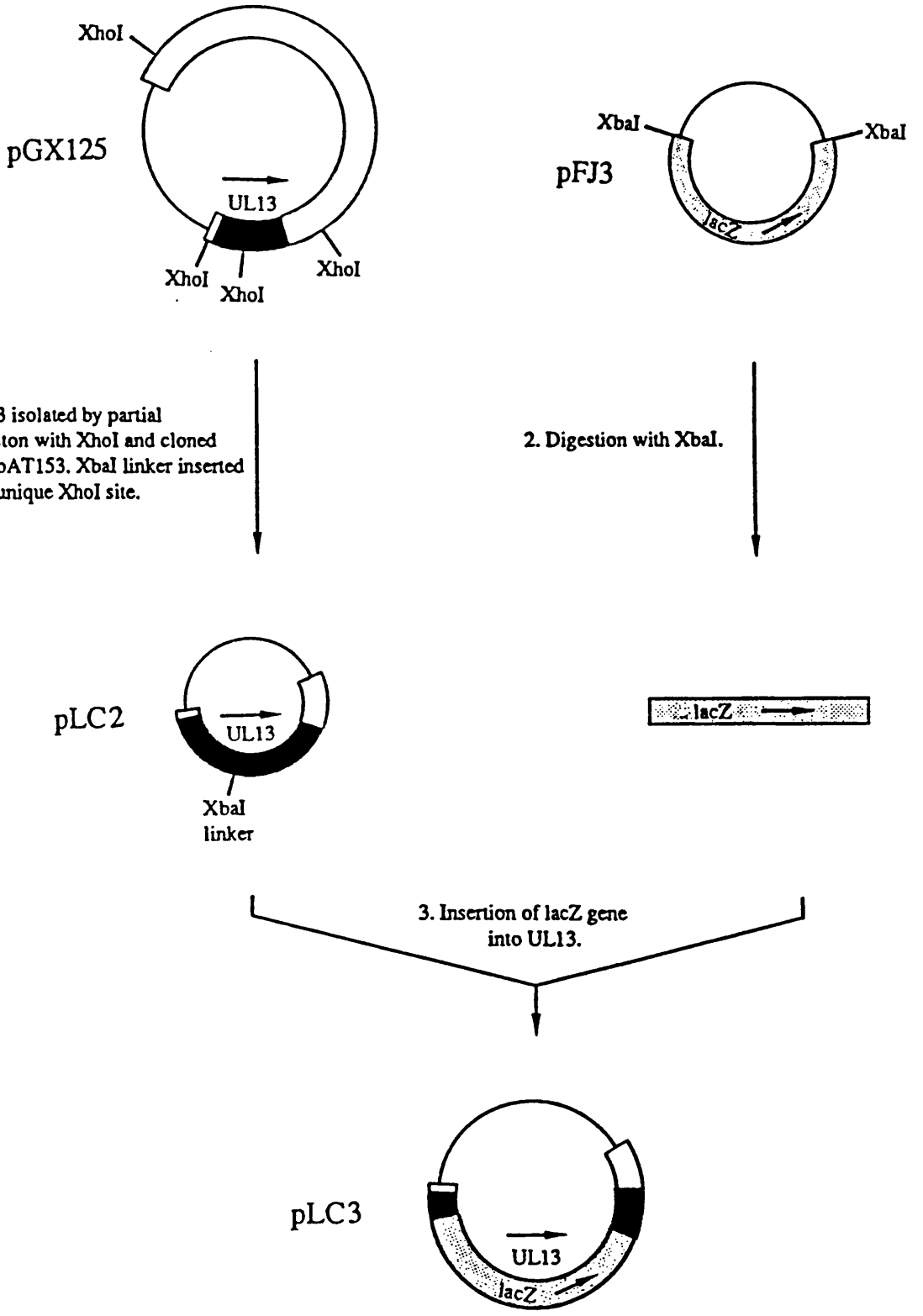
pLC3 contains the UL13 ORF disrupted by the insertion of a *lacZ* gene and was constructed as shown in Figure 5.1.

The source of the UL13 gene (residues 28504 to 26950) was the plasmid pGX125 which contains the *Kpn*I *f* fragment of HSV-1 (17789 to 28626) cloned into the vector pAT153. pGX125

Figure 5.1 Construction of pLC3.

pGX125, which contains the *Kpn*I *f* fragment of HSV-1, was digested with *Xho*I and the 2.2 kbp *Xho*I fragment, containing the intact UL13 ORF, was purified. This fragment was cloned into the complementary *Sal*I site of pAT153 to give pLC1 (not shown) which was then linearised at the unique *Xho*I site, within the UL13 ORF, and an *Xba*I linker inserted (pLC2). The *lacZ* gene, excised from pFJ3 using *Xba*I, was then inserted into pLC2 using the *Xba*I linker to give a plasmid containing a disrupted UL13 ORF (pLC3).

The solid area represents the UL13 ORF. The shaded areas indicate the *lacZ* gene.


pGX125
XhoI
UL13
XhoI
XhoI
XhoI
pFJ3
XbaI
XbaI
lacZ
1. UL13 isolated by partial digeston with XhoI and cloned into pAT153. XbaI linker inserted into unique XhoI site.
2. Digestion with XbaI.
pLC2
UL13
XbaI linker
lacZ
3. Insertion of lacZ gene into UL13.
pLC3
UL13
lacZ

contains four *Xho*I sites, two of which (26347 to 26352 and 28555 to 28560) border the UL13 gene and one (28058 to 28063) which lies in the middle of the gene. A partial digestion of pGX125 with *Xho*I was carried out and the resulting restriction fragments were separated by electrophoresis on a minigel. The 2.2 kbp restriction fragment (26347 to 28555), which contains the entire UL13 gene, was excised from the gel, purified and subcloned into the complementary *Sal*I site of pAT153, to give pLC1. Cloning into the *Sal*I site destroyed the terminal *Xho*I sites of the 2.2 kbp fragment, leaving pLC1 with a single *Xho*I site, situated within the UL13 gene. pLC1 was cut at this unique *Xho*I site, the linearised plasmid was blunt-ended and a phosphorylated *Xba*I linker was inserted. The plasmid was cut with *Xba*I and religated to ensure that only one copy of the linker had been inserted. This plasmid (pLC2) was then linearised with *Xba*I and the *E. coli lacZ* gene, which had been cut from pFJ3 using *Xba*I, was inserted to give pLC3.

The UL13 gene overlaps extensively with its neighbours (see Figure 1.10). The transcript site (residue 27048) and the promoter of the downstream gene, UL12, lie within the UL13 coding region and the UL13 ORF is predicted to have an out of frame overlap of 83 codons with the coding region of its right neighbour UL14. The position of the *lacZ* insert (at residue 28058) is approximately 1 kbp upstream from the transcript start site of UL12 and is outwith the coding region of UL14. Thus, the insertion is not expected to

interfere with the transcription of the neighbouring genes.

The *lacZ* insert disrupts the UL13 ORF at amino acid 148 which is just upstream of the start of the catalytic domain, at amino acid 157 (Smith & Smith, 1989). A protein consisting of the 148 N terminal amino acids may be expressed in the insertion mutant and translation may also be possible (depending upon a suitably placed promoter) from an initiation codon present at amino acid 230. This latter protein would consist of the UL13 catalytic domain which lacked motifs I to IV and which would therefore be inactive.

5.1.2. Cotransfection and Plaque Purification of UL13-*lacZ* Virus

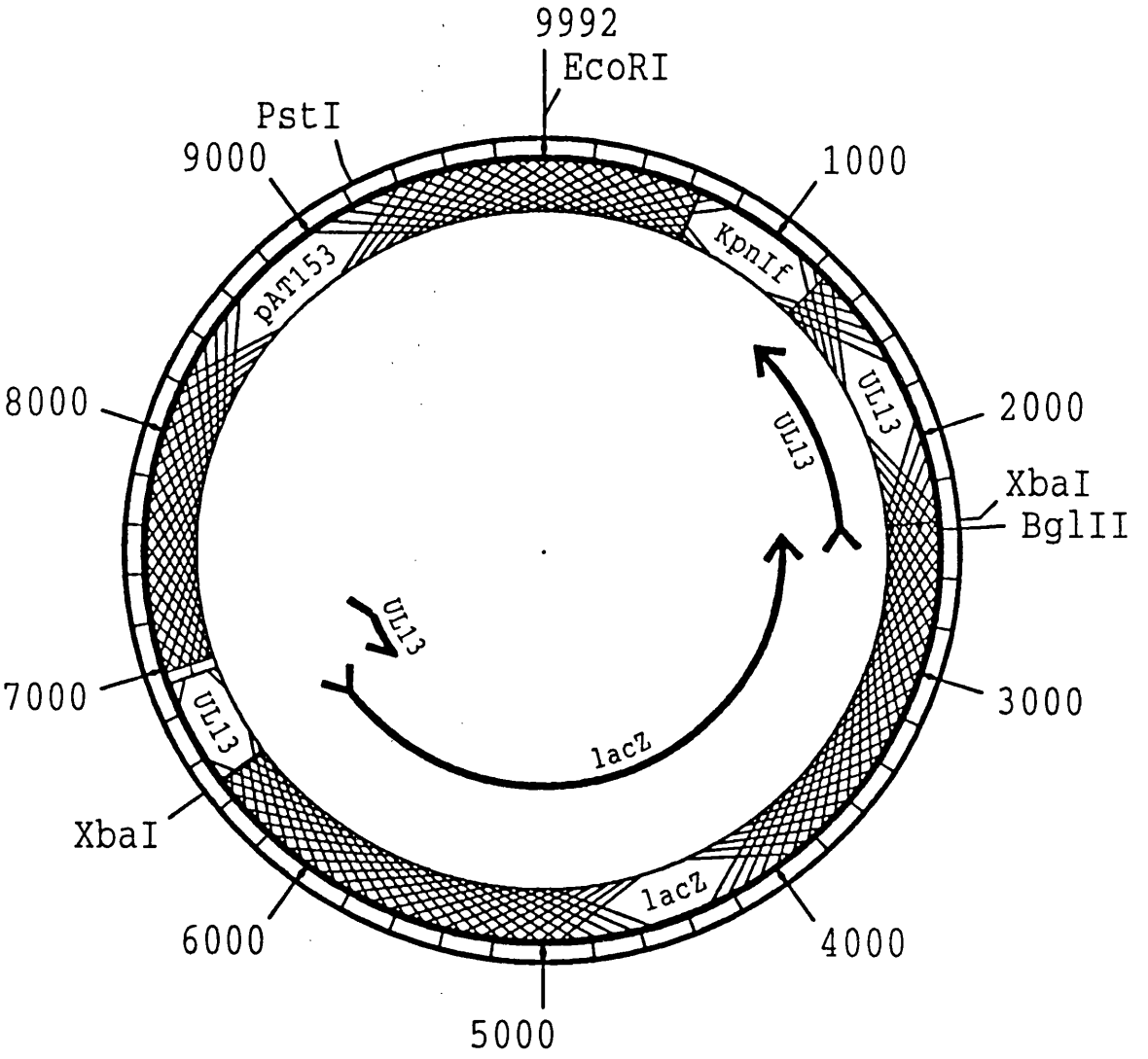
pLC3 was linearised with *Pst*I (Figure 5.2) and cotransfected into BHK cells with *wt* DNA (see section 3.4.1.). The cotransfections were harvested and titrated in duplicate; one set was stained with Giemsa stain and the other was overlaid with Agar/X-Gal (for the detection of "blue" plaques). This was repeated several times without success. The DNA was then cotransfected into BHK cells from four different sources. Recombinant virus was generated in only one of these batches with a recombination percentage of approximately 0.4% (which was lower than that observed in the construction of the US3-*lacZ* insertion mutant). "Blue" plaques were picked from plates containing large numbers of *wt* plaques and were carried through five rounds of plaque purification before

Figure 5.2 Structure of pLC3.

The structure of pLC3 was drawn using the PLASMIDMAP program. The scale is in nucleotides and starts at the first nucleotide of the *EcoRI* site in the vector pAT153. The plasmid is 9992 nucleotides in length.

The position of the orientation of the *lacZ* insert, within the UL13 gene, is shown (nucleotides 6484 to 2367).

The position of several restriction enzyme sites are also indicated (*EcoRI*, *Xba*I, *Bgl*I and *Pst*I). The *Pst*I site was used to linearise the plasmid prior to co-transfection.


9992
EcoRI
PstI
9000
1000
pAT153
KpnIf
UL13
2000
XbaI
BglII
8000
UL13
UL13
7000
3000
lacZ
UL13
XbaI
lacZ
6000
4000
5000

they were deemed to be pure. After restriction enzyme analysis stocks were grown of three isolates - A54, A510 and A515.

It should be noted that these three isolates originated from the same co-transfection plate and therefore cannot be regarded as independent.

5.1.3. Restriction Enzyme Analysis of UL13-*lacZ* Virus DNA

The genome structure of the UL13-*lacZ* virus was determined by digestion of ^{32}P-labelled DNA with *Xba*I and *Bgl*II.

The UL13 gene lies within the *Xba*I c fragment of the *wt* genome (Figure 5.3(a)). Insertion of the *lacZ* gene introduces two additional *Xba*I sites into the genome (Figure 5.3(b)). Thus, the *Xba*I c fragment (34 kbp) is replaced by three novel fragments: one of 17.4 kbp (N'), which runs below the *f* fragment (24.7 kbp); one of 16.5 kbp (N'''), which runs above the *g* fragment (10.6 kbp); and one of 4.1 kbp (N'') representing the *lacZ* insert, which runs below *g*. These changes in the *Xba*I restriction profile of the A54, A510 and A515 isolates are shown in Figure 5.4.

The position of the *Bgl*II sites in the *wt* and the UL13-*lacZ* genomes is shown in Figure 5.5. The insertion of the *lacZ* gene introduces a novel *Bgl*II site resulting in the loss of the *o* fragment (5.3 kbp) and the generation of two novel

Figure 5.3 **Location of *Xba*I sites surrounding the UL13 gene in the *wt* and UL13-*lacZ* virus.**

The genomes of HSV-1 *wt* (a) and the UL13-*lacZ* virus (b) are shown, with the unique regions represented by solid lines and the repeat elements as open boxes. The dotted line represents the boundary between the L and the S segments.

The vertical lines mark the position of *Xba*I restriction sites and the letters represent the names of individual restriction fragments.

N', N'' and N''' indicate the location of novel fragments in the UL13-*lacZ* genome.

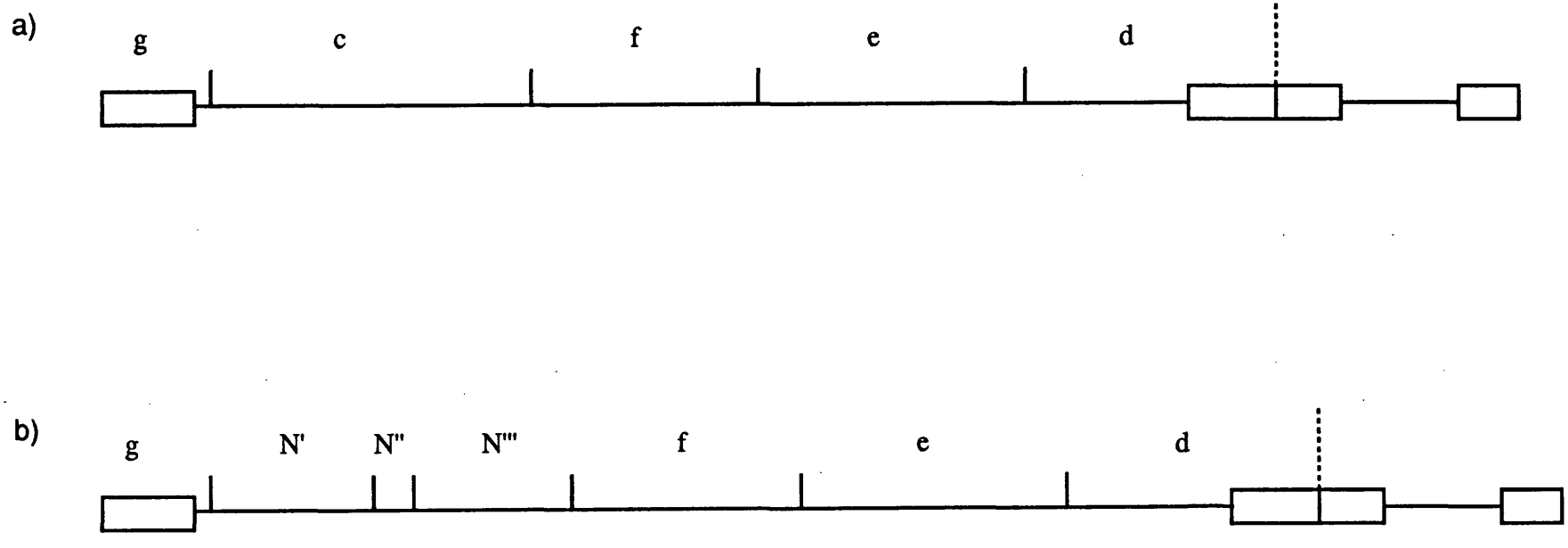

a)
g
c
f
e
d
b)
g
N'
N''
N'''
f
e
d

Figure 5.4 ***Xba*I restriction profile of *wt* virus and the UL13-*lacZ* isolates.**

^{32}P-labelled viral DNA was cut with *Xba*I and the fragments separated on a 0.6% agarose gel. The gel was dried and exposed to X-Omat XS-1 film. Lane 1; *wt* virus, lanes 2, 3 and 4; A54, A510 and A515, respectively. The names of the *wt* restriction fragments are to the left of the *wt* lane. Empty arrowheads (▷) indicate *wt* fragments which have been lost while filled arrowheads (▶) indicate the presence of novel fragments.

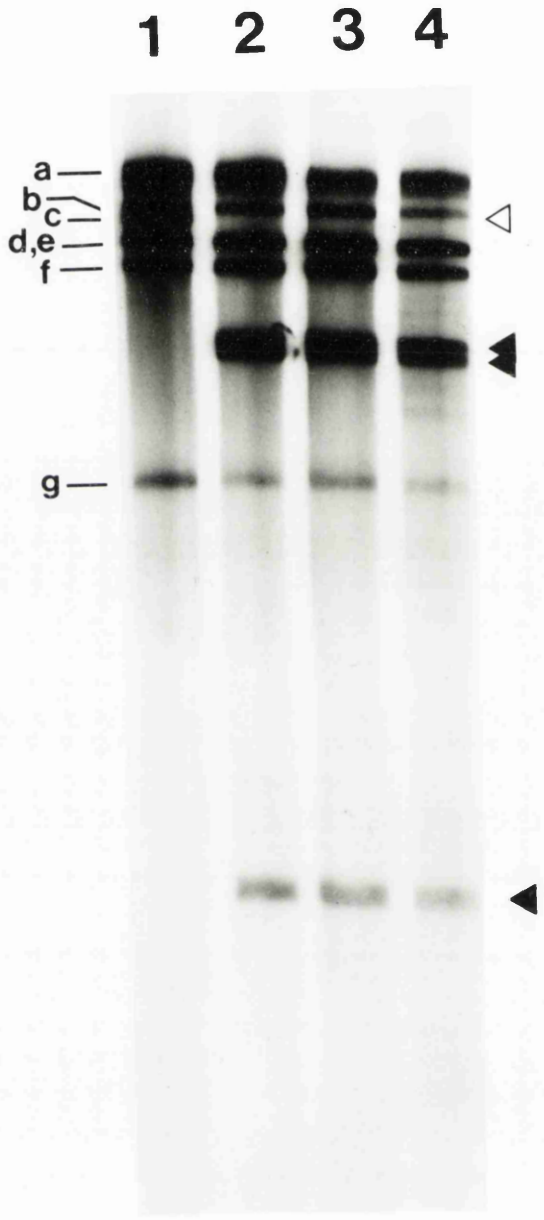

1
2
3
4
a
b
c
d,e
f
g

Figure 5.5 **Location of *Bgl*II sites surrounding the UL13 gene in the *wt* and UL13-*lacZ* virus.**

The genomes of HSV-1 *wt* (a) and the UL13-*lacZ* virus (b) are shown, with the unique regions represented by solid lines and the repeat elements as open boxes. The dotted line represents the boundary between the L and the S segments.

The vertical lines mark the position of *Bgl*II restriction sites and the letters represent the names of individual restriction fragments.

N' and N'' indicate the location of novel fragments in the UL13-*lacZ* genome.

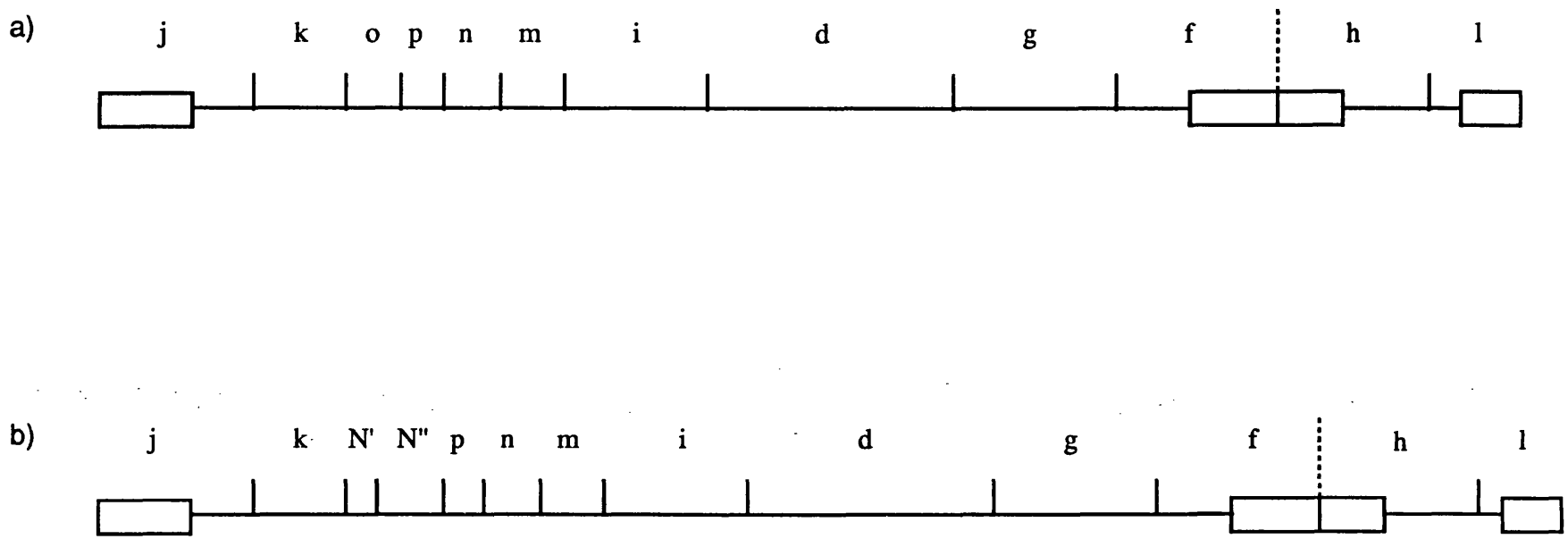
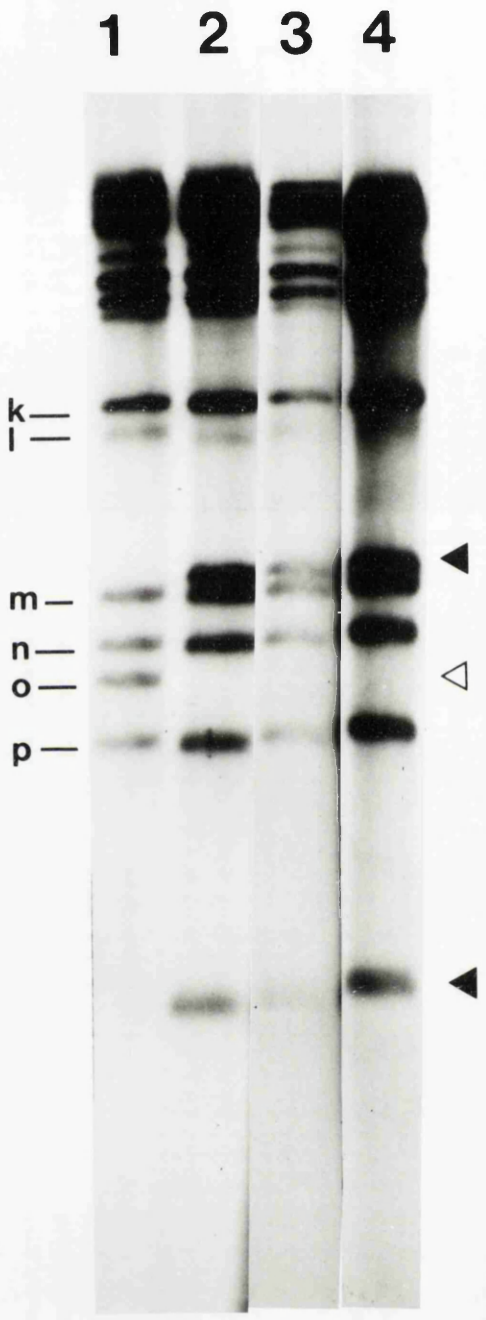

a)
j
k
o
p
n
m
i
d
g
f
h
l
b)
j
k
N'
N"
p
n
m
i
d
g
f
h
l

Figure 5.6 ***Bgl*II restriction profile of *wt* virus and the UL13-*lacZ* isolates.**

^{32}P-labelled viral DNA was cut with *Bgl*II and the fragments separated on a 0.8% agarose gel. The gel was dried and exposed to X-Omat XS-1 film. Lane 1; *wt* virus, lanes 2, 3 and 4; A515, A510 and A54, respectively. The names of the *wt* restriction fragments are to the left of the *wt* lane. Empty arrowheads (▷) indicate *wt* fragments which have been lost while filled arrowheads (▶) indicate the presence of novel fragments.


1
2
3
4
k
l
m
n
o
p

fragments, one of 6.7 kbp (N''), which runs just above *m* (6.4 kbp) and one of 2.7 kbp (N') which runs below the *p* fragment (4.6 kbp). The loss of the *o* fragment and the appearance of the bands above *m* and below *p* are shown in Figure 5.6. (The variation in the size of the *l* fragment is due to the fact that it represents the termini of the short region of the genome (see section 4.1.3.).

These results confirmed that the *lacZ* gene had been inserted at the correct position in the UL13 ORF.

5.2. GROWTH PROPERTIES OF THE UL13-*LACZ* VIRUS

The fact that a UL13-*lacZ* virus had been purified and stocks grown indicated that the UL13 product, like the US3 gene product, is not required for virus growth in tissue culture. Differences in the growth of the UL13-*lacZ* virus, compared to *wt*, were investigated:

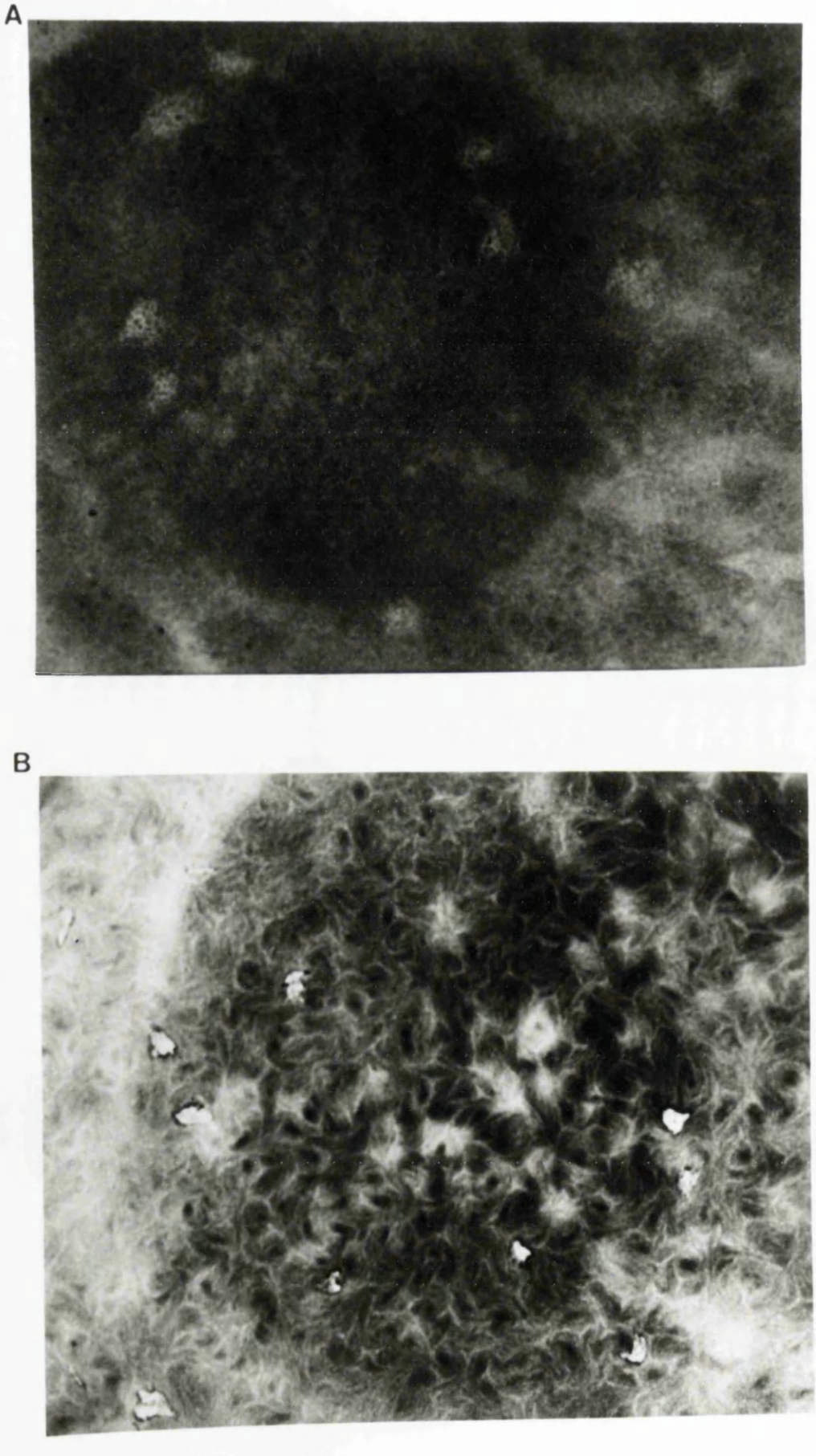
5.2.1. Plaque morphology

The UL13-*lacZ* isolates produced plaques which were similar to those of the US3-*lacZ* isolates, that is, they were slightly smaller than *wt* plaques and their borders were more sharply defined (Figure 5.7).

Figure 5.7 Plaque morphology of the UL13-*lacZ* virus.

Morphology of (a) *wt* and (b) UL13-*lacZ* A54 isolate plaques on BHK cells. The viruses were titrated in parallel and after two days were stained with Giemsa stain and photographed.

The bar represents approximately 2mm.


A
B

5.2.2. Growth curve at a high moi

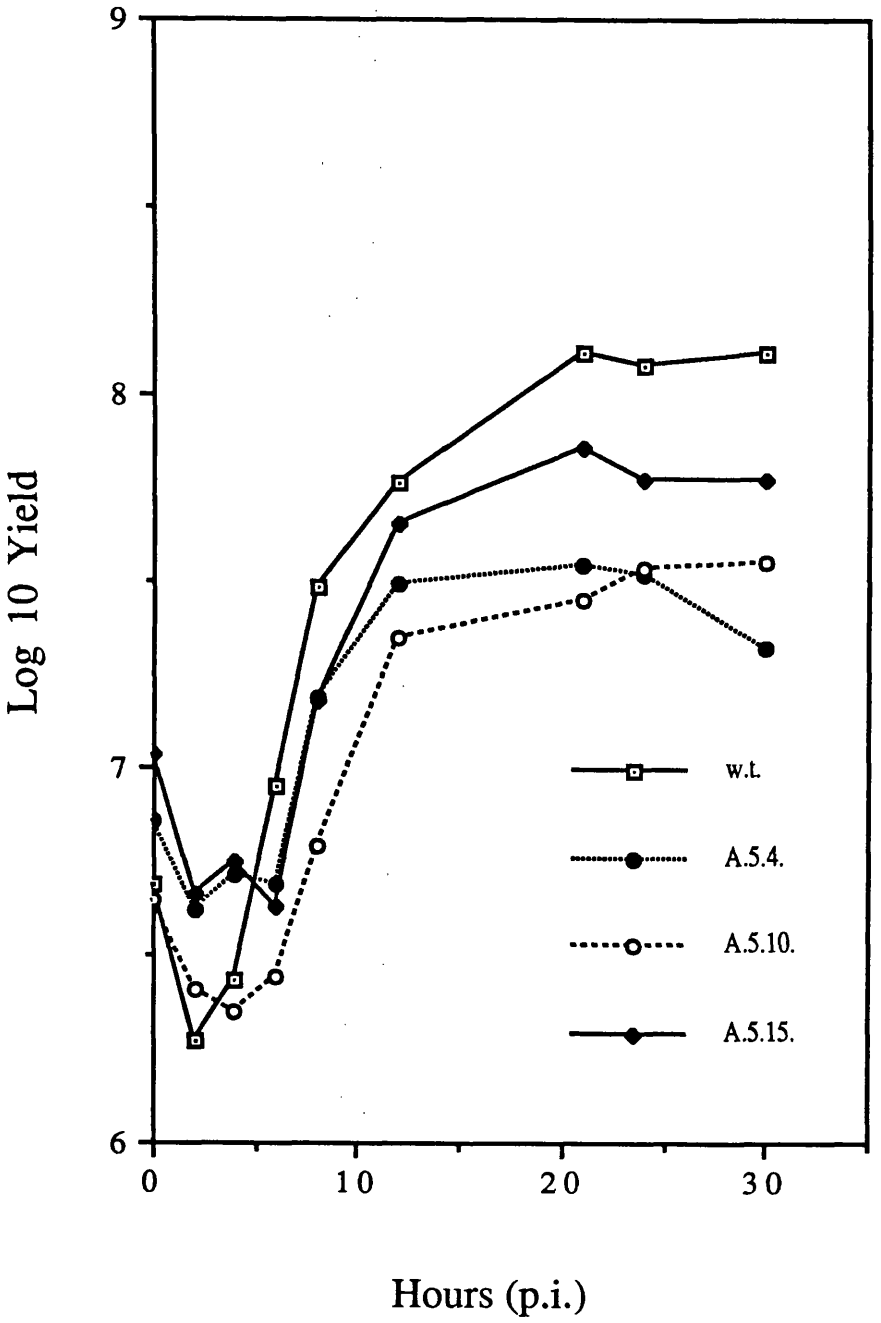
BHK cells were infected with HSV-1 *wt* or with the three UL13-*lacZ* isolates at a moi of 10 and harvested over a period of 30 hours. The growth curves are shown in Figure 5.8.

The three isolates grew to titres which were 2.2-fold (A515), 3.7-fold (A510) or 6.2-fold (A54) less than that of *wt*, indicating that the disruption of the UL13 gene has little effect on virus growth. This experiment was repeated in order to determine if the differences in the growth of the three isolates are reproducible. The final yields of the isolates were found to be 1.2-fold (A515), 15.9-fold (A510) and 3.0-fold (A54) less than that of *wt*. (The difference for A510 in this experiment is unusually high and cannot be explained.) Thus, it is apparent from these results that the A515 isolate consistently grows to higher titres than the other two isolates. One possible explanation for this increased growth is that the A515 isolate stock is contaminated with *wt* virus. The stock was therefore used to set up a low multiplicity infection which was titrated, overlaid with EMC_5/X-Gal and examined for the presence of *wt* plaques. Restriction enzyme profiles of ^{32}P-labelled A515 DNA were also examined closely for the presence of *wt* fragments. However, no evidence of *wt* contamination could be found. Another possible explanation for the differences in the growth of the three isolates is that the A54 and A510 isolates possess an additional mutation which is responsible

Figure 5.8 Growth curve at a high moi.

BHK cells were infected with *wt* virus or with one of the three UL13-*lacZ* isolates (A54, A510 or A515) at a moi of 10. The cells were incubated at 37°C and harvested over a period of 30h. The samples were subjected to ultrasonic disruption and titrated.

Virus yield represents the titre x sample volume.


9
8
7
6
0
10
20
30
Log 10 Yield
Hours (p.i.)
w.t.
A.5.4.
A.5.10.
A.5.15.

for their reduced yields. Such mutations, if present, would be expected to be point mutations or small insertions/deletions, as the restriction enzyme profiles provide no evidence of gross structural differences between the genomes of the three isolates (Figures 5.4 and 5.6).

5.2.3. Growth curve at a low moi

BHK cells were infected at a moi of 0.001 and harvested over a period of 72h. The virus yields are shown in Figure 5.9.

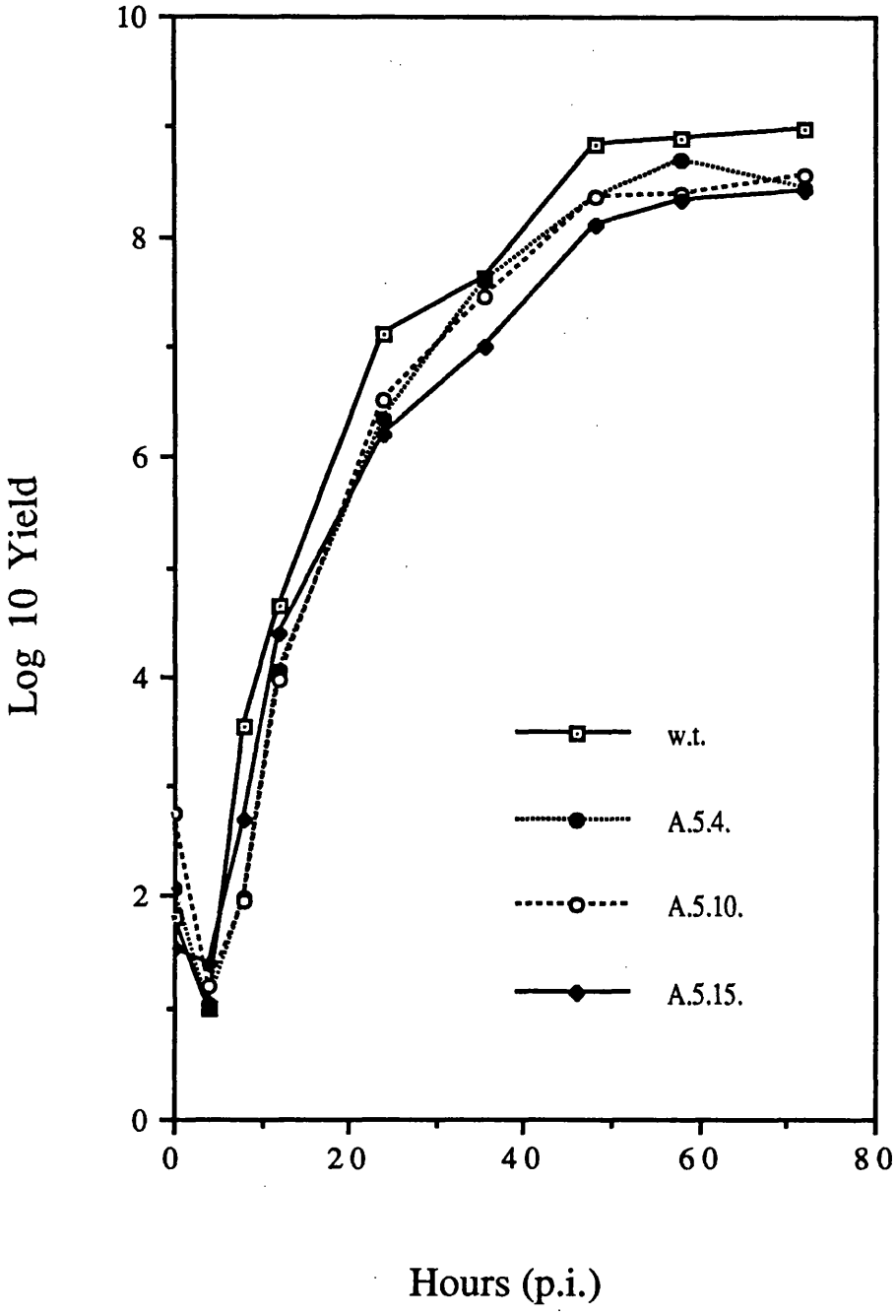
The three isolates gave final yields which were 3.6-fold (A54), 2.8-fold (A510) or 4.0-fold (A515) less than that of *wt*. Similar results were obtained when the experiment was repeated (3.0-fold for A54, 2.5-fold for A510 and 2.5-fold for A515). These experiments therefore confirm that disruption of the UL13 gene does not result in a significant reduction in virus growth compared to *wt*.

It is interesting to note that, unlike the experiment with the US3-*lacZ* virus (section 4.2.3.) the ratios between the *wt* and UL13-*lacZ* isolate yields in this experiment are very similar to those observed following high multiplicity infection (section 5.2.2.), indicating that the slight reduction in growth which follows one round of UL13-*lacZ* viral DNA replication is insufficient to affect the quality of virus entering further rounds.

Figure 5.9 Growth curve at a low moi.

BHK cells were infected with *wt* virus or with one of the three UL13-*lacZ* isolates (A54, A510 or A515) at a moi of 0.001. The cells were incubated at 37°C and harvested over a period of 72h. The samples were subjected to ultrasonic disruption and titrated.

Virus yield represents the titre (pfu/ml) x sample volume.


10
8
6
4
2
0
0
20
40
60
80
Log 10 Yield
Hours (p.i.)
w.t.
A.5.4.
A.5.10.
A.5.15.

It is also apparent from these results that all three isolates grew to similar yields, that is, the small differences in the growth of the isolates, observed during the high multiplicity infection (section 5.2.2.) are eliminated following several rounds of replication. The reason for this is unclear.

5.2.4. Growth of UL13-*lacZ* virus at different temperatures

The temperature sensitivity of the UL13-*lacZ* virus was examined by infecting BHK cells with *wt* or the three UL13-*lacZ* isolates at a multiplicity of 10 and incubating overnight at 31°C, 37°C, 38.5°C or 39.5°C. The virus yields obtained and the ratios of the *wt* yields to the isolate yields are shown in Table 5.1.

The growth of the UL13-*lacZ* isolates was only slightly reduced compared to *wt*, over all the temperatures, and followed a very similar pattern to that of *wt*, that is, maximal yields were reached at 37°C and 31°C with yields decreasing as the temperature was raised. Thus, the UL13-*lacZ* virus is not temperature-sensitive.

Once again, it was noted that the A54 and A510 yields were consistently lower than the A515 yields (see 5.2.2.).

Table 5.1 Growth of UL13-*lacZ* virus at different temperatures

BHK cells were infected at a moi of 10 with *wt* or one of the three UL13-*lacZ* isolates (A54, A510 and A515) and incubated overnight at 31°C, 37°C, 38.5°C or 39.5°C. The infected cells were harvested and titrated on BHK cells at 37°C.

Virus yield represents the titre (pfu/ml) multiplied by the sample volume.

The growth of the UL13-*lacZ* isolates, at each temperature, was compared with that of *wt* by calculating the ratio of *wt* yield:isolate yield.

Temp.	Virus Yield (p.f.u./ml)				Ratio of Yields		
	wt	A54	A510	A515	wt : A54	wt : A510	wt : A515
31°C	$3.5x10^8$	$5.2x10^7$	$3.6x10^7$	$7.5x10^7$	6.8	9.8	4.7
37°C	$3.5x10^8$	$3.4x10^7$	$4.9x10^7$	$1.9x10^8$	10.3	7.1	1.9
38.5°C	$7.8x10^7$	$4.2x10^6$	$6.7x10^6$	$1.7x10^7$	18.6	11.6	4.6
39.5°C	$3.7x10^6$	$2.4x10^5$	$2.1x10^5$	$9.0x10^5$	15.6	17.9	4.1

5.2.5. Growth of UL13-*lacZ* virus on different cell types

BHK, HFL or Vero cells were infected with *wt* virus or one of the UL13-*lacZ* isolates at a moi of 10 and incubated at 37°C overnight. The resulting yields and the ratios of *wt* to isolate yields are shown in Table 5.2.

On BHK cells the yields of the UL13-*lacZ* isolates were, on average, 4.4-fold (A54), 3.0-fold (A510) and 1.7-fold (A515) less than that of *wt*. These ratios are all within the range expected from previous results (see 5.2.2.) and confirm that, on BHK cells, the growth of the UL13-*lacZ* isolates is only slightly reduced compared to *wt*. On HFL cells, the yields of the three isolates were slightly reduced, compared to those obtained on BHK cells, while the *wt* yields remained unchanged. Thus, the corresponding *wt*:isolate ratios were slightly higher than for BHK cells (6.5 for A54, 5.7 for A510 and 3.0 for A515). On Vero cells, the isolate yields were considerably reduced compared to *wt* resulting in higher ratios of 8.8 for A54, 13.4 for A510 and 7.5 for A515.

Thus the UL13-*lacZ* virus displays a slight preference for BHK cells over HFL and Vero cells.

It should be noted that, once again, the A515 isolate grew to higher titres than the A54 or A510 isolates, irrespective of the cell type used.

Table 5.2 Growth of UL13-*lacZ* virus on different cell types

BHK, HFL and Vero cells were infected at a moi of 10 with *wt* or one of the three UL13-*lacZ* isolates (A54, A510 and A515) and incubated overnight at 37°C. The infected cells were harvested and titrated on BHK cells.

Virus yield represents the titre (pfu/ml) multiplied by the sample volume.

The growth of the UL13-*lacZ* isolates on each cell type was compared with that of *wt* by calculating the ratio of *wt* yield:isolate yield.

Cell Type	Expt. No.	Virus Yield				Ratio of Yields		
		wt	A54	A510	A515	wt : A54	wt : A510	wt : A515
BHK	1	$1.2x10^8$	$1.8x10^7$	$9.3x10^7$	$1.5x10^8$	4.4	3.0	1.7
	2	$9.5x10^8$	$2.5x10^8$	$2.0x10^8$	$2.4x10^8$			
	3	$4.0x10^8$	$1.5x10^8$	$1.4x10^8$	$9.1x10^8$			
HFL	1	$4.3x10^8$	$4.9x10^7$	$6.1x10^7$	$1.3x10^8$	6.5	5.7	3.0
	2	$9.8x10^8$	$1.5x10^8$	$1.6x10^8$	$2.2x10^8$			
	3	$1.7x10^8$	$4.1x10^7$	$4.2x10^7$	$1.3x10^8$			
VERO	2	$1.0x10^8$	$7.7x10^6$	$7.2x10^6$	$1.0x10^7$	8.8	13.4	7.5
	3	$2.7x10^8$	$5.9x10^7$	$2.1x10^7$	$5.4x10^7$			

5.2.6. Growth of UL13-*lacZ* virus on "resting" cells

The UL13-*lacZ* isolates were grown on "resting" cells in order to determine if the viability of the UL13-*lacZ* virus is dependent on the presence of a factor found in exponentially growing BHK cells which complements the mutation in the UL13 gene.

Both "resting" and exponentially growing cells were infected with HSV-1 *wt* or the three UL13-*lacZ* isolates, in duplicate, at a moi of 10, incubated at 37°C overnight, harvested and titrated. The results are shown in Table 5.3. [This experiment was carried out at the same time as the US3-*lacZ* virus experiments (section 4.2.7.)].

As mentioned previously (section 4.2.7.) the *wt* yields on the exponentially growing BHK cells were lower than expected from earlier experiments, resulting in slightly lower *wt*:isolate ratios. On "resting" cells the isolate yields were similar to those observed on the exponentially growing cells except for two plates which gave considerably reduced yields (9.2 x 10^5 pfu/ml for A510 and 6.0 x 10^5 pfu/ml for A515). As in the US3-*lacZ* experiments, it was assumed that the cells on these two plates had died and the yields were not used for calculating the ratios. Thus, despite the uncharacteristic growth of *wt* on exponentially growing cells, it is clear that the growth of the UL13-*lacZ* virus is not significantly impaired on "resting" cells, indicating that the function of

Table 5.3 Growth of UL13-*lacZ* virus on "resting" cells

Exponentially growing and "resting" BHK cells were infected at a moi of 10 with *wt* or one of the three UL13-*lacZ* isolates (A54, A510 and A515) and incubated overnight at 37°C. The infected cells were harvested and titrated.

Virus yields represent the titre multiplied by the sample volume.

The growth of the UL13-*lacZ* isolates was compared with that of *wt* by calculating the ratio of *wt* yield:isolate yield.

* indicates unusually low yields which were ignored when the yield ratios were calculated.

Cell Type	Virus Yield				Ratio of Yields		
	wt	A54	A510	A515	wt : A54	wt : A510	wt : A515
Exponential	9.1×10^7	4.2×10^7	3.7×10^7	7.6×10^7	2.2	2.2	1.2
	6.6×10^7	2.8×10^7	3.6×10^7	5.8×10^7			
"Resting"	2.0×10^8	2.0×10^7	2.0×10^7	$^*6.0 \times 10^5$	6.0	7.75	1.7
	1.1×10^8	3.2×10^7	$^*9.2 \times 10^5$	9.3×10^7			

the UL13 gene product is not complemented by a factor present only in exponentially growing BHK cells.

These results also suggest that the UL13-*lacZ* virus will be viable *in vivo*, as "resting" cells are thought to resemble the cells naturally infected with HSV-1 more closely than exponentially growing cells (see section 4.2.7.).

[Once again, it was noted that the A515 isolate grew to higher titres than the A54 or A510 isolates.]

5.3. NEUROVIRULENCE OF THE UL13-*lacZ* INSERTION MUTANT

The neurovirulence of the A515 isolate of the UL13-*lacZ* insertion mutant was compared to that of the *wt* virus, as described in section 4.9. (This work was kindly carried out by Dr Lesley Robertson.) The results, shown in Table 5.4, indicate that the A515 virus is viable *in vivo* and, with an LD_{50} of 5×10^2 pfu/animal, is only slightly less neurovirulent than *wt* ($LD_{50} \leqslant 10^{1.5}$ pfu/animal). To verify that animal death was due to the A515 virus, the viral DNA present within the brains of A515 infected mice which had died was analysed, as before, (section 4.9., work carried out by Dr Christine MacLean). The restriction profile was characteristic of the A515 stock virus and no *wt* virus was detected.

Thus, the absence of the UL13 gene product does not

Table 5.4 Neurovirulence of the UL13-*lacZ* insertion mutant

Three-week old BALB/c mice were infected intracranially with either *wt* or the A515 isolate of the UL13-*lacZ* insertion mutant and the number of deaths (out of a total of 4) scored for each virus dose. The LD_{50} of each virus was determined by plotting the % dead animals against the virus dose (graphs not shown).

Virus	Dose (pfu/animal)						LD_{50} (pfu/animal)
	10^7	10^6	10^5	10^4	10^3	10^2	
wt	N.D.[a]	N.D.	N.D.	N.D.	4/4	4/4	$\leq 10^{1.5}$
A515	4/4	4/4	4/4	4/4	3/4	1/4	5×10^2

a Not Done

neurovirulence not
significantly reduce virus growth in tissue culture.

5.4. PARTICLE COUNTS

Particle counts were carried out on purified virion preparations, which are cleaner than SV stocks and therefore give more reliable results.

Both *wt* and UL13-*lacZ* A515 virions were prepared (as described in section 3.1.5.) and the number of particles present in a sample of each preparation counted. Virion samples were also titrated, in parallel, and the particle:pfu ratios calculated.

The results, shown in Table 5.5, indicate that there is no significant difference between the *wt* and A515 ratios. This result is not unexpected as the growth of the A515 isolate is only slightly less than that of *wt*.

5.5. ANALYSIS OF UL13-*lacZ* VIRUS-INDUCED POLYPEPTIDES

5.5.1. Analysis of [^{35}S]-methionine-labelled infected cell extracts

The polypeptides induced by *wt* and the UL13-*lacZ* virus were labelled with [^{35}S]-methionine, separated on a 5-12.5% gradient polyacrylamide gel and compared. Two differences

Table 5.5 Particle:pfu ratio of UL13-*lacZ* virus

The number of particles/ml present in two *wt* and A515 virion preparations were divided by the titres to give the particle:pfu ratios.

Particle counts were carried out by Mr J Aitken.

Virus	Virion Prep.	Particles/ml	Titre (pfu/ml)	Particle:pfu Ratio
wt	1	$2.6x10^{11}$	$7.9x10^{9}$	32.9 : 1
	2	$1.6x10^{10}$	$1.4x10^{10}$	1.1 : 1
A515	1	$1.7x10^{9}$	$1.8x10^{9}$	0.9 : 1
	2	$1.4x10^{10}$	$2.9x10^{8}$	48.3 : 1

were expected in the UL13-*lacZ* virus polypeptide profiles. Firstly, the 57K protein which corresponds to the UL13 gene product (Cunningham *et al.*, 1992) should not be produced by the UL13-*lacZ* virus, and secondly, there should be a novel protein of approximately 120K representing the β-galactosidase protein. In addition, a novel protein of approximately 25K, representing the C-terminal 288 amino acids of the UL13 protein, may be present. However, the expression of this protein is speculative, depending on a suitably placed promoter.

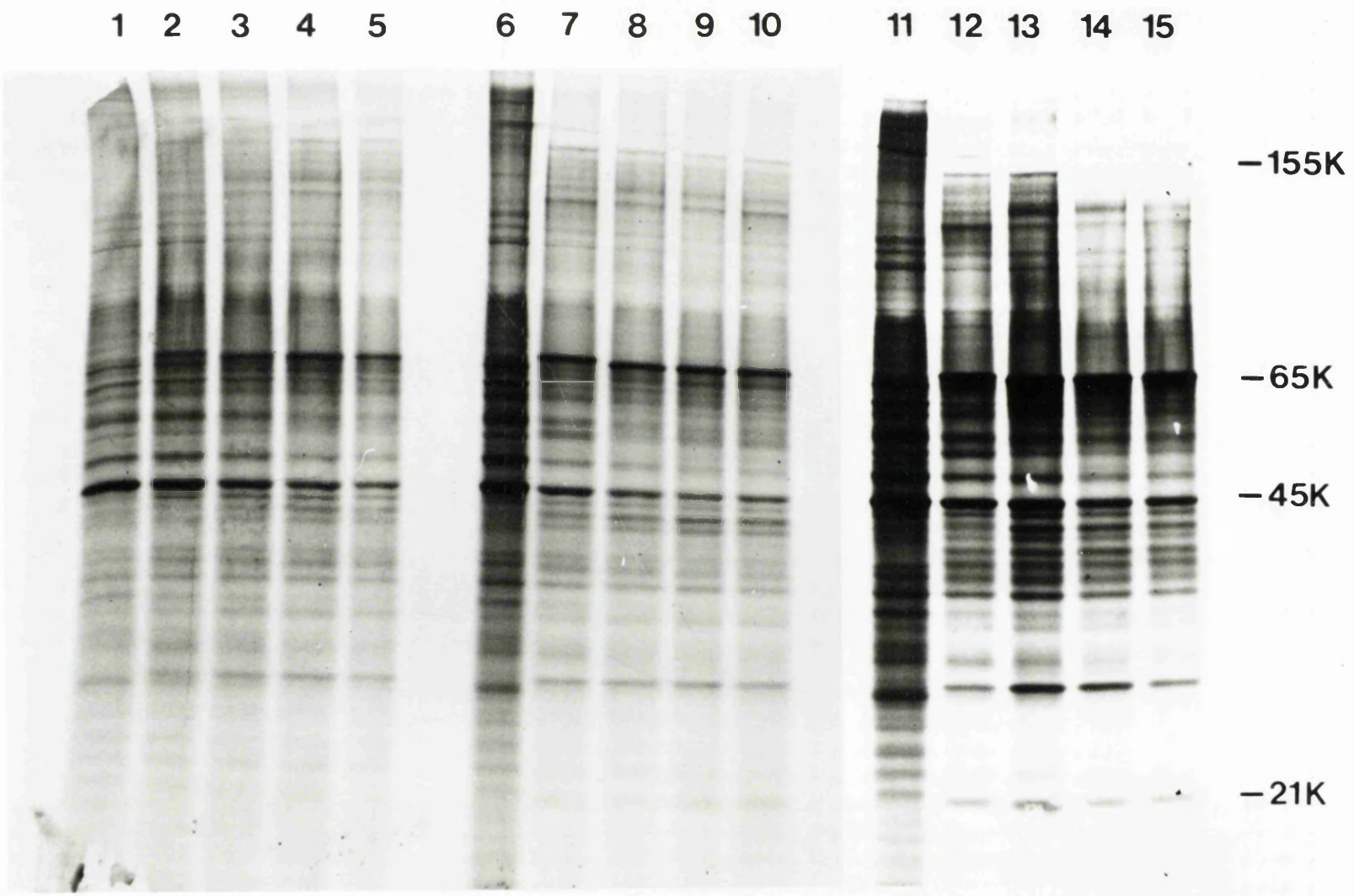
Figure 5.10 shows the *wt* A54, A510 and A515 induced proteins labelled during a 4h-7h pulse (lanes 1-5), a 7h-10h pulse (lanes 6-10) and an overnight label (lanes 11-15). None of the predicted changes in the UL13-*lacZ* virus were observed in any of the three isolates, during any of the labels. It is possible that the proteins of interest have been masked by co-migrating proteins or are present in only small quantities.

One difference that was noted in the UL13-*lacZ* profiles was a slight increase in the mobility of Vmw65, the UL48 gene product (indicated in Figure 5.10). This may be caused by a change in the post-translational modification of the protein, for example, phosphorylation, glycosylation or cleavage. However, it is possible that this apparent decrease in molecular weight is an artifact of this experiment as silver-stained UL13-*lacZ* virion preparations do not show a

Figure 5.10 **[^{35}S]-methionine-labelled polypeptides induced by *wt* virus and the UL13-*lacZ* isolates.**

BHK cells were either mock-infected (lanes 1, 6 and 11) or infected with *wt* (lanes 2, 7 and 12), A54 (lanes 3, 8 and 13), A510 (lanes 4, 9 and 14) or A515 (lanes 5, 10 and 15) and labelled with [^{35}S]-methionine at 4-7h p.i. (lanes 1-5), 7-10h p.i. (lanes 6-10) or overnight (lanes 11-15). The cells were harvested and the proteins separated on a 5-12.5% gradient gel.

Known MWs are indicated to the right of lane 15.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
155K
65K
45K
21K

corresponding shift in the mobility of Vmw65 (see Figure 5.11).

5.5.2. Analysis of [^{35}S]-methionine Labelled Virions.

The UL13 gene product is known to be a constituent of the virion (Cunningham *et al.*, 1992). The polypeptide profiles of UL13-*lacZ* A515 virions and *wt* virions were therefore compared. (The A515 isolate was chosen as it has a slight growth advantage over the other two isolates and should therefore produce slightly greater numbers of virions.)

To visualise the virion proteins, the infected cells were labelled, *in vivo* with [^{35}S]-methionine. The labelled virions were then purified on a Ficoll 400 gradient and their proteins separated on an SDS-9% polyacrylamide gel. However, the signals from the labelled proteins were very weak (even after long exposures) suggesting that either the labelling was inefficient or that the virion yield was poor. Thus, the expected loss of the UL13 protein from the A515 virions could not be visualised.

5.5.3. Silver-staining of UL13-*lacZ* Virion Proteins

Another approach used to investigate the expression of UL13 in the UL13-*lacZ* virus was to silver-stain virion preparations.

Both virions and light (L) particles of *wt* and the UL13-*lacZ* A515 isolate were purified. These preparations were fractionated on an SDS 9% polyacrylamide gel which was then silver-stained (see 3.5.2.b.). A 57K protein, which corresponds to the observed size of the UL13 gene product (Cunningham *et al.*, 1992), was present in both *wt* virions and L particles, but was absent from the A515 preparations (see Figure 5.11). These results therefore confirm that the UL13 gene product is not synthesized in the UL13-*lacZ* virus and also provides evidence that the UL13 protein is present in the virion in appreciable quantities. In addition, the fact that the UL13 protein is present in both virions and L particles suggests that the protein is a constituent of the tegument or envelope, as L particles do not contain capsids. In further experiments, carried out by Dr H. W. McL. Moss, the membranes were stripped from *wt* virions and L particles and the proteins fractionated on an SDS-polyacrylamide gel which was then silver-stained. The 57K UL13 protein was still present in these preparations indicating that UL13 must be a tegument protein (data not shown). [The electrophoresis and the silver staining of the gel shown were carried out by Dr H. W. McL. Moss.]

5.6. *In vitro* PHOSPHORYLATION OF UL13-*lacZ*-INFECTED CELL EXTRACTS AND VIRIONS

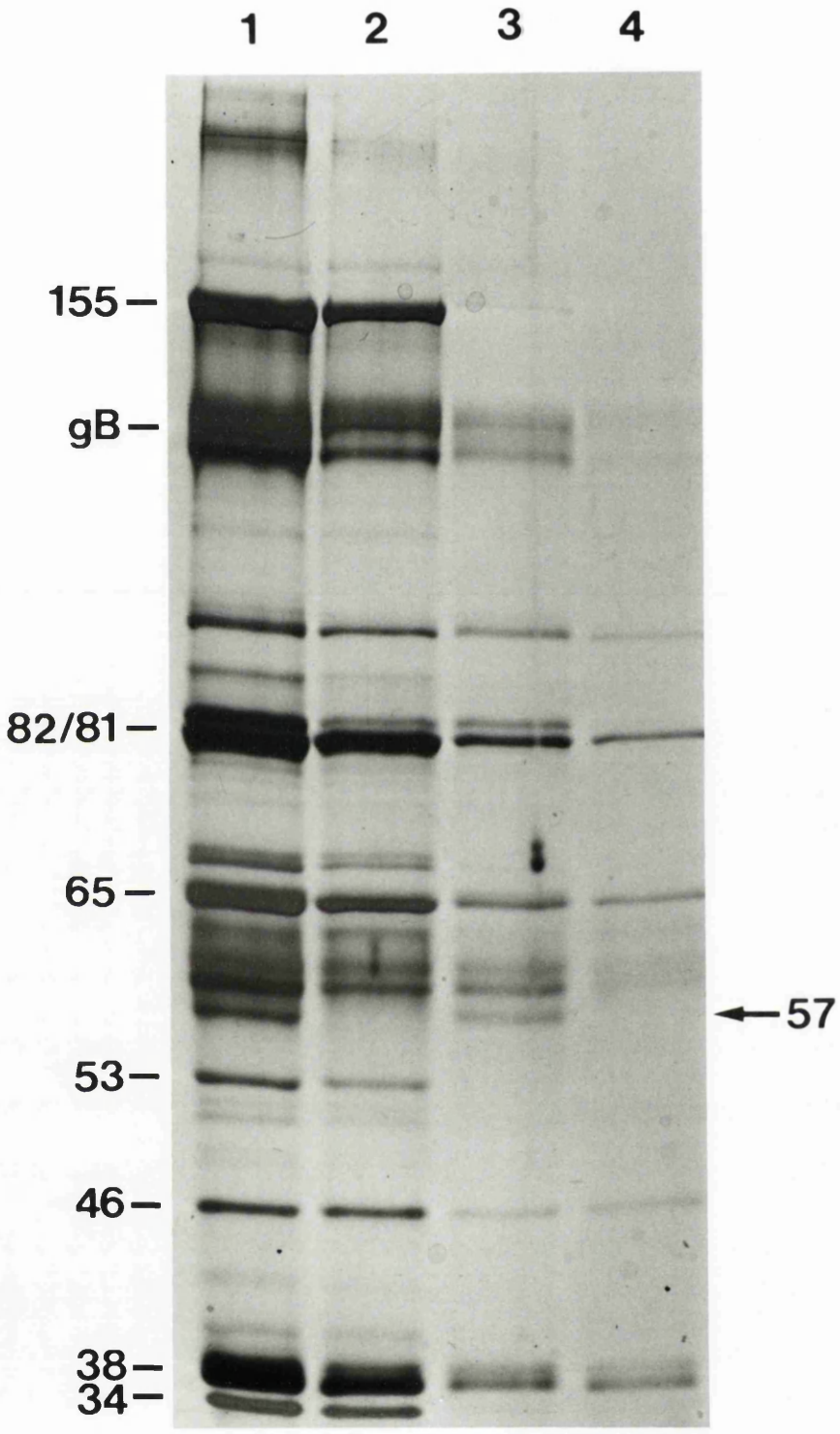
The experiments described in this section were carried out in conjunction with Dr H. W. McL. Moss.

Figure 5.11 **Silver-stained preparations of *wt* and UL13-*lacZ* A515 virions and L-particles.**

Virions (lanes 1 and 2) and L-particles (lanes 3 and 4) were purified from *wt*- (lanes 1 and 3), or A515- (lanes 2 and 4) infected cells and separated on an SDS 9%-polyacrylamide gel. The gel was then silver-stained, as described in section 3.5.2. (b), and a copy of the gel was transferred from duplicating film (Kodak, X-Omat Duplicating Film).

Known virion proteins are indicated to the left of lane 1 and the 57K UL13 gene product is indicated, with an arrow, to the right of lane 4.

The staining of these preparations was carried out by Dr H. W. McL. Moss.


1
2
3
4
155
gB
82/81
65
57
53
46
38
34

5.6.1. *In vitro* Phosphorylation of Nuclear Extracts of Infected Cells.

Nuclear extracts were used for these experiments as the UL13 protein had previously been shown to localise in the nuclei of infected cells (Cunningham *et al.*, 1992).

BHK cells were infected with *wt* or one of three UL13-*lacZ* isolates and incubated at 37°C for 5h. Nuclear extracts were prepared and aliquots were incubated with [γ-^{32}P]-ATP in the presence of different concentrations of salt (ranging from 0 to 1.5M). The proteins were then separated on an SDS 9% polyacrylamide gel and the phosphorylation profiles of the two viruses compared to determine if the UL13 gene plays a role in virus-induced protein phosphorylation.

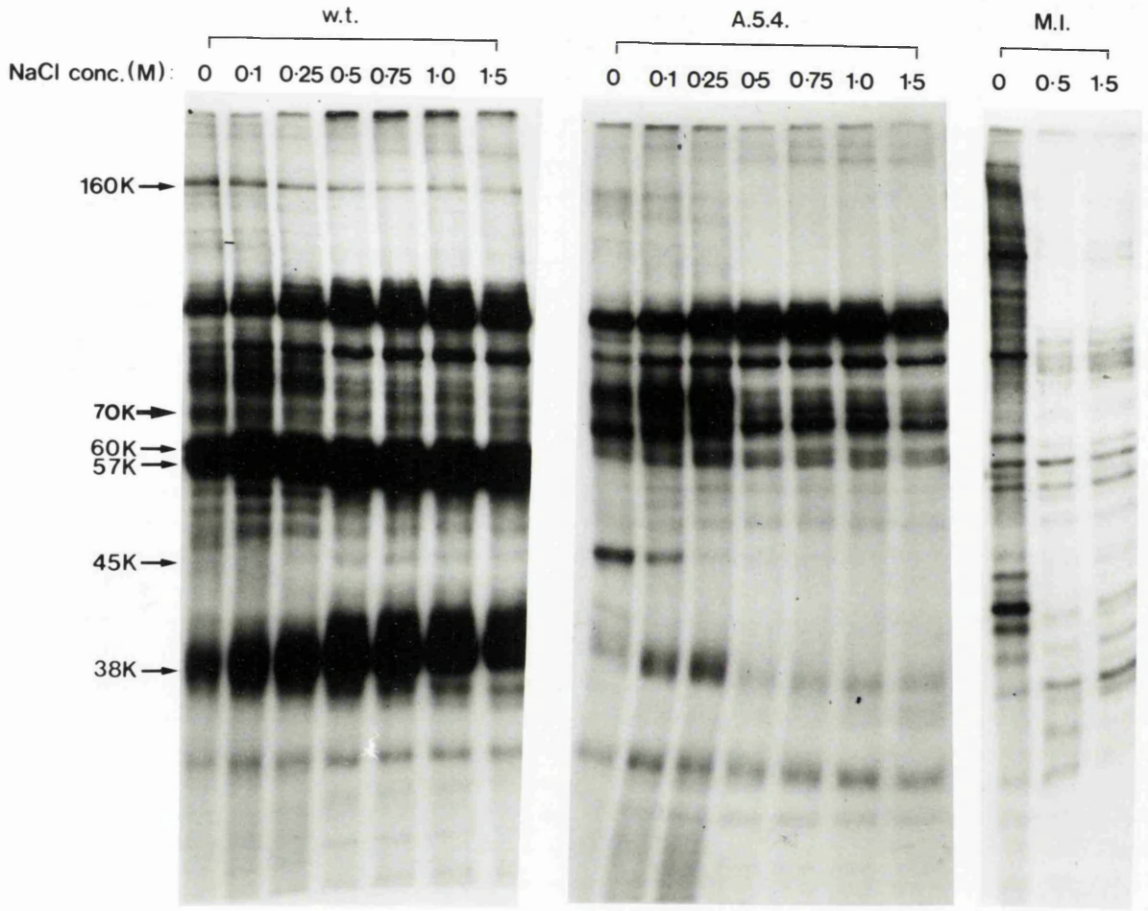
Figure 5.12 shows the phosphorylation profiles of *wt*, UL13-*lacZ* A54, and MI extracts. The *wt* extracts contain many phosphoproteins among which is the prominently labelled 57K UL13 phosphoprotein. The intensity of this band increases as the salt concentration increases, up to the 1.5M maximum, which is consistent with the previously observed optimal salt concentration for UL13 phosphorylation (Cunningham *et al.*, 1992).

Examination of the UL13-*lacZ* A54 phosphorylation profile reveals several differences compared to *wt*. The most noticeable difference is the complete absence of the 57K UL13

Figure 5.12 ***In vitro*** **phosphorylation of nuclear extracts from *wt* and UL13-*lacZ*-infected cells, at a range of salt concentrations.**

BHK cells were mock-infected or infected with *wt* or the A54 isolate for 5h at 37°C. Nuclear extracts were prepared which were then incubated with [γ-^{32}P]-ATP in the presence of a range of salt concentrations. The phosphoproteins were separated on a 9% gel. The phosphoproteins which were absent or underphosphorylated in the UL13-*lacZ* extracts are indicated, with arrows, to the left of the *wt* tracks.

This autoradiograph was prepared by Dr H. W. McL. Moss.


w.t.
A.5.4.
M.I.
NaCl conc.(M):
0 0·1 0·25 0·5 0·75 1·0 1·5
0 0·1 0·25 0·5 0·75 1·0 1·5
0 0·5 1·5
160K
70K
60K
57K
45K
38K

phosphoprotein, at all salt concentrations. There is also a reduction in the phosphorylation of several other proteins, especially at the higher salt concentrations. The apparent molecular weights of these proteins have been estimated to be 160K, 60K, 45K and 38K. The 38K phosphoprotein has been mapped (by Dr H. W. McL. Moss), using intertypic recombinants, to gene UL49 (Coulter *et al.*, 1993) and is equivalent to the previously observed virion/tegument protein VP22 (Elliott & Meredith, 1992). The 160K protein is presumed to be viral in origin, as there is no corresponding band in MI extracts, and may represent Vmw175 or a breakdown product of the 273K UL36 phosphoprotein. The origins of the other two substrates are unknown, although a protein of MW 60K is present in MI extracts suggesting that the 60K substrate could be a cellular protein.

The most straightforward explanation for the observed reduction in phosphorylation of these proteins is that they are acting as substrates for a protein kinase which is induced by the UL13 gene, directly or indirectly. As the UL13 gene has been predicted to encode a serine/threonine kinase it is considered likely that this protein kinase activity is encoded by the UL13 gene itself.

It is assumed that the observed phosphorylation of the 160K and the 38K proteins in the lower salt concentration A54 extracts is due primarily to the action of cellular kinases which are optimally active at these low salt concentrations,

although there does appear to be a reduction in the degree of labelling compared to *wt* suggesting that the UL13 protein kinase also contributes to the phosphorylation of these proteins at low salt concentrations.

Another noticeable difference between the *wt* and A54 phosphoprotein profiles is the apparent increase in the phosphorylation of two proteins in the A54 extracts: a 45K protein in the 0-0.1 M NaCl extracts and a 70K protein in all A54 extracts. The 45K protein may be equivalent to the 45K protein mentioned above, which exhibits a reduction in phosphorylation in A54 extracts with higher salt concentrations or it may represent a novel phosphoprotein. The identity of the 70K protein has not yet been determined. The reason for the increased labelling of these proteins is not known: either the proteins are more extensively phosphorylated or there are increased amounts of the proteins present.

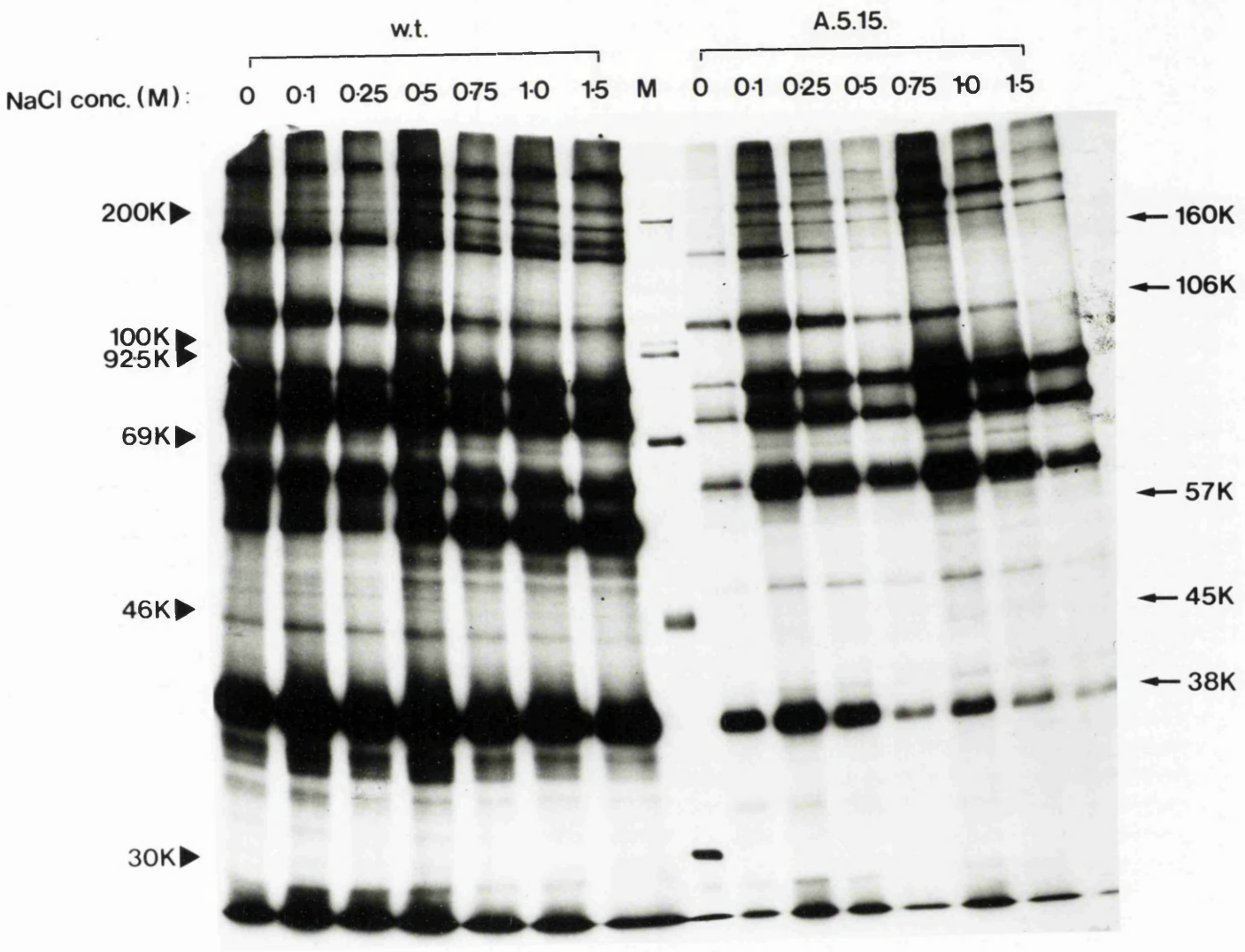
5.6.2. *In vitro* Phosphorylation of Virion Preparations

As the UL13 protein is known to be a constituent of the virion (Cunningham *et al.*, 1992; section 5.5.3.), the *wt* and UL13-*lacZ* A515 virion proteins were incubated with [γ-^{32}P]-ATP and subjected to SDS-PAGE. The resulting phosphoprotein profiles were then compared and differences noted (see Figure 5.13).

Figure 5.13 ***In vitro*** **phosphorylation of** ***wt*** **and UL13-*lacZ* virions.**

Wild-type and UL13-*lacZ* A515 virions were purified, treated with NP40 and incubated with [γ-^{32}P]-ATP, in the presence of a range of salt concentrations. The MWs of the marker proteins (M) are shown to the left of the *wt* tracks. The phosphoproteins which were absent or underphosphorylated in the UL13-*lacZ* virions are indicated, by arrows, to the right of the A515 tracks.

This autoradiograph was prepared by Dr H. W. McL. Moss.


w.t.
A.5.15.
NaCl conc. (M):
0 0·1 0·25 0·5 0·75 1·0 1·5 M 0 0·1 0·25 0·5 0·75 1·0 1·5
200K
100K
92·5K
69K
46K
30K
160K
106K
57K
45K
38K

As expected, the prominent 57K UL13 protein, present in the *wt* virions was absent from the UL13-*lacZ* A515 virions and there was also a marked decrease in the phosphorylation of a 160K protein, a 106K protein, a 45K protein and a 38K protein, especially at the higher salt concentrations.

The 38K protein, as previously mentioned (section 5.6.1.), has been shown to be the product of the UL49 gene and it is assumed that the 160K and 45K phosphoproteins are equivalent to the underphosphorylated proteins of similar size observed in the nuclear extracts (section 5.6.1.). The origin of the novel 106K phosphoprotein is unknown. The 60K phosphoprotein, which was labelled in the nuclear extracts (section 5.6.1.), does not appear to be present in these purified virion preparations supporting the suggestion that this is a cellular protein.

The 45K and 70K proteins which are hyperphosphorylated in the A54 nuclear extracts (see section 5.6.1.) were absent from the A515 virion preparation. This indicates that these phosphoproteins are not constituents of the virion and also suggests that there are two distinct 45K phosphorylated species in nuclear extracts.

These results provide further evidence that the UL13 gene encodes a virion protein kinase.

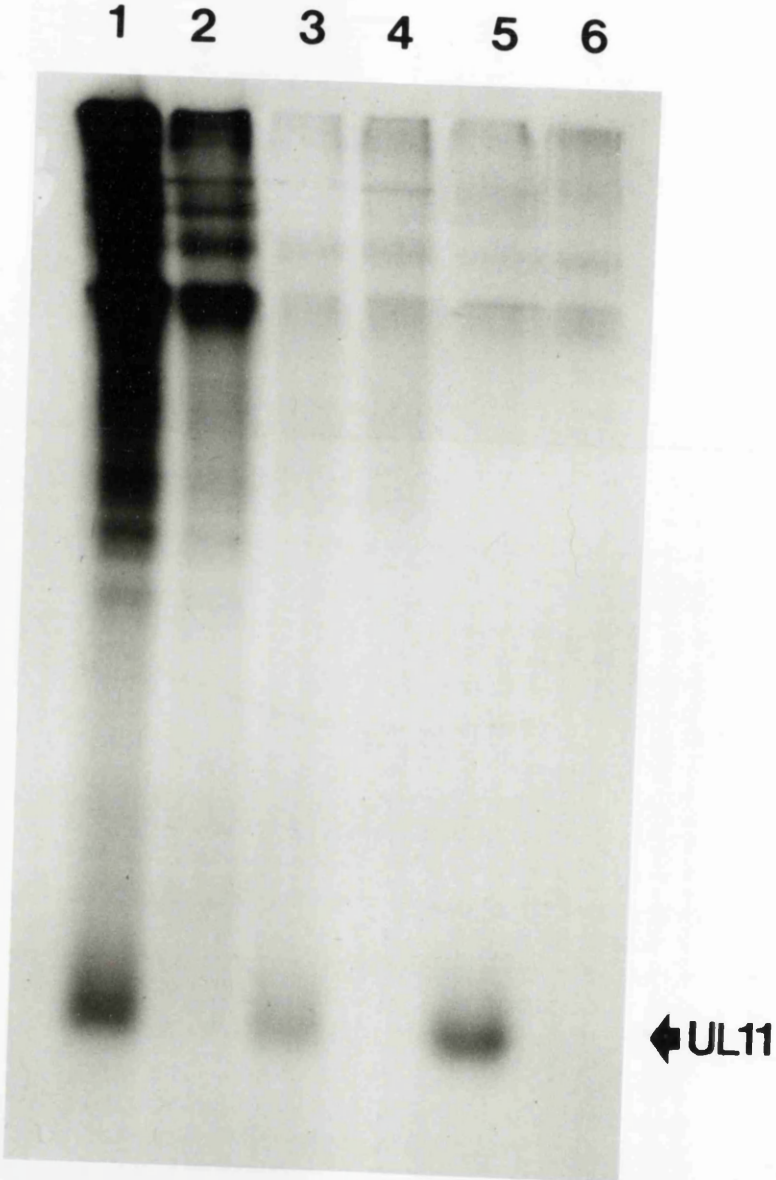
5.6.3. Investigation of the Phosphorylation of the UL11 Gene Product

Work carried out in this laboratory by Dr C. A. MacLean has identified the product of the UL11 gene of HSV-1 as a 13-16K myristylated phosphoprotein which is located in the virion tegument (MacLean *et al.*, 1989; 1992). As the UL13 gene product is a constitutent of the virion and has been shown to phosphorylate a 38K virion protein (VP22, the product of gene UL49) it was thought possible that the UL11 protein may represent another substrate of the UL13 protein kinase activity. [The UL11 protein would not have been detected in the previous *in vitro* phosphorylation experiments (sections 5.6.1. and 5.6.2.) due to its low molecular weight.] The phosphorylation state of the UL11 protein in UL13-*lacZ* virions was therefore determined by immunoprecipitating the protein from virions which had been labelled, *in vitro*, with [γ-^{32}P]-ATP. (The anti-UL11 sera was a gift from Dr C. A. MacLean.) The phosphorylation state of the UL11 protein in US3-*lacZ* virions was also determined.

Figure 5.14 shows that a prominently labelled phosphoprotein is immunoprecipitated from all three virion preparations (*wt* virions, lane 1; US3-*lacZ* A57 virions, lane 3; and UL13-*lacZ* A515 virions, lane 5). The fact that this protein is absent from samples which contain the peptide against which the antisera had been raised confirms that this protein is the product of the UL11 gene. Thus, it is apparent that neither

Figure 5.14 **Immunoprecipitation of the phosphorylated UL11 virion protein from *wt*, UL13-*lacZ*, and US3-*lacZ* virions.**

Wild-type virions (lanes 1 and 2), US3-*lacZ* A57 virions (lanes 3 and 4), and UL13-*lacZ* A515 virions (lanes 5 and 6) were treated with NP40 and incubated with [γ-^{32}P]-ATP. The phosphorylated virions were then incubated with an anti-peptide antiserum raised against UL11 in the absence of peptide (lanes 1, 3 and 5) or in the presence of peptide (lanes 2, 4 and 6) and loaded onto a 7.5-15% gradient gel. The UL11 gene product is indicated with an arrow.


1
2
3
4
5
6
UL11

the UL13 nor the US3 protein kinase is solely responsible for the phosphorylation of the UL11 virion protein. [It is not possible to determine if the two HSV-1 protein kinases contribute to the phosphorylation of the UL11 protein as the amount of protein loaded onto each track was not standardised.]

CHAPTER 6: CONSTRUCTION AND INITIAL CHARACTERISATION OF A UL13-US3 DOUBLE INSERTION MUTANT

6. UL13-US3 DOUBLE INSERTION MUTANT

The main purpose behind the construction of a UL13-US3 double insertion mutant was to determine if there is any degree of cooperativity between the two HSV-1 protein kinases. If cooperativity does exist then a considerable decrease in virus yields would be expected from a virus in which both protein kinase genes had been disrupted. This was investigated as described below.

6.1. CO-INFECTION AND PLAQUE PURIFICATION OF THE DOUBLE MUTANT

An HSV-1 mutant with a *lacZ* insertion in both the US3 and the UL13 genes was generated by a double recombination between the DNA of the two single *lacZ* insertion mutants. This was achieved by co-infecting BHK cells, in duplicate, with 5 pfu/cell of both the US3-*lacZ* virus (A57 isolate) and the UL13-*lacZ* virus (A515 isolate). The cells were incubated at 37°C overnight, harvested, subjected to ultrasonic disruption and titrated. The expected progeny from this co-infection are the two single *lacZ* insertion mutants ("blue" plaques), the double *lacZ* insertion mutant ("blue" plaques) and also *wt* virus ("white" plaques). The frequency of "white" plaques present should give an indication of the likely frequency of double mutant plaques. In this experiment, the percentage of "white" plaques was calculated to be 15%. Therefore out of the 62 plaques which were picked, approximately 9 might be

expected to possess the double mutant genotype, assuming this was viable. The DNAs of the 62 plaques were analysed by digestion with *Bam*HI (see next section) and 4 plaques were found to produce the restriction profile expected for the double mutant. These plaques were picked from the same plate and are probably related. The four plaques were carried through four successive rounds of plaque purification and stocks were grown of two isolates, A42 and A44.

6.2. RESTRICTION ENZYME ANALYSIS OF THE DOUBLE INSERTION MUTANT

The genome structure of the double mutant was analysed by digestion of ^{32}P-labelled DNA with *Bam*HI or with *Hin*dIII.

The location of *Bam*HI restriction sites in the *wt* virus, the UL13-*lacZ* virus and the US3-*lacZ* virus genomes are shown in Figure 6.1. (a), (b) and (c). The predicted *Bam*HI sites in the double mutant genome are shown in Figure 6.1 (d). The UL13 and US3 genes of HSV-1 lie within the *Bam*HI *a* and *n* fragments, respectively. The *lacZ* insert possesses an additional *Bam*HI site. Thus, in the double *lacZ* insertion mutant the *a* and *n* fragments are expected to be replaced by four novel fragments (Figure 6.1. (d)). The two fragments which replace the *a* (12.5 kbp) fragment are a 10.2 kbp fragment (N_2) which runs just above the b fragment (10.1 kbp) on an agarose gel, and a 6.4 kbp fragment (N_1) which runs slightly below the *j* fragment (6.5 kbp). The two fragments

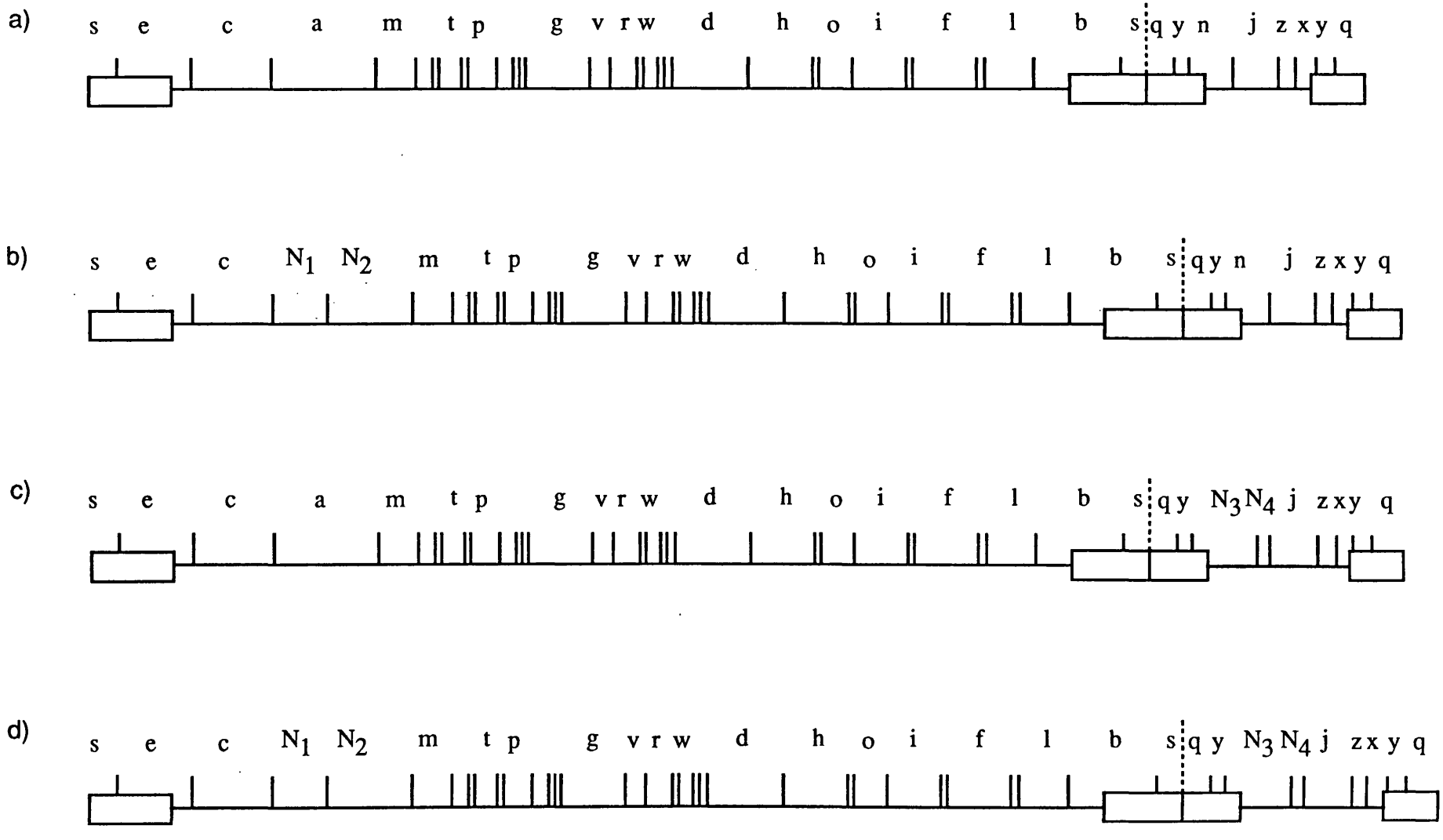
Figure 6.1 **Location of *Bam*HI restriction sites in the *wt*, the UL13-*lacZ*, the US3-*lacZ* and the UL13-US3-*lacZ* genomes.**

The genomes of HSV-1 *wt* (a), the UL13-*lacZ* virus (b), the US3-*lacZ* virus (c) and the UL13-US3 double mutant (d) are shown. The unique regions are represented by solid lines and the repeat elements as open boxes. The dotted line represents the boundary between the L and the S segments.

The vertical lines mark the position of *Bam*HI restriction sites and the letters represent the names of individual restriction fragments (Davison, 1981).

N_1 and N_2 indicate the location of novel fragments in the UL13-*lacZ* genome and also at the UL13 locus of the double mutant.

N_3 and N_4 indicate the location of novel fragments in the US3-*lacZ* genome and also at the US3 locus of the double mutant.


a)
s e c a m t p g v r w d h o i f l b s q y n j z x y q
b)
s e c N1 N2 m t p g v r w d h o i f l b s q y n j z x y q
c)
s e c a m t p g v r w d h o i f l b s q y N3 N4 j z x y q
d)
s e c N1 N2 m t p g v r w d h o i f l b s q y N3 N4 j z x y q

replacing the *n* (4.9 kbp) fragment are an 8.4 kbp fragment (N_3) which runs between the *e* (8.9 kbp) and *f* (8.1 kbp) fragments and a 0.6 kbp fragment (N_4) which runs below *l* (0.76 kbp).

Figure 6.2 shows the *Bam*HI restriction profile for the *wt* virus and the A42 and A44 isolates. The profile for the A42 isolate is as expected for the double mutant showing all the changes mentioned above except for the presence of the 0.6 kbp fragment which is assumed to have run off the bottom of the gel. The A44 isolate, however, while gaining the expected novel fragments, also contains the *a* and the *n* *wt* fragments.

These results were checked by digesting the DNA with *Hin*dIII. The location of the *Hin*dIII sites in the *wt* virus, the UL13-*lacZ* virus, the US3-*lacZ* virus and the double mutant are shown in Figure 6.3.

The UL13 gene lies within the *Hin*dIII *j* fragment and the US3 gene lies within the *Hin*dIII *n* fragment. The *lacZ* insert contains two *Hin*dIII sites. Therefore, in the double mutant (see Figure 6.3 (d)) the *j* fragment (11.8 kbp) will be replaced by two fragments; one of 12.1 kbp (N_2) which will run below the *h* fragment (12.4 kbp) and one of 3.8 kbp (N_1), which will run between the *o* fragment (2.9 kbp) and the *n* fragment (4.9 kbp). A very small fragment of approximately 20 bp will also be produced but is not seen on the gel. The

Figure 6.2 ***Bam*HI restriction profile of *wt* virus and the UL13-US3 double insertion mutant.**

^{32}P-labelled viral DNA was cut with *Bam*HI and the fragments separated on a 0.8% agarose gel. The gel was dried and exposed to X-Omat XS-1 film. Lane 1; *wt* virus, lanes 2 and 3; A42 and A44 isolates, respectively. The names of the *wt* restriction fragments are to the left of the *wt* lane. Empty arrowheads (▷) indicate *wt* fragments which have been lost while filled arrowheads (▶) indicate the presence of novel fragments.

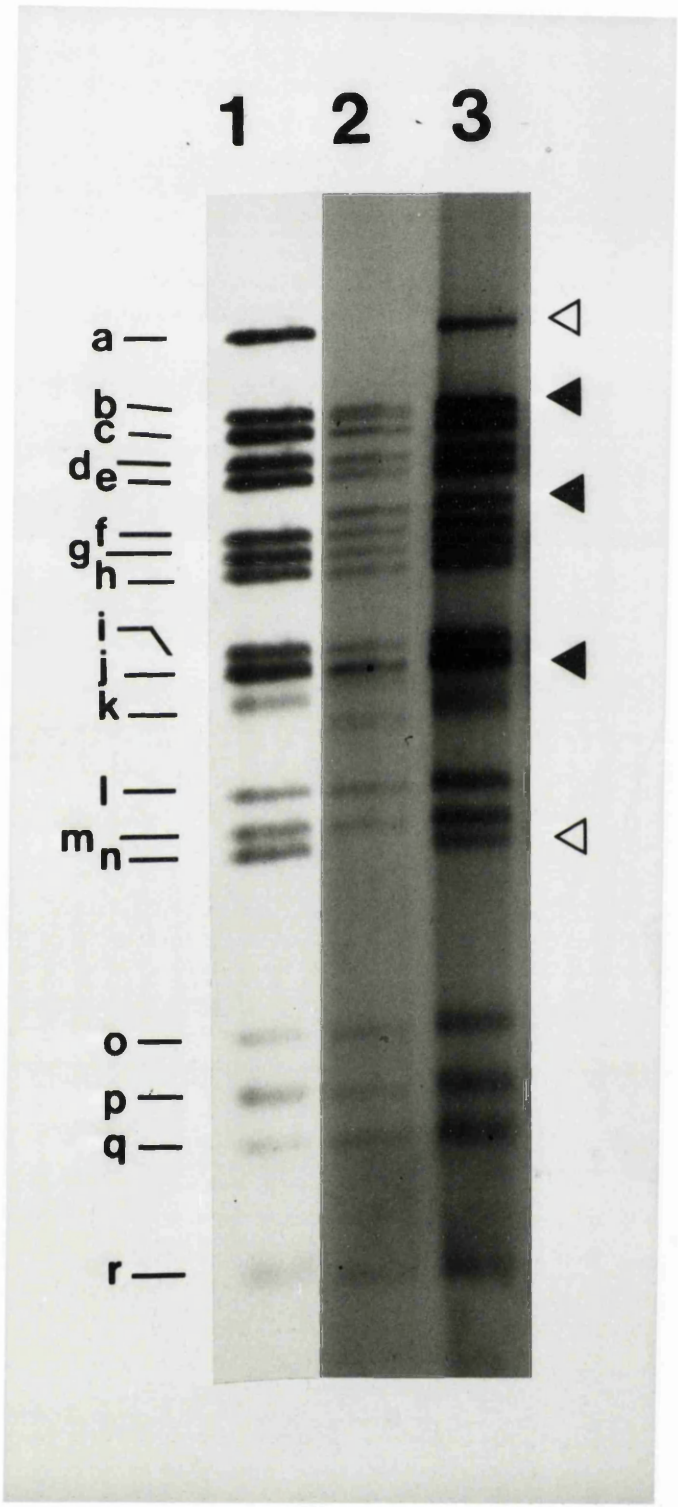

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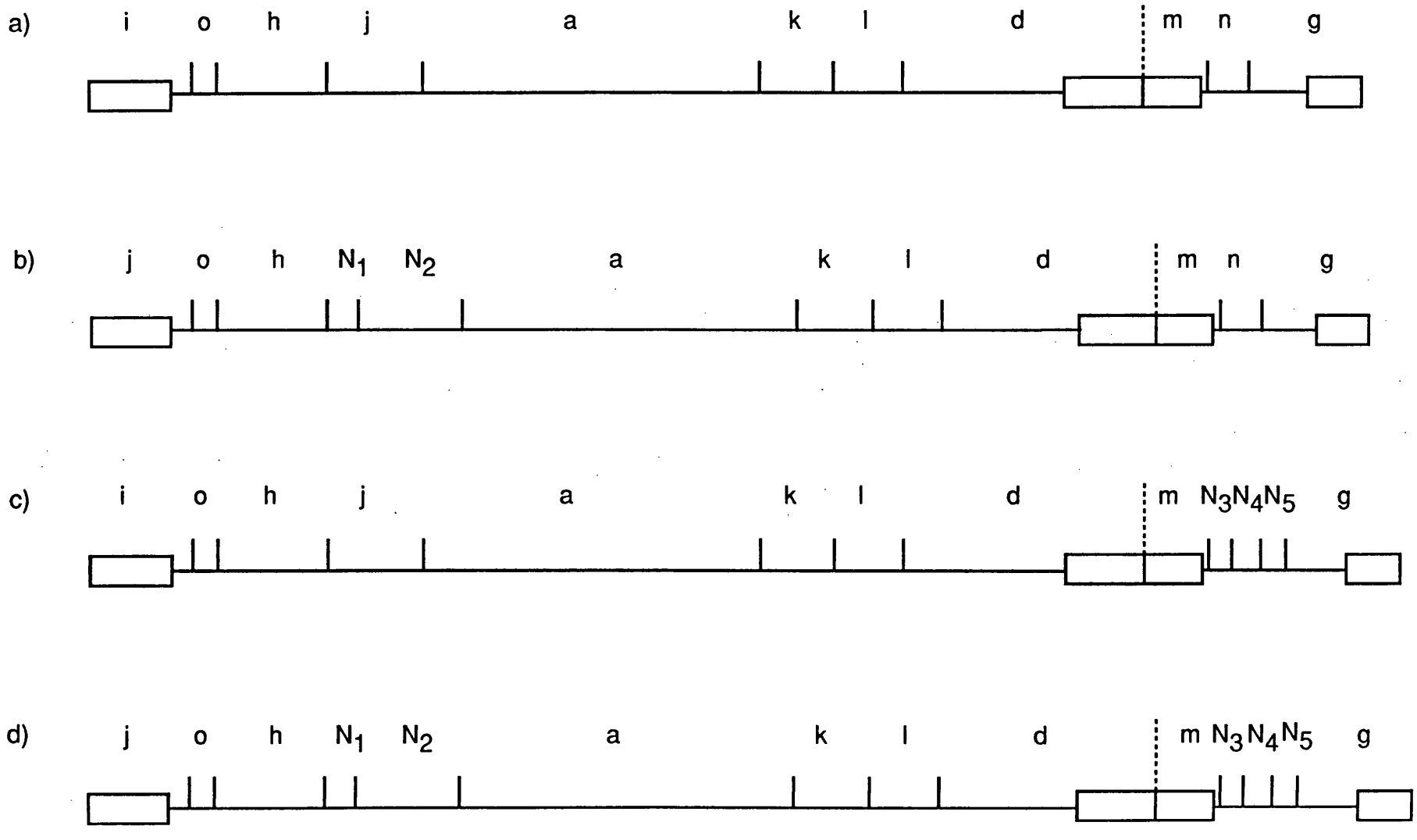
Figure 6.3 **Location of *Hin*dIII restriction sites in the *wt*, the UL13-*lacZ*, the US3-*lacZ* and the UL13-US3-*lacZ* genomes.**

The genomes of HSV-1 *wt* (a), the UL13-*lacZ* virus (b), the US3-*lacZ* virus (c) and the UL13-US3 double mutant (d) are shown. The unique regions are represented by solid lines and the repeat elements as open boxes. The dotted line represents the boundary between the L and the S segments.

The vertical lines mark the position of *Hin*dIII restriction sites and the letters represent the names of individual restriction fragments (Wilkie, 1976; Davison, 1981).

N_1 and N_2 indicate the location of novel fragments in the UL13-*lacZ* genome and also at the UL13 locus of the double mutant.

N_3, N_4 and N_5 indicate the location of novel fragments in the US3-*lacZ* genome and also at the US3 locus of the double mutant.


a)
i o h j a k l d m n g
b)
j o h N1 N2 a k l d m n g
c)
i o h j a k l d m N3N4N5 g
d)
j o h N1 N2 a k l d m N3N4N5 g

US3-*lacZ* insertion will result in the loss of the *n* fragment (4.9 kbp) and the generation of three novel fragments; one which will co-migrate with the 3.8 kbp fragment generated by the UL13-*lacZ* insertion (N_4) and two 2.6 kbp fragments (N_3 and N_5) which will run below the o fragment as a two-molar band.

Figure 6.4 shows the *Hin*dIII restriction profiles for the *wt*, A42 and A44 viruses. The A42 profile contains the changes predicted above but, once again, the A44 profile contains the predicted novel fragments while retaining the *wt* fragments. The obvious explanation for these results is that the A44 virus preparation is contaminated with *wt* virus, despite the four rounds of purification. While this is possible, no *wt* plaques were observed when the stocks were titrated and the low titre of the stock (see later) would also suggest that *wt* virus is not present. In view of these results the A44 isolate can not be classed as a double mutant.

6.3. GROWTH PROPERTIES OF THE DOUBLE MUTANT

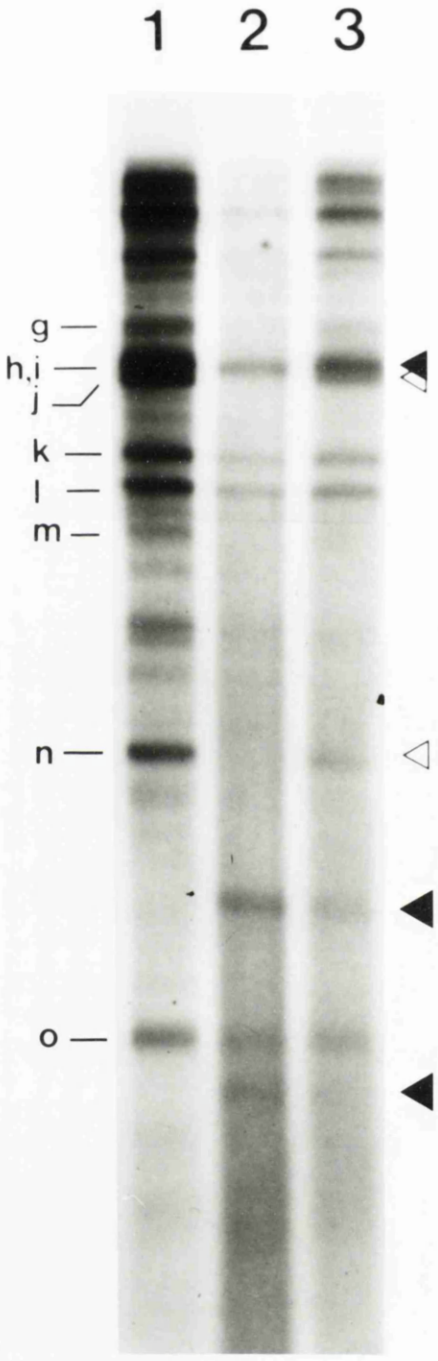
6.3.1. Plaque Morphology

The plaques formed by the A42 (and A44) isolates were much smaller than those produced by *wt* or by the two single *lacZ* insertion mutants, with maximum diameters equivalent to 20-30% of the maximum diameter of *wt* plaques.

Figure 6.4 ***Hin*dIII restriction profile of *wt* virus and the UL13-US3 double insertion mutant.**

^{32}P-labelled viral DNA was cut with *Hin*dIII and the fragments separated on a 0.8% agarose gel. The gel was dried and exposed to X-Omat XS-1 film. Lane 1; *wt* virus, lanes 2 and 3; A42 and A44 isolates, respectively. The names of the *wt* restriction fragments are to the left of the *wt* lane*. Empty arrowheads (▷) indicate *wt* fragments which have been lost while filled arrowheads (▶) indicate the presence of novel fragments.

* Bands not named represent fragments which have been only partially digested.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g
h,i
j
k
l
m
n
o

6.3.2. Titres of Virus Stocks

The titre for the A42 stock was 2.8 x 10^7 pfu/ml which was considerably lower than that of the *wt* stock (1.5 x 10^{10} pfu/ml). (The titre for the A44 isolate was even lower, at 4.0 x 104 pfu/ml.)

6.3.3 Particle Counts

The number of particles present in stocks of the A42 isolate and *wt* were counted and divided by the titres to give the particle:pfu ratios. The results, shown in Table 6.1, show that the A42 virus exhibits a much larger particle:pfu ratio (3643:1) than that of the *wt* stock (16:1) indicating that the A42 virus stock contains a greater number of non-infectious particles.

6.3.4. Growth Curve at a Low moi

BHK cells were infected with *wt* virus or A42 isolate at a moi of 0.001 and harvested over a period of 72 hours. The growth curves are shown in Figure 6.5.

The growth of the double mutant is reduced approximately 800-fold compared to *wt*. This reduction is considerably greater than would be predicted from a simple combination of the growth properties of the two single mutants, but is consistent with the very high particle:pfu ratio of the A42

Table 6.1 Particle:pfu ratio of UL13-US3 double mutant

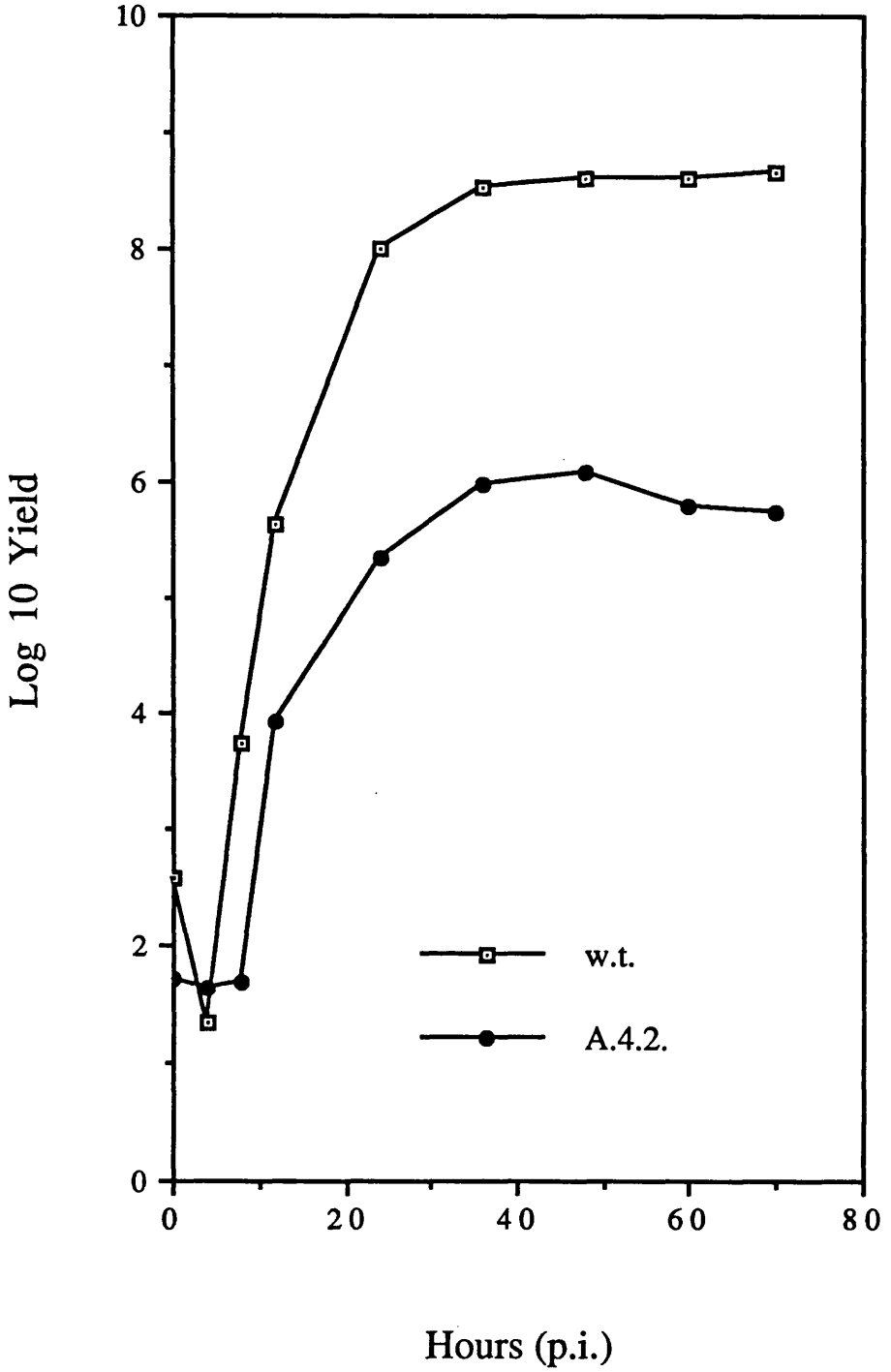
The number of particles/ml present in stocks of the *wt* and A42 viruses were divided by the titres to give the particle:pfu ratios.

Particle counts were carried out by Mr J Aitken.

Virus	Particles/ml	Titre (pfu/ml)	Particle:pfu Ratio
wt	2.3×10^{11}	1.5×10^{10}	16.0 : 1
A42	1.0×10^{11}	2.8×10^{7}	3643 : 1

Figure 6.5 Growth curve at a low moi.

BHK cells were infected with *wt* virus or with the A42 UL13-US3-*lacZ* isolate at a moi of 0.001 and harvested over a period of 72h. The samples were subjected to ultrasonic disruption and the virus yields calculated (titre x sample volume).


10
8
6
4
2
0
0
20
40
60
80
Log 10 Yield
Hours (p.i.)
w.t.
A.4.2.

stock.

These results may be interpreted in two ways: either there is a considerable degree of cooperativity between the two viral protein kinases in *wt* HSV-1, or another deleterious mutation has been inadvertently introduced into the genome. The construction of a revertant virus could resolve this.

(It should be noted that, during the low moi growth curve, the A44 isolate reached yields similar to those of the A42 isolate (data not shown)).

CHAPTER 7: DISCUSSION

7. DISCUSSION

The aim of the research presented in this thesis was to investigate the role of two HSV-1 genes, US3 and UL13, during the HSV-1 lytic cycle. At the start of this research, both US3 and UL13 had been predicted to encode protein kinases due to the presence of characteristic amino acid sequence motifs (McGeoch & Davison, 1986; Smith & Smith, 1989; Chee *et al.*, 1989). The US3 gene had been shown indeed to encode a novel protein kinase, present in the cytoplasm of HSV-1 infected cells. This protein kinase had been purified and its enzymatic properties characterised (Purves *et al.*, 1986a). However, the substrates of the kinase and its functions were unknown. In contrast to US3, protein kinase activity had not been assigned to the UL13 gene product although the protein had been shown to correlate with a novel protein kinase activity present in the nuclei of HSV-1 infected cells (Cunningham *et al.*, 1992). Thus, the specific aims of this project were (1) to identify the substrates of the US3 protein kinase, (2) to determine if the UL13 gene product possesses protein kinase activity, and if so, (3) to identify its substrates.

The tools used in this research were three HSV-1 mutants which were constructed by inserting the *lacZ* gene of *Escherichia coli* into the US3 gene, the UL13 gene, or both the US3 and UL13 genes. The US3-*lacZ* and UL13-*lacZ* viruses were characterised with respect to their growth properties

and polypeptide synthesis and the protein kinase activity of the viruses was investigated by *in vitro* phosphorylation experiments. The UL13/US3 double *lacZ* insertion mutant underwent only limited characterisation due to a lack of time.

The conclusions reached about the substrates and the functions of the US3 and UL13 gene products are discussed below.

7.1. US3-*lacZ* INSERTION MUTANT

The US3-*lacZ* mutant was constructed by cotransfection of plasmid DNA containing the disrupted US3 gene with *wt* HSV-1 DNA. The plaques formed by the recombinant virus, which turned blue in the presence of X-Gal, were picked and carried through 5 to 6 rounds of plaque purification. Three US3-*lacZ* isolates were chosen for further investigation and stocks grown. These stocks were deemed to be pure on the basis of (i) a lack of *wt* fragments following restriction enzyme digestion of the viral DNA and (ii) the absence of *wt* plaques following titration of the US3-*lacZ* virus stocks. However, following the *in vivo* neurovirulence experiments, the three US3-*lacZ* isolates were found to be contaminated with a low level of *wt* virus. Thus, all of the US3-*lacZ* experiments described in this thesis were, presumably, carried out with low levels of *wt* virus present, and the results are therefore not truly representative of a US3-negative virus.

The viability of the recombinant US3-*lacZ* virus indicated that the US3 gene is not essential for virus growth in tissue culture. (The low level of *wt* contamination cannot, alone, account for the observed virus titres.) This result is consistent with that of Longnecker & Roizman (1987) who reported the construction of a US3 deletion mutant. However the US3-*lacZ* mutant differs from the deletion mutant in its growth properties. Longnecker & Roizman (1987) reported that the US3- deletion mutant grew to similar titres as *wt*, however the US3-*lacZ* virus (despite the presence of *wt* virus) exhibits a significant reduction in growth compared to *wt*. This difference in the growth of the two mutants may be due to the different host cells used - Vero cells for the US3- deletion mutant and BHK cells for the US3-*lacZ* mutant. Thus, the US3-*lacZ* virus was grown on different cell types to determine if a US3-negative virus exhibits a host range phenotype. The US3-*lacZ* virus grew on all the cell types tested, although titres did vary depending on the cell type. On cells which gave lower US3-*lacZ* titres (3T6, BSC-1 and one batch of Vero cells) the *wt* yields were also reduced and the difference between the two viruses was negligible, while on cells which supported good virus growth (BHK, HFL, Flow 2002 and one batch of Vero cells) a significant reduction in US3-*lacZ* virus titres, compared to *wt*, was observed. Thus, in contrast to the US3-deletion mutant of Longnecker & Roizman (1987), a reduction in the growth of the US3-*lacZ* virus, compared to *wt*, on Vero cells is observed.

Further characterisation of the growth properties of the US3-*lacZ* virus revealed that the virus is slightly temperature sensitive, being more tolerant than *wt* of lower temperatures (31°C) but more sensitive to higher temperatures of 38.5°C and 39.5°C. The mutant was found to grow well on "resting" cells indicating that virus growth is not dependent upon a factor present in actively dividing cells. The *in vivo* growth experiments revealed that the US3-*lacZ* virus exhibits a reduced neurovirulence, following intracerebral infection, with an LD_{50} of 1 x 10^4 pfu/animal compared to the *wt* LD_{50} of $\leqslant 10^{1.5}$ pfu/animal. This LD_{50} value is lower than that reported by Meignier *et al.* (1988) for the US3-deletion mutant following corneal infection (1.8 x 10^6 pfu/animal) but is greater than that reported recently by Nishiyama *et al.* (1992) for an HSV-2 US3-*lacZ* mutant which was only 10-fold less virulent than *wt* following corneal or intracerebral infection. Following analysis of the virus present in the brains of the mice inoculated with the US3-*lacZ* virus significant quantities of *wt* virus was discovered, which led to the discovery of a low level of *wt* contamination within the US3-*lacZ* virus stocks. It is therefore anticipated that the neurovirulence of a purified US3-*lacZ* virus will be reduced further. Latency experiments were not performed with this mutant. However, both Meignier *et al.* (1988) and Nishiyama *et al.* (1992) have reported that HSV-1 and HSV-2 US3-negative viruses are able to establish and maintain latency.

Alterations in the polypeptide synthesis of the US3-*lacZ* virus were investigated by [^{35}S]-methionine-labelling of US3-*lacZ* and *wt* infected cells, followed by SDS-PAGE. The polypeptide profiles of the two viruses were then compared. The absence of, or (allowing for the low level of *wt* contamination) a reduction in the amount of a 68K protein was expected in the US3-*lacZ* polypeptide profile, as the MW of the US3 gene product has previously been observed to be 68K (Purves *et al.*, 1986a; Frame *et al.*, 1987). However, no differences were observed between the US3-*lacZ* and *wt* profiles. This is probably due to comigration of the US3 protein with other proteins. The US3-*lacZ* virion proteins were also analysed as Zhang *et al.* (1990) had reported that the US3 protein is a component of virions. Both US3-*lacZ* and *wt* virions were purified and the proteins separated by SDS-PAGE and visualised by silver-staining. However, once again, no difference between the *wt* and US3-*lacZ* profiles was observed. Immunoprecipitation and immunoblotting experiments were also carried out on extracts of US3-*lacZ* or *wt* infected cells using antisera raised against the C-terminus of the US3 protein. These antisera had previously been shown to react with a 68K protein in purified preparations of the US3 protein (Frame *et al.*, 1987). However, in these experiments, the antisera failed to recognise the *wt* US3 protein. This is probably due to the fact that in these experiments the *wt* US3 protein was present as a minor protein in infected cell extracts rather than as a purified preparation, as in the

experiments of Frame *et al.* (1987). In addition, the affinity of the antisera may have reduced with age. It is also possible that the lack of detection of US3 is due to a poor HSV-1 infection.

DNA synthesis by the US3-*lacZ* virus was examined as any reduction in the amount of DNA synthesised or packaged by the virus would result in a decrease in the number of infectious particles which could account for the observed reduction in the growth of the US3-*lacZ* virus. Thus, the total amount of DNA synthesised by US3-*lacZ* and *wt* were measured, together with the amount of DNA encapsidated and the amount of DNA released into the medium. The *wt* and US3-*lacZ* measurements were then compared statistically using the Students *t*-test. The total DNA levels for the two viruses were not significantly different indicating that there is no gross reduction in the synthesis of US3-*lacZ* DNA compared to *wt*. The results for the encapsidated and released DNA proved to be contradictory to those of the total and released DNA, with an approximate 2-fold reduction in the amount of US3-*lacZ* encapsidated DNA classed as statistically significant while similar reductions in the amount of total DNA or released DNA (which must also be encapsidated) were not. Disregarding the inconsistencies in the statistical analysis (probably caused by insufficient data), it was concluded that the small reduction in DNA levels detected for the US3-*lacZ* virus could not account for the observed reduction in virus growth. This conclusion is supported by the particle:pfu ratio of the US3-*lacZ* virus which is significantly greater than that of *wt* implying that the reduction in US3-*lacZ* growth is not due to

a decrease in the number of DNA-containing virus particles but to an increase in the proportion of non-infectious particles. Consistent with this rise in non-infectious particles is the observation that, during the lag phases of both the high and low multiplicity growth curves, the US3-*lacZ* virus titres do not decrease as much as those of wt, suggesting that the US3-*lacZ* virions are impaired for cell entry. The lag phase was therefore investigated further, taking samples at 30 minute intervals for the first 5 hours p.i., however, in this experiment a significant difference was not observed between the titres of the two viruses. Thus, a conclusion cannot be reached about the ability of the US3-*lacZ* virions to enter the host cells.

In order to identify potential substrates of the US3 protein kinase extracts of US3-*lacZ* and *wt* infected cells were labelled *in vitro* with γ-^{32}P-ATP and the phosphoproteins separated by SDS-PAGE. In the cytoplasmic extracts of cells infected with *wt* for a period of 5 hours at 37°C, a 68K phosphoprotein was observed which was found to be absent from the equivalent extracts of US3-*lacZ* infected cells. It was concluded that this phosphoprotein probably represents the US3 protein kinase. It should be noted that the 68K US3 protein kinase was not present in nuclear extracts of *wt*-infected cells, which is consistent with the findings of Purves *et al.* (1986a). In addition, the US3 protein was also absent from cytoplasmic extracts of cells which had been infected with *wt* for 16h at 31°C, suggesting that either the

protein has been degraded by this stage in the infection or is no longer phosphorylated. This observation, combined with the fact that the US3 mRNAs are known to be present early in infection, suggests that the US3 protein kinase is active during the initial stages of infection and may therefore be involved in altering the environment of the host cell in order to facilitate viral infection. As US3 is present only in alphaherpesviruses such a function could help determine the host range of the virus.

The absence of the 68K US3 protein kinase was not the only difference observed in the US3-*lacZ* phosphorylation profile, there was also a reduction in the phosphorylation of several other proteins with estimated MWs of 200K, 80K and 30K. The identity of these potential substrates has not yet been established, although it is considered likely that the 30K protein represents the 30K UL34 gene product which has recently been reported to be a substrate of the US3 protein kinase (Purves *et al.*, 1991, 1992). The UL34 protein is a virion protein which is essential for virus replication in tissue culture, although its phosphorylation by US3 is not essential (Purves *et al.*, 1991). During infection with a US3 deletion mutant the UL34 phosphoprotein, which localises to the plasma membrane, was reported to be replaced with four faster migrating phosphoproteins (MW 25-35K) which are not structurally related to the UL34 protein. Purves *et al.* (1992) postulate that the US3 protein kinase is responsible for maintaining these proteins in their unphosphorylated

states, perhaps by regulating the activity of cellular kinases associated with cellular membranes.

Identification of the other potential US3 protein kinase substrates, observed during the *in vitro* labelling experiments, will be of importance in elucidating the precise role of the US3 protein kinase during HSV-1 infection.

7.2. UL13-*lacZ* INSERTION MUTANT

In contrast to US3, the HSV-1 UL13 gene has homologues in all three herpesvirus subfamilies (Smith & Smith, 1989; Chee *et al.*, 1989) suggesting that it was acquired early in herpesvirus evolution, and may play a generally important role in the life cycle of the virus. Thus, at the start of this research, it was considered possible that a UL13-negative mutant would not be viable in tissue culture. Access therefore had been arranged to two UL13 expressing cell lines (constructed by S. Weller and J. Lang) but, as it turned out, these were not needed. The UL13-*lacZ* insertion mutant was constructed by homologous recombination between plasmid DNA, containing the *E. coli lacZ* gene inserted into the UL13 ORF, and *wt* HSV-1 DNA. Recombinant "blue" plaques were picked and purified indicating that the UL13 gene is not essential for virus growth in tissue culture.

The growth properties of the UL13-*lacZ* mutant were investigated. Growth curves carried out at both high and low

multiplicities revealed that the virus grows to titres similar to *wt* but produces smaller plaques. This result differs from that recently reported for an HSV-1 UL13-deletion mutant which was found to be impaired in both growth and plaque formation (Purves & Roizman, 1992). However, growth experiments on a PRV mutant which contains an oligonucleotide insertion in the PRV UL13 homologue revealed no significant differences in either plaque size or titre compared to *wt* PRV (de Wind *et al.*, 1992). The UL13-*lacZ* virus does not display a marked *ts* phenotype and was able to replicate on all cell types tested although titres were slightly reduced on Vero cells. The virus also grew well on "resting" cells. In an experiment to test the pathogenicity of the UL13-*lacZ* virus in mice by intracerebral inoculation, the LD_{50} was found to be only slightly increased compared to *wt*. Thus, it is concluded that the UL13-*lacZ* virus is not significantly impaired for growth either *in vitro* or *in vivo*.

The UL13 gene product has been recognised as a 55-57 KDa phosphoprotein, present in HSV-1 virions (Cunningham *et al.*, 1992; Overton *et al.*, 1992). The virion proteins of the UL13-*lacZ* virus were therefore examined by comparing the silver stained polypeptide profiles of UL13-*lacZ* and *wt* purified virions. A protein of approximately 57K, which corresponds to the observed MW of the UL13 gene product, was found to be absent from the UL13-*lacZ* virions and is therefore presumed to represent the UL13 gene product. The ability to detect the unlabelled UL13 protein indicates that

the protein is a relatively abundant component of HSV-1 virions.

Cunningham *et al.* (1992) reported that the UL13 gene product is phosphorylated by a novel protein kinase activity present in the nuclei of infected cells and suggested that the activity was actually encoded by UL13 with the observed phosphorylation representing an autophosphorylation reaction. To investigate this, nuclear extracts of infected cells and purified virion preparations were incubated with γ-^{32}P-ATP *in vitro* and the phosphoproteins separated by SDS-PAGE. As expected, the strongly phosphorylated 57K UL13 protein was absent from the UL13-*lacZ* phosphoprotein profile. In addition, there was a reduction in the phosphorylation of several other proteins, with estimated MWs of 160K, 106K, 60K, 45K and 38K, indicating that the UL13 gene product does indeed possess protein kinase activity. This conclusion is supported by the recent reports that the VZV and PRV homologues of UL13 also display protein kinase activity (Ng & Grose, 1992; DeWind *et al.*, 1992). One of the potential substrates of the UL13 protein kinase, the 38K protein, has been mapped, using intertypic recombinants, to gene UL49, the product of which has recently been reported to be a tegument protein, previously identified as VP22 (Elliott & Meredith, 1992). The identity of the other underphosphorylated proteins have not yet been determined.

Another difference observed between the *wt* and US3-*lacZ*

phosphoprotein profiles was the apparent hyperphosphorylation of two proteins, with estimated MWs of 45K and 70K, in the A54 nuclear extracts. These two proteins have not yet been identified, although they do not appear to be virion proteins (see section 5.6.2.) and it is considered likely that the 45K phosphoprotein is distinct from the phosphoprotein of the same MW which exhibits reduced phosphorylation in both UL13-*lacZ* nuclear extracts and virions. The reason for the increased labelling of these proteins is not known: either the proteins are more extensively phosphorylated or there are increased amounts of the proteins present. It is interesting to note that Purves & Roizman (1992) have recently reported the presence of a prominently labelled 70K protein in extracts of cells infected with a UL13-negative virus. This protein was identified as one of the five post-translationally modified forms of Vmw68 (ICP22) (MWs 70-82K), the products of gene US1 (α22). Further analysis revealed that the increased labelling of the 70K protein was due, not to an increase in the phosphorylation state of the protein but, to an increase in the amount of protein present. Purves & Roizman (1992) therefore concluded that UL13 is responsible for the post-translational processing of Vmw68 to the apparent higher MW species. Vmw68 has been reported to be involved in extending the host range of HSV-1 in tissue culture and also appears to regulate late protein synthesis (Sears *et al.*, 1985). Thus, Purves & Roizman (1992) propose that UL13 modifies Vmw68 late in infection thereby altering the protein's function and perhaps regulating protein

synthesis. A late function for UL13 would not be unexpected as the gene has previously been assigned to the βγ temporal class (Costa *et al.*, 1983) or, more recently, to the γ temporal class (Overton *et al.*, 1992). However, the presence of UL13 in the virion also suggests that the protein kinase might play a role early in infection or may be active in the virion itself.

Recent work on the HCMV UL13 homologue UL97 has revealed that the product of this gene is responsible for the phosphorylation of the antiviral analogue ganciclovir (Littler *et al.*, 1992; Sullivan *et al.*, 1992). The activity of ganciclovir is dependent upon the formation of the triphosphate, which is a strong inhibitor of the viral DNA polymerase. In many herpesviruses, this initial phosphorylation of ganciclovir is catalysed by viral thymidine kinase, however, in HCMV, which does not appear to encode a thymidine kinase, it is the UL97 gene product which is responsible for the phosphorylation. It is not known if this function of UL97 has evolved to compensate for the absence of an HCMV-encoded thymidine kinase or if phosphorylation occurs due to a chance similarity between ganciclovir and one of its natural substrates. Whichever is the case, this function has important implications for antiviral drug design. It is not known if the HSV-1 UL13 protein kinase possesses a similar activity.

7.3. UL13-US3 DOUBLE INSERTION MUTANT

A UL13-US3 double insertion mutant was constructed by crossing the US3-*lacZ* DNA with the DNA of the UL13-*lacZ* virus during a co-infection of BHK cells. The plaques formed by the recombinant virus were very small, approximately 20-30% of the diameter of *wt* plaques, and the growth of the virus following a low multiplicity infection was found to be approximately 800-fold less than that of *wt*. This reduction in growth is considerably more than would be expected from a simple combination of the growth properties of the UL13-*lacZ* virus and the US3-*lacZ* virus (contaminated with *wt*).

There are several possible explanations for this. Firstly, the two protein kinases may have common substrates and can therefore substitute for each other in the single insertion mutants. However, this is not thought likely to be the case as the two protein kinases have very different substrate specificities (Purves *et al.*, 1986b; Cunningham *et al.*, 1992) and the *in vitro* phosphorylation experiments carried out during the course of this research have not identified common substrates. Secondly, there is the possibility that the large reduction in growth is caused by an additional mutation inadvertantly introduced into the genome of the double mutant during its construction. This possibility could be eliminated by the construction of a revertant. Another possible explanation for the large reduction in growth is that the two HSV-1 protein kinases play similar roles in the virus life cycle.

De Wind *et al.* (1992) have reported that a PRV mutant containing oligonucleoside insertions in the PRV UL13 and US3 homologues is greatly reduced in both growth and plaque size. The construction of a UL13/US3 double deletion mutant has also been reported by Purves & Roizman (1992) - the virus is viable in tissue culture but no other details of its growth properties have been reported.

Further characterisation of this mutant has not been possible due to a lack of time.

7.4. CONCLUSIONS AND FUTURE WORK

The work presented in this thesis has confirmed that the US3 protein kinase is non-essential for virus growth in tissue culture, although its absence does cause a significant reduction in virus titres. Three potential substrates of the kinase have been detected, one of which probably corresponds to the UL34 gene product, identified as a substrate of the US3 protein kinase by Purves *et al.* (1992). This work has also shown that the UL13 gene is non-essential for virus growth, despite its conservation throughout the three herpesvirus families, and that the product of this gene is a relatively abundant component of the HSV-1 virion. It has been demonstrated that the UL13 protein possesses protein kinase activity and five potential substrates have been detected. One of these substrates has been identified as the 38K UL49 protein which is a tegument protein, designated

VP22. Finally, preliminary experiments with the UL13/US3 double insertion mutant have suggested that there is a degree of cooperativity between the two HSV-1 protein kinases.

Despite this work and the results published recently on US3 and UL13, little is still known about the role of these protein kinases during HSV-1 infection. Further investigation of the potential substrates of the two kinases is therefore of importance. Identification of potential viral substrates may be possible by gene mapping, using intertypic recombinants, as with the 38K UL49 gene product. Alternatively, identification may be achieved through recognition of the protein by antisera previously raised against specific HSV-1 proteins. Once identified, the functions of the substrates should give vital clues as to the roles played by the kinases. In cases where the function of the substrate is not known, as with the UL49 gene product, HSV-1 mutants with lesions in the appropriate gene can be constructed and their phenotypes closely examined in order to determine gene function.

Investigating the location of the protein kinases and their substrates throughout the course of infection should also provide valuable information about their functions. This may be achieved through the use of specific antibodies, which are then recognised by a second antibody labelled in such a way as to be visible by microscopy (immunofluorescence).

Other experiments to be carried out include (1) the construction of deletion mutants, to eliminate foreign DNA, (2) the generation of revertants for the three *lacZ* insertion mutants, to ensure that the phenotypes of the viruses are due to the introduced mutations and, (3) further characterisation of the UL13/US3 double insertion mutant, including extensive analysis of the growth properties of the muant and *in vitro* phosphorylation experiments.

Characterisation of the three HSV-1 mutants constructed during this research has yielded information regarding the functions of the US3 and UL13 gene products.

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