Characterisation of the Protein Kinase Domain of the Large Subunit of Herpes Simplex Virus Ribonucleotide Reductase.

by

Jillian Katherine Cooper

A thesis presented for the Degree of Doctor of Philosophy

in

The Faculty of Science at the University of Glasgow

Institute of Virology,
Church Street,
Glasgow,
G11 5JR

August 1994

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

For Mum and Dad.

Table of Contents

Summary

Ribonucleotide reductase catalyses the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates, this enzyme plays a central role in nucleotide metabolism and is essential for DNA replication. Herpes simplex virus type 1 (HSV-1) ribonucleotide reductase is composed of two subunits, a 136kDa large subunit (R1) and a 36kDa small subunit (R2). The large subunits of the herpes simplex (type1 and type2) members of the alphaherpesvirus family are unusual in that they have a 325 amino terminal extension which shows no homology to any other reductase large subunit sequenced (Nikas *et al.*, 1986). Both HSV-1 R1(Conner *et al.*, 1992b) and HSV-2 R1(Chung *et al.*, 1989; Chung *et al.*, 1990) autophosphorylate and in HSV-2 R1 the autophosphorylating activity maps to the unique amino terminal region (Luo *et al.*, 1991; Ali *et al.*, 1991). The herpes simplex viruses are similar in many aspects, their pathology is similar and both are neurotropic, becoming latent in sensory ganglia. These similarities are reflected in the conservation of genes between the two types. Primary sequences of all the HSV-1 genes has been determined (McGeoch *et al.*, 1988) and corresponding in the HSV-2 genes sequenced a high degree of homology has been noted. R1 is unusual in that it shows a varying degree of conservation, with a carboxy terminal portion which is 80% conserved between HSV-1 and HSV-2 and an amino terminal region which shows only 38% conservation. The lack of conservation of this region raised the question of how the autophosphorylating/kinase activities of the two differ.

To determine if this lack of conservation explains the differences that were observed in both the autophosphorylating and transphosphorylating activities of HSV-1 and HSV-2 R1, HSV-2 R1 was overexpressed in *E.coli* and purified to apparent homogeneity using the same expression system and purification scheme used for HSV-1 R1. Analysis of *E.coli* expressed HSV-2 R1 showed that it has properties similar to those described for HSV-1 R1 (Conner*et al.*, 1992b). Autophosphorylation of HSV-2 R1 is similar to that of HSV-1 R1 in its stimulation by basic polypeptides. Like HSV-1 R1 the HSV-2 large subunit is susceptible to proteolytic cleavage. Proteolytic cleavage products of a similar Mwt are observed in type 1 and type 2 R1 extracts. Previously it has been shown that removal of portions of the amino terminal region of HSV-1 R1 did not affect *in vitro* reductase activity. Cleavage may be important in the modulation of the amino terminal-associated activity allowing it to interact with and modulate the activities of other proteins. In contrast to previous observations (Chung *et al.*, 1989), transphosphorylation of histones by the HSV-2 R1 expressed in *E.coli* was not observed. That HSV-2 or HSV-1 R1 do not transphosphorylate can not be ruled out as the substrate may be very specific, protein kinases can have very specific substrates, it is also possible that a co-factor is required to

promote transphosphorylation.

The unique amino terminal region of HSV-1 R1, like that of HSV-2 R1, contains the intrinsic autophosphorylating activity. Mutations were made in HSV-1 R1 to localise and identify regions of R1 important in its autophosphorylating activity. The autophosphorylating activity was shown to reside within the first 257 amino acids of HSV-1, at least 70 amino acids of the unique region of R1 can be deleted without affecting the ability of this region to autophosphorylate. Mutation of the amino terminal region of R1 has identified regions which when mutated by the insertion of a linker either increase or decrease the autophosphorylating activity. An increased autophosphorylating activity was observed with two independent insertion mutants located within a similar region. This region may function normally to down-regulate the autophosphorylating activity possibly by masking sites of catalytic importance or sites of autophosphoryation, a conformational change may normally be required to overcome this inhibitory region. A deletion mutant mapping to the insertion sites at which increased autophosphorylation was observed also had increased autophosphorylating activity, providing further evidence that sequences downstream of these residues are involved in the control of the autophosphorylating activity.

Insertions which decrease activity map to regions upstream of the proposed nucleotide binding site of the unique amino terminal region, it has been postulated that this region in the HSV-2 R1 amino terminus is an important component of a stable kinase doamin (Luo and Aurelian, 1991). A secondary structure prediction by Nikas *et al.*, (1986) showed that this region may form a compact hydrophobic β-sheet structure. Mutation of this region may simply lead to a decreased activity by the destabilisation of a region structurally important. An internal deletion mutant, which removes 93 amino acids of the unique region completely destroys activity, sites of either catalytic importance or sites of phosphorylation map to within this region.

Comparison of the localisation of the amino terminal region in stably transfected cell lines with the localisation of full length R1 in infected cells showed that the localisations are similar, although in the cell lines the pattern of localisation was more defined, being almost exclusively cytoplasmic, with some nuclear localisation being observed The reductase and kinase regions of HSV-1 R1 do not appear to have different subcellular localisations. Preliminary analysis of the ability of HSV-1 to replicate in cell lines expressing the amino terminal region suggest that this region may impair virus replication. Further analysis is required to determine how replication is blocked.

The unique region of HSV R1 may have a function in the pathogenicity of these viruses the unusual pattern of expression of the large subunit mediated by an unusual promoter region may allow R1 to be expressed in the absence of any *de novo* gene expression.

Cleavage may be important in releasing the amino terminal region from the reductase portion of R1 allowing it to interact with other proteins and the autophosphorylating activity may allow this region to be self-regulatory. An autophosphorylating activity has been described for a protein which does not function as a protein kinase and this activity is thought to be a way in which this protein regulates its own function (Csermely *et al.*, 1991). The autophosphorylation of R1 may be important in the regulation of a non-kinase activity. The role of the unique region in the virus life cycle will be determined by the construction of a mutant in which this region has been deleted.

Abbreviations.

A	Adenine
AA	amino acid
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney
bp	base pairs
C	cytosine
C-terminal	carboxy terminal
cAMP	cyclic adenosine monophosphate
CAT	chloroamphenicol acetyltransferase
cGMP	cyclic guanosine monophosphate
Ci	Curie
cpe	cytopathic effect
Da	dalton
DAG	diacylglycerol
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'deoxyguanosine-5'-triphosphate
dl	deletion
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxyribonucleoside-5-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
dx	dexamethasone
E	early
E.coli	*Eschericia coli*
EBV	Epstein-Barr virus
ECl	enhanced chemiluminescence
EDTA	sodium ethylenediamine tetra-acetic acid
eIF-	elongation factor
Et	ethidium
FPLC	fast protein liquid chromotography
G	guanine
g	gram

GCG	genetics computer group
GMEM	Glasgow's modified eagles medium
GTP	guanosine-5'-triphosphate
HCMV	human cytomegalovirus
Hepes	4-(2-hydroxyethyl)-1-piperizine ethane
HHV6	human herpesvirus 6
HHV7	human herpesvirus 7
HRP	horse radish peroxidase
hsp	heat shock protein
HSV	herpes simplex virus
ICP	infected cell protein
IE	immediate early
In	insertion
IPTG	isopropyl-D-thiogalactoside
IRL	internal repeat long
IRS	internal repeat short
K	kilo
Kb	kilobase pairs
L	late
l	litre
LAT's	latency associated transcripts
LTR	long terminal repeat
M	molar
mg	milligram
ml	millilitre
mM	millimolar
MMLV	Moloney murine leukaemia virus
mRNA	messenger ribonucleic acid
mRNA	messenger RNA
n	nano
N-terminal	amino terminal
OD	optical density
Ori	origin
PAGE	polyacrylamide gel electrophoresis
PDGFr	platelet derived growth factor receptor
PEG	polyethylene glycol
pfu	plaque forming units
poly(A)	polyadenylic acid
PRV	pseudo rabies virus

RGB	resolving gel buffer
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulphate
SGB	stacking gel buffer
snRNP	small nuclear ribonucleic acid binding protein
syn	syncitial plaque morphology locus (syn^+=non-syncitial; syn^-=syncitial)
T	thymidine
TFII	transcription factor (pol II)
tk	thymidine kinase
TRL	long terminal repeat
TRS	short terminal repeat
ts	temperature sensitive
TWEEN-20	polyoxyethylene-sobitanmonolaurate
u	unit
UL	unique long
US	unique short
uv	ultra violet
v	volts
Vmw	apparent molecular weight of virus induced polypeptide
vol	volumes
VZV	varicella zoster virus
wt	wild type
μ	micro

AMINO ACID SYMBOLS

A alanine	G glycine	M methionine	S serine
C cysteine	H histidine	N asparagine	T threonine
D aspartate	I isoleucine	P proline	V valine
E glutamate	K lysine	Q glutamine	W trypophan
F phenylalanine	L leucine	R arginine	Y tyrosine

(The three letter code abbreviations are underlined).

Chapter 1. Introduction

1A Herpesvirus biology

1A.1 The herpesviruses

1A.1.1 Classification

The Herpesviridae are a large and diverse family of viruses, at least seven of which are known to infect humans (Roizman, 1990). Initial classification of herpesviruses was made according to the virus host range, duration of the reproductive cycle, the cytopathology of the virus and the characteristics of the latent infection (Roizman and Furlong, 1974). More recently, herpesviruses have been re-classified according to their genome structure (Roizman, 1982) see Figure 1A.1.

Alpha-herpesviruses e.g. HSV-1, HSV-2 and VZV
This class of herpesviruses has a varied host range both *in vivo* and *in vitro* some have a wide host range others are more restricted. The reproductive life cycle is short. These viruses are very cytopathic, infection spreading rapidly in tissue culture. The majority of this class form latent infections in sensory ganglia.

Beta-herpesviruses e.g. HCMV
The in vivo host range of this class is very narrow, frequently species and genus restricted. *In vitro* and *in vivo* these viruses are not very cytopathic, the virus replicates slowly and infected cells become enlarged (cytomeglia). Viral DNA can remain stable in both the nucleus and the cytoplasm of the cell allowing establishment of cells carrying viral DNA. Latency occurs in secretory glands, lymphoreticular cells and kidneys.

Gamma-herpesviruses e.g EBV
Like beta-herpesviruses this class is very host restricted, replication occurring only in the same host family to which the virus belongs. Replication *in vitro* can occur in lymphoblastoid cells, lytic replication is observed in some epithelial and fibroblast cells. The viruses tend to be specific for B- or T- lymphocytes. In lymphocytes replication is

often blocked at a prelytic stage, the viral genome persisting in the cell with minimum viral gene expression, cell death occurs without virus production. The replicative cycle and cytopathogenicity of this family of herpesviruses is variable. Latency is established in lymphoid tissue.

Analysis of the complete genome of the herpesvirus channel catfish virus showed that this virus, originally thought to belong to the alphaherpesvirus family belongs to a new taxonomic group (Davison, 1992).

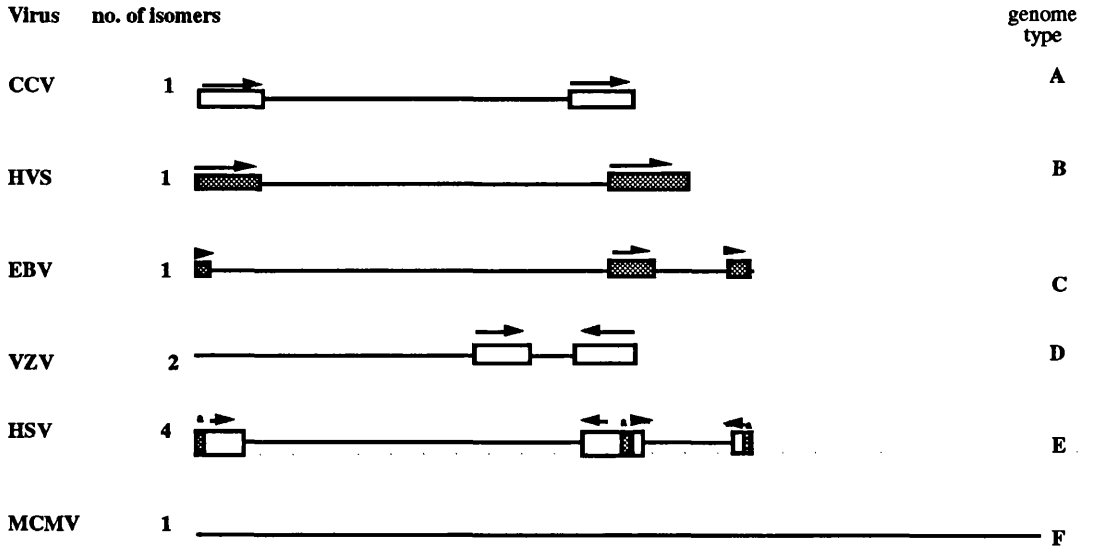


Figure 1A.1 Classification of herpesviruses according to genome arrangement. Examples of genomes types A to F are channel catfish virus (CCV), herpesvirus saimiri (HVS), Epstein-Barr virus (EBV), varicella-zoster virus (VZV), herpes simplex virus (HSV) and murine cytomegalovirus (MCMV) respectively. Unique sequences in the genomes are shown as lines, arrowed lines show possible inversions. Open boxes are large (> 1Kb) repeats, shaded boxes are repeats of small reiterated sequences: arrows above the boxes denote whether repeats are direct or inverted. The small terminal direct and internal inverted repeats of HSV (the "a" sequences) are indicated. The number of possible isomers of each molecule are also given. (Reproduced from Roizman, 1982)

1A.1.2 Pathogenicity of the human herpesviruses

Herpesviruses cause a range of clinical disorders, some of which are described below.

HSV-1 causes vesicular lesions of the mouth, lips and nasal membranes, ocular keratitis and occasionally more severe symptoms such as encephalitis, particularly in immunocompromised individuals (Smith *et al.*, 1941; Gallardo, 1943; Rawls, 1985).

HSV-2 is the main agent of sexually transmitted genital herpes. It is closely related to HSV-1 and some overlaps in their clinical manifestations occur (Whitley, 1985). HSV-2

* Vmw175 was shown to specifically be associated with light particles (virions which lack a nucleocapsid) and not virions by Szilagyi and Cunningham (1991), it can therefore be concluded that the association of Vmw175 with virions described by Yao and Courtney (1989) was due to contaminating light particles in the virion preparations.

has also been implicated in the aetiology of cervical cancer. HSV-1 and HSV-2 often establish latency in trigeminal and sacral ganglia respectively, the periodic recurrence of lytic infection leading to outbreaks of lesions at peripheral sites (Klein, 1982; Knox *et al.*, 1982; Hill, 1985).

VZV is the causative agent for chickenpox (varicella), a disease resulting from primary infection normally found in children, and of shingles (herpes zoster), a localised vesicular condition occurring in adults which appears to be caused by reactivated, latent VZV (Weller *et al.*, 1958; Gleib, 1985).

HCMV infections can cause complications in immunosuppressed individuals and in patients with acquired immune deficiency syndrome (AIDS). It is also sometimes associated with neurological damage in neonates (Alford and Britt, 1984). Infection of the vast majority of the human population, however, results only in a mild, sub-clinical condition.

EBV infects B-lymphocytes and is the causative agent of infectious mononucleosis. It is associated with Burkitt's lymphoma, nasopharyngeal carcinoma and lymphomas in immunosuppressed individuals (Neiderman *et al.*, 1976; Miller 1985).

HHV6 was first isolated from immunosuppressed patients but it can infect T-cells *in vitro* (Salahudin *et al.*, 1986). It is linked to the childhood illness exanthum subitum (Roseala infantum; Yamanishi *et al.*, 1988).

HHV7 is present in CD4+T cells and is purified from peripheral blood mononuclear cells under conditions promoting T-cell activation (Frenkel *et al.*, 1990). The role of HHV7 in human disease is, as yet, unknown.

1A.1.3 Virus Structure

All herpesviruses have a similar structure: an outer lipid membrane surrounds an amorphous, proteinaceous structure known as the tegument; the tegument in turn surrounds the icosahedral nucleocapsid (Roizman and Furlong, 1974). The virus envelope contains at least eight virus-encoded glycoproteins. The tegument is the least well defined part of the virion, several virus proteins have been shown to be associated with this part of the virus: the immediate early gene transactivator Vmw65 (Batterson and Roizman, 1983); the early gene transactivators Vmw175 (Yao and Courtney, 1989) and Vmw110 (Yao and Courtney, 1992), the host shut-off protein (UL41) which down regulates host cell protein synthesis (Kwong *et al.*, 1988) and the protein kinase encoded by UL13 (Cunningham *et al.*, 1992).

Seven virus encoded proteins form the nucleocapsid, a structure composed of 150 hexameric and 12 pentameric capsomers, see Figure 1A.2.

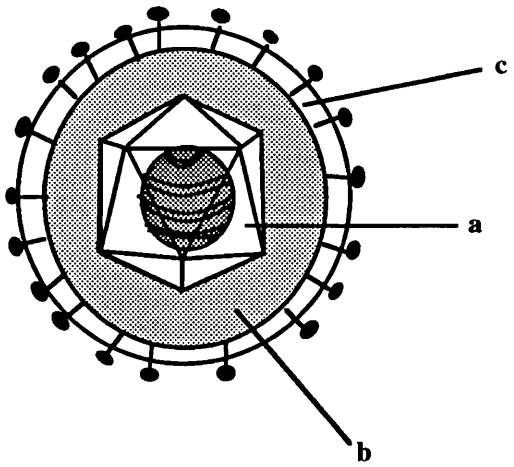


Figure 1A.2 The structure of HSV-1. The virion is composed of three main components a) shows the nucleocapsid surrounding the virus genome, b) shows the virus tegument and c) shows the outer lipid membrane.

1A.1.4 HSV-1 genome organisation

The genome of HSV-1 is a linear duplex DNA molecule (Becker *et al.*, 1968; Roizman and Furlong, 1974) with a molecular weight of 152Kb (McGeoch *et al.*, 1988). The HSV-1 genome is composed of two covalently linked segments, long (L) and short (S), each flanked by inverted repeat sequences (R); terminal (TRL or TRS) or internal (IRL or IRS), (Sheldrick and Berthelot, 1974). The inverted repeats RL and RS share a 400bp a sequence (McGeoch *et al.*, 1988) which is the minimum terminal repetitive sequence required for optimal circularisation (Wadsworth *et al.*, 1975). One or more copies of the a sequence are located internally at the joint between the long and short segments in the opposite orientation to the terminal a sequence (Sheldrick and Berthelot, 1974; Wadsworth *et al.*, 1975; Wagner and Summers, 1978).

The HSV-1 genome exists as four isomers (Figure 1A.3), present in equimolar amounts in virus DNA preparations (Delius and Clements, 1976; Wilkie, 1976). The a sequence was originally thought to mediate isomerisation but mutants which lack an a sequence can also recombine indicating that the a sequence is not essential for genome recombination (Varmuza and Smiley, 1985; Weber *et al.*, 1988).

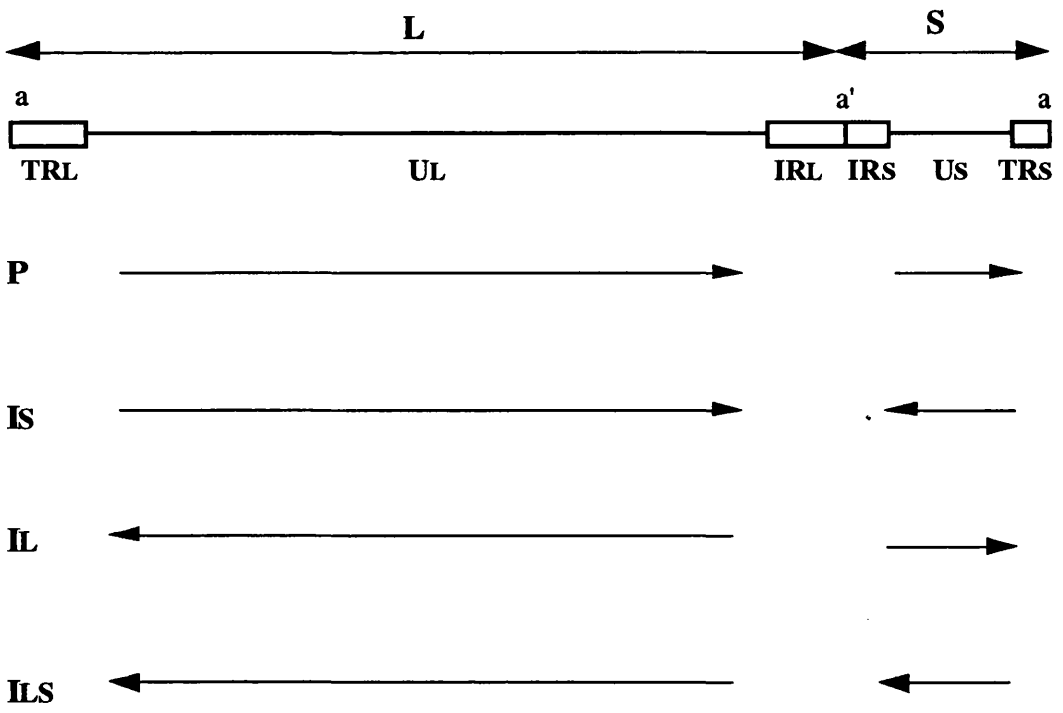


Figure 1A.3 Isomers of HSV-1. The "a" sequence present as a direct repeat at the termini of the genome and, in an inverted orientation, at the L-S junction allows the HSV-1 genome to rearrange into four possible isomers. The four isomers shown are present in equimolar amounts and are designated P (prototype), IS (inverted short), IL (inverted long) and ILS (inverted L and S). (Reproduced from McGeoch, 1987)

1A.1.5 HSV-1 gene arrangement

The complete sequence of HSV-1 has been determined (McGeoch *et al*. 1988) giving a total genome length of 152260 residues. The genome has a high G+C content of 68.3% ranging from 64% in US to 80% in RS. To date 74 open reading frames have been identified. Functions are known for several HSV-1 genes and predictions have been made about function of others by the comparison of their predicted amino acid sequence with the sequences of proteins of known function. The majority of the open reading frames, 56, are located in the unique long region (UL) of the HSV-1 genome. All the genes involved in viral DNA replication and nucleotide metabolism map to the unique long region. Of the 13 open reading frames located within the unique short region (US) the majority, 8, are associated with the virion. The open reading frames for the transactivators Vmw175 and Vmw110 are located in the repeat regions flanking the unique short region (RS) and unique long region (RL) respectively. One recently identified gene, RL1, which encodes ICP34.5, the neurovirulence factor (McGeoch and Barnett, 1991; Chou *et al.*, 1990; Chou and Roizman, 1992), maps to the internal repeat region its open reading frame bridging the IRS and IRL, see Figure 1A.4.

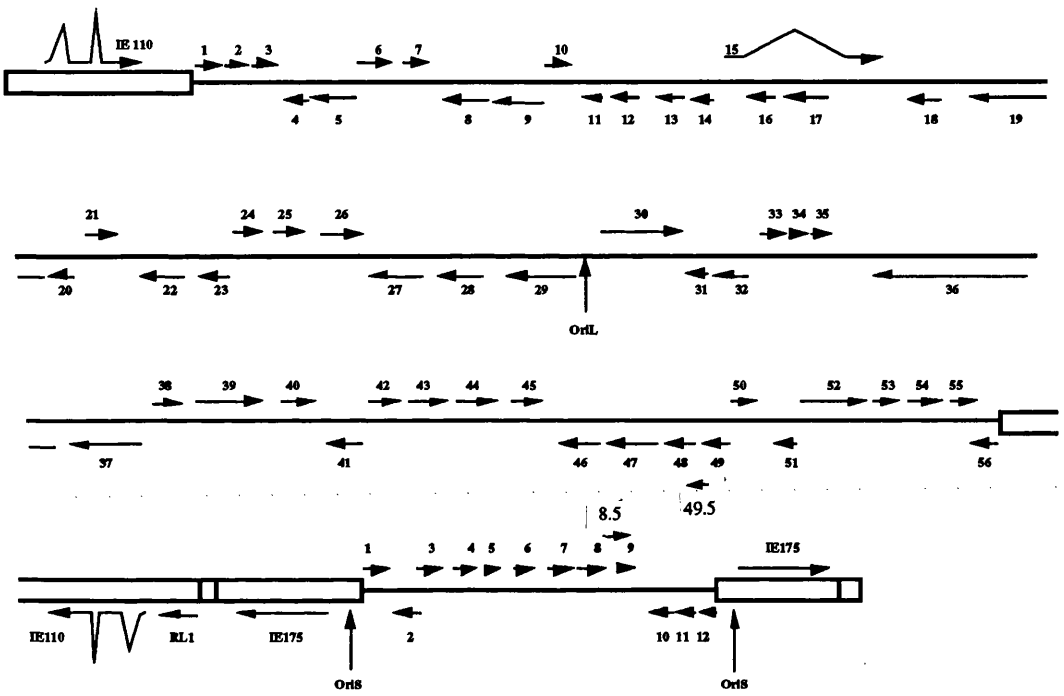


Figure 1A.4 Organisation of genes in HSV-1. The HSV-1 genome is shown on four successive lines, unique regions are represented by solid lines and major repeat regions as open boxes. The size and orientation of proposed open reading frames (ORFs) are shown by arrows. The origins of replication are also indicated. (Reproduced with modifications from McGeoch, 1988a)

Table 1A.1. Properties of HSV-1 encoded proteins (Reproduced from McGeoch *et al.*, 1993)

Genes of HSV-1

gene	Function	essential
RL1	Neurovirulence factor (ICP34.5)	ne
RL2	IE protein; transcriptional regulator (ICP0, Vmw110)	ne
UL1	Glycoprotein L; complexes with glycoprotein H (UL22)	e?
UL2	Uracil-DNA glycosylase	ne
UL3	Function unknown	ne
UL4	Function unknown	ne
UL5	Component of DNA helicase-primase essential for DNA replication	e
UL6	Role in virion morphogenesis (VP11-12?)	e
UL7	Function unknown	
UL8	Component of DNA helicase-primase complex essential for DNA replication	e
UL9	*Ori*-binding protein essential for DNA replication; DNA helicase	e
UL10	Function unknown; probable integral membrane protein	ne
UL11	Myristylated tegument protein; role in envelopment and transport to nascent virions	ne
UL12	Deoxyribonuclease; role in maturation/packaging of nascent DNA	e
UL13	Tegument protein (VP18.8); probable protein kinase	ne
UL14	Function unknown	—
UL15	Function unknown; possible NTP-binding motifs	e?
UL16	Function unknown	ne
UL17	Function unknown	e?
UL18	Capsid protein (VP23)	—
UL19	Major capsid protein (VP5)	e
UL20	Integral membrane protein; role in egress of nascent virions	e/ne
UL21	Function unknown	—
UL22	Virion surface glycoprotein H; complexes with glycoprotein L (UL1); role in cell entry	e
UL23	Thymidine kinase	ne
UL24	Function unknown	ne
UL25	Virion protein	e
UL26	Proteinase, acts in virion maturation; N-terminal portion is capsid protein (VP24)	e
UL26.5	Internal protein of immature capsids (VP22A.); processed by UL26 proteinase	e
UL27	Virion surface glycoprotein B; role in cell entry	e
UL28	Role in capsid maturation/DNA packaging	e
UL29	Single-stranded DNA-binding protein essential for DNA replication (ICP8)	e
UL30	Catalytic subunit of replicative DNA polymerase; complexes with UL42 protein	e
UL31	Function unknown	
UL32	Function unknown	
UL33	Role in capsid maturation/DNA packaging	e
UL34	Membrane-associated phosphoprotein; substrate for US3 protein kinase	
UL35	Capsid protein	
UL36	Very large tegument protein (VP1-3, Vmw273)	e
UL37	Function unknown; may have DNA-binding role	—
UL38	Capsid protein (VP19C)	e
UL39	Ribonucleotide reductase large subunit (ICP6, Vmw136, R1)	e/ne

Table 1 continued

UL40	Ribonucleotide reductase small subunit (Vmw38, R2)	e/ne
UL41	Virion protein; host shutoff factor	ne
UL42	Subunit of replicative DNA polymerase; increases processivity; complexes with UL30 protein	e
UL43	Function unknown; probable integral membrane protein	ne
UL44	Virion surface glycoprotein C; role in cell entry	ne
UL45	Virion protein	ne
UL46	Modulates IE gene transactivation by UL48 protein	ne
UL47	Tegument protein (VP13-14?); modulates IE gene transactivation by UL48 protein	ne
UL48	Major tegument protein; transactivates IE genes (VP16, Vmw65, αTIF)	e
UL49	Tegument protein (VP22)	—
UL49A	Possible membrane glycoprotein	e?
UL50	Deoxyuridine triphosphate	ne
UL51	Function unknown	± e
UL52	Component of DNA helicase-primase complex essential for DNA replication	e
UL53	Glycoprotein K	e?
UL54	IE protein; post-translational regulator of gene expression (ICP27, Vmw63)	e
UL55	Function unknown	ne
UL56	Function unknown	ne
LAT	Family of transcripts, some extending into R_S, expressed in latency; function unknown; protein coding capacity uncertain	ne
RS1	IE protein; transcriptional regulator (ICP4, Vmw175)	e
US1	IE protein; function unknown (ICP22, Vmw68)	e/ne
US2	Function unknown	ne
US3	Protein kinase; phosphorylates UL34	ne
US4	Virion surface glycoprotein G	ne
US5	Virion surface glycoprotein J?	ne
US6	Virion surface glycoprotein D; role in cell entry	e
US7	Virion surface glycoprotein I	ne
US8	Virion surface glycoprotein E	ne
US9	Virion protein	ne
US10	Virion protein	ne
US11	Virion protein; ribosome-associated in infected cell	ne
US12	IE protein; function unknown (ICP47, Vmw12)	ne
UL49.5	Membrane protein	ne
US8.5	Function unknown	ne

1A.2 Entry of HSV-1 into cells

Entry of HSV-1 into cells is pH independent (Wittels and Spear, 1991) indicating that the entry of HSV-1 does not occur by endocytosis. Currently, it is believed that HSV-1 enters the cell by the direct fusion of the virion envelope with the plasma membrane. Several studies have identified virion components involved in the attachment of the virus to the cell and the subsequent penetration of the virus. As yet no specific receptor, other than the cell surface proteoglycan heparin sulphate, involved in the interaction with either HSV-1, or HSV-2, has been identified, both viruses have a wide host range, the receptor involved is likely a common cell receptor.

1A. 2.1 Attachment of virus to cell

Initial attachment to the cell surface is dependent on two virus glycoproteins, gC and gB, and a common cell surface proteoglycan, heparin sulphate (Herold *et al.*, 1992). Heparin inhibits the binding of HSV-1 to cells (WuDunn and Spear, 1989; Shieh *et al.*, 1992). Two cell lines resistant to infection by HSV-1 have been isolated both contain mutations in the pathway involved in heparin sulphate synthesis (Shieh *et al.*, 1992: Gruenheid *et al.*, 1993). Adsorption of HSV-1 to these lines is drastically reduced demonstrating the importance of heparin sulphate in attachment of virus to the plasma membrane.

Binding of the viral glycoproteins gB, gC and gH to the cell surface was demonstrated by affinity purification of cellular membrane proteins labelled with biotin (Kuhn *et al,* 1990). Biotinylated cell surface proteins were affinity purified with avidin, incubated with virion lysates and viral proteins interacting with the cell surface proteins were identified by Western blot analysis using an HSV-1 specific rabbit antisera. The Mwts of the proteins interacting with the cell surface proteins were found to correspond to the virus glycoproteins gB, gC and gH, the identity of these proteins was confirmed using mono-specific antisera raised against gB, gC and gH.

Initial attachment is mediated by the virus glycoprotein gC. Interactions between gC and heparin have been demonstrated; gC can be purified on heparin affinity columns (Shieh *et al.*, 1992). An HSV-1 mutant in gC shows an impaired ability to bind to cells (Herold *et al.*, 1991). Further evidence for the role of gC in the initial attachment of the virus to the cell came from analysis of spontaneous mutants which were resistant to neomycin, a drug which interferes with the binding of virus to the cell (Langeland *et al.*, 1987); all neomycin resistant strains contained alterations in the gC open reading frame (Langeland *et al.*, 1990; Campadelli-Fiume *et al.*, 1990) Virus infection can be neutralised by anti-gC antibodies (Svennerholm *et al.*, 1991; Fuller and Spear, 1985). The glycoproteins, gB and gH, also play a role in virus penetration of the cell. Mutants in gC show impaired binding to the cell, in the absence of gC, cell binding can be partially complemented by gB, another glycoprotein which interacts with heparin (Kuhn *et al.*, 1990).

1A.2.2 Penetration of cell membrane

HSV-1 mutants have been isolated which bind to the cell surface but fail to penetrate the plasma membrane; mutants in gD bind to the cell membrane but fail to penetrate (Ligas and Johnson, 1988; Johnson and Ligas, 1988). Glycoprotein D is essential for virus-cell membrane fusion and for the fusion (syncytia) of infected cells. In addition, gB is involved in membrane fusion, mutation of this gene demonstrated directly the role of this

glycoprotein on viral penetration, mutants become attached to the cell but fail to penetrate (Cai *et al.*, 1987). Mutation of gH inhibits virus penetration of the cell but not the attachment of the virus to the cell (Forrester *et al.*, 1992) .

1A.3 The HSV-1 regulatory cascade

HSV-1 genes fall into three temporal classes whose expression is co-ordinately regulated and sequentially ordered in a cascade fashion (Honess and Roizman, 1974). The five immediate early genes; IE1, IE2, IE3, IE4 and IE5 and the large subunit of ribonucleotide reductase are the first genes to be transcribed after infection (Clements *et al.*, 1977). Transcription of all the immediate early genes with the exception of Vmw110 (Godowaski and Knipe, 1985;Weinheimer and McKnight, 1987) is then down regulated in response to synthesis of IE3 (Vmw175) (O'Hare and Hayward, 1985b; Beard *et al.*, 1986; Gelman and Silverstein, 1987; Roberts *et al.*, 1988). Expression of Vmw175, the major regulatory protein of the virus, is continuously required for expression of early genes (Dixon and Schaffer, 1980; Preston, 1979; Watson and Clements, 1978; 1980). Finally, once DNA replication has been initiated, late genes are expressed (Holland *et al.*, 1980). Optimal levels of late gene expression require the presence of both Vmw175 and Vmw63 (McCarthy *et al.*, 1989; McMahan and Schaffer, 1990; Rice and Knipe, 1988; Su and Knipe, 1989; Sacks *et al.*, 1985). Late gene products are mainly structural polypeptides. The regulatory cascade of HSV-1 is shown in Figure 1A.5.

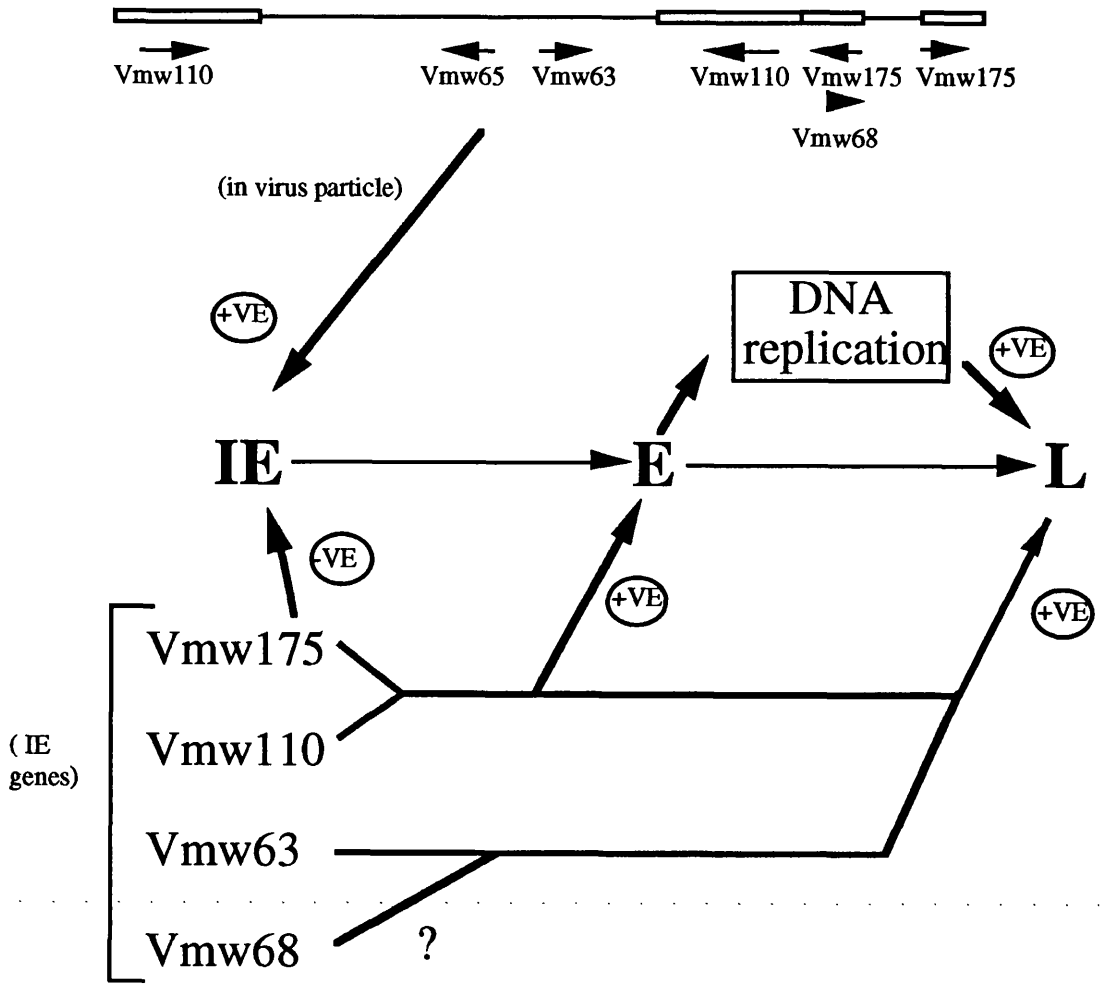


Figure 1A.5 HSV-1 regulatory cascade. Gene expression in HSV-1 falls into three distinct classes, immediate early (IE) gene expression is stimulated by the virion protein Vmw65. Following expression of the immediate early gene product, Vmw175, early (E) gene expression is initiated. Finally once DNA replication has been initiated the late (L) genes are expressed. The down regulation of immediate early gene expression by Vmw175 is also shown.
(Reproduced with modifications from an original diagram by R. D. Everett)

1A.3.1 Immediate early gene expression

1A.3.1.1 Cis-acting sequences.

(a) TAATGARAT /Vmw65

Vmw65 is a component of the HSV-1 virion which specifically up-regulates transcription from HSV-1 immediate early promoters. Fusion of the promoters of immediate early genes to the HSV thymidine kinase gene showed that increases in tk activity was now independent of synthesis of viral gene products (Post *et al.*, 1981). Mutants incapable of releasing viral DNA have immediate early transactivating activity, indicating that the transactivator was located in the virion tegument (Batterson and Roizman, 1983). The

virion component responsible for immediate early transactivation, Vmw65, was identified (Campbell *et al.,* 1984) and found to be present in significant quantities, between 500-1000 molecules per virion (Heine *et al .,* 1974). The upstream element which responds to Vmw65 transactivation of immediate early genes was identified by sequence comparison (Mackem and Roizman, 1982a,b; Whitton and Clements, 1984). The sequence TAATGARAT appears in several copies upstream of the immediate early genes in both HSV-1 and HSV-2, and confers Vmw65 inducibility to otherwise non-responsive genes (Preston *et al.,* 1984; Bzik and Preston, 1986).

Vmw65 alone does not interact with DNA (McKnight *et al.,* 1987; Marsden *et al .,* 1987). Reports by O'Hare and Goding, (1988) and Preston *et al.,* (1988) described the identification of the cellular component which mediates transactivation of immediate early genes by Vmw65. The cellular factor, Oct-1, belongs to the pou homeodomain family of transcription factors (Herr *et al.,* 1988; O'Hare *et al.,* 1988). A closely related homeodomain protein Oct-2 interacts with the TAATGARAT element but fails to produce Vmw65 specific transactivation, demonstrating the specificity of the Vmw65/Oct-1 interaction (Gerster and Roeder, 1988; apRyhs *et al.,* 1989). A third, recently purified (Kristie and Sharp, 1993) and cloned cellular factor (Wilson *et al.,* 1993), is important in the TAATGARAT complex formation, this factor, HCF (Gerster and Roeder, 1988; Kristie *et al.,* 1989; Xiao and Capone, 1990; Katan *et al.,* 1990), promotes assembly of Vmw65 in the transactivating complex. HCF interacts with Vmw65 and brings the virus transactivator to the target TAATGARAT site whereupon binding of Oct-1 the fully functional complex is formed.

Transactivation by Vmw65

The carboxy terminal acidic tail, a region of 79 amino acids, forms the transcriptional activation domain of Vmw65 (Ace *et al.,* 1988; Cousens *et al.,* 1989; Greaves and O'Hare, 1989; Treizenberg *et al.,* 1988a). This region when coupled to the DNA binding domain of Gal 4 produces similar levels of transactivation to the HSV-1 TAATGARAT/Oct-1/HCF/Vmw65 complex (Sadowski *et al.,* 1988). The 79 amino acid transactivation domain of Vmw65 can be further separated into two functionally distinct regions (Reiger *et al .,* 1993; Walker *et al.,* 1993), the N- and C-terminal portions of the transactivation domain (which each form a predicted helical structure). Both the N- and C-terminal regions function as transcriptional activators (Walker *et al.,* 1993), full activity requiring both. Interactions between the Vmw65 transactivation domain and the basal transcription factor TFIIB have been demonstrated (Lin and Green, 1991; Lin *et al.,* 1991). The interaction between TFIIB and Vmw65 is not sufficient for full activation , *in vitro* transcription assays demonstrated that a factor associated with the TFIID complex (TBP) is required for full activity (Brou *et al.,* 1993; Tanese *et al.,* 1991). A TAF (transcriptional

activating factor), a factor which mediates the effect of an upstream activator on the pre-initiation complex, TAFII40 from Drosophilla, interacts with the TFIIB/Vmw65 complex (Goodrich *et al.,* 1993), see Figure 1A. 6. Studies by Walker *et al.,* (1993) identified a 135kDa polypeptide which appears to be specifically associated with the activation domain, possibly another TAF; Goodrich *et al.,* (1993) have proposed that there may be another TAF interaction.

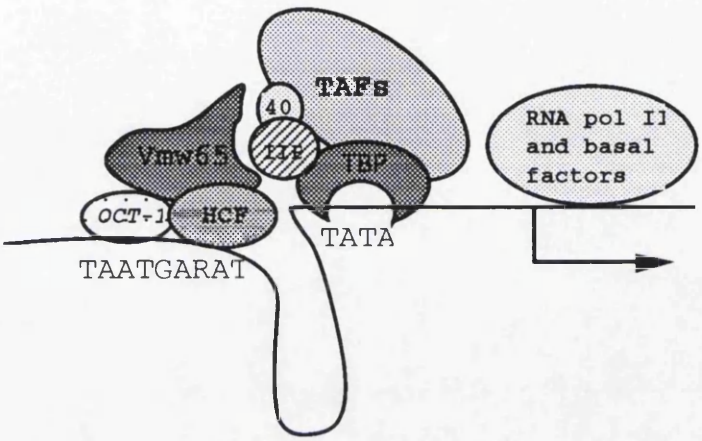


Figure 1A.6 Interactions between Vmw65 and the basal transcription machinery. Vmw65 interactions with the basal transcription machinery and the upstream activator Oct-1, interactions between Vmw65 and TFIIB and TAFII40 are also shown. (Modification of diagram by Goodrich *et al.,* 1993)

1A.3.1.3 GA-rich motifs/ ets binding proteins

In addition to the TAATGARAT element which mediates the response to Vmw65 (Preston and Tanhill, 1984; Bzik and Preston, 1986) an additional GA-rich motif is important in immediate early gene expression, as shown for the expression of Vmw175 (Triezenberg *et al,* 1988b). Analysis of the upstream region of Vmw175 identified an element, a hexamer, GCGGAA, which, in addition to TAATGARAT, is required for optimal levels of Vmw175 expression. The proteins binding to this element belong to the ets family of DNA binding proteins. The interaction with the Vmw175 upstream region involves two distinct proteins, a DNA binding protein GABPα and a transactivating protein GABPβ (LaMarco *et al.,* 1991; Thompson *et al.,* 1991). Regions which bind GABPα and GAPBβ are also found upstream of IE110, IE63 and IE68/12. Formation of the IEC (Vmw65/Oct-1/HCF) is essential for mediating a response to Vmw65, GABPα and GABPβ promote the formation of the IEC on TAATGARAT. The distance separating the TAATGARAT from the GA rich motif determines the ability of the GA-rich binding proteins (GABPα and GABPβ) to promote IEC formation. The orientation of the GA-rich motif is also important (Bailey and Thompson, 1992).

1A.3.2 Immediate early gene products

The immediate early genes of HSV-1 are transcribed in the absence of viral gene expression (Honess and Roizman, 1974; Clements *et al.*, 1977). Five immediate early genes have been identified; IE-1, IE-2, 1E-3, 1E-4 and IE-5. The expression of these genes and the functions of the gene products will be described in the following Sections.

Glasgow nomenclature		Chicago nomenclature	
Gene	**Product**	**Gene**	**Product**
IE-1/IE110	Vmw110	α0	ICP0
IE-2/UL54	Vmw63	α27	ICP27
IE-3/IE175	Vmw175	α4	ICP4
IE-4/US1	Vmw68	α22	ICP22
IE-5/US12	Vmw12	α47	ICP47

Table 1A.2 Nomenclature of HSV-1 immediate early genes and their products.

1A.3.2 1 Vmw175

Vmw175 is a large, phosphorylated, nuclear-localised protein (Pereira *et al.*, 1977) which exists as a homodimer (Metzler and Wilcox, 1985), and is essential for viral replication, being continuously required for early and late gene expression (Preston, 1979; Watson and Clements, 1980). Vmw175 is also important in the autoregulation of IE gene expression, O'Hare and Hayward (1985b) showed that Vmw175 can repress both basal and Vmw65 induced expression from the Vmw175 promoter.

Comparison of HSV-1 Vmw175 with the VZV homologue lead to the identification of five conserved structural regions, regions 2 and 4 showing the greatest homology (McGeoch *et al;* 1986; Davison and McGeoch, 1986). Regions of Vmw175 important in the transactivation of early and late gene expression and for repression of immediate early gene expression have been mapped by insertional and deletion mutagenesis (Paterson and Everett, 1988). Mutation of region 2 showed this region was important in both the transactivation of the early genes, in the synergy with Vmw110 and the repression of immediate early gene expression. Mutation of region 4 also affected the transactivation of early genes in the presence of Vmw110.

Transactivation by Vmw175

Transactivation by Vmw175 is observed with promoters in which the only recognisable cis-acting element is the TATA box (Imbalzano *et al.*, 1991; Mavromara-Nazos *et al.*, 1986; Shapira *et al.*, 1987). Several studies have investigated the interaction of Vmw175 with the early promoters, tk (Papavassilou and Silverstein, 1990; Kristie and Roizman, 1986) and gD (Everett, 1984; Tedder *et al.*, 1989; Faber and Wilcox, 1986). Conflicting results were obtained, although Vmw175 interacts with the tk promoter, no specific contact sites, as described for the gD promoter, were identified. These observations led to the proposal that Vmw175 may mediate transactivation by directly contacting the basal transcription apparatus (RNA pol II and the TFII factors which recruit pol II to the TATA box). Vmw175 can restore activity to a promoter in which upstream activator sequences have been deleted (Imbalzano *et al.*, 1991). A cellular globin gene inserted into the tk locus of HSV-1 (Panning and Smiley, 1989) behaves like a virus early gene during infection; it was later demonstrated that expression of the globin gene was dependent on functional Vmw175, supporting the proposal that early genes do not have a specific cis acting element which mediates their transactivation by Vmw175 (Smiley and Duncan, 1992). Vmw175 directly contacts two basal factors, TFIID (the TATA binding protein) and TFIIB and promotes the formation of a stable preinitiation complex (Smith *et al.*, 1993). Vmw175 shares properties found in other viral transactivators, adenovirus E1A (Lee *et al.*, 1991) and EBV Zta (Lieberman and Berk, 1991), which do not directly contact DNA but promote preinitiation complex assembly.

Repression of immediate early genes

Vmw175 binds specifically to the sequence ATCGTCNNNYCGRC, found at the site of transcript initiation in the immediate early promoters (Faber and Wilcox, 1986). Binding to the immediate early promoters of IE1, IE2 (Kristie and Roizman, 1986) as well as to its own promoter (Faber and Wilcox, 1988; Katter-Cooley and Wilcox, 1989; Kristie and Roizman, 1986; Muller, 1987; O'Hare and Hayward, 1987) has been demonstrated. Vmw175 interaction with the regulatory regions of the immediate early promoters leads to the repression of their expression (O'Hare and Hayward, 1985b; Gelman and Silverstein, 1987a, b). In addition Vmw175 has been shown to repress the LAT (latency associated transcript) promoter (Batchelor and O'Hare, 1990). The mechanism of Vmw175 repression was investigated by Gu *et al.*, (1993). In the presence of Vmw175 SP-1 activated transcription was inhibited whereas basal transcription was unaffected leading to the proposal that repression, like activation, is mediated by contacts with the basal transcription complex, which in turn inhibits SP-1 stimulation of transcription, consistent with earlier observations that Vmw175 inhibits Vmw65 activation of immediate early promoters (O'Hare and Hayward, 1985b). The interactions of Vmw175 which promote and repress gene expression are shown in Figure 1A.7.

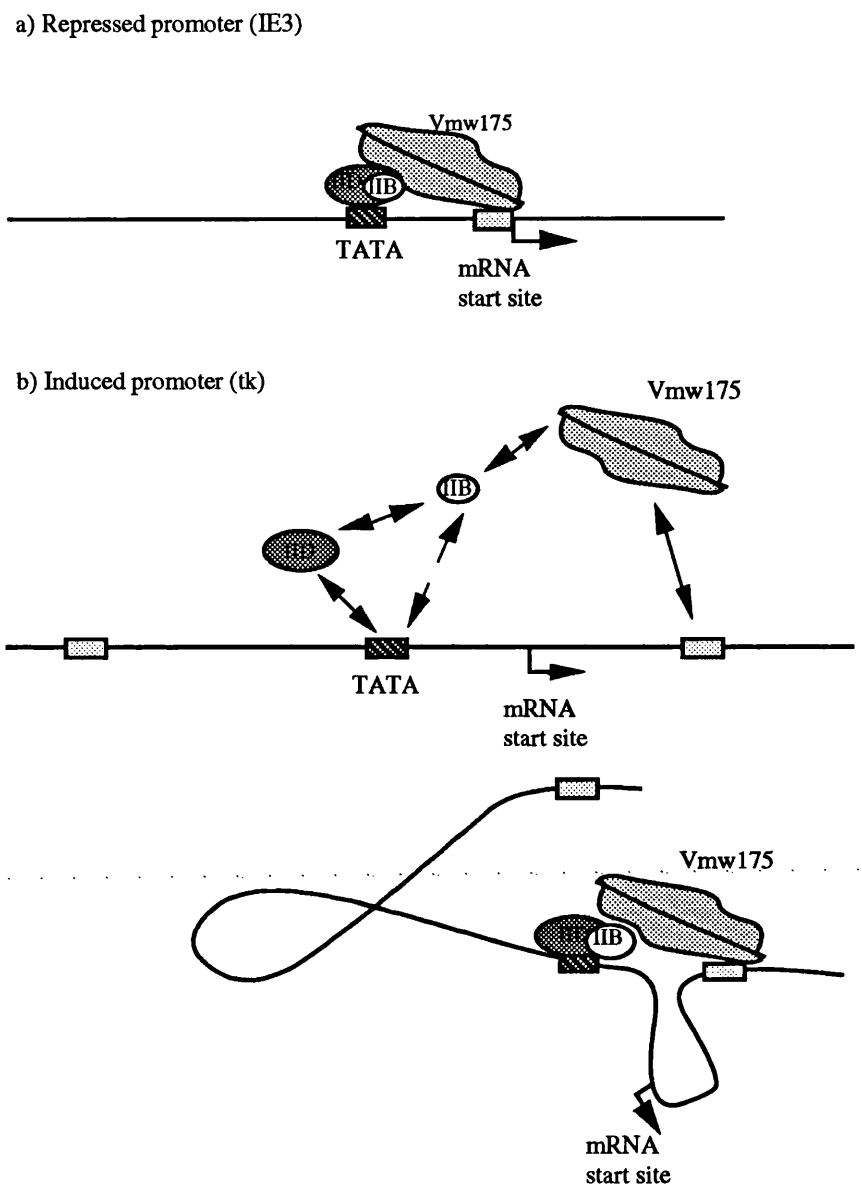


Figure 1A.7 Model showing different activities of Vmw175. a) Shows how Vmw175 repression of activated transcription may occur by blocking the site of transcription initiation b) Shows how the many relatively non-specific Vmw175 binding sites act to increase the level of transcription via contacts with TFIIB by promoting formation of the pre-initiation complex assembly. (Reproduced from Smith *et al.*, 1993)

1A.3.2.2 Vmw110

Vmw110 is a 110kDa nuclear phosphoprotein (Ackermann *et al.*, 1984; Pereira *et al.*, 1977). Analysis of the function of Vmw110 as a transactivator using transient transfection assays with reporter constructs showed that Vmw110 is a transactivator of all classes of virus genes as well as cellular genes (Everett, 1984; Gelman and Silverstein, 1985; Gelman and Silverstein, 1986; Mavromara-Nazos *et al.*, 1986; Mosca *et al.*, 1987; Quinalan and Knipe, 1985; Sekulovich *et al.*, 1988). Although not absolutely required for early and late

gene expression, Vmw110 is required for optimal levels of expression, achieved only when both Vmw110 and Vmw175 are present (O'Hare and Hayward, 1985a). Mutational analysis of Vmw110 identified several regions important in function . Mutations fell into two categories: those which effect its synergy with Vmw175 and those which are involved in the transactivating activity. Two regions important for the synergy with Vmw175 were identified (Everett, 1989). Removal of the zinc finger of Vmw110, located towards the amino terminal region of the protein destroyed transactivating activity (Everett, 1989).

To determine the role of Vmw110 in a productive HSV-1 infection, mutants in the Vmw110 loci have been constructed (Stow and Stow, 1986; Sacks and Schaffer, 1987). Mutants lacking Vmw110 fail to grow as efficiently as wild-type and replication is host-range and multiplicity dependent. To determine the effect on virus gene expression and regions of Vmw110 required for its function defined mutations in Vmw110 were introduced into the virus (Cai and Schaffer, 1992; Chen and Silverstein, 1992). Expression of two early gene products, UL29 and the large subunit of ribonucleotide reductase, and three late proteins; gB, gC and ICP5, were significantly reduced, confirming that, as found in transient transfection assays (O'Hare and Hayward, 1985a), Vmw110 is required for efficient early and late gene expression. In contrast to transient transfection data, immediate early gene expression in the Vwm110 mutants was unaffected, Cai and Schaffer, (1992) proposed that Vmw110 transactivation of immediate early genes is significant only in the absence of functional Vmw65. In a similar study Chen and Silverstein, (1992) also found that early and late gene expression was decreased in Vmw110 mutants (lacking a functional zinc finger) and expression of immediate early gene, IE63, was reduced.

1A.3.2.3 Vmw63

The immediate early protein Vmw63, like Vmw175, is essential for virus replication. Analysis of the function of Vmw63 in the regulation of HSV-1 gene expression indicated its role in post-transcriptional processes (Sandri-Goldin and Mendoza, 1992; McLauchlan *et al.*, 1992). HSV-1 mutants in Vmw63 display a number of phenotypes; the expression of late gene products is significantly reduced (McCarthy *et al.*, 1989; McMahan and Schaffer, 1990; Rice and Knipe, 1990; Sacks *et al.*, 1985); immediate early gene products accumulate (Smith *et al.*, 1992) and DNA synthesis decreases (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice and Knipe, 1990). To determine the role of Vmw63 in late gene expression Smith *et al.*, (1992) analysed the levels of transcription by comparing the rate of initiation of transcription to levels of mRNA and protein. Levels of late gene protein and mRNA were reduced in cells infected with the Vmw63 mutant whereas the amount of transcript produced (initiated) was unaltered, it was concluded that Vmw63

plays a role in the processing of the nascent RNA. Vmw63 plays a role in HSV-1 transcript polyadenylation, a factor which influences the efficiency of late transcript polyadenylation in infected cells (McLauchlan *et al.*, 1989) is absent in cells infected with an Vmw63 mutant (McLauchlan *et al.*, 1992). Vmw63 promoter independent activation of gene expression has been demonstrated with other viral regulatory sequences (Chapman *et al.*, 1992). The localisation of the major DNA binding protein, UL29, into replication compartments in infected cells is altered in cells infected with a mutant in Vmw63, suggesting a role for Vmw63 in the localisation of UL29 (Curtin and Knipe, 1993).

Defined deletions in Vmw63 mapped regions of Vmw63 required for both its transactivating and transrepression activities (McCarthy *et al.*, 1989; Rice and Knipe, 1990). The C-terminal portion of Vmw63 is important in both repression and activation, mutants in this region fail to down-regulate early gene expression and activate late gene expression (Rice *et al.*, 1993). More defined mutational analysis separated the regions of the C-terminus involved in transrepression and transactivation, mutants which affect only the transactivation function of Vmw63 have been isolated, all map to the C-terminal region, the final 78 amino acids being critical for function (Rice and Lam, 1994). Transrepression was affected in some mutants isolated but the affect of the mutation was dependent on how it was constructed, substitution mutations in the C-terminal region which destroys transactivation but not transrepression have been isolated, whereas an insertional mutation at the same site destroys both activities (Rice and Lam, 1994). Mutation of the amino terminal portion of Vmw63 reduces the efficiency of viral DNA replication (Rice *et al.*, 1993).

1A.3.3 Early gene expression

Early gene expression peaks between 5-7 hr post-infection and is dependent on the presence of the immediate early transactivator Vmw175 (Preston, 1979; Watson and Clements, 1977). A functional TATA box is the only cis-acting sequence absolutely required for early gene expression (Everett, 1984), Vmw175 transactivation is mediated by its direct interaction with the TATA associated initiation complex (Smith *et al.*, 1993). Early gene expression is down-regulated upon initiation of DNA replication, Vmw63 plays a role in this down-regulation (Sekulovich *et al.*, 1988; McCarthy *et al.*, 1989; Rice and Knipe, 1990; Rice and Knipe, 1994).

1A.3.4 Early gene products

All HSV-1 genes involved in nucleotide metabolism and DNA replication are expressed at early times post-infection.

1A.3.4.1 Enzymes involved in nucleotide provision

The HSV-1 encoded enzymes involved in nucleotide provision are shown below (Figure 1A.8), and the importance of these enzymes in viral replication will be discussed.

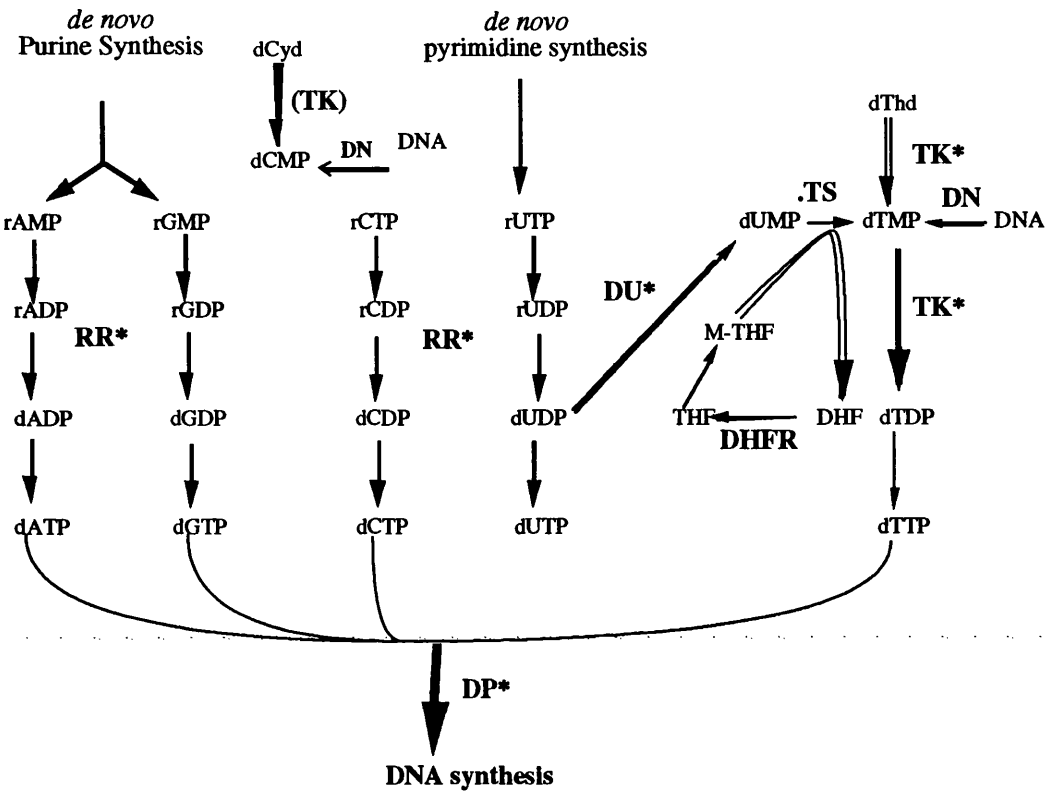


Figure 1A.8 Enzymes involved in nucleotide metabolism encoded by HSV-1. RR (ribonucleotide reductase), DU (dUTPase), tk (thymidine kinase), DN (DNase), and DP (DNA polymerase) are all encoded by HSV-1. DHFR (dihydrofolate reductase) is provided by the cell. * encoded by HSV-1. (Reproduced from Morrison, 1991)

a) Thymidine kinase

Thymidine kinase (tk) provides dTMP, dCMP and dTDP for DNA synthesis. HSV-1 thymidine kinase shows a relaxed substrate specificity when compared to the cellular enzyme, it can, for example, phosphorylate dCyd (Perera and Morrison, 1970; Jamieson and Subak-Sharpe, 1974; Jamieson *et al.*, 1974). Because of the relaxed substrate specificity, its potential as an antiviral target was investigated. Elion *et al.*, (1977) showed that an acyclic nucleoside analogue, acyloguanosine, was converted to a monophosphate by HSV-1 tk, this analogue is an efficient and specific inhibitor of HSV-1 DNA polymerase, inhibition occurring by premature chain termination. It is, to date, the only low toxicity antiviral known to be effective against HSV. Mutants in tk have been isolated, although non-essential for replication in tissue culture, tk mutants have been shown to have reduced pathogenicity (Coen *et al.*, 1989; Tenser *et al.*, 1989).

b) dUTPase

dUTPase catalyses the hydrolyses of dUTP to dUMP and pyrophosphate, reducing dUTP pools in the cell. An activity unique to cells infected with HSV-1 was detected (Wohlrab and Francke, 1980) which differed from the cellular enzyme in that it has low levels of activity on dCTP and dTTP compared to the cellular enzyme (Caradonna and Cheng, 1981). As found for tk, mutants in dUTPase are viable in cell culture (Fischer and Preston, 1986), but important in virus pathogenicity (Pyles *et al.*, 1992).

c) DNAse (alkaline nuclease)

An HSV-1 DNAse activity was first noted by Keir and Gold, (1963). The HSV-1 DNAse has been purified (Banks *et al.*, 1983) and shown to exist as a phosphorylated monomer (Banks *et al.*, 1985; Thomas *et al.*, 1988). Its function is to provide nucleotides through the degradation of cellular DNA (Nutter *et al.*, 1985). Mutants in the DNAse have been isolated, limited replication was observed in tissue culture indicating that it is an essential virus function (Moss, 1986). In another DNAse mutant (Weller *et al.*, 1990) the DNAse was found to play a role in the egress of capsids from the nucleus, synthesis of viral DNA was reduced and the amount of capsids which fail to package DNA was dramatically increased (Snao *et al.*, 1993). It has been proposed that the DNAse may play a role in the cleavage of the DNA prior to encapsidation.

d) Ribonucleotide reductase

Ribonucleotide reductase is discussed in detail in section 1D.

1A.3.4.2 HSV-1 DNA replication

1A.3.4.2.1 Origins of DNA replication

Within the HSV-1 genome there are three origins of replication belonging to two classes: OriL, located in the unique long region of the genome and OriS, located in the short inverted repeats (Spaete and Frenkel, 1982; Stow, 1982; Stow and McMonagle, 1983; Vlasny and Frenkel, 1982; Weller *et al.*, 1985). Plasmids containing either the OriS or OriL regions become amplified during HSV-1 infection (Challberg, 1986). The sequences of the origins are closely related; both contain inverted repeat sequence with a central AT-rich sequence (Weller *et al.*, 1985; Murchie and McGeoch, 1982). Elements essential in OriS function have been identified (Lockson and Galloway, 1988; Deb and Doelberg, 1988; Hernadez *et al.*, 1991; Weir and Stow, 1990). UL9 binding sites and some flanking sequences contain binding sites for both SP-1 and NF-1 are essential for replication (Wong and Schaffer, 1991).

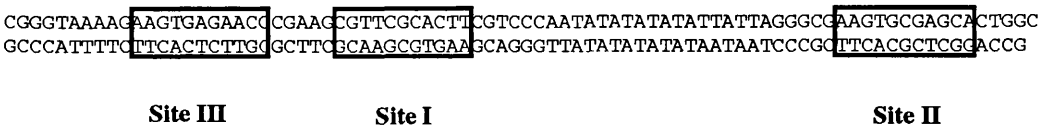


Fig 1A.9 UL9 binding sites in OriS. The three UL9 binding sites in OriS are shown, sites 1 and 2 are essential for DNA replication. (Modification of diagram by Challberg, 1991)

1A.3.4.2.2 Gene products and their role in replication

HSV-1 genes involved in DNA replication were first identified by Challberg, (1986) using an HSV-1 origin dependent plasmid system. Seven HSV-1 gene products were found to be involved in replication (see Table 1A.3 below).

Gene	Function
UL30	DNA polymerase
UL42	Polymerase accessory protein
UL5	Helicase/primase
UL8	Helicase/primase
UL52	Helicase/primase
UL9	Origin binding protein
UL29	Major DNA binding protein

Table 1A.3 HSV-1 genes directly involved in viral DNA replication and their function in replication.

Although HSV-1 encodes many genes required for DNA replication cellular factors must also play a role as yet no HSV-1 gene product has been identified with DNA ligase or topoisomerase activity.

a) UL9, origin binding protein

UL9 binds to the origins of replication, three binding sites in OriS have been identified; two high affinity sites and one low affinity binding site (Weir *et al.*, 1989; Olivo *et al.*, 1988; Elias and Lehman, 1988; Elias *et al.*, 1990). UL9 binding to the high affinity sites is essential for DNA replication, mutation of these sites eliminates or greatly reduces replication. In contrast mutation of the low affinity binding site has little effect on replication (Lockson and Galloway, 1988; Hernadez *et al.*, 1991; Weir and Stow, 1990). UL9 exists as a dimer (Bruckner *et al.*, 1991; Fierer and Challberg, 1992), the DNA

* UL42, the polymerase accessory factor, was first mapped by Parris *et al.,* (1988).

binding domain of UL9 maps to a region containing a putative coiled-coil (Deb and Deb, 1991), binding of UL9 to the high affinity sites is co-operative (Elias *et al.*, 1990). UL9 contains an intrinsic helicase activity, it has been postulated that UL9 plays a critical role in the initial melting of the origin, which then allows the recruitment of the HSV-1 DNA replication apparatus (Bruckner *et al.*, 1991).

b) UL30, the DNA Polymerase

A DNA polymerase activity unique to HSV-1 infected cells was first described by Keir and Gold, (1963). The HSV-1 DNA polymerase has properties similar to those of other DNA polymerases: (1) replication uses a specific primer to initiate replication, oligoribonucleotides were found to initiate HSV-1 DNA replication; (2) the Km values for dNTPs in the HSV-1 DNA polymerase was found to be lower than that of the cellular enzyme indicating the higher dNTP binding affinity of the HSV-1 DNA polymerase, this ensures that viral replication is favoured over cellular DNA replication; (3) the HSV-1 enzyme possess 3′ —> 5′ exonuclease activity, an activity important in the proof reading of nascent DNA (Knopf, 1979; Powell and Purifoy, 1977) and homology to the 5′—>3′ exonuclease activity of DNA polymerases has been described within the N-terminal portion of the HSV-1 DNA polymerase (Crute and Lehman, 1989). Purified DNA polymerase is associated with UL42, (Vaughan *et al.*, 1984; Gallo *et al.*, 1988) the polymerase accessory protein, and the major DNA binding protein, UL29.

c) UL42 , the Polymerase accessory factor

The role of the DNA Polymerase/UL42 interaction was first proposed by Gallo *et al.*, (1989), purification of the HSV-1 DNA polymerase leads to a decrease in polymerase activity, full activity is recovered by the addition of UL42 to the assay (Gottlieb *et al.*, 1990). DNA polymerase exists as a heterodimer with UL42 in infected cells, the interaction is independent of any other viral protein (Gottlieb *et al.*, 1990). Regions of UL42 required for polymerase interaction map to the carboxy terminal portion of UL42 (Digard and Coen, 1990; Stow, 1993; Hart and Boehme, 1992; Digard *et al.*, 1993; Tenney *et al.*, 1993). Mutational analysis has mapped sites of the DNA polymerase which interact with UL42 to the C-terminal region of DNA polymerase. A putative helical structure located in the C-terminal region of the DNA polymerase was proposed to be the site of interaction with UL42 (Tenney *et al.*, 1993; Digard *et al.*, 1993). Optimal processivity by DNA polymerase and UL42 is only achieved in the presence of the major DNA binding protein UL29 (Hernadez and Lehman, 1990; Gottlieb *et al.*, 1990).

d) UL29, the major DNA binding protein

The major DNA binding protein of HSV-1, UL29, is essential for HSV-1 DNA replication (Weller *et al.*, 1983; Littler *et al.*, 1983; Conley *et al.*, 1981). UL29 is important in both

DNA replication and in late gene expression. Properties of UL29 include: (1) binding to single stranded DNA *in vitro* and *in vivo* (Ruyechan, 1983); (2) localisation to the cell nucleus and the formation of replication compartments (Gao and Knipe, 1992); (3) down-regulation of viral gene expression (Godowski and Knipe, 1986); (4) stimulation of late gene expression (Gao and Knipe, 1991). UL29 shares properties found in the *E.coli* single-stranded DNA binding protein; in its ability to disrupt duplexes of complementary homopolymers and to protect single stranded DNA from nucleases, UL29 binds to single stranded DNA and to a lesser extent duplex DNA (Powell and Purifoy, 1976; Knipe *et al.*, 1982; Ruyechan and Weir, 1984). Upon translocation to the nucleus UL29 interacts with the nuclear matrix to form pre-replicative sites, initiation of replication brings about the formation of replication compartments, the formation of which is completely dependent on a functional UL29 product (deBruyn and Knipe, 1988). Within the UL29 open reading frame a potential zinc finger was identified, zinc binding is critical for UL29 function (Gupte *et al.*, 1991), the DNA binding domain of UL29 has been mapped to the putative zinc finger (Gao and Knipe, 1989; Leinbach and Heath, 1988).

UL29 mediates the melting of small region of duplex DNA (Boehmer and Lehman, 1993). End-labelled oligonucleotides annealed to single stranded DNA are displaced by UL29. By labelling oligonucleotides differing in size it was demonstrated that UL29 can displace these with almost equal efficiency. Displacement reactions were found to require saturation quantities of UL29 increasing with increased size of oligonucleotide. Duplexes of greater than 350 bases were not displaced.

e) UL5/UL8/UL52 ,the helicase/primase complex

A novel helicase/primase activity is induced in cells infected with HSV-1 (Crute *et al.*, 1988). The helicase/primase activity unique to HSV-1 infected cells has been isolated, three HSV-1 gene products; UL8, UL5 and UL52 are required for this activity (Crute *et al.*, 1989; Crute and Lehman, 1991; Dodson *et al.*, 1989). Although the helicase/primase activity of HSV-1 normally exits as a tripartite complex, UL5 and UL52 alone contain full activity (Calder and Stow, 1990; Dodson and Lehman, 1991). UL5 contains a structural region common to helicases (Gorbalenya *et al.*, 1988; Hodgman, 1988; McGeoch *et al.*, 1988; Lane, 1988), indicating that the other component essential for helicase activity, UL52, may be required to mediate a conformational change essential for UL5 helicase activity.

In vitro assays for lagging strand DNA synthesis showed UL8 was essential. It was proposed that UL8 is important in the stabilisation of the primers with the template (Sherman *et al.*, 1992). The roles of the HSV-1 replication proteins are summarised in Figure 1A.10.

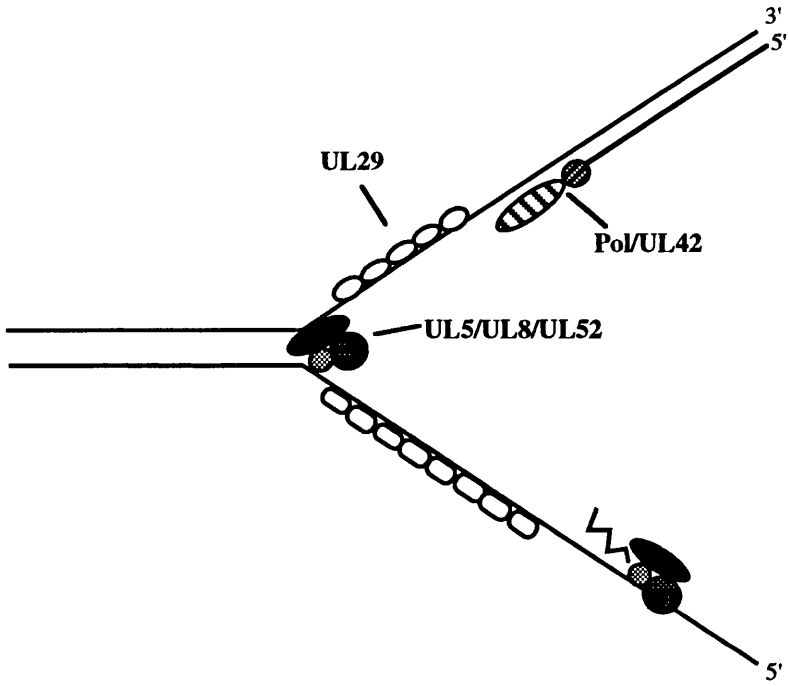


Figure 1A.10 HSV-1 proteins, their role in replication. Diagram summarises the model for DNA replication and the role of each of the HSV-1 gene products in leading (5′ to 3′) and lagging (3′ to 5′) strand synthesis. (Modification of diagram by Challberg, 1991)

1A.3.5 A replication/transcription relationship in HSV-1

DNA replication initiates true late gene expression. Localisation of the major DNA binding protein, UL29, into the replication compartments is disrupted in an HSV-1 mutant in Vmw63 (Curtin and Knipe, 1993). Both UL29 (Godowaski and Knipe, 1986) and Vmw63 (Knipe *et al.*, 1993) play a role in the down-regulation of early gene expression and the stimulation of late gene expression (Gao and Knipe, 1991). UL29 binding to DNA may mediate Vmw63 interaction with the transcription/processing apparatus. Transcription factors can also play a role in DNA replication (Lue and Kornberg, 1993) and replication factors can also influence transcription; factors involved in origin recognition in yeast inhibit gene expression (Micklem *et al.*, 1993), a similar effect is observed with Vmw63 which down-regulates early gene expression, although no direct interaction between Vmw63 and any of the HSV-1 replication proteins has been demonstrated, the decrease in DNA synthesis observed in cells infected with a Vmw63 null mutant implies that Vmw63 may play either a direct or indirect role in viral DNA replication. A feature common to Vmw63 and UL29 is their ability to bind zinc. Zinc binding domains are known to play a role in protein-nucleic acid interactions and recently, their role in mediating protein-protein interactions has been described. The cellular elongation factor, TFIIS contains an unusual zinc binding motif which is critical in its interaction with RNA Pol II, an interaction which is important in the regulation of read-

through (Agarwal *et al.*, 1991) and in nascent transcript cleavage (Reines, 1992; Reines and Mote, 1993). Whether similar interaction between RNA Pol II and the HSV-1 transcriptional regulators/replication proteins mediates or alters Pol II activity is unknown.

1A.3.6 Late gene expression

Late gene expression falls into two distinct classes; leaky-late and true late. Leaky late gene expression can be observed in the absence of viral DNA replication (Blair and Wagner, 1986; Godowaski and Knipe, 1986; Holland *et al.*, 1980; Pederson *et al.*, 1981) whereas true late gene expression occurs once viral DNA replication has been initiated (Holland *et al.*, 1980).

1A.3.6.1 Leaky-late gene expression

The immediate early gene products, Vmw175, Vmw110 and Vmw63 are all required for optimal levels of leaky late gene expression seen prior to DNA replication (Costa *et al.*, 1985; Everett, 1986; Sekulovich *et al.*, 1988; Su and Knipe, 1989). Cis-acting sequences required for the expression of leaky late genes include a functional TATA box and an origin sequence, optimal levels of leaky-late gene expression post DNA replication require the initiation of DNA replication (Johnson and Everett, 1986). A cis-acting sequence which binds the cellular factor YY1 (Mills *et al.*, 1994) plays a critical role in the expression of the leaky late class of genes (Chen *et al.*, 1992), deletion of the YY1 binding site in the VP5 promoter reduced levels of expression by 40-fold (Chen *et al.*, 1992). YY1 has been proposed to be a major determinant of late gene subclass, binding sites exist in the gB and gC as well as the VP5 leaky-late promoters, this appears to be a feature unique to this particular subclass (Chen *et al.*, 1992).

1A.3.6.2 True late gene expression

Like the leaky late genes, true late genes require the immediate early gene products Vmw110, Vmw175 and Vmw63 (Deluca *et al.*, 1984; Deluca and Schaffer, 1985; Sacks *et al.*, 1985; Mavromara-Nazos *et al.*, 1986). The promoters of both gC (Homa *et al.*, 1986) and US11 (Johnson and Everett, 1986) have been investigated, the only cis-acting requirements found were a functional TATA box and an Ori sequence. Expression only occurs once DNA replication is initiated (Holland *et al.*, 1980; Wagner, 1985). Post-transcriptional processes also control levels of late gene expression (McLauchlan *et al.*, 1989; 1992)

1A.4 Virus maturation

1A.4.1 Capsid formation

Capsids are composed of seven virus proteins (VP5, VP19C, VP21, VP22A., VP23, VP24 and VP26) of which two, VP21 and VP24, are produced by the proteolytic cleavage of a polyprotein precursor by the virion protease, an activity which resides in the VP22A. (Liu and Roizman, 1991, 1992; Preston *et al.,* 1992). The outer icosahedral shell is composed of VP5, VP19C, VP23 and VP26. VP22A forms the internal scaffold of the capsid, cleavage of this protein is thought to be critical in the packaging of the virus DNA. A temperature sensitive mutant in VP22A at the non-permissive temperature still forms capsids but fails to incorporate the DNA (Preston *et al.,* 1983).

1A.4.2 DNA packaging

Concatameric virus DNA undergoes cleavage at the "a" sequence present at the termini of the virus genome (Davison and Wilkie, 1981; Mocarski and Roizman, 1982; Varmuza and Smiley, 1985; Nasseri and Mocarski, 1988). DNA is then packaged into the capsid, a process dependent on the proteolytic cleavage of VP22A, the "a" sequence also plays a role in packaging (Kaemer *et al.,* 1981; Vlazny and Frenkel, 1981) and recently the importance of the viral DNAse in the packaging of DNA has been demonstrated (Snao *et al.,* 1993).

1A.4.3 Virion maturation

The capsid and tegument then become enveloped. Where envelopment occurs is at present unknown, two models have been proposed; envelopment occurring as the capsid is transported across the nuclear membrane or, whilst the capsid is transported through the cytoplasm. A mutant in the UL20 gene of HSV-1, a gene thought to encode a membrane-associated protein, shows a high accumulation of capsids in the space between the inner and outer lamellae of the nuclear membrane with a reduced number of naked capsids in the cytoplasm (Baines *et al.,* 1991). Disruption of Golgi function in the cell leads to an increased accumulation of cytoplasmic capsids (Cheung *et al.,* 1991; Whealy *et al.,* 1991), indicating that envelopment requires proper Golgi function. Of the virus glycoproteins, which are found distributed throughout the cell, gD has been shown to inhibit HSV-1 fusion with membranes thereby preventing mature virions from re-entering the cell (Campadelli-Fume *et al.,* 1990). It is obvious therefore that the virion as it is transported through the cell should avoid membranes containing gD, it has been postulated that special

structures, virosomes, exist whose role is to transport the virion through the cell. Recently *in vitro* enveloped particles have been identified which lack capsids and DNA, these particles designated light (L) particles (Szilagyi and Cunningham, 1991: McLauchlan and Rixon, 1992) are thought to assemble independently of virion maturation.

1A.5 Selected virus/host interactions

1A.5.1 Shut-off factor (UL41)

Infection of cells by HSV-1 and HSV-2 results in the down regulation of host protein synthesis (Fenwick and McMenamin, 1984; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987). The infected cell factor responsible is a component of the virion, infecting cells with UV-irradiated virus (Fenwick and Clark, 1982; Fenwick and Walker, 1987; Schek and Bachenheimer, 1985; Strom and Frenkel, 1987) or infecting in the presence of actinomycin D (Schek and Bachenheimer, 1985; Strom and Frenkel, 1987), does not effect host mRNA destabilisation during infection. Using mutants in the host shut-off locus, it was found that not only is cellular mRNA half-lifedecreased, viral mRNAs have increased stability and accumulate as infection proceeds (Krikorian and Read, 1991; Kwong and Frenkel, 1987; Oraskar and Read, 1987, 1989; Strom and Frenkel, 1987). The gene encoding this function has been mapped (Kwong *et al.*, 1988) and the infected cell protein has been shown to exist in multiple forms according to its phosphorylation state (Read *et al.*, 1993). How UL41 interacts with the mRNA and promotes its degradation is unknown.

1A.5.2 Neuropathogenicity

A region of HSV-1 which spans the long internal and short internal repeat region is important in determining the neuropathogenicity of the virus (MacLean *et al,* 1991; Taha *et al,* 1990; Thompson *et al,* 1986; Thompson *et al,* 1983). The gene, RL2, whose product, ICP34.5, plays an essential role in the neuropathogenicity of the virus (McGeoch and Barnett 1991; Chou *et al,* 1990; Chou and Roizman, 1992) maps to this region of the genome. ICP34.5 plays a primary role in determining the neuropathogenicity of HSV-1 (Chou *et al,* 1990; Chou and Roizman, 1992; Bovolan *et al,* 1994), mutation of the ICP34.5 reading frame reduces the neuropathogenicity of HSV-1. Differences in the effect of these mutations are observed between strains, in strain F the introduction of a stop codon into the ORF of ICP34.5 leads to a reduction in neurovirulence (Chou and Roizman, 1992) which is greater than that observed for strain 17^+ (Bovolan *et al,* 1994). Bovolan *et al.*, (1994) have suggested that the difference observed may in part be explained by the different strains of mice used for the analysis of these mutants.

1A.5.3 HSV-1 Latency

Following infection, HSV-1 replicates prior to entry into the nerve axon. The virus is then transported to the cell body of the neuron by retrograde fast axonal transport, how the virus particle is transported is unknown. Acute ganglionic infection is observed, a stage at which the virus can be recovered from ganglionic homogenates, prior to the establishment of latency, a stage at which no virus can be recovered from homogenates. Factors which stimulate the reactivation of the virus include; stress, UV-irradiation, hypothermia, trauma at the original site of infection and nerve root section. Upon reactivation the virus is transported to the peripheral site of infection where virus replication can occur. Lesion development can occur at the peripheral site but this is not necessarily always the case (Reviewed by Hill, 1985)

1A.5.3.1 Establishment of latency

Mutants in HSV-1 have been isolated which effectively establish latency but which fail to reactivate from the latent state, these include mutants in the enzymes involved in nucleotide provision; thymidine kinase (Coen *et al,* 1989; Tenser *et al,* 1989); ribonucleotide reductase (Jacobson *et al,* 1989) and dUTPase (Pyles *et al,* 1992). Expression , as analysed by fusion of promoters to β-galactosidase, of genes from different classes of viral gene has been observed in the ganglia (Ho and Mocarski, 1988). There is no evidence for viral replication during the acute phase of infection in the ganglia, suggesting that a comparison of events occurring during lytic replication and those occurring during acute ganglia infection can not be made (Speck and Simmons, 1991). A recent model has proposed that cells which do not support HSV-1 replication contain a repressor of immediate early gene expression (Lillycrop *et al.,* 1991). The presence/absence of this repressor determines the ability of the virus to replicate or enter a latent state. Lillycrop *et al.,* (1991) found that Oct-2, a factor closely related to Oct-1 can interact with the TAATGARAT sequences in the promoters of the immediate early genes and down-regulate expression of these genes, this factor exists in neuronal cells derived from the dorsal root ganglia.

1A.5.3.2 Maintenance of the latent state

The latency-associated transcripts which are encoded by an 8.0Kb segment from the long repeat region (IRS and TRS) of the HSV genome are the only transcripts present during HSV latent infection (Stevens *et al.,* 1987; Spivak and Fraser, 1987). The LAT transcript is antisense to the Vmw110 transcript. Although there are several forms of LAT transcript, 8.0Kb (Dobson *et al.,* 1988; Zwaagstra *et al.,* 1990) 2.0Kb and 1.5Kb and 1.45Kb (Rock *et*

al., 1987; Spivak and Fraser, 1987; Stevens *et al.*, 1987; Wagner *et al.*, 1988), the 2.0Kb transcript is the most abundant and can be detected during lytic infection (Krause *et al.*, 1988; Spivak and Fraser, 1987; Spivak *et al.*, 1991). The 1.5Kb and 1.45Kb LATs are thought to be derived from the splicing of the 2.0Kb LAT (Spivak *et al.*, 1991; Wagner *et al*, 1988; Wechsler *et al.*, 1988).

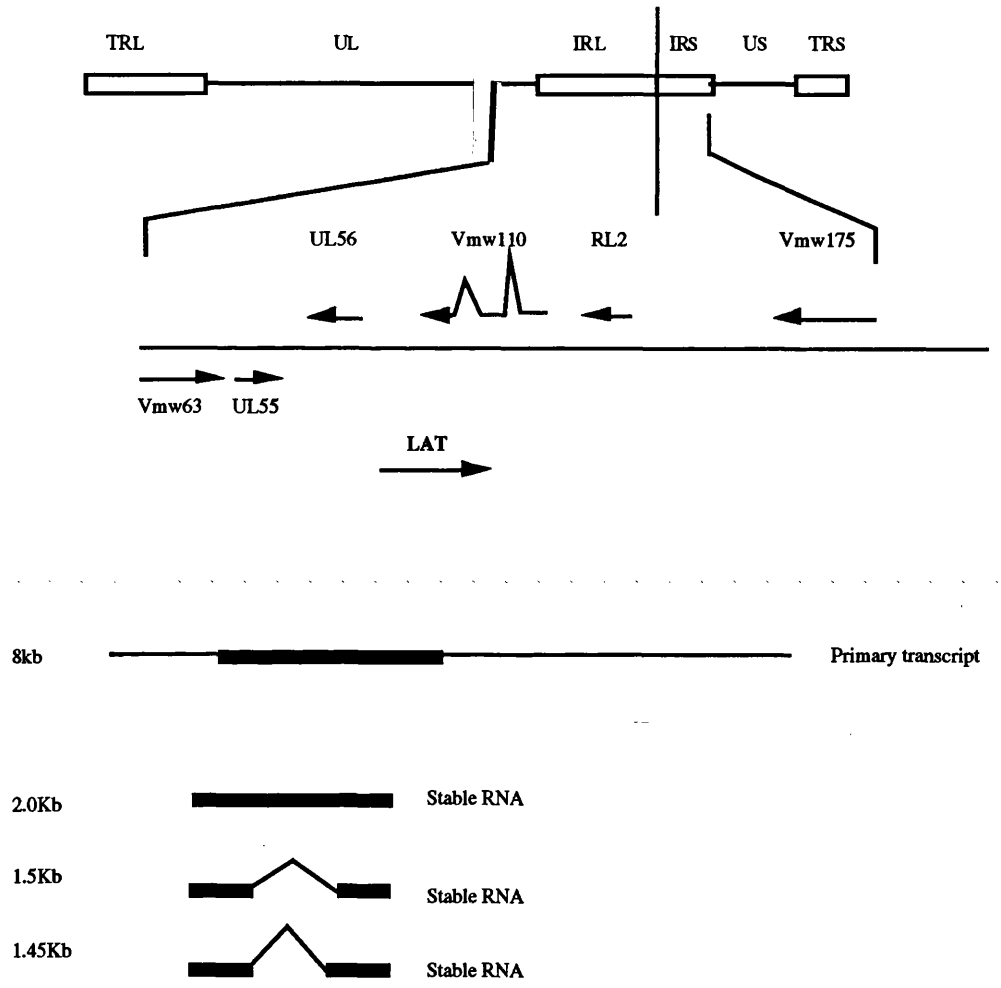


Figure 1A.11 Transcription around the LAT region of the HSV-1. The position of the LAT transcripts within the HSV-1 genome is shown, as are the positions of the immediate early gene products Vmw175, Vmw63 and Vmw110 which is antisense to the LAT transcript. The LAT transcripts which have been identified are shown, the primary transcript of 8.0Kb, the spliced short 1.5 and 1.45Kb transcripts and the stable intron from the primary transcript (2.0Kb)
(Reproduced with modifications from Fraser *et al.*, 1992)

1A.5.3.3 Expression of LATs

Regulatory regions responsible for the tissue-specific expression of the LATs have been identified (Batchelor and O'Hare, 1990, 1992; Dobson *et al.*, 1989; Leib *et al.*, 1991; Zwaagstra *et al.*, 1989, 1990, 1991). Deletion of sequences 5′ to the LAT TATA box and the cAMP response element (CRE) showed that both cis-acting elements are required for

expression (Dobson *et al.*, 1989). A more detailed analysis by Rader *et al.*, (1993) investigated by site-directed mutagenesis the precise role of both the TATA box and the CRE element in LAT expression during different stages of the latent life cycle. The CRE element was found to be important in the expression of the LAT during reactivation whereas mutation of the TATA box decreased the number of neurons expressing the LATs. In a separate study Nicosa *et al.*, (1993) have shown that the expression of the 2.0Kb (major LAT species) was detectable when both the TATA and CRE elements were mutated; no 1.5Kb LAT was observed which led them to propose that a TATA-less promoter expresses the 2.0Kb form in the absence of a functional TATA box and that the TATA promoter expresses LAT during latency and the TATA-less promoter functions during reactivation.

1A.5.3.4 Reactivation

Reactivation is thought to be mediated by the immediate early gene product Vmw110. In HSV-1 strain KOS deletion of the Vmw110 ORF impaired the ability of this virus to reactivate (Leib *et al,* 1989), in contrast the mutant in strain 17^+ showed only a reduction in the kinetics of reactivation and not the ability of the virus to reactivate (Clements and Stow, 1989). Using an *in vitro* model the importance of Vmw110 in the reactivation of HSV-2 has been demonstrated (Harris *et al*; 1989; Zhu *et al,* 1990).

1B Protein kinase structure and cellular processes controlled by phosphorylation

1B.1 Protein kinase classification/structure

1B.1.1 Protein kinase classification

Protein kinases have been classified according to the acceptor amino acid group (Hunter, 1991), see Table 1B.1.

Classification	Acceptor amino acid	Euk/pro
Serine/threonine	Serine or threonine	E/P
Tyrosine	tyrosine	E
Histidine	histidine/arginine/lysine	E/P
Cysteine	cysteine	P
Aspartyl/glutamyl	acyl groups	P

Table 1B.1 Classification of protein kinases.

Both protein serine/threonine and tyrosine kinases are well characterised, over 100 kinases belonging to this two categories have been identified, both by the purification of the kinases and by cloning using probes to regions of protein kinases which are well conserved (Hunter, 1987). The serine/threonine and tyrosine kinases are found in eukaryotic cells, the majority of protein kinases in the eukaryotic cell belong to the serine/threonine class of kinase. Protein kinases capable of phosphorylating serine/threonine and tyrosine have been identified (e.g. Wee1$^+$ (Featherstone and Russell, 1991), Clk and Nek (Ben-david *et al.*, 1991), YPK1 (Dailey *et al.*, 1990) and SpK1 (Stern *et al.*, 1991). Autophosphorylation of Wee1$^+$, followed by phosphoamino acid analysis showed that phosphotyrosine and phosphoserine were present in equal amounts. Protein kinases capable of phosphorylating histidine, arginine and lysine exist in eukaryotic cells as proteins with phosphate on these residues have been isolated, but no protein kinase responsible has been isolated (Pesis *et al.*, 1988; Levy-Favatier *et al.*, 1987).

1B.1.2 Protein kinase structure; subdomains and their role in catalysis

Comparison of the sequences of a large number of protein kinases led to the identification of discrete regions of conserved amino acids important in catalysis (Hanks *et al.,* 1988). Although both the serine/threonine and tyrosine kinases share subdomains,stretches of amino acids have been identified which are characteristic to a particular class (Hanks *et al.,* 1988). Table 1B.2 shows the amino acid residues in each kinase subdomain and their predicted/known function in catalysis.

Motif	Number	Function
GTGSFG	I	Binds B-PO_4 of ATP
K	II	Binds α and B-PO_4s of ATP
E	III	Ion pairs with Lysine (domain II)
RDLKPEN	VI	Catalytic loop
		R, ligand for Thr(P)
		D, catalysis
		K, catalysis
		N, stabilises loop
DFG	VII	Chelates Mg^{2+}
D	VIII	Hydrogen bonds
E	IX	Ion pairs to VII
R	X	Ion pairs to IX

Table 1B.2 Protein kinase subdomains and their role in catalysis. The residues involved in protein kinase subdomain formation are shown with their predicted/known role in catalysis.

a) Subdomain I

A nucleotide binding site, a motif common in all enzymes which bind nucleotides, first identified by Rossmann *et al.,* (1974). The binding site is a highly conserved structure, however the only amino acids which are highly conserved and which are the main characteristic feature of a nucleotide binding domain is a triad of glycines which form a loop structure important in the binding of the phosphate groups of the nucleotide (Rossmann *et al.,* 1974; Branden, 1980; Jurnak, 1985). The structure of the nucleotide binding site of cAMP-dependent protein kinase is shown in Figure 1B.1.

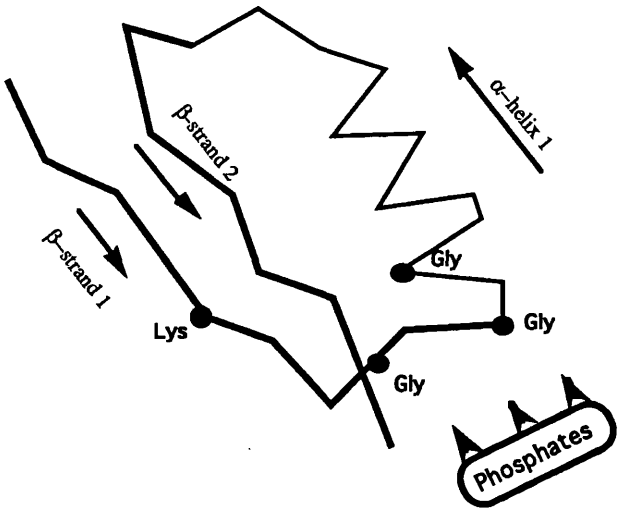


Figure 1B.1 Tertiary structure of nucleotide binding domain of cAMP-dependent protein kinase (PKA). The glycine triad directly involved in the interaction of ATP with the protein kinase is shown. The triad is flanked by a β-strand structure and an α-helix. The position of the three ATP phosphates is shown with respect to the glycine triad. (Reproduced with modifications from Taylor *et al.*, 1990)

b) Subdomain II

Downstream of the nucleotide binding site a lysine residue is located, normally spaced within 25 amino acids of the glycine triad. The importance of this residue in catalysis has been demonstrated, an ATP analogue, p-fluorosulfonylbenzoyladenosine (FSBA), irreversibly binds to this lysine (Zoller *et al.*, 1981). Mutation of this lysine leads to a loss of kinase activity (Chen *et al.*, 1987; Chou *et al.*, 1987). The lysine residue forms an ion pair with a conserved glutamate which brings the lysine into close proximity with the α and β phosphates of Mg-ATP.

c) Subdomain III

An invariant glutamate residue located downstream of the lysine in domain II (see above), which together with the glycine-rich loop bind and correctly position Mg-ATP. Mutation of the glutamate demonstrated that this residue is critical in catalysis (Gibbs and Zoller, 1991).

d) Subdomain VI

Subdomain 6 shows the greatest divergence between the serine/threonine kinases and the tyrosine kinases (Hanks *et al.*, 1988), this region can be used to determine the amino acid specificity of the kinase. All kinases contain two conserved amino acids in this region which vary according to the substrate for serine kinases, an aspartate and an arginine. The aspartate positions the hydroxyl acceptor group and the arginine stabilises the catalytic loop by forming a hydrogen bond with the aspartate. A lysine (serine/threonine) or

arginine (tyrosine) then mediates the transfer of the γ phosphate from ATP to the hydroxyl group.

e) Subdomain VII

Highly conserved subdomain, three amino acids; phenylalanine, aspartate and glycine interact with Mg^{2+} to correctly orientate the phosphate to be transferred (Zheng *et al.*, 1993). Interaction between the substrate Mg^{2+} and this site neutralises the charge on the aspartate reducing the hydrophobicity of the active site and facilitating phosphate transfer. Mutation of the aspartate in this subdomain in yeast TpK1 greatly reduced activity (Gibbs and Zoller, 1991a). The site of phosphate transfer and residues involved are shown in Figure 1B.2.

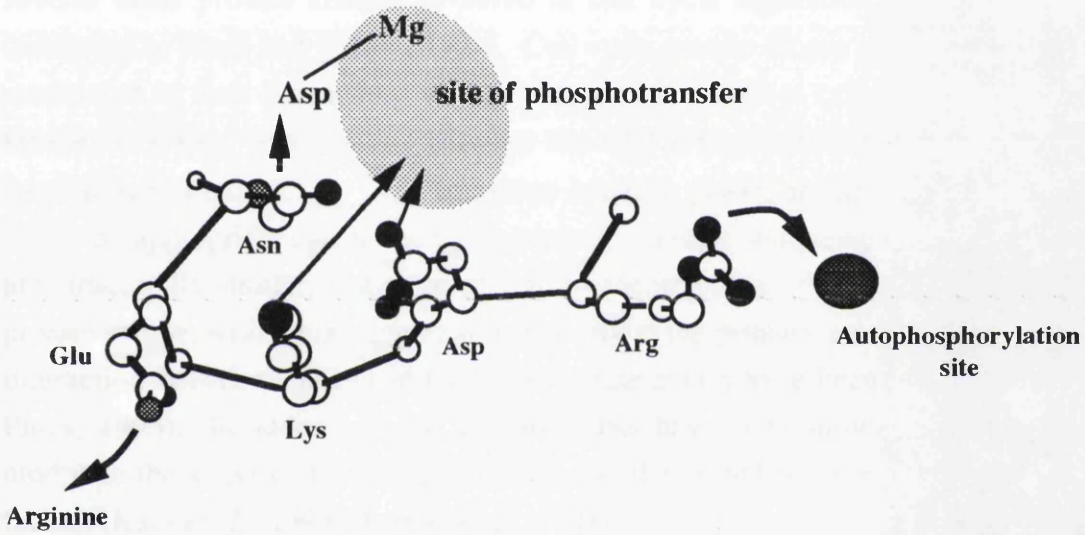


Figure 1B.2 Catalytic domain of cAMP dependent protein kinase. Residues directly involved in catalysis are shown. (Reproduced from Taylor *et al.*, 1992)

f) Subdomain VIII

This domain is highly conserved in tyrosine kinases, like subdomain 6 this region is an indicator of substrate specificity. The exact role of this region is unknown, the glutamate forms an ion pair with a conserved arginine in region 10, an interaction which is believed to play a key structural role. Mutation of this region leads to significant reduction in activity (Gibbs and Zoller, 1991b).

1B.2 Cellular events controlled by protein kinases

Protein kinases play a. decisive role in many cellular processes; cell mobility, cell division, gene expression, translation and metabolism, a few are discussed in the following Sections.

1B.2.1 Cell Cycle

A protein kinase essential in the cell cycle was first identified in yeast. This 34kDa protein kinase, cdc2, plays an important role in the progression of the cell from G1 to the S (synthesis) phase and from the G2 to the M (mitotic) phase. Control of cdc2 during the different phases of the cell cycle is dependent on a family of proteins, the cyclins (Lew and Reed, 1992; Fitch *et al.*, 1992; Richardson *et al.*, 1992). Since the discovery of cdc2, several other protein kinases involved in cell cycle regulation have been identified (reviewed in Pines and Hunter, 1991). Cell cycle protein kinase activities are, like cdc2, modulated by their interactions with cyclins and are therefore called the cyclin dependent kinases (CDKs). Interactions between the CDKs and their corresponding cyclin are specific (see Figure1B.3). The transition between phases in the cell cycle is mediated when the appropriate cyclin and CDK interact. (Fang and Newport, 1991). The CDKs are structurally similar, just large enough to encompass all the conserved elements of a protein kinase, with a high degree of variability at the primary amino acid level. Sites of interaction between CDKs and their appropriate cyclin have been mapped (reviewed in Pines, 1993). In addition to the cyclins CDKs have been shown to interact with and modulate the activity of other cell proteins e.g. the retinoblastoma protein and its related factors (Kato *et al.*, 1993; Hannon *et al.*, 1993).

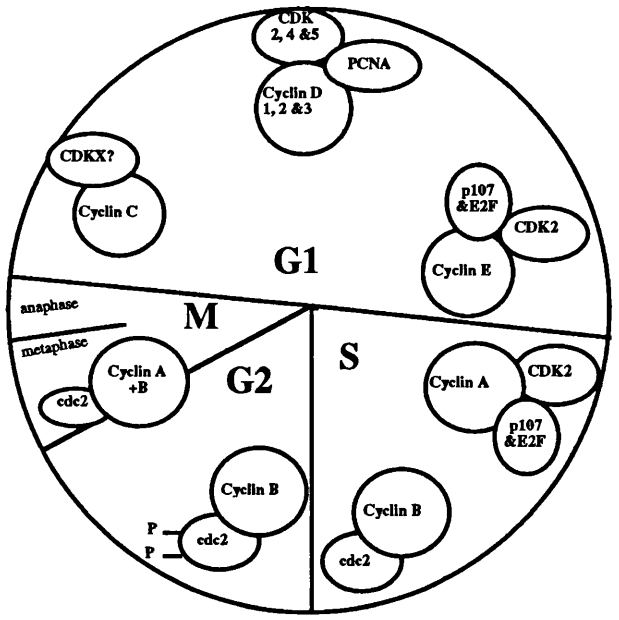


Figure 1B.3 The control of the cell cycle by protein kinases. Progression through each stage of the cell cycle, M-phase, S-phase, G1 and G2-phases is controlled by protein kinases. The activity of the protein kinase is controlled by their interactions with cyclins. The protein kinases active at a particular phase of the cell cycle are shown in the pie-shaped diagram, some protein kinases are active in more than one phase of the cell cycle, this activity is dependent on the interaction of the cyclin dependent protein kinases with a particular cyclin present during the phase. (Reproduced from Pines, 1993)

1B.2.2 DNA replication/repair

a) DNA replication

Replication of DNA in the eukaryotic genome is tightly regulated and restricted to the S-phase of the cell cycle. Fusion of S-phase cells to G1 cells induces synthesis of DNA in the G1 cells (Rao and Johnson, 1970). From these experiments it was concluded that there is an inducer of DNA synthesis which accumulates during G1 (Rossow *et al.*, 1979). Replication is thought to be induced by the p34(cdc2) kinase (McVey *et al.*, 1989; D'Urso *et al.*, 1990; Blow and Nurse, 1990; Fang and Newport, 1991). Analysis of SV40 replication in vitro showed that a factor RPA-32, part of the trimeric RPA complex, is phosphorylated during or just prior to DNA replication. Phosphorylation occurs during the S-phase of the cell cycle (Dutta and Stilman, 1992). Dutta and Stilman, (1992) demonstrated RPA-32 phosphorylation by the cdc2 protein kinase, RPA-32 phosphorylation by cdc2 stimulates DNA replication and unwinding in extracts from G1 cells.

An additional factor involved in DNA replication, PCNA (proliferating cell nuclear antigen), is important in both DNA replication and repair and associates with D-cyclins and the D1-cyclin associated kinase (Xiong *et al.*, 1992). Although the phosphorylation of

PCNA has not been demonstrated, the phosphorylation state of the cyclin with which it becomes associated may play a role in the function of this protein.

b) DNA repair

In yeast a protein kinase, Dun-1, is induced by agents which promote DNA damage (Zhou and Elledge, 1993). The phosphorylation of this protein increases with DNA damage. The importance of Dun-1 autophosphorylation has been demonstrated, mutation of residues in the nucleotide binding or catalytic domains of this protein kinase, followed by transfections with the plasmid expressing these mutants fail to rescue yeast strains carrying a Dun-1 mutation. It has been proposed that Dun-1 plays a primary role in the eukaryotic SOS response. The action of DUN-1 is thought to be mediated either by its direct interaction with the basal transcription machinery or by the modulation of a factor which itself interacts with the basal machinery.

1B.2.2 Control of transcription by protein kinases

a) Basal transcription

The synthesis of mRNA in eukaryotes is carried out by RNA polymerase II, a multi-subunit enzyme that together with seven general transcription factors (TFIIA, IIB, IIC, IID, IIE, IIF and IIH) initiates transcription from Pol II promoters. The C-terminal domain (CTD) of RNA pol II contains multiple tandem repeats of a consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, this region has been shown to be essential for Pol II activity (Young, 1991). Pol II exists as two forms RNA Pol IIA and a phosphorylated form, RNA Pol IIO, Laybourn and Dahmus, (1990) demonstrated that the phosphorylation state of Pol II changes as the transcription cycle proceeds. Pol IIA preferentially associates with the preinitiation complex (Lu *et al.*, 1991), whereas Pol IIO is found mainly in elongation complexes (Payne *et al.*, 1989). Phosphorylation is thought to displace Pol II from the promoter triggering elongation. A protein kinase which can phosphorylate Pol II has been found which co-purifies with TFIIH, a five polypeptide complex (Lu *et al.*, 1992). Full kinase activity of this TFIIH is achieved upon formation of a stable preinitiation complex. The yeast, *saccharomyces cerevisae* contains a homologue of TFIIH, which also has CTD specific kinase activity (Gileadi *et al.*, 1992).

b) Upstream Activators

Brief external stimuli by polypeptide hormones, cytokines and neurotransmitters can lead to long term alterations in gene expression by signal regulated transcription factors. Of the transcription factors known, members of the Bzip family (Vinson *et al.*, 1989) are one the best characterised families. The AP-1 and CREB/ATF proteins which bind to TRE (TPA response elements) and CRE (cAMP response elements) respectively have been

extensively studied (Angel and Karin, 1991; Montminy *et al.*, 1990; Karin, 1990). AP-1 exists as either a heterodimer (c-fos/c-jun) or as a homodimer (c-jun/c-jun), heterodimers are more stable and therefore more transcriptionally active (Angel and Karin, 1991). AP-1 activity is stimulated by polypeptides, growth factors, cytokines and neurotransmitters, signalling occurring via membrane associated protein kinases or by phospholipid turnover (Cantley *et al.*, 1991). AP-1 activity is stimulated by increased transcription of c-fos or by post-translational modification of c-jun. Phosphorylation of c-jun occurs in the amino terminal region of the protein, a region in which the transactivation domain is located, this modification of c-jun has been shown to stimulate transactivation by c-jun by 5-10 fold (Binetruy *et al.*, 1991; Smeal *et al.*, 1992). AP-1 activity is down regulated by casein kinase II, sites in c-jun have been identified (Lin *et al.*, 1992), phosphorylation of these sites reduces DNA binding activity (Smeal *et al.*, 1992; Boyle *et al.*, 1991).

CREB activity is controlled by protein kinase A (PKA) (Montminy *et al.*, 1990). Increases in intracellular levels of cAMP determine the activity of PKA, cAMP binds to the regulatory subunit promoting dissociation of the catalytic subunit and therefore activation of PKA. The catalytic subunit can then translocate to the nucleus where it can phosphorylate CREB. The activity of CREB is dependent on its phosphorylation state, phosphorylation of CREB leads to a 10-20 fold increase in CREB transactivating activity (Gonzalez and Montminy, 1989; Hagiwara *et al.*, 1992). Phosphorylation is thought to activate CREB by altering the conformation of the transactivation domain. The importance of phosphorylation of CREB has been demonstrated in a transgeneic mouse in which the CREB PKA phosphorylation site has been mutated, these mice display a dwarf phenotype, an indicator of abnormal pituitary function (Struthers *et al.*, 1991).

1B.2.3 Control of RNA processing by protein kinases

The snRNP (small nuclear RNA binding protein) protein, U1-70K, is phosphorylated (Wooley *et al.*, 1982; Woppmann *et al.*, 1990). The role of phosphorylation in the regulation of splicing, both constitutive and alternative, has been proposed (Tazi *et al.*, 1992; Mermoud *et al.*, 1992). It is believed that the dephosphorylation of splicing factors is required for the cleavage and ligation of pre mRNA, a step which like the initial formation of the splicesome is ATP dependent (Tazi *et al.*, 1992). A kinase activity co-purifying with the U1-70K protein has recently been described (Woppmann *et al.*, 1993). The kinase activity associates with snRNP particles, in particular the U1-70K protein although similar kinase activities were found in other snRNP complexes. The kinase activity is not dependent on the RNA component of the snRNP. Another complex, the alternative splicing factor (ASF) was also phosphorylated by this co-purifying kinase

activity. The sites for phosphorylation are arginine and serine rich, a domain which is common to the splicing factors and RNA binding proteins.

1B.2.4 Phosphorylation and subcellular localisation

The intracellular localisation of Rab4, a guanine nucleotide binding protein, thought to be involved in the regulation of intracellular transport pathways, is dependent on its phosphorylation state. Rab4, which is normally associated with endosomes, when phosphorylated by cdc2 (Bailly *et al.*, 1991) dissociates from the endosome membranes (Van der Sluijs *et al.*, 1992a,b). The interaction of the protein kinase MARCKS with membranes is also determined by its phosphorylation state, only when unphosphorylated can it associate with membranes (Hartwig *et al.*, 1992). Phosphorylation of the growth factor receptor, PDGFr, provides a signal for the recruitment and formation of a large signal-transduction complex capable of signalling from the plasma membrane to the nucleus (Blenis, 1993; Davis, 1993). PDGFr phosphorylation plays an essential role in altering the spatial distribution of specific enzymes allowing them to contact their relevant substrates.

1B.2.5 Control of translation by phosphorylation

Covalent modification of translational machinery components by phosphorylation is a principal means of regulating protein synthesis in eukaryotic cells. In particular the phosphorylation of the alpha subunit of initiation factor eIF-2, a key factor required for protein synthesis, is one of the best characterised translational control mechanisms. Phosphorylation of eIF-2 alpha leads to inhibition of protein translation (Moldave, 1985; Hershey, 1991). Protein kinases which phosphorylate eIF-2 alpha have been identified, the activity of of these kinases is dependent on: extracellular stimulation, availability of metabolites and viral infection (Pestka *et al.*, 1987; Hinnebusch, 1990; Sen and Lengyel, 1992). Protein kinases which are activated by viral infection are the RNA dependent eIF-2 alpha protein kinases (PKR), there is also a family of protein kinases which are stimulated by haemin deficiency , the haemin-regulated eIF-2 alpha protein kinases and, a family of general eIF-2 alpha protein kinases involved in the general control of translation.

eIF-2 forms a ternary complex, eIF-2·GTP·methionyl-tRNA$_i$ whose formation is necessary for the binding of the initiator methionyl-tRNA$_i$ to the 40S ribosomal subunit, following binding eIF2 is released as an eIF-2·GDP complex. eIF-2 is then recycled prior to another round of initiation. Phosphorylation of eIF-2 alpha inhibits its interaction with eIF-2B, an interaction essential in the exchange of GDP for GTP, which is required to recycle an

active eIF-2 alpha subunit. (Moldave, 1985; Hershey, 1991). The stimuli and kinases involved in eIF-2 alpha phosphorylation are summarised in Figure 1B.4.

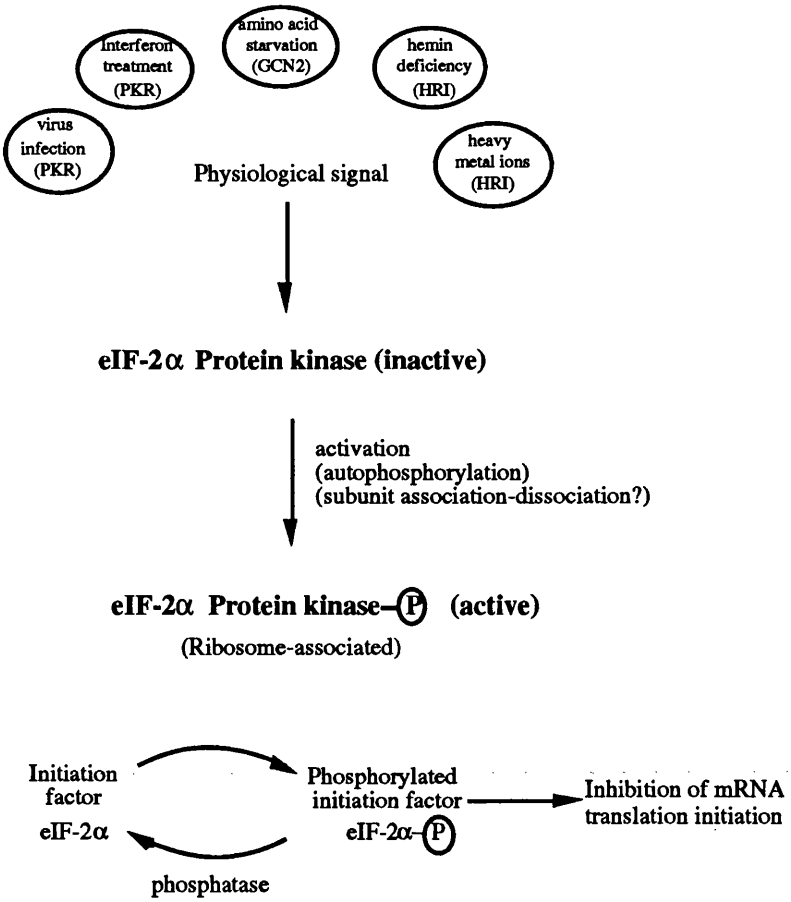


Figure 1B.4 Control of translation by phosphorylation of eIF-2 alpha. Several physiological signals have been shown to effect translation by the phosphorylation of eIF-2 alpha. The signals which stimulate eIF-2 alpha are shown and the protein kinases which mediate eIF-2 alpha phosphorylation are indicated (PKR, RNA-dependent protein kinase; GCN2, yeast general physiological protein kinase; HRI, haemin regulated protein kinase). Phosphorylation of eIF-2 alpha inhibits complex formation with eIF-2B., preventing the interaction of the initiating tRNA with the small ribosomal subunit. (Reproduced from Samuel, 1993)

eIF-4F is a three subunit complex that binds to 5′ cap structure of eukaryotic mRNAs and facilitates ribosome binding by unwinding secondary structure in the mRNA 5' noncoding region. A component of the eIF-4F complex, eIF-4E is a phosphoprotein. Reagents which stimulate protein kinase C increase eIF-4E phosphorylation (Xu *et al.*, 1993; Morley *et al.*, 1993). The phosphorylation of eIF-4E has been proposed to promote its association with eIF-4 alpha and eIF-4 gamma, assembly of the eIF-4F complex, this complex formation is thought to be an important translational control mechanism (Morley *et al.*, 1993).

Translation of specific transcripts can be specifically inhibited. Translation of the protamine transcript is regulated during germ cell development. A 18kDa

phosphoprotein which binds to the 3′ untranslated region of this message inhibiting its translation, the interaction is specific for this transcript. Dephosphorylation of the 18kDa protein leads to realease of the transcript and its subsequent translation (Yunhee *et al.*, 1993).

1B.3 Protein kinases and their role in signal transduction

Protein kinases play an integral role in signal transduction. Interaction between a polypeptide growth factor and its receptor can lead to long term changes in the cell.

1B.3.1 Receptor tyrosine kinases

In response to a ligand binding a receptor tyrosine kinase, the receptor phosphorylates itself on tyrosine. Phosphorylation of individual residues on the receptor allows it to interact with cytoplasmic signalling molecules, these in turn mediate the pleitrophic responses of the cell to the growth factor. Regions of the signalling molecules which interact with the phosphotyrosine residues on receptors have been characterised (Matsuda *et al.*, 1990), the interaction sites on these molecules are structurally similar and have been called SH2 domains (src homology domain). Many proteins have been shown to have SH2 domains, these include; GAP (GTPase activating protein), cytoplasmic kinases, phospholipases and cytoskeletal-binding proteins.

Stimulation of platelet-derived growth factor β-receptor (PDGFβr) mediates the interaction of phosphotidylinositol-3-kinase (PtdIns-3-kinase), GAP, phospholipase C and c-Src with this receptor (Kypta *et al.*, 1990). The interaction between the PDGFβr and PtdIns-3-kinase leads to increased Ras activity, Ras activation is important in the induction of mitogenesis (Cai *et al.*, 1991), Ras activity is stimulated by its interaction with PtdIns-3-kinase (Sjolander *et al.*, 1991). Intracellular protein kinase activity is also stimulated by growth factors, examples included raf-1, protein kinase C and the MAP kinase. Raf-1 plays an important role in serum-induced proliferation, gene expression mediated by serum stimulation has been demonstrated for the c-fos transactivator (Sternberg and Horvitz, 1991). Figure 1B.5 shows the interaction of PDGFβr with its signalling molecules and the effect of these interactions.

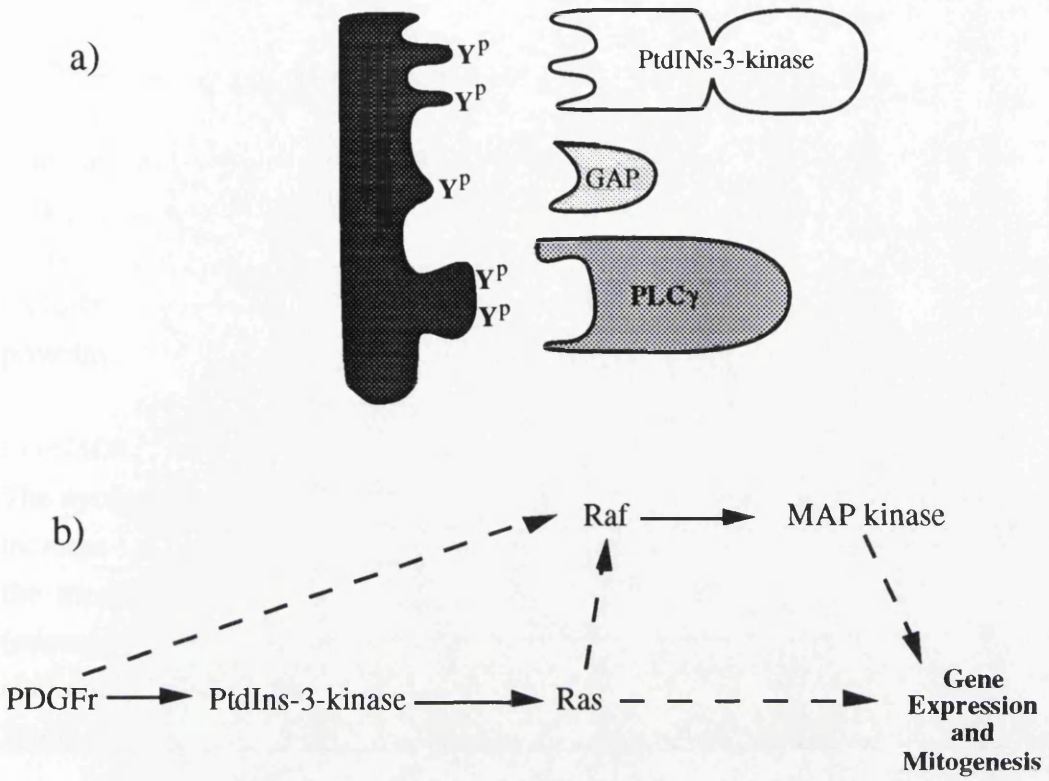


Figure 1B.5 Interaction of PDGFβr with its signalling molecules and effects of these interactions. The interactions of the PDGFβr with its signalling molecules is shown in a) The interaction between PDGFβr and PtdIns-3-kinase, PLCγ and GAP are shown. The effect of the interaction between PDGFβr and PtdIns-3-kinase is shown in b) PtdIns-3-kinase interaction with PDGFβr leads to increased Ras activity in the cell, the increase in Ras in turn leads to the stimulation of both the Raf and MAP serine/threonine kinases which in turn leads to the stimulation of gene expression and mitogenesis.
Reproduced with modifications from a) Blenis and Resh, 1993 and b) Davis,1993)

1B.3.2 Signal transduction via cyclic nucleotides (cAMP and cGMP)

a) cAMP

Interaction between hormones (e.g. thyroid-simulating hormone, epinephrine, and leutinising hormone) with their receptors leads to an increase in intracellular cAMP. The receptors may directly mediate cAMP production by being associated with the enzyme adenylate cyclase, which catalyses the formation of cAMP from ATP, or indirectly through a G protein (GTP binding protein). Indirect activation of adenylate cyclase is mediated by receptor binding ligand which alters its conformation allowing the receptor to interact with the G protein which in turn leads to displacement of GDP by GTP and in turn conformationally changes the G-protein allowing it to interact with and stimulate adenylate cyclase.

b)Protein kinase A

In the absence of cAMP protein kinase A exists as an inactive tetramer composed of two catalytic subunits and two regulatory subunits. Binding of cAMP to the regulatory subunit, leads to the dissociation of the catalytic subunit which, in turn leads to the activation of the catalytic subunit (Granot *et al.*, 1980). All physiological responses to cAMP are dependent on protein kinase A. Many Substrates for protein kinase A have been identified these include; enzymes involved in cellular metabolism, ribosomal proteins and structural proteins.

c) cGMP

The synthesis of cGMP by guanylate cyclases is stimulated by peptide hormones or by the increase in cellular levels of nitric oxide (NO). There are two forms of guanylate cyclases; the membrane associated (stimulated by peptide hormones) or the cytoplasmic form (stimulated by NO). The intracellular levels of NO are controlled by the enzyme NO synthetase, this enzyme is activated by glucocorticoids (Radomski *et al.*, 1990), increased levels of NO in turn lead to increased cGMP synthesis.

d) Protein kinase G

Protein kinase G, like protein kinase A, is regulated by its interaction with a cyclic nucleotide. Little is known about protein kinase G substrates and the affect of their phosphorylation on the cell.

1B.3.3 Diacylglycerol/Ca^{2+} and protein kinase C

a) Diacylglycerol +

Protein kinase C is activated via diacylgycerol (DAG). Both growth factor receptors and cytoplasmic growth factor-activated tyrosine kinases (Rhee and Choi, 1992) stimulate protein kinase C activity. Inositol 1,4,5-triphosphate (IP3) mediates the affect of the receptor/ligand interaction. Following stimulation of a receptor the conformation of the receptor becomes altered allowing it to interact with G proteins which in turn interact with and stimulate the activity of phospholipase C, a membrane associated enzyme which hydrolyses phosphatidylcholine (or phosphotidylinositol 4,5-bisphosphate) to produce DAG (Exton, 1990; Billah and Anthes, 1990). IP3 promotes the release of Ca^{2+} from intracellular calcium stores, calciosomes which are modified portions of the endoplasmic reticulum (Berridge, 1993), calcium also activates protein kinase C.

b) Protein kinase C

Protein kinase C exist as multiple subspecies in a number of tissues (Kikkawa *et al.*, 1989). Each subspecies has distinct enzymological characteristics and differential tissue expression and intracellular localisation. The subspecies show varied requirements for Ca^{2+} and phospholipid metabolites (Ogita *et al.*, 1992; Koide *et al.*, 1992). DAG binding to protein kinase C promotes its autophosphorylation which in turn promotes the phosphorylation of exogenous substrates. The diversity of protein kinase C makes it a versatile regulator in the cell.

1C Viral encoded protein kinases

The protein kinases encoded by the herpesviruses and vaccinia virus will be discussed. Although protein kinases have been described for other viruses, such as retroviruses, the transduced protein kinases of retroviruses are of cellular origin and will not therefore be discussed.

1C.1 HSV protein kinases

Several early reports described kinase activities associated with herpesviruses; HSV (Rubenstein *et al.*, 1972; Lemaster and Roizman, 1980); PRV (Tan, 1975) and equine herpesvirus (Randall *et al.*, 1972). Lemaster and Roizman, (1980) showed that the kinase activities of HSV were associated with the capsid-tegument of virions. Purification of a virion-associated kinase activity from infected cells was first described by Stevely *et al.*, (1985). The PRV virion-associated protein kinase activity was stimulated by virion disruption, suggesting that the kinase(s) was part of the virion tegument/capsid and not the virus envelope. Several protein kinase activities were described, two activities were found to have properties identical to cellular casein kinases; in their elution profiles from a phosphocellulose column, their ability to utilise GTP as a phosphate donor and their inhibition of activity by heparin.

Partial purification of virus specific kinase activity was first described by Katan *et al.*, (1985). Both the HSV-1 and the PRV kinases show an increase in activity during virus growth (Purves *et al.*, 1986). Virus gene expression is essential for induction of the kinase activity; in cells treated with cycloheximide prior to infection or, in cells infected with UV-irradiated virus, the kinase activity was not induced. In HSV-1 the ts mutant tsK (when at non-permissive temperature produces a nonfunctional form of Vmw175 preventing early and late gene expression) at the non-permissive temperature failed to induce the kinase activity (Purves *et al,* 1986). The protein kinases of herpesviruses and their substrates where identified are shown in Table 1C.1.

Virus	Gene	Family	Substrate
HSV-1	US3	US3	UL34
VZV	ORF66	US3	N/D
HSV-1	UL13	UL13	Vmw68 (IE protein) VP22 (capsid protein)
VZV	ORF47	UL13	ORF62 (Vmw175 homologue)
PRV	N/D	UL13	N/D
HCMV	UL97	UL13	Gancyciclovir (nucleoside analogue)

Table 1C.1 Herpesvirus protein kinases and their substrates. Table shows the kinase, the gene encoding the kinase where determined, the family to which the kinase belongs and the substrates of the kinase where identified. N/D=not determined

1C.1.1 US3 kinase family

a) HSV-1 US3

The US3 protein kinase is a cytoplasmic protein kinase found in small quantities in the virion. Confirmation that US3, an HSV-1 gene predicted to encode a protein kinase (McGeoch and Davison, 1986), encodes a protein kinase came from analysis of an HSV-1 mutant with this gene deleted (Purves *et al.*, 1991). Cytoplasmic extracts from cells infected with wt HSV-1, the US3 deletion mutant or a revertant of the deletion were fractionated and compared for kinase activity by FPLC elution profiles. In cells infected with the US3 deletion mutant the virus-specific kinase activity was absent, extracts from cells infected with the revertant showed the reappearance of this activity confirming that the kinase is encoded by US3.

A substrate for the US3 protein kinase was identified by comparison of phosphoprotein profiles from *in vivo* labelled cytoplasmic extracts prepared from cells infected with wt HSV-1, the US3 deletion mutant and its revertant (Purves *et al.*, 1991). A phosphoprotein of 30kDa in WT infected cell extracts was absent in extracts from cells infected with the US3 deletion mutant, a 34kDa phosphoprotein not observed in wt infected cell extracts appears in extracts of cells infected with the deletion mutant. The presence/absence of the 30kDa and 34kDa phosphoproteins is dependent on the presence of a functional US3 open reading frame.

Intertypic recombinants mapped the gene encoding the 30kDa substrate to the unique long region of the genome, between the UL31-35 genes. Of the four potential genes, the UL34 gene was favoured since the predicted mwt of the polypeptide was within the observed Mwt range. An HSV-1 insertion mutant was constructed; using a Vmw175 epitope the UL34 polypeptide was tagged. The 30kDa phosphoprotein reacted with anti-Vmw175

antisera, confirming that it was the product of UL34. Within UL34 a potential phosphorylation site for US3 was identified, mutation of this site altered the mobility of the 30kDa phosphoprotein, providing further evidence that UL34 is the US3 substrate.

Interactions between the US3 protein kinase and the UL34 substrate have been partially characterised by Purves *et al.,* (1992). The 34kDa phosphoprotein observed only in lysates from cells infected with the US3 deletion mutant does not react with anti-UL34 antisera, confirming this product is not an alternative processed form of UL34. Several novel phosphoproteins have been observed in lysates from cells infected with the US3 deletion mutant; a 33kDa protein was invariably observed and occasionally other phosphoproteins were apparent. Novel phosphoproteins seen in the US3 deletion mutant are observed only when UL34 is not phosphorylated; similar patterns are observed in a mutant in which the site of phosphorylation in UL34 is mutated (Purves *et al.,* 1992). In its unphosphorylated state UL34 exists as a complex , phosphorylation of UL34 leads to the disruption of this complex.

Purified virions contain the UL34 gene product and UL34 is also associated with cell membranes. The localisation of UL34 to cell membranes is not dependent on its phosphorylation state (Purves *et al.,* 1992). The exact role of the US3 protein kinase in viral replication has yet to be determined, no significant affect on viral replication has been observed with mutants in the US3 ORF (Purves *et al.,* 1992).

b) VZV ORF66

ORF66 of VZV is the homologue of HSV-1 US3, the homology between these genes was first noted by McGeoch and Davison, (1986). Immunoprecipitations of *in vivo* phosphate labeled extracts from cells infected with a recombinant vaccinia virus expressing ORF66 and from VZV infected cells demonstrated that the 48kDa ORF66 gene product is phosphorylated. However, Stevenson *et al,* (1994) failed to show *in vitro* phosphorylation of the recombinant vaccinia expressed protein. The intracellular localisation of ORF66 was found, like US3, to be exclusively cytoplasmic (Stevenson *et al.,* 1994).

c) PRV homologue

The US3 homologue in PRV has been cloned and mapped to the unique short region of PRV (Zhang *et al.,* 1990). The degree of homology to the HSV-1 gene is varied; showing the greatest homology toward the C-terminal catalytic domain and high degree of divergence toward then N-terminal domain. The protein kinase activity of this gene product was demonstrated by immunoprecipitation followed by in vitro kinase assays of extracts from PRV infected cell extracts (Zhang *et al.,* 1990). Like the HSV-1 US3 gene product, the PRV US3 protein kinase is cytoplasmic and is detectable in small amount with

the PRV virion. Purified PRV US3 protein kinase phosphorylates virion proteins (Zhang *et al.*, 1990).

1C.1.2 UL13 and homologues

This protein kinase, conserved throughout the alpha-,beta- and gamma-herpesviruses, was first identified at the UL13 locus of HSV-1 (Chee *et al.*, 1989; Hanks *et al.*, 1988; McGeoch and Davison, 1986; Smith and Smith, 1989); in VZV ORF 47; PRV gene has not as yet been defined; in HCMV the UL97 gene; in EBV the BGLF4 gene and in HHV6 the 15R gene. The conservation of this protein kinase suggests it may play a critical regulatory role in herpesvirus replication. The protein kinases found within the UL region appear to belong to a novel class of protein kinase and may therefore be good candidates for the development of an antiviral, effective against all herpesviruses. To date, protein kinase activity has been demonstrated for UL13 of HSV-1 (Cunningham *et al.*, 1992, Purves and Roizman 1992; Coulter *et al.*, 1993); for VZV ORF47 (Ng and Grose, 1992) and for the PRV UL13 homologue (de Wind *et al.*, 1992).

a) HSV-1 UL13

A protein kinase activity associated with this gene was first described by Cunningham *et al.*, (1992). Nuclear extracts form mock or HSV-1 infected cells were assayed *in vitro* for kinase activity, a major phosphoprotein of 57kDa unique to infected cell nuclear extracts was observed. This phosphoprotein was first observed at 2-3 hr post-infection and levels peaked at 5 hr post-infection, remaining constant after 5 hr.

HSV-1/HSV-2 intertypic recombinants mapped the gene encoding the 57kDa phosphoprotein, to between UL9 and UL15. Immunoprecipitations using an anti-UL13 fusion antisera, followed by *in vitro* kinase assays produced a 57kDa band unique to precipitates from infected cells (Coulter *et al.*, 1993). The UL13 protein kinase is associated with the virion, disruption of pure virion preparations followed by *in vitro* kinase assays produces a major phosphoprotein which co-migrates with the 57kDa observed in infected cell nuclear extracts. Immunoprecipitation of disrupted virion preps followed by in vitro kinase assays provided further proof that this major virion-associated phosphoprotein was identical to the 57kDa product of the UL13 gene observed in the nuclei of infected cells (Coulter *et al.*, 1993).

Two mutants in UL13 have been described; an insertion mutant in which the open reading frame was destroyed by insertion of a lacZ expression cassette (Coulter *et al.*, 1993) and a deletion mutant in which the entire UL13 locus was deleted (Purves and Roizman, 1992). These mutants were used to characterise substrates of this kinase activity. Comparisons of

nuclear and virion-associated phosphoprotein profiles from cells infected with either the wt or the UL13 mutant virus were made. Two major phosphoproteins were altered by destruction of the UL13 gene; the UL13 gene product itself (57kDa) and a virion-associated 38kDa protein The 38kDa phosphoprotein in cells infected with wt virus shows an almost identical kineticsof phosphate incorporation to the UL13 encoded 57kDa protein. Intertypic recombinants were used to identify the UL13 38kDa substrate, the gene encoding the 38kDa phosphoprotein was found to be UL49 (VP22) which encodes a major tegument protein previously described by Elliot and Meredith, (1992).

The deletion mutant in which the entire UL13 ORF is removed was used by Purves and Roizman, (1992) to characterise potential substrates for the UL13 protein kinase. In addition, the possible relationship between the UL13 and US3 protein kinase activities of HSV-1 was investigated using a mutant in which both open reading frames were deleted. Several phosphoproteins were altered in whole cell, *in vitro* labelled, extracts from BHK cells infected with the UL13 deletion mutant. The most striking difference was observed in the pattern of phosphorylation of a viral associated 70kDa band. In the absence of the UL13 protein kinase this band accumulates and higher Mwt phosphoproteins of ~80kDa are reduced, in contrast to *in vitro* labelled extracts from cells infected with wt HSV-1 in which the higher Mwt bands are more predominant than the lower Mwt 70kDa phosphoprotein. Western blot analysis was used to identify the viral protein phosphorylated by the UL13 kinase, antisera specific to ICP22 (IE68) reacted with both the 70kDa and the higher Mwt species. It has been proposed that the UL13 protein kinase is essential for post-translational modification of the ICP22 protein. Confirmation that ICP22 is the 70kDa came from analysis of a mutant in the ICP22 locus, the 70kDa and associated species were absent in extracts from cells infected with this mutant (Purves and Roizman, 1992).

In both studies the effect of the mutations in the UL13 locus on viral replication was investigated, in both cases the mutations did not appear to have a profound effect on viral replication. To date, two separate viral proteins have been proposed as substrates for the UL13 kinase activity; one an immediate early protein, ICP22 (IE68), whose function is, at present, unknown and the other, a virion tegument protein UL49 (Elliot and Meredith, 1992).

b) VZV ORF 47

The VZV homologue of HSV-1 UL13, open reading frame 47, a gene of the unique long region of VZV was identified as a possible protein kinase (Smith and Smith, 1989). Immunoprecipitation, followed by *in vitro* kinase assay, using an anti-ORF47 fusion antisera demonstrated that the 54kDa product of ORF47 is a functional protein kinase (Ng

and Grose, 1992). Phosphoamino acid analysis showed that VZV ORF 47 predominantly phosphorylates serine residues and to a lesser extent threonine. The intracellular localisation of VZV ORF47 appears to differ from HSV-1 UL13, showing a cytoplasmic localisation with a strong perinuclear association. Like HSV-1 UL13,ORF 47 is associated with the virion (Stevenson *et al.,* 1994).

Co-immunoprecipitation experiments using ant-ORF47 specific antisera and antisera to known VZV phosphoproteins (ORFs 4, 61, 66 and 63) identified a substrate for the ORF47 protein kinase in infected cells. Co-precipitation of ORF62 with ORF47 followed by *in vitro* kinase assays demonstrated that ORF62 is a substrate of the ORF47 protein kinase, ORF62 is the Vmw175 homologue of VZV, it is a major transcriptional activator in VZV.

c) PRV UL13 Homologue

Unlike HSV-1 and VZV, the complete genomic sequence of PRV has not been determined. A UL13 homologue in PRV was identified by random mutagenesis of a 49Kbp region of the UL segment, cloned into a cosmid vector (de Wind *et al.,* 1992). The mutagenesis method, first described by de Wind *et al.,* (1990), randomly inserts oligonucleotides, containing translational stop codons in all three reading frames and an EcoR1 site (no EcoR1 site in PRV genome), throughout the region of interest. Viral mutants were constructed by co-transfecting the linearised mutated DNA with wt viral DNA. Using an HSV-1 UL13 probe, and low stringency conditions, two cosmids containing the mutated PRV UL13 homologue were isolated, one in 5′ region and the other in the 3′ region. The PRV homologue was *in vitro* transcribed and translated and found to produce a protein within the predicted Mwt range (41kDa). Transient transfection assays were performed to show that the PRV UL13 homologue possesses protein kinase activity. Using a VSV glycoprotein epitope the PRV UL13 open reading frame was tagged allowing the protein to be easily immunoprecipitated, and assayed for kinase activity *in vitro.* To show that kinase activity was a function of UL13 homologue from PRV, and not a minor contaminating kinase, a mutant was constructed in which the putative lysine involved in phosphotransfer (lys 103) was mutated to a methionine; this mutant immunoprecipitated but failed to autophosphorylate.

(d) HCMV UL97

Unlike the alpha-herpes homologues of UL13, the HCMV UL97 does not act as a protein kinase (Littler *et al.,* 1992; Sullivan *et al.,* 1992). Phosphorylation of an antiviral nucleoside analogue, ganciclovir, is observed *in vitro* by the recombinant UL97 gene product (Littler *et al.,* 1992), truncation of the N-terminal region of the UL97 ORF did not effect ganciclovir phosphorylating activity, confirming that the region with protein kinase homology is sufficient for activity. Marker rescue experiments of HCMV isolates resistant

to ganciclovir identified UL97 as the gene responsible for ganciclovir phosphorylation (Sullivan *et al.*, 1992). A region important for UL97 phosphorylation of ganciclovir is located at a site homologous to the nucleotide binding site of PKA (Sullivan *et al.*, 1992).

1C.2 Vaccinia virus protein kinase

B1R

Vaccinia virus genes with regions of homology to known protein kinases were first described by Howard and Smith, (1989). Several reports have described a protein kinase activity associated with vaccinia virions (Paoletti and Moss, 1972; Kleiman and Moss, 1973, 1975, 1975). The vaccinia virus protein kinase is tightly associated with the virus core, two virus proteins in heat inactivated extracts, of 11kDa and 37kDa, are transphosphorylated. The B1R ORF shows homology to protein kinases with residues characteristic of a serine/threonine kinase.

The B1R gene has been overexpressed and its *in vitro* protein kinase activity characterised (Banham and Smith, 1992; Lin *et al.*, 1992). Lin *et al.*, (1992) expressed the B1R gene as a glutathionine-S-transferase fusion, affinity-purified protein autophosphorylates, and in addition, transphosphorylation of the exogenous substrates casein and histones was observed Confirmation that the kinase activity was a function of the B1R gene product was provided by assaying a mutant in which a lysine, predicted to be involved in phosphate transfer, was mutated to a glutamine; neither autophosphorylating or transphosphorylating activity was observed. Preferred sites of phosphorylation by the B1R kinase activity have been determined; in autophosphorylation the preferred site is a threonine, in transphosphorylation both serine and threonine were phosphorylated. Purified virions extracted and fractionated into envelope and core fractions were found, by Western analysis, to contain the B1R protein kinase. Two proteins, both components of the 40S ribosomal subunit, are transphosphorylated by B1R (Banham *et al.*, 1993). Phosphorylation of these proteins is observed early in infection.

Figure 1C.1 Comparison of viral protein kinases. Comparison was carried out using University of Wisconsin GCG pileup program. The protein kinases belonging to the unique short region of alphaherpesviruses (HSV1Us3, VZV ORF 66) are compared to the UL13 family of protein kinases conserved throughtout the herpesvirus classes (α: HSV1 UL13; VZV 47; β: HCMV UL97; HHV6 15R; γ: EBV Bglf4), the vaccinia virus protein kinase B10R, the proposed vaccinia protein kinase B12R and the large subunit of HSV-1 ribonucleotide reductase (HSV-1 R1). Pileup shows that there is limited conservation of residues between viral protein kinases. The large subunit of ribonucleotide reductase shows little similarity to other viral protein kinases. Residues involved in catalysis, where identified, are bolded.

```
           1                                                  50
 Hsv1us3   .......... .......... .......... .......... ..........
  VZV 66   .......... .......... .......... .......... ..........
Hsv1ul13   .......... .......... .......... .......... ..........
  VZV 47   .......... .......... .......... .......... ..........
Hcmvul97   MSSALRSRAR SASLGTTTQG WDPPPLRRPS RARRRQWMRE AAQAAAQAAV
Hhv6_15r   .......... .......... .......... .......... ..........
   Bglf4   .......... .......... .......... .......... ..........
 VV BIOR   .......... .......... .......... .......... ..........
 VV BI2R   .......... .......... .......... .......... ..........
HSV-1 R1   .......... .......... .......... .......... ..........

           51                                                 100
 Hsv1us3   .......... .......... .......... .......... ..........
  VZV 66   .......... .......... .......... .......... ..........
Hsv1ul13   .......... .......... .......... .......... ..........
  VZV 47   .......... .......... .......... .......... ..........
Hcmvul97   QAAQAAAAQV AQAHVDENEV VDLMADEAGG GVTTLTTLSS VSTTTVLGHA
Hhv6_15r   .......... .......... .......... .......... ..........
   Bglf4   .......... .......... .......... .......... ..........
 VV BIOR   .......... .......... .......... .......... ..........
 VV BI2R   .......... .......... .......... .......... ..........
HSV-1 R1   .......... .......... .......... .......... ..........

           101                                                150
 Hsv1us3   .......... .......... .......... .......... ..........
  VZV 66   .......... .......... .......... .......... ..........
Hsv1ul13   .......... .......... .......... .......... ..........
  VZV 47   .......... .......... .......... .......... ..........
Hcmvul97   TFSACVRSDV MRDGEKEDAA SDKENLRRPV VPSTSSRGSA ASGDGYHGLR
Hhv6_15r   .......... MDNGVETPQG QKTQPINLPP VRKKLRK... ..........
   Bglf4   .......... .......... .......... .......... ..........
 VV BIOR   .......... .......... .......... .......... ..........
 VV BI2R   .......... .......... .......... .......... ..........
HSV-1 R1   .......... .......... .......... .......... ..........

           151                                                200
 Hsv1us3   ......MACR KFCRVYGGQG RRKEEAVPPE TKPSRVFPHG PFYTPAEDAC
 VZV 66    .......... .......... .......... .......... ...MNDVDAT
Hsv1ul13   .......... ........MD ESRRQRPAGH VAANL..SPQ GARQRSFKDW
  VZV 47   .......... .......... .....MDADD TPPNLQISPT AGPLRS....
Hcmvul97   CRETSAMWSF EYDRDGDVTS VRRALFTGGS DPSDSVSGVR GGRKRPLRPP
Hhv6_15r   .......... ...HEGLGKG VKRKLFAEDS SPLKKQISAC SDME.TLSSP
   Bglf4   .......... .......... .......... .......... ..........
 VV BIOR   .......... .......... .......... .......... ..........
 VV BI2R   .......... .......... .......... .......... ..........
HSV-1 R1   .......... .......... .......... .......... ..........
```

Figure 1C.1 continued

```
          201                                                       250
 Hsv1us3  LDSPPPETPK PSHTTPPSEA ERLCHLQEIL AQMYGNQDYP IEDDPSADAA
  VZV 66  DTFVGQGKFR GAISTSPSHI MQTCGFIQ.. ......QMFP VEMSPGIESE
Hsv1ul13  LASYVHSNPH GASGRPSGPS LQDAAVSRSS HGSRHRSGLR ERLRAGLSRW
  VZV 47  .....HHNTD GHEPNATAAD QQERESTNPT HGCVNHP... ...WANPSTA
Hcmvul97  LVSLARTPLC RRRVGGVDAV LEENDVELRA E........S QDSAVASGPG
Hhv6_15r  VKS.....EC ESRSASLDES FGKCKHEIAC DCSAIEELLC HESLLDS.PM
   Bglf4  .......... .......... ...MDVNMAA ELSPTNSSSS GELSVSPEPP
 VV BIOR  .......... .......... .......... .......... ..........
 VV BI2R  .......... .......... .......... .......... ..........
HSV-1 R1  .MASRPAASS PVEARAPVGG QEAGGPSAAT QGEAAGAPLA HGHHVYCQRV

          251                                                       300
 Hsv1us3  DDVDEDAPDD VAYPEEYAEE LFLPGDATGP LIGANDHIPP PCGASPPGIR
  VZV 66  DDPNYDVNMD IQSFNIF... .......... .......... ..........
Hsv1ul13  RMSR..SSHR RASPETPGTA AKLNRPPLR. RSQAALTAPP SSPSHILTLT
  VZV 47  TCME..SPER SQQTSLFLLK HGLTRDPIHQ RERVDVFPQF NKPPWVFRIS
Hcmvul97  RIPQPLSGSS GEESATAVEA DSTSHDDVHC TCSNDQIITT SIRGLTCDPR
Hhv6_15r  KLSNAHTIFS SNKWKLELEK IIASKQIFLD MSENAELAAY G..ETLCNLR
   Bglf4  RETQAFLG.. ....KVTVID YFTFQHKHLK VTNIDDMTET ..........
 VV BIOR  .......... .......... .......... .......... ..........
 VV BI2R  .......... .......... .......... .......... ..........
HSV-1 R1  NGVMVLSDKT PGSASYRISD SNFVQCGSNC TMIIDGDVVR GRPQDPGAAA

          301                                                       350
 Hsv1us3  RRSRDEIGAT GFTAEE.... ..LDAMDREA ARAISRGGKP PSTMAKLVTG
  VZV 66  .......... ....DG.... ..VHETEAEA SVALCAEARV GINKA.....
Hsv1ul13  RIRKLCSPVF ..AINPALHY TTLEIPGARS FG........ ........GS
  VZV 47  KLSRLIVPIF ..TLNEQLCF SKLQIRDRPR FA........ ........GR
Hcmvul97  MFLRLTHPEL CEL...SISY LLVYVPKEDD FCHKICYAVD MSDESYRLGQ
Hhv6_15r  IFEKISSPFL FDVQSEERSY SVVYVPHNKE LCGQFCQPEK TMARV..LGV
   Bglf4  .......... .......... LYVKLPENMT RCDHLPITCE .....YLLGR
 VV BIOR  ....MDSGIY ETPINYKKSN VSAVSVNNTI FVT....... ..........
 VV BI2R  .......... .......... .......... .......... MESFKYCFDN
HSV-1 R1  SPAPFVAVTN IGAGSDGGTA VVAFGGTPRR SAGTSTGTQT ADVPTEALGG

          351                                                       400
 Hsv1us3  MGFTIHGALT PGSEGCVFDS SHPDYPQRVI VKAGWYTSTS HEARLLRRLD
  VZV 66  .GFVILKTFT PGAEGFAFAC MDSKTCEHVV IKAGQRQGTA TEATVLRALT
Hsv1ul13  GGYGDVQLIR EHKLAVKTIK EKEWFAVELI ATLL.VGECV LRAGRTH...
  VZV 47  GTYGRVHIYP SSKIAVKTMD SRV.FNRELI NAIL.ASEGS IRAGERL...
Hcmvul97  GSFGEVWPLD RYRVVKVARK HSETVLTVWM SGLIR.TRAA GEQQQPP...
Hhv6_15r  GAYGKVFDLD ..KVAIKTAN EDESVISAFI AGVIR.AKSG ADLLSHE...
   Bglf4  GSYGAVYAHA DNATVKLYDS VTELYHELMV CDMIQIGKAT AEDGQDK...
 VV BIOR  ...GGLFINN SNSTIVVNNM EKLDIYKDKQ WSIIEMPMAR VYHGIDS...
 VV BI2R  DGKKWIIGNT LYSGNSILYK VRKNFTSSFY NYVMKIDHKS HKPLLSEIRF
HSV-1 R1  PPPPPRFTLG GGCCSCRDTR RRSAVFGGEG DPVGPAEFVS DDRSSDSDSD

          401                                                       450
 Hsv1us3  HPAILPLLDL HVVSGVTCL. ......VLPK YQADLYTYLS R.....RLNP
  VZV 66  HPSVVQLKGT FTYNKMTCL. ......ILPR YRTDLYCYLA A.....KRN.
Hsv1ul13  ..NI...... RGFIAPLGFS LQQRQIVFPA YDMDLGKYIG QLASLRTTNP
  VZV 47  ..GI...... SSIVCLLGFS LQTKQLLFPA YDMDMDEYIV RLSRRLTIPD
Hcmvul97  ..SLVGTGVH RGLLTATGCC LLHNVTVHRR FHTDMFHHDQ W.......KL
Hhv6_15r  ..CVIN.... .NLLISNSVC MSHKVSLSRT YDIDLHKFED W.......DV
   Bglf4  ..ALVDY... ......LSAC TSCHALFMPQ FRCSLQDYGH W.......HD
 VV BIOR  ..TFGMLYFA GGLSVTEQYG NLEKNNEISC YNPRTNKWFD ISYTIYKISI
 VV BI2R  YISVLDPLTI DNWTRERGIK YLAIPDLYGI GETDDYMFFV IKNLGRVFAP
HSV-1 R1  DSEDTDSETL SHASSDVSGG ATYDDALDSD SSSD...... ..........
```

Figure 1C.1 Continued

```
          451                                                       500
 Hsv1us3  LGRPQIAAVS RQLLSAVDYI HRQ.GIIHRD IKTENIFINT PE.......D
  VZV 66  LPICDILAIQ RSVLRALQYL HNN.SIIHRD IKSENIFINH PG.......D
Hsv1ul13  SVSTALHQCF TELARAVVFL NTTCGISHLD IKCANILVML RS.DAVSLRR
  VZV 47  HIDRKIAHVF LDLAQALTFL NRTCGLTHLD VKCGNIFLNV DNFASLEITT
Hcmvul97  ACIDSYRRAF CTLADAIKFL NHQCRVCHFD ITPMNVLIDV NPHNPSEIVR
Hhv6_15r  RNVMNYYSVF CKLADAVRFL NLKCRINHFD ISPMNIFLN. ..HKKEIIFD
   Bglf4  GSIEPLVRGF QGLKDAVYFL NRHCGLFHSD ISPSNILVDF .TDTMWGMGR
 VV BIOR  SSLCKLNNVF YVFSKDIGYV EKYDGAWKLV HDRLPAIKAL STSPY.....
 VV BI2R  KDTESVFEAC VTMINTLEFI HSR.GFTHGK IEPRNILIRN K........R
HSV-1 R1  .......... ....DSLQID GPVCRPWSND TAPLDVCPGT PGPGADAGGP

          501                                                       550
 Hsv1us3  ICLGDFGAAC FVQGSRSSPF PYGIAGTIDT NAPEVLAGDP YTTTVDIWSA
  VZV 66  VCVGDFGAAC FPVDINANRY .YGWAGTIAT NSPELLARDP YGPAVDIWSA
Hsv1ul13  AVLADFSLVT LNSNSTIARG QFCLQEPDLK SPR....MFG MPTALTTANF
  VZV 47  AVIGDYSLVT LNTYSLCTRA IFEVGNPS.H PEH....VLR VPRDASQMSF
Hcmvul97  AALCDYSLSE PY.......P DYNERCVAVF QET....GTA RRIPNCSHRL
Hhv6_15r  AVLADYSLSE MH.......P NYNGTCAIAK EYD....KNL QLVPISRNKF
   Bglf4  LVLTDYGTAS LH.......D RNKMLDVRLK SSK....GRQ LYRLYCQREP
 VV BIOR  .......... .......... .......... .......... ..........
 VV BI2R  LSLIDYSRTN KLYKSGNSHI DYNEDMITSG NINYMCVDNH LGATVSRRGD
HSV-1 R1  SAVDPHAPTP EAGAGLAADP AVARDDAEGL SDPRPRLGTG TAYPVPL...

          551                                                       600
 Hsv1us3  GLVIFETAVH NASLFSAPRG PKRGP.CD.. ...S..QITR IIRQAQVHVD
  VZV 66  GIVLFEMATG QNSLFE..RR GLDGN.CD.. ...SERQIKL IIRRSGTHPN
Hsv1ul13  HTLVGHGYNQ PPELLVKYLN NERAEFTN.. ...HRLKHDV GLA......V
  VZV 47  RLVLSHGTNQ PPEILLDYIN GTGLTKYT.. ...GTLPQRV GLA......I
Hcmvul97  RECYHPAFRP MPL........ .QKLLICD.. ...PHARFPV AGLR.RYCMS
Hhv6_15r  CDMFNPGFRP LVA........ .NAMILVN.. ...VCGAFDG ENNPLRHCNL
   Bglf4  FSIAKDTYKP LCLLSKCYIL RGAGHIPD.. ...PSACGPV GAQTAL..RL
 VV BIOR  .......... .......... .......... .......... ..........
 VV BI2R  LEMLGYCMIE WFGGKLPWKN ESSIKVIK.. ...QKKEYKK FIATFFEDCF
HSV-1 R1  .ELTPENAEA VARFLGDAVN REPALMLEYF CRCAREETKR VPPRTFGSPP

          601                                                       650
 Hsv1us3  EFSPHPESRL TSRYRSRAAG NNRPPYTRPA WTRYY....K MDIDVEYLVC
  VZV 66  EFPINPTSNL RRQYIGLAKR SSRKPGSRPL WTNLY....E LPIDLEYLIC
Hsv1ul13  DLYALGQTLL ELVVSVYVAP SLGVPVTRFP GYQYFNNQLS PDFALALLAY
  VZV 47  DLYALGQALL EVILLGRLPG QLPISVHRTP HYHYYGHKLS PDLALDTLAY
Hcmvul97  ELSALGNVLG FCLM..RLLD ...RRGLDEV RMGT..E..A LLFKHAGAAC
Hhv6_15r  DLCAFAQVVL SCVL..RMTD ...KRGCREA QLYY..E..K RLFALANEAC
   Bglf4  DLQSLGYSLL YGIM..HLAD STHKIPYPNP DMGF..DRSD PLYFLQFAAP
 VV BIOR  .......... .......... .......... .......... ..........
 VV BI2R  PEGNEPLELV RYIELVYTLD YSQTPNYDRL RRLFIQD... ..........
HSV-1 R1  RLTEDDFGLL NYALVEMQRL CLDVPPVPPN AYMPY..... ..........

          651                                                       700
 Hsv1us3  KALTFDGALR PSAAELLCLP LFQQK..... .......... ..........
  VZV 66  KMLSFDARHR PSAEVLLNHS VFQTLPDPYP NPMEVGD... ..........
Hsv1ul13  RCVLHPALFV .NSAETNTHG LAYDVPEGIR RHLRNPKIRR AFTDRCINYQ
  VZV 47  RCVLAPYILP .SDIPGDLNY NPFIHAGELN TRISRNSLRR IFQCHAVRYG
Hcmvul97  RALENGKLTH CSDACLLILA AQMSYGACLL GEHGAALVSH TLRFVEAKMS
Hhv6_15r  RLNPLKYPFA YRDACCKVLA EHVVLLGLLF YRDVVEIYEK LYDFLDERGE
   Bglf4  KVVLLEVLSQ MWN.....LN LDMGLTSCGE SPCVDVTAEH MSQFLQ.WCR
 VV BIOR  .......... .......... .......... .......... ..........
 VV BI2R  .......... .......... .......... .......... ..........
HSV-1 R1  .......... .......... .......... .......... ..........
```

Figure 1C.1 Continued

```
          701                                                       750
 Hsv1us3  .......... .......... .......... .......... ..........
  VZV 66  .......... .......... .......... .......... ..........
Hsv1ul13  HTHKAILSSV ALPPELKPLL VLVSRLCHTN PCARHALS.. ..........
  VZV 47  VTHSKLFEGI RIPASLYPAT VVTSLLCHDN SEIRSDHPLL WHDRDWIGST
Hcmvul97  SCRVRAFRRF YHECSQTMLH EYVRKNVERL LATSDGLYLY NAFRRTTSII
Hhv6_15r  FGSRDLFEAT FLNNSKLTRR QPIREGLASL QSSEYGEKLL HDLRELFLIN
   Bglf4  SLKKRFKESY FFNCRPRFEH PHLPGLVAEL LA...DDFFG PDGRRG....
 VV BIOR  .......... .......... .......... .......... ..........
 VV BI2R  .......... .......... .......... .......... ..........
HSV-1 R1  .......... .......... .......... .......... ..........

          751          765
 Hsv1us3  .......... .....
  VZV 66  .......... .....
Hsv1ul13  .......... .....
  VZV 47  .......... .....
Hcmvul97  CEEDLDGDCR QLFPE
Hhv6_15r  STADLDKDTS SLFHM
   Bglf4  .......... .....
 VV BIOR  .......... .....
 VV BI2R  .......... .....
HSV-1 R1  .......... .....
```

1C.3 HSV-1-induced phosphorylation

There are 35 phosphorylated viral specific polypeptides (Wilcox *et al.,* 1980) a few have been characterised. Cellular protein phosphorylation patterns are altered by infection with HSV-1, HSV-1, like vaccinia virus, alters the pattern of ribosomal protein phosphorylation during infection (Kennedy *et al.,* 1981; Masse *et al.,* 1990).

1C.3.1 Phosphorylation of immediate early polypeptides of HSV-1

The immediate early polypeptides Vmw175 (Pereira *et al.,* 1977), Vmw110 (Ackermann *et al.*, 1984), Vmw63 (Wilcox *et al.,* 1980) and Vmw68 (Wilcox *et al.,* 1980; Purves *et al.,* 1992) are phosphorylated in vivo. The point at which these proteins exist as phosphoproteins in the infected cell was investigated by Wilcox *et al.,* (1980). Phosphorylation of Vmw175 occurs very soon after infection whereas optimal levels of Vmw63 phosphorylation is not observed until 2 hr post-infection. It was suggested by Wilcox that differences in the pattern of phosphorylation of the immediate early proteins were due to different protein kinase requirements; the kinase which phosphorylates Vmw175 being present in an active form early in infection, whereas the protein kinase required for Vmw63 phosphorylation only becomes active several hours into the productive infection. In addition, Wilcox *et al.,* (1980) showed that the abundance of the

phosphorylated form of Vmw63 varied throughout infection, phosphate cycling on and off this particular protein, a similar situation was suggested for Vmw110.

Of the immediate early polypeptides the importance of phosphorylation in determining activity is known only for Vmw175. The phosphorylation state of Vmw175 has been shown to effect the interaction of Vmw175 with immediate early, early and late leader sequences (Papavassiliou *et al.*, 1991). Unphosphorylated Vmw175 having a greater affinity for immediate early leader sequences and phosphorylation appears to be essential for complex formation on early and late leader sequences.

1C.3.2 Phosphorylation of non-glycosylated membrane proteins

Two non-glycosylated membrane proteins are phosphoryalted in vivo; UL34 the substrate of the US3 protein kinase (Purves *et al.*, 1991; Purves *et al.*, 1992) and UL31 (Chang and Roizman, 1993). The effects of phosphorylation on UL34 have been discussed (see Section 1C.1.2), briefly, in the absence of UL34 phosphorylation several new phosphoproteins are observed, the lack of phosphorylation of UL34 does not however appear to impair the replication of the virus. UL31, which contains casein kinase II, cAMP kinase and protein kinase C phosphorylation sites is phosphorylated *in vivo* (Chang and Roizman, 1993). This protein associates with the nuclear matrix and is essential for viral replication. The importance of phosphorylation on the activity of this protein is presently unknown.

1C.3.3 Phosphorylation of capsid proteins

The capsid protein, VP26, the product of the UL35 ORF is phosphorylated, this protein appears in three forms depending on its extent of phosphorylation, the phosphorylated form is the most abundant in the virion (McNabb and Courtney, 1992). The importance of phosphorylation in the localisation of this protein, to discrete areas of the nucleus, and in the formation of mature virions is unknown. This protein, although not essential for capsid formation, appears to play a role in the formation of a mature virion particle.

The capsid-associated polypeptide, VP22 (ICP35) is phosphorylated *in vivo* (Braun *et al.*, 1984). This family of differentially processed polypeptides attaches to mature capsid particles, attachment is however completeley dependent on the packaging of the DNA into the capsid.

1C.3.4 Phosphorylation of RNA Pol II in HSV-1 infected cells

The phosphorylation of RNA Pol II is altered in cells infected with HSV-1 (Rice *et al.*, 1994). Instead of the typical phosphorylated form, RNA Pol IIO, a new form which migrates between the fully phosphorylated and the unphosphorylated form appears, Rice *et al.*, (1994) have called this faster migrating phosphorylated species RNA pol IIi (intermediate). The localisation of RNA pol II in infected cells was investigated (Rice *et al.*, 1994), pol II co-localised with UL29, the major DNA binding protein involved in the formation of HSV-1 replication compartments. Phosphorylation of pol II is thought to be important in the removal of histones from promoters allowing the de-repression of the promoter (Peterson *et al.*, 1991). Rice *et al.*, proposed that by preventing the full phosphorylation of RNA pol II the virus may alter the ability of Pol II to interact with normal cellular promoters, allowing the preferential expression of virus genes since in the lytic state the viral genome is not associated with histones (Leinbach and Summers, 1980; Muggeridge and Fraser, 1986). The aberrant pattern of Pol II phosphorylation was shown to require the expression of an immediate early gene (Rice *et al.*, 1994).

1D Ribonucleotide reductase

1D.1 The need for an HSV-1 encoded enzyme.

The cellular ribonucleotide reductase is active only in actively dividing cells, viral replication in terminally differentiated neurons and non-dividing cells is completely dependent on the virus enzyme. Mammalian ribonucleotide reductase exists as a tetramer composed of dimers of the two subunits, the large subunit (R1) and the small subunit (R2) (Reichard, 1988; Wright *et al.*, 1990; Hurta and Wright, 1992). The activity of the mammalian enzyme correlates with the onset of DNA replication during the S-phase of the cell cycle (Lewis *et al.*, 1978; Weber, 1983); control of the activity is dependent on the synthesis of the small subunit during S-phase (Wright *et al.*, 1992; Bjorkland *et al.*, 1990). Once R2 is expressed, reductase activity is controlled by phosphorylation of R2. Phosphorylation is important in the regulation of ribonucleotide reductase in the mammalian cell (Albert and Nodzenski, 1989; Hurta and Wright, 1993). Recently, the direct phosphorylation of R2 by both $p34^{cdc2}$ and CDK2 protein kinases has been demonstrated (Chan *et al.*, 1993). The activity of both protein kinases is dependent on the cell being in a particular phase of the cell cycle.

1D.2 HSV-1 ribonucleotide reductase

A ribonucleotide reductase activity associated with HSV-1 was first suggested when it was found that HSV-1 replication was resistant to hydroxyurea, a potent inhibitor of the cellular reductase activity (Cohen *et al.*, 1977). The HSV-1 enzyme exists as a tetramer of the type $\alpha 2\beta 2$, composed of two dimers of each of the subunits, the 136kDa large subunit, and the 38kDa small subunit (Frame *et al.*, 1985; Bachetti *et al.*, 1984; Ingemarson and Lankinen, 1987). The activity of the HSV-1 enzyme differs from that of the cellular enzyme, it does not have an absolute requirement for Mg^{2+} or ATP, and it is not allosterically regulated (Cohen, 1972; Ponce de Leon *et al.*, 1977; Huszar and Bachetti, 1981; Langelier *et al.*, 1978). The activities of the other herpesvirus ribonucleotide reductases are similar to that of HSV-1; PRV (Lankinen *et al.*, 1982), VZV (Spector *et al.*, 1987), and EBV (Henry *et al.*, 1978). Comparison of the amino acid sequences of the herpesvirus ribonucleotide reductases with the sequences of the mouse, clam T4 and *E.coli* enzymes showed that there is conservation throughout species (Eriksson and Sjoberg, 1988). The reducing power of HSV-1 ribonucleotide reductase is supplied by thioredoxin

(Darling *et al.*, 1988), a small cellular protein which contains an oxidation-reduction-active disulphide provided by cysteine residues (Holmgren, 1968), the disulphide acts as a hydrogen donor for ribonucleotide reductase (see Figure 1D.1).

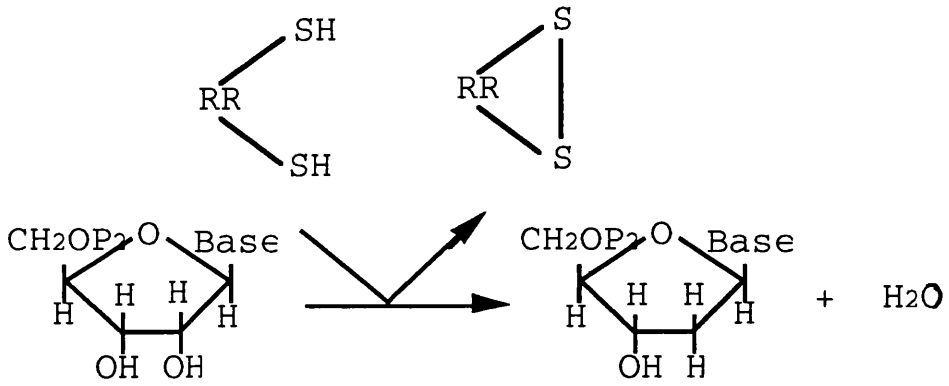


Figure 1D.1 Catalytic mechanism of ribonucleotide reductase. Ribonucleoside diphosphates are reduced to deoxyribonucleosides diphosphates by ribonucleotide reductase (RR). Reduction is mediated by the oxidation of sulphydryl groups in ribonucleotide reductase (RR-$(SH)_2$).

1D.3 Ribonucleotide reductase and virus pathogenicity.

Mutants in the HSV-1 ribonucleotide reductase large and small subunits have been used to determine the importance of this enzyme in viral replication, both lytic and latent. Its role in lytic replication in tissue culture was first determined using two temperature sensitive mutants, ts1207 containing a single point mutation in the large subunit (Preston *et al.*, 1984; Nikas *et al.*, 1990), and ts1222 which contains a frame shift in the small subunit, R2 (Preston *et al.*, 1988). The mutant ts1222 fails to produce any detectable viral specific reductase activity at both the permissive and the non-permissive temperature. In actively dividing cells the growth of ts1222 is essentially similar to that of the wt virus, in contrast, growth on serum starved, or confluent cells, is impaired severely. In dividing cells, when the cellular enzyme is active, the mutation in the HSV-1 reductase is complemented (Preston *et al.*, 1988). A similar phenotype was found for the R1 mutant ts1207 at the non-permissive temperature (Preston *et al.*, 1984). *In vivo* studies using these mutants showed that ribonucleotide reductase activity is essential for neuropathogenicity, the mutants fail to replicate following intracranial inoculation of mice (Cameron *et al.*, 1988).

Insertion and deletion mutants in the HSV-1 R1 locus have also been constructed (Goldstein and Weller, 1988a; Goldstein and Weller, 1988b). The pathogenicity of both mutants has been determined (Jacobson *et al.*, 1989; Yamada *et al.*, 1991). The deletion mutant, ICP6Δ, in which the entire R1 open reading frame is deleted was tested for its

ability to replicate in mice. Explantation of ganglia from mice infected with ICP6Δ followed by co-cultivation of the ganglia with permissive cells failed to produce any detectable virus, demonstrating the importance of ribonucleotide reductase in either the establishment, maintenance or reactivation of HSV-1 (Jacobson *et al.*, 1989). Using the the lacZ insertion mutant (Goldstein and Weller, 1988a), Yamada *et al.*, (1991) showed that this reductase null mutant could successfully establish latency, and that it was the reactivation of this virus that was specifically impaired; mice latently infected with this mutant were superinfected with HSV-2, several days after superinfection, ganglia were explanted and the number of reactivating HSV-1 insertion mutants scored by staining resultant plaques with β-galactosidase. Blue plaques were detected, indicating that the mutant viral genomes were present in the ganglia, and that reductase activity is not essential in either the establishment or the maintenance of the virus in the ganglia (Yamada *et al.*, 1991). In the guinea-pig epithelial model the R1 mutants show no growth impairment when compared to wt virus (Turk *et al.*, 1989), which raises the question of which animal model more closely mimics the human situation.

1D.4 Proteolytic cleavage of R1 in infected cells.

The large subunit of ribonucleotide reductase is proteolytic cleaved in certain cell lines during lytic replication (Ingemarson and Lankinen, 1987; Lankinen *et al.*, 1989). The cleavage of R1 in infected cells was first noted in Vero cells, full length R1 with the molecular weight 136kDa degrades to 110, 93 and 81kDa forms (Ingemarson and Lankinen, 1987). Cleavage of R1 is inhibited by the protease inhibitor N^{α}-P-tosyl-L-lysine chloromethylketone (TCLK), a serine and sulphydryl protease inhibitor. Cleavage of R1 in infected Vero cell extracts is inhibited by the addition of 3T3 cell extracts, cleavage is therefore cell line specific. Cleavage of R1 is also observed in infected BHK-21 cells, multiple bands are observed with antibodies directed against R1 (Preston *et al.*, 1984; Huszar and Bacchetti, 1983; Huszar *et al.*, 1983). Ingemarson and Lankinen, (1987) proposed that at least one cleavage product, the 93kDa, contains reductase activity: extracts in which no full length R1 could be detected, but the 93kDa form was present, contain HSV-1 specific reductase activity. Further degradation to the 81kDa product results in no detectable reductase activity. The proteolytic fragments of R1 have been mapped using a series of antisera raised against fragments of the R1 open reading frame fused to β-galactosidase (Lankinen *et al.*, 1989). Sites of proteolytic cleavage map to the region between the unique N-terminal region (Nikas *et al.*, 1986) of R1 and the C-terminal portion homologous to other R1 proteins, only antibodies which were raised against the conserved portion of R1 reacted with the 93kDa and 81kDa fragments, showing that only the N-terminal portion of R1 is removed (Lankinen *et al.*, 1989). The cleavage of the R1 to at

least one fragment, the 93kDa , does not impair reductase activity (Conner *et al.*, 1992a). Using proteases, chymotrypsin and trypsin, to cleave full length R1 expressed in *E.coli* (Furlong *et al.*, 1991) it was shown that the interaction of the C-terminal cleavage products, which lack the N-terminal region, with R2 was unimpaired and they possess full reductase activity, showing the N-terminal region of R1 does not play a role in the reductase activity (Conner *et al.*, 1992a). Conner *et al.*, (1993) mapped minimal regions of R1 required for its interaction with R2, and for its dimerisation, using randomly generated truncations of R1. Interaction between R1 and R2 requires a region in R1 between amino acids 349 and 373 and the dimerisation of R1 requires amino acids between 420 and 457, although Conner *et al.*, (1993) indicated that other regions of R1 were most likely involved in dimerisation. The dimerisation domain of HSV-1 R1 contains a stretch of leucines which could potentially form a zipper-like structure, the corresponding region in HSV-2 is also thought to play a role in R1 dimerisation (Chung *et al.*, 1991).

1D.5 HSV ribonucleotide reductase as an antiviral target

HSV-1 ribonucleotide reductase activity can specifically be inhibited by a synthetic peptide, YAGAVVNDL, this peptide represents the extreme C-terminal region of the small subunit, R2. The nonapeptide inhibits HSV ribonucleotide reductase activity in vitro (Dutia *et al.*, 1986; Cohen *et al.*, 1986), inhibition of reductase activity is specific to the HSV enzyme, no affect on the cellular enzyme was observed. Inhibition occurs by preventing the interaction of the two subunits of ribonucleotide reductase (McClements *et al.*, 1988; Darling *et al.*, 1988). By linking a photoaffinity tag to the peptide its interaction with the large subunit was demonstrated (Paradis *et al.*, 1988). The potential of this nonapeptide as a general inhibitor of all herpesvirus ribonucleotide reductases has been demonstrated (Dutia *et al.*, 1986).

1D.6 Expression of R1

1D.6.1 HSV-1

Like the immediate early genes of HSV (types 1 and 2) the large subunit of ribonucleotide reductase is expressed in the presence of cycloheximide (Watson and Clements, 1977). Expression of HSV-1 R1 is optimal in the presence of Vmw110 (Goldstein and Weller, 1988a; Sze and Herman, 1992). The R1 gene and upstream regions were transfected with the virus transactivators Vmw65, Vmw175 and Vmw110 and expression of R1 was monitored by immunofluoresence, only when the cell lines were transfected with a

construct expressing Vmw110 was expression of R1 observed (Goldstein and Weller, 1988a).

Sze and Herman, (1992) analysed R1 promoter activity using HSV-1 mutants in which the R1 promoter region was fused to the *E.coli* lacZ gene allowing expression to be monitored by determining β-galactosidase activity. To determine levels of R1 expressed at immediate early times, infections were carried out in the presence of cycloheximide the cycloheximide block was then removed and the β-gal activity produced by the R1 promoter was compared to levels of β-gal produced from a mutant in which β-gal expression was driven by the Vmw175 promoter. The β-gal activity produced by the R1 promoter were considerably lower than observed for the Vmw175 promoter, and it was therefore concluded that the R1 promoter is only weakly activated by Vmw65. Confirmation of this came from analysis of R1 expression in cell lines containing both the R1 promoter/LacZ hybrid gene and Vmw65 under the control of the metallothionein promoter. When expression of Vmw65 was induced by the addition of Zn^{2+} to the medium, only moderate increases in β-gal activity were observed. From these analyses it was concluded that the weak induction of HSV-1 R1 is mediated by a weak Oct-1/Vmw65 complex forming on the imperfect TAATGARAT motif (AATGGGAT , see Figure 1D.3).

Further investigations of R1 expression were made in the cell line containing the R1/lacZ hybrid in the absence of Zn^{2+}. Cells were infected with HSV-1 mutants in either Vmw175 or Vmw110, and the ability of these mutants to induce expression of β-gal from the R1 promoter was compared to levels produced by infection with wt HSV-1. Induction was optimal with the Vmw175 mutant, and greatly reduced in infections with the Vmw110 mutant, again showing the importance of Vmw110 in maximal R1 expression and confirming the results of Goldstein and Weller, (1988a).

```
                    SP-1                Oct-1/TAATGARAT?
HSV-1   AACGTCCTTTGGGCGGGAGGAAGCCACAAAATGCAAATGGGATACATGGA
                || ||     || ||||     | | ||||||||||||| ||||||
HSV-2   GTGTGTCTCTGAAATGGCGGAAAC--CGACATGCAAATGGGATTCATGGA
                        GA-rich
                          AP-1
HSV-1   AGGAACACACCCCCGTGACTCAGGACATCGGTGTGTCCTTTTGGGTTTCA
        ||||||||||||||||||| || ||  | ||||  |  |  ||
HSV-2   CACGTTACACCCCCCTGACTCAGGAGATAGGCATATCCTCCTTAGATTG
                                                    AP-1

                                              TATA box
HSV-1   CTGA-AACTGGCCCGCGCCCCACCCCTGCGCGATGTGGATAAAAAGCCAG
        ||  |  ||  |  ||| |||||||||||  |  |  |||| |||||||.|
HSV-2   CTCAGCACACGATCGCACCCCACCCCTGTGTGCCGGGGAT-AAAAGCCAA
```

Figure 1D.3 Comparison of the R1 promoter regions of HSV-1 and HSV-2. Elements previously identified, important in Vmw65 induction, the TAATGARAT and GA-rich motifs are shown in bold, AP-1 elements and SP-1 elements are also highlighted.

1D.6.2 HSV-2 R1 expression

HSV-2 R1 expression has been analysed by both immunofluoresence and by transient transfection analysis (Wymer *et al.*, 1989). Enhanced expression of HSV-2 R1, as analysed by immunofluoresence, was observed when plasmids expressing Vmw65 and Vmw110 were co-transfected with construct containing the entire R1 open reading frame and upstream promoter region. A region, from -533 to +103, was found to confer the response to Vmw65. Co-transfection with a Vmw110 expressing construct also increased R1 promoter activity. As observed in the analysis of expression by immunofluoresence, the R1 promoter failed to respond to Vmw175. Induction of expression from the R1 promoter is dependent on orientation. When placed in the opposite orientation the R1 upstream region fails to respond to either Vmw110 or Vmw65. In several cell lines investigated, Hep2 (human laryngeal carcinoma cells), 293 (adenovirus transformed cells) and in primary astrocytes, transactivation by Vmw65 was demonstrated; in contrast, induction by Vmw110 was found to be cell-type specific.

Wymer *et al.*, (1989) identified several cis-response elements in the R1 promoter; several SP-1 binding sites, two AP-1 binding sites, a TAATGARAT-like motif, an octamer motif overlapping the putative TAATGARAT, a single GA-rich element and a ATCGTC site,

which may bind Vmw175. Virus induced proteins were shown to interact with the R1 promoter (Wymer *et al.*, 1989). Competition retardation analysis using oligonucleotides spanning the Octamer/TAATGARAT with mutations in the Octamer, TAATGARAT, or both, demonstrated the importance of the Octamer motif in the formation of the viral-specific complexes, oligonucleotides mutated in the Octamer motif failed to compete for the virus specific factor(s). Using *in vitro* translated Oct-1 and Vmw65 the viral-specific complexes were shown to contain Vmw65 (Wymer *et al.*, 1989). Further analyses of the Octamer, TAATGARAT and GA-rich elements of the R1 promoter were made using mutants of a R1/CAT hybrid construct, allowing the contribution of each element in mediating the response to Vmw65 to be investigated, the affect of these mutations is shown in table 1D.1.

	Wild-type	Fold activation	Mutant	Fold activation
GA rich motif	GGCGGAAAC	26	GGCTTAAAC	21
Octamer motif	ATGCAAAT	26	AGCCAAAT	14
	ATGCAAAT	26	ATGCACCT	2
GARAT	AAATGGGATTC	26	AAATGGTCTTC	2

Table 1D.1 Analysis of cis-acting sequences in the HSV-2 R1 promoter

As shown in the table above, mutation of both the Octamer motif, and the GARAT element, reduce Vmw65 transactivation. The GA-rich element mutant showed only a slight reduction in activation. In the HSV-2 R1 promoter Vmw65 transactivation is mediated by a imperfect TAATGARAT element.

1D.6.3. Analysis of the AP-1 motifs in the HSV-2 R1 promoter

Within the R1 promoters of both HSV-1 and HSV-2 AP-1 sites have been identified, a single site in HSV-1 and two in HSV-2 (Wymer *et al.*, 1989). AP-1 (activator-protein 1) sites bind the cellular protein c-jun, which exists as a homodimer. C-jun has also been shown to exist as a heterodimer with c-fos, the heterodimer is a stronger activator of transcription. AP-1 elements in the HSV-2 R1 promoter are functional (Wymer *et al.*, 1992); transfections of F9 cells (essentially AP-1 negative) with the R1 promoter/CAT hybrid, and c-jun, enhanced CAT activity by 20-25 fold, and further increases were observed with the addition of c-fos in equimolar concentrations to c-jun. Gel retardation analysis using *in vitro* translated c-jun confirmed the interaction of this protein with the R1 promoter.

1D.7 The unique amino terminal region of HSV R1

HSV-1 and HSV-2 R1 are unique among the herpesviruses in that they both contain an amino terminal extension of around 300 amino acids. The function of this region has been extensively studied in HSV-2. The unique N-terminal region of HSV is shown in Figure 1D.2.

Figure 1D.2 Comparison of amino acid sequences of HSV (1 and 2) R1 with that of other large subunits. Comparisons were made using the University of Wisconsin (GCG) GAP program. The sequences of HSV-1 and HSV-2 R1 are compared to the sequences of VZV, EBV, HVS, HCMV, yeast, vaccinia (VV) and E.coli ribonucleotide reductase large subunits. Residues conserved throughout the alphaherpesviruses (HSV-1, HSV-2 and VZV) are shown in bold.

```
          1                                                  50
 HSV-1 R1  MASRPAASSP VEARAPVGGQ EAGGPSAATQ GEAAGAPLAH GHHVYCQRVN
 HSV-2 R1  MANRPAASAL AGARSPSERQ EPREPEVAPP G......... GDHVFCRKVS
  VZV R1   .......... .......... .......... .......... ..........
  EBV R1   .......... .......... .......... .......... ..........
  HVS R1   .......... .......... .......... .......... ..........
Yeast R1   .......... .......... .......... .......... ..........
   VV R1   .......... .......... .......... .......... ..........
Ecoli R1   .......... .......... .......... .......... ..........
HCMV R1    .......... .......... .......... .......... ..........

          51                                                 100
HSV-1 R1  GVMVLSDKTP GSASYRISDS NFVQCGSNCT MIIDGDVVRG RPQDPGAAAS
HSV-2 R1  GVMVLSSDPP GPAAYRISDS SFVQCGSNCS MIIDGDVARG HLRDLEGATS
  VZV R1   .......... .......... .......... .......... ..........
  EBV R1   .......... .......... .......... .......... ..........
  HVS R1   .......... .......... .......... .......... ..........
Yeast R1   .......... .......... .......... .......... ..........
   VV R1   .......... .......... .......... .......... ..........
Ecoli R1   .......... .......... .......... .......... ..........
 HCMV R1   .......... .......... .......... .......... ..........

          101                                                150
HSV-1 R1  PAPFVAVTNI GAGSDGGTAV VAFGGTPRRS AGTSTGTQ.. .........T
HSV-2 R1  TGAFVAISNV AAGGDGRTAV VALGGTSGPS ATTSVGTQTS GEFLHGNPRT
  VZV R1   .......... .......... .......... .......... ..........
  EBV R1   .......... .......... .......... .......... ..........
  HVS R1   .......... .......... .......... .......... ..........
Yeast R1   .......... .......... .......... .......... ..........
   VV R1   .......... .......... .......... .......... ..........
Ecoli R1   .......... .......... .......... .......... ..........
 HCMV R1   .......... .......... .......... .......... ..........

          151                                                200
HSV-1 R1  ADVPTEALGG PPPPPRFTLG GGCCSCRDTR RRSAVFGGEG DPVGPAEFVS
HSV-2 R1  PEPQGPQAVP PPPPPPFPWG HECCARRDAR ......GGAE KDVGAAESWS
  VZV R1   .......... .......... .......... .......... ..........
  EBV R1   .......... .......... .......... .......... ..........
  HVS R1   .......... .......... .......... .......... ..........
Yeast R1   .......... .......... .......... .......... ..........
   VV R1   .......... .......... .......... .......... ..........
Ecoli R1   .......... .......... .......... .......... ..........
 HCMV R1   .......... .......... .......... .......... ..........
```

Figure 1D.2 Continued

```
          201                                                        250
HSV-1 R1  DDRSSDSDSD DSEDTD.... ..SETLSHAS SDVSGGATYD DALDSDSSSD
HSV-2 R1  DGPSSDSETE DSDSSDEDTG SGSETLSRSS SIWAAGATDD DDSDSDSRSD
  VZV R1  .......... .......... .......... .......... ..........
  EBV R1  .......... .......... .......... .......... ..........
  HVS R1  .......... .......... .......... .......... ..........
Yeast R1  .......... .......... .......... .......... ..........
   VV R1  .......... .......... .......... .......... ..........
Ecoli R1  .......... .......... .......... .......... ..........
 HCMV R1  .......... .......... .......... ......MNPA DADEEQRVSS

          251                                                        300
HSV-1 R1  DSLQIDGPVC RPWSNDTAPL ........DV CPGTPGPGAD AGGPSAVDPH
HSV-2 R1  DSVQPDVVVR RRWSDGPAPV AFPKPRRPGD SPGNPGLGAG TGPGSATDPR
  VZV R1  .......... .......... .......... .......... ..........
  EBV R1  .......... .......... .......... .......... ..........
  HVS R1  .......... .......... .......... .......... ..........
Yeast R1  .......... .......... .......... .......... ..........
   VV R1  .......... .......... .......... .......... ..........
Ecoli R1  .......... .......... .......... .......... ..........
 HCMV R1  VPAHRCRPGR IPSRSAETET EESSAEVAAD TIGGDDSELE EGPLPGGDKE

          301                                                        350
HSV-1 R1  APTPEAGAGL AADPAVARDD AEGLSDPRPR LGTGTAYPVP LELTPENAEA
HSV-2 R1  AS...ADSDS AAHAAAPQAD VAPVLDSQPT VGTDPGYPVP LELTPENAEA
  VZV R1  .......... .......... .......... .......... ..........
  EBV R1  .......... .......... .......... .......... ..........
  HVS R1  .......... .......... .......... .......... ..........
Yeast R1  .......... .......... .......... .......... ..........
   VV R1  .......... .......... .......... .......... ..........
Ecoli R1  .......... .......... .......... .......... ..........
 HCMV R1  ASAGNTNVSS GVACVAGFTS GGGVVSWRPE SPSPDGTPSV LSLTRDSGPA

          351                                                        400
HSV-1 R1  VARFLGDAVN REPALMLEYF CRCAREETKR VPPRTFGSPP RLTEDDFGLL
HSV-2 R1  VARFLGDAVD REPALMLEYF CRCAREESKR VPPRTFGSAP RLTEDDFGLL
  VZV R1  .......... .......... .....MEFKR IFNTVHDIIN RLCQHGYKEY
  EBV R1  .......... .......... .......... .......... ..........
  HVS R1  .......... .......... .......... .......... ..........
Yeast R1  .......... ...MFVYKRD GRQEKVAFDK ITARVSRLCY GLDSDHVDPV
  VV R1   .......... ...MFVIKRN GYKENVMFDK ITSRIRKLCY GLNTDHIDPI
Ecoli R1  .........M NQNLLVTKRD GSTERINLDK IHRVLDWAAE GLHNVSISQV
 HCMV R1  VPSRGGRVSS GLSTFNPAGA TRMELDSVEE EDDFGASLCK VSPPIQATRM

          401                                                        450
HSV-1 R1  NYALVEMQRL CLDVPPVPPN AYMPYYLREY VTRLVNGFKP LVSRSARLYR
HSV-2 R1  NYALAEMRRL CLDLPPVPPN AYTPYHLREY ATRLVNGFKP LVRRSARLYR
  VZV R1  .......... .....IIPPE STTPVELMEY ISTIVSKLKA VTRQDERVYR
  EBV R1  .......... .........M ATTSHVEHEL LSKLIDELKV KANSDPEADV
  HVS R1  .......... .......... ....MSQETI ISNLIDMLKV SAGWDREANE
Yeast R1  EITQKVISGV YPGVTTIELD NLAAETAATM TTKH.PDYAI LAAR......
   VV R1  KIAMKVIQGI YNGVTTVELD TLTAEIAATC TTQH.PDYAI LAAR......
Ecoli R1  ELRSHI..QF YDGIKTSDIH ETIIKAAADL ISRDAPDYQY LAAR......
 HCMV R1  LMGKKCHCHG YWGKFRFCGV QEPARELPSD RNALWREMDT VSRHSAGLGS

          451                                                        500
HSV-1 R1  ILGVLVH... .LRIRTREAS FEEWLRSKEV ALDFGLTERL R.....EH.E
HSV-2 R1  ILGILVH... .LRIRTREAS FEEWMRSKEV DLDFGLTERL R.....EH.E
  VZV R1  CCGELIH... .CRINLRSVS METWLTSP.. ..ILCLTPRV R.....QAIE
  EBV R1  LAGRLLHRLK AESVTHTVAE YLEVFSDKFY DE..EFFQMH R.....DELE
  HVS R1  ISGRLFHKLM DMSSTETISQ YMSLFGPLLE PHILEFIQNY E.....QEID
Yeast R1  IAVSNLHKQT EKVFSTVVQQ LHDYVNPKTD KPAPMISDKI Y.....DIVM
   VV R1  IAVSNLHKET KKLFSEVMKD LFNYVNPKNG KHSPIISSIT M.....DVVN
Ecoli R1  LAIFHLRKKA YGQFEP..PA LYDHV.VKMV EMGKYDNHLL E.....DYTE
 HCMV R1  FRLFQLIMRH GPCLIRHSPR CDLLLGRFYF KANWARESRT PLCYASELCD
```

Figure 1D.2 Continued

```
          501                                                        550
HSV-1 R1  AQLVILAQAL DHYDCLIHST PHTLVERGLQ SALKYEEFYL ...KRFGGHY
HSV-2 R1  AQLMILAQAL NPYDCLIHST PNTLVERGLQ SALKYEEFYL ...KRFGGHY
  VZV R1  GRRDEIRRAI ..LEPFLKDQ YPALATLGLQ SALKYEDFYL ...TKLEEGK
  EBV R1  TRVSAFAQSP A......... YERIVSSGYL SALRYYDTYL YVGR...SGK
  HVS R1  EVCLEYRASY D......... FMCLRNCGIL PAKRFYDTYV LPPRTEMNGK
Yeast R1  KHKDELDSAI IYDRDFTYNF F......... .GFKTLERSY LL..RIDGKV
   VV R1  KYKDKLNSVI IYERDFSYNY F......... .GFKTLEKSY LL..KINNKI
Ecoli R1  EEFKQMDTFI DHDRDMTFSY A......... .AVKQLEGKY LVQNRVTGEI
 HCMV R1  ESVRRFVLRH MEDLPKLAEE TARFVELAGC WGLYAAILCL DKVCRQLHGQ
```

1D.7.1 HSV-2 R1 kinase activity

Kinase subdomains were identified within the unique N-terminus of both HSV-1 and HSV-2 R1 with varying degrees of conservation between the two (Chung *et al.*, 1989). Regions located to the C-terminal part of the unique N-terminus show greater homology to the residues described by Hanks *et al.*, (1988). Probably one of the most striking features of the N-terminal domain is the apparent lack of the lysine involved in phosphate transfer from the nucleotide binding site to the substrate. In HSV-1 no lysines are found in the region downstream of the proposed nucleotide binding site, and in HSV-2 the spacing of the lysine initially proposed (lys 177) is some 60 amino acids downstream of the nucleotide binding site, greatly exceeding the average spacing of between 14-23.

A kinase activity associated with HSV R1 was first demonstrated by Chung *et al.* (1989). Using monoclonal antibodies against HSV-2 R1 Chung *et al.*, (1989) demonstrated that kinase assays of immunoprecipitates from either infected or transiently transfected cells produced a phosphoprotein of a Mwt identical to that of R1. Confirmation that the observed phosphoprotein was HSV-2 R1 came from comparisons of phosphoproteins immunoprecipitated from cells containing a frame shift mutation in R1, only cells containing the wild-type expression vector produced the 140kDa phosphoprotein. Phosphoamino acid analysis showed that HSV-2 R1 belongs to the serine/threonine class of protein kinase. In autophosphorylation reactions serine is the favoured site of phosphate transfer. The kinase activity was shown to be a feature of the unique N-terminal region of HSV-2 R1, expression of the first 446 amino acids followed by immunocomplex kinase assay produced a phosphoprotein of 65kDa, this phosphoprotein was competed for using the peptide against which the monoclonal antibody used to precipitatewas raised. It was concluded that the phosphorylation of R1 occurs either in the absence of external factors, or that the activity phosphorylating R1 must be strongly associated.

It has been reported that HSV-2 R1 is myristylated (Chung *et al.*, 1990). Immunoprecipitation of R1 from either infected or transfected cells grown in the presence of [^{3}H] myristic acid demonstrated that R1 was labelled with [^{3}H]. The myristylation of R1 lead Chung and colleagues to propose that the HSV-2 R1 activity may associate with the plasma membrane, and that it may be related to the growth factor receptor family of protein kinases which are also myristylated. Within the N-terminus of HSV-2 R1 (and HSV-1 R1) there is a region rich in acidic amino acids. This is a feature common to the growth factor receptor kinases and is important in the control of their activity (Morrison *et al.*, 1989). Polylysine activation of growth factor receptor kinases is thought to be mediated by their interaction with the highly acidic stretches of amino acids (Gatica *et al.*, 1987; Morrison *et al.*, 1989). Increases in the kinase activity of HSV-2 R1 were observed in the presence of polylysine, but only in the presence of manganese. HSV-2 R1 transphosphorylates exogenous substrates, but only in the presence of polylysine and manganese.

Regions important in the kinase activity of HSV-2 R1 were mapped by analysis of deletion mutants of HSV-2 R1 expressed in *E.coli* (Luo *et al.*, 1991). The mutations made are shown in the table below (Table 1D.2).

Amino acids expressed	**Activity**
1-445	++
1-416	++
1-340	++

Table 1D.2 Analysis of truncations of the HSV-2 R1 N-terminal region.

Kinase assays were performed on immunoprecipitates of lysed *E.coli* extracts. The immunoprecipitates produced a phosphoprotein of a Mwt substantially smaller than predicted, 29kDa compared to the predicted 53kDa. The 29kDA product was shown to be HSV-2 R1 specific by performing competition experiments, immunoprecipitation of [^{35}S] labelled methionine protein from *E.coli* extracts was effectively competed by the addition of infected cell extracts, but not mock infected cell extracts. Assays for autophosphorylating activity and transphosphorylating activity using deletions of the N-terminus from its C-terminus showed that only the first 340 amino acids were required for activity. Investigation of conditions optimal for autophosphorylation and transphosphorylation of the 29kDa showed that manganese was absolutely required for both activities.

Mutations were introduced into the predicted subdomain I (nucleotide binding region) and II (essential lysine) to investigate the importance of these regions in HSV-2 R1 kinase activity (Luo and Aurelian, 1992). To determine if the nucleotide binding region is important, a glycine positioned in the middle of this proposed site, was converted to a proline. The role of the lysine located more than 70 amino acids downstream of the nucleotide binding region was investigated by converting this residue to a leucine. Internal deletions in the R1 open reading frame were also made; between amino acids 106-178 removing the proposed subdomains I and II (mentioned above), amino acids 86-106 removing the region proposed by Chung *et al.*, 1990 to be transmembrane, and amino acids 106-445. Mutants were assayed for autophosphorylating activity and the Km value for each determined. Both the site directed mutants autophosphorylated and their Km values were slightly increased. Deletion of residues 86-106, and 106-445, destroyed activity and the deletion removing amino acids 106-178 was greatly impaired although autophosphorylation was observed. Further analysis of this mutant showed that the activation of the kinase activity by manganese had been destroyed it was proposed that this region mediates the interaction with manganese.

1D.7.2 Intracellular localisation of the HSV-2 R1/ amino terminal region

The intracellular localisation of HSV-2 R1 from infected cells was first determined by crude subcellular fractionation (Chung *et al.*, 1989). Cells were labelled with [^{35}S] methionine and fractionated into soluble, cytoskeletal and nuclear fractions. The amount of R1 in each fraction was determined by immunoprecipitation, the majority of R1 was located within the soluble or cytoskeletal fractions and was only observed in the nuclear fraction at later times post-infection. R1 localising to purified plasma membrane fractions was demonstrated by immunoprecipitation. All samples of R1 localising to the soluble, cytoskeletal and plasma membrane fractions were shown to possess autophosphorylating activity.

Immunofluorescence showed the large subunit localising to the cytoplasm in cells infected with HSV-2 (Wymer *et al.*, 1989). Stably transformed cell lines expressing the large subunit showed R1 localising to the plasma membrane as well as the cytoplasm (Luo and Aurelian, 1992).

1D.7.3 Autophosphorylation of HSV-1 R1

Autophosphorylation of HSV-1 R1 has been demonstrated (Conner *et al.*, 1992b). Several truncations in R1 were used to localise both the site of phosphorylation, and the intrinsic

protein kinase activity. A truncation in which the amino terminal 245 amino acids were removed shows no ability to autophosphorylate, indicating that either the site of phosphorylation or part of the active site of the kinase was contained within the amino terminal 245 amino acids. Using the truncation described in section 3A (Results and Discussion), dC449, expressing the first 449 amino acids of HSV-1 R1 it was demonstrated that this region contains both the site of phosphorylation and all the residues required for autophosphorylation. Conditions optimal for autophosphorylation have been determined, activity is almost completely dependent on the presence of Mn^{2+}, a divalent cation which like Mg^{2+} interacts with residues involved in phosphate transfer, by correctly orienting the phosphate to be transferred (Zheng *et al.*, 1990). A 10-fold increase in activity is observed in the presence of the basic polypeptide protamine, the exact mechanism by which this basic polypeptide acts to stimulate activity is unknown. Both ATP and GTP can act as a source of phosphate for R1 autophosphorylation; GTP effectively inhibits ^{32}P uptake. The dual nucleotide specificity of this enzyme is a feature shared with the UL13 protein kinase of HSV-1. Non-hydrolysable ATP analogues such as 5′ adenylylimidophosphate (AMP-PNP) and 5′ fluorosulfonylbenzoyladenosine (FSBA) inhibit R1 autophosphorylation, FSBA inhibits protein kinase activity by inactivating the lysine residue involved in phosphate transfer, normally FSBA labels protein kinases at this residue (Kamps *et al.*, 1984; Russo *et al.*, 1985). FSBA inhibits the autophosphorylating activity of HSV-1 R1 but does not appear to bind to R1, this has been observed for another protein kinase, the BCR protein kinase (Maru and Wittle, 1991).

Analysis of the kinetics of R1 autophosphorylation demonstrated that R1 autophosphorylation is an intramolecular reaction, other molecules of R1 are not phosphorylated. In addition, by analysis of phosphate incorporation it was shown that there is only one site of phosphorylation (Conner *et al.*, 1992b). To determine the ability of HSV-1 R1 to transphosphorylate, exogenous substrates, as used for HSV-2 R1 (Chung *et al.*, 1989) were added to pure preparations of HSV-1 R1. Initially, histone transphosphorylation was demonstrated but further analysis showed that a truncation in which the first 245 amino acids of R1 were removed also contained transphosphorylating activity. This indicated that the transphosphorylating activity of full length R1 was most likely a contaminant. The transphosphorylating activity was separated from the autophosphorylating activity by FPLC. The transphosphorylating activity problems highlight the problems encountered in the analysis of a protein kinase even when expressed in *E.coli* and purified to apparent homogeneity. It is possible that a transphosphorylating activity is associated with the HSV-1 R1 but the substrate is highly specific or, a co-factor is required for the activity.

1E. Aims of research

The initial aim of the work presented in this thesis was to locate and mutate regions of the HSV-1 R1 large subunit which are important in the autophosphorylating activity. The autophosphorylating activity was mapped to the unique N- terminus and found to reside within the first 449 amino acids of R1. Insertional mutation of this region was carried out, it was hoped that these analyses would help to identify key catalytic regions or residues of th eautophosphorylating activity.

To directly compare the autophosphorylating activities of HSV-1 and HSV-2 R1, HSV-2 R1 was overexpressed in *E.coli* and purified to apparent homogeneity using the same purification scheme that had been determined for HSV-1 R1.

Cell lines were constructed which express the N-terminal region of HSV-1 R1 to complement mutations introduced into this region in the virus. The importance of the N-terminal region in virus replication will be determined by the construction of an HSV-1 mutant lacking this region.

Chapter 2. Materials and Methods

2A Materials

2A.1 Cells

2A.1.2 Eukaryotic cells

(a) *BHK-21 clone 13* cells (McPherson and Stoker, 1962), a fibroblast cell line derived from baby hamster kidney cells were used for the growth and assay of HSV-1 strain 17 syn$^+$.
(b) *Rat-1* cells, a rat fibroblastoid 3T3-like cell line (Botchan *et al.*, 1976) were used for both stable and transient transfections.

2A.1.2 Bacterial cells

E. Coli strain DH5 (*supE* 44,*hsdR* 17, *recA* 1,*endA* 1, *gyrA* 96, *thi*-1 *relA* 1), a recombination deficient strain (Hanahan, 1983)were used for the growth of plasmid DNA Strain JM101 (*supE, thi* Δ (*lac-proAB*) F′ [*traD* 36 *proAB*$^+$ *lac* I^q *lacZ* ΔM15] were used for the selection of recombinants into vector containing amber mutations (Messing, 1979). Expression of proteins was carried out in strain BL21(DE3) (*hsdS gal* (λ I*ts* 857 *ind* 1 *S* am 7 *nin* 5 *lac* UV5-T7 gene1), a strain containing a copy of T7 RNA polymerase carried on λ DE3 integrated into the chromosome of BL21 (Studier and Moffat, 1986)

2A.2 Viruses

HSV-1 Glasgow strain 17 syn$^+$, which forms non-syncytial plaques on BHK cells (Brown *et al.*, 1973) and the temperature sensitive mutant tsK, which has a temperature sensitive lesion in Vmw175, produces five functional polypeptides; Vmw110, Vmw63, Vmw136, Vwm68 and Vmw12 at the non-permissive temperature (39°C) (Marsden *et al.*, 1976) were obtained from department stocks maintained by Mary Murphy.

2A.3 Tissue culture medium

a) BHK cells were grown in Glasgow Modified Eagle's medium (Busby *et al.*, 1964) supplemented with 100 units/ml streptomycin, 100 units/ml penicillin, 0.002% (w/v) phenol red. The growth medium used (ETC10) consisted of 80% Eagles, 10% tryptose phosphate and 10% newborn calf serum.

b) Rat-1 cells were grown in Dulbecco's medium (DMEM, Gibco laboratories) supplemented with 100units/ml penicillin 100units/ml streptomycin, and 20mM glutamine. Foetal calf serum was added to a final concentration of 10%.

2A.4 Medium used for harvesting cells

a) PBS, which contained 170mM NaCl, 3.4mMKCl, 10mMNaHPO4, 2mM KH_2PO_4 (pH 7.2).

b) versene, PBS supplemented with 0.6mMEDTA and 0.0015% (w/v) phenol red.

c) Trypsin, 25% Difco trypsin dissolved in Tris-saline (140mM NaCl, 30mM KCl, 280mM Na_2 HPO_4, 1mg/ml dextrose, 25mM Tris-HCl (pH7.4), 0.005% (w/v) phenol red supplemented with 100 units/ml penicillin and 100 units/ml streptomycin).

2A.5 Bacterial culture medium

DH5 and JM101 strains of bacteria were grown on L-broth (10g/l NaCl, 10g/l Difco bactotryptone and 5g/l yeast extract pH 7.5). The BL21 (DE3) strain used for protein expression were grown in 2YT (5g/l NaCl, 6g/l difco Bactotryptone, 10g/l yeast extract).

2A.6 Plasmids

pET8C : plasmid carries the bacteriophage T7 Ø 10 promoter and Ø terminator, plasmid contains a unique BamH1 cloning site.

pYN1: (Nikas, 1986). The HSV-1 R1 open reading frame contained on a XhoI /HindIII fragment derived from the HindIII K fragment of the HSV-1 genome.

pRR1: (Nikas, 1986). The HSV-2 R1 open reading frame cloned on a SstI/XhoI fragment from HSV-2 strain HG52 cloned into a pUC vector.

2A.7 Reagents

Oligonucleotides were synthesised in this department by Dr. John McLauchlan using a Biosearch 8600 DNA synthesizer.

Most analytical grade chemicals were purchased from BDH Chemicals UK or Sigma Chemical Co.; ammonium persulphate and TEMED were obtained from Bio-Rad Laboratories; boric acid from Koch-Light Laboratories; cesium chloride from Melford Laboratories Ltd.; nitrocellulose paper from Scleicher and Schuell Inc.; nylon membrane (Hybond-N), ECL Western detection reagents and megaprime random priming kit from Amersham LKB Ltd; dNTPs from Pharmacia; G418 (Geneticin) from Gibco; DOTAP transfection reagent from Boehringer Mannheim; Geneclean kit from Bio 101 inc; IPTG from Promega; polylysine, protamine and histones from Sigma..

2A.8 Radiochemicals

All radiochemicals were obtained from Amersham at the following specific activities

5′ [α-^{32}P] dNTPs, 3000Ci/mmol
5′ [γ-^{32}P] ATP, 5000Ci/mmol
L-^{35}S-methionine, around 1000Ci/mmol

2A.9 Enzymes

Proteinase K, DNA polymerase holoenzyme, T4 polynucleotde kinase, T4 DNA ligase were purchased from Boehringer Mannheim; restriction enzymes were purchased from either Boehringer Mannheim or New England Biolabs; RNase A and lysozyme were purchased from Sigma; Klenow fragment polymerase was expressed by Dr. E. Telford and purified in the department. Sequenase™ T7 polymerase and sequenase kits were obtained from United States Biochemical.

2A.10 Antibodies

2A.10.1 Polyclonal and Monoclonal

Both the polyclonal and the monoclonal antibodies used were a kind gift from members of lab 106 in this institute. The polyclonal antibodies F1 and F3, prepared by fusing various regions of the R1 ORF to β–galacosidase were prepared by Dr. H. Lankinen (Lankinen *et al.*, 1993). Polyclonal antibodies 77 and 106 raised against full length HSV-1 R1 and a

truncated R1 polypeptide (AA 245-1037), were prepared by Jill Murray. The monoclonal antibody, mab1026, raised against full length R1 was also prepared by Dr. H. Lankinen (Lankinen *et al.*, 1993), the epitope for this monoclonal maps to between AA 289 and 313.

2A.10.2 Commercial antibodies

Anti-mouse-FITC conjugated antibodies and Protein-A sepharose were both obtained from Sigma immunochemicals.

2A.11 Commonly used buffers

Coomassie brilliant Bluestain	2mg/ml Coomassie Brilliant Blue in methanol:H_2O:acetic adic (200:200:28).
50x Denhardt's	1% Polyvinylpyrrolidone, 1% BSA, 1% Ficol.
5x gel loading buffer:	1x TBE, 1% SDS, 50% glycerol, bromophenol blue.
10x Mung bean buffer	300mM sodium acetate (pH 4.5), 500mM NaCl, 10mM $ZnCl_2$, 50% glycerol
2xCSK	200mM NaCL, 600mM sucrose, 20mM pipes (pH6.8), 6mM $MgCl_2$, 2mM EDTA
Denaturing solution:	0.5M NaOH, 1.5M NaCl.
Neutralising solution:	1.5M NaCl, 0.5M Tris-HCl (pH7.2), 0.001M .. EDTA.
2xHBS:	280mM NaCl, 50mM Hepes, 1.5mM Na_2HPO_4 (pH 7.1).
Hybridisation Solution:	6x SSC, 5x Denhardt's, 0.1% SDS, 50µg/ml calf thymus DNA.
2xPK	100mM Tris-HCL (pH8.0), 20mM EDTA (pH8.0), 20mM NaCl, 0.4% SDS.
RGB (resolving gel buffer):	181.5g Tris, 4g SDS, 1l H_20, (pH8.9).
SDS boiling mix	1ml SGB, 0.8ml 25%SDS, 0.5ml β-mercaptoethanol, bromophenol blue.
SDS tank buffer	6.32g Tris, 4g glycine, 1g SDS, 1l H_2O.
SGB (stacking gel buffer)	59g Tris, 4g SDS, 1l H_2O, (pH6.7).
20x SSC	174g/l NaCl, 88.2g/l Trisodium Citrate.
20x TBE	2.5M Tris, 800mM boric acid, 54mM EDTA.
TE	10mM Tris (pH 7.5), 1mM EDTA (pH8.0).
5x TBS	24.2g Tris, 292.4g NaCl, 2l H_2O (pH 7.5).
Blotting buffer (Westerns)	25mM Tris, 192mM glycine,

	20% methanol (pH8.3).
TTBS	20mM Tris, 500mM NaCL, 0.05% Tween (pH 7.5).
Blocking Solution (Westerns)	20mM Tris, 500mM NaCl, 5% marvel.
Solution 1	50mM glucose, 25mM Tris (pH8.0), 10mM EDTA (pH8.0).
Solution 2	0.2M NaOH, 1% SDS.
Solution 3	6ml 5M potassium acetate, 1.15ml glacial acetic acid, 2.85ml dH_20.
50x TAE	242g Tris, 57.1ml glacial acetic acid, 100ml 0.5M EDTA (pH8.0).

2B Methods

2B.1 Preparation of DNA

2B.1.1 Small scale 'miniprep'

An overnight culture was prepared by inoculating 2mls of LB supplemented with ampicillin (100µg/ml) with a single colony and incubating at 37°C. The overnight culture was pelleted by brief centrifugation at 15000 rpm (benchtop microcentrifuge). Pellets were resuspended in 100µl of solution 1 and incubated for 5 min on ice, 200µl solution 2 was added, carefully mixed, and incubated for a further 10 min at room temp. Finally, 150µl solution 3 was added, mixed and cell debris was pelleted by centrifugation at 15000 rpm for 15 min. Supernatants were removed and the DNA precipitated by adding 2 volumes of 100% ethanol, pellets were washed in 70% ethanol and dried under vacuum. Dry DNA pellets were routinely resuspended in 50µl of dH_2O.

2B.1.2 Large scale preparation of DNA

A starting culture was prepared by inoculating 10ml of LB-amp with a stab from a glycerol stock and incubating overnight at 37°C. The 10ml overnight culture was used to inoculate 300ml of LB-amp and grown for 6 hr; chloramphenicol was added to a final concentration of 50µg/ml and the culture was grown overnight. Bacteria were pelleted by centrifugation at 9000 rpm (Sorvall GS3 rotor) for 10 min at 4°C. Pellets were resuspended in 10ml of ice cold solution 1 containing lysozyme at a final concentration of 1mg/ml and incubated for 10 min on ice. Solution 2 was added (20mls), carefully mixed and incubated for a further 15 min at room temp. Finally, 15ml of solution 3 was added and incubated for a further 10 min on ice. The lysate was centrifuged at 15000 rpm (Sorvall SS34 rotor) to remove cell debris. Nucleic acids were precipitated from the lysate supernatant by the addition of an equal volume of isopropanol (propan-1-ol) and incubating for 30 min at room temp, followed by centrifugation at 3000 rpm (Beckman benchtop) to pellet. Pellets were drained and air dried for 30 min. The nucleic acid pellets were then resuspended in 10.5ml of dH_2O. Cesium chloride was added to a final concentration of 1mg/ml, 0.5ml of ethidium bromide (10mg/ml) was added and debris removed by centrifugation at 3000 rpm (Beckman benchtop) for 10 min. The resulting solution was centrifuged for 48 hr at 44000 rpm. Supercoiled DNA was isolated and ethidium bromide removed by several extractions with water saturated butan-1-ol. DNA

was dialysed against TE and its concentration was determined by spectrophotometry OD_{260} 1.0=50µg DNA/ml).

2B.2 Cloning methods

2B.2.1 Restriction digests

a) Vector digests

In the preparation of vector backbone the plasmid was digested using the specified manufacturers' buffers, to reduce vector background routinely 1u of calf intestinal phosphatase was added to the digest and incubated for 2 hr at the recommended temperature The phosphatase was inactivated by the addition of an equal volume of 2xPK buffer containing proteinase K (100µg/µl). DNA was then purified by 2 phenol extractions 1 chloroform extraction and ethanol precipitation (2.5 volumes ethanol, 1/10 vol 3M sodium acetate pH7.0).

b) Miniprep Digests

Miniprep digests; typically 5µl of total DNA isolated was digested with 1u of enzyme, again using the manufacturers specified buffers, RNase A was added to the digest to a final concentration of 10µg/ml to remove contaminant RNA. Digests were carried out for 1 hr at the recommended temperature.

c) Partial restriction digests

A starting mix containing routinely 15µg of plasmid DNA in a total volume of 150µl (1x concentration of restriction enzyme buffer) was aliquoted between 9 1.5ml eppendorf tubes. Restriction enzyme was added, 0.5u, to the first tube (total volume 30µl) mixed gently by pipetting, 15µl of this mix was removed and added to tube 2, again the contents were mixed and 15µl removed and added to tube 3, this was repeated till final tube. Tubes 1-9 were incubated at 37°C, tube 10 was incubated on ice, after 30 min all reactions were stopped by the addition of 0.2M EDTA. Agarose gel loading buffer was added to the digests and the partial digests were resolved on a 1% agarose gel (1x TAE). Linear bands were identified by comparing the partial digests to linear and uncut plasmid DNA.

2B.2.2 Isolation of restriction fragments from agarose gels

Slab gels of 0.8-1.5% agarose in 1xTAE containing EtBr at a final concentration of 0.5µg/ml were run submerged at up to 12V/cm. Samples were loaded in 1/5 vol loading buffer. Gels were run for 1-3 hr, till bromophenol blue almost ran off the end and examined and photographed with long-wave uv illumination. Bands were removed and

DNA eluted using geneclean. DNA was eluted from glassmilk beads into a final volume of 20µl.

2B.2.3 Blunt-ending DNA

a) End filling with Klenow

DNA cut with a restriction enzyme which produced a 5′ overhang was precipitated, dried and resuspended in 16µl of dH_2O; 2µl of the appropriate restriction buffer, 1µl of 2mM dNTPs and 0.5u of Klenow were added and the reaction incubated at room temperature for 30 min. If further restriction digestion was to be carried out the Klenow was inactivated at 65°C for 20 min and the reaction mixture was cooled before the addition of the appropriate restriction enzyme.

b) Mung bean exonuclease

DNA was resuspended in 16µl dH_2O, 2µl of 10x mung bean buffer and 20u of mung bean exonuclease were added. The reaction was incubated at 30°C for 30 min, the reaction volume was then made up to 100µl after 2.0M NaCl was added to a final concentration of 0.2M. The reaction mix was then extracted twice with phenol chloroform and ethanol precipitated.

2B 2.4 Ligations

a) 'Sticky ends'

Ligation of 'sticky' ended DNA (typically ratio insert to vector 5:1) was carried out in the manufacturers specified buffer, to improve efficiency of ligation fresh ATP was added to a final concentration of 1mM, 1u of ligase was used per ligation. Ligations were incubated at 16°C overnight.

b) Blunt ends

Ligations of blunt end DNA (typically ratio of insert to vector 1:1) was performed in the presence of polyethylene glycol 6000 which was added to a final concentration of 4%. Ligations were incubated overnight at 16°C.

c) Ligation of Oligonucleotides

Phosphorylated oligonucleotides were heated to 100°C for 5 min and left to cool to room temperature for 20 min. The oligonucleotides were added to the phosphatased vector DNA and ligated overnight at 16°C. Oligonucleotide linkers being blunt-end ligated were precipitated prior to ligation by the addition of 2 vol 100% ethanol and 1/10th vol 3M sodium acetate (pH 7.0); precipitated DNA was washed in 70% ethanol and dried by

speedvac. DNA was resuspended in dH_2O and digested with an excess of the linker site enzyme. DNA was again precipitated and ligated (in the absence of extra PEG).

2B.2.5 Preparation and transformation of competent *E.coli*

2B.2.5.1 Preparation of Competent cells

A 100ml culture was prepared by inoculating 2YT-amp with 1ml of an overnight culture of *E.coli* (routinely *E.coli* strain DH5) and grown till the OD (λ=600nm) reached 0.5 (usually between 2-2.5 hrs). Cells were pelleted by centrifugation at 3000 rpm (Beckman benchtop) for 5 min, the pellet was then resuspended in 1/2 the original culture vol of 100mM CaCl2, 1mM $MgCl_2$ and incubated for 40 min on ice. Cells were re-pelleted and resuspended in 1/50 of the original culture vol of 100mM $CaCl_2$/1mM $MgCl_2$. Cells were left on ice for 4 hr before being used for transformations. Competent *E.coli* were kept for several days at 0°C.

2B.2.5.2 Transformation of competent *E.coli*

Competent *E.coli*, 150µl, were transformed with various dilutions (1/10, 1 and 10) of the ligation mixes (vector alone, insert alone ± phosphotase(if used)), incubated on ice for 45 min, heat shocked at 42°C for 40 seconds and incubated a further 5 min on ice before being plated onto LB-amp (100µg/ml) plates. Plates were incubated at 37°C overnight.

2B.3 DNA Sequencing

2B.3.1 Preparation of template DNA

Template DNA was prepared essentially as described in 2B.1.1. Miniprep DNA was denatured by adding 4µl 2M NaOH and 4µl 2mM EDTA(pH 8.0) and incubating at 90°C for 5min. Denatured DNA was precipitated by adding 1/10th vol 3M sodium acetate (pH5.0) and 2.5 vol 100% ethanol, precipitation was carried out at room temp. After 10 mins the DNA was pelleted by centrifugation at 15000 rpm (benchtop microfuge) for 20 min. Pellets were washed in 70% ethanol and vacuum dried.

2B.3..2 Purification of primers (oligonucleotides)

Oligonucleotides were received in a concentrated ammonia solution, the oligonucleotides were heated to 55°C for 5 hr to deprotect. Ammonia wasthen removed by spinning the

oligonucleotide solutions overnight in a speedvac. Pellets were resuspended in 40µl of denaturing loading buffer (- dyes) heated to 100°C for 5 min and cooled on ice, 1/4 of the total oligonucleotide synthesised was then loaded onto a 15% denaturing polyacrylamide gel (loading buffer containing dyes was run alongside the oligo to monitor the progress) and run at 35 watts till the bromophenol blue was near the bottom of the gel. Oligonucleotides were visualised by UV shadowing, bands were isolated and the gel slice was sheared by spinning through a perforated 0.5 ml eppendorf into a 1.5 ml eppendorf. The oligonucleotide was eluted by adding 1ml of dH_2O and incubating overnight at 37°C. Polyacrylamide fragments were removed by spinning the solution through a spinex tube. The oligonucleotide solution was phenol/chloroform extracted twice and ethanol precipitated. Pellets were resuspended in dH_2O and the yield determined by measuring optical density at 260 and 280.

2B.3.3 Annealing of primer to denatured template

Denatured DNA was resuspended in 7µl of dH_2O. 2µl of annealing buffer and 2µl of primer solution (9ng/reaction) was added to the template DNA and incubated for 30 mins at 37°C. Annealed DNA was used directly for sequencing.

2B.3.4 Sequencing (T7)

Method essentially as described by Sanger, (1977). To the annealed DNA 2µl of labelling mix (20µM dGTP, 20µM dCTP, 20µM dTTP), 1µl 0.3M DTT, 10µCi [α-^{35}s] dATP, and 3u T7 DNA polymerase was added, mixed gently and incubated for 5 min at room temp. Termination mixes (15µM of each of the four ddNTP 150µM dNTPs) were aliquoted into a microtitre plate (2.5µl per well) and warmed to 37°C for 1min. Labelling reactions were then added to each of the four termination reactions and incubated for 10 min at 37°C. Reactions were stopped by the addition of 5µl of stop solution (acrylamide gel loading buffer). The reaction mixtures were heated to 100°C for 2 min prior to loading on a single strength denaturing polyacrylamide gel (8%).

2B.3.5 Electrophoresis of sequencing reactions

All sequencing reactions were run through a 8% denaturing polyacrylamide gel which had been pre-run at 70 watts prior to loading of samples. Once loaded the samples were run at 80 watts for the estimated period of time to give the sequence of interest between 50-100 nucleotides from the bottom of the gel. The gel was then removed and in the case of ^{35}-S dATP no prior fixing was given before drying.

2B.4 Southern blot analysis and hybridisation

2B.4.1 Preparation of DNA from mammalian cells

A large tissue culture flask, (750ml) ~5 x10^7 cells per flask, was washed twice with versene, cells harvested by trypsinisation. Cells were pelleted and washed twice in ice-cold PBS and resuspended in 1 vol of digestion buffer (100mM NaCl, 10mM Tris-Cl (pH 8.0), 25mM EDTA (pH 8.0), 0.5% SDS and 0.1mg/ml proteinase K). All samples were incubated at 50°C with rotation in a hybridisation oven for 16 hrs. Nucleic acids were extracted by phenol/chloroform/isoamyl alcohol extraction. DNA was then precipitated by adding 1/2 vol 7.5M ammonium acetate and 2 vol ethanol, DNA was pelleted immediately and washed in 70% ethanol. Pellets were resuspended in TE and gently shaken at 65°C to facilitate solubilisation RNA was then removed by adding 0.1% SDS and RNase A (10μg/ml) and incubating at 37°C for 1 hr.

The quality of the DNA was determined by runing a small sample on a 0.7% agarose gel, any samples which produced DNA which had a greater electrophoretic mobility than uncut phage λ were discarded. Restriction digests were carried out using 10μg DNA.

2B.4.2 Transfer of DNA onto nylon membranes

The gel was treated for 10 min with 0.2M HCl to denature the DNA. After acid treatment the gel was rinsed several times in dH_2O to remove all traces of acid and incubated for 2x 45 min in neutralising solution The gel was then placed on a piece of 3MM pre-wetted in 2xSSC on a support allowing the 3MM to contact the transfer buffer. The gel was surrounded with nescofilm to ensure that buffer can only pass through the gel. The nylon membrane cut to be slightly larger than the gel was soaked in transfer buffer (10x SSC) and placed on top of the inverted gel, two pieces of 3MM cut to the same size as the gel and soaked in 2xSSC were placed on top of the membrane. A stack of paper towels were then placed on top and weighted to improve transfer. Transfer was carried out overnight.

The nylon membrane was removed and rinsed in 6xSSC, DNA was UV crosslinked to the membrane using a Stratalinker.

2B.4.3 Preparation of random primed probe

The appropriate fragment was purified from a 1xTAE agarose gel using geneclean. The Amersham megaprime kit was used for the production of high specific activity probes.

Purified DNA, 25ng, was mixed with random primers and heated to 100°C for 5 min the mix was cooled to room temperature the three 'cold' dNTPs were added to a final concentration of 0.5 mM. The fourth dNTP was added as 50µ Ci [α^{32}-P] dCTP 2 u of Klenow was added and the reaction incubated for up to 30 min at 37°C, 1µl 0.2M EDTA (pH8.0) was added to terminate the reaction.

Unincorporated label was removed by purification of the probe through a 1ml G50 sepharose column. Samples were collected and each fraction cherenkov counted to determine position of probe elution. Samples were pooled and usually the whole probe was used for a hybridisation.

2B.4.4 Hybridisation of Southern blot

Blots were incubated with 5ml of prehybridisation solution at 65°C for 2 hr. The probe was denatured by boiling for 5 min and placed on ice. The probe was mixed with 0.5 ml of prehybridisation solution and carefully added to the hybridisation tube. Hybridisations were typically carried out overnight. Blots were then washed for 20 min in 2xSSC/0.1% SDS, 20 min in 1xSSC/0.1 %SDS and for 20 min in 0.1xSSC/0.1% SDS. Blots were air dried, wrapped in clingfilm and autoradiographed.

2B.5 Overexpression of proteins

2B.5.1 T7 Expression system

The T7 expression system is completely selective, T7 RNA polymerase recognises promoter sequences completely different from those recognised by *E.coli* RNA polymerase. In some cases (Studier and Moffat, 1985) transcription by T7 polymerase is so active that the majority of transcription in the host is carried out by the T7 RNA polymerase. The T7 expression vector chosen for expression of HSV R1 and truncations of this gene is one of the pET (plasmid for expression by T7 RNA polymerase) vector series which contains a T7 promoter (from the phage gene 10), unique cloning site and the T7 terminator sequence. A modified bacterial strain, BL21 (*F^- ompT, R_B^-, m_B^-)* a B strain, which as a B strain lacks the *lon* protease and in addition lacks the *ompT* outer membrane protease was used for protein expression. The particular derivative of BL21 used, BL21(DE3), contains an integrated copy of phage λ in which the *int* gene of the phage has been disrupted by insertion of the T7 RNA polymerase gene under the control of the UV5 promoter allowing selective induction of RNA polymerase expression upon addition of isopropyl-ß-D thiogalactopyranoside (IPTG) (Studier and Moffat, 1986).

2B.5.2 Small scale induction of protein synthesis

E.coli strain BL21 were transformed with the expression vector, colonies were picked and grown overnight in 10ml of 2YT. Glycerol stocks were made from the fresh overnight cultures and stored at -70°C. For small scale inductions a stab of the glycerol stock was used to inoculate 1ml of 2YT-amp the culture was grown for 1 hr at 37°C and the culture was used to streak a fresh LB-amp plate. The LB-amp plate was incubated overnight at 37°C to allow colonies to develop. Colonies were used to inoculate 5ml of 2YT-amp (150μg/ml) this culture was grown at 37°C till the optical density (λ=600) of the culture reached between 0.4-0.5 . IPTG was added to a final concentration of 150μg/ml to induce protein expression. *E.coli* were pelleted and resuspended in 1ml dH_2O and 0.5ml 3x boiling mix. Protein expression was analysed by Western blotting or Coomassie staining SDS-PAGE.

2B.5.3 Large scale induction of protein synthesis

As for small scale induction except the 5ml starter culture was used to inoculate 300ml of 2YT amp. Again, the culture was grown at 37°C till the optical density reached 0.5, at this point IPTG was added and the culture was transferred to 26°C. Cultures were grown for a further 2 hr post-induction. Bacteria were pelleted by centrifugation at 2000 rpm (Sorvall GS3 rotor) for 10 min at 4°C. Pellets were resuspended in 10ml of 25mM Hepes (pH 7.6)/ 1mM DTT (in some cases the protease inhibitor PMSF was added) and stored overnight at -70°C prior to lysis.

2B.5.4 Lysis of Bacteria

Lysozyme was added to the 10ml prep of whole *E.coli* to a final concentration of 250μg/ml and incubated on ice for 30 min or till lysis was obvious. The lysates were then spun at 18000 rpm (Sorvall SS34 rotor) for 20 min at 4°C Lysates were then ammonium sulphate precipitated.

2B.6 Protein purification

2B.6.1 Ammonium sulphate precipitation of protein

Ammonium sulphate was added to the desired final concentration, precipitations were carried out over a period of 30-45 min the salt being added gradually over this time. All precipitations were carried out on ice with constant stirring. Precipitates were pelleted by centrifugation at 15000 rpm (Sorvall SS34) for 20 min at 4°C. Supernatants were carefully

removed and the pellets were re-spun for a further 5 min to remove any traces of supernatant. Pellets were carefully resuspended in 1ml of 25 mMHepes/1mM DTT and stored at -70°C.

2B.6.2 FPLC

Ammonium sulphate fractions were in 1ml aliquots were spun for 1 min to remove all debris prior to loading on the column. The column was loaded in 2x500µl samples. 1ml fractions were collected and tested for protein by Western blot analysis, for further FPLC analysis all positive fractions were pooled.

2B.7 Analysis of proteins

2B.7.1 Coomassie stained gels

Protein samples were mixed with 3x boiling mix and heated to 100°C for 3 min. Samples were then loaded onto an SDS-polyacrylamide gel and run till the bromophenol blue of the boiling mix ran off the gel. Commonly 10 or 12.5% resolving gels were run. Gels were treated with Coomassie stain for 15 min and destained till the majority of background staining was removed (only protein bands being visible).

2B.7.2 Western blot analysis

Protein samples were resolved by electrophoresis and proteins were electroblotted onto nitrocellulose for 2 hr at 250 mA. The nitocellulose filter was then blocked using a 5% marvel solution (in 1xTBS) for 1 hr at room temp. Blocking solution was removed and primary antibody (typically diluted at 1/100 or 1/200) in TTBS (0.5% tween TBS solution) was added and the blot incubated for a minimum of 2 hr. Blots were then washed 2x 5 min in TTBS to remove any excess primary antibody before adding the secondary antibody: protein A-peroxidase conjugate in the case of polyclonal antibodies and an anti-mouse-peroxidase conjugate in the case of monoclonal antibodies, and incubated for 1 hr at room temp. Excess secondary antibody was removed and the blot washed 2x5 min in TTBS and 2x 5 min in TBS. Bands were visualised by adding a mixture of 100ml TBS containing 60µl of 30% H_2O_2 and 20ml ice cold methanol containing 60mg of horse radish peroxidase colour development reagent (Bio-Rad). Bands were developed over 30 min maximum.

The Amersham ECL detection method used in several blots, using this method of detection the blots were treated as above but the final TBS washes were emitted. Blots were mixed

with equal volumes of the two developing reagents for 1 min at room temp. The blot was then sandwiched between cling film at autoradiographed.

2B.7.3 Immunoprecipitation

Antigens in ammonium sulphate fractions were immunoprecipitated and analysed for kinase activity. Crude extracts were mixed with 5-10µl of primary antibody (~10 mg) and incubated for 3 hr at 4°C using an end over end rotator. Protein-A sepharose (CLB4) was added and incubation carried out for a further hour at 4°C. The protein A sepharose-antigen complex was pelleted by brief centrifugation at 1000 rpm and the supernatants removed. Pellets were washed 2x 20 min in 200µl of dilution buffer (10mM Tris·Cl (pH7.6), 150mM NaCl, 0.5% NP40) and given a final was in TS solution. An equal volume of boiling mix (1x) was added to sepharose pellets and heated to 100°C for 10 min. Eluted proteins were then analysed by SDS-PAGE.

2B.8 *I n vitro* kinase assays

2B.8.1 Partially purified protein

Fractions from FPLC columns were assayed by adding Mn^{2+} to a final concentration of 1mM. 10µCi γ^{32}-PATP was added and reactions were incubated for 20 min at room temperature. Reactions were terminated by adding 1/2 sample vol of 3x Boiling mix and heating to 90°C for 3 min. Samples were resolved by SDS -PAGE and gels were routinely Coomassie stained for 10 min and destained overnight to reduce background. Gels were vacuum dried and bands were visualised by autoradiography.

2B.8.2 *In vitro* kinase assays on immunocomplexed antigen

The protein A-sepharose bound antigen after washing several times in 25mM hepes (pH7.6) was resuspended in 50µl of kinase reaction buffer (25mM hepes pH 7.6, 1mM Mn^{2+} , 0.1mg/ml protamine and 20µCi γ^{32}-PATP) reactions were incubated for 20 min at room temperature and excess isotope was removed by washing several times in 25mM hepes. Proteins were eluted from the sepharose in 1x boiling mix and resolved by SDS-PAGE.

2B.9 Tissue Culture

Cells were routinely maintained in 750 ml flasks. Rat-1 cells when 90% confluent were sub-cultured; monolayers were washed in versene (2x20ml) and trypsinised by adding 5ml of diluted trypsin solution, trypsinisation was carried out at 37°C for 5 min. Cells were dispersed by repeated pippetting and added to 20ml of DMEM.

2B.10 Infections

2B.10.1 Preparation of stocks of infectious virus

An 80% confluent monolayer of BHK cells was seeded with 10^6 pfu of wt HSV-1 per roller bottle in 40ml of GMEM (a moi of 0.003pfu/cell) and incubated for 2-4 days until cells exhibited obvious cpe. The cells were shaken into medium and pelleted at 3000 rpm (Beckman benchtop) for 15 min at 4°C. Cell associated virus (CAV) was prepared by sonicating the pellet in 2ml of medium. Cell released virus (CRV) was pelleted from supernatant at 1200 rpm (Sorvall GSA rotor) for 2 hr at 4°C and gently resuspended in 2ml supernatant and sonicated in a sonibath. Sterility checks were performed by streaking blood agar plates and incubating at 37°C for 5 days. Virus stocks were stored at -70°C.

2B.10.2 Titration of virus stocks

Virus samples were serially diluted in 10-fold dilutions to a final dilution of 10^{-9}. Duplicate plates of BHK cells were infected with 100µl of 10^{-4} to 10^{-9}. The virus was left to adsorb for 1 hr at 37°C before the inoculum was removed and the plates were overlaid with methylcellulose medium (containing 1.5% methylcellulose) and incubated for 3-5 days at 37°C. Giemsa stain was then added to the methylcellulose medium, left for 1 hr and washed off. The plaques could then be counted and the virus titre established.

2B.10.3 Virus growth curves

Tissue culture plates (30mm) were seeded at a density of $5x10^5$ cells per plate and incubated overnight. The following day, the cells ($1x10^6$/plate) were infected with 5pfu/cell in a total volume of 200µl. The virus was left to adsorb for 1 hr at 37°C before the medium was removed. Cells were washed twice with medium (without serum) and overlayed with 1.5ml growth medium. Cell released virus was harvested at appropriate times by removing medium, pelleting cell debris and harvesting supernatant. Virus samples were stored at -70°C till virus production was analysed by titration on BHK monolayers.

2B.11 Transfections

2B.11.1 Transient transfections

Rat-1 cells were seeded at $4x10^6$ cells per 10cm plate and left overnight. The DNA solution (10μg total) was diluted with 2x HBS. DOTAP (lipofection reagent) transfection reagent was diluted in a separate vial, the DNA solution was then added to the DOTAP solution, mixed by vortexing and left at room temp for 10 min. After the 10 min incubation the solution was added carefully to the monolayers. The transfection reagent was left on cells overnight. Monolayers were washed several times in serum free DMEM and finally 10mls of medium was added to the monolayers. Cells were left for a further 48 hr before harvesting and analysis

2B.11.2 Cell lines

Rat-1 cells were transfected using the method outlined above. Once the monolayers became confluent neomycin resistant cells were selected for; 400μg/ml G418 was added to the cells and selection was continued until individual colonies appeared which could be isolated easily using a cloning ring (4mm). Individual clones were grown in multiwell plates and then expanded, neomycin was maintained in the medium throughout expansion.

2B.12 Immunolocalisation

2B.12.1 Preparation of cells

Sterile coverslips were placed in a 5cm plate and cells were seeded at 10^5/plate to give a sub-confluent covering the next day. Coverslips were removed and washed several times in PBS. Cells were fixed in 3.7 % paraformaldehyde in CSK for 10 min at room temperature. Cells were permeabilised by incubation in CSK/TritonX-100 for 15 min at room temperature.

2B.12.2 Labelling cells

Coverslips were washed several times in ice-cold PBS. The primary antibody was then added and incubated for 1hr at 37°C. Excess primary antibody was removed by washing in PBS, secondary antibody was then added and incubated at 37°C for 1 hr. Excess antibody was again removed by washing in PBS, coverslips were then mounted onto slides using 50% glycerol (in PBSA).

Chapter 3. Results and Discussion

3A Overexpression of the HSV-1 R1 unique amino terminal region and characterisation of autophosphorylating activity

The amino terminal region of HSV-2 R1 exhibits protein kinase activity (Chung *et al.*, 1989). Comparison of the unique amino terminal regions of HSV-1 and HSV-2 shows this region is the least conserved part of the HSV R1 (Nikas *et al.*, 1988; Chung *et al.*, 1989; see Figure 3A.1).

```
HSV-1   1    MASRPAASSPVEARAPVGGQEAGGPSAATQGEAAGAPLAHGHHVYCQRVN   50
HSV-2   1    MANRPAASALAGARSPSERQEPREPEVAPPG          GDHVFCRKVS   41

HSV-1   51   GVMVLSDKTPGSASYRISDSNFVQCGSNCTMIIDGDVVRGRPQDPGAAAS   100
HSV-2   42   GVMVLSSDPPGPAAYRISDSSFVQCGSNCSMIIDGDVARGHLRDLEGATS   91

HSV-1   101  PAPFVAVTNIGAGSDGGTAVVAFGGTPRRSAGTSTGTQ           T   139
HSV-2   92   TGAFVAISNVAAGGDGRTAVVALGGTSGPSATTSVGTQTSGEFLHGNPRT   141

HSV-1   140  ADVPTEALGGPPPPPRFTLGGGCCSCRDTRRRSAVFGGEGDPVGPAEFVS   189
HSV-2   142  PEPQGPQAVPPPPPPPFPWGHECCARRDAR      GGAEKDVGAAESWS   185

HSV-1   190  DDRSSDSDSDDSEDTD      SETLSHASSDVSGGATYDDALDSDSSSD   233
HSV-2   186  DGPSSDSETEDSDSSDEDTGSGSETLSRSSSIWAAGATDDDDSDSDSRSD   235

HSV-1   234  DSLQIDGPVCRPWSNDTAPL        DVCPGTPGPGADAGGPSAVDPH   275
HSV-2   236  DSVQPDVVVRRRWSDGPAPVAFPKPRRPGDSPGNPGLGAGTGPGSATDP   285

HSV-1   276  APTPEAGAFLAADPAVARDDAEGLSDPRPRLGTGTAYPVPLELTPENAEA   325
HSV-2   286  AS   ADSDSAAHAAAPQADVAPVLDSQPTVGTDPGYPVPLELTPENAEA   332
```

Figure 3A.1 Comparison of the amino acid sequences of the amino terminal regions of HSV-1 and HSV-2. Comparisons were made using the University of Wisconsin GCG Gap program, the upper sequence is HSV-1 R1 and the lower sequence is HSV-2 R1. The comparison shows that the amino terminal regions are more divergent than the rest of the protein which shows homology to the sequences of other herpesvirus ribonucleotide reductase large subunits which have been sequenced. Up to amino acid 300, the sequences show only 38% homology whereas the rest of the protein shares 90% homology.

Within the amino terminal region of HSV-2, putative protein kinase subdomains, a feature common to even the most divergent protein kinases (Hanks *et al.*, 1988), were identified.

As shown in Figure 3A.2, the conservation of these subdomains in the HSV-1 amino terminal region is less than observed for the amino terminal region of HSV-2.

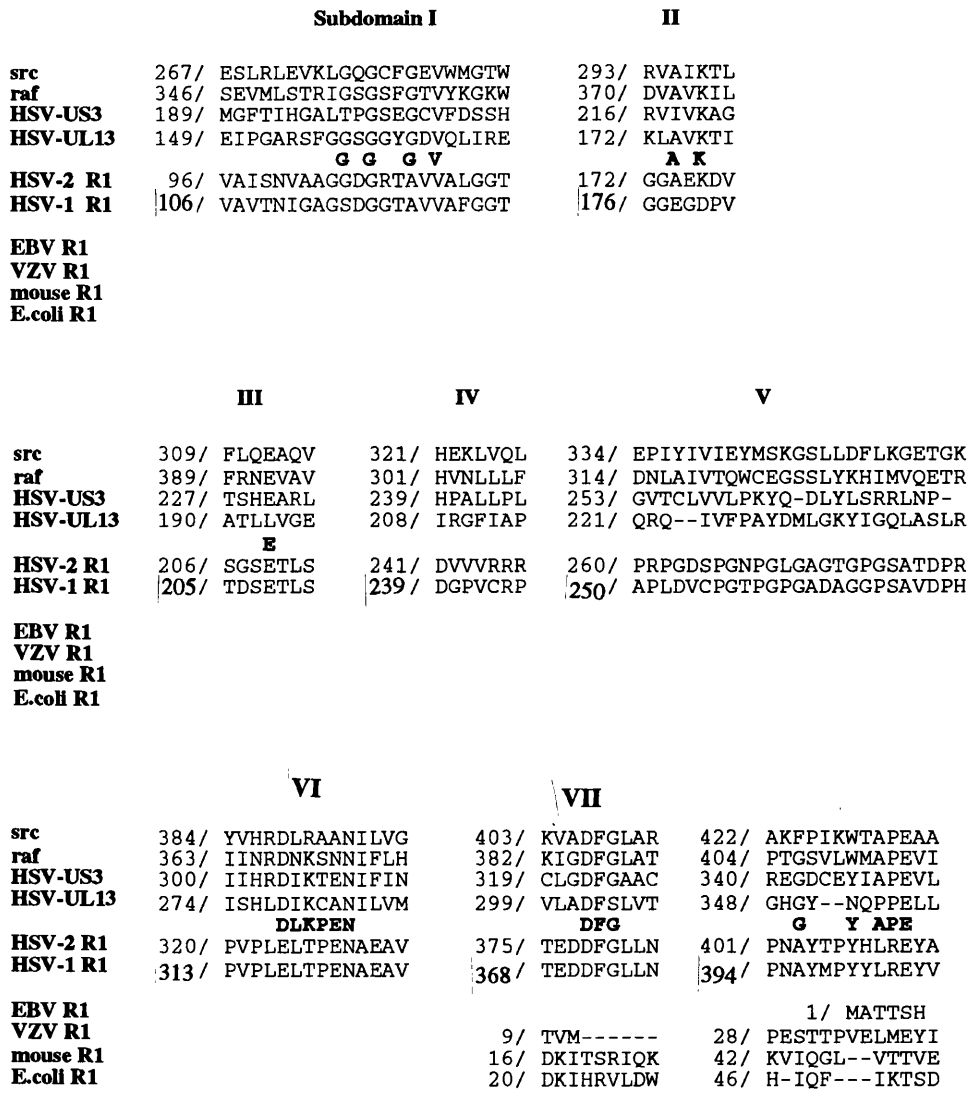


Figure 3A.2 Alignment of the amino terminal regions of HSV-1 R1 and HSV-2 R1 with other protein kinase subdomains R1 is aligned with protein kinases from both the serine/threonine kinase family (the cellular kinase raf and two other HSV-1 protein kinases US3 and UL13) and the tyrosine kinase family (src). In addition, the sequences of HSV-1 and HSV-2 R1 were compared to the sequences of other ribonucleotide reductase large subunits (of other herpesviruses, the mammalian protein and to the *E.coli* protein)

To determine if the amino terminal region of HSV-1 contained protein kinase activity, even in the apparent absence of certain conserved regions, full length R1 overexpressed in *E.coli* and purified to apparent homogeneity was assayed for protein kinase activity. The ability of pure R1 to either autophosphorylate or to transphosphorylate was determined (Conner *et*

al., 1992b). Under the assay conditions used, autophosphorylation but not transphosphorylation was observed.

3A.1 Over-expression of the amino terminal region of HSV-1

The amino terminal region of R1 from HSV-1 strain 17^+ was subcloned from a pUC based vector, pYN1, containing the entire R1 open reading frame (see Figure 3A.3).

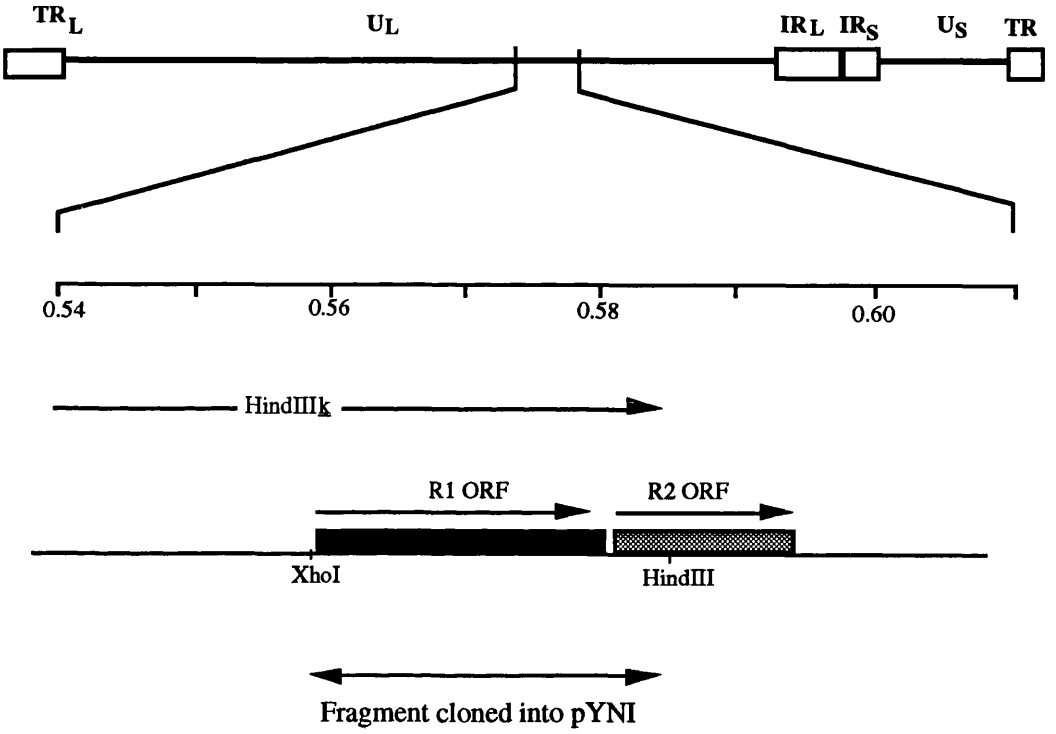


Figure 3A.3 Locus of both the large and small subunits of HSV-1 RR in the HSV-1 genome. The large and small subunit genes are both located in the unique long region of the HSV-1 genome, transcript mapping showed the mRNAs of the two subunits as 3′ co-terminal. Restriction sites used to clone the R1 open reading frame are indicated, the entire R1 open reading frame contained in the HindIII fragment k was removed using XhoI and HindIII sites located 5′ and 3′ respectively.

To determine whether the amino terminal region of HSV-1 R1 contains the intrinsic protein kinase activity (Conner *et al.*, 1992b.), a vector was constructed for the expression of a N-terminal truncation in *E.coli.* A vector containing the HSV-1 R1 open reading frame, pYN1, from the HSV-1 Hind III k fragment of strain 17^+ was initially converted to pYN1-SD a plasmid in which the Shine-Dalgarno region of *E.coli* leader sequences were placed within the optimal spacing for efficient translation from the initiating methionine of R1 (Shine and Dalgarno, 1974). The oligonucleotides inserting the Shine-Dalgarno sequences upstream of the R1 initiating methionine were inserted between a BalI site in the R1 gene located between the first and second codons and an EcoR1 site located in the pUC vector polylinker (Figure 3A.4)

The truncation in the R1 ORF was then made by digesting pYN1-SD with the enzyme BstX1, this site was then rendered blunt using mung bean exonuclease prior to digestion with HindIII (Figure 3A.4). To ensure that translation terminated at the predicted amino acid in the R1 ORF, oligonucleotides containing translational termination codons, in all three reading frames, were ligated into the BstX1/HindIII cut plasmid, the oligonucleotides contained a half HindIII site at their 3´ end ensuring they were orientated correctly.

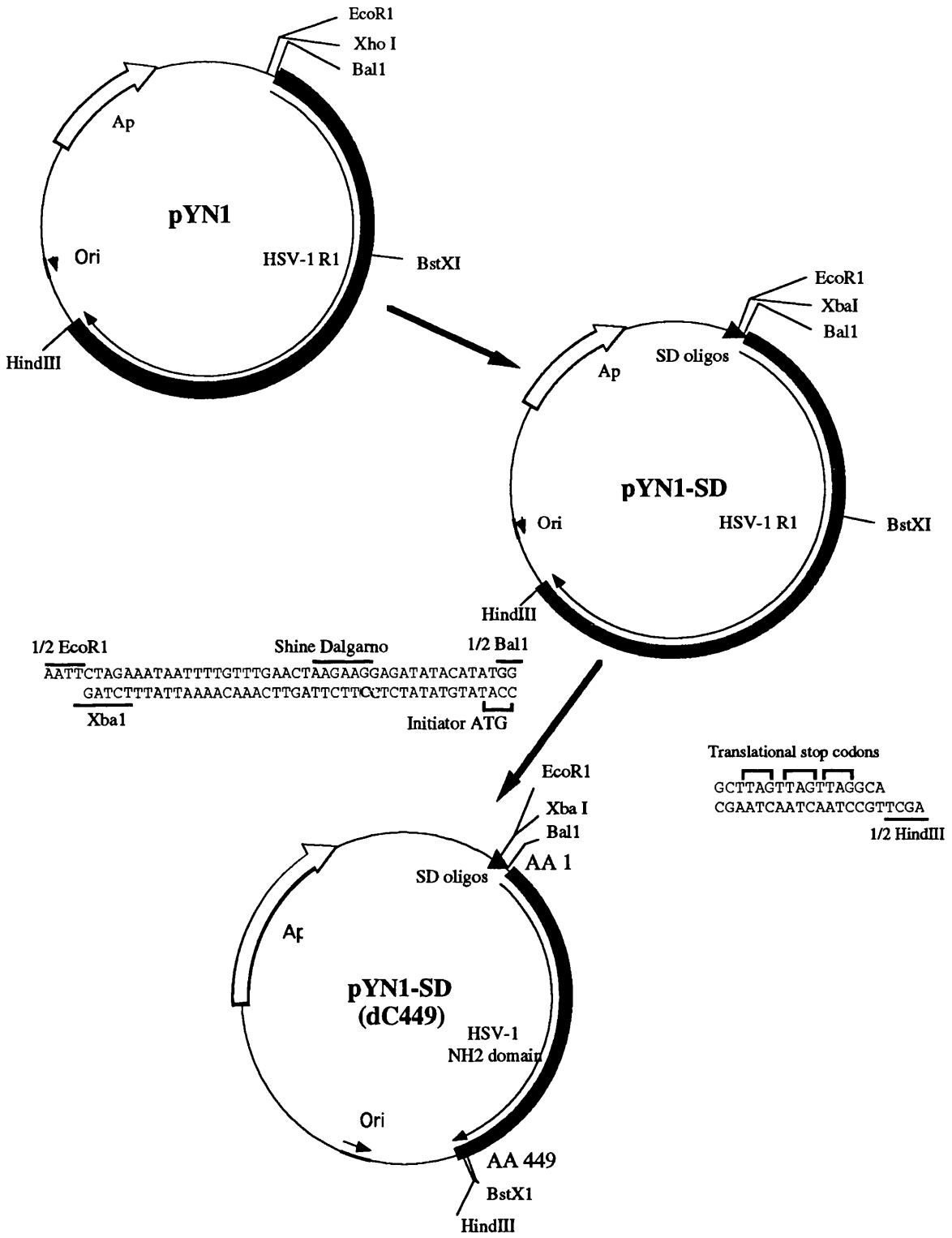


Figure 3A.4 Construction of pYN1-SD (dC449). The vector pYN1-SD (dC449) was constructed by producing an altered pUC vector pYN1-SD in which oligonucleotides containing the *E.coli* Shine-Dalgarno sequence were placed at a distance optimal for initiation of translation from the R1 initiation codon. A BstX1 site located at codon 449 was utilised to make the truncation. Both the Shine-Dalgarno and the translational termination oligonucleotides are shown.

The truncated R1 open reading frame was subcloned into a pET (T7) expression vector in which the unique BamH1 cloning site had been converted into a unique HindIII site. The unique HindIII site was used to clone the truncated R1 ORF, containing AA's 1-449 on an Xba1/HindIII fragment (Figure 3A.5).

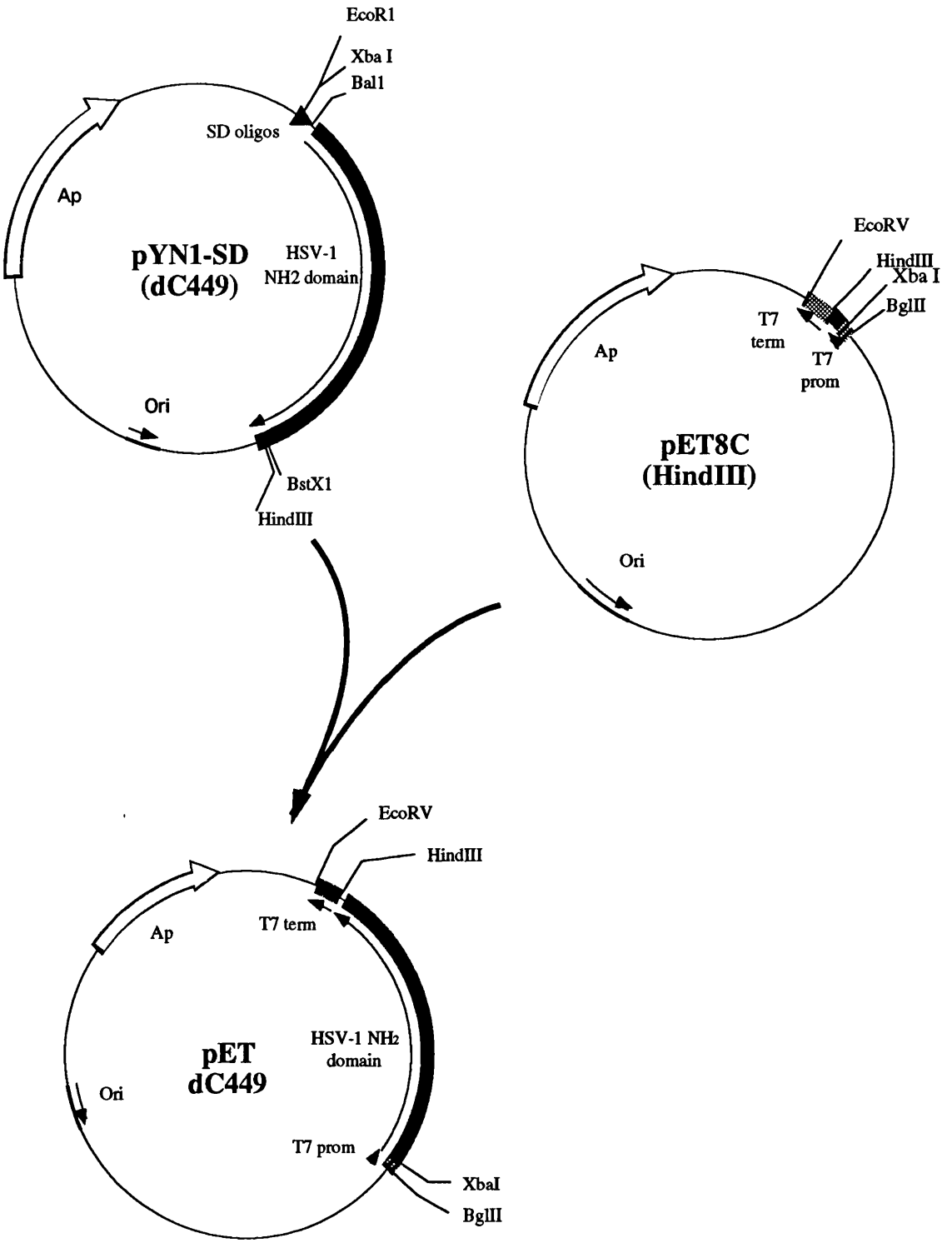


Figure 3A.5 Construction of pETdC449. The pET expression vector, pET(HindIII) was constructed by first removing the vector HindIII site by end-filling with Klenow. The new HindIII cloning site was made by end filling the BamHI site and ligating HindIII linkers. The truncation expression vector, finally pETdC449 was made by ligating the 1.4Kb Xba1/HindIII fragment of the R1 ORF from pYNI-SD(dC449) into pET(HindIII) cut with Xba1 and HindIII.

3A.2 Induction of synthesis of the HSV-1 R1 truncation

To determine whether pETdC449 expresses the amino terminal region of HSV-1 R1, a small scale culture (5ml) was grown till the O.D.(λ= 600) reached 0.5, IPTG was then added to a final concentration of 150μg/ml and the culture was grown for a further 2 hr. The induction of expression of the HSV-1 truncation can be seen in the Coomassie stained gel in Figure 3A.6 (a), in the extracts which have been induced (lane 1) unique bands are visible, the upper band indicated falls into the Mwt range predicted for the truncation (predicted Mwt ~46kDa). Confirmation that this band is the truncated HSV-1 R1 polypeptide came from Western blot analysis (Figure 3A.6 (b)). Small scale induction of single colonies of *E.coli* transformed with vector alone (lane 2) or with the vector expressing the HSV-1 truncation (lanes 3 and 4, two separate isolates) were subjected to SDS-PAGE and transferred onto nitrocellulose and analysed by Western blot. The blot was incubated with a monoclonal antibody (mAb 1026), an antibody raised against full length HSV-1 R1 whose epitope maps to an exposed loop located between amino acids 289 and 323, a region spanned by the truncated polypeptide (Lankinen *et al.*, 1993). The blot shows the appearance of unique bands in the induced *E.coli* containing pET dC449 (lanes 3 and 4 Figure 3A.6(b)). The lower band observed in the induced track in the Coomassie gel is most likely related to the amino terminal region of HSV-1 R1 as this region contains several sites for proteolytic cleavage, and as will be discussed later, this truncation is very susceptible to cleavage. The absence of the lower band in the Western blot can be explained by the loss of the monoclonal antibody epitope which maps to an unstable loop structure, that has been predicted to separate the unique region of R1 from the region at which homology to other reductase large subunits begins. Lower Mwt cleavage products will most likely lack this epitope and therefore will not be recognised by this antibody. The truncated R1 polypeptide will be referred to as dC449, this nomenclature identifies the amino acid at which the R1 open reading frame is truncated, dC indicates that the truncation was made from the carboxy end of R1.

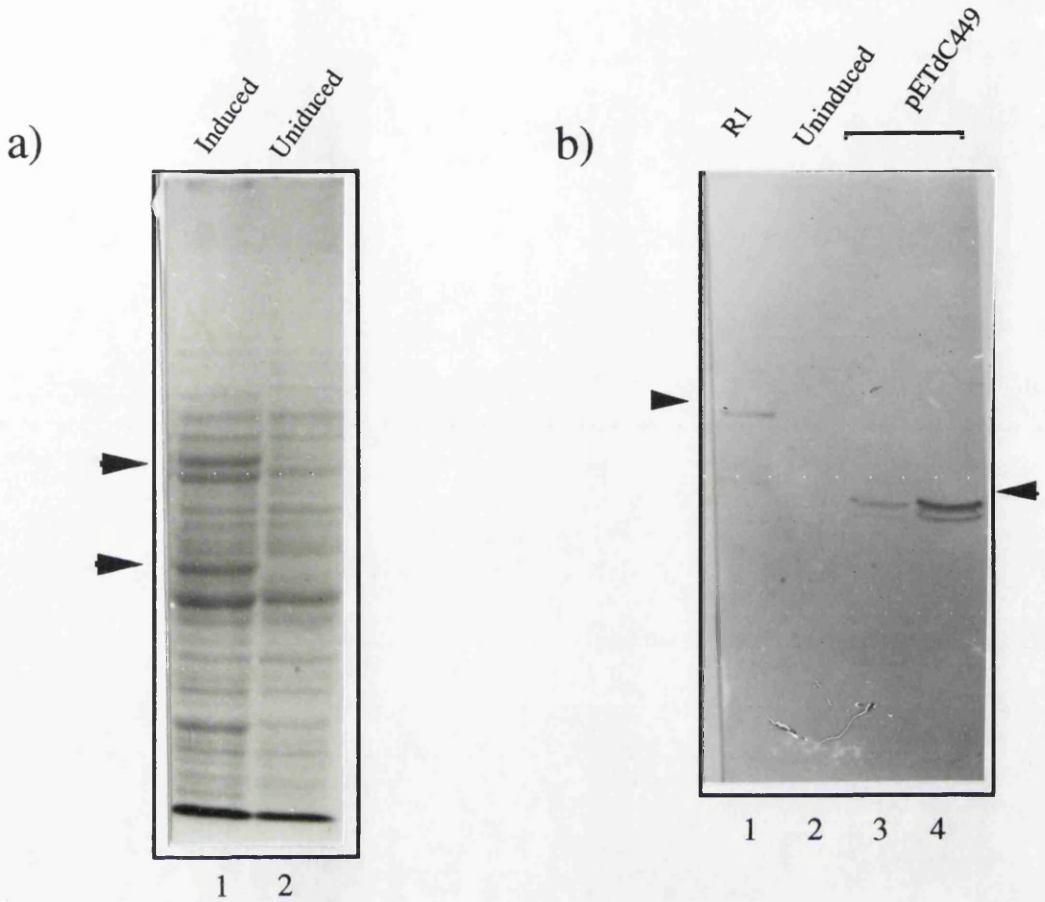


Figure 3A.6 Induction of expression of truncated R1 (first 449 amino acids of HSV-1 R1).

a) Coomassie stained gel (10%) showing the protein profiles from *E.coli* strain *BL21 (DE3)* transformed with the vector pETdC449. Lane 1 shows the profile for induced *E.coli* (IPTG added to a final concentration of 150µg/ml) and lane 2 shows the profile for uninduced *E.coli.* The bands unique to the induced extract are indicated.

b) Western blot of whole extracts of *E.coli* either uninduced (lane2),or induced (lanes 3 and 4), probed with monoclonal antibody 1026, lane 1 is the positive control, R1. The bands unique to induced extracts is indicated.

3A.3 Optimisation of induction conditions

Levels of expression of proteins in the T7 expression system vary with each protein, in particular, the length of induction and the temperature at which induction is carried out can determine the amount of protein synthesised. For full length R1, the optimal level of expression was observed at 2 hr post-induction carried out at 26°C (Furlong *et al.,* 1991) . Optimal times and temperatures for harvesting the cultures expressing dC449 were determined. Small scale cultures were induced at either 26°C or 37°C and aliquots were

removed at various time points and after the final aliquot was collected samples were subjected to SDS-PAGE, transferred onto nitrocellulose and probed with the polyclonal 106 (raised against AA245-1137 of HSV-1 R1), see Figure 3A.7. The optimal temperature for induction was found to be 26°C (blot a, Figure 3A.7), dC449 has increased stability at 26°C (lanes 4 and 5, blot (a)), whereas at 37°C after 2 hr dC449 becomes unstable (lanes 4 and 5, blot (b)). Levels of dC449 expression peak between 1 and 2 hr post-induction (compare lanes 4 and 5 of blot a, Figure 3A.7). The optimum conditions for dC449 expression are similar to those described for the full length protein (Furlong *et al.*, 1991).

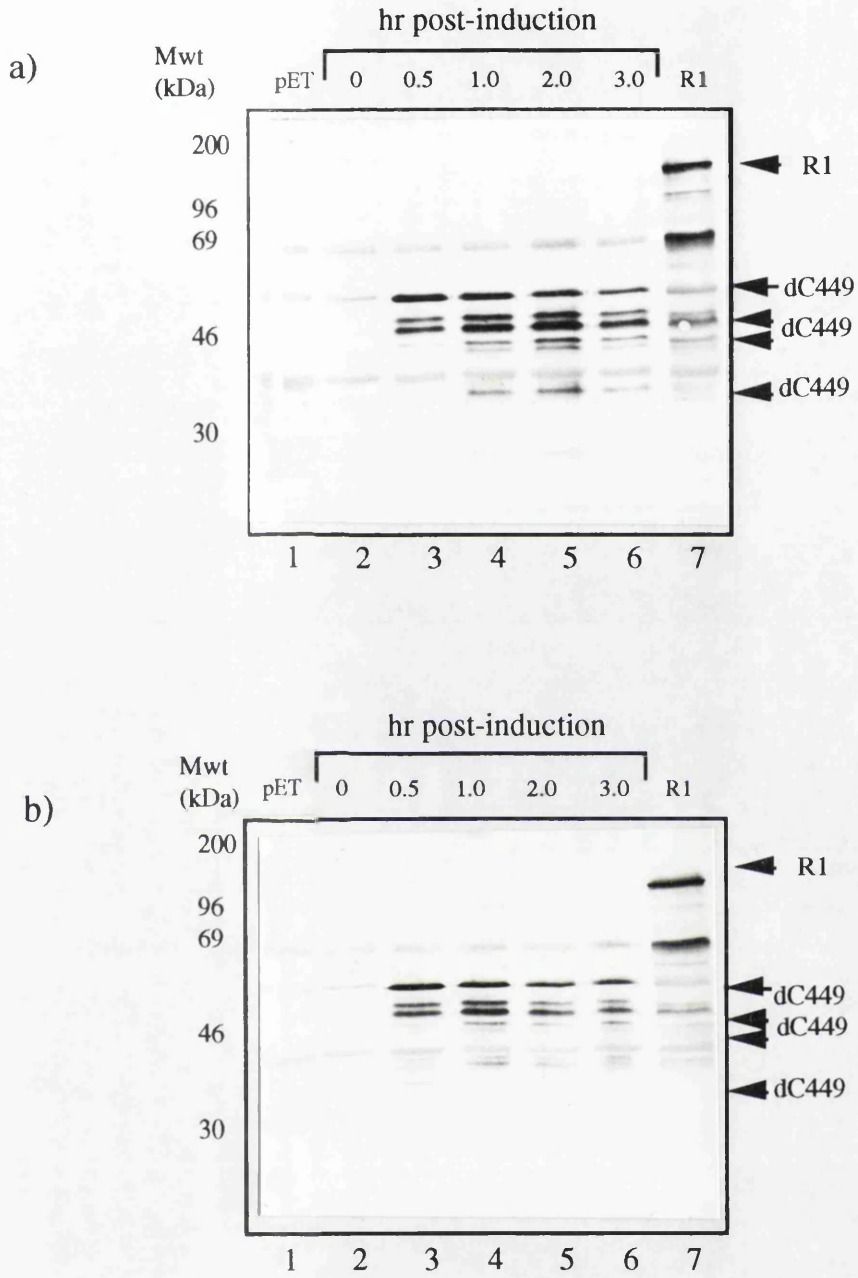


Figure 3A.7 Western blots showing optimal induction conditions. Western blots of small scale inductions, 1ml aliquots of the starting culture were removed at various times indicated after the addition of IPTG to the culture.

a) Blot of extracts from *E.coli (BL21 (DE3))* transformed with pETdC449 and induced with IPTG at 26°C, lane 2 is *E.coli* transformed with the control plasmid pET8C, lanes 2 to 6 are pETdC449 transformed *E.coli* harvested at 0, 0.5, 1.0, 1.5 and 2.0 hr post-induction and lane 7 is a positive control, full length R1, for the antisera.

b) Blot of extracts from *E.coli* induced at 37°C, lanes are as described above for the induction at 26°C. Induced bands specific to *E.coli* containing pETdC449 are indicated.

3A.4 Partial protein purification of HSV-1 truncation

The solubility of dC449 was determined by lysing *E.coli* prepared from a large scale induction (300ml culture). Samples of the lysate were subjected to SDS-PAGE, transferred to nitrocellullose and analysed by Western blot (Figure 3A.8, lane 2). Lysates were ammonium sulphate precipitated to concentrate the protein, the final concentration optimal for precipitation of the truncation was determined by precipitating aliquots of lysate from a large scale preparation. The final concentration of ammonium sulphate added for optimal precipitation was 45%, Figure 3A.8 shows the increase in precipitation (compare lanes 4, 6, 8 and 10) of dC449 with increasing ammonium sulphate.

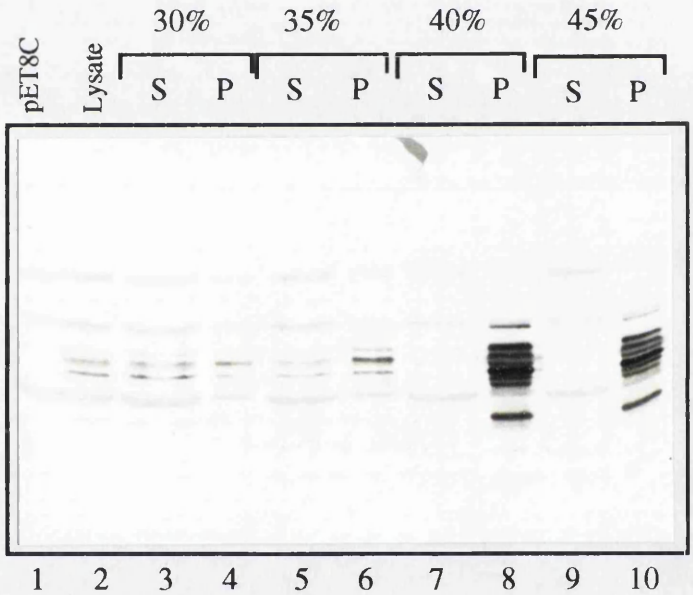


Figure 3A.8 Western blot showing solubility of dC449 and optimisation of ammonium sulphate precipitation. *E.coli* cells transformed with pETdC449 were lysed, insoluble material was removed by centrifugation and the lysate was ammonium sulphate precipitated by adding various concentrations of ammonium sulphate from 30% (lane 3) to 45% (lane 10). Precipitates (lanes 4, 6, 8 and 10) were compared to the supernatants (lanes 3, 5, 7 and 9), 5µg of protein from extracts was loaded onto a 12.5% SDS-PAGE.

Following precipitation, initial attempts at purification of dC449 used the protocol determined for full length R1 (Furlong *et al.*, 1991), the ammonium sulphate fraction was passed through a heparin Affi-gel FPLC (Bio-Rad) column. This purification method was unsuitable for the truncation as it had no affinity for this matrix. Because the amino terminal region carries a high negative charge, a mono-Q FPLC anion exchange column was used to partially purify the truncation; no further purification steps were attempted. The mono-Q elution profile for dC449 is shown by Western blot (Figure 3A.9), the blot

was probed with the N-terminus specific antibody F1, raised against amino acids 1 to 145. Elution of the truncation is first clearly observed in fraction 19 (lane 5) and peaks in fraction 23 (lane 9). Bands highlighted are the full length dC449 (the upper band) and major truncated products (two lower bands).

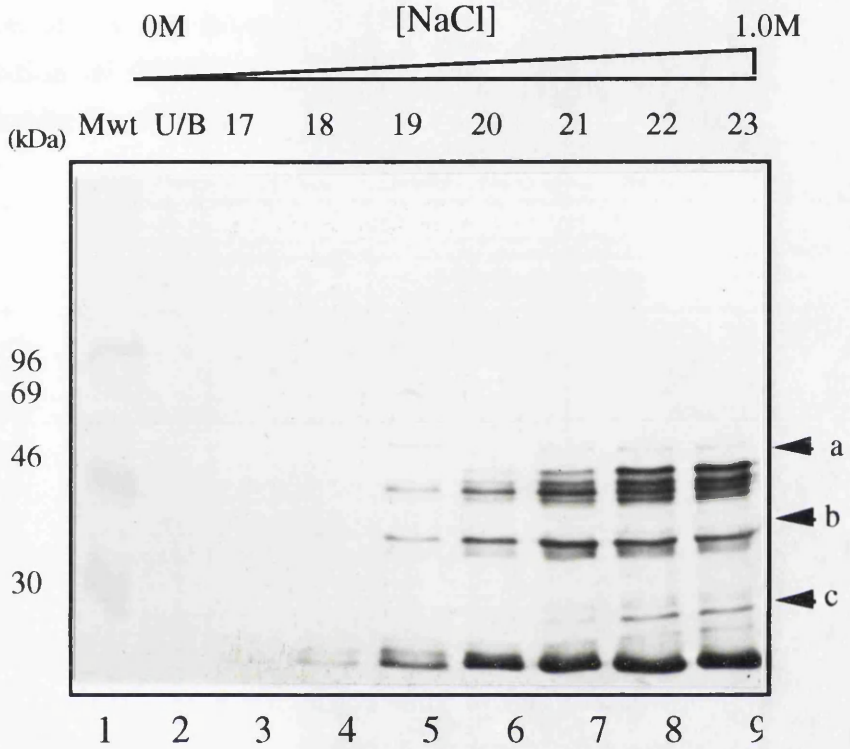


Figure 3A.9 Western blot showing FPLC purification of dC449. Ammonium sulphate concentrated protein was partially purified by FPLC using a mono-Q column. Fractions from the column were tested for the presence of the truncation : 20µl samples from each fraction were subjected to SDS-PAGE, transferred to nitrocellulose and analysed by Western blot using a polyclonal antisera (F1) raised against the amino terminal 145 amino acids of R1. Lane 2 is the unbound (flow-through) fraction and lanes 3- 9 are samples from the mono-Q run. Major bands unique to the mono-Q fractions are highlighted.

3A.5 Assay for autophosphorylation of dC449

3A.5.1 In ammonium sulphate precipitates

Initial screening for autophosphorylation of dC449 was carried out using ammonium sulphate precipitates from large scale cultures. Assays were carried out by adding manganese to a final concentration of 1mM and protamine, a basic polypeptide which stimulates the autophosphorylation of the full length protein (Conner *et al.*, 1992b), see

Figure 3A.10. The optimal ammonium sulphate concentration for precipitation dC449 was found to be 45% (Figure 3A.8). Comparison of autophosphorylation assays (Figure 3A.10) carried out on both uninduced (lane 1) and induced extracts (lane 2) shows that there are phosphoproteins unique to induced extracts which are equivalent to dC449 and two of its degradation products, previously observed by Western analysis of dC449 extracts, shown in Figure 3A.9. Contaminating bands are visible in uninduced extracts, one band co-migrates with band b (lane 2), explaining the higher level of autophosphorylation observed in the induced lane where the contaminant *E.coli* band and dC449 co-migrate. Although phosphorylation of dC449 can be observed in crude ammonium sulphate fractions contamination by *E.coli* kinases is a problem.

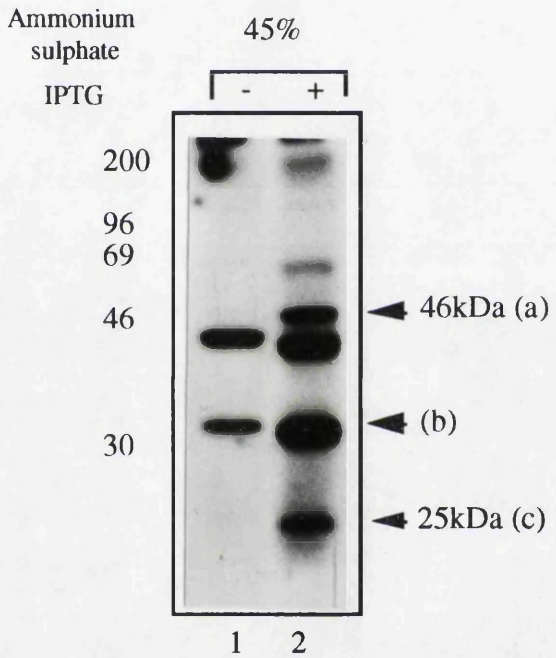


Figure 3A.10 Autoradiograph of *in vitro* kinase assays of crude ammonium sulphate fractions. Large scale preparations of induced and uninduced *E.coli* transformed with pETdC449, were lysed and ammonium sulphate precipitated. Precipitated protein was resuspended in 25mM Hepes pH7.6. Equal amounts of precipitated protein (5µg) were assayed for autophosphorylating activity by adding Mn^{2+} (0.5mM), protamine (0.1mg/ml) and 10 µCi γ^{32}PATP. Lanes 1 shows the phosphoprotein profile for uninduced *E.coli* precipitated with 45% ammonium sulphate and lanes 2 shows the phosphoprotein profile for induced *E.coli* precipitated with 45% ammonium sulphate. Bands related to dC449 are indicated.

3A.5.2 In partially purified mono-Q fractions

In addition to Western blot analysis, to determine the position of dC449 autophosphorylating activity eluting from the mono-Q column, kinase assays were performed on the fractions shown to contain dC449 by Western analysis. Equal volumes

(20µl of a 1ml fraction) of each fraction were assayed for their ability to autophosphorylate. As described in 3A.5.1, assays were carried out in the presence of 1mM manganese and 0.1mg/ml protamine. Figure 3A.11 shows the increase in a major phosphoprotein (band a), activity peaking at fraction 20 (lane 6). Lower Mwt bands (b and c) are also observed, although the level of autophosphorylation is low in comparison to band a, the peak elution activity of these bands differ from that of band a; peak elution of b is observed in fractions 18 and 19 (lanes 4 and 5) and, peak elution of band c is observed in fractions 19 to 21(lanes 5 to 7). A higher Mwt phosphoprotein, previously observed in crude ammonium sulphate fractions co-elutes with dC449, and its phosphorylating activity peaks in the same fraction as full length dC449 (fraction 20, lane 6). The full length R1 sample used as a control in this assay has undergone severe proteolytic cleavage most likely due to several freeze-thaws; storage at -70°C rapidly increases R1 degradation (Ingemarson and Lankinen, 1987). Proteolytic cleavage products of R1 which co-migrate with those produced by dC449 degradation are observed (most obvious with bands a and b, although in the R1 sample band a migrates slightly faster because band a in dC449 extracts represents the full 449 amino acid R1 truncation). The autophosphorylation activity of dC449 does not therefore appear to directly correlate with the fraction at which optimal elution of the truncation is observed as determined by Western blot analysis (Figure 3A.9). Assays were carried out on untreated fractions, the final concentration of NaCl in each fraction may affect the activity of the truncation in an autophosphorylation assay; full length R1 autophosphorylation is inhibited by NaCl at concentrations greater than 250mM.

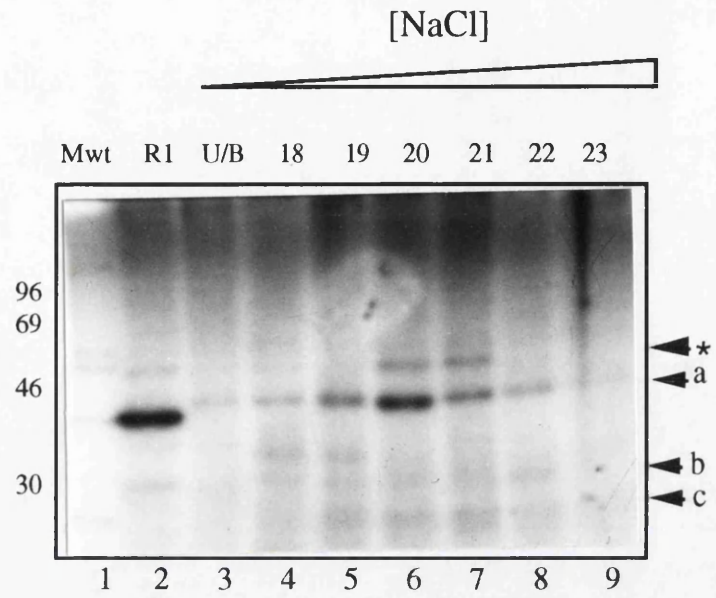


Figure 3A.11 Autoradiograph showing kinase assays of mono-Q fractions. Lanes 3 to 9 are fractions from an FPLC mono-Q column assayed for kinase activity in the presence of Mn^{2+}(0.5mM) and protamine(0.1mg/ml). Lane 3 is a sample from the unbound fraction and lanes 4 to 9 are fractions 18 to 23 from the column and represent the fractions across which the elution of the truncation is observed by Western blot analysis. Samples are compared to a pure preparation of HSV-1 R1 in lane 2. Bands which represent the autophosphorylated dC449 and its cleavage products are indicated (a to c).

Confirmation that phosphoproteins observed above are related to dC449 came from a comparison of Western blot analysis of FPLC fractions with kinase assays carried out on immunocomplexed dC449 from fractions spanning the peak elution fraction. Bands which react with the antisera 106 (raised against amino acids 245 to 1137) in the fractions (fractions 22 to 28, lanes 4 to 10 Figure 3A.12(a)) are also phosphorylated in the immunocomplex kinase assay (lanes 5 and 6, Figure 3A.12(b)). The autophosphorylating activity of dC449 correlates with its elution profile, the greatest level of autophosphorylation is observed in the peak elution fraction, fraction 24.

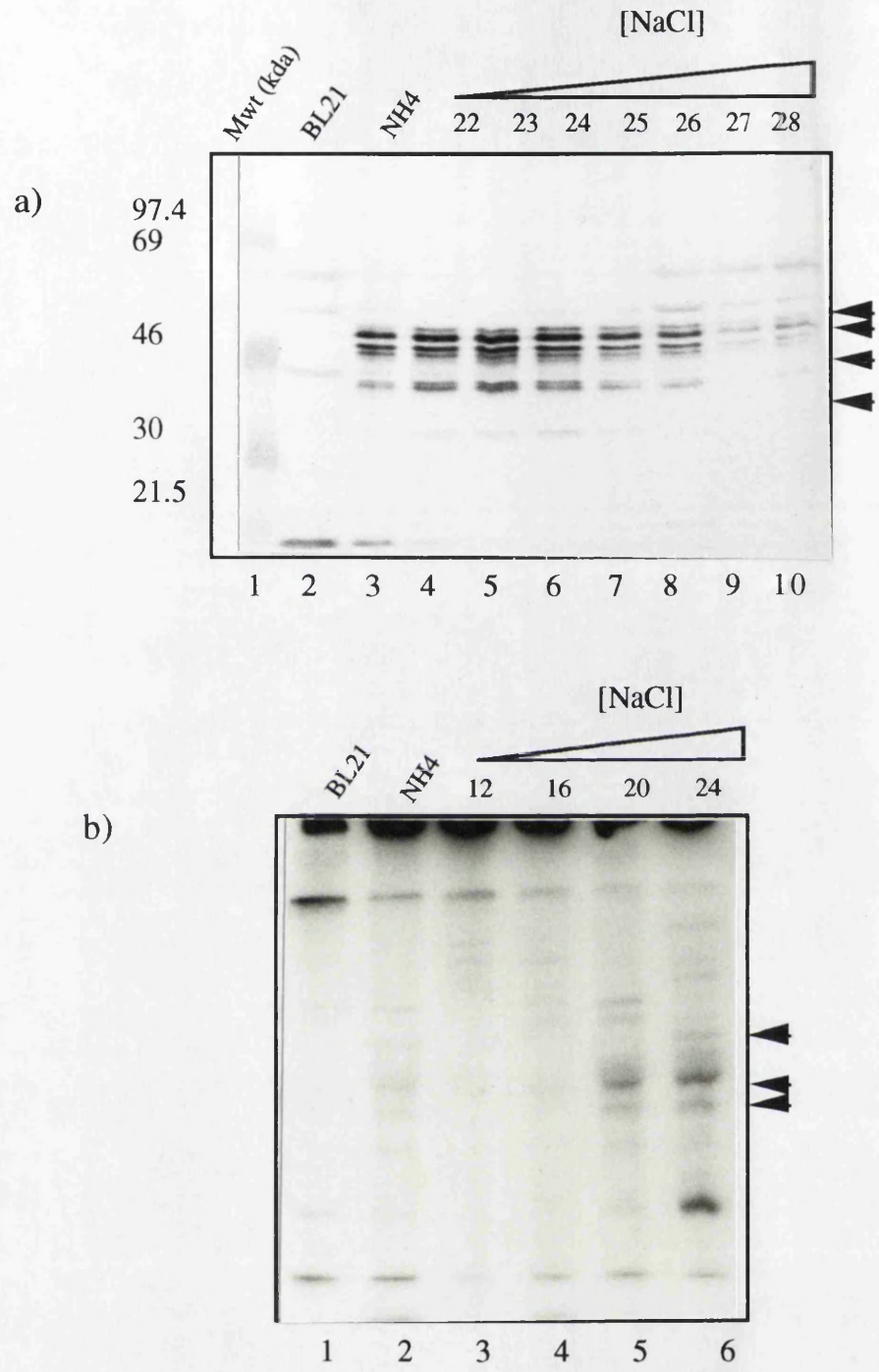


Figure 3A.12 Western blot and autoradiograph comparing dC449 positive FPLC fractions with phosphoproteins produced in an immunocomplex *in vitro* kinase assay

Fractions from an FPLC mono-Q run were analysed for peak elution of dC449 by Western blot assay of fractions

a) Shows Western blot using polyclonal antibody 106, FPLC fractions (lanes 4 to 10) are compared to the ammonium sulphate fraction (lane 3) and to extracts from *E.coli* containing the control plasmid pET8C (lane 2). Fractions were also assayed for their ability to autophosphorylate by immunocomplex kinase assay

b) Shows the autoradiograph of such an assay of Mono-Q FPLC fractions (lanes 3 to 6, fractions 12, 16, 20 and 28), on ammonium sulphate fractions from dC449 expressing *E.coli* (lane 2) and from *E.coli* transformed with the control plasmid pET8C (lane 1).

3A.6 Discussion

The first 449 amino acids of HSV-1 R1 autophosphorylate showing that this region contains both the intrinsic protein kinase activity of HSV-1 R1 and the site of autophosphorylation. This region,dC449, when expressed in *E.coli* is proteolytically unstable. Several bands unique to induced extracts are observed in Western blots of fractions eluted from a mono-Q anion exchange column. Cleavage is a feature of HSV-1 R1, occurring in infected cells (Ingemarson and Lankinen, 1987). Sites of proteolytic cleavage have been mapped, all were found to be located within the amino terminal 1/3 of the protein (Lankinen *et al.*, 1988; Conner *et al.*, 1992a). Cleavage of the R1 molecule does not affect the reductase activity (Conner *et al.*, 1992a), demonstrating that the amino terminal region plays no role in the *in vitro* reductase activity. That the truncation dC449 expressed in *E.coli* was unstable was not therefore a surprise. Protease inhibitors used to reduce cleavage were ineffective suggesting that, as found for an HSV-2 amino terminal truncation expressed in *E.coli* (Luo *et al.*, 1991), that cleavage occurs *in vivo*. The cleavage sites located within the amino terminal region of HSV-1 R1 which have been mapped (Conner *et al.*, 1992a) are shown in Figure 3A.13.

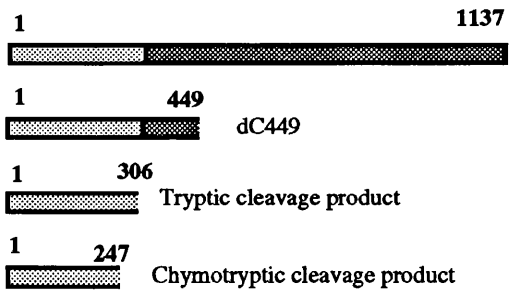


Figure 3A.13 Proteolytic cleavage sites in HSV-1 R1. The unique N-terminus of HSV-1 R1 is shown as the lighter shaded areas. Sites of cleavage with respect to dC449 are shown.

Three main, novel, phosphoproteins are observed in *in vitro* kinase assays of crude extracts from *E.coli* expressing the amino terminal 449 amino acids of HSV-1 R1; the full length 51kDa, and two cleavage products of 45kDa and 25kDa respectively. Both the full length dC449 and the 25kDa cleavage products are obvious in ammonium sulphate extracts, whereas further mono-Q purification is required to clearly observe the 45kDa cleavage product, which in ammonium sulphate fractions is probably co-migrating with an *E.coli* phosphoprotein. Problems encountered with contaminating *E.coli* phosphoproteins in

kinase assays of ammonium sulphate fractions were overcome by both FPLC fractionation and immunoprecipitation.

Comparisons of phosphoproteins produced from fractions assayed from an FPLC column with the same fractions analysed by Western blot analysis showed that the peak phosphoprotein fraction differed from the peak elution fraction. That the phosphoproteins are related to the dC449 truncation and its cleavage products was shown by assaying immunocomplexed polypeptides from the fractions spanning the peak elution fraction. The activity was greatest in the peak fraction, differences observed by the two methods of analysis can in part be explained by the different assay conditions. In an immunocomplex kinase assay the proteins are assayed in a buffer containing equal concentrations of manganese and salt, unlike crude assays in which the salt concentration is varied across the gradient used to elute the proteins. Exact kinetic analysis and comparison of the dC449 truncation with the full length HSV-1 R1 was attempted but problems were encountered in the quantification of the truncation because of its impurity and its instability.

3B Mutagenesis of the HSV-1 amino terminal region

3B.1 Introduction

Little is known about the regions of the R1 amino terminal domain which are critical for the kinase activity. Regions of potential importance were first identified using amino acid sequence comparison by Chung *et al.*, (1989). However, data from this group suggest that the regions initially thought to be important for substrate binding, in particular the putative nucleotide binding region, when mutated did not significantly reduce HSV-2 R1 autophosphorylating activity (Luo and Aurelian, 1992). To locate possible regions of importance for the HSV-1 activity, mutants of the N-terminal region were constructed.

3B.2 Construction of mutations

Mutations throughout the amino terminal region were made by inserting a 12 base pair oligonucleotide (pCCC**GAATTC**GGGp), containing an EcoR1 site, into blunt restriction sites located throughout the amino terminal 449 amino acids. The wild type expression vector, pETC449, contains an EcoR1 restriction site located in the vector backbone in a non-essential region; this site was destroyed by end filling with Klenow. Partial digests were made in the EcoR1-negative plasmid using three different restriction enzymes; NaeI, RsaI and SmaI. The three blunt restriction enzymes chosen have several recognition sites in the 1.4Kb fragment of the R1 truncation Partial digestion was carried out as described in Materials and Methods. Digests were resolved through a 1% Tris-acetate agarose gel, linear bands were isolated, gene cleaned and treated with calf intestinal phosphatase to reduce vector background. Oligonucleotides were annealed, phosphorylated and ligated into the linearised plasmids. Sites of insertion into the R1 open reading frame are summarised in Figure 3B.1.

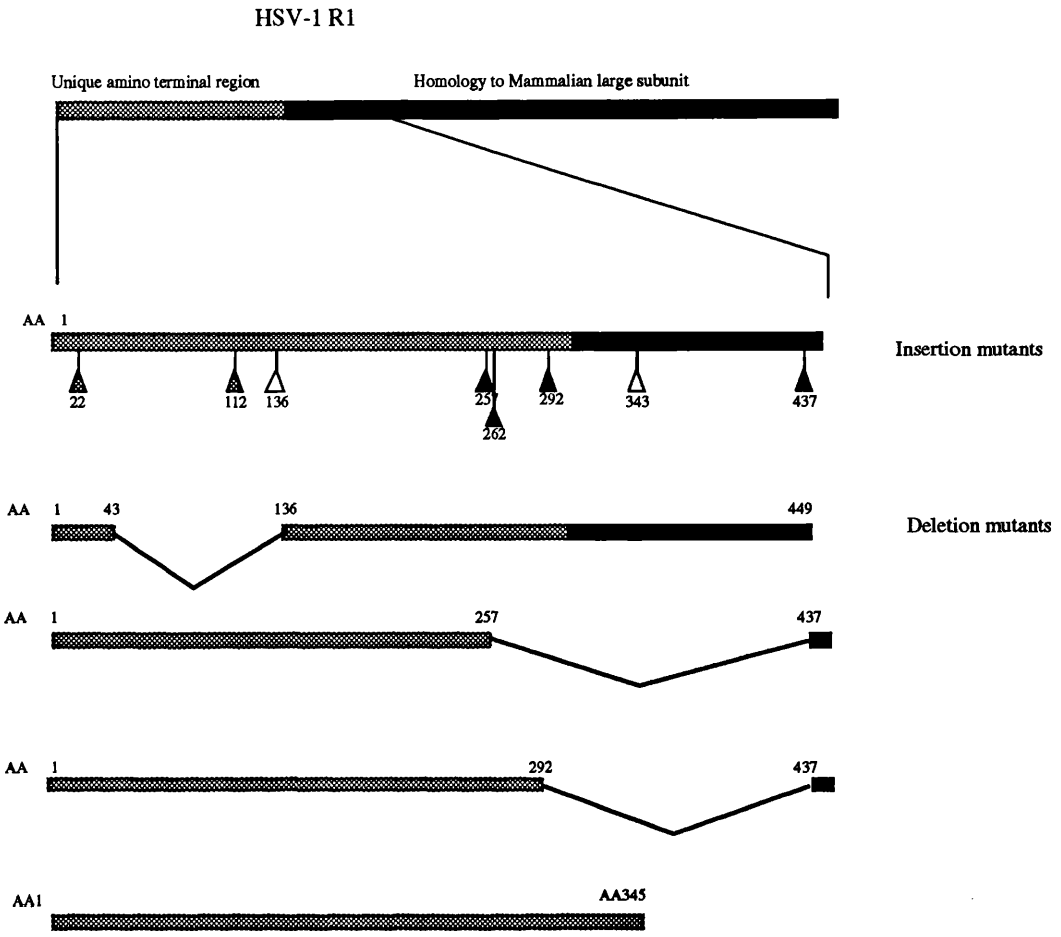


Figure 3B.1 Location of insertion/deletion mutants in the unique amino terminal region of HSV-1 R1. Amino acid residues at which oligonucleotides were inserted are indicated by triangles, the restriction site used to create the insertion is colour coded for each triangle. Insertion into NaeI sites are shown as grey triangles; into RsaI as white triangles and into SmaI sites as black triangles.

Clones containing insertions were screened for by restriction digestion with EcoR1. The site of insertion was crudely mapped by double digestion with XbaI/EcoR1 or HindIII/EcoR1, and the exact location of the insertion confirmed by double-stranded dideoxy DNA sequence analysis.

3B.3 Expression of insertion and deletion mutants

Insertion and deletion mutant plasmids were transformed into *BL21 (DE3)* and colonies expressing mutants were screened for by small scale induction. Large scale preparations of each mutant were lysed and proteins were concentrated by ammonium sulphate precipitation. The level of expression of each mutant was then assessed by Western blot analysis of equal amounts of ammonium sulphate precipitated protein. Levels of expression of each mutant varied, with the lowest expression level observed for the

insertion mutant In112 (Figure 3B.2(a), lane 4), expression of the other insertion mutants is comparable to levels observed for dC449 (lane 2). Expression of the deletion mutants, also compared to dC449 (Figure 3B.2 (b)), was also varied. The truncations have an increased stability compared to dC449, with fewer cleavage products observed, compare lanes 4 and 5 with lane 2. The antibody used to analyse the expression of the truncations was raised against an R1 truncation in which the amino terminal 245 amino acids had been deleted, the expression of dl257 appears to be very low, with only a weak band being apparent, this deletion must therefore lack most of the epitopes for the antibody used and it is possible that the amount of protein expressed is not accurately determined by Western analysis with this particular antibody.

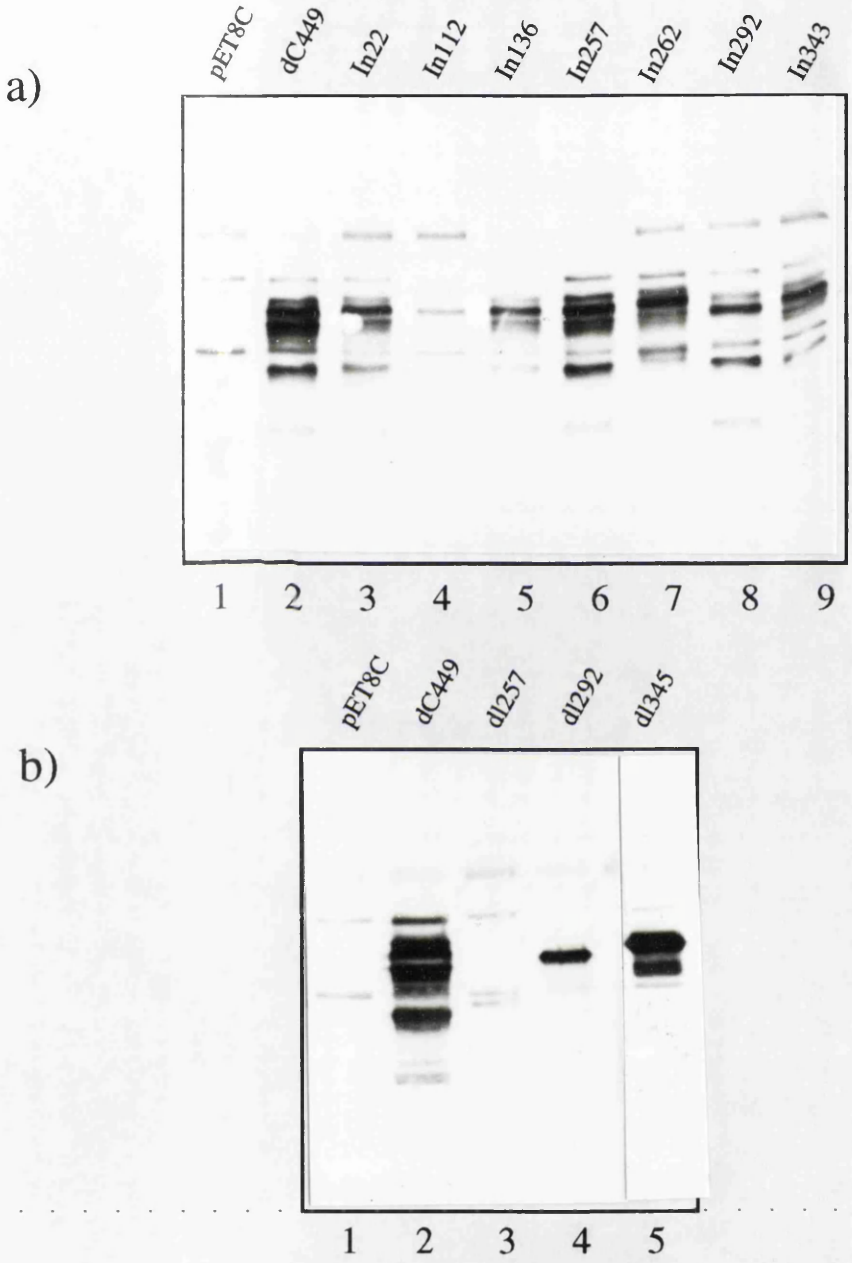


Figure 3B.2 Expression of insertion and deletion mutants. Levels of expression of both insertion and deletion mutants were compared by Western blot analysis of ammonium sulphate fractions, equal final concentrations of protein, 3μg, were resolved by SDS-PAGE, transferred onto nitrocellulose and probed with the polyclonal antibody 106

a) Shows Western blot of insertion mutants lanes 3 to 9 were compared to that of dC449 (lane 2) and lane 1 is *E.coli* transformed with the control vector pET8C.

b) Shows Western blot analysis of deletion mutants, lanes 3 to 5, compared to dC449 (lane 2), lane 1 as in (a).

3B.4 Developing an assay for analysis of mutants

3B.4.1 Immunoprecipitations

To easily assay the mutants, a method for rapid determination of kinase activity was developed. Several polyclonal antibodies raised against HSV-1 R1 were screened for their ability to immunoprecipitate full length R1 and truncations of R1. The antisera chosen for precipitations, antibody 106, was raised against a truncation of R1 in which the first 245 amino acids are removed, the epitopes for this antibody will all therefore map downstream of amino acid 245, the interaction between this antibody and the mutants is unlikely to affect the autophosphorylation reaction.

Conditions for precipitation were optimised using full length R1; varying concentrations of antibody, from 5 µl to 50 µl were incubated with ammonium sulphate precipitated fractions of R1 and the ability to immunoprecipitate R1 was then assessed by Coomassie staining an SDS-PAGE (10%) of the precipitates (shown in Figure 3B.3). Figure 3B.3 shows that immunoprecipitation of full length R1 using the polyclonal antibody 106 is optimal with as little as 10µl (compare lane 7 with 8 to 10 in 3B.3(a)) of the antibody serum. With 5µl of antibody, R1 precipitation is observed, but levels are not optimal when compared to other Ab concentrations (compare lane 6 with lanes 7 to 10). By limiting the amount of antibody in immunoprecipitations, the amount of antigen precipitated will be less than optimal for the amount of antigen added, this allows differences in the levels of the mutants expressed to be compensated for, as in most cases the antigen should always be in excess. Because of the lack of a suitable purification scheme for the N-terminus the immunoprecipitation assay is the most accurate way to determine the affect of the mutation on the ability of this region to autophosphorylate.

In addition, the ability of the polyclonal antibody to precipitate a truncated form of R1, dN245, which lacks the amino terminal region (deleted from amino acids 1 to 245), was determined.* This particular deletion shows no ability to autophosphorylate (Conner *et al.*, 1992b) and can therefore act as a control in the immunoprecipitation/kinase assay. Precipitation of the truncation in the immune serum is shown in Figure 3B.3 (b) (see lane 9), the amount of protein precipitated from the 33% ammonium sulphate fraction is comparable to the amount of R1 precipitated from the ammonium sulphate R1 fraction (lanes 6 and 7).

* The deletion mutant dN245 is precipitated by pre-immune serum, this is probably due to a non-specific interaction with the sepharose matrix to which protein A is coupled (J. Conner, personal communication).

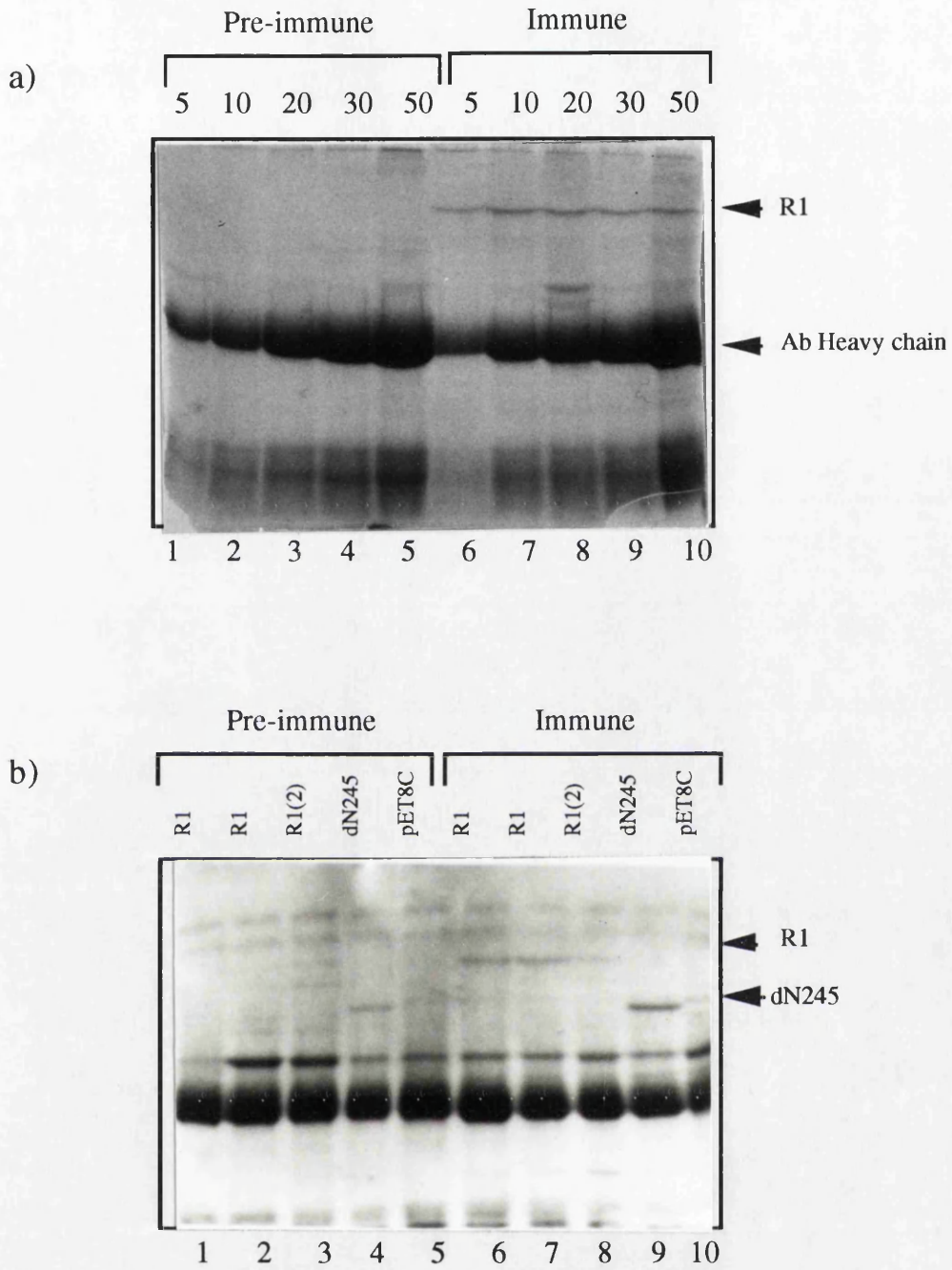


Figure 3B.3 Optimisation of immunoprecipitations.

a) Shows a Coomassie stained gel of the titration of the polyclonal antibody 106. Lanes 1 to 5 are precipitations of 35% ammonium sulphate fraction of HSV-1 R1 using 5µl (lane 1) to 50 µl (lane 5) of pre-immune serum, the exact amount of serum used is indicated at the top of the lanes. Lanes 6 to 10 are precipitations carried out with serum after immunisation with the truncated R1 polypeptide dN245 , as with the pre-immune serum the amount of serum used ranged between 5µl (lane 6) and 50µl (lane 10).

b) Shows a Coomassie stained gel of immunoprecipitations carried out with different ammonium sulphate fractions of full length R1 for both HSV-1 and HSV-2 and with an ammonium sulphate fraction of the truncation dN245 in which the amino terminal 245 amino acids of HSV-1 R1 are deleted. Lanes 1 to 5 are precipitations carried out with pre-immune serum and lanes 6 to 10 are precipitations carried out with immune serum (106). Lanes 1 and 6 and lanes 2 and 7 are precipitations of two different ammonium sulphate fractions of full length HSV-1 R1, lanes 3 and 8 are precipitations of an ammonium sulphate fraction of HSV-2 R1. The precipitation of dN245 is shown in lanes 4 and 9.

Kinase assays were carried out on immunoprecipitates from ammonium sulphate fractions from E.coli expressing full length R1, the amino terminal truncation dN245 and the carboxy terminal truncation dC345 (first 345 amino acids of HSV-1 are expressed). Equal volumes of ammonium sulphate precipitated protein fractions were immunoprecipitated with the polyclonal antibody 106, and the precipitates were assayed for autophosphorylating activity, see Figure 3B.4.

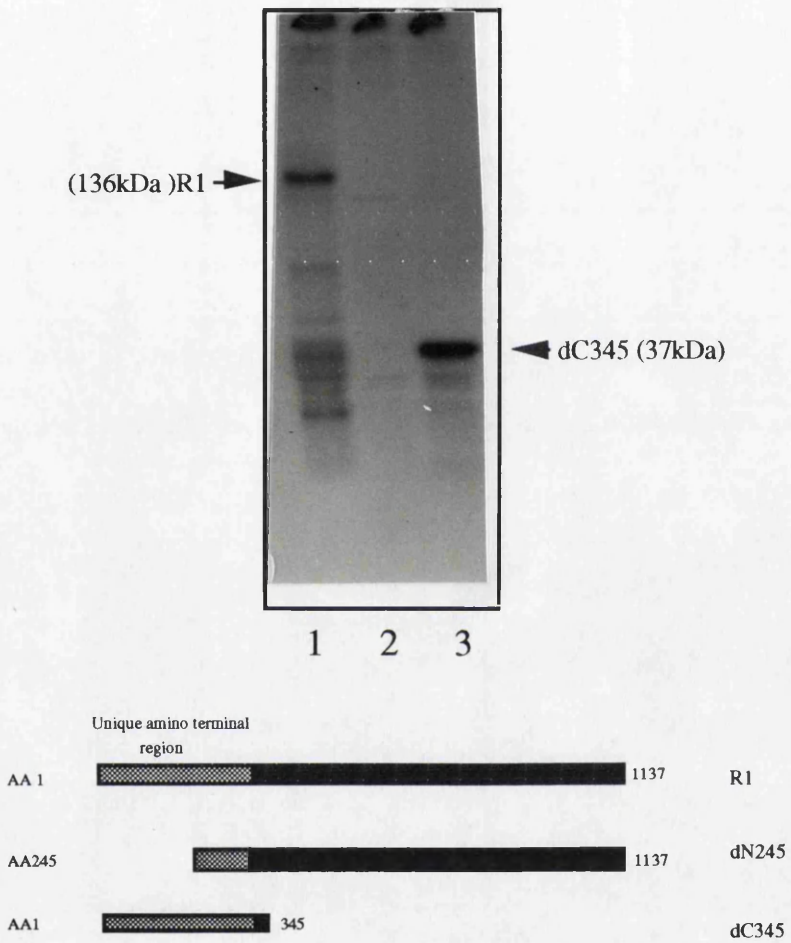


Figure 3B.4 Autoradiograph of immunoprecipitation/kinase assay of ammonium sulphate fractions for R1 and truncated R1. Ammonium sulphate fractions of full length R1 (lane 1), N-terminal truncated R1 (lane 2) and C-terminal truncated R1 (lane 3) were precipitated with 5µl of polyclonal antisera 106. Following precipitation and washes to remove non-specific proteins the immobilised antigen was incubated in kinase assay buffer for 30 min at room temp. Samples were resolved by 10% SDS-PAGE. The truncations are shown, residues of R1 expressed are for dC345 (AA1-345) and for dN245 (AA245-1037)

Only samples from ammonium sulphate fractions of full length R1 and dC345 show strong phosphoprotein bands following kinase assay, compare lanes 1 and 3 to the amino terminal truncation in lane 2. Bands common to full length R1 and dC345 are observed, indicating that cleavage occurs at the same sites in both. This assay provides further evidence that the ability of R1 to autophosphorylate resides in the amino terminal region.

3B.5 Assay for autophosphorylation of insertion mutants

Equal volumes of total ammonium sulphate fractions were used for immunoprecipitations and the optimal amount of antibody determined by precipitation of full length R1, was used for all mutants. Precipitates were assayed for kinase activity using the method developed for the full length protein.

Mutations which both decrease and increase the kinase activity of the amino terminal region of HSV-1 R1 were isolated. Sites of insertion which decreased activity include an insertion at amino acid 22 (In22, lane 3, Figure 3B.5(a)) and insertions at 112 (In112, lane 4 , gel (a)). Further downstream, insertions at amino acids 292 and 343 (Figure 3B.5 (b) lanes 4 and 5) also decreased activity when compared to wild-type (lane 2, both gels). A significant increase in the autophosphorylation of the immunocomplexed truncation was observed when insertions were made at amino acids 257 and 262, compare lanes 6, gel(a) and lane 3, gel (b) to the initial construct in lanes 2 of both gels. In addition, a deletion mutant which removes amino acids 257 to 434 of the truncation open reading frame shows a significant increase in the ability to autophosphorylate. That the increased autophosphorylation of this particular deletion is probably a direct result of the deletion and not enhanced resistance to proteolysis can be seen by comparing this particular truncation with two other deletions which show decreased sensitivity to proteolysis (compare Dl 257 (Figure 3B.5 lane7, gel (b)), with Dl 292 (lane 8, gel (b)), and Dl 345 (lane 9, gel (b)). The internal deletion mutant Dl43-136 (lane 6, gel (b)), is completely inactive.

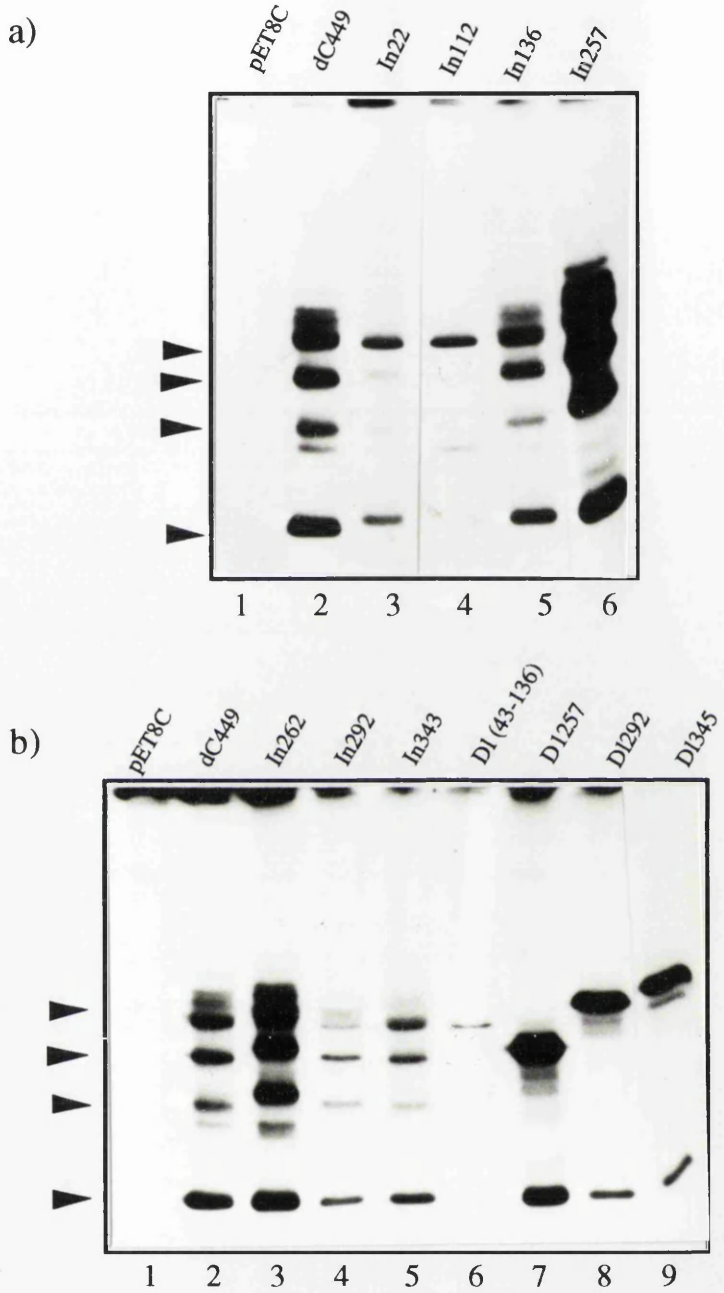


Figure 3B.5 Autoradiographs of immunoprecipitation/ kinase assays of insertion and deletion mutants in the HSV-1 R1 amino terminal region.

a) Shows an autoradiograph of an SDS-PAGE (12.5%) of immunoprecipitates from ammonium sulphate fractions of insertion mutants assayed for their ability to autophosphorylate. Sites of insertion for each mutant are indicated at the top of the gel, phosphoproteins highlighted are those unique to extracts containing dC449 and dC449 mutants.

b) shows an autoradiograph of both insertion (lanes 3, 4 and 5) and deletion mutants (lanes 6, 7, 8 and 9), again bands unique to extracts expressing the amino terminal region of HSV-1 R1 are highlighted.

The ability of the insertion mutants to be precipitated by the antibody 106 was determined by analysing the immunoprecipitates by Western blot. Precipitates were resolved by SDS-PAGE, transferred onto nitrocellulose and the membrane was probed with the R1 N-terminal specific antibody F1 (raised against AA1-145 of HSV-1 R1). Figure 3B.5(b) shows the Western blot, all insertion mutants which showed an altered activity are precipitated by 106; those which decrease activity, In112 and In136 (lanes 5 and 6, respectively); those which increase activity, In 257 and In262 (lanes 7 and 8, respectively) and those which do not appear to affect activity, In 22, In 43 and In292 (lanes 3, 4 and 9 respectively). This analysis confirms that the differences observed between mutants is in fact due to the mutations created rather than an altered ability to be precipitated.

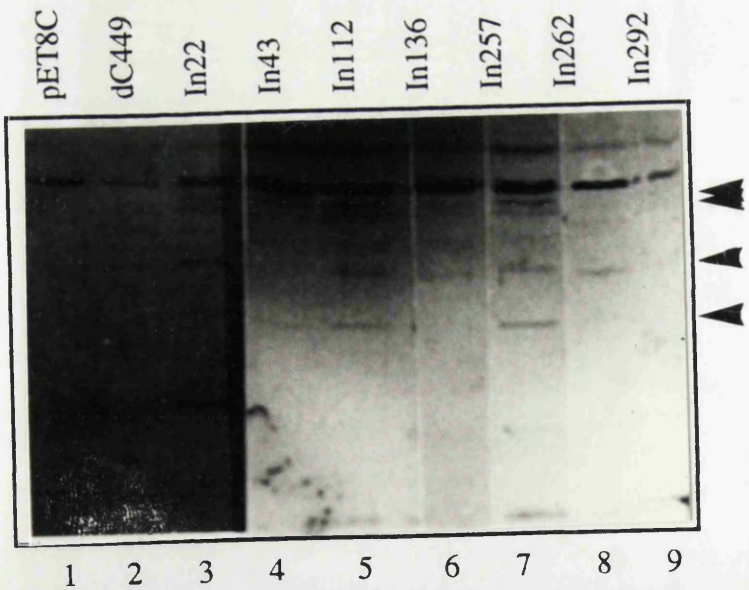


Figure 3B.5(b). Western blot showing the amount of protein precipitated for each insertion mutant. The precipitation of each insertion mutant (lanes 3 to 9) is compared to the amount of protein precipitated from the wild-type (dC449, lane 2). Lane 1 is the negative control, extracts from pET8C transformed *E. coli*.

3B.6 Discussion

The sites of insertion into the amino terminal domain with respect to the localisation of regions potentially important in catalysis is shown below (Figure 3B.6).

```
1       MASRPAASSPVEARAPVGGQEAGGPSAATQGEAAGAPLAHGHHVYCQRVN
                    In22 GQEAGDFPGGPS      In43 GHHVWGNSPCQRVN

51      GVMVLSDKTPGSASYRISDSNFVQCGSNCTMIIDGDVVRGRPQDPGAAAS

                Subdomain 1
101     PAPFVAVTNIGAGSDGGTAVVAFGGTPRRSAGSTGTQTADVPTEALGGPP
          In112 NIGAGDFPGSDG       In136 STGGDFPTQTA

                              Subdomain 2
151     PPPRFTLGGGCCSCRDTRRRSAVFGGEGDPVGPAEFVSDDRSSDSDSDDS

          Subdomain 3                          Subdomain 4
201     SETDSETLSHASSDVSGGATYDDALDSDSSSDDSLQIDGPVCRPWSNDTA

              Subdomain 5
251     DLPVCPGTPGPGADAGGPSAVDPHAPTPEAGAGLAADPAVARDDAEGLSD
   In257 PVCPGDFPGTPG                  In292 PAVARGNSPDAEG
        In262 TPGPGNSPGADAG

                Subdomain 6
301   PRLGTGTAYPVPLELTPENAEA
```

Figure 3B.6 Insertions into the amino terminal region of HSV-1 R1 compared to the localisation of sites potentially important in catalysis. Regions important in catalysis in protein kinase, subdomains, and their predicted location in the amino terminal region are shown. Insertions into the amino terminal region and the sequences created by these insertions are also shown below and bolded.

Mutations were constructed throughout the N-terminal domain of HSV-1 R1 to locate potential regions of importance in the R1 autophosphorylating activity. A 12 base pair oligonucleotide was inserted into blunt restriction sites located within the 449 amino acid open reading frame of HSV-1 R1. To analyse the autophosphorylating activity of the mutants an immunoprecipitation assay was developed using a polyclonal antibody raised against a truncation of HSV-1 R1. Optimal precipitation of full length R1 was used to determine the amount of antisera required for effective precipitation whilst keeping the antibody in limiting amounts so that differences in the levels of expression of the mutants could be compensated for in the assay.

Mutants were isolated with a variety of phenotypes; 1) with no affect on the activity; 2) those which impair but do not completely destroy activity and 3) those which significantly increase activity. Mutations which decrease activity map upstream and within the proposed nucleotide binding site located around amino acid 111 to 123. Insertion at amino acid 22 reduces activity, this may be a region important in the formation of a structurally

stable kinase domain, this region has been predicted to form a hydrophobic β-sheet structure (Nikas *et al.*, 1986). The insertion into the predicted nucleotide binding site at amino acid 112 did not destroy activity, as found for the insertion at amino acid 22 the activity was significantly reduced, this may however be due to reduced expression of this particular mutant. That activity is not completely destroyed by mutation of the predicted nucleotide binding site may in part be explained by the conservation of a glycine in the construction of this mutant (Figure 3B.7). Mutation of the HSV-2 nucleotide binding site leads to decreased autophosphorylating activity (Luo and Aurelian, 1992). It is possible that this site in both HSV-1 and HSV-2 is not the actual nucleotide binding site. No other conserved nucleotide binding site is located within the first 449 amino acids of HSV-1 R1. It is however possible that R1 has a non-consensus nucleotide binding site, as has been observed for other protein kinases, a protein kinase with no apparent nucleotide binding site has been reported (Herman *et al.*, 1991). The role of the region located upstream of the nucleotide binding site in the autophosphorylating activity is unknown, this region in the HSV-2 R1 N-terminal domain is thought to be important in the stability of this domain, deletions in this region lead to the instability of the N-terminal domain when expressed in *E.coli* and in eukaryotic cells (Luo *et al.*, 1991). It was proposed by Luo *et al.*, (1991) that this region is important in the formation of a structurally stable protein kinase domain.

```
AA 104      VAVTNGAGSDGGTAVVA
In112       VAVTNGAGDFPPGSDG
                   *
```

Figure 3B.7 Mutation of the proposed nucleotide binding site in the HSV-1 amino terminal region. The sequence of the proposed nucleotide binding site is shown above, the glycine triad is highlighted. The residues inserted into the nucleotide binding site in the mutant In112 are shown below, the glycine which could in part complement this mutation is highlighted by an asterix.

Mutations around amino acid 257 lead to a several fold increase in activity, a mutation located at amino acid 262 also shows an increase in activity but not as large as observed for the insertion at 257. Both these insertion sites are located close to or within the predicted kinase subdomain 5, a region in protein kinases of, as yet, unknown function. The amino acids inserted into both sites are similar to those inserted into another site located at amino acid 292, that these particular insertions create another phosphorylation site, thereby increasing the observed phosphorylation, is unlikely. That a region involved in the down-regulating of the protein kinase exists is not surprising as it is rarely of benefit for a protein kinase activity to be optimal without some means of regulation. A region in the insulin receptor has been identified which controls autophosphorylation, mutation of this region leads to increased autophopshorylation (Sung *et al.*, 1994). The down-regulatory region in

leads to increased autophopshorylation (Sung *et al.*, 1994). The down-regulatory region in the HSV-1 R1 protein kinase may simply act to mask either the site catalytically important, or it could be speculated that a co-factor interaction with this region is required *in vivo* to overcome the inhibitory effect of this region. There are several well documented cases where a protein kinase structure is altered upon interaction with a stimulatory co-factor. such an interaction may be important in the N-terminus activity.

Deletion mutants, both internal, between amino acids 43 and 136, and carboxy terminal to amino acid 257 and 292 were also produced and analysed for their autophosphorylating activity. As found with the insertion mutant at amino acid 257, the deletion from 257 led to an increased autophosphorylating activity. This mutant has increased stability, with fewer cleavage products observed. A comparison of the 257 deletion with the mutant in which the carboxy terminal amino acids from amino acid 292 were deleted, which also has increased stability shows that the deletion from amino acid 257 does appear to have a direct affect on the kinase activity. It appears that the deletion mutations are removing amino acids located downstream of 257 which are involved in down-regulating activity. Deletion of amino acids 43 to 136 leads to a complete loss in activity indicating that within this region residues crucial for R1 autophosphorylating activity are located.

3C Overexpression of HSV-2 R1 and characterisation of kinase activity

3C.1. Introduction

To compare accurately the protein kinase activities associated with HSV-1 and HSV-2 R1, both proteins were expressed in *E.coli* and purified using a similar purification scheme. The HSV-1 R1 protein kinase activity would appear to differ from that of HSV-2, in that unlike HSV-2 which has previously been shown to transphosphorylate exogenous substrates (Chung *et al.*, 1989), there does not appear to be a similar activity associated with HSV-1 R1 (Conner *et al.*, 1992b).

The HSV-2 R1 kinase activity was characterised using the full length R1 immunoprecipitated from infected human epidermoid 2 cells (Hep2) and from cells transiently transfected with a construct constitutively expressing HSV-2 R1: the kinase activity was shown to be stimulated by manganese and polylysine (Chung *et al.*, 1990). Recently, mutations in HSV-2 have shown that the site proposed to be involved in nucleotide binding and the lysine residue thought to be involved in phosphate transfer are dispensable, mutants in this region showed a significant but not a complete loss in activity (Luo and Aurelian, 1992).

A transphosphorylating activity, originally thought to be associated with HSV-1 R1 was shown to be a contaminant when truncations which lacked the N-terminus and the ability to autophosphorylate were shown to transphosphorylate. It was therefore possible that the transphosphorylation activity associated with HSV-2 R1 was also a contaminant, or, that the purification scheme used for HSV-1 removed a co-factor essential for this activity. To determine if the differences reflected the different assay conditions used, HSV-2 R1 was overexpressed in *E.coli* purified to near homogeneity and assayed for both autophosphorylating and transphosphorylating activity under the same conditions used to characterise HSV-1 R1.

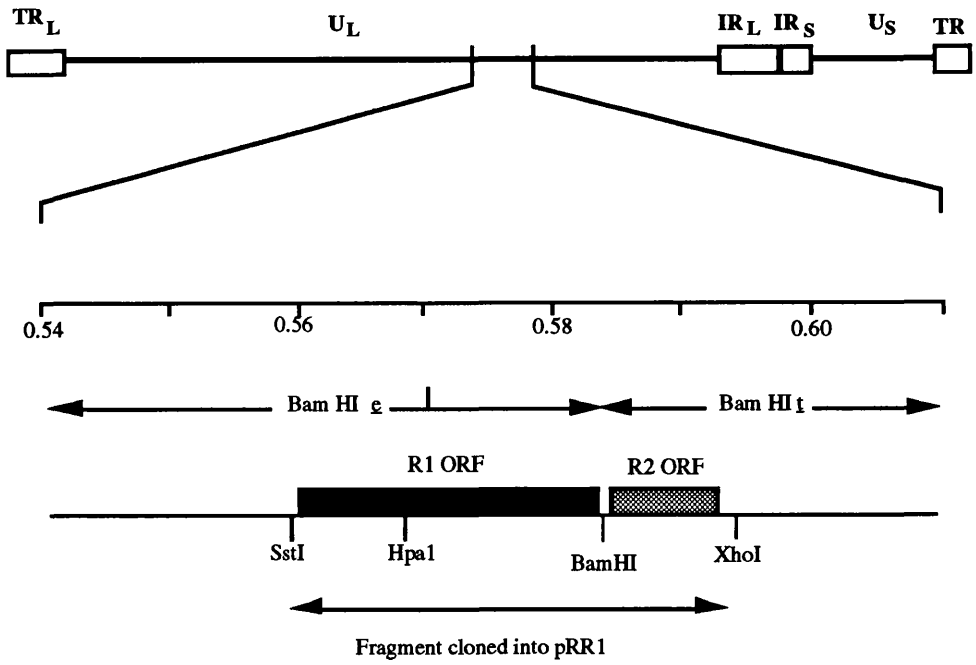


Figure 3C.1 Locus of HSV-2 R1 and R2. The loci of the large and small subunits of HSV-2 RR are shown. Both map to the unique long region of the genome, and like HSV-1, the transcripts for R1 and R2 are 3′ co-terminal. Sites used to initially clone the entire R1 open reading frame into the pUC vector, pRR1 are indicated.

3C.2 Construction of vector for the overexpression of HSV-2 R1 in *E.coli*

The strategy for the construction of the expression vector for HSV-2 R1 was essentially the same as described for pYN1-SD (Figure 3A.2). Oligonucleotides containing the *E.coli* Shine-Dalgarno sequence were placed upstream of the HSV-2 R1 initiation codon within the predicted optimal spacing for efficient translation. The entire reading frame of HSV-2 R1 in the pUC based vector pUCRR1 was digested with Ball (site falls within the second codon of R1) and EcoR1 (a site within the pUC polylinker). The oligonucleotides were cloned into this blunt Ball/sticky end plasmid. Insertion of the oligonucleotides was determined by screening minipreps with Xba1 and dideoxy DNA sequencing confirmed the oligonucleotides were correctly positioned. The entire open reading frame of HSV-2 R1 was then cloned into a pET vector (Figure 3C.2). The R1 open reading frame was removed from the plasmid pRR1-SD on a Xba1/BamH1 fragment and ligated into the T7 expression vector pET8C cut with Xba1 and BamH1.

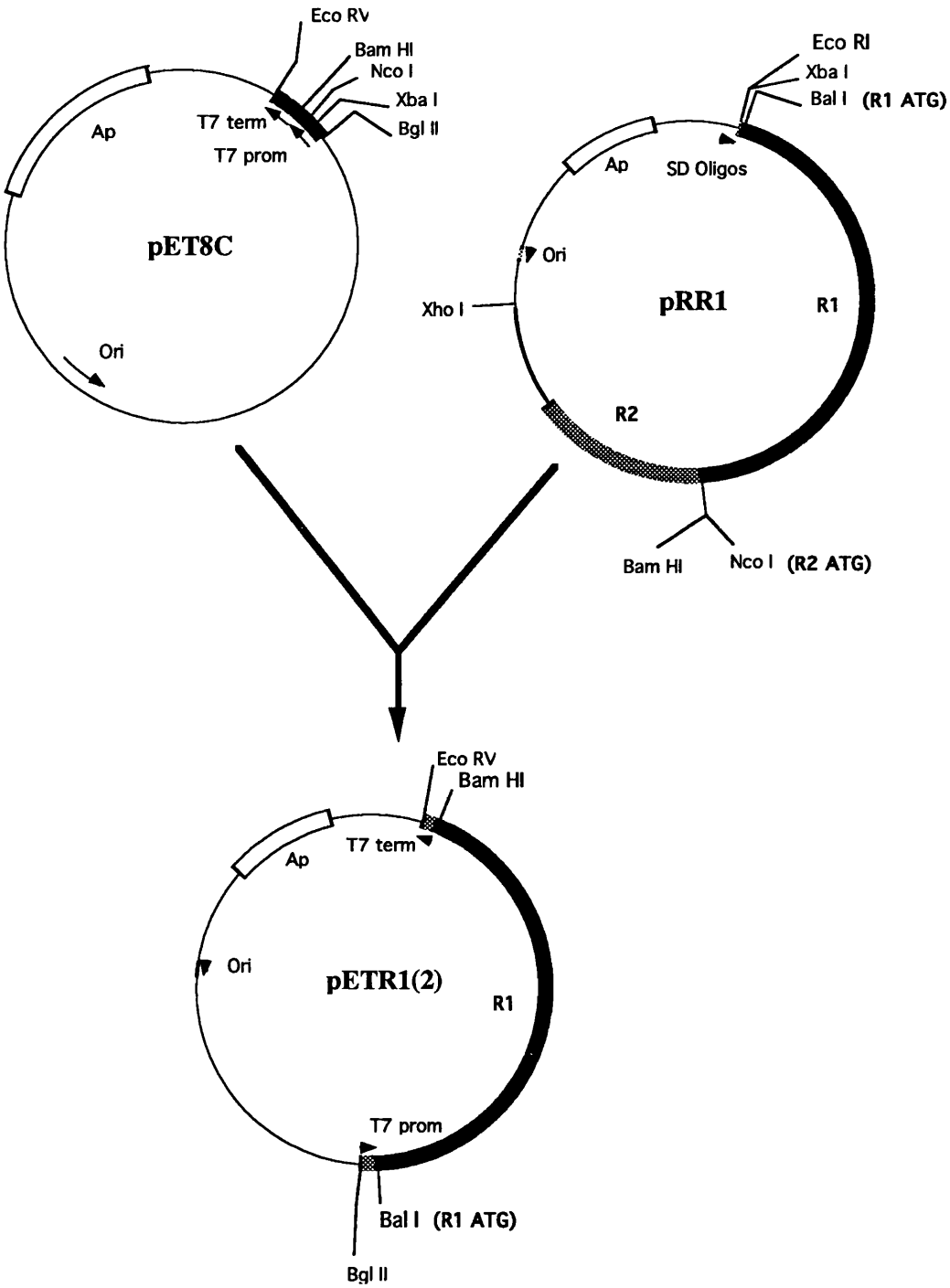


Figure 3C.2 Construction of HSV-2 T7 expression vector. Plasmid pRR1-SD was constructed essentially as described for pYN1-SD (Figure 3A.2). Shine-Dalgarno oligonucleotides were ligated into pRR1 which had been cut with Xba1 and Bal I. The R1 open reading frame was then removed on an Xba1/BamH1 fragment, the Xba1 site was created by the ligation of the Shine-Dalgarno oligonucleotides and the BamH1 site located downstream of the termination codon of R1, and ligated into pET8C cut with Xba1 and BamH1.

3C.3 Induction of expression of HSV-2 R1

Plasmid pETR1(2) was transformed into *E.coli* strain *BL21 (DE3)* and expression of R1 was induced by the addition of IPTG (150ug/ml final concentration). A time course of a small scale preparation was carried out to determine the optimal hr post-induction to harvest cultures. Expression was determined by Western blot analysis using a polyclonal antibody (22029) raised against HSV-1 R1 which cross-reacts with HSV-2 (see Figure 3C.3). Induction of expression can be detected by Western blot analysis within 1 hr post-induction, as observed for HSV-1 R1 (Furlong *et al.*, 1991), the level of expression peaks at 2 hr post-induction (lane 6) and decreases by 3 hr post-induction (lane 8). Additional background bands were also detected.

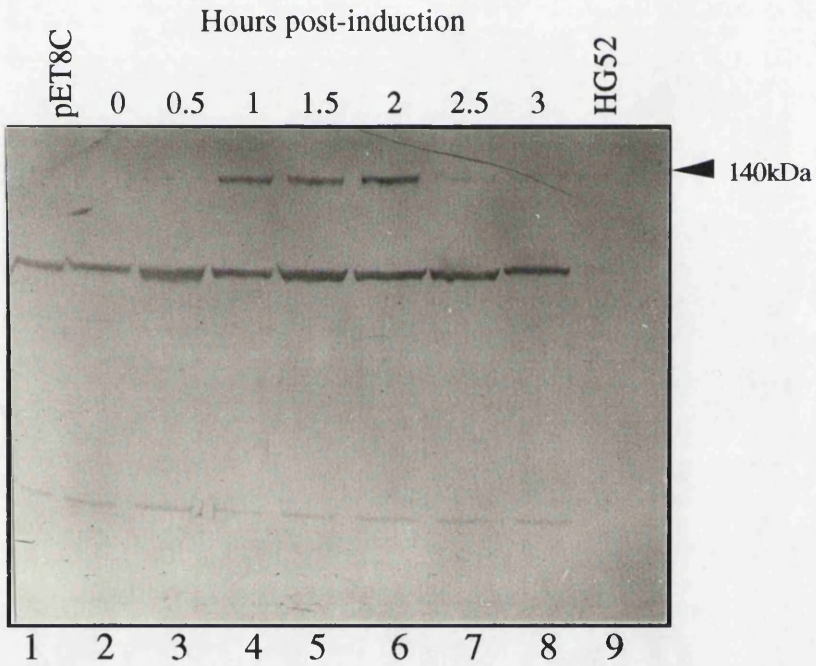


Figure 3C.3 Induction of expression of HSV-2 R1. *E.coli,* strain *BL21 (DE3)* transformed with pETR1(2), were grown to an optical density of 0.5. IPTG was added to the culture to a final concentration of 150µg/ml and cultures were grown at 26°C. Samples (1ml) were then removed at 30 min intervals up to 3 hr post-induction. Samples were subjected to SDS-PAGE (10%) transferred to nitrocellulose and analysed by Western blot using an antisera (20209) initially raised against HSV-1 R1 but which cross reacts with HSV-2 R1. Lanes 2 to 8 are samples of *E.coli* removed from 0 min post-induction (lane 2) to 3 hr post-induction (lane 8). An uninduced control is shown (lane 1) and an HG52 extract was used for a positive control (lane 9).

3C.4 Purification of HSV-2 R1

The concentration of ammonium sulphate optimal for precipitation of HSV-2 R1 was determined, like HSV-1 R1 precipitation was optimal at a final concentration of 35%
HSV-1 R1 was purified to apparent homogeneity using a heparin Affi-gel column (Furlong *et al.*, 1991), this column was tested for its ability to bind HSV-2 R1. A 10l starting culture was used for the purification of HSV-2 R1, ammonium sulphate precipitates were loaded onto a heparin Affi-gel column, the elution of HSV-2 R1 from this column is shown in Figure 3C.4. The Coomassie stained gels show that R1 is the major protein interacting with the matrix, like HSV-1 R1 (Furlong *et al.*, 1991), HSV-2 R1 was purified using a single column.

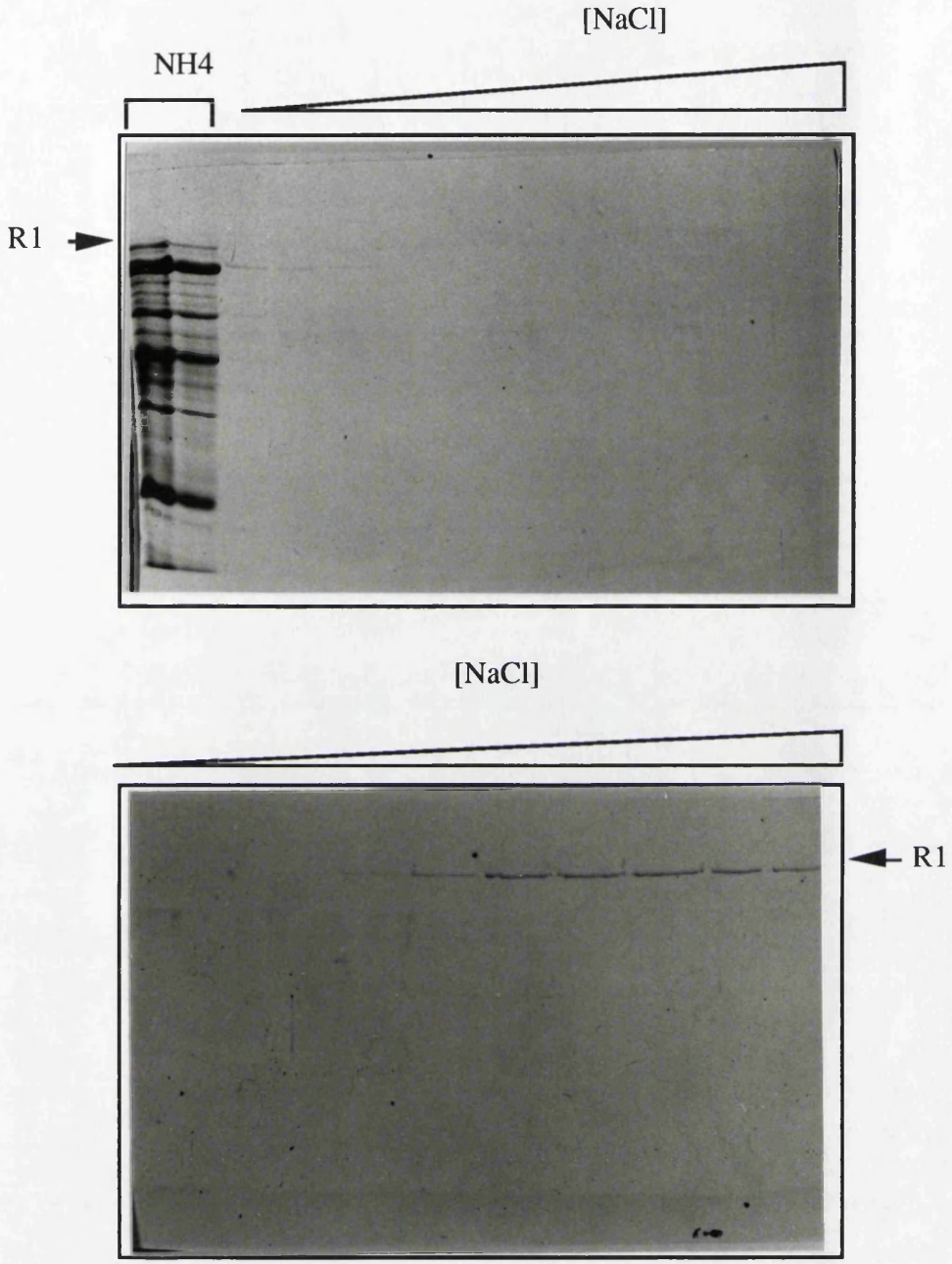


Figure 3C.4 Purification of HSV-2 R1. Coomassie stained gels of elution profile of ammonium sulphate fractions from *E.coli* expressing HSV-2 R1. The starting ammonium sulphate fraction is shown in the upper gel, the rest of the gel represents fractions from the heparin Affi-gel column, the elution of R1 is shown in the final fractions in the lower gel. R1 is highlighted.

3C.5 Assay for autophosphorylating activity associated with HSV-2 R1

HSV-2 R1 preparations purified using a Heparin Affi-gel FPLC column were assayed for autophosphorylation. Samples were assayed under the conditions found to be optimal for HSV-1 kinase activity (25mM Hepes pH 7.6, 1mM Mn^{2+}, 0.1mg/ml protamine) although no NaCl was added. The results of such an assay are shown in Figure 3C.5. Of particular interest are the two bands which co-elute from the FPLC column, most apparent in lane 4; the lower molecular weight band showing a greater activity than the full length upper band. It is likely that the lower Mwt band is a degradation product, similar analyses of HSV-2 R1 by Chung *et al.*, (1991) showed a 55kDa major cleavage product in HSV-2 R1 kinase assays.

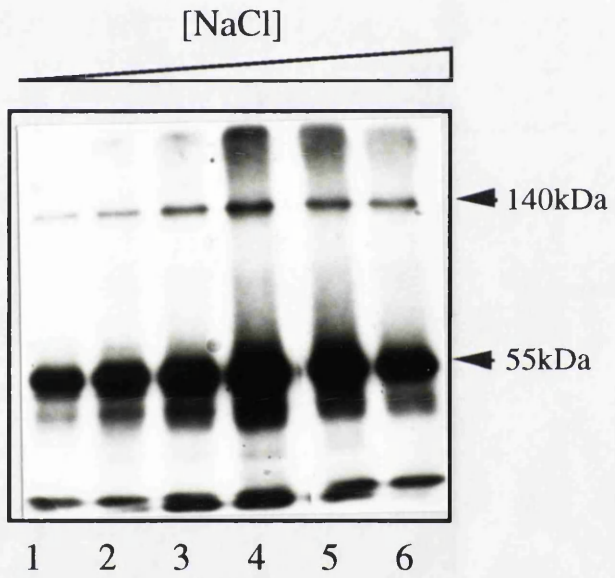


Figure 3C.5 Autoradiograph of kinase assays of FPLC (heparin Affi-gel column) fractions. Samples of equal volume from each 1ml fraction eluted from the heparin Affi-gel column were assayed for autophosphorylating activity. Samples were assayed in the presence of Mn^{2+} (1mM) and protamine (0.1mg/ml). Bands which are HSV-2 R1 associated are indicated.

3C.5.1 Optimisation of HSV-2 autophosphorylation

Full length HSV-1 R1 autophosphorylation is optimal in 250mM NaCl and 1mM Mn^{2+}. Activity is further stimulated by the addition of the basic polypeptide protamine to a final concentration of 0.1mg/ml (Conner *et al.*, 1992). The effect of salt and manganese on HSV-2 R1 autophosphorylation was investigated and compared to the effect of salt and manganese on HSV-1 R1 autophosphorylation (see Figure 3C.6). Various concentrations

of NaCl were assayed for the affect on autophosphorylation (Figure 3C.6 (a)); no significant affect on the autophosphorylation of HSV-2 R1 was observed, compare lanes 3, 5, 7 and 9, although the pattern of the phosphoproteins associated with HSV-2 R1 (55kDa and 25kDa) appears to be altered by the final salt concentration in the assay, the 55kDa band becomes more predominant as the salt concentration is increased (lane 9), explaining the differences observed in the kinase assays of the heparin Affi-gel fractions (Figure 3C.6), where the 55kDa band is more active than the full length protein; autophosphorylation of the 55kDa band is optimal in high salt. The 25kDa bands observed in both HSV-1 and HSV-2 R1 autophosphorylation assays are unaffected by the salt concentration in the assay; this band probably represents a stable amino terminal domain cleavage product. A band of a similar Mwt is observed in kinase assays of dC449. The relationship between the 25kDa band and HSV-2 R1 could not be conclusively determined due to the lack of amino terminal specific antisera for HSV-2 R1. A 29kDa HSV-2 R1 cleavage product is observed in extracts from*E.coli* expressing the HSV-2 amino terminal truncation (Luo *et al.,* 1991); it is therefore likely that the 25kDa protein observed in assays with full length R1 is also R1 specific . The full length HSV-1 R1 activity in this analysis, like that of full length HSV-2 R1, did not appear to be significantly altered by salt concentration, but was optimal in the absence of salt. A band observed in HSV-2 assays of between 90-110kDa, highlighted by an asterix, may be an intermediate cleavage product.

There is an absolute requirement for manganese for protein kinase activity, that autophosphorylation of both HSV-1 and HSV-2 R1 is observed in the absence of added Mn^{2+} (Lanes 1 and 2, gel b, Figure 3C.6) indicates that there are trace amounts of either Mn^{2+} or Mg^{2+} in ths samples. Large increases in the autophosphorylating activity of HSV-1 R1 are observed by the addition of relatively low concentrations of Mn^{2+} (0.5mM). Figure 3C.6 (b) shows a comparison of the effect of Mn^{2+} on the autophosphorylating activity of HSV-2 (lanes 2, 4, 6 and 8, gel b) and HSV-1 R1 (lanes 1, 3, 5 and 7). Both are stimulated by the addition of Mn^{2+} to a final concentration of 0.5mM (lane 3, HSV-1 R1; lane 4, HSV-2 R1). The autophosphorylation of the HSV-2-specific 55kDa cleavage product is also dependent on manganese, this band only becomes apparent upon the addition of manganese to the assay (lane 4). Both the HSV-1 and HSV-2 25kDa cleavage products show a stimulation by the addition of manganese to the assay, for HSV-2 compare lanes 2 and 4, the activity of the 25kDa band is increased by the addition of Mn^{2+}; the increase observed is not as large as the increase detected with the full length R1, it is possible that the 25kDa fragment has a higher affinity for Mn^{2+} or Mg^{2+}.

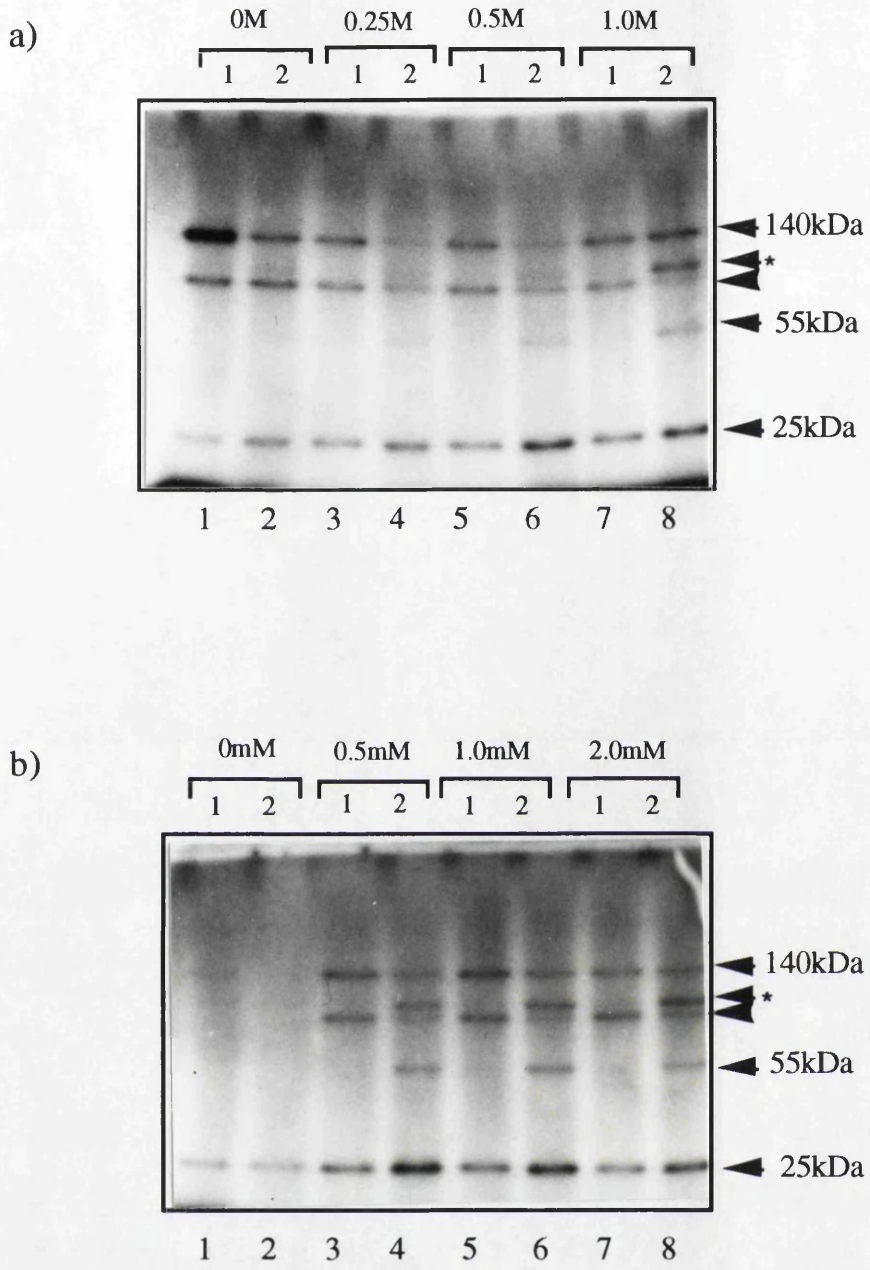


Figure 3C.6 Autoradiographs showing optimisation of HSV-2 R1 autophosphorylation, compared to HSV-1 R1.

a) Shows the autoradiograph of phosphoproteins produced in various final concentrations of NaCl by *in vitro* kinase assays of HSV-1 and HSV-2 R1 resolved by SDS-PAGE (10%). Lanes 2, 4, 6 and 8 are HSV-2 R1 autophosphorylation assays carried out in 0mM, 250mM, 500mM and 1M NaCl, and lanes 3, 5, 7 and 9 are HSV-1 R1 autophosphorylated in the same final salt concentrations as HSV-1 R1. Bands highlighted in this autoradiograph are full length R1, upper band (both HSV-1 and HSV-2), and several lower Mwt cleavage products.

b) Shows an autoradiograph of the phosphoproteins resolved by SDS-PAGE (10%) in various final concentrations of manganese. Lane 1, 3, 5 and 7 are HSV-1 R1 autophosphorylated in assay buffer containing a final concentration of manganese of 0mM, 0.5mM, 1.0mM and 2.0mM respectively. Lanes 2, 4, 6 and 8 are HSV-2 R1 related phosphoproteins assayed in the same final concentrations of manganese as described for HSV-1. Again, phosphoproteins highlighted represent full length R1 (HSV-1 and HSV-2) and several lower Mwt cleavage products. sizes were determined using reference markers

3.C.5.2 Stimulation of HSV-2 autophosphorylation by basic polypeptides

Basic polypeptides stimulate the autophosphorylating activity of growth factor receptor protein kinases (Gatica *et al.*, 1987; Rosen and Lebwohl, 1988; Morrison *et al.*, 1989). HSV-2 R1 purified from infected or transfected cells by immunoprecipitation autophosphorylates and transphosphorylates; both activities are stimulated by polylysine (Chung *et al.*, 1990). In contrast, HSV-1 R1 overexpressed in *E.coli* and purified to homogeneity is not stimulated by polylysine, although the basic polypeptide protamine has been shown to stimulate activity as much as 10-fold (Conner *et al.*, 1992b). To determine whether HSV-2 R1 overexpressed in *E.coli* can be stimulated in a similar fashion, both polylysine and protamine were assayed for their ability to stimulate autophosphorylation. Various concentrations of both polylysine (from 0 to 1mM) and protamine (from 0 to 0.2mg/ml) were added to the standard kinase reaction buffer. Phosphoproteins were subjected to SDS-PAGE and bands were visualised by autoradiography (see Figure 3C.7). Polylysine showed no stimulation of autophosphorylation of either the full length (140kDa) or the intermediate truncation (55kDa) at any concentration tried, see lanes 1 to 6 of Figure 3C.7 (a). At a concentration greater than 0.05mM (lane 3) the autophosphorylation of both the full length HSV-2 R1 and the 55kDa fragment was inhibited significantly. In contrast, the phosphorylation of the 25kDa fragment is observed at concentrations of polylysine as high as 1.0mM. Protamine stimulates the autophosphorylation activity of both the full length HSV-2 R1 and the 25kDa fragment; for full length R1 stimulation is observed (upper band indicated) at concentrations between 0.05mg/ml (lane 2) and 0.1mg/ml (lane 3), and inhibition of activity at a concentration of 0.2mg/ml (lane 4). The lower band indicated, 25kDa, shows an initial increase in activity at a concentration of 0.05mg/ml (lane 2) and decreases at higher concentrations of protamine (0.1mg/ml, lane 3) to an activity comparable to that observed in the absence of protamine (lane 1). Protamine, a basic polypeptide, may mediate the stimulation of activity of a protein kinase by making the sites of phosphorylation more accessible to phosphorylation. Autophosphorylation of the intermediate 55kDa cleavage product is inhibited by protamine, in this polypeptide the sites of either autophosphorylation or catalytic residues may be readily accessible compared to either the full length or the 25kDa product. In the 25kDa, fragment the active site may be masked by C-terminal sequences, or the 29kDa fragment could be structurally more stable. The basic polypeptides will have a greater effect on the ability of either the full length or 25kDa product to autophosphorylate.

Alternatively, since the pattern of phosphorylation for the 25kDa fragment and the full length protein is similar, the 25kDa may result from the cleavage of full length R1 after

autophosphorylation has occurred, indeed phosphorylation could stimulate the proteolytic cleavage.

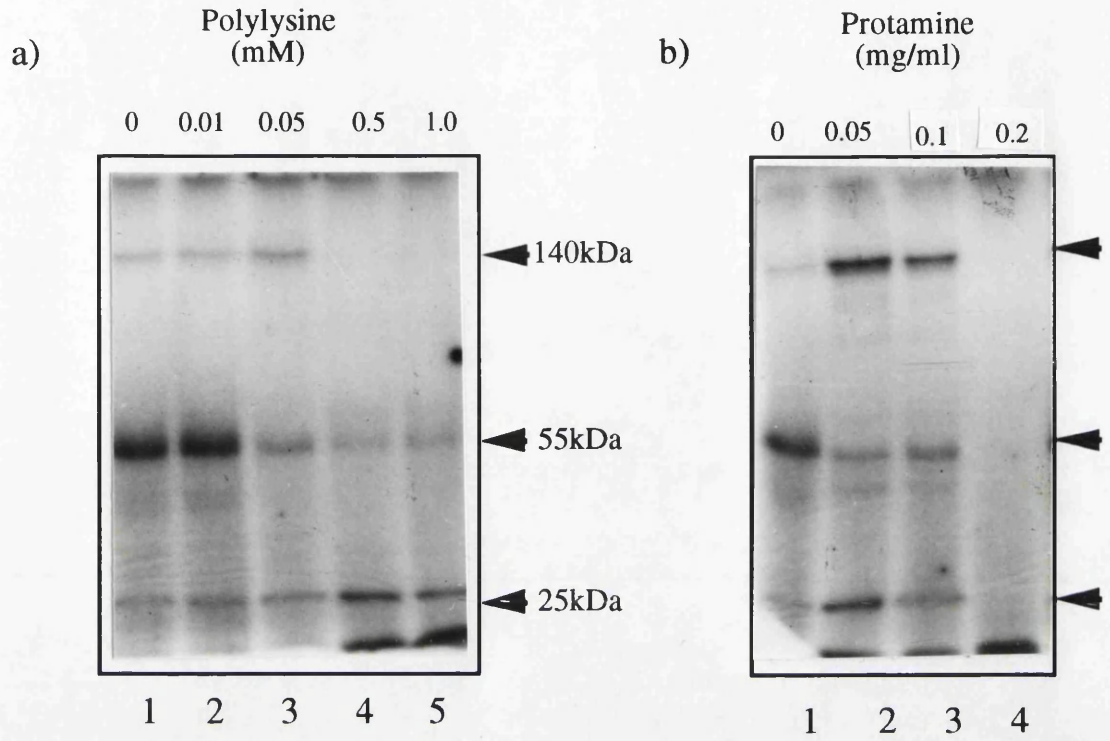


Figure 3C.7 Stimulation of HSV-2 autophosphorylation. HSV-2 R1 autophosphorylation was assayed in the presence of two basic polypeptides, polylysine and protamine to determine their effects on autophosphorylation. Bands related to HSV-2 R1 are highlighted equal volumes of a pure HSV-2 R1 preparation were used in each assay.
a) Shows the effect of polylysine on the autophosphorylation activity. Lane 1 is R1 in the absence of polylysine, lanes 2 to 5 are R1 assayed in the presence of polylysine; 0mM (lane 2), 0.01mM (lane 3), 0.05mM (lane 3), 0.5mM (lane 4) and 1.0mM (lane 5).
b) Shows the effect of protamine on HSV-2 R1. Lane 1 is R1 in the absence of protamine and lanes 2 to 4 contain protamine at a final concentration of 0.05mg/ml, 0.1mg/ml and 0.2mg/ml. Activity is stimulated at concentrations of 0.05mg/ml (lane 2) and 0.1mg/ml (lane 3) and severely inhibited at 0.2mg/ml (lane 4).

3C.6 Assay for transphosphorylation by HSV-2 R1

A transphosphorylating activity associated with HSV-2 R1 has previously been described (Chung *et al.*, 1989). To determine whether HSV-2 R1 expressed in *E.coli* contains a similar activity, transphosphorylation assays were carried out under various conditions found previously to promote the transphosphorylation of exogenous substrates commonly used to characterise a protein kinase. As shown above, the autophosphorylation of HSV-2 R1 can been enhanced by the addition of protamine to the assay, optimal levels of autophosphorylation were observed when protamine was added to a final concentration of 0.05mg/ml To determine whether protamine or polylysine can stimulate a transphosphorylating activity of HSV-2 R1, in transphosphorylation assays both basic

polypeptides were included, and compared to the ability of HSV-2 R1 to transphosphorylate in their absence. Transphosphorylation assays were carried out with histones as a potential substrate, a substrate which has previously been shown to be transphosphorylated by HSV-2 R1 purified by immunoprecipitation from infected cells (Chung *et al.*, 1989). Equal amounts of an FPLC purified preparation of HSV-2 R1 were incubated in a kinase assay mixture, in certain reactions the basic polypeptides protamine (see lanes 2 and 5) and polylysine (lanes 3 and 6) were added to determine whether these act to stimulate the transphosphorylation of histones (lanes 4, 5 and 6), reactions were incubated for 20 min at room temperature. Phosphoproteins were resolved by SDS-PAGE (10%), see Figure 3C.8. Comparison of lanes 1, 2 and 3 with those which contain histones, lanes 4, 5 and 6, shows there is no differences in the phosphoprotein profile, if histones had been transphosphorylated new bands of between 25-35kDa would have been observed. There is, however, a slight increase in phosphorylation of the HSV-2 polypeptides in the presence of histones, most notably for the truncated species in the absence of a basic polypeptide, see lane 4 (compare to lane 1) and for full length R1 in the presence of protamine (0.1mg/ml), compare lane 5 to lane 2. Histones, like protamine are basic polypeptides, so the stimulation of the autophosphorylating activity of HSV-2 R1 may be similar to that observed for protamine, but dependent on protamine for the stimulation of full length R1.

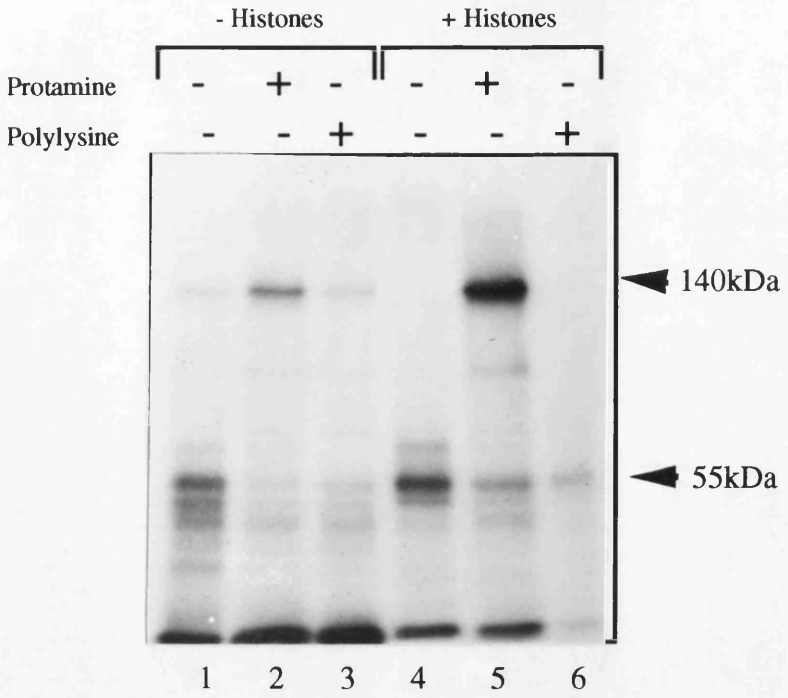


Figure 3C.8 Autoradiograph of assay for transphosphorylation of histones by HSV-2 R1. HSV-2 R1 was assayed for transphosphorylating activity under various conditions which are known to stimulate the autophosphorylating activity of this protein. Lane 1 and 4 shows the assay in the absence of basic polypeptides, lane 4 also contains histones (0.1mg/ml, histone type III) at a final concentration of 5µg per reaction , lanes 2 and 5 are transphosphorylation assays carried out in the presence of protamine (0.1mg/ml), lane 5 also contains histones. Lanes 3 and 6 are transphosphorylation assays carried out in the presence of polylysine (40mM). Bands highlighted are those related to HSV-2 R1.

3C.7 Discussion

The kinase activity of the HSV-2 R1 was originally thought to differ from that of HSV-1 (Chung *et al.*, 1989). To make an accurate comparison of the activities of the two, HSV-2 R1 was over-expressed in *E.coli* using the T7 expression system used for the full length HSV-1 R1 (Furlong *et al.*, 1991). HSV-2 R1 was then purified to apparent homogeneity using the heparin Affi-gel matrix that was successful in the purification of the HSV-1 R1. Previously, HSV-2 R1 purified by immunoprecipitation from infected cells was shown to be completely dependent on manganese for activity (Chung *et al.*, 1989; Chung *et al.*, 1990), analysis of the HSV-1 R1 kinase activity showed it was also dependent on manganese (Conner *et al.*, 1992b). The HSV-2 R1 autophosphorylating activity was compared to HSV-1 R1 autophosphorylating activity over a variety of salt and manganese concentrations. In the experiments comparing the effects of sodium chloride on both HSV-1 R1 and HSV-2 R1, the activity showed no significant differences over the range of salt concentrations tried. Both full length HSV-1 and HSV-2 R1 showed an absolute

requirement for manganese as did the 55kDa HSV-2 specificpolypeptide. Both the HSV-1 and HSV-2 25kDa polypeptide autophosphorylated in the absence of exogenous Mn^{2+} suggesting that trace amounts of either Mn^{2+} or Mg^{2+} is enough to promote its autophosphorylation. The divalent cation may interact strongly with the 25kDa polypeptide.

The effects of basic polypeptides on the autophosphorylation of HSV-1 and HSV-2 R1 were found to differ; the basic polypeptide polylysine has previously been shown to stimulate HSV-2 R1 kinase activity (Chung *et al.*, 1990), whereas protamine was found to stimulate the activity of the HSV-1 R1 (Conner *et al.*, 1992b). The stimulation of the *E. coli* expressed HSV-2 activity was investigated, unlike previously, the only basic polypeptide which showed stimulation of the autophosphorylation of full length HSV-2 was protamine, polylysine showed no effect at low concentrations and at higher concentrations inhibited autophosphorylation. As found for the amino terminal region of HSV-1 R1 and for the full length HSV-1 R1, the HSV-2 enzyme degrades, two main cleavage products are commonly observed; of 55kDa and 25kDa. The affect of basic polypeptides on the phosphorylation of these cleavage products is varied; for the 55kDa cleavage product, neither polylysine or protamine were found to have a stimulatory affect, and for the 25kDa cleavage product autophosphorylation is stimulated by both protamine and polylysine. Cleavage products associated with HSV-2 R1 have been previously noted; 55kDa (Chung *et al.*, 1989) and the smaller 25kDa product (Luo *et al.*, 1992). Differences in the autophosphorylation pattern of these cleavage products when compared to full length R1 may represent different accessibility's of either the active site or the site of phosphate transfer in these truncations. The interaction of protamine or polylysine with the 55kDa cleavage product does not affect the autophosphorylation of this polypeptide, this cleavage product may have adopted a structure which allows the autophosphorylation to be optimal and failure of either polylysine or protamine to stimulate autophosphorylation suggests that interaction with these sites can not be made more accessible, optimal phosphorylation is observed in the absence of the basic polypeptides. Formation of the 25kDa cleavage product, which may be the stable protein kinase domain of R1 may lead to a more tightly complexed molecule in which, as proposed for the full length, molecule autophosphorylation can be stimulated by basic polypeptides.

HSV-2 R1 differs from that of HSV-1 R1 in that it can transphosphorylate exogenous substrates (Chung *et al.*, 1989; Chung *et al.*, 1990). The substrate chosen for this assay of the *E.coli* expressed HSV-2 R1, histones, was previously used to analyse the transphosphorylating activity of HSV-1 R1 (Conner *et al.*, 1992b). Initially transphosphorylating activity associated with HSV-1 R1 was shown to be a contaminant when the amino terminal truncated R1 dN245 was also shown to have

transphosphorylating activity. The transphosphorylating assay of HSV-2 R1 expressed in *E.coli* showed that HSV-2 R1 did not posses any transphosphorylating activity with histones as a substrate. The autophosphorylation of the HSV-2 R1 was however affected by the addition of histones to the assay, in the presence of protamine histones lead to an even greater increase in autophosphorylating activity. Whereas in the absence of basic polypeptides, or in the presence of polylysine, histones acted to inhibit autophosphorylation. Histone inhibition of HSV-2 R1 autophosphorylation has been noted previously for a 29kDa amino terminal truncation of HSV-2 R1 expressed in *E.coli* (Luo *et al.*, 1991). In contrast to the full length R1 autophosphorylation, the phosphorylation of the 55kDa cleavage product was not affected by histones, which raises the obvious question of whether or not this phosphoprotein is directly related to HSV-2 R1. Without any HSV-2 amino terminal specific antisera this protein can not conclusively be shown to be related to HSV-2 R1. Comparison of the profiles of type 1 and type 2 R1 purified by the same purification scheme suggests that the 55kDa protein is not a contaminant *E.coli* protein which also binds to the column, as this protein would also be observed in the HSV-1 preparations. It is, however, possible that it is an *E.coli* protein which specifically interacts with HSV-2 R1. The 25kDa cleavage product is a feature of both HSV-1 and HSV-2 R1. In the case of HSV-1 that this protein is a cleavage product was shown by Western blot analysis, this product is observed in preparations of both the dC449 truncation and with full length R1. The 25kDa protein may be a result of R1 cleavage after phosphorylation has occurred.

Stimulation of protein kinase activity by both histones and protamine has been noted for the cellular protein kinase, caesin kinase II (Palen and Traugh, 1991). For casein kinase II it has been proposed that transient interactions between this protein kinase and specific intracellular compounds stimulate the autophosphorylating activity of casein kinase II, autophosphorylation leading to the up-regulation of the transphosphorylating activity of this enzyme.

Comparison of the activities of the type 1 and type 2 R1 *in vitro* shows that they are similar, it is possible they also play a similar *in vivo* function.

3D Construction and analysis of cell lines expressing the HSV-1 R1 unique amino terminal region

3D.1 Construction of expression vector

To constitutively express the HSV-1 R1 N-terminal region, the unique amino terminal region was cloned into a retroviral vector. The vector chosen contains two copies of the Moloney murine leukaemia virus LTR. Transcription is initiated from a TATA box in the U5 region of the retroviral LTR and a polyadenylation signal is located in the repeat (R) region of the downstream LTR. The truncated R1 open reading frame, amino acids 1-564 was cloned into the vector on a Xba1/Sal-1 fragment. The original vector was cut with BamH1 and HindIII, both sites were rendered blunt using Klenow; the vector was then treated with calf intestinal phosphatase to reduce vector background in ligation. The R1 truncation was prepared by Xba1/Sal-1 digestion of pYN1-SD (Figure 3A.4) (Xba1 site located within the T7 promoter, Sal1 site located between amino acids 564 and 565), see Figure 3D.1.

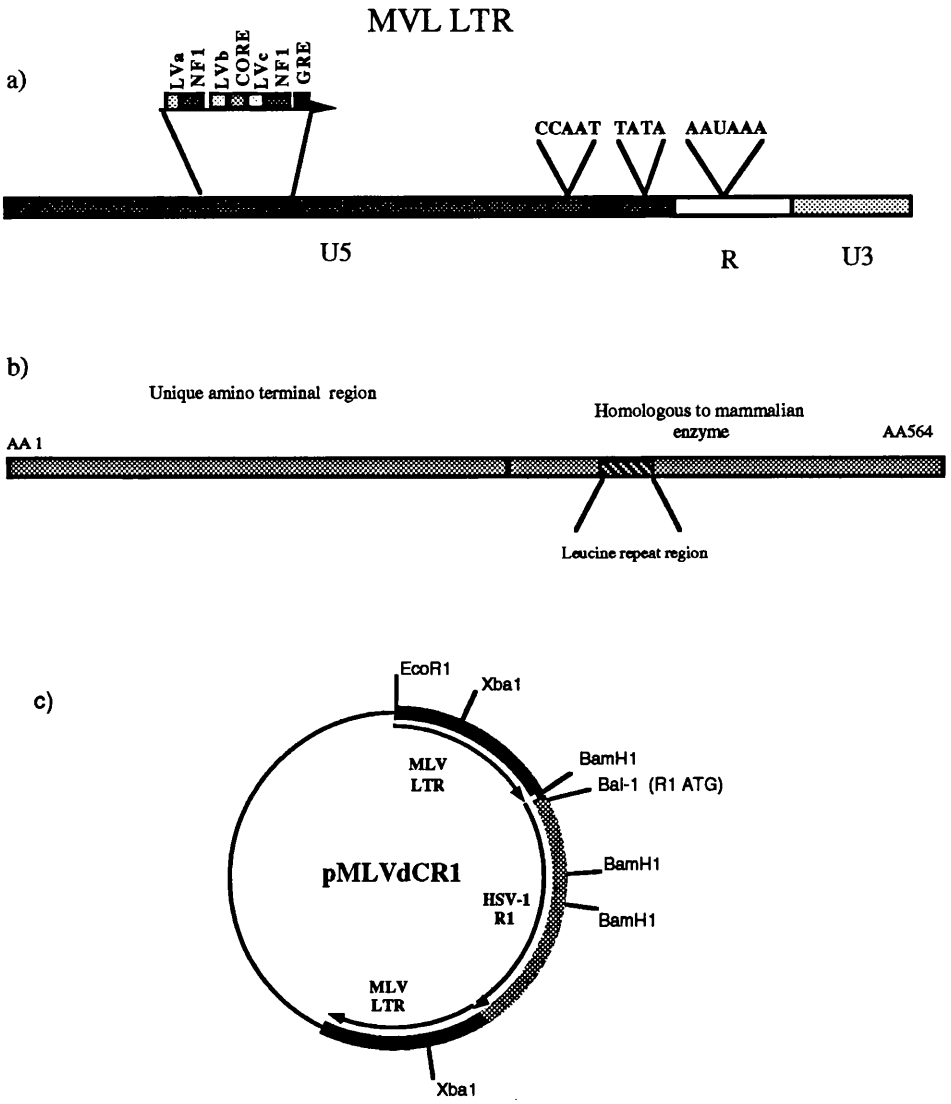


Figure 3D.1 Construction of vector for the expression of HSV-1 R1 in mammalian cells.

a) Shows the Moloney murine leukaemia virus long terminal repeat region. In the unique 5′ region, sites for upstream activator binding are located, these include the glucocorticoid response element (GRE), nuclear factor 1 (NF1) and the leukaemia virus specific binding factors (LVa, LVb and LVc). The TATA box is also located within the unique 5 region and the polyadenylation signal is located within the repeat (R) region of the LTR.

b) Shows the region of HSV-1 cloned into the retroviral vector, this region includes the unique amino terminal region (AA 1-325) and the leucine repeat region located downstream of the unique region in the region which shows the greatest conservation to the HSV-2 enzyme (98%), and where homology to the mammalian R1 enzyme begins.

c) Shows the vector used to express the amino teminal region in mammalian cells, the truncated R1 open reading frame was cloned into this vector on an XbaI/SalI fragment derived from pYN1 (see Figure 3A.4).

3D.2 Construction of cell lines

Expression from the plasmid containing the amino terminal region of HSV-1 R1 was first confirmed by Western blot analysis, cell lines were then constructed. Plasmid pMLVdCR1 was co-transfected into Rat-1 cells with p61, a plasmid in which the neomycin gene is expressed from an HSV-1 tK promoter (see Figure 3D.2). Transfections were carried out with varying concentrations of plasmid; ratio of pMLVdCR1:p61 ranging from 1:10 to $1:10^3$. DNA was transfected overnight, plates were then washed with serum-free medium. Once plates became confluent, G418 (Gibco) was added to the plates at a final concentration of 400μg/ml; the concentration of G418 used was determined by optimising the amount required to kill Rat-1 cells within 7 days, at this concentration, resistant colonies are able to proliferate with minimum impairment caused by G418. Colonies were grown until it was possible to pick them easily using a cloning ring (typically 7-14 days). Clones were expanded and frozen stocks made prior to analysis.

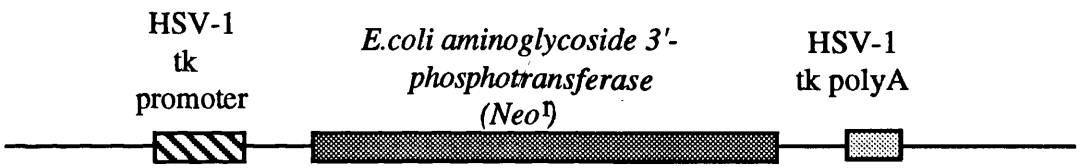


Figure 3D.2 Neomycin resistant cassette in plasmid p61. The neomycin resistance gene, from the *E.coli* transposable element Tn5, aminoglycoside 3'-phosphotranferase was expressed from the HSV-1 tk gene promoter, downstream processing was mediated by the HSV-1 tk polyadenylation sequences.

3D.3 Analysis of cell lines

3D.3.1 Expression of truncation in neomycin resistant cell lines

Each cell line was screened for the expression of the truncation by Western blot analysis. Cells were seeded on 10cm culture plates and allowed to reach confluence. To determine whether expression could be enhanced by a synthetic glucocorticoid, (MLV LTR U5 region contains a glucocorticoid response element), dexamethasone was added to the medium at a final concentration of 10μg/ml. Cells were incubated with dexamethasone for 24hr prior to harvesting. Monolayers were harvested as described in Materials and Methods and extracts were resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and the blot was probed with the polycolonal antisera 77 raised against full length R1 expressed in *E.coli*. The Western blot (using Amersham ECL detection (Figure 3D.3a,b)) shows bands unique to cell lines when compared to the parental cell line, Rat-1,

within the predicted Mwt range of 65kDa. The majority of cell lines do not show induced levels of expression in the presence of dexamethasone suggesting that in the majority of cases the GRE element does not respond to dexamethasone (see Figure 3D.3a,b). Two lines are stimulated by dexamethasone, line 1A and 3A (lanes 5 and 9, gel a). The high precentage of positives is probably due to the efficiency of the transfection reagent used, a lipofection reagent DOTAP and the final ratio of pMLVdCR1:p61 used was $1:10^3$. *

* Two independently transfected plates of Rat-1 cells were used for the isolation of the cell lines. The isolated clones, a and b, refer to the different plates transfected.

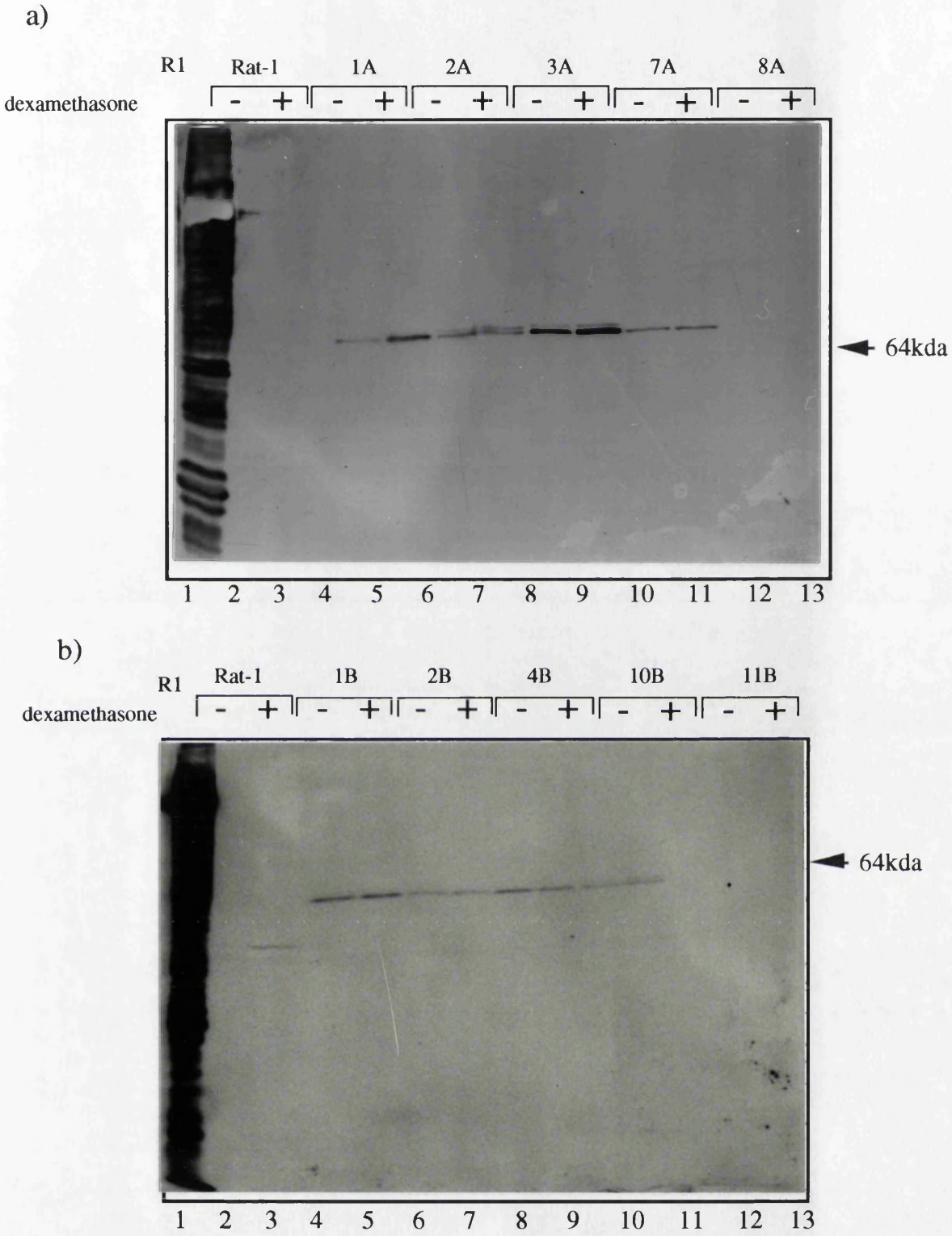


Figure 3D.3 Western blot of extracts from cell lines. Neo^r cell lines, ten shown above, were plated out on 100mM plates and grown till confluent, the synthetic glucocorticoid dexamethasone was added to a final concentration of 10µg/ml (samples shown in lanes 3, 5, 7, 9, 11 and 13) 24hr prior to harvest. Plates were washed twice in ice cold PBS and harvested in 500µl of 1x boiling mix, 100µl of each sample was loaded onto a 10% SDS-PAGE, transferred to nitrocellulose and probed with a polyclonal antibody, 77, raised against full length *E.coli* expressed HSV-1 R1. a) Shows Western blot of A isolates, b) Shows Western blot of B isolates

3D.3.2 Copies of R1 truncation integrated into the cellular genome

Genomic DNA was prepared as described in Materials and Methods. DNA, 10μg, was digested overnight with Xba1 or EcoR1. Xba1 sites are located within the LTRs flanking the truncated R1, digests with Xba1 will give an indication of whether the promoter and downstream regions are intact and how the plasmid has integrated into the genome. EcoR1 has a single site in the vector backbone, digests with EcoR1 should therefore give an indication of the number of copies of insert/cell and also whether the plasmid has inserted into the genome as concatamers. Concatameric DNA will produce a band of 5.6Kb, the size of the plasmid, in an EcoR1 digest. Digests were resolved overnight on a 0.8% 1xTBE gel. The gel was photographed to determine the extent of digestion and then transferred onto a nylon membrane. The blot was hybridised overnight with an R1 specific DNA probe (see Figure 3D.4).

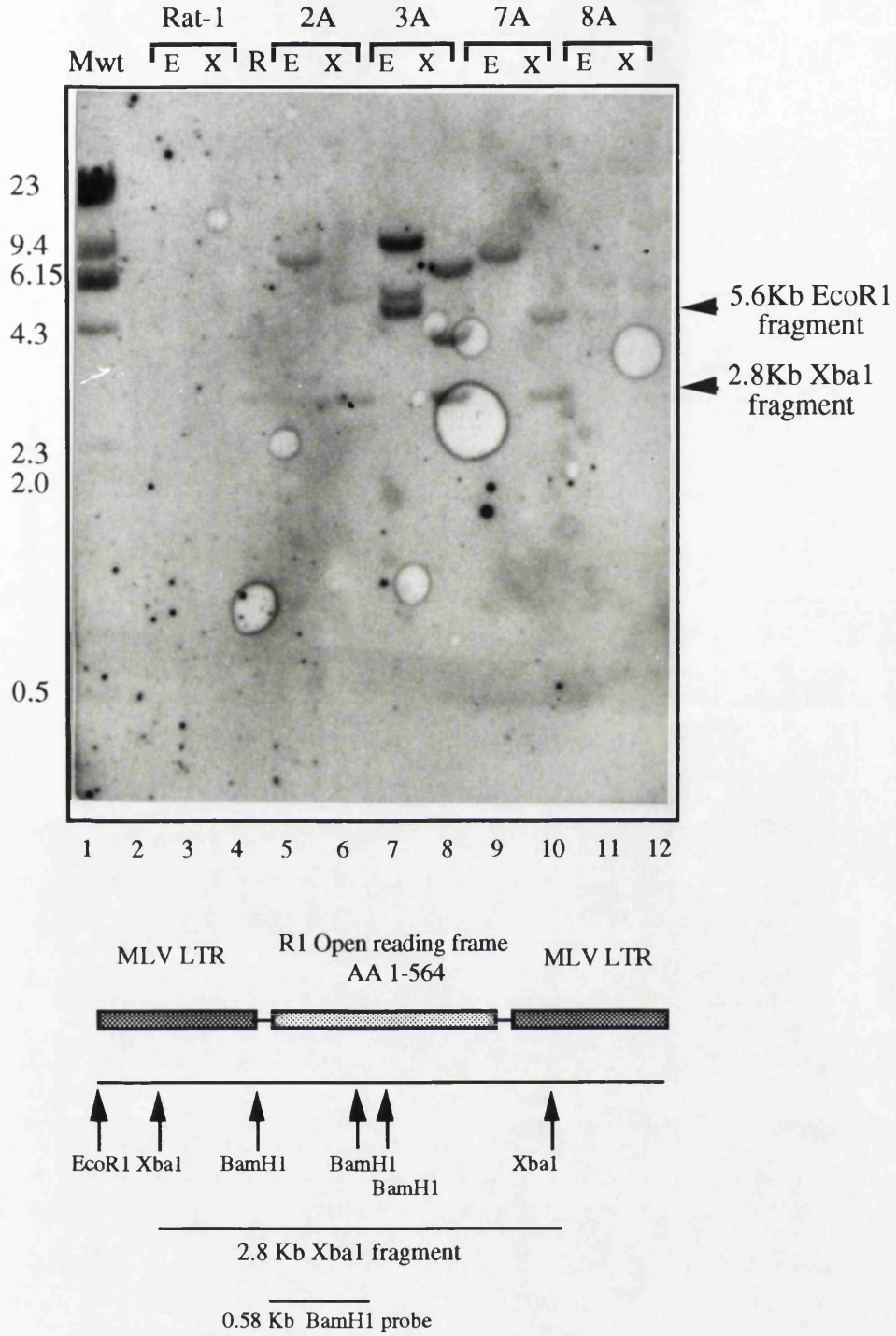


Figure 3D.4 Southern blot of genomic DNA isolated from cell lines. Genomic DNA, 10μg, was digested with the restriction enzymes Xba1(X) or EcoR1(E). Lanes 5 and 6 are DNA isolated from line 2A and digested with Xba1 or EcoR1 respectively, lanes 7 and 8 are line 3A, lanes 9 and 10 are 7A and lanes 11 and 12 are line 8A. Transformed cell lines are compared to control Rat-1 cells, lanes 2 and 3. Lane 4 (R) is a reconstruction containing 1 genome equivalent of pMLVdCR1 DNA. The location of restriction sites, EcoR1 and Xba1 with respect to their location in the R1 truncation and LTRs, used to digest genomic DNA is shown, as are the BamH1 sites used for the 0.58Kb probe.

The Southern blot shows that several cell lines contain the R1 insert in multiple copies. The highest plasmid copy number of the four lines analysed is observed in line 3A (lane 7,

Xba1 digest and lane 8, EcoR1 digest); when compared to the genome equivalent reconstruction in lane 4 it is apparent that in this cell line the plasmid has integrated as multiple copies; other lines analysed, 2A (lanes 5 and 6), 7A (lanes 9 and 10) and 8A (lanes 11 and 12) are positive but the copy number is not as high as in 3A. The restriction enzymes used, EcoR1 and Xba1, cleave once or twice per plasmid respectively. In lines 2A, 3A and 7A bands of 2.8Kb are produced in an Xba1 digest, indicating that in these lines the plasmid has integrated in such a way that the upstream and downstream LTR sequences are maintained at least partially. In an EcoR1 digest of these lines both 2A and 7A contain one major band, this indicates that in these lines the plasmid has integrated into a single site, that a band of 5.6Kb is absent in both these lines suggests that the plasmid has not integrated as a concatamer. From the restriction analysis of 2A and 7A it appears that there is only one copy of the truncated R1 open reading frame integrated per cell. The patterns of restriction digests for 2A and 7A are similar, the EcoR1 fragments co-migrate (Lane 5, line 2A and lane 9, line 7A), differences in the Xba1 digests are however apparent (lane 6, line 2A and lane 10, line 7A); the presence of more than one band in the Xba1 digests is most likely to be a result of incomplete digestion, that digests differ shows that the lines are not related. *

In line 3A several bands are apparent in both the EcoR1 and the Xba1 digests (lane 7, EcoR1 digest and lane 8, Xba1 digest). In the EcoR1 digest a band of 5.6Kb is present, this band which represents linear plasmid DNA indicates that the plasmid has integrated as concatamers. In addition to the 5.6Kb species, bands of a higher Mwt are apparent, these can in part be explained by partial digestion, see Figure 3D.5. The slowest migrating band in the EcoR1 digest has a Mwt greater than 9.4Kb and could be an 11.2Kb band produced by a partial. The middle band which cannot be explained by a partial digestion could be produced by the cleavage in a terminal plasmid at the site of integration and in a flanking genomic DNA. Several bands apparent in the Xba1 digest can be explained by the partial digestion of the concatameric DNA, a band migrating between 9.4Kb and 6.15Kb is probably an 8.4Kb partial fragment. As proposed for the EcoR1 pattern the middle band in the Xba1 digest probably represents cleavage in the genomic DNA and in the terminal plasmid of the concatamer.

* The highest copy number of the four lines analysed is seen in line 3A; when compared to the genome equivalent in lane 4 it is apparent that in this cell line the plasmid has integrated at least 100 times; other lines analysed, 2A, 7A and 8A are.positive but the copy number is not as high as 3A, estimates of copy number for each line are for 2A, 10; for 7A 50 copies/cell and for line 8A less than 1 copy/cell.

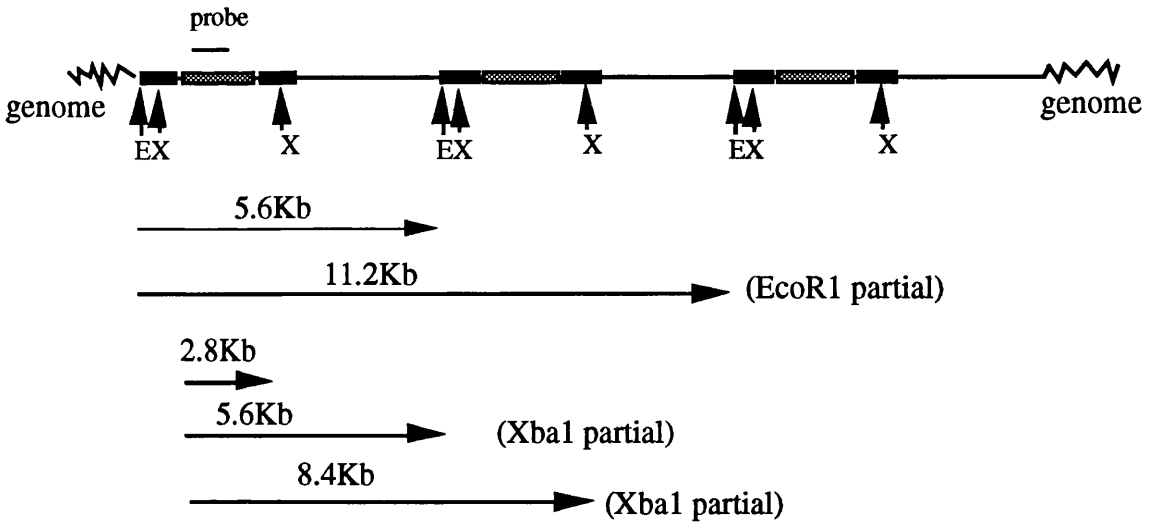


Figure 3D.5 Possible partial digest bands in line 3A. The pattern of partial restriction bands occurring in line 3A which appears to be the only line analysed which has integrated the plasmid as concatamers is shown.

The Southern blot data confirms the Western blot data in that the high expression line 3A has the highest DNA copy number with the plasmid integrated as concatamers.

A weak signal is observed in line 8A (Figure 3D.4, lanes 11 and 12), in this particular line the plasmid copy number is less than one per cell. The DNA is present but the expression of the R1 truncation was not apparent by Western analysis (see Figure 3D.3).

3D.4 Sub-cellular localisation of the amino terminal region in cell lines

3D.4.1 Crude subcellular fractionation

Cells were seeded on 10cm plates, plates were set up in duplicate for whole cell fraction comparisons and the effect of dexamethasone by increasing the amount of protein, on localisation was also determined. Monolayers to be fractionated were incubated in hypotonic buffer, homogenised and spun for 1 hr at 100K. Pellets were resuspended in 0.9ml of 1x boiling mixture; soluble fractions were made up to a volume of 0.6ml and 0.3ml 3x boiling mixture was added. Whole cell extracts were incubated and harvested in 0.6ml of isotonic buffer. Equal volumes of each sample; whole, particulate and soluble were loaded onto a 10% SDS-PAGE. Proteins were transferred onto nitrocellulose and probed with polyclonal antibody 77.

Figure 3D.6 shows the autoradiograph of the Western blot (using Amersham ECL detection). In the high expression line, 3A, the majority of the amino terminal region

localises to the soluble fraction (lane 7); when expression is enhanced by dexamethasone a substantial amount of the amino terminal region is detected in the particulate fraction (lane 9).

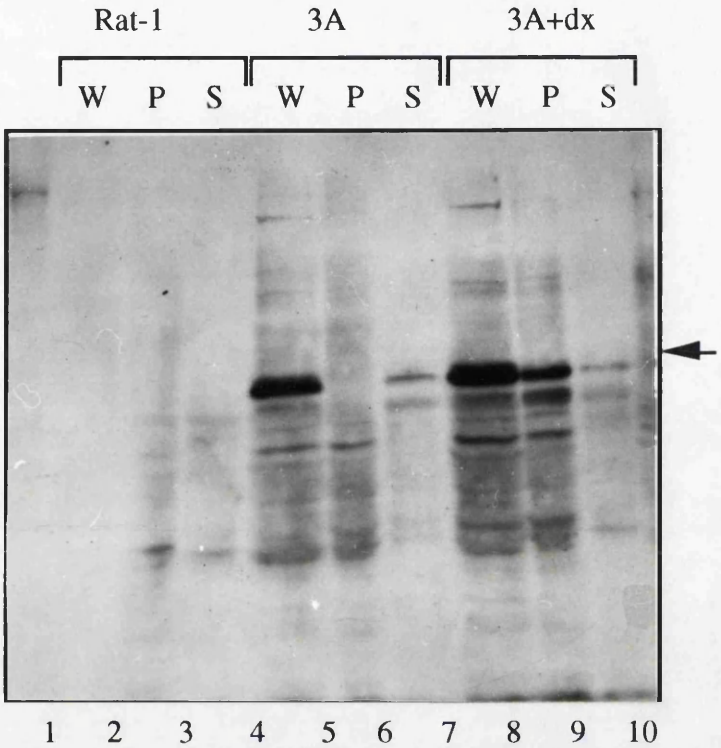


Figure 3D.6 Western blot of cell line 3A separated into particulate and soluble fractions. Equal total numbers of cells were harvested, for whole fractions (W, see lanes 2, 5 and 8), monolayers were harvested in 1X boiling mix, fractions of cells were prepared by harvesting cells, lysing and removing insoluble material by centrifugation, pellets were resuspended in a volume of buffer equivalent to that of the supernatant.

3D.4.2 Localisation of HSV-1 R1

The intracellular localisation of R1 in both infected cells and in cell lines was investigated by immunoflouresence. The localisation of R1 in cells infected with the wt and the mutant tsK was determined. In a tsK infection at the non-permissive temperature, immediate early genes are over-expressed, R1 although not a typical immediate early is also over-expressed. Using this virus the effect of the small subunit, R2 on the localisation of R1 can be determined. During infection R1 undergoes proteolytic cleavage (Ingermarson and Lankinen, 1987), it is possible that once it has undergone cleavage the localisation of the cleavage products may differ from that of full length R1, by comparing the localisation of the amino terminal region in cell lines to its localisation in the infected cell any role of the C-terminal sequences on its localisation can be determined.

3D.4.2.1 Localisation of R1 in infected cells

Infections using either wt or tsK were carried out in BHK cells seeded onto coverslips. Coverslips were then incubated with the R1 specific monoclonal antibody 1026. Infections using the tsK mutant were carried out at either the non-permissive temperature (39°C) or the permissive temperature (31°C) and allowed to proceed for 4 hr. Infections with wt virus were carried out at 37°C, and to allow expression of R2, infections were carried out for 8 hr the pattern of R1 localisation in wt infected cells is shown in Figure 3D.7 (a and b)

Figure 3D.7 (a) Mock infected BHK cells
(photographed at 40x magnification)

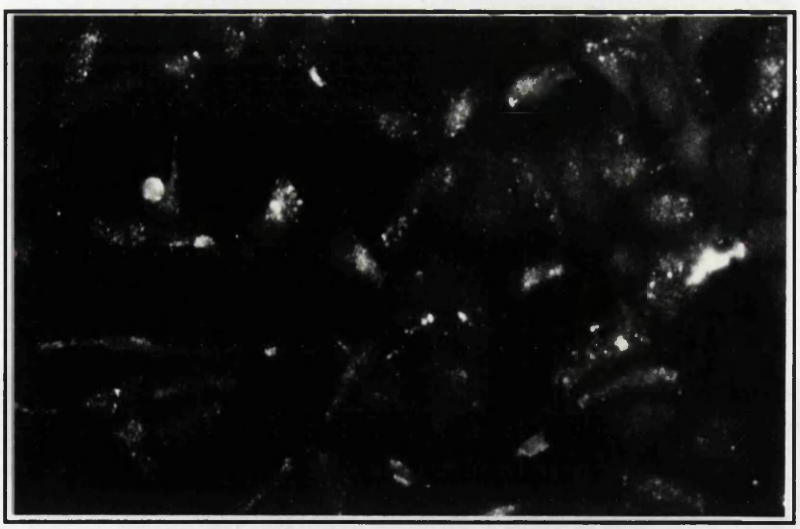


Figure 3D.7 (b) wt infected cells. Localisation of R1 in cells infected with wt virus at 10pfu/cell, fixed at 8 hr post-infection. Stained with mAb 1026.
(photographed at 40x magnification)

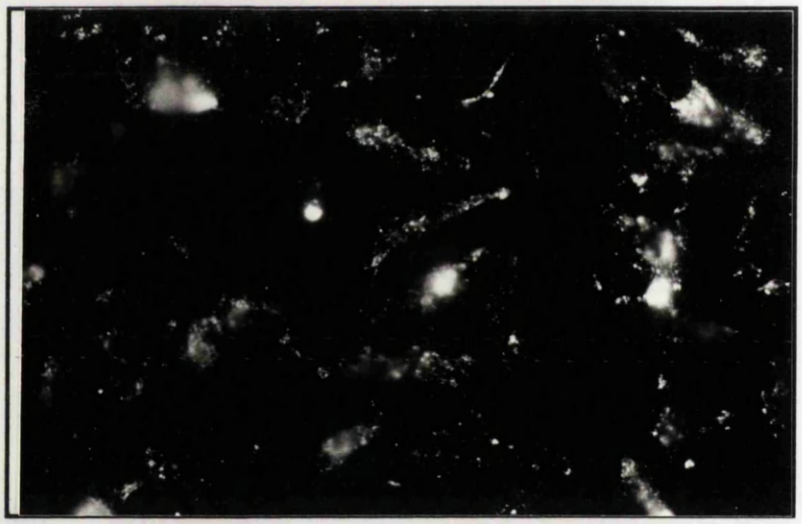


Figure 3D.7 (c) wt infected cells.(31°) Localisation of R1 in cells infected with wt virus at 10pfu/cell, fixed at 8 hr post-infection. Stained with mAb 1026. **(photographed at 40x magnification)**

In cells infected with the wt virus R1 localises throughout the cell. The pattern of staining is punctuate with discrete spots of fluorescence observed. Differences in the level of staining between cells can be explained by differences in the uptake of virus between cells, cells showing weak fluorescence being infected with fewer virus particles.

3D.4.2.2 Localisation of R1 in BHK cells infected with tsK

The localisation of R1 in cells infected with tsK at both the permissive and non-permissive temperature was determined, see Figure 3D.8 (a and b, permissive temperature) and Figure 3D.9 (a and b, nonpermissive temperature)

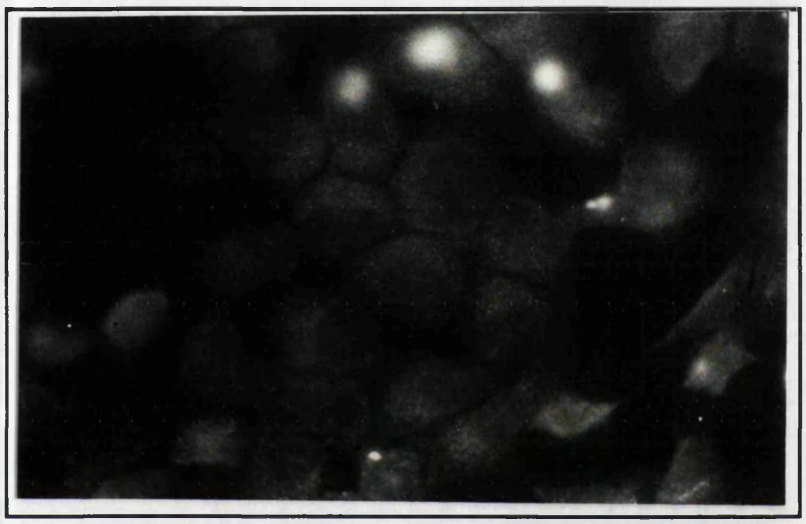


Figure 3D.8 (a) Mock infected BHK cells (31˚C)
(photographed at 60x magnification)

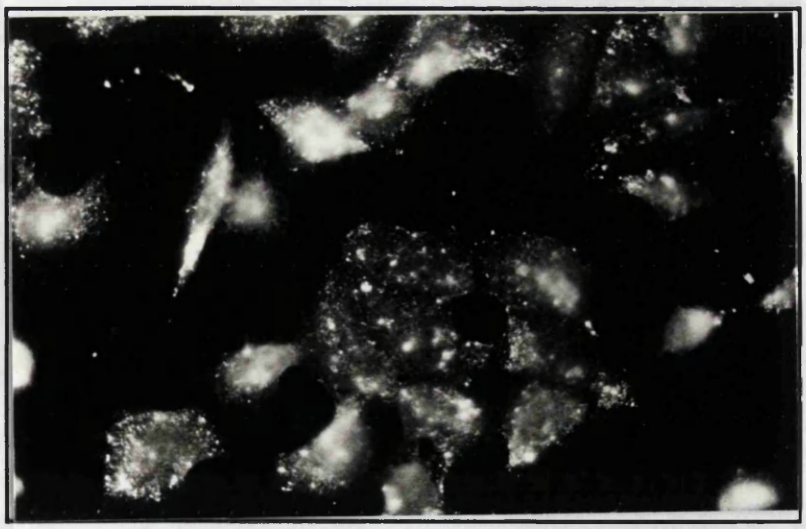


Figure 3D.8 (b) BHK cells infected with tsK (31˚C). Cells were Infected with tsK at infection was allowed to proceed for 4 hr before fixing, cells were stained with mAb1026. **(photographed at 60x magnification)**

Mock infected cells show a grainy background. In cells infected with tsK at the permissive temperature, as found for infection with wt, R1 is localised to discrete sites located throughout the cell, with in some cells a concentration of antigen is observed around the nucleus.

Figure 3D.9 Mock infected BHK cells (39˚C).
(photographed at 60x magnification)

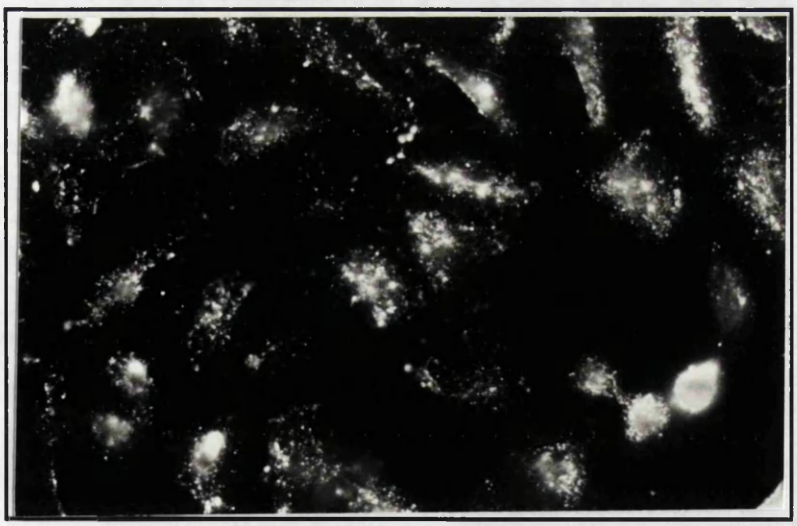


Figure 3D.9 (b) BHK cells infected with tsK (39˚C). Cells were infected with tsK, infection was allowed to proceed for 4 hr before fixing. Cells were stained with mAB 1026.
(photographed at 60x magnification)

The localisation of R1 in cells infected with tsK at 39˚C is similar to that observed in cells infected with tsK at the permissive temperature and cells infected with wt virus. The

localisation of R1 does not appear to be affected by the presence of the small subunit which is absent in the tsK non-permissive infection. In all infections the localisation is throughout the cell, and is in some cells a concentration around the nucleus.

3D.4.2.3 Localisation of N-terminal region in stably transformed cell lines

With the cleavage of R1 between the unique N-terminal region and the conserved reductase regions it is possible that the N-terminal region may show a different intracellular localisation to full length R1. Using the stably transformed cell lines the localisation of the N-terminal region was investigated. Cells were seeded onto coverslips and allowed to become 60-70% confluent before being paraformaldehyde fixed, cells were then stained with the R1 specific monoclonal antibody used in infected cells. The localisation of the N-terminal region was investigated in three independently isolated cell lines, which were shown to express this truncation (section 3D.4), the localisation of this region is shown in Figure 3D.10 (a to d).

Figure 3D.10 (a) Rat-1 cells stained with mAb 1026
(photographed at 100x magnification)

Figure 3D.10 (b) Localisation of N-terminal region in line 2A.
(photographed at 100x magnification)

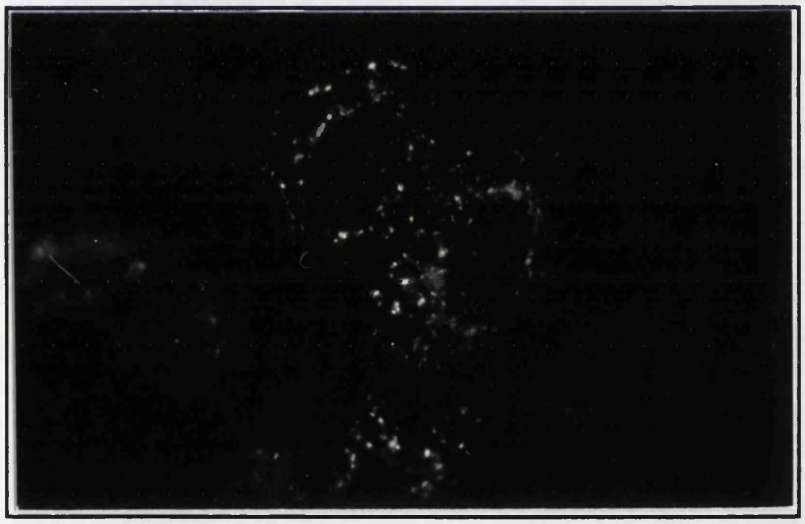


Figure 3D.10 (c) Localisation N-terminal region in cell line 3A.
(photographed at 100x magnification)

Figure 3D.10 (d) Localisation of N-terminal region in cell line 8A.
Cell lines (a to d) were stained with mAb 1026
(photographed at 100x magnification)

In all cell lines investigated the N-terminal region shows an almost exclusive cytoplasmic localisation. As found for R1 in infected cells the N-terminal region localises to discrete areas of the cytoplasm, the pattern in the cell lines is however more punctate; the punctate spots are more discrete than observed in the BHK infected cells, it is unlikely, although possible, that the cell lines (Rat-1 compared to BHK) may in some way influence the localisation. There is, as observed in some infected cells, a concentration of the punctate spots in the vicinity of the nucleus, and in certain cells weakly fluorescent spots are observed within the nucleus.

3D.5 Growth of HSV-1 on cell lines.

The effect of the constitutive expression of the N-terminal region of R1 on the growth of HSV-1 was determined. Cell lines were infected with 5pfu/cell of HSV-1 (17+) and the amount of cell-released virus produced after certain time points was determined. Infections were allowed to proceed for 72 hr. A representative growth curve for such an experiment is shown below, Figure 3D.11, viral replication on the cell lines was compared to its replication on the parental cell line Rat-1.

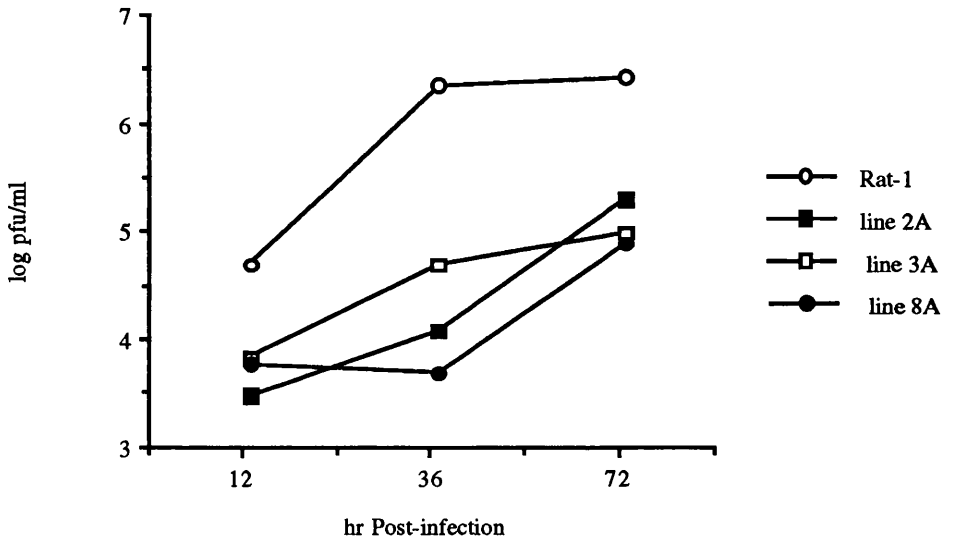


Figure 3D.11 Growth of HSV-1 on cell lines expressing the N-terminal region of HSV-1 R1. Three independently isolated cell lines were analysed for the effect of constitutive expression of the R1 N-terminal region, the lines tested 2A, 3A and 8A are compared to the parental line Rat-1, the key indicates the relevant curves for each line.

Compared to the parental line Rat-1 all three cell lines analysed show a reduced ability to replicate HSV-1, or possible to release HSV-1. The amount of virus produced at between 12-36 hr is considerably lower than observed for Rat-1 cells. Throughout infection the cell lines were monitored for effects of infection, in particular it was noted that even after 36 hr there was no obvious cytopathic effect, and at 72 hr the amount of visible plaques apparent were low, the plaque number for the 72 hr timepoint was counted for each line, the line 2A contained the largest number of plaques (>200/60mm plate) and line 3A having the lowest number of plaques (<100/60mm plate). The plaques appeared morphologically normal. The amount of plaques produced at the 72hr time point does not correlate with amount of virus released, as analysed by the single step growth curve, the virus titre does not show a significant increase between the 36 hr time point when no plaques were observed and the 72 hr time point when plaques become apparent. It is possible that the few plaques observed are virus replicating in cells which are neomycin resistant.but lack the N-terminal region. The virus may effectively adsorb to the cells in the 1 hr period normally used for infections and later be released into the medium without penetrating the cell. Further experiments are needed to determine the exact mechanism by which the cell lines reduce cell-released virus yields, it may be that the block is only in the release of the virus from these lines and that had the cell-associated virus been determined the yield may not differ.

3D.6 Discussion

The association of the HSV-2 R1 N-terminal region with plasma membranes has been demonstrated (Chung *et al.*, 1990), to determine if the HSV-1 R1 N-terminal region shows a similar localisation the intracellular localisation of both full length R1 in the infected cell and the N-terminal region in stably transformed cell lines was determined. The pattern of localisation in both was similar, localising to discrete areas of the cytoplasm with a concentration of foci around the nucleus. The identity of these sites has yet to be determined. In the infected cell R1 shows a very strong staining around the nucleus this could possibly be the Golgi. However in the cell lines this very strong peri-nuclear staining is not observed although the foci are more concentrated around the nucleus. It is possible that these discrete areas are small cytoplasmic organelles, similar to endosomes, small membrane structures are found in the cytoplasm. Like the mammalian reductase large subunit of HSV-1 R1 localises to the cytoplasm (Engstrom *et al.*, 1984) although the mammalian enzyme is distributed throughout the cytoplasm rather in foci. The crude fractionation experiment, in which cells were separated into soluble and particulate fractions showed, that in cell lines investigated, the N-terminal region although mainly soluble was present in the particulate fraction. This may indicate that the association of the N-terminal region with membranes is transient or weak, dissociation from membranes occurring relatively easily. Membrane association may be mediated by a lipid, through anchoring mediated by the lipid. HSV-2 R1 myristolyation (Chung *et al.*, 1990) and potential lipid modification have been identified in HSV-1 R1.

Perhaps one of the most interesting findings in the analysis of the stably transformed cell lines is their ability to impair the replication of HSV-1. The amount of virus released from cell lines infected with HSV-1 is reduced by 100-fold when compared to the parental cell line, Rat-1. Virus release is also delayed even after 72 hr the amount of virus released can not be compared to the amount of virus released after 12 hr in the Rat-1 cells. To determine the exact role of the N-terminal region in blocking viral replication several experiments are needed to determine the efficiency of uptake into these cells as well as the expression of the different virus genes.

* A region rich in aspartic acid residues located between 190 and 234 may mediate the interaction of the N-terminus with the basic polypeptides. This region also rich in serine residues probably contains the site of the N-terminal region autophosphorylation.

4. General Discussion

The large subunit of HSV ribonucleotide reductase is unusual in: a) its pattern of expression, being expressed in the presence of cycloheximide, unlike the small subunit which is a typical early gene (Clements *et al.*, 1977), expression of R1 does not require *de novo* synthesis of viral proteins, b) the simplex members of the alphaherpesviruses subfamily contain a 325 amino acid extension at their amino terminus, which shows no homology to any other reductase large subunits which have been sequenced (Nikas *et al.*, 1988; Chung *et al.*, 1989), this region is not required for *in vitro* reductase activity (Conner *et al.*, 1992).

R1 autophosphorylation: self-regulation of protein kinase activity or another activity?
Comparison of the autophosphorylating activities of HSV-1 and HSV-2 R1 both overexpressed in *E.coli* and purified to apparent homogeneity using the same purification scheme, showed that there are little, if any, differences in the autophosphorylation activities. Basic polypeptides which stimulate protein kinase autophosphorylation were assayed for their ability to stimulate HSV-2 R1 autophosphorylation. In contrast to previous reports (Chung *et al.*, 1990), where polylysine was shown to stimulate HSV-2 R1 autophosphorylation only protamine, the basic polypeptide reported to stimulate HSV-1 R1 autophosphorylation (Conner *et al.*, 1992b), had any affect on the autophosphorylation of full length HSV-2 R1. HSV-2 R1 autophosphorylation was further stimulated by the addition of histones to the assay, this further increase in actvity was observed only in the presence of protamine. Histone stimulation of protein kinase autophosphorylation has been reported previously; autophosphorylation of casein kinase II is stimulated by protamine, addition of histones to the assay leads to a further increase in activity (Palen and Traugh, 1991). Autophosphorylation of protein kinase C is also stimulated by both histones and protamine (Chauhan and Chauhan, 1992). This stimulation of protein kinase C autophosphorylation by basic polypeptides is thought to be physiologically important. Interactions between protein kinase C and basic polypeptides *in vivo* may modulate protein kinase C activity in the absence of its phospholipid activators. Protamine, a polypeptide rich in arginine residues, can stimulate both HSV-1 and HSV-2 R1 autophosphorylation *in vitro*. *In vivo* R1 autophosphorylation could be controlled by its interaction with cellular proteins rich in basic residues.*

Autophosphorylation is a way in which a protein can self-regulate its activity, it is an activity commonly associated with protein kinases. Autophosphorylation reactions have

also been described for proteins which are not protein kinases. Protein kinase autophosphorylation can be specifically stimulated by the binding of a regulatory ligand. With the dsRNA-dependent protein kinase the autophosphorylation is completely dependent on its interaction with dsRNA, only once dsRNA binds to this kinase and the kinase autophosphorylates can exogenous substrates be phosphorylated (Thomas and Samuel, 1993). Growth factor receptor autophosphorylation occurs only after it binds a specific ligand. Intramolecular autophosphorylation is a mechanism by which many protein kinases control their transphosphorylating activity. A serine/threonine protein kinase which specifically phosphorylates a protein phosphatase becomes 10-fold more active towards exogenous substrates after its autophosphorylation (Guo *et al.*, 1993).

Autophosphorylation has also been observed in proteins which do not function as protein kinases. The eukaryotic heat shock response protein, hsp90, autophosphorylates (Csermely and Kahn, 1991). Phosphorylation of hsp90 was shown to be substrate-specific, no exogenous substrates tried were phosphorylated by hsp90. The autophosphorylation of hsp90 was shown to be stimulated by basic polypeptides, in particular histones, the interaction with histones has been proposed to be physiologically important. A conformational change induced by the phosphorylation of hsp90 may mediate its interaction with steroid receptors and other proteins (Csermely *et al.*, 1993). Notably the hsp90 ATP binding site shows no homology to ATP binding sites in protein kinases, but is however typical for a heat shock protein. The autophosphorylation of R1 may be important in modulating a non-kinase activity associated with the N-terminus, that R1 may not be a protein kinase can not therefore be ruled out.

An alternative catalytic core in the HSV R1 kinase?

The intrinsic autophosphorylating activity of HSV-1 R1 (Conner *et al.*, 1992b) is a feature of the unique amino terminal region, as previously shown for HSV-2 R1 the unique amino terminus (Luo *et al.*, 1991; Luo and Aurelian 1992; Ali et *al.*, 1992). Regions important in the autophosphorylating activity of HSV-1 R1 were mapped by both insertion and deletion mutagenesis. C-terminal deletion of the unique amino terminal region to amino acid 257 showed that the basic catalytic core must be completely contained within this region. C-terminal deletion to amino acid 257 removes amino acids which have been postulated to be of catalytic importance (Chung *et al.*, 1989). That the 257 deletion is active indicates that original predictions are inaccurate. A protein kinase catalytic core normally spans some 200 amino acids, that the HSV-1 catalytic core is contained within the region between the proposed nucleotide binding motif at amino acid 114 and residue 257, a distance of around 140 amino acids is unlikely *

* and therefore the N-terminal region of HSV-1 R1 must contain an alternative nucleotide binding site.

Residues critical for protein kinase function, subdomains, were identified firstly by sequence comparisons (Hanks *et al.,* 1988) and latterly by site-directed mutagenesis (Gibbs and Zoller, 1991). A protein kinase can be separated into two functional regions. An ATP binding region and a region which directly facillitates phosphate transfer. The ATP binding site typically contains a glycine triad, which directly interacts with ATP, and downstream a lysine and a glutamic acid residue which stabilise the binding of ATP. The lysine and glutamic acid residues are essential for protein kinase activivty. Residues that are practically invariant in all protein kinases are shown in Figure 4.1(a). There are rare examples of protein kinases which do not have a typical nucleotide binding glycine triad, with some lacking one or two of the glycines (Taylor, 1992). Mutation of this region in several protein kinases can produce a range of affects on kinase activity, activity can be completely destroyed (Odawara et *al.,* 1989), or significantly up-regulated (Pelley *et al.,* 1989). Protein kinase has been described which lack one, two or all three glycines (Herman *et al.,* 1991). It is possible that in both HSV-1 and HSV-2 R1 the nucleotide binding motif predicted is incorrect, and that another region upstream of the predicted site binds ATP. Residues which are crucial in protein kinase function are; a lysine typically located within the 23 amino acids downstream of the nucleotide binding site and a glutamic acid residue. Together they form an ion pair which promotes the formation of a stable complex between the kinase and the phosphate, mutation of the lysine destroys activity (Gibbs and Zoller, 1991).

Insertion into the proposed nucleotide binding site of the R1 unique amino terminal region leads to decreased activity, however as mentioned previously the expression of this particular mutant was poor, so differences can in part be explained by the reduced amount of this particular mutant. Deletion of a region spanning the nucleotide binding site, from amino acids 43 to 136 completely destroys activity, indicating that this region is essential for R1 autophosphorylation, and it is likely that this region contains residues important in catalysis. Mutation of both the proposed nucleotide binding site and downstream lysine residue in HSV-2 R1, only slightly reduces the activity (Luo and Aurelian, 1992); indicating that phosphate transfer is mediated by either another residue or that the lysine prediction is incorrect.

The autophosphorylating activities of HSV-1 and HSV-2 R1 are similar, it is therefore reasonable to assume that their catalytic residues will be conserved. Within the N-terminus of HSV-1 R1 there is only one lysine and in HSV-2 there are two, one of which is positionally conserved with HSV-1. There is no glutamic acid residue within the normal spacing observed downstream of these lysines in HSV-1 and HSV-2, instead an aspartic acid residue, another acidic amino acid, is located. It is possible that in the tertiary structure the loss of a ethyl group (see figure 4.1 (c)) may be compensated for by the lysine

being in closer proximity to the aspartic acid residue. Potential residues located within the first 257 amino acids of HSV-1 R1 are shown in Figure 4.1 (b), the residues chosen as potential catalytic sites are conserved in HSV-2 R1 although the spacings between the residues differs slightly.

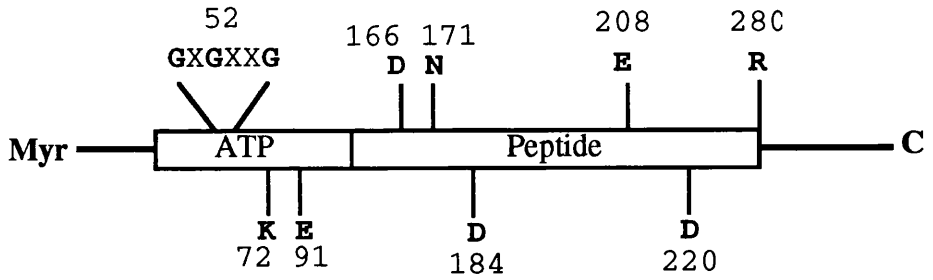
Notably, insertion into amino acid 22, some 100 amino acids upstream of the nucleotide binding site leads to a large reduction in activity. This region of the N-terminus may either be catalytically or structurally important in the autophosphorylation reaction, and it is possible that this region may fall into or close to another potential nucleotide binding site.

In vivo latency experiments with N-terminal HSV-1 mutants will determine the importance of this region in the establishment, maintenance and reactivation of the virus in the latent life cycle. If the current model for latency used for this type of analysis, the mouse foot-pad model, fails to show any differences with the N-terminal mutants compared to the wild-type, other models (dexamethasone and hypothermia induced reactivation models for example) which stimulate reactivation of HSV-1 in ganglia before ganglia explantation may produce different results. *In situ* hybridisation experiments could be used to determine the effect of mutation in the N-terminal region has on the expression of viral genes during reactivation.

If, as possible, mutation of the N-terminal region fails to have an affect on virus latency then biochemical analysis will be needed to determine the role of this region. Initially the interactions of this region with cellular/viral proteins will need to be characterised by co-precipitation experiments to determine if there are any strong interactions (pull down experiments with the N-terminus coupled to a matrix is an alternative way to carry out this type of analysis). The identity of the protein(s) interacting with the N-terminus can then be identified using the yeast two hybrid system allowing the interacting protein cDNA to be isolated and sequenced.

Once the N-terminal region has been purified to apparent homogeneity kinetic analysis can be carried out. With its purification the identification of the autophosphorylation site and residues involved in catalysis can be determined by site-directed mutagenesis. Sites which could be mutated include residues located around amino acids 257 which appear to function normally to down regulate activity and also, the lysine located at amino acid 59 which may be the actual site of phosphate transfer.

a)



b)

```
HSV-1 1 MASRPAASSPVEARAPVGGQEAGGPSAATQGEAAGAPLAHGHHVYCQRVN
        || |||||    || |  `||   |  |  |          | || |  |
HSV-2 1 MANRPAASALAGARSPSERQEPREPEVAPPG          GDHVFCRKVS

HSV-1 51 GVMVLSDKTPGSASYRISDSNFVQCGSNCTMIIDGDVVRGRPQDPGAAAS
         ||||||   || | ||||||| |||||||| |||||||  ||   |   | |
HSV-2 42 GVMVLSSDPPGPAAYRISDSSFVQCGSNCSMIIDGDVARGHLRDLEGATS

HSV-1101 PAPFVAVTNIGAGSDGGTAVVAFGGTPRRSAGTSTGTQ            T
           |||  |  || || ||||| |||   || || |||             |
HSV-2 92 TGAFVAISNVAAGGDGRTAVVALGGTSGPSATTSVGTQTSGEFLHGNPRT

HSV-1 140 ADVPTEALGGPPPPPRFTLGGGCCSCRDTRRRSAVFGGEGDPVGPAEFVS
                  ||||| |  |  ||  |  |       ||    || ||  |
HSV-2 142 PEPQGPQAVPPPPPPPFPWGHECCARRDAR      GGAEKDVGAAESWS

HSV-1 190 DDRSSDSDSDDSEDTD       SETLSHASSDVSGGATYDDALDSDSSSD
          |  ||||   ||   |       |||||  ||    |||  ||  |||| ||
HSV-2 186 DGPSSDSETEDSDSSDEDTGSGSETLSRSSSIWAAGATDDDDSDSDSRSD

HSV-1 234 DSLQIDGPVCRPWSNDTAPL          DVCPGTPGPGADAGGPSAVDPH
          || |  |  |||| ||                || || ||  |  || ||
HSV-2 236 DSVQPDVVVRRRWSDGPAPVAFPKPRRPGDSPGNPGLGAGTGPGSATDP

HSV-1 276 APTPEAGAFLAADPAVARDDAEGLSDPRPRLGTGTAYPVPLELTPENAEA
          |    |    ||  |    |     |  |  ||   |||||||||||||
HSV-2 286 AS   ADSDSAAHAAAPQADVAPVLDSQPTVGTDPGYPVPLELTPENAEA
```

c)

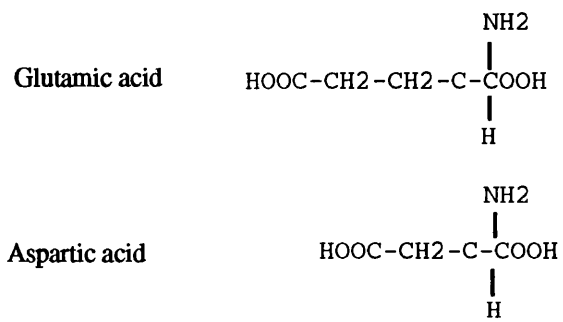


Figure 4.1 An alternative catalytic core in HSV-1 R1 amino terminal region. Residues which are conserved i n protein kinases are shown in (a), residues similar to those identified in (a), with the exception of a glutamic acid residue located within the N-terminal domains of both HSV-1 and HSV-2 R1 which may be catalytically important are shown in (b)in bold. The structure of glutamic acid and aspartic acid is shown in (c)

Regulation of HSV-1 kinase activity

A region which may be important in the control of R1 autophosphorylation has been identified; insertions into amino acid 257 and 262 increased R1 autophosphorylation, most apparent in the 257 insertion mutant, and C-terminal deletion to amino acid 257 also produced a significant increase in autophosphorylating activity. The region identified which down-regulates R1 autophosphorylation maps to between amino acids 257 and 292. Comparison of the autophosphorylating activities of the 257 deletion mutant with that of the 292 deletion shows that the increased activity of 257 is due to the removal of the additional C-terminal amino acids. The identification of a region which acts normally to down-regulate autophosphorylation is not surprising, in many protein kinases a region may impair autophosphorylation by masking either the active site or site of phosphate transfer.. In the human insulin receptor (a tyrosine kinase) a region of deletion of 115 amino acids lead to a increased ability of this receptor to autophosphorylate (Sung *et al.*, 1994), it has be postulated that this region acts to down-regulate receptor activity in the absence of insulin, insulin binding promotes a conformational change in this region. In the HSV R1 N- terminal domain a ligand interaction may be required to stimulate autophosphorylation by inducing of a conformational change which then releases active sites from inhibition by amino acids located around 257.

The importance of proteolytic cleavage in the R1 kinase activity

HSV-1 R1 undergoes proteolytic cleavage in infected cells, cleavage of R1 occurs in the region between the unique N-terminus and the conserved reductase portion of the protein (Lankinen *et al.*, 1989). The N-terminal region of R1 can be removed without affecting the *in vitro* reductase activity (Conner *et al.*, 1992a). This raises the question of the importance of the N-terminus in the *in vivo* reductase activity. The reductase regions of R1 may act to modulate the N-terminus kinase activity by either promoting or preventing its interaction with other proteins before the release of this region by proteolysis. Within the reductase portion of R1 there is a leucine repeat region (Chung *et al.*, 1991), which has been shown to be important in R1 dimerisation (Conner *et al.*, 1993), recently a cellular serine/threonine protein kinase with a leucine repeat region toward its C-terminus has been identified (Ing *et al.*, 1994; Ezoe*et al.*, 1994), the importance of this leucine repeat region in the activity or function of this cellular kinase is currently unknown. R1 dimerisation may modulte N-terminus autophosphorylation by preventing its interaction with other proteins or stimulatory ligands. Cleavage of R1 may alter N-terminal autophosphorylation, releasing the N-terminus and thereby promoting its interaction with its substrate(s). The expression of R1 at immediate early times in infection suggests that R1 may be an important virus regulatory protein, R1 may in fact be essential in the modulation of another immediate early protein function, by directly or indirectly influencing the phosphorylation state of an immediate early gene product. Of the four immediate early proteins which are

phosphorylated both Vmw63 and Vmw110 phosphorylation has been shown to occur later in infection than Vmw175 phosphorylation, which appears to be phosphorylated immediately after translation (Wilcox *et al.*, 1980). Vmw63 an arginine rich protein is a potential candidate for phosphorylation by the N-terminal kinase *in vivo*: arginine rich polypeptides stimulate the activity of both HSV-1 (Conner *et al.*, 1992b) and HSV-2 R1(section 3C.5.2). Alternatively, the substrate could be a cellular protein whose phosphorylation is required to modulate the functions of the immediate early proteins.

The N-terminus: its role in neurotropism

Both HSV-1 and HSV-2 R1 are expressed as immediate early virus proteins, the expression of R1 mRNA is not blocked by cycloheximide whereas R2 is a typical early virus gene, no mRNA is observed in the presence of cycloheximide (Clements *et al.*, 1977). The presence of R1 at immediate early times suggests that it plays a regulatory role as other HSV-1 immediate early gene products are known to be involved in regulation of virus gene expression in some way. Interestingly, consensus DNA sequences required for AP-1 binding have been identified within the promoter of both HSV-1 and HSV-2 R1. Transient expression assays with a plasmid containing the HSV-2 R1 promoter fused to the CAT reporter gene showed that expression of CAT was increased by co-transfection with a construct expressing c-jun (the DNA binding part of the typically bipartite complex), further increases were observed when the c-fos (strong transactivator part of complex) was also co-transfected (Wymer *et al.*, 1992). In other neurotropic viruses AP-1 elements have been identified in regulatory regions. In JC virus, a neurotropic virus which causes the demyelinating disease progressive multifocal leukoencephalopathy , AP-1 sites were shown to bind proteins from cell nuclear extracts; it was proposed that binding to this site may play a role in the control of basal expression from the promoter/enhancer (Amemiya *et al.*, 1992). In addition AP-1 may be involved in enabling HCMV (a lymphotropic herpesvirus) to replicate in the central nervous system (seen in patients with AIDS); a number of AP-1 elements have been identified in the HCMV immediate early gene 1 enhancer region (Sambucetti *et al.*, 1989).

C-fos expression has been extensively studied in the mouse nervous system reviewed in (Morgan and Curran, 1991), and recently a transgeneic mouse was made in which the c-fos promoter was placed upstream of the lacZ open reading frame (Smeyne *et al.*, 1992). Levels of c-fos were dramatically increased in the nervous system following treatment of these mice with various stimuli including the convulsants. Natural stimuli such as light promoted c-fos expression in localised regions of the brain, ganglia and retina (Smeyne *et al.*, 1992). C -fos is increased within trigeminal ganglia after explantation (Sharp *et al.*, 1989). It is possible that upon neuronal damage or stimulation that HSV R1 is expressed. The immediate early polypeptide Vmw110 contains within its intron several AP-1 sites

(Hayward, 1993), it is also possible that this intron functions as an enhancer region and Vmw110 expression may be stimulated by c-fos and c-jun induction in the neuron. Together Vmw110 and R1 may be important in the modulation of cellular proteins which in promote the expression of other virus genes. HSV-1 mutants in Vmw110 show only an impaired ability to reactivate from latency (Clements and Stow, 1989), Vmw110 is still believed to be important in efficient reactivation. C-fos and c-jun stimulation occurs when a cell is about to enter apoptosis, it seems plausible that if a neuron is undergoing apoptosis that the R1 and Vmw110 expression may allow a more efficient reactivation in this particular situation.

A mechanism by which HSV-1 R1 may act to modulate gene expression has recently become apparent; Smith *et al.*, 1994 have demonstrated that HSV-2 R1 interacts with protein which controls the turnover of GTP by the cellular protein p21ras. The GTPase activating protein, GAP120 is co-precipitated with R1 from cell lines expressing HSV-2 R1. In the presence of R1 GAP120 phosphorylation is stimulated, the phosphorylation of GAP120 prevents it interacting with and inactivating p21ras. The interaction between HSV-2 R1 and GAP120 is a function of the N-terminal domain, deletion part of the N-terminal region prevents this R1 interaction. Increased p21ras activity has recently been shown to be important in the expression of all genes, its effect on gene expression is thought to be mediated by preventing repression of transcription by p53 a tumor suppressor protein which represses transcription by its interaction with a basal transcription factor, the TATA binding protein (Abdellatif *et al.*, 1994). Alternatively this interaction may be a way in which the HSV-1 and HSV-2 R1 N-terminal regions differ.

There are many aspects of both the maintenance and reactivation stages of the latent state which may ultimately be controlled by the phosphorylation state of certain proteins. With very little current knowledge of the exact steps or phases which switch the virus into the lytic cycle it is difficult to speculate how the N-terminal region may be important.

Future work

The exact role of the N-terminal region will be determined by the construction of a deletion mutant in which only the N-terminus of reductase has been deleted, the construction of this mutant is currently underway. The exact effect of this mutation on virus replication will hopefully help to provide an explanation for the simplex specificty of this region. Further

mutants can then be constructed using the mutations isolated which up-regulate and down regulate activity *in vitro*.

The stable deletion isolated which has increased activity is a particularly good candidate for future structural studies, which in turn will provide valuable insights into potential catalytic residues.

References

Abdellatif, M., MacLellan, W. R. and Schneider, M. D. **1994** p21 Ras as a govenor of global gene expression. *J. Biol. Chem.* **269** 15432-15426.

Ace, C. I., Dalrymple, M. A., Ramsay, F. H., Preston, V. G. and Preston, C. M. **1988** Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65 *J. Gen. Virol.* **69** 1641-1650.

Ackermann, M., Braun, D. K., Pereira, L., and Roizman, B. **1984** Characterization of herpes simplex virus type 1 0, 4 and 27 with monoclonal antibodies. *J. Virol* **52** 108-118

Agarwal, K., Baek, K. H., Joen, C. J., Miyamoto, K., Ueno, A. and Yoon, H. S. **1991** Stimulation of transcript elongation requires both the zinc finger and RNA polmerase II binding domains of TFIIS. *Biochem.* **30** 7842-7851.

Albert, D. A. and Nodzenski, E. **1989** M2 subunit of ribonucleotide reductase is a target of cyclic AMP-dependent protein kinase. *J. Cell. Physio.* **1989** 129-136.

Alford, C. A. and Britt, W. J. **1984** Cytomegalovirus. In "Virology" (Ed, B. N. Fields), Raven Press New York 629-660.

Ali, M. A., Parkash, S. S. and Jariwalla, R. J. **1992** Localization of the antigenic sites and intrinsic protein kinase domain within a 300 amino acid segment of the ribonucleotide reductase large subunit from herpes simplex virus type 2. *Virology* **187** 360-367.

Amemiya, K., Traub, R., Durham, L., and Major, E. O. **1992** Adjacent nuclear factor-1 and activator protein binding sites in the enhancer of neurotropic JC virus. *J. Biol. Chem.* **267** 14204-14211

Angel, P. and Karin, M. **1991** The role of jun, fos and the AP-1 complex in cell-proliferation and transformation. *Biochim. Biophys. Acta* **1072** 129-157

apRhys, C. M., Ciufo, D. M., O'Neill, E. A., Kelly, T. J. and Hayward, G. S. **1989** Overlapping octamer and TAATGARAT motifs in the VF65-response elements in herpes simplex virus immediate-early promoters represent independent binding sites for cellular nuclear factor III. *J. Virol.* **63** 2798-2812.

Bacchetti, S., Evelegh, M. J., Muirhead, B., Sarton, C. S. and Huszar, D. **1984** Immunological characterization of herpes simplex virus type 1 and 2 polypeptides involved in viral ribonucleotide reductase activity. *J. Virol.* **49** 591-593

Bailey, A. C. and Thompson, R. **1992** A sequence-specific DNA-binding protein recognising a GA-rich element co-operates with Oct-1 at the herpes simplex virus IE3 promoter. *Intervirology* **34** 74-85.

Bailly, E., McCaffery, M., Touchot, N., Zahraouyi, A., Gould, B. and Bornens, M. **1991** Phosphorylation of two small GTP-binding proteins of the Rab family by p34cdc2. *Nature* **350** 715-718

Baines, J. D., Ward, P. L., Campadelli-Fiume, G. and Roizman, B. **1991** The UL20 gene of herpes simplex virus encodes a function necessary for viral egress. *J. Virol.* **65** 6414-6424.

Banham, A. H. and Smith, G. L. **1992** Vaccinia virus B1R encodes a 34-kDa serine/threonine protein kinase that localizes in cytoplasmic factories and is packaged into virions. *Virology* **191** 803-812.

Banham, A. H., Leader, D. P. and Smith, G. L. **1993** Phosphorylation of ribosomal proteins by the vaccinia virus B1R protein kinase. *FEBS Letts.* **321** 27-31.

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

Banks, L. M., Purifoy, D. J. M., Hurst, P.-F., Killington, R. A. and Powell, K. L. **1983** Herpes simplex virus non-structural proteins. IV. Purification of the virus-induced deoxyribonuclease and characterization of the enzyme using monoclonal antibodies. *J. Gen. Virol.* **64** 2249-2260.

Batchelor, A. H. and O'Hare, P. **1990** Regulation and cell-type specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1. *J. Virol.* **64** 3269-3279.

Batchelor, A. H. and O'Hare, P. **1992** Localisation of cis-acting sequence requirements in the promoter of the latency-associated transcript of herpes simplex virus type 1 required for cell-type-specific activity. *J. Virol.* **66** 3573-3582.

Batterson, W. and Roizman, B. **1983** Characterization of the herpes simplex virion-associated factor responsible for the induction of α genes. *J. Virol.* **46** 371-377.

Beard, P., Faber, S., Wilcox, K. W. and Pizer, L. I. **1986** Herpes simplex virus immediate early infected-cell polypeptide 4 binds to DNA and promotes transcription. *Proc. Natl. Acad. Sci. USA* **83** 4016-4020.

Becker, Y., Dym, H. and Sanov, I. **1968** Herpes simplex virus DNA. *Virology* **36** 184-192.

BenDavid, Y., Letwin, K., Tannock, L., Bernstein, A. and Pawson, T. **1991** A mammalian protein kinase with potential for serine/threonine and tyrosine phosphorylation is related to cell cycle regulators. *Embo J.* **10** 317-325.

Berridge, M. **1993** Inositol triphosphate and calcium signalling. *Nature* **361** 315-325.

Billah, M. M. and Anthes, J. C. **1990** The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem. J.* **269** 281-291.

Binetruy, B. Smael, T. and Karin, M. **1991** Ha-ras augments c-jun activity and stimulates phosphorylation of its activation domain. *Nature* **351** 122-127.

Bjorklund, S., Skog, S., Tribukait, B. and Thelander, L. **1990** S-phase specific expression of mammalian ribonucleotide reductase R1 and R2 subunit mRNAs. *Biochem.* **29** 5452-5458.

Blair, E. D. and Wagner, E. K. **1986** A single regulatory region mediates both cis activation and trans activation of the herpes simplex virus VP5 promoter in transient expression assays in vivo. *J. Virol.* **60** 460-469.

Blenis, J. **1993** Signal transduction via MAP kinases: proceed at your own RSK. *Proc. Natl. Acad. Sci. USA.* **90** 5889-5892.

Blenis, J. and Resh, M. D. **1993** Subcellular localisation specified by protein acylation and phosphorylation. *Curr. Opin. Cell. Biol.* **5** 984-989.

Blow, J. J. and Nurse, P. **1990** A cdc2-like protein is involved in the initiation of DNA replication in xenopus egg extracts. *Cell* **62** 855-862.

Boehmer, P. E. and Lehman, I. R. **1993** Herpes simplex virus type 1 ICP8: helix destabilizing properties. *J. Virol.* **67** 711-715.

Bolovan, C. A., Sawtell, N. M. and Thompson, R. L. **1994** ICP34.5 mutants of herpes simplex virus type 1 strain 17syn+ are attenuated for neurovirulence in mice and for replication in confluent primary mouse embryo cell cultures. *J. Virol.* **68** 48-55.

Botchan, M., Topp, W and Sambrook, J. **1976** The arrangement of simian virus 40 sequences in the DNA of transformed cells. *Cell* **9** 269-287.

Boyle, W. J., Smeal, T., Defize, L. H. K., Angel. P., Woodgett, J. R., Karin, M. and Hunter, T. **1991** Activation of protein kinase C decreases phosphorylation of c-jun at sites that negatively regulate its DNA-binding domain. *Cell* **64** 573-584.

Branden, C-I. **1980** Relation between structure and function of α/β proteins. *Quart. Rev. Biochem.* **13** 317-330.

Braun, D. K., Roizman, B. and Pereira, L. **1984** Characterization of posttranscriptional products of herpes simplex virus gene 35 proteins binding to the surface of full but not empty capsids. *J. Virol.* **49** 142-153.

Brou, C., Chaudray, S., Davidson, I., Lutz, Y., Wu, J., Egly, J-M, Tora, L. and Chambon, P. **1993** Distinct TFIID complexes mediate the effect of different transcriptional activators. *Embo J.* **12** 489-499.

Brown, S. M., Ritchie, D.A. and Subak-Sharpe, J. H. **1973** Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. *J. Gen. Virol.* **18** 329-346.

Brucker, R. C., Crute, J. J., Dobson, M. S. and Lehman, I. R. **1991** The herpes simplex virus 1 origin binding protein: a DNA helicase. *J. Biol. Chem.* **265** 11227-11232.

Busby, D. W. G., House, W. and McDonald, J. R. **1964** In "Virology Techniques". Churchill, London.

Bzik, D. J. and Preston, C. M. **1986** Analysis of the DNA sequences which regulate the transcription of herpes simplex virus immediate early gene 3:DNAsequences required for enhancer-like activity and response to transactivation by a virion polypeptide. *Nucleic Acids Res.* **14** 929-943.

Cai, H., Szeberenyi, J. and Cooper, G. M. **1990** Effect of a dominant inhibitory Ha-ras mutation on mitogeneic signal transduction in NIH 3T3 cells. *Mol. Cell. Biol.* **10** 5314-5323.

Cai, W. and Schaffer, P. A. **1992** Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early and late genes in productively infected cells. *J. Virol.* **66** 2904-2915.

Calder, J. M. and Stow, N. D. **1990** Herpes simplex virus helicase-primase. The UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities. *Nucleic Acid. Res.* **25** 2573-3578.

Cameron, J. M., McDougall, I., Marsden, H. S., Preston, V. G., Ryan, D. M. and Subak-Sharpe, J. H. **1988** Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus and a valid antiviral target. *J. Gen. Virol.* **69** 2607-2612.

Campadelli-Fiume, G., Stripe, D., Boscaro, A., Avitabile, E., Foa-Tomasi, L., Barker, D. and Roizman, B. **1990** Glycoprotein C-dependent attachment of herpes simplex virus to susceptible cells leading to productive infection. *Virology* **178** 213-222.

Campbell, M. E., Palfreyman, J. W. and Preston, C. M. **1984** Identification of herpes simplex DNA sequences which encode a trans-acting polypeptide responsible for stimulation of immediate early transcription *J. Mol. Biol.* **180** 1-19

Cantley, L.C., Auger, K.R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. **1991** Oncogenes and signal transduction. *Cell* **64** 281-302.

Cardonna, S. and Cheng, Y. S. **1981** Induction of uracil-DNA glycosylase and dUTP nucleotide hydrolase activity in herpes simplex-infected human cells. *J. Biol. Chem.* **256** 9834-9837.

Challberg, M. D. **1991** Herpes simplex virus DNA replication. *Seminars in Virol.* **2** 247-256.

Challberg, M.D. **1986** A method for identifying the viral genes required for herpesvirus DNA replication. *Proc. Natl. Acad. Sci. USA.* **83** 9094-9098.

Chan, A. K., Litchfield, D. W. and Wright, J. A. **1993** Phosphorylation of ribonucleotide reductase R2 protein: *In vivo* and *in vitro* evidence of a role for $p34^{cdc2}$ and CDK2 protein kinases. *Biochemistry* **32** 12835-12840.

Chang, Y. E. and Roizman, B. **1993** The product of the UL31 gene of herpes simplex virus 1 is a nuclear protein which partitions with the nuclear matrix. *J. Virol.* **67** 6348-6356.

Chapman, C. J., Harris, J. D., Hardwicke, M. A., Sandri-Goldin, R. M., Collins, M. K. L. and Latchman, D. S. **1992** Promoter independent activation of heterologous gene expression by the herpes simplex virus immediate-early protein ICP27. *Virology.* **186** 573-578.

Chauhan, V. P. S. and Chauhan, A. **1992** Protamine induces autophosphorylation of protein kinase C: Stimulation of protein kinase C-mediated protamine phosphorylation by histone. *Life Sci.* **51** 537-544.

Chee, M. S., Lawrence, G. L., Barrell, B. G. **1989** Alpha-, beta- and gammaherpesviruses encode a putative phosphotransferase. *J. Gen. Virol.* **70** 1151-1160.

Chen, J and Silverstein, S. **1992** Herpes simplex viruses with mutations in the gene encoding ICP0 are defective in gene expression. *J. Virol.* **66** 2916-2927.

Chen, S., Mills, L., Perry, P., Riddle, S., Wobig, R., Lown, R. and Millette, R. L. **1992** Transactivation of the major capsid protein gene of herpes simplex virus type 1 requires a cellular transcription factor. *J. Virol.* **66** 4304-4314.

Chen, W. S., Lazar, C. S., Poenie, M., Tsein, R. Y., Gill, G. N. and Rosenfeld, M. G. **1987** Requirement for intrinsic protein tyrosine kinase in the immediate and late actions of the EGF receptor. *Nature* **328** 820-823.

Cheung, P., Banfield, B. W. and Tufaro, F. **1991** Brefeldin A arrests the maturation and egress of herpes simplex virus particles during infection. *J. Virol.* **65** 1893-1904.

Chou, C. K., Duil, T. J., Russell, D. S., Gherzi, R., Lebwohl, A. U. and Rosen, O. M. **1987** Human insulin receptors mutated at the ATP- binding site lack protein tyrosine kinase activity and fail to mediate postreceptor effects of insulin. *J. Biol. Chem.* **262** 1842-1847.

Chou, J and Roizman, B. **1992** The γ1 34.5 gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programmed cell death in neuronal cells. *Proc. Natl. Acad. Sci. USA.* **89** 3266-3270.

Chou, J., Kern, E. R., Whitley, R. J. and Roizman, B. **1990** mapping of herpes simplex virus-1 neurovirulence to γ1 34.5, a gene nonessential for growth in culture. *Science* **250** 1262-1266.

Chung, T. D., Luo, J., Wymer, J. P., Smith, C. C. and Aurelian, L. **1991** Leucine repeats in the large subunit of herpes simplex virus type 2 ribonucleotide reductase (RR;ICP10) are involved in RR activity and subunit complex formation. *J. Gen. Virol.* **72** 1139-1144.

Chung, T. D., Wymer, J. P., Kulka, M., Smith, C. C.and / Aurelian, L. **1990** Myristylation and polylysine mediated activation of the protein kinase domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *Virology* **179** 168-178.

Chung, T. D., Wymer, J. P., Smith, C. C., Kulka, M. and Aurelian, L. **1989** Protein kinase activity associated with the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *J. Virol* **63** 3389-3398.

Clements, G. B. and Stow, N. D. **1989** A herpes simplex virus type 1 mutant containing a deletion within immediate early gene 1 is latency-competent in mice. *J. Gen. Virol.* **70** 2501-2506.

Clements, J. B., Watson, R. J. and Wilkie, N. M. **1977** Temporal regulation of herpes simplex type 1 transcription: location of transcripts on the viral genome. *Cell* **12** 275-285.

Coen, D. M., Kosz-Vnenchak, M., Jacobson, J. G., Leib, D. A., Bogard, C. L., Schaffer, P. A., Tyler, K. L. and Knipe, D. M. **1989** Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proc. Natl. Acad. Sci. USA* **86** 4736-4740.

Cohen, E. A., Gaudreau, P., Brazeau, P. and Langelier, Y. **1986** Specific inhibition of herpesvirus ribonucleotide reductase by a nonapeptide derived from the carboxy terminus of subunit 2. *Nature* **321** 441-443.

Cohen, G. **1972** Ribonucleotide reductase activity of synchronised KB cells infected with herpes simplex virus. *J. Virol.* **9** 408-418.

Cohen, J. C., Henry, B. E., Randall, C. C. and O'Callaghan, D. J. **1977** Ribonucleotide reductase activity in hydroxyurea resistant herpesvirus replication. *Proc. Soc. Exp. Biol. Med.* **155** 395-399.

Conley, A. F., Knipe, D. M., Jones, P. C. and Roizman, B. **1981** Molecular genetics of herpes simplex virus VII. Characterization of a temperature-sensitive mutant produced by *in vitro* mutagenesis and defective in DNA synthesis and accumulation of gamma polypeptides. *J. Virol.* **37** 191-206.

Conner, J., Cooper, J., Furlong, J. and Clements, J. B. **1992b** An autophosphorylating but not transphosphorylating activity is associated with the unique N-terminus of the herpes simplex virus type 1 ribonucleotide reductase large subunit. *J. Virol* **66** 7511-7516.

Conner, J., Furlong, J., Murray, J., Meighan, M., Cross, A., Marsden, H. and Clements, J. B. **1993** Herpes simplex virus type 1 ribonucleotide reductase large subunit: regions of the protein essential for subunit interaction and dimerisation. *Biochemistry* **32** 13673-13680.

Conner, J., Macfarlane, J., Lankinen, H., and Marsden, H. **1992a** The unique N terminus of the herpes simplex virus type 1 large subunit is not required for ribonucleotide reductase activity. *J. Gen. Virol.* **73** 103-112.

Costa, R. H., Draper, KJ. G., Devi-Rao, G., Thompson, R. L. and Wagner, E. K. **1985** Virus-induced modification of the host cell is required for expression of the bacterial chloroamphenicolacetyl transferase gene controlled by a late herpes simplex virus promoter (VP5). *J. Virol.* **56** 19-30.

Coulter, L. J., Moss, H. W. M., Lang, J. and McGeoch, D. J. **1993** A mutant of herpes simplex virus type 1 in which the UL13 protein kinase gene is disrupted. *J. Gen. Virol.* **74** 387-395.

Cousens, D. J., Greaves, R., Goding, R. C. and O'Hare, P. **1989** The C-terminal 79 amino acids of the herpes simplex virus regulatory protein, Vmw65, efficiently activates transcription in yeast and mammalian cells in chimeric DNA-binding proteins. *Embo J.* **8** 2337-2342.

Crute, J. J. and Lehman, I. R. **1989** Herpes simplex-1 DNA polymerase. Identification of an intrinsic 5' to 3' exonuclease with ribonuclease H activity. *J. Biol. Chem.* **264** 19266-19270.

Crute, J. J. and Lehman, I. R. **1991** Herpes simplex virus-1 helicase-primase. Physical and catalytic properties. *J.Biol. Chem.* **266** 4484-4488.

Crute, J. J., Mocarski, E. S. and Lehman, I. R. **1988** A DNA helicase induced by herpes simplex virus type 1. *Nucl. Acid. Res.* **16** 6586-6596.

Crute, J. J., Tsurumi, T., Zhu, L., Weller, S. K., Olivo, P. D., Challberg, M. D., Mocarski, E. S. and Lehman, I. R. **1989** Herpes simplex virus helicase-primase: a complex of three herpes encoded gene products. *Proc. Natl. Acad. Sci. USA.* **86** 2186-2189.

Csermely, P. and Kahn, C. R. **1991** The heat shock protein (hsp-90) possesses an ATP binding site and an autophosphorylating activity. *J. Biol. Chem.* **266** 4943-4950.

Csermely, P., Schnaider, T., Cheatman, B., Olson, M. O. J. and Kahn, C. R. **1993** Insulin induces the phosphorylation of nucleolin. A possible mechanism of insulin induced efflux from nuclei. *J. Biol. Chem.* **268** 9747-9752.

Cunningham, C., Davison, A. J., Dolan, A., Frame, M. C., McGeoch, D. J., Meredith, D. M., Moss, H. W. M. and Orr, A. C. **1992** The UL13 virion protein of herpes simplex virus type 1 is phosphorylated by a novel virus-induced protein kinase. *J. Gen. Virol.* **73** 303-311.

Curtin, K. D. and Knipe, D. M. **1993** Altered properties of the herpes simplex virus ICP8 DNA-binding protein in cells infected with ICP27 mutant viruses. *Virology.* **196** 1-14.

Dailey, D., Schieven, G. L., Lim, M. Y., Marquardt, H., Gilmore, T., Thorner, J. and Martin, G. S. **1990** Novel yeast protein kinase (YPK1 gene product) is a 40-kilodalton

phosphotyrosyl protein associated with protein-tyrosine kinase activity. *Mol. Cell. Biol.* **1990** 6244-6256.

Darling, A. J., MacKay, E. M., Ingemarson, R. and Preston, V. G. **1988** Reconstitution of herpes simplex virus type 1 ribonucleotide reductase activity from the large and small subunits. *Virus Genes.* **2** 163-176.

Darling, A. J., McKay, E. M., Ingermarson, R and Booth, B. **1990** Herpes simplex virus-encoded ribonucleotide reductase: evidence for the association/reassociation of the holoenzyme. *Virus Genes* **3** 367-372.

Davis, R. **1993** The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* **268** 14553-14556.

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

Davison, A. J. and McGeoch, D. J. **1986** Evolutionary comparisons of the S segments in the genomes of herpes simplex virus type 1 and varicella zoster virus. *J. Gen. Virol.* **67** 597-611.

Davison, A. J. and Wilkie, N. M. **1981** Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. *J. Gen. Virol.* **55** 315-318.

Deb, S. and Deb, S. P. **1991** A 269-amino acid segment with a pseudo leucine zipper and helix-turn-helix motif codes for the sequence specific DNA-binding domain of herpes simplex virus type 1 origin binding protein. *J. Virol.* **65** 2829-2938.

Deb, S. and Doelberg, M. **1988** A 67-base pair segment from the Ori-S region of herpes simplex virus type 1 encodes origin function. *J. Virol.* **62** 2516-2519.

deBruyn, K. A and Knipe, D. M. **1988** Formation of DNA replication compartments in herpes virus-infected cells require a viral DNA binding protein. *Cell.* **55** 857-868.

Delius, H. and Clements, J. B. **1976** A partial denaturation map of HSV type 1 DNA: evidence for inversions of the unique DNA regions. *J. Gen. Virol.* **33** 125-133.

Deluca, N. A. and Schaffer, P. A. **1985** Activation of immediate early and late promoters by temperature-sensitive and wild type forms of herpes simplex virus type1 protein ICP4. *Mol. Cell. Biol* **5** 1997-2008.

Deluca, N.A., Courtney, M. A. and Schaffer, P. A. **1984** Temperature-sensitive mutants in herpes simplex virus type 1 ICP4 permissive for early gene expression. *J. Virol.* **52** 767-776.

Desai, P., Ramarkishnan, R., Lin, Z. W., Osak,B., Glorioso, J. C. and Levine, M. **1993** The RR1 gene of herpes simplex virus type 1 is uniquely transactivated by ICP0 during infection. *J. Virol.* **67** 6125-6135.

deWind, N., Domen, J. and Berns, A. **1992** Herpesviruses encode an unusual protein serine/threonine kinase which is nonessential for growth in cultured cells. *J. Virol.* **66** 5200-5209.

Digard, P. and Coen, D. M. **1990** The binding site of the herpes simplex virus polymerase for the viral UL42 protein. *J. Biol. Chem.* **265** 17393-17396.

Digard, P., Bebrin, W. R., Weisshart, K. and Coen, D. M. **1993** The extreme C terminus of herpes simplex DNA polymerase is crucial for functional interaction with processivity factor UL42 and for viral replication. *J. Virol.* **67** 258-264.

Dixon, R. A. F. and Schaffer, P. A. **1980** Time-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein VP175. *J.Virol.* **36** 189-203.

Dobson, A. T., Serdarati, F., Devi-Rao, G., Flanagan, M. T., Farrell, M. J., Stevens, J. G., Wagner, E. K., and Feldman, L. T. **1989** Identification of the latency-associated promoter by expression of rabbit beta-globin mRNA in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. *J. Virol.* **63** 3844-3851.

Dodson, M. S. and Lehman, I. R. **1991** Association of DNA helicase-primase activities with a subassembly of the herpes simplex virus type 1 helicase-primase composed of UL5 and UL52 gene products. *Proc. Natl. Acad. Sci. USA.* **88** 1105-1109.

Dodson, M. S., Crute, J. J., Bruckner, R. C. and Lehman. I. R. **1989** Overexpression and assembly of the herpes simplex virus type 1 helicase-primase in insect cells. *J. Biol. Chem.* **264** 20835-20838.

Durso, M., Zucchi, I., Ciccodicola, A., Palmieri, G., Abidi, F. E. and Schlessinger, D. **1990** Human glucose-6-phosphate dehydrogenase gene carried on a yeast artificial chromosome encodes an active enzyme in monkey cells. *Genomics* **7** 531-534.

Dutia, B. M., Frame, M. C., Subak-Sharpe, J. H., Clark, W. N. and Marsden, H. S. **1986** Specific inhibition of herpesvirus ribonucleotide reductase by synthetic peptides. *Nature* **321** 439-441.

Dutta, A. and Stillman, B. **1992** *cdc2* family of kinases phosphorylate a human cell DNA replication factor, RPA, and activate DNA replication. *Embo J.* **11** 2189-2199.

Elias, P. And Lehman, I. R. **1988** Interaction of origin binding protein with an origin of replication of herpes simplex virus type 1. *Proc. Natl. Acad. Sci. USA.* **85** 2959-2963.

Elias, P., Gustafsson, C. M. and Hammerstein, O. **1990** The origin binding protein of herpes simplex virus type 1 binds co-operatively to the viral origin of replication. *J. Biol. Chem.* **265** 17167-17173.

Elion, G. B., Furman, P. A., Fyfe, J. A., deMirannda, P., Beauchamp, L. and Schaffer, H. J. **1977** Selectivity of action of an antiherpetic agent, 9-(2-hydoxyethoxymethyl) guanine. *Proc. Natl. Acac. Sci. USA.* **74** 5716-5720.

Elliot, G. D. and Meredith, D. M. **1992** The herpes simplex virus type 1 tegumant protein VP22 is encoded by gene UL49. *J. Gen. Virol.* **73** 723-726.

Eriksson, S. and Sjoberg, B-M **1989** Ribonucleotide reductase. In allosteric enzymes 1889-217. (Ed. G. Herve) Boca Raton: CRC press.

Everett, R. D. **1984** Transactivation of transcription by herpesvirus products: requirement for two HSV-1 immediate-early polypeptides for maximum activity. *Embo. J.* **3** 3135-3141.

Everett, R. D. **1986** The products of herpes simplex virus type 1 (HSV-1) immediate-early genes 1, 2 and 3 can activate HSV-1 genes in trans. *J. Gen. Virol.* **68** 2507-2513.

Everett, R. D. **1989** Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *J. Gen. Virol.* **70** 1185-1205.

Everrett, R. D. **1987** A detailed mutational analysis of Vmw110, a trans-acting transcriptional activator encoded by herpes simplex virus type 1. *Embo. J.* **6** 2069-2076.

Exton, J. H. **1990** Signalling through phosphatidylcholine breakdown. *J. Biol. Chem.* **265** 1-4.

Ezoe, k., Lee, S-T., Strunk, K. M. and Spritz, R. A. **1994** PTK1, a novel protein kinase required for proliferation of human melanocytes. *Oncogene* **9** 935-938.

Faber, S. W. and Wilcox, K. W. **1986** Association of the herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences. *Nucleic Acids Res.* **14** 6067-6083.

Faber, S. W. and Wilcox, K. W. **1988** Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation sites. *Nucleic Acids Res.* **16** 555-570.

Fang, F. and Newport, J. W. **1993** Distinct roles of CDK2 and cdc2 in RP-A phosphorylation during the cell cycle. *J. Cell. Sci.* **106** 983-994.

Featherstone, C. and Russell, P. **1991** Fission yeast p107 (wee1) mitotic inhibitor is a tyrosine/serine kinase. *Nature* **349** 808-811.

Fenwick, M. L. and Clark, J. **1982** Early and delayed shut-off of host protein synthesis in cells infected with herpes simplex virus. *J. Gen. Virol.* **71** 411-418.

Fenwick, M. L. and McMenamin, M. M. **1984** Early virion-associated suppression of cellular protein synthesis by herpes simplex virus is accompanied by inactivation of mRNA. *J. Gen. Viol.* **65** 1225-1228.

Fenwick, M. L. and Walker, M. J. **1987** Suppression of synthesis of cellular macromolecules by herpes simplex virus. *J. Gen. Virol.* **41** 37-51.

Fierer, D. S. and Challberg, M. D. **1992** Purification and characterization of UL9, the herpes simplex virus type 1 origin-binding protein. *J. Virol.* **66** 3986-3995.

Fisher, F. B. and Preston, V. G. **1986** Isolation and characterisation of herpes simplex type 1 mutants which fail to induce dUTPase activity. *Virology* **148** 190-197.

Fraser, N. W., Block, T. M. and Spivak, J. G. **1992** The latency-associated transcripts of herpes simplex virus: RNA in search of function. *Virology* **191** 1-8.

Gallo, M. L., Jackwood, D. H., Murphy, M., Marsden, H. S. and Parris, D. S. **1988** Purification of the herpes simplex virus type 1 65-kilodalton DNA-binding protein: properties of the protein and evidence of its association with the virus-induced DNA polymerases. *J. Virol.* **62** 2874-2883.

Fitch, I., Dahmann, C., Surana, U., Amon, A., Nasmyth, K., Goetsch, L., Byers, B. and Futcher, B. **1992** Characteriztion of four B-type cyclin genes of the budding yeast saccharomyces cerevisiae. *Mol. Biol. Cell.* **3** 805-818.

Forrester, A., Farrell, H., Wilkinson, G., Kaye, J., Davis-Poynter, N. and Minson, T. **1992** Construction and properties of a mutant of herpes simplex virus type 1 and gylcoprotein H coding sequences deleted. *J. Virol.* **66** 341-348.

Frame, M. C., Marsden, H. S. and Dutia, B. M. **1985** The ribonucleotide reductase induced by herpes simplex virus type 1 involves minimally a complex of two polyletides (136K and 38K). *J. Gen. Virol.* **66** 1518-1587.

Frenkel, N., Schirmer, E. C., Wyatt, L. S., Kalsafanas, G., Roffman, E., Darovich, R. M. and June, C. H. **1990** Isolation of a new herpes virus from CD4+ T-cells . *Proc. Natl. Acad. Sci. USA.* **87** 748-752.

Fuller, A. O. and Spear, P. G. **1985** Specificities of monoclonal antibodies and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack inhibition by potent neutralising antibodies. *J. Virol.* **55** 475-482.

Furlong, J., Conner, J., Mclauchlan, J., Lankinen, H., Galt, C., Marsden, H. and Clements, J. B. **1991** The large subunit of herpes simplex virus type 1 ribonucleotide reductase: expression in *Escherichia coli* and purification. *Virology* **182** 846-851.

Gallardo, E. **1943** Primary herpes simplex kerititis : clinical and experimental study. *Arch. Opthamology.* **30** 217-220.

Gallo, M. L., Dorsky, D. I., Crumpacker, C. S. and Parris, D. S. **1989** The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. *J. Virol.* **63** 5023-5029.

Gao, M. and Knipe, D. M. **1989** Genetic evidence for multiple nuclear functions of the herpes simplex virus ICP8 DNA-binding protein. *J. Virol.* **63** 5285-5267.

Gao, M. J. and Knipe, D. M. **1991** Potential role for herpes simplex virus DNA replication protein in stimulation of late gene expression. *J. Virol.* **65** 2666-2675.

Gao, M. J. and Knipe, D. M. **1992** Distal protein sequences can affect the function of a nuclear localization signal. *Mol. Cell. Biol.* **12** 1330-1339.

Gatica, M., Allende, C. C., Antonelli, M. and Allende, J. E. **1987** Polylysine-containing peptides, including the carboxy-terminal segment of the human c-Ki-ras 2 protein, affect the activity of some key membrane enzymes. *Proc. Natl. Acad. Sci. USA* **84** 324-328.

Gelman, I. H. and Silverstein, S. **1985** Identification of immediate-early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. *Proc. Natl. Acad. Sci. USA* **82** 5265-5269.

Gelman, I. H. and Silverstein, S. **1986** Co-ordinate regulation of herpes simplex virus gene expression is mediated by the functional interaction of two immediate early gene products. *J. Mol. Biol.* **191** 395-409.

Gelman, I. H. and Silverstein, S. **1987** Herpes simplex immediate-early promoters are responsive to virus and cell trans-acting factors. *J. Virol.* **61** 2286-2296.

Gerster, T. and Roeder, R. G. **1988** A herpesvirus trans-activating protein interacts with transcriptional factor OTF-1 and other cellular proteins. *Proc. Natl. Acad. Sci. USA.* **85** 6347-6351

Gibbs, C. S. and Zoller, M. J. **1991** Rational scanning mutagenesis of a protein kinase identifies functional regions involved in catalysis and substrate interactions. *J. Biol. Chem.* **266** 8923-8931.

Gileadi, O., Feaver, W. J. and Kornberg, R. D. **1992** Cloning of a subunit of yeast RNA polymerase II transcription factor b and CTD kinase. *Science* **257** 1389-1391.

Gleib, L. D. **1985** Varicella-zoster virus. In "Virology" (Ed, B. N. Fields). Raven press New York.

Godowaski, P. J. and Knipe, D. M. **1986** Identification of a herpes simplex virus that represses late gene expression from parental viral genomes. *J. Virol.* **55** 357-365.

Goldstein, D. H. and Weller, S. K **1988b** Factor(s) present in herpes simplex virus type 1 infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterisation of an ICP6 deletion mutant. *Virology* **166** 41-51.

Goldstein, D. H. and Weller, S. K. **1988a** Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensible for virus growth and DNA synthesis: Isolation and characterisation of ICP6 lacZ insertion mutant. *J. Virol.* **62** 196-205.

Gonzalez, G. A. and Montminy, M. R. **1989** Cyclic-AMP stimulates somatostatin gene transcription by phosphorylation of CREB by serine -133. *Cell* **59** 657-680.

Goodrich, J. A., Hoey, T., Hunt, C. J., Adom, A. and Tijan, R. **1993** Drosophilla TAFII 40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* **75** 719-730.

Gorbalenya, A. E., Koonin, E. B., Donchenko, A. P. and Blinov, V. M. **1988** A conserved NTP-motif in putative helicases. *Nature* **333** 22-

Gottlieb, J., March, A. I., Coen, D. M. and Challberg, D. M. **1990** The herpes simplex virus 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. *J. Virol.* **64** 5976-5987.

Granot, J., Mildvan, A. S., Hiyama, K., Kondo, H. and Kaiser, E. T. **1980** magnetic resonance studies of the effect of the regulatory subunit on metal and substrate binding to the catalytic subunit of bovine heart protein kinase. *J. Biol. Chem.* **255** 4569-4573.

Greaves, R., and O'Hare, P. **1989** Separation of requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virus regulatory protein Vmw65. *J. Virol.* **63** 1641-1650.

Grueinheid, S., Gatzke, L., Meadows, H. and Tufaro, F. **1993** Herpes simplex virus infection and propogation in a mouse L cell mutant lacking heparan sulfate proteoglycans. *J. Virol.* **67** 93-100.

Gu., B., Rivera-Gonzalez, R., Smith, C. A. and DeLuca, N. A. **1993** Herpes simplex virus infected cell polypeptide 4 preferentially represses SP-1 activated over basal transcription from its own promoter. *Proc. Natl. Acad. Sci. USA* **90** 9528-9532.

Guo, H., Reddy, S. A. G. and Damuni, Z. **1993** Purification and characterization of an autophosphorylation-activated protein serine threonine kinase that phopsphorylates and inactivates protein phosphotase 2A. *J. Biol. Chem.* **268** 11193-11198.

Gupte, S. S., Olson, J. and Ruyechan, W. T. **1991** The major herpes simplex virus type 1 DNA-binding protein is a zinc metalloprotein. *J. Biol. Chem.* **266** 11413-11416.

Hagiwara, M., Alberts, A., brindle, P. Meinkoth, J., Fermisco, J., Deng, T., Karin, M., Shenolikar, S. and Montminy, M. **1992** Traanscription attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell* **70** 105-113.

Hanahan, D. **1983** Studies on transformation of *E.coli* with plasmids. *J. Mol. Biol.* **166** 557-580.

Hanks, S. K., Quinn, A. M. and Hunter, T. **1988** The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241** 42-52.

Hanks, S. K. and Quinn, A. M. **1991** Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. *Methods. Enzym.* **200** 38-62.

Hannon, G. J., Demetrick, D. and Beach, D. **1993** Isolation of the Rb-related p130 through its interaction with CDK2 and cyclins. *Genes Dev.* **7** 2378-2391.

Harris, R. A., Everett, R. D., Zhu, X., Silverstein, S. and Preston, C. M. **1989** Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an *in vitro* latency system. *J. Virol.* **63** 3513-3115.

Hart, G. J. and Boehme, R. E. **1992** The effect of the UL42 protein on the DNA polymerase activity of the catalytic subunit of the DNA polymerase encoded by herpes simplex virus type 1. *FEBS Lett.* **305** 97-100.

Hartwig, J. H., Thelen, M., Rosen, A., Janmey, P. A., Nairn, A. C. and Ackerem, A. **1992** MARCKs is an actin filament croslinking protein regulated by protein kinaseC and calcium-calmodulin. *Nature* **356** 618-622.

Hayward, G. S. **1993** Immediate-early gene regulation in herpes simplex virus. *Sem. Virol.* **4** 15-25.

Heine, J. W., Honess, R. W., Cassai, E. and Roizman, B. **1974** Proteins specified by herpes simplex virus XII. The virion polypeptides of type 1 strains. *J. Virol.* **14** 640-651

Henry, B. E., Glaser, R., Hewelson, J. and O'Callaghan, D. J. **1978** Expression of altered ribonucleotide reductase activity associated with the replication of the Epstein-Barr virus. *Virology* **89** 262-271.

Herman, P. K., Stack, J. H., Demodena, J. A. and Emr, S. D. **1991** A novel protein kinase homolog essential for protein sorting to the yeast lysosome-like vacuole. *Cell* 425-437.

Hernadez, T. R. and Lehman, I. R. **1990** Functional interaction between the herpes simplex-1 DNA polymerase and UL42 protein. *J. Biol. Chem.* **265** 11227-11232.

Hernadez, T. R., Dutch, R. E., Lehman, I. R., Gustafson, C. and Elias, P. **1991** Mutations in aherpes simplex virus type 1 origin that inhibit replication. *J. Virol.* **65** 1649-1652.

Herold, B. C., WuDunn, D., Montgomery, R. I., Esko, J. D. and Spear, P. G **1992** Glycoprotein C of herpes simplex virus type 1 plays a principle role in the adsorption of virus to cells and in infectivity. *J. Virol.* **65** 1090-1098.

Herr, W., Strum, R. A., Clerc, R. G., Corcoran, L. M., Baltimore, D., Sharpe, P. A., Ingraham, H. A., Rosenfeld, M. G., Finney, M., Rurken, G. and Horivitz, H. R. **1988** The pou domain: a large conserved region in the mammmalian pit-1, oct-1, oct-2 and *Caenorhabditis elegans unc*-86 gene products. *Genes Dev.* **2** 1513-1516.

Hershey, J. W. B. **1991** Translational control in mammalian cells. *Ann. Rev. Biochem.* **60** 717-755.

Hill, T. J. **1985** Herpes simplex virus latency, in the herpesviruses, Vol 3 (Roizman B, Ed) 175-240. Plenum press New York.

Hinnebusch, A. G. **1990** Involvement of an initiation factor and protein phosphorylation in translational control of *GCN4* mRNA. *Trends Biochem.* **15** 148-152.

Ho, D. Y. and Mocarski, E. B. **1989** Herpes simplex virus latent RNA (LAT) is not required for latent infection in the mouse. *Proc. Natl. Acad. Sci. USA* **86** 7596-7600.

Ho, D. Y. and Mocarski, E. S. **1988** β-galactosidase as a marker in the peripheral and neural tissue of the herpes simplex virus-infected mouse. *Virology* **167** 279-283.

Hodgman, T. C. **1988** A new superfaminly of replicative proteins. *Nature* **333** 22-23.

Holland, L., Anderson, K., Shipman Jr, C. and Wagner, E. **1980** Viral DNA synthesis is required for the efficient expression of specific HSV-1 mRNA species. *Virology* **101** 10-24.

Honess, R. W. and Roizman, B. **1974** Regulation of herpesvirus macromolecular synthesis I. Cascade regulation of the synthesis of three group of viral protein. *J. Virol.* **14** 8-19.

Horikoshi, N., Maguire, K., Kralli, A., Maldonado, E., Reinberg, D. and Weinmann, R. **1991** Direct interaction between adnovirus E1A protein and the TATA box binding transcription factor IID. *Proc. Natl. Acad. Sci. USA.* **88** 5124-5128.

Howard, S. T. and Smith, G. L. **1989** Two early vaccinia virus genes encode polypeptide related to protein kinases. *J. Gen. Virol.* **70** 3187-3201.

Hunter, T. **1987** A thousand and one protein kinases. *Cell* **50** 823-829.

Hunter, T. **1991** Protein kinase classification. *Methods Enzymol* **200** 3-81.

Hurta, R. A. R., Greenberg, A. H. and Wright, J. A. **1993** Transfroming growth factor beta 1 selectively regulates ornithine decarboxylase gene expression in malignant H-ras transformed fibrosarcoma cell lines. *J. Cell. Physio.* **156** 272-279.

Huszar, D. and Bacchetti, S. **1981** Partial purification of the ribonucleotide reductase induced by herpes siimplex virus infection of mammalian cells. *J. Virol.* **37** 580-588.

Huszar, D., Beharry, S. and Bacchetti, S. **1983** herpes simplex virus induced ribonucleotide reductase: development of antibodies specific for the enzyme. *J. Gen. Virol.* **64** 1327-1335.

Imbalzano, A. N., Coen, D. M. and Deluca, N. A. **1991** Herpes simplex virus transactivator ICP4 operationally substitutes for the cellular transcription factor SP-1 for the efficient expression of the viral thymidine kinase gene. *J. Virol* **65** 565-574.

Ing, Y. L., Leung, I. W. L., Heng, H. H. Q, Tsui, L-C and Lassam, N. J. **1994** MLK-3: identification of a widely-expressed protein kinase bearing an SH3 domain and a leucine zipper-basic region domain. *Oncogene* **9** 1745-1750.

Ingemarson, R. and Lankinen, H. **1987** The herpes simplex virus type 1 ribonucleotide reductase is atight complex of the type $\alpha_2\beta_2$ composed of 40K and 140k proteins which latter show multiple forms due to proteolysis. *Virology* **156** 417-422.

Jacobson, J. G., Leib, D. A., Goldstein, D. J., Bogard, C. L., Schaffer, P. A., Weller, S. K. and Cohen, D. M. **1989** A herpes simplex ribonucleotide reductase deletion mutant is defective for productive acute and reactivatable infections in mice and for replication in mouse cells. *Virology* **173** 276-283.

Jacobson, J. G., Leib, D. A., Goldstein, D., Bogard, C. L., Schaffer, P. A., Weller, S. K. and Coen, D. M. **1989** A herpes simplex virus ribonuclotide reductase deletion mutant is defective for productive acute and reactivatable latent infections of mice and for replication in mouse cells. *Virology* **173** 276-283.

Jamieson, A. T. and Subak-Sharpe, J. H. **1974** Biochemical studies on the herpes simplex virus-specified deoxypyrimidine kinase activity. *J. Gen. Virol.* **24** 481-492.

Jamieson, A. T., Gentry, G. A. and Subak-Sharpe, J. H. **1974** Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. *J. Gen. Virol.* **24** 465-480.

Johnson, D. C. and Ligas, M. W. **1988** Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. *J. Virol.* **62** 4605-4612.

Johnson, P. A. and Everett, R. D. **1986** The control of herpes simplex virus type 1 late gene transcription: a `TATA-box'/ Cap site region is sufficient for fully efficient regulated activity. *Nucleic Acdis Res.* **14** 8247-8264.

Jurnak, F. **1985** Structure of the GDP domain of EF-Tu and location of the amino acids homologous to *ras* oncogene proteins. *Science* **230** 32-

Kamps, M. P. and Sefton, B. M. **1986** Neither arginine nor histidine can carry out the function of lysine-295 in the ATP-binding site of $p60^{src}$. *Mol. Cell. Biol.* **6** 751-762.

Karin, M. **1992** Signal transduction from cell-surface to nucleus in development and disease. *FASEB J.* **6** 2581-2590.

Katan, M. Haigh, A., Verijzer, C. P., Van der Vhet, P. C. and O'Hare, P. **1990** Characterisation of acellular factor which interacts functionally with Oct-1 in the assembly of a multicomponent transcription complex. *Nucleic Acids Res.* **18** 6871-6880.

Katan, M., Stevely, W. S. and Leader, D. P. **1985** Partial purification and characterization of a new phosphoprotein kinase from cells infected with pseudorabies virus. *Eur. J. Biochem.* **152** 57-65.

Kato, J., Matsushime, H., Hiebert, S. W. , Ewen, M. E. and Sherr, C. J. **1993** Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes and Dev.* **7** 331-342.

Katter-Cooley, P. and Wilcox, K. W. **1989** Characteirization of the DNA-binding properties of herpes simplex virus regulatory protein ICP4. *J. Virol.* **63** 696-704.

Keir, H. M. and Gold, E. **1963** Deoxyribonucleic acid nucleotidyl transferase and deoxyribonuclease from cultured cells infected with herpes simplex virus. *Biochimica et Biophysica acta* . **72** 263-276.

Kennedy, I. M., Stevely, W. S. and Leader, D. P. **1981** Phosphorylation of ribosomal proteins in hamster fibroblasts infected with pseudorabies virus or herpes simplex virus. *J. Virol.* **39** 359-366.

Kikkawa, U., Kishimoto, A. and Nishizuka, Y. **1989** The protein kinase C family: heterogeneity and its implications. *Ann. Rev. Biochem.* **58** 31-44.

Klein, R. T. **1982** The pathogenesis of acute latent and recurrent herpes simplex virus infections: brief review. *Arch. Virol.* **72** 143-168.

Kleinman, J. H. and Moss, B. **1973** Protein kinase activityfrom vaccinia virus: solubilization and separation into heat-labile and heat-stable components. *J. Virol.* **12** 684-689.

Kleinman, J. H. and Moss, B. **1975a** Purification of a protein kinase and two phosphate acceptor proteins from vaccinia virions. *J. Biol. Chem.* **250** 2420-2429.

Kleinman, J. H. and Moss, B. **1975b** characteization of a protein kinase and two phosphate acceptors from vaccinic virions. *J. Biol. Chem.* **250** 2430-2437.

Knipe, D. M., Quinalan, M. P. and Spang, A. E. **1982** Characterisation of two conformational forms of the major DNA-binding protein encoded by herpes simplex virus 1 *J. Virol.* **44** 736-741.

Knof, K. **1991** Properties of herpes simplex virus DNA polymerase and characterisation of its associted exonuclease activity. *Eur. J. Biochem* **98** 231-244.

Knox, S. R., Corey, L., Blough, H. A. anf Lerner, A. W. **1982** Historical findings in subsets from a high socieconomic group who have genital infections with herpes simplex virus. *Sex. Trans. Dis.* **19** 15-20.

Koide, H., Ogita, K., Kikkawa, U. and Nishizuka, Y. **1992** Isolation and characterization of the epsilon subspecies of protein kinase C from rat brain. *Proc. Natl. Acad. Sci. USA* **89** 1149-1153.

Krause, P. R., Croen, K. D., Straus, S. E. and Ostrove, J. M. **1988** detection and characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia. *J. Virol.* **62** 4819-4823.

Krikorian, C. R. and Read, G. S. **1991** An in vitro mRNA degradation system to study the virion host shutoff function of herpes simplex virus. *J. Virol* **65** 112-122.

Kristie, T. M. and Roizman, B. **1986** α4, the major regulatory protein of herpes simplex virus type 1 is stably and specifically associated with promoter-regulatory domains of α genes and of selected other viral genes. *Proc. Natl. Acad. Sci. USA.* **83** 3218-3222.

Kristie, T. M. and Sharp, P. A. **1993** Purification of the cellular C1 factor required for the stable recognition of the Oct-1 homeodomain by the herpes simplex alpha-trans-induction factor (VP16). *J. Biol. Chem.* **268** 6525-6534.

Kristie, T. M., LeBowitz, J. H. and Sharp. P. A. **1989** The octomer-binding proteins form multi-protein-DNA complexes with HSV α TIF regulatory protein. *Embo J.* **8** 4229-4238.

Kuhn, J. E., Kramer, M. D., Willenbacher, W., Weiland, U. and Lorentzen, E. U. **1990** Identification of herpes simplex virus type 1 glycoproteins interacting with the cell surface. *J. Virol.* **64** 2491-2497.

Kwong, A. D. and Frenkel, N. **1987** Herpes simplex virus-infected cells contain a function(s) that destabilises both host and viral mRNAs. *Proc. Natl. Acad. Sci. USA.* **84** 1926-1930.

Kwong, A. D., Kruper, J. A. and Frenkel, N. **1988** Herpes simplex virus virion host shutoff function. *J.Virol.* **62** 912-921.

Kypta, R. M., Goldberg, Y., Ulug, E. T. and Courtneidge, S. A. **1990** Association between the PDGF receptor and members of the src family of tyrosine kinases. *Cell* **1990** 481-492.

LaMarco, K., Thompson, C. C., Byers, B. P., Walton, E. M. and McKnight, S. L. **1991** Identification of the Ets-and notch-related subunits in GA binding protein. *Science* 789-792.

Lane, D. **1988** Enlarged family of putative helicases. *Nature* **334**

Langeland, N., Holmsen, H., Lillehaug, J. R. and Haarr, L. **1987** Evidence that neomycin inhibits binding of herpes simplex virus type 1 to the cellular receptor. *J. Virol.* **61** 3388-3393.

Langeland, N., Oyan, A. M., Marsden. H. S., Cross, A., Gloriosos, J. C., Moore, L. J. and Haarr, L. **1990** Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved in adsorption to the cellular receptor. *J. Virol.* **64** 1271-1277.

Langelier, Y. and Buttin, G. **1978** Characterisation of ribonucleotide reductase induction in BHK-21/C13 syrian hamster cell line upon infection with herpes simplex virus (HSV). *J. Gen. Virol.* **57** 21-31.

Lankinen, H., Everett, R., Cross, A., Conner, J. and Marsden, H. S. **1993** Epitope mapping identifies an exposed loop between the unique amino- and conserved carboxy-domains of the large subunit of herpes simplex virus type 1 ribonucleotide reductase. *J. Gen. Virol.* **74** 1871-1877.

Lankinen, H., Graslund, A. and Thelander, L. **1982** Induction of a new ribonucleotide reductase after infection of mouse L cells with pseudorabies virus. *J. Virol.* **41** 893-900.

Lankinen, H., Telford, E., MacDonald, D. and Marsden, H. **1989** The unique N-terminal domain of the large subunit of herpes simplex virus ribonucleotide is prefferentially sensitive to proteolysis. *J. Gen. Virol.* **70** 3159-3169.

Laybourn, P. J. and Dahmus, M. E. **1990** Phosphorylation of RNA polymerase IIa occurs subsequent to interaction with the promoter and before initiation of transcription. *J. Biol. Chem.* **265** 13165-13173.

Lee, W. S., Kao, C. C., Bryant, G. O., Liu, X and Berk, A. J. **1991** Adenovirus E1A activation binds the basic repeat in the TATA box transcription factor. *Cell* **67** 365-376.

Leib, D. A., Nadeau, K. C., Rundle, S. A. and Schaffer, P. A. **1991** The promoter of the latency associated transcripts of herpes simplex virus type 1 contains a functional cAMP response elemement: Role of the latency-associated transcripts and cAMP in reactivation of viral latency. *Proc. Natl. Acad. Sci. USA* **88** 48-52

Leinbach, S. S. and Heath, L. S. **1988** A carboxy-terminal peptide of the DNA-binding protein ICP8 of herpes simplex virus contains a single-stranded DNA-binding site. *Virology* **166** 10-16.

Leinbach, S. S. and Summers, W. C. **1980** The structure of the herpes simplex virus type 1 DNA as probed by micrococcal nuclease digestion. *J. Gen. Virol.* **51** 45-59.

Lemaster, S and Roizman, B. **1980** Herpes simplex virus phosphoproteins. II. Characterization of the virion protein kinase aand the polypeptides phosphorylated in the virion. *J. Virol.* **35** 798-811.

Levy-Favatier, F., delpech, M. and Kruh, J. **1987** characterization of an arginine-specific protein kinase tightly bound to rat liver DNA. *Eur. J. Biochem.* **166** 617-621.

Lew, D. J. and Reed, S. I. **1992** A proliferation of cyclins. *Trends. Cell Biol.* **2** 77-80.

Lieberman, P. M. and Berk, A. J. **1991** The Zta trans-activator protein stabilises TFIID association with promoter DNA by direct protein-protein interaction. *Genes. Dev.* **5** 2441-2454.

Ligas, M. W. and Johnson, D. C. **1988** A herpes simplex mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to cells but is unable to penetrate into cells. *J. Virol.* **62** 1486-1494.

Lillycrop, K. A., Dent, C. L., Wheatley, S. C., Beech, M. N., Ninkina, N. N., Wood, J. N. and Latchman, D. S. **1991** The octamer-binding protein Oct-2 represses HSV-1 immediate-early genes in cell lines derived from latently infectable sensory neurons. *Neuron* **7** 387-390.

Lin, A. N., Frost, J., Deng, T. L., Smeal, T., Alalawi, N., Kikkawa, U., Hunter, T., Brenner, D. and Karin , M. **1992** Caesin kinase-II is a negative regulator of c-jun DNA-binding and AP-1 activity. *Cell.* **70** 777-789.

Lin, M., Chen, W. and Broyless, S. S. **1992** The vaccinia virus B1R is a serine/threonine kinase. *J. Virol.* **66** 2717-2723.

Lin, Y-S, Ha, I., Reinberg, D. and Green, M. **1991** Binding of the general transcription factor TFIIB to an acidic activating region. *Nature* **353** 569-571.

Lin, Y. S. and Green, M. R. **1991** Mechanism of action of an acidic transcriptional activator in vitro. *Cell* **64** 971-981.

Lissoos, T. W., Beno, D. W. A. and Davis, B. H. **1993** 1,25-dihydroxyvitamin-D3 acutely activates Raf kinase and Raf perinuclear translocation via a protein-kinase-C dependent pathway. *Clinical Res.* **41** 161.

Littler, E., Purifoy, D., Minson, A and Powell, K. L. **1983** Herpes simplex virus non-structural proteins . III. Function of the major DNA binding protein. *J. Gen. Virol.* **64** 983-995.

Littler, E., Stuart, A. D. and Chee, M. S. **1992** Human cytomegalovirus UL97 open reading frame encodes a protein that phosphorylates the antiviral nucleoside analogue ganciclovir. *Nature* **358** 160-162.

Liu, F and Roizman, B. **1992** Differentiation of multiple domains in the herpes simplex virus proteas encoded by the U_L26 gene. *Proc. Natl. Acad. Sci. USA.* **89** 2076-2080.

Liu, F. and Roizman, B. **1991** The herpes simplex virus 1 gene encoding a protease contains within its coding region the more abundnt substrate *J. Virol.* **65** 5149-5156.

Lockson, D. and Galloway, D. A. **1988** Sequence and structural requirements of a herpes simplex DNA replication origin. *Mol. Cell. Biol.* **8** 4018-4027.

Lu, H., Flores, O., Weinmann, R. and Reinberg, D. **1991** The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. *Proc. Natl. Acad. Sci. USA* **88** 1004-1008.

Lu, H., Zawel, L., Fisher, L., Egly, J. M. and Reinberg, D. **1992** Human general transcription factor IIH phosphorylates the C-terminal domain of RNA polymerase II. *N ature* **358** 641-645.

Lue, N. F. and Kornberg, R. D. **1993** A possible role for the yeast TATA-element-binding protein in DNA replication. *Proc. Natl. Acad. Sci. USA* **90** 8018-8022.

Luo, J-H., Smith, C. C., Kulka, M. and Aurelian, L. **1991** A truncated protein kinase domain of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) expressed in *Escherichia coli. J. Biol. Chem.* **266** 20976-20983.

Luo, J. H. and Aurelian, L. **1992** The transmembrane helical segment but not the invariant lysine is required for the kinase activity of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10). *J. Biol. Chem.* **267** 9645-9653.

MacClean, A. R., Ul-Fareed, M., Robertson, L., Harland, J. and Brown, S. M. **1991** Herpes simplex virus type 1 deletion variants 1714 and 1716 pinpoint neurovirulence related sequences in glasgow strain 17^+ between immediate early gene 1 and the 'a' sequence. *J. Gen. Virol.* **72** 631-639.

Mackem, S. and Roizman, B. **1982a** Differentiation between α promoter and regulatory regions of herpes simplex virus 1: the functional domains and sequence of a movable α regulator. *Proc. Natl. Acad. Sci. USA* **79** 4917-4921.

Mackem, S. and Roizman, B. **1982b** Structural features of the herpes simplex α genes 4, 0 and 27 promoter regulatory sequences which confer α regultion on chimeric thymidine kinase genes. *J. Virol* **44** 939-949.

MacPherson, I and Stoker, M. **1962** Polyoma transformation of hamster cell clones-an investigation of genetic factors affecting all competence. *Virology* **16** 147-151.

Marsden, H. S. , Campbell, M. E. Haarr, L., Frame, M. C. Parris, D. S., Murphy, M., Hope, R. G., Muller, M. T. and Preston, C. M. **1987** The 65,000 Mr DNA-binding and virion trans inducing proteins of herpoes simplex type 1. *J. Virol* **61** 2428-2437.

Marsden, H. S., Crombie, I. K., Subak-Sharpe, J. H. **1976** Control of protin synthesis in herpes virus-infected cells: analysis of the polypeptides induced by wild type and sixteen temperature-sensitive mutants. *J. Gen. V irol.* **31** 347-372.

Maru, Y. and Wittle, O. N. **1991** The *BCR* gene encodes a novel serine/threonine kinase activity within a single exon. *Cell* **67** 459-468.

Masse, T., Garcin, D., Jacquemont, B. and Madjar, J. J. **1990** Ribosome and protein synthesis modifications after infection of human epidermoid carcinoma cells with herpes simplex virus type 1. *Mol. General. Genet.* **220** 377-388.

Matsuda, M., Mayer, B. J., Fukui, Y. and Hanafusa, H. **1990** Binding of transforming protein P47(gag-crk), to a broad range of phosphotyrosine-containing proteins. *Science* **248** 1537-1539.

Mavromara-Nazos, P., Silver, S., Hybenthal-Voss, J., McKnight, J. L. C. and Roizman, B. **1986** Regulation of herpes simplex virus 1 genes: α gene sequence requirements for transient induction of indictor genes regulated by β or late ($\gamma 2$) promoters. *Virology* **149** 152-164.

McCarthy, A. M., McMahan, L. and Schaffer, P. A. **1989** Herpes simplex virus type 1 ICP27 mutants exhibit altered patterns of transcription and are DNA deficient. *J. Virol.* **63** 18-27.

McClements, W., Yamanaka, G., Garsky, V., Perry, H., Bacchetti, S., Colonno, R and Stein, R. B. **1988** Oligopeptides inhibit the ribonucleotide reductase of herpes simplex virus by causing subunit separation. *Virology* **162** 270-273.

McGeoch, D. J. and Barnett, B. C. **1991** Neurovirulence factor. *Nature* **353** 609.

McGeoch, D. J., Dalrymple, M. A., Davison, A. J., Dolan, A., Frame, M. C., Perry, D., Scott, J. E. and Taylor, P. **1988a** The complete sequence of the long unique region in the genome of herpes simplex virus type 1. *J. Gen. Virol.* **69** 1531-1574.

McGeoch, D. J., Barnett, B. C. and MacLean, C. A. **1993** Emerging functions of alphaherpesvirus genes. *Seminars in Virol.* **4** 125-134.

McGeoch, D. J., Dalyrmple, M. A., Dolan, A., McNab, D., Derry, L. J., Taylor, P. and Challberg, M. D. **1988b** Structures of herpes simplex virus type 1 genes required for the replication of virus DNA. *J. Virol.* **62** 444-453.

McGeoch, D. J., Dolan, A., Donald, S. and Braver, D. H. K. **1986** Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1. *Nucleic. Acids. Res.* **14** 1727-1744.

McKnight, S. L., Kristie, T. M. and Roizman, B. **1987** Binding of the virion protein mediating gene induction in herpes simplex virus 1 infected cells at its cis site requires cellular proteins. *Proc. Natl. Acad. Sci. USA* **84** 7061-7065.

McLauchlan, J. and Rixon, F. J. **1992** Characterization of enveloped tegument structures (L particles) produced by alphaherpesviruses: integrity of the tegument does not depend on the presence of capsid or envelope. *J. Gen. Virol.* **73** 269-276.

McLauchlan, J., Phelan, A., Loney, C., Sandri-Goldin, R. M. and Clements, J. B. **1992** Herpes simplex virus IE63 acts at the posttranscriptional level to stimulate viral mRNA 3' processing. *J. Virol.* **66** 6939-6945.

McLauchlan, J., Simpson, S. and Clements, J. B. **1989** Herpes simplex virus induces a processing factor that stimulates poly(A) site usage. *Cell* **59** 1093-1105.

McMahan, L. and Schaffer, P. A. **1990** The repressing and enhancing functions of the herpes simplex regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection. *J. Virol.* **64** 3471-3458.

McNabb, D. S. and Courtney, R. J. **1992** Identification of the herpes simplex virus type 1 protein encoded by the UL35 open reading frame. *J. Virol.* **66** 2653-2663.

McVey, D., Brizuela, L., mohr, I., Marshak, D. R., Gluzman, Y. and Beach, D. **1989** phosphorylation of large tumour antigen by cdc2 stimulates SV40 DNA replication. *Nature* **341** 503-507.

Mermoud, J., Cohen, P and Lamond, A. **1992** A ser/thr protein phosphotase required for both catalytic steps of pre-mRNA splicing. *Nucleic Acids Res.* **20** 5263-5269.

Messing, J. **1979** A multipurpose cloning system based on the single-stranded DNA bacteriophage M13. *Rec. DNA Tech. Bull.* **2** 43-48.

Metzler, D. W. and Wilcox, K. W. **1985** Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. *J. Virol.* **55** 329-337.

Micklem, G., Rowley, A., Harwood, J., Nasmyth, K. and Diffley, J. F. X. **1993** Yeast origin recognition complex is involved in DNA replication and transcriptional silencing. *Nature* **366** 87-89.

Miller, G. **1985** Epstein-Barr virus. In "Virology" (Ed, B. N. Fields), Raven Press, New York, 563-589.

Mills, L. K. Shi, Y. and Millette, R. L. **1994** YY1 is the cellular factor shown previously to bind regulatory regions of several leaky-late ($\beta\gamma$, $\gamma 1$) genes of herpes simplex virus type 1. *J. Virol.* **68** 1234-1238.

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

Moldave, K. **1985** Eukaryotic protein synthesis. *Ann. Rev. Bioch.* **54** 1109-147.

Montminy, M. R., Gonzale, G. A. and Yamamoto, K. **1990** Regulation of cAMP-inducible genes by CREB. *Trends. Neurosci.* **13** 184-188.

Morgan, J. I. and Curran, T. **1991** Stimulus-transcription coupling in the nervous system: involovement of the inducible oncogenes fos and jun. *Ann. Rev. Neurosci.* **14** 421-451.

Morley, S. J., Rau, M., Kay, J. E. and Pain, V. M. **1993** Increased phophorylation of eukaryotic initiation factor 4 alpha during early activation of T lymphocytes correlates with increased initiation factor 4F complex formation. *Eur. J. Biochem.* **218** 39-48.

Morrison, B. D., Feltz, S. M. and Pessin, J. E. **1989** Polylysine specifically activates the insulin-dependent insulin receptor protein kinase. *J. Biol. Chem.* **264** 9994-10001.

Mosca, J. D., Bednarik, D. P., Raj, N. B. K., Rosen, C. A., Sodroski, J. G., Haseltine, W. A., Hayward, G. S. and Pitha, P. M. **1987** Activation of human immunodeficiency virus by herpes virus infection: identification of a region within the long terminal repeat that responds to a trans-acting factor encoded by herpes simplex virus. *Proc. Natl.Acad. Sci. USA* **84** 7408-7412.

Moss, H. **1986** The herpes simplex virus type 2 alkaline DNase is essential for replication and growth. *J. Gen. Virol.* **67** 1173-1178.

Muller, M. T. **1987** Binding of the herpes simplex virus type 1 gene product ICP4 to its own transcription start site. *J. Virol.* **61** 858-865.

Murchie, M. J. and McGeoch, D. J. **1982** DNA sequence analysis of an immediate-early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950 to 0.978). *J. Gen. Virol.* **62** 1-15.

Nasseri, M. and Mocarski, E. S. **1988** The cleavage recognition signal is contained with sequences surrounding the a-a junction in herpes simplex virus DNA. *Virology* **167** 25-30.

Neiderman, J., Miller, G., Pearson, H. and Pagno, J. **1976** Patterns of excretion of Epstein-Barr virus in saliva and other oropharangeal sites during infectious mononucleosis. *N. Eng. J. Med.* **294** 1365-1369.

Ng, T. I. and Grose, C. **1992** Serine protein kinase associated with Varicella-Zoster virus ORF 47. *Virology* **191** 9-18.

Nicosa, M., Deshmane, S. L., Zabolotny, J. M., Valyi-Nagy, T. and Fraser, N. W. **1993** Herpes simplex virus type 1 latency-associated transcript (LAT) promoter deletion mutants can express a 2-kilobase transcript mapping to the LAT region. *J. Virol.* **67** 7276-7283

Nikas, I., Darling, A. J., Lankinen, H. M., Cross, A. M., Marsden, H. M. and Clements, J. B. **1990** A single amino acid substitution in the large subunit of herpes simplex virus ribonucleotide reductase which prevents subunit interaction. *J. Virol.* **71**

Nikas, I., McLauchlan, J., Davison, A. J., Taylor, W. R. and Clements, J. B. **1986** Structural features of ribonucleotide reductase. *Proteins: Struc. Func, Genet.* **1** 376-384.

Nutter, L., Grill, S. and Cheng, Y. C. **1985** The sources of deoxyribonucleotides for virus DNA synthesis in herpes simplex virus type 2 (HSV-2) infected Hela cells. *Fed. Proc.* **44** 431.

O'Hare, P. and Hayward, G. S. **1985a** Evidence for a direct role for the 175,000 and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the treansactivation of delayed-early promoters. *J. Virol.* **53** 751-760.

O'Hare, P. and Hayward, G. S. **1985b** Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early expression in a pathway involving positive and negative feedback regulation. *J. Virol.* **56** 723-733.

O'Hare, P. and Goding, C. R. **1988** Herpes simplex virus regulatory elements and the immunoglobulin octomer domain bind a common factor and are both targets for transactivation. *Cell* **52** 435-445.

O'Hare, P. and Hayward, G. S. **1987** Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate early gene by three virus encoded trans activating factors. *J. Virol* **61** 190-199.

O'Hare, P., Goding, C. R. and Haigh, A. **1988** Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. *Embo J.* **7** 4231-4238.

Odawara, M., kadowaki, T., Yamamoto, R., Shibasaki, Y., Tobe, K., Accili, D., Bevins, C., Mikami, Y., Matsuura, N., Akanuma, Y., Takaku, F., Taylor, S. I. and Kasuga, M. **1989** Human diabetes associated with a mutation in the tyrosine kinase domain of the insulin receptor. *Science* **245** 66-68.

Ogita, K., Miyamoto, S. I., Yamaguchi, K., Koide, H., Fujisawa, N., Kikkawa, U., Sahara, S., Fukami, Y. and Nishizuka, Y. **1992** Isolation and characterisation of delta-subspecies of protein kinase C from rat brain. *Proc. Natl. Acad. Sci. USA* **89** 1592-1596.

Olivo, P. D., Nelson, N. J. and Challberg, M. D. **1988** Herpes simplex virus DNA replication : The UL9 gene encodes an origin binding protein. *Proc. Natl. Acad. Sci. USA* **85** 5414-5418.

Oraskar, A. A. and Read, G. S. **1987** A mutant of herpes simplex virus type 1 exhibits increased stability of immediate-eartly (alpha) mRNAs. *J. Virol.* **61** 604-606.

Oraskar, A. A. and Read, G. S. **1989** Control of mRNA stability by the virion host shutoff function of herpes simplex virus. *J. Virol.* **63** 1897-1906.

Parris, D. S., Cross, A., Haarr, L., Orr, A. and Marsden, H. S. **1988** Identification of the gene encoding the 65-kilodalton DNA-binding protein of herpes simplex virus type 1. *J. Virol.* **62** 818-825.

Palen, E. and Traugh, J. A. **1991** Phosphorylation of casein kinase II. *Biochem.* **30** 5586-5590.

Panning, B. and Smiley, J. R. **1989** Regulation of cellular genes transduced by herpes simplex virus. *J. Virol.* **63** 1929-1937.

Paoletti, E. and Moss, B. **1972** Protein kinases and specific phosphate acceptor proteins associated with vaccinia virus cores. *J. Virol.* **10** 417-424.

Papavassiliou, A. G. and Silverstein, S. J. **1990** Interaction of cell and virus proteins with DNA sequences encompassing the promoter/regulator and leader regions of the herpes simplex virus thymidine kinase gene. *J. Biol. Chem.* **265** 9402-9412

Papavassiliou, A. G., Wilcox, K. W. and Silverstein, S. J. **1991** The interaction of ICP4 with cell/infected cell factors and its state of phosphorylation modulate differential recognition of leader sequences in herpes simplex virus DNA. *Embo J.* **10** 397-406.

Paradis, H., Gaudreau, P., Brazeau, P. and Langelier, Y. **1988** Mechanism of inhibition of herpes simplex virus (HSV) ribonclotide reductase by a nonopeptide corresponding to the carboxy-terminus of its subunit 2. *J. Biol. Chem.* **263** 16045-16050.

Paterson, T. and Everret, R. D. **1988** Mutational dissection of the HSV-1 immediate early protein Vmw 175 involved in transcriptional transactivation and repression. *Virology* **166** 186-196.

Payne, J. M., Laybourn, P. J and Dahmus, M. E. **1989** The transition of RNA polymerase II from initiation to elongation is associated with phosphorylkation of the carboxy-terminall domain of subunit IIA. *J. Biol. Chem.* **264** 19621-196229.

Pederson, M. M., Taley-Brown, S. and Millette, R. L. **1981** Gene expression of herpes simplex virus III. Effect of arabinosyladenine on viral polypeptide synthesis. *J. Virol.* **38** 712-719.

Pelley, R. J., Maihle, N. J., Boerkoel, C., Shu, H.-K., Carter, T. H., Moscovici, C. and Kung, H.-J. **1989** Disease tropism of c-erb: effects of carboxy-terminal tyrosine and internal mutations on tissue-specific tropism. *Proc. Natl. Acad. Sci. USA* **86** 7164-7168.

Pereira, L., Wolff, M.H., Fenwick, M. and Roizman, B. **1977** Regulation of herpesvirus macromolecular synthesis v. properties of two polypeptides made in hSV-1 and HSV-2 infected cells. *Virology* **77** 733-749.

Perera, P. A. and Morrison, S. M. **1970** Evidence for the induction of a new deoxycytidine kinase in cells infected with herpes virus. *Bioch. J.* **117** 21-22.

Pesis, K. H., Wei, Y., Lewis, M. and Matthews, H. R. **1988** Phosphohistidine is found in basic nuclear proteins of polcephalum physarum. *FEBS Letts.* **239** 151-154.

Pestka, S. and Langer, J. A. **1987** Interferons and their actions. *Ann. Rev. Biochem.* **56** 727-777.

Peterson, C. L., Kruger, W. and Herkowitz, I. **1991** A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SINI *Cell* **64** 1135-1143.

Pines, J. **1993** Cyclins and cyclin-dependent protein kinases: take your partners. *Trend. Bioch. Sci.* **18** 195-197.

Pines, J. and Hunter, T. **1991** Cyclin-dependent kinases: a new cell cycle motif?. *Trends. Cell Biol.* **1** 117-121.

Ponce de Leon, M., Eisenberg, R. J. and Cohen, G. H. **1977** Ribonucleotide reductase from herpes simplex virus (types 1 and 2) infected and uninfected KB cells: properties of the partially purified enzymes. *J. Gen. Virol.* **26** 547-553.

Post, L. E., Mackem, S. and Roizman, B. **1981** Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with α gene promoters. *Cell* **24** 555-565.

Powell, K. L. and Purifoy, D. J. M. **1976** DNA-binding proteins in duced by herpes simplex virus type 2 in Hep-2 cells. *Virology* **66** 217-228.

Powell, K. L. and Purifoy, D. J. M. **1976** DNA-binding proteins induced by herpes simplex virus type 2 in HEp-2 cells. *J. Virol.* **19** 717-731.

Preston, C. M. **1979** Control of herpes simplex virus type 1 mRNA synthe sis in cells infected with wild-type virus or the temperature sensitive mutant tsK *J. Virol.* **29** 275-284.

Preston, C. M. and Tannahil, C.M. **1984** Effects of orientation and postion on the activity of a herpes simplex virus immediate-early gene far-upstream region. *Virology* **137** 439-444.

Preston, C. M., Cordingley, M. G. and Stow, N. D. **1984** Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate early gene. *J. Virol* **50** 708-716.

Preston, C. M., Frame, M. C. and Campbell, M. C. **1988** A complex formed between cell componemts and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence *Cell* **52** 425-434

Preston, V. G., Coater, J. A. V. and Rixon, F. J. **1983** Identification and characterisation of a herpes simplex virus gene product required for encapsidaton of virus DNA. *J. Virol.* **45** 1056-1064.

Preston, V. G., Darling, A. J. and McDougall, I. M. **1988** The herpes simpplex virus type 1 temperature sensitive mutant ts1222 has a single base pair deletion in the small subunit of ribonucleotide reductase. *Virology* **167** 458-467.

Preston, V. G., Palfreyman, J. W. and Dutia, B. M. **1984** Identification of a herpes simplex type 1 polypeptide which is a component of the virus-induced ribonucleotide reductase. *J. Virol.* **65** 1457-1466.

Preston, V. G., Rixon, F. J., McDougall, I. M., McGregor, M. and AlKobaisi, M.F. **1992** Processing of the herpes simplex virus assembly protein ICP35 near its carboxy terminal end requires the product of the whole U_L26 reading frame. *Virology* **186** 87-98.

Purves, F. C. and Roizman, B. **1992** The UL13 gene of herpes simlex virus 1 encodes the functions for posttranslational processing associated with phosphorylation of the regulatory protein α22. *Proc. Natl. Acad. Sci. USA* **89** 7310-7314.

Purves, F. C., Katan, M., Stevely, W. S., and Leader, D. P. **1986** Characterization of the induction of a new protein kinase in cells infected with herpesviruses. *J. Gen. Virol.* **67** 1049-1057.

In vivo characterization of site-directed mutataions in the promoter of the herpes simplex virus type 1 latency-associated transcripts.

Purves, F. C., Lognecker, R. M., Leader, D. P. and Roizman, B. **1987** Herpes simplex virus type 1 protein kinase encoded by open reading frame US3 which is not essential for virus growth in cell culture. *J. Virol.* **61** 2896-2901.

Purves, F. C., Spector, D. and Roizman, B. **1991** The herpes simplex virus 1 protein kinase encoded by the US3 gene mediates posttranscriptional modifiction of the phosphoprotein encoed by the UL34 gene. *J. Virol.* **65** 5757-5764.

Purves, F. C., Spector, D. and Roizman, B. **1992** UL34 the target of the herpes simplex virus US3 protein kinase is a membrane protein which in its unphosphorylated state is associated with novel phosphoproteins. *J. Virol.* **66** 4295-4303.

Pyles, R. B., Sawtell, N. M. and Thompson, R. L. **1992** Herpes simplex virus type 1 dUTPase mutants are attenuated for neurovirulence, neuroinvasiveness, and reactivation from latency. *J. Virol.* **66** 6706-6713.

Quinalan, M. P. and Knipe, D. M. **1985** Stimulation of expression of a herpes simplex DNA binding protein by two viral functions. *Mol. Cell. Biol.* **5** 957-963.

Rader, K. A., AcklndBerglund, C. E., Miller, J. K., Pepose, J. S. and Leib, D. A. *J. Gen. Virol.* **74** 1859-1869.

Radomski, M. W., Palmer, R. M. J. and Moncada, S. **1990** An L-arginine/nitric oxide pathway present in human platelets regulates aggregation. *Proc. Natl. Acad. Sci. USA* **87** 5193-5197.

Randall, C. C., Rogers, H. W., Downer, D. N. and Gentry, G. A. **1972** Protein kinase activity in equine herpesvirus. *J. Virol.* **9** 216-222.

Rao, P. N. and Johnson, R, T. **1970** Mammalian cell fusion: studies on the regulation of DNA synthesis and mitosis. *Nature* **225** 159-164.

Rawls, W. G. **1985** Herpes simplex virus. In "Virology" (Ed, B. N. Fields), Raven Press, New York.

Reichard, P. **1988** Interactions between deoxyribonucleotide and DNA synthesis. *Ann. Rev. Biochem.* **57** 349-374.

Reiger, J. L., Shen, F. and Treizenberg, S. J. **1993** Pattern of aromatic and hydrophobic amino acids critical for one of two subdomains of the VP16 transcriptional acivator. *Proc. Natl. Acad. Sci. USA* **90** 883-887.

Reines, D. **1992** E longation factor-dependent transcript shortening by template-engaged RNA polymerase II. *J. Biol. Chem.* **267** 3795-3800.

Reines, D. and Mote, J. Jr. **1993** Elongation factor SII-dependent transcription by RNA polymerase II through a sequence-specific DNA-binding protein. *Proc. Natl. Acad. Sci. USA.* **90** 1917-1921.

Rhee, S. G. **1991** Inositol phospholipid-specific phospholipase C: interaction of the gamma 1 isoform with tyrosine kinase. *Trends Biochem.* **16** 297-301.

Rice, S .A. and Knipe, D. M. **1990** Genetic eidence for two distinct transactivation functions of the herpes simplex viruds α protein ICP27. *J. Virol.* **64** 1704-1715.

Rice, S. A. and Knipe, D. M. **1988** Gene-specific transactivation by herpes simplex virus type 1 α protein ICP27. *J. Virol.* **62** 3814-3823.

Rice, S. A. and Lam, V. **1994** Amino acid substitution mutations in the herpes simplex virus ICP27 protein define an essential gene regulation function. *J. Virol.* **68** 823-833.

Rice, S. A., Lam, V. and Knipe, D. M. **1993** The acidic amino-terminal region of herpes simplex virus type 1 alpha protein ICP27 is required for an essential lytic function. *J. Virol.* **67** 1778-1787.

Rice, S. A., Long, M. C., Lam, V. and Spencer, C. A. **1994** RNA polymerase II is aberrantly phosphorylated and localized to viral replication compartments following herpes simplex virus infection. *J. Virol.* **68** 988-1001.

Rice, S.A. and Knipe, D.M. **1988** Gene-specific transactivation by herpes simplex virus type 1 α protein ICP27. *J. Virol.* **62** 3814-3823.

Richardson, H., Lew, D. J., Henze, M., Sugimoto, K. and Reed, S. I. **1992** Cyclin-B homologs in *saccharomyces cerevsiae* function in S-phase and G(2). *Genes. Dev.* **6** 2021-2034.

Roberts, M. S. , Boundy, A., O'Hare, P., Pizzatro, M. C. Ciufo, D. M. and Hayward, G. S. **1988** Direct correlation between a negative autoregulatory response element at the CAP site of the herpes simplex virus type 1 IE175 (α 4) promoter and a specific binding site for IE175 (ICP4) protein. *J. Virol* **62** 4307-4320

Rock, D. L., Nesburn, A. B., Ghiasi, H., Ong, J., Lewis, T. L., Lockensard, J. R. and Weschler, S. L. **1987** Detection of latency related viral RNAs in trigeminal ganglia of rabbits latently infected with herpes simplex virus type 1. *J. Virol.* **61** 3820-3826.

Roizman, B. **1982** The family herpesviridae: general description, taxonomy and classification. In "The Herpesviruses" vol 1 (Ed, B. Roizman). Plenum Press. New York 1-23.

Roizman, B. **1990** Herpesviridae- a brief introduction, in : "Virology" **2** 1787-1792, (eds; Fields, B. N. and Knipe, D. M.), Raven Press, New York.

Roizman, B. and Furlong, D. **1974** The replication of herpesviruses. In comprehaensive Virology vol 3 (Eds. H. Frankel-Conrat and R. R. Wagner) Plenum press, New York 229-403.

Roller, R. J. and Roizman, B. **1994** A herpes simplex virus 1 US11-expressing cell line is resistant to herpes simplex virus infection at a step in viral entry mediated by glycoprotein D. *J. virol.* **68** 2830-2839.

Rosen, O. M. and Lebwohl, D. E. **1988** Polylysine activates and alters the divalent cation requirements of the insulin receptor protein tyrosine kinase. *FEBS. Letts.* **231** 397-401.

Rossmann, M. G., Moras, and Olsen, K. **1974** Chemical and biological evolution of nucleotide binding proteins. *Nature* **250** 194

Rossow, P. W., Riddle, V. C. and Pardec, A. B. **1979** Synthesis of labile serum-dependent protein in early G1 controls animal cell growth. *Proc. Natl. Acad. Sci. USA* **76** 4446-4450.

Rubenstein, A. S., Gravell, M. and Darlington, R **1972** Protein kinase in enveloped herpes simplex virions. *Virology* **50** 287-290.

Samuel, C. E. **1993** The eIF-2α protein kinases, regulators of translation in eukaryotes from yeasts to humans. *J. Biol. Chem.* **268** 7063-7606.

Sen, G. C. and Lengyel, P. **1992** The interferon system. *J. Biol. Chem.* **267** 5017-5020.

Ruychen, W. T. and Weir, A. C. **1984** Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. *J. Virol.* **52** 727-733.

Ruyechan, W. T. **1983** The major herpes simplex virus DNA-binding protein holds single-stranded DNA in an extended configuration. *J. Virol.* **46** 727-733.

Sacks, W. R. and Schaffer, P. A.. **1987** Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. *J. Virol.* **61** 829-839.

Sacks, W. R., Greene, C. C., Ashman, D. P. and Schaffer, P. A. **1985** Herpes simplex virus type 1 ICP27 is an essential regulatory protein. *J. Virol.* **55** 796-805.

Sadowaski, I., Ma, J., Treizenberg, S. and Ptaschne, M. **1988** Gal4-VP16 is an unusally potent transcriptional activator. *Nature* **335** 563-564.

Salahudin, S. Z., Albasutri, D. V., Markem, P. D., Josephs, S. F., Struzeneggar, S., Kaplan, M., Halligan, G., Bibenfeld, P., Wong-Staal, F., Kramanski, B. and Gallo, C. **1986** Isolation of a new virus HBLV in patients with lympho proliferative disorders. *Science* **234** 596-601.

Sambucetti, L. C., Cherrington, J. M., Wilkinson, G. W. G. and Mocarski, E. S. **1989** NF-κB activation of the cytomegalovirus enhancer is mediated by the viral transactivator and by T cell stimulation. *Embo J.* **8** 4251-4258.

Sandri-Goldin, R. M. and Mendoza, G. E. **1992** A herpes simplex virus regulatory protein appears to act post-transcriptionally by affecting mRNA processing. *Genes. Dev.* **6** 848-863

Schek, N. and Bachenheimer, S. L. **1985** Degradation of cellular mRNAs induced by a viriono-associated factor during herpes simplex virus infection of vero cells. *J. Virol.* **55** 601-610.

Sekulovichj, R. E., Leary, K. and Sandri-Goldin, R. M. **1988** The herpes simplex virus type 1 α protein ICP27 can act as a trans-repressor or trans-activator in combination with ICP4 and ICP0. *J. Virol.* **62** 4510-4522.

Shapira, M., Homa, F. L., Glorioso, J. C. and Levine, M. **1987** Regulation of the herpes simplex virus type 1 late (γ) glycoprotein C gene: sequences between base pairs -34 to +39 control transient expression and responsiveness to transactivation by the products of immediate-early (α) 4 and 0 genes. *Nucleic Acids Res.* **15** 3097-3111.

Sharp, F. R., Griffith, J., Gonalez, M. F. and Sagar, S. M. **1989** Trigeminal nerve section induces fos-likeimmunoreactivity (FLI) in brainstrem and decreases FLI in sensory cortex. *Mol. Brain. Res.* **6** 217-220.

Sheih, M-T, WuDunn, D., Montgomery, R. I., Esko, J. D. and Spear., P. G. **1992** Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell. Biol.* **116**

Sheldrick, P. and Berthelot, N. **1974** Inverted repetitions in the chromosome of HSV. Cold Spring Harbor Symp. Quant. Biol. **39** 667-678.

Sherman, G., Gottleib, J. and Challberg, M. D. **1992** The UL8 subunit of the herpes simplex virus helicase-primase complex is required for efficient primer utilization. *J. Virol.* 4884-4892.

Shine, J. and Dalgarno, L. **1975** Determination of cistron specificity in bacterial ribosomes. *Nature* **254** 34-36.

Sjolander, A., Yamamoto, k., Huber, B. E. and Lapetina, E. G. **1991** Association of p21(ras) with phosphotidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA.* **88** 7980-7912.

Smeal, T., Binetruy, B., Mercola, D., Groverbardwick, A., Heidecker, G., Rapp, U.R. and Karin, M. **1992** Oncoprotein-mediated signalling cascade stimulates c-jun activity by phosphorylation of serine 63 and serine 73. *Mol. Cell. Biol.* **12** 3507-3513.

Smeyne, R. J., Schilling, K., Robertson, L., Luk, D., Oberdick, J., Curran, T. and Morgan, J. I. **1992** Fos-lacZ transgenic mice: mapping sites of gene induction in the nervous system. *Neuron* **8** 13-23.

Smiley, J. R. and Duncan, J. **1992** The herpes simplex virus type 1 immediate-early polypeptide ICP4 is required for expression of globin genes located in the viral genome. *Virology* **190** 538-541.

Smith, C. A. , Bates, P. Rivera-Gonvalez, R. Gu, B. and Deluca, N. **1993** ICP4 the major transcriptional regulatory protein of herpes simplex vir us type 1 forms a tripartite complex with TATA -binding protein and TFIIB. *J. Virol.* **67** 4676-4687

Smith, C. C., Luo, J. H., Hunter, J. C. R., Ordonez, J. V. and Aurelian, L. **1994** The transmembrane domain of the large subunit of HSV-2 ribonucleotide reductase (ICP10) is required for protein kinase activity and transformation-related signaling pathways that result in ras activation. *Virology* **200** 598-612.

Smith, I.L., Hardwicke, M.A. and Sandri-Goldin, R.M. **1992** Evidence that the herpes simplex virus immediate early protein ICP27 acts post-transcriptionally during infection to regulate gene expression. *Virology* **186** 74-86.

Smith, M. L., Lenette, E. M. and Reames, H. R. **1941** Isolation of the virus herpes simplex and demonstration of intranuclear inclusions in the case of acute encephalitis. *Am. J. Pathol.* **17** 55-56.

Smith, R. F. and Smith, T. F. **1989** Identification of new protein kinase-related genes in three herpesviruses, herpes simplex virus, varicella-zoster virus, and Epstein-Barr virus. *J. Virol.* **63** 450-455.

Snao, L., Rapp, L. M. and Weller, S. K. **1993** Herpes simplex virus 1 alkaline nuclease is required for efficient egress of capsiods from the nucleus. *Virology* **196** 146-162.

Souvignet, C. and Chambaz, E. M. **1990** Phospholipid independent phosphorylation of protamine by protein kinase C: effects of polyanions. *Cellu. Signalling.* **2** 171-176.

Spaete, R.R. and Frenkel, N. **1982** The herpes simplex virus amplicon: a new eukaryotic defective-virus cloning amplifying vector. *Cell* **30** 295-304.

Speck, P. G. and Simmons, A. **1991** Divergent molecular pathways of productive and latent infection with a virulent strain of herpes simplex virus type 1. *J. Virol.* **65** 4001-4005.

Spector, T., Stonehuerner, J. G., Biron, K. K. and Averett, E. R. **1987** Ribonucleotide reductase induced by varicella-zoster virus. Characterization of its inhibition. *Biochem. Pharm.* **36** 4341-4346.

Spivak, J. G. and Fraser, N. W. **1987** Detection of herpes simplex virus type 1 transcripts during latent infection in mice. *J. Virol.* **61** 3841-3847.

Spivak, J. G., Woods, G. M. and Fraser, N. W. **1991** Identification of a novel latency-specific splice donor signal within the herpes simplex virus type 1 2.0-kilobase latency-associated (LAT): translation inhibition of LAT open reading frames by the intron within the 2.0-kilobase LAT. *J. Virol.* **65** 6800-6810.

Stern, D. F., Zheng, P., Beider, D. R. and Zerillo, C. **1991** SpK1, a new kinase from saccharomyces cerevisiae, phosphorylates proteins on serine, threonine and tyrosine. *Mol. Cell. Biol.* **1991** 987-1001.

Sternberg, P. W. and Horvitz, H. R. **1991** Signal transduction during C. elegans vulval induction. *Trends Genetics* **7** 366-371.

Stevely, W. S., Katan, M., Stirling, V., Smith, G., and Leader, D. **1985** Protein kinase activities associated with the virions of pseudorabies and herpes simplex virus. *J. Gen. Virol.* **676** 661-673.

Stevens, J. G., Wagner, E. K., Devi-Rao, G. B., Cook, M. L. and Feldman, L. T. **1987** RNA complementary to a herpes simplex virus gene mRNA is prominent in latently infected neurons. *Science* **235** 1056-1059.

Stevenson, D., Colman, K. L. and Davison, A. J. **1994** Characterization of the putative protein kinases specified by varicella-zoster virus genes 47 and 66. *J. Gen. Virol.* **75** 317-326.

Stow, N. D. **1982** Localisation of an origin of DNA replication within the TRs/IRs repeated region of the herpes virus type 1 genome. *Embo J.* **1** 863-867.

Stow, N. D. **1993** Sequences at the C-terminus of the herpes simplex virus type 1 UL30 protein are dispensible for DNA polymerase activity but not for viral origin-dependent DNA replication. *Nucleic Acids Res.* **21** 87-92.

Stow, N.D. and McMonagle, E.C. **1983** Characterisation of the TRs/IRs Origin of replication of herpes simplex virus type 1. *Virology* **130** 427-438.

Stow, N.D. and Stow, E.C. **1986** Isolation and characterisation of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. *J. Gen. Virol.* **67** 2571-2585.

Strom, T. and Frenkel, N. **1987** Effects of herpes simplex virus on mRNA stability. *J. Virol.* **61** 2198-2207.

Struthers, R.S., Vale, W.W., Arias, C., Sawehenko, P. E. and Montminy, M. R. **1991** Somatotrophin hydroplasia and dwarfism in transgenic mice expressing a non-phosphorylated CREB mutant. *Nature* **350** 622-624.

Studier, F. W. and Moffat, B. A. **1986** Use of bacteriophage T7 polymerase to direct high-level expression of cloned genes. *J. Mol. Biol.* **189** 113-

Su, L. and Knipe, D.M. **1989** Herpes virus α protein ICP27 can in hibit or augment viral gene transactivation. *Virology* **170** 496-504.

Sullivan, V., Talarico, C. L., Stanat, S. G., Davis, M., Coen, D. M. and Biron, K. K. **1992** A protein kinase homologue controls phosphorylation of ganciclovir in human cytomegalovirus-infected cells. *Nature* **358** 162-164.

Sung, C. K.,Wong, K. Y., Yip, C. C., Hawley, D. M. and Goldfine, I. D. **1994** Deletion of residues 485-599 from the human insulin receptor abolishes antireceptor antibody binding and influences tyrosine kinase activation. *Mol. Endcr.* **8** 315-324.

Svennerholm, B., Jeansson, S., Vahlne, A and Lycke, E. **1991** Involvement of glycoprotein C (gC) in adsorption of herpes simplex virus type 1 (HSV-1) to the cell. *Arch. Virol.* **120** 273-279.

Swain, M. A. and Galloway, D. A. **1986** Herpes simplex virus specifies two subunits of ribonucleotide reductase encoded by 3' coterminal transcripts. *J. Virol.* **57** 802-808.

Sze, P. and Herman, R.C. **1992** The herpes simplex virus type 1 ICP6 gene is regulated by a "leaky" early prom oter. *Virus Res.* **26** 141-152.

Szilagyi, J. F. and Cunningham, C. **1991** Identification and characterization of a novel non-infectious herpes simplex virus related particle. *J. Gen. Virol.* **72** 661-668.

Taylor, S. S., Knighton, D. R., Zheng, J., Ten Eyck, L. F. and Sowadski, J. M. **1992** Structure framework for the protein kinase family. *Ann. Rev. Cell Biol.* **8** 79-94.

Taha, M. Y., Brown, S. M., Clements, G. B. and Graham, D. I. **1990** The JH26045 deletion variant of herpes simplex virus type 2 (HG52) fails to produce necrotizing encephalitis following intracranial innoculation of mice. *J. Gen. Virol.* **71** 1597-1601.

Tan, K. B. **1975** Comparitive study of protein kinase associated with animal viruses. *Virology* **64** 566-570.

Tanese, N., pugh, B. F. and Tijan, R. **1991** Coactivators for proline activator purified fom the multisubunit human TFIID complex. *Genes Dev.* **5** 2212-2224.

Taylor, S. S., Buechler, J. A., and Yonemoto, W. **1990** cAMP-dependent protein kinase: framework for a diverse family of enzymes. *Ann Rev. Biochem.* **59** 971-1006.

Tazi, J., Daugeron, M., Cathala, G., Brunel, C. and Jeanteur, P. **1992** Adenosine phosphothioates (ATPalphas and ATPtau S) differentially affect the two steps of mammalian pre-mRNA splicing. *J. Biol. Chem.* **267** 4322-4326.

Tedder, D. G. , Everret, R. d. , Wilcox, K. W., Beard, P. and Pizer, L. I. **1989** ICP4-binding sites in the promoter and coding regions of the herpes simplex virue gD gene contribute to activation of in vitro transcription by ICP4. *J. Virol* **63** 2510-2520.

Tedder, D. G. and Pizer, L. I. **1988** Role for DNA-protein interaction of the herpes simplex virus glycoprotein D gene. *J. Virol.* **62** 4661-4672.

Tenney, D. J., Micheletti, P. A., Stevens, J. T., Hamatake, R. K., Matthews, J. T., Sanchez, A. R., Hurlburt, W. W., Bifano, M. and Cordingley, M. G. **1993** Mutations in the C terminus of herpes simplex virus type 1 DNA polymerase can affect binding and stimulation by its acessory protein UL42 without affecting basal polymerase activity. *J. Virol.* **67** 543-547.

Tenser, B. R., Edris, W. A. and Hay, K. A. **1993** Neuronal control of herpes simplex virus latency. Virology **195** p337-347.

Tenser, R. B., Hay, K. A. and Edris, W. A., **1989** Latency-associated transcript but not reactivatable virus is present in sensory ganglion neurons after inocultion of thymidine kinase-negative mutants of herpes simplex virus type 1. *J. Virol.* **63** 2861-2865.

Thomas, M. S., Gao, M., Knipe, D. M. and Powell, K. L. **1992** Association between the herpes simplex virus major DNA-binding proten and alkaline nuclease. *J. Virol.* **1992** 1152-1161.

Vaughan, P. J., Banks, L. M., Purifoy, D. J. M. and Powell, K. L. **1984** Interactions between herpes simplex DNA-binding proteins. *J. Gen. Virol.* **65** 2033-2041.

Thompson, C. C., Brown, T. A. and McKnight, S. L. **1991** convergence of Ets- and notch-related structural motifs in a heterodimeric DNA binding complex. *Science* **253** 762-768.

Thompson, R. L. and Stevens, J. G. **1983** Biological characterisation of a herpes simplex virus intertypic recombinant which is completely and specifically non-neurovirulent. *Virology* **131** 171-179.

Thompson, R. L., Rogers, S. K. and Zerhusen, M. A. **1989** Herpes simplex virus neurovirulence and productive infection of neural cells is associated with a function that maps between 0.82 and 0.832 map units on the HSV-1 genome. *Virology* **172** 435-450.

Towler, D. A., Gordon, J. I., Adams, S. P. and Glaser, L. **1988** The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* **57** 69-99.

Treizenberg, S. J., Kingsbury, R. C. and McKnight, S. L. **1988a** Functional disection of VP16, the trans-activator of herpes simplex virus immediate early gene expression. *Genes Dev.* **2** 718-729.

Treizenberg, S. J., LaMarco, K. L. and Mcknight, S. L. **1988b** Evidence of DNA:protein interactios that mediate HSV-1 immediate early gene activation by VP16. *Genes Dev.* **2** 730-742.

Turk, S. R., Kik, N. A., Birch, G. M., Chiego, D. J. Jr. and Shipman, C. Jr. **1989** Herpes simplex virus type 1 ribonucleotide reductase null mutants induce lesions in guinea pigs. *Virology* **73** 733-735.

Van der Sluijs, P., Hull, M., Huber, L. A., Male, P., Boud, B. and Mellman, I. **1992a** Reversible phosphorylation-dephosphorylation determines the localisation of rab4 during the cell cycle. *Embo. J.* **11** 4379-4389.

Van der Sluijs, P., Hull, M., Webster, P., Male, P., Goud, B. and Mellman, I. **1992b** The small GTP-binding protein rab4 controls an early sorting event in the endocytic pathway. *Cell* **70** 729-740.

Varmuza, S. L. and Smiley, J. R. **1985** Signals for site-specific cleavage of HSV DNA: Maturation involves two separate cleavage events distal to the recognition sequences. *Cell* **41** 793-802.

Vinson, C. R., Sigler, P. B. and McKnight, S. L. **1989** Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* **246** 911-916.

Vlazney, D. A. and Frenkel, N. **1981** Replication of herpes simplex virus DNA: localization of replication signals within defective virus genomes. *Proc. Natl. Acad. Sci. USA* **78** 742-746.

Wadsworth, S., Jacob, R. J. and Roizman, B. **1975** Anatomy of herpes simplex virus DNA II. Size composition and arrangement of inverted repeats. *J. Virol.* **5** 1487-1497.

Wagner, E. K. **1985** Individual HSV transcripts: Characterization of specific genes. In "The Herpesviruses", Vol 3, (Ed, B. Roizman), Plenum Press, 45-104.

Wagner, E. K. and Summers, W. C. **1978** Structure of the joint region and the termini of the DNA of herpes simplex virus type 1. *J. Virol.* **27** 374-387.

Wagner, E. K., Flanagan, W. M., Devi-Rao, G. Zhang, Y. F., Hill, J. M., Anderson, K. P. and Stevens, J. G. **1988** The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. *J. Virol.* **62** 4577-4585.

Walker, S., Greaves, R. and O'Hare, P. **1993** Transcriptional-activation by the cacidic domain of Vmw65 requires the integrity of the domain and involves additional determinants distinct from those necessary for TFIIB binding. *Mol. Cell. Biol.* **13** 5233-5244.

Wang, D. and Hawley, D. K. **1993** Identification of a 3′ —> 5′ exonuclease activity associated with human RNA polymerase II. *Proc. Natl. Acad. Sci. USA.* **90** 843-847.

Watson, R. J. and Clements, J. B. **1978** Characterisation of transcription-deficient temperature-sensitive mutants of herpes simplex virus type 1. *Virology* **91** 364-379.

Watson, R. J. and Clements, J. B. **1980** A herpes simplex virus type 1 function continously required for early and late virus RNA synthesis. *Nature* **285** 329-330.

Weber, G. **1983** Biochemical strategy of cancer cells and the design of chemotherapy: G.H.A. Clowes memorial lecture. *Canc. Res.* **43** 3466-3493.

Weber, P.C., Challberg, M. D., Nelson, N. J., Levine, M. and Glorioso, J. C. **1988** Inversion events in the HSV-1 genome are directly mediated by the viral DNA replication machinery and lack sequence specificity. *Cell* **54** 369-381.

Wechsler, S. L., Nesburn, A. B., Watson, R., Slanina, S. M. and Ghiasi, H. **1988** Fine mapping of the latency-related gene of herpes simplex virus type 1: alternative splicing produces distinct latency-realted RNAs containing open reading frames. *J. Virol.* **62** 4051-4058.

Weinheimer, S. D. and McKnight, S. L. **1987** Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. *J. Mol. Biol.* **195** 819-833.

Weir, H. M. and Stow, N. D. **1990** Two binding sites for the herpes simplex virus type 1 UL9 protein are required for efficient activity of the OriS replication origin. *J. Gen. Virol.* **71** 1379-1385.

Weir, H. M., Calder, J. M. and Stow, N. D. **1989** Binding of the herpes simplex virus type 1 UL9 gene product to an origin of viral DNA replication. *Nucleic. Acids. Res.* **17** 1409-1425.

Weizhong, C., Person, S., Warner, S. C., Zhou, J. and Deluca, N. A. **1987** Linker-insertion nonsense and restriction-site deletion mutations in the gB glycoprotein gene of herpes simplex virus type 1. *J. Virol.* **61** 714-721.

Weller, S. K., Aschman, D. P., Sacks, W. R., Coen, D. M. and Schaffer, P. A. **1983** Genetic analysis of temperature sensitive mutants of HSV-1: the combined use of complementation and physical mapping for cistron assignment. *Virology* **130** 290-305.

Weller, S. K., Sparado, A., Schaffer, J. E., Murray, A. W. , Maxam, A. M. and Schaffer, P. A. **1985** Cloniong, sequencing and functional analysis of OriL, a herpes simplex virus type 1 origin of DNA synthesis. *Mol. Cell. Biol.* **5** 930-942.

Weller, S. k., Seghatoleslami, R. M., Shao, L., Rowse, D. and Carmicheal, E. P. **1990** The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: Isolation and characterisation of a lacZ mutant. *J. Gen. Virol.* **71** 2941-2952.

Weller, T. H., Whitton, H. M. and Bell, E. J. **1958** The etiological agents of varicella and herpes zoster: isolation, propogation and cultural characteristics *in vitro* . *J. Exp. Med.* **108** 843-868.

Whealy, M. E., Card, J. P., Meade, R. P., Robbins, A. K. and Enquist, L. W. **1991** Effect of brefeldin A on alphaherpesvirus membrane protein glycoylation and virus egress. *J. Virol.* **65** 1066-1081.

Whitley, R. J. **1985** Epidemiology of herpes simplex viruses. In "The Herpesviruses" , Vol. 3, (Ed, B. Roizman). Plenum press 1-44.

Whitton, J. L. and Clements, J. B. **1984** Replication origins and a sequence involved in co-ordinate induction of the immediate early gene family are conserved in an intergenic region of herpes simplex virus. *Nucleic Acids Res.* **12** 2061-2074.

Wilcox, K. W., Kohn, A., Skayanskaya, E. and Roizman, B. **1980** Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some polypeptides and can alter their affinity for DNA. *J. Virol.* **33** 167-182.

Wilkie, N. M. **1976** Physical maps of herpes simplex virus type 1 DNa for restriction endonucleases HindIII, HpaII and XbaI. *J. Virol.* **20** 222-233.

Wilson, A. C., LaMarco, K., Peterson, M. G., and Herr, W. **1993** The VP16 accesory protein HCF is a family of polypeptides processed from a large precursor protein. *Cell* **74** 115-125.

Wittels, M. and Spear, P. G. **1991** Penetration of cells by herpes simplex virus does not require a low pH-dependent endocytic pathway. *Virus. Res.* **18** 271-290.

Wohlrab, F. and Francke, B. **1980** Deoxyribopyrimidine triphosphatase activity specific for cells infected with herpes simplex virus type 1. *Proc. Natl. Acad. Sci. USA.* **77** 1872-1876.

Wong, S. W. and Schaffer, P. A. **1991** Elements in the transcriptional regulatory region flanking herpes simplex virus type 1 OriS stimulate origin function. *J. Virol.* **65** 2601-2611.

Woppmann, A., Patschinsky, T., Bringmann, P., Godt, F. and Luhrmann, R. **1990** Characterisation of human and murine snRNP proteins by two-dimensional gel

electrophoresis and phosphopeptide analysis of U1-specific 70K protein variants. *Nucleic Acids. Res.* **18** 4427-4438.

Woppmann, A., Will, C. L., Kornstadt, U., Zuo, P., Manley, J. L. and Luhrmann, R. **1993** Identification of an snRNP-associated kinase activity that phosphorylates arginine/serine rich domains typical of splicing factors. *Nucleic Acids Res.* **21** 2815-2822.

WuDunn, P. and Spear, P. G. **1989** Initial interaction of herpes simplex virus with cells binding to heparan sulfate. *J. Virol.* **63** 52-58.

Wymer, J. P., Chung, T. D., Chang, Y-N, Hayward, G.S. and Aurelian, L. **1989** Identification of immediate-early-type cis-response elements in the promoter of the ribonucleotide reductase large subunit from herpes simplex virus type 2. *J. Virol* **63** 2773-2784.

Wymer, J. P., Colette, M. J., Aprhys, Chung, T. D., Feng, C-P., Kulka, M. and Aurelian, L. **1992** Immediate-early and functional AP-1 cis-response elements are involved in the transcriptional regulation of the lagre subunit of herpes simplex virus type2 ribonucleotide reductase (ICP10). *Virus. Res.* **23** 253-270.

Xiao, P. and Capone, J. P. **1990** A cellular factor binds to the herpes simplex vir us type 1 transactivator Vmw65 and is required for Vmw 65-dependent protein-DNA complex assembly with Oct-1 *Mol. Cell. Biol.* **10** 4974-4977

Xiong, Y, Zhang, H. and Beach, D. **1992** D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell* **71** 505-514.

Xu, Z., Dholakia, J. H. and Hille, M. B. **1993** Maturation hormone induced an increase in the translational activity of starfish oocytes coincident with the phosphoryaltion of the mRNA cap binding protein, eIF-4E, and the activation of several kinases. *Dev. Genetics* **14** 424-439.

Yamada, Y., Kimura, h., Morishima, T., Daikoku, T., Maeno, K. and Nishiyama, Y. **1991** The pathogenecity of ribonucleotide reductase null mutants of herpes simplex virus type 1 in mice. *J. infect. Dis.* **164** 1091-1092.

Yamanishi, K., Okuna, T., Shiraki, K., Takahashi, M., Kondo, T., Asano, Y. and Kurata, T. **1988** Identification of the human herpesvirus 6 as a casal agent for exanthem subitum. *Lancet* **1** 1056-1067.

Yao, F. and Courtney, R. J. **1989** A major transcripttional regulatory protein (ICP4) of herpes simplex virus type 1 is associted with purified virions *J. Viol* **63** 3338-3344.

Yao, F. and Courtney, R. J. **1992** Association of ICp0 but not ICP27 with purified virions of herpes simplex virus type 1. *J. Virol.* **66** 2709-2716.

Young, R. A. **1991** RNA polymerase II. *Ann. Rev. Biochem.* **60** 689-715.

Yunhee, K. K. and Hecht, N. B. **1993** Binding of a phosphoprotein to the 3′ untranslated region of the mouse protamine 2 mRNA temporally represses its translation. *Mol. Cell. Biol.* **13** 6547-6557.

Zhang, G., Stevens, R. and Leader, D. P. **1990** The protein kinase encoded by the short region of pseudorabies virus: Description of the gene and identification of its product in virions in infected cells. *J. Gen. Virol.* **71** 1757-1765.

Zheng, J., Knighton, D. R., Ten Eyck, L. F., Karlsson, R., Xuong, N-H., Taylor, S. S. and Sowadski, J. M. **1993** Crystal structure of the catalytic subunit of cAMP-dependent protein kinase complexed with MgATP and peptide inhibitor. *Biochem.* **32** 2154-2161.

Zhou, Z and Elledge, S. J. **1993** *Dun1* encodes a protein kinase that controls the DNA damage response in yeast. *Cell* **75** 1197-1127.

Zhu, X., Chen, J., Young, C. S. A. and Silvestein, S. **1990** Reactivition of latent herpes simplex virus by adenovirus recombinants encoding mutant IE-0 gene products. *J. Virol.* **64** 4489-4498.

Zoller, M.J., Nelson, N. C. and Taylor, S. S. **1981** Affinity labeling of cAMP-dependent protein kinase with *p*-fluorosulfonylbenzoyladenosine. *J. Biol. Chem.* **256** 10837-10842.

Zwaagstra, J. C., Ghiasi, H., Nesburn, A. B. and Wechsler, S. L. **1991** Identification of a major regulatory sequence in the latency associated transcript (LAT) promoter of herpes simplex virus type 1 (HSV-1). *Virology* **182** 287-297.

Zwaagstra, J. C., Ghiasi,, H., Slanina, S. M., Nesburn, A. B., Wheatley, S. C., Lillycrop, K., Wood, J., Latchman, D. S., Patel, K. and Wechsler, S. L. **1990** Activity of herpes simplex virus type 1 latency-associated transcript (LAT) promoter in neuron-derived cells: Evidence for neuron specificity and for a large LAT transcript. *J. Virol.* **64** 5019-5028.

Zwaagstra, J., Ghiasi, H., Nesburn, A. B. and Wechsler, S. L. **1989** In vitro promoter actvity associated with the latency-associated transcript gene of herpes simplex virus type 1. *J. Gen. Virol.* **70** 2163-2169.