STUDIES ON HERPES SIMPLEX VIRUS TYPE 1 LATENCY IN TISSUE CULTURE CELLS.

by

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SUMMARY.

After primary infection, herpes simplex virus type 1 (HSV-1) remains latent in neurons of the host. To facilitate studies on HSV-1 latency, models of latency have been developed in tissue culture.

The HSV-1 protein Vmw65 is an essential structural component of the virus tegument. Following fusion of the virus envelope with the cell membrane Vmw65 is released into the cell and forms a complex with cellular factors which bind to TAATGARAT (where R is a purine) elements upstream of the viral immediate early (IE) genes. Once the complex has bound to DNA, the acidic carboxy-terminal domain of Vmw65 stimulates transcription by recruiting components of the RNA polymerase II transcription initiation complex to the promoter.

The mutant *in*1814 has a 4 amino acid insertion in Vmw65 which disrupts its ability to form the transactivation complex, as a consequence levels of IE transcription are reduced. During infection of cells with *in*1814 at low multiplicity of infection (MOI), only a minor proportion of infecting viruses undergo replication, whereas the majority of viral genomes enter a quiescent state. These quiescent genomes can be reactivated by expression within the cell of the HSV-1 encoded protein Vmw110 and probably by other herpesvirus proteins functionally equivalent to Vmw110. At high MOI *in*1814 replicates as efficiently as wild type virus.

To perform structural and functional studies on the quiescent *in*1814 genomes, it is necessary to permit infection of cultured cells at high MOI. An *in vitro* latency system has been developed by D.R.S. Jamieson and C.M. Preston using two modifications which further reduce the expression of viral IE proteins, thereby enabling infection of human foetal lung (HFL) cells with *in*1814 at high MOI without extensive cell destruction. Firstly, the regulatory region controlling transcription of the gene encoding the IE protein Vmw110 has been replaced by the Moloney murine leukaemia virus (MMLV) enhancer. Since the MMLV enhancer is a relatively inefficient promoter in HFL cells the resulting mutant, named *in*1820, exhibits a phenotype equivalent to that of a Vmw110 deletion mutant of *in*1814 when titrated on HFL cells. Another modification is pretreatment of the cells with human interferon α (IFNα). Treatment of cells with IFNα results in an antiviral state against HSV-1 which has been attributed to reduced viral IE gene expression.

Previous studies demonstrated that infection of IFNα–treated HFL cells with *in*1820 at high MOI results in high efficiency retention of viral genomes in a transcriptionally repressed state. The purpose of the study reported here was to extend previous analysis by D.R.S. Jamieson on the structure of the *in*1820 genome during latency *in vitro*, and to characterise early events in the establishment of latency.

Initial experiments confirmed previous data showing that the *in*1820 genome is nonlinear in the *in vitro* latency system, as was reported to occur in neurons *in vivo*. Quantification of Southern blots revealed that after an initial input MOI of 120 particles of *in*1820 per cell, approximately 84% of the total nuclear viral DNA was nonlinear and that on average each cell retained approximately 12 viral genomes.

Upon reactivation of latent *in*1820 by superinfection of latently infected HFL cells in the presence of an inhibitor of DNA replication, the reactivated genomes remained nonlinear, demonstrating that a change from a nonlinear to a linear form is not a requirement for reactivation. The configuration of the linear genomes also remained unchanged upon reactivation.

During *in vitro* latency, the nonlinear viral genomes were more sensitive to nucleases than the linear genomes. Linearity and resistance to nucleases is indicative of genomes which are not uncoated and are thus unable to circularise. Comparison of rates of uncoating after infection of BHK, HeLa, CV-1 and HFL cells revealed that uncoating appeared significantly slower in HFL cells than in the other cell types tested.

Latent HSV-1 DNA in the brainstems of mice is associated with nucleosomes in a chromatin structure. Previous studies examining the structure of *in*1820 in the *in vitro* latency system showed that the thymidine kinase (TK) gene is not bound by nucleosomes with regular spacing. The study reported here also showed the absence of a regular chromatin structure on the TK gene and furthermore that the nature of the inhibitor of DNA replication used to prevent the spread of nonlatent virus was not a significant variable. Fascinatingly, the LAT / Vmw110 encoding region, the only region transcriptionally active during latency *in vivo*, was in a regular chromatin structure in the *in vitro* latency system. However, when the LAT / Vmw110 encoding region and the TK gene were examined in a parallel experiment, both regions exhibited a possible regular nucleosomal structure suggesting that a regular nucleosomal structure exists on the entire genome and is not confined to the LAT / Vmw110 region. Thus the previous inability to detect chromatin on the TK gene may have been caused by differences in the arrangement of nucleosome between experiments or experimental differences in the ability to detect nucleosome-associated DNA.

Infection of HFL cells with light particles (L-particles) or virions of *in*1814 which have been inactivated by irradiation with ultraviolet (UV) light, made the cells less permissive for plaque initiation upon subsequent infection with *in*1853 (*in*1853 is *in*1814 with the *Escherichia coli lac* Z marker gene recombined into the TK locus).

Infection of HFL cells with UV-irradiated *in*1814 L-particles had no detectable effect on adsorption, penetration and uncoating of *in*1853, indicating an inhibition of viral gene expression similar to that caused by the action of IFNα. However, the release of a

soluble factor into the culture medium by HFL cells infected with UV irradiated viral particles could not be detected.

Northern blot analysis showed reduced levels of viral IE mRNAs after infection of HFL cells previously infected with UV irradiated *in*1814 L-particles. The reduction in levels of the viral IE mRNAs did not require protein synthesis, implying that UV irradiated particles were activating previously existing factors rather than inducing transcription of cell genes. In addition, it was demonstrated that the virion-host shutoff factor present in the UV inactivated viral particles was not responsible for their inhibitory effect on plaque initiation.

The ability of *in*1814 to establish latency *in vitro* with high efficiency is reflected in its high particle / PFU ratio. The particle / PFU ratio of *in*1814 varies between cell types and is particularly high in HFL cells. An investigation was undertaken to determine whether slow uncoating was the cause of the high particle / PFU ratio observed in HFL cells and hence an important property in the establishment of *in vitro* latency. Slow uncoating of the virus might give more time for components of the virion to modify the cellular environment before release of the viral DNA into the cell nucleus. During natural HSV-1 infection *in vivo* virus particles fuse with nerve endings at the periphery and the nucleocapsids travel along the nerve axon to release the viral genomes into the cell nucleus. The relatively long distance between the peripheral nerve ending and the neuronal cell body causes a delay between infection at the cell surface and release of the viral DNA into the nucleus. The inhibition of *in*1853 plaque initiation in HFL cells previously infected with UV inactivated *in*1814 may be interpreted as supporting the hypothesis that viral proteins modify the cell, predisposing the cell to being latently infected.

In order to control uncoating of *in*1814, a temperature sensitive uncoating defect was cloned from the HSV-1 mutant *ts*1213 and recombined into the *in*1814 genome, yielding a virus named *in*1815 which did not release viral DNA into the nuclei of cells infected at 39.5°. Uncoating of *in*1815 was controlled by temperature shift.

One important characteristic of latency of *in*1814 in HFL cells is unresponsiveness of the viral genome to activators of transcription such as N,N'-hexamethylene-bis-acetamide (HMBA). HMBA overcomes the defect in *in*1814 based viruses if applied early in infection. However, during the first 7 hours of infection the virus gradually enters a latent state which is unresponsive to HMBA.

Infecting HFL cells with *in*1814 or *in*1815 and maintaining at 39.5° for 7 hours, followed by the addition of HMBA and downshift to 31°, showed that unlike *in*1814, *in*1815 did not become unresponsive to HMBA at 39.5°. The rate of silencing of the *in*1815 genome after downshift to 31° was not greater after 7 hours at 39.5° than after 1 hour at 39.5°, indicating that the factors responsible for silencing the viral genome did not

accumulate to higher levels at 39.5°. No evidence was found to suggest that slow uncoating causes increased efficiency of establishment of latency.

ABBREVIATIONS.

ABBREVIATION.	FULL NAME.
2,5-A	2'-5'-oligoadenylates
<	less than
α	alpha
ACG	acycloguanosine
ADP	adenosine-5'-diphosphate
AIDS	acquired immunodeficiency syndrome
AMP	adenosine-5'-monophosphate
ANS	autonomic nervous system
Ara-C	arabinofuranosyl cytosine
β	beta
BHK	baby hamster kidney
bp	base pair(s)
BSA	bovine serum albumin
BVDU	(E)-5-(2-bromovinyl)-2'-deoxyuridine
cAMP	cyclic adenosine-5'-monophosphate
CAT	chloramphenicol acetyl transferase
cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CDTA	trans-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid
CH	cycloheximide
cm	centimetre(s)
CMC	carboxymethyl cellulose
CNS	central nervous system
CPE	cytopathic effect
DEP	diethyl pyrocarbonate
dl	deletion mutant
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	2'-deoxynucleoside-5'-triphosphate
DR	direct repeat
dsDNA	double-stranded deoxyribonucleic acid
dsRNA	double-stranded ribonucleic acid

DTT	dithiothreitol
dTTP	2'-deoxythymidine-5'-triphosphate
dUMP	2'-deoxyuridine-5'-monophosphate
dUTP	2'-deoxyuridine-5'-triphosphate
dUTPase	deoxyuridine triphosphatase
E	early
E.coli	*Escherichia coli*
EBV	Epstein-Barr virus
EDTA	sodium ethylenediamine tetra-acetic acid
eg.	example
eIF	eukaryotic translation initiation factor
γ	gamma
g	glycoprotein
G+C	guanosine plus cytosine
GAS	gamma-activated site
GMEM	Glasgow modified Eagle's medium
GTP	guanosine-5'-triphosphate
GTPase	guanosine triphosphatase
HCF	host cell factor
HCMV	human cytomegalovirus
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HFL	human foetal lung
HHV	human herpesvirus
HIV	human immunodeficency virus
HMBA	N,N'-hexamethylene-bis-acetamide
hr(s)	hour(s)
hsp	heat shock protein
HSV	herpes simplex virus
HVS	herpesvirus saimiri
ICP	infected cell polypeptide
IE	immediate-early
IETC	immediate-early gene transactivation complex
IFN	interferon
IL	interleukin
IM	infectious mononucleosis
in	insertion mutant
IRF	interferon regulated factor
IR_L	internal repeat of the long region
IR_S	internal repeat of the short region

ISGF	interferon-stimulated gene factor
ISRE	interferon-stimulated response element
kb	kilobase(s)
kbp	kilobase pair(s)
kDa	kilodalton(s)
KS	Kaposi's sarcoma
L	late
L-particles	light particles
L-terminus	terminus of the long region
LAP	latency-active promoter
LAT	latency-associated transcript
LD_{50}	dose producing 50% fatality
LiDS	lithium dodecyl sulphate
LTR	long terminal repeat
M	molar
mA	milliamperes
Mab	monoclonal antibody
MCMV	murine cytomegalovirus
MELC	myeloid erythroleukaemia cell
MEM	modified Eagle's medium
µg	microgram(s)
mins	minutes
MIR	major internal repeat
mJ	millijoules
µl	microlitre(s)
ml	millilitre(s)
mLAT	minor hybridising latency-associated transcript
mM	millimolar
MMLV	Moloney murine leukaemia virus
MN	micrococcal nuclease
MOI	multiplicity of infection
MOPS	3-(N-morpholino)propanesulphonic acid
MPC	magnetic particle concentrator
mRNA	messenger ribonucleic acid
ND	not determined
NGF	nerve growth factor
nm	nanometre
NPT	non-permissive temperature
nt	nucleotide(s)

OD	optical density
ORF	open reading frame
ori_L	origin of replication in the long region
ori_S	origin of replication in the short region
PAA	phosphonoacetic acid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit(s)
PI	plaque isolate
PIPES	piperazine-NN'-bis-2-ethanesulphonic acid
PKC	protein kinase C
PKR	interferon-induced protein kinase
PML	promyelocytic leukaemia (-associated protein)
PMSF	phenylmethylsulphonyl fluoride
pol	polymerase
poly(A)	polyadenosine
poly(A)$^+$	polyadenylated
poly(A)$^-$	nonpolyadenylated
pRB	retinoblastoma-associated protein
RNA	ribonucleic acid
RNAP II	ribonucleic acid polymerase II large subunit
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
S-terminus	terminus of the short region
SDS	sodium dodecyl sulphate
SN	staphylococcal nuclease
snRNA	small nuclear ribonucleic acid
snRNP	small nuclear ribonucleoprotein particle
ssDNA	single stranded deoxyribonucleic acid
Stats	signal transducers and activators of transcription
SV40	simian virus 40
TCA	trichloroacetic acid
TF	transcription factor
TK	thymidine kinase
TMP	thymidine-5'-monophosphate
TNTC	too numerous to count
TPA	phorbal 12-myristate 13-acetate

Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
TR_L	terminal repeat of the long region
TR_S	terminal repeat of the short region
ts	temperature sensitive
U_L	long unique region
U_S	short unique region
UV	ultraviolet
UV-*in*1814 (eg.)	ultraviolet light-inactivated *in*1814
V	virion DNA
v/v	volume/volume ratio
vhs	virion host shutoff
Vmw	molecular weight of viral protein in kilodaltons
VP	virion protein
VSV	vesicular stomatitis virus
VZV	varicella zoster virus
w/v	weight/volume ratio
w/w	weight/weight ratio
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
°	degrees centigrade

1. INTRODUCTION.

1.1. THE HERPESVIRUSES.

1.1.1. Characteristics of the herpesviruses.

The herpesviruses produce a variety of diseases in humans and are widespread in other vertebrates (Minson, 1989). However, each herpesvirus is usually limited to a single host species in nature. They are united by their structural architecture and mode of replication. Structurally, a herpesvirus virion is 120-300 nm in diameter and consists of a single dsDNA genome ranging from 125-240 kbp in size surrounded by an icosahedral nucleocapsid. The entire virion is surrounded by a lipid envelope and between the envelope and the capsid exists amorphous material which constitutes the tegument (figure 1.3.).

The mode of replication of the herpesviruses exhibits several unifying characteristics: 1) viral DNA synthesis and nucleocapsid assembly occur in the nucleus of the infected cell. 2) Enzymes for viral nucleic acid metabolism are encoded by the virus. 3) Lytic infection results in the death of the cell. 3) Latent infections can be established.

The genes encoded by the herpesviruses include about 40 'core' genes which show amino acid sequence homology between all of the herpesviruses and are believed to result from a common evolutionary progenitor (table 1.1.; Figure 1.4.; Davison, 1993). The core genes encode the structural proteins and proteins involved in virion morphogenesis, nucleotide metabolism and DNA packaging. Presumably it is the remaining genes which result in the individual characteristics of each virus.

The herpesviruses have been divided into three subfamilies, alpha, beta and gamma, depending mainly upon their biological characteristics (Minson, 1989).

Alphaherpesviruses are distinguished by their ability to infect a wide range of tissue culture cells *in vitro*, their rapid growth cycle, and their ability to establish latency in nervous tissues. Humans are natural hosts for 3 alphaherpesviruses: herpes simplex virus types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV).

The betaherpesviruses grow less rapidly in tissue culture and *in vivo* and are limited by the cell types which they are capable of infecting. The betaherpesviruses known to naturally infect humans are human cytomegalovirus (HCMV), and human herpesviruses 6 and 7 (HHV-6 and HHV-7). The cytomegaloviruses gain their name from the characteristic cytopathic effect (CPE) which they induce, since infected cells become extremely large and round. The sites at which the betaherpesviruses establish latency has

been difficult to identify, however the observation that HCMV is transmitted by blood products suggests that latency may occur in a circulatory cell type. Latency of HCMV has been observed in cultured granulocyte-macrophage progenitor cells (Kondo *et al.*, 1994), and in addition, the major population of virally infected cells in the blood of mice infected with murine cytomegalovirus are mononuclear phagocytes (Stoddart *et al.*, 1994), thus macrophages and their progenitor cells have been implicated as sites of betaherpesvirus latency (Sinclair and Sissons, 1994).

Gammaherpesviruses are distinguished by their ability to replicate and establish latency in lymphoid cells. The gammaherpesviruses infecting humans are Epstein-Barr virus (EBV) and possibly human herpesvirus 8 (HHV-8).

1.1.2. The role of herpesviruses in human disease.

HSV.

Initial infection with HSV occurs *via* the mucous membranes of the mouth, genitals, throat or eyes (Minson, 1989; Ginsberg, 1990). Replication ensues at the site of entry and virus spreads to the local lymph nodes. Replication is usually localised to the site of infection, however viraemia occurs occasionally, resulting in replication in distant organs. Infection usually occurs during the first 6-18 months of life and is most often inapparent, however 10-15% of those infected develop herpetic gingivostomatitis (vesicles on the oral mucous membranes or mucocutaneous border).

During the primary infection, virus is taken up by nerve endings and travels to the nerve cell bodies in the sensory ganglia (predominantly the trigeminal ganglia in oral gingivostomatitis), where it establishes a latent infection for the lifetime of the host despite the presence of circulating anti-HSV antibodies. Reactivation can be triggered later in life by a variety of stimuli including trauma, hormonal changes or illness, thereby resulting in viral replication at the site of the primary infection.

Two serotypes of HSV exist: HSV-1 is primarily associated with oral infections while HSV-2 is predominantly associated with genital infections. However, both serotypes are capable of replication and latency at either anatomical location but recurrent reactivation of HSV-1 is favoured in the trigeminal ganglia and HSV-2 reactivation is favoured in the spinal ganglia innervating the genitals, thus the two HSV serotypes appear to have evolved to optimise their spread at separate anatomical sites.

Complication with HSV infections include fatal encephalitis in neonates and immuno-compromised patients, and blindness resulting from primary infection in the eye.

VZV.

The primary disease caused by VZV is called chickenpox or varicella. Virus enters by the respiratory tract and replicates in the local lymph nodes (Minson, 1989; Ginsberg, 1990). Viraemia results, and after an incubation period of 14-15 days a fever ensues and vesicles appear on the skin. The primary disease can be particularly severe in adults, however infection is so common that most people are infected before the age of 20.

During primary infection, VZV establishes latency in sensory ganglia of the spinal or cranial nerves where it persists for the lifetime of the host in the presence of neutralising antibodies. As with HSV, trauma and immune suppression can trigger reactivation. Reactivation leads to shingles (also called herpes-zoster), which is manifested as vesicles and severe pain at the distribution of the infected nerve.

HCMV.

HCMV is the most common cause of severe intra-uterine infections (Pass, 1985; Minson, 1989; Ginsberg, 1990; Borucki and Pollard, 1994). Although 0.5–2.4% of newborn babies are infected with HCMV (in the USA), clinical symptoms only occurs in approximately 5–10% of those infected. Severity of clinically apparent HCMV infections vary from mild to severe, however approximately one third suffer permanent neurological damage.

It is common for asymtomatic adults to secrete virus, thus HCMV establishes latent infections. There is mounting evidence that macrophage progenitor cells and macrophages are the sites of HCMV latency (Sinclair and Sissons, 1994).

EBV.

Infection with EBV occurs *via* the oral route, after which it replicates in the epithelium of the mouth and salivary gland ducts (Rickinson *et al.*, 1985; Minson, 1989; Ginsberg, 1990). It is believed that EBV infects B-lymphocytes associated with the oropharangeal epithelial tissues and that the infected B-lymphocytes circulate around the body and provide a source of latent infection.

Infection usually occurs within the first five years of life and produces subclinical disease, however increased hygiene in recent years has caused infection of a greater proportion of the population to be delayed until adolescence. When EBV infection occurs at ages above 15 years, approximately 50% of patients exhibit the clinical manifestations of the disease known as infectious mononucleosis (IM) or glandular fever. Symptoms of

IM include enlarged lymph nodes and spleen, abnormal lymphocytes in the blood and fatigue which can last for months. IM patients secrete EBV for weeks after the primary infection.

EBV can immortalise B-lymphocytes *in vitro*, and in addition EBV-infected immortalised B-lymphocytes can be isolated from the blood of EBV-infected individuals, thus the B-lymphocyte is a major site of EBV latency. EBV sequences are commonly found in at least 2 types of tumours, Burkitt's lymphoma and nasopharangeal carcinoma, thus the immortalisation functions of EBV can facilitate the development of certain tumours.

HHV-6 and HHV-7.

HHV-6 was first isolated from the blood of patients with lymphoproliferative disorders or AIDS (Salahuddin *et al.*, 1986). Up to 90% of the population are asymptomatically infected with HHV-6, however infection in infants sometimes causes a mild skin rash termed exanthum subitum (Yamanishi *et al.*, 1988; Okuna *et al.*, 1989), and acute febrile illness in young children (Pruksananonda *et al.*, 1992; Hall *et al.*, 1994). HHV-6 can replicate in $CD4^+$ T-lymphocytes, and there is evidence that the monocyte is a site of latency. Despite its lymphotropism HHV-6 has been classified as a betaherpesvirus, since it shares amino acid sequence similarities and overall gene organisation with HCMV, and infects a wide range of cell types other than lymphocytes (Lawrence *et al.*, 1990; Neipel *et al.*, 1991; Nicholas and Martin, 1994).

HHV-7 shares a number of common features with HHV-6, including replication in T-lymphocytes (Frenkel *et al.*, 1990; Berneman *et al.*, 1992; Lusso *et al.*, 1994), a similar genome organisation (Lindquester and Pellett, 1991; Gompels *et al.*, 1995; Secchiero *et al.*, 1995) and 50-60% nucleotide identity in the genomic regions which have been examined (Berneman *et al.*, 1992). HHV-7 has also been isolated from the blood and saliva of immunosuppressed and healthy subjects (Frenkel *et al.*, 1990; Wyatt and Frenkel, 1992). Although the association of HHV-7 with any disease has yet to be resolved, there is evidence that HHV-7 may be associated with some cases of exanthum subitum (Portolani *et al.*, 1995).

HHV-8.

Recent attempts to identify infectious agents associated with the Kaposi's sarcoma (KS) lesions suffered by AIDS patients have demonstrated the existence of unique viral DNA sequences with close homology to regions of the EBV genome (Memar *et al.*,

1995). Sequences of the new herpesvirus, termed HHV-8, are present in all AIDS-related and sporadic forms of KS and have been demonstrated in peripheral blood mononuclear cells of KS^+ patients, thus the presence of HHV-8 DNA appears essential for the aetiology of KS. HHV-8 has yet to be isolated or demonstrated to have immortalising or transforming ability.

1.1.3. Human herpesvirus genomes.

Herpesviruses possess large single dsDNA genomes which vary in size, G+C content and in the arrangment of their major repeat regions (figure 1.1.; McGeoch, 1989).

The genome of HSV-1 is approximately 152 kbp, with a G+C content of 68.3% (McGeoch *et al.*, 1985, 1986, 1988a; Perry and McGeoch, 1988). It consists of 2 covalently joined segments termed the long (L) and short (S) regions, representing 82% and 18% of the genome respectively. Both the L and S regions are divided into a unique region (U_L or U_S) flanked by repeats in opposite orientation to each other. The internal repeat in the L region is termed IR_L and the terminal repeat is termed TR_L, likewise the internal and terminal repeats of the S region are termed IR_S and TR_S respectively. At each of the termini there is an approximately 250-350 bp direct repeat termed the *a* sequence, which is present in variable numbers at the L-terminus and one copy at the S-terminus (Wilkie, 1976; Wagner and Summers, 1978; Roizman, 1979; Davison and Wilkie, 1981). There is also at least one copy of the *a* sequence in the joint between the L and S regions which is in opposite orientation to that of the terminal *a* sequences. Both of the genomic termini possess an overhanging residue leaving a 3' hydroxyl group free (Mocarski and Roizman, 1982).

The L and S regions can invert relative to each other so that virion DNA consists of equimolar proportions of 4 isomers (figure 1.2.; Hayward *et al.*, 1975; Morse *et al.*, 1977; Roizman, 1979), one of which has been assigned as the prototype to facilitate mapping (Roizman, 1979; McGeoch *et al.*, 1988a). Since the genes in the repeat regions are diploid, the internal inverted repeats can be deleted and the virus remains viable in tissue culture, however the resulting deletion mutants produce progeny whose L and S segments cannot invert relative to each other (Poffenberger *et al.*, 1983; Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986). Deletion mutants have been constructed which are fixed in one of all the 4 isomeric forms. Recombination between the *a* sequences is the mechanism which generates the 4 isomers (Mocarski and Roizman, 1982; Chou and Roizman, 1985).

Since a small proportion of virion DNA is circular and circularisation of linear viral genomes occurs rapidly after entry into the nucleus, it is likely that the termini of the genome are in close proximity or possibly held together in the virion (Poffenberger and

Figure 1.1. Sizes and structures of human herpesvirus genomes. Repeat regions are represented by open rectangles with the orientation of the repeats indicated by arrows. Unique regions are represented by horizontal lines.
Abbreviations are as follows :

U_L	unique long.
U_S	unique short.
T/IR_L	terminal / internal long repeat.
T/IR_S	terminal / internal short repeat.
D_L	direct repeat left.
D_R	direct repeat right.
MIR	major internal repeat.

Adapted from Anderson (1991).

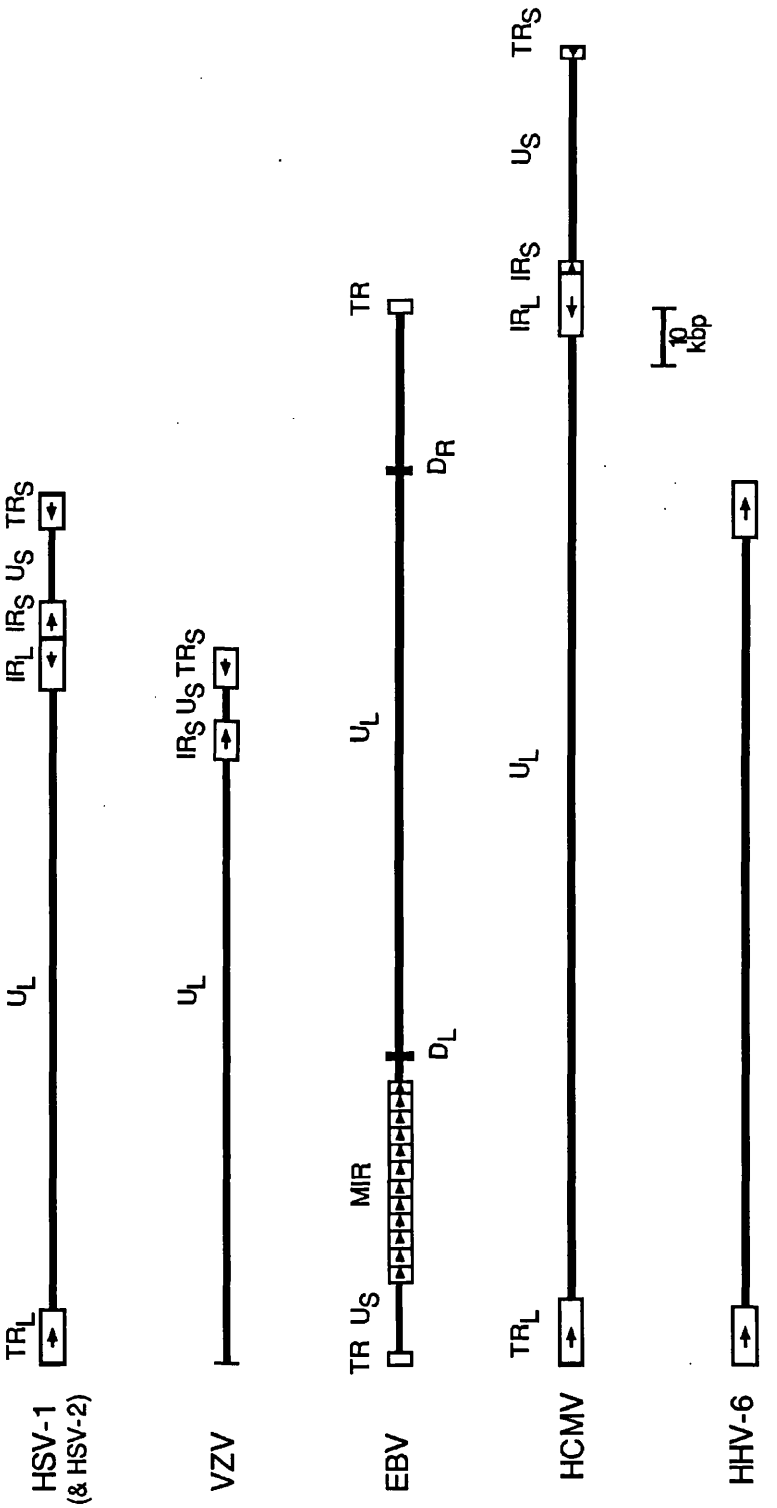
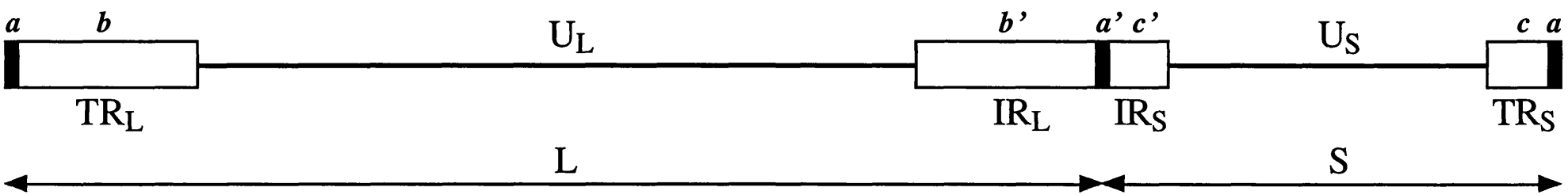
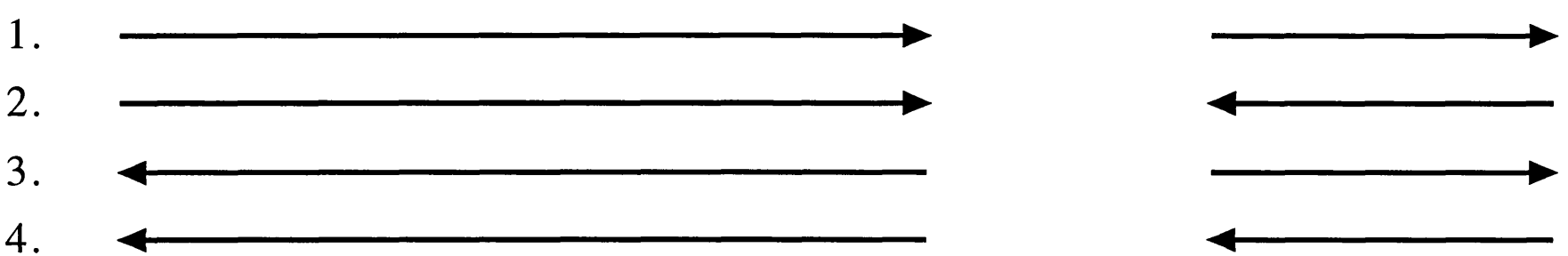

HSV-1
(& HSV-2)
TR$_L$
U$_L$
IR$_L$ IR$_S$ U$_S$ TR$_S$
VZV
U$_L$
IR$_S$ U$_S$ TR$_S$
EBV
TR U$_S$
MIR
D$_L$
U$_L$
TR
D$_R$
HCMV
TR$_L$
U$_L$
IR$_L$ IR$_S$ U$_S$ TR$_S$
10 kbp
HHV-6

Figure 1.2. Gross structure of the HSV genome showing *a* sequences and possible isomeric forms. (A) The HSV genome displaying the positions and orientations of the repeated sequences. *b'* and *c'* are identical to *b* and *c*, but in inverse orientation. *a'* and *a* are repeat elements in inverse orientation and variable in copy number. There are 1-5 *a* sequences at the L-terminus and the joint between the L and S segments, whereas there is only 1 *a* sequence at the S-terminus. (B) The 4 possible isomeric forms resulting from different orientations of the U_L and U_S regions. The larger arrows represent the orientations of the U_L segments and the smaller arrows represent the orientations of the U_S segments.

A .



B .



Roizman, 1985; Roizman and Sears, 1993). Virion DNA is also reported to contain nicks and gaps (Frenkel and Roizman, 1972; Wilkie, 1973).

The genomes of HSV-1 and HSV-2 are closely related and colinear. Coding sequences of HSV-1 and HSV-2 show 70-80% similarity (McGeoch *et al.*, 1987), whereas the noncoding regions are more greatly diverged (Davison and Wilkie, 1981; Whitton and Clements, 1984; McGeoch *et al.*, 1991). HSV-2 also contains a slightly higher G+C content, 69%, compared to 68.3% in HSV-1 (McGeoch *et al.*, 1988a). A major difference between the 2 viruses has been noted in their U_S4 genes, which encodes gG (McGeoch *et al.*, 1987). The HSV-2 U_S4 is 1460 bp larger than its HSV-1 counterpart, encoding 2 additional domains in the gG protein.

The VZV genome is approximately 125 kbp with a G+C content of 46.0% (Davison and Scott, 1986). The gross VZV genome structure is similar to that of HSV only the inverted repeats flanking the U_L segment are only 88 bp compared to the 9.2 kbp repeats of HSV, in addition the VZV genome is not terminally redundant (Davison, 1984). As with HSV, both termini end in a 1 nt 3' overhang. Four isomers of the VZV genome exist, however, approximately 95% of virion genomes contain 1 orientation of U_L.

The genome of EBV strain B95-8 is approximately 172 bp with a G+C content of 59.5%, however these values are not accurate since strain B95-8 has acquired a spontaneous deletion. Taking the deletion into account the size is likely to be around 186 kbp (Raab-Traub *et al.*, 1980; Baer *et al.*, 1984; Laux *et al.*, 1985). The genomic termini consist of several directly repeated copies of a 540 bp sequence. Several repeat elements are located internally in the EBV genome, these include D_L and D_R which are 1 kbp direct repeats at the extremities of the U_L segment, and the major internal repeat (MIR), which is a tandemly reiterated 3072 bp sequence separating the U_L and U_S segments.

The HCMV genome has a very similar structure to the HSV genome but is much larger, with a size of 230 kbp and G+C content of 56% (Weststrate *et al.*, 1983; Chee *et al.*, 1990). Four isomers of HCMV are possible, and the genome contains an element equivalent to the *a* sequence (Spaete and Mocarski, 1985).

The HHV-6 genome is 159 kbp with a G+C content of 43% (Gompels *et al.*, 1995). The HHV-6 genome consists of a 143 kbp unique segment flanked by 8 kbp direct repeats. The terminal direct repeats are themselves flanked by 350 bp elements with similar structure to human telomeres. The HHV-7 genome has a very similar structure to the genome of HHV-6, with 50-60% sequence identity (Berneman *et al.*, 1992; Secchiero *et al.*, 1995).

1.1.4. The HSV-1 *a* sequence.

The HSV-1 *a* sequence is highly conserved but contains variable numbers of repeat elements. The *a* sequence is tandemly reiterated 1-5 times at the L-terminus, but only one copy exists at the S-terminus (Wilkie, 1976; Wagner and Summers, 1978). The HSV-1 strain F *a* sequence has been analysed in some detail, and consists of the following elements: a 20 bp direct repeat (DR1), a 65 bp unique sequence (U_b), 19-23 copies of a 12 bp direct repeat (DR2), 2-3 copies of a 37 bp direct repeat, and a 58 bp unique sequence (U_c) followed by another DR1 (Mocarski and Roizman, 1981). The *a* sequence can therefore be represented as:

$$DR1–U_b–DR2_n–DR4_m–U_c–DR1$$

Adjacent *a* sequences share the intervening DR1 (Mocarski and Roizman, 1981). The size of the *a* sequence varies from strain to strain and is partly determined by the numbers of copies of DR2 and DR4. Since the terminal *a* sequence of the L component contains a truncated DR1 consisting of 18 bp and a one nucleotide 3' overhang, whereas the *a* sequence of the S-terminus ends with a DR1 consisting of only 1 bp and one 3' overhang, a complete DR1 is formed upon circularisation (Mocarski and Roizman, 1982). The *a* sequence contains *cis*-acting signals for circularisation, cleavage of concatemers into single unit length molecules, and encapsidation (Mocarski and Roizman, 1982; Vlazny *et al.*, 1982; Varmuza and Smiley, 1985). In addition, it contains the promoter for ICP34.5 (Chou and Roizman, 1986).

1.1.5. HSV virion structure.

The HSV virion is approximately 120-300 nm in diameter and consists of 3 distinct structures, namely capsid, tegument and envelope (figure 1.3.; Rixon, 1993; Beers *et al.*, 1994). The capsid is an icosahedral structure in the centre of the virion, in which the viral genome resides. The tegument is an apparently amorphous layer surrounding the capsid. Surrounding the tegument is the external lipid envelope with protruding glycoprotein spikes. Over half of the over 70 proteins encoded by HSV-1 are believed to be present in the virion or involved in virion assembly.

The 100-110 nm diameter icosahedral capsid is composed of 162 capsomeres (Schrag *et al.*, 1989). Three types of capsid can be isolated from cells infected with HSV (Gibson and Roizman, 1972). Type A capsids are empty capsids, type B capsids lack DNA but contain a core of proteinacious material, whereas type C capsids contain the viral DNA. Since the three capsid types represent different stages of nucleocapsid

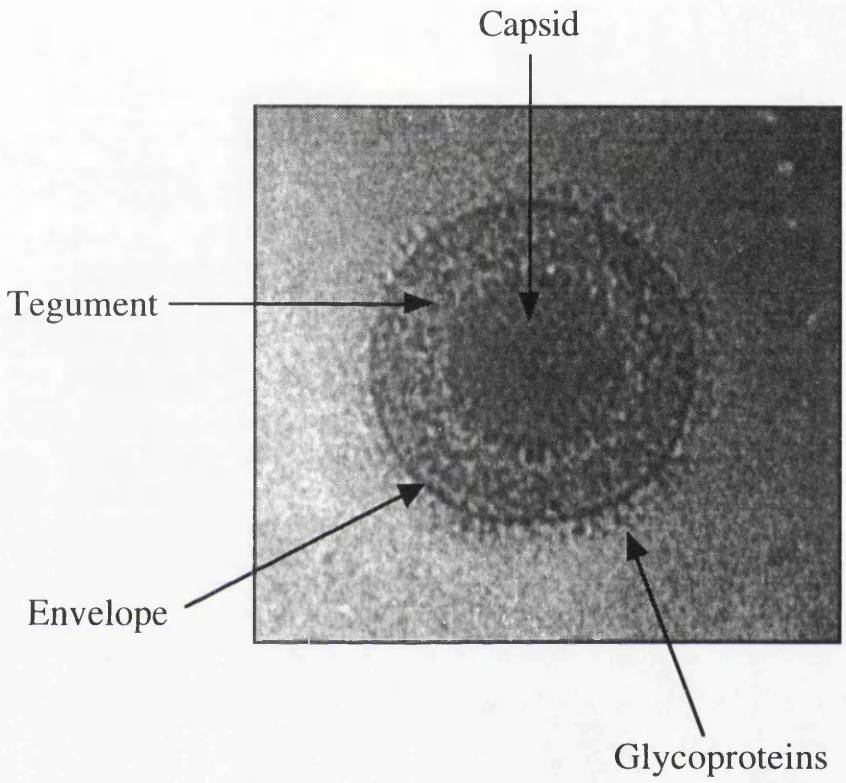


Figure 1.3. The HSV virion. A cryo-electron micrograph of the HSV virion is shown with the positions of the capsid, tegument, envelope and glycoprotein spikes indicated by arrows. The virion is magnified approximately 230, 000-fold. Photograph supplied by F.J. Rixon.

maturation and DNA packaging, their analysis has shed some light on these processes. Type A capsids are believed to be the result of abortive attempts to package DNA (Newcomb and Brown, 1989; Newcomb *et al.*, 1989; Newcomb and Brown, 1991). Type A capsids consist of 5 viral structural proteins, namely VP5 (encoded by U_L19) which is the capsomer protein (Heine *et al.*, 1974; Vernon *et al.*, 1981; Shrag *et al.*, 1989), VP19c (U_L38) which binds to DNA (Braun *et al.*, 1984), VP23 (U_L18) which possibly connects adjacent capsomeres (Schrag *et al.*, 1989), VP24 (U_L26) which is believed to be a remnant of the scaffold proteins which are necessary for capsid assembly and lost upon packaging of the DNA (Davison *et al.*, 1992), and VP26 (U_L35) which is required for mechanical stability (Newcomb and Brown, 1991). Type B capsids differ in protein content from the type A capsids in that they contain VP22a ($U_L26.5$) and small amounts of VP21 (U_L26), and these 2 proteins form the scaffold which is transiently associated with the capsids prior to packaging of the DNA (Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988; Newcomb and Brown, 1989; Schrag *et al.*, 1989; Newcomb and Brown, 1991). Type C capsids contain the viral DNA, since the scaffold proteins are lost after packaging, the polypeptide content of type C capsids is similar to that of the type A capsids (Gibson and Roizman, 1974; Rixon *et al.*, 1988; Sherman and Bachenheimer, 1988; Newcomb and Brown, 1989).

The tegument lies between the capsid and envelope and often appears variable in size and shape upon examination under the electron microscope, however, the variable appearance may be a product of sample preparation (Rixon, 1993). The tegument is probably not as chaotic as was previously thought, since specific interactions of various tegument components have recently been uncovered (Smibert *et al.*, 1994; Elliott *et al.*, 1995). The tegument contains many viral factors whose direct introduction into the cell by virus-cell fusion facilitates the process of infection. Examples include Vmw65 (Ace *et al.*, 1988, 1989), the virion host shutoff factor (Kwong *et al.*, 1988), and possibly the IE proteins Vmw175 and Vmw110 (Yao and Courtney, 1989, 1992; Yang and Courtney, 1995). There are probably many other viral gene products in the tegument which are nonessential but play important qualitative roles in replication, examples of which include protein kinases (Coulter *et al.*, 1993). Vmw65 plays an important qualitative role in stimulating IE transcription and also has an essential structural role (Ace *et al.*, 1988, 1989; Weinheimer *et al.*, 1992).

At least 11 virally-encoded glycoproteins reside in the virion envelope (Spear, 1993). The glycoproteins are involved in adsorption, penetration, membrane fusion, envelopment, prevention of superinfection and egress. In addition, the glycoproteins are important targets for the immune response. Three glycoproteins, gB, gD and gH are indispensable for growth in tissue culture. It is likely that the glycoproteins function in envelopment by specifically interacting with virus tegument proteins (Zhu and Courtney, 1994).

1.2. THE LYTIC CYCLE OF HSV.

1.2.1. Early stages of infection.

The early stages of HSV infection can be summarised as follows (Roizman and Sears, 1993; Spear, 1993). The HSV virion binds to a specific receptor on the cell surface. Binding is followed by fusion of the viral membrane with the cell plasma envelope, thereby releasing the contents of the tegument and the nucleocapsid containing the DNA into the cytoplasm. The virion core migrates to the nucleus where the viral genome is released into a nuclear pore. Components of the tegument play an important qualitative role in modifying the cell and in stimulating viral gene expression, thus enabling efficient viral replication.

Attachment occurs by specific binding of the virion glycoproteins to receptors on the cell surface (Spear, 1993). There are 11 HSV-1 glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM), of which 6 are dispensable for infection of most tissue culture cells (gC, gE, gG, gI, gJ and gM). A characteristic feature of HSV attachment is that several glycoprotein-receptor interactions can be utilised in tissue culture. A possible reason for the redundancy of the majority of the viral glycoproteins in tissue culture is that HSV encounters a wide range of cell types *in vivo*, some of which are highly polarised and might express only a limited subset of HSV receptors, thus the glycoproteins dispensable in tissue culture might be crucial for infection of particular cell types *in vivo* (Roizman and Sears, 1993).

Heparin sulphate has been identified as a major cellular receptor for HSV-1 by interacting with the viral gC, however weak binding is still observed with gC mutants. Binding *via* heparin can also occur in the absence of gC by at least one other glycoprotein, gB.

Penetration involves fusion of the viral and cellular surface membranes. Viral glycoproteins gB, gD and gH are essential for the fusion process (Spear, 1991). Once inside the cytoplasm, the viral core containing the DNA is transported to the nucleus by the cellular cytoskeleton (Kristensson *et al.*, 1986).

Very little is known about the molecular events involved in uncoating and release of the viral genome into the nuclear pore. The major tegument protein Vmw273 plays an essential role in uncoating, since the uncoating mutation in the strain HFEM mutant *ts*B7 maps to this protein (Batterson *et al.*, 1983). In cells infected with *ts*B7 at the non-permissive temperature (NPT), DNA-containing nucleocapsids can be observed accumulating at the nuclear pores and when the cells are shifted to a permissive temperature the DNA is extruded from the nucleocapsids into the nuclei.

Once released into the nucleus, the viral genome is converted to a nonlinear form by pre-existing factors (Poffenberger and Roizman, 1985).

1.2.2. Regulation of viral gene expression.

Since the initial sequence analysis of the HSV-1 genome, several further open reading frames (ORFs) have been identified. Thus the total number of known HSV-1 genes is well in excess of 70 and gradually increasing (table 1.1.; figure 1.4.). HSV genes are transcribed by the host RNA polymerase II in discrete subnuclear compartments (Roizman and Sears, 1993; Rice *et al.*, 1994). Recent data suggests that the large subunit of RNA polymerase II exhibits an unusual pattern of phosphorylation in HSV infected cells (Rice *et al.*, 1994), and that Vmw68 is required for this modification (Rice *et al.*, 1995). The significance of RNA pol II phosphorylation in the regulation of HSV gene expression has yet to be determined, however it is important to note that Vmw68 is required for the normal viral transcription pattern (Rice *et al.*, 1995). HSV transcripts are similar to those of the host, thus they are capped at the 5' end, polyadenylated at the 3' end, and methylated internally. Only 5 HSV transcription units are known to yield spliced products, namely IE1 (encoding Vmw110), IE4 (Vmw68), IE5 (Vmw12), U_L15 and the latency-associated transcript (LAT). Translation of viral mRNA takes place in the cytoplasm.

HSV gene expression is regulated temporally *via cis*-acting regulatory sequences in the 5' proximal regions of the genes and also by 3'-end mRNA processing (Roizman and Sears, 1993; Beers *et al.*, 1994). On the basis of their time of transcription, dependence upon viral regulatory proteins and viral DNA replication, the HSV genes are divided into three major classes: immediate early (IE), early (E) and late (L).

The 5 IE genes are the first viral genes to be transcribed. IE transcription is stimulated by pre-existing factors, thus they are the only genes expressed in the presence of inhibitors of protein synthesis. With the exception of Vmw12, the IE genes are regulators of subsequent viral gene expression. The IE proteins Vmw175, Vmw110 and Vmw63 perform an autoregulatory function by downregulating IE gene expression.

The E genes are dependent upon the IE genes for their expression but do not require viral DNA replication. The E gene products are predominantly involved in nucleic acid metabolism and DNA synthesis; examples include thymidine kinase (TK), ribonucleotide reductase, deoxyuridine triphosphatase (dUTPase) and the DNA polymerase.

The L genes can be divided into 2 groups, L_1 genes are only partially inhibited by inhibitors of viral DNA synthesis (examples are gB, gD and ICP34.5) whereas L_2 genes are wholly dependent on DNA synthesis (gC, for example). The L proteins are mainly virion structural proteins or factors involved in virion formation.

Table 1.1. Designation, function and status of HSV-1 strain 17 genes. Genes marked in bold and underlined are conserved in all 3 herpesvirus subfamilies. The positions of the origins of replication are shown, as are the nucleotide positions of the genomic segments and central *a* sequence. The status of each gene in cell culture is indicated as follows: E = essential; NE= non-essential; E? = probably essential; NE? = probably nonessential; (E) = mutants are viable but severely disabled; E/NE = essential under certain conditions. Compiled by A.J. Davison and C.A. MacLean.

GENE	FUNCTION OF PROTEIN	STATUS
RL1	Neurovirulence factor (ICP34.5)	NE
RL2 (3 exons)	IE protein; regulator of gene expression (Vmw110, ICP0)	NE
LAT	Latency-associated transcript; probably not protein coding	NE
(UL starts at 9213)		
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 complex; possesses helicase motifs	E
UL6	Minor capsid protein	E
UL7	Function unknown	E?
UL8	Component of DNA helicase-primase complex	E
UL9	*Ori*-binding protein essential for DNA replication	E
UL10	Virion surface glycoprotein M	NE
UL11	Myrisylated tegument protein; role in virion envelopment	NE
UL12	Deoxyribonuclease; role in maturation / packaging of DNA	(E)
UL13	Tegument protein; probable protein kinase	NE
UL14	Function unknown	E
UL15 (2 exons)	Role in DNA packaging; putative terminase component	E
UL16	Function unknown	NE
UL17	Function unknown	E
UL18	Capsid protein (VP23); component of intercapsomeric triplex	E
UL19	Major capsid protein (VP5); forms hexons and pentons	E
UL20	Integral membrane protein; role in egress of nascent virions; host range phenotype; *syn* locus	E/NE
UL21	tegument protein	NE
UL22	Virion surface glycoprotein H; complexes with glycoprotein L (UL1); role in cell entry	E
UL23	Thymidine kinase	NE
UL24	Function unknown; *syn* locus	NE
UL25	Capsid-associated tegument protein	E
UL26	Protease; acts in virion maturation; N-terminal portion is capsid protein (VP24)	E
UL26.5	Internal protein of immature capsids (VP22a); processed by UL26 protease	(E)
UL27	Virion surface glycoprotein B; role in cell entry; *syn* locus	E

UL28	Role in DNA packaging	E
UL29	Single-stranded DNA-binding protein	E
(centre of *oriL* is at 62473 / 62474)		
UL30	Catalytic subunit of replicative DNA polymerase; complexes with UL42 protein	E
UL31	Function unknown	E
UL32	Function unknown	E?
UL33	Role in DNA packaging	E
UL34	Membrane-associated phosphoprotein; substrate for US3 protein kinase	E?
UL35	Capsid protein (VP26); located on tips of hexons	E?
UL36	Very large tegument protein; role in uncoating	E
UL37	Tegument protein	E?
UL38	Capsid protein (VP19c); component of intercapsomeric triplex	E
UL39	Ribonucleotide reductase large subunit (Vmw136, ICP6, RI)	E/NE
UL40	Ribonucleotide reductase small subunit (Vmw38, R2)	E/NE
UL41	Tegument 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	Tegument / envelope protein	NE
UL46	Tegument protein; modulates IE transactivation by UL48 protein	NE
UL47	Tegument protein; modulates IE transactivation by UL48 protein	NE
UL48	Tegument protein; transactivates IE genes (Vmw65, VP16, α-TIF)	E
UL49	Tegument protein	NE?
UL49A	Envelope protein disulphide-linked to tegument	NE?
UL50	Deoxyuridine triphosphatase	NE
UL51	Function unknown	(E)
UL52	Component of DNA helicase primase complex	E
UL53	Glycoprotein K	(E)
UL54	IE protein; post-transcriptional regulator of gene expression (Vmw63, ICP27)	E
UL55	Function unknown	NE
UL56	Function unknown	NE

(IRL starts at 117158)		
LAT	Latency-associated transcript; probably not protein coding	NE
RL2 (3 exons)	IE protein; regulator of gene expression (Vmw110, ICP0)	NE
RL1	Neurovirulence factor (ICP34.5)	NE
(Left hand of *a* sequence is at 125970)		
(Internal *c* sequence starts at 126371)		
RS1	IE protein; transcriptional regulator (Vmw175, ICP4)	E
(Centre of *ori*S is at 131997)		
(US starts at 132603)		
US1	IE protein; required for virally-induced phosphorylation of RNA pol II; host range phenotype (Vmw68, ICP22)	E/NE
US2	Function unknown	NE
US3	Protein kinase; phosphorylates UL34 protein	NE
US4	Virion surface glycoprotein G	NE
US5	Proposed glycoprotein J	NE
US6	Virion surface glycoprotein D; role in cell entry	E
US7	Virion surfacer glycoprotein I; complexed with glycoprotein E (US8) in F_C receptor	NE
US8	Virion surface glycoprotein E; complexed with glycoprotein I (US7) in F_C receptor	NE
US8A	Function unknown	NE
US9	Tegument protein	NE
US10	Virion protein	NE
US11	Virion protein; ribosome-associated in infected cells	NE
US12	IE protein, interferes vith antigen presentation (Vmw12, ICP47)	NE
(TRS starts at 145583)		
(Centre of *ori*S is at 146233)		
RS1	IE protein; transcriptional regulator (Vmw175, ICP4)	E
(Last nucleotide is 152259)		

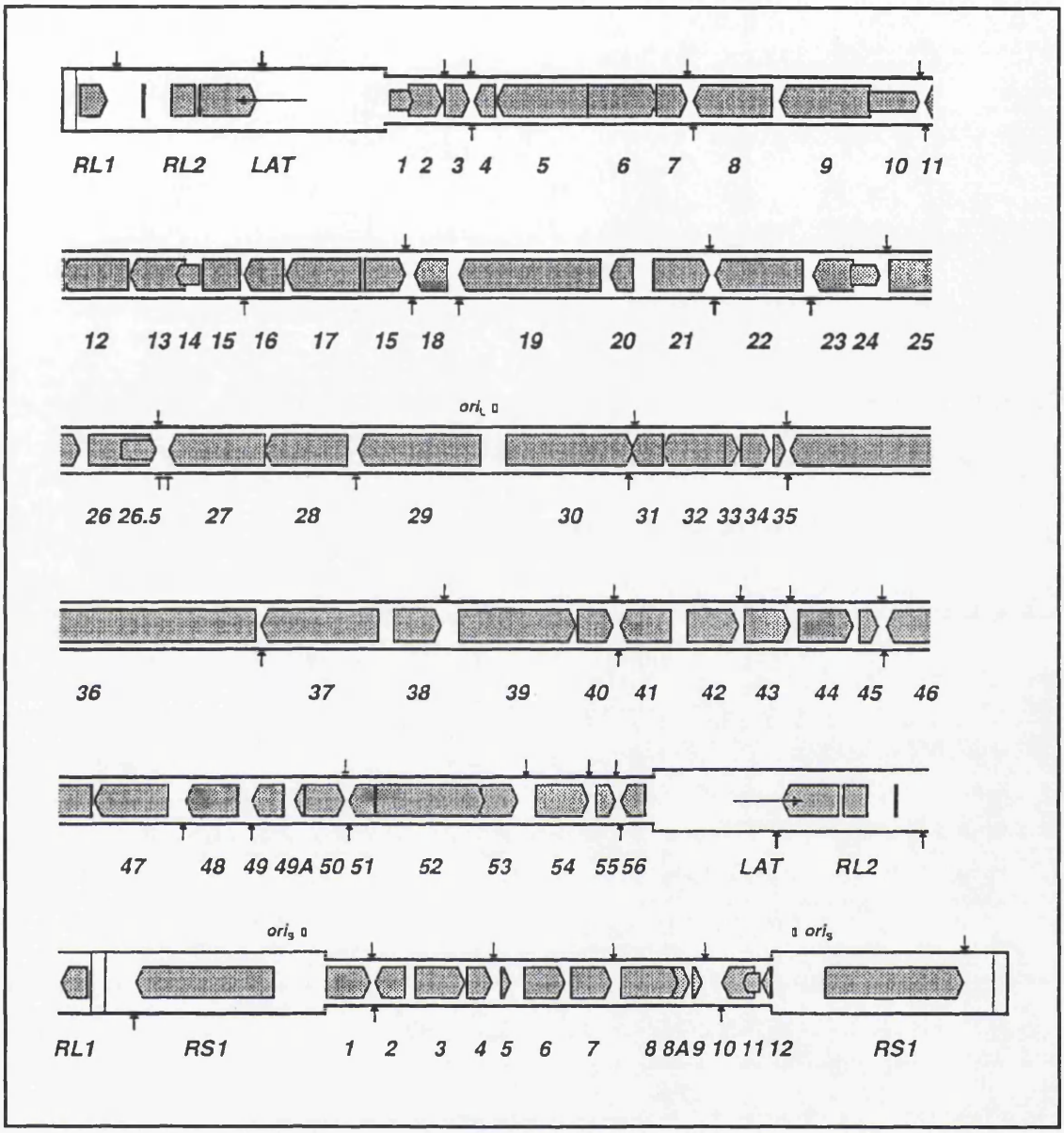


Figure 1.4. HSV-1 gene organisation. The genome is divided into 6 panels 25 kbp long, with the exception of the 6th panel which is longer. The repeat segments are depicted as wider than the unique segments. Protein coding regions are represented by shaded horizontal arrows with the direction of transcription indicated by the direction of the arrow. LAT transcription is indicated by a plain horizontal arrow. The vertical arrows show the positions of polyadenylation sites within the appropriate strand. The positions of the 3 replication origins are also depicted. Adapted from Davison (1993).

1.2.2.1. Regulation of IE transcription.

The HSV IE genes are defined as the only viral genes transcribed in the presence of cycloheximide (Everett, 1987). It follows that IE transcription is regulated by pre-existing viral and cellular factors rather than by viral transactivator proteins synthesised *de novo* after infection, as are the E and L genes. Another crucial characteristic of the IE genes is their ability to be transactivated by the tegument protein Vmw65 (O'Hare, 1993; Spector *et al.*, 1993). After fusion of the viral particle with the cell, Vmw65 is transported to the nucleus where it complexes with 2 cellular factors, thereby forming the IE transactivation complex (IETC). IETC binds *cis*-acting sequences upstream of all of the viral IE genes. Once IETC is bound to DNA, the acidic carboxy-terminal domain of Vmw65 increases the rate of transcription by interacting with components of the basal transcriptional apparatus assembled around the TATA-box.

In early studies it was observed that infection of cell lines stably transfected with the Vmw175 promoter controlling the TK gene led to a stimulation of TK expression, even in the presence of cycloheximide (Post *et al.*, 1981). The ability of UV-inactivated virus to induce TK expression and the ability of the temperature sensitive uncoating mutant *ts*B7 to induce TK at the NPT strongly indicated that components of the virus particles were stimulating gene expression (Batterson and Roizman, 1983; Batterson *et al.*, 1983). The consensus *cis*-acting sequence necessary for induction by the virion factor was identified as TAATGARAT (where R is a purine; Mackem and Roizman, 1982a, b, c; Kristie and Roizman, 1984; Preston *et al.*, 1984; Gaffney *et al.*, 1985; Bzik and Preston, 1986; O'Hare and Goding, 1988; Preston *et al.*, 1988). In addition to the TAATGARAT elements, G+C-rich elements responsible for the basal levels of IE transcription were identified (Kristie and Roizman, 1984). The G+C-rich elements are binding sites for the transcription factor Sp1.

The ORF which encodes the protein capable of stimulating IE transcription was identified by cotransfecting an IE-TK construct with HSV DNA fragments (Campbell *et al.*, 1984). The virion transactivator protein is the gene product of U_L48, named Vmw65 (Dalrymple *et al.*, 1985; Pellett *et al.*, 1985). Vmw65 is present in the virion tegument at an abundance of about 500-1000 copies per virion (Spear and Roizman, 1972; Heine *et al.*, 1974).

It was apparent that Vmw65 transactivated IE genes *via* a complex with cellular factors (McKnight *et al.*,. 1987; Gerster and Roeder, 1988; O'Hare *et al.*, 1988; O'Hare and Goding, 1988; Preston *et al.*, 1988; Triezenberg *et al.*, 1988; Spector *et al.*, 1990). The cellular factor Oct-1 and another previously unknown cell factor termed host cell factor (HCF) are essential components of IETC.

Oct-1 is a homeobox protein which interacts with the octamer elements upstream of many ubiquitously expressed genes involved in morphology, polarity and differentiation,

the consensus octamer element being ATGCAAAT (Herr, 1992). Binding sites for Oct-1 are also found in the immunoglobulin promoters and promoters of other viruses such as SV40, adenovirus and the IE promoters of most other alphaherpesviruses. The transcription factor Oct-2 is expressed in B-lymphocytes and activates transcription *via* the octamer elements upstream of the immunoglobulin genes, however Oct-2 is incapable of forming a complex with Vmw65 (Stern *et al.*, 1989). Oct-1 and Oct-2 belong to a family of homeobox proteins termed POU proteins, which share a 150-160 amino acid sequence called the POU domain. The POU domain can be divided into 2 subdomains, the POU-homeo subdomain and the POU-specific subdomain. The POU-homeo subdomain contacts the TAAT portion of the octamer element. The POU-homeo subdomain is important for DNA binding and for complex assembly with Vmw65. The POU-specific subdomain has no intrinsic DNA binding activity, but when linked to the POU-homeo subdomain contacts ATGC in the octamer element and provides sequence specificity. The GARAT motif is not necessary for Oct-1 to bind to DNA by itself. However, since GARAT is very highly conserved in the HSV IE gene promoters it seems probable that Vmw65 makes contact with GARAT when in IETC, but that binding is extremely weak in the absence of Oct-1 and HCF. Evidence for Vmw65 binding to DNA has been reported (Kristie and Sharp, 1990; Stern and Herr, 1991). Oct-1 binding to the HSV IE gene promoters alone has only a small effect on transcription (O'Hare and Goding, 1988), thus the Vmw65 component of IETC is crucial for stimulating transcription.

Several lines of evidence have implied that the highly acidic carboxy-terminal domain of Vmw65 is the region of IETC important for stimulating transcription. (1) When the carboxy-terminal domain of Vmw65 was fused to the DNA-binding domain of the yeast transcription factor GAL4, an extremely potent activator was produced (Sadowski *et al.*, 1988). (2) A Vmw65 protein lacking the carboxy-terminal domain was capable of complex formation but not of transactivation (Greaves and O'Hare, 1989). (3) The carboxy-terminal domain of Vmw65 interacts specifically with components of the basal transcription initiation apparatus (Stringer *et al.*, 1990; Lin and Green, 1991; Walker *et al.*, 1993). (4) The acidic domain has amino acid sequence similarities to other transcriptional activators (Cress and Triezenberg, 1991).

The third component of the IETC, HCF, was previously unidentified and has therefore only been investigated relatively recently. HCF is unable to bind the octamer element, but binds to Vmw65. The HCF-Vmw65 complex can only bind to Oct-1 which is already bound to DNA, thus the sequential order of complex formation has been determined. The initial step is Oct-1 binding to the octamer elements upstream of the viral IE genes, Vmw65 complexed with HCF then associates with the DNA-bound Oct-1, thereby forming IETC. A cDNA encoding HCF has been cloned (Wilson *et al.*, 1993), and it is encoded by a single gene located on the X chromosome (Wilson *et al.*, 1995a). The function of HCF in uninfected cells is unknown and the protein shows no homology

to any other protein. When the cDNA is expressed in human cells the resulting products comprise a series of proteins with molecular weights ranging from 110-300 kDa, thus it appears that the smaller products arise by processing of the larger 300 kDa protein. The HCF amino acid sequence reveals a highly conserved 26-amino acid sequence repeated 6 times in the HCF precursor protein. The repeat alone is sufficient to induce cleavage of a heterologous protein (Wilson *et al.*, 1995b). Alternative splicing of the HCF mRNA and cleavage of the precursor protein is likely to be important for regulation of HCF activity. HCF is expressed most abundantly in foetal and placental tissues and in tissue culture cells, suggesting that it has a role in cell proliferation (Wilson *et al.*, 1995b). In adults HCF is most abundant in the kidney, however it is not abundant in the brain, a site of HSV latency.

The finding that the HSV IE genes are dependent upon viral and cellular factors for high levels of expression has raised important questions as to the role of Vmw65, octamer binding proteins and HCF in the HSV life cycle, latency in particular. Since it seems likely that insufficient IE gene expression leads to latency, loss of Vmw65, lack of HCF or Oct-1, or the binding of transcriptional repressors to the octamer elements upstream of the HSV IE genes are all possible determinants of the outcome of HSV infection (section 1.3.5.).

The construction of the HSV-1 mutant *in*1814, which has a 4 amino-acid insertion in Vmw65 rendering it unable to interact with other components of IETC and thereby unable to transactivate IE genes, has enabled an assessment of the role of Vmw65 in the virus life cycle to be made (Ace *et al.*, 1988, 1989). The insertion in *in*1814 leads to an approximately 10-fold reduction in viral IE gene expression (Ace *et al.*, 1989). In tissue culture, *in*1814 is impaired in its ability to initiate plaque formation at low multiplicities of infection (MOIs), resulting in a high particle / PFU ratio. Preston and co-workers have undertaken to investigate the genomes which do not undergo replication in the belief that they enter a latent state at least partially resembling that *in vivo* (Harris and Preston, 1991; Jamieson *et al.*, 1995). At high MOIs, the defect in *in*1814 is overcome and replication ensues normally, presumably due to residual IE gene expression. *In*1814 replicates with reduced efficiency *in vivo* but establishes latency and reactivates normally (Steiner *et al.*, 1990; Ecob-Prince *et al.*, 1993a), thus both *in vivo* and *in vitro*, loss of IETC results in an increased propensity to establish a latent rather than lytic infection.

1.2.2.2. The IE proteins.

There are 5 HSV IE proteins, namely Vmw175 (encoded by IE3, named ICP4 in American terminology), Vmw110 (IE1, ICP0), Vmw63 (IE2, ICP27), Vmw68 (IE4, ICP22) and Vmw12 (IE5, ICP47).

Vmw175.

Vmw175 is an essential viral polypeptide required for expression of the E genes and repression of the IE genes, thus it is capable of transactivation or repressing RNA polymerase II-mediated transcription. The essential regulatory function of Vmw175 is clearly demonstrated by the HSV-1 mutant *ts*K which overproduces IE polypeptides at the NPT and is unable to enter into the E phase of lytic infection (Preston, 1979a, 1979b). Mutant *ts*K has a single amino acid substitution in the DNA-binding domain of Vmw175 which completely abolishes transactivation activity at the NPT (Davison *et al.*, 1984).

The mechanism by which Vmw175 activates or represses transcription involves binding to DNA (Faber and Wilcox, 1986; Kristie and Roizman, 1986; Everett *et al.*, 1991a; Pizer *et al.*, 1991). The consensus sequence for binding is RTCGTCNNYNYSG (where R is a purine, Y is a pyrimidine, S is C or G and N is any base; Faber and Wilcox, 1986; DiDonato *et al.*, 1991; Everett *et al.*, 1991a; Pizer *et al.*, 1991), however this sequence is highly degenerate and nonspecific interactions with DNA suffice for transcriptional activation (Smiley *et al.*, 1992; Gu and DeLuca, 1994). Site-specific DNA binding near the transcription initiation site is required for repression, however (Roberts *et al.*, 1988; Gu *et al.*, 1993; Michael and Roizman, 1993).

Vmw175 is a target for phosphorylation and ADP-ribosylation (Preston and Notarianni, 1983; Xia *et al.*, 1996). It has been demonstrated that different electrophoretic forms of Vmw175 differ in the ability to bind to DNA (Michaeli *et al.*, 1988) and possibly in the ability to form cell factor-DNA complexes (Papavassiliou *et al.*, 1991). In infected cells, Vmw175 normally exists as a 350 kDa dimer (Metzler and Wilcox, 1985; Shepard *et al.*, 1990).

The genes repressed by Vmw175 include LAT (Batchelor and O'Hare, 1994), ORF-P (Bohenzky *et al.*, 1993; Yeh and Shaffer, 1993; Lagunoff and Roizman, 1994, 1995) and IE3 itself (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985b). Removal of the Vmw175 binding site induces changes in the pattern of expression of the gene, such as expression earlier during infection and loss of dependence on DNA synthesis (Koop *et al.*, 1993; Rivera-Gonzalez *et al.*, 1994).

Vmw175 acts in concert with, and physically interacts with Vmw110 to stimulate transcription (Everett, 1984b; Yao and Shaffer, 1994). In addition, Vmw175 activity is modulated by Vmw63 since Vmw175 synthesised in the absence of Vmw63 is impaired in its ability to repress transcription from the ORF-P promoter and exhibits impaired ability to bind to DNA (Samaniego *et al.*, 1995). The distribution of Vmw175 within the infected cell is also altered by Vmw63 (Zhu and Schaffer, 1995), thus there is mounting evidence that the IE proteins act synergistically and regulate one-another's activity.

Vmw175 on the IE3 binding site forms a complex with the TATA-binding protein (TBP) and TFIIB, suggesting that this complex is responsible for repression of

transcription (Smith *et al.*, 1993; Kuddus *et al.*, 1995). The mechanism by which the tripartite complex inhibits transcription has yet to be determined.

Vmw110.

IE1, the gene encoding Vmw110, is located entirely within the R_L regions of the genome and is therefore diploid (Perry *et al.*, 1986). Unusually for an HSV gene, IE1 contains 2 introns. A virus devoid of both introns has been constructed but a resulting phenotype has not yet been demonstrated (Everett, 1991; Natarajan *et al.*, 1991), thus a function of Vmw110 mRNA splicing has never been demonstrated.

Vmw110 is a nuclear phosphoprotein which binds to calf-thymus DNA-cellulose columns in crude nuclear extracts (Pereira *et al.*, 1977; Hay and Hay, 1980; Ackerman *et al.*, 1984; Everett *et al.*, 1991b). A crucial feature of Vmw110 is that it is capable of transactivating all of the 3 classes of HSV genes, and also many cellular genes (Everett, 1984b, 1986, 1988; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a, b; Quinlan and Knipe, 1985; Gelman and Silverstein, 1986; Cai and Schaffer, 1989, 1992; Jang *et al.*, 1991). Vmw110 and Vmw175 act synergistically to activate the E and L viral genes (Everett, 1984b, 1986, 1988; O'Hare and Hayward, 1985a, b; Gelman and Silverstein, 1986). The synergistic action of Vmw110 and Vmw175 might be *via* a direct interaction of the two proteins (Yao and Schaffer, 1994).

Although not essential for virus replication, Vmw110 mutants exhibit increased particle / PFU ratios after infection of tissue culture cells at low MOI (Everett, 1986; Stow and Stow, 1986, 1989). In addition to its replication-enhancing function, Vmw110 is the sole requirement for reactivation of HSV in *in vitro* latency systems (Russell and Preston, 1986; Harris *et al.*, 1989; Harris and Preston, 1991). Mutants of Vmw110 are also impaired in their ability to reactivate from latency in animal models (Clements and Stow, 1989; Leib *et al.*, 1989; Cai *et al.*, 1993), thus in addition to facilitating replication, Vmw110 plays a specific qualitative role in reactivation from latency. The observation that Vmw110 mutants are capable of reactivation, albeit at reduced efficiency, and that Vmw110 mutants replicate with similar abilities to wild type virus in the U2OS cell line (Yao and Schaffer, 1995), suggests that cell factors can substitute functionally for the Vmw110 reactivation function.

Vmw110 belongs to a class of viral and cellular proteins which contain a characteristic C_3HC_4 arrangement of cysteines and histidines which binds zinc ions, the 'RING' finger. The RING finger is essential for the full function of Vmw110 (Everett, 1986, 1988, 1989; Harris *et al.*, 1989; Everett *et al.*, 1995a). All of the alphaherpesviruses examined to date encode a protein containing the RING finger motif, and a few of them have been demonstrated to be interchangeable between viruses

(Moriuchi *et al.*, 1994; Everett *et al.*, 1995b). The exact function or mechanism of action of the RING finger is unknown.

Vmw110 binds strongly and specifically to a 135 kDa cellular protein in infected cells (Meredith *et al.*, 1994, 1995). Micro-sequencing of peptides from the 135 kDa protein, followed by hybridisation screening of cDNA librarys with 'guessmer' probes derived from the amino acid sequence, has recently enabled cloning of the entire cDNA encoding the 135 kDa protein (R.D. Everett and M.R. Meredith, unpublished data). The 135 kDa protein has 2 regions of homology to the ubiquitin-specific protease family of enzymes, a family of proteases which de-ubiquitinise proteins. The precise role of the 135 kDa protein in virus infection has yet to be determined, however it is possible that by interating with the 135 kDa protein, Vmw110 inactivates a protein whose normal function is to remove ubiquitin from a protein targeted for proteolysis. The function of the 135 kDa protein might also be related to the interaction of Vmw110 with ND10 domains (section 1.3.8.4.). Identification of the targets of the 135 kDa protein should facilitate our understanding of the role of Vmw110 in virus infection.

Vmw63.

Vmw63 is an essential regulatory protein which is required for the switch from E to L viral gene expression and for efficient DNA replication (Sacks *et al.*, 1985; Sekulovich *et al.*, 1988; McCarthy *et al.*, 1989; Su and Knipe, 1989; McMahan and Schaffer, 1990; Rice and Knipe, 1990; Curtin and Knipe, 1993). Vmw63 acts synergistically with Vmw175 and Vmw110 to stimulate or repress gene expression (Everett, 1986; Rice and Knipe, 1988; Sekulovich *et al.*, 1988; Hardwicke *et al.*, 1989; Su and Knipe, 1989), but has also been demonstrated to be capable of transactivating the gB promoter in the absence of other viral gene products and therefore possesses some transactivation capability by itself (Rice and Knipe, 1988).

A crucial activity of Vmw63 is its effects on mRNA 3' processing and stability. Vmw63 is responsible for the selective usage of L viral poly(A) sites in infected cells (McLauchlan *et al.*, 1989, 1992). In addition, it has been demonstrated that Vmw63 stabilises labile cellular mRNAs by a mechanism that is dependent on the 3' end processing and poly(A) signals (Mosca *et al.*, 1987, 1992; Brown *et al.*, 1995). The observation that Vmw63 is capable of binding to the 3' mRNA processing signals of labile mRNAs (Brown *et al.*, 1995), suggests that Vmw63 modulates gene expression by selectively targeting and stabilising mRNA.

Vmw63 is believed to contribute to the overall shutoff of host gene expression by virtue of its ability to inhibit pre-mRNA splicing. Since spliced genes are widespread in the cell genome but scarce in the HSV genome, the inhibition of splicing by Vmw63

might enable the selective expression of viral RNA over host RNA. HSV infection leads to inhibition of host cell splicing (Schröder *et al.*, 1989; Sandri-Goldin and Mendoza, 1992; Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994). In addition, infection with wild type virus but not Vmw63 mutants leads to the redistribution of components of the spliceosome termed small nuclear ribonucleoprotein particles (snRNPs), from their diffuse speckled pattern in the nuclei of uninfected cells to discrete clusters on the nuclear periphery (Martin *et al.*, 1987; Phelan *et al.*, 1993). Vmw63 is required for the inhibition of host cell splicing and localises in the redistributed snRNP-containing nuclear structures (Sandri-Goldin and Mendoza, 1992; Phelan *et al.*, 1993; Hardy and Sandri-Goldin, 1994), thus it was proposed that the redistribution of snRNPs by Vmw63 is related to the inhibition of host cell splicing during HSV infection. In a recent study a *ts* viral Vmw63 mutant was observed to redistribute snRNPs without inhibiting host cell splicing (Sandri-Goldin *et al.*, 1995), thus redistribution of snRNPs is not sufficient for inhibition of splicing *per se* and another Vmw63-induced function is required.

Vmw68.

Vmw68 is a nuclear protein which is phosphorylated by the U_L13 gene product in infected cells (Purves *et al.*, 1992, 1993). Vmw68 mutants are highly attenuated *in vivo* (Meingner *et al.*, 1988; Poffenberger *et al.*, 1994), and exhibit cell-type dependent defects in tissue culture (Sears *et al.*, 1985; Poffenberger *et al.*, 1993; Rice *et al.*, 1995). Vmw68 mutants grow well in Vero cells, but in nonpermissive cell types such as human foetal lung (HFL) cells expression of the E proteins is delayed and expression of the L proteins is delayed and reduced, whereas DNA replication is unaffected.

In a recent study it was demonstrated that Vmw68 is required for the accumulation of an unusually phosphorylated form of the RNA pol II large subunit (RNAP II), named IIi (Rice *et al.*, 1995). It is possible that IIi has altered promoter specificity that is crucial for transcriptional regulation HSV genes. In HFL cells infected with Vmw68$^-$ mutants transcription of the L genes is greatly reduced, however the viral transcription pattern in Vero cells infected with Vmw68$^-$ mutants is normal even though IIi is not produced, thus it appears that the requirement for IIi is dependent upon other host cell factors.

Vmw12.

Unlike the other HSV IE proteins, Vmw12 is located in the cytoplasm of infected cells and is not phosphorylated (Preston, 1979b; Hay and Hay, 1980; Marsden *et al.*,

1982). Virus mutants defective in Vmw12 grow normally in tissue culture (Mavromaro-Nazos *et al.*, 1986), suggesting that Vmw12 plays a specific role in pathogenesis *in vivo*. Consistent with a role for Vmw12 *in vivo*, it was recently reported that Vmw12 prevents antigen presentation to $CD8^+$ T-lymphocytes (York *et al.*, 1994).

1.2.2.3. Regulation of early gene expression.

Early studies on the regulation of E gene promoters employed the use of transformed cells stably transfected with the HSV TK gene controlled by its native 5' promoter sequences (Everett, 1987). TK was expressed at low levels in the cell genome, but was strongly stimulated upon infection with HSV by a mechanism which involved the IE proteins (Leiden *et al.*, 1976), especially Vmw175 (Kit *et al.*, 1978). Expression from the ε-globin promoter controlling TK was also stimulated by HSV, thus the stimulation also appeared to act on non-viral promoters (Everett, 1985). Microinjection of various deletion constructs of the TK promoter gene into Xenopus oocytes was subsequently used to identify the regulatory sequences important for TK activity. A 110 bp region upstream of the TK mRNA cap site, consisting of a TATA box, a CAAT motif and two Sp1 binding sites were important for full TK expression (McKnight *et al.*, 1981; McKnight and Kingsbury, 1982; Jones *et al.*, 1985). The use of transient transfection techniques were employed to examine the regions of the gD promoter sufficient for full induction by the HSV IE genes. Two Sp1 sites and a TATA box were identified in an 83 bp region of the gD promoter necessary for full activity, however no single region was demonstrated as crucial for inducibility (Everett, 1983, 1984). The validity of the transfection experiments was confirmed by the construction of viruses with modified TK promoters. The TK gene was regulated with similar characteristics in the context of the viral genome as it was after transfection (Coen *et al.*, 1986). Thus Vmw175 is essential for activation of the E genes (Everett, 1983; O'Hare and Hayward, 1984; Eisenberg *et al.*, 1985; ElKareh *et al.*, 1985), and in addition Vmw110 is also capable of apparently nonspecific activation (Everett, 1984b; Gelman and Silverstein, 1985; O'Hare and Hayward, 1985a; Quinlan and Knipe, 1985). Some investigators have reported that Vmw175 and Vmw110 are capable of efficient activation by themselves (Gelman and Silverstein, 1985; O'Hare and Hayward, 1985), while in other studies Vmw175 and Vmw110 exhibited low activity by themselves, but acted synergistically to give strong activation when together (Everett, 1984b, 1986).

1.2.2.4. Regulation of late gene expression.

The L genes of HSV encode components of the virion, and factors required for virion assembly, DNA packaging and egress. The L genes can be divided into 2 classes depending upon their expression in the presence of inhibitors of viral DNA synthesis: leaky L genes (L_1) are expressed at low levels in the presence of inhibitors of viral DNA synthesis, whereas expression of the true L genes (L_2) is undetectable in the absence of DNA synthesis (Spector *et al.*, 1993).

Experiments in which the HSV TK gene was fused to the VP5 promoter (an L_1 gene; Dennis and Smiley, 1984) or U_L49.5 promoter (an L_2 gene; Silver and Roizman, 1985) and used to stably transfect TK^- cells demonstrated that basal expression of an L gene in the cell genome is lower than that of an E gene (the TK promoter), and that like an E gene, the VP5 promoter is stimulated upon infection with TK^- HSV. However both of the viral L promoters were still expressed after infection with HSV in the presence of the viral DNA replication inhibitor phosphonoacetic acid (PAA), thus the L genes were regulated as E genes when in the cell genome (Silver and Roizman, 1985). Clearly L genes are regulated differently in the context of the viral genome than in the cell genome.

The mechanism of repression of the L genes prior to, and activation after the onset of DNA synthesis is unknown at present. Evidence has been reported which suggests that ICP8, the ssDNA binding protein essential for viral DNA synthesis, may be a negative regulator of L gene expression, since the ICP8 *ts* mutant KOS1.1 *ts*18 exhibited low level expression of the L_2 gC gene at the NPT in the presence of inhibitors of DNA synthesis (Godowski and Knipe, 1985).

Like the E genes, the HSV L genes are not expressed in cells infected with *ts* Vmw175 mutants at the NPT (Preston, 1979a). Vmw110 can transactivate all classes of genes, including L genes, in addition, the IE protein Vmw63 can activate an L_1 gene (VP5) in concert with Vmw175 or Vmw110, but does not cause activation on its own and actually represses an L_2 gene (gC), when expressed with Vmw175 or Vmw110 (Sekulovich *et al.*, 1988).

The L gene promoters are the simplest of all the classes of HSV genes, at a superficial level seeming to consist solely of a TATA-box (Johnson and Everett, 1986; Homa *et al.*, 1988; Flanagan *et al.*, 1991). However, the transcribed, untranslated leader sequences may contain elements necessary for high level expression (Mavromara-Nazos and Roizman, 1989; Godowski *et al.*, 1994). Godowski *et al.* identified an element common to the nontranslated leader sequences of many L genes which was capable of binding a 35 kDa cellular protein and conferring transcriptional activation (Godowski *et al.*, 1994).

Huang *et al.* observed a 40 kDa protein bind to the –6 to +8 region of the U_L19 promoter (Huang *et al.*, 1996), and Mills *et al.* have identified binding sites for the factor

YY1 in the upstream regions of many L_1 genes (Mills *et al.*, 1994). Although the L gene promoters contain binding sites for cellular transcription factors, the mechanism by which the virus modulates expression of the L genes during viral replication remains unknown.

1.2.3. Alterations of host metabolism.

HSV infection is known to alter the host cellular metabolism in a number of ways.

Overall shutoff of host gene expression.

Infection with HSV leads to the overall inhibition of cellular DNA, RNA and protein synthesis (Roizman *et al.*, 1965; Fenwick, 1984). The 'shutoff' of cellular metabolism by HSV can been divided into 2 stages, that which is mediated by a virion component and occurs very early in infection (early shutoff), and that which is mediated by a viral protein synthesised *de novo* (delayed shutoff; Fenwick and Clarke, 1982). The observations that UV-irradiated virus induces the early shutoff of cellular protein synthesis (Fenwick and Walker, 1978; Fenwick and Clarke, 1982; Fenwick and McMenamin, 1984), and that the shutoff occurs when *de novo* viral gene expression is prevented by chemical inhibitors (Schek and Bachenheimer, 1985; Storm and Frenkel, 1987), demonstrated that the early shutoff is caused by components of the virus particle. The virion host shutoff (*vhs*) function induces the degradation within the cytoplasm of host as well as viral mRNAs (Fenwick and McMenamin, 1984; Schek and Bachenheimer, 1985; Storm and Frenkel, 1987), thereby causing rapid mRNA turnover and facilitating the changes in expression of the kinetic classes of viral genes. A functional U_L41 gene of HSV-1 is necessary for *vhs* activity (Kwong *et al.*, 1988; Fenwick and Everett, 1990a, b; Smibert and Smiley, 1990; Read *et al.*, 1993), and was demonstrated to be the only viral gene product required to induce the degradation of CAT mRNA expressed from a transfected reporter construct (Pak *et al.*, 1995).

Actinomycin D chase experiments in which the *in vivo vhs*-induced degradation of an mRNA was followed using RNase protection employing probes to protect 5' or 3' regions of the reporter mRNA demonstrated that the 5' portion was preferentially degraded (Karr and Read, abstract 202, 20th International Herpesvirus Workshop, Groningen, The Netherlands, 1995). Moreover, in an *in vitro* translation system *vhs* was demonstrated to induce a single endonucleolytic cleavage at one of several sites located 30-60 nt from the 5' end of the mRNA, thus removing the 5' cap structure (Elgadi *et al.*, abstract 201, 20th International Herpesvirus Workshop, Groningen, The Netherlands, 1995). The cleavage event occurred much less efficiently on an uncapped RNA and was

inhibited by cap analogues, indicating that the cap structure was important for *vhs* activity. Evidence was found to suggest that the cap-dependent cleavage event was a primary event in the degradation process, and that *vhs* also induces cleavage in the target RNA at apparently random sites by a cap-independent process. It remains to be determined whether the *vhs* factor has its own RNase activity or whether it functions *via* a cellular RNase.

The U_L13-encoded protein kinase contributes to mRNA instability in infected cells (Overton *et al.*, 1994). Since phosphorylation of *vhs* was unaffected in lysates of virions from a U_L13 mutant, the U_L13 product does not appear to induce mRNA instability by phosphorylating *vhs*.

Vmw63 contributes to the shutoff of host gene expression by inhibiting the processing of pre-mRNAs (section 1.2.2.3.; Hardwicke and Sandri-Goldin, 1994).

Cellular genes activated by HSV.

Although HSV infection causes the overall inhibition of cellular gene expression, the expression of a proportion of cellular genes remains unaffected and some are stimulated (Smiley *et al.*, 1991) . The cellular stress proteins are induced in response to HSV infection (Notarianni and Preston, 1982; LaThangue *et al.*, 1984; Latchman *et al.*, 1987; Russell *et al.*, 1987a). A comparison of the abilities of adenovirus 5, HSV-1, SV40 and vaccinia virus to induce 3 species of the 70 kDa heat shock protein (*hsp*) family revealed that only adenovirus 5 and HSV-1 were capable of induction, and only one of the 70 kDa *hsp's* were induced, suggesting that the induction was not a general response to viral infection but was specific (Phillips *et al.*, 1991). Induction of the 70 kDa *hsp* by HSV-1 was transient, and was followed by repression. It has been noted that temperature sensitive HSV-1 Vmw175 mutants which overproduce IE proteins induce high levels of stress proteins at the NPT (Notarianni and Preston, 1982). An HSV-1 mutant encoding a truncated Vmw175 failed to induce the stress response, therefore the abnormal form of Vmw175 expressed by the *ts* mutants was responsible for the induction (Russell *et al.*, 1987a). The HSV IE proteins are capable of specifically inducing cellular genes, thus Vmw175 induces ubiquitin B but not ubiquitin A or C (Latchman *et al.*, 1987; Kemp and Latchman, 1988b), Vmw110 induces *c-jun* and the AP-1 transcription factor (Jang *et al.*, 1991), Vmw63 is required for the accumulation of a protein termed p40 which is expressed in transformed cells (Estridge *et al.*, 1989). In addition to the IE proteins, Vmw65 is capable of transactivating cellular genes controlled by octamer elements which resemble a viral TAATGARAT regulatory sequence, for example the gene encoding the U3 snRNA is transactivated by Vmw65, but not the genes encoding the U1 or U2 snRNAs (Kemp and Latchman, 1988a; Latchman, 1991). Cellular gene induction can

occur in the absence of protein synthesis (Kemp *et al.*, 1986), not only *via* transinduction by Vmw65 but also by binding to a specific receptor on the cell surface (Preston, 1990).

The *Alu* repeats are the most abundant family of mobile short interspersed elements (SINEs) in the human genome. There are nearly 10^6 copies of the *Alu* repeats per genome, compromising approximately 5% of the total DNA, each repeat being approximately 300 nt in length. *Alu* elements transpose *via* an RNA intermediate transcribed by RNA pol III, however, the majority are transcriptionally silent due to the absence of the necessary *cis*-acting regulatory sequences. RNA pol III transcription of the *Alu* repeats is stimulated by infection with adenovirus 5 or HSV-1 (Jang and Latchman, 1989; Panning and Smiley, 1994; 1995), thus activation of *Alu* transcription might be a normal cellular response to viral infection. Induction by HSV-1 requires *de novo* viral gene expression and occurs when transcription is limited to the IE genes (Panning and Smiley, 1994). Deletions which inactivated each of the five IE genes had no effect on *Alu* induction, therefore 2 or more HSV IE proteins suffice to stimulate *Alu* transcription. In contrast to a previous finding (Jang and Latchman, 1992), Vmw63 was not required for induction (Panning and Smiley, 1994). The exact mechanism of stimulation of *Alu* transcription is unknown, however transactivation might be due to the direct action of viral transactivators, alterations of cell chromatin or methylation, or alteration of the cell growth signalling pathways. The role of the *Alu* SINEs in the life cycle of both cells and virus is unknown, however, it has been reported that expression of *Alu* elements transfected into HeLa cells inhibits proliferation (Sakamoto *et al.*, 1991), hence it was suggested that one role of the *Alu* elements is to alter the cell cycle, reduce cell growth and limit viral replication (Panning and Smiley, 1995).

ICP34.5.

Mutants of HSV-1 defective in ICP34.5 have an LD_{50} increased by approximately 10^5-fold in rodents (Whitley *et al.*, 1993). The requirement for ICP34.5 varies between cell types, but ICP34.5 is crucial for efficient replication in neurons, hence its importance in virulence. In human SK-N-SH neuroblastoma cells infected with ICP34.5$^-$ mutants the onset of viral DNA replication triggers premature shutoff of protein synthesis and inhibition of viral replication, whereas wild type virus does not trigger the shutoff (Chou and Roizman, 1992). It was proposed that ICP34.5 functions by preventing the viral infection-induced stress response which leads to inhibition of protein synthesis. In non-neuronal cells such as Vero cells, premature shutoff is not induced by HSV infection and ICP34.5 is not required. The carboxy-terminal domain of ICP34.5 has homology to the carboxy-terminal domains of the murine protein MyD116, a protein expressed in myeloid leukaemia cells induced to differentiate by IL-6, and the hamster protein GADD34, which

is expressed in response to growth arrest and DNA damage (Chou and Roizman, 1994). The carboxy-terminal domain of ICP34.5 is required to preclude the premature shutoff in infected cells, and the carboxy-terminal domain of the murine Myd116 protein can substitute functionally for its viral counterpart (Chou and Roizman, 1994; He *et al.*, 1996), thus ICP34.5 appears to mimic the action of Myd116.

The translation initiation factor eIF-2α is phosphorylated, and hence inactivated, in cells infected with ICP34.5$^-$ mutants, in contrast to cells infected with wild type virus or mock-infected cells where it is not phosphorylated (Chou *et al.*, 1995). In cells infected with ICP34.5$^-$ mutants the dsRNA-dependent interferon (IFN)-induced protein kinase (PKR; section 1.5.) was found to associate with a 90 kDa protein, whereas in wild type infected cells association with the 90 kDa protein occurred only at very low levels. Since activated PKR shuts-off protein synthesis by phosphorylating eIF-2α, it was proposed that in the absence of ICP34.5, PKR complexes with p90 and shuts-off protein synthesis by phosphorylating eIF-2α.

Modification of RNA polymerase II.

Infection with HSV leads to an unusually phosphorylated form of the RNA pol II large subunit (RNAP II) and its recruitment to subnuclear viral replication compartments (Rice *et al.*, 1994). It was suggested that the unusually phosphorylated form of RNAP II could account for the differences between regulation of genes in the cell genome and in the context of the viral genome. Thus, a cellular gene is inactive when in the cell genome during viral infection, but remains active in the context of the viral genome. It was demonstrated that the IE protein Vmw68 is required for the modification of RNAP II and establishment of the normal viral transcription program (Rice *et al.*, 1995).

1.2.4. DNA Replication.

HSV DNA replication occurs in approximately 200 discrete compartments in the cell nucleus. The formation of the replication compartments is dependent on the U_L29 gene product, ICP8 (De Bryn Kops and Knipe, 1988). DNA replication occurs by a rolling circle mechanism and results in the production of large head-to-tail concatemers (Jacob and Roizman, 1977; Jacob *et al.*, 1979; Jongeneel and Bachenheimer, 1981).

There are 3 palindromic origins of DNA replication in the HSV-1 genome which are capable of binding the U_L9 gene product (figure 1.4.; Vlazny and Frenkel, 1981; Stow, 1982; Stow and McMonagle, 1983; Quinn and McGeoch, 1985; Weller *et al.*, 1985;

HSV gene	Function	Reference
U_L30	DNA polymerase.	Coen *et al.*, 1984.
U_L29	ssDNA-binding protein; anchors the polymerase to the replication complex.	Boehmer and Lehman, 1993.
U_L9	*Ori*-binding protein.	Boehmer *et al.*, 1993.
U_L42	DsDNA binding protein; increases processivity of DNA replication.	Digard *et al.*, 1993.
U_L8, U_L5 and U_L52	Form the helicase-primase complex in equimolar amounts.	Crute *et al.*, 1989; Calder *et al.*, 1992.

Table 1.2. HSV genes essential for replication of viral DNA and their proposed functions.

Lockshon and Galloway, 1986). There is one copy of ori_S in each of the S segment repeats, which directs unidirectional DNA replication. Another replication origin, ori_L, is located in the U_L segment and directs bidirectional replication. Ori_L or one of the ori_S origins can be deleted without affecting virus viability.

There are 7 viral genes which are essential for viral DNA replication in tissue culture. The genes essential for DNA replication were identified by cotransfecting plasmids containing a viral replication origin with cloned fragments of the HSV genome and assessing the ability of the viral genes to replicate the origin-containing plasmid (Challberg, 1986; McGeoch *et al.*, 1988b; Wu *et al.*, 1988). The viral genes necessary for efficient DNA replication in the assay and their proposed functions are presented in table 1.2.

There are many virally encoded enzymes involved in nucleotide metabolism which are necessary for efficient virus replication *in vivo*, but not essential for replication in tissue culture cells. The virally-encoded enzymes involved in nucleotide metabolism are especially important for replication in neurons which are non-dividing and therefore have low natural pools of the cellular enzymes with equivalent functions.

The alkaline DNase (U_L12) complexes with the U_L29 gene product and cleaves the *a* sequence during packaging (Weller *et al.*, 1990; Thomas *et al.*, 1992). The uracil DNA-glycosylase (U_L2) is involved in DNA repair and proof reading (Caradonna *et al.*, 1987; Pyles and Thompson, 1994). This enzyme removes dUTP residues and deaminated cytosine residues in DNA and is presumably of importance since the HSV genome contains a high G+C content. The viral ribonucleotide reductase consists of 4 subunits, 2 from U_L39 and 2 from U_L40, and converts ribonucleotides to deoxyribonucleotides thereby producing sufficient deoxyribonucleotides for viral DNA synthesis (Ingemarson and Lankinen, 1987; Idowu *et al.*, 1992). TK (U_L23) phosphorylates thymidine to TMP (Jamieson and Subak-Sharpe, 1974; Efstathiou *et al.*, 1989), TMP is then converted to dTTP by cellular enzymes and the viral ribonucleotide reductase. The viral dUTPase (U_L50; Preston and Fisher, 1984; Fisher and Preston, 1986; Pyles *et al.*, 1992), hydrolyses dUTP to dUMP, thereby preventing incorporation of dUTP into DNA and providing a pool of dUMP for conversion to dTMP by thymidylate synthase.

1.2.5. Cleavage and packaging of DNA.

Capsid assembly and packaging of the viral DNA occurs in the nucleus. Viral DNA is packaged into type B capsids which contain the 'scaffold' proteins: the U_L26 and $U_L26.5$ gene products and the self-cleavage products of U_L26 (Rixon *et al.*, 1988; Liu and Roizman, 1991a, b, 1992; Davison *et al.*, 1992; Dilanni *et al.*, 1993).

Viral DNA lacking free ends, such as circular or head-to-tail concatemeric DNA, is cleaved into unit length virion genomes and packaged into pre-formed capsids by a tightly coupled mechanism involving amplification of *a* sequences (Deiss and Frenkel, 1986). The ultimate result is the production of a packaged viral genome with a free S-terminus containing a DR1 consisting of a single bp and a 1 nt 3' extension, and an L-terminus consisting of 1-5 copies of the *a* sequence ending in a DR1 containing 18 bp and a 1 nt 3' extension, such that a single DR1 is produced when the ends are joined together (Mocarski and Roizman, 1982).

Stow *et al.* noted that the ability of a plasmid containing a viral *oris* to be packaged by a helper virus required the presence of *a* sequences in the plasmid (Stow *et al.*, 1983). Subsequently Deiss *et al.* identified 2 regions in the U_b and U_c regions of the *a* sequence, termed *pac*1 and *pac*2 respectively, which were important for packaging and are conserved among several herpesviruses (Deiss *et al.*, 1986). Deiss *et al.* proposed a model for the packaging and cleavage process (Deiss *et al.*, 1986; Roizman and Sears, 1993). The model proposes that a protein binds to an *a* sequence and in turn binds to a site on the capsid, the DNA is then extruded into the capsid until another *a* sequence in identical orientation comes to be packaged, whereupon 1 of the juxtaposed *a* sequences is

cleaved. The cleaved *a* sequence is then repaired and duplicated by the double-strand break and gap repair mechanism proposed by Szostak *et al.* (Szostak *et al.*, 1983). Following duplication, cleavage occurs within the shared DR1 sites of the *a* sequences. The model predicts that the region of DNA packaged is predicted by the distance between 2 direct repeats of the *a* sequence, consistent with the finding that the internal *a* sequences can be deleted without fully inhibiting packaging (Poffenberger *et al.*, 1983; Poffenberger and Roizman, 1985; Jenkins and Roizman, 1986). However, defectives containing over 17 direct repeats of the *a* sequence can be detected in virions from defective viruses (Frenkel *et al.*, 1976), in addition there is some evidence that shorter than full-length DNAs are packaged but not enveloped (Vlazny *et al.*, 1982), thus it has been suggested that complete filling of the capsid is required for correct cleavage and maturation to occur.

Viral mutants unable to cleave and encapsidate DNA typically accumulate type B capsids and concatemeric viral DNA under nonpermissive conditions. The full array of viral gene products required for cleavage and encapsidation and their exact functions are unknown, however genes required include U_L6, U_L15, U_L25, U_L26, U_L28, U_L32 and U_L33 (Preston *et al.*, 1983; Addison *et al.*, 1984, 1990; Sherman and Bachenheimer, 1987, 1988; Al-Kobaisi *et al.*, 1991; Poon and Roizman, 1993; Baines *et al.*, 1994; Patel *et al.*, 1996). Chou and Roizman have identified 2 viral proteins which can specifically bind to the *pac*2 element of the *a* sequence (Chou and Roizman, 1989), and in addition, the capsid protein VP19c is capable of binding to DNA (Braun *et al.*, 1984), thus these viral proteins might play important roles in the cleavage and encapsidation process.

The capsid is enveloped by budding through patches of tegument proteins and immature glycoproteins on the inner nuclear membrane. The virion reaches the Golgi where the glycoproteins are glycosylated to their mature form (Darlington and Moss, 1969; Schwartz and Roizman, 1969; Johnson and Spear, 1982). It remains unclear whether the envelope present on released virions is derived from the inner nuclear membrane or derived from the Golgi membrane, since two possible pathways of envelopment can be envisaged (Rixon, 1993). The first pathway predicts that after budding through the inner nuclear membrane the envelope is lost by fusion with the outer nuclear membrane and that the virion envelope is formed by fusion with the Golgi membrane. In the second pathway, after gaining the envelope from the inner nuclear membrane the nucleocapsid enters a vesicle derived from the outer nuclear membrane. The vesicle-enclosed virion fuses with the Golgi membrane thereby releasing the virion into the Golgi and preserving the envelope derived from the inner nuclear membrane.

1.3. HSV LATENCY.

1.3.1. General aspects of latency.

HSV remains in a latent state within neurons innervating the site of primary infection (Stevens and Cook, 1971; Nesburn *et al.*, 1972; Stevens *et al.*, 1972; Walz *et al.*, 1974). In many individuals, reactivation occurs spontaneously in latently infected neurons and virus returns to the periphery to produce clinical lesions, thereby augmenting the spread of the virus to other individuals. During primary infection, HSV adsorbs to sensory nerve endings at the peripheral site of replication and the nucleocapsid is released into the nerve axon *via* fusion of the viral and cell membranes. The nucleocapsid is transported to the nerve cell body in the ganglia by axonal transport (Cook and Stevens, 1973). When in the cell body the viral genome is uncoated, released into the neuronal nucleus and converted to a nonlinear form (Rock and Fraser, 1983, 1985; Efstathiou *et al.*, 1986). The time between infection at the neuronal cell surface and entry of the nucleocapsid into the nucleus after infection of the mouse *via* the footpad was estimated to be approximately 20-24 hours (Cook and Stevens, 1973). Once in the nucleus, the viral genome is either converted to the latent state and stably retained for the life span of the host, or enters into the lytic cycle of replication. During latency, transcription of the genome is confined to the LAT region, thus the viral IE promoters, which are characterised by their strong activities in the absence of protein synthesis, are repressed.

1.3.2. Sites of latency.

1.3.2.1. Neuronal sites of latency.

In 1929, Goodpasture wrote "It seems to me probable from experimental and clinical facts that herpetic virus does reside in a latent state within the human body and specifically in the nervous tissues, perhaps primarily within nerve cells of the ganglia; and that neuronal disturbances are frequently the basis of subsequent outbreaks." Goodpasteur made an intelligent deduction from the observation that the major proportions of human herpetic eruptions occur from the peripheral distribution of the fifth cranial nerve, and that injury to nerves (Cushing, 1905), as well as stress, illness and administration of toxic substances caused reactivation.

More direct evidence that nerve ganglia harbour latent HSV came in 1971 when Stevens and Cook reactivated HSV-1 from the spinal ganglia of mice months after inoculation into the footpad (Stevens and Cook, 1971). Latently infected murine ganglia were explanted and cocultivated with permissive tissue culture cells. Explantation caused reactivation of HSV-1 from the ganglia leading to destruction of the cultured cells. Virus could not be detected in ganglia which were homogenised and assayed for infectious virus immediately after explantation from latently infected mice, and in addition, ultrastructural and immunofluorescence analyses failed to detect virus-specific products in latently infected ganglia. A novel latent state was therefore occurring rather than a low level of replication. Later, Stevens and co-workers demonstrated the presence of latent HSV in the trigeminal ganglia of rabbits after inoculation in the eye (Nesburn *et al.*, 1972; Stevens *et al.*, 1972). Using the cocultivation technique on human ganglia obtained *post mortem*, numerous groups have demonstrated the presence of latent HSV-1 in humans (Bastian *et al.*, 1972; Baringer and Swoveland, 1973; Plummer, 1973; Warren *et al.*, 1977; Warren *et al.*, 1978; Lonsdale *et al.*, 1979). Baringer isolated HSV-2 from three separate human sacral ganglion isolates, consistent with the observation that HSV-2 reactivations occur predominately at the genital mucosa, and in one subject HSV-1 and HSV-2 were recovered from the trigeminal and sacral ganglia respectively (Baringer, 1974). HSV-1 has been reactivated from the peripheral ANS of mice (Price *et al.*, 1975) and humans (Warren *et al.*, 1978), therefore latency is not restricted to the sensory ganglia. HSV-1 has also been reactivated from the trigeminal nerve roots taken from human cadavers (Warren *et al.*, 1982), suggesting that clinically latent HSV may not be confined to the ganglia. Latency is established in ganglia innervating the site of inoculation (Walz *et al.*, 1974), and after replication in the local sensory ganglia the virus travels to the CNS *via* the nerve axons (Cook and Stevens, 1973). Inoculation of HSV-1 into the cornea (Baringer and Griffith, 1970), brain (Tanaka *et al.*, 1994) or induction of viraemia (Cook and Stevens, 1976) leads to a severe productive infection in the CNS of mice, after which latent virus can be detected in multiple neuronal tissues (Cook and Stevens, 1976; Tanaka *et al.*, 1994). It seems likely that latency can be established in virtually any nervous tissue that is exposed to an adequate concentration of virus. The trigeminal and spinal ganglia are the most frequent sites of reactivation in humans, producing lesions at the peripheral mucosae thereby facilitating the spread of the virus.

HSV-1 has been reactivated from CNS tissue from only a very small fraction of inoculated mice (Knotts *et al.*, 1973; Cook and Stevens, 1976; Cabrera *et al.*, 1980). In the study by Cabrera *et al.*, 90% of mice had a productive infection in the brain after inoculation of HSV-1 into the cornea (Cabrera *et al.*, 1980). Reactivation after explant / cocultivation occurred in trigeminal ganglia from 95% of the mice that had recovered from the acute infection but reactivation occurred in CNS tissue from only 5%. However,

HSV-1 DNA was detected in the CNS tissues from 30% of the recovered mice, suggesting that latency was readily established in the CNS but reactivation was less efficient than in trigeminal ganglia. HSV-1 DNA has also been detected in human brain tissue (Sequiera *et al.*, 1979; Fraser *et al.*, 1981). Reactivation from human CNS tissue has never been demonstrated, making it difficult to assess the significance of latency in the CNS in human disease.

The cell type(s) within latently infected ganglia which harbour the HSV genome is an important issue. Cook *et al.* examined explanted ganglia of mice by immunofluoresence labelling with HSV-1-specific antibodies (Cook *et al.*, 1974). After explantation to induce reactivation, HSV-1 antigens appeared initially in neurons and at later times in the surrounding cells. When rabbit anti-HSV-1 antibodies were present in the culture medium of explanted ganglia, virus was restricted to the neurons, implying that reactivation occurred solely in the neurons. After explantation and cocultivation in medium containing [^{3}H]-thymidine, incorporation of ^{3}H occurred at above background levels only in neurons. Neurons do not replicate their own DNA, therefore the incorporation must have been due to HSV-1 DNA replication. After the [^{3}H]-thymidine had been removed and replaced with unlabelled thymidine, the radioactive label moved into the surrounding cells. It was deduced that the neurons were the sole site of reactivation after explantation of the ganglia. McLennan and Darby infected mice in the footpad with *ts* mutants of HSV-1 and allowed the infections to proceed to latency (McLennan and Darby, 1980). The nerves of cervical ganglia were severed in order to induce reactivation *in vivo* and three days later the ganglia were removed and immunohistochemically stained for HSV-1 antigen. The body temperature of mice was nonpermissive for the mutants, therefore the reactivated virus was not expected to spread from the site of latency. Staining occurred only in neurons, supporting the data of Cook *et al.* (Cook *et al.*, 1974). The data of McLennan and Darby should be interpreted with caution for two reasons: first, the *ts* mutants did not replicate in the ganglia as would occur in a natural infection with wild-type virus, and as it had previously been shown that HSV-1 reaches ganglia *via* the nerve axons and not the blood or lymph (Cook and Stevens, 1973), virus would have been unable to reach the non-neuronal cells and establish latency; and, second, the latent HSV-1 was reactivated by nerve section, a treatment which selectively affects neurons and not the non-neuronal supporting cells. Although the methods used by McLennan and Darby can be brought into question, it should be stated that work by others has proved that the final interpretation that latency in sensory ganglia occurs solely in neurons was probably correct. Kennedy *et al.* observed that reactivation of HSV-1 and HSV-2 occurred initially in neurons after explantation and dissociation of the DRG of latently infected mice (Kennedy *et al.*, 1983). Neurons were unambiguously identified with a neuron-specific antibody. Detection of LAT by *in situ* hybridisation has facilitated the detection of cells

latently infected with HSV-1 (Stroop *et al.*, 1984; Deatly *et al.*, 1988) or HSV-2 (Tenser *et al.*, 1991). LAT expression is largely confined to neurons but some studies have reported hybridisation to LAT occurring in non-neuronal cells of the nervous system (Deatly *et al.*, 1988; Tenser *et al.*, 1991).

1.3.2.2. Non-neuronal sites of latency.

There is evidence that HSV can establish latency in cell types other than neurons. Extra-neuronal sites of latency have been identified by their ability to produce virus after explantation / cocultivation. However, this technique does not distinguish latency from persistent low-grade virus replication and a number of criteria have therefore been used which a virus-tissue interaction must exhibit in order to be considered a latent interaction. 1) During a latent infection, infectious virus is not detectable in cell-free homogenates prepared immediately after explantation (Stevens and Cook, 1971). 2) Virus appears immediately after cocultivation of persistently infected tissue, whereas in latently infected tissue there is a prolonged delay between explantation and the release of virus (Scriba, 1981). 3) Persistent infections are cleared by exposure to an inhibitor of viral DNA replication in the culture medium during cocultivation (Scriba, 1981), whereas the rate of reactivation from latently infected tissue is unaffected after removal of the inhibitor (Scriba, 1981; Cook and Brown, 1987; Al-Saadi *et al.*, 1988)

HSV-1 or HSV-2 was induced to reactivate from the explanted footpads of mice months after inoculation at that site (Al-Saadi *et al.*, 1983; Subak-Sharpe *et al.*, 1984a, b). As with latently infected ganglia, infectious virus was not detectable in the footpads homogenised and assayed for infectivity immediately after explantation, but was reactivated after culturing. When mice were administered acycloguanosine (ACG) for prolonged periods before explant, reactivation was not precluded (Clements and Subak-Sharpe, 1988). Treatment of explanted footpads *in vitro* with ACG or PAA in the culture medium also failed to affect the appearance of virus after the inhibitors were removed (Al-Saadi *et al.*, 1988), thus a novel latent state was occurring rather than a persistent low level of replication. Footpads of mice do not contain neuronal cell bodies, therefore latency must have been in non-neuronal cells. The cellular foci of reactivation in the mouse footpad were identified by *in situ* hybridisation to viral RNA in sections taken from cultured footpads (Clements and Jamieson, 1989). HSV-1-specific RNA first appeared in basal cells of hair follicles, cells of the hair root sheath, epithelial cells of sebaceous glands and in cells of the epidermis.

HSV-1 and HSV-2 have been recovered from the footpads of guinea pigs during the latent phase of infection (Scriba, 1977). However, reactivation from the guinea pig footpad differs from that which occurs in the mouse footpad as HSV-2 clearly persists as

a replicative rather than latent infection (Scriba, 1981). Analyses of reactivation from the guinea pig footpad suggested persistent replication in all three of the criteria listed above (Scriba, 1981).

Another possible non-neuronal site of HSV latency is the cornea (Shimeld *et al.*, 1982; Cook *et al.*, 1987; Claoué *et al.*, 1990; Cook *et al.*, 1991b). Interestingly, several groups have reported transcripts antisense to Vmw110 mRNA in the corneas of latently infected animals (Cook *et al.*, 1991a; Abghari *et al.*, 1992). In addition, regions of the LAT promoter which stimulate transcription specifically in neuronal cells are also active in corneal cells (Perng *et al.*, 1994b), thus transcription of the LAT may be regulated with similar characteristics in corneal cells as it is in neurons.

When IE transcription of HSV-1 is inhibited, the genome is stably retained in cultured non-neuronal cells in a form resembling latency (Harris and Preston, 1991; Jamieson *et al.*, 1995; this thesis). The presence of VZV transcripts in satellite cells of latently infected human sensory ganglia suggests that VZV can establish latency in non-neuronal cells, thus giving credibility to the hypothesis that HSV is capable of latency in non-neuronal cells (Croen *et al.*, 1988; Croen and Straus, 1991).

1.3.3. Animal models.

The animals most frequently used to study HSV latency are mice, rabbits and guinea pigs, although no single model mimics the situation in the natural human host exactly. Any finding concerning latency which is suggested by *in vitro* experimentation ultimately requires confirmation in animal models.

After inoculation of animals with HSV, latency was established in the nervous tissue innervating the site of inoculation (Stevens and Cook, 1971; Stevens *et al.*, 1972; Cook and Stevens, 1973; Walz *et al.*, 1974). Surviving animals continued to harbour latent virus which was reactivated by cocultivation of the ganglia with permissive tissue culture cells. Cocultivation of ganglia has proved an invaluable technique for assessing the latency-competence of viruses and for identifying sites of latency. However, the relevance of molecular events following cocultivation to reactivation *in vivo* is dubious. The models of *in vivo* reactivation described below are valuable methods of examining the host-virus interaction, but explantation / cocultivation of mouse ganglia is a better model for molecular studies.

Inoculation of mice in the ear led to a latent infection in the cervical dorsal root ganglia (Hill *et al.*, 1975). A small proportion of latently infected mice exhibited spontaneous recurrence leading to erythryma or vesicles at the site of inoculation. A greater proportion of latently infected mice could be induced to reactivate by exposing the ear to stimuli such

as UV light, ice or cellophane stripping (Blyth *et al.*, 1976; Hill *et al.*, 1978; Harbour *et al.*, 1983).

Sawtell and Thompson used transient hyperthermia to reactivate HSV-1 from trigeminal and lumbosacral ganglia of mice latently infected after inoculation at one of several sites (Sawtell and Thompson, 1992a). Hyperthermia caused rapid *in vivo* reactivation, but reactivation occurred from only a very small proportion of neurons in the ganglia. The hyperthermia-induced reactivation technique showed that a LAT^- mutant reactivated less frequently from the trigeminal ganglia than a LAT^+ virus (Sawtell and Thompson, 1992b). The difference in reactivation frequencies was attributed to a difference in the abilities to establish latency rather than a difference in reactivation abilies *per se*.

The administration of $CdSO_4$ led to reactivation of HSV-1 from the trigeminal ganglia of latently infected mice (Fawl and Roizman, 1993). Reactivation was not induced by zinc, nickel or manganese, indicating that reactivation was not caused by the metallothioneins and might be caused by $CdSO_4$ specifically inhibiting the factors which maintain the virus in the latent state.

The proportion of mice which survived inoculation in the eye was increased by passive immunisation 24 hours prior to infection (Shimeld *et al.*, 1989). Treatment with the immune-suppressors cyclophosphamide and dexamethasone resulted in ocular shedding in 50% of the animals.

After inoculation in the eye and establishment of latency, reactivation was induced in the trigeminal ganglia of rabbits by iontophoresis of epinephrine (Kwon *et al.*, 1981) or treatment with cyclophosphamide and dexamethasone (Haruta *et al.*, 1989). The rabbit eye model has been useful in demonstrating the role of LAT in efficient reactivation (Hill *et al.*, 1990; Farrell *et al.*, 1993; Bloom *et al.*, 1994).

The guinea pig has been used in a model for genital herpes. Inoculation in the vagina or urethra resulted in a self-limiting primary infection with replication in the lumbosacral ganglia leading to latency in the dorsal root ganglia (Stanberry *et al.*, 1982, 1985). Symptomatic and asymptomatic occurrences occurred after the primary infection and the frequency of such occurrences decreased with time. The primary infection was similar after inoculation with HSV-1 or HSV-2, however spontaneous reactivation occurred more frequently with HSV-2 and the guinea pig model thus apparently mimicked genital herpes in humans.

While *in vivo* models have provided important information on the pathogenesis of HSV infection and the role of viral factors in latency, their use in the dissection of the molecular mechanisms controlling latency is limited because of the complexity of the contribution of host factors and the immune system to latency, they are expensive and only a small proportion of neurons are induced to reactivate simultaneously. An *in vitro* latency system in which all of the latent genomes can be induced to reactivated

simultaneously is clearly desirable to enable characterisation of establishment, maintenance and reactivation of latency at the molecular level. Molecular interactions observed in *in vitro* systems should ideally be confirmed by experimentation *in vivo*.

1.3.4. *In vitro* models.

Studies on latency with animal models are hindered by the small amount of latently infected tissue available and by experimental inadequacies imposed by the use of animals. To facilitate studies on latency, *in vitro* models have been developed. An *in vitro* latency system should exhibit the following characteristics (1) Viral genomes should persist in cell nuclei in the absence of infectious virus. (2) Viral genomes should be nonlinear, as they are *in vivo*. (3) Transcription of the genomes should be repressed or at most limited to the LAT region. (4) Latent virus should be capable of reactivation to produce infectious virus.

In vitro latency systems can be divided into three types, those that use (1) primary cultures from the ganglia of rodents, (2) cell-lines of neuronal origin, or (3) non-neuronal tissue culture cells.

1.3.4.1. *In vitro* models using primary neuronal cultures.

The most significant *in vitro* system using primary neuronal cultures was developed by C.L.Wilcox and co-workers. Cervical ganglia from neonatal rats were treated with collagenase, dissociated onto a growth surface and treated with the mitotic inhibitor fluorodeoxyuridine to reduce the non-neuronal cell population to less than 5% of the total cell population (Wilcox and Johnson, 1987). The cultures were infected with HSV-1 and incubated in the presence of anti-HSV-1 antiserum to minimise the number of cultures destroyed by viral CPE. The presence of nerve growth factor (NGF) in the culture medium was neccesary not only for the survival of the neurons but also to maintain the virus in the latent state. Infection with 1 PFU per cell resulted in 63% of the cultures surviving the initial infection with 53% of the surviving cultures containing reactivatable virus. The application of ACG for 7 days after infection instead of anti-HSV-1 antiserum enabled MOIs of up to 5 PFU per cell to be used with 100% of the cultures surviving and 100% of the surviving cultures retaining reactivatable virus (Wilcox and Johnson, 1988). Viral antigens were not detected during the latent phase but appeared in all neurons within 24 hours after NGF depletion, indicating that all the neurons contained latent virus (Wilcox and Johnson, 1988). The 2.0 kb LAT and its 1.5 kb splicing derivative were the only viral transcripts detected during the latent phase (Doerig *et al.*, 1991b; Smith *et al.*,

1994). An antigen encoded by LAT ORF-2 (section 1.3.9.) was reported to be present during latent infection of the neuronal cultures (Doerig *et al.*, 1991a), but as identification of ORF-2 products *in vivo* and further characterisation in neuronal cultures has not been forthcoming, definite conclusions about the putative LAT translation products are unavailable. Probably the most significant information gathered from the system of Wilcox was that depletion of NGF from the culture medium, stimulation of cAMP-dependent second messenger pathways or activation of protein kinase C (PKC) resulted in reactivation (Wilcox and Johnson, 1987; Wilcox *et al.*, 1990; Smith *et al.*, 1992).

In a system developed by Wigdahl *et al.*, isolated rat foetal dorsal root ganglia were pretreated with IFNα and the replication inhibitor (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) for 1 day prior to infection with up to 2.5 PFU of HSV-1 per cell (Wigdahl *et al.*, 1983). The infected cultures were maintained for 7 days at 37° in the presence of the inhibitors, after which time the inhibitors were removed and the cultures shifted to 40.5°. The virus remained in an apparently latent state as long as the cultures remained at the elevated temperature. Reactivation was triggered by downshift to 37°. Southern blot analysis of DNA from the system of Wigdahl *et al.* revealed that the predominant population of viral genomes were linear (Wigdahl *et al.*, 1984a). Further structural and biochemical analysis of the system have not been forthcoming.

1.3.4.2. *In vitro* models using neuron-derived cell lines.

Clone C1300 mouse neuroblastoma cells are nonpermissive for HSV replication compared to other mouse cell-types (Vahlne and Lycke, 1978; Wheatley *et al.*, 1990). A C1300 clone highly resistant to HSV-1 was obtained by repeatedly exposing C1300 cells to progressively higher MOIs of virus (Nilheden *et al.* 1985a). The highly resistant clone was infected with 1 PFU of HSV-1 per cell and passaged in the presence of neutralising HSV-1 antibody without cell destruction (Nilheden *et al.*, 1985b). Reactivation of latent HSV-1 by superinfection with HSV-2 showed that the latent virus was diluted upon passage, indicating that the genomes were retained in a nonreplicating state (Nilheden *et al.*, 1985b). The restriction of HSV-1 in C1300 cells is caused by a reduction in viral IE transcription dependent on the TAATGARAT elements upstream of the IE genes (Kemp *et al.*, 1989, 1990). Transfection of plasmids containing octamer elements relieved the restriction of replication in C1300, indicating that the transfected binding sites were competing with the TAATGARAT elements in the viral genomes for a transcriptional repressor which bound to the viral TAATGARAT elements (Kemp *et al.*, 1990). The reduction of viral IE transcription in C1300 cells is probably caused by neuronal forms of Oct-2 binding to the TAATGARAT elements and preventing formation of the Vmw65-containing transactivation complex, as has been shown in other non-permissive cell lines

derived from neonatal rat dorsal root ganglia fused to C1300 cells (ND cells; section 1.3.5.; Lillycrop *et al.*, 1991; Wheatley *et al.*, 1991; Lillycrop and Latchman, 1992; Lillycrop *et al.*, 1994). After infection of ND3 cells with 1 PFU of HSV-1 per cell, Vmw175 was detected in only 1.5% of the cells (Wheatley *et al.*, 1990). Transcription from the five viral IE genes was not detected in nuclear run-on assays but transcripts antisense to Vmw110 mRNA, not formally identified as LAT, were present at high levels (Wheatley *et al.*, 1990). Infection of the neuron-derived cell lines which expressed high levels of Oct-2 led to a latent viral state which resembled latency in neurons *in vivo*. Cell lines such as ND3 could be used for *in vitro* studies on latency, however latency in ND cells has not been characterised in detail.

1.3.4.3. *In vitro* models using non-neuronal tissue culture cells.

O'Neill *et al.* examined the possibility of HSV-2 entering a latent state in cultured HFL cells when virus replication was inhibited with arabinofuranosyl cytosine (Ara-C; O'Neill *et al.*, 1972). HFL cells were pretreated with Ara-C for 24 hours, infected with HSV-2 and maintained at 37° for 7 days in the presence of Ara-C. After removal of the inhibitor, infectious virus was undetectable for a 5 to 6 day period, defined as the latent period. Latent HSV-2 was not eliminated by prolonged exposure to Ara-C, indicating that a latent rather than persistent state was occurring. After latency was established with 1 PFU of HSV-2 per cell, 0.025% of the cells produced infectious virus. Later it was found that the latent state could be maintained in the absence of Ara-C if the cultures were shifted to 40° after removal of the inhibitor (O'Neill, 1977). Latent HSV-2 was reactivated by downshift to 37° or by superinfection with HCMV (Colberg-Poley *et al.*, 1979). After superinfection with HCMV of cultures latently infected with 0.2 PFU of HSV-2 per cell, 1.6 % of the cells contained viral RNA and 0.3% of the cells produced infectious virus (Colberg-Poley *et al.*, 1981). *Ts* mutants of HCMV defective in DNA replication retained the ability to reactivate latent HSV-2 at the NPT, suggesting that an IE or E function of HCMV induced reactivation (Colberg-Poley *et al.*, 1981). A closely related system was used to establish latency of HSV-1 (Wigdahl *et al.*, 1981). Latent HSV-1 was reactivated by incubation at 37°, superinfection with HCMV or superinfection with *ts* mutants of HSV-2 (Wigdahl *et al.*, 1981, 1982a). The antiviral action of human leukocyte IFNα acted synergistically with the HSV-1-specific antiviral BVDU to enable high initial MOIs to be used without destruction of cell monolayers (Wigdahl *et al.*, 1982b). With an MOI of 2.5 PFU of HSV-1 per cell, 1-3% of the cells harboured at least one latent genome which was induced to reactivate by incubation at 37° or superinfection with HCMV (Wigdahl *et al.*, 1982b). HSV-1 latency was established in cultured rat or human sensory neurons using a similar technique to that used to establish latency in HFL cells (Wigdahl

et al., 1983, 1984b). Analysis of the HSV-1 genome during latency in HFL cells and rat sensory neurons revealed a copy number of 0.25-0.5 genomes per haploid cell genome equivalent in HFL cells and 8-10 genomes per haploid cell genome equivalent in neurons (Wigdahl *et al.*, 1984a). No alteration in size or molarity of the HSV-1 terminal or joint DNA fragments was detected compared to virion DNA, suggesting that the latent viral genomes were nonintegrated, linear and nonconcatemeric (Wigdahl *et al.*, 1984a). The efficiency of establishment of latency was increased to 1-5% of the cells containing reactivatable virus by use of ACG in combination with IFNα (Scheck *et al.*, 1986, 1987). Superinfection of cultures containing latent HSV-2 with HSV-1 in the presence of BVDU, or superinfection of latent HSV-1 with HCMV in the presence of various DNA replication inhibitors, did not lead to reactivation, thus at least a subset of early-late gene products was required for reactivation (Scheck *et al.*, 1987, 1989). During the latent phase, 2% of the cells contained HSV-1-specific RNA (Scheck *et al.*, 1989).

Russell and Preston developed an *in vitro* latency system which exploited the failure of HSV-2 to replicate at 42° (Russell and Preston, 1986). HFL cells were infected at 37° with up to 0.03 PFU of HSV-2 per cell, shifted to 42° and incubated for 6 days, after which time the cultures were returned to 37° (Russell and Preston, 1986; Preston and Russell, 1991). Upon downshift to 37°, the virus remained latent, as defined by the absence of viral TK activity, CPE, or infectious virus for up to 6 days (Russell and Preston, 1986; Russell *et al.*, 1987b; Russell, 1989; Preston and Russell, 1991). Almost all of the initial input inoculum was competent for reactivation by superinfection (Russell and Preston, 1986; Preston and Russell, 1991). When HSV-1 insertion and deletion mutants were tested for the ability to induce reactivation, the Vmw110 deletion mutant *dl*1403 did not induce reactivation (Russell and Preston, 1986; Russell *et al.*, 1987b) and, furthermore, it was demonstrated using recombinant adenoviruses that Vmw110 was the only HSV-1 protein required to induce reactivation (Harris *et al.*, 1989). The finding that Vmw110 caused reactivation was a revolutionary discovery that implicated Vmw110 in reactivation from latency *in vivo* and supported the validity of the *in vitro* latency system. Once established, the latent state was insensitive to activators of transcription other than Vmw110, such as Vmw175 or Vmw65, as demonstrated by the inability of *dl*1403 or UV-inactivated *ts*K to induce reactivation (Russell and Preston, 1986; Russell *et al.*, 1987b), thus the viral genomes were in a novel transcriptionally repressed state. In further support of the relevance of *in vitro* latency, and in contrast to the systems described above which used inhibitors to induce latency, the viral genomes were retained in a nonlinear configuration (Preston and Russell, 1991). Conversion to the nonlinear configuration during the establishment of latency in HFL cells was slow, being completed 4 days after infection. Virtually 100% of the input PFU of virus was capable of reactivation, corresponding to about 10% of the latently infected cells. Quantification of Southern blots revealed a viral genome copy number of 4.5 genomes per latently infected cell, thus a

proportion of the retained genomes were unable to reactivate. Latent virus was diluted with the cells upon subculture, hence viral DNA did not replicate with the cell DNA, and it was also noted that reactivation could not be induced by treatments which alter the cell metabolic state such as application of dimethyl sulphoxide (DMSO), phorbal 12-myristate 13-acetate (TPA) or ultraviolet (UV) light, thus the viral genomes were stable to alterations in the cell metabolic state (Russell and Preston, 1986). A small background of spontaneous reactivation was observed, suggesting that under certain conditions the latent genomes were induced to reactivate by cell factors or did not establish latency fully.

The system of Russell and Preston exhibits several characteristics which are advantageous over the inhibitor-induced systems. (1) The higher temperature of 42° negated the requirement for the use of chemical inhibitors. (2) The latent state was stable at 37°, whereas in the inhibitor-induced system reactivation occurred rapidly after downshift to 37°. (3) A greater proportion of cells harboured a reactivatable virus, 10% compared to 1-5% in the systems of Scheck *et al.* (Scheck *et al.*, 1986, 1987). (4) The latent, reactivatable genomes were nonlinear, in contrast to the inhibitor-induced system where the genomes were linear (Wigdahl *et al.*, 1984a). (5) Vmw110 was the sole requirement for reactivation, whereas reactivation from inhibitor-induced latency involved at least a subset of early-late gene products (Scheck *et al.*, 1987, 1989).

It was hypothesised that two populations of genomes exist in the inhibitor-induced systems, one which reactivates upon downshift to 37° and one which reactivates after superinfection with HCMV (Shiraki and Rapp, 1986). The number of cells that reactivated virus after temperature reduction decreased rapidly with time, whereas the number that reactivated by HCMV superinfection was steady (Shiraki and Rapp, 1986). Preston and Russell proposed that the latter represented virus latency in the 42°-induced system (Preston and Russell, 1991); such a proposal might indicate that latent viral genomes in the inhibitor-induced systems which are reactivated by superinfection are nonlinear, and that the 37°-reactivatable genomes are linear but present at such a high abundance as to swamp-out detection of the nonlinear genomes in Southern blots (Wigdahl *et al.*, 1984a).

The HSV-1 mutant *in*1814 has a 4 amino-acid insertion in Vmw65 which renders it unable to stimulate IE transcription but does not impair the structural role of the protein (Ace *et al.*, 1989). Transcription of the viral IE genes is reduced approximately 10-fold in cells infected with *in*1814, greatly reducing its cytotoxicity (Ace *et al.*, 1989). After infection of tissue culture cells at low MOI, *in*1814 exhibits a high particle / PFU ratio which is particularly high in HFL cells (Ace *et al.*, 1989). The particle / PFU ratio of *in*1814 in HFL cells was 1.7×10^5 particles per PFU compared to 11 particles per PFU for wild-type strain 17, thus after infection of cells with *in*1814, the majority of viruses did not form plaques. The mutation in *in*1814 did not alter the rate of entry of the viral

genomes into the cell nuclei or the stability of the viral genomes once they had reached the nuclei, hence the genomes were retained in the cell nuclei (Ace *et al.*, 1989).

The low infectivity and cytotoxicity of *in*1814 enabled establishment of latency in HFL cells after infection at an MOI of 5 particles per cell (Harris and Preston, 1991). *In*1814 established latency in HFL cells efficiently at 37° and was retained at 1-8 copies per cell in a nonlinear form. The presence of a viral replication inhibitor during latency was necessary to prevent replication and spread of the small background of nonlatent virus, and Ara-C was deemed a suitable inhibitor as it did not affect the reactivation ability or configuration of the latent genomes. Viral genes, as exemplified by TK and LAT, were repressed during latency in HFL cells, when examined by the highly sensitive polymerase chain reaction (PCR) assay (Anderson, 1991). Lack of LAT expression in HFL cells was deemed to be because neuronal transcription factors necessary for LAT transcription were absent in HFL cells (Anderson, 1991; Harris and Preston, 1991). It was of interest to note that incubation of cells infected with 1814R (the revertant of *in*1814) at 42° inhibited plaque formation by 1000-fold, compared to a 4-fold reduction on *in*1814, suggesting incubation at 42° might cause latency by disrupting transactivation by Vmw65 (Harris and Preston, 1991).

Several researchers have postulated that latency in neurons is caused by a block to IE transcription, possibly *via* a disruption of transactivation by Vmw65 (Roizman and Sears, 1987; Kristie and Roizman, 1988; Kemp *et al.*, 1990; Lillycrop *et al.*, 1991; Sears *et al.*, 1991; Roizman and Sears, 1993; Lillycrop *et al.*, 1994; Hagmann *et al.*, 1995). It is therefore possible that in tissue culture the mutation in *in*1814 mimics the disruption of transactivation in neurons, thereby leading to latency (Ace *et al.*, 1989; Harris and Preston, 1991).

The *in vitro* latency system using *in*1814 was limited to low initial MOIs because of residual cytotoxicity and IE transcription at high MOI (Harris and Preston, 1991). An *in vitro* system in which the viral DNA could be detected in Southern blots without previous banding in CsCl gradients was needed to facilitate molecular studies on the latent genomes, hence the *in vitro* system was modified by incorporating steps which further reduced cytotoxicity and IE transcription (Jamieson, 1993; Jamieson *et al.*, 1995). HFL cells were pretreated with IFNα, a treatment which is known to reduce HSV IE transcription (Mittnach *et al.*, 1988; Oberman and Panet, 1989; DeStasio and Taylor, 1990). In addition, *in*1814 was modified by replacing the promoter controlling transcription of IE1 with the MMLV LTR, yielding the virus named *in*1820. As the MMLV LTR is not active under IE conditions in HFL cells, *in*1820 behaves as if deleted for IE1 (Jamieson *et al.*, 1995). Mutants of Vmw110 have a similar phenotype to *in*1814 in cell culture (Stow and Stow, 1986; Everett, 1989), hence the plaque-initiation defect in *in*1814 is probably caused by lower expression of Vmw110 (Jamieson *et al.*, 1995).

Reduced expression of Vmw110 from *in*1820 leads to an exceptionally high particle / PFU ratio and low cytotoxicity in HFL cells, thus an increased proportion of virus-cell interactions lead to latency (Jamieson, 1993; Jamieson *et al.*, 1995). Latency was established by infecting IFNα-treated HFL cells with 1 PFU of *in*1820 per cell and incubating the cultures at 37° for 2 or 3 days in the presence of Ara-C.

Insertion of *lacZ* under the control of IE promoters into nonessential regions of the *in*1820 genome enabled the retention of the virus to be assessed quantitatively. An extrapolation of the number of blue cells formed after reactivation of cultured latently infected at low MOIs with the number of genomes detected in Southern blots in cultures infected at high MOI enabled the demonstration that most viral genomes were competent for reactivation (Jamieson *et al.*, 1995). This was a crucial finding because for the first time it could be stated with certainty that the genomes in an *in vitro* latency system detected in Southern blots were templates for reactivation and were thus biologically relevant.

After infection with 1 PFU per cell, nonlinear *in*1820 genomes were retained in HFL cells at high enough abundance to be detected in Southern blots without previous banding on CsCl gradients (Jamieson, 1993), as was required in previous *in vitro* latency systems using HFL cells (Harris and Preston, 1991; Preston and Russell, 1991), thus molecular studies on the structure of the latent genomes were facilitated by use of *in*1820.

Assessment of the fate of the input *in*1820 DNA during the course of latency showed that the quantity of intracellular nuclear viral genomes fell by 3-fold during the first 3 days of infection, but the nonlinear genomes remaining after 2 days infection were stable (Jamieson *et al.*, 1995). The input DNA not converted to the nonlinear form could not be detected in the cytoplasm or culture medium and presumably was degraded.

Regularly spaced nucleosomes could not be detected on the TK gene of latent *in*1820 genomes, as assayed by micrococcal nuclease (MN) digestion and Southern blotting, however the viral TK gene was bound by regularly spaced nucleosomes when in the cell genome, thus the TK gene does not have an intrinsic property which precludes the formation of regularly spaced nucleosomes during latency (Jamieson *et al.*, 1995). The nucleosome organisation of the genomes during latency in HFL cells appeared to differ fundamentally from the arrangement *in vivo* where the latent genomes are predominantly in a regular structure (Deshmane and Fraser, 1989). It was suggested that the absence of a regular nucleosome arrangement on genomes latent in HFL cells could be explained by the apparent inefficiency of reactivation from nervous tissue where the number of genomes detected in Southern blots far exceeds the number of neurons from which reactivation is induced, thus the chromatin-associated genomes may not be competent for reactivation and might be biologically irrelevant. The genomes in the *in vitro* system might represent the small proportion of genomes which can be reactivated *in vivo*.

*In*1820 genomes became more sensitive to MN as the virus proceeded to latency, as compared to shortly after infection and to a virus transcribing in the presence of Ara-C (Jamieson, 1993). It was proposed that the increase in sensitivity was due to structural changes which occurred as a result of entry into the nonlinear, latent state and that transcribing viruses do not have such changes conferred upon them.

1.3.5. Establishment.

The level of IE gene expression during the early stages of infection probably determines whether an infection proceeds to the lytic cascade observed in tissue culture cells or enters into the latent state (Roizman and Sears, 1987; Kristie and Roizman, 1988; Kemp *et al.*, 1990; Roizman and Sears, 1990; Lillycrop *et al.*, 1991; Sears *et al.*, 1991; Lillycrop *et al.*, 1994). When IE transcription is below a threshold level, the genome is converted to the latent state, whereas if IE transcription is above the threshold, viral replication ensues and presumably the neuron is killed, as invariably occurs during infection of tissue culture cells. The observation that there is no sensory loss at the site of recurrent lesions is difficult to reconcile with neuronal cell killing (Gominak *et al.*, 1990), thus it is disputable whether reactivation leads to the death of the neuron.

All virus mutants tested are capable of establishing latency, including *in*1814 which has a 4 amino-acid insertion in Vmw65 preventing transactivation of IE genes (Steiner *et al.*, 1990; Harris and Preston, 1991; Ecob-Prince *et al.*, 1993a, b), thus none of the virus gene products identified so far are required for establishment of latency. The apparent inability of TK, Vmw63, Vmw110 and Vmw175 mutants to establish latency was probably caused by defective replication, leading to lower inocula of virus reaching sensory ganglia and inefficient replication after reactivation, rather than inability to establish latency (Clements and Stow, 1989; Coen *et al.*, 1989; Efstathiou *et al.*, 1989; Leib *et al.*, 1989).

Latency can be established in non-neuronal tissue culture cells only after artificial manipulation (Wigdahl *et al.*, 1981; Russell and Preston, 1986; Harris and Preston, 1991; Jamieson *et al.*, 1995), thus, if the natural block is at the IE level, neurons must contain factors that inhibit viral IE transcription. Inhibition of IE transcription *in vivo* might be caused by neuronal forms of Oct-2 binding to the TAATGARAT elements upstream of the IE genes and repressing transcription, as has been observed in transformed cell lines of neuronal origin (Kemp *et al.*, 1990; Lillycrop *et al.*, 1991, 1993), or the viral transactivator Vmw65 may be lost during passage over the relatively long distance between the neuronal cell surface and the neuronal nucleus, so that when the viral genome enters the cell nucleus IE transcription is not stimulated by Vmw65 (Roizman and Sears, 1987).

HSV-1 transcription was repressed in neuron-derived cell lines in a manner dependent on the TAATGARAT elements (Kemp *et al.*, 1990). Repression of the HSV IE promoters was mediated by the homeobox protein Oct-2 binding to the TAATGARAT elements and inhibiting transactivation by Vmw65 (Lillycrop *et al.*, 1991, 1993). Oct-2 is present in B-cells and in neuronal cells, but the predominant forms in the two cell types differ due to alternative splicing of the transcript (Wirth *et al.*, 1991; Lillycrop and Latchman, 1992; Stoykova *et al.*, 1992). Although all forms can activate a simple octamer element (Wirth *et al.*, 1991), they differ in their effect in the context of the TAATGARAT elements in the viral genome (Lillycrop and Latchman, 1992). The B-cell form of Oct-2, named Oct-2.1, activates transcription, whereas the neuronal forms, Oct-2.4 and 2.5, cause repression (Lillycrop and Latchman, 1992). Recently it was reported that Oct-2 could not be detected in sensory neurons, but the factors required for Vmw65-mediated activation, Oct-1 and HCF, were detected, suggesting that repression by Oct-2 is not an important mechanism of IE gene repression *in vivo* (Hagmann *et al.*, 1995). Further clarification of the pattern of Oct-2 expression *in vivo* is required before conclusions can be made about its role in latency.

The Vmw65-induced complex formed by extracts of permissive HeLa, 3T3 cells or brain can be resolved into 3 distinct bands in DNA-protein complex electrophoretic mobility shift assays (Hagmann *et al.*, 1995). However, electrophoresis of complexes formed from extracts of sensory neurons revealed that the lower mobility complex was absent (Hagmann *et al.*, 1995). It was suggested that alternative processing of HCF or the absence of a fourth factor in sensory neurons could account for the absence of the lower mobility complex, and the different complex could be incapable of transactivation. It is of interest to determine the cause of the unusual Vmw65-induced complex in sensory neurons, and whether it is capable of transactivation.

If Vmw65 is lost over the distance between the neuronal cell surface and the cell nucleus, neurons infected *via* the periphery, where the distance travelled by the nucleocapsid is measured in centimetres, would be more likely to be latently infected than neurons infected from adjacent neurons, where the distance between the cell surface and the nucleus is a few micrometers. The theory that latency is caused by prevention of Vmw65 transactivation would explain why HSV IE genes are stimulated by a complex between cell factors and a component of the virion tegument rather than by enhancer elements dependent solely upon cellular transcription factors for high levels of expression (Roizman and Sears, 1993). An attempt was made to determine whether expression of Vmw65 causes reactivation or precludes the establishment of latency. The gene encoding Vmw65 was placed under control of the mouse metallothionein promoter and recombined into the HSV-1 genome (Sears *et al.*, 1991). The recombinant virus was inoculated into mice and Vmw65 expression induced at various stages of infection by administering $CdSO_4$. Induction of Vmw65 during latency did not cause reactivation of virus, neither

did induction at the time of inoculation. Similar results were obtained using transgenic mice containing the mouse metallothionein promoter controlling Vmw65. It was concluded that the absence of Vmw65 cannot alone account for the establishment of latency. The data of Sears *et al.* are somewhat confusing given the finding that the metallothionein promoter is not expressed during latency (Lokensgard *et al.*, 1994), and that $CdSO_4$ causes reactivation (Fawl and Roizman, 1993).

The HSV-1 IE genes show a degree of transcriptional activity even in the absence of Vmw65, as demonstrated by use of the HSV-1 mutant *in*1814 which has a 4 amino-acid insertion in Vmw65 preventing formation of the transactivation complex (Ace *et al.*, 1989). The defect in *in*1814 is overcome after infection at high MOI, presumably because of residual IE transcription, thus MOI might be an important determinant of the outcome of infection in neurons *in vivo*.

1.3.6. Maintenance.

The HSV-1 genome was retained as a nonlinear episome associated with nucleosomes during latency (Rock and Fraser, 1983, 1985; Efstathiou *et al.*, 1986; Mellerick and Fraser, 1987; Deshmane and Fraser, 1989). Latent DNA was stable over several months in mice (Efstathiou *et al.*, 1986), and presumably remains in humans until death. The lack of any free ends would prevent degradation by exonucleases, and association with nucleosomes might also prevent degradation. In *in vitro* latency systems, nonlinear DNA was preferentially retained and linear DNA not converted to the nonlinear form was degraded (Preston and Russell, 1991; Jamieson *et al.*, 1995). Thus nonlinearity and association with nucleosomes may contribute to DNA stablility during latency.

The number of viral genomes retained per latently infected neuron is unclear at present. In trigeminal ganglia harbouring latent virus, there is an estimated 0.1-1 genomes per cell (Puga *et al.*, 1978; Cabrera *et al.*, 1980; Efstathiou *et al.*, 1986; Rock and Fraser, 1986). This is the copy number for the total number of cells in a ganglion, but as neurons only constitute approximately 10% of the total number of cells in a ganglion, there is likely to be more than one genome per latently infected neuron. Only 3% of neurons in a latently infected ganglion express LAT (Rock *et al.*, 1987), thus if expression of LAT is taken as a marker for latent infection the number of genomes per latently infected neuron is likely to be much higher. However, data from studies using *in situ* PCR indicate that the number of latently infected neurons exceeds the number of neurons expressing levels of LAT detectable by *in situ* hybridisation (Gressens and Martin, 1994; Ramakrishnan *et al.*, 1994; Mehta *et al.*, 1995). Until recently, individual cells latently infected with HSV could only be identified *in situ* by hybridisation to LAT RNA or transcripts produced from reporter genes linked to the LAT promoter. Attempts to detect latent HSV DNA by

in situ hybridisation have failed, probably because of the low copy number of HSV genomes per latently infected cell, or perhaps because the latent genome is in a structure or compartment inaccessible to probes under current conditions of section preparation and hybridisation (Stevens, 1989). The inability to detect latent HSV DNA by *in situ* hybridisation left it unclear whether HSV can establish a latent infection without expressing LAT. In non-neuronal cells the neuron-specific LAT promoter is unlikely to be active during latency (Zwaagstra *et al.*, 1990; Anderson, 1991; Leib *et al.*, 1991; Zwaagstra *et al.*, 1991; Batchelor and O'Hare, 1992; Kenny *et al.*, 1994), and detection of latently infected cells in non-neuronal tissues *in situ* is therefore limited to the detection of HSV antigens or transcripts after explantation (Clements and Jamieson, 1989). The highly sensitive technique of *in situ* PCR is now being used to identify latently infected cells in ganglia irrespective of whether transcripts are detectable (Gressens and Martin, 1994; Ramakrishnan *et al.*, 1994; Mehta *et al.*, 1995). The assay of Mehta *et al.* was sensitive enough to detect a single copy cell gene in 50% of neurons (Mehta *et al.*, 1995). In three separate studies the number of neurons containing a viral genome detected by *in situ* PCR was greater than the number of LAT^+ neurons detected by *in situ* hybridisation suggesting that not every latently infected neuron contained LAT (Gressens and Martin, 1994; Ramakrishnan *et al.*, 1994; Mehta *et al.*, 1995). However, because of the sensitivity of *in situ* PCR and the relative insensitivity of *in situ* hybridisation, the discrepancy between HSV DNA^+ and LAT^+ neurons may have been caused by neurons containing HSV DNA detectable by *in situ* PCR but expressing levels of LAT undetectable by *in situ* hybridisation. Dual labelling *in situ* RT-PCR for the detection of LAT and *in situ* PCR for the detection HSV DNA will determine if neurons contain HSV DNA without expressing LAT. *In situ* PCR will facilitate an accurate assessment of the number of viral genomes per latently infected neuron, and in addition it will give greater insight into the sites of latency and enable detection of HSV DNA in ganglia infected with LAT^- mutants .

Sympathetic and sensory neurons are dependent for growth and survival upon NGF synthesised and released by the tissues which they innervate (Thoenen and Barde, 1980; Bandtlow *et al.*, 1987). After binding to the nerve cell surface, NGF and its receptor are internalised and transported to the nerve cell body leading to induction of expression of cell genes (Johnson *et al.*, 1987; Lindsay and Harmar, 1989). It has long been documented that damage to the nerve terminals of latently infected neurons causes reactivation of HSV (Cushing, 1905; Goodpasteur, 1929; Cook *et al.*, 1974; Walz *et al.*, 1974; Hill *et al.*, 1978; Tenser *et al.*, 1988), One possible mechanism by which damage to nerve terminals causes reactivation is by preventing NGF or possibly other factors released by the target tissues from reaching the neuronal cell bodies. The continued presence of NGF is necessary for maintenance of latency in sympathetic or sensory

neurons cultured *in vitro* (Wilcox and Johnson, 1987, 1988; Wilcox *et al.*, 1990). Depletion of NGF for a little as 1 hour resulted in reactivation from cultured ganglia (Wilcox *et al.*, 1990). The species-specific anti-human NGF receptor monoclonal antibody Mab-20.4 caused reactivation in human but not rat neuronal cultures, and in addition the monoclonal antibody Mab-192 which binds to the rat NGF receptor but does not block its function did not cause reactivation from rat ganglia. 6-hydroxydopamine, which is toxic for sympathetic nerve terminals, caused reactivation of HSV from latently infected cultures *via* a mechanism which was partially overcome by the addition of higher levels of NGF. When levels of 6-hydroxydopamine that cause destruction of the nerve terminals were applied, NGF did not overcome the effect, hence 6-hydroxydopamine appeared to reactivate latent virus by blocking the transport of NGF. Colchicine, which inhibits microtubule polymerisation leading to a blockage of axonal transport (Johnson *et al.*, 1987), also induced reactivation. Inhibition of translation for 1 hour was sufficient to cause reactivation, indicating that continually synthesised factors keep the viral genome in the latent state. Exposure of latently infected cultured ganglia to UV light failed to cause reactivation from latency, even at high doses that damaged the cells, however UV damaged cultures retained the ability to reactivate virus after NGF deprivation, further supporting the concept that reactivation occurred as a result of specific molecular events rather than because of the general state of the cells. In summary, NGF specifically causes the induction of factors necessary to maintain HSV in a latent state within cultured ganglia, and probably in ganglia *in vivo*. Reactivation by NGF deprivation acts *via* activation of one of several second-messenger pathways that lead to reactivation (Smith *et al.*, 1992).

Levels of Oct-2 were increased 3-4-fold in cultured rat sensory neurons in response to NGF stimulation, as measured by a DNA electrophoretic mobility shift assay (Wood *et al.*, 1992). The increase in Oct-2 DNA binding was paralleled by a 2-fold increase in Oct-2 mRNA. Levels of the POU proteins Oct-1 and Brn-3 and the non-POU transcription factor TFIIIC were unaffected by NGF, thus NGF appeared to cause a specific upregulation of Oct-2. At least one of the mechanisms by which NGF maintains HSV in the latent state might be to constantly upregulate levels of Oct-2 transcription. After tissue damage at the periphery, insufficient levels of NGF would reach the ganglia and hence Oct-2 levels would decrease below levels required to keep the latent genome repressed, thus viral IE transcription would ensue and the replicative cycle would begin.

1.3.7. Reactivation.

Latent HSV can be triggered to reactivate *in vivo* by fever, stress, sunlight, administration of toxic substance and local stimulation at the peripheral site innervated by

latently infected neurons. As discussed in section 1.3.6., NGF deprivation of neurons is one possible mechanism by which reactivation can be induced.

Vmw65 is not present during the initial stages of reactivation from latency, thus IE transcription is initiated by a change in the pool of cellular transcription factors within the latently infected neuron. The ability of *in*1814 to reactivate from latency after explantation clearly demonstrates that transactivation by Vmw65 is not necessary for reactivation (Steiner *et al.*, 1990; Ecob-Prince *et al.*, 1993a, b). Induced expression of Vmw65 by administration of $CdSO_4$ to latently infected transgenic mice containing the mouse metallothionein promoter controlling Vmw65 expression did not cause any difference in the recovery of virus (Sears *et al.*, 1991), and in addition the latent genomes in *in vitro* systems are also insensitive to Vmw65 (Russell and Preston, 1986; Harris and Preston, 1991).

Vmw110 was the sole requirement for reactivation in *in vitro* latency systems developed by Preston and co-workers (Russell *et al.*, 1987b; Harris *et al.*, 1989; Harris and Preston, 1991). In addition a Vmw110 deletion mutant entered a latent state in tissue culture in a way resembling *in vitro* latency of *in*1814 (Stow and Stow, 1989). A role for Vmw110 in reactivation *in vivo* is demonstrated by the inefficient capability of IE1 mutants to reactivate from latently infected ganglia, thus Vmw110 is not essential for reactivation from latency but facilitates this process (Clements and Stow, 1989; Leib *et al.*, 1989; Cai *et al.*, 1993). Initial studies examining the latency capabilities of Vmw110 mutants were complicated by the fact that LAT was mutated in addition to Vmw110, thus a phenotype could not be attributed unequivocally to the mutation in Vmw110 (Clements and Stow, 1989; Leib *et al.*, 1989). Insertion of IE1 and its control elements between U_L26 and U_L27 of a LAT$^-$ / Vmw110$^-$ mutant restored the replication and reactivation capability of the virus to half that of wild-type virus, demonstrating that Vmw110 plays a role in reactivation (Cai *et al.*, 1993). Vmw110 facilitates, but is not essential for reactivation, thus it seems likely that cell transcription factors can mimic its function. Evidence that Vmw110 represents a class of transcription factors which mimic its reactivation function is manifold. (1) Vmw110 mutants can reactivate from latency (Clements and Stow, 1989; Leib *et al.*, 1989). (2) The defect in *in*1814 varies during the cell cycle (Daksis and Preston, 1992). (3) The defect of Vmw110 mutants varies greatly between cell types (Stow and Stow, 1986; Everett, 1989; Yao and Schaffer, 1995). An osteosarcoma cell line appears to express a factor that substitutes functionally for Vmw110 (Yoa and Schaffer, 1995). The isolation and characterisation of such a factor will finally test the hypothesis that cell factors related to Vmw110 can reactivate HSV from latency.

A function encoded by the LAT region plays a role in facilitating reactivation (section 1.3.9.), the mechanism of action of the LAT on reactivation is unknown.

The compounds N,N'-hexamethylene-bis-acetamide (HMBA) and DMSO can facilitate reactivation in explanted cultures (section 1.3.8.2.; Harbour *et al.*, 1983; Whitby *et al.*, 1987; Bernstein and Kappes, 1988; Leib *et al.*, 1989). These compounds also overcome the defect of *in*1814 in tissue culture by stimulating transcription (section 1.4.; McFarlane *et al.*, 1992), thus one possible mechanism by which they facilitate reactivation is to substitute for the lack of Vmw65.

No viral gene product which is essential for the establishment of latency has yet been identified. However, any viral factor which is required for efficient replication in neurons plays an important role in the pathogenesis of HSV latency. The phenotype of TK mutants exemplifies the the role of virus replication in reactivation. TK is specifically required for replication in nondividing neuronal cells (Tenser and Dunstan, 1979; Coen *et al.*, 1989; Efstathiou *et al.*, 1989), thus during infection of laboratory animals with TK^- mutants, virus replication occurs at the periphery and virus reaches the innervating neurons. Replication does not occur in ganglia, however, due to the absence of TK, even though latency is established. Although activation of the genomes of latent TK^- mutants is likely to occur, the neurons are too nonpermissive for a productive infection to ensue resulting in the poor reactivation phenotype.

1.3.8. The structure of HSV genomes during latency.

1.3.8.1. Genome configuration.

The predominant population of HSV genomes in virus particles are linear DNA molecules (Poffenberger and Roizman, 1985; McGeoch, 1989). Several groups have endeavoured to determine the configuration of the genome during latency in neurons. There are several possible arrangements including linear, circular, linear concatemeric or circular concatememeric episomal DNA, or integration into the cell genome either as a single copy or a concatemer (figure 1.5.).

Puga *et al.* examined the sizes of the terminal restriction endonuclease fragments of HSV-1 during latency in mouse trigeminal ganglia (Puga *et al.*, 1984). *Eco*RI fragments fractionated by reverse phase column chromatography and subjected to Southern blotting revealed the presence of several sizes of terminal restriction fragments suggesting extensive genome rearrangement or integration into the host DNA at a limited number of integration sites.

Analysis of HSV-1 DNA in human brain tissue by Southern blotting revealed that in some cases the entire genome was present and in others only part of the genome was present (Fraser *et al.*, 1981). Terminal restriction fragments could be detected in some

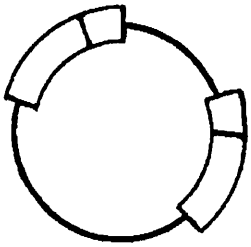
Figure 1.5. Possible configurations of HSV genomes during latency. The predominant population of genomes in HSV virions are linear (Poffenberger and Roizman, 1985; McGeoch, 1989). During latency, the joint *Bam*HI restriction fragments are present at double the molarity of the unique fragments (Rock and Fraser, 1985; Efstathiou *et al.*, 1986), indicating that the ends are joined together. As the latent genomes are episomal (Mellerick and Fraser, 1987), the most likely configurations are single or concatemeric circular molecules. Adapted from Anderson (1991).

UNIT LENGTH :

1. Linear

ENDLESS :

2. Circular



3. Concatemeric

4. Integrated, concatemeric

5. Integrated, linear

cases, suggesting the DNA was in a linear non-integrated form. In contrast Rock and Fraser detected most, if not all of the genome in mouse brains, but terminal restriction fragments were not detected implying that the genomes were not linear unit length molecules (Rock and Fraser, 1983). The molar ratio of restriction fragments from the joint region : unique fragments of HSV-1 in murine brains was determined by densitometric analysis of Southern blots (Rock and Fraser, 1985). The ratio of joint fragments to unique fragments was 1 : 1 for virion DNA, 1.6 : 1 during the acute phase of infection and 2 : 1 during latency in trigeminal ganglia. The ratio of 2 : 1 suggests a circular, concatemeric, integrated concatemeric or single genomes integrated *via* the unique sequences of the genome.

Efstathiou *et al.* showed that the HSV-1 genome was present in human trigeminal ganglia in an endless form (Efstathiou *et al.*, 1986). All four isomers were detected in murine brainstems and the genome was stably retained over a four month period. The genomic termini were detected in mouse brainstems during the acute phase of infection.

The HSV-1 genome was separated from the chromosomal DNA of latently infected mouse brains by CsCl buoyant density gradient centrifugation (Mellerick and Fraser, 1987). CsCl buoyant density gradient centrifugation separates DNA molecules on the basis of differences in their G+C contents. EBV DNA was not separated from cell DNA from a cell line known to harbour an integrated EBV genome, thus the HSV-1 genome was existing in mouse brains in an unintegrated form.

It is now generally accepted that the HSV genome is an endless episomal molecule during latency, although it remains unclear whether the genomes are single circular molecules or concatemers. The data of Fraser *et al.* and Puga *et al.* are difficult to explain (Fraser *et al.*, 1981; Puga *et al.*, 1984), however, linear DNA in their latently infected samples may have been caused if reactivation had occurred in the latently infected tissues. Conversion of the virus genome to the nonlinear configuration occurs during the early stages of lytic infection in tissue culture cells and is caused by pre-existing factors (Poffenberger and Roizman, 1985). During acute infection there is a mixture of endless concatemeric genomes present as replicative intermediates, and linear genomes packaged in nucleocapsids.

The retention of nonlinear HSV DNA in *in vitro* latency models has been reported by Preston and co-workers (Harris and Preston, 1991; Preston and Russell, 1991; Jamieson *et al.*, 1995) and this is in contrast to the apparently related systems of Wigdahl *et al.*, in which the viral DNA was linear (Wigdahl *et al.*, 1984a). In light of the finding that the HSV genome is nonlinear during latency in neurons, an *in vitro* latency system representing true latency should contain nonlinear rather than linear DNA.

1.3.8.2. Methylation.

Methylation of DNA plays an important role in the regulation of gene transcription, methylation usually correlating with gene inactivity (Razin and Cedar, 1991). Numerous studies have sought to identify whether methylation of HSV DNA is a mechanism by which transcription of the genome is regulated during latency. Chemicals known to cause gross demethylation of the cellular genome, such as 5-azacytidine (Jones and Taylor, 1980), HMBA, DMSO and L-ethionine (Christman *et al.*, 1980), have been utilised to investigate the effect of DNA demethylation on reactivation of HSV from latency (Harbour *et al.*, 1983; Whitby *et al.*, 1987; Bernstein and Kappes, 1988; Leib *et al.*, 1989). Application of DMSO to the ears of mice latently infected with HSV-1 after inoculation at that site caused reactivation in innervating cervical ganglia (Harbour *et al.*, 1983). 5-azacytidine and L-ethionine increased the incidence of reactivation of HSV-1 when added to the culture medium of cultured, dissociated latently infected mouse ganglia, and the presence of DMSO and 5-azacytidine caused earlier detection of virus (Whitby *et al.*, 1987). HSV-2 was recovered earlier and from a greater percentage of latently infected guinea pig dorsal root ganglion and external genital skin cultures when HMBA was present in the culture medium (Bernstein and Kappes, 1988). The failure of an HSV-1 Vmw110 mutant to reactivate from cultured murine ganglia was circumvented by DMSO (Leib *et al.*, 1989). It is probable that these hypomethylating substances have additional effects on the cell besides demethylation of DNA (Tamame *et al.*, 1983; Schafer and Priest, 1984). Reactivation of HSV could be caused by a change in regulation of cell functions rather than demethylation of viral DNA. HMBA overcomes the defect in the HSV-1 mutant *in*1814 if applied early after infection, whereas 5-azacytidine does not (McFarlane *et al.*, 1992). The effect of HMBA on *in*1814 appears not to be *via* a mechanism involving demethylation of viral DNA, but by a direct stimulation of IE transcription (McFarlane *et al.*, 1992; McFarlane, 1993), supporting the view that hypomethylating agents may facilitate reactivation by mechanisms not involving demethylation of viral DNA.

During the latent phase of infection in an *in vitro* latency system which utilised a persistently infected lymphoblastoid T-cell line, the HSV-1 genome was extensively methylated (Youssoufian *et al.*, 1982), this is in contrast to the productive phase when the genome was not methylated. Methylated CG residues were not detected in the *in vitro* latency system of Jamieson *et al.* when a *lacZ* gene insertion in the viral genome was examined (Jamieson *et al.*, 1995). Cleavage of DNA extracted from murine brainstems with restriction enzymes which fail to cut DNA at methylated sites revealed that the HSV-1 genome was not extensively methylated during latency in neurons *in vivo* (Dressler *et al.*, 1987). The three enzymes used by Dressler *et al.* were estimated to cut at 3% of the total CG residues of the viral genome, some of which were at sites likely to be

preferentially methylated to control transcriptional activity. Methylation at all sites was not excluded and the resolution was limited due to the low copy number of viral genomes in neuronal tissue. DNA methylation is unlikely to cause the gross transcriptional repression of the HSV genome during latency in neurons.

Deamination of 5-methylcytosine in CG dinucleotides produces TG or CA. Lymphotropic herpesviruses (EBV and HVS) are deficient in CG and contain excess TG and CA, possibly as the result of continuous methylation of their genomes (Honess *et al.*, 1989). Neurotropic herpesviruses (HSV-1, HSV-2 and VZV) and HCMV contain the expected number of CG dinucleotides predicted from the mononucleotide compositions, implying that methylation is not important for transcriptional regulation of these viruses, at least at the gross level. Retrospective sequence analysis is in agreement with the data of Dressler *et al.*, indicating that methylation of CG residues is not an important mechanism of gene silencing in HSV (Dressler *et al.*, 1987; Honess *et al.*, 1989). The herpesviruses predicted to be regulated by methylation, such as EBV and HVS, establish latency in dividing cells where efficient *de novo* methylation is possible, whereas *de novo* methylation in non-dividing neurons is likely to be inefficient making methylation an unlikely cause of transcriptional regulation of HSV during latency (Honess *et al.*, 1989).

1.3.8.3. Nucleosome organisation.

Eukaryotic cell DNA is wound around nucleosomes (Kornberg and Lorch, 1992). Regions of DNA organised with nucleosomes can be identified by the use of deoxyribonucleases such as MN or staphylococcal nuclease (SN) which cut between nucleosomes but are incapable of cleaving DNA wound around nucleosome cores. Partial digestion of chromatin with MN or SN yields a series of DNA fragments with sizes multiples of approximately 200 bp, thus digestion with MN and SN has proved a useful tool for studying nucleosomes binding to viral DNA. The association of viral genomes with nucleosomes has also been detected by electron microscopic examination of virus nucleoprotein complexes extracted from infected cells (Griffith, 1975; Pignatti and Cassai, 1980) and by electrophoretic analysis of polypeptides associated with virus nucleoprotein extracts (Pignatti and Cassai, 1980).

The genomes of polyoma virus and SV40 were organised in nucleosomes during lytic infection (Griffith, 1975). During productive infection in adenovirus infected cells, parental viral genomes were bound by nucleosomes but progeny DNA was non-nucleosomal (Daniell *et al.*, 1981). The EBV genome was associated with nucleosomes in non-producing cell lines, but after reactivation from latency and in productive cell lines only a small proportion was associated with nucleosomes (Shaw *et al.*, 1979). Studies on the HSV genome during productive infection have shown that nucleosomes were

undetectable (Mouttet *et al.*, 1979; Pagnatti and Cassai, 1980; Lentine and Bachenheimer, 1990) or as with EBV, most DNA was non-nucleosomal with a small background of nucleosome-associated DNA (Leinbach and Summers, 1980; Hall *et al.*, 1982; Muggeridge and Fraser, 1986). The HSV-1 genome was associated with nucleosomes in a chromatin structure during latency in the brainstems of mice (Deshmane and Fraser, 1989). All regions of the latent HSV-1 genome examined, including the region transcriptionally active during latency, were in a structure indistinguishable from cell chromatin when analysed by MN digestion. The presence of nucleosomes may be required for long term stability of the HSV genome during latency (Efstathiou *et al.*, 1986; Deshmane and Fraser, 1989). The fact that nucleosomes are present on the latent HSV-1 genome but are absent during lytic infection makes it feasible that nucleosomes contribute to silencing the genome during latency. However, a number of promoters which are active in the cell genome do not remain active in the viral genome during long term latency both *in vivo* (Lokensgard *et al.*, 1994) and in *in vitro* latency systems (Smith *et al.*, 1994; Jamieson *et al.*, 1995), thus transcriptional silencing during latency may involve more than nucleosome-mediated repression.

1.3.8.4. Transcriptional repression.

Gene repression can be caused by the binding of repressor proteins to specific DNA sequences (Herschbach and Johnson, 1993). It has been proposed that during the establishment of latency IE transcription is repressed by neuronal forms of the octamer-binding transcription factor Oct-2 binding to the viral TAATGARAT elements and preventing *trans*-activation by Vmw65 (Lillycrop *et al.*, 1991; Wood *et al.*, 1992). Oct-2 is upregulated in NGF treated neurons, whereas Oct-1 is unaffected (Wood *et al.*, 1992). As NGF is necessary for the maintenance of latency, maintenance of Oct-2 levels may provide a mechanism for the action of NGF, inferring that Oct-2 remains bound to the genome during latency.

A novel regulatory sequence in the long repeat region of the HSV genome distinct from the LAT core promoter can drive expression of LAT or reporter genes over long distances during latency and thus contains an element which prevents promoter inactivation (section 1.3.9.; Dobson *et al.*, 1990; Lokensgard *et al.*, 1994). Fascinatingly, the MMLV LTR contains an element which is at least partially functionally equivalent to the HSV element and can drive long-term expression during latency when placed downstream of the neuron-specific LAT core promoter at the gC locus (Lokensgard *et al.*, 1994) or when placed by itself as far as 500 bp downstream of the LAT polyadenylation signal (Dobson *et al.*, 1990), but when placed alone at the gC locus is repressed (Lokensgard *et al.*, 1994). The HSV-specified element responsible for overcoming

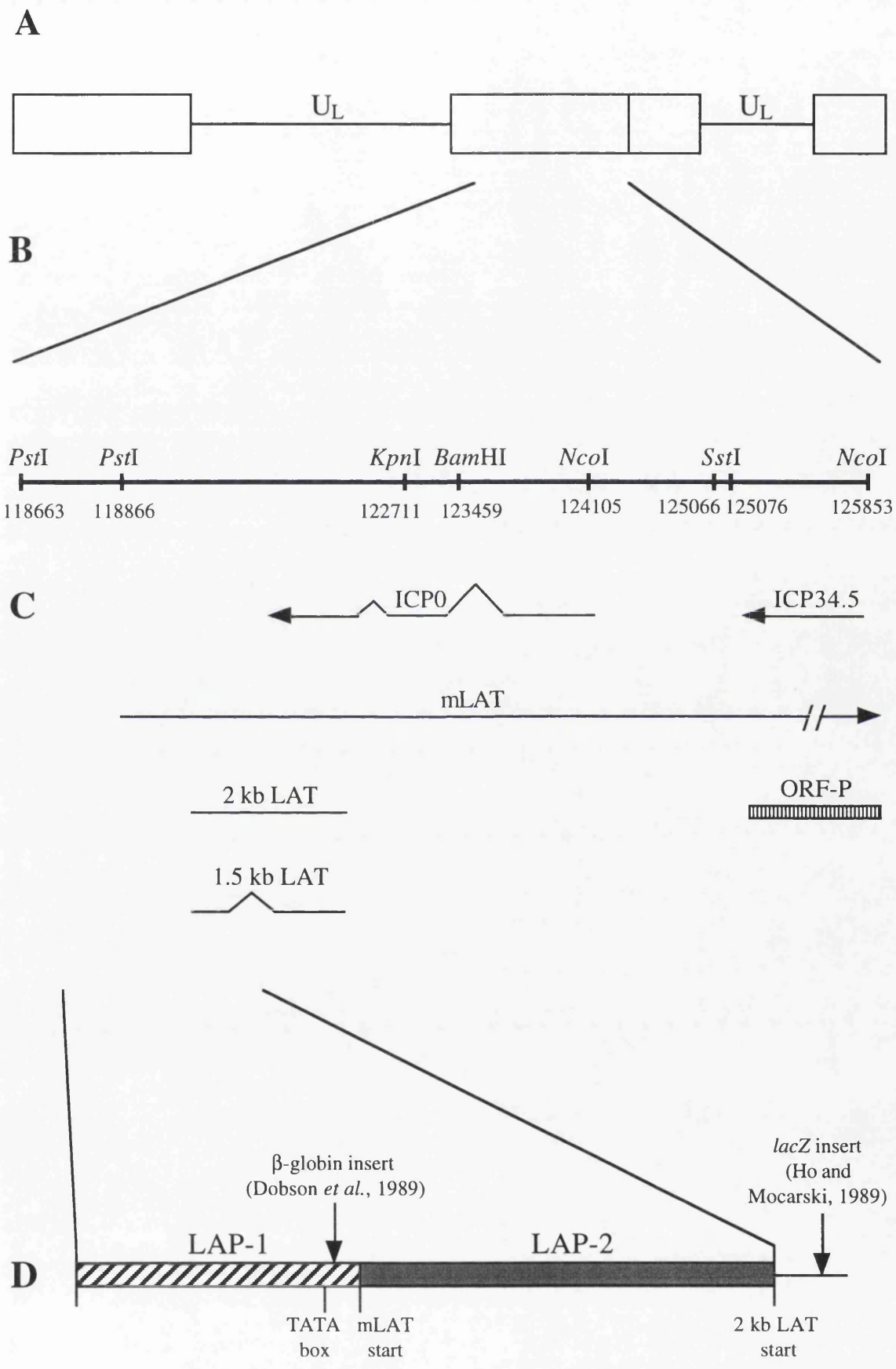
repression of the genome and its precise location within the HSV long repeat region remains to be characterised, although evidence from various studies suggests that it is located downstream of the LAT cap site (Lokensgard *et al.*, 1994). The effects on DNA structure of the element responsible for maintaining LAT transcription might give clues to the mechanism of repression of the viral genome during latency.

The location of the HSV genome within the nucleus might play a crucial role in latency. Recently, HSV-1 DNA was found to co-localise with nuclear structure known as ND10 domains during the early stages of lytic infection (Maul *et al.*, abstract 133, 20th International Herpesvirus Workshop, Groningen, The Netherlands, 1995; R.D. Everett, personal communication). The function of ND10 domains is unknown. One component of ND10, named PML, contains a RING finger domain similar to that of Vmw110 (Goddard *et al.*, 1991). In promyelocytic leukaemia cells a chromosomal translocation fuses the RING finger of PML with the retinoic acid receptor, inferring that PML and other components of ND10 play a role in cell differentiation (Kakizuka *et al.*, 1991). Treatments which cause reactivation of HSV, such as hyperthermia (Sawtell and Thompson, 1992a), administration of $CdSO_4$ (Fawl and Roizman, 1993) or expression of Vmw110 (Harris *et al.*, 1989; Harris and Preston, 1991; Cai *et al.*, 1993; Minigawa *et al.*, 1994) all cause disruption of ND10 (Maul *et al.*, 1993; Maul, 1995; Maul *et al.*, abstract 133, 20th International Herpesvirus Workshop, Groningen, The Netherlands, 1995) whereas treatment of cells with the IFNs, which inhibit the onset of lytic infection, causes an increase in the number of ND10 domains (Guldner *et al.*, 1992; Korioth *et al.*, 1995). It appears that disruption of ND10 is required for efficient lytic infection and that factors present in ND10 and their interaction with Vmw110 might play an important role in latency and reactivation. It is of interest to know if HSV DNA is located in ND10 domains during latency.

1.3.9. Viral transcription during latency.

In early studies HSV transcripts were detected in latently infected neurons (Galloway *et al.*, 1979, 1982; Stroop *et al.*, 1984). Detailed analysis of transcription using *in situ* hybridisation and northern blotting revealed that the only detectable transcripts arose from around IE1 and were antisense to Vmw110 mRNA (Croen *et al.*, 1987; Deatly *et al.*, 1987; Spivack and Fraser, 1987; Stevens *et al.*, 1987; Gordon *et al.*, 1988; Krause *et al.*, 1988; Steiner *et al.*, 1988; Stevens *et al.*, 1988). The most abundant latency-associated transcript (LAT) was around 2 kb in size, and a smaller 1.5 kb molecule was also observed at about 50% abundance to the 2 kb LAT (Rock *et al.*, 1987; Spivack and Fraser, 1987; Stevens *et al.*, 1987; Krause *et al.*, 1988; Wagner *et al.*, 1988a, b). Both the 2 kb LAT and the 1.5 kb LAT overlapped and were antisense to about 750 residues of

Figure 1.6. Transcription and regulatory elements of the LAT region. (A) Depiction of the HSV-1 genome. (B) The internal copy of the long repeat region is expanded showing the restriction endonuclease sites relevant to the studies in the thesis presented here. (C) Transcripts arising from the LAT region and their directions of transcription. The 2 kb LAT is believed to be a splicing product from the mLAT, however the spliced mLAT exon has not been detected and recent evidence suggests that the 2 kb LAT may also arise independently of the mLAT *via* LAP-2-mediated transcription (Chen *et al.*, 1995). The position of ORF-P is also indicated (Bohenzky *et al.*, 1993; Lagunoff and Roizman, 1994, 1995). (D) Two regulatory elements controlling LAT transcription, LAP-1 and LAP-2. LAP-1 mediates LAT transcription during latency and LAP-2 is important for transcription during lytic infection. The positions of the β-globin insertion downstream of the LAT TATA box in the recombinant virus made by Dobson *et al.* (1989) and the *lacZ* insertion downstream of LAP-2 in the recombinant virus made by Ho and Mocarski (1989) are indicated.


A
U_L
U_L
B
*Pst*I
*Pst*I
*Kpn*I
*Bam*HI
*Nco*I
*Sst*I
*Nco*I
118663
118866
122711
123459
124105
125066
125076
125853
C
ICP0
ICP34.5
mLAT
2 kb LAT
ORF-P
1.5 kb LAT
D
β-globin insert
(Dobson *et al.*, 1989)
lacZ insert
(Ho and
Mocarski, 1989)
LAP-1
LAP-2
TATA
box
mLAT
start
2 kb LAT
start

the 3' end of Vmw110 mRNA (figure 1.6.; Rock *et al.*, 1987; Spivack and Fraser, 1987; Stevens *et al.*, 1987; Krause *et al.*, 1988; Wagner *et al.*, 1988a, b; Spivack *et al.*, 1991). The LATs were largely confined to the nuclei of neurons and were poly(A)$^-$ (Rock *et al.*, 1987; Stevens *et al.*, 1987; Wagner *et al.*, 1988a). The transcription pattern was similar during latency in humans (Krause *et al.*, 1988; Steiner *et al.*, 1988), mice (Spivack and Fraser, 1987; Stevens *et al.*, 1987) and rabbits (Rock *et al.*, 1987). In one study virus strain and host species affected the relative abundance of the 2 kb and 1.5 kb LATs (Wagner *et al.*, 1988b). During lytic infection in tissue culture or acute infection of animal brains the 2 kb LAT was much less abundant than during latency and the 1.5 kb LAT was undetectable (Spivack and Fraser, 1987; Krause *et al.*, 1988; Steiner *et al.*, 1988; Wagner *et al.*, 1988b).

Cloning of partial LAT cDNAs from latently infected trigeminal ganglia enabled the identification of a 550 nucleotide sequence spliced from the 2 kb LAT forming the 1.5 kb LAT (Spivack *et al.*, 1991). Interestingly, the splice signals forming the 1.5 kb LAT differ from the usual eukaryotic splice signal in that the 5' end of the LAT intron is GC instead of GT. It was suggested that the unusual signal accounted for the neuronal specificity of the splicing reaction and acted inefficiently during latency, causing the greater abundance of the 2 kb LAT over the 1.5 kb LAT. Removal of the intron from the 2 kb LAT may enable efficient translation of putative downstream ORFs: such ORFs can be translated from denatured RNA *in vitro* but their translation products have yet to be demonstrated *in vivo*.

Several groups have reported hybridisation in latently infected ganglia with probes from regions immediately adjacent to both the 5' and 3' ends of the 2 kb LAT, and these transcripts were named the minor hybridising LAT (mLAT; Krause *et al.*, 1988; Wagner *et al.*, 1988a; Mitchell *et al.*, 1990a). The mLAT was detected by *in situ* hybridisation but was present at levels undetectable in northern blots, appearing to be far less stable than the major abundance LATs (Krause *et al.*, 1988; Wagner *et al.*, 1988a; Mitchell *et al.*, 1990a). A TATA box-containing promoter located 700 bp upstream of the 5' end of the LAT was identified by its ability to drive expression of heterologous genes during latency (Dobson *et al.*, 1989), lytic infection (Dobson *et al.*, 1989), and in transfection assays (Zwaagstra *et al.*, 1989; Batchelor and O'Hare, 1990; Zwaagstra *et al.*, 1990). Deletion of the promoter 700 bp upstream of the LAT from the HSV genome abolished expression of the major LAT and the mLAT (Javier *et al.*, 1988; Dobson *et al.*, 1989; Izumi *et al.*, 1989; Mitchell *et al.*, 1990b). Transcription from the LAT promoter terminated at the first polyadenylation signal located 8.77 kb from the TATA box, thus the mLAT was colinear with and overlapped the LAT, antisense to IE1 and ICP34.5, and was poly(A)$^+$ (Dobson *et al.*, 1989; Devi-Rao *et al.*, 1991). It seemed likely that the mLAT was spliced to produce the abundant LAT, however the 6.77 kb derivative of such a splicing event was not detected, presumably due to instability (Dobson *et al.*, 1989; Devi-Rao *et al.*, 1991).

When a clone of LAT DNA was inserted into the *E.coli lacZ* gene within a replicating eukaryotic expression vector, resulting transcription gave rise to high levels of LAT and relatively lower levels of spliced β-galactosidase mRNA (Farrell *et al.*, 1991). Sequencing of the spliced product revealed that splicing had occurred at the consensus donor and acceptor sites. The LAT appeared to be an unusually stable intron that itself contained an intron.

The regulatory region upstream of the mLAT transcription start site is a functional transcriptional promoter containing the usual consensus RNA polymerase II elements, including a TATA box. Elements controlling transcription from the promoter upstream of the mLAT include several Sp1 sites (Batchelor and O'Hare, 1992), two cAMP response elements (Leib *et al.*, 1991; Kenny *et al.*, 1994), a CAAT homology (Batchelor and O'Hare, 1992), regions which confer neuronal specificity (Zwaagstra *et al.*, 1990, 1991; Batchelor and O'Hare, 1992), a binding site for an activator protein termed the LAT promoter binding factor (LPBF; Zwaagstra *et al.*, 1991), and a repressive Vmw175 binding site located on the transcription start site (Batchelor and O'Hare, 1990; Batchelor *et al.*, 1994). The element regulating LAT expression exhibits two crucial characteristics, firstly it is capable of overcoming the transcriptional repression which occurs on the rest of the genome, and secondly it has neuronal specificity. Fascinatingly, the LAT TATA-containing promoter and upstream sequences were only capable of driving expression during the early stages of latency but were unable to drive long-term transcription when placed away from the LAT region at the gC locus (Lokensgard *et al.*, 1994). The MMLV LTR was unable to drive long-term expression when placed at the gC locus by itself, but when paired with the upstream LAT promoter long-term transcription initiated within the LTR (Lokensgard *et al.*, 1994). The MMLV LTR was thus able to overcome repression of the genome during latency and the upstream LAT promoter provided the required neuronal transcription factor binding sites. The LAT region of the HSV-1 genome is thought to contain a DNA element functionally equivalent to the MMLV LTR. The following evidence suggests that the region of the HSV-1 genome capable of overcoming repression is located between the LAT start site and the start of the 2 kb LAT: 1) A virus with the rabbit β-globin gene immediately downstream of the LAT TATA box was able to express rabbit β-globin during latency (Dobson *et al.*, 1989). 2) A virus with the *lacZ* gene downstream of the LAT TATA box with 1.5 kbp of the downstream sequences deleted was unable to give long term expression (Margolis *et al.* 1992), as the entire sequences upstream of the LAT TATA box were present the long-term expression element cannot be upstream of the TATA box. 3) The MMLV LTR remained active during latency when placed by itself 500 bp downstream of the LAT polyadenylation signal (Dobson *et al.*, 1990) but was not active when placed by itself at a distance from the LAT region at the gC locus (Lokensgard *et al.*, 1994). Clearly proximity to the LAT region conferred activity on the MMLV LTR. The region between the mLAT start site and the 2 kb LAT

start site is a novel TATA-less promoter active during latency in mouse ganglia and in transfection assays (Goins *et al.*, 1994).

The novel latency-active promoter downstream of the mLAT start site and upstream of the 2 kb LAT was named the latency-active promoter-2 (LAP-2) and the TATA-containing upstream promoter named LAP-1 (figure1.6.; Goins *et al.*, 1994). LAP-2 contains elements common to eukaryotic housekeeping gene promoters, was downregulated by Vmw175 and Vmw110 and was 5-10 fold less active than LAP-1 in transfection assays (Goins *et al.*, 1994). Transcription from LAP-2 started at or near the 5' end of the 2 kb LAT, implying that the 2 kb LAT can be transcribed as a single unit independent of LAP-1 and the mLAT (Goins *et al.*, 1994). Previous studies have supported transcription initiation at the start of the 2 kb LAT: Ho and Mocarski inserted *lacZ* at +137 relative to the start of the 2 kb LAT and found that mRNA initiated near the 5' end of the stable LATs rather than at the LAT TATA box (Ho and Mocarski, 1989). The splice sites forming the 2 kb LAT in the clones described by Spivack *et al.* did not correlate with consensus splice site usage in that the 5' end was one nucleotide removed from the proposed splice donor sequence and the 3' end mapped 79 bp upstream of the splice acceptor site (Spivack *et al.*, 1991). Recent data suggests both LAP-1 and LAP-2 are required for maximal LAT expression during latency (Chen *et al.*, 1995). LAP-2 contains the elements causing expression during lytic infection, whereas LAP-1 did not contribute to expression during lytic infection. LAP-1 was the element most influential on 2 kb LAT during latency. LAP-1 and LAP-2 may be independent elements required for expression of LAT at different stages of infection. It is of interest that LAP-1 remained active during latency in the absence of LAP-2, therefore the element within the LAT region proposed by Feldman to drive long-term expression (Lokensgard *et al.*, 1994), must be downstream of the region designated as LAP-2 by Glorioso (Goins *et al.*, 1994). Clearly LAP-1 and the long-term expression element act in concert to enable long-term transcription during latency. Further analysis is required to identify and characterise the long-term expression element in HSV functionally equivalent to the MMLV LTR, and to determine how LAT expression and splicing is regulated during lytic and latent infection.

Several ORFs have been identified in the LAT DNA sequence, however antibodies raised against synthetic peptides from the predicted proteins have never demonstrated the presence of a LAT-encoded protein *in vivo* (Wagner *et al.*, 1988a; Wechsler *et al.*, 1989). Doerig *et al.* reported a LAT antigen expressed in the *in vitro* latency system of Wilcox and co-workers utilising primary neuronal cultures from foetal dorsal-root ganglia treated with ACG (Doerig *et al.*, 1991a). Antibodies were raised against a bacterially expressed fusion protein consisting of part of the ORF designated by Wagner *et al.* as ORF-2 (Wagner *et al.*, 1988a). The antibody stained latently infected cultures, but although the predicted size of the protein was 33 kDa, the antibody recognised an 80 kDa protein. The antigen observed by Doerig *et al.* has never been identified *in vivo* and until such an

identification is made should be considered an unfortunate artefact of their experimental system.

Two LAT ORFs are conserved between HSV-1 strains F, 17(syn$^+$) and KOS, making it feasible that they code for functional proteins (Wechsler *et al.*, 1989). Translation of one ORF has been induced *in vitro*, although translation occurred only after the transcripts had been heat denatured (Spivack *et al.*, 1991). Translation of the two conserved LAT ORFs would be inhibited by the secondary structure and AUGs present in the intron of the 2 kb LAT. It was proposed that splicing to produce the 1.5 kb LAT regulates translation of the LAT ORFs enabling translation during latency, an attractive hypothesis because the 1.5 kb LAT has only been demonstrated during latency and is absent during lytic infection.

LAT sequences of HSV-1 and HSV-2 are dissimilar except for the promoters and regions overlapping IE1 (McGeoch *et al.*, 1991). Previously identified ORFs are not conserved between HSV-1 and HSV-2 and both virus strains show no signs of the three nucleotide bias in nucleotide composition characteristic of HSV-1 coding sequences. Sequence comparison between the LAT regions of HSV-1 and HSV-2 provided evidence against a LAT-encoded protein.

The inability to detect a LAT-encoded protein, the nuclear localisation and lack of a poly(A) tail of the LAT and the lack of any ORFs conserved between HSV-1 and HSV-2 argues against a protein encoded by the major abundance LATs, however a LAT-encoded protein might be expressed transiently at a specific stage of infection such as during the early stages of establishment of or reactivation from latency, hence the protein would be undetectable during latency. The possibility of translation from an as yet undetected RNA or ORF cannot be discounted.

Lagunoff *et al.* identified 16 potential ORFs predicted to encode 50 or more codons within the mLAT and examined 5 of the ORFs by in-frame insertion of an epitope from an HCMV-encoded protein (Lagunoff *et al.*, 1994). One ORF designated ORF-P (figure 1.6.), was detected in extracts from lytically infected tissue culture cells using antibodies specific for the HCMV epitope. Insertion of a second in-frame epitope into ORF-P resulted in the detection of a slower migrating protein, confirming the authenticity of ORF-P. In addition, another group has detected an RNA transcript arising from ORF-P, and have identified its promoter (Bohenzky *et al.*, 1993). ORF-P is antisense to and comprises almost all of the gene encoding ICP34.5. It is of interest that mutations affecting ICP34.5 also disrupt ORF-P and that such mutants exhibit a reduced capacity to establish and reactivate from latency (Whitley *et al.*, 1993), thus ORF-P has raised considerable interest as having a potential role in latency. Regulation of ORF-P exibits charateristics expected of a latency-active gene, since it is expressed under IE conditions, is repressed by Vmw175 and its promoter contains a potential binding site for Vmw65, thus ORF-P is transcriptionally repressed during lytic infection and might be expressed

during latency (Lagunoff and Roizman, 1994, 1995). Although ORF-P translation products have not been detected in latently infected tissue to date, the low level of expression observed in tissue culture suggests that expression might be undetectable during latency. During late times of infection in tissue culture, the epitope-tagged ORF-P was modified to forms which migrated more slowly during electrophoresis and which assumed distinct structures in the cell nuclei (Lagunoff and Roizman, 1995).

The main approach used to investigate the function of LAT has been to examine the phenotypes of mutants which do not express a stable LAT during latency in animal models. In numerous studies, the inability to express a stable LAT during latency did not affect the ability to establish and maintain latency (Ho and Mocarski, 1989; Sederati *et al.*, 1989; Block *et al.*, 1990; Hill *et al.*, 1990; Deshmane *et al.*, 1993; Perng *et al.*, 1994a). A common phenotype observed with many, but not all mutants was impaired reactivation (Steiner *et al.*, 1989; Hill *et al.*, 1990; Perng *et al.*, 1994a). The inability to produce a stable LAT during latency did not necessarily correlate with impaired reactivation (Ho and Mocarski, 1989; Block *et al.*, 1990; Deshmane *et al.*, 1993). Regulation of the LAT region is complex and is dependent on different regulatory elements during lytic infection and latency (Goins *et al.*, 1994; Lokensgard *et al.*, 1994; Chen *et al.*, 1995). It is of interest to note that some LAT mutants unable to express a stable LAT during latency can express transcripts from the LAT region during lytic infection (Block *et al.*, 1990), hence transcription from the LAT region was not completely disrupted when transcription of a stable LAT during latency was prevented, thus it is possible that mutations which prevented LAT expression during latency did not all impair the function of the LAT region which facilitates reactivation. The region of the LAT transcription unit which facilitates reactivation requires further identification and characterisation.

A LAT$^-$ mutant of strain KOS which expresses β-galactosidase from the LAT promoter established 80% fewer β-galactosidase-expressing neurons during latency in the trigeminal ganglia of mice when compared to the wild type parent, and reactivated 80% less efficiently after the mice were exposed to hyperthermia (Sawtell and Thompson, 1992b). However, no difference was observed in the establishment or reactivation of latency from the lumbrosacral ganglia, indicating that the reactivation impairment of the LAT$^-$ mutant was dependent upon the site of latency. Analysis of colocalisation of β-galactosidase with HSV antigen during the establishment of latency revealed a higher degree of colocalisation in trigeminal but not the lumbrosacral ganglia infected with the LAT$^-$ mutant. It was suggested that LAT facilitated repression of viral replication, thus in ganglia infected with the LAT$^-$ mutant a greater proportion of cells were lytically infected and killed, resulting in a poor ability to establish latency and an impaired ability to reactivate. Another group reported that a LAT$^-$ mutant of strain 17(syn$^+$) was delayed in reactivation from trigeminal ganglia of mice, but the KOS LAT$^-$ mutant was not impaired

in reactivation from the lumbosacral ganglia (Devi-Rao *et al.*, 1994), thus supporting the hypothesis that LAT$^-$ mutants have a site-dependent phenotype.

The mechanism by which LAT facilitates reactivation is unknown. LAT might act as a functional RNA or might encode an as-yet undetected protein. Early suggestions that LAT functions as an antisense molecule by downregulating translation of Vmw110 mRNA (Stevens *et al.*, 1987) was disproved by the finding that LAT mutants establish latency and are impaired for reactivation rather than biased towards lytic infection. As LAT mutants are capable of establishing and maintaining latency, it is clear that LAT plays a qualitative rather than essential role in latency.

1.4. EFFECT OF HMBA ON *in*1814.

HMBA is of interest since it dramatically overcomes the defective phenotype of *in*1814 in tissue culture, restoring its particle / PFU ratio close to that of wild type virus (McFarlane *et al.*, 1992, McFarlane, 1993). HMBA also enhances reactivation of HSV from explanted ganglia (Bernstein and Kappes, 1988).

HMBA belongs to a class of compounds, including DMSO and hypoxanthine, which are united by their ability to induce terminal differentiation of myeloid erythroleukaemia cells (MELCs) and certain tumour cell lines (Friend *et al.*, 1971; Palfrey *et al.*, 1977; Collins *et al.*, 1980; Reuben *et al.*, 1980; Marks *et al.*, 1987). Treatment of MELCs with HMBA leads to a dramatic change in gene expression in the cells, ultimately resulting in greatly increased expression of the α and β^{maj}-globin genes. The exact mechanism by which HMBA initiates the changes in gene expression are not fully understood, however HMBA binds to the cell surface and causes a change in the cell-surface potential (Arcangeli *et al.*, 1993). Early changes induced by HMBA include a decrease in levels of *c-myc* and *c-myb* mRNAs, an increase in *c-fos* mRNA and a transient rise in p53 mRNA (Ramsay *et al.*, 1986; Richon *et al.*, 1989). However, these early changes in *c-myc*, *c-myb*, *c-fos* and p53 are not sufficient for irreversible commitment to differentiation (Richon *et al.*, 1989). Forced constitutive expression of *c-myb* in MELCs makes the cells unable to differentiate (Copolla and Cole, 1986; Dmitrovsky *et al.*, 1986; Clarke *et al.*, 1988), and dexamethasone, which inhibits HMBA-induced differentiation, prevents the early decrease in *c-myb* induced by HMBA (Ramsay *et al.*, 1986), thus the downregulation of *c-myb* appears necessary for differentiation to occur.

Exposure of MELCs to HMBA or DMSO results in extensive demethylation of the cell genome (Razin *et al.*, 1986, 1988). Exposure to HMBA in the presence of inhibitors of methylation results in inhibition of MELC differentiation (Razin *et al.*, 1988). However, HMBA-induced differentiation is inhibited in the presence of cycloheximide even though hypomethylation occurs normally, thus although DNA hypomethylation appears to be essential for HMBA-induced MELC differentiation, it is not sufficient *per se*.

There is evidence that PKC is a component of the HMBA-induced differentiation pathway. HMBA and DMSO cause an initial increase, followed by a decrease in diacylglycerol, one of the triggers of PKC activity (Michaeli *et al.*, 1992; Durkin *et al.*, 1992). Phorbol esters which mimic diacylglycerol, inhibit differentiation (Melloni *et al.*, 1987; Falleto *et al.*, 1985). Isoform-specific translocation of PKCδ and PKCε from the cytosol to the membrane occurs early during differentiation, and failure to do so is associated with resistance to HMBA-induced differentiation (Leng *et al.*, 1993).

Recent molecular studies on the induction of MELC differentiation have focused on the control of the cell cycle (Kiyokawa *et al.*, 1994; Marks *et al.*, 1994). MELCs exposed to HMBA exhibit a prolonged G_1-phase (Gambari *et al.*, 1978, 1979). Researchers have suggested that the prolongation of the G_1-phase by HMBA enables a larger proportion of the cells to leave the cell cycle and undergo differentiation (Marks *et al.*, 1994). HMBA-treatment results in a 20-fold decrease in cdk4 protein levels, a 3-fold increase in cyclin D3 levels and a 3-4-fold increase in levels of underphosphorylated pRB (Richon *et al.*, 1992; Kiyokawa *et al.*, 1994). The inhibition of cdk4 activity appears to be a crucial step in differentiation, since over-expression of cdk4 in transfected cells leads to an inhibition of HMBA-induced differentiation (Kiyokawa *et al.*, 1994). Cdk4 may be required for phosphorylation of pRB and p107, which in their phosphorylated forms are unable to complex with and inactivate transcription factors such as E2F. The transcription factor E2F transactivates genes required for progression into the cell cycle, such as *c-myc*, *c-myb* and DNA polymerase α. Thus HMBA leads to underphosphorylation of proteins such as pRB and p107, thereby resulting in prolonged G_1-phase and differentiation rather than entry into S-phase and mitosis. Consistent with the hypothesis that HMBA acts by altering the cell cycle, HMBA-treament of MELCs causes a loss of E2F DNA-binding activity, an increase in levels of p107 protein and the emergence of a new form of E2F (Richon and Ventaperez, 1996).

Reactivation of HSV in animal models is stimulated by HMBA and DMSO (section 1.3.8.2.; Harbour *et al.*, 1983; Whitby *et al.*, 1987; Bernstein and Kappes, 1988; Leib *et al.*, 1989). Since other hypomethylating substances such as 5-azacytidine and L-ethionine also facilitate reactivation (Whitby *et al.*, 1987; Stephanopoulos *et al.*, 1988), the ability of HMBA and DMSO to facilitate reactivation was attributed to their hypomethylating activity. However, HMBA and DMSO dramatically overcome the defective phenotype of *in*1814 in tissue culture, whereas 5-azacytidine does not (McFarlane *et al.*, 1992), thus the mode of action of these substances on reactivation of HSV from latency has been brought into question.

The particle / PFU ratio of *in*1814 is close to that of wild type virus during infection in the presence of HMBA (McFarlane *et al.*, 1992). Previous exposure of cells to HMBA is unable to stimulate *in*1814 titre, thus the changes on the cell induced by HMBA are transient. *In*1814 titre gradually becomes insensitive to HMBA over the first 6 hours of infection, since after approximately 6 hours infection HMBA does not stimulate the titre above that which occurs in its absence. HMBA therefore has an effect on *in*1814 similar to that of Vmw65, since Vmw65 is also unable to stimulate *in*1814 titre at 6 hours post-infection (Harris and Preston, 1991). The concentration of HMBA required for maximal stimulation of *in*1814 titre (5 mM) is similar to the concentration which causes maximal induction of MELC differentiation. In addition, other inducers of MELC differentiation

are also capable of stimulating the titre of *in*1814 (McFarlane and Preston, 1992; C.M. Preston, unpublished data), suggesting a common mechanism of action between the induction of MELC differentiation and the stimulation of *in*1814 titre.

HMBA increases levels of viral IE mRNAs in HFL cells infected with *in*1814 by approximately 2-fold, suggesting that HMBA exerts its effect on *in*1814 by stimulating IE gene expression (McFarlane *et al.*, 1992). The effect on viral IE mRNA levels occurs in the presence of cycloheximide, thus HMBA acts transiently on pre-existing factors rather than by inducing the expression of cellular genes.

The particle / PFU ratio of the Vmw110 deletion mutant *dl*1403 is not restored to that of wild type by HMBA (McFarlane *et al.*, 1992), neither are Vmw175-defective mutants able to forms plaques in the presence of HMBA (McFarlane *et al.*, 1992; McFarlane, 1993), thus it is unlikely that HMBA acts by inducing factors that functionally substitute for Vmw110 or Vmw175.

The ability of HMBA to stimulate gene expression is dependent on the location of the template DNA. HMBA stimulates gene expression from IE, E and L HSV promoters and from the HCMV major IE and SV40 promoters when in transfected plasmids, thus the effect of HMBA on gene expression appears relatively non-sequence-specific (McFarlane, 1993). However, when introduced into the cell genome, the HSV IE and E promoters are unresponsive to HMBA but remain responsive to transactivation by Vmw65 and the viral IE proteins. The non-sequence-specific action of HMBA suggests that it stimulates gene expression by altering a global process involved in gene expression, possibly by enhancing transcription initiation (McFarlane, 1993).

One possible mechanism by which HMBA could increase the accumulation of viral IE mRNAs, is to promote the formation of a transactivation complex at TAATGARAT elements. HMBA might either modify cell components enabling them to transactivate *via* TAATGARAT or enable the mutated Vmw65 to form the viral transactivation complex with Oct-1 and HCF. However, an investigation into the formation of complexes at TAATGARAT using gel-retardation experiments did not reveal any difference in TAATGARAT binding complexes in untreated and HMBA-treated cells (McFarlane, 1993), thus HMBA does not appear to stimulate HSV IE gene expression by inducing a TAATGARAT-binding transactivation complex.

Although it seems possible that HMBA increases levels of viral IE mRNAs by stimulating transcription, McFarlane was unable to demonstrate that HMBA stimulated transcription mediated by RNA pol II or RNA pol III in *in vitro* transcription assays (McFarlane, 1993). It was suggested that the inability to detect a stimulation of transcription was due to the highly transient nature of the alterations induced by HMBA.

In summary, HMBA is known to alter gene expression in cells and at least some of these alterations have been uncovered, however, the mechanism by which it stimulates HSV IE gene expression and overcomes the defect in *in*1814 is not yet fully understood.

1.5. THE INTERFERONS AND THEIR ANTIVIRAL ACTION AGAINST HSV.

IFN was first identified as a soluble factor released by chick egg chorioallantoic membrane upon exposure to heat-inactivated influenza virus (Isaacs and Lindenman, 1957). The soluble factor conferred resistance to virus infection when used to treat to other cells.

The IFNs are produced by a wide variety of cells and have marked effects on cellular gene expression leading to modulation of growth and differentiation and an antiviral state (Samuel, 1991; Sen and Lengyel, 1992).

Two types of IFN exist, type I includes IFNα and IFNβ and type II is IFNγ. Almost all cell types are capable of producing the type I IFNs, whereas type II is produced solely by T lymphocytes and natural killer cells. Type I IFNs are induced by virus infection, bacteria, mycoplasma and exposure to certain lymphokines such as colony-stimulating factor-1, interleukin (IL)-1, IL-2 and tumour necrosis factor. Inducers of type II include IL-2 and any antigen to which the organism is sensitised.

All of the type I IFN genes are clustered on the short arm of human chromosome 9 (Weissmann and Weber, 1986). There are over 20 copies of IFNα and just one copy of IFNβ. All of the type I IFN genes lack introns, whereas the single gene encoding IFNγ contains 3 introns and is located on the long arm of chromosome 12.

IFNs α and β share the same receptor, whereas IFNγ uses a separate receptor (Merlin *et al.*, 1985). However, expression of the human IFNα receptor on mouse cells confers responsiveness to certain subtypes of human IFNα but not to human IFNβ, inferring that additional receptor molecules might be involved in the binding of IFNβ (Uzé *et al.*, 1990).

Induction of the IFN genes occurs at the transcriptional level and does not require protein synthesis (Hiscott *et al.*, 1995). The IFN genes contain *cis*-acting sequences at their 5' ends which bind transcriptional activators which are activated in response to the appropriate stimuli. The 5' regulatory region of the IFNβ gene is the best characterised of the IFN promoters, containing a 110 nucleotide regulatory element with multiple binding site for both activators and repressors. In the uninduced state, the IFNβ promoter is bound by numerous transcriptional repressors, whereas upon virus infection a large array of activators bind to their specific sites, replacing the repressors. The activators include IRF (IFN-regulated factor)-1, the NFκB / Rel family of transcription factors, the ATF-2 / CREB family and HMG 1/Y. The signal for the activators to bind to the IFNβ gene regulatory regions are not fully understood in most cases, however NFκB binding can be induced by the action of PKR (Williams, 1995). In response to activation by dsRNA produced in virus-infected cells and possibly by other virus-induced stimuli, PKR

phosphorylates IκB which in its dephosphorylated state complexes with and represses NFκB. Thus phosphorylation of IκB releases NFκB enabling transcriptional activation of NFκB-dependent genes.

The binding of IFNα to its receptor activates latent transcriptional activators termed Stats (signal transducers and activators of transcription; Levy, 1995). In uninduced cells, Stat1 and Stat2 are located in the cytoplasm, whereas upon IFNα-treatment they are tyrosine phosphorylated by a member of the JAK family of receptor-bound kinases (Janus kinases), assemble into multimeric complexes and translocate to the nucleus. Stat1 and Stat2 form a complex termed ISGF3 (IFN-stimulated gene factor 3) by binding to the constitutively expressed DNA-binding IRF p48. ISGF3 binds to the ISREs (IFN-stimulated response elements) upstream of all IFNα-induced genes and stimulates their transcription. The individual biological responses generated by each individual IFN can be explained by the functional overlap and complex regulation of their signal transducers and the interferon-stimulated gene regulatory elements, for example phosphorylated Stat1 alone can also activate IFNγ-induced genes containing the gamma-activated site (GAS), thus GAS-containing promoters are activated by both IFNα and IFNγ.

The IFNs are known to induce the synthesis of more than 30 proteins, some of which are undetectable in untreated cells and some of which are increased only slightly in response to IFN. A proportion of the IFN-induced genes are only induced by one IFN, whereas others are induced by all 3 types.

The molecular basis for the antiviral state induced by the IFNs has been extensively studied and has revealed 3 major mechanisms of resistance to virus replication so far, the Mx system, the 2',5'-A oligoadenylate synthetase-induced pathway and the dsRNA-dependent IFN-induced protein kinase pathway. These 3 mechanisms will be reviewed briefly.

The human MxA protein accumulates in the cytoplasm of type I IFN-treated cells. MxA possess antiviral activity against influenza (Pavlovic *et al.*, 1995), measles (Schnorr *et al.*, 1993), VSV (Pavlovic *et al.*, 1995; Schwemmle *et al.*, 1995) and Thogoto virus (Pavlovic *et al.*, 1995). All mammalian species examined synthesise one or more Mx proteins in response to the type I IFNs, however the mechanism of action of the Mx proteins differs between species and the action of any single Mx protein differs between the target viruses. Mx proteins are believed to exert their antiviral functions by binding to and inactivating polymerase proteins of negative-stranded RNA viruses. The MxA protein inhibits different steps of the multiplication cycles of influenza A virus, VSV and measles virus. In the case of VSV, primary transcription of the input genome is inhibited (Staehli and Pavlovic, 1991; Schnorr *et al.*, 1993). Influenza A virus is inhibited at an unknown step between primary transcription and genome amplification (Pavlovic *et al.*, 1992). MxA appears to affect measles virus glycoprotein synthesis, rather than transcription or

genome replication (Schnorr *et al.*, 1993). Mx proteins are GTP-binding proteins with GTPase activity that is essential for their function (Pitossi *et al.*, 1993). Although the mechanism that GTP binding and hydrolysis plays in the activity of the Mx proteins is not fully understood, a recent study indicated that the binding of GTP to MxA, and not GTP hydrolysis, was necessary for antiviral activity against VSV (Schwemmle *et al.*, 1995).

The enzyme 2',5'-oligoadenylate synthetase is induced by type I and type II IFNs (Samuel, 1991). When activated by dsRNA, 2',5'-oligoadenylate synthetase catalyses the formation of 2',5'-oligoadenylates (abbreviated to 2,5-A), a family of oligonucleotides with the structure $ppp(A2'p5')_nA$, where $n \geq 2$. In turn, 2,5-A binds to and activates RNase L, which proceeds to cleave single stranded viral and cellular RNAs, thereby inhibiting viral replication. The enzyme 2',5'-phosphodiesterase performs a regulatory role by catalysing the conversion of 2,5-A to AMP and ATP. The 2,5-A system acts selectively against the picornaviruses.

PKR is induced by type I IFNs and activated by the binding of dsRNA. Activation of PKR leads to autophosphorylation, and when in the activated state PKR phosphorylates the α subunit of the translation initiation factor eIF-2α, thereby leading to inhibition of translation (Samuel, 1993). Viruses including adenovirus, EBV, HIV, influenza, poliovirus, reovirus and vaccinia have evolved intriguing and varied mechanisms for preventing the antiviral function of PKR (Katze, 1993). Mechanisms of inhibition of PKR include the production of RNAs which specifically bind to and inactivate PKR (adenovirus, EBV and HIV), the activation of cellular pathways which repress PKR activity (influenza and poliovirus), and the production of viral proteins which bind and sequester the dsRNA activator molecules (reovirus and vaccinia).

Viruses with DNA genomes, including HSV, are relatively resistant to the action of the IFNs compared to the RNA viruses. The production of infectious HSV progeny is reduced by approximately 100-fold in IFN-pretreated cells in culture. However, the mechanism of action of the IFNs against HSV appears not to occur *via* one of the well characterised IFN-induced pathways described above, but by a specific inhibition of IE transcription (Mittnacht *et al.*, 1988; Oberman and Panet, 1988; LaMarco and McKnight, 1989; DeStasio and Taylor, 1990). Researchers have consistently found that adsorption, penetration and uncoating of HSV is unaffected in IFN-pretreated cells (Mittnacht *et al.*, 1988; Oberman and Panet, 1989; DeStasio and Taylor, 1990), but that levels of IE and E transcripts are reduced (Mittnacht *et al.*, 1988; Oberman and Panet, 1988; DeStasio and Taylor, 1990). The reduction in HSV IE mRNA levels occurs as the result of an inhibition of transcription initiation rather than a slowing down transcript-elongation or degradation of the RNA (Mittnacht *et al.*, 1988; DeStasio and Taylor, 1990). However, while viral genes in the context of the viral genome are affected by IFN, cellular genes are insensitive (Mittnacht *et al.*, 1988; Oberman and Panet, 1989; DeStasio and Taylor,

1990). In addition, the ability of a plasmid expressing Vmw65 to stimulate transcription from a cotransfected plasmid containing an HSV IE promoter was circumvented by IFN pretreatment, thus it was proposed that IFN inhibits HSV by specifically disrupting transactivation by Vmw65 (DeStasio and Taylor, 1990).

The observation that the Vmw65 mutant *in*1814 is more sensitive to IFNα than its revertant 1814R is apparently inconsistent with the hypothesis that IFN acts by disrupting Vmw65 (Jamieson *et al.*, 1995), since if IFN acts solely on the Vmw65-containing complex one would expect *in*1814 to be insensitive to IFN. It is of importance that IFN acts against SV40 (Brennan and Stark, 1983) and MCMV (Gribaudo *et al.*, 1993) by inhibiting IE transcription, and that the HCMV promoter is sensitive to IFN when in the HSV genome (C.M. Preston, unpublished data). In the case of MCMV, the major IE promoter is largely dependent upon pre-existing cellular transcription factors, including NFκB, ATF, AP-1 and IRFs, thus it is unlikely that IFN acts by disrupting viral transactivators. In addition, the HCMV promoter is devoid of TAATGARAT elements but is sensitive to IFN when in the HSV genome (C.M. Preston, unpublished data). Recent data suggests that IFN acts specifically on an IE promoter in the viral genome, but that the same promoter in the cell genome is insensitive (C.M. Preston, unpublished data). Thus, IFN prevents transcription specifically from IE promoters in the viral genome by a mechanism that is not just specific for Vmw65. One possible model which might explain the specific action of IFN on viral IE genes proposes that IFN treatment results in the inactivation of cellular transcription factors specifically within the compartments of viral transcription.

The objectives of the study described in the thesis presented here were to further analyse the *in vitro* latency system of Jamieson *et al.* (section 1.3.4.3.; Jamieson, 1993; Jamieson *et al.*, 1995), in other words to investigate the structure of HSV genomes after latency was established in HFL cells pretreated with IFNα and infected with the double mutant *in*1820, which contains a non-transactivating Vmw65 and is virtually devoid of Vmw110 expression in this cell type. In addition to the structural studies, investigations were also made into the possibility that virus structural components modify HFL cells thereby facilitating the establishment of latency, and into the possibility that delayed virus uncoating mimics infection of neurons *in vivo* and leads to latency.

2. MATERIALS.

2.1. Tissue culture cells.

HFL Flow 2002 cells (ICN Flow) were normally supplied at passage 16 and renewed after 8-15 further passages. HFL cells were used for *in vitro* latency experiments.

BHK-C13 cells obtained from C.M. Preston and originally described by MacPherson and Stoker (1962) were used for growth and titration of virus stocks.

WS-HeLa and CV-1 cells were supplied by the Institute cytology facility.

2.2. Tissue culture media.

All cell types were propagated in Glasgow Modified Eagle's Medium (GMEM) (Busby *et al.*, 1964). GMEM was diluted from a 10× concentration and supplemented to a final concentration of 0.26% (w/v) sodium bicarbonate, 1× MEM non-essential amino acids, 100 units/ml penicillin, 100 µg/ml streptomycin, 4 mM L-glutamine. Further modification depended on the cell type for which the medium was to be used, as is shown below.

EF_{10} 10% (v/v) foetal calf serum.
ETC_{10} 10% (v/v) tryptose phosphate broth, 10% (v/v) newborn calf serum.
EF_{10} was used for propagation of HFL, WS-HeLa and CV-1 cells.
ETC_{10} was used for propagation of BHK cells.

During titrations of HSV-1, secondary plaque formation was prevented by overlaying cell monolayers with one of the following media.

CMC/EF_{10} (HFL only) EF_{10} containing 1.5% (w/v) carboxymethylcellulose sodium salt.

CMC/ETC_{10} (BHK) ETC_{10} containing 1.5% (w/v) carboxymethylcellulose sodium salt.

EHu_5 (HFL and BHK) Eagle's medium containing 5% (v/v) pooled human serum.

Agar overlay containing X-gal (BHK)	Eagle's medium containing 10% (v/v) newborn calf serum, 0.5% (w/v) Noble agar and 225 µg/ml X-gal. X-gal was dissolved in DMSO at a concentration of 150 mg/ml and added just before use.

The remaining media are listed below.

Tryptose phosphate broth	2% (w/v) bactotryptose, 0.2% (w/v) bactodextrose, 0.5% (w/v) NaCl, 0.25% (w/v) Na_2HPO_4.
Tris-saline	140 mM NaCl, 30 mM KCl, 280 mM Na_2HPO_4, 1 mg/ml glucose, 5 mM Tris-HCl (pH 7.4), 0.001% (w/v) Phenol red, 100 u/ml penicillin, 100 µg/ml streptomycin.
Trypsin	0.25% (w/v) trypsin dissolved in Tris-saline.
Versene	600 mM EDTA in PBS A, 0.0015% (w/v) phenol red.
PBS A	170 mM NaCl, 3.4 mM KCl, 1 mM Na_2HPO_4, 2 mM KH_2PO_4. pH 7.2.
PBS B	6.8 mM $CaCl_2$.
PBS C	4.9 mM $MgCl_2$.
PBS A+B+C	8 parts PBS A: 1 part PBS B : 1part PBS C.

2.3. Reagents used in tissue culture.

ACG, Ara-C, Brefeldin-A, cycloheximide, HMBA, human lymphoblastoid IFNα and PAA were all obtained from Sigma Chemical Company Ltd.

2.4. Virus stocks.

HSV-1 viruses were derived from wild-type strain 17 (syn^+).

The table overleaf summarises properties of the *in*1814 based viruses used for the research presented in this thesis.

*Ts*K (Marsden *et al.*, 1976), *dl*1403 (Stow and Stow, 1986), *dl*1403/β-gal (*dl*1403 with an *E.coli lac* Z marker gene recombined into U_L 43, constructed by C.M. Preston, unpublished data) and all of the viruses described in table 2.1. were

Virus	Vmw65 transactivation	IE-1 promoter	HCMV-*LAC Z*	Reference
*in*1814	−	wild type	−	Ace *et al.* (1989).
1814R	+	wild type	−	Ace *et al.* (1989).
*in*1853	−	wild type	TK locus	C.M.Preston, unpublished data.
*in*1863	+	wild type	TK locus	C.M.Preston, unpublished data.
*in*1820	−	MMLV LTR	−	Jamieson *et al.* (1995).
*in*1825	+	MMLV LTR	−	Jamieson *et al.* (1995).
*in*1883	−	MMLV LTR	TK locus	Jamieson *et al.* (1995).
*in*1830	−	MMLV LTR	U_L43	C.M.Preston, unpublished data.

Table 2.1. Summary of *in*1814 and *in*1820-based viruses.

supplied by C.M. Preston.

The temperature sensitive uncoating mutant *ts*1213 was supplied by V.G. Preston (V.G. Preston, unpublished data).

*In*1850 is *in*1814 with a 600 bp *Pvu*I fragment from the HSV-1 LAT promoter upstream of the *E.coli lacZ* gene inserted into the TK locus (C.M. Preston, unpublished data).

2.5. Isolation of nuclei from tissue culture cells.

TK lysis buffer	10 mM Tris HCl pH 7.5, 2 mM $MgCl_2$, 150 mM NaCl, 0.5% (v/v) Nonidet P-40.
Sucrose wash	10 mM Tris HCl pH 7.5, 2 mM $MgCl_2$, 10% (w/v) sucrose. Protein contamination was removed by the addition of DEP to 0.1% (v/v) with vigorous shaking followed by autoclaving. Tris HCl was added after DEP treatment and autoclaving.

2.6. Digestion of cell nuclei with nucleases.

DNase I storage buffer	10 mM Tris HCl pH 7.5, 5 mM $MgCl_2$, 50% (v/v) glycerol.

MN was stored in sterile distilled water.

The reaction buffers for digestion of nuclei with DNase I and MN were as follows.

DNase I	0.25 M sucrose, 10 mM PIPES, 10 mM NaCl, 3 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM PMSF.
MN	0.25 M sucrose, 20 mM PIPES, 10 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM 2-mercaptoethanol, 0.1 mM PMSF.

2.7. Purification of DNA from tissue culture cells.

SDS / Proteinase K buffer	20 mM Tris HCl pH 7.5, 2.5 mM EDTA pH 7.5, 0.5% SDS, 100 µg/ml proteinase K.
Phenol : chloroform	1 volume redistilled phenol : 1 volume chloroform.

2.8. Purification of poly(A)-containing RNA from tissue culture cells.

Lysis/binding buffer	100 mM Tris HCl pH 8.0, 500 mM LiCl, 10 mM EDTA pH 8.0, 1% (w/v) LiDS, 5 mM DTT.

Wash buffer with LiDS	10 mM Tris HCl pH 8.0, 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS.
Elution solution	2 mM EDTA pH 8.0.

2.9. Enzymes.

Restriction endonucleases were supplied by Boehringer Mannheim or New England Biolabs.

Klenow enzyme was supplied by Boehringer Mannheim.

T4 DNA ligase was from New England Biolabs.

2.10. Electrophoresis of DNA.

10× TBE buffer	90 mM Tris, 1 mM EDTA, 90 mM boric acid. pH 8.3.
Ficoll loading buffer	10% (w/v) ficoll, 5× TBE, 0.1% (w/v) bromophenol blue.

2.11. Electrophoresis of RNA.

10× MOPS buffer	0.4 M MOPS, 0.1 M CH_3COONa, 10 mM EDTA. pH 7.2.
Sample loading buffer	50% (v/v) deionised formamide, 1× MOPS buffer, 7% (w/v) formaldehyde, 0.1% (w/v) bromophenol blue.

2.12. Southern transfer and hybridisation.

Alkaline transfer solution	0.4 M NaOH, 0.6 M NaCl.
Neutralising solution	0.5 M Tris HCl, 1 M NaCl. pH 7.0.
0.8M Sodium phosphate buffer pH 7.4	0.7 M Na_2HPO_4, 0.1 M NaH_2PO_4.
Southern pre- / hybridisation buffer	0.5 M sodium phosphate buffer pH 7.4, 7% SDS.
20× SSC	3M NaCl, 0.3 M sodium citrate.
Membrane wash buffer	0.2× SSC, 0.1% SDS.

2.13. Northern transfer and hybridisation.

20× SSPE	3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA. pH 7.4.
50× Denhardt's	1% (w/v) BSA, 1% (w/v) ficoll 400, 1% (w/v) polyvinylpyrrolidone.
Northern pre- / hybridisation buffer	5× SSPE, 50% (v/v) deionised formamide, 1% (w/v) SDS, 5× Denhardt's, 10 µg/ml sheared denatured calf thymus DNA (northern pre-hybridisation buffer only).

2.14. Purification of DNA from agarose gels.

Sephaglas	Sephaglas BP 20% (w/v) suspended in 6 M NaI, 50 mM Tris HCl pH 8.0, 0.05% Na_2SO_3, 10 mM CDTA.
Gel solubiliser	6 M NaI, 50 mM Tris HCl pH 8.0, 0.05% Na_2SO_3, 10 mM CDTA.
Wash buffer	20 mM Tris HCl pH 8.0, 1 mM CDTA, 0.1 mM NaCl, 60% (v/v) ethanol.
Elution buffer	10 mM Tris HCl, 1 mM EDTA.

2.15. Radiochemicals.

α-[^{32}P] labelled dNTPs at a specific activity of 10 mCi/ml were supplied by Amersham International plc.

2.16. Labelling of probes by random primer extention.

Oligo reaction mix	250 mM Tris HCl pH 8.0, 25 mM $MgCl_2$, 50 mM 2-mercaptoethanol, 1 M HEPES pH 6.6, 30 OD units/ml random sequence hexadeoxyribonucleotides.

2.17. Plasmids.

Plasmids containing cloned DNA fragments were supplied by C.M. Preston.

2.18. Bacterial strains and culture media.

E.coli. strain K12 DH-1 (Hanahan, 1983) was grown in L-broth.

L-broth	1% (w/v) NaCl, 1% (w/v) bactopeptone, 0.5% (w/v) yeast extract.

For isolation of single bacterial colonies, L-broth containing 1.5% agar was used to give a solid substrate.

When required, bacterial growth media was supplemented with 100 µg/ml ampicillin (SmithKline Beecham Pharmaceuticles).

2.19. Small scale preparation of plasmid DNA from *E.coli.*

STET	5% (v/v) Triton X-100, 50 mM EDTA pH 8.0, 50 mM Tris HCl pH 8.0, 10% (w/v) sucrose.

2.20. Large scale preparation of plasmid DNA from *E.coli.*

Buffer P1	100 µg/ml RNase A, 50 mM Tris HCl, 10 mM EDTA. pH 8.0.
Buffer P2	200 mM NaOH, 1% SDS.
Buffer P3	3 M CH_3COOK pH 5.5.
Equilibration buffer	0.15 % (v/v) Triton X-100. pH 7.0.
Wash buffer	1 M NaCl, 50 mM MOPS, 15% (v/v) ethanol. pH 8.5.
Elution buffer	1.25 M NaCl, 50 mM Tris HCl, 15% (v/v) ethanol. pH 8.5.

2.21. Transfection of DNA into tissue culture cells.

2× HEBS	40 mM HEPES NaOH pH 7.05, 0.28 M NaCl, 2 mg/ml D-glucose, 10 mM KCl, 1.4 mM Na_2HPO_4.

2.22. Staining of tissue culture monolayers for plaque assay.

Giemsa's stain improved R66 solution was supplied by BDH Chemicals.

2.23. Staining of tissue culture monolayers for β-galactosidase.

β-galactosidase stain	PBS A containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% (v/v) Nonidet P-40 and 1 mg/ml X-gal. X-gal was dissoved in DMSO at a concentration of 40 mg/ml and added just before use.

2.24. Suppliers.

Reagents were obtained from the companies listed below.

Amersham International plc, BDH Chemicals, Boehringer Mannheim, Fisons Scientific Equipment, Gibco BRL, ICN Flow, Pharmacia Chemicals, Sigma Chemical Company, SmithKline Beecham Pharmaceuticals.

3. METHODS.

3.1. Tissue culture.

HFL cells were grown in tissue culture flasks at 37° in an atmosphere of 5% CO_2 : 95% air. When grown to full confluence, usually after 2 or 3 days at 37°, the cells were used immediately or moved to 31° and used after a further 1–4 days. Each tissue culture flask had a growth surface of 175 cm^2 and contained approximately 2×10^7 cells at full confluence. Passage of HFL cells was carried out as follows.

Medium was poured from a flask of confluent HFL cells and the cell monolayer washed with 20 ml versene. The versene was poured away and 25 ml 1 volume trypsin : 4 volumes versene was added to the flask. After briefly rinsing the cells in the trypsin / versene approximately 23 ml was poured from the flask leaving 2 ml remaining. When the majority of cells were visibly dislodged from the growth surface 10 ml EF_{10} was added and the cells aspirated and split 1 : 4 to seed other tissue culture flasks, or used to seed tissue culture dishes. One flask yielded a sufficient number of cells to seed 25 35 mm diameter tissue culture dishes.

WS-HeLa and CV-1 cells were propagated in the same way as HFL cells, with the only difference that trypsin was diluted with versene in the ratio 1 volume trypsin : 1 volume versene.

BHK-C13 cells were grown in tissue culture roller bottles at 37° in an atmosphere of 5% CO_2 : 95% air. At full confluence, usually after 2 or 3 days at 37°, the cells were used immediately or moved to 31° and used after a further 1-4 days. Each tissue culture roller bottle had a growth surface of 850 cm^2 and at full confluence contained approximately 2×10^8 cells. Passage of BHK cells was carried out as follows.

Medium was poured from a roller bottle of confluent BHK cells and the cell monolayer washed with 40 ml versene. The versene was poured away and 40 ml 1 volume trypsin : 1 volume versene was added. After briefly rinsing the cells in the trypsin / versene, approximately 37 ml was poured off leaving 3 ml remaining. The monolayer was observed until the majority of cells were dislodged and the cells immediately aspirated in 10 ml ETC_{10} and split 1 : 10 to seed other roller bottles, or used to seed tissue culture dishes. One roller bottle yielded a sufficient number of cells to seed 250 35 mm diameter tissue culture dishes.

Monolayers seeded in tissue culture dishes were ready for use after incubation at 37° for 16–30 hrs. All cell types grew to similar densities hence the number of cells present on any given size dish was the same for all cell types.

diameter of dish	number of cells upon confluence	volume of medium per dish
35 mm	1×10^6	2 ml
60 mm	3×10^6	6 ml
90 mm	7.5×10^6	15 ml

Each well on 24-well tissue culture plates was seeded with cells suspended in 1 ml of medium and contained 2×10^5 cells after growth to full confluence.

3.2. Preparation of HSV-1 stocks.

Virus seed stocks used for inoculating BHK cells in tissue culture roller bottles were prepared by inoculating a plaque isolate onto a BHK monolayer in a 60 mm diameter tissue culture dish and incubating at the appropriate temperature. When severe CPE was evident the cells were harvested, sonicated and the virus titre was determined.

Tissue culture roller bottles seeded with BHK cells and grown to 80-100% confluence were each inoculated with 2×10^6 PFU of seed stock in 50 ml ETC_{10} and incubated at the appropriate temperature. During the growth of *in*1814-based viruses 3 mM HMBA was included in the growth medium for the first 16–24 hrs of infection, after which the medium was replaced with 40 ml ETC_{10}. During growth of *in*1820 based viruses and HSV-1 viruses which did not contain the Vmw65 mutation of *in*1814, the presence of HMBA was not necessary hence the cells were maintained in the 50 ml inoculum throughout infection.

When the infected cells exhibited severe CPE they were dislodged into the growth medium and distributed into centrifuge bottles. The cellular material was removed by centrifugation in a Sorvall GSA rotor at 2000 rpm for 15 minutes at 4°. Sorvall GSA rotors were centrifuged in a Sorvall RC5-B centrifuge. The resulting pellets were processed to release the cell-associated virus and the supernatants centrifuged to concentrate the extracellular virus.

The cell pellets were resuspended in ETC_{10} (0.5 ml for each roller bottle of BHK cells infected), pooled, and bath sonicated thoroughly to release the cell-associated virus. The cellular debris was pelleted by centrifugation at 2500 rpm for 10 minutes at 4° in a Sorvall RT6000B benchtop centrifuge and the supernatant stored at –70° for future use as a virus stock.

The extracellular virus particles were concentrated by centrifugation in a Sorvall GSA rotor at 12000 rpm for 2 hrs at 4°. The pellets were gently resuspended in ETC_{10} (0.5 ml for each roller bottle of BHK cells infected), pooled, bath sonicated to disperse aggregated virus and cell debris and stored at –70° for future use as a virus stock.

3.3. Purification of HSV-1 light particles.

The technique used for purification of extracellular light particles (L-particles) was modified from the method first described by Szilágyi and Cunningham (1991).

Tissue culture roller bottles seeded with BHK cells were inoculated with HSV-1 as described in section 3.2. When severe CPE was evident the cells were removed from the growth surface by shaking the roller bottle and the medium containing the cells distributed in centrifuge bottles. The cellular material was removed by centrifugation in a Sorvall GSA rotor at 5000 rpm for 15 minutes at 4°. In order to concentrate the extracellular L-particles and virions the supernatants were centrifuged in a Sorvall GSA rotor at 12000 rpm for 2 hrs at 4°. After centrifugation the supernatants were discarded and the pellets overlaid with 1 ml Eagle's medium without phenol red for every three infected roller bottles of BHK cells processed. The pellets were left to soften on ice with gentle agitation for 6 hrs, gently aspirated and left on ice with gentle agitation for a further 16 hrs. Gradual resuspension of the viral particles over a 22 hr period negated the need for vigorous aspiration or bath sonication and hence minimised damage to the fragile L-particles. The resuspended pellets were layered onto 35 ml pre-formed 5-15% gradients of ficoll 400 dissolved in Eagle's medium without phenol red. The resuspended pellets were distributed on the gradients such that each gradient took L-particles and virions from 3 or 4 roller bottles. The L-particles and virions were separated in the gradients by centrifugation in a Sorvall AH629 rotor at 12000 rpm for 2 hrs at 4°. AH629 rotors were centrifuged using a Sorvall OTD50B ultracentrifuge. L-particles were removed by side puncture and extraction with a surgical needle and syringe and pelleted by centrifugation in a Sorvall AH629 rotor at 12000 rpm for 16 hrs at 4°. The pellets were resuspended by overlaying with 100 µl Eagle's medium without phenol red for each roller bottle of infected BHK cells processed and incubating on ice for 6 hrs with occasional agitation. L-particles were stored at −70°.

3.4. Titration of HSV-1 stocks.

Titres of HSV-1 stocks were determined by plaque assay on BHK cells. HSV-1 stocks were serially diluted 10-fold and 200 µl of each dilution used to infect a BHK monolayer in a 35 mm diameter tissue culture dish. Usually each dilution was titrated in duplicate. After 1 hr adsorption with rocking every 20 minutes to prevent drying of the monolayers the inocula were removed and the cells overlaid with 2 ml per dish of EHu_5 or CMC/ETC_{10}. Monolayers were incubated at the appropriate temperature until plaques were easily visible under a low magnification light microscope. The medium was removed and approximately 1 ml Giemsa stain added to each dish. After staining for a minimum of 30 minutes at room temperature the cells were washed with water and the plaques counted under a dissection microscope.

Titres of HSV-1 mutants containing the Vmw65 mutation of *in*1814 were standardised by titration on BHK cell monolayers with 3 mM HMBA in the culture medium. The presence 3 mM HMBA during the early stages of infection has previously been shown to restore the particle / PFU ratio of *in*1814 close to that of wild type HSV-1 (McFarlane *et al.*, 1992). After the 1 hr adsorption period the inocula were removed and the monolayers overlaid with 2 ml per dish of EHu_5 containing 3 mM HMBA or 2 ml per dish of CMC/ETC_{10} containing 3 mM HMBA. Cells were incubated, stained and counted as described in the paragraph above.

HSV-1 mutants containing the *in*1814 mutation and which expressed β-galactosidase were titrated on HFL monolayers in the absence of HMBA. Because mutants with the *in*1814 phenotype titrate in a nonlinear manner in the absence of HMBA, 3-fold rather than 10-fold dilutions were used for titrations. HFL monolayers seeded on 24-well dishes were infected with 100 µl of dilution per well. After 1 hr adsorption with rocking every 15 minutes the inocula were removed and the monolayers overlaid with 1 ml per well of EHu_5 or 1 ml per well of CMC/EF_{10}. After incubation for 20–24 hrs the monolayers were fixed and stained for β-galactosidase activity.

3.5. Inactivation of HSV-1 by irradiation with UV light.

When cell released HSV-1 preparations were to be UV-irradiated, it was first necessary to replace the ETC_{10} in which the virus was suspended with Eagle's medium without phenol red, since phenol red in ETC_{10} absorbs UV light and reduces the efficiency of UV-inactivation.

0.8 ml of a cell released HSV-1 preparation was added to 10 ml Eagle's medium without phenol red in a 30 ml glass corex tube and centrifuged in a Sorvall SS34 rotor at 15000 rpm for 1 hr at 4°. Sorvall SS34 rotors were centrifuged in a Sorvall RC-5B centrifuge. The supernatant was discarded and the virus pellet washed briefly with 5 ml Eagle's medium without phenol red. The pellet was resuspended in 0.8 ml Eagle's medium without phenol red and bath sonicated to disperse aggregated virus particles. The sample was ready for UV-irradiation. Gradient purified HSV-1 L-particle or virion preparations were UV-irradiated without previous treatment.

A 40 µl fraction of the sample to be irradiated was removed, foetal calf serum was added to a final concentration of 10% and the fraction stored at –70°. The remainder of the sample was distributed into 35 mm diameter tissue culture dishes, ≤0.4 ml being applied to each dish. The dishes were placed in a UV Stratalinker 1800 (Stratagene) with the lids off and subjected to 5 exposures of 80 mJ of UV radiation. The dishes were removed from the Stratalinker between each exposure and rocked to distribute the virus preparation and prevent drying. After the final exposure, foetal calf serum was added to a final concentration of 10% and the samples mixed and stored at –70°. Subsequently, the

UV-irradiated sample and the untreated fraction were titrated in parallel to assess the drop in virus titre caused by the UV-irradiation. A reduction in titre of $>10^5$-fold was consistently observed.

3.6. *In vitro* latency.

In this thesis is described the analysis of an *in vitro* latency system for HSV-1 which was designed and characterised by D.R.S. Jamieson and C.M. Preston (Jamieson., 1993; Jamieson *et al.*, 1995). The *in vitro* latency system is modified from an earlier system of Harris and Preston (1991).

Latency of *in*1820 in HFL cells was established as follows.

HFL cells which had been seeded in a 35 mm tissue culture dish 16–24 hrs previously were treated with IFNα by replacing the growth medium with 2 ml EF_{10} containing 1000 u/ml IFNα and incubating at 37° for 16–20 hrs. The IFNα was removed thoroughly and the cells given 2 × 2 ml washes with EF_{10}. An inoculum of *in*1820 in 200 µl EF_{10} was applied to the monolayer and the virus allowed to adsorb to the cells by incubation at 37° for 1 hr with rocking every 20 minutes. When the adsorption period was complete, 2 ml EF_{10} containing 50 µg/ml Ara-C was added and incubation continued at 37° for 2 or 3 days, during which time the virus entered the state referred to in this thesis as '*in vitro* latency'. When latency was established in HFL cells grown on 60 mm plates, volumes were scaled up accordingly. The technique used to establish *in vitro* latency is summarised in figure 3.1.

*In*1820 was reactivated from *in vitro* latency by superinfection with HSV-1. The EF_{10} containing Ara-C was removed from a latently infected HFL cell monolayer in a 35 mm dish and the superinfecting virus in 200 µl EF_{10} containing 50 µg/ml Ara-C was applied. After 1 hr adsorption at 37° with rocking every 20 minutes, 2 ml EF_{10} containing 50 µg/ml Ara-C was added and incubation continued at 37° for a further 6 or 7 hrs.

3.7. Isolation of nuclei from tissue culture cells.

During the isolation of nuclei from tissue culture cells, aggregation of nuclei and the activity of endogenous nucleases was minimised by storing the samples on ice between manipulations and by chilling the buffers on ice for ≥45 minutes before use.

Culture medium was removed from a monolayer of cells grown in a 35 mm or 60 mm diameter tissue culture dish and the cells washed with 2 ml or 6 ml respectively of PBS A+B+C. The cells were harvested in 1 ml PBS A+B+C using a rubber policeman, transferred to a 1.5 ml vial and pelleted by centrifugation at 13000 rpm for 45 seconds in a benchtop microfuge. The supernatant was removed with a Pasteur pipette and the cell pellet resuspended in the residual PBS A+B+C by vortex mixing. 1 ml TK lysis buffer was added, the cells distributed by vortex mixing and left on ice for 5 minutes to allow

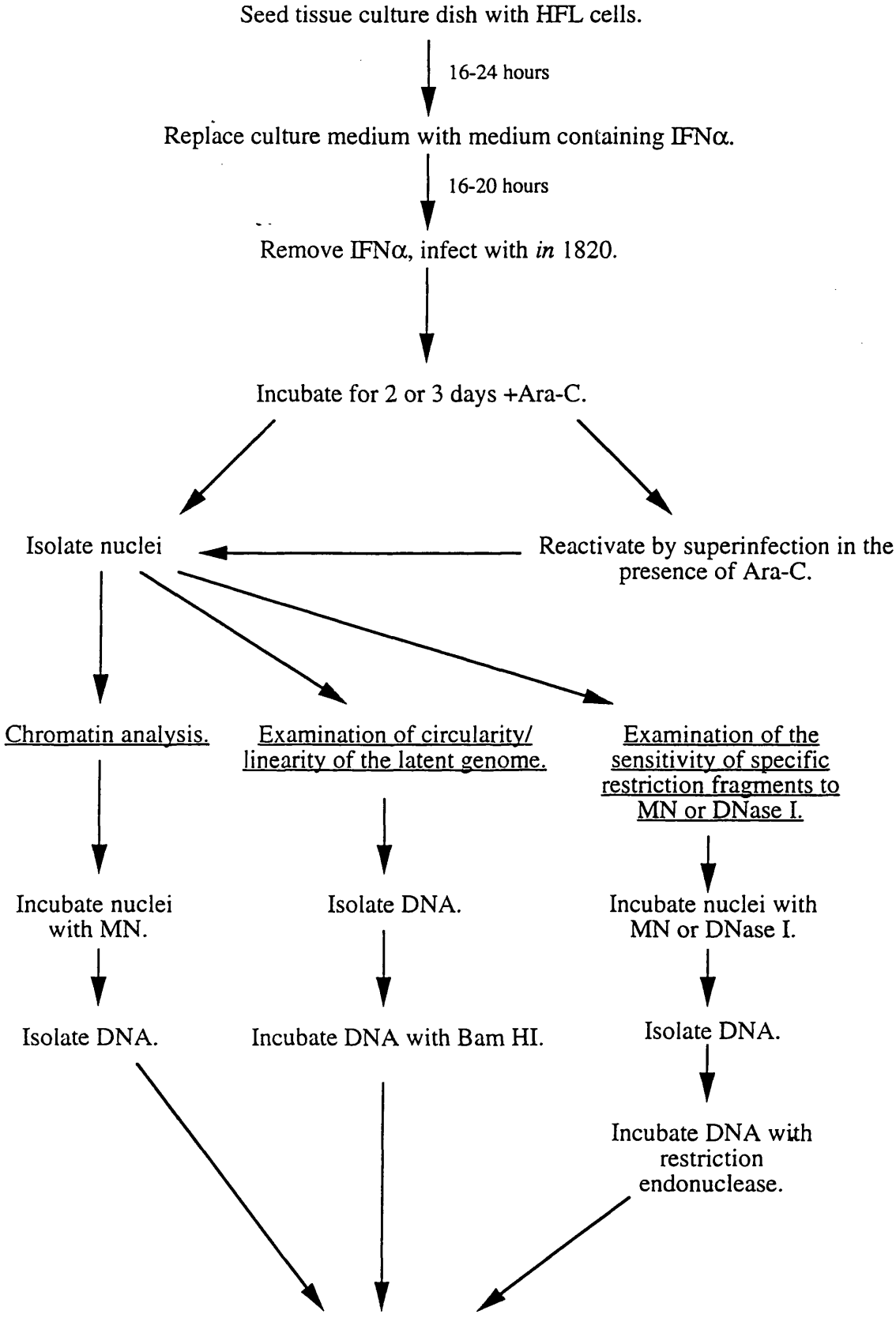


Figure 3.1. Summary of the *in vitro* latency system and the techniques used to examine the structure of the latent / reactivated genomes.

lysis of the cells. Nuclei were pelleted by centrifugation at 13000 rpm for 45 seconds, the lysis buffer containing the cytoplasmic material was removed and either discarded or the DNA purified for Southern blot analysis. The nuclei were resuspended in the residual TK lysis buffer by vortex mixing and 1 ml sucrose wash was added followed by further vortex mixing to distribute the nuclei. Nuclei were pelleted through the sucrose wash by centrifugation at 13000 rpm for 45 seconds, the sucrose wash was removed and the nuclei resuspended in the residual sucrose wash by vortex mixing. The nuclei were ready for incubation with MN or DNase I; alternatively the nucleic acid was purified immediately, as described below and summarised in figure 3.1.

3.8. Digestion of tissue culture cell nuclei with nucleases.

Nuclei isolated from cells cultured in a 35 mm or 60 mm diameter tissue culture dish were resuspended in 150 µl MN or DNase I reaction buffer and vortex mixed thoroughly. The reaction was started by the addition of MN dissolved in sterile distilled water or DNase I dissolved in storage buffer without glycerol to various concentrations followed by brief vortex mixing and incubation in a 37° waterbath for various times. The reaction was stopped by placing the nuclei on ice and immediately adding EDTA pH 7.5 to a final concentration of 12 mM.

3.9. Digestion of naked viral DNA with MN.

Purified *in*1850 DNA was supplied by C. M. Preston. 2 µg *in*1850 DNA in 7 µl H_2O was added to 50 µl MN reaction buffer. 10 µl of various concentrations of MN dissolved in H_2O was added and the sample placed in a 37° waterbath for 5 minutes. The reaction was stopped by adding EDTA pH 7.5 to a final concentration of 17 mM and the sample made to a volume of 500 µl with H_2O. The sample was phenol : chloroform extracted and the DNA purified as described in section 3.10. The purified DNA pellet was resuspended in 20 µl H_2O and half the sample electrophoresed in a 1.5% agarose gel in order the assess the extent of nuclease digestion.

3.10. Purification of DNA from tissue culture cells.

Nuclei isolated from cells cultured in a 35 mm or 60 mm diameter tissue culture dish were incubated in 0.5 ml SDS / proteinase K buffer at 37° overnight or at 45° for 3–5 hrs. After MN or DNase I digestion, 350 µl SDS / proteinase K buffer made at a 1.43× concentration was added to the nuclei suspended in 150 µl reaction buffer to give the final concentration of SDS / proteinase K buffer specified in section 2.7.

After digestion with SDS / proteinase K buffer, the sample was extracted twice with phenol : chloroform and once with chloroform. Extractions were carried out by

adding 0.5 ml phenol : chloroform or chloroform, followed by vigorous mixing. The phases were separated by centrifugation at 13000 rpm for 10 minutes in a benchtop microfuge and the aqueous phase containing the nucleic acid was removed to a separate vial. After the chloroform extraction, CH_3COONa pH 7.0 was added to a final concentration of 0.3 M and the nucleic acid was precipitated by adding 1 ml ethanol and vigorously mixing the sample. Precipitated nucleic acid was pelleted by centrifugation at 13000 rpm for 10 minutes. After removal of the supernatant, the pellet was washed by adding 0.5 ml 70% (v/v) ethanol and gently mixing. The pellet was collected at the bottom of the vial by centrifugation at 13000 rpm for 5 minutes, the supernatant removed and the pellet allowed to dry by lying the vial on the bench with the lid open for ≥45 minutes. The dried pellet was subject to restriction endonuclease cleavage or for chromatin analysis was resuspended in sterile distilled water containing 10 µg/ml RNase A and incubated at 37° for 30 minutes. DNA was stored at –20°.

To purify DNA from the cytoplasmic fraction, the lysate from cells cultured in a 35 mm or 60 mm diameter tissue culture dish was made up to the concentration of SDS / proteinase K buffer shown in section 2.7 and divided into 2 × 0.5 ml fractions. DNA was purified as with nuclear DNA. The resuspended purified DNA pellets from the two halves of the sample were pooled.

3.11. Purification of poly(A)-containing RNA from tissue culture cells.

Poly(A)-containing RNA was purified from tissue culture cells using the Dynabeads Oligo $d(T)_{25}$ (Dynal) kit. The use of Dynabeads Oligo $d(T)_{25}$ relies on base pairing between poly(A) residues at the 3' end of most mRNA and the oligo (dT) residues covalently coupled to the surface of magnetic polystyrene beads. The beads can be separated from solution with a magnetic particle concentrator (MPC).

Culture medium was removed from a monolayer of cells cultured in a 90 mm diameter tissue culture dish and the cells washed with 15 ml PBS A+B+C which had previously been cooled to 4°. The cells were harvested in 5 ml PBS A+B+C with a rubber policeman, transferred to a 50 ml polypropylene conical tube and pelleted by centrifugation at 1500 rpm for 10 minutes at 4° in a Sorvall RT6000B benchtop centrifuge. The supernatant was removed and the pellet resuspended in 1 ml PBS A+B+C and transferred to a 1.5 ml vial. The cells were pelleted by centrifugation at 13000 rpm for 45 seconds in a benchtop microfuge, the supernatant removed and the cell pellet stored at –70°. Without prior thawing, the pellet was resuspended in 0.5 ml lysis / binding buffer and passed through a 21 gauge syringe needle 3 times to shear the DNA and reduce the viscosity of the solution. Dynabeads Oligo $d(T)_{25}$ from 260 µl of a 10 mg/ml suspension in PBS A were concentrated in an MPC and washed once with 200 µl lysis / binding buffer. The lysis / binding buffer was discarded, the vial removed from the MPC and the Dynabeads Oligo $d(T)_{25}$ resuspended in the cell lysate. Binding of poly(A)-containing

RNA to the Dynabeads Oligo $d(T)_{25}$ was allowed by incubation at room temperature for 5 minutes with occasional agitation. The vial was placed in the MPC, the lysate removed from the Dynabeads Oligo $d(T)_{25}$ / RNA and the Dynabeads Oligo $d(T)_{25}$ / RNA given 3 washes with 0.5 ml wash buffer with LiDS. The final wash was removed thoroughly and the poly(A)-containing RNA eluted from the Dynabeads Oligo $d(T)_{25}$ by removing the vial from the MPC and resuspending the Dynabeads Oligo $d(T)_{25}$ / RNA in 20 µl elution solution followed by incubation in a 65° waterbath for 2 minutes. The vial was placed in the MPC and the supernatant containing the eluted poly(A)-containing RNA removed to a separate vial and stored at –70°.

3.12. Cleavage of DNA with restriction endonucleases.

Pellets of nuclear or cytoplasmic nucleic acid isolated from tissue culture cells cultured in a 35 mm or 60 mm diameter tissue culture dish were resuspended in 40–100 µl of a suitable buffer containing 10 units of restriction enzyme and 10 µg/ml RNase A. Reaction conditions were optimised according to the suppliers recommendations. Digests were usually incubated at 37° overnight.

Cleavage of plasmid DNA was carried out according to the manufacturers instructions, incubation at 37° for 3–5 hrs being sufficient. The inclusion of RNase A was only necessary for cleavage of plasmid DNA from small scale preparations.

For cleavage of DNA with more than one restriction endonuclease which required buffers that were incompatible, the DNA was incubated with one enzyme, adjusted to a volume of 0.5 ml with sterile distilled water, extracted with phenol : chloroform and subject to the DNA purification procedure described in section 3.10. The cleaved, purified DNA pellet was ready for incubation with another restriction endonuclease.

3.13. Electrophoresis of DNA.

Agarose gels for Southern blotting or separation of cleaved plasmid DNA fragments had the dimensions 260 mm × 160 mm and a volume of 300 ml. Gels consisted of 0.7–1.5% (w/v) agarose, 1× TBE buffer, 0.5 µg/ml ethidium bromide and were electrophoresed horizontally in 1× TBE buffer with 1 cm of buffer above the gel. DNA was electrophoresed at 16 mA overnight or at 6 mA over 2 days, or until the bromophenol blue had migrated approximately 13 cm.

Before loading on the gel, ficoll loading buffer was added to each DNA sample to a 1× final concentration.

Electrophoresed DNA was visualised by transillumination with UV light. Short wavelength UV light was used for gels which were to be blotted. Gels used to separate fragments for cloning or to be radiolabelled for use as probes were transilluminated with long wavelength UV light. Agarose gels were photographed using Polaroid 667 film.

3.14. Electrophoresis of RNA.

Agarose gels for northern blotting had the dimensions 260 mm × 160 mm and a volume of 200 ml. Gels consisted of 1.2% (w/v) agarose, 1× MOPS buffer, 2% (w/v) formaldehyde and were electrophoresed horizontally in 1× MOPS buffer with 1 cm of buffer above the gel. RNA was electrophophoresed at 30 mA overnight or until the bromophenol blue had migrated approximately 13 cm. Gel tanks used for electrophoresis of RNA were previously treated overnight with 0.1% (v/v) DEP and were used solely for electrophoresis of RNA. The glass plate on which the gel was cast and which supported the gel during electrophoresis was also used exclusively for RNA.

Before loading on the gel, sample loading buffer was added to each sample in the ratio 5 volumes RNA sample : 11 volumes sample loading buffer and the RNA denatured by heating to 100° for 1 minute followed by incubation on ice for 10 minutes.

A sample of whole cell RNA was electrophoresed alongside poly(A)-containing RNA to enable positions of the rRNAs to be visualised after staining with ethidium bromide.

After electrophoresis, the gel was cut as required and placed in a container with 1500 ml distilled water. After gentle agitation for 10 minutes at room temperature, the water was poured away and another 1500 ml distilled water added followed by gentle agitation for a further 10 minutes at room temperature. Agitation with distilled water removed formaldehyde from the gel. The water was poured away, 500 ml distilled water containing 2 μg/ml ethidium bromide was added and agitation continued for 10 minutes at room temperature to allow the ethidium bromide to enter the gel and bind the RNA. The RNA was visualised by transillumination with short wavelength UV light and photographed using Polaroid 667 film.

3.15. Southern transfer and hybridisation.

The method used to transfer DNA onto hybridisation membranes was the alkaline transfer capillary blotting technique adapted by Reed and Mann (1985) from the technique described by Southern (1975).

The electrophoresed, photographed gel was cut to the required size and the DNA denatured by immersion in 400 ml alkaline transfer solution for 15–30 minutes immediately prior to blotting. Gels used for the analysis of chromatin (sections 4.10–4.12.) were depurinated before soaking in alkaline transfer solution. Depurination was achieved by soaking the gel in 400 ml 0.25 M HCl for 5 minutes with occasional agitation followed by a brief rinse in deionised water.

A bijou rack was placed in an enamel tray and a glass plate 190 mm × 157 mm placed onto the rack. A strip of Whatman 3 mm thick chromatography paper cut to 320 mm × 157 mm was placed across the length of the plate and a strip 187 mm × 190 mm

across the breadth. Alkaline transfer solution was poured over the chromatography papers to soak them, leaving the two ends hanging over the plate to act as wicks. The gel was placed onto the chromatography papers and any air pockets between the gel and the paper were removed by rolling a clean pipette over the gel. A piece of GeneScreen *Plus* hybridisation transfer membrane (NEN Research Products) cut to the exact size of the gel was placed in alkaline transfer solution, allowed to soak up the solution by capillary action and placed onto the gel ensuring that the correct side of the membrane contacted the gel. Air spaces between the hybridisation membrane and the filter papers were removed and six pieces of Whatman filter paper cut to the exact size of the gel and saturated in alkaline transfer solution were placed onto the membrane. Dry paper hand towels cut to the size of the sandwich were placed onto the filter papers and the sandwich weighted with a glass plate and a 500 ml bottle filled with water. Finally, 300 ml alkaline transfer solution was added to the enamel tray and the apparatus left overnight to allow transfer of the DNA onto the membrane.

When transfer was complete, the membrane was placed in 100 ml neutralising solution for 15 minutes with occasional agitation. The membrane was daubed with absorbent tissue to remove excess neutralising solution and the DNA immediately cross-linked to the damp membrane by exposure to 120 mJ of UV light in a UV Stratalinker 1800. The membrane was hybridised immediately, or stored on the bench for future use.

During hybridisation, constant mixing and temperature control was maintained by use of a Hybaid oven. All buffers and the Hybaid hybridisation tubes were heated to 70° in the oven before use. The temperature was kept at 70° as stringently as possible throughout the following procedure.

The membrane was rolled in a Hybaid nylon support membrane, placed inside a hybridisation tube and pre-hybridised in 50 ml Southern pre-hybridisation buffer for 1–2 hrs. After removal of the Southern pre-hybridisation buffer, 20 ml Southern hybridisation buffer was added to the tube and agitation continued for 3–5 hrs. Purified, denatured [^{32}P]-labelled probe was added to the Southern hybridisation buffer and agitation continued for 16–24 hrs.

The membrane was washed for 45 minutes in 50 ml of a solution equivalent to the Southern pre-hybridisation buffer, followed by 3 × 30 minute washes with 100 ml membrane wash buffer.

The hybridised membrane was removed from the tube, daubed with an absorbent tissue to remove excess liquid and exposed to autoradiographic film.

3.16. Northern transfer and hybridisation.

The method used to transfer RNA onto hybridisation membranes and to hybridise the bound RNA with DNA probes was described in the GeneScreen *Plus* transfer and detection protocols booklet (NEN Research Products).

The photographed gel was blotted onto GeneScreen *Plus* using the technique described for Southern transfer but with 10× SSPE as the transfer solution.

After transfer, the membrane was rinsed briefly in 2× SSPE, dried at room temperature and baked at 80° for 2 hrs to remove traces of formaldehyde.

Hybridisation was at 42° in a Hybaid oven. The oven, buffers and hybridisation tubes were warmed to 42° before hybridisation. The membrane was pre-hybridised in 50 ml northern pre-hybridisation buffer for 2–4 hrs at 42°, the northern pre-hybridisation buffer was removed and 20 ml northern hybridisation buffer was added and incubation continued for 1–3 hrs. The purified, denatured [^{32}P]-labelled DNA probe was added and agitation continued for 16–24 hrs.

The membrane was washed in 50 ml 2× SSPE for 15 minutes at 42°, removed from the hybridisation tube, transferred to a plastic container and subsequently washed by agitation in 200 ml 2× SSPE for 15 minutes at room temperature, 400 ml 2× SSPE / 2% SDS in a 65° water bath for 45 minutes and finally in 200 ml 0.1× SSPE at room temperature for 15 minutes.

The membrane was dried by daubing with an absorbent tissue and exposed to autoradiographic film.

3.17. Labelling of probes by random primer extension.

DNA was radiolabelled with α-[^{32}P] dNTPs by extension of hybridised random sequence hexadeoxyribonucleotides with Klenow DNA polymerase (Feinberg and Vogelstein, 1983).

The DNA fragment to be radiolabelled was made to a volume of 22 µl in a 1.5 ml vial and denatured by placing in a boiling water bath for 2 minutes followed by incubation on ice for 10 minutes. The solution was collected at the bottom of the vial by brief centrifugation and 10 µl oligo reaction mix was added followed by 2 µl 0.1% BSA, 2.5 µl each of two 4 mM dNTPs, 5 µl each of the remaining two α-[^{32}P]-labelled dNTPs (specific activity 10 mCi/ml) and 1 µl Klenow enzyme (2 units/µl). The labelling reaction mixture was incubated at room temperature overnight or at 37° for 2 hrs.

The proportion of [^{32}P] incorporated into DNA was assessed as follows. 0.5 µl of the labelling reaction was diluted in 10 µl H_2O and 3 µl of the dilution spotted onto each of 2 Whatman grade 1 2.5 cm diameter filter papers. One of the filters papers was placed in 100 ml 10% TCA for 10 minutes at room temperature with occasional agitation, the remaining filter paper was left untreated. During the wash in 10% TCA, fragments of DNA remain bound to the filter paper, whereas unincorporated dNTPs are soluble in acid. The 10% TCA was discarded, 50 ml ethanol was added and the filter paper rinsed by swirling the solution. The ethanol was discarded and the filter paper rinsed again in 50 ml ethanol. Both filter papers were dried under a 275 Watt infra-red reflector lamp, placed in scintillation vials with 5 ml Ecoscint A (national diagnostics) and the radioactivity was

determined in a scintillation counter. The incorporation was determined as the percentage of radioactivity on the 10% TCA-washed filter paper compared to the total (100%) radioactivity on the untreated filter paper. Only labelling reactions with incorporation >30% were used.

[^{32}P]-labelled DNA was separated from unincorporated [^{32}P]-labelled dNTPs by passage through a 10 ml column of swollen medium sephadex G-50 (Pharmacia Chemicals).

The purified DNA probe was denatured by adding NaOH to a final concentration of 0.15 M and incubating at room temperature for 15 minutes.

3.18. Purification of DNA from agarose gels.

DNA was purified from agarose gel slices using the Sephaglas BandPrep Kit (Pharmacia Chemicals).

The band containing the DNA to be purified was excised from the agarose gel during visualisation with long wavelength UV light and transferred to a 1.5 ml vial. 250 µl gel solubiliser was added and the vial placed in a 60° water bath until the gel slice had dissolved completely. If the gel slice had not dissolved after 15 minutes, a further 200 µl gel solubiliser was added and incubation continued. The container of Sephaglas was shaken to produce an even suspension and 5 µl added to the dissolved agarose gel slice for each µg of DNA to be purified. The DNA was allowed to bind to the Sephaglas by incubation at room temperature for 5 minutes with agitation every minute. The Sephaglas with bound DNA was pelleted by centrifugation at 13000 rpm for 10 seconds in a benchtop microfuge and the supernatant discarded. The pellet was washed by resuspending the Sephaglas in a volume of wash buffer 8× the volume of Sephaglas added above and pelleted again by centrifugation at 13000 rpm for 10 seconds. The washing procedure was repeated twice to give a total of three washes. After the final wash had been removed, the vial was flicked to partially disperse the Sephaglas and the pellet dried by lying the vial on the bench for ≥45 minutes. DNA was eluted from the Sephaglas by resuspending in a volume of elution buffer 2× the volume of Sephaglas used and incubating at room temperature for 5 minutes with agitation every minute. The suspension was centrifuged at 13000 rpm for 1 minute and the supernatant containing the DNA removed to a fresh vial. The elution step was repeated to maximise the yield of DNA.

3.19. DNA Probes.

The diagrams in figures 3.2. and 3.3. indicate the positions of DNA probes to detect sequences within the LAT / IE1 and TK regions respectively.

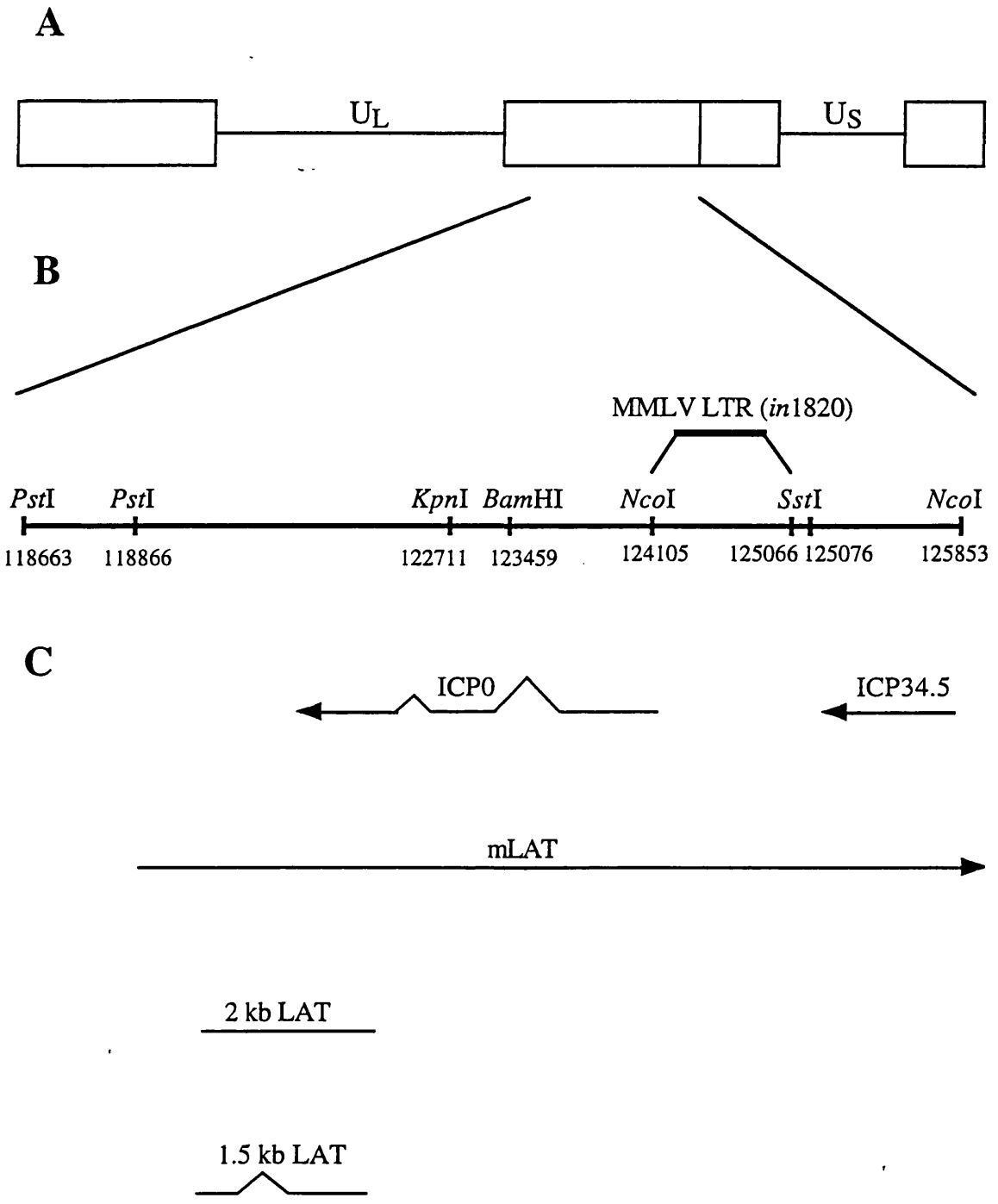


Figure 3.2. Location of probe fragments at the LAT / IE1 region of the genome. (A) Representation of the HSV-1 genome showing the location of the unique long and unique short regions. (B) The internal copy of the long repeat region expanded with the the location of the restriction endonuclease sites relevant to the studies in the thesis presented in the thesis presented here indicated. The replacement of the wild type IE1 promoter with the MMLV LTR, yielding the *in*1820-based viruses, is depicted (details on the construction of *in*1820 are published in Jamieson *et al.* 1995). (C) Transcripts arising from the LAT / IE1 region and their positions relative to the restriction sites.

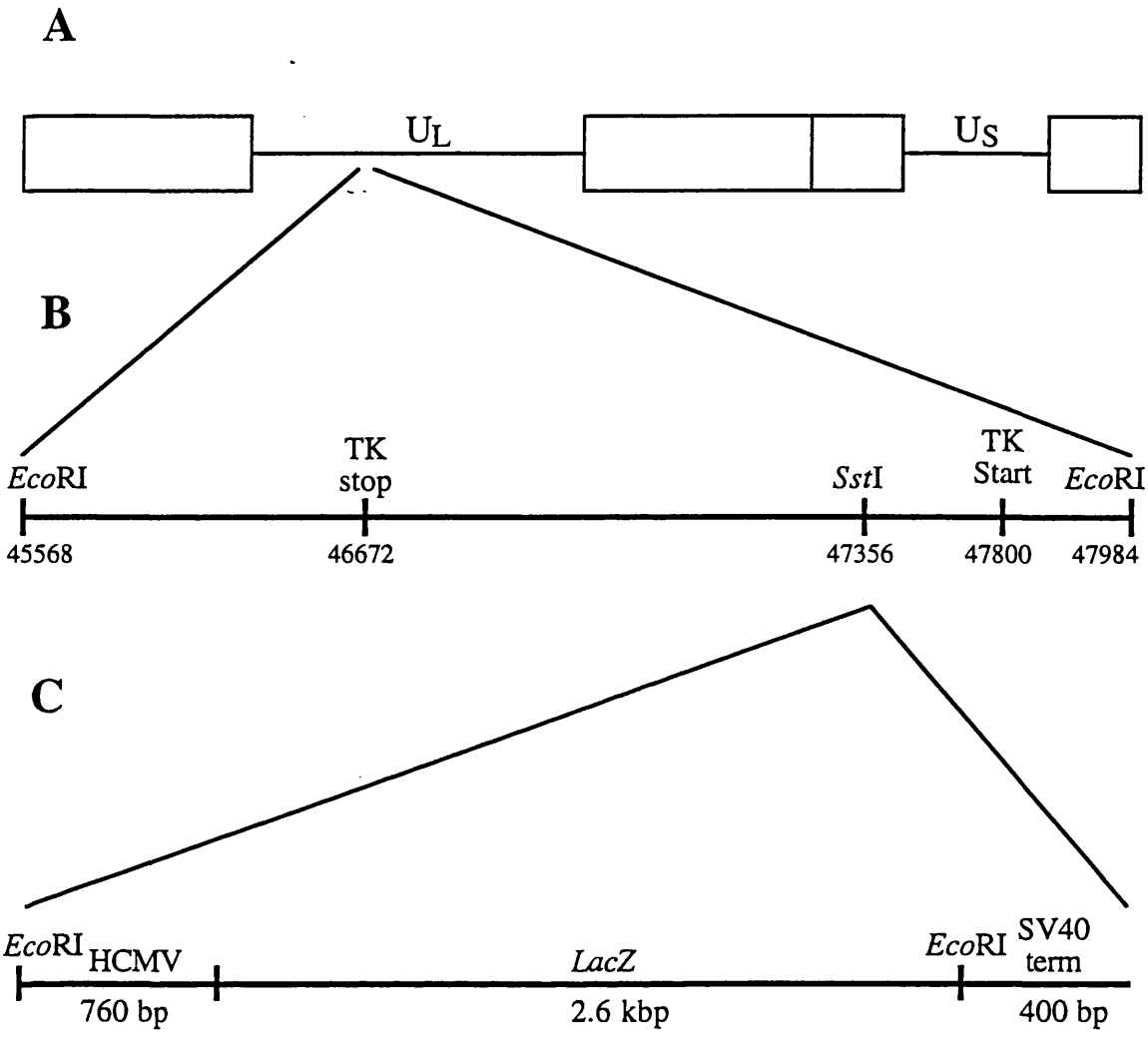


Figure 3.3. Location of the *Eco*RI restriction endonuclease cleavage sites at the TK locus and its modification by the insertion of the HCMV IE promoter controlling *lacZ*. (A) Representation of the HSV-1 genome showing the location of the unique long and unique short regions. (B) The *Eco*RI fragment encompassing U_L23, encoding the TK gene, expanded from U_L region. (C) The position of insertion of the HCMV IE promoter controlling transcription of the *E.coli lacZ* gene with the SV40 termination signal. With viruses wild type at the TK locus, *Eco*RI digestion yields a single fragment of 2.42 kbp, viruses containing the HCMV / *lacZ* insertion produce 2 *Eco*RI fragments, one of 1.03 kbp and one of 1.80 kbp.

3.20. Autoradiography.

Hybridised membranes were exposed at –70° to X-omat S film (Kodak) with a DuPont Cronex Lightning Plus intensifying screen.

3.21. Stripping of DNA probes from hybridisation membranes.

Membranes were boiled for 30 minutes in a solution of 10 mM Tris HCl pH 7.5, 1 mM EDTA and 1% SDS. To assess the efficiency of stripping, the membrane was exposed to autoradiographic film.

3.22. Quantification of hybridisation.

Hybridisation was quantified using a Molecular Dynamics PhosphorImager with Molecular Dynamics ImageQuant software.

To calculate the relative proportions of nonlinear and linear molecules of latent *in*1820, the relative band peak areas of hybridisation to the joint and terminal fragments were determined for latent (JLAT and TLAT respectively) and virion (JVIR and TVIR respectively) DNA. The ratio of joint / terminus for latent DNA was adjusted for the hybridisation bias observed in virion DNA.

$$\text{JLAT} / \text{TLAT} \times \text{TVIR} / \text{JVIR} = x$$

As each nonlinear molecule has two joints, and each linear molecule contributes one joint, the number of nonlinear genomes per linear genome was $0.5(x-1)$.

The number of *in*1820 genomes per cell was calculated by comparing hybridisation to the joint fragments of the latent sample to hybridisation to the virion DNA standards, which were of known mass. The method used to calculate the number of *in*1820 genomes per cell was as follows.

Because the majority of virion genomes contain 1 copy of the joint fragment and most latent genomes contained 2 copies, it was first necessary to adjust the band peak areas of hybridisation to the joint of latent DNA to that which would have occurred from an equivalent mass of virion DNA.

The contribution of hybridisation to the joint from linear genomes in the latent DNA sample (*Jlinear*) was calculated from the J : T ratio of hybridisation to virion DNA. The hybridisation to the joint from the latent sample was then adjusted to give the hybridisation that would have occurred from an equivalent mass of virion DNA.

(Total hybridisation to joint – *Jlinear*) ÷ 2 = Adjusted hybridisation to the joint from the nonlinear genomes

Total hybridisation to joint of latent sample, from an equivalent mass of virion DNA = *Jlinear* + adjusted hybridisation to the joint from the nonlinear genomes

The value of hybridisation to the joints of the virion DNA samples which were of known mass, were used to calculate the mass (*M*) of latent DNA represented by the adjusted hybridisation to the joint from an equivalent mass of virion DNA.

The number of genomes in the sample was then calculated using *M*, the molecular mass of HSV-1 DNA (96×10^6 g/mol) and Avagadro's constant (6×10^{23} /mol).

$$6 \times 10^{23}(M) \div 96 \times 10^6 = \text{number of genomes.}$$

The number of genomes per cell = number of genomes in the sample ÷ number of cells from which the DNA sample was extracted.

3.23. Ligations.

Ligation reactions with a total volume of 5 µl consisted of various ratios of vector : insert, 1× ligase buffer and 0.5 units of T4 DNA ligase. The ingredients were kept chilled on ice while the reactions were being set up. Ligations were incubated at 15° for 20–24 hrs before being used to transform *E.coli*. For each ligation experiment, a control ligation of vector without insert was performed in parallel to determine the background of vector ligated to itself.

3.24. Preparation of competent *E.coli*.

1 ml of an *E.coli* K12 DH-1 culture which had been grown in L-broth overnight was added to 1 ml 80% (v/v) glycerol and stored at –20° for use as a seed stock.

10 ml L-broth was inoculated with 20 µl seed stock and shaken overnight at 37°. The following day, 90 ml L-broth was inoculated with 2.5 ml of the overnight culture and shaken at 37° until the bacteria had grown to an OD_{590} of 0.2, usually after 1.5–2 hrs. The cells were pelleted by centrifugation at 5000 rpm for 10 minutes at 4° in a Sorvall SS34 rotor, resuspended in 40 ml 100 mM $CaCl_2$ which had previously been cooled to 4° and incubated on ice for 1–2 hrs. The DH-1 bacteria were pelleted by centrifugation at 5000 rpm for 10 minutes at 4° in a Sorvall SS34 rotor, resuspended in 800 µl 100 mM $CaCl_2$ and stored on ice. The DH-1 bacteria were then competent and could be stored at 4° for up to 20 hrs before transformation.

3.25. Transformation of *E.coli.*

50 µl of a competent *E.coli* K12 DH-1 preparation was added to a ligation reaction and incubated on ice for 1–3 hrs. The bacteria were heat shocked by placing the vial in a 42° waterbath for 1 minute 15 seconds, added to 0.5 ml L-broth and shaken at 37° for 1 hrs 15 minutes to enable the transformed cells to express β-lactamase. 150 µl was spread in triplicate on the surface of 1.5% agar / L-broth / ampicillin set in 8.5 cm diameter Sterilin Petri dishes (Bibby Sterilin Ltd) and incubated at 37° overnight.

3.26. Small scale preparation of plasmid DNA from *E.coli.*

Colonies were picked with a sterile plastic pipette tip, inoculated into 2 ml L-broth containing ampicillin and shaken at 37° overnight. The plasmid copy number was amplified by adding chloramphenicol to a concentration of 20 µg/ml and shaking at 37° overnight. 1.5 ml of a chloramphenicol-treated culture was transferred to a 1.5 ml vial and the bacteria pelleted by centrifugation at 6500 rpm for 2 minutes in a benchtop microfuge. After the supernatant had been discarded, 100 µl 9 volumes STET : 1 volume 10 mg/ml lysozyme was added and the sample vortex mixed. Lysis of the *E.coli* was completed by incubation in a boiling water bath for 1 minute 15 seconds. The bacterial debris was pelleted by centrifugation at 13000 rpm in a benchtop microfuge and the supernatant containing the plasmid DNA was added to a vial containing 400 µl 0.3 M CH_3COONa pH 7.0. DNA was precipitated by adding 0.5 ml isopropanol and incubating at –20° for ≥1 hr. The precipitated DNA was pelleted by centrifugation at 13000 rpm for 10 minutes in a benchtop microfuge, the supernatant removed and the pellet washed by adding 0.5 ml 70% (v/v) ethanol and gentle mixing. The pellet was collected at the bottom of the vial by centrifugation at 13000 rpm for 5 minutes, the 70% ethanol removed and the pellet allowed to dry by lying the tube on the bench with the lid open for ≥45 minutes. The plasmid DNA pellet was resuspended in 33 µl of H_2O and 5 µl was used for incubation with the required restriction enzyme(s) in the presence of 10 µg/ml RNase A, followed by agarose gel electrophoresis.

3.27. Large scale preparation of plasmid DNA from *E.coli.*

Large scale preparation of plasmid DNA from *E.coli* was achieved with the Quiagen Plasmid Midi kit (Quiagen Inc.).

A glycerol stock of DH-1 bacteria harbouring the plasmid to be purified was streaked onto the surface of 1.5% agar / L-broth / ampicillin using a platinum loop. Colonies were grown by incubation at 37° overnight. A single colony was picked, inoculated into 10 ml L-broth containing ampicillin and shaken at 37° overnight. 100 ml L-broth containing ampicillin was inoculated with 1ml of the overnight culture and shaken

at 37° overnight. The plasmid content of the bacteria was amplified by adding chloramphenicol to 20 µg/ml and shaking at 37° overnight.

The bacterial cells were pelleted by centrifugation in a GSA rotor at 8000 rpm for 5 minutes, the pellet was resuspended thoroughly in 4 ml buffer P1 and transferred to a 40 ml plastic centrifuge tube. 4 ml buffer P2 was added, the sample mixed by gently inverting the tube 6 times and incubated at room temperature for 5 minutes. 4 ml buffer P3 which had previously been cooled to 4° was added and the sample mixed gently and incubated on ice for 15 minutes with occasional gentle agitation. Debris from the lysed bacteria was pelleted by centrifugation in a Sorvall SS34 rotor at 16000 rpm for 15 minutes at 4° and the supernatant containing the plasmid DNA promptly transferred to a Quiagen-tip 100 which had been previously equilibrated with 4 ml buffer QBT. After the supernatant had passed through the column, the resin plus bound plasmid DNA was given 2 × 10 ml washes with buffer QC and the DNA eluted with 5 ml buffer QF into a 30 ml glass Corex centrifuge tube. Plasmids were precipitated by adding 3.5 ml isopropanol and mixing. The plasmid DNA was pelleted by centrifugation in an SS34 rotor at 10000 rpm for 30 minutes at 4°. After removal of the supernatant, the pellet was washed briefly with 5 ml 70% (v/v) ethanol and dried by lying the tube on the bench for ≥45 minutes. The dried pellet was resuspended in 2 × 250 µl of sterile distilled H_2O and stored at –20°.

3.28. Determination of DNA concentration.

Concentration and purity of plasmid preparations was determined by measuring the absorbence at wavelengths of 260 nm and 280 nm in a Beckman Du-62 spectrophotometer. An OD of 1 at 260 nm was taken as corresponding to a concentration of 50 µg/ml. The ratio of the OD at 260 nm : OD at 280 nm gives an indication of purity and is 1.8 for an ideal aqueous solution of dsDNA.

3.29. Transfection of DNA into tissue culture cells.

DNA was transfected into tissue culture cells by the calcium phosphate precipitation method adapted from the technique described by Stow and Wilkie (1976).

HSV-1 DNA for transfections was prepared as follows. 100 µl of a cell released virus preparation was incubated at 37° overnight with SDS / proteinase K buffer in a total volume of 500 µl. The sample was extracted with phenol / chloroform and the DNA isolated as described in section 3.10. The purified viral DNA pellet was resuspended by adding 50 µl H_2O and incubating at 4° overnight.

HSV-1 DNA was cotransfected with plasmid DNA into BHK cells in 35 mm diameter tissue culture dishes.

BHK cells used for transfections were seeded the previous day and had grown to 80–100% confluence.

The transfection mix was made and stood at room temperature for 5 minutes before use.

Transfection mix.

calf thymus DNA	2.75 µg
HSV-1 DNA	0.1–0.3 µg
linear plasmid DNA	0.2–0.6 µg
HEBS	1×
$CaCl_2$	136 mM
Total volume	132 µl

The transfection mix was dripped onto a BHK cell monolayer in a 35 mm dish and the cells incubated at 37° for 40–45 minutes with rocking every 10 minutes to prevent drying. 2 ml ETC_{10} was added and incubation continued at 37° for 3–4 hrs.

The medium was poured from the dish and the monolayer washed with 2 ml ETC_{10}. The ETC_{10} was removed thoroughly and the monolayer overlaid with 0.3 ml 25% DMSO / 1× HEBS and incubated at room temperature for 4 minutes with frequent rocking. The DMSO / HEBS was removed thoroughly and after 2 × 2 ml washes with ETC_{10}, 2 ml ETC_{10} was added and the cells incubated at the appropriate temperature until plaques were visible under a low magnification light microscope.

3.30. Construction of HSV-1 recombinants.

HSV-1 recombinants were constructed by cotransfection of purified viral DNA with a linearised plasmid encoding the mutation or insertion flanked by viral sequences. Plasmids were linearised by cleavage with a restriction enzyme, usually at a cleavage site within the vector sequences. Recombination between the viral sequences flanking the mutation and the homologous sequences in the viral DNA yielded the desired recombinant virus.

The temperature sensitive uncoating mutant *in*1815 was constructed by cotransfection of *in*1814 DNA with pLR1 which had been linearised with *Hind*III. After plaques had appeared, the cells were harvested, bath sonicated, diluted serially in 10-fold steps and 200 µl of each dilution was used to infect a BHK cell monolayer in a 35 mm tissue culture dish. After the 1 hr adsorption period the inocula were removed and the cells overlaid with 2 ml per dish of ETC_{10} containing 3 mM HMBA and incubated at 31° for 16 hrs. The ETC_{10} containing 3 mM HMBA was removed and the cells overlaid with

2 ml per dish of agar overlay without X-gal and incubated at 31° until plaques were visible under a low magnification light microscope. Plaques were picked in a volume of 15 µl with a micropipette, transferred to 500 µl ETC_{10} and bath sonicated. The virus in each plaque isolate was screened for temperature sensitivity by inoculating 100 µl onto each of two BHK cell monolayers in a single well of two replicate 24-well tissue culture plates. After the 1 hr adsorption period the inocula were removed and the cells overlaid with 1 ml per well of CMC/ETC_{10} containing 3 mM HMBA. One of the plates was incubated at 31° and the other at 39.5°. The monolayers at 31° were incubated for 4 days and the monolayers at 39.5° were incubated for 2 days before staining with Giemsa. A plaque isolate which contained temperature sensitive virus was subject to 2 further plaque purifications before being grown as a seed stock.

To ensure that the virus in the positive plaque isolate contained the same temperature sensitive lesion as *ts*1213, it was subject to the coinfection / recombination experiment with *ts*K and *ts*1213 described in section 4.29. To ensure *in*1815 had a defect in uncoating at 39.5°, it was subjected to the Southern blot assay described in section 4.30.

The temperature sensitive mutation in *in*1815 was rescued by cotransfection of *in*1815 DNA with a plasmid containing the *Kpn*I C fragment from wild type HSV-1. After plaques had appeared, the cells were harvested, bath sonicated, diluted serially into 10-fold dilutions and 200 µl of each dilution was used to infect a BHK cell monolayer in a 35 mm diameter tissue culture dish. After the 1 hr adsorption period the inocula were removed and the cells overlaid with 2 ml per dish of CMC/ETC_{10} containing 3 mM HMBA and incubated at 39.5° for 2 days. Plaques were picked and subjected to 2 plaque purifications at 39.5°. To ensure that the $Vmw65^-$ phenotype was retained, the response of the viral titre to HMBA during infection of BHK cells was assessed (section 4.31.). The rescuant of *in*1815 was named *in*1816.

To construct HSV-1 mutants expressing β-galactosidase, DNA isolated from the virus in which the insertion was to be made was cotransfected with a linearised plasmid containing the *lacZ* gene with desired controlling elements and flanking sequences. After plaques had appeared, the cells were harvested, bath sonicated, diluted serially into 10-fold dilutions and 200 µl of each dilution was used to infect a BHK cell monolayer in a 35 mm diameter tissue culture dish. After the 1 hr adsorption period the inocula were removed and the cells overlaid with 2 ml per dish of ETC_{10} containing 3 mM HMBA and incubated at the appropriate temperature for 16 hrs. The ETC_{10} containing 3 mM HMBA was removed and the cells overlaid with 2 ml per dish of agar / X-gal overlay and incubated at the appropriate temperature until plaques were visible under a low magnification light microscope. Replicating plaques formed by viruses expressing β-galactosidase were visualised as staining blue in the presence of X-gal. Blue plaques were picked in a volume of 15 µl with a micropipette, transferred to 1 ml ETC_{10}, bath

sonicated and subjected to plaque purifications until approximately 50% of plaques were stained blue by the agar / X-gal overlay.

Plaque isolates containing virus which expressed β-galactosidase were screened by Southern blotting to ensure the correct sizes of restriction endonuclease fragments at the region of modification. 500 µl of plaque isolate was added to a monolayer of BHK cells in a well of a 24-well tissue culture plate and the cells incubated at the appropriate temperature for 1 hr. After the adsorption period, 500 µl ETC_{10} containing 6 mM HMBA was added to the well and incubation continued until the cells exhibited severe CPE. The medium was removed, 200 µl SDS / proteinase K buffer was added to the monolayer and the cells incubated at 37° overnight. The following day, 150 µl of the cell extract was placed in a 1.5 ml vial. The cell extract was made to a final concentration of 0.3 M CH_3COONa pH 7.0 and a total volume of 500 µl. The sample was extracted with phenol / chloroform and the DNA purified as described in section 3.10.

The purified DNA pellet was resuspended by adding 25 µl of H_2O and incubating at 4° overnight. The resuspended pellet was incubated at 37° overnight with 5 units of the appropriate restriction endonuclease(s) and 10 µg/ml RNase A in a total volume of 50 µl. The next day, 5 µl of the digested infected cell DNA was further digested with 5 units of the restriction endonuclease(s) for 3 hrs at 37° in a total volume of 20 µl. 6 µl of 5× ficoll loading buffer was added and 10 µl electrophoresed in an agarose gel and subjected to Southern blotting and hybridisation.

3.31. Staining of tissue culture monolayers for β-galactosidase.

Medium was removed from a cell monolayer in a 35 mm diameter tissue culture dish and the cells washed with 2 ml PBS A. To fix the cells, the monolayer was overlaid with 1 ml PBS A containing 1% glutaraldehyde and incubated at room temperature for 45–60 minutes. The glutaraldehyde was removed and after two 2 ml washes with PBS A, 1 ml β-galactosidase stain was added and the monolayer incubated at 37° until blue foci were easily visible under a low magnification light microscope (usually after 3–5 hrs). The β-galactosidase stain was removed, the monolayer washed with 2 ml distilled water and blue foci counted under a dissection microscope.

4. RESULTS.

OBJECTIVES.

The objectives of the study described here were to extend previous structural analysis of the viral genomes in the *in vitro* latency system described by Jamieson *et al.* (described in section 1.3.4.3.; Jamieson, 1993; Jamieson *et al.*, 1995), and to investigate important early events in the establishment of *in vitro* latency.

CHAPTER 1.

Structural studies on HSV-1 during latency in IFNα-treated HFL cells.

4.1. *In*1820 genomes were retained in a nonlinear form.

The HSV-1 genome is a nonlinear episome during latency in neurons (Rock and Fraser, 1983; Rock and Fraser, 1985; Efstathiou *et al.*, 1986). In order to determine whether *in*1820 DNA was nonlinear in the *in vitro* latency system, IFNα-treated HFL cells were infected with 75 particles of *in*1820 per cell and incubated for 3 days in the presence of Ara-C. Nuclear DNA was purified, cleaved with *Bam*HI, electrophoresed alongside known amounts of virion DNA, blotted and hybridised to radiolabelled DNA fragment from a region of the MMLV LTR (figure 3.2.). Use of the MMLV LTR probe enabled detection of the joint and L-terminal *Bam*HI restriction fragments and also minor species caused by variation in the number of copies of the *a* sequence.

Hybridisation showed a predominance of the viral *Bam*HI restriction fragments joining the long and short regions of the genome and a reduction in the L-terminal *Bam*HI fragments, indicating that the ends of the genomes were joined together (figure 4.1., lanes 3, 7 and 11). The degree of conversion to a nonlinear form was quantified by comparison with hybridisation to purified virion DNA (lanes 1–2, 5–6 and 9–10). Table 4.1. shows results of quantification of the degree of non-linearity of latent *in*1820 obtained from two separate experiments, the autoradiographs of which are shown in figures 4.3. and 4.5. The values for the 6 latent *in*1820 samples ranged between 75% and 92% nonlinear, the average was 84% nonlinear.

Table 4.1. also shows the results of calculations to determine the average number of genomes retained per cell. *In* 1820 genomes were retained between a range of

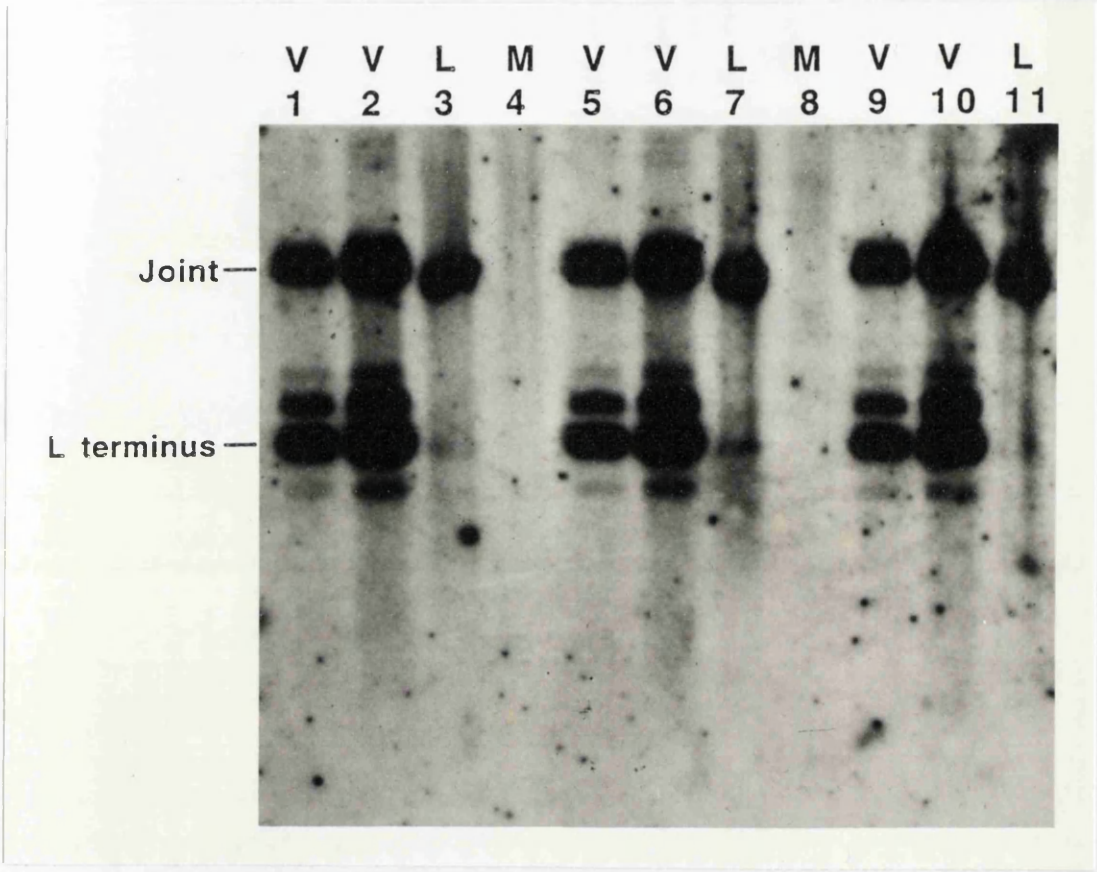


Figure 4.1. Configuration of viral genomes during *in vitro* latency. IFNα-treated HFL cells in 60 mm diameter tissue culture dishes were infected with 75 particles of *in*1820 per cell (lanes 3, 7 and 11) or mock infected (lanes 4 and 8) and overlaid with EF_{10} containing 50 μg/ml Ara-C. After incubation at 37° for 3 days the nuclei were isolated and the DNA purified. DNA was cleaved with *Bam*HI and half of each sample electrophoresed in a 0.7% agarose gel alongside 1.5 ng (lanes 1, 5 and 9) and 5.0 ng (lanes 2, 6 and 10) *in*1825 virion DNA standards. Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 455 bp *Bam*HI / *Bfr*I fragment from the MMLV LTR (figure 3.2.). Positions of the joint and L-terminal *Bam*HI restriction fragments are indicated. L: latently infected sample; M: mock infected sample; V: virion DNA.

Figure	4.3		4.5			
Lane	4	11	1	2	3	7
% Nonlinear	84	90	86	76	92	75
Average % nonlinear	84					
Copy number (genomes /cell)	6.3	5.6	14.7	12.0	15.6	18.8
Average copy number (genomes /cell)	12.2					

Table 4.1. Configuration and copy number of latent *in*1820 genomes. Hybridisation to the latent *in*1820 DNA samples and virion DNA standards in figures 4.3. and 4.5. was quantified. The percentage of latent *in*1820 genomes which were converted to a nonlinear form and the number of genomes retained per cell were calculated by comparison to the virion DNA standards .

6.3 and 18.8 genomes per cell with an average of 12.2 genomes per cell. The data in table 4.1. are derived from experiments in which the input MOI was 110 particles per cell, thus it is probable that approximately 90% of the input DNA was lost. For an accurate determination of the percentage of viral DNA which was retained during latency, a direct comparison with DNA isolated from the virus inoculum was required. Jamieson *et al.* showed that input *in*1820 genomes not retained in the cell nuclei were undetectable in the cytoplasm or culture medium and were presumably degraded, although the nonlinear DNA was stable for up to 4 days after infection (Jamieson *et al.*, 1995).

Retention of nonlinear HSV genomes in *in vitro* latency systems using HFL cells has been observed previously (Harris and Preston, 1991). *In*1814 was retained as a

nonlinear molecule after infection of HFL cells at low MOI and maintenance in the presence of Ara-C or aphidicolin. The experiments described in this thesis confirm the observation made by Jamieson that nonlinear *in*1820 genomes are retained in IFNα-treated HFL cells after infection at high MOI and maintenance in the presence of Ara-C (Jamieson, 1993). Previous studies showed that most *in*1883 genomes retained after 3 days infection were competent for reactivation, as measured by β-galactosidase expression after superinfection (Jamieson *et al.*, 1995). Most of the retained *in*1820 genomes were converted to a nonlinear form and retained in virtually all of the cells in a state resembling latency.

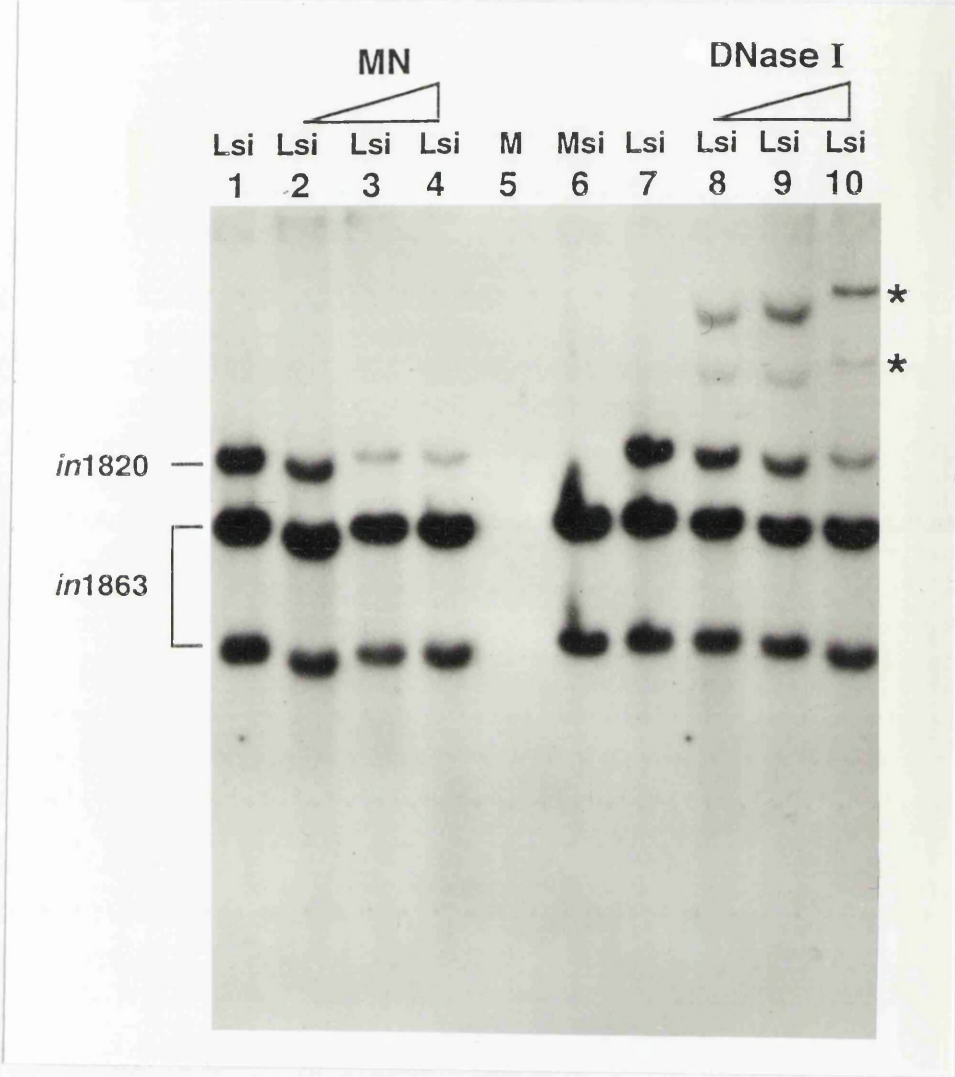
Approximately 16% of latent *in*1820 DNA was linear (table 4.1.). A characterisation of the structural differences between the linear and nonlinear DNA is described later in this chapter.

4.2. *In*1820 genomes reactivated from *in vitro* latency by superinfection were more sensitive to nucleases than the superinfecting genomes.

Jamieson demonstrated an increase in MN sensitivity of the *in*1820 TK gene as the virus proceeded to latency (Jamieson, 1993). To examine whether insensitivity to MN or DNase I was restored after superinfection and reactivation with *in*1863 (*in*1863 encodes a wild-type Vmw65 and has the HCMV major IE promoter controlling the *E.coli lacZ* gene at the TK locus), IFNα-treated HFL cells were infected with 75 particles of *in*1820 per cell and incubated for 3 days in the presence of Ara-C. The cultures were superinfected with 5 PFU of *in*1863 per cell and incubated in the presence of Ara-C. At 8 hours post-superinfection the nuclei were isolated and incubated with various concentration of MN or DNase I for various times or were untreated. The DNA was purified, cleaved with *Eco*RI, electrophoresed, blotted and hybridised to a radiolabelled *Eco*RI n fragment spanning the HSV-1 TK gene. The HCMV IE promoter / *lacZ* insert in the TK gene of *in*1863 enabled the *Eco*RI restriction fragments to be resolved from the wild-type restriction fragment of *in*1820 (figure 3.3.; figure 4.2.; positions of *in*1820 and *in*1863 TK gene restriction fragments are indicated).

The TK gene of *in*1820 was more sensitive to MN and DNase I than the TK gene of *in*1863 (lanes 2–4 and 8–10), therefore resistance to nucleases was not restored by reactivation. As both *in*1820 and *in*1863 were transcriptionally active, the difference in nuclease sensitivity of their genomes was not caused by a difference in transcriptional activity but was most likely to have been caused by the difference in the total period of time that the viruses had been infecting the cells, 3 days in the case of

Figure 4.2. Relative nuclease sensitivities of viral genomes reactivated from latency and genomes of a superinfecting virus. IFNα-treated HFL cells in 60 mm diameter tissue culture dishes were infected with 75 particles of *in*1820 per cell (lanes 1–4 and 7–10) or mock infected (lanes 5 and 6) and overlaid with EF_{10} containing 50 μg/ml Ara-C. After incubation at 37° for 3 days the cells were superinfected with 5 PFU of *in*1863 per cell (lanes 1–4 and 6–10) or mock superinfected (lane 5) and incubated at 37° in the presence of 50 μg/ml Ara-C. At 7 hours post-superinfection, the nuclei were isolated and digested with MN (lanes 2–4) or DNase I (lanes 8–10) or were untreated (lanes 1 and 5–7). DNA was purified, digested with *Eco*RI and half of each sample electrophoresed in a 1% agarose gel. Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 2.4 kbp *Eco*RI n fragment from the HSV-1 TK gene (figure 3.3.). MN digestion was as follows: 1 unit for 3 minutes (lane 2), 3 units for 5 minutes (lane 3) or 22 units for 10 minutes (lane 4). DNase I digestion was as follows: 1 unit for 3 minutes (lane 8), 2 units for 5 minutes (lane 9) or 5 units for 20 minutes (lane 10). Positions of *in*1820 and *in*1863-specific restriction fragments are indicated. * indicates the positions of partial *Eco*RI digestion products. Lsi: latent / superinfected sample; M: mock infected / mock superinfected sample; Msi: mock infected / superinfected sample.


MN
DNase I
Lsi Lsi Lsi Lsi M Msi Lsi Lsi Lsi Lsi
1 2 3 4 5 6 7 8 9 10
*
*
in1820
in1863

*in*1820 and 8 hours with *in*1863. There was a small proportion of *in*1820 DNA in the nuclei which remained intact after extensive exposure to nucleases (lanes 4 and 10).

4.3. Nonlinear viral genomes were more sensitive to nucleases than linear genomes.

Jamieson observed that *in*1820 genomes became more sensitive to MN as the virus proceeded to latency and that transcriptionally active genomes did not enter the nuclease-senstive state (Jamieson, 1993). The nuclease-sensitive state was attributed to a structure specific for the latent genomes. The finding in section 4.2. that differences in nuclease sensitivity was dependent on the time of infection suggested that sensitivity to nucleases may be determined by uncoating of the viral genomes. There was a proportion of latent DNA which was highly resistant to nucleases, indicative of two separate populations of genomes. A comparison of the nuclease sensitivities of the nonlinear and linear latent viral DNA populations was undertaken in order to determine if they represented the two separate nuclease sensitive and nuclease resistant types. IFNα-treated HFL cells were infected with 110 particles of *in*1820 per cell and incubated for 3 days in the presence of Ara-C. Nuclei were isolated and digested with various concentrations of MN or DNase I for various times or were untreated. The DNA was purified, electrophoresed alongside virion DNA, blotted and hybridised to a radiolabelled fragment from the MMLV LTR.

The nonlinear DNA was the nuclease sensitive fraction and the linear DNA was nuclease resistant (figure 4.3. lanes 5–7 and 12–14; table 4.2.). The nuclease resistant linear viral DNA was probably from nonuncoated genomes associated with the nuclear pores or membranes which co-purified with the nuclei. It was clear, however that a significant proportion of the genomes (84%) were stably retained as nonlinear, uncoated, transcriptionally repressed, reactivatable molecules and were thus biologically relevant. The data of Jamieson (1993) can now be interpreted in light of the finding that differences in nuclease sensitivity are caused by differences in uncoating. Experiments which examined the nuclease sensitivity of viral DNA after infection with Vmw65$^+$ viruses were limited to the short term due to cytotoxicity (Jamieson, 1993), but it seems likely that transcriptionally active viruses would become nuclease sensitive if long term experiments were possible.

The experiment presented in figure 4.3. and table 4.2. examined the nuclease sensitivity of the MMLV LTR in the *in*1820 long repeats. To examine whether the joint regions were in a structure distinct from the rest of the genome, rendering them more sensitive to nucleases, the hybridisation membrane depicted in figure 4.3. was stripped and re-hybridised to a radiolabelled 2.4 kbp *Eco*RI n fragment from the HSV-1 TK

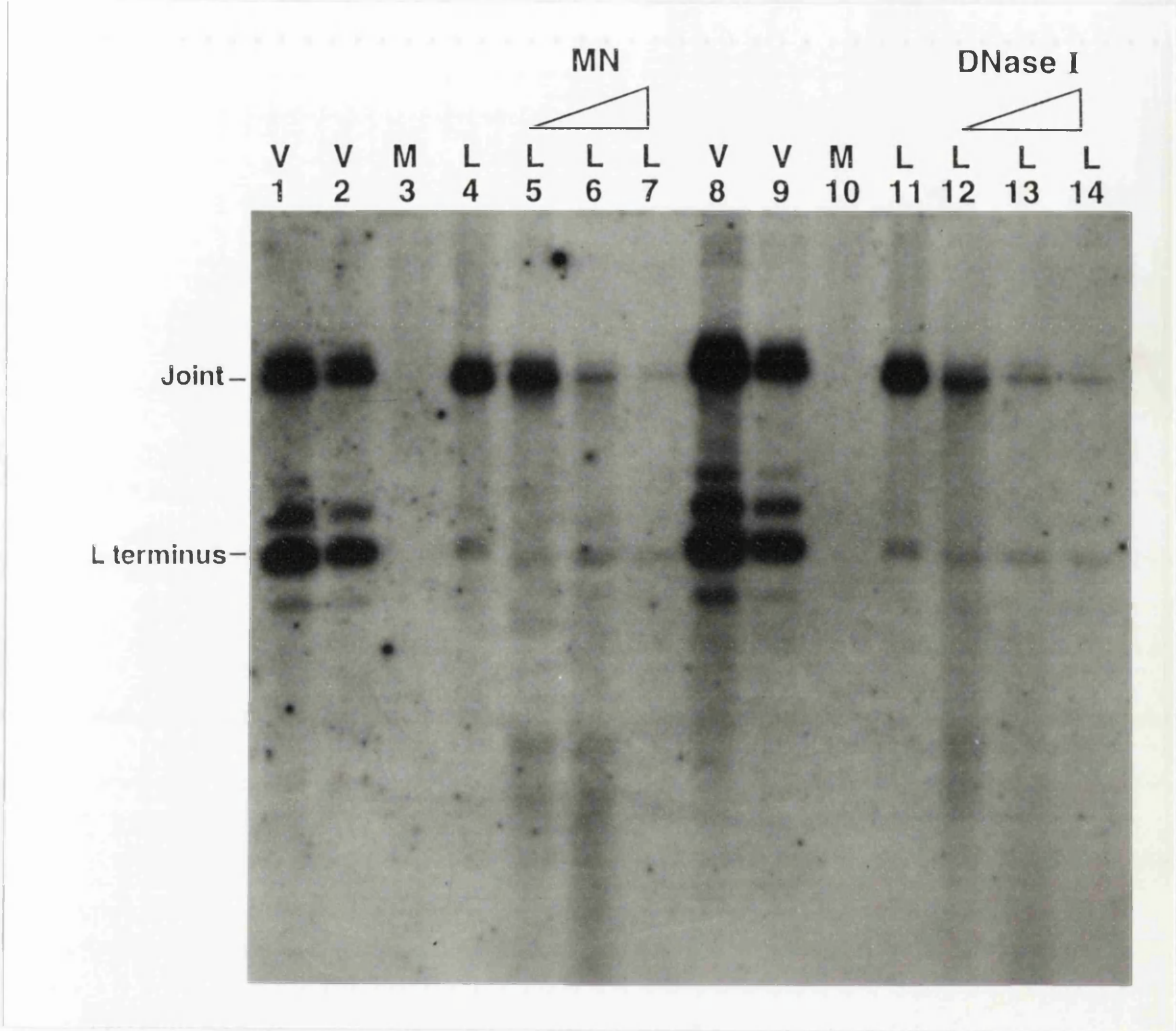


Figure 4.3. Relative nuclease sensitivities of nonlinear and linear viral genomes. IFNα-treated HFL cells in 60 mm diameter tissue culture dishes were infected with 110 particles of *in*1820 per cell (lanes 4–7 and 11–14) or mock infected (lanes 3 and 10) and overlaid with EF_{10} containing 50 μg/ml Ara-C. After incubation at 37° for 3 days the nuclei were isolated and digested with MN (lanes 5–7) or DNase I (12–14) or were untreated (lanes 3–4 and 10–11). DNA was purified, digested with *Bam*HI and half of each sample electrophoresed in a 0.7% agarose gel alongside 1.5 ng (lanes 2 and 9) and 5.0 ng (lanes 1 and 8) *in*1825 virion DNA standards. Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 455 bp *Bam*HI / *Bfr*I fragment from the MMLV LTR. MN digestion was as follows: 1 unit for 3 minutes (lane 5), 3 units for 5 minutes (lane 6) or 22 units for 10 minutes (lane 7). DNase I digestion was as follows: 1 unit for 3 minutes (lane 12), 2 units for 5 minutes (lane 13), or 5 units for 20 minutes (lane 14). Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. L: latently infected sample; M: mock infected sample; V: virion DNA.

Nuclease	MN			DNase I			
Enzyme units	0	1	3	0	1	2	5
Incubation time (mins)	0	3	5	0	3	5	20
% hybridisation to joint	100	62	12	100	32	9.8	8.5
% hybridisation to L terminus	100	124	114	100	132	56	72

Table 4.2. Nuclease sensitivities of *in*1820 joint and L-terminal *Bam*HI restriction fragments. Hybridisation to the nuclease treated and untreated latently infected nuclear DNA samples in figure 4.3. was quantified and the relative amounts of hybridisation to the joint and L-terminal restriction fragments determined. The non-nuclease treated samples represent 100% DNA.

gene, resulting hybridisation is presented in figure 4.4. Quantification revealed that the nuclease sensitivity of the TK gene, which is located in the unique long region of the genome, did not significantly differ from the joint region (table 4.3.). Sensitivity to nucleases was presumable a property of the entire nonlinear genomes and was not confined to the joints.

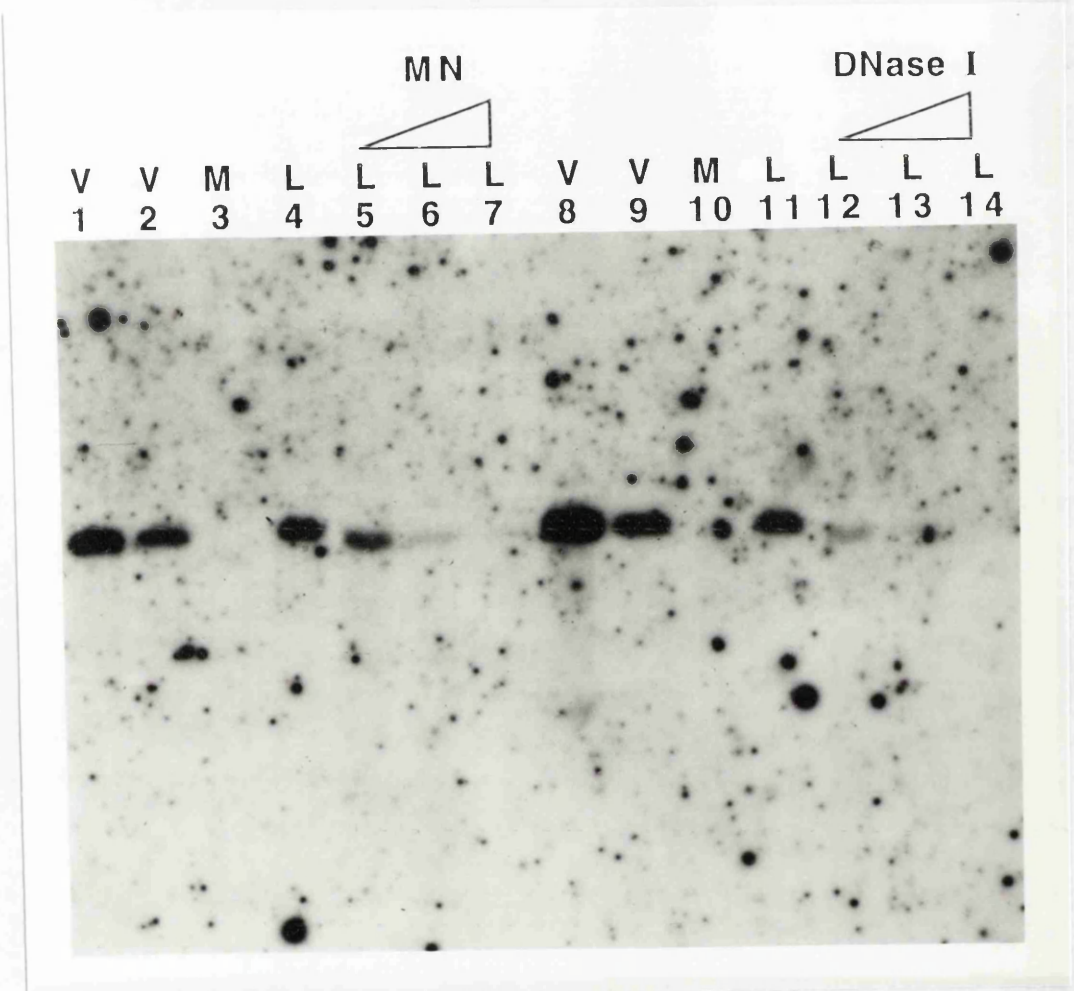


Figure 4.4. Nuclease sensitivity of the *in*1820 TK gene. The hybridisation membrane from the experiment described in section 4.3. (figure 4.3.) was stripped and re-hybridised to a [^{32}P]-labelled 2.4 kbp *Eco*RI n fragment from the HSV-1 TK gene. L: latently infected sample; M: mock infected sample; V: virion DNA.

Nuclease	MN			DNase I	
Enzyme units	0	1	3	0	1
Incubation time(mins)	0	3	5	0	3
% TK gene	100	62	9	100	27

Table 4.3: Nuclease sensitivity of the *in*1820 TK gene. Hybridisation to the nuclease treated and untreated latently infected nuclear DNA samples shown in figure 4.4. was quantified and the relative amounts of hybridisation determined. The non-nuclease treated samples represent 100% DNA.

4.4. Nonlinear viral genomes were templates for reactivation.

Superinfection of latently infected HFL cells with viruses expressing Vmw110 resulted in activation of the HCMV enhancer and the Vmw110 promoter in the latent genomes, and resumption of viral replication (Jamieson, 1993; Jamieson *et al.*, 1995). To investigate whether a change in configuration of the nonlinear genomes occurred after reactivation, the configuration of the *in*1820 genomes were determined after superinfection with *in*1863, a virus which expresses Vmw110, or *dl*1403, an IE1 deletion mutant which does not reactivate viruses from *in vitro* latency. IFNα-treated HFL cells were infected with 110 particles of *in*1820 per cell and incubated for 3 days in the presence of Ara-C. Cultures were superinfected with 5 PFU per cell of *in*1863 or *dl*1403 and incubated for 7 hours in the presence of Ara-C. Nuclei were isolated and the DNA purified, electrophoresed alongside virion DNA, blotted and hybridised to a radiolabelled fragment from the MMLV LTR.

No significant change in configuration occurred after superinfection with either virus (figure 4.5.; table 4.1.). The nonlinear genomes were templates for reactivation and did not require previous conversion to the linear form to allow viral gene expression. Presumably the linear molecules would have been converted to the

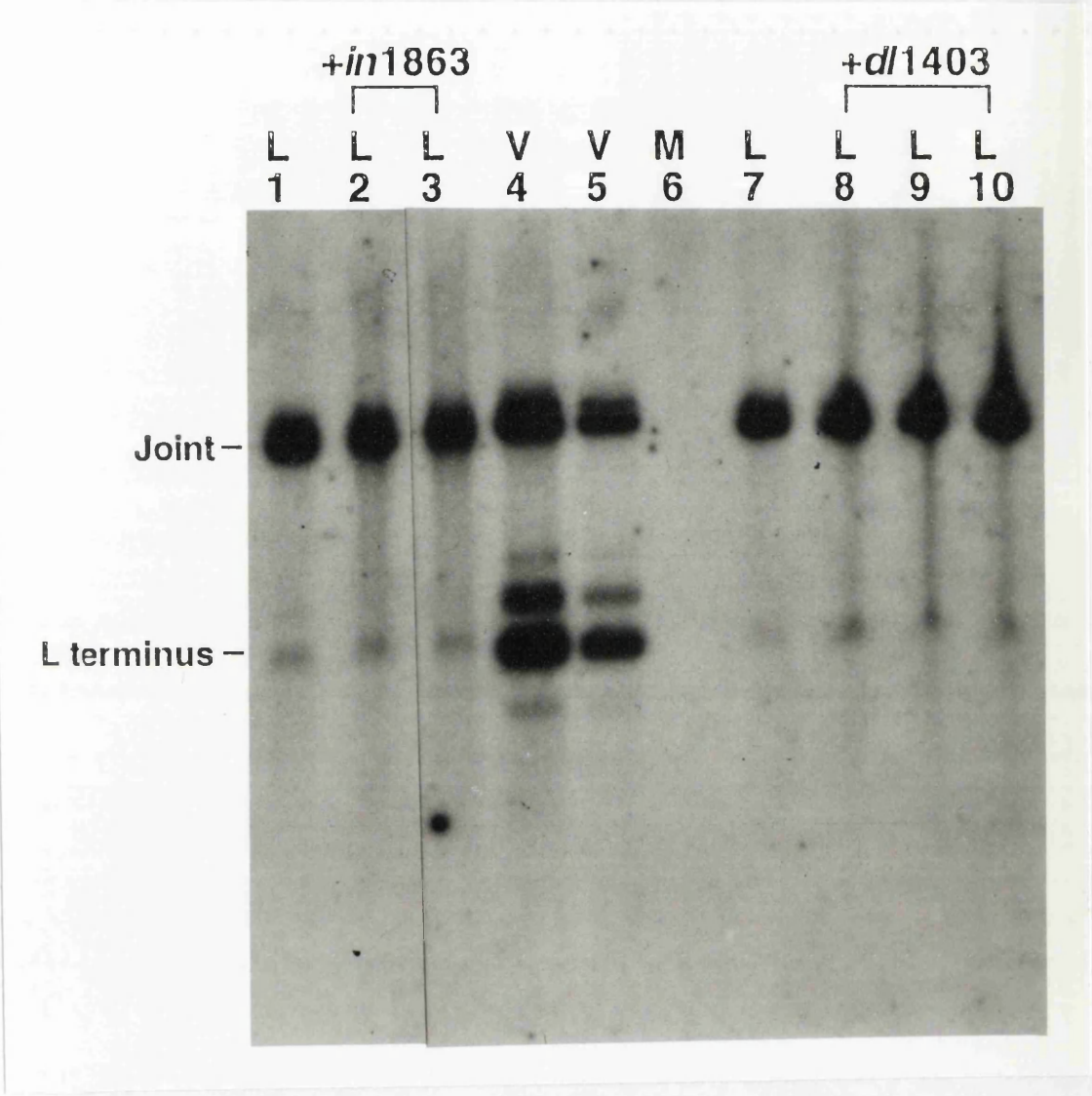


Figure 4.5. Configuration of viral genomes after reactivation. IFNα-treated HFL cells in 60 mm diameter tissue culture dishes were infected with 110 particles of *in*1820 per cell (lanes 1–3 and 7–10) or mock infected (lane 6) and overlaid with EF_{10} containing 50 μg/ml Ara-C. After incubation at 37° for 3 days, the cultures were superinfected with 5 PFU per cell of *in*1863 (lanes 2–3) or *dl*1403 (lanes 8–10) or were mock superinfected (lanes 1 and 7) and incubated at 37° in the presence of 50 μg/ml Ara-C. At 7 hours post-superinfection the nuclei were isolated and the DNA was purified. DNA was cleaved with *Bam*HI and half of each sample electrophoresed in a 0.7% agarose gel alongside and 5.0 ng (lane 4) and 1.5 ng (lane 5) *in*1825 virion DNA strandards. Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 455 bp *Bam*HI / *Bfr*I fragment from the MMLV LTR. Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. L: latently infected sample; M: mock infected sample; V: virion DNA.

nonlinear form if superinfection induced uncoating. The linear molecules remained linear, therefore uncoating was not induced by superinfection.

4.5. HFL cells contained fewer nonlinear, uncoated viral genomes than BHK, HeLa and CV-1 cells at a set time after infection.

The presence of linear, nonuncoated viral genomes after 3 days infection of HFL cells indicated that uncoating was unexpectedly slow. To investigate whether slow viral uncoating was a peculiar property of HFL cells, the rate of conversion to the nonlinear form, sensitivity to DNase I and amount of total nuclear viral DNA was compared after infection of HFL, BHK, HeLa and CV-1 cells. HFL, BHK, HeLa or CV-1 cells were infected with 75 particles of *in*1820 per cell and incubated in the presence of Ara-C. At 7 hours post-infection, nuclei were isolated and treated with DNase I or were untreated, DNA was purified, digested with *Bam*HI, electrophoresed alongside virion DNA, blotted and hybridised to a radiolabelled fragment from the MMLV LTR. Resulting hybridisation is shown in figure 4.6. Hybridisation was quantified and the relative proportions of nonlinear and linear viral genomes calculated by comparison to the virion DNA standards. The relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples between the cell types is presented in figure 4.7. The proportions of nonlinear viral genomes within each cell type and the percentage of genomes remaining after DNase I digestion is presented in table 4.4.

HFL cells contained a lower proportion of nonlinear molecules than the other cell types, 28% of the genomes in HFL cells being nonlinear compared with 88% in BHK cells, 52% in HeLa cells and 52% in CV-1 cells (figure 4.6.; figure 4.7). A comparison of numbers of nonlinear genomes between the cell types revealed that the number of nonlinear genomes present in HFL cells was approximately 7-fold lower than in BHK cells, 6-fold lower than in HeLa cells and 4-fold lower than in CV-1 cells (figure 4.7.). A lower proportion of nonlinear molecules in HFL cells indicated that uncoating or conversion to the nonlinear form occured more slowly in HFL cells than in the other three cell types. The genomes in HFL cell nuclei were more resistant to DNase I digestion than in the other cell types, supporting the view that differences in the number and proportion of nonlinear molecules was due to differences in rates of uncoating (table 4.4.).

The lower proportion of nonlinear genomes in HFL cells could have been caused by the a slower rate of virus penetration across the cell membrane or migration to the nuclei. If entry into the nuclei was limited by slower penetration across the cell surface and migration to the nuclei, then the viral genomes would have had less time to

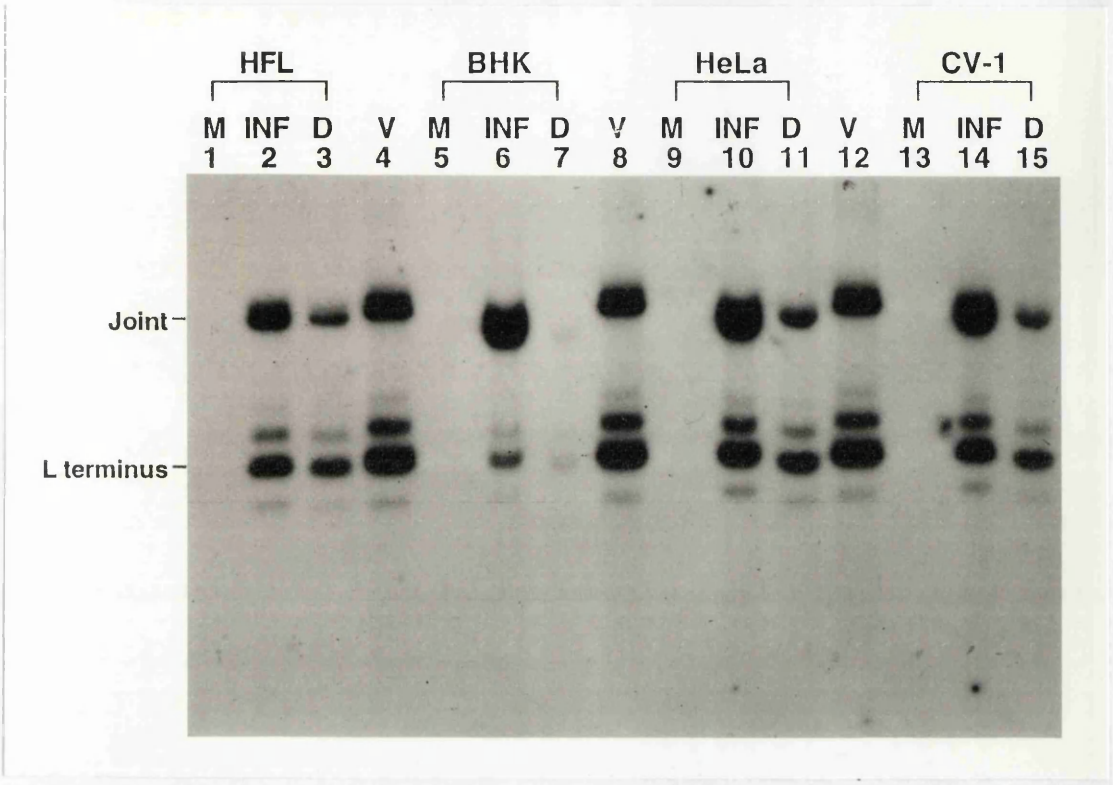


Figure 4.6. Comparison of the configuration of viral genomes after infection of HFL, BHK, HeLa and CV-1 cells. HFL, BHK, HeLa or CV-1 cells in 35 mm diameter tissue culture dishes were infected with 75 particles of *in*1820 per cell (samples in lanes 2–3, 6–7, 10–11 and 14–15 were from infected HFL, BHK, HeLa and CV-1 cell nuclei respectively) or mock infected (samples in lanes 1, 5, 9 and 13 were from mock infected HFL, BHK, HeLa and CV-1 cell nuclei respectively), overlaid with medium containing 50 µg/ml Ara-C and incubated at 37°. At 7 hours post-infection the nuclei were isolated and digested with 10 units of DNase I for 20 minutes (lanes 3, 7, 11 and 15) or were untreated (lanes 1–2, 5–6, 9–10 and 13–14). DNA was purified, digested with *Bam*HI and half of each sample electrophoresed in a 0.7% agarose gel alongside standards of *in*1825 virion DNA (lanes 4, 8 and 12). Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 455 bp *Bam*HI / *Bfr*I fragment from the MMLV LTR. Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. D: infected, DNase I digested sample; INF: infected, untreated sample; M: mock infected sample; V: virion DNA.

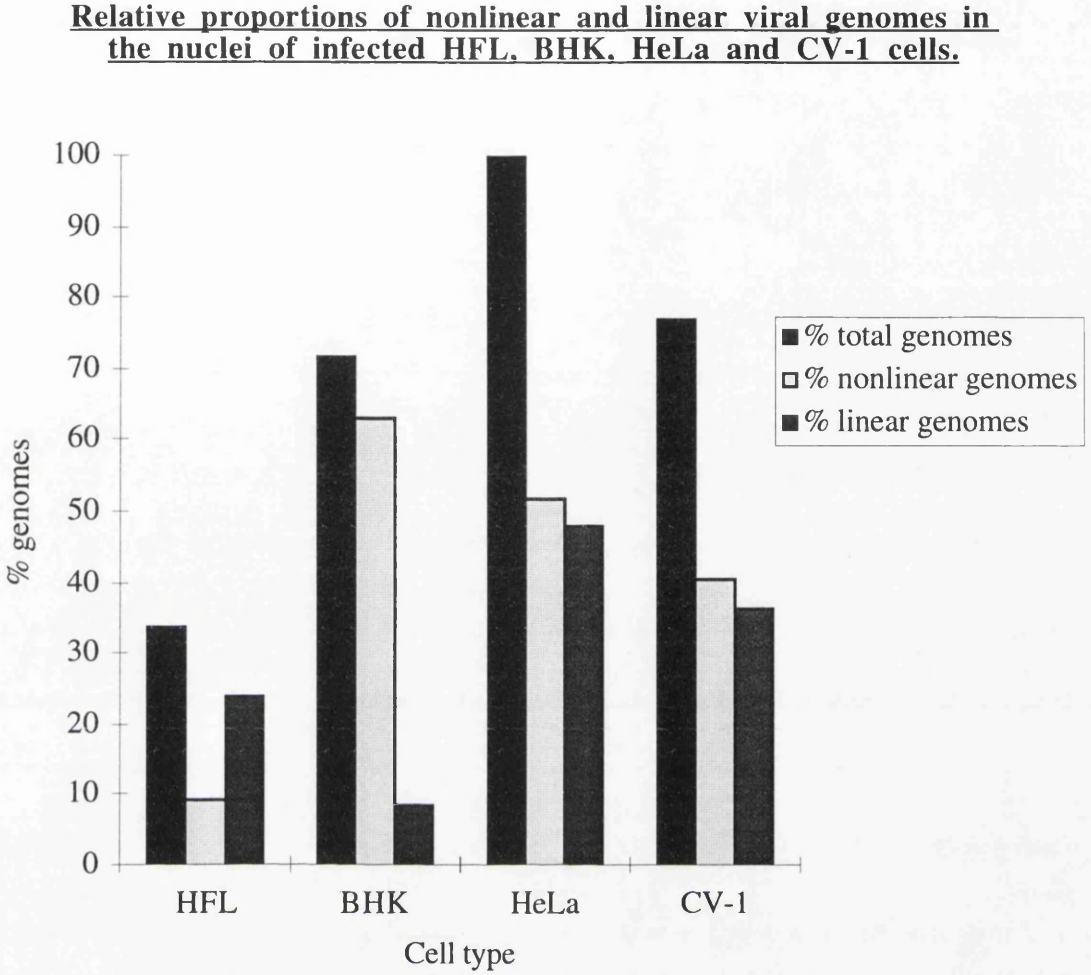


Figure 4.7. Relative proportions nonlinear and linear viral genomes in the nuclei of different infected cell types. Hybridisation in figure 4.6. was quantified and the relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples determined by comparison with the purified virion DNA standards. The total number of genomes in HeLa cell nuclei, which contained the greatest total number of genomes compared to the other three cell types, represents 100%.

Cell type	HFL	BHK	HeLa	CV-1
% genomes nonlinear	28	88	52	52
% genomes remaining after DNase I digestion	47	4.8	24	21

Table 4.4. Configuration and DNase I sensitivity of viral genomes in different infected cell types. Hybridisation in figure 4.6. was quantified and the percentage of nonlinear genomes in the non-nuclease treated samples calculated by comparison to the purified virion DNA standards. The percentage of genomes remaining after DNase I digestion was determined by comparison with the untreated samples.

uncoat and a lower proportion of nonlinear molecules would be expected. If a reduced rate of penetration and migration to the nuclei was the case, HFL cells should contain a greater number of genomes in the cytoplasm. To test the possibility that penetration and migration to the nuclei occurred more slowly in HFL cells than in BHK, HeLa and CV-1 cells, a comparison of viral DNA in the cytoplasm at a set time after infection was performed. HFL, BHK, HeLa or CV-1 cells were infected with 75 particles of *in*1820 per cell in triplicate and incubated in the presence of Ara-C. At 7 hours post-infection nuclei were isolated and the DNA in the TK lysis buffer purified, digested with *Bam*HI, electrophoresed, blotted and hybridised to a radiolabelled fragment from the MMLV LTR. Hybridisation showed that levels of viral DNA in the cytoplasm of HFL cells was not greater than in the other cell types (figure 4.8.), thus slower rate of penetration and migration to the nuclei was unlikely to be the cause of the lower proportion of nonlinear molecules in HFL cells.

In summary, HFL cells contained significantly fewer nonlinear, uncoated viral genomes than BHK, HeLa and CV-1 cells at a set time point after infection and this difference might be caused by differences in rates of virus uncoating.

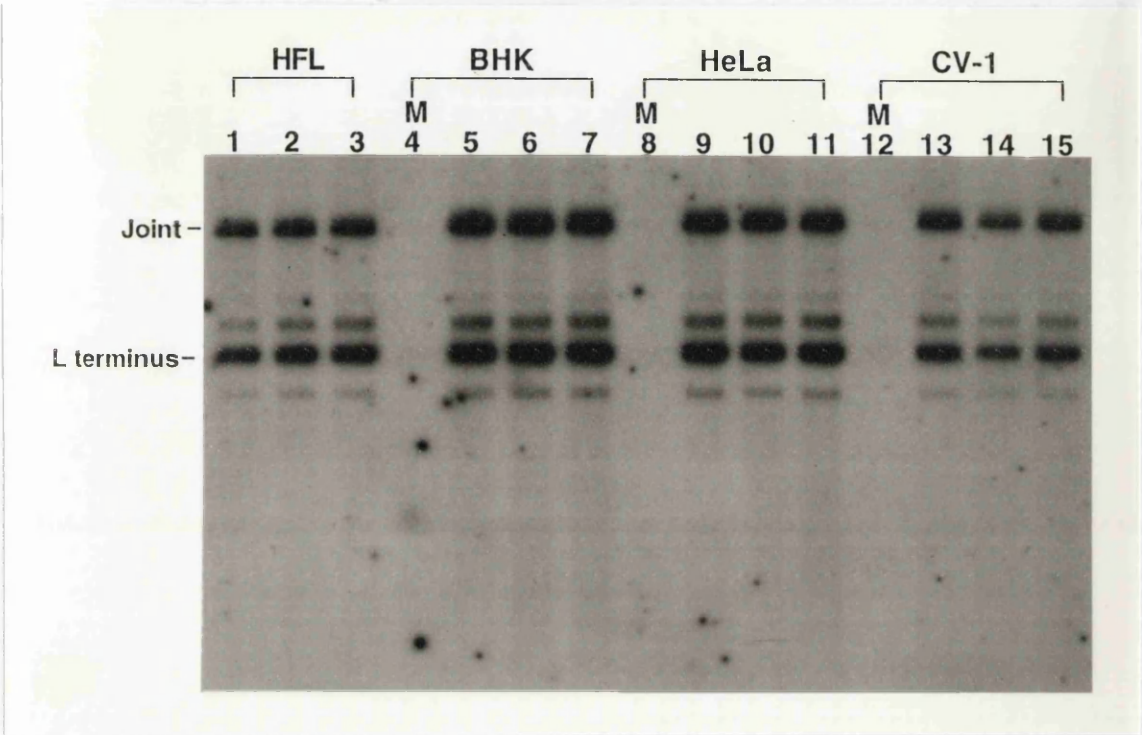


Figure 4.8. Comparison of the numbers of viral genomes in the cytoplasm after infection of HFL, BHK, HeLa and CV-1 cells. HFL, BHK, HeLa or CV-1 cells in 35 mm diameter tissue culture dishes were infected with 75 particles of *in*1820 per cell (samples in lanes 1–3, 5–7, 19–11 and 13–15 were from infected BHK, HeLa and CV-1 cells respectively) or mock infected (samples in lanes 4, 8, and 12 were from mock infected BHK, HeLa and CV-1 cells respectively), overlaid with medium containing 50 µg/ml Ara-C and incubated at 37°. At 7 hours post-infection, the cells were lysed with TK lysis buffer, DNA was purified from the resulting lysate, digested with *Bam*HI and electrophoresed in a 1.2% agarose gel. Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 455 bp *Bam*HI / *Bfr*I fragment from the MMLV LTR. Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. M: mock infected sample.

4.6. The *in*1814 mutation and HMBA did not exert their effects by inhibiting virus uncoating.

The data in section 4.5. suggested that virus uncoating was unusually slow in HFL cells, hence in this and the next three sections experiments are described which investigate the relationship between virus uncoating and *in vitro* latency. Mutants of HSV-1 defective in expression of Vmw110 exhibit a particularly high particle / PFU ratio in HFL cells (Stow and Stow, 1986; Ace *et al.*, 1989; Everett, 1989). After infection of neurons *in vivo* , viral nucleocapsids are transported along the nerve axons leading to a delay between infection at the neuronal cell surface and release of the viral genomes into the nuclei (Cook and Stevens, 1973), hence it was tempting to postulate that slow uncoating in HFL cells was mimicking the situation *in vivo*. An investigation to determine if the *in*1814 mutation, HMBA, IFNα or Ara-C act by altering the rate of virus uncoating is described in this and the next three sections..

The virus protein Vmw65 is a major component of the virion tegument and as such might play a role in virus uncoating. It seemed possible that the mutation in *in*1814 affects virus replication by blocking uncoating in addition to preventing transactivation of IE genes and that HMBA might overcome the defect in *in*1814 by facilitating uncoating. An investigation was undertaken to examine the effect of the *in*1814 mutation and HMBA on virus uncoating by comparing the rates of conversion of the genomes of *in*1814 and its revertant 1814R to the nonlinear form after infection in the presence or absence of HMBA. HFL cells were infected with 0.5 PFU of *in*1814 or 1814R per cell and incubated for 7 hours in the presence of Ara-C with or without 5 mM HMBA. Nuclei were extracted and were digested to a sufficient extent with DNase I to degrade most of the cell DNA visualised on the agarose gel, or were untreated. DNA was purified, digested with *Bam*HI, electrophoresed, blotted onto a hybridisation membrane and hybridised to a radiolabelled DNA fragment from the HSV-1 IE1 promoter (figure 3.2.). Resulting hybridisation is presented in figure 4.9. Quantification of the relative proportions of nonlinear and linear genomes in the non-nuclease treated samples is presented in figure 4.10. The percentage of nonlinear molecules within the non-nuclease treated samples and the percentage of genomes remaining after DNase I digestion is presented in table 4.5.

If the rate of uncoating was stimulated in the presence of HMBA the proportion of nonlinear molecules would be increased and the DNase I sensitivity of the genomes increased. The proportion of nonlinear molecules was not increased by HMBA, neither was the DNase I sensitivity of the genomes increased (figure 4.10.; table 4.5.), thus it is unlikely that HMBA facilitated uncoating. A comparison of the total number of genomes in HMBA treated samples with the total number of genomes in the untreated samples showed that HMBA did not significantly alter virus adsorption, penetration or

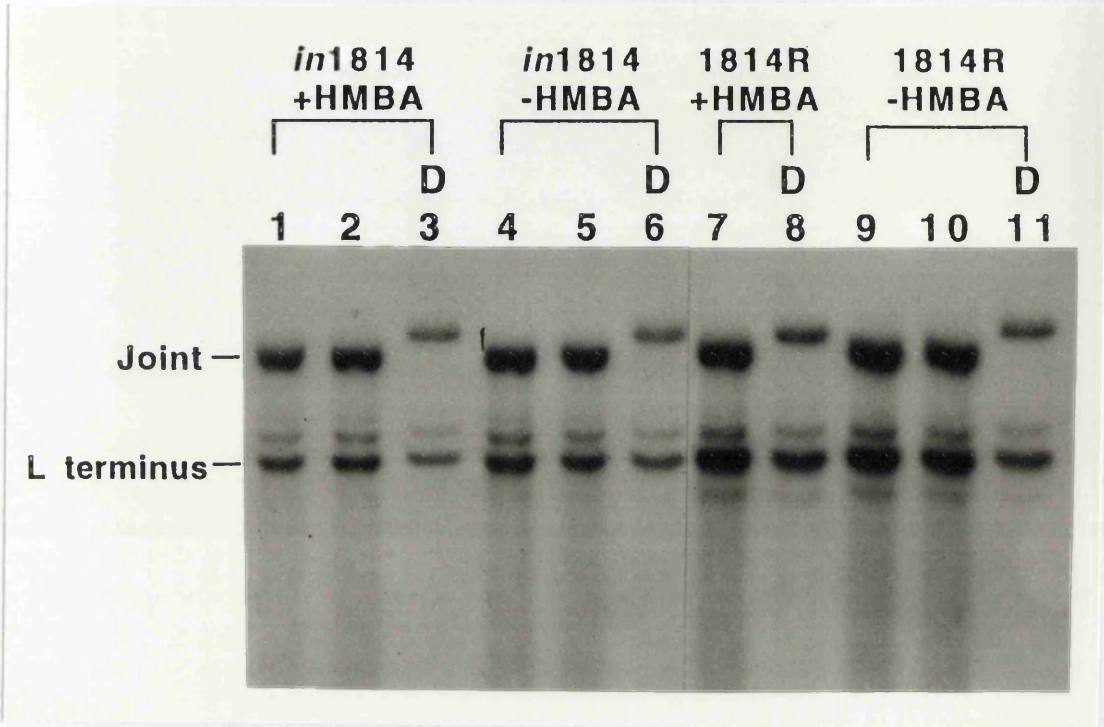


Figure 4.9. Effect of the *in*1814 mutation and HMBA on virus uncoating. HFL cells in 35 mm diameter tissue culture dishes were infected with 0.5 PFU per cell of *in*1814 (lanes 1–6) or 1814R (lanes 7–11), overlaid with EF_{10} containing 50 µg/ml Ara-C with (lanes 1–3 and 7–8) or without (lanes 4–6 and 9–11) 5 mM HMBA, and incubated at 37°. At 7 hours post-infection the nuclei were isolated and digested with 5 units of DNase I for 20 minutes (lanes 3, 6, 8 and 11) or were untreated (lanes 1–2, 4–5, 7 and 9-10). DNA was purified, digested with *Bam*HI, electrophoresed in a 1.2% agarose gel, blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled fragment specific for the 960 bp *Bam*HI / *Sst*I fragment spanning the promoter of IE1 (figure 3.2.). Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. The difference in mobility of the joint fragments between DNase I treated and untreated samples was caused by differences in the total amounts of DNA electrophoresed. D: DNase I digested sample.

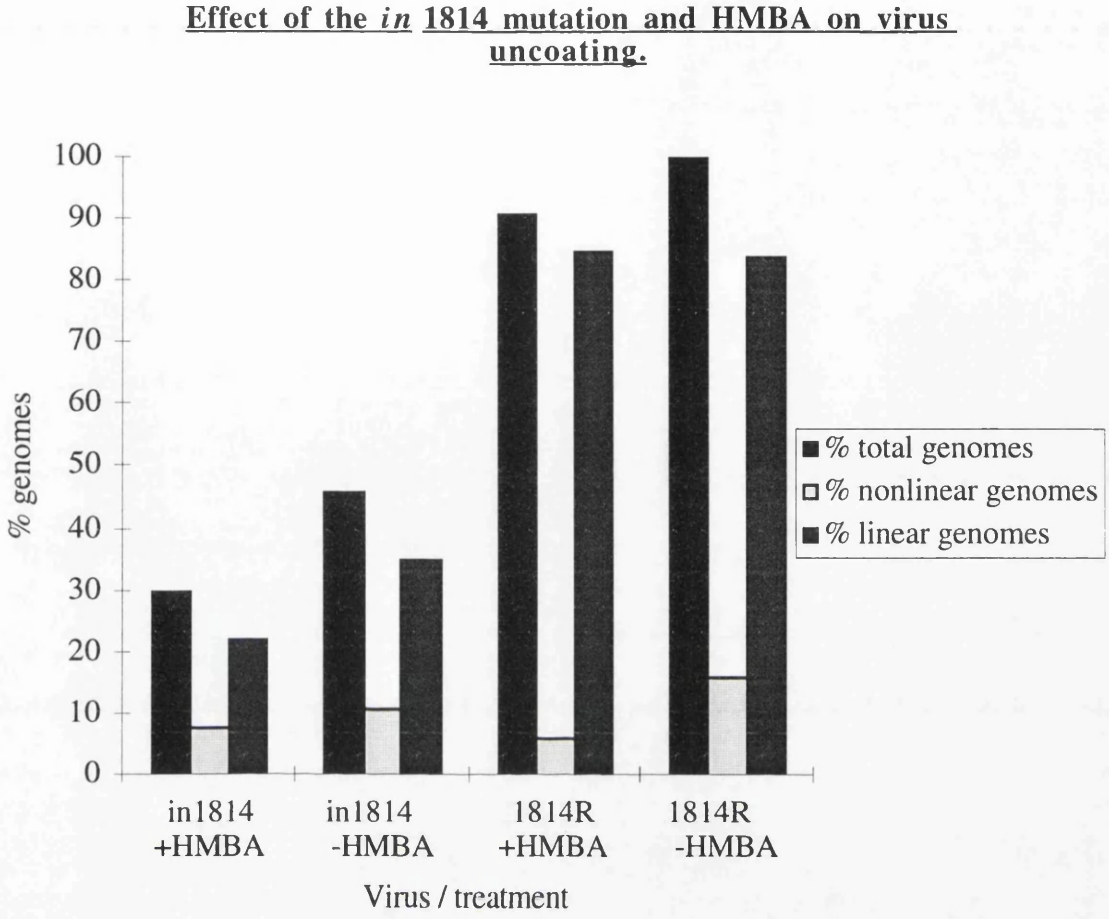


Figure 4.10. Effect of the *in*1814 mutation and HMBA on virus uncoating. Hybridisation shown in figure 4.9. was quantified and the relative proportions of nonlinear and linear genomes in the non-nuclease treated samples determined. The hybridisation bias for virion DNA used to calculate the relative proportions of nonlinear and linear genomes was taken from hybridisation shown in figures 4.3. and 4.5. The total number of genomes in the nuclei from cells infected with 1814R in the absence of HMBA, which contained the greatest total number of genomes compared to the other samples, represents 100%.

Virus ±HMBA	*in*1814 +HMBA		*in*1814 –HMBA		1814R +HMBA	1814R –HMBA	
% nonlinear	30	22	21	27	7	16	15
Average % nonlinear	26		24		7	16	
% genomes remaining after DNase I digestion	43		41		36	33	

Table 4.5. Effect of *in*1814 mutation and HMBA on virus genome configuration and DNase I sensitivity. Hybridisation in figure 4.9. was quantified and the percentage of nonlinear genomes in the non-nuclease treated samples calculated by comparison to the purified virion DNA standards. The percentage of genomes remaining after DNase I digestion was determined by comparison with the untreated samples. The slow mobilities of the joint fragments in the DNase I-digested samples compared to the non-nuclease treated samples was caused by differences in the total amounts of DNA loaded on the gel.

migration of the genomes to the nuclei. If the *in*1814 mutation inhibited uncoating, the proportion of nonlinear genomes in *in*1814 infected samples would have been smaller than in 1814R infected samples. The data presented in table 4.5. demonstrated that the proportion of nonlinear genomes was not smaller in *in*1814 infected samples compared to 1814R infected samples: *in*1814 was 24% nonlinear in the absence of HMBA whereas 1814R was 16% nonlinear . The total number of viral genomes in the nuclei of 1814R infected samples was approximately 2.5-fold higher than in *in*1814 infected samples (table 4.5.). Ace *et al.* demonstrated that the *in*1814 mutation had no significant effect on entry of the viral genomes into the nuclei of infected cells (Ace *et al.*, 1989), thus the difference described in the study described here may be explained by experimental error in dilution or titration of the virus preparations. It should be noted that MOI was calculated in terms of PFU / cell rather than particles / cell and that the *in*1814 preparation was titrated in the presence of 3 mM HMBA to overcome the particle / PFU defect. An accurate determination of the effect of the *in* 1814 mutation on

entry of viral genomes into nuclei would require the cells to be infected with equal numbers of particles of *in*1814 or 1814R. Lower proportions of nonlinear DNA in the 1814R infected samples might be related to cytotoxicity caused by expression of IE proteins. In the 1814R samples infected in the presence of HMBA IE gene expression would have been stimulated by both Vmw65 and HMBA, only 7% of the genomes were nonlinear, supporting the theory that cytotoxicity reduced the number of nonlinear molecules. The data presented in this thesis supports previous work by others who demonstrated that HMBA and the Vmw65 mutation of *in*1814 exert their effects by directly altering levels of IE transcription (Ace *et al.*, 1989; McFarlane *et al.*, 1992).

4.7. The antiviral state caused by IFNα-treatment did not act *via* an alteration of virus uncoating.

It has been reported previously that the antiviral action of IFNα on HSV-1 is *via* a specific inhibition of IE transcription (Mittnach *et al.*, 1988; Oberman and Panet, 1989; DeStasio and Taylor, 1990). To determine whether IFNα-treatment causes inhibition of virus adsorption, penetration, migration to the nucleus, uncoating or conversion of the genomes to the nonlinear configuration, the total number of genomes and proportion of nonlinear viral genomes after infection of IFNα-treated and mock-treated HFL cells was compared using the Southern blot assay. HFL cells were treated with 1000 u/ml of IFNα in the culture medium for 24 hours, or were mock-treated. The cells were infected with 75 particles of *in*1820 per cell and incubated in the presence of Ara-C. At 24 hours post-infection the nuclei were extracted and digested with DNase I or untreated. DNA was purified, digested with *Bam*HI, electrophoresed, blotted and hybridised to a radiolabelled DNA fragment from the MMLV LTR. Resulting hybridisation is presented in figure 4.11. Relative proportions of nonlinear genomes and linear genomes in the non-nuclease treated samples were quantified and presented in figure 4.12. The percentage of nonlinear molecules in the non-nuclease treated samples and percentage of genomes remaining after DNase I digestion was quantified and presented in table 4.6.

If IFNα-treatment reduced adsorption, penetration or uncoating, a smaller proportion of total numbers of genomes and a smaller proportion of nonlinear genomes would be expected in IFNα-treated cells. Quantification presented in figure 4.12. showed that the total number of genomes was increased by 1.4-fold and the proportion of nonlinear genomes was increased by 1.5-fold in the nuclei of IFNα-treated cells. The antiviral action of IFNα was not on adsorption, penetration, migration to the nucleus,

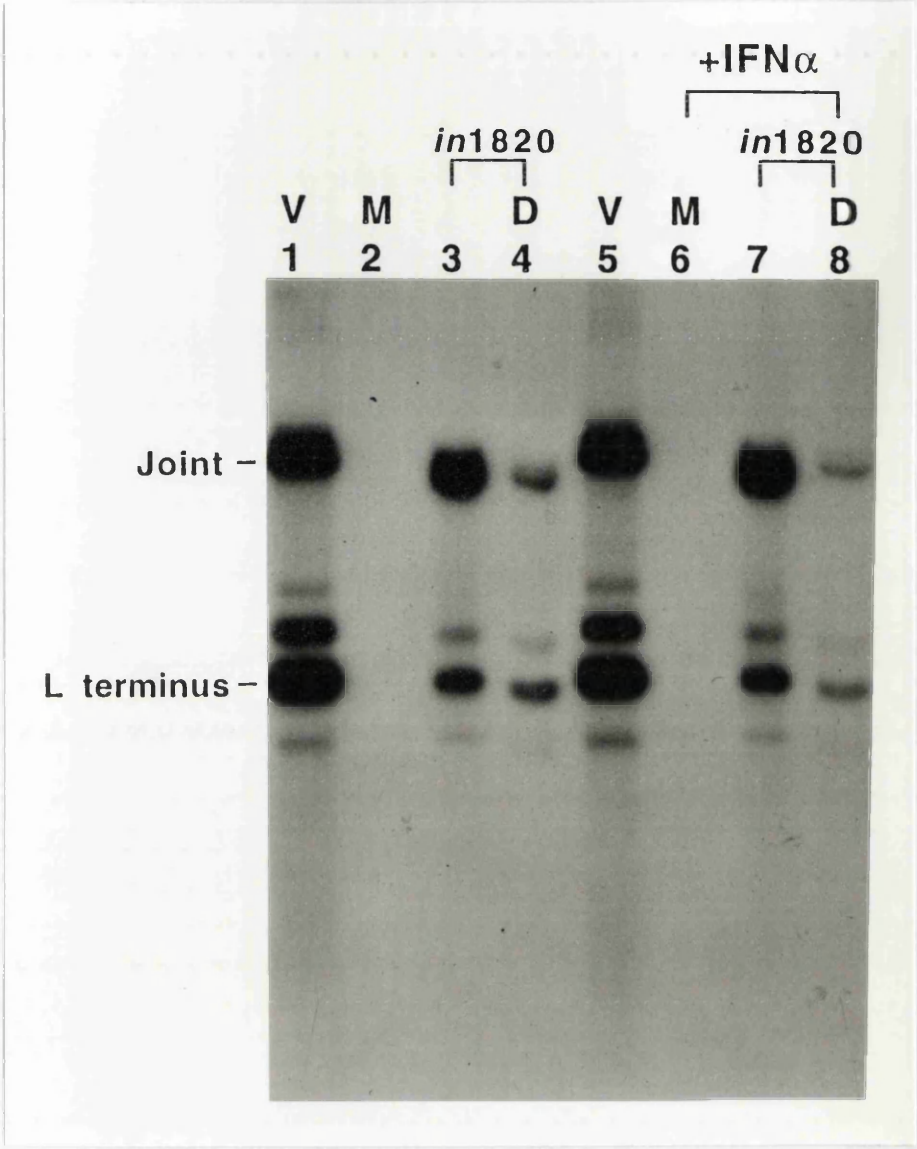


Figure 4.11. Effect of IFNα pretreatment on virus uncoating. HFL cells in 35 mm diameter tissue culture dishes were treated overnight with 1000 u/ml of IFNα in the culture medium (lanes 6–8) or were mock treated (lanes 2–4). The monolayers were infected with 75 particles of *in*1820 per cell (lanes 3–4 and 7–8) or mock infected (lanes 2 and 6), overlaid with EF_{10} containing 50 µg/ml Ara-C and incubated at 37°. At 24 hours post-infection the nuclei were isolated and digested with 10 units of DNase I for 20 minutes (lanes 4 and 8) or were untreated (lanes 3 and 7). DNA was purified, digested with *Bam*HI and electrophoresed in a 0.8% agarose gel alongside 1.5 ng standards of *in*1825 virion DNA (lanes 1 and 5). Electrophoresed DNA was was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 455 bp *Bam*HI / *Bfr*I fragment from the MMLV LTR. Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. D: DNase I digested sample; M: mock infected sample; V: virion DNA.

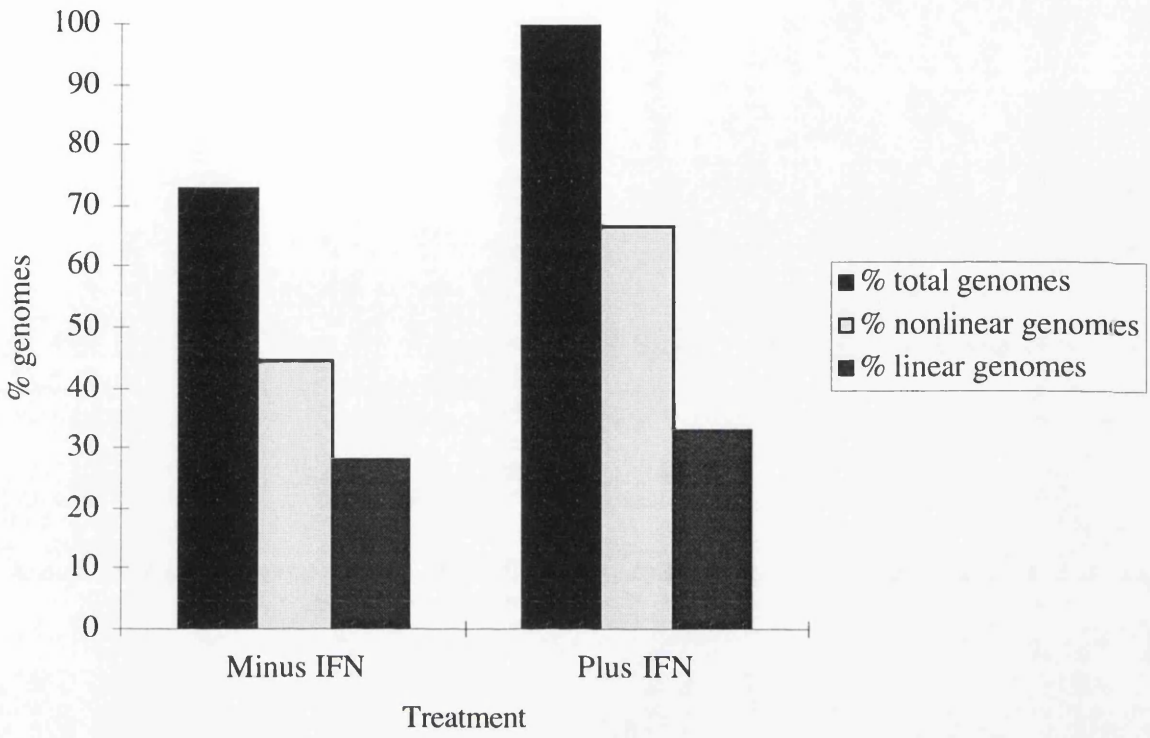


Figure 4.12. Relative proportions of nonlinear and linear viral genomes in the nuclei of IFNα-treated and mock-treated infected HFL cells. Hybridisation in figure 4.11. was quantified and the relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples quantified by comparison to the virion DNA standards.The total number of genomes in the nuclei from the IFNα-treated cells, which contained the greatest total number of genomes compared to the non-IFNα-treated sample, represents 100%.

± IFNα	–IFNα	+IFNα
% nonlinear	61	67
% genomes remaining after DNase I digestion	27	9.5

Table 4.6. Configuration and DNase I sensitivity of viral genomes in IFNα-treated and mock treated HFL cells. Hybridisation in figure 4.11. was quantified and the percentage of genomes which were nonlinear in the non-nuclease treated samples calculated by comparison to the purified virion DNA standards. The percentage of genomes remaining after DNase I digestion was determined by comparison with the untreated samples.

uncoating or conversion of the genomes to the nonlinear configuration, but was on IE transcription, as reported by others. The increase in total numbers of genomes and proportion of nonlinear genomes in IFNα-treated cells might have been caused by the protective effect of IFNα reducing the number of cells dying from viral CPE and floating into the culture medium. Viral genomes in the dead cells would not have been detected in the assay, leading to a lower signal in the untreated samples.

4.8. Conversion of viral genomes to the nonlinear configuration did not require *de novo* protein synthesis.

Little is known about the mechanism by which HSV-1 genomes are converted to the nonlinear configuration after infection. To examine whether uncoating and conversion of viral genomes to the nonlinear configuration was dependent on viral or cellular gene products synthesised *de novo* or was caused by pre-existing cell or viral factors, the rate of conversion to the nonlinear configuration was compared after infection of HFL or BHK cells in the presence of Ara-C or cycloheximide. HFL or BHK cells were infected with 75 particles of *in*1820 per cell and incubated in the presence of Ara-C or cycloheximide. At 7 hours post-infection nuclei were isolated and

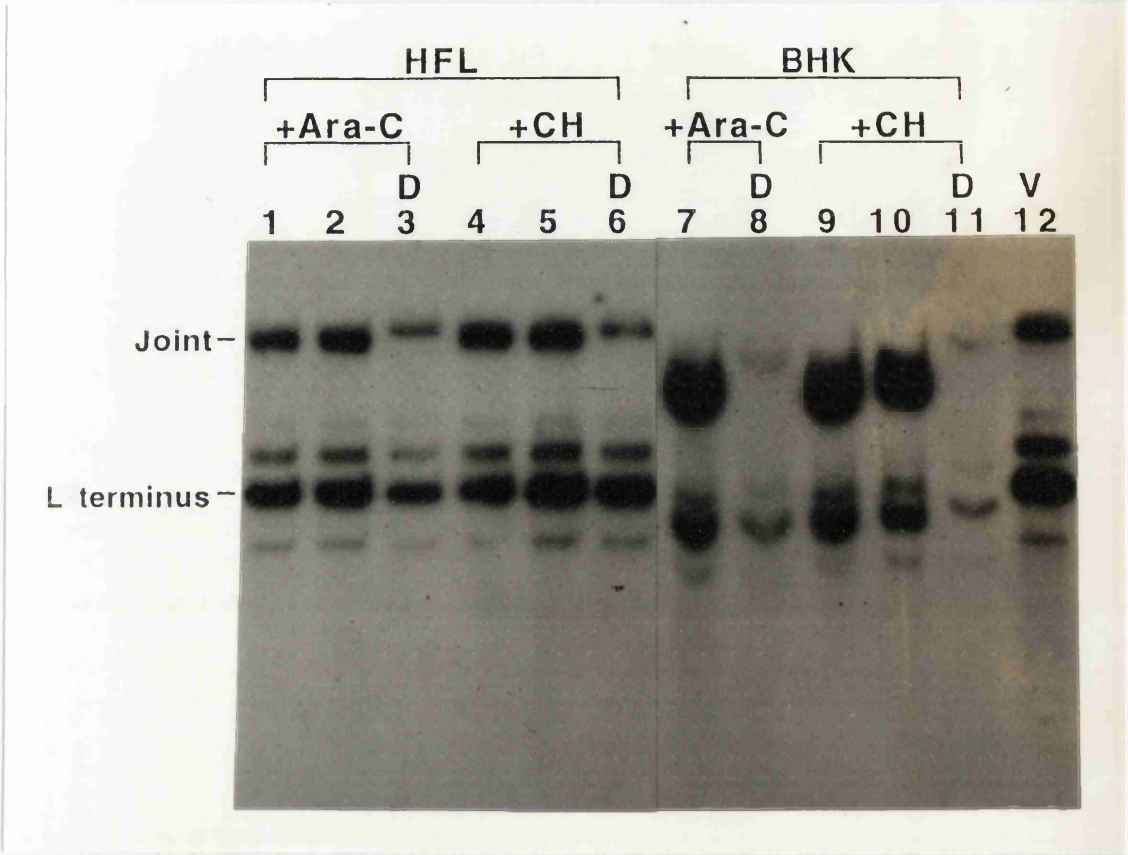


Figure 4.13. Effect of inhibition of protein synthesis on virus genome uncoating and conversion to the non-linear form. HFL or BHK cells in 35 mm diameter tissue culture dishes were infected with 75 particles of *in*1820 per cell (samples in lanes 1–6 and 7–11 were from infected HFL and BHK cells respectively), overlaid with medium containing 50 µg/ml Ara-C (lanes 1–3, 7 and 8) or 50 µg/ml cycloheximide (lanes 4–6 and 9–11) and incubated at 37°. At 7 hours post-infection the nuclei were isolated and digested with 5 units of DNase I for 20 minutes (lanes 3, 6, 8 and 11) or were untreated (lanes 1–2, 4–5, 7, and 9–10). DNA was purified, digested with *Bam*HI, electrophoresed in a 1.2% agarose gel alongside a 1.5 ng *in*1825 virion DNA standard (lane 12), blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 455 bp *Bam*HI / *Bfr*I fragment from the MMLV LTR. Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. D: DNase I digested sample; V: virion DNA.

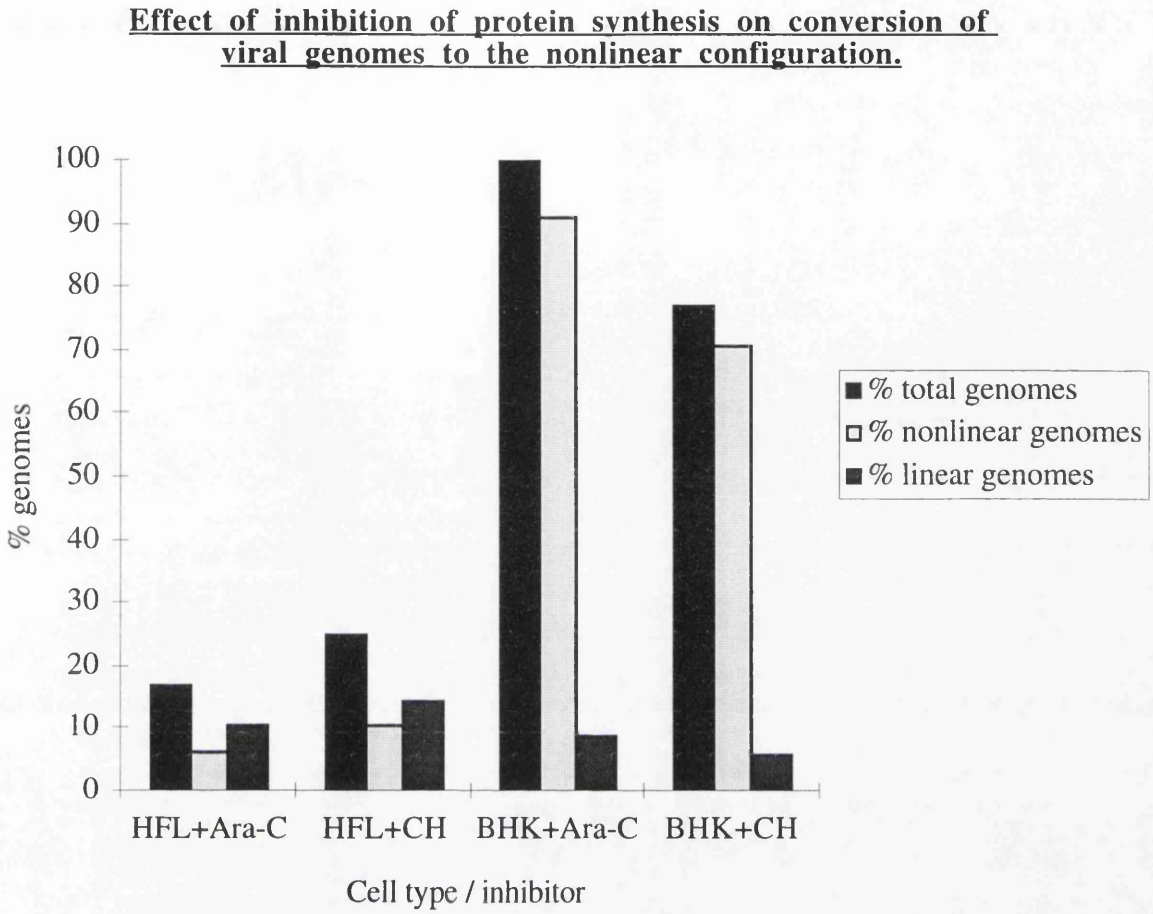


Figure 4.14. Relative proportions of nonlinear and linear viral genomes after infection in the presence or absence of protein synthesis. Hybridisation shown in figure 4.13. was quantified and the relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples quantified by comparison to the virion DNA standards. The total number of genomes in the BHK cell nuclei infected +Ara-C, which contained the greatest total number of genomes compared to the other samples, represents 100%.

Cell type / inhibitor	HFL +Ara-C	HFL +CH	BHK +Ara-C	BHK + CH
Average % nonlinear	38	43	91	92
Average % genomes remaining after DNase I digestion	53	60	5.2	3.5

Table 4.7: Configuration and DNase I sensitivity of viral genomes after infection in the presence or absence of protein synthesis. Hybridisation in figure 4.13. was quantified and the percentage of genomes which were nonlinear in the non-nuclease treated samples calculated by comparison to the purified virion DNA standards. The percentage of genomes remaining after DNase I digestion was determined by comparison with the untreated samples.

digested with DNase I or untreated. DNA was purified, digested with *Bam*HI, electrophoresed in an agarose gel, blotted and hybridised to a radiolabelled DNA fragment from the MMLV LTR. Resulting hybridisation is presented in figure 4.13. The relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples was quantified and presented in figure 4.14. The percentage of nonlinear genomes in the non-nuclease treated samples and genomes remaining after DNase I digestion is presented in table 4.7.

The proportion of nonlinear genomes within the nuclei of both cell types was not significantly altered when protein synthesis was inhibited by cycloheximide (figures 4.13. and 4.14.). The proportion of genomes remaining after DNase I digestion was similar after infection in the presence of Ara-C or infection in the presence of cycloheximide, indicating that the number of uncoated genomes was unaffected by inhibition of protein synthesis (table 4.7.). It was concluded that uncoating and conversion of the viral genomes to the nonlinear form was not dependent on *de novo* protein synthesis but was caused by factors pre-existing in the cell or associated with incoming virus particles. It was noted again that BHK cells contained approximately 9-

fold more uncoated, nonlinear viral genomes than HFL cells (figure 4.14.) and that the viral genomes in BHK cells were approximately 13-fold more sensitive to DNase I (table 4.7.), supporting the concept that rate of uncoating differs greatly between the two cell types.

4.9. *Ts*1213 genomes were not uncoated or converted to the nonlinear configuration at the NPT.

Temperature sensitive uncoating mutants of HSV-1 have been characterised by their inability to release viral DNA from nucleocapsid cores, to produce any virus-specific products or to be fully complemented by other mutants at the NPT (Knipe *et al.*, 1981; Batterson *et al.*, 1983). The HSV-1 mutant *ts*1213 contains a *ts* lesion which has been mapped to the major tegument protein encoded by U_L36 and has a phenotype consistent with that of an uncoating mutant (V.G. Preston, personal communication). In order to confirm that *ts*1213 is an uncoating mutant and assess the validity of the Southern blot assay for uncoating utilised in sections 4.5.–4.8., *ts*1213 was examined in the assay. BHK cells were infected with 0.5 PFU of *ts*1213 or 1814R per cell and incubated at 31° or 39.5° in the presence of Ara-C. At 7 hours post-infection nuclei were isolated and digested with DNase I or untreated. DNA was purified, digested with *Bam*HI, electrophoresed alongside purified virion DNA, blotted and hybridised to a radiolabelled DNA fragment from the 5' coding sequences of IE1 (figure 3.2.). Resulting hybridisation is presented in figure 4.15. The relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples was quantified and is presented in figure 4.16. The percentage of nonlinear genomes in the non-nuclease treated samples and the percentage of genomes remaining after DNase I digestion is presented in table 4.8.

At 31°, *ts*1213 DNA was converted to the nonlinear configuration at a similar rate to 1814R (figure 4.15., lanes 2 and 4; figure 4.16.). At 39.5°, the NPT for *ts*1213, 1814R DNA was converted to the nonlinear configuration with comparable efficiency to that at 31°, but *ts*1213 showed no evidence of conversion to the nonlinear configuration (figure 4.15., lanes 8 and 10; figure 4.16.). The total numbers of genomes in the nuclei after infection with *ts*1213 at the NPT was about 30% of the total numbers of genomes after infection at 31°, indicating that the ability of the linear genomes to co-purify with the nuclei was related to uncoating. The percentage of genomes remaining after DNase I treatment was approximately 1.7-fold greater in the sample infected with *ts*1213 at the NPT than in samples infected under permissive conditions implying that the genomes were more resistant to DNase I but retained a degree of sensitivity. The

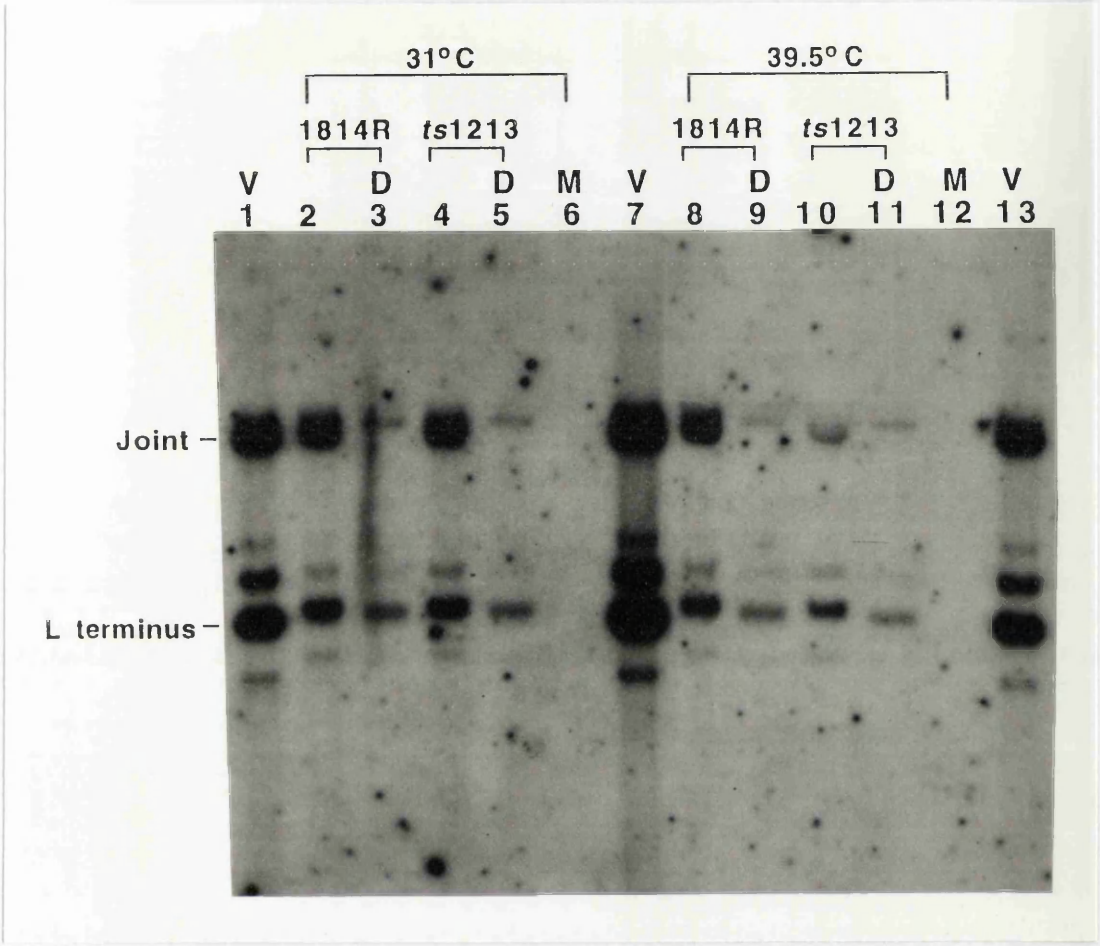


Figure 4.15. Configuration of *ts*1213 genomes after infection at the NPT. BHK cells in 35 mm diameter tissue culture dishes were infected with 0.5 PFU per cell of 1814R (lanes 2–3 and 8–9) or *ts*1213 (lanes 4–5 and 10–11) or mock infected (lanes 6 and 12), overlaid with EF_{10} containing 50 µg/ml Ara-C and incubated at 31° (lanes 2–6) or 39.5° (lanes 8–12). At 7 hours post-infection the nuclei were isolated and digested with 10 units of DNase I for 20 minutes (lanes 3, 5, 9 and 11) or were untreated (lanes 2, 4, 8 and 10). DNA was purified, digested with *Bam*HI and half of each sample electrophoresed in a 0.7% agarose gel alonside 1.0 ng *in*1825 virion DNA standards (lanes 1, 7 and 13). Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 640 bp *Bam*HI / *Nco*I fragment from the 5' coding sequences of IE1 (figure 3.2.). Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. D: DNase I digested sample; M: mock infected sample; V: virion DNA.

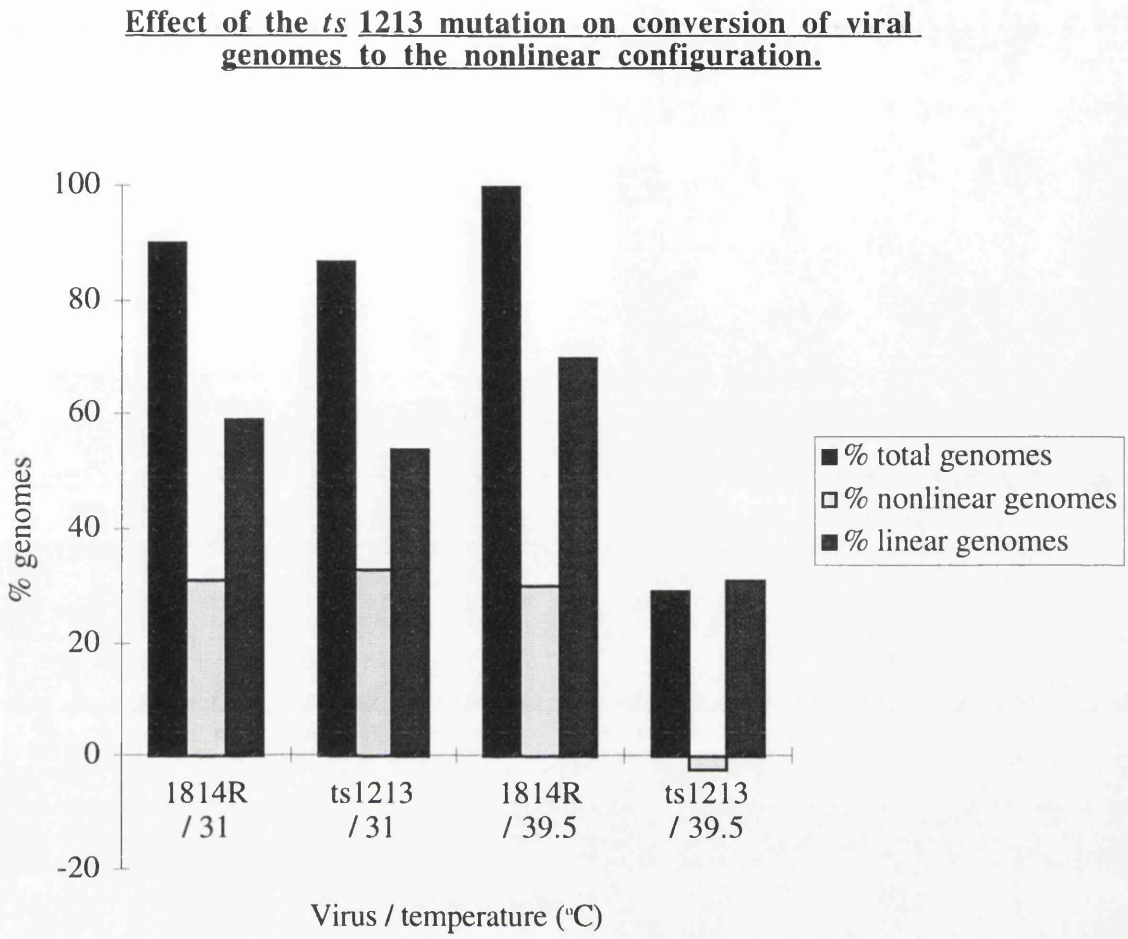


Figure 4.16. Effect of the *ts*1213 mutation on the relative proportions of nonlinear and linear viral genomes after infection at the permissive and non-permissive temperatures. Hybridisation in figure 4.15. was quantified and the relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples quantified by comparison to the virion DNA standards. The total number of genomes in nuclei from cells infected with 1814R at 39.5°, which contained the greatest total number of genomes compared to the other samples, represents 100%.

Temperature (°)	31		39.5	
Virus	1814R	*ts*1213	1814R	*ts*1213
% nonlinear	34	38	30	–7
% genomes remaining after DNase I digestion	ND	38	26	55

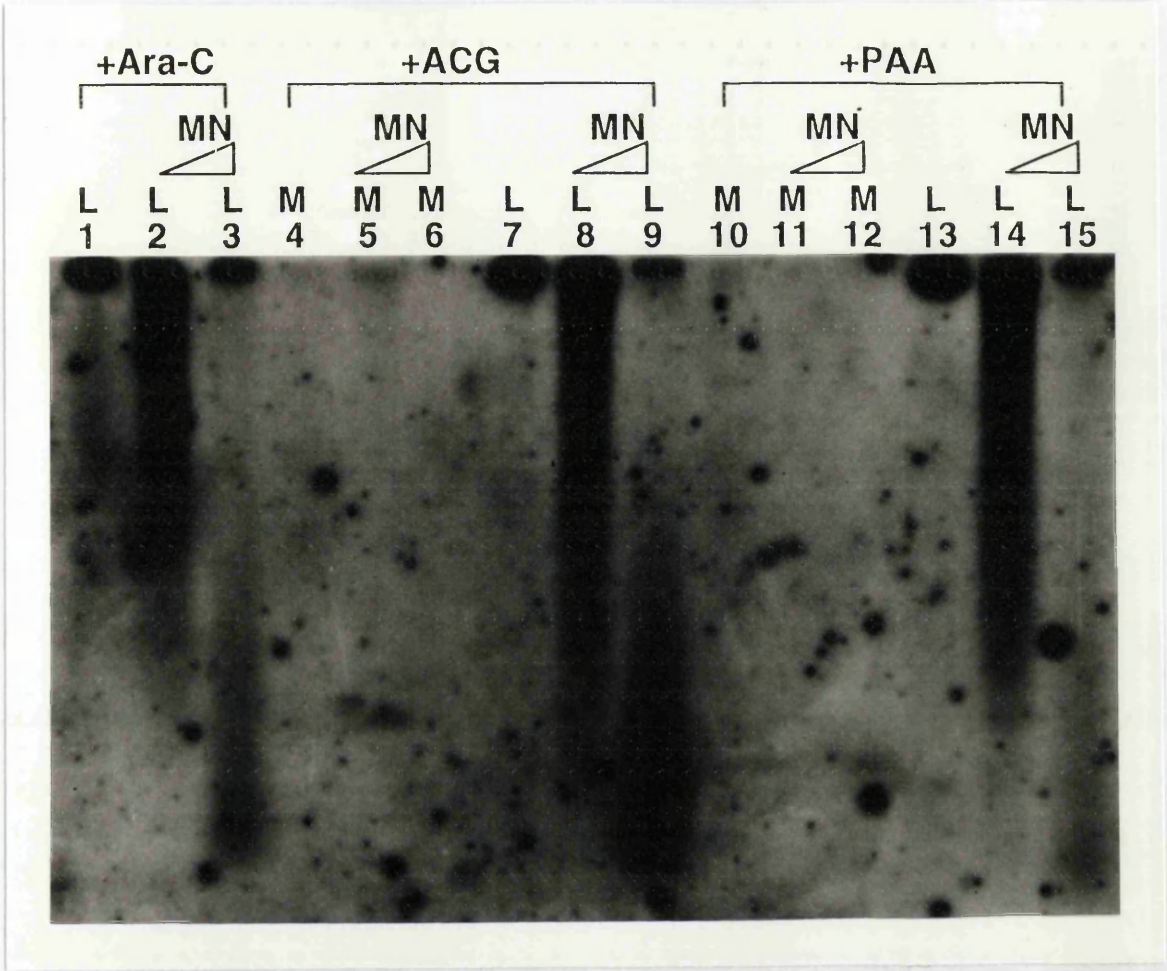
Table 4.8. Configuration and DNase I sensitivity of *ts*1213 genomes after infection at the permissive and non-permissive temperatures. Hybridisation in figure 4.15. was quantified and the percentage of genomes which were nonlinear in the non-nuclease treated samples calculated by comparison to the purified virion DNA standards. The percentage of genomes remaining after DNase I digestion was determined by comparison with the untreated samples. ND; not determined due to interfering background.

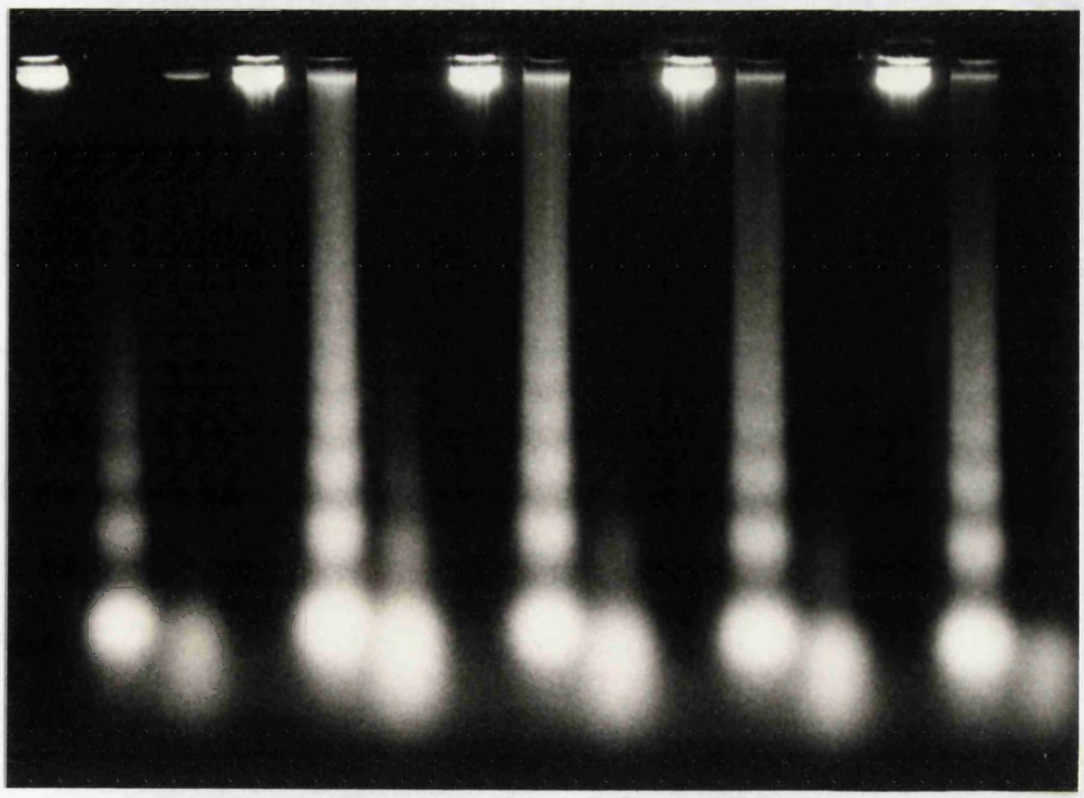
*ts*1213 genomes exhibited a phenotype at 39.5° expected of an uncoating mutant, they were not converted to the nonlinear form, did not associate with nuclei as efficiently as in normal infection and were resistant to DNase I, giving credibility to the Southern blot assay for uncoating and confirming that *ts*1213 is an uncoating mutant.

4.10. Non-nucleosomal pattern of MN digestion of the TK gene was not altered by the specificity of the DNA replication inhibitor.

Partial digestion of eukaryotic chromatin with MN produces a series of DNA fragments with sizes multiples of about 200 bp (figure 4.17., lower panel). HSV-1 DNA in the brainstems of latently infected mice was digested by MN into a series of fragments chracteristic of cell chromatin (Deshmane and Fraser, 1989). Previous work demonstrated that the viral TK gene produced heterogeneously sized DNA fragments after partial MN digestion of nuclei from latently infected HFL cells (Jamieson, 1993; Jamieson *et al.*, 1995). The TK gene was bound by regularly spaced nucleosomes when in the cell genomes of a stably transfected cell line, demonstrating that the HSV-1 TK

Figure 4.17. MN digestion of the viral TK gene during latency in the presence of Ara-C, ACG or PAA. IFNα-treated HFL cells in 35 mm diameter tissue culture dishes were infected with 75 particles of *in*1820 per cell (lanes 1–3, 7–9 and 13–15) or mock infected (lanes 4–6 and 10–12) and overlaid with EF_{10} containing 50 μg/ml Ara-C (lanes 1–3), 5 μM ACG (lanes 4–9) or 50 μg/ml PAA (lanes 10–15). After incubation at 37° for 3 days the nuclei were extracted and digested with MN (lanes 2–3, 5–6, 8–9, 11–12 and 14–15) or were untreated (lanes 1, 4, 7, 10 and 13). DNA was purified, electrophoresed in a 1.5% agarose gel, blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 2.4 kbp *Eco*RI n fragment from the HSV-1 TK gene. MN digestion was as follows: 5 units for 10 minutes (lanes 2, 5, 8, 11 and 14) or 10 units for 10 minutes (lanes 3, 6, 9, 12 and 15). Upper panel shows the autoradiograph, lower panel shows the ethidium bromide-stained gel. L: latently infected sample; M: mock infected sample.


+Ara-C
+ACG
+PAA
MN
MN
MN
MN
MN
L L L M M M L L L M M M L L L
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15



DNA sequences did not preclude the formation of chromatin (Jamieson *et al.*, 1995). It appeared that during latency *in vitro*, *in*1820 genomes were either bound by irregularly spaced nucleosomes or were in a completely different structure from the majority of cell chromatin.

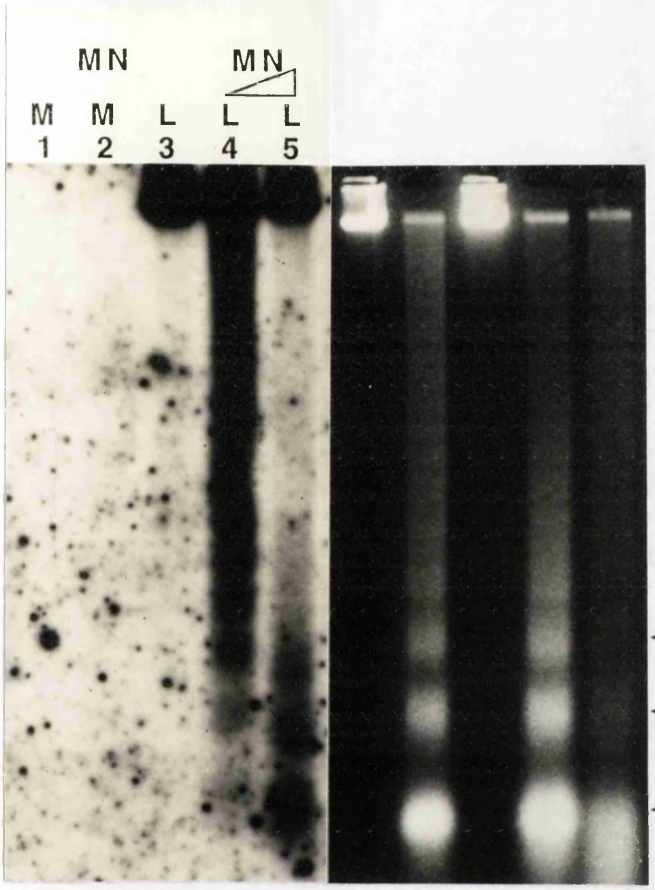
Cellular histone synthesis is tightly coupled to DNA replication. To examine the possibility that a reduction in cell histone pools by Ara-C treatment prevented a normal pattern of nucleosome spacing on the latent viral genomes, the pattern of MN digestion was examined after latency in the presence of ACG or PAA, which have more specific action on viral DNA replication. IFNα-treated HFL cells were infected with 75 particles of *in*1820 per cell and incubated in the presence of Ara-C, ACG or PAA. At 3 days post-infection, nuclei were extracted and partially digested with MN or were untreated. DNA was purified, electrophoresed, blotted onto a hybridisation membrane and hybridised to the HSV-1 *Eco*RI n fragment spanning the TK gene. The cell DNA was digested into a characteristic 'ladder' of nucleosome-associated DNA fragments, as visualised in the ethidium bromide-stained gel (figure 4.17., lower panel). Resulting hybridisation demonstrated that the latent genomes were not bound by regularly spaced nucleosomes during latency in the presence of Ara-C, ACG or PAA (figure 4.17., upper panel). Inhibition of cell DNA replication and intracellular histone pools by Ara-C did not preclude a regular nucleosome arrangement. The absence of regularly spaced nucleosomes on the *in*1820 genomes was therefore not a secondary consequence of inhibition of histone synthesis.

4.11. A chromatin-like pattern of MN digestion was detected at the region of the genome encoding Vmw110 and LAT.

Only the TK gene had been examined in previous studies using MN digestion to detect chromatin on viral genomes in the *in vitro* latency. An investigation was undertaken to determine if the region of the genome which is transcriptionally active during latency *in vivo* formed a chromatin structure during *in vitro* latency. IFNα-treated HFL cells were infected with 120 particles of *in*1820 per cell and incubated in the presence of Ara-C. At 3 days post-infection nuclei were extracted and partially digested with MN or were untreated. DNA was purified, electrophoresed, blotted onto a hybridisation membrane and hybridised to a radiolabelled 3.8 kbp *Pst*I / *Kpn*I fragment from the IE1 / LAT region (figure 3.2.).

The IE1 / LAT region was digested into DNA fragments which produced a ladder characteristic of cell chromatin (figure 4.18., left panel). The presence of chromatin at the region of the genome transcriptionally active *in vivo* and the inability

Figure 4.18. MN digestion of the LAT / IE1 encoding region. IFNα-treated HFL cells in 35 mm diameter tissue culture dishes were infected with 120 particles of *in* 1820 per cell (lanes 3–5) or mock infected (lanes 1 and 2) and incubated at 37° in the presence of 50 μg/ml Ara-C. At 3 days post-infection the nuclei were isolated and digested with MN (lanes 2, 4 and 5) or were untreated (lanes 1 and 3). DNA was purified, half of each sample electrophoresed in a 1.5% agarose gel, blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 3.8 kbp *Pst*I / *Kpn*I fragment from the LAT / IE1 region (figure 3.2.). MN digestion was as follows: 5 units for 2 minutes (lanes 2 and 4) or 10 units for 10 minutes (lane 5). Left panel shows an autoradiograph, right panel shows the ethidium bromide-stained gel. Positions of DNA fragments which were associated with 1, 2 or 3 nucleosomes are indicated. M: mock infected sample; L: latently infected sample.


MN
MN
M
M
L
L
L
1
2
3
4
5
Trinucleosome
Dinucleosome
Mononucleosome

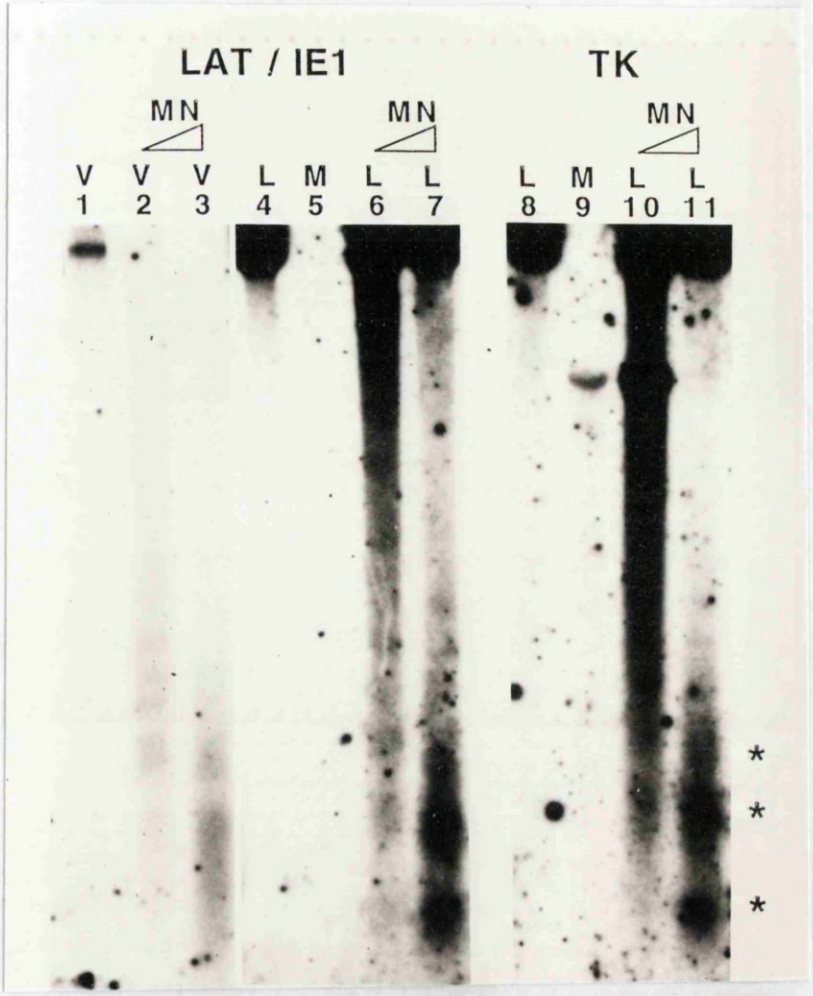
to detect chromatin at the TK gene implied that specific regions of the genome were forming chromatin more readily than others. Differential formation of chromatin might reflect transcriptional regulation of the genome during latency. The bands on the autoradiograph (figure 4.18., left panel) did not correspond exactly with the nucleosomal bands from the bulk of cell DNA observed in the ethidium bromide-stained gel (figure 4.18., right panel). The viral nucleosome-associated DNA fragments migrated faster than the nucleosome-associated fragments from the cell DNA, noticable particularly with the di- and tri- nucleosome-associated fragments. A possible explanation for the difference in migration speeds between viral DNA fragments and cell nucleosome-associated DNA fragments was that nucleosomes on the viral genomes were more closely spaced than on the cell DNA. Other possible causes of the difference in migration speeds between viral cell nucleosome-associated DNA might be high G+C content or unusual structural properties of the HSV sequences, or alternatively by an alteration of their positions during transfer from the gel onto the hybridisation membrane. A control of hybridisation to a cell gene performed in parallel with hybridisation to the IE1 / LAT region was required to confirm a genuine difference in migration speeds between viral and cellular protein-associated DNA fragments.

4.12. Chromatin was detected on the TK gene and the IE1 / LAT region when examined in parallel.

The apparent difference in chromatin organisation between the TK gene and the IE1 / LAT region may have been caused by differences between experiments. The TK gene and the IE / LAT region were examined in parallel. In addition, naked viral DNA digested with MN was examined alongside samples probed for IE1 / LAT in order to assess whether the ladder of DNA fragments observed in figure 4.18. was due to chromatin or sequence-specific nuclease digestion. IFNα-treated HFL cells were infected with 120 particles of *in* 1820 per cell and incubated in the presence of Ara-C. At 2 days post-infection nuclei were extracted and partially digested with MN or were untreated. DNA was purified, electrophoresed alongside partially MN-digested or non-digested naked virion DNA and blotted onto a hybridisation membrane. The hybridisation membrane was divided into two parts, one part was hybridised to a radiolabelled 3.8 kbp *Pst*I / *Kpn*I fragment from the IE1 / LAT region and the the remaining part of the membrane was hybridised to a radiolabelled 2.4 kbp *Eco*RI n fragment from the TK gene.

A regular pattern was apparent in the samples probed for the TK gene and the samples probed for the IE1 / LAT region, indicating that the arrangement of

Figure 4.19. MN digestion of the TK gene and LAT / IE1 encoding region. IFNα-treated HFL cells in 35 mm diameter tissue culture dishes were infected with 120 particles of *in*1820 per cell (lanes 4, 6, 7, 8, 10 and 11) or mock infected (lanes 5 and 9) and incubated at 37° in the presence of 50 μg/ml Ara-C. At 2 days post-infection the nuclei were isolated and digested with MN (lanes 5, 6, 7, 9, 10 and 11) or untreated (lanes 4 and 8). DNA was purified, electrophoresed in a 1.5% agarose gel alongside untreated (lane 1) or MN digested (lanes 2 and 3) naked *in*1850 virion DNA, blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 3.8 kbp *Kpn*I / *Pst*I fragment from the region of the HSV-1 genome encoding LAT / IE1 (lanes 1–7) or a 2.4 kbp *Eco*RI n DNA fragment from the HSV-1 TK gene (lanes 8–11). MN digestion was as follows: 0.08 units for 5 minutes (lane 2), 0.1 units for 5 minutes (lane 3), 10 units for 2 minutes (lanes 5, 6, 9 and 10) or 10 units for 10 minutes (lanes 7 and 11). Upper panel shows the autoradiograph, lower panel shows the ethidium bromide-stained gel. *; positions of DNA fragments which were associated with 1, 2 or 3 nucleosomes. M: mock infected sample; L: latently infected sample; V: virion DNA.


LAT / IE1
TK
MN
MN
MN
V 1
V 2
V 3
L 4
M 5
L 6
L 7
L 8
M 9
L 10
L 11
*
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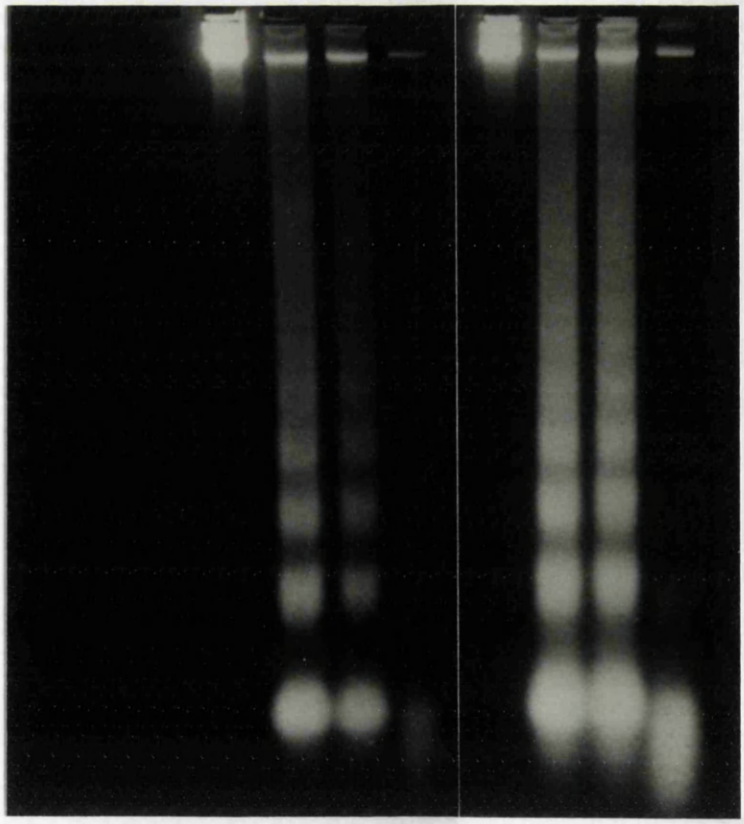


nucleosomes was similar on both regions and presumably on the entire genome (figure 4.19., upper panel). As in the experiment described in section 4.11., the viral nucleosome-associated DNA fragments migrated faster than the nucleosome-associated DNA fragments from the cell DNA, indicating that nucleosomes on the viral genomes might be more closely spaced than on the cell DNA. The presence of discrete bands in the MN-digested naked virion DNA samples indicated that sequence-specific digestion was occurring in the assay. As the products of sequence-specific MN-digestion did not exactly correspond to the apparent nucleosome-associated fragments observed in the IE1 / LAT probed samples and as the nucleosomal bands in the TK-hybridised samples and the IE1 / LAT-hybridised samples were superimposable, the banding pattern in the MN-digested latently infected samples was probably caused by nucleosome positioning. Previous apparent differences in chromatin organisation of the TK gene and the IE1 / LAT region were caused by differences in chromatin arrangement between experiments or differences in the ability to detect the nucleosomal ladder of DNA fragments.

CHAPTER 2.

The inhibition of HSV-1 infectivity in HFL cells pretreated with UV-inactivated *in*1814.

4.13. Infectivity of *in*1853 was reduced in HFL cells pretreated with UV-inactivated *in*1814.

The study described in section 4.5. demonstrated that at a set time after infection HFL cell nuclei contained fewer uncoated, nonlinear viral genomes than most other cell types, possibly as a result of slower virus uncoating. An investigation to determine whether the *in*1814 mutation, HMBA or IFNα exert their effects on *in vitro* latency by altering virus uncoating was described, the results of which were negative. It was considered possible that the slow uncoating in HFL cells was related to the more efficient establishment of latency in these cells (Wigdahl *et al.*, 1981, 1982b, 1984b; Russell and Preston, 1986; Stow and Stow, 1986; Ace *et al.*, 1989; Everett, 1989; Harris and Preston, 1991). One possible mechanism by which slow virus uncoating could lead to latency is if components of the virus particle alter the intracellular environment, thus converting the cell to a state in which latency is the outcome of infection. The delay between fusion of the virion with the cell surface and release of the genome into the nuclei of HFL cells might allow more time for the components of the virion to modify the cell than in other cell types, and this hypothesis is possibly similar to the situation during establishment of latency after infection of the mouse *via* the footpad, where the delay between infection of nerve axons at the periphery and entry into the neuronal cell nuclei was estimated to be between 20 and 24 hours (Cook and Stevens, 1973). Slow virus uncoating in HFL cells might therefore mimic establishment of latency *in vivo*. In the chapter presented here, a study is described which was aimed at investigating the possibility that virus structural components modify HFL cells to a state in which latency is the outcome of infection.

To test the hypothesis that a structural component of virus particles modifies cells to make them more liable to be latently infected, HFL cells were treated with a preparation of *in*1814 which had been UV-irradiated to eliminate its capacity to express genes, and the effect on the ability of the cells to support plaque formation upon subsequent infection with *in*1853 was examined. *In*1853 expresses β-galactosidase under the control of the HCMV major IE promoter, enabling the rapid and sensitive assay of plaque formation by staining the monolayers for β-galactosidase activity. Previous UV-irradiation of the

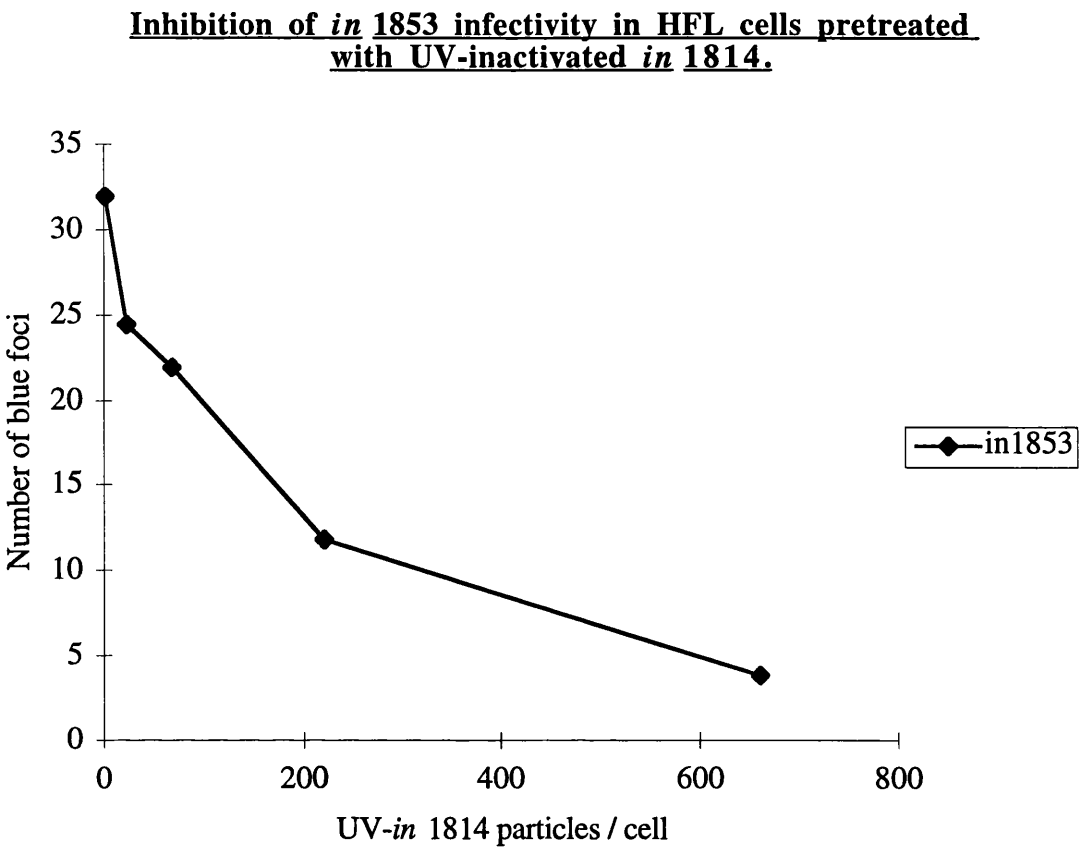


Figure 4.19. Inhibition of *in*1853 infectivity in HFL cells pretreated with UV-inactivated *in*1814. HFL cells in 24-well tissue culture dishes were treated with UV-inactivated *in*1814 at various MOIs or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment the monolayers were superinfected with 100 PFU of *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with EHu_5 and incubated 37°. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The value for the mock-pretreated sample is the average of 8 samples, the remaining values are the averages of duplicates.

*in*1814 was necessary to prevent the expression of IE genes which would complement the superinfecting *in*1853.

HFL cell monolayers were treated with various amounts of UV-irradiated *in*1814 or mock-treated and incubated at 37°. At 6 hours post-treatment the cells were infected with 100 PFU of *in*1853 per monolayer of 2×10^5 cells. The titre of *in*1814-based viruses in HFL cells in the absence of HMBA is far less than in BHK cells in the presence of HMBA, therefore to ensure that an easily countable number of plaques were produced, *in*1853 was previously titrated in HFL cells in the absence of HMBA. The infected monolayers were incubated for a further 24 hours and then fixed, stained for β-galactosidase activity and the resulting numbers of blue foci of virus replication per well was determined. The average numbers of blue foci per monolayer is presented in figure 4.19.

*In*1853 infectivity was inhibited by previous treatment with UV-*in*1814, in a manner which was dependent on the initial input of UV-irradiated particles. An 8-fold inhibition was observed after infection with 660 particles of UV-*in*1814 per cell.

The ability of UV-*in*1814 to inhibit *in*1853 infectivity suggested that components of virus particles modified the cells to a state in which latency was the outcome of infection. In the chapter described here, a characterisation of the inhibition of infectivity by UV-*in*1814 is described.

4.14. UV-*in*1814 light-particles retained the ability to cause inhibition of *in*1853 infectivity.

Novel HSV-1-related particles termed light-particles (L-particles) have been described (Szilágyi and Cunningham, 1991; McLauchlan and Rixon, 1992; Rixon *et al.*, 1992; Szilágyi and Berriman, 1994). L-particles are similar in structure to HSV-1 virions but lack the nucleocapsid and DNA, thus they consist only of tegument and envelope and are non-infectious (Szilágyi and Cunningham, 1991; Szilágyi and Berriman, 1994). The protein composition of L-particles is similar to that of virions except that the nucleocapsid proteins are absent, however it has been reported that Vmw175 in extracellular virus preparations is located exclusively in the L-particles and not the virions (Szilágyi and Cunningham, 1991; McLauchlan and Rixon, 1992). In addition, L-particles frequently contain inclusion vesicles which consist of a low electron density material surrounded by an envelope with no obvious glycoprotein spikes (Szilágyi and Berriman, 1994). The role of L-particles in the HSV life cycle, if any, is unknown and their production *in vivo* has never been demonstrated.

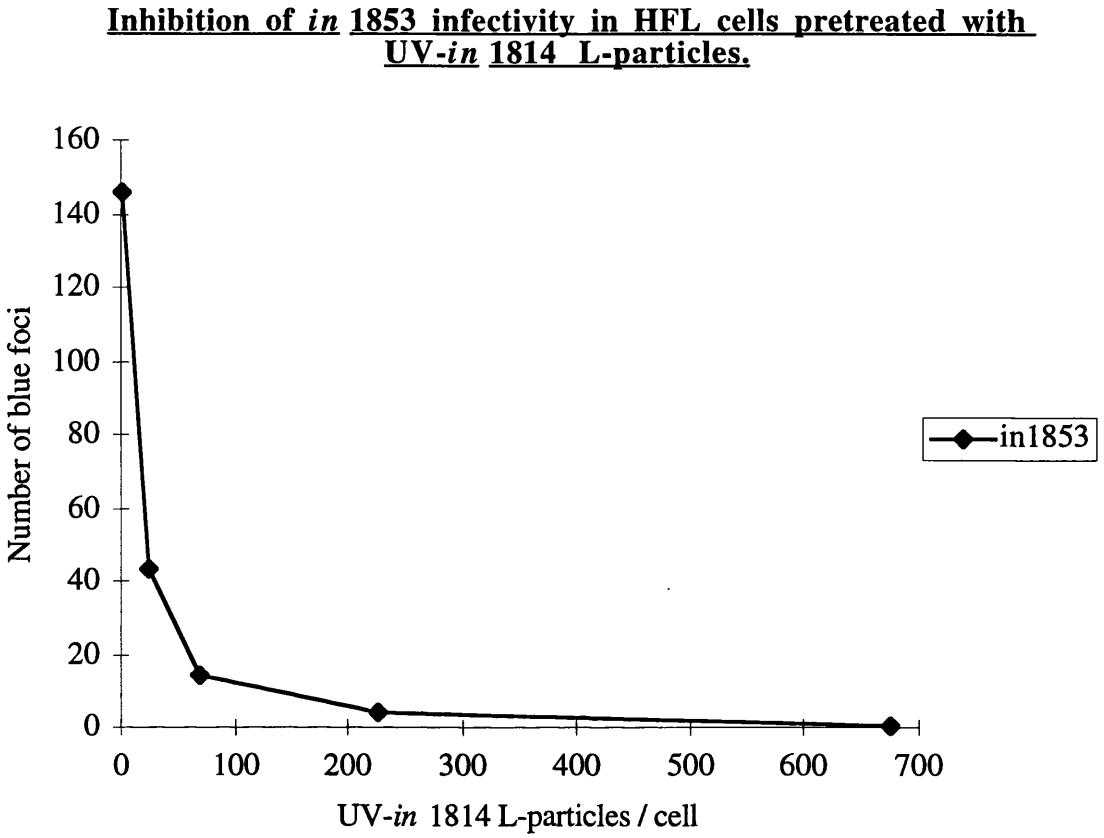


Figure 4.20. Inhibition of *in*1853 infectivity in HFL cells pretreated with UV-*in*1814 L-particles. HFL cells in 24-well tissue culture dishes were treated with UV-*in*1814 L-particles at various MOIs or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment the monolayers were superinfected with 75 PFU of *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with EHu_5 and incubated 37°. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The value for the mock-pretreated sample is the average of 8 samples, the remaining values are the averages of duplicates.

If components of the virus membrane or tegument were responsible for the inhibition of *in*1853 infectivity by UV-*in*1814, then L-particles would retain the ability to cause the inhibition. The effect on *in*1853 infectivity of pretreating HFL cells with *in*1814 L-particles was examined in the same way as described for UV-*in*1814. L-particle preparations are contaminated with virions at a level of 0.1-0.5% (Szilágyi and Cunningham, 1991), thus prior UV-irradiation of the L-particles was required to prevent viral gene expression, especially of Vmw110, from complementing *in*1853.

HFL cell monolayers were treated with various MOIs of UV-*in*1814 L-particles or mock-treated and incubated at 37°. At 6 hours post-treatment the cells were infected with 75 PFU of *in*1853 per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA), incubated for a further 24 hours and then fixed, stained for β-galactosidase activity and the numbers of blue foci per monolayer were determined. The average numbers of blue foci of virus replication per monolayer were counted and are presented in figure 4.20.

*In*1853 infectivity was inhibited in HFL cells pretreated with UV-*in*1814 L-particles to a greater extent than the inhibition caused by pretreatment with UV-*in*1814. After treatment with 675 UV-*in*1814 L-particles per cell, *in*1853 infectivity was inhibited by approximately 140-fold, almost reaching levels undetectable in the assay. In section 4.13., 660 UV-*in*1814 particles per cell gave an 8-fold inhibition, whereas in the section described here an 8-fold inhibition was estimated to occur after treatment with approximately 70 UV-*in*1814 L-particles per cell, thus UV-*in*1814 L-particles caused a stronger inhibition than UV-*in*1814 and reached maximal inhibitory activity at lower MOIs. The observation that L-particles gave greater inhibition of infectivity than the virus preparation suggested that components of the tegument or envelope are responsible for their inhibitory activity. Alternatively, the greater inhibition by L-particles may have been caused by the lower proportion of virions in the L-particle preparation compared to the virus preparation, leading to a lower proportion of treated cells expressing viral proteins and complementing the infectivity defect of *in*1853. Although UV-irradiation eliminated the titre of virus and L-particle preparations, residual gene expression cannot be discounted.

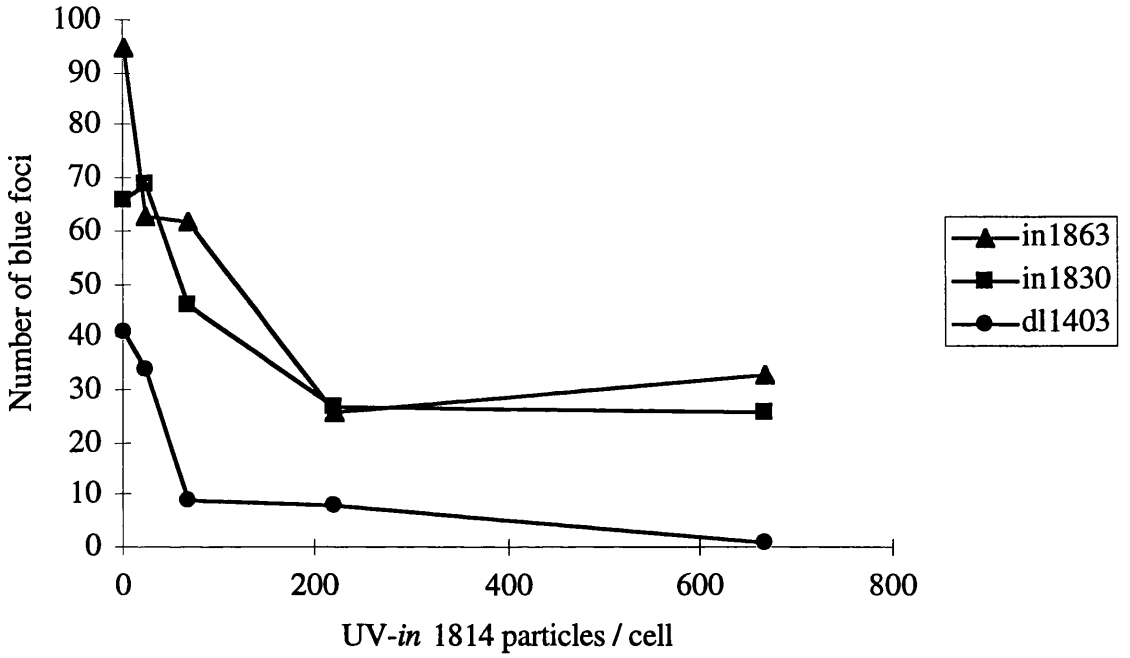
4.15. Infectivities of *in*1863, *in*1830 and *dl*1403 were reduced in HFL cells pretreated with UV-*in*1814 or UV-*in*1814 L-particles.

If Vmw65 overcomes the inhibition of infectivity in HFL cells pretreated with UV-*in*1814 L-particles or virus, then a virus containing wild-type Vmw65 would be insensitive to the inhibition. The sensitivity of *in*1863 (an essentially wild-type virus with

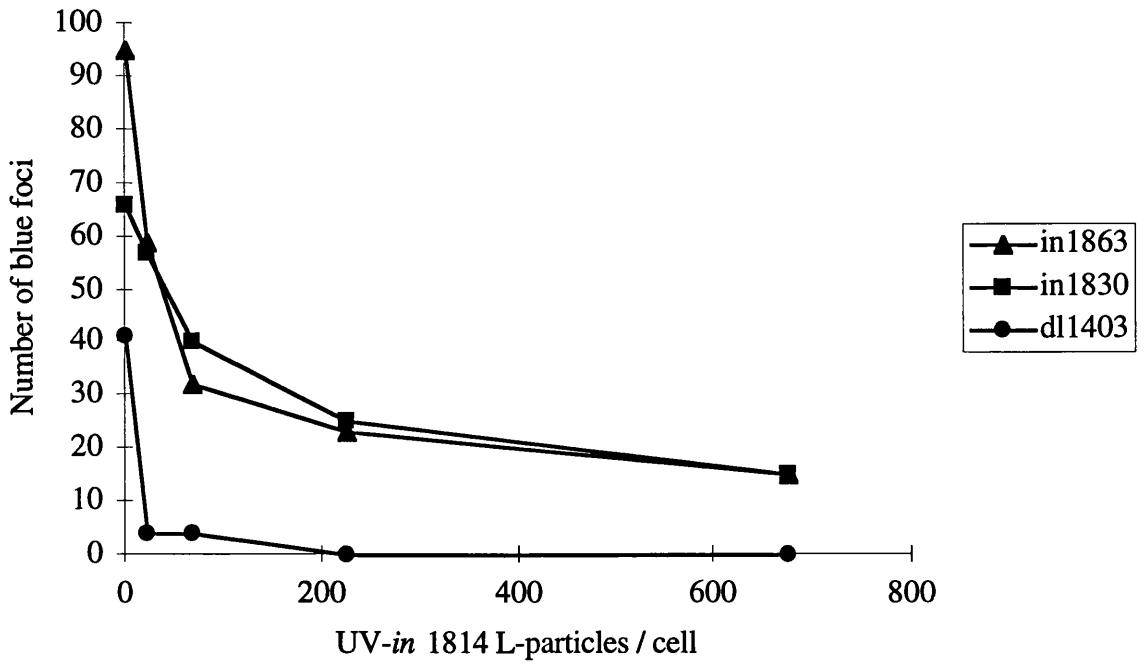
Figure 4.21. Inhibition of *in*1863, *in*1830 and *dl*1403/β-gal infectivity in HFL cells pretreated with UV-*in*1814 or UV-*in*1814 L-particles. HFL cells in 24-well tissue culture dishes were treated with UV-*in*1814 or UV-*in*1814 L-particles at various MOIs or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment each monolayer was infected with 100 PFU of *in*1863, *in*1830 or *dl*1403 per well (MOI was determined from the titres in HFL cells in the absence of HMBA) overlaid with EHu_5 and incubated 37°. At 24 hours post-infection with the the non-UV-inactivated viruses the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The value for the mock-pretreated sample is the average of 4 samples, the remaining values are from single determinations. (A) Effect of UV-*in*1814 on infectivity; (B) effect of UV-*in*1814 L-particles on infectivity.

Inhibition of *in* 1863, *dl* 1403/β-gal and *in* 1830 infectivity in HFL cells pretreated with UV-*in* 1814 virus or L-particles.

A UV-*in* 1814



B UV-*in* 1814 L-particles



the HCMV enhancer controlling transcription of *lacZ* at the TK locus), *in*1830 (*in*1820 with the HCMV enhancer controlling transcription of *lacZ* at the U_L43 locus) and *dl*1403/β-gal (*dl*1403 with the HCMV enhancer controlling transcription of *lacZ* at the U_L43 locus) to the inhibition of infectivity in HFL cells pretreated with UV-*in*1814 or UV-*in*1814 L-particles was examined.

HFL monolayers were treated with various MOIs of UV-*in*1814 or UV-*in*1814 L-particles or were mock-treated and incubated at 37°. At 6 hours post-treatment the cells were infected with 100 PFU of one of the *lacZ*-containing viruses per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA), incubated for a further 24 hours and then fixed, stained for β-galactosidase activity and the resulting numbers of blue foci of virus replication per well were counted. The numbers of blue foci of virus replication is presented in figure 4.21.

Infectivity of all of the viruses was inhibited in HFL cells pretreated with UV-*in*1814 virus or L-particles. *In*1863 was inhibited approximately 6-fold in HFL cells pretreated with 675 UV-*in*1814 L-particles per cell, thus Vmw65 did not overcome the inhibition. *Dl*1403 was especially sensitive to the inhibition, with a greater than 40-fold inhibition occurring in monolayers pretreated with UV-*in*1814 L-particles at MOIs above 225 particles per cell. *In*1830 was inhibited only 2.5-fold in monolayers pretreated with 225 UV-*in*1814 L-particles per cell. Mutant *in*1830 is effectively Vmw110$^-$ under IE conditions in HFL cells, thus it is unclear why *in*1830 was more resistant to the inhibition than *dl*1403. As in previous experiments described in the chapter presented here, UV-*in*1814 L-particles caused greater inhibition of all three viruses than did UV-*in*1814 (comparison of graphs A and B).

4.16. Inhibition of *in*1853 infectivity in HFL cells pretreated with UV-*in*1814 L-particles or virus reached a maximum at 6 hours post-treatment with the UV-inactivated viral particles.

To examine how the inhibition of infectivity in HFL cells pretreated with UV-*in*1814 virus or L-particles varied over time, HFL monolayers were treated with 65 UV-*in*1814 particles or L-particles per cell, or were mock infected and incubated at 37°. At various times post-treatment the monolayers were infected with 100 PFU of *in*1853 per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA) and incubated at 37°. To allow for the drop in titre of the *in*1853 inoculum over time, mock-treated monolayers were infected with the *in*1853 dilution at each time point and the dilution was stored on ice between time points. At 24 hours post-infection with the final time point of *in*1853 infection, the monolayers were fixed and stained for β-galactosidase

Table 4.9. Time course of the inhibition of *in*1853 infectivity in HFL cells pretreated with UV-*in*1814 virus or L-particles. HFL cells in 24-well tissue culture dishes were treated with 65 particles per cell of UV-*in*1814 or UV-*in*1814 L-particles, either alone or co-infected with 100 PFU of *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA), or were mock-treated, overlaid with EF_{10} and incubated at 37°. At various times post-treatment the monolayers were infected with 100 PFU *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with EHu_5 and incubated 37°. To allow for the drop in titre of the *in*1853 inoculum over time, mock-infected monolayers were infected with the *in*1853 dilution at each time point and the dilution was stored on ice between time points. At 24 hours post-infection with the final time point of *in*1853 infection, the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The value for the mock-pretreated samples is the average of duplicates, the remaining values are the from single determinations. (A) Samples pretreated with UV-*in*1814 L-particles. (B) Samples preinfected with UV-*in*1814.

A

Time of infection of the cells with *in*1853, post-treatment with UV-*in*1814 L-particles (hours)		0	2	4	6	9
Pretreatment	Mock (number of plaques)	400	253	169	151	154
	UV-*in*1814 L-particle (number of plaques)	153	12	6	2	5
Fold-inhibition of *in*1853 infectivity		2.6	24	22	52	31

B

Time of infection of the cells with *in*1853, post-treatment with UV-*in*1814 (hours)		0	2	4	6	9
Pretreatment	Mock (number of plaques)	400	253	169	151	154
	UV-*in*1814 (number of plaques)	158	28	14	6	14
Fold-inhibition of *in*1853 infectivity		2.5	9	12	25	11

activity. The resulting numbers of blue foci of virus replication per well were counted and the average values and the fold-inhibition at each time point presented in table 4.9.

The inhibitory effect on *in*1853 infectivity increased over time and reached a maximum at 6 hours post-treatment with the UV-inactivated particles. The observation that the inhibition increased after infection suggested that a gradual rather than immediate change occurred in the cells after treatment with UV-inactivated viral particles.

4.17. The components of UV-*in*1814 L-particles responsible for the inhibition of *in*1853 infectivity were removed by centrifugation.

The inhibition of *in*1853 infectivity in HFL cells treated with UV-*in*1814 L-particles or virus might have been caused by soluble factors in the UV-inactivated preparations rather than viral particles. To assess whether the inhibition was caused by viral particles, an L-particle preparation was centrifuged and the resulting supernatant and resuspended pellet were tested for their ability to inhibit *in*1853 infectivity. As a control for any loss of inhibitory activity due to loss of infectivity of the particles over time, an aliquot was stored at 4° during the centrifugation and tested for the ability to inhibit *in*1853 infectivity.

HFL cells were treated with 3 µl of the supernatant, pellet or 4°-stored L-particles per monolayer of 2×10^5 cells (corresponding to an MOI of 6600 particles per cell of the original preparation) and incubated at 37°. At 7 hours post-treatment the cells were infected with 100 PFU of *in*1853 per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA) and incubated at 37°. At 24 hours post-infection with *in*1853, the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The average values for each treatment is presented in figure 4.22.

The resuspended pellet and the sample stored at 4° caused a 10-fold inhibition of *in*1853 infectivity, whereas the supernatant caused no significant inhibition, thus the inhibitory factors were pelleted during centrifugation and were almost certainly associated with the viral particles.

It is notable in this experiment that the inhibitory activity of the L-particles was lower than in previous experiments, an MOI of 6600 L-particles per cell giving a 10-fold inhibition of infectivity compared to the experiment described in section 4.14. where an MOI of 100 L-particles per cell was sufficient to cause an equivalent inhibition. The differences in inhibitory activity between experiments was probably caused by differences in the qualities of the L-particle preparations.

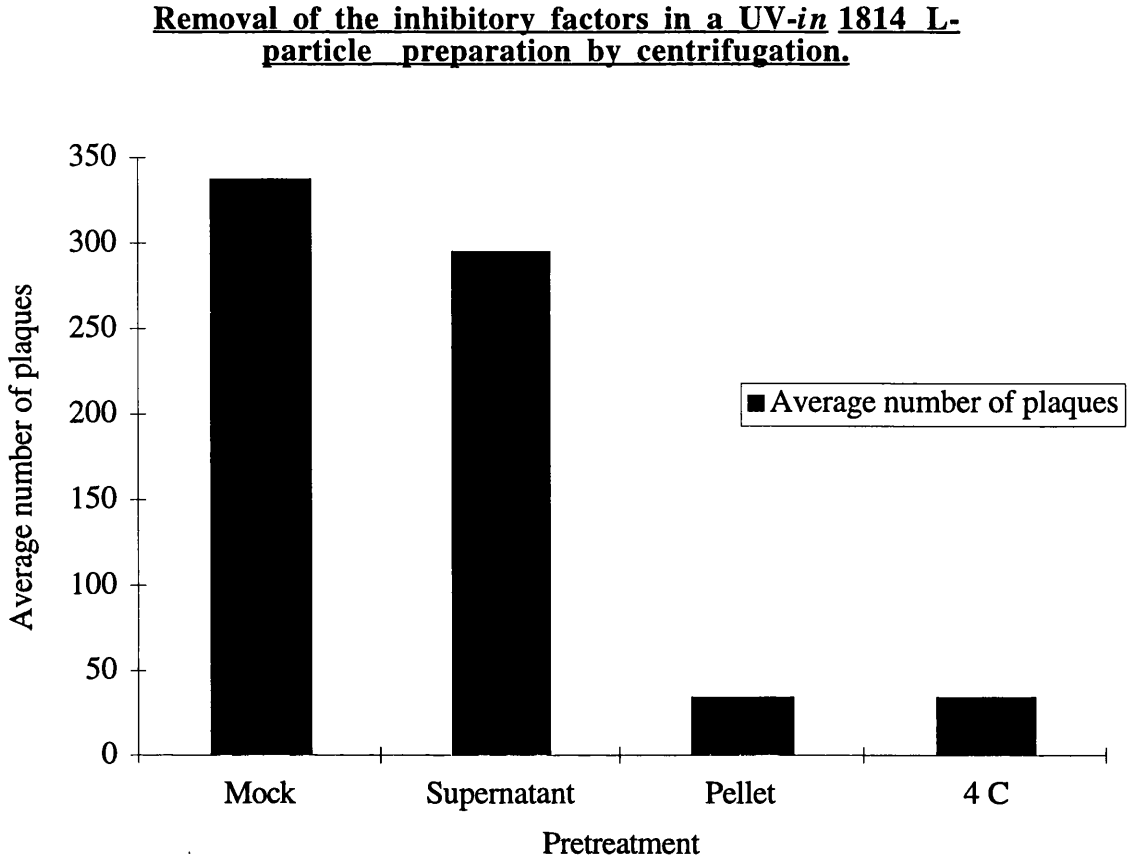


Figure 4.22. Removal of the inhibitory factors in a UV-*in*1814 L-particle preparation by centrifugation. 100 µl of an UV-*in*1814 L-particle preparation was centrifuged at 12000 rpm for 16 hours in an AH629 rotor. The resulting pellet was resuspended in 100 µl Eagle's A+B without phenol red and both the supernatant, the resuspended pellet and an aliquot of the L-particle preparation which were stored at 4° during the centrifugation were examined for their ability to inhibit *in*1853 infectivity when used to pretreat HFL cells. HFL cells in 24-well tissue culture plates were treated with 3 µl per well (corresponding to an MOI of 6600 particles per cell of the original preparation) of the supernatant, pellet or 4°-stored light particles, overlaid with EF_{10} and incubated at 37°. At 7 hours post-treatment, the cells were superinfected with 100 PFU *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with CMC/EF_{10} and incubated at 37°. At 24 hours post-infection with *in*1853, the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The value for the mock-pretreated samples is the average of 4 samples, the remaining samples are the averages from triplicates.

4.18. HMBA only partially overcame the inhibition of *in*1853 infectivity in HFL cells pretreated with UV-*in*1814.

The particle / PFU ratio of *in*1814 preparations are restored to levels similar to those of wild-type HSV-1 in the presence of 3 mM HMBA, thus when present at the initial stages of infection, HMBA can almost completely overcome repression of *in*1814 genomes (McFarlane *et al.*, 1992). The restoration of *in*1814 infectivity by HMBA is the result of a stimulation of IE transcription, substituting for Vmw65 transactivation however, during the first 6 hours of infection the defect in *in*1814 cannot be overcome by HMBA or Vmw65, thus once established, the latent state is unresponsive to HMBA or Vmw65. One possible interpretation of the observation that latent virus is unresponsive to HMBA is that *in*1814 infection leads to an antiviral state in the cells which leads to latency and that this antiviral state cannot be overcome by HMBA. This interpretation is supported by the observation that UV-inactivated viral particles induce an antiviral state and that the antiviral state acts against *in*1863, suggesting that it is not overcome by Vmw65.

In order to assess whether the repression of *in*1853 infectivity in HFL cells treated with UV-*in*1814 can be overcome by HMBA, HFL cells were treated with 65 UV-*in*1814 particles per cell or mock-treated and incubated at 37°. At 6 hours post-treatment the monolayers were infected with various amounts of *in*1853 (input PFUs shown were determined from the titre in HFL cells in the presence of HMBA) and overlaid with EHu_5 or EHu_5 containing 5 mM HMBA. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. Values are presented in table 4.10(A). In addition, to determine the inhibition that occurred in the absence of HMBA it was necessary to perform an experiment in parallel in which the input PFU of *in*1853 was determined using the titre in the absence of HMBA (table 4.10.(B)).

In the presence of HMBA, *in*1853 infectivity was inhibited by at least 5-fold in HFL cells pretreated with UV-*in*1814 (A). In the absence of HMBA the inhibition was 19-fold (B), thus HMBA was not able to overcome fully the inhibition of infectivity by the UV-viral particles. Like latency of *in*1814 in untreated cells, the antiviral state induced by the UV-viral particles dominates over HMBA and Vmw65.

Table 4.10. HMBA partially overcame the inhibition of infectivity in HFL cells pretreated with UV-*in*1814. (A) HFL cells in 24-well tissue culture dishes were treated with 65 particles of UV-*in*1814 per cell or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment the monolayers were superinfected with various amounts of *in*1853 per well (PFUs shown were determined from the titres in HFL cells in the presence of HMBA), overlaid with EHu_5 or EHu_5 containing 5 mM HMBA and incubated 37°. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. Values are the results of single determinations. (B) Monolayers were pre-treated as indicated in the table and described above with the only difference that the input PFU was as determined from the titre in HFL cells in the absence of HMBA. Values are the averages of triplicates.

A

Input PFU of *in*1853 (+HMBA titre)		200	67	33	20
Treatment	**–HMBA**	0	0	0	0
	+HMBA	200	66	27	21
	+UV-*in*1814 / +HMBA	35	13	2	1
Fold-inhibition		5	5	14	21

B

Input PFU of *in*1853		100
Pretreatment	**Mock**	95
	+UV-*in*1814	5
Fold-inhibition		19

4.19. The inhibition of infectivity in HFL cells pretreated with UV-*in*1814 was only partially overcome by co-infection with *ts*K.

The particle / PFU ratio of *in*1814 is restored to levels similar to those of wild-type virus in cells co-infected with *ts*K or UV-irradiated *ts*K (Ace *et al.*, 1989; Harris and Preston, 1991). Almost all input *in*1820 infectivity in the *in vitro* latency system is competent for reactivation by superinfection with *ts*K (Jamieson *et al.*, 1995).

To assess whether the inhibition of infectivity in HFL cells pretreated with UV-*in*1814 can be overcome by co-infection with *ts*K at a NPT for *ts*K, HFL cells were treated with 65 particles of UV-*in*1814 per cell or mock-treated. After incubation for 6 hours, the monolayers were superinfected with 500 or 1000 PFU of *in*1853 per monolayer (MOI was determined from the titre in BHK cells in the presence of HMBA) and immediately superinfected with 0.5 PFU of *ts*K per cell or mock-superinfected. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. Values are presented in table 4.11.

Superinfection with *ts*K reactivated only a proportion of the *in*1853 which was inhibited in the UV-*in*1814 particle pretreated cells, thus in this respect the apparently latent state induced in UV-viral pretreated cells appeared to differ from latency of *in*1814-based viruses in untreated cells.

Input PFU of *in*1853		500	1000
Treatment	***in*1853 alone**	2	2
	+*ts*K	TNTC	TNTC
	+UV-*in*1814 / +*ts*K	150	200

Table 4.11. The inhibition of infectivity in HFL cells pretreated with UV-*in*1814 was only partially overcome by co-infection with *ts*K. HFL cells in 24-well tissue culture dishes were treated with 65 particles of UV-*in*1814 per cell or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment the monolayers were superinfected with 500 or 1000 PFU of *in*1853 per well (PFUs shown were determined from the titres in BHK cells in the presence of HMBA) and immediately superinfected with 0.5 PFU of *ts*K per cell or mock-superinfected, overlaid with EHu_5 and incubated 37°. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. Values are the results of single determinations. Mock-infected samples are values for single determinations, all other values are the averages of duplicates. TNTC: too numerous to count.

4.20. *In*1853 infectivity inhibited in UV-*in*1814 L-particle-pretreated HFL cells was not reactivated by superinfection with *ts*K at 2 days post-infection.

The inability of co-infection with *ts*K to restore the infectivity of *in*1853 to wild-type levels in UV-viral particle-treated HFL cells might have been caused by the inhibitory activity of the UV-viral particle treatment acting on *ts*K and hindering the efficiency of complementation. An experiment was carried out to assess whether the *in*1853 genomes which were inhibited in UV-viral particle-treated HFL cells could be reactivated by superinfection after 2 days infection, when the inhibitory activity of the UV-viral particle treatment might have subsided.

HFL cells were treated with 65 UV-*in*1814 L-particles per cell or mock-treated. At

Treatment	*in*1853 alone	+*ts*K	+UV-*in*1814 L-particles +*ts*K
Average number of plaques	0	32	7

Table 4.12. *In*1853 infectivity inhibited by UV-*in*1814 L-particles was not reactivated by superinfection with *ts*K at 2 days post-infection. HFL cells in 24-well tissue culture dishes were treated with 65 UV-*in*1814 L-particles per cell or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment the monolayers were superinfected with 100 PFU *in*1853 per well (PFU was determined from titres in BHK cells in the presence of HMBA), overlaid with EF_{10} containing 50 μg/ml Ara-C and incubated 37°. At 2 days post-infection with *in*1853 the monolayers were superinfected with 0.5 PFU of *ts*K per cell or mock-superinfected, overlaid with EHu_5 and incubated at 37°. After incubation for a further 24 hours the cells were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. Values are the averages of triplicates.

6 hours post-treatment the monolayers were infected with 100 PFU of *in*1853 per monolayer (MOI was determined from the titre in BHK cells in the presence of HMBA) and incubated in the presence of Ara-C. At 2 days post-infection with *in*1853 the monolayers were superinfected with 0.5 PFU of *ts*K per cell or mock superinfected. After incubation for a further 24 hours the cells were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The average values are presented in table 4.12.

The infectivity which was inhibited by UV-*in*1814 L-particle pretreatment was not fully reactivated to give a number of plaques equal to the number of plaques in monolayers mock-pretreated. It appeared that the *in*1853 inhibited in HFL cells pretreated with UV-*in*1814 L-particles was at least partially permanently lost and could not be reactivated by *ts*K.

4.21. The inhibition of *in*1853 infectivity in HFL cells treated with UV-*in*1814 remained for at least 2 days.

In section 4.20. it was found that *in*1853 inhibited in HFL cells pretreated with UV-*in*1814 L-particles was not reactivated by superinfection with *ts*K at 2 days post-infection. In the section described here, the possibility that the inhibition of infectivity in HFL cells remained after 2 days, thereby hindering the ability of *ts*K to cause reactivation, is examined.

HFL cells were treated with various MOIs of UV-*in*1814 or mock-treated. At 6 hours, 1 day or 2 days post-treatment the cells were infected with 100 PFU of *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA). At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The average values are presented in figure 4.23.

The inhibition of *in*1853 infectivity remained for 1 and 2 days in HFL cells treated with UV-*in*1814. It is probable, therefore, that the inability to reactivate *in*1853 fully by superinfection with *ts*K was caused by the previous UV-*in*1814 viral particle treatment inhibiting *ts*K, thus superinfection with *ts*K was not a reliable method of reactivating *in*1853 from HFL cells pretreated with UV-*in*1814. Alternatively, the inability of superinfection with *ts*K to fully reactivate the *in*1853 could have been explained if a proportion of the *in*1853 genomes in the UV-*in*1814 pretreated cells were degraded or converted to a form unresponsive to *ts*K superinfection.

4.22. Tissue culture medium taken from HFL cells treated with UV-*in*1814 L-particles did not confer significant inhibition of *in*1853 infectivity when used to treat other HFL cells.

HFL cells treated with UV-inactivated viral particles might release soluble factors, such as IFNα, which could cause the reduced ability of the cells to support HSV infection. As IFNα acts by reducing viral IE transcription, the hypothesis that repression of *in*1814 genomes in HFL cells occurs *via* an IFNα-induced pathway was considered possible.

HFL cells were treated with 800 UV-*in*1814 L-particles per cell or mock-treated, washed with EF_{10} to remove any un-adsorbed L-particles, and overlaid with EF_{10}. After incubation for 6 hours the medium was removed and stored and the cells were infected with 100 PFU of *in*1853 per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA). At 24 hours post-infection with *in*1853 the cells were fixed,

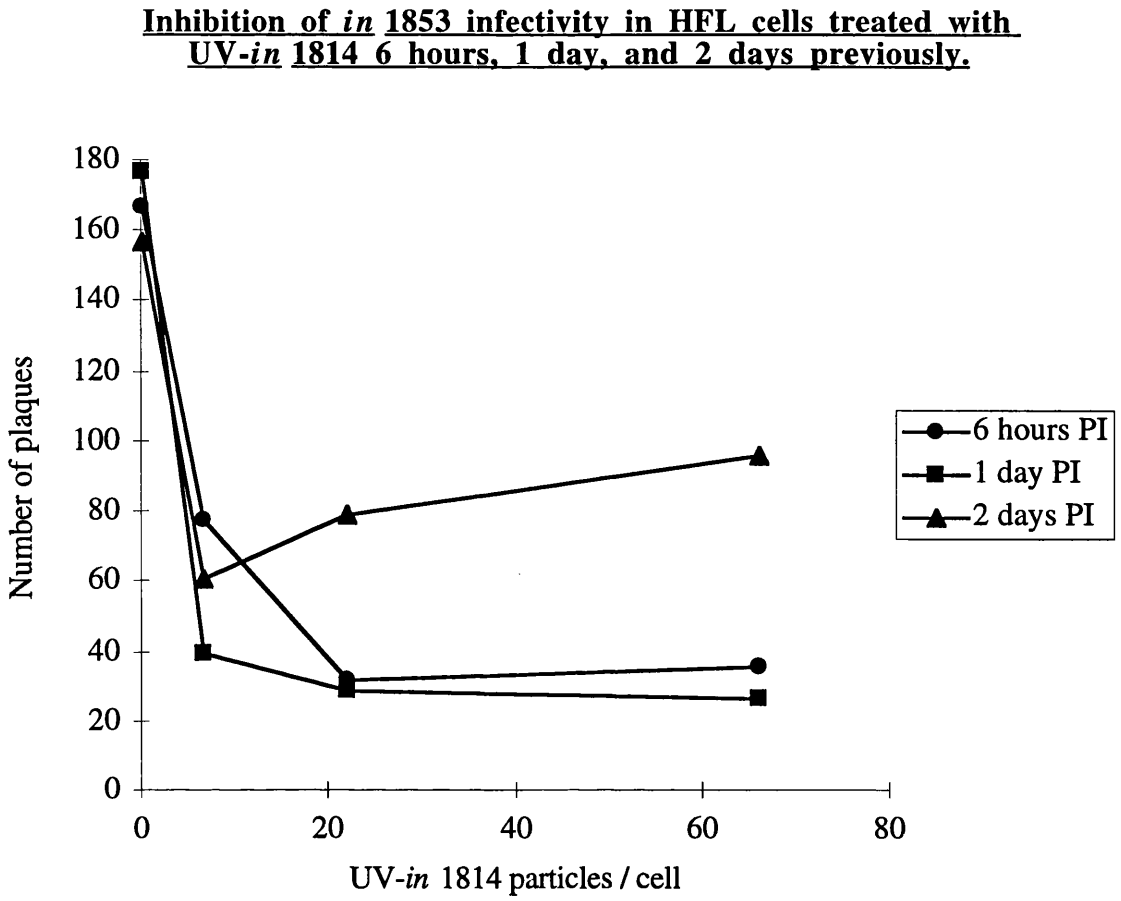
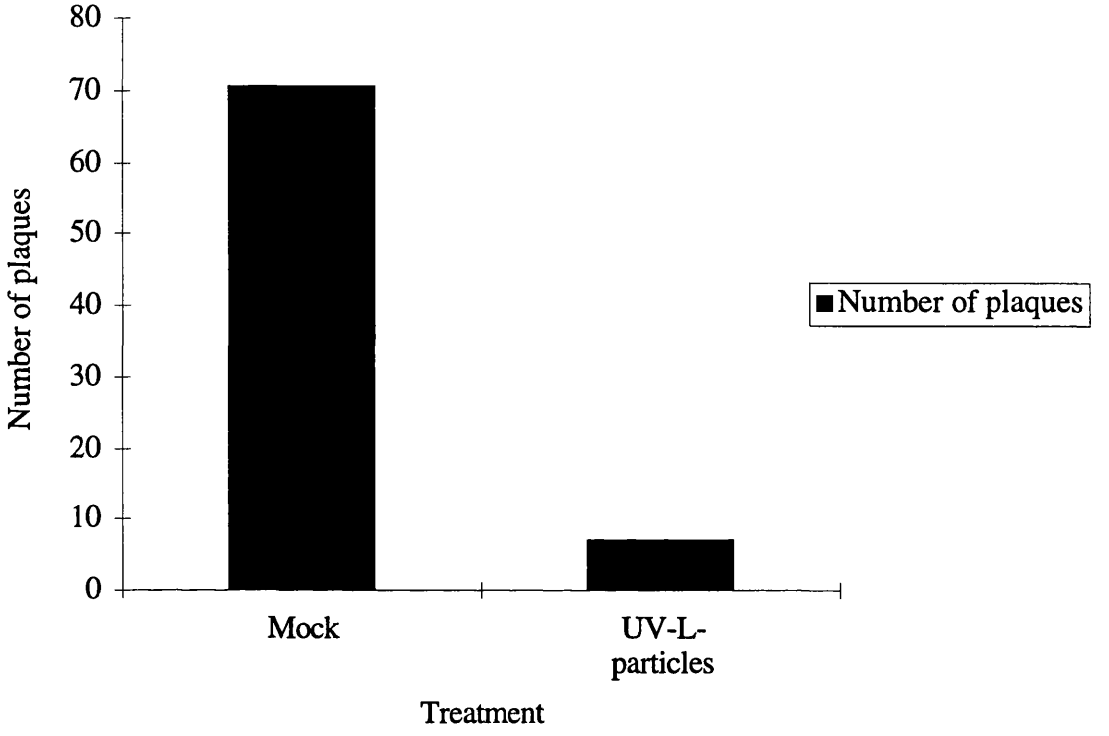


Figure 4.23. Inhibition of *in*1853 infectivity after infection of HFL cells 6 hours, 1 day and 2 days post-treatment with UV-*in*1814. HFL cells in 24-well tissue culture dishes were treated with various MOIs of UV-*in*1814 or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours, 1 day or 2 days post-treatment the cells were superinfected with 100 PFU of *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with EHu_5 and incubated at 37°. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. Values for the mock-pretreated samples are the averages of 6 samples, the remaining samples are the averages from duplicates.

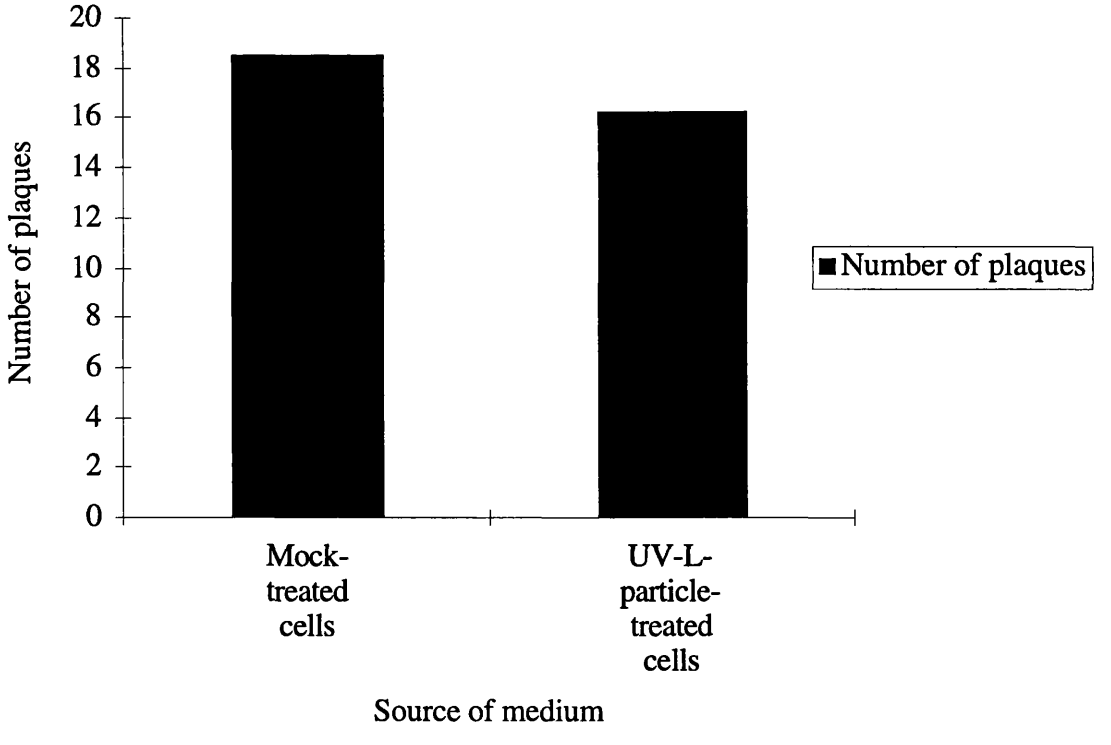
Figure 4.24. Tissue culture medium taken from HFL cells treated with UV-*in*1814 L-particles did not confer significant inhibition of *in*1853 plaque initiation when used to treat other HFL cells. (A) HFL cells in 24-well tissue culture dishes were treated with 800 UV-*in*1814 L-particles per cell or mock-treated, washed once with 1 ml of EF_{10} per well, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment the medium was removed, stored, and the cells infected with 100 PFU of *in*1853 per well (PFU was determined from the titre in HFL cells in the absence of HMBA), overlaid with CMC/EF_{10} and incubated at 37°. At 24 hours post-infection with *in*1853 the cells were fixed, stained for β-galactosidase activity and the blue foci of virus replication counted. (B) The medium from the mock-treated or L-particle-treated HFL cells was immediately applied to HFL cell monolayers in a separate 24-well tissue culture plate and the cells incubated at 37° overnight. The cells were infected with 100 PFU of *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with CMC/EF_{10} and incubated at 37°. At 24 hours post-infection with *in*1853 the cells were fixed, stained for β-galactosidase activity and the blue foci of virus replication counted. Values presented are the averages numbers of foci of virus replication per well from 4 samples.

Absence of soluble factors released by HFL cells treated with UV-*in* 1814 L-particles.

A Initial inhibition



B Inhibitory effect of medium



stained for β-galactosidase activity and the blue foci of virus replication counted. The medium from the mock-treated or UV-L-particle treated HFL cells were immediately applied to separate HFL cell monolayers and incubation was continued overnight. The medium-treated cells were infected with 100 PFU of *in*1853 per monolayer and at 24 hours post-infection the cells were fixed, stained for β-galactosidase activity and the blue foci of virus replication counted. The average numbers of plaques per monolayer are presented in figure 4.24.

In the initial inhibition experiment (figure 4.24. A) *in*1853 infectivity was inhibited 10-fold in the UV-L-particle treated HFL cells. However, when other HFL cells were exposed overnight to medium taken from the UV-*in*1814 L-particle treated HFL cells, no significant inhibition was conferred to those cells (figure 4.24. B), thus a soluble factor released from HFL cells in response to infection with UV-*in*1814 L-particles was not detected.

4.23. Pretreatment of HFL cells with UV-*in*1814 L-particles did not exert its affect on *in*1853 infectivity by altering virus adsorption, penetration, migration to the nuclei, uncoating or DNA stability.

The observation that cells become nonpermissive to superinfection after treatment with high MOIs of UV-inactivated virus has been made in previous studies by other research groups (Vahlne *et al.*, 1979; Johnson and Ligas, 1988). After one study, it was suggested that gD brought into the cell with the UV-inactivated particles caused the nonpermissive state by sequestering receptors required for fusion of the virus with the cell, thus preventing penetration of superinfecting viruses (Johnson and Ligas, 1988). The hypothesis that gD inhibits superinfection is supported by the observation that cell lines constitutively expressing gD are nonpermissive (Campadelli-Fiume *et al.*, 1988; Johnson and Spear, 1989; Campadelli-Fiume *et al.*, 1990). In addition, a cell line constitutively expressing the viral structural component encoded by U_S11 was nonpermissive due to a blockage in virus penetration (Roller and Roizman, 1994). The inhibition of virus replication in the U_S11 expressing cell line also appeared to be *via* an interaction with gD. It is a possibility that the blockage to virus infection in UV-*in*1814 viral particle treated HFL cells observed in the preceding sections was occurring before viral IE transcription, possibly as a result of sequestration of gD receptors by gD brought into the cell with the UV-inactivated viral particles. Thus the effect of pretreating HFL cells with UV-*in*1814 on the levels of viral DNA in the nuclei after infection with *in*1853 was examined using the Southern blot assay described in chapter 1 of the results section of the thesis described here.

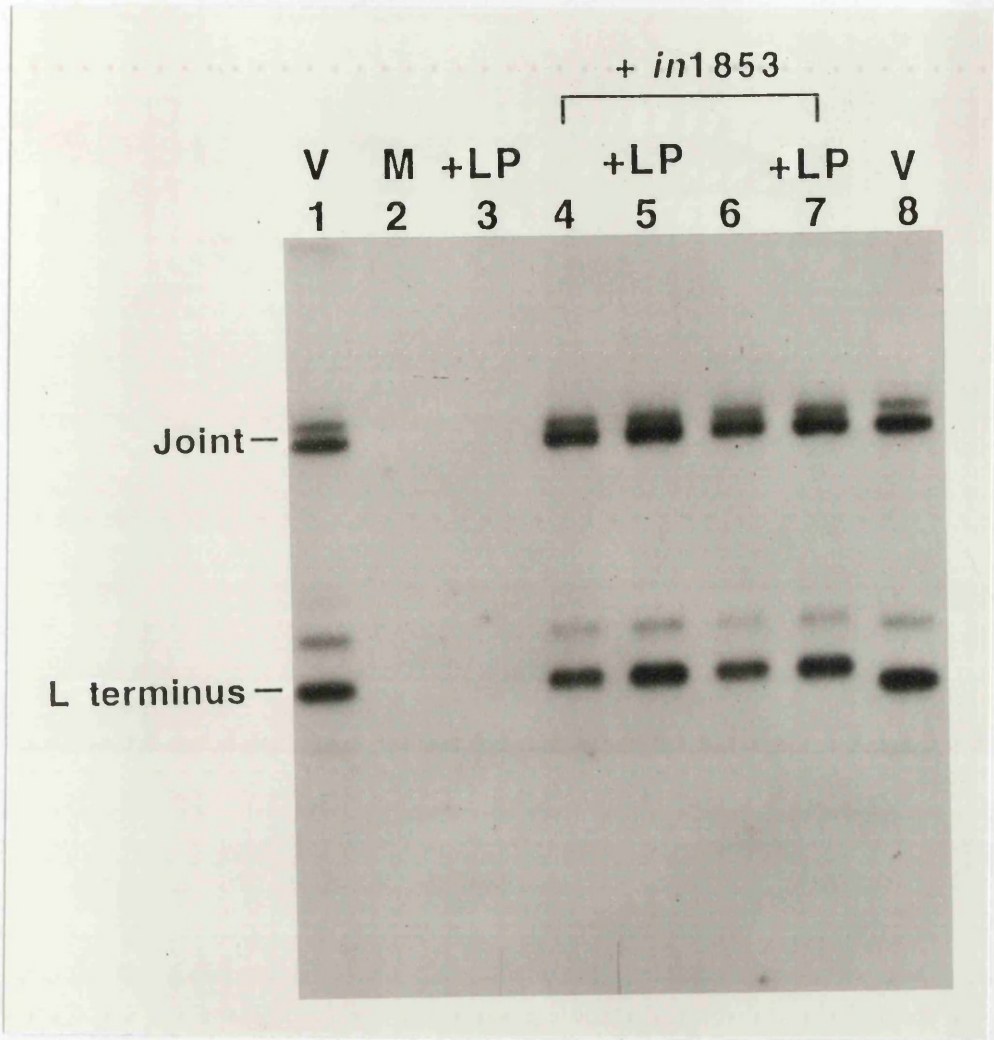


Figure 4.25. Adsorption, penetration, migration to the nuclei and uncoating of *in*1853 in HFL cells pretreated with UV-*in*1814 L-particles. HFL cells in 35 mm diameter tissue culture dishes were treated with 225 UV-*in*1814 L-particles per cell (lanes 3, 5 and 7) or mock-treated (lanes 2, 4 and 6), overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment, the cells were superinfected with 1 PFU of *in*1853 per cell (lanes 4–7) or mock superinfected (lanes 2 and 3) and incubated at 37° in the presence of 50 µg/ml Ara-C. At 24 hours post-treatment, the cell nuclei were isolated and the DNA purified, cleaved with *Bam*HI and half of each sample electrophoresed in a 0.7% agarose gel alongside 1.0 ng of *in*1814 (lane 1) and *in*1825 (lane 8) DNA. Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 640 bp *Bam*HI / *Nco*I fragment from the 5' coding sequences of IE1 (figure 3.2.). Positions of joint and L-terminal *Bam*HI fragments are indicated. The slower migration of the L-terminal fragment from *in*1825 is due to the replacement of the IE1 promoter with the MMLV LTR (lane 8). LP: UV-*in*1814 L-particle pretreated sample; M: mock-pretreated sample; V: virion DNA.

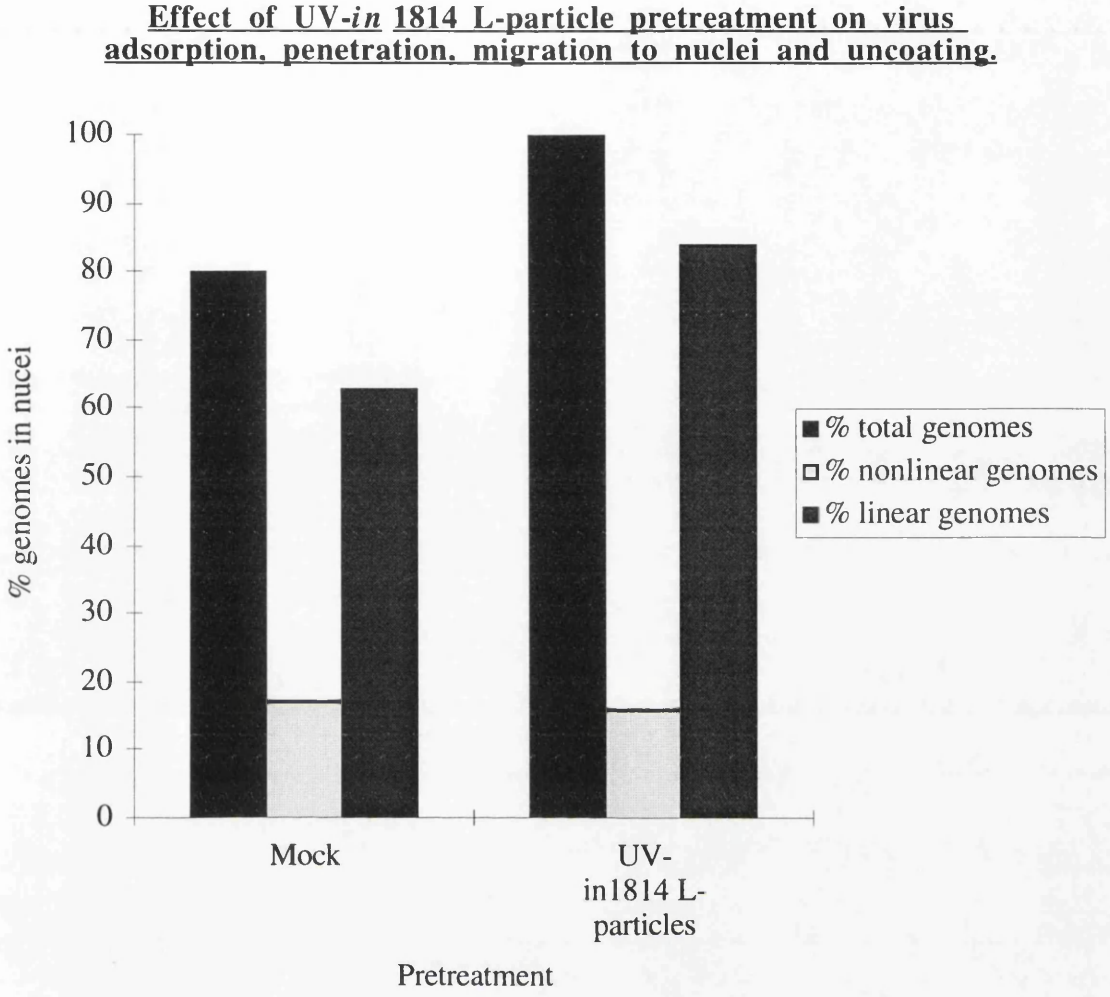


Figure 4.26. Relative proportions of nonlinear and linear viral genomes in the nuclei after infection of UV-*in*1814 L-particle pretreated or mock-pretreated HFL cells. Hybridisation in figure 4.25. was quantified and the relative proportions of nonlinear and linear genomes determined by comparison with the purified virion DNA standards. The total number of genomes in the UV-*in*1814 L-particle pretreated HFL cell nuclei, which contained the greatest total number of genomes compared to mock-pretreated cells, represents 100%.

HFL cells were treated with 225 UV-*in*1814 L-particles per cell or mock-treated. At 6 hours post-treatment the cells were superinfected with 1 PFU of *in*1853 per cell or mock superinfected and incubated in the presence of Ara-C. At 24 hours post-superinfection, cell nuclei were isolated and the DNA purified, cleaved with *Bam*HI and half of each sample electrophoresed alongside virion DNA. Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a radiolabelled fragment from the 5' coding sequences of IE1 which detects the joint and L-terminal *Bam*HI restriction fragments. Resulting hybridisation is shown in figure 4.25. Quantification of the relative proportions of nonlinear and linear genomes is presented in figure 4.26.

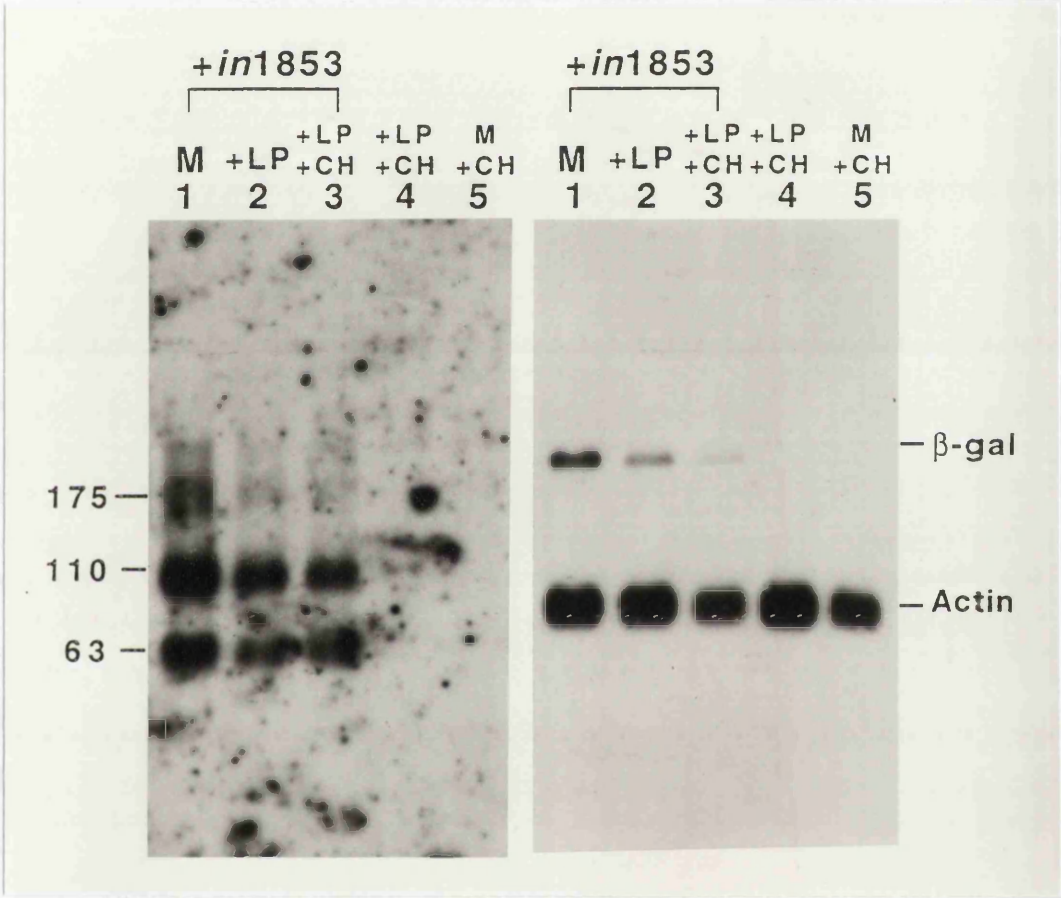
UV-*in*1814 L-particle pretreatment did not decrease the amount of *in*1853 DNA in the nuclei, neither was the degree of nonlinearity significantly altered. Thus the mechanism of inhibition of superinfection did not, therefore, appear to be *via* an inhibition of adsorption, penetration, migration to the nuclei, uncoating or DNA stability. In the experiments described in this thesis, the nonpermissive state induced by UV-virus pretreatment appeared to differ from that observed by Johnson and Ligas (1988).

4.24. Levels of viral IE mRNAs were reduced by 2-fold in HFL cells pretreated with UV-*in*1814 L-particles, and the reduction in IE mRNA levels did not require *de novo* protein synthesis.

As virus adsorption, penetration, migration to nuclei or DNA stability was not significantly altered in UV-*in*1814 L-particle pretreated cells (section 4.23.), it follows that the inhibition of plaque initiation in such cells was caused by an alteration of viral gene expression. Inhibition of HSV IE transcription causes a reduced ability to initiate plaque formation, as demonstrated by the phenotype of *in*1814 and the action of IFNα, thus one possible mechanism by which the UV-inactivated viral particles might exert their effects is to cause an inhibition of viral IE gene expression. Levels of viral IE mRNAs after infection with *in*1853 were compared in HFL cells pretreated or mock-pretreated with UV-*in*1814 L-particles. The effect of inhibiting protein synthesis during the UV-*in*1814 L-particle pretreatment was also examined.

HFL cells were treated with 265 UV-*in*1814 L-particles per cell or mock-treated and overlaid with medium with or without cycloheximide. At 5 hours post-treatment the cells were infected with 10 PFU of *in*1853 per cell or mock superinfected in the presence of cycloheximide. At 5 hours post-infection with *in*1853, poly(A)-containing RNA was extracted, each sample was divided into 2 portions, electrophoresed in an agarose gel and blotted onto a hybridisation membrane. The hybridisation membrane was divided in two,

Figure 4.27. Viral IE mRNAs in HFL cells pretreated with UV-*in*1814 L-particles. HFL cells in 90 mm diameter tissue culture dishes were treated with 265 UV-*in*1814 L-particles per cell (lanes 2–4) or mock-treated (lanes 1 and 5), overlaid with EF_{10} with (lanes 3–5) or without (lanes 1 and 2) 50 μg/ml cycloheximide and incubated at 37°. At 5 hours post-treatment the cells were superinfected with 10 PFU of *in*1853 per cell (lanes 1–3) or mock superinfected (lanes 4 and 5) in the presence of 50 μg/ml cycloheximide, overlaid with EF_{10} containing 50 μg/ml cycloheximide and incubated at 37°. At 5 hours post-infection with *in*1853, poly(A)-containing RNA was extracted. Each sample was divided into 2 halves, electrophoresed in a 1.2% agarose gel and blotted onto a hybridisation membrane. The hybridisation membrane was divided in two, one half was hybridised to a [^{32}P]-labelled 670 bp *Eco*RI / *Pvu*II DNA fragment from a plasmid containing the cloned *Xho*I C fragment from HSV-1, specific for the mRNA encoding Vmw175, a 700 bp *Asp*718 / *Bam*HI fragment from the plasmid pJR3, specific for Vmw110 mRNA and a 1.2 kbp *Bam*HI / *Sal*I fragment from plasmid HpaSV, specific for Vmw63 mRNA (left panel). The remaining half of the membrane was hybridised to a 500 bp *Eco*RI / *Hind*III fragment from plasmid B47#19, specific for β-galactosidase mRNA and an 840 bp *Bgl*II / *Xba*I fragment from plasmid pMG1, specific for γ-actin mRNA (right panel). The positions of mRNAs are indicated. CH: pretreatment was in the presence of cycloheximide; LP: sample pretreated with UV-*in*1814 L-particles; M: mock infected sample.


+in1853
+in1853
M
+LP
+LP +CH
+LP +CH
M +CH
1
2
3
4
5
175
110
63
β-gal
Actin

and one half was hybridised to radiolabelled DNA fragments specific for Vmw175, Vmw110 and Vmw63 mRNAs (figure 4.27., left panel), while the remaining half of the membrane was hybridised to radiolabelled DNA fragments specific for β-galactosidase and γ-actin mRNAs (figure 4.27., right panel). Hybridisation to γ-actin in parallel enabled accurate quantification by allowing adjustment of viral IE mRNA levels for the total amount of RNA loaded in each track. The resulting autoradiographs are presented in figure 4.27.

Levels of all of the viral IE mRNAs were reduced by approximately 2-fold in HFL cells pretreated with UV-*in*1814 L-particles (comparison of lanes 1 and 2), but the reduction in IE mRNA levels was not significantly altered when protein synthesis was inhibited by cycloheximide during the pretreatment (comparison of lanes 2 and 3), thus the inhibition was presumably mediated through pre-existing factors. The reduction in levels of viral IE mRNAs might account for the reduced ability of HSV to form plaques in cells pretreated with UV-*in*1814 L-particles, however, whether levels of IE mRNA were reduced by an alteration of transcription or mRNA stability cannot be distinguished by northern blotting. It was noted that levels of β-galactosidase mRNA transcribed from the HCMV major IE promoter were reduced by a similar extent to those of the HSV mRNAs, and since the HCMV major IE promoter does not contain TAATGARAT elements (C.M. Preston, personal communication), the reduction in IE mRNA levels was not caused solely by TAATGARAT-specific inhibition of transcription.

4.25. A version of *in*1814 defective in the *vhs* function was constructed.

A possible explanation for the reduced levels of viral IE mRNA after infection of HFL cells pretreated with UV-*in*1814 L-particles was that *vhs* proteins carried into the cells by the UV-*in*1814 L-particles were causing degradation of viral mRNA. To test the hypothesis that *vhs* was responsible for the ability of UV-viral particles to induce the uninfectious state, a *vhs*$^-$ version of *in*1814 was constructed and in section 4.26. was examined for the ability to cause inhibition of *in*1853 infectivity when UV-irradiated and applied to HFL cells.

Plasmid pMJ78, containing a fragment encompassing the region of the HSV-1 genome encoding U_L41 (encoding the *vhs* protein) with a 935 bp fragment of U_L41 replaced by the HCMV major IE promoter controlling the *E.coli lacZ* gene (figure 4.28.), was constructed and recombined into the *in*1814 genome by cotransfection (pMJ78 was kindly constructed and supplied by M.J. Nicholl and C.M. Preston). Fourteen plaque isolates from plaques stained blue by X-gal were screened for the presence of the HCMV / *lacZ* insert by Southern blotting (figure 4.29.). All but one of the plaque isolates

Figure 4.28. Construction of a *vhs*$^-$ version of *in*1814. An 8.1 kbp *Eco*RI / *Xho*I fragment of the HSV-1 *Eco*A fragment, encompassing U_L41, was cloned yielding plasmid pMJ74 (all plasmids used to construct *vhs*$^-$ *in*1814 were constructed by M.J. Nicholl and C.M. Preston). Plasmid pMJ74 was digested with *Bam*HI and *Pst*I and an *Xho*I / *Bgl*II / *Xba*I linker was inserted yielding plasmid pMJ75 which has a 935 bp deletion in U_L41. A 3.8 kbp *Xba*I (partial) / *Xho*I fragment from plasmid pMJ101 (Jamieson *et al.*, 1995) consisting of the HCMV major IE promoter controlling the *E.coli lacZ* gene was ligated between the *Xba*I and *Xho*I sites in pMJ75, yielding pMJ78. Plasmid pMJ78 was cotransfected with *in*1814 DNA and β-galactosidase containing plaques were isolated and screened for the presence of the modification in pMJ78. A: the HSV-1 genome; B: the region of the genome cloned into pMJ74 and the probe used for screening recombinants; C: positions of the deletion in U_L41 and the insertion of HCMV / *lacZ* in pMJ78. The orientation of insertion of the HCMV / *lacZ* fragment was unknown.

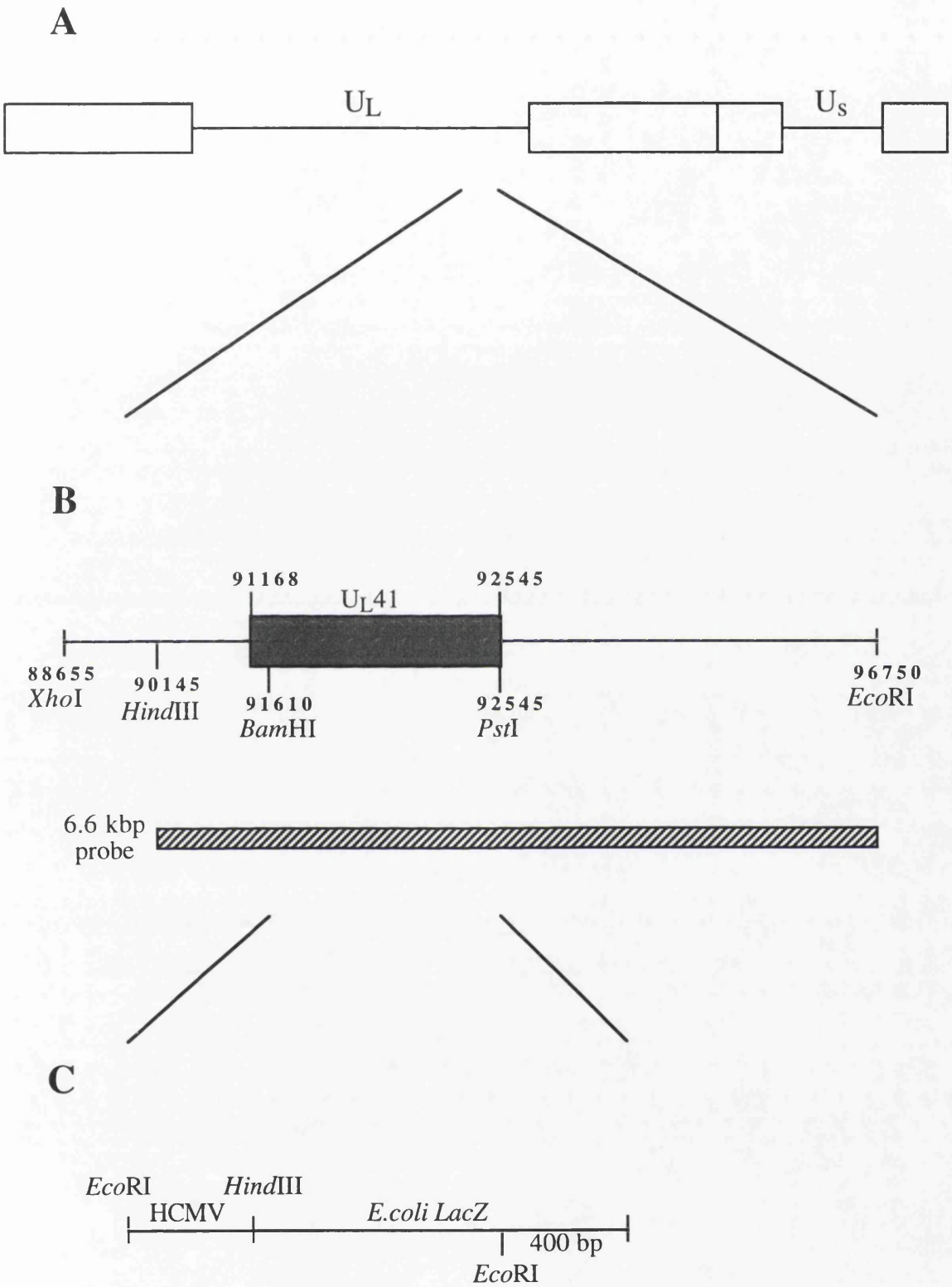
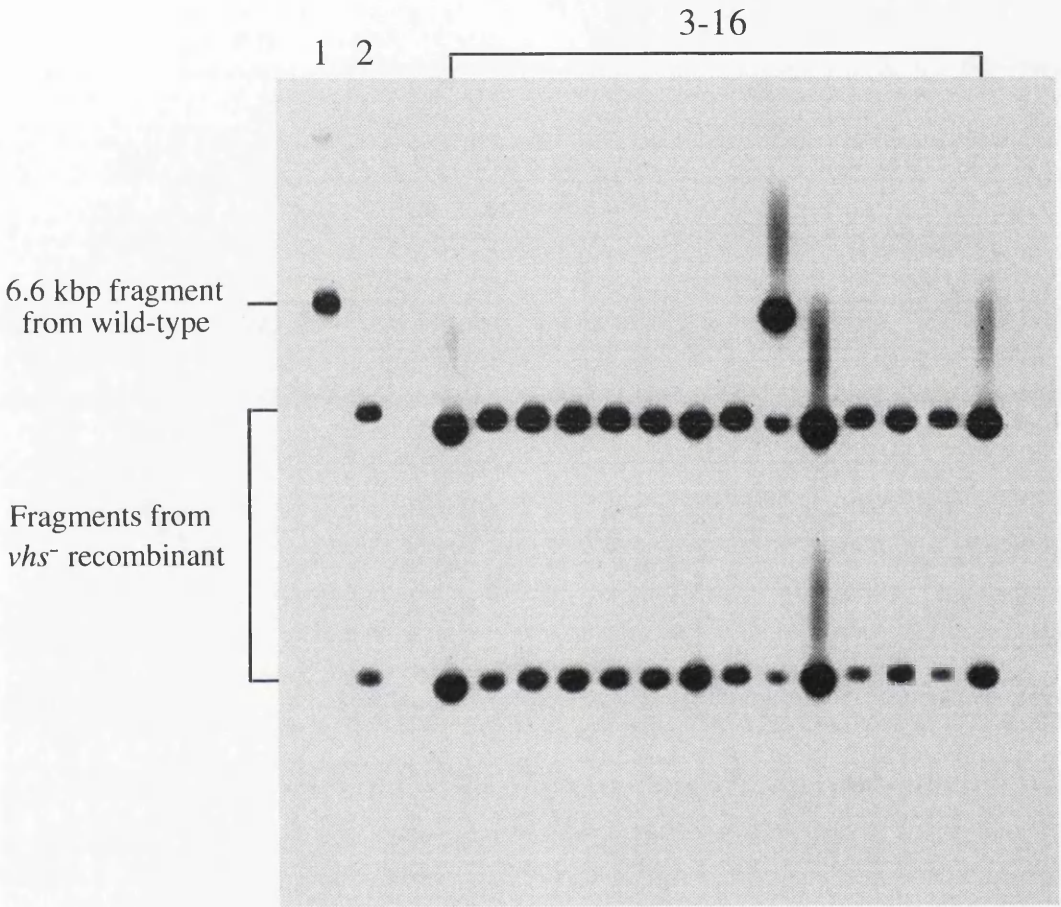

A
U_L
U_S
B
91168
92545
U_L41
88655
*Xho*I
90145
*Hind*III
91610
*Bam*HI
92545
*Pst*I
96750
*Eco*RI
6.6 kbp
probe
C
*Eco*RI
*Hind*III
HCMV
E.coli LacZ
400 bp
*Eco*RI

Figure 4.29. Screening of plaque isolates for the *lacZ* insertion in U_L41. *In*1814 DNA and pMJ78 were cotransfected into BHK cells and the monolayers were incubated at 37°. After plaques had appeared the cells were harvested, bath sonicated and titrated in 10-fold serial dilutions in the presence of X-gal. Blue plaques were picked, subjected to 4 rounds of plaque purification each selecting for β-galactosidase expression by staining with X-gal, and then screened for the presence of the deletion in U_L41 and the HCMV / *lacZ* insert. Fourteen plaques were picked, inoculated onto BHK cell monolayers in 24-well tissue culture plates and incubated in the presence of HMBA. When severe CPE was evident the medium was removed and the DNA extracted from the cells, digested with *Eco*RI and *Hind*III, and a proportion was electrophoresed in a 0.8% agarose gel, blotted onto a hybridisation membrane and hybridised to a radiolabelled 6.6 kbp *Eco*RI / *Hind*III fragment spanning the wild-type HSV-1 U_L41 gene (figure 4.28.). Lane 1: plasmid pMJ74 containing the wild-type *Eco*RI / *Hind*III fragment spanning U_L41 digested with *Eco*RI and *Hind*III; lane 2: plasmid pMJ78 containing the HCMV / *lacZ* insert digested with *Eco*RI and *Hind*III; lanes 3-16: plaque isolates digested with *Eco*RI and *Hind*III. A scanned autoradiograph is presented. The positions of the wild-type and recombinant *Eco*RI / *Hind*III fragments are indicated. All of the plaque isolates contained virus with the HCMV / *lacZ* insert. Isolate 9 was a mixture of wild-types and recombinants. The exact sizes of the fragments of the recombinants depended upon the orientation of the HCMV / *lacZ* insert in pMJ78, which was unknown. If the HCMV enhancer was in the left orientation, the *Eco*RI / *Hind*III fragments would have been 4.6 kbp and 1.5 kbp, if in the right orientation the fragments would have been 4.2 kbp and 1.9 kbp. Isolate 3 was grown as a virus stock and used for further experimentation.


3-16
1 2
6.6 kbp fragment from wild-type
Fragments from *vhs*⁻ recombinant

contained pure recombinant virus. Details on the construction of *vhs*⁻ *in*1814 is presented in the legends to figures 4.28. and 4.29.

4.26. The *vhs* function did not cause the reduced infectivity of *in*1853 in HFL cells pretreated with UV-*in*1814 L-particles.

If *vhs* caused the inhibition of infectivity in HFL cells pretreated with UV-*in*1814, then the *vhs*⁻ *in*1814 mutant would be unable to cause the inhibition.

To examine whether UV-*vhs*⁻ *in*1814 L-particles retained the ability to modify HFL cells and convert them to a state less able to be infected with *in*1853, HFL cells were treated with UV-*in*1814 L-particles or UV-*vhs*⁻ *in*1814 L-particles at various MOIs or mock-treated. At 6 hours post-treatment the monolayers were infected with 100 PFU of *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA) and incubated at 37°. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The results of the inhibition experiment are presented in table 4.13.

UV-inactivated L-particles from *vhs*⁻ *in*1814 gave inhibition of *in*1853 infectivity at a level comparable to that of UV-*in*1814 L-particles in pretreated HFL cells (table 4.13., comparison of inhibition in HFL cells pretreated with 3000 particles per cell), thus *vhs* did not cause the inhibition of infectivity in HFL cells pretreated with UV-*in*1814 L-particles. It should be noted that this is the result from a single experiment and the L-particle preparations appear less effective than in previous experiments, giving only a 4-fold inhibition with 10^4 particles per cell.

Pretreatment	MOI (particles / cell)	Number of plaques	Fold inhibition
Mock	–	52	–
UV-*in*1814 L-particles	3000	18.5	2.8
UV-*vhs*⁻ *in*1814 L-particles	10	36	1.4
	300	34	1.5
	3000	15	3.5
	10000	13	4

Table 4.13. Inhibition of *in*1853 infectivity in HFL cells pretreated with UV-*vhs*⁻ *in*1814 L-particles. HFL cells in 24-well tissue culture dishes were treated with UV-*in*1814 L-particles or UV-*vhs*⁻ *in*1814 L-particles at various MOIs or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment the monolayers were infected with 100 PFU of *in*1853 per well (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with CMC/EF_{10} and incubated at 37°. At 24 hours post-infection with *in*1853 the monolayers were fixed, stained for β-galactosidase activity and the number of blue foci per well was determined. The value for the mock-pre-infected samples is the average of 4 samples, the values from the samples pretreated with UV-*in*1814 L-particles is the average of duplicates, the remaining values are from single determinations.

CHAPTER 3.

Studies on *in vitro* latency with a temperature sensitive uncoating mutant.

4.27. A restriction fragment encompassing the temperature sensitive uncoating mutation in *ts*1213 was cloned yielding plasmid pLR1.

The hypothesis that delayed uncoating leads to latency in HFL cells can be tested by inserting a *ts* uncoating mutation into the genome of *in*1814 and controlling uncoating by temperature shift. If delayed uncoating leads to latency, the virus would be expected to be repressed more efficiently at the NPT than at the permissive temperature. The *ts* mutant *ts*1213 has a phenotype consistent with that of an uncoating mutant and, like its counterpart *ts*B7, its mutation is located within the tegument protein encoded by U_L36 (V.G. Preston, unpublished data; Batterson *et al.*, 1983). In this chapter the construction of a version of *in*1814 containing the *ts* lesion of *ts*1213, and an assessment of whether it is repressed during infection at the NPT, is described.

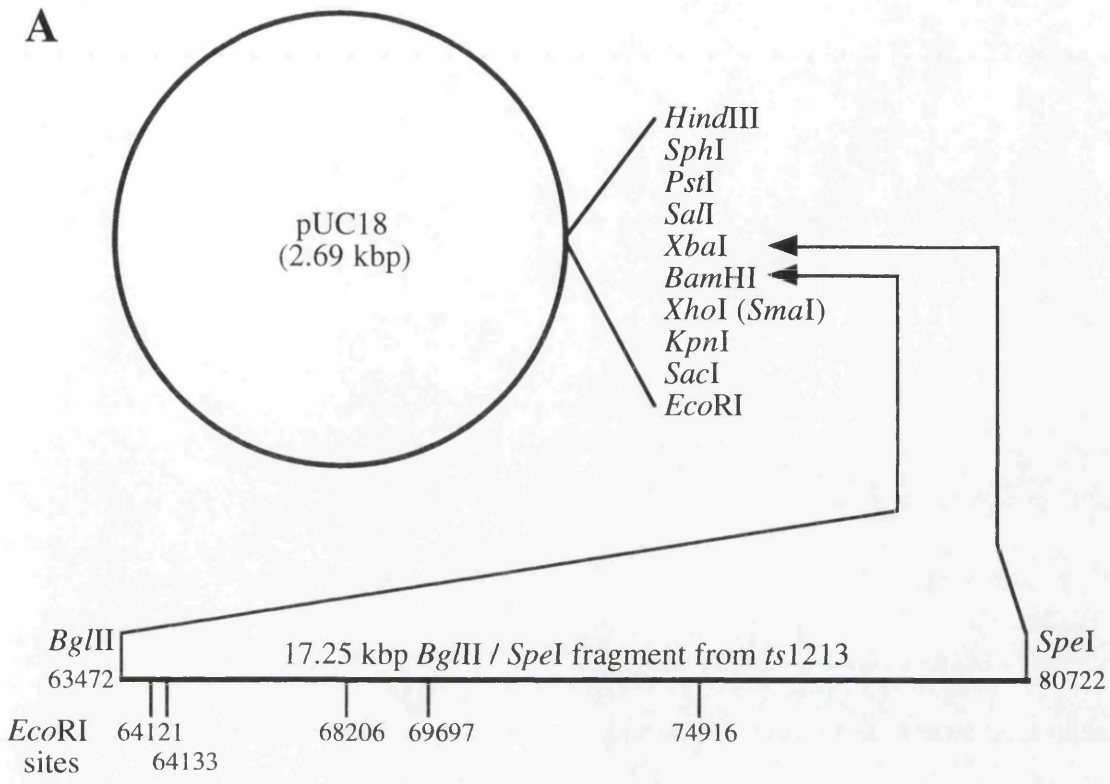
Plasmid pUC18*lacZ* (Jamieson *et al.*, 1995) was digested with *Bam*HI and *Xba*I to remove the *lacZ* insert and the pUC18 fragment was purified for use as a vector. A 17.25 kbp *Bgl*II / *Spe*I DNA fragment from the *ts*1213 genome, encompassing the *ts* mutation, was ligated into the pUC18 vector, yielding plasmid pLR1. Colonies were screened by digestion with *Eco*RI. From 48 colonies screened one contained the correct plasmid. A summary of the construction of pLR1 and its *Eco*RI digestion profile is shown in figure 4.30.

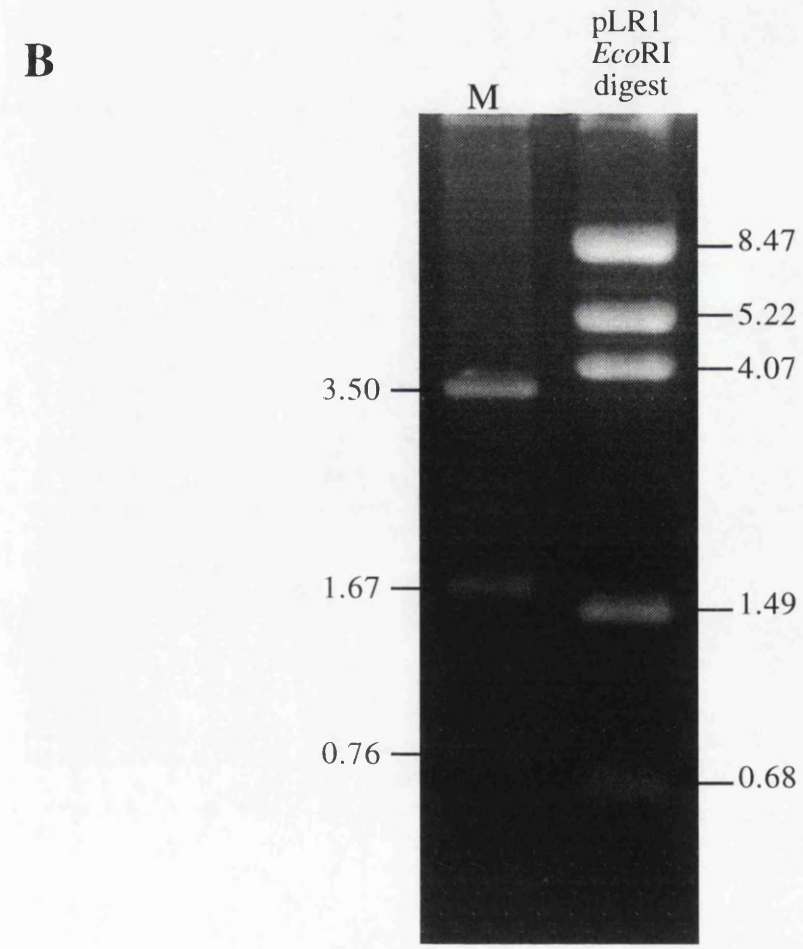
4.28. Plasmid pLR1 did not rescue the temperature sensitive lesion in *ts*1213.

To examine whether pLR1 contains the *ts*1213 *ts* lesion, the ability of pLR1 to rescue *ts*1213 was assessed by cotransfection of pLR1 with *ts*1213 DNA.

*Ts*1213 DNA was cotransfected into BHK cells together with pGX122 digested with *Kpn*I or pLR1 digested with *Hind*III. Plasmid pGX122 contains the *Kpn*I C fragment from wild-type HSV-1, which encompasses the corresponding region mutated in *ts*1213. After incubation at 31° for 6 days, the cells were harvested in the culture

Figure 4.30. Cloning of the temperature sensitive mutation in *ts*1213 yielding plasmid pLR1. (A) Strategy for cloning of the temperature sensitive mutation in *ts*1213. A 17.25 kbp *Bgl*II / *Spe*I fragment from *ts*1213 spanning the *ts* lesion in U_L36 was ligated between the *Bam*HI and *Xba*I sites of the pUC18 vector from pUC18*lacZ* (Jamieson *et al.*, 1995). The vector sequences were obtained from pUC18*lacZ* because the juxtaposition of the *Bam*HI and *Xba*I sites in the native form of pUC18 would hinder efficient restriction endonuclease cleavage. Note that the *Sma*I site was removed during the construction of pUC18*lacZ* (Jamieson *et al.*, 1995). (B) *Eco*RI digestion profile of pLR1. A scanned photograph of an ethidium bromide-stained 0.7% agarose gel is presented. M; plasmid pTK1 digested with *Bam*HI and *Sma*I as size markers. Sizes of the expected DNA restriction fragments are indicated in kbp.


A
pUC18
(2.69 kbp)
HindIII
SphI
PstI
SalI
XbaI
BamHI
XhoI (SmaI)
KpnI
SacI
EcoRI
BglII
63472
17.25 kbp BglII / SpeI fragment from ts1213
SpeI
80722
EcoRI sites
64121
64133
68206
69697
74916


B
M
pLR1
EcoRI
digest
8.47
5.22
4.07
3.50
1.67
1.49
0.76
0.68

Experiment	Plasmid	Titre at 31° (PFU per ml)	Titre at 39.5° (PFU per ml)
1	pGX122	3.7×10^7	3.2×10^6
	pLR1	5.2×10^6	<50
	None	2.3×10^7	50
2	pGX122	5.6×10^6	3.0×10^5
	pLR1	7.2×10^6	<50
	None	1.5×10^7	<50

Table 4.14. Marker rescue of *ts*1213 with pLR1 or pGX122. BHK cells in 35 mm diameter tissue culture dishes were cotransfected with 0.2 μg of *ts*1213 DNA plus 4 μg (experiment 1) or 8 μg (experiment 2) of pGX122 digested with *Kpn*I or pLR1 digested with *Hind*III. After incubation at 31° for 6 days, the cells were harvested in the culture medium, bath sonicated and the virus titres were determined in BHK cells in the presence of 3 mM HMBA at 31° or 39.5°.

medium, bath sonicated and the virus titres were determined at 31° or 39.5°. The average titres are presented in table 4.14.

Plasmid pGX122 rescued the mutation in *ts*1213, as demonstrated by the observation that approximately 10% of the viruses produced from the cotransfection were capable of forming plaques at 39.5°. However, pLR1 DNA did not rescue *ts*1213 at levels above the background represented by *ts*1213 DNA transfected alone, therefore pLR1 probably contains the *ts*1213 mutation.

4.29. The *ts* uncoating mutation encoded by pLR1 was recombined into the *in*1814 genome, yielding *in*1815.

To recombine the *ts* mutation of *ts*1213 into the *in*1814 genome, pLR1 which had been linearised by digestion with *Hind*III was cotransfected with *in*1814 DNA into BHK cells. After incubation at 31° for 4 days the cells were harvested, bath sonicated and 10-fold serial dilutions were used to infect BHK cell monolayers. After incubation at 31° in the presence of HMBA for 4 days, 36 plaques were picked. The plaque isolates were screened for temperature sensitivity by inoculating a proportion onto BHK cells in duplicate 24-well tissue culture plates and incubating one of the plates at 31° and the other at 39.5°. After plaques had appeared, the monolayers were stained and the numbers of plaques were determined. One of the plaque isolates contained *ts* virus and was subjected to 2 plaque purifications and grown as a seed stock. The *ts* virus in the plaque isolate was examined by coinfection with *ts*K or *ts*1213 to ensure that its *ts* lesion was the same as that in *ts*1213. If the seed stock contained the same mutation as *ts*1213 it would recombine with *ts*K but not *ts*1213.

BHK cells were infected with 2.5 PFU per cell of various combinations of the plaque isolate (PI), *ts*K or *ts*1213 and incubated at 31° for 24 hours. The cells were harvested, bath sonicated and the titres determined at 31° and 39.5° in the presence of HMBA. The titres are presented in table 4.15.

The plaque isolate rescued the mutation in *ts*K, but not *ts*1213, therefore it contains the mutation in *ts*1213. The virus in the plaque isolate, which is *in*1814 with the *ts* lesion of *ts*1213, was named *in*1815.

Virus	Titre at 31°	Titre at 39.5°
PI alone	1.1×10^7	<500
*Ts*1213 alone	1.6×10^8	<500
*Ts*K alone	4.8×10^7	<500
PI + *ts*1213	1.8×10^7	<500
PI + *ts*K	2.7×10^7	5.8×10^5
*Ts*1213 + *ts*K	3.4×10^7	8.6×10^5

Table 4.15. Coinfection / recombination of *in*1815 with *ts*1213 or *ts*K. BHK cells in 35 mm diameter tissue culture dishes were infected with 2.5 PFU per cell of various combinations of the plaque isolate (PI), *ts*K or *ts*1213 and incubated at 31° for 24 hours. The cells were harvested into the culture medium, bath sonicated, and the virus titres at 31° and 39.5° were determined in the presence of 3 mM HMBA.

4.30. *In*1815 exhibited a phenotype expected of an uncoating mutant, as determined in the Southern blot assay.

In order to confirm that *in*1815 is an uncoating mutant, *in*1815 was examined in the Southern blot assay described in chapter 1. BHK cells were infected with 0.5 PFU of *in*1814 or *in*1815 per cell and incubated at 31° or 39.5° in the presence of Ara-C. At 7 hours post-infection nuclei were isolated and digested with DNase I or were untreated. DNA was purified, digested with *Bam*HI, electrophoresed alongside purified virion DNA, blotted, and hybridised to a radiolabelled DNA fragment from the 5' coding sequences of IE1. Resulting hybridisation is presented in figure 4.31. The relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples were quantified and are presented in figure 4.32. The percentages of nonlinear genomes in the non-nuclease treated samples and genomes remaining after DNase I digestion are presented in table 4.16.

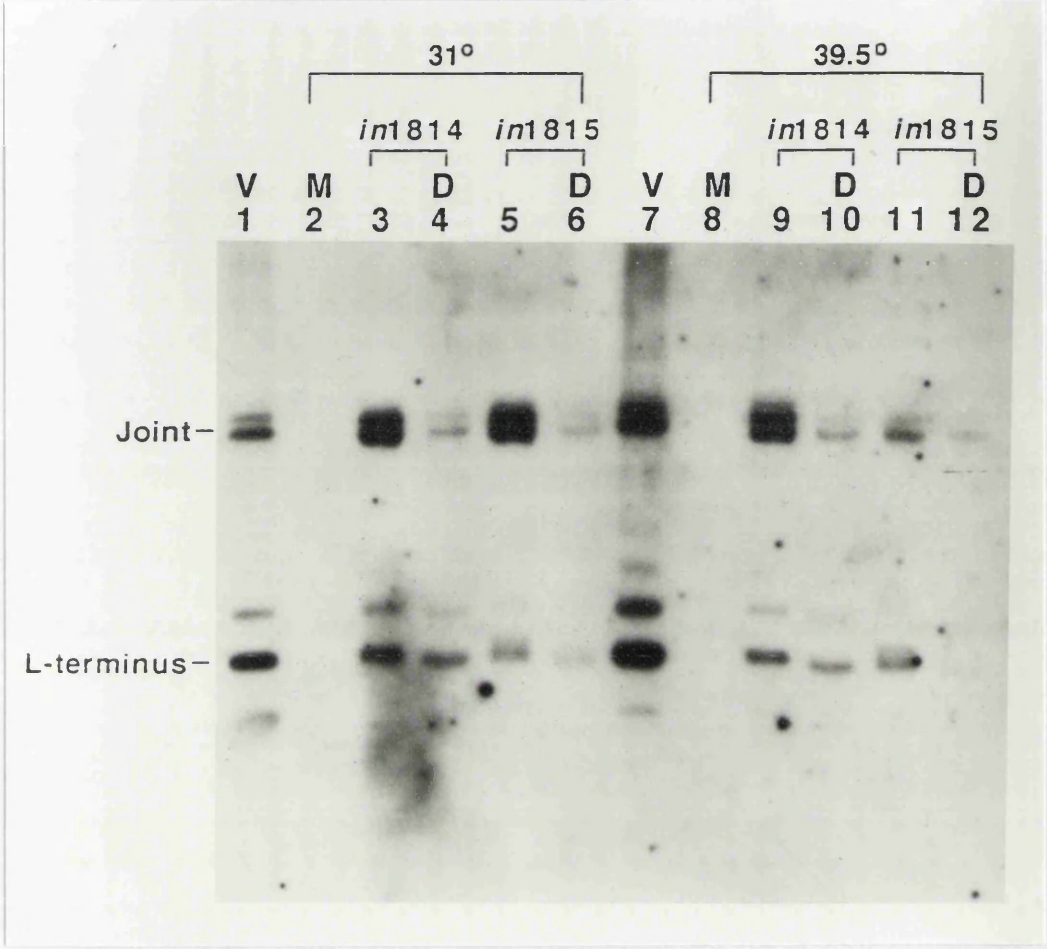


Figure 4.31. Configuration of *in*1815 genomes after infection at the NPT. BHK cells in 35 mm diameter tissue culture dishes were infected with 0.5 PFU per cell of *in*1814 (lanes 3–4 and 9–10) or *in*1815 (lanes 5–6 and 11–12) or mock infected (lanes 2 and 8), overlaid with EF_{10} containing 50 µg/ml Ara-C and incubated at 31° (lanes 2–6) or 39.5° (lanes 8–12). At 7 hours post-infection nuclei were isolated and digested with 5 units of DNase I for 20 minutes (lanes 4, 6, 10 and 12) or were untreated (lanes 3, 5, 9 and 11). DNA was purified, digested with *Bam*HI and half of each sample was electrophoresed in a 0.7% agarose gel alongside 1.0 ng *in*1814 virion DNA standards (lanes 1 and 7). Electrophoresed DNA was blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 640 bp *Bam*HI / *Nco*I fragment from the 5' coding sequences of IE1. Positions of joint and L-terminal *Bam*HI restriction fragments are indicated. D: DNase I digested sample; M: mock infected sample; V: virion DNA.

Demonstration that *in* 1815 is an uncoating mutant using the Southern blot assay.

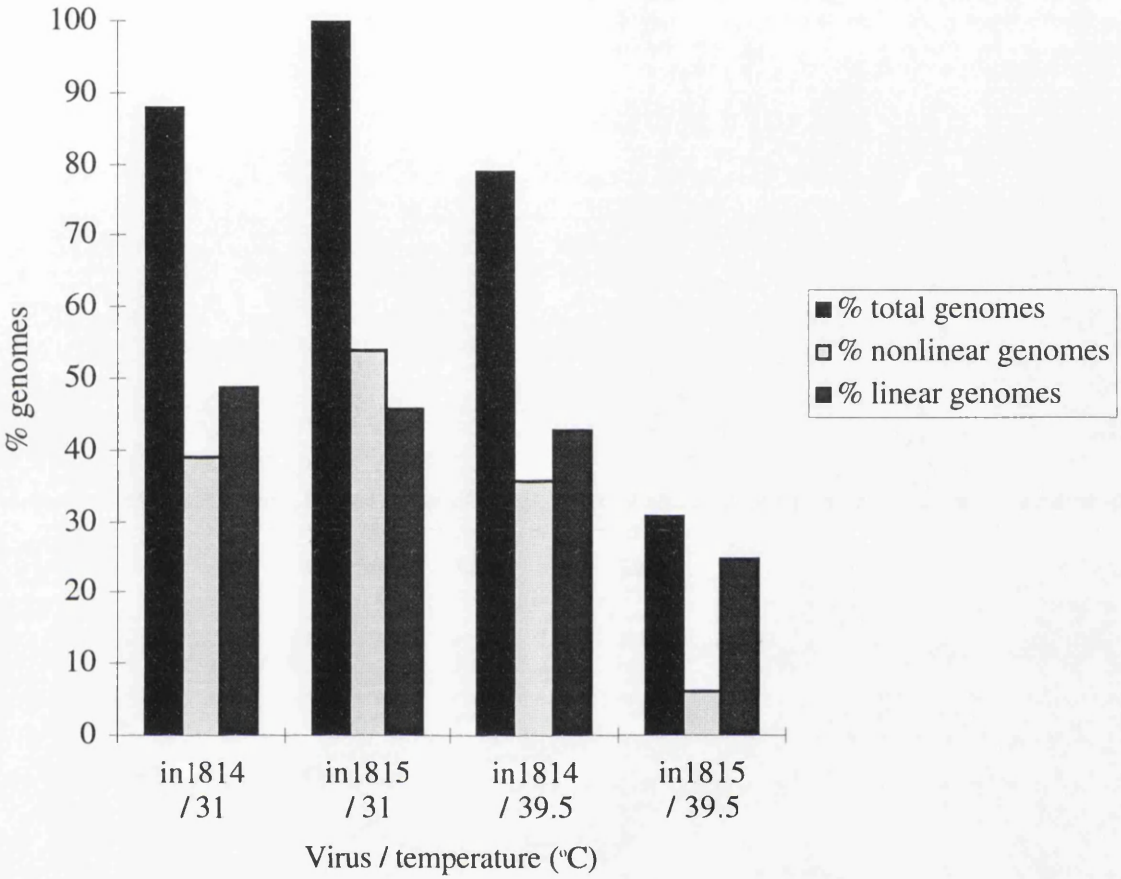


Figure 4.32. Relative proportions of nonlinear and linear *in*1815 genomes after infection at the permissive and non-permissive temperatures. Hybridisation in figure 4.31. was quantified and the relative proportions of nonlinear and linear viral genomes in the non-nuclease treated samples quantified by comparison to the virion DNA standards. The total number of genomes in nuclei from cells infected with *in*1815 at 31°, which contained the greatest total number of genomes compared to the other samples, represents 100%.

Temperature (°C)	31		39.5	
Virus	*in*1814	*in*1815	*in*1814	*in*1815
% nonlinear	45	52	46	30
% genomes remaining after DNase I digestion	23	21	33	60

Table 4.16. Configuration and DNase I sensitivity of *in*1815 genomes after infection at the permissive and non-permissive temperatures. Hybridisation in figure 4.15. was quantified and the percentage of genomes which were nonlinear in the non-nuclease treated samples calculated by comparison to the purified virion DNA standards. The percentage of genomes remaining after DNase I digestion was determined by comparison with the untreated samples.

At 31°, *in*1815 DNA was converted to the nonlinear configuration at a similar rate to *in*1814 DNA (figure 4.31., lanes 3 and 5; figure 4.32.). At 39.5°, the NPT for *in*1815, *in*1814 DNA was converted to the nonlinear configuration with comparable efficiency to that at 31°, but conversion of *in*1815 DNA to the nonlinear configuration was impaired (figure 4.31., lanes 9 and 11; figure 4.32.). The total numbers of genomes in the nuclei after infection with *in*1815 at the NPT was about 30% of the total numbers of genomes after infection at 31°. The percentage of genomes remaining after DNase I treatment was over 2-fold greater in the sample infected with *in*1815 at the NPT than in samples infected under permissive conditions, implying that the genomes were more resistant to nucleases. The *in*1815 genomes exhibited a phenotype at 39.5° expected of an uncoating mutant: they were not converted to the nonlinear form, did not associate with nuclei as efficiently as in normal infection and were resistant to DNase I. Mutant *in*1815 thus exhibited a similar phenotype to *ts*1213 in the uncoating assay.

4.31. *In*1815 did not become insensitive to HMBA at the NPT as efficiently as its revertant *in*1816, or *in*1814.

During the first 6 hours of infection in HFL cells, *in*1814 genomes gradually enter a latent state which cannot be overcome by activators of transcription such as HMBA or Vmw65, but if either of these two activators are added at the beginning of infection the defect in *in*1814 is overcome and the virus forms plaques with normal efficiency (Harris and Preston, 1991; McFarlane *et al.*, 1992). If delayed uncoating leads to latency, then *in*1815 might be expected to become unresponsive to HMBA at 39.5° more rapidly than *in*1814. A series of 7 experiments were carried out to determine whether *in*1815 establishes latency more efficiently at 39.5° than *in*1814. In addition to comparing *in*1815 with *in*1814, *in*1815 was compared with its rescuant, *in*1816, which has the *ts* mutation restored but retains the mutation in Vmw65. The *ts* uncoating mutation in *in*1815 was restored by cotransfecting *in*1815 DNA with linearised plasmid pGX122 which contains the *Kpn*I C fragment from wild type HSV-1, encompassing region of the mutation in *ts*1213. Plaques were selected at 39.5° and plaque purified to yield the rescuant *in*1816 which should be equivalent to *in*1814. To enable virus titres to be determined by staining monolayers for β-galactosidase activity, the *lacZ* gene under the control of the HCMV major IE enhancer was recombined into a nonessential region of the *in*1815 genome yielding *in*1817 (a detailed description of the construction of *in*1817 is described below), and the repression of *in*1817 at 39.5° was compared to that of *in*1853 (*in*1814 with the *lacZ* gene under the control of the HCMV major IE enhancer inserted into the TK gene).

HFL cell monolayers were infected at 39.5° with 10-fold serial dilutions of an *in*1815, *in*1814, *in*1816, *in*1817 or *in*1853 preparation. Immediately after the 1 hour adsorption period or after 7 or 24 hours at 39.5° the culture medium was replaced with medium containing 3 mM HMBA and the monolayers were shifted to 31°. The monolayers were left at 31° for 4–6 days and stained with Giemsa or for 2 days and stained for β-galactosidase activity, plaques were counted and virus titres were determined. The results from 7 separate experiments are presented in table 4.17.

The purpose of these experiments was to assess whether *in*1815 became repressed more rapidly than *in*1814 during incubation at 39.5°. If uncoating leads to latency, then during incubation at 39.5° the titre of *in*1815 would be expected to drop more rapidly than *in*1814.

At 39.5°, viruses containing the uncoating mutation were not repressed as efficiently as viruses not containing the uncoating mutation, as was demonstrated by the smaller decrease in the titre of *in*1815 compared to *in*1814 or *in*1816 after incubation at 39.5° for 7 hours or 24 hours (table 4.17.), thus delayed uncoating did not lead to latency.

Table 4.17. Repression of *in*1815 during infection of HFL cells at 39.5°. HFL cell monolayers were infected at 39.5° with 10-fold serial dilutions of an *in*1815, *in*1814, *in*1816, *in*1853 or *in*1817 preparation. Either immediately after the adsorption period, or after incubation at 39.5° for 7 hours or 24 hours the culture medium was removed and replaced with medium containing 3 mM HMBA and the monolayers were shifted to 31°. The monolayers were left at 31° for 4–6 days and stained with Giemsa (experiments 1–5) or for 2 days and stained for β-galactosidase activity (experiments 6 and 7), plaques were counted and the virus titres were determined. Results are presented from 7 separate experiments. * indicates titre was determined entirely at 31°. ND; not determined.

EXPERIMENT NUMBER	MUTANT	TITRE 1 hr 39.5°	TITRE 7 hrs 39.5°	TITRE 24 hrs 39.5°	FOLD DECREASE 7 hrs	24 hrs
1	*in*1815	5.6×10^9	1.3×10^9	ND	4	ND
2	*in*1814	2.0×10^8*	1.2×10^7	ND	17	ND
	*in*1815	8.9×10^8	8.7×10^8	ND	1	ND
3	*in*1814	5.9×10^7	4.6×10^6	3.0×10^6	13	20
	*in*1815	1.7×10^{10}	7.8×10^9	3.1×10^{10}	2	0.5
4	*in*1814	4.4×10^8	3.6×10^6	3.6×10^6	122	122
	*in*1815	1.4×10^{10}	3.5×10^9	8.3×10^9	4	2
5	*in*1814	7.7×10^8	3.5×10^7	ND	22	ND
	*in*1815	1.3×10^{10}	3.3×10^9	ND	4	ND
	*in*1816	8.1×10^9	2.2×10^8	ND	37	ND
6	*in*1853	1.6×10^9	1.8×10^8	ND	9	ND
	*in*1817	6.3×10^9	2.9×10^9	ND	2	ND
7	*in*1853	1.1×10^{10}	1.4×10^9	ND	8	ND
	*in*1817	7.2×10^9	2.4×10^9	ND	3	ND

Table 4.18. Repression of *in*1815 upon downshift to 31° after 1 hour or 7 hours incubation at 39.5°. HFL cells in 35 mm diameter tissue culture dishes were infected at 39.5° with 10-fold serial dilutions of an *in*1814, *in*1816, or *in*1815 preparation and overlaid with EF_5 containing 2% pooled human serum. Monolayers were immediately shifted to 31° (A) or were maintained at 39.5° for a further 6 hours before being downshifted (B). At 0 hours, 3 hours or 6 hours after downshift the culture medium was removed and the monolayers overlaid with CMC/EF_{10} containing 3 mM HMBA. After incubation at 31° for 4 days the plaques were counted and the virus titres were determined.

A. Downshift to 31° after 1 hour at 39.5°.

Virus	Time HMBA added after downshift to 31° (hours)	Titre (PFU / ml)	Fold decrease in titre
	0	7.7×10^8	0
*in*1814	3	5.4×10^7	14
	6	2.6×10^7	30
	0	8.1×10^9	0
*in*1816	3	3.1×10^8	26
	6	1.7×10^8	48
	0	1.3×10^{10}	0
*in*1815	3	7.0×10^8	19
	6	1.9×10^8	68

B. Downshift to 31° after 7 hours at 39.5°.

Virus	Time HMBA added after downshift to 31° (hours)	Titre (PFU / ml)	Fold decrease in titre (compared to +HMBA after 1 hour at 39.5°)
	0	3.5×10^7	22
*in*1814	3	3.0×10^7	26
	6	3.5×10^7	22
	0	2.2×10^8	37
*in*1816	3	2.4×10^8	34
	6	1.7×10^8	48
	0	3.3×10^9	4
*in*1815	3	1.4×10^9	9
	6	4.8×10^8	27

If delayed uncoating facilitates the establishment of latency, but the action of HMBA dominates over the repression of the viral genomes when present immediately after uncoating, then the assay described above might not have detected an increased rate of repression of *in*1815 at 39.5°. To determine whether an increased rate of repression occurs after downshift to 31°, HFL cells were infected with *in*1814, *in*1816, or *in*1815 at 39.5° and the rate of repression of the viruses after downshift to 31° was compared by adding HMBA at various times after downshift.

HFL cells were infected at 39.5° with 10-fold serial dilutions of an *in*1814, *in*1816, or *in*1815 preparation and overlaid with EF_5 containing 2% pooled human serum. Monolayers were immediately shifted to 31° or were maintained at 39.5° for a further 6 hours before being downshifted. At 0 hours, 3 hours or 6 hours after downshift the culture medium was removed and the monlolayers were overlaid with CMC/EF_{10} containing 3 mM HMBA. After incubation at 31° for 4 days the plaques were counted and the virus titres were determined. The resulting titres are presented in table 4.18.

Upon downshift immediately after the adsorption period, *in*1815 was repressed at a similar rate to *in*1814 and *in*1816, thus *in*1815 had retained the *in*1814 mutation during its construction (table 4.18.A). After incubation at 39.5° for 7 hours, *in*1815 had become less repressed than *in*1814 or *in*1816, 4-fold as compared to 22-fold and 37-fold respectively. While the repression of *in*1814 and *in*1816 was completed after incubation at 39.5° for 7 hours, repression of *in*1816 was not complete (table 4.18.B). If delayed uncoating leads to latency, then the rate of repression of *in*1815 upon downshift after 7 hours at 39.5° would be greater than after downshift immediately after adsorption. The rate of repression of *in*1815 upon downshift to 31° after incubation at 39.5° for 7 hours was not greater than upon downshift after 1 hour at 39.5°. No evidence was found in this study to suggest that delayed uncoating leads to latency.

4.32. Treatment of HFL cells with UV-*in*1815 L-particles made the cells less permissive for *in*1853 plaque initiation.

The observation that *in*1815 does not become repressed at 39.5° is difficult to reconcile with the finding that cells treated UV-inactivated viral particles become less permissive for plaque initiation. One possible explanation was that *in*1815 had acquired a mutation during its construction which prevents its ability to induce the nonpermissive state. In order to ensure that *in*1815 retained the ability to inhibit plaque initiation when UV-irradiated and used to treat HFL cells, UV-*in*1815 L-particles were tested for their ability to inhibit plaque initiation.

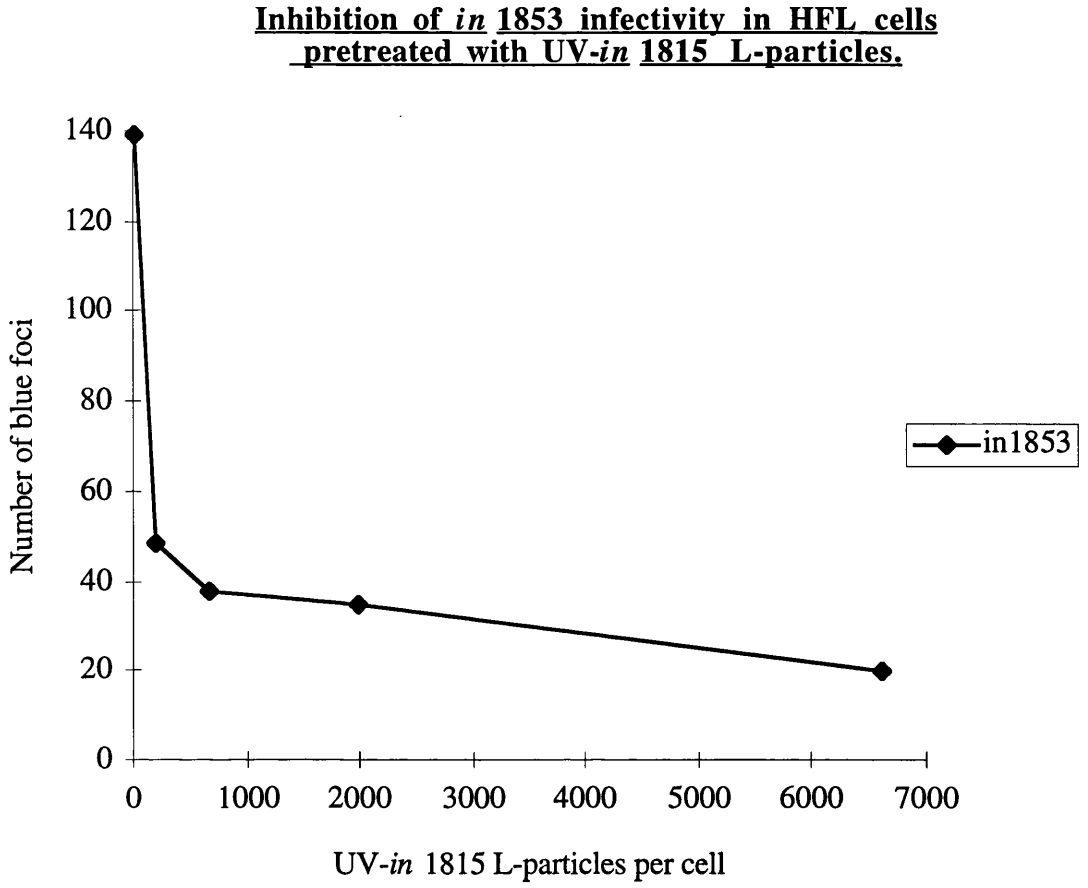


Figure 4.33. Inhibition of infectivity in HFL cells pretreated with UV-*in*1815 L-particles. HFL cell monolayers were treated with various MOIs of UV-*in*1815 L-particles or mock-treated, overlaid with EF_{10} and incubated at 37°. At 6 hours post-treatment the cells were infected with 100 PFU of *in*1853 per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with EHu_5, incubated for a further 24 hours and then fixed, stained for β-galactosidase activity and the average number of blue foci per monolayer was determined. The value for the mock infected sample is the average of 4 samples the remaining values are the averages of duplicates.

HFL cell monolayers were treated with various MOIs of UV-*in*1815 L-particles or mock-treated and incubated at 37°. At 6 hours post-treatment the cells were infected with 100 PFU of *in*1853 per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA), incubated for a further 24 hours and then fixed, stained for β-galactosidase activity and the number of blue foci per monolayer was determined. The average numbers of blue foci of virus replication per monolayer is presented in figure 4.33.

*In*1853 plaque initiation was inhibited up to 7-fold in HFL cells pretreated with UV-*in*1815 L-particles, thus *in*1853 does not have a unique characteristic which prevents its ability to induce the nonpermissive state.

4.33. Pretreatment of HFL cells with non-UV-inactivated *in*1815 L-particles at the NPT stimulated *in*1853 plaque initiation.

A possible explanation for the observation that HFL cells treated with UV-inactivated viral particles become less permissive for plaque initiation, but that *in*1815 did not become repressed at 39.5°, is that the inhibitory activity of the particles is a product of UV-irradiation. In previous studies viral particles required UV-irradiation to prevent IE gene expression complementing *in*1853, however the requirement of prior UV-irradiation of the particles might be circumvented by pretreating the cells with *in*1815 at the NPT, under circumstances where the virus cannot uncoat and express its genome. The effect of pretreating HFL cells with non-UV-irradiated *in*1815 L-particles at 39.5° on *in*1853 plaque initiation was investigated.

HFL cell monolayers were treated with various MOIs of non-UV-inactivated *in*1815 L-particles or mock-treated and incubated at 39.5°. After incubation for a further 5 hours the cells were infected with 100 PFU of *in*1853 per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA), incubated for a further 24 hours and then fixed, stained for β-galactosidase activity and the average number of blue foci per monolayer was determined. Values are presented in figure 4.34.

At MOIs of up to about 5 particles per cell there was a slight inhibition of *in*1853 infectivity, but infectivity was stimulated by higher MOIs. The stimulation of *in*1853 plaque initiation in HFL cells pretreated with non-UV-irradiated *in*1815 L-particles indicated that the nonpermissive state induced by the UV-irradiated particles was the result their UV irradiation. Alternatively, *ts*1213 might have 'leaked' during incubation at the NPT at sufficient levels to complement *in*1853.

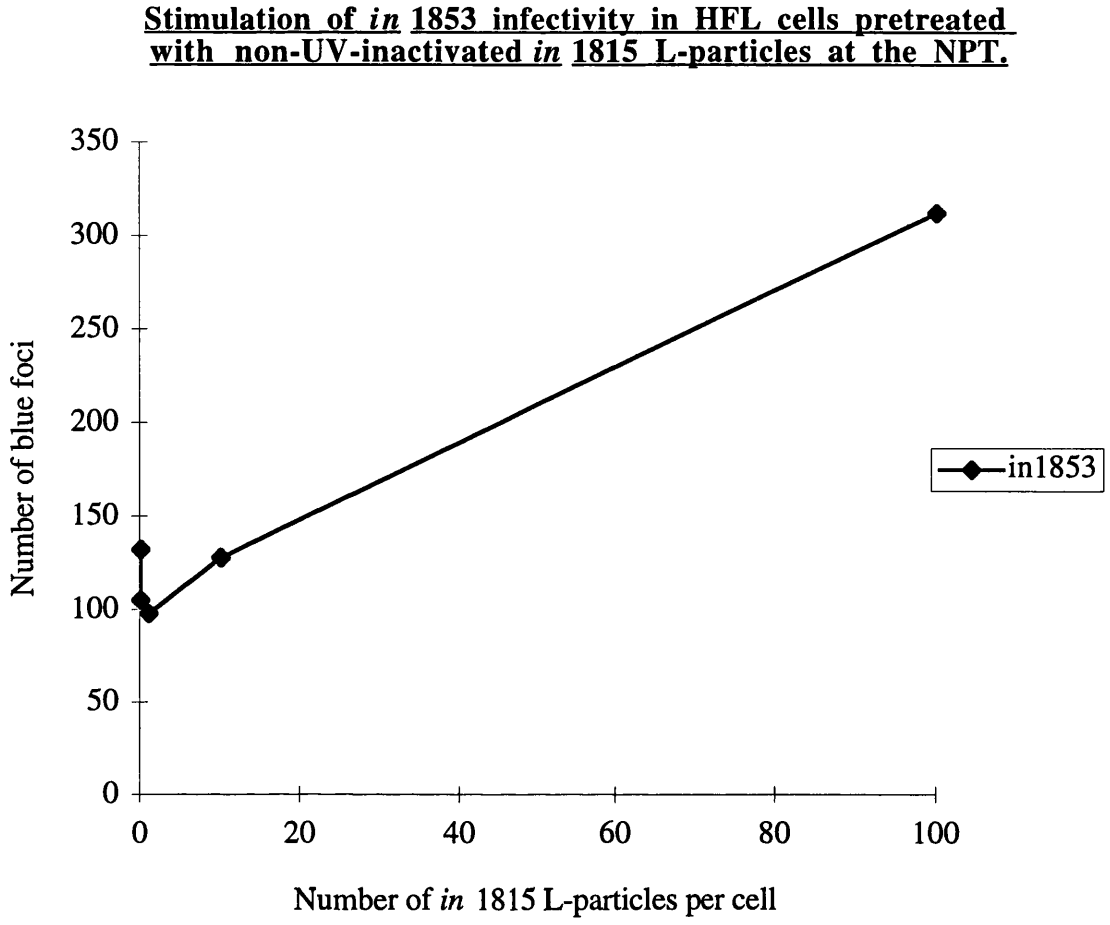


Figure 4.34. Stimulation of infectivity in HFL cells pretreated at 39.5° with non-UV-inactivated *in*1815 L-particles. HFL cell monolayers were treated with various MOIs of non-UV-inactivated *in*1815 L-particles or mock-treated and overlaid with EF_{10}. After incubation for a further 5 hours the cells were infected with 100 PFU of *in*1853 per monolayer (MOI was determined from the titre in HFL cells in the absence of HMBA), overlaid with CMC/EF_{10}, incubated for a further 24 hours and then fixed, stained for β-galactosidase activity and the average number of blue foci per monolayer was determined. All incubations were at 39.5°, media were preheated to 39.5° before use and all manipulations were carried out in an atmosphere maintained at 37°. The value for the mock infected sample is the average of 4 samples the remaining values are the averages of triplicates.

4.34. Infection of HFL cells with *in*1815 at the NPT resulted in the induction of an IFNα-induced cellular gene.

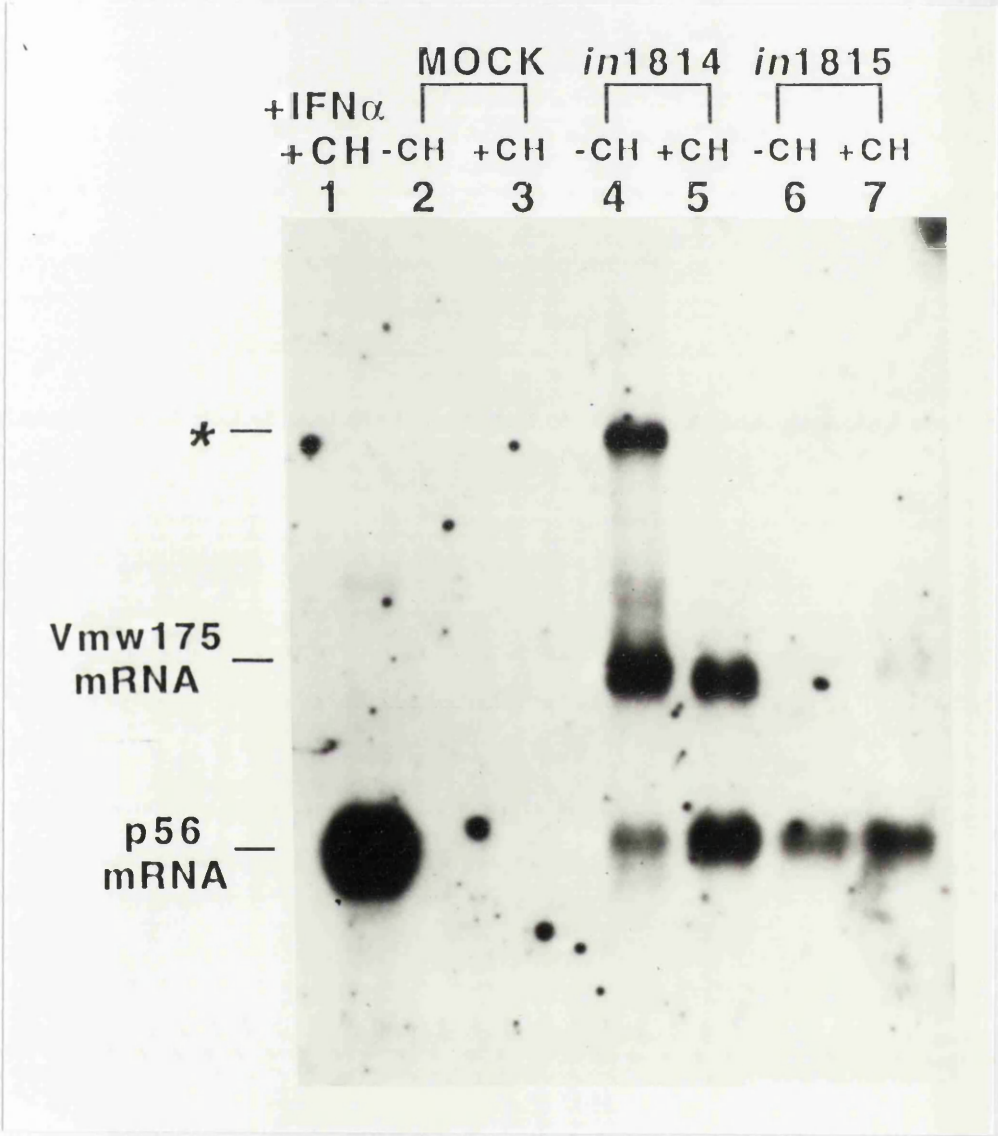
Infection with HSV results in the overall inhibition of host protein synthesis (Fenwick, 1984; Roizman and Sears, 1993), however transcription of a small number of cell genes is induced (Kemp *et al.*, 1986; Latchman *et al.*, 1987; Jang and Latchman, 1989; Jang *et al.*, 1991; Latchman, 1991; Smiley *et al.*, 1991). The majority of cell genes induced by HSV are stimulated by viral gene products synthesised *de novo*, but at least some do not require protein synthesis hence their induction is triggered by the binding of virus to the cell surface, entry into the cell or uncoating (Kemp *et al.*, 1986; Preston, 1990). Previously it was reported that a 56 kDa protein (p56) was induced in HFL cells by HSV infection, and that induction occurred in the absence of viral protein synthesis (Preston, 1990). A protein of the same apparent molecular weight is induced by the IFNs (Rubin *et al.*, 1988). Since IFN-induced genes are activated in response to virus infection it seemed possible that the 56 kDa protein induced by IFN was identical to that induced by HSV. A cDNA encoding the IFN-induced 56 kDa protein has been isolated (c56; Chebath *et al.*, 1983) and was used as a probe to investigate whether c56 RNA is induced by HSV and whether *in*1815 retains the ability to cause the induction. As IFNα pretreatment facilitates the establishment of latency in HFL cells, IFNα-induced factors are candidates as possible mediators of the repression of *in*1814.

HFL cells were infected with 10 PFU per cell of *in*1814 or *in*1815 and incubated at 39.5° in the presence or absence of cycloheximide. In parallel, a monolayer was overlaid with medium containing cycloheximide and 1000 u/ml of IFNα and incubated at 39.5°. At 6 hours post-infection poly(A)-containing RNA was extracted, electrophoresed in an agarose gel, blotted onto a hybridisation membrane and hybridised to radiolabelled c56 (supplied by V.G. Preston, Chebath *et al.*, 1983) and a DNA probe specific for Vmw175 RNA. Resulting hybridisation is presented in figure 4.35.

RNA complementary to c56 was induced by both *in*1814 and *in*1815 at 39.5°, in the presence or absence of protein synthesis. The observation that *in*1815 induced c56 RNA at the NPT indicated that induction was triggered by a stage of infection prior to uncoating of the genome, such as adsorption or entry into the cell. C56 RNA was strongly induced by IFNα treatment and the induction occurred in the absence of protein synthesis, thus the induction of c56 by HSV appeared to be *via* an intermediate signal and not *via* the release of IFNα. As expected, Vmw175 RNA was reduced in cells infected with *in*1815 at the NPT compared to *in*1814 at the same temperature however, small amounts of Vmw175 were present suggesting that *in*1815 had leaked.

The observation that *in*1815 activates an IFNα-induced pathway at the NPT, combined with the fact that it did not become repressed efficiently at 39.5° argues against

Figure 4.35. Induction of an IFNα-induced cellular gene during infection with *in*1815 at the NPT. HFL cells in 90 mm diameter tissue culture dishes were infected at 39.5° with 10 PFU per cell of *in*1814 (lanes 4 and 5) or *in*1815 (lanes 6 and 7) or were mock infected (lanes 2 and 3) in the presence (lanes 3, 5 and 7) or absence (lanes 2, 4 and 6) of 50 μg/ml cycloheximide, overlaid with EF_{10} with or without cycloheximide and incubated at 39.5°. In parallel, a monolayer was overlaid with EF_{10} containing 1000 u/ml IFNα and incubated at 39.5° (lane 1). At 6 hours post-infection poly(A)-containing RNA was extracted, half of each sample was electrophoresed in a 1.2% agarose gel, blotted onto a hybridisation membrane and hybridised to a [^{32}P]-labelled 330 bp *Bgl*II / *Ssp*I fragment from plasmid c56 which contains a cDNA of the IFN-induced 56 kDa protein (supplied by V.G. Preston, Chebath *et al.*, 1983), and to a Vmw175-specific 670 bp *Eco*RI / *Pvu*II fragment from plasmid X1, containing the HSV-1 *Xho*I C fragment. The positions of c56 and Vmw175 RNAs are indicated. * indicates the position of a large c56 or Vwm175-specific RNA produced in the presence of *de novo* viral protein synthesis.


MOCK
in1814
in1815
+IFNα
+CH -CH +CH -CH +CH -CH +CH
1 2 3 4 5 6 7
*
Vmw175 mRNA
p56 mRNA

the possibility that the repression is mediated by the IFN-induced 56 kDa protein, or probably any other IFNα-induced factor.

It is tempting to speculate that the 56 kDa protein observed to be induced by HSV in previous studies (Preston, 1990) is identical to a protein of the same apparent molecular weight that is induced by IFNα (Chebath *et al.*, 1983; Rubin *et al.*, 1988). Both proteins have the same apparent molecular weight, are induced by IFNα and do not require *de novo* protein synthesis for their induction.

4.35. The *E.coli lacZ* gene under the control of the HCMV major IE promoter was inserted into a nonessential region of the *in*1815 genome, yielding *in*1817.

To facilitate studies with *in*1815 and assess the extent to which it leaks at the NPT, a version which expresses β-galactosidase under the control of the HCMV major IE promoter was constructed.

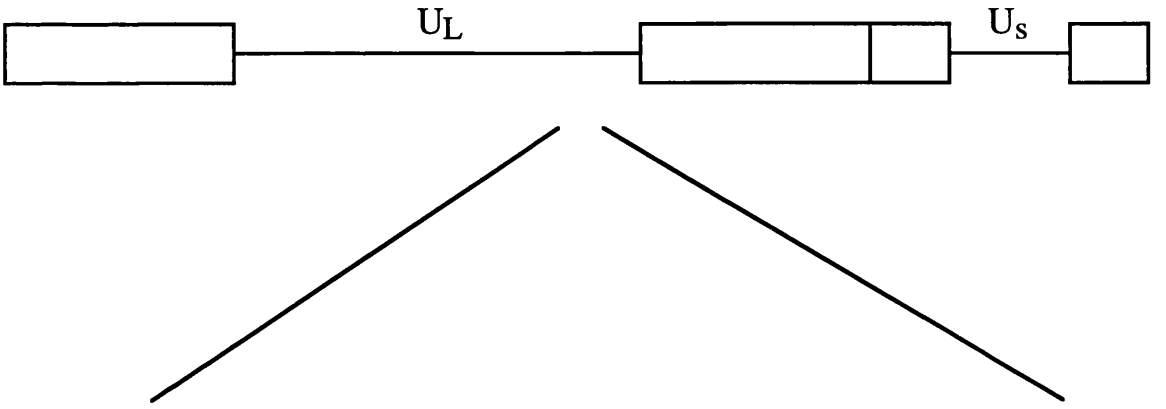
The *lacZ* gene was inserted into the U_L43 gene, mutants of which have no demonstrable phenotype *in vitro* or *in vivo* (MacLean *et al.*, 1991), by cotransfecting the plasmid B3#76 with *in*1815 DNA. Twenty-four plaque isolates from plaques stained blue by X-gal were screened for the presence of the HCMV / *lacZ* insert by Southern blotting (figure 4.37.). All of the plaque isolates contained pure recombinant virus. Details on the construction of *in*1817 are presented in the legends to figures 4.36. and 4.37.

4.36. *In*1817 leaked at the NPT.

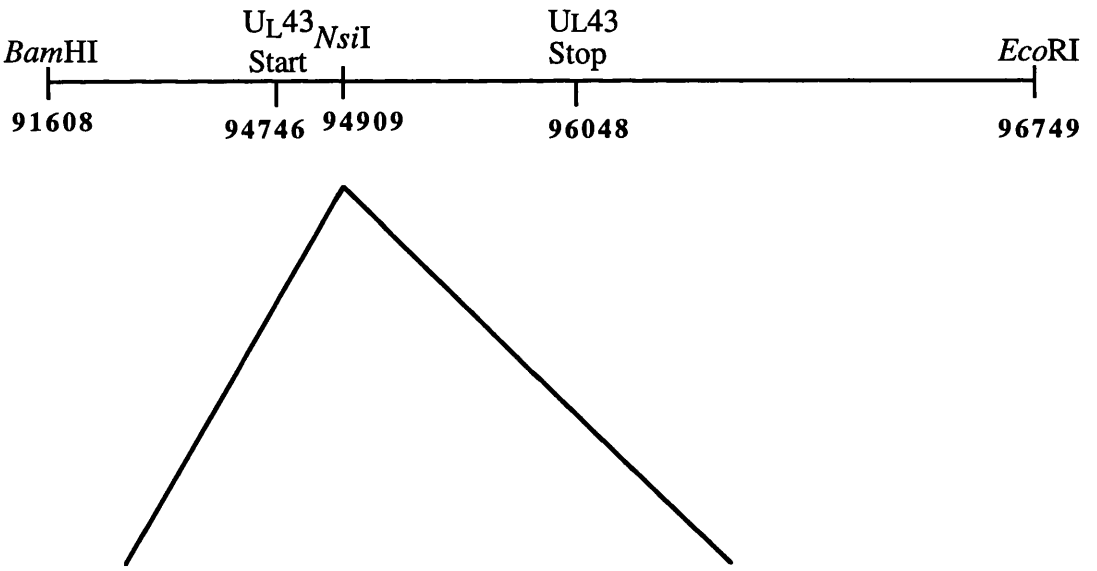
In the experiment described in section 4.33., an attempt was made to demonstrate the inhibition of plaque initiation in HFL cells pretreated with non-UV-irradiated L-particles by using *in*1815 L-particles at the NPT; it was found that the non-UV-irradiated L-particles stimulated plaque initiation. However, it is possible that the *ts* uncoating mutation in *in*1815 leaks at the NPT, leading to the expression of viral IE proteins that would complement a superinfecting virus. Staining *in*1817-infected cell monolayers with X-gal enabled an assessment of the proportion of PFU which leak at the NPT, thus although plaques are not formed by viruses containing the *ts*1213 mutation at the NPT the mutation might also act at a late stage of replication, enabling expression of viral proteins but preventing the virus from spreading from cell to cell and forming plaques. Staining *in*1817-infected monolayers with X-gal enabled single lytically infected cells to be

Figure 4.36. Insertion of *lacZ* into the *in*1815 genome. An *Xho*I / *Bgl*II / *Xba*I linker was ligated into the unique *Nsi*I site within the U_L43 coding sequences of clone p35 (MacLean *et al.*, 1991), yielding A994#29. Clone p35 consists of a 5.1 kbp *Bam*HI (position 91608 in the HSV-1 genome) / *Eco*RI (96749) fragment of the HSV-1 genome cloned into the *Bam*HI / *Eco*RI sites of pUC19 (MacLean *et al.*, 1991). An *Xba*I / *Xho*I fragment from plasmid pMJ101 (Jamieson *et al.*, 1995) consisting of the HCMV major IE promoter controlling the *lacZ* gene with SV40 terminator sequences was ligated into the *Xba*I / *Xho*I sites of A994#29 to yield B3#76 (B3#76 was constructed and kindly supplied by C.M. Preston). Plasmid B3#76 was cotransfected into BHK cells with *in*1815 DNA and β-galactosidase expressing plaques were screened for the presence of the insertion in U_L43. A: The HSV-1 genome. B: The region of the genome cloned in p35. C: The position of insertion of HCMV / *lacZ* in B3#76.

A


UL
Us

B


BamHI
UL43 Start
NsiI
UL43 Stop
EcoRI
91608
94746
94909
96048
96749

C

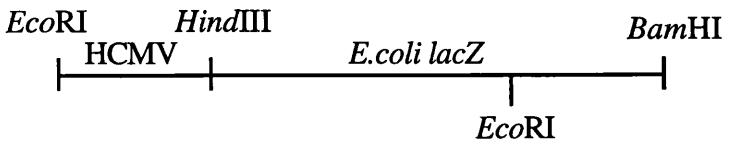
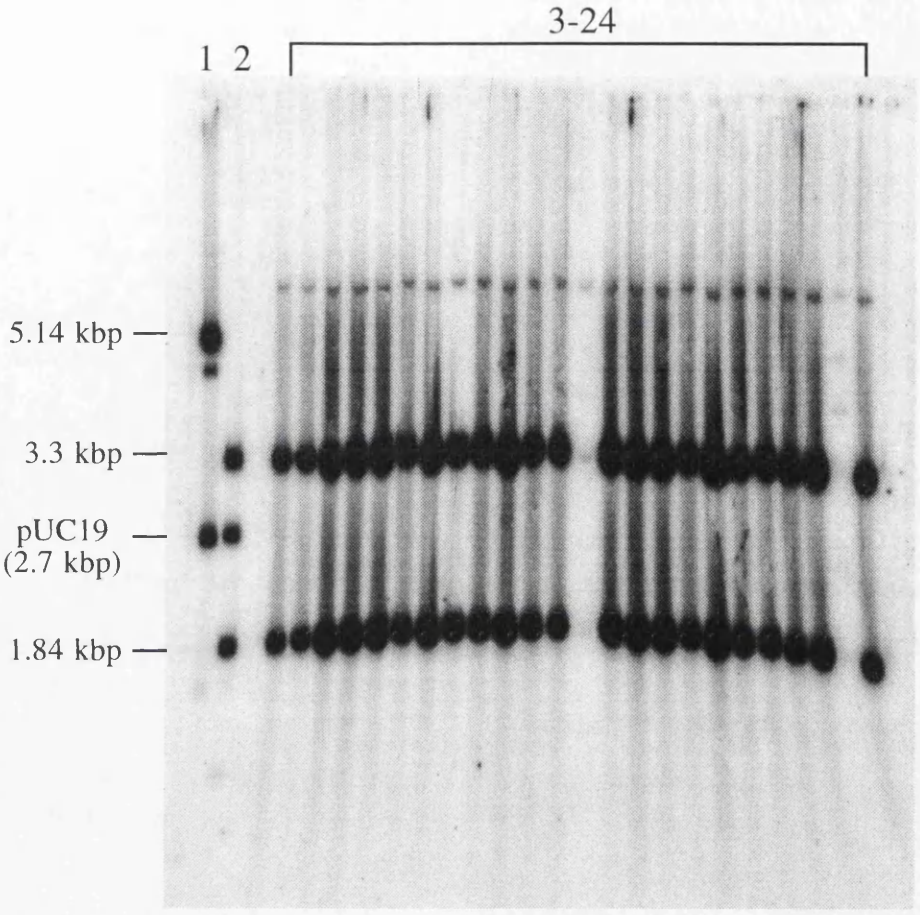

EcoRI
HindIII
BamHI
HCMV
E.coli lacZ
EcoRI

Figure 4.37. Screening of *in*1815 plaque isolates for the *lacZ* insertion in U_L43. *In*1815 DNA and B3#76 were cotransfected into BHK cells and incubated at 31°. After plaques had appeared the cells were harvested, bath sonicated and titrated in 10-fold serial dilutions in the presence of X-gal. Blue plaques were picked, subjected to 4 rounds of plaque purification (each selecting for β-galactosidase expression by staining with X-gal), and then screened for the presence of the HCMV / *lacZ* insert in U_L43. Twenty-four plaques were picked, inoculated onto BHK cell monolayers in 24-well tissue culture plates and incubated at 31° in the presence of HMBA. When severe CPE was evident the medium was removed and the DNA was extracted from the cells, digested with *Eco*RI, *Hind*III and *Bam*HI. A proportion was electrophoresed in a 0.8% agarose gel, blotted onto a hybridisation membrane and hybridised to radiolabelled plasmid p35 containing the wild-type HSV-1 U_L43 gene (figure 4.36.). Lane 1: Plasmid p35 containing the wild-type U_L43 digested with *Eco*RI, *Hind*III and *Bam*HI. Lane 2: Plasmid B3#76 containing the HCMV / *lacZ* insert digested with *Eco*RI *Hind*III and *Bam*HI. Lanes 3-26: Blue plaque isolates digested with *Eco*RI, *Hind*III and *Bam*HI. The positions of the wild-type and recombinant fragments and the pUC19 vector are indicated. All of the plaque isolates contained virus with the HCMV / *lacZ* insert. There is no hybridisation to isolates 13 and 23, probably due to loss of the DNA pellets during extraction. Isolate 3 was grown as a virus stock and used for further experimentation. A scanned autoradiograph is presented.


3-24
1 2
5.14 kbp
3.3 kbp
pUC19
(2.7 kbp)
1.84 kbp

Temperature (°C)	Input PFU	Number of blue cells	
		Alone	+HMBA
31	10^3	0	1398
	10^4	4	ND
	10^5	300	ND
	10^6	ND	ND
39.5	10^3	0	95
	10^4	2	784
	10^5	24	ND
	10^6	329	ND

Table 4.19. Assessment of the leakiness of *in*1817 at the NPT. HFL cells in 35 mm diameter tissue culture dishes were infected at 31° or 39.5° with 10^6, 10^5, 10^4 or 10^3 PFU of *in*1817 per monolayer. The infected cells were overlaid with EF_{10} containing 2.5 μg/ml Brefeldin A or EF_{10} containing Brefeldin A and 3 mM HMBA and incubated at 31° or 39.5°. At 19 hours post-infection the monolayers were stained for β-galactosidase activity and the number of blue cells per monolayer was determined.

counted.

HFL cells were infected at 31° or 39.5° with various MOIs of *in*1817, overlaid with medium containing Brefeldin A or medium containing Brefeldin A and HMBA and incubated at 31° or 39.5°. At 19 hours post-infection the monolayers were stained for β-galactosidase activity and the number of blue cells per monolayer was determined and

presented in table 4. 19.

In the presence of HMBA, the number of *in*1817-infected cells expressing viral genes in monolayers infected at 39.5° was approximately 6% of the number of cells expressing viral genes at 31°. In the absence of HMBA 15% of the *in*1817 leaked, thus up to 15% of *in*1817 leaked at the NPT. *In*1817 does not form plaques at 39.5°, thus the *ts* uncoating mutation presumably also acts at a late stage of replication thereby preventing the production of infectious virus in cells infected with leaky virus.

In light of the finding that *in*1815 leaked at the NPT, is seems probable that the stimulation of *in*1853 in HFL cells pretreated with non-UV-inactivated *in*1815 L-particles at 39.5° (section 4.33.) was caused by contaminating virions in the L-particle preparation leaking, expressing IE genes and complementing the *in*1853.

In the experiments examining the ability of *in*1815 to enter the repressed, latent state at 39.5° (section 4.31.), the small proportion of virus which became repressed at 39.5° might have been caused by leaking virus entering the latent state. The fact remains that *in*1815 did not enter the latent state at 39.5° as efficiently as *in*1814 or *in*1816, hence the interpretation that delayed uncoating did not lead to latency remains unaffected.

5. DISCUSSION.

It was previously reported that viral genomes entered a latent state in tissue culture cells when viral IE transcription was abrogated (Harris and Preston, 1991; Jamieson, 1993). In the first chapter of the results section of the thesis presented here, a study is described which was aimed at delineating the structure of HSV-1 genomes during latency in HFL cells after viral IE transcription and cytotoxicity had been reduced by pretreating the cells with IFNα, and by use of the mutant *in*1820. *In*1820 has two modifications, an insertion in Vmw65 rendering it unable to transactivate IE genes, and a modification in the promoter controlling transcription of IE1, resulting in a Vmw110$^-$ phenotype under IE conditions in HFL cells.

An investigation into the possibility that virus structural components biochemically modify HFL cells to a state which makes latency the outcome of infection is described in the second chapter, and in the final chapter an attempt was made to determine whether slow virus uncoating leads to latency.

As with related *in vitro* latency systems using HFL cells (Harris and Preston, 1991; Preston and Russell, 1991), and during latency *in vivo* (Rock and Fraser, 1983, 1985; Efstathiou *et al.*, 1986) the predominant population of retained viral genomes were in a nonlinear form (sections 4.1, 4.3 and 4.4.). The nonlinear viral genome configuration observed in the *in vitro* latency system described in the thesis presented here is in contrast to the system of Wigdahl *et al.* who established latency of wild type HSV-1 in HFL cells by pretreating the cells with IFNα and BVDU for 24 hours prior to infection with up to 2.5 PFU of virus per cell (Wigdahl *et al.*, 1984a). After incubation at 37° for 7 days the virus was maintained in the latent state in the absence of the inhibitors if the cells were shifted to 39.5–40.5°. Reactivation was induced by superinfection with HCMV or by downshift to 37°. The predominant population of latent viral genomes in the system of Wigdahl *et al.* was linear (Wigdahl *et al.*, 1984a), suggesting that the basis for repression of viral replication fundamentally differs from latency described by Preston and co-workers (Russell and Preston, 1986; Harris and Preston, 1991; Jamieson *et al.*, 1995). In the system studied here (Jamieson *et al.*, 1995), the linear population of viral genomes resulted from incomplete uncoating of input virus particles, thus it is tempting to speculate that the block to viral replication in the systems utilising inhibitors and high temperature occurs as a result of inhibition of uncoating of input genomes or by inhibition of a stage of the replicative cycle post-cleavage or encapsidation of *de novo* synthesised DNA. As has been suggested previously (Shiraki and Rapp, 1986; Preston and Russell, 1991), two populations of genomes might exist in the system of Wigdahl *et al.*, one which is blocked by incubation at 39.5–40.5° and reactivated by downshift to 37° and one which is reactivated by superinfection with HCMV. It seems possible that the

HCMV-reactivated genomes in the system of Wigdahl *et al.* are in a nonlinear state resembling the latent genomes in the system of Jamieson *et al.*, but that the nonlinear population is of such low abundance relative to the linear population that their detection in Southern blots is prevented. The HCMV-reactivated genomes in the systems using inhibitors and elevated temperature remain stable over time, but the 37°-reactivated genomes decrease in number (Shiraki and Rapp, 1986), thus the hypothesis that these two populations of genomes represent nonlinear and linear DNA respectively is supported by the observation that nonlinear genomes in the system of Jamieson *et al.* are stable and the linear genomes are preferentially degraded (Jamieson *et al.*, 1995).

Virtually all of the genomes detected in the *in vitro* latency system were competent for reactivation by superinfection (Jamieson *et al.*, 1995). That virtually all the detectable genomes were competent for reactivation enabled the demonstration that the nonlinear population are templates for reactivation and that conversion to a linear form is not required as a prerequisite for the genomes to express genes and resume replication (section 4.4.), thus nonlinearity *per se* is not responsible for repressing the latent genomes. It seems likely that nonlinearity plays an important role in preventing degradation by exonucleases and may be required to enable other changes to occur which stabilise or repress the genomes.

A striking finding which arose from the structural studies was that HFL cells contained a greater proportion of non-uncoated genomes than other cell types at a set time after infection, and a smaller total amount of DNA (sections 4.5. and 4.8.). Use of the *in vitro* latency system enabled experiments to be carried out in which infections proceeded for 3 days, however non-uncoated genomes were still present at 3 days post-infection, thus uncoating appeared particularly slow in HFL cells. Alternatively, the high proportion of non-uncoated genomes might be explained if linear DNA is degraded particularly efficiently in HFL cells before it is stabilised by conversion to the nonlinear configuration and associated with nucleosomes. Specific degradation of linear DNA has been observed previously (Jamieson *et al.*, 1995), and might itself be a result of inefficient uncoating. The hypothesis that degradation of virus DNA occurs more efficiently in HFL cells than in other cell types was supported by the observation that infected HFL cells contained fewer viral genomes in both the nuclei and cytoplasm than the other cell types tested (section 4.5.).

In previous studies by Jamieson it was observed that latent genomes were more sensitive to MN when compared to *in*1820 shortly after infection or to genomes transcribing in the presence of Ara-C (Jamieson, 1993). It was suggested that latent genomes were sensitive to MN as a specific result of their latent state and that transcribing viruses remained MN-resistant because they did not have such changes conferred upon them. In the study described in the thesis presented here, the linear portion of genomes in

the *in vitro* latency system were resistant to nucleases and were thus defined as resulting from non-uncoated input genomes (section 4.3.), thus the nuclease-sensitive state was a result of uncoating rather than conversion to the latent form. The interpretation of Jamieson was probably confused by the unusually high proportion of non-uncoated virus genomes which remain in the nuclei of infected HFL cells. Another possible cause of the high resistance of transcribing viral genomes to MN was that the ability of the cells to uncoat incoming virus particles was reduced by the cytotoxicity of viral gene expression, and had long-term experiments been possible with transcribing viruses, they too would have entered a nuclease-sensitive state.

Infectivity of HSV-1 in HFL and BHK cells is similar but HFL cells contained up to 9-fold fewer nonlinear, uncoated viral genomes (sections 4.5 and 4.8.). It follows that a large proportion of viral genomes in HFL cells do not undergo replication and are degraded, but in BHK cells these genomes are converted to the nonlinear configuration and retained, whereas input genomes destined to undergo replication are stable in both HFL and BHK cells. The observation that only a small proportion of input viral genomes undergo replication has been made previously (Jacob and Roizman, 1977).

Virus uncoating was assayed by hybridising Southern blots to probes which detected the joint and L-terminal *Bam*HI restriction fragments of the genome (sections 4.5.–4.9., 4.23. and 4.30.). Conversion to the nonlinear configuration was expected to occur rapidly after uncoating, therefore the amount of nonlinear genomes was indicative of the rate of uncoating. The sensitivity of the nuclear viral DNA to digestion with DNase I was also used to assess the degree of uncoating. Genomes of the temperature sensitive uncoating mutant *ts*1213 exhibited a phenotype at the NPT expected of an uncoating mutant: they were not converted to the nonlinear configuration, did not associate with nuclei as efficiently as in normal infection and were relatively resistant to DNase I (section 4.9.). The Southern blot assay was thus a valid and useful method of assessing the effect on uncoating of treatments which alter HSV-1 replication, such as IFNα and HMBA.

Uncoating and conversion to the nonlinear configuration was found to result from the action of pre-existing cell factors or factors brought in with virus particles (section 4.8.), as has been observed in other laboratories (Poffenberger and Roizman, 1985). Uncoating was unaffected by the *in*1814 mutation (section 4.6.), IFNα pretreatment (section 4.7.) or HMBA (section 4.6.), thus while uncoating possibly varies between cell types, no evidence was found that rate of uncoating is a target for treatments which modify virus infectivity.

In agreement with studies by Jamieson, initial data indicated that the TK gene was not associated with regularly spaced nucleosomes (section 4.10.; Jamieson, 1993). In addition, using replication inhibitors with a specific action on viral DNA replication, it was demonstrated that the absence of regularly spaced nucleosomes on the latent genomes

was not a secondary consequence of Ara-C inhibiting histone synthesis. The data in the thesis presented here is in agreement with those of Daniell *et al.* who demonstrated that the regular nucleosome arrangement on parental adenovirus DNA during infection was not prevented when histone synthesis was inhibited with butyrate (Daniell *et al.*, 1981), thus even when *de novo* histone synthesis is prevented, cells contain sufficient pools of histones to bind incoming virus genomes and form a regularly spaced structure.

A chromatin-like pattern of MN digestion was detected on the LAT / IE1 region of *in*1820 genomes in the *in vitro* latency system (section 4.11.), indicating that the latent genomes were in a similar structure to the bulk of latent HSV-1 DNA in the brainstems of mice (Deshmane and Fraser, 1989). To the knowledge of the author of the thesis presented here, there are no published reports to date of regular nucleosome arrangement on the viral genomes in any other *in vitro* latency system. In order to convert to the nonlinear configuration and associate with nucleosomes, the viral genomes must be free from components of the nucleocapsid and available to components of the cell. Association with nucleosomes may increase the stability of the latent genomes and might also contribute to their transcriptional repression. Nucleosome-associated DNA fragments from latent viral DNA partially digested with MN appeared to have migrated faster in agarose gels than nucleosome-associated DNA fragments from cell DNA, an observation which was also made with latent viral DNA from mice (Deshmane and Fraser, 1989). One interpretation of the discrepancy between the migration speeds of viral and cellular nucleosome-associated DNA fragments is that nucleosomes were more closely spaced on viral DNA than on the cell DNA, however there are other possible technical reasons for the discrepancy which render this interpretation speculative, such as alteration of electrophoretic mobility caused by high G+C content or alteration of the positions of the nucleosome-associated DNA fragments during transfer from the agarose gels onto the hybridisation membranes.

The detection of a regular nucleosome arrangement at the LAT / IE1 region of the genome when no such pattern had been previously detected at the TK locus, indicated that the LAT / IE1 region formed a structure bound by regularly spaced nucleosomes in preference to other regions of the genome; an exciting possibility because the LAT / IE1 region is the only region of the viral genome to be transcriptionally active during latency *in vivo*, thus nucleosome positioning might play a crucial role in transcriptional regulation of this region. However, in the final experiment, when the LAT / IE1 region and the TK gene were examined in parallel, a regular arrangement was found on both regions indicating that the regular pattern is present on the entire genome and that the previous inability to detect chromatin on the TK gene was caused by differences in chromatin arrangements between experiments or by differences in the ability to detect the ladder of DNA fragments (section 4.12.). In experiments using Southern blotting and hybridisation of MN-digested DNA to detect chromatin, small variations in the arrangement of

nucleosomes and variations in the ability to resolve the heterogeneously sized nucleosome-associated DNA fragments have a large bearing on the interpretation of the data. Chromatin was detected on the TK gene only once, and it is possible that the effects seen in figure 4.19. lane 11 are due to unfortunate background on the autoradiograph.

There are two possible reasons why chromatin was not detected on the latent viral genomes in previous studies and in some of the experiments described here. One possible cause was that the ability to detect the ladder of DNA fragments in Southern blots varied between experiments. Another possibility was that the state of the cells varied between experiments in such a way as to alter the ability of nucleosomes to form a regular binding pattern, perhaps as a result of variations in levels of histone pools. In light of the previous finding that in a parallel experiment chromatin was detected on the TK gene when in the cell genome but not when in the latent genome (Jamieson *et al.*, 1995), the latter hypothesis is most likely to be the true explanation. As explained above, small variations in nucleosome positioning on the genomes and in the techniques used to detect chromatin have a large bearing on the interpretation of the data. Although inhibition of histone synthesis by Ara-C did not affect the inability the detect chromatin on the TK gene in the latent genome, Ara-C was added at 1 hour post-infection and might not have had time to exert a large enough effect on the levels of histone pools to alter the nucleosomal arrangement.

Since neurons are nondividing, do not undergo DNA synthesis and therefore contain low histones pools, the formation of a regular nucleosome arrangement on HSV genomes during the initial stages of latency in neurons might also be expected to occur slowly.

It has been observed that *in*1814 and other mutants defective in Vmw110 have exceptionally high particle / PFU ratios in HFL cells (Stow and Stow, 1986; Ace *et al.*, 1989; Everett, 1989). In addition, other *in vitro* latency systems have utilised HFL cells (Wigdahl *et al.*, 1981; 1982b, 1984b; Russell and Preston, 1986; Harris and Preston, 1991) thus HFL cells seem particularly suited to *in vitro* latency experiments. One possible interpretation of the observation that HFL cells contained fewer nonlinear, uncoated genomes after infection than other cell types, is that virus uncoating occurs more slowly in HFL cells than in the other cell types. During establishment of latency in mouse dorsal root ganglia after infection in the footpad, transport of the virus nucleocapsids along the nerve axons results in a 20-24 hour delay between binding of the virus to the cell surface and entry of the virus nucleocapsid into the neuronal cell body (Cook and Stevens, 1973), thus slow uncoating of HSV in HFL cells might mimic establishment of latency in ganglia *in vivo*. Researchers in other laboratories have hypothesised previously that axonal transport facilitates the establishment of latency by resulting in the loss of Vmw65 (Roizman and Sears, 1987). In the latter two chapters of the results section in the

thesis presented here, investigations were carried out to determine whether a virus structural component modified cells and induced latency, and to determine whether slow virus uncoating gives the antiviral state an advantage over viral replication, thereby leading to latency.

The observation that HFL cells treated with UV-inactivated *in*1814 were less permissive for plaque initiation upon subsequent infection with HSV-1 appeared to support the concept that components of viral particles modify cells, causing the establishment of latency (sections 4.13–4.15.).

The inhibition of plaque initiation in HFL cells pretreated with UV-*in*1814 L-particles did not occur *via* an inhibition of adsorption, penetration, migration to the nuclei or uncoating (section 4.23.), thus the nonpermissive state appeared to be the result of an inhibition of viral IE gene expression. Levels of the viral IE mRNAs were reduced in UV-*in*1814 L-particle-pretreated HFL cells by a mechanism which did not require protein synthesis (section 4.24.), whether as a result of inhibition of transcription or RNA stability is unclear at present. The *vhs* function of HSV-1 was a possible component of the viral particles which could have reduced the viral IE mRNA levels by causing their degradation, however, UV-inactivated L-particles from a *vhs*$^-$ version of *in*1814 retained the ability to induce the nonpermissive state in HFL cells (section 4.26.), demonstrating that *vhs* was not responsible for the inhibition.

The observation that the antiviral state induced by the UV-*in*1814 viral particles was not overcome by HMBA (section 4.18.) and acted against a virus expressing wild-type Vmw65 (section 4.15.) suggested that the antiviral state induced by the UV-inactivated particles was similar to that which occurs during latency of *in*1814 in normal cells, where the virus becomes unresponsive to these activators during the first 6 hours of infection. However, the apparently latent state induced in HFL cells pretreated with UV-inactivated viral particles appeared to differ from latency of *in*1814 in untreated HFL cells, in that reactivation of all the input virus was not induced by superinfection with *ts*K (sections 4.19.–4.21.). One possible explanation for the inability to reactivate the latent virus in UV-*in*1814 treated HFL cells was that the inhibitory activity of the UV-inactivated particles was acting against the *ts*K, lowering the efficiency of reactivation (section 4.21.).

Adsorption, penetration, migration to the nuclei and uncoating were unaffected in HFL cells pretreated with UV-*in*1814 L-particles (section 4.23.), thus the inhibition of plaque initiation appeared to differ from that observed by Johnson and Ligas who reported that gD brought into the cells with the UV-inactivated particles caused a nonpermissive state by sequestering gD receptors required for fusion of the virus with the cells (Johnson and Ligas, 1988).

In the experiments of Johnson and Ligas, human R970 cells were treated with UV-inactivated gD-containing or gD$^-$ virus at 4° and whilst being maintained at 4° were

infected with HSV-1 or HSV-2 (Johnson and Ligas, 1988). The infected cells were shifted to 37° and the infectivity assayed by labelling with [^{35}S]methionine, immunoprecipitating TK and examining the amount of labelled TK after electrophoresis. TK was undetectable in cells pretreated with over 5000 particles of the gD-containing virus per cell, but not in cells pretreated with the gD$^-$ virus. Infectivity was not inhibited when the UV-inactivated gD-containing virus was added after the infectious virus, thus it was concluded that pretreatment of the cells with UV-inactivated gD-containing virus inhibited subsequent infection with HSV by sequestering gD receptors, and that treatment of the cells with the gD$^-$ virus failed to bring about the nonpermissive state because of their lack of gD. The interpretation that gD inhibits superinfection was supported by the observation that cell lines constitutively expressing gD are nonpermissive (Campadelli-Fiume *et al.*, 1988; Johnson and Spear, 1989; Campadelli-Fiume *et al.*, 1990). It is of interest to speculate why the inhibitory activity of the UV-inactivated viral particles observed by Johnson and Ligas differed from the nonpermissive state reported in the thesis presented here. If the inhibition was caused by gD brought into the cell with the UV-inactivated viral particles, the inhibitory activity would be expected to have reached maximum activity immediately after the adsorption period. In the study described in the thesis presented here, the inhibitory nonpermissive state in the UV-inactivated viral particle treated HFL cells increased over the first 6 hours after treatment (section 4.16.), indicating that a gradual biochemical change was occurring in the cells. The difference between the experiments described by Johnson and Ligas and those presented in the thesis described here may lie in the fact that in the experiments by Johnson and Ligas penetration of the cells by the UV-inactivated viral particles was prevented by incubation at 4°, thus at the time of adding the infectious virus the UV-inactivated particles were still present on the cell surface, whereas in the experiments in the study described here penetration of the UV-inactivated particles was expected to have been completed by the time the infectious virus was added. After adsorption of the UV-inactivated viral particles with the cells, gD would remain on the cell surface and might retain the ability to inhibit further infection of the cells, as demonstrated by the nonpermissivity of the gD-expressing cell lines (Campadelli-Fiume *et al.*, 1988; Johnson and Spear, 1989; Campadelli-Fiume *et al.*, 1990). However, in most of the experiments described in the thesis presented here the infectious virus was added 6 hours after treatment with the UV-inactivated particles, thus levels of gD on the cell surface might have decreased to levels below that required to give inhibition of adsorption. In addition, MOIs of UV-inactivated viral particles used in the experiments presented here were less than those used by Johnson and Ligas, contributing further to the difference in amount of gD brought into the cells. It is possible that UV-inactivated viral particles inhibit infection by two mechanisms, one which involves the inhibition of penetration by gD and one which occurs after fusion of the UV-inactivated particles with the cells. Whether gD caused the

nonpermissive state in the study described here could be tested using a UV-inactivated gD^- version of *in*1814.

When uncoating of *in*1815 was prevented by infection at 39.5°, the viral titre remained responsive to HMBA (section 4.31.). In addition, the rate at which the genomes became unresponsive to HMBA after downshift to 31° was not increased compared to infected cells downshifted to 31° immediately after the 1 hour adsorption period at 39.5° (section 4.31.), thus delayed uncoating did not lead to latency in HFL cells.

The inability of *in*1815 to become unresponsive to HMBA at 39.5° might be explained if the components of the viral particles which induce the repressed state were not released at 39.5°. It was demonstrated that the uncoating mutant *ts*B7 released Vmw65 after infection at 39.5° (Batterson and Roizman, 1983), but did not release a gene product of U_L26 which raises the mutation frequency of cells (Shillitoe *et al.*, 1993; Das *et al.*, 1994), thus components of the tegument can be released during infection at 39.5° but the nucleocapsid and its contents remain intact, as would be expected if uncoating is prevented. If the repression of the viral genome was induced by components of the nucleocapsid or components associated with the virus DNA, then latency would not have been induced at 39.5°. However, delayed uncoating caused by the mutation in *in*1815 was expected to be a reliable representation of the delay in uncoating in HFL cells, thus it is unlikely that slow uncoating in HFL cells facilitates the establishment of latency. The possibility that the repression is induced by a viral transcript synthesised *de novo* has been discounted by the finding that viral defectives containing only an origin of replication, a packaging signal and the *lacZ* gene are shut-off (C.M. Preston, unpublished data), thus the basis for repression of Vmw65 mutants remains elusive.

The observation that delayed uncoating did not induce latency, combined with the finding that virus inhibited in UV-*in*1814 viral particle treated HFL cells was not fully reactivated by superinfection with *ts*K, indicates that the apparent latent state induced in UV-*in*1814-pretreated cells differs from latency of *in*1814 in normal infection. The ability of UV-*in*1814 viral particles to induce the nonpermissive state may be a by-product of UV-irradiation, possibly resulting from UV-damaged DNA or proteins inducing a stress response in the cells, making them more resistant to infection.

Future work.

Now that it has been demonstrated that at least some specific regions of the latent genomes are associated with regularly spaced nucleosomes, a characterisation of the role of chromatin in latency should be performed. To what extent do nucleosomes repress the latent genomes, and is the chromatin detected on the viral genomes identical to the usual chromatin found on the majority of cell DNA? It is of interest to know whether nucleosomes need to be removed from the viral genomes for reactivation to occur and if so, which viral or cell gene products mediate their removal. Another important issue is the mechanism of action of the DNA element proposed to overcome the repression of the latent genomes and drive LAT expression *in vivo* (Lokensgard *et al.*, 1994). Although LAT is not expressed in the *in vitro* latency system (Anderson, 1991), the long-term expression element might exist as a hypersensitive-site, or other alteration of chromatin. Thus it should be determined whether the nucleosomes are arranged uniformly on the genomes or whether there are differences in chromatin organisation between different regions.

The mechanism by which the latent genomes are silenced in HFL cells is of crucial importance. If the factors responsible for repressing the latent genomes are induced by HSV infection, they could be identified by cloning cDNAs from HSV-infected cells and performing differential screening using radiolabelled cDNA from infected and uninfected cells as probes. However, the work described in the thesis presented here and work by others (C.M. Preston, unpublished data) suggest that the repression of *in*1814 in HFL cells does not require protein synthesis, and therefore is caused by pre-existing factors. One possible method of identifying the pathway by which the repression occurs, might be to isolate a version of *in*1814 which is unable to enter latency, to map the location of the mutated gene within the viral genome and characterise its product.

The location of the latent genomes within the cell nuclei might cause their repression by preventing the access of transcription factors. HSV DNA localises in ND10 domains during the early stages of lytic infection (Maul *et al.*, abstract 133, 20th International Herpesvirus Workshop, Groningen, The Netherlands, 1995; R.D. Everett, personal communication), thus it is of interest to know whether the viral DNA is located in ND10 domains during latency. The finding that treatments which cause reactivation, such as expression of Vmw110 (Harris *et al.*, 1989; Harris and Preston, 1991; Cai *et al.*, 1993; Minigawa *et al.*, 1994), administration of $CdSO_4$ (Fawl and Roizman, 1993) and hyperthermia (Sawtell and Thompson, 1992) cause redistribution of ND10 (Maul *et al.*, 1993; Maul, 1995; Maul *et al.*, abstract 133, 20th International Herpesvirus Workshop, Groningen, The Netherlands, 1995) implicates a role for ND10 domains in latency.

Until the inhibition of infectivity in HFL cells pretreated with UV-*in*1814 viral particles is demonstrated with non-UV-irradiated particles, the inhibition cannot be unequivocally considered a *bona fide* property of the viral particles rather than a by-product of their UV-irradiation. UV-irradiation might be avoided by using PREP particles, viral particles similar to L-particles which are produced during infection in the presence of viral DNA replication inhibitors or by DNA replication mutants at the NPT (Dargan *et al.*, 1995). The particle / PFU ratio of PREPs ranges from 6×10^5 to 3.8×10^8 particles per PFU, thus interference from residual viral IE gene expression complementing superinfecting viruses might be low enough to obviate the need for UV-irradiation.

Despite the finding that delayed uncoating did not lead to latency in HFL cells, it remains tempting to postulate that the delay between infection at the cell surface and entry of the viral DNA into the nuclei of sensory neurons *in vivo* plays a role in the establishment of latency. It would be interesting to determine the effect of delaying uncoating in neuronal cells.

Clearly an investigation of the role of LAT is not possible with the *in vitro* latency system as it stands, since LAT is not expressed (Anderson, 1991). The absence of LAT expression might be explained by the absence of the appropriate neuronal transcription factors in HFL cells. However, LAT expression might be induced if the LAT controlling elements are replaced with elements active during latency in HFL cells. To date no such element has been found (C.M. Preston, unpublished data). A subset of VZV RNAs have been demonstrated in satellite cells of latently infected human sensory ganglia (Croen *et al.*, 1988; Croen and Straus, 1991), suggesting that VZV is capable of latency in non-neuronal cells and that the promoters of these genes are active when the rest of the genome is repressed. A valid experiment would be to insert one of the latency-active genes of VZV into the genome of *in*1820 and examine whether expression occurs during latency in HFL cells.

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Quiescent viral genomes in human fibroblasts after infection with herpes simplex virus type 1 Vmw65 mutants

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The development and utilization of a tissue culture system for the analysis of quiescent, nonreplicating herpes simplex virus type 1 (HSV-1) genomes is described. It was demonstrated previously that the HSV-1 Vmw65 mutant *in*1814, which is impaired for immediate early (IE) transcription, was retained for many days in human fetal lung (HFL) fibroblasts in a quiescent 'latent' state. Molecular analysis of the viral genome was not possible, however, due to residual expression of IE proteins and consequent cytotoxicity at high m.o.i. In the study reported here, IE transcription was reduced further by pretreatment of cells with interferon-α (IFN-α) and by the the use of mutant *in*1820, a derivative of *in*1814 in which the Vmw110 promoter was replaced by the Moloney murine leukaemia virus (Momulv) enhancer. The Momulv enhancer was not expressed under IE conditions; thus *in*1820 was more impaired for replication than *in*1814 and behaved as if deficient for both Vmw65 and Vmw110. In cells pretreated with IFN-α and subsequently infected with *in*1820 cytotoxicity was overcome, enabling a tissue culture system to be developed in which all cells stably retained at least one quiescent viral genome. To assist the analysis of gene expression, *in*1820 was further modified by insertion of the *Escherichia coli lacZ* gene controlled by the human cytomegalovirus enhancer (mutant *in*1883) or the HSV-1 immediate early Vmw110 promoter (*in*1884). Expression of β-galactosidase was not detected after infection of IFN-α-pretreated cells with *in*1883 or *in*1884 but could be induced in almost all cells containing a viral genome, by superinfection of cultures. *In*1820-derived viruses were retained for at least 9 days and were not reactivated by subculture of cells. A regular arrangement of nucleosomes, as found in cellular chromatin, was not detected on the viral genome at the thymidine kinase locus. The non-linear genome was a template for reactivation with no requirement for prior conversion to a linear form. A small number of remaining linear genomes resulted from incomplete uncoating of input virus.

Introduction

Transcription of herpes simplex virus type 1 (HSV-1) immediate early (IE) genes is stimulated by the virus structural protein Vmw65 (also known as VP16 or α-TIF), resulting in efficient production of five IE proteins shortly after infection (Post *et al.*, 1981; Campbell *et al.*, 1984). Vmw65 forms a complex at the IE-specific sequence TAATGARAT (R is a purine) with at least two cellular proteins, Oct-1 and HCF, thereby bringing the C-terminal activating region of Vmw65 into proximity with cellular factors required for initiation of transcription (McKnight *et al.*, 1987; O'Hare & Goding, 1988; Preston *et al.*, 1988; Triezenberg *et al.*, 1988; Sadowski *et al.*, 1988; Stern *et al.*, 1989; Katan *et al.*, 1990; Kristie & Sharp, 1990; Wilson *et al.*, 1993). Three of the IE proteins, Vmw175 (ICP4), Vmw110 (ICP0) and Vmw63 (ICP27), are known to be important regulators of viral gene expression. Vmw175 and Vmw63 are essential for virus replication, exerting effects at the transcriptional and post-transcriptional levels, respectively (Preston, 1979*b*; Watson & Clements, 1980; Dixon & Schaffer, 1980; Sacks *et al.*, 1985; Everett, 1987; Sandri-Goldin & Mendoza, 1992), whereas Vmw110 is not essential but enables virus replication to be initiated efficiently, especially after infection at low m.o.i. (Stow & Stow, 1986; Sacks & Schaffer, 1987; Everett, 1989). Vmw110 is a potent activator of gene expression which acts on a wide variety of virus- or plasmid-borne promoters in a manner that is apparently not sequence-specific (Everett, 1984; O'Hare & Hayward, 1985; Cai & Schaffer, 1992; Chen & Silverstein, 1992).

At present, three properties of Vmw65 have been

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documented. The protein is a major structural component of the virus tegument and is therefore required for the formation of mature virions (Heine *et al.*, 1974; Ace *et al.*, 1988; Weinheimer *et al.*, 1992). More recently, Vmw65 has been shown to associate with the virion host shutoff (vhs) protein (Smibert *et al.*, 1994). The third function, activation of IE transcription, has been studied in great detail at the molecular level but only one virus mutant defective in this process, *in*1814, has been described to date (Ace *et al.*, 1989). The Vmw65 protein specified by *in*1814 is deficient in transactivation of IE transcription due to the presence of a four-amino-acid insertion within a domain required for binding to Oct-1 and HCF (Ace *et al.*, 1988; Greaves & O'Hare, 1990; Hayes & O'Hare, 1993). As a consequence of reduced IE transcription, *in*1814 is unable to initiate productive infection efficiently and thus, at low m.o.i., only a small proportion (0·1–1%, depending on the cell type) of virus–cell interactions result in virus replication (Ace *et al.*, 1989; Daksis & Preston, 1992). The vast majority of infected cells harbour the *in*1814 genome in a stable quiescent state that has been termed '*in vitro* latency' (Harris & Preston, 1991). By 1 day after infection, *in*1814 genomes become insensitive to activation by Vmw65 or hexamethylene bisacetamide (HMBA), even though these agents effectively complement the defect in virus replication if applied at the time of infection (Ace *et al.*, 1989; Harris & Preston, 1991; McFarlane *et al.*, 1992). It is possible, however, to recover *in*1814 from cultures several days after initial infection by superinfection with HSV-1, provided the superinfecting virus expresses functional Vmw110 (Harris & Preston, 1991). Entry of *in*1814 into the quiescent state is probably due mainly to reduced synthesis of Vmw110, since mutants deleted for the Vmw110 gene have a phenotype similar to that of *in*1814 (Stow & Stow, 1986, 1989; Everett, 1989).

HSV latency in the peripheral nervous system of animals and humans is characterized by repression of all regions of the genome except for that encoding the latency-associated transcripts (LATs) (Croen *et al.*, 1987; Spivack & Fraser, 1987; Stevens *et al.*, 1987; Fraser *et al.*, 1992). It has been suggested that a block to IE transcription, possibly imposed by inhibitory TAATGARAT-binding proteins, is primarily responsible for the abortion of lytic replication and hence the establishment of latency (Roizman & Sears, 1987; Kristie & Roizman, 1988; Kemp *et al.*, 1990; Lillycrop *et al.*, 1991, 1994; Sears *et al.*, 1991). In support of this hypothesis, *in*1814 establishes latency after inoculation into mice even though productive infection in neurons is impaired, suggesting that IE gene expression is not required for latency (Steiner *et al.*, 1990; Valyi-Nagy *et al.*, 1991; Ecob-Prince *et al.*, 1993). The phenotype of *in*1814 in tissue culture cells is therefore apparently similar to that observed in mice: productive replication is reduced and virus retention in a latent state is favoured. *In*1814 is the only virus mutant currently available to investigate the effects of reducing IE gene expression in tissue culture cells; therefore studies on the structure and function of the quiescent *in*1814 genomes are relevant to an understanding of the suppression of gene expression that occurs during latency.

At present, the structural basis for the inactivity, and insensitivity to inducers, of quiescent *in*1814 genomes retained in fibroblasts is unclear. It is known that viral DNA is sequestered in a non-linear, probably circular, form analogous to that found during latency *in vivo* (Rock & Fraser, 1983; Efstathiou *et al.*, 1986; Harris & Preston, 1991), but information is lacking on the nature of viral nucleoprotein complexes, either at specific promoters or the entire genome. Analysis of this problem presents technical difficulties because although the predominant outcome of infection is failure of *in*1814 to replicate, the small number of productive interactions can form a significant background, especially if viral DNA replication occurs, thereby obscuring detection of the quiescent genomes. Furthermore, at high m.o.i. *in*1814 is cytotoxic due to expression of IE proteins (Johnson *et al.*, 1994). We describe here methods which reduce IE transcription from viruses containing the *in*1814 mutation, enabling the establishment of viable human fibroblast cultures containing at least one viral genome per cell. The methodology has been used to obtain novel information on the structure and expression of the quiescent genome.

Methods

Cells. Flow 2002 human fetal lung (HFL) fibroblasts (Flow Laboratories) were propagated in Eagle's medium supplemented with fetal calf serum at 10% (EF10) or 5% (EF5) or with 5% human serum (EHu5). BHK-21 (clone 13) cells were grown in Eagle's medium supplemented with 10% new born calf serum and 10% tryptose phosphate. Human 143 thymidine kinase deficient (TK^-) cells, transformed to a TK^+ phenotype by transfection of plasmid pTK1 (encoding the HSV-1 TK gene; Wilkie *et al.*, 1979), were kindly provided by M. McFarlane (University of Glasgow, Molecular Biology, UK) and grown in EF10 containing 0·1 mM-hypoxanthine, 16 μM-thymidine, 0·4 μM-aminopterin and 3 μM-glycine. All cell culture media contained penicillin (100 units/ml) and streptomycin (100 μg/ml).

Plasmids. To prepare HSV recombinants containing the *Escherichia coli lacZ* gene inserted within the TK gene, plasmid pMJ27 was first constructed. The *Hin*dIII site within the vector sequences of pGX166 (pTK1 modified by the insertion of an *Xho*I linker at the *Sac*I site within the TK coding sequences; kindly provided by V. G. Preston) was destroyed by cleavage, end-filling with Klenow enzyme and religation, to yield pGX166ΔH3. The *E.coli lacZ* gene plus simian virus 40 (SV40) promoter and enhancer was excised from plasmid pFJ3 (Rixon & McLauchlan, 1990) as a 4073 bp *Bam*HI–*Xba*I fragment and cloned between the *Bam*HI and *Xba*I sites of pUC18 (previously modified by insertion of an *Xho*I linker into the *Sma*I site) to yield pUC18lacZ. The *lacZ* gene, together with the SV40 promoter and

polyadenylation signals, was excised from pUC18lacZ as a *Sal*I–*Xho*I fragment and cloned into the *Xho*I site of pGX166ΔH3. A plasmid in which the direction of *lacZ* transcription was opposite to that of TK was selected and designated pMJ27. Plasmid pMJ27 contains unique *Xba*I and *Hin*dIII sites flanking the SV40 promoter plus enhancer, and has a unique *Xho*I site downstream of the SV40 polyadenylation signal.

The human cytomegalovirus (HCMV) Towne strain enhancer was cloned as a 760 bp *Sau*3AI fragment (Stinski & Roehr, 1985) from plasmid pHD101-4 (kindly provided by E. Blair, Wellcome Research Laboratories, Beckenham, UK) into the *Bam*HI site of pUC18 (from which the *Sph*I site had been removed by treatment with Klenow enzyme and religation), excised as an *Eco*RI (end-filled)–*Hin*dIII fragment and cloned between the *Xba*I (end-filled) and *Hin*dIII sites of pMJ27 to replace the SV40 enhancer, yielding plasmid pMJ101. The HSV-1 Vmw110 promoter was excised as an 836 bp *Bbv*I (end-filled)–*Sac*I fragment from pJR3 (Everett, 1984) and cloned between the *Sph*I (Klenow-treated) and *Sac*I sites of pUC18. The promoter was then removed as an *Eco*RI (end-filled)–*Hin*dIII fragment and cloned between the *Xba*I (end-filled) and *Hin*dIII sites of pMJ27 to yield plasmid pMJ102. The structures of pMJ27, pMJ101 and pMJ102 are shown in Fig. 1.

Viruses. The HSV-1 strain 17 mutant *in*1814 contains a 12 bp insertion in the coding sequences for Vmw65 (Ace *et al.*, 1989). Mutant *in*1820 was derived from *in*1814 essentially by replacing both copies of a 971 bp *Nco*I–*Sac*I region containing the promoter for Vmw110 [nucleotide positions 124105–125076 in the inverted long repeat (IR_L) copy; Perry & McGeoch, 1988] with a 760 bp DNA fragment containing the Moloney murine leukaemia virus (Momulv) enhancer and promoter (Lang *et al.*, 1983). A detailed description of the construction of *in*1820 will be presented elsewhere. 1814R was constructed by rescue of the Vmw65 mutation of *in*1814 (Ace *et al.*, 1989) and *in*1825 was similarly produced by rescue of the Vmw65 mutation of *in*1820.

To construct mutants *in*1883 and *in*1884, *in*1820 DNA was cotransfected into BHK cells with *Sac*I-cleaved pMJ101 and pMJ102, respectively. Progeny viruses expressing β-galactosidase (β-gal) were identified by the development of blue plaques in the presence of X-Gal and purified by three rounds of enrichment for *lacZ*-containing viruses. Final plaque isolates were grown as small scale cultures and DNA was purified from infected cells, cleaved with *Eco*RI and analysed by Southern transfer and hybridization, using ^{32}P-radiolabelled 2·4 kbp *Eco*RI n fragment, spanning the TK gene, as probe. Insertion of *lacZ* disrupts *Eco*RI n, yielding species of 1·0 kbp and either 2·0 kbp or 5·0 kbp. Virus isolates showing the correct pattern, and lacking detectable hybridization to the parental 2·4 kbp fragment after long autoradiographic exposures, were grown as virus stocks.

The genotypes of the viruses used can be summarized as follows: *in*1814, Vmw65$^-$, *in*1820: Vmw65$^-$/110prom→Momulv; *in*1825, Vmw65$^+$/110prom→Momulv; *in*1883, Vmw65$^-$/110prom→Momulv/TK$^-$[HCMV-β-gal]; *in*1884, Vmw65$^-$/110prom→Momulv/TK$^-$[110-prom-β-gal].

The HSV-1 mutant *ts*K contains a temperature-sensitive lesion in the immediate early protein Vmw175 (ICP4) (Preston, 1979*b*; Davison *et al.*, 1984).

Virus preparations were standardized by titration on BHK cells in the presence of 3 mM-HMBA (McFarlane *et al.*, 1992) and, in addition, virus particle concentrations were determined (Ace *et al.*, 1989). Preparations of *in*1820-based viruses used in this study contained approximately 100 particles per p.f.u.

Infection of cells. HFL cell monolayers, initially consisting of approximately 6×10^5 cells per 35 mm diameter Petri dish, were pretreated for 16–20 h with 10^3 units of human lymphoblastoid interferon-α (IFN-α, Sigma)/ml, after which time the cell number had risen to 8×10^5 per plate. Culture medium was removed thoroughly, cells were washed by addition of 2 ml EF5 and infected in 0·2 ml EF5. After 1 h at 37 °C, 2 ml EF5 containing 50 μg/ml cytosine arabinoside (AraC) was added and incubation continued at 37 °C for 2 or 3 days. For superinfection, culture medium was removed thoroughly and 2 ml EF5 added. Cells were incubated at 37 °C for 1–2 h, culture medium was again removed carefully and 0·2 ml EF5 containing 8×10^5 p.f.u. of *ts*K was added. After incubation at 37 °C for 1 h 2 ml EHu5, or 2 ml EF5 containing 2·5 μg/ml Brefeldin A (Sigma), was added. Monolayers were incubated for 22–24 h and stained for β-gal activity, or for 2 days and stained with Giemsa.

Analysis of DNA. The 'nuclear' fraction was prepared by lysis of cells with Nonidet P40 (NP40) and centrifugation through 10% sucrose, and DNA was extracted by proteinase K treatment and phenol–chloroform extraction (Preston, 1979*a*; Harris & Preston, 1991). Equilibrium buoyant density gradient centrifugation of DNA was carried out as described previously (Harris & Preston, 1991). Precipitated DNA from individual 35 mm diameter plates or pooled gradient fractions was digested with *Bam*HI and electrophoresed on 0·6% or 0·7% (w/v) agarose gels in TBE (90 mM-Tris, 90 mM-boric acid, 1 mM-EDTA pH 8·3). DNA fragments were capillary transferred to GeneScreen Plus membrane (NEN, Du Pont) in 0·4 M-NaOH, 0·6 M-NaCl and UV-crosslinked to the matrix by irradiation in a Stratalinker (Stratagene). Membranes were incubated at 70 °C in hybridization solution (0·5 M-NaH_2PO_4–Na_2HPO_4 buffer, 7% SDS pH 7·4) for 2–3 h prior to addition of denatured DNA fragments, radiolabelled with ^{32}P by random primer extension (Feinberg & Vogelstein, 1983), in hybridization solution. The probes used were: purified HSV-1 *Eco*RI n, spanning the TK gene; *Bam*HI k, spanning the joint; or a 500 bp *Bfr*I–*Sma*I fragment from the Momulv enhancer (Lang *et al.*, 1983). Hybridization was continued at 70 °C for 20–24 h and membranes were washed once at 70 °C with hybridization solution followed by two washes at 70 °C with 0·2 × SSC/0·1% SDS (1 × SSC is 0·15 M-NaCl, 0·015 M-sodium citrate), each wash taking 45 min. Membranes were washed briefly with deionized water, dried and exposed to X-omat S film (Kodak) for autoradiography using an intensifying screen. Standard DNA for hybridization was extracted from partially purified *in*1820 virus particles released into culture medium. A portion was digested with *Bam*HI and ribonuclease A, extracted with phenol–chloroform, ethanol precipitated and dissolved in deionized water. The DNA concentration was determined spectrophotometrically, and the measured value confirmed by electrophoresis of uncleaved *in*1820 DNA with linearized plasmids of known concentrations.

Quantification of hybridization was achieved by phosphorimage analysis, using Molecular Dynamics ImageQuant software. To calculate genome copy numbers and the relative proportions of linear and non-linear DNA, measured values of band peak areas were first corrected for variations in transfer and hybridization efficiencies of joint and terminal fragments by comparison with *in*1820 DNA standards. If x and y are the corrected values for joint and terminal fragments, respectively, the relative number of linear genomes was y and non-linear genomes $0{\cdot}5(x-y)$. The total number of genomes was given by the sum of linear and non-linear molecules.

Nuclease digestion of nuclei. Nuclei prepared as described above were resuspended in 10 mM-Tris–HCl pH 7·5, 2 mM-$MgCl_2$, 10% (w/v) sucrose, and micrococcal nuclease (MN; Pharmacia P-L) or DNase I (DN-EP; Sigma) added at various concentrations. MN digestion was carried out in 20 mM-PIPES pH 7·0, 0·25 M-sucrose, 10 mM-NaCl, 1 mM-$MgCl_2$, 1 mM-$CaCl_2$, 5 mM-β-mercaptoethanol, 0·1 mM-PMSF. DNase I digestion was carried out in 10 mM-PIPES pH 7·0, 0·25 M-sucrose, 10 mM-NaCl, 3 mM-$MgCl_2$, 5 mM-β-mercaptoethanol, 0·1 mM-PMSF. After incubation at 37 °C for fixed times of 2–30 min, reactions were stopped by addition of ice-cold EDTA to a final concentration of 12 mM. Samples were digested by addition of 0·4 ml 20 mM-Tris–HCl pH 7·5, 2·5 mM-EDTA, 0·5% SDS and 100 μg/ml proteinase K and

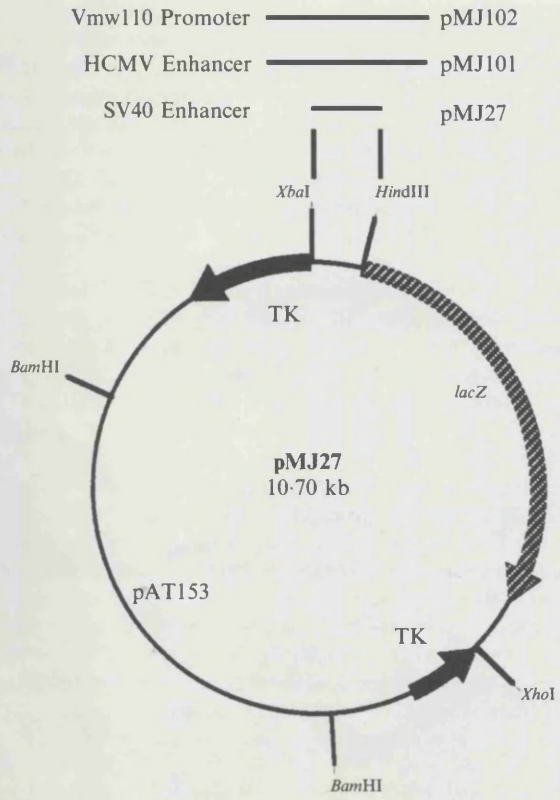

Fig. 1. Structures of pMJ27, pMJ101 and pMJ102.

incubation at 37 °C for 5–16 h. DNA was extracted and either electrophoresed directly on 2% agarose gels or, after cleavage with *Bam*HI, on 0·6% agarose gels. Southern transfer and hybridization was carried out as described above. DNA analysed on 2% gels was partially depurinated by incubation of the gel in 0·25 M-HCl for 15 min prior to transfer.

Analysis of IE RNA. Monolayers of HFL cells were infected with 10 p.f.u. of virus per cell in the presence of 50 μg of cycloheximide/ml. After incubation at 37 °C for 5 h in the continuous presence of cycloheximide, RNA was extracted and analysed by dot-blot hybridization using radiolabelled probes specific for the IE genes or γ-actin, as described previously (Daksis & Preston, 1992).

Histochemical staining for β-galactosidase. Culture medium was removed and monolayers fixed by addition of 1% glutaraldehyde, dissolved in PBS, for 1 h at room temperature. After removal of glutaraldehyde, monolayers were washed twice with PBS, and reaction mixture (5 mM-potassium ferricyanide, 5 mM-potassium ferrocyanide, 2 mM-$MgCl_2$, 0·01% NP40, 1 mg/ml X-Gal, all dissolved in PBS) was added. After incubation for an appropriate time (usually 3–5 h) at 37 °C, monolayers were washed with water. Monolayers were counterstained by addition of Carmalum stain [25 g/l carmine; 25 g/l aluminium potassium sulphate; 2·5% (v/v) glacial acetic acid] and incubation at 4 °C for 1–2 days, washed with water, dried and mounted.

Results

*Properties of mutants in*1820, *in*1883 *and in*1884

As described in the Introduction, we wished to examine the quiescent viral genomes which are retained in HFL cells after infection with *in*1814. We considered that it

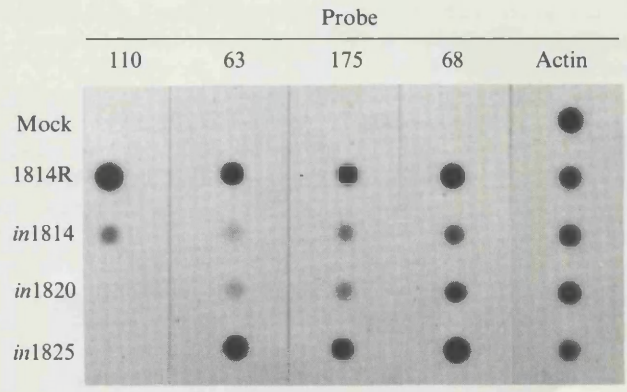

Fig. 2. IE RNA synthesis. HFL cells were infected with 10 p.f.u. of virus/cell or mock-infected, and incubated at 37 °C for 5 h in the presence of 50 μg of cycloheximide/ml. RNA was extracted, 1 μg was applied to nitrocellulose membranes and hybridization with gene-specific probes was carried out.

was essential to meet two criteria: first, for structural studies the viral genome must, realistically, be present at one or more copies per cell; and, second, evidence must be provided that the genomes detected were biologically relevant. In practice, the latter proposition demanded the demonstration that most of the quiescent genomes detected were capable of resuming gene expression in response to an appropriate stimulus. Since there was no model in which these objectives had been attempted or achieved, it was necessary to develop a new cell culture system for the study of quiescent genomes. Extending our unpublished observations, which were in agreement with those reported by Johnson *et al.* (1992, 1994), that production of IE proteins resulted in cell degeneration, we attempted further to reduce IE transcription after infection of HFL cells with *in*1814. The first step involved specifically reducing Vmw110 synthesis, since mutants deleted only for this gene can also persist in a quiescent state (Stow & Stow, 1989). Mutant *in*1820 was derived from *in*1814 by replacement of the Vmw110 gene promoter with the Momulv promoter and enhancer. Additional insertion, at the TK locus, of the *E. coli lacZ* gene controlled by either the HCMV enhancer or the HSV-1 Vmw110 promoter yielded *in*1883 and *in*1884, respectively. When titrated on HFL cells, *in*1820 and its derivatives were more impaired for replication than *in*1814, with virus stocks exhibiting an approximately 10-fold higher particle:p.f.u. ratio (results not shown). This observation was extended by analysis of IE RNA accumulation after infection of HFL cells in the presence of cycloheximide (Fig. 2). As shown previously (Ace *et al.*, 1989; Daksis & Preston, 1992), levels of IE RNAs were lower in *in*1814-infected cells compared with 1814R-infected cells. The amounts of IE RNAs encoding Vmw175, Vmw68 and Vmw63 were similar in *in*1814- and *in*1820-infected cells, but IE RNA encoding Vmw110

was not detectable in *in*1820-infected cells. HFL cells infected with *in*1825, the rescuant of *in*1820 at the Vmw65 locus, also showed no detectable production of Vmw110 RNA. Thus, the Momulv enhancer was not recognized as an IE promoter, with the consequence that *in*1820 behaved as a Vmw65/Vmw110 double mutant in HFL cells. A detailed description of the properties of *in*1820 and *in*1825 will be published elsewhere.

Viruses with mutations in Vmw110 or Vmw65 exhibit low titres in plaque assays because initiation of productive replication is extremely inefficient at low m.o.i. (Stow & Stow, 1986; Sacks & Schaffer, 1987; Ace *et al.*, 1989; Everett, 1989). This causes difficulties in accurately assessing the effective input m.o.i., as discussed by Cai & Schaffer (1992). To overcome this problem, we standardized virus preparations by titration on BHK cells in the presence of 3 mM-HMBA, which allows *in*1814 and *in*1820 to be assayed essentially as wild-type viruses (McFarlane *et al.*, 1992). This procedure provided a sound basis for comparison of virus stocks but, as shown below, a value approximately three times higher was obtained by co-infection with 1 p.f.u. of the HSV-1 mutant *ts*K per cell and subsequent incubation at 37 °C. Co-infection with *ts*K provides both Vmw65 and Vmw110, thereby fully complementing the defects of *in*1820. When 'p.f.u.' was measured by complementation with *ts*K, particle:p.f.u. ratios of *in*1814- and *in*1820-based mutants were equivalent to those of wild-type HSV-1 stocks.

Use of IFN-α pretreatment to reduce IE gene expression

Although the use of *in*1820, instead of *in*1814, increased the number of virus particles that could be added to monolayers, cell degeneration still occurred after infection with 10^6 p.f.u. per plate (approximately 1 p.f.u. per cell). Further reduction of IE gene expression was achieved by pretreatment of cells with IFN-α, a procedure known to inhibit IE transcription and reduce cell killing (Mittnach *et al.*, 1988; Oberman & Panet, 1989; Johnson *et al.*, 1992).

Monolayers were pretreated with 10^3 units of IFN-α/ml for 16–20 h prior to infection with 1814R, *in*1814, *in*1820 or *in*1883 (Table 1). The titre of 1814R was reduced by approximately 7-fold after IFN-α pretreatment. The titre of *in*1814 was reduced to an even greater extent, although application of 10^6 p.f.u. resulted in cell destruction irrespective of IFN-α pretreatment. When infected with 10^6 p.f.u. of *in*1820, however, IFN-α-pretreated monolayers withstood infection and produced only five plaques per plate, whereas at lower m.o.i. no plaques were observed. The use of *in*1883, which permitted subsequent staining for β-gal expression, provided a more sensitive means of detecting viral replication or gene expression but even in this case only 16 'plaques', some of which were single cells, were observed on plates infected with 3×10^5 p.f.u of *in*1883.

Table 1. *Inhibition of HSV replication by* IFN-α *pretreatment*

Virus	Inoculum (p.f.u. per plate)	Plaque numbers* −IFN-α	Plaque numbers* +IFN-α
1814R	1×10^3	CPE†	196
1814R	1×10^2	98	14
*in*1814	1×10^6	CPE	CPE
*in*1814	1×10^5	146	1
*in*1820	1×10^6	CPE	5
*in*1820	3×10^5	CPE	0
*in*1820	1×10^5	38	0
*in*1883‡	3×10^5	CPE	16
*in*1883	1×10^5	72	3
*in*1883	3×10^4	14	0

* Values represent the means of duplicate or triplicate determinations.

† CPE: extensive cytopathic effect prevented estimation of plaque numbers.

‡ Plaques, or single cells, were counted after reaction of *in*1883-infected monolayers with X-Gal.

Pretreatment with IFN-α thus permits the survival of cultures after infection with at least 1 p.f.u. of *in*1820 per cell. Since the titre of *in*1820 after complementation with *ts*K was approximately 3-fold higher than the value measured on BHK cells in the presence of HMBA, it follows that an input m.o.i. of 3×10^5 p.f.u. per monolayer of 8×10^5 cells should deliver an average of at least one genome per cell. Under these circumstances, molecular analysis of the genome should be possible, provided the small number of potentially active genomes (16 after infection with 3×10^5 p.f.u. per plate; Table 1) was not amplified by replication. An inhibitor of DNA replication, AraC, was therefore added after infection of IFN-α-pretreated monolayers.

To determine whether IFN-α pretreatment or the presence of AraC exerted significant influence on the retention of virus, HFL cells were infected with 30 p.f.u. of *in*1883, incubated with or without AraC for 2 days, then superinfected with *ts*K (Table 2). Co-infection with *ts*K resulted in 85 plaques on untreated cultures or 65 on IFN-α-pretreated monolayers. *In*1883 was recovered by superinfection after 2 days, as expected from previous studies (Harris & Preston, 1991); a process referred to as 'reactivation' hereafter. Treatment with AraC did not reduce the reactivation of *in*1883 and IFN-α pretreatment had only a small effect. Crucially, reactivation of *in*1883 from cells treated with both agents (140 plaques) was close to that observed in untreated cells (178 plaques), demonstrating that the combination of IFN-α pretreatment and AraC addition did not significantly affect retention and reactivation of the *in*1883

Table 2. *Complementation of in 1883 by tsK* *

Time of superinfection	AraC	Plaques on monolayers† −IFN-α	Plaques on monolayers† +IFN-α
0 h‡	−	85	65
2 days	−	178	113
2 days	+	174	140

* Monolayers of HFL cells, either pretreated with IFN-α or mock-pretreated, were infected with 30 p.f.u. of *in*1883 and incubated at 37 °C with or without AraC. After 2 days, monolayers were washed and superinfected with 8×10^5 p.f.u. of *ts*K per plate, overlaid with EHu5 and incubated at 37 °C for 24 h. Plaques were identified by expression of β-gal. No plaques were detected on monolayers infected with *in*1883 alone.

† Values represent the means of triplicate determinations.

‡ Monolayers were co-infected with 30 p.f.u. of *in*1883 and 8×10^5 p.f.u. of *ts*K.

Table 3. *Stability of the virus–cell interaction**

Treatment	Plaques per monolayer† mock si	Plaques per monolayer† si *ts*K
Superinfect day 3	0	169
Subculture 1:3 on day 3, superinfect on day 6‡	0	55
Subculture 1:3 on day 6, superinfect on day 9§	0	13

* IFN-α-pretreated HFL cultures were infected with 40 p.f.u. of *in*1883 and maintained at 37 °C for 3 days in the presence of AraC. Monolayers were washed and incubation continued at 37 °C for a further 3 days. Cultures were mock-superinfected (mock si) or superinfected with 8×10^5 p.f.u. of *ts*K per plate (si *ts*K) and maintained at 37 °C for 24 h prior to staining for β-gal.

† Values represent the means of between three and nine determinations.

‡ After 3 days in the presence of AraC, monolayers were washed, trypsinized and replated at one third of the original density. On day 6, cultures were mock-superinfected or superinfected with 8×10^5 p.f.u. of *ts*K per plate and maintained at 37 °C for 24 h prior to staining for β-gal.

§ Monolayers subcultured on day 3 were again subcultured 1:3 on day 6 and superinfected on day 9.

genome. Analogous results were obtained when infections were initiated with *in*1820 or *in*1884 (results not shown). The increase in recovery of virus by superinfection over the 2 day period is thought to be due to gradual uncoating of the viral genome (see below, Fig. 5): low-level replication is unlikely since the presence of AraC at a concentration known to inhibit viral DNA synthesis did not prevent the effect and, furthermore, no increase in DNA levels was detectable over the 2 day period (Fig. 5).

The stability of retention of *in*1820-based viruses was investigated (Table 3). IFN-α-pretreated monolayers were infected with 40 p.f.u. of *in*1883 per plate and, after 3 days in the presence of AraC, monolayers were superinfected with *ts*K or mock-superinfected. No virus replication or expression of β-gal was detected on untreated plates, but superinfection with *ts*K yielded an average of 169 plaques per plate. Subculture and reseeding at 3-fold dilution after 3 days in the presence of AraC did not result in reactivation of *in*1883. Once monolayers had become confluent, superinfection with *ts*K yielded approximately one-third of the initial number of plaques per plate. A further cycle of subculture again failed to reactivate virus, and the number of plaques per plate reactivated by superinfection with *ts*K was again reduced by a factor of approximately three. Therefore, trypsinization, subculture and regrowth of HFL cells did not reactivate quiescent *in*1883, emphasizing that repression of gene expression could not be overcome by the changes in cell metabolism which occur during subculture. There was also no evidence for co-replication with the host cell, since reactivation competent cells were diluted in approximately the same ratio as the total cell population. The *in*1883 genome remained within cells and could be reactivated for at least 9 days after infection.

Retention of the viral genome after infection at high m.o.i.

The information from the previous sections was used to develop a system in which each HFL cell should contain at least one quiescent viral genome. Infection of IFN-α-pretreated monolayers with 30 p.f.u. of *in*1883 yielded 140 plaques after superinfection with *ts*K (Table 2); thus addition of 3×10^5 p.f.u. of *in*1883 should result in almost all cells in a monolayer of 8×10^5 cells receiving, on average, one reactivatable genome. To demonstrate directly the validity of this extrapolation, IFN-α-pretreated monolayers were infected with 3×10^4 p.f.u. or greater of *in*1883 per plate, maintained in the presence of AraC for 2 days and superinfected with *ts*K. To prevent intercellular spread of virus, Brefeldin A was present during superinfection (Cheung *et al.*, 1991). Control experiments showed that in cells treated with Brefeldin A, β-gal and its reaction product were restricted to individual cells (results not shown). After superinfection, monolayers were stained for the presence of β-gal (Fig. 3).

In cultures infected with 3×10^5 p.f.u. of *in*1883 per plate, very few β-gal-expressing cells were observed on mock-superinfected cells (Fig. 3*a*), whereas almost all cells expressed the enzyme after superinfection with *ts*K (Fig. 3*b*). When the initial inoculum was reduced to 1×10^5 p.f.u. per plate (Fig. 3*c*), approximately 95% of cells expressed β-gal after superinfection, and at 3×10^4 p.f.u. per plate (Fig. 3*d*) the proportion of β-gal-expressing cells was approximately 33%, as determined by counting on colour photographs of stained cells. Very similar results were obtained after initial infection with

Table 4. *Relationship between genome copy number and reactivatable virus*

*in*1883 (p.f.u. per plate)	Genomes retained after 3 days*	β-Galactosidase-expressing cells†		Genomes per reactivating cell
		mock si	si *ts*K‡	
3×10^4	$3{\cdot}7 \times 10^5$ (2)§	0·6 (6)§	$2{\cdot}7 \times 10^5$	1·4
1×10^5	$1{\cdot}0 \times 10^6$ (4)	5·6 (6)	$7{\cdot}6 \times 10^5$	1·3
3×10^5	$2{\cdot}3 \times 10^6$ (2)	32·2 (6)	$8{\cdot}0 \times 10^5$	2·9

* Data from Southern blots, as shown in Fig. 4, were used to calculate genome copy number in IFN-α-pretreated HFL cell cultures infected with *in*1883. The results were correlated with analysis of β-gal-expressing cells, as shown in Fig. 3.

† Monolayers infected with *in*1883 were stained for β-gal after mock-superinfection (mock si) or superinfection with 8×10^5 p.f.u. of *ts*K per plate (si *ts*K) in the presence of Brefeldin A.

‡ Calculated from the percentage of cells staining for β-gal and the measured average cell density of 8×10^5 cells per monolayer.

§ Mean value, number of determinations in brackets.

*in*1884 (Fig. 3*e–f*). In addition, superinfection with 1814R (essentially wild-type HSV-1) and incubation for 7 h in the presence of Brefeldin A, or AraC, reactivated *in*1883 or *in*1884 as efficiently as superinfection with *ts*K (results not shown).

The extrapolation from results obtained at low m.o.i. was therefore verified directly. It was possible to produce cultures with each cell containing at least one reactivatable genome and with the background of cells harbouring potentially replicative genomes contributing less than 0.01% of the population. The results also demonstrate that the HCMV enhancer and Vmw110 promoter were not detectably active, except in a small number of cells.

Structure and copy number of DNA

Previous experiments showed that viral DNA was sequestered as a non-linear molecule when HFL cells were infected with *in*1814 at an m.o.i. of approximately 0·1 p.f.u. per cell and subsequently maintained in the presence of AraC or aphidicolin (Harris & Preston, 1991). The configuration of viral DNA was therefore investigated after infection of IFN-α-pretreated HFL cells with 10^6 p.f.u. of *in*1820 per plate and maintenance in the presence of AraC. DNA was extracted from cell nuclei, cleaved with *Bam*HI and enriched for viral sequences by equilibrium bouyant density gradient centrifugation. Hybridization with the joint-spanning *Bam*HI k fragment from HSV-1 revealed a preponderance of joint fragment after 1 day in culture (Fig. 4*a*, lane 1) and an almost complete absence of termini after 2 days (lane 2), demonstrating conversion to a non-linear form.

To obtain an estimate of viral genome copy number, DNA was extracted from nuclei of cells pretreated with IFN-α, infected with 3×10^4 or 1×10^5 p.f.u. of *in*1883 and maintained in the presence of AraC for 7 h or 3 days. Hybridization was carried out on unfractionated DNA using a radiolabelled DNA fragment from the Momulv enhancer, thereby avoiding problems of cross-hybridization with cellular sequences which can occur with HSV joint-spanning fragments (Fig. 4*b*). This probe detected the joint-spanning fragment, the L terminus and minor species containing additional 'a' sequences, and a minor band of unknown origin smaller than the L terminus. The results again demonstrated that the DNA was predominantly non-linear (Fig. 4*b*, lanes 6 and 7). Quantification was carried out on the data shown in Fig. 4(*b*) and on four independent experiments, and the total amount of viral DNA was compared with the number of reactivation competent cells as estimated from proportions of β-gal-containing cells after superinfection with *ts*K (Table 4). After infection with 3×10^4 and 1×10^5 p.f.u. of *in*1883 per plate, the situations in which β-gal expression is proportional to input m.o.i., cultures retained, respectively, $3{\cdot}7 \times 10^5$ and $1{\cdot}0 \times 10^6$ *in*1883 DNA molecules per monolayer of 8×10^5 cells. The estimated numbers of β-gal-expressing cells upon superinfection with *ts*K were $2{\cdot}7 \times 10^5$ (33%) and $7{\cdot}6 \times 10^5$ (95%), as derived from experiments represented in Fig. 3; thus cultures contained 1·3–1·4 genomes per reactivated cell. Most genomes detectable at 3 days after infection were therefore competent for reactivation, as measured by expression of β-gal after superinfection.

This conclusion is crucial because it can be stated with certainty that the genomes detected biochemically were templates for reactivation. As a consequence, studies on the structure of the quiescent genome in HFL cells and the changes that occur upon reactivation will be meaningful since they pertain to biologically relevant material. It is important, therefore, to define the limits within which the conclusion is based. The values presented rely on calculations that are individually subject to error, but over a number of experiments using 1×10^5 or fewer p.f.u. of *in*1883 and, to a lesser extent, *in*1884, the genome copy per reactivating cell varied only between 1·0 and 2·0. The proportion of non-linear genomes at 3 days post-infection ranged between 70%

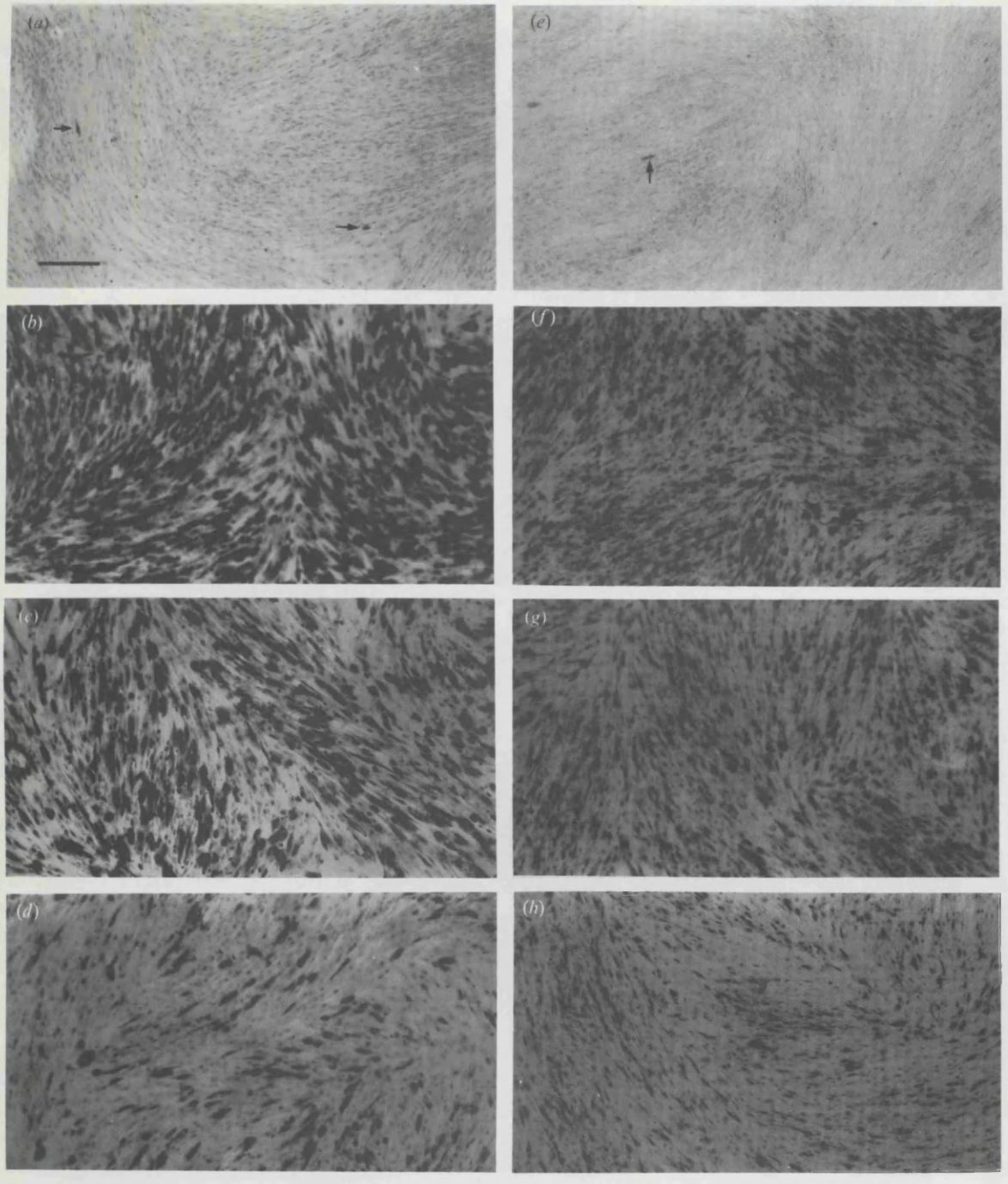

Fig. 3. Expression of β-gal after superinfection. Cultures were pretreated with IFN-α and infected with *in*1883 or *in*1884 for 48 h in the presence of AraC. Monolayers were stained for β-gal 24 h after mock-superinfection (*a*, *e*) or superinfection with 8×10^5 p.f.u. of *ts*K per plate (*b*, *c*, *d*, *f*, *g*, *h*) in the presence of 2·5 μg/ml Brefeldin A. Cultures were initially infected with *in*1883 at 3×10^5 (*a*, *b*), 1×10^5 (*c*) or 3×10^4 (*d*) p.f.u. per plate, or *in*1884 at 3×10^5 (*e*, *f*), 1×10^5 (*g*) or 3×10^4 (*h*) p.f.u. per plate. Cells expressing β-gal on mock-superinfected plates (*a*, *e*) are labelled with arrows. Bar represents 120 μm.

and > 95%, thus the number of non-linear genomes per reactivating cell was, in some cases, lower than presented in Table 4 and closer to one.

Comparison of β-gal-expressing cell numbers (Table 4) with values for plaque formation (Table 2) revealed a discrepancy: after superinfection with *ts*K, cultures infected with 3×10^4 p.f.u. of *in*1883 yielded approximately $2{\cdot}7 \times 10^5$ β-gal-expressing cells yet an input of

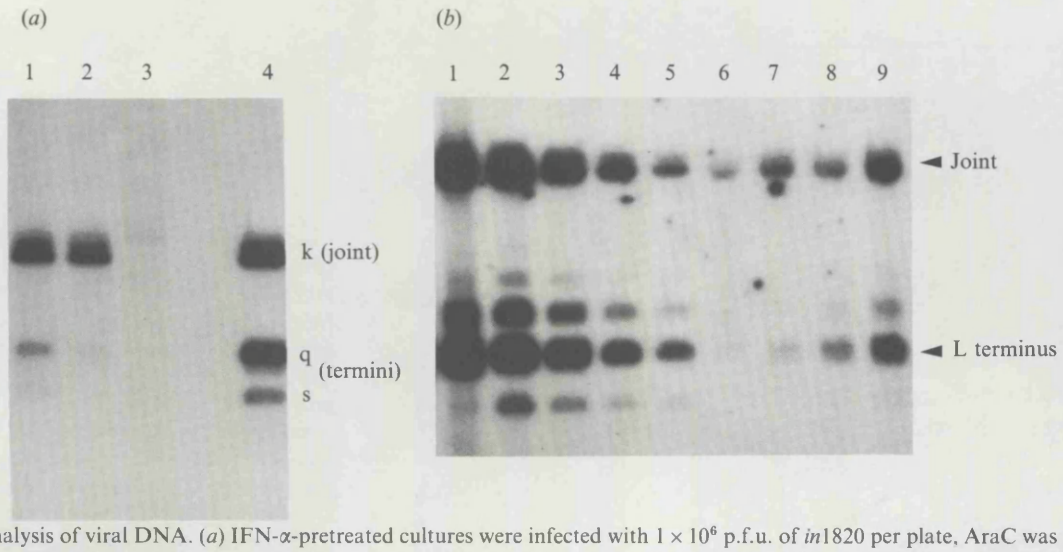

Fig. 4. Analysis of viral DNA. (*a*) IFN-α-pretreated cultures were infected with 1×10^6 p.f.u. of *in*1820 per plate, AraC was added, and DNA was extracted after 1 (lane 1) or 2 (lane 2) days. Viral DNA was cleaved with *Bam*HI and partially purified by equilibrium buoyant density gradient centrifugation prior to Southern transfer and hybridization, using radiolabelled HSV-1 *Bam*HI k fragment as probe. Mock-infected cell DNA of equivalent density (lane 3), and 1·5 ng of *in*1820 DNA (lane 4) were also analysed. (*b*) IFN-α-pretreated cultures were infected with 3×10^4 (lanes 6 and 8) or 1×10^5 (lanes 7 and 9) p.f.u. of *in*1883 per plate and nuclear DNA was extracted at 7 h (lanes 8 and 9) or 3 days (lanes 6 and 7) post-infection. Standards consisted of 3×10^7 (4·5 ng, lane 2), 1×10^7 (lane 3), 3×10^6 (lane 4) or 1×10^6 (lane 5) copies of *in*1820 DNA. DNA extracted from 1×10^5 p.f.u. of *in*1820 was applied to lane 1. The probe was a Momulv-specific fragment.

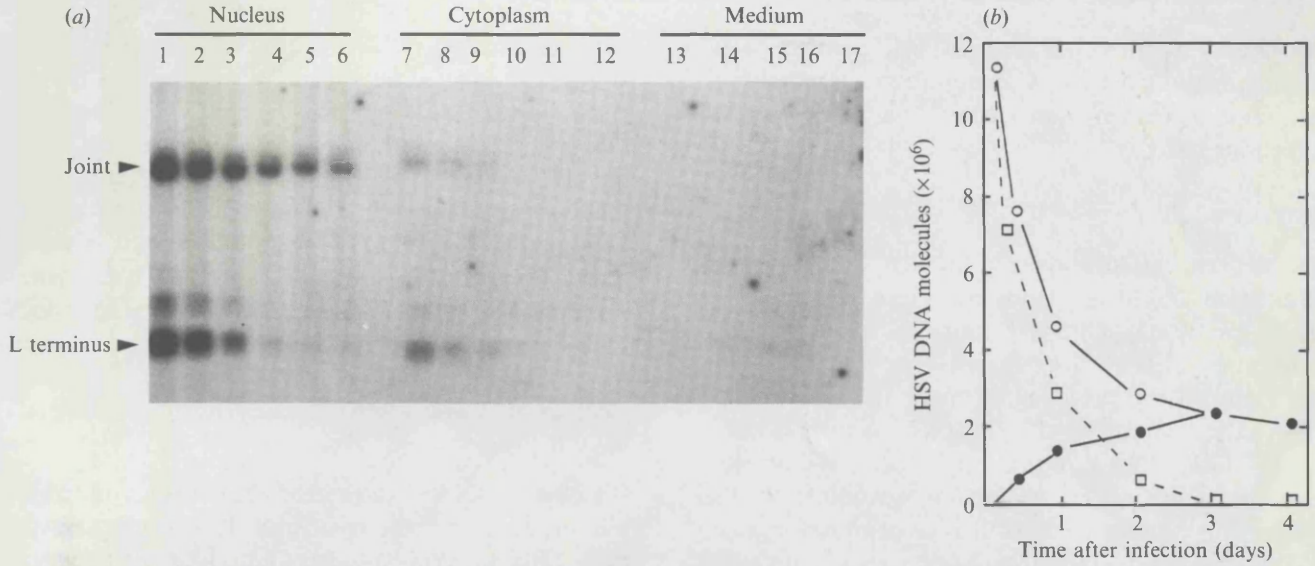

Fig. 5. Fate of *in*1883 DNA. (*a*) IFN-α-pretreated cultures were infected with 3×10^5 p.f.u. of *in*1883 per plate and DNA was extracted from the nuclear fraction (lanes 1–6), NP40 supernatant plus sucrose wash (lanes 7–12) or culture medium (lanes 13–17) at 2 h (lanes 1, 7 and 13), 7 h (lanes 2, 8 and 14), 1 day (lanes 3, 9 and 15), 2 days (lanes 4, 10 and 16), 3 days (lanes 5, 11 and 17) or 4 days (lanes 6 and 12) after infection. The probe was a Momulv-specific fragment. (*b*) The signals from lanes 16 were quantified by phosphorimage analysis, and the amounts of total (○), linear (□) and non-linear (●) *in*1883 DNA plotted.

30 p.f.u. per plate resulted in approximately 100 plaques (140 in the experiment described in Table 2), instead of the 270 expected. This observation was found to be due to a suppressive effect of the human serum used to prevent virus spread during virus titration. Cultures were co-infected with 30 p.f.u. of *in*1883 and 8×10^5 p.f.u. of *ts*K, overlaid with various combinations of serum and Brefeldin A, and incubated at 37 °C for 24 h prior to staining for β-gal. Monolayers overlaid with EHu5 alone yielded 73 plaques (mean of triplicate determinations), whereas those overlaid with EF5 containing 2·5 μg/ml Brefeldin A resulted in 151 stained cells. EHu5 containing Brefeldin A gave 57 stained cells. The presence of human serum lowered the 'titre' by a factor of more than two, by an unknown mechanism, thereby accounting for the differences between the reactivation efficiencies derived

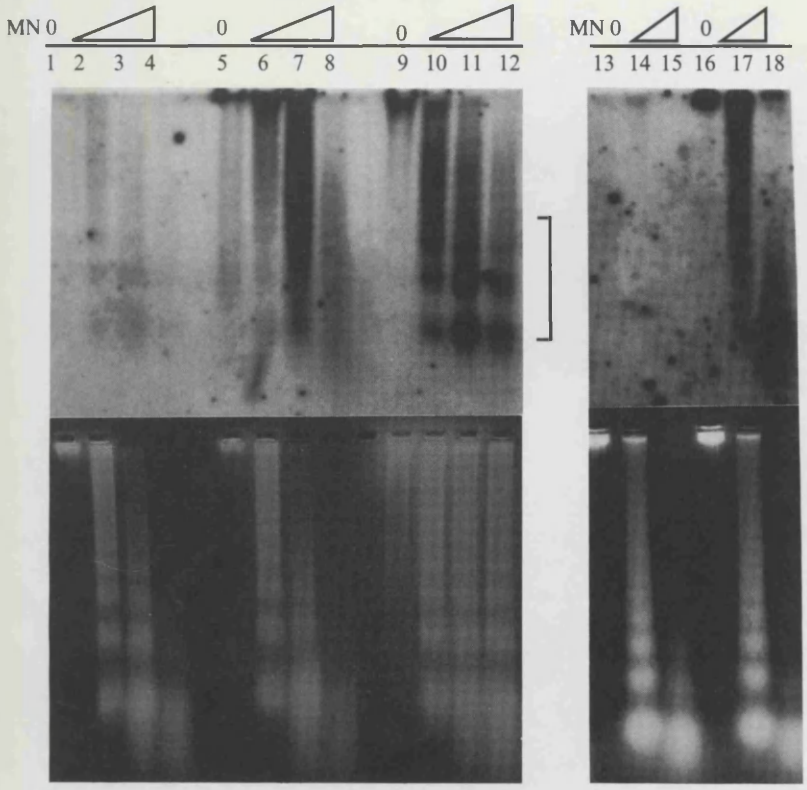

Fig. 6. Digestion of viral DNA with MN. IFN-α-pretreated cells were infected with 1×10^6 p.f.u. of *in*1820 per plate and nuclei prepared after 3 days. Nuclei were digested with MN, DNA was extracted and electrophoresed on a 2% (w/v) agarose gel. The probe was *Eco*RI n, spanning the TK gene. In lanes 1–8, AraC was added after infection, whereas in lanes 13–18 5 μM-acyclovir was added. DNA was from mock-infected cells (lanes 1–4 and 13–15), latently infected cells (lanes 5–8 and 16–18) or 143 TK^- cells transformed with pTK1 (lanes 9–12). Nuclei were treated at 37 °C with no MN (lanes 1, 5, 9, 13 and 16), 7.5 units for 2 min (lanes 2, 6 and 10), 22·5 units for 10 min (lanes 3, 7 and 11), 45 units for 30 min (lanes 4, 8 and 12), 5 units for 2 min (lanes 14 and 17), or 10 units for 10 min (lanes 15 and 18). Lower panels show ethidium-bromide-stained gels, upper panels show autoradiographs. Nucleosomal DNA fragments are bracketed.

from plaque numbers or stained cell counts. 'Reactivation', as used in Table 4, therefore strictly applies to activation of gene expression rather than the production of infectious virus.

Fate of input DNA

The experiment shown in Fig. 4(*b*) also includes hybridization to DNA from an input inoculum of 1×10^5 p.f.u. of *in*1883 (lane 1) and DNA present at 7 h after infection of IFN-α-pretreated cells with 3×10^4 and 1×10^5 p.f.u. in the presence of AraC (lanes 8 and 9). Over five determinations, 1×10^5 p.f.u. corresponded to $1{\cdot}07 \times 10^7$ input DNA molecules, of which $2{\cdot}9 \times 10^6$ were detected in the nuclear fraction at 7 h post-infection. The 7 h value thus exceeded the number of genomes retained after 3 days (1×10^6), and an explanation was sought for this unexpected finding.

IFN-α-pretreated cells were infected with 3×10^5 p.f.u. of *in*1883, washed twice with EF5 after 1 h at 37 °C, and overlaid with EF5 containing AraC. At various times after addition of virus to cells, monolayers were harvested and nucleic acids extracted from the nuclear fraction, the NP40 supernatant plus 10% sucrose wash of crude nuclei (cytoplasmic fraction), and from the culture medium. Nucleic acids were cleaved with *Bam*HI plus ribonuclease A and analysed by Southern hybridization (Fig. 5*a*). Viral DNA was predominantly in the nuclear fraction at all times, although some linear molecules were found in the cytoplasmic fraction up to 1 day after infection. No viral DNA was detected in the culture medium at any time. When the data from Fig. 5(*a*) were expressed graphically (Fig. 5*b*), it was found that the total amount of *in*1883 DNA fell by approximately 3-fold during the first 2 days of infection, but that the number of non-linear genomes present at 2 days remained stable. Input DNA not converted to the non-linear form during the course of the experiment was presumably degraded, since it could not be recovered in the cytoplasmic fraction or growth medium.

Regular nucleosome arrangement cannot be detected at the TK locus

Cellular DNA is organized into chromatin, of which the nucleosome is the basic unit. It has been shown that HSV DNA forms a chromatin-like structure when sequestered in mouse brain stem (Deshmane & Fraser, 1989). To investigate whether *in*1820 DNA was organized similarly in HFL cells, nuclei from IFN-α-pretreated cells infected with *in*1820 and maintained in the presence of AraC were partially digested with MN, the fragments separated by electrophoresis, and hybridization carried out using radiolabelled *Eco*RI n from the TK gene (Fig. 6). As a control, nuclei from human 143 TK^- cells stably transformed to a TK^+ phenotype by transfection of the HSV-1 TK gene were analysed in parallel.

Examination of ethidium-bromide-stained gels demonstrated that cellular chromatin was digested to the characteristic 'ladder' of DNA fragments. When viral

DNA was examined by hybridization, however, material of heterogeneous sizes was observed, with no evidence for the presence of regularly spaced nucleosomes (Fig. 6, lanes 5–8). The HSV-1 TK gene, when present in 143 TK^- cells, was organized into chromatin (Fig. 6, lanes 9–12), demonstrating that there are no intrinsic features of the TK gene which preclude the formation of regularly packaged nucleosomes.

Maintenance of cells in the presence of AraC might have reduced cellular histone pools due to inhibition of DNA synthesis, thereby preventing the formation of properly spaced nucleosomes on viral DNA. IFN-α-pretreated, *in*1820-infected HFL cells were therefore treated with 5 μM-acyclovir, which specifically inhibits HSV DNA synthesis. Nuclear DNA was analysed after digestion with MN (Fig. 6, lanes 13–18), but again no evidence was obtained for a chromatin-like structure of the *in*1820 genome. Similar results were obtained when phosphonoacetic acid was used to prevent HSV DNA replication (results not shown).

These experiments show that the *in*1820 genome, at the TK locus, was not arranged into the regular structure characteristic of cellular chromatin.

Non-linear DNA is a template for reactivation

Superinfection of cultures resulted in activation of expression from the HCMV enhancer (in *in*1883) and the Vmw110 promoter (in *in*1884) (Fig. 3). To investigate whether reactivation was accompanied by alteration of the non-linear genome configuration, IFN-α-treated monolayers were infected with *in*1820, maintained for 2 days in the presence of AraC and superinfected with 1814R in the continuous presence of AraC. At 8 h after infection, nuclear DNA was prepared, cleaved with *Bam*HI and analysed by Southern hybridization, using the probe specific for the Momulv enhancer (Fig. 7). Reactivation did not result in a change in the relative proportions of non-linear and linear molecules (lanes 1 and 2), even though this process was known to activate gene expression. The non-linear genome was therefore the template for reactivation, and conversion to a linear form was not a prerequisite for this event to occur. In addition, the residual linear DNA remained linear throughout the 8 h period after infection.

Linear DNA represents non-uncoated genomes

Even after 2–3 days in the presence of AraC a small amount of *in*1820 DNA was linear (Figs 4, 5 and 7). To determine the nature of this material, nuclear DNA was prepared from HFL monolayers pretreated with IFN-α, infected with *in*1820 and incubated for 2 days in the presence of AraC. Nuclei were incubated with MN or

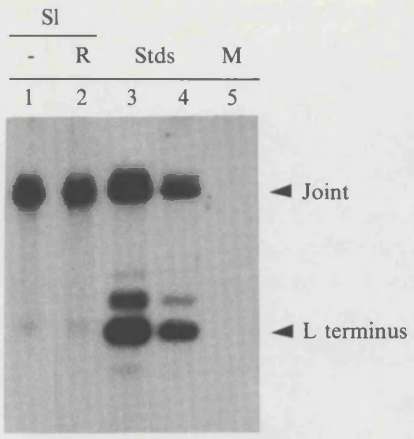

Fig. 7. Structure of viral DNA after reactivation. IFN-α-pretreated cultures were infected with $1{\cdot}5 \times 10^6$ p.f.u. of *in*1820 per plate and after 3 days cultures were mock-infected or infected with 5 p.f.u. of 1814R per cell and incubated at 37 °C for 8 h in the presence of AraC. DNA was extracted, cleaved with *Bam*HI, followed by hybridization using a Momulv-specific probe. Lane 1, mock-superinfected cells; lane 2, cells superinfected with 1814R; lane 5, uninfected cells infected after 3 days with 1814R. Lanes 3 and 4 show hybridization to 3×10^7 and 1×10^7 copies of *in*1820 DNA, respectively.

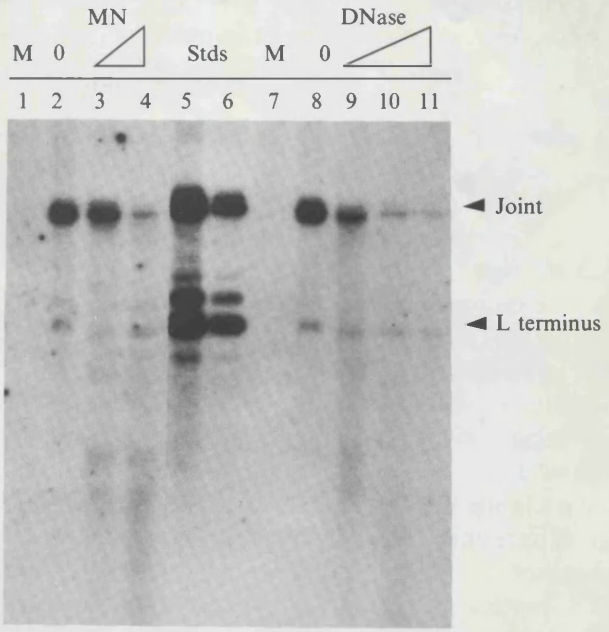

Fig. 8. Nuclease sensitivity of the viral genome. IFN-α-pretreated cultures were infected with $1{\cdot}5 \times 10^6$ p.f.u. of *in*1820 per plate and nuclei were digested with MN or DNase I. DNA was purified, cleaved with *Bam*HI and electrophoresed. The probe was a Momulv-specific fragment. DNA from mock-infected cells is shown in lanes 1 and 7. DNA from latently infected cells was digested with no enzymes (lanes 2 and 8), MN at 1 unit for 3 min (lane 3) or 3 units for 5 min (lane 4), or DNase I at 1 unit for 3 min (lane 9), 2 units for 5 min (lane 10) or 5 units for 20 min (lane 11). Lanes 5 and 6 show hybridization to 3×10^7 and 1×10^7 copies of *in*1820 DNA, respectively.

DNase I, and DNA was extracted, cleaved with *Bam*HI and analysed by Southern hybridization, using the Momulv-specific fragment as probe (Fig. 8). As digestion

proceeded, hybridization to the joint-spanning fragment decreased more sharply than to the terminal fragment until the residual, essentially nuclease resistant, viral DNA (lanes 4 and 11) was composed only of the linear form with joint and terminal fragments essentially equimolar. This result demonstrates that the small amount of linear DNA remaining in cells at 2–3 days after infection presumably originated from particles that had not undergone full uncoating and therefore protected the DNA from the action of nucleases.

Discussion

We describe the development of a cell culture system in which the HSV genome was stably retained in a quiescent state in human fibroblasts at high efficiency without cell destruction. The crucial feature is the incorporation of steps designed to reduce IE gene expression, namely the use of viruses with mutations in Vmw65 and the Vmw110 promoter together with pretreatment with IFN-α. It appears that cytotoxicity during nonpermissive infection with HSV-1 (strain 17) was due mainly to expression of IE proteins and that virus structural components, including the vhs factor, were not deleterious at the multiplicities used. This conclusion agrees with that reached in studies by Johnson *et al.* (1992, 1994), who also reported that pretreatment with IFN-α reduced cytotoxicity by HSV-1. Reactivation by superinfection was almost as efficient in IFN-α-pretreated cultures as in untreated cultures (Table 2). For investigation of genome structure, which realistically demands the presence of at least one genome per cell, it was necessary to include an inhibitor of DNA synthesis to prevent replication, and hence amplification, of the low background of potentially replicative virus. The origin of this virus is unclear: a small population of cells may be inherently more permissive for *in*1820, or a proportion of cells may receive a larger number of virus particles (possibly due to aggregation of virions) and thus overcome the block to replication. It is important to emphasize that the use of IFN-α pretreatment and AraC did not fundamentally affect the interaction of *in*1820 with the host cell; these agents merely enabled the system to be adapted for use at an m.o.i. of 1 p.f.u. or greater per cell and thus for analysis of the viral genome.

Pretreatment of cells with IFN-α inhibited plaque formation by *in*1814 more severely, in percentage terms, than 1814R (Table 1). The blocks to IE transcription imposed by the Vmw65 mutation and IFN-α pretreatment were thus additive in their effects on virus replication, a result apparently at variance with the view that IFN-α pretreatment inhibits transactivation by Vmw65 (DeStasio & Taylor, 1990). If this were the case, it might be expected that *in*1814 and related viruses with impaired transactivation would be resistant to the action of IFN-α. Two possible explanations exist. The insertion mutation in the *in*1814 genome may not completely inactivate Vmw65, leaving a residual activity which is the target for IFN-α. Although we have failed to detect activity of the mutant Vmw65 in functional assays *in vivo* or *in vitro* (Ace *et al.*, 1988, 1989), it remains possible that it can form an unstable complex with Oct-1 and HCF in the infected cell and hence activate IE transcription to some extent. Alternatively, IFN-α may reduce IE transcription in a manner that does not involve Vmw65. This possibility is implied by the observation that expression from the HCMV enhancer, which is active under IE conditions, is also inhibited by IFN-α pretreatment (Johnson *et al.*, 1992; C. M. Preston, unpublished observations). Perhaps IFN-α prevents the onset of IE transcription in a manner analogous to the effect of the agent on SV40 early transcription (Brennan & Stark, 1983).

During the quiescent state, expression of β-gal from the HCMV enhancer and Vmw110 promoter could not be detected in the vast majority of cells, indicating that these elements were not active. In contrast, enzyme was readily detected in the few cells which presumably had produced sufficient amounts of IE proteins. The block to IE gene expression could easily account for the absence of transcription from the remainder of the viral genome since early and late genes are inactive without IE proteins. The HCMV enhancer (present in *in*1883) is, however, expressed in the absence of IE proteins and is not dependent upon functional Vmw65 (C. M. Preston, unpublished observations). The lack of detectable β-gal expression in *in*1883-infected cells implies that the block to gene expression also applies to the HCMV enhancer and thus may not be strictly promoter-specific. Once established, repression of gene expression is stable since genomes remain quiescent even during cell subculture. The cell-cycle related factor(s) which mimic Vmw110 action (Cai & Schaffer, 1991) or influence IE transcription (Daksis & Preston, 1992; Ralph *et al.*, 1994) are thus unable to reactivate quiescent genomes from *in*1820-based viruses.

No similar studies on the fate and structure of HSV DNA in the absence of IE transcription have been carried out, thus it is difficult to make detailed comparisons with the work of others. B. L. Wigdahl and colleagues pretreated HFL cells with IFN-α and found that infection with HSV-1 at up to 2·5 p.f.u. per cell could be tolerated without cell degeneration. Following incubation for 7 days in the presence of an inhibitor of viral DNA synthesis, virus could not be detected provided cultures were maintained at a supraoptimal temperature of 39·5–40·5 °C (Wigdahl *et al.*, 1982). Temperature downshift to 37 °C resulted in virus

replication. Linear HSV genomes were found in cultures (Wigdahl *et al.*, 1984), in contrast to the findings reported here. From our studies, the presence of linear DNA implies that the genome was present in virus particles that had not been fully uncoated or had been packaged *de novo* into nucleocapsids. The problem in interpreting the data of Wigdahl *et al.* (1984) is that cultures contained more than 0.5 genomes per cell, but not more than 3% of cells produced virus, even when reactivation was induced by superinfection (Wigdahl *et al.*, 1982, 1984). It was therefore not possible to state that the linear genomes were templates for reactivation since a small proportion of non-linear molecules would not have been detected. In our experiments, the close correlation between reactivation and copy number demonstrates that non-linear DNA is the relevant form.

During latency in animals and humans, HSV DNA in the peripheral and central nervous systems is non-linear (Rock & Fraser 1983; Efstathiou *et al.*, 1986), and conversion of input genomes to a non-linear form seems to occur in the absence of viral DNA replication (Slobedman *et al.*, 1994). In addition, genomes extracted from the brain-stem were found to be packaged into a chromatin-like structure (Deshmane & Fraser, 1989). While these data clearly describe the structure of the major forms of the latent genome, the DNA copy number per latently infected cell again far exceeds the number of cells from which virus can be reactivated. In the peripheral nervous system, it has been estimated that there are tens or hundreds of genomes per latently infected neuron, yet virus can be reactivated from only a proportion of neurons (Rock & Fraser, 1983; Efstathiou *et al.*, 1986; Simmons *et al.*, 1992; Kosz-Vnenchak *et al.*, 1993; Ecob-Prince & Hassan, 1994). HSV can be recovered from the central nervous system only at very low efficiency (Roizman & Sears, 1987); thus it is not possible to be certain that the chromatin-like genomes are able to reactivate.

Surprisingly, full uncoating of the *in*1820 genome was slow, only reaching completion 2–3 days after infection. The existence of large numbers of non-uncoated genomes has been recognized previously (Jacob & Roizman, 1977), but long-term examination of the progression to uncoating of all genomes has not been possible due to viral replication and/or cell destruction. The early block imposed by inhibition of IE transcription has enabled us to demonstrate that almost all genomes that remain in cells eventually become uncoated. It should be noted that the rate of uncoating of strain 17-derived viruses in HFL cells was slower than found in other cell types (Poffenberger & Roizman, 1985; L. H. Robinson, unpublished observations), but that neither IFN-α pretreatment nor the insertion mutation in Vmw65 affected the process (Oberman & Panet, 1989; L. H. Robinson, unpublished observations). A large proportion (up to 80%) of genomes which become associated with cells are lost, presumably due to degradation since they could not be detected in cytoplasm or culture medium (Fig. 5*a*, *b*). The way in which viral DNA is degraded and the apparently selective resistance of non-linear molecules is unclear at present, and we are not aware of any comparable studies using different cell types and virus strains.

At the structural level, our results indicate that the *in*1820 genome undergoes some of the events that normally occur prior to DNA synthesis. Circularization of the genome occurs even in the absence of protein synthesis (Poffenberger & Roizman, 1985). The acquisition of sensitivity to MN and the existence of a structure unlike chromatin has been described previously during productive infection (Leinbach & Summers, 1980; Muggeridge & Fraser, 1986), although the most abundant form of DNA was resistant to nuclease digestion. Nonetheless, the genome we detect is transcriptionally inactive, and the basis for the block is unclear. Non-linearity *per se* was not the primary reason for the absence of gene expression, since non-linear molecules were templates for superinfection-induced reactivation, but circularization may be required to initiate further structural changes involved in silencing the genome. Formation of a tight chromatin structure, at least at the TK locus, does not occur and we have found no CpG methylation of the CCGG sites in the β-gal gene of *in*1883 (C. M. Preston, unpublished observations). Future studies will use the approaches described here to investigate the overall organization of the quiescent viral genome and to examine specific regions in detail.

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Summary of *in*1814 and *in*1820-based viruses.

Virus	Vmw65 transactivation	IE-1 promoter	HCMV-*LAC Z*	Reference
*in*1814	−	wild type	−	Ace *et al.* (1989).
1814R	+	wild type	−	Ace *et al.* (1989).
*in*1853	−	wild type	TK locus	C.M.Preston, unpublished data.
*in*1863	+	wild type	TK locus	C.M.Preston, unpublished data.
*in*1820	−	MMLV LTR	−	Jamieson *et al.* (1995).
*in*1825	+	MMLV LTR	−	Jamieson *et al.* (1995).
*in*1883	−	MMLV LTR	TK locus	Jamieson *et al.* (1995).
*in*1830	−	MMLV LTR	U_L43	C.M.Preston, unpublished data.