STRUCTURAL STUDIES ON THE HERPES SIMPLEX VIRUS TYPE 1 GENOME DURING LATENCY IN TISSUE CULTURE CELLS

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Unless otherwise stated the results were obtained by the author's own efforts.

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SUMMARY

Herpes simplex virus (HSV) has the pathogenic property of establishing latency during primary infection of man. Following the primary infection at a peripheral site, virions travel via the axons to the neuronal cell bodies in sensory or autonomic ganglia where latency is established and maintained. The virus can be reactivated, return down the axon to the periphery, replicate, be shed and transmitted to other individuals. This gives rise to clinically important recurrent disease, namely herpes labialis, keratitis, uveitis and genitalis. Reactivation is of increasing importance in immunocompromised individuals. Encephalitis, presumed to be due to reactivation of latent virus from the trigeminal ganglion, is the most serious manifestation with a mortality rate of about 50% if untreated and considerable morbidity in half of the survivors.

The infecting virions possess the tegument protein Vmw65 which *trans*-activates the immediate early (IE) genes, thereby initiating the productive cycle. This cycle displays a co-ordinated sequential expression of IE, early and late genes. The IE gene products are important in the regulation of further viral gene expression and may determine whether a neurone supports a replicative or latent infection.

The HSV-1 mutant *in*1814 has a mutation in the gene encoding Vmw65, with a consequent reduction in IE mRNA and hence proteins in cells infected with this virus. On infection of human foetal lung (HFL) fibroblasts at a multiplicity of infection (moi) of 5 particles per cell, *in*1814 readily establishes latency rather than undergoing lytic infection. At this moi 10% - 20% of cells retain at least one latent genome. To investigate the latent genome at the molecular level however, it was considered desirable that at least one if not more copies should be retained per cell. The *in vitro* latency system has been modified to enable a higher genome copy number to be attained.

The addition of more than 5 - 10 particles of *in*1814 per HFL cell causes degeneration of the monolayer even when lytic infection is blocked by the presence of cytosine arabinoside, an inhibitor of DNA replication. It was reasoned that further measures to reduce IE protein levels after infection might reduce the cytopathic effect and thus enable a higher moi to be used.

IE gene expression was further reduced firstly, by replacement of the normal Vmw110 promoter in *in*1814 with the murine Moloney leukaemia virus enhancer to produce *in*1820 and secondly, by pretreatment of HFL cells with human lymphoblastoid interferon alpha (IFN-α), a known inhibitor of IE gene transcription. It was possible to add 100 particles of *in*1820 per cell to IFN-α treated cultures and retain an intact monolayer with only 5 plaques forming. The additive effect of the mutation to produce *in*1820 and IFN-α pretreatment thus allows a high initial moi to be tolerated by the monolayer.

Previous investigators have shown that reactivation of *in*1814 requires the presence of Vmw110. The temperature sensitive mutant *ts*K, which expresses Vmw110 at the nonpermissive temperature, was found to reactivate the double mutant *in*1820 when superinfected into IFN-α pretreated HFL cells harbouring the latent virus. This was as efficient as reactivation of *in*1814 from untreated cells.

In vivo latent HSV-1 DNA is non-linear and, with the exception of the latency associated transcripts, it is transcriptionally silent. The latent genome is therefore very different in terms of its physical structure and transcription pattern to the productively infecting DNA. In the modified *in vitro* latency system progression from the linear to the non-linear configuration over 48 hours was demonstrated by the gradual disappearance of the terminal sequences of the infecting DNA with persistence of the joint sequences. Using techniques that are able to resolve large DNA molecules, further characterisation of this latent non-linear genome was undertaken.

A probe from the thymidine kinase (TK) gene was able to specifically detect latent viral DNA in the *in vitro* latency system in the presence of host cell DNA . Using this probe and comparing hybridisation to an *in*1820 DNA standard the amount of viral DNA from a latently infected monolayer was at least 15 viral genomes per host cell.

Reactivation of latent genomes was demonstrated by the expression of the *lacZ* gene inserted into the mutant virus. Cells expressing the enzyme product β-galactosidase became blue when provided with the appropriate substrate. Superinfection with wild type virus was used to induce reactivation and the spread of reactivated virus from cell to cell, which would normally destroy the monolayer, prevented by brefeldin A. A cell that stains blue must therefore have contained a silent latent genome that had become transcriptionally active. It was shown that the majority of the cells in the monolayer possessed a latent genome that could be reactivated. A

superinfecting virus in which the Vmw110 gene has been deleted, *dl*1403, was unable to activate the latent genome.

Previous studies have shown that cellular DNA exists in close association with a skeletal nuclear substructure, the nuclear matrix or cage. Nuclear matrix preparations from latently infected HFL cells were made, and it was demonstrated that the viral genome was closely associated with this structure and co-purified with it.

The chromatin structure, characteristic of all eukaryotic DNA, can be visualised by the formation of a ladder of multimeric units of nucleosomes when digested with micrococcal nuclease. Nucleosomes, and by inference a chromatin structure, were shown in cells transformed with the HSV-1 TK gene but, surprisingly, this was not true of the latent viral genome. Further studies with micrococcal nuclease showed that the latent genome was more sensitive to digestion. This sensitivity did not alter when the genome was reactivated by superinfection and the superinfecting virus remained relatively resistant to digestion even when prevented from replicating despite the fact that it was transcriptionally active.

This research has developed an *in vitro* model of HSV-1 latency and has permitted some structural analysis of the latent genome. It is a suitable model for the further investigation of latency and has the potential to answer some of the questions which will ultimately facilitate the systematic development of therapies to disrupt or disable the latent virus.

ABBREVIATIONS

A	adenine
aa	amino acid
ara-C	cytosine β-D-arabinofuranoside
BHK	baby hamster kidney
bp	base pairs
BSA	bovine serum albumin
BVDU	(*E*)-5-(2-bromovinyl)-2'-deoxyuridine
C	cytosine
cAMP	cyclic 3':5' adenosine monophosphate
CAT	chloramphenicol acetyltransferase
CH	cycloheximide
Ci	Curie(s)
CNS	central nervous system
cpe	cytopathic effect
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
DEP	diethyl pyrocarbonate
DRG	dorsal root ganglion
EEB	electroelution buffer
dGTP	2'-deoxyguanosine-5'-triphosphate
dl	deletion
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
ds	double stranded
dTTP	2'-deoxythymidine-5'-triphosphate
EDTA	ethylenediamine tetra-acetic acid disodium salt
G	guanine
gD	glycoprotein D
h	hour(s)
HAT	hypoxanthine aminopterin thymidine
HEL	human embryo lung
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HFL	human foetal lung
HCMV	human cytomegalovirus

HMBA	hexamethylene bisacetamide
HSV	herpes simplex virus
HSV-1	HSV type 1
HSV-2	HSV type 2
IE	immediate early
IEC	immediate early complex
IFN-α	human lymphoblastoid interferon-α
in	insertion
IRF	interferon response factor
IR_L	internal repeat long
IR_S	internal repeat short
ISGF3	IFN stimulated gene factor 3
ISRE	interferon stimulated response element
iu	international units
kb	kilobase(s)
kbp	kilobase pairs
kd	kilodaltons
L	late
LAT(s)	latency associated transcript(s)
LPBF	LAT promoter binding factor
MEM	minimum essential media
MHC	major histocompatability complex
min	minute(s)
MoMuLV	Moloney murine leukaemia virus
moi	multiplicity of infection
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NRD	negative regulatory domain
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
pfu	plaque forming unit(s)
PIPES	piperazine-N,N'-bis-2-ethanesulphonic acid
PMSF	phenylmethylsulphonylfluoride
PNS	peripheral nervous system
PRD	positive regulatory domain
PSG	penicillin, streptomycin and glutamine
RI	refractive index

RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
s	seconds
ss	single stranded
TBS	Tris buffered saline
TCA	trichloroacetic acid
TE	Tris EDTA
TK	thymidine kinase
Tris	tris(hydroxymethyl)aminomethane
TR_L	terminal repeat long
TR_S	terminal repeat short
ts	temperature sensitive
U_L	unique long
U_S	unique short
UV	ultraviolet light
Vmw	molecular weight of viral polypeptide in kilodaltons
wt	wild type
Xgal	5-Bromo-4-Chloro-3-indolyl-β-D-galactopyranoside

1. INTRODUCTION

1.1. The herpesviruses

1.1.1. Structure

The herpesvirus family has more than 80 members. They share the same structural features in their virions and the pathogenic property of latency. The virion, which has a mean diameter of about 215nm (Schrag *et al.*, 1989), is composed of 4 elements (Wildy *et al.*, 1960; Roizman and Furlong, 1974; Roizman, 1990). The core contains a single, linear, double stranded DNA genome and is surrounded by the capsid, the two elements together forming the nucleocapsid. The packaged DNA is in a liquid crystalline state and is uniformly dense filling the whole of the space, which has a radius of about 43nm, enclosed by the capsid (Booy *et al.*, 1991). The capsid is a thick-shelled icosahedron of 14 to 15nm (Booy *et al.*, 1991) comprised of 162 capsomeres made up of 150 hexamers and 12 pentamers. The tegument surrounds the capsid and is an amorphous matrix of protein which varies in amount between different herpesviruses. The whole is enclosed in a triple layered lipid membrane structure, the envelope, derived from the host cell nuclear membrane but containing HSV glycoprotein molecules projecting from its surface (Stannard *et al.*, 1987).

1.1.2. Classification

Subdivision of the herpesviruses is based on their biological properties of host range, reproductive cycle, cytopathology and latent characteristics. This gives rise to three subfamilies, the *alphaherpesvirinae*, *betaherpesvirinae* and *gammaherpesvirinae* (Roizman, 1982a) and these are described in table 1.1. This subdivision is better than one determined by genome structures because the biological properties dominate any other single set of properties and it produces a minimum number of subfamilies. The sequence data that are emerging are largely compatible with these subdivisions although there are some discrepancies. On the basis of sequence data, HHV-6 should be grouped with HCMV in the *betaherpesvirinae* subfamily but on the basis of permissivity of lymphoid cell lines, it is assigned to the *gammaherpesvirinae* subfamily.

SUBFAMILY		
Alphaherpesvirinae		
Host range	*in vivo*: generally to the natural host *in vitro*: variable	Examples: Human herpesvirus 1 (HSV-1) Human herpesvirus 2 (HSV-2)
Reproductive cycle	short	Human herpesvirus 3 (VZV)
Cytopathology	rapid spread in cell culture with mass destruction	
Latent infection	frequently, but not exclusively, in ganglia	
Betaherpesvirinae		
Host range	*in vivo*: narrow, often the species or genus of host *in vitro*: best in fibroblasts	Examples: Human herpesvirus 5 (HCMV) Human herpesvirus 6 (HHV-6)
Reproductive cycle	relatively long	
Cytopathology	slowly progressive lytic foci in cell culture; frequent cytomegalia *in vitro and in vivo*	
Latent infection	possibly in secretory glands, lymphoreticular cells, and other tissues	
Gammaherpesvirinae		
Host range	*in vivo*: narrow, usually the family or order of natural host *in vitro*: replicative in lymphoblastoid cells; lytic in some epithelioid and fibroblastoid cells	Example: Human herpesvirus 4 (Epstein-Barr virus)
Reproductive cycle	variable	
Cytopathology	variable	
Latent infection	frequently in lymphoid tissue	

TABLE 1.1: SUBDIVISION OF HERPESVIRUSES ACCORDING TO THEIR BIOLOGICAL PROPERTIES (Roizman, 1982a)

1.1.3. Pathology

There are seven human herpes viruses recognised to date and the pathology of six of these has been extensively described (Roizman, 1982b; 1983; 1985; Roizman and Lopez, 1985; Fields and Knipe, 1990). HSV-1 infection most commonly presents with gingivostomatitis and conjunctivitis and is usually a mild childhood illness. HSV-1 is able to establish latent infection in the sensory ganglia which innervate the site of the primary infection. Humoral and cell mediated immunity is established but this does not affect the latent virus and it has an uncertain role in limiting reactivation. Periodic reactivation of the latent virus produces recurrences in the distribution of the ganglia, principally herpes labialis ("cold sores") and herpes keratitis or uveitis, leading causes of blindness. Spread from the meningeal branches of the trigeminal nerve or via the olfactory pathway and connections in the limbic system to the temporal lobes, either during primary infection or reactivation, can lead to encephalitis which is a very serious complication with both high mortality and morbidity. This is particulary important in patients who have lymphoproliferative diseases, AIDS or are on immunosuppressive therapy following organ or bone marrow transplantation. These patients are also at risk of severe widespread infection involving the gastrointestinal tract and the respiratory system.

Primary infection with HSV-2 is a sexually transmitted disease and is the usual cause of herpes genitalis although in recent studies 20 - 40% of genital isolates have been found to be HSV-1 (Peutherer *et al.*, 1982; Corey *et al.*, 1983; Scoular *et al.*, 1990). HSV-2 also establishes latency in the relevant sensory ganglia and produces troublesome recurrent infections. An important complication is neonatal infection following transmission of the virus from maternal genital lesions to the infant during parturition.

Varicella-zoster virus (VZV) has man as its only host and is the cause of chicken pox (varicella) as the primary illness and shingles (zoster) when the infection is reactivated from latent sensory ganglia. Varicella can have serious complications in those who are immunocompromised, with generalised dissemination to the skin and viscera. Zoster is a feature of increasing age and immunosuppression and commonly leads to painful, debilitating post herpetic neuralgia. This virus too causes encephalitis although it is usually less serious than herpes simplex encephalitis. In immunocompetent patients, VZV encephalitis is more likely to be caused by immune mediated indirect mechanisms rather than by a productive viral infection typical of HSV (Kennedy *et al.*, 1988; 1990).

Epstein Barr virus infection is often asymptomatic but is the cause of infectious mononucleosis. Infection in immunocompromised individuals can cause severe lymphoproliferative disease with extensive infiltration of immature B lymphocytes into organs and tissues. The virus is able to transform human lymphocytes and it is associated with Burkitts lymphoma and nasopharyngeal carcinoma (Miller, 1990).

Human cytomegalovirus infection is usually benign and subclinical but is a recognised cause of meningitis and is especially important in the immunosuppressed patient notably after renal transplantation and in patients with AIDS. The predilection of HCMV for leucocytes is an important consideration in blood tranfusion and organ grafting. It is a major cause of congenital abnormalities which may be related to its susceptibility to reactivate in the uterine cervix.

Human herpes virus 6 was first described as an infection of peripheral blood lymphocytes in patients with lymphoproliferative disorders (Salahuddin, 1986). Infection is widespread (Briggs *et al.*, 1988) but apart from a transient childhood illness, exanthema subitum and a mononucleosis with cervical lymphadenopathy, it does not have any known pathology.

Human herpes virus 7 has been isolated from CD4+ T cells of a healthy individual following their incubation under conditions promoting activation (Frenkel *et al.*, 1990).

1.1.4. Genome structures

The genomes of the herpes viruses differ widely in size, base composition and the arrangement of reiterated sequences (Roizman, 1990). The gross structures of the six human herpes viruses which are linear DNA molecules when isolated from virions are shown in figure 1.1.

Sequence data are complete for HSV-1 strain 17 (McGeoch *et al.*, 1985; 1986; 1988; Perry and McGeoch, 1988); VZV (Davison and Scott, 1986); EBV strain B95-8 (Baer *et al.*, 1984) and HCMV strain AD169 (Chee *et al.*, 1990). Base composition ranges from a G + C content of 43% for HHV-6 (Lopez and Honess, 1990) to 68.3% and 69% for HSV-1 (McGeoch *et al.*, 1988) and HSV-2 (Roizman, 1982a) respectively. Furthermore, base composition is very variable within the genome. For example, the G + C content of the 6.6kbp R_S of HSV-1 is 79.5% (McGeoch *et al.*, 1986).

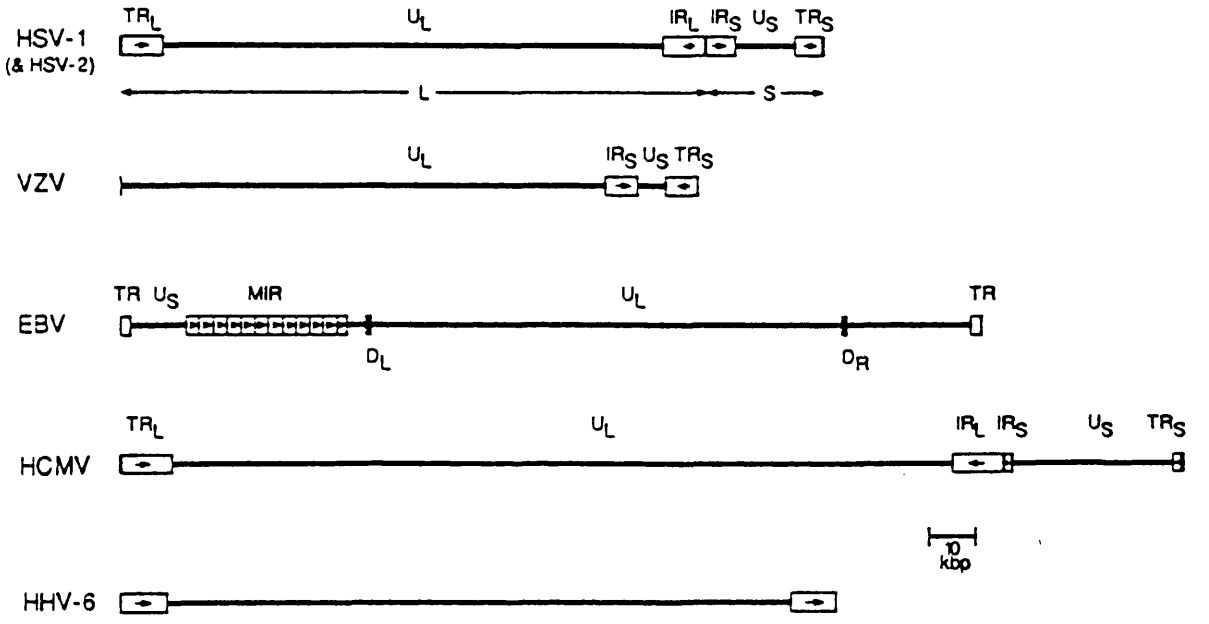


FIGURE 1.1: GROSS STRUCTURES OF THE HUMAN HERPESVIRUSES.

The genome structures of six of the seven known human herpesviruses. Each linear genome is shown with unique sequences as lines and repeat elements as boxes with relative orientations indicated by arrows. After McGeoch, 1989.

MIR = major internal repeat
U_L = unique long U_S = unique short
D_L = direct repeat left D_R = direct repeat right
I/TR_L = internal/terminal repeat long I/TR_S = internal/terminal repeat short

Observations under the electron microscope of viral DNA spreads led to a proposed arrangement of the HSV-1 genome (Sheldrick and Berthelot, 1974) which has since been confirmed (McGeoch *et al.*, 1985; 1986; 1988; Perry and McGeoch, 1988). In the native state it is a linear duplex and there are 152,260 residues in each strand. It has two covalently linked regions termed long (L) and short (S) each with unique sequences, U_L and U_S, flanked by repeat sequences, R_L and R_S. At the genomic termini these are denoted as TR_L and TR_S and where they meet internally, to form the joint, as IR_L and IR_S. The internal repeat is identical to but inverted with respect to the terminal repeat. Each terminus has one overhanging residue with a free 3' hydroxyl group (Mocarski and Roizman, 1982). R_L and R_S are distinct except for a 400bp direct repeat, the "a" sequence, at the termini and one or more copies in an opposite orientation at the joint (Wagner and Summers, 1978; Davison and Wilkie, 1981). The repeat regions have been designated as ab, b'a', ac and a'c' (Wadsworth *et al.*, 1975). Families of short, directly repeated sequences of variable copy number between 10 and 100 bp have been found, usually in R_L, in all large scale sequencing studies. The relative orientation of U_L and U_S with respect to the joint has the potential to produce four isomers which have been identified from terminal and junction fragments and partial denaturation profiles (Hayward *et al.*, 1975) and found in equimolar amounts in the DNA from plaque purified virions (Roizman, 1979). One isomer has been designated as the prototype and is the arrangement used for mapping and sequence determination. The other three isomers are produced by the inversion of S (I_S), the inversion of L (I_L) and the inversion of both S and L (I_{SL}). All isomers are able to participate in a productive (Davison and Wilkie, 1983) or a latent infection (Wigdahl *et al.*, 1984a; Efstathiou *et al.*, 1986). The gross organisation of the HSV-1 genome is shown in figure 1.2.

1.1.5. Genome contents

The open reading frames corresponding to the proposed HSV-1 genes are shown in figure 1.3. The U_L region has 58 genes and the U_S region 12 genes. The repeat segment R_L contains 2 genes, R_L1 and R_L2, coding for the neurovirulence factor ICP34.5, also known as $\gamma_1$34.5, (Chou *et al.*, 1990; McGeoch and Barnett, 1991) and the transcriptional regulator Vmw110 respectively. The repeat segment R_S, contains the IE175 gene. Thus the whole genome has at least 76 ORFs encoding 73 distinct proteins. There are possibly others arising from very small or extensively overlapping genes and those with complex intron/exon arrangements. Downstream of the IE110 gene in R_L is the initiation site of the LATs (Stevens *et al.*, 1987) whose function is

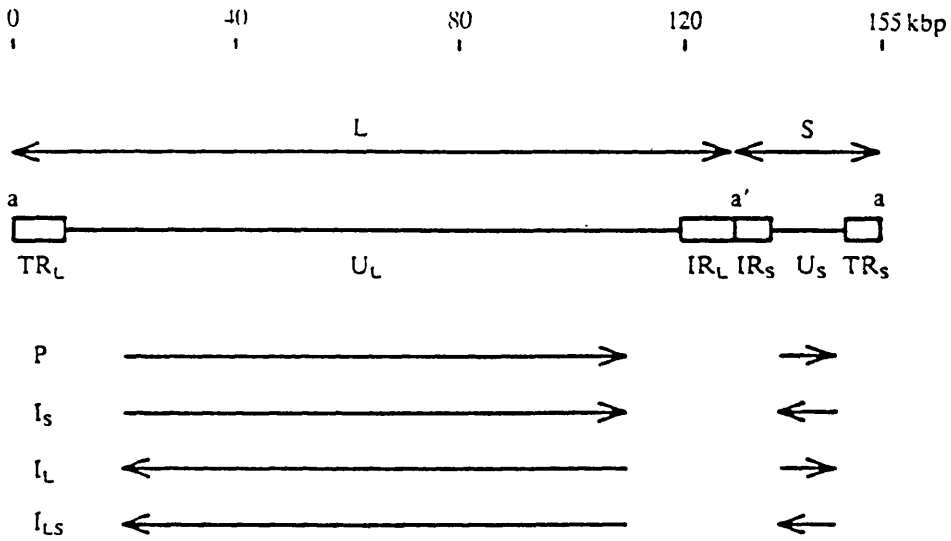


FIGURE 1.2: GROSS ORGANISATION OF THE HSV-1 GENOME

A conventional representation of the HSV-1 genome is shown, with the unique (U_L and U_S) and the major repeat (TR_L and TR_S) sequences as solid lines and open boxes respectively. The locations of the L and S segments are marked. Terminal a sequences and the internal, opposite orientation a' sequence are indicated. The copy number of a' sequences present at the junction between the L and S components of the genome is variable although often equal to one. The four isomers as described in the text are drawn beneath. From McGeoch, 1987.

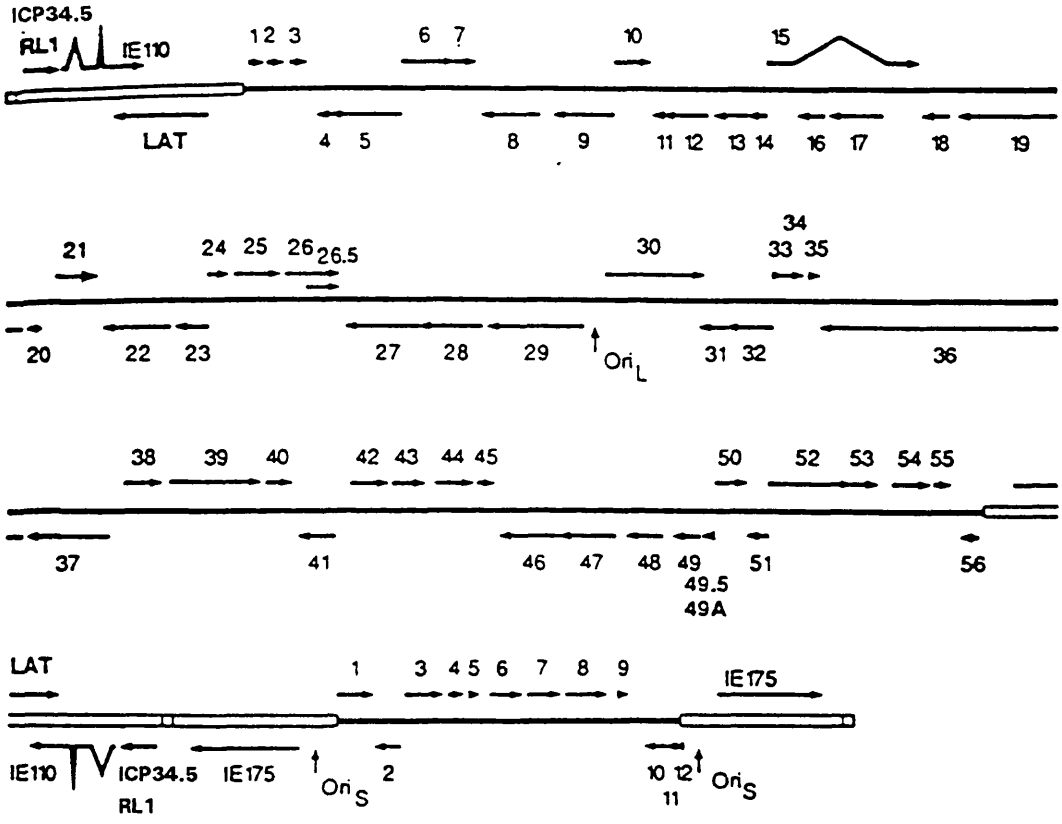


FIGURE 1.3: ORGANISATION OF THE GENES IN THE GENOME OF HSV-1

The prototype orientation represented on four lines. The locations of open reading frames are shown by arrows with splicing within the coding regions indicated. In the top three lines, genes U_L1 - U_L56 are shown as 1 - 56 and in the bottom line genes U_S1 - U_S12 as 1 - 12. Locations of origin of DNA replication are also indicated. Reproduced from McGeoch, 1989 with the addition of $U_L26.5$ (Liu and Roizman, 1991a), $U_L49.5/49A$ (Barnett *et al.*, 1992; Barker and Roizman, 1992) $ICP34.5/RL_1$ (Chou and Roizman, 1990; McGeoch *et al.*, 1991) and LATs (Stevens *et al.*, 1987).

obscure and for which no protein product has been reported yet. Most genes possess independent promoters and generate unspliced mRNA. All temporal classes of genes require host cell RNA polymerase II for their expression. They are expressed as immediate early (IE) genes, early genes and late genes (Honess and Roizman, 1974). The IE genes do not require *de novo* protein synthesis for their promoters to be recognised. The early genes encode a number of proteins many of which are required for DNA replication. The products of the late genes are largely involved in the assembly and maturation of virions.

The genes can be categorised into those which are involved in (i) control, (ii) nucleic acid metabolism, replication and repair and (iii) structure and assembly of virions. Controlling genes include the five IE genes. Of these IE110 (R_L2), IE175 (R_S1) and U_L54 (Vmw63) are transcriptional regulators whose products control expression of the later genes and the IE genes themselves (Everett, 1987). The other two IE genes, U_S1 (Vmw68) and U_S12 (Vmw12) have not had a function assigned to them yet. The product of U_L48, Vmw65, has an important regulatory role (Campbell *et al.*, 1984). It functions with one or more cellular proteins through specific upstream sequences to activate the expression of the IE genes (O'Hare and Goding, 1988; Preston *et al.*, 1988). It has been reported that the products of U_L46 and U_L47, another tegument protein (McLean *et al.*, 1990), modulate the activity of Vmw65 (McKnight *et al.*, 1987) but this has only been confirmed for the latter (Zhang *et al.*, 1991). Another regulatory protein U_L41, a tegument component (McLauchlan *et al.*, 1992a), shuts off host cell macromolecular synthesis by degrading mRNA (Kwong *et al.*, 1988) and U_S11, also a structural protein, is a site and conformation specific RNA binding protein which associates with the 60S subunit of infected cell ribosomes (Roller and Roizman, 1991; 1992). Two other genes, U_L13 (Smith and Smith, 1989) and U_S3 (McGeoch and Davison, 1986), which may code for protein kinases, have a role in controlling or modulating gene expression but this has not been clearly defined to date.

Enzymes involved in nucleic acid metabolism include thymidine kinase, the product of U_L23 (Kit and Dubbs, 1963; Munyon *et al.*, 1972), ribonucleotide reductase, composed of a large and a small subunit (Frame *et al.*, 1985) encoded by U_L39 and U_L40 respectively (McLauchlan and Clements, 1983; Preston *et al.*, 1984) and deoxyuridine triphosphatase encoded by U_L50 (Preston and Fisher, 1984). Transfection of HSV-1 DNA fragments into cultured cells carrying plasmids with the HSV DNA replication origin have shown the absolute requirement for 7 genes in replication (Challberg, 1986) which are: U_L5, U_L8, U_L9, U_L29, U_L30, U_L42, and U_L52. The DNA-binding properties of the U_L37 protein and the kinetics of its

appearance suggest that it may be involved in the late events in viral replication (Shelton *et al.*, 1990). The deoxyribonuclease from U_L12 (Banks *et al.*, 1985) is not essential for viral DNA synthesis but may have a role in the processing or packaging of viral DNA into infectious virions (Weller *et al.*, 1990). A DNA repair enzyme, uracil DNA glycosylase, is encoded by U_L2 (Mullaney *et al.*, 1989).

The virion contains at least 30 different species of protein molecules (Dargan, 1986; Roizman and Sears, 1990). Three different types of capsids have been recognised by electron microscopy in the nuclei of infected cells. They are also distinguishable by their protein composition. Full or C capsids, which have a core of genomic DNA, are composed of a major protein VP5, encoded by U_L19 (Costa *et al.*, 1984) and four other polypeptides: VP19c, VP23, VP24 and VP26, encoded by U_L38 (Braun *et al.*, 1984; Rixon *et al.*, 1990); U_L18 (Rixon *et al.*, 1990); U_L26 (Davison *et al.*, 1992) and U_L35 (Davison *et al.*, 1992) respectively. Intermediate or B capsids, which are the progenitors of both C and A or empty capsids, have an additional two proteins. These are the scaffolding proteins VP22a from the $U_L26.5$ gene (Preston *et al.*, 1983; Liu and Roizman, 1991a) and VP21 produced by self cleavage of the U_L26 gene product (Liu and Roizman, 1991a; 1991b). A number of gene products may be involved in the packaging process since the formation of full capsids is affected by mutations in the genes U_L6 (Sherman and Bachenheimer, 1987); U_L25 (Addison *et al.*, 1984); U_L28 (Addison *et al.*, 1990); U_L32 (Sherman and Bachenheimer, 1987) and U_L33 (Al-Kobasi *et al.*, 1991). The tegument also has a major protein, Vmw65, the product of U_L48; the very large tegument protein VP1, encoded by U_L36 (Batterson *et al.*, 1983; McGeoch *et al.*, 1988) and other species encoded by U_S9 (Frame *et al.*, 1986) and U_S10 (Rixon and McGeoch, 1984). The myristylated tegument protein, U_L11, may have a role in the envelopment and transport of nascent virions (Baines and Roizman, 1992; Maclean *et al.*, 1992). Another major tegument protein is the highly phosphorylated VP22 encoded by U_L49 (Elliot and Meredith, 1992). There are a number of gene products whose hydropathy profiles suggest they are membrane proteins and include U_L10, U_L20, U_L34, U_L43, U_L45, and $U_L49.5$/49A. They are all poorly characterised. Finally, on the surface there are at least 10 glycoproteins (Roizman and Sears, 1990; Hutchinson *et al.*, 1992) which have been denoted as gB (U_L27), gC (U_L44), gD (U_S6), gE (U_S8), gG (U_S4), gH (U_L22), gI (U_S7), gJ (U_S5), gK (U_L53) and gL (U_L1).

In addition, a region of R_L extending into R_S encodes the LATs which are the only transcripts detectable in latently infected cells (Stevens *et al.*, 1987). Their function is unknown and their capacity for translation is uncertain.

There are two sites and three loci which are potential origins of replication (Roizman and Sears, 1990). One site, ori_L, lies between U_L29 and U_L30 and the other, ori_S, in R_S. All the functional signals at the R_S origin of replication occur within a 100bp viral DNA fragment. A prominent feature of this region is an almost perfect palindromic sequence of 45bp containing 18 A or T residues at its centre (Stow and McMonagle, 1983). The "a" sequence plays a part in packaging in that it has elements involved in cleavage of the concatemers generated by replication (Stow *et al.*, 1983), and two viral proteins have been identified which bind specifically to the "a" sequence and might have a role in encapsidation (Chou and Roizman, 1989). The role of the joint itself is undetermined but it is of interest that there are no transcripts spanning the junction and there is no physical separation of the L and S segments during the normal infective process.

Experiments analysing host range mutants have demonstrated that at least 20 genes are dispensible for growth in tissue culture (Roizman and Sears, 1990). As is apparent from the foregoing discussion there are still 14 genes whose function remains totally unknown.

1.1.6. Lytic infection

The major events of the viral life cycle are well documented (Roizman and Sears, 1990). HSV-1 attaches to cells by interacting with heparan sulphate which is covalently linked to cell surface proteoglycans (Shieh *et al.*, 1992). Although it is not essential for attachment this process is facilitated by the viral glycoprotein, gC (Herold *et al.*, 1991). Other glycoproteins are probably required for successful penetration of the cell since mutants with lesions in the genes for gB (Cai *et al.*, 1988), gD (Ligas and Johnson, 1988) and gH (Forrester *et al.*, 1992) which are able to attach to the cell surface nevertheless have severly reduced infectivity. In addition, gL is required for the correct expression of gH (Hutchinson *et al.*, 1992). Entry is gained by a pH-independent fusion of the virion envelope with the cell membrane (Wittels and Spear, 1991) and the capsid is transported, possibly by microtubules (Kristensson *et al.*, 1986), to the nuclear pores. At the pores and by a process dependent on the activity of the tegument protein VP1 (Batterson *et al.*, 1983), the viral DNA is released into the nucleus.

Within an hour of infection the synthesis of host cell macromolecules is inhibited and as the production of cellular proteins, RNA and DNA gradually declines there is an

increase in viral material. Early "host shut-off" is mediated by the product of U_L41, a tegument protein (Fenwick and Walker, 1978; Kwong *et al.*, 1988; McLauchlan *et al.*, 1992a) whereas secondary or delayed shut off is mediated by the expression of the viral genes (Fenwick and Clark, 1982).

The lytic cycle is characterised by a cascade of gene expression involving the synthesis of the IE gene products followed by the activation of the early genes and then the late genes (Honess and Roizman, 1974). In the infected cell, the viral genes are transcribed by cellular RNA polymerase II. The virion tegument protein, Vmw65, initiates the cascade by selectively activating viral IE promoters shortly after infection (O'Hare and Goding, 1988; O'Hare *et al.*, 1988; Preston *et al.*, 1988). All IE gene promoters contain one or more copies of a far upstream element with the sequence TAATGARAT (where R is a purine) which is essential for transactivation mediated by Vmw65. Vmw65 associates with cellular factors to form the IEC which binds specifically to the TAATGARAT (O'Hare and Goding, 1988; Preston *et al.*, 1988) as illustrated in figure 1.4. The cellular protein Oct-1 which normally binds to an octamer motif similar to the TAATGARAT (O'Hare *et al.*, 1988), is a constituent of the IEC and is altered by association with Vmw65 to bind to the viral *cis*-acting element (O'Hare *et al.*, 1988). Vmw65 is a phosphoprotein whose N terminal 410 aa are necessary for complexing with the cellular proteins (Greaves and O'Hare, 1990) and whose acidic C terminal region is responsible for transactivation (Greaves and O'Hare, 1989). Vmw65 is not absolutely required for the initiation of infection but it greatly enhances its efficiency, especially at low moi (Ace *et al.*, 1989). There are other upstream *cis*-acting elements in IE genes which enhance their transcription even in the absence of Vmw65 (Cordingley *et al.*, 1983).

The IE proteins, Vmw110, Vmw175 and Vmw63 play important roles in lytic cycle gene expression, controlling both the early and the late genes (Everett, 1986; 1987). The other IE proteins, Vmw68 and Vmw12, are poorly understood. Vmw110 is a potent transactivator and in transfection assays it activates transcription from IE, E and late promoters of HSV-1 (O'Hare and Hayward, 1985a;1985b; Everett, 1986). It is not essential in tissue culture but confers a growth advantage on the virus (Stow and Stow, 1986; Sacks and Schaffer, 1987). It may be able to interact synergistically with Vmw175 (Everett, 1987). Vmw175 is a major regulatory protein of HSV-1 (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a) and is essential for activation of the early promoters and progression of the lytic cycle (Preston, 1979; DeLuca *et al.*, 1985; Persson *et al.*, 1985). It effects transcriptional activation by binding within a promoter

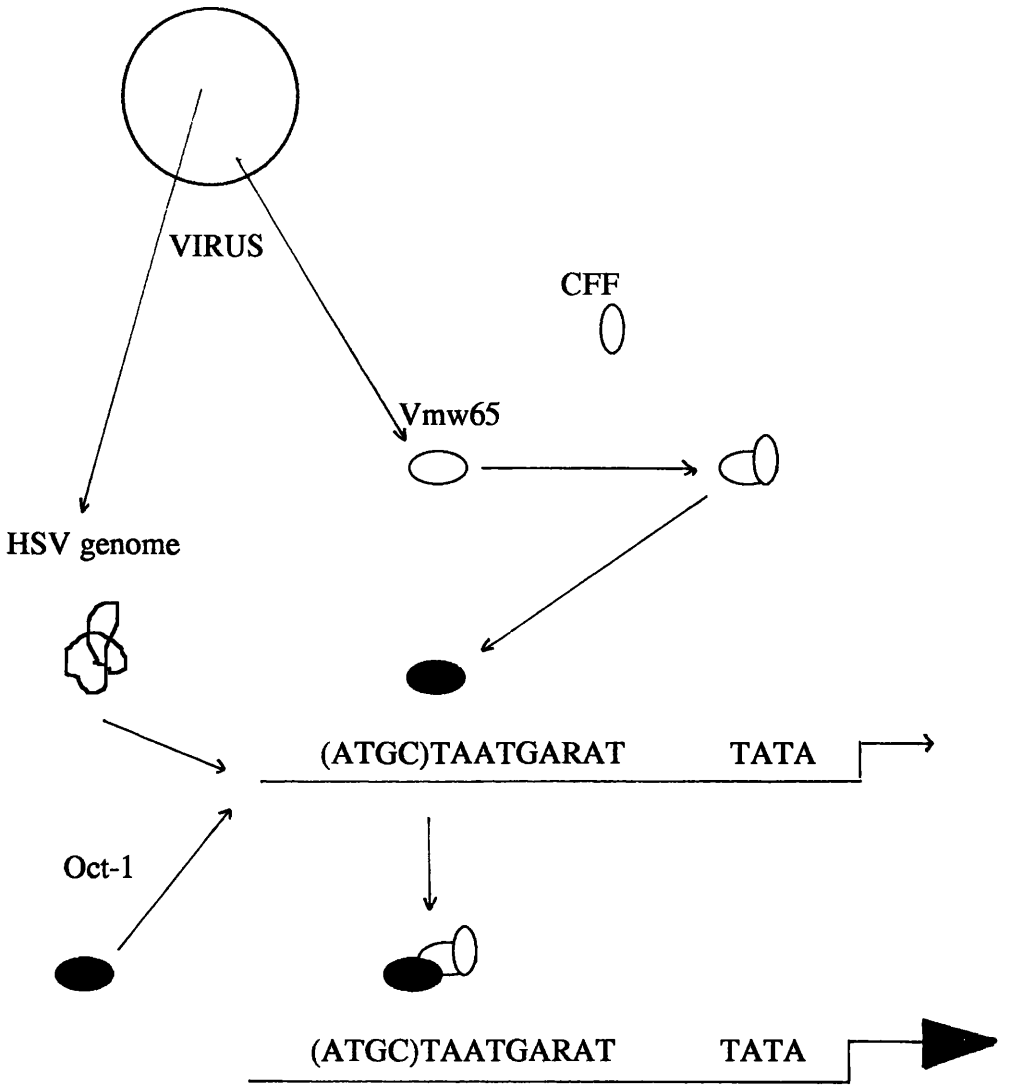


FIGURE 1.4: REGULATORY REGIONS OF THE IE GENES SHOWING INTERACTION OF TRANSACTIVATING ELEMENTS

Upon entry to the cell, Vmw65 in the tegument of the virion complexes with complex forming factor (CFF; Katan *et al.*, 1990). This possibly occurs in the cytoplasm. Once in the nucleus, octamer sites in the IE genes are occupied by Oct-1. The Vmw65-CFF complex recognises Oct-1 in the context of the flanking GARAT signal locating the acidic activation domain onto the IE genes and thereby activating transcription by promoting assembly of an initiation complex. It is possible that the interaction with Oct-1 occurs after Vmw65 complexes with CFF and the transcriptional target.

region (Tedder *et al.*, 1989). The DNA-binding domain is located within a highly conserved region of about 230 residues near to the N terminus (Everett *et al.*, 1990; 1991). Vmw175 represses its own synthesis, probably by binding at its own transcriptional start site (DeLuca and Schaffer, 1988), and the products of the other IE genes (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985b). Vmw63 is also essential for viral growth. Deletion mutants over express the IE gene products and under express the late gene products suggesting that Vmw63 is involved in the switch from early to late gene expression (McCarthy *et al.*, 1989). Vmw63 may act at the posttranscriptional level by selectively stimulating viral mRNA 3' processing (McLauchlan *et al.*, 1992b; Smith *et al.*, 1992). The early and late genes of HSV-1 do not possess virus specific promoter sequences but have sequences corresponding to the binding sites of cellular transcription factors (Johnson and Everett, 1986; McKnight and Tjian, 1986).

Within 30 minutes of infection the linear genome loses its free ends (Poffenberger and Roizman, 1985). Viral DNA synthesis starts between 3 and 5 hours after infection and peaks between 9 and 11 hours (Rixon *et al.*, 1983). Molecules of more than unit length DNA appear as head to tail concatemers generated by a rolling circle mechanism (Jacob and Roizman, 1977; Jongeneel and Bachenheimer, 1981) and eventually form large tangled masses of viral DNA filling the whole of the host cell nucleus (Rixon *et al.*, 1983). The capsid shell assembles with the scaffolding proteins VP22a and VP21, the latter derived from the U_L26 protease, to form the B capsid. The protease cleaves itself to release VP21 and VP24. Mature C capsids are created by the proteolytic degradation of VP22a and VP21 from the B capsids and the packaging of DNA. Empty A capsids result if there is a failure to package DNA. As it is packaged the newly replicated DNA is cleaved into unit length molecules (Deiss and Frenkel, 1986). Capsids must acquire tegument and envelope before leaving the cell but the details of these events are poorly understood. Nevertheless it seems that the initial stage involves budding of the capsid into the space between the inner and outer nuclear membranes (Darlington and Moss, 1968). The Golgi complex is implicated in the maturation of the virions since eventual release to the exterior occurs by exocytosis from Golgi-derived vesicles (Johnson and Spear, 1982). The U_L20 membrane protein is vital for the successful delivery of the mature virion to the cell surface (Baines *et al.*, 1991).

At 18 to 20 hours after infection mature virions are released from the cell.

1.2. Latency

1.2.1. Latent infection

The phenomenon of latency has been well described since Harvey Cushing's publication of his observations in 1905 following surgical procedures on the trigeminal nerve (Cushing, 1905). In 1929 it was hypothesised that the virus remains in a non-infectious state within sensory ganglia (Goodpasture, 1929) and this has now been substantiated by many investigators (Wildy, 1967; Cook and Stevens, 1973). The virus reaches the sensory ganglion by intra-axonal transport in the appropriate nerve that innervates the site of the peripheral infection (Wildy, 1967; Cook and Stevens, 1973).

The ability to persist in their hosts in a state of latency with only occasional interruptions in the form of lytic infections is a characteristic feature of all herpesviruses. Such recurrent infections in humans are primarily responsible for the prevalence of these viruses. Generally, the herpesviruses are lytic but during the primary infection it appears that a few cells do not undergo the viral lytic replication cycle and establish a lifelong latent state with the virus instead. Since Vmw175 specifically down regulates its own promoter by up to 50-fold, dominating Vmw65 and Vmw110 mediated upregulation in transient co-transfection assays (Roberts *et al.*, 1988), it is tempting to speculate that this might be significant in altering the course of the infection and directing it towards latency *in vivo*. The mechanism by which the switch from a lytic to a latent pathway occurs is not understood but it is likely to be the result of an interplay between cellular and viral factors and the immune response. Generally, latent virus is harboured in neural tissue and especially in neurones of sensory ganglia (Kennedy *et al.*, 1983). Latently infected sensory neurones account for only about 1% of the cells in the ganglion. Despite the predilection for neural tissue there are reports of non-neuronal sites of latency such as the mouse footpad for HSV-2 (Clements and Subak-Sharpe, 1988) and the mouse cornea for HSV-1 (Abghari and Stulting, 1988). It is clear that latency is not established in all cells capable of being infected which suggests that the cell phenotype is important to this process. There also appears to be a well established role for the immune response in the maintenance of the latent state as well as during acute infection since immunocompromised or immunosuppressed patients are particularly vulnerable to severe recurrent disease. Latency can be operationally defined as the absence of or inability to recover infectious particles from tissues or cells that harbour the virus. Latency has to be distinguished

from persistent infection where there is continued low level viral replication and infectious particles are recoverable from disrupted cells. Persistent infection occurs with chronic hepatitis B, human immunodeficiency virus or subacute sclerosing panencephalitis.

In the past 20 years an enormous amount of thought and research has been devoted to latency but an understanding at the molecular level remains elusive. It has been reduced to a tripartite process, namely establishment and maintenance of latency and reactivation from latency. To investigate the phenomenon itself as well as these aspects in particular, a variety of methods involving both animal and tissue culture models have been employed.

1.2.2. Investigation of latency in animal models

A number of animal models have been developed to facilitate the study of herpesvirus latency. These include the mouse, rat, guinea pig, rabbit and monkey although the mouse is by far the most commonly used. A variety of routes of inoculation have been used in these animals, such as the peripheral sites of the eye (Nesburn *et al.*, 1967; Rock and Fraser, 1983; 1985); ear pinna (Hill *et al.*, 1975; 1978); footpad (Stevens and Cook, 1971; Scriba, 1975) and vagina (Scriba and Tatzber, 1981) as well as the intracerebral (Hill *et al.*, 1975); intraperitoneal (Richards *et al.*, 1981) and intravenous (Cook and Stevens, 1976) routes. Following inoculation at peripheral sites the virus enters the relevant sensory nerve and travels to the corresponding ganglion where latency is established (Wildy, 1967; Cook and Stevens, 1973). To be useful as a model the host must not only have the capacity to retain the virus in a latent state but at a predictable site. The mouse eye has been a good model for producing a high incidence of latent infections (Rock and Fraser, 1983). The severity of the acute infection may affect the quantity of viral DNA in the CNS that is detectable 2 - 5 months afterwards (Cabrera *et al.*, 1980; Rock and Fraser, 1983). The virus replicates at the inoculation site and travels via the ophthalmic branches of the trigeminal nerve supplying the cornea to the trigeminal ganglion where it can be recovered by macerating the tissue a few days later. The virus may also reach the CNS by travelling along the trigeminal root to the brain stem or by ascending the facial nerve (Anderson and Field, 1983). Neither spontaneous nor experimentally induced reactivation occurs readily in the model, which makes it suitable for investigating the establishment and maintenance of, but not reactivation from, the latent state. The rabbit eye model has also been used and although it supports latent infection, frequent spontaneous recurrences (Nesburn *et al.*, 1967) are likely to make the interpretation of

data difficult in some experimental situations. Inoculation of the mouse footpad (Stevens and Cook, 1971) leads to establishment of latency in the sacral ganglia following transport along branches of the sciatic nerve. This model is also limited by the failure of latently infected animals to suffer recurrent disease when stimulated (Stevens and Cook, 1973) and thus again is not suitable to study reactivation. In contrast, the guinea pig footpad model (Scriba, 1975) is subject to spontaneous recurrence of clinical disease which in some circumstances could be also disadvantageous. Intradermal or subcutaneous injection of virus in the pinna of the mouse ear produces erythema or paralysis (Hill *et al.*, 1975; 1978) and the second, third and fourth cervical dorsal root ganglia become sites of established latent infection. Reactivation can be induced from the mild trauma caused by stripping cellophane tape off the ear (Hill *et al.*, 1978). This is associated with observable clinical signs, similar to those in the human host, but only about 30% of animals show recurrent disease following this stimulus. HSV-1 infection of the guinea pig vagina leads only to mild symptoms, a low level of persistence in the sacral ganglia and no recurrences (Scriba and Tatzber, 1981). This is quite unlike HSV-2 genital infection of the guinea pig which invades and persists in the sensory ganglia much more efficiently and readily establishes recurrent lesions. However, peripheral persistence in the vagina complicates the interpretation of recurrence (Scriba and Tatzber, 1981) since a recurrence rate of up to 100% may be associated with only a 60% incidence of latent infection in the lumbosacral dorsal root ganglia (Stanberry *et al.*, 1982). This may reflect the lack of sensitivity of the methods used. Another problem with this model is the finding that the recurrent genital lesions are often sterile (Stanberry *et al.*, 1982).

The intracerebral route is not a natural route of herpesvirus infection. When mice were inoculated with HSV-1 directly into the right hemisphere more mice were killed than from the same dose inoculated intradermally (Hill *et al.*, 1975). The difference between the pathogenicity of HSV-1 and HSV-2 following intradermal inoculation was not so great when the intracerebral route was used (Hill *et al.*, 1975). Others have found HSV-2 to be more neurovirulent than HSV-1 with a greater ability to spread and cause disease within the CNS (Richards *et al.*, 1981). No evidence of latency within the CNS was reported although the establishment of latency following intracerebral inoculation of mice with ts mutants has been shown by others (Lofgren *et al.*, 1977). The intraperitoneal route (Richards *et al.*, 1981) is also an unnatural route of infection and this makes such models inappropriate for further studies. Haematogenous spread of herpes simplex infections does occur in man albeit rarely, but no direct seeding into the mouse CNS occured from haematogenous spread and it was probable that infection was first established in the sensory ganglia (Anderson and Field, 1983). This accords

with the earlier reports that viraemia caused by injection of virus into the tail vein of mice was followed by the establishment of latency principally in sensory ganglia although latent virus could be recovered from the brains of a minority (Cook and Stevens, 1976). Interestingly, latent infection was not apparent in any of the extraneural tissues examined but was demonstrated in the adrenal medulla. This tissue has a neural origin and contains sympathetic ganglion cells, in agreement with the hypothesis that latent infections are generally limited to nervous tissues and more specifically to the neurones in these tissues although there may be exceptions.

In man, latent infection may be activated by a number of varied stimuli which include fever, sunlight and trauma. Trigeminal sensory root section performed as a treatment for trigeminal neuralgia is also able to provoke herpetic lesions in man (Carton and Kilbourne, 1952) and this observation has been adapted to effect reactivation of latent virus in animal models. Homogenisation of latently infected DRG of mice beyond 2 weeks following inoculation of the footpad does not result in detection of the infecting virus unlike explantation of the whole ganglia (Cook and Stevens, 1971; Walz *et al.*, 1974). Thus homogenisation after neurectomy can be used as a means of measuring reactivation. Cutting the lumbosacral roots of the sciatic nerve of latently infected murine ganglia reactivated virus in 30% of the organs (Walz *et al.*, 1974). This observation established sciatic nerve section as a reproducible model of viral reactivation *in vivo*, and this technique has been extended to other sites (Price and Schmitz, 1978). Neurectomy causes a profound alteration in the metabolic state of neurones including an increase in RNA synthesis (Langford *et al.*, 1980). Such a change is part of the general chromatolytic or axon reaction which may be related to loss of NGF being supplied from the periphery (Tenser *et al.*, 1988).

The first demonstration that HSV can induce a latent infection in ganglia was in 1971 (Stevens and Cook, 1971). Mice were inoculated in the rear footpad and those that recovered from the initial paralysis were sacrificed at intervals up to 4 months post infection. The spinal ganglia were either explanted and maintained as organ cultures, with the supernatant being regularly examined for the presence of virus, or homogenised and assayed for virus. Organ cultures were positive for HSV between 7 and 14 days but no virus was detected following homogenisation (Stevens and Cook, 1971). This established explantation of ganglia as a model of viral reactivation *in vitro*. The mouse, however, does not naturally experience recurrent disease unlike the herpetic rabbit eye (Nesburn *et al.*, 1967). Explantation of the trigeminal ganglia of latently infected rabbits (Stevens *et al.*, 1976) and superior cervical and vagus ganglia of humans (Warren *et al.*, 1978) also effects reactivation. Co-cultivation techniques of

chopped latently infected neural tissue with cultures of appropriate indicator cell monolayers has also been used to demonstrate the presence of virus in mice (Cook and Stevens, 1976), in rabbits (Knotts *et al.*, 1973) and in humans (Baringer, 1974; Warren *et al.*, 1978). Culture of dissociated cells from the mouse DRG has been used to demonstrate reactivation in neurones identified by a neurone specific immunofluoresence marker (Kennedy *et al.*, 1983).

Despite the use of explant reactivation as a method to investigate the phenomenon of reactivation from the latent state it must be borne in mind that it is totally unlike any of the usual stimuli which effect reactivation *in vivo*. Since it is such a drastic manipulation of the neurone it may not accurately mimic the subtle processes normally involved in the natural event and therefore be of questionable interpretation.

It is clear that one of the major problems with animal models, apart from cost, is that none of them exactly matches the condition in the human host. Some of the more experimentally suitable animals in terms of size and economy are often less permissive and commonly fail to mimic latency in either being very difficult, if not impossible to reactivate or the opposite extreme of being too easy. To establish latency in some animals requires high doses of virus and others infected at low doses suffer from high mortality rates. The different patterns of regulated viral gene expression in neuronal cells need to be well understood if the mechanisms controlling latency and reactivation are to be unravelled. However, the number of neurones available from latently infected ganglia *in vivo* are insufficient for such detailed study. Thus no one animal model fulfils the criteria to provide material to study latency in its entirety although it might allow a particular aspect to be investigated.

1.2.3. Investigation of latency in tissue culture models

Animal models not only fail to mimic the clinical features of latent infection in humans but are also subject to many variables that are difficult to manage in the experimental situation. In addition, animal experiments are limited by the low number of cells in the sensory ganglia that retain a latent genome that can be reactivated and the inaccessibility of neurones *in situ*. Tissue cultures have the attraction of being pure, can be visualised directly and permit the manipulation of the cellular environment to a much greater extent. The ideal *in vitro* latency model would have:-

(i) host cells of a neuronal origin
(ii) cells able to survive viral infection

(iii) no infectious virus in surviving cells
(iv) complete viral genomes persisting in surviving cells
(v) viral genomes persisting as non-linear, non-integrated structures
(vi) the latency associated transcripts expressed
(vii) virus genomes that can be reliably reactivated by a defined manipulation
(viii) a high proportion of cells with a latent genome that can be reactivated

Various tissue culture models have been described. Wigdahl and co-workers have developed models based on cultured cells including primary neurones, infected in the presence of inhibitors of replication and at 40°C. It is well established that HSV cannot replicate in tissue culture cells at temperatures of 41 - 42°C (Crouch and Rapp, 1972; Marcon and Kucera, 1976). The mechanism for this could be the inactivation of an essential viral protein normally involved in directing the lytic process or a cellular response to the supraoptimal temperature. Either mechanism could leave the genome open to be directed down the latent pathway. HEL fibroblast cultures were infected with HSV-1. Replication was blocked by ara-C and the viral genome maintained in the repressed state by subsequently raising the temperature from 37 to 40.5°C after removal of the inhibitor (Wigdahl *et al.*, 1981.) Activation of HSV-1 replication could be effected by either reducing the temperature or superinfecting the cultures with HCMV. Infectious centre assays indicated that only 0.0002 to 0.02% of the cell population contained a viral genome that could be activated. Subsequent experiments showed that about 10% of the HEL fibroblasts expressed HSV-1 antigens early after activation (Wigdahl *et al.*, 1982a). To further characterise the viral genome it was essential to increase the number of cells containing it that could be activated into the replication cycle. By using combined BVDU and IFN-α treatment, the maximum number of surviving HEL fibroblasts containing an HSV-1 genome that could be "reactivated" to produce infectious virus was increased to between 1 and 3% (Wigdahl *et al.*, 1982b). BVDU inhibits viral DNA polymerase after phosphorylation by thymidine kinase but permits the complete expression of IE genes. The cell culture type was broadened to include cells of a neuronal origin. Dissociated rat foetus sensory ganglia neurones were grown as an essentially pure culture by using antimitotic drugs to eradicate non-neuronal cells (Wigdahl *et al.*, 1983). The culture was pretreated for 24 hours with BVDU and human IFN-α prior to infection with HSV-1 at 2.5 pfu per cell. The virus became undetectable 3 days after infection and remained undetectable if the inhibitor treatment was continued. The virus could also be maintained in this non-replicating state by continued incubation at 40.5°C even though the inhibitors had been removed. It was suggested that this represented the latent state. Once the incubation temperature of the culture was shifted down to 37°C, replication ensued and by

analogy this was construed as representing reactivation. Infectious virus was detectable at 1 - 3 days following inhibitor removal and reduction of the temperature to 37°C, and by 4 - 7 days extensive cytopathic effect was observed. No infectious centre assays were performed but at 3 days following the removal of the inhibitor and at 37°C, 50% of the cells showed virus specific immunofluorescence. This was in contrast with the previous experiments where only about 10% of the HEL fibroblast population expressed HSV-1 antigens early after this "reactivation" (Wigdahl *et al.*, 1982a). The experiments were extended to examine the state of the HSV-1 genome during establishment and maintenance of repressed infection in HEL fibroblasts; during maintenance of repressed infection in rat foetal neurones and during reactivation (Wigdahl *et al.*, 1984a). It was considered likely that many of the infected rat foetal neurones contained more than one HSV-1 genome equivalent by finding that there were 8 - 10 copies of most HSV-1 *Bam* HI DNA fragments per haploid cell genome equivalent. There was a much greater retention of latent virus in the neurones than in the fibroblasts. By examining restriction endonuclease digests of partly purified total cell DNA from latently infected cultures and comparing them with similar digests of reconstructed mixtures of purified HSV-1 virion DNA, no alteration in the size or molarity of the viral terminal or junction DNA fragments was detected. This suggested that the predominant form was non-concatemeric and linear. The authors concur that the results do not preclude the possibility of a minor undetectable, yet important, HSV-1 DNA species being retained in an integrated or non-integrated plasmid or concatemeric form and they could not completely exclude the possibility of integration of the viral genome into the host cell DNA. The rat is not a natural host for latent HSV but a later paper (Wigdahl *et al.*, 1984b) describes this *in vitro* model using human foetus sensory neurones as the host cell type. No detailed characterisation has been reported.

Apart from the serious doubt as to whether repressed replication of the virus by non-physiological inhibitors which permit continued expression of IE genes or by supraoptimal temperatures is the same as being latent, there are two other important criticisms of these results. Firstly, the viral genome predominantly persists in the linear configuration and, secondly, no accurate transcription patterns have been reported and thus the nature of viral gene expression in the repressed state is undetermined. Therefore the model does not fulfil the criteria set out in (v) and (vi) above.

It appears that latency may occur in the non-neuronal cells of the cornea (Schimeld *et al.*, 1982; Abghari and Stulting, 1988) and an *in vitro* tissue culture model has been

described using rabbit corneal epithelial cells, keratocytes and endothelial cells grown separately in culture and infected at 0.1 pfu per cell (Cook and Brown, 1986). At an incubation temperature of 42°C and using acycloguanosine to eliminate any ongoing low level replication, two of these cell types were considered to support a latent infection using a moi of 0.001 pfu per cell (Cook and Brown, 1987). Latent infection was defined as the absence of infectious virus at 42°C and release of infectious virus following transfer to 37°C. Infectious virus was also released by intratypic superinfection up to 29 days post infection demonstrating the continuing presence of input viral DNA in the culture. Furthermore, the resident "latent" genomes were capable of recombining with the superinfecting virus. The question arises as to whether the repressed virus is the same as a latent virus and there are no reports of the transcription patterns of the genome nor of its structure.

An *in vitro* latency system without the need for chemical inhibitors of viral replication has been described (Russell and Preston, 1986). Virus replication in HFL fibroblasts (essentially equivalent to the HEL cells used by Wigdahl and co-workers) infected with HSV-2 at 0.003 pfu per cell was suppressed by infection at the supraoptimal temperature of 42°C. When the culture was transferred to the normal growth temperature of 37°C infectious virus remained undetectable for at least 6 days. The virus could be reactivated by intertypic superinfection at 38.5°C with ts mutants of HSV-1 or with HCMV but not by cell subculture or treatments altering cell metabolism. The HSV-1 mutant *ts*K, which has a mutation in the gene encoding Vmw 175 and hence only produces IE proteins at the non-permissive temperature, was equally efficient at reactivation as a late ts mutant. The reactivation efficiency was very high and reproducible suggesting that one or more of the IE proteins, excluding Vmw175, is responsible for initiating reactivation. Further studies (Russell *et al.*, 1987) with another mutant of HSV-1, *dl*1403, which has a deletion of most of the sequence encoding Vmw110, showed that it was able to establish latency as efficiently as wild type HSV-1 yet it was unable to reactivate HSV-2 when used to superinfect latently infected cultures. This suggested a role for Vmw110 in reactivation in this *in vitro* model and this conclusion has been borne out by the results of others (Harris *et al.*, 1989). Vmw110 expressed by adenovirus recombinants was able to reactivate HSV-2 from latently infected HFL monolayers in the absence of any other known HSV proteins (Harris *et al.*, 1989). Furthermore, a region in the second exon of the gene encoding Vmw110 was shown to be important for this process (Harris *et al.*, 1989). Investigations into the structure of the latent genome were able to demonstrate that the joint fragment was present at approximately twice the molar concentration of fragments from unique regions suggesting fusion of the termini (Preston and Russell, 1991).

Thus the latent HSV-2 DNA was present in the cell nucleus in a non-linear configuration in keeping with that previously demonstrated *in vivo* for HSV-1 (Rock and Fraser, 1983; 1985). There are at least three differences between this model and those discussed above which rely upon inhibitors to induce "latency". Firstly, reactivation in the latter requires more than the presence of HSV IE proteins and not merely the provision of Vmw110 (Scheck *et al.*, 1987), secondly, a reduction in temperature to 37°C is sufficient to lead to viral replication and thirdly, the DNA is predominantly linear. It may be that there are two populations of viral genomes in the inhibitor induced model, one population liable to replicate when the temperature is reduced and the other requiring superinfecting virus to enter the lytic cycle (Shiraki and Rapp, 1986). The system developed by Russell and colleagues may harbour virus largely as the second population.

An extension of this tissue culture system has been reported (Harris and Preston, 1991). The HSV-1 mutant *in*1814 which has a 12bp insertion in the Vmw65 gene with the consequent abolition of its transinducing properties, can be retained in HFL fibroblasts after infection at a low moi and incubation at 42°C. It too, like HSV-2, is reactivated by the provision of Vmw110 from a superinfecting virus. However, latency could also be established by *in*1814 at the physiological temperature of 37°C and because it has low cytotoxicity, at a higher moi of 5 particles per cell. In the presence of ara-C, an inhibitor of DNA synthesis, the viral DNA was maintained at 1 to 8 copies per latently infected cell in a non-linear configuration. Thus the absence of transinduction by Vmw65 predisposes the virus to latency in this system and the genome is in a form analagous to that found *in vivo* (Rock and Fraser, 1983; 1985). These findings support the hypothesis that the failure of transactivation by Vmw65 leads to abortion of the lytic process and directs the virus towards latency. No gene expression from the *in*1814 genome was detectable during latency at 37°C using the polymerase chain reaction with primers specific for thymidine kinase or LATs (Anderson, 1991). This is in contrast to the situation *in vivo*, although it may be explicable on the grounds of the neurone specific nature of the LAT promoter. During latent infection, LATs are only found in cells of a neuronal origin (Batchelor and O'Hare, 1990).

Another tissue culture system based on neuronal cells has been described (Wilcox and Johnson, 1987; 1988). Primary sympathetic neuronal cultures were prepared by dissociating the superior cervical ganglia of prenatal rats and growing them in standard media containing 20μM fluorodeoxyuridine. This antimitotic agent reduces the non-neuronal cell population to less than 5% and produces neuronal densities of

approximately 5000 - 8000 per culture. The cultures could be maintained for up to five weeks after removing the fluorodeoxyuridine and inoculation with HSV-1 without evidence of viral infection. After removing the antimitotic agent the cultures were infected with HSV-1 and following adsorption the viral inoculum was removed and replaced with either medium or medium containing 2% anti-HSV antiserum. Cultures surviving at 14 days post infection were rinsed to remove the anti-HSV antibodies if appropriate. Addition of antibodies to HSV after virus inoculation was not necessary to establish latency but it greatly reduced the number of cultures destroyed by lytic infection. Reactivation was produced by the addition of antibodies to NGF to the surviving cultures. The model satisfied the operational definition of latency (Price, 1986) they selected. At a moi of 0.03 pfu per cell and without the anti-HSV antiserum 48% of the cultures were lost and 11% of the surviving cultures retained latent virus. If the cultures were treated with HSV neutralising antiserum for 2 weeks after the inoculation the moi could be increased to 1 pfu per cell and 53% of the surviving cultures retained the latent virus. Further increments in the moi led to the total destruction of the cells (Wilcox and Johnson, 1987). If the cultures were treated for 12 hours prior to and 7 days post infection with inhibitors which block viral DNA synthesis such as acyclovir or phosphonoacetic acid a higher moi of 5 pfu per cell could be achieved (Wilcox and Johnson, 1988). This procedure totally prevented lytic infection and was considered a rational means to prevent spread and increase the survival and number of cells with latent HSV. At a moi of 5 pfu per cell there was 100% survival and 100% reactivation of the cultures. During the latent phase no viral specific antigens were detected but after 24 hours of NGF deprivation they were in all of the neurones suggesting that the majority harboured latent HSV-1. The discovery that NGF was required to maintain latency in cultures of rat sympathetic neurones was extended and shown that it was also required to maintain latency in cultures of sensory neurones obtained from DRG of rats, monkeys and humans (Wilcox *et al.*, 1990). The neurones of human origin were cultured from aborted foetuses at less than 10 weeks gestation which may not mimic the host cell in the adult. A further finding was that when the human neuronal cell cultures were deprived of NGF there was 100% reactivation of latent HSV. Since the major clinical reservoirs of latent HSV in man are the sensory ganglia and especially the sensory neurones of the trigeminal and DRG, this tissue culture model should be a close approximation in terms of the host cells to the *in vivo* situation. Subsequent workers have used strand specific Northern blot and RNase protection hybridisation analyses to investigate viral transcription during the latent infection of foetal rat DRG neurones *in vitro* (Doerig *et al.*, 1991a). In their system the 2kb RNA was the major species detected although the 1.5kb and 1.45kb species were also present albeit in minor quantities. The LAT was also present 12

hours after reactivation had been initiated by depriving the culture of NGF. The gD mRNA, an mRNA characteristic of the productive infection, was not detected during the latent state but was detected after reactivation had been triggered by NGF deprivation (Doerig *et al.*, 1991a). In another study the more controversial finding of a protein designated a latency-associated viral antigen, LAA, encoded by a region corresponding to the LATs was reported. (Doerig *et al.*, 1991b). This product has not been described *in vivo*. A number of questions remain as to the comparability of this model to the *in vivo* situation, including the unnatural host and the presence of the LAA, but the most outstanding is whether the configuration of the viral genome is linear or non-linear in the *in vitro* neuronal latency system. Studies on this aspect have not been reported.

Unfortunately, the amounts of viral DNA available from primary cultures of sensory neurones are insufficient for extensive study at the molecular level. C1300 mouse neuroblastoma cells bear many of the characteristics of differentiated neurones including the development of neurites and the synthesis of neural enzymes and transmitters (Augusti-Tocco and Sata, 1969; Schubert *et al.*, 1969; Amano *et al.*, 1972). They are relatively non-permissive for the lytic growth of HSV (Vahlne and Lycke, 1978) and this has been assigned to a failure of transcription of IE genes following infection (Kemp and Latchman, 1989). However no transcriptional patterns of LATs were detected in these cells during non-permissive lytic infection and thus some doubt was expressed as to whether the model was analogous to viral gene regulation *in vivo* (Wheatley *et al.*, 1990). Such cell lines may be able to yield latent DNA in sufficient quantities for detailed study and indeed immortalised cell lines derived from neonatal rat DRG fused with a C1300 neuroblastoma cell line have been used to extend investigations of latency *in vitro* (Wheatley *et al.*, 1990). Neonatal rat DRG were cultured in the presence of cytosine arabinoside for 3 days to kill the dividing non-neuronal cells such as fibroblasts and Schwann cells and leave a pure population of non-dividing neurones. These primary neuronal cells were fused with N18 TG2 cells, a HAT-sensitive neuroblastoma cell line derived from C1300 cells, and immortalised HAT-resistant cells were selected in medium containing HAT to produce the ND series of immortalised DRG cell lines (Wood *et al.*, 1990). They retained many properties of the parental DRG neurones such as the ability to synthesise neuropeptides and glycolipid surface markers and respond electrophysiologically to sensory neurone activators and displayed features characteristic of the neurones rather than other ganglion cells (Dodd and Jessell, 1985). They also possessed rat specific markers emphasising their lineage from this animal. One of these cell lines, ND3, was non-permissive for HSV infection at a low moi of 1 pfu per cell to an extent

comparable to the C1300 cells as determined by the low level of immunofluorescent staining with antibody to the HSV-1 IE protein Vmw 175. No transcriptional products from any of the 5 IE genes were detected in nuclear run on assays whereas the converse was shown in permissive 3T3 cells. A selected clone in which the *Hpa* I - *Sst* I fragment containing the IE110/LAT region had been cloned between the promoters for T3 and T7 RNA polymerase was used to infer the presence of the LATs. As the IE110 and LAT genes are on opposite strands, these polymerases will transcribe ssRNA complementary to either IE110 mRNA or LAT RNA. The RNA preparations were hybridised with the radiolabelled products of nuclear run on assays and only the transcription of LAT RNA was detectable in infected ND3 cells and none of the IE110 mRNA. The latter was present in permissive cells. Sensory ganglia do not provide sufficient material to measure the rates of IE transcription during latency *in vivo* so it is not known whether IE mRNAs are not transcribed or rapidly degraded. The authors suggest that this *in vitro* model system is suitable for studying the processes regulating the interaction of HSV with neuronal cells and the establishment of latency *in vivo*. Again this immortalised cell line may not necessarily equate with the natural host cell. To date no data pertaining to the structure of the latent genome in this model have been published. Thus once again the question of how such a system based on abnormal cells relates to the *in vivo* situation still needs to be fully addressed.

1.2.4. Organisation of the latent genome

The structure of the HSV-1 genome during latency in peripheral nerve ganglia and the CNS of mice has been investigated (Rock and Fraser, 1983). Mice were infected with 1 x 10^6 pfu of HSV-1 by corneal scarification. At 2 months post infection explanted cultures of the trigeminal ganglia all gave reactivation but there was no reactivation following explantation of CNS tissue i.e. brain stems, cerebella or cerebra at the same time. This was despite virus being recovered from the same CNS sites at 6 days post infection. The DNA from infected ganglia and brain tissue was used for the analysis of the viral DNA by Southern blot hybridisation. Most, if not all of the viral genome was detected in the latently infected brain tissue which implied that the inability to reactivate the virus was not due to incomplete or defective genomes in the CNS as suggested by others (Cabrera *et al.*, 1980) but more a reflection of the nature of the host cell or the latent virus or both. Using a nick translated ^{32}P-labelled *Bam* HI SP joint-spanning fragment of the viral genome hybridised to *Bam* HI digests of tissue DNA, the viral DNA from both brain tissue at 2 months post infection and pooled trigeminal ganglia at 5 months post infection appeared to lack the terminal fragments of the genome (Rock and Fraser, 1983). This was not due to a lack of sensitivity because

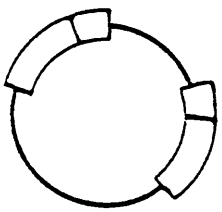
latently and acutely infected brains had SP joint fragments of similar intensity with terminal fragments of differing intensity. In contrast, acutely infected brain tissue DNA when compared with virion DNA showed terminal fragments increasing in size by between about 300 and 500 bp consistent with a reiteration of the "a" sequence found during the productive infection of tissue cultures by others (Post *et al.*, 1980) and in submolar concentrations with a ratio to the joint region of 0.3 as determined by densitometric analysis. This too has been previously observed in acutely infected tissue culture and attributed to the presence of concatemers during replication (Jacob *et al.*, 1979). The results are not compatible with any model where the latent genome persists as a unit length linear molecule. They imply that the viral DNA undergoes a biochemical change in the process of becoming latent. There was no difference in configuration between the latent DNA from the PNS and the CNS and thus the marked difference in reactivation following explant culture cannot be attributed to a failure of the genome to complete this structural change. The loss of the terminal fragments could be due to a deletion of the termini, integration of the DNA via its ends, circularisation of unit length DNA or the linking together of genomes to produce long linear or circular concatemers which may or may not integrate into the host genome (see figure 1.5). A subsequent report (Rock and Fraser, 1985) which examined these possibilities in the same model, showed that the loss of the free ends was accompanied by a quantitative relative increase in the joint fragments. The structure of the HSV-1 genome dictates that circularisation or concatenation will produce a further joint sequence from fusion of the termini. To determine the number of copies of joint sequences to unique sequences the *Bam* HI digests of infected tissue were hybridised with nick translated ^{32}P-labelled *Bam* HI SP and B HSV-1 DNA fragments. The SP fragment hybridises to the joint and termini and the B fragment hybridises to the B and E fragments derived from the long repeat plus some unique sequences of the digested genome. Autoradiographs showed that the intensity of the terminal S and P bands from acutely infected tissue was reduced compared with virion DNA and absent from the latently infected brains and trigeminal ganglia. Quantification by densitometric scanning showed that the molar ratio of joint to unique sequences, taken as 1 in the virion, increased to 1.6 in acute infection and to 2 in the latent state. This confirmed the findings of the earlier experiments. Thus *in vivo* the latent genome cannot be a unit length linear molecule, neither can it have integrated by the termini since they no longer exist. On the basis of these results it is clearly non-linear and could be a unit length circular molecule or a circular or large linear concatemer. Integration remains a possibility but it would need to be via unique sequences otherwise the ratio of joint to unique sequences would fall from 2 to 1. These findings were confirmed in latently infected murine brain stem, spinal cord and cervical ganglia following inoculation via

UNIT LENGTH:

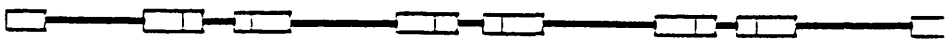
1. Linear

ENDLESS:

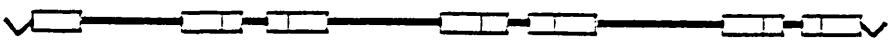
2. Circular



3. Concatemeric



4. Integrated, concatemeric



5. Integrated, linear

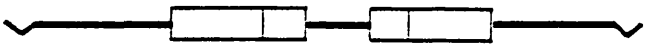


FIGURE 1.5: HYPOTHETICAL STRUCTURES OF THE LATENT HSV GENOME

The unit length, linear genome of the virion has the potential to be organised into a number of forms as is evident from the diagram. As discussed in the text, current evidence suggests that the latent genome is a circular episome.

the ear (Efstathiou *et al.*, 1986). This non-linear DNA was qualitatively and quantitatively stable in mouse neural tissue over a 4 month period, indicating that it was the genuinely latent genetic material rather than DNA persisting from the initial infection which would have been degraded. The viral DNA from human trigeminal ganglia was also shown to be endless and in all 4 isomeric configurations (Efstathiou *et al.*, 1986).

An earlier study (Fraser *et al.*, 1981) investigated the HSV-1 DNA in human brain tissue by endonuclease digestion, separation of the products by gel electrophoresis and hybridisation with labelled HSV-1 fragments. The method was able to detect 1 copy of the viral genome in 200 cells. In some samples only part of the genome was detected and in others the terminal fragments could be detected suggesting a linear non-integrated form. This is completely different to the results from the *in vivo* studies on the latent viral genome in the mouse PNS and CNS (Rock and Fraser, 1983; 1985 Efstathiou *et al.*, 1986) and the human PNS (Efstathiou *et al.*, 1986) described above and it has not been repeated nor confirmed by others.

Other investigators of the structure of the latent viral genome *in vitro* have reported it to be predominantly non-integrated, linear and non-concatemeric (Wigdahl *et al.*, 1984a). This was the first description of the characterisation of the HSV genome in a tissue culture model in which replication could be maintained in a repressed state and activation could be produced. It remains an unanswered question as to whether the use of drugs and elevated temperature to repress replication genuinely mimics the latent state *in vivo*. The DNA from HEL fibroblasts latently infected with HSV-1 was partially purified and digested with either *Xba* I, *Bam* HI or *Hind* III. The digest was subjected to electrophoresis, Southern blotting and hybridisation with a ^{32}P-radiolabelled HSV-1 *Bam* HI SP (K) DNA fragment to detect the joint and terminal fragments of the HSV-1 genome. There were no detectable changes in the molecular weights of the HSV-1 terminal fragments obtained by these three restriction endonucleases. Digestion of the DNA from latently infected rat foetal neurones with *Bam* HI produced terminal DNA fragments similar in size to those obtained from a *Bam* HI digestion of HSV-1 virion DNA. Densitometric scanning of the autoradiographs of the restriction digested DNA isolated from either infected HEL fibroblasts or rat foetal neurones and hybridised with the *Bam* HI SP (K) DNA fragment demonstrated that the molar ratios of the joint SP (K) to the terminal S or P DNA fragments resembled those obtained by a similar digestion of HSV-1 virion DNA in reconstruction experiments. These results suggested that the genome was largely non-integrated, non-concatemeric and linear rather than circular when latent in

fibroblasts and cells of a neuronal origin. The findings contrast with those following *Bam* HI digestion of the DNA from productively infected rat foetal neurones 24 hours after infection. Hybridisation with the *Bam* HI SP (K) DNA fragment showed the joint markedly increased in comparison with the terminal fragments and this was presumed to be due to the presence of replicating DNA concatemers.

The genome structure has been investigated in other tissue culture models of HSV-1 and HSV-2 latency (Harris and Preston, 1991; Preston and Russell, 1991). The DNA from HFL fibroblasts infected with 0.03 pfu per cell of HSV-2 was digested with *Bam* HI and the viral DNA separated from host cell DNA by equilibrium density gradient centrifugation. This technique exploits the differences in the G + C content between human and viral DNA making it possible to separate the HSV-1 genome from the host cell DNA. The ^{32}P-labelled joint-spanning fragment *Bam* HI g was used to probe for the joint and two terminal fragments, *Bam* HI g, u and v respectively. Only the joint was detectable in latently infected material unlike the DNA from reconstructed controls or productively infected cells. Densitometric analysis of the latent genome joint showed an average molarity of 1.85 with respect to the unique fragment *Bam* HI l. This result suggested a circular or concatenated molecule or one integrated at a position other than the termini. Both the formation of a closed circular structure or integration via the unique sequences but not the repeat sequences would give a joint to unique sequence ratio of 2. Two other important observations from this work were made. Firstly, the loss of the termini was a slower process than the fusion of termini occurring during a productive infection (Poffenberger and Roizman, 1985) and secondly, there were up to 8 copies per latently infected cell. This work was extended to include the HSV-1 mutant *in*1814 (Harris and Preston, 1991). The virus readily established latency at a moi of 5 particles per cell if the culture was kept at 42°C. The viral DNA was examined for the presence of the joint fragment, *Bam* HI k and the terminal fragments, *Bam* HI q and s, as latency was established. At 5 hours the termini were still present albeit in lower concentrations than the joint and by 2 and 4 days the joint only was detected. The calculated copy number was between 1 and 8 copies per latently infected cell. The conclusion was that the latent *in*1814 genome was retained in low abundance in a non-linear configuration. These results are in accord with the *in vivo* situation (Rock and Fraser, 1983; 1985) and are in contrast with the *in vitro* model which uses drugs to repress replication (Wigdahl *et al.*, 1984a). The validity of the latter model as representative of the latent state *in vivo* has already been questioned.

The HSV-1 genome can also be separated from host cell DNA by CsCl buoyant density gradient centrifugation in acutely and latently infected mice (Mellerick and

Fraser, 1987). Most of the viral DNA from acutely infected brain tissue was present in the band at the buoyant density of virion DNA. The small amount that associated with the mouse DNA could be released easily by recentrifugation suggesting that it had merely become trapped. This DNA could be composed of the high molecular weight replicative intermediates seen during HSV DNA synthesis (Jacob and Roizman, 1977). Similarly the majority of the latent viral DNA banded at the buoyant density of virion DNA but the small quantity remaining associated with the chromosomal DNA could not be disentangled and was considered artifactual. Since the latent genome was found primarily in an extrachromosomal state, resisted trapping suggesting it was compact and lacked free ends (Rock and Fraser 1983; 1985; Efstathiou *et al.*, 1986), it was postulated (Mellerick and Fraser, 1987) to exist as a circular episome.

An interesting corollary occurs in EBV infection. In non-producer cell lines the EBV genome lacks the terminal fragments (Heller *et al.*, 1981) and it has been shown that the majority of latent EBV DNA is episomal and circular (Lindahl *et al.*, 1976).

Eukaryotic nuclear DNA is associated with histones to form chromatin. The proteins in the chromatin complex are believed to have a role in the regulation of gene activity (Weisbrod, 1982; Weintraub, 1985). The fundamental packaging unit of chromatin is the nucleosome which can be revealed by micrococcal nuclease digestion (Kornberg, 1977). The enzyme attacks the linker regions between the nucleosomes with the histones protecting the core. Thus partial digests produce a series of fragments, each an integral multiple of the nucleosomal repeat unit which can be separated by gel electrophoresis. A typical nucleosomal cellular DNA digest produces a ladder of fragments (Noll, 1974), each a multiple of approximately 180bp (Shaw *et al.*, 1976) reduced to a core monomer of 140bp as the digestion progresses (Whitlock and Simpson, 1976). Mice infected by corneal scarification and retaining latent viral DNA in the trigeminal ganglia as determined by explant assay were used as sources to investigate the genome structure (Deshmane and Fraser, 1989). Nuclear material from the CNS containing >3pg per μg of cellular DNA had to be used because of the inadequate sensitivity of the technique. Micrococcal nuclease digestion produced a typical nucleosomal pattern from all the major regions in the latent DNA as determined by hybridisation with total HSV-1 virion genomic DNA and subgenomic cloned fragments. In contrast, some of the viral DNA in acutely infected brain stems was either resistant to nuclease digestion or was randomly digested to produce a smear down the gel. This latter pattern is suggestive of a naked DNA molecule or one not packaged into nucleosomes. Others have also found that during acute infection in tissue culture (Leinbach and Summers, 1980) or in the mouse CNS (Muggeridge and

Fraser, 1986) only a small fraction of the viral DNA is in a nucleosomal form which implies that at least while replicating the HSV-1 DNA is predominantly in structures other than nucleosomes. It has been proposed (Deshmane and Fraser, 1989) that in the latent state viral transcription is disrupted by the association with cellular histones to form chromatin as a host defence mechanism or to confer long term stability on the genome. The latent genome in the mouse CNS is remarkably resistant to reactivation (Cabrera *et al.*, 1980) which may be a consequence of being converted to chromatin. However, in the PNS the latent genome is reactivated with ease (Cabrera *et al.*, 1980) and might reflect a continuing non-nucleosomal arrangement. Unfortunately insufficient material was available in the PNS for this to have been studied.

1.2.5. Transcriptional activity of the latent genome

There had been a number of reports of the presence of viral transcripts during latent HSV-1 infection of the mouse CNS (Stroop *et al.*, 1984), latent HSV-2 infection of guinea pig sensory ganglia (Tenser *et al.*, 1982) and human sensory ganglia (Galloway *et al.*, 1982) and for the HSV-1 protein product Vmw175 in latently infected rabbit ganglia (Green *et al.*, 1981) before the discovery of the LATs in latently infected mouse sensory neurones (Stevens *et al.*, 1987). Using *in situ* hybridisation and confirming the findings with Northern blotting, cloned probes representing the complete viral genome identified transcripts arising from the long repeat regions. It was shown that the major species of RNA hybridised to a probe from the complementary strand to the IE110 gene and that it had a predominantly nuclear localisation. This was in contrast with acute infection where the transcript was more evenly distributed throughout the cell, and it suggested that the RNA was confined to the nucleus during the latent state. The transcripts could be recovered in the poly(A)$^-$ fraction following oligo(dT)-cellulose chromatography of RNA preparations from latently infected whole ganglia of mice inoculated in the footpad with HSV-1 strain KOS(M) (Wagner *et al.*, 1988a). A poly(A)$^+$ RNA species initially detected in low abundance was subsequently shown to be a contaminant (Wagner *et al.*, 1988b). The major species accounted for more than 90% of the transcripts detected and was shown by agarose gel electrophoresis to be no larger than 2.3kb (Wagner *et al.*, 1988a). The minor species corresponded to a band between 1.8 and 2.0kb. Diffuse signals from RNA originating beyond the nominal 3' and 5' ends of the major LAT were also seen but were of no more than 300 - 350 bases in length and accounted for less than 5% of the major species. No other viral transcripts were detected although species transcribed at low levels or rapidly degraded could not be excluded. The transcripts were mapped using subclones of DNA from the region of the HSV genome around the IE110 gene.

The 5' end of the molecule was characterised in detail and the DNA sequence encoding the transcript analysed. The 5' end of the LAT was located 510 bases to the left of a *Kpn* I site at 0.783 map units and the 3' end just within a 310bp *Sma* I fragment 660 - 970bp to the right of the *Sal* I site at 0.790 map units. It was suggested that the smaller species was a processed form of the major one. The abundance was calculated as 2 x 10^4 - 5 x 10^5 molecules per infected cell and similar results were subsequently reported in latently infected human trigeminal ganglia (Stevens *et al.*, 1988). Only very low levels were detected during the productive infection of cultured rabbit skin cells (Wagner *et al.*, 1988b) which may indicate that the promoter control regions governing the expression of LATs have neuronal specificity.

There are at least 3 poly(A)$^-$ transcripts, usually designated 2.0kb, 1.5kb and 1.45kb, present in latently infected trigeminal ganglia (Spivack and Fraser, 1987) and their genomic location is shown in figure 1.6. These were detected by a virion DNA probe in Northern blots of latently infected tissue following corneal inoculation of mice. The size of these transcripts is at variance with the estimated size in the earlier report based on KOS-M infection (Stevens *et al.*, 1987) but technical differences as well as the different route of infection and site of latency, might account for this. The transcripts were found to accumulate suggesting synthesis during the latent state. They were partly collinear and all mapped to a 3.0kb *Pst* I - *Mlu* I subfragment of *Bam* HI B, partially overlapping by a maximum of 950 bases the 2684 bases of the Vmw110 mRNA which is transcribed in the opposite direction. The 2.0kb transcript was detectable at considerably reduced levels in acutely infected ganglia and in cultured CV-1 cells. The RNAs were present in latent infections with the F, KOS and HFEM strains of HSV-1.

The sequence of the HSV-1 DNA encoding the LAT has been scrutinised for features to suggest it could be eventually translated into a protein product (Wagner et al., 1988a). A 2400 base sequence from a *Sph* I site located to the left of the *Kpn* 1 site at 0.783 map units to the *Sma* I site at 0.794 map units, 660 bases to the right of the *Sal* I site at 0.790 map units, was analysed. There are some features of an RNA polymerase II promoter in the sequence 5' of the cap site and within 86 bases upstream of this cap site there are two potential CAAT box homologies and one possible TATA box homologue (figure 1.7). A pyrimidine rich region including a 26 base run of thymines exists immediately upstream of these homologues and a role in tissue specificity was suggested. Translation initiation signals exist in all three reading frames but only two have codon patterns considered consistent with protein coding (Fickett,

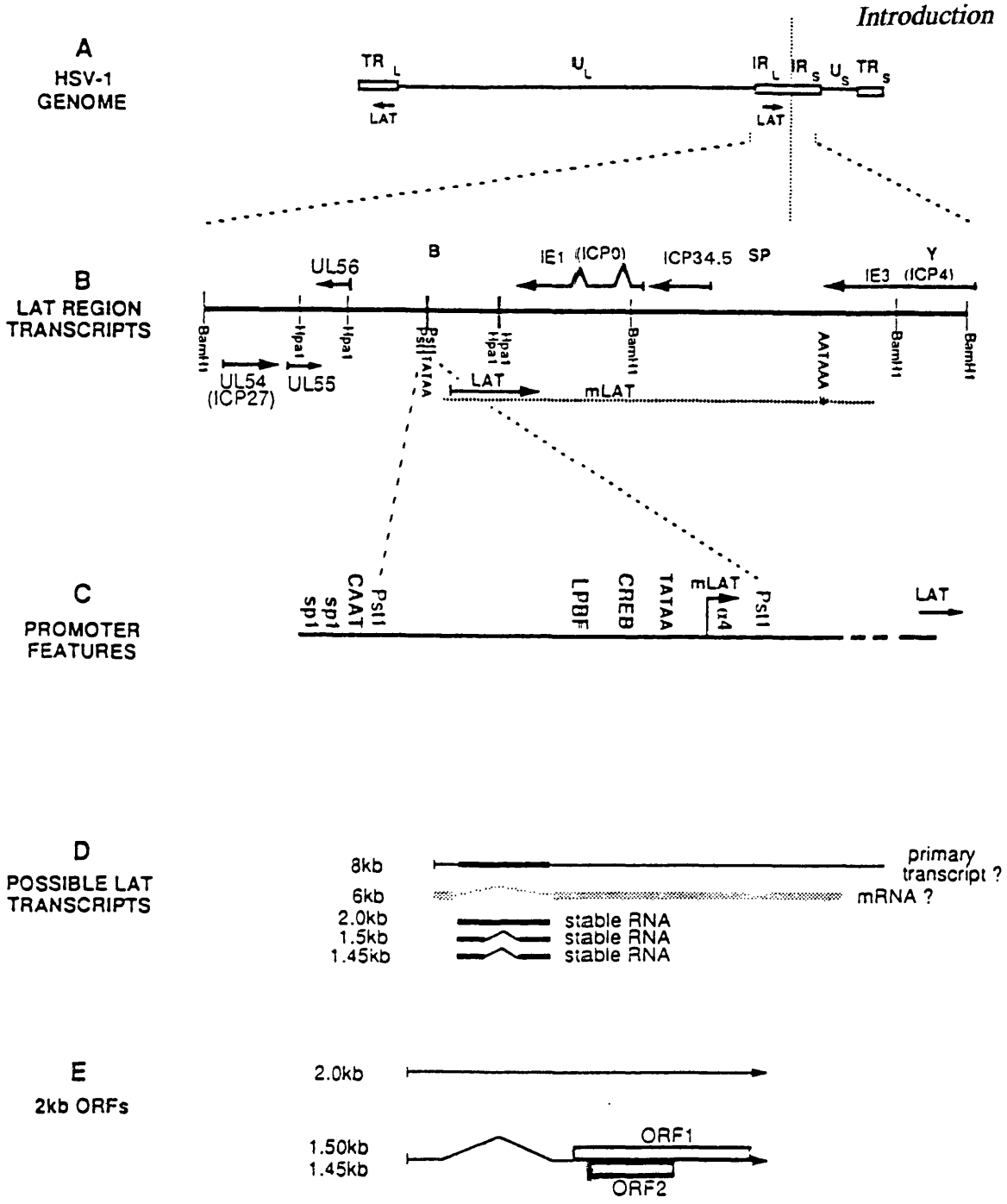


FIGURE 1.6: THE LAT REGION OF HSV-1

The LAT region and the LATs are discussed in the text. The 6.0kb LAT which would result from splicing if the 2.0kb is an intron has not been detected and there is no convincing evidence for protein products from the transcripts. Reproduced from Fraser *et al.*, 1992.

1982). The first, ORF 1, beginning at base 1160 and extending to base 2074 potentially codes for 305aa. The 3' region of this ORF is complementary to 360 bases at the 3' end of the ORF for Vmw110 (Perry *et al.*, 1986). Comparison with the sequence data of the same region of strain 17syn$^+$ (Perry *et al.*, 1986) shows a high degree of homology. The second, ORF 2, is initiated at base 625 and remains open for 207 bases representing 69aa.

Apart from the poor TATA and CAAT box homologies already noted there are no obvious promoter elements within the first 195 nucleotides upstream of the 5' end of the LAT. Nevertheless a sequence analysis of the gene and flanking regions from strain F found potential promoter elements present 700bp further upstream. Some of the sequence elements upstream of LAT are shown in figure 1.7. There is an excellent consensus TATA box at −687, a good CAAT box at −817 and SP1 binding sites at −589, −863 and −887 (Wechsler *et al.*, 1989). Another potential promoter was found just before ORF 1 and ORF 2 with a TATA box at 826 surrounded by 4 SP1 sites (Wechsler *et al.*, 1989). This would however give an RNA slightly smaller than has been detected. Similar sequence information was found in the gene of strain 17syn+ (Wechsler *et al.*, 1988). Examining the LATs from rabbit trigeminal ganglia the two most abundant RNAs appeared to share 5' and 3' ends and to be the result of alternative splicing (Wechsler *et al.*, 1988). These products were 2.0kb and 1.3 - 1.5kb with the smaller LAT 2 having at least one intron spliced from LAT 1. No consensus polyadenylation sequence was found within 4000 nucleotides of the nominal 3' end of the LATs although a small amount of *in situ* hybridisation beyond the 3' end of LATs and within the *Bam* HI SP fragment has been reported (Rock *et al.*, 1987a).

A minimal promoter region necessary for the constitutive activity of the LAT gene in neuronal and non-neuronal cells has been mapped between −161 and −2 (Zwaagstra *et al.*, 1991). A LAT promoter binding factor (LPBF) was identified which bound specifically to a core palindrome within the region and appeared to have a major regulatory role. Deletion of the palindrome led to loss of LPBF binding and an 8 - 30 fold fall in promoter activity (Zwaagstra *et al.*, 1991).

A recombinant virus in which the rabbit β-globin gene coding sequences had been inserted immediately downstream of the upstream potential polymerase II promoter sequence expressed β-globin mRNA but no LATs in latently infected mouse ganglia whereas another recombinant in which this TATA box had been removed did not express the LATs (Dobson *et al.*, 1988). Such a large distance between a TATA box

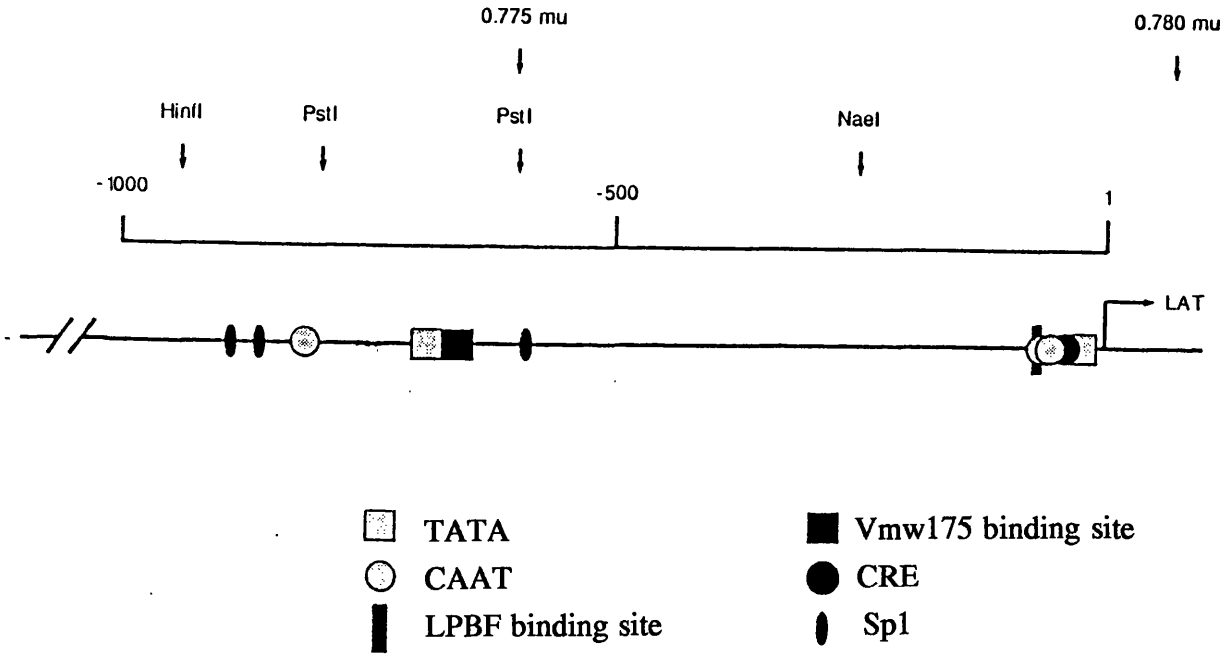


FIGURE 1.7: DNA SEQUENCE ELEMENTS UPSTREAM OF LAT

Sequence analysis has shown the presence of promoter elements 5' of the LAT cap site namely a TATA box and 2 CAAT boxes but as discussed in the text these are poor homologies. In addition there is a cAMP response element (CRE) and an LPBF binding site. The functional LAT promoter is located 700bp upstream of the 5' end of LAT with an excellent consensus TATA box at −687, a good CAAT box at −817, Sp1 motifs and a Vmw 175 binding site.

and the 5' end of its transcript would be unusual but might confer regulatory properties. With the LAT promoter at this location, RNA upstream of the LAT must be synthesised and degraded during the latent phase. Low levels of such RNA were observed by *in situ* hybridisation but not by Northern blotting (Dobson *et al.*, 1988). The RNA generated during a productive infection was used to detect a transcript extending from the LAT promoter to a proposed poly A signal 8.5kb downstream (Dobson *et al.*, 1988) which suggests that the LAT may be processed from a larger product. A larger transcript from a region of the genome adjacent to the LATs had been reported earlier as detected by *in situ* hybridisation and appeared to be weakly transcribed during latency (Deatly *et al.*, 1987). Later a large sized transcript at low abundance in both acutely and latently infected mice was detected with a LAT probe (Mitchell *et al.*, 1990). Thus the LAT could be transcribed from conventional promoter elements far upstream of the transcription unit which encodes the stable species. This finding implies that the LATs which are observed are derived from an unstable primary transcript originating distal to the TATA box 700bp upstream of the cap site and extending to the poly A sequence almost 5kb downstream of the 3' end of the LAT. If this is the case and considering that the stable LAT is bounded by splice sites, not polyadenylated and confined to the nucleus, it has many features of an intron. Using LAT expression plasmid constructs other investigators have reported that LAT is a stable intron (Farrell *et al.*, 1991).

The mutant X10-13 which has a deletion in the 5' end of the LAT transcription unit does not express any detectable LATs nor any other viral transcripts during latent infection in mouse neurones (Javier *et al.*, 1988). This observation suggests that all viral transcripts present during latency are related to the expression of the major LAT and that there are no independent transcription units. This was in keeping with the demonstration of the collinearity of the smaller 1.5 and 1.45kb LATs with the major species except for a 730 base intron (Wagner *et al.*, 1988b). Following corneal inoculation of rabbits with the McKrae strain of HSV-1, the latently infected trigeminal ganglia were shown to harbour the smaller 1.5kb transcript, derived by splicing a 730 base intron from the major LAT. This represented up to 50% of the total viral RNA. A similar proportion was found using the KOS(M) strain in the rabbit and with several strains in the mouse. This was different to the proportion arising from latent infection of the mouse with KOS(M) where the smaller transcript accounted for less than 10% of the total LAT (Wagner *et al.*, 1988). Evidently both the strain of the virus and the host have a role in the processing or the stability of these transcripts which must ultimately be a reflection of DNA sequence variations and host factors. It implies that the relative proportion of the spliced LAT is not a determinant of latency *per se*. The spliced

species was not present in acute infection indicating that splicing occurs after the latent state has been established in mouse or rabbit neurones. Using oligonucleotide probes and confirming the findings with mung bean nuclease protection assays the splice acceptor site was accurately identified. The excision of the intron located between bases 297 and 1033 installs ORF 2 within 250 bases of the transcriptional start site and brings the translational leader length into the same range as other HSV-1 mRNAs.

Direct characterisation of the LATS using a combination of cDNA and PCR cloning demonstrated an intron spliced from the 2.0kb LAT to produce at least one of the smaller LATs (Spivack *et al.*, 1991). The 5' end of this intron has a novel GC rather than the usual GT and it was proposed that this structure within the 2.0kb LAT may be part of a neuronal specific control mechanism yielding the transcripts present in sensory neurones during latent infection and reactivation but absent during the viral replication cycle.

A number of suggestions have been advanced for a role for the LATs (Stevens *et al.*, 1987). Firstly, the RNA may act as a messenger for a protein that is involved in the early stages of the productive cycle but because it is restricted to the nucleus translation is prevented and the productive cycle is aborted. Secondly, the protein product may be required to maintain the latent state and, thirdly, the RNA acts as an antisense molecule to regulate the expression of the IE110 gene by reducing or even eliminating the pool of Vmw110 mRNA.

Although the LATs have ORFs which may suggest a protein product none encoded by these ORFs have been reported *in vivo*. The LATs have 2 ORFs, denoted ORF 1 and ORF 2, common to all 3 sequences of strain F, 17syn$^+$ and KOS (Wechsler *et al.*, 1989). The larger of the two, ORF 1, has a code highly conserved among HSV-1 strains (Wagner *et al.*, 1988a) which some have argued is suggestive of a protein product. It has the coding potential for 305 aa.

If the larger transcript hypothesis is accepted then the LATs will have conventional promoter regions with the translational leader of appropriate length following splicing of the 2.0kb LAT to the 1.5kb LAT. This again is suggestive of a protein product and indeed if, as proposed, the polyadenylation site is 8.5kb from the cap site many more candidate proteins become possible.

Antibodies have been raised to several polypeptides corresponding to different portions of the ORF 1 but no viral protein has been identified (Wechsler *et al.*, 1989).

In another report an antiserum raised in rabbits to a 14aa peptide synthesised from the ORF 1 of the LAT failed to detect any antigens by immunohistochemistry of acutely or latently infected ganglia (Wagner *et al.*, 1988a). Both of these reports refer to unpublished observations. Nevertheless, there have been publications of protein products arising from the LATs. The presence of the IE viral protein Vmw175 in the trigeminal ganglia of latently infected rabbits detected by an indirect immunofluorescence assay using a monospecific antibody (Green *et al.*, 1981) has been difficult to reproduce. Moreover rabbits are subject to frequent spontaneous reactivation which clouds the interpretation. Alternatively, there may be a species dependent translation of LATs since the Vmw175 mRNA was undetected in latently infected mouse ganglia (Deatly *et al.*, 1987). An antiserum has been raised in rabbits against a bacterially expressed fusion protein containing part of a LAT encoded polypeptide (Doerig *et al.*, 1991b). This chimeric protein was produced from the plasmid pATH LAT 2 encoding a chimeric gene, part of which has the carboxy-terminal portion of the LAT ORF 1. Antiserum was also raised to the protein product of another chimeric gene in the plasmid pATH ICP0 which possesses the carboxy-terminal part of the third exon of the IE protein Vmw110. Apart from the virally encoded aa these two proteins are otherwise identical. Using immunocytochemical techniques an antigen recognised by the anti-LAT 2 antiserum was demonstrated in primary neurones latently infected *in vitro*. This antigen, LAA, was not detected by the same techniques in mock infected neuronal cultures, neuronal cultures infected with the LAT deletion mutant *dl*1403 or productively infected Vero cells. The mutant, *dl*1403, has a *Sal* I - *Xho* I deletion in both copies of the LAT gene and was able to establish latent infection *in vitro* with 100% reactivation following NGF deprivation. On the other hand the anti-Vmw110 antiserum whilst not reacting to the latently or mock infected cultures did to the productively infected Vero cells. Furthermore, analysis of the product in Western immunoblots demonstrated a protein in whole cell extracts from latently infected neurones recognised by the anti-LAT 2 antiserum with a mass of 80kd. This was absent in mock infected cultures or in productively infected Vero cells. A 45kd protein of varying intensity was also identified and it was suggested to be a degradation product. Another difficulty was the large discrepancy between the experimental finding of an 80kd protein and the predicted 33kd protein. This may be explicable if larger ORFs are generated by RNA splicing.

There are a number of observations which mitigate against the LATs producing a protein product during latency. Firstly, there has been no unequivocal demonstration of such a product *in vivo*. Secondly, the RNA is located within the nucleus rendering it untranslatable. Thirdly, no convincing evidence of any polyadenylated species has

been found (Wagner *et al.*, 1988b) despite earlier reports (Puga and Notkins, 1987; Wagner *et al.*, 1988a) and, fourthly, there is no polyadenylation signal within 900bp of the 3' end of the major transcript (Perry *et al.*, 1986). This last observation may be undermined by the identification of related transcripts in latently infected ganglia. These have been identified by *in situ* hybridisation (Dobson *et al*,. 1989; Mitchell *et al.*, 1990) or Northern blotting (Zwaagstra *et al.*, 1990) and shown to extend downstream of the major LAT 3' end to a polyA signal 8.5kb from the LAT TATA box. Since RNAs from this region were shown to be polyadenylated in productively infected cells (Dobson *et al.*, 1989) there is reason to believe that this may well be the case in neurones too. An alternative possibility would be a splicing event to generate an ORF with a polyadenylated tail.

The experimental data need not only to be explained but also confirmed before a role for a LAT protein product in either the maintenance of latency or reactivation can be entertained. It has been hypothesised that the LAA could enhance reactivation. This might be the reason for the delayed reactivation of some LAT mutants in explant assays (Clements and Stow, 1989; Leib *et al.*, 1989a; 1989b; Steiner *et al.*, 1989) or the reduced efficiency of reactivation *in vivo* (Hill *et al.*, 1990; Trousdale *et al.*, 1991). Other LAT mutants have the same reactivation kinetics as LAT^+ viruses (Ho and Mocarski, 1989; Block *et al.*, 1990) which could be because there are only minor or no alterations to the ORF 1. If the antigen is present during latency it would presumably need to be inactive until triggered by other events of the reactivation process.

The suggestion that the LATs might suppress expression of IE110 transcripts to which it is partially complementary by an antisense mechanism is attractive because IE110 gene expression is inextricably involved with the initial events surrounding infection (Stow and Stow, 1986) and also appears to have a role in reactivation (Leib *et al.*, 1989b). A deletion mutant within the gene encoding the transcriptional activator Vmw110 was shown to replicate poorly at low moi (Stow and Stow, 1986). Others have shown that although deletion mutants of Vmw110 can establish latency they fail to reactivate efficiently or not at all from murine trigeminal ganglia (Leib *et al.*, 1989b). The relative abundance and the nuclear localisation of the LATs is also in favour of such a mechanism but a major difficulty with the suggestion is that the sense transcript (Vmw110 mRNA) has never been detected during latency. It may be that any sporadic expression of the sense transcript during latency is immediately so overwhelmed by the antisense RNA that it is beyond the current methods of detection. Experiments in which plasmid constructs expressing LAT, Vmw110 and the reporter gene luciferase driven by the HSV-1 thymidine kinase promoter were co-transfected into rabbit skin

cells showed LAT to inhibit transactivation by Vmw110 (Farrell *et al.*, 1991). A number of mechanisms as to how this might occur were suggested. Firstly, the LAT sequesters the IE110 transcript in the nucleus preventing translation, secondly, the RNA duplex formed by LAT and the IE110 mRNA may lead to modification of the IE110 transcript to render it untranslatable or its protein product non-functional or, thirdly, the duplex is a target for degradation by RNA nucleases (Farrell *et al.*, 1991).

The hypothesis that LATs were capable of preventing productive infection by the inhibition of Vmw110 expression through an antisense mechanism has been weakened by the evidence from a number of LAT mutants being able to establish latent infection in animal models. The LAT mutant TB1 is able to establish latency and reactivate with the same efficiency as the parental virus (Block *et al.*, 1990). There were no detectable HSV-1 transcripts in mouse ganglia latently infected with this mutant and although a 0.7 - 0.8kb RNA was apparent in productively infected CV-1 cells it did not accumulate *in vivo* (Block *et al.*, 1990). The authors of the report argue that as disruption of the LATs does not transcriptionally derepress other viral genes a functional role based on an antisense mechanism seems unlikely. They admit that neither a readthrough transcript including sequences downstream of the insert nor the possibility of the insert conferring instability on the transcript *in vivo* could be excluded. The LAT mutant, 1704, can establish latent infection and be maintained without detectable levels of LATs for more than 6 weeks in trigeminal ganglia (Steiner *et al.*, 1989) as measured by the ability to reactivate the virus by explantation. It is difficult to envisage how the null mutants *dl*LAT1.8 (Leib *et al.*, 1989a) and *dl*1403 (Clements and Stow, 1989) can establish a latent infection if Vmw110 antisense repression is the sole means of establishing and maintaining the latent state.

1.3. Potential factors contributing to the latent state

1.3.1. Viral replication

The role of replication in establishing latency is difficult to assess. Results based on inhibited replication are difficult to interpret because ts mutants (Watson *et al.*, 1980) may leak and immune serum (Klein, 1980) or antiviral drugs (Klein *et al.*, 1979) may not be completely effective in preventing viral replication. Using deletion and nonsense mutations in the Vmw63 or Vmw175 genes, three non-replicating viruses were unable to reactivate by co-cultivation of explanted ganglia (Leib *et al.*, 1989b).

Furthermore, one of these, the Vmw175 mutant, could not be reactivated using superinfection of dissociated ganglionic neurones with a replication incompetent virus either and there was no detectable viral DNA in the ganglia (Leib *et al.*, 1989b). Thus the absence of replication at the inoculation site rendered the virus unable to establish reactivatable latency. However, since the input virus would be unable to replicate, the DNA of a latent infection might be below the limits of detection. The findings could also be explained if Vmw63 and 175 have a role later in latency. Findings based on the ability of the mutant *in*1814 to establish latent infection in mouse trigeminal ganglia without detectable replication in the eye (Steiner *et al.*, 1990) support the hypothesis that replication during the initial infection is not a prerequisite for a latent infection. This is in accord with the results of others who were able to detect HSV DNA by a polymerase chain reaction assay in mouse ganglia infected by corneal inoculation 30 days or more previously with replication incompetent mutants, including those with mutations in the IE175 gene, of HSV-1 (Katz *et al.*, 1990). Another mutant which was unable to replicate due a deletion in the IE175 gene was shown to establish latency and stably express a cloned reporter gene in mouse ganglia following inoculation in the footpad (Dobson *et al.*, 1990). Similarly, inhibition of the early stages of replication did not prevent a latent infection being established *in vitro* (Russell *et al.*, 1987). Thus neither productive infection nor viral DNA replication is necessary for the viral DNA to be established and maintained in the ganglia. Replication at the site of inoculation merely increases the number of genomes available to enter the latent state.

Ecob-Prince and others (1993a) identified sensory neurones harbouring latent virus *in vivo* by *in situ* hybridisation. They were able to show that equal pfu of *in*1814, the rescued revertant and the wt virus varied in their ability to establish latency and this was determined by the number of particles in the inoculum. Thus whereas only 3% of the neurones in the DRG were positive for LATs following infection with wt virus this could be increased to about 20% using more particles of the less neurovirulent mutant *in*1814. Increasing the number of particles of wt virus killed the host. As in the observations above these results also show that even though a virus has limited ability to replicate or is unable to form plaques this does not prevent it from establishing latency.

1.3.2. Failure of IE gene expression

The virion structural protein Vmw65 (Heine *et al.*, 1974) which is also a transactivator of the viral IE promoters in newly infected cells, has three essential regulatory domains. The 80aa C-terminal portion is a powerful activator domain

(Sadowski *et al.*, 1988) while one internal domain contacts the DNA and one or more accessory cellular proteins, and another directs specific interaction with helix 2 of the DNA recognition motif within the homeobox domain of Oct-1 (Stern and Herr, 1991). A stable DNA bound protein:protein complex, the IEC, is formed which targets the response elements within viral promoters and initiates the transcription of IE genes within 20 minutes of infection (Mosca *et al.*, 1985) without the need for viral protein synthesis. There are two distinct types of Vmw65 response elements (apRhys *et al.*, 1989) the most familiar being the *cis*-acting TAATGARAT (R=purine) sequence (Preston C.M. *et al.*, 1984; Whitton and Clements, 1984; Bzik and Preston, 1986) in the 5' regulatory region. A single copy of either from the IE175 and IE110 promoter is sufficient to confer responsiveness to Vmw65 (apRhys *et al.*, 1989). As the structural and activation properties of Vmw65 are determined by different domains within the protein it was possible to construct a mutant which only affects the transinducing activity (Ace *et al.*, 1988). The mutant, *in*1814, has a 12bp insertion in the gene encoding Vmw65 which abolishes its ability to transinduce IE promoters as shown by superinfection of cells transfected with a plasmid containing CAT controlled by the IE175 promoter (Ace *et al.*, 1989). The role of the protein as a major structural component in virion assembly is apparently unaffected by the mutation. The mutation also abolished the ability of Vmw65 to complex with cell proteins and the specific regulatory element TAATGAGAT of IE68/12 (Ace *et al.*, 1989). The migration of the viral genome to the nucleus was unimpaired but the absolute level of the IE mRNA was reduced by up to 4-fold in the case of IE110, equivalent to the effect on the rate of synthesis of the protein (Ace *et al.*, 1989). Although *in*1814 was able to infect cells normally at a high moi its plaque forming ability was reduced and it had a much higher p:pfu ratio than wt virus on both BHK and HFL cells. On the latter cell type the ratio was 11 for the wt virus and 1.7 x 10^5 for the mutant. The defect could be partially overcome by prior transfection of a plasmid containing the IE110 gene or by prior infection with UV inactivated HSV *ts*K, which supplies functional Vmw65 in *trans*. Thus although the functional role of Vmw65 is redundant at a moi of 100 particles per cell, its transinducing ability is critical for successful productive infection at a low moi of 1 particle per cell. Even in the absence of Vmw65 there was a basal level of transcription of IE genes which suggests that the primary determinant of IE gene expression is not the transactivator itself. The requirement for Vmw65 at a low moi, as may occur in a natural infection, suggests that there is a threshold level of IE gene products required to effect a productive infection and that this protein has a place in guaranteeing it. It is hypothesised that latency is the ensuing outcome if there is a block to transcriptional activity early in the infection. Using *in*1814 in a tissue culture model it was shown that at a moi of up to 5 particles per cell the viral genome was

maintained at a low copy number in a non-linear configuration (Harris and Preston, 1991). Moreover, the defect could not be complemented by Vmw65 once latency had been established but the silent genome remained sensitive to transactivation and hence reactivation by Vmw110. The fact that *in*1814 established latency at 42°C in tissue culture as efficiently as wt virus on the basis of particles (Harris and Preston, 1991) implies that the insertion does not affect entry or uncoating. The inference is that the reduction of IE transcription by 5 - 10 fold, characteristic of this mutant, is critical for the switch from the lytic to the latent pathway. Using the same mutant in the mouse eye model (Steiner *et al.*, 1990), latency was shown to be established shortly after the arrival of virus in the trigeminal ganglia, some 24 to 48 hours post infection. It was as efficient as the parent or the rescued revertant on the basis of equal pfu but not particles. The efficiency of reactivation by explantation was similarly demonstrated. A cell line of stably transformed mouse L cells constitutively expressing a mutated Vmw65 which lacks the acidic activation domain and hence its transactivating property (Friedman *et al.*, 1988) has a much diminished ability to support HSV-1 replication and this is thought to be due to reduced IE gene expression by dominant interference from the endogenous mutated Vmw65. It appears that the mutant protein competes with the incoming Vmw65 for important cellular factors and transactivation is inhibited. The phenotype of *in*1814 in tisssue culture can be dramatically reversed by a short exposure to HMBA early after infection (McFarlane *et al.*, 1992). There was an increased accumulation of IE mRNA following the exposure indicating a direct stimulation of IE transcription and presumably a primary effect on gene regulation again emphasising the importance of IE gene expression in determining the outcome of infection. The results of the studies with *in*1814 *in vitro* (Harris and Preston, 1991) and *in vivo* (Steiner *et al.*, 1990), are all in accord with the view that a failure of IE gene transcription is the normal route to latency (Kemp *et al.*, 1990).

One hypothesis that could explain failure of IE gene transcription is absence of the viral transactivating factor Vmw65 (Roizman and Sears, 1987). Evidence has been reported which does not support this hypothesis (Sears *et al.*, 1991). The establishment of latency could not be precluded nor could latency be terminated by supplying Vmw65 in *trans* from a recombinant virus.

The replication of HSV is restricted in mouse neuroblastoma C1300 cells (Vahlne and Lycke, 1977). Early investigators found evidence for an inhibitor of replication which was not due to the induction of IFN (Vahlne and Lycke, 1978). The restriction could be overcome by pretreatment of the cells with sodium butyrate which was accompanied by an increase in the transcription of viral IE genes following infection

(Ash, 1986; Kemp and Latchman, 1989). This finding was the first demonstration that a block to HSV IE gene transcription exists in neuronal cells and suggested that the failure of the lytic cycle was a consequence of failed IE gene expression. Subsequently it was shown, using a reporter gene driven by an IE promoter, that the transcriptional repression is mediated by the octamer motif in IE promoters (Kemp *et al.*, 1990). A factor specific to neurones which binds to the TAATGARAT elements and thereby attenuates the activity of IE viral promoters was identified in the ND series of cell lines from immortalised primary rat DRG neurones (Wheatley *et al.*, 1991). The cellular transcription factor Oct-1 (Fletcher *et al.*, 1987) binds to ATGCAAAT in cellular gene promoters (Parslow *et al.*, 1984) and to TAATGARAT in viral IE gene promoters (O'Hare and Goding, 1988; Preston *et al.*, 1988). Plasmids consisting of promoters driving the CAT gene are expressed very weakly in non-permissive ND cells (Kemp *et al.*, 1990) but their activity is greatly enhanced by the co-transfection of plasmids containing isolated TAATGARAT elements of the viral IE or the octamer motifs of cellular gene promoters (Kemp *et al.*, 1990; Wheatley *et al.*, 1991). Furthermore, transfection of these elements increased the permissivity of neuronal cells for HSV infection (Kemp *et al.*, 1990; Wheatley *et al.*, 1991). These findings strongly suggested the presence of an inhibitor that is titrated away by the addition of excess octamer or TAATGARAT motifs. The ND cell line and DRG were shown to contain Oct-2 mRNA (Lillycrop *et al.*, 1991) which has been previously demonstrated to be present in embryonic sensory ganglia and adult neuronal tissues of the rat (He *et al.*, 1989) and brain extracts of the mouse (Scholer *et al.*, 1989). An additional octamer/TAATGARAT-binding protein absent from permissive cells was identified as the lymphocyte- and neurone-specific octamer-binding protein Oct-2 (Lillycrop *et al.*, 1991). Down regulation of IE expression could be achieved by transfection of plasmids that express Oct-2 (Lillycrop *et al.*, 1991) confirming its properties as a repressor in neuronal cells. Oct-2 acts as an activator in B lymphocytes (Muller *et al.*, 1988) suggesting that the neurone must either modify the protein or it is involved with another factor to be rendered a repressor. Thus Oct-2 has the potential to bind to the TAATGARAT to form a complex that would prevent the binding of either Oct-1 or the IEC to the IE promoters thereby reducing IE transcription, aborting the lytic cycle and switching the infection into latency. The RNA encoding Oct-2 is alternatively spliced in both B cells (Wirth *et al.*, 1991) and neuronal cells (Lillycrop and Latchman, 1992) to yield multiple protein isoforms when translated. The isoforms Oct 2.4 and 2.5 are the most abundant in neurones and repress the promoters containing the octamer/TAATGARAT motifs. Since the isoforms 2.1, 2.2 and 2.3 are activators in non-neuronal cells but repressors in neurones it is hypothesised that the conversion

from activator to repressor is mediated by the high levels of the inhibitory 2.4 and 2.5 isoforms (Lillycrop and Latchman, 1992).

The studies with *in*1814 are strongly supportive of the hypothesis that IE transcription is the key in determining the outcome of an infection. The existence of repressors such as Oct-2, with the potential to repress IE transcription in neurones, is also suggestive, as are the results of the experiments of IFN on HSV replication whose effect is directed at the same point in the viral life cycle (section 1.4.4).

1.3.3. Nerve growth factor

NGF is required by sympathetic and sensory neurones *in vitro* and *in vivo* to maintain normal function and also, depending on their age and cell type, to survive (Thoenen and Barde, 1980). The manifold properties of NGF are probably mediated ultimately through its effects on transcriptional regulation (Lindsay and Harmer, 1989; Chao, 1992). A role for NGF in the latent state of HSV has been suggested on the basis of two observations. Firstly, that latency is only clearly apparent in NGF dependent neurones and, secondly, that axotomy or central rhizotomy, which diminishes or abolishes NGF retrograde transport is a very powerful stimulus to reactivation. Wilcox and Johnson were able to show that 24 hours after NGF deprivation of latently infected rat sympathetic neurones HSV-1 or HSV-2 antigens were readily detected in virtually all of the cells. By 48 - 72 hours the cytopathic effect was evident and infectious virus could be recovered (Wilcox and Johnson, 1988). During the latent phase the cells had normal morphological features and no infectious virus either in the culture supernatants or in association with the cells after lysis was apparent. No immunohistochemical viral specific antigens were detected. This observation that NGF was required to maintain latency in cultures of rat sympathetic neurones was also seen in cultures of sensory neurones from the DRG of rats, monkeys and humans (Wilcox *et al.*, 1990). Upon depleting NGF by addition of anti-NGF serum and then replacing it with medium containing excess NGF they were able to show that interruption of the neuronal supply for only 1h led to reactivation of the virus, indicating that the latent virus was very sensitive to changes in the concentration or binding of NGF. The sensitivity was species specific. A species specific monoclonal antibody, Mab-20.4, which blocks NGF binding to the human receptor was able to reactivate latent HSV from human but not rat sensory neurones. Moreover, Mab-192 which despite binding to the rat sensory neurone receptor does not prevent NGF binding to it as well was unable to produce reactivation. It appears therefore, that the effects of NGF on HSV latency are mediated by NGF binding to the NGF receptor

and that the maintenance of latency is susceptible to cellular functions controlled by NGF. When NGF binds to its receptor it is internalised and both factor and receptor are transported to the neuronal cell body. It is possible to interfere chemically with the steps in this process for example by the addition of 6-hydroxydopamine, which destroys sympathetic nerve terminals or colchicine, which prevents retrograde transport. Either of these treatments was able to effect reactivation of latent HSV from cultured sympathetic neurones. A brief period lasting only 1 hour of protein or RNA synthesis inhibition using cycloheximide or 5,6,dichloro-1-D-furanosylbenzimidazole respectively also resulted in reactivation in 100% of the cultures which suggests that a short lived factor may exist in neurones either to maintain the *status quo* or to repress reactivation. This supports other studies (Tenser *et al.*, 1988) where damage inflicted on the neuronal target or to the terminals reactivated virus, suggesting that the tissue actively synthesises a factor necessary to maintain latency. To test whether deprivation of NGF is only a means of inflicting a more general injury and thereby perturbing maintenance to effect reactivation, the cultures were exposed to UV irradiation from doses causing no morphological damage to those which killed the cells. Since no reactivation occurred at any dose it is clear that not all forms of cell injury will waken the latent virus. The effect of NGF deprivation on the viability of the neurone is reversible for 18 hours. This is in contrast to the effect on the latent virus which cannot be reversed after 1 hour of deprivation, suggesting that the virus is able to come out of latency and replicate by responding early to a warning signal that the cell's viability might be under threat. The nature of the NGF signal which maintains latency or suppresses reactivation has not been clarified.

In mobility shift assays using nuclear extracts from adult rat DRG neurones it was shown that Oct-2 protein levels were increased by 3 - 4 fold when the cells were grown in the presence of NGF (Wood *et al.*, 1992). There was a parallel increase in the Oct-2 mRNA but the Oct-1 mRNA was unaffected. This differential up-regulation by NGF provides a mechanism for the repression of the viral IE genes during the establishment and maintenance of latency consistent with the hypothesis that Oct-2 specifically inhibits IE gene expression in neurones. Conversely, the loss of the supply of NGF by a failure of retrograde transport as would occur in axonal damage would lead to a reduction in the levels of the inhibitory Oct-2 and the viral genome would become susceptible to activation by an ensuing rise in IE gene products such as Vmw110 (Leib *et al.*, 1989b).

1.3.4. Latency associated transcripts

A number of mutants have been used to investigate the possible role of the LATs in latency and these are shown in figure 1.8. The mutant X10-13 (Javier *et al.*, 1988) is an HSV-1 x HSV-2 recombinant with predominantly HSV-1 genetic material. It has a sequence deletion in the LAT which includes the characterised LAT promoter 700bp upstream of the 5' end (Dobson *et al.*, 1988; Wechsler *et al.*, 1989). It does not express any LATs or other viral transcripts detectable by *in situ* hybridisation or Northern blotting yet is able to establish latent infection following footpad inoculation of mice and be maintained in the latent state (Javier *et al.*, 1988). Since both mutant and wt virus could be recovered with equal frequency from explanted lumbar ganglia it was deduced that expression of LATs is not required for establishment of or reactivation from the latent state in mouse neurones. However, although reactivation *in vitro* was unaffected (Javier *et al.*, 1988) X10-13 has been shown to reactivate poorly *in vivo* (Hill *et al.*, 1990). Latency was established in the rabbit eye model but the virus could not be efficiently reactivated after iontophoresis of epinephrine onto the cornea (Hill *et al.*, 1990). The deletion was restored by marker rescue to yield virus XC-20 which expresses LATs and reactivates at a significantly higher rate, comparable to that of the wt virus. Both X10-13 and XC-20 were able to establish latency in equivalent numbers of neurones. It was concluded that latent phase transcription of HSV-1 facilitates ganglionic reactivation and subsequent ocular shedding of the reactivated virus. There was no difference between the two viruses in the low level of spontaneous reactivation. There appear to be basic physiological differences between spontaneous and induced reactivation in that intact corneal nerves need to be present to achieve epinephrine induced reactivation but not for indirect reactivation elicited by systemic disturbances such as intravenous cyclophosphamide or dexamethasone. It could be that the LATs are important for direct activation *in vivo* such as caused by sunlight or trauma whereas they have no importance in indirect activation produced by more generalised stimuli such as immunosuppression or pyrexia. It is also feasible that different processes operate in the lumbosacral ganglia to those in the trigeminal ganglia. The difference in the kinetics of reactivation was not apparent when rabbit trigeminal ganglia (Hill *et al.*, 1990) or murine lumbosacral ganglia (Javier *et al.*, 1988) were co-cultivated. Thus reactivation *in vivo* might well be an entirely different process to explant reactivation *in vitro* with a role for the LATs in the former but not the latter.

The interpretation of the results of the experiments using the mutant X10-13 does not account for the possibility of the HSV-2 sequences compensating for the LAT deletion. The recombinant has not been sufficiently well characterised so that even

though the deletion does leave the body of the LAT intact and expression is undoubtedly reduced the conclusions can only be suggestive.

The mutant TB1 is derived from HSV-1 strain HFEM and has 400bp of λ DNA in place of a 168bp deletion within the transcribed portion of the LAT gene (Block *et al.*, 1990). The disruption occurs between bases +839 and +1007 with respect to the RNA start site of the 1.5 - 2.0kb LATs. A 0.7 - 0.8kb RNA was expressed during productive infection in tissue culture but no HSV-1 transcripts were detected in latently infected mouse ganglia or in tissue culture. Nevertheless, the mutant was able to establish latent infection and reactivate as efficiently as the parental or revertant strains from explanted trigeminal ganglia latently infected following corneal inoculation of mice (Block *et al.*, 1990). As the mutant is unable to produce full length LATs they cannot be essential for the establishment of or reactivation from latency as determined by explant cultivation although the smaller 0.7 - 0.8kb product present during lytic infection *in vitro* but not detectable *in vivo* may be functional (Block *et al.*, 1990). Therefore if the LATs have a biological function it must include the region between the conventional promoter (Dobson *et al.*, 1989) and the disruption in TB1. This encompasses a stretch of some 1600 nucleotides and would implicate the first 838bp of the LATs in the process of reactivation. The results do not preclude the possibility of sequences downstream of the insertion site being responsible for reactivation. Furthermore, as the mutant has the coding potential for the putative larger transcript it might be produced at low levels with the possibility of the derived transcripts being present in undetectable yet functional amounts. The absence of the 2.0 and 1.5kb LATs in latent infection with TB1 could still be explicable on the grounds of them being processing products or introns from a functionally important larger transcript (Dobson *et al.*, 1989; Mitchell *et al.*, 1990).

The LAT defective HSV-1 mutant, RH142, has both copies of the LAT disrupted by the insertion of the *E. coli lacZ* gene under LAT promoter control (Ho *et al.*, 1989). LATs were not detectable in either latently or productively infected cells although β-galactosidase expression was readily detectable in sensory neurones of latently infected mice. This mutant was able to grow in cultured cells as well as become established and maintained in the latent state as determined by explant reactivation, in the trigeminal ganglia of mice (Ho *et al.*, 1989). The reactivation kinetics and efficiency were the same as those of the parent. This again indicates that LATs are dispensible for these processes to occur.

The LAT defective HSV-1 mutant, 1704, has a 3758bp deletion in IR_L and the adjacent unique region (Junejo *et al.*, 1991) thus removing most of the IR_L copy of the LATs coding region. In addition it has a 942bp deletion immediately upstream of the coding region of the TR_L copy (Junejo *et al.*, 1991) removing most of the proposed promoter (Wechsler *et al.*, 1988; 1989) but not extending into the 5' end of the LATs. The deletions do not extend into the IE110 gene. The virus was predicted to be a null mutant incapable of producing any putative large or derived transcripts Thus as expected, none of the recognised LATs were detected in the latent state in mice by Northern blotting or *in situ* hybridisation implying that the 942bp deletion may contain the important regulatory elements for the transcripts. The mutant was able to replicate in trigeminal ganglia with kinetics similar to those of the parent virus (17^+) and to establish latent infection as determined by reactivation from explants (Steiner *et al.*, 1989). Since LATs were not detected it was concluded that they are not essential, at least at detectable levels, for the establishment of latency. However, 1704 is slower to reactivate from latently infected explants than the parental strain HSV 17^+ (Steiner *et al.*, 1989) in that 100% of explanted trigeminal ganglia reactivated 17^+ within 7 days but it was 31 days before 89% of the ganglia reactivated 1704. This was the first demonstration of delayed reactivation with a defined mutant and suggested a role for LATs in this process. A role based on an antisense mechanism was considered unlikely since if this were the case a mutant expressing no LATs would be expected to reactivate more rapidly rather than more slowly as was observed. The potential value of accumulated transcripts in favouring reactivation by preparing the genome in a state of readiness for the event was considered more likely. Other explanations for the observations such as the deletion resulting in latency being established in fewer neurones or that there are other unidentified genes which play a part in reactivation and are affected by the deletions were not excluded.

The KOS-derived LAT mutant *dl*LAT 1.8 is also a null mutant expressing no LATs as determined by *in situ* hybridisation (Leib *et al.*, 1989a). The mutation, affecting both copies, removes the putative promoter region (Wechsler *et al.*, 1988), the transcriptional start site and 1015bp of the DNA sequence specifying LATs (Leib *et al.*, 1989a). The mutation completely removes both copies of the LATs (Leib *et al.*, 1989). It is replication competent and capable of establishing latent infection in the mouse ocular model (Leib *et al.*, 1989a). The levels of viral DNA present in latently infected tissue were comparable to those in wt virus infection. Despite being replication competent in the mouse ocular model it reactivated from explanted ganglia with lower efficiency (49%) than the LAT^+ parent (94%) or the rescued virus (85%)

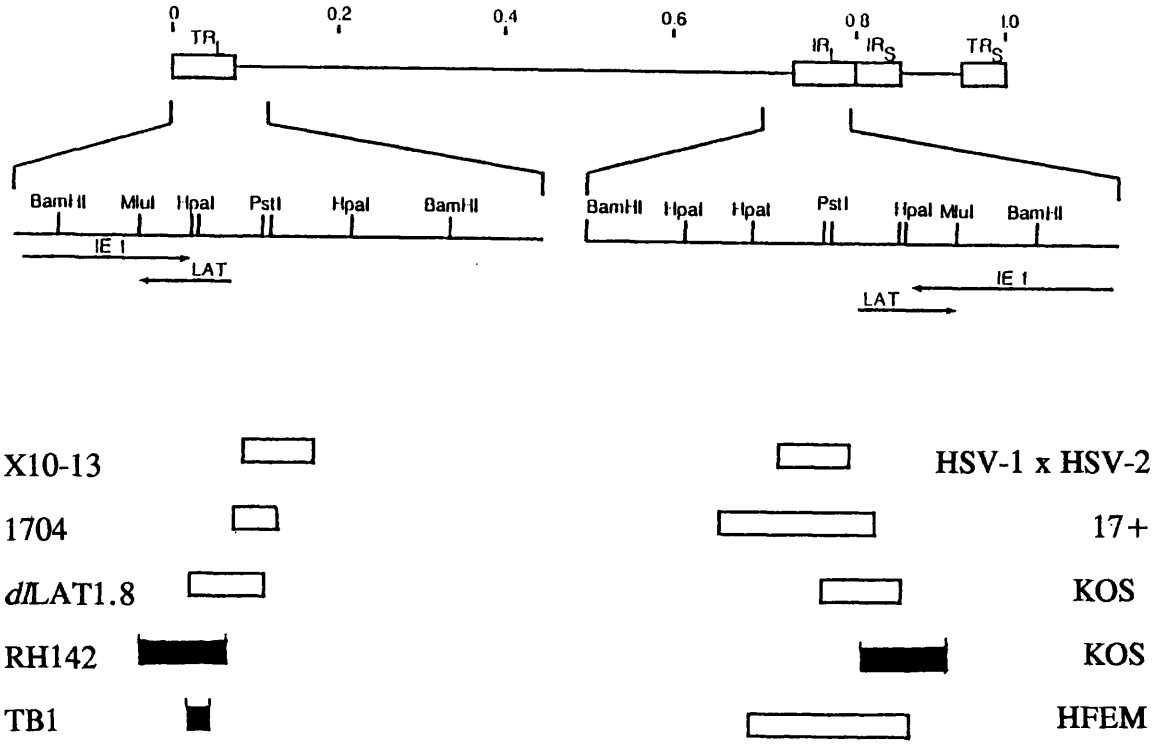


FIGURE 1.8: LOCATION OF VARIOUS MUTATIONS IN THE LAT REGION OF VIRUSES USED IN STUDIES OF LAT FUNCTION

The HSV-1 prototype genome arrangement is shown with *Bam* HI B and E enlarged. The five mutants described in the text are illustrated. X10-13 is an HSV-1 x HSV-2 recombinant predominantly HSV-1 except between 0.396 - 0.404 map units which was derived from HSV-2 and in addition a spontaneous deletion of 1.2kb within both long repeats at sites between 0.019 - 0.076 map units and 0.743 - 0.808 map units (Javier *et al.*, 1988). 1704 is an HSV-1 variant deleted in TR_L and IR_L/U_L (MacLean and Brown, 1987; Junejo *et al.*, 1991). The deletions are 942bp and 3758bp respectively. *dl*LAT1.8 has a 1.8kb deletion which removes the putative LAT promoter and 1015bp of the sequences specifying LAT (Leib *et al.*, 1989a). The mutant RH142 has the *lacZ* gene fused to putative LAT promoter-regulatory sequences 136bp downstream of the transcription start site in both copies of the LAT gene following a deletion from +137 to +509 relative to the putative start site (Ho and Mocarski, 1989). TB1 is derived from HFEM, which has a 4.1kb deletion within the *Bam* HI B fragment and therefore only one complete copy of LATs and contains an additional 440bp segment of bacteriophage λ DNA in place of a 168bp deletion in the transcribed portion of the remaining LAT gene (Block *et al.*, 1990).

again suggesting a role for LATs in this process (Leib *et al.*, 1989a). The absence of ORF 1 in this mutant as well as in 1704 is not the explanation for their impaired reactivation kinetics since the disruption in TB1 also removes the start of ORF 1 and it reactivates normally. The levels of viral DNA present in latently infected tissue were comparable to those after wt infection although a difference in structure or modification might account for the altered biological activity. There was no change in neurovirulence in terms of mortality but microscopic examination of acutely infected tissue revealed more extensive cpe suggesting that higher titres were reached early in infection, presumably because the genes are less efficiently repressed as latency is established. The increased viral damage may result in fewer viable latent neurones and thus explain the reduced frequency of reactivation. The results indicate that LATs are not essential for establishment of or reactivation from latency but they may have a role in the efficiency of the latter process. This explanation might also account for the delayed expression of lytic phase transcription in explanted murine ganglia infected with LAT$^-$ viruses compared with wt virus controls (Sederati *et al.*, 1989).

The possibility of reactivation being delayed in some cases as a result of fewer neurones becoming latently infected has been addressed experimentally (Ecob-Prince *et al.*, 1993b). In this study reactivation *in vivo* following neurectomy was determined by the induction of Vmw110 mRNA although viral antigens were undetectable and the release of infectious virus was not investigated. None of the neurones which expressed Vmw110 mRNA expressed LATs unlike lytically infected tissue where both RNAs could be detected in a single cell. This raises the possibilities of the down-regulation of LATs in reactivation (Rock *et al.*, 1992) due to the expression of an IE gene such as IE175 (Batchelor and O'Hare, 1990), reactivation occurring from latently infected cells in which LATs have become undetectable or reactivation from latently infected cells in which LATs have never been expressed.

The observations made with the various LAT mutants argue against a role for the LATs in the establishment or maintenance of latency although there has been one report which suggests there may be a site dependent function for the LATs in establishing latency (Sawtell and Thompson, 1992). Direct comparison of the relative number of latent infections established by LAT$^+$ and LAT$^-$ mutants in which the LAT promoter drives the *E. coli lacZ* gene during latency showed that the frequency of reactivation by hyperthermia was dependent on the anatomical site. The reactivation frequency of LAT$^-$ mutants was reduced by 80% in the trigeminal ganglia but unaffected in the lumbosacral ganglia of mice compared with wt virus frequencies. The likely explanation is a reduction in establishment of latency since the LAT$^-$ mutants were

shown to establish about 80% fewer latent infections in trigeminal ganglia compared with the LAT^+ mutants but with the same or greater frequency of latent infections in the lumbosacral ganglia. It was concluded that the LATs promote the establishment of latency in the trigeminal ganglia but not in the lumbosacral ganglia of mice (Sawtell and Thompson, 1992). The *in vitro* findings in studies of reactivation are inconclusive. The mutants X10-13, TB1 and RH142 have wt virus reactivation profiles whereas the other LAT mutants 1704 and *dl*LAT 1.8 do not. The lack of correlation of LAT^+ neurones with reactivation (Ecob-Prince *et al.*, 1993) also suggests that these transcripts have no role in this phenomenon. Thus despite the transcriptional activity of the latent genome the question as to whether the products have any part at all to play in the latent state still remains unanswered.

1.3.5. Vmw110

Vmw110 is one of the first proteins synthesised in a productive infection and is a potent transcriptional activator of all three classes of genes, a property first demonstrated in transfection assays (Everett, 1984). It can act alone or with Vmw175 to stimulate gene expression. O'Hare and Hayward proposed that Vmw175 and Vmw110 together may mediate a switch between lytic and latent infection (O'Hare and Hayward, 1985). They studied the *trans*-acting regulatory components of HSV in a transient assay system by analysis of the expression of recombinant constructs. They speculated that if the inhibitory action of Vmw175 and the stimulatory action of Vmw110 on the Vmw175 regulatory region also operated on the Vmw110 regulatory region then a dual action could effect a balanced control of IE expression. An alteration in the balance could favour latency. In addition reactivation could be effected by an interruption of the Vmw175 repressor function.

The mutant *dl*1403 has a 2kb deletion within the gene encoding Vmw110 in TR_L and IR_L (Stow and Stow, 1986) and also includes part of the LATs. It is predicted to produce a polypeptide consisting of the original N-terminal 105aa and 56aa from a different reading frame. It has reduced virulence but is still able to become latent in sensory ganglia following footpad inoculation and reactivate from explants albeit much more slowly than the wt or the marker rescued virus (Clements and Stow, 1989). Thus the presence of functional Vmw110 is not an absolute requirement for reactivation in murine ganglia. Comparison of the reactivation kinetics of *dl*1403 with the wt virus may not be valid. Lacking Vmw110 it will probably enter the replication cycle more slowly and spread less efficiently through the ganglia. The result may well be fewer neurones ultimately becoming latently infected which could account for the altered

reactivation kinetics. Other investigators found that three different deletion mutants of Vmw110, including *dl*1403, varied in their ability to establish and reactivate from the latent state (Leib *et al.*, 1989b). Two of the mutants, *dl*x0.7 and *dl*x3.1, established latency but did not reactivate from standard explant cultures (Leib *et al.*, 1989b). This was not due to poor growth since addition of DMSO to explanted ganglia increased the reactivation of *dl*x3.1 but did not improve its growth characteristics (Leib *et al.*, 1989b). Vmw110 is important for growth in cell culture at low moi (Stow and Stow, 1986). Deletion mutants replicate poorly in tissue culture at low moi but have wt virus patterns of gene expression at high moi. As Vmw110 is important at low moi this might be the reason for the latency incompetence of these viruses since the reactivation process may in effect be equivalent to an infection at low multiplicity. All three deletions also differ in relation to the LATs and the authors were tempted to speculate that in the absence of Vmw110, the more LAT sequences removed the more the virus became latency competent.

In vitro experiments with adenovirus recombinants expressing Vmw110 have shown that this gene product alone is sufficient to reactivate latent HSV-2 (Harris *et al.*, 1989). The transactivation domain of Vmw110 appears to be the same domain required for reactivation (Zhu *et al.*, 1990). These findings confirm the results of earlier investigations into the properties of ts insertion or deletion mutants of HSV *in vitro* (Russell *et al.*, 1987). The Vmw110 deletion mutant, *dl*1403, was unable to reactivate latent HSV-2 unlike *ts*K or *in*1411 which fail to produce functional Vmw175. Similarly latent *in*1814 *in vitro* can be reactivated by superinfection with wt HSV-1 but not with *dl*1403 showing that it too requires Vmw110 to effect reactivation (Harris and Preston, 1991). Vmw110 appears to be a generalised transactivator not specific for promoter sequences. Spontaneous reactivation of latent virus may depend on activation of either this transactivator, if it has been modified during the establishment and maintenance of the latent state, or of cellular factors that mimic generalised transactivators.

Oct-2 may also have a role in reactivation since reduced levels of inhibitory Oct-2 might allow reactivation to take place. The effect of NGF which is required to maintain Oct-2 might be an important mediator of the reactivation stimulus (Wood *et al.*, 1992).

The possibility that IE proteins might affect gene expression very early in infection exists since Vmw175 has been shown to be present in certain virus related particles known as L-particles (Szilagyi and Cunningham, 1991)

1.3.6. Host factors

Reactivation of latent virus by co-cultivation or homogenisation of tissue from the mouse PNS is readily achieved unlike mouse CNS tissue in which it is very difficult or impossible (Deatly et al., 1988). *In situ* hybridisation experiments demonstrated that HSV-1 transcripts from the same region of the genome are present in both of these tissues when latently infected (Deatly *et al.*, 1988) which suggests that the difference in reactivation cannot be attributed to a different transcription pattern between the two during latency. It may be that subtle differences in the structure or in modifications of the latent DNA occur between the CNS and PNS such that host differences become important determinants of reactivation in explanted material.

DMSO can induce the reactivation of the Vmw110 deletion mutant *dl*x3.1 (Leib *et al.*, 1989b), can induce recurrent disease in latently infected mice (Harbour *et al.*, 1983) and increases the frequency of reactivation of HSV-1 from latently infected ganglia (Whitby *et al.*, 1987). DMSO has effects on cellular processes (de la Torre, 1983) so it is suggested that an alteration in the host mediated by DMSO leads to reactivation. The related compound HMBA also enhances the reactivation of latent HSV in explanted ganglia but not replication in tissue culture (Bernstein and Kappes, 1988). It has however been shown to reverse the phenotype of *in*1814 in tissue culture (McFarlane *et al.*, 1992), due to increased IE mRNA accumulation. Since this effect was apparent in the presence of cycloheximide it must involve pre-existing cellular or viral components. It is interesting to speculate that stimulation of IE mRNA production has a part to play in the mechanism of reactivation from latency *in vivo*.

1.4. Interferon

1.4.1. Classification

Interferon was discovered in 1957 (Isaacs and Lindenmann, 1957) as a factor which is produced in virally infected cells and capable of converting other cells into a virus resistant state. It has subsequently been shown that it is not a single entity but a potent and multifunctional family of many members which can rightly be described as cytokines. These are small proteins which are able to influence gene expression and cell physiology. Cytokines are synthesised in response to a number of stimuli and, following secretion, interact with receptors on other cells to regulate cellular

proliferation and differentiation. Those which are produced in and regulate the immune system form a subdivision known as lymphokines. Human IFNs have been grouped into two types and four antigenically distinct classes (Samuel, 1991; Sen and Lengyel, 1992). Type I IFNs include the members of the α, β and ω classes and type II the γ class. The α interferons originate from leucocytes and the class is composed of at least 22 subtypes, each subtype having 70% of their 165 or 166 amino acids in common (Finter, 1991). There is only one member in each of the β and ω (formerly designated as α-2) IFN classes and these originate from fibroblasts. The fourth class, γ IFN, also has only one human member and is very different to all the other IFNs. Since it originates from lymphocytes it is more accurately described as a T cell lymphokine.

IFNs are host specific and have a wide range of modulatory effects including those on the immune system and on the proliferative activity of cells as well as on viral replication. They have a powerful part to play in the immediate response to invasion by viruses, being found in the blood and the tissues during the acute phase of infection.

The antiviral defence may be the main function of IFNs α and β since they are induced by viruses and by naturally occuring as well as synthetic dsRNA (Field *et al.*, 1970). RNA viruses are more potent inducers of IFN than DNA viruses. It has been suggested that dsRNA might be an intermediate product in viral replication boosting interferon production even further during infection of the host cell. Other biological stimuli which enhance IFN-α or -β biosynthesis include bacteria, mycoplasma, protozoa, certain cytokines and growth factors. IFN-γ on the other hand is induced by antigenic stimulation of sensitised lymphocytes.

Human IFN-α, -β and -ω genes form a cluster on the short arm of chromosome 9 and all lack introns. Human IFN-α usually consists of a 166 aa primary protein following maturation in which a 23aa signal sequence is cleaved off the precursor protein. Unlike IFN-β or -γ it is not glycosylated (Weissmann and Weber, 1986).

It is probable that all cell types are capable of IFN production although only when induced. Viral infection activates factors which bind to the virus responsive regions of the human IFN-β (Goodbourn *et al.*, 1985) and IFN-α promoters (Ryals *et al.*, 1985). The former has regulatory elements known as PRDI, PRDII and NRDI sequences (Goodbourn and Maniatis, 1988) whereas the latter has only PRDI-like elements in common (MacDonald *et al.*, 1990). The exact sequence of the PRD confers specificity of induction to a particular virus or a transcriptional activator such as IRF-1 but the

genes may require other activators which bind to the GAAATG sequence in the promoter (MacDonald *et al.*, 1990). The earliest intermediates in IFN induction are IFN mRNAs (Wagner and Huang, 1965) which can be extracted from induced cells and translated in cell free systems (Reynolds *et al.*, 1975). The synthesis of IFN is shut off after induction (Sehgal and Gupta, 1980) and translatable IFN mRNAs are rapidly and selectively degraded (Sehgal *et al.*, 1978). Transcriptional and posttranscriptional controls regulate IFN production (Taylor and Grossberg, 1990).

1.4.2. Interferon induced proteins

IFNs secreted from producing cells bind to cell surface receptors (Aguet and Mogensen, 1983) and trigger multiple pathways of signal transduction which can be distinguished by their susceptibility to various inhibitors. IFN-α and -β bind to the same receptor (Aguet and Blanchard, 1981). The bound IFN is not internalised but eventually released from the receptor intact (Aguet, 1980). The rate of transcription of IFN activatable genes increases within minutes of the cells coming into contact with IFN (Friedman *et al.*, 1984). It peaks within a few hours and some genes may continue to be transcribed at the same rate whereas others may decline even in the presence of IFN (Gupta *et al.*, 1974; Friedman *et al.*, 1984). The relative transcription rates depend on the concentration of IFN (Friedman and Stark, 1985). A consensus *cis*-acting DNA sequence, the ISRE, conferring inducibility by IFNα/β (Levy *et al.*, 1986; 1988) is present in all 5' flanking regions of IFNα/β inducible genes examined to date. ISGF3 is required for the transcriptional activation of the ISRE-containing genes (Kessler *et al.*, 1988) and is present in the cytoplasm as two inactive protein subunits Levy *et al.*, 1989). The α subunit of ISGF3 is activated following the interaction of IFN with the receptor at the cell surface. The mediator is unknown. The activated α subunit combines with the γ subunit to form active ISGF3 (Levy *et al.*, 1989) which is translocated to the nucleus (Levy *et al.*, 1989) and binds to the consensus DNA site (Dale *et al.*, 1989).

There are at least 20 well characterised IFN inducible proteins (Staeheli, 1990) but many others occur at low abundance and it is likely that some have not yet been identified. Continued exposure to IFN may be required to ensure that the full repertoire of proteins is produced. Two IFN inducible proteins that may play a part in the antiviral activity of IFN have been extensively studied. The first, oligo 2',5'adenylate synthetase becomes active in the presence of dsRNA (Zilberstein *et al.*, 1978). It converts ATP to 2'-5'$(A)_n$ where n extends from 1 to about 15 (Dougherty *et al.*, 1980). This product activates latent RNase L which degrades mRNA (Baglioni *et al.*,

1978). The second is a dsRNA activatable protein kinase (Zilberstein *et al.*, 1978). It phosphorylates the α subunit of the peptide initiation factor eIF-2 (Zilberstein *et al.*, 1978) and as a result initiation of mRNA translation is inhibited (De Beneditti and Baglioni, 1984). Another well characterised IFN induced protein is the Mx protein (Staeheli *et al.*, 1984; Staeheli and Haller, 1985). IFN-α induces class I MHC antigens which is not dependent on the presence of dsRNA (Fellous *et al.*, 1982). It does not significantly induce class 2 antigens unlike IFN-γ which induces both of these classes (Collins *et al.*, 1984).

IFN induces the synthesis of other proteins but these are largely of unknown function (Staeheli, 1990).

1.4.3. Interferon and the antiviral state

The antiviral properties of IFN are well documented. Injected IFN is able to protect from some viral diseases (Stewart, 1979) and mice treated with antiserum to mouse IFNs are killed by amounts of virus several hundred fold less than controls (Gresser *et al.*, 1976a; 1976b). Viral replication can be impaired at concentrations as low as 3×10^{-14}M (Kawakita *et al.*, 1978). The antiviral state is non-specific and a number of mechanisms may operate at various stages in the replication cycle of a virus including penetration, uncoating (a general term applied to all events that set the stage for the parental viral genome to express its functions), transcription, translation and assembly of progeny viruses. It seems likely that several steps are affected and that some viruses will be more susceptible at certain stages than others.

The dependence of oligo 2',5'adenylate synthetase and the protein kinase on the presence of dsRNA has led many to postulate the existence of dsRNA intermediates in viral replication. The activation of these pathways could therefore lead to the destruction of viral mRNA or inhibition of its translation. It has been reported that oligo 2',5'adenylate synthetase is induced in mouse trigeminal ganglia during the acute phase of infection with HSV together with interferon activity and that both activities disappear once latent infection is established (Sokawa *et al.*, 1980). Another investigation (Lewis, 1988) in which clones of an IFN resistant murine cell line were infected with vesicular stomatitis, Mengo virus and reovirus, found that the effect of IFN on the oligo 2',5'adenylate synthetase and eIF-2 kinase pathways did not correlate with their antiviral properties. In particular, one clone had greatly elevated levels of oligo 2',5'adenylate synthetase but no antiviral state and another had an activated antiviral state but no elevation of oligo 2',5'adenylate synthetase activity. Thus these

two enzyme systems alone are not sufficient to explain the antiviral properties of IFN and other mechanisms are likely to contribute. This is also suggested by the finding that certain strains of HeLa cells constitutively express high levels of oligo 2',5'adenylate synthetase but require IFN treatment to acquire the antiviral state (Meurs *et al.*, 1981). It has been proposed that the *in vivo* levels of $2'5'(A)_n$ can be determined by agents other than oligo 2',5'adenylate synthetase. The present experimental evidence appears to show that functional oligo 2',5'adenylate synthetase is not an absolute requirement for the antiviral state to be established by IFN. On the other hand, a functional eIF-2 system does appear to correlate with the antiviral activity of some virus cell systems (Whitaker-Dowling and Youngner, 1986). The mechanisms dependent on dsRNA activated pathways may be more important for RNA viruses than DNA viruses and some viruses may be able to overcome some of the effects of interferon. This might be relevant to HSV infection during which inhibitors of RNase L are formed which would impair the effect of the oligo 2',5'adenylate synthetase pathway (Cayley *et al.*, 1984). This could also account for the relatively high doses of IFN required to inhibit the growth of herpes simplex compared with other viruses (Cayley *et al.*, 1984).

The Mx protein in the mouse which is induced by IFN-α and -β is able to establish the antiviral state against influenza virus (Staeheli *et al.*, 1984). A human counterpart induced by IFN-α has been described (Staeheli and Haller, 1985).

The induction of MHC antigens (Heron *et al.*, 1978) may contribute to the antiviral response by enhancing the lytic effect of cytotoxic T lymphocytes which is virus specific and histocompatability antigen restricted (Hood *et al.*, 1983; Schwartz, 1985). The antigenic threshold might be breached by the IFN induction and since MHC antigens present viral antigens to T cells this would elicit an efficient T cell response and an important host defense mechanism to virus infection.

1.4.4. Interferon and herpes simplex virus replication

Studies using cultured fresh human monocytes (Linnavuori and Hovi, 1983) found that replication following infection with HSV was either aborted or highly restricted at a high moi of 1 pfu per cell but not at a low moi of 10^{-4} pfu per cell. IFN production was evident in those cells infected at the high moi but not in those infected at the low moi. Furthermore, the productively infected monocytes differentiated into macrophages, a process prohibited by infection at a high moi of HSV and also by exogenous IFN. Antisera to IFN-α/β enhanced the rate of replication and production

of HSV. These results suggest that IFN induced in monocytes by HSV prevents replication of the virus either directly or by inhibiting differentiation into macrophages or both. Extensive cell death was not evident at the higher moi from which it could be inferred that IFN reduced the cytopathic effect

Early studies of HSV-1 products in infected mouse L-cells had found that IFN inhibits HSV replication at an early stage in the viral life cycle. This was evident from the decreased levels of the early proteins, thymidine kinase and DNA polymerase, of the replicating virus (Panet and Falk, 1983). Later, the expression of early genes was shown to be inhibited suggesting that the inhibition occurred prior to or during early protein synthesis (Domke *et al.*, 1985). These findings were confirmed in human macrophages and in addition reduced immunofluorescence of the IE protein, Vmw175, was observed (Domke-Opitz *et al.*, 1986). Reduced translation of IE proteins in IFN treated mouse macrophages has also been shown by other investigators although the synthesis of Vmw175 mRNA was only moderately inhibited (Straub *et al.*, 1986). These results indicated that IFN primarily acts at the translational level. Some studies have suggested different mechanisms to explain the effects of IFN. Munoz and Carrasco were unable to show any gross inhibition of viral protein synthesis by IFN but rather the production of defective virions (Munoz and Carrasco, 1984) while others found IFN blocked the late stages of morphogenesis with reduced or delayed synthesis of glycoproteins D and B (Chatterjee *et al.*, 1985). In the latter experiments cloned IFN was used and at a dose of only 100 units per ml which might explain the different results to those of their predecessors. Pretreatment of cultured mouse splenic macrophages with murine IFN-α/β for 18 hours led to a stable inhibition of HSV-1 replication for more than 50 hours (Mittnacht *et al.*, 1988). There was no difference between IFN pretreated cultures and controls in the quantity of viral DNA present in nuclei shortly after infection indicating that there was no inhibition in the uptake of the virus or transport to the nucleus and no breakdown of the viral genome. The analysis of viral DNA, RNA and protein synthesis in these cultures identified the expression of the IE genes as the major target of IFN mediated inhibition. Immunofluorescence studies using a monoclonal antibody specific for Vmw175 showed a marked decrease in the nuclear fluorescence of the viral antigen indicative of an inhibition early in the replicative cycle, and the analysis of nuclear run off transcripts demonstrated a significant decrease in IE transcription rates following the pretreatment (Mittnacht *et al.*, 1988). Using probes specific for the Vmw175 gene and examining the transcripts on Northern blots the inhibition appeared to be to the onset of transcription. It was suggested that this inhibition *per se* is responsible for the depression of early gene expression which had been observed previously (Domke *et al.*, 1985). In contrast, the

cellular actin gene and overall transcriptional activity was comparable in controls and pretreated cultures at all times (Mittnacht *et al.*, 1988). A similar finding with another DNA virus, SV40, has also been reported where the block mediated by IFN was specific for the onset of but not ongoing transcription (Brennan and Stark, 1983). Possible explanations as to how IFN might achieve this effect on IE genes include blocked uncoating of the viral genome rendering it inaccessible to transcription, direct inhibition of the IEC or a direct negative effect on IE gene transcription by an IFN induced cellular activity. In an attempt to narrow down these possibilities the effect of IFN-α pretreatment on HSV-1 infection of human HEp-2 cells was investigated (Oberman and Panet, 1989). They were able to confirm the earlier findings and hypothesised that an uncoated viral genome would be more sensitive to DNase digestion than the input DNA. This hypothesis is based on the supposition that the parental viral nucleoprotein undergoes a structural change at the initiation of transcription and that this change is reflected in an increased sensitivity to digestion by pancreatic DNase I. They found that virion DNA in the absence of nuclei was completely resistant to DNase I but at 1 hour post infection had become extremely sensitive. This was interpreted as evidence that the genome had become uncoated. Since IFN had no effect on this sensitivity they argue that its mode of action is not related to the uncoating of the viral genome but is primarily at the initiation of IE gene transcription. This limits the site of IFN action even further. Outstanding possibilities which remain include the involvement of cellular anti-HSV factors induced in IFN treated cells to reduce IE gene mRNA production or the effect of IFN on Vmw65 which has a particular role in transactivating IE genes. A role at this point in the viral replication cycle would satisfactorily explain why pretreatment with IFN is required for it to be effective and why a higher moi is able to overcome it.

The specific effect of IFN on the transactivation event has been explored further (De Stasio and Taylor, 1990). Both human recombinant IFN-α and IFN-γ were shown to inhibit viral replication at an early stage in human cell lines as evident by reduced IE mRNA accumulation (De Stasio and Taylor, 1990). Northern blotting demonstrated that the inhibition involved IE genes and when the IE response element was linked to a reporter gene as for example in p110CAT, IFN inhibited the transactivation. The transcription of an unrelated gene in the presence of Vmw65 was unaffected by IFN treatment. There was no abnormal degradation of mRNA by IFN treatment. The site of IFN action thus has been narrowed down to a direct effect either on the transactivator Vmw65 or on the IEC, the interaction of the transactivator with the IEC or a modification of the IEC following its assembly. The effect of IFN-α treatment on the level of Oct-1 activity in a transformed human B lymphoblastoid cell line (Daudi

cells) has also been investigated (Dent *et al.*, 1991). Oct-1 is a ubiquitous DNA-binding protein which plays an essential part in gene expression (Affolter *et al.*, 1990). IFN-α treatment reduced the levels of this protein and specifically inhibited gene expression from octamer containing promoters. Mobility shift assays demonstrated that treatment for 48 hours but not 24 hours decreased the level of the Oct-1 protein-DNA complex. The authors argue that down-regulation of Oct-1 may be responsible for the failure of IE transcription following IFN-α treatment with the consequent abortion of the viral lytic cycle.

It has not yet been possible to distinguish experimentally between the various hypotheses for the effect of IFN on the early events in the viral life cycle.

2. MATERIALS

2.1. Virus stocks

The HSV-1 viruses used were all derived from the parent HSV-1 strain 17 (Brown *et al.*, 1973) and supplied by C.M. Preston. The mutant *in*1814 has a 12bp insertion in the gene encoding Vmw65 (Ace *et al.*, 1989) and 1814R is the rescued revertant (Ace *et al.*, 1989). The mutant *in*1820, constructed by C.M. Preston, is derived from *in*1814 with the Vmw110 promoter replaced by the MoMuLV enhancer. The mutant *in*1863 has the *E. coli lacZ* gene under the control of the CMV enhancer inserted in the TK gene of 1814R and *in*1883 is derived from the same insertion in the TK gene of *in*1820. The mutant *in*1835 has the *E.coli lacZ* gene under the SV40 promoter replacing the LAT promoter in *in*1814. Insertion of the *E.coli lacZ* gene under the control of the Vmw110 promoter into the TK gene of *in*1820 yielded the mutant *in*1884. The mutant *dl*1403 has a 2kb deletion within both the TR_L and IR_L copies of the gene encoding Vmw110 (Stow and Stow, 1986) and *ts*K has a temperature-sensitive mutation in the Vmw175 gene in which a single C:G bp in the wt gene is replaced by T:A in the mutant gene (Davison *et al.*, 1984).

2.2. Tissue culture cells

Two cell types were used. BHK-21 clone 13 (BHK) cells were as described by earlier investigators (MacPherson and Stoker, 1962) and HFL cells were Flow 2002 cells, provided by Flow laboratories.

2.3. Tissue culture media

EF10	Eagles BHK 21 Glasgow MEM without L-glutamine, tryptose phosphate broth and sodium bicarbonate (Gibco BRL) supplemented with 0.25% $NaHCO_3$, 100 iu/ml penicillin, 100

	μg/ml streptomycin, 4mM L-glutamine and 10% foetal calf serum (Gibco BRL).
ETC10	Eagles BHK 21 Glasgow MEM without L-glutamine, tryptose phosphate broth and sodium bicarbonate (Gibco BRL) supplemented with 0.25% $NaHCO_3$, 100 iu/ml penicillin, 100 μg/ml streptomycin, 4mM L-glutamine, 10% newborn calf serum (Gibco BRL) and 10% tryptose phosphate broth.
EHu2	EF10 with 2% human serum instead of 10% foetal calf serum
EHu5	EF10 with 5% human serum instead of 10% foetal calf serum
Trypsin	0.25% (w/v) trypsin (Difco) in Tris-saline
Versene	0.6M EDTA in PBSA containing 0.0015% (w/v) phenol red

2.4. Solutions

alkaline transfer solution	0.4M NaOH, 0.6M NaCl
neutralising solution	0.5M Tris HCl pH 7.0, 1M NaCl
PBSA	170mM NaCl, 3.4mM KCl, 1mM Na_2HPO_4, 2mM KH_2PO_4 and buffered to pH 7.2
PBSB	6.8mM $CaCl_2$
PBSC	4.9mM $MgCl_2$
PBS	8 parts PBSA : 1 part PBSB : 1 part PBSC
20 x SSC	3M NaCl, 0.3M sodium citrate. Filtered.

20 x SSPE	3.6M NaCl, 0.2M Na_2HPO_4-NaH_2PO_4 buffer pH 7.4, 20mM EDTA
TBS	140mM NaCl, 10mM KCl, 10mM Tris HCl and 1mM $MgCl_2$ pH 7.0
Sucrose solutions	all sucrose solutions were treated with 250μl of DEP to destroy endogenous nucleases and then autoclaved to inactivate the DEP.

2.5. Reagents

The reagents used were of the highest purity and obtained from the following suppliers:

BDH Chemicals, Beecham Research Laboratories, Bethesda Research Laboratories, Bio-Rad Laboratories, Fluka, Kochlight Limited, May and Baker, Melford Laboratories, Merck, Sharp and Dohme International, Pharmacia Chemicals and Sigma Chemical Company Limited.

2.6. Radiochemicals

These were all supplied by Amersham International plc. Specific Activities were 40 - 60 Ci/mmol for [methyl-^{3}H]thymidine and 3000 Ci/mmol for [α-^{32}P]dNTPs.

2.7. Commonly used buffers

L buffer	0.1M EDTA pH8.0, 10mM Tris HCl pH 7.6 and 20mM NaCl
lysis buffer	10mM Tris HCl pH 7.5, 2mM $MgCl_2$, 10mM NaCl and 0.5% Nonidet P40

TE	10mM Tris HCl, 1mM EDTA pH 7.5
TNE	10mM Tris HCl pH 7.5, 1mM EDTA pH 7.5 and 10mM NaCl

2.8. Electrophoresis buffers

EEB	5mM sodium acetate, 1mM EDTA, 40mM Tris HCl pH 7.8
TBE	90mM Tris, 1mM EDTA, 90mM boric acid pH 8.3
5 x TBE loading buffer	A few grains of bromophenol blue powder added to a 1:2 dilution of 10 x TBE in glycerol

2.9. Hybridisation mixtures

50 x Denhardt's solution	1% (w/v) ficoll, 1% (w/v) BSA fraction V, 1% (w/v) polyvinylpyrollidone
DNA/RNA prehybridisation mixture	50% formamide (deionised using 'Amberlite' MB-1 monobed mixed resin immediately before use), 5 x SSPE, 5 x Denhardt's solution, 100μg/ml of sonicated and denatured calf thymus DNA. pH adjusted to 7.5 with 1M HCl as measured by indicator paper
DNA/RNA hybridisation mixture	50% deionised formamide, 5 x SSPE, 1 x Denhardt's solution, 100μg/ml of sonicated and denatured calf thymus DNA. pH adjusted to 7.5 with 1M HCl as measured by indicator paper
DNA/DNA hybridisation mixture	7% SDS, 0.5M Na_2HPO_4/NaH_2PO_4 buffer pH 7.4

2.10. Reaction mixtures

histochemical reaction mixture (stain) for β-galactosidase activity	1mg/ml 5-Br-4-Cl-3-indolyl-β-D-galactopyranoside (X-gal), 5mM potassium ferricyanide, 5mm potassium ferrocyanide, 2mM $MgCl_2$ and 0.02% Nonidet P40. The X-gal was dissolved in dimethylsulphoxide (DMSO) at 40mg/ml and then diluted into the reaction mixture
micrococcal nuclease reaction mixture	0.25M DEP treated sucrose, 0.1mM PMSF, 20mM PIPES pH 7.0, 1mM $MgCl_2$, 1mM $CaCl_2$, 10mM NaCl and 5mM 2-mercaptoethanol added immediately before use. The PMSF was made as a fresh 50mM stock solution in methanol and diluted to 0.5mM in distilled water immediately prior to use
oligolabelling reaction mixture	250mM Tris HCl and 25mM $MgCl_2$ buffered to pH 8.0, 50mM β-mercaptoethanol, 1M HEPES pH 6.6 and 27 OD units/ml of a random mixture of hexamer oligonucleotides
proteinase K reaction mixture	20mM Tris HCl pH 7.5, 2.5mM EDTA pH 7.5, 0.5% SDS and proteinase K at either 100μg/ml or 250μg/ml
TK reaction mixture	50mM Na_2HPO_4/NaH_2PO_4 buffer pH 6.0, 10mM $MgCl_2$, 5mM ATP, 100μM dTTP and 10μCi [methyl-^{3}H]thymidine

2.11. Enzymes

Klenow fragment DNA polymerase and proteinase K were supplied by Boehringer Mannheim GmbH and micrococcal nuclease by Pharmacia LKB Biotechnology. Restriction enzymes were supplied by Boehringer Mannheim GmbH or New England Biolabs, Inc.

2.12. Other materials

Bradford reagent	0.01% coomassie brilliant blue G, 0.0003% w/v SDS, 4.75% v/v ethanol and 8.5% w/v phosphoric acid.
BSA	provided by C.M. Preston
IFN-α	Human Lymphoblastoid Interferon; Hu IFN-α-Ly supplied by Sigma Chemical Company, Ltd. Dissolved at 10^6 iu/ml in sterile water and stored in aliquots at −70°C. One unit of IFN-α protects 50% of indicator cells from viral cytopathology.
Carmalum stain (Kirkpatrick's modification)	This was provided by the pathology department at the Institute of Neurological Sciences, Glasgow where it was made according to the following recipe: 25g carmine was added to 25ml glacial acetic acid and 600ml distilled water. After standing for 20min, the mixture was boiled for 1h, and 25g aluminium potassium sulphate added after cooling. 400ml of distilled water was added and the mixture reboiled, cooled and filtered. One crystal of thymol was added. Stored at 4°C
Giemsa	1.5%(v/v) suspension of Giemsa stain in glycerol obtained from BDH Laboratory supplies heated to 56°C for 2h and diluted with an equal volume of methanol.

3. METHODS

3.1. Cell culture

HFL cells were grown in EF10 in 175cm^2 plastic culture flasks at 37°C in an atmosphere of 5% CO_2 and 95% air. The monolayers were harvested by washing once with 10ml of versene, once with a 10ml 1:1 mix of versene:trypsin and suspension in 10ml of fresh EF10. The homogeneous cell suspension was used to seed subsequent monolayers. For further passage, the cell suspension was subcultured at 1:5 dilution into flasks each containing 50ml of EF10. To seed monolayers on tissue culture plates, 10ml of the cell suspension was diluted to 50ml in EF10 and 2ml, 5ml or 15ml pipetted onto 35mm, 50mm or 90mm diameter tissue culture plates respectively and incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Monolayers were usually confluent within 24h.

A confluent monolayer of HFL cells in a 175cm^2 flask has about 1.5 x 10^7 cells and on a 35mm, 50mm or 90mm diameter plastic tissue culture dish approximately 10^6, 2 x 10^6 and 7 x 10^6 cells respectively.

3.2. Preparation of a herpes simplex virus stock

BHK cells from a seed stock of 10^7 cells per ml were grown in 850cm^2 plastic roller bottles. Each of four bottles received 8 x 10^7 cells in 150ml of ETC10 and were incubated at 37°C overnight until at least 50% confluent. The medium was replaced with 50ml ETC10 containing approximately 2 x 10^6 pfu of *in*1820 seed stock and incubation was continued for a further 3 days at 31°C when extensive cpe was apparent. The bottles were shaken to disperse the cells and the combined medium centrifuged at 2500rpm for 10 minutes in a GSA rotor in a Sorvall RC5B centrifuge. This procedure separated the virus preparation into cell released virus in the supernatant and virus still associated with the cells in the pellet. The supernatant was centrifuged at 12000rpm in a GSA rotor for 2h at 4°C and the pellet resuspended in 10ml of the supernatant. The viral stock was dispensed into small aliquots and stored at -70°C. Only cell released virus was used.

3.3. Titration of a herpes simplex virus stock

The virus stocks were titrated on confluent monolayers of both BHK and HFL cells grown on 35mm diameter tissue culture dishes. Serial 10-fold dilutions were made in ETC10 for titration on BHK cells and in EF10 for titration on HFL cells. Monolayers in triplicate from which the medium had been withdrawn were inoculated with 200μl of each dilution. Following absorption at 37°C for 1h, the infected monolayer was overlaid with EHu2 in the case of BHK cells and EHu5 in the case of HFL cells and incubation continued for 3 days. The medium was removed and the monolayer stained with Giemsa at room temperature for 20 minutes. The tissue culture dishes were rinsed under running water and the plaques counted using a microscope. The number of pfu per ml was determined.

3.4. The *in vitro* latency system

The system described below is a modification of that described by earlier investigators (Russell and Preston, 1986; Harris and Preston, 1991).

Typically, confluent HFL monolayers grown on 35mm, 50mm or 90mm diameter tissue culture plates were treated with IFN-α at 10^3 units per ml in 2ml, 5ml and 15ml of EF10 respectively. Treatment was continued at 37°C for 18h and then the medium aspirated with a Pasteur pipette and the monolayer washed with fresh EF10 to remove all residual IFN-α. The cells were inoculated with virus usually at 120 particles per cell in a volume of 200μl, 400μl or 1.2ml according to the size of the plate. The monolayers were incubated for 1h at 37°C with rocking at 20 minute intervals to allow the virus to adsorb to and penetrate the cells. For the determination of plaque numbers, EHu5 was added to make up to the requisite volume and incubation at 37°C continued for 72h, medium was poured off, the plates stained with Giemsa for 20min and gently washed under running water. Plaques were counted under a dissecting microscope. For the analysis of DNA, EF10 containing ara-C at 50μg/ml was added to make up to the requisite volume and incubation continued at 37°C for 72h. Ara-C is included in the medium to prevent "non-latent" virus from replicating. It is an analogue of deoxyribonucleotides and as ara-CTP acts as a competitive inhibitor of dCTP in the DNA polymerase reaction or as an incorporated analogue

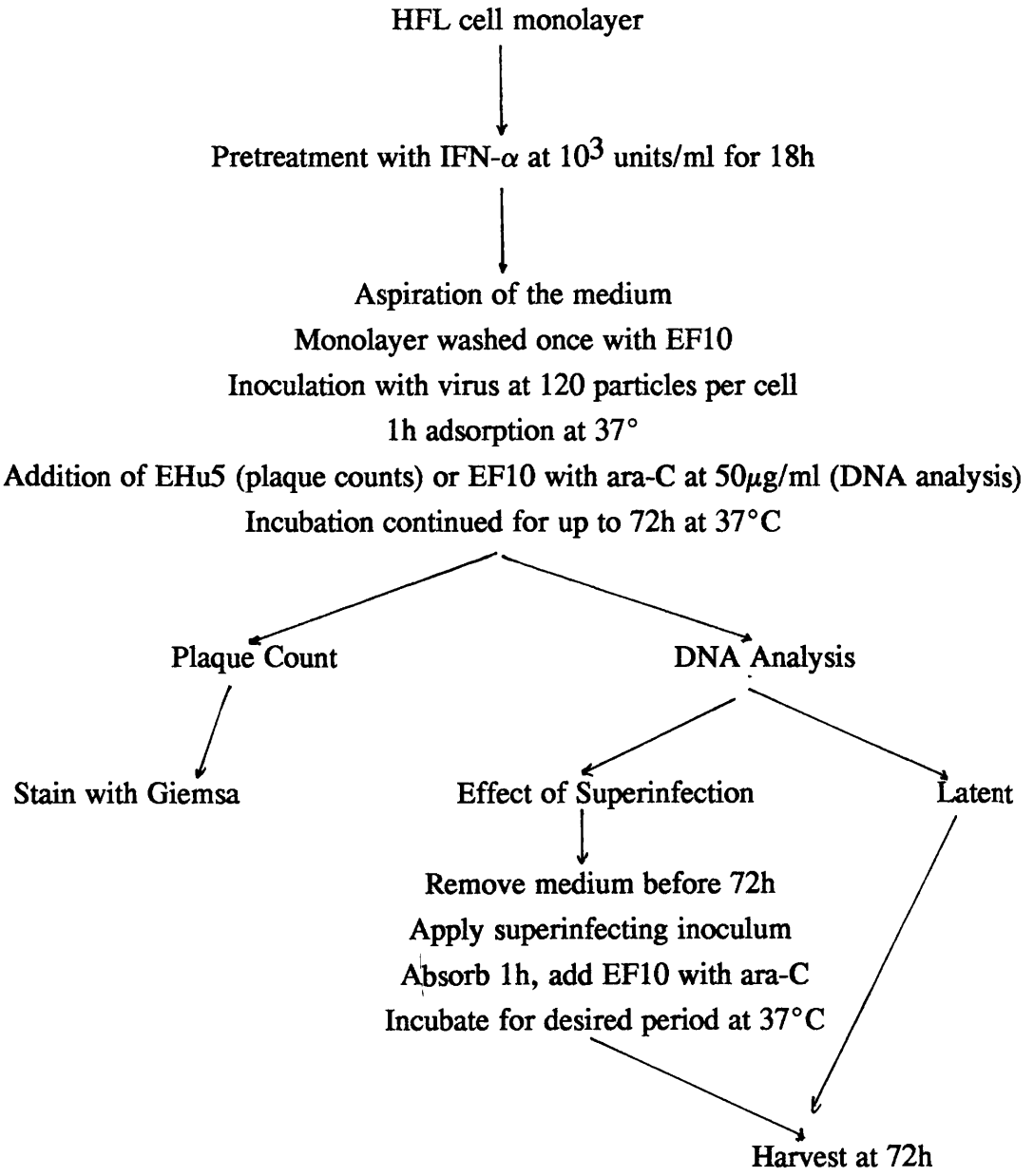


FIGURE 3.1: FLOW DIAGRAM OF PROCEDURE FOR THE *IN VITRO* HSV-1 LATENCY MODEL

into DNA impedes its synthesis (Cozzarelli, 1977). From the known p:pfu ratio of 1.4 x 10^5 to 10^6 and infecting at 120 particles per cell between 0.012% and 0.086% of the genomes will be potentially productive. This will not affect the molecular studies but if the virus were allowed to replicate and spread it would create a significant background problem. The cells were harvested. If the monolayer was to be superinfected, the incubation period was appropriately curtailed to permit the removal of the medium, application of the superinfecting inoculum, absorption for 1h and then the incubation continued at 37°C in the presence of EF10 containing ara-C at 50μg/ml. At 72h total incubation time the cells were harvested. The model system is shown as a flow diagram in figure 3.1.

3.5. Preparation of nucleic acids

3.5.1. Preparation of nuclear DNA

The monolayers were harvested at 72h by pouring off the medium carefully and washing once by addition and removal of ice cold PBS. For a 50mm diameter plate, 1ml of fresh ice cold PBS was added and the cells scraped into this using a silicone rubber policeman. The resulting cell suspension was transferred to a 1.5ml reaction vial using a Pasteur pipette and the cells collected by centrifugation at 6500rpm for 30s in a benchtop microfuge. The supernatant was discarded and the cell pellet resuspended in 1ml of ice cold lysis buffer. This was incubated at 4°C for 5min with intermittent mixing on a vortex mixer. The nuclei were pelleted by centrifugation at 13000rpm for 2min in a benchtop microfuge and the supernatant discarded. The pellet was mixed on a vortex mixer and resuspended in 0.5ml of proteinase K reaction mixture containing 250μg/ml proteinase K. Incubation was continued for 18h at 37°C. Sodium acetate, pH 7.0, was added to 0.3M and the nucleic acids purified by one extraction with phenol/chloroform (1:1v/v) and one extraction with chloroform. The DNA was precipitated by addition of 1ml of ice cold ethanol. The mixture was inverted, centrifuged in a benchtop microfuge at 13000rpm for 2min and the pellet washed in 70% ethanol to remove any excess salt. Finally, it was allowed to dry overnight in air at room temperature and stored at −20°C.

When cells were harvested from 90mm diameter plates, the nuclear pellet was resuspended in 10ml of proteinase K reaction mixture in a 50ml polypropylene conical tube. The volumes used for purification were adjusted accordingly and the DNA

precipitated by the addition of two volumes of ice cold ethanol. It was then collected by centrifugation at 2500rpm for 20min in a benchtop microfuge at 25°C.

3.5.2. Preparation of cytoplasmic RNA

Monolayers of HFL cells were grown on 50mm diameter plates and variously treated with IFN-α and infected as described for the *in vitro* latency model above. The medium was poured off and the cells washed with ice cold PBS. 1ml of fresh ice cold PBS was added and the cells scraped into this using a silicone rubber policeman. The suspension was transferred into a 1.5ml reaction vial using a Pasteur pipette and centrifuged at 6500rpm in a benchtop microfuge for 30s. The supernatant was drawn off and the remaining pellet resuspended in 67μl of cold 10mM Tris HCl pH 7.0 and 1mM EDTA. Following the addition of 7.5μl of 5% Nonidet P40, the suspension was mixed on a vortex mixer and incubated on ice for 5min. A further 7.5μl of 5% Nonidet P40 was added, the sample mixed again and centrifuged at 6500rpm in a benchtop microfuge for 2min. The supernatant which contains cytoplasmic RNA was taken up as a 50μl aliquot into a 1.5ml sterile reaction vial containing 45μl of 20 x SSC and 30μl of formaldehyde. The solution was mixed and incubated, after clamping the vial in a metal holder, for 15min at 60°C and then stored at −70°C. A 2.5μl aliquot, diluted to 250μl in sterile water, was taken for quantification.

3.6. Preparation of nuclear matrices

Nuclei, prepared by lysing cells from monolayers as described previously in section 3.5.1 but washed and collected in TBS, were resuspended in 1ml of ice cold 10% DEP treated sucrose containing 10mM Tris HCl pH 7.5 and centrifuged at 6500rpm for 4min and the supernatant discarded. This procedure effectively removed the detergent NP40 and prohibited excessive clumping of the nuclei. The pellet was resuspended in 300μl of 10mM NaCl/25mM Tris HCl pH 8.0 and mixed on a vortex mixer. It was mixed again following the addition of 3μl of 10% Triton X-100. Following the addition of 300μl of 4M NaCl, the sample was kept on ice for 5min with intermittent gentle mixing by inversion. The sample, now viscous, was taken up in a Pasteur pipette and layered onto a 100μl 40%w/v sucrose/25mM Tris HCl pH 8.0 cushion in a 1.5 ml reaction vial. The vial was inserted into an adapter and centrifuged in an SS34 rotor at 18000rpm for 20min at 4°C. The nucleoids, which appeared as a greyish aggregate at the interface with the cushion, were removed in a volume of 100μl by

being drawn up into a pipette tip. The salt was eliminated by a repeat of this procedure after the nucleoids had been resuspended in 1ml of ice cold 25mM Tris HCl pH 8. Matrix preparation was based on a previously published method (Cook *et al.*, 1982).

3.7. Preparation of nucleosomal DNA

Cells were harvested and lysed as described in section 3.5.1. The nuclei were resuspended in 1ml of ice cold 10% w/v sucrose, 10mM Tris HCl pH7.5 and 2mM $MgCl_2$ and centrifuged for 4min at 6500rpm in a benchtop microfuge. The supernatant was discarded and micrococcal nuclease reaction mixture added to the pellet. The nuclei were dispersed in the reaction mixture using a vortex mixer and kept on ice. At 0min micrococcal nuclease was added and incubation at 37°C commenced for the predetermined time interval. The volume of reaction mixture was adjusted so that when the enzyme was added the total volume was 150μl. The reaction was stopped by placing the sample on ice and adding EDTA to 12mM. The sample was made up to 500μl by the addition of proteinase K reaction mixture with a final concentration of proteinase K at 100μg/ml and incubated at 37°C for 3h. DNA was extracted from the sample as described in section 3.5.1.

3.8. Preparation of latent viral genomes

HFL cells were grown on 90mm diameter tissue culture plates and treated in accordance with the *in vitro* latency model described in section 3.4. The cells were washed with 15ml PBS and collected in 1ml of PBS into a 1.5ml reaction vial. They were pelleted by centrifugation at 6500rpm for 30s in a benchtop microfuge and the supernatant discarded. The pellet was resuspended in ice cold L buffer to a concentration of 5 x 10^7 cells per ml and warmed to 42°C. 1% low melting point agarose cooled to 42°C was mixed in an equal volume with the cells and quickly pipetted into the moulds of a block former. When set, the blocks were expelled into 50ml polypropylene conical tubes containing 50 volumes of L buffer with 1% SDS and proteinase K at 1mg/ml. The blocks were incubated at 50°C for 2 days. At the end of the incubation period the blocks were washed three times in 10mM EDTA pH 8.0 and stored in 50 volumes of the same at 4°C. For digestion with *Ase* I the blocks were washed 10 times in 10mM EDTA to remove all traces of proteinase K, equilibrated in 600μl of restriction enzyme buffer for 15min and incubated with 200 units of enzyme

for 4h at 37°C. Prior to electrophoresis all blocks were soaked twice for 30min in 50 volumes of TE buffer.

3.9. Purification and characterisation of nucleic acids

3.9.1. Restriction enzyme digestion

DNA recovered from a 50mm diameter tissue culture plate was routinely digested in a volume of 100μl of reaction mixture containing the appropriate buffer for the restriction enzyme as specified by the manufacturer. The material obtained from a 90mm diameter plate was digested in a volume of 500μl. The digestion was usually carried out overnight at 37°C. When complete and if not used immediately the digest was stored at −20°C.

3.9.2. Phenol/chloroform extraction of DNA

To separate DNA from protein, the nucleic acid was extracted from samples by the addition of sodium acetate, pH 7.0, to 0.3M followed by an equal volume of a 1:1 distilled phenol:chloroform mixture, mixed on a vortex mixer and centrifuged. Samples in a 1.5ml reaction vial were centrifuged in a benchtop microfuge at 13000rpm for 2min whereas those in 50ml polypropylene conical tubes were centrifuged in a Beckman GPR centrifuge at 2000rpm for 20min. The aqueous phase was taken and extracted with an equal volume of chloroform to remove any residual phenol. The DNA in aqueous solution was precipitated by the addition of 2 - 3 volumes of ice cold ethanol, agitated by hand and centrifuged at 13000rpm for 2min in a benchtop microfuge or at 2500rpm for 20min in a Beckman GPR centrifuge. The pellet was washed once with 70% ethanol to remove the excess salt and dried in air at room temperature. Subsequently, the pellet was either dissolved in distilled water containing 10μg of ribonuclease A for 3h at room temperature and then stored at −20° C or subjected to a restriction enzyme digest as required.

3.9.3. Ultracentrifugation of DNA in a caesium chloride gradient

Separation of HSV DNA from cellular DNA in preparations of latently infected cells was achieved by ultracentrifugation of the preparation through a caesium chloride gradient of RI 1.4 at 25°C. The principle of this separation is the exploitation of the high G + C content of the viral DNA relative to the host cell genome. Latently

infected cellular DNA derived from 90mm diameter plates was digested to completion with *Bam* HI in a reaction volume of 500μl and mixed with a working solution of CsCl (1.465g/ml) to a volume of 5ml and a final RI of 1.4. adjusted by either the addition of water or CsCl solid. The mixture was transferred to a TV865B centrifuge tube, balanced with stock CsCl of RI 1.4 and the remaining space in the neck of the tube filled with liquid paraffin. The neck was sealed with a stopper, capped and crimped with a crimper. The samples were centrifuged in a TV865B vertical rotor at 40000rpm for 18h at 15°C in a Sorvall OTD50B ultracentrifuge. The gradient was collected in fractions of 10 drops into wells in a 24 well plate by piercing the bottom of the clamped tube with a 21 gauge needle. The RI of each fraction was determined and those between 1.4010 and 1.4030 were pooled. The pooled fractions were placed into dialysis membrane tubing (prepared by boiling in 5mM EDTA for 5min) and dialysed against TNE for 3h at 4°C. The buffer was changed once and the dialysis continued overnight. The volume of the sample was reduced to approximately 1ml by emptying the contents of the tubing into a 15ml conical tube, adding 2 volumes of butan-2-ol and centrifuging for 4min at 2000rpm. The upper phase was discarded and the residual butan-2-ol evaporated by incubating the open tube for 3h at 55°C. The final material, in aliquots of 0.5ml, was transferred to 1.5ml reaction vials and the DNA precipitated by the addition of sodium acetate, pH 7.0, to 0.3M and 2 volumes of ice cold ethanol. After standing at −20°C for 1 - 2h the sample was centrifuged at 13000rpm for 2min in a benchtop microfuge and the pellet washed in 70% ethanol and air dried at room temperature overnight. The dried pellet was dissolved in 40μl of distilled water containing 1μg of ribonuclease, left for 3h at room temperature and stored at −20°C.

3.9.4. Electroelution of DNA fragments

The requisite DNA was run in a 0.6% agarose minigel and the appropriate fragment excised when visualised under long wave UV light. It was eluted onto dialysis membrane (previously boiled in 5mM EDTA for 5min) in EEB using an electroelution chamber at 20mA per sample at 4°C for 1.5h. The volume of EEB in the well was reduced to 200μl and the DNA was removed from the dialysis membrane by vigorous pipetting. The volume of the sample was made up to 400μl with distilled water and sodium acetate, pH 7.0, to 0.3M. The DNA was extracted with 400μl of 1:1 v/v phenol:chloroform, 400μl of chloroform and precipitated in 0.5ml of propan-2-ol. After standing at room temperature for 1 - 2h the DNA fragment was collected by centrifugation in a bench top microfuge for 10min at 13000rpm. The pellet was washed in 0.5ml of 100% ethanol, dried and dissolved in 15μl deionised water. This stock solution was diluted 10-fold prior to use.

3.9.5. Quantification of nucleic acids

The relative intensities of DNA bands for comparative purposes were measured by storage phosphor technology. Membranes to which radiolabelled DNA had been hybridised and prepared for autoradiography as described in section 3.13, were wrapped in clingfilm and exposed to a storage phosphor screen overnight. The screen was transferred to a Molecular Dynamics 400B PhosphorImager™, scanned and the created image analysed using Molecular Dynamics ImageQuant software.

RNA was quantified by measuring the OD at 260nm in a Beckman Du-62 spectrophotometer. Estimation was based on 1mg/ml of RNA giving 25 OD units at 260nm. DNA was quantified by measuring the OD of *in*1820 DNA at 260nm.

3.10. Separation of DNA by gel electrophoresis

3.10.1. Minigels

Small amounts of DNA were analysed by electrophoresis in 50ml of either 0.6% or 0.8% agarose in 1 x TBE and cast in a 100mm x 70mm x 7mm minigel kit. Loading buffer was added to the sample and 10μl loaded into the well. Electrophoresis in 1 x TBE was conducted at 40mA. At 45min, ethidium bromide was added to the buffer at 2μg/ml to stain the gel. At 1h the gel was visualised under UV illumination and photographed on Polaroid 667 film.

3.10.2. Large agarose gels

Horizontal slab gels were used for Southern transfer of DNA. Agarose, 0.6 - 1%, in 300ml of 1 x TBE containing 0.5μg/ml ethidium bromide was cast on the sand blasted face of a 260mm x 160mm x 7.5mm glass plate. Samples were loaded in 1 x loading buffer and the wells topped up with 1 x TBE. The tank was filled with 1 x TBE containing 0.5μg of ethidium bromide to be level with but not over the surface of the gel. Electrophoresis was conducted at 8 - 18mA for 16 - 20h. The gel was visualised under UV illumination and photographed on Polaroid 667 film.

For the analysis of nucleosomal DNA, gels were cast as 2% agarose in a 3:1 ratio of 1.5% agarose and 0.5% NuSeive® GTG agarose. Otherwise the conditions were as above.

Separation of high molecular weight latent DNA was performed in a Rotaphor® Type V electrophoresis chamber. Gels of between 0.7% and 1% agarose in 300ml of 0.25 - 1 x TBE containing 0.5μg of ethidium bromide were cast in the frame. Agarose blocks containing embedded DNA, prepared as described in section 3.8, were inserted into the wells and the ramp along the well line trimmed to lie flush with the surface of the gel. The electrophoresis was conducted in 0.25 x TBE under various permutations of temperature, switching interval, angle and voltage for up to 72h.

3.11. Hexanucleotide (random) primer extension

A modified method of random primer extension described by Feinberg and Vogelstein (1983) was used. Approximately 10ng of DNA was made up to 21μl in distilled water, heated in a dri-block at 100°C for 10min, cooled on ice for 5min and centrifuged at 6500rpm in a benchtop microfuge for 20s. To this was added 2μl of 0.1% BSA, 10μl of oligolabelling reaction mixture, 2.5μl each of 0.4mM dATP and dCTP, 5μl (50μCi) each of [α-^{32}P]-dGTP and [α-^{32}P]-dTTP and 2μl (4 units) of labelling grade Klenow fragment DNA polymerase. Incubation was continued overnight at room temperature. The radiolabelled DNA fragment was purified by passage through a Sephadex® G-50 Medium column.

3.11.1. Determination of percentage incorporation

A 0.5μl volume of the oligolabelling mixture was diluted to 10μl in water and 3μl spotted onto Whatman No. 1 filter paper discs in duplicate. The discs were allowed to dry and one washed once in 10% w/v TCA, once in 5% w/v TCA and twice in ethanol. It was dried under a heat lamp. Both discs were placed in scintillation vials containing 10ml Ecoscint A and the radioactivity determined in a scintillation counter. Incorporation was measured as the percentage of radioactivity on the washed disc compared with the unwashed disc. At least 30% incorporation was found to be required for a successful outcome to hybridisation.

3.11.2. Purification of radiolabelled DNA fragments

A 10ml column was prepared from 1g of Sephadex® G-50 Medium swollen in 30ml distilled water for at least 3h. The oligolabeling reaction mixture containing the DNA fragment was mixed with 50μl of 1:1 phenol:chloroform and the aqueous phase

applied to the column. The eluate was collected as fractions of 5 drops in 1.5ml reaction vials and those harbouring the peak of radioactivity pooled as a total volume of 1.5ml. The sample was denatured by the addition of 33μl of 6M NaOH for 10min at room temperature.

3.12. Southern transfer and hybridisation

3.12.1. Transfer of DNA from agarose gels to a membrane

The technique used is a modification of that described by Southern (1975). Following electrophoresis in agarose gels the DNA was transferred to GeneScreen*Plus* hybridisation membrane (Du Pont) by alkaline transfer as described by Chomczynski and Qasba (1984). After cutting off the wells and trimming, the gel was soaked in alkaline transfer solution for 30min. It was inverted and laid on two sheets of moistened Whatman 3MM paper, which acted as a wick by being placed at right angles to each other on a glass plate and supported in a tray. The GeneScreen*Plus* hybridisation membrane was cut to the size of the gel, immersed in transfer solution and laid on top of the gel. Any air bubbles between the gel and the membrane were expelled by rolling with a glass tube. Capillary transfer was achieved by sequentially layering two wet and four dry sheets of Whatman No 1 paper and a 5cm stack of absorbent paper towels, all cut to the size of the gel. A glass plate and a weight were placed on the top of the stack and the transfer effected over a period of 16 - 24h. The towels and filter papers were discarded and the membrane lifted away from the gel. The membrane was soaked for 15min in neutralising solution and allowed to dry at room temperature until just moist. The DNA was crosslinked to the membrane by exposure to 120000 microjoules/cm^2 of ultra violet light irradiation in a Stratalinker UV Crosslinker 1800 and then dried at room temperature.

To effect the transfer of high molecular weight DNA, depurination was carried out by incubation of the gel in 0.25M HCl for 15min prior to alkaline treatment.

3.12.2. Application of RNA to a membrane

Total cell RNA was diluted to 3.0, 1.0 and 0.3μg in 50μl of 20 x SSC and applied to a Schleicher and Schull BA 85 nitrocellulose membrane using a dot - blot manifold. Prior to the application the membrane was soaked in distilled water followed by 20 x SSC and placed on top of a sheet of Whatman No. 1 filter paper soaked in 20 x SSC

and the two inserted into the manifold. 50μl of the diluted sample was applied to each well. The blot was dried for 2h in air and then baked in a vacuum oven at 80°C for a further 2h.

3.12.3. DNA/DNA hybridisation

This was conducted either in a shaking water bath or a Hybaid hybridisation oven. For the shaking water bath method, the dry membrane was placed into a polythene bag and preincubated with 50ml of hybridisation buffer for 2h at 70°C. The denatured radiolabelled DNA fragment was added to 18.5ml of hybridisation buffer containing 33 μl of 6M HCl, to neutralise the denaturing NaOH, and warmed to 70°C. The whole was added to the membrane in the bag and incubation at 70°C continued with shaking for 24 - 72h.

The radioactive hybridisation solution was poured off, the membrane removed from the bag and washed once in 400ml of fresh hybridisation buffer and twice in 400ml solutions of 0.2 x SSC and 0.1% SDS. Each wash was at 70°C and for 45min. After washing, the membrane was rinsed under running water, dried in air at room temperature and put up for autoradiography.

For the hybridisation oven method, the membrane was rolled up with Hybaid support mesh sheeting and inserted into a 300mm x 35mm Hybaid hybridisation bottle. Incubation with 50ml of hybridisation buffer was performed for 2h at 70°C and then the buffer poured out. The denatured radiolabelled DNA fragment was added to 18.5ml of hybridisation buffer containing 33μl of 6M HCl and warmed to 70°C. The whole was poured into the hybridisation bottle and incubation at 70°C continued for 24 - 72h.

The radioactive hybridisation solution was poured off and the membrane washed once in 50ml of fresh hybridisation buffer and twice in 50ml of 0.2 x SSC / 0.1% SDS. Each wash was at 70°C and for 45min. After washing, the membrane was removed from the bottle, rinsed under running water, dried in air at room temperature and put up for autoradiography.

3.12.4. DNA/RNA hybridisation

This was conducted in a shaking water bath. The baked nitrocellulose membrane was placed into a polythene bag and preincubated with 15ml of prehybridisation buffer

for 2h at 42°C. Denatured radiolabelled DNA was added to 8.5ml of hybridisation buffer containing 33μl of 6M HCl and warmed to 42°C. The whole was added to the membrane in the bag and incubation at 42°C continued with shaking for 24 - 72h.

The radioactive hybridisation solution was poured off, the membrane removed from the bag and washed twice in 500ml of 2 x SSC / 0.1% SDS at room temperature for 15min, twice in 500ml of 0.1 x SSC / 0.1% SDS at room temperature for 15min and twice in 500ml of 0.1 x SSC / 0.1% SDS at 70°C for 15min. After washing, the membrane was rinsed under running water, dried in air at room temperature and put up for autoradiography.

3.13. Autoradiography

The dried membrane holding the radiolabelled hybridised DNA was placed on a glass plate and enclosed in polythene sheeting. It was put into a cassette with Kodak X-OMAT S film in front and an image intensifying screen on top. The sheeting and the intensifying screen were wiped with Kodak intensifying screen cleaner and antistatic solution before the cassette was made light proof and put at −70°C. The film was developed in a Kodak X-OMAT Processor Model ME-3.

3.14. Estimation of protein concentration

Protein concentration was measured using the method described by Bradford (1976). 1ml of Bradford reagent was added to 25μl of the sample in a plastic cuvette. The cuvette, covered with Nesco film, was inverted several times and stood at room temperature for 15min. The absorbance at 595nm was measured in a spectrophotometer and compared with a standard curve constructed from absorbance at the same wavelength of concentrations ranging from 0 - 120μg of BSA.

3.15. Determination of thymidine kinase activity

The supernatant recovered following lysis and centrifugation of the cells as described in section 3.5.1. was assayed for thymidine kinase activity. Up to 12μl of

the sample was incubated at 30°C for 3h in TK reaction mixture to a total volume of 50 μl. The reaction was stopped by the addition of 10μl of 100mM EDTA pH 7.5 and 1mM thymidine and heated in a dri-block at 100°C for 5min. The sample was cooled on ice for 5min and centrifuged at 13000rpm for 2min in a benchtop centrifuge. 50μl of the supernatant was spotted onto Whatman DE81 discs and dried under a heat lamp. The discs were immersed twice in 40mM ammonium formate pH 4.0 and 10μM thymidine at 37°C for 10min and followed by two washes of 5min each in 100% ethanol. The discs were dried under a heat lamp, placed into scintillation vials with 10ml of Ecoscint A and their radioactivity determined in a scintillation counter.

3.16. Staining monolayers for β-galactosidase activity

Cells infected with viruses which are carrying the *lacZ* gene can be stained for the activity of the gene product, β-galactosidase. Brefeldin A, which prevents egress of the virus from the cell, was added to the medium at 3μg/ml in all cases where potentially lytic virus would spread from cell to cell and destroy the monolayer.

The medium overlying cells grown on 35mm diameter tissue culture plates was poured off and the monolayer washed thoroughly with 2ml ice cold PBS. The cells were fixed with 1% glutaraldehyde in PBS for 1h at 4°C. The fixative was washed off with two washes of PBS and 2ml of histochemical reaction mixture for β-galactosidase activity added. Staining was allowed to proceed for at least 1h at 37°C and then the reaction mixture was washed off several times with distilled water. Positively stained cells are blue. β-galactosidase liberates 5-bromo 4-chloro 3-indolyl from X-gal which is oxidised by ferricyanide to form a blue indigo dye (Pearson *et al.*, 1963). Ferrocyanide prevents further oxidation to a colourless indigo compound. Occasionally crystals formed on the plates but they were easily removed by incubation at 37°C for 2h in a solution equivalent to the β-galactosidase stain without X-gal followed by further washing with water. The fixed cell sheets were then stained for 12h with a counterstain, Carmalum, to reveal all the cells in a contrasting pink colour. The stain was washed off with distilled water and the plates dried upside down in air at room temperature. Coverslips were mounted in aqua mount and the blue cells counted under a dissecting microscope. Photographs were taken using a Zeiss Axioskop with an MC 80 camera set at 12 volts. One blue and two neutral density filters were needed in the lighting conditions during daytime. Kodak Gold II ASA 100 film for colour prints was used throughout.

4. RESULTS

4.1. Objectives

Herpes simplex virus is characterised by the pathogenic property of establishing latency in the neuronal cells of sensory and autonomic ganglia during primary infection of man. Once established, latency can be maintained for the lifetime of the host. However, the latent virus can be reactivated and replicate giving rise to clinically important recurrent disease. The objectives of this study were to characterise a model of HSV-1 latency which would allow investigation of the process at the molecular level and demonstrate some structural features of the latent genome in the model. The experimental work was based on improvement of an *in vitro* model of HSV latency in HFL cells that has been described previously (Russell and Preston, 1986; Harris and Preston, 1991). The final system is shown schematically in figure 3.1. It has been demonstrated (Harris and Preston, 1991) that infecting HFL monolayers with 5 particles of *in*1814 per cell resulted in 10 - 20% of the cells retaining at least one latent genome. Modifications were designed with the specific intention of being able to increase the moi to achieve a high genome copy number per cell but without causing cell death. Two modifications were introduced. Firstly, the HFL cell monolayer was pretreated with IFN-α to reduce the expression of IE genes (Mittnacht *et al.*, 1988) and secondly, *in*1820 which produces Vmw110 at low levels when infecting HFL cells, was used. A Vmw110 deletion mutant has been shown to favour a non-lytic interaction with HFL cells rather than plaque formation at low moi (Stow and Stow, 1989). Reactivation was effected by superinfection with a virus which expressed Vmw110.

Having established latency at a high moi of up to 120 particles per cell and demonstrated a copy number of at least one latent genome per cell, further experiments were carried out to examine the structural features of the latent genome in the model more closely. Current evidence suggests that the latent HSV genome *in vivo* exists as a non-linear molecule (Rock and Fraser, 1983; 1985; Efstathiou *et al.*, 1986), probably an episome (Mellerick and Fraser, 1987), in a chromatin-like arrangement (Deshmane and Fraser, 1989). Both of these features have been examined in the *in vitro* model, the former by looking for the loss of the termini and the latter by examination of the pattern produced by digestion with micrococcal nuclease. Preliminary investigations into the size of the latent DNA molecule and also into the relationship of the genome to the nuclear substructure of the host cell have been performed.

4.2. Mutant *in*1820 has increased propensity to establish latency *in vitro*

The mutant *in*1814 has a 12bp insertion in the gene encoding Vmw65, the virion tegument protein which transinduces IE gene transcription (Ace *et al.*, 1989). As a consequence of the mutation, the ability of Vmw65 to transinduce IE gene expression and to form a protein-DNA complex with cell proteins and the IE specific regulatory element TAATGARAT (where R is a purine residue) is abolished. However accumulation of IE mRNA, and in particular Vmw110 mRNA, although reduced is not eliminated. This renders the transinduction of IE transcription by Vmw65 unimportant at high moi when many of the particles, although unable to form plaques, are able to participate normally in the early stages of infection (Ace *et al.*, 1989). Thus the addition of greater than 5 - 10 particles of *in*1814 per HFL cell results in the degeneration of the monolayer even when lytic infection is blocked by the presence of ara-C, an inhibitor of DNA replication. Ara-C, used to prevent the breakthrough of replicating virus in previous experiments, did not affect the establishment of latency in the *in vitro* system (Harris and Preston, 1991). It was hypothesised that further measures to reduce IE protein levels after infection might reduce the cytopathic effect (Johnson *et al.*, 1992) and thus enable a higher moi to be used. A further mutation was introduced into *in*1814 by replacement of the normal Vmw110 promoter with the MoMuLV LTR enhancer which is expressed very poorly in HFL cells to give mutant *in*1820. It has been found that *in*1820 produces undetectable levels of Vmw110 mRNA under IE conditions (J. Daksis and C. M. Preston, personal communication). The effect of these mutations on the particle per pfu ratio is shown in table 4.1.

Confluent monolayers of HFL cells on 30mm diameter tissue culture plates were inoculated with virus in a 200μl inoculum which was allowed to adsorb for 1h. The medium was then made up to 2ml with EHu5. This prevents extracellular spread of the virus by neutralizing any released virions. It does not prevent the intercellular spread of virus which eventually forms a plaque. Thus the plaque arises from one infected cell which has become productively infected and the virions have spread via cell to cell contact into the neighbouring fibroblasts. Infection was allowed to proceed at 37°C for 48h before the monolayers were inspected for plaque formation.

From the results presented in table 4.1, it is evident that the same number of plaques (6) resulted from 0.0001 particles per cell of 1814R as 1 particle per cell of

VIRUS	PARTICLES PER CELL	PLAQUES PER PLATE
1814R	0.1	cpe
	0.01	>200
	0.001	36
	0.0001	6
*in*1814	10	cpe
	1	11
	0.1	1
	0.01	0
*in*1820	10	148
	1	6
	0.1	0
	0.01	0

TABLE 4.1: THE MUTANTS *in*1814 AND *in*1820 HAVE REDUCED PLAQUE FORMING ABILITY ON HFL CELLS COMPARED WITH 1814R

Each 30mm diameter plate of confluent HFL cells was inoculated with a 200μl viral inoculum and maintained at 37°C for 1h to allow adsorption and penetration of virus. 1.8ml of EHu5 was added, incubation continued for 48h, the monolayer stained with Giemsa and the plaques counted. Plaque numbers greater than 200 per plate cannot be counted accurately because of coalescence but are indicated as >200 since the monolayers were still intact, unlike those largely destroyed by the cpe.
(The figures are from a single experiment but it was repeated with consistent results)

*in*1820 which represents a 10^4-fold increase in the particle per pfu (p:pfu) ratio. The rescued virus 1814R, is essentially a wild type HSV control (Ace *et al.*, 1989). The increase in the p:pfu ratio of *in*1820 compared with *in*1814 is at least 2-fold in that 1 particle per cell of *in*1814 produces twice as many plaques as 1 particle per cell of *in*1820. A similar result is seen in the data from the untreated cells in table 4.2. Here the number of plaques (49) from 0.001 particles per cell of 1814R is of the same order as the number (38) from 10 particles per cell of *in*1820 i.e an increase in the ratio by a factor of 10^4. As for *in*1814, 10 particles per cell produced nearly 4 times as many plaques as the same moi of *in*1820. This is in accord with unpublished observations

(C.M. Preston, personal communication) in which the p:pfu ratio on HFL cells is 10^5 - 5 x 10^5 for *in*1814 whereas it is 10^6 for *in*1820 and 10 - 50 for 1814R. It is concluded that the effect of the double mutation is to decrease the propensity of the virus to form plaques on HFL cell monolayers.

4.3. IFN-α pretreatment inhibits replication of *in*1814 and *in*1820

IFN-α is known to inhibit IE gene transcription (Mittnacht *et al.*, 1988; Oberman and Panet, 1988; 1989). Therefore the expected effect of pretreating HFL monolayers with IFN-α would be to reduce the IE transcription of the infecting virus and thus its cytopathogenicity. The results of the pretreatment of cells with 10^3 units of IFN-α/ml for 16h prior to infection with virus are presented in table 4.2. The IFN-α was washed out with EF10 immediately prior to inoculation with a 200μl viral inoculum as described above.

The results show that pretreatment of the cells with IFN-α at 10^3 units/ml reduced the plaque forming ability of 1814R, *in*1814 and *in*1820. The effect was more marked at lower moi in that 0.001 particles per cell of 1814R were more effectively prevented from forming plaques than 0.01 particles of the same. Thus the plaque forming ability of *in*1820 can be even further reduced. Pretreatment of cells with IFN-α makes it possible to infect with *in*1820 at a higher moi than *in*1814 without the production of plaques. From table 4.2 it can be seen that even at 100 particles per cell *in*1820 still produces only 5 plaques. These plaques represent virus which does not undergo latent infection, unlike the majority of the infecting particles, and although only a small percentage of the total such viruses need to be prevented from replicating. The replication inhibitor ara-C was used to achieve this when required.

As discussed in section 1.4, IFN-α and IFN-γ are very different in their biological activities. Their modes of action are likely to differ too in that combination treatments have been reported to give rise to a synergistic enhancement of antiviral activities (Samuel and Knutson, 1983; Czarniecki *et al.*, 1984). One paper (Klotzbucher *et al.*, 1990) reported that pretreatment of mouse splenic macrophages with murine IFN-γ led to a dose dependent reduction in HSV-1 yield comparable to that obtained with IFN-α/β. The data suggested a translational inhibition of IE gene expression by IFN-γ, unlike IFN-α/β which appeared to inhibit IE gene expression at the transcriptional level. The

use of IFN-γ as well as IFN-α in inhibiting plaque formation on HFL monolayers was explored. The results are presented in tables 4.2 and 4.3. It is evident from table 4.2 that IFN-γ is not as effective as IFN-α in further reducing the plaque forming abilities of the mutants *in*1814 or *in*1820 and the data in table 4.3 confirms this for the mutant *in*1814 and also demonstrates that there is no cumulative effect of IFN-α and IFN-γ. The discrepancy between these results and others (Klotzbucher *et al.*, 1990) might be explicable on the grounds of a higher dose causing partial reversal of HSV-1 growth inhibition i.e. maximum inhibition was observed at 200-500 units of IFN-γ/ml which declined when higher doses were used (Klotzbucher *et al.*, 1990). The use of IFN-γ has no place in the *in vitro* system being developed with *in*1820 infection of HFL cells.

VIRUS	PARTICLES PER CELL	PLAQUES PER PLATE		
		NO IFN-α	IFN-α	IFN-γ
1814R	0.1	cpe	>200	cpe
	0.01	>200	98	>200
	0.001	49	7	69
*in*1814	100	cpe	>200	cpe
	10	146	1	42
	1	1	0	2
	0.1	1	0	0
*in*1820	100	cpe	5	cpe
	30	>200	0	147
	10	38	0	5
	1	1	0	0

TABLE 4.2: PRETREATMENT WITH IFN-α REDUCES THE TITRE OF 1814R, *in*1814 AND *in*1820

30mm diameter plates of confluent HFL cells were mock pretreated or pretreated with IFN-α or IFN-γ at 10^3 units/ml for 16h. After washing the monolayer, a 200μl viral inoculum was applied and maintained at 37°C for 1h to allow adsorption and penetration of virus. 1.8ml of EHu5 was added, incubation continued for 48h, the monolayer stained with Giemsa and the plaques counted. Plaque numbers greater than 200 per plate cannot be counted accurately because of coalescence but are indicated as >200 since the monolayers were still intact, unlike those largely destroyed by the cpe.

In the tables 4.2 and 4.3 it is evident that the mutants are more sensitive to the inhibitory effects of IFN-α than the revertant 1814R which has a wild type phenotype. The data in table 4.2 show that pretreatment with IFN-α produces a reduction in the plaque forming ability of *in*1820 by more than 200-fold and between a 100 and 200-fold reduction in the plaque forming ability of *in*1814. In contrast it only reduces the plaque forming ability of 1814R by about 5-fold. It appears that the introduction of the mutations has enhanced the susceptibility of the virus to the inhibitory effects of IFN-α.

The reduced efficiency of the plaque forming ability of *in*1814, apparent when comparing the tables 4.1, 4.2 and 4.3, is thought to be a consequence of declining viral infectivity resulting from repeated thawing and freezing of virus stocks. To overcome this viral stocks were stored as small aliquots and used twice at the most.

VIRUS	PARTICLES PER CELL	PLAQUES PER PLATE			
		NO IFN	IFN-α	IFN-γ	IFN-α + -γ
1814R	0.1	cpe	>200	cpe	>200
	0.01	>200	142	>200	126
	0.001	30	17	76	23
*in*1814	1000	>200	6	cpe	25
	100	10	1	52	1
	10	0	0	0	0

TABLE 4.3: PRETREATMENT WITH IFN-γ HAS NO EFFECT ON THE REPLICATION OF *in*1814 IN HFL CELLS

30mm diameter plates of confluent HFL cells were mock pretreated or pretreated with IFN-α, IFN-γ or IFN-α and IFN-γ together at 10^3 units/ml for 16h. After washing the monolayer, a 200μl viral inoculum was applied and maintained at 37°C for 1h to allow adsorption and penetration of virus. 1.8ml of EHu5 was added, incubation continued for 48h, the monolayer stained with Giemsa and the plaques counted. Plaque numbers greater than 200 per plate cannot be counted accurately because of coalescence but are indicated as >200 since the monolayers were still intact, unlike those largely destroyed by the cpe.

From these experiments it has been shown that a high moi of at least 30 particles per cell can therefore be achieved without plaque formation by infecting with the double mutant *in*1820 following pretreatment of HFL cells with 10^3 units/ml of IFN-α. An even higher moi of 100 should be possible if the low background of breakthrough virus capable of causing plaques but not cpe is controlled with an inhibitor of viral replication such as ara-C.

4.4. The effect of IFN-α pretreatment persists for at least 72 hours

As discussed in section 1.4, the effect of IFN is mediated by the induction of permanent changes in the cell, part of which is attributable to the synthesis of new proteins.

PARTICLES OF *in*1814 PER CELL	PLAQUES PER PLATE, INFECTED 0h POST IFN-α PRETREATMENT		PLAQUES PER PLATE, INFECTED 72h POST IFN-α PRETREATMENT	
	NO IFN-α	IFN-α	NO IFN-α	IFN-α
100	62	0	79	10
10	2	0	24	0
1	0	0	1	0
0.1	0	0	0	0

TABLE 4.4: THE EFFECT OF IFN-α PRETREATMENT PERSISTS FOR AT LEAST 72h FOLLOWING REMOVAL.

Eight 30mm diameter plates of confluent HFL cells were pretreated with IFN-α at 10^3 units/ml for 16h. A further 8 were mock pretreated. After washing the monolayers, either a 200μl viral inoculum of *in*1814 was added or 2ml of fresh EF10 was added for 72h, removed and then a 200μl viral inoculum of *in*1814 applied. Following inoculation the monolayer was maintained at 37°C for 1h to allow adsorption and penetration of virus. 1.8ml of EHu5 was added, incubation continued for 48h, the monolayer stained with Giemsa, and the plaques counted. The figures given are from a single experiment but it was repeated with consistent results.

If this is the case in this system, removal of IFN-α after pretreatment should not lead to an immediate loss of its effect on the inhibition of the infecting virus. Monolayers pretreated with IFN-α at 10^3 units/ml for 16h were thoroughly washed with medium to remove all traces of the IFN and maintained for a further 72h at 37°C prior to infection with *in*1814. The plaque forming ability of the mutant was determined 48h later. The results are given in table 4.4. The reduced plaquing efficiency of the virus compared with the earlier experiments is explained above.

The results show that an effect on the plaque forming ability of *in*1814 after the washing out of IFN-α is still present 72h later. This suggests that the antiviral mechanism of IFN-α on HSV-1 infection of HFL cells is via long term induced changes rather than an effect that requires its continued presence. This would include the induction of new proteins or modifications such as phosphorylation to those proteins already in existence.

4.5. Investigation of the duration of pretreatment with IFN-α

To investigate the minimum period of interaction to achieve maximum inhibition by IFN-α, HFL monolayers were pretreated with IFN-α for various lengths of time up to 16h prior to infection. It can be seen from the results given in table 4.5 that there is an effect on the plaque forming ability of *in*1814 even with a short exposure to IFN-α pretreatment in that the number of plaques was nearly halved by a 2h IFN-α pretreatment interval. By 5h this number was reduced to 18%. To completely abolish the plaque forming ability of *in*1814 a longer exposure was required which in this experiment was extended to 16h. Thus the effects of IFN-α require continued stimulation in the initial stages of establishing the antiviral state which becomes unnecessary once the changes have been fully induced. These observations suggest that changes induced by the pretreatment can accumulate during the early part of the process, in support of the contention that the effects of IFN-α include permanent modifications such as in the absolute levels of the mediators by the synthesis of new proteins or the rate of synthesis of existing proteins as well as alterations in their activities, for example by phosphorylation.

PLAQUES PER PLATE AFTER 16h PRE-TREATMENT		PLAQUES PER PLATE AFTER 5h PRE-TREATMENT		PLAQUES PER PLATE AFTER 2h PRE-TREATMENT		PLAQUES PER PLATE AFTER 0h PRE-TREATMENT	
NO IFN-α	IFN-α	NO IFN-α	IFN-α	NO IFN-α	IFN-α	NO IFN-α	IFN-α
118	0	113	21	103	59	170	167

TABLE 4.5: THE EFFECT OF IFN-α PRETREATMENT FOR 0h, 2h, 5h and 16h ON THE PLAQUE FORMING ABILITY OF *in*1814

Each 30mm diameter plate of confluent HFL cells was pretreated with IFN-α at 10^3 units/ml for 16h, 5h, 2h or 0h. After washing the monolayer, a 200μl viral inoculum of 100 particles of *in*1814 per cell was applied and maintained at 37°C for 1h to allow adsorption and penetration of virus. 1.8ml of EHu5 was added, incubation continued for 48h, the monolayer stained with Giemsa and the plaques counted. Results of a single experiment.

4.6. Combined effects of HMBA and IFN-α

It has been shown that the presence of HMBA in the cell culture medium is able to overcome the replication defect of *in*1814 (McFarlane *et al.*, 1992). The effect can be achieved by an exposure to the agent for as short as 1.5h early after infection and appears to result from an increase in the production of IE mRNA. Since the action of IFN-α also appears to be at the level of IE mRNA production, it was of interest to determine whether one of these reagents was dominant over the other. This might provide further clues as to their modes of action. HFL monolayers were pretreated with IFN-α as usual but the viral inoculum on some monolayers contained 5mM HMBA, which was maintained at the same concentration throughout the incubation. Some monolayers which were inoculated with virus and incubated without HMBA present were treated with HMBA 24h later. The results are presented in table 4.6.

PARTICLES PER CELL OF *in*1814	PLAQUES PER PLATE				
	NO IFN-α NO HMBA	IFN-α NO HMBA	NO IFN-α HMBA	IFN-α HMBA	IFN-α HMBA (24h)
100	cpe	>200	cpe	cpe	>200
10	>200	1	cpe	173	6
1	18	0	>200	7	0
0.1	0	0	>200	1	0
0.01	0	0	28	0	0

TABLE 4.6: THE CUMULATIVE EFFECT OF HMBA AND IFN-α ON THE PLAQUE FORMING ABILITY OF *in*1814.

30mm diameter plates of confluent HFL cells were pretreated with or without IFN-α at 10^3 units/ml for 16h. After washing the monolayers a 200μl viral inoculum of *in*1814 with or without 5mM HMBA was added and the monolayer was maintained at 37°C for 1h to allow adsorption and penetration of virus. Following adsorption and penetration of virus 1.8ml of EHu5 was added with or without 5mM HMBA and the incubation continued for 48h. On 5 plates, HMBA to 5mM was not added to the inoculum nor the EHu5 until 24h afterwards. The monolayer was stained with Giemsa, and the plaques counted. The figures given are from a single experiment but it was performed twice.

The results confirm that HMBA is very effective in overcoming the mutation in *in*1814 in the absence of IFN-α pretreatment (columns 1 and 3). However in those cells which have been pretreated with IFN-α, the number of plaques generated by HMBA treatment (column 4) is much the same as those from cells that have had no pretreatment (column 1) although less than those cells that have had HMBA treatment only (column 3). Thus it appears that HMBA can partially overcome the effect of IFN-α if given early in infection. Neither of these agents is totally dominant over the other i.e. in the presence of IFN-α HMBA is still effective but IFN-α is also inhibitory in the presence of HMBA. The negative effect of IFN-α and the positive effect of HMBA on the replicating ability of *in*1814 combine to produce a result similar to the phenotype under normal conditions. The results in column 5 show that by 24h HMBA is

ineffective. This finding is unlikely to be due to the pretreatment with IFN-α since the loss of responsiveness of *in*1814 to HMBA in the absence of IFN-α with time has already been reported (McFarlane *et al.*, 1992). It is more likely to be because the viral genome has become latent in which condition it is refractory to all transactivators with the exception of Vmw110 (Harris and Preston, 1991).

4.7. Reactivation of virus from IFN-α pretreated cells is as efficient as from untreated cells

Previous investigators have shown that reactivation of *in*1814 requires the presence of Vmw110 (Harris and Preston, 1991). They demonstrated that *in vitro* the retained virus *in*1814 could be reactivated by superinfection with the temperature sensitive mutant *ts*K which expresses Vmw110 at the non-permissive temperature in a multiplicity dependent manner. This had been demonstrated earlier in a forerunner of the *in vitro* latency system, in which latent HSV-2 was also reactivated by *ts*K in a highly efficient manner (Russell and Preston, 1986). To investigate whether this was also true in the *in vitro* latency model using IFN-α pretreated monolayers infected with *in*1820, latently infected HFL cells which had either been pretreated with IFN-α or left untreated were superinfected with *ts*K. As shown in table 4.7 superinfection with *ts*K at 0.1 pfu per cell was able to reactivate the double mutant *in*1820 in IFN-α pretreated cells as efficiently as *in*1814 from untreated cells. At 0.1 particles per cell it is expected that 2000 cells will have retained one particle with the potential of forming a plaque based on an estimated p:pfu value of 50. Superinfection at 0.1 pfu per cell of *ts*K at the non-permissive temperature should therefore produce 200 plaques. The numbers of plaques produced in the experiment approximates to this figure.

Thus IFN-α pretreatment does not affect reactivation by *ts*K. It is concluded that the genome is not irreversibly inactivated by this pretreatment. Furthermore in addition to overcoming the IFN-α effect, *ts*K is also able to overcome the defect in Vmw65 and the reduced Vmw110 expression inherent in *in*1820. Unlike *ts*K, HMBA is incapable of completely reversing the effects of IFN-α and defective Vmw65 (table 4.6).

It is also apparent that the plaquing efficiency of *ts*K at 31°C was unaffected by IFN-α pretreatment. Although IFN-α had been removed 24h prior to infection with *ts*K, table 4.4 shows that the effect of IFN-α on *in*1814 persists for up to 72h following

its removal. It may be that *in*1814 is more sensitive to inhibition by IFN-α than *ts*K as is shown with the wt virus in table 4.2. Maintainance of the culture at 31°C following infection with *ts*K might also inhibit the effect of IFN-α pretreatment on the virus.

VIRUS	PARTICLES PER CELL	SUPERINFECTION	TEMP (°C)	PLAQUES PER PLATE	
				NO IFN-α	IFN-α
*in*1814	0.1	mock	38.5	0	0
*in*1814	0.1	0.1 pfu per cell *ts*K	38.5	110	82
*in*1820	0.1	mock	38.5	1	0
*in*1820	0.1	0.1 pfu per cell *ts*K	38.5	177	193
mock	0	0.1 pfu per cell *ts*K	38.5	0	0
mock	0	20 pfu *ts*K	31	16	18
mock	0	200 pfu *ts*K	31	184	111

TABLE 4.7: *ts*K REACTIVATES VIRUS EFFICIENTLY FROM IFN-α PRETREATED CELLS

HFL cells were grown on 30mm diameter plates pretreated with IFN-α at 10^3 units/ml for 16h or untreated. After washing, the cells were inoculated with a 200μl viral inoculum and the monolayer was maintained at 37°C for 1h to allow adsorption and penetration of virus. 1.8ml of EF10 was added, incubation continued for 24h, medium removed and the cells superinfected with a 200μl inoculum of *ts*K. This virus was allowed to adsorb and penetrate the cells for 1h, 1.8ml of EHu5 was added and the incubation continued for a further 48h or 72h at the non-permissive (38.5°C) or permissive (31°C) temperatures respectively. Plates were stained with Giemsa and the plaques counted. The figures given are from a single experiment but it was performed twice.

4.8. IFN-α pretreatment reduces IE mRNA accumulation

To confirm that IFN-α pretreatment reduces IE mRNA accumulation as reported by others (Mittnacht *et al.*, 1988; Oberman and Panet, 1988), in HFL cells, dot blot

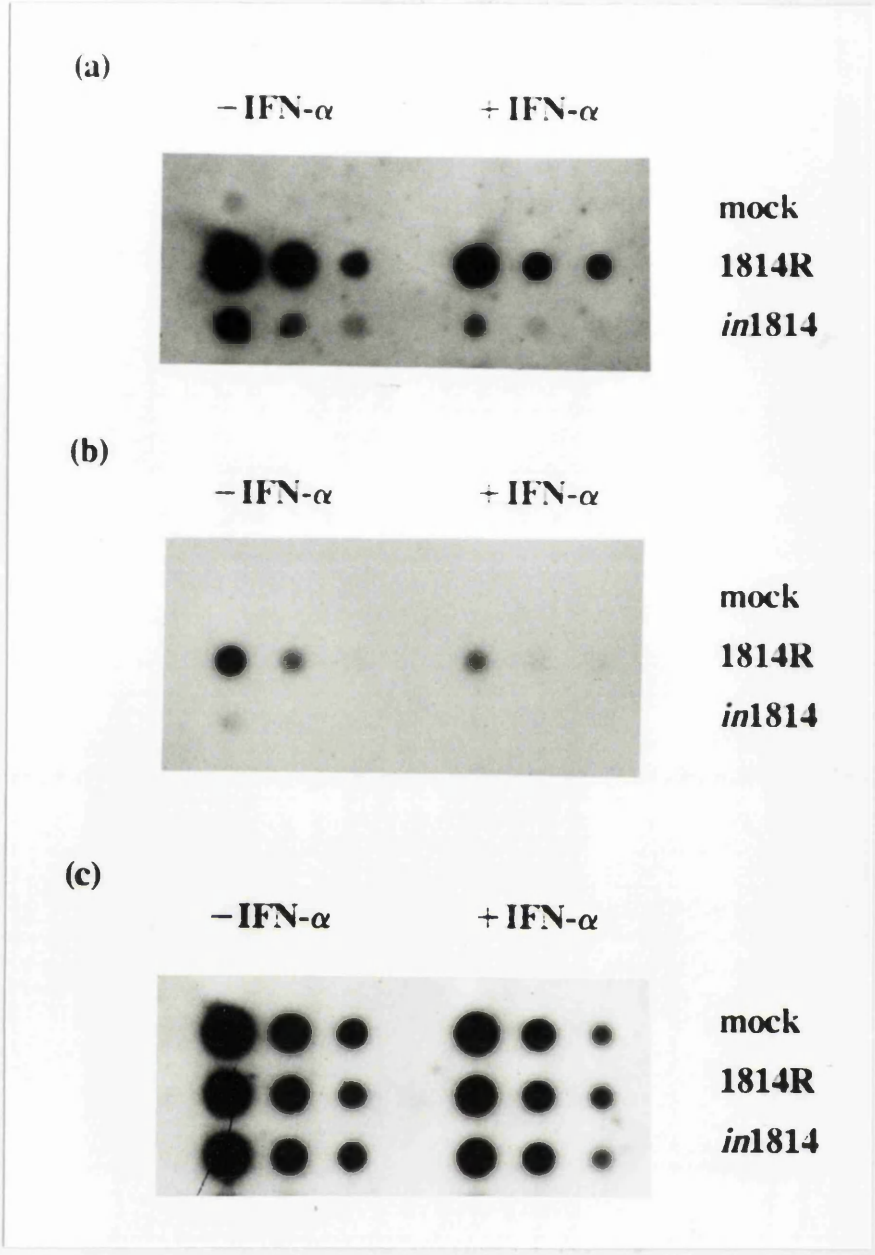


FIGURE 4.1: PRETREATMENT WITH IFN-α REDUCES THE ACCUMULATION OF Vmw110 mRNA.

HFL cells were grown on 50mm diameter plates and mock pretreated or pretreated with IFN-α at 10^3 units/ml for 16h. After removal of the IFN-α by washing, the monolayers were inoculated with virus at 150 particles per cell together with cycloheximide at 25μg/ml in a 400μl inoculum and maintained at 37°C for 1h to allow adsorption and penetration of virus. 5ml of EF10 containing cycloheximide at 25μg/ml was added and incubation continued for another 2h. RNA was prepared, diluted to 3.0, 1.0 and 0.3μg in 50μl of 20 x SSC and applied sequentially left to right to a nitrocellulose membrane. The membrane was hybridised with a ^{32}P-labelled DNA fragment from the Vmw110 (a) and (b) or actin (c) genes. Panels (a) and (b) show different exposures of the same blot.

hybridisation was performed. Monolayers infected with *in*1814 or 1814R in the presence of the protein synthesis inhibitor cycloheximide were harvested and the RNA extracted. The total amount of RNA was determined in each extract and various amounts were immobilised on a nitrocellulose membrane for hybridisation with specific probes. The DNA fragment from the IE110 gene was used as a marker for IE mRNA and from the actin gene as a marker for host cell mRNA. The results are shown in figure 4.1. In figure 4.1a it is evident that a 1μg dilution of total RNA from IFN-α pretreated cells infected with *in*1814 has approximately the same amount of Vmw110 mRNA as a 0.3μg dilution of total RNA from untreated similarly infected cells. Thus there is at least a 3-fold reduction in Vmw110 mRNA following pretreatment with IFN-α. The effect is not so clear at this exposure for cells infected with 1814R but the shorter exposure shown in figure 4.1b demonstrates that the effect is essentially the same. The effect of IFN-α pretreatment on the general level of mRNA synthesis as exemplified by actin mRNA and shown in figure 4.1c is not to the same extent.

4.9. Cycloheximide does not inhibit the effect of IFN-α

If the mechanism of IFN-α action is mediated via the synthesis of new proteins, the addition of the protein synthesis inhibitor, cycloheximide, should indirectly inhibit the effect of IFN-α. Experimental conditions were set up such that the effect of IFN-α could be inhibited but not potential lytic infection following its removal. This was achieved by pretreating the monolayers with both IFN-α and cycloheximide. HFL cells are unable to withstand exposure to cycloheximide for a period greater than 5h but it has been shown in table 4.5 that IFN-α is over 80% effective by this time. Before the inoculation with virus the monolayers were washed repeatedly to remove all traces of cycloheximide which, if still present, would prevent protein synthesis essential to a productive infection. The results are given in table 4.8.

The results show that the presence of cycloheximide does not alter the ability of IFN-α to inhibit the effect it has on *in*1814 or *in*1820. Thus the action of IFN-α does not require the synthesis of new proteins while it is exposed to the cells.

VIRUS	PARTICLES PER CELL	PLAQUES PER PLATE			
		NO IFN-α NO CH	IFN-α NO CH	NO IFN-α CH	IFN-α CH
*in*1814	100	cpe	cpe	cpe	cpe
	10	cpe	135	cpe	cpe*
	1	14.5	1	20.5	1.5
	0.1	0	0	0	0
*in*1820	100	cpe	159	cpe	88.5
	10	27	0	31.5	1.5
	1	1	0	1	0

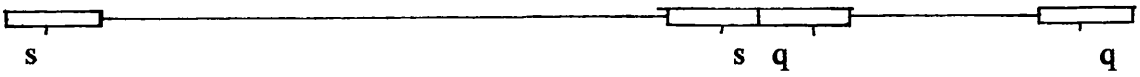
TABLE 4.8: THE EFFECT OF IFN-α AND CYCLOHEXIMIDE ON THE PLAQUE FORMING ABILITY OF *in*1814 AND *in*1820

HFL monolayers were cultured on 30mm diameter plates and pretreated with IFN-α at 10^3 units/ml and cycloheximide at 25μg/ml in various combinations for 5h. The cells were washed four times at 37°C with EF10 by gentle rocking of each plate for 2min and blotting them dry between each wash. The viral inoculum was applied in 200μl and the monolayer was maintained at 37°C for 1h to allow adsorption and penetration of virus. Following this, 1.8ml of EHu5 was added and the incubation continued for 48h. The overlay was poured off, the monolayer stained with Giemsa, and the plaques counted. The figures shown are mean values of duplicate samples. (*much less extensive than cpe in the absence of IFN-α and presence of CH)

4.10. Configuration of the viral DNA in the high multiplicity latency model

The *in vitro* latency model is based on a reduction of IE gene expression achieved by using a virus that expresses a mutant Vmw65, replacement of the Vmw110 promoter with the MoMuLV enhancer and pretreatment of the cells with IFN-α. At the moi used some virus will not go latent but remains potentially lytic and thus ara-C is included in the medium to prevent replication of this background virus. Superinfection with *ts*K shows that reactivation can be achieved with high efficiency. *In vivo*, latent HSV-1 DNA bears the characteristic of being non-linear (Rock and Fraser, 1983; 1985; Efstathiou *et al.*, 1986) and is transcriptionally silent with the

exception of the latency associated transcripts (Stevens *et al.*, 1987). Other investigators have suggested that it is maintained as a circular episome (Mellerick and Fraser, 1987). Hence the latent genome is structurally distinct to the DNA of a productively infecting virus. Earlier studies using an *in vitro* model and a low moi of 5 particles per cell (Harris and Preston, 1991), demonstrated that *in*1814 DNA is retained at relatively low abundance in a non-linear configuration during latency. It is important to know whether in the modified system of IFN-α pretreatment and using a higher moi of the virus *in*1820, the latent viral genome is also largely in the non-linear configuration. HFL monolayers pretreated with IFN-α were infected at a moi of 50 particles of *in*1820 per cell and incubated in the presence of ara-C for 24 or 48h. The DNA was extracted, cleaved with *Bam* HI and fractionated on a caesium chloride gradient to separate viral from host cell DNA. The samples containing the viral DNA underwent electrophoresis on an agarose gel, were blotted onto a nylon membrane and hybridised with a ^{32}P-radiolabelled DNA fragment. The fragment used was the purified joint spanning fragment, HSV-1 *Bam* HI k, radiolabelled to high specific activity by the random primer extension method. Progression from the linear to the non-linear configuration over 48h is evident by the gradual disappearance of the terminal sequences but the persistence of the joint sequences as is shown in figure 4.2. Although most of the termini had disappeared by 24h the process was not complete until 48h had elapsed. At a lower moi of 5 particles per cell and using *in*1814 (Harris and Preston, 1991) the first sign of disappearence of the termini was apparent at 5h but it was a full 48h post infection before they were undetectable. Latent HSV-2 produced by infecting HFL cells at the supraoptimal temperature also becomes an endless molecule (Preston and Russell, 1991) but in contrast to HSV-1 this developed more slowly and was not complete until 4 days post infection. Since the joint became bimolar relative to the unique sequences (Preston and Russell, 1991), it appears that the loss of the termini is due to their fusion to yield an additional joint. The simplest model to explain this phenomenon is circularisation of the linear genome. In the experiment shown in figure 4.2 the lack of *Bam* HI terminal sequences is not due to the protocol since terminal fragments were not lost following fractionation on caesium chloride gradients of the DNA samples which contain a mixture of *Bam* HI digested mock infected HFL cell DNA and virion DNA (data not shown). Pretreatment with IFN-α had no apparent effect on the physical form adopted by the genome in that the same pattern was observed in both pretreated and untreated cells (data not shown). The doublet hybridisation signal which occurs at the joint is due to a variation in the number of reiterated "a" sequences. The reason for the differences in the intensities of the signals corresponding to the two termini and joint fragments is not clear but it is



k = s + q

Diagram to show the *Bam* HI restriction fragments k, q and s of the HSV-1 genome

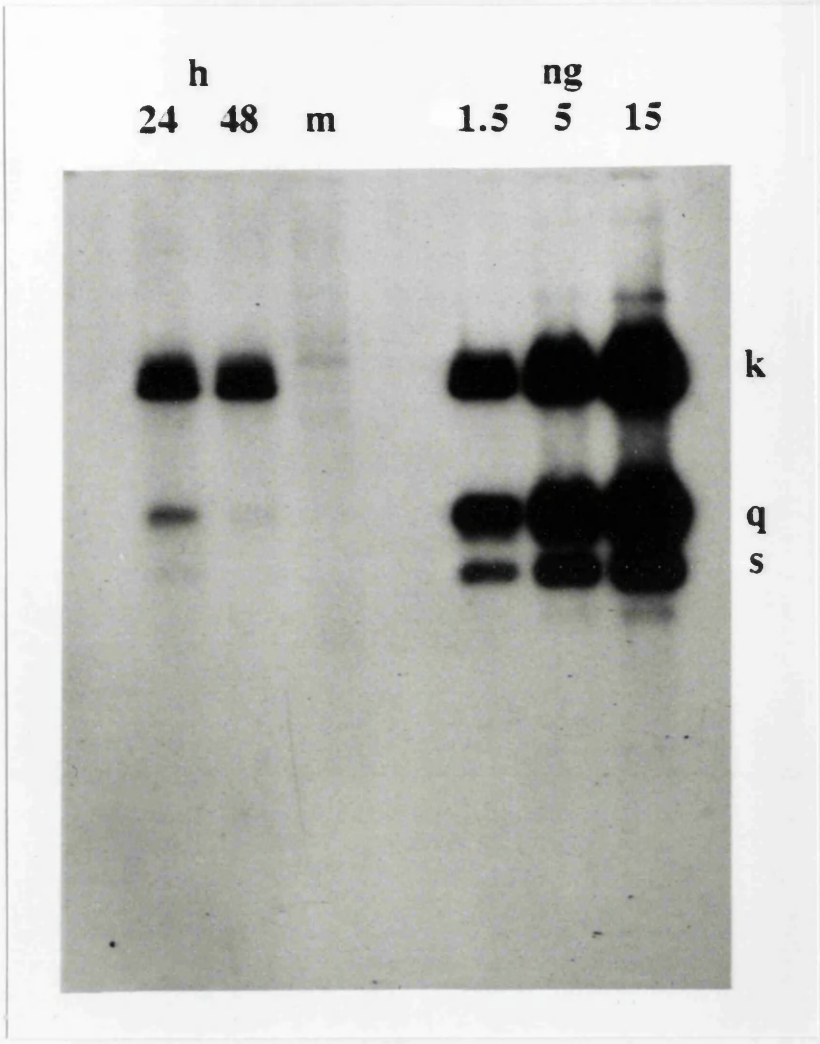


FIGURE 4.2: THE *in*1820 GENOME ADOPTS THE NON-LINEAR CONFIGURATION AFTER 48h IN THE *IN VITRO* LATENCY SYSTEM.

HFL cells were grown on 90mm diameter plates and pretreated with IFN-α at 10^3 units/ml for 16h. After the removal of the IFN-α the plates were infected with *in*1820 at 70 particles per cell or mock (m) infected and the monolayers were maintained at 37 °C for 1h to allow adsorption and penetration of virus. EF10 medium to 15ml per plate and containing ara- C at 50μg/ml was added and incubation was continued for 24 or 48h. The cells were harvested, DNA extracted from nuclei and cleaved with *Bam* HI at 100 units per sample in a total volume of 500μl. Viral DNA was separated from the host cell DNA by fractionation on a caesium chloride gradient, repurified and half of the total electrophoresed in a 1% agarose gel and analysed by Southern blotting. The DNA was hybridised with the ^{32}P-radiolabelled fragment *Bam* HI k which recognises sequences at the joint (k) and termini (q and s). Standards containing 1.5, 5 and 15ng of *in*1820 DNA are also shown.

probably a reflection of the hybridisation procedure. The intensities are different again in figure 4.20 where the band corresponding to the termini is more intense than the band corresponding to the joint.

It is concluded that the modifications utilised do increase the propensity to undergo latent infection and the genome continues to behave in a fashion analagous to that seen at a lower moi (Harris and Preston, 1991) and as demonstrated *in vivo* (Rock and Fraser 1983; 1985; Efstathiou *et al.*, 1986). The result gives credence to the model as mimicking one of the hallmarks of latent infection *in vivo.*

4.11. Viral DNA can be specifically detected in the presence of host cell DNA

Fractionation of DNA extracted from the infected cells on caesium chloride gradients into viral and host cell specific nucleic acid components prior to further investigation by hybridisation was performed to eradicate non-specific binding of the ^{32}P-radiolabelled *Bam* HI k fragment to cellular sequences. It is however, undesirable as it imposes limitations on the yield of the viral DNA and the number of samples that can be readily analysed. As the non-linear configuration has been demonstrated in this model (figure 4.2) only the presence of the viral DNA, rather than its structure, needs to be shown for many experiments. Ideally it should be possible to identify specifically the viral DNA in the extracts without having to resort to removing the host cell DNA. This has been achieved using a pTK1 2.4kb fragment, encompassing the TK gene, as probe. This hybridises to the p fragment from a *Bam* HI digest of HSV-1. DNA from infected cells was prepared and cleaved with *Bam* HI but the caesium chloride centrifugation step was omitted. Hybridisation of the ^{32}P-radiolabelled fragment from pTK1 was specific for the viral DNA even in the presence of host cell DNA as is illustrated in figure 4.3. The viral DNA was clearly identified by hybridisation of the pTK1 fragment to the *Bam* HI digested standard of 25ng *in*1820 (lanes s1 and s2) and there was no non-specific binding to cellular sequences in the mock infected unfractionated extract (lane m). In the latently infected cell extracts viral DNA was clearly identified. The unfractionated band represents 8% of the original material and the fractionated band 25% of the original material. Since the bands in lanes 1 and 3 are of approximate intensity it follows that about 68% of the *in*1814 DNA was lost by fractionation. The band in lane 4 is only at the most half the intensity of the band in

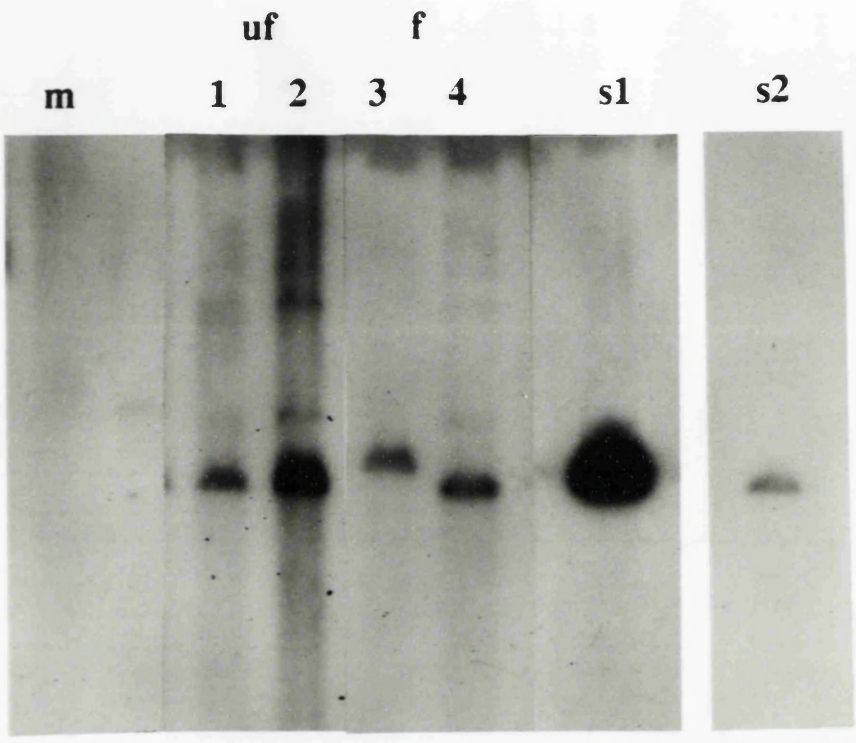


FIGURE 4.3: THE HSV-1 GENOME CAN BE DETECTED SPECIFICALLY BY THE ^{32}P-RADIOLABELLED DNA FRAGMENT pTK1 IN THE PRESENCE OF HOST CELL DNA

DNA was extracted from infected HFL monolayers grown on 90mm diameter plates and cleaved with *Bam* HI at 100 units per sample in a total volume of 500μl. 250μl of each sample was fractionated on a caesium chloride gradient. Following the electrophoresis of 40μl of unfractionated (uf) and 125μl fractionated (f) material in a 0.6% agarose gel and Southern blotting, the DNA was hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1. Lanes 1 - 4 are from HFL cells pretreated with IFN-α at 10^3 units per ml for 16h, infected at 70 particles per cell for 24h and incubated in EF10 containing ara-C at 50μg/ml. Lanes 1 and 3 represent material from *in*1814 and lanes 2 and 4 from *in*1820 infected cells. The autoradiograph was exposed for 48h. Also shown is a standard of 25ng *in*1820 DNA exposed for both 48h (s1) and 2.5h (s2) and a mock (m) infected sample. The bands in lanes 3 and 4 migrate differently because of the large amount of DNA loaded into the wells.

lane 2 which means that over 80% of the *in*1820 DNA was lost by fractionation. Thus fractionation on caesium chloride gradients reduces the yield of latent genomes considerably but it is no longer necessary and hence more viral DNA will be available from the samples in the next generation of experiments. By comparison with the 25ng *in*1820 standard following a 2.5h exposure of the autoradiograph with the 48h exposure, the unfractionated DNA in lane 1 contains at least 1.25ng of *in*1814 DNA and in lane 2 probably 2.5ng if not more of *in*1820 DNA. This is from 8% of the original material i.e. from 560 000 cells since 7 x 10^6 cells form the monolayer on a 90mm diameter plate. As 10^7 genomes approximates to 1.5ng, 1.25ng represents about 8 330 000 genomes. In other words this latency model has at least 15 viral genomes per host cell and justifies introducing the modifications to the system.

It has been demonstrated that the *in vitro* model of latency that has been developed has the features of the latent genome *in vivo* in that it is non-linear and it extends to more than 15 genomes per cell which should allow further investigation of latency at the molecular level.

4.12. Latent viral genomes can be reactivated from most of the cells in an infected monolayer.

To investigate further the presence of at least one viral genome in each latently infected cell in a monolayer a recombinant virus possessing the *lacZ* gene was used. The virus, *in*1884, has the IE110 promoter driving the *lacZ* gene inserted into the TK gene of *in*1820. Expression of the inserted gene can be observed directly by the activity of its enzyme product, β-galactosidase. The presence of β-galactosidase can be demonstrated by the blue colouration of the cell produced when the enzyme is provided with the appropriate substrate. Since the latent genome is transcriptionally silent except for the LATs (Stevens *et al.*, 1987) the recombinant virus can be used to investigate gene expression during latency. Reactivation can be effected by superinfection with 1814R. To stop the spread of reactivated virus from cell to cell in the monolayer brefeldin A is included in the medium after the superinfecting virus has adsorbed. Brefeldin A (Pelham, 1991) blocks release of virions into the extracellular medium. It does not affect nucleocapsid formation but no enveloped virus particles are observed in the cytoplasm. Published studies indicate that brefeldin A induced retrograde movement of molecules from the Golgi to the endoplasmic reticulum early in infection arrests the ability of the host cell to support maturation and egress of enveloped viral

particles (Cheung *et al.*, 1991). Unlike ara-C, brefeldin A will allow the reactivated genome to replicate and thus the signal will be higher and more easily detected. The blue colour arising from β-galactosidase activity is a marker for each cell which has retained a latent viral genome and has been reactivated by the superinfecting virus. As is shown in table 4.9 and figure 4.4, the presence of β-galactosidase is evident in the majority of the cells of a latently infected monolayer superinfected with 1814R, unlike the latently infected monolayer that has been mock superinfected and in which few cells stain blue (figure 4.5). The result shows that the IE110 promoter has been silenced by the establishment of the latent state. Although mock superinfection produces about 500 blue cells and presumably represents a background of breakthrough virus, this accounts for less than 0.1% of the total cells infected and does not significantly affect the results from this model nor their interpretation. As is seen in table 4.2 infection with 100 particles of *in*1820 per cell in an IFN-α pretreated monolayer still gives rise to the formation of 5 plaques. This similarly represents breakthrough virus although the numbers of plaques are only one hundredth of the breakthrough blue cells. The observation suggests that either there are genomes which have not undergone latent infection which are capable of transcribing but are unable to form detectable plaques or alternatively, it is a reflection of the sensitivity of the β-galactosidase detection assay compared with plaque formation in this system. It has been found using *in*1884 and looking for blue plaques under EHu5 that there are small plaques approximating in number to blue cells produced in the presence of brefeldin A (C.M. Preston, personal communication). At a lower moi of 7.5 particles per cell only about 20% of the cells become blue when superinfected with 1814R (table 4.9 and figure 4.6) This suggests that at this moi only 20% of the cells harbour a latent genome that can be reactivated, consistent with the results of previous investigators (Harris and Preston, 1991) who found that at a moi of 5 particles of *in*1814 per HFL cell between 13 and 21% of cells contained a potentially active genome that could be reactivated by superinfection with *ts*K. Co-infection of *in*1884 and 1814R produce similar levels of blue cells (table 4.10) as are produced by the superinfection experiments (table 4.9). This result shows that superinfection is highly efficient at reactivating the genome to equate with the activity from a genome that has not been switched into the latent pathway but whose predisposition has been overcome by the co-infecting virus.

Therefore the high moi latency tissue culture model does give rise to at least one latent viral genome per cell which is transcriptionally silent but can be induced to be transcribed by superinfection. It is hypothesised that this induction of transcription is a model for reactivation of the latent viral genome *in vivo*.

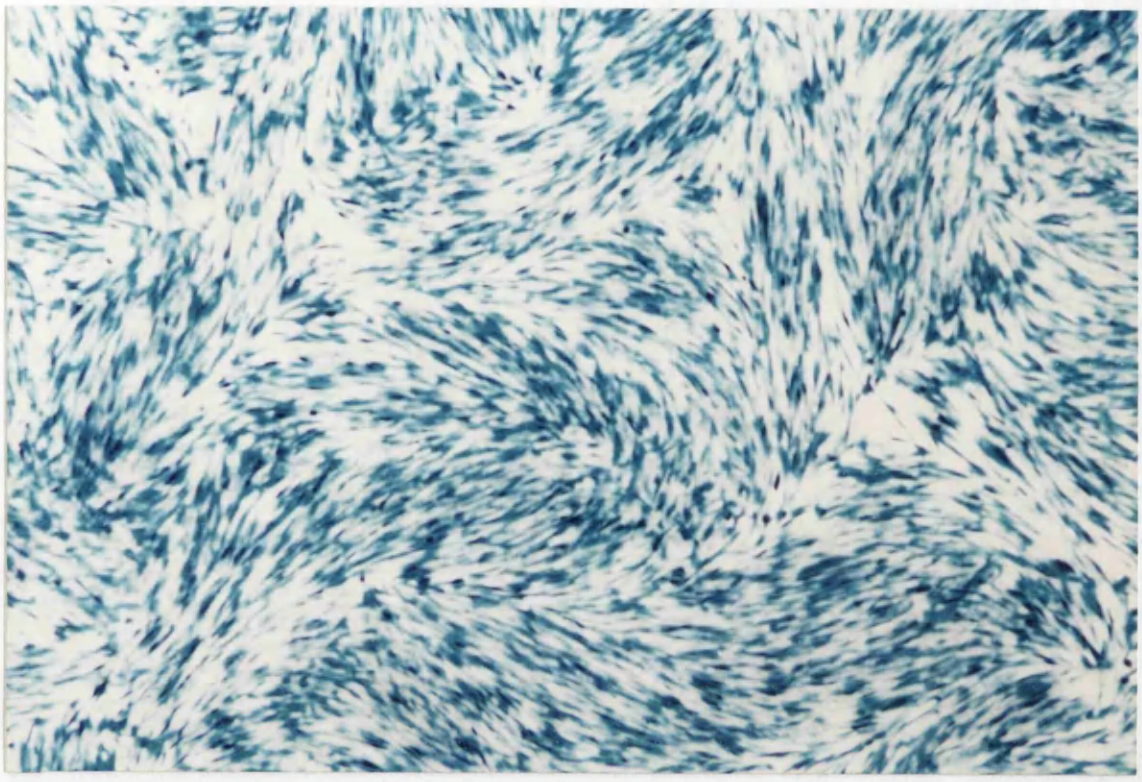


FIGURE 4.4: β-GALACTOSIDASE ACTIVITY IN LATENTLY INFECTED CELLS AT HIGH MOI SUPERINFECTED WITH 1814R

HFL cells were grown on 30mm diameter plates and pretreated with IFN-α at 10^3 units/ml for 16h. After the removal of the IFN-α the monolayers were infected with *in*1884 at 75 particles per cell and and the monolayer was maintained at 37°C for 1h to allow adsorption and penetration of virus. Medium EF10 containing ara-C at 50μg/ml was added and incubation continued for 64h. At 64h, the monolayers were thoroughly washed with EF10 to remove the ara-C and superinfected with the 1814R at 5 pfu per cell. After 1h to allow adsorption and penetration of the superinfecting virus, EF10 containing brefeldin A at 3μg/ml was added and incubation continued for a further 8h. The plates were then washed twice with ice cold PBS, fixed with 1% glutaraldehyde, washed again with PBS and stained for the presence of β-galactosidase. Positively stained cells are blue. The monolayers were counterstained with carmalum which renders the remaining cells which are negative for β-galactosidase, pink. Magnification x 2.5.

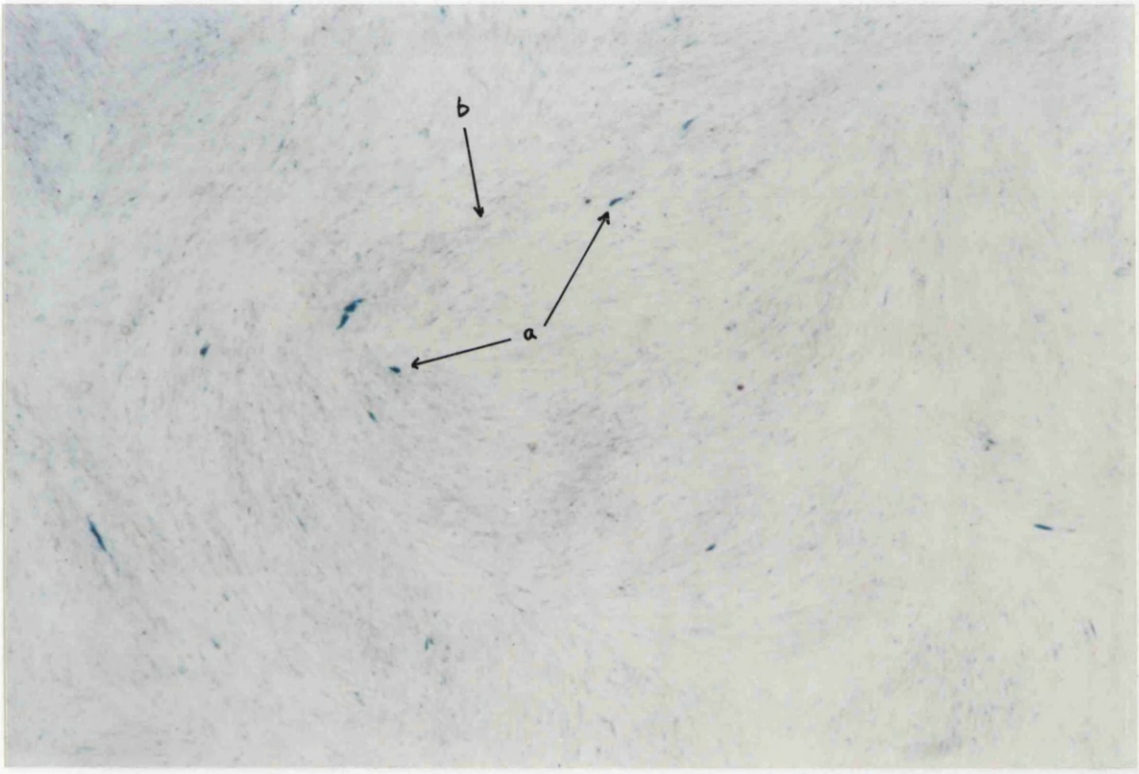


FIGURE 4.5: β-GALACTOSIDASE ACTIVITY IN MOCK SUPERINFECTED LATENTLY INFECTED CELLS

Latency was established in HFL cells with *in*1884 and β-galactosidase assayed as described in the legend to figure 4.4, but were mock superinfected after 64h in the presence of brefeldin A at 3μg/ml. Positive blue cells (a) and background blue staining (b) also found on mock treated monolayers are indicated by arrows. Magnification x 2.5.

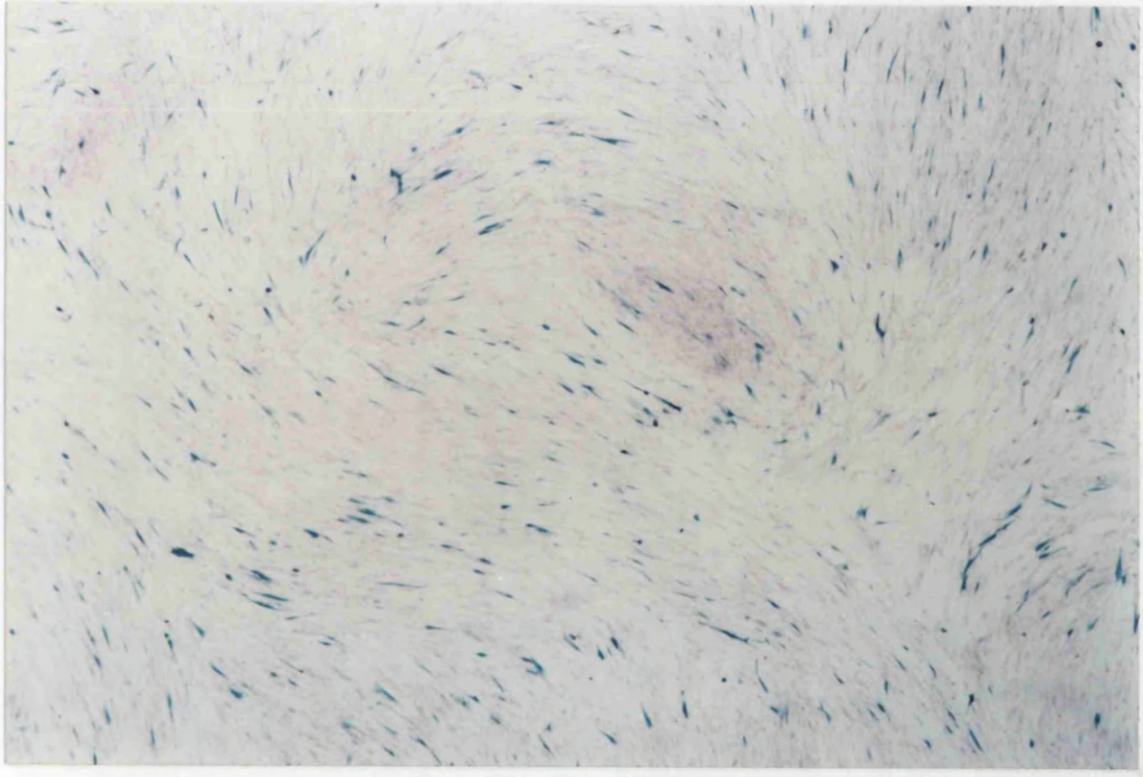


FIGURE 4.6: β-GALACTOSIDASE ACTIVITY IN CELLS LATENTLY INFECTED AT LOW MOI SUPERINFECTED WITH 1814R

Latency was established in HFL cells with *in*1884 and β-galactosidase assayed as described in the legend to figure 4.4, except a lower moi of 7.5 particles of *in*1884 per cell was used. Magnification x 2.5.

It has been reported (Kosv-Vnenchak *et al.*, 1992, Abstracts of the 17th International Herpesvirus Workshop, Edinburgh, Scotland.) that optimal immediate early and early HSV gene expression in cultured trigeminal ganglia requires the synthesis of viral DNA. To investigate whether this is the case in the tissue culture latency model, reactivation was effected in the presence or absence of the DNA synthesis inhibitor ara-C. As is shown in the results in table 4.9, when ara-C was omitted following superinfection there is no difference in the number of blue cells obtained compared with superinfection in the presence of ara-C. At a high moi a difference might not be apparent since the result is qualitative rather than quantitative in that one active genome will render a cell as blue as a cell with many active genomes. However, at lower moi ranging from 7.5 - 0.075 particles per cell the result is the same in that there is still no difference in the number of blue cells following superinfection of latently infected monolayers in the presence or absence of ara-C. The data shown in table 4.10 give the number of blue cells resulting from co-infecting with these two viruses at two moi of *in*1884 as in the superinfecting experiments. It is the result of a single experiment and the proportion of blue cells arising from 75 and 7.5 particles of *in*1884 is lower than would be expected if compared with the data in table 4.9. It is nevertheless clear from these two tables of data that superinfection is an efficient means of reactivating latent virus since the number of blue cells resulting from a superinfection is not less than those from a co-infection in which the predisposition of *in*1884 to latency has been negated by the simultaneous presence of 1814R. These findings suggest that viral DNA synthesis is not required for activation of the IE110 promoter upon superinfection, and imply that reactivation of the entire genome is not dependent upon genome replication.

PARTICLES PER CELL OF *in*1884	SUPERINFECTION	ARA-C	BREFELDIN A	NUMBER OR PROPORTION OF BLUE CELLS PER PLATE
75	mock	+	+	540 (~0.05%)
75	mock	–	+	423 (~0.04%)
75	1814R @ 5 pfu/cell	+	+	>95%
75	1814R @ 5 pfu/cell	–	+	>95%
7.5	1814R @ 5 pfu/cell	+	+	~20%
7.5	1814R @ 5 pfu/cell	–	+	~20%
0.75	1814R @ 5 pfu/cell	+	+	~5%
0.75	1814R @ 5 pfu/cell	–	+	~5%
0.075	1814R @ 5 pfu/cell	+	+	~1000 (~0.1%)
0.075	1814R @ 5 pfu/cell	–	+	~1000 (~0.1%)
mock	1814R @ 5 pfu/cell	–	+	0

TABLE 4.9: β-GALACTOSIDASE ACTIVITY FOLLOWING SUPERINFECTION OF LATENTLY INFECTED MONOLAYERS

Latency was established in HFL cells with *in*1884 and β-galactosidase assayed as described in the legend to figure 4.4 except that the moi was varied and the monolayers were superinfected with mock or with 1814R at 5 pfu per cell in the presence of brefeldin A at 3μg/ml and in the presence or absence of ara-C at 50μg/ml. Blue, positively stained cells, were counted or determined as a proportion of the total number of cells on the plate.

PARTICLES PER CELL OF *in*1884	CO-INFECTION	ARA-C	BREFELDIN A	APPROXIMATE PROPORTION OF BLUE CELLS PER PLATE
500	1814R @ 5 pfu/cell	+	+	100%
500	1814R @ 5 pfu/cell	−	+	100%
75	1814R @ 5 pfu/cell	+	+	30%
75	1814R @ 5 pfu/cell	−	+	70%
7.5	1814R @ 5 pfu/cell	+	+	50%
7.5	1814R @ 5 pfu/cell	−	+	30%

TABLE 4.10: β-GALACTOSIDASE ACTIVITY FOLLOWING CO-INFECTION OF MONOLAYERS

HFL cell monolayers were co-infected with *in*1884 and 1814R at the specified multiplicities in the presence of brefeldin A at 3μg/ml and in the presence or absence of ara-C at 50μg/ml. The monolayers were washed at 8h post infection and cells expressing β-galactosidase detected as descibed in the legend to figure 4.4.

4.13. Vmw110 is required for the reactivation and expression of the *lacZ* Gene

Evidence that the transcriptional activator Vmw110 has a role in the reactivation of latent virus has been described above (section 1.3.5). In cells containing reactivated *in*1884, Vmw110 is provided by the superinfecting virus 1814R. It follows that if Vmw110 is required for the reactivation of latent *in*1884, a superinfecting virus such as *dl*1403 with a mutation in the Vmw110 gene (Stow and Stow, 1986), should be unable to effect reactivation and this will be apparent by the failure of the superinfecting virus to turn latently infected cells blue when stained for β-galactosidase activity. Monolayers latently infected with *in*1884 at 75 particles per cell were mock superinfected or with *dl*1403 or 1814R. Reactivation was determined by quantifying the number of blue cells produced. The results are presented in table 4.11. Even with a high multiplicity of 5 pfu per cell the superinfecting virus *dl*1403 is still incapable of reactivating the latent virus *in*1884. The result demonstrates the requirement for Vmw110 in this system to bring about transcriptional activation of the latent genome.

PARTICLES PER CELL OF *in*1884	SUPERINFECTION	BLUE CELLS PER PLATE
75	mock	387 (~0.04%)
75	*dl*1403 @ 5 pfu/cell	729 (~0.07%)
75	1814R @ 5 pfu/cell	>90%

TABLE 4.11: β-GALACTOSIDASE ACTIVITY FOLLOWING SUPERINFECTION OF LATENTLY INFECTED MONOLAYERS WITH *dl*1403

Latency was established in HFL cells with *in*1884 and β-galactosidase assayed as described in the legend to figure 4.4 except that the monolayers were superinfected with mock, *dl*1403 or 1814R at 5 pfu per cell and following adsorption and penetration of the virus incubated in the presence of brefeldin A at 3μg/ml. Blue, positively stained cells, were counted or determined as a proportion of the total number of cells on the plate.

The results presented in section 4.12. and figure 4.5. demonstrate that the IE110 promoter driving the *lacZ* gene is inactive when *in*1884 is in the latent state. The data presented in table 4.11 suggest that it is insensitive to Vmw65, which normally activates it. A feature of the latent herpesvirus genome is, with the exception of the LATs, its transcriptional silence (Stevens et al., 1987). Thus other promoters are inactive during latency *in vivo*. The inactivity of other promoters during latency *in vitro* was examined. The virus *in*1883 is derived from *in*1820 and has the inserted *lacZ* gene under the control of the HCMV promoter. It was used to examine the activity of the HCMV promoter during latency in the *in vitro* model. Monolayers latently infected with *in*1883 were mock superinfected or with *dl*1403 or 1814R. The data in table 4.12 show that *in*1883 behaves in the same manner as *in*1884, confirming the transcriptional silence of the genome during latency with respect to another promoter. It was apparent during this experiment that the cells infected with *in*1883 and reactivated by 1814R became blue at a much more rapid rate and more intensely than their *in*1884 infected counterparts. This observation is explained by the greater potency of the HCMV promoter relative to the IE110 promoter. In a similar experiment the virus *in*1835 was also used as the latently infecting virus. This mutant is derived from *in*1814 and has the *E. coli lacZ* gene under the control of the SV40 promoter replacing the LAT promoter. It too did not produce many blue cells following a mock superinfection, although just above a background count, and yet could also be reactivated efficiently if superinfected with 1814R. This result shows that another promoter gives the same effect as the HCMV promoter and the IE110 promoter, that the site of insertion is not critical and that *in*1814 can be used as well as *in*1820.

4.14. Thymidine kinase activity as a marker for reactivation of the latent genome

During latency in animal systems the TK gene, in common with the rest of the genome except LATs, becomes silent. Using the available mutants it is possible to investigate the activity of this gene during latency and after reactivation in the *in vitro* model. The mutant *in*1820 has a normal TK gene but the superinfecting virus, *in*1863, has a disruption in the TK gene. The mutant *in*1863 is otherwise wild type. Thus any TK activity that is measured must reflect transcription from the *in*1820 genome. Monolayers latently infected with *in*1820 were superinfected with *in*1863 and the cells harvested at either 1h or 8h later following adsorption. The supernatant after lysis of the cells was retained for assay of TK activity. The results are presented in table 4.13.

VIRUS	SUPERINFECTION	NUMBER OR PROPORTION OF BLUE CELLS PER PLATE
*in*1883 @ 0.5 pfu/cell	mock	823 (~0.08%)
*in*1883 @ 0.5 pfu/cell	*dl*1403 @ 5 pfu/cell	868 (~0.09%)
*in*1883 @ 0.5 pfu/cell	1814R @ 5 pfu/cell	>95%
*in*1884 @ 75 p/cell	mock	243 (~0.02%)
*in*1884 @ 75 p/cell	*dl*1403 @ 5 pfu/cell	436 (~0.04%)
*in*1884 @ 75 p/cell	1814R @ 5 pfu/cell	>95%
*in*1835 @ 50 p/cell	mock	~1%
*in*1835 @ 50 p/cell	1814R @ 20 pfu/cell	>95%

TABLE 4.12: THE HMCV (*in*1883) IE110 (*in*1884) AND SV40 (*in*1835) PROMOTERS ARE INACTIVE IN THE *IN VITRO* LATENCY MODEL.

Latency was established in HFL cells with *in*1884 and β-galactosidase assayed as described in the legend to figure 4.4 and in addition latency was also established with *in*1883 or *in*1835. The titre of *in*1883 was too low for a particle count, therefore the titre on BHK cells in the presence of HMBA was used. The monolayers were superinfected with mock, *dl*1403 or with 1814R at 5 pfu per cell in the presence of brefeldin A at 3μg/ml. Blue, positively stained cells, were counted or determined as a proportion of the total number of cells on the plate.

EXPERIMENTAL CONDITIONS	CPM/μg PROTEIN		
latent mock / mock superinfected	153	96	364
latent *in*1820 / mock superinfected	nd	nd	299
latent *in*1820 / *in*1863 superinfected 1h	186	191	378
latent *in*1820 / *in*1863 superinfected 8h with ara-C	592	972	3093*
latent *in*1820 / *in*1863 superinfected 8h without ara-C	1272	1244	4168
co-infected *in*1820 and *in*1863 8h with ara- C	686	635	3482
co-infected *in*1820 and *in*1863 8h without ara- C	927	848	2546

TABLE 4.13: THYMIDINE KINASE ACTIVITY OF LATENT AND REACTIVATED *in*1820

HFL monolayers were grown on 50mm diameter plates and pretreated with IFN-α at 10^3 units/ml. Cells were mock or latently infected with *in*1820 at 120 particles per cell for 63h or 70h. At 63h or 70h infected cells were mock superinfected or superinfected with *in*1863 at 7.5 pfu per cell. At 63h uninfected cells were co-infected with *in*1820 at 120 particles per cell and *in*1863 at 7.5 pfu per cell. Ara-C was used at 50μg/ml. At 72h all the plates were harvested and the cells lysed. The lysate was retained for the determination of thymidine kinase activity. The results of three experiments are shown and each figure is the mean of duplicate assays with the exception of the co-infected samples which were only performed singly and the result marked with an asterisk since the replicate sample gave a highly anomalous result of 13684 cpm/μg protein. nd = not done

The results given in the table 4.13 and compared with mock infected cells show that HFL cells latently infected with *in*1820 have no detectable viral TK activity. This was expected in view of the absence of expression of the *lacZ* gene from the *in*1883, *in*1884 and *in*1835 genomes during latency (sections 4.12 and 4.13). After superinfection for 1h with *in*1863 there was still no thymidine kinase activity measurable, but by 8h a 10 fold increase in the enzyme activity from background was observed. This activity was at the same level as that produced by co-infecting the two viruses for 8h, showing that the reactivation of *in*1820 by *in*1863 is efficient. The absence of ara-C leads to a slightly greater activity as would be expected if the reactivated virus is permitted to replicate and consequently provide more expressible DNA.

4.15. Latent genome size and organisation

The genome of HSV-1 is a double stranded DNA molecule that is unit length and linear. The latent HSV-1(F) genome lacks the terminal restriction fragments *Bam* HI s and p but has a bimolar joint fragment *Bam* HI sp generated by the fusion of s and p. This has been shown to be true in the *in vitro* latency system where the joint spanning *Bam* HI k fragment of HSV-1 strain 17 is generated by the fusion of the terminal fragments s and q (Harris and Preston, 1991 and section 4.10). It follows that the latent genome must be organised into an endless form. There are a number of physical structures which could give rise to an endless form and these are illustrated in figure 1.5. They are an autonomous circular molecule of either unit length or as a concatemer, a linear molecule of either unit length or concatemeric and integrated into the host cell genome or an autonomous linear concatemer. Current evidence favours an autonomous circular molecule of unknown size (Mellerick and Fraser, 1987).

It is possible using field inversion electrophoresis technology to separate large molecules of DNA and, furthermore, large linear molecules from circular molecules of the same size. Linear molecules have a greater mobility than circular molecules although the precise mechanisms underlying the migratory properties of these molecules is not understood (Hightower and Santi, 1989). Nuclear material of infected cells was embedded in agarose and electrophoresed in a field subjected to inversion. To avoid the viral genomes becoming entangled with the high molecular weight cellular DNA and thus impeding their progress in the gel, the agarose blocks were exposed to *Ase* I. No restriction site exists for this enzyme in the *in*1820 genome and it will

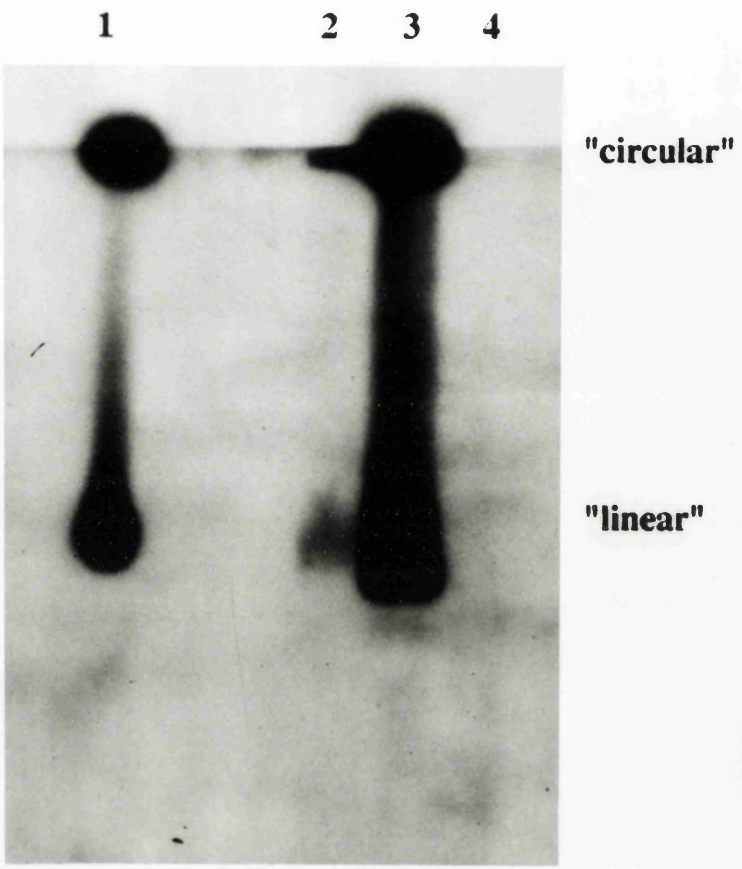


FIGURE 4.7: SEPARATION OF COMPLETE LYTIC AND LATENT VIRAL GENOMES FROM HOST CELL DNA

Mock (lane 4), lytically (lane 3) or latently (lane 2) infected cells embedded in agarose blocks were digested with proteinase K and then with 200 units of *Ase* I for 4h prior to loading on a 1% agarose gel. Other lytically infected cells (lane 1) embedded in agarose blocks were digested with proteinase K but not *Ase* I. Lytically infected cells had been infected with 1814R at 15 pfu per cell for 6h and latently infected cells, following IFN-α pretreatment, with *in*1820 at 120 particles per cell in the presence of 50μg/ml ara-C for 48h before harvesting. Field inversion electrophoresis with a field strength of 120 - 180 volts on a logarithmic scale was performed using a Rotaphor at 13°C over 48h. The other parameters were a switching interval of 5 - 90s on a linear scale and a switching angle of 235° - 250° on a logarithmic scale. The samples were electrophoresed from the front of the chamber and the field moved with the power on. The gel was depurinated prior to Southern blotting and hybridisation with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

therefore remain intact whether circular or linear whereas the host genome will be extensively digested. The result is seen in figure 4.7.

No hybridisation to mock infected HFL cell was detected (lane 4). In lane 1 there is a large amount of viral DNA high up in the gel as well as lower down. The lower band corresponds to a lambda ladder marker of 145.5 kb (not shown) when the gel was photographed under ultraviolet illumination. This approximates to 150kb and presumably represents the unit length 1814R genome. The DNA at the top of the gel must represent higher orders of organisation during replication such as rolling circles. Since 1814R is vulnerable to digestion with *Ase* I it appears as a smear in lane 3 which runs beyond the unit length representing digestion to fragments less than 150kb. In lane 2 there are two distinct populations of *in*1820 DNA. The lower band is unit length linear DNA but at the top of the gel there is a band of DNA of a different organisation. It is suggested that this is the latent *in*1820 genome. It is not possible to determine whether this is a unit length or concatemeric circular molecule nor whether it is integrated or autonomous. No quantitative comparison between the two bands is valid because the differences in physical organisation of the DNA might affect efficiency of transfer to the hybridisation membrane.

The utilisation of field inversion electrophoresis was technically very demanding and frustrating. One early experiment was very suggestive of an autonomous latent genome migrating more slowly than the viral DNA from lytically infected cells but despite intensive efforts to optimize the experimental conditions this could not be repeated. It should be possible to refine the technique to a much higher degree and the results, a typical example of which is shown in figure 4.7, would be much improved. The technology opens up the possibility of exploring and exploiting the differences in structure between lytic and latent genomes and once the optimal conditions for separating the two have been determined it should be feasible to investigate the temporal aspects of the changes between the structures and how this relates to the establishment of and reactivation from latency.

4.16. Latent viral DNA is associated with a nuclear substructure

The existence of a nucleoskeleton has been hypothesised as a necessary structure to organise the chromatin in the nucleus. However, the presence of such a structure has

been controversial because the various candidates which include matrices, scaffolds and cages have all been isolated under very non-physiological conditions. One method involves extraction of the subnuclear components using high concentrations, such as 2M, of salt. This yields a variety of complexes termed ghosts, matrices, scaffolds, folded chromosomes and nuclear cages. Others have claimed that the structures detected microscopically are merely results of coagulation arising during the isolation procedures. Thus sceptics have argued that the nuclear substructures are isolation artefacts with no counterparts *in vivo* (Cook, 1988). It is not surprising therefore that there is little agreement as to the nature of the nucleoskeleton which is synonymously called the nuclear matrix or the nuclear scaffold.

Conditions approximating physiological have been used to demonstrate successfully that essentially all active polymerases are attached to a nuclear structure (Jackson and Cook, 1986a; 1986b; Jackson *et al.*, 1988) and that this is the active site of replication and transcription (Jackson and Cook 1985b; 1986a). This structure has been termed the nucleoskeleton (Jackson and Cook 1985a; 1985b) to distinguish it from those isolated under hypertonic conditions.

Nascent DNA is looped by attachment to the skeleton and interest has centred on whether this is determined by specific sequences. If attachment is specific then the sequence involved should resist nucleolytic attack and pellet with the nuclear substructure. If there is no specificity in the attachment then there will be neither enrichment nor depletion of a particular sequence in the pellet. Specific association has been demonstrated by incubating matrices with pure DNA fragments (Cockerill *et al.*, 1987) and matrix-associated regions have been functionally defined (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Webb *et al.*, 1991) The attachment hypothesis provides the nucleoskeleton with an active rather than a passive role. Gene activation and inactivation is envisaged as being related to attachment and detachment to the skeleton which is dynamic, existing in different forms and in a constant flux between assembly and disassembly. Nuclear matrix proteins may be expressed in a cell specific manner (Fey and Penman, 1988). Thus the nucleoskeleton has a potential role in gene expression through the association with DNA via the matrix-associated regions (Cockerill and Garrard, 1986) and the cell specific proteins of which it is composed.

Integrated viral genes in cell lines transformed by polyoma virus or avian sarcoma virus lie close to the point of attachment to the nuclear substructure (Cook *et al.*, 1982). The *in vitro* tissue culture model provides the opportunity to investigate

whether the herpesvirus genome associates with the nucleoskeleton either during a productive or latent infection and if this regulates its expression.

To investigate the possibility of the HSV-1 genome associating with the nuclear matrix, nuclear preparations were made from cells variously infected with *in*1820. The sub-nuclear structure, the matrix, was extracted from the nuclei using a high concentration of salt and collected on a sucrose cushion. To show that the nuclei had been further fractionated both the pellet and the supernatant arising from the matrix preparation were assayed for protein content by the Bradford method (Bradford, 1976). The pellet fraction yielded up to 17μg of protein and the supernatant up to 40μg. The combined pellet and supernatant protein content approximates to about 10% of the total cellular protein which is the predicted amount in a nuclear preparation. The photograph of the stained gel (figure 4.8a) shows that the cellular DNA is largely present in the pellet with very little appearing in the supernatant i.e. the reverse of the protein distribution, thereby validating the method used as a successful fractionation technique.

Figure 4.8a shows that the DNA is confined to the pellet fraction with the exception of lane 6 where more DNA is present in the supernatant. This is due to contamination of the supernatant fraction with material from the corresponding pellet fraction. This was evident during the preparation and is because of the viscosity of the matrix which makes a clean separation difficult. This difficulty is also the reason for the loss of the DNA from lane 5 which was also apparent during the preparation. The material from one sample is shown in two lanes (4) and the outside lane of these two is shown in part (b) since this lane contains the DNA. Thus all the DNA in the nucleus is contained within the nuclear subfraction. The viral DNA is also confined to this fraction as is shown in figure 4.8b. This co-purification of the viral genome and the host cell matrix is apparent in the early stages of latent infection at 1h (lanes 9 and 10), 8h (lanes 7 and 8) as well as when latency is fully established by 72h (lanes 1-4).

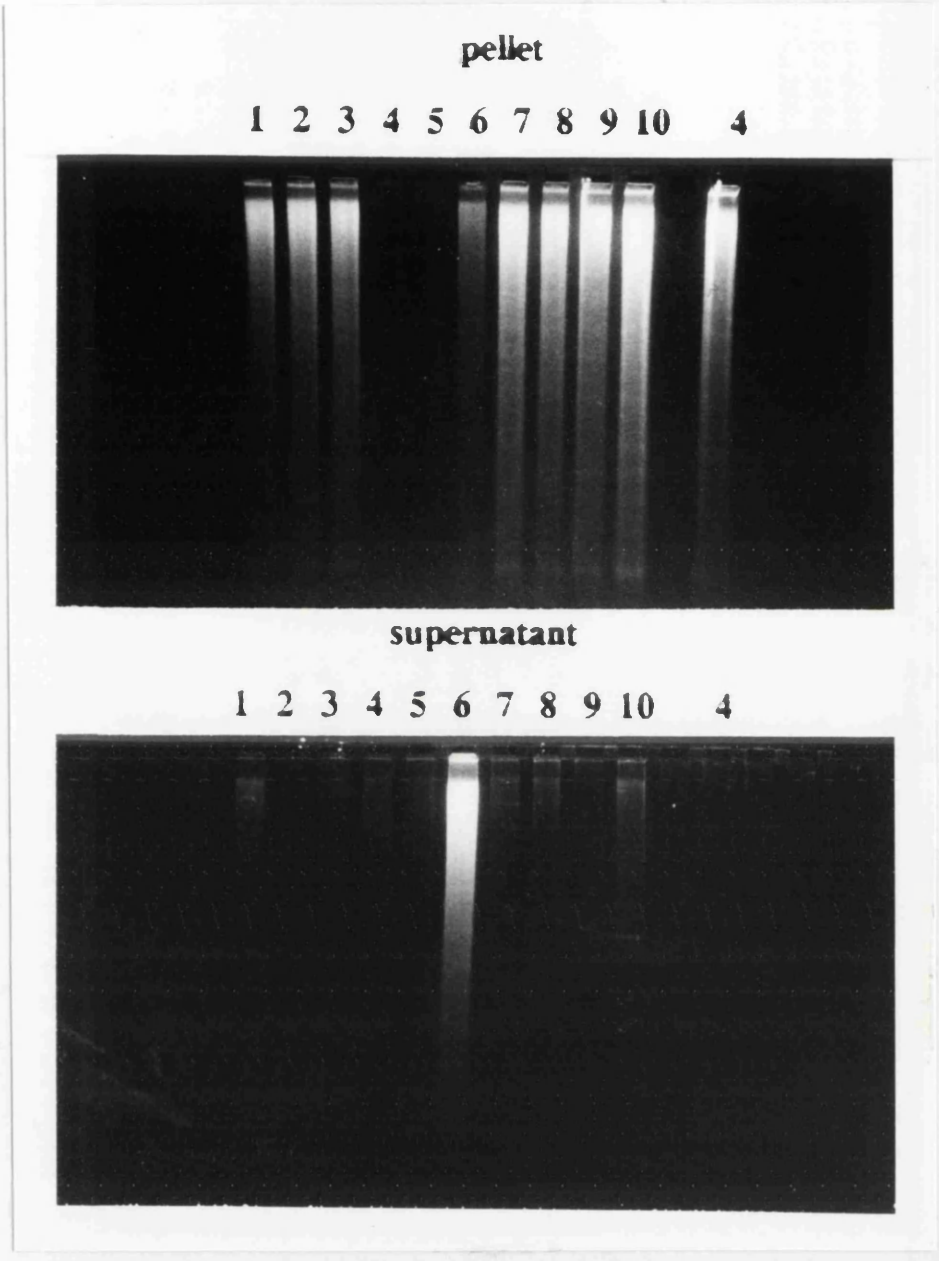


FIGURE 4.8(a): UNDER CONDITIONS PREDISPOSING TO LATENCY THE VIRAL DNA IS ASSOCIATED WITH THE NUCLEAR SUBSTRUCTURE

IFN-α pretreated monolayers were infected with *in*1820 for 1h (lanes 9 and 10), 8h (lanes 7 and 8) or 72h (lanes 1 - 6) in the presence of ara-C. The samples in lanes 5 and 6 were from monolayers superinfected with *in*1863 8h prior to harvesting. Matrices were prepared and also the top half of the supernatant fraction retained. Both supernatant (250μl) and pellet (100μl) fractions were incubated with proteinase K overnight, the DNA extracted, cleaved with 30 units of *Eco* RI for 3h and 25μl electrophoresed in a 1% agarose gel for 18h. The gel was photographed under ultraviolet light. The top half of the panel is material from the pellet fraction and the bottom half from the supernatant fraction.

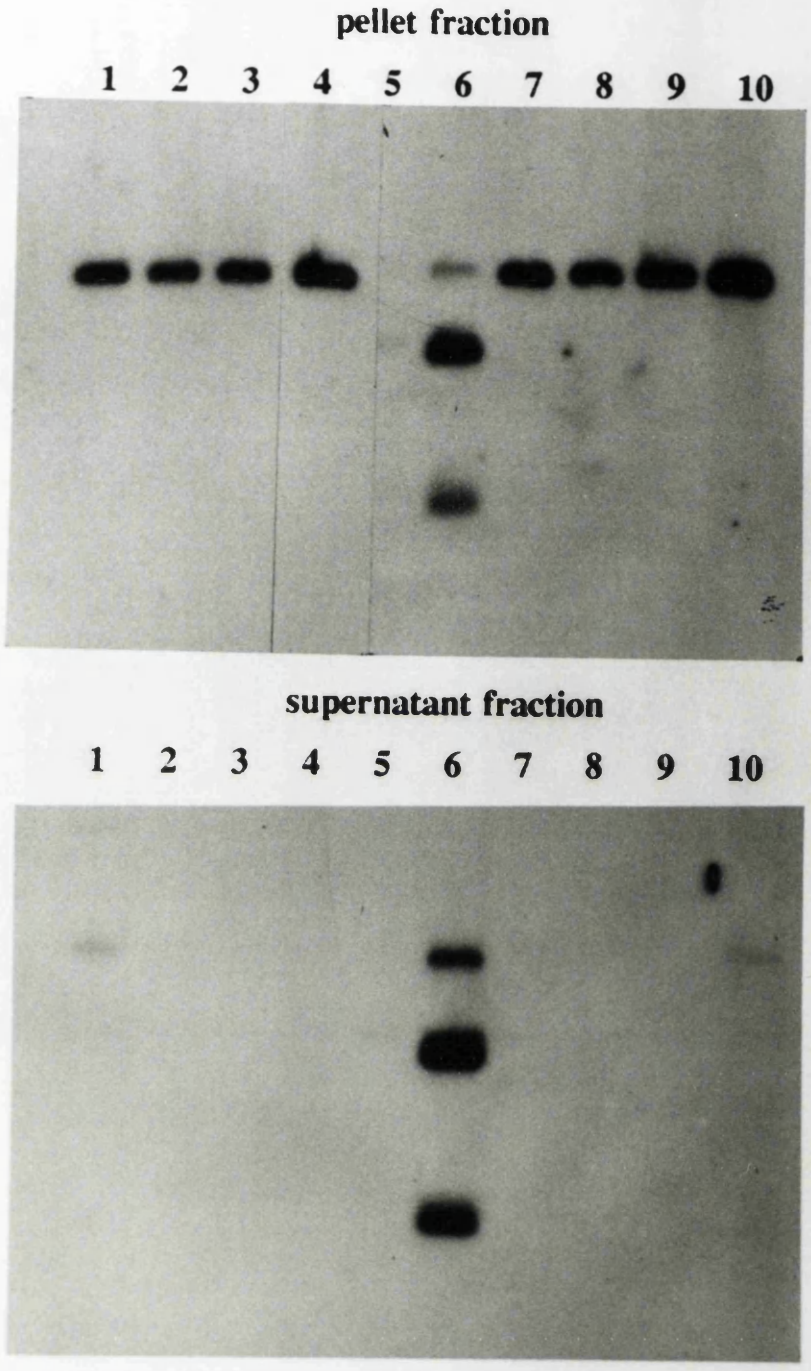


FIGURE 4.8(b): UNDER CONDITIONS PREDISPOSING TO LATENCY VIRAL DNA IS ASSOCIATED WITH THE NUCLEAR SUBSTRUCTURE

The gel shown in figure 4.8(a) was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

The results of a similar experiment to investigate whether an association with the matrix also occurs during a lytic infection are shown in figures 4.9a and 4.9b. The revertant 1814R was used to infect cells in the presence or absence of replication and protein synthesis inhibitors. When replication was allowed to continue for 8h after inoculation viral DNA was present both in the pellet and supernatant fractions, lanes 1 and 2, as would be expected. The DNA present in the supernatant fraction presumably represents progeny genomes. This distribution between matrix and nucleoplasm was prevented by the presence of cycloheximide as is shown in lanes 5 and 6. Lanes 3 and 4 show that inhibition of replication by ara-C confined the productively infecting genome to the nuclear substructure. Since 1.5ng represents 10^7 genomes, the intensity of the band in lane 12 approximates to 16 x 10^7 genomes. The monolayer was infected at 800 particles per cell which is 160 x 10^7 particles per plate and half of the preparation was run on the gel. Thus the intensity of the bands in the pellet fractions is, as expected, greater than the intensity of the band in lane 12. Taken together the results shown in figures 4.8b and 4.9b indicate that viral DNA associates with the host cell nuclear substructure early in infection irrespective of whether it then becomes productive or latent.

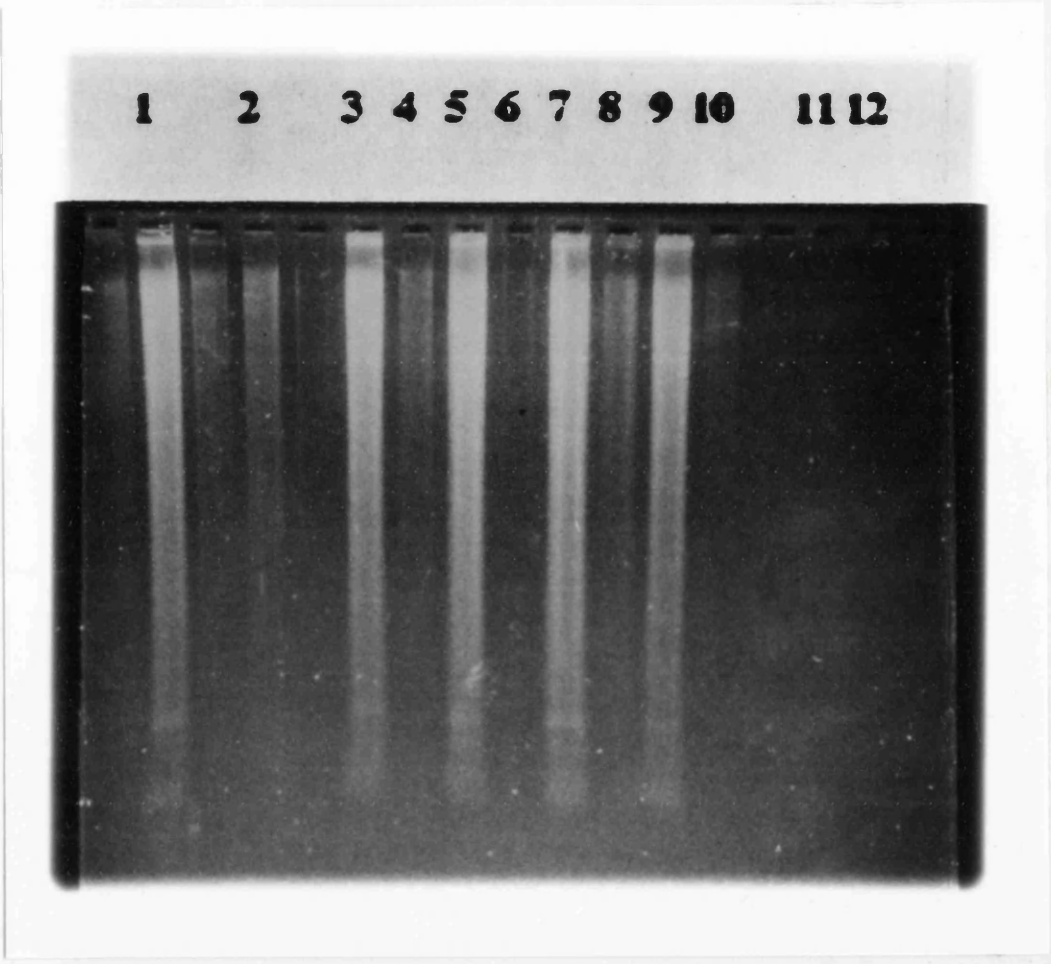


FIGURE 4.9a: WILD TYPE VIRAL DNA IS ASSOCIATED WITH THE NUCLEAR SUBSTRUCTURE EARLY IN INFECTION

Monolayers were inoculated with 1814R at 800 particles per cell (lanes 1 - 8) or mock infected (lanes 9 and 10) and incubated for a total of 8h under various conditions with or without inhibitors of protein synthesis, effected by cycloheximide or replication, effected by ara-C. No inhibitors were present in lanes 1 and 2, ara-C was present in lanes 3, 4, 7 and 8 and cycloheximide was present in lanes 5, 6, 7 and 8. Pellet or matrix (p) and supernatant (s) fractions were prepared and are denoted by odd and even numbers respectively. The fractions were incubated with proteinase K overnight, the DNA extracted, dissolved in 100μl of buffer, cleaved with 50 units of *Eco* RI for 2.5h and 50μl electrophoresed in a 0.6% agarose gel for 18h. Two standards of 5 and 25ng of DNA were run in lanes 11 and 12 respectively. The gel was photographed under ultraviolet light. Material apparent between lanes 1 and 2 arises from overspill of DNA from lane 1.

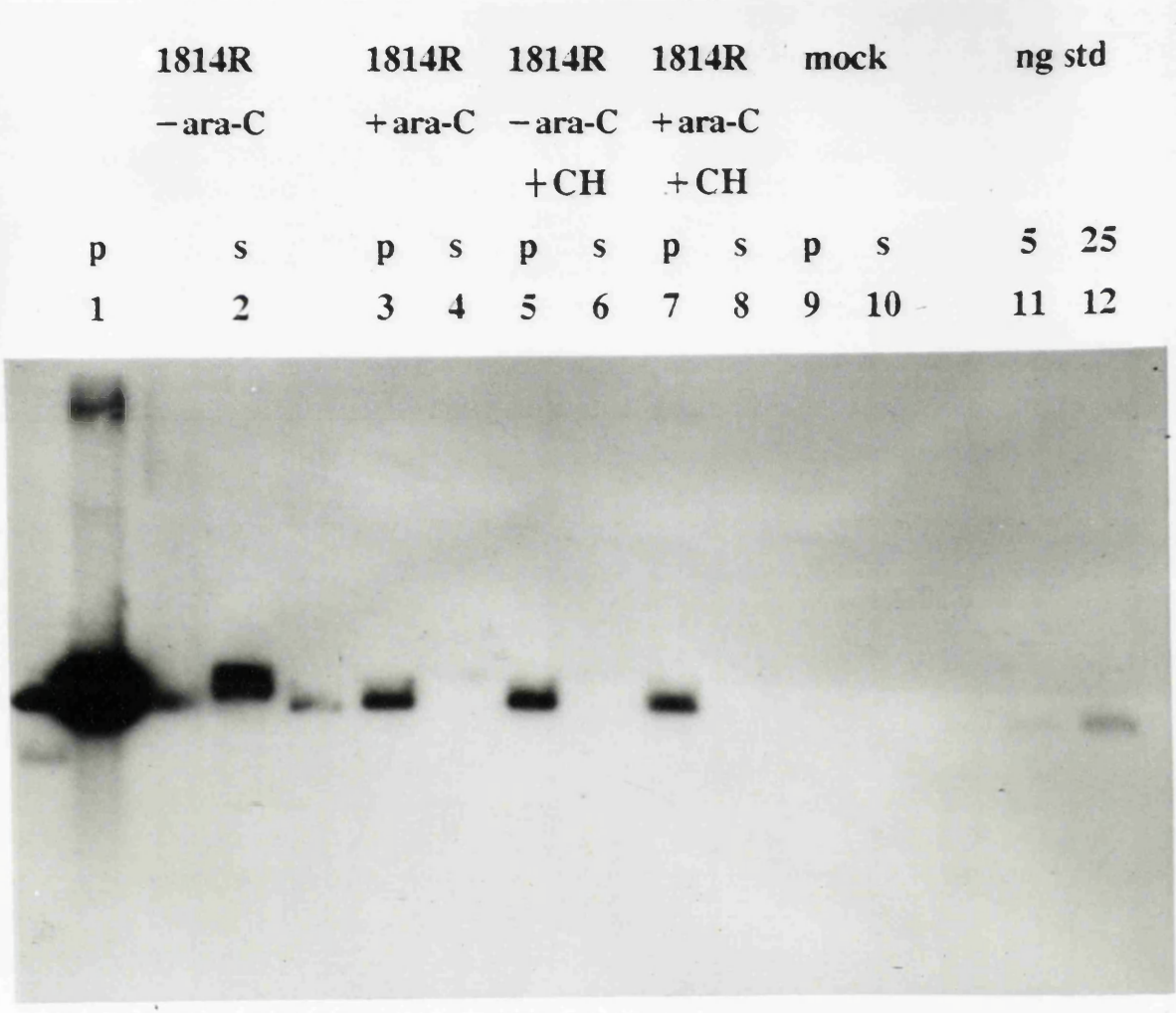


FIGURE 4.9b: WILD TYPE VIRAL DNA IS ASSOCIATED WITH THE NUCLEAR SUBSTRUCTURE EARLY IN INFECTION

The gel shown in figure 4.9a was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

4.17. The latent viral genome *in vitro* does not have a chromatin-like structure

Earlier investigators have found that during latency the viral DNA is associated with nucleosomes in a chromatin structure (Deshmane and Fraser, 1989). Micrococcal nuclease attacks the linker regions between the nucleosomes in chromatin and generates a series of multimeric units based on the 140bp nucleosomal core (Whitlock and Simpson, 1976). These can be visualised as a ladder when run in a 2% gel. If the number of units of the enzyme or the incubation time is increased a more extensive digestion occurs with fewer large multimers and many more smaller ones produced. To examine whether the HSV-1 DNA in the *in vitro* latency model associates with histones to form chromatin, HFL monolayers variously infected with virus were subjected to micrococcal nuclease digestion. The results are shown in figures 4.10a, 4.10b, 4.11a and 4.11b. The photographs of the stained gels, figures 4.10a and 4.11a, show the nucleosomal ladder produced by digestion of all the extracted DNA. However, the viral DNA whether in the early stages of infection or when latent, as shown in figure 4.10b, does not give rise to this pattern. In addition, there is much more viral DNA near the origins of those tracks from the early stages of infection (lanes 1 - 12) than in the tracks from a latent infection (lanes 13 - 15). This observation suggests that the viral DNA is more resistant to digestion early in infection than when latent and this is investigated later. The reduced amount of hybridisation in lane 13 (figure 4.10b) is probably a result of poor transfer of high molecular weight DNA to the membrane since there is a reasonable quantity present in the gel well prior to Southern blotting (figure 4.10a). Depurination of the DNA (see section 4.15) was not performed for this experiment.

To confirm that the non-nucleosomal structure of the viral DNA is not merely a feature of the TK gene, pTK1 transformed 143TK$^-$ cells which have the TK gene under the control of the TK promoter, were also subjected to micrococcal nuclease digestion. This cell line consists of a mixture of colonies from 143TK$^-$ cells transformed with pTK1 and was donated by M. McFarlane. Figure 4.11b shows that the TK gene in this cell line is nucleosomal, in contrast with the same gene in latently infected untransformed cells.

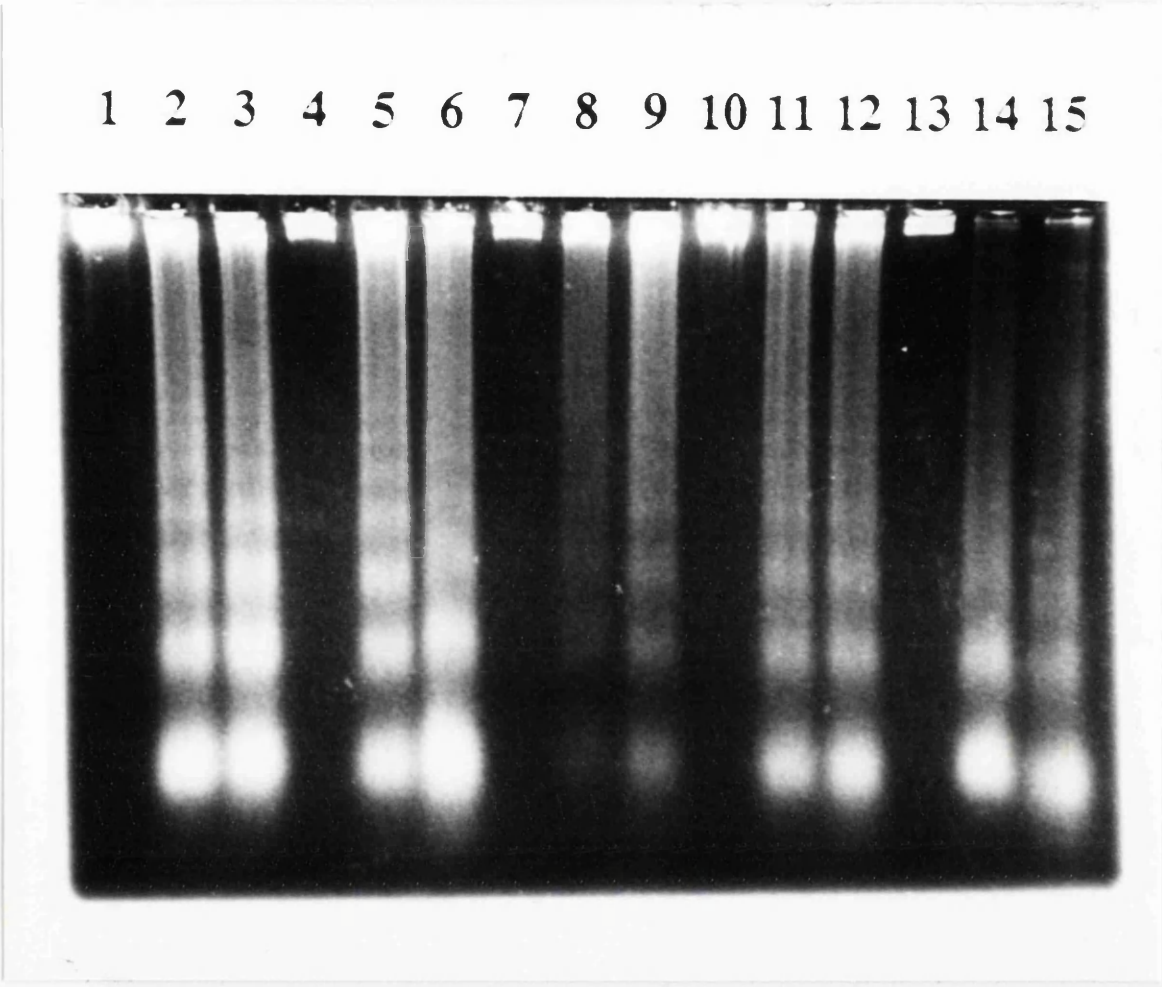


FIGURE 4.10a: TOTAL DNA FROM HSV-1 INFECTED HFL CELLS PRODUCES A NUCLEOSOMAL PATTERN WHEN DIGESTED WITH MICROCOCCAL NUCLEASE.

HFL monolayers on 50mm diameter plates and variously infected with virus were harvested, lysed and their DNA subjected to micrococcal nuclease digestion. The extracted DNA was redissolved in 100μl of water containing 10μg of ribonuclease and 50μl was electrophoresed in a 2% gel composed of 1.5% NuSieve®, 0.5% agarose and ethidium bromide at 0.5μg/ml. The gel was photographed under ultra violet light. Lanes 1, 2, 3,7,8 and 9 were infected with wild type HSV-1 at 120 particles per cell. Lanes 4, 5, 6 and 10 to 15 were infected with *in*1820 at 120 particles per cell. The virus was present for 1h (lanes 1 - 6), 8h (lanes 7 - 12) or had established latency (13 - 15). Lanes 7 - 15 had been treated with ara-C but only lanes 13 - 15 pretreated with IFN-α. Lanes 1, 4, 7, 10 and 13 were digested for 0 min, lanes 2 ,5, 8, 11 and 14 for 2 min and lanes 3, 6, 9, 12 and 15 for 5 min with 7.5 units of micrococcal nuclease.

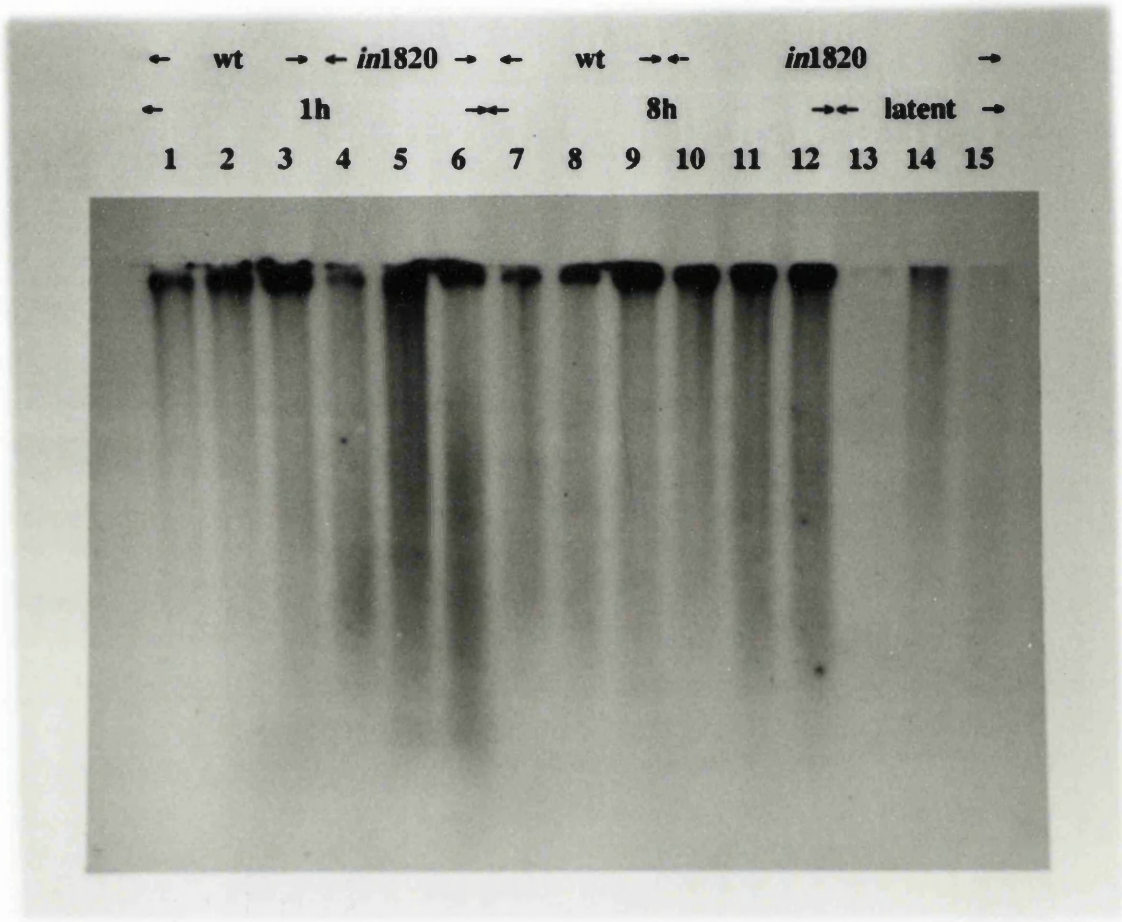


FIGURE 4.10b: VIRAL DNA DOES NOT FORM NUCLEOSOMES WHEN DIGESTED WITH MICROCOCCAL NUCLEASE.

The gel shown in figure 4.10a was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

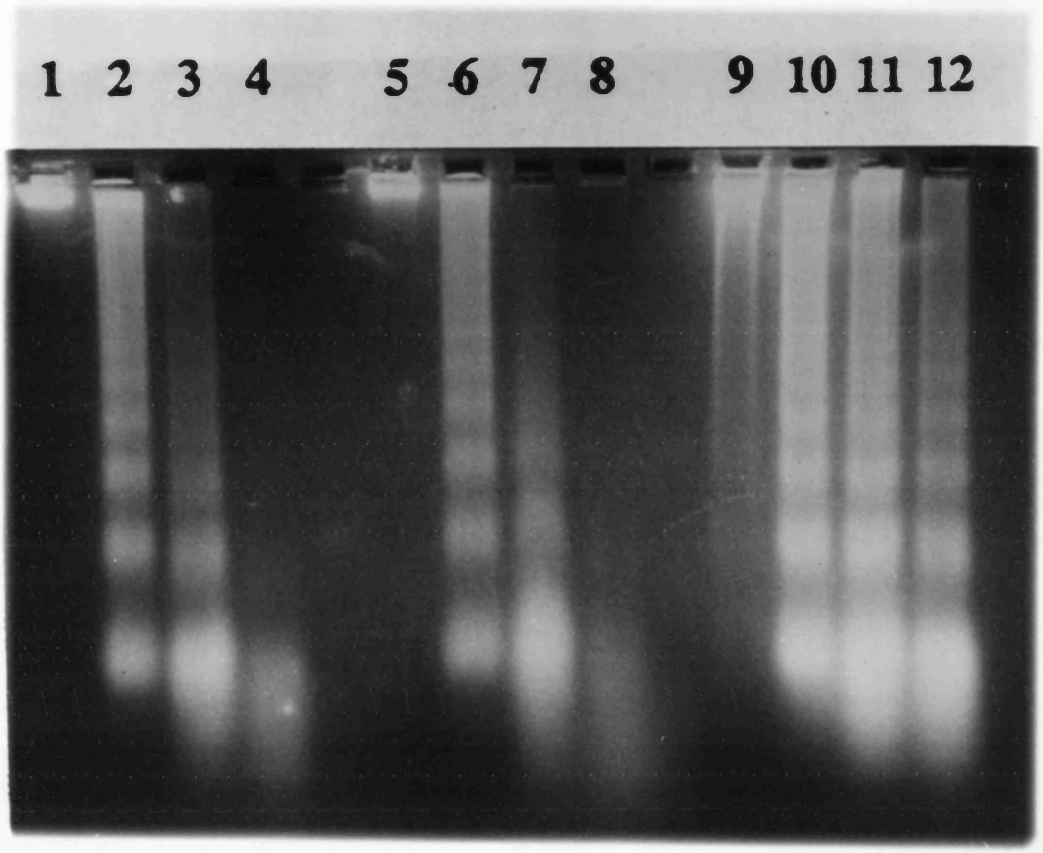


FIGURE 4.11a: TOTAL DNA FROM MOCK AND LATENTLY INFECTED HFL CELLS AND IN A pTK1 TRANSFORMED CELL LINE IS NUCLEOSOMAL

HFL cells mock (lanes 1 - 4) or latently infected with *in*1820 at 120 particles per cell (lanes 5 - 8) or pTK1 transformed cells (lanes 9 - 12) were grown on 50mm diameter plates, harvested, lysed and their DNA subjected to micrococcal nuclease digestion. Lanes 1, 5 and 9 were digested for 0 min, lanes 2 ,6 and 10 for 2 min with 7.5 units, lanes 3, 7 and 11 for 10 min with 22.5 units and lanes 4 ,8 and 12 for 30 min with 45 units, of micrococcal nuclease. The extracted DNA was redissolved in 100μl of water containing 10μg of ribonuclease and 30μl was electrophoresed in a 2% gel composed of 1.5% NuSieve®, 0.5% agarose and ethidium bromide at 0.5μg/ml. The gel was photographed under ultra violet light.

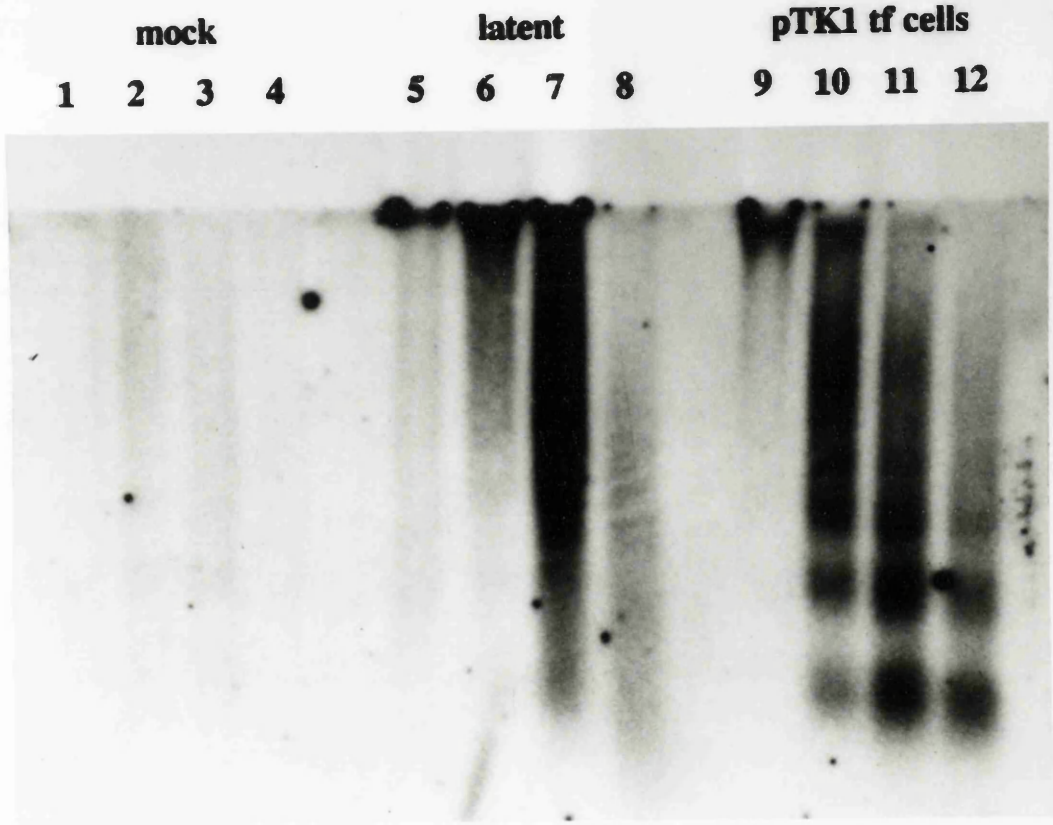


FIGURE 4.11b: THE TK GENE IS NUCLEOSOMAL IN A pTK1 TRANSFORMED CELL LINE BUT NOT IN THE LATENT VIRAL GENOME

The gel shown in figure 4.11a. was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

4.18. The latent viral genome is more sensitive to micrococcal nuclease digestion than in the early stages of infection

Although some of the proteins associated with the viral genome including the DNA-binding and nucleocapsid proteins have been described, very little is known about the proteins which are associated with the intracellular viral genome. The term "uncoating" has been used to describe the processes involved in the preparation of the input genome for expression and this envisages some form of change to the nature or distribution of the proteins on the viral DNA. It is likely that during transcription, replication and latency the DNA will have a different arrangement of these proteins. The nature of these proteins, the degree or extent of their association may well be reflected in the sensitivity of the genome to digestion with nucleases. Increased sensitivity to digestion with DNase I has been reported to be associated with changes in conformation (Axel *et al.*, 1978; Elgin, 1982) and others have documented parental HSV-1 DNA being converted within the first hour of infection to a form sensitive to DNase I and suggested to be concomitant with the "uncoating" of the genome (Oberman and Panet, 1989). The result shown in figure 4.10b. suggests that early in infection the viral genome is more resistant to micrococcal nuclease digestion than when it has become latent. To explore this further, the DNA from *in*1820 infected cells digested with micrococcal nuclease was further cleaved with *Eco* RI. This yields a 2.4kb band which will hybridise with its counterpart, the 2.4kb *Eco* RI fragment, pTK1. The degree of sensitivity to micrococcal nuclease will be reflected in the amount of DNA recovered in this band. The more sensitive the DNA is, the less will be seen on hybridisation. Figures 4.12a and 4.12b show that the latent viral genome is much more sensitive to digestion than the genome which has only been in the cells for 2.5h. The degree of sensitivity is displayed in table 4.14. It shows that early in infection 42% of the viral DNA was digested by a 30 minute exposure to 45 units of micrococcal nuclease whereas in latently infected cells 80% of the viral DNA was digested by the same treatment. Also shown in table 4.14 are the absolute values of the viral DNA early and late in infection but before digestion with micrococcal nuclease. There was a loss of 35% of the viral DNA when latency had been establised at 72h compared with the amount of DNA present shortly after infection.

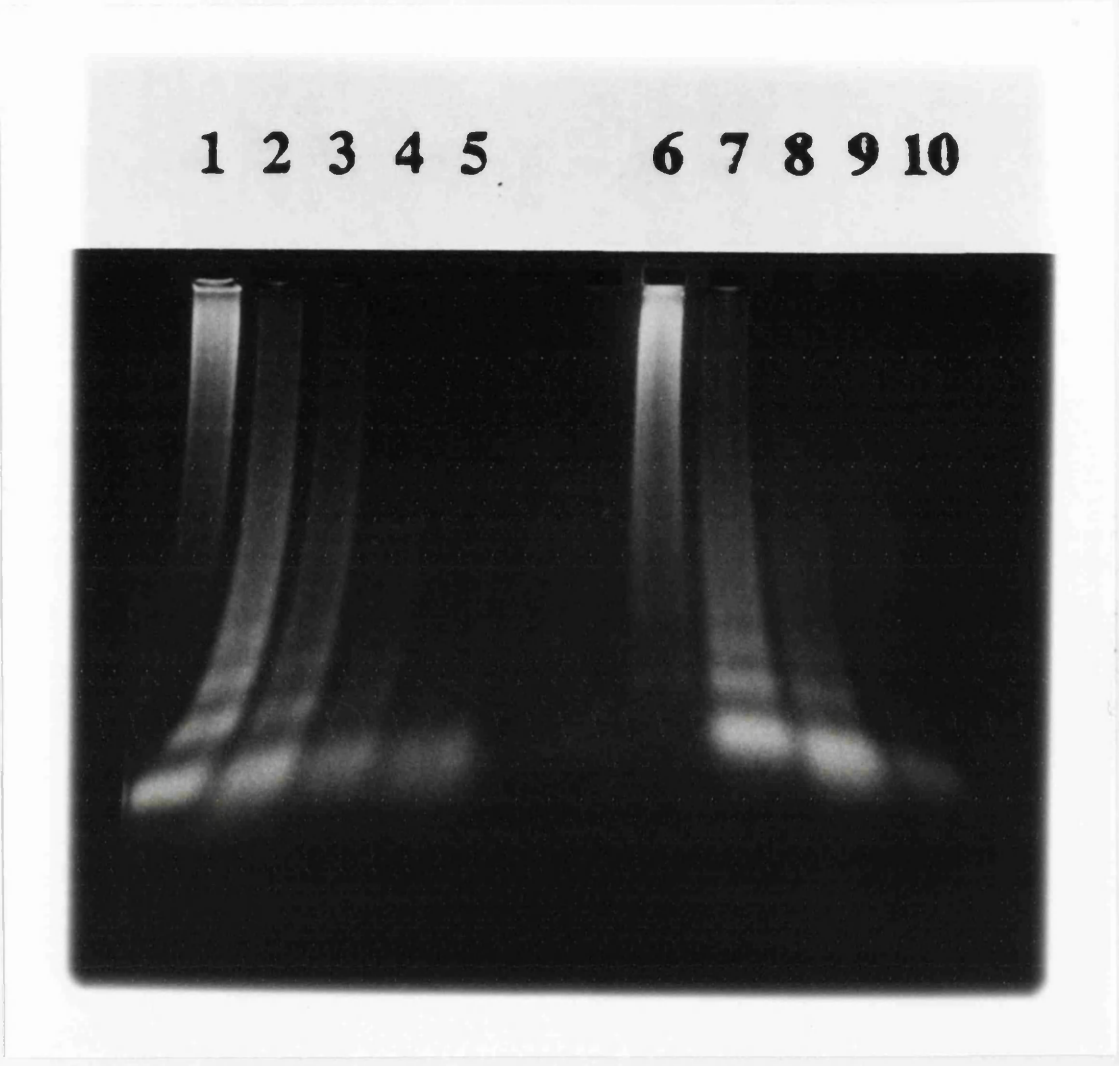


FIGURE 4.12a: THE LATENT VIRAL GENOME IS MORE SENSITIVE TO MICROCOCCAL NUCLEASE DIGESTION THAN SHORTLY AFTER INFECTION

HFL monolayers on 50mm diameter plates were infected with *in*1820 at 120 particles per cell. Following adsorption and penetration of the virus for 1h, the infection was continued for a further 1.5h (lanes 1 - 5) or 72h i.e. into latency (lanes 6 - 10). The lysed nuclei were digested with micrococcal nuclease as follows: lanes 1 and 6, 0 units for 0 min; lanes 2 and 7, 7.5 units for 2 min; lanes 3 and 8, 22.5 units for 5 min; lanes 4 and 9, 22.5 units for 15 min and lanes 5 and 10 with 45 units for 30 min. The extracted, digested DNA was cleaved with 10 units of *Eco* RI for 12h in a 100μl reaction and 25μl electrophoresed in a 1% agarose gel for 18h. The gel was photographed under ultra violet light.

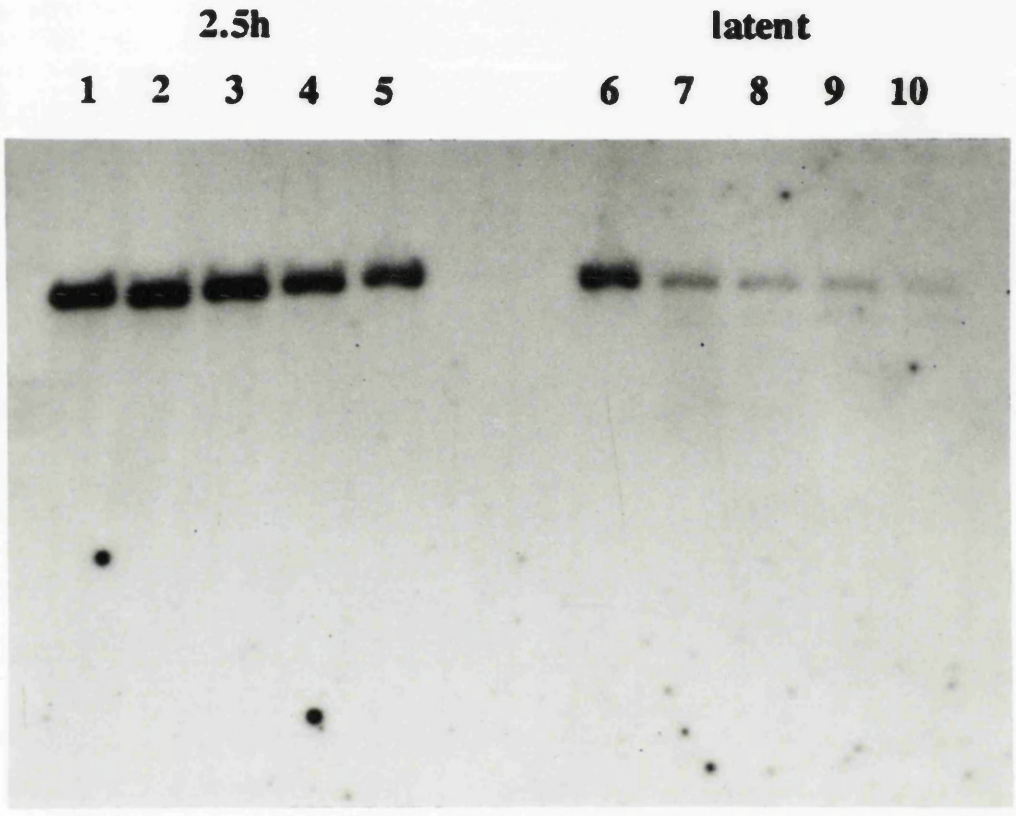


FIGURE 4.12b: THE LATENT VIRAL GENOME IS MORE SENSITIVE TO MICROCOCCAL NUCLEASE DIGESTION THAN SHORTLY AFTER INFECTION

The gel shown in figure 4.12a. was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

DURATION OF INFECTION	EARLY (1.5h)					LATENT (72h)				
ENZYME UNITS	0	7.5	22.5	22.5	45	0	7.5	22.5	22.5	45
DIGESTION TIME (min)	0	2	5	15	30	0	2	5	15	30
% OF DNA PRESENT	100	108	101	71	58	100	32	26	30	20

ABSOLUTE VALUE AT 0 MINUTES		% LOSS OF DNA
EARLY (1.5h)	LATENT (72h)	
34043	22136	35

TABLE 4.14: QUANTIFICATION OF THE SENSITIVITY OF THE VIRAL GENOME TO MICROCOCCAL NUCLEASE SHORTLY AFTER INFECTION AND DURING LATENCY

The DNA bands shown in the autoradiograph depicted in figure 4.12b. were quantified by storage phosphor technology. The amount of DNA present in the band at 0 min and 0 units is arbitrarily defined as 100%.

Since there is a marked difference in the sensitivity of the genome when latent compared with soon after infection it was decided to follow the change in sensitivity as the genome proceeds to latency. The results are shown in figures 4.13a and 4.13b and table 4.15. HFL cells were infected with *in*1820 under conditions predisposing to latency. The monolayers were harvested and the nuclei extracted at 1, 8, 16, 24 and 72h after adsorption and penetration of virus and the DNA digested with micrococcal nuclease. The data show that a significant change in the sensitivity to micrococcal nuclease had occurred by 16h, when 74% of the viral DNA had been digested, and this change persisted to 72h when there was still 70% digestion of the viral DNA. The DNA had acquired nearly all of this significant sensitivity to micrococcal digestion at 8h in the absence of IFN when 63% had been digested, which suggests that a structural change in the genome as it becomes latent occurs by about 8h following adsorption and penetration of the virus. The loss of viral DNA relative to the amount at 1h after infection and as latency was established is also shown in table 4.15. This was considerable at 78% and is much more than the 35% lost in the earlier experiment shown in table 4.14. In the data from table 4.14 the first value was from material infected for half and hour longer than the corresponding value in table 4.15 which would account for part of the difference. It is evident that as latency is established there is a significant loss of the input DNA presumably destroyed by cellular processes. The result at 8h in the presence of IFN was not consistent with the others and it was suspected that there was some loss of DNA in the sample digested with 0 units as can be appreciated from lane 5 in figure 4.13a and would account for the anomaly. This was confirmed by the results shown in figures 4.14a and 4.14b and table 4.16.

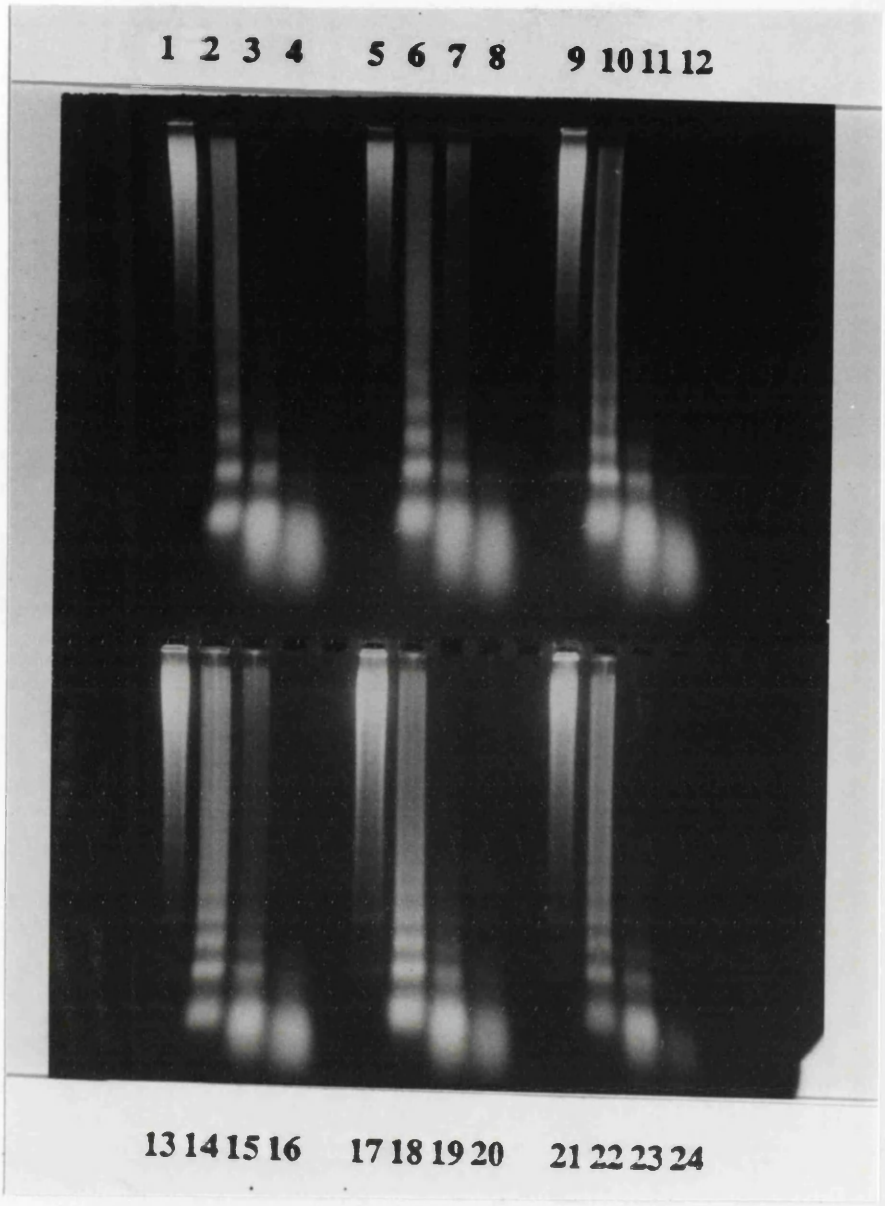


FIGURE 4.13a: INCREASE IN SENSITIVITY TO MICROCOCCAL NUCLEASE DIGESTION AS THE VIRAL GENOME PROCEEDS TO LATENCY.

Nuclei from cells infected with *in*1820 at 120 particles per cell from 1h to 72h were subjected to digestion with micrococcal nuclease. Each successive digest in a quartet from left to right is with 0 units for 0 min (lanes 1, 5, 9, 13, 17 and 21); 7.5 units for 2 min (lanes 2, 6 ,10, 14, 18 and 22); 22.5 units for 10 min (lanes 3, 7, 11, 15, 19 and 23) and 45 units for 30 min (lanes 4, 8, 12, 16, 20 and 24). Following digestion the DNA was extracted and cleaved with 10 units of *Eco* RI for 18h in a 100μl reaction and 25μl electrophoresed in a 1% agarose gel for 18h. The gel was photographed under ultra violet light. Comigrating bands in the undigested samples arise from *Eco* RI restriction of cellular DNA

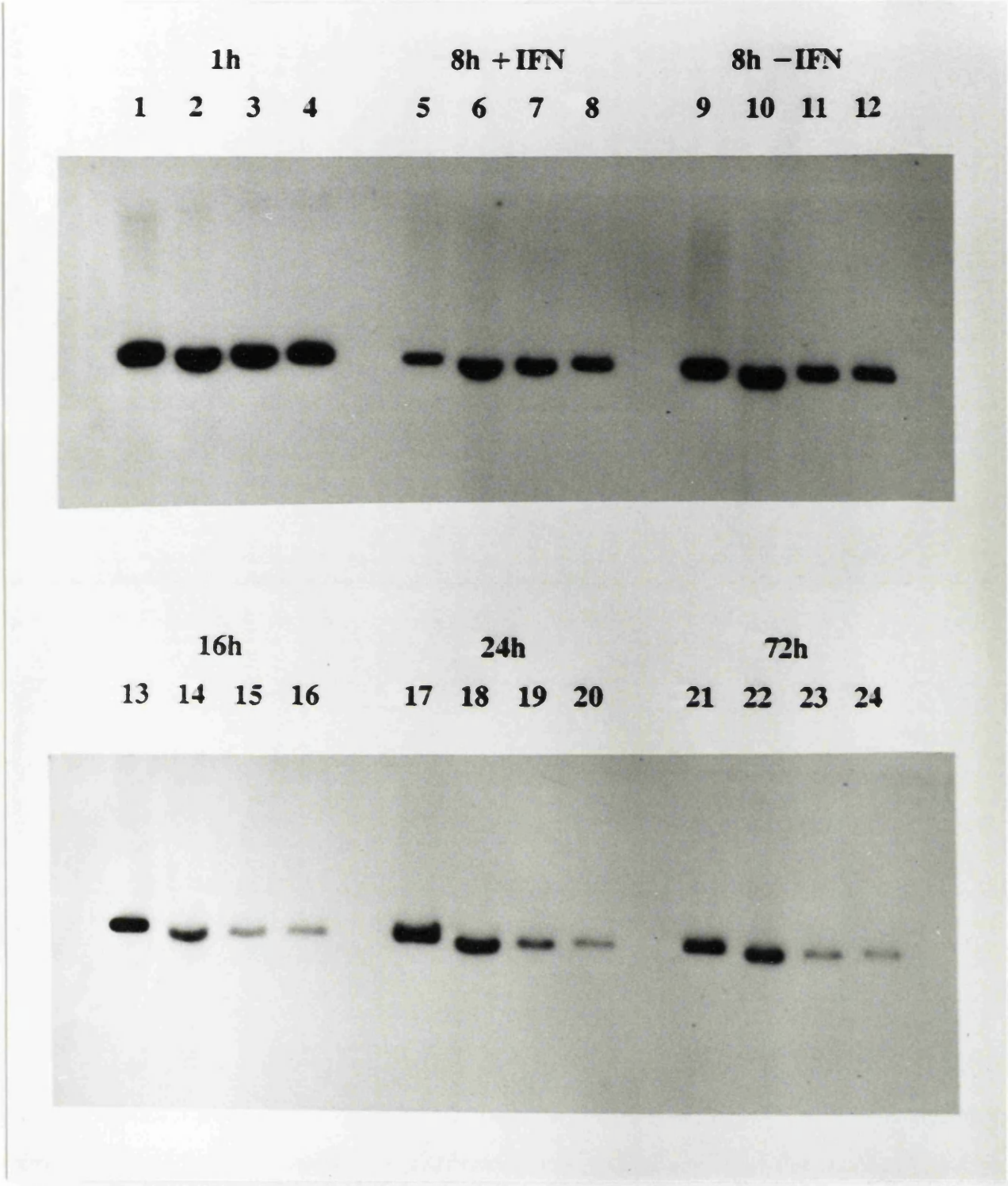


FIGURE 4.13b: INCREASE IN SENSITIVITY TO MICROCOCCAL NUCLEASE DIGESTION AS THE VIRAL GENOME PROCEEDS TO LATENCY.

The gel shown in figure 4.13a. was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

UNITS (u) OF ENZYME/TIME (min) OF INCUBATION	% VIRAL DNA PRESENT AT VARIOUS TIMES FOLLOWING INFECTION					
	1h	8h +IFN	8h −IFN	16h	24h	72h
0 u/0 min	100	100	100	100	100	100
7.5 u/2 min	73	181	64	68	59	82
22.5 u/10 min	75	135	38	33	21	38
45 u/30 min	59	110	37	26	12	30

ABSOLUTE VALUE AND % OF VIRAL DNA LOST AT VARIOUS TIMES FOLLOWING INFECTION PRIOR TO DIGESTION WITH MICROCOCCAL NUCLEASE					
	1h	8h −IFN	16h	24h	72h
0 u/0 min	354182	198364	124877	130331	76801
% loss relative to 1h	100	44	65	63	78

TABLE 4.15: QUANTIFICATION OF MICROCOCCAL NUCLEASE SENSITIVITY OF THE VIRAL GENOME AS IT PROGRESSES TO LATENCY

The DNA bands shown in the autoradiograph depicted in figure 4.13b. were quantified by storage phosphor technology. The DNA band present at 0 min and 0 units is arbitrarily defined as 100%.

IFN-α has been used in the *in vitro* latency model to further reduce Vmw110 expression and thereby enhance the tendency of *in*1820 to proceed to the latent form. The effect of IFN-α pretreatment on the acquisition of increased sensitivity to micrococcal nuclease by the viral genome was investigated. From the results shown in figures 4.14a and 4.14b and table 4.16 it is apparent that at 8h there is no difference in sensitivity between monolayers pretreated with IFN-α and those untreated subsequently infected with *in*1820. The result is similar to that in table 4.15 from an 8h infection in the absence of IFN. Thus 63 - 64% of the viral DNA had become sensitive to digestion with micrococcal nuclease at 8h after penetration of the virus whether IFN was present or not. There was no change in the absolute amounts of viral DNA at 8h in IFN-α treated cells compared with untreated cells.

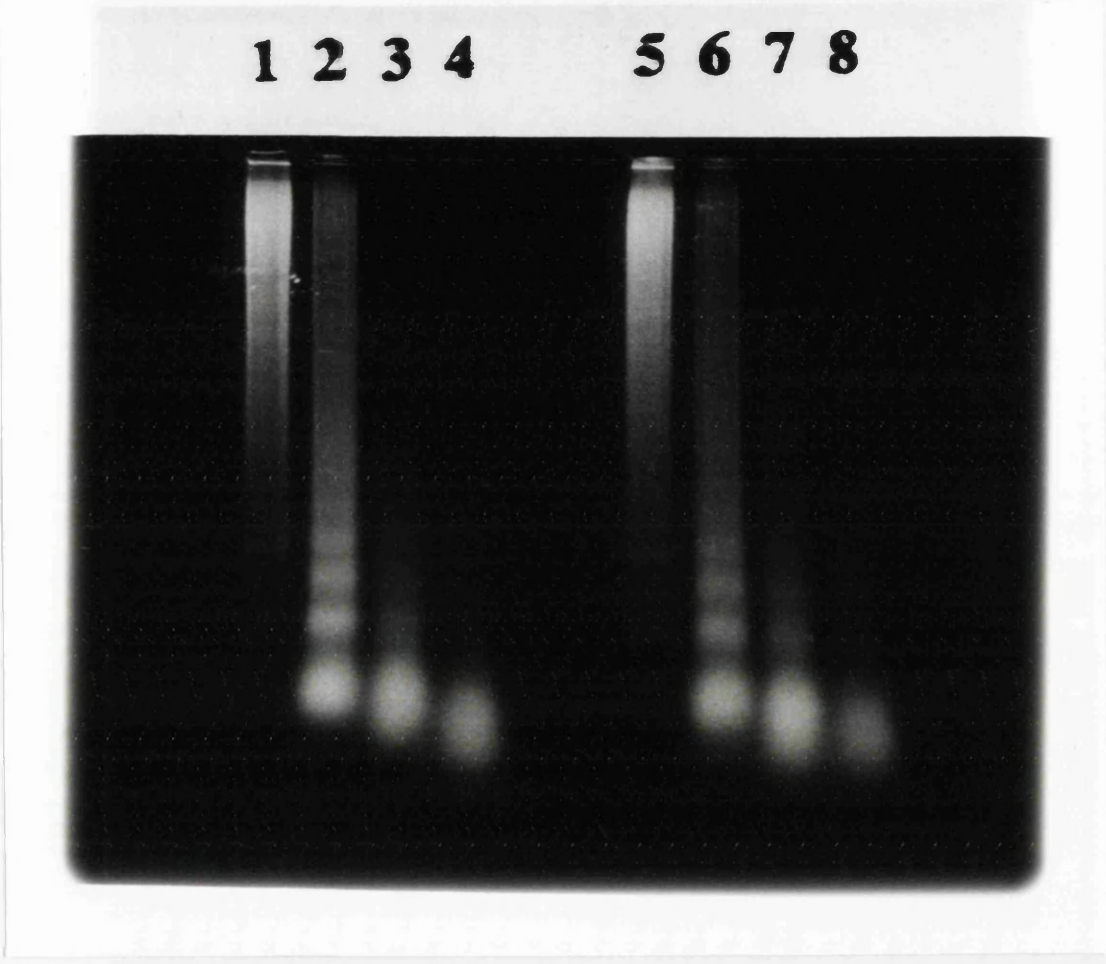


FIGURE 4.14a: THE EFFECT OF IFN-α ON THE MICROCOCCAL NUCLEASE SENSITIVITY OF THE VIRAL GENOME 8h AFTER INFECTION

HFL cells grown on 50mm diameter plates and either pretreated with IFN-α at 10^3 units/ml or untreated were infected with 120 particles per cell of *in*1820 for 8h in the presence of ara-C at 50μg/ml. Nuclei were digested with micrococcal nuclease as follows: 0 units for 0 min (lanes 1 and 5); 7.5 units for 2 min (lanes 2 and 6); 22.5 units for 10 min (lanes 3 and 7) and 45 units for 30 min (lanes 4 and 8). Following digestion the DNA was cleaved with 10 units of *Eco* RI for 18h in a 100μl reaction and 25μl electrophoresed in a 1% agarose gel for 18h. The gel was photographed under ultra violet light.

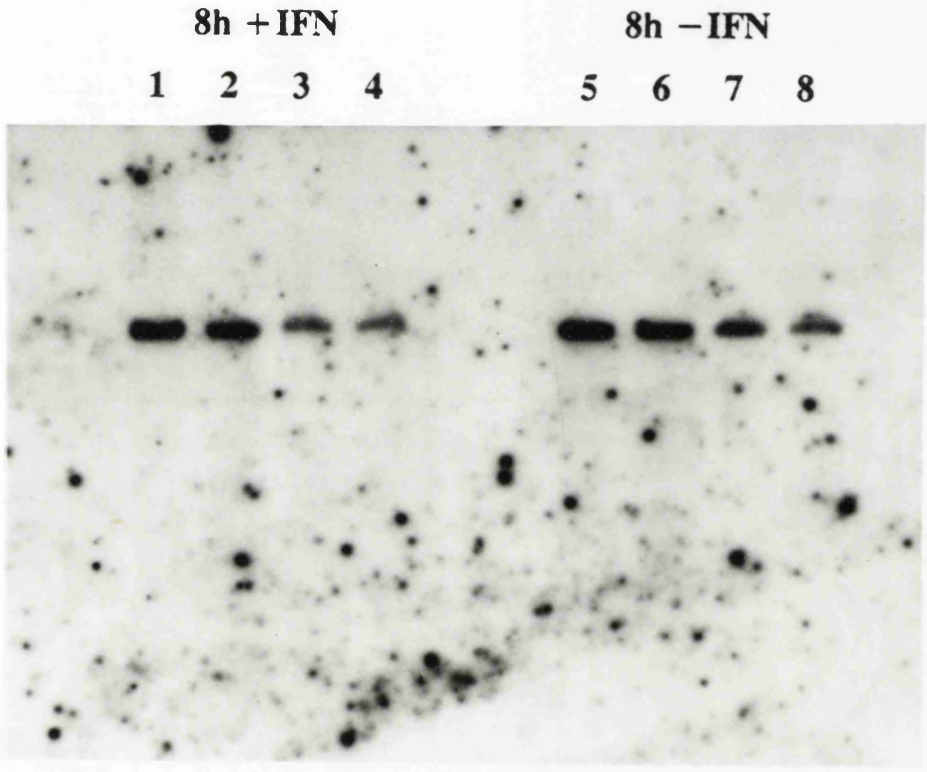


FIGURE 4.14b: THE EFFECT OF IFN-α ON THE MICROCOCCAL NUCLEASE SENSITIVITY OF THE VIRAL GENOME 8h AFTER INFECTION

The gel shown in figure 4.14a. was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

UNITS (u) OF ENZYME/TIME (min) OF INCUBATION	% VIRAL DNA PRESENT FOLLOWING INFECTION FOR 8h	
	NO IFN-α	IFN-α
0 u/0 min	100	100
7.5 u/2 min	83	84
22.5 u/10 min	44	26
45 u/30 min	36	36

ABSOLUTE VALUE AT 0 MINUTES		% LOSS OF DNA
8h −IFN	8h +IFN	
55195	54432	1.4

TABLE 4.16: QUANTIFICATION OF THE EFFECT OF IFN-α ON THE MICROCOCCAL NUCLEASE SENSITIVITY OF THE VIRAL GENOME 8h AFTER INFECTION

The DNA bands shown in the autoradiograph depicted in figure 4.14b. were quantified by storage phosphor technology. The DNA band present at 0 min and 0 units is arbitrarily defined as 100%.

4.19. Micrococcal nuclease sensitivity of reactivated latent DNA

Earlier experiments (section 4.14 and table 4.13) have demonstrated that *in*1863 is able to reactivate latent *in*1820. The mutant *in*1863 is wild type virus with the HCMV promoter driving the *lacZ* gene inserted into the TK gene. Cleavage with *Eco* RI produces a 2.0kb and a 1.0kb fragment from the TK gene due to the presence of an *Eco* RI site in the inserted gene. Since similar digestion of *in*1820 produces a 2.4kb fragment from the TK gene it is possible to identify both viruses when present together by hybridisation with the 2.4kb pTK1 ^{32}P-radiolabelled fragment. Experiments were undertaken to investigate the micrococcal nuclease sensitivity of the reactivated latent

genome. The micrococcal nuclease sensitivity of the reactivated *in*1820 genome and the *in*1863 genome after superinfection, but without replication, for 1h and 8h is shown in figure 4.15b and table 4.17. The upper bands in the first quartet represent the TK gene of the latent *in*1820 after 1h superinfection with *in*1863. It was more sensitive to micrococcal nuclease digestion than the middle and lower bands which represent the TK gene from the superinfecting virus. This is shown by the digestion of 63% of the latent genome by 45 units of micrococcal nuclease at 30 minutes compared with only 35% of the superinfecting genome. In the second quartet the effect of superinfection for 8h is shown. The upper band from the latent virus, now presumably accessible for transcription, showed the same sensitivity i.e. 63% digestion as the latent genome whereas the superinfecting genome, also transcriptionally active, remained relatively resistant with only 33% at a maximum being digested after the same incubation. The results show that the latent genome, despite being reactivated, still remains sensitive to the nuclease. The superinfecting genome however, continues to be relatively resistant even after 8h.

To illustrate this point further the experiment above was repeated with different viruses so that the latent genome sensitive to micrococcal nuclease digestion will be apparent in the middle and lower bands of a quartet and the relatively resistant transcribing input DNA in the upper band of a quartet. Latent *in*1884 was reactivated by superinfection with 1814R. *Eco* RI digestion of 1814R will produce a 2.4kb fragment and of *in*1884 two smaller fragments, of size 2.0 and 1.0kb, when hybridised with pTK1. This is the reverse situation of the experiment in figures 4.15a and 4.15b and table 4.17 and is shown in figures 4.16a and 4.16b and table 4.18. The result confirmed the findings of continuing sensitivity of the latent genome even when reactivated with 66% and 83% digestion occuring at the end of the defined incubation time after 1h and 8h from superinfection respectively and the relative resistance of the superinfecting non-replicating viral DNA with 34% and 44% digestion under the same conditions. In figure 4.16b two exposures of the same blot are shown. The difference in sensitivity between the latent and the superinfecting virus can be appreciated by comparing the two panels since the intensity of the band in the lower panel representing 1814R has been made comparable to the intensity of the middle band in the upper panel which represents the latent virus *in*1884.

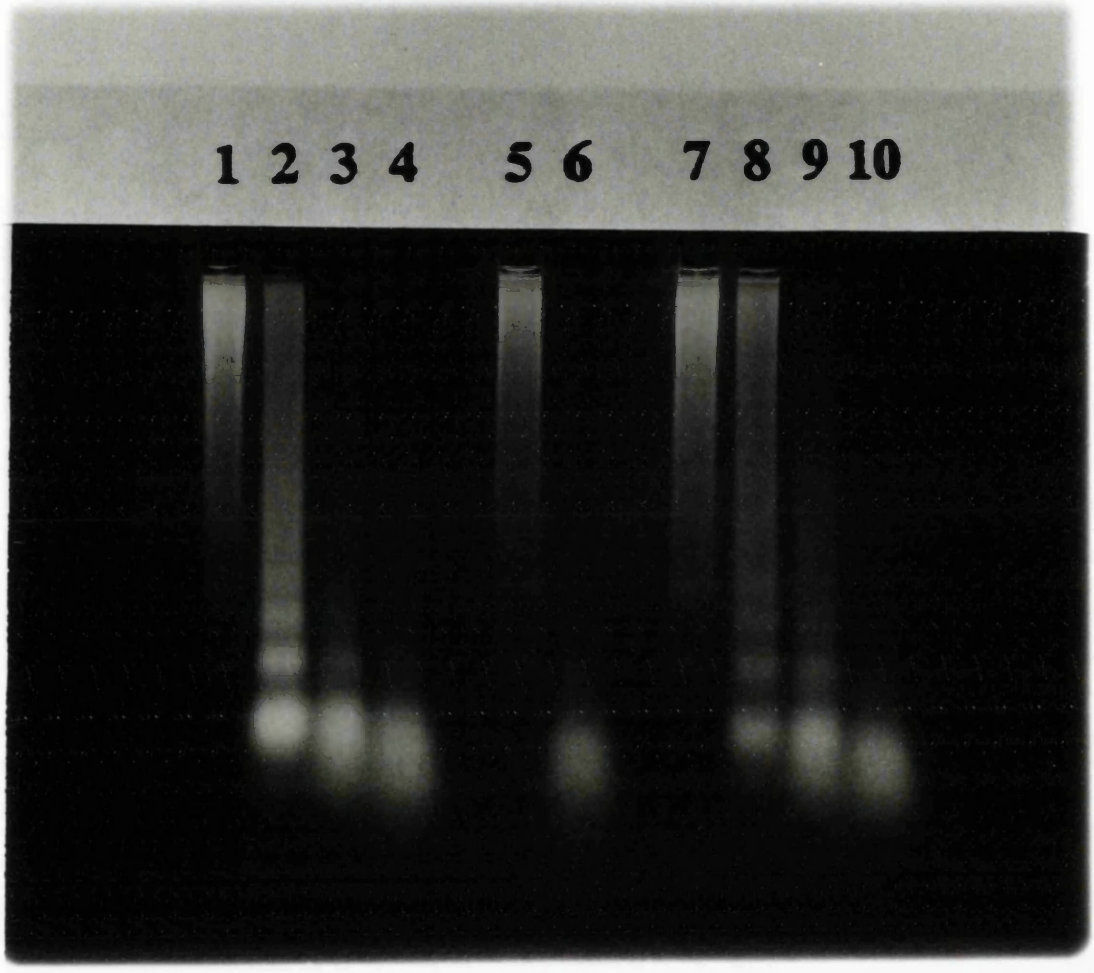


FIGURE 4.15a: MICROCOCCAL NUCLEASE SENSITIVITY OF REACTIVATED AND SUPERINFECTING GENOMES IN THE PRESENCE OF ARA-C

HFL monolayers on 50mm diameter plates mock (lanes 5 and 6) or latently infected with *in*1820 at 120 particles per cell (lanes 1 - 4 and 7 - 10) were superinfected with 7.5 pfu per cell of *in*1863 and maintained for 1h (lanes 1 - 4) or 8h (lanes 7 - 10) in the presence of ara-C. Digestion with micrococcal nuclease was as follows: lanes 1, 5 and 7, 0 units for 0 min; lanes 2 and 8, 7.5 units for 2 min; lanes 3 and 9, 22.5 units for 10 min and lanes 4, 6 and 10, 45 units for 30 min. All the samples were cleaved with 10 units of *Eco* RI in a 100μl reaction for 18h and 25μl was run in a 1% agarose gel. The gel was photographed under ultra violet light.

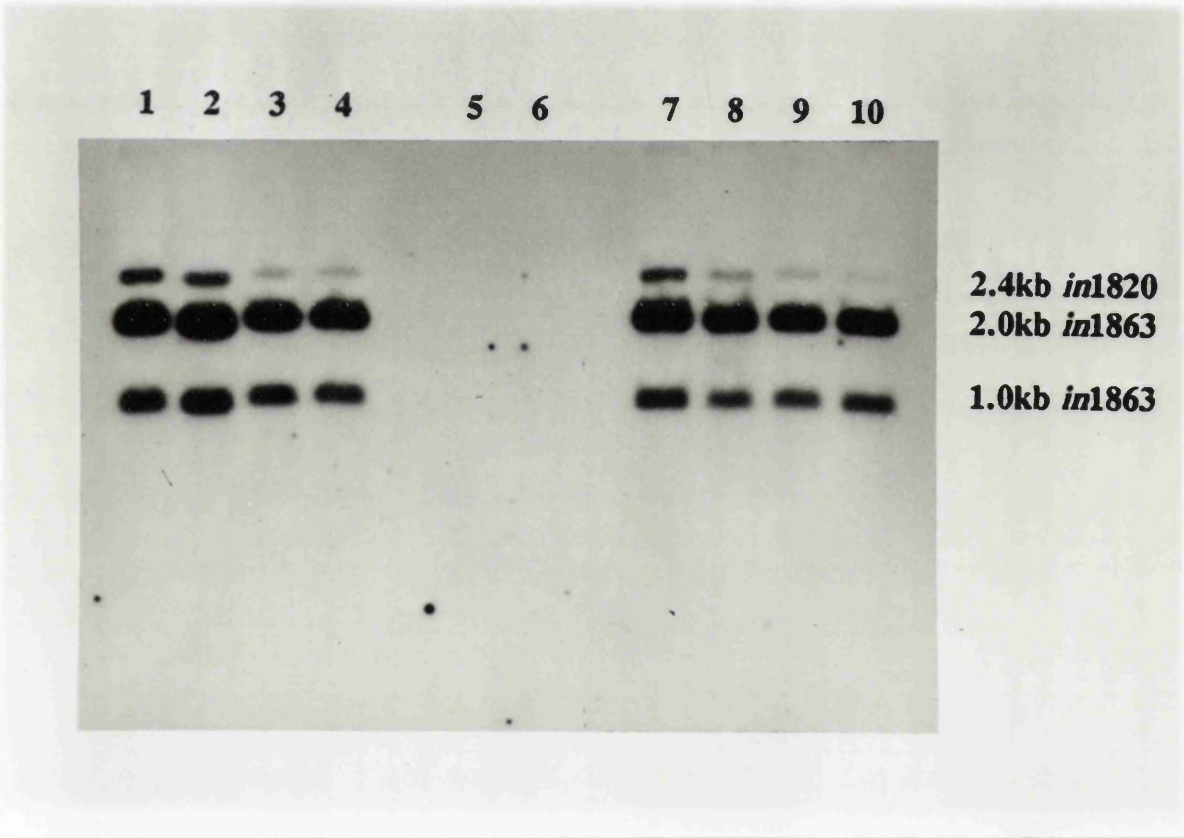


FIGURE 4.15b: MICROCOCCAL NUCLEASE SENSITIVITY OF REACTIVATED AND SUPERINFECTING GENOMES IN THE PRESENCE OF ARA-C

The gel shown in figure 4.15a. was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

DURATION OF SUPERINFECTION		1h				8h			
ENZYME UNITS (u)		0	7.5	22.5	45	0	7.5	22.5	45
INCUBATION TIME (min)		0	2	10	30	0	2	10	30
%DNA	2.4kb	100	77	38	27	100	50	35	27
	2.0kb	100	123	78	65	100	64	60	67
	1.0kb	100	133	82	66	100	68	69	82

TABLE 4.17: QUANTIFICATION OF MICROCOCCAL NUCLEASE SENSITIVITY OF REACTIVATED AND SUPERINFECTING GENOMES IN THE PRESENCE OF ARA-C

The DNA bands shown in the autoradiograph depicted in figure 4.15b were quantified by storage phosphor technology. The DNA band present at 0 min and 0 units is arbitrarily defined as 100%.

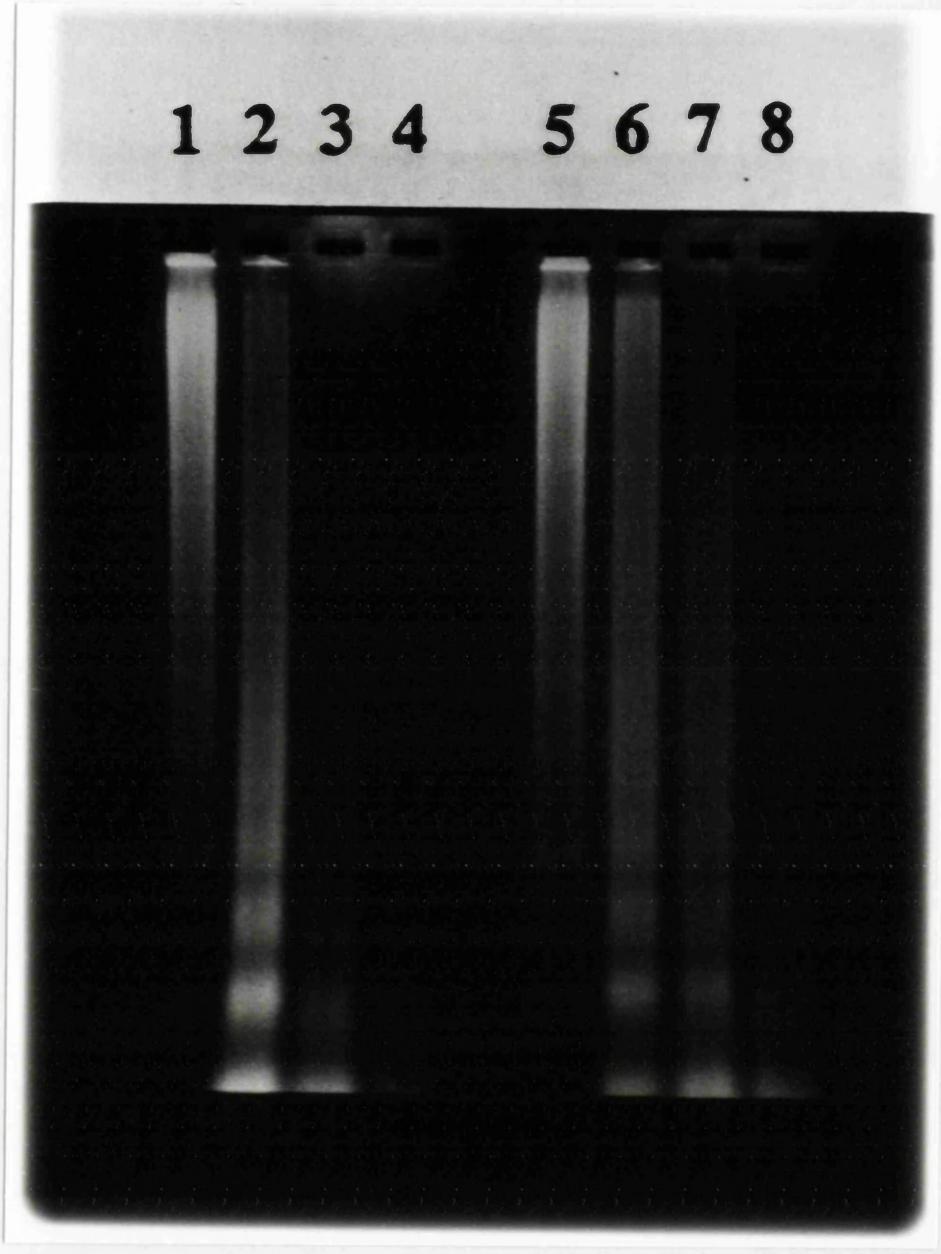


FIGURE 4.16a: MICROCOCCAL NUCLEASE SENSITIVITY OF REACTIVATED AND SUPERINFECTING GENOMES IN THE PRESENCE OF ARA-C

HFL monolayers on 50mm diameter plates latently infected with *in*1884 at 120 particles per cell were superinfected with 7.5 pfu per cell of 1814R and maintained for 1h (lanes 1 - 4) and 8h (lanes 5 - 8) in the presence of ara-C. Digestion with micrococcal nuclease was as follows: lanes 1 and 5, 0 units for 0 min; lanes 2 and 6, 7.5 units for 2 min; lanes 3 and 7, 22.5 units for 10 min and lanes 4 and 8, 45 units for 30 min. The samples were cleaved with 10 units of *Eco* RI for 18h in a 100μl reaction and 25μl run in a 1% agarose gel. The gel was photographed under ultra violet light.

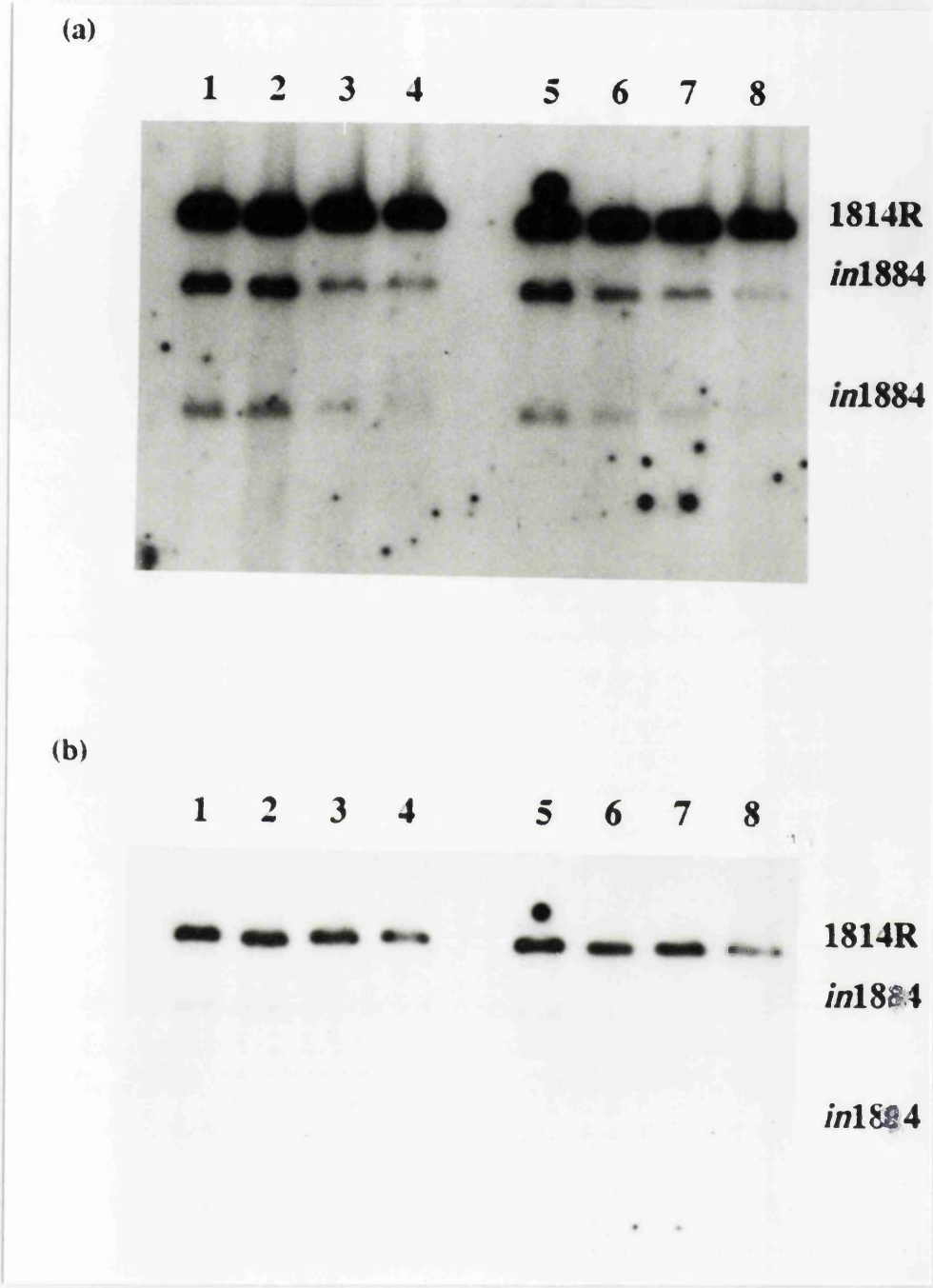


FIGURE 4.16b: MICROCOCCAL NUCLEASE SENSITIVITY OF REACTIVATED AND SUPERINFECTING GENOMES IN THE PRESENCE OF ARA-C

The gel shown in figure 4.16a was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1. The lower panel is a shorter exposure of the upper panel to enable a direct comparison of the sensitivities between the middle band of the upper panel representing *in*1884 and the upper band of the lower panel representing 1814R to be shown.

DURATION OF SUPERINFECTION		1h				8h			
ENZYME UNITS (u)		0	7.5	22.5	45	0	7.5	22.5	45
INCUBATION TIME (min)		0	2	10	30	0	2	10	30
%DNA	2.4kb	100	97	91	66	100	75	81	56
	2.0kb	100	102	56	34	100	35	29	17

TABLE 4.18: QUANTIFICATION OF MICROCOCCAL NUCLEASE SENSITIVITY OF REACTIVATED AND SUPERINFECTING GENOMES IN THE PRESENCE OF ARA-C

The DNA bands shown in the autoradiograph depicted in figure 4.16b were quantified by storage phosphor technology. The DNA band present at 0 min and 0 units is arbitrarily defined as 100%.

Another experiment with 1814R, at a lower moi of 0.75 pfu per uninfected cell was performed to demonstrate that the relative resistance of 1814R when used as the superinfecting virus shown in figure 4.16b and table 4.18 was not a consequence of the high multiplicity, 7.5 pfu per cell, employed. The results are shown in figure 4.17b and table 4.19. They demonstrated that even at a lower moi the transcribing, albeit non-replicating, superinfecting genome retained the pattern of relative resistance compared with the transcribing, previously latent genome. Thus 70% of the viral DNA from latently infected cells is sensitive to micrococcal nuclease digestion either at 1h or 8h following superinfection whereas only 53% and 38% of the superinfecting DNA is sensitive under the same conditions and at the same respective times.

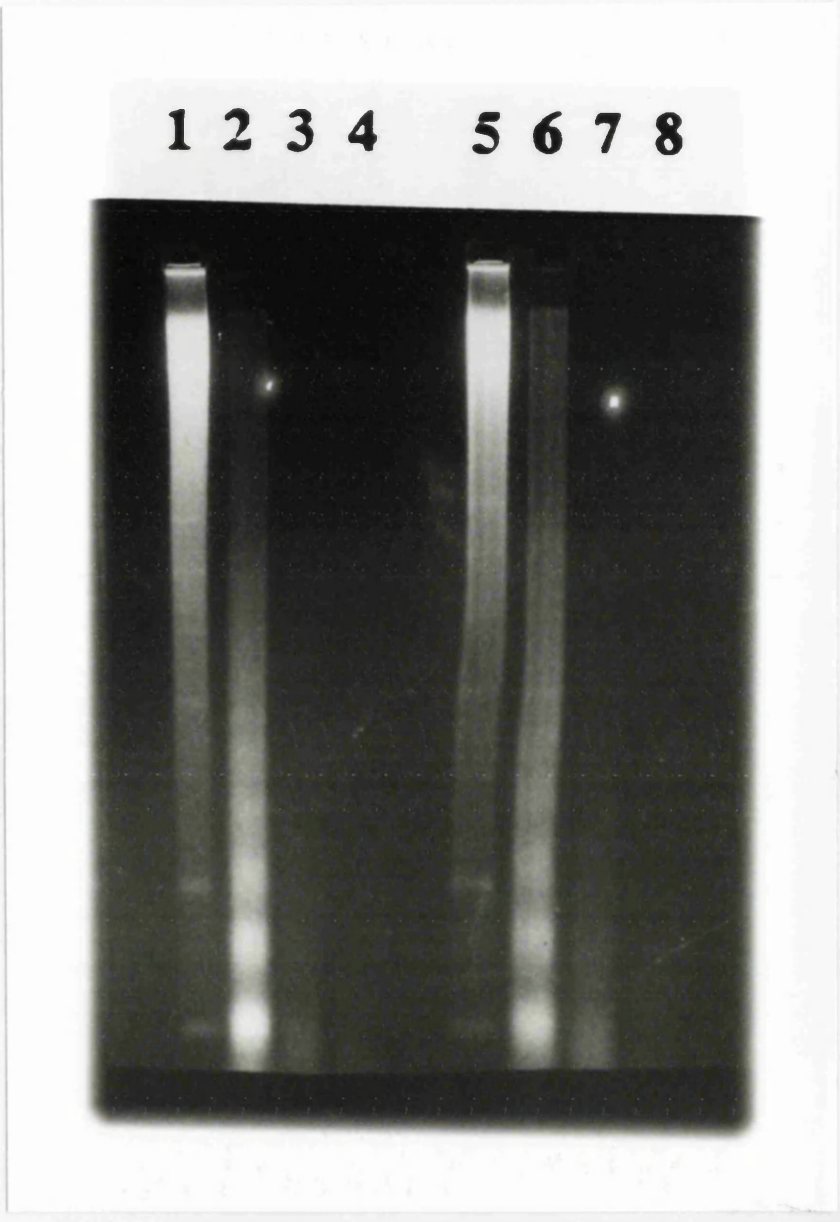


FIGURE 4.17a: THE 1814R GENOME IS RELATIVELY RESISTANT TO MICROCOCCAL NUCLEASE DIGESTION AFTER INFECTION AT LOW MOI.

HFL monolayers on 50mm diameter plates were infected for 1h (lanes 1 - 4) and 8h (lanes 5 - 8) with 0.75 pfu per cell of 1814R in the presence of ara-C. Digestion with micrococcal nuclease was as follows: lanes 1 and 5, 0 units for 0 min; lanes 2 and 6, 7.5 units for 2 min; lanes 3 and 7, 22.5 units for 10 min and lanes 4 and 8, 45 units for 30 min. The samples were cleaved with 10 units of *Eco* RI for 18h in a 100μl reaction and 25μl run in a 1% agarose gel. The gel was photographed under ultra violet light.

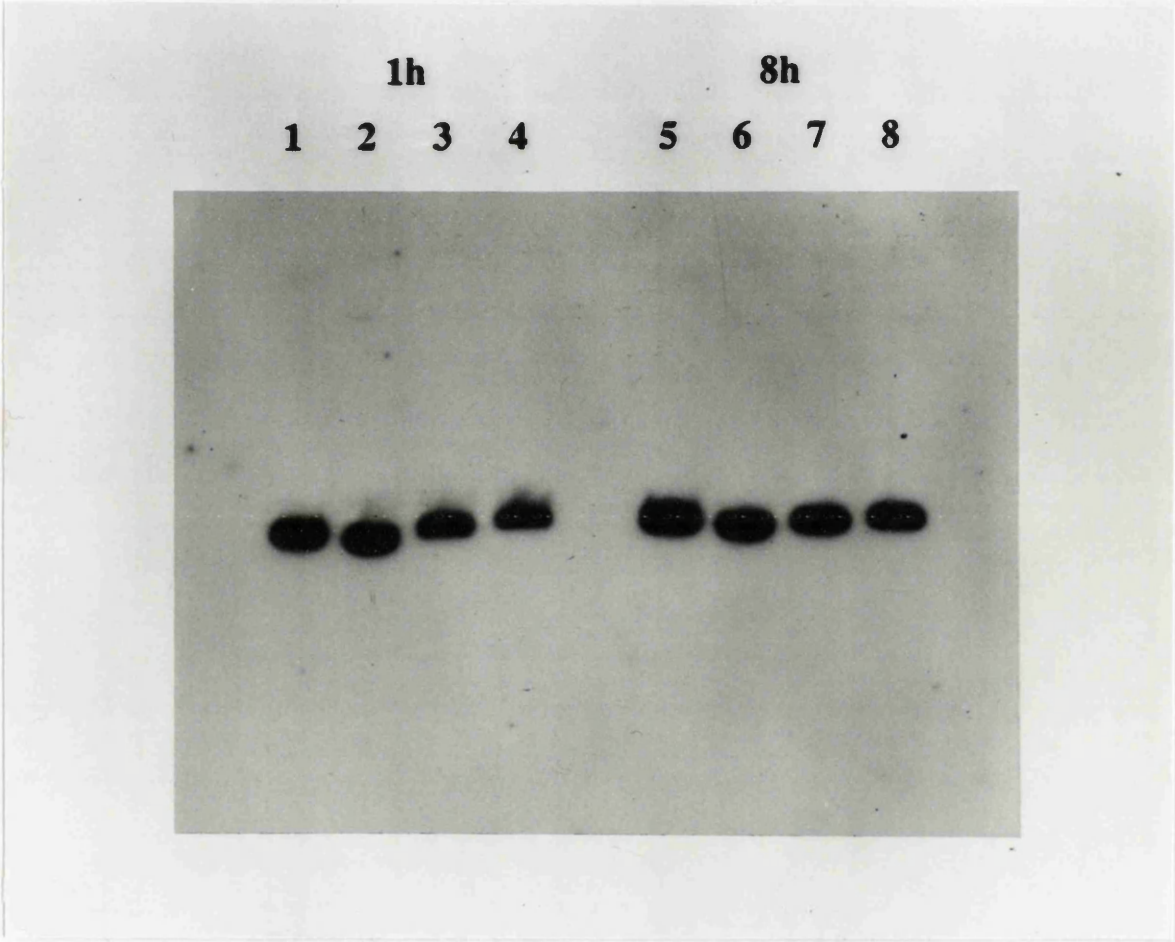


FIGURE 4.17b: THE 1814R GENOME IS RELATIVELY RESISTANT TO MICROCOCCAL NUCLEASE DIGESTION AFTER INFECTION AT LOW MOI.

The gel shown in figure 4.17a was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1.

DURATION OF INFECTION	1h				8h			
ENZYME UNITS (u)	0	7.5	22.5	45	0	7.5	22.5	45
INCUBATION TIME (min)	0	2	10	30	0	2	10	30
%DNA	100	74	49	47	100	72	70	62

TABLE 4.19: QUANTIFICATION OF THE RELATIVE RESISTANCE OF THE 1814R GENOME TO MICROCOCCAL NUCLEASE DIGESTION AFTER INFECTION AT LOW MOI.

The DNA bands shown in the autoradiograph depicted in figure 4.17b were quantified by storage phosphor technology. The amount of DNA present in the band at 0 min and 0 units is arbitrarily defined as 100%.

4.20. Micrococcal nuclease sensitivity of replicating DNA

Micrococcal nuclease sensitivity is evident by 8h into a latent infection with *in*1820, figure 4.13b and table 4.15. Such sensitivity is not acquired by either *in*1863 or 1814R after the same interval, figures 4.15b, 4.16b and 4.17b and tables 4.17, 4.18 and 4.19. These latter two are not as predisposed to latency as *in*1820 since they express IE genes in the same amounts as wild type HSV-1. Thus it is in the progress to latency in the presence of ara-C that the genome becomes more sensitive to digestion with micrococcal nuclease. The predisposition of *in*1820 to a latent infection can be overcome by simultaneous infection with a virus supplying the IE gene product Vmw110 such as 1814R or *in*1863. This has been demonstrated by the expression of the *lacZ* gene in all cells co-infected with *in*1884 and 1814R (table 4.10). HFL monolayers were simultaneously infected with *in*1820 and *in*1863 for 8h in the absence of IFN-α pretreatment. The micrococcal nuclease sensitivity of the genomes of the two viruses when co-infected and maintained for 8h in the presence or absence of ara-C was investigated and is shown in figure 4.18b and table 4.20. Under these two different conditions both viral genomes have the same sensitivity to micrococcal nuclease but they are either both resistant, as in the presence of ara-C when over 80% remains

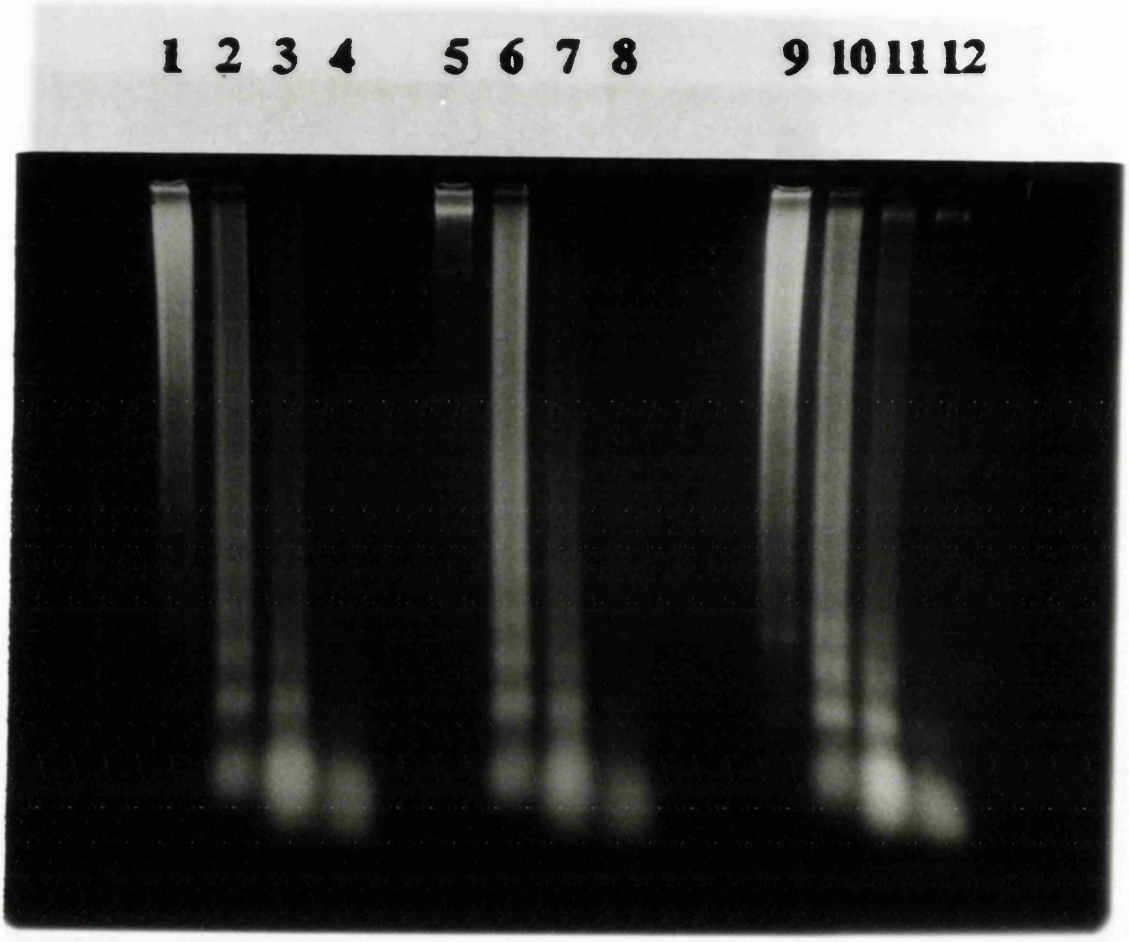


FIGURE 4.18a: THE *in*1820 AND *in*1863 GENOMES HAVE THE SAME MICROCOCCAL NUCLEASE DIGESTION PATTERNS WHEN CO-INFECTED FOR 8h.

HFL monolayers on 50mm diameter plates were co-infected for 8h with 120 particles per cell of *in*1820 and 7.5 pfu per cell of *in*1863 in the presence (lanes 1 - 4) or absence (lanes 9 - 12) of ara-C or mock infected (lanes 5 - 8). Digestion with micrococcal nuclease was as follows: lanes 1, 5 and 9, 0 units for 0 min; lanes 2, 6 and 10, 7.5 units for 2 min; lanes 3, 7 and 11, 22.5 units for 10 min and lanes 4, 8 and 12, 45 units for 30 min. Following cleavage with 10 units of *Eco* RI for 18h in a 100μl reaction, 30μl were run in a 1% agarose gel. The gel was photographed under ultra violet light.

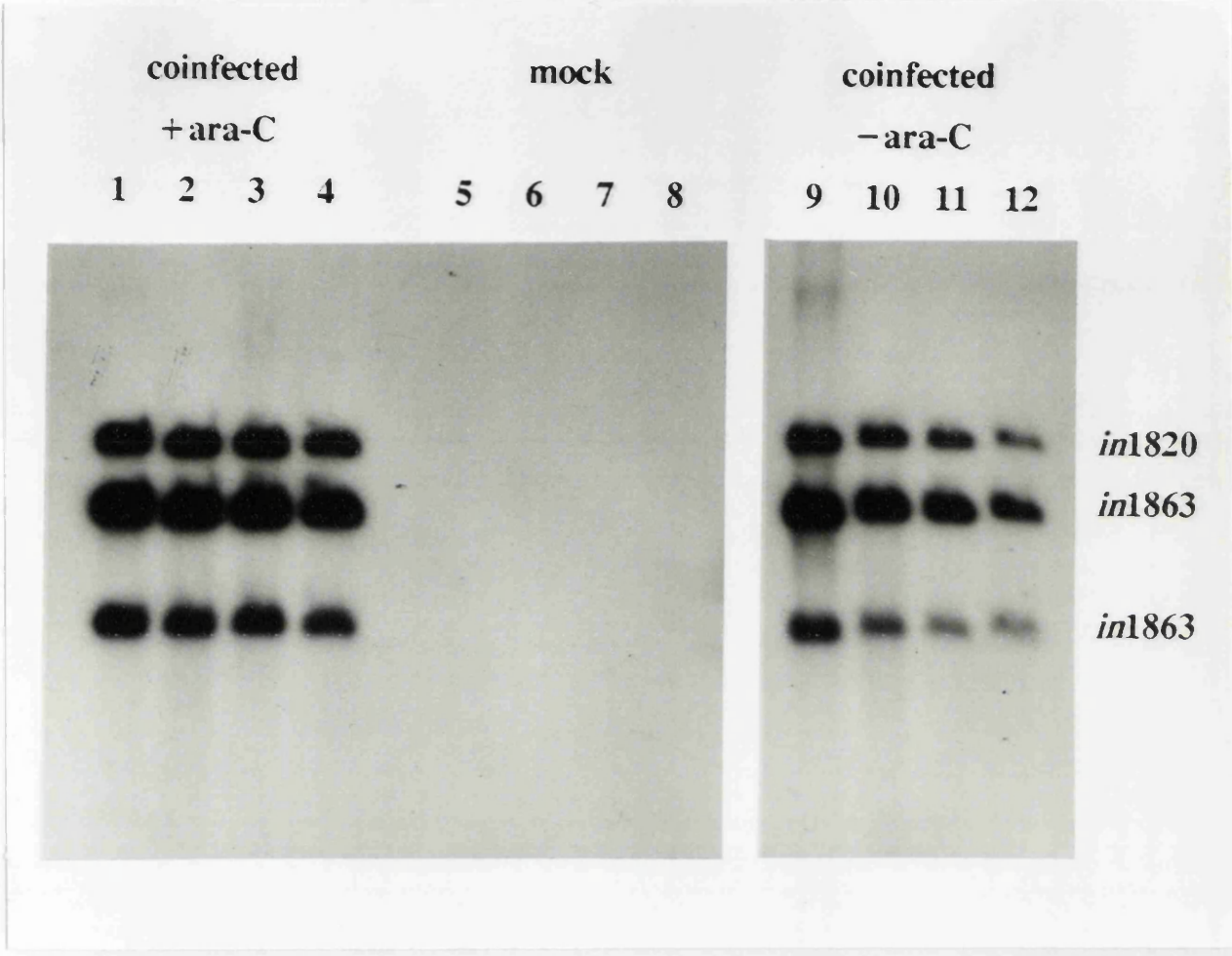


FIGURE 4.18b: THE *in*1820 AND *in*1863 GENOMES HAVE THE SAME MICROCOCCAL NUCLEASE DIGESTION PATTERNS WHEN CO-INFECTED FOR 8h.

The gel shown in figure 4.18a was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1. The panel showing lanes 9 - 12 was derived from a 1/2h exposure in contrast with the panel showing lanes 1 - 8 which was obtained from a 22h exposure.

DURATION OF CO-INFECTION		8h+ara-C				8h−ara-C			
ENZYME UNITS (u)		0	7.5	22.5	45	0	7.5	22.5	45
INCUBATION TIME (min)		0	2	10	30	0	2	10	30
%DNA	2.4kb (*in*1820)	100	99	66	81	100	52	43	32
	2.0kb (*in*1863)	100	106	68	80	100	53	41	27
	1.0kb (*in*1863)	100	107	81	85	100	54	34	24

VIRUS	ABSOLUTE VALUE AT 0 MINUTES		INCREASE IN DNA (FOLD)
	8h +ara-C	8h −ara-C	
*in*1820 (2.4kb)	35176	1038787	29.5
*in*1863 (2.0kb)	110190	3093414	28
*in*1863 (1.0kb)	46549	1343852	29

TABLE 4.20: QUANTIFICATION OF SENSITIVITIES OF THE *in*1820 AND *in*1863 GENOMES TO MICROCOCCAL NUCLEASE DIGESTION WHEN CO-INFECTED FOR 8h.

The DNA bands shown in the autoradiograph depicted in figure 4.18b were quantified by storage phosphor technology. The amount of DNA present in the band at 0 min and 0 units is arbitrarily defined as 100%.

undigested, or they are both sensitive, as in the absence of ara-C with between 68% and 76% being digested. In the absence of ara-C most of the DNA will be progeny DNA, in contrast with co-infection in the presence of ara-C where it is only input virus DNA. When replication is permitted both viruses appeared to replicate at the same rate with an increase in viral DNA after 8h of about 30-fold (table 4.20).

Both the reactivated and superinfecting viral genomes in all the data shown had been prevented from replicating by the presence of ara-C. To investigate whether replication is associated with an alteration in the sensitivity to nuclease digestion,

monolayers retaining latent *in*1820 were reactivated by superinfection with *in*1863 in the absence of ara-C. The results are presented in figures 4.19a and 4.19b and table 4.21. They show that the replicated genome is more sensitive to nuclease digestion than when prohibited by ara-C from entering the replicative cycle. The micrococcal nuclease sensitivity acquired by the input DNA as it progresses to replication is the same as that of the latent DNA. Thus the latent genome and the replicating genome have the same degree of sensitivity to micrococcal nuclease digestion. A possible explanation is that they share similar structural properties such as circularisation in that it has been shown that the *in vitro* latent genome is non-linear (section 4.10) and that during a lytic infection rolling circle intermediates are formed *en route* to replication (Roizman and Sears, 1990). Alternatively, the protein coating of the genome in both stages, although different, nevertheless confers a sensitivity not present when the DNA is only being transcribed. Another suggestion is that in establishing latency the genome acquires a potentially replicative structure either in terms of its configuration or protein coating but the actual process of replication does not occur. In other words the latent genome is ready for replication as soon as the appropriate signal, hypothesised to be Vmw110, is received. Another interesting observation shown in table 4.21 was that the latent viral genome replicated following superinfection at a significantly greater rate than the superinfecting virus. This was surprising since following co-infection the replication rates are identical, table 4.20. The result suggests that either the latent virus is capable of replicating more efficiently than the input virus, which could be a reflection of its physical state, or that *in*1863 is more sensitive to the residual effects of IFN-α than the reactivated *in*1820.

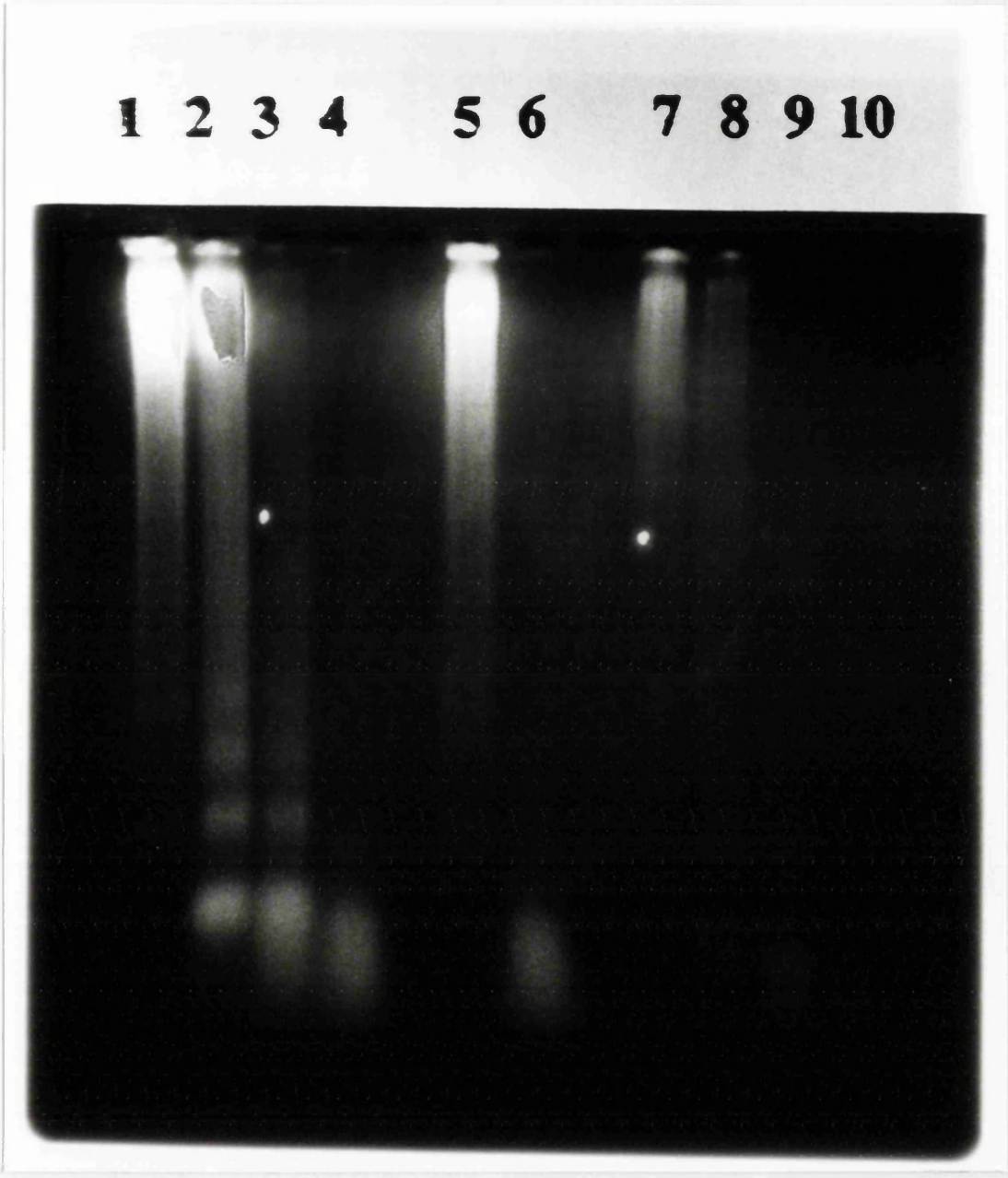


FIGURE 4.19a: THE REPLICATING SUPERINFECTING GENOME IS AS SENSITIVE TO MICROCOCCAL NUCLEASE DIGESTION AS THE LATENT GENOME.

HFL monolayers on 50mm diameter plates latently infected with *in*1820 at 120 particles per cell were superinfected with *in*1863 at 7.5 pfu per cell and maintained for 8h in the presence (lanes 1 - 4) or absence (lanes 7 - 10) of ara-C or mock infected (lanes 5 and 6). Digestion with micrococcal nuclease was as follows: lanes 1, 5 and 7, 0 units for 0 min; lanes 2 and 8, 7.5 units for 2 min; lanes 3 and 9, 22.5 units for 10 min and lanes 4,6 and 10, 45 units for 30 min. Following cleavage with 10 units of *Eco* RI for 18h in a 100μl reaction, 25μl (lanes 1 - 6) or 5μl (lanes 7 - 10) were run in a 1% agarose gel. The gel was photographed under ultra violet light.

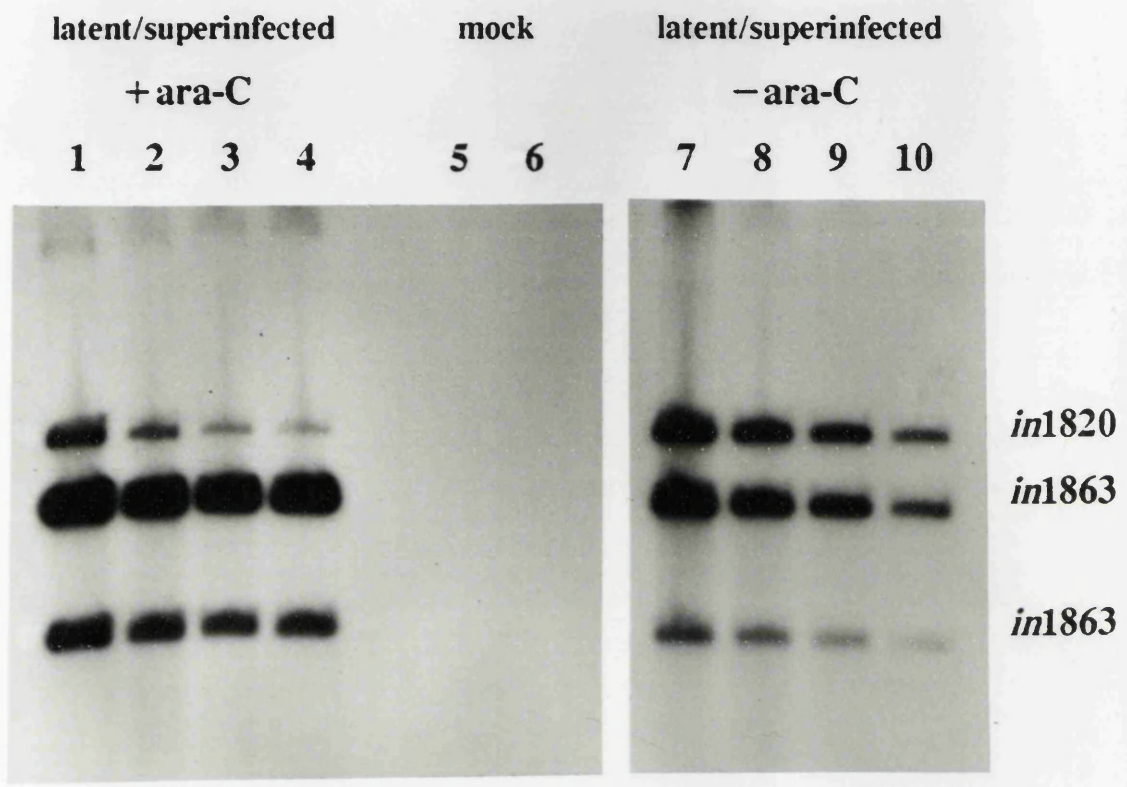


FIGURE 4.19b: THE REPLICATING SUPERINFECTING GENOME IS AS SENSITIVE TO MICROCOCCAL NUCLEASE DIGESTION AS THE LATENT GENOME.

The gel shown in figure 4.19a was blotted and the membrane hybridised with the ^{32}P-radiolabelled 2.4kb fragment from pTK1. The panel showing lanes 7 - 10 was derived from a 4h exposure in contrast with the panel showing lanes 1 - 6 which was obtained from an 18h exposure.

CONDITIONS OF SUPERINFECTION		ARA-C PRESENT				ARA-C ABSENT			
ENZYME UNITS (u)		0	7.5	22.5	45	0	7.5	22.5	45
INCUBATION TIME (min)		0	2	10	30	0	2	10	30
%DNA	2.4kb (*in*1820)	100	48	30	25	100	66	46	22
	2.0kb (*in*1863)	100	67	54	58	100	65	47	23
	1.0kb (*in*1863)	100	70	56	58	100	71	50	27

VIRUS	ABSOLUTE VALUE AT 0 MINUTES		INCREASE IN DNA (FOLD)
	8h +ara-C	8h −ara-C	
*in*1820 (2.4kb)	9665	92035	9.5
*in*1863 (2.0kb)	71598	123567	1.7
*in*1863 (1.0kb)	15546	20718	1.3

TABLE 4.21: QUANTIFICATION OF THE SENSITIVITIES OF THE REPLICATING SUPERINFECTING GENOME AND THE LATENT GENOME TO MICROCOCCAL NUCLEASE DIGESTION .

The DNA bands shown in the autoradiograph depicted in figure 4.19b were quantified by storage phosphor technology. The amount of DNA present in the band at 0 min and 0 units is arbitrarily defined as 100%.

4.21. Presence of linear and circular molecules following latent infection at high multiplicities of infection

The MoMuLv enhancer that has been inserted into the viral genome of *in*1814 to produce *in*1820 lies in the long terminal repeat. The effect of cleaving the genome with *Bam* HI and hybridising with ^{32}P-radiolabelled "Moloney enhancer fragment" will produce one 5.75kb band if the molecule is circular since only *Bam* HI k fragments containing the MoMuLv enhancer will be generated and two bands of 5.75kb and 3.05kb if linear since the MoMuLv enhancer will be present in both the *Bam* HI k and

s fragments. Other viral genomes such as *in*1863 which do not have the MoMuLv enhancer will be undetected by the "Moloney enhancer fragment". It is therefore possible to demonstrate the relative amounts of the circular and linear forms of *in*1820 by quantification of the bands arising from the joint and terminal fragments. The results given in figure 4.20 and table 4.22 were based on a single experiment and only tentative conclusions are drawn. As table 4.22 shows cells latently infected with 75 or 120 particles of *in*1820 retained between 81 and 72% of the viral DNA as circular molecules. This is less than the number of circular molecules shown in figure 4.2 where nearly all the genomes were circular and in which monolayers were grown on 90mm plates and may have received a slightly lower moi. In addition the result in figure 4.2 arises from material that had been fractionated on caesium chloride gradients. The lower moi in table 4.22 has a higher proportion of circular molecules. The observed changes on superinfection with *in*1863 or *dl*1403 were small and may not be significant. The results from the micrococcal nuclease digestion experiments showed no change in sensitivity when superinfected with *in*1863 and this has been interpreted as demonstrating no major structural change occurring. If this interpretation is correct it is not surprising that *dl*1403 does not significantly alter the population of circular to linear molecules either. The results of this experiment suggest that at a moi of 120 particles per cell up to 28% of the molecules have remained linear. These might not be genuinely latent molecules and could account for the 20 - 30 % of resistance to micrococcal digestion consistently found in cells latently infected at this moi. Evidence to support this suggestion has recently been obtained (L.H. Robinson, personal communication) and is described in the discussion.

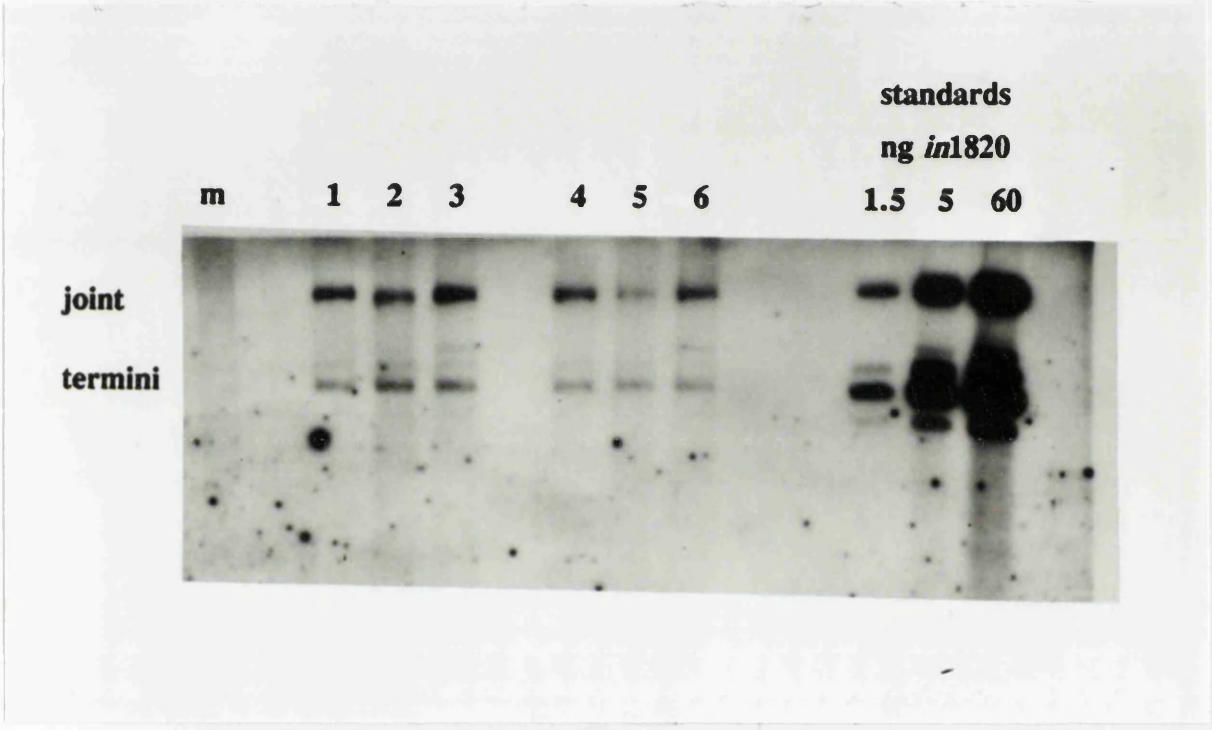


FIGURE 4.20: CIRCULAR AND LINEAR GENOMES DURING LATENT INFECTION WITH *in*1820 AT HIGH MOI AND FOLLOWING SUPERINFECTION WITH *in*1863 OR *dl*1403.

HFL monolayers on 50mm plates latently infected with *in*1820 at 75 and 120 particles per cell were superinfected for 8h with 7.5 pfu per cell of either *in*1863 or *dl*1403 in the presence of ara-C. The samples were cleaved with 20 units of *Bam* HI for 18h in a 100μl reaction and 25μl run in a 1% agarose gel. The ^{32}P-radiolabelled pm1sp6 440bp *Bfr* I / *Bam* HI fragment was used for the hybridisation. The upper band in each lane originates from non-linear DNA and the lower band in each lane from linear DNA.

PARTICLES PER CELL	120			75		
SUPERINFECTING VIRUS	NONE	*in*1863	*dl*1403	NONE	*in*1863	*dl*1403
% CIRCULAR MOLECULES	72	52	82	81	68	95

TABLE 4.22: QUANTIFICATION OF THE PERCENTAGE OF CIRCULAR GENOMES DURING LATENCY AND REACTIVATION WITH *in*1863 AND *dl*1403.

The DNA bands shown in the autoradiograph depicted in figure 4.20 were quantified by storage phosphor technology and the percentage of circular molecules as a proportion of the total number of genomes determined.

5. DISCUSSION

Each subfamily of the herpesviruses, although sharing common morphological properties and the ability to establish latent infection, has a distinct pattern of gene organisation and regulation. Each possesses its own particular transactivator genes with characteristic elements in the promoters of the IE and E genes. Thus the mechanisms underlying lytic infection and those involved in the establishment and maintenance of latency are likely to be specific to each subfamily. IE genes are transcribed shortly after infection even in the absence of protein synthesis or presence of protein synthesis inhibitors (Clements *et al.*, 1977). The HSV-1 genome encodes five IE genes, namely IE175, IE110, IE68, IE63 and IE12, and the expression of at least three of these is critical to the outcome of the infection. Thus IE175 and IE63 are essential for the subsequent activation of the E and L genes and hence productive infection, IE175 and IE110 are potent but differentially acting transactivators and IE175 is able to down regulate its own and other promoters. Upon infection of cells the virion delivers its tegument protein Vmw65 which complexes with the cellular Oct-1 DNA binding protein and other factors to specifically activate the IE promoters. Evidence is accumulating that the decision as to whether infection proceeds to lysis or latency is taken at the initial stages of infection prior to any substantial expression of the infecting genome.

One of the most important sources of evidence to support this hypothesis comes from experiments based on the mutant *in*1814. This virus has a defective Vmw65 (Ace *et al.*, 1988), is unable to transinduce IE gene expression (Ace *et al.*, 1989) and is predisposed to latent infection *in vitro* (Harris and Preston, 1991) and *in vivo* (Steiner *et al.*, 1990). The *in vitro* findings have been confirmed (section 4.2 and table 4.1). At higher moi the predisposition to latency is overcome (table 4.1). It is proposed that this is because there is a basal rate of transcription of IE genes independent of activation by Vmw65 which exceeds threshold levels at higher moi and drives the infection into the productive cycle. If this argument is correct it implies that a low moi leads to only a low level of expression of the IE genes and latency ensues. Thus the level or rate of expression of the IE genes is suspected to be the critical factor in determining the outcome of an infection with HSV-1. To analyse latency at the molecular level it is desirable to achieve levels of latent infection greater than one latent genome in about 20% of the cells as has already been reported (Harris and Preston, 1991). Ideally, the level of latent infection would be in 100% of the cells but to do this

the moi has to be increased. If expression of IE genes is the critical factor then in addition to loss of the activator Vmw65, measures to reduce the basal rate of IE gene transcription should further favour the latency pathway and permit higher moi without ensuing cpe. The mutant *in*1820 has, as well as the Vmw65 mutation, the IE110 promoter substituted by the MoMuLV enhancer. This would be expected to reduce the transcriptional activity of the IE110 gene in cells of a human origin with a concomitant fall in cytopathogenicity. Others have shown that *in*1820 produces undetectable levels of Vmw110 mRNA under IE conditions (J.I. Daksis and C.M. Preston, personal communication). This prediction has been borne out by the reduced plaquing ability of the double mutant (section 4.2 and table 4.1) and it permits a higher moi than previously used with *in*1814. The result lends further support to the hypothesis that reduced IE gene expression is the key to the switch to latent infection.

TK^- HSV mutants are able to establish latency in mouse trigeminal ganglia (Coen *et al.*, 1989). The expression of the lytic genes of these mutants during the establishment of latent infection is restricted (Kosz-Vnenchak *et al.*, 1990). Although the LATs were detected there were minimal levels of productive gene and particularly IE gene expression. Why the mutation should affect IE gene expression is not clear. Nevertheless, severely reduced IE gene expression is again associated with latent infection as has been shown with the mutants *in*1814 and *in*1820 (section 4.2 and table 4.1). In addition these observations demonstrate that the predisposition to latency associated with reduced IE gene expression evident with *in*1814 and *in*1820 is not restricted to an inhibitory mechanism directed by a mutant Vmw65.

IFN has been shown to reduce HSV-1 IE gene expression. The transcription of the genes encoding IE175 (Mittnacht *et al.*, 1988) and IE110 (De Stasio and Taylor, 1990) is inhibited by this cytokine. Pretreatment of the host cells with IFN offers yet another means of reducing IE transcription from the incoming genome if the mechanism is at least in part different to a direct effect on Vmw65. The replication of both the mutants *in*1814 and *in*1820 as well as the rescued virus 1814R has been shown to be inhibited by IFN-α pretreatment but the mutants are more sensitive to the cytokine than the revertant (section 4.3 and tables 4.2 and 4.3). Similarly, in table 4.7, *ts*K at the permissive temperature was not as sensitive as *in*1814 or *in*1820 to the effect of IFN-α. Reduction in IE gene expression appears to enhance the susceptibility of virus to the inhibitory effects of IFN-α pretreatment. Further investigation has shown that pretreatment with IFN-α reduces the accumulated amount of mRNA, at least in terms of the IE110 gene, in this model (section 4.8 and figure 4.1). This is in accord with the data of other investigators who have demonstrated that IFN-α inhibits transcription

(Mittnacht *et al.*, 1988). Once more the increased efficiency of latent infection is associated with reduced IE gene expression adding more weight to the hypothesis.

The mechanism as to how IFN-α might reduce the level of IE gene transactivation has been explored by a number of investigators but no definitive conclusions have been reached (section 1.4.4). It is clearly later than the stage of uncoating in agreement with an earlier report (Obermann and Panet, 1989). If the IFN-α effect were absolute and prevents uncoating only there would be no difference in the plaquing ability between *in*1814 and *in*1820 following IFN-α pretreatment. The additional defect in the latter virus can only be apparent once the virus has become transcriptionally active, for which the genome presumably must be uncoated. The plaquing ability of *in*1820 is less than *in*1814 following IFN-α pretreatment (section 4.3 and tables 4.2 and 4.3). Neither is the inhibitory effect of IFN-α operating solely and directly on Vmw65. If it were then the effect of IFN-α on the rescued virus 1814R would be expected to reduce it to the plaquing ability of *in*1814 which it does not (tables 4.2 and 4.3) and since *in*1814 has a defect in Vmw65 and already is unable to transactivate the IE genes any further inhibitory activity at this site will be ineffective. As tables 4.2, 4.3 and figure 4.1 show, IFN-α has a cumulative effect on the plaquing ability and IE110 mRNA production of the Vmw65 mutants. The results from later experiments also argue against an uncoating defect induced by IFN-α pretreatment. If the genome were not uncoated it would not be expected to be sensitive to superinfection nor to digestion with micrococcal nuclease. Sensitivity of the genome to both of these manipulations has been shown, to superinfection in figure 4.4 and tables 4.7 and 4.9 and to micrococcal nuclease digestion in figure 4.12b and table 4.14. Furthermore, as is shown in figures 4.14b and table 4.16, there is no difference in the sensitivity of the genome to micrococcal nuclease digestion from cells latently infected in the presence or absence of IFN-α pretreatment.

These results suggest that the site of action of IFN-α in the *in vitro* model is either on the components of the transcription complex which determines basal transcriptional activity or a direct effect on IE gene transcription. This could be achieved by modification of pre-existing cellular factors, the induction of new proteins or a combination of both. A potential target is Oct-1. Down-regulation of this protein by IFN-α has been reported and hypothesised as the cause of IE gene transcription failure (section 1.3.2; Dent *et al.*, 1991). The results presented in section 4.3 and tables 4.2 and 4.3 are consistent with this hypothesis. The possibility that IFN-α pretreatment affects the components of the IEC was investigated in the *in vitro* model using gel retardation assays. No effect on Oct-1 was demonstrated but the method may not be

sufficiently sensitive to detect a change in the amount and the data are not shown. An alternative up-regulation of the repressor Oct-2 (section 1.3.2) seems unlikely since this octamer-binding protein is lymphocyte- and neurone- specific and probably not present in HFL cells although no formal investigations have been reported to establish this latter statement.

The mutant *ts*K, which expresses the IE110 gene normally, is able to reactivate the double mutant from IFN-α pretreated cells in a highly efficient manner (section 4.7 and table 4.7). The monolayers initially infected with *in*1820 were superinfected with *ts*K 24h later. The effect of IFN-α is still apparent at 24h following its removal (section 4.4 and table 4.4). After 24h the latent genome of *in*1814 is no longer sensitive to transactivation by Vmw65 (Harris and Preston, 1991) thus the provision of Vmw110 by the superinfecting virus is not only able to overcome the defect in Vmw65 but also the anti viral effect of IFN-α. This is further evidence that the inhibitory mechanism of IFN-α must operate prior to transcription of the IE genes.

The results presented in section 4.4 and table 4.4 show that the effect of IFN-α pretreatment is still present at 72h following its removal. This suggests that the stimulus induces irreversible changes in the cell which could be accounted for either by modifications to existing proteins by a secondary messenger or induced new proteins or the induction of new proteins which themselves mediate the IFN-α effect. A whole range of effects on mRNA production following IFN treatment has been documented including the continued accumulation of some mRNAs but not others after it has been removed (Friedman *et al.*, 1984). The effect of IFN-α as an anti viral agent is apparent at 2h of treatment although maximal levels of inhibition are not established until the cells have been pretreated for 16h (section 4.5 and table 4.5). This is in keeping with the suggestions made above as to how the IFN-α effect is mediated. Induced proteins which correlate with the development of the anti viral state have been seen to accumulate, albeit at different rates, within a few hours of treatment with IFN (Rubin and Gupta, 1980; Gupta, 1981). The finding that cycloheximide does not prevent the effect of IFN-α pretreatment on viral replication (section 4.9 and table 4.8) implies that it does not depend on the synthesis of new proteins. This is difficult to envisage in the light of experimental evidence which has shown that inhibition of RNA synthesis with actinomycin D prevents IFN-α from establishing the anti viral state (Gupta, 1981) and the hypotheses developed to explain its anti viral activity (section 1.4.3). In the experiment detailed in table 4.8, IFN-α pretreatment and cycloheximide exposure were simultaneous and both were washed out prior to infection of the monolayer. If the secondary messenger for mediating IFN-α can be generated without

protein synthesis it may remain in the cells even though the stimulus is removed. Once the protein synthesis inhibition is relieved the secondary messenger will then be able to induce the necessary proteins to effect the desired response. As the cycloheximide is removed just before inoculation there still might be opportunity for the induction of new proteins to interfere with the productive infection of the virus. This would reconcile the data presented in table 4.8 with the prevailing evidence and hypotheses concerning IFN action. This could be investigated by determining the IE mRNA levels rather than plaque formation when cycloheximide is present throughout the course of the experiment.

IFN-α pretreatment reduces the level of IE mRNA (section 4.8, figure 4.1). This is likely to lead to reduced levels of IE proteins. If there is a critical level of IE protein which determines whether an infection proceeds to lysis or latency then IFN-α may be effective in favouring latency by further depressing IE protein levels below this threshold amount, or as would occur in a population of infected cells, shift the curve describing the normal distribution of IE protein concentration to the left. If HMBA operates in the opposite direction serving to elevate the IE protein levels and shift the curve to the right, this would in effect restore the phenotype. This is what occurs in the presence of these two agents, neither of which is dominant over the other (section 4.6 and table 4.6).

The question that arises is how IE gene expression might be overcome or inhibited *in vivo* so that latency rather than lysis occurs. A relative loss in the tegument protein Vmw65 is a possibility especially if the distance from the periphery where infection occurs to the site of gene transcription in the neuronal nucleus is large. In traversing this distance by axoplasmic transport, opportunity would be available for this transcriptional activator to be modified or even destroyed. The neurone is unique with its long axon and distant cell body relative to the epithelial surface which provides a possible explanation as to why latency could be favoured in such cells. This suggestion is however difficult to sustain if latency can be established in other cells. Reports of latent infection in extra neural tissues have been published (sections 1.2.1 and 1.2.3.) although no clear distinction between *bona fide* latent infection and viral persistence was made in some of these experiments. In an experiment designed to test the hypothesis that latent infection of sensory neurones results from the failure of Vmw65 to be transported from the nerve ending to the nucleus it was concluded that the absence of the viral transactivating factor alone could not account for the establishment of latency (Sears *et al.*, 1991).

For the putative VZV IE genes, ORFs 4, 61, 62 and 63 see Davison and McGeoch, 1986; Shiraki and Hyman, 1987.

For the homologue of Vmw65, ORF 10 found in VZV see Davison and Scott, 1986.

For the three EBV proteins, BZLF-1 , BMLF-1 and BRLF-1 see Countryman and Miller, 1985; Lieberman *et al.*, 1986 and Hardwick *et al.*, 1988.

For HCMV ORFs U_L123, U_L122, U_L36-38 and U_S3 see Sternberg *et al.*, 1985; Kouzarides *et al.*, 1988 and Weston, 1988.

For the major IE region of HCMV see Stinski *et al.*, 1983.

There is good evidence that repression of IE gene expression operates in neurones (section 1.3.2). Cell specificity of repressors such as Oct-2 would account for the restriction of latent infection to certain host cells such as the neurone. Since octamer binding proteins have a role in the formation and activity of the IEC, a repressor mechanism at this point in the viral life cycle would be highly effective in switching off gene expression. This site would also have room for other repressors that might be available as a result, for example, from stimulation by IFN.

The role of the IE genes of other human herpesviruses in switching between a lytic and latent infection has not been investigated. This is largely because there is a paucity of knowledge about temporal gene expression in these viruses. The VZV genome has the putative IE genes, ORFs 4, 61, 62 and 63 but these have been identified primarily through sequence homology to the HSV genes namely U_L54 (Vmw63), IE110, IE175 and U_S1(Vmw68) respectively. A homologue of Vmw65, ORF 10, is also found in VZV. VZV latency differs in a number of respects to HSV-1 latency not least in that it is largely established in the non-neuronal cells of the sensory ganglia. At least three EBV proteins, BZLF-1, BMLF-1 and BRLF-1, induced early in the lytic replicative cycle have been identified as transactivators of gene expression with a distinct spectrum of activation effects on EBV promoters. One of these, BMLF-1, is analogous to HSV-1 IE63 and may have a role in activation from latency. The latent state of EBV is different to HSV-1 in that there has to be ongoing replication to maintain the copy number per cell as the B lymphocyte continues to divide. HCMV IE gene expression is predominantly from ORFs U_L123, U_L122, U_L36-38 and U_S3. The U_L123 and U_L122 proteins originate from two contiguous regions called IE1 and IE2 which form part of the major IE region. Approximately 88% of the IE viral RNA hybridises to this region. The major IE promoter (MIEP) directs the expression of both IE1 and IE2. The IE1 protein transactivates the MIEP, several heterologous promoters and augments the transactivating activity of the 84kd IE2 protein. The IE2 protein is able to transactivate homologous and heterologous promoters alone or augmented by IE1. The IE2 transcripts encode several mRNAs which translate into proteins of 84, 55 and 40kd. The 55kd protein activates the MIEP whereas the 84 and 55kd proteins down regulate the MIEP. Thus HCMV has functionally similar major IE proteins to those in HSV. All of these IE gene products undoubtedly have a role in HCMV early gene regulation but their place if any, in the pathway leading to a latent infection has not been established.

Once HSV-1 latency has been established the viral genome is distinguished from the input DNA in terms of its organisation and expression (sections 1.2.3 and 1.2.4).

Most of the experimental evidence to date indicates that the latent genome has a non-linear configuration. The most likely explanation for the data is circularisation of unit length molecules. In the *in vitro* model the terminal fragments are lost as latency becomes established (section 4.10 and figure 4.2) which is consistent with latency in a similar model at lower moi (Harris and Preston, 1991) and with HSV-2 latency *in vitro* (Preston and Russell, 1991). That this *in vitro* model mimics the non-linear organisation of the viral DNA during latency *in vivo*, which might be reasonably considered as a defining feature of a latent infection, enhances its suitability as a model system for in depth investigations into this process at the molecular level. Evidence is also available to suggest that the latent non-linear molecule is present as a circular genome, an episome (section 1.2.3). The results with the *in vitro* model (section 4.15 and figure 4.7) also suggest that the non-linear DNA is autonomous which again supports the contention that this is a valid model of latency.

Little is known about the non-linearity or otherwise of the other human herpesviruses during latency. In EBV transformed lymphocytes the latent genome is a circular episome analogous to what is proposed for the latent HSV-1 genome *in vitro* and *in vivo*. Circularisation occurs within 24h of infection of these B lymphocytes (Hurley and Thorley-Lawson, 1989) which are non-permissive for EBV replication which is a similar time course for HSV-1 to become non-linear as latency is established in the *in vitro* model (section 4.10).

The replication of HSV-1 probably proceeds via a rolling circle mechanism (Roizman and Sears, 1990) and this may well apply to the other members of the subfamily. It is claimed that a proportion of HSV is circular in the particle (Poffenberger and Roizman, 1985). The linear molecules of EBV DNA isolated from cultured producer cells probably arise from circular intermediates (Shaw, 1985). Similarly VZV adopts circular forms via the genomic termini during infection although whether this is a covalent link or one held together by a protein has not been established (Kinchington *et al.*, 1985). Replication of HCMV is also considered likely to occur via a rolling circle mechanism (Stinski, 1991). It could be argued that organisation into a non-linear configuration is a prerequisite for the efficient replication of the infecting DNA. By extending this argument, latent viral DNA in a non-linear and specifically circular arrangement could be in a state suitable for replication. If this is the case then it would appear that the latent pathway has circumvented the block in the lytic pathway to bring the genome to a condition in which it is immediately ready to replicate once the correct stimulus is received.

The concept of the nucleoskeleton as a structure similar to the cytoskeleton has been the subject of much controversy because of the highly artificial conditions under which it has been purported to have been isolated or visualised. Most preparations have used high salt extraction methods because chromatin tends to aggregate under isotonic conditions. Sceptics have contended that such high salt extraction methods produce artifactual structures which cannot be related to the *in vivo* situation and have dismissed the evidence for the existence of a nuclear substructure. The problem has been compounded by the fact that the artifacts produced under high salt conditions arise partly from the aggregation of nascent DNA and polymerases, vital elements of the replication machinery. These difficulties and objections have been overcome by first encapsulating cells in agarose microbeads prior to lysing their membranes in a "physiological" buffer (Jackson *et al.*, 1988).

In early experiments, nascent DNA was found to be tightly associated with a nuclear substructure termed the nuclear matrix (Berezney and Coffey, 1975). Numerous reports have followed claiming to show that replicating DNA attaches to the nuclear matrix (Wanka *et al.*, 1977; Dijkwel *et al.*, 1979) but the question of artifactual structures had remained as a nagging doubt. However, using gentler techniques the findings have been supported and although only residual chromatin remained attached to the intermediate filament most of the polymerase activity was retained suggesting that the active enzyme was attached to the skeleton (Jackson *et al.*, 1988). Subsequently the active polymerases were shown to be focally clustered within the nucleus and each focus to fire in a synchronous manner (Cook, 1991). This is further evidence in favour of an underlying structure serving to organise and integrate polymerase activity. Recently the sites of replication have been visualised under electron microscopy to be attached to the skeleton. The focal sites of synthesis persist even after eluting the majority of the chromatin and these have been shown to contain DNA polymerase-α and proliferating cell nuclear antigen (Hozak *et al.*, 1993).

It is arguable from the above findings that the high salt methods are valid as a means of extracting the nuclear substructure and these were used to investigate the association of the viral DNA with it in the *in vitro* model. As is discussed in section 4.16 and shown in figures 4.8b and 4.9b the viral DNA is associated with the nuclear substructure very soon after infection and must do so almost immediately after entering the nucleus. There was no detectable difference in this association between viral DNA isolated at 1h, 8h or 72h as it progresses to the latent state. Association of DNA with the nuclear skeleton may be a prerequisite to replication and this was demonstrated in the early stages of an infection which would under normal conditions be productive

(figure 4.9b). Thus the viral genome appears to adopt a topological position as soon as it reaches the nucleus which is maintained even though there are further organisational changes to be undertaken as it becomes latent. This may be because the spatial arrangement is vital for these subsequent changes to occur or to place the DNA in a position ready to reactivate and replicate as soon as the appropriate stimulus is received or the host conditions become favourable. The attachment of the DNA is not dependent on nucleosomal organisation since it is not detached even though 90% of the chromatin is removed (Jackson *et al.*, 1988). If the viral DNA had to acquire the relevant cellular proteins to become nucleosomal in order to associate with the nucleoskeleton, that association must occur within 1h of infection (figure 4.8b).

The formation and maintenance of chromatin as occurs in all eukaryotic DNA is essential for it to be compacted within the nucleus. In addition the position of nucleosomes on the DNA template has the potential to facilitate regulated transcription of the genes. There is a large body of experimental evidence to suggest that the association of regulatory elements with histones will preclude their recognition by transactivating factors (Felsenfield, 1992). Whereas some transcription factors are able to bind to the DNA when it is wrapped around the histone core others are prevented from binding because of the severe deformation of the structure. Thus the organisation of the DNA into chromatin imposes structural hindrances to the interaction of transcription factors with their recognition elements. It follows that DNA that is non-nucleosomal might be more susceptible to transactivation and hence in a state of heightened readiness for gene expression.

The discovery of viral DNA being organised into nucleosomes akin to eukaryotic DNA was first reported in extracts of SV40 infected cells (Griffith, 1975). Although bearing some identity with cellular nucleosomes differences have been reported (Bellard *et al.*, 1976). An interesting difference is that many of the viral nucleosomes are at some distance apart from one another connected by bridges of naked DNA of irregular length as revealed by electron microscopy and confirmed by nuclease digestion (Bellard *et al.*, 1976). There have been conflicting reports of viral chromatin lacking histone H1 (Bellard *et al.*, 1976; Varshavsky *et al.*, 1976; Ponder *et al.*, 1978) but since this particular histone is associated with the region of DNA between the nucleosomes it might explain the variable spacing of nucleosomes in the viral DNA.

The structure of the genome from a number of DNA viruses has been investigated in terms of its nucleosomal organisation by digestion with micrococcal nuclease. During the whole of their productive life cycles and in their virions, SV40 and polyoma

virus nucleoprotein complexes digested with micrococcal nuclease yield a ladder of nucleosomal fragments (Griffith, 1975; Ponder *et al.*, 1978). In contrast, EBV DNA during the whole of the productive cycle, at least in a producer cell line, is nonnucleosomal (Shaw *et al.*, 1979). However, in the latent state it is different and the majority of the EBV DNA appears to become associated with nucleosomes and organised into chromatin (Shaw *et al.*, 1979; Dyson and Farrell, 1985). Another variation occurs with adenovirus type 2 which appears to adopt a chromatin arrangement only during part of the replication cycle (Dery *et al.*, 1985). With this virus the typical nucleosomal pattern following micrococcal nuclease digestion was only generated from intranuclear parental DNA (Tate and Philipson, 1979; Sergeant *et al.*, 1979) and not from the virion (Tate and Philipson, 1979). At 1h post infection the viral DNA produced a smear following digestion but at 6h up to 50% of the viral DNA had a nucleosomal pattern similar to that of the cellular chromatin. Such a pattern was not seen in partially uncoated virus or isolated cores. In cells productively infected with HSV-2 the viral DNA exists in either form. Nucleosomes accounted for 37% of the replicating HSV-2 nucleoprotein at 10 - 12h post infection which suggests that the DNA is only organised into nucleosomes at a late stage (Hall *et al.*, 1982). This is in contrast with HSV-1 lytic infection in tissue culture cells (Mouttet *et al.*, 1979; Leinbach and Summers, 1980; Sinden *et al.*, 1982) and in mice (Muggeridge and Fraser, 1986) where the majority of the replicating DNA is clearly nonnucleosomal. One report suggests that most of the regions of the HSV-1 genome during latency are organised into nucleosomes and that this might be a mechanism of silencing gene expression or that the silenced genome is vulnerable to being converted into chromatin (Deshmane and Fraser, 1989). It seems that the existence of viral chromatin is not a universal characteristic of DNA viruses and that viral transcription commonly occurs on nonnucleosomal DNA.

The demonstration of the nonnucleosomal pattern in micrococcal nuclease digests of HSV-1 DNA at 1h and 8h after infection in the *in vitro* model (section 4.17 and figures 4.10b and 4.11b) is in agreement with the findings of other investigators as cited above. These results are with respect to the TK gene and it is inferred that a similar situation prevails throughout the rest of the genome during replication although it is possible that a small stretch of DNA not associated with nucleosomes has been detected only. The persistence of this pattern in the latent genome (section 4.17 and figures 4.10b and 4.11b) is however in stark contrast to the published data (Deshmane and Fraser, 1989). The material analysed in this report was taken from the brain stems of mice infected by corneal scarification and shown to harbour latent virus by explant reactivation assay. The sensitivity of their method only permitted them to analyse

samples containing >3pg of latent HSV-1 DNA per μg of micrococcal nuclease digested cellular DNA and much of the material was therefore unsuitable. It was assumed that the samples harbouring lower concentrations would give similar results. In addition the viral DNA was not digested at the same rate as the cellular chromatin and this delay might reflect different conformational structures. The latent HSV-1 genome in the brain stem is remarkably resistant to reactivation (Cabrera *et al.*, 1980) which might be a reflection of its acquisition of a chromatin structure and may be the explanation for the different result in the *in vitro* model (section 4.17 and figures 4.10b and 4.11b). In the latter case it has been possible to reactivate the latent genome consistently (sections 4.7 and 4.12, tables 4.7 and 4.9 and figure 4.4) which might be easier to achieve if the genome is nonnucleosomal. An alternative explanation to the genome being nonnucleosomal is that the nucleosomal organisation fails to protect it against micrococcal nuclease attack. Some investigators have shown that although micrococcal nuclease digestion of DNA from adenovirus produces a digest of heterogeneous lengths it is nevertheless clearly nucleosomal when viewed by electron microscopy (Vayda *et al.*, 1983; Mirza and Weber, 1982). To resolve this paradox it has been suggested that the nucleosomes are formed from precursor proteins into prochromatin which could be converted to chromatin by proteolytic processing (Mirza and Weber, 1982). It is hypothesised that prochromatin protects the DNA against micrococcal nuclease digestion. Since there is no difference in the micrococcal nuclease digestion pattern between HSV-1 DNA in the early stages of infection and when latent, it appears that the latent genome is in a state in which it can rapidly commence replication when favourable conditions prevail. Thus these results once more suggest that during latency the genome has been arrested at a stage just short of replication and is poised to switch into replication as soon as the appropriate signals arrive. The pathway to this stage is not necessarily the same pathway as followed in the normal course of replication. The data given in table 4.21, where the latent DNA was replicated much more efficiently than the superinfecting viral genome, support this hypothesis.

Further analysis at other sites in the DNA needs to be carried out to show that the nonnucleosomal organisation is present throughout the latent genome and not merely at the TK gene. An extension of these investigations into the organisation of proteins on the DNA of the latent genome would be with similar analyses using DNase I. Nonnucleosomal DNA has increased sensitivity to this enzyme (Eissenberg *et al.*, 1985). Regions exist at intervals in chromatin which are free of nucleosomes and the DNA at these sites has been found to have relative sensitivity to cleavage by DNase I. They have been termed DNase hypersensitive sites and are frequently found at or near

the 5' end of the gene which suggests a possible role in activation of transcription. It has been found that both active and potentially active genes are preferentially sensitive to digestion by DNase I with localised sites of hypersensitivity. The high degree of sensitivity compared to the surrounding chromatin is not necessarily limited to DNase I and may apply to micrococcal nuclease also (Kaye *et al.*, 1984). In this paper it is reported that the enhanced sensitivity to micrococcal nuclease was not only relative to inactive genes but to naked DNA as well. Thus genes which are open or accessible for expression are, it would seem, simultaneously vulnerable to attack from nucleases. There have been many reports of more rapid or extensive cleavage by nucleases in the transcribed regions of chromatin compared with untranscribed controls (Kornberg and Lorch, 1992). A more rapid digestion to the nucleosomal ladder by micrococcal nuclease is indicative of enhanced susceptibility to digestion of the linkers between nucleosomes following activation of the gene, whereas a loss of the nucleosomal ladder and the resultant smear is more likely to arise from enhanced susceptibility to digestion within the nucleosome. Such altered patterns of digestion are very suggestive of a fundamental structural change once the DNA is transcribed and this is likely to take place prior to or concomitant with the initiation of transcription. Identical DNA sequences can differ in DNase I sensitivity depending on their locations. Furthermore, although DNase hypersensitive sites lack nucleosomal structure, there is evidence that non histone proteins bind in and around these sites. Thus conformational differences or a change in the nature of the proteins covering the DNA may be responsible for the enhanced susceptibility to nuclease attack.

Latent viral DNA in the *in vitro* model has been shown to acquire an increased sensitivity to digestion by micrococcal nuclease with respect to the TK gene (section 4.18 and figures 4.12b, 4.13b and 4.14b and tables 4.14, 4.15 and 4.16) and it is hypothesised that this pertains to the rest of the genome. This is relative to DNA shortly after infection (figure 4.12b and table 4.14) and also to DNA that has been prevented from replicating by ara-C but is nevertheless transcriptionally active (figures 4.15b, 4.16b and 4.19b and tables 4.17, 4.18 and 4.21). If transcription is suppressed as happens in the model to favour latency, sensitivity is still acquired at about 8h as the genome proceeds to the latent state (figure 4.13b and table 4.15). It is suggested that the increased sensitivity is a reflection of the structural changes that have occurred as the genome has become latent in terms of its non-linearity and possibly its associated proteins too. The transcribing but not replicating genome does not have such changes conferred upon it and maybe for this reason it remains relatively resistant. The replicating genomes will have a population of circular intermediates unlike those merely transcribing which might be the explanation for the difference in sensitivity. If

this suggestion is true it again envisages the latent genome to be in an arrested state immediately prior to replicating and in a heightened condition ready to respond to the incoming signal (table 4.21).

It could be argued that the DNA becomes more sensitive to micrococcal nuclease merely as a result of uncoating. Uncoating is a non-specific term to embrace all the events after the virus has penetrated the cell until its genome is in a state of readiness to express its functions. In herpesvirus infection the capsid is presented at the nuclear pores and the viral DNA or a DNA-protein complex released into the nucleus. It is unlikely that free DNA would exist in the nucleus after uncoating since it is a highly charged molecule. It is more likely that following uncoating it either retains some viral proteins or associates with others. All the preparations of viral DNA used in the micrococcal digests are nuclear so it would have to be argued that the genome entering the nucleus 2.5h post inoculation (figure 4.12b and table 4.14) has not been fully uncoated and continues to be resistant to digestion. This argument would also have to be applied to *in*1863 and *in*1884 at 8h post infection, since under non-replicating conditions they too remain resistant (figures 4.15b and 4.16b and tables 4.17 and 4.18). Furthermore *in*1863 is transcriptionally active at 8h as demonstrated by its ability to reactivate latent *in*1820 (table 4.13). It is difficult to imagine how a genome can be transcriptionally active and not be completely uncoated. At this stage it is, by definition, uncoated.

During latency HSV-1, HSV-2, pseudorabies virus and bovine herpesvirus all express large amounts of RNA from a single region in their genomes, overlapping and antisense to one of the IE genes (Stevens *et al.*, 1987; Rock *et al.*, 1987b; Cheung, 1989; Croen *et al.*, 1991). The role of these LATs is still obscure although most of the evidence suggests that they have no absolute part to play in the establishment or maintenance of latency (section 1.3.4). On the other hand there are some reports which indicate that these RNAs might be important for efficient reactivation from the latent state (section 1.3.4). In latent VZV infection five regions of the genome are expressed to yield RNAs in very low abundance (Croen and Straus, 1991). They are found in cells other than neurones within the sensory ganglia (Croen *et al.*, 1988). This is different to the expression of LATs. Latent VZV reactivates much less efficiently than HSV-1 and it is tempting to speculate that this is related to the absence of LATs or that its site precludes it from being subject to the same neuronal factors as HSV-1. EBV transformed lymphocytes also display limited transcription of the viral genome. Genes expressed in cell lines include a family of six nuclear antigens (EBNAs), the latent membrane protein (LMP) and three RNAs which do not appear to

be translated. Only one of these, EBNA-1, which is essential to maintain the copy number has been shown to be present in all latently infected cells. Latent EBV infection is not strictly comparable to latent HSV-1 infection so no parallels between the two patterns of gene expression during latency can be drawn.

In the *in vitro* latency model viral gene expression has been silenced, at least with respect to four promoters. These are the Vmw110, TK, HCMV and SV40 promoters (sections 4.12 and 4.14, figure 4.5, tables 4.9, 4.12 and 4.13) and this is presumed to extend to the rest of the genome. This is characteristic of the latent state *in vivo* with the exception of the LATs. There is reason to believe that the LATs would not be expressed in the *in vitro* model. Firstly, they are likely to be neurone specific (Batchelor and O'Hare, 1990) and secondly, which may be related, they were undetectable in the predecessor of the model which was also based on HFL cells (Anderson, 1991). If this is true, although not investigated, it further demonstrates that HSV-1 latency can be established, maintained and reactivation effected without expression of LATs. However the role of LATs, if any, in the efficiency of reactivation remains undetermined and cannot be explored in this model.

The model has been shown to retain the latent viral genome which can be efficiently reactivated (section 4.7 and table 4.7). It has also been shown that it is possible to latently infect every cell in a monolayer with at least one genome that becomes transcriptionally silent and can be manipulated to become active as determined by the activity of β-galactosidase, the product of the *lacZ* gene (section 4.12, figure 4.4 and tables 4.9, 4.11 and 4.12). It is suggested that this mimics the switching on of the latent genome when reactivated. Similarly the latent genome can be manipulated to express the thymidine kinase gene (section 4.14 and figure 4.13), again suggested to mimic the situation occurring during reactivation. The superinfecting virus, *ts*K which efficiently reactivates *in*1820 (section 4.7 and table 4.7), provides Vmw110 at the non-permissive temperature. Latent *in*1884 and *in*1883 cannot be reactivated by *dl*1403 which lacks Vmw110 (section 4.13 and tables 4.11 and 4.12). It is concluded that latent HSV-1 in the *in vitro* model requires Vmw110 for reactivation. This is consistent with the earlier findings (Harris and Preston, 1991) and with much of the experimental data published to date (section 1.3.5). Thus the silent latent genome remains sensitive to transactivation by Vmw110. A comparison can be made with the change from latent to lytic EBV infection. This is mediated by expression of the gene BZLF-1 which is silent during latency and is a transactivator of some EBV promoters. In some ways the reactivation of HSV-1 in the model is analogous to the initiation of the lytic pathway following the initial infection. The balance between a lytic or latent

infection has been tipped in favour of the latter by the inhibition of IE and specifically IE110 gene expression. This again suggests that the latent genome has arrived at the point of entering the productive cycle albeit by an alternative pathway, awaiting the arrival of the transactivator Vmw110. Since reactivation inevitably involves replication of the genome, and this by a rolling circle mechanism, it would be expected that circular molecules will persist even though the genes have become active. In figure 4.20, circular molecules are shown to be present during latency and superinfection with *in*1863 or with *dl*1403. The presence of ara-C during superinfection prevented replication in this experiment so that there are no linear molecules generated by entry into the productive cycle. Even in the latent infection there are a number of linear molecules still present at a level of up to 28% (table 4.22). This is surprisingly high in view of the earlier data. It might account for the residual micrococcal nuclease resistance once latency has been established. As is presented in the tables 4.14, 4.15, 4.16, 4.17, 4.18 and 4.21 micrococcal nuclease sensitivity usually levels out at about 20 - 30% of the total viral DNA. It is postulated that sensitivity is a property of the circular molecule and the residual resistance is due to the persistence of linear molecules from the time of the original infection. Support for this hypothesis has been provided by the subsequent experiments of others in which latent *in*1820 was digested with micrococcal nuclease, restricted with *Bam* HI and hybridised with the Moloney enhancer fragment. Micrococcal nuclease sensitivity was largely confined to the band generated by circular molecules with little change in the band generated by the linear DNA (L.H. Robinson, personal communication).

6. FUTURE WORK

The *in vitro* model that has been described has been shown to be valid for the further investigation of latency at the molecular level and should contribute to an understanding of the biology of this phenomenon. The work has potential for exploring further aspects of the physical organisation of the latent genome, examination of viral gene expression and its control during latency and analysis of the mechanism of reactivation.

Having established that the latent genome is circular (section 4.10 and figure 4.2) and can be separated from linear molecules by field inversion electrophoresis (section 4.15 and figure 4.7) it should be possible to refine the technology and use it to demonstrate unit length or concatenated structures during latency. It might also be a useful tool to analyse other changes in the latent genome following reactivation if this alters the mobility of the molecule under these conditions. The proportion of circular to linear molecules needs to be examined in greater depth using the MoMuLv enhancer fragment (section 4.21) at a lower initial moi. Since these methods differentiate circular and linear molecules it would be possible to examine the sensitivity of these two forms to micrococcal nuclease digestion under different conditions and to establish if it is the linear form which accounts for the residual resistance to digestion.

It should be possible to further characterise the association of the viral DNA with the nucleoskeleton. Cleavage with restriction enzymes could be used to investigate whether a particular sequence, which would become enriched in the pellet fraction, attaches it to the matrix. The effects of reactivation could also be explored if there is a specific association of the genome with the substructure. The properties of drugs which might interfere with the attachment and thus have potential therapeutic use could also be investigated.

Nucleosomal organisation has only been demonstrated in relation to the TK gene. This should be extended to other genes to determine whether this is a property of the genome as a whole during latency.

The susceptibility of the genome to micrococcal nuclease should also be related to other viral genes and compared with cellular genes. The sensitivity to other nucleases

such as DNase I could provide further information concerning the structural aspects of the genome.

The potential of the β-galactosidase detection assay is considerable since it allows a clear demonstration of the transcribing activity of the genome at least with respect to the *lacZ* gene. The technique could be used to look at particular aspects of Vmw110 in effecting reactivation, the effects of drugs on preventing latency or reactivation and whether drugs are able to overcome the effect of IFN.

The model does not allow investigation of the LATs since they are not produced. If the model could be modified to express LATs this would open up a whole new avenue of experimentation and investigation. This might be achieved by the removal of the neurone specific region and its replacement with an enhancer able to drive LAT expression in HFL cells or adaptation of the system to include infection of neuronal cells.

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