HERPES SIMPLEX VIRUS LATENCY

IN CULTURED CELLS

BY

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SUMMARY

HERPES SIMPLEX VIRUS LATENCY IN CULTURED CELLS

The molecular basis of herpes simplex virus (HSV) latency has been investigated by analysis of a latent interaction between HSV and cells in culture.

An *in vitro* latency system for HSV, based on previous work by E. Notarianni and C.M. Preston, has been characterised, in which incubation at a supraoptimal temperature converts HSV to a latent state within tissue culture cells. Human foetal lung (HFL) cells were infected at low multiplicity with HSV and, following adsorption, the cultures were shifted to 42°C for 6 days, then downshifted to a temperature permissive for HSV replication for a further 4 to 6 days. During the latter incubation period no virus was usually detectable and the HSV was considered to be in a latent state. HSV could be reactivated from this latent state at high efficiency by intertypic superinfection of the cultures with HSV mutants or with human cytomegalovirus.

To define the HSV gene products involved in latency, the behaviour of various temperature-sensitive (*ts*), insertion (*in*) and deletion (*dl*) mutants of HSV in the *in vitro* latency system was examined. The rationale behind this approach is that mutants which fail to become established in a latent state, or which fail to reactivate latent HSV, must lack functions involved in establishment or reactivation, respectively. Two mutants of HSV type 1 (HSV-1) used in these studies, *ts*K and *in*1411, do not synthesise active immediate early (IE) polypeptide Vmw175 and are blocked at a very early stage of the virus replication cycle, and a third mutant of HSV-1, *dl*1403, does not produce IE polypeptide Vmw110, but otherwise exhibits a pattern of protein synthesis indistinguishable from that of wt HSV-1. All mutants tested were able to establish latency in HFL fibroblasts and could be reactivated by intertypic superinfection with HSV or with human cytomegalovirus, showing that no viral DNA synthesis and little or no viral

gene expression is necessary for the establishment of latency *in vitro*, and that at most the viral proteins involved are IE polypeptides Vmw12, Vmw63, Vmw68, Vmw175 or Vmw110, the early polypeptide Vmw136, and, possibly, components of the input virion. Reactivation of latent HSV-2 was achieved by superinfection with *ts*K or *in*1411. However, superinfection with *dl*1403 failed to reactivate latent HSV-2 as a consequence of a deletion in the region of the genome encoding Vmw110, strongly suggesting that Vmw110, which is known to regulate gene expression by *trans*-activation, is required for reactivation in the *in vitro* latency system. The results presented do not indicate whether Vmw110 acts alone or in conjunction with one or more of the virion components and/or the other IE polypeptides, excluding Vmw175. Harris *et al*. (1989) have recently shown that Vmw110 alone can reactivate latent HSV *in vitro*. One possibility is that a block in viral gene expression occurs at a very early stage in the viral cycle, either as the direct cause or as a consequence of establishment of latency, and that the block can be released by the Vmw110 gene product, thereby allowing HSV to continue into the lytic cycle.

The latent state of HSV DNA *in vivo* appears to be 'endless' and is therefore either circular, concatemeric or integrated via regions of the genome other than the termini. A recent report shows that the majority of latent HSV DNA *in vivo* is extrachromosomal, suggesting that latent HSV DNA *in vivo* is not likely to be integrated into cellular DNA. The physical nature of the HSV DNA in the *in vitro* latency system described has been determined. The relative proportion of latent HSV genomes, initially present *in vitro* at 0.03-0.1 copies per cell, was selectively increased and the presence of joint and terminal fragments of HSV in latent HSV DNA was investigated by the use of a modified Southern hybridisation procedure. During the 42^{o}C incubation period the HSV DNA is present in an 'endless' configuration, suggesting that the state of the latent HSV genome is 'endless' *in vitro*.

These findings are discussed in terms of relevance to HSV latency *in vivo*, and of possible molecular mechanisms involved in HSV latency.

ABBREVIATIONS

A	adenine
ACV	acyclovir
Ad	adenovirus
ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
Ara-C	cytosine arabinoside
bp	base pairs
BHK	baby hamster kidney
BHV-1	bovine herpes virus type 1
BL	Burkitt's lymphoma
BMLF	BamHI-M left reading frame
BMRF	BamHI-M right reading frame
BSA	bovine serum albumin
BZLF	BamHI-Z left reading frame
BVDU	(E)-5-(2-bromovinyl)-2'-deoxyuridine
C	cytosine
cpm	counts per minute
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanidine-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DBP	DNA-binding protein
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dl	deletion
D_L	direct repeat, left
D_R	direct repeat, right
ds	double stranded
DTT	dithiothreitol
E (E/L)	early (early/late) (class of HSV gene)
EA	early antigen
EBNA	Epstein-Barr virus nuclear antigen
EBV	Epstein-Barr virus
EDTA	sodium ethylenediamine tetra-acetic acid
G	guanine
HBLV	human B-lymphotropic virus

HCMV	human cytomegalovirus
HSV	herpes simplex virus
HMBA	hexamethylenebisacetamide
ICP	infectious cell protein
IE	immediate early (class of HSV gene)
IEC	immediate early complex
IFN	interferon
IgG	immunoglobulin G
in	insertion
IR	internal repeat
k	kilo
L	late (class of HSV gene)
L	long (HSV genome segment)
LAT	latency associated transcript
LMP	latent membrane protein
LT	latency transcript
MA	membrane antigen
m.o.i.	multiplicity of infection
M_R	relative molecular weight
mRNA	messenger ribonucleic acid
MIR	major internal repeat
MTR	morphological transformation region
N	unspecified nucleotide
NFIII	nuclear factor III
NGF	nerve growth factor
NPC	nasopharyngeal carcinoma
NPT	non-permissive temperature
OD	optical density
ori	origin of DNA replication
ORF	open reading frame
OTF	octamer binding transcription factor
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pfu	plaque forming units
poly(A)$^+$	polyadenylic acid
PT	permissive temperature
R	purine
R_L	long segment repeat sequences
R_S	short segment repeat sequences
RNA	ribonucleic acid

RNase	ribonuclease
RR	ribonucleotide reductase
S	short (HSV genome segment)
ss	single stranded
SDS	sodium dodecyl sulphate
T	thymine
TEMED	N,N,N',N'-tetramethylethylene diamine
TIF	*trans*-inducing factor
TK	thymidine kinase
TPA	tetradecanoylphorbol acetic acid
TR	terminal repeat sequence
Tris	tris(hydroxymethyl)aminomethane
ts	temperature sensitive
U	unique sequence
U_L	long segment unique sequences
U_S	short segment unique sequences
UV	ultra-violet
VA	viral antigen
VCA	viral capsid antigen
Vmw	M_R of viral polypeptide
VP	virion protein
VZV	varicella zoster virus
wt	wild type

1. INTRODUCTION

The research presented in this thesis concerns the characterisation of a latency system for herpes simplex virus (HSV) in cultured cells and the analysis of molecular aspects of HSV latency in the system. The biology and genomic structures of the human herpesviruses are described in INTRODUCTION Section 1.1.; Sections 1.2. and 1.3. of the INTRODUCTION deal with the biological and molecular features of lytic and latent interactions of HSV with the host cell.

1.1. THE HUMAN HERPESVIRUSES

1.1.1. Herpesviruses - Description and Classification

Members of the animal virus family Herpesviridae are structurally similar and possess a double-stranded linear DNA genome of relative molecular weight (M_R) ranging between $80\text{-}150 \times 10^6$.

In terms of the structural components of the virion, herpesviruses can be identified by the presence of an envelope, tegument, capsid and core (DNA-protein complex) (Wildy *et al.*, 1960; Epstein, 1962b). The envelope, the outermost structure of the herpesvirion, is derived from nuclear and other cellular membranes (Morgan *et al.*, 1959; Epstein, 1962a; Siminoff and Menefee, 1966; Shipkey *et al.*, 1967; Nii *et al.*, 1968). It has a typical trilaminar appearance (Epstein, 1962b), and contains surface projections approximately 8nm in length (Wildy and Watson, 1963). The tegument is defined as the structure located between the capsid and the envelope, and electron microscopy reveals it as a layer of amorphous material in thin section (Epstein, 1962b; Roizman and Furlong, 1974; Spear, 1980) and sometimes as a fibrous structure in negatively stained virions (Wildy *et al.*, 1960). The amount of tegument can vary from virion to virion, even in the same cell (Fong *et al.*, 1973) and appears, at least in part, to be determined by the virus strain (McCombs, 1971). The icosadeltahedral capsid, which has been characterised in most detail for herpes simplex virus type 1, is 100-110nm in diameter.

It contains 12 pentameric capsomeres and 150 hexameric capsomeres of 12nm diameter and 14nm length with a deep central indentation approximately 4nm wide tapering from the outer surface towards the capsomere base (Wildy et al., 1960). The herpesvirus core is situated within the capsid and contains the deoxyribonucleic acid (Epstein, 1962b; Zambernand and Vatter, 1966). The core is of toroidal conformation with a central cylindrical structure (Furlong et al., 1972). Several models have been proposed to account for the electron microscopic appearance of the core, the most favoured of which is that proposed by Furlong and coworkers (1972) in which the viral DNA, in a 4-5nm thick nucleoprotein filament, is wound around the cylindrical structure.

The classification of the 80 or so herpes viruses comprising the family Herpesviridae into the Subfamilies Alpha-, Beta-, and Gamma-herpesvirinae, was conducted by the Herpes Study Group appointed by the International Committee on Taxonomy of Viruses (Roizman et al., 1981), and is based upon the biological properties of the viruses, including duration of reproductive cycle, cytopathology, characteristics of latent infection and host range. A summary of the characteristics of the herpesvirus Subfamilies, and designation, to these Subfamilies, of the human herpesviruses, with which this thesis is mainly concerned, is shown in Table 1.1. Note that the newly isolated human B lymphotropic virus (human herpes virus 6) has not yet been unequivocally designated to a Subfamily, but recent evidence suggests that it is a member of the betaherpesvirinae.

1.1.2. Human Herpesviruses and Disease

Herpes simplex virus (HSV) primary infection, which may produce clinical or sub-clinical infection in children, is thought to be responsible for the presence of antibodies to HSV type 1 (HSV-1) in 90% of the adult population. A significant proportion of adults also have antibodies to HSV type 2 (HSV-2) and a correlation exists between increased sexual activity and the incidence of HSV-2 primary infection (Whitley, 1985). Both HSV-1 and HSV-2 are spread by direct

TABLE 1.1. CHARACTERISATION OF HERPESVIRUS SUBFAMILIES: DESIGNATION OF HUMAN HERPESVIRUSES TO SUBFAMILIES

SUBFAMILY 1 ALPHAHERPESVIRINAE

Relatively short reproductive cycle; *in vitro* rapid spread of infection.
Cytolytic - destruction of susceptible cells.
Latent infections established primarily in ganglia.

Examples: Herpes simplex virus type 1 (human herpes virus 1)
Herpes simplex virus type 2 (human herpes virus 2)
Varicella zoster virus (human herpes virus 3)

SUBFAMILY 2 BETAHERPESVIRINAE

Restricted host range.
Relatively short reproductive cycle; *in vitro* slowly progressing infection.
Cytomegalic - frequent enlargement of infected cells.
Latent infections established in secretory glands, lymphoreticular cells, kidneys and other tissues.

Example: Human Cytomegalovirus (human herpes virus 5)

SUBFAMILY 3 GAMMAHERPESVIRINAE

Host range restricted to family or order of natural host.
In vitro replication in lymphoblastoid cells; some cause lytic infections in epitheloid and fibroblastic cells.
Latent infection established frequently in lymphoid tissue.

Example: Epstein-Barr Virus (human herpes virus 4)

contact. Following primary infection HSV can establish a latent infection in the neurons of sensory ganglia or brain tissue (Baringer and Swoveland, 1973; Fraser et al., 1981). Reactivation may result in asymptomatic infection or in periodic muco-cutaneous infections; in general, HSV-1 infection is associated with cold sore lesions of the lips (herpes labialis) and the eyes (herpes keratitis), while HSV-2 is more usually associated with genital lesions (herpes genitalis) (Whitley, 1985). The possibility of a causal relationship between HSV-2 and cervical neoplasia stemmed from epidemiological studies, which suggested a higher prevalence of HSV-2 antibodies among women suffering from cervical neoplasia than in matched controls, and, indirectly, from the demonstration of oncogenic potential of HSV for rodents. However, the serological results of a prospective study carried out by Vonka and associates (1986a) on more than 10,000 women aged 25 to 45 years do not lend support to the hypothesis that HSV-2 is involved in the pathogenesis of cervical neoplasia. HSV has, rarely, been associated with serious generalised infections in newborns or meningitis, myelitis or a fatal non-epidemic encephalitis of adults (Whitley, 1985).

A primary infection with varicella zoster virus (VZV) usually results in the childhood disease known as chicken pox, which is a generalised infection at the skin surface; the disease can be spread by the respiratory route and direct contact. VZV can become established in a latent state in dorsal root ganglia and the spinal cord during primary infection. Upon reactivation of latent VZV, the cutaneous disease herpes zoster (shingles), a disease primarily of adults, may be produced. In adults and in a small percentage of children, VZV infection may cause herpes zoster encephalitis or meningitis (Kennedy, 1987).

Human cytomegalovirus (HCMV) infection normally results in a benign asymptomatic disease, although congenital HCMV infections may result in mental retardation. Infectious virus can be reactivated from transfused blood or transplanted organs in recipient immunosuppressed patients in which, therefore, clinically apparent infections occur frequently. The consequences of active infection are

variable but generally include fever, pneumonia, mononucleosis and hepatitis, or combinations of these (Hamilton, 1982).

Epstein-Barr virus (EBV) has been detected in African Burkitts lymphoma (BL) and nasopharyngeal carcinoma (NPC) tissues, although its role in the production of these neoplasms has not been resolved. Although EBV infection is often asymptomatic, the virus is the causative agent of infectious mononucleosis, otherwise known as glandular fever. EBV is able to establish a latent infection in the lymphocytes of patients with infectious mononucleosis, and reactivation may produce recurrences of the disease symptoms (Epstein and Achong, 1986; Dambaugh et al., 1986).

The human B-lymphotropic virus (HBLV), or human herpes virus 6, has been isolated from patients with lymphoproliferative diseases and infants with roseola infantum; some of these patients were also seropositive for human immunodeficiency virus type 1 (Salahuddin et al., 1986; Downing et al., 1987; Tedder et al., 1987; Yamanishi et al., 1988).

1.1.3. Human Herpesviruses and Cellular Transformation

Analysis of the transforming abilities of HSV and HCMV was initiated because of sero-epidemiological studies which suggested a link between these herpesviruses and cervical carcinomas. HSV in which the normal capacity to grow and kill infected host cells is eliminated can induce morphological transformation of rodent cells (Duff and Rapp, 1971). The viral DNA is successively lost on passage of transformed cells (Minson et al., 1976), suggesting a 'hit and run' mechanism for transformation (Skinner, 1976). Specific DNA fragments of HSV-1, HSV-2 and HCMV have been identified which have transforming ability but are not necessarily retained within the transformed cell: MTRI (morphological transformation region I) (XbaI f [0.29 to 0.45mu] and BglII i [0.311 to 0.415mu]) of HSV-1 DNA (Reyes et al., 1979); MTRII (BglII n [0.58 to 0.62mu]) of HSV-2 DNA (Reyes et al., 1979; Macnab and McDougall, 1980; Galloway and McDougall, 1981; Cameron et al., 1985); MTRIII (BglII c

[0.54 to 0.58mu]) of HSV-2 DNA (Peden et al., 1982; Jariwalla et al., 1983); and, HCMV MTR (HindIII E subfragment [0.123 to 0.14]) of HCMV DNA (Galloway et al., 1984; Nelson et al., 1984). The transforming regions of HSV-1, HSV-2 and HCMV DNA are not homologous. There is no evidence for the existence of an HSV-coded transforming protein (Cameron et al., 1985); specific subfragments from the transforming regions of HSV and HCMV are sufficient for transformation and yet too small to code for proteins (Galloway et al., 1984; Jones et al., 1986). The mechanism of transformation is likely to be complex; cis or trans activation or alteration of expression of cellular genes, oncogenes or endogenous retrovirus genes might occur via enhancement, mutagenesis or gene amplification (Sugden, 1986; Macnab, 1987). Interestingly, the transformed cells are oncogenic in the host animal and immunocompetent mice.

EBV infection, which has been associated with NPC and BL, immortalises infected B lymphocytes in vitro as it does in vivo. In contrast to the situation with HSV and HCMV, the immortalised cells contain the complete viral genome and multiple viral functions appear to be required for induction and maintenance of cell proliferation. 20 to 25% of the genome is required to initiate and maintain immortalisation (Mark and Sugden, 1982). Several virally encoded proteins, or candidates for virally encoded proteins, have been identified in transformed cells, at least 2 of which, Epstein-Barr virus nuclear antigen I (EBNAI) and EBNA2, have been assigned putative functions in immortalisation (Section 1.3.5.2.).

1.1.4. Genome Structures of Human Herpesviruses

Herpesviruses have also been classified on the basis of gross characteristics of the genome (Roizman et al., 1981). The structures of the double-stranded linear DNA genomes of HSV and other human herpesviruses are illustrated in Figure 1.1.

The HSV genome, which consists of double-stranded DNA containing random single strand breaks, has a molecular weight of about 100×10^6 (Becker et al., 1968; Kieff et al.,

Figure 1.1. **STRUCTURES OF THE GENOMES OF THE HUMAN HERPESVIRUSES**

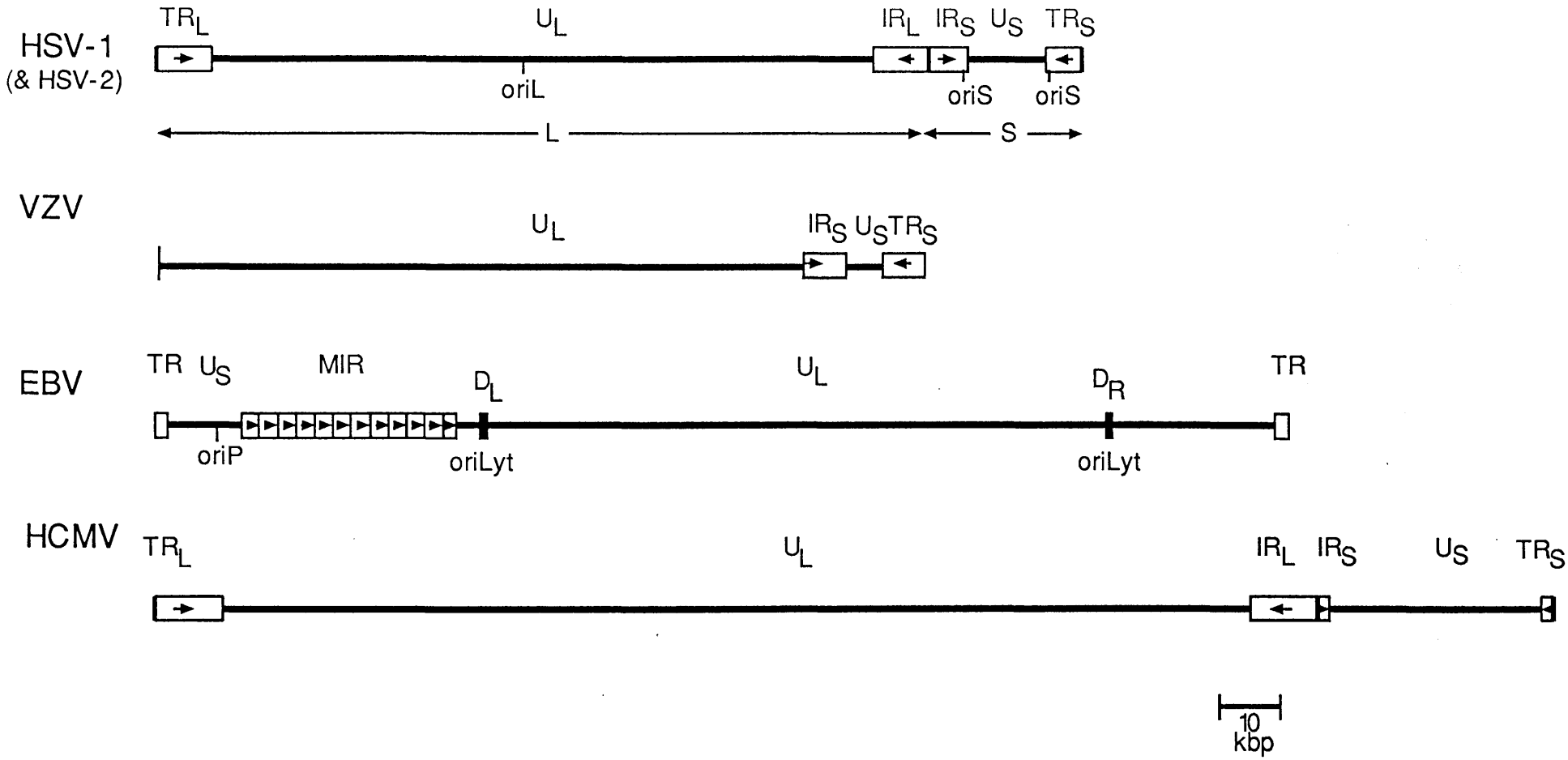
The genomes are represented as linear molecules. The bold lines represent unique sequences and the boxes represent repeat sequences, with arrows indicative of the relative sequence orientations. The genomes are described in Section 1.1.4.

ABBREVIATIONS:

Sequences; U_L - unique, long
U_S - unique, short
I/TR_L - internal/terminal repeat, long
I/TR_S - internal/terminal repeat, short
MIR - major internal repeat
D_L - direct repeat, left
D_R - direct repeat, right

ori - origin of DNA replication

This figure was adapted from McGeoch (1989), with the permission of D. McGeoch.

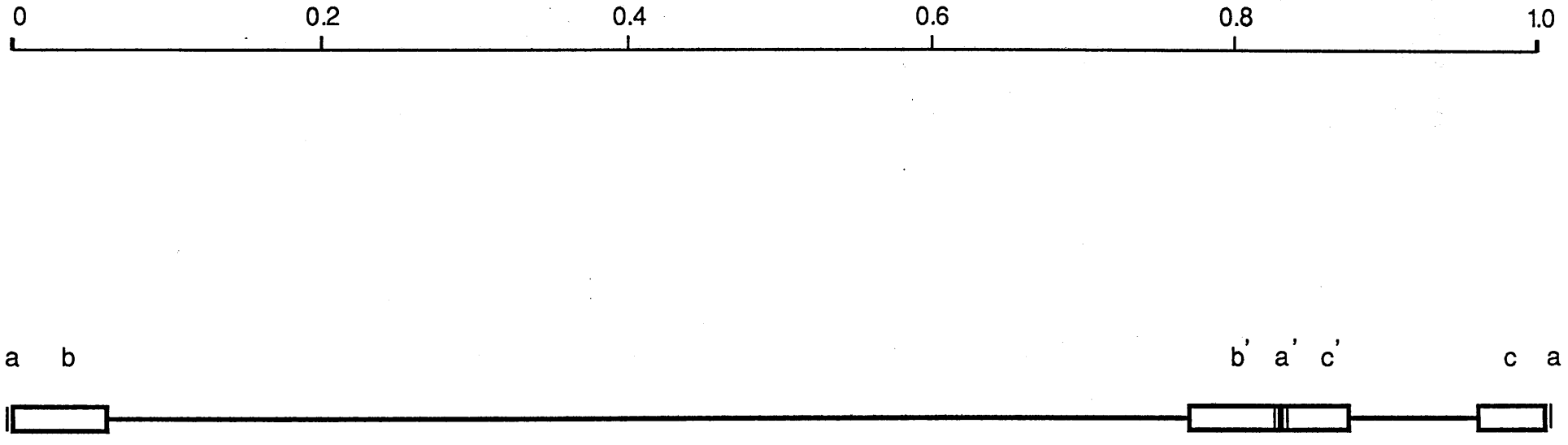

HSV-1
(& HSV-2)
TRL
UL
IRL
IRS
US
TRS
oriL
oriS
oriS
L
S
VZV
UL
IRS
US
TRS
EBV
TR
US
MIR
DL
UL
DR
TR
oriP
oriLyt
oriLyt
HCMV
TRL
UL
IRL
IRS
US
TRS
10
kbp

1971; Wilkie, 1973; Wilkie et al., 1974; Clements et al., 1976). HSV-1 (strain 17) DNA contains 152,260 base pairs (bp). The overall base composition of HSV-1 DNA is 68.3% G+C (McGeoch et al., 1988), whilst that of HSV-2 DNA is 69% G+C as determined by buoyant density centrifugation (Goodheart et al., 1968; Kieff et al., 1971). The HSV-1 genome consists of 2 covalently linked segments L (long) and S (short) each comprising a unique DNA sequence (U_L and U_S) bounded by inverted repeats (R_L and R_S) (Sheldrick and Berthelot, 1974). The inverted repeats of L are designated ab and a'b', each of approximately 9200bp (HSV-1), while those of S are designated a'c' and ac, and are each 6600bp (HSV-1) (Wadsworth et al., 1975; Perry and McGeoch, 1988; McGeoch et al., 1986; Figure 1.2.). The 'a' sequence, of 400bp in HSV-1 (17) and 250bp in HSV-2 (333) (Davison and Wilkie, 1981), is present as a direct repeat at the termini of the genome and, also, in inverted orientation at the L-S junction (Grafstrom et al., 1974, 1975; Sheldrick and Berthelot, 1974; Wadsworth et al., 1975, 1976). Several copies of this sequence may occur at the L terminus and at the L-S junction (Wagner and Summers, 1978). The L and S segments invert relative to each other about the mutual joint region during infection. Consequently, there are 4 types of HSV DNA molecule, present in equimolar amounts in populations of virion DNA (Sheldrick and Berthelot, 1974; Hayward et al., 1975; Clements et al., 1976; Delius and Clements, 1976; Wilkie and Cortini, 1976; Skare and Summers, 1977; Roizman, 1979), which appear to be functionally equivalent (Davison and Wilkie, 1983a; Longnecker and Roizman, 1986).

The HCMV genome, of M_R 150-155x10^6, consists of some 235,000bp (Geelen et al., 1978; Lakeman and Osborn, 1979) with a base composition of 57-58% G+C (Crawford and Lee, 1964; Plummer et al., 1969). The gross structure of the HCMV genome is similar to that of HSV, with 2 unique regions (U_L and U_S) flanked by 2 pairs of dissimilar inverted repeats (R_L and R_S). Furthermore, an 800bp sequence similar to the HSV 'a' sequence is present at the L-S joint and in variable copy number at the S segment termini (LaFemina and Hayward, 1980; Tamashiro et al, 1984; Spaete and Mocarski, 1985), and 4 isomers of the HCMV genome are present in equimolar

Figure 1.2. **THE RELATIVE POSITIONS OF THE a, b and c SEQUENCES IN THE LONG AND SHORT REGIONS OF THE HSV GENOME.**

The positions of a, b and c sequences in the genome are shown. Note that a', b' and c' sequences are equivalent to a, b and c sequences, respectively, in inverted orientation. One or more copies of the a sequences are present at the termini of the L and S components of the HSV genome (Figure 1.1.).


0
0.2
0.4
0.6
0.8
1.0
a
b
b'
a'
c'
c
a

amounts in virion populations (Kilpatrick and Huang, 1977; LaFemina and Hayward, 1980).

The genome of VZV, of M_R 80x10^6 (Dumas et al., 1980; Ecker and Hyman, 1982), is composed of 124,884bp (Davison and Scott, 1986) of overall base composition 46% G+C (Ludwig et al., 1972; Davison and Scott, 1986). Its gross structure is similar to that of HSV and HCMV, except that the inverted repeat sequences flanking U_L are only 88.5bp (compared to 9200bp in HSV-1), there is no proper terminal redundancy, and only 5% of VZV virion DNA contains L in inverted orientation (Davison, 1984), the remainder consisting of equimolar amounts of 2 isomeric genome arrangements which differ only with respect to the relative orientation of S (Dumas et al., 1981; Ecker and Hyman, 1982).

EBV, which has a genome of M_R 115x10^6 (Given and Kieff, 1978) and of 172,282bp with 60% G+C content (strain B95-8; Baer et al., 1984), has a different genomic organisation, consisting of a short and a long unique region (U_S and U_L), which are maintained in a unique orientation relative to each other (Given and Kieff, 1978; Skare and Strominger, 1980), and, which are divided by up to 12 direct sequence repeats of about 3000bp (MIR) (Rymo and Forsbloom, 1978; Given and Kieff, 1979; Hayward et al., 1980). The EBV genome is terminally redundant with up to 5 copies of a 550bp sequence, randomly distributed across both termini in linear virion DNA (Given et al., 1979; Kintner and Sugden, 1979).

The genomes of HSV, HCMV, VZV and EBV are alike in possessing families of multiple copies of short, directly repeated sequences. Each group of repeats has a distinct sequence, and the repeats vary in copy number and size, for example, from the 12bp direct repeat which occurs 18 times within the HSV-1 (17) 'a' sequence (Davison and Wilkie, 1981), to the 125bp Not I repeats in EBV (Jones and Griffin, 1983). In HSV, short direct repeats, which have been observed in U_S, R_S and R_L, are generally GC rich, with an asymmetry of purine versus pyrimidine content of the strands, and a tendancy towards strings of G on 1 strand (McGeoch et al., 1985; Davison and Wilkie, 1981: Rixon et al., 1984; Perry and McGeoch, 1988).

Complete nucleotide sequences are known for HSV-1 (McGeoch et al., 1985; McGeoch et al., 1986; McGeoch et al., 1988, Perry and McGeoch, 1988), EBV (Baer et al., 1984) and VZV (Davison and Scott, 1986). The sequence of the S segment and parts of the L segment unique and repeat regions of HCMV have been reported (Weston and Barrell, 1986; Kouzarides et al., 1987), and most of the U_S region of the HSV-2 genome has also been sequenced (McGeoch et al., 1987).

The genome of HSV-1 is predicted to possess 72 genes, densely arranged and encoding 70 distinct proteins. There are 12 genes in U_S and 56 in U_L, while R_S and R_L each contain 1 well-characterised gene encoding the transcriptional control proteins IE Vmw175 and IE Vmw110 respectively, as shown in Figure 1.3. (Easton and Clements, 1980; McGeoch et al., 1986; McGeoch et al., 1988; McGeoch, 1989). The genomes of HSV-1 and HSV-2 share extensive sequence homology in approximately 50% of sequences, as shown by DNA hybridisation analysis (Kieff et al., 1972) and electron microscope studies (Kudler et al., 1983) and are very similar in physical structure, although U_S of HSV-2 is 1500-3000bp longer than that of HSV-1 (Davison, 1981). The least homologous regions include the repeat sequences of L and most of S (Wilkie et al., 1979; Peden et al., 1982). Evidence that the genomes are collinear comes from experiments utilising intertypic recombinants in which certain functions such as thymidine kinase (Halliburton et al., 1980), DNA polymerase (Chartrand et al., 1979) and the IE genes (Easton and Clements, 1980) were shown to map in equivalent locations in HSV-1 and HSV-2, and, from molecular hybridisation analysis which identified regions of homology in the HSV-1 and HSV-2 genomes (Davison and Wilkie, 1983b), as well as from sequence data (McGeoch et al., 1987).

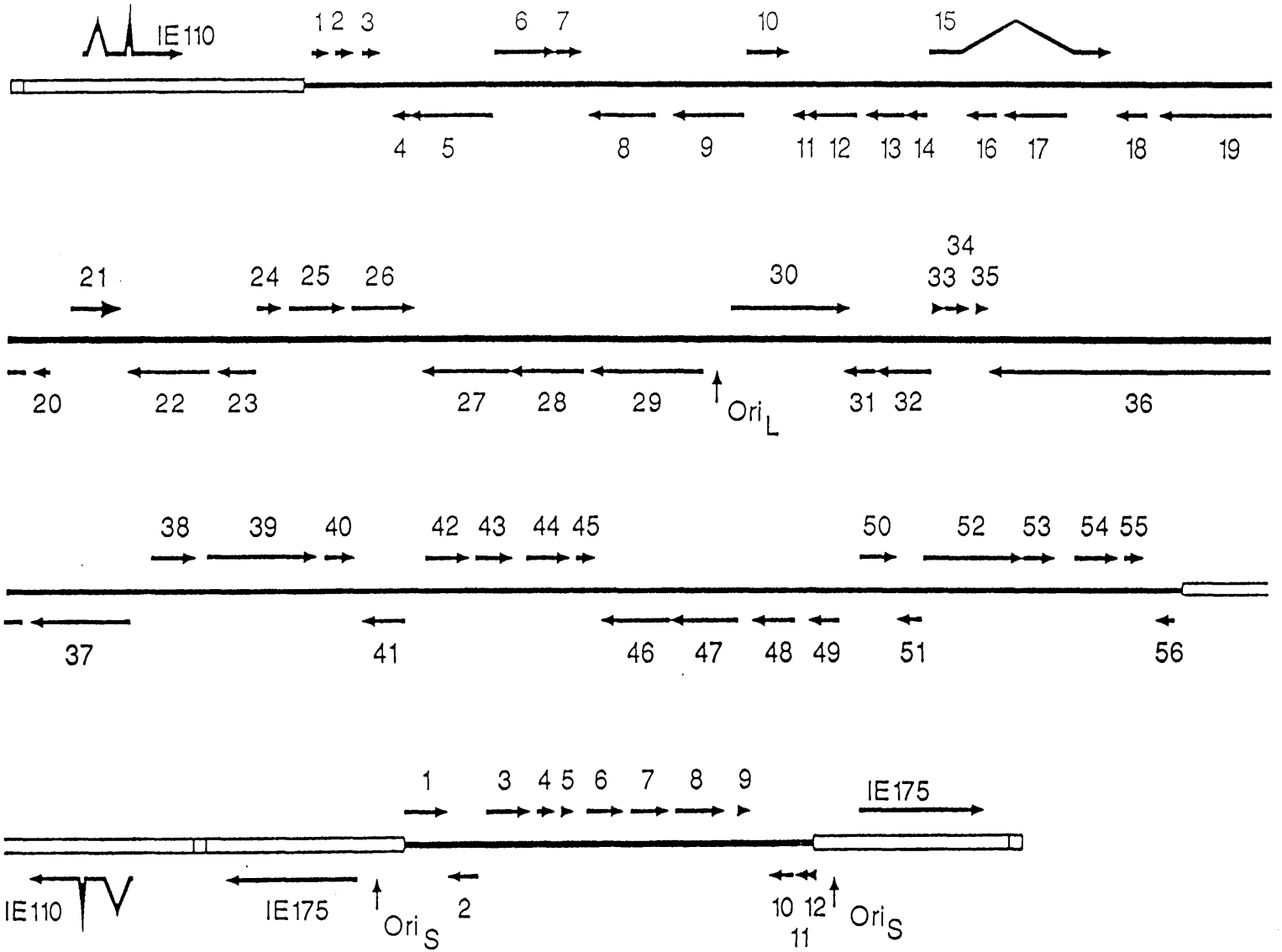
The other human herpesviruses show only 5% homology to each other at the DNA level (Huang and Pagano, 1974). However, regions of homology, as reflected in the sequence of the proteins encoded and the relative organisation of the genes, have been identified for HSV-1, VZV, EBV and HCMV. Homology of amino acid sequences, predicted by computer analysis of the nucleotide sequence (Taylor, 1986), occurs to varying degrees within the L segments of all these

Figure 1.3. THE LOCATION OF GENES IN THE HSV-1 GENOME

The locations of reading frames (shown by arrows) in the HSV-1 genome (represented on 4 lines, 40kbp per line), and reproduced from McGeoch (1989) with the kind permission of the author. Reports concerning the functions of the HSV-1 genes are referenced in McGeoch et al. (1988a) McGeoch (1989) and in relevant sections of the thesis.

IE110/IE-1 - IE transcriptional regulator, Vmw110; UL2 - Uracil-DNA glycosylase; UL3,4 - Unknown; UL5 - DNA helicase component, DNA replication; UL6 - Presumed virion component; UL7 - Unknown; UL8 - DNA replication, function unknown; UL9 - DNA replication, ori-binding protein; UL10 - Unknown, possible membrane-inserted protein; UL11 - Unknown; UL12 - Deoxyribonuclease; UL13 - Predicted protein kinase; UL14,15,16,17,18 - Unknown; UL19 - Major capsid protein (ICP5, VP5); UL20, 21 - Unknown; UL22 - Glycoprotein H; UL23 - TK; UL24 - Unknown; UL25 - Virion protein; UL26 - DNA packaging; UL27 - Glycoprotein B; UL28 - Probably structural; UL29 - ssDNA binding protein required for replication (DBP); UL30 - Replicative DNA polymerase; UL31 - Unknown; UL32 - Probably structural UL33 - Structural, packaging; UL34 - Possible virion protein; UL35 - Unknown; UL36 - Very large tegument protein; UL37 - Unknown; UL38 - Capsid assembly; UL39 - RR large subunit; UL40 - RR small subunit; UL41 - Presumed virion protein; UL42 - DNA binding protein, replication; ① UL44 - Glycoprotein C; UL45,46,47 - Unknown; UL48 - Major tegument protein, transactivates IE genes; UL49 - Unknown; UL50 - Deoxyuridine triphosphate; UL51 - Unknown; UL52 - DNA replication, function unknown; UL53 - Possible membrane protein; UL54/IE-2 - IE transcriptional regulatory protein, Vmw63; ② IE175/IE-3 - IE transcriptional regulatory protein, Vmw175; US1/IE4 - IE protein Vmw68, function unknown; US2 - Unknown; US3 - Protein kinase; US4 - Glycoprotein G; US5 - Unknown, possible glycoprotein; US6 - Glycoprotein D; US7 - Glycoprotein I; US8 - Glycoprotein E; US9 - Virion protein; US10 - Virion protein; US11 - Unknown: US12/IE-5 - IE protein, Vmw12, unknown.

① U_L43 - unknown.
② $U_L55,56$ - unknown.


IE110
1 2 3
6 7
10
15
4 5
8
9
11 12
13 14
16
17
18
19
21
24 25 26
30
34
33 35
20
22
23
27
28
29
Ori L
31 32
36
38
39
40
42 43 44 45
50
52
53
54 55
37
41
46
47
48
49
51
56
1
3 4 5 6 7 8 9
IE175
IE110
IE175
Ori S
2
10 12
11
Ori S

genomes. There are no similarities in the predicted coding capacity and organisations of the U_S regions of VZV, HCMV and EBV, HSV-1 and HCMV, or, HSV-1 and EBV (Baer et al., 1984; Weston and Barrell, 1986; Davison and Scott, 1986; McGeoch et al., 1986; Davison and McGeoch, 1986; Davison and Taylor, 1987; McGeoch et al., 1988). However, despite large differences in % G+C content, the genomes of HSV-1 and VZV, which are both alphaherpesviruses, contain homologies in both S and L. There is considerable reorganisation of the genes in the S segment relative to each other in HSV-1 and VZV, while the gene arrangement in VZV L corresponds well to that of HSV-1 L from the HSV-1 isomer in which the long region is inverted compared to the accepted prototype isomer (Davison and Scott, 1986; McGeoch et al., 1986; Davison and McGeoch, 1986; McGeoch et al., 1988).

1.2. HERPES SIMPLEX VIRUS - THE LYTIC CYCLE

1.2.1. Initial Stages of Infection

HSV infection is initiated upon adsorption of the virus to the host cell and penetration of the virions through the cell membrane. Adsorption to the host cell is presumably brought about by an interaction between cellular receptors and viral glycoproteins exposed at the virion surface, and there is evidence to suggest the involvement of glycoproteins gB, gC, gD and gE in this event (Para et al., 1982; Johnson et al., 1984; Fuller and Spear, 1985). Penetration occurs predominantly by fusion of the viral envelope with the plasma membrane (Morgan et al., 1968; Para et al., 1980) and experiments utilising HSV temperature sensitive (ts) mutants tsB5 and tsJ12, which fail to process gB to its mature forms at the non-permissive temperature (NPT), have implicated this glycoprotein in the membrane fusion process (Sarmiento et al., 1979; Little et al., 1981; DeLuca et al., 1982). The occurrence of penetration defective mutants with ts lesions in a region of the genome outwith gB coding sequences suggests the involvement of other viral polypeptides in penetration (Addison et al., 1984). Internalised capsids are transported to the nuclear pores. Release of the HSV genome from the capsid into the nucleus requires a viral function but not de novo RNA and protein synthesis, suggesting involvement of viral structural component(s) and/or host cell factors (Hochberg and Becker, 1968). Characterisation of a ts mutant defective in release of viral DNA indicated a role for a virion component in the release of HSV DNA (Batterson et al., 1983).

1.2.2. Effect on Host Macromolecular Synthesis

Infection of cells with HSV has an inhibitory effect on the synthesis of cellular DNA, RNA and protein (Fenwick, 1984) which is generally more pronounced in cells infected with HSV-2 than with HSV-1 (Powell and Courtney, 1975; Pereira et al., 1977; Fenwick et al., 1979; Schek and Bachenheimer,

1985), although HSV-2 strain HG52 is an exception (Marsden et al., 1978). Protein synthesis inhibition is mediated by at least two factors; a virion-associated component, which is non-essential for growth of virus in tissue culture, and a protein synthesised later in infection, which can function in the absence of a functional virion component and is essential for the full inhibitory effect on host protein synthesis (Honess and Roizman, 1975; Fenwick and Clark, 1982; Read and Frenkel, 1983). Disaggregation of polyribosomes, degradation of cellular mRNA and inhibition of RNA synthesis accompany the suppression of cellular protein synthesis (Fenwick and McMenamin, 1984) and are also mediated by a component of the virion, although protein synthesis is also required for host mRNA degradation in some cell types (Fenwick and Clark, 1982; Nishioka and Silverstein, 1977, 1978; Schek and Bachenheimer, 1985). The virion-associated inhibition of host protein synthesis is clearly non-essential for virus replication since viable mutants with mutations affecting host shut-off have been isolated (Read and Frenkel, 1983); the mutation of one mutant mapped to a 265bp fragment within a region of the genome including gene UL41. It is not yet clear whether the UL41 gene is involved directly in host shut-off (Kwong et al., 1988) or is involved in mRNA stabilisation (Fenwick and Owen, 1988).

In addition to the HSV-induced host shutoff effect, certain cellular genes are activated during HSV infection. Expression of some cellular genes is reported to be enhanced during normal HSV infection (LaThangue et al., 1984; MacNab et al., 1985; Patel et al., 1986; Kemp et al., 1986), while overexpression of an abnormal form of Vmw175 activates the expression of cellular stress genes (Notarianni and Preston, 1982; Russell et al., 1987). In this regard it is of interest that HSV immediate early gene products (Section 1.2.3.) have been shown to activate certain cellular promoters, such as the rabbit B globin gene promoter (Everett, 1984a, 1985).

1.2.3. Transcription and Gene Products

Transcription of HSV genes is directed by eukaryotic cellular RNA polymerase II (Alwine et al., 1974; Ben-Zeev and Becker, 1977) and other cellular factors, as well as viral factors. Therefore, HSV mRNAs have some features in common with eukaryotic cellular mRNAs and also with mRNAs of other nuclear-replicating DNA viruses (Wagner, 1984, 1985; McGeoch, 1986, 1988a): a cap at the 5' end; polyadenylation at the 3' end, signalled by AATAAA in the gene (Silverstein et al., 1976); and a leader sequence between the cap and the translation initiation site. Methylation of HSV mRNA, by a mechanism resembling that of the host cell, may also occur (Bartoski and Roizman, 1976).

In terms of the transcriptional organisation of the HSV genome, genes are found in both orientations on the genome, each with its own promoter (McGeoch et al., 1988a; McGeoch, 1989; Figure 1.3.). Gene overlap is a common feature, for example, RNA mapping data has identified families of transcripts with identical 3' ends (Rixon and McGeoch, 1984; Draper et al., 1986), and transcripts with overlapping 5' ends on opposite strands (Wilkie et al., 1980). Splicing, although relatively uncommon, occurs within some HSV transcripts, notably those of IEs Vmw110 (Perry et al., 1986) Vmw68 and Vmw12 (Watson et al., 1981; Rixon and Clements, 1982) and U_L15 (McGeoch et al., 1988a). Expression of the HSV genome during lytic infection occurs in a coordinate and sequential manner to produce three main temporal classes of transcripts, immediate early (IE), early (E) and late (L). There is no obvious clustering of genes along the genome on the basis of class (McGeoch et al., 1986; McGeoch et al., 1988a).

The viral genes transcribed initially in a lytic HSV infection, designated IE or alpha (α) genes, have been defined as those genes expressed in the absence of prior (de novo) viral protein synthesis (Kozak and Roizman, 1974; Clements et al., 1977; Jones and Roizman, 1979). Although under such conditions the IE mRNAs accumulate in relatively large amounts, they are normally expressed only transiently in a lytic infection (Clements et al., 1977; Watson et al.,

1979; Anderson et al., 1980). Functional IE gene products are required for progression to the E and L phases of gene expression (Honess and Roizman, 1974, 1975; Preston, 1979a,b; Everett, 1984a; O'Hare and Hayward, 1985); L genes also require viral DNA synthesis for maximal expression (Swanstrom and Wagner, 1974; Powell et al., 1975; Jones and Roizman, 1979; Holland et al., 1980; Conley et al., 1981; Pederson et al., 1981). The absence of viral DNA synthesis affects two classes of HSV genes, the E/L genes (also known as $\beta\gamma$ γ_1, and "leaky" late) and the "true" late genes (γ or γ_2) (Roizman and Batterson, 1985; Wagner, 1985; Harris-Hamilton and Bachenheimer, 1985). Although viral DNA synthesis is required for maximum expression of E/L genes, expression is detected early in infection and occurs at moderately high levels in the absence of DNA synthesis (Gibson and Spear, 1983; Johnson et al., 1986). In contrast, "true" late gene expression is severely reduced in the absence of viral DNA synthesis (Jones and Roizman, 1979: Holland et al., 1980; Hall et al., 1982), but is, nevertheless, detectable with sensitive assays (Godowski and Knipe, 1985; Johnson et al., 1986). HSV gene expression is generally controlled at the level of transcription initiation, and certain HSV gene products, notably the IE polypeptides, are involved in transactivation, repression and autoregulation of transcription (Everett, 1987a).

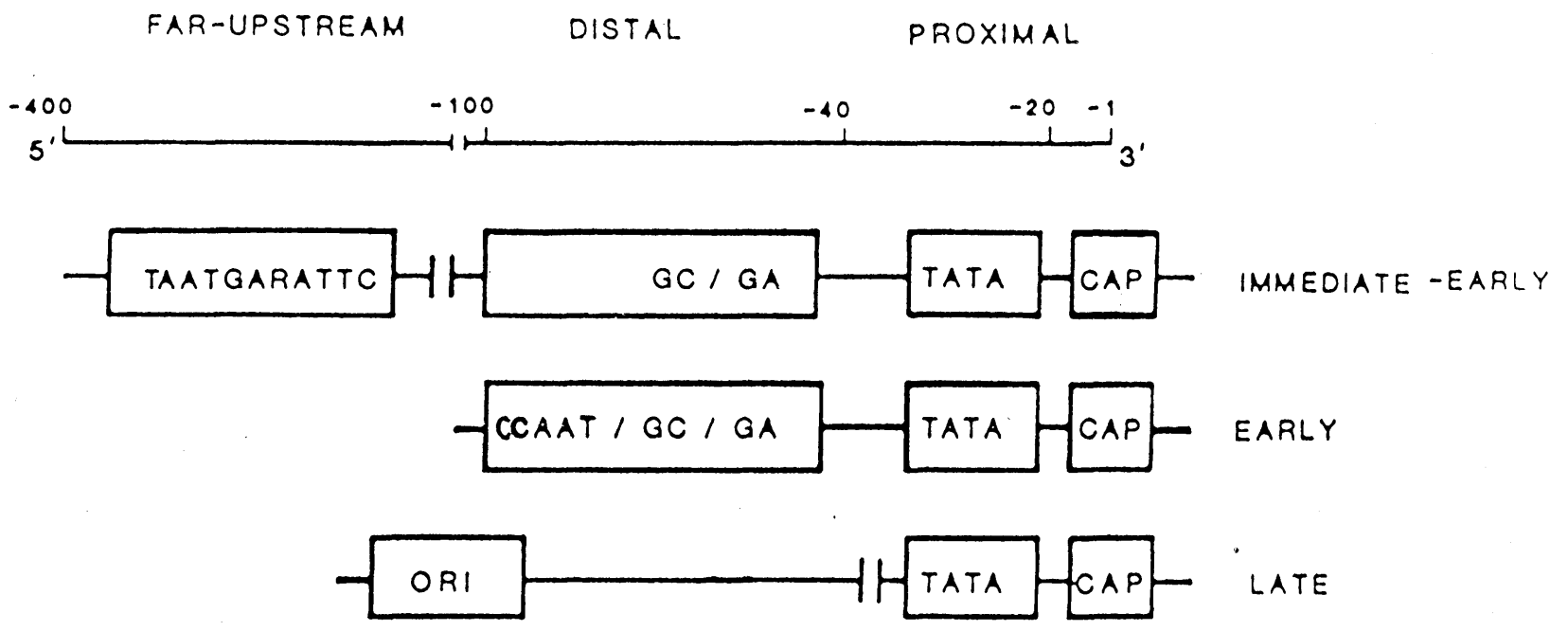
The control of transcription of the HSV IE, E and L genes, the promoter elements required for efficient expression of genes in IE, E (including E/L) and L kinetic classes, diagrammed in Figure 1.4., and the functions of HSV gene products, are considered in the following sections.

1.2.3.1. Regulation of Immediate Early Gene Expression

There are 5 major IE genes of HSV-1 designated IE-1 to IE-5, or, α0, α27, α4, α22 and α47. The IE genes code for polypeptides Vmw110, Vmw63, Vmw175, Vmw68 and Vmw12, named on the basis of apparent molecular weight as estimated by SDS-PAGE, and also known as ICP0, ICP27, ICP4, ICP22 and ICP47, respectively. The IE polypeptides were initially mapped by analyses of virus genomes and polypeptides

Figure 1.4. **DNA SEQUENCE REQUIREMENTS FOR IE, E AND L PROMOTER ACTIVITY.**

IE promoter regions contain a capsite, a TATA box, distal promoter elements, including GC- and GA- rich motifs, and a far-upstream element required for activation by the virion component, Vmw65, which includes the consensus sequence TAATGARATTC. E promoters include only the distal and proximal promoter sequences. Late promoters may include only a TATA box and capsite region on a replicating template. This figure was reproduced from Johnson and Everett (1986) with the kind permission of Dr. R.D. Everett.


FAR-UPSTREAM
DISTAL
PROXIMAL
-400
-100
-40
-20
-1
5'
3'
TAATGARATTC
GC / GA
TATA
CAP
IMMEDIATE -EARLY
CCAAT / GC / GA
TATA
CAP
EARLY
ORI
TATA
CAP
LATE

expressed by HSV-1/HSV-2 intertypic recombinants (Preston et al., 1978). The locations of the genes for the IE polypeptides were confirmed by RNA mapping and in vitro translation of IE mRNAs (Watson et al., 1979) as well as by hybridisation studies which, in addition, established the transcriptional orientation of the IE genes (Clements et al., 1979; Mackem and Roizman, 1980). Four of the IE genes are associated with the repeat regions of the HSV genome, as illustrated in Figure 1.5. IE-1 and IE-3 are diploid due to their respective positions in the repeat regions flanking U_L and U_S. The coding sequences of IE-4 and IE-5 lie at either end of U_S, but the promoter and regulatory regions of these genes are identical and are located in R_S. IE-1, IE-4 and IE-5 yield primary transcripts which are spliced (Watson et al., 1981; Rixon and Clements, 1982; Perry et al., 1986) in contrast to IE-2 and IE-3 (Rixon et al., 1982; Whitton et al., 1983).

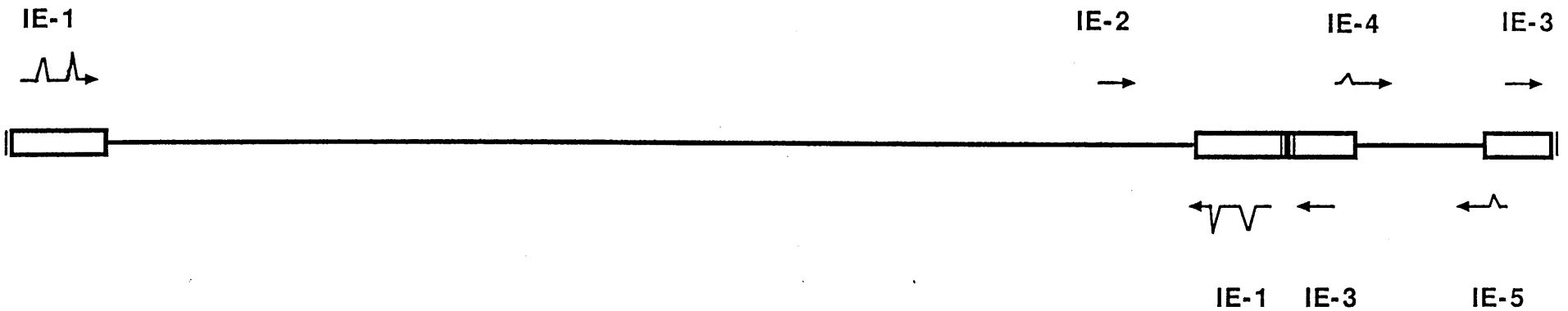
Functional transcription regulatory domains of the IE genes have been defined by determining the levels of expression from an indicator gene linked to sequences from the 5' non-transcribed and non-translated leader portions of the viral IE genes. Such hybrid plasmids have been recombined into HSV thymidine kinase minus (TK^-) genomes (Post et al., 1981), incorporated into eukaryotic cellular genomes (Post et al., 1981, 1982; Mackem and Roizman, 1982a,b,c; Kristie and Roizman, 1984; Mosca et al., 1985), microinjected into Xenopus oocytes (Cordingley et al., 1983), or transfected into eukaryotic cells (Cordingley et al., 1983; Lang et al., 1984; Preston et al., 1984; Preston and Tannahill, 1984; Kristie and Roizman, 1984). Indicator genes utilised, selected for the relative ease with which the gene products can be assayed as a measure of gene expression, include viral TK, chloramphenicol acetyl transferase and interferon. These investigations have demonstrated that the regions upstream of the IE mRNA cap sites can be dissected into at least 2 components, a promoter region and a far upstream region. The IE upstream promoter-regulatory components have been sequenced (Mackem and Roizman, 1982a,c; Murchie and McGeoch, 1982; Whitton et al., 1983; Whitton and Clements, 1984a) and multiple

Figure 1.5. **GENOMIC LOCATIONS OF THE HSV-1 IMMEDIATE EARLY TRANSCRIPTS.**

The relative locations and transcriptional orientations of the IE mRNAs are shown. References for mapping data are as follows:

IE-1 - Perry *et al.*, 1986.
IE-2 - Mackem and Roizman, 1982c.
IE-3 - Rixon *et al.*, 1982.
IE-4 and IE-5 - Rixon and Clements, 1982.

The spliced regions of the immediate early mRNAs are indicated.


IE-1
IE-2
IE-4
IE-3
IE-1
IE-3
IE-5

cis-acting elements have been identified which respond to a variety of cellular and trans-acting factors.

Promoter sequences present within 110 bases upstream of IE-3 and 69 bases upstream of IE-4/5 initiation sites, impart upon indicator genes the capacity to be expressed, but not to be regulated as an IE gene (Mackem and Roizman, 1982a,c; Cordingley et al., 1983). A TATA box is located 20-30 bases upstream of the mRNA initiation site (Mackem and Roizman, 1982c) analogous in position to the TATA homology ($\overset{T}{C}ATAT\overset{T}{A}AAG$) which is important in the efficiency and positioning of transcriptional initiation and is present 5' to many RNA polymerase II transcribed genes (Proudfoot, 1979; Benoist et al., 1980; Benoist and Chambon, 1981). In IE-3, for example, sequences -37 to -16, which are non-essential for promoter function in Xenopus oocytes but appear to be important for precise initiation of transcription, include at -29 to -22 the sequence TATATGAG (Cordingley et al., 1983).

The far upstream sequences of HSV-1 and HSV-2 IE genes, positioned between -331 and -110 in IE-3, impart upon the indicator gene plus promoter sequences the capacity to be expressed at higher than the basal levels of expression produced by the promoter alone and the ability to be regulated as an IE gene (Post et al., 1981; Mackem and Roizman, 1982a,c; Cordingley et al., 1983); both effects act at the transcriptional level, in an orientation-independent manner and at distances of up to 1000 bases (Lang et al., 1984; Preston and Tannahill, 1984). IE far upstream regions also contain elements which respond to negative regulation by IE polypeptide Vmw175 as discussed below in the context of IE protein functions.

Positive regulation of IE gene expression is mediated in trans by a virion structural component, a conclusion based on observations that expression from IE promoter-regulatory regions, but not from E gene upstream regions, could be augmented by infection with an HSV mutant which could penetrate cells but was incapable of expressing IE genes (Post et al., 1981; Mackem and Roizman, 1982a; Batterson and Roizman, 1983; Batterson et al., 1983; Cordingley et al., 1983; Preston and Tannahill, 1984;

Kristie and Roizman, 1984). Identification of the gene encoding the HSV-1 virion trans-inducing factor (TIF) was achieved by analysis of TK activity in cells co-transfected with TK genes linked to IE-3 promoter-regulatory regions along with specific HSV sequences which together represented the entire HSV genome (Campbell et al., 1984). The gene, HSV-1 UL48, located in U_L of the HSV genome, has been sequenced (Dalrymple et al., 1985; Pellett et al., 1985) and encodes a phosphorylated protein of apparent M_R of 65,000 (by SDS-PAGE), identified by Campbell and coworkers (1984) and termed Vmw65 (also known as VP16), which is a major structural component of the virion tegument (Marsden et al., 1978; Lemaster and Roizman, 1980) expressed late in the HSV lytic cycle (Marsden et al., 1976; Hall et al., 1982). In vitro mutagenesis studies have identified regions of Vmw65 which appear to be involved in trans-induction (Triezenberg et al., 1988b; Ace et al., 1988) and which, incidentally, are distinct from those regions of the protein essential for virus assembly (Ace et al., 1988). The importance of Vmw65 activity in the HSV lytic cycle has recently been demonstrated by construction and analysis of an HSV-1 mutant defective in Vmw65-mediated stimulation of IE gene expression. The mutant was found to have a particle to pfu ratio at least 100-fold higher than that of wtHSV-1 in baby hamster kidney cells and up to 10^5-fold higher in human foetal lung cells (Ace et al., 1989).

IE far-upstream regulatory domains contain at least 1 and frequently multiple copies of an AT-rich element of consensus TAATGARATTC (R=purine) (Mackem and Roizman, 1982a,c; Cordingley et al., 1983; Whitton and Clements, 1984a; Preston et al., 1984; Kristie and Roizman, 1984; Figure 1.4.) which comprises at least part of a cis-site for the Vmw65-mediated induction of IE genes (Campbell et al., 1984; Gaffney et al., 1985; Bzik and Preston, 1986). Functional analyses of deletion mutations in IE far upstream regions suggest that a single TAATGARAT consensus signal located at position -115 to -106 (TAATGGAAT) in IE-3 can respond efficiently to Vmw65 induction (O'Hare and Hayward, 1987), yet, similar studies suggest a requirement for additional sequence elements in the IE far upstream regions

to maximise Vmw65-mediated induction at a TAATGARATTC motif located at -265 to -256 (Preston et al., 1984; Kristie and Roizman, 1984; Bzik and Preston, 1986). Far upstream elements notable for enhancer-like activity (Mackem and Roizman, 1982a; Lang et al., 1984), distinct from homologues of the TAATGARATTC consensus, include multiple GC-rich and GA-rich elements (Figure 1.4.). The regulatory region of IE-3 contains six copies of the GC-rich sequence 5'GGGCGG3', which is a binding site for the eukaryotic cellular transcription factor Spl (Jones and Tjian, 1985; Briggs et al., 1986). Two of the Spl binding motifs are required for basal levels of expression from the IE-3 regulatory region and others may be involved in facilitating the response to Vmw65 (Post et al., 1981; Jones et al., 1985; McKnight and Tjian, 1986; Triezenberg et al., 1988a; Dynan and Tjian, 1983). The GA-rich elements, generally found in close proximity to at least one TAATGARATTC consensus, have been implicated in IE gene regulation (Kristie and Roizman, 1984) and shown to increase TAATGARATTC-mediated responsiveness to Vmw65 (Bzik and Preston, 1986; Triezenberg et al., 1988a).

Despite the sequence specificity of Vmw65 action, the protein has no substantial affinity either for DNA containing the TAATGARATTC motif or non-specifically for ds DNA (Marsden et al., 1987). Various studies have shown that Vmw65 forms a complex (IEC) with at least 2 cellular proteins and the TAATGARATTC domain (McKnight et al., 1987; Preston et al., 1988; O'Hare and Goding, 1988; Gerster and Roeder, 1988) and that formation of the complex correlates directly with Vmw65-mediated stimulation of IE gene expression (O'Hare and Goding, 1988; Ace et al., 1988). An affinity purified transcription factor OTF-1 not only binds to the TAATGARATTC domain, but is required in the formation of IEC (Gerster and Roeder, 1988). OTF-1, which is functionally equivalent to transcription factor NFIII, occurs in a wide variety of different cell types and is involved in the regulation of RNA polymerase II-transcribed genes, RNA polymerase III gene-specific transcription (Rosales et al., 1987; Sollner-Webb, 1988) and adenovirus DNA replication (Prujin et al., 1986). OTF-1 interacts with the conserved octamer sequence motif ATGCAAAT and stimulates

in vitro transcription via such interaction (Fletcher *et al.*, 1987; Scheidereit *et al.*, 1987). A homologue of the octamer motif overlaps the 5' end of many TAATGARATTC elements of IE genes (O'Hare and Goding, 1988; Prujin *et al.*, 1986). Gerster and Roeder (1988) have recently reported that both the octamer sequence (ATGCTAAT) and the GATAT moiety of the IE-1 gene element ATGCTAATGATAT are required for the formation of IEC, and, Preston and coworkers (1988) have demonstrated that the sequence TAATGAGAT is sufficient for complex formation. Complementation studies, in which purified OTF-1 was added to nuclear extracts depleted of endogenous OTF-1, showed that at least one cellular factor in addition to OTF-1 and Vmw65 is required for the efficient formation of IEC (Gerster and Roeder, 1988).

On the basis of these findings and on the evidence that Vmw65 possesses an acidic carboxy terminus (Dalrymple *et al.*, 1985) required for activation of transcription from the IE-3 gene promoter (Triezenberg *et al.*, 1988), Gerster and Roeder (1988) have proposed the possibility that the role of OTF-1 and the other cellular factor(s) involved is to position the activation domain of Vmw65 on the herpes IE promoters. Furthermore, these authors suggest that the requirement for the GARAT motif as well as the octamer element in IEC formation may ensure that only the viral IE and not all other cellular octamer-containing genes are responsive to *trans*-activation by Vmw65.

1.2.3.2. Properties and Functions of Immediate Early Gene Products

Since protein synthesis inhibitor treatment of HSV-1 infected cells at the time of infection results in transcription of a limited set of HSV genes (the IE genes) and the overproduction of IE mRNAs, but no E or L gene transcription, it was postulated that the IE mRNA translation products had important functions in the early stages of the viral lytic infection. The functions of the IE proteins have been investigated utilising mutant viruses with ts mutations, insertions or deletions in the IE genes and utilising cloned IE genes.

Vmw175

The IE-3 gene product, Vmw175, is post-translationally modified by phosphorylation and poly(ADP-ribosyl)ation, can exist in 3 forms distinguishable by differences in electrophoretic mobility, and accumulates in the nucleus (Pereira et al., 1977; Preston and Notarianni, 1983). A mutant, tsK, has been isolated which contains a mutation in IE-3 such that the gene product possesses a ts lesion (Preston, 1979a; Preston, 1981). At the NPT Vmw175 fails to be processed normally to the forms of lower electrophoretic mobility and migration to the nucleus is impaired. These properties, which are reversible upon downshift of tsK-infected cells (Preston, 1979b), are correlated with the production of a restricted polypeptide profile of tsK at the NPT in which only the IE polypeptides, including the aberrant Vmw175, and Vmw136 are produced (Marsden et al., 1976; Preston, 1979b). Functional Vmw175 is, therefore, essential for the transition from the IE to the E and L phases of transcription and, since temperature upshift of tsK-infected cells reverses the migration characteristics and processing of Vmw175 and arrests the normal pattern of viral polypeptide and mRNA synthesis, the functional polypeptide is probably required for most of the viral lytic cycle and not just transiently (Preston, 1979b; Watson and Clements, 1980). Other mutants containing ts lesions in Vmw175 (tsD, tsT) possess phenotypes comparable to tsK at the NPT in that the RNA species produced are very similar (Watson and Clements, 1978), IE polypeptides are overproduced and Vmw175 shows increased electrophoretic mobility and impaired migration to the nucleus. Polypeptides detected in tsD and tsT infected cells at the NPT include limited amounts of some non-IE gene products as well as the IE proteins (Preston, 1979b). An insertion mutant, in1411, has an 8bp oligonucleotide linker insertion in IE-3, which also creates an in-frame amber termination codon in the Vmw175 coding sequences, and therefore does not produce IE polypeptide Vmw175. in1411, as well as HSV mutants containing deletions in IE-3, are not viable under non-permissive conditions, fail to induce the synthesis of early and late proteins and to autoregulate

immediate-early protein synthesis, and are therefore phenotypically similar to tsK (N.D. Stow and E.C. Stow, personal communication; Russell et al., 1987).

The essential role of Vmw175 in promoter activation during the transition from IE to E and L gene expression has also been demonstrated in transfection experiments. Transcription from E gene (gD/TK) promoters was detected following infection with HSV-1; trans-activation of E gene promoters was not induced by HSV-1 infection in the presence of the protein synthesis inhibitor cycloheximide, or, by infection with tsK at the NPT (Everett, 1983; El Kareh et al., 1985). In short term transfection assays, in which genes containing E regulatory regions were cotransfected into cells with plasmids containing IE genes, Vmw175 was shown to be capable of E promoter trans-activation; the level of promoter activation varied according to the assay system used (Everett, 1984a, 1986; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986). An enhanced level of transcription from E promoters was observed in the additional presence of Vmw110 (DeLuca and Schaffer, 1985; Everett, 1986; O'Hare and Hayward, 1985a). The E gene sequence elements required for trans-activation by Vmw175 are discussed in Section 1.2.3.3..

The role of Vmw175 in autoregulation or repression of IE genes has been studied by assay of the level of expression from genes under IE promoter control cotransfected into cells with plasmids expressing Vmw175. It was shown that both constitutive and induced levels of transcription from the IE-3 promoter could be repressed by Vmw175 (O'Hare and Hayward, 1985b), and that, whereas low doses of Vmw175 stimulated transcription from IE-1, IE-3 and IE-4/5 promoters, increasing doses of Vmw175 resulted in reduction in transcription from these IE promoters (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986). Parenthetically, there is also evidence for an involvement of later gene products in autoregulation or repression of IE gene transcription (Dixon and Schaffer, 1980; Godowski and Knipe, 1986).

The various roles of Vmw175, in the transactivation of E and L gene transcription, in autoregulation and in the

repression of IE gene expression, could be mediated by the formation of specific Vmw175-DNA complexes. Several investigators have reported that incubation of radiolabelled DNA with HSV-infected cell extracts (Kristie and Roizman, 1986a,b; Muller, 1987) or partially purified preparations of Vmw175 (Beard et al., 1986; Faber and Wilcox, 1986, 1988; Michael et al., 1988) can result in the formation of stable protein-DNA complexes that contain Vmw175 and specific viral DNA sequences. Muller (1987) reported that Vmw175 binds specifically to a sequence at the transcriptional start site of IE-3; complex formation between Vmw175 and the start site sequence are not detected prior to 2h post-infection, which is around the time of repression of IE-3 transcription. A sequence in the same region of the IE-3 gene was previously reported to be involved in the response to autoregulation (O'Hare and Hayward, 1987). Therefore, formation of the complex is implicated in the autoregulation of IE genes (Muller, 1987). Vmw175, resolved from other proteins by SDS-PAGE, transferred to nitrocellulose and renatured, was able to bind to specific DNA fragments (Michael et al., 1988). This finding, together with results from recent investigations using DNA affinity chromatography to purify Vmw175 and DNase protection analysis, demonstrated that Vmw175 binds directly to specific DNA sequences in the absence of any other virus- or cellular-encoded proteins (Kattar-Cooley and Wilcox, 1989). The target sequences involved in the Vmw175-DNA complex formation associated with autoregulation and repression of IE gene expression and with stimulation of E gene expression, include the hexanucleotide sequence 5'-ATCGTC-3' (Faber and Wilcox, 1986).

The regions of the Vmw175 polypeptide important for the repression of the IE-3 promoter, and for transactivation of an HSV E gene promoter in synergy with HSV IE protein Vmw110, have been investigated by construction of small in-frame insertion and deletion mutants of a plasmid-borne copy of IE-3 and subsequent analysis of the activity of the resultant mutant polypeptides in transient transfection assays. Large stretches of the protein are apparently relatively unimportant for either function. The two regions most sensitive to disruption correlate to sequences

conserved between Vmw175 and VZV 140K, a transactivating protein of VZV with homology to HSV Vmw175 (Paterson and Everett, 1988).

Vmw110

HSV-1 IE-1 gene product Vmw110 is a predominantly nuclear, phosphorylated protein (Pereira et al., 1977; Hay and Hay, 1980; Ackermann et al., 1984). Virus mutants containing ts lesions in Vmw110 have not been isolated. Variants of HSV-1 and HSV-2 have been isolated which contain only one copy of the IE-1 gene (Poffenberger et al., 1983; Harland and Brown, 1985), and viruses containing deletions in TR_L and IR_L copies of the Vmw110 gene have been constructed (Stow and Stow, 1986; Sacks and Schaffer, 1987). HSV-1 mutant dl1403 contains a 2000bp deletion in both copies of IE-1 and specifies a truncated form of the polypeptide consisting of the original N-terminal 105 amino acids followed by a reading frame not used by Vmw110. The IE-1 gene product is not absolutely essential for virus growth in tissue culture, since dl1403 was able to replicate and produce plaques on BHK cells, but at low m.o.i. (for example, 0.01 pfu/cell) on BHK cells the effect of the deletion is a 20-100 fold reduction in virus yields compared to wt HSV-1; growth and plaquing efficiencies of dl1403 are reduced even further on HFL cells. The effect is largely overcome at higher virus doses and viral polypeptide synthesis, DNA replication, DNA encapsidation and production of viral particles do not differ significantly in dl1403 or wt HSV-1 infected cells; as a result stocks of dl1403 exhibit significantly higher particle to pfu ratios than wt HSV-1 stocks (Stow and Stow, 1986). Interestingly, the defect at low m.o.i. in HFL cells can be complemented by HCMV and VZV, as discussed in Section 4.4.5. (Stow and Stow, 1989).

A positive role for Vmw110 in E promoter activation has been demonstrated in transient expression assays; Vmw110 was shown to be a potent trans-activator of IE, E and L classes of viral genes (Everett, 1984a; Mavromara-Nazos et al., 1986; Quinlan and Knipe, 1985) and also to act synergistically, in some systems, with Vmw175 in the

activation of a variety of viral promoters (DeLuca and Schaffer, 1985; Everett, 1986; O'Hare and Hayward, 1985a) and cellular promoters (Everett, 1984a; O'Hare and Hayward, 1985a).

In transfection experiments, insertion or deletion mutant alleles of Vmw110 expressed from plasmids were tested for the ability to activate genes under E (gD) promoter control. Several regions of the Vmw110 polypeptide required for its ability to activate gene expression were defined (Everett, 1987b) and it was apparent that the relative importance of such regions was dependent upon the presence or absence of Vmw175. A region crucial for Vmw110 function in the absence of Vmw175, in the second exon of IE-1, includes a potential zinc finger domain (Berg, 1986). A region towards the carboxyl terminus was more important for Vmw110 function in the presence of Vmw175.

Mutant HSV-1 viruses were constructed with insertion and deletion mutations in IE-1 and, growth and plaque formation of the mutants were examined, as well as their ability to activate a transfected HSV E (gD) gene promoter. The effect produced by mutations in Vmw110, which occurred at the level of gene expression, was a reduction in the efficiency of plaque formation by HSV-1, the extent of which was dependent upon the position of the mutation within the polypeptide, the multiplicity of infection and the cell type. Analysis of the mutant FXE, which is phenotypically similar to dl1403, indicated that the region of Vmw110 affected by mutation in FXE is essential for Vmw110 function in plaque formation and in the ability to trans-activate a transfected HSV E promoter (Everett, 1989). In FXE, this region of the Vmw110 polypeptide lacks 45 amino acids spanning the potential metal-binding domain which was previously shown, in transfection experiments, to be functionally important for trans-activation by Vmw110 (Everett, 1987b).

Interestingly, despite the ability of Vmw110 alone to trans-activate E gene expression in transfection experiments, the protein appears to be incapable of fulfilling this function in a virus containing a ts lesion in Vmw175 during infection at the NPT (Preston, 1979a,b).

There is evidence for interference by the Vmw175 ts mutant protein on the normal transport of Vmw110 into the nucleus (Knipe and Smith, 1986), on Vmw110-induced activation of gene expression (Gelman and Silverstein, 1986) and for a trans-dominance of the tsK form of Vmw175 over the wt protein (Gelman and Silverstein, 1986). However, since a virus with a deletion in IE-3 (d120) which expresses a severely truncated Vmw175 protein is also unable to grow in normal cell lines and exhibits an IE polypeptide profile (DeLuca et al., 1985), it seems that Vmw110 by itself is unable to stimulate E gene expression efficiently during HSV infection.

Vmw63

HSV-1 Vmw63, the gene product of IE-2, is a phosphoprotein and localises to the nucleus of infected cells (Wilcox et al., 1980; Knipe et al., 1987). Virus mutants containing ts lesions in Vmw63 have been isolated; at the NPT, Vmw175 and Vmw63 are overproduced, normal levels of E and some E/L proteins are synthesised and viral DNA is replicated, but production of "true" L polypeptides is severely restricted (Sacks et al., 1985). Mutant viruses with deletions in IE-2 have been constructed which lack the transcriptional start site as well as portions of the promoter and coding sequences of the gene. Vmw63-specific transcripts and proteins were not specified, and the viruses were replication incompetent. The deletion mutant phenotypes were characterised by the synthesis of greatly reduced levels of viral DNA, overexpression of E proteins, reduced levels of E/L proteins and absence of "true" L proteins; alterations in viral protein synthesis appeared to occur at the level of transcription (McCarthy et al., 1989).

Analysis, by transient expression assay, of the capacity of Vmw63 to trans-activate transcription has yielded variable data. Although the results of several investigators suggest that Vmw63 alone does not possess significant trans-activation potential (Everett, 1984a; Mavromara-Nazos et al., 1986), a recent report has demonstrated trans-activation of transcription from an E gene promoter (gB) (Rice and Knipe, 1988). There is more

abundant evidence that Vmw63 has the capacity to repress the expression of viral genes (IE and E) (Gelman and Silverstein, 1987: Rice and Knipe, 1988; Sekulovich et al., 1988), and to stimulate further the expression of genes under L promoter control (Everett, 1986; Sekulovich et al., 1988; Rice and Knipe, 1988), when the expression of these genes is induced by Vmw175 and Vmw110.

Taken together, these observations are consistent with the hypothesis that Vmw63 is not involved directly in viral DNA synthesis, but that the synthesis of reduced levels of viral DNA in the absence of Vmw63 is a consequence of the aberrant regulation of viral genes, the products of which are involved in DNA synthesis. It has therefore been suggested that Vmw63 has an essential function in the modulation of E and L gene transcription (McCarthy et al., 1989).

Vmw68

The protein Vmw68 (the product of HSV-1 IE gene 4) is nuclear and phosphorylated (Pereira et al., 1977; Hay and Hay, 1980; Ackermann et al., 1984). A virus mutant has been constructed which contains a 500bp deletion in IE-4. The truncated IE-4 gene specifies a protein of M_R 30,000 which does not possess the carboxyl terminal one-third of the Vmw68 protein (Post and Roizman, 1981; Sears et al., 1985a). The growth properties of the IE-4 deletion mutant vary between different cell lines, a finding which led to the proposal that a host cell factor can substitute for the function of Vmw68 in some cell lines. It has been suggested that the reduction in viral growth observed in certain mutant-infected cell lines can be attributed to a lack of L viral gene products (Sears et al., 1985a).

Vmw12

In contrast to the other IE polypeptides, Vmw12 is non-nuclear and unphosphorylated (Pereira et al., 1977; Hay and Hay, 1980; Marsden et al., 1982). It has been demonstrated that IE-5 is not an essential gene for viral growth in tissue culture, since viable deletion mutants which lack the entire IE-5 gene have been isolated

(Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

1.2.3.3. Regulation of Early Gene Expression

Early gene expression, which reaches a maximum level at 4h to 6h post adsorption, requires prior synthesis of IE gene products (Honess and Roizman, 1974; Preston, 1979a,b); E gene promoters respond to transactivation by Vmw175 and Vmw110 (Everett, 1984a; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1985; Quinlan and Knipe, 1985; DeLuca and Schaffer, 1985).

Early gene promoter sequence requirements for the activation of transcription in *trans* by viral gene products have been analysed by determining the levels of expression from an indicator gene linked to mutagenised E promoter sequences (of the TK gene, UL23, and of the gD gene, US6) and microinjected into *Xenopus* oocytes (McKnight *et al.*, 1981, 1982; Jones *et al.*, 1985) or transfected into eukaryotic cells (Everett, 1983, 1984b). Additionally, mutagenised E (TK gene) promoter sequences have been introduced into the HSV-1 genome and analysed for the ability to function as promoters (Coen *et al.*, 1986). Such investigations have demonstrated that the E promoters of the TK and gD genes contain several functional regions, including a cap site, a TATA box, and upstream GA-rich elements and GC-rich (Sp1-binding) regions; an inverted CCAAT motif is also present in the promoter of the TK gene (McKnight and Kingsbury, 1982; Everett, 1983, 1984b; Eisenberg *et al.*, 1985; El Kareh *et al.*, 1985; Coen *et al.*, 1986). The relative importance, in quantitative terms, of these regions in E gene expression was dependent upon the assay system utilised; in the virus, the upstream promoter region of the TK gene was functionally less important, whereas the TATA box and cap site regions are especially important for full activation of genes under the control of the TK and gD promoters (Coen *et al.*, 1986; Everett, 1984b; Figure 1.4.). Results, from analysis of the activities of deletion and insertion mutants within the gD promoter region after induction during HSV-1 infection and in response to

activation by linkage of the promoter sequences to the SV40 enhancer, showed that mutations which resulted in a reduction of the response to *trans*-activation by viral gene products also resulted in a reduction of the response to *cis*-activation by the SV40 enhancer. Promoter sequence requirements in *trans*- and *cis*- activation of transcription are therefore equivalent (Everett, 1984b; Figure 1.4.). No domain in the E TK gene specifically responsive to *trans*-activation by viral gene products has been identified. These findings imply that IE gene products activate E gene transcription either by binding sequences recognised by cellular transcription factors or by altering the activity of cellular transcription factors (Eisenberg *et al*., 1985; Coen *et al*., 1986). There is evidence to suggest an important role for the TATA box in E gene expression in infected cells. Analysis of transcription from hybrid SV40 promoters, in which the TATA box region of gD (responsive to *trans*-activation) was substituted for the TATA box element of SV40 (poorly responsive), demonstrated that the gD TATA box could confer, upon the SV40 promoter, the ability to be efficiently *trans*-activated by HSV gene products (Everett, 1988). Furthermore, the most severe effect on E gene transcription in infected cells was observed with mutations affecting the TATA box element of the promoter region (Coen *et al*., 1986).

1.2.3.4. Regulation of Late Gene Expression

Late genes require HSV DNA replication for maximal expression; expression is first detected at 2h to 3h post adsorption (note that DNA synthesis is initiated approximately 2h post adsorption and reaches maximum levels at approximately 8h post adsorption) and the accumulation of L gene products reaches its maximum by 10h to 16h post adsorption (Munk and Sauer, 1964; Roizman, 1969). Although the requirement for Vmw175 and Vmw110 in true late gene expression is not clear, activation of a late promoter (L42) by cotransfection with IE genes has been detected in transient assay systems (DeLuca and Schaffer, 1985; Mavromara-Nazos *et al*., 1986).

Promoter sequence requirements for L gene expression have been delineated in a transient assay system (Johnson and Everett, 1986b), in which "true" L gene (US11) promoter deletions were located on plasmids containing an HSV-1 origin of replication (ori_S), which enables replication of transfected plasmid DNA in the presence of infecting HSV (Johnson and Everett, 1986a; Stow and McMonagle, 1983), and activation of gene expression was determined. DNA promoter sequence elements required for fully efficient regulated expression of US11 lie within 31bp of the RNA cap site, and therefore consist only of a proximal TATA box and cap-site region (Johnson and Everett, 1986b; Figure 1.4.). The promoter sequences required for expression of the gC gene were demonstrated to be similar (Homa et al., 1986). The conversion of an E HSV gene (gD) into a L gene, in terms of its characteristics of expression, was achieved by removal of sequences 5' to its TATA box, thus confirming that a proximal TATA box is sufficient for L gene expression (Johnson and Everett, 1986b). The promoter sequences necessary for induction of L promoters by Vmw175 and Vmw110 are also contained within the 31bp region upstream of the RNA cap site (Johnson and Everett, 1986b).

1.2.3.5. Herpes Simplex Virus - Encoded Polypeptides

The functions and properties of the HSV IE polypeptides are discussed in Section 1.2.3.2. Enzymes and proteins involved in DNA replication are encoded, primarily, by HSV E genes and are dealt with in Section 1.2.4. Interestingly, the E gene product DBP (major DNA binding protein) appears to have a role in the general repression of HSV gene transcription in the absence of viral DNA synthesis (Godowski and Knipe, 1983, 1986) as well as a role in HSV DNA replication.

There are at least 30 different species of virion structural proteins, encoded primarily, but not exclusively, by the HSV L genes, including the HSV glycoproteins, capsid and tegument proteins. There are seven known HSV-1 glycoproteins; gB (encoded by UL27), gC (UL44), gD (US6) (Spear, 1976), gE (US8) (Baucke and Spear, 1979), gG (US4),

gH (UL22) (Marsden et al., 1978, 1984; Frame et al., 1986; McGeoch et al., 1987, 1988) and gI(US7) (Longnecker et al., 1987). Furthermore, an ORF in US possesses the potential to encode a glycoprotein (McGeoch et al., 1985). There exist HSV-2 homologues to all the HSV-1 glycoproteins, although gG-2 contains an insert of DNA which is absent from gG-1 (McGeoch, 1989). Mutant studies have shown that gB and gH are essential for growth in tissue culture (Little et al., 1981; Weller et al., 1983a; Buckmaster et al., 1984; Gompels and Minson, 1986), but that several of the glycoproteins, including gC (Heine et al., 1974) gE, gG, gI and US5 (Longnecker and Roizman, 1986; Longnecker et al., 1987; Harland and Brown, 1988) are non-essential. Certain of the glycoproteins have roles in viral adsorption and penetration, as discussed in Section 1.2.1. The HSV capsid consists of at least 6 polypeptides, including the major capsid protein, of M_R 155,000 (ICP5, VP5), encoded by UL19 (Marsden et al., 1978; Morse et al., 1978; McGeoch et al., 1988a); the polypeptide p40, encoded by UL46, is a component of empty capsids, and is discussed in Section 1.2.4. in the context of encapsidation (Addison, 1986; Rixon et al., 1988). HSV tegument polypeptides are generally virion proteins not classified as capsid species or glycoproteins and are released by solubilisation of the virion with non-ionic detergent (Lemaster and Roizman, 1980; Roizman and Furlong, 1974; Spear, 1980). The IE transcriptional transactivator Vmw65 is a major constituent of the tegument (Marsden et al., 1978; Lemaster and Roizman, 1980). Other constituents of the tegument include, the extremely large product of gene UL36, and proteins encoded by US9 and US10 (Batterson et al., 1983; Rixon and McGeoch, 1984; Frame et al., 1986b).

1.2.4. Replication, Encapsidation and Assembly

Circularisation of linear HSV DNA molecules occurs following entry into the cell nucleus (Jacob and Roizman, 1977), perhaps via direct ligation of the termini (Davison and Wilkie, 1983b; Poffenberger and Roizman, 1985). DNA replication results in the formation of large head to tail

concatemers and therefore probably occurs by a rolling circle mechanism (Jacob *et al.*, 1979; Jongeneel and Bachenheimer, 1981). Electron microscopic analysis suggested that the HSV genome contained one or more specific sequences at which DNA replication initiated (Friedman *et al.*, 1977). Characterisation of two classes of defective genomes has identified three origins of replication (Figure 1.1.) (Vlazny and Frenkel, 1981; Spaete and Frenkel, 1982), two of which (*ori*$_S$; Class I defectives) are within IR_S and TR_S sequences, localised to the non-transcribed region between IE genes 3 and 4 (Stow and McMonagle, 1983), while the third (*ori*$_L$; Class II defectives) lies in U_L sequences between the DNA polymerase gene and the DBP gene (Weller *et al.*, 1985; Quinn and McGeoch, 1985; Lockshon and Galloway, 1986). HSV-1 *ori*$_S$ contains an imperfect palindrome, each arm consisting of 21bp, with a central A-T rich sequence which, along with sequences outside the palindrome, is essential for *ori* function (Stow and McMonagle, 1983; Stow, 1985). HSV-2 contains two copies of *ori*$_S$ within almost identical 137bp direct repeats (Whitton and Clements, 1984). A nuclear protein from HSV-1 infected cells has been demonstrated to bind directly to an 18bp region across one end of the palindrome of *ori*$_S$ DNA (Elias *et al.*, 1986). HSV-1 *ori*$_L$ contains a perfect palindrome with arms of 72bp and a central AT-rich sequence with high homology to *ori*$_S$, and HSV-2 *ori*$_L$ contains a 136bp palindrome very similar in sequence to HSV-1 *ori*$_L$ (Weller *et al.*, 1985; Quinn and McGeoch, 1985; Lockshon and Galloway, 1986). The significance of the presence of three *ori* sites in the HSV genome is not known. Indeed, studies with mutant viruses have shown that deletion of *ori*$_L$ or one copy of *ori*$_S$ has little or no effect on viral replication in cultured cells (Longnecker and Roizman, 1986; Polvino-Bodnar *et al.*, 1987). Furthermore, since *ori*$_S$ and *ori*$_L$ share extensive sequence homology, and since equivalent proteins are necessary for *ori*$_S$- and *ori*$_L$- dependent replication (see below), it is likely that *ori*$_S$ and *ori*$_L$ are functionally equivalent.

The induction of virus-specific replicative functions by HSV was inferred from the requirement of helper viruses to replicate defective genomes or plasmids containing an HSV

origin of replication (Vlazny and Frenkel, 1981; Stow, 1982) and these functions have been analysed by characterisation of mutant viruses. Functions essential to HSV DNA replication include DNA polymerase (UL30) (Keir and Gold, 1963; Chartrand et al., 1979, 1980), DBP (UL29) (Conley et al., 1981; Weller et al., 1983), alkaline exonuclease (UL12) (Keir and Gold, 1963; Preston and Cordingley, 1982; Banks et al., 1983; Moss, 1986) and ribonucleotide reductase (RR) (UL39 and UL40) (Cohen, 1972; Dutia, 1983), while enzymes involved in DNA metabolism but non-essential for virus growth in tissue culture include dUTPase (UL50) (Wohlrab and Francke, 1980; Preston and Fisher, 1984), uracil-DNA glycosylase (UL2) (Mullaney et al., 1989) and TK (UL23) (Kit and Dubbs, 1963; Jamieson et al., 1974), although the latter may assume a more important role in non-dividing cells (Field and Wildy, 1978). TK and RR catalyse reactions in the biosynthesis of DNA precursors. The latter consists of two subunits, Vmw136 and Vmw38, encoded by separate but contiguous genes (UL39, UL40) (Preston et al., 1984; Bacchetti et al., 1984; Frame et al., 1985; McLauchlan and Clements, 1983). The HSV DNA polymerase, a protein of M_R 136,000 involved directly in viral DNA replication, differs from most eukaryotic polymerases by virtue of its 3' to 5' exonuclease activity which may have a proof-reading effect (Powell and Purifoy, 1977; Purifoy and Powell, 1981; Knopf, 1979). It has been suggested that DBP, a protein of M_R 128,000 which has been shown to dissociate the strands of duplex DNA *in vitro* (Powell et al., 1981) acts in a replication complex with DNA polymerase and HSV exonuclease, since ts lesions in purified DBP impair the function of the two enzymes at the NPT (Littler et al., 1983). Additional proteins involved in HSV DNA synthesis include, possibly, DNA topoisomerase(s) (Biswal et al., 1983; Muller et al., 1985) and protein kinases encoded by US3 and UL13, the latter mapped by virtue of its sequence homology to other known protein kinase genes (McGeoch and Davison, 1986).

It has been shown that five cloned restriction fragments of HSV-1 DNA together can supply all of the *trans*-acting functions essential for replication of plasmids containing ori_S or ori_L when cotransfected into Vero cells

(Challberg, 1986). Based on these findings, a transient complementation assay has been developed which, in combination with sequencing data, has enabled the identification of seven HSV genes which are both necessary and sufficient for origin-dependent replication. Two of these genes encode the viral DNA polymerase and DBP (Wu et al., 1988). The other five genes, designated UL5, UL8, UL9, UL42 and UL52, are predicted to encode proteins of M_R 99,000, 80,000, 94,000, 51,000 and 114,000 (McGeoch et al., 1988b). The UL42 gene product has been identified as a previously recognised DNA-binding factor found in extracts of HSV-1 infected cells (Parris et al., 1988). Evidence has recently been presented which indicates that the UL9 gene product, of M_R 82,000, is an origin-binding protein (Olivo et al., 1988). UL5 is a component of the DNA helicase found in infected cells (Crute et al., 1988; McGeoch et al., 1989). The products of genes UL5, UL8, UL9 and UL52 are located in the nucleus, appear early after infection and are expressed despite inhibition of viral DNA synthesis, which is consistent with the idea that they are the products of early genes (Olivo et al., 1989).

There is evidence to suggest that the HSV 'a' sequence is involved in inversion of the L and S components of the HSV-1 genome relative to each other (Section 1.1.4.), and in cleavage and packaging of the viral genome. The 'a' sequence of HSV-1 strain 17 has the format: $(DR1)(U_a)(DR2)_{18}(U_b)(DR1)$, where U_a and U_b are unique sequences and DR1 and DR2 are direct repeats of 17 and 12bp, respectively (Davison and Wilkie, 1981). DR2 was proposed to be a *cis*-acting sequence for isomerisation, which occurs via intermolecular and intramolecular recombination (Mocarski et al, 1980; Mocarski and Roizman, 1982; Chou and Roizman, 1985), although, the HSV-2 'a' sequence does not possess DR2 repeats (Davison and Wilkie, 1981) and isomerisation of the genome of some HSV-1 mutants lacking 'a' sequences has been demonstrated (Pogue-Geile et al., 1985; Longnecker and Roizman, 1986). Cleavage of concatemeric DNA into unit length molecules occurs as a prerequisite to or concomittant with the packaging of DNA into capsids. A role for the 'a' sequence in cleavage and packaging of DNA was suggested by

the finding that progeny of plasmid molecules which contain HSV ori_S (Stow, 1982) were encapsidated, in the presence of helper virus, only when an 'a' sequence was also located on the plasmid molecule (Stow et al., 1983). The 'a' sequence at both the L and S termini of the HSV genome ends in a partial copy of the DR1 element, the complete sequence being recreated by ligation of L and S termini (Davison and Wilkie, 1981; Mocarski and Roizman, 1982). Varmuza and Smiley (1985) proposed that the generation of termini was via 2 distinct cleavage events and that the mechanism of cleavage involved: introduction of ss nicks, possibly in Ub and Uc, followed by strand repair synthesis, a model which could also account for doubling of 'a' sequences; or, the introduction of ds nicks in the 'a' sequence, at L-S junctions positioned in the same orientation, and subsequent production of molecules with or without 'a' sequences, the latter being rapidly degraded. Ts mutants have been isolated which are defective in both cleavage and packaging events (tsl204, tsl201) (Preston et al., 1983; Addison, 1986). A defect in the processing of a major structural (tegument) protein, p40 (Vmw40), a product of gene UL26 (Preston et al., 1983; McGeoch et al., 1988a) to forms of lower electrophoretic mobility, was observed in cells infected with tsl201 at the NPT; the unprocessed proteins became associated with empty capsids which accumulated in the cell nuclei in large numbers (Preston et al., 1983). A mutant tsl203, which makes but fails to package viral DNA at the NPT, also assembles partially cored capsids, but, in contrast to tsl201, tsl203 processes p40 normally at the NPT (Matz et al., 1983). It has recently been shown that p40 is present in empty capsids, but is not a major component of full capsids or of mature virions. It has, therefore, been suggested that p40 becomes transiently associated with capsids at an early stage in their assembly and that its removal from capsids is linked with the process of packaging (Rixon et al., 1988). Nucleocapsids are enveloped at the inner lammella of the nuclear membrane; only capsids containing an approximate genome equivalent of DNA are enveloped and transported to the cytoplasm as mature virions (Roizman and Furlong, 1974; Vlazny et al., 1982; Stow

et al., 1986).

The outcome of a lytic infection of HSV is the production of progeny HSV particles and lysis of the infected cell, and is, therefore, in contrast to that of a latent HSV infection, which will be discussed below.

1.3. HERPES SIMPLEX VIRUS LATENT INFECTION

The capacity to establish a latent infection appears to be a general property of herpesviruses. Latent viral infection occurs between episodes of acute disease and involves the persistence of virus within specific tissues such that infectious virus is undetectable and there are no overt signs of viral replication, cell damage or disease. The latent infections produced by HSV types 1 and 2, which will be considered together, and EBV are the most studied of those produced by viruses of the Herpesviridae.

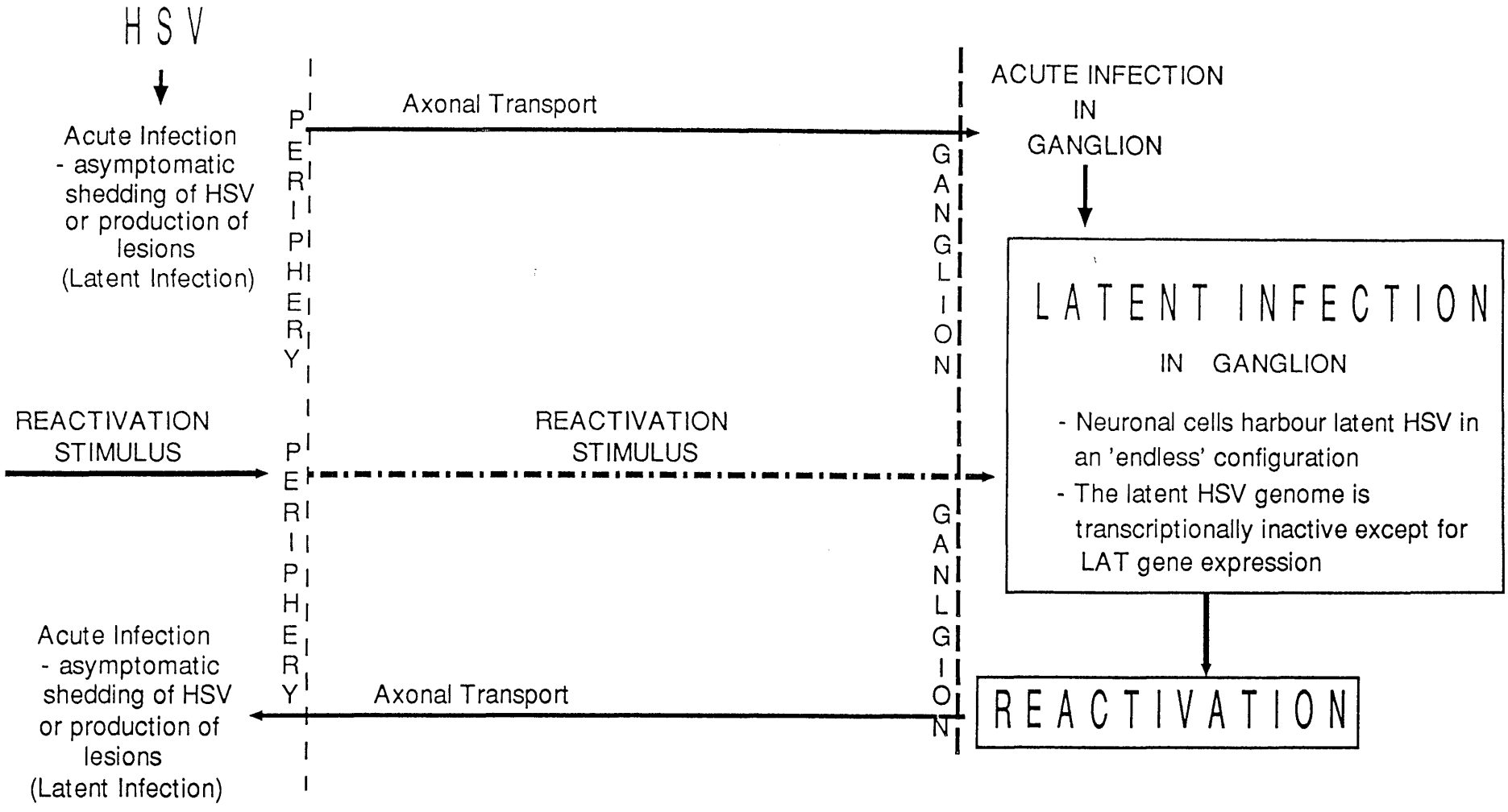
1.3.1. Herpes Simplex Virus Latency - Animal Model Systems

HSV establishes latent infections in both man and experimental animals. Following primary peripheral HSV infection, or inoculation, of skin, mucous membrane or eye, HSV travels through associated sensory nerves; there is evidence, from studies in mice and rabbit models, that travel from the site of infection to the primary site of latency in the peripheral nervous system occurs via retrograde axonal transport. The virus becomes established in a latent state in neuronal cells of the corresponding sensory ganglia or other nervous tissue. Reactivation from the latent state results in productive infection in previously latently infected neurons, the subsequent reappearance of infectious virus in the locality of the primary infection and asymptomatic shedding of infectious virus (recurrence) or the production of lesions (recrudescences) at the periphery (Goodpasture, 1929; Stevens, 1975; Wildy et al., 1982; Blyth and Hill, 1984; Hill, 1985; Stanberry, 1986; Sugden, 1986; Roizman and Sears, 1987; Baichwal and Sugden, 1988).

The basic pattern of establishment of latent HSV infection is similar in various experimental animals. The mouse footpad model was the first latency system to be developed (Stevens and Cook, 1971, 1973a,b; Walz et al., 1974). HSV inoculation of a mouse rear footpad results in a local cutaneous infection and, following centripetal movement of virus through the peripheral and central nervous

Figure 1.6. SCHEMATIC REPRESENTATION OF HSV LATENCY IN VIVO.

A schematic representation of HSV latency in vivo, based on evidence from animal model latency systems and information from HSV latent infections of humans. Details of HSV latency are presented in Section 1.3..


HSV
Acute Infection
- asymptomatic shedding of HSV or production of lesions
(Latent Infection)
PERIPHERY
Axonal Transport
GANGLION
ACUTE INFECTION IN GANGLION
LATENT INFECTION
IN GANGLION
- Neuronal cells harbour latent HSV in an 'endless' configuration
- The latent HSV genome is transcriptionally inactive except for LAT gene expression
REACTIVATION STIMULUS
REACTIVATION STIMULUS
PERIPHERY
GANGLION
REACTIVATION
Acute Infection
- asymptomatic shedding of HSV or production of lesions
(Latent Infection)
Axonal Transport
PERIPHERY
GANGLION

systems to the brain, an acute infection develops in the nervous system, the outcome of which may be temporary or permanent posterior paralysis and possibly death of the mouse; surviving mice which recover from paralysis within 3 weeks are used for studies of latency. During the acute infection (1-10 days post inoculation) HSV can be recovered from sciatic nerve, sacrosciatic spinal ganglia, dorsal roots, spinal cord and brain. Thereafter, infectious HSV cannot be demonstrated in homogenates of nervous tissues. Explantation and cocultivation of the nervous tissues with indicator cell monolayers results in the reappearance of infectious HSV (Stevens and Cook, 1971), thereby demonstrating that HSV is harboured in a latent state in these tissues.

Latent HSV infection can be established in the lumbosacral dorsal root ganglia of guinea pigs subsequent to rear footpad or vaginal inoculation (Scriba, 1975, 1976). In mouse (Knotts et al., 1974; Walz et al., 1974) and rabbit (Stevens et al., 1972) eye models, HSV resides in a latent state in the trigeminal ganglia after infection of the cornea via scarification or inoculation. Latent infections have also been established in the trigeminal ganglia and cervical dorsal root ganglia of mice following lip and ear inoculation, respectively (Walz et al., 1974; Hill et al., 1975).

Reactivation of HSV and recurrence or recrudescence occurs spontaneously in some animal systems, including the rabbit eye model (Nesburn et al., 1977) and the mouse ear model (Hill et al., 1975), as well as the guinea pig model system for genital HSV infection (Scriba, 1976). Reactivation of HSV to produce an acute infection in the ganglia can be induced by neurectomy in certain systems (Walz et al., 1974; Price and Schmitz, 1978). Reactivation can also be induced in some systems by exposure of the exterior to various physical and chemical stimuli as described in Section 1.3.3.3., or exposure to systemic stimuli, which, in the mouse ear model for instance, result in the reappearance of infectious HSV in the skin of the ear and in the cervical dorsal root ganglia (Hill, 1985).

Animal model latency systems in which recurrence

and/or recrudescence occurs spontaneously or can be induced have been useful in exploration of the mechanism(s) responsible for recurrent herpetic disease, whereas systems in which spontaneous recurrences are rare or not inducible have been used in the study of viral and host factors involved in establishment and maintenance of latent disease. In vitro cell culture latency systems have also been developed and utilised to elucidate the molecular nature of the latent HSV infection, as will be discussed in Section 1.3.6. Ultimately, findings from in vivo and in vitro studies must be confirmed in humans wherever possible.

1.3.2. Establishment of Latency in Ganglia

1.3.2.1. Sites of Latency

The idea of a connection between production of herpetic lesions and the nervous system initially arose from clinical observations that lesions tended to recur in the same locality. Involvement of the sensory ganglia in development of herpetic lesions was suggested by the observations that there was a correlation between the sites of recrudescence and histological changes in the related ganglia (Goodpasture and Teague, 1923) and that destruction of trigeminal ganglia resulted in a corresponding inability to produce lesions (Cushing, 1905). In addition, various clinical and experimental observations led to the theory that herpesvirus resides in a latent state in the cells of the sensory ganglia and other nervous tissues (Goodpasture, 1929; Carton and Kilbourne, 1952; Carton, 1953; Paine, 1964).

Direct evidence has come from experimentation in mouse, rabbit and guinea pig systems (Stevens and Cook, 1971; Walz et al., 1974; Stevens et al., 1972; Knotts et al., 1973; Baringer and Swoveland, 1974; Scriba, 1975, 1976) and, importantly, from human tissues (Plummer, 1973; Bastian et al., 1972; Baringer and Swoveland, 1973; Rodda et al., 1973) since, as described in Section 1.3.1., infectious HSV (Warren et al., 1978) can be reactivated from sensory ganglia following explantation of the nervous tissues and organ culture in the

presence of indicator monolayer cultures. The presence of HSV DNA has also been demonstrated in such tissues (Walz et al., 1976; Puga et al., 1978; Cabrera et al., 1980; Fraser et al., 1981; Rock and Fraser, 1983; Kennedy et al., 1983; Puga et al., 1984; Stroop et al., 1984). Depending on the site of primary infection, HSV can establish a latent infection in any type of nerve ganglion, including autonomic ganglia in mice (Price et al., 1975a) and humans (Warren et al., 1978). Furthermore, latent infection has been demonstrated in the central nervous system of rabbits and mice (Plummer et al., 1970; Knotts et al., 1973; Cook and Stevens, 1976; Cabrera et al., 1980) by explant culture, and HSV DNA has been detected in human brain tissue (Sequiera et al., 1979; Fraser, 1981). Infectious HSV was recovered, by explantation and cocultivation, from brain tissue of 5% and from trigeminal ganglia of 95% of latently infected mice, although HSV DNA was detected in brain in 30% of the mice which harboured latent HSV in their trigeminal ganglia. There may, therefore, be a difference in the ability to reactivate HSV, by explantation and cocultivation, between the central and peripheral nervous systems (Cabrera et al., 1980).

Certain experimental findings have been put forward as evidence to suggest that latent HSV may be harboured in non-neural tissues. For instance, the phenomenon of shedding of infectious virus in the absence of clinical disease (recurrence) in body secretions of humans or rabbits (Kaufman, 1967; Douglas and Couch, 1970) or in peripheral tissues of mice (Hill et al., 1980), and, the detection of infectious HSV following explantation and cocultivation of tissues of the mouse vagina (Walz et al., 1977), ear (Hill et al., 1980) and footpad (Al-Saadi et al., 1983), of clinically normal rabbit corneas (Cook et al., 1987) and of tissues of the guinea pig footpad (Scriba, 1977) and genital regions (Scriba, 1976, 1981; Scriba and Tatzber, 1981; Stanberry et al., 1985b,c). As yet, there has been no detection of HSV in homogenates or explant cultures of human peripheral tissues, such as skin from sites of frequent recurrent HSV infection (Findlay and MacCallum, 1940; Rustigian, et al., 1966), although herpes lesions have been

observed in denervated areas of skin (Hoyt and Billison, 1976) and herpes virus has been isolated from human corneal explants in culture (Shimeld et al., 1982; Tullo et al., 1985).

In guinea pigs, spontaneous reactivation of HSV from latency often takes place with concurrent and almost continuous production of the disease at the periphery (Scriba, 1977). HSV was detected in skin explants of guinea pig footpads which had been surgically manipulated to destroy the nerve supply to, and thus prevent establishment of latent infection in, corresponding ganglia; a low incidence of spontaneous recurrent disease was also demonstrated (Scriba, 1981). However, Scriba (1981) found that phosphonoacetic acid (PAA) or acycloguanosine (ACG) eliminated HSV infection in peripheral tissue but did not eliminate the ability of latent HSV to reactivate in ganglia following explantation, and suggested that HSV exists in a productive state in the peripheral tissues of guinea pigs, as opposed to a latent state.

In a recent study by Bernstein and Kappes (1988), the addition of a demethylating agent, hexamethylenebisacetamide (HMBA), to explant cultures of latently infected guinea pig dorsal root ganglia, genital skin and uterine cervix increased the recovery of HSV. It was suggested that, since HMBA had no effect on HSV replication in vitro, it probably induced reactivation via demethylation in both neural and non-neural tissues and, therefore, that persistent infection in the non-neural tissues was probably equivalent to the latent infection of dorsal root ganglia and not a low level chronic productive infection that would have been unaffected by demethylation (Bernstein and Kappes, 1988).

It has been postulated by Hill and coworkers that the presence of HSV in the skin of latently infected mice exhibiting no clinical disease could reflect the presence of microfoci of infection from an input of virus from the primary site of latency (sensory ganglia) rather than a latent infection of the skin from which virus had reactivated (Hill et al., 1980). In mice latently infected in the cervical ganglia the initial site of inoculation (ear skin) was denervated (cervical nerve section) and then the

denervated ears were stripped with cellophane tape to induce recurrent disease; infectious virus was isolated from the skin very rarely and recurrent disease was not seen (Hill et al., 1983), implying that recurrent disease in the mouse requires an intact nerve supply to the skin. Hill and coworkers (1983) suggested that in these experiments the virus isolated had originated from minor nerve supplies to the pinna from the trigeminal or jugular ganglia, or was present in the skin before nerve section, and reflected chronic viral multiplication in an environment made susceptible by the process of enervation or by incomplete enervation, rather than a latent infection of the peripheral tissues.

Clements and Subak-Sharpe (1988) and Al-Saadi et al. (1988) have recently presented compelling evidence that HSV can establish latent infection in non-neuronal cells as well as neuronal cells. Infectious HSV can be recovered from explant cultures of the footpad (site of inoculation) and/or the sensory ganglia of mice latently infected with differents strains or ts mutants of HSV-1 and HSV-2 (Al-Saadi et al., 1983; Clements and Subak-Sharpe, 1983; Subak-Sharpe et al., 1984a,b; Al-Saadi, 1984). Recovery of virus from the footpad following explantation and cocultivation was not prevented by treatment of infected mice with acycloguanosine (ACG, an inhibitor of HSV which can eradicate persistent but not latent infections), or by section of the nerves of the inoculated leg, or by nerve section and ACG treatment combined (Clements and Subak-Sharpe, 1988; Al-Saadi et al., 1988). It was concluded that, since nucleated neuronal cell bodies are absent from the footpad, latent HSV can reside in cells other than neurons. Furthermore, viral RNA has been detected in a sub-population of epithelial cells of the explanted footpad by in situ hybridisation analysis using HSV-specific probes. The footpad cell types in which HSV can establish latency have been identified as the basal cells and root sheath cells of hair follicles, as well as epithelial cells of the sebaceous glands and cells within the epidermis (Clements and Jamieson, 1989).

Therefore, although it is clear that the predominant

site of latency of HSV in humans and in experimental animal model systems is the dorsal root ganglion, HSV latency has also been demonstrated in non-neuronal tissues in the mouse, an observation which may or may not be relevant to HSV latency in humans.

1.3.2.2. Nerve Tissue Cells Harbouring Latent HSV

Electron microscopic studies have demonstrated that, during the acute phase of HSV infection in the nervous system of mice, Schwann, satellite and other supporting cells are infected abortively or are resistant to HSV infection (Dillard et al., 1972; Stevens and Cook, 1973a; Cook and Stevens, 1973; Knotts et al., 1974). The majority of evidence suggests that latent HSV resides in the neuronal cell body. Indirect support for this hypothesis comes from the observations that in mice and rabbits HSV can be reactivated (by explantation) from ganglia, which contain neuronal cell bodies, but not from nerve roots, which do not, both tissues otherwise containing equivalent cell types (Baringer and Swoveland, 1973; Cook et al., 1974). Immunofluorescence and electron microscopy studies on latently infected ganglia implanted in millipore chambers and transplanted into syngeneic mice have shown that virus antigen and virus particles were first detected in neuronal cells; thymidine incorporation into virus DNA also commenced in neurons rather than satellite cells. Kennedy and associates (1983) also detected HSV antigens initially in neurons, identified by antineuronal monoclonal antibody, as opposed to satellite cells, of dissociated cultures from latently infected mouse ganglia. In situ hybridisation analyses showed that viral DNA could be detected in the nuclei of neurons prior to detection in satellite cells of ganglia from mice (Cook et al., 1974) and rabbits (zur Hausen and Schulte-Holthausen, 1975) and that viral mRNA could be detected first in the neurons of human ganglia (Galloway et al., 1979). In the studies of McLennan and Darby (1980), ts mutants of HSV-1 were used to latently infect mice (which have a core temperature of 38.5^{o}C, the restrictive temperature for mutant growth) and were

reactivated by peripheral nerve section *in vivo* or by explantation at the restrictive temperature *in vitro*; since the spread of reactivated virus from the site of activation was not possible and detection of viral antigens was limited to neuronal cells, it was concluded that latent HSV must reside in the neuronal cells. An HSV transcript has recently been detected in latently infected ganglia, in the absence of acute infection, which is localised to the nuclei of neuronal cells (Stevens *et al.*, 1987; Section 1.3.4.2.).

1.3.2.3. Factors Affecting Establishment of Latency

HSV growth in peripheral infection is normally of limited duration and can be affected by genetic attributes of the virus, the route of infection, host resistance to HSV infection and immunological mechanisms which remove virus from the peripheral site and terminate the acute infection (Nash and Wildy, 1983). The extent of productive infection at the periphery influences the amount of virus available to enter the nerve endings and probably, thereby, the incidence of latent infection in neuronal cells. The incidence of latent infection is usually greater after symptomatic virus disease (Harbour *et al.*, 1981), appears to be related to the severity of the peripheral infection (Tullo *et al.*, 1982) and is therefore influenced by the immune status of the host (Walz *et al.*, 1976; Tullo *et al.*, 1982). Suppression of virus multiplication at the mouse periphery, by application of phosphonoacetic acid at the site of inoculation, has been shown to correlate with a drop of virus titre in the corresponding ganglia (Klein and Destefano, 1981). However, there is evidence that there is no prerequisite for viral replication in the establishment of a latent infection in the ganglia. Treatment of mice pre- and post- primary inoculation with anti-HSV antiserum, to the extent that virus was never isolated from skin or ganglia, did not prevent the establishment of latency (Sekizawa, 1980), although it is possible that in these studies virus replication was not entirely suppressed.

The incidence of infectious HSV in the ganglia of animal model latency systems tends to follow a set pattern;

for instance, in the mouse footpad model of Stevens and Cook (1971) infectious virus can be isolated from sensory ganglia for up to about 10 days post-inoculation (Cook and Stevens, 1973), suggesting that a productive infection may occur in the ganglia, but is not demonstrable thereafter. The immune status of the host influences the virus titre but does not eliminate the acute phase of the ganglionic infection (Openshaw, et al., 1979; Kino et al., 1982). There have been some reports that, following the period of productive infection of the ganglia, there is a background incidence of isolation of infectious virus, estimated at 1% (Blyth and Hill, 1984), at 10% (Stevens et al., 1975) and at 20% (after 6 months, in this instance reduced from 80% at 1 month) (Schwartz et al., 1978). Electron microscopy studies have also demonstrated the presence of virions in a low percentage of neurons in latently infected rabbit trigeminal ganglia (Baringer and Swoveland, 1974), although this may reflect the presence of reactivated virus since spontaneous reactivation occurs frequently in the rabbit.

The production of infectious virus in nerve tissue is likely to affect the number of neurons harbouring latent HSV. Even so, since ts DNA-negative mutants of HSV can cause latent infection in ganglia and brains of mice, albeit at reduced levels (Lofgren et al., 1977; McLennan and Darby, 1980; Watson et al., 1980; Al-Saadi et al., 1983), and on the assumption that the DNA phenotype of the mutants was fully restricted, it would appear that productive viral infection of nervous tissues is not essential for latency to occur.

In conclusion, the factors affecting establishment of latency are complex. The extent of latent infection appears to be influenced by the level of viral replication, both at the periphery and in the ganglia, which may be controlled by various immunological and other factors (Walz et al., 1976; Openshaw et al., 1979; Kino et al., 1982; Tullo et al., 1982; Kapoor et al., 1982). However, it has not been possible to determine absolutely whether or not viral replication at the periphery or in the ganglia is essential for the establishment of latency.

1.3.3. Factors Affecting Maintenance of Latency, Reactivation and Recurrence/Recrudescence

1.3.3.1. The Physiological State of the Neuronal Cell

An important factor in maintenance of latent HSV infection in neuronal cells is the physiological state of the neuron as it relates to permissiveness to HSV replication (Price and Schmitz, 1978). The neuron is a fully differentiated cell with specialised functions; in the rat, some 30,000 genes are expressed exclusively in brain tissue and there is evidence to suggest that control of neuronal-specific gene expression is via specific identifier sequences (Sutcliffe et al., 1984) and/or via alternative RNA processing (Rosenfeld et al., 1984). The neuronal cell is long-lived and non-dividing. Therefore, cellular DNA synthesis occurs only as a consequence of DNA repair mechanisms (Sanes and Okun, 1972; Ishiwaka et al., 1978) and large areas of the genome remain untranscribed. The relatively quiescent state of the neuronal genome, in terms of DNA replication and transcription, might permit the virus to reside in a similarly inactive state.

HSV reactivation can be induced upon cutting or damage of nerves or nerve root, as occurs, for instance, during explantation of nervous tissues. The mechanism(s) by which explantation of latently infected tissue induces reactivation probably involves an alteration in the physiological state of the neuron, perhaps due to an effect of changes in the levels of components in the extra- and intra- cellular environments, such as immunological factors (Section 1.3.3.2.) and nerve growth factors (Section 1.3.5.) or, alternatively, via an effect of cellular repair processes, which result in increased transcription, protein synthesis and DNA synthesis and which are activated by nerve section (Watson, 1974; Grafstein, 1975; Hill, 1984). Neurons in the central nervous system have a relatively reduced ability to regrow and repair nerve processes (Grafstein, 1975), so that if reactivation does involve increased gene expression and DNA synthesis via cellular repair mechanisms, this might account for the finding that reactivation is a

less frequent event in the central nervous system than in the peripheral nervous system, as mentioned in Section 1.3.2.1. (Hill, 1984).

1.3.3.2. Immunological Influences

Maintenance of HSV in a latent state in neuronal cells *in vivo* might be due to an active effect of a host factor(s), possibly a component of the immune system, rather than the physiological state of the neuron *per se*. A role for immunological mechanisms in the maintenance of HSV in a latent state has therefore been examined (Nash, 1981; Wildy *et al.*, 1982; Wildy and Gell, 1985; Stanberry, 1986).

Experiments in which latently infected ganglia in millipore chambers were transplanted into the peritoneal cavities of immune and non-immune mice, removed after 4 days and assayed for the presence of HSV antigens and HSV DNA, suggested that antibody (anti-herpes IgG) was important in the maintenance of latent HSV infection, because the ganglia from immune mice developed only small discrete foci compared to the extensive areas of virus replication in ganglia from non-immune mice (Stevens and Cook, 1974). However, as the initial transplantation of ganglia could have reactivated HSV, it is likely that the results reflect the effect of antibody on the spread of reactivated virus rather than the effect on maintenance of latency. Two pieces of data make it unlikely that neutralising antibody plays a major role in maintenance: in humans there is no correlation between the level of neutralising antibodies and the frequency of recurrent disease, and pre-existing antibody to HSV-1 does not prevent recurrence of HSV-2 genital disease (Corey *et al.*, 1980; Reeves *et al.*, 1981); in mice, inoculated in the lip or cornea with HSV-1 then passively immunised with anti-HSV-1 antibody, a latent infection developed in the trigeminal ganglia which was maintained even after clearance of antibody from the circulation (Sekizawa *et al.*, 1980).

Suppression of cell-mediated immunity in latently infected immunocompetent mice, by treatment with cyclophosphamide and/or X-irradiation, resulted in reactivation of HSV in up to 70% of animals even in the

presence of high concentrations of neutralising antibody, suggesting a possible involvement of cell-mediated immunity in maintenance of latency, although it is also possible that the treatments per se reactivate HSV (Openshaw et al., 1979; Sekizawa et al., 1980). In guinea pigs it has been found that animals that do not have recrudescences appear to be immunologically competent, while those that have recrudescences have impaired cell-mediated immunity to herpes (Donnenberg et al., 1980). It has also been reported that humans undergoing recrudescence of HSV skin lesions have impaired cell-mediated immunity, which manifests itself in a failure of T lymphocytes to produce macrophage inhibition factor specifically in response to herpes antigens (Shillitoe et al., 1977). Furthermore, interferon production, or associated immune events, appears to protect against recurrences of herpes labialis (Cunningham and Merigan, 1983). However, these experiments do not distinguish between the factors that maintain the latent HSV infection in the neuron and factors that are involved in the control of events following reactivation until production of herpetic lesions.

1.3.3.3. Reactivation and Recurrence/Recrudescence

In humans, reactivation can be induced by a variety of stimuli including exposure to ultraviolet light, fever, stress and hormonal changes (Stevens, 1980; Wildy, 1982; Hill, 1985), and, in animals reactivation can be induced by exposure of the periphery to various physical and chemical stimuli, as discussed below.

Three main theories have been proposed by Hill and Blyth (1976; Hill, 1985), from their investigation of the mouse ear model of latency, to explain the ability of external stimuli to induce reactivation of virus from an internal site and to account for events leading to the presence of infectious HSV and/or the production of recrudescent lesions at the periphery. In the "skin trigger" theory, a peripheral stimulus produces local alterations in the tissues, leading to increased susceptibility of the tissue to HSV infection and/or depression of local defence

mechanisms. As a result, microfoci of HSV infection already present at the periphery (either as a latent infection or derived from reactivation of latent infection in the ganglia) grow and produce a clinical lesion. The "ganglion and skin trigger" theory proposes that, following a peripheral stimulus, reactivation of the latent infection in the ganglion results in the production of infectious virus, and that this virus, or subviral particles, proceeds along the associated nerves to the periphery. Local alterations from a peripheral stimulus, as described, enable the incoming virus to replicate in epidermal cells and produce a clinical lesion. The "ganglion trigger" theory suggests that peripheral stimuli can reactivate HSV from the ganglia to produce shedding of infectious virus but no recurrent disease at the periphery unless, either, a sufficient amount of virus is produced so as to overwhelm the peripheral defence mechanisms, or, the host has a defective immune response. There is evidence that the "ganglion and skin trigger" theory provides the most accurate account of induced recurrent HSV disease. It has also been suggested that HSV reactivation in the ganglion is a constant occurrence, although the infection does not always produce a herpetic lesion (Hill and Blyth, 1976; Hill, 1985). Since productive HSV infection destroys host cells and since there is no evidence for a decrease in the number of cells harboring latent HSV with increasing recurrences, it has been suggested that, whenever a recurrent lesion occurs, HSV produced travels up the peripheral nerve and latently infects more neurons, the so-called "round trip" hypothesis (Klein, 1976).

In the mouse model, peripheral stimuli (skin triggers) which lead to the reappearance of infectious HSV in the skin of the ear and in the cervical dorsal root ganglia include exposure of the peripheral site of inoculation to UV light (Blyth *et al.*, 1976), repeated applications of cellophane tape (Hill *et al.*, 1983) or chemicals such as dimethylsulphoxide (DMSO) or xylene (Harbour *et al.*, 1983). There is some evidence for an effect on local immune responses and/or the susceptibility of skin cells to infection with HSV in response to such peripheral

stimuli, which might involve depletion of Langerhans cells (macrophage-like cells) in the epidermis and the production of prostaglandins (hormone-like substances produced locally in tissues) (Sprecher and Becker, 1988; Hill, 1985). Injection of prostaglandin E_2 also induces infectious virus in the skin (Blyth et al., 1976).

Reactivation in the ganglion presumably results from the disruption of the processes which maintain HSV in a latent state. To resolve the molecular mechanisms involved in both maintenance of latency and reactivation, it is essential to examine the action of the stimuli which trigger reactivation in the ganglion.

Reactivation can be induced experimentally by several techniques including dissection of the nerve root associated with the latently infected ganglion (Walz et al., 1974; Price and Schmitz, 1978) and explantation of latently infected ganglia or other nervous tissue (Stevens et al., 1971). The possible effects of nerve damage on the physiological state of neurons have been outlined (Section 1.3.3.1.). Reactivation from explanted latently infected ganglia can be enhanced by cocultivation of intact or dissociated ganglia on permissive cell monolayers (Stevens et al., 1972; Knotts et al., 1973; Wohlenberg et al., 1979; Harbour et al., 1981), by intertypic superinfection (Thomas et al., 1985) of ganglia or by exposure of ganglia to demethylating agents (Bernstein and Kappes, 1988; Stephanopoulos et al., 1988).

Methylation of cytosine bases in DNA is associated with transcriptional inactivity. DMSO, which can cause hypomethylation of cellular DNA (Christman et al., 1977), has been shown to induce reactivation of HSV in ganglia (Hill et al., 1983; Harbour et al., 1983); 5-azacytidine, which functions to prevent methylation of DNA and therefore is effective in replicating cells, and HMBA, a demethylation agent, both induce reactivation of latent HSV from explants of neural and non-neural tissues (Bernstein and Kappes, 1988; Stephanopolous et al., 1988). Since only a very small region of the HSV genome (LAT) is transcribed during latent infection (Stevens et al., 1986; Section 1.3.4.2.), one possibility is that HSV is maintained in a latent state in

neurons by extensive methylation; exposure of latently infected cells to demethylating agents might cause activation of HSV gene expression directly or indirectly by conversion of the host cell to a permissive state for HSV replication via cellular gene activation. Latent EBV is heavily methylated (Kintner and Sugden, 1981; Diala and Hoffman, 1983; Saemundson et al., 1984); 5-azacytidine has been shown to induce the EBV lytic cycle in latently EBV-infected cell lines (Ben-Sasson and Klein, 1981) and activation of the lytic cycle of EBV by known inducers of differentiation has been associated with an active DNA demethylation mechanism (Szyf et al., 1985). There is also evidence for extensive methylation of the HSV genome in an in vitro latency system utilising human lymphoblastoid cells of T-cell origin (Youssoufian et al., 1982; Section 1.3.6.), of the herpes saimiri genome integrated into the genome of lymphoid tumour cells (Desrosiers et al., 1979), as well as of an inactive TK gene in mouse cells transformed by HSV, which could be activated by treatment with 5-azacytidine (Clough et al., 1982). Nonetheless, Dressler et al. (1987) have reported that latent HSV-1 is not extensively methylated in vivo.

An interesting observation has been the retrieval of HSV genetic information from latently infected ganglia, both in vitro (Brown et al., 1979; Lewis et al., 1984) and in vivo (Thomas et al., 1985), by heterotypic HSV superinfection. Previous observations had suggested that ganglia colonised with one strain of herpes were resistant to establishment following superinfection by another strain. However, the results of Thomas et al. (1985) suggest that it is possible to superinfect a latently infected ganglionic neuronal cell with a heterotypic HSV strain and that the superinfecting virus acts in trans to induce reactivation of the latent HSV. Superinfection experiments might aid in a resolution of the events involved in reactivation.

1.3.4. The Molecular Biology of HSV Latency

1.3.4.1. The Latent HSV Genome

Several groups have reported the detection of HSV or HSV DNA sequences in neurological tissue from latently infected animals (Stevens and Cook, 1971; Stevens et al., 1972; Knotts et al., 1973; Walz et al., 1976; Puga et al., 1978; Brown et al., 1979; Cabrera et al., 1980; Fraser et al., 1981; Puga et al., 1984; Rock and Fraser, 1983; Kennedy et al., 1983; Stroop et al., 1984). From the results of infectious centre assays, performed on cells from dissociated dorsal root ganglia of mice acutely (6 to 8 days post inoculation) or latently (6 weeks to 15 months post inoculation) infected with HSV, it was estimated that infectious virus could be recovered from 1.0% of the cells from the acute phase and 0.1% of the cells from the latent phase (Walz et al., 1976). As the neuron is accepted to be the site of latent HSV (Cook et al., 1974; McLennan and Darby, 1980) and the dorsal root ganglion contains 5% to 10% neurons, approximately 2% of the neuronal cells harboured latent HSV which could be reactivated (Walz et al., 1976). In a similar study, Kennedy and coworkers (1983) deduced that, in mice latently infected with HSV, 0.4% of neuronal cells from dissociated dorsal root ganglia harboured a latent HSV genome with reactivation potential. Obviously, sequences representing the entire HSV genome must reside in at least this proportion of latently infected neurons. Southern hybridisation studies using cloned HSV DNA fragments as probes have demonstrated that DNA sequences from all regions of the genome are detectable in latently infected tissue from mice and humans (Rock and Fraser, 1983; Fraser et al., 1981; Efstathiou et al., 1986), as a consequence of which, defective genomes are unlikely to play a major role in HSV latency.

Levels of viral-specific DNA have been measured, in trigeminal ganglia of mice during the acute and latent stages of infection, by analysis of the reassociation kinetics of a trace of high specific activity ^{125}I-labelled virus DNA mixed in liquid phase with a vast excess of

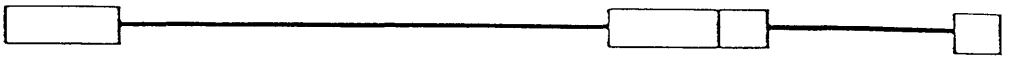
ganglionic DNA. DNA was detected at 1.2 to 2.0 genome equivalents per cell during the acute stage and at approximately 0.1 genome equivalents per cell during the latent stage (Puga et al., 1978). Southern hybridisation analyses have produced estimates, for the mean number of latent HSV genomes in mice ganglion, of 0.015 to 0.15 (Rock and Fraser, 1983) and of 0.78 (Efstathiou et al., 1986) copies per cell genome equivalent. Latent HSV DNA has also been detected in the mouse central nervous system (Rock and Fraser, 1983; Stroop et al., 1984; Efstathiou et al., 1986), at mean concentrations of 0.26 copies per cell for brainstem tissue and 0.19 copies per cell for spinal cord tissue, and in human trigeminal ganglia, at 0.01 to 0.1 viral genomic copies per cell genome equivalent (Efstathiou et al., 1986). Since 5-10% of cells in mice ganglia are neuronal (Walz et al., 1976) and assuming that only 0.4-2% of neurons harbour latent HSV, each latently infected cell could contain up to 3900 copies of the latent genome. This may be an overestimate because evaluation of the percentage of neurons which harbour latent HSV was based on the amount of virus reactivated following explantation and cocultivation of ganglia, while it is possible that HSV resides in some neurons in a latent state from which it may not be induced to reactivate by this procedure, such as has been demonstrated in brain tissues of mice (Cabrera et al., 1980) and in ganglia of humans (Brown et al., 1979). Recent in situ hybridisation analysis studies have revealed that a latency associated transcript (Section 1.3.4.2.) is present in 3% of neurons from the trigeminal ganglia of rabbits latently infected with HSV-1 (Rock et al., 1987a). The significance of the copy number per cell of latent HSV will be considered in Section 5.0..

Analysis of the physical structure of HSV DNA from mouse and human neurological tissues may provide useful information concerning the molecular mechanisms involved in HSV latency. Latent HSV genomes could exist as unit length, linear molecules, characteristic of virion DNA, or in an alternative state (Figure 1.7.). Circularisation or concatemerisation of the genome results in the loss of the terminal restriction endonuclease fragments and the

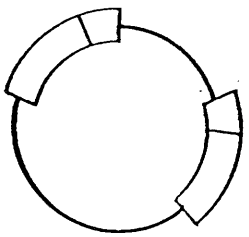
Figure 1.7. SCHEMATIC REPRESENTATION OF POSSIBLE PHYSICAL STATES OF THE LATENT HSV GENOME

Virion DNA is unit length and linear, whereas latent HSV DNA is endless, contains bimolar joint fragments and may therefore be circular, concatemeric, concatemeric and integrated into the cellular genome, or unit length, linear and integrated into the cellular genome via regions of the viral genome other than the termini (Rock and Fraser, 1983; Rock and Fraser, 1985; Efstathiou et al., 1985).

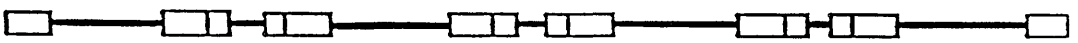
UNIT LENGTH, LINEAR:



ENDLESS:

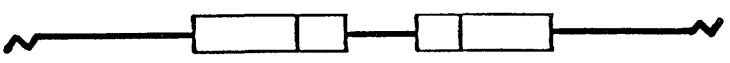


Circular



Concatemeric

Integrated, Concatemeric



Integrated, Linear

consequent production of an additional joint fragment, such that the molar ratio of joint to unique sequences tends to 2:1. This characteristic has been exploited in analysis of the latent state of the genome; cloned radiolabelled DNA fragments representing the joint and terminal regions of HSV have been used to probe DNA from latently infected tissue in Southern hybridisation analyses.

In a report by Puga et al. (1984), novel-sized DNA fragments, hybridising to an HSV terminal fragment probe, were detected in DNA, from trigeminal ganglia of latently infected mice, enriched for viral DNA by reverse phase chromatography. The finding led these workers to conclude that structural changes, such as may result from extensive gene rearrangement or integration into a limited number of sites in the cellular genome via the terminal regions, had occurred in the genome concomitantly with the establishment of latency.

However, in contrast to these findings, Rock and Fraser (1983, 1985), also in an analysis of DNA from trigeminal ganglia and brains of latently infected mice, demonstrated a lack of terminal fragments of HSV DNA in the latently infected tissues, despite the presence of HSV sequences from other regions of the genome (Rock and Fraser, 1983). Virion joint fragments were shown to be present at an approximately 2:1 molar ratio with respect to unique sequences of HSV (Rock and Fraser, 1985). It was therefore proposed that the majority of HSV DNA which resides in latently infected cells exists in an "endless" state and was not unit length and linear, extensively rearranged or integrated into the cellular genome via the terminal fragments.

Efstathiou et al. (1986) confirmed the presence of HSV DNA in an "endless" configuration in DNA from the trigeminal ganglia, brainstems and spinal cords of latently infected mice, showed that the viral DNA was maintained in a stable form in these tissues over a 4 month period and, further, demonstrated that HSV DNA from human trigeminal ganglia was also harboured in an "endless" state. All 4 isomeric forms of the HSV genome were present in tissues from both latently infected mice and humans (Efstathiou et

al., 1986). It is possible, therefore, that the novel fragments detected by Puga et al. (1984) were entirely cellular, since there are homologies between cellular DNA sequences and HSV DNA sequences (Peden et al., 1982; Puga et al., 1982), and it may be either that the procedure used had enriched for specific cellular DNA sequences, or that latent HSV infection had caused amplification of certain cellular DNA sequences which were then enriched for by the technique employed.

The presence of "endless" molecules of HSV in latently infected cells could be indicative of circularisation or concatemerisation of the genome or of integration of the genome into the cellular chromosome either as a concatemer or via regions of the genome other than the termini (Figure 1.7.). In an attempt to distinguish between possibilities, Mellerick et al. (1987) examined the HSV-1 genome in both acutely and latently infected mice by caesium chloride buoyant density centrifugation. It was found that most HSV-1 specific DNA from latently infected mouse brains banded at the buoyant density of virion DNA. A small fraction banded at the density of the mouse chromosomal DNA, although the amount of hybridisation of this fraction to an HSV-1 specific probe did not significantly exceed that which arose due to the cross-hybridisation of HSV-1 and uninfected mice brain DNA. No latent HSV-1 banded as extrachromosomal DNA following re-centrifugation of the chromosomal DNA fraction in caesium chloride, suggesting that the latent HSV-1 DNA is not an extended replicating intermediate but rather a compact form that resists trapping. Mellerick et al. (1987) therefore concluded that the majority of the latent HSV DNA exists in an extrachromosomal state in the mouse model, probably in a circular episomal form, although this has yet to be confirmed.

The majority of HSV DNA in a productive infection would be likely to be replicated and linear, and indeed, it has been shown that HSV-1 DNA in acutely infected brain tissue is in the linear configuration characteristic of virion DNA (Rock and Fraser, 1983). Therefore, the majority of HSV DNA in the brain and trigeminal ganglia of latently

infected animals and humans is unlikely to be participating in a productive infection, since the evidence suggests that latent HSV DNA is "endless".

1.3.4.2. HSV Gene Expression in Latently Infected Cells

A competent viral genome can reside in a latent state within a cell which is potentially capable of allowing it to multiply only if viral or host cell factors are present which function to establish and/or maintain the latent state. The theory that HSV has developed functions involved in latency is based on the argument that it is advantageous for HSV to reside in a latent state from which spontaneous reactivation may occur because latent HSV, in its protective site in cells of the nervous system, can avoid immune elimination and thereby provide a significant reservoir for production of the herpetic lesions which are involved in dissemination of the virus in the human population.

There have been 2 main approaches to the determination of which, if any, HSV genes are involved in latency; investigation of the HSV genes essential for establishment, maintenance and/or reactivation, and an investigation of the HSV genes expressed by the latent viral genome.

The former approach initially centred upon a study of the ability of various ts HSV mutants to establish and maintain a latent state in the nervous system of the mouse, the core temperature of which (38.5^{o}C) is restrictive for these mutants. Studies utilised the available HSV-1 and HSV-2 ts mutants and the mouse latency model system (Lofgren *et al.*, 1977; Watson *et al.*, 1980; Al-Saadi *et al.*, 1983); mutant virus detectable following explant and cocultivation of appropriate tissues at the permissive temperature for mutant growth was judged to have established latency. Two classes of mutants were identified, those which established latency efficiently, and those which did not. Since HSV-1 and HSV-2 mutants both capable and incapable of DNA synthesis at the restrictive temperature could establish a latent infection by these criteria, viral DNA synthesis was proposed to be irrelevant to the establishment of latency. A

loose correlation between paucity of virus-specific morphological products at the restrictive temperature and latent infections was apparent; also, there was no obvious clustering of genes apparently associated with latent infection (Watson et al., 1980; Al-Saadi et al., 1983). Watson and associates (1980) proposed that the expression of Vmw175 is a prerequisite for establishment of the latent state, on the basis that an HSV-1 mutant, tsK, in which a ts mutation involves the gene encoding IE Vmw175, was unable to establish a latent infection in mice by their criteria. The mutant tsI, which produces most HSV polypeptides and synthesises DNA at the NPT, was also apparently unable to establish a latent infection, which suggested that additional viral genes, produced late in infection, could be involved in establishment. In subsequent studies, a revertant of tsI, RI-1, which produces wt yields of virus at 38.5^{o}C (the NPT for tsI), was tested for its ability to establish a latent state in lumbosacral ganglia of mice following footpad inoculation; RI-1 was able to establish a latent state in the ganglia but appeared to be ts for reactivation by explantation and cocultivation. This finding suggests that tsI contains multiple ts mutations, one of which might effect a gene required for reactivation, but does not indicate whether or not tsI possesses a mutation in a gene essential for the establishment of latency (Cook et al., 1986). Complexities in the interpretation of data from such studies are discussed in Section 4.3.6.; the subsequent availability of insertion and deletion mutants of HSV provided better material for latency studies in animals. The ability of a deletion or insertion mutant of HSV to establish a latent state in a mouse or rabbit experimental model system following peripheral inoculation would suggest that there was no absolute requirement in latency for the function(s) affected by the mutation. Preliminary studies with a TK^{-} mutant, selected by growth in arabinofuranosylthymine (ara-T) which inhibits replication of TK^{+} HSV, had suggested a requirement for TK in efficient establishment, although a role for other possible defects in the TK^{-} mutants could not be excluded (Tenser et al., 1979). However, it has recently been demonstrated, utilising a

well-characterised TK^- mutant, HSV-1(F) 305, with a 700bp deletion in the TK gene (Post et al., 1981), that the viral TK gene is not essential for establishment of latency; interestingly, this TK^- mutant was able to establish a latent infection in rabbits but was unable to establish latency in mice (Meignier et al., 1988). Experiments utilising insertion and deletion mutants of HSV have shown that genes for IE Vmw110 (Clements and Stow, 1989), IE Vmw12 (Meignier et al., 1988), IE Vmw68, TK (Sears et al., 1985a,b; Meignier et al., 1988), glycoproteins G and E, α viral protein kinase (Meignier et al., 1988) and also the oriL region of the HSV genome (Polvino-Bodnar et al., 1987) are not essential for establishment (or reactivation) of latency.

In attempts to define HSV genes expressed by the latent viral genome, various techniques have been employed including hybridisation procedures for the detection of HSV RNA and immunological procedures for the detection of HSV proteins.

In the liquid-phase hybridisation studies of Puga and coworkers (1978), in which HSV-specific RNA was measured in murine trigeminal ganglia during the acute and latent stages of infection, virus-specific RNA was found at the acute stage but not during the latent stage of infection. However, due to the relatively low level of sensitivity of this method, limited transcription of the latent genome could have occurred and escaped detection (Puga et al., 1978). Indeed, there have since been various reports of viral gene expression during HSV latency.

HSV polypeptides were detected in latently infected trigeminal ganglia of rabbits by indirect immunofluorescence using antisera to HSV-1, and specifically the IE polypeptide Vmw175 was detected using monospecific antisera to Vmw175 (Green et al., 1981). In subsequent studies, no Vmw175 protein was detected in the trigeminal ganglia of mice using antisera to Vmw175 and the avidin-biotin complex immunoperoxidase method (Pepose et al., 1986), and, furthermore, no Vmw175 mRNA was detected, using in situ hybridisation analysis with cloned radioactively labelled fragments, in the trigeminal ganglia of rabbits (Rock et

al., 1987a). It is, however, possible that Vmw175 is produced during establishment of latency in neurons of the rabbit trigeminal ganglia and persists in these cells, although spontaneous reactivation cannot be ruled out as an explanation for the detection of Vmw175 in latently infected trigeminal ganglia of rabbits.

Despite a report that enzymatically active HSV-specific TK could be detected in sensory ganglia of latently infected mice (Yamamoto et al., 1977), it has been shown, as previously mentioned, that the early HSV-1 gene TK is not essential for establishment of a latent infection (Sears et al., 1985b; Meignier et al., 1988) and, furthermore, that TK mRNA is undetectable in latently infected mouse, rabbit or human ganglia by in situ hybridisation analysis (Stevens et al., 1987; Puga and Notkins, 1987; Deatly et al., 1987; Rock et al., 1987a; Steiner et al., 1988).

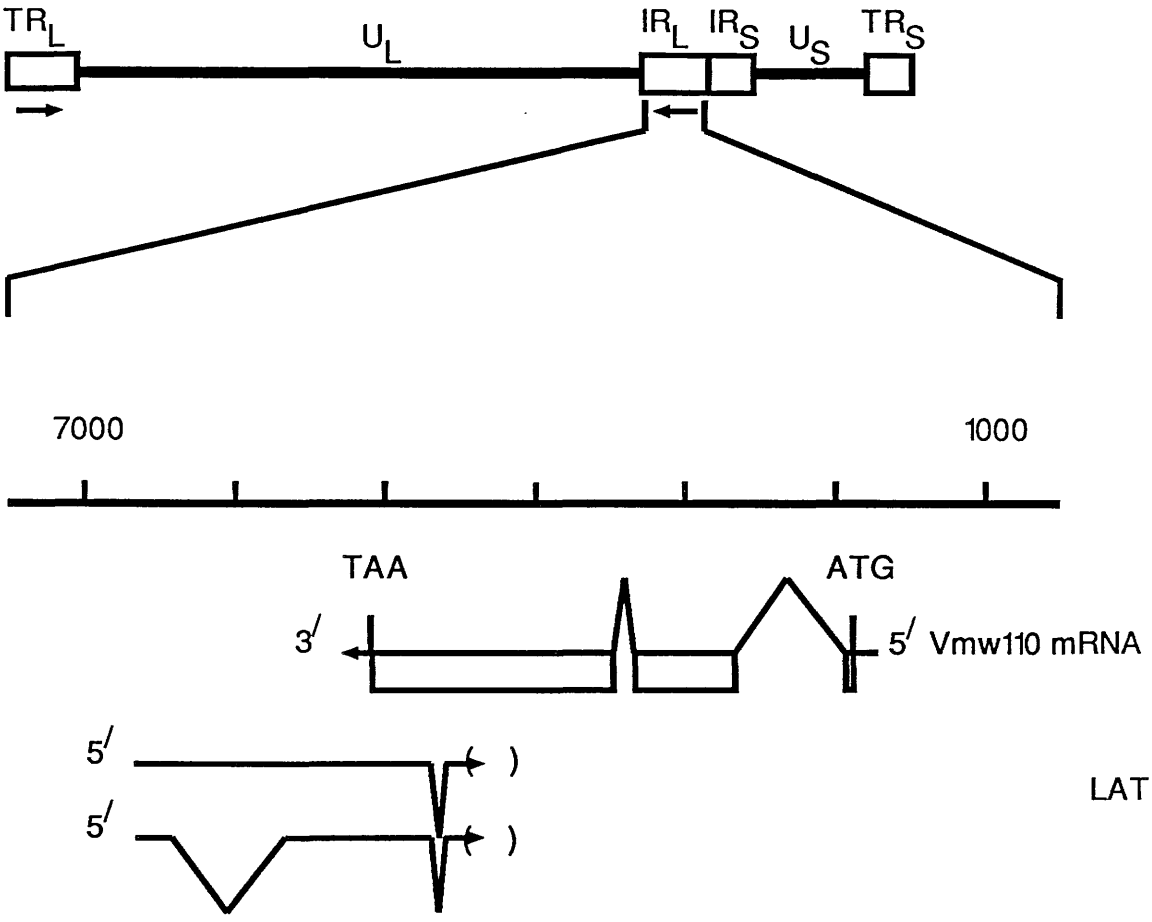
In situ hybridisation studies using cloned fragments representing the HSV genome have, however, demonstrated that HSV-2 specific RNA is present in latently infected human paravertebral ganglia removed from humans at autopsy (Galloway et al., 1979; 1982) and in latently infected trigeminal ganglia of guinea pigs (Tenser et al., 1982). HSV-1 specific RNA has been detected in the trigeminal ganglia and olfactory systems of mice (Stroop et al., 1984; Stevens et al., 1987; Deatly et al., 1987; Puga and Notkins, 1987) and in human trigeminal ganglia (Steiner et al., 1988) during HSV-1 latent infection.

A recent major development has been the discovery, initially by Stevens and associates (1987) using in situ hybridisation with probes from defined regions of the genome, that the viral-specific RNA detectable in the sensory ganglia of latently infected mice, rabbits and humans hybridises to the R_L region of the HSV-1 genome which encodes the IE polypeptide Vmw110 (Stevens et al., 1987; Puga and Notkins, 1987; Deatly et al., 1987, 1988; Rock et al., 1987a; Steiner et al., 1988). Surprisingly, the RNA is transcribed from the strand opposite from that encoding Vmw110 (Stevens et al., 1987; Rock et al., 1987a; Figure 1.8.). This RNA, known as the latency-associated

Figure 1.8. THE GENOMIC LOCATION OF THE LATENCY-ASSOCIATED TRANSCRIPT

The location of LAT (Section 1.3.4.2.) in the IR_L region of the HSV genome is shown relative to the position of Vmw110 mRNA. Spliced regions are shown as raised sections; all copies of LAT are spliced. The exact position of the 3' end of LAT is not known. Numbering of nucleotides begins at the IR_L junction 'a' sequence (Perry et al., 1986).

This figure was kindly provided by R. Harris.


TR_L
U_L
IR_L
IR_S
U_S
TR_S
7000
1000
TAA
ATG
3′
5′
Vmw110 mRNA
5′
5′
LAT

transcript(s) (LAT), was localised to the neuronal nucleus and was present in relatively high abundance (Stevens et al., 1987; Deatly et al., 1987, 1988; Rock et al., 1987a; Steiner et al., 1988).

There are at least 1 and possibly 2 LATs; an abundant major LAT detected in approximately 3% of the neurons from latently infected rabbit trigeminal ganglia which hybridises to the HSV-1 BamHI B restriction fragment and a less abundant LAT present in less than 0.3% of the neurons which hybridises to the HSV-1 BamHI K fragment (Rock et al., 1987a). At least 2 latency-related RNA bands, of 1.8 to 2.3kb and of 1.2 to 1.5kb, have been identified by Northern blot analysis of RNA from latently infected trigeminal ganglia. These RNAs map within the HSV-1 BamHI restriction fragment B and partially overlap the 3' terminus of IE-1 on the opposite DNA strand (Spivack and Fraser, 1987). The 1.8-2.3kb RNA has also been detected at low levels in HSV-1 infected cultured cells and in trigeminal ganglia of mice during the acute phase of infection, in contrast to the 1.2-1.5kb RNA which is not detectable in HSV-1 infected cultured cells and which is only found in mice during the latent phase of infection (Spivack and Fraser, 1987; Spivack and Fraser, 1988a). Studies of Spivack and Fraser (1988a) suggested that the 1.8-2.3kb RNA was not regulated as an IE gene in cultured cells, since protein synthesis inhibition was shown to prevent the expression of the LAT gene, but this conclusion was not supported by the studies of Wagner et al. (1988b) in which expression of the LAT gene occurred despite protein synthesis inhibition. Furthermore, during latent infection the LATs appear to be expressed in the absence of detectable levels of IE gene expression (Stevens et al., 1987; Rock et al., 1987a; Spivack and Fraser, 1987) suggesting that the LATs are transcribed as IE transcripts. The genomic location of the abundant 1.8-2.3kb RNA has been determined by Northern blot hybridisation using radioactively-labelled subclones of the BamHI B fragment and, more accurately, by S1 nuclease and primer extension analyses. The 5' end is located approximately 1210 nucleotides downstream from the 3' end of IE-1, while the 3' end, which cannot be determined precisely as there are no

polyadenylation sites close to the mapped end, overlaps IE-1 by approximately 1000 nucleotides, (Wagner et al., 1988; Wechsler et al., 1988). Recent fine mapping studies indicate that the 1.8-2.3kb and 1.2-1.5kb RNAs appear to share their 5' and 3' ends and that they are produced by alternative splicing (Wechsler et al., 1988; Wagner et al., 1988; Figure 1.8.); splicing to produce the 1.2-1.5kb RNA appears to be specific to latently infected neurons (Spivack and Fraser, 1988a; Wagner et al., 1988). The LAT detected by in situ hybridisation with the HSV-1 BamHI K restriction fragment, located to the right of the BamHI B restriction fragment, is physically separated from the BamHI B associated transcripts; this transcript maps to the 5' end of IE Vmw110 and might represent an additional minor exon resulting from splicing in the 3' region of the major LAT to within BamHI-K (Wechsler et al., 1988), but it is present in such small amounts that its size has not yet been determined by Northern blot analysis (Rock et al., 1987a).

The sequences of the major LAT are known for HSV-1 strains 17 and KOS, the homology between which is high (Perry and McGeoch, 1988; Wagner et al., 1988a). The main features of the major LAT sequence are as follows. Interestingly, there is an RNA polymerase III B element homology (Galli et al., 1981) at the cap site, although there is no A element 5' to this region (Wagner et al., 1988a). The sequence 5' to the cap site was proposed by Wagner and associates (1988a) to have some features of an RNA polymerase II promoter, including a weak TATA box homology approximately 25 bases 5' of the cap site and 2 elements of poor homology to CCAAT boxes (CAGTA and CACT). However, computer analysis of the sequence upstream of the cap site of LAT in HSV-1 17 syn^{+} (Perry and McGeoch, 1988) suggested that the first potential TATA box was as far away as position -686 (note that the predicted 5' end of the major LAT TCCAGGTAG is designated position 1, Wechsler et al., 1988), that the first likely CCAAT box began at position -817 and that three GC boxes (Sp1-binding sites) were located starting at positions -886, -862 and -589. A second potential promoter region, including a TATA box beginning at position +827 and upstream accompanying GC rich

regions, was located just upstream of the longest potential open reading frame in the LAT gene. Potential splicing sites were located at positions predicted by hybridisation data (Wechsler et al., 1988; Wagner et al., 1988b). There are no consensus polyadenylation signals in close proximity to the 3' end of LAT (Perry and McGeoch, 1988; Wagner et al., 1988a; Wechsler et al., 1988), a finding which correlates well with the results of Wagner et al. (1988a) who showed that the majority of the 1.8-2.3kb RNA is present in the poly $(A)^-$ fraction of RNA from latently infected cells. However, Wagner et al. (1988a) also reported that a small amount of the 1.2-1.5kb RNA species detected by Northern blot hybridisation can be eluted in the poly $(A)^+$ fraction. Although there are open reading frames (ORFs) of significant size within the genomic sequence, the codon contents of these ORFs are not characteristic of other parts of the HSV genome, and it has therefore been suggested that LAT is unlikely to encode a protein (Perry and McGeoch, 1988). Furthermore, analysis of latently infected cells using antipeptide antisera raised against predicted sequences of the LAT ORF has not resulted in the detection of a protein product from this region of the genome (E. Wagner, quoted at the 1988 Herpesvirus Workshop, Los Angeles, USA).

Several roles have been proposed for the major LAT gene in HSV latency (Stevens et al., 1987). An attractive possibility is for LAT gene expression to produce an effect on the expression or processing of the mRNA encoding Vmw110, since the LAT gene overlaps the IE-1 gene and because there is evidence that Vmw110 is involved in the regulation of HSV gene expression, absence of which might, therefore, result in the establishment of a latent state (Section 5.0.). LAT RNA might be involved in antisense suppression of Vmw110 production by 3' or 5' complementarity (Green et al., 1986; Kim et al., 1985) or as a trans-acting factor in the regulation of expression from IE-1 or other HSV genes. In favour of the involvement of LAT RNA are its localisation to the nuclei of cells from latently infected ganglia, as shown by in situ hybridisation analyses (Stevens et al., 1987; Rock et al., 1987a; Deatly et al., 1987, 1988; Steiner et al., 1988), and the apparent absence, in studies using

antipeptide antisera, of a protein product in latently infected cells. However, as previously mentioned, there are putative open reading frames within the LAT gene nucleotide sequence (Perry and McGeoch, 1988; Wagner et al., 1988a) and therefore, if there is synthesis, at low levels, of LAT gene product(s), such protein(s) might be involved in latency in some regulatory capacity influencing the expression of HSV genes. A further possibility, suggested by Rock and coworkers (1987), is that transcription of the LAT gene might have a cis-acting effect on sequences in its proximity, perhaps preventing the transcription of IE-1 or other HSV genes.

It is worth noting that a similar transcript has been detected in the neuronal cells of rabbit trigeminal ganglia latently infected with bovine herpesvirus (BHV-1) which maps to a region of the BHV-1 genome encoding a major IE gene, indicating the possibility of a role for such transcripts in both HSV and BHV-1 latency. Even so, there is no evidence for a high degree of sequence conservation between the LAT RNAs, as probes containing the HSV-1 and BHV-1 LAT genes failed to cross-hybridise (Rock et al., 1987b). Interestingly, a 1400bp sequence without an assigned function exists in the genome of VZV at a position corresponding to the LAT region of HSV-1 (Davison and Scott, 1986; Perry and McGeoch, 1988).

Work is currently underway to determine the importance of the LAT gene in HSV latency. Recent studies in which HSV mutants, apparently incapable of expressing LAT, have been tested for the ability to establish a latent infection imply that LAT is not essential for HSV latency (Wagner et al., 1988a; Steiner et al., 1989).

1.3.5. The Molecular Biology of EBV Latency

Lymphocytes latently infected with EBV in vitro or in vivo proliferate indefinitely in tissue culture, retain the complete EBV genome in episomal form and express a limited set of the approximately 80 to 90 genes encoded by the EBV genome (Dambaugh et al., 1986). Some latently infected lymphocytes become permissive for EBV replication at low

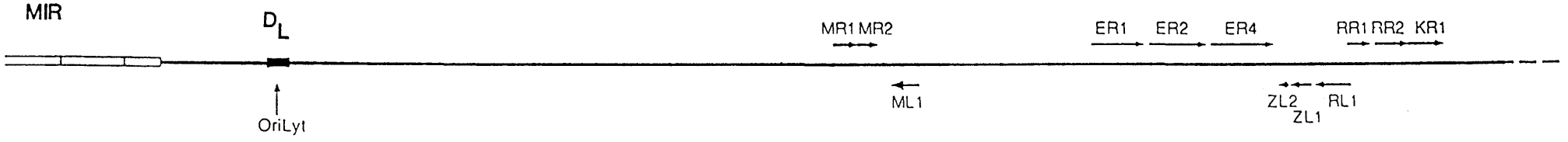
frequency, while certain EBV infected cell lines, such as Namalwa (an African BL cell line) and IB4 (a cell line established by infection and growth transformation of normal human foetal lymphocytes with EBV B95-8), are almost or completely non-permissive for productive infection (Klein and Dombos, 1973; King et al., 1980) and are therefore used for analysis of viral genes involved in latency. EBV, in immortalised B cells Raji (a Burkitt's lymphoma non-producer cell line) and B95-8 (a producer cell line isolated from marmoset lymphocytes transformed with EBV B95-8 DNA), has been induced to enter the lytic cycle by treatment with various chemicals, by superinfection with EBV, or by transfection of specific EBV DNA fragments into latently infected cells. Activation of the latent genome results in the production of over 50 mRNAs (King et al., 1981; Hummel and Kieff, 1982); polyadenylated RNAs expressed during latent infection are not detected in productively infected cells (Hummel and Kieff, 1982). Specific transcripts, from regions of the EBV genome including BamHI M, BamHI Z and BamHI R (Figure 1.9.), are classified as IE RNAs since they can be produced in the absence of protein synthesis (Sample et al., 1984; Biggin, M., Dyson, P.J. and Farrell, P.J., Herpesvirus Workshop, Ann Arbor, Michigan, 1985). In B95-8 infected cells induced to permissive infection with TPA, Hummel and Kieff (1982) identified a persistent early class of RNAs and a late class of RNAs, the abundances of which were relatively resistant or sensitive, respectively, to viral DNA synthesis inhibition. Genes have been identified which code for transcription trans-activators, proteins involved in replication of the EBV genome and glycoproteins (McGeoch, 1989). An origin of replication (oriLyt), composed of elements of 321bp and 374bp separated by a 263bp non-essential region and containing multiple repeats, has been identified, which functions in lytic infection (Figures 1.1., 1.9.). EBV replicated during the lytic phase is in concatemeric configuration (Hammerschmidt and Sugden, 1988). The onset of the lytic cycle leads irreversibly to cell death.

Figure 1.9. **GENOMIC LOCATIONS OF IMMEDIATE EARLY TRANSCRIPTS OF EPSTEIN-BARR VIRUS**

Part of the MIR and the U_L regions of the EBV genome (see also Figures 1.1. and 1.10.) with the locations of EBV IE RNAs BZLF1, BRLF1, BMLF1 and BMRF1 and an oriLyt sequence are shown.

Abbreviations are as follows:

YR1	- BamHI Y rightward reading frame 1
MR1 (MR2)	- BamHI M rightward reading frame (BMRF) 1 (2)
ML1	- BamHI M leftward reading frame (BMLF) 1
ER1 (ER2,ER3)	- BamHI E rightward reading frame 1 (2,3)
ZL1 (ZL2)	- BamHI Z leftward reading frame (BZLF1) 1 (2)
RL1	- BamHI R leftward reading frame (BRLF1) 1
RR2 (RR3)	- BamHI R rightward reading frame 2 (3)
KR1	- BamHI K rightward reading frame 1
oriLyt	- origin of DNA replication during lytic cycle at direct repeat left (D_L)
MIR	- major internal repeat consisting of direct 3072bp repeats (open box regions)


MIR
D_L
MR1 MR2
ER1 ER2 ER4
RR1 RR2 KR1
OriLyt
ML1
ZL2 RL1
ZL1

1.3.5.1. The Latent EBV Genome

Latently infected immortalised lymphocytes usually contain more than one copy of the complete EBV genome (zur Hausen et al., 1970; Nonoyama and Pagano, 1971, 1973; Kawai et al., 1973). Nearly all of the human B-type, lymphoid cell lines established in continuous culture maintain a characteristic number of latent EBV genomes, for example, the Raji cell line contains 50 to 60 copies of EBV per cell (Nonoyama and Pagano, 1973; Pritchett et al., 1976). In virions EBV DNA is linear, whereas the bulk of EBV DNA in most latently infected cell lines is in the form of covalently closed circular episomes (Nonoyama and Pagano, 1972; Tanaka and Nonoyama, 1974; Lindahl et al., 1976; Kaschka-Dierich et al., 1977), formed by covalent linkage between the terminal repeat (TR) sequences of the genome (Dambaugh et al., 1980). EBV DNA in latently infected cells is thought to be replicated by cellular DNA polymerase (Summers and Klein, 1976; Colby et al., 1980). The DNA multiplies in tandem with the replication of the B lymphocyte, but the stable retention of a constant number of genomes in actively dividing cells cannot be explained simply by the semi-conservative replication of viral DNA as an integral part of the host chromosome (Adams, 1987). Doubling of EBV DNA occurs early during the cellular S phase of non-producer Raji cells (Hampar et al., 1974); electron microscopy studies suggest that episomal forms of the EBV DNA act as independent replicons (Gussander and Adams, 1984). A Meselson-Stahl density transfer experiment indicated that the majority of latent EBV DNA molecules each replicate once during the cell cycle (Adams, 1987). A single replication origin, oriP, has been identified which is required for EBV plasmid replication (Yates et al., 1984, 1985; Reisman et al., 1985) (Figure 1.10.). In cell lines harbouring one or few copies of the EBV molecule, such as Namalwa (which contains only one copy; Pritchett et al., 1976) and IB4 (which contains several episomal copies; King et al., 1980), it has been possible to investigate the integration of EBV DNA into the cellular genome. In Namalwa and IB4 the EBV genomes were found to be collinear with

Figure 1.10. GENOMIC LOCATIONS OF EPSTEIN-BARR VIRUS LATENCY-ASSOCIATED TRANSCRIPTS

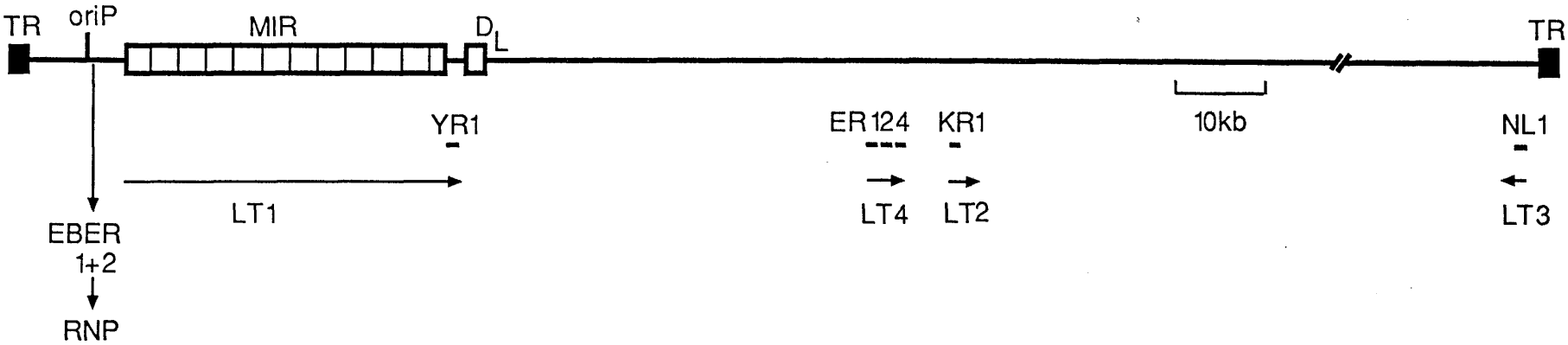
Regions of EBV associated with gene expression during latency include BamHl WYH (LTl), BamHI K (LT2), BamHI Nhet (LT3) and BamHI E (LT4). The locations of EBV latency-associated transcripts within these regions (YRl, ERl, ER2, ER4, KRl and NLl - named according to the following format; YRl = BamHI Y rightward reading frame 1 [BYRFl]) are shown.

The translation products of the transcripts are as follows:

YRl - EBNA2 (EBV nuclear antigen 2)
ERl - EBNA3A (EBNA3)
ER2 - EBNA3B (EBNA4)
ER4 - EBNA3C (EBNA6)
KRl - EBNAl
NLl - LMP (latency membrane protein)

The small non-polyadenylated RNAs, EBERl and EBER2, which are incorporated into ribonucleoprotein particles (RNP), and the oriP origin of replication are also shown.

TR = terminal repeat
MIR = major internal repeat


TR
oriP
MIR
D_L
TR
YR1
ER 124
KR1
10kb
NL1
LT1
LT4
LT2
LT3
EBER
1+2
RNP

linear virion DNA, however, recombination of EBV DNA into the cellular genome had occurred on Namalwa chromosome 1 and IB4 chromosome 4 (Henderson et al., 1983). EBV DNA integration into the IB4 genome was not via TR, and is likely to have been via an episomal intermediate. Integration of EBV DNA via TR into the Namalwa genome was accompanied by a deletion of more than 15kb of host DNA (Matsuo et al., 1984; Kieff et al., 1984). The biological significance of EBV DNA integration has not yet been resolved.

1.3.5.2. EBV Gene Expression in Latently Infected Cells

Latent EBV DNA is heavily methylated, unlike virion DNA (Kintner and Sugden, 1981; Diala and Hoffman, 1983; Saemundson et al., 1984), which is, perhaps, significant, since only a limited number of regions of the genome are expressed during latent infection. Four of the regions known to code for latent polyadenylated transcripts are: BamHI WYH (LT1), BamHI K (LT2), BamHI Nhet (LT3), BamHI E (LT4) (Figure 1.10.). These regions give rise to at least six different nuclear proteins and the latent membrane protein LMP.

EBNA1, a protein of M_R 65,000-80,000 encoded by the first rightward reading frame of BamHI K (BKRF1) (Sample et al., 1986; Speck and Strominger, 1985), binds to repetitive sites within a cis-acting element of EBV, a putative ori sequence (oriP) (Figure 1.10.); both trans-acting EBNA1 and oriP are required for the replication of plasmids derived from EBV (Yates et al., 1984, 1985; Reisman et al., 1985; Rawlins et al., 1985). Furthermore, since EBNA1 is a trans-acting activator of transcription from promoters linked to a cis-acting component of oriP, it may be required for the expression of viral genes, or possibly cellular genes containing EBNA1 binding sites, in the transformed cell (Reisman et al., 1985; Reisman and Sugden, 1986; Sugden, 1986).

EBNA2, a protein of M_R 88,000 (Dambaugh et al., 1984; Hennessy and Kieff, 1983, 1985), is encoded by BYRF1 (Rowe et al., 1985; Sample et al., 1986; Figure 1.10.). EBV P3HR-1

virus, which contains a 6800bp deletion within LT1 such that there is no production of EBNA2, is unable to transform lymphocytes (Miller et al., 1974; Ragona et al., 1980; Skare et al., 1985; Volsky et al., 1984; Dambaugh et al., 1986). There is evidence for the involvement of EBNA2 in initiation of cell growth transformation (Rickinson et al., 1987).

The BNhetL1 open reading frame in LT3 encodes the latent membrane protein (LMP) (Figure 1.10.), of M_R 58,000, which possesses potential transmembrane domains and an acidic carboxy terminus of 200 amino acids that appears to be located in the inner aspect of the plasma membrane of latently infected cells (Hennessy et al., 1984; Fennewald et al., 1984; Mann et al., 1985). It has been proposed that LMP, like EBNA2, could be involved in the stimulation of cell DNA synthesis and maintenance of cell proliferation (Wang et al., 1985) and could be responsible for the response of immune T cells which leads to immune cytolysis and suppression of the growth of EBV infected lymphocytes (LYDMA reactivity) (Thorley Lawson and Israelsohn, 1987).

The LT4 region contains 3 open reading frames, BERF1, BERF2b and BERF4 (Figure 1.10). The functions of the translation products, termed EBNA3A (also designated EBNA3), EBNA3B (EBNA4) and EBNA3C (EBNA6) (Hennessy et al., 1986; Petti et al., 1988; Ricksten et al., 1988), are unknown. The EBV-immortalised, non-producer cell line IB4 lacks EBNA4, which is, therefore, clearly non-essential for the latent state.

A latency-associated protein termed EBNA5 or "leader protein", again of unknown function, is translated from multiple exons in MIR, the set of large internal repeats in LT1 (Sample et al., 1986; Speck et al., 1986; Dillner et al., 1986).

Additionally, a gene which spans the ends of the EBV genome and which is, therefore, complete only when the genome is circular, encodes the so-called terminal protein which is expressed during latency and which is likely to be a membrane-inserted species (Laux et al., 1988).

Two small non-polyadenylated RNAs transcribed from U_S, EBER1 and EBER2, which are incorporated into ribonucleoprotein particles, are also detectable in

EBV-latently infected cells (Figure 1.10.). Interestingly, EBER1 and EBER2 are able to substitute for VAI and VAII in adenovirus replication (Bhat and Thimmappaya, 1983); these RNAs are similar to adenovirus VA RNAs in terms of size (King et al., 1981; Lerner et al., 1981) and their transcription by RNA polymerase III (Rosa et al., 1981; Arrand and Rymo, 1982). Possible roles for EBER1 and EBER2 in EBV latency have not yet been characterised; EBER1 and EBER2 are expressed in greater abundance in productively infected cells and are of the early RNA class (Hummel and Kieff, 1982).

1.3.5.3. Reactivation of Latent EBV

Most clones of EBV-immortalised lymphoblasts release infectious EBV, but do so inefficiently and at such low levels that detection of cell-released EBV is rare, although virus can be detected following lethal X-irradiation and cocultivation of the cells with nontransformed B lymphocytes (Wilson and Miller, 1979; Sugden, 1984). EBV latency can be overcome in producer and non-producer cell lines by exposure to the tumour promoter TPA or to other chemical inducers (Gerber, 1972; zur Hausen et al., 1978; Jeang and Hayward, 1983; Faggioni et al., 1986; Boos et al., 1987). Superinfection of EBV latently infected cells with a variant of non-transforming EBV P3HR-1 can also activate latency (Henle et al., 1970), a property which has been correlated with the presence of heterogeneous EBV DNA fragment BamHI WZhet in the EBV P3HR-1 variant genome (Miller et al., 1984; Countryman and Miller, 1985). Transfection into latently infected cells of EBV DNA fragments containing BZLF1 (a subfragment of BamHI WZhet, BamHI-Z left reading frame) (Figure 1.9.), under the control of sequences known to promote and enhance mRNA transcription in eukaryotic cells, induced early EBV antigen production in Raji cells and production of infectious virus in another latently infected cell line (Countryman and Miller, 1985; Takada et al., 1986; Chevallier-Greco et al., 1986; Grogan et al., 1987) via the activity of the IE BZLF1 gene product (Countryman et al., 1987).

EBV trans-activator R, an EBV IE protein encoded by BamHI R leftward ORF (BRLF1) (Figure 1.9.), activates expression of an EBV cytoplasmic early antigen in cotransfection experiments (Hardwick et al., 1988). The expression of EBV nuclear IE antigens encoded by BamHI M rightward (BMRF1) and leftward (BMLF1) ORFs (Figure 1.9.) has been correlated with trans-activation of transcription from heterologous promoters and, although the BMLF1 encoded trans-activator does not activate EBV E antigen expression (Hardwick et al., 1988), expression of an EBV activatable E promoter was shown to depend upon the presence of both the BZLF1 and BMLF1 gene products (Chevallier-Greco et al., 1986). A possible role for these proteins in the regulated expression of the EBV genome has, therefore, been suggested (Oguro et al., 1987).

Nevertheless, the BZLF1 gene product is both necessary and sufficient for activation of the EBV lytic cycle induced by superinfection and by transfection of EBV DNA fragments, which presumably involves switch on of E EBV genes by the BZLF1 IE transactivator protein. Surprisingly, then, the mechanism of activation by TPA seems to require de novo synthesis of cellular proteins, since neither BamHI Z, BamHI M, nor any other region of the EBV genome contains genes which are induced by TPA in the presence of cycloheximide (Laux et al., 1988). Cellular and viral DNA hypomethylation have also been associated with induction of the EBV lytic cycle (Bensasson and Klein, 1981; Szyf et al., 1985).

1.3.6. HSV Latency in Cultured Cells

In vitro HSV-host cell interactions which have been described include the following: lytic infections, in which infectious virus is produced with subsequent destruction of the host cell; persistent or carrier infections, in which expression of viral antigens and production of infectious virus occur continuously, but cultures are maintained; abortive infections, in which there may be limited expression of the viral genome but no production of infectious virus; and latent infections, in which the

complete viral genome resides within the host cell, limited gene expression may occur and no infectious virus is detectable, but the HSV genome can be reactivated with subsequent production of infectious virus. HSV infection can also result in the transformation of host cells, as described in Section 1.1.3.

The development and characterisation of *in vitro* cell culture systems for HSV which reproduce the *in vivo* latent virus-host cell interaction is essential for analysis of the intracellular molecular mechanisms involved in latency and reactivation. While the absence of variable parameters involved in the modulation and control of virus replication, such as serum antibody, cell-mediated immunity, interferon (IFN) and hormones, could be levied as a criticism of the use of *in vitro* latency systems to study latency at the molecular level, it is, in fact, these same characteristics that make such systems inherently useful due to their innate simplicity and relative reproducibility. It has been proposed that an ideal HSV latency system would possess the following characteristics: survival of infected cells; absence of infectious particles in the surviving cultures; persistence of the complete viral genome in the surviving cells; induction of the synthesis of infectious virus by some manipulation of the culture (Levine *et al.*, 1980). It has also been suggested that *in vitro* latency should involve cells of neuronal origin, since HSV resides in a latent state in neuronal cells *in vivo*. However, there is recent evidence that HSV can be harboured in a latent state in non-neuronal cells (Clements and Subak-Sharpe, 1988; Al-Saadi *et al.*, 1988; Clements and Jamieson, 1989). Non-productive *in vitro* HSV-cell interactions have been established in transformed neuronal cell lines (Adler *et al.*, 1978; Doller *et al.*, 1979; Levine *et al.*, 1980), permissive and semi-permissive cells of neuronal origin (Wigdahl *et al.*, 1984a,b; Nilheden *et al.*, 1985; Wilcox and Johnson, 1987; Biswal *et al.*, 1988) and permissive and semi-permissive cells of non-neuronal origin (O'Neill *et al.*, 1972, 1977; Wigdahl *et al.*, 1982b; Scheck *et al.*, 1986; Shiraki and Rapp, 1986; Wrzos and Rapp, 1987; Youssouffian *et al.*, 1982; Notarianni, 1986; Russell and Preston, 1986).

Inhibition of the cytocidal potential of HSV in permissive cells of non-neuronal or neuronal origin relies on transient or continuous exposure of the infected cell cultures to antiviral inhibitors and/or suppressive temperatures. Herpes simplex viruses possessing no cytocidal capabilities under appropriate conditions, such as viruses possessing lethal ts, insertion or deletion mutations, or UV-irradiated HSV, have also been used to infect permissive cells to achieve non-productive interactions. The salient features of certain *in vitro* latency systems are described in the following section.

A non-productive interaction between HSV-1 and a line of chemically transformed neuronal cells (Bl03) has been characterised (Adler *et al.*, 1978) and demonstrated to be a multiplicity- and temperature-dependent phenomenon, with minimum virus yields at a low multiplicity of infection (0.01 pfu/cell) and an elevated temperature (39^{o}C) (Levine *et al.*, 1980). Inability of the virus to replicate was not due to failure of the virus to adsorb to the cell surface and there was no apparent presence of a viral inhibitor in the cells. HSV DNA synthesis was not detectable. Thymidine kinase, DNA polymerase and virus structural proteins were produced (Adler *et al.*, 1978), but the high molecular weight glycoproteins normally produced in HSV infections were absent (Levine *et al.*, 1980). HSV-1 nucleic acid sequences were, therefore, retained within the cells, although it was not established whether the entire HSV genome was harboured in the cells since attempts to recover infectious virus were not successful.

Advantage has been taken of the restriction of HSV replication in mouse C1300 neuroblastoma cells, compared to other murine cells, in the development of an *in vitro* latency system. Following the observations that low multiplicity HSV infection produces C1300 cell cultures which only occasionally or persistently release virus into the culture fluid and that a C1300 cell has to be infected with more than one HSV particle to produce progeny virus (Vahlne *et al.*, 1981), Nilheden *et al.* (1985) produced clones of C1300 cells with increased resistance to HSV following exposure of the cells to successively increasing

m.o.i.'s of HSV for a great number of cell passages. The clones tolerated higher m.o.i.'s of HSV, produced lower yields of progeny virus and higher activities of a non-interferon HSV inhibitor, although growth rate and HSV adsorptive and penetrative capacities did not differ from those of the parental cells. Culture of the hyper-resistant cells in the presence of HSV-neutralising antibody for one or two passages after infection and further passage without antibody resulted in a latent virus-cell interaction; progeny HSV-1 could be rescued by HSV-2 superinfection, though retrieval of HSV-1 decreased with passage of the latently infected cells.

Youssoufian et al. (1982) have described a system utilising a lymphoid cell line (CEM) persistently infected with HSV-1, usually productive in terms of infectious virus and viral antigen but also demonstrating two transient latent stages when no infectious virus or viral antigen were detectable. Virus production could be induced from cells in the transient latent periods by a single treatment with the mitogen phytohaemagglutinin. The latent state could be made permanent by a single treatment of the non-producing cells with the mitogen concanavalin A. Viral DNA, present in these latently infected cells at approximately 1 copy per genome equivalent, was shown, by restriction endonuclease cleavage studies, to be heavily methylated at CG dinucleotides, in contrast to the DNA from CEM cells productively infected with HSV-1 which contained 40 to 80 copies of non-methylated viral DNA. This finding is of particular interest in view of the recent reports of enhanced reactivation of latent HSV from explanted neural tissues with demethylating agents (Bernstein and Kappes, 1988; Stephanopoulos et al., 1988). Furthermore, although most, if not all, of the viral genome was present in cells from productive and latent stages, a terminal fragment of the short region of HSV-1 DNA was distinctly underrepresented in DNA from the latent cells but not in DNA from the producer cells, indicating an endless configuration for latent DNA in the lymphoid cell line, perhaps comparable to the endless state of latent DNA in vivo (Youssoufian et al., 1982).

A latent state of HSV-2 has been established in a

human neuroblastoma cell line (SMS-KCNR) by exposure of the infected cells to antiherpetic acyclovir (ACV) and human IFN for 3 days; thereafter, the cells could be propagated without any antiherpetic agents. No infectious virus was detected in the cell cultures, although almost 60% of the cell population contained an HSV genome, as judged by *in situ* hybridisation analysis. Infectious HSV-2 could be rescued following extensive treatment of the latently infected cells with bromodeoxyuridine, which resulted in differentiation and maturation of the cells to flat cell types. Two intracellular polypeptides with M_R 85,000 and 67,000 were reactive with hyperimmune anti-HSV-2 rabbit serum, but it is not clear whether they were HSV-2 gene products. Two cellular enzymes, involved in metabolism of neurotransmitters, were expressed at a higher level in latently infected cells than in mock-infected control cells, and one possibility is that IE HSV proteins, which are capable of stimulating cellular promoters stably integrated into the host cell genome, may have activated the expression of the genes encoding the enzymes (Biswal *et al.*, 1988).

An interesting latency system has been described by Wilcox and Johnson (1987), in which primary sympathetic neuronal cultures, inoculated with HSV and incubated in medium containing 2% anti-HSV antiserum for 14 days, were subsequently maintained for up to 5 weeks without evidence of viral infection. Deprivation of nerve growth factor (NGF) induced the reactivation of infectious HSV, indicating that the cells contained the full complement of HSV information. This *in vitro* latency model has suggested a possible role for NGF in the regulation of latency *in vivo*; nerve damage is known to stimulate the reactivation of HSV *in vivo* and it is postulated that such damage could result in reduction or cessation of retrograde transport of NGF to the neuronal soma and, therefore, that reactivation *in vivo* could be a consequence of NGF deprivation (Wilcox and Johnson, 1987).

HSV latency systems, in which HSV is maintained in a repressed state for a variable number of days by treatment with chemical inhibitors followed by incubation at elevated temperatures (39.5 to 40.5^{o}C), have been set up in cultures of human diploid fibroblasts (O'Neill *et al.*, 1972), human

neurons (Wigdahl et al., 1984b) and rat sensory neurons (Wigdahl et al., 1984a). Inhibitors, or combinations of inhibitors, used include, cytosine arabinoside (ara-C) (O'Neill et al., 1972), (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU) plus IFN (Wigdahl et al., 1982b) or ACV plus IFN (Scheck et al., 1986), which will all inhibit DNA synthesis (Silagi, 1965), and the protein synthesis inhibitor cycloheximide (Shiraki and Rapp, 1986). Interestingly, ts mutants of HSV-1 (DNA positive phenotype) have been maintained in a repressed state in HEL cells, for at least 40 days, by incubation at the elevated temperature of 40.5°C (Wrzos and Rapp, 1987). Productive HSV infection can be activated several days after lowering the temperature of incubation or within 24h following intertypic superinfection (Colberg-Poley et al., 1979).

In the system described by O'Neill et al. (1972), HEL cells pretreated with ara-C were infected with HSV (at 0.1 to 0.5 pfu/cell) and maintained in growth media plus ara-C for 7 days; infectious virus was not detectable for 5-11 days following removal of inhibitor. HSV RNA was not detectable in these cells, within the limits of the in situ hybridisation assay utilised (Colberg-Poley et al., 1981). Elevation of the temperature from 37°C to 40.5°C for HSV-1 (Wigdahl, 1981), or to 39.5°C for HSV-2, at the time of ara-C reversal resulted in a prolongation of HSV latency for at least 128 days post infection (O'Neill, 1977). In the presence of ara-C, "empty", non-enveloped, herpesvirus-like particles were present in nuclei with fragmented chromatin, and, following removal of ara-C and temperature elevation, no virions were found (Hung et al., 1984). Subsequent reduction of the temperature to 37°C at any time during the latent period resulted in reactivation of infectious virus after an incubation period at 37°C of at least 11 days (O'Neill, 1977).

Reactivation was also achieved within 24h after superinfection of quiescent cultures at 39.5°C or 40.5°C with wt HCMV and with ts mutants of HCMV or HSV-2 (Colberg-Poley et al., 1979; Colberg-Poley et al., 1981; Wigdahl et al., 1982a). The involvement of early viral functions in the reactivation event is likely since HSV was

reactivated from its repressed state within 12h of wt HCMV superinfection, before newly synthesised HCMV was apparent (Colberg-Poley et al., 1981), and by superinfection with ts DNA-negative mutants of HCMV and HSV and with HSV mutants capable of expressing only early genes at the NPT (Colberg-Poley et al., 1981; Wigdahl et al., 1982). Superinfection with a transcription negative ts mutant (tsB7) was unable to induce reactivation, suggesting that viral adsorption and penetration were not sufficient to trigger the reactivation event (Scheck et al., 1987). Scheck and associates (1987) also found that HSV-1 superinfection, of cultures harbouring HSV-2 in a repressed state, in the presence of BVDU, which selectively inhibits transcription of late and a subset of early HSV-1 genes, failed to reactivate HSV-2 replication, thereby suggesting that early, as well as immediate early, HSV-1 genes are required for HSV-2 reactivation in this system. Additionally, although there is no direct evidence, Vonka and associates (1986b) have suggested that cellular DNA synthesis, or associated processes, play a key role in the activation of latent HSV, on the basis that HSV reactivation can be induced by methods known to induce cell DNA synthesis, including HCMV superinfection (Stinski, 1983).

Infectious centre assays (performed at 37^{o}C) of cells from cultures in which HSV was in a repressed state suggested that the proportion of cells harbouring an HSV genome that can be reactivated was 0.0002% to 0.02% (O'Neill et al., 1972; Colberg-Poley et al., 1981); at 12h post HCMV superinfection (at 0.2 pfu/cell), before newly synthesised HCMV was apparent, 0.01% of cells (initially infected with HSV-2 at 0.02 pfu/cell) expressed HSV RNA (Colberg-Poley et al., 1981).

The system was, therefore, modified in an attempt to increase the percentage of cells harbouring a latent HSV genome (Wigdahl et al., 1982b). HEL cells, rat neurons or human neurons were pretreated with BVDU in combination with IFN, infected at high multiplicity (2.5 pfu per cell) with HSV and incubated for 7 days in the presence of the inhibitors. Infectious virus was not detectable for at least 9 days in surviving human neurons following inhibitor

removal (Wigdahl et al., 1984b), but was detected within 3 days of inhibitor removal in HEL and rat neuron cultures. However, HSV could be maintained in a repressed state in HEL and rat neuron cultures for at least 60 days and 15 days, respectively, by temperature elevation to 40.5^{o}C after inhibitor removal (Wigdahl et al., 1984a). Reactivation of infectious HSV could be induced, via temperature reduction or HCMV superinfection, in at least 1% to 3% of the surviving HEL cells and indirect immunofluoresence studies and blot hybridisation analyses indicated that, 10 hours after HCMV superinfection at 1 pfu per cell, 10% of surviving cells were positive for HSV antigens (Wigdahl et al., 1982b). HSV specific immunofluorescence was apparent in approximately 50% of rat neuronal cells, treated in the same manner, 3 days after inhibitor removal and incubation at 37^{o}C (Wigdahl et al., 1983). Southern hybridisation studies demonstrated that, on average, HEL cultures contained 0.25 to 0.5 copies, and rat neuron cultures contained 8 to 10 copies, of most, if not all, HSV fragments, per haploid cell genome equivalent. There was no detectable alteration in size or molarity of the HSV terminal or junction DNA fragments and, therefore, the predominant form of the HSV genome was unit length and linear (Wigdahl et al., 1984a), in contrast to the endless state of the latent HSV genome in human peripheral and mouse central and peripheral nervous system tissues (Rock and Fraser, 1983, 1985; Efstathiou et al., 1986).

2. MATERIALS

2.1. Tissue Culture Cells

Human foetal lung (HFL) cells and human foreskin fibroblasts were supplied by Dr. B. Carritt and Dr. M. Hodgins, respectively, and were used in HSV latency experiments. BHK-21/Cl3 cells were derived by Macpherson and Stoker (1962) and were used routinely in the preparation and titration of HSV stocks.

2.2. Tissue Culture Cell Media

Cells were routinely grown in Eagle's medium (GMEM, Gibco) (Busby et al., 1964) which contained 6mM glutamine, 0.25% sodium bicarbonate, 100units/ml of penicillin, 100ug/ml of streptomycin, and other supplements as listed below:

EF10 or EF5	Eagle's medium containing 10% or 5% foetal calf serum (Gibco) and 1% non-essential amino acids
ETC10	Eagle's medium containing 10% calf serum and 10% tryptose phosphate
EHu5	Eagle's medium containing 5% human serum

Other media used in tissue culture are as follows:

PBSA	170mM NaCl, 3.4mM KCl, 2mM KH_2PO_4, 10mM Na_2HPO_4 (pH 7.2)
PBS	(Phosphate-Buffered Saline), PBSA plus $CaCl_2H_2O$ and $MgCl_26H_2O$ both at 1g/l
TBS	(Tris-Buffered Saline), 140mM NaCl, 10mM KCl, 10mM Tris, 1mM $MgCl_2$
Tris-Saline	140mM NaCl, 30mM KCl, 280mM Na_2HPO_4, 1mg/ml glucose, 0.001% (w/v) phenol red, 25mM Tris HCl (pH 7.4), 100units/ml penicillin, 100ug/ml

	streptomycin
Trypsin	0.25% (w/v) trypsin in Tris-Saline
Versene	0.6mM EDTA in PBSA containing 0.002% (w/v) phenol red

2.3. Virus Stocks

HSV-2 wild type (wt) strain HG52, HSV-1 wt strain 17, and temperature sensitive (ts) mutants of HSV-2 (ts3, ts5, ts9, ts11) and HSV-1 (tsI, tsK, [both of syncytial plaque morphology]) were obtained from stocks maintained in the Institute of Virology by M. Murphy. The virus u.v.tsK is tsK which was irradiated with ultraviolet light, by Dr. C. Preston, to prevent gene expression and reduce the titre by a factor of $5x10^5$. HSV-2 wt strains UW268 and 186 were initially obtained from Dr. P. Schaffer, and HSV-2 wt strain 333 was initially obtained from Dr. F. Rapp. The HSV-1 deletion mutant dl1403 and the dl1403 marker rescued viruses (Stow and Stow, 1986) were supplied by E.C. Stow. The HSV-1 insertion mutant in1411 (Russell et al., 1987) was provided by E.C. Stow.

Human Cytomegalovirus (HCMV) strain AD169 was supplied by Dr. K. Fletcher.

Adenoviruses strain 2 (Ad2) and strain 5 (Ad5) were provided by Dr. R. Hay.

2.4. Bacterial Strains

Escherichia coli K12 DH-1 (rec A1, nal A, r_R^-, m_R^-, endoI^-, B^-, rel A1) (Hanahan, 1983) was used throughout.

2.5. Bacterial Culture Media

Bacteria were routinely grown in L.Broth [1% (w/v) NaCl, 1% (w/v) Bactopeptone, 0.5% (w/v) yeast extract] and L.Broth Agar [L.Broth plus 1.5% (w/v) agar].

2.6. Plasmids

Genomic libraries of cloned HSV DNA fragments were

established in the Institute of Virology. Clones were grown from seed stocks supplied by Dr. V. Preston.

2.7. Enzymes

Restriction enzymes and DNA polymerase I were purchased from Bethesda Research Laboratories and used according to the suppliers recommendations. Lysozyme, proteinase K, deoxyribonuclease I (bovine pancreas) and ribonuclease A (bovine pancreas) were purchased from Sigma (London) Chemical Company Limited. The large (Klenow) fragment of DNA polymerase was purchased from Boehringer Corporation Limited.

2.8. Chemicals

Chemicals used were of the highest purity available and were obtained from Sigma Chemical Company, BDH Chemicals, or an alternative supplier. Other suppliers were: Kochlight Limited, (boric acid, acrylamide, caesium chloride, sodium hydroxide, TCA [trichloroacetic acid]); Pharmacia Fine Chemicals, (sephadex G50, ficoll, hexadeoxyribonucleotides [pdN6]); Boehringer Corporation Limited (Tris [tris hydroxymethyl aminomethane]); L.C. Services Coorporation, (TPA [12-O-tetradecanoyl phorbol 13 acetate]).

2.9. Radiochemicals

Amersham supplied all radiochemicals utilised. Specific activities were 40-60 Ci/mmol for [^{3}H]-thymidine, >800Ci/mmol for [^{35}S]-methionine, and, 3000Ci/mmol for the 5'-alpha-[^{32}P]-deoxynucleoside triphosphates.

2.10. DNA Electrophoresis Buffers

EEB	(Electroelution Buffer), 40mM Tris, 5mM sodium acetate, 1mM EDTA (pH 7.8)
TBE	90mM Tris, 90mM Boric Acid,

1mM EDTA (pH 8.3)

TBE Loading Buffer
49% (v/v), 1xTBE, 1% (w/v) SDS, 50% (v/v) glycerol, 0.1% (w/v) bromophenol blue

E Buffer
36mM Tris, 30mM NaH_2PO_4, 1mM EDTA (pH 7.8)

Dye-Ficoll (Loading Buffer)
10% (w/v) Ficoll, 0.1M EDTA, 5xE Buffer, Bromophenol Blue

2.11. Polypeptide Electrophoresis Buffers

Tank Buffer
0.1% SDS, 52mM Tris, 53mM Glycine

Gel Buffer
0.4% SDS, 1.5M Tris pH 8.9

Stacking Gel Buffer
0.4% SDS, 0.5M Tris pH 6.7

ESB
(Electrophoresis Sample Buffer), 30% (v/v) Glycerol, 5.7% (v/v) Stacking Gel Buffer, 11uM beta-mercaptoethanol

2.12. Lysis Buffers

TK Lysis Buffer
20mM Tris HCl (pH 7.5), 2mM $MgCl_2$, 10mM NaCl, 0.5% (v/v) Nonidet P40

Nuclei Lysis Solution I
50mM Tris HCl (pH 7.8), 10mM EDTA, 100mM NaCl, 0.4% SDS

Nuclei Lysis Solution II
100mM Tris HCl (pH 7.8), 50mM EDTA, 200mM NaCl, 0.8% SDS

STET:Lysozyme (9:1)
50mM Tris HCl (pH 8.0), 50mMEDTA, 8% (w/v) sucrose, 5% (v/v) Triton X100, 0.1% (w/v) lysozyme

2.13. DNA Blot Hybridisation Solutions

Gel Soak I
3M NaCl, 1M NaOH

Gel Soak II	1.2M NaCl, 2M Tris (pH 8.0)
SSC	150mM NaCl, 15mM Trisodium citrate
TS	(Transfer Solution), 0.6M NaCl, 0.4M NaOH
NS	(Neutralising Solution), 1M NaCl, 0.5M Tris HCl (pH 7.0)
Hybridisation Solution	0.02M Tris HCl (pH 7.5), 6xSSC, 50xDenhardts, 1mM EDTA, 0.5% SDS, 50ug/ml denatured calf thymus DNA
Pre-Hybridisation Solution	6.6xSSC, 5xDenhardts, 0.5% SDS, 18ug/ml denatured calf thymus DNA
Denhardt's Solution	0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrollidine, 0.02% (w/v) BSA (Denhardt, 1966)

2.14. Radio-Labelling Solutions

Nick Translation Buffer	0.5M Tris HCl (pH 7.5), 0.1M $MgCl_2$, 1 mM DTT, 500ug/ml BSA
PE Reaction Mix (Solution A):(Solution B):(Solution C) 2 : 5 : 3	(Primer Extension) Reaction Mix,
Solution O	1.25M Tris Hcl (pH 8.0), 0.125M $MgCl_2$
Solution A	Solution O plus 0.25M betamercaptoethanol, 20uM dTTP and 20uM dATP
Solution B	2M Hepes pH 6.6
Solution C	Hexadeoxyribonucleotides (pDN6) at 90 OD units/ml in 10mM Tris HCl (pH 7.5), 1mM EDTA

2.15. Other Solutions

Fix	1% glacial acetic acid, 9% methanol
Destain	5% methanol, 7% glacial acetic acid

Giemsa	1.5% (w/v) suspension of Giemsa in glycerol, heated at 56°C for 120 min and diluted with an equal volume of methanol

3. METHODS

3.1. Cell Culture

Monolayer cultures of human foetal lung (HFL) cells and of human foreskin fibroblasts (HFF) were grown in EF10 medium at 37°C in an atmosphere of 5% CO_2, 95% air. The cultures were passaged every 2 to 3 days; cells from confluent monolayers were harvested by addition of 10ml of a 1:1 solution of trypsin:versene, followed by 10ml of medium, and resuspended cells were split and used to seed monolayers. Confluent monolayers of cells yielded approximately $6x10^7$ cells per 175cm^2 plastic tissue culture flask, $1x10^6$ cells per 35mm diameter Petri dish and $3x10^6$ cells per 50mm diameter Petri dish.

Baby hamster kidney (BHK-21 [C13]) cell monolayers were grown and maintained in ETC10 medium at 37°C in an atmosphere of 5% CO_2, 95% air. Cultures were passaged every 2 to 3 days, as described above. Confluent monolayers yielded approximately $3x10^8$ cells per 850cm^2 plastic roller bottle, $1x10^6$ cells per 35mm diameter Petri dish and $3x10^6$ cells per 50mm diameter Petri dish.

For analysis of plaque formation, 5% human serum was substituted for foetal calf or calf serum (EHu5).

3.2. Virus Stocks

3.2.1. Herpes Simplex Virus

Stocks of herpes simplex virus were propagated in BHK cells, with the exception of in1411 which was grown in M65 cells (Davison and Stow, 1985) by N.D. Stow and E.C. Stow.

Cell monolayers in plastic roller bottles were infected with virus at an m.o.i. of 0.01 pfu per cell or with plaque isolate material, in 100ml of ETC10. Infected cultures were maintained at 31°C for 3-4 days until the appearance of extensive cytopathic effect (CPE). The bottles were shaken vigorously to dislodge the cells into 20ml medium. Centrifugation, at 996g for 15min at 4°C, produced

a pellet containing cell-associated virus (CAV) and a supernatant containing cell-released virus (CRV). The CAV-containing pellet was sonicated in 1ml of ETC10, to release the virus, and recentrifuged at 996g for 15min at 4°C to remove cell debris. CAV in the supernatant was stored in aliquots at -70°C. The CRV-containing supernatant was centrifuged at 13,200g for 2h at 4°C and the pellet of CRV was resuspended in a small volume of ETC10, sonicated, and stored at -70°C. Sterility checks (on blood agar plates) were performed on CRV and CAV stocks, and the viral stocks were titrated on BHK cells. It should be noted that HSV-2 infections in cell culture produce relatively low concentrations of CRV and that stocks used were therefore only CAV.

3.2.2. Human Cytomegalovirus (HCMV)

Stocks of HCMV strain AD169 were produced by infection of HFL monolayers at 0.01 pfu per cell. The infected cultures were incubated at 37°C for 1.5 to 2h to allow viral adsorption and penetration. The inoculum was removed, the monolayers were washed in EF5 medium, overlaid with fresh EF5 and incubated at 37°C for several days. Medium was changed if it became acidic. The medium was removed from cultures in which extensive CPE was apparent (5 to 7 days pi), and stored at -70°C. Fresh medium was added to the cells and incubation was continued. This procedure was repeated daily until all the cells detached into the medium (10 days pi). Stocks were pooled, sonicated and stored at -70°C.

3.3. Titration of Virus

HSV stocks, with the exception of in1411, were titrated on BHK cell monolayers, and HCMV stocks were titrated on HFL cell monolayers. Serial 10-fold dilutions of virus were made in ETC10 or EF10, and 0.2ml samples of each dilution were inoculated onto cell monolayers from which growth medium had been aspirated. Following an adsorption period of 1h (HSV) or 1.5h (HCMV) at a temperature

permissive for viral growth (31°C, HSV ts mutants; 37°C, HSV and HCMV), the cells were overlaid, either with EHu5 (HSV), to neutralise unadsorbed virus and thereby prevent the formation of secondary HSV plaques, or with EF5 (HCMV). Incubation was continued at the appropriate temperature for 2-4 days (HSV) or 8-10 days (HCMV) until plaques were of sufficient size. The medium was then removed and the cells were stained with Giemsa Stain. After the stain was washed off, the viral plaques, clearly visible under a dissecting microscope, were scored for each dilution, and the number of plaque-forming units of virus per ml was calculated.

3.4. Plaque Isolates

Plaque isolates were picked under EF10 from infected cell monolayers containing separated plaques. The plaque isolate was sonicated and stored in 0.5ml of EF10. Isolates were titrated on BHK cells.

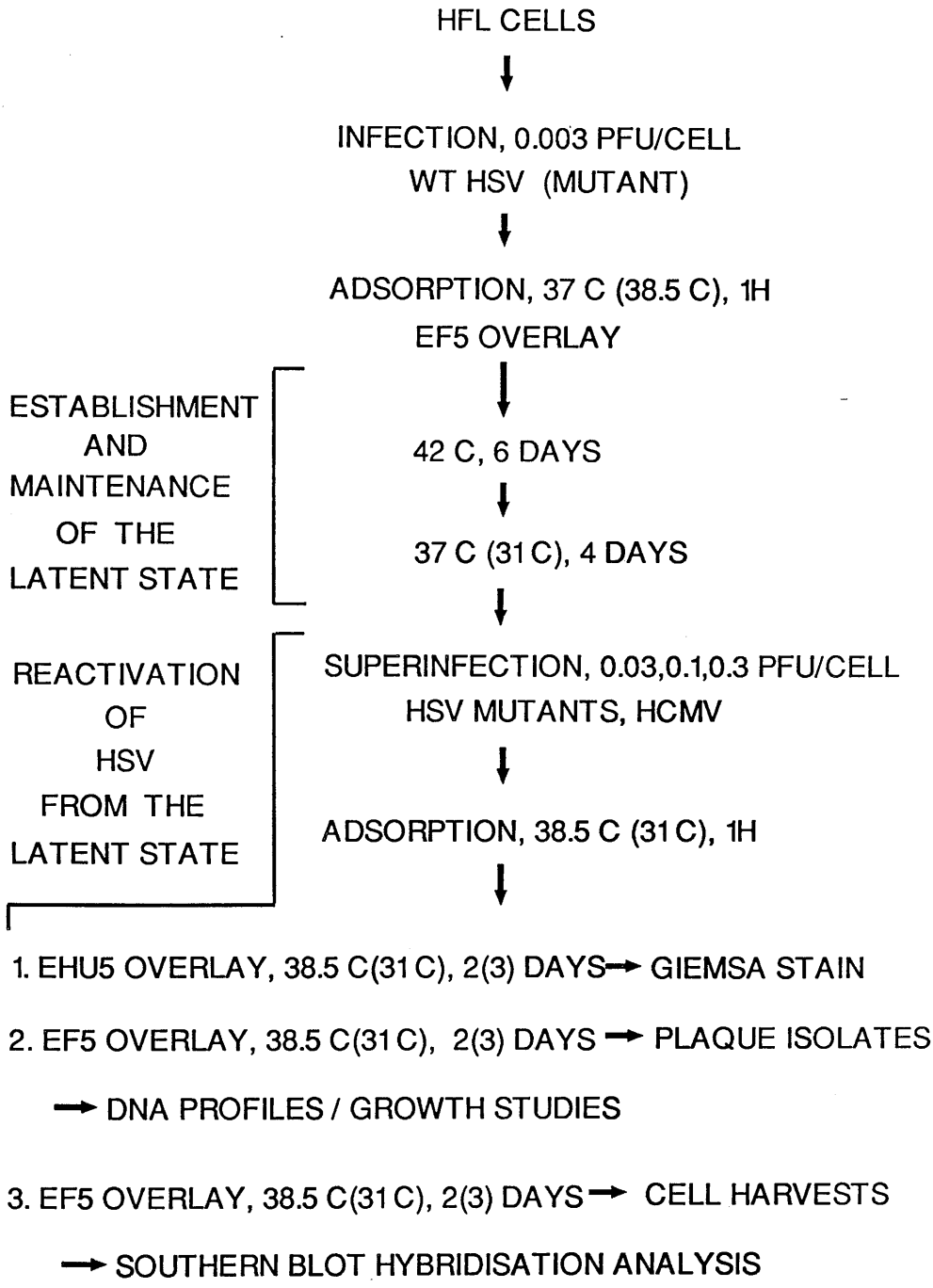
3.5. Growth Curves

Confluent monolayers of HFL cells in 35mm diameter Petri dishes were infected with virus at 0.003 pfu per cell. Virus was allowed to adsorb to and penetrate the cells for 1h at 38.5°C and infected cells were washed once with EF5, overlaid with EF5 and incubated at 38.5°C for 16, 24 and 48h. At the appropriate time points, samples were harvested, by scraping into the medium with sterile rubber policemen, sonicated and titrated on BHK cells.

3.6. Outline of in vitro Latency System for HSV

The basic in vitro latency system for HSV is outlined schematically in Figure 3.1.. Confluent monolayers of HFL cells on 35mm diameter Petri dishes were infected with HSV at 0.003 pfu per cell (3000 pfu per dish). The virus was allowed to adsorb to and penetrate the cells for 1h at 37°C, the remaining virus inoculum was aspirated and the monolayers were washed once in EF10. Infected monolayers were overlaid with EF5 and incubated at 42°C for 6 days.

FIGURE 3.1. **SCHEMATIC REPRESENTATION OF THE IN VITRO LATENCY SYSTEM FOR HSV**



Medium changes were performed at two day intervals as rapidly as possible at room temperature (RT) using EF5 which was prewarmed to 42°C. Cultures were shifted to 37°C (or 31°C) for a further 4 days, during which time the medium was changed every day or on alternate days. Throughout this 37°C incubation, sample monolayers were scraped into the culture medium, sonicated thoroughly, and tested for the presence of infectious virus by plaque assay. Superinfection was carried out after the 4 day incubation at 37°C. Superinfecting virus was allowed to adsorb to and penetrate the cells for 1 to 1.5h at 38.5°C (or 31°C). The cells were overlaid with EHu5, incubated for 2 days at 38.5°C (or 31°C), stained with Giemsa Stain and then examined for the presence of viral plaques.

3.7. Preparation of Competent Bacteria

100ul of a glycerol stock of Escherichia coli strain DH-1 was added to 10ml of L-Broth and incubated overnight (o/n) at 37°C. A 2ml sample of this overnight culture was added to 200ml L-Broth which was then shaken at 37°C for 2.5 to 3h until the OD_{630} had reached 0.2. The culture was placed on ice for 10min prior to centrifugation at 830g for 15min. The L-Broth supernatant was removed, and the pellet was resuspended in a half volume of ice cold 100mM $CaCl_2$ and left on ice for 1h prior to recentrifugation. The resultant pellet was resuspended in 2ml of 100mM $CaCl_2$.
The DH-1 bacteria were competent at this stage and were either stored o/n at 0°C or transformed directly.

3.8. Transformation of DH-1

Plasmid DNA (ampicillin^R) samples were mixed with 100ul aliquots of competent bacteria, set on ice for 1h with occasional mixing, then heated to 42°C for 1.5min and added to 2ml of warm L-Broth. The mixture was shaken for 90min at 37°C. 0.2ml aliquots of the suspension were plated out in 90mm plastic dishes containing agar plus ampicillin at 50ug/ml. The plates were incubated at 37°C o/n. Colonies were picked and stored at 4°C o/n or at -20°C in a mixture

containing 40% glycerol and 1% bactopeptone.

3.9. Small Scale Preparation of Plasmid DNA

Transformed DH-1 colonies were picked and resuspended in 2ml of L-Broth plus ampicillin (50ug/ml). The cultures were shaken at 37°C for 5 to 6h until dense. Chloramphenicol was added to a final concentration of 25ug/ml and the cultures were left shaking at 37°C o/n. The cultures were brought to RT and 1.5ml of each was centrifuged at 2,900g for 5min. The supernatant was removed, and the pellet was resuspended by vortexing in 100ul of a 9:1 solution of STET:Lysozyme (10mg/ml). The mixture was immediately heated at 100°C for 1min, and then centrifuged at 11,600g for 10min. The supernatant was added to 0.4ml of 0.3M sodium acetate, and 0.5ml of isopropanol was added. After storage at -20°C for 30min, the DNA was pelleted by centrifugation, redissolved in 30ul of de-ionised water (DW) and stored at -20°C. The DNA was analysed by restriction enzyme analysis.

3.10. Large Scale Preparation of Plasmid DNA

Quantities shown are for a 200ml culture.

An overnight culture of transformed DH-1 (20ul) in L-Broth (10ml) plus ampicillin (50ug/ml) was grown at 37°C, and 1ml of this culture was added to 200ml of L-Broth and shaken at 37°C for several hours. Chloramphenicol was added at a concentration of 25ug/ml and the cultures were shaken o/n at 37°C. The cultures were centrifuged at 4,800g for 10min. The pellet was resuspended in 5ml of 25% sucrose/ 50mM Tris-HCl, pH 8.0. 2.5ml of 5mg/ml lysozyme was added and the mixture was set on ice for 30min. Following the addition of 2ml of 250mM EDTA, pH 7.5, the mixture was left on ice for 5min. 1.5ml of 5M NaCl and 1.5ml of 20% SDS were added, and the mixture was mixed gently and placed on ice for 2 to 3h prior to centrifugation at 36,900g for 1h at 4°C. The supernatants were phenol/chloroform extracted and ethanol precipitated (see below). The pellet of nucleic acid plus protein was dissolved in 9ml of 20mM Tris HCl, pH 7.5, 100mM NaCl, 1mM EDTA. Degradation of RNA and protein was

achieved by treatment with 20ul of 5mg/ml RNAase A at 37°C for 2 to 4h, followed by proteinase K at 100ug/ml at 37°C for 2 to 16h. The volume was increased to 4.5ml, and DNA was phenol:chloroform extracted, then isopropanol precipitated (see below). The DNA pellet was redissolved in 300ul of DW. Plasmid DNA was stored at -20°C.

3.11. Phenol:Chloroform Extraction of DNA

Purification of DNA from protein contaminants was achieved by extraction of DNA in phenol:chloroform. DNA in aqueous solution was mixed with an equal volume of phenol:chloroform (1:1), agitated (gentle agitation is required for extraction of large DNA fragments and total cellular DNA, to prevent shearing of DNA), and centrifuged. Centrifugation was at 11,600g for 5min with sample volumes of <1.5ml, or at 1,557g for 15min with sample volumes of >5ml. The aqueous phase containing the DNA was re-extracted in phenol:chloroform, and then extracted once in chloroform to remove contaminating phenol. DNA was ethanol/isopropanol precipitated.

3.12. Ethanol and Isopropanol Precipitation of DNA

DNA in aqueous solution was mixed with 2 volumes of ethanol and sodium acetate (0.3M final concentration), and placed at -20°C for >2h. As an alternative, larger DNA fragments were precipitated in an equal volume of isopropanol, and sodium acetate (0.3M final concentration) left at RT for >2h, to reduce contamination with DNA fragments of <20 base pairs which are not precipitated in isopropanol. Following precipitation, DNA was pelleted by centrifugation, with the exception of total cellular DNA, the globular mass of which was lifted out of the ethanol with a pipette. DNA was washed in ethanol, recentrifuged, dried and redissolved in DW.

3.13. Restriction Enzyme Digestion

DNA was digested in a solution containing 1xRE Buffer

(HSB, MSB) and 0.01% BSA, or commercial RE Buffer [BRL], and restriction enzyme (1 unit/ug DNA, BRL). For the preparation and analysis of DNA fragments, or, for the analysis of *in vivo*-labelled DNA fragments the digestions, in 20ul or 150ul volumes, were normally carried out at 37°C for 4h.
For the digestion of high molecular weight total cellular DNA, in volumes ranging from 45ul to 900ul, the DNA was incubated with 1 unit of restriction enzyme per ug of DNA at 37°C o/n; a further 0.5 units of enzyme per ug of DNA was then added and incubation was continued for a further 4h period.

3.14. Electrophoresis of DNA

3.14.1. Minigels

Analysis of small amounts of DNA utilised horizontal, 0.5% agarose, 50ml gels in TBE Buffer (100mmx70mmx7mm). DNA samples plus TBE Loading Buffer in a final volume of 10ul were loaded into gel slots and electrophoresed in TBE Buffer at constant current, 40mA for 1h. Following electrophoresis, the gel was stained in TBE Buffer plus ethidium bromide (4ug/ml) for 15 min. DNA was visualised by exposure of the gel to ultraviolet light and photographed on Polaroid 667 film.

3.14.2. Large Agarose Gels

Analysis and preparation of DNA restriction fragments utilised horizontal, 1% agarose, 300ml gels in E Buffer (260mmx160mmx7.5mm) plus ethidium bromide (0.5ug/ml). Southern transfer of DNA samples to GeneScreen Plus Hybridisation Transfer Membrane (DuPont) was from horizontal, 0.6% agarose, 200ml gels (260mmx160mmx5mm) in E Buffer plus ethidium bromide (0.5ug/ml). DNA samples in Dye-Ficoll were electrophoresed in E Buffer at constant current, 40mA for 16-18h. DNA was visualised by exposure of the gel to ultraviolet light and photographed on Polaroid 667 film. Gels for autoradiography were dried on a glass plate in an oven at 60°C.

3.15. Electroelution of DNA Fragments

DNA was electroeluted from agarose to dialysis membrane in EEB in an electroelution apparatus at 20mA per sample for 1h. The DNA was removed from the dialysis membrane in 200ul of EEB. The volume was increased to 400ul by the addition of 160ul of DW and 40ul of 3M sodium acetate. The DNA was phenol:chloroform extracted, then isopropanol precipitated. The DNA pellet was redissolved in DW.

3.16. Estimation of DNA Concentration

Endonuclease-digested DNA samples were electrophoresed in minigels with DNA of known concentration. DNA bands were visualised under uv irradiation. Approximate estimations of DNA concentrations were obtained by densitometric analysis of DNA bands on negative Polaroid 665 film.

3.17. Radiolabelling of DNA

3.17.1. In vivo Labelling of Viral DNA

The method used was that of Lonsdale (1979), as modified by Park (1983).

Confluent BHK cell monolayers in 1.5cm diameter Linbro wells ($2x10^5$ cells per well) were infected with 3-5 pfu per cell of virus in 100ul volume and incubated at 37°C for 1h to allow viral adsorption and penetration. The inoculum was removed, and monolayers were washed twice in Phosphate-Free Medium, then overlaid with 250ul of Phosphate-Free Medium for 4h at 37°C. At 5h pi 50uCi of [^{32}P]-orthophosphate (PBS II carrier-free) in 300ul of phosphate-free medium was added to each well and incubation was continued at 37°C for 24-48h, until 70-100% CPE was apparent. Cells were lysed by addition of 250ul of 0.5% SDS per well and careful pipetting up and down, then transferred to 1.5ml vials. The DNA was phenol:chloroform extracted,

then ethanol precipitated on dry ice for 30min. The DNA pellet was resuspended in 10mM Tris HCl, pH 7.5, 1mM EDTA plus 0.25mg/ml RNase A in a final volume of 100ul, and incubated at 37°C for 2h. Samples were stored at -20°C.

3.17.2. In vitro ^{32}P-Labelling and Preparation of DNA Probes

Nick Translation (Rigby et al., 1977).

A solution of 0.25 to 0.5ug of DNA in 2ul; 2.5ul of 10x Nick Translation Buffer plus non-radioactive dATP and dTTP, each at 20uM; and 2ul of DNAase ($2x10^{-5}$ mg/ml); was incubated on ice for 2 to 3min and diluted to 17ul. Then, 3ul (30uCi) each of [-^{32}P]dGTP and [-^{32}P]dCTP and 2 ul of E.Coli polymerase I (1 unit/ul) were added and the solution (final volume 25ul) was incubated at 14°C for 2 to 3h.

Hexanucleotide Primer Extension

(Feinberg and Vogelstein, 1983).

DNA (0.1ug) in 20ul was boiled for 10min, cooled at RT and centrifuged briefly.

A 50ul solution consisting of the 20ul DNA, 10ul of Reaction Mix, 400ug/ml of BSA, 5ul (50uCi) of [-^{32}P]dGTP or [-^{32}P]dCTP, 2uM of non-radioactively labelled dCTP or dGTP (as appropriate), and 2.5 units of Klenow DNA polymerase, was incubated at RT o/n.

Preparation of ^{32}P-Labelled DNA for Hybridisation

The probes were purified by passage through a 5ml Sephadex G-50 (medium) column to separate the labelled DNA from the unincorporated triphosphates. The probe was eluted in approximately 800ul of 10mM Tris HCl, pH 7.5, 1mM EDTA.

The specific activity was usually of the order of $1x10^{6}$ to $1x10^{7}$cpm/ug of DNA by nick translation, or, $4x10^{8}$cpm/ug of DNA by primer extension.

The radiolabelled probe in 800ul volume was denatured by addition of 200ul of 1N NaOH at RT for 10min. The solution was neutralised by addition to 8.8ml of Hybridisation Solution plus 200ul of 1M HCl.

3.18. Extraction of Nuclear and Cytoplasmic DNA

Monolayer cultures were scraped from tissue culture plates into 0.5ml of PBS by the use of sterile rubber policemen. The cells were pelleted by centrifugation at 2,900g for 1min and the PBS was removed. 200ul of TK Lysis Buffer was added to the cell pellets, which were resuspended by vortexing, placed at 4°C for 5min, and vortexed once more. The lysed cell suspensions were then centrifuged at 11,600g for 1min. The pellet represented the nuclear fraction and the supernatant represented the cytoplasmic fraction.

The cytoplasmic fraction, in a solution of 10mM EDTA pH 7.5, 100mM sodium chloride and in a final volume of 500ul, was phenol:chloroform extracted and ethanol precipitated. RNA was degraded by treatment with RNAase A at 100ug/ml for 3h at 37°C.

Nuclei were incubated in 250ul of Nuclei Lysis Solution I containing 200ug/ml proteinase K for 16-20h at 37°C, and phenol:chloroform extracted. Nucleic acids were precipitated in ethanol and dissolved in DW. RNA was degraded by RNAase A (100ug/ml) treatment at 37°C for 3h.

3.19. Preparation of Whole Cell Fraction from which to Extract DNA

In this instance, the technique employed had to be such that the infectivity of viral particles in the preparation was not destroyed.

Cells were harvested by scraping (sterile rubber policemen) into 0.5ml of TBS. The preparation was frozen at -140°C and thawed at 37°C three times. For each sample, the presence of infectious viral particles was determined by titration of 20ul of cell lysate on BHK cells. 0.5ml of Nuclei Lysis Solution II containing 200ug/ml proteinase K was added and the sample was incubated at 37°C for 16-20h. Samples were phenol:chloroform extracted, nucleic acids were precipitated in ethanol and pellets were dissolved in DW. RNA was removed by treatment with 100ug/ml of RNAase A for 3h at 37°C.

3.20. Ultracentrifugation of DNA in a Caesium Chloride Gradient

Separation (on the basis of density) of viral DNA sequences of interest from the bulk of cellular DNA sequences (with the exception of high G + C sequences) was achieved by ultracentrifugation of DNA through a CsCl gradient of RI 1.4 (density 1.707g/ml). Restriction enzyme-digested, HSV-infected cellular DNA in aqueous solution was mixed with a solution of CsCl to a final RI of 1.4 (density 1.707g/ml), and a final volume of approximately 18ml. The mixture was transferred to a TV865B ultracentrifugation tube and topped up with liquid paraffin. The tube was sealed by the use of a crimping apparatus. The sample, and control balance sample, were centrifuged in a TV865B vertical rotor at 128,618g for 18h at 15^{o}C. The sample was fractionated by piercing the bottom of the tube with a syringe needle and collecting fractions of 10 drops. The RI of these fractions was measured. Control experiments with ^{3}H-thymidine labelled HSV-2 infected cell DNA digested with the appropriate restriction enzyme, indicated that the HSV DNA was present in fractions of RI 1.4036 to 1.4002. Therefore, fractions of RI 1.4036 to 1.4002 were pooled and either, re-centrifuged, or, dialysed o/n in TE to remove CsCl. Fresh dialysis medium was added and dialysis was continued for a further 3h. The pooled, dialysed fractions were then concentrated to a volume of 1ml by agitation in butanol, followed by removal of the upper layer of butanol, and incubation at 65^{o}C to evaporate the remaining butanol. The 1ml sample was divided equally into 2 parts and ethanol precipitated. The DNA pellet was redissolved in 40ul of DW.

3.21. DNA Blot Hybridisation

3.21.1. Transfer of DNA from Agarose to Membrane (Southern, 1975)

DNA fragments separated according to size by electrophoresis, were denatured, transferred to GeneScreen

Plus Hybridisation Transfer Membrane (DuPont) and immobilised.

After electrophoresis, the agarose gel was pre-soaked for 30min in 0.25N HCl (partial depurination of the DNA) (Wahl et al., 1979). The gel was placed in Gel Soak I for 45min (cleavage and separation of DNA strands) and then in Gel Soak II for 45min (neutralisation). After each step the gel was rinsed briefly in DW.

The agarose gel, containing denatured DNA fragments, was laid upon Whatman's 3MM paper which had been pre-wet with transfer solution and arranged upon a horizontal support such that it formed a wick for the transfer solution within which the support was set. GeneScreen Plus membrane, cut to the same dimensions as the gel, was pre-soaked in transfer solution and placed, B-side down upon the agarose gel. The membrane was covered sequentially with 2 pieces of similarly sized Whatman's 3MM paper, a stack of paper towels, and a 1kg weight. DNA transfer was allowed to proceed for 16 to 18h.

Transfer was carried out in a 6xSSC solution. Following transfer the membrane was placed in 0.4N NaOH for 1h, then soaked in a solution of 0.2M Tris-HCl pH 7.5 - 2xSSC. The membrane was dried at RT for at least 2h.

An alternative method used to transfer DNA fragments to GeneScreen Plus is the Alkaline Transfer Method, which was described by Chomczynski and Qasba (1984). The agarose gel was presoaked for 30min in TS (alkaline denaturation). Transfer was carried out as described above, however, TS was substituted for 6xSSC. Following transfer the membrane was neutralised for 15min in NS, with occasional shaking, and dried at RT for at least 2h.

3.21.2. DNA-DNA Hybridisation

The GeneScreen Plus membrane (plus covalently-bound DNA) was incubated in 100ml of Pre-Hybridisation solution for >2h at 68°C with constant agitation. This solution was then removed and replaced by Hybridisation Solution plus denatured, ^{32}P-labelled DNA probe in a final volume of 10ml. The hybridisation was carried out at 68°C, for 16 to 20h,

with constant agitation of the membrane. The hybridisation solution was removed and the membrane was washed briefly in 2xSSC at RT, 3 times in a solution of 2xSSC, 0.2% SDS at 68°C for 30min with agitation, then twice in cold tap water for several seconds. The membrane was blotted onto tissue paper and laid on a plate, covered with a sheet of thin polythene, a sheet of X-Omat S photographic film, and an intensifier screen. The film was exposed at -70°C for appropriate periods.

3.22. Analysis of Polypeptides

3.22.1. Pulse-Labelling of Cells

Confluent monolayers of HFL cells in 15mm diameter Linbro wells ($2x10^5$ cells per well), were mock-infected or infected with 10 pfu of virus per cell in 100ul. Following adsorption and penetration of the virus for 1h at 37°C, the infected cultures were overlaid with prewarmed EF5 (900ul) and incubation was continued at the appropriate temperature. Cultures incubated at 38.5°C or 42°C were pulse-labelled at 5h pi, whereas, cultures incubated at 31°C were pulse-labelled at 9.5h pi; monolayers were washed once in prewarmed PBS prior to the addition of PBS containing [^{35}S]-methionine (50uCi/ml) and Actinomycin D (1ug/ml). After 30min, the radio-labelled medium was removed, the monolayers were washed in 0.5ml of ice cold PBS, and then in 0.5ml of ice cold 10mM Tris HCl, pH 7.6, 2.5mM EDTA. The cells were harvested in 50ul of ice cold 10mM Tris HCl, pH 7.6, 2.5mM EDTA, transferred to a 1.5ml vial, mixed with 25ul ESB, and heated at 100°C for 15min. Samples were stored at -70°C or analysed immediately.

3.22.2. SDS-Polyacrylamide Gel Electrophoresis

[^{35}S]-methionine-labelled polypeptides were electrophoresed in vertical SDS-polyacrylamide gels plus stacking gels (10% acrylamide/1% DATD) in Tank Buffer.

The gel solution consisted of 13.3ml of 30% acrylamide/3% DATD solution, 10ml of gel buffer, and 0.5ml

of a 10% (w/v) ammonium persulphate solution (freshly prepared) in a final volume of 40ml. 40ul of TEMED was mixed gently into the gel solution which was then poured into a sandwich of glass plates (260mmx160mmx1mm) to fill 75% of the space. Using a Pasteur pipette, 20ml of a 1/4xGel Buffer solution was immediately and carefully layered onto the surface of the gel solution, which was then left to set for >1h. The stacking gel solution consisted of 4ml 30% acrylamide/3% DATD, 6ml of Stacking Gel Buffer, and 0.5ml of 10% (w/v) ammonium persulphate solution in a final volume of 20ml. 25ul of TEMED was mixed into the solution and immediately, following removal of the 1/4xGel Buffer solution, the surface of the gel was rinsed with 5ml of stacking gel solution, which was then removed. The remaining space between the glass plate sandwich was filled with stacking gel solution and a Teflon comb was inserted in the top. Approximately 5-15min later, when the stacking gel had polymerised, a small volume of Tank Buffer was layered gently along the top of the comb, which was then eased out of the stacking gel. The slots produced were washed twice with Tank Buffer. Samples were electrophoresed in Tank Buffer at 35mA, constant current, for 3-4h, or until the dye front had reached the base of the gel. The gel sandwich was removed from the kit, and the gel was separated from the stacking gel and immersed in a 50:50 mix of Fix:Destain at RT for 1-2h. The gel was heat-dried under vacuum, then exposed to Agfa film.

3.23. Determination of Thymidine Kinase (TK) Activity

Cells from 35mm diameter Petri dishes were harvested as described in section, except that the TK Lysis Buffer contained 6.5mM beta-mercaptoethanol. The supernatants representing the cytoplasmic fractions were assayed for TK activity immediately or stored at -70^{o}C.

20ul of the cytoplasmic fraction (1/10 of the total fraction) was analysed in a reaction solution of 50ul consisting of 100mM sodium phosphate (pH 6.0), 10mM magnesium chloride, 5mM ATP, 100uM dTTP and 100uCi/ml [^{3}H]-thymidine. The samples were incubated at 31^{o}C for 3h.

The reaction was terminated by addition of 5ul of a solution of 1mM thymidine, 5mM EDTA (pH 7.0), and incubation at 90°C for 3-4min. The solution was cooled on ice, and centrifuged at 11,600g for 3-4min. 20ul samples of supernatant were spotted onto Whatmann DE81 discs which were then washed in a solution of 4mM ammonium formate, 10mM thymidine at 37°C for three 10min periods. The discs were rinsed twice in absolute alcohol, dried under a heat lamp and placed into scintillation vials containing 3ml of EcoScint A (National Diagnostics). Radioactive counts were measured in a scintillation counter.

ESTIMATION OF DNA CONCENTRATION IN EXPERIMENTS FROM SECTION 4.5.

Approximate estimations of HSV DNA concentrations were obtained by densitometric analysis of autoradiographs using a Joyce-Loebl scanning densitometer. Areas beneath the peaks of the densitometric tracing were measured by the use of a programme, DENS, written by Dr. P. Taylor for a DEC PDP 11/44 computer linked to a digitizing table.

4. RESULTS AND DISCUSSION

4.1. OBJECTIVES

The ultimate aim of the research described in this thesis is to elucidate the molecular basis for HSV latency by the use of an *in vitro* latency system. The system was developed from preliminary work by Dr. C. Preston and Dr. E. Notarianni and is outlined schematically in Figure 3.1. and described in Section 3.6. Essentially, HFL cells are infected with HSV at low multiplicity, incubated at 42°C for 6 days to convert HSV to a latent state, and then incubated at 37°C for 4 days, during which period the latent state is maintained. Experimental reactivation of latent HSV is induced by superinfection with HSV or HCMV.

There were four major objectives of the project: characterisation of an *in vitro* latency system for HSV; identification of the viral factors involved in establishment of latency; identification of the viral factors involved in reactivation of HSV from its latent state; determination of the physical nature of the latent HSV genome.

4.2. CHARACTERISATION OF AN IN VITRO LATENCY SYSTEM FOR HSV

4.2.1. Introduction

The *in vitro* latency system described below relies upon the use of supraoptimal temperatures to convert HSV to a latent state. It is well-established that HSV, especially HSV-2, cannot replicate in tissue culture cells at temperatures of 41°C to 42°C and there have been several reports of non-productive interactions of HSV with host cells induced by incubation of infected cells at these supraoptimal temperatures (Crouch and Rapp, 1972; Darai and Munk, 1973; Marcon and Kucera, 1976; Levine *et al.*, 1980; Notarianni, 1976). Abortive HSV-2 infections of permissive human and rat cells have been induced by incubation at 42°C for 8 days; HSV DNA synthesis and infectious virus

production were not detectable at 42°C. The surviving cell populations contained HSV information, as shown by the appearance of virus specific antigens and the rare production of infectious virus during subsequent incubation at 37°C. It was noted that some of the surviving cells acquired properties characteristic of transformed cells, such as resistance to HSV superinfection and altered morphology, and certain of the transformed rat cells were tumorigenic for neonatal rats (Darai and Munk, 1973; Marcon and Kucera, 1980). Notarianni (1986) reported that incubation at 41.5-42°C of human embryo lung cells infected at low m.o.i. with HSV resulted in the production of a non-infectious state for HSV-2 which could be maintained at the permissive temperature of 37°C for 12 to 17 days; the latent virus could be reactivated by inter-typic superinfection. Only a small proportion of the cells containing HSV genetic information acquired some properties associated with transformation; the ability to grow in soft agar and/or altered morphologies. Since, in the vast majority of infected cells the HSV-2 genome was harboured in a non-infectious state from which it could be induced to reactivate, it was of interest to examine the virus-cell interaction in the context of latency.

4.2.2. Conversion of HSV to a Non-Infectious State in Cultured Cells

Confluent monolayers of HFL cells, in 35mm diameter Petri dishes (approximately 10^6 cells/plate), were infected with wt HSV-2 strain HG52 at 0.003 pfu/cell, and following adsorption and penetration of virus, the cultures were incubated at 42°C for 6 days and then at 37°C for 4 days. Throughout the latter incubation at 37°C, and a further 2 day incubation at 38.5°C, (both temperatures are permissive for HSV replication), cultures were tested for the presence of infectious virus.

Sample monolayers were scraped into the culture medium, sonicated thoroughly, and subjected to plaque assay analysis on BHK cells at 37°C. The results indicated that infectious virus was rarely detected. On the occasions when

infectious virus was detected in a latently infected monolayer at 37°C, it appeared to arise from 1 or 2 plaques. These plaques presumably resulted from HSV-2 infection of an HFL cell in which the virus had not stabilised in a non-infectious state and was therefore able to replicate when the cells were shifted to the permissive temperature of 37°C. In all experiments, therefore, it was important to maintain untreated, latently infected cultures as controls to assess the level of `spontaneous' reactivation.

In 468 control cultures from 12 experiments, 4 monolayers (arising from 2 experiments) showed spontaneous reactivation of 1 or 2 plaques after incubation for 6 days at 42°C followed by 4 days at 37°C. Furthermore, after a further 2 days at 38.5°C, 3 monolayers (arising from 1 experiment) out of 81 control cultures spontaneously reactivated virus. Infectious virus was never detected in cell sonicates from monolayers which showed no evidence of HSV-2 cytopathic effect. Thus, over a 6 day period at temperatures permissive for HSV-2, infectious virus was undetectable in 9 out of 12 experiments, and was not reactivated at significant levels, compared with the efficiency obtained by experimental reactivation (see later) in the other 3.

The vast majority of infectious HSV-2 particles initially applied to the HFL cells was therefore rendered non-infectious by incubation at 42°C, and since, as described in Section 4.2.4., it is possible to experimentally reactivate HSV-2 from these cultures, the virus-cell interaction is considered here to be latency.

4.2.3. Permissiveness of Virus, Cells and Growth Medium Following 42°C Treatment

The absence of detectable HSV-2, after incubation of HFL cells latently infected with HSV-2 (0.003 pfu/cell) at temperatures permissive for HSV, could have been due to stable alterations of the virus and/or of the cell, induced by 42°C-treatment, resulting in a non-permissive system.

To test this possibility, the growth of wt HSV-2, spontaneously reactivated virus, or, an experimentally

reactivated virus (see below) from plaque isolate material, was compared in normal HFL cells and cells which had been incubated for 6 days at 42°C followed by 4 days at 37°C (Table 4.1.). The results show that wt HSV-2, the spontaneously reactivated virus and the experimentally reactivated virus grew equally well in both cell systems.

Therefore, non-permissiveness of cells or changes in the properties of the virus did not account for the failure to detect HSV-2 in latently infected cultures at 37°C.

A further possibility was that, during incubation of HSV-2 infected cultures at 42°C, an inhibitor of HSV growth might be produced by the cells or by the virus, which was needed only transiently to convert HSV to a latent state. This was tested by assay of the inhibitory effects of growth medium, from mock and latently infected cultures, upon the infectivity of a sample of HSV-2.

HFL cell monolayers were mock-infected or infected with HSV-2 at 0.003 pfu/cell and, following adsorption at 37°C, the cultures were incubated at 42°C. At 1 day post-infection fresh growth medium (EF5) was added to the cultures and, subsequently, aspirated after 1 or 2 days in contact with the cell cultures at 42°C. 2ml samples of this growth medium were added to 500 pfu of HSV-2 and incubated at 37°C for 1h. The inoculum was then used to infect BHK cell monolayers at 37°C, and plaques produced were counted 2 days post-infection.

The results in Table 4.2. seem to indicate an inhibitory effect of growth medium from mock infected and latently infected cultures on the infectivity of HSV-2. However, control samples of growth medium incubated at 42°C in the absence of mock- or latently-infected cultures produced an identical effect (Table 4.2.).

Therefore, it would appear that incubation of growth medium at 42°C resulted in an alteration of the medium such that it was unable to support the replication of HSV-2. It is possible that this property of 42°C-treated medium, in combination with incubation of HSV-infected cells at 42°C for 6 days, might be involved in the conversion of HSV to a latent state.

Table 4.1. PERMISSIVENESS OF HEAT-TREATED AND NORMAL UNTREATED HFL CELLS TO INFECTION WITH WT AND REACTIVATED HSV-2

TREATMENT OF CELLS	TIME POST-INFECTION (h)	P.F.U./ml WT HSV-2	REACTIVATED HSV-2	SPONTANEOUSLY REACTIVATED HSV-2
HEAT-TREATED	16	1.8×10^5	1.5×10^5	1.5×10^5
	24	6.3×10^6	1.0×10^7	3.2×10^6
	48	1.7×10^8	1.3×10^8	4.3×10^7
NORMAL, UNTREATED	16	1.5×10^5	1.5×10^5	1.5×10^5
	24	8.4×10^6	7.5×10^6	5.5×10^6
	48	2.5×10^8	1.6×10^8	1.5×10^8

Normal untreated or heat-treated (pre-incubated at 42°C for 6 days followed by 4 days at 37°C) HFL cells were infected with 0.003 pfu/cell of wt HSV-2 or with virus isolated by superinfection of latently infected cultures with tsKsyn (reactivated virus). After various periods of incubation at 37°C, cell monolayers plus growth medium were harvested, sonicated and titrated on BHK cells.

Table 4.2. EFFECTS OF 42°C-TREATED MEDIA ON THE INFECTIVITY OF HSV-2[a]

VOLUME OF MEDIUM (MLS)	SOURCE OF MEDIUM: MOCK-INFECTED CELLS[b]		HSV-2 INFECTED CELLS[b]		MEDIUM ONLY[c]		UNTREATED MEDIUM
	1[d]	2	1	2	1	2	
2.00	17[e]v[f]	0	14 v	0	30 s	0	
1.50	40 s	12 v	43 s	20 s	34 s	4 v	
1.00	75 s	40 s	70 s	58 s	95 n	29 s	
0.50	102 n	105 n	136 n	165 n	126 n	105 n	
0.25	189 n	128 n	128 n	158 n	170 n	100 n	
2.00							191n

a The sample of medium to be tested was made up to a final volume of 2ml with fresh EF5, incubated at 37°C for 1h with 500 pfu of HSV-2, then added to BHK cell monolayers. Plaques were counted after 2 days at 37°C.

b EF5 incubated at 42°C on mock infected or HSV-2 infected cell monolayers for 1 or 2 days.

c EF5 incubated at 42°C for 0, 1 or 2 days.

d Number of days incubation of medium at 42°C.

e Number of plaques of HSV-2.

f Plaque size: v, very small; s, small; n, normal.

4.2.4. Experimental Reactivation Induced by Superinfection

For the virus-cell interaction of the system described here to be considered as latency, it was essential to demonstrate reactivation of HSV-2. It has previously been shown that latent HSV can be reactivated in culture by superinfection with HSV ts mutants or with HCMV ts mutants (Colberg-Poley et al., 1979, 1981; Lewis et al., 1984; Wigdahl et al., 1982a,b; Notarianni, 1986).

4.2.4.1. Superinfection of Latently-Infected Cultures by ts Mutants of HSV-1

Cell monolayers, latently infected with HSV-2 (0.003 pfu/cell), which had been incubated for 6 days at 42°C and 4 days at 37°C, were superinfected at 38.5°C with HSV-1 ts mutants tsKsyn or tsIsyn. Both of these mutants, in contrast to HSV-2 strain HG52, form syncytial plaques on HFL cells. The HSV-1 mutant tsK has a mutation in the coding sequences for Vmw175 and synthesises only IE proteins at 38.5°C, whereas tsIsyn is a late mutant which produces viral DNA as well as most polypeptides at the NPT (Section 1.3.4.2.; Marsden et al., 1976; Preston, 1979a; Preston, 1981).

Cultures were superinfected with the ts mutants at multiplicities of 0.03, 0.1 or 0.3 pfu/cell, overlaid with growth medium containing neutralising human serum (EHu5), and plaques were counted after incubation for 2 days at 38.5°C (Table 4.3.). Following superinfection with either mutant, cell monolayers contained plaques which were predominantly non-syncytial, resembling HSV-2. The number of plaques increased approximately in proportion to the amount of ts mutant, but it should be noted that m.o.i. values of 1 or more gave reduced plaque numbers due to cell killing (Section 4.2.4.4.).

4.2.4.2. Confirmation of the Identity of Reactivated Virus

To confirm that the non-syncytial, non-ts virus reactivated by superinfection with tsKsyn was HSV-2 rather than revertants or intertypic recombinants of tsKsyn, DNA

Table 4.3. REACTIVATION OF LATENT HSV-2 BY SUPERINFECTION

INITIAL INFECTION	M.O.I.[a]	SUPERINFECTION tsKsyn[b]	tsIsyn	HCMV	Ad2	Ad5	uvtsK
HSV-2[c]	0.03	109[d](3)[e]	116 (1)	94	0	0	0
HSV-2	0.10	212 (3)	213 (0)	332	0	0	0
HSV-2	0.30	504 (4)	TNTC[f]	TNTC	0	0	0
MOCK	0.30	4 (4)	0	0	0	0	0

a Pfu per cell of superinfecting virus.

b Superinfecting virus; uvtsK is tsK which has been u.v. irradiated to reduce the titre by a factor of 5×10^5.

c Initial m.o.i. was 0.003 pfu per cell.

d Number of plaques per plate.

e Numbers in parentheses represent the number of syncytial plaques.

f TNTC, too numerous to count (>500 plaques).

from reactivated virus plaque isolates was labelled *in vivo* with [^{32}P]-orthophosphate and restricted with BamHI (Figure 4.1.), as described by Lonsdale (1979). All of the 8 virus samples analysed contained DNA characteristic of HSV-2, with no evidence for the presence of revertants or recombinants. Size variation of fragments g, p, w and the band containing y and z was observed, as previously reported in plaque isolates of HSV-2 (Buchman *et al.*, 1980; Davison and Wilkie, 1981; Maitland *et al.*, 1982; Chaney *et al.*, 1983).

4.2.4.3. HCMV Superinfection of Latently-Infected Cultures

HFL cells infected with HSV-2 (0.003 pfu/cell) were incubated at 42°C for 6 days and 37°C for 4 days prior to superinfection with HCMV. Superinfection was at 0.03, 0.10 and 0.30 pfu/cell. Following adsorption for 1.5h at 37°C, the cultures were incubated at 37°C for 2 days.

The results in Table 4.3. indicate that HCMV was as efficient as the HSV-1 *ts* mutants in its ability to reactivate latent HSV-2, in agreement with previous findings in a related *in vitro* latency system (Colberg-Poley *et al.*, 1979, 1981; Wigdahl *et al.*, 1981); the virus produced was identified as HSV-2 by its ability to form plaques on BHK cells, which are non-permissive for HCMV.

4.2.4.4. Cytotoxicity at Higher Superinfecting M.o.i.

Theoretically, superinfection of each latently infected cell with at least 1 infectious viral particle (under conditions non-permissive for growth of the superinfecting virus) should subsequently give rise to plaques, the number of which would directly reflect the number of cells harbouring latent HSV in reactivatable form.

Table 4.4. shows the results of an experiment in which cells were infected with HSV-2 at 0.003 pfu/cell and incubated at 42°C for 6 days and then at 37°C for 4 days, prior to superinfection with *ts*Ksyn or HCMV at 0.03, 0.10, 0.30, 1.0, or 3.0 pfu/cell. At 2 days post-superinfection, the cells were harvested, sonicated and titrated on BHK cells. Superinfection of latently infected cultures with

Figure 4.1. DNA PROFILES OF REACTIVATED VIRUS

Autoradiograph of ^{32}P-labelled BamHI-digested DNA from reactivated virus samples (lanes 3 to 10 [read left to right]). Cells were infected with plaque isolates of virus reactivated from latently infected cultures by superinfection with tsKsyn. Control lanes contain DNA from cells infected with wt HSV-2 (HG52) (lane 2) or wt HSV-1 (17) (lanes 1 and 11). DNA samples were labelled *in vivo* with [^{32}P]-orthophosphate and restricted with BamHI. *In vivo* - labelling, electrophoresis and autoradiography were performed by Dr. C.M. Preston. The fragments which vary in size are labelled.

HSV-2

REACTIVATED VIRUS

HSV-1

HSV-1

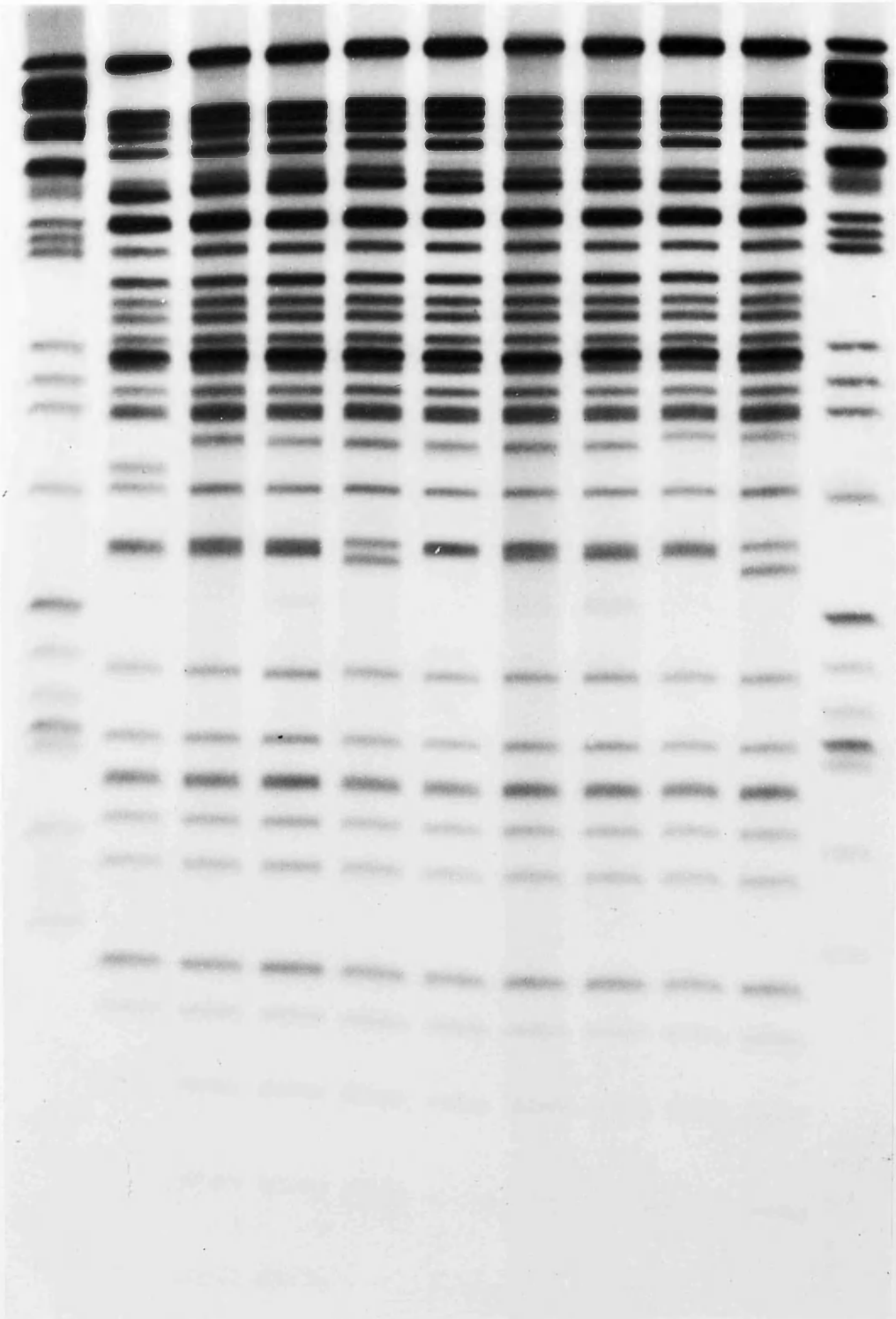


Table 4.4. REDUCED YIELD OF REACTIVATED VIRUS AT HIGHER SUPERINFECTING MULTIPLICITIES[a]

INITIAL INFECTION	M.O.I.[b]	SUPERINFECTION tsKsyn[c]	SUPERINFECTION HCMV
HSV-2[d]	0.03	2.9×10^{6}[e]	8.1×10^{5}
HSV-2	0.10	2.1×10^{6}	7.5×10^{5}
HSV-2	0.30	6.1×10^{5}	2.6×10^{5}
HSV-2	1.00	3.4×10^{4}	2.3×10^{5}
HSV-2	3.00	3.8×10^{3}	4.5×10^{4}
MOCK	3.00	0	0

a Latent infections were established with HSV-2 in HFL cells and reactivation was achieved by superinfection with tsKsyn or HCMV. 2-3 days post superinfection the cells were harvested into the 2ml of bathing medium per plate, sonicated and titrated on BHK cells.

b Pfu per cell of superinfecting virus.

c Superinfecting virus.

d Initial m.o.i. was 0.003 pfu per cell.

e Pfu per ml of cell harvest material.

tsKsyn at m.o.i. values of 0.3 or more, and with HCMV at m.o.i. values of greater than 0.3 gave reduced yields of reactivated HSV-2.

This experiment illustrates the problem of cell killing which occurs at the higher superinfecting multiplicities and which is probably due to a toxic effect on the cells of non-infectious viral particles in the inoculum. Therefore, at such multiplicities, the number of plaques formed would not accurately reflect the number of cells in which HSV was harboured in a latent and reactivatable state.

4.2.5. High Efficiency of Experimental Reactivation

The number of plaques produced following superinfection at a specific m.o.i. can vary by at least 3-fold. From the data presented in Table 4.3., it is clear that a high proportion of the infectious HSV-2 particles were reactivated by superinfection. For example, in cultures of 10^6 cells initially infected with 3000 pfu of HSV-2, an average of 209 plaques were detected after superinfection with 0.1 pfu/cell of tsKsyn or tsIsyn, and 332 were observed after superinfection with 0.1 pfu/cell of HCMV. This finding suggests that the efficiency of reactivation is very high, since only 10% of HFL cells harbouring HSV-2 had received a potentially infectious superinfecting virus particle. The data in Table 4.13. suggest an approximately 3-fold lower efficiency of reactivation of HSV-2 by superinfection with tsKsyn for this experiment.

To further investigate the efficiency of reactivation, lower initial doses of HSV-2 were used (Table 4.5.). The results in Table 4.5. show that 15-34% of the initial infectious HSV-2 could be reactivated by superinfection with 0.3 pfu/cell of tsKsyn, and imply that a greater proportion (50-100%) could have been reactivated but for the problem of cytotoxicity at higher multiplicities of tsKsyn (Section 4.2.4.4.). This calculation could be affected by the presence of non-infectious HSV-2 or tsKsyn particles, as discussed in Section 4.2.9..

The calculation of reactivation efficiency depends

Table 4.5. EFFICIENCY OF REACTIVATION OF HSV-2 FROM LATENTLY INFECTED CULTURES BY SUPERINFECTION WITH tsKsyn[a]

EXPT. NO.	ACTUAL PFU APPLIED TO PLATE	PLAQUES REACTIVATED	% INITIAL HSV-2 REACTIVATED
1	61, 84	13, 12	18
1	210, 220	37, 27	15
2	42, 53	13, 14	29
2	103, 104	35, 34	34

a Latent infections were established in HFL cells at low initial m.o.i.and reactivation was achieved by superinfection with tsKsyn at 0.3 pfu/cell.

upon the well-established observation that replication and spread of HSV-2 to form new foci of infection does not occur at 42°C (Crouch and Rapp, 1972; Darai and Munk, 1973; Marcon and Kucera, 1976; Levine et al., 1980; Notarianni, 1986). Addition of neutralising rabbit anti-HSV serum (a gift of Dr. A. Cross) to the growth medium during the first 2 days of incubation at 42°C did not affect the number of plaques produced after superinfection with tsKsyn, suggesting that HSV-2 did not replicate and spread at 42°C in the system reported here (Table 4.6.). (It should be noted that spread of virus to adjacent cells would not be prevented by the presence of antiserum, but that such foci would yield only 1 plaque upon superinfection).

The calculation of reactivation efficiency would also be affected by the replication and spread of HSV-2 to form new foci of infection at 37°C, a temperature permissive for HSV replication. The following experiment attempted to determine the level of replication and spread of the virus at 37°C. HFL cells were infected with HSV-2 at 0.003 pfu/cell and incubated at 42°C for 6 days at 42oC and then at 37°C for 1, 2, 3 or 4 days. The cultures were superinfected with tsKsyn at a m.o.i. of 0.03 pfu/cell at the NPT. The results from Table 4.7. show that there is no significant difference in the numbers of plaques of virus reactivated by tsKsyn superinfection (0.03 pfu/cell) following 1, 2, 3 and 4 days incubation at 37°C, thus implying that there is no spread of HSV-2 during the 37°C period to form new foci of infection. This correlates with the infrequency of detection of infectious virus during 37°C incubation (Section 4.2.2.).

Taken together, these results imply that a relatively high efficiency of experimental reactivation can be achieved.

4.2.6. Subculture of Latently Infected Cultures

To investigate whether alterations in cell growth state could reactivate HSV-2, latently infected HFL cells were subcultured by trypsinisation and replating on new Petri dishes at half the original cell density. Monolayers

Table 4.6. EFFICIENCY OF REACTIVATION FROM LATENTLY INFECTED CULTURES PRETREATED WITH NEUTRALISING ANTISERUM[a]

SUPERINFECTION	M.O.I.[b]	ANTISERUM DILUTION 0	1/100[c]	1/250
tsKsyn	0.03	40[d](0)e	27 (1)	32 (1)
tsKsyn	0.10	116 (2)	98 (1)	100 (2)
MOCK	0.00	0	0	0

a Latent infections were established with 0.003 pfu/cell of wt HSV-2 in HFL cells. The cultures were overlaid with EF5 or with EF5 plus rabbit anti-HSV antiserum for the first 2 days postinfection at 42°C, and overlaid with EF5 for a further 4 days at 42°C and 4 days at 37°C. The cultures were then superinfected with tsKsyn and plaques were counted after 2 days at 38.5°C.

b Pfu per cell of superinfecting virus.

c Antiserum dilutions. In control experiments: 1/100 dilution neutralised 90% of infectious HSV-2 and 1/250 dilution neutralised 50% of infectious HSV-2.

d Number of plaques per plate.

e Numbers in parentheses represent the number of syncytial plaques.

Table 4.7. SUPERINFECTION OF LATENTLY-INFECTED CULTURES AFTER 1, 2, 3, AND 4 DAYS INCUBATION AT 37°C[a]

DAYS, TEMP.[b]	PLAQUES PER PLATE
6,42°C and 1,37°C	100 (3)c
6,42°C and 2,37°C	168 (5)
6,42°C and 3,37°C	172 (5)
6,42°C and 4,37°C	110 (0)

a Cultures were infected with HSV-2 at 0.003 pfu/cell, incubated at 42°C for 6 days and 37°C for 1, 2, 3, or 4 days, and then superinfected with tsKsyn at 0.03 pfu/cell at 38.5°C.
Plaques were counted after 2 days.

b The number of days of incubation at 42°C and at 37°C prior to superinfection.

c Numbers in parentheses represent the number of syncytial plaques.

were grown to confluence and tested for the presence and reactivation of HSV-2.

HFL cell monolayers on 35mm Petri dishes, infected with HSV-2 at 0.003 pfu/cell, were incubated at 42°C for 6 days followed by 37°C for 4 days, prior to subculture. Confluent monolayers of passaged cells were re-subcultured, or, superinfected with tsKsyn at 0.3 pfu/cell and incubated at 38.5°C for 2 days.

No infectious virus was recovered in the growth medium or after harvesting, sonication and titration of cells, but superinfection with tsKsyn was able to reactivate HSV-2 (Table 4.8.). The number of plaques produced per plate after superinfection with tsKsyn declined as cells were subcultured, although HSV-2 could still be reactivated after three passages.

This experiment shows that not only is HSV maintained in a non-infectious state in tissue culture cells for at least 13 days at a temperature permissive for HSV replication, but that this non-infectious state is not disrupted upon subculture of infected cell monolayers.

4.2.7. Chemical or Ultraviolet Light Treatment of Latently Infected Cultures

Various other manipulations of latently infected cells were carried out to test the stability of the virus-cell interaction, including exposure to UV irradiation and to the chemical compounds 5-azacytidine or 12-O-tetradecanoyl-phorbol-13-acetate (TPA).

UV irradiation has been shown to stimulate the reactivation of latent HSV-1 *in vivo* (Blyth *et al.*, 1986), for example, in the mouse ear model, in which initial infection with HSV in the ear results in the establishment of a latent HSV infection in the cervical ganglia, exposure of the ear to UV irradiation results in the reappearance of infectious virus in the ganglia 1 to 5 days later (Harbour *et al.*, 1983).

5-azacytidine induces the expression of genes by inhibiting the methylation of newly synthesised DNA (Jones, 1984; Taylor *et al.*, 1984), and can not only induce the

Table 4.8. MAINTENANCE AND REACTIVATION OF HSV-2 AFTER SUBCULTURE OF LATENTLY INFECTED CELLS[a]

Number of plaques /plate after superinfection with 0.3 pfu/cell of tsKsyn or mock superinfection

	ORIGINAL		PASS 1[b]		PASS 2		PASS 3	
EXPT. NO.	Mock	tsK	Mock	tsK	Mock	tsK	Mock	tsK
1	0	183 (9)[c]	0	115 (7)	ND[d]	ND	ND	ND
2	0	307 (6)	0	240 (8)	0	95 (6)	0	23 (2)
3	0	244 (3)	0	125 (3)	0	31 (0)	0	23 (2)

a Latent infections were established with 0.003 pfu/cell of wt HSV-2 in HFL cells.

b At each passage, cell monolayers were trypsinised and reseeded at half the original density. Superinfection was carried out at 38.5°C when cell monolayers were confluent and plaques were counted after 2 days.

c Numbers in parentheses represent the number of syncytial plaques.

d ND, no data.

reexpression of the HSV TK gene from mouse cells transformed with the viral information in an inactive state (Clough et al, 1982) but can also activate the EBV replicative cycle in latently infected human lymphoid cell lines (Ben-Sasson and Klein, 1981).

TPA affects latent EBV by enhancing the expression of various transcripts and inducing the virus to enter the replicative cycle (zur Hausen et al., 1978; Jeang and Hayward, 1983; Faggioni et al., 1986; Boos et al., 1987).

HFL cells infected with HSV-2 (0.003 pfu/cell) were incubated at 42°C for 6 days and 37°C for 4 days, and then exposed to various chemicals or to ultraviolet irradiation. The concentrations and time periods of treatments to which latently infected cells were exposed, as well as other experimental details, are described in Table 4.9. It should be noted that since the ability of 5-azacytidine to demethylate DNA requires replication of DNA, the latently infected cells were passaged once during exposure to 5-azacytidine.

None of the treatments tested were able to induce the reactivation of HSV-2 from its latent state in vitro, as discussed in Section 4.2.9.

4.2.8. Specificity of Virus-Cell Interaction

To determine whether establishment and maintenance of a latent state in vitro was exclusively a feature of an interaction between HFL cells and HSV-2 strain HG52, the cell type and virus strain were varied.

HSV-2 strain HG52 (0.003 pfu/cell) could be converted efficiently to a latent state in another cell line, human foreskin fibroblasts, upon incubation at 42°C for 6 days. Incubation was continued at 37°C for a further 4 days, during which time no plaques of reactivated virus were detectable. The presence of latent HSV-2 in these cells was demonstrated by superinfection of cultures with 0.03, 0.10 or 0.30 pfu/cell tsKsyn and incubation at 38.5°C for 2 days, which resulted in the appearance of plaques of reactivated virus in the cell monolayers (Table 4.10.)

Three additional strains of HSV-2, strains 333, 186

Table 4.9. CHEMICAL AND PHYSICAL TREATMENTS APPLIED TO LATENTLY INFECTED CULTURES

TREATMENT	EXPERIMENTAL DETAILS
UV-Irradiation (240 nm) Distance = 200cm Exposure = 30sec, 1,2,3 or 5 min Energy output = 32erg/mm^2/sec	EF5 was aspirated from latently infected cell monolayers and replaced by warm PBS. Petri dish lids were removed and the cells exposed to UV light. EF5 was reapplied and cultures were incubated at 38.5°C for 2 days.
TPA Concentration = 200ng/ml	EF5 was removed from monolayers and replaced with TPA in warm PBS. EF5 was reapplied and the cultures were incubated at 38.5°C for 2 days.
5-Azacytidine Concentrations = 0.75,1.5,3.0 or 6.0uM	EF5 was removed from monolayers, replaced with 5-azacytidine in EF5 and incubated at 37°C for 2 days. The cells were then trypsinised and replated at half the original cell density in EF5 plus 5-azacytidine. Monolayers were stained after a further 4 days at 37°C.

HFL cells were infected with HSV-2 at 0.003 pfu per cell and incubated in EF5 at 42°C for 6 days and then at 37°C for a further 4 days, prior to exposure to the various physical and chemical treatments outlined above.

Table 4.10. ESTABLISHMENT OF DIFFERENT STRAINS OF HSV-2 IN A LATENT STATE IN HUMAN FIBROBLASTIC CELL LINES HFL AND HFF[a]

	CELL LINE				
	HFF	HFL			
M.O.I.[b] Super-Infection	HSV-2 Strain				
	HG52	HG52	333	186	UW268
0.03	67[c](ND)[d]	40 (2)	12 (1)	8 (1)	8 (1)
0.10	142 (ND)	127 (3)	52 (5)	23 (2)	16 (1)
0.30	TNTC[e]	TNTC	133 (10)	44 (3)	89 (8)

a Cultures were infected with 0.003 pfu/cell of wt HSV-2 virus strains, incubated at 42°C for 6 days and 37°C for 4 days. Superinfection of cultures with tsKsyn was carried out at 38.5°C; and plaques were counted after 2 days.

b Pfu of superinfecting virus per cell.

c Number of plaques per plate.

d Numbers in parentheses represent the number of syncytial plaques.

e TNTC, too numerous to count.

and UW268, were compared with HSV-2 strain HG52 for the ability to establish a latent interaction with HFL cells. HFL cells were infected with HSV (0.003 pfu/cell) and incubated at 42°C for 6 days and then 37°C for 4 days. There was no evidence of plaques of spontaneously reactivated virus in the monolayers during the 37°C period. The presence of latent virus in these cultures was demonstrated by the appearance of plaques of reactivated HSV-2 following tsKsyn superinfection at 0.03, 0.10, or 0.30 pfu/cell at 38.5°C. All HSV strains tested became established in a latent state in HFL cells and could be experimentally reactivated (Table 4.10.). Results in Table 4.12. show that HSV-1 strain 17 is also able to establish a latent state in this system; reactivation of latent HSV-1 was achieved by HCMV superinfection at 0.02 pfu/cell.

4.2.9. Discussion

In the system described, HSV is efficiently converted to a non-productive state in human fibroblasts by incubation at 42°C, and can be maintained in this state at permissive temperatures for viral growth for at least 13 days; the level of spontaneous reactivation is low. The virus-cell interaction produced can be classified as latency since HSV is maintained in a non-infectious state under conditions permissive for virus replication and can be experimentally reactivated by superinfection.

In the use of HFL cells and supraoptimal temperatures to suppress virus replication, the system described (Notarianni, 1986; Russell and Preston, 1986) is similar to work published by O'Neill (1977), who has examined HFL cells after infection with HSV-2 at 39.5 to 40°C, but found that all cultures rapidly produced virus when downshifted to 37°C. It is likely, therefore, that the higher temperature of 42°C may be important for establishment of the latent state. It is important to note that here the latent state is defined as the period, albeit short, at the permissive temperature of 37°C rather than 39.5 to 40.5°C used in inhibitor-treated repressed cells.

It is possible that conversion of HSV to a latent

state by the use of supraoptimal temperature is a consequence of the cellular stress response, which is known to be induced in human embryo lung cells incubated at 42°C (Notarianni, 1986; Figure 4.2.). The stress response is manifested at the level of transcription and leads to repression of transcription of most cellular genes and the overexpression or positive induction of expression of a set of genes termed cellular stress (or heat shock) genes (Notarianni, 1986). A latent state might be achieved through the effect of alterations in cell metabolism brought about by the cellular stress response, or, as a direct result of the cellular stress response upon viral gene expression. The apparent inability of 42°C-treated growth medium to support an efficient lytic infection of HSV is another consideration, and further investigation of the properties of the 42°C-treated medium would be of some interest.

It has been argued that *in vitro* latency systems should involve neuronal cells, which are highly differentiated and non-dividing and which represent the predominant site of latency for HSV. However, since there is recent evidence to suggest that HSV establishes latency in non-neuronal cells (Clements and Jamieson, 1989; Clements and Subak-Sharpe, 1988; Al-Saadi *et al.*, 1988) it may be the metabolic state of the neuron rather than its extreme differentiation *per se* that is of importance in producing the latent state. Therefore, the use of fibroblasts in the latency system described rather than neuronal cells may not be an important variable.

Production of a latent viral state might be a general feature of interaction between HSV and cells following 42°C treatment; HSV-2 established a latent state in both HFL and HFF cells, and HSV-1 established a latent state in HFL cells. The HFL and HFF cells utilised in the system were chosen partly because of their ability to survive incubation at the supraoptimal temperature of 42°C for 6 days. Cook and Brown (1986, 1987) have since reported that HSV-1 can be maintained in a non-productive state in cultured rabbit corneal cells (epithelial cells, keratocytes and endothelial cells) during incubation of infected cultures at 41.5°C or 42°C and for several days during the subsequent 37°C

incubation period. Cook and Brown (1986, 1987) considered that HSV was latent during the period of incubation at 41.5°C or 42°C, which are temperatures non-permissive for viral growth, rather than during the period at 37°C in which infectious virus was not detectable. Cellular stress proteins were induced in the rabbit corneal cells at 41.5°C (Cook and Brown, 1986). Superinfection of infected-cultures with an HSV-1 deletion mutant, following 42°C treatment, resulted in the isolation of virus with the wild-type DNA structure, at least a proportion of which was assumed to be reactivated input virus; in addition recombinants between the HSV-1 genomes harboured in the corneal cells and the superinfecting HSV-1 genomes were isolated. The significance of the system of Cook and Brown (1986, 1987) is in the demonstration that incubation at supraoptimal temperatures can convert HSV to a latent state in corneal cells in which, there is evidence to suggest, HSV can be harboured in a latent state *in vivo* (Shimeld *et al.*, 1982; Tullo *et al.*, 1985; Cook *et al.*, 1987).

The latent state produced is apparently very stable, inasmuch as it is not susceptible to disruption by the various chemical and physical manipulations described, the effects of which, *in vitro*, would be generally manifested in alterations of cellular or viral metabolism.

UV-irradiation of cells latently infected with HSV *in vitro* did not induce reactivation of HSV. The primary effect of ultraviolet irradiation on the DNA of cultured cells would be a photolytic action causing adjacent pyrimidine rings, particularly thymines, to coalesce in pairs, thereby weakening the bonds between the 2 strands of the double helix and leading to distortion of the helix and inhibition of replication and transcription of DNA. *In vivo*, UV-irradiation at the periphery induces reactivation of HSV from latently infected ganglia (Blyth *et al.*, 1976).

It is unlikely that the effects of direct UV-irradiation of cultured cells would be equivalent to those produced in ganglionic cells following UV-irradiation at the periphery.

On the assumption that the 5-azacytidine treatment of

latently infected cells demethylated the HSV genome, demethylation is apparently unable to induce sufficient HSV gene expression for progression from the latent to the lytic cycle, implying that the latent state of the HSV genome is not a result of extensive methylation of the genome. Although this does not correlate with the *in vivo* findings that 5-azacytidine and the demethylation agent HMBA induce reactivation of latent HSV from explants of neural and non-neural tissues (Bernstein and Kappes, 1988; Stephanopoulos *et al.*, 1988), it is consistent with the findings of Dressler *et al.* (1987) that latent HSV is not heavily methylated *in vivo*. The inability of TPA treatment to induce reactivation suggests that the mechanism of reactivation, and thus possibly the latent state, of HSV differs from that of EBV; the latent EBV genome is reported to be extensively methylated and may be reactivated by treatment with demethylation agents as well as with TPA.

HSV is not reactivated by passage of the latently infected cultures and it was noted that the proportion of cells harbouring a latent HSV genome decreased upon cell passage. This would seem to suggest that replication of the latent HSV genome does not occur concomitantly with cellular DNA replication. However, a decrease in the proportion of cells harbouring latent HSV DNA would also be observed if the cells latently infected with HSV were unable to divide, or if the cells and the latent virus were able to divide but death of a proportion of the latently infected cells occured. Consequently, the replicative nature of the latent HSV cannot be inferred from this experiment.

Reactivation of HSV from its latent state can be induced rapidly by intertypic superinfection, as previously shown by Notarianni (1986) and as reported for other *in vitro* latency systems (Colberg-Poley, 1979, 1981; Wigdahl, 1982a,b). In experiments reported here, superinfection, by viruses *ts*K or HCMV, of HSV-infected cells incubated at 42^{o}C for 6 days and 37^{o}C for a further 4 days, reactivated HSV-2 rapidly and efficiently.

Although the efficiency of reactivation calculated suggests that both the establishment of latency and reactivation from latency of HSV occur with high frequency

in the system described, involvement of non-infectious viral particles present in initial infecting and in superinfecting inoculums would affect this calculation. For instance, if non-infectious HSV-2 particles were maintained in a latent state and were reactivated by tsKsyn superinfection, complementation of the superinfecting virus by the reactivated virus, and vice versa, would culminate in the formation of a plaque. One argument against this occurrence is that HCMV reactivates latent HSV-2 as efficiently as tsKsyn. There is also an approximate correlation between HSV genome copy number per cell and the number of infectious HSV-2 particles (Section 4.5). Alternatively, should non-infectious particles of tsKsyn and of HCMV have been able to supply the functions essential for triggering the reactivation event, then a higher proportion of latently infected cells would have received a potentially reactivating viral particle than calculated according to the number of infectious viral particles. Assuming that the particle to pfu ratio of tsKsyn is between 10:1 and 100:1; if 0.3 pfu/cell is the superinfecting m.o.i. then each cell would receive 3 to 30 non-infectious particles per cell. Unless less than 1 of the non-infectious particles per cell is capable of producing functions required for reactivation, a 3-fold lower level of reactivation would not be expected at a 3-fold lower multiplicity of superinfection. Since a 2- to 3-fold lower level of reactivation is observed following superinfection of latently infected cells at 0.1 pfu/cell compared to superinfection at 0.3 pfu/cell, then it may be concluded that less than 1 out of 3, and possibly less than 1 out of 30, non-infectious particles is capable of reactivating latent HSV. Complementation studies of Messer (1978) suggested that some non-infectious particles of HSV-2 mutant viruses were effective in producing plaques if complemented by another HSV-2 mutant and were therefore capable of producing some viral functions. However, in HSV-1 infections, the proportion of cells expressing IE gene 3, as determined by immunofluorescence studies, correlated approximately to the number of pfu initially applied to the cells (N.D. Stow, personal communication). IE-3 gene expression is positively regulated by the same mechanism as

expression of the other IE genes, therefore it is unlikely that HSV-1 non-infectious viral particles express the IE genes. Since, from the results presented in this thesis (Section 4.4.) there is a requirement for at least one of the IE polypeptides in the reactivation of HSV from its latent state *in vitro* by superinfection with HSV-1 mutants, then it is unlikely that non-infectious particles of superinfecting HSV-1 mutants are capable of inducing reactivation.

Therefore, the implication of the results presented is that there is a high efficiency of establishment and reactivation in the latency system: 30-100% of input HSV-2 is able to establish a latent state from which reactivation may be induced upon superinfection with the HSV-1 mutant *ts*Ksyn. In the system of Wigdahl and coworkers (1981, 1982a,b), HSV, converted to a latent state by the use of viral inhibitors and temperature elevation, can be induced to reactivate by temperature reduction or by intertypic superinfection with ts mutants of HCMV or with HSV. However, the percentage recovery of input virus following superinfection is relatively low, (1% to 3%), despite an initial infecting m.o.i. of 2.5 pfu/cell and a superinfecting m.o.i. of 1 pfu/cell, suggesting a low efficiency either of establishment of latency or of experimental induction of reactivation. It was shown that a significant proportion of the cells harboured HSV in a latent state not reactivatable by superinfection or temperature reduction, as judged by the presence of HSV antigens following superinfection or temperature reduction and the level of detection of HSV genomes in the cells (Wigdahl *et al*., 1982b, 1984a,b). Thus, the efficiency of conversion of HSV to a latent state and the efficiency with which latent HSV can be experimentally reactivated appear to be significantly lower in this system in comparison to the system described in this thesis.

In summary, the latency system described fulfills all of the criteria proposed by Levine *et al*. (1980) inasmuch as, infected cells survive and harbour the complete HSV genome in a non-infectious state, and infectious virus can be recovered following manipulation of the culture by

superinfection. Although the system described is similar to, and indeed partly based on, previous studies by others, differences exist in the methodology used and results obtained. It is not clear whether these variations are a matter of degree or if the virus-cell interactions are fundamentally different; this will be discussed in later sections. The system has the advantages of simplicity, relying solely on the use of supraoptimal temperature to convert HSV to a latent state, as well as a high level of reproducibility and a high efficiency of establishment and reactivation. The following sections are concerned with analysis of the latent viral-cell interaction.

4.3. VIRAL FACTORS INVOLVED IN ESTABLISHMENT OR MAINTENANCE OF LATENCY

4.3.1. Introduction

The approaches taken in analysis of the viral factors involved in establishment or maintenance of latency *in vitro* are essentially equivalent to the approaches used in the *in vivo* experiments outlined in Section 1.3.4.2., namely, identification of the HSV genes required in latency via examination of the behaviour of HSV mutants in the latency system, and, analysis of HSV gene expression in latently infected cells.

In the former studies, the ability of a mutant to become established in a latent state *in vitro* was assessed by its capacity to maintain a non-infectious state, during incubation at a permissive temperature for HSV replication for at least 6 days, from which it could be induced to reactivate upon superinfection. The purpose of these studies was to elucidate viral factors non-essential for HSV latency, and thereby, by a process of elimination, to identify viral factors which might be involved in establishment or maintenance of the latent state.

Preliminary analysis of viral gene expression in latently infected cells involved the use of a sensitive enzyme assay to detect the presence of functional gene products from the HSV E TK gene.

4.3.2. Analysis of Latently Infected Cultures for Viral TK Activity

Latent HSV gene expression was investigated by analysis of TK enzyme activity in latently infected cells. Since only a small proportion (0.3%) of HFL cells harbour a latent HSV genome in the system described, the initial m.o.i. of input HSV was increased 100-fold or 300-fold to give a higher proportion (30% or 100%) of cells containing latent HSV and, thereby, to increase the probability of detecting gene expression from the latent virus, should it

occur. The viral TK assay is sensitive enough to detect the presence of the enzyme in cells initially infected at 0.01 pfu per cell (C.I. Ace, personal communication). Therefore, TK assays were performed on HFL cell cultures infected with wt HSV-2 strain HG52 at 0.3 or 1.0 pfu/cell, which had been incubated at 42°C for 6 days.

The results (Table 4.11.) indicate that no viral thymidine kinase activity above background levels for mock infected cells was detectable in these latently infected cultures, suggesting that viral TK is not required for the maintenance of the latent state *in vitro*. This result also suggests that transcription, translation or processing of viral TK is inhibited in latently infected cells, or that the TK polypeptide is altered in such a way as to destroy its enzymatic activity. The HSV TK gene is a typical E HSV gene in terms of its promoter and regulatory regions, so, if there is a block in transcription of this gene in latently infected cells, it is probable that expression of other E HSV genes is similarly affected.

4.3.3. HSV-2 ts Mutants and Establishment of Latency

The HSV-2 mutants tested for the ability to establish latency *in vitro* were *ts*3, *ts*5, *ts*9 and *ts*11. With respect to the properties of these mutants at the NPT: *ts*3 and *ts*5 are DNA positive; *ts*9 and *ts*11 are DNA negative; the best characterised are *ts*5, which is defective in a late function at the NPT (38.5°C), and *ts*9, which is defective in the uncoating process as well as in a late function at the NPT (39.5°C) (Messer, 1978; Marsden *et al*., 1976).

HFL cells were infected with *ts*3, *ts*5, *ts*9 or *ts*11 at 0.003 pfu/cell, and, following a 1h adsorption period at the NPT, the cultures were incubated at 42°C for 6 days and then at 31°C for 4 days.

During the 31°C period, no plaques were observed in any of the cultures, demonstrating that replication of the mutants was repressed by incubation at 42°C, as described previously for the wt HSV-2 strains (Section 4.2.8.). Superinfection of *ts*5- and *ts*9- infected cultures with HCMV at 31°C resulted in the appearance of plaques, indicating

Table 4.11. VIRAL THYMIDINE KINASE ASSAY OF LATENTLY INFECTED CULTURES[a]

INITIAL INFECTION		TK ACTIVITY
Virus	M.o.i.	cpm/Cell
Mock	0	0.03
HSV-2	0.3	0.04
HSV-2	1.0	0.03
Positive Control[b]		1.50

a, HFL cells were mock-infected or infected with HSV-2 at 0.3 or 1.0 pfu per cell and incubated in EF5 at 42°C for 6 days. Cultures were harvested and thymidine kinase assays were performed on 20ul of the cytoplasmic fraction from each sample. The results are shown in cpm/cell. The data are from 1 experiment.

b A positive control for the TK assay, a sample known to possess thymidine kinase activity.

that reactivation of these mutants had occurred (Table 4.12.). There was, however, reduced recovery of the HSV-2 mutant ts9 compared to recovery of wt HSV-2 and ts5, as discussed in Section 4.3.6.. Virus was not recovered from the latently infected cultures upon HCMV superinfection at the non-permissive temperature; titration of cell sonicates on BHK cells at 31°C and 38.5°C showed that the virus reactivated following HCMV superinfection at 31°C of the latently infected cultures was unable to form plaques on BHK cell monolayers at the NPT. Therefore, the reactivated virus was parental ts virus, rather than revertant virus. These results indicate that both ts5 and ts9 were able to establish a latent infection in vitro.

HCMV superinfection at 31°C of ts3- and tsll-infected cultures did not result in the production of plaques in the cell monolayers (Table 4.12.). However, titration of cell sonicates, from the HCMV superinfected cultures, on BHK cell monolayers at 31°C resulted in the production of plaques of ts virus (plaques were not produced upon titration of cell sonicates at 38.5°C). One possible explanation of these findings is a decreased plaquing efficiency of ts3 and tsll mutant viruses on HFL cells compared to BHK cells. Indeed, a comparison of the titres of the ts3 and tsll mutant viruses on BHK cells and on HFL cells revealed that the mutants produced 100-fold less plaques on HFL cells. These findings suggest that both ts3 and tsll were converted to a latent state in this in vitro system, and were induced to reactivate by HCMV superinfection, but that plaques of reactivated virus were not produced due to the reduced plaquing efficiency of these mutants on HFL cells.

In conclusion, all of the HSV-2 ts mutants tested, in addition to wt HSV-2, were able to establish a latent infection in vitro. Therefore, assuming that these mutants are as restricted in their gene expression at 42°C as at the non-permissive temperature of 38.5°C, growth and spread of input virus is not a prerequisite for the interaction to occur.

Table 4.12. ESTABLISHMENT OF LATENCY BY HSV ts MUTANTS[a]

SUPER-INFECTION	INITIAL INFECTION HSV-2	ts3	ts5	ts9	ts11
HCMV	49[b](13)[c]	0	87 (25)	13 (5)	0
MOCK	0	0	0	0	0

SUPER-INFECTION	INITIAL INFECTION HSV-1	tsKsyn	tsIsyn
HCMV	62 (11)	31 (10)	95 (26)
MOCK	0	0	0

a Monolayers were initially infected with 0.003 pfu/cell of wt HSV-1, wt HSV-2, or ts mutants of HSV, incubated at 42°C for 6 days, 31°C for 4 days and superinfected with 0.02 pfu/cell of HCMV or mock-superinfected. Plaques formed after incubation at 31°C for 3 days were counted.

b Mean number of plaques per plate.

c Standard error of the means of at least 3 determinations.

4.3.4. HSV-1 Ts Mutants and Establishment of Latency

The ability of wt HSV-1 to establish a latent infection *in vitro* was referred to in Section 4.2.8. (Table 4.12.). The HSV-1 *ts* mutants tested for the ability to establish latency were *ts*Isyn and *ts*Ksyn. The phenotypes of these mutants at the NPT have been described elsewhere (Sections 1.2.3.2., 1.3.4.2. and 4.2.4.1.).

4.3.4.1. Polypeptide Profile of tsK at 42°C.

To determine whether *ts*K produces the same polypeptide profile at 42°C as at 38.5°C, cultures of HFL cells were infected with *ts*Ksyn at 10 pfu/cell and cultures were pulse-labelled after incubation at 42°C or 38.5°C for 4h, or, after incubation at 31°C for 8h. Labelled, infected cell polypeptides were analysed by SDS-PAGE. The results shown in Figure 4.2. suggest that the polypeptide profiles of the mutant at 42°C and at the NPT of 38.5°C are similarly restricted.

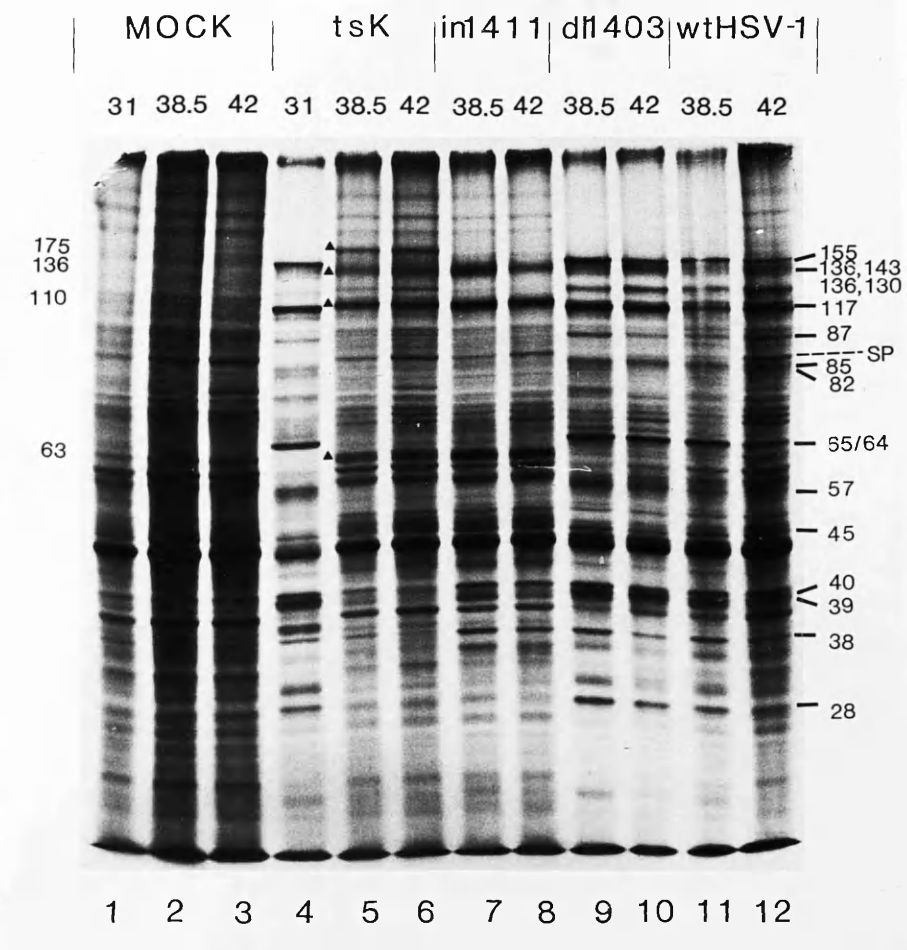
4.3.4.2. Establishment of Latency by tsKsyn and tsIsyn

HFL cells were infected with *ts*Isyn or *ts*Ksyn at 0.003pfu/cell and, following adsorption and penetration at 38.5°C, the cultures were incubated at 42°C for 6 days and at 31°C for 4 days.

Plaques were not observed in any of the infected, 42°C treated cultures during subsequent incubation at the permissive temperature of 31°C, demonstrating repression of replication of these mutants as a result of the 42°C incubation. In both cases, however, superinfection with HCMV at 31°C resulted in the production of plaques, indicating that reactivation of HSV had occurred (Table 4.12.). The reactivated virus from *ts*I- and *ts*K-infected cultures was shown to have retained the ts phenotype when cell sonicates were titrated on BHK cells at 31°C and 38.5°C, and, as with the HSV-2 mutants, virus was not recovered from the latently infected cultures upon HCMV superinfection at the

Figure 4.2. **VIRAL POLYPEPTIDE PROFILES AT 42°C**

HFL cell monolayers were mock-infected (lanes 1, 2 and 3), or infected with tsK (4, 5 and 6), in1411 (lanes 7 and 8), dl1403 (lanes 9 and 10) or wt HSV-1 (17) (lanes 11 and 12). Cultures were pulse-labelled after incubation at 38.5°C (lanes 2, 5, 7, 9 and 11) or 42°C (lanes 3, 6, 8, 10 and 12) for 4h, or, at 31°C (lanes 1 and 4) for 8h. SP (⊷) is a cellular stress protein. HSV early and late (—) and immediate early polypeptides (▲) are labelled.


MOCK
tsK
in1411
dl1403
wtHSV-1
31 38.5 42 31 38.5 42 38.5 42 38.5 42 38.5 42
175
136
110
63
155
136,143
136,130
117
87
SP
85
82
65/64
57
45
40
39
38
28
1 2 3 4 5 6 7 8 9 10 11 12

non-permissive temperature.

Therefore, HSV-1 mutants tsI and tsK were able to establish a latent infection in vitro. These findings support the notion that growth and spread of input virus is not necessary for establishment and maintenance of latency in vitro. The results with tsKsyn also illustrate that little or no viral gene expression is required for establishment and maintenance, and suggest that, at most, only the early polypeptide Vmw 136 and the IE polypeptides are necessary, with the possible exception of IE Vmw175 which is produced by tsK as an abnormal polypeptide at the non-permissive temperature (Preston, 1979). Further investigation was carried out by the use of mutants in1411 and dl1403.

4.3.5. HSV-1 Insertion and Deletion Mutants

The phenotype of in1411 is similar to that of tsK (at the non-permissive temperature), since gene expression is limited to the IE polypeptides Vmw12, Vmw63, Vmw68 and Vmw110, plus Vmw136; the results in Figure 4.2. show polypeptide profiles for in1411-infected HFL cells 4h post infection at temperatures of 38.5°C and at 42°C. Cultures were infected with in1411 (0.003 pfu/cell) and incubated at 42°C for 6 days followed by downshift to 37°C for 4 days. After this period, it was not possible to demonstrate the presence of latent virus by superinfection with HCMV, but this result was probably due to the failure of in1411 to form plaques since Vmw175 is essential for virus growth. Latent in1411 could be reactivated, however, by superinfection with tsI or ts5 (Table 4.13.). In these cases, plaques presumably resulted from reciprocal complementation and, possibly, recombination between in1411 and the superinfecting mutant tsI or ts5. As expected, tsK failed to reactivate in1411 since complementation between these 2 viruses does not occur.

in1411 is, therefore, able to establish a latent infection in the in vitro system, and this result confirms that Vmw175 is not required for establishment or maintenance of latent HSV-2 in vitro.

Table 4.13. ESTABLISHMENT OF LATENCY BY in1411 AND dl1403[a]

SUPER-INFECTION	M.O.I.[b]	INITIAL INFECTION: HSV-1	dl1403	in1411	HSV-2	MOCK
HCMV	0.01	27	7	0	23	0
HCMV	0.03	98	19	0	47	0
tsKsyn	0.03	41	15	0	33	0
tsKsyn	0.10	ND[c]	108	0	83	0
tsKsyn	0.30	273	235	0	213	0
tsIsyn	0.03	19	34	25	48	0
tsIsyn	0.10	77	113	81	146	0
tsIsyn	0.30	ND	299	206	ND	0
ts5	0.03	38	44	18	40	0
ts5	0.10	230	180	107	152	0
ts5	0.30	>400	>400	>400	>400	0
MOCK	0.00	0	0	0	0	0

a Cultures were infected with 0.003 pfu/cell of wt HSV-1, wt HSV-2 or in1411, or with 0.0003 pfu/cell of dl1403, incubated at 42°C for 6 days and 37°C for 4 days. Superinfection was carried out at 38.5°C; and plaques were counted after 2 days.

b Pfu of superinfecting virus per cell.

c ND, no data.

The mutant dl1403 is unable to produce IE polypeptide Vmw110, but otherwise produces a pattern of protein synthesis indistinguishable from that of wt HSV-1 (Stow and Stow, 1986) at temperatures of 38.5°C and 42°C (Figure 4.2.). To establish latency with dl1403 it was necessary to use a tenfold lower input multiplicity than usual, as stocks of this virus have a higher number of particles which do not form plaques (Stow and Stow, 1986) but cause cell death *in vitro*. HFL cells were therefore infected with dl1403 at 0.0003 pfu/cell, and incubated at 42°C for 6 days and 37°C for 4 days. It was found that dl1403 was reactivated efficiently upon superinfection with HCMV, tsK, tsI or ts5 and, surprisingly, that the number of plaques recovered was equivalent to that found in cultures initially infected with a tenfold higher multiplicity of wt HSV-1 (Table 4.13.). This was because in dl1403 stocks many of the particles which cannot form plaques *per se* can nevertheless produce plaques when complemented by another non-replicating virus (Stow and Stow, 1989).

The major point to be made here is that the mutant dl1403 was able to establish a latent state *in vitro* and, therefore, that the absence of IE polypeptide Vmw110 does not preclude the establishment of latency.

4.3.6. Discussion

Care must be taken in the interpretation of results from studies utilising HSV mutants in *in vitro* latency systems and in *in vivo* latency systems. It is recognised that mutants which are not detectable following induction of reactivation in *in vivo* model systems might lack the ability to replicate efficiently at the periphery of the host organism, the ability to be transported to the ganglion (or the site of latency), the ability to become established in a latent state, the ability to be reactivated upon explantation or cocultivation of latently infected tissue, or, the ability to replicate in the indicator cell monolayers; in *in vitro* latency systems such mutants might lack the ability to establish or maintain a latent state, the ability to grow efficiently in the tissue culture cells,

or, the ability to be reactivated from the latent state. The main point is that, in latency studies of this sort, no conclusions can be drawn, concerning viral factors involved in establishment or maintenance of latency, simply on the basis of a failure to recover latent mutant viruses from the system. However, mutants which are able to establish a latent infection *in vivo* or *in vitro,* and to be reactivated from the latent state, obviously produce all the functions required for establishment and maintenance of a latent infection. Therefore, functions which such mutants are unable to produce are not essential for establishment or maintenance of latency. Where ts mutants are utilised, this approach is, of course, dependent upon retention of the restricted phenotype of the mutants, characterised in tissue culture at the appropriate NPT, in latency systems. In this respect, the *in vitro* latency system described here has an advantage over *in vivo* systems, because control experiments can indicate whether or not a ts mutant has the same phenotype in tissue culture at the supraoptimal temperature of 42^{o}C and at the NPT for viral replication. More recent studies *in vivo* and *in vitro* have avoided such a problem by the use of genetically engineered HSV mutant viruses (Section 1.3.4.2.). In the studies described, the HSV-1 mutants in1411 and dl1403, constructed by Stow and Stow (1987, 1988; Russell *et al.*, 1987), were utilised.

The ts mutants tested for their ability to establish a latent state in vitro were HSV-2 mutants *ts*3, *ts*5, *ts*9 and *ts*11, and HSV-1 mutants *ts*K and *ts*I. These mutants have previously been analysed for their ability to establish a latent infection *in vivo* in studies of the mouse footpad latency system; *ts*3 and *ts*9 were recoverable only from the footpad, *ts*11 was recovered only from the dorsal root ganglia, and *ts*5 was not recoverable from either the footpad or the dorsal root ganglia in initial studies (Al-Saadi *et al.*, 1983) but recent work found that *ts*5 was recoverable from explant material and therefore capable of becoming established in a latent state (S. Batra, personal communication); *ts*K and *ts*I were not recoverable in explanted material (Lofgren *et al.*, 1977; Watson et al, 1980; Al-Saadi *et al.*, 1983). Although *ts*3 and *ts*11 were not

recoverable from explant material *in vivo*, evidence from the studies presented here suggests that *ts*3 and *ts*11 are host range mutants in tissue culture, with plaquing efficiencies some 100-fold lower on HFL compared to BHK cells, and it is, therefore, possible that the inability to recover the viruses from latently infected explant tissue was due to a restricted ability to replicate in certain cells following explant.

All of the ts mutants tested were able to become established in a latent state including the HSV-2 mutant *ts*9, which is defective in uncoating and has greatly impaired gene expression at the NPT. It is, therefore, possible that viral gene expression is not a requirement for establishment and maintenance of the latent state. However, there was a reduced recovery of *ts*9, compared to wt HSV-2, following HCMV superinfection of cultures latently infected with the mutant. Thus, the possibility arises that viral gene expression is advantageous, although clearly not essential, for establishment of latency. However, the effect of cellular factors on uncoated virus held for a prolonged period in the cell is not known, and it is possible that *ts*9 may become uncoated within the HFL cells during incubation at 42^{o}C and therefore produce some gene expression, albeit restricted due to the secondary late mutation. The levels of recovery of the mutant following HCMV superinfection could, then, be a reflection of the proportion of *ts*9 which had become uncoated in the HFL cells. Clearly, it is not possible to deduce the requirement for HSV viral gene expression in latency from the experiment utilising *ts*9. In the *in vitro* latency system described, the bulk of wt HSV-2 DNA which likely includes latent HSV genomes, are localised to the nucleus and exist in an endless configuration during the 42^{o}C incubation period (Section 4.5.), therefore, for *ts*9 to be harboured in an equivalent state, the mutant virus must become uncoated during 42^{o}C incubation, prior to circularisation and localisation of the genome to the nucleus. A less probable alternative is that, HSV may be harboured in a non-productive state in cells in either of two different forms, as uncoated virus in the cytoplasm or as naked DNA in the nucleus, both forms of virus being

efficiently reactivated by superinfection.

The most informative results were those obtained by the use of mutants tsKsyn, in1411 and dl1403. Taken together, these results suggest that little or no viral gene expression is required for establishment, since mutants expressing only IE genes and Vmw136 are converted to a latent state, and furthermore that there is no requirement for expression of IE gene 3 (coding for Vmw175) and IE gene 1 (coding for Vmw110). These findings do not contradict the results of recent studies carried out in vivo which indicated several HSV genes which were not essential for establishment or reactivation of latency, including glycoproteins G and E, the viral protein kinase, thymidine kinase, and, significantly, the genes encoding IE Vmw68 and IE Vmw12 (Meignier et al., 1988; Sears et al., 1985a,b). Furthermore, recent studies using the in vivo latency mouse footpad model system showed that the mutant dl1403 is capable of establishing a latent infection in vivo, demonstrating the lack of a requirement for IE Vmw110 in latency (Clements and Stow, 1989).

In vitro studies on expression of viral genes in latently infected cells are at a preliminary stage; the functional E TK enzyme is not detectable in cells infected with HSV-2 at low m.o.i. during 42°C incubation. Therefore, there is a block in the production of the functional E TK gene product which may occur at the level of transcription, RNA processing, translation or protein modification. This finding implies that production of other HSV gene products could be similarly affected. Furthermore, the E TK gene is, presumably, not required for the maintenance of the latent state. Although the inability to detect the functional E TK in cells at 42°C might appear to contradict the finding that HSV-infected cells had the same polypeptide profiles during incubation at 42°C as at 37°C, it should be noted that polypeptide analysis was carried out on cells infected with HSV at higher multiplicities (10pfu/cell) and during the first 24h of incubation at 42°C, in contrast to the TK assay which was carried out on cells infected with HSV at m.o.i. of less than 1 pfu/cell and following 6 days at 42°C. It

would seem, therefore, that either an increase in the period of 42°C incubation is correlated with a decrease in HSV transcription and/or, wt HSV behaves differently in high multiplicity infection of cells compared to low multiplicity infections during incubation at 42°C. In this respect it is worth noting that cultures infected at relatively high m.o.i. and incubated at 42°C exhibit higher levels of spontaneous reactivation than cultures infected at low m.o.i.. Perhaps then, there is a correlation between the m.o.i., the level of HSV gene expression and the amount of spontaneous reactivation. This will be discussed in the context of *in vivo* latency in Section 5.0..

In vivo studies suggest that the majority of the latent HSV genome remains transcriptionally inactive; only one region of the genome (LAT) is actively transcribed. Recent mutant studies have suggested that the LAT gene is not essential for establishment, maintenance or reactivation of HSV latency *in vivo* (Wagner *et al.*, 1988a; Javier *et al.*, 1988; Steiner *et al.*, 1989). Expression of LAT in the *in vitro* latency system has not yet been analysed, therefore, it is not known whether there is a role for LAT in HSV latency *in vitro*. LAT will be discussed further in Section 5.0.

The findings of the above studies, utilising the *in vitro* latency system, suggest that the pattern of HSV gene expression during the 42°C incubation period is restricted and that little or no viral gene expression is required for establishment or maintenance of a latent state *in vitro*. Suppression of viral gene expression could occur as a result of inhibition by cellular or viral factors (including only IE proteins, E protein Vmw136, virion components or LAT) or, the lack of essential factors involved in viral gene expression; it is also possible that at least some of the viral genes are inaccessible for transcriptional activation by virtue of their association with chromatin, as discussed in Section 5.0.

4.4. VIRAL FACTORS INVOLVED IN REACTIVATION

4.4.1. Introduction

The reactivation of HSV from its latent state *in vivo*, which has been discussed in Section 1.3.3. may be induced by many, seemingly unrelated, stimuli; the molecular mechanisms involved in reactivation have not yet been defined.

Reactivation of HSV from *in vitro* latency systems that rely upon infection of cells in the presence of inhibitors of virus replication for establishment, and incubation at supraoptimal temperatures for maintenance of latency (O'Neill *et al.*, 1972; O'Neill, 1977; Wigdahl *et al.*, 1981, 1982a, 1983), can be induced by superinfection with HSV *ts* mutants, or HCMV ts mutants, which are unable to synthesise virus DNA and express only IE and E virus genes at the NPT (Colberg-Poley *et al.*, 1979, 1981; Lewis *et al.*, 1984; Wigdahl *et al.*, 1982a,b).

The latent virus-cell interaction described in this thesis is not susceptible to disruption by the chemical and physical treatments tested (Section 4.2.7.; Table 4.9.). However, as previously demonstrated by Notarianni (1986), reactivation of HSV can be induced by infection with HSV mutants, including *tsK* which produces only the IE polypeptides at the NPT; HSV is efficiently reactivated from its latent state *in vitro* by intertypic superinfection with HSV-1 *ts* mutants *tsK* or *tsI* (Tables 4.3., 4.13.), the HSV-2 *ts* mutant *ts5* (Table 4.13.), or by superinfection with HCMV (Tables 4.3., 4.13.) (Section 4.3.). Therefore, reactivation can be induced by the physical processes of viral infection and/or the presence of HSV or HCMV virion components and/or the expression of HSV or HCMV genes; the presence of HSV IE proteins and/or virion components are sufficient for reactivation induced by HSV superinfection.

The approach taken to determine the viral factors involved in reactivation of HSV from its latent state was to examine the ability of various well-defined HSV mutants to induce reactivation. Cultures latently infected with wt

HSV-2 were superinfected with mutant viruses and the subsequent appearance of plaques of reactivated HSV-2 was taken to indicate that reactivation had occurred.

4.4.2. Reactivation of Latent HSV

To establish whether viral functions, or the physical processes of viral infection, were responsible for the disruption of the latent state which occurred upon superinfection of latently infected cultures, the ability of adenovirus or UVtsK to reactivate latent HSV was investigated.

Adenoviruses were chosen for this study, partly to determine whether the ability to induce reactivation of latent HSV *in vitro* might be specific to herpesviruses, and partly because early in infection adenoviruses produce polypeptide E1A which, like HSV IE Vmw175, is a transactivator and involved in the temporal regulation of gene expression. UVtsK virus is tsK which has been irradiated with ultraviolet light to decrease the titre by a factor of 5×10^5 and to prevent detectable viral gene expression. The virion component of HSV, Vmw65, which is responsible for transactivation of IE gene expression, retains its function despite uv-irradiation (Preston *et al.*, 1984).

Cultures infected with HSV-2 (0.003pfu/cell) and incubated for 6 days at 42°C and 4 days at 37°C, which therefore harboured latent HSV-2 genomes, were superinfected with adenoviruses 2 or 5, or UVtsK. Within the time course of these latency experiments plaques of adenovirus would not be formed. However, as previously documented (Table 4.3.) no plaques of reactivated virus (or superinfecting virus) were formed in the cultures after 2 to 3 days at 38.5°C.

The inability of the adenoviruses to induce the formation of plaques of reactivated virus indicates that no adenovirus function produced is capable of inducing reactivation of latent HSV. The finding that UVtsK was unable to reactivate latent HSV-2 shows that none of the virion components of HSV, including Vmw65, are directly responsible for the reactivation of latent HSV-2 by HSV superinfection. Together these results indicate that

expression, specifically, of HSV or HCMV genes, rather than the physical processes of viral adsorption and penetration or the activity of virion components, is necessary for the induction of reactivation.

These findings, in combination with the observation that tsK was able to reactivate HSV-2 at the non-permissive temperature as efficiently as the late mutant tsI (Table 4.3), suggest that one or more of the polypeptides synthesised by tsK at the non-permissive temperature is responsible for the reactivation of latent HSV-2 induced by HSV superinfection. As previously described, tsK produces only the IE polypeptides and the E polypeptide Vmw136 at the non-permissive temperature. TsK transcribes the IE gene 3, coding for Vmw175, but produces an abnormal Vmw175 protein with altered electrophoretic mobility (Preston, 1979b), which, unlike wt Vmw175, is unable to autoregulate transcription of IE gene 3, or stimulate transcription of the E and L genes of HSV (Preston, 1979a; Dixon and Schaffer, 1980; Watson and Clements, 1980). Therefore, the finding that tsK superinfection reactivated latent HSV also suggests that the presence of functional Vmw175 is not a prerequisite for reactivation.

To determine which of the IE gene products were involved in the reactivation of latent HSV it was initially proposed to transfect plasmids containing DNA encoding IE polypeptides into latently infected cells, followed by monitoring of the cell cultures for the presence of reactivated virus. In control experiments DNA was applied to cell monolayers in calcium chloride precipitates or in DEAE dextran and the cells were permeabilised by treatment with DMSO or glycerol to facilitate the entry of the DNA into the cell; protoplast fusion was also used in an attempt to transfect the HFL cells (a review of these techniques has been presented by Spandidos and Wilkie, 1984). However, it was found that HFL cells did not take up extracellular DNA by any of the transfection techniques employed. It is well-documented that transfer of DNA into human diploid cells by transfection procedures is very inefficient. Therefore, in an alternative approach, experiments examining the ability of HSV mutants to induce reactivation were

extended to include the insertion mutant in1411 and the deletion mutant dl1403.

4.4.3. Reactivation Induced by Superinfection with in1411

The HSV-1 mutant in1411 was used in these studies to further investigate possible involvement of Vmw175 in the reactivation event, as in1411 is unable to produce IE Vmw175.

Cultures latently infected with HSV-2 were superinfected with in1411 at various m.o.i. At 0.1 pfu/per cell, in1411 formed a small number of plaques on mock-infected HFL cells due to the presence of revertants which probably arose by recombination with the resident gene in the helper cells, M65, used for propagation of in1411 (N. D. Stow and E. C. Stow, personal communication).

However, it is clear from the data in Table 4.14. that in1411 was able to reactivate latent HSV-2, confirming that IE polypeptide Vmw175 is not required for reactivation. This finding also substantiates the possibility that one or more of the HSV-1 IE polypeptides, or the E polypeptide Vmw136, is involved in the reactivation event.

4.4.4. Superinfection of Latently Infected Cultures with dl1403

The mutant dl1403 is unable to produce IE polypeptide Vmw110, and was therefore used in experiments to determine whether there was any involvement of Vmw110 in reactivation.

Mutant dl1403 forms plaques on HFL cells, albeit at lower efficiency than on BHK cells (Stow and Stow, 1986). When this virus was used for reactivation, no increase in plaque number was observed on cell cultures harbouring latent HSV-2 compared with mock-infected cell cultures which had been incubated at 42^{o}C for 6 days and at 37^{o}C for 4 days (Table 4.14.), and all plaques showed the characteristic morphology produced by dl1403 (Stow and Stow, 1986), with no evidence for replication of HSV-2. The ability of dl1403 to replicate means that the sensitivity of detection of reactivation is low. Thus by use of 0.001 pfu of dl1403 per

Table 4.14. REACTIVATION OF LATENT HSV-2 BY HSV-1 MUTANTS[a]

SUPERINFECTION	M.O.I.[b]	INITIAL INFECTION: MOCK	INITIAL INFECTION: HSV-2
tsKsyn	0.03	3(3)[c]	78(3)
tsKsyn	0.10	3(3)	231(3)
in1411	0.03	ND[d]	231
in1411	0.10	14	>500
dl1403	0.0003	79	70
dl1403	0.0010	176	169

a Cultures were mock-infected or infected with 0.003 pfu/cell (ts and in mutants) or 0.03 pfu/cell (dl1403) of wt HSV-2 and incubated at 42°C for 6 days followed by 37°C for 4 days. Superinfection was carried out at 38.5°C and plaques were counted after 2 days.

b Pfu of superinfecting virus per cell.

c Values in parentheses represent the number of syncytial plaques.

d ND, no data.

Figure 4.3. **THE GENOMIC LOCATIONS OF HSV-2 HindIII L AND CROSS-HYBRIDISING HSV-1 HindIII FRAGMENTS**

The relative positions of the HSV-2 HindIII L fragment and the HSV-1 HindIII e, b, n, and g fragments, which cross-hybridise with the HSV-2 HindIII L probe, are illustrated. The scale in map units is shown above.


0
0.2
0.4
0.6
0.8
1.0

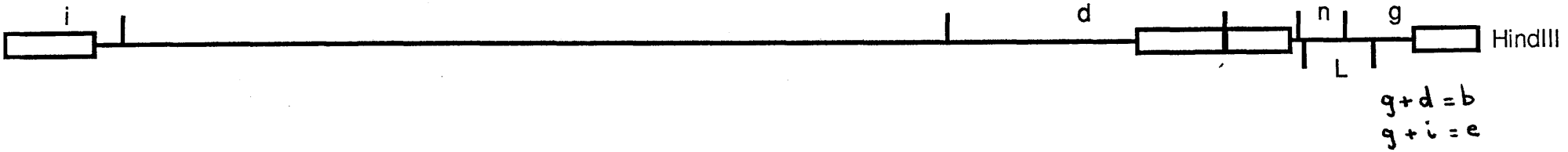
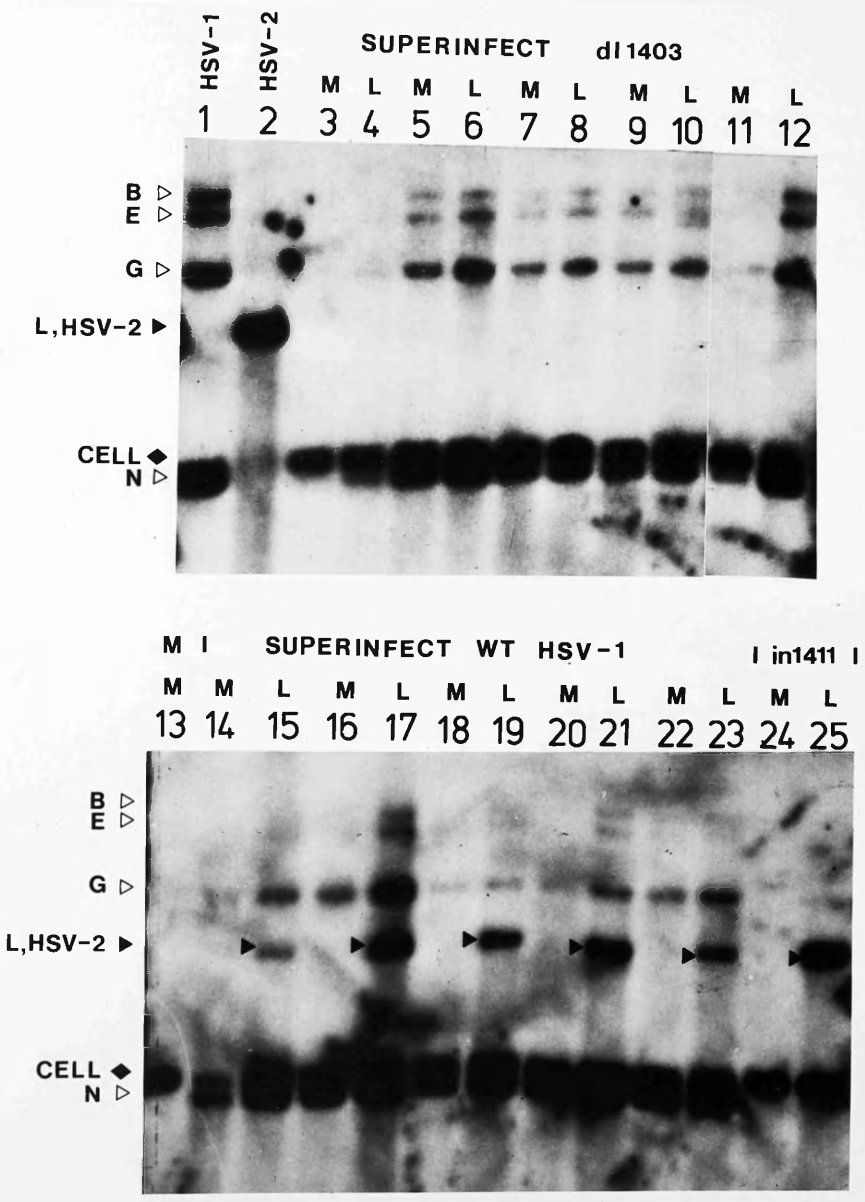

i
d
n
g
HindIII
L
q+d=b
q+i=e

Figure 4.4. DNA FROM SUPERINFECTED CULTURES

HFL cells were mock-infected (M) or infected with 0.03 pfu of HSV-2 per cell (L), and incubated at 42°C for 6 days followed by 37°C for 4 days. Cultures were superinfected with dl1403 (lanes 3 to 12), wt HSV-1 (lanes 14 to 23), in1411 (lanes 24 and 25), or mock-superinfected (lane 13). Cytoplasmic DNA was extracted, cleaved with HindIII L and hybridised to radiolabelled HSV-2 HindIII L. The multiplicity of superinfection was 0.0003 (lanes 14 and 15), 0.001 (lanes 16, 17, 24 and 25), 0.003 (lanes 3, 4, 18 and 19), 0.01 (lanes 5, 6, 20 and 21), 0.03 (lanes 7, 8, 22 and 23), 0.1 (lanes 9 and 10) or 0.3 (lanes 11 and 12) pfu per cell. HSV-1-specific (▷), HSV-2-specific (▶) and a background cellular band (◆) are labelled.

Autoradiograph - 3 day exposure.


HSV-1
HSV-2
SUPERINFECT dl1403
M L M L M L M L M L
1 2 3 4 5 6 7 8 9 10 11 12
B
E
G
L,HSV-2
CELL
N
M I SUPERINFECT WT HSV-1 I in1411 I
M M L M L M L M L M L M L
13 14 15 16 17 18 19 20 21 22 23 24 25
B
E
G
L,HSV-2
CELL
N

cell only 0.1% of the initial HSV-2, or 30 plaques, would be expected and this would be difficult to detect over a background of 176 plaques (Table 4.14.). The value of 30 reactivated plaques represents a minimum estimate since, as shown in Table 4.13. and discussed above, many particles of dl1403 unable to form plaques alone could be complemented by reactivated HSV-2. On balance, the results shown in Table 4.14. suggest that dl1403 fails to reactivate latent HSV-2.

4.4.4.1. Analysis of Reactivation in Hybridisation Studies

The apparent inability of dl1403 to reactivate latent HSV-2 was investigated by Southern hybridisation analysis.

HFL cells, mock-infected or infected with HSV-2 at 0.03 pfu/cell and incubated for 6 days at 42°C and for 4 days at 37°C, were then superinfected with dl1403, in1411 or wt HSV-1 at various m.o.i.. Cultures were harvested after 2 to 3 days at 37°C, when extensive cytopathic effect was observed, and DNA was extracted from the cytoplasmic fractions and digested with HindIII. DNA fragments were separated by electrophoresis and the presence of HSV-2 in these samples was determined by Southern hybridisation of the DNA using cloned HSV-2 HindIII L fragment (Figure 4.3.), derived from U_S, as a probe. This fragment migrates differently upon electrophoresis from the HSV-1 HindIII fragments B, E, G, and N (Figure 4.3.), with which it shares homology.

Figure 4.4. shows that HSV-2 DNA was present in latently infected cultures subsequently superinfected with HSV-1 or in1411 but was not detected in cultures which had been superinfected with dl1403. The presence of cross-hybridising HSV-1 bands demonstrates that wt HSV-1 and dl1403 replicated efficiently.

To ensure that the failure of dl1403 to reactivate HSV-2 was due to the deletion in the coding sequences for Vmw110, virus isolates generated by rescue of dl1403 with a small DNA fragment derived entirely from the Vmw110 gene (Stow and Stow, 1986) were tested for the ability to reactivate latent HSV. Cultures latently infected with HSV-2 were superinfected with these revertants and incubated at

Figure 4.5. **DNA FROM SUPERINFECTED CULTURES**

HFL cells were mock-infected (M) or infected with 0.03 pfu of HSV-2 per cell (L), and incubated at 42°C for 6 days followed by 37°C for 4 days. Superinfection was with 4 independent isolates of virus obtained by rescue of dl1403 with a cloned DNA fragment, R1 (lanes 1 and 2), R2 (lanes 3 and 4), R3 (lanes 5 and 6), R4 (lanes 7 and 8), or with dl1403 (lanes 9 to 12). Cytoplasmic DNA was extracted, cleaved with HindIII and hybridised to radiolabelled HSV-2 HindIII L. The multiplicity of superinfection was 0.003 (lanes 1 to 8), 0.03 (lanes 9 and 10) or 0.3 (lanes 11 and 12) pfu per cell. HSV-1 (▷) and HSV-2 (▶) bands are labelled.

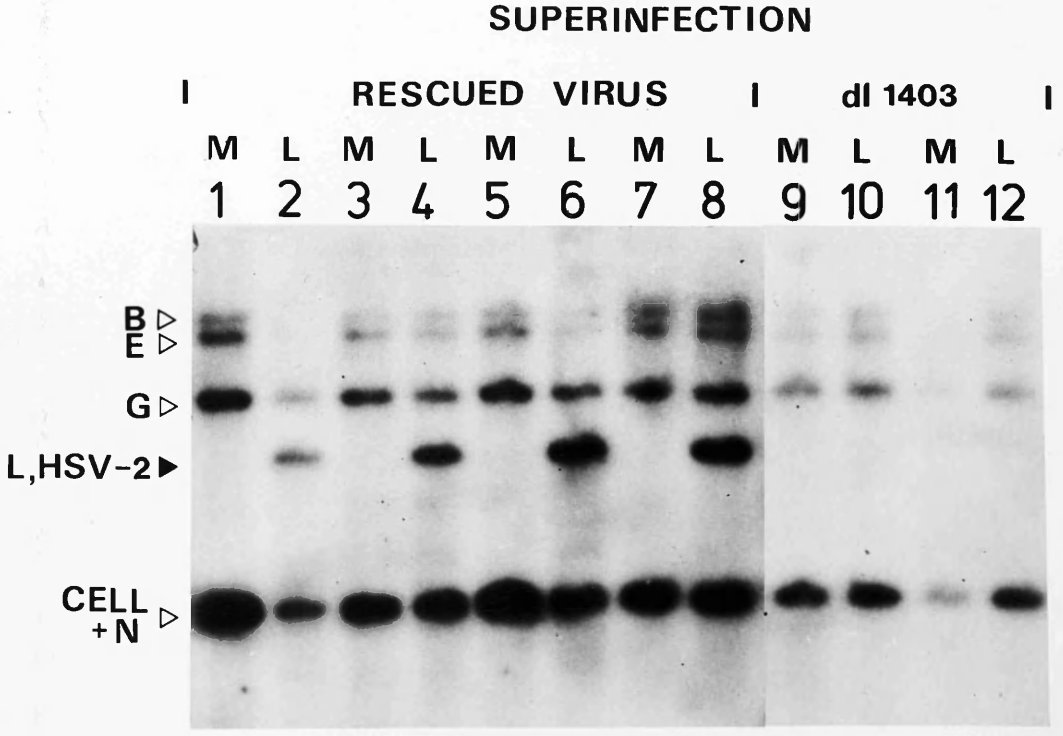
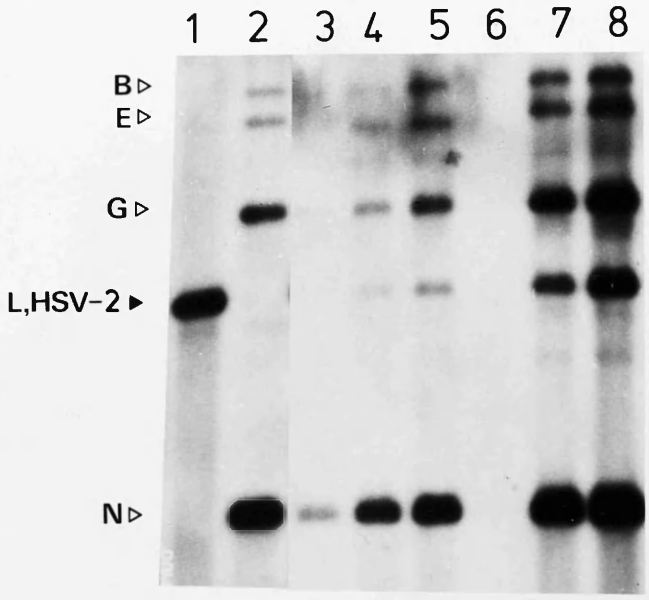

SUPERINFECTION
I
RESCUED VIRUS
I
dl 1403
I
M L M L M L M L M L M L
1 2 3 4 5 6 7 8 9 10 11 12
B
E
G
L,HSV-2
CELL
+N

Figure 4.6. CO-REPLICATION OF HSV-2 AND dl1403

HFL cells were infected with 3 pfu of dl1403 (lanes 3 to 5) or rescued virus R4 (lanes 6 to 8) plus 0.1pfu of HSV-2 per cell. Cytoplasmic DNA was extracted after incubation of cultures at 37°C for 2h (lanes 3 and 6), 8h (lanes 4 and 7) or 18h (lanes 5 and 8), cleaved with HindIII, and hybridised to radiolabelled HSV-2 HindIII L. HSV-2 DNA (lane 1) or HSV-1 DNA (lane 2) were also analysed. HSV-1 (▷) and HSV-2 (▶) bands are labelled.


1 2 3 4 5 6 7 8
B
E
G
L,HSV-2
N

37°C for 2 days. Southern blot analysis, using as a probe [^{32}P]-labelled HSV-2 fragment HindIII L, of HindIII-digested DNA derived from the cytoplasmic extracts of these cultures, showed that the four independently isolated revertants reactivated HSV-2 efficiently (Figure 4.5.).

Therefore, the deletion in the gene encoding Vmw110 is responsible for the failure of dl1403 to reactivate HSV-2. It should be noted that the deletion in dl1403 also affects the 3' end of the LAT gene, and this will be discussed below.

The possibility remained that dl1403 superinfection was able to reactivate HSV-2 from latently infected cells, but that the presence of dl1403 inhibited the replication of reactivated HSV-2, which was therefore undetectable.

Although it is clear from the presence of HSV-1-specific fragments that dl1403 replicated in HFL cells infected at less than 1 pfu/cell (Figures 4.4., 4.5.), it was possible that dl1403 inhibited the replication of HSV-2 when both viruses were present in the same cell. HFL cell cultures were coinfected with 3 pfu of dl1403 or rescued virus plus 0.1 pfu of HSV-2 per cell, and the virus was allowed to adsorb to and penetrate the cells for 1h at 37°C. Incubation was continued at 37°C. The cultures were harvested at 2h, 8h, and 20h post-infection. Southern blot analysis was performed on HindIII-digested DNA derived from the cytoplasmic extracts of these cultures. The probe was [^{32}P]-labelled HindIII L of HSV-2.

The results in Figure 4.6. show that HSV-2 replicated in both cases, showing that interference with HSV-2 replication by dl1403 did not account for the failure to reactivate latent virus. As expected from previous studies, dl1403 replicated less efficiently, or more slowly, than rescued virus in HFL cells, and HSV-2 was affected in the same way (Stow and Stow, 1986; Sacks and Schaffer, 1987)

4.4.5. Discussion

The results presented suggest that reactivation induced by superinfection with HSV involves only the HSV IE genes and/or virion components. By contrast, the results of

Scheck et al. (1987), utilising a system in which HSV is converted to a repressed state by treatment of infected cultures with chemical inhibitors followed by incubation at elevated temperatures, suggested that early, as well as immediate early, HSV-1 genes were required for reactivation of HSV-2 by HSV-1 superinfection in this system. Therefore, it is apparent that the non-productive state of HSV produced in latency systems utilising inhibitors and elevated temperatures differs from that produced in systems which rely solely on the use of supraoptimal temperatures to achieve a latent state for HSV, at least in terms of requirements for reactivation.

The finding that, in contrast to wt HSV-1 or tsK, dl1403 did not reactivate HSV indicates that the region of the genome affected by the deletion, which includes most of the coding sequences for Vmw110 (Figure 4.7.), is essential for reactivation induced by HSV-1 superinfection. Therefore, the results strongly suggest that Vmw110 is required for reactivation.

It is not clear from the results whether Vmw110 acts alone or in conjunction with one or more of the virion components and/or the other HSV IE polypeptides, although, from the results of superinfection with in1411, there is no requirement for at least IE polypeptide Vmw175 in reactivation. In order to resolve the issue, it was necessary to introduce the gene encoding Vmw110 into latently infected cells and determine the ability of Vmw110 to stimulate reactivation. Transfection of DNA into HFL cells was unsuccessful by the techniques employed (Section 4.4.2.), therefore, construction of a virus vector expressing Vmw110 was desirable. Since wt Ad5 superinfection does not induce HSV reactivation, the virus vector selected was an Ad5 mutant dlE1,3, which is deleted for the E1 and E3 regions (Haj-Ahmad and Graham, 1986), kindly supplied by Dr. F. Graham. Unfortunately, insertion of the IE-1 gene sequences into dlE1,3 was not completed within the time limits of the project. However, adenovirus vectors expressing Vmw110 under the control of the IE-1 promoter (O_{PRO}-O) or the adenovirus major late promoter (MLP-O) have been constructed by Zhu et al. (1988), and recent studies by

R.A. Harris and C.M. Preston utilising O_{PRO}-O and MLP-O have indicated that both viruses are capable of reactivating latent HSV, suggesting that Vmw110 can trigger the reactivation event in the absence of the other IE polypeptides (Harris et al., 1989).

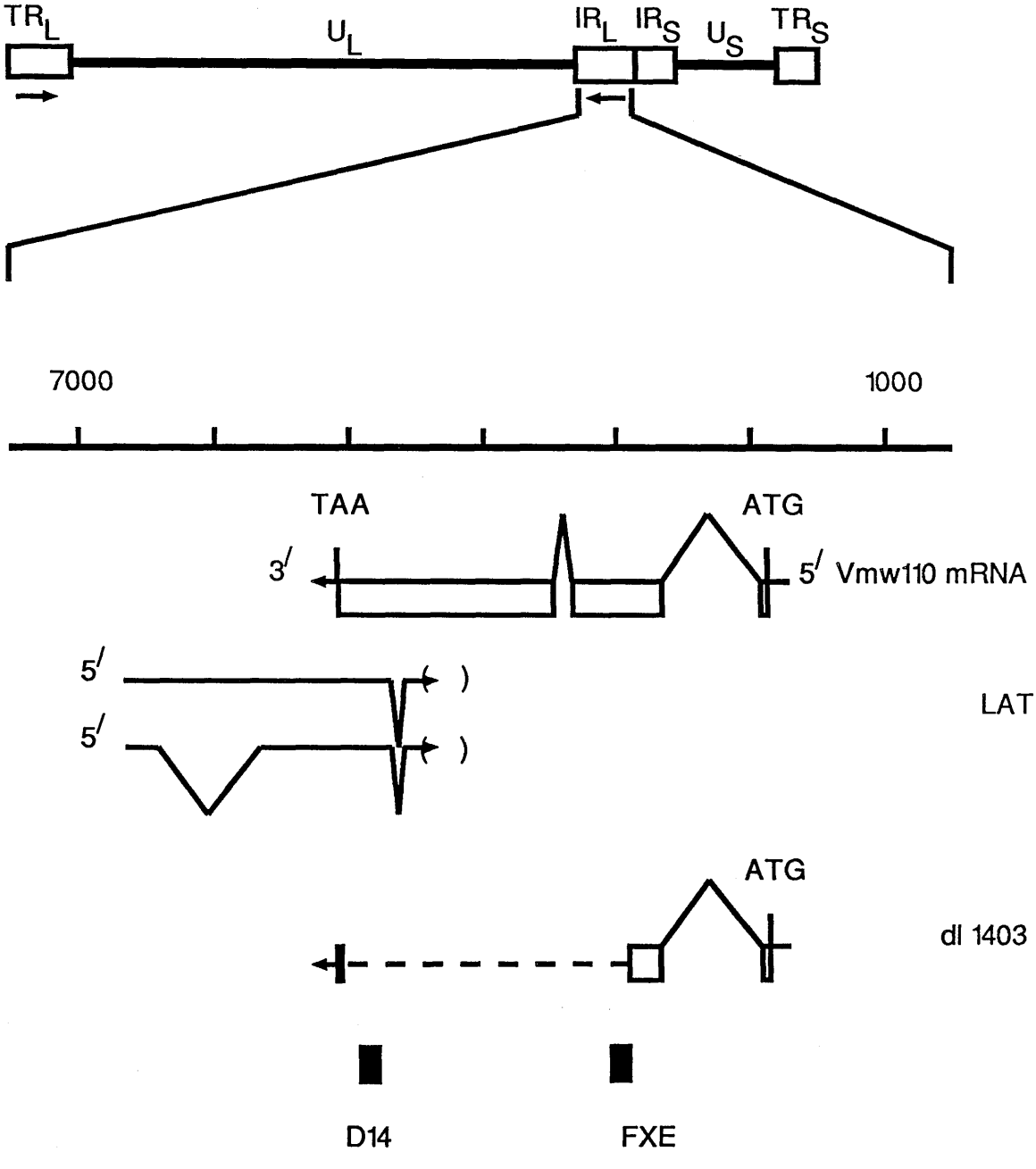
Since the deletion in dl1403 extends through the IE-1 gene to the LAT gene (Figure 4.7.), it was possible that LAT was also required for reactivation. Evidence that LAT production by the superinfecting virus is not a requirement for the reactivation event has been provided by Harris et al. (1989): reactivation of HSV was induced by superinfection of latently infected cultures with adenovirus recombinants, which expressed Vmw110 but which did not produce LAT due to deletions in the promoter and the 5' portions of the coding sequences for the LAT gene, and with HSV-1 mutant D14 (Figure 4.7.), which contains a deletion in the LAT gene such that 41 amino acids are missing from the carboxy terminus of the longer potential reading frame in LAT; reactivation was not achieved by superinfection with an HSV-1 mutant incapable of expressing Vmw110 due to deletions which did not affect the LAT gene. Therefore, the presence of Vmw110 alone is sufficient to stimulate reactivation of HSV from latently infected cultures (Harris et al., 1989).

The ability of Vmw110 to stimulate expression, presumably at the level of transcription, from a variety of HSV promoters and heterologous promoters introduced into cells by transfection, has been demonstrated. The degree of trans-activation can be increased in the presence of Vmw175, but to date there are no examples of eukaryotic or animal genes which fail to respond to Vmw110; there appears to be a general lack of sequence specificity in the promoters activated by Vmw110 during transfection assays (Everett, 1983, 1984a,b). Evidence for the importance of a region containing a potential metal-binding domain in the transactivation function of Vmw110 and in viral growth was recently provided by Everett (1988, 1989) (Section 1.2.3.2.). R.A. Harris and C.M. Preston have analysed the ability of HSV-1 mutant FXE (Figure 4.7.), which lacks the region containing the potential metal-binding site (Everett, 1989; Section 1.2.3.2.), to reactivate HSV in the in vitro

Figure 4.7. **POSITIONS OF DELETIONS IN HSV-1 MUTANTS dl1403, FXE AND D14 IN RELATION TO LAT AND TO THE IE Vmw110 GENE**

The locations of Vmw110 mRNA and LAT are shown in relation to the positions of deletions in HSV-1 mutants dl1403, FXE and D14. Spliced regions are shown as raised sections; all copies of LAT are spliced. The exact position of the 3' end of LAT is not known. Filled boxes and dashed lines represent deleted regions. Numbering of nucleotides begins at the IR_L junction 'a' sequence (Perry et al., 1986).

This figure was kindly provided by Ruth Harris.


TR_L
U_L
IR_L
IR_S
U_S
TR_S
7000
1000
TAA
ATG
3'
5'
Vmw110 mRNA
5'
5'
LAT
ATG
dl 1403
D14
FXE

latency system. Superinfection of latently infected cultures with FXE did not reactivate HSV, which, therefore, implies that Vmw110 reactivates HSV via its ability to stimulate gene expression (Harris et al., 1989).

HCMV superinfection was also able to induce HSV reactivation; if reactivation by HCMV and HSV superinfection occur via similar mechanisms, the HCMV gene function involved in reactivation would be expected to share some properties with the HSV IE Vmw110 polypeptide. Interestingly then, HCMV, but not UV-irradiated HCMV, is capable of complementing low multiplicity infections of the dl1403 mutant. Therefore, HCMV produces a function, which is not a virion component, capable of substituting for the Vmw110 function in HSV-1 infections (Stow and Stow, 1989). It seems probable that the HCMV function involved in reactivation and the HCMV function which substitutes for Vmw110 function in complementation of dl1403 are one and the same. Although there are no obvious amino acid sequence homologies between HCMV IE gene products and Vmw110, the HCMV IE gene products are likely candidates for the complementation of dl1403, since complementation can occur in cells in which HCMV infection is blocked at an early stage and the major IE polypeptide accumulates (Stow and Stow, 1989). Furthermore, like Vmw110, the HCMV IE gene products stimulate gene expression in a manner that is not specific to promoter sequences (Everett, 1984a,b). It would, therefore, be of interest to determine the ability of individual HCMV IE gene products to induce reactivation of HSV from the in vitro latency system.

It should be noted the VZV possesses the potential to complement dl1403 infections, implying that VZV encodes a polypeptide which substitutes for Vmw110 functions, possibly the VZV 61 gene product which shares some amino acid homology with Vmw110 (Davison and Scott, 1986; Perry et al., 1986), and it is, therefore, possible that superinfection of latently infected cultures with VZV would also result in induction of the reactivation event (Stow and Stow, 1989).

Since superinfection of latently infected cultures with dl1403, which does not produce Vmw110 but which otherwise has a polypeptide profile similar to that of wt

HSV, failed to reactivate HSV, it may be concluded that the HSV transcriptional trans-activators Vmw175, Vmw63 and Vmw65 were unable to trigger the reactivation event. Stimulation of reactivation by HSV superinfection is therefore due to a specific effect of Vmw110. Due to the lack of target sequence specificity of stimulation, it has been suggested by Harris et al. (1989) that Vmw110 functions either, by increasing template availability in a relatively non-specific manner, perhaps by displacing other bound proteins from a latent genome that is maintained in a state resembling inactive chromatin, or, by activating gene expression at a post-transcriptional stage, perhaps acting on a specific viral or cellular transcript to trigger reactivation.

Latency in vivo is characterised by episodes of reactivation of HSV, and the in vitro latency system also exhibits a low level of 'spontaneous' reactivation (Section 4.2.2.; Russell and Preston, 1986). Active Vmw110 is not the trigger for reactivation in such situations since it is presumably not present before these events occur and is not introduced extrinsically. Therefore, 'spontaneous' reactivation must be induced either by the formation of active Vmw110, which could occur as a result of modification of the polypeptide or its transcript or stimulation of transcription, or by cellular processes which substitute for the Vmw110 trans-activation function. Support for the latter possibility comes from the observation that latent dl1403 can be reactivated by HCMV (Section 4.2.4.3.), since no Vmw110 is present under these circumstances.

The ability of a transactivator protein to reactivate HSV from its latent state indicates a possible block in viral gene expression of the HSV genome. The inability of dl1403 superinfection of latently infected cultures to stimulate reactivation of HSV from the latency system suggests that functional Vmw110 (or the HSV-2 equivalent, Vmw118) is not being produced in HSV-1 (HSV-2) latently infected cells. Furthermore, the failure of dl1403 superinfection to reactivate HSV suggests the possibility that the gene encoding Vmw118, the HSV-2 functional equivalent and homologue of Vmw110, is not accessible to

activation by Vmw65, Vmw175 and Vmw63 which stimulate expression from IE-1 in transfection studies (Mackem and Roizman, 1982; O'Hare and Goding, 1988; Ace et al., 1989) and that, therefore, in the latent DNA the IE-1 gene, and possibly the other IE genes, is maintained in a transcriptionally inactive state. However, it is also possible that these transactivators stimulate IE-1 transcription but that functional Vmw110 is unable to be expressed.

The implication is that HSV is maintained in a latent state due to suppression of viral gene expression which may be the cause or effect of conversion of HSV to a latent state, and can be reactivated by induction of viral gene expression, in this case by the introduction of Vmw110 or its functional equivalent in HCMV.

4.5. THE STATE OF THE LATENT HSV GENOME

4.5.1. Introduction

The physical nature of the latent HSV-1 genome has been determined by Southern hybridisation analysis of DNA from animal model systems and from human tissue, using DNA probes which span the joint and terminal regions of the genome (Rock and Fraser, 1983; Efstathiou *et al.*, 1985). The reported state of latent HSV-1 DNA *in vivo* is 'endless', as indicated by a predominance of joint fragments, at a 2:1 molar ratio compared to unique HSV sequences, and a corresponding underrepresentation of the terminal fragments. The latent HSV DNA is, therefore, circular, concatemeric or integrated via regions of the genome other than the termini (Figure 1.7.).

A similar strategy has been employed in analysis of the state of the HSV genome in the *in vitro* latency system. This analysis presented problems due to the low number of latent HSV genomes per cell genome equivalent. Therefore, the relative proportion of HSV DNA was increased in two ways. First, the initial input m.o.i. of HSV to the system was increased 10-fold or 30-fold. Superinfection of the cultures, following incubation at 42^{o}C for 6 days and 37^{o}C for 4 days showed that the number of latent HSV genomes was increased proportionally (Table 4.15.), although at higher m.o.i. the level of spontaneous reactivation was such that it occurred in most cultures and therefore interfered with detection of latent genomes. Even at the multiplicities used a relatively high proportion of cultures had to be discarded due to the presence of spontaneously reactivated virus (for example, see Table 4.16.). Secondly, since HSV DNA has a high G+C content and therefore a greater buoyant density than most cellular DNA sequences, HSV DNA was separated from the bulk of cellular DNA sequences in infected 42^{o}C-treated cultures by equilibrium centrifugation in CsCl gradients. To facilitate separation, DNA from 42^{o}C-treated cultures was cleaved with *Bam*HI prior to centrifugation.

The state of the latent HSV genome in the *in vitro*

Table 4.15. REACTIVATION OF LATENT HSV-2 FROM CULTURES INITIALLY INFECTED AT 0.03 PFU/CELL[a]

EXPERIMENT NUMBER (Relates to data in Figure Number)	SUPERINFECTION Virus	M.o.i.	REACTIVATED VIRUS PLAQUE NUMBER Actual	Expected[b]
1. (Figure 4.12., 4.13.)	HCMV	0.001	28	30
2. (Figure 4.9 .)	tsKsyn	0.03	660	900
3.	tsKsyn	0.003	72	90

a HFL cell monolayers were infected with HSV-2 at 0.03 pfu/cell and incubated in EF5 at 42°C for 6 days and then at 37°C for 4 days. The cultures were then superinfected with HCMV or tsK at various multiplicities and overlaid with EHu5. After 2 days at 38.5°C, the cultures were stained with Giemsa stain and plaques were counted.

b Expected plaque numbers were calculated on the assumption that 100% of input virus is able to establish a latent infection and be induced to reactivate upon superinfection with HCMV or tsKsyn.

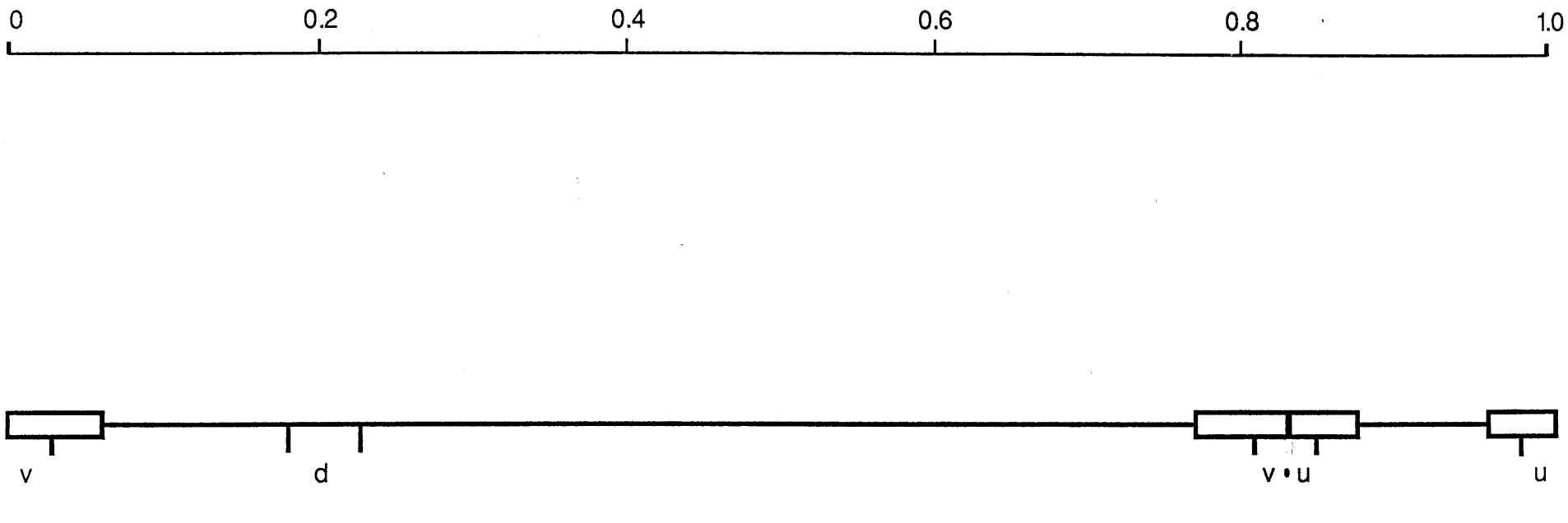
Table 4.16. SPONTANEOUS REACTIVATION IN LATENTLY INFECTED CULTURES MONITORED BY PLAQUE ASSAY OF CELL LYSATES

TREATMENT OF CELLS PRIOR TO HARVESTING (Days, Temperature)	NUMBER OF CULTURES CONTAINING SPONTANEOUSLY REACTIVATED VIRUS
6, 42°C + 2, 37°C	3 out of 18
6, 42°C + 4, 37°C	6 out of 22
6, 42°C + 6, 37°C	7 out of 18

HFL cells were infected with HSV-2 at 0.03 pfu/cell and incubated in EF5 at 42°C for 6 days and then at 37°C for 2, 4, or 6 days at 37°C. The cultures were then harvested and frozen and thawed 3 times. 20ul samples of cell lysates were titrated on BHK cells in EF5 and cultures were scored as positive or negative for spontaneously reactivated virus. The data are from 1 experiment.

Figure 4.8. THE GENOMIC LOCATIONS OF HSV-2 FRAGMENTS BamHI g, BamHI u, BamHI v AND BamHI d.

The HSV-2 BamHI fragments illustrated are the terminal fragments u and v, the joint fragment g (g=u+v), and fragment d which is located in U_L. The scale in map units is shown above.


0
0.2
0.4
0.6
0.8
1.0
v
d
v • u
u
BamHI
v + u = g

latency system was investigated for two reasons. Primarily, it might elucidate some of the molecular mechanisms involved in the latent viral-cell interaction, but, it is also necessary to determine whether the physical nature of the latent genome is identical *in vitro* and *in vivo* and, therefore, whether the viral-cell interaction *in vitro* is comparable to that *in vivo*.

4.5.2. Detection of HSV DNA Joint Fragments in HSV-2 Infected HFL Cells Incubated at 42°C for 4 Days

The state of the HSV genome was analysed in HFL cell cultures which had been infected with HSV-2 at 0.03 or 0.1 pfu/cell and incubated at 42°C for 4 days. Nuclear DNA extracted from these cultures was digested with BamHI and density-gradient banded in caesium chloride to remove the bulk of the cellular DNA sequences from DNA fragments of densities ranging from 1.709 to 1.746, which include HSV DNA. DNA fragments of such densities were separated on the basis of electrophoretic mobility and analysed by Southern hybridisation, utilising [^{32}P]-labelled (by nick translation) plasmid pBamg DNA as a probe. Plasmid pBamg consists of the HSV-2 fragment BamHI g, derived from the joint region of the HSV-2 genome (Figure 4.8.), cloned into pAT153. In control experiments, hybridisation, to BamHI-digested cellular DNA plus linear HSV-2 DNA, of the pBamg probe and of HSV-2 fragment BamHI g was compared. The results in Figures 4.9.(b) and 4.9.(c) indicate that both the probes hybridised to the HSV-2 BamHI g (joint) fragments (g=v+u) and the HSV-2 BamHI v (terminal) and BamHI u (terminal) fragments (note that the terminal BamHI u and v fragments are approximately the same size and often comigrate), and that, in addition, the pBamg probe hybridised to certain cellular DNA sequences.

Figure 4.9.(a) shows that DNA samples, from HSV-2 infected HFL cells incubated at 42°C for 4 days, contain DNA which hybridises to pBamg, has the same electrophoretic mobility as HSV-2 BamHI g, and which is not present in mock-infected HFL cells incubated at 42°C for 4 days. There were no detectable sequences hybridising to pBamg of the

Figure 4.9. DETECTION OF HSV JOINT FRAGMENTS IN HFL CELLS INFECTED WITH HSV-2 AND INCUBATED AT 42°C FOR 4 DAYS

(a) Lanes 1, 2, 3, and 4 contain $4.5x10^7$, $1.5x10^7$, $4.5x10^6$, $1.5x10^6$ BamHI-digested HSV-2 genomes, respectively.

Lane 10 contains $4.5x10^7$ copies of BamHI-digested pBamg plasmid DNA (HSV-2 BamHI g plus vector sequences).

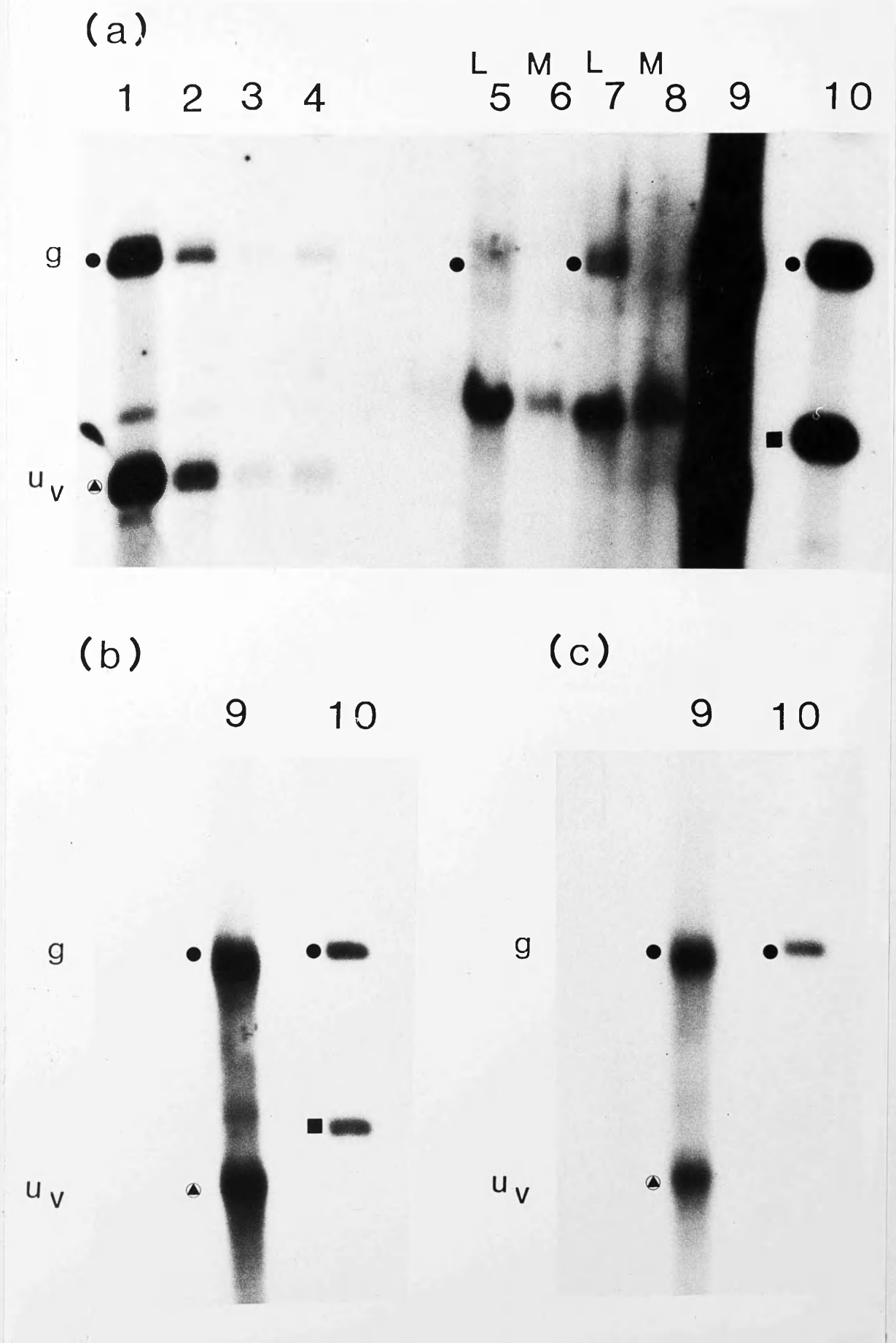
Lanes 5 to 9 contain BamHI-digested nuclear DNA of densities 1.709 to 1.746, from cell cultures which had been incubated at 42°C for 4 days. In lanes 5 and 7, the DNA is from $3x10^7$ HFL cells infected with HSV-2 at 0.03 or 0.1 pfu/cell respectively; in lanes 6 and 8, the DNA is from $3x10^7$ mock-infected HFL cells; in lane 9, the DNA is from $3x10^7$ mock-infected cells, plus HSV-2 infected cells from a lytic infection.

Southern hybridisation of DNA fragments separated by electrophoresis used, as a probe, ^{32}P-labelled pBamg plasmid DNA.

Bands representing HSV-2 BamHI g (●), u and v (◓) are marked. The band depicted by (■) represents the vector sequences of plasmid pBamg.

(b) Experimental details of samples in lanes 1 and 2 are as for lanes 9 and 10, respectively, of Figure 4.9.(a).

(c) Experimental details are as for Figure 4.9.(b), except that the ^{32}P-labelled probe used in Southern hybridisation analysis was the HSV-2 fragment BamHI g.


(a)
L M L M
1 2 3 4 5 6 7 8 9 10
g
u_v
(b)
9 10
g
u_v
(c)
9 10
g
u_v

same electrophoretic mobility as the HSV-2 BamHI v and u fragments in DNA from mock- or HSV-2- infected HFL cells incubated at 42°C for 4 days. A control sample containing mock-infected DNA plus linear HSV-2 genomes, which had also been BamHI-digested and density-gradient banded, contained DNA fragments representing HSV-2 BamHI fragments g, v and u, illustrating that BamHI fragments v and u were not lost during processing of DNA samples.

It seems, therefore, that HSV-2 infected cultures incubated at 42°C for 4 days contain HSV-2 DNA joint fragments but do not contain detectable HSV-2 terminal fragments. It is, therefore, possible that the HSV-2 genome persists in an endless configuration. Alternatively, the HSV DNA could persist as unit length, linear molecules, integrated into the cellular genome via the repeat regions of the HSV DNA, although there was no detection of the novel-sized terminal fragments which would be so generated. It is likely, therefore, that the HSV-2 genome is in an 'endless' configuration in these cultures and is therefore either circular, concatemeric or integrated via regions of the genome other than the termini.

Reconstructions showed (via densitometric analysis) that in a DNA sample derived from $3x10^7$ HFL cells initially infected with 0.1 pfu/cell of HSV-2, the HSV-2 joint fragments were present at approximately $5x10^6$ copies. Assuming 100% of the pfu applied to the cells becomes established in a latent state, at least $3x10^6$ HSV-2 genomes would be present in the latently infected sample. If the latent HSV-2 DNA is endless, $6x10^6$ copies of the joint fragment and no terminal fragments would be expected. Since the level of HSV joint fragments detected in infected 42°C-treated cultures is approximately the same as the level predicted, the DNA detected is likely to be the DNA which establishes a latent state.

4.5.3. Determination of the Molar Ratio of HSV Joint Sequences to HSV Unique Sequences in HSV-2 Infected Cells Incubated at 42°C for 4 days

Further evidence was sought for the presence of HSV-2 DNA in an endless configuration in HSV-2 infected cells

which had been incubated at 42°C for 4 days. The molar ratio of HSV-2 joint sequences to HSV-2 unique sequences was determined by Southern hybridisation analysis of the DNA using, as probes, the HSV-2 BamHI g fragment representing the joint sequences, plus the cloned HSV-2 BamHI d fragment, derived from the U_L region of the HSV-2 genome and therefore representing a unique HSV-2 sequence (Figure 4.8.).

Nuclear DNA was extracted from HFL cells infected with HSV-2 at 0.03 pfu/cell which had been incubated for 4 days at 42°C. DNA from such cultures was digested with BamHI and density-gradient banded in caesium chloride. DNA fragments of densities ranging from 1.709 to 1.746, were electrophoresed in agarose gels and Southern hybridisation was performed.

Figure 4.10.(a) shows the results of a DNA analysis, with probe BamHI g, indicating that, as in Figure 4.9.(a), HSV-2 joint fragments, but not termini, are present in HSV-2 infected cultures following incubation at 42°C for 4 days. The initial m.o.i. was 0.03 pfu/cell and therefore the expected number of HSV-2 genomes was at least 10^6. If the HSV-2 molecules were endless the number of joint fragments present would be $2x10^6$, compared to the detected level in (estimated via densitometry) Figure 4.10.(a) of 10^7 copies of joint fragments. Therefore, 5-fold more joint fragments were detected than predicted. The factors affecting the calculations made to determine levels of latent DNA are discussed below (Section 4.5.6.).

The membrane autoradiographed and photographed for Figure 4.10.(a) was stripped of the BamHI g probe and reprobed with a mixture of ^{32}P-labelled BamHI g and BamHI d fragments, in equal concentrations and at approximately equal specific activities. Bands representing BamHI g and BamHI d are present in the lane containing DNA from HSV-2 infected 42°C treated cells. Densitometric analysis of these bands indicates that the molar ratio of bands representing BamHI g to bands representing BamHI d is between 1.1:1.0 and 1.5:1.0. The molar ratio of these bands in reconstruction samples is 0.8:1.0. Therefore, the calculated ratio of BamHI g to BamHI d sequences is between 1.4:1.0 and 1.9:1.0. A more accurate estimate of the molar ratio of joint to unique sequences cannot be obtained due to the close proximity of

Figure 4.10. DETERMINATION OF THE MOLAR RATIO OF HSV JOINT FRAGMENTS TO HSV UNIQUE FRAGMENTS IN HSV-2 INFECTED HFL CELLS INCUBATED AT 42°C FOR 4 DAYS

(a) Lanes 1, 2 and 3 contain $1.5x10^7$, $4.5x10^6$, and $1.5x10^6$ BamHI-digested HSV-2 genomes, respectively.

Lane 6 contains $1.5x10^7$ copies of BamHI-digested plasmid pBamg (HSV-2 BamHI g plus vector sequences).

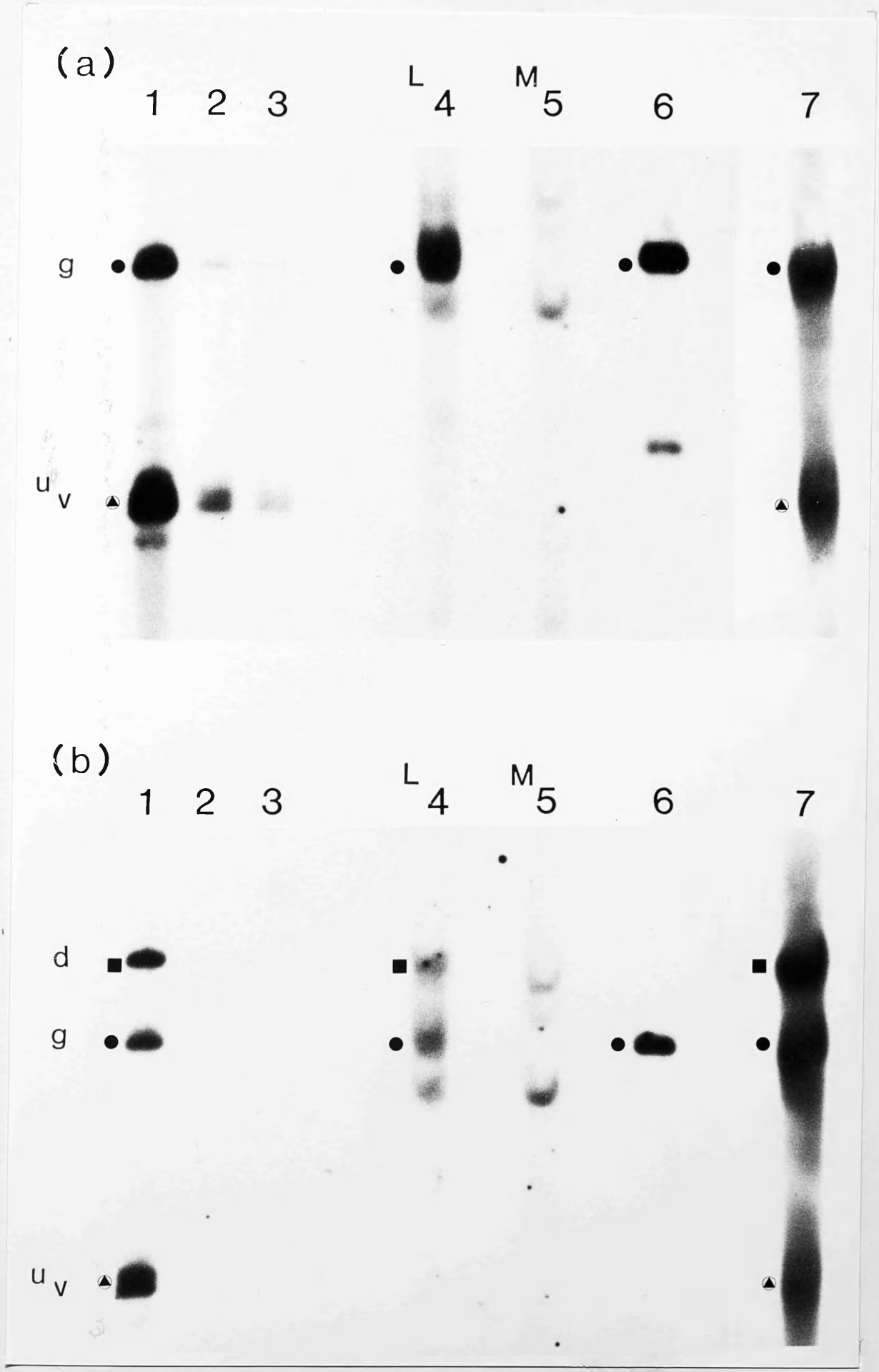
Lanes 4, 5 and 7 contain BamHI-digested nuclear DNA of densities 1.709 to 1.746, from cell cultures which had been incubated at 42°C for 4 days. In lane 5, the DNA is from $3x10^7$ mock-infected HFL cells; in lane 4, the DNA is from $3x10^7$ HFL cells infected with HSV-2 at 0.03 pfu/cell; in lane 7, the DNA is from $3x10^7$ mock-infected cells, plus HSV-2 infected cells from a lytic infection.

Southern hybridisation of DNA fragments separated by electrophoresis used ^{32}P-labelled HSV-2 fragment BamHI g as a probe.

Bands representing HSV-2 BamHI fragments g (●), u and v (⏢) are labelled.

(b) Experimental details are the same as for Figure 4.10.(a) except that Southern hybridisation analysis utilised ^{32}P-labelled HSV-2 fragments BamHI g and BamHI d.

Bands representing HSV-2 BamHI fragments d (■), g (●), u and v (⏢) are identified.


(a)
1
2
3
L
4
M
5
6
7
g
u
v
(b)
1
2
3
L
4
M
5
6
7
d
g
u
v

the BamHI g and BamHI d bands and a cross-hybridising cellular band and due to the diffuseness of the bands. Therefore, HSV-2 joint fragments are present in higher molar ratios than HSV unique sequences, at best approaching bimolarity compared to unique HSV-2 sequences. It is, therefore, likely that viral genomes from HSV-2 infected HFL cells incubated at 42°C for 4 days are in an 'endless' configuration and are not unit length, linear molecules integrated into the cellular genome via the repeat regions of the genome. No distinction can be made between circular, concatemeric or integrated states from this data.

4.5.4. Analysis of HSV DNA Sequences in HFL Cells Latently Infected with HSV-2

Although the HSV-2 genome was analysed following incubation of infected cells for 4 days at 42°C, in the *in vitro* latency system HSV is considered to be latent during the 37°C period, within which infectious virus is not usually detectable even though 37°C is a permissive temperature for HSV replication.

Therefore, to determine the state of the latent HSV genome *in vitro*, DNA was extracted from cytoplasmic and nuclear fractions of HSV-2 infected cultures which had been incubated at 42°C for 6 days and then at 37°C for a further 4 days. To check for spontaneous reactivation, DNA from the cytoplasmic fraction of each culture was analysed for the presence of HSV-2 DNA by digestion with BamHI and Southern hybridisation analysis using the HSV-2 fragment BamHI g as a probe (the probe was ^{32}P-labelled by hexanucleotide random priming to increase specific activity and incorporation of label and thereby increase the sensitivity of detection of fragments hybridising to BamHI g). Lane 6 in Figure 4.11.(a) shows the presence of HSV-2 DNA, and therefore the corresponding nuclear fraction was discarded. Nuclear DNA, from several cultures of cells in which the presence of spontaneously reactivated virus was not apparent by Southern hybridisation analysis of the corresponding cytoplasmic fractions, was digested with BamHI. The latent HSV genomes were concentrated and separated from the bulk of the

Figure 4.11. DETECTION OF HSV DNA SEQUENCES IN HFL CELLS LATENTLY INFECTED WITH HSV-2

(a) Lanes 1, 2 and 21 contain $1.5x10^7$, $4.5x10^6$ and $1.5x10^7$ BamHI-digested HSV-2 genomes, respectively.

Lanes 11 and 13 contain BamHI-digested plasmid pBamg (HSV-2 BamHI g plus vector sequences) at $1.5x10^7$ copies.

Lanes 3 to 10 and 14 to 20 contain BamHI-digested DNA from the cytoplasmic fractions of $3x10^6$ HFL cells infected with HSV-2 at 0.03 pfu/cell and incubated at 42°C for 6 days and at 37°C for a further 4 days.

Lane 12 contains BamHI-digested DNA from the cytoplasmic fractions of $3x10^6$ mock-infected HFL cells.

Southern hybridisation of DNA fragments separated by electrophoresis utilised ^{32}P-labelled HSV-2 fragment BamHI g as a probe.

Bands representing HSV-2 BamHI fragments g (■), u and v (▲) are indicated.

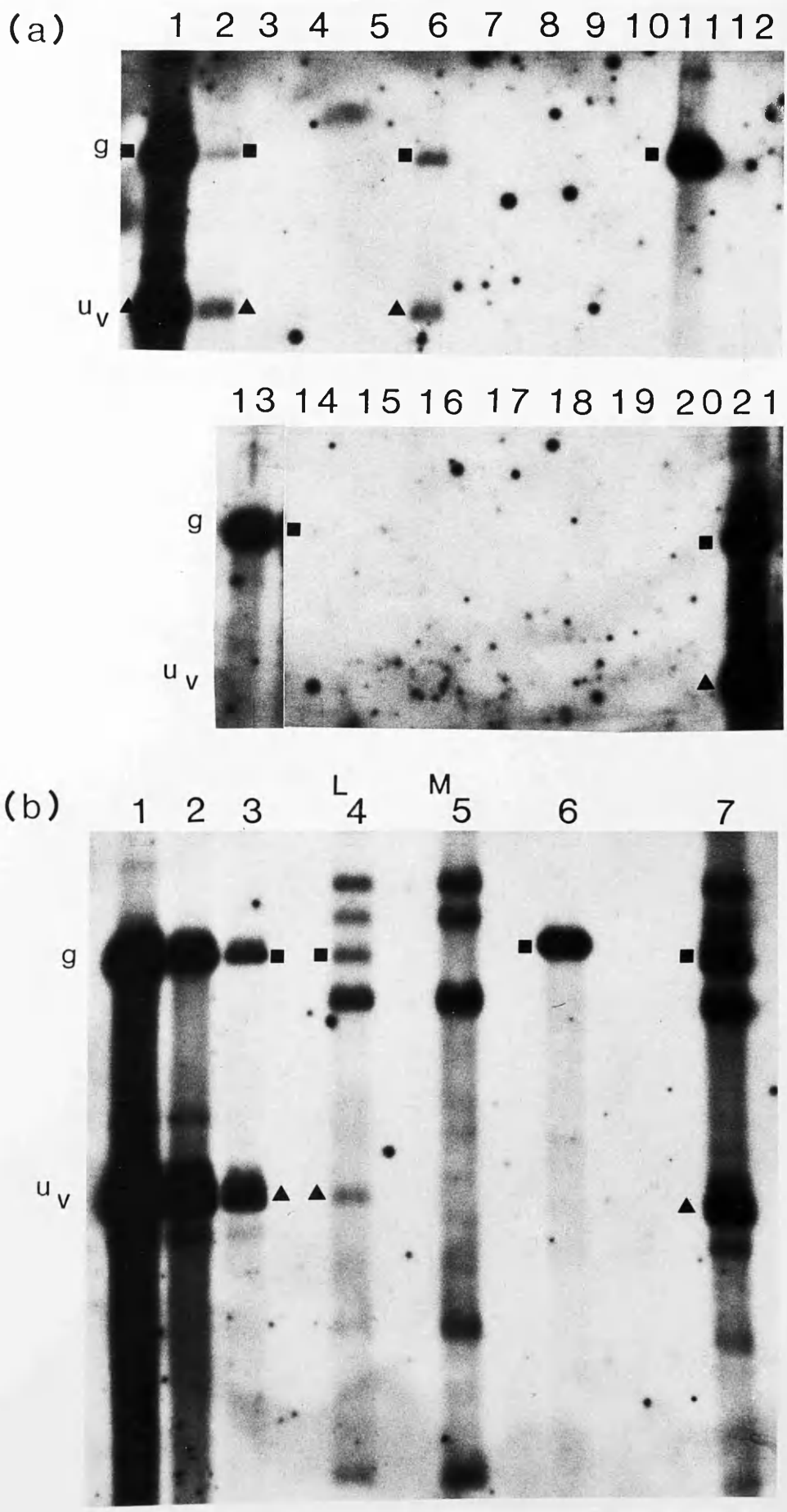
(b) Lanes 1, 2 and 3 contain BamHI-digested HSV-2 genomes at $1.5x10^7$, $4.5x10^6$ and $1.5x10^6$ copies, respectively.

Lane 6 contains BamHI-digested plasmid pBamg (HSV-2 BamHI g plus vector sequences) at $1.5x10^7$ copies.

Lanes 4, 5 and 7 contain BamHI-digested nuclear DNA of densities 1.709 to 1.746, from cell cultures which had been incubated at 42°C for 6 days and at 37°C for a further 4 days. In lanes 5 and 4, the DNA is from $4.2x10^7$ HFL cells mock-infected or infected with HSV-2 at 0.03 pfu/cell, respectively; in lane 7, the DNA is from $4.2x10^7$ mock-infected HFL cells, plus HSV-2 infected cells from a lytic infection.

Southern hybridisation analysis of DNA used the ^{32}P-labelled HSV-2 fragment BamHI g as a probe.

Bands representing HSV-2 BamHI g (■), u and v (▲) are marked.


(a)
1 2 3 4 5 6 7 8 9 10 11 12
g
uv
13 14 15 16 17 18 19 20 21
g
uv
(b)
1 2 3 L 4 M 5 6 7
g
uv

CALCULATION

By densitometric analysis there are approximately: 3.6×10^6 Joint fragments and 3.0×10^6 Terminal fragments in the latently infected sample.

Assumption: Each terminal fragment is from a unit length linear molecule.

Note: Terminal fragments are bimolar in this experiment since BamHI v is approximately the same size as BamHI u.

Therefore, there are 1.5×10^6 unit length linear genomes with a total of 1.5×10^6 joint fragments. This leaves 2.1×10^6 joint fragments (ie. 3.6×10^6 minus 1.5×10^6).

Assumption: Each of the 2.1×10^6 joint fragmentsis from an 'endless' HSV genome.

Therefore, there are 1.1×10^6 'endless' HSV genomes, and a total of 2.6×10^6 (ie. 1.5×10^6 plus 1.1×10^6) HSV genomes in the latently infected sample.

cellular DNA sequences by ultracentrifugation twice in a caesium chloride gradient. Southern hybridisation analysis of the DNA was carried out using the BamHI g fragment of HSV-2 as a probe.

Figure 4.11.(b) indicated that HSV-2 was present in the latently infected samples and that both joint and terminal HSV-2 sequences were represented. Since joint fragments were present at $3.6x10^6$ copies and terminal fragments were present at $3.0x10^6$ copies, $2.6x10^6$ HSV-2 molecules were present in the latently infected samples (compared to the predicted number of $1.3x10^6$ molecules), of which $1.5x10^6$ were unit, length and linear and $1.1x10^6$ were endless. The possibility that the HSV-2 genomes detected were representative of a small amount of spontaneously reactivated HSV-2 could not be dismissed. The level of detection of spontaneously reactivated virus in DNA from cytoplasmic fractions was approximately $4.5x10^5$ copies of HSV-2. Therefore, if DNA from reactivated virus was present in less than half of the cytoplasmic fractions of the cultures in this experiment at less than or equal to $4.5X10^5$ copies it might have escaped detection but could have accounted for the $2.6x10^6$ copies of HSV-2 detected in the latently infected cultures.

To overcome this difficulty, a more sensitive assay for the level of spontaneous reactivation was required. Therefore, HSV-2 infected (0.03 pfu/cell) cultures, which had been incubated at 42°C for 6 days and then at 37°C for a further 2, 4 or 6 days, were harvested, frozen and thawed 3 times, and a sample of the lysate was titrated on BHK cells at 37°C. Control reconstruction experiments implied that 60% of infectious virus present in the latently infected cultures would be detected. The number of cultures, from this experiment, in which spontaneous reactivation was detected by titration of cell lysates is shown in Table 4.16.; it should be noted that the level of spontaneous reactivation in these cultures is higher than expected from previous data (Section 4.2.2.), which might be an anomaly of the particular experiment or might be due to the increased sensitivity of detection of spontaneous reactivation by the method employed. Total cellular DNA from cultures in which

spontaneously reactivated virus was not detectable was pooled, BamHI digested, ultracentrifuged twice on caesium chloride gradients and analysed by Southern hybridisation using the HSV-2 fragment BamHI g as a probe.

Figure 4.12. shows that no HSV-2 DNA was detectable in these cultures despite a sensitivity of detection of at least 4.5×10^5 copies of HSV DNA and expected copy numbers for latent HSV-2 of 1.4×10^6 (lane 5), 7.2×10^5 (lane 6) and 10^6 (lane7). Bimolar joint fragments would have been present at 2.7×10^6, 1.4×10^6 and 2×10^6 copies, respectively.

This suggests that the HSV DNA sequences detected in the experiment shown in Figure 4.11. were representative of spontaneously reactivated virus.

Therefore, the approach taken did not permit the detection of latent HSV-2 during incubation at 37°C in the *in vitro* latency system. To detect latent HSV, either the proportion of latent HSV genomes per cell genome equivalent must be increased, or the sensitivity of detection of the Southern hybridisation technique employed must be increased.

4.5.5. The Fate of the HSV Genome in HFL Cells during Incubation at 42°C

From the data discussed so far, it appeared that the concentration of HSV-2 genomes was much decreased in cultures that had been incubated at 42°C for 6 days and then at 37°C for 2, 4 or 6 days in comparison with cultures that had only been incubated at 42°C for 4 days. Therefore, to determine the fate of the HSV DNA detectable during the 42°C period, a time course analysis was performed. DNA was extracted from the nuclear fractions of HSV-2 infected cultures which had been incubated for 1, 2, 3, 4, 5 or 6 days at 42°C. The DNA from 42°C treated cultures was BamHI-digested, centrifuged in CsCl gradients, electrophoresed, and Southern hybridisation was performed with the ^{32}P-labelled HSV-2 BamHI g probe.

Analysis of DNA from the cytoplasmic fractions showed that no HSV-2 DNA was detectable in these fractions and, therefore, the majority of the HSV DNA present in cultures at 42°C was confined to the nucleus. Figure 4.13. indicates

Figure 4.12. **ANALYSIS OF DNA FROM HFL CELLS LATENTLY INFECTED WITH HSV-2 AND SCREENED FOR THE PRESENCE OF SPONTANEOUSLY REACTIVATED VIRUS BY PLAQUE ASSAY**

Lanes 1, 2 and 3 contain BamHI-digested HSV-2 genomes at $1.5x10^7$, $4.5x10^6$ and $1.5x10^6$ copies, respectively.

Lane 8 contains BamHI-digested plasmid pBamg (HSV-2 BamHI g plus vector sequences) at $1.5x10^7$ copies.

Lanes 4 to 7 contain BamHI-digested whole cell DNA of densities 1.709 to 1.746. In lane 4, the DNA is from $4.5x10^7$ mock-infected HFL cells incubated at 42°C for 6 days and at 37°C for 4 days; in lanes 5, 6 and 7, the DNA is from $4.5x10^7$, $2.4x10^7$ and $3.3x10^7$ HFL cells, respectively, infected with HSV-2 at 0.03 pfu/cell and incubated at 42°C for 6 days and then at 37°C for 2, 4 or 6 days, respectively; in lane 9, the DNA is from mock-infected HFL cells incubated at 42°C for 6 days and at 37°C for 4 days, plus HSV-2 infected HFL cells from a lytic infection.

Southern hybridisation analysis of DNA used the ^{32}P-labelled HSV-2 fragment BamHI g as a probe.

Bands representing HSV-2 BamHI g (●), u and v (▲) are indicated.

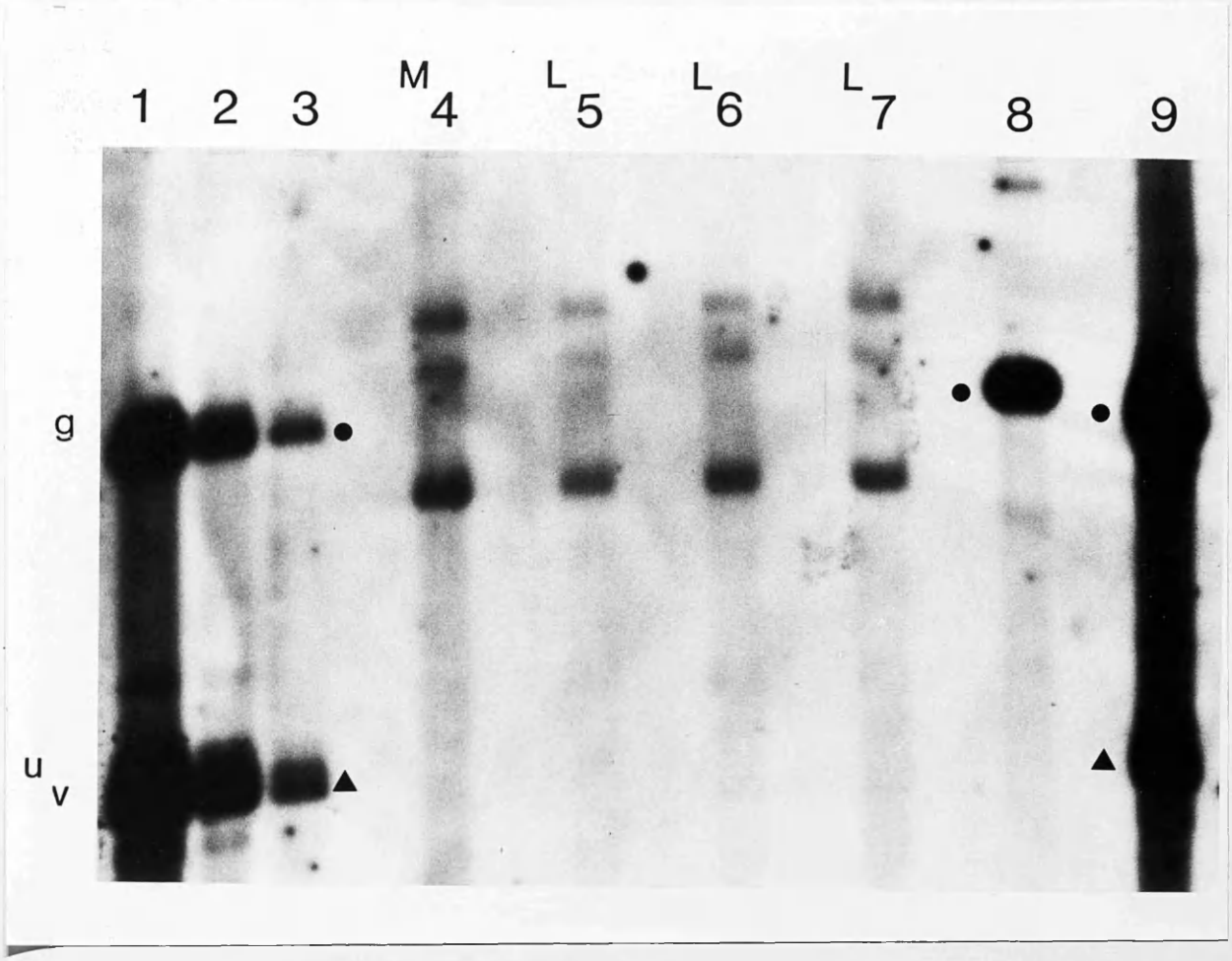

1
2
3
M
4
L
5
L
6
L
7
8
9
g
u
v

Figure 4.13. **DETECTION OF THE HSV GENOME IN HFL CELLS DURING INCUBATION AT 42°C**

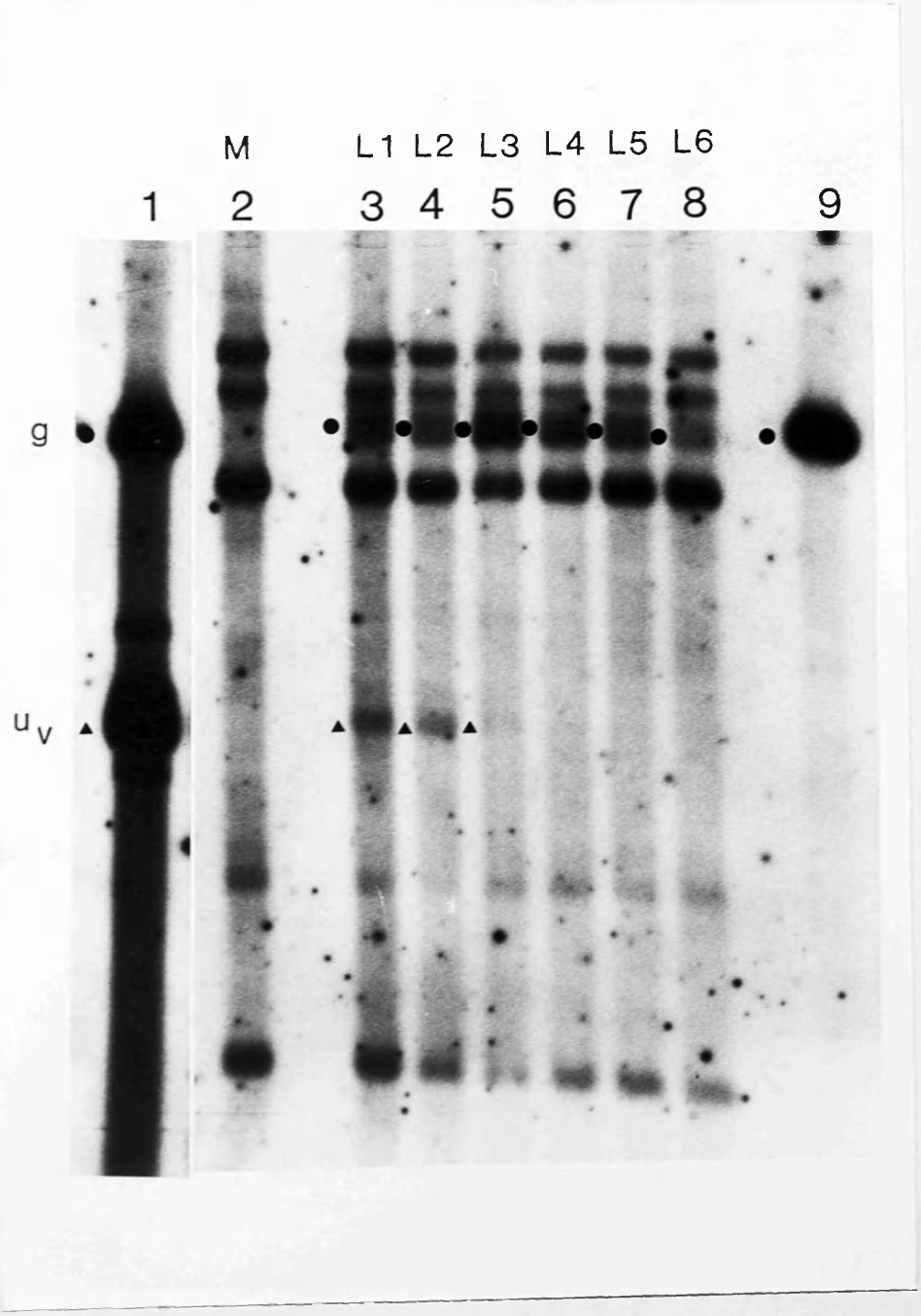
Lane 1 contains BamHI-digested HSV-2 genomes at $1.5x10^7$ copies.

Lane 9 contains BamHI-digested plasmid pBamg (HSV-2 BamHI g plus vector sequences) at $1.5x10^7$ copies.

Lanes 2 to 8 contain BamHI-digested nuclear DNA of densities 1.709 to 1.746. In lane 2, the DNA is from mock-infected HFL cells incubated at 42°C for 6 days. In lanes 3 to 8 the DNA is from $1.8x10^7$ HFL cells infected with HSV-2 at 0.03 pfu/cell and incubated at 42°C for 1, 2, 3, 4, 5 and 6 days respectively.

Southern hybridisation analysis of DNA used ^{32}P-labelled HSV-2 fragment BamHI g as a probe.

Bands representing HSV-2 BamHI g (●), u and v (▲) are labelled.


M
L1 L2 L3 L4 L5 L6
1 2 3 4 5 6 7 8 9
g
u v

CALCULATION

Estimations of DNA concentrations were obtained by densitometric analysis. The values for the number of unit length, linear HSV genomes, the number of 'endless' genomes and the number of HSV genomes present in total were derived in the same way as outlined on the page facing p139.

a change in the conformation of HSV-2 genomes during incubation at 42°C. The DNA from HSV-2 infected cells incubated at 42°C for 1 day contained both joint fragments (at $6.3x10^5$ copies) and terminal fragments (at $4.7x10^5$ copies) of HSV-2 DNA; a total of $4.3x10^5$ HSV-2 genomes were present, of which approximately 50% were unit length and linear ($2.3x10^5$ molecules) and 50% ($2.0x10^5$) were endless. Similarly, the DNA from HSV-2 infected cells incubated at 42°C for 2 days contained $6x10^5$ copies of joint and $4.4x10^5$ copies of terminal fragments, and therefore a total of 4.1 HSV-2 genomes of which $2.2x10^5$ were unit length and linear, while $1.9x10^5$ were endless. After the third day, DNA from HSV-2 infected cells incubated at 42°C contained approximately 3-fold greater levels of HSV-2 joint fragments ($2.5x10^6$) and there was a marked decrease of terminal fragments. No terminal HSV-2 fragments were detectable in DNA from HSV-2 infected cells incubated at 42°C for 4 or 5 days. These findings imply a change in the state of the HSV-2 DNA from linear to 'endless' during the first 4 days of incubation at 42°C. There was a gradual reduction in the level of HSV-2 joint fragments, relative to the level of the cross-hybridising cellular fragments, in DNA from HSV-2 infected cells during incubation at 42°C; from $2.5x10^6$ on day 3, to $1.1x10^6$ on day 4, to $0.7x10^6$ on day 5 and to $0.25x10^6$ on day 6. It would therefore seem that the number of HSV-2 DNA molecules decreases during 42°C incubation and this could partially account for the inability to detect latent HSV-2 DNA during the 37°C period.

In summary, HSV-2 DNA from cells incubated at 42°C for 1 to 6 days is found predominantly in the nucleus. The HSV-2 DNA appears to change from unit length linear genomes into endless molecules during a 3 day incubation at 42°C; after 6 days of incubation at 42°C, the level of (endless) molecules of HSV-2 in infected cells appears to be decreased.

4.5.6. Discussion

Incubation of HSV-infected cells at the supraoptimal temperature of 42°C results in conversion of the HSV genome

to an 'endless' molecule. The results presented suggest that the proportion of genomes converted to an endless state increases with the number of days of incubation of infected cultures at 42°C, until by the 4th day at 42°C all the HSV DNA appears to be endless. The level of spontaneous reactivation following downshift, to a permissive temperature for HSV, of infected cultures decreases with an increasing number of days (up to six) incubation of infected cultures at 42°C (C.M. Preston, personal communication; Notarianni, 1986). Therefore, there is a temporal correlation between achievement of a stable latent state for HSV and the conversion of HSV DNA to an endless conformation.

It is likely that circularisation of HSV-2 occurs during incubation of infected cultures at 42°C in view of the fact that, circularisation would be the first step both in integration into the cellular genome of HSV DNA via regions of the viral genome other than the termini and in the production of concatemers by a rolling circle mechanism. It is not possible to distinguish between circular, concatemeric or integrated states in these experiments. However, since the molar ratio of joint fragments (BamHI g) to unique fragments (BamHI d) tends to 2:1, following 4 days incubation of HSV-2 infected cultures at 42°C, this argues against a model in which a copy of the viral genome integrates into cellular DNA via the joint regions to form novel-sized junction fragments. It is known from mutant studies that replication is not essential for establishment of latency in the system described (Section 4.3.) and, since there is a reasonable correlation between the numbers of genomes detected and the expected numbers of genomes present, it is unlikely that replicative concatemeric structures account for the presence of endless molecules of HSV.

The results from superinfection of latently infected cultures (initial m.o.i. 0.003 pfu/cell) suggested that at least 30% and up to 100% of the pfu applied to the cells was able to become established in a latent state from which reactivation was inducible by superinfection (Section 4.2.5.). Superinfection of control cultures, from

experiments in which HSV-2 at an initial multiplicity of 0.03 pfu/cell was converted to a latent state in HFL cells by incubation at 42°C (Table 4.15.), indicated that the number of plaques of reactivated virus increased in proportion to the increased amount of input virus. The copy numbers of HSV-2 genomes detected in infected cultures incubated at 42°C were compared with predicted copy numbers of HSV-2 genomes. In the experiments described in Sections 4.5.2. (DNA from cultures incubated for 4 days at 42°C) and 4.5.5. (1,2 and 4 days at 42°C) there was a good correlation between detected and predicted copy numbers of HSV-2 genomes; in the experiments described in Sections 4.5.4. (4 days at 42°C), 4.5.5. (3 days at 42°C) and 4.5.3. (4 days at 42°C) detected copy numbers of HSV-2 genomes were 2-fold, 2.4-fold and 5-fold, respectively, higher than predicted copy numbers; while, in the experiments described in Section 4.5.5. (5 days at 42oC) and Sections 4.5.5. (6 days at 42°C) detected HSV-2 genome copy numbers were 0.6-fold and 0.2-fold, respectively, lower than predicted copy numbers. The presence in some experiments of higher HSV-2 genome copy numbers than predicted could be due to inaccuracies in the measurement of HSV DNA concentrations for reconstruction samples and/or of input virus titres. Replication of HSV during 42°C incubation in culture is also a possibility, although there is evidence from several sources that replication of HSV at 42°C is unlikely to occur (Crouch and Rapp, 1972; Darai and Munk, 1973; Marcon and Kucera, 1976; Notarianni, 1986). It could be argued that the higher copy number of HSV molecules detected was not a result of HSV replication if a correlation was observed between the levels and states of viral DNA detected in wt HSV infected cultures incubated at 42°C, viral DNA from cultures infected with a ts replication defective mutant and incubated at 42°C, viral DNA from wt HSV infected cultures treated with inhibitors of replication (for example, phosphonoacetic acid) and incubated at 42°C, and, viral DNA from wt HSV infected cultures harvested early during incubation at 42°C. Another explanation for the detection of higher levels of HSV genomes than predicted would be the presence of molecules of virus which are not capable of forming plaques during a

lytic infection of cultured cells but which are capable of establishing a latent state from which, however, reactivation cannot be induced by HSV superinfection. It is noteworthy that cells latently infected with HSV in vivo apparently contain rather high numbers of viral genomes (Section 1.3.4.1.). Therefore, in both in vivo and in vitro latency systems, the DNA detected may or may not represent latent HSV genomes potentially capable of being induced to reactivate.

There is an apparent decrease in the copy number of HSV-2 genomes during the latter period of incubation at 42°C. The latent HSV DNA is undetectable during the period at 37°C. This could be explained if a proportion of the HSV-2 fragments detected during 42°C incubation was accounted for by the presence of non-infectious or defective HSV DNA, unable to establish a latent state and degraded during incubation at the supraoptimal temperature. This explanation does not account for the inability to detect the predicted level (on the basis of initial infecting pfu and reactivation efficiencies) of latent HSV-2 DNA during 37°C incubation. A low recovery of HSV DNA following the manipulations required to concentrate sufficient quantities of the DNA was a possibility, although control experiments suggested that the efficiency of recovery was relatively high. Despite the inability to detect HSV DNA at 37°C, on the basis of the evidence presented above, particularly from the time course experiment, it is not unreasonable to propose that the state of the HSV genome remains endless during the latent period at 37°C, although this has yet to be confirmed.

Wigdahl et al. (1982b), utilising an in vitro latency system which relies upon treatment with viral inhibitors and elevated temperatures to establish and maintain a latent state for HSV, detected unit length and linear HSV DNA molecules, suggesting a difference between the non-productive states of HSV produced by treatment with inhibitors and with elevated temperatures and by treatment with elevated temperatures alone. Differences in the efficiency of and requirements for reactivation by superinfection between the systems have been mentioned in

the preceding results sections. It is thought that differences in the m.o.i. utilised in the systems could be an important parameter. In the in vitro latency system described here, low m.o.i.'s were used and, there was a reasonable correlation between the amount of HSV-2 DNA detected (during 42^{o}C incubation), the amount of virus reactivated by superinfection and the amount of pfu initially used to infect the cultures. In the system described by Wigdahl et al. (1982b), high m.o.i. was used, as reflected by the amount of DNA detected, but the reactivation efficency was comparatively low. Supposing that the latent HSV genomes are those which can be induced to reactivate in the system of Wigdahl et al. (1982b) and are therefore present at relatively low copy number, perhaps, then, the state of the latent HSV DNA is endless but is masked by the presence of unit, length linear HSV molecules not involved in latency.

It is widely accepted that the latent HSV genome resides in a endless state in vivo and the detection of HSV genomes in an endless configuration is regarded as a criterion for the classification of HSV as latent in in vitro systems. The evidence presented indicates that the state of the latent genome in the in vitro latency system described in this thesis might be endless. There is recent evidence to suggest that in vivo the latent HSV-1 genome is extrachromosomal (Mellerick and Fraser, 1987) and, furthermore, has a nucleosomal structure similar to that of cellular chromatin, as determined by micrococcal nuclease digestion (Deshmane and Fraser, 1989). Since in vivo and in vitro transcription of latent HSV genomes is restricted, it is possible that the latent genome may be associated with transcriptionally inactive chromatin structures. This will be discussed further in Section 5.

5.0. FINAL DISCUSSION

HSV latency, both *in vivo* and in the *in vitro* latency system described in this thesis, appears to involve a relationship between the virus and its host cell such that the virus exists in a non-replicative state and exhibits a restricted pattern of gene expression; the latent HSV genome resides in an endless configuration *in vivo*, associated with nucleosomes, and probably also resides in an endless state *in vitro*.

The observations that the latent HSV genome does not express the full repertoire of genes involved in lytic infection (Stevens *et al.*, 1987; Section 4.) and that the HSV Vmw110 trans-activator protein is capable of stimulating the reactivation event *in vitro* (Russell *et al.*, 1987a; Harris *et al.*, 1989), suggest that latency is controlled at the level of gene expression. The following discussion considers the possibility of control of HSV latency at the level of IE gene expression since these proteins are the first to be produced during lytic infection and since the IE proteins Vmw175, Vmw110 and Vmw63 are involved in control of HSV gene expression in *trans*. The possible importance of levels of Vmw110 and Vmw175 gene expression in the lysis/latency decision of HSV was proposed by O'Hare and Hayward (1985b).

The properties of the IE proteins are detailed in Section 1.2.3.2.: only the salient points are reiterated here. IE polypeptide Vmw175, which has the capacity to stimulate E and L transcription and is involved in the negative regulation of IE gene expression (Everett, 1984a, 1986; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a,b; Gelman and Silverstein, 1986), is essential for the transition from IE to later phases of HSV gene expression that occurs during lytic infection (Preston, 1979b). IE polypeptides Vmw63 and Vmw110 are also transactivators of gene expression (Everett, 1984a; Mavromara-Nazos *et al.*, 1986; Quinlan and Knipe, 1985; Rice and Knipe, 1988; Everett, 1986; Sekulovich *et al.*, 1988) and Vmw63 appears to be essential for modulation of E and L gene transcription (McCarthy *et al.*, 1989). Vmw110 is not absolutely essential

for virus growth in tissue culture since, at relatively high m.o.i., replication of the HSV-1 mutant dl1403, which does not express Vmw110, is similar to that of wt HSV-1. However, in low multiplicity dl1403 infections the efficiency of plaque formation is severely reduced in a cell-dependent manner (Stow and Stow, 1986). A similar phenotype is exhibited by HSV-1 mutant in1814, which is defective in transinduction of IE transcription as a result of a 12bp insertion in the gene encoding the transinducing factor Vmw65. In comparison to IE RNA levels in wt HSV-1-infected cells, in in1814-infected cells the accumulation of IE-1 and IE-2 RNA was reduced 4 to 5 fold, whereas the level of IE-4 RNA was reduced only 2-fold and IE-3 RNA was unaffected (Ace et al., 1989). Ace et al. (1989) suggested that the inability of in1814 to replicate at low m.o.i. could result from failure to produce sufficient levels of IE proteins for initiation of lytic infection and that Vmw65 functions to ensure that such 'threshold' levels are attained. In view of this, it may be significant that IE genes are not expressed at detectable levels by latent HSV in vivo (Stevens et al., 1987), that functional HSV-1 IE Vmw110 (or HSV-2 Vmw118) is not, apparently, present in latently-infected cells in vitro, and that IE Vmw110, which is not essential for the establishment of latency, can stimulate reactivation of latent HSV in vitro (Russell et al., 1987; this thesis; Harris et al., 1989). It is particularly intriguing that in low multiplicity infections of both in1814 and dl1403, as well as in low multiplicity infections of wt HSV following incubation at 42°C, virus can be maintained in a non-productive state for several days at 37°C and then reactivated to form plaques by superinfection with HCMV (Stow and Stow, 1989; Ace, Harris and Preston, personal communication; Notarianni, 1986; Russell and Preston, 1986; Russell et al., 1987; this thesis). Complementation of in1814 by Vmw110 has also been demonstrated (Ace et al., 1989). Both the levels of spontaneous reactivation in the in vitro latency system and the plaquing efficiencies of the mutants dl1403 and in1814 increase relative to the initial m.o.i.. Therefore, it is plausible that incubation of cells at the supraoptimal temperature of 42°C renders HSV DNA

incapable of synthesising sufficient levels of IE proteins required for progression to the lytic cycle, and that the presence of Vmw110, or its proposed cellular, HCMV or VZV functional counterpart (see Section 4.4.5.), initiates the lytic cycle and/or restores the ability of the latent HSV genome to synthesise the appropriate amounts of IE polypeptides (perhaps by a mechanism similar to Vmw110 complementation of in1814 [Ace et al., 1989]).

Leib et al. (1989), recently reported the results of an investigation into the roles of the IE proteins Vmw175, Vmw110 and Vmw63 in the establishment and reactivation of ganglionic latency in a mouse ocular model. Viruses with deletion or nonsense mutations in the genes encoding Vmw175 and Vmw63 were unable to establish a latent state, probably due to the inability of the viruses to replicate at the site of inoculation. Virus mutants dlx3.1 and dlx0.7, with deletions in the coding and non-coding sequences of Vmw110, were able to become established in a latent state but were deficient in reactivation. In vivo studies with dl1403 have indicated that there is no absolute requirement for Vmw110 in establishment or reactivation, although there was a reduced level of recovery of dl1403 compared to wt virus in vivo (Leib et al., 1989; Clements and Stow, 1989). The trigger for reactivation in vivo may be functionally equivalent to Vmw110 or could act at the level of IE gene expression resulting in the production of 'threshold' quantities of IE proteins, including Vmw110, for progression into the lytic cycle.

Roizman and Sears (1987) proposed a model, based on the apparent presence of the HSV genome in multiple copies in each neuron that harbors latent virus (Cabrera et al., 1980; Puga et al., 1978; Rock and Fraser, 1983; Rock and Fraser, 1985; Efstathiou et al., 1986), in which the balance between the latent state and viral gene expression, leading to lytic replication, depends upon the copy number of viral genomes per cell. The authors suggested that the effect of reactivation stimuli on neuronal cells is to amplify latent viral genomes and that viral gene expression ensues when the copy number of genomes in individual cells reaches a specific level at which point the lytic cycle proceeds. The

most obvious mechanism for the proposed activation of the lytic cycle by the accumulation of high genome numbers, with respect to the evidence discussed above, assumes low levels of, at least, IE expression from each genome and a cumulative effect of the gene products. A model in which reactivation is dependent upon genome copy is not inconsistent with the observations that spontaneous reactivation from the in vitro latency system described occurs at increased levels when cells are initially infected at high m.o.i. and that the plaque forming defects of dl1403 and in1814 can be overcome at high m.o.i.. A mechanism for controlled amplification of the viral genome involving a host polymerase and a specific origin of DNA replication was postulated by Roizman and Sears (1987), based on findings from EBV latency in which replication of the latent genome involves a viral origin of replication (oriP), the EBNA-1 protein and cellular DNA polymerase (Yates et al., 1985; Reisman et al., 1985; Rawlins et al., 1985; Summers and Klein, 1976; Colby et al., 1980). It is important to note that latent EBV multiplies in tandem with replication of the B lymphocyte (Adams, 1987) and since neuronal cells do not divide the mechanism for proposed amplification of latent HSV would differ, at least in this respect, from the mechanism for multiplication of latent EBV DNA.

Control of latency at the level of IE gene expression would require suppression of IE gene expression at transcriptional or post-transcriptional levels.

The theory that absence of Vmw65 and the consequent suppression of IE gene transcription results in the establishment of a latent state was proposed by Kristie and Roizman (1988) and has been supported by Ace et al. (1989) on the basis of evidence from analysis of mutant in1814, including the ability of in1814 to reside in a state resembling latency in infections at low m.o.i.. It was originally suggested that in neuronal cells the loss or inactivation of Vmw65 could occur during transport of the HSV nucleocapsid over the comparatively great distance from the neuronal cell surface to the neuronal nucleus (Kristie and Roizman, 1988). However, an alternative mechanism for loss or inactivation of Vmw65 must be postulated since there

is convincing evidence that HSV can exist in a latent state in cells other than neuronal cells both *in vivo* (Clements and Subak-Sharpe, 1988; Al-Saadi *et al.*, 1988; Clements and Jamieson, 1989) and *in vitro* (Section 1.3.6.; Notarianni, 1986; Section 4.0.).

A possible role for LAT in the control of latency at the level of IE gene expression was suggested by the findings that the LAT gene was the only region of the genome expressed in latently infected cells and that the LAT RNA was antisense to the mRNA of the IE Vmw110 protein (Stevens *et al.*, 1987; Rock *et al.*, 1987a) which has the potential for *trans*-activation of gene expression and which reactivates HSV from its latent state *in vitro*. A negative effect of LAT on Vmw110 production might result in the establishment of HSV in a latent state; possible mechanisms for such an effect by LAT were outlined in Section 1.3.4.2.. However, recent experiments utilising HSV mutants which express Vmw110 but which fail to produce detectable levels of LAT, due to deletions in the region 5' to the LAT template, have suggested that there is no prerequisite for LAT in the establishment, or reactivation, of HSV latency (Steiner *et al.*, 1989). It is nonetheless interesting that LAT is the only transcript detectable in cells latently infected with HSV and the selective expression of the LAT gene may occur via the presence of distinct cis-acting upstream transcriptional control elements and differences in methylation patterns or chromatin structure.

Another possible mechanism for control of latency at the level of HSV IE gene expression is DNA methylation. EBV has been shown to be extensively methylated and can be reactivated by treatment with demethylation agents (Bensasson and Klein, 1981; Szyf *et al.*, 1985). Although there is evidence to suggest that HSV DNA is not extensively methylated *in vivo* (Dressler *et al.*, 1987), demethylation agents stimulate reactivation of HSV from its latent state (Bernstein and Kappes, 1988; Stephanopoulos *et al.*, 1988). It is therefore conceivable that only specific regions of the HSV genome involved in control of expression, perhaps including the promoter regions of IE genes, are methylated and that demethylation results in activation of these genes

and, thereby, in initiation of the lytic cycle.

There is recent evidence that most latent HSV-1 DNA, including the transcriptionally active LAT region, has a nucleosomal structure similar to that of cellular chromatin and that, in contrast, during acute infection of mice the majority of viral DNA is not associated with nucleosomes (Deshmane and Fraser, 1989). It is noteworthy that the latent EBV is predominantly nucleosome-associated, whereas, in producer line P3HR-1, the majority of EBV DNA is nonnucleosomal (Shaw et al., 1979; Dyson and Farrell, 1985). It is possible that the transcriptional inactivity of the majority of the latent HSV genome is a consequence of formation of a particular chromatin structure. A correlation exists between transcriptionally active regions of DNA and regions which are DNase-hypersensitive (Gross and Garrard, 1988); the analysis of DNase hypersensitive sites within HSV-1 latent DNA is reported to be underway (Deshmane and Fraser, 1989).

In conclusion, evidence to date is consistent with a mechanism for control of latency at the level of IE gene expression. Suppression of IE gene expression from the latent HSV genome could be the result of absence or loss of the IE trans-inducing factor Vmw65, the presence of a cellular/viral repressor, selective DNA methylation or the association of the latent HSV genome with chromatin in a transcriptionally inactive state. Reactivation could be induced upon attainment of sufficient levels of IE gene expression, which could be achieved by activation of, or substitution for, IE gene expression by cellular factors, removal of repressor activities or an increase in genome copy number.

Experiments utilising the in vitro latency system described in this thesis and considered to be of importance for further investigation of the molecular basis for HSV latency encompass three main areas: analysis of the mechanism by which Vmw110 stimulates reactivation of HSV from its latent state, analysis of the pattern and control of gene expression from the latent HSV genome, and determination of the state of the latent HSV genome.

The probability of Vmw110 action at the level of viral gene expression has been discussed in Section 4.4.5.. Vmw110-mediated stimulation of expression from the HSV genome present in latently infected cells could occur directly or, indirectly via the effect of Vmw110 on cellular gene expression. To distinguish between the possibilities, RNA species produced in latently infected cell cultures could be analysed, in a time course experiment following the introduction of the Vmw110 gene into latently infected cells and utilising protein synthesis inhibitor treatment or, following direct introduction of the Vmw110 protein into the latently infected cells in the presence of a protein synthesis inhibitor. Also, due to similarities between the in vitro latency system and low multiplicity infections of mutants dl1403 and in1814, it would be of interest to determine the mechanism for 'reactivation' of the mutants. Since in1814 can be complemented by Vmw110, investigation of the mechanism of complementation could yield information concerning the properties of Vmw110. Identification of the genes activated by Vmw110 during stimulation of reactivation might suggest mechanisms for the prevention of reactivation of HSV.

Since the levels of IE gene expression might be crucial in the lysis/latency decision of HSV in vitro and in vivo, an investigation of the levels of IE transcripts or IE gene products in cells latently infected with HSV would be appropriate. The production of LAT may be a criterion for the classification of wt HSV as latent in vitro. The expression of the LAT gene and the possible role of LAT in establishment and maintenance of latency in vitro, should, therefore, be analysed; the results of Harris et al. (1989) indicate that LAT gene expression is not essential in the reactivation event. There appear to be differences in viral gene expression between high multiplicity and low multiplicity HSV infections at 42°C; analysis of viral gene expression in cells infected at low m.o.i. might be more relevant because conversion of HSV to a latent state in the in vitro latency system described apparently relies upon low m.o.i.. IE gene expression and the production of LAT in latently infected cells could be analysed by Northern blot

or *in situ* hybridisation or, by the DNA polymerase chain reaction method.

Confirmation of the state of the latent HSV genome in the *in vitro* latency system described is essential because, like LAT production, the presence of HSV DNA in an endless configuration could be considered as a criterion for a virus-cell interaction *in vitro* equivalent to that of the HSV latent interaction *in vivo*. Since HSV DNA is not detectable in cells during the 37°C period following incubation at 42°C for 6 days, the amount of HSV genomes per cell must be increased in order to determine the state of the latent HSV genome; perhaps this could be achieved, without affecting the levels of spontaneous reactivation, by utilising mutant viruses for the initial infection. Furthermore, investigation of the association of the latent HSV genomes with chromatin, by micrococcal nuclease digestion, and analysis of regions of DNase hypersensitivity and of CpG methylation, by digestion with *Msp*I and *Hpa*II restriction enzymes, might provide information regarding the transcriptional status of the latent HSV genome *in vitro*.

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