Human cytomegalovirus origin-dependent DNA synthesis

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SUMMARY

Human cytomegalovirus (HCMV) origin-dependent DNA synthesis has not been as well studied as herpes simplex virus type 1 (HSV-1) DNA synthesis, partly due to the difficulty of manipulating HCMV permissive cell lines in tissue culture. Seven virus encoded proteins are required for HSV-1 origin-dependent DNA synthesis. Of the seven essential proteins, the six replication fork proteins, comprising the helicase/primase (UL5/UL8/UL52), the DNA polymerase (UL30/UL42) and a single stranded DNA binding protein (UL29), are conserved throughout the *Herpesviridae*. The seventh protein required for HSV-1 origin-dependent DNA synthesis is the origin-binding protein, UL9, which is conserved among the alphaherpesviruses and the roseolovirus genus. HCMV origin-dependent DNA synthesis requires the six conserved replication fork proteins and five other HCMV encoded proteins. HCMV does not encode an obvious counterpart to UL9.

In this thesis, transient replication assays were used to further investigate the initiation of HCMV origin-dependent DNA synthesis. In order to simplify the transient transfection assay, alternatives to human fibroblast cell lines were tested for their ability to support HCMV DNA synthesis.

A transfected HCMV origin-containing plasmid was replicated by HCMV strain AD169 infection in Vero cells which did not apparently support the replication of the infecting genomes. The replication of independent origin-containing plasmids, and the failure of control plasmids to be replicated, confirmed that replication was specific to the HCMV origin sequences and independent of the vector used. Replication of an unmethylated origin-containing plasmid confirmed that the bacterial methylation patterns on the plasmid DNA were not responsible for its aberrant replication. Inhibitors were used to demonstrate

that DNA synthesis was directed by the viral DNA polymerase. An assay exploiting the ability of simian virus 40 (SV40) T-antigen to recruit cellular DNA polymerases to direct SV40 origin-dependent DNA synthesis was used to confirm that the concentrations of inhibitors used did not abrogate the activity of the cellular (nuclear) replicative polymerases.

In contrast to previously published data, genomic DNA synthesis was observed in HCMV strain AD169 infected 293 cells and in HCMV strain Towne infected 293 and Vero cells. Therefore, the ability of HCMV to replicate in 293 and Vero cells was examined by virus growth assay and electron microscopic examination of infected cells. Neither Vero cells infected with HCMV strain AD169 or Towne, nor 293 cells infected with HCMV strain AD169, supported the production of HCMV progeny as measured by plaque formation or by electron microscopy (E.M.). HCMV particles were observed by E.M. at 140 h p.i. in 10% of 293 cells infected with HCMV strain Towne.

An HCMV origin-containing plasmid was replicated by the HSV-1 replication fork proteins in the presence of the HCMV auxiliary proteins (TRS1, UL36-38, UL84, UL112-113 and UL122-123). Replication was dependent on intact origin sequences and was directed by the HSV-1 DNA polymerase. The HCMV major immediate early proteins (IE1/2) were sufficient to mediate replication of an HCMV origin-containing plasmid by the HSV-1 replication fork proteins, addition of UL36-38 significantly increased the efficiency of replication. Stop mutants in pSVH (expressing the major immediate early proteins) and pZP8 (expressing UL36-38) and a deletion mutant in pZP8 were used to demonstrate that IE2-86 and UL38 likely provided functions important for origin-dependent DNA synthesis. UL84, which specifies a putative origin-specific function (Sarisky & Hayward, 1996), was not required for HCMV origin-containing plasmid replication in this assay.

Simian cytomegalovirus (SCMV) strain Colburn is more closely related to HCMV than HCMV is to HSV-1, therefore, the ability of SCMV superinfection to replicate an HCMV origin-containing plasmid was examined. SCMV infection directed the replication of an HCMV origin-containing plasmid in the absence of any HCMV proteins, addition of plasmids expressing UL84 did not increase the replication efficiency. The model system of replication of a varicella-zoster virus (VZV) origin-containing plasmid by HSV-1 superinfection was used to demonstrate that the addition of a cognate origin-binding protein to such "cross-complementation" assays increased replication efficiency. In an experiment in which the level replication of an HCMV origin-containing plasmid by SCMV strain Colburn was below the limits of detection, addition of HCMV IE1/IE2 and UL36-38 increased the efficiency of replication to a detectable level.

The results of experiments using HSV-1 plasmids or SCMV virus to provide replication fork proteins to replicate an HCMV origin containing plasmid indicate that proteins provided by the IE1/IE2 locus and the UL36-38 locus, but not UL84, perform important origin specific roles in these assays.

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Unless otherwise stated, the data presented in this thesis are the results of my own efforts.

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ABBREVIATIONS

aa	amino acid(s)
AP	assembly proteins
BSA	bovine serum albumin
°C	degrees Centigrade
CAV	cell associated virus
Ci	Curie
CLB	cell lysis buffer
CRV	cell released virus
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytidine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dNTP	2'-deoxynucleoside-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DEAE	diethylamino-ethyl
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double stranded
DTT	dithiothreitol
EBNA-1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetra-acetic acid (disodium salt)
EGTA	ethylene glycol-bis(β-amino-ethylether)N,N,N',N' tetra acetic acid
E.M.	electron microscopy
FCS	foetal calf serum
gB or gH	glycoprotein B or glycoprotein H
h	hour(s)
HCMV	human cytomegalovirus
HeBS	hepes-buffered saline
hepes	N-[2-hydroxyethyl]piperazine-N'-[2-ethane sulphonic acid]
HHV-	human herpesvirus-
HIV	human immunodeficiency virus

h p.i.	hours post infection
h p.t.	hours post transfection
HF	human fibroblast(s)
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
IE	immediate early
IF	immunofluorescence
IgG	immunoglobulin G
kbp	kilobase pairs
l	litre(s)
lacZ	β-galactosidase
M	molar
MCMV	murine cytomegalovirus
mC-BP	minor capsid protein binding protein
mCP	minor capsid protein
MCP	major capsid protein
mcs	multiple cloning site
ml	millilitre(s)
mM	millimolar
mm	millimetre(s)
m.o.i.	multiplicity of infection
mRNA	messenger RNA
NBCS	newborn calf serum
NEB	New England BioLabs
ng	nanogram(s)
nm	nanometre(s)
NP40	Nonidet-P 40 detergent
nt	nucleotide(s)
NTB	nick translation buffer
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
p.f.u.	plaque forming units
PFA	phosphonoformic acid

PML	promyelocytic leukaemia
POD	PML oncogenic domain
RCMV	rat cytomegalovirus
RhCMV	rhesus cytomegalovirus
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RSB	reticulocyte standard buffer
SCMV	simian cytomegalovirus
SCP	smallest capsid protein
SDS	sodium dodecyl sulphate
SRT	smallest replicator transcript
ss	single stranded
SSC	standard saline citrate
STET	buffer containing SDS, Triton X-100, Tris-HCl and EDTA
SV40	simian virus 40
TAE	buffer containing Tris, EDTA, and acetic acid
TBE	buffer containing Tris, boric acid and EDTA
TE	buffer containing Tris-HCl and EDTA
TRITC	tetramethyl rhodamine isothiocyanate
Triton X-100	octyl phenoxy polyethoxyethanol
TS	Tris buffered saline
TSB	transformation and storage buffer
UV	ultra-violet
V	Volt(s)
vRNA	virus-associated RNA
v/v	volume per volume (ratio)
VZV	varicella-zoster virus
w/v	weight per volume (ratio)
X-gal	5-bromo-4-chloro-3-indoyl β-D-galactopyranoside
μCi	microCurie(s)
μg	microgram(s)
μl	microlitre(s)

Chapter 1: Introduction

This thesis is concerned with origin-dependent DNA synthesis in the herpesvirus, human cytomegalovirus (HCMV). I will first provide a general introduction to the *Herpesviridae*, noting the characteristics which are common to all members of the family and describing the human herpesviruses identified thus far. This will be followed by a brief overview of the lytic cycle of HCMV.

Much of our knowledge of HCMV DNA synthesis has come from our knowledge of herpes simplex virus 1 (HSV-1), so DNA synthesis in HSV-1 will be reviewed, followed by a description of DNA synthesis in Epstein-Barr virus (EBV) and HCMV. In part, studies into HCMV replication have been hampered by its restricted growth in tissue culture and therefore I will briefly review HCMV replication in cultured cells.

1.1 Characteristics of the *Herpesviridae*

The general characteristics of the *Herpesviridae* have been reviewed in the recent report of the International Committee on the Taxonomy of Viruses (ICTV) (Minson *et al.*, 2000), and by Davison and Clements (1998). The *Herpesviridae* are a large family of enveloped viruses with double-stranded DNA genomes packaged within an icosahedral capsid. The *Herpesviridae* encode most of the proteins required to direct origin-dependent viral genomic DNA synthesis in the nuclei of infected cells. Viral DNA is packaged into a preformed proteinaceous capsid which is surrounded in turn by a tegument layer and a lipid envelope containing viral glycoproteins. Release of virus from infected cells results in cell death. The *Herpesviridae* are also characterised by their ability to establish latent infections following a primary infection.

Classification

There are three subfamilies within the *Herpesviridae*: the *Alpha*, *Beta*, and *Gammaherpesvirinae*. Classification into a subfamily of the *Herpesviridae* was originally

on the basis of the biological properties of the virus. However, as more sequence data is produced, classification will ultimately be on the basis of DNA sequence. The classification of the *Herpesviridae* has recently been presented in the ICTV report (Minson *et al.*, 2000).

The *Alphaherpesvirinae* tend to be fast growing viruses able to infect a wide range of cell types in tissue culture and to establish latent infections primarily in sensory ganglia. *Betaherpesvirinae* are generally the slowest growing and most host restricted of the *Herpesviridae* in tissue culture, infection is usually associated with the formation of large cells with intranuclear inclusions. *Betaherpesvirinae* are maintained in latent form in spleen cells, monocytes and possibly other cell types. *Gammaherpesvirinae* are slightly less host restricted *in vitro* and are able to establish latent infections in lymphoid cells. Subfamilies of the *Herpesviridae* may be subdivided into genera; *Cytomegalovirus*, *Muromegalovirus* and *Roseolovirus* are the three genera of the *Betaherpesvirinae*.

Human herpesviruses (HHVs)

Eight herpesviruses of man have been isolated to date, including members of all three subfamilies, reviewed in *Fields Virology* by Roizman and Sears (1996), Mocarski (1996), Kieff (1996), Cohen and Straus (1996), Pellett and Black (1996) and Frenkel and Roffman (1996).

HSV-1 (HHV-1) is an alphaherpesvirus of the *Simplexvirus* genus. HSV-1 commonly causes cold sores and may rarely be responsible for encephalitis. Following primary infection, HSV-1 can establish a latent infection in sensory ganglia from which the virus can reactivate. Herpes simplex virus 2 (HSV-2 or HHV-2) is a *Simplexvirus*, closely related to HSV-1, which is also able to establish a latent infection in sensory ganglia, but which manifests a productive infection in the form of genital lesions. The third human alphaherpesvirus is varicella-zoster virus (VZV or HHV-3) which is the prototype *Varicellovirus*. Primary infection with VZV causes chicken pox, following which the virus

maintains a latent infection in sensory neurones. Reactivation from latency can cause a more severe illness known as shingles.

Three members of the *Betaherpesvirinae* are human herpesviruses. HCMV (HHV-5) is the most intensively studied member of the *Cytomegalovirus* genus of the *Betaherpesvirinae*. HCMV infection is usually asymptomatic in immune-competent individuals but can cause severe neurological damage in congenitally infected infants. HCMV infection in immuno-suppressed individuals, such as individuals infected with human immunodeficiency virus (HIV) and transplant recipients, can cause HCMV retinitis and other complications. HCMV infection is probably responsible for nearly 10% of cases of infectious mononucleosis with possible complications of this including pneumonia, hepatitis and aseptic meningitis. Human herpesvirus type 6 (HHV-6) is a *Roseolovirus* causing *exanthem subitum* in children. HHV-6 has also been associated with multiple sclerosis. Human herpesvirus type 7 (HHV-7) is the second member of the *Roseolovirus* genus. Similarly to HCMV, both HHV-6 and HHV-7 may reactivate in patients following organ transplantation.

Two gammaherpesviruses of humans have been isolated. EBV (HHV-4) is a *Lymphocryptovirus* which establishes a latent infection in B-lymphocytes. EBV is the common cause of infectious mononucleosis, and is associated with Burkitts lymphoma, Hodgkins disease and nasopharyngeal carcinoma. Human herpesvirus type 8 (HHV-8 or Kapsosi's sarcoma associated herpesvirus) is a *Rhadinovirus* which may establish latency in T or B lymphocytes. HHV-8 is associated with Kaposi's sarcoma, normally manifesting as skin lesions but which is a severe complication in individuals infected with HIV. HHV-8 infection may be associated with the progression of HIV infection to AIDS.

Genome arrangement

The genomes of the *Herpesviridae* are linear double-stranded DNA molecules ranging in size from 125 to over 240 kbp, containing from 70 to over 200 open reading

frames. Herpesvirus genomes contain various arrangements of unique sequences and repeated elements, giving rise to seven distinct genome types which are represented schematically in figure 1.1 and have been described by Roizman (1996) and by McGeoch and Davison, (1995). There is no correlation between the type of genome arrangement and the subfamily to which a virus belongs. HCMV has a 230 to 240 kbp genome which, like HSV-1 and HSV-2, falls into a group 6 arrangement. Group 6 genomes have two stretches of unique sequences (U_L and U_S), each flanked by inverted repeats (IRL and TRL flanking U_L, and IRS and TRS flanking U_S). The long (TRL + U_L + IRL) and short (IRS + U_S + TRS) segments of group 6 genomes are able to invert relative to each other so that an HCMV genome can exist in one of four possible isomeric forms. An additional repeated element, the 'a'-sequence, is found at the genomic termini and the junction between the long and short segments.

Gene expression

Gene expression in the *Herpesviridae* is strictly controlled. The three temporal classes of genes expressed during infection are immediate early (α), early (β1 and β2), and late (γ1 and γ2). Genes are transcribed in the infected cell nucleus by cellular RNA polymerase II.

Particle structure

Herpesvirus particles consist of a core composed of the viral genome packaged in a liquid-crystalline array within a twenty faced (icosahedral) capsid assembled from virus encoded proteins. The capsid is surrounded by an amorphous tegument layer, also composed of virus proteins. Tegumented virus is enclosed in a host-cell derived lipid envelope containing virus glycoproteins. A cryo-electron micrograph showing an HCMV particle is shown with a schematic representation of the particle (figure 1.2).

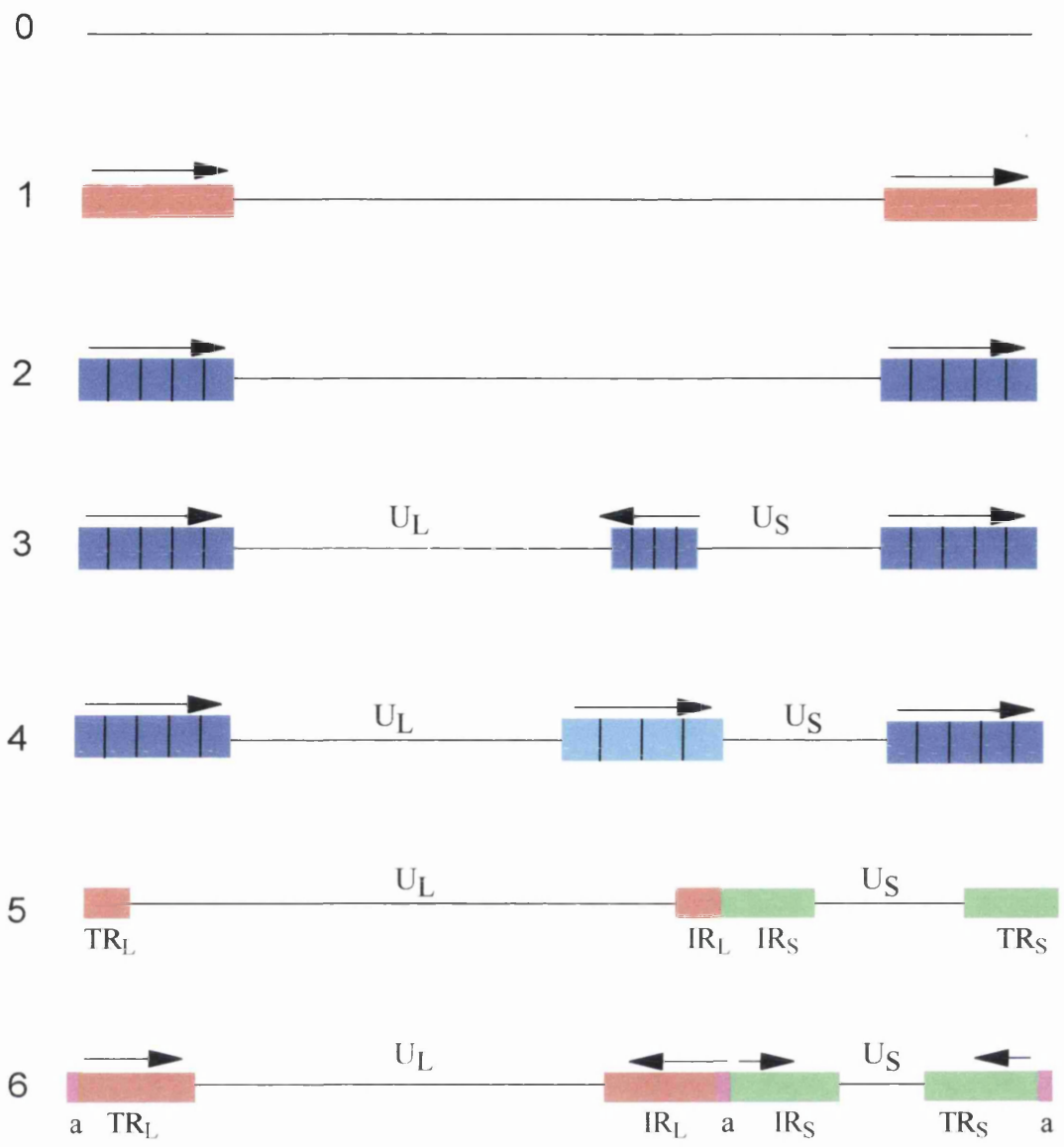


Figure 1.1 Genome arrangements in the *Herpesviridae*, (Davison & McGeoch, 1995; Roizman, 1996).

Group 0 genomes have a single unique region, e.g. tree-shrew herpesvirus.

Group 1 genomes have a single unique region flanked by a direct, terminal repeat, e.g. channel catfish herpesvirus, HHV6 and HHV7.

Group 2 genomes have a single unique region flanked by a group of direct terminal repeats, e.g. herpesvirus saimiri.

Group 3 genomes have two unique regions separated by and flanked by a group of direct repeats, e.g. cottontail rabbit herpesvirus.

Group 4 genomes have two unique regions separated by a group of internal repeats. At each end of the genome is a group of direct terminal repeats unrelated to the internal repeats, e.g. Epstein-Barr virus.

Group 5 genomes have two unique regions, each unique region is flanked by an inverted repeat. The repeat regions flanking the long and short unique regions (U_S and U_L) are not related. The repeats flanking U_L are quite short, and only the S segment has a high frequency of inversion. A virus with this genome arrangement is varicella-zoster virus.

Group 6 genomes have two unique regions, each unique region is flanked by an inverted repeat. The repeat regions, I/TR_L and I/TR_S flanking U_L and U_S respectively, are not related. An additional repeat, the 'a' sequence, is found at the genomic termini and at the junction between the internal repeats (IR_L and IR_S). Both the L and S segments show high frequency inversion. Herpes simplex virus type 1 and type 2 as well as human cytomegalovirus have this type of genome arrangement.

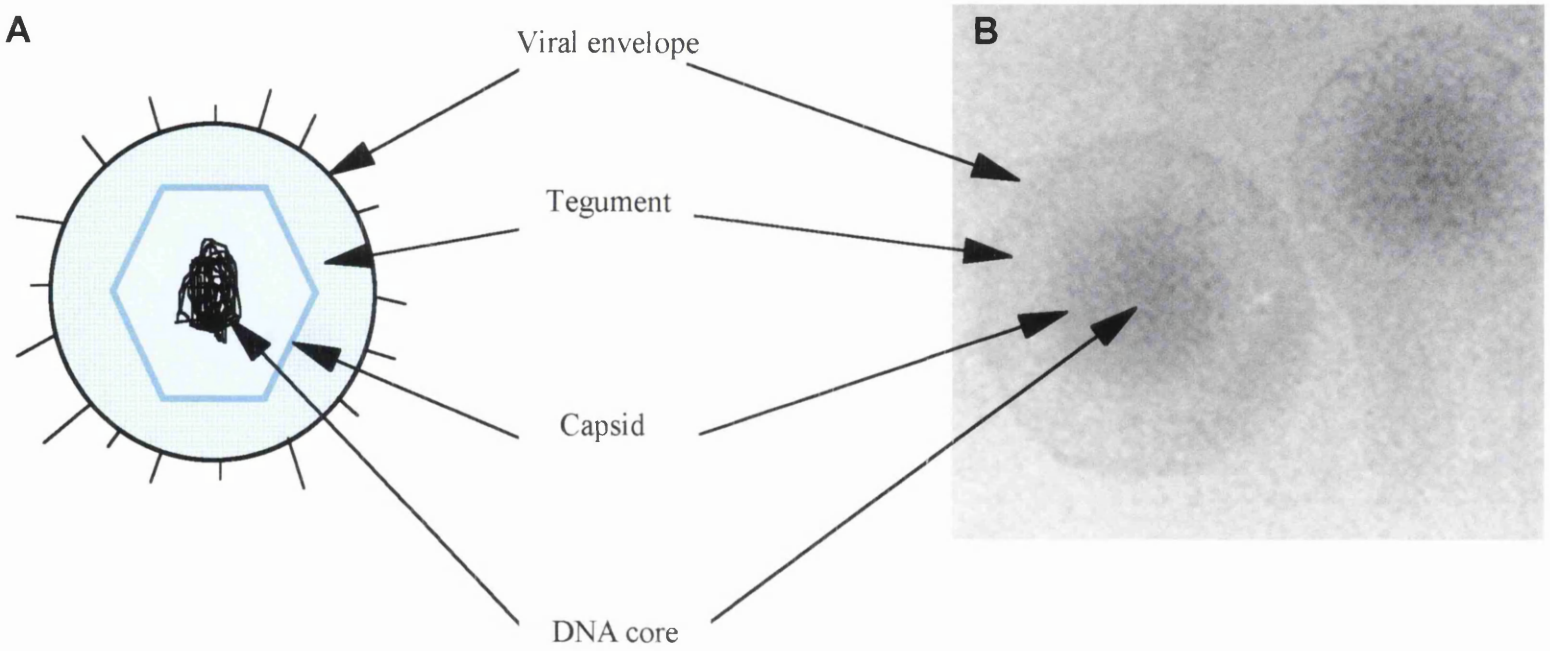


Figure 1.2 Particle structure of the *Herpesviridae*.

Panel A shows a schematic representation of a herpesvirus particle showing the DNA core, the proteinaceous capsid and tegument enclosed within the viral envelope containing viral glycoproteins. The HCMV capsid is approximately 125 nm in diameter.

Panel B shows a cryo-eletron micrograph of two HCMV particles. The positions of the components of the virus particle are shown. This image was provided by D Bhella.

1.2 The lytic cycle of HCMV

Herpesvirus replication has been reviewed by Mocarski, (1993), Roizman and Sears (1993), Liebowitz and Kieff (1993) and Davison and Clements (1998). The HCMV lytic cycle will be described here, and differences between HCMV replication and HSV-1 replication will be noted.

HCMV genome

The HCMV genome was represented in figure 1.1. HCMV genes have been named according to their position within the genome. The TRL region of the HCMV strain AD169 genome (GenBank accession number X17403) spans approximately 11.2 kbp containing fourteen loci. The genes in the 167 kbp U_L region are named UL1 to UL132. The IRS region is approximately 2.5 kbp long and contains one open reading frame. The US region contains 36 genes in 35.4 kbp of DNA. Usually the protein product of an open reading frame is named after the gene that encodes it, so the protein product of the UL84 gene is known as UL84. Several proteins are additionally named on the basis of their size or function, for example, the protein product of the UL83 gene is also known as pp65 or the lower matrix protein. The HCMV genome potentially encodes over 200 proteins and contains one copy of an origin of lytic phase DNA synthesis, oriLyt.

The published HCMV genome sequence is that of HCMV strain AD169 (Chee, 1989). It has since been shown that the genomes of the HCMV Towne laboratory strain and the HCMV Toledo clinical isolate differ from that of HCMV strain AD169. In fact, the AD169 genome possibly lacks open reading frames found in the Towne genome, and both laboratory strains have lost open reading frames (up to nineteen) found in more recent clinical isolates (Cha *et al.*, 1996). Another indication of the heterogeneity of HCMV is the finding that the virus genome used to produce the AD169 genomic sequence lacks DNA (approximately 1 kbp) found in most stocks of HCMV strain AD169 (Dargan *et al.*, 1997).

Attachment

The first events in the HCMV lytic cycle *in vitro* involve binding of the virus to the target cell, and its penetration. As has been demonstrated for other herpesviruses, initial interactions of HCMV with the cell surface may involve interactions with heparin, probably mediated by viral glycoprotein B (Compton *et al.*, 1993).

Virus adsorption also involves the binding of a viral glycoprotein to a specific cell surface receptor(s). HCMV has been shown to bind to some cellular membrane proteins of 30 to 34 kDa, and the presence of these proteins in cell membranes has been shown to be indicative of the ability of HCMV to be internalised (Nowlin *et al.*, 1991). Unlike HSV-1, for which cellular receptors have been identified (reviewed by Campadelli-Fiume et al., 2000), the identity of the HCMV cellular receptor(s) remains unknown. HCMV replication in tissue culture is quite cell type restricted, and the lack of a suitable cell surface receptor is one point at which the virus life cycle may be blocked.

Internalisation and uncoating

Following attachment, HCMV is internalised by membrane fusion. Binding of glycoprotein H (gH), in complex with glycoprotein L, to a 92.5 kDa phosphorylated cellular glycoprotein may be involved in the fusion mechanism (Keay *et al.*, 1989). HSV-1 penetration into a target cell involves gH and other virus glycoproteins (reviewed by Rajcani & Vojvodova, 1998). Attachment of HCMV to the cell surface has been shown to induce transcription of several cellular mRNAs and to increase detectable NF-κB binding activity (Boldogh *et al.*, 1991; Yurochko *et al.*, 1995). This latter response can be stimulated on addition of purified HCMV gB to cells (Yurochko et al., 1997).

After internalisation, capsids migrate to the nucleus by an unknown mechanism. DNA is released into the nucleus, and the genome circularises and is replicated (McVoy & Adler, 1994). HCMV genomes, like those of other herpesviruses, localise adjacent to pre-existing nuclear structures known as promyelocytic leukaemia (PML) oncogenic

domains (PODs), which are thought to be the site of viral gene expression (Ishov & Maul, 1996). Some tegument proteins migrate to the nucleus with the capsid, and the upper matrix protein, pp71, can increase expression from promoters containing ATF and AP-1 sites, including the HCMV major immediate early promoter (Liu & Stinski, 1992).

Temporal pattern of HCMV gene expression

HCMV mRNAs are transcribed in the nucleus by the cellular transcription machinery, then translated in the cytoplasm, also using the host-cell machinery. Gene expression in the herpesviruses is strictly controlled in three temporal classes; immediate early, early and late (Roizman, 1996; Mocarski, 1996).

The herpesvirus immediate early gene products are the first to be synthesised in infected cells, and most of these are involved in the expression of subsequent classes of genes. HCMV immediate early gene expression is mainly confined to four loci; IE1/IE2 (UL122-123), IRS1/TRS1, UL36-38, and US3 (reviewed by Fortunato & Spector, 1999). At least one product of the US3 locus is thought to play a role in immune evasion by sequestration of some MHC class I molecules in the endoplasmic reticulum of the infected cell (Jones *et al.*, 1996; Ahn *et al.*, 1996). US3 is non-essential for virus growth in tissue culture (Jones & Muzithras, 1992). IE1 and IE2 together are able to activate expression of some early genes, while expression from other early promoters may require cooperation with other immediate early gene products (Stenberg *et al.*, 1990; Pizzorno *et al.*, 1998). Expression of the HCMV major immediate early proteins (IE1/IE2), as well as expression of some HCMV early genes, has been shown to be enhanced by the HCMV tegument proteins pp71 and UL69 (homologue of the HSV-1 IE63 post-transcriptional activator) (Liu & Stinski, 1992; Winkler *et al.*, 1994).

Following immediate early protein synthesis, the early genes are expressed. Early genes are mainly involved, directly or indirectly, in DNA metabolism and in the modulation of host cell metabolism. Efficient expression of the HCMV encoded proteins required for

origin-dependent DNA replication requires the immediate early products of IE1/IE2, UL36-38, IRS1/TRS1 and at least one of the early gene products of the UL112-113 locus (Iskenderian *et al.*, 1996).

Finally, expression of late genes is either greatly stimulated by, or dependent upon, viral DNA synthesis. Most late proteins are structural or have some role in particle assembly or DNA packaging.

DNA replication

HCMV DNA replication is described in more detail in section 1.4. Briefly, HCMV DNA replication, as in other herpesviruses, is thought to be initiated at a specific region of the genome, the origin, known in HCMV as oriLyt. The virus replication fork proteins are recruited, and semi-conservative DNA synthesis is postulated to proceed via an initial amplification of a circular template by theta replication, followed by rolling circle replication to produce concatemeric products. HCMV DNA synthesis occurs in distinct virus replication compartments which are derived from cellular PODs.

Capsid assembly

Less is known about capsid assembly and DNA packaging in HCMV than in HSV-1, and much of our knowledge of the processes in HCMV is inferred from our knowledge of HSV-1 (Gibson, 1996). The HCMV structural proteins, MCP (major capsid protein), mCP (minor capsid protein), mC-BP (minor capsid protein binding protein), AP (uncleaved assembly proteins) and SCP (smallest capsid protein), localise to the nucleus. The HCMV structural proteins are homologous to the HSV-1 proteins VP5, VP23, VP19c, VP22a and VP26 respectively. MCP interacts with AP in hexamers and pentamers. mCP interacts with mC-BP in a 2:1 ratio to form structures resembling HSV-1 triplexes.

MCP and the assembly proteins are organised into pentamers and hexamers which may be linked through interactions with the triplexes in precursor capsids. The precursor capsid (preB-capsid) contains the MCP, mCP, mC-BP and AP. The preB-capsid matures

into a virion following self-cleavage of the assembly proteins, DNA packaging, and exit of the cleavage products of the assembly proteins. The smallest capsid protein (SCP) is probably added to the virus particle following DNA packaging.

DNA packaging

The mechanism of DNA packaging has not been elucidated. DNA packaging requires enzymes to specifically cleave one complete genome from replicated, concatemeric DNA, and to pump that DNA into a capsid. Seven HSV-1 encoded proteins involved in DNA packaging have been identified, these are UL6, UL15, UL25, UL28, UL32, UL33, and UL17 (reviewed in Homa & Brown, 1997). The HSV-1 proteins have been more intensively studied than the HCMV proteins. Viral mutants in UL15, UL28 and UL33 have been shown to be defective in DNA packaging (Poon & Roizman, 1993; Addison *et al.*, 1990; al-Kobaisi *et al.*, 1991). UL15 and UL28 have been shown to interact, possibly to form a complex similar to the bacterial T4 terminase (Abbotts *et al.*, 2000). UL25 has been shown to be non-essential for the cleavage of concatemeric DNA, but to be required for packaging (McNab *et al.*, 1998). The remaining packaging proteins, including UL25, may be involved in the formation of a 'portal' on the capsid, facilitating the association of DNA with the capsid and the retention of packaged DNA within the particle.

Homologues of the HSV-1 proteins involved in DNA packaging have been identified in HCMV, these are UL104, UL89, UL77, UL56, UL52, UL51, and UL93 respectively (Chee *et al.*, 1990). HCMV UL89 has some sequence homology with the bacteriophage T4 terminase protein and can bind to HCMV packaging signals (Underwood *et al.*, 1998; Wang & McVoy, 1999). UL56 has been shown to be a DNA binding protein specific for the HCMV packaging signals, which shows ATP-independent nuclease activity (Bogner *et al.*, 1998). Furthermore, benzimidazole ribonucleotides, which inhibit packaging of viral DNA, act on UL56 and UL89. In HCMV infected cells treated with benzimidazole ribonucleotides, concatemeric DNA is not cleaved into individual genomes,

supporting the proposition that UL56 and UL89 are most likely involved in resolving concatemeric DNA into genome lengths (Krosky *et al.*, 1998; Underwood *et al.*, 1998). HCMV UL56 has also been shown to localise, in a phosphonoacetic acid sensitive manner, to viral replication compartments and to interact with the viral DNA polymerase (UL44), thus "implicating that DNA replication is coupled with packaging" (Giesen et al., 2000).

The electrostatic repulsions between closely packed DNA strands may be partially neutralised by the polyamines which are also packaged into HCMV capsids (Gibson *et al.*, 1984), and the DNA may adopt a partially liquid crystalline structure (Bhella *et al.*, 2000). By analogy with HSV-1, capsid assembly may be completed when the smallest capsid protein (SCP) decorates the outside of mature capsids. SCP may interact with the tegument.

Virus maturation and egress

The tegument is an amorphous protein layer associated with the viral capsid. The HCMV tegument is predominantly composed of pp65, pp71, UL32, UL47, and UL48. It has been proposed (Gibson, 1996) that tegument may be added to HCMV particles at a distended region of the nuclear membrane known in HHV-6 infected cells as the 'tegusome' (Roffman *et al.*, 1990). A more recent report suggests that tegumentation of HCMV particles may occur, at least in part, in a cytoplasmic compartment (Sanchez *et al.*, 2000). HCMV virions have been shown to contain phospholipids, and various non-structural proteins including a protein kinase, phosphatases and a DNA polymerase (reviewed in Gibson, 1996). Recently, specific viral RNA molecules have been reported to be packaged within HCMV particles, though the relevance of this is unclear (Bresnahan & Shenk, 2000).

HCMV particles acquire an envelope when crossing the inner nuclear membrane which may be lost on crossing the outer nuclear membrane (reviewed by Mocarski, 1996). Particles are probably re-enveloped following passage through the golgi apparatus, and

virus likely exits the cell via exocytosis. The envelope contains most of the virus encoded glycoproteins and possibly some host-cell membrane proteins (reviewed by Gibson, 1996; Britt & Mach, 1996). In addition to infectious virus particles, HCMV infected cells also produce two major types of non-infectious particles, these are dense bodies and non-infectious enveloped particles (NIEPS) (Irmiere & Gibson, 1983). Dense bodies are composed mainly of pp65 surrounded by an envelope containing viral glycoproteins. NIEPS are less abundant than dense bodies and contain capsids lacking the densely staining DNA core.

1.3 Herpesvirus DNA replication

1.3.1 HSV-1 lytic-phase DNA replication

Lytic-phase DNA replication has been much more extensively studied in HSV-1 than in any other herpesvirus, and investigations into DNA replication in the other herpesviruses have, to some extent, been directed by our knowledge of HSV-1 (reviewed in Boehmer & Lehman 1997).

HSV-1 origins

Early electron micrographs of replicating HSV-1 DNA provided evidence that HSV-1 DNA synthesis begins at defined regions within the genome. The locations of three origins of lytic-phase DNA synthesis were later identified by transient replication assays (Stow, 1982; Weller *et al.*, 1985). The transient replication assays examined the replication of transfected plasmids containing fragments of HSV-1 DNA following superinfection with HSV-1. Total cellular DNA was digested with a restriction enzyme to linearise the origin-containing plasmid, and with *Dpn*I to degrade input plasmid DNA. The presence of an HSV-1 origin of replication within the insert allowed the amplification of the plasmid, and the generation of *Dpn*I resistant molecules which were detected by Southern blotting.

The HSV-1 genome has three sequence blocks which contain all the *cis*-acting signals required to serve as origins for DNA replication; one origin, oriL, is represented in a single copy near the centre of the long unique region of the genome; the other origin, oriS, is in the short repeats of the genome and is thus present twice in inverted orientation (Weller *et al.*, 1985; Stow, 1982). The HSV-1 origins consist of an essential core region flanked by sequences which augment origin activity and contain transcription factor binding sites (Stow & McMonagle, 1983; Wong & Schaffer, 1991). The core regions of oriL and oriS are closely related, although there are some size and sequence differences. The locations and compositions of the HSV-1 lytic origins are shown in figure 1.3. Neither oriS nor oriL is absolutely essential for virus growth in tissue culture (Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993). Because of a tendency for plasmids containing oriL to delete in bacteria, most studies have been performed on oriS, and it is oriS that will be described in more detail here.

HSV-1 oriS has a core region of approximately 80 bp containing an imperfect palindrome of 45 bp (Stow & McMonagle, 1983; Lockshon & Galloway, 1988). The palindrome is composed of an 18 bp A/T region flanked by inverted repeats (imperfect) which are binding sites for the UL9 protein (Elias & Lehman, 1988; Weir *et al.*, 1989). The UL9 binding sites flanking the A/T palindrome are termed 'box I' and 'box II'. Box I (CGTTCGCACT) is a very high affinity binding site for UL9 and box II (TGCTCGCACT) is a high affinity site; UL9 has approximately five times greater affinity for box I than box II. Close to box I is another UL9 binding site, box III; UL9 has 1000 times lower affinity for box III than box I. oriL is more symmetrical than oriS- the central region of oriL is a perfect palindrome of 145 bp with the 20 bp A/T region flanked on both sides by a copy of box I. While these differences might possibly allow the two types of origin to function in the initiation of different stages of lytic-phase DNA replication, it is not known whether the two origins actually differ in their activity.

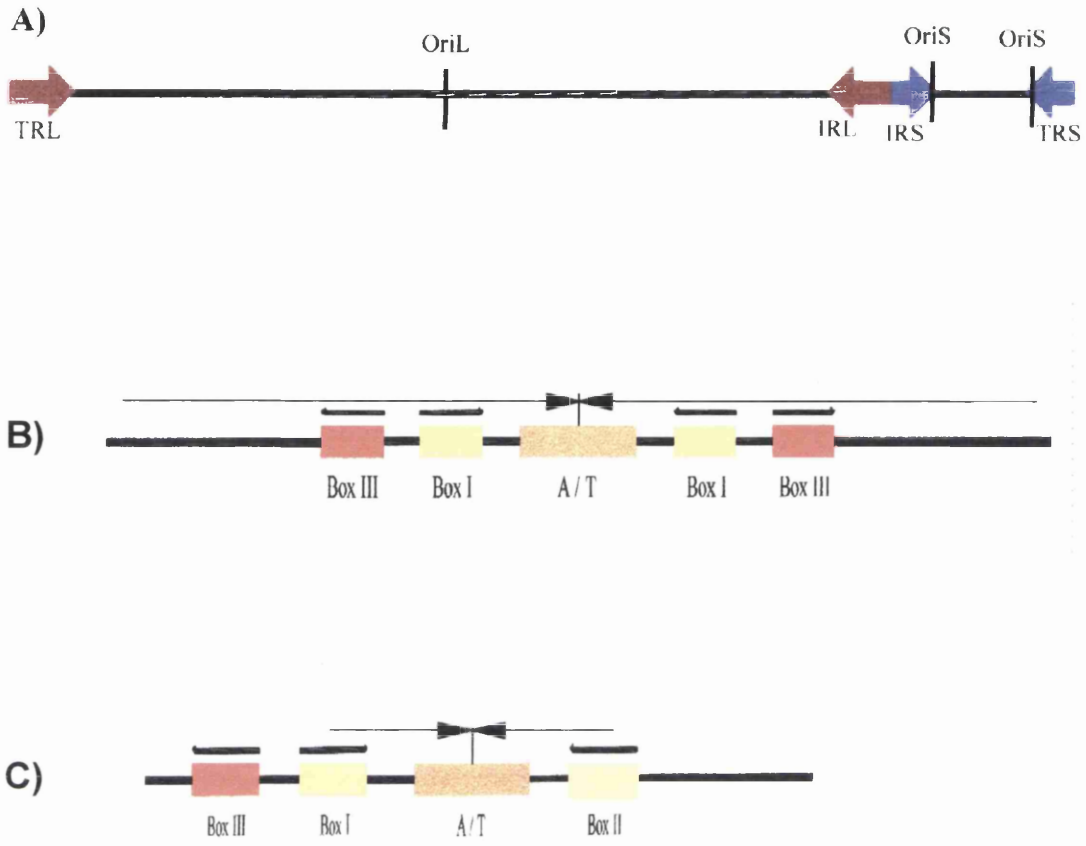


Figure 1.3 HSV-1 origin structures.

A) The location of the HSV-1 lytic origins of DNA replication (oriL and oriS) within the HSV-1 genome.

B) The core of oriL is a 144 bp perfect palindrome. The main features of the origin are the 20 bp A/T sequence and boxes I and III.

C) The core of oriS contains a 45 bp imperfect palindrome. The 18 bp A/T sequence is flanked by one copy and box I and one copy of box II.

The palindromic sequences are denoted by arrows (——►).

DNA replication proteins

Seven virus proteins were identified as having direct, essential roles in HSV-1 origin-dependent genomic DNA replication (Challberg, 1986; Wu *et al.*, 1988; Heilbronn & zur Hausen, 1989). Transient replication assays were used to identify UL5, UL8, UL9, UL29, UL30, UL42 and UL52 as having essential functions in HSV-1 origin-dependent DNA synthesis. Initially, the transient replication assays involved the cotransfection of cosmids containing fragments of HSV-1 DNA with a plasmid containing an HSV-1 origin. This assay identified six fragments of HSV-1 DNA which encoded functions required for HSV-1 origin-dependent DNA synthesis (Challberg, 1986). Systematic subcloning and mutagenesis allowed the identification of ten open reading frames, including those for three known immediate early activators of gene expression, which were required for optimal activity. None of the three transactivators was absolutely essential, thus it was hypothesised that only the seven early genes had direct and essential functions in HSV-1 DNA synthesis (Wu *et al.*, 1988). This was confirmed using temperature-sensitive viral mutants (reviewed by Weller, 1991 and Boehmer & Lehman, 1997) and in transient replication assays in which the proteins were expressed from plasmids with constitutive promoters and downstream regulatory regions (Heilbronn & zur Hausen, 1989).

UL9 is the HSV-1 origin-binding protein; it is essential for DNA replication and is presumed to have an obligatory role in initiation (described later). The remaining six proteins, often referred to as the replication fork proteins, are likely to function both in initiation and in elongation. The replication fork proteins comprise the DNA polymerase holoenzyme (UL30 and UL42), the single-stranded DNA binding protein (UL29) and the helicase/primase (UL5, UL8 and UL52). These six proteins are conserved throughout the *Herpesviridae* (table 1.1). In contrast to the replication fork proteins, homologues of the origin-binding protein, UL9, are only found in the *Alphaherpesvirinae* and the *Roseolovirus* genus of the *Betaherpesvirinae*.

	HSV-1	EBV	HCMV
Helicase	UL5	BBLF4	UL105
Helicase / primase accessory factor	UL8	BBLF2 / 3	UL102
Single-stranded DNA binding protein	UL29	BALF2	UL57
DNA polymerase	UL30	BALF5	UL54
DNA polymerase accessory factor	UL42	BMRF1	UL44
Primase	UL52	BSLF1	UL70

Table 1.1 Six replication fork proteins conserved throughout the *Herpesviridae*. Transient replication assays were used to determine the virus encoded proteins required for DNA synthesis in HSV-1, EBV and HCMV (Wu et al., 1988; Heilbronn & zur Hausen, 1989; Fixman et al., 1992; Pari & Anders, 1993). In addition to the six replication fork proteins conserved throughout the herpesviruses, one or more other virus encoded proteins are required for virus origin dependent DNA synthesis.

The HSV-1 DNA polymerase consists of two subunits which can be co-purified (Gallo *et al.*, 1989). UL30 is the catalytic subunit of the HSV-1 DNA polymerase and has some activity in the absence of the accessory protein (Marcy *et al.*, 1990). As well as its DNA polymerase activity, UL30 has a proof-reading 3'-5' exonuclease and RNase H activity (Crute & Lehman, 1989). The polymerase accessory protein, UL42, is a double-stranded DNA binding protein which increases the processivity of the polymerase (Gottlieb *et al.*, 1990; Digard *et al.*, 1993).

The helicase/primase is a heterotrimer composed of one copy of each of UL5, UL8, and UL52 (Crute & Lehman, 1991). Helicase/primase activity is specified by UL5 and UL52 (Calder & Stow, 1990; Dodson & Lehman, 1991). The third member of the helicase/primase complex is UL8, which interacts with and modulates the activity of UL5/UL52 (Crute *et al.*, 1989; Tenney *et al.*, 1994). UL8 also interacts with the DNA polymerase and the origin-binding protein (Marsden *et al.*, 1997; McLean *et al.*, 1994).

The origin-specific function in HSV-1 DNA replication is provided by UL9 (Elias & Lehman, 1988; Olivo *et al.*, 1988). UL9 is a sequence specific DNA binding protein with helicase activity, which exists in solution as a homodimer (Bruckner *et al.*, 1991). As well as an interaction with UL8, UL9 interacts with UL29 and UL42 (Boehmer & Lehman, 1993; Monahan *et al.*, 1998).

The single stranded DNA binding protein, UL29 (also known as ICP8), cooperatively binds to single stranded DNA (Boehmer & Lehman, 1993). UL29 probably interacts with the polymerase accessory protein, and is required for optimum polymerase activity (Vaughan et al., 1984; Ruyechan & Weir, 1984). It also stimulates the helicase/primase, and the helicase activity of the origin-binding protein (Hamatake *et al.*, 1997; Boehmer *et al.*, 1993).

The sites and significance of the interactions amongst the replication proteins, and with HSV-1 DNA, have been extensively investigated and the findings have been recently

reviewed, (Stow, 2000)

Host cell encoded functions

Some essential host cell enzymes are presumed to be required in herpesvirus DNA replication. For example, HSV-1 does not encode a topoisomerase and would therefore require a host-cell topoisomerase to remove supercoils from the replicating circular DNA. As HSV-1 does not encode a DNA ligase, the host cell enzyme must be used to ligate Okazaki fragments on the lagging strand. In a temperature-sensitive baby hamster kidney (BHK) cell line with a mutant in the regulator of chromosome condensation (Rcc1), HSV-1 failed to replicate and infecting genomes remained linear, implicating a requirement for circularisation prior to viral DNA replication, and an essential role for Rcc1 in HSV-1 infection in BHK cells (Umene & Nishimoto, 1996).

Non-essential enzymes involved in DNA metabolism

HSV-1 also encodes several proteins involved in nucleotide metabolism which are non-essential in tissue culture but may play more important roles during an *in vivo* infection (reviewed by Boehmer & Lehman, 1997). A ribonucleotide reductase, composed of UL39 and UL40 subunits, converts ribonucleotide diphosphates to the corresponding deoxyribonucleotide diphosphates. Thymidine kinase, a homodimer of UL23, is able to phosphorylate thymidine and deoxycytidine. Thymidine kinase also activates the antiviral drug acyclovir. A uracil N-glycosylase, encoded by UL2, is involved in DNA repair. The HSV-1 dUTPase, UL50, converts dUTP to dUMP. The thymidine kinase, uracil N-glycosylase and the dUTPase may all be required for efficient reactivation from latency (Coen *et al.*, 1989; Pyles & Thomsen, 1994; Pyles *et al.*, 1992). An alkaline exonuclease, UL12, is non-essential for DNA replication or late gene expression, but may be involved in resolving branched DNA structures to facilitate DNA packaging (Shao *et al.*, 1993).

Pathway of HSV-1 DNA synthesis

While the HSV-1 DNA replication origins and the proteins required for origin-dependent DNA synthesis have been well studied, there are still very large gaps in our basic understanding of the mechanisms of genomic DNA replication.

HSV-1 DNA is thought to be replicated in two phases by two different mechanisms (reviewed by Boehmer & Lehman, 1997). Theta form replication is proposed to be employed in a first phase of replication, involving the amplification of the circular template, because the initial accumulation of HSV-1 DNA proceeds more rapidly than would be predicted by single event rolling circle replication (Zhang *et al.*, 1994). Ultimately, the products of HSV-1 DNA replication are head-to-tail concatemers, suggesting that a second phase of DNA synthesis may be rolling circle replication.

Rolling circle replication has been reconstituted *in vitro* using a circular single-stranded DNA template partially annealed to a complementary strand with a free 5'-poly-dT tail (Falkenberg *et al.*, 2000). DNA synthesis required only the polymerase holoenzyme and the two catalytic subunits of the helicase/primase complex (UL5 and UL52). Replication products were double-stranded concatemeric DNA, and competition assays demonstrated that leading and lagging strand synthesis occurred concurrently. Clearly, the HSV-1 replicative machinery is capable of directing rolling circle DNA replication, but the use of such artificial templates cannot provide us with a model for initiation, or determine if this method of DNA replication actually occurs during HSV-1 infection.

Initiation of HSV-1 DNA replication

UL9 is likely to provide the origin-specific function to initiate the formation of a bi-directional replication fork, as required for theta replication. In the current model for the origin-specific initiation of HSV-1 DNA synthesis, it is believed that dimeric UL9 binds cooperatively to each of the two high affinity sites within the origin (boxI and boxI/boxII)

(Fierer & Challberg, 1992). The two bound dimers are, by virtue of the spacing between the binding sites, located on the same side of the DNA helix (Lockshon & Galloway, 1988), and the cooperativity of binding of UL9 to the two sites suggests that the bound dimers may interact with each other (Fierer & Challberg, 1995). The A/T tract between the bound UL9 dimers is distorted and looped out (Koff *et al.*, 1991). The distortion of the origin may expose single-stranded DNA which can be bound by UL29. Bound UL29 may interact with and stimulate the helicase activity of UL9 which can then unwind the DNA helix (Lee & Lehman, 1997), exposing more single-stranded DNA which can be coated with UL29.

Following unwinding of the origin by UL9, the DNA polymerase and the helicase/primase complex are probably recruited, possibly involving interactions of UL8 and UL42 with UL9 and/or between UL8 and UL30. Recruitment of the HSV-1 DNA polymerase to the replication fork may displace UL9 from the origin, allowing the helicase/primase to continue to unwind the origin and to lay down primers for DNA synthesis on the lagging strand. The interactions amongst the replication fork proteins presumably allow coordination of helicase and polymerase activity on the leading strand, and primase and polymerase activity on the leading and lagging strand. During theta replication, DNA synthesis would proceed in both directions around the circular HSV-1 genome. UL9 can interact with the cellular polymerase α-primase, and it has been proposed that instead of the viral helicase/primase, this cellular enzyme may be responsible for synthesising the primer for leading strand DNA synthesis (Lee *et al.*, 1995).

The mechanism for switching from theta to rolling circle replication is unknown, however, a late protein product of the UL9 gene, OBPC, may be involved. OBPC contains the C-terminal DNA binding domain of the full-length UL9 protein, but it lacks helicase activity and is unable to interact with the other HSV-1 replication proteins, thus OBPC can function as a potent inhibitor of origin-dependent HSV-1 DNA synthesis (Baradaran *et al.*, 1996). OBPC could thus enable a switch to rolling circle replication by preventing

re-initiation of theta replication.

Model systems for rolling circle replication (reviewed by Kornberg & Baker, 1992), are initiated at a free 3'-OH end on a DNA molecule, generated by cleavage, or 'nicking', of one strand of a circular DNA duplex. The mechanism for the initiation of rolling circle replication in HSV-1 is unknown, however, UL9 may not be required. UL9 has not been reported to have any DNA nicking activity, and a virus containing a temperature sensitive mutation in UL9 can replicate to wild-type levels if infected cells are shifted to the non-permissive temperature late in infection (Blumel & Matz, 1995).

Elongation in rolling circle synthesis is probably similar to that described for theta replication, except that a circular DNA strand acts as the template for leading strand synthesis, while the displaced concatemeric, single-stranded product of leading strand synthesis acts as the template for lagging strand synthesis.

1.3.2 Other herpesviruses encode sequence-specific origin-binding proteins

Other alphaherpesviruses, *e.g.*, VZV, pseudorabies virus, Marek's disease virus and equine herpesvirus 1, encode UL9-like origin-binding proteins which are essential for origin-dependent DNA replication, and which have distinct binding sites within their origins (Elias *et al.*, 1986; Martin & Deb, 1994; Wu *et al.*, 1996; Dijkstra *et al.*, 1997). The degree of similarity between the VZV origin-binding protein (product of gene 51) and the HSV-1 origin-binding protein (UL9) is such that the VZV origin-binding protein can functionally substitute for UL9 in a transient replication assay (Webster et al., 1995).

The UL9-like origin-binding protein seems to be unique to the *Alphaherpesvirinae* and the roseoloviruses. HHV-6B, a *Roseolovirus*, encodes an 'alphaherpesvirus-like' origin-binding protein, the product of the U73 gene (Inoue *et al.*, 1994). The core HHV-6B lytic origin is approximately 400 bp in length and contains two binding sites for the origin-binding protein, separated by an A/T rich region (Dewhurst *et al.*, 1994). The two HHV-6 origin-binding protein binding sites are similar sequences to box I and box II of

HSV-1 oriS (Inoue *et al.*, 1994).

Epstein-Barr virus has two distinct origins, oriLyt and oriP. OriP is required to maintain a latent infection, during which viral DNA replication is coordinated with the cell cycle (Yates *et al.*, 1985; Yates & Guan, 1991). OriP contains a central unwinding element flanked by EBNA-1 binding sites. In its ability to bind to specific sites within the origin and to facilitate recruitment of the DNA replicative machinery, EBNA-1 is functionally equivalent to UL9, although it lacks any intrinsic helicase activity. The replicated products of oriP dependent replication are circular molecules, so replication may be exclusively by the theta mechanism, (Yates *et al.*, 2000; Ceccarelli & Frappier, 2000). EBV oriLyt and lytic-phase DNA replication are described below.

1.3.3 Epstein-Barr virus lytic-phase DNA replication

Lytic origin

An EBV origin of lytic-phase DNA replication (oriLyt) was first identified in strain B95-8 (Hammerschmidt & Sugden, 1988). While in EBV strain B95-8, oriLyt is present only once, most other strains of EBV have two functional copies of the oriLyt. OriLyt in EBV is larger and more complex than the lytic origins of HSV-1. The core of oriLyt has two essential regions, separated by approximately 260 bp, flanked by auxiliary regions which contribute to replication efficiency. Part of the larger of the two essential regions of oriLyt can be substituted with the HCMV major immediate early promoter/enhancer (Hammerschmidt & Sugden, 1988). The minimal essential origin contains seven binding sites for the Zta transactivator and two binding sites for the Rta transactivator (Schepers *et al.*, 1993a;b). The lytic origin of EBV is represented schematically in figure 1.4.

Lytic-phase DNA replication

Transient replication assays were used to define the EBV proteins required for EBV oriLyt dependent DNA replication. Originally, six potential homologues of the HSV-1

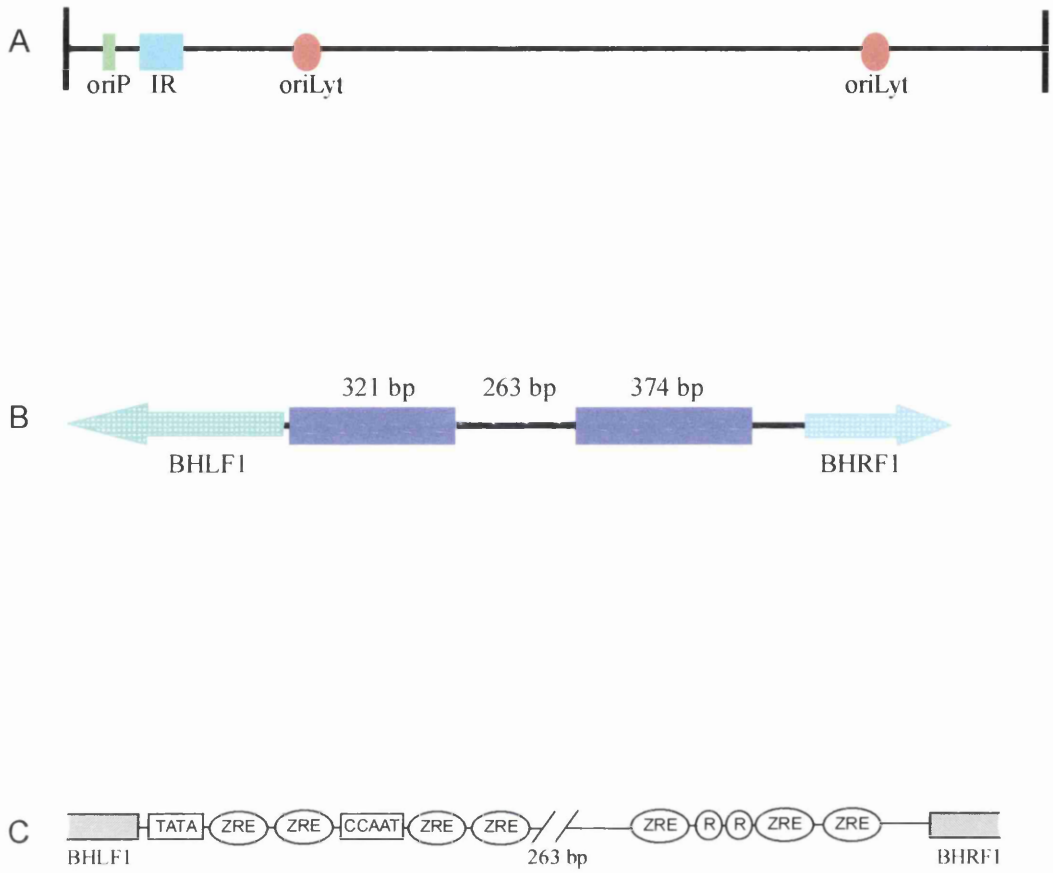


Figure 1.4 Schematic representation of EBV oriLyt.

A) The positions of the latent origin of DNA replication (oriP), the internal repeats (IR) and the origins of lytic DNA replication (oriLyt) within the EBV strain M-ABA genome are shown. EBV strain B95-8 contains only the left-hand copy of oriLyt.

B) The EBV oriLyt has two essential regions separated by a dispensable region. The 321 bp essential region contains the BHLF1 promoter. The 374 bp essential region contains two short A/T rich palindromes and an enhancer element.

C) Notable features of the two essential regions include the TATA and CCAAT boxes of the BHLF1 promoter and binding sites for the viral Zta (ZRE) and Rta (R) transactivators.

These diagrams are adapted from Fixman *et al.*, 1992 and Schepers *et al.*, 1993 (a and b).

replication fork proteins, as well as the three lytic-phase transactivators (Zta, Mta and Rta), and a function supplied by the *Sal*I-F fragment of EBV genomic DNA, were identified as being essential for oriLyt dependent DNA replication (Fixman *et al.*, 1992). Subsequently, it was demonstrated that the function supplied by *Sal*I-F was a uracil-DNA glycosylase which was not required when the other replication proteins were expressed constitutively (Fixman *et al.*, 1995).

No sequence homologue of the HSV-1 origin-binding protein was identified, but when the HSV-1 replication fork proteins were tested for their ability to direct EBV oriLyt dependent DNA replication, Zta and Rta were the only EBV encoded proteins which were additionally required (Fixman et al., 1995). Mutations of the Zta binding sites within the origin prevent origin-dependent DNA replication. Replacing Zta binding sites in oriLyt, in an EBV origin-containing plasmid, with binding sites for the bovine papillomavirus-1 E2 transactivator, abrogated origin function. Replication of this mutated EBV origin-containing plasmid was rescued when a chimeric protein consisting of the DNA binding and dimerization domain of E2 was fused to the transactivation domain of Zta, thus it is thought to be the transactivation domain of Zta which is required for efficient DNA replication (Schepers *et al.*, 1993b). The transactivation domain of Zta has subsequently been shown to interact with the EBV helicase/primase complex, analogous to the interactions between the HSV-1 origin-binding protein and helicase/primase (Gao *et al.*, 1998).

While this evidence suggests an origin-specific role for Zta in EBV oriLyt dependent DNA replication, it is interesting to note that in an omission assay with expression plasmids for the six EBV replication fork proteins, the putative uracil DNA glycosylase, and the three lytic-phase transactivators, neither Zta nor Rta was absolutely essential for DNA replication (Fixman *et al.*, 1995).

1.4 Human cytomegalovirus DNA replication

In contrast to HSV-1, the mechanism for the initiation of HCMV lytic-phase DNA replication and the proteins which serve an origin-specific function remain poorly understood. Possible mechanisms for the initiation of oriLyt dependent DNA replication will be reviewed in section 1.4.5. Once an HCMV replication fork has been established, elongation is assumed to proceed in the same way for HCMV as described for HSV-1, utilising the six conserved replication fork proteins (table 1.1). The proteins in HCMV, however, have been much less well characterised than their HSV-1 counterparts.

1.4.1 Cytomegalovirus lytic origins of DNA replication

A region of the HCMV genome containing an origin of lytic-phase DNA replication was first proposed following the observation that limited DNA synthesis in HCMV infected cells treated with ganciclovir was confined to one region of the genome (Hamzeh *et al.*, 1990). Ganciclovir is active only in HCMV infected cells where it inhibits viral DNA synthesis by acting as a chain terminator when incorporated into DNA. Subsequent to the identification of the ganciclovir amplified region, lytic origins of DNA replication in murine (MCMV), simian (SCMV), rat (RCMV) and human cytomegaloviruses were identified by transient replication assays, (Masse *et al.*, 1997; Anders & Punturieri, 1991; Vink *et al.*, 1997; Anders *et al.*, 1992).

Like the EBV oriLyt, the cytomegalovirus lytic origins studied so far are much larger (>1 kbp) and more complex than the HSV-1 origins. All the cytomegalovirus origins sequenced to date lie directly upstream of the gene encoding the single-stranded DNA binding protein, but share little sequence homology. Several common features are present within the cytomegalovirus and roseolovirus (HHV-6) origins, as shown in figure 1.5. Although the core origin of HHV-6 is only approximately 400 bp in length, flanking regions contribute to origin activity (Dewhurst *et al.*, 1994) and have been included in figure 1.5.

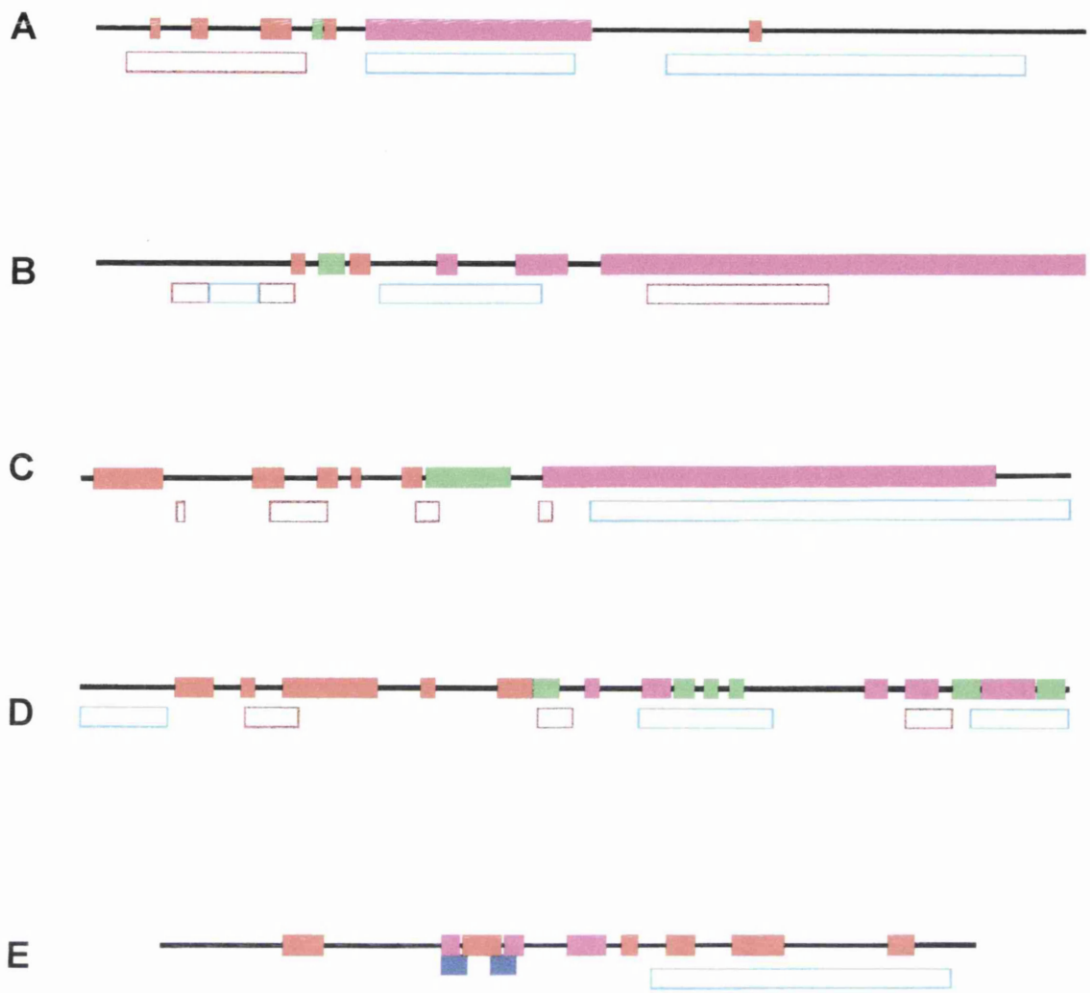


Figure 1.5 Comparisons of the lytic origins of the *Betaherpesvirinae*.

The lytic origins of rat cytomegalovirus (A), human cytomegalovirus (B), murine cytomegalovirus (C), simian cytomegalovirus (D), and human herpesvirus-6 (E). Approximately 1500 bp of oriLyt is shown for the rat, murine and simian viruses. Approximately 2000 bp of oriLyt for human cytomegalovirus and 1000 bp of oriLyt for HHV6 are shown.

Sequence blocks shown correspond to regions of oriLyt which are mainly:

- direct repeats
- indirect repeats
- A/T rich
- G/C rich
- asymmetric C rich
- origin-binding protein binding sites

The structures of these lytic origins have been described by Masse *et al.*, (1997) and Vink *et al.*, (1997).

There are distinct G+C and A+T rich elements and numerous sets of direct and indirect repeats, including multiple binding sites for a variety of cellular transcription factors, especially Sp1 binding sites in HCMV and RCMV or AP-2 binding sites in MCMV.

In contrast to the ability of HSV-1 superinfection to direct the replication of a transfected VZV origin-containing plasmid (Stow & Davison, 1986), the murine, simian and human cytomegalovirus lytic origins lack sufficient similarity to be functionally interchangeable (Anders & Punturieri, 1991; Masse *et al.*, 1997).

1.4.2 HCMV oriLyt DNA requirements

As previously described, a region of HCMV containing a putative oriLyt was first identified following the observation that limited DNA synthesis was detectable in one genomic region in ganciclovir treated cells (Hamzeh *et al.*, 1990). Ganciclovir is phosphorylated by HCMV UL97 phosphotransferase (Sullivan *et al.*, 1992; Littler *et al.*, 1992) and can then be incorporated by the DNA polymerase into the replicating DNA, where it acts as a chain terminator. From the structure of the ganciclovir terminated DNAs, Hamzeh *et al.* (1990) were able to conclude that DNA synthesis proceeds bidirectionally away from oriLyt. Transient replication assays were subsequently used to show that plasmids containing sequences surrounding and including the ganciclovir amplified region were replicated in HCMV infected cells, and thus represented a true oriLyt (Anders & Punturieri, 1991; Anders *et al.*, 1992). HCMV oriLyt has been examined in detail and the minimal core region of the origin was located to an approximately 1.5 kbp region between nt 91751 and nt 93299, (Zhu *et al.*, 1998).

The core region of HCMV oriLyt consists of two essential regions (I and II) of 364 bp and 166 bp separated by an approximately 400 bp non-essential sequence. The central non-essential segment and sequences upstream of essential region I contribute significantly to origin function, sequences downstream of essential region II contribute less. The essential regions of HCMV oriLyt as well as the smallest replicator transcript (SRT) and

the virus-associated RNAs (vRNAs) are described below and in figure 1.6.

Essential region I (nt 92209 - 92573) contains the 'Y-block' element, a polypyrimidine tract (nt 92471 - 92501), which is immediately upstream of the region of termination of transcription of the SRT (Huang *et al.*, 1996). Origin-containing plasmids with mutations in the 'Y-block', generated so as to conserve spacing of flanking regions, were used to confirm that this element is essential for origin function (Huang *et al.*, 1996). Region I also contains numerous repeated sequences, of which a 29 bp repeat consisting of an inverted pair of ATF-CREB sites was reported to be the most important component in the left half of essential region I (Zhu *et al.*, 1998).

Essential region II (nt 92979 - 93145) has been less intensively studied than region I. Region II contains Sp1 binding sites and has been shown to contribute to SRT promoter activity (Huang *et al.*, 1996; Zhu *et al.*, 1998). Region II also contains part of vRNA2 (Prichard *et al.*, 1998).

vRNAs are two small oriLyt derived RNAs reported to be covalently integrated into the origin regions of some packaged HCMV genomes (Prichard *et al.*, 1998). Using the data provided for the sizes and spacing of the vRNAs, and the definition of DNA fragments which the vRNAs can hybridise to, it is suggested that the 500 nucleotide long vRNA1 lies between nt 93799 - 94340, and that the 300 nucleotide long vRNA2 lies between nt 92949 - 93290. vRNA2 is located approximately 250 bp upstream of the transcription start site for the smallest replicator transcript (SRT) at nt 92686. Both SRT and vRNA2 are complementary to the same strand (Prichard *et al.*, 1998).

SRT refers to a series of non-polyadenylated early transcripts, with a common 5' end and loosely defined 3' ends, which are transcribed from HCMV oriLyt between nt 92686 and 92431-92474 (Huang *et al.*, 1996). SRT transcription is cycloheximide sensitive but PFA insensitive, and is probably directed by host cell RNA polymerase II, under the control of an HCMV activated promoter region between nt 92688 - 92786.

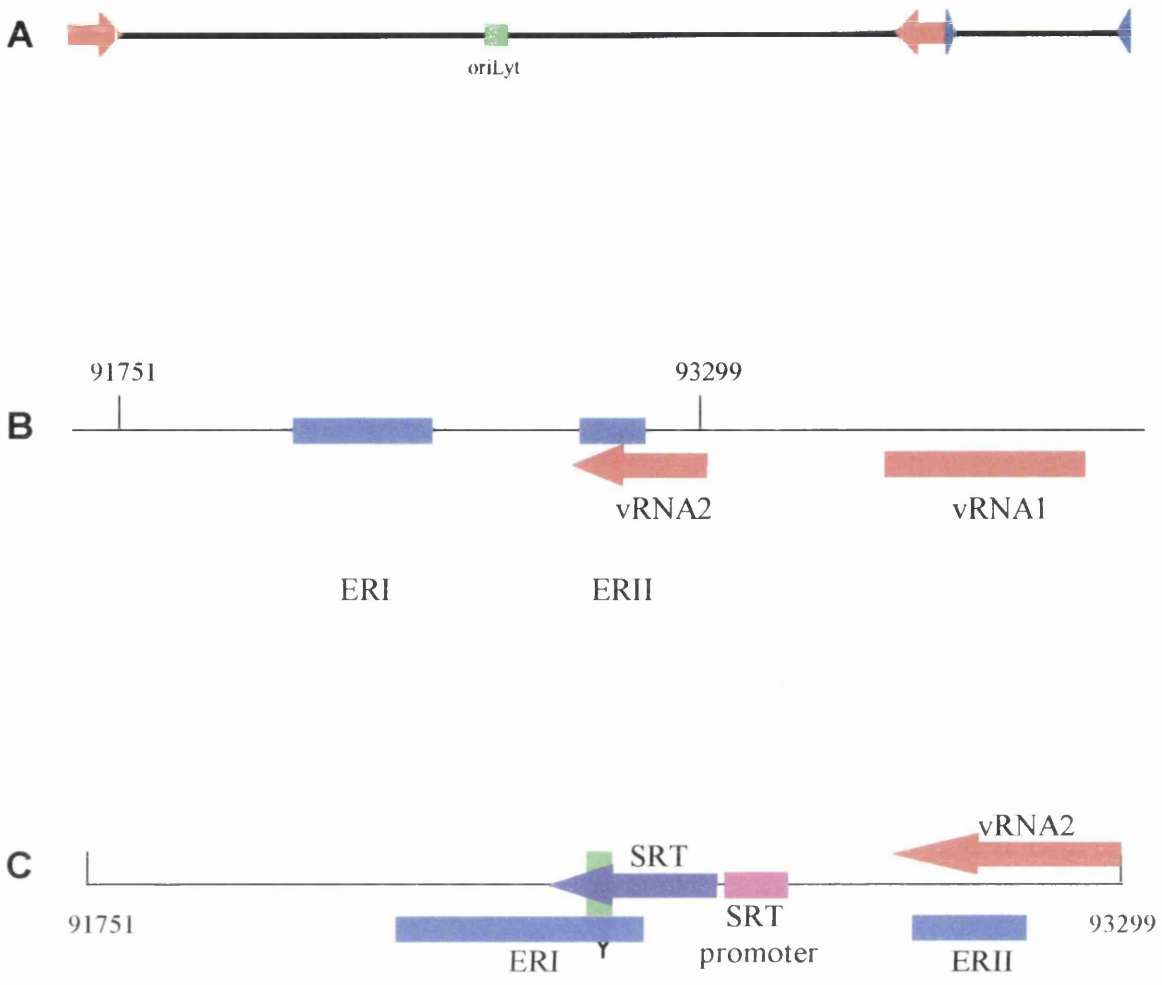


Figure 1.6 Features of HCMV oriLyt.

A) The location of oriLyt within the HCMV genome.

B) The location of the essential regions (ERI and ERII) (Zhu *et al.*, 1998) and the positions of stably incorporated viral RNA molecules (vRNAs) (Prichard *et al.*, 1998) within HCMV oriLyt.

C) The core of HCMV oriLyt lies between nt 91751 - 93299 (Zhu *et al.*, 1998). The core of the origin overlaps both essential regions and vRNA2. The smallest replicator transcript (SRT) refers to a series of RNA molecules transcribed from the origin under the control of the SRT promoter (Huang *et al.*, 1996). The SRT molecules have a common 5' end but variable 3' ends, as represented by the arrowhead. Transcription of SRT terminates at, or shortly downstream of, the 'Y-block' (Y), a 45 nucleotide long polypyrimidine tract (Huang *et al.*, 1996).

Regions further downstream of the minimal promoter were shown to augment promoter activity.

Models for initiation of HCMV oriLyt dependent DNA replication, and possible roles for the vRNAs and the SRT will be described later (section 1.4.5). However, it should be noted that a deletion (Δ92574 - 92979) which removed the SRT promoter and half of the SRT gene, replicated to 48% of the level achieved by the wild-type origin (Zhu *et al.*, 1998). The fact that origin-containing plasmids propagated in bacteria, which presumably lack covalently incorporated vRNA sequences, can be replicated, suggests that vRNAs are also unlikely to serve an absolutely essential role in the initiation of HCMV origin-dependent DNA synthesis.

1.4.3 HCMV DNA replication protein requirements

Replication fork proteins

Pari and Anders (1993) described a set of eleven loci which encode functions sufficient (and essential) for HCMV origin-dependent DNA replication in a transient replication assay. As expected, the eleven loci included homologues of the six herpesvirus conserved replication fork proteins, a DNA polymerase (UL54) and polymerase accessory protein (UL44), a single-stranded DNA binding protein (UL57), and three putative subunits of a helicase/primase complex (UL70, UL102, and UL105).

The HCMV DNA polymerase and polymerase accessory subunit have sequence homology with their HSV-1 counterparts (Ertl & Powell, 1992). The catalytic subunit, UL54, is a 5'-3' DNA polymerase with 3'-5' exonuclease activity, and, on the basis of sequence homology, a possible RNase H activity (Huang, 1975; Nishiyama *et al.*, 1983; Marcy *et al.*, 1990; Anders & McCue, 1996). The polymerase accessory protein was originally identified as an early-class DNA binding protein in SCMV infected cells (Gibson *et al.*, 1981). UL54 and UL44 were expressed in insect cells and were shown to form a stable complex. Co-expression of UL54 with UL44 resulted in increased processivity

compared with the activity of UL54 alone (Ertl & Powell, 1992).

Helicase activity is thought to be encoded by UL105 (Smith *et al.*, 1996) and primase activity by UL70. These assignments are on the basis that the two loci contain conserved catalytic motifs and have extensive sequence homology with their proposed HSV-1 counterparts, UL5 and UL52 respectively (Chee *et al.*, 1990; Martignetti *et al.*, 1991). The UL70 and UL105 proteins are essential for HCMV origin-dependent DNA replication, but do not activate expression of the remaining essential replication proteins (Anders & McCue, 1996). More recently, a heterotrimeric complex containing UL70, UL105 and UL102, analogous to the helicase/primase complex of HSV-1, has been purified (Anders & McCue, 1996). UL102 was also shown to be essential for HCMV origin-dependent DNA replication, and while the two proteins share obvious sequence similarity in only one region, UL102 is believed to be the HCMV helicase/primase accessory factor homologous to HSV-1 UL8 (Smith & Pari, 1995a). The HCMV single-stranded DNA binding protein homologous to HSV-1 UL29, is UL57 (Kemble *et al.*, 1987; Anders, 1990).

Auxiliary loci

The remaining five loci, UL36-38, UL84, UL112-113, UL122-123, and IRS1/TRS1, were termed auxiliary loci. The known functions of the HCMV auxiliary proteins have been reviewed (Colberg-Poley, 1996; Stenberg, 1996). Four of the five auxiliary loci (UL36-38, UL112-113, UL122-123 and IRS1/TRS1) are thought to play key roles in transactivating the expression of the replication fork proteins (Iskenderian *et al.*, 1996). The HCMV auxiliary loci do not include an obvious sequence homologue of the HSV-1, HHV-6 or HHV-7 origin-binding proteins. The positions of the auxiliary loci in the HCMV genome, and a schematic diagram representing the well characterised immediate early and early spliced products of UL36-38 and UL122-123 (IE1/2), are shown in figure 1.7. The reported functions of the auxiliary loci are described below, and their

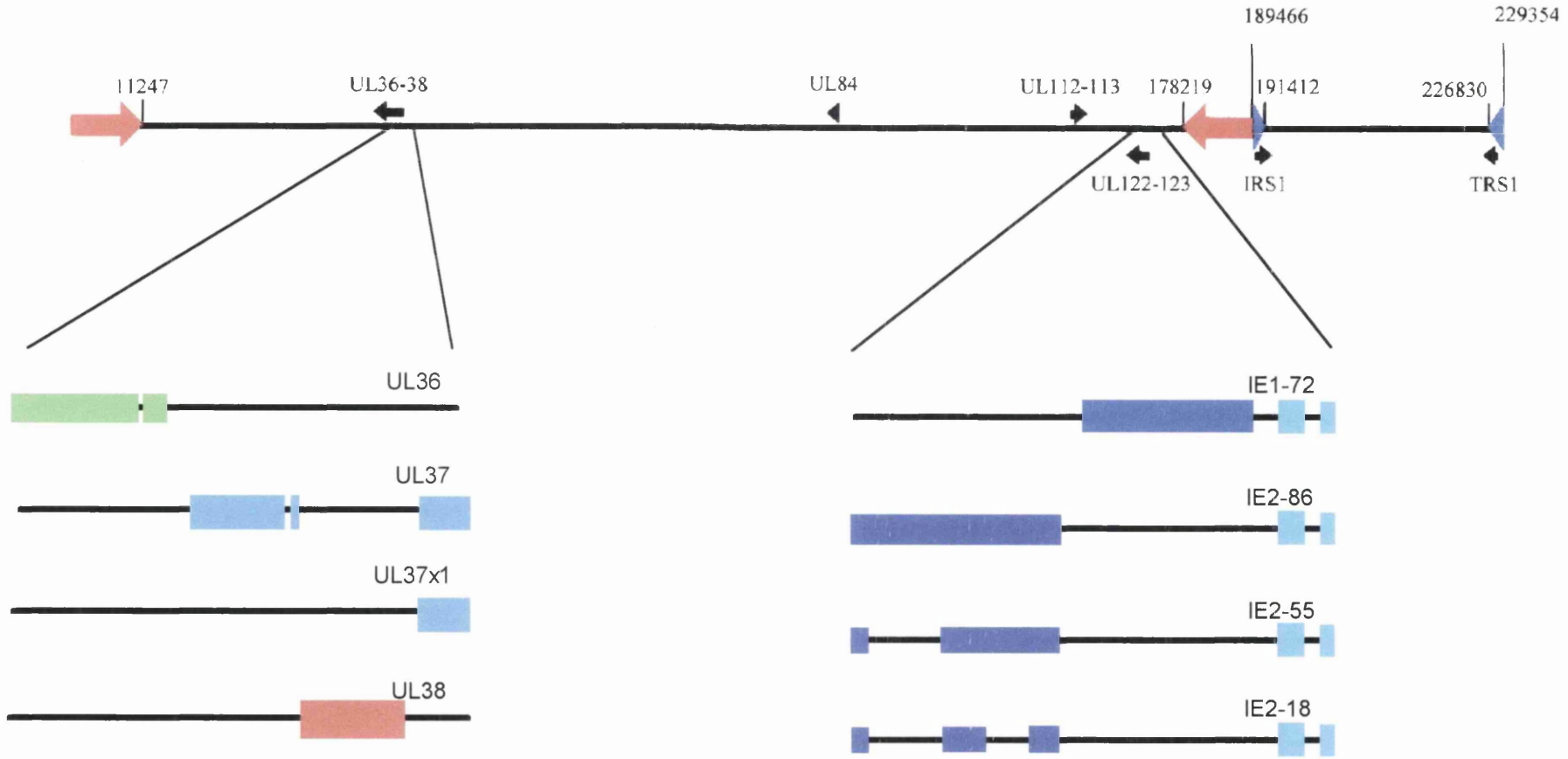


Figure 1.7 Representation of the HCMV auxiliary loci essential for HCMV origin dependent DNA replication (Pari & Anders 1993). The 229 kbp HCMV strain AD169 genome (GenBank accession number X17403) is illustrated with the locations and the direction of transcription of the five HCMV auxiliary loci. UL84 and IRS1/TRS1 are produced from unspliced mRNAs. The UL36-38 and UL122-123 (IE1/2) proteins are produced from spliced mRNAs. The coloured blocks represent the regions of mRNA which are translated for the named protein. The UL112-113 locus produces spliced mRNAs from which four proteins are translated. The structures of the UL112-113 mRNAs have not been definitively determined.

requirements in transient replication assays will be described later (section 1.4.4).

UL36-38 is a complex spliced locus encoding at least four proteins; UL36, UL37, UL37x1 and UL38 (Kouzarides et al., 1988; Tenney & Colberg-Poley, 1991). The protein product of UL36 is a member of the US22 gene family, but the protein has not been purified. Two contradictory reports have been published regarding the requirement UL36 for HCMV growth in tissue culture. It was first shown, using antisense oligonucleotides to inhibit splicing of UL36 and UL37 transcripts, that both proteins are required for HCMV DNA replication in HF cells (Smith & Pari, 1995b). More recently, however, an HCMV recombinant lacking UL36 was shown to have wild-type growth kinetics in HF cells (Patterson & Shenk, 1999). UL37 is a transmembrane glycoprotein which shares an N-terminal acidic domain with UL37x1; the acidic domain is involved in transcriptional activation (Colberg-Poley *et al.*, 1998). No function has yet been assigned to UL38, but one early (1.35 kb) and one early:late transcript (2.5 kb) from UL38 have been detected (Tenney & Colberg-Poley, 1991).

UL84 is an early protein which has been shown to co-immunoprecipitate with IE2-86 from HCMV infected human fibroblast cells (Spector & Tevethia, 1994). More recently, purified UL84 has been shown to interact with purified IE2-86 (McCue & Anders, 1998). UL84 does not cooperate with the other auxiliary loci to transactivate expression of the replication fork proteins (Iskenderian *et al.*, 1996), rather it has been shown to be a strong inhibitor of some IE2 mediated transcriptional activation (Gebert *et al.*, 1997). UL84 has also been shown to be essential for the formation of HCMV DNA replication compartments (Sarisky & Hayward, 1996), and an abstract was presented at an International Herpesvirus Workshop reporting that UL84 could function as an RNA endonuclease (Sarisky & Hayward, 1996b), although these latter data have not been published.

The UL112-113 locus encodes four known DNA binding nuclear phosphoproteins, some of which are capable of cooperating with IE1/2, UL36-38, and TRS1 to activate expression of some HCMV early genes (Iskenderian *et al.*, 1996). At least one product of UL112-113 is required for recruiting the replication fork proteins to precursors of viral DNA replication compartments (Ahn et al., 1999).

TRS1 is an immediate early gene, a member of the US22 gene family, mainly contained within the short repeat region (terminal) of the genome. IRS1 is mainly contained within the short repeat region (internal) of the genome and so is homologous to TRS1. The N-terminal 549 aa of IRS1 (846 aa) and TRS1 (788 aa) are encoded by the short repeat regions of the genome. The unique sequences of the two proteins, encoded by the short unique region of the genome, retain 55% identity. TRS1 acts together with IE2-86 to activate expression of UL44, and can cooperate with other HCMV immediate early proteins to activate expression of proteins required for DNA replication (Stasiak & Mocarski, 1992; Iskenderian *et al.*, 1996).

The UL122-123 locus (expressing the major immediate early proteins, IE1/2) is the most extensively studied of the auxiliary loci. The region encodes at least four immediate early proteins and one late protein (Stinski *et al.*, 1983; Jahn *et al.*, 1984; Stenberg *et al.*, 1984). Major immediate early transcripts are spliced such that IE1 and IE2 have the same 5' exons and the proteins share the same 85 N-terminal amino acids. IE1/2 are transcriptional activators which can interact with a number of cellular and viral proteins (reviewed by Fortunato & Spector, 1999). The only IE1 protein is IE1-72, the major IE2 protein is IE2-86, but mRNAs for IE2-55 and IE2-18 have been seen in infected HF cells treated with cycloheximide, and in the case of IE2-18, in infected monocytes (Stinski *et al.*, 1983; Kerry *et al.*, 1995).

IE2-86 alone can modulate transcription, for example, in repressing expression from the major immediate early promoter. IE2 can also act in concert with IE1 to upregulate

expression of certain viral genes, and can cooperate with other HCMV immediate early and early proteins to modulate gene expression (Pizzorno *et al.*, 1988; Stenberg *et al.*, 1990; Iskenderian *et al.*, 1996; Gebert *et al.*, 1997). Direct binding to DNA has been demonstrated for IE2-86 but not for IE1. However, no single, defined IE2-86 binding sequence has been identified (Pizzorno & Hayward, 1990; Arlt *et al.*, 1994; Huang *et al.*, 1994; Huang & Stinski, 1995; Schwartz *et al.*, 1994; Scully *et al.*, 1995). A 14 bp element ($CGGN_8CCG$) within HCMV oriLyt is a potential binding site for IE2-86 (Huang et al., 1996).

IE2-86 appears to have several modes of action. It can modulate expression in a promoter dependent fashion by direct DNA binding, by interaction with cellular transcription factors, or by interactions with components of the cellular transcriptional machinery (Hagemeier et al., 1992, 1994; Caswell et al., 1993; Chiou et al., 1993; Spector & Tevethia, 1994; Lang et al., 1995; Schwartz et al., 1996; Yoo et al., 1996). An HCMV virus lacking IE1 was shown to have normal growth when it was used to infect HF cells at a high multiplicity, showing that IE1 is non-essential for HCMV growth in tissue culture (Greaves & Mocarski, 1998). During low multiplicity infection of HF with the HCMV-IE1 mutant, there was delayed accumulation of the HCMV polymerase accessory protein (compared to wild-type infection) and HCMV replication compartments were not formed. It is not known how high multiplicity infection of HF with the HCMV-IE1 mutant compensates for the absence of IE1.

Some of the auxiliary loci are involved in modifying nuclear structures within infected cells, and may therefore be required for the formation of HCMV DNA replication compartments. Infecting viral DNA is targeted to the nucleus where it accumulates adjacent to pre-existing nuclear structures called PODs, which are the site of HCMV immediate early gene expression (Ishov *et al.*, 1997; Ahn & Hayward, 1997). The distribution of cellular proteins within the PODs is altered as a result of virus infection, and,

following the dispersal of the PML antigen from the PODs by IE1 (Wilkinson *et al.*, 1998), HCMV DNA replication compartments begin to form at the periphery of PODs.

The formation of viral DNA replication compartments has been reported to be dependent on the presence of UL84 (Sarisky & Hayward, 1996). Some product of the UL112-113 locus has subsequently been shown to cause the redistribution of the HCMV DNA polymerase from a diffuse nuclear distribution into distinct compartments (Ahn *et al.*, 1999). The UL112-113 directed redistribution of UL44 has been shown to be enhanced in the presence of the remaining replication fork proteins. Thus, UL112-113 has been proposed to function, in part, by recruiting the replication fork proteins to the replication compartments (Ahn *et al.*, 1999). IE2 has been shown to become incorporated into HCMV DNA replication compartments after dispersal of IE1 (Ahn *et al.*, 1999).

1.4.4 HCMV transient replication assay

Sarisky and Hayward (1996) used HCMV replication proteins supplied from plasmids under the control of constitutive promoters and downstream regulatory signals to further examine the trans-acting functions with a direct role in HCMV DNA replication. In order to further exclude the possibility that any of the auxiliary proteins were solely required for efficient gene expression, the UL69 post-transcriptional activator (homologous to HSV-1 IE63) was also tested in these assays. The authors examined the ability of different combinations of proteins to direct replication of an HCMV origin-containing plasmid in transient replication assays in either Vero or HF cells. The published results of the transient replication assays (Sarisky & Hayward, 1996) are summarised in table 1.2.

In "omission" assays, all eleven HCMV DNA replication loci and an HCMV origin-containing plasmid were transfected together in a transient replication assay, then the auxiliary loci were individually omitted from the transfection and the replication of the origin-containing plasmid was examined. "Omission" assays were also done in the additional presence of the UL69 post-transcriptional activator.

A)

	1. Omission assay in Vero cells (no UL69)	2. Omission assay in Vero cells (with UL69)	3. Omission assay in HF cells (no UL69)
UL84	Essential (1 %)	Essential (4 %)	Essential (1 %)
UL36 - 38	Essential (1 %)	61 %	Essential (1 %)
UL112 - 113	31 %	29 %	39 %
IE1 / IE2	5 %	77 %	Essential (1 %)
IRS1 / TRS1	63 %	53 %	93 %

B)

	1. Addition assay with EBV proteins in Vero	2. Addition assay with HCMV proteins and UL69 in Vero cells
UL84	Positive	Essential
UL69	Negative	Always included
UL36 - 38	Negative	Not essential
UL112 - 113	Not tested	Essential
IE2	Not tested	Essential
IRS1 / TRS1	Not tested	Not essential

Table 1.2 Requirements for HCMV auxiliary loci in transient replication assays (Sarisky & Hayward, 1996). Different combinations of the five HCMV auxiliary loci were tested for the ability to direct replication of a transfected HCMV origin-containing plasmid in Vero or HF cells, when all of the replication proteins were constitutively expressed. Replication fork functions were provided from expression plasmids specifying the six replication fork proteins of either EBV or HCMV, and where indicated, an expression plasmid for HCMV UL69 was also present. For the omission assays (A), the amount of plasmid replicated following the individual omission of one of the HCMV auxiliary loci was expressed as a percentage of the amount of plasmid replicated in presence of all of the expression plasmids. A plasmid is scored as essential if the signal obtained when the plasmid was omitted was less than (or equal to) the signal from the negative control, obtained when plasmids for all six replication fork proteins were omitted from the transfection. The addition assays (B) were performed in Vero cells using replication fork proteins from plasmids specifying the EBV or HCMV proteins. In the assay using EBV replication fork proteins (1), replication of the HCMV origin-containing plasmid was observed on addition of UL84 alone. In the assay using the HCMV replication fork proteins (2), replication required other auxiliary loci in addition to UL84, so the minimal set of auxiliary loci required for HCMV origin-containing plasmid replication is shown.

In Vero cells, without UL69, omission of UL84 or UL36-38 abrogated replication. Omission of IE1/2 reduced replication efficiency to a very low level. In the presence of UL69 in Vero cells, omission of UL84 abolished replication, but none of the other auxiliary loci was absolutely essential. An "omission" assay done in HFFF2 cells, without UL69, showed that omission of UL84 or UL36-38 or IE1/2 abrogated replication (table 1.2, panel A).

An "addition" assay was carried out in Vero cells using the six HCMV replication fork proteins and UL69. Various combinations of auxiliary loci were tested for their ability to mediate HCMV origin-containing plasmid replication. In this assay, the minimum requirement for replication was the addition of UL84, UL112-113 and IE2 (table 1.2 panel B).

A third type of assay tested the ability of the constitutively expressed EBV replication fork proteins to direct replication of an HCMV origin-containing plasmid in Vero cells. Replication was observed on addition of UL84 alone (table 1.2 panel B).

Finally, the formation of HCMV DNA replication compartments by cotransfection in Vero cells was shown to be dependent on the presence of UL84. Replication compartments were observed following transfection of expression plasmids for the replication fork proteins with UL69, UL84, IE2 and UL112-113.

UL69 clearly modified the requirements for the auxiliary loci in these transient replication assays, possibly removing the requirement for UL36-38. However, it is important to note that it was not identified as one of the eleven loci required for HCMV origin-dependent DNA replication in the original transient replication assays (Pari & Anders, 1993).

1.4.5 Initiation of HCMV DNA synthesis

Prichard *et al.* (1998) proposed that HCMV DNA replication may proceed in two phases, suggesting that an initial round of amplification might take place in the subset of

genomes containing covalently incorporated vRNAs. The authors propose that an RNase H like enzyme would specifically nick or degrade the RNA moiety of the persistent RNA:DNA hybrid, leaving a free 3' end of RNA or DNA from which DNA synthesis could be initiated. The second round of replication is then proposed to be initiated by a transcriptional event, as described below, and may use genomes either with or without covalently incorporated RNA.

The results described above (section 1.4.4), regarding the absolute requirement for UL84 in HCMV oriLyt dependent DNA replication, the reported RNA endonuclease properties of UL84 protein, and the discovery of origin derived transcripts (SRT), have led to the proposal that UL84 may be involved in the initiation of the second phase of replication (Prichard *et al.*, 1998) which might proceed by a mechanism similar to the initiation of mitochondrial heavy strand DNA replication.

The two strands of mitochondrial DNA are not replicated simultaneously. Initially, RNA synthesis proceeds from the light strand promoter located near the mitochondrial 'D-loop' region. If transcription continues around the template, an RNA transcript is released. Alternatively, the transcript may be processed once it has crossed the origin of heavy strand DNA synthesis (O_H) within the 'D-loop'. Cleavage of the RNA at O_H is carried out by a nuclear-encoded, site-specific mitochondrial RNA processing enzyme (reviewed in Kornberg & Baker, 1992). A stable RNA:DNA hybrid can be formed between the primer RNA transcript and the GC rich sequence at O_H, and DNA synthesis can proceed. Hybrid formation is not only dependent on the GC rich sequence, but also on the mitochondrial RNA polymerase, as heterologous RNA polymerases can synthesise the transcript, but not promote hybrid formation (Xu & Clayton, 1995).

By analogy with mitochondrial heavy strand initiation, it is proposed that the SRTs form stable hybrids with the origin sequences at the 'Y-block', and that an RNA endonuclease, possibly encoded by UL84, cleaves the RNA strand of the hybrid leaving a

short RNA with a free 3'-OH which can be used to prime DNA synthesis.

In the initiation of mitochondrial heavy strand DNA synthesis, RNA:DNA hybrid formation is not simply a function of the RNA and DNA sequences (Xu & Clayton, 1995), it specifically requires transcription to be directed by the mitochondrial RNA polymerase. An alternative role for UL84 might therefore be to stabilise the HCMV RNA:DNA hybrid at oriLyt.

The differences in requirements for the remaining HCMV auxiliary loci in HF and Vero cells (Sarisky & Hayward, 1996) is possibly related to the presence of different host cell transcription factors being involved in SRT synthesis in the different cell lines. The requirement for IE1/IE2 in HF cells (Sarisky & Hayward, 1996) may be associated with the presence of potential IE2-86 binding sites in the origin (Huang *et al.*, 1996).

With such a complex origin region, and differing requirements for *trans*-acting factors in different cell lines, as well as the cell type specific differences seen in IE gene expression, it seems possible that there may not be a single unique mechanism for the initiation of HCMV DNA replication. If the transcription of the SRT is involved in the priming of DNA synthesis, it is possible to envisage that the promoter can be activated by various combinations of cellular and/or viral proteins.

While the mechanism of initiation of HCMV origin-dependent DNA synthesis has not been identified, the evidence reviewed here strongly implicates UL84 as serving an essential origin-specific function. Four of the five HCMV auxiliary loci, but not UL84, are required for efficient early gene expression, and UL84 was the only auxiliary protein which was absolutely essential for HCMV origin-dependent DNA synthesis in transient replication assays. UL84 was the only HCMV protein tested which was able to direct replication of a transfected HCMV origin-containing plasmid by the EBV replication fork proteins. Sarisky and Hayward (1996), reported that UL84 was obligatory for origin-dependent DNA amplification and suggested that it was "a key viral component in promoting the initiation

of HCMV oriLyt-directed DNA replication".

1.5 Range of cells permissive for HCMV infection *in vitro*

HCMV DNA replication has been difficult to study partially due to the restricted range of easily manipulated, fully permissive cell lines available. While *in vivo*, HCMV can infect almost all organ systems, the virus can normally be propagated *in vitro* only in human fibroblast cell lines such as HFL1 (human foetal lung) and HFFF2 (human foetal foreskin fibroblast). HCMV has also been reported to infect a wide range of other cell types *in vitro*, although the infection tends to be abortive (reviewed by Sinzger & Jahn, 1996). It has been shown that the growth restrictions in tissue culture may be partly due to passaging laboratory stocks in fibroblast cells (Waldman *et al.*, 1989; Brown *et al.*, 1995; Sinzger *et al.*, 1999). Passaging HCMV in tissue culture may be associated with gross changes, especially large-scale deletions, within the virus genome (Cha *et al.*, 1996), and loss of certain genes may, as seen for MCMV (Cavanaugh *et al.*, 1996) result in altered cell tropisms.

An early investigation into the range of cell types permissive for HCMV infection showed that three HCMV strains could abortively infect primary bovine embryonic fibroblasts, and productively infect Vero cells (Waner & Weller, 1974). Later it was demonstrated that only a small fraction of HCMV infected Vero cells supported immediate-early gene expression, but that no viral DNA replication was detected (LaFemina & Hayward, 1988). LaFemina and Hayward (1988) conducted a comprehensive investigation into cytomegalovirus immediate-early gene expression and DNA replication in a variety of cell lines. Twenty-one cell lines were examined for their ability to support the replication of HCMV strain Towne, SCMV strain Colburn and MCMV strain Smith. The results of this investigation with respect to HCMV infection are summarised in table 1.3.

	Cell type infected	Characteristics	IE (a)	DNA (b)
Human	HF	Diploid fibroblast	++++	++++
	NT2/D1	Teratocarcinoma	-	-
	NT2/D1 + RA	Retinoic acid differentiated NT2/D1	+++	+++
	143	Osteosarcoma, TK-	+++	+
	MG63	Osteosarcoma	+++	+
	PBL	Peripheral blood lymphocytes	-	-
	FOS	Megakaryocyte-like	-	-
	HL60	Premyelocytic leukaemia	-	-
	NBE	HF, SV40 T Ag+	-	-
	293	HEK, Adenovirus E1A+	-	-
Simian	AGMKF	African green monkey kidney fibroblasts	++	-
	CyMF	Cynomolgous monkey fibroblasts	++	-
	RhMF	Rhesus monkey fibroblasts	++	-
	Vero	AGMK, immortalised	-	-
	CV1	AGMK, immortalised	ND	ND
Murine	MEF	Mouse embryo fibroblasts	++	-
	BALB/c-3T3	Mouse, immortalised	++++	-
	F9	Mouse teratocarcinoma	-	-
	PYS	Parietal yolk sac, transformed	-	-
	Ltk-	Mouse, transformed TK-	-	-

Table 1.3 IE gene expression and DNA replication in HCMV strain Towne infected cells. Adapted from LaFemina & Hayward, 1988.

(a) levels of IE gene expression determined by synthesis of ^{35}S-methionine labeled IE68 after cycloheximide reversal in cells infected with 50 p.f.u./cell of HCMV.

(b) levels of DNA replication determined by synthesis of ^{32}P-labelled HCMV genomic DNA in cells infected with 20 p.f.u./cell of HCMV

HCMV infection of HF cells defines the fully permissive ++++ level of synthesis.

+++ denotes a level of replication which is 30 - 70 % of the fully permissive level.

++ denotes a level of synthesis which is 10 - 30 % of the fully permissive level.

+ denotes a level of synthesis which is 3 - 10 % of the fully permissive level.

- denotes a level of synthesis which is less than 3 % of the fully permissive level.

ND, not done.

Only four human cell lines; HF, two osteosarcoma cell lines (143 and MG63) and a retinoic acid-differentiated teratocarcinoma cell line (NT2/D1), supported HCMV DNA replication. Ten of the cell lines examined supported immediate-early gene expression. These include three murine and three simian cell lines in addition to the four human cell lines previously mentioned. The block to immediate early gene expression has not been clearly identified as, at least in 'non-permissive' 293 and Vero cells, immediate-early proteins were synthesised following transfection of a plasmid encoding the HCMV major immediate-early proteins under the control of the HCMV major immediate-early promoter and downstream regulatory signals (LaFemina & Hayward, 1988).

Interestingly, SCMV and MCMV were more promiscuous in their ability to replicate in cell lines of heterologous species than HCMV. Treatment of some cells in tissue culture, *e.g.*, NT2 human teratocarcinoma, with retinoic acid or short chain fatty acids (e.g. sodium butyrate or sodium proprionate) sometimes rendered cells more permissive to infection with HCMV (LaFemina & Hayward, 1986; Wu *et al.*, 1994)..

HCMV has been shown to productively infect primary chimpanzee skin fibroblasts (Perot et al., 1992), and human trophoblasts (Hemmings *et al.*, 1998). HCMV can also infect bone marrow stem cells, although virus is only produced following cell differentiation (Minton *et al.*, 1994).

1.6 Aims of this project

My aim in this project was to investigate the initiation of HCMV origin-dependent lytic-phase DNA replication. Since, at the time I began this project, UL84 seemed the most likely HCMV product providing an origin-specific function, my initial objective was to investigate its role in the initiation of origin-dependent DNA synthesis. In order to have an assay for UL84 function, I first attempted to set up the assay of Pari and Anders (1993) using transient transfection of an HCMV origin-containing plasmid with plasmids expressing the six HCMV replication fork proteins and the five auxiliary loci in HF cells.

My attempts to establish the Pari and Anders assay in HF cells were unsuccessful. I therefore tested the ability of other cell lines to support HCMV origin-dependent DNA synthesis. I also used heterologous replication fork proteins, supplied by HSV-1 plasmids or virus superinfection, or supplied by SCMV superinfection, to set up alternative assays to investigate the initiation of HCMV origin-dependent DNA synthesis.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

Most chemicals were obtained from BDH Laboratory supplies, Poole, Dorset and were of AnalaR grade.

Other sources of chemicals and reagents are listed:

Boehringer Mannheim GmbH, Germany	Tris base
Melford Laboratories Ltd., Suffolk	caesium chloride
Prolabo, Fontenay Sur Bois	boric acid, chloroform, glacial acetic acid, glycerol, isopropanol, methanol, sodium chloride
Sigma Chemicals Company, St. Louis, Mo.	bovine serum albumin, chloramphenicol, deoxyribonucleic acid (calf thymus), ficoll, hepes, isoamyl alcohol, phosphonoformic acid, polyvinylpyrrolidone, Triton X-100

2.1.2 Enzymes

Most restriction enzymes were obtained from Boehringer Mannheim. Other sources of enzymes are listed:

Boehringer Mannheim	proteinase K
New England BioLabs (NEB)	DNA polymerase I, T4 DNA ligase, T4 DNA polymerase

	*Bst*EII, *Dpn*II, *Dpn*I, *Sex*AI, *Scr*FI
Sigma	deoxyribonuclease I (DNase I)
	hen egg white lysozyme, protease XIV
	ribonuclease TI (RNase TI)
	ribonuclease A (RNase A)

2.1.3 Radiochemicals

α–^{32}P deoxyribonucleoside triphosphates (dNTPs) were obtained from Amersham at 3000 Ci mMole^{-1} (10 μCi μl^{-1})

2.1.4 Plasmids

The following plasmids were obtained from D Anders at the Department of Biomedical Sciences, SUNY, New York (Pari & Anders, 1992). The plasmids express the HCMV DNA replication proteins under the control of their native promoters and downstream signals, the HCMV strain AD169 DNA fragments are cloned into pGEM7zf.

pZP3	IRS1 locus (nt 189500-193108)	
pZP8	UL36-38 locus (nt 42045-53104)	
pZP13	UL84 locus (nt 120505-123363)	
pZP24	UL112-113 locus (nt 159699-163028 and 163950-164152)	
pSVH	UL122-123 major immediate early locus from HCMV strain Towne	
pSP50	HCMV origin-containing plasmid (nt 89795-94860)	
pZP7	UL44	Polymerase accessory protein (nt 54118-56701)
pZP18	UL102	Helicase:primase accessory protein (nt 146036-151125)
pZP20	UL105	Helicase (nt 150312-155121)
pZP29	UL70	Primase (nt 99700-103827)
pZP33	UL54	DNA polymerase (nt 76642-81435)
pZP37	UL57	single-stranded DNA binding protein (nt 78262-92576)

The nucleotide coordinates used here, and throughout this thesis, are from the published sequence for HCMV strain AD169 in GenBank accession number X17403, (http://www2.ncbi.nlm.nih.gov/irx/cgi-bin/birx_doc?genbank+5340494), unless otherwise noted.

pMA1 contains a 6.8 kbp oriLyt fragment of HCMV strain Towne in pUC18, this was obtained from M McVoy, Department of Pediatrics, Medical College of Virginia, Richmond Virginia.

pCMV10 is based on pUC19, and contains a multiple cloning site immediately downstream of the human cytomegalovirus major immediate early promoter, as well as simian virus 40 (SV40) splicing and polyadenylation signals (Stow *et al.*, 1993). pElacZ contains a β-galactosidase open reading frame cloned into pCMV10.

Plasmids expressing the HSV-1 core replication machinery were obtained from N Stow. The following HSV-1 replication plasmids consist of the open reading frame cloned from HSV-1 strain 17 +, into pCMV10 (Stow *et al.*, 1993):

pE5	HSV-1 UL5	helicase
pE8	HSV-1 UL8	helicase-primase accessory factor
pE29	HSV-1 UL29	single stranded DNA binding protein
pE30	HSV-1 UL30	polymerase
pE42	HSV-1 UL42	polymerase accessory factor
pE52	HSV-1 UL52	primase

Plasmids containing fragments of VZV (Dumas strain) DNA were obtained from N Stow, Institute of Virology, Glasgow. pVO2 containing a VZV lytic origin of DNA replication cloned into pAT153 has been previously described (Stow & Davison, 1986). pEV51 and pEV51R contain the open reading frame for the VZV origin binding protein in the forward or reverse (pEV51R) orientation expressed from pCMV10.

pCMV10, pElacZ and pAT153 (derived from pBR322) were obtained from N Stow, Institute of Virology, Glasgow. A pAT153 derived plasmid containing the *Hin*dIII D fragment of HCMV DNA was obtained from D Dargan, Institute of Virology, Glasgow.

2.1.5 Eukaryotic cells and culture media

Tissue culture media, antibiotics and supplements were obtained from Gibco BRL unless stated otherwise. Foetal calf serum (catalogue number 10106-169) and new-born calf serum (catalogue number 16010-084) were obtained from Gibco BRL and are mycoplasma and virus free. Most cell lines were obtained from J Mitchell in the cytology department in the Institute of Virology.

The original source of the cells and the passage numbers at which they were used in experimental work are listed:

BHK 21 C13	Syrian hamster kidney fibroblasts
	from N Stow, used at passage 25-50
CV-1	African green monkey kidney fibroblasts
	from ECACC, used up to passage 30 from recovery
HFL1	human embryonal lung fibroblasts
	from Flow, used at passage 21-40
HFFF2	human foetal foreskin fibroblasts
	from ECACC, used at passage 15-40
U-373 MG	human glioblastoma astrocytoma
	from ECACC, used up to passage 30 from recovery
293	human embryonal kidney cells transformed with sheared Ad5 DNA
	from G Brown, used at passage 10-30
Vero	African green monkey kidney fibroblasts
	from ECACC, used at passage 20-40

The ECACC is the European cell and culture collection at Porton Down, Salisbury.

Wash (DPS) is Dulbecco's modified Eagle's medium supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

HFL1 and HFFF2 were cultured in DPS supplemented with 10% foetal calf serum and 2 mM L-glutamine (DC10). U-373 MG were cultured in DC10 medium supplemented with 1mM sodium pyruvate MEM.

BHK 21 C13, CV-1, Vero and 293 were cultured in BHK medium supplemented with 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin and 10% foetal calf serum (EC10) or 5% foetal calf serum (EC5) during transfections.

Transfection medium is DPS supplemented with 5% foetal calf serum (DC5). Transfection medium was supplemented with 1mM sodium pyruvate for U-373 MG cells.

Human fibroblast (HF) methyl-cellulose overlay is 0.9x Dulbecco's modified Eagle's medium containing 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 1.5 % carboxy-methyl-cellulose, 10 % foetal calf serum, 1 mM sodium pyruvate and 1x non-essential amino acids. BHK 21 C13 methyl-cellulose overlay is 0.9x Glasgow modified Eagle's medium containing 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 1.5 % carboxy-methyl-cellulose, and 5 % new-born calf serum.

Trypsin is 0.25% trypsin in Tris-buffered saline (TS) supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 15 μg ml^{-1} phenol red. Versene is 0.02% EDTA and 15 μg ml^{-1} phenol red in phosphate-buffered saline A (PBS-A). Versene/trypsin is a 4:1 (v/v) mixture.

Storage medium is Dulbecco's modified Eagle's medium (or Glasgow modified Eagle's medium for BHK 21 C13 cells) with foetal calf serum and glycerol in a 5:4:1 v/v ratio.

2.1.6 Viruses

The following herpesvirus strains were used in the experimental work:

Herpes simplex virus type 1 strain 17+ was obtained from Institute of Virology stocks.

Cytomegalovirus strains AD169 and Towne were obtained from D Dargan. Cytomegalovirus strain Colburn was obtained from A S Tyms, London. Cytomegalovirus strain Colburn was originally isolated from a human brain (Huang *et al.*, 1978). Despite its isolation from a human brain, the virus is most similar to an African green monkey cytomegalovirus and it is usually referred to as SCMV (Gibson, 1983).

2.1.7 Prokaryotic cells and culture media

Most plasmids were maintained in *Escherichia coli* strain DH5α. Bacteria used for the production of unmethylated plasmid DNAs were *Escherichia coli* strain SCS110 which has mutant alleles for DNA adenine methylase and DNA cytosine methylase. Competent SCS110 cells were obtained from Stratagene, UK.

L-broth is 1% w/v NaCl, 1% w/v Difco tryptone, 0.5% w/v Difco yeast extract in distilled water. LB agar is L-broth containing 1% w/v Difco bacto agar.

2.1.8 Solutions

The compositions of solutions in routine use are described:

blot wash	2x SSC, 0.1% w/v SDS
boiling mix	6% w/v SDS, 30% v/v stacking gel buffer, 30% v/v glycerol, 0.3% w/v bromophenol blue, 210 mM β-mercaptoethanol
calf thymus DNA (blotting)	2 mg ml^{-1} in TE pH 8.0, denatured by boiling for 20 minutes
calf thymus DNA (transfection)	3 mg ml^{-1} in TE pH 8.0, proteinase K treated

2x cell lysis buffer (2x CLB)	10 mM Tris-HCl (pH 7.5), 1.2% w/v SDS, 1 mM EDTA (pH 8.0)
Chloroform:isoamyl alcohol	24:1 chloroform: isoamyl alcohol
50x Denhardt's solution	1% w/v BSA, 1% w/v ficoll 400, 1% w/v polyvinylpyrrolidone
DNase dilution buffer	50% v/v glycerol, 50 mM NaCl
5x DNA loading dyes	5x running buffer with 50% w/v sucrose, 0.25% w/v bromophenol blue
gel soak I	0.6 M NaCl, 0.2 M NaOH
gel soak II	0.6 M NaCl, 1.0 M Tris-HCl (pH8.0)
hybridisation mix (final concentrations)	5x Denhardt's solution, 0.05% w/v SDS, 1 mM EDTA (pH8.0), 6x SSC, 20 mMTris-HCl (pH7.5), 50 $\mu g\ ml^{-1}$ calf thymus DNA (blotting)
hepes buffered saline (HeBS)	21 mM hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5.5 mM D(+)glucose, adjusted to pH 6.95 - 7.15 with NaOH
IE1/IE2 wash (PBS-T)	0.005% Tween in PBS
IE1/IE2 antibody diluent	1% (w/v) BSA in IE1/IE2 block
IE1/IE2 block	5% (w/v) Marvel in PBS-T
IF fix	2 % (v/v) formaldehyde, 2 % (v/v) sucrose in PBS
IF permeabilisation buffer	0.5 % NP40 (v/v), 10 % (w/v) sucrose in PBS
lacZ fix	2 % (v/v) formaldehyde, 0.2 % (v/v) glutaraldehyde in PBS

lacZ stain	5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.5 mg ml^{-1} X-Gal in PBS
20x Loening's buffer	815 mM NaH_2PO_4, 720 mM Tris, 20 mM EDTA
10x nick translation buffer (10x NTB)	500 mM Tris-HCl (pH 7.5), 10 mM DTT 100 mM $MgSO_4$, 0.1 mM dATP, 0.1 mM dTTP, 0.5 mg ml^{-1} BSA
phosphate buffered saline A (PBS-A)	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4
phosphate buffered saline complete (PBS)	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 6.8 mM $CaCl_2$, 4.9 mM $MgCl_2$
pre-hybridisation buffer	6x SSC, 5x Denhardt's solution, 0.1% w/v SDS, 20 µg ml^{-1} calf thymus DNA (blotting)
protease (Sigma XIV)	20 mg ml^{-1} in TE (pH 7.5), pre-digested for 1 hour at 37°C
proteinase K	20 mg ml^{-1} in TE (pH 7.5)
reticulocyte standard buffer (RSB)	10 mM Tris-HCl (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$
200x RNase	10,000 U ml^{-1} ribonuclease T1, 1 mg ml^{-1} ribonuclease A in TE (pH 7.5)
Resolving gel buffer (4x)	1.5 M Tris-HCl (pH 8.9), 0.4% w/v SDS
Sample buffer	30% v/v boiling mix
Stacking gel buffer (4x)	488 mM Tris-HCl (pH 6.8), 0.4% w/v SDS

20x standard saline citrate (20x SSC)	3 M NaCl, 300 mM trisodium citrate
STET buffer	8% w/v sucrose, 0.5% v/v Triton X-100, 10 mM Tris-HCl (pH8.0), 50 mM EDTA
sucrose reagent	250 mM sucrose, 2 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5)
50x TAE	2 M Tris-acetate, 100 mM EDTA
Tank buffer	52 mM Tris, 53 mM glycine, 0.1% w/v SDS
10x TBE	890 mM Tris, 10mM EDTA, 890 mM boric acid
TE (pH 7.5)	10 mM Tris-HCl (pH 7.5), 1 mM EDTA
TE (pH8.0)	10 mM Tris-HCl (pH8.0), 1 mM EDTA
transformation and storage buffer (TSB)	10% w/v PEG 3350, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in L-broth
Tris buffered saline (TS)	137 mM NaCl, 5 mM KCl, 5.5 mM D(+)glucose, 0.7 mM Na_2HPO_4, 25 mM Tris-HCl (pH 7.4)
Triton reagent	0.5% v/v Triton X-100, 62.5 mM EDTA (pH 8.0), 50 mM Tris-HCl (pH 8.0)

2.1.9 Antibodies

The following antibodies and detection reagents were obtained from Sigma Immunochemicals (unless otherwise indicated) and were used at the stated dilutions.

A polyclonal antibody to the HCMV major immediate early proteins was obtained from D Dargan, Institute of Virology, Gasgow. This was used at a 1:5 dilution.

Protein-A peroxidase: 1:1000

Goat anti-mouse IgG (Whole molecule) Cy^{TM}3 conjugate from Amersham Pharmacia Biotech: 1:500

D42 is a mouse monoclonal antibody to HCMV UL84 protein obtained from E S Huang, Department of Medicine, University of North Carolina at Chapel Hill, North Carolina (unpublished). This was used in immunofluorescence at a 1:500 dilution.

2.1.10 Miscellaneous materials

Hybond-N and sephadex G-50 were obtained from Amersham Pharmacia Biotech. 3 mm filter paper was obtained from Whatman International Limited.

The phosphorimaging screens and scanner system were from BioRad.

Dialysis tubing was obtained from Medicell International Limited.

BEEM capsules and reagents used in preparation of samples for electron microscopy were from TAAB laboratories.

2.2 Methods

2.2.1 Growth and maintenance of eukaryotic cells

Cell culture

Cells were grown in 175 cm^2 flasks containing 50 ml of the appropriate medium. Unless otherwise stated, cells were maintained at 37°C in an atmosphere containing 5% CO_2. Cells were harvested at 95-100% confluency by washing once with versene and once with versene/trypsin (293 cells were given two versene washes without trypsin) followed by resuspension in 9 ml of growth medium. Cells were split 1:8-1:6 for faster growing cells (BHK 21 C13 and Vero) or 1:6-1:4 for slower growing cells (CV-1, HFL1, HFFF2, U-373 MG and 293).

Cells were seeded into 35 mm petri dishes at approximate densities of 2x10^5 (Vero, or BHK 21 C13), 4x10^5 (293 or CV-1) or 6x10^5 (HFFF2, HFL1 or U-373 MG) cells in 2

ml growth medium (DC10 or EC10) to be approximately 80 - 90% confluent at the time of transfection or infection. Cells were incubated for approximately 16-24 hours prior to use.

Cells for use in immunofluorescence were seeded into 35 mm petri dishes containing sterile coverslips (13 mm diameter). Cells were routinely screened for the presence of mycoplasma by J Mitchell, and were never found to contain any mycoplasma.

Cell storage and recovery

Cells were harvested at 85-90% confluency, as described above. Cells were resuspended in 10 ml of storage medium and aliquoted in 1.5 ml amounts into cryo-storage vials and frozen slowly at -70°C before being transferred to liquid nitrogen for long term storage.

Cells were recovered from liquid nitrogen by warming to room temperature and seeding into a 25 ml flask containing 10 ml of growth medium and incubated as described.

2.2.2 Growth and maintenance of virus stocks

Growth, titration and storage of cytomegalovirus strains AD169, Towne and Colburn

Growth medium from flasks of HFFF2 cells at 80-90% confluency was replaced with 50 ml fresh growth medium containing approximately $1x10^5$ p.f.u. of the appropriate strain of cytomegalovirus and incubation was continued. At three days post infection, the inoculum was discarded and 30 ml of fresh medium was added. Medium containing virus was collected at 9, 11, 13 and 15 days post infection by decanting into sterile tubes. Fresh medium was added to each flask. Cell debris was pelleted by centrifugation at 2000 rpm (Beckman GPR centrifuge) for 10 minutes at room temperature. Virus was aliquoted and stored at -70°C in glass vials.

High titre stocks of AD169 were obtained by pelleting virus for 2 hours at 12000 rpm in a GSA rotor (Sorvall) and resuspending the pellet in 1/30th the original volume DC10.

Stocks of AD169 and Towne were not obviously unstable over several cycles of freeze/thawing. Colburn stocks were less stable and once thawed were not refrozen.

Cytomegaloviruses were titrated on 90% confluent HFFF2 monolayers in 35 mm dishes. Cells were infected with 100 µl of virus serially diluted in DPS. Adsorption was for 45 minutes at 37°C. Following adsorption, cells were overlayed with 2 ml growth medium and incubation was continued for 5-7 days. When cytopathic effect was first observed in cells infected at the highest multiplicity, growth medium was replaced with 2 ml of HF methyl-cellulose overlay and incubation was continued for a further 5-7 days. Cells were stained by direct addition of 1.5 ml Giemsa stain containing methanol to the methyl-cellulose overlay followed by incubation for 48 hours at 4°C. Plaques were counted on a dissecting microscope following removal of Giemsa stain and inactivated virus by washing with water.

Growth, titration and storage of HSV-1

Medium from BHK 21 C13 cells at approximately 80% confluency in 175 cm^2 flasks was replaced with 4 ml of DC5 containing approximately $4x10^6$ pfu of HSV-1 strain 17+. Virus was adsorbed to the monolayer for 45 minutes at 37°C. 40 ml medium was added per flask and incubation was continued for approximately four days at 31°C.

Cells were harvested by gently tapping the flask then decanting the medium into a sterile falcon tube. Cell debris was pelleted by centrifugation at 2000 rpm (Beckman GPR centrifuge). Supernatant was decanted into a sterile GSA bottle. Virus was pelleted from the supernatant by centrifugation at 12000 rpm (Sorvall GSA rotor) for 2 hours at 4°C. The pellet was resuspended by sonication in 1/10th volume of DC5. Aliquots were frozen and stored at -70°C in glass vials as cell released virus (CRV).

The cell pellet was resuspended in 1/5th volume of EC5. The resuspended cells were extensively sonicated to release cell associated virus (CAV). CAV was cleared by centrifugation for 2 minutes at 2000 rpm (Beckman GPR centrifuge), supernatant was

aliquoted and stored in glass vials at -70°C.

Monolayers of BHK 21 C13 cells were infected with 100 µl of virus serially diluted in wash. Adsorption was for 45 minutes at 37°C and 5% CO_2. Following adsorption, cells were overlayed with 2 ml BHK methyl-cellulose overlay and incubation was continued for a further 2-3 days. Overlay was removed and cells were stained by addition of Giemsa stain to the plates for 48 hours at 4°C. Plaques were counted on a dissecting microscope following removal of Giemsa stain by washing with water.

2.2.3 Growth, maintenance and manipulation of prokaryotic cells

Growth and storage of E. coli

Starter cultures of *E. coli* strain DH5α or SCS110 were grown overnight at 37°C by inoculation of 5 ml L-broth, containing appropriate antibiotic, with approximately 5 µl of a DMSO stock. DMSO stocks were prepared by freezing 900 µl saturated bacterial culture with 100 µl DMSO. DMSO stocks were stored at -70°C.

Preparation and transformation of competent E. coli (Chung *et al.*, 1988)

An overnight culture of bacteria grown in the absence of antibiotic was used to inoculate 50 ml of L-broth. The culture was grown at 37°C with shaking until the exponential growth phase was reached. Bacteria were pelleted in sterile falcon tubes at 2500 rpm (Beckman GPR centrifuge) for 10 minutes at 4°C. Supernatant was discarded and the bacterial pellet was resuspended in 1/10th volume of ice-cold TSB:DMSO and incubated at 4°C for 10 minutes. Competent bacteria were immediately aliquoted (100 µl) into sterile vials and flash-frozen in dry ice with ethanol. Aliquots were stored at -70°C until required.

Competent bacteria were thawed by hand. Plasmid DNA was added to the cells (no more than 1 µg per aliquot) and cells were incubated at 4°C for 30 minutes. 900 µl of TSB:DMSO:glucose was added to the cells which were subsequently incubated at 37°C

with shaking for one hour. Bacteria were plated onto LB agar plates containing an appropriate antibiotic, and transformants were selected.

Storage and transformation of commercially available cells

Competent *E. coli* strain SCS110 cells were used to produce unmethylated plasmid DNA. These cells were stored at -70°C until required. Cells were transformed according to the manufacturer's (Stratagene) protocol, and transformants were selected.

Selection of transformed bacteria

Transformed bacteria were plated onto solid LB agar plates containing an appropriate antibiotic and incubated overnight at 37°C. Overnight cultures were then set up by 'picking' colonies into L-broth containing an appropriate antibiotic. DNA was prepared by the STET method (as described in section 2.2.6), then screened by restriction endonuclease analysis.

2.2.4 Manipulation of plasmid DNA

The following is an outline of the techniques used to produce the novel plasmids described in this thesis. The origin-containing plasmids, pCMVori, pCMVoriΔXhoI, pADori, and pADoriΔXhoI, are based on pCMV10 and pAT153 and contain HCMV DNA fragments subcloned from the *Hin*dIII D fragment of HCMV strain AD169 obtained from D Dargan. These origin-containing plasmids are described in figure 2.1.

Restriction enzyme digestion for cloning DNA fragments

Approximately 20 μg of plasmid DNA was incubated for 3 hours with 50 units of the desired restriction enzyme in 100 μl of the appropriate restriction buffer (1x) and at the optimum temperature for the enzyme. The digest was extracted once with phenol, once with chloroform:isoamyl alcohol, then finally ethanol precipitated and resuspended in TE pH 7.5.

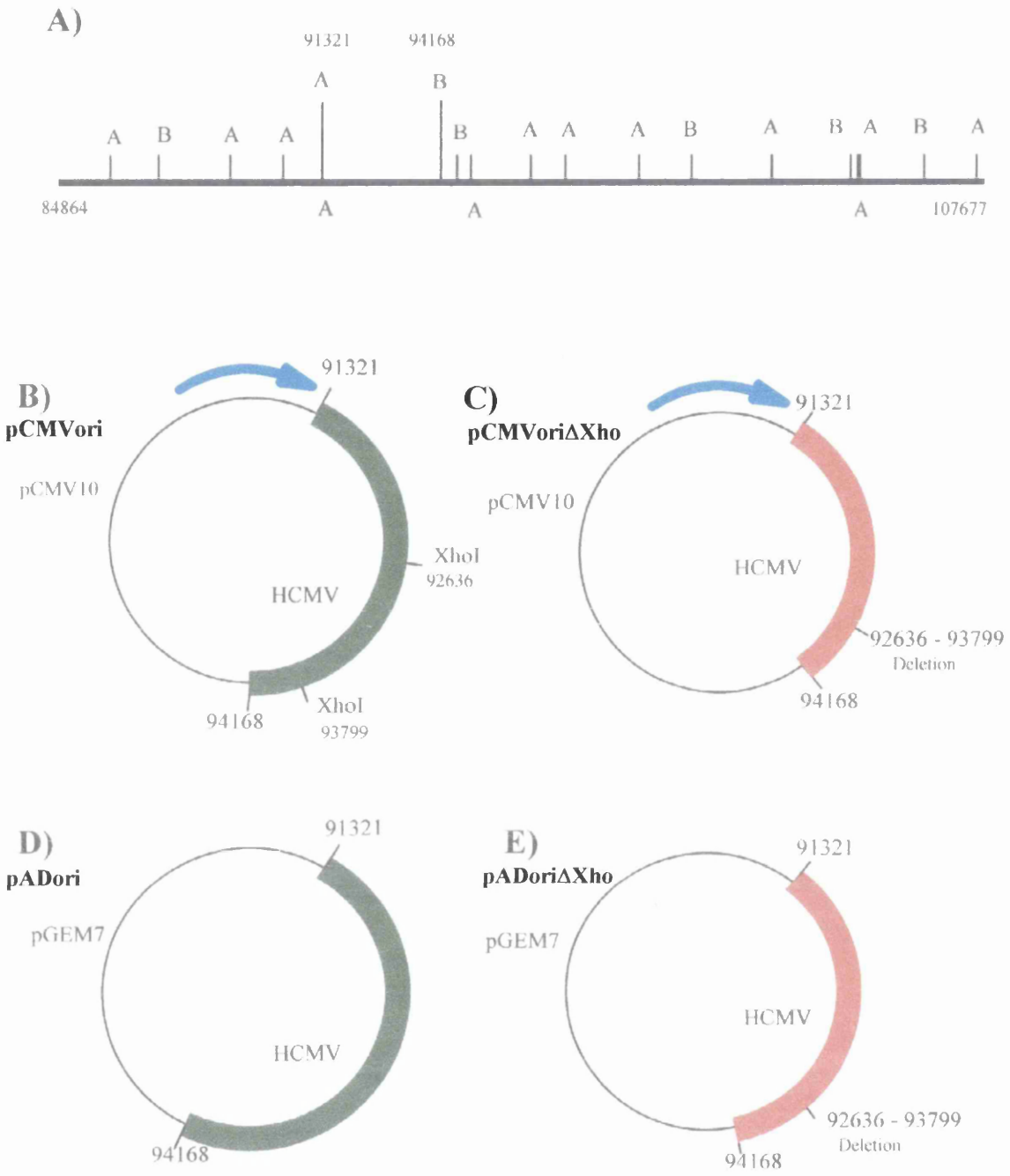


Figure 2.1 HCMV origin containing plasmids.

A) The HCMV *Hin*dIII D fragment spans nt 84864 - 107677. The location of recognition sequences for *Aat*II (A) and *Bst*EII (B) are shown.

B) The 2.8 kbp *Aat*II-*Bst*EII fragment (nt 91321-94168) was inserted into *Hin*dIII digested pCMV10 to generate plasmid pCMVori.

C) pCMVori was digested with *Xho*I to remove a 1.2 kbp fragment (nt 92636-93799), generating plasmid pCMVoriΔXho

D) The 2.8 kbp of HCMV DNA spanning oriLyt were subcloned from pCMVori into *Hin*dIII digested pGEM7, creating plasmid pADori.

E) The 1.4 kbp of HCMV DNA spanning the deleted oriLyt were subcloned from pCMVoriΔXho into *Hin*dIII digested pGEM7, creating plasmid pADoriΔXho.

The orientation of the HCMV major immediate early promoter in pCMV10 based plasmids is denoted by a blue arrow.

Blunt ending

Digested DNA was incubated for 30 minutes at 37°C with 3 units of T4 DNA polymerase in 50 µl of 1x T4 DNA polymerase buffer containing 100 µg/ml BSA and 0.2 mM each of dATP, dTTP, dCTP, and dGTP. The digest was extracted once with phenol, once with chloroform:isoamyl alcohol, and finally ethanol precipitated and resuspended in TE pH 7.5.

Linker ligation

Approximately 5 µg of blunt ended DNA was incubated overnight at room temperature with 2 units of T4 DNA ligase in 1x T4 DNA ligase buffer with approximately 0.5 µg of linker DNA. The reaction volume was increased to 130 µl with distilled water and the ligase was inactivated by heating to 70°C for 10 minutes.

Preparation of required enzyme fragment

DNA was digested for three hours at the appropriate temperature with 50 units of restriction enzyme in 150 µl of the appropriate restriction enzyme buffer.

DNA fragments were then separated on 1% agarose gel in TAE (as described in section 2.2.7). The gel was examined under long wave ultra violet (UV) light and a block of the gel containing the desired fragment was cut out and transferred to clean dialysis tubing with 1 ml of fresh TAE. The DNA fragment was electroeluted from the agarose slice at 200 V for 2 hours.

The DNA was purified on a 0.4 ml DEAE-Sephacel column. The DEAE-Sephacel was washed with 3 ml TE (pH 7.5) containing 0.1 M NaCl. Electroeluted DNA in TAE was loaded onto the column, washed with 5 ml TE (pH 7.5) containing 0.1 M NaCl, and eluted with two 0.4 ml aliquots of TE (pH 7.5) containing 1 M NaCl. DNA was concentrated by ethanol precipitation and resuspension in a small volume of TE pH 7.5

Preparation of vector

5 μg of plasmid vector was digested for 3 hours at 37°C with 30 units of restriction enzyme and 1 unit of calf intestinal phosphatase in 50 μl of 1x restriction enzyme buffer. EGTA was added to a final concentration of 20 mM and the digest was extracted once with phenol, once with chloroform:isoamyl alcohol, then ethanol precipitated and the DNA resuspended in TE pH 7.5.

Ligation of fragment and vector

Approximately 1 μg of vector and 1 μg of fragment were incubated overnight at 4°C with 1 unit of T4 DNA ligase in 20 μl of 1x NEB ligase buffer. The ligation was extracted once with phenol, once with chloroform:isoamyl alcohol, ethanol precipitated and the DNA was resuspended in TE pH 7.5.

2.2.5 Preparation of mutant pSVH and pZP8 plasmids

pSVH was used in the generation of pSVHΔXhoI, pSVHΔBglII, pSVHΔSexAI and pSVHΔBglIIΔSexAI. pZP8 was used in the generation of pZP8ΔBstEII, pZP8ΔEcoNI and pZP8ΔRsrII. Plasmid pZP8ΔRsrII was generated by digesting pZP8 to completion with *Rsr*II, then religating. The plasmid was transformed into competent bacteria as described above. The pSVH mutants and the remaining pZP8 mutants were generated by inserting 'amber' *Xba*I linkers at the named restriction enzyme site. The 'amber' *Xba*I linkers used in this thesis have the sequence CTAGTCTAGAGACTAG. The linkers contain an *Xba*I restriction site (TCTAGA) and the UAG 'amber' stop codon in each of the three forward (and three reverse) reading frames, thus forcing the termination of protein synthesis. The insertion of these linkers was confirmed by *Xba*I digestion of the mutant plasmids produced. The pZP8 and pSVH mutants are described in figure 4.10 and 4.13.

Insertion of 'amber' linkers

Approximately 5 μg of plasmid DNA was digested with the appropriate restriction enzyme, blunt ended and phosphatased as described. 1 μg of digested DNA was incubated overnight at room temperature with 2 units of T4 DNA ligase and 0.5 μg of phosphorylated *XbaI* amber linkers in 50 μl of 1x T4 DNA ligase buffer. The ligation was extracted once with phenol, once with chloroform:isoamyl alcohol, ethanol precipitated and resuspended in TE pH 7.5. Bacteria were transformed with plasmid DNA as described above (section 2.2.3).

2.2.6 Preparation of plasmid DNA

STET method for DNA extraction from bacterial cultures (Sambrook *et al.*, 1989)

Bacteria were pelleted from 1 ml of an overnight culture by centrifugation for 2 minutes at 13000 rpm (MSE microfuge). Supernatant was discarded and bacteria were resuspended in 100 μl STET buffer. Bacterial suspensions were digested for approximately 30 seconds at room temperature with approximately 1.4 μg/ml of lysozyme. Samples were boiled for 1 minute. Cell debris was pelleted by centrifugation for 10 minutes at 13000 rpm (MSE microfuge). Cell debris was removed and DNA was precipitated with 100 μl isopropanol then resuspended at 37°C for 30 minutes in 2x RNase in TE pH 8.0.

Large-scale preparation of plasmid DNA

An overnight culture (4 ml) of bacteria grown at 37°C in L-broth containing a suitable antibiotic was inoculated into 350 ml of LB plus antibiotic in a 2 l conical flask. The culture was incubated at 37°C overnight with shaking. Bacteria were pelleted at 8000 rpm for 10 minutes (Beckman GSA rotor). The bacterial pellet was washed in TE pH 8.0 and repelleted at 5000 rpm for 5 minutes (Beckman SS34 rotor). The pellet was resuspended in 2 ml sucrose reagent then digested with approximately 2.5 mg ml^{-1} lysozyme for 30 minutes at 4°C. Triton reagent (3.2 ml) plus 0.25 M EDTA pH 8.0 (0.8 ml) was

added to lysed cells and incubation was continued at 4°C for 15 minutes.

Cellular debris was removed by pelleting at 35000 rpm (Sorvall T865 rotor) for 30 minutes at 4°C. The supernatant was decanted into a clean 15 ml tube, 200 µl of ethidium bromide (10 mg ml^{-1} in water) was added and the volume made up to 7.5 ml with sterile water. Caesium chloride (7.5 g) was added to give a final density of 1.55-1.60 g ml^{-1}. The DNA solution was transferred to a T1270 tube and the tube was filled with liquid paraffin and sealed. Gradients were centrifuged at 44000 rpm (Sorvall T1270 rotor) at 16°C for at least 36 hours.

After centrifugation, two bands were clearly visible on the gradient; the upper band consisted of open circle and nicked DNA, the lower band was supercoiled plasmid DNA. The supercoiled plasmid DNA was extracted with an 18 gauge needle and 2 ml syringe and was transferred to a clean 5 ml tube. Ethidium bromide was removed from the plasmid DNA by four extractions with 1.5 ml isoamyl alcohol. Caesium chloride was removed by two rounds of dialysis against 3 litres of TE pH 7.5. The concentration of plasmid DNA was determined on a spectrophotometer by measuring absorbance at 260 nm, using the fact that an absorbance of 1.0 at 260 nm corresponds to a concentration of 50 µg/ml of double-stranded DNA. The identity of the purified plasmid DNA was confirmed by restriction enzyme analysis.

2.2.7 Agarose gel electrophoresis

Agarose was dissolved by boiling in the appropriate buffer. The dissolved agarose was cooled to hand hot then ethidium bromide was added to a final concentration of 0.5 µg/ml. The gel was poured into a mould with a comb and allowed to set. The comb was removed from the gel and the gel was placed in a gel tank containing the appropriate buffer. DNA samples were mixed with loading buffers to give the samples the same composition as the gel running buffer and samples were loaded into wells. Gels were run at 10 to 50V for 3 hours to overnight.

2.2.8 Calcium phosphate transfection of mammalian cells

Calcium phosphate transfection with hepes-buffered saline (adapted from Stow & Wilkie, 1976)

Cells for transfection were set up as previously described. In a sterile 15 ml tube, 26 μg DNA (plasmid with calf thymus carrier DNA) was mixed with 1 ml of hepes-buffered saline (HeBS). 56 μl of 2.5M $CaCl_2$ was added per ml of HeBS, and a precipitate was allowed to form for five minutes at room temperature while medium was removed from cell monolayers. When multiple plates were to receive the same precipitate, the reaction was scaled up appropriately. 0.4 ml of precipitate was added to each monolayer and incubation was continued for 40 minutes. 2 ml of DC5 was added to each monolayer and incubation was continued. Four hours after transfection (h p.t.), cells were washed and 'boosted' as described below.

Medium was removed from 293 cells, the monolayers were washed twice with 2 ml of DPS, and incubation was continued in DC5. U-373 MG cells were boosted by removing medium, washing with DPS, incubating with 1 ml of 15% v/v glycerol in HeBS for 2 minutes at room temperature. Glycerol was removed and the cells were washed.

All other cells were 'boosted' by removing medium, washing with DPS, incubating for 5 minutes at room temperature with 25% (v/v) DMSO in HeBS, followed by washing once with DPS. DC5 was added to boosted monolayers (2 ml per dish) and incubation was continued.

Preparation of calf thymus DNA for transfection

Calf thymus DNA was dissolved in TE pH 8.0 at 3 mg ml^{-1}. Proteinase K was added to a final concentration of 100 μg/ml and SDS was added to a final concentration of 5 % (w/v). Proteinase digestion was at 55°C for one hour. After digestion, NaCl and EDTA were added (to final concentrations of 0.3 M and 5 mM respectively), then the DNA

was extracted with equal volumes of phenol, phenol:chloroform:isoamyl alcohol (25:24:1), chloroform:isoamyl alcohol. DNA was finally precipitated with 2.5 volumes of ethanol overnight at -20°C, then pelleted by centrifugation at 8000 rpm and 4°C for 30 minutes in a Beckman SM24 rotor. The DNA pellet was washed with 70 % ethanol:30 % 0.3 M sodium acetate (pH 7.0) then vacuum dried and resuspended in 1x RNase in TE (pH 7.5) at a final concentration of 3 mg ml^{-1}.

Optimisation of conditions by β-galactosidase assay

The optimum pH of HeBS used in transfection was determined for each batch of buffer prepared. Generally, five 200 ml samples in the pH range 6.95 - 7.15 were removed as the pH of a batch of HeBS was adjusted with NaOH. Transfection efficiency of the samples was compared by transfection of pElacZ into cells followed by staining for β-galactosidase activity. The buffer which gave the best transfection efficiency was used in subsequent experiments. The optimum DNA concentration in the precipitate was similarly determined by staining for β-galactosidase activity.

In situ β-galactosidase assay

Medium was removed from monolayers and the cells were washed with 2 ml PBS. Cells were fixed at room temperature for 5 minutes with 1 ml of lacZ fix. Fix was removed and cells were washed with PBS. 1 ml of lacZ stain was added to each 35 mm dish and plates were incubated overnight at 37°C. Cells were examined by light microscopy and the number of positive cells (blue cells) per field of view was counted. Five fields of view (approximately 3 mm^2) were counted for each 35 mm dish, and the average number of positive cells was determined. Transfection efficiencies were typically in the range of 1-2% for HFFF2, HFL1 and CV-1 cells, in the range of 2-5% in Vero, BHK 21 C13 and U-373 MG cells, and in the range of 25-30% in 293 cells.

2.2.9 Transient replication assay

Cells were transfected as described above. Infection was done at 6 hours post transfection. Medium was removed from monolayers and virus was added in a final volume of 100 µl. 293 cells were infected in a final volume of 500 µl. Mock infected plates were treated with wash without virus. Following adsorption of virus for 45 minutes, 2 ml of DC5 was added to each monolayer and incubation was continued as required.

Total-cellular DNA preparation

Medium was removed and cells were washed with 2 ml TS. Protease digestion was carried out at 37°C with 0.5 mg/ml protease XIV in 1x CLB for 3-6 hours. NaCl was added to 0.3 M and EDTA was added to 3.6 mM. Lysates were sequentially extracted with an equal volume of phenol then chloroform:isoamyl alcohol and precipitated overnight with 2.5 volumes of ethanol at -20°C. DNA was pelleted by centrifugation at 8000 rpm and 4°C for 20 minutes in a Beckman SM24 rotor. The DNA pellet was washed with 70 % ethanol:30 % 0.3 M sodium acetate (pH 7.0) then vacuum dried and resuspended in 1x RNase in TE (pH 7.5). Approximately 25% of the DNA from a 35 mm plate was digested for analysis by Southern blot.

Preparation of cell nuclei

Cells were scraped into the supernatant medium then transferred to a clean 10 ml tube and pelleted for 2 minutes at 1000 rpm (Beckman GPR centrifuge) at room temperature. Cells were resuspended by vortexing in RSB containing 0.5% (v/v) NP40. Nuclei were pelleted for 3 minutes at 1000 rpm at room temperature. Cytoplasmic supernatant was discarded and the nuclear pellet was resuspended in 2 ml RSB, repelleted and the supernatant discarded. Nuclei were resuspended in 1 ml TE (pH 7.5) and digested in 1x CLB with 0.5 mg/ml protease XIV at 37°C for 3 - 6 hours. DNA was extracted as described above.

2.2.10 Preparation of radio-labelled probe (Rigby *et al.*, 1977)

250 ng of plasmid DNA was incubated with 10 μCi each of α ^{32} P dCTP and α ^{32} P dGTP, 2 units of DNA polymerase I and $1x10^{-7}$ mg DNaseI at 16°C for 90 minutes in a final volume of 30 μl of 1x NTB (containing 'cold' dATP and dTTP).

The reaction mixture was extracted once with an equal volume of phenol. The radiolabelled probe was separated from unincorporated nucleotides by column chromatography over G50 Sephadex. Plasmid was denatured by incubation in 0.16 M NaOH at room temperature for 10 minutes, neutralised with HCl and the probe was added to 8.6 ml of hybridisation buffer heated to 68°C and used immediately.

2.2.11 Southern blotting (Southern, 1975)

Suitably digested DNA samples were separated by electrophoresis at 20-40 V for 16-24 hours through a 0.8% (w/v) agarose slab gel in Loening's buffer containing 0.5 μg/ml ethidium bromide. The gel was photographed under long wave UV light.

DNA was denatured by shaking the gel for 45 minutes in gel soak I. The alkali was neutralised by shaking the gel for 45 minutes in gel soak II. DNA was transferred overnight onto nitrocellulose membrane by capillary blotting with 6x SSC at room temperature.

The membrane was marked, rinsed with 6x SSC then crosslinked by exposure to 120 mJ/cm UV light. Blocking of the membrane was with 100 ml pre-hybridisation buffer in a sealed hybridisation bag at 68°C with shaking for at least 2 hours.

The pre-hybridisation fluid was discarded, the hybridisation mix was added using an 18 gauge needle and 10 ml syringe. The bag was resealed and hybridisation was carried out for 16-24 hours at 68°C with shaking.

The hybridisation fluid was discarded and the blot was washed three times at 68°C with shaking for 20 minutes each with 300 ml blot wash. The blot was rinsed with water

then blotted dry.

The blot was then exposed to a BioRad FluorS phosphoimaging screen. The image was developed and manipulated on a BioRad Personal FX FluorS MultiImager and the associated software.

2.2.12 Quantitation of DNA in bands from Southern blotting

A selection box was drawn around a marker band on a blot to be quantified. The box was then copied over different areas of the blot to be quantified, including an area of background. The software calculated the 'counts' in each boxed region and a report was printed. For data presentation, the background was subtracted from the signal for each boxed region. The number of counts generated by the marker bands (corresponding to a known amount of DNA) was used to calculate the amount of DNA in a test area. Measurements were only taken from exposures where no saturation of pixels had occurred.

2.2.13 Western blotting to detect HCMV major immediate early proteins

Protein samples were mixed with 1/5 volume of boiling mix then boiled for 2-3 minutes prior to separation by SDS-PAGE (Marsden *et al.*, 1978). Gels with a 10% running gel (39:1 acrylamide to bisacrylamide) and a 5% stacking gel (19:1 acrylamide to biacrylamide) were run in tank buffer at 150 V using a BioRad 'Mini-protean' kit.

Prior to blotting, the gel, blotting pads, filter paper and membrane were soaked for 10 minutes in chilled Towbin buffer (Towbin et al., 1979). Gels were blotted using a BioRad transfer kit in chilled Towbin buffer at 150V for 45 minutes. The membrane was subsequently blocked, probed and exposed.

The blotted membrane was blocked in IE1/IE2 block for 2 hours at 37^{0}C with gentle agitation. The blocked membrane was washed five times with 50 ml of IE1/IE2 wash at room temperature for a total of 15 minutes.

The membrane was incubated for 2 hours at 37°C with gentle agitation in diluted IE1/IE2 polyclonal antibody. The blot was washed as before.

The membrane was finally incubated in protein-A peroxidase in IE1/IE2 wash with 1% (w/v) BSA for one hour at 37°C. The blot was washed as before with a final wash in PBS followed by deionised water. The membrane was reacted with ECL reagent according to the manufacturers protocols. The blot was exposed to X-Omat film and developed.

2.2.14 Indirect immunofluorescence to detect UL84

Medium was removed from cells and monolayers were washed with 2 ml PBS. Cells were fixed by incubation in IF fix for 10 minutes at room temperature, then washed three times with PBS and permeabilised by incubation in IF permeabilisation buffer for 10 minutes at room temperature. Cells were washed three times for 5 minutes each with PBS:FCS, then incubated for 90 minutes at room temperature with antibody D42 diluted in PBS:FCS. Cells were washed three times for 5 minutes each with PBS:FCS then incubated for 90 minutes at room temperature in the dark with Cy3 conjugated goat anti-mouse antibody diluted in PBS:FCS. After antibody incubation, cells were rinsed with PBS:FCS then washed five times for 5 minutes each with PBS:FCS. Prior to mounting, samples were rinsed twice with PBS then once with deionised water. Coverslips were mounted onto slides with a drop of CitiFluor and then sealed using clear nail varnish.

Samples were examined at 543 nm on a Nikon Microphot fluorescent microscope and images were captured and processed using a 'CCD' camera and the associated IPLab software.

2.2.15 Preparation of samples for electron microscopy (E.M.)

HCMV infected or mock infected cells in petri dishes were scraped into the supernatant media then transferred to clean 10 ml tubes and pelleted for 2 minutes at 1000 rpm (Beckman GPR centrifuge). Supernatant was discarded, the cell pellet transferred, in a small amount of PBS, to a clean BEEM capsule and spun for 3 minutes at 1000 rpm to seat the pellet. Supernatant was discarded and 0.5 ml of E.M. grade glutaraldehyde (2.5% v/v in PBS) was carefully added to each sample. Samples were stored at 4°C until required,

then processed and examined by Jim Aitken on a JEOL 100S microscope.

Chapter 3: HCMV replication in epithelial and non-human fibroblast cells

3.1 Introduction

Human cytomegalovirus growth *in vitro* is almost completely restricted to cell lines of human origin, although low yields of infectious progeny virus have reportedly been obtained from primary chimpanzee skin fibroblasts (Perot *et al.*, 1992). Cell lines fully permissive for HCMV growth *in vitro* can be difficult to culture or manipulate.

Table 1.3 summarised the results of an extensive investigation into the blocks to immediate early gene expression and DNA replication by human (Towne) cytomegalovirus in cells of human, murine and simian origin (LaFemina & Hayward, 1988). Only three cell lines (all human) of the nineteen additional different lines examined supported HCMV Towne DNA replication to a level greater than 3% of the level in fully permissive human fibroblast (HF) cells.

3.2 Results

3.2.1 Replication of an HCMV origin-containing plasmid in HCMV strain AD169 infected Vero cells

Studies into human cytomegalovirus DNA replication might be facilitated if easily manipulated cell lines could be used for experimentation. Sarisky and Hayward (1996) showed that Vero cells transfected with plasmids expressing the HCMV replication proteins, UL44, UL54, UL57, UL70, UL102, UL105, UL84, UL112-113 and IRS1/TRS1, from the SV40 early promoter, and with plasmids expressing IE1/2 and UL36-38 from the HCMV major immediate early promoter, were able to support replication of an HCMV origin-containing plasmid. I therefore re-examined the ability of Vero cells to support HCMV origin-dependent DNA synthesis, initially by testing for their ability to support the replication of a transfected HCMV origin-containing plasmid (pSP50) following

superinfection with HCMV strain AD169.

Duplicate monolayers of Vero cells were set up in 35 mm dishes and transfected with 1.6 μg/dish of pSP50. One dish of cells was infected with 0.25 p.f.u./cell of HCMV, the other dish was mock infected. Total cellular DNA was prepared 116 h p.i. and digested with *Xba*I (which linearises pSP50) and *Dpn*I (which cuts at *dam* methylated GATC sites). The input plasmid DNA used here had *dam* methylated GATC sites as it was prepared from the *dam*+ *E. coli* strain DH5α. DNA which has been synthesised in eukaryotic cells is not *dam* methylated. *Dpn*I digestion is therefore a useful tool, digesting only DNA synthesised in bacteria, thus allowing a distinction to be made between unreplicated, input plasmid (which is degraded by *Dpn*I) and plasmid replicated in eukaryotic cells (which is *Dpn*I resistant).

Fragments were separated by electrophoresis, blotted and hybridised to ^{32}P-labelled pAT153. The blot was exposed to a phosphorimager screen and a phosphorimage (figure 3.1) was acquired using a Bio-Rad Personal Molecular Imager and Quantity One software.

A band resistant to *Dpn*I cleavage, of the same size as linearised input plasmid was detected in DNA from infected cells, demonstrating that Vero cells support the replication of a transfected HCMV origin-containing plasmid upon superinfection with HCMV strain AD169. No replicated pSP50 was detected in mock infected cells. The bands at the bottom of the blot are the result of hybridisation of the probe to the unreplicated, *Dpn*I digested input plasmid.

3.2.2 Time-courses of DNA replication

(a) HCMV strain AD169 time-courses

Following the observation that AD169 infected Vero cells could support the replication of an HCMV origin-containing plasmid, the replication of helper virus genomes was examined. Vero, CV-1, 293, BHK 21 C13, and U-373 MG cells were investigated for

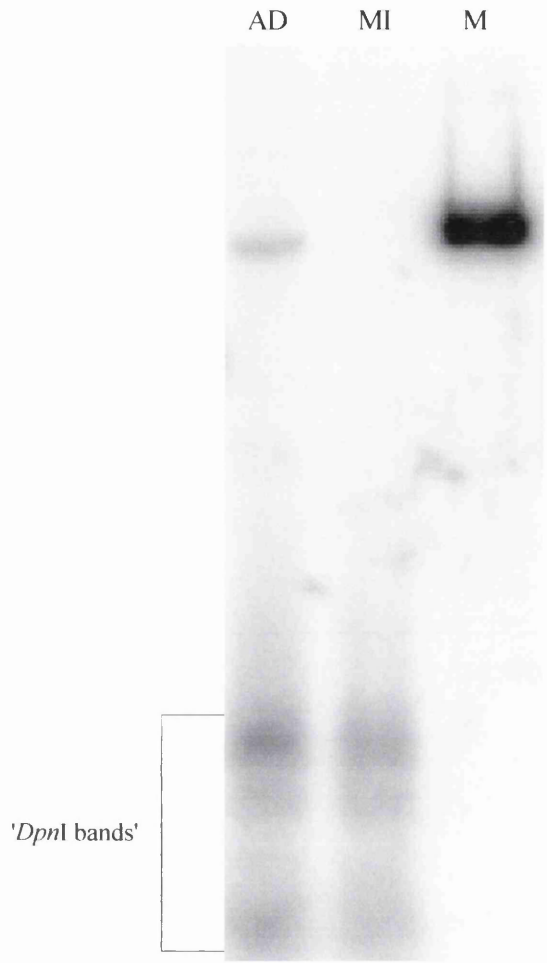


Figure 3.1 Replication of pSP50 in HCMV infected Vero cells.

Vero cells were transfected with 1.6 μg/dish of pSP50. Cells were infected with 0.25 p.f.u./cell of HCMV (AD) or mock infected (MI). DNA was prepared 116 h p.i.. 25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and the blot hybridised to ^{32}P labeled pAT153. '*Dpn*I bands' were produced by *Dpn*I degradation of the unreplicated, *dam* methylated input plasmid DNA.

4 ng of *Eco*RI digested pSP50 was loaded (M).

their ability to support replication of a transfected HCMV origin-containing plasmid and the replication of the superinfecting HCMV strain AD169 genomes. Fully permissive HFFF2 and HFL1 cell lines were also examined.

Vero, CV-1, 293, BHK 21 C13, U-373 MG, HFFF2 and HFL1 monolayers were set up and transfected as described. Cells were transfected with 1.6 μg/dish of pSP50 and five dishes for each cell line were superinfected with 1 p.f.u./cell of HCMV. Infected cells were harvested at 0, 24, 72, 124 and 168 h p.i.. One dish for each cell line was mock infected and harvested at 168 h p.i..

Total cellular DNA was prepared and digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis and ethidium bromide stained gels were examined by long wave ultraviolet light before blotting to confirm efficient recovery and digestion of DNA. Gels were blotted and the DNA was hybridised to ^{32}P-labelled pSP50. Figure 3.2 shows that ^{32}P-labelled pSP50 should detect 10.8 and 4.2 kbp fragments of *Eco*RI digested HCMV DNA and 5.6 and 2.4 kbp in fragments of *Eco*RI digested pSP50. Phosphorimages (figures 3.3 - 3.6) were generated as previously described.

Figures 3.3a. shows an example of an ethidium bromide stained gel, the blot produced from this gel is shown in figure 3.3b. In HFFF2 cells (figure 3.3b) the amount of HCMV genomic DNA increased from 24 h p.i. to 168 h p.i.. Replicated pSP50 was detected by 72 h p.i., and reached a maximum level at 124 h p.i.. Similarly, 293 cells (figure 3.3b) supported the replication of both pSP50 and AD169 genomic DNA. Input HCMV genomic DNA was detectable at 0 h p.i. but the signal had decreased by 24 h p.i.. Accumulation of replicated genomic DNA was observed from 24 to 168 h p.i.. pSP50 was replicated by 72 h p.i., and increased in amount from 124 to 168 h p.i.. In HFFF2 and 293 cells, as in all the cell lines examined, the mock infected samples showed no bands corresponding to either replicated pSP50 or to HCMV genomic DNA.

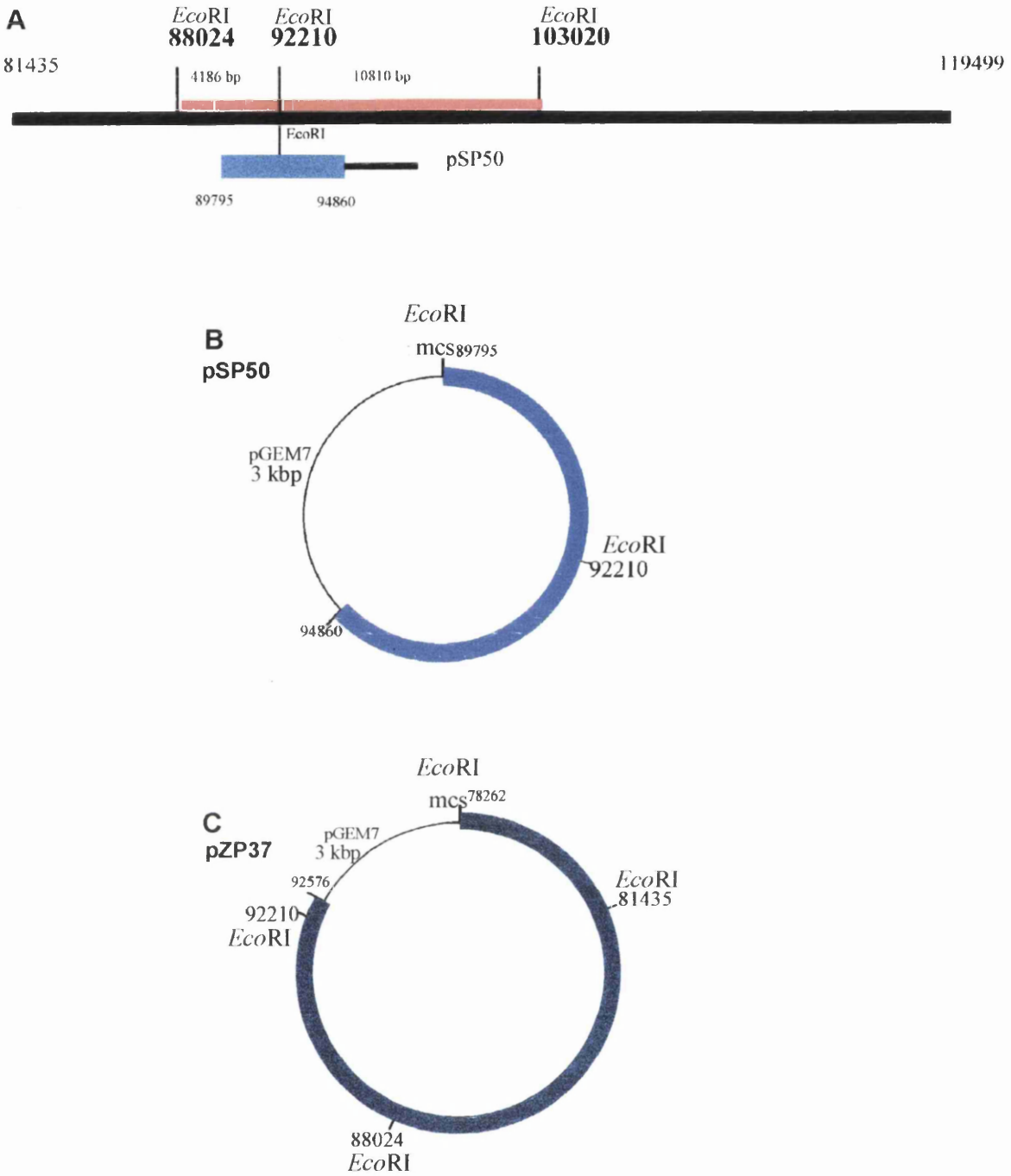


Figure 3.2 *Eco*RI restriction maps of the HCMV origin region in the HCMV genome, pSP50 and pZP37.

A) The HCMV genome digested with *Eco*RI and hybridised to pSP50 (B) produces bands of 4.2 kbp and 10.8 kbp.

B) pSP50 digested with *Eco*RI yields fragments of 2.4 kbp and 5.6 kbp. When hybridised to pSP50, both bands are detected. When hybridised to pAT153, only the 5.6 kbp fragment, containing the vector sequences, is detected.

C) pZP37 digested with *Eco*RI yields fragments of 2.7 kbp, 6.6 kbp, 4.2 kbp and 3.3 kbp. When hybridised to pAT153, only the 3.3 kbp fragment, containing the vector sequences, is detected.

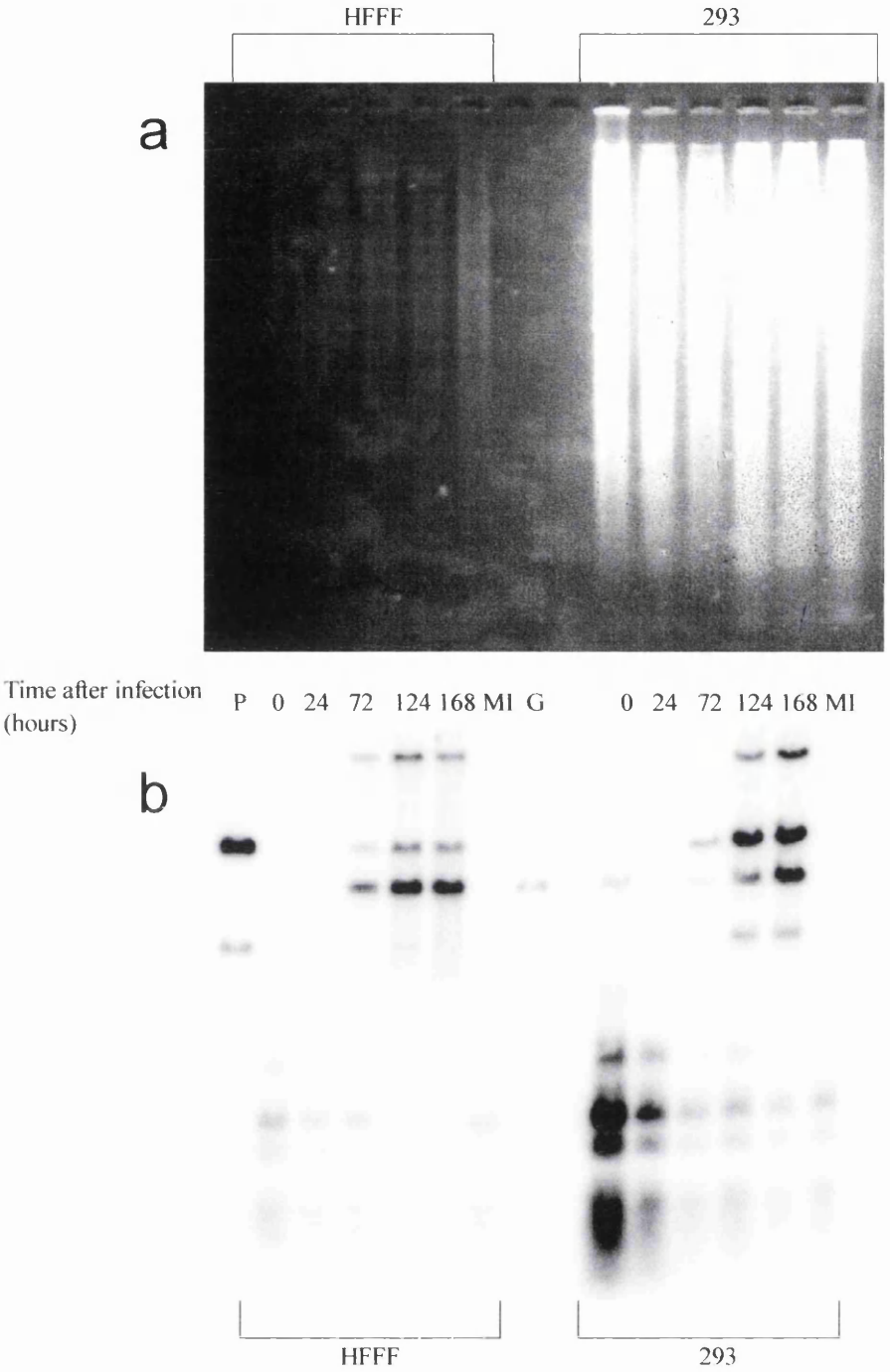


Figure 3.3 Time-course of infection of HFFF2 and 293 cells with HCMV strain AD169.

Cells were transfected with 1.6 μg/dish pSP50 plasmid and infected with 1 p.f.u./cell of HCMV strain AD169. Infected cells were collected at various times after infection and total cellular DNA was prepared. 25% of the DNA from a 35 mm dish of cells was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and hybridised to ^{32}P-labelled pSP50.

Panel (a) shows the ethidium bromide stained gel corresponding to the Southern blot in panel (b).

150 ng of *Eco*RI digested HCMV genomic DNA (G) and 4 ng of *Eco*RI digested pSP50 (P) were loaded as markers. A mock infected sample for each cell type is included (MI).

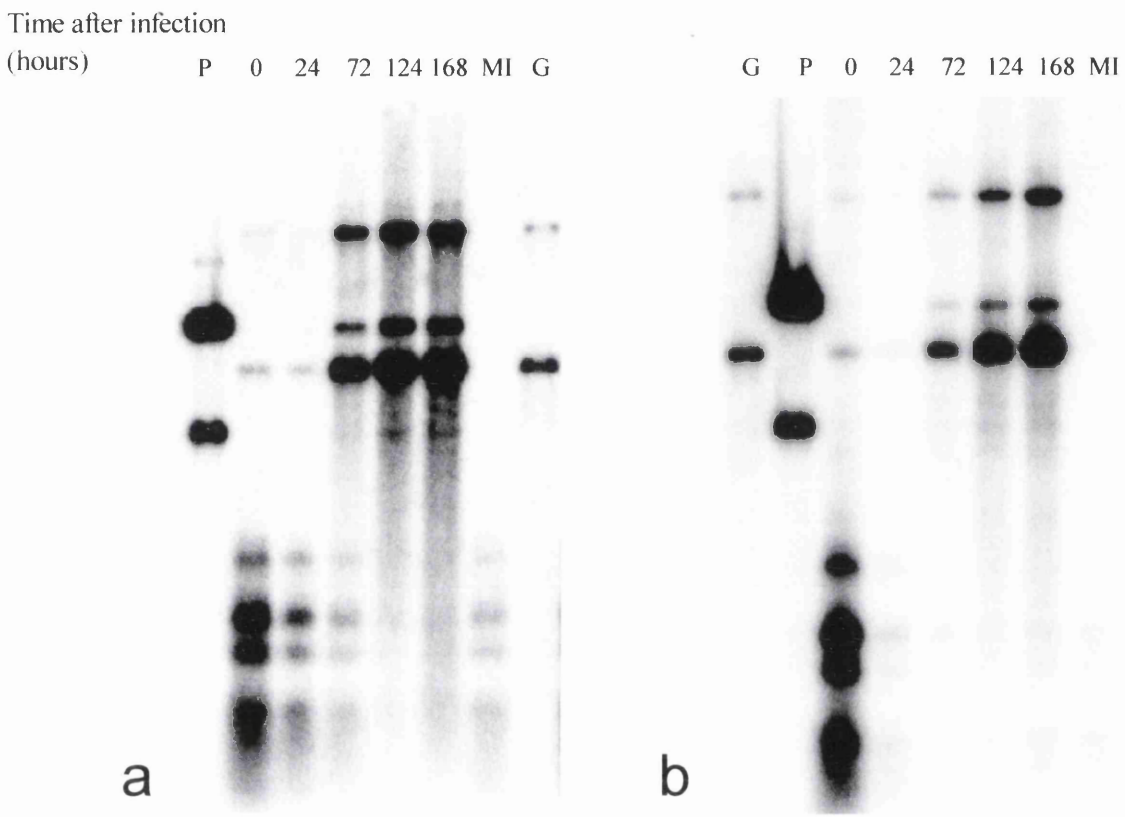


Figure 3.4 Time-courses of infection of HFL1 (a) and U-373MG cells (b) with HCMV strain AD169.

Cells were transfected with 1.6 μg/dish pSP50 plasmid and infected and processed as described in the legend to figure 3.3.

Markers for *Eco*RI digested HCMV genomic DNA (G) and pSP50 (P) and a mock infected sample for each cell type (MI) are included.

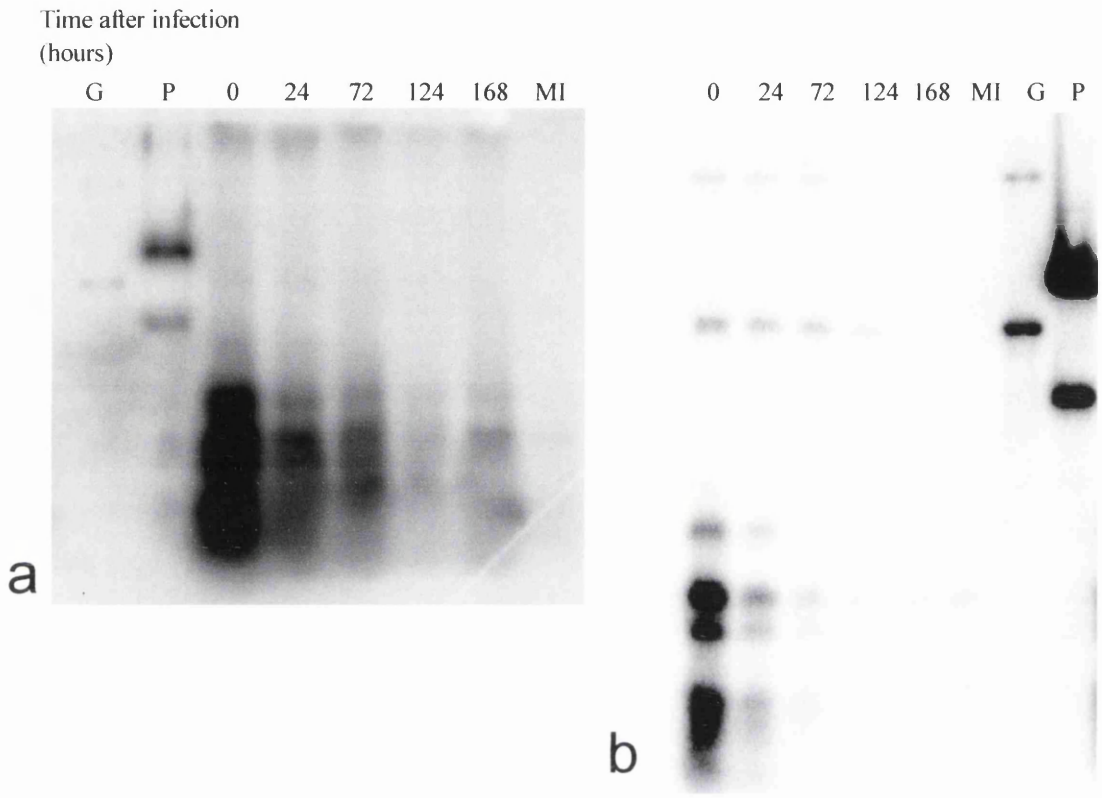


Figure 3.5 Time-courses of infection of BHK 21 C13 (a) and CV-1 cells (b) with HCMV strain AD169.

Cells were transfected with 1.6 μg/dish pSP50 plasmid and infected and processed as described in the legend to figure 3.3.

Markers for *Eco*RI digested HCMV genomic DNA (G) and pSP50 (P) and a mock infected sample for each cell type (MI) are included.

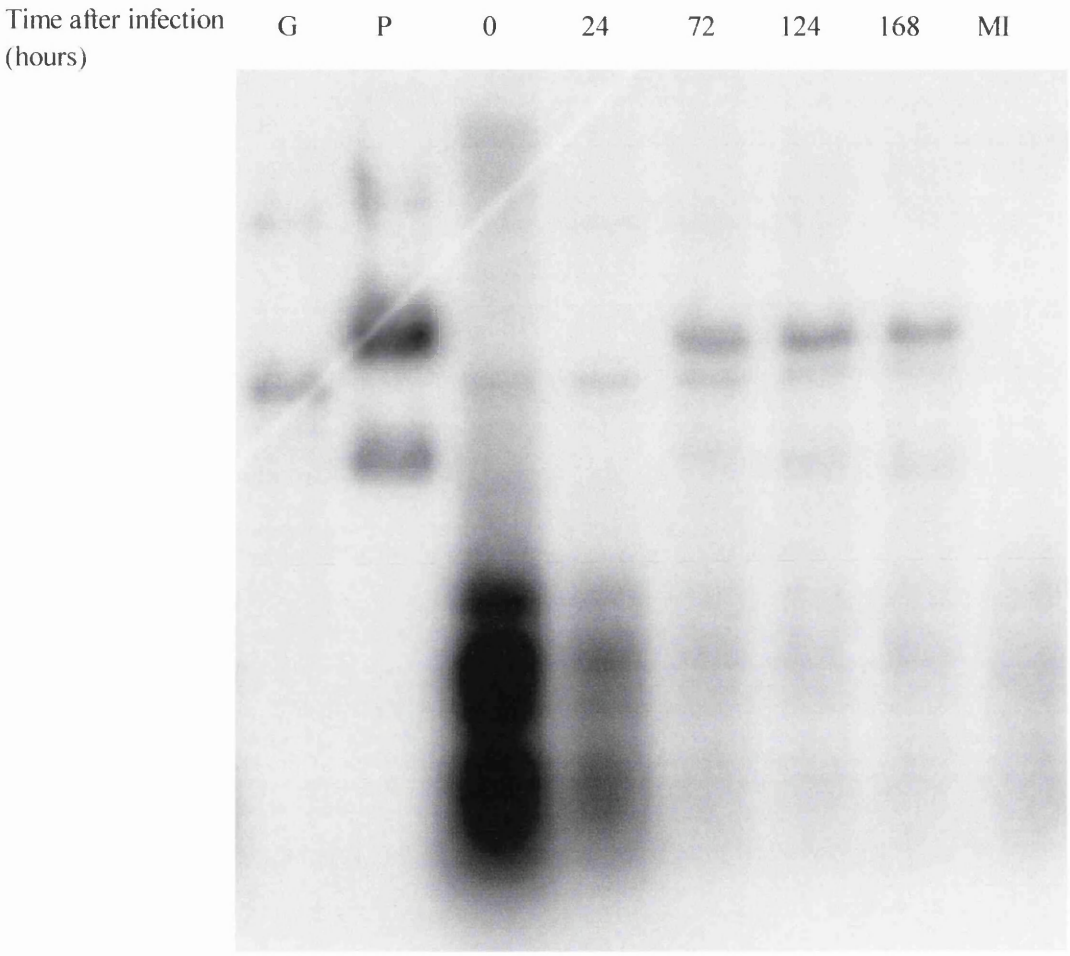


Figure 3.6 Time-course of infection of Vero cells with HCMV strain AD169.

Cells were transfected with 1.6 μg/dish pSP50 and infected and processed as described in the legend to figure 3.3.

Markers for *Eco*RI digested HCMV genomic DNA (G) and pSP50 (P) and a mock infected lane (MI) are included.

The replication of HCMV and pSP50 in HFL1 (a) and U-373 MG (b) cells is shown in figure 3.4. In both cell lines, the amount of HCMV genomic DNA increased from 24 h p.i. to 168 h p.i.. Replicated pSP50 was observed by 72 h p.i. and increased in amount at 124 h p.i.. A further increase in replicated pSP50 was observed in U-373 MG cells by 168 h p.i.. In contrast to the above, BHK 21 C13 (a) and CV-1 (b) cells (figure 3.5) showed no detectable accumulation of HCMV genomic DNA and no replication of the origin-containing plasmid DNA.

A third phenotype was exhibited by Vero cells (figure 3.6) which supported the replication of transfected pSP50 in the absence of detectable genomic DNA replication. HCMV genomic DNA was detectable at decreasing levels from 0 to 168 h p.i.. In contrast, replicated pSP50 was readily detectable from 72 h p.i. to 168 h p.i..

Summary

Three phenotypes were observed. In 293, U-373 MG, HFFF2 and HFL1 cells, HCMV strain AD169 genomic DNA and a transfected origin-containing plasmid both replicated. In CV-1 and BHK 21 C13 cells, there was no detectable replication of either the origin-containing plasmid or the infecting helper virus genomes. Finally, in Vero cells, HCMV directed the replication of the origin-containing plasmid in the absence of detectable genomic DNA synthesis.

These findings agree with those of LaFemina and Hayward (1988) for the replication of HCMV genomic DNA in all the cell lines except 293 cells which were previously described as being negative. The ability of Vero cells to support replication of the origin-containing plasmid, in the apparent absence of genomic DNA synthesis, was a novel, unexpected result.

(b) HCMV strain Towne time-courses

LaFemina and Hayward (1988) used HCMV strain Towne in their investigations. Since different strains of HCMV may have only 95% identity at the nucleotide sequence

level, it is possible that their behaviour in different cell lines might vary. The replication of HCMV strain Towne was examined to determine whether interstrain differences could account for the apparent discrepant result in 293 cells, and whether replication of pSP50 in Vero cells was peculiar to HCMV strain AD169.

Time-courses of replication of pSP50 and HCMV strain Towne in HFFF2, 293, Vero, and U-373 MG cells were carried out as described for AD169 time-course experiments, except that infections were with 1 p.f.u./cell of HCMV strain Towne. Samples were collected at 0, 24, 72, 119 and 166 h p.i.. One dish for each cell line was mock infected and harvested at 166 h p.i.. Total cellular DNA was prepared and processed as previously described for HCMV strain AD169 time-courses, phosphorimages are shown in figures 3.7 and 3.8.

Replication of both HCMV Towne genomic DNA and pSP50 DNA was clearly detectable in HFFF2 cells (figure 3.7a), 293 cells (figure 3.7b) and U-373 MG cells (figure 3.8a). In Vero cells, (figure 3.8b) replicated pSP50 was detected from 72 h p.i., and in this case, plasmid replication was accompanied by a small accumulation of genomic DNA.

Summary

HCMV strain Towne directed replication of both its own genomic DNA and a transfected HCMV origin-containing plasmid in HFFF2, Vero, 293 and U-373 MG cells. These results differ from LaFemina and Hayward (1988) in that replication of HCMV strain Towne genomic DNA was previously not detected in 293 or Vero cells.

(c) Comparison of AD169 and Towne replication

HFFF2, U-373 MG, 293 and Vero cells all support HCMV origin-dependent DNA synthesis directed by HCMV infection. HCMV strain AD169 and Towne behaved similarly with regard to both genomic and plasmid DNA replication in all cell lines tested, except that Vero cells appeared to support a very low level of HCMV strain Towne genomic DNA synthesis, but no synthesis of strain AD169 DNA. The behaviour of the two strains of

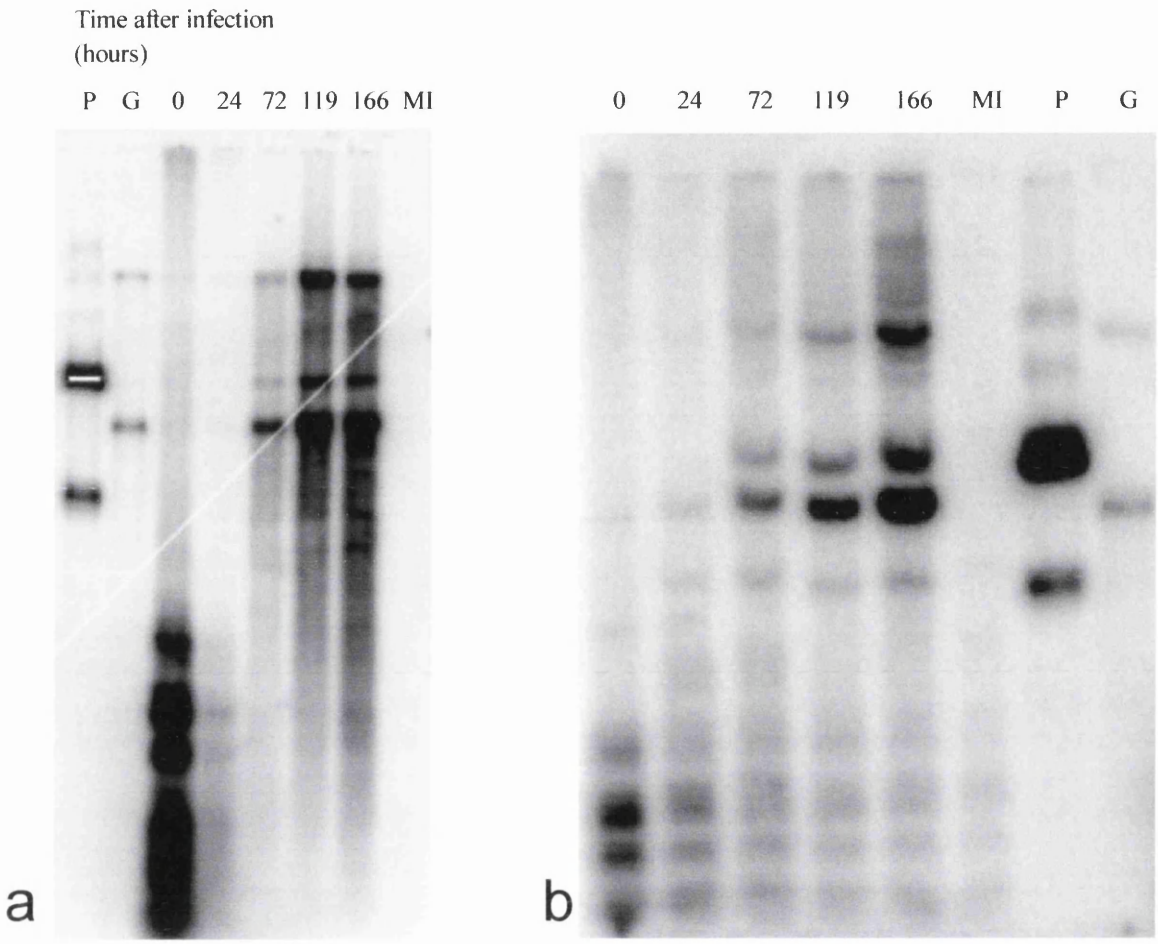


Figure 3.7 Time-courses of infection of HFFF2 (a) and 293 cells (b) with HCMV strain Towne.

Cells were transfected with 1.6 μg/dish pSP50 and infected with 1 p.f.u./cell of HCMV strain Towne. Infected cells were collected at various times after infection and total cellular DNA was prepared. 25% of the DNA obtained from a 35 mm dish of cells was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and hybridised to ^{32}P-labelled pSP50.

Markers for *Eco*RI digested HCMV genomic DNA (G) and pSP50 (P) and a mock infected sample for each cell type (MI) are included. The white line at the centre of the larger pSP50 marker band (panel a), and throughout this thesis, is indicative of saturated pixels.

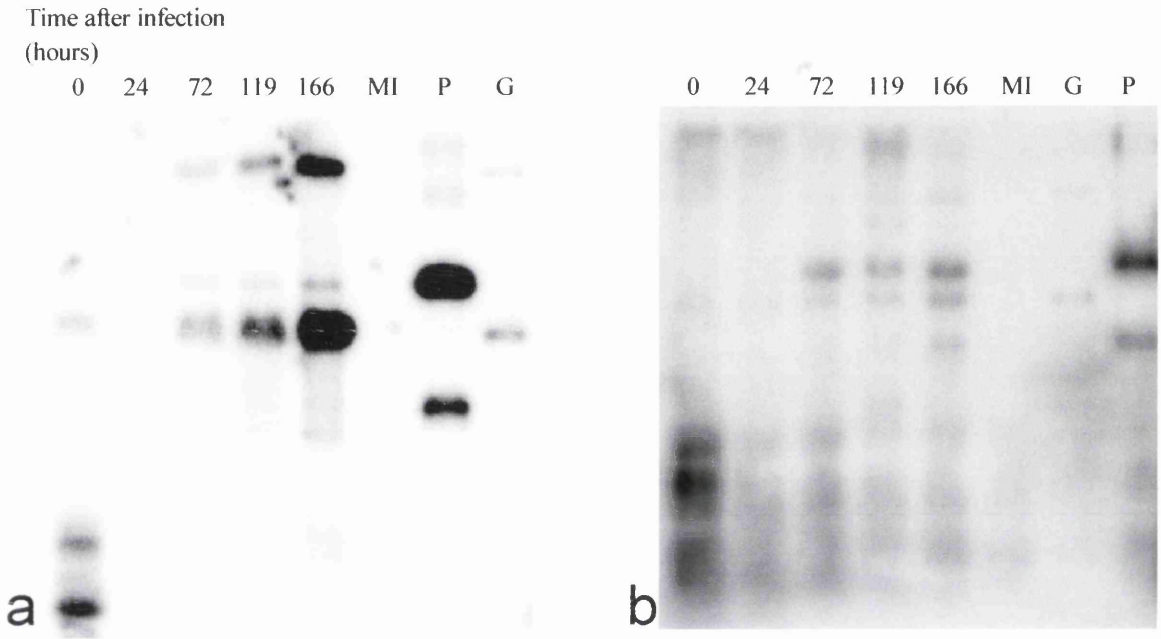


Figure 3.8 Time-courses of infection of U-373 MG (a) and Vero cells (b) with HCMV strain Towne.

Cells were transfected with 1.6 μg/dish pSP50 and infected and processed as described in the legend to figure 3.7.

Markers for *Eco*RI digested HCMV genomic DNA (G) and pSP50 (P) and a mock infected sample for each cell type (MI) are included.

virus in the different cell lines was compared by using the Quantity One software (as described in section 2.2.12) to quantify the signals corresponding to genomic DNA in these 'positive' cell lines.

Figure 3.9 shows the accumulation in infected cells of (a) AD169 genomic DNA and (b) Towne genomic DNA. 293 and U-373 MG cells support both AD169 and Towne genomic DNA replication to levels comparable to those seen in the normally used permissive HFFF2 cell line. In these experiments, Vero cells supported HCMV strain Towne DNA replication to approximately 30% of the level accumulated in infected HFFF2 cells.

While the data presented in figure 3.9 are derived only from the images presented in this thesis, the trends described are typical of repeat experiments. The one exception to this is that the replication of HCMV strain Towne DNA in HFFF2 cells shown in figure 3.7a is lower than usually observed.

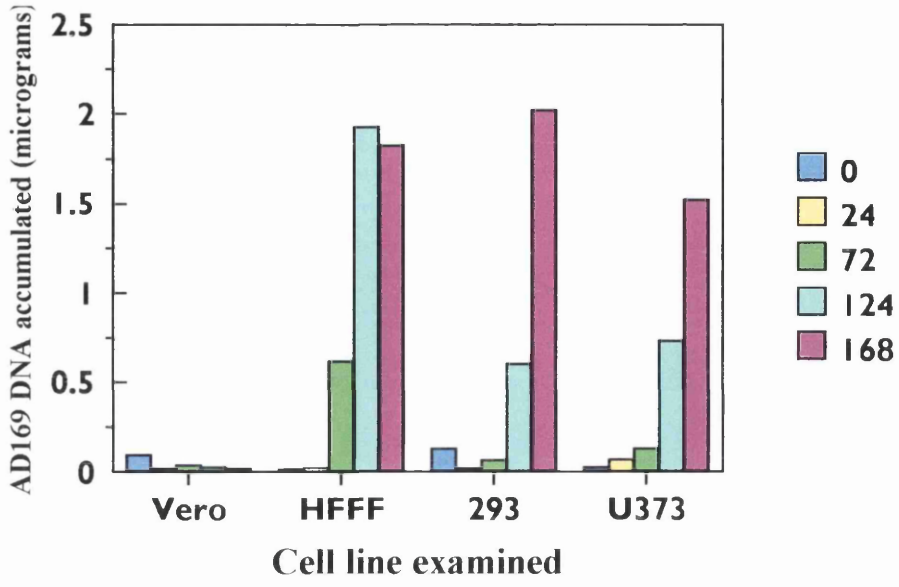
3.2.3 Specificity of replication

As Vero cells infected with AD169 supported the replication of a transfected plasmid but not detectable replication of helper virus genomes, it was considered important to confirm that the DNA synthesis observed required the HCMV DNA polymerase and the intact HCMV origin sequences in the plasmid.

(a) Sensitivity to DNA polymerase inhibitors

The HCMV DNA polymerase is inhibited by phosphonoformic acid (PFA) and phosphonoacetic acid (PAA) (Nishiyama *et al.*, 1983; Oberg, 1983). In order to confirm that the plasmid DNA replication in HCMV infected Vero cells was directed by the viral DNA polymerase, the replication of pSP50 in HCMV infected cells treated with PFA and PAA was examined.

a



b

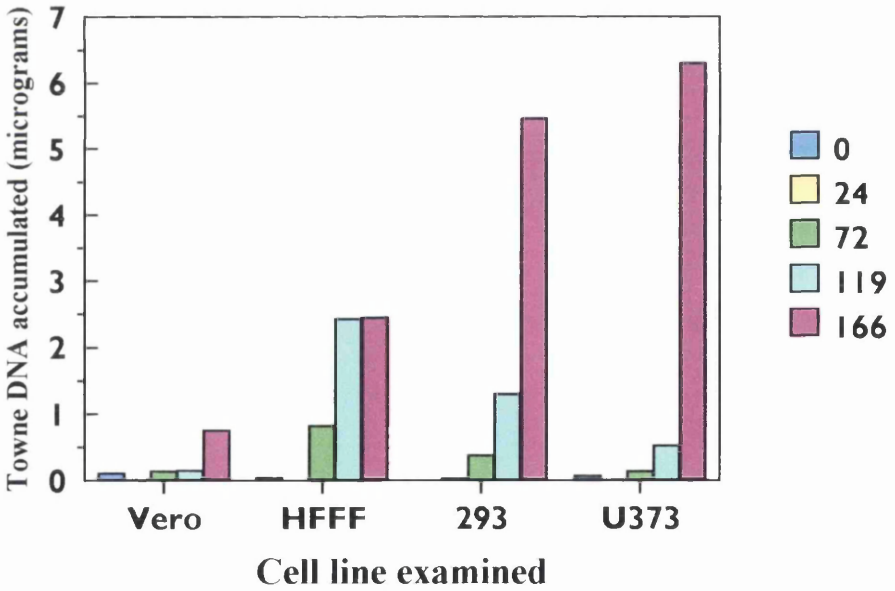


Figure 3.9 Graphic representation of the amount of genomic DNA accumulated in (a) AD169 or (b) Towne infected Vero, HFFF2, 293 and U-373 MG cells.

The data were obtained from the scans in figures 3.3b, 3.4b and 3.6-3.8. The amount of HCMV genomic DNA markers loaded on each gel is consistent and was therefore used to calculate the amount of DNA accumulated. The amount of replicated genomic DNA is calculated in micrograms.

Effect of PFA and PAA on pSP50 replication

HFFF2, 293 and Vero cells were transfected with pSP50; three dishes of cells for each cell line were superinfected with HCMV and one dish of each was mock infected. After virus adsorption, the mock infected dish and one infected dish for each cell line were given 2 ml DC5. One dish of infected cells for each line was given DC5 supplemented with 200 μg/ml PAA and one dish of infected cells was given DC5 supplemented with 250 μg/ml PFA. Samples were harvested at 112 h p.i.. Total cellular DNA was prepared and digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis, blotted and hybridised to ^{32}P-labelled pAT153. A phosphorimage was produced as described.

Figure 3.10 shows that in the absence of inhibitor, pSP50 was replicated in all three cell lines following HCMV infection. Replication of pSP50 in infected cells was prevented in the presence of the DNA polymerase inhibitors PAA and PFA.

Effect of PFA and PAA on SV40 origin-dependent DNA synthesis

While the herpesvirus DNA polymerases have been reported to be up to one hundred times more sensitive to PAA and PFA than the cellular DNA polymerases, the sensitivity of the cellular enzymes to these phosphonic acids is reportedly cell and tissue type dependent (Nishiyama *et al.*, 1983; Kornberg & Baker, 1992). To confirm that the concentration of the inhibitors used was not affecting the major cellular replicative DNA polymerases in 293 and Vero cells, these cells were investigated for their ability to support non-herpesviral DNA synthesis in the presence of the viral DNA polymerase inhibitors, PAA and PFA.

pSV3-neo contains an SV40 origin of replication and constitutively expresses SV40 T-antigen. pSV3-neo can be replicated in human and simian cells by the cellular polymerases α and δ when T-antigen is expressed in a suitably phosphorylated state to interact with and unwind the SV40 origin (Kelly, 1988).

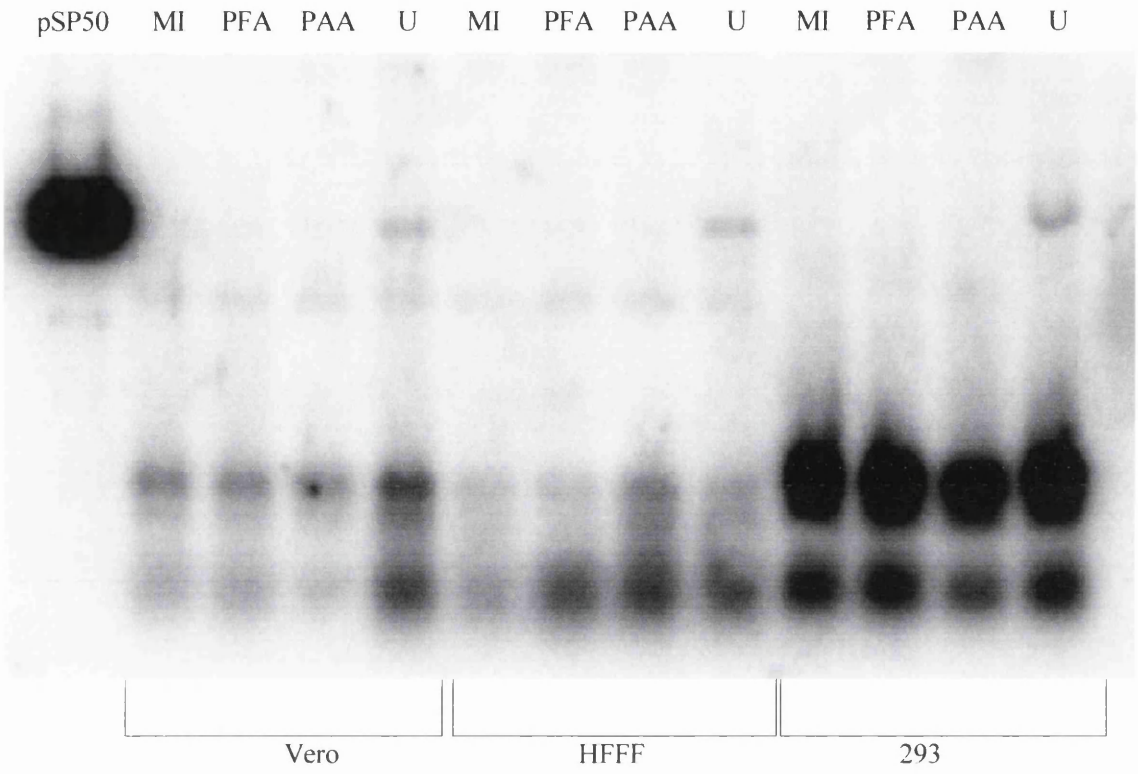


Figure 3.10 Effect of PFA and PAA on pSP50 replication.

Vero, HFFF and 293 cells were transfected with 1.6 μg/dish pSP50 and infected with 0.25 p.f.u./cell of HCMV. Cells were incubated in medium containing either 250 μg/ml of PFA (PFA) or 200 μg/ml of PAA (PAA). DNA from mock infected plates (MI) and from infected cells incubated in medium without inhibitor (U) was also prepared. Total cellular DNA was prepared 112 h p.i.. 25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and the blot hybridised to ^{32}P-labelled pAT153.

4 ng of *Eco*RI digested pSP50 was loaded as a marker (pSP50).

Monolayers of Vero and 293 cells were transfected with 1.6 μg/dish pSV3-neo. Cells were mock infected and replicate dishes for each cell line were given either 2 ml DC5, or DC5 supplemented with 200 μg/ml PAA, or DC5 supplemented with 250 μg/ml PFA. Samples were collected after 112 hours and processed as described for the previous experiment, the result is shown in figure 3.11.

The effect of PFA on DNA synthesis driven by the cellular DNA polymerases was minimal. The presence of PAA had a slight inhibitory effect on the replication of pSV3-neo although this was much less than its effect on the replication of pSP50 (figure 3.10).

Summary

The replication of pSP50 in Vero and 293 cells superinfected with HCMV was sensitive to viral DNA polymerase inhibitors at concentrations which did not abrogate the activity of the cellular DNA polymerases α and δ, indicating that the replication is most likely carried out by the viral DNA polymerase.

(b) Dependence on the HCMV origin sequences

pSP50 contains approximately 5 kbp of AD169 DNA which includes the HCMV origin of lytic-phase DNA replication (oriLyt). In order to demonstrate that the plasmid replication observed in HCMV infected Vero cells was dependent on the HCMV origin, other plasmids containing complete or partial origins, and the vectors into which the origin sequences are cloned, were tested for their ability to be replicated following AD169 superinfection.

pSP50 and pGEM7 replication

pSP50 contains an HCMV oriLyt fragment cloned into the vector pGEM7. In order to show that the vector sequences of pSP50 were not responsible for its replication, the replication of pSP50 and pGEM7 were compared.

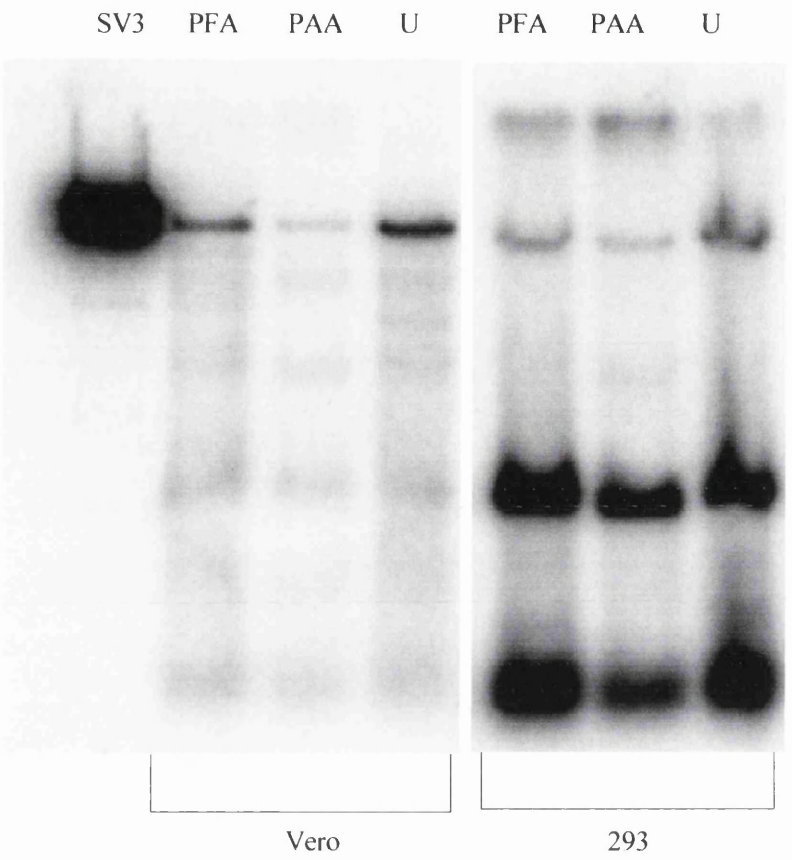


Figure 3.11 Effect of PAA and PFA on SV40 origin-dependent DNA synthesis.

Vero and 293 cells were transfected with 1.6 μg/dish pSV3-neo. Cells were incubated in medium containing either 250 μg/ml of PFA (PFA) or 200 μg/ml of PAA (PAA) or in medium without inhibitor (U). Total cellular DNA was harvested 112 h p.t.. 25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and the blot hybridised to ^{32}P-labelled pAT153.

4 ng of *Eco*RI digested pSV3-neo was loaded as a marker (pSV3).

Vero and HFFF2 cells were cotransfected with pSP50 and pGEM7. One dish of cells for each line was superinfected with HCMV, the other dish was mock infected. Total cellular DNA was prepared 112 h p.i. and digested with *Eco*RI and *Dpn*I. *Eco*RI digestion linearises pGEM7 so that a single band of 3 kbp is produced which is clearly distinguishable from the 5.6 kbp *Eco*RI fragment of pSP50 containing the vector sequences (see figure 3.2b). Fragments were separated by electrophoresis, blotted and hybridised to ^{32}P-labelled pAT153.

Figure 3.12 shows that pSP50 replicates in HCMV infected HFFF2 and Vero cells but that pGEM7 was not replicated.

pSP50 and pZP37 replication

pZP37 contains approximately 14 kbp of AD169 DNA sequences in the same vector (pGEM7) as pSP50. The sequences in pZP37 overlap HCMV oriLyt so that pZP37 contains only approximately 40 % of the essential minimal origin as defined by Anders *et al.* (1992).

Duplicate Vero and U-373 MG monolayers were cotransfected with pSP50 and pZP37. One dish of cells for each cell line was infected with HCMV, the other dish was mock infected. Samples were collected 112 h p.i.. Total cellular DNA was prepared and digested with *Eco*RI and *Dpn*I, and processed as previously described. Figure 3.2c shows that the vector sequences of *Eco*RI digested pZP37 are within a 3.3 kbp fragment detected in the marker lane in figure 3.13

Figure 3.13 shows that pSP50 was replicated in both AD169 infected Vero and U-373 MG cells, but that pZP37 was not replicated.

pCMVori replication

pCMVori is an HCMV origin-containing plasmid with HCMV sequences (nucleotides 91321-94168) subcloned from a *Hin*dIII D containing plasmid into pCMV10. The construction of pCMVori and its derivatives was described in figure 2.1.

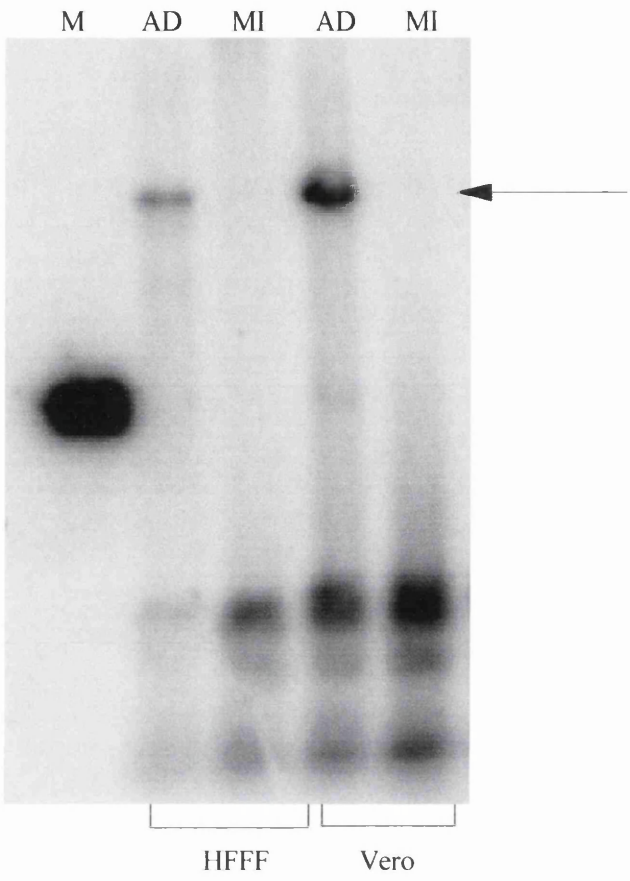


Figure 3.12 Specificity of replication to origin sequences - pGEM7.

HFFF and Vero cells were cotransfected with 1.6 μg/dish each of pGEM7 and pSP50. Cells were infected with 0.25 p.f.u./cell of HCMV (AD) or mock infected (MI). Total cellular DNA was prepared 112 h p.i.. 25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated through a 0.8% agarose gel which was blotted as described and the blot hybridised to ^{32}P-labelled pAT153. 4 ng of *Eco*RI digested pGEM7 was loaded as a marker (M).

◄— Position of *Eco*RI digested pSP50 marker band.

Figure 3.13 Specificity of replication to intact origin sequences - pZP37.

U373MG and Vero cells were cotransfected with 1.6 μg/dish each of pZP37 and pSP50. Cells were infected with 0.25 p.f.u./cell HCMV. DNA from infected (AD) and mock infected (MI) cells was harvested 112 h p.i.. 25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and the blot hybridised to ^{32}P-labelled pAT153.

4 ng each of *Eco*RI digested pZP37 (pZP37) and pSP50 (pSP50) were loaded as markers.

HFFF2 and Vero cells were transfected with either pCMVori or pCMV10. One dish of each duplicate transfection was infected with HCMV, the other dish was mock infected. Total cellular DNA was prepared 90 h p.i. and digested with *Xba*I and *Dpn*I. *Xba*I digestion linearises both plasmids so that the size difference is based on the insert. pCMV10 (lacking an insert) is 3.8 kbp, and pCMVori is 6.6 kbp.

Figure 3.14 shows that pCMVori was replicated in AD169 infected HFFF2 and Vero cells and not in mock infected cells. No replication of pCMV10 was detected.

pADori and pMA1 replication

pADori contains the origin sequences from pCMVori subcloned into pGEM7, pADoriΔXho contains the mutated origin from pCMVoriΔXho cloned into pGEM7. *Xho*I deletion removes 1160 bp of oriLyt spanning 'essential region II' as defined by Zhu *et al.* (1998). pMA1 contains an HCMV oriLyt derived from HCMV strain Towne.

Duplicate monolayers of Vero cells were either cotransfected with pADori and pADoriΔXho or transfected with pMA1. One of the duplicate plates was infected with HCMV, the other was mock infected and incubation was continued for 112 h. Total cellular DNA was prepared and digested with *Xba*I and *Dpn*I. Fragments were separated by electrophoresis, blotted, and hybridised to ^{32}P-labelled pAT153. *Xba*I digestion linearises all three plasmids, generating fragments of 9.4 kbp (pMA1), 5.8 kbp (pADori) and 4.7 kbp (pADoriΔXho). The blot was exposed to a phosphorimager screen and an image (figure 3.15) was produced.

pADori and pMA1 were replicated in HCMV infected Vero cells. The deleted origin-containing plasmid, pADoriΔXho, was not replicated.

Summary

The replication of transfected plasmids by HCMV superinfection in Vero cells was dependent on an intact HCMV origin.

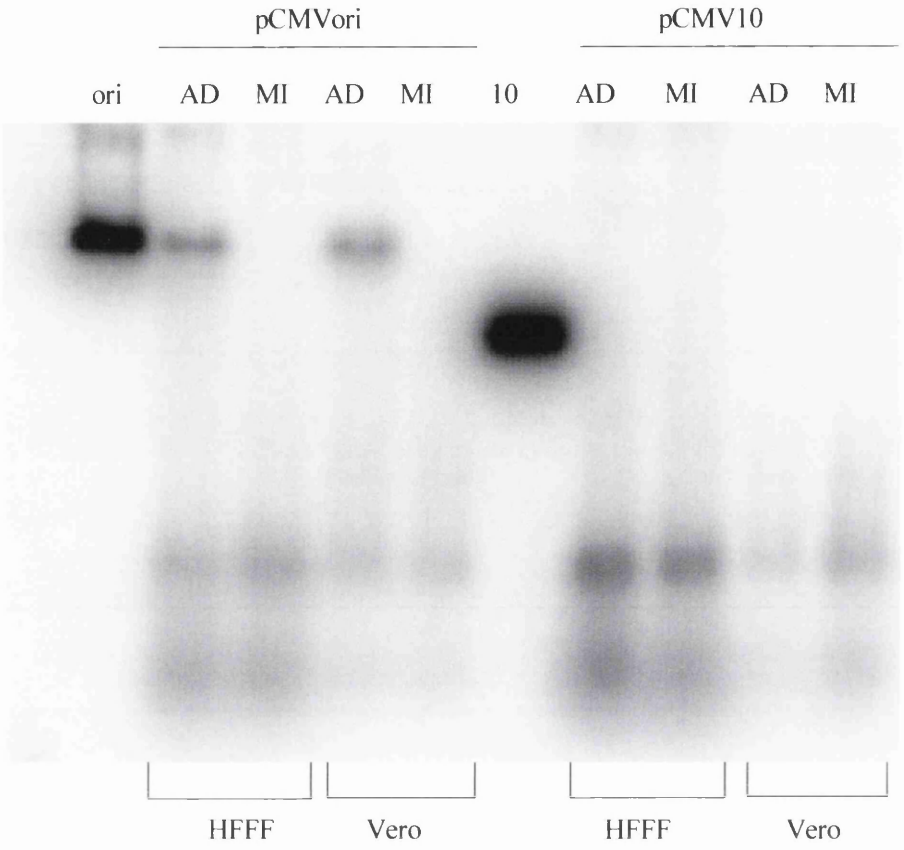


Figure 3.14 Replication of an independent origin containing plasmid - pCMVori.

HFFF and Vero cells were transfected with 1.6 μg/dish of either pCMVori or pCMV10. Cells were infected with 0.25 p.f.u./cell of HCMV (AD) or mock infected (MI). Total cellular DNA was prepared at 90 h p.i.. 25% of the DNA from a 35 mm dish was digested with *Xba*I and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and the blot hybridised to ^{32}P-labelled pAT153.

4 ng each of *Xba*I digested pCMVori (ori) and pCMV10 (10) were loaded as markers.

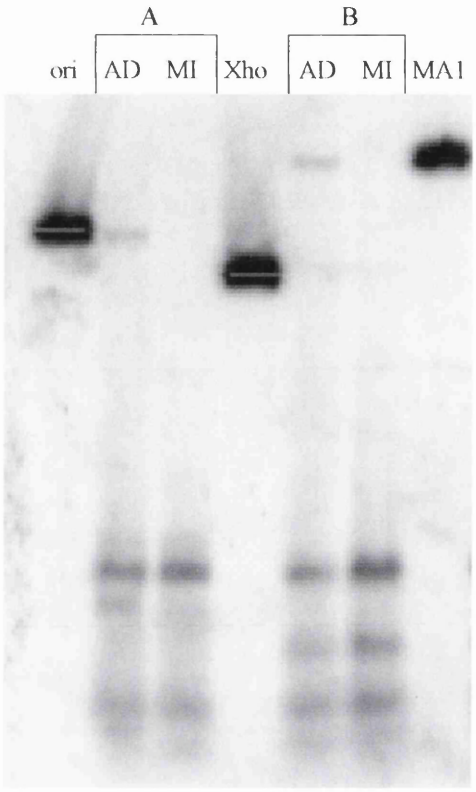


Figure 3.15 Replication of two independent origin containing plasmids and a mutated origin containing plasmid.

Vero cells were either cotransfected with 1.6 μg/dish of each of pADori and pADoriΔXho (A), or transfected with 1.6 μg/dish pMA1 (B). Cells were infected with 0.25 p.f.u./cell of HCMV (AD) or were mock infected (MI). Total cellular DNA was prepared 112 h p.i.. 25% of the DNA from a 35 mm dish was digested with *Xba*I and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and the blot hybridised to ^{32}P-labelled pAT153.

4 ng of *Xba*I digested pADori (ori), pADoriΔXho (Xho) and pMA1(MA1) were loaded as markers.

(c) Effect of bacterial DNA methylation

Plasmid DNA purified from *E. coli* DH5α bacteria has been methylated by DNA adenine and DNA cytosine methylases (*dam* and *dcm*) at the A residue in GATC sequences and at both C residues in $CC^{A}/_{T}GG$ sequences respectively. *Dam* methylation is used by bacteria to differentiate replicated from non-replicated DNA for purposes of DNA repair, and in the control of replication. The function of *dcm* methylation is unknown.

Eukaryotic DNA is methylated at the cytosine of some CG sequences- a process involved in the control of gene transcription. Methylation or undermethylation of DNA can alter the way DNA binding proteins interact with DNA.

The presence of bacterial methylation on the transfected origin-containing plasmid DNA, but not the superinfecting HCMV genomes, might provide an explanation for the different behaviours of the two DNAs in transient replication assays in Vero cells.

Preparation of unmethylated plasmid DNA

pSP50 was purified from a bacterial strain (SCS110) lacking both *dam* and *dcm* methylases. The unmethylated state of the DNA was confirmed by restriction analysis. Figure 3.16 compares the restriction profiles of methylated and unmethylated pSP50 DNA digested with *Dpn*II (cleaves only when its restriction site, GATC, is not *dam* methylated) or with *Scr*FI (cleaves only when its restriction site, CCNGG, is not *dcm* methylated). Figure 3.16 shows that DNA prepared from *E. coli* strain SCS110 is cleaved by *Dpn*II, while DNA prepared from strain DH5α is uncleaved. *Scr*FI cleaved DNA prepared from strain SCS110 at more sites than it cleaved DNA prepared from strain DH5α. DNA prepared from *E. coli* strain DH5α was *dam* and *dcm* methylated, while, as expected, DNA prepared from strain SCS110 was not methylated.

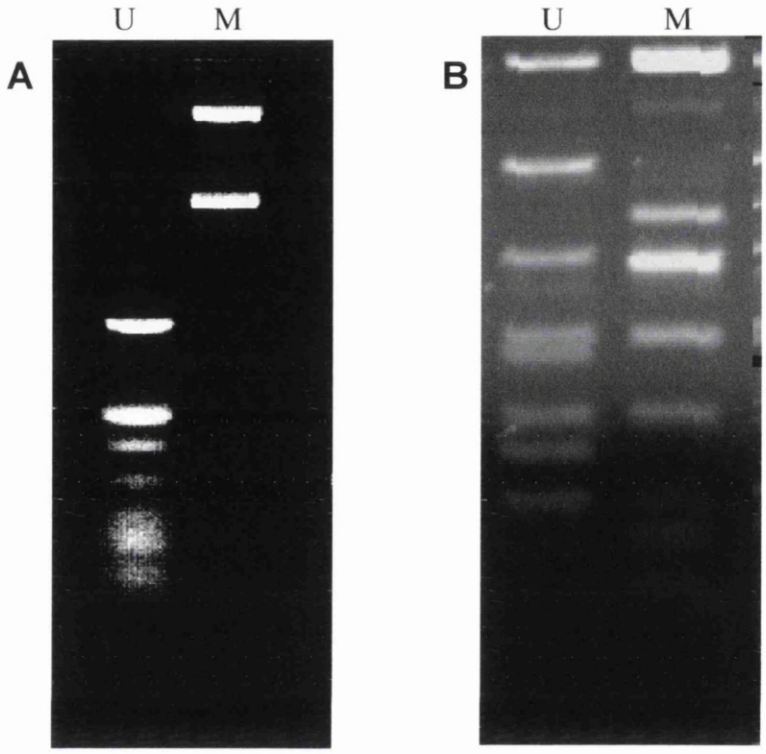


Figure 3.16 Digestion to confirm methylation state of plasmid DNA

A) The restriction profile of unmethylated (U) and methylated (M) pSP50 when digested with *Dpn*II.

B) The restriction profile of unmethylated (U) and methylated (M) pSP50 when digested with *Scr*FI.

1 μg of each plasmid was digested for 1 hour at 37^{0}C with 10 units of enzyme. 0.5 μg of each plasmid digest was subjected to electrophoresis through a 1 % agarose gel. The gel was photographed under UV light.

Replication of unmethylated origin-containing plasmid DNA

Two dishes each of Vero and 293 cells were transfected with unmethylated pSP50 (*dam-/dcm-*). One dish for each was infected with HCMV, the other was mock infected. *Dpn*I digestion could not be used to differentiate between input plasmid and replicated plasmid, so to remove some of the background from unreplicated input DNA, nuclear DNA was prepared instead of total cellular DNA. Nuclei were collected 160 h p.i., DNA was prepared, digested with *Eco*RI, and analysed by Southern blot as previously described.

In figure 3.17, the pSP50 detected in mock infected cells is the unmethylated input plasmid. Less unmethylated input plasmid was detectable in Vero cells than in 293 cells. The relative amounts of pSP50 observed in mock infected 293 and Vero cells corresponds with the relative amounts of *Dpn*I bands seen in the two cell lines in previous experiments (*e.g.* figure 3.10), and probably reflects differences in the uptake and stability of transfected DNA in the two cell lines.

The increase in intensity of the pSP50 band in AD169 infected cells compared with mock infected cells confirms that unmethylated pSP50 was replicated in infected Vero and 293 cells. Origin-containing plasmid DNA replication in Vero cells is therefore not a consequence of the plasmid DNA being *dam* or *dcm* methylated.

3.2.4 Production of virus progeny and electron microscopic examination of infected cells

293 cells clearly support the replication of infecting genomic DNA of HCMV strains AD169 and Towne. In order to investigate how far the replication cycle proceeds, cells were examined for the production of progeny virus by virus yield assay and electron microscopic examination of infected cell pellets.

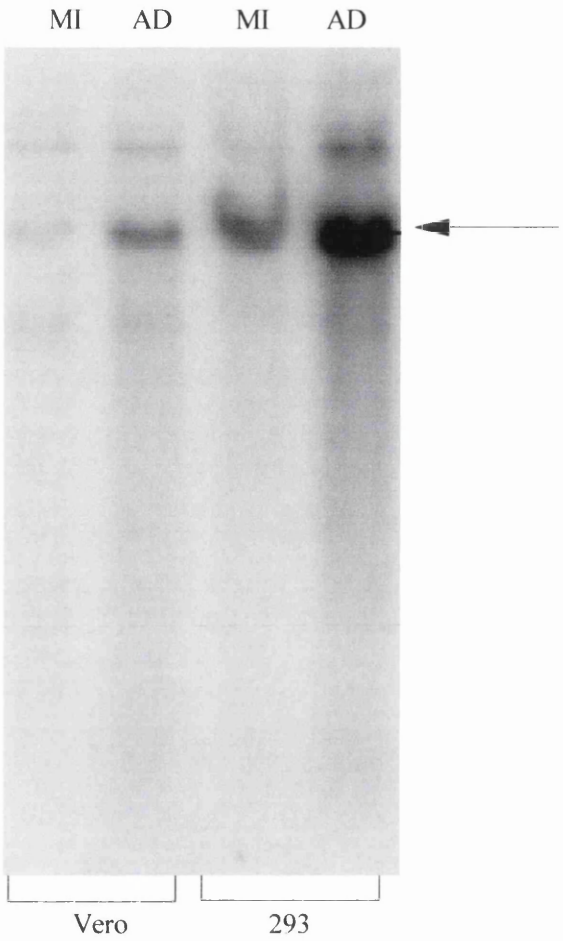


Figure 3.17 Replication of unmethylated pSP50.

Vero and 293 cells were transfected with 1.6 μg/dish of unmethylated pSP50. Cells were infected with 0.25 p.f.u./cell of HCMV (AD) or mock infected (MI). Nuclear DNA was prepared 160 h p.i.. 25% of the DNA from a 35 mm dish was digested with *Eco*RI. Fragments were separated by electrophoresis through a 0.8% agarose gel which was blotted as described and the blot hybridised to ^{32}P-labelled pAT153.

◄——— Position of *Eco*RI digested pSP50.

(a) Production of viral progeny

HCMV strain AD169

HFFF2, Vero and 293 cells were infected with 1 p.f.u./cell of HCMV strain AD169. Virus was adsorbed to the cells for 45 minutes at 37°C. After virus adsorption, the inoculum was removed and monolayers were washed twice with 2 ml of DPS per dish, then fed 2 ml of DC5 and the incubation was continued. Samples were collected by scraping cells into the supernatant medium then extensively sonicating the cell suspension. Samples were collected at 0, 26 and 110 h p.i., stored at -70°C until required, then sonicated and titrated on HFFF2 monolayers as described. Virus yields from a single experiment are plotted in figure 3.18.

In all three cell lines, an initial drop in virus titre corresponding to the eclipse phase was observed. At 110 h p.i., progeny virus was detected from HFFF2 cells while a further decrease in titre was observed from both 293 and Vero cells.

HCMV strain Towne

Monolayers of Vero and 293 cells infected with 1 p.f.u./cell of HCMV strain Towne, were incubated, harvested and titrated as described above. Results from a single experiment are plotted in figure 3.19.

In both cell lines, an initial drop in titre was observed at 26 h p.i.. By 110 h p.i., the titre from Vero cells had dropped further while the titre from 293 cells increased slightly. Although this experiment does not include HCMV strain Towne growth in HFFF2 cells, these were used for the production of the viral stocks and would be expected to yield a curve similar to that obtained for HCMV strain AD169 infection.

Summary

Vero cells do not support the growth of either HCMV strain AD169 or Towne as determined by virus yield assay. Although strain AD169 did not grow in 293 cells, the

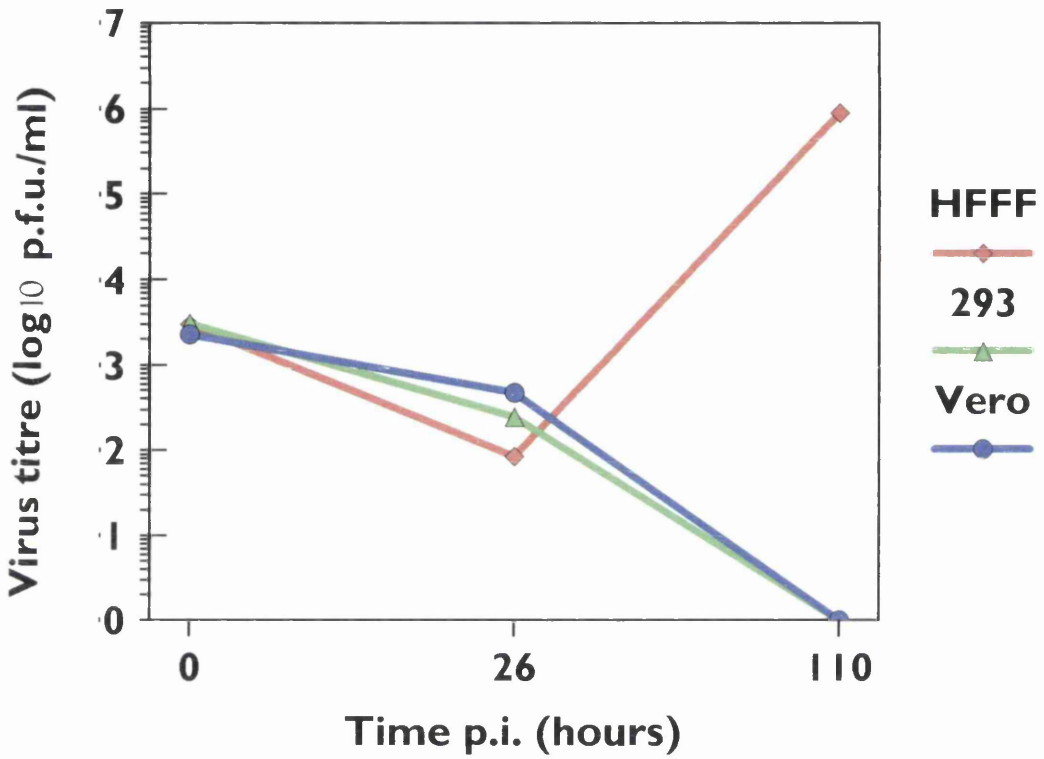


Figure 3.18 Production of infectious HCMV strain AD169.
Vero, 293 and HFFF2 cells were infected with 0.5 p.f.u./cell of HCMV strain AD169. Cell sonicates were collected at various times after infection and titrated on HFFF2 cells.

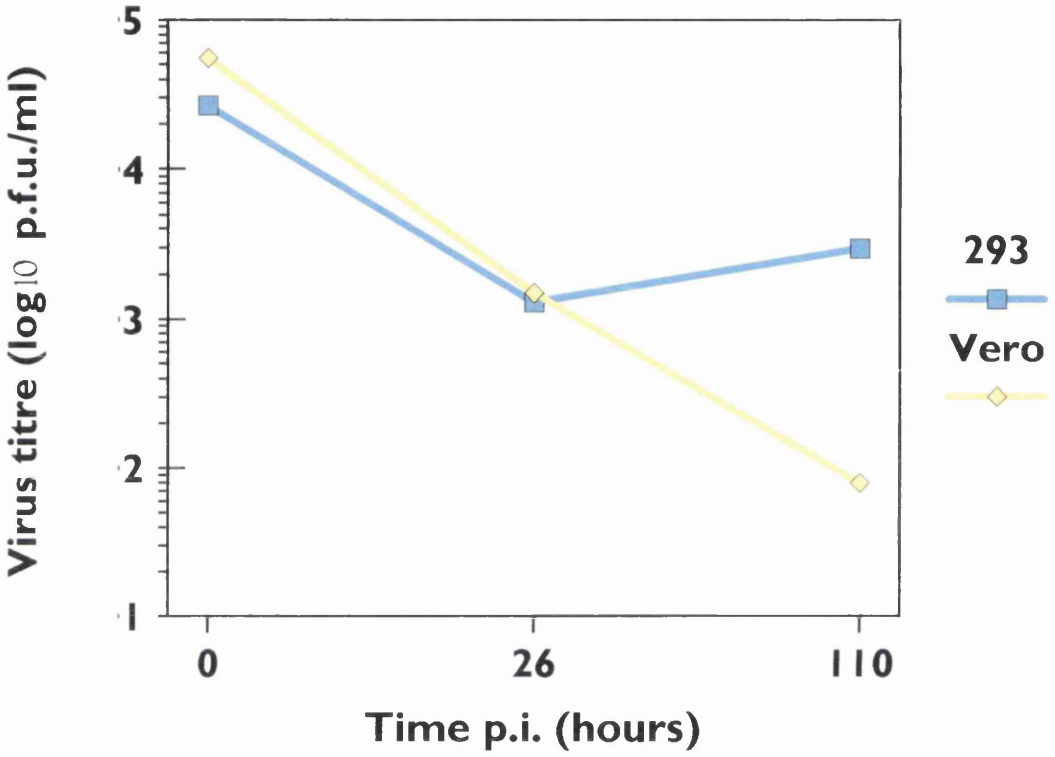


Figure 3.19 Production of infectious HCMV strain Towne.
Vero and 293 cells were infected with 1 p.f.u./cell of HCMV strain Towne. Cell sonicates were collected at various times after infection and titrated on HFFF2 cells.

small increase in the titre obtained from strain Towne infected 293 cells between 26 and 110 h p.i. suggests that limited growth of this virus may have occurred, even though the final yield obtained was less than the input. Before concluding that 293 cells support limited growth of HCMV strain Towne, these virus yield assays must clearly be repeated.

(b) Examination of infected cells by electron microscopy (EM)

To further investigate how far the growth cycle of HCMV proceeds in 293 cells, infected cell sections were examined by transmission electron microscopy (EM).

50 mm dishes of HFFF2, Vero and 293 cells were set up overnight to be approximately 80 % confluent when infected. Monolayers were infected with 2 p.f.u./cell of HCMV strain AD169, or Towne, or mock infected. Cells were fed 5 ml of DC5 per dish and incubation was continued at 37°C.

Cell pellets were prepared 140 h p.i. and stored under 2.5 % glutaraldehyde in PBS (v/v) at 4°C until the pellets were processed for examination by transmission electron microscopy. The preparation, examination and photography of cell sections was performed by Mr. Jim Aitken. At least fifty nuclei were examined for each section. Negatives from the electron microscope were scanned by D Bhella. Images of infected cell sections are shown in figures 3.20 - 3.22. The data presented here are the result of one experiment taken at one time point and should be repeated and expanded to confirm that the results are not an aberration.

Figure 3.20 shows HCMV strain AD169 (a) and Towne (b) infected HFFF2 cells. In the mock infected sample, no virus was detected. All the nuclei examined from the AD169 and Towne infected cell pellets contained virus particles.

Figure 3.21 shows HCMV strain AD169 (a) and Towne (b) infected Vero cells. No virus particles were seen in any AD169, Towne or mock infected Vero cells.

Figure 3.22 shows HCMV strain Towne infected 293 cells. Approximately 10 % of nuclei examined contained virus particles and two or three extracellular virus particles were

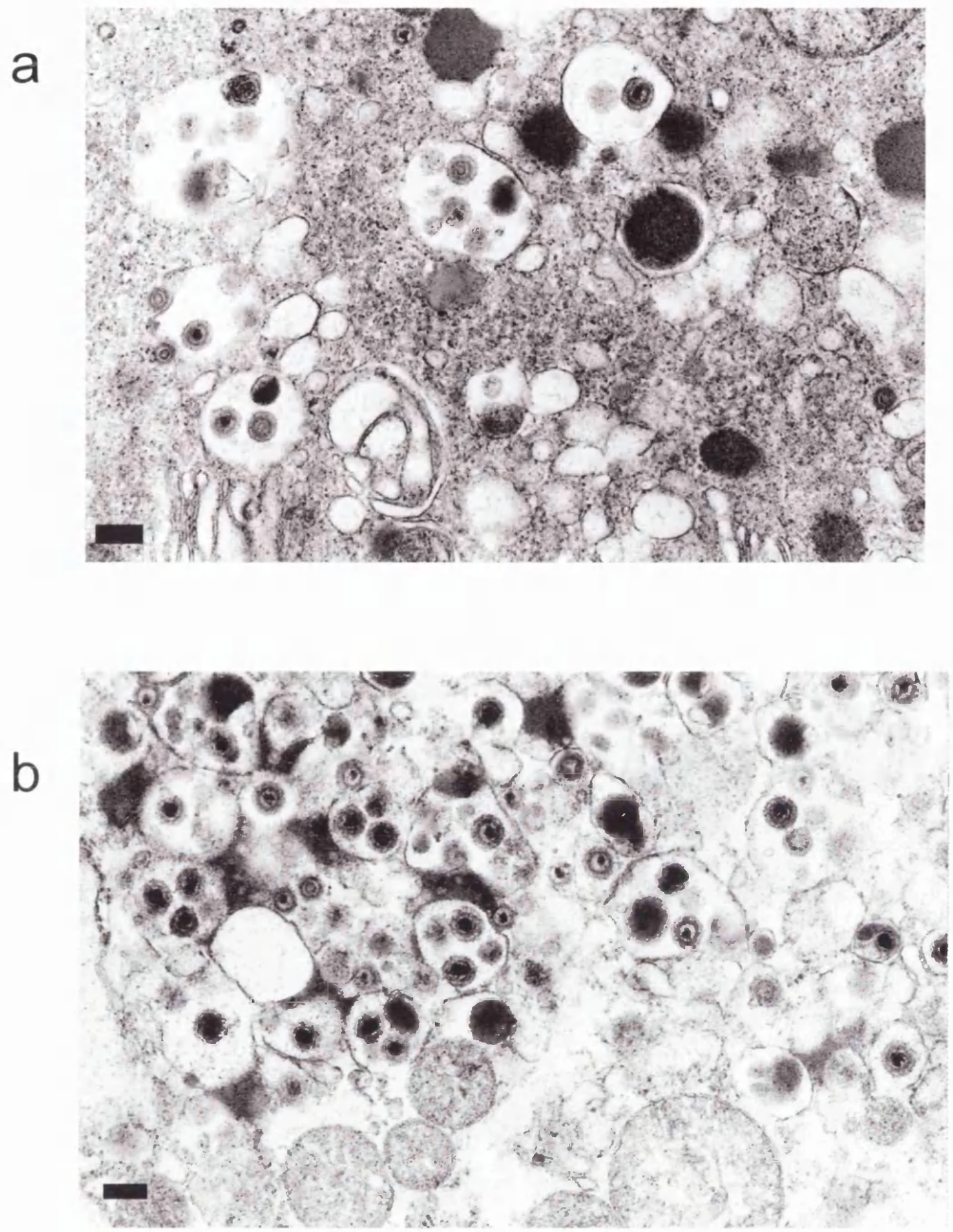


Figure 3.20 Electron micrographs of cytomegalovirus infected HFFF2 cells.

HFFF cells were infected with 2.5 p.f.u. per cell of HCMV strain AD169 (a) or with 2.5 p.f.u. per cell of HCMV strain Towne (b). Cell pellets were collected 140 h p.i.. Pellets were dehydrated, stained with uranyl acetate and counter-stained with lead citrate. 70 nm thick sections were examined at 10,000 times magnification.

The bar in the bottom left-hand corner of each picture represents approximately 250 nm.

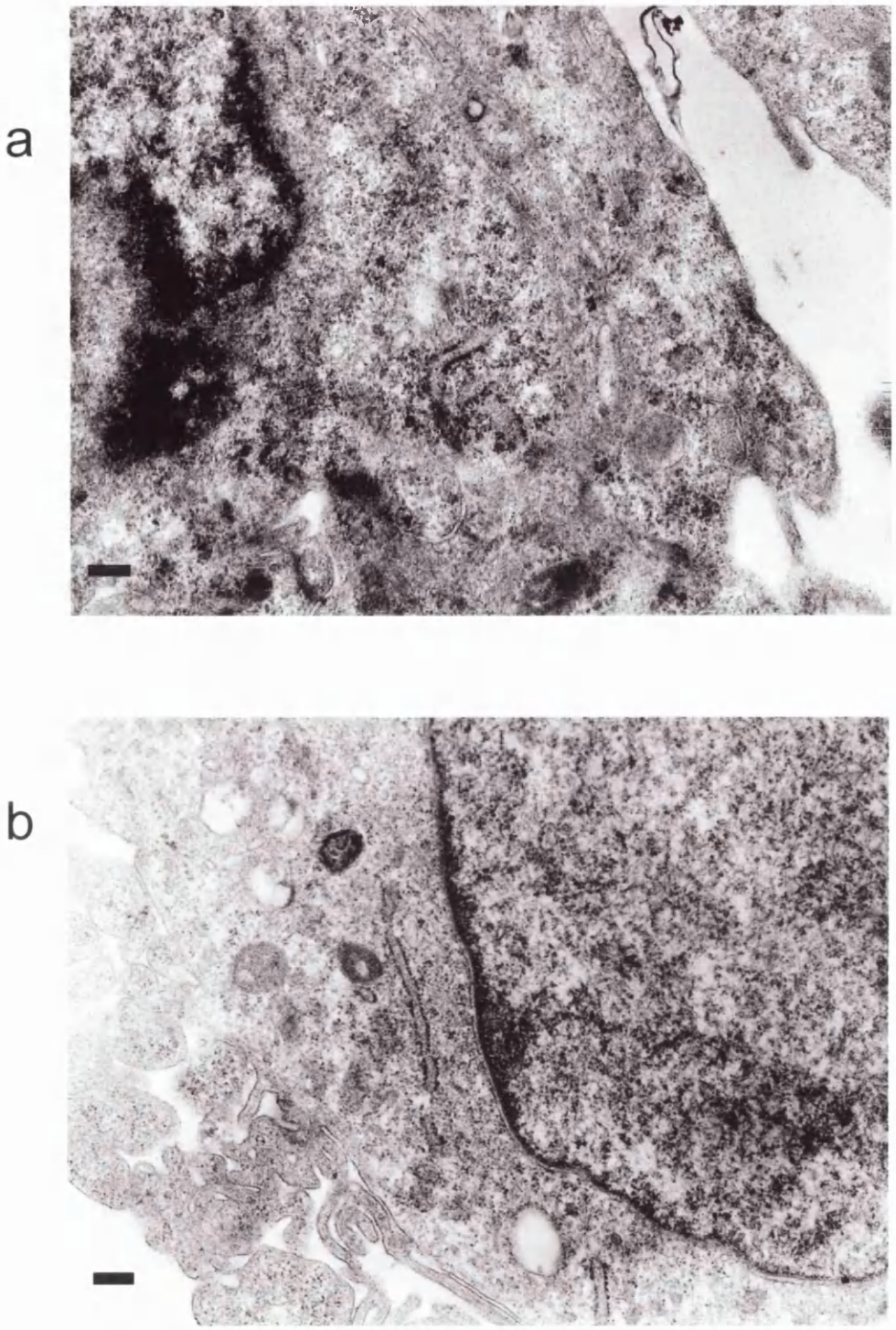


Figure 3.21 Electron micrographs of cytomegalovirus infected Vero cells.

Vero cells were infected with 2.5 p.f.u. per cell of HCMV strain AD169 (a) or with 2.5 p.f.u. per cell of HCMV strain Towne (b). Cell pellets were collected 140 h p.i.. Pellets were dehydrated, stained with uranyl acetate and counter-stained with lead citrate. 70 nm thick sections were examined at 10,000 times magnification.

The bar in the bottom left-hand corner of each picture represents approximately 250 nm.

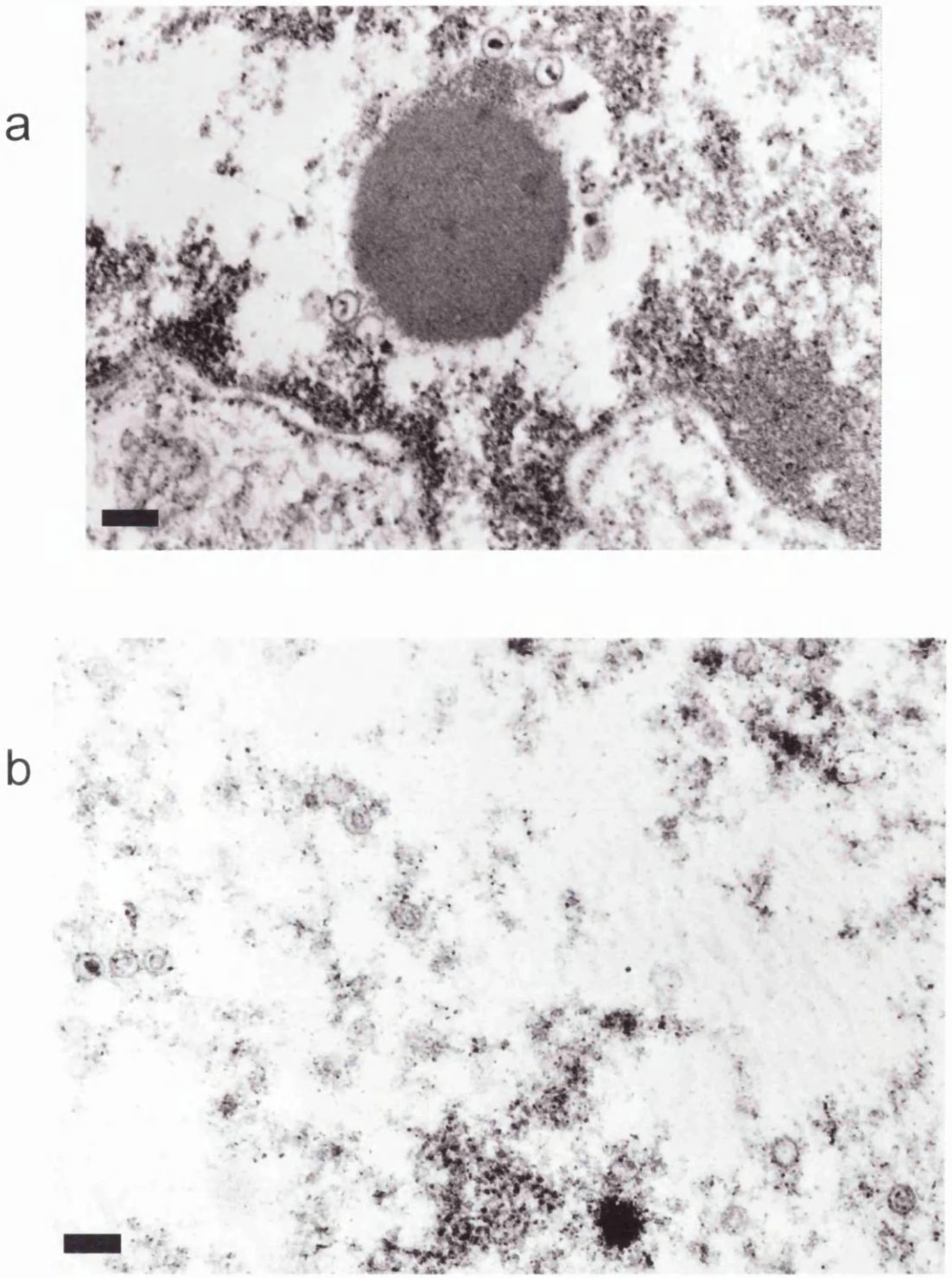


Figure 3.22 Electron micrographs of cytomegalovirus infected 293 cells.

293 cells were infected with 2.5 p.f.u. per cell of HCMV strain Towne. Cell pellets were collected 140 h p.i.. Pellets were dehydrated, stained with uranyl acetate and counter-stained with lead citrate. 70 nm thick sections were examined at (a) 10,000 or (b) 20,000 times magnification.

The bar in the bottom left-hand corner of each picture represents approximately 250 nm.

seen. Cells which contained virus particles seemed to be clumped together in the block.

During the examination of HCMV strain AD169 infected 293 cells, only one nucleus (of >100 nuclei examined) was found containing capsids. No virus was seen in mock infected 293 cells.

Summary

No virus was observed in any mock infected sample. HCMV particles were not observed in any sample of infected Vero cells, but all the nuclei in the infected HFFF2 samples contained virus particles. 293 cells contained HCMV strain Towne particles in approximately 10 % of cells examined by electron microscopy, but only one nucleus from over one hundred AD169 infected 293 cells examined contained any particles.

3.3 Discussion

3.3.1 Replication of an HCMV origin-containing plasmid in HCMV strain AD169 infected Vero cells

An HCMV origin-containing plasmid was shown to be replicated in Vero cells in the absence of detectable accumulation of helper virus genomes (figure 3.6). The data presented in section 3.2.3 confirmed that plasmid replication was dependent on the HCMV DNA polymerase and intact HCMV origin sequences, but independent of the presence of bacterial DNA methylation.

The most simple explanation for the apparently different behaviour of plasmid and genomic DNA is that because of the high background signal from input genomes, a low level of replication may have been missed. The use of Southern blot analysis means that an unambiguous increase in the signal from genomic DNA is necessary in order to conclude that replication has taken place.

In CV-1 cells, a very marked decrease in the amount of HCMV genomic DNA was observed, presumably as non-infectious input viral DNA was degraded. The decrease in

HCMV genomes detected in Vero cells was slower than the decrease in CV-1 cells, suggesting that a low level of DNA synthesis may have been occurring in Vero cells. The almost constant level of genomic DNA detected between 2 and 6 days post infection might represent a balance between genome replication and degradation. To analyse this further, time-course experiments could be done in HCMV strain AD169 infected Vero cells in the presence and absence of PFA. If higher levels of HCMV DNA accumulated at late times in the absence of inhibitor, this would suggest that DNA synthesis was occurring at a low level.

Other possible explanations for the replication of an HCMV origin-containing plasmid but apparently not HCMV strain AD169 genomes in Vero cells, are discussed below.

(a) Relative copy numbers

Genomic DNA was supplied by infection with 1 p.f.u./cell of HCMV (one infectious genome per cell). Since the particle:p.f.u. ratios of my virus stocks were in the range of 100-300:1, this may mean that more than one genome actually reached the nucleus of each infected cell. Nevertheless, as 1 μg of pSP50 corresponds to approximately $1x10^{11}$ plasmid molecules, it is probable that many more copies of the HCMV origin-containing plasmid than HCMV genomes were delivered. If the HCMV replication proteins in transfected and superinfected cells are present in limiting amounts, it is possible that preferential replication of the plasmid sequence might occur. However, this postulated inhibition of genomic replication by origin-containing plasmids would only be expected to be observed in these time-course experiments if the plasmid were taken up by the majority of cells.

A time-course for HCMV DNA replication in Vero cells which had not been transfected was essentially identical to that presented in figure 3.6, showing that the lack of detectable HCMV DNA accumulation in transfected cells was not solely due to inhibition

by the origin-containing plasmid (N. Stow, unpublished observation).

(b) DNA methylation

As previously discussed, methylation of DNA in prokaryotic and eukaryotic cells may be used to regulate the replication or transcription of DNA. The plasmid DNA used has bacterial *dam* and *dcm* methylation which is absent from viral genomes, but lacks the CpG methylation which is found on eukaryotic DNA. The presence of bacterial DNA methylation was not responsible for HCMV origin-containing plasmid replication in HCMV infected Vero cells since unmethylated plasmid DNA was replicated (figure 3.17). The absence of CpG methylation from the origin-containing plasmid DNA is unlikely to have any effect on replication since it was reported that CpG methylation of HCMV genomes in infected cells did not occur (LaFemina & Hayward, 1986). However, the effect of CpG methylation on pSP50 replication could be tested by examining the replication of plasmid DNA which has been methylated by the bacterial CpG methylase, *Sss*I.

(c) Circularisation

Transfected plasmid molecules are circular, while infecting HCMV genomes are linear and are circularised prior to DNA replication (McVoy & Adler, 1994). Circularisation of infecting HCMV genomes is one of the earliest steps in DNA replication, first detectable at about 4 h p.i. (McVoy & Adler, 1994), and reaching a maximum by 16 h p.i.. The failure of HCMV genomic DNA to circularise has previously been observed following infection of non-permissive Balb/c-3T3 cells (LaFemina & Hayward, 1983) when four HCMV early polypeptides were synthesised, indicating that at least in this case, linear HCMV genomes are available as a template for RNA transcription. It is possible that a protein required for efficient circularisation of HCMV genomic DNA is absent from, or not synthesised in, some 'non-permissive' cells such as Vero cells. Thus the HCMV replication proteins may be expressed from linear DNA and be able to replicate the circular plasmid molecules but not linear HCMV genomes.

I used field inversion gel electrophoresis (FIGE) followed by Southern blotting in an attempt to separate and detect circular and linear HCMV genomes (McVoy & Adler, 1994), but these experiments were unsuccessful and the above hypothesis was not tested.

3.3.2 HCMV strain Towne DNA replicates more efficiently in the cell lines tested than strain AD169

In contrast to strain AD169, HCMV strain Towne genomic DNA synthesis was detected following infection of Vero cells, (figure 3.8b). Also, although the data were not the result of a direct comparison of replication efficiencies, more HCMV strain Towne genomic DNA than AD169 DNA accumulated in infected 293 and U-373 MG cells. An investigation into the replication of human cytomegalovirus in SCID-hu mice (Brown *et al.*, 1995) also revealed differences in the replicative abilities of HCMV strain AD169 and Towne. HCMV strain Towne and strain AD169 from one source were able to replicate in the mice, while AD169 from a second source could not. The observed difference in DNA replication of the two HCMV strains studied here in Vero cells, may be due to interstrain sequence differences.

Because both HCMV strains are able to replicate an HCMV origin-containing plasmid in Vero cells, reported differences in the origin sequences of HCMV strain AD169 and Towne (Chen *et al.,* 1996), in immediate early gene expression (Gibson, 1981), or possible differences in expression of the DNA replicative machinery of the two viruses, are unlikely to account for their different behaviours in Vero cells. However, HCMV strain Towne has at least one sequence block which is absent from the AD169 sequence (Cha *et al.*, 1996), and the presence of additional genes in the Towne strain might allow the virus to be less host-cell restricted.

Sequence variation between the two strains is particularly pronounced at the 'a'-sequence (Mocarski *et al.*, 1987). As well as having roles in cleavage/packaging of replicated concatemeric DNA at the L-S junction, the 'a'-sequence has been implicated in

the circularisation of infecting linear genomes, and it would therefore be of interest to determine whether strain AD169 and Towne differ in their ability to circularise following infection of Vero cells.

3.3.3 HCMV strain Towne DNA replicates in cells which have previously been reported to not support HCMV DNA synthesis

The results presented in section 3.2.2, showing that Vero and 293 cells can support the replication of HCMV strain Towne genomic DNA to a level greater than 3% of the level detected in a fully permissive infection of human fibroblast (HF) cells, differ from previously reported findings, (LaFemina & Hayward, 1986; 1988).

To exclude the possibility that my cell lines were not actually Vero or 293 cells, I checked my 293 cells by immunofluorescence using a commercially available antibody, to confirm that they expressed Adenovirus E1A. In addition, I used Vero cells from two sources in the early experiments and these gave identical results. The possibility that HCMV driven replication was confined to a small number of HF cells contaminating the 293 and Vero cultures can be excluded because of the high levels of replication of the origin-containing plasmid observed in these cells.

In this thesis, DNA replication was examined following superinfection of cells which had been transfected, while LaFemina and Hayward (1986; 1988) infected untransfected cells. Transfection itself is unlikely to have caused the differences seen, and indeed, similar analysis of strain AD169 replication in untransfected Vero and 293 cells yielded essentially the same results as I observed in transfected cells (N Stow, personal communication).

Altered tropisms of clinical HCMV isolates, associated with changes in the viral genomes, have been reported to occur following tissue culture adaptation of the viruses (Sinzger *et al.*, 1999), and laboratory strains of HCMV have been reported to have lost up to 19 genes found in recent clinical isolates (Cha *et al.*, 1996). It is therefore conceivable

that my HCMV strain Towne stocks differ from those used by LaFemina and Hayward (1986; 1988). Resolution of the differences in behaviour reported here might be achieved by exchange of virus and cell stocks

3.3.4 Production of virus progeny and electron microscopic examination of infected cells

The data presented here (figures 3.18 - 3.22) as growth curves and electron micrographs were from preliminary experiments which need to be repeated in order to confirm the results, especially those concerning the replication of HCMV strain Towne in 293 cells.

Neither Vero cells infected with HCMV strain AD169 or Towne, nor 293 cells infected with HCMV strain AD169, supported the production of HCMV progeny as measured by plaque assay or by electron microscopy (EM).

HCMV particles were, however, observed by EM at 140 h p.i. in 10% of 293 cells infected with HCMV strain Towne. The observation of HCMV particles in only one nucleus of over one hundred HCMV strain AD169 infected 293 cells examined suggests that the particles in Towne infected cells are unlikely to be input virus. This data would seem to indicate another clear difference between the behaviour of HCMV strains AD169 and Towne in tissue culture. However, to confirm that *de novo* synthesis of proteins and DNA is required for HCMV strain Towne particle formation, infected cells should be incubated both with and without cycloheximide or phosphonoformic acid and analysed by electron microscopy.

It would be interesting to further investigate the block to the replication cycle in HCMV Towne and AD169 infected Vero and 293 cells, and to determine whether all the cells in the population behave similarly, or if DNA synthesis occurs in only a minority of cells in a population. Western blotting or indirect immunofluorescence against infected Vero and 293 cells could be done with a panel of antibodies to HCMV early and late

proteins. In order to verify that the proteins are synthesised *de novo* and are not components of the inoculum, proteins from infected cells incubated in cycloheximide or phosphonoformic acid should be screened in parallel.

Chapter 4: Transient replication assay

4.1 Introduction

The cis-acting signals and trans-acting proteins required for viral DNA synthesis have now been identified for representative members of the Alpha-, Beta-, and Gammaherpesviruses.

A lytic origin for HSV-1 DNA replication was identified by transfecting plasmids containing fragments of HSV-1 DNA into cells which were infected with HSV-1. Subsequently, the cotransfection method developed by Challberg (Challberg, 1986) was used to identify seven HSV-1 proteins directly involved in HSV-1 origin-dependent DNA synthesis (Wu *et al.*, 1988)

Epstein-Barr virus is the prototype human Gammaherpesvirus. An EBV origin of lytic phase DNA replication (oriLyt) was identified, as were EBV encoded homologues of the six HSV-1 replication fork proteins (Hammerschmidt & Sugden, 1988; Fixman *et al.*, 1992). The replication fork proteins are the helicase-primase complex, the DNA polymerase holoenzyme and the single-stranded DNA binding protein. In addition to the EBV replication fork proteins, Zta, Mta and Rta are required for EBV oriLyt dependent DNA synthesis in a cotransfection assay. The functional conservation of the six replication fork proteins between HSV-1 and EBV is such that in a cotransfection assay, the HSV-1 proteins, supplemented with Zta and Rta, directed EBV oriLyt dependent DNA synthesis, (Fixman *et al.*, 1995).

An origin of HCMV lytic-phase DNA replication (oriLyt) has been identified, (Anders *et al.*, 1992) and a transient replication assay was used to identify eleven HCMV loci required for origin-dependent DNA synthesis (Pari & Anders, 1993). The eleven loci identified included homologues of the six herpesviral replication fork proteins. The remaining five HCMV loci identified were termed auxiliary loci. Four of the auxiliary loci were shown to cooperate in the activation of expression from the HCMV DNA polymerase

accessory protein promoter (Iskenderian *et al.*, 1996). The effect of the fifth auxiliary locus (UL84) on gene expression was small.

Sarisky and Hayward (1996) reported that the six EBV replication fork proteins together with HCMV UL84 were able to activate the HCMV origin in transfected Vero cells, suggesting that of the HCMV auxiliary loci, only UL84 provided an essential origin-specific function.

4.2 Results

4.2.1 Replication of an HCMV origin-containing plasmid by the HCMV replicative machinery

The HCMV transient replication assay (Pari & Anders, 1993) requires the transfection of twelve plasmids into human foetal foreskin fibroblasts. I endeavoured to use this assay to further investigate HCMV origin-dependent DNA synthesis, but, despite numerous attempts, was unable to achieve replication of the HCMV origin-containing plasmid by cotransfection of HF cells with the set of plasmids used by Pari and Anders (1993).

As 293 and Vero cells were shown to be permissive for HCMV origin-dependent DNA synthesis (section 3.2.2 of this thesis), and because of their relatively high efficiency of transfection, the HCMV transient replication assay was attempted in these cells. Again, no replication of an HCMV origin-containing plasmid was detected following cotransfection with plasmids encoding the eleven essential replication loci.

4.2.2 Replication of an HCMV origin-containing plasmid by the HSV-1 core replication machinery

As it had previously been demonstrated that the EBV replication fork proteins were functionally interchangeable with the replication fork proteins of both HSV-1 and HCMV (Fixman *et al.*, 1995; Sarisky & Hayward, 1996), the ability of the HSV-1 replication fork

proteins to replicate an HCMV origin-containing plasmid in the presence of the HCMV auxiliary loci was investigated.

(a) Replication of pSP50 by the HSV-1 core replication machinery

The six open reading frames encoding the HSV-1 replication fork proteins UL5, UL8, UL29, UL30, UL42 and UL52 are contained in plasmids pE5, pE8, pE29, pE30, pE42 and pE52. Plasmid pE9 expresses the HSV-1 origin-binding protein (UL9) and a functional copy of oriS is contained within pS1. The HSV-1 plasmids were used in combination with the HCMV origin-containing plasmid (pSP50) and plasmids encoding the HCMV auxiliary proteins (pZP3 encoding IRS1/TRS1, pZP8 encoding UL36-38, pZP13 encoding UL84, pZP24 encoding UL112-113, pSVH encoding IE1/IE2, pCV84 constructed to constitutively express UL84).

Expression of UL84 in transfected cells

To confirm that pCV84 expresses UL84, immunofluorescent labelling was used to detect UL84 in transfected cells. pZP13 was transfected with the remaining ten replication plasmids to ensure expression from the UL84 promoter. Expression of UL84 from the HCMV major immediate early promoter (in pCV84) did not require addition of any other HCMV loci.

293 cells were set up and transfected as previously described. One dish of cells (a) was transfected with 0.4 μg/dish each of pZP3, pZP7, pZP8, pZP13, pZP18, pZP20, pZP24, pZP29, pZP33 and pZP37 and with 1.6 μg/dish each of pSVH and pSP50. The cells were fixed 120 h p.t.. One dish of cells (b) was transfected with 0.8 μg/dish of pCV84 and fixed 48 h p.t.. Cells were permeabilised then probed with the UL84 monoclonal antibody, D42, followed by Cy™3 conjugated goat anti-mouse IgG.

Cells were examined by fluorescent microscopy at 543 nm. Images were captured in black and white with a CCD camera system and were converted to pseudocolour using

the associated software. The fluorescent images shown in figure 4.1 indicate that in both instances UL84 could be detected with the monoclonal antibody. In cells transfected with either pZP13 or pCV84, the staining is predominantly nuclear, but is not in clearly defined replication compartments. Cells transfected with a plasmid containing the UL84 open reading frame in reverse orientation (pCV84(-)) did not stain red (data not shown). Non-expressing cells in the fields of view shown in figure 4.1, which are not stained red, also confirm the specificity of antibody binding.

pSP50 is replicated by the HSV-1 replication fork proteins and the HCMV auxiliary proteins

Vero cells were transfected with a mixture containing the six HSV-1 replication fork plasmids supplemented with either (a) 0.4 μg/dish each of pE9 and pS1, (b) 0.4 μg/dish of pCV84 and 1.6 μg/dish of pSP50, or (c) 0.4 μg/dish each of pZP3, pZP8, pZP13 and pZP24 and 1.6 μg/dish each of pSVH and pSP50. Total cellular DNA was prepared at 46 h p.t.. DNA was processed as previously described, hybridised to ^{32}P-labelled pAT153, and an image obtained (figure 4.2).

As expected, pS1 was replicated by the HSV-1 replication machinery and the HSV-1 origin-binding protein. Similarly, pSP50 was replicated after cotransfection of the six HSV-1 plasmids with the full set of five HCMV auxiliary loci with UL84 supplied by pZP13. pSP50 was not, however, replicated by cotransfection of the HSV-1 core replication plasmids with a plasmid that constitutively expresses UL84.

The HSV-1 replication fork proteins, like their EBV homologues, are therefore able to replicate an HCMV origin-containing plasmid in the presence of the HCMV auxiliary proteins. In contrast to the findings with EBV, UL84 alone does not appear to be able to mediate replication in the presence of the HSV-1 replication fork proteins.

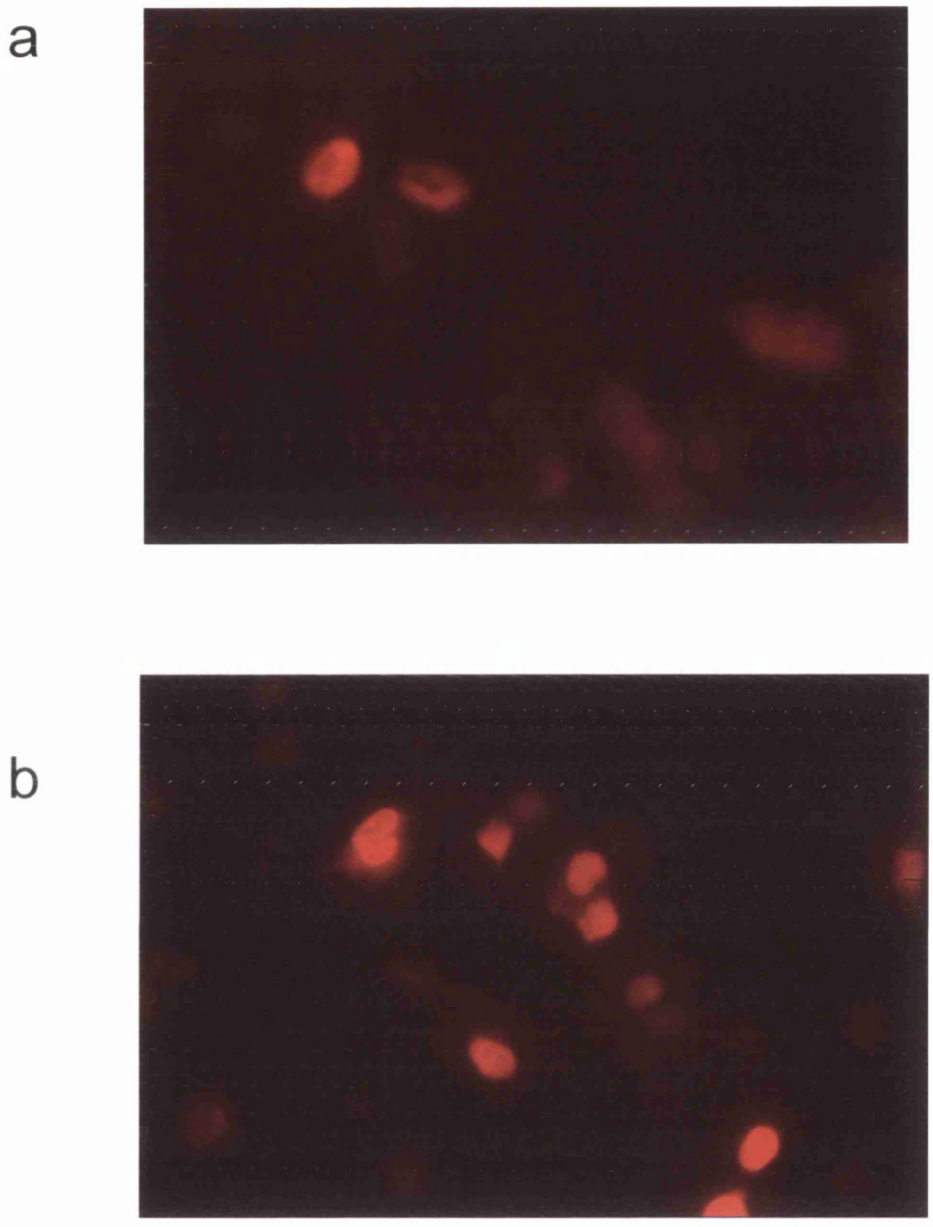


Figure 4.1 Expression of UL84 in transfected 293 cells.

293 cells were transfected with (a) 0.4 μg/dish each of pZP3, pZP7, pZP8, pZP13, pZP18, pZP20, pZP24, pZP29, pZP33 and pZP37 and 1.6 μg/dish each of pSVH and pSP50 or with (b) 0.8 μg/dish of pCV84.

Cells were fixed 120 (a) or 48 (b) h post transfection. Cells were permeabilised and probed with D42 (monoclonal UL84 antibody) then Cy™3 conjugated goat anti-mouse IgG. Slides were examined by fluorescent microscopy at 543 nm. Digital images were captured and processed as described.

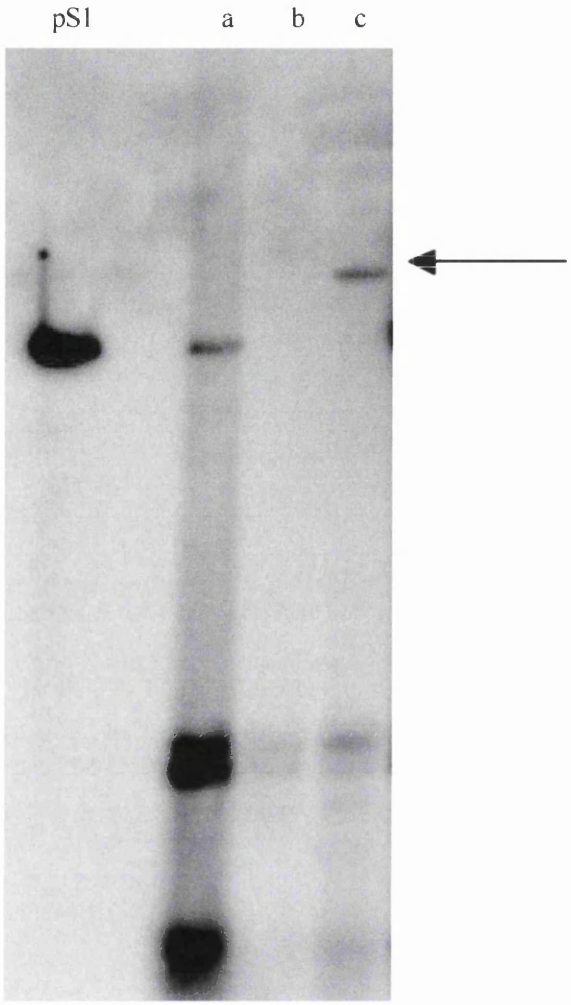


Figure 4.2 Replication of pSP50 by the HSV-1 replicative machinery.

Vero cells transfected with 0.4 μg/dish each of the HSV-1 replication fork plasmids (pE5, pE8, pE29, pE30, pE42 and pE52) with the addition of: (a) 0.4 μg/dish each of pS1 and pE9, (b) 1.6 μg/dish pSP50 and 0.4 μg/dish pCV84, (c) 1.6 μg/dish pSP50 and 0.4 μg/dish each of the HCMV auxiliary plasmids (pZP3, pZP8, pZP13 and pZP24) and 1.6 μg/dish of pSVH.

25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of *Eco*RI digested pS1 was loaded as a marker (pS1).

◀—— Shows the position of an *Eco*RI digested pSP50 marker band.

(b) Sensitivity of replication to PAA

To confirm that the replication of pSP50 (figure 4.2) was due to the HSV-1 DNA polymerase, the sensitivity of replication to PAA was determined.

Vero monolayers were transfected with pCMVori, the six HSV-1 replication plasmids, and the HCMV auxiliary plasmids. After the DMSO boost, cells were fed with unsupplemented medium or with medium supplemented with 200 μg/ml of PAA. At 46 h p.t., total cellular DNA was prepared and processed as described previously.

Figure 4.3 shows that in the absence of polymerase inhibitor, pCMVori was replicated. Cells treated with PAA failed to support the replication of pCMVori, confirming that the replication observed was due to the HSV-1 DNA polymerase.

(c) Replication is specific to HCMV origin sequences

To confirm that the replication observed was due to the HCMV origin sequences, three origin-containing plasmids and one deleted origin-containing plasmid were tested in the cotransfection assay.

Vero cells were transfected with the six HSV-1 replication fork plasmids and the HCMV auxiliary plasmids together with 0.8 μg/dish of pSP50, pADori, pADoriΔXhoI or pMA1.

Total cellular DNA was prepared 46 h p.t. and digested with *Xba*I (linearises each of the test plasmids), and *Dpn*I. Fragments were separated by electrophoresis, blotted and hybridised as before. The blot was exposed to a phosphorimager screen and a phosphorimage was produced (figure 4.4).

As observed following HCMV superinfection in transfected Vero cells (figures 3.1 and 3.15), in this experiment pSP50, pADori and pMA1 were all replicated, the deleted origin-containing plasmid, pADoriΔXhoI, was not. Replication of an HCMV origin-containing plasmid by the HSV-1 replication fork proteins is therefore not peculiar

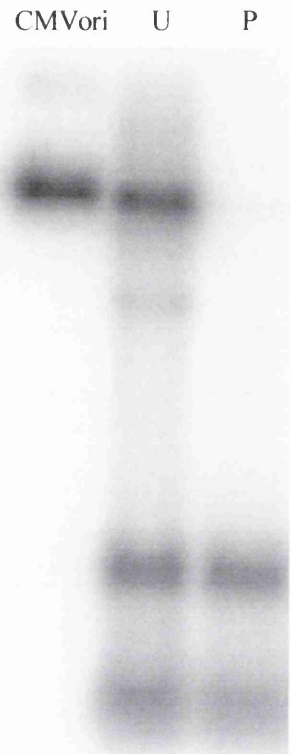


Figure 4.3 Sensitivity of replication to PAA.

Vero cells transfected with 0.4 μg/dish each of the HSV-1 replication fork plasmids, 1.6 μg/dish of pCMVori and 0.4 μg/dish of each of the five HCMV auxiliary plasmids. Cells were incubated in unsupplemented media (U) or media containing 200 μg/ml PAA (P).

25% of the DNA from a 35 mm dish was digested with *Xba*I and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of *Xba*I digested pCMVori was loaded as a marker (CMVori)

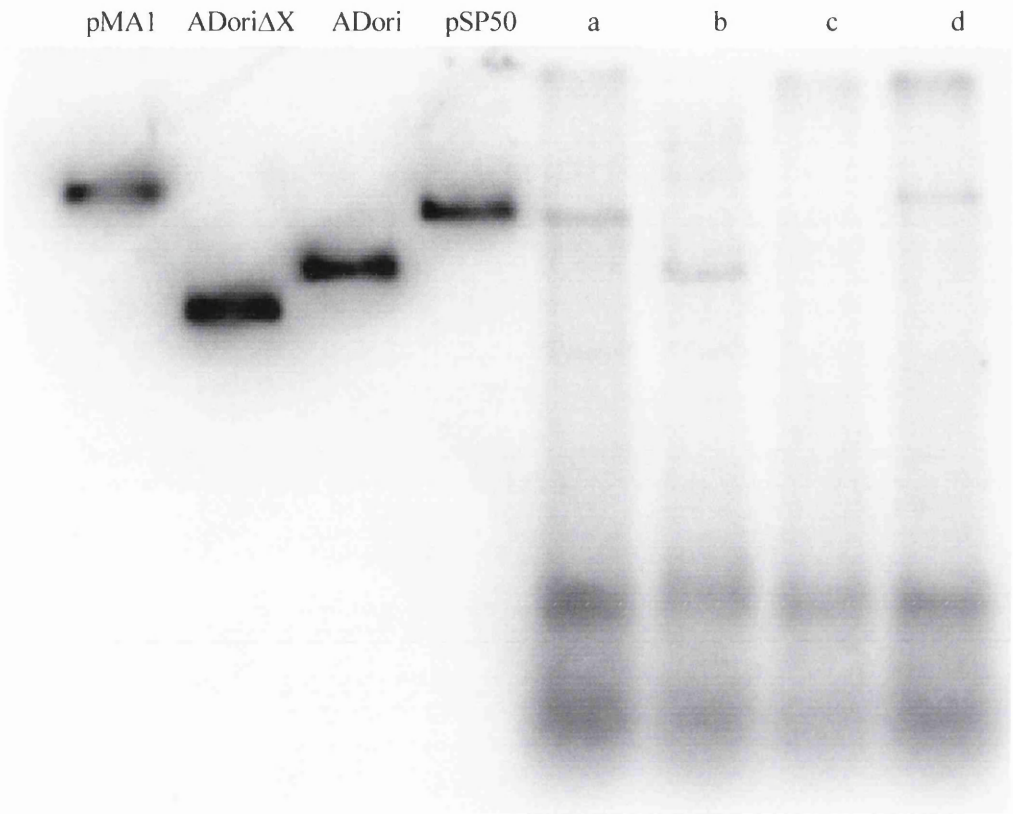


Figure 4.4 Replication is specific to HCMV origin sequences.

Vero cells transfected with 0.4 μg/dish each of the HSV-1 replication fork plasmids and 0.4 μg/dish of each of the five HCMV auxiliary plasmids.

0.8 μg/dish of the appropriate origin containing plasmid was added; (a) pSP50, (b) pADori, (c) pADoriΔXho, or (d) pMA1.

25% of the DNA from a 35 mm dish was digested with *Xba*I and *Dpn*I. Fragments were separated through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng each of *Xba*I digested pSP50 (pSP50), pADori (ADori), pADoriΔXho (ADoriΔX) and pMA1 (pMA1) were loaded as markers.

to pSP50 and is, furthermore, dependent on an intact HCMV lytic origin sequence.

4.2.3 HCMV functions required for replication of an HCMV origin-containing plasmid by the HSV-1 replication fork proteins

The results presented in section 4.2.2 demonstrated that the HSV-1 replication fork proteins are able to replicate an HCMV origin-containing plasmid in the presence of the five HCMV auxiliary loci. Further work was therefore done to define which of the HCMV auxiliary loci are essential in the HSV-1 driven transient replication assay.

(a) Replication is not dependent on UL84 or IRS1/TRS1

An omission assay was done to determine whether UL36-38, UL84, or IRS1/TRS1 were important for the replication of pSP50 by the HSV-1 replication fork proteins.

Vero cells were transfected with the HSV-1 replication fork plasmids, pSP50, and the five HCMV auxiliary plasmids (a). (b) pZP3, (c) pZP8 or (d) pZP13 were individually omitted from the transfection mix. At 46 h p.t., total cellular DNA was prepared and processed for analysis by Southern blotting as previously described, and an image was produced.

Figure 4.5 shows that omission of pZP8 significantly inhibited the replication of pSP50. Omission of pZP13 or pZP3 did not reduce the amount of plasmid replicated.

(b) pSVH provides functions for replication of pSP50 by the HSV-1 replication proteins

Vero cells were set up and transfected with the HSV-1 replication fork plasmids, pSP50, and all five possible combinations of four HCMV auxiliary plasmids (0.4 μg/dish of each) *i.e.*, each of the five HCMV auxiliary plasmids was omitted in turn from the transfected plates. Total cellular DNA was prepared, digested with *Eco*RI and *Dpn*I, and processed as previously described to produce the image in figure 4.6.

It was previously shown (figure 4.5) that there was no decrease in the amount of pSP50 replication when either UL84 or ISR1/TRS1 were omitted from the transient

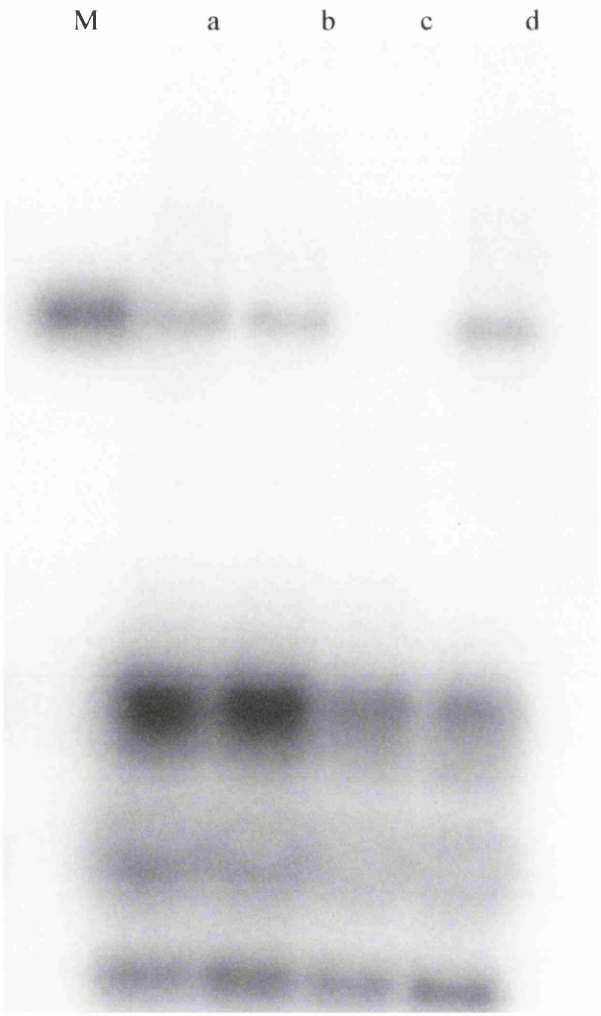


Figure 4.5 Replication is not dependent on UL84 or IRS1/TRS1.

Vero cells were transfected with 0.4 μg/dish of each of the HSV-1 replication fork plasmids, 1.6 μg/dish of pSP50 and pSVH and 0.4 μg/ dish of each of pZP3, pZP8, pZP13 and pZP24 with the following omissions; (a) no omission, (b) pZP3, (c) pZP8 and (d) pZP13.

25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of EcoRI digested pSP50 was loaded as a marker (M).

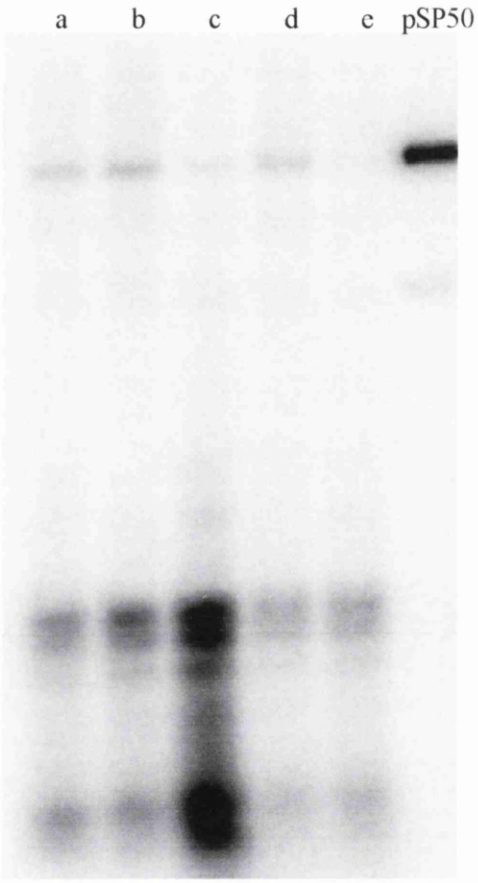


Figure 4.6 Replication is dependent on pSVH.

Vero cells transfected with 0.4 μg/dish of each of the HSV-1 replication fork plasmids, 1.6 μg/dish of pSP50 and 0.4 μg/dish of each of pZP3, pZP8, pZP13, pZP24 and pSVH with the following omissions; (a) pZP13, (b) pZP3, (c) pZP8, (d) pZP24 and (e) pSVH.

25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of *Eco*RI digested pSP50 was loaded as a marker (pSP50)

replication assay. The level of replication in the absence of either pZP3 or pZP13 can therefore be taken as the positive control level. Figure 4.6 shows that omission of pZP24, (d), did not reduce the replication of pSP50 by the HSV-1 replication fork proteins. Omission of pSVH (e) abrogated replication of pSP50, and although the effect was less marked, omission of pZP8 (c) also caused a large drop in replication efficiency.

The UL112-113 products (expressed from plasmid pZP24) are, like UL84 and IRS1/TRS1, dispensable in this assay, whereas one or more of the proteins encoded by pSVH and pZP8 appear to play more important roles.

(c) pSVH is sufficient to complement replication of pSP50 by the HSV-1 core replication proteins

Vero cells were set up and transfected as previously described with a mix containing the HSV-1 replication fork plasmids and pSP50 in the presence or absence of pSVH. At 48 h p.t., total cellular DNA was prepared and analysed as previously described.

Figure 4.7 shows that although pSP50 was not replicated by the HSV-1 replication machinery alone (a), replication was observed on addition of pSVH (b).

One (or more) of the protein products of pSVH is therefore both necessary and sufficient to mediate replication of an HCMV origin-containing plasmid by the HSV-1 replication fork proteins.

(d) Replication efficiency is significantly increased by pZP8

Vero monolayers were transfected with a mix containing pCMVori, the HSV-1 replication fork plasmids and pSVH. Plates were additionally transfected with individual plasmids pZP3, pZP8, pZP13 or pZP24, or with pZP8 in combination with pZP3, pZP13 or pZP24.

Total cellular DNA was prepared 46 h p.t., digested with *Xba*I and *Dpn*I, and analysed by Southern blot hybridised to ^{32}P-labelled pAT153. The blot was exposed to a phosphorimager screen, the image generated is shown in figure 4.8.

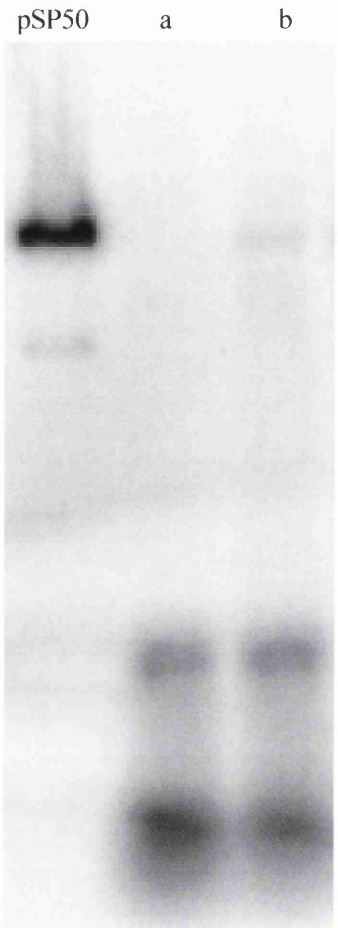


Figure 4.7 pSVH is sufficient to complement the HSV-1 replication fork proteins for replication of pSP50.

Vero cells were transfected with 0.4 μg/dish of each of the six HSV-1 core replication plasmids, 1.6 μg/dish of pSP50 and either (a) no addition, or (b) 0.4 μg/dish of pSVH.

25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of *Eco*RI digested pSP50 was loaded as a marker (pSP50)

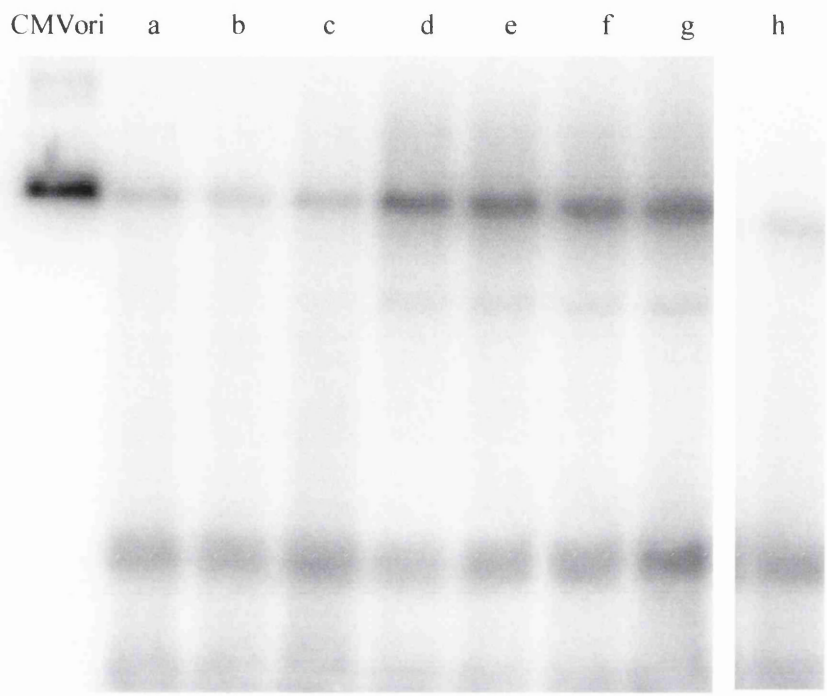


Figure 4.8 Replication efficiency is significantly increased by pZP8.

Vero cells transfected with 0.4 μg/dish of each of the HSV-1 replication fork plasmids and pSVH, and 1.6 μg/dish each of pCMVori and 0.4 μg/dish of each of (a) pZP3, (b) pZP13, (c) pZP24, (d) pZP8, (e) pZP3 and pZP8, (f) pZP13 and pZP8, (g) pZP24 and pZP8 or (h) no additional plasmids.

25% of the DNA from a 35 mm dish was digested with *Xba*I and *Dpn*I. Fragments were separated through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of *Xba*I digested pCMVori (CMVori) was loaded as a marker.

As before, the HCMV origin-containing plasmid, pCMVori, was replicated by the HSV-1 replication machinery supplemented with pSVH (h). Addition of pZP3 (a), pZP13 (b), or pZP24 (c) did not increase the amount of pCMVori replicated, in contrast, addition of pZP8 (d) did significantly increase the amount of replicated plasmid observed. Addition of pZP3, pZP13 or pZP24 (lanes e, f, or g respectively) to a mix containing both pSVH and pZP8 did not further increase the amount of plasmid replicated.

Thus one or more of the products of pZP8 can significantly enhance the replication of an HCMV origin-containing plasmid by the HSV-1 replication fork proteins and pSVH.

(e) Replication fork functions could not be provided by HSV-1

In order to simplify this assay, HSV-1 virus was tested for its ability to replicate pSP50 in the presence of the HCMV auxiliary loci.

Vero cells were transfected with pSP50, pZP8 and pSVH. Replication fork proteins were provided by infection with 20 p.f.u./cell of HSV-1 (X) or by cotransfection of the six HSV-1 replication fork plasmids (P).

Total cellular DNA was prepared 46 h p.t. and digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis, blotted and hybridised to ^{32}P-labelled pAT153. The blot was exposed to a phosphorimager screen and scanned (figure 4.9).

As before, the HSV-1 replication fork plasmids in the presence of pZP8 and pSVH were able to replicate an HCMV origin-containing plasmid. Remarkably, HSV-1 virus was unable to function in this assay.

(f) The replicative function of pSVH is attributable to IE2

The major immediate early locus contained in pSVH is a complex region reported to specify at least five transcripts. To elucidate which of the major immediate early proteins activate the replication of an HCMV origin-containing plasmid in conjunction with the HSV-1 replication fork proteins, mutants in pSVH were made and tested. The construction of pSVH amber mutants is described in figure 4.10.

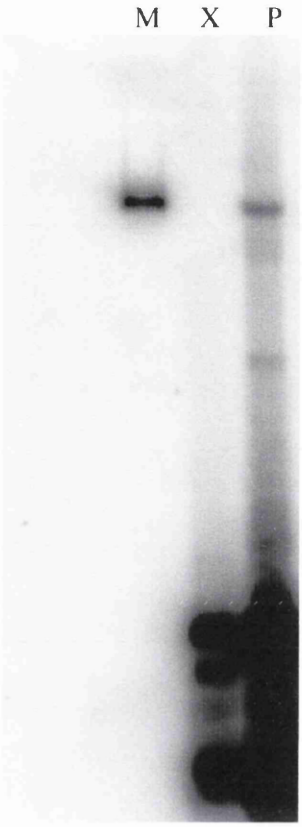


Figure 4.9 HSV-1 virus does not provide replicative machinery to replicate pSP50.

Vero cells were transfected with 1.6 μg/dish of pSP50 and 0.4 μg/dish of each of pSVH and pZP8. Replicative machinery was provided by transfection of 0.4 μg/dish of each of the six HSV-1 replication fork plasmids (P) or by infection with 20 p.f.u./cell of HSV-1 (X).

25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of *Eco*RI digested pSP50 was loaded as a marker (M).

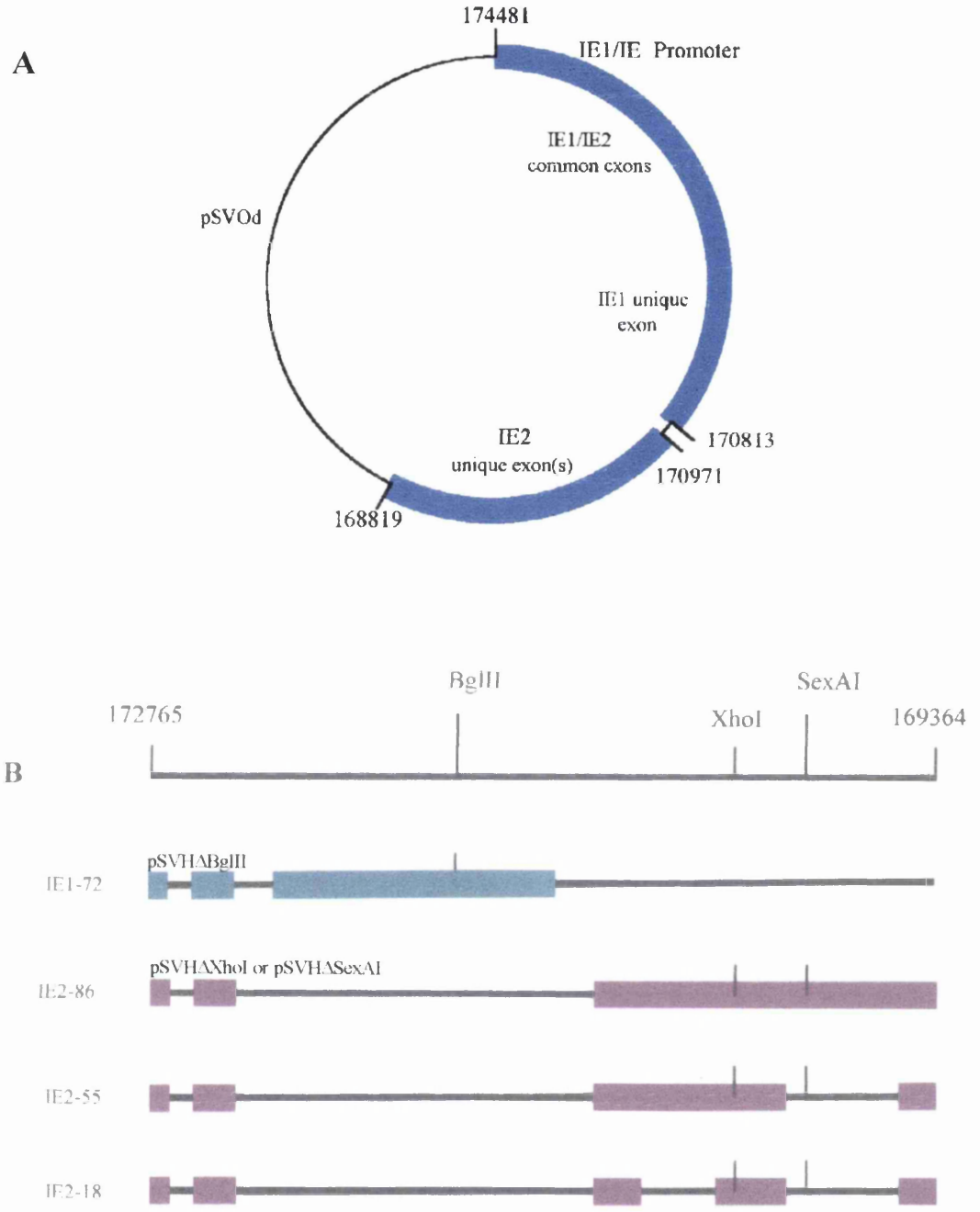


Figure 4.10 pSVH mutants.

A) The pSVH plasmid contains two overlapping fragments of DNA from HCMV strain Towne which span the IE1/2 locus (Stenberg *et al.*, 1990).

B) The coding region for IE1/2 is shown along with the restriction enzymes used in the construction of pSVH mutants. The single and double mutants were made by inserting an *Xba*I amber linker at the site named. The colored blocks show the regions of the spliced mRNAs which are translated for each protein.

Expression of IE1 and IE2 from pSVH derived plasmids

To confirm that plasmids pSVHΔBglII (intact IE2 locus) and pSVHΔXhoI (intact IE1 locus) produce the appropriate proteins, samples of HCMV infected or transfected cells were reacted with an IE1/IE2 polyclonal antibody in a Western blot.

293 cells were set up and transfected with 1.6 μg/dish of pSVH, pSVHΔBglII or pSVHΔXhoI, or infected with 1 p.f.u./cell of HCMV strain AD169 or Towne, or mock infected.

Cells were harvested 24 h p.i.. Proteins were separated by electrophoresis through a 10% SDS-polyacrylamide gel. 10 % of the cells from a 35 mm dish were loaded per lane. Duplicate gels were run, one gel was stained to check that similar amounts of protein were loaded in each lane (data not shown), the other gel was electroblotted as described. The blot was reacted with anti-IE1/IE2 followed by peroxidase conjugated protein-A. The blot was developed using ECL reagent and exposed to X-Omat film. The film was scanned and its image is shown in figure 4.11.

Both IE1 and IE2 were detected in cells infected with AD169 or Towne, or transfected with pSVH. The pSVH transfected cells showed proteins which co-migrated with those in HCMV strain Towne infected cells. This was expected since the HCMV DNA sequences in pSVH were cloned from HCMV strain Towne. The electrophoretic mobilities of IE1 and IE2 in cells infected with strain AD169 were slightly different from those in cells infected with strain Towne, in agreement with previous results (Gibson, 1981).

pSVHΔBglII expressed IE2-86 but not IE1-72, while pSVHΔXhoI expressed IE1-72 but not IE2-86. The two pSVH mutant plasmids therefore express the expected proteins.

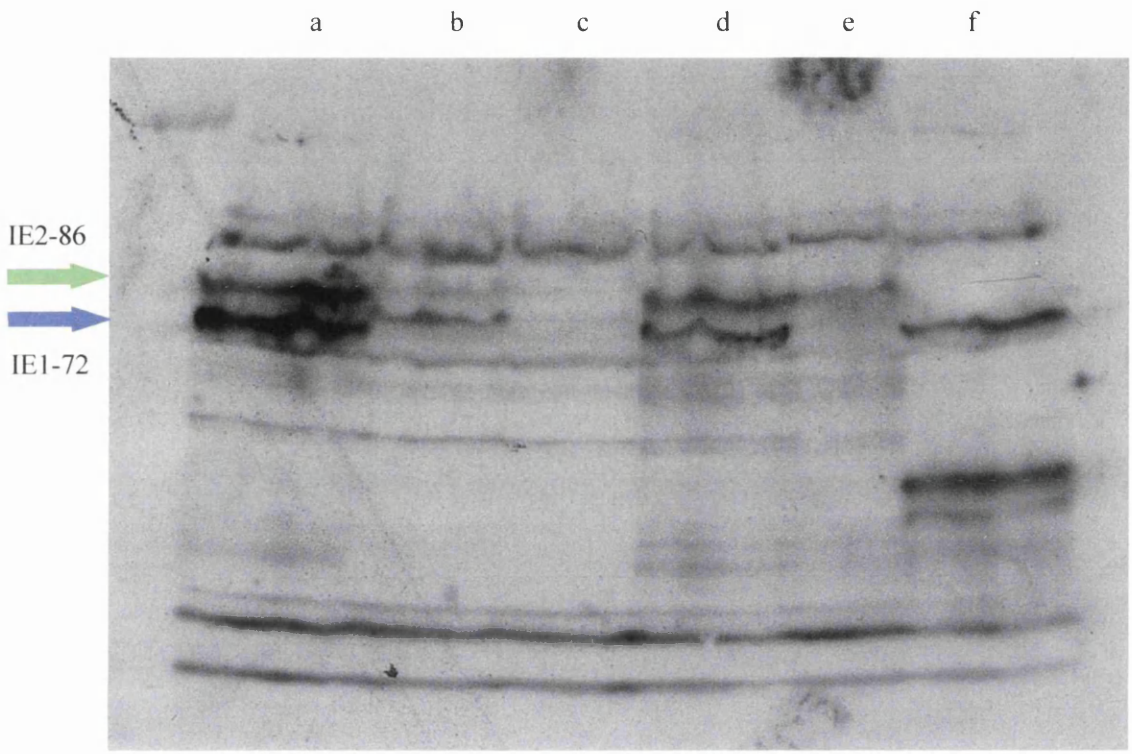


Figure 4.11 Expression of IE1/IE2 from HCMV and pSVH plasmids.

293 cells were either infected with 1 p.f.u/cell of HCMV strain Towne (a), strain AD169 (b) or mock infected (c), or transfected with either 1.6 μg/dish of pSVH (d), pSVHΔBglII (e), or pSVHΔXhoI (f).

Cells were harvested 24 h p.i. and 10% of the cells from a 35 mm dish were subjected to electrophoresis through a 10% SDS-polyacrylamide gel which was electroblotted and hybridised to polyclonal IE1/IE2 followed by horseradish peroxidase conjugated protein-A. The blot was developed with ECL reagent and exposed to X-Omat film.

Replicative function of pSVH resides within the IE2 locus

The pSVH mutants were tested for function in the transient replication assay.

Vero cells were set up and transfected with a mix containing the HSV-1 replication fork plasmids, pSP50 and pZP8 with (a) no addition, (b) pSVH, (c) pSVHΔXhoI, (d) pSVHΔBglII, or (e) pSVHΔXhoI and pSVHΔBglII. Total cellular DNA was prepared 46 h p.t., analysed by Southern blot as described, and a phosphorimage was produced, figure 4.12.

As before, replication of pSP50 was directed by the HSV-1 replication fork proteins with pSVH and pZP8 (b); pZP8 alone was insufficient to promote HCMV origin-dependent DNA synthesis by the HSV-1 replication fork proteins (a). Addition of pSVHΔBglII (d) to the transfection mix allowed transactivation of pSP50, while addition of pSVHΔXhoI (c) did not support transactivation. Transactivation was observed when both IE mutants were transfected together (e).

These results indicate that the products of pZP8 alone are insufficient to mediate replication of an HCMV origin-containing plasmid by the HSV-1 replication fork proteins. Furthermore, in the presence of pZP8, replication of pSP50 was observed when a plasmid expressing IE2 but not IE1 was used, demonstrating that IE1 is not required for activation of the HCMV origin. Transactivation in the presence of both IE mutants confirmed that neither deleted protein had a dominant inhibitory effect. It can therefore be concluded that the IE2 locus plays an important role in activating the HCMV origin in this assay.

(g) pSVH and pZP8 functions responsible for transactivating replication

IE2 mutants

The IE2 locus generates at least four transcripts, the most abundantly expressed of these (at immediate early times, in HF cells) is IE2-86. To determine whether IE2-86 is responsible for the replicative function of pSVH, a mutant was made (pSVHΔSexAI) that

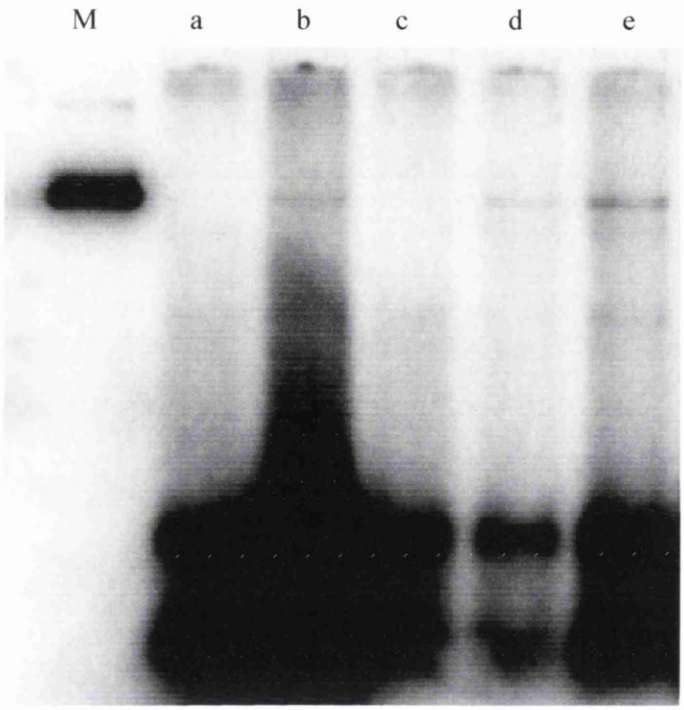


Figure 4.12 Replicative function of pSVH resides within the IE2 locus.

Vero cells were transfected with 0.8 μg/dish of pSP50, with 0.4 μg/dish of each of the six HSV-1 replication fork plasmids and pZP8 with the addition of 0.4 μg/dish of the following; (a) no addition, (b) pSVH, (c) pSVHΔXhoI, (d) pSVHΔBglII and (e) pSVHΔXhoI and pSVHΔBglII.

25 % of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of *Eco*RI digested pSP50 was loaded as a marker (M).

was predicted to abrogate IE2-86 and L40 (a late transcript) and maintain intact IE2-55 and IE2-18 transcripts. This mutant was tested for function in the cotransfection assay. To ensure that any effect seen using the IE2-86 mutant was not due overexpression of IE1, a double mutant lacking both IE1 and IE2-86 (pSVHΔBglIIΔSexAI) was also made (figure 4.10) and tested (see below).

UL36-38 mutants

The UL36-38 locus in pZP8 expresses at least four known proteins. To determine which of the products of the UL36-38 locus were necessary for stimulating replication of pSP50 by the HSV-1 replication fork proteins and IE2, pZP8 mutants were made and tested.

The construction of pZP8 amber mutants, and an *Rsr*II deletion mutant, is described in figure 4.13.

Vero cells were transfected with the six HSV-1 replication fork plasmids and pSP50 alone (a) or with the addition of HCMV auxiliary plasmids in combinations outlined in table 4.1.

	LANE										
	a	b	c	d	e	f	g	h	i	j	locus or mutation
pSVH		X	X	X	X	X	X				IE1/IE2
pSVHΔSexAI									X		IE2-86 (L40) mutant
pSVHΔSexAIΔBglII										X	IE1 and IE2-86 (L40) mutant
pZP8			X					X	X	X	UL36-38
pZP8ΔBstEII				X			X				UL36 mutant
pZP8ΔEcoNI					X		X				UL37 and UL37x1 mutant
pZP8ΔRsrII						X	X				UL37, UL37x1 and UL38 mutant

Table 4.1 The plasmids added (X) to the transfection mix containing 0.4 μg/dish of each of the six HSV-1 core replication plasmids and 1.6 μg/dish of the HCMV origin-containing plasmid. The column labels correspond to the lanes of the phosphorimage shown in figure 4.14. The proteins expressed by, or the mutations in, the pSVH and pZP8 derived plasmids are described.

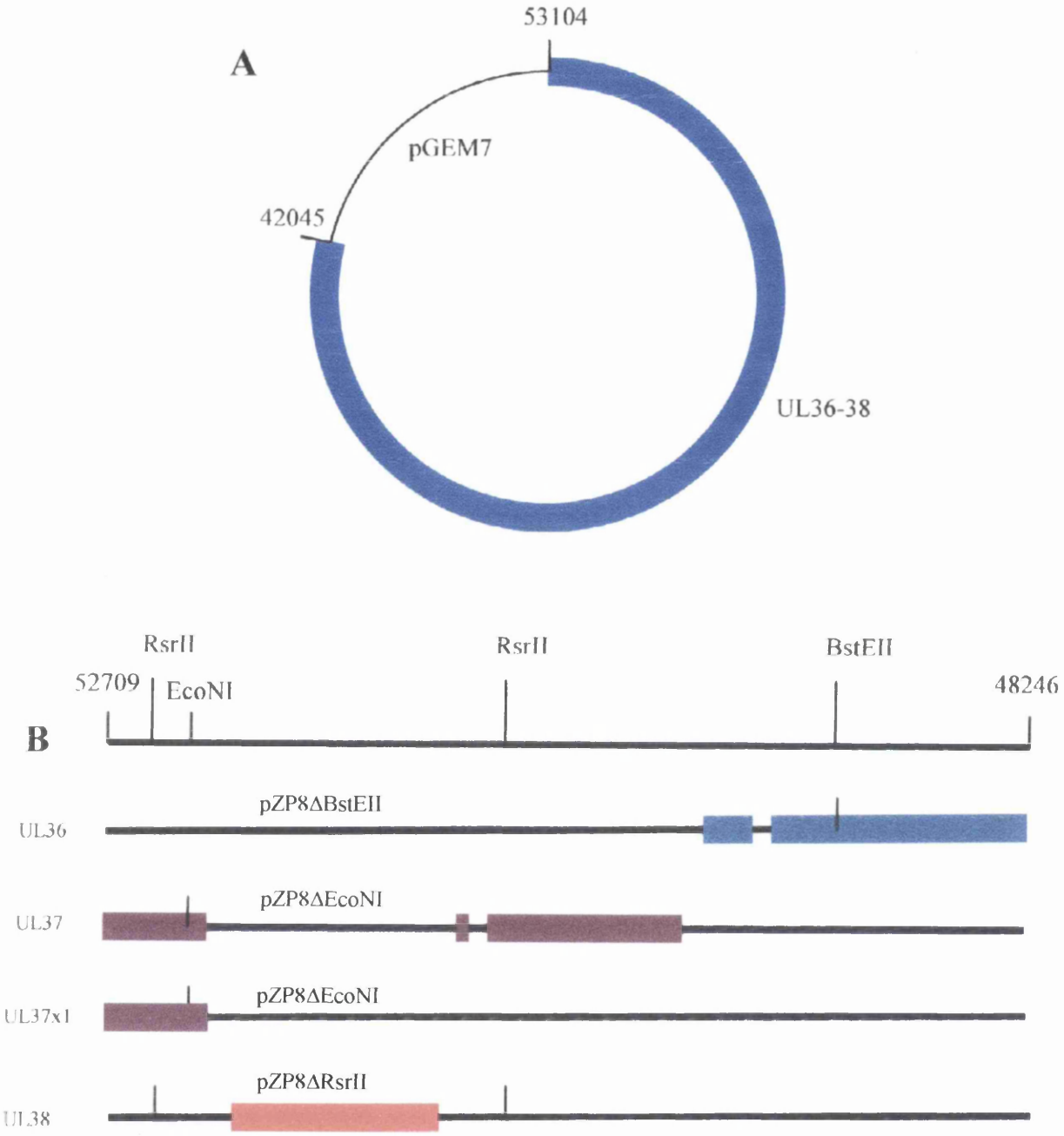


Figure 4.13 pZP8 mutants.

A) The pZP8 plasmid contains 11.0 kbp of HCMV DNA spanning the UL36-38 locus (Pari *et al.*, 1993). B) The UL36-38 coding region contained in pZP8, showing the restriction enzymes used in the construction of the pZP8 mutants. The colored blocks show the translated regions of the spliced mRNAs from UL36-38. Two of the mutant pZP8 plasmids (to abrogate UL36 and UL37/UL37x1) were made by inserting an *Xba*I amber linker at the appropriate site within pZP8. The UL38 mutant plasmid was made by deleting the sequences of pZP8 between the two *Rsr*II sites shown.

Total cellular DNA was prepared 46 h p.t. and digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis, blotted and hybridised to ^{32}P-labelled pAT153. The blot was exposed to a phosphorimager screen and a phosphorimage was acquired as previously described. The image is shown in figure 4.14.

The HSV-1 core replication proteins alone, as before, were insufficient to replicate pSP50 (a), replication was observed when pSVH (b), but not pZP8 (h), was individually present. A combination of pSVH and pZP8 (c) resulted in the highest level of replication.

A level of replication similar to the level of replication with pSVH alone was observed when pZP8ΔRsrII (f) was transfected. Replication was, however, stimulated when pZP8ΔBstEII (d) or pZP8ΔEcoNI (e) were transfected. Replication was also stimulated when all three pZP8 mutants were added to pSVH and the HSV-1 core replication plasmids (g), demonstrating that the products of pZP8ΔRsrII do not have a dominant inhibitory effect.

Cotransfection of the six HSV-1 replication fork plasmids and pZP8 with either pSVHΔSexAI (i) or pSVHΔBglIIΔSexAI (j) failed to complement replication of pSP50.

These results show that the important protein contributed by pSVH is IE2-86 (or possibly L40). The fact that the pZP8 derivative plasmids affecting the UL36 or UL37/UL37x1 proteins supported stimulated levels of DNA replication, whereas the plasmid affecting UL37/UL37x1 and UL38 did not, suggests that UL38 is the only function supplied by pZP8 which is required for enhanced levels of replication.

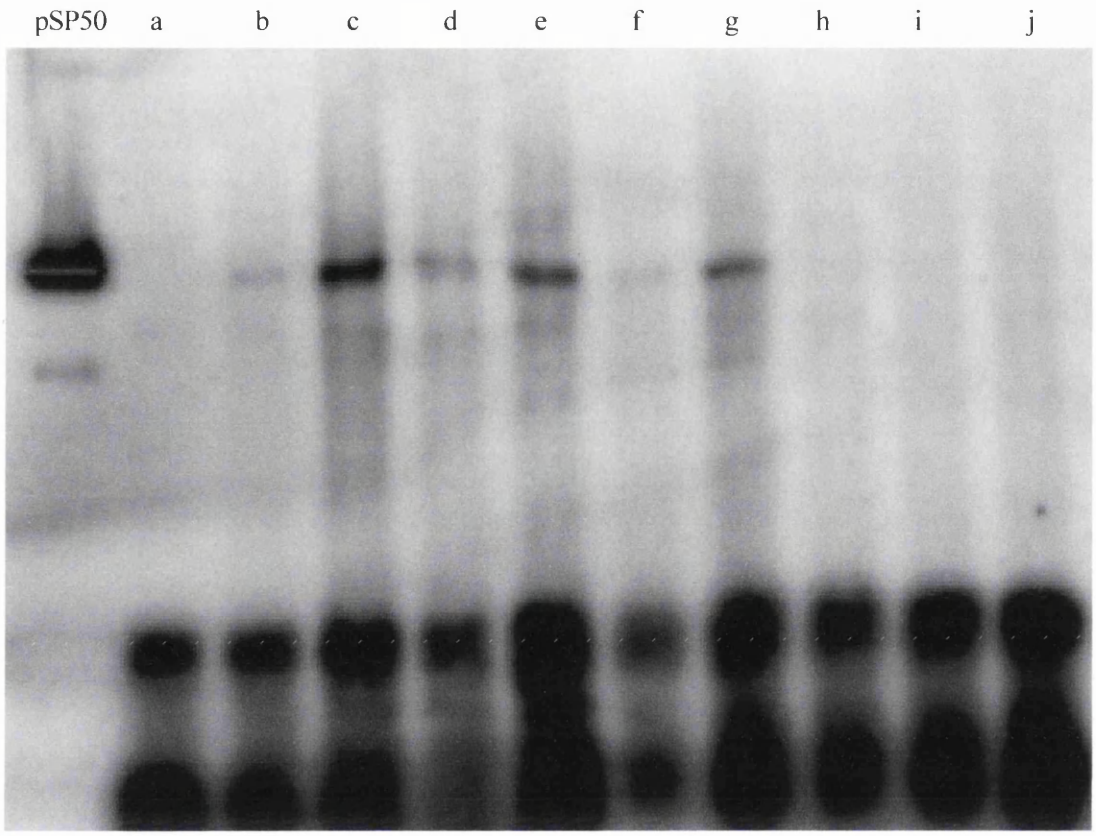


Figure 4.14 IE2-86 and UL38 function in mediating replication of pSP50 by the HSV-1 replication fork proteins.

Vero cells were transfected with 0.4 μg/dish of each of the six HSV-1 replication fork plasmids, 1.6 μg/dish of pSP50 and 0.4 μg/dish of the following plasmids: (a) no addition, (b) pSVH, (c) pSVH and pZP8, (d) pSVH and pZP8ΔBstEII, (e) pSVH and pZP8ΔEcoNI, (f) pSVH and pZP8ΔRsrII, (g) pSVH and pZP8ΔBstEII, pZP8ΔEcoNI and pZP8ΔRsrII, (h) pZP8, (i) pZP8 and pSVHΔSexAI and (j) pZP8 and pSVHΔBglIIΔSexAI.

25% of the DNA from a 35 mm dish was digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis through a 0.8% agarose gel and blotted as described. The blot was hybridised to ^{32}P-labelled pAT153.

4 ng of *Eco*RI digested pSP50 was loaded as a marker (pSP50)

4.3 Discussion

As I was unable to achieve replication of an HCMV origin-containing plasmid in a transient replication assay using plasmids obtained from David Anders, I decided to investigate the ability of the HSV-1 replication fork proteins to replicate an HCMV origin-containing plasmid when cotransfected with the HCMV auxiliary proteins. In an omission assay using the six HSV-1 replication fork proteins, only IE1/2 was absolutely essential for replication, although omission of UL36-38 resulted in a very large drop in replication efficiency. The other auxiliary loci were dispensable.

IE1/2 alone was able to mediate replication by the HSV-1 replication fork proteins (figure 4.7). Individual addition of UL84 or UL36-38 to the replication fork proteins was also tested but neither was able to mediate replication (figure 4.1 and 4.11). In an addition assay with the HSV-1 replication fork proteins and IE1/2, addition of UL36-38 greatly enhanced the replication efficiency. Individual addition of the other auxiliary loci did not result in a significant increase in replication efficiency (figure 4.8).

4.3.1 Requirements for replication of an HCMV origin-containing plasmid by the HSV-1 replication fork proteins

Whilst my results have demonstrated that there is not an absolute requirement for UL84, it should be noted that this is in the context of the replication of an HCMV origin-containing plasmid by the HSV-1 replication fork proteins, and that there may be differences in HCMV DNA replication during a normal virus infection.

My failure to reproduce the work of Pari and Anders (1993) may have been due to a low transfection efficiency in HFFF2 and HFL1 cells. However, when the assay was performed in 293 cells, which transfect with much greater efficiency and have been shown to be permissive for HCMV DNA replication (figure 3.3), no replication of the origin-containing plasmid was ever detected. A mutation in one (or more) of the

replication plasmids would prevent the assay working, but while none of the plasmids was sequenced, a mutation seems unlikely as the plasmids were obtained directly from D Anders in whose hands they were functional. A third possible reason for failure to achieve plasmid replication in the transient replication assay might have been in my use of well-established cell lines instead of the newly cultured foetal foreskin fibroblasts used by Pari and Anders (1993).

When tested for its ability to substitute for the HSV-1 replication fork plasmids, HSV-1 superinfection failed to direct HCMV DNA replication. One possible explanation for this is that the HCMV auxiliary proteins may be significantly less adept than UL9 at recruiting the HSV-1 replication fork proteins, so that HSV-1 viral DNA would be preferentially replicated in this assay. It would be interesting to determine whether in the presence of UL36-38 and IE1/2, an HSV-1 UL9 null mutant could activate an HCMV oriLyt. Another possible explanation for the failure of HSV-1 to activate HCMV oriLyt is that virus infection might prevent expression of, or sequester, host cell proteins required for the initiation of HCMV DNA synthesis.

Both IE1/2 and UL36-38 produce spliced transcripts. IE1/2 produces one late and four immediate-early transcripts, and U36-38 produces one early and three immediate early transcripts (reviewed by Fortunato & Spector, 1999, also see figures 4.10 and 4.13). Derivatives of the pSVH (IE1/2) and pZP8 (UL36-38) plasmids containing amber mutations, and a deletion mutant, were constructed to abrogate expression of certain of the IE1/2 or UL36-38 proteins. Because some of the characterised protein products of IE1/2 and UL36-38 are involved in the regulation of gene expression, double mutants and combinations of mutant plasmids were tested to ensure that the effects seen were due to the mutation of the individual protein rather than due to a secondary effect on gene expression. The mutant plasmids enabled the identification of IE2-86 (or L40) and UL38 as the gene products which were most active in this HSV-1/HCMV transient replication assay.

IE2-86 is the major product of the HCMV major immediate early region two (IE2). It is a promiscuous, non-sequence specific trans-activator of gene expression capable of autoregulation (Pizzorno *et al.*, 1988; Stenberg *et al.*, 1990; Gebert *et al.*, 1997). IE2-86 can modulate transcriptional activation, and hence gene expression, by direct binding to promoter sequences or by interaction with one of a plethora of cellular proteins (reviewed by Fortunato & Spector, 1999). No function has yet been attributed to the UL38 protein. To confirm the requirement for the two HCMV proteins, plasmids expressing only IE2-86 and UL38 should be constructed and tested in this assay.

4.3.2 Possible mechanisms of initiation of HCMV DNA replication

The requirement for IE2-86 in this assay is most interesting in the light of the observation that a small RNA molecule, termed the smallest replicator transcript (SRT), is transcribed from the HCMV oriLyt region (Huang *et al.*, 1996). The candidate promoter for the SRT contains putative IE2-86 binding sites, deletion of some of which have been reported at a recent International Herpesvirus Workshop to abrogate origin function (Kiehl *et al.*, 2000). It has been proposed that the initiation of HCMV DNA replication, like mitochondrial heavy strand DNA replication, may require an origin derived transcript (Prichard *et al.*, 1998). IE2-86 may be responsible for directing transcription through the origin, and UL38 might possibly activate this function or stabilise the RNA:DNA hybrid. Since UL84 seems absolutely essential for HCMV origin-dependent DNA synthesis in the presence of either the HCMV or EBV replication fork proteins, it may provide some function that is required by the HCMV and EBV replication fork proteins but not the HSV-1 proteins. The HSV-1 replication fork proteins may encode some enzyme function not encoded by the HCMV or EBV proteins, *e.g.*, the HSV-1 DNA polymerase has an RNase H activity (Marcy *et al.*, 1990) while no such activity has been confirmed for the HCMV DNA polymerase. Alternatively, the HSV-1 proteins may allow initiation of DNA replication by a mechanism which does not require UL84.

Similarly, the HSV-1 replication fork proteins were used to substitute for the EBV replication fork proteins to drive replication of an EBV oriLyt containing plasmid. In this assay, addition of the Zta and Rta transactivators allowed replication of the EBV origin-containing plasmid (Fixman *et al.*, 1995). Zta has subsequently been shown to interact with the EBV helicase/primase complex (Gao *et al.*, 1998) and is now accepted as being the origin-specific function encoded by EBV. Interestingly, in a transient replication omission assay using the EBV replication fork proteins, the EBV uracil-DNA glycosylase and the three lytic cycle transactivators, neither Zta nor Rta was absolutely essential to direct replication of an EBV origin-containing plasmid, while omission of Mta almost completely abrogated replication (Fixman *et al.*, 1995). Thus, while transient replication assays have proven extremely useful in the initial identification of virus encoded functions required for DNA replication, in complex systems such as HCMV and EBV, careful consideration should be given to the interpretation of all available data before any final conclusions are reached.

My results contrast with those obtained in transient replication assays using either the EBV or HCMV replication fork proteins expressed from constitutive promoters to activate HCMV oriLyt (Sarisky & Hayward, 1996 and summarised in table 1.2). The data presented by Sarisky and Hayward (1996) led to the conclusion that UL84 was the only HCMV encoded auxiliary protein with an absolutely essential role in HCMV oriLyt dependent DNA replication. However, the authors also demonstrated that in an omission assay in HF cells transfected with the HCMV replication fork proteins, three HCMV auxiliary loci, UL36-38, UL84 and IE1/IE2, were essential for HCMV origin-containing plasmid replication.

The HCMV lytic cycle in tissue culture is more protracted than that of HSV-1, and seems to be intimately associated with the cell cycle and cell type infected, thus HCMV may have developed multiple mechanisms to initiate or control the initiation of DNA

replication. It is easy to imagine that when taken out of the context of a virus infection, a control mechanism could be deregulated, leading to less strictly controlled initiation of DNA replication, and possibly to less stringent protein requirements. One example of the association of HCMV lytic replication with intracellular conditions, is the ability of HCMV infected stem cells in tissue culture to support HCMV replication only when the cells are differentiated (Minton *et al.*, 1994). If HCMV is able to initiate DNA replication by more than one mechanism, it may be difficult to determine which, if any, is the preferred one without studying the cell types infected *in vivo*.

Chapter 5: Replication of an HCMV origin-containing plasmid by a simian cytomegalovirus

5.1 Introduction

Several cytomegaloviruses of non-human mammals have been identified, including viruses which infect monkeys, mice, rats and guinea pigs (reviewed in Plummer, 1973; Staczek, 1990). The lytic origins of DNA replication of simian cytomegalovirus (SCMV) strain Colburn (Anders & Punturieri, 1991) and murine cytomegalovirus (MCMV) strain K181 (Masse *et al.*, 1997) have been identified and sequenced. The cytomegalovirus origins which have been studied are large, complex, and share little sequence identity. There are, however, several blocks within the origins whose features are conserved throughout the cytomegaloviruses.

It has been reported (Masse *et al.*, 1997) that plasmids containing cytomegalovirus lytic origins are only replicated *in vitro* in cells infected with the virus type from which the origin sequences were obtained. A plasmid containing an HCMV oriLyt was thus only replicated in HCMV infected human fibroblasts, not in Colburn or K181 infected human, murine or simian fibroblasts.

HCMV is more closely related to SCMV than to EBV or HSV-1 (McGeoch *et al.*, 2000). Therefore, the failure of SCMV to support the replication of pSP50 in transfected human fibroblasts is in surprising contrast to the ability of transfected EBV or HSV-1 replication fork proteins to support HCMV origin-dependent DNA synthesis in the presence of the HCMV auxiliary factors (Sarisky & Hayward, 1996; section 4.2 of this thesis). Thus, while the replication fork proteins of HCMV and SCMV are likely to be very similar, it seems that the origin-specific proteins may be quite different. The ability of SCMV infection to replicate an HCMV origin-containing plasmid was therefore re-examined.

5.2 Results

5.2.1 pSP50 is replicated in Colburn infected HFFF2 cells

As it had previously been reported that UL84 was the only HCMV encoded factor required to mediate replication of an HCMV origin-containing plasmid by the EBV replication fork proteins (Sarisky & Hayward, 1996), the ability of SCMV to direct replication of pSP50 in the presence of UL84, expressed from its own promoter (pZP13) or from the HCMV major immediate early promoter (pCV84), was examined.

Triplicate dishes of HFFF2 cells were transfected with 1.6 μg/dish of pSP50 (a), 1.6 μg/dish of pSP50 and 1.6 μg/dish of pZP13 (b), or 1.6 μg/dish pSP50 and 1.6 μg/dish of pCV84 (c). One dish of each triplicate transfection was infected with 0.25 p.f.u./cell of HCMV (A), one dish was infected with 0.25 p.f.u./cell of Colburn strain cytomegalovirus (C), the third dish was mock infected (M).

Total cellular DNA was prepared 96 h p.i., digested with *Eco*RI and *Dpn*I, and processed as previously described. The phosphorimage is shown in figure 5.1.

Colburn cytomegalovirus infection directed the replication of transfected pSP50 in the absence of any HCMV encoded transactivator (C:a). The level of replication in Colburn infected cells was much lower than the level achieved by HCMV infection (A:a). When pSP50 was cotransfected with a plasmid expressing UL84 (either from pZP13 (C:b) or pCV84(C:c)), there was no significant change to the amount of pSP50 replicated following superinfection with Colburn.

Cotransfection of pSP50 with pZP13 (A:b) did not significantly alter the amount of pSP50 replicated by HCMV infection, but cotransfection with pCV84 (A:c) resulted in lower levels of replication. No pSP50 replication was detected in the mock infected samples.

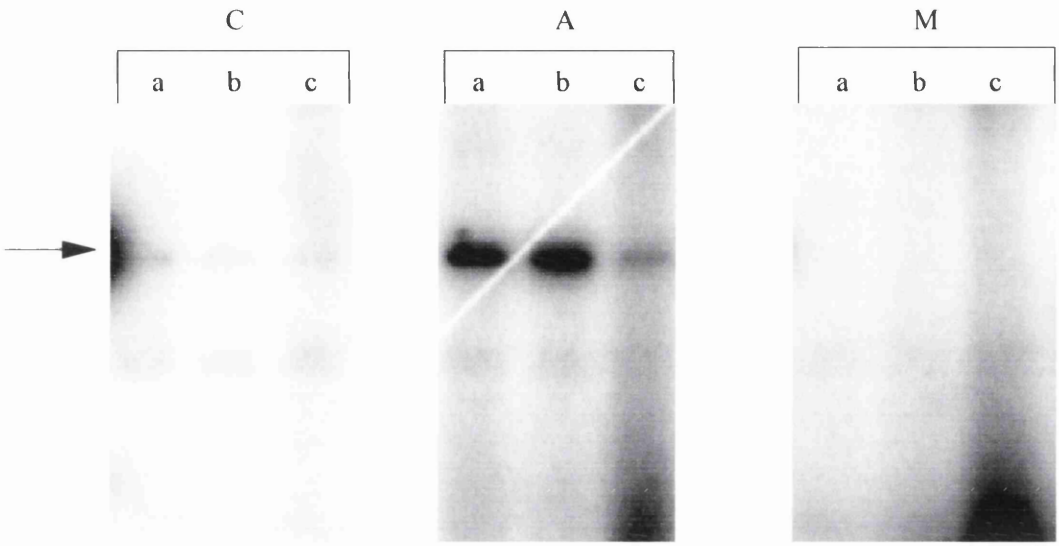


Figure 5.1 Replication of an HCMV origin-containing plasmid (pSP50) by Colburn cytomegalovirus.

HFFF2 cells were transfected with 1.6 μg/dish of pSP50 either (a) unsupplemented or with the addition of (b) 1.6 μg/dish of pZP13 or (c) 1.6 μg/dish of pCV84.

Cells were either infected with 0.25 p.f.u./cell of Colburn (C), infected with 0.25 p.f.u./cell of HCMV (A) or mock infected (M).

Total cellular DNA was prepared 96 h p.i. digested with *Eco*RI and *Dpn*I and fragments were separated by electrophoresis through a 0.8% agarose gel, blotted and hybridised to ^{32}P-labelled pAT153.

The position of *Eco*RI digested pSP50 marker band is shown →

Colburn cytomegalovirus infection can therefore supply all the functions necessary to replicate an HCMV origin-containing plasmid, and the amount of DNA replicated is not increased on addition of UL84.

5.2.2 Replication of a VZV origin-containing plasmid by HSV-1

SCMV infection activated an HCMV origin-containing plasmid in the absence of any HCMV encoded auxiliary factor, suggesting that SCMV encodes a protein capable of activating an HCMV origin. SCMV superinfection resulted in a lower level of replication than was obtained following superinfection with HCMV, so the origin-specific proteins may be functionally interchangeable but preferentially specify their cognate origins. Addition of the putative HCMV origin-specific protein (UL84) to the transient replication assay did not increase the amount of plasmid DNA replicated by SCMV.

In a similar "cross-complementation" assay, HSV-1 infection of cells transfected with a plasmid containing an origin of VZV DNA replication (pVO2) results in amplification of the VZV origin-containing plasmid (Stow & Davison, 1986). To determine the effect of the addition of a defined cognate origin-binding protein in a heterologous assay system, the effect of the VZV origin-binding protein (expressed from plasmid pEV51) on the efficiency of replication of a VZV origin-containing plasmid (pVO2) following HSV-1 superinfection was investigated. A plasmid containing the open reading frame for the VZV origin-binding protein in reverse orientation (pEV51R) was used as a control.

Duplicate monolayers of 293 cells were transfected with 0.4 μg/dish of pVO2 (a), 0.4 μg/dish each of pVO2 and pEV51 (b), or 0.4 μg/dish each of pVO2 and pEV51R (c). One dish of each duplicate transfection was infected with 10 p.f.u./cell of HSV-1, the other was mock infected. One dish of cells was transfected with 0.4 μg of pVO2 and 1.6 μg of pEV51 and infected with 10 p.f.u./cell of HSV-1 (d).

Total cellular DNA was prepared at 20 h p.i. and digested with *Eco*RI and *Dpn*I. Fragments were separated by electrophoresis, blotted and hybridised to ^{32}P-labelled pAT153. The blot was exposed to X-Omat film, and the film was developed and scanned to produce the image shown in figure 5.2.

HSV-1 infection directed replication of a transfected VZV origin-containing plasmid (a). Cotransfection of pVO2 with pEV51 increased the efficiency of replication in a dose dependent manner (b,d). Addition of pEV51R slightly decreased the amount of pVO2 replicated (c).

Thus, while HSV-1 infection alone replicated a VZV origin-containing plasmid, addition of increasing amounts of the cognate origin-binding protein to the assay resulted in an increased replication efficiency.

5.2.3 Stimulation of pSP50 replication by Colburn cytomegalovirus

The increase in the amount of replication of a VZV origin-containing plasmid by HSV-1 in the presence of increasing amounts of the VZV origin-binding protein suggested that the transactivation of the HCMV origin-containing plasmid by Colburn cytomegalovirus might be enhanced by addition of an HCMV origin-specific function. The data presented in section 4.2 demonstrated a direct role for some of the proteins encoded by pSVH and pZP8, but not UL84, in HCMV lytic origin-dependent DNA synthesis in the presence of the HSV-1 replication fork proteins. The ability of pSVH and pZP8 to enhance replication of pSP50 in Colburn cytomegalovirus infected cells was therefore examined.

A single experiment in which similar levels of replicated pSP50 were detected in all HCMV infected samples showed that replicated pSP50 was only detected in the Colburn infected samples which received pZP8 and pSVH (data not shown). While the level of pSP50 replication was lower in this experiment than in figure 5.1, (where detectable replication of pSP50 by SCMV occurred in the absence of additional plasmids and in the presence of pCV84), addition of pZP8 and pSVH had a stimulatory effect on the

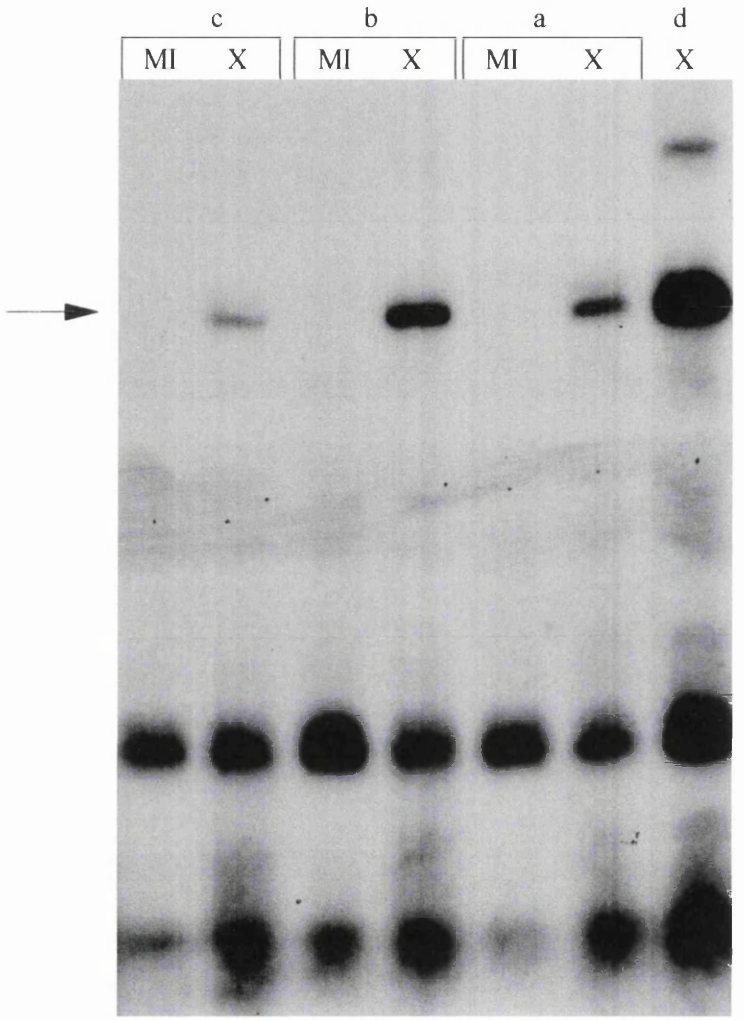


Figure 5.2 Replication of a VZV origin-containing plasmid by HSV-1.

293 cells were transfected with 0.4 μg/dish of pVO2 with either no addition (a) or with the addition of 0.4 μg/dish of pEV51R (c), 0.4 μg/dish of pEV51 (b) or 1.6 μg/dish of pEV51 (d). Cells were either mock infected (MI) or infected with 10 p.f.u./cell of HSV-1 (X). Total cellular DNA was prepared 20 h p.i., digested with *Eco*RI and *Dpn*I and fragments were separated by electrophoresis through 0.8% agarose gel, blotted and hybridised to ^{32}P-labelled pAT153.

The position of the *Eco*RI digested pVO2 marker band is shown ⟶

replication of pSP50 by Colburn cytomegalovirus superinfection.

Had more time been available, this experiment using Colburn cytomegalovirus to replicate pSP50 would have been repeated and extended to include the pSVH and pZP8 mutants described earlier in order to identify the individual proteins responsible.

5.3 Discussion

HSV-1 and VZV are both human Alphaherpesviruses, the origins, and origin-binding proteins of which, are so closely related that in a transient replication assay, a VZV origin-containing plasmid is replicated following superinfection with HSV-1 virus. Nevertheless, HSV-1 replicates an HSV-1 origin-containing plasmid 20-30 times more efficiently than it replicates a VZV origin-containing plasmid (Stow & Davison, 1986).

Cotransfection of a plasmid expressing the VZV origin-binding protein increased the amount of VZV origin-containing plasmid replicated following HSV-1 superinfection (figure 5.2), demonstrating that addition of the cognate origin-binding protein can increase replication efficiency in such a "cross-complementation" assay.

SCMV strain Colburn had previously been reported to be unable to direct replication of an HCMV origin-containing plasmid (Masse *et al.*, 1997), but the data presented here show that low levels of activation can occur (figure 5.1).

Similar "cross-complementation" experiments to that done with the VZV/HSV-1 model system were therefore carried out to determine whether any HCMV protein could be identified which enhanced replication from HCMV oriLyt following SCMV superinfection. SCMV driven replication of an HCMV origin-containing plasmid was not enhanced on addition of plasmids expressing UL84, as might have been expected if UL84 provided the HCMV origin-binding protein function.

In an experiment in which the level of replication of the HCMV origin-containing plasmid by SCMV strain Colburn was below the limits of detection, addition of HCMV IE1/IE2 and UL36-38 increased the efficiency of replication to a detectable level. Thus, as

with the assays using the HSV-1 replication fork proteins, the IE1/2 and UL36-38 loci appear to perform an HCMV oriLyt specific function. These studies should be extended using the pSVH and pZP8 derivatives previously described (figure 4.10 and 4.13).

The major immediate early regions from cytomegaloviruses of rhesus macaques (RhCMV), rats and mice have been sequenced and shown to have extensive amino acid homology to the major immediate early region 2 of human cytomegalovirus (Messerle *et al.*, 1992; Sandford *et al.*, 1993; Barry *et al.*, 1996). In the case of RhCMV and HCMV, the IE2 proteins are 48% identical (Barry *et al.*, 1996). All the cytomegalovirus origins described to date contain several repeated sequence motifs including possible binding sites for cellular transcription factors, which might act in conjunction with IE2-86 (or its homologues in the other cytomegaloviruses) in initiating cytomegalovirus DNA replication. In the light of these findings and the data presented in section 4.2 of this thesis, it would be interesting to re-examine the ability of the other cytomegaloviruses to transactivate each others origins in transient replication assays.

Chapter 6: Conclusions

6.1 Replication of HCMV genomes in epithelial and non-human fibroblast cells

In chapter 3, I have shown that both 293 cells and Vero cells are capable of supporting HCMV DNA synthesis, and that 293 cells might support, to a very limited extent, the production of infectious HCMV strain Towne particles. Further investigations into the block to HCMV replication in these cell lines could yield important information about the HCMV life cycle and its requirements for interactions with the host cell.

Figure 3.8b shows that HCMV strain Towne genomic DNA accumulates in infected Vero cells. While there was no detectable accumulation of HCMV strain AD169 genomes in Vero cells, it is unclear whether this is due to a failure to detect accumulation of DNA, or a failure of the virus to synthesise DNA. Further work could be undertaken to resolve this. In order to see a small amount of genomic DNA replication, unreplicated input genomes would need to be clearly distinguishable, or separated from newly synthesised DNA. This might be achieved by *in vivo* labelling of replicating DNA in infected cells. Labelled DNA could subsequently be hybridised to HCMV genomic DNA. If this was not sensitive enough, HCMV DNA synthesis might be confined to the origin region by incubating infected cells in medium containing ganciclovir.

Ganciclovir is an effective HCMV antiviral which acts on viral DNA replication, but does not inhibit the viral DNA polymerase, so a series of short DNA fragments from the origin region are produced in ganciclovir treated HCMV infected cells (Hamzeh *et al.*, 1990). Ganciclovir terminated DNAs could be separated from input genomes by electrophoresis of undigested DNA and detected directly with a radiolabelled probe, alternatively, *in vivo* labelled ganciclovir DNAs could be hybridised to a DNA fragment containing HCMV origin sequences, such as pSP50. Phosphonoformic acid inhibition

would be used to confirm that incorporation of label was directed by the HCMV DNA polymerase. The detection of ganciclovir terminated DNA would confirm that origin-dependent DNA synthesis was occurring.

I have proposed that HCMV strain AD169 DNA may fail to be replicated in Vero cells if the infecting genomic DNA is not circularised. Detection of ganciclovir terminated DNAs would not necessarily preclude this possibility as initiation and limited DNA synthesis might be possible on a linear template. The ability of HCMV strain AD169 DNA to circularise should thus be tested by FIGE, which allows the separation of high molecular weight DNA molecules (McVoy & Adler, 1994).

The replication efficiency of different human cytomegalovirus strains in different cell lines could also be examined in greater detail. For example, combining Southern blot data with indirect immunofluorescence might make it possible to assess whether a small amount of virus replication takes place in the majority of Vero and 293 cells, or if a wild type level of DNA synthesis is achieved in a small minority of these cells. In cells which support HCMV DNA replication, but not infectious virus production, it would also be interesting to find out at what point the lytic cycle is blocked.

6.2 Initiation of HCMV origin-dependent DNA synthesis

Two novel transient replication assay systems have been described (section 4.2 and 5.2) and used to identify HCMV encoded proteins with possible origin-specific functions during HCMV DNA synthesis.

Firstly, an assay using the HSV-1 replication fork proteins to replicate an HCMV origin-containing plasmid was described. In this assay there was no absolute requirement for UL84 to initiate DNA synthesis at an HCMV origin, in fact, omission of UL84 from an assay in which the four remaining HCMV auxiliary loci were present did not significantly reduce the replication of the origin-containing plasmid (figure 4.5). IE2-86 was able to link the HSV-1 replication fork proteins to the HCMV origin (figure 4.7), and UL36-38 was

the only one of the three remaining auxiliary loci which, individually, significantly increased the amount of replication observed (figure 4.8).

The second assay system used transfection followed by superinfection with Colburn cytomegalovirus (SCMV). SCMV was shown to be able to replicate an HCMV origin-containing plasmid (figure 5.1). In a model system used to investigate the action of a cognate origin-binding protein in the presence of heterologous replication fork proteins, *i.e.* the replication of a VZV origin-containing plasmid by HSV-1 virus, replication was shown to be enhanced on addition of the cognate origin-binding protein (figure 5.2). Addition of UL84 did not affect the amount of replication observed (figure 5.1). Data from a preliminary experiment suggested that addition of IE1/IE2 and UL36-38 to the Colburn 'superinfection assay' does increased the amount of HCMV origin-containing plasmid replicated by Colburn cytomegalovirus infection (not shown).

The use of a simian cytomegalovirus to replicate an HCMV origin-containing plasmid could provide a useful additional assay for the study of HCMV DNA replication as the two cytomegaloviruses are more closely related than HCMV is to either HSV-1 or EBV. However, infection with Colburn cytomegalovirus is a much more complex system than transfection with plasmids expressing the herpesviral replication fork proteins, and any results obtained must be carefully interpreted.

The fact that Colburn cytomegalovirus (SCMV) is able to replicate an HCMV origin-containing plasmid in the absence of any HCMV encoded proteins suggests that SCMV encodes at least one protein which can provide an HCMV origin-specific function, although this should be confirmed using plasmids containing mutant origin sequences or plasmids containing only vector sequences. It would also be interesting to determine which protein encoded by SCMV is specific for the HCMV origin in a transient replication assay, and, if appropriate, to identify the DNA sequences which are required. The ability of HCMV infection to replicate an SCMV origin-containing plasmid could also be examined.

In the two transient replication assay systems I have described, I found that there was no absolute requirement for UL84, but that one or more of the major immediate early proteins, probably IE2-86, displays some HCMV origin-specific properties. These results clearly contrast with those published by Sarisky and Hayward (1996), who reported that of the HCMV auxiliary loci, only UL84 was absolutely essential for origin-dependent DNA replication. UL84 has also been shown to be an inhibitor of HCMV replication (Gebert *et al.*, 1997), a phenotype similar to the inhibition of HSV-1 DNA replication by UL9 (Stow *et al.*, 1993). However, UL84 has been shown to interact with IE2-86, (Spector & Tevethia, 1994) and the inhibition of HCMV replication by UL84 may be due to the effects of UL84 on transcriptional transactivation by IE2-86, whereas the inhibition of HSV-1 DNA replication by UL9 is thought more likely to be at the point of UL9 binding to the HSV-1 origin and its interactions with the HSV-1 replication fork proteins. The reported RNase H activity of UL84 (Sarisky & Hayward, 1996b) has not been published, and the data of Sarisky and Hayward (1996) have not been confirmed or extended.

At the recent International Herpesvirus Workshop it was reported (Kiehl *et al.*, 2000) that mutation of an IE2-86 binding site within a region flanking the core of HCMV oriLyt abrogated origin function. This flanking region could be functionally substituted with regions containing clusters of IE2-86 binding sites (*e.g.*, the UL112-113 promoter), furthermore, there are reportedly several IE2-86 binding sites overlapping functionally important regions of the core of oriLyt. This recent report supports my data in suggesting an important role for IE2-86 in the initiation of HCMV oriLyt dependent DNA synthesis. Further evidence in support of my data comes from the fact that in a transient replication assay, UL84, UL112-113 and IE2 were the minimum set of auxiliary loci required for HCMV origin-dependent DNA synthesis in Vero cells in the presence of the HCMV replication fork proteins and UL69 (Sarisky & Hayward, 1996). The authors also reported that in HF cells in the presence of the full set of eleven replication proteins, UL84,

UL36-38 and IE1/2 were absolutely essential for HCMV origin-dependent DNA synthesis. The full set of HCMV replication fork proteins and IE2-86, UL84 and TRS1 have been over-expressed using a semliki forest virus (SFV) expression system in mammalian cells (McCue and Anders, 1998). The UL38 protein should probably also be similarly overexpressed, purified, and its function examined. It would also be most interesting to characterise possible interactions of IE2-86 with the replication fork proteins, and with UL84 and UL38. The ability of IE2-86, UL84 and UL38 to bind independently or together to specific DNA sequences (or RNA:DNA hybrid regions) within HCMV oriLyt should be examined in detail.

In order to resolve the question of which proteins are required for initiation of HCMV origin-dependent DNA synthesis, much further investigation is needed. Plasmids expressing only UL38 and IE2-86 should be constructed and tested in the transient replication assays described here, and the specific protein domains of IE2-86 and UL38 involved in DNA replication should be determined.

When considered together, previously published work showing an essential role for UL84 in HCMV DNA replication, and my data showing a direct role for IE2-86 in HCMV DNA replication, as well as the presence of RNA molecules within and transcribed from the origin, tend to suggest that the initiation of HCMV lytic-phase DNA synthesis may proceed by more than one mechanism. It is possible that different mechanisms for the initiation of DNA replication may be employed in different cell types, or at different stages in the cell cycle. However, testing such a hypothesis will probably require the construction of HCMV mutants and their examination in different cell lines that more closely resemble those infected *in vivo*. Construction of HCMV IE2 mutants has proven extremely difficult, and once constructed, a separation of the effects of the mutation on HCMV gene expression from any direct effect on origin-dependent DNA synthesis will be extremely difficult. Cell free systems for HCMV oriLyt dependent DNA synthesis should also assist in the

identification of proteins that perform origin-specific functions. Such systems, when supplemented with extracts from various cell types, may also reveal the existence of different initiation mechanisms. Finally, cell free systems may allow detailed dissection of the molecular events during initiation of HCMV origin-dependent DNA synthesis.

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