EXPRESSION AND CHARACTERIZATION OF FOUR HERPES SIMPLEX VIRUS TYPE 1 GENE PRODUCTS REQUIRED FOR THE REPLICATION OF VIRAL DNA

by

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SUMMARY

The work presented in this thesis is concerned with the study of the products of four genes of herpes simplex virus type-1 (HSV-1). These genes, UL5, UL8, UL9 and UL52, are members of a set of seven HSV-1 genes which are essential for the replication of HSV-1 DNA in tissue culture.

Three of these seven genes encode products which are abundant in HSV-1 infected cells and have been relatively well characterised. These are UL29 which encodes the major single-stranded DNA binding protein, UL30 which encodes the viral DNA polymerase, and UL42 which encodes a double-stranded DNA binding protein, M_r 65 000. The protein products of the remaining four genes, UL5, UL8, UL9 and UL52, are of low abundance in HSV-1 infected cells and at the outset of this work had not been identified. Since then the UL9 gene product has been identified as an origin-binding protein which recognises specific DNA sequence elements (Olivo *et al.*, 1988; Weir *et al.*, 1989), and the UL5, UL8 and UL52 proteins have been purified as a complex from HSV-1 infected cells which has DNA helicase and DNA primase activities (Crute *et al.*, 1989).

To facilitate their identification and subsequent characterization, the UL5, UL8, UL9 and UL52 gene products were individually expressed in recombinant viruses. These four open reading frames (ORFs) were individually expressed under the control of the HSV-1 immediate-early (IE) 3 gene promoter in the temperature sensitive HSV-1 mutant *ts*K. A lesion within the IE3 gene of this mutant results in the overproduction of IE gene products at the non-permissive temperature (NPT). Thus, products of the inserted replication genes could be expressed in the absence of other DNA replication proteins at the NPT. Each ORF was also expressed under the control of the polyhedrin promoter in recombinant baculoviruses constructed by Dr N D Stow.

Insertion of these ORFs into *ts*K recombinant viruses allowed the products of the UL5, UL8, UL9 and UL52 genes to be identified as novel polypeptides on SDS-polyacrylamide gels. The apparent molecular weights of the over-expressed proteins corresponded closely to those predicted from DNA sequence analysis. The gene products were also identified immunologically by reactivity with antisera raised against peptides corresponding to the C-termini of their predicted amino acid sequences (a kind gift of Dr M D Challberg). On the basis of [^{32}P]-

orthophosphate labelling experiments, none of the four proteins appeared to be significantly phosphorylated, .

Based upon the identification of a UL5, UL8 and UL52 protein complex in HSV-1 infected cells (Crute *et al.*, 1989), attempts were made to assemble this complex *in vivo*, initially by infecting BHK cells with the *ts*K recombinant viruses, and subsequently by infecting insect cells with recombinant baculoviruses. The enzymatic activities of the complex were investigated using the baculovirus system. Insect cells were infected with either parental baculovirus or the UL5, UL8 and UL52 recombinants, individually or in all possible combinations. Extracts from the infected cells were fractionated by phosphocellulose column chromatography assayed for DNA-dependent ATPase, DNA helicase and DNA primase activities. Novel peaks of each of these activities were detected in extracts from cells triply infected with the UL5, UL8 and UL52 recombinants or doubly infected with UL5 and UL52 recombinants. These activities, which were not present in any of the other extracts, eluted at 150-200mM NaCl, similar to that reported for the HSV-1 helicase-primase detected in cells infected with *wt* HSV-1 (Crute *et al.*, 1988).

SDS-PAGE analysis of the phosphocellulose column fractions revealed that the UL5, UL8 and UL52 proteins co-eluted with the induced activities, at salt concentrations differing from those at which any of these proteins eluted when expressed alone. This result is indicative of an interaction between the three proteins. The UL5 and UL52 proteins co-eluted in a similar fashion when expressed in the absence of the UL8 protein, at a position co-incident with the novel peaks of enzymatic activity. The UL8 protein is therefore not required for the novel DNA-dependent ATPase, DNA helicase and DNA primase activities associated with the complex, and moreover, the UL5 and UL52 proteins together are able to form a functional complex in the absence of the UL8 protein.

The intracellular localization of the HSV-1 origin-binding protein (OBP), which is encoded by gene UL9, and the HSV-1 helicase-primase complex were studied by indirect immunofluorescence in BHK cells infected with the *ts*K recombinant viruses. OBP expressed in the absence of other DNA replication proteins localized within the nucleus where it exhibited a diffuse pattern with discrete foci also present. Localization of the helicase-primase complex was investigated by fluorescently staining the UL52 protein subunit. When expressed alone, the UL52 protein exhibited a cytoplasmic, perinuclear staining pattern.

This pattern remained unchanged when the UL52 protein was co-expressed with either the UL5 or UL8 protein. However, when the UL5, UL8 and UL52 proteins were expressed together the UL52 protein was efficiently localized within the nucleus. These results suggest that the UL8 protein, which is not required for the enzymatic activities of the complex, may have a role in facilitating entry of an active complex into the cell nucleus.

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ABBREVIATIONS

The Bases

A	adenine	C	cytosine
G	guanine	T	thymine
U	uracil	N	any base
R	purine	Y	pyrimidine

NTP, rNTP	ribonucleoside 5'-triphosphate
NDP	ribonucleoside 5'-diphosphate
NMP	ribonucleoside 5'-monophosphate
dNTP	2'-deoxynucleoside 5'-triphosphate
ddNTP	2'-dideoxynucleoside 5'-triphosphate

The Amino Acids

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

Others

AcNPV	*Autographa californica* Nuclear Polyhedrosis Virus
AIDS	Acquired Immunodeficiency Disease Syndrome
APS	ammonium persulphate
BCdR	5'bromo-deoxycytidine
BHK	baby hamster kidney (cells)
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
BrdCTP	5'bromo-deoxycytidine 5'-triphosphate
°C	degrees Celsius
CAV	cell associated virus

Ci	Curie
CLB	cell lysis buffer
cm	centimetre
CPE	cytopathic effect
cpm	counts per minute
CRV	cell released virus
C-terminus	carboxyl terminus (of a peptide)
Da	Daltons
DEAE	diethylamino-ethyl
DEPC	diethylpyrocarbonate
DC	DNA cellulose (buffer)
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
E	early (gene)
EBV	Epstein-Barr Virus
E.coli	*Escherichia coli*
EDTA	ethylenediamine tetra-acetic acid (sodium salt)
EtBr	Ethidium bromide
FITC	Fluorocein isothiocyanate
FPLC	Fast Protein Liquid Chromatography
g	grams
GMEM	Glasgow modification of Eagle's medium
h, hr	hours
HCMV	human cytomegalovirus
HeBS	HEPES buffered saline
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonate
HHV	human herpesvirus
hpi	hours post infection
hplc	high performance liquid chromatography
HRP	horse radish peroxidase
HSV	herpes simplex virus
Hu	human
ICP	infected cell protein
IE	immediate early (gene)
IEC	immediate early complex

Ig	immunoglobulin
IP	immunoprecipitation
IR	internal repeat
k	kilo
kb	kilobase
l	litre
L	late (gene)
L	long (segment of the HSV chromosome)
LAT	latency associated transcript
LB	Luria's bacterial growth buffer
M	Molar
M_r	relative molecular mass
mDBP	major DNA binding protein
mg	milligram
min	minute(s)
MIR	major internal repeat
ml	millilitre
mm	millimetre
mM	millimolar
moi	multiplicity of infection
MOPS	3-(N-morpholine)propanesulphonic acid
mRNA	messenger ribonucleic acid
NBCS	newborn calf serum
ng	nanogram
nm	nanometre
NPT	non-permissive temperature
N-terminus	Amino terminus (of a peptide)
OBP	origin binding protein
OD	optical density
ORF	open reading frame
ORI	origin of DNA replication
Pi	orthophosphate
PAA	phosphonoacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
pfu	plaque forming units
PMSF	phenylmethylsulphonyl fluoride

POL	polymerase
PT	permissive temperature
RGB	resolving gel buffer
RNA	ribonucleic acid
RNase	ribonuclease
RP-A, RP-C	replication proteins A and C
rpm	revolutions per minute
RR	ribonucleotide reductase
RSB	resuspension buffer
S	short (segment of HSV chromosome)
SDS	sodium dodecyl sulphate
sec	second(s)
S.f.	*Spodoptera frugiperda*
SSB	single stranded DNA binding protein
ssDNA	single stranded DNA
STET	sucrose, Tris, EDTA, TritonX100 buffer
SSC	sodium chloride/citrate buffer
syn	syncytial plaque morphology locus
	(syn^+ = non-syncytial, syn^- = syncytial)
SV40	simian virus 40
TBE	Tris Borate EDTA electrophoresis buffer
TBS	Tris buffered saline
TBST	Tris buffered saline plus Tween 20
TE	Tris EDTA buffer
TEMED	n,n,n',n'-tetramethylethylene diamine
TK	thymidine kinase
TM	Tris magnesium
TR	terminal repeat
Tris	Tris [hydroxymethyl] amino-methane
ts	temperature sensitive
U	unique (segment of the HSV chromosome)
UL	unique long (gene)
US	unique short (gene)
UV	ultraviolet
V	volts
vhs	virus host shut-off (gene)
Vmw	viral protein of molecular weight
vol/vols	volume(s)

VP	viral protein
VZV	varicella zoster virus
W	Watts
w/v	weight per volume
wt	wild type
XC	xylene cyanol
μ	micro

CHAPTER 1: INTRODUCTION

1A. GENERAL PROPERTIES OF HERPESVIRUSES

1. Structure and Classification of Herpesviruses

Herpes simplex virus type 1 is a member of the family of viruses, *Herpesviridae*. Members of this group are large DNA-containing viruses which are diverse in their biological properties and have been isolated from a wide variety of hosts (Roizman, 1982).

Distinct morphological features are common to all members. The virus particle is 150-200nm in diameter and is composed of four distinct elements. The genomic DNA, in the range M_r $80x10^6$ to $150x10^6$, is contained within an electron dense core (Epstein, 1962). The core is surrounded by an icosahedral capsid assembled from 162 capsomeres, 150 hexameric and 12 pentameric (Wildy *et al.*, 1960; Schrag *et al.*, 1989) which is in turn surrounded by an amorphous proteinaceous layer (Morgan *et al.*, 1959; Schwartz and Roizman, 1969) now referred to as the tegument (Roizman and Furlong, 1974). The outermost structure is the envelope, a trilaminar membrane (Wildy *et al.*, 1960) derived from host cell nuclear and plasma membrane (Nii *et al.*, 1968; Asher *et al.*, 1969). Virally encoded glycoproteins which, by electron microscopy, can be observed as 8nm spikes, protrude from the surface of the virion envelope (Wildy and Watson, 1962; Spear and Roizman, 1972).

The *Herpesviridae* have historically been classified, on the basis of biological properties, into three subfamilies i.e. alpha-, beta- and gamma-herpesviruses (Roizman, 1982; Matthews, 1982).

Alphaherpesviruses are neurotropic, exhibit a wide *in vitro* host range and a relatively short reproductive cycle causing rapid destruction of permissive cells in tissue culture. Latent infection is frequently established in ganglia. Included in this group are HSV-1, HSV-2 and varicella-zoster virus (VZV). Betaherpesviruses exhibit a restricted host range and a relatively long reproductive cycle. In a productive infection cells frequently become enlarged (cytomegalia). Latent infections may be established in a variety of tissues. Included in this group is human cytomegalovirus (HCMV). Gammaherpesviruses are lymphotropic, specific for B or T lymphocytes and have a narrow host range. Latent infection is frequently established in lymphoid tissue though reproductive cycle and cytopathology are variable. Epstein-Barr virus (EBV) is a member of this group.

In addition to biological properties, comparison of genome organisation and DNA sequence are of increasing importance in the determination of evolutionary relationships between members of the Herpesviridae. Classification by biological properties alone of the recently identified human herpesvirus 6 (HHV-6), originally termed human B lymphotropic virus (Salahuddin *et al.*, 1986), would place it amongst the gammaherpesviruses. However, limited DNA sequence analysis of the HHV-6 genome has shown it to be more closely related to members of the betaherpesvirus group (Lawrence *et al.*, 1990).

2. Epidemiology of HSV and Other Human Herpesviruses

To date, seven herpesviruses are known to infect the human host, namely HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6 and HHV-7.

HSV types 1 and 2 cause cutaneous lesions and are transmitted by close physical contact. HSV-1 is associated with those of the lips, known as cold sores (herpes labialis), and less commonly with those of the eye (herpes keratoconjunctivitis) whereas HSV-2 is largely associated with genital lesions (herpes genitalis), (reviewed by Whitley, 1985). Though infection is generally mild, rare cases of encephalitis or severe general infection can arise in newborn or immunocompromised individuals. Following a primary infection a latent infection of HSV is frequently established in the neuronal cells of sensory ganglia and the brain where the viral genome is maintained in a non-replicating state (Stevens and Cook, 1971; Baringer and Sworeland; 1973, Cook *et al.* 1974; Fraser *et al.* 1981,). Since terminal fragments have not been detected the genome is thought to be maintained in an 'endless' form (Rock and Fraser, 1983; 1985; Efstathiou *et al.*, 1986) existing extrachromosomally as a circle or possibly a concatemer (Mellerick and Fraser, 1987). Expression from the latent viral genome is very limited though a virus-specific transcript has been detected in the nuclei of cells latently infected with HSV (Stevens *et al.*, 1987; Rock *et al.*, 1987; Spivack and Fraser, 1987; Steiner *et al.*, 1988; Wagner *et al.*, 1988a). This transcript is termed the latency-associated transcript (LAT) and has been found to be transcribed from a region downstream of the IE1 gene within the long repeat. DNA sequence analysis suggests that a protein is unlikely to be encoded within this region (Perry and McGeoch, 1988). No unique polyadenylated LAT messages or encoded polypeptide have been identified (Wagner *et al.*, 1988a;b). Mutants of HSV lacking expression of LAT retain the ability to establish a latent infection (Javier *et al.*, 1988). Although not absolutely required for

establishment of, and reactivation from, the latent state LATs may have a role in controlling the frequency of reactivation (Leib *et al.*, 1989b; Steiner *et al.*, 1989; Hill *et al.*, 1990).

Animal and *in vitro* cell culture models have been used to study requirements for the establishment and maintenance of a latent HSV infection (Wigdahl *et al.*, 1982; Shiraki and Rapp, 1986; Russell and Preston, 1986). Although specific requirements for establishment have not been defined (Polvino-Bodnar *et al.*, 1987; Meignier *et al.*, 1988; Russell *et al.*, 1987), it has been demonstrated *in vitro* that the viral immediate-early protein Vmw110 is involved in reactivation of the latent viral genome (Russell *et al.*, 1987; Harris *et al.*, 1989). The role of Vmw110 *in vivo*, however, is less clear. A mutant of HSV-1, *dl*1403, with deletions in both copies of the IE1 gene is able to establish and reactivate from the latent state (Clements and Stow, 1989). A study of several IE1 deletion mutants by Leib *et al.* (1989a) revealed some, but not all, to be deficient in their ability to establish and reactivate from a latent infection in the mouse ocular model.

HSV-2 has been associated with human squamous cell carcinoma of the cervix although expression of HSV gene products occurs only in a small proportion of cervical carcinoma tissues (reviewed by Rawls, 1985; Macnab, 1987). Evidence of a direct role for HSV in the development of cancer is inconclusive. Infection with HSV can result in the amplification of heterologous DNA sequences within the host cell (Matz *et al.*, 1985). Transient transfection experiments and infection with mutants of HSV-1 have revealed that amplification of SV40 DNA sequences within the host cell requires SV40 T antigen and all but one of the seven HSV-1 DNA replication proteins [HSV-1 origin-binding protein not required](Matz, 1989; Heilbronn and ZurHausen, 1989; Heilbronn *et al.*, 1990). This suggests that amplification of host DNA sequences can occur in the absence of viral DNA synthesis and that HSV-1 replication proteins are able to interact with other viral or cellular proteins in mediating this effect. Although HSV does not appear to encode a transforming oncogene, regions of the genome clearly have the ability to transform cells in tissue culture (Reyes *et al.*, 1979; Galloway and McDougall, 1983). Small fragments of DNA lacking any complete protein coding sequence are capable of transformation and no particular part of the HSV genome appears to be consistently retained or expressed in transformed cells (Cameron *et al.*, 1985; Galloway and McDougall, 1983). Transformation by HSV has been proposed to occur by a 'hit and run' mechanism whereby normal

cellular gene regulation is disrupted due to temporary interaction with the virus thus allowing further events in the transformation process to occur (Skinner, 1976; Galloway and McDougall, 1983). Consistent with this, HSV has been shown to have the potential to act as a mutagen (Clarke, 1991) and to cause up-regulation of transformation-specific polypeptides (Macnab, 1987).

VZV manifests as two major diseases. Chicken pox (varicella), normally a childhood disease, is characterized by skin lesions or pustules, and shingles (herpes zoster) arises from reactivation of a latent VZV infection resulting in the development of a painful rash, the incidence and severity of which increases with age or with immunosuppression of the host (Kennedy, 1987).

EBV causes mild infection in children but may result in infectious mononucleosis if occurring as a primary infection of adolescents and adults. Establishment of a latent infection in lymphoid and epithelial cells is frequent. A direct role for EBV in the development of Burkitt's lymphoma as well as a link with nasopharyngeal carcinoma has been shown (Epstein and Achong, 1986).

Infection with HCMV is generally asymptomatic, detectable only by an elevated antibody titre, although the virus may cause infectious mononucleosis or, as a congenital infection, may result in mental retardation (Hamilton, 1982). A latent infection may be established in a variety of tissues including kidney, secretory glands and lymphoreticular cells.

The recently identified HHV-6 was first isolated from patients with AIDS or lymphoproliferative diseases. HHV-6 is thought to grow in both B and T lymphocytes (Salahuddin *et al.*, 1986; Lopez *et al.*, 1988) and has been reported as the causative agent of exanthem subitum, a transient disease of children (Yamanishi *et al.*, 1988) though no clear association with disease in adults has been described.

HHV-7 has only very recently been identified. Isolated from the $CD4^+$ T-lymphocytes of a healthy individual, HHV-7, like HHV-6 and HCMV, appears to be induced to replicate during the course of T cell proliferation (Frenkel *et al.*, 1990a;b).

3. HSV-1 Genome Structure and Organisation

The linear, double-stranded DNA genome of HSV-1 has a molecular weight of 100×10^6 (Becker *et al.*, 1968; Kieff *et al.*, 1971) and may be regarded as two covalently linked components, named the long (L) and short (S) regions (**Figure 1a**). Each region consists largely of unique

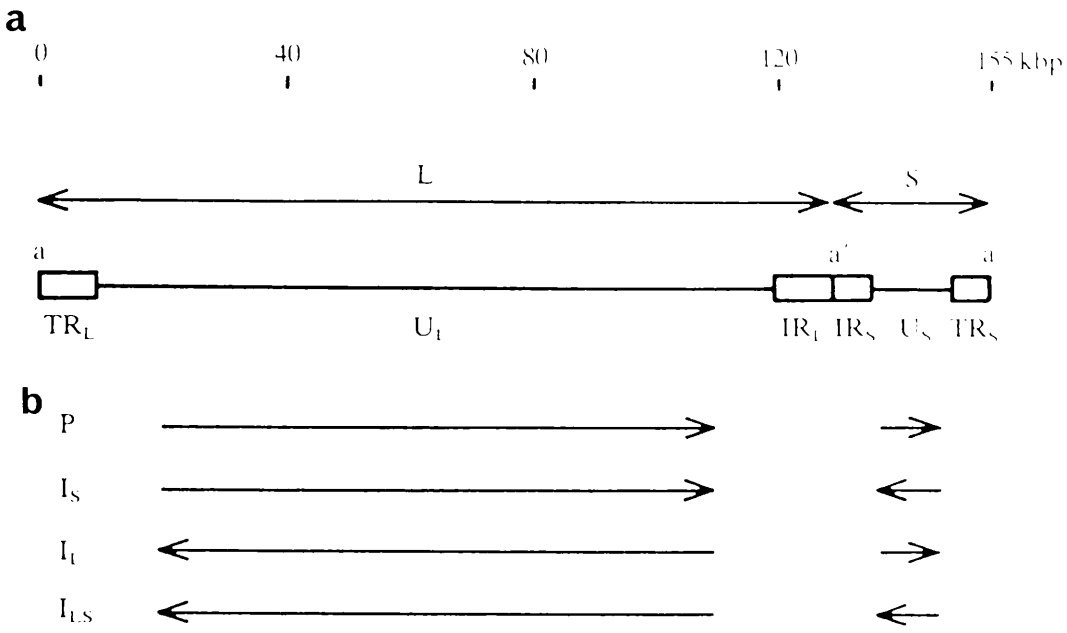


Figure 1. Organization of the HSV-1 Genome

(a) The viral genome which comprises two covalently joined segments, L and S. Each consists of an unique sequence (U_L and U_S respectively, shown as solid lines) flanked by a pair of inverted repeat sequences (respectively TR_L and IR_L, IR_S and TR_S, shown as open boxes). A directly repeated 400bp sequence, termed the *a* sequence, is present at each terminus and also, in inverted orientation, at the 'joint' between L and S.

(b) Preparations of HSV-1 DNA contain equimolar amounts of four sequence orientation isomers which differ in the relative orientations of L and S. These are designated P (arbitrary prototype), I_S (inverted S), I_L (inverted L) and I_{LS} (inverted L and S), (reproduced from McGeoch, 1987).

sequences (U_L and U_S respectively) bracketed by inverted repeats. U_L is flanked by a pair of inverted repeat elements, termed R_L, and referred to as TR_L and IR_L to indicate their terminal or internal location. Similarly, U_S is flanked by repeat elements IR_S and TR_S. The R_L and R_S sequences are distinct. At the genome termini there occurs a direct repeat of 400bp, termed the *a* sequence. A single copy of the *a* sequence is found at the S terminus and one or more copies at the L terminus. In addition, a variable number of copies of the *a* sequence occur in inverted orientation at the L-S junction (Roizman, 1979). At each genome terminus there is a single residue 3' overhang (Mocarski and Roizman, 1982a; b).

Preparations of HSV-1 DNA have been found to contain equimolar amounts of four sequence orientation isomers, differing in the relative orientation of L to S. These are designated P (prototype), I_S (inversion of S), I_L (inversion of L) and I_{SL} (inversion of S and L) (**Figure 1b**), (Hayward *et al.*, 1975; Delius and Clements, 1976; Wilkie and Cortini, 1976; Roizman, 1979).

The genome of HSV-1 strain 17 syn$^+$ has been completely sequenced (McGeoch *et al.*, 1985; 1986; 1988b; Perry and McGeoch, 1988; Rixon and McGeoch, 1985). It contains 152,260 bp with a mean base composition of 68.3% (G+C). The genomes of three other human herpesviruses have now also been completely sequenced.

The VZV genome contains 124,884 bp with a mean base composition of 46% (G+C) (Davison and Scott, 1986). In gross structure VZV resembles HSV although the inverted repeats flanking U_L are markedly smaller (88bp in VZV versus 9200bp in HSV) and no element corresponding to the *a* sequence is apparent. Four sequence orientation isomers have been identified although one orientation of U_L is 20-fold more prevalent (Davison, 1984).

The EBV genome contains 172,282 bp with a mean base composition of 59% (G+C) (Baer *et al.*, 1984). However, a 13.6 kbp deletion has been identified in the EBV strain which was sequenced suggesting a complete genome of around 186 kbp (Raab-Traub *et al.*, 1980). Organisation of the EBV genome differs markedly from HSV and VZV. The termini are formed from several direct repeats, variable in number, and a set of large, directly repeated elements form a major internal repeat (MIR) towards one end of the genome. The MIR and terminal repeats flank the long and short unique sequences. Within U_L, towards its extremities, lie a further two identical repeats, DR_L and DR_R, which contain families of related tandemly repeated

sequences (Raab-Traub *et al.*, 1980; Laux *et al.*, 1985).

The genome of HCMV is the largest of the human herpesviruses and contains 230 kbp with a mean base composition of 56% (G+C) (Chee *et al.*, 1990). Though larger than HSV, its gross structural arrangement is similar including the presence of a repeat element at the genome termini and L-S junction corresponding to the HSV *a* sequence (Weststrate *et al.*, 1983; Spaete and Mocarski, 1985).

Sequence determination of the remaining three human herpesvirus genomes is at present incomplete. However, HSV-2 gross structure is very similar to that of HSV-1 and the genomes of HSV types 1 and 2 are highly related and closely colinear (Davison and Wilkie, 1981; 1983; Swain and Galloway, 1983; Swain *et al.*, 1985; Lockshon and Galloway, 1986; Draper *et al.*, 1986; McGeoch *et al.*, 1987; Worrad and Caradonna, 1988).

The genome of HHV-6 is estimated to contain 161.5 kbp with a mean base composition of 41% (G+C). It comprises a central unique region flanked by direct repeats DR_L and DR_R at left and right termini. An internal tandem reiteration occurs within the unique region towards the right terminus (Martin *et al.*, 1991). DNA sequence analysis thus far has revealed the genome of HHV-6 to be most closely related to that of HCMV (Lawrence *et al.*, 1990).

DNA sequence analysis and earlier mapping data of the HSV-1 genome led to the identification of 72 open reading frames (ORFs) which potentially encode 70 distinct polypeptides. Fifty-six genes lie within U_L, termed UL1-UL56, and 12 within U_S, termed US1-US12. Genes IE1 and IE3 lie within R_L and R_S respectively and are thus each represented twice (**Figure 2**; McGeoch *et al.*, 1985; 1988b). This total of HSV-1 genes may be incomplete. Reports of other possible genes include a transcribed ORF spanning the origin of viral DNA replication, *ori*$_S$ (Hubenthal-Voss *et al.*, 1987) and the latency-associated transcript (LAT) described earlier (Section 1A.2.). The region upstream of gene IE1 is reported to encode a polypeptide termed ICP34.5 which is important for virus neurovirulence (Ackerman *et al.*, 1986; Chou and Roizman, 1986; Chou and Roizman, 1990; Taha *et al.*, 1990).

Functions have been assigned to many of the polypeptides encoded by HSV-1 genes and include roles in transcriptional regulation, DNA replication and virion structure and assembly. The functions of approximately one-third of HSV-1 genes remain at present

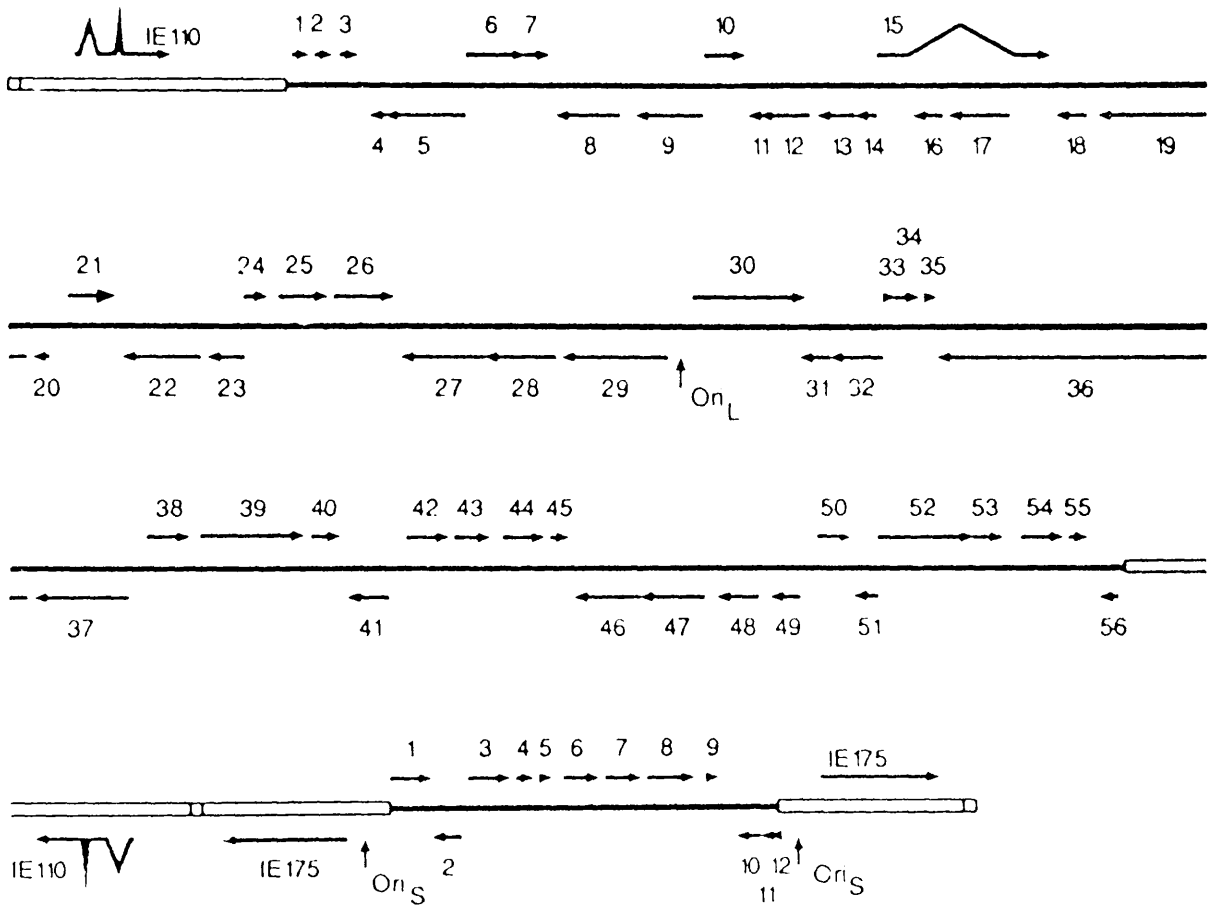

IE110
1 2 3
6 7
10
15
4 5
8 9
11 12 13 14
16 17
18 19
21
24 25 26
30
34
33 35
20
22 23
27 28 29
On L
31 32
36
38 39 40
42 43 44 45
50
52 53 54 55
37
41
46 47 48 49
51
56
1 3 4 5 6 7 8 9
IE175
IE110
IE175
On S
2
10 12
11
On S

Figure 2. Genetic Content of the HSV-1 Genome

The organization of the HSV-1 genome is represented on four successive lines of 40kb each. Locations of open reading frames are shown by arrows, with splicing within coding regions indicated. Genes UL1-UL56, marked as 1-56, are shown on the first three lines and on the fourth line are genes US1-US12, marked as 1-12. Locations of origins of DNA replication (ori_S and ori_L) are also indicated (reproduced from McGeoch, 1989).

undefined. Eleven of the genes in U_S together with nine genes in U_L have been shown to be non-essential for viral growth in tissue culture (Umene, 1986; Weber *et al.*, 1987; Longnecker and Roizman, 1986; 1987).

The genomes of the alphaherpesviruses HSV-1 and VZV exhibit extensive conservation. The major orientation of VZV U_L corresponds to the I_L isomers of HSV-1 and exhibits an extensively colinear gene arrangement. The genome of the gammaherpesvirus EBV is greatly diverged from that of HSV-1 and VZV, in both gross genome structure and organisation. However,approximately 29 of the 80 EBV genes and 40 of the 208 HCMV ORFs have clearly identified homologues in HSV and VZV (Baer *et al.*, 1984; Davison and Taylor, 1987; McGeoch *et al.*, 1988b; McGeoch 1989; Chee *et al.*, 1990).

1B. LYTIC CYCLE OF HSV-1 INFECTION

1. Initiation of Infection

Infection of a cell with HSV begins with viral adsorption to and penetration of the cell membrane. These are separable functions mediated by virally encoded glycoproteins which protrude from the viral envelope. Initial binding of HSV appears to be to heparan sulphate on the cell surface membrane (WuDunn and Spear, 1989). However, a recent report suggests that HSV-1 may penetrate the cell following attachment to the high affinity receptor for fibroblast growth factor (FGF) since uptake of the virus is greatly reduced in the presence of inhibitors which bind the receptor. In addition, transfection of the FGF receptor into a non-expressing cell line non-permissive for HSV-1 resulted in susceptibility of the cells to viral infection (Kaner *et al.*, 1990).

Following attachment, fusion of the viral envelope with the plasma membrane of the cell occurs (Morgan *et al.*, 1968), liberating the viral capsid into the cytoplasm (Fuller and Spear, 1985). Several viral glycoproteins appear to be involved in these events (Fuller and Spear, 1985; Johnson *et al.*, 1984; Para *et al.*, 1982; McGeoch and Davison, 1986; Gompels and Minson, 1986). A role for glycoprotein C in attachment and penetration by HSV-1 has recently been proposed (Langeland *et al.*, 1990; Campadelli-Fiume *et al.*, 1990; Herold *et al.*, 1991). Glycoprotein D has been shown to be essential for virus penetration (Ligas and Johnson, 1988; Johnson and Ligas, 1988) and glycoprotein B has been implicated in viral penetration and cell fusion

(Sarimiento *et al.*, 1979; Manservigi *et al.*, 1977; Cai *et al.*, 1988). In addition temperature sensitive syncytial plaque morphology mutations have been mapped to the gene encoding gB (DeLuca *et al.*, 1982; Bond *et al.*, 1982; Addison *et al.*, 1984; Bzik *et al.*, 1984; Ruyechan *et al.*, 1979).

Once inside the cell the capsid is transported to the nuclear pore where the viral DNA is uncoated and released into the nucleus (Batterson and Roizman, 1983; Knipe *et al.*, 1981).

2. Host Macromolecular Synthesis During HSV Infection

Lytic infection of permissive cells with HSV results in a rapid decrease in the synthesis of host cell polypeptides (reviewed by Fenwick, 1984). Inhibition of host cell DNA synthesis occurs concomitantly with replication of the viral genome (Roizman and Roane, 1964; Kaplan and Ben-Porat, 1963).

Reduction in synthesis of the majority of host cell polypeptides (Sydiskis and Roizman, 1967; 1968) arises from disaggregation of polyribosomes and degradation of host mRNA (Nishioka and Silverstein, 1977; 1978; Schek and Bachenheimer, 1985). Host shut-off may be divided into two distinct phases i.e. 'early shut-off', mediated by a virion component, during which host polyribosomes and mRNAs are rapidly degraded, and 'delayed shut-off', dependent upon viral gene expression, during which host mRNAs are further degraded (Nishioka and Silverstein, 1977).

Mutants of HSV deficient in early or virion-associated host shut-off (*vhs*) have been isolated. These mutants retain a delayed shut-off function (Read and Frenkel, 1983). Stability of both host and viral mRNAs is increased in cells infected with *vhs* mutants. The *vhs* factor appears to be involved in disaggregation of polyribosomes as well as non-specific degradation of host and viral mRNAs (Kwong and Frenkel, 1987; Oroskar and Read, 1987).

The degree of host shut-off induced by different strains of HSV types 1 and 2 varies and it has been proposed that the mechanism may differ between the two viruses (Hill *et al.*, 1983; 1985). Notably, most strains of HSV-2 appear to inhibit host protein synthesis more severely than HSV-1 strains (Pereira *et al.*, 1977; Schek and Bachenheimer, 1985).

The *vhs*$^-$ mutation has been mapped to the UL41 ORF (Kwong *et al.*, 1988). Cloning of this ORF from HSV-2 strain G, which encodes a particularly effective *vhs* function (Fenwick and Owen, 1988), into

HSV-1 strain 17, which is less efficient in early shut-off, was found to confer efficient *vhs* function (Fenwick and Everett 1990). It has been proposed that the wildtype polypeptide encoded by UL41 may interact with a cellular protein, acting together to destabilise both host and viral mRNA (Kwong and Frenkel, 1989).

Although synthesis of the majority of host proteins is inhibited upon infection with HSV, the production of cellular stress and heat shock proteins is stimulated (LaThangue *et al.*, 1984; Patel *et al.*, 1986; Kemp *et al.*, 1986).

3. Regulation of HSV Gene Expression

Lytic infection of cells in tissue culture results in expression of HSV-1 genes as a temporal cascade in three broad phases i.e. immediate-early (IE or alpha), early (E or beta) and late (L or gamma) (Honess and Roizman, 1974; Clements *et al.*, 1977).

HSV genes are transcribed by host RNA polymerase II (Alwine *et al.*, 1974; Preston and Newton, 1976; Costanzo *et al.*, 1977). Synthesis of specific mRNAs correlates closely with the kinetics of expression of the encoded proteins indicating that regulation of HSV gene expression is largely under transcriptional control (Zhang and Wagner, 1987; Smith and Sandri-Goldin, 1988).

Immediate-early gene products are the first to be expressed, appearing around 1 h p.i. Expression can occur in the absence of *de novo* protein synthesis and is shut-off by 3-4 hpi (Kozak and Roizman, 1974; Clements *et al.*, 1977; Jones and Roizman, 1979).

Early gene products can be observed shortly after the appearance of IE proteins and prior to the onset of DNA replication (Honess and Roizman, 1974; Clements *et al.*, 1977; Wagner, 1985).

Late gene products are expressed after the onset of DNA replication (Honess and Roizman, 1974; Jones and Roizman, 1979; Clements *et al.*, 1977).

(a) Expression of Immediate-Early Genes

Five immediate-early polypeptides are specified by HSV-1, namely Vmw110, Vmw175, Vmw63, Vmw68 and Vmw12. RNA mapping and DNA sequence analyses have identified the genes encoding these polypeptides as IE1, IE3, UL54, US1 and US12 respectively (See **Figure 2**; Clements *et al.*, 1979; Rixon *et al.*, 1982; Murchie and McGeoch, 1982; McGeoch *et al.*, 1985; 1986; 1988b; Perry *et al.*, 1986). Genes IE1 and IE3 lie entirely within R_L and R_S respectively and are thus diploid.

US1 and US12 have identical promoters located within R_S but distinct coding sequences within U_S. UL54, mapping within U_L, is the only IE gene not associated with the major repeat regions of the viral genome.

Regulation of IE gene expression has been extensively studied. DNA sequence analysis has revealed several *cis*-acting elements located upstream of the transcriptional initiation site of IE genes which are recognised by both cellular and viral *trans*-acting factors (Mackem and Roizman, 1980; 1981; Murchie and McGeoch, 1982; Whitton *et al.*, 1983; Whitton and Clements, 1984a). Deletion analyses of IE gene promoters have defined two distinct domains i.e. a minimal promoter region containing a 'TATA-box', located 20-30 nucleotides upstream from the mRNA cap site (Mackem and Roizman, 1982a; b; c; Cordingley *et al.*, 1983; C M Preston *et al.*, 1984), and an upstream regulatory region containing multiple *cis* elements. These *cis* elements include some which respond to cellular transcription factors to increase efficiency of transcription eg. 'GC boxes' recognised by the transcriptional activator Sp1 (Jones and Tjian, 1985; Briggs *et al.*, 1986), as well as the consensus sequence element $^{5'}$TAATGARAT$^{3'}$, essential for *trans*-induction by the HSV virion tegument polypeptide Vmw65, encoded by UL48 (Mackem and Roizman, 1982b; C M Preston *et al.*, 1984; Kristie and Roizman, 1984; Campbell *et al.*, 1984; Dalrymple *et al.*, 1985; Gaffney *et al.*, 1985; Bzik and Preston, 1986; O'Hare and Hayward, 1987a). Following entry of the HSV virion into the host cell, the Vmw65 tegument protein becomes localized within the cell nucleus where it is able to transinduce HSV IE genes and establish a lytic infection.

The Vmw65 polypeptide has been shown to lack the ability to bind directly to DNA (Marsden *et al.*,1987) and its transactivation activity is thought to be mediated by protein-protein interactions with cellular transcription factors which specifically recognise and bind the TAATGARAT sequence consensus within IE gene promoters (Ace *et al.*, 1988; Cousens *et al.*, 1989). The Vmw65 polypeptide forms a complex with at least two cellular proteins, termed the Immediate-Early Complex (IEC), which binds specifically to the TAATGARAT element and can be assembled *in vitro* (McKnight *et al.*, 1987; Preston *et al.*, 1988; O'Hare and Goding, 1988; Gerster and Roeder, 1988; Kristie *et al.*, 1989; Ace *et al.*, 1988). Formation of this complex correlates with expression from IE gene promoters (O'Hare and Goding, 1988; Ace *et al.*, 1988). The cellular transcription factor OTF-1 (NF-III, TRF), which recognises the octamer element $^{5'}$ATGCAAAT$^{3'}$, has been shown both to bind to the

TAATGARAT element and to interact with the Vmw65 polypeptide (Pruijn *et al.*, 1986; Fletcher *et al.*, 1987; O'Hare and Goding, 1988). The C-terminal 80 amino acids of the Vmw65 polypeptide, which comprise a highly acidic domain, are necessary for stimulation of transcriptional activity but dispensable for the formation of IEC (Triezenberg *et al.*, 1988a; Sadowski *et al.*, 1988; Cousens *et al.*, 1989; Greaves and O'Hare, 1989). A region close to the N-terminus, within amino acids 141-185, appears to be necessary and sufficient for the formation of IEC (Triezenberg *et al.*, 1988a; Werstuck and Capone, 1989a; b). An HSV-1 mutant, *in*1814, which lacks the ability to form IEC due to a lesion in gene UL48 is viable in tissue culture but defective for growth at low m.o.i.. This suggests that *trans*-induction of IE gene expression by Vmw65 is particularly important for initiation of infection at low m.o.i. (Ace *et al.*, 1989).

A GA-rich sequence element has been identified within the upstream regulatory region of the IE3 gene which binds a cellular factor, and, though unrelated to the operation of the TAATGARAT element, may function in synergy with it (Triezenberg *et al.*, 1988a; b; O'Hare and Hayward, 1987; Bzik and Preston, 1986).

The products of HSV genes UL46 and UL47 have been implicated in the modulation of *trans*activation of IE gene expression by Vmw65 (McKnight *et al.*, 1987). A deletion mutant lacking the UL47 gene appears to exhibit reduced levels of IE gene *trans*-induction by Vmw65. However, deletion mutants of HSV lacking either UL46 , UL47 or both ORFs are viable in tissue culture and produce Vmw65 in amounts indistinguishable from wild-type HSV (Zhang *et al.*, 1991).

In addition to Vmw65 and cellular transcription factors, expression from IE gene promoters is regulated by the viral IE proteins Vmw110 and Vmw175 (O'Hare and Hayward, 1985a;b; 1987; Gelman and Silverstein, 1987a;b).

(b) Functions of Immediate-Early Gene Products

The IE proteins Vmw175, Vmw110, Vmw68 and Vmw63 are phosphorylated and localise to the cell nucleus. Vmw12, however, is not phosphorylated and localises in the cytoplasm (Pereira *et al.*, 1977; Marsden *et al.*, 1978; 1982; Fenwick and Walker, 1979; Hay and Hay, 1980; Wilcox *et al.*, 1980; Ackerman *et al.*, 1984).

(i) Vmw175

Studies of *ts* mutants of HSV-1 have shown that functional Vmw175 is required for the stimulation of expression of both early and late

genes and additionally for the repression of IE gene expression. A lesion in gene IE3 of the mutant *tsK* results in the production of aberrant Vmw175 and the over-production of IE gene products at the NPT. Early and late gene expression is not induced with the exception of the large subunit of ribonucleotide reductase, encoded by UL39 (Preston, 1979a; Watson and Clements, 1980).

In transfection assays Vmw175 has been shown to stimulate transcription from an HSV-1 early gene promoter linked to a reporter gene (Everett, 1984a; 1986; Quinlan and Knipe, 1985; O'Hare and Hayward, 1985a; Gelman and Silverstein, 1986). This transactivation activity is synergistically enhanced when Vmw175 is co-transfected with Vmw110 (DeLuca and Schaffer, 1985; O'Hare and Hayward, 1985a; Everett, 1986). Expression from an IE gene promoter linked to the reporter gene has been shown to be repressed by Vmw175 (O'Hare and Hayward, 1985b). However, when the amounts of the Vmw175-expressing plasmid transfected are low the promoters of IE genes IE3, US1 and US12 are stimulated (DeLuca and Schaffer, 1985; Gelman and Silverstein, 1986).

Although most IE proteins are able to bind DNA (Hay and Hay, 1980; Metzler and Wilcox, 1985) only Vmw175 exhibits sequence specificity in doing so, binding the consensus motif $^{5'}$ATCGTC$^{3'}$ (Beard *et al.*, 1986; Muller, 1987). This motif occurs in the promoter regulatory regions of genes IE1, IE3 and UL54 (Faber and Wilcox, 1988; Kristie and Roizman, 1986; Paterson and Everett, 1988) and has been implicated in the repression of expression from IE promoters, most convincingly from the IE3 gene promoter (Gelman and Silverstein, 1986; 1987; DeLuca and Schaffer, 1985; 1988; O'Hare and Hayward, 1985b; 1987; Roberts *et al.*, 1988; Muller, 1987; Faber and Wilcox, 1986a; 1988; Beard *et al.*,1986). Vmw175 is able to bind sequences other than the ATCGTC motif (Kristie and Roizman, 1986; Michael *et al.*, 1988). However, no sequences specifically responsive to Vmw175, or any other viral or cellular transactivator have been identified in early or late gene promoters (Everett, 1984a; Coen *et al.*, 1986; Michael *et al.*, 1988; Kattar-Cooley and Wilcox, 1989).

(ii) Vmw110

Though not absolutely essential for growth in tissue culture, mutants of HSV-1 constructed with large deletions in both copies of gene IE1 grow poorly at low moi, indicating that a lack of Vmw110 reduces the ability to establish a productive infection (Stow and Stow, 1986; Sacks and Schaffer, 1987). At high moi behaviour of the mutant

is indistinguishable from wild-type. A consequence of the reduced ability of the mutant to initiate infection at low m.o.i. is that the ratio of particles:pfu is raised (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989).

Vmw110 is a potent transactivator of both viral promoters of all classes and cellular promoters, and can act independently or in synergy with Vmw175 (Everett, 1984a; 1985; 1986; Quinlan and Knipe, 1985; Mavromara-Nazos *et al.*, 1986; O'Hare and Hayward, 1985a; DeLuca and Schaffer, 1985). Vmw110, in the absence of Vmw175, has been shown to reactivate HSV-2 from cells latently infected *in vitro* (Russell *et al.*, 1987b; Harris *et al.*, 1989).

(iii) Vmw63

Vmw63 is essential for growth in tissue culture since deletion mutants of HSV-1 which fail to express the protein are unable to replicate on non-complementing cell lines (McCarthy *et al.*, 1989). Temperature-sensitive mutants of HSV with lesions in UL54 overproduce Vmw63 and an aberrant form of Vmw175 at the NPT (Sacks *et al.*, 1985; Rice and Knipe, 1988). They express early genes and replicate their genomes but early-late gene expression is reduced and no true-late genes are induced. Deletion mutants over-produce early gene products and also fail to induce true-late genes. These observations suggest a role for Vmw63 in the regulation of early and late gene expression and perhaps also of IE gene expression (McCarthy *et al.*, 1989).

In transfection assays Vmw63 has been shown to transactivate transcription from an early gene promoter (Rice and Knipe, 1988). In addition, Vmw63 has been shown to transactivate a number of early gene promoters in synergy with Vmw110 and Vmw175, and conversely to repress transcription from some promoters synergistically transactivated by Vmw110 and Vmw175 (Sekulovich *et al.*, 1988; Everett, 1986; Rice and Knipe, 1988). Together with Vmw110 and Vmw175, Vmw63 therefore appears to be involved in both down-regulation and induction of HSV gene expression.

(iv) Vmw68

Though Vmw68 is non-essential for growth in tissue culture, a deletion mutant of HSV lacking the C-terminal one-third of the polypeptide grows poorly in certain cell types (Post and Roizman, 1981) and exhibits decreased neurovirulence in mice (Sears *et al.*, 1985). Impaired growth appears to be due to reduced late gene expression, implying a role for Vmw68 in the regulation of late gene

expression (Sears *et al.*, 1985).

(v) Vmw12

Vmw12, is not essential for growth since deletion mutants which fail to produce the Vmw12 polypeptide grow as efficiently as wild-type HSV (Longnecker and Roizman, 1986; Umene, 1986; Brown and Harland, 1987).

(c) Expression of Early Genes

Early gene expression begins after the appearance of functional IE gene products and is dependent upon promoter transactivation by the IE proteins (Honess and Roizman, 1974; Clements *et al.*, 1977; Wagner, 1985; Weinheimer and McKnight, 1987; Zhang and Wagner, 1987). The products of the genes in this group can be divided into two subgroups on the basis of their expression kinetics ie. early (β_1) and delayed-early (β_2). Genes of both subgroups are expressed prior to the onset of DNA replication though delayed-early genes are not fully expressed until after. UL39, which encodes the large subunit of ribonucleotide reductase, is classed as an early gene but appears 'intermediate' between early and immediate-early since its expression can be detected prior to activation of its promoter by IE proteins (Roizman and Batterson, 1985; Wymer *et al.*, 1989).

Linker scanning mutagenesis of the promoter and upstream regulatory regions of the HSV-1 TK gene has defined functional elements which include a 'TATA-box', GA- and GC-rich sequences and 'CCAAT-box' elements (McKnight and Kingsbury, 1982; 1984b; Eisenberg *et al.*, 1985; El Karah *et al.*, 1985).Cellular transcription factors are known to interact with these elements (Jones *et al.*, 1985; Graves *et al.*, 1986). Mutational analysis of early gene promoters has revealed no sequence elements specific for transactivation by HSV gene products suggesting that stimulation of early gene expression by IE proteins is mediated indirectly through interaction with cellular transcription factors (Everett, 1984b; Eisenberg *et al.*, 1985; Coen *et al.*, 1986).

(d) Expression of Late Genes

Late gene expression is dependent upon the presence of functional IE and E gene products and upon viral DNA synthesis, which is brought about by a subset of early gene products. Prevention of DNA synthesis by metabolic inhibitors or by temperature-sensitive mutations results in failure to efficiently express late genes (Honess and Roizman, 1974; Powell *et al.*, 1975; Holland *et al.*, 1980; Conley *et al.*,

1981; Pederson *et al.*, 1981; Swanstrom and Wagner, 1974). Variation in the kinetics of protein expression exists between members of this group, which, as a consequence, can be divided into two subgroups ie. leaky-late ($gamma_1$) and true-late ($gamma_2$). Genes of both subgroups are fully expressed only following the onset of DNA replication. Some degree of expression of leaky-late genes occurs before DNA synthesis is initiated though true-late gene products are undetectable at this time (Roizman and Batterson, 1985; Wagner, 1985). Functional Vmw175 has been shown to be continually required for the expression of late genes (Watson and Clements, 1980) and as with early promoters no sequences specific for transactivation by viral gene products can be identified in late gene promoters (Everett, 1984b).

The promoters of HSV late genes US11, UL19 and UL44 have been shown to respond to transactivation by IE proteins (Costa *et al.*, 1985; Shapira *et al.*, 1987; Johnson, 1987). Detailed mutational analysis of the promoters of genes US11 and UL44 has defined a region 31 bp upstream from the transcription start site to be necessary and sufficient for initiation of transcription. This region, which contained the 'TATA-box' and the mRNA cap site, was functional only when an activated origin of DNA replication was present in *cis* (Johnson and Everett, 1986a; Homa *et al.*, 1986).

Late gene promoters lack the upstream regulatory sequences present in IE and E gene promoters. 'Early' regulation of gene US6, which encodes the virion glycoprotein D, may be converted to 'late' regulation by the removal of these upstream sequences to leave a promoter region containing only a 'TATA-box' and mRNA cap site whose expression is then dependent upon the activity of an origin of replication in *cis* (Johnson and Everett 1986b). The inability of a promoter to respond to cellular factors which stimulate IE and E gene expression therefore appears to result in late expression.

The role of DNA replication in the expression of late genes is not completely clear. The increase in template copy number following the onset of DNA replication contributes to increased late gene expression. However, in transfection experiments (Johnson and Everett, 1986a), mRNA accumulation was significantly greater than could be explained by the template copy number alone. An origin of replication may provide an essential *cis*-acting function, particularly in the absence of sequence elements recognised by cellular transcription factors, or the replicative machinery may be involved. Alternatively, an opportunity for transcription to proceed may be presented following structural

- Studies of genome replication in HSV and pseudorabies virus (PRV) have shown similarities between the two viruses.

changes arising during the replicative event (Johnson and Everett, 1986a; Mavromara-Nazos and Roizman, 1987).

4. Replication of HSV DNA

Following the release of viral DNA into the infected cell nucleus the genome rapidly becomes circularised (Jacob and Roizman, 1977; Ben-Porat and Veach, 1980; Poffenberger and Roizman, 1985). This is thought to be achieved via direct ligation of the two terminal *a* sequences (Davison and Wilkie, 1983; Poffenberger *et al.*, 1983). 3' single base extensions present at both the L and S termini may facilitate this ligation event (Mocarski and Roizman, 1982a; b; Davison and Rixon, 1985). Following entry into the nucleus immediate-early and early viral gene products are expressed and synthesis of viral DNA begins.

Analysis of replicative intermediates has revealed several important features of the DNA synthetic process. HSV replicative intermediates, pulse labelled in infected tissue culture cells with [^{3}H]-thymidine, characteristically sediment more rapidly in neutral sucrose gradients than do unit genome lengths of viral DNA (Jacob and Roizman, 1977). Restriction enzyme digestion of labelled HSV replicative intermediate DNA has shown it to be 'endless' ie. terminal fragments cannot be detected and are thus presumed to be joined head-to-tail (Jacob *et al.*, 1979; Jongeneel and Bachenheimer, 1981). The rate of sedimentation in neutral sucrose gradients is variable amongst replicative intermediates. Those present early in infection, around 2-3 hpi, sediment at up to twice the rate of unit length viral DNA. Later, at 4-6 hpi, replicative intermediates sediment 100 times faster than unit length DNA (Jacob and Roizman, 1977; Ben-Porat and Tokazewski, 1977). It has been proposed that those of the first group represent an initial amplification of the viral genome template as circular monomers (Ben-Porat and Tokazewski, 1977). Intermediates arising later in infection have been observed, by electron microscopy, as 'large tangled masses' (Ben-Porat and Rixon, 1979). Their sedimentation properties and lack of genome termini suggest, at least at late times of infection, that replication of viral DNA may occur by a rolling-circle mechanism which generates concatemers of the viral genome linked head-to-tail (Jacob *et al.*, 1979). Unit genome lengths are subsequently cleaved and packaged into virions (Vlazny *et al.*, 1982).

Electron microscopy and alkaline agarose gel electrophoresis of the products synthesized by infected cell extracts on a pre-formed

replication fork suggests that they arise by a rolling-circle mechanism of replication (Rabkin and Hanlon, 1990). The sedimentation properties of replicative intermediates present late in infection might, however, be due to topological linkage of daughter molecules or to homologous recombination between replicating genomes (Jongeneel and Bachenheimer, 1981). This latter hypothesis is supported indirectly by reported high levels of homologous recombination exhibited by HSV in infected cells (Smiley *et al.*, 1980; Schaffer *et al.*, 1974; Honess *et al.*, 1980). The high frequency of recombination appears closely linked to viral DNA replication process (Weber *et al.*, 1988). Rolling-circle replication of DNA and homologous recombination between daughter molecules are however, not mutually exclusive.

During the course of DNA replication the two segments of the viral genome, L and S, invert relative to each giving rise to four structural isomers. All isomers arise in equimolar amounts and are functionally equivalent (Preston *et al.*, 1978; Poffenberger *et al.*, 1983; Jenkins and Roizman, 1986). Although the *a* sequence has been shown to be sufficient for inversion, recombination between other sequences invertedly repeated in the genome has also been shown to cause inversion (Pogue-Geile *et al.*, 1985; Jenkins *et al.*, 1985; Varmuza and Smiley, 1985; Weber *et al.*, 1987; 1988).

The HSV genome specifies both *cis*- and *trans*-acting functions involved in the replication of viral DNA. These will be discussed in some detail in the next section of the introduction (Section 3).

5. Packaging of HSV DNA and Assembly of Mature Virions

The *a* sequence has been shown to contain all the necessary *cis*-acting elements required for site-specific cleavage and packaging of progeny viral genomes. Insertion of an additional *a* sequence generates novel termini at the position of insertion (Smiley *et al.*, 1981; Mocarski and Roizman, 1982a; b; Varmuza and Smiley, 1985). Heterologous DNA containing an *a* sequence and an HSV origin has been shown to become packaged and propagated as a defective genome in the presence of helper virus (Stow *et al.*, 1983; 1986; Deiss and Frenkel, 1986).

The events of cleavage and packaging are closely coupled although the precise order of events at the *a* sequence is not completely resolved (Ladin *et al.*, 1980; Vlazny *et al.*, 1982; Deiss and Frenkel, 1986; Addison, 1986). **Figure** 3 illustrates the *a* sequence, the limits of which are marked by a direct repeat (17-21bp), DR_1, present

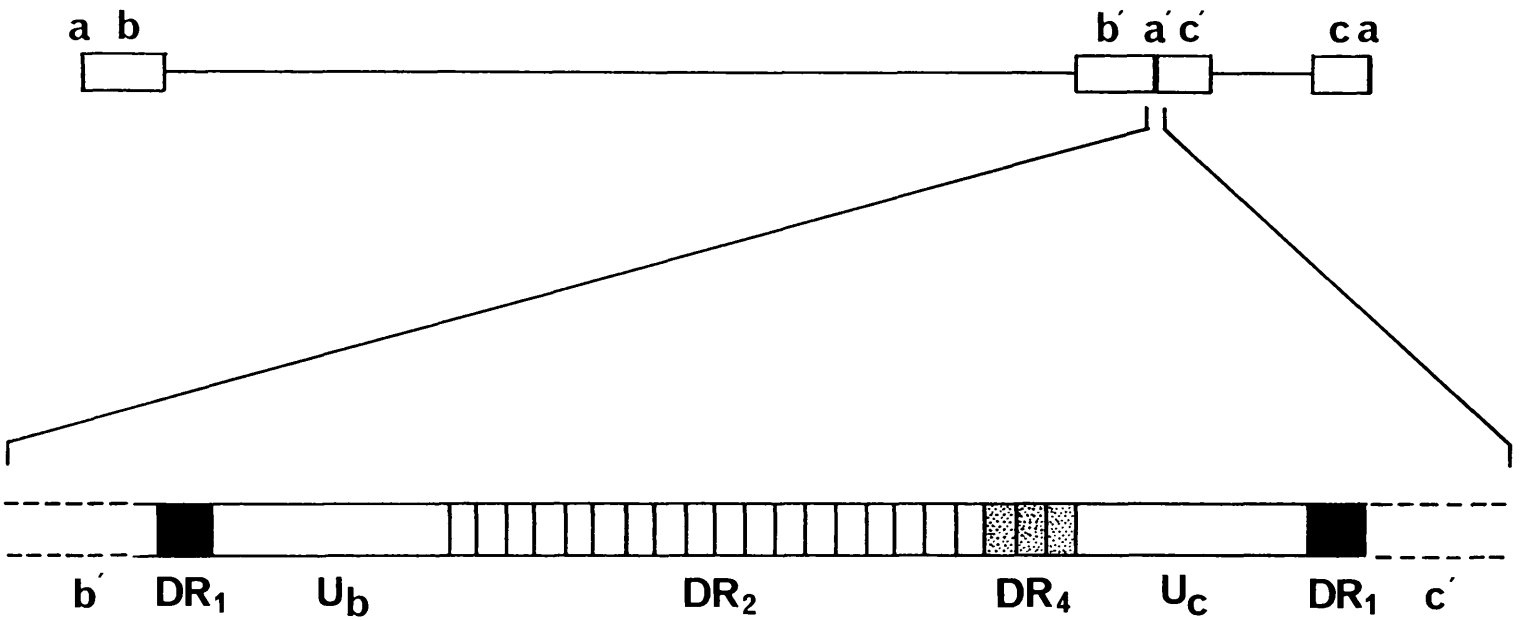

a b
b´ a´ c´
c a
b´
DR1
Ub
DR2
DR4
Uc
DR1
c´

Figure 3. The HSV-1 *a* sequence

The upper part of the figure shows the HSV-1 genome. Solid lines represent unique sequences. Inverted repeats are indicated as *b*, *b'*, *c* and *c'* (TR_L, IR_L, IR_S and TR_S respectively). Organization within a copy of the *a* sequence from the L/S junction (*a'*) is shown below and comprises the following:

1. Unique sequences U_b and U_c respectively located proximal to *b'* and *c'* sequences.

2. A direct repeat of 17-21bp, DR_1 (filled boxes), located at either end of the *a* sequence.

3. A directly repeated 12bp element, DR_2 (open boxes), present in 1-22 copies.

4. A directly repeated 37bp element, DR_4 (stippled boxes), present in 0-3 copies.

at each end of the sequence. Direct repeats DR_2 and DR_4, which vary in copy number, occur internally (Davison and Wilkie, 1981; Mocarski and Roizman, 1981). The DR_2 element has been implicated in high frequency site-specific recombination during genome isomerization (Chou and Roizman, 1986). However, the HSV-2 *a* sequence does not contain DR_2 elements (Davison and Wilkie, 1981). Adjacent to the DR_1 elements lie two locally unique regions, U_b and U_c, proximal to repeat sequences *b* (R_L) and *c* (R_S) respectively. Deletion analysis of the *a* sequence has indicated that these unique sequences are important for cleavage and packaging of viral DNA (Varmuza and Smiley, 1985) and that the two sequences function in concert (Deiss *et al.*, 1986). Although the nucleotide sequence of the *a* sequence is variable between HSV-1 and HSV-2, two highly conserved sequences are apparent, termed pac-1 and pac-2, which are located in U_b and U_c respectively (**Figure 3**).

Models for the events occurring at the *a* sequence during cleavage and packaging have been proposed (Varmuza and Smiley, 1985; Deiss *et al.*, 1986). DR_1 elements at the L and S termini of the HSV genome are incomplete and may be ligated to form a novel joint sequence such that tandem *a* sequences share a DR_1 element. Concatemeric molecules of DNA generated by rolling-circle replication will contain this novel joint sequence which will arise once every unit genome length. A putative cleavage/packaging complex may recognise the novel sequence each time it occurs in a particular orientation, and cleave at the shared DR_1 element to generate unit length genomes. However, specifically constructed defective genomes with an *a* sequence at only one terminus have, following DNA replication, been shown to have an *a* sequence at both termini. This suggests that duplication may occur, perhaps following a staggered cleavage across an *a* sequence followed by repair synthesis to generate two full *a* sequences.

Packaging of unit genome lengths appears to occur concurrently with cleavage, and to be initiated by cleavage at an *a* sequence. The free terminus thus generated may be inserted into a pre-formed capsid structure into which the DNA is continuously packaged until cleavage at the next similarly orientated *a* sequence occurs. This second cleavage event completes one round of packaging and initiates another.

Conserved motifs within the *a* sequence are probably recognized by a putative *trans*-acting cleavage/packaging complex. Proteins from

HSV-infected cells have been shown to bind to the conserved sequence pac-2 when linked to DR_1 (Chou and Roizman, 1989). Both components are required for formation of the sequence-specific protein-DNA complex. Protein does not bind to pac-2 or DR_1 alone, or when pac-1 is substituted for pac-2 linked to DR_1. Purification of the proteins which form this complex revealed three major polypeptide species which included the DNase encoded by UL12. The product of UL36, VP1, may also form part of this complex (Lemaster and Roizman, 1980) as well as another protein of M_r140 000. Cellular DNA-binding proteins may also be involved in directing the activity of the complex to its required site. A host protein has been observed to bind specific sequences at the genome termini of HCMV which are homologous to the HSV-1 *a* sequence (Kemble and Mocarski, 1989).

Analysis of *ts* mutants defective in cleavage and packaging of progeny viral genomes has suggested possible roles for the products of HSV-1 genes UL26 and UL33 in these processes. Mutant *ts*1201 fails to process the UL26 gene product, p40, which as a result, remains associated with empty capsids which accumulate in the nucleus (Preston *et al.*, 1984; McGeoch *et al.*, 1988b). Another mutant, *ts*1233, which has a lesion in UL33, assembles partially cored capsids and also fails to process p40 (Al-Kobaisi, 1989).

Although viral or heterologous DNA, of various lengths and containing an *a* sequence may be packaged into a capsid, only those capsids containing approximately genome lengths of DNA acquire an envelope and leave the nucleus (Vlazny *et al.*, 1982). This suggests that structural alterations to the capsid occur following encapsidation of a full length genome. Capsids become enveloped by budding through the inner lamella of the nuclear membrane which is rich in virally encoded glycoproteins (Roizman and Furlong, 1974). Envelopment is followed by transportation, via the golgi apparatus, to the cytoplasmic membrane where mature virions are subsequently released from the cell.

1C. *CIS*- AND *TRANS*-ACTING ELEMENTS REQUIRED FOR REPLICATION OF HSV DNA

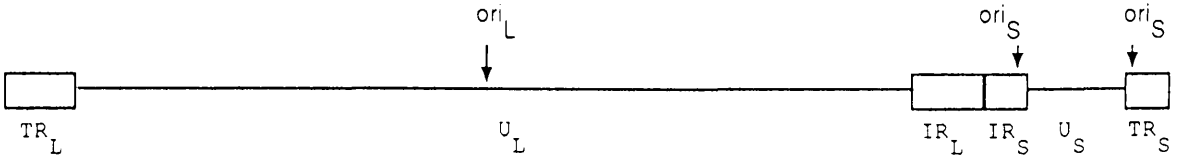
1. *Cis* Elements

Regions of the HSV genome corresponding to putative origins of replication were first observed by electron microscopy of replicating HSV DNA (Friedmann *et al.*, 1977). DNA replication forks and loops were

apparent at three positions in the genome suggesting that three distinct origins of replication were present. The existence and nature of *cis*-acting origins of replication was confirmed by analysis of defective genomes arising during serial passage of HSV at high moi. Maintenance of these defective genomes in virus stocks suggested that they contained *cis*-acting signals required for initiation of synthesis and for cleavage and packaging of DNA (Frenkel *et al.*, 1975). Analysis of these genomes by restriction enzyme digestion revealed them to consist of tandem repeats of HSV DNA sequences. On the basis of these sequences defective genomes could be divided into two groups, classes I and II. Members of both groups contained copies of the *a* sequence. Class I molecules additionally contained sequences from the short region (Denniston *et al.*, 1981; Frenkel *et al.*, 1976; 1980; 1981; Vlazny and Frenkel, 1981) whilst sequences from the middle of the long unique region were present in class II genomes (Schroder *et al.*, 1975; Kaerner *et al.*, 1979; 1981; Frenkel *et al.*, 1980; Vlazny *et al.*, 1982, Locker *et al.*, 1982; Spaete and Frenkel, 1982). These observations suggested the presence of origins of replication within both the L and S segments of the HSV-1 genome (Vlazny and Frenkel, 1981). These were subsequently termed ori_L and ori_S.

(a) *Ori*$_S$

Fragments containing the S region sequences present in class I defective genomes were cloned from the wild-type HSV-1 genome and analysed by Stow (1982). A transient replication assay was developed in which plasmids containing fragments to be tested for *cis*-acting origin function were transfected into tissue culture cells and helper functions supplied in *trans*, either by super-infecting with *wt* HSV-1 or by co-transfection with *wt* HSV-1 DNA. Only plasmids containing functional HSV origin sequences allowed amplification of the linked plasmid vector sequences. This system located an origin of replication within a 995bp fragment mapping entirely within R_S. Further systematic analysis of this 995bp fragment (Stow and McMonagle, 1983) defined a 90bp region in R_S which contained all necessary *cis*-acting sequences for the initiation of DNA synthesis (**Figure 4**). The products of plasmid replication in these analyses were shown to be high molecular weight molecules of DNA consisting of tandem copies of the complete plasmid, which suggested a rolling-circle mechanism of replication. This origin of replication was termed *ori*$_S$ and, since it lies within R_S, two identical copies are present in the HSV-1 genome (Stow,


ori L
ori S
ori S
TR L
U L
IR L
IR S
U S
TR S

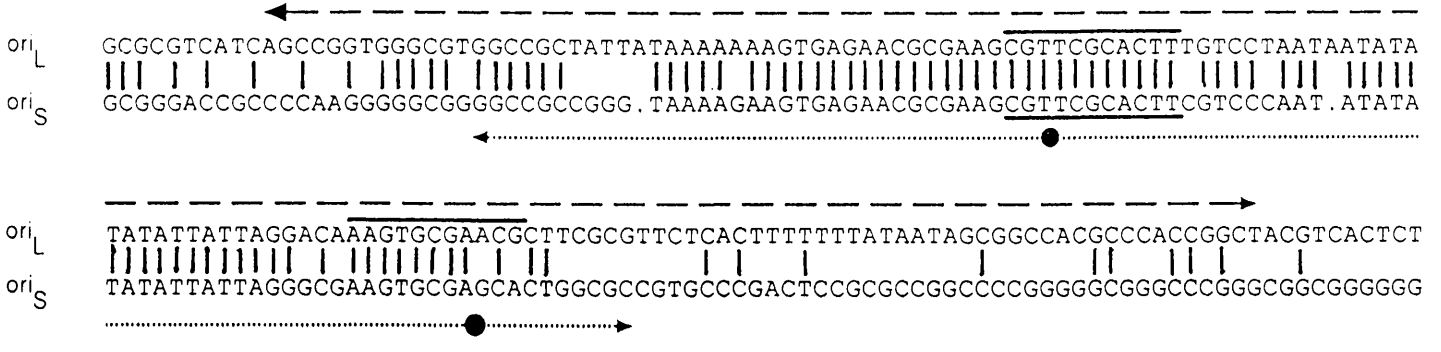

ori L GCGCGTCATCAGCCGGTGGGCGTGGCCGCTATTATAAAAAAAGTGAGAACGCGAAGCGTTCGCACTTTGTCCTAATAATATA
ori S GCGGGACCGCCCCAAGGGGGCGGGGCCGCCGGG.TAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAAT.ATATA
ori L TATATTATTAGGACAAAGTGCGAACGCTTCGCGTTCTCACTTTTTTTATAATAGCGGCCACGCCCACCGGCTACGTCACTCT
ori S TATATTATTAGGGCGAAGTGCGAGCACTGGCGCCGTGCCCGACTCCGCGCCGGCCCCGGGGGCGGGCCCGGGCGGCGGGGGG

Figure 4. HSV-1 Origins of Replication

The upper part of the figure shows the HSV-1 genome with positions of the HSV-1 origins of replication indicated. The lower part of the figure shows aligned sequences of *ori*$_L$ and *ori*$_S$. Vertical lines represent conserved bases. Arrowed, dashed lines above the *ori*$_L$ sequence indicate the limits of the 144bp palindrome and arrowed, dotted lines below the *ori*$_S$ sequence indicated the limits of the minimal *ori*$_S$ as defined by Stow and McMonagle, 1983. The limits of the *ori*$_S$ palindrome are indicated by the closed circles. The conserved 11bp motif is indicated by the horizontal lines above the *ori*$_L$ sequence and below the *ori* $_S$ sequence.

1982; Stow and McMonagle, 1983). *Ori*$_S$ lies in an intergenic region between two divergently transcribed immediate-early genes (IE3 and US1/US12) and is surrounded by the promoters and upstream regulatory sequences of these genes. *Ori*$_S$ has been reported to be contained within a transcribed open reading frame (Hubenthal-Voss *et al.*, 1987; Hubenthal-Voss and Roizman, 1988). The role of this transcript in DNA replication, if any, remains to be determined. Cellular proteins appear to bind the transcript at its initiation site (Roller *et al.*, 1989). A protein translated from this transcript, however, has not been detected and indeed the protein-coding capacity of this transcript remains disputed (McGeoch *et al.*, 1988b).

A prominent feature of the minimal *ori*$_S$ sequence is an almost perfect 45bp palindrome containing 18 consecutive A and T residues at its centre (Stow and McMonagle, 1983). Both arms of the palindrome and the central A-T rich region are essential for *ori*$_S$ function in both HSV-1 and HSV-2 (Stow, 1982; Stow and McMonagle, 1983; Lockshon and Galloway, 1988). In contrast to these results Deb and Doelberg (1988) reported that one arm of the palindrome was dispensable for replication although subsequent studies on the role of protein binding sites within the origin region (see Section 1C.3(c)) suggest that this is unlikely.

HSV-2, strain HG52 contains four copies of *ori*$_S$, since within both IR$_S$ and TR$_S$ a sequence of 137bp containing the origin is tandemly duplicated (Whitton and Clements, 1984b). Analysis of HSV-2 (strain 333) *ori*$_S$ has defined a minimum functional core region of 75bp within which lies a 56bp palindrome containing a central A-T rich tract. The DNA sequence of HSV-2 *ori*$_S$ is very similar to that of HSV-1 *ori*$_S$ with 73 of the 75 residues identical within this core region (Lockshon and Galloway, 1988).

VZV *ori*$_S$ also exhibits DNA sequence homology to *ori*$_S$ of HSV-1. It contains a palindromic region with a central AT-rich tract and can initiate amplification of linked plasmid sequences in response to HSV-1 replication factors supplied in *trans* (Stow and Davison, 1986).

(b) *Ori*$_L$

Sequences from the centre of U$_L$ which are present in class II defective genomes and are thought to contain a functional origin have also been analysed. However, these studies were initially hindered by the tendency of this region of the HSV-1 genome to suffer deletion when cloned in bacteria (Spate and Frenkel, 1982; Weller *et al.*, 1985).

Cloned monomeric units of class II defective genomes which had suffered this deletion were able to replicate if passaged in the presence of helper virus. It was found that under these conditions the deletion had invariably been repaired, presumably by recombination with the helper virus. This suggested that sequences essential for origin activity were located within the deletion prone region of approximately 100bp (Spate and Frenkel, 1982). This second HSV origin of replication (*ori*$_L$) was first sequenced using defective genomes from HSV strain Angelotti (Gray and Kaerner, 1984). A 296bp region prone to deletion upon cloning was identified. This region which contained, in tandem, two long palindromic sequences was found not to be the simplest form of *ori*$_L$. *Ori*$_L$ was subsequently sequenced using virion DNA of two strains of HSV-1, KOS and 17 (Weller *et al.*, 1985; Quinn and McGeoch, 1985). Finally *ori*$_L$ was successfully cloned in an undeleted form within a 425bp fragment into yeast which facilitated detailed functional analysis (Weller *et al.*, 1985). This cloned *ori*$_L$ was shown to be functional in a plasmid amplification assay when HSV-1 helper functions were supplied in *trans* (Weller et al. 1985).

DNA sequence analysis of the *ori*$_L$ region of strains KOS and 17 revealed a perfect 144bp palindrome exhibiting striking homology (85%) to *ori*$_S$ such that the *ori*$_S$ palindrome entirely aligned with the central region of *ori*$_L$. Sequence homology extends beyond the *ori*$_S$ palindrome, approximately 40bp to one side (Figure 4; Quinn and McGeoch, 1985; Weller *et al.*, 1985). Within these regions of homology *ori*$_S$ and *ori*$_L$ exhibit 85% sequence identity. HSV-2 *ori*$_L$ has also been cloned and sequenced. It contains a 136bp almost perfect palindrome and exhibits 88% homology with HSV-1 *ori*$_L$ (Lockshon and Galloway, 1986). The *ori*$_L$ region of strain Angelotti (described above) contains two tandem copies of this palindrome.

Ori$_L$, like *ori*$_S$, is located between two divergently transcribed genes. These are UL29 and UL30, encoding major single-stranded DNA binding protein (mDBP) and viral DNA polymerase respectively, both of which have essential functions in the replication of HSV DNA. It is not known whether this arrangement of genes is of special significance for origin function.

An 11bp sequence present at one end of the HSV-1 *ori*$_S$ palindrome is completely conserved in HSV-2 and VZV *ori*$_S$, and also occurs once in each arm of *ori*$_L$ in HSV-1 and HSV-2 (**Figure 4**; Weller

et al., 1985; Stow and Davison, 1986). A closely related sequence is present at the other end of the *ori*$_S$ palindrome in HSV-1 and HSV-2. These sequences coincide with regions within HSV-1 *ori*$_S$ shown to be protected from DNase I digestion by an HSV encoded origin-binding protein (Elias *et al.*, 1986; Elias and Lehman, 1988). This origin-binding protein has since been identified as the product of the HSV-1 gene UL9 (Olivo *et al.*, 1988; Weir *et al.*, 1989).

The significance of the HSV-1 genome possessing three origins of replication is unclear. *Ori*$_S$ and *ori*$_L$ appear to be functionally equivalent. Mutants lacking *ori*$_L$ are viable in tissue culture indicating that *ori*$_L$ is dispensable for viral DNA replication (Polvino-Bodnar *et al.*, 1987) and indeed the alphaherpesvirus VZV does not posses an *ori*$_L$, only two copies of *ori*$_S$ (Davison and Scott, 1986; Stow and Davison, 1986). Mutants lacking one copy of *ori*$_S$ are also viable although mutants lacking both copies of *ori*$_S$ have not been isolated (Longnecker and Roizman, 1986; Smith *et al.*, 1989). These observations imply that viral DNA replication in tissue culture requires at least one copy of *ori*$_S$ or alternatively, at least two origin sequences.

Regions flanking *ori*$_S$ appear to effect a modest stimulation of DNA replication in plasmid amplification assays (Stow, 1982; Stow and McMonagle, 1983; Lockshon and Galloway, 1988). A recent analysis (Wong and Schaffer, 1991) has demonstrated an 80-fold reduction in origin function following deletion of sequences flanking *ori*$_S$, although it is not clear why the magnitude of this effect was much greater than that observed by other workers. These sequences include the transcriptional regulatory elements of genes IE3 and IE4/5. Competition experiments revealed that factors binding these regulatory elements are likely to be involved in an auxiliary capacity in *ori*$_S$ function (Wong and Schaffer, 1991). Therefore, in common with other eukaryotic origins of DNA replication (e.g. SV40, adenovirus) HSV-1 *ori*$_S$ appears to contain two primary functional components. These are a core region and an auxiliary component, composed of an enhancer or promoter element which functions in transcription as well as DNA replication (DePamphilis, 1988). Efficiency of initiation of DNA synthesis may be increased by transcription factors which recognise and bind the auxiliary component of *ori*$_S$ or, as observed for *E. coli oriC* (Baker and Kornberg, 1988), transcription near *ori*$_S$ may additionally facilitate melting of the duplex.

Although HSV origins have been defined in transient assays there is no direct evidence of initiation of DNA synthesis at these sites.

However, by analogy with other systems it seems very likely to be the case.

2. *Trans*-Acting Functions

Initial attempts to identify virally encoded *trans*-acting proteins required for DNA synthesis involved the analysis of candidate enzymatic activities induced in HSV-infected cells. The first virally encoded activities identified in this way were the viral DNA polymerase (Keir and Gold, 1963; Hay *et al.*, 1971), thymidine kinase (Kit and Dubbs, 1963) and alkaline exonuclease (Morrison and Keir, 1968, Hay *et al.*, 1971). Many others followed, including the major single-stranded DNA-binding protein (Bayliss *et al.*, 1975; Purifoy and Powell, 1976), origin-binding protein (Elias *et al.*, 1986), ribonucleotide reductase (Cohen, 1972), uracil DNA-glycosylase (Caradonna and Cheng, 1981), dUTPase (Wohlrab and Francke, 1980), protein kinase (Blue and Stobbs, 1981; Purves *et al.*, 1987) and most recently, DNA helicase and DNA primase activities (Crute *et al.*, 1988; 1989). As will be described later, not all of these activities turned out to be required for viral DNA replication in tissue culture.

(a) Identification of Genes Encoding *Trans*-Acting Functions

Identification of genes specifying *trans*-acting functions essential for the replication of viral DNA was initially approached using conditional-lethal mutants of HSV defective in DNA synthesis (Schaffer *et al.*,1987). Approximately ten complementation groups of *ts* mutants have been isolated which are DNA-negative (DNA^-) at the NPT.

A complementary genetic approach has been the use of a transient replication assay in which fragments of HSV DNA are tested for their ability to support the amplification of an HSV origin-containing plasmid when co-transfected into cells in tissue culture (Challberg, 1986). By systematic subcloning, deletion and enzyme cleavage of the transfected fragments essential regions of the genome were defined. Relating these to the genomic DNA sequence identified seven HSV-1 open reading frames, ie. UL5, UL8, UL9, UL29, UL30, UL42 and UL52, as necessary and sufficient for origin-dependent DNA synthesis (**Table 1**; Wu *et al.*, 1988; McGeoch *et al.*, 1988a). In addition, there was a requirement for immediate early genes to stimulate the expression of these seven ORFs. This requirement could be met by various combinations of IE proteins Vmw110, Vmw175 and Vmw63, none of which was obligatorily required, suggesting that their involvement was indirect.

HSV-1 gene (1)	Predicted Protein M_r	Homologous VZV gene (2)	Homologous EBV gene (3)	Homologous HCMV gene (4)
UL5	98,710	55	BBLF4	UL105
UL8	79,921	52	?	?
UL9	94,246	51	?	?
UL29	128,341	29	BALF2	UL57
UL30	136,272	28	BALF5	UL54
UL42	51,156	16	?	?
UL52	114,416	6	BSLF1	UL70

Table 1. HSV-1 Genes Required for DNA Replication

(adapted from McGeoch, 1987)

(1) The seven HSV-1 genes were identified as essential for DNA synthesis in transient transfection assays (Challberg, 1986; Wu *et al.*, 1988).

(2), (3) and (4) show homologous genes in VZV, EBV and HCMV respectively, identified by sequence comparisons (McGeoch *et al.*, 1988b; Chee *et al.*, 1990)

'?' indicates a lack of any reading frame exhibiting significant sequence homology.

Detailed mapping of *ts* mutants with DNA$^-$ phenotypes has identified mutations which occur within each of the seven genes identified as essential for DNA replication in the transient assay (Dixon and Schaffer, 1980; Purifoy *et al.*, 1977; Purifoy and Powell, 1981; Chartrand *et al.*, 1980; Coen *et al.*, 1984; Conley *et al.*, 1981; Weller *et al.*, 1983b; 1987; Littler *et al.*, 1983; Zhu and Weller, 1988; Marchetti *et al.*, 1988). Temperature-sensitive mutations which render the virus defective for DNA replication at the NPT have been mapped to other genes suggesting that Vmw175 and the large subunit of ribonucleotide reductase may indirectly participate in the synthesis of viral DNA (Preston, 1979b; Dixon and Schaffer, 1980).

Targeted null mutations have now been isolated for most of the seven essential DNA replication genes which should aid their functional characterization. These deficient viruses can be propagated on cell lines expressing the *wt* gene product (Goldstein and Weller, 1988c; Zhu and Weller, 1988; Carmichael and Weller, 1989, Johnson *et al.*, 1991).

The correlation between the genes identified in the transient assay and by analysis of *ts* mutants suggests that all HSV-1 genes directly involved in the replication of viral DNA in tissue culture have now been identified. In support of this, the level of plasmid amplification sustained by the seven essential open reading frames appeared only marginally lower than that sustained by five restriction fragments comprising most of the HSV-1 genome. However, it is probable that certain genes which appear not to affect or have only a minor effect on viral DNA synthesis in tissue culture (e.g. thymidine kinase, ribonucleotide reductase) may be essential for DNA replication *in vivo* (Field and Wildy, 1978; Cameron *et al.*, 1988; Goldstein and Weller, 1988a; b).

Ori$_S$ and *ori*$_L$ appear functionally equivalent in plasmid amplification assays and no viral gene products have been identified as being specific to either origin. However, although the same proteins may be involved, mechanisms operating at these origins show differences.

Open reading frames homologous to each of the seven HSV-1 DNA replication genes have been identified in the genome of the alphaherpesvirus VZV. Clear counterparts for the genes UL5, UL29, UL30 and UL52 only, have been recognised in the genomes of betaherpesvirus, HCMV and gammaherpesvirus, EBV (**Table 1**; McGeoch, 1987; Chee *et al.*, 1990).

3. *Trans*-Acting Functions Directly Involved in HSV DNA Replication

Three of the seven HSV-1 genes identified as essential for replication encode products which are abundant in HSV-1 infected cells and which were first recognized several years ago and have been relatively well characterised. These are UL29 (*dbp*) which encodes the major single-stranded DNA-binding protein (mDBP) (Conley *et al.*, 1981; Quinn and McGeoch, 1985), UL30 (*pol*) which encodes the viral DNA polymerase (POL)(Chartrand *et al.*, 1979; Quinn and McGeoch, 1985; Gibbs *et al.*, 1985), and UL42 which encodes a double-stranded DNA-binding protein, M_r62 000-65 000 found to associate with POL (Wu *et al.*, 1988; McGeoch *et al.*, 1988a; Parris *et al.*, 1988). The products of the remaining four genes are present in very low abundance in infected cells and have only recently been identified with the aid of expression systems. The product of the UL9 gene has recently been shown to be an origin-binding protein which recognises specific sequence elements within HSV origins (Olivo *et al.*, 1988; Weir *et al.*, 1989). The UL5, UL8 and UL52 gene products have been reported to form a complex in infected cells which exhibits DNA dependent ATPase, DNA dependent GTPase, DNA helicase and DNA primase activities (Crute *et al.*, 1989) and are the subject of much of the work described in this thesis.

(a) DNA Polymerase Holoenzyme (Encoded by Genes UL30 and UL42)

A novel activity with properties distinguishable from those of the host DNA polymerases was described in HSV-infected cells (Keir and Gold, 1963; Keir *et al.*, 1966; Hay *et al.*, 1971; Weissbach *et al.*, 1973). This novel activity was shown to be virally encoded and essential for the replication of viral DNA by analysis of *ts* and drug-resistant mutants (Dixon and Schaffer, 1980; Purifoy *et al.*, 1977; Purifoy and Powell, 1981; Chartrand *et al.*, 1980; Coen *et al.*, 1982; 1984; Hay and Subak-Sharpe, 1976; Honess and Watson, 1977; Schaffer *et al.*, 1978; Crumpacker *et al.*, 1980; Furman *et al.*, 1981; Jofre *et al.*, 1977; Knipe *et al.*, 1979).

HSV DNA polymerase (POL), the product of gene UL30 (*pol*), has been purified to homogeneity and the catalytic activity shown to reside in a single polypeptide chain M_r 140 000 (Powell and Purifoy, 1977; Knopf, 1979; O'Donnell *et al.*, 1987a). This closely corresponds to M_r 136 000 predicted from DNA sequence analyses (Gibbs *et al.*, 1985; Quinn and McGeoch, 1985). Active HSV POL has been translated *in*

vitro (Dorsky and Crumpacker, 1988) and also expressed in yeast (Haffey *et al.*, 1988) and in a recombinant baculovirus (Marcy *et al.*, 1990) confirming that the enzyme is functional in the absence of other HSV proteins. HSV POL is related to other DNA polymerases which are members of the mammalian DNA polymerase α family (Gibbs *et al.*, 1985; 1988; Larder *et al.*, 1987). HSV POL possesses a 3'-5' exonuclease proof-reading activity (Knopf, 1979; Marcy *et al.*, 1990) and a 5'-3' exonuclease/RNase H activity (Crute and Lehman, 1989, Marcy et *al.*, 1990), analogous to that of *E.coli* DNA polymerase I which specifically degrades RNA/DNA heteroduplexes or duplex DNA in a 5'-3' direction during removal of RNA primers or DNA repair (Kornberg, 1980).

A *ts* mutant with a lesion in the N-terminal region of the protein has been shown to encode a DNA polymerase which is thermolabile, both *in vivo* and *in vitro* (Aron *et al.*, 1975; Purifoy and Powell, 1981). Other *ts* lesions and mutations to drug-resistance which have been finely mapped and sequenced fall predominantly within the carboxy-terminal half of the *pol* gene product. This region exhibits homology with the amino acid sequences of many prokaryotic and eukaryotic DNA polymerases (Gibbs *et al.*, 1985; 1988; Larder *et al.*, 1987; Wong *et al.*, 1988) and the mutations most frequently occur within a proposed nucleotide-binding site.

A number of phenotypically distinct mutants of HSV-1 (strain KOS) have been isolated which exhibit abnormal drug resistance or sensitivity. Mutant *ts*C4 exhibits limited resistance to acyclovir and hypersensitivity to phosphonoacetic acid (PAA). The *ts* mutation has been mapped by intertypic marker rescue experiments (Chatrand *et al.*, 1980). Subsequent mapping by rescue with cloned restriction fragments of HSV-1 DNA revealed distinct *ts* and PAA hypersensitivity lesions within the *pol* gene (Coen *et al.*, 1984). Temperature sensitivity and PAA resistance of mutant *ts*D9 have been mapped to a single region within the *pol* locus distinct from those mapped for *ts*C4. A third distinct lesion in *pol* (*ts*C7) was also mapped (Chartrand *et al.*, 1980; Coen *et al.*, 1984).

Mutations in *pol* which confer aphidicolin resistance have been isolated and mapped to two regions near the 3' end of HSV POL which are highly conserved in DNA polymerases (Hall and Woodward, 1989; Hall *et al.*, 1989). The site to which aphidicolin binds overlaps the nucleotide-binding site of the enzyme. The nucleotide binding site of HSV POL is located between amino acid residues 597 and 961. This region comprises three of the most highly conserved domains of the

protein, at least two of which are directly involved in substrate binding (Gibbs *et al.*, 1985; 1988; Knopf, 1986; Larder *et al.*, 1986; 1987; Tsurumi *et al.*, 1987).

PAA, which is an analogue of pyrophosphate, binds to a site on HSV POL also overlapping the nucleotide-binding site but not the aphidicolin-binding site. These results indicate that functionally distinct domains of HSV POL exist and that their organisation is complex (Hall *et al.*, 1989). Directed mutations within *pol* have revealed several complementation groups and confirm the existence of separable domains of the enzyme (Marcy *et al.*, 1990). Close communication between functional domains may occur since the 3'-5' exonuclease activity associated with HSV POL is as sensitive to PAA as is DNA polymerase catalytic activity (O'Donnell *et al.*, 1987a).

Most DNA-dependent DNA polymerases have been found to exist in a complex with one or more non-covalently linked proteins whose presence is necessary for optimal function. HSV-2 POL frequently purifies with another polypeptide of M_r 50,000-60,000 (Powell and Purifoy, 1977; Vaughan *et al.*, 1985). In HSV-1, an equivalent protein has been identified as the product of gene UL42. Antisera raised against the UL42 protein co-precipitated UL42 protein and HSV POL, indicating that the two proteins form a stable complex (Gallo *et al.*, 1988; Parris *et al.*, 1988). Sedimentation and gel filtration analyses are consistent with this complex existing as a heterodimer of DNA polymerase (catalytic subunit) and UL42 protein (accessory subunit) (Gottlieb *et al.*, 1990). The complex has been assembled *in vitro* by association of products translated from synthetic mRNAs transcribed from cloned genes (Gallo *et al.*, 1989; Digard and Coen, 1990) and *in vivo* in insect cells co-infected with recombinant baculoviruses individually expressing each gene product (Gottlieb *et al.*, 1990). This indicates that formation of the complex does not require additional HSV proteins.

When separated from UL42 protein by purification through a glycerol gradient, the POL catalytic subunit exhibited greatly reduced activity on an activated DNA template. Addition of purified UL42 protein stimulated activity 4-10 fold but not in the presence of anti-UL42 MAb. These observations suggest a specific role for UL42 protein in stimulating catalytic activity of POL (Gallo *et al.*, 1989).

A comparison of the catalytic properties of HSV POL-UL42 protein complex with those of the individual subunits purified from HSV-1 infected cells or expressed in insect cells infected with recombinant

baculoviruses has shown that the UL42 protein increases processivity of the catalytic subunit when a template of singly primed single-stranded M13 DNA is used (Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990). Products of the POL-UL42 protein complex were almost exclusively equivalent to full-length M13 DNA molecules whereas those produced by POL alone were much smaller. Addition of an equimolar amount of UL42 protein to purified POL prior to commencement of the reaction resulted in the generation of full-length products. The POL-UL42 protein complex was also found to utilize significantly fewer primers than POL alone, but with considerably greater efficiency. In template challenge experiments, where a second template was added to a committed replication reaction on an M13 template, products synthesised by POL alone originated from both M13 and the ϕX174 challenge template and few of these were full-length molecules. POL-UL42 protein complex, however, synthesised almost exclusively full-length M13 molecules indicating that the complex remains associated with its template and its DNA polymerase activity is highly processive. It has therefore been proposed that the UL42 protein serves as a processivity factor for the viral DNA polymerase and that a heterodimer of UL30 and UL42 gene products represents the functional HSV DNA polymerase holoenzyme (Gottlieb *et al.*, 1990). The region of POL involved in interaction with the UL42 protein has been mapped to a region conserved in the DNA polymerase α family, including the polymerases encoded by VZV and EBV. This region, near the C-terminus, is distinct from those involved in substrate binding (Digard and Coen, 1990).

Although classed as a member of the DNA polymerase α family, HSV DNA polymerase has been shown to be more closely related to DNA polymerase *δ* than to α itself (Boulet *et al.*, 1989). In addition to amino acid sequence similarity, both exist as heterodimers of a large catalytic subunit and a small accessory subunit (Bauer and Burgers, 1988; Lee *et al.*, 1984). Unlike DNA polymerase α, both exhibit 3'-5' exonuclease activity and are highly processive.

The UL42 protein, M_r 62 000-65 000, is phosphorylated and is approximately 20 times as abundant as POL in HSV-1 infected cells (Gottlieb *et al.*, 1990). It binds in a non-cooperative and sequence independent manner to double-stranded DNA (Marsden *et al.*, 1987). The significance of its DNA-binding properties in the replication of viral DNA is unclear and it is not known whether uncomplexed UL42 protein has any role. The UL42 protein appears to bind tightly and

preferentially to the ends of duplex DNA and this increased affinity for duplex ends may be related to its role in stimulating DNA polymerase processivity (Gottlieb *et al.*, 1990). By analogy with accessory subunits of other DNA polymerases, the UL42 protein may act as a 'clamp' which decreases the possibility of dissociation of the enzyme and the template (Mace and Alberts, 1984; Huber *et al.*, 1987a; b).

Nuclear localisation is an intrinsic property of both POL and UL42 protein. However, the localization of these proteins to characteristic replication compartments depends upon the presence of mDBP and ongoing viral DNA synthesis (deBruyn Kops and Knipe, 1988; Goodrich *et al.*, 1990).

(b) Major Single-Stranded DNA-Binding Protein (Encoded by Gene UL29)

In early studies a polypeptide of estimated M_r 130 000 (ICP8, mDBP) was identified as a major protein induced in HSV infected cells (Honess and Roizman, 1973; Powell and Courtney, 1975). Analysis of *ts* mutants later identified this protein as the product of HSV-1 gene *dbp* (UL29) and demonstrated that it was essential for the replication of viral DNA (Conley *et al.*, 1981; Weller *et al.*, 1983; Littler *et al.*, 1983; Dixon *et al.*, 1983; Godowski and Knipe, 1983; 1985; 1986; Leinbach *et al.*, 1984; Holland *et al.*, 1984). The estimated molecular weight of the HSV major single-stranded DNA-binding protein (mDBP) is M_r 128 341, predicted from DNA sequence analysis (Quinn and McGeoch, 1985). mDBP binds preferentially and co-operatively to single-stranded DNA with a stoichiometry of one mDBP molecule bound per 12 nucleotides (Bayliss *et al.*, 1975; Powell and Purifoy, 1976; Ruyechan, 1983; Lee and Knipe, 1983; O'Donnell *et al.*, 1987b; Gao *et al.*, 1988). mDBP binds DNA such that protein-coated DNA filaments are formed. In the absence of DNA, mDBP molecules aggregate into a filamentous form (O'Donnell *et al.*, 1987b). Binding of mDBP to ssDNA is thought to stabilise the structure of the replication fork (Powell *et al.*, 1981; Ruyechan, 1983; Ruyechan and Weir, 1984; Lee and Knipe, 1983; O'Donnell *et al.*, 1987b). In addition, mDBP causes a decrease in the melting temperature of poly(dA.dT) duplex DNA (Powell *et al.*, 1981) and may facilitate strand separation.

mDBP, in the absence of other HSV proteins, has been reported to stimulate the activity of HSV DNA polymerase on activated DNA templates (Ruyechan and Weir, 1984; Gottleib *et al.*, 1990: Hernandez

and Lehman, 1990). On singly primed single-stranded templates mDBP has been shown to effect a modest stimulation of the DNA polymerase catalytic subunit (Gottleib *et al.*, 1990) and additionally the polymerase holoenzyme (Hernandez and Lehman, 1990).

By analysis of *ts* mutations, the DNA-binding domain of mDBP was suggested to be in the C-terminal region of the polypeptide (Weller *et al.*, 1983). Analysis of truncated mDBP polypeptides translated *in vitro* confirmed this location (Leinbach and Heath, 1988). Fine mapping of *ts* mutations has defined a region between amino acid residues 348 and 450 as important for DNA-binding (Gao *et al.*, 1988) and proteolytic digestion of mDBP has identified two regions required for DNA-binding (Wang and Hall, 1990). One region, between residues 332 and 564, contains a zinc-binding motif and may participate in intra- or intermolecular interactions contributing to co-operative DNA-binding (Wang and Hall, 1990; Gao *et al.*, 1988; Leinbach and Heath, 1988). The presence of zinc, however, does not appear necessary for DNA binding, either by mDBP or an expressed fragment of the protein containing this motif (Leinbach and Heath, 1988; Ruyechan *pers. comm.*). The other region, towards the C-terminus, between residues 571-1160, was identified as the core DNA-binding site of mDBP (Wang and Hall, 1990). Both regions are conserved in homologous proteins encoded by herpesviruses VZV and EBV.

HSV mDBP inherently localises to the nucleus of the infected cell. Following its synthesis in the cytoplasm it becomes attached to the cytoplasmic framework and is then transported to the nucleus where it associates with the nuclear matrix (Fenwick *et al.*, 1978; Knipe and Spang, 1982; Quinlan and Knipe, 1983). The precise distribution of mDBP is regulated, at least in part, by the physiology of the infected cell and the status of viral DNA replication (Knipe and Spang, 1982; Quinlan *et al.*, 1984). In the absence of DNA replication mDBP is localised in regularly distributed discrete areas termed pre-replicative sites. As viral replication proceeds mDBP becomes localised in randomly distributed globular structures termed replication compartments. The size and number of replication compartments increases throughout the process of viral DNA replication until most of the nucleus is included. A minor proportion of mDBP molecules remain at pre-replicative sites, possibly serving as a reservoir (Quinlan *et al.*, 1984). The distribution of sites at which viral DNA is synthesised is coincident with the distribution of replication compartments (Lee and Knipe, 1983; Rixon *et al.*, 1983; Randall and Dinwoodie, 1986; deBruyn Kops and Knipe, 1988).

When DNA synthesis is inhibited mDBP does not localise within replication compartments but is present at pre-replicative sites. Following reversal of the block in DNA synthesis mDBP redistributes to the replication compartments (Quinlan *et al.*, 1984; Randall and Dinwoodie, 1986; deBruyn Kops and Knipe, 1988). Treatment with DNase I results in considerable reduction in the amount of mDBP present in replication compartments suggesting an interaction between mDBP and replicating viral DNA at these sites (Quinlan *et al.*, 1984; Randall and Dinwoodie, 1986).

Under conditions permitting viral DNA replication, HSV-1 DNA polymerase, UL42 protein and origin-binding protein each exhibit an intracellular localisation pattern similar to that of mDBP (Olivo *et al.*, 1989). DNA polymerase and UL42 protein have been shown to co-localise to replication compartments within the same cell. However, this localisation is dependent upon the expression of functional mDBP (Randall and Dinwoodie, 1986; Thomas *et al.*, 1988; Goodrich *et al.*, 1990; Bush *et al.*, 1991). HSV alkaline exonuclease and IE protein Vmw175 are also distributed in the infected cell nucleus similarly to mDBP (Thomas *et al.*, 1988; Randall and Dinwoodie, 1986).

These observations suggest a major role for mDBP in the recruitment of HSV DNA replication proteins to sites at which synthesis of viral DNA occurs. It therefore appears that organised sites for HSV DNA synthesis exist as is also the case for cellular DNA replication although their relationship remains to be determined (Huberman, 1987; deBruyn Kops and Knipe, 1988). In HSV-1 infected cells the sites at which cellular DNA replication occurs become redistributed such that they coincide with pre-replicative sites of viral DNA synthesis (deBruyn Kops and Knipe, 1988). In addition, certain cellular proteins are redistributed such that they co-localize with mDBP (Wilcock and Lane, 1991). When viral DNA synthesis is inhibited by PAA components of the cellular DNA replication apparatus remain at these sites and continue to synthesise cellular DNA. Redistribution of cellular DNA replication structures did not occur in cells infected with *ts* mutants of HSV-1 with a lesion in gene UL29. These studies therefore indicate a role for mDBP in the organisation of cellular as well as viral DNA replication proteins at discrete sites within the infected cell nucleus (deBruyn Kops and Knipe, 1988; Bush *et al.*, 1991).

Some *ts* mutants of HSV with lesions in UL29 exhibit sensitivity to metabolic inhibitors of HSV DNA polymerase. This may reflect a physical interaction between mDBP and POL which modulates the

catalytic activity of DNA polymerase (Chiou *et al.*, 1985). Specific mutations directed to various regions within UL29 have revealed several mutant phenotypes of mDBP (Gao and Knipe, 1989). DNA negative mutants encoding mDBP with defects in DNA-binding, nuclear localisation or both functions were isolated. Thus, DNA-binding and nuclear localisation appear to be separable functions of the protein. Also, mutants were identified which were unable to replicate DNA but encoded mDBP which could bind DNA *in vitro* and was localised to the nucleus *in vivo*, suggesting that other essential functions may be mediated by the protein. These could include protein:protein interactions with viral or cellular factors involved in the replication of viral DNA or be related to the melting of DNA (Gao *et al.*, 1988; Gao and Knipe, 1989).

The N-terminal domain of mDBP exhibits limited amino acid sequence similarity with mammalian proliferating cell nuclear antigen (PCNA) (Matsumoto *et al.*, 1987). The intracellular localisation of PCNA is regulated by the status of cellular DNA replication and its presence is required for the catalysis of DNA synthesis by mammalian DNA polymerase *δ* (Bravo-MacDonald and Bravo, 1985). T4 gene 32 protein is similarly essential for replication of the bacteriophage DNA and is also a multi-functional protein which exhibits single-stranded DNA-binding activity and participates in protein:protein interactions with other T4 gene products (Alberts *et al.*, 1983; Nossal and Alberts, 1983). Like PCNA and T4 gene 32 protein, mDBP appears to have several important roles in DNA metabolism.

(c) Origin-Binding Protein (Encoded by Gene UL9)

An activity in HSV-1 infected cells which interacted specifically with an HSV-1 origin of replication was first described by Elias *et al.* (1986). To identify the gene encoding this activity HSV-1 ORFs essential for the replication of viral DNA were cloned and expressed in insect cells using a baculovirus vector (Olivo *et al.*, 1988) or in BHK cells using, as a vector, an HSV-1 mutant, *ts*K (Weir *et al.*, 1989; This thesis). In the latter case the ORFs were inserted under the control of the IE3 gene promoter. Since *ts*K is blocked at the IE stage of infection these inserted ORFs could be expressed at NPT in the absence of other DNA replication genes. These studies showed that the UL9 gene encoded origin-binding activity (Olivo *et al.*, 1988; Weir *et al.*, 1989). Antisera raised in rabbits to synthetic decapeptides corresponding to the predicted C-terminal amino acid sequences of

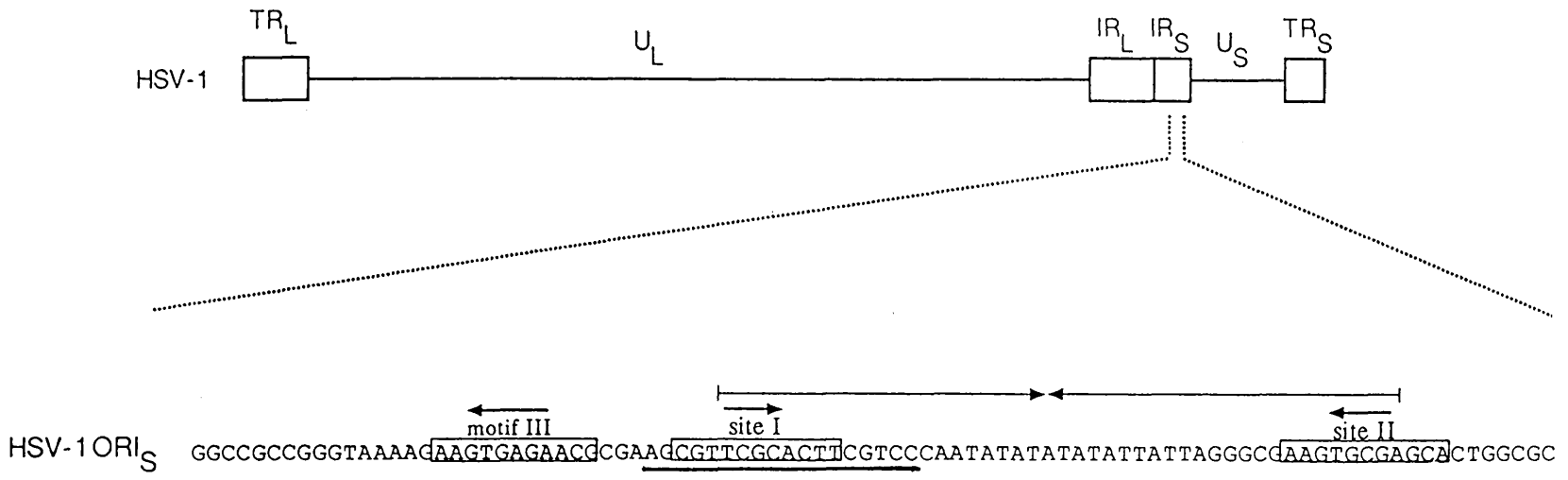
four HSV-1 replication genes (UL5, UL8, UL9 and UL52) confirmed that HSV origin-binding protein (OBP) was encoded by gene UL9 since protein-DNA complexes containing *ori*$_S$ and the origin-binding activity were immunoprecipitated only by the anti-UL9 antiserum (Olivo *et al.*, 1988). Similarly complexes between the origin-binding protein isolated from HSV-1 infected cells and a DNA fragment containing *ori*$_S$, exhibited reduced mobility in a gel retardation assay, and were further retarded by addition of anti-UL9 antiserum (Weir *et al.*, 1989).

DNA sequence analysis has predicted an M_r 94,246 for the UL9 gene product (McGeoch *et al.*, 1988a). HSV origin-binding activity purified to homogeneity from HSV-1 infected cells exhibited an apparent M_r 83,000 (Elias and Lehman, 1988). HSV-1 OBP immunoprecipitated from HSV-1 infected cells or from insect cells infected with a recombinant baculovirus expressing the UL9 gene exhibited M_r 82,000 (Olivo *et al.*, 1988). It is not known whether these proteins correspond to the complete predicted ORF. The experiments with anti-peptide anti-sera show that the C-terminal sequences must be present.

Origin recognition is an intrinsic property of the UL9 protein (Olivo *et al.*, 1988; Elias and Lehman, 1988). HSV OBP was originally shown, by DNase I foot-printing, to protect an 18bp region spanning the left-hand end of the HSV-1 *ori*$_S$ palindrome, as depicted in **Figure 5** (Elias *et al.*, 1986). Within this 18bp protected sequence (binding site I) lies an 11bp sequence ($^{5'}$CGTTCGCACTT$^{3'}$), which is conserved in *ori*$_S$ of HSV-2 and VZV and *ori*$_L$ of HSV-1 and HSV-2 (Weller *et al.*, 1985; Stow and Davison, 1986; Lockshon and Galloway, 1986; 1988). Two additional almost identical copies of this 11bp sequence exist within the minimal HSV-1 *ori*$_S$ (Stow and McMonagle, 1983). One of these occurs in inverted orientation in the right arm of the palindrome (site II), and the other in inverted orientation adjacent to site I outside the palindrome at the left-hand side (motif III). Purified OBP was subsequently shown to bind site II in addition to site I although the affinity for site I was 10 times greater than for site II (Elias and Lehman, 1988). This difference in affinity is probably a reflection of the difference between the nucleotide sequences of the 11bp motifs.

DNase I protection studies and methylation interference experiments identified an 8bp sequence ($^{5'}$GTTCGCAC$^{3'}$) as being crucial for sequence specific binding. It has been proposed that this sequence comprises two overlapping pentameric repeats ($^{5'}$GT$^{T}/_{G}$CG$^{3'}$).

(a)


HSV-1
TR_L
U_L
IR_L
IR_S
U_S
TR_S
HSV-1ORI_S
motif III
site I
site II
GGCCGCCGGGTAAAAGAAGTGAGAACGCGAAGCGTTCGCACTTCGTCCCAATATATATATATTATTAGGGCGAAGTGCGAGCACTGGCGC

(b)

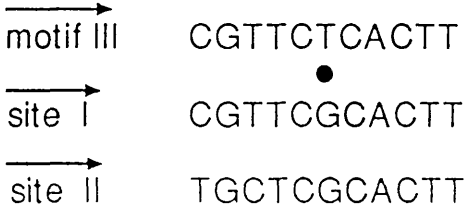

motif III
CGTTCTCACTT
site I
CGTTCGCACTT
site II
TGCTCGCACTT

Figure 5. OBP Binding Sites Within *Ori*$_S$

The 90bp sequence of the HSV-1 minimal *ori*$_S$, as defined by Stow and McMonagle (1983), is shown in panel **a**. The 45bp palindrome is indicated by the arrows. Conserved sites I and II and motif III are also indicated. The region protected from DNase I digestion, as reported by Elias *et al.*, (1986) which incorporates site I, is underlined.

The sequences of sites I, II and motif III are aligned, in the same orientation, in panel **b**. The single base difference between site I and motif III is indicated by the dot. In mutational analysis of site I, a G-T substitution was introduced (Weir and Stow, 1990).

In double stranded form:

$^{5'}$-GT$^{T}/_{G}$CG

GC$^{T}/_{G}$TG-$^{5'}$

The symmetry of this element is consistent with UL9 protein binding as a dimer, each monomer recognizing the pentameric motif (Koff and Tegtmeyer, 1988). In support of this hypothesis purified OBP has been found to exist as a dimer in solution (M D Challberg, unpublished results, reviewed by Challberg and Kelly, 1989).

Deletion and mutational analyses of HSV-1 *ori*$_S$ have shown site I to be essential for the initiation of replication. The results indicated a direct relationship between the ability of UL9 protein to bind site I and *ori*$_S$ function (Deb and Deb, 1989; Weir and Stow, 1990). Binding of OBP was abolished by a single point mutation in site I at one of the two G residues present in the overlap of the inverted pentameric motifs (**Figure** 5, Weir and Stow, 1990). These G residues have been shown to form essential contacts with OBP (Koff and Tegtmeyer, 1988) and the behaviour of the point mutant confirms the importance of one of the GC base pairs in sequence-specific binding of HSV OBP.

Deletion of site II resulted in a residual origin activity of approximately 5% (Lockshon and Galloway, 1988; Weir and Stow, 1990). In contrast, Deb and Doelberg (1988) have reported that site II is dispensable for efficient initiation of replication. The reason for this discrepancy is not clear. VZV *ori*$_S$, which lacks site II, replicates with only 5% of the efficiency of HSV *ori*$_S$ in response to HSV helper functions (Stow and Davison, 1986). This is consistent with a requirement for two functional binding sites for efficient initiation of DNA replication (Weir and Stow, 1990). An isolated motif III fails to bind OBP *in vitro* although its sequence differs from that of site I by only one nucleotide, an essential OBP-contacting G residue, and its deletion results in only a slight (2-fold) reduction in *ori*$_S$ function (Weir and Stow, 1990). Approximately 20bp to the left of site I in HSV-2 *ori*$_S$ have also been shown to be required for efficient replication to be initiated (Lockshon and Galloway, 1988). It is possible that motif III may contribute to the co-operative binding of OBP to *ori*$_S$ or this region may contain some other important sequence element.

DNase I foot-print analysis has shown that OBP binds *ori*$_S$ co-operatively (Elias and Lehman, 1988). Introduction of two point mutations to a synthetic oligonucleotide representing the site I sequence caused a 100-fold decrease in affinity of OBP whereas the same mutations within a fragment containing the complete *ori*$_S$ sequence resulted in only a 2-fold reduction in affinity for this site

(Elias *et al.*, 1990). Such co-operative binding by OBP to *ori*$_S$ may well be mediated by protein:protein interactions. The C-terminal one-third of OBP contains the DNA-binding domain of the protein (Weir *et al.*, 1989). The N-terminal regions of OBP molecules bound to DNA could possibly be involved in interactions with each other and other proteins (Elias and Lehman, 1988). Following interaction of OBP with *ori*$_S$ the pattern of DNase I cleavage at non-protected positions becomes altered suggesting that binding causes a conformational change (Elias *et al.*, 1990). Insertion of various numbers of AT dinucleotides into the centre of the *ori*$_S$ palindrome causes cyclic changes in origin function suggesting that sequences other than those recognised by OBP are important for activity (Lockshon and Galloway, 1988). Maxima in origin function correspond to insertion of integral numbers of helical turns of DNA suggesting that in addition to nucleotide sequence specificity in relative orientation of OBP binding sites may be required for efficient origin function. OBP molecules bound to sites I and II may interact to cause a conformational change, possibly bending the AT-rich tract and rendering it accessible to the remaining components of the HSV-1 replicative machinery. Local unwinding of the AT region may also occur. Relevant to this is the observation that the N-terminal region of OBP contains several motifs including some involved in NTP hydrolysis which are conserved in many proteins which have DNA helicase activity (Gorbalenya *et al.*, 1989; Gorbalenya and Koonin, 1989). OBP has in fact been shown to exhibit DNA helicase activity *in vitro* using partially double-stranded substrates (Bruckner *et al.*, 1991; M D Challberg, unpublished results; This thesis). Whether OBP is capable of unwinding duplex DNA at HSV origins of replication remains to be demonstrated.

By analogy with other systems, recognition and binding of the origin by OBP may represent the primary event in the initiation of replication of HSV DNA. However, verification of this direct role is difficult in the absence of an *in vitro* system. Following binding OBP is likely to be involved in the assembly of a preinitiation complex and subsequent initiation of DNA synthesis (Elias *et al.*, 1990).

Asymmetry in OBP affinity for its binding sites in *ori*$_S$ could impart unidirectionality in initiation of replication. In contrast, the presence of identical OBP-binding sites in the two arms of HSV *ori*$_L$ may suggest that replication from this origin is bidirectional.

(d) Helicase-Primase Complex (Encoded by Genes UL5, UL8 and UL52)

A novel DNA-dependent ATPase activity induced in HSV-1 infected cells was first described by Crute *et al.* (1988). This activity was distinguishable from host activities by its elution pattern during phosphocellulose column chromatography and its nucleoside triphosphate specificity.

DNA-dependent ATPase activity is a property of DNA helicases which couple hydrolysis of ATP to unwinding of duplex DNA, and the partially purified HSV-1 induced DNA-dependent ATPase activity was also shown to exhibit DNA helicase activity (Crute *et al.*, 1988). DNA helicase activity was assayed using a model substrate which consisted of a radio-labelled oligonucleotide annealed to a circular single-stranded DNA molecule. Products of an unwinding reaction in the presence of helicase and ATP (or other NTP) were resolved on non-denaturing polyacrylamide gels. Rapid migration of the labelled oligonucleotide into the gel is diagnostic of helicase activity. Unwound hybrid substrate DNA remains at the top of the gel. Modifications of the substrate DNA may allow further characterization of the helicase e.g. activity may vary with the length of duplex to be unwound, the presence of 5' and/or 3' tail on the fragment to be displaced, or with DNA:RNA hybrids or RNA duplex substrates. HSV DNA helicase activity was shown to be dependent upon the presence of a free, single-stranded tail at the 3' end of the fragment to be displaced suggesting 5'-3' translocation of the enzyme on the strand to which it is bound (single-stranded circle)(Crute *et al.*, 1988). Purification of HSV DNA helicase to near homogeneity revealed three major polypeptides; M_r 120,000, 97,000 and 70,000. DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities also co-purified with these proteins (Crute *et al.*, 1989). Likely candidates to encode the M_r 120,000, 97,000 and 70,000 proteins were HSV-1 essential replication genes UL52, UL5 and UL8 respectively, which at the time had no known function. Antisera raised to the products of these genes (Zhu and Weller, 1988; Olivo *et al.*, 1989) were found to be immunoreactive with the co-eluting proteins thus demonstrating that the HSV-1 helicase-primase complex is encoded by genes UL52, UL5 and UL8 (Crute *et al.*, 1989). Because of the low abundance of the helicase-primase complex in HSV-1 infected cells several more recent studies have employed the UL5, UL8 and UL52 proteins over-expressed in insect cells using recombinant baculoviruses (Olivo *et al.*, 1989; Dodson *et al.*, 1989; Calder and Stow, 1990; This Thesis). Like UL9, these ORFs

have also been over-expressed in BHK cells using the mutant *ts*K (This thesis).

Functional HSV-1 helicase-primase complex has been assembled *in vivo* in insect cells by triple infection with three recombinant baculoviruses individually expressing the UL5, UL8 and UL52 proteins (Dodson *et al.*, 1989; Calder and Stow, 1990). DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities of the over-expressed complex are identical to those of complex synthesised in HSV-1 infected cells (Dodson *et al.*, 1989). However, co-infection experiments have demonstrated that the UL8 protein is dispensable for all four enzymatic activities and that a complex formed between the UL5 and UL52 proteins is virtually indistinguishable in its properties from the complex containing all three subunits (Calder and Stow, 1990; Dodson and Lehman, 1991; This Thesis).

Since the UL8 protein is not required for any of the known enzymatic activities of the complex its role is rather unclear. It is possible that it may confer other enzymatic activities to the complex or be involved in transportation and/or stability of the complex within the infected cell. Indeed intracellular localization studies described in this thesis on the UL5, UL8 and UL52 proteins over-expressed in BHK cells suggest that the UL8 protein must be present to allow efficient entry of the UL5 and UL52 subunits, which specify the enzymatic activities, into the nucleus. This may reflect assembly of the helicase-primase complex as a heterotrimer in the cytoplasm and its transportation intact into the nucleus.

No enzymatic activities have been detected for the individual UL5, UL8 and UL52 proteins (Calder and Stow, 1990; Dodson and Lehman, 1991). The amino acid sequence of the UL5 protein nevertheless contains motifs which are conserved in proteins known to exhibit DNA or RNA helicase activity (Hodgeman, 1988; Gorbalenya and Koonin, 1989). However, DNA helicase activity is not an intrinsic property of the UL5 protein (Calder and Stow, 1990; Dodson and Lehman, 1991). This suggests that the UL52 protein may somehow facilitate DNA helicase activity of the UL5 subunit.

A DNA primase activity reported to be induced in HSV-1 infected cells by Holmes *et al.* (1988) is apparently unrelated to that of the helicase-primase complex (Crute *et al.*, 1989). This activity is now thought to be due to release of RNA polymerase from Vero cell mitochondria upon HSV-1 infection (Tsurumi and Lehman, 1990).

4. *Trans*-Acting Functions Indirectly Involved in HSV DNA Replication

Several genes controlling the expression of essential DNA replication proteins or involved in nucleotide metabolism can be considered as having functions indirectly concerned with HSV DNA synthesis.

Immediate-early proteins Vmw175, Vmw110 and Vmw63, due to their role in early gene transcription, are indirectly involved in the synthesis of viral DNA. Although these IE transactivators were employed by Wu *et al.* (1988) in transient assays which identified the seven DNA replication genes there was no obligatory requirement for any one of them. Moreover, Heilbronn and Zur Hausen (1989) achieved amplification in the absence of all 3 proteins by attaching the seven DNA replication genes to the strong HCMV major IE promoter. Thus none of these proteins is directly required for viral DNA synthesis. It is interesting to note, however, that during viral DNA synthesis Vmw175 localizes to the same replication compartments as several of the DNA replication proteins (Randall and Dinwoodie, 1986).

The HSV-1 thymidine kinase, first described by Kit and Dubbs (1963), phosphorylates thymidine, thymidylate and deoxycytidine (Jamieson and Subak-Sharpe, 1974; Chen and Prusoff, 1978) It is non-essential for viral growth in cultured, dividing cells but is required for growth in serum starved cells in order to supply TTP for DNA synthesis. In growing cells TTP is provided by host pathways (Jamieson *et al.*, 1984). TK has also been shown to be non-essential for the establishment of HSV latency in mice although TK^- mutants do not reactivate as efficiently as *wt* HSV (Efstathiou *et al.*, 1989). Phosphorylation of nucleoside analogues by TK can lead to inhibition of HSV infection by affecting the DNA replicative machinery (Rapp and Wigdahl, 1983). Herpesvirus thymidine kinases have recently been shown to have evolved from eukaryotic deoxycytidine kinase rather than thymidine kinase (Harrison *et al.*, 1991).

Ribonucleotide reductase (RR) catalyses the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates during the synthesis of DNA precursors (Cohen, 1972; Thelander and Reichard, 1979; Dutia, 1983). The virus-encoded enzyme is composed of two subunits, the interaction of which is essential for activity. These are large (RR_1) and small (RR_2) subunits, encoded by HSV-1 genes UL39 and UL40 respectively (V G Preston *et al.*, 1984; Bachetti *et al.*, 1984, Frame *et al.*, 1985; McLauchlan and Clements, 1983; McGeoch *et*

al., 1988b). Mutants with lesions in UL40 or UL39 exhibited greatly reduced pathogenicity in mice (Cameron *et al.*, 1988). The HSV-1 mutant *ts*1207 harbours a lesion in UL39 and fails to grow in tissue culture at NPT (V G Preston *et al.*, 1984). In contrast, an insertional mutation within UL39 does not affect viral growth in dividing cells. However, under conditions where infected cells were serum starved or incubated at high temperature (39.5°C) viral growth was severely compromised although small amounts of viral DNA were synthesised (Goldstein and Weller, 1988a; b). Growth of *ts*1207 was also severely compromised at 39.5°C but small amounts of newly synthesised viral DNA were detected (Goldstein and Weller, 1988b). The growth of viral mutants with lesions in RR therefore appears to depend upon the state of the infected cell. Host pathways, which in dividing cells are available for the synthesis of dNTPs, may be unavailable in non-dividing cells or be rendered inactive at high temperatures and the viral enzyme is therefore essential under these conditions. Viral RR may be similarly required for efficient replication *in vivo* in certain cell types.

Deoxyuridine triphosphatase, encoded by HSV-1 gene UL50, catalyses conversion of dUTP to dUMP plus pyrophosphate (Wohlrab and Francke, 1980; Preston and Fisher, 1984). This may be more important for allowing minimal incorporation of dUTP into nascent DNA than for the supply of dUMP for thymidine synthesis. The enzyme is not required for viral growth in tissue culture (Fisher and Preston, 1986).

HSV-encoded uracil DNA-glycosylase was first described by Caradonna and Cheng (1981). It is involved in DNA repair, removing misincorporated uracil, is dispensable for viral growth in tissue culture and is encoded by HSV-1 gene UL2 (Worrad and Caradonna, 1988; Mullaney *et al.*, 1989).

HSV-encoded exonuclease was first described by Keir and Gold (1963) as an increased alkaline nuclease activity induced in HSV infected cells. Following purification, the enzyme was found to consist of a single polypeptide which exhibited 5'-3' exonuclease activity as well as an endonuclease activity (Morrison and Keir, 1968; Hoffman and Cheng, 1979; Strobel-Fidler and Francke, 1980; Hoffman, 1981). The enzyme is encoded by gene UL12 (McGeoch *et al.*, 1986b; Draper *et al.*, 1986). Conflicting studies with *ts* mutants have indicated this enzyme to be either required or dispensable for viral growth and DNA synthesis (Moss *et al.*, 1979; Francke and Garret, 1982; Moss, 1986).

More recent studies (Weller *et al.*, 1990) with an exonuclease null mutant clearly show that the enzyme is essential for viral growth but not for the synthesis of viral DNA. Possible roles suggested for the exonuclease include the provision of dNTPs for DNA synthesis, cleavage of concatemers and an involvement in recombination (Weller *et al.*, 1990). HSV exonuclease is localised in replication compartments within the cell nucleus at which viral DNA synthesis occurs (Vaughan *et al.*, 1985). Alkaline exonuclease activity has been found to co-precipitate with HSV DNA polymerase (Randall and Dinwoodie, 1986) and to be reduced in amount in cells infected with a *pol ts* mutant (Littler *et al.*, 1983).

Topoisomerase I nicks and rejoins single strands of the DNA duplex acting as a swivel ahead of an advancing replication fork. This activity has been detected in HSV-1 infected cells and purified virions (Biswal *et al.*, 1983, Leary and Francke, 1984; Muller *et al.*, 1985). Fractionation of purified virions suggests that the activity may be a component of the tegument or envelope (Muller *et al.*, 1985). Topoisomerase I activity has also been reported to co-purify with HSV DNA polymerase (Biswal *et al.*, 1983). It is not clear whether the topoisomerase activities described in these papers are host or virally encoded, or whether they play an essential role in viral DNA synthesis.

Protein Kinase activity is induced upon HSV-1 infection of cells in tissue culture (Blue and Stobbs, 1981). HSV-1 gene US3 has been shown to encode a protein kinase activity which is dispensable for growth in tissue culture (Frame *et al.*, 1987; Purves *et al.*, 1987). Although the substrates for this enzyme are unknown they may include proteins involved directly or indirectly in the replication of HSV DNA (eg. POL, UL42 protein). A protein kinase function has also been proposed for the product of HSV-1 gene UL13 (Smith and Smith, 1989).

Additional virus-encoded functions indirectly involved in replication of HSV DNA may exist, together with directly or indirectly involved host functions. A role for host topoisomerase II activity induced in HSV-1 infected cells has been proposed in the replication of viral DNA (Ebert *et al.*, 1990). Other activities related to the synthesis of DNA and which are likely to be involved in viral DNA replication include a DNA ligase. The identification of host functions essential for viral DNA synthesis probably awaits the development of an origin-dependent cell-free system for HSV-1 DNA replication.

1D. MECHANISMS OF DNA REPLICATION

A similar common sequence of events leads to initiation of origin-dependent DNA synthesis in many prokaryotes and eukaryotes (Kornberg, 1980; Alberts, 1984).

An initial complex is formed following specific recognition and binding of DNA sequence elements within an origin of replication by an initiator protein. Melting and/or bending of a small region of the origin results in an open complex. The duplex is further unwound by a DNA helicase to allow formation of a pre-priming complex within the resulting replication bubble. RNA primers are then synthesised and subsequently elongated as nascent DNA strands by DNA polymerase.

As intracellular parasites, viruses are dependent upon the host cell for their development and in order to replicate their DNA they use either the replicative machinery of the host or encode their own replication proteins. Bacteriophages M13 and ϕX174 of *E. coli* rely almost completely on the machinery of the host cell to replicate whereas bacteriophage T4 encodes most if not all of the proteins directly involved in the replication of its genome. Similarly in the mammalian cell, simian virus 40 (SV40) encodes only one of the proteins required for the replication of its genome and thus relies on the host machinery whereas HSV-1 appears likely to encode most of the functions directly involved in the replication of viral DNA. By studying the replication of small, well defined viral chromosomes many events involved in the chromosomal replication of their bacterial and mammalian hosts have been illuminated.

Proteins involved in replication of the *E. coli* chromosome have been studied most extensively and have been used to reconstitute synthesis of *E. coli* DNA *in vitro* (Kornberg, 1980). Replication of SV40 DNA has also been well studied and origin-dependent DNA synthesis has recently been achieved *in vitro* using purified proteins (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990). Biochemical and functional characterization of these proteins has identified their specific roles in DNA synthesis, some of which are described briefly in the following sections.

DNA Synthesis Initiator Proteins

Replication of the *E. coli* chromosome is initiated at a unique site termed *oriC* which includes all the necessary *cis*-acting sequences within a 245bp core region and is highly conserved amongst Enterobacteriaceae (Oka *et al.*, 1980; Zyskind *et al.*, 1983). Mutational analyses have revealed two types of sequence element within *oriC* which have essential roles in the initiation of DNA synthesis, namely four 9bp tandem repeats towards the right end and three 13bp A-T rich tandem repeats at the left end of *oriC* (**Figure 6a**; Oka *et al.*, 1984).

The nonamer elements are recognised and co-operatively bound by *E. coli* initiator protein, dnaA and are therefore termed 'dnaA boxes' (Fuller *et al.*, 1984; Kaguni and Kornberg, 1984). Electron microscopy and DNase I footprinting studies have demonstrated the formation of an initial dnaA-*oriC* complex where the negatively supercoiled *oriC* element is wrapped around a central core of 20-40 dnaA protein monomers (Fuller and Kornberg, 1983; Funnell *et al.*, 1987). Supercoiling of *oriC* is a requirement for initiation of replication (Funnell *et al.*, 1986) and confers increased affinity for dnaA protein (Fuller and Kornberg, 1983). Following recognition by dnaA protein, the 13mers are melted sequentially to form the open complex. This process requires hydrolysis of ATP to ADP by dnaA protein although dATP or CTP may serve as substitute substrates (Bramhill and Kornberg, 1988a; b). Formation of the open complex is stimulated by HU histone-like protein (Dixon and Kornberg, 1983; Ogawa *et al.*, 1985). Subsequently dnaB-C protein complex is directed into the melted region by dnaA protein to bidirectionally extend the replication bubble and facilitate formation of a pre-priming complex.

Replication of the SV40 genome is also initiated at a single origin and proceeds bidirectionally. The viral genome comprises 5.2 kbp of circular, double-stranded DNA which is complexed with histones to form a minichromosome essentially indistinguishable in structure from host chromatin. The only virally encoded gene product required for SV40 origin-dependent replication is T Antigen; a multifunctional phosphoprotein of M_r 82,000. SV40 therefore depends upon the host cell replicative machinery for synthesis of its genome (Tegtmeyer, 1972, Stillman, 1989).

The SV40 origin comprises a 64bp core region containing all

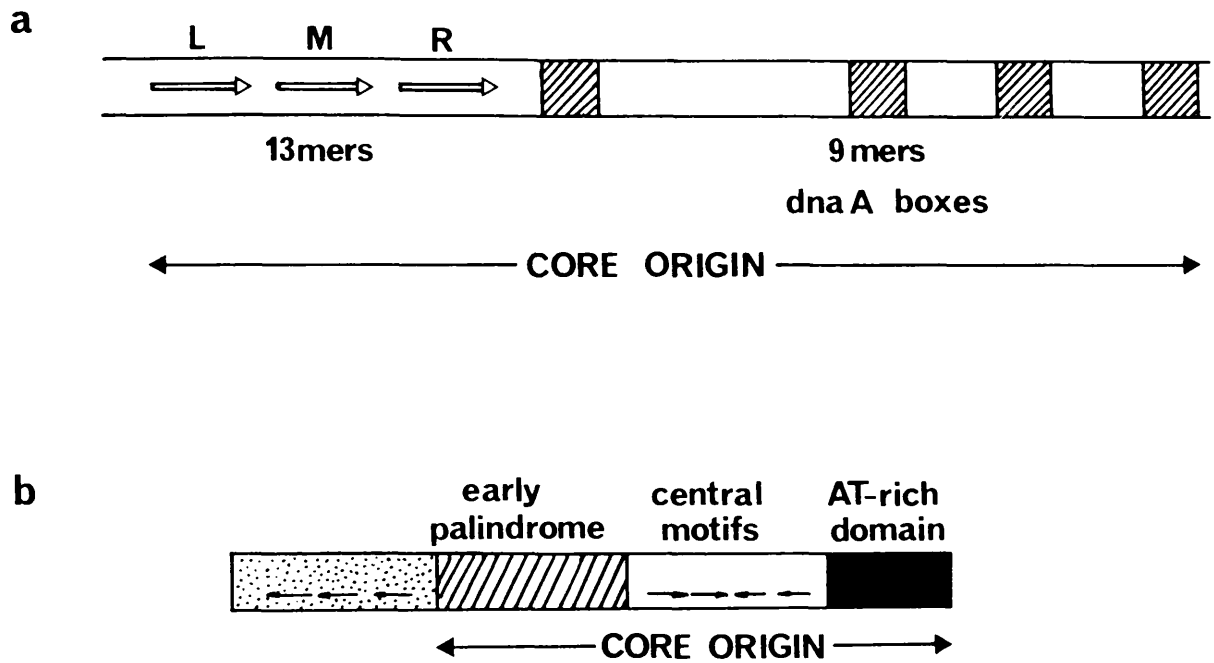


Figure 6. Organization of Origins of Replication in *E. coli* and SV40

Minimal origin sequences are indicated by the large, solid arrows. (a)*E. coli oriC* contains three 13 mer elements (L, M and R), shown as open arrows, within an AT-rich region. Hatched boxes represent 9mer motifs which are bound by dnaA protein.

(b)The SV40 core origin contains three domains as indicated. T antigen binding sites (GAGGC motifs) are indicated by the small, solid arrows within the central domain and also to the left of the early palindrome.

Minimal origins are schematically represented. *E coli oriC* and SV40 core origin comprise 245 bp and 64 bp respectively.

necessary *cis*-acting elements required for the replication of viral DNA *in vitro* (**Figure 6b**; Deb *et al.*, 1986a; Stillman *et al.*, 1985; Li *et al.*, 1986; Smale and Tjian, 1986; Dean *et al.*, 1987a). Genetic and mutational analyses have identified three functionally distinct domains within the core origin. The relative spacing of these domains as well as their sequence is critical to their function (Deb *et al.*, 1986a). The central domain of the core contains four copies of the motif $^{5'}$GAGGC$^{3'}$ arranged within a 27bp inverted repeat. These motifs are recognised by T antigen and each is bound by a single T antigen monomer (Tjian, 1978; DeLucia *et al.*, 1983; Mastrangelo *et al.*, 1985). Flanking the central domain are an imperfect palindromic region, termed the early palindrome, and a 17bp AT-rich domain. Additional flanking sequences include an additional T antigen binding site which increases the efficiency of replication approximately 2-fold both *in vivo* and *in vitro* (Stillman *et al.*, 1985; DeLucia *et al.*, 1986; Li *et al.*, 1986). Adjacent *cis*-acting transcriptional enhancer elements and sequences recognised by cellular transcription factors appear to have less effect on replication *in vitro*. However, although transcription *per se* is not required *in vivo*, efficiency of initiation may be increased by perturbing local chromatin structure (Stillman *et al.*, 1985; Li *et al.*, 1986; Smale and Tjian, 1986; DePamphillis, 1988). T antigen functions as an initiator protein and a DNA helicase in the replication of SV40 DNA

(Stahl *et al.*, 1986). In the presence of ATP, T antigen binds the central pentamer motifs to form a multimeric structure in which 12 T antigen monomers surround the entire core region as a double hexamer (Dean *et al.*, 1987c; Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988; Mastrangelo *et al.*, 1989). Local melting of the palindromic region and deformation of the AT-rich domain then follows. The AT-rich domain has been proposed as a centre for DNA bending, its sequence being critical to its conformation (Deb *et al.*, 1986b), and in addition, this domain has been shown to undergo structural alterations following sequence specific binding of T antigen to $^{5'}$GAGGC$^{3'}$motifs (Borowiec and Hurwitz, 1988). Cellular proteins have been reported to bind the early palindrome and the AT-rich region and conformational changes have been demonstrated within the AT-rich domain. However, roles for these proteins in the replication of SV40 DNA, if any, have not been established (Baur and Knippers, 1988; Borowiec and Hurwitz, 1988; Traut and Fanning, 1988; Fanning *et al.*, 1988). In the presence of ATP, single-stranded DNA-binding and topoisomerase activities, T antigen may then be loaded

into the open complex and its DNA helicase activity activated for bidirectional unwinding.

2. Extending the Replication Bubble and Priming of Nascent DNA Strands

(a) DNA-Strand Unwinding by DNA helicase

Unwinding of duplex DNA is a prerequisite for DNA synthesis. Following initial strand separation DNA helicases extend the replication bubble in an energy-dependent reaction where hydrolysis of 5' nucleoside triphosphate is coupled to enzymatic unwinding of duplex DNA. DNA strand unwinding at an origin of replication or at an advancing replication fork may be mediated by distinct helicases or a single enzyme (Geider and Hoffman-Berling, 1981). Displacement of the strands provides a template for DNA primase to synthesize RNA primers which can subsequently be elongated by DNA polymerase.

The *E. coli* chromosome encodes at least eight DNA helicases, each of which has a specialized role in DNA replication, repair or recombination. At least three DNA helicases, namely Rep, dnaB and n' proteins are present at the *E. coli* replication fork. DnaB is regarded as the principal replicative helicase involved in propagation of the fork (reviewed by Matson and Kaiser-Rogers, 1990). DnaB and n' protein are components of the *E. coli* primosome, located on the lagging strand template (Kornberg, 1988).

A complex of dnaB-dnaC proteins is directed into the open complex by dnaA protein following separation of the DNA strands at *oriC*. The DNA helicase activity of dnaB protein then unwinds duplex DNA in both directions (Bramhill and Kornberg, 1988a; b). Unwinding of partial duplex DNA substrates by dnaB helicase is dependent upon the presence a 3' single-stranded tail on the fragment to be displaced and can be stimulated either by *E. coli* single-stranded DNA-binding protein (SSB) or by SSB and dnaG primase (LeBowitz and McMacken, 1986). This suggests a 5'-3' polarity in activity, compatible with advancement of the primosome at the replication fork. These observations suggest that in addition to unwinding *oriC*, dnaB is active at the replication fork and translocates along the lagging strand, displacing strands which are subsequently maintained in an extended conformation, presumably by SSB.

E. coli n' protein is also a DNA helicase but acts in a 3'-5' direction, opposing movement of the primosome. Activity is dependent

upon the presence of SSB (Lee and Marians, 1987). Helicase activities of dnaB and n' proteins have been proposed to be coupled at the replication fork with n' protein acting as a 'DNA translocase', pumping DNA through the complex of replication proteins to aid overall movement of the primosome and extending the template DNA into a configuration suitable for copying by DNA polymerase (Matson and Kaiser-Rogers, 1990).

The *E. coli* Rep protein also has DNA helicase activity with 3'-5' polarity, again dependent upon SSB. The protein is proposed to bind to the leading strand and assist dnaB protein in unwinding duplex DNA ahead of the advancing replication fork (Yarranton and Gefter, 1979). In addition to Rep protein, *E. coli* DNA helicase II may also be involved in this unwinding (Kornberg, 1988).

In the replication of SV40 DNA, T antigen, in addition to its role in origin recognition and initial strand separation, has been shown to act as a DNA helicase (Stahl *et al*, 1986) and as such is also involved in progression of the SV40 replication fork (Hurwitz *et al.*, 1990).

OBP of HSV-1 also exhibits DNA helicase activity (Bruckner *et al.*, 1991; This Thesis). However, the relationship of this activity to the mechanism of HSV-1 DNA synthesis awaits further study. DNA helicase activity of the HSV-1 helicase-primase complex requires the presence of a 3' single-stranded tail on the fragment to be displaced from a partial duplex substrate (Crute *et al.*, 1988) suggesting 5'-3' translocation along the lagging strand template at the advancing HSV-1 replication fork.

(b) Priming of Nascent DNA Strands

Nascent DNA strands are most commonly initiated by an RNA primer, generally 4-12 nucleotides in length, which is usually synthesized specifically by a DNA primase (Kornberg, 1988). Deoxyribonucleotides may also be accepted into synthesis of a primer by some primases. Alternatively, an RNA polymerase transcript may serve as a primer for DNA synthesis. Indeed RNA polymerase transcription is essential for priming DNA synthesis initiated at primary origins of replication of bacteriophage T4 (Mosig, 1987). The leading strand should require priming only once, at the initiation of its synthesis whereas the lagging strand will require multiple priming. Once synthesized, primers are elongated by DNA polymerase.

A distinct mechanism of priming DNA synthesis which does not involve synthesis of an RNA primer is employed by adenovirus.

Cytosine residues at the 5' ends of the linear viral genome are covalently linked to adenovirus terminal protein. During initiation of replication the viral DNA polymerase covalently bonds dCMP (derived from dCTP) to terminal protein in a template -dependent reaction. Subsequently, the 3' OH of the protein bound C residue acts as a primer for unidirectional extension by DNA polymerase as the template strand is copied. Synthesis of each strand of the viral genome is therefore initiated and proceeds separately from opposite termini (Nagata *et al.*, 1983a;b). A similar mechanism is also used by bacteriophage ϕ29 of *B. subtillis.*

In SV40, the positions of primers synthesized during DNA replication have been mapped and this has allowed the precise definition of synthetic events occuring at the viral origin of replication (Hay and DePamphilis, 1982). Following opening of the origin, synthesis of the leading strands is primed in opposing directions. Bidirectional m ovement of the replication forks exposes the lagging strand templates and Okazaki fragments are primed and extended towards the origin. The origin of replication therefore corresponds to a transition point between continuous and discontinuous synthesis of each of the two daughter strands.

(c) Coupling of DNA Primase with Other Enzymatic Activities

DNA helicase and DNA primase activities are frequently coupled in prokaryotes (Matson and Kaiser-Rogers, 1990). The *E. coli* primosome comprises a multi-subunit complex of replication proteins, including dnaB and n' proteins and dnaG primase, which assembles at specific sites on single-stranded DNA (**Figure 7**). A role has been proposed for the n' protein in directing primosome assembly at these sites (Abarzua *et al.*, 1984; Soeller *et al.*, 1984). In addition to its DNA helicase function, dnaB protein may direct *E. coli* dnaG primase towards the replication fork for synthesis of RNA primers (Baker *et al.*, 1986; 1987).

DNA helicase and DNA primase activities are also coupled in bacteriophages T4 and T7. Duplex DNA at the T4 replication fork is unwound in an energy dependent reaction by gene 41 helicase moving 5'-3' along the lagging strand template (Liu and Alberts, 1981, Venkatesan *et al.*, 1982). In addition to its function as a DNA helicase, gene 41 protein complexes with gene 61 protein to form the T4 primosome. In the absence of gene 41 helicase, primer synthesis by gene 61 primase is greatly reduced and those primers which are

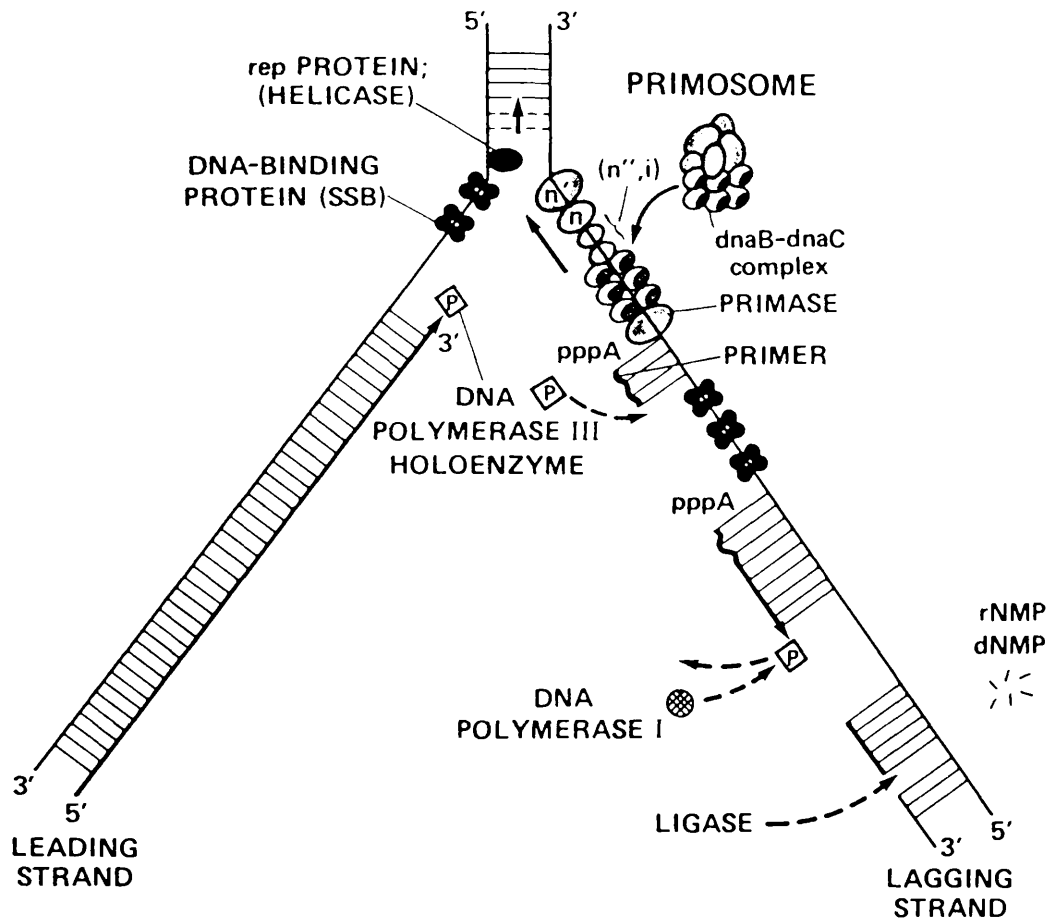


Figure 7. The *E. coli* DNA Replication Fork

The schematic diagram shows enzymes involved in DNA synthesis initiated from *E. coli oriC*. Replication is continuous for one strand (leading) and discontinuous for the other (lagging) (reproduced from Kornberg, 1982).

synthesized fail to be elongated. Hence, both gene 41 and gene 61 proteins are required for functional primer synthesis (Nossal and Alberts, 1983).

In bacteriophage T7, DNA helicase and DNA primase activities are properties of a single polypeptide, namely gene 4 protein (G4P). Two forms of this protein exist, of M_r 56,000 and 63,000. Both complex with T7 DNA polymerase, the smaller form acting as a processive DNA helicase in a 5'-3' direction and stimulating synthesis of RNA primers by the larger form (Nakai and Richardson, 1988; Bernstein and Richardson, 1988a;b;1989).

Coupling of DNA helicase and DNA primase activities in prokaryotes appears striking and ensures co-ordinated synthesis of leading and lagging strands. Results presented in this thesis and reported recently (Calder and Stow, 1990; Dodson and Lehman, 1991) suggest that this phenomenon exists in HSV-1. Whether it extends to include other eukaryotes awaits the isolation of those enzymes. The replication of SV40 DNA *in vitro* requires mammalian DNA polymerase α for lagging strand synthesis. POL α, which is not highly processive, is associated with a DNA primase activity. This coupling co-ordinates primase and polymerase functions.

3. Elongation of Nascent DNA Strands

(a) DNA Polymerases

E. coli

Once synthesized, RNA primers are subsequently elongated by DNA polymerase. Elongation of both leading and lagging strands initiated at *oriC in vitro* requires more than 20 *E. coli* proteins. RNA primers synthesised by the primase subunit of the primosome are elongated into nascent DNA strands by DNA polymerase III holoenzyme, continuously on the leading strand template and discontinuously on the lagging strand template. Replication of both strands has been proposed to be carried out simultaneously by a replisome which includes a single dimeric DNA polymerase associated with the primosome and one or more helicases (Kornberg, 1980; 1982; 1988; **Figure 8**). In a scheme of concurrent replication, leading strand synthesis must always be ahead of that of the lagging strand. Looping the lagging strand template 180° (perhaps around a DNA polymerase molecule) achieves 3'-5' orientation of both strands at the replication fork. A primer generated on the lagging strand can be extended by

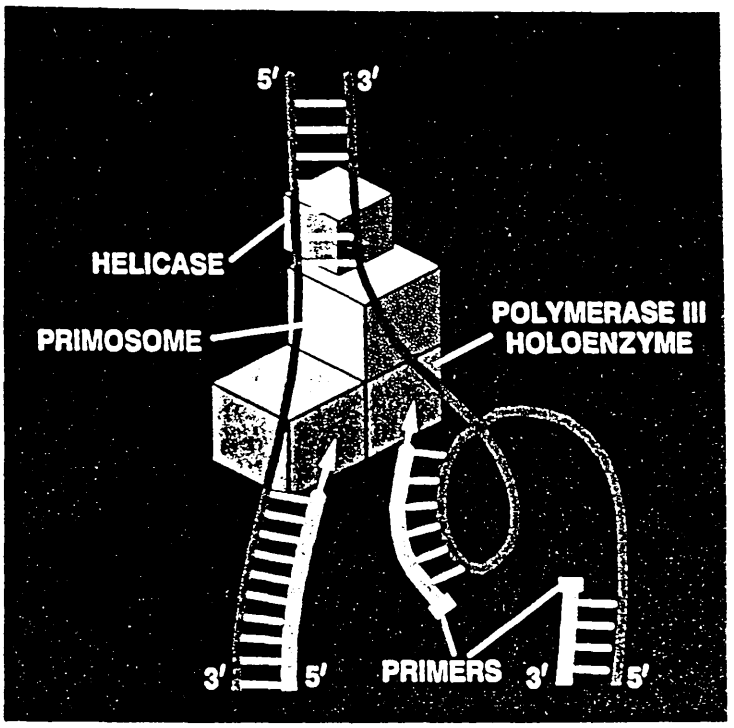


Figure 8. Concurrent Replication of Leading and Lagging Strands in E. coli

The figure represents a scheme for concurrent replication of both template strands by a dimeric polymerase associated with primase and helicase activities (reproduced from Kornberg, 1982). Note that looping of the lagging strand allows synthesis of both nascent strands to occurr in the same direction as the advancing replication fork.

DNA polymerase until synthesis approaches the 5' end of the previous Okazaki fragment (approximately 1000 nucleotides) after which the template strand is released and unlooped. Continued synthesis of the leading strand has meanwhile generated a fresh template for synthesis of the next Okazaki fragment on the lagging strand. Movement of the primosome can therefore occur in the same direction as elongation of the leading strand allowing concurrent leading and lagging strand synthesis and co-ordination of polymerase and primosome functions.

SV40

The development of a cell-free system for SV40 origin-dependent DNA replication demonstrated the absolute requirement for T antigen (Li and Kelly, 1984; 1985; Stillman and Gluzman, 1985; Wobbe *et al.*, 1985). Fractionation and purification of mammalian cell extracts has now identified seven cellular factors which, together with T antigen, are necessary and sufficient for the reconstitution of SV40 origin-dependent DNA replication *in vitro* (Weinberg *et al.*, 1990). These host cellular proteins include replication protein A (RP-A), DNA polymerase α-primase complex (POL α), proliferating cell nuclear antigen (PCNA), replication protein C (RP-C), topoisomerase I, the catalytic subunit of protein phosphatase 2A ($PP2A_c$) and DNA polymerase *δ* (POL *δ*).

Two mammalian DNA polymerases are required for the synthesis of leading and lagging strands, namely POL *δ* and POL α (Prelich *et al.*, 1987; Decker *et al.*, 1987; Wold *et al.*, 1988; Murakami *et al.*, 1986b). POL α, which is not highly processive (Downey *et al.*, 1988; Hohn and Grosse, 1987), is composed of four polypeptide subunits M_r180,000, 70,000, 58,000 and 48,000. The largest subunit contains the 5'-3' DNA polymerase active site and the two smallest subunits form a DNA primase enzyme (Fry and Loeb, 1986; Campbell, 1986). POL α specifically interacts with SV40 T antigen (Smale and Tjian, 1986; Gannon and Lane, 1987) and through this interaction may determine the host specificity of the virus (Murakami *et al.*, 1986). In addition to 5'-3' DNA polymerase activity, POL *δ* possesses 3'-5' exonuclease activity, which provides a proof-reading function, but lacks DNA primase activity (Byrnes *et al.*, 1976; Lee *et al.*, 1980; 1984; Byrnes, 1985; Crute et *al.*, 1986; So and Downey, 1988). A mammalian protein M_r 37,000, known as PCNA, has been identified as a factor which stimulates catalysis of DNA synthesis by POL *δ* but not POL α (Prelich *et al.*, 1987a;b; Bravo *et al.*, 1987; Downey *et al.*, 1988). POL *δ* and PCNA together form a highly processive DNA polymerase (Tan *et al.*,

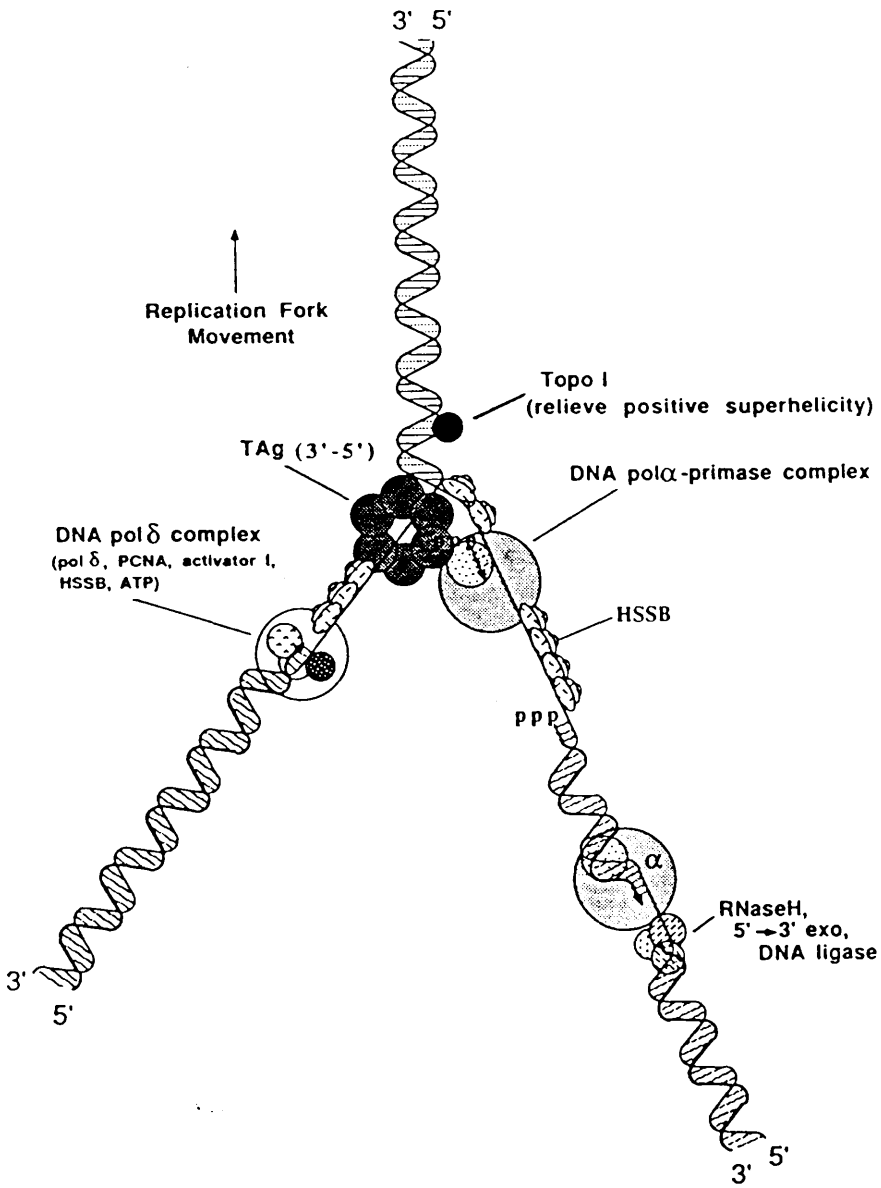


Figure 9. The SV40 DNA Replication Fork

The diagram shows the enzymes involved in the replication of SV40 DNA and their relative position at the advancing replication fork. HSSB and Activator 1 are also known as RP-A and RP-C respectively (reproduced from Hurwitz *et al.*, 1990).

1986; Downey *et al.*, 1988). PCNA is required for efficient DNA replication *in vitro* since in the absence of PCNA only aberrant lagging strands are synthesised (Prelich *et al.*, 1987a; Prelich and Stillman, 1988). PCNA has been shown to localise within the cell to sites at which DNA synthesis occurs (Bravo and Macdonald-Bravo, 1985; Madsen and Celis, 1985).

A two polymerase model has been proposed for the replication of SV40 DNA. Nascent strands are primed on both template strands by POL α. The lagging strand continues to be elongated by POL α whereas leading strand synthesis is catalyzed by POL *δ*-PCNA complex (Tsurimoto and Stillman, 1989a;b; Hurwitz *et al.*, 1990). Proteins expected to be present at the SV40 replication fork are shown in **Figure 9.**

Models for the concurrent synthesis of leading and lagging strands have been proposed by Stillman (1989; **Figure 10**), and Hurwitz *et al.* (1990; **Figure 11**) based on mechanisms previously proposed for *E. coli* (Kornberg, 1982; 1988) and bacteriophage T4 (Nossal and Alberts, 1983). The rate of fork movement is determined by the DNA helicase activity of T antigen. Displacement of the lagging strand template allows POL *δ*, PCNA and RP-C protein complex access to the leading strand template. Meanwhile, the lagging strand either becomes wrapped around a DNA polymerase molecule (**Figure 10**) or is maintained in extended configuration by RP-A (HSSB) (**Figure 11**). At the origin, POL α bound to T antigen synthesises primers on both template strands. The leading strand is elongated continuously by POL *δ* whilst primers on the lagging strand template are elongated by POL α until the 3' end of the nascent DNA strand is proximal to the 5' end of the preceding Okazaki fragment at which point POL α is released and its DNA primase activity reactivated.

The experimental evidence to date suggests that replication of HSV-1 DNA requires only the single viral DNA polymerase. It is not known whether concomitant synthesis of leading and lagging HSV-1 DNA strands actually occurs. If so, it may involve dimerization of the polymerase. With the development of a cell-free system for HSV-1 DNA synthesis this question may be more easily be addressed.

(b) DNA Polymerase Accessory Proteins

Proteins which supply accessory functions to the DNA polymerase form part of the multi-subunit complex at the replication fork. In general, these proteins, together with the catalytic subunit(s), form

Figure 10. **Model I: Concurrent Replication of Leading and Lagging Strands in SV40**

The diagram illustrates a proposed model (Stillman, 1989) of a eukaryotic DNA replication fork, based on that previously suggested for E.coli. Two DNA polymerases, α and *δ*, form a complex with their accessory proteins to respectively synthesize lagging and leading strands. Looping of the lagging strand template around a polymerase molecule is proposed. SV40 T antigen appears to act as a DNA helicase to advance the replication fork.

Figure 11. **Model II: Concurrent Replication of Leading and Lagging Strands in SV40**

The figure illustrates a proposed model (Hurwitz *et al.*, 1990) of concurrent synthesis of SV40 DNA strands, based on that proposed for bacteriophage T4. With the aid of accessory proteins, DNA polymerases α and *δ* respectively synthesize lagging and leading strands. Polymerase and primase functions are co-ordinated by looping of the lagging strand template. DNA-strand unwinding at the replication fork may be performed by T Antigen.

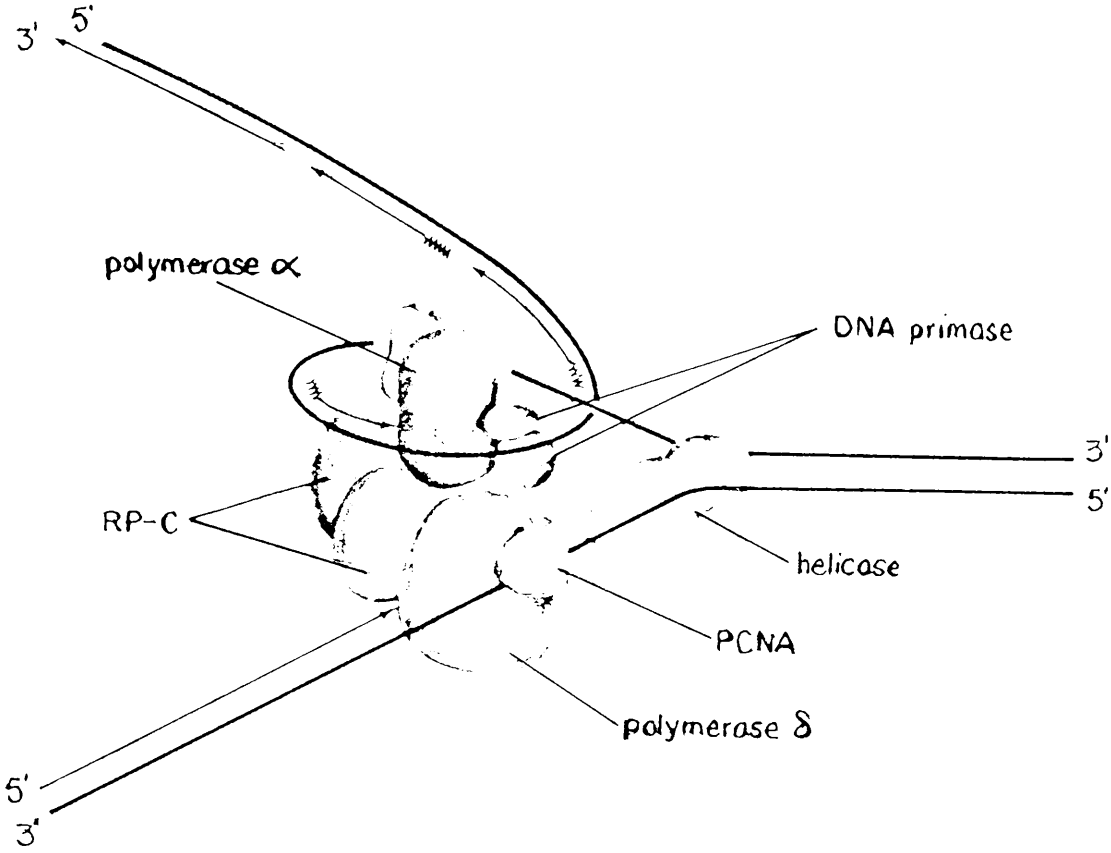


Figure 10

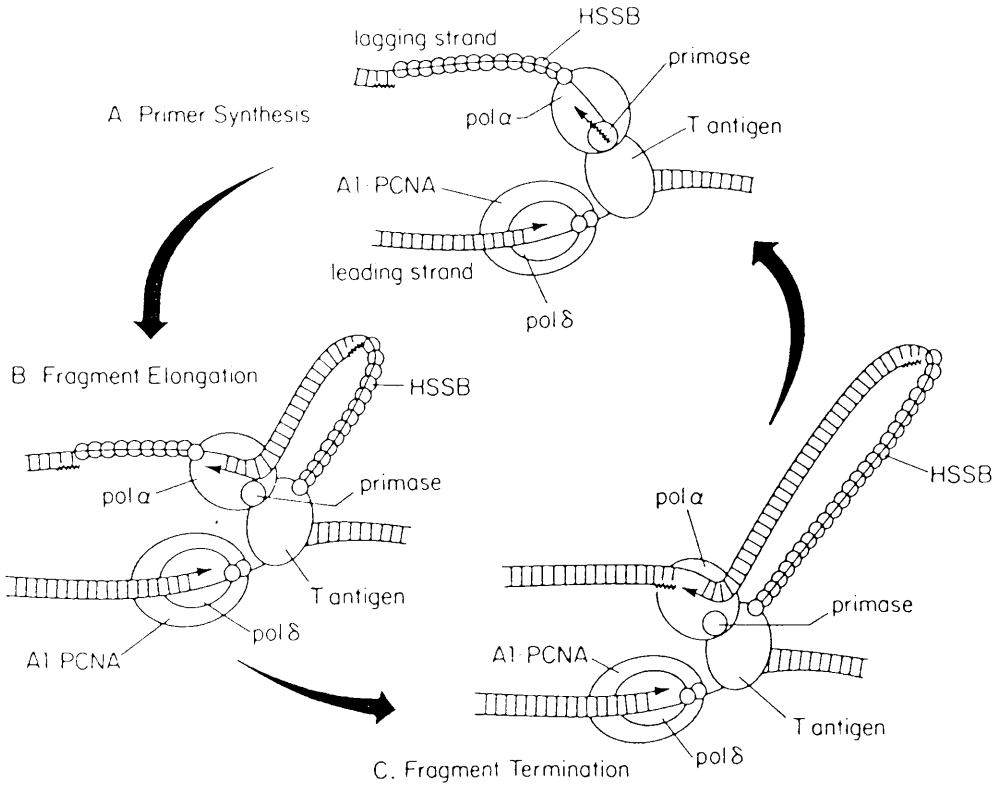


Figure 11

the DNA polymerase holoenzyme. The accessory proteins generally conferring high processivity upon catalytic activity (Kornberg, 1988).

The β subunit (M_r 37,000) of the *E. coli* DNA polymerase III holoenzyme is required for highly processive activity. One β subunit is present in each catalytic core of the asymmetric polymerase dimer. Numerous auxiliary subunits may associate with one or other catalytic core to facilitate either continuous synthesis on one strand or discontinuous on the other (Kornberg, 1988).

Three T4 proteins supply accessory functions to the bacteriophage DNA polymerase, namely gene 44, 45 and 62 proteins. When complexed with the catalytic subunit (gene 43 protein) these proteins increase processivity of the polymerase in an ATP dependent manner by acting as a 'clamp' to prevent dissociation of the polymerase from the DNA template (Huang *et al.*, 1981; Mace and Alberts, 1984). Together all four proteins form the highly processive T4 DNA polymerase holoenzyme (Jarvis *et al.*, 1989a;b; Capson *et al.*, 1991).

Replication of SV40 DNA *in vitro* requires the cellular factor PCNA for highly processive leading strand synthesis by mammalian DNA polymerase *δ* (Tan *et al.*, 1986; Downey *et al.*, 1988). A second accessory factor, RP-C, is also required for *in vitro* DNA synthesis. This factor has been shown to bind preferentially to primer ends and to increase the affinity of POL *δ* for such ends by at least 10-fold. Lack of PCNA and/or RP-C results not only in a loss of leading strand synthesis but also aberrant lagging strand synthesis suggesting that replication of both strands is co-ordinated (Prelich *et al.*, 1987a; Lee *et al.*, 1988; Prelich and Stillman, 1988; Tsurimoto and Stillman, 1989a;b).

As described above (section 1C.3(a)), the HSV-1 UL42 gene product, which is essential for replication of viral DNA, has been shown to act as an accessory protein, conferring increased processivity upon the viral DNA polymerase (Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990).

(c) Single-Stranded DNA Binding Proteins

Following initial strand separation and extension of the replication bubble by DNA helicases the displaced strands are maintained in an extended conformation by single-stranded DNA binding proteins. This function, which is essential for DNA synthesis, provides a template for DNA primase and DNA polymerase.

E. coli single-stranded DNA binding protein (SSB) is essential for

replication of the *E. coli* chromosome and is proposed to bind the leading and lagging strand template strands ahead of the advancing DNA polymerase at the replication fork (Kornberg, 1980; 1982; 1988).

T4-encoded single-stranded DNA binding protein, gene 32 protein, is also essential for replication of the bacteriophage DNA *in vitro* and *in vivo* (Nossal and Alberts, 1983). Gene 32 protein possesses a helix-destabilizing activity which melts secondary structures formed by single-stranded DNA. Removal of these structures ahead of the advancing DNA polymerase stimulates processivity of DNA synthesis. Gene 32 protein, by binding single-stranded DNA, is also proposed to stabilize the T4 replication fork, providing a loading site for gene 41 DNA helicase and a conformation of DNA compatible with concurrent leading and lagging strand synthesis by a dimeric DNA polymerase (Nossal and Alberts, 1983; Alberts, 1984). Protein:protein interactions have been demonstrated between gene 32 protein and at least 10 other T4 proteins which have roles in DNA replication and recombination (Formosa *et al.*, 1983).

In vitro synthesis of SV40 DNA requires the single-stranded DNA-binding activity of RP-A (Human single-stranded DNA-binding protein), a complex of three polypeptide subunits M_r 70,000, 32,000 and 14,000 (Wold and Kelly, 1988, Fairman and Stillman, 1988). RP-A is proposed to bind single-stranded DNA following unwinding of the duplex and establishment of the SV40 replication fork (Hurwitz *et al.*, 1990).

Major single-stranded DNA-binding protein (mDBP), encoded by HSV-1 gene UL29, is essential for the replication of viral DNA and is probably involved in stability of the HSV-1 replication fork. mDBP has been shown to stimulate processivity of the viral DNA polymerase (Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990). It also localizes to regions of viral DNA synthesis within the infected cell and is required for the co-localization of other HSV replication proteins to these sites (Quinlan *et al.*, 1984; deBruyn Kops and Knipe, 1988). Like T4 gene 32 protein, HSV-1 mDBP therefore appears to perform several functions in DNA synthesis.

(d) Topoisomerases

Positive supercoiling of duplex DNA arises ahead of the advancing replication fork as a consequence of the actions of DNA helicases and single-stranded DNA-binding proteins. Topoisomerases release this torsional stress by decreasing the linking number of the DNA. Type I

topoisomerases nick a single strand, rotate and reseal the duplex to decrease the linking number by one. Type II topoisomerases, also known as gyrases, cut and reseal both strands of the duplex to reduce the linking number by two. Type II topoisomerases are involved in the separation of completely synthesized daughter DNA molecules.

Topoisomerase I activity is absolutely required *in vitro* for the replication of SV40 DNA, presumably to relieve superhelical tension ahead of the proceeding replication fork (Weinberg *et al.*, 1990). However, *in vitro*, SV40 daughter strands were not separated. *In vivo* this is likely to involve topoisomerase II activity (Sundin and Varshavsky, 1980; Weinberg *et al.*, 1990).

Topoisomerases are also likely to be involved in the replication of HSV-1 DNA. Induction of topoisomerase I activity in HSV-1 infected cells has been demonstrated (Muller *et al.*, 1985) and a role for host topoisomerase II in the replication of HSV-1 DNA has been proposed (Ebert *et al.*, 1990). However, whether the virus encodes topoisomerase activity has not been conclusively established.

(e) Completion of DNA Synthesis

DNA synthesis is completed when two replication forks moving in opposite directions meet. Alternatively, synthesis may be halted at a specific termination sequence, as in *E. coli*.

To complete replication of the progeny strands degradation of ribonucleotide primers, subsequent filling-in of gaps by DNA polymerase and ligation of DNA fragments is required. Enzymes likely to be involved in such events include RNase H and/or 5'-3' exonuclease, for removal of nucleotides, and a DNA ligase. Some or all of these activities have been included in the proposed models for *E. coli* and SV40 DNA replication (**Figures 7 and 9**; Kornberg, 1988; Hurwitz *et al.*, 1990). These events are likely to be closely linked to the advancing synthetic machinery at the replication fork.

RNase H activity is an intrinsic property of HSV-1 DNA polymerase (Crute and Lehman, 1989) which is likely to be involved in removal of ribonucleotides which prime synthesis of the lagging strand. However, a virally encoded DNA ligase has not been identified.

In order that complete molecules may be duplicated they may be replicated in a circular form (e.g. SV40), as concatemers, primed by a terminal protein, or, in the case of linear chromosomes, contain telomeric ends which are synthesized by a specific telomerase. The

final processes for circular molecules replicated as theta forms involves separation of the two daughter molecules, and for concatemers produced by a rolling circle mechanism, cleavage into unit length molecules.

4. Rolling Circle Replication

The bulk of HSV-1 DNA synthesis is thought to proceed by a rolling circle mechanism since newly synthesized viral DNA appears to exist as long head-to-tail tandem concatemers (Section 1B.4; Jongeneel and Bachenheimer, 1981; Jacob *et al.*, 1979). In a rolling circle mechanism a double-stranded circularized genome may be specifically nicked on one strand. The nicked strand is then displaced by DNA helicase and maintained in an extended conformation by single-stranded DNA-binding protein. Further displacement provides a template for DNA primase and DNA polymerase. Subsequent assembly of replication proteins on the template DNA strands establishes a replication fork which is continuously and unidirectionally propagated such that head-to-tail concatemers are synthesized (**Figure 12**). Replication forks established in this way are otherwise indistinguishable from those established at a bi-directional origin of replication such as occurs in the case of SV40.

Many of the proteins involved in the replication of *E. coli* and bacteriophage DNA have been isolated and characterized *in vitro* with the aid of nicked circular DNAs produced experimentally by DNase treatment. Replication of the genome of bacteriophage ϕX174 by a rolling-circle mechanism has been demonstrated using *E. coli* DNA replication proteins (reviewed by Kornberg, 1982). Such templates may also prove useful in the structural and functional analysis of the HSV-1 replication fork.

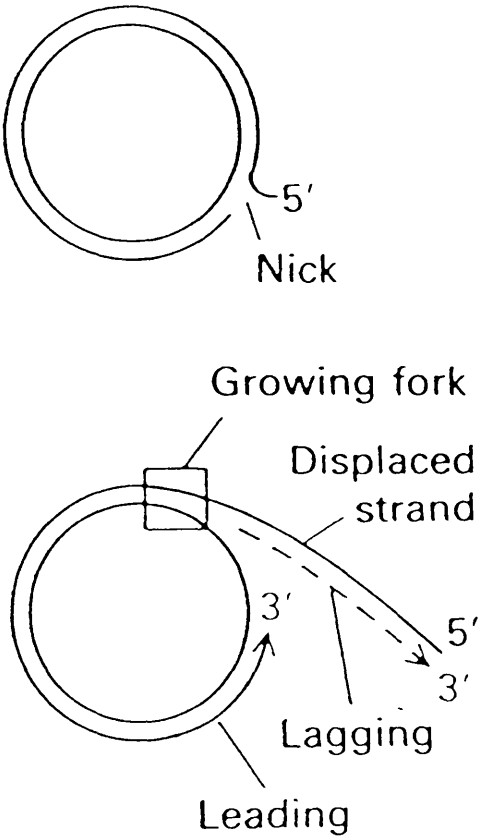


Figure 12. Rolling-Circle Replication

In a rolling-circle mechanism a double-stranded circular DNA molecule may be replicated as a concatemer to produce unit length molecules joined head-to-tail. A specific nick in one strand provides a loading site for DNA helicase and subsequently a template for DNA primase and DNA polymerase.

2A. MATERIALS

1. Reagents

Most analytical grade chemicals were purchased from either BDH Ltd. or Sigma Chemical Co. Ltd.. Exceptions to this included:

Beecham Research Labs. Ltd.	-ampicillin
Bio-Rad Labs. Ltd.	-ammonium persulphate
	-TEMED (N,N,N',N',tetramethyl-enediamine)
Du Pont Ltd.	-En^{3}Hance autoradiography enhancer
Fluka Chemicals Ltd.	-HPLC grade dimethylsulphoxide
	-formaldehyde
James Burrough (FAD) Ltd.	-absolute alcohol 100
Koch Light Labs.	-boric acid
	-caesium chloride
May and Baker Ltd.	-acetic acid (glacial)
	-chloroform
	-glycerol
	-hydrochloric acid
	-methanol
Pharmacia LKB Ltd.	-DEAE Sephacel
	-ficoll 400
	-2'deoxyribonucleoside 5'triphosphates
	-ribonucleoside 5'triphosphates
	-poly (dT)
	-d(N)$_6$ random polymer

2. Miscellaneous Materials

Miscellaneous materials and their suppliers are indicated as follows:

Bethesda Research Labs.	-nuclease-free BSA solution
	-Immunoprecipitin (suspension of fixed *Staphylococcus aureus*, for precipitation of immunoglobulin).
Bio-Rad Labs Ltd.	-Bio-gel A1.5m agarose bead suspension
	-Protein assay dye reagent
Kodak Ltd.	-XS1 autoradiographic film
	-TriX pan 400 35mm film
Medicell International Ltd.	-dialysis membrane
New England Biolabs	-restriction enzyme linker-oligonuclotides
	-M13mp18 ssDNA
	-universal primer, 17mer
Pierce	-Protein micro-assay solutions
Promega Biotech	-immunoblot qualified BSA
Schleicher and Schuell	-nitrocellulose membrane
United States Biochemicals	-universal primer, 17mer
Wacker Chemicals	-wacker saline GF 38

3. Solutions

CLB (cell lysis buffer):0.5% SDS, 20mM Tris.HCl (pH 7.5), 2mM EDTA.
Chloroform (nucleic acid extraction): chloroform:isoamyl alcohol, 24:1
50x Denhardt's reagent: 1% ficoll 400, 1% polyvinylpyrrolidine, 1% BSA
Extraction Buffer A: 10mM Hepes.NaOH (pH 7.9), 10mM KCl, 1.5mM $MgCl_2$, 0.5% NP40, 0.5mM DTT, 0.5mM PMSF.
Extraction Buffer C: 20mM Hepes.NaOH (pH 7.9), 25% glycerol,1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 0.5mM PMSF.

Gel soak I: 0.2M NaOH, 0.6M NaCl
Gel soak II: 1M Tris.HCl (pH 7.5), 0.6M NaCl
Giemsa stain: 1.5% Giemsa in glycerol, heated to 50°C for 2hr and diluted with an equal volume of methanol.
HeBS Buffer: 137mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 5,5mM D-glucose, 21mM Hepes (to pH 7.05 with NaOH).
Hybridisation Buffer: 6x SSC, 5x Denhardt's reagent, 0.05% SDS, 50μg/ml denatured calf thymus DNA,20mM Tris.HCl(pH 7.5), 1mM EDTA.
10x Kinase Buffer: 0.5M Tris.HCl(pH 7.6), 100mM $MgCl_2$, 50mM DTT, 1mM EDTA, 1mM spermidine.
5x Ligase Buffer: 250mM Tris.HCl(pH 7.5), 50mM $MgCl_2$,100mM DTT, 5mM ATP, 250μg/ml BSA.
LB(Loening's Buffer): 36mM Tris, 36mM $NaH_2PO_4.2H_2O$, 1mM EDTA.
5x LB gel loading buffer: 50% sucrose, 0.01% BPB in 5x LB.
NTE: 10mM Tris.HCl (pH 7.5), 100mM NaCl, 0.5mM EDTA
PBS A: 170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4 (pH 7.2)
PBS: PBS A supplemented with 6.8mM $CaCl_2$ and 4.9mM $MgCl_2$.
Phosphocellulose column Buffer B: 20mM Hepes.NaOH (pH 7.6), 1mM DTT, 1mM EDTA, 1mM EGTA, 10% glycerol, 0.5mM PMSF, (containing NaCl as indicated).
Prehybridisation Buffer: 6x SSC, 5x Denhardt's reagent, 0.1% SDS, 20μg/ml denatured calf thymus DNA.
RSB: 10mM Tris.HCl (pH 7.5), 10mM KCl, 1.5mM $MgCl_2$, 1mM DTT, 0.5mM PMSF.
SDS-PAGE Buffers;
a)Boiling mix: 30% SGB, 30% glycerol, 15% β-mercapto-ethanol, 6% SDS, 0.03% BPB
sample buffer: one-third dilution of boiling mix
b)4x RGB (resolving gel buffer): 1.5M Tris.HCl (pH 8.9), 0.4% SDS
c)4x SGB (stacking gel buffer): 0.488M Tris.HCl (pH 6.7), 0.4% SDS
d)Tank buffer: 52mM Tris base, 53mM glycine, 0.1% SDS
20x SSC: 3M NaCl, 0.3M trisodium citrate
STET Buffer: 8% sucrose, 0.5% Triton X-100, 10mM Tris.HCl (pH 8), 50mM EDTA.
Sucrose Reagent: 0.25M sucrose, 50mM Tris.HCl (pH 8), 2mM $MgCl_2$
5x Tailing Buffer: 0.5M Potassium Cacodylate (pH 7.2),10mM $CoCl_2$ 1mM DTT
TBE: 90mM Tris base, 89mM boric acid, 1mM EDTA
5xTBE gel loading buffer:50% sucrose, 0.01% BPB in 5xTBE
TBS: 10mM Tris.HCl (pH 8), 150mM NaCl
TBST: TBS supplemented with 0.05% Tween 20
TE: 10mM Tris.HCl (pH 7.5), 1mM EDTA
TM Buffer: 10mM Tris.HCl (pH 7.5), 10mM $MgCl_2$
Tris saline: 25mM Tris, 140mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 1mg/ml dextrose
Trypsin: 0.25% trypsin in Tris saline
Versene: 0.6mM EDTA in PBS A plus 0.02% phenol red

4. Enzymes

Restriction endonucleases and compatible buffers were supplied by Bethesda Research Labs., Northumbria Biologicals Ltd. and Boehringer Mannheim. Exonuclease *Bal*31 and terminal deoxynucleotide transferase were supplied by Bethesda Research Labs. Calf intestinal phosphatase,

T4 DNA ligase, large (Klenow) fragment of DNA polymerase I, DNA polymerase I and proteinase K were obtained from New England Biolabs and T7 DNA Polymerase from Pharmacia LKB Ltd.. DNase, RNase, pronase and lysozyme were purchased from Sigma Chemical Co. Ltd..

5. Radiochemicals

Biochemical radionuclides were obtained from Amersham International plc.

5'[α-^{32}P] deoxyribonucleoside triphosphates	3000Ci/mmol (10μCi/μl)
5'[gamma-^{32}P] adenosine triphosphate	5000Ci/mmol (10μCi/μl)
5'[α-^{35}S] deoxyadenosine thiotriphosphate	1000Ci/mmol (10μCi/μl)
[^{35}S]-L-methionine	800Ci/mmol (15μCi/μl)

6. Antibodies

Anti-peptide antisera which had been raised in rabbits to decapeptides from the carboxyl termini of the HSV-1 genes UL5, UL8, UL9 and UL52 were generously supplied by Dr M D Challberg (Olivo *et al.* 1989).
Horse radish peroxidase (HRP) conjugated swine anti-rabbit immunoglobulin was purchased from Promega Biotech and fluoroscein (FITC) conjugated swine anti-rabbit immunoglobulin from Sigma Chemical Co. Ltd.

7. Cells

BHK 21 clone 13 (BHK) cells, a continuous cell line derived from baby hamster kidney (MacPherson and Stoker, 1962) were routinely used for the growth of *wt* HSV-1, *ts*K and *ts*K recombinant viruses, the preparation of infected cell extracts and immunofluorescence studies.
A continuous line of *Spodoptera frugiperda* cells (*S.f.*; Brown and Faulkner, 1977) was routinely used for the growth of *wt* and recombinant AcNPV and the preparation of infected cell extracts.

8. Tissue Culture Media

Tissue culture media and supplements were supplied by Gibco Ltd.. Plastics were supplied by Gibco Ltd. and Sterilin Ltd..

ETC10: Glasgow modified Eagle's medium (GMEM) (Busby *et al.* 1964), supplemented with 100 units/ml penicillin, 100μg/ml streptomycin, 0.2μg/ml amphotericin, 10% tryptose phosphate broth and 10% new born calf serum.

EC5: GMEM supplemented with antibiotics as above plus 5% new born calf serum.

EC5Hu2: EC5 additionally supplemented with 2% Human serum.

'WASH': GMEM supplemented with antibiotics only, as above.

TC100/5:TC100 medium supplemented with 100units/ml penicillin, 100μg/ml streptomycin and 5% foetal calf serum.

9. Viruses

Herpes simplex virus type 1 (HSV-1) Glasgow strain 17 *syn*$^+$(Brown *et al.* 1973), the wild-type virus used in these studies, and the temperature sensitive mutant, *tsK syn*$^+$ (Marsden *et al.* 1976) which was used in the construction of recombinant viruses, were obtained from institute stocks.

Recombinant baculoviruses were constructed using wild-type *Autographa californica* nuclear polyhydrosis virus (AcNPV) as the parent by Dr N D Stow, from whom both *wt* and recombinant AcNPV viruses were obtained.

10. Bacteria

Plasmids were propagated in *E. coli.* strain DH5 (F^-, recA1, endA1, hsdR17 (r_K^-, m_K^+), SupE44, λ^-, thi-1, gyrA, relA1). Competent DH5 cells ready for transformation were purchased from Bethesda Research Labs..

11. Bacterial Culture Media

Bacteria were grown in L-broth (10g/l Bactopeptone, 5g/l yeast extract (pH 7.5), sterilised by autoclaving). Agar plates contained 1.5% w/v agar in L-broth and where appropriate the medium was supplemented with ampicillin to a final concentration of 50μg/ml.

12. Plasmids

Plasmids were kindly provided by members of the institute as indicated:

p23 (Dr C M Preston). Derived from a plasmid containing the HSV-1 *Bam*HI *p* fragment cloned into the *Bam*HI site of the vector, pAT 153. A 357 bp *Bam*HI-*Hin*dIII fragment specifying the HSV-1 IE3 gene upstream regulatory sequences, promoter and mRNA 5'end was inserted into an unique *Xho*I site created within the TK gene coding region regenerating the unique *Xho*I site at the end of the fragment corresponding to the mRNA leader. Genes were cloned into this *Xho*I site.

pUL59 (Dr N D Stow). Comprises an *Mlu*I fragment of HSV-1 DNA (nucleotides 12127-15162), containing the UL5 gene, cloned as a *Bam*HI fragment (blunt-ended) into the unique, blunt-ended *Xho*I site of p23.

pC6 (Dr N D Stow). Comprises an *Sst*II-*Mst*II fragment of HSV-1 DNA (nucleotides 17850-20488), containing the UL8 gene, cloned as a *Bam*HI fragment (blunt-ended) into the unique, blunt-ended *Xho*I site of p23.

pIE9 (Dr N D Stow). Comprises an *Sst*II-*Nar*I fragment of HSV-1 DNA (nucleotides 20666-23539), containing the UL9 gene, cloned as an *Xho*I fragment into the unique, *Xho* I site of p23.

In these three plasmids, pUL59, pC6 and pIE9, fragments were cloned such that the inserted gene was transcribed, under the control of the IE gene 3 promoter, in the same direction as TK.

pUL522 (Dr N D Stow). Comprises the HSV-1 *Hpa*I *q* fragment (nucleotides 108883-112510), containing the UL52 gene, blunt end ligated into the filled-in *Sal*I site of the vector plasmid pUC8

pUL51 (Dr N D Stow). Comprises the HSV-1 *Mlu*I fragment (nucleotides 12127-15162), containing the UL5 gene, cloned into the vector plasmid pUC8 as a *Bam*HI fragment.

pUL82 (Dr N D Stow). Comprises the HSV-1 *Sst*II-*Mst*II fragment (nucleotides 17850-20488), containing the UL8 gene, cloned into the vector plasmid pUC8 as a *Bam*HI fragment.

p301 (Dr N D Stow). Comprises the HSV-1 *Sst*II-*Nar*I fragment (nucleotides 20666-23539), containing the UL9 gene, cloned into the vector plasmid pUC8 as an *Eco*RI fragment.

p*Bam*p(pGX 153) (Dr N D Stow). Comprises the HSV-1 *Bam*HI *p* fragment cloned into the *Bam*HI site of the vector plasmid pAT 153.

pAT 153 (Twigg and Sherrat, 1980) and **pUC8** (Viera and Messing, 1982) vector plasmids were obtained from Institute stocks.

2B. METHODS

1. Tissue Culture

a. BHK cells were routinely passaged, every 2-3 days, in 850cm^2 roller bottles in ETC10 at 37°C in an atmosphere of 5% CO_2 in air (Macpherson and Stoker 1962). Confluent monolayers were resuspended, after two versene washes and brief trypsinisation (0.25% trypsin), in 20ml of ETC10. Cells remained viable for up to 5 days when stored at 4°C.

b. *S.f.* cells were routinely passaged, every 2-3 days, in 175cm^2 tissue culture flasks in TC100/5 at 28°C in air. Confluent monolayers were dislodged by vigorous shaking into 10ml of TC100/5. When stored at 4°C cells remained viable for at least 24hr.

For long term storage, cells were suspended in medium containing 10% glycerol and 25% foetal calf serum and aliquots frozen in liquid nitrogen. Cells were generally passaged 20-30 times after which fresh stocks were recovered.

2. Preparation of Stocks of Infectious Virus

a. Wild-type HSV-1 and HSV-1 *ts* mutant viruses were propagated in BHK cells. Cell monolayers in roller bottles were infected with virus at a m.o.i. of 0.01 pfu per cell in 40ml EC5. Infected cultures were maintained at 31°C for 3-5 days until an extensive cytopathic effect was exhibited. The cells were shaken into the medium and pelleted at 1500 rpm for 10 min at 4°C (Beckman GPR centrifuge). Cell associated virus (CAV), released into the medium after sonication of the cell pellet in 5ml EC5 per roller bottle, was stored in aliquots at -70°C. Cell released virus (CRV) was pelleted from the supernatant at 12000 rpm for 12hr at 4°C (Sorvall GSA rotor), gently resuspended in EC5 and stored in aliquots at -70°C.

b. Stocks of *wt* and recombinant AcNPV were propagated in *S.f.* cells. Cell monolayers in 175cm^2 tissue culture flasks were infected at a m.o.i. of 4pfu per cell, incubated at room temperature for 1hr and then overlaid with 30ml of TC100/5 and incubated at 28°C for 2-4 days until the cells exhibited an obvious cytopathic effect. The cells were shaken into the medium and pelleted at 1500 rpm for 10 min at 4°C (Beckman GPR centrifuge). Cell released virus in the supernatant was aliquoted and stored at -70°C.

Sterility checks were performed by streaking virus preparations on blood agar plates and incubating at 31°C for 3 days.

3. Titration of virus stocks

a. Stocks of *wt* HSV-1, *ts*K and *ts*K recombinant viruses were titrated on BHK cell monolayers in 50mm or 35mm tissue culture Petri dishes. The growth medium was removed from the plates and 0.1ml aliquots of 10-fold serial dilutions of virus in GMEM wash were inoculated onto the cell monolayers. After 1hr absorption time the cells were overlaid with EC5Hu2 to prevent secondary plaque formation, incubated at the appropriate temperature for 2-3 days then stained by adding an equal volume of Giemsa stain to the medium. After 15 min the stain was washed off and the plaques counted using a dissecting microscope.

b. Stocks of *wt* and recombinant AcNPV were titrated on *S.f.* cell monolayers in 35mm tissue culture Petri dishes. After removal of growth medium these were inoculated with 10-fold serial dilutions of virus in TC100 and incubated at room temperature for 1hr. The inoculum was removed and the cells overlaid with 2ml of a warm mixture (37°C) containing equal volumes of 3% molten low gelling temperature agarose (Sea-Plaque) and TC100/5, followed (when set) by 1ml of liquid TC100/5. The infected cells were incubated at 28°C for 3-4 days and then stained for 24hr with 0.5ml of a solution of 1 part 0.4% Neutral Red plus 24 parts TC100/5. Plaques were counted by inverting the plates on a light box.

4. Preparation of HSV-1 DNA

HSV-1 DNA was prepared essentially as described by Wilkie (1973). Briefly, infected cells were pelleted as described in 2a). Cell-associated virus (CAV) was extracted from the pellet by resuspension in RSB containing 0.5% NP40. Cell-released virus (CRV) was pelleted from the growth medium supernatant. CAV and CRV pellets were resuspended in TE and 1/25 vol. of 250mM EDTA and 1/40 vol. of 20% SDS added to disrupt the particles. Viral DNA was phenol:chloroform extracted then either digested with RNase A+T and precipitated with 2.5 vols. of ethanol or purified by caesium chloride (CsCl) equilibrium gradient centrifugation. Viral DNA (in TE) was stored at -20°C.

5. Preparation of Total Infected Cell DNA

After thoroughly removing the medium from infected BHK cell monolayers were overlaid cell lysis buffer (CLB) containing 0.5mg/ml pronase and incubated at 37°C for 3-5hr. DNA was subsequently phenol:chloroform extracted and precipitated with ethanol. DNA pellets were resuspended in TE (50-200μl) containing RNase A (5μg/ml) and RNase T_1 (50units/ml) and stored at -20°.

6. Transfection of Cells with DNA

Recombinant viruses were generated by co-transfecting BHK cell monolayers in 50mm dishes with viral and plasmid DNA by the calcium phosphate precipitation method (Corsalo and Pearson 1981).
0.5μg of HSV-1 *tsK* DNA, 1μg of plasmid DNA cut with either *Bam*HI (pUL59, pC6, p206) or *Sca*I (pIE9) and 35μg sonicated calf thymus DNA as carrier were mixed with 1ml HeBS buffer (pH 7.05) in 15ml Falcon tubes. After addition of 70μl of 2M $CaCl_2$ to each tube a fine precipitate was apparent. Monolayers from which the growth medium had been removed were inoculated in duplicate with 0.4ml of the calcium phosphate precipitate, incubated 1hr at 31°C and overlaid with 4ml EC5. Following a further 3hr incubation the transfected monolayers were 'boosted' with DMSO (Stow and Wilkie, 1976). Medium was removed from the plates and each was rinsed with GMEM wash. 1ml of 20% DMSO in HeBS buffer was added per plate for 4 min, decanted, and the cells washed again. One set of transfections was finally overlaid with EC5, the other with EC5Hu2 and incubated at 31°C for 3 days. On those plates overlaid with human serum, progeny plaques were counted. From those plates overlaid with EC5 only, recombinant virus progeny were selected and enriched.

7. Selection and Enrichment of HSV-1 *tsK* Recombinant Viruses

The synthetic thymidine analogue 5'bromo-deoxycytidine (BCdR) was used as a selective agent for the growth of TK deficient (recombinant) viruses. Transfected cells were harvested into the growth medium, disrupted by sonication, and the progeny virus titrated on BHK cells in the absence or presence of BCdR (100μg/ml) to allow the proportion of TK deficient virus to be determined. The progeny from the transfected monolayers was then enriched for TK^- virus by two serial passages at low moi (0.005 pfu/cell) in the presence of BCdR and then plaque purified by limiting dilution as described above. Incubations were performed at 31°C throughout. The

structure and purity of the recombinant viruses were confirmed by restriction enzyme analysis.

8. Plaque Purification of *tsK* Recombinant Viruses

Viruses were plaque purified by limiting dilution. Progeny virus was diluted to approximately $2x10^2$ pfu/ml and seven serial 2-fold dilutions made. Twelve 0.1ml samples of each dilution were used to inoculate BHK cell monolayers in micro-titre wells. After 1hr absorption at 31°C each monolayer was overlaid with 0.1ml of EC5 and incubated at 31°C for 3 days. Wells were screened, using a dissecting microscope, for the presence of single virus plaques. The cells and medium of such wells were harvested by resuspension and passaged on BHK cell monolayers in linbro wells. After approximately 3 days at 31°C, when a cytopathic effect was evident, the medium of each well was removed to individual sterile glass vials and stored as virus stock at -70°C. From the infected cell monolayer, total cellular DNA was prepared to allow analysis of viral genome structure.

9. Restriction Enzyme Digestion of DNA

Restriction enzyme digests of DNA were performed using commercial restriction enzymes (1-5 units/μg DNA) and buffers. Usually 1μg of DNA was incubated in a final volume of approximately 40μl for 3-4hr at the recommended temperature.

10. Deletion of Plasmid DNA with Nuclease Bal31

Plasmid pUL522 DNA (20μg) was linearised by digestion with *Hin*dIII in a final volume of 0.5ml for 5hr at 37°C then serially extracted with phenol and chloroform and precipitated with 2.5 vols. of ethanol. Pelleted DNA was lyophilised and resuspended in 180μl H_2O. 45μl of 5xBal31 buffer (100mM Tris.HCl (pH 8), 60mM $MgCl_2$, 60mM $CaCl_2$, 5mM EDTA, 1M NaCl) and 1 unit of nuclease Bal31 were added and a 40μl sample (t_o) immediately removed from the mixture into 350μl of 0.2M EGTA (pH8), vortexed and serially extracted with phenol and chloroform and stored on ice. The remainder of the mixture was incubated at 31°C. Samples were withdrawn at various time points from 2 min to 20 min and treated as above. All samples were then precipitated with 2.5 vols. of ethanol, the pellets lyophilised and resuspended in 22μl of H_2O. 5ul of each sample was digested with *Eco*RI and electrophoresed through a mini-agarose gel to monitor the extent of the deletion.

11. Ligation of DNA to linker oligonucleotides

Bal31 digested plasmid DNA was treated with 1 unit of calf intestinal phosphatase for 1hr at 37°C, to prevent plasmid religation in the absence of linker oligonucleotides, then extracted with phenol and chloroform and precipitated with 2.5 vols. of ethanol. The lyophilised DNA (2.5μg) was then resuspended in 30μl H_2O and recircularized at room temperature overnight in the presence of phosphorylated *Bam*HI 8bp linker molecules in a 40μl reaction containing ligase buffer and 1 unit of T4 DNA ligase.

After ligation, the DNA was phenol and chloroform extracted, ethanol precipitated and the lyophilised pellet resuspended in 20μl H_2O prior to transformation of *E. coli*.

12. Cloning of DNA Fragments

Restriction fragments of DNA containing genes to be expressed in *tsK* were cloned into the unique *Xho*I site of the plasmid p23. as *Bam*HI fragments, except for the UL9 gene which was inserted by direct ligation into the site as an *Xho*I fragment. The inserts were excised from plasmids containing the UL5, UL8 and UL52 genes. 5μg plasmid JC1 DNA, which contains the UL52 gene as a *Bam*HI fragment, was digested with *Bam*HI, phenol and chloroform extracted and precipitated with 2.5 vols. of ethanol. The lyophilised pellet was resuspended in 20μl H_2O to which 2 units T4 DNA polymerase, all four dNTPs to a final concentration of 0.3mM and T4 polymerase buffer were added to give a 24μl reaction. The *Bam*HI cohesive ends were allowed to fill in at room temperature overnight and the required fragment subsequently purified by agarose gel electrophoresis. The expression vector plasmid was linearised by digestion with *Xho*I and the cohesive ends filled in as above, then phenol and chloroform extracted, ethanol precipitated and the lyophilised pellet resuspended in H_2O. Vector DNA (1μg) was treated with calf intestinal phosphatase as described above, re-extracted, then mixed with fragment DNA (2.5μg) and incubated overnight in a 40μl reaction in 1xligase buffer containing 1 unit of T4 DNA ligase. Ligated DNA was phenol and chloroform extracted, ethanol precipitated and the lyophilised pellet resuspended in 20μl H_2O, prior to transformation of *E. coli*. Plasmids pUL59 and pC6 which contain the UL5 and UL8 genes in p23 were similarly constructed by Dr N D Stow. Plasmid pIE9 was constructed by direct ligation of the UL9 gene (cloned as an *Xho*I fragment) into the *Xho*I site of p23.

13. Transformation of Competent *E.coli*

Competent *E.coli* DH5 bacterial cells were purchased from BRL. A 20μl aliquot of these, stored at -70°C, was thawed and left on ice for 10 min. 5μl ligated DNA was added to the cells, gently mixed and the incubation on ice continued a further 30 min. The cells were then heat shocked at 42°C for 90 sec then transferred onto ice for 1 min. 0.5ml of L-broth was added, gently mixed, and the cells then incubated at 37°C for 1hr. The cells were then serially diluted in L-broth and 100μl samples of undiluted, 10^{-1} and 10^{-2} dilutions spread onto ampicillin plates and incubated at 37°C overnight. Colonies were picked and also grown in 5ml of L-broth containing 50μg/ml ampicillin. The cultures were shaken overnight at 37°C and small scale plasmid preparations made.

14. Small Scale Plasmid Preparation (Mini-Prep)

From 5ml cultures of transformed bacterial colonies, 1ml was taken and the cells pelleted by centrifugation for 10 sec at 12000 rpm (MSE microfuge) and resuspended in 0.1ml of STET buffer by vortexing. To this 16μl of fresh lysozyme (10mg/ml in H_2O) was added and the samples boiled for 50 sec. Immediately after boiling the samples were centrifuged at 12000 rpm for 10 min (MSE micro-fuge) and the soft pellets removed. To the supernatant 0.1ml of isopropanol was added and the samples incubated at -20°C for 5 min. The precipitated DNA was pelleted, lyophilised and resuspended in 200μl of TE plus RNase A (5μg/ml) plus RNase T_1 (10 units/ml). Mini-prep plasmid DNA was stored at -20°C and 5μl used for restriction enzyme analysis.

15. Large Scale Plasmid Preparation

5ml overnight cultures were prepared by inoculation of L-broth containing 50μg/ml ampicillin with either 10μl of bacterial stock (stored in 50% glycerol or 7% DMSO at -70°C) or a colony picked from an ampicillin agar plate.

Overnight cultures were then added to 400ml L-broth containing 50ug/ml ampicillin in 2 litre conical flasks and shaken at 37°C until the optical density (OD) reached 0.6 at 650nm. Plasmid DNA was amplified by the addition of 1ml chloramphenicol solution (34mg/ml in absolute alcohol) to the culture and shaking continued overnight.

Bacterial cells were pelleted at 6000 rpm (Sorvall GS3 rotor) for 5 min, washed in 8ml of TE and re-pelleted at 5000 rpm (Sorvall SM24

rotor) for 5 min. Pelleted cells were resuspended in 2ml sucrose reagent by vortexing, 0.4ml of fresh lysozyme (20mg/ml in H_2O) was added and the cells incubated at room temperature for 30 min. 0.8ml of 0.25M EDTA and 3.2ml of Triton reagent were then added and the incubation at room temperature continued for a further 15 min, after which the cells were centrifuged at 35000 rpm for 30 min at 4°C (Sorvall Ti50 or Type 65 rotor). The supernatant was transferred to a 15ml "Falcon" tube and the volume brought to 7.5ml with H_2O. 7.5g of caesium chloride (CsCl) was added and dissolved by inversion of the tube giving a final density of 1.55-1.6g/ml. After the addition of 0.2ml ethidium bromide (10mg/ml) the mixture was transferred to plastic centrifuge tubes and centrifuged at 44000 rpm for 18hr at 15°C (Sorvall TV865 rotor). DNA bands were visualised under long wave UV light and the lower band containing supercoiled plasmid DNA was removed with a hypodermic needle and syringe. Ethidium bromide was removed by successive extractions (usually four) with an equal volume of isoamyl alcohol until no fluorescence due to ethidium bromide was detected under long wave UV light. The plasmid DNA was dialysed twice against a 1000-fold excess of TE buffer for 2-3hr prior to storage at -20°C.

Plasmid DNA was quantified by comparison with standard DNA of known concentration. A small quantity of plasmid DNA (<1μg) was linearised with an appropriate restriction enzyme and electrophoresed through an agarose gel alongside a standard sample of purified plasmid DNA of known concentration quantified by UV absorption at 260nm (where an absorbance value of 1.0 corresponds to 50μg/ml ds DNA). The gels were stained with EtBr, visualised by UV transillumination and photographed on Polaroid 665 film. The relative concentrations of DNA in each band were determined by scanning densitometry (Hoefer GS-360 scanning densitometer) of the resulting negative and the amount of DNA present was calculated from the area under the scan peaks.

Some DNA samples were directly quantified by UV spectroscopy as above.

16. Gel Electrophoresis of Nucleic Acids

(a) Non-denaturing Agarose Gels

Horizontal slab gels (21.5cm x 16.5cm) of 0.6% to 1.0% agarose were prepared in either 1xTBE or 1xLB containing 0.5μg/ml ethidium

bromide. Prior to electrophoresis, DNA samples were mixed with 1/5 volume 5xTBE or 5xLB loading buffer as appropriate. After loading, gels were electrophoresed at 100V for 3hr (TBE gels) or 40V overnight (LB gels).

(i) Purification of DNA Restriction Fragments from Non-Denaturing Agarose Gels

To purify a resolved DNA fragment, the DNA was visualised under long wave UV light and the required band excised from the gel. The gel slice was placed inside a bag made from a piece of boiled dialysis tubing containing 0.5ml of 0.5xTBE. The closed bag was immersed in a shallow layer of 0.5xTBE in an electrophoresis tank and electroeluted at 150V for 2-3hr. The DNA in solution was purified on a 0.4ml bed volume DEAE sephacel column. The column was washed before and after loading the DNA sample with 5ml TE buffer containing 0.1M NaCl. The DNA was then eluted with 2 aliquots of 0.25ml TE buffer containing 1M NaCl, precipitated with ethanol and the lyophilised pellet resuspended in TE.

(b) Non-denaturing Polyacrylamide Gels

For the resolution of low molecular weight DNA fragments, detection of DNA-protein complexes in a gel retardation assay or displaced labelled oligonucleotide in a helicase assay, 5-10% polyacrylamide gels (acrylamide:bisacrylamide, 19:1, or 55:1 in the gel retardation assay) were run in 1xTBE. 1.5mm thick gels were prepared in vertical glass plate sandwiches, polymerised with 0.001 vol. of TEMED and 0.01 vol. of 10% ammonium persulphate (APS). DNA fragments and helicase assay samples were loaded as for non-denaturing agarose gels. Gel retardation assay samples were loaded in their own loading buffer. Gels were electrophoresed at 150-300V for 1-3hr and the resolved products visualised by ethidium bromide staining or autoradiography.

(c) Denaturing Polyacrylamide Gels

The products of DNA sequencing and DNA primase assay reactions were resolved on denaturing polyacrylamide gels run in 1xTBE. A gel mix was prepared which contained 6% acrylamide (acrylamide:bisacrylamide, 19:1), previously deionised, and 8.3M urea in 0.5xTBE and stored in the dark at 4°C for up to 1 month. 0.35mm thick gels were prepared in long vertical glass plate sandwiches using a mix

containing 72μl each of 25% APS and TEMED and 60ml of gel mix. 2-3μl reaction samples were boiled for 2-3 min in formamide dye mix and immediately loaded into the thoroughly rinsed wells of the gel. Electrophoresis was at 1 Watt/25cm^2 until the BPB reached the bottom of the gel. Gels were either fixed in 10% acetic acid and oven dried (120°C) onto one glass plate previously coated with wacker solution, or transferred directly onto Whatman 3MM chromatography paper and dried at 80°C under vacuum.

17.Synthesis and Purification of Oligonucleotides

Oligonucleotides were synthesised within the institute by Dr J McLauchlan on a Biosearch 8600 DNA Synthesiser from which the DNA was eluted in 1ml of ammonia. Following incubation at 55°C for 5hr, the ammonia was removed by lyophilisation. The DNA was resuspended in 50μl of H_2O and half removed to a fresh tube and stored at -20°C. The remainder was mixed with an equal volume of 90% formamide in 1xTBE then boiled for a few minutes and loaded onto a denaturing 15% polyacrylamide gel (acrylamide:bisacryl-amide, 24:1) containing 8M urea and 1xTBE (this type of gel was suitable for the purification of 15-100mer oligonucleotides). Formamide dye mix (2μl) was loaded into wells adjacent to those containing the oligonucleotide sample as a migration marker and the gel electrophoresed in 1xTBE at 250-300V for 4-5hr, until the BPB had almost reached the bottom of the gel. The gel was then transferred onto cling film and the DNA viewed over a silica gel thin layer chromatography plate with an angled short wave UV lamp. The DNA in the gel absorbed UV light thus casting a shadow on the fluorescent chromatographic plate. The required band was excised and the DNA eluted into 0.5ml of elution buffer(0.5M NH_4OAc, 10mM $MgCl_2$, 0.1% SDS and 1mM EDTA) overnight at 37°C with constant agitation. The DNA solution was sequentially extracted with phenol:chloroform (1:1) and chloroform then ethanol precipitated. The DNA was pelleted, lyophilised and resuspended in H_2O. The Absorbance values at 260nm and at 280nm of the recovered DNA were determined and its concentration estimated taking an A_{260} value of 1 as equivalent to 20ug/ml of oligonucleotide DNA.

Single-stranded oligonucleotides synthesised:

45mer; $^{5'}$ACTCTAGAGGATCCCCGGGTACGTTATTGCATGAAAGCCCGGCTG$^{3'}$

15mer; $^{5'}$GTCTTCCTGCCCCAT$^{3'}$

18. Plasmid DNA sequencing

Plasmid DNA was sequenced by the dideoxy chain termination sequencing protocol (Sanger *et al.*, 1977) using denatured plasmid DNA templates.

10ug of plasmid DNA, purified by caesium chloride gradient, was denatured in 0.2M NaOH for 5 min at room temperature, NH_4OAc added to 0.3M then precipitated with ethanol. The pellet was washed with 75% ethanol lyophilised and resuspended in 20µl H_2O.

(a) Dideoxy sequencing

2µl of denatured plasmid DNA was annealed with 10ng of primer oligonucleotide in a final volume of 10µl containing 20mM Tris and 20mM $MgCl_2$ at 37°C for 30 min. 2.5µl each of four termination mixes (i.e., G, A, T and C) were aliquoted into individual microtitre wells. These mixes contained 150µM dGTP, dATP, dCTP and dTTP and 15µM of either dideoxy (dd)ATP (A mix), ddCTP (C mix), ddGTP (G mix) or ddTTP (T mix) in 20mM $MgCl_2$, 40mM Tris.HCl (pH 7.5) and 50mM NaCl. The annealed reactions were radio-labelled by incubation at room temperature for 5 min with 1µl 0.1mM DTT, 2µl labelling mix (2µM dGTP, dCTP, and dTTP), 30µCi [^{35}S]-dATP and 2 units of T7 DNA polymerase. 3.4µl of labelled, annealed reactions were transferred to each of the four termination mixes and incubated at 37°C for 15 min. Reactions were stopped by the addition of 4µl of formamide dye mix then boiled for 2-3 min before loading onto a denaturing polyacrylamide gel for analysis.

19. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially as described by Marsden *et al.* (1976). Stock solutions of 30% acrylamide with 5% or 2.5% cross-linking agent (acrylamide:bisacrylamide, 19:1 and 39:1 respectively) were prepared in H_2O. 1.5mm thick gels were prepared in vertical glass plate sandwiches. A resolving gel of 9% acrylamide with 2.5% cross-linker was prepared in 1xRGB, on top of which was polymerized a stacking gel of 5% acrylamide (with 5% cross-linker) in 1xSGB. Mini-protein gels were similarly prepared using Bio-Rad mini-protein gel plates.

Protein samples were boiled for 5 min in sample buffer, immediately loaded onto the gel and electrophoresed in tank buffer at 40-60mA for 3hr or 8-12 mA overnight. Gels were fixed by shaking for 45 min in methanol:H_2O:acetic acid, 50:50:7, and either dried at

80°C under vacuum onto Whatman filter paper or shaken for 45 min in En^{3}Hance autoradiography enhancer and for 30 min in H_2O before drying. Dried gels were exposed to autoradiographic film either at room temperature or -70°C ('EnH^{3}anced' gels).

20. Preparation of Sonicated Calf Thymus DNA (Gel Retardation Assay)

A solution of calf thymus DNA in H_2O (approximately 5mg/ml) was sonicated using a probe sonicator three times for 30 sec at 100W, with 30 sec intervals on ice. The sonicated DNA was sequentially extracted three times with phenol followed by three times with ether, then dialysed overnight against 1 litre of H_2O. The Absorbance at 260nm was measured and the DNA concentration was adjusted as required with H_2O prior to storage at -20°C (1 Absorbance unit at 260nm is equivalent to 50μg/ml double stranded DNA).

21. Preparation of Denatured DNA (Southern Blots)

Calf thymus or salmon sperm DNA, used as a carrier in DNA:DNA and RNA:DNA prehybridisation and hybridisation buffers, was prepared by dissolving the double-stranded DNA in sterile H_2O at a concentration of 2-5mg/ml and boiling for 15 min. The resulting solution of denatured DNA was stored at 4°C.

22. Preparation of Activated Calf Thymus DNA (ATPase Assay)

Calf thymus DNA was dissolved in 10mM Tris.HCl (pH 7.5) and 5mM $MgCl_2$ at a concentration of 2.5mg/ml and incubated with pancreatic DNase I (40ng DNase I/mg DNA) at 60°C for 5 min essentially as described by Moss (1982). The DNA was then extracted with phenol and dialysed against 10mM Tris.HCl (pH 7.5) overnight.

23. Radioactive Labelling of DNA

(a) 5' end labelling of single-stranded oliginucleotides

Synthetic oligonucleotide (30ng) was resuspended in kinase buffer containing 50μCi of [gamma-^{32}P]ATP and 5 units of T4 polynucleotide kinase in a final volume of 25μl and incubated at 37°C for 2hr. 4μl of 0.25M EDTA was then added and the mixture heated to 65°C followed by slow cooling to room temperature. Labelled DNA was sequentially extracted with phenol and chloroform. The DNA was precipitated with ethanol, lyophilised, resuspended in an appropriate volume of TE buffer and stored at -20°C.

(b) 3' end labelling of single-stranded oligonucleotides

Synthetic oligonucleotide (30ng) was resuspended in tailing buffer containing 40 μCi of [α-^{32}P]dATP and 25 units of terminal deoxynucleotide transferase in a final volume of 30μl and incubated at 18°C for 1hr, then sequentially extracted with phenol and chloroform and precipitated with ethanol. The lyophilised pellet was resuspended in an appropriate volume of TE and stored at -20°C.

(c) Internal Labelling of Plasmid DNA by Nick Translation

Plasmid DNA (100-200ng) was radiolabelled by nick-translation (Rigby *et al.* 1977) in the presence of 20μCi each of [α-^{32}P]dGTP and [α-^{32}P]dCTP. Incubation was in a final volume of 25μl NT buffer (50mM Tris.HCl (pH 7.5), 10mM $MgCl_2$, 1mM DDT, 40μM dATP, 40μM dTTP, 50μg/ml BSA) with 5x10^{-7}μg/ml DNase I added for 5 minutes at 37°C followed by 1 unit of *E.coli* DNA polymerase I for a further 2-3hr at 14°C. The volume was brought to 125μl with H_2O, extracted with phenol and the aqueous phase loaded onto a Sephadex G-50 column in order to separate labelled plasmid DNA from unincorporated nucleotides.

(d) Internal Labelling of Plasmid DNA by Primer Extension

The method used was as described by Feinberg and Vogelstein, 1983. 10-100ng of plasmid DNA was boiled for 10 min in 20μl H_2O and subsequently reacted overnight in the presence of 20μg BSA, 50μCi [α-^{32}P]dGTP plus 2μM dCTP (or 50μCi [α-^{32}P]dCTP plus 2μM dGTP) and 2 units of large fragment of *E.coli.* DNA polymerase I in a final volume of 50μl (5xPE buffer is **2A:5B:3C**, where **A**= 1.2M Tris.HCl (pH 8.0), 120mM $MgCl_2$, 0.2M β-mercaptoethanol, 0.4mM dATP, 0.4mM dTTP, **B**= 2M Hepes.NaOH (pH 6.6) and **C**= 50 A_{260} units $d(N)_6$ random hexanucleotide primers in 556μl TE). Labelled DNA was separated from unincorporated triphosphate as described for nick translated plasmids.

24. <u>Southern Transfer of DNA to Nitrocellulose</u>

Restriction enzyme digested DNAs for Southern transfer (Southern, 1975) were run on agarose gels in 1xLB. Following electrophoresis the gel was denatured first in Gel Soak I for 1hr then neutralised in Gel Soak II for 1hr. The DNA was blotted overnight onto a pre-soaked nitrocellulose membrane using 6xSSC, a wick of Whatman 3mm paper and a weighted capillary stack of absorbent paper towelling. The filter was then air dried and baked at 80°C for 2hr in a vacuum oven prior

to hybridisation.

25. Electroblot Transfer of Protein to Nitrocellulose

Polypeptides for electroblot transfer (Towbin *et al.*, 1979) were electrophoresed on SDS-polyacrylamide mini-gels. The gel was washed for a few minutes in electroblotting buffer to remove the SDS then placed on Whatman 3MM paper. The top surface of the gel was covered with a pre-soaked nitrocellulose membrane plus more 3MM paper and the whole assembly inserted into a Bio-Rad 'Mini Trans-Blot Cell', following the manufacturers instructions. Polypeptides were electro-blotted onto the nitrocellulose membrane either for 2hr at 150mA or overnight at 40mA.

26. DNA:DNA hybridisation

Nitrocellulose membranes were incubated in plastic bags with 100ml of prehybridisation buffer and shaken for 1-3hr at 65°C. The probe was prepared by denaturing 10^7-10^8 cpm of nick translated DNA in 0.2M NaOH (final volume 1.2ml) for 10 min neutralised with HCl before adding 8.8ml hybridisation buffer. Prehybridisation buffer was replaced with the hybridisation buffer containing the denatured, labelled DNA and the membranes incubated with shaking at 65°C overnight. They were then washed three times for 30 min in 1xSSC/0.25% SDS at 65°C and finally blotted dry with tissue paper and exposed to autoradiographic film.

27. Protein Detection using Antibodies

Nitrocellulose membranes were incubated with blocking buffer (1%BSA in TBST), whilst shaking for 30 min at room temperature. Primary rabbit anti-sera were diluted 1:40-1:50 in TBST and placed with the membranes in small bags and incubated for 4-15hr with constant agitation. The membranes were washed three times for 10 min in TBST then incubated with Horse Radish Peroxidase (HRP)-conjugated anti-rabbit IgG anti-serum diluted 1:2500 in TBST for 30 min-1hr. After washing again and allowing the membranes to dry briefly, protein:antibody recognition was visualised by the addition of HRP colour development solution (Promega). Once the desired intensity of colour was reached, membranes were washed thoroughly in running deionised H_2O then air dried and stored in the dark, wrapped in plastic and aluminium foil.

28. Fractionation of Infected Cells for Protein Localisation Studies

Cells were fractionated by a procedure modified from that described by Dignam *et al.* (1983).

BHK cell monolayers in 90mm tissue culture dishes were infected with 5-10 pfu/cell of *ts*K or *ts*K recombinant viruses at 38.5°C and labelled with 100μCi/plate of [^{35}S]-L-methionine as described in Results. The cells were then harvested and washed in PBS. *S.f.* cell monolayers in 90mm tissue culture dishes were infected with 5-10 pfu/cell of *wt* or recombinant AcNPV at 28°C, labelled with 100μCi of [^{35}S]-L-methionine at 24hr post-infection and at 30hr the cells harvested and washed in Tris Saline.

A sample of the washed cells was removed and lysed directly in sample buffer providing an extract containing total cellular proteins. The remaining cells were pelleted and resuspended in 0.1ml of buffer A or RSB then NP40 added to 0.5% and the cells lysed on ice for 10 min. The nuclei were pelleted by centrifugation at 12000rpm (MSE microfuge) and the supernatant retained as the cytoplasmic fraction. The nuclei were either resuspended in H_2O, to give a total nuclear fraction, or extracted with 0.1ml of buffer C containing 0.6M NaCl on ice for 30 min then centrifuged at 70000 rpm for 30 min at 4°C (Beckman TLA 100.2 rotor). The resulting supernatant and pellet represented nuclear extract and nuclear pellet respectively.

For SDS-PAGE analysis, samples of cytoplasmic and nuclear extracts and nuclear pellet were lysed in sample buffer (final conc 1x).

29. Preparation of Infected Cell Extracts for Biochemical Studies

BHK and *S.f.* cell monolayers were infected , labelled and harvested as above.

(a) Cytoplasmic extracts

Cells were resuspended in RSB (0.1ml/90mm dish, 0.3ml/140mm dish) then NP40 added to 0.5% and the cells lysed on ice for 10 min. The nuclei were removed by centrifugation at 6500 rpm (micro-centrifuge) for 2 min and the supernatant retained, flash-frozen in a bath of solid CO_2 and methanol and stored at -70°C as cytoplasmic extract. The nuclei were pelleted at 12000 rpm for 2 min, then extracted.

(b) Nuclear extracts

Pelleted nuclei were resuspended in buffer C (0.1ml/90mm dish, 0.3ml/140mm dish) and proteins eluted on ice for 30 min. The

extracted nuclei were centrifuged at 70000 rpm for 30 min at 4°C (Beckman TLA 100.2 rotor) and the supernatant flash frozen and stored at -70°C as nuclear extract.

(c) Total cellular extracts

Cells were resuspended directly in buffer C containing 0.6M NaCl (0.1ml/90mm dish, 0.3ml/140mm dish) then NP40 added to 0.5% and the cells incubated on ice for 30 min. Following centrifugation at 70000 rpm for 30 min at 4°C (Beckman TLA 100.2 rotor) the supernatant was retained, flash frozen and stored at -70°C as total cellular extract.

All extracts retained enzymatic activity for at least 12 months.

(d) Dialysis of High Salt Extracts

Prior to phosphocellulose column chromatography it was necessary to reduce the NaCl concentration of nuclear and total cellular extracts. Extracts were dialysed against a 400-fold excess of column buffer B containing 50mM NaCl for 2-3hr at 4°C, with one change of buffer. Dialysed extracts were centrifuged briefly (5 min, MSE microfuge) to remove any precipitate prior to loading onto phosphocellulose columns.

30. Preparation of Infected Cell Extracts for Immunoprecipitation

BHK cell monolayers in 35mm tissue culture dishes were infected with *ts*K or *ts*K recombinant viruses at 38.5°C and labelled as indicated at 6-8hpi with 20μCi of [^{35}S]-L-methionine. At 8-10hpi the plates were washed with ice-cold PBS and the cells scraped into 0.5ml of IP extraction buffer (0.1M Tris.HCl (pH 8), 10% glycerol, 0.5% NP40, 0.5% sodium deoxycholate, 0.2mM PMSF) and incubated at 4°C for 1hr. The lysed cells were centrifuged at 12000 rpm for 15 min (micro-centrifuge) and the supernatant (IP extract) retained, flash frozen and stored at -70°C prior to immunoprecipitations.

31. Immunoprecipitation

Proteins were precipitated from IP extracts by a procedure modified from that described by Kessler, 1975.

50μl of IP extract was incubated on ice for 2hr with 1μl of undiluted rabbit anti-peptide antiserum (supplied by Dr M D Challberg) then 10μl of sheep anti-rabbit IgG antiserum added and incubation on ice continued for 1hr. Finally 50μl of Immunoprecipitin (fixed *Staphylococcus aureus* cells) was added and mixed on ice for 1hr then the mixture centrifuged at 12000 rpm for 30

sec (micro-centrifuge). The pellet was washed 4 times by resuspension in 0.5ml IP wash buffer (0.5M LiCl, 0.1M Tris.HCl (pH8), 1% β-mercaptoethanol). The final pellet was resuspended in 50μl of sample buffer and boiled for 5 min. Following centrifugation at 12000 rpm for 5 min (micro-centrifuge) the supernatant was analysed by SDS-PAGE. Later experiments revealed the use of the sheep anti-rabbit IgG step prior to the addition of immunoprecipitin to be unnecessary and this was subsequently omitted.

32. Estimation of Protein Concentration

Estimates of total protein concentration in infected cell extracts were obtained using Bio-Rad protein assay dye reagent. 5μl of cell extract was diluted in 1ml of H_2O and 0.8ml of this taken and mixed with 0.2ml of dye reagent and left at room temperature for 10 min. Absorbance at 595nm was measured for each sample and also for similarly treated BSA solutions of known concentration. Total protein concentration of samples was estimated from a plot of absorbance at 595nm against concentration for the BSA standards.

Estimates of total protein concentration in phosphocellulose column fractions were obtained using Pierce Micro Protein Assay System. 20μl of fraction was diluted to a volume of 0.5ml with H_2O, an equal volume of assay reagent added and the mixture incubated at 60°C for 1hr, then slowly cooled to room temperature. Absorbance at 562nm was measured for samples and BSA solutions of known concentration. Total protein concentration was estimated from a plot of absorbance at 562nm against concentration for the BSA standards.

33. Fractionation of Infected Cell Polypeptides

(a) Phosphocellulose Column Chromatography

Whatman P11 phosphocellulose was precycled and equilibrated according to the manufacturers instructions and stored at 4°C in column buffer B with 0.1% sodium azide added.

From a 2ml sterile syringe the plunger was removed and replaced with a plug of sterile glass wool. The syringe was held upright and phosphocellulose slurry added to give a bed volume of 2ml. After washing thoroughly with column buffer B containing 50mM NaCl, dialysed nuclear or total cellular extract was loaded onto the column and the flow through collected and re-loaded twice more. The column was then washed with 10ml of buffer B plus 50mM NaCl and proteins subsequently eluted with a 15ml linear gradient of increasing NaCl

concentration. For this, 1ml aliquots of buffer B containing NaCl increasing in 25mM steps from 50mM up to 400mM were applied sequentially to the column. Finally, a 2M wash (2ml) was applied. Fractions (usually 1ml) were collected, flash frozen and stored at -70°C. All procedures were carried out at 4°C.

Activity in these fractions was retained for at least 10 months.

(b) FPLC MONO-Q Ion-Exchange Chromatography

Using a Pharmacia FPLC apparatus, a MONO-Q ion-exchange column (1ml bed volume) was equilibrated with column buffer B containing 50mM NaCl. Dialysed infected cell extract was applied and the column washed with buffer B plus 50mM NaCl. Proteins were eluted with an increasing linear gradient of NaCl from 50mM up to 500mM followed by a 1M wash. Approximately 30 1ml fractions were collected, flash frozen and stored at -70°C.

34. Assay for DNA Dependent ATPase Activity

DNA dependent ATPase activity was assayed by a procedure modified from those described by Crute *et al.*, (1988) and Clark *et al.*, (1981). Reaction mixes (50μl) contained 20mM Tris.HCl (pH 7.5), 10% glycerol, 3.5mM $MgCl_2$, 100μg/ml BSA, 5mM DTT and 0.05mM ATP plus 1.5 μCi [gamma-^{32}P]ATP, 0.1μg activated calf thymus DNA and 20μl enzyme fraction. These were incubated at 33°C for 40 min after which 150μl of 2% activated charcoal in 50mM HCl/5mM H_3PO_4 was added, mixed by vortexing and left to stand for 5 min. The reactions were then centrifuged for 2 min at 12000 rpm and 120μl of the supernatant transferred to a fresh tube. Free phosphate liberated into the supernatant by the hydrolysis of ATP was counted and, by comparison with total radioactivity initially present in the reaction, the amount of ATP hydrolysis was calculated.

35. Assay for DNA helicase activity

Helicase activity was assayed essentially as described by Crute *et al.* (1988).

Helicase substrate was prepared by radioactively labelling 30ng of a 45 mer single stranded oligonucleotide (see Section 2B.20) either at the 5' end with T4 polynucleotide kinase in the presence of [gamma-^{32}P]ATP or at the 3' end with terminal deoxynucleotide transferase in the presence of [α-^{32}P]dATP. Labelled oligonucleotide was then annealed with an equimolar amount of single stranded M13mp18 DNA in

TM buffer containing 30mM NaCl. The mixture was first heated to 65°C for a few minutes then allowed to cool slowly to room temperature and remain there for at least 30 min. The annealed substrate, which contains a 3' tail (23 nucleotides at 5' end of the 45mer are complementary to M13 whilst the remaining 22 non-complementary nucleotides form 3'single-stranded tail), was then purified by gel exclusion chromatography on a Bio-gel A1.5m agarose bead column.

A second helicase substrate, without a 3' tail (used in assaying helicase activity of the UL9 protein) was prepared by first annealing a 17 mer single stranded oligonucleotide (universal sequencing primer) to single stranded M13mp18 DNA as described above. The 3' end of the oligonucleotide was labelled with T7 DNA polymerase in the presence of [α-^{32}P]dCTP and unlabelled dGTP followed by a chase in the presence of unlabelled dCTP. Labelled substrate containing a complementary region extending 20bp was purified on a Sephadex G-50 column.

Helicase assay reactions (40μl) contained 20μl enzyme fraction, 20ng of helicase substrate and either 4μl of 10x helicase buffer 1 (0.2M Tris.HCl (pH 7.5), 0.1M $MgCl_2$, 5mM DTT, 20mM ATP and 1mg/ml BSA) or 8μl of 5x helicase buffer 2 (0.1M Tris.HCl (pH 7.5), 17.5mM $MgCl_2$, 25mM DTT, 15mM ATP, 0.5mg/ml BSA and 50% glycerol). Incubation was for 2hr at 37°C and the products were analysed by electrophoresis through a non-denaturing 10% polyacrylamide gel containing in 1xTBE.

36. Assay for DNA Primase Activity

DNA primase activity was assayed essentially as described by Crute *et al.* (1989) using an indirect procedure in which any RNA primers formed are elongated by a DNA polymerase in the presence of an appropriate labelled dNTP.

Reactions (30μl) contained 10μl enzyme fraction and 6μl of 5x primase buffer (250mM Tris.HCl (pH8.7), 17.5mM $MgCl_2$, 25mM DTT, 5mM ATP, 0.5mg/ml BSA, 50% glycerol) plus 0.2μg poly(dT), 22μM dATP, 1μCi [α-^{32}P]dATP and 1 unit of the large fragment of *E.coli* DNA polymerase I. Incubation was at 37°C for 2hr. Reactions were then incubated with 0.5mg/ml proteinase K at 37°C for 15 min and the products of the reaction sequentially extracted with phenol and chloroform and precipitated with ethanol. Following lyophilisation the products were resuspended in formamide dye mix. Samples were boiled for 2-3 min before analysis on a denaturing 6% polyacrylamide gel.

37. Assays for Protein:DNA Interaction

(a) DNA Cellulose Columns

DNA cellulose columns were used, as described by Alberts and Herrick, 1971, to investigate the DNA binding properties of proteins present in phosphocellulose column fractions.

Lyophilised single-stranded DNA cellulose (Sigma), was soaked for 3hr in DC buffer (10mM Tris.HCl (pH 7.5), 50% glycerol, 5mM EDTA, 5mM β-mercaptoethanol), washed twice by resuspension in the same buffer and stored at -20°C or at 4°C for up to 1 month.

Pre-soaked DNA cellulose was equilibrated in DC buffer containing 50mM NaCl and a 5ml disposable Bio-Rad column prepared containing a bed volume of 0.3ml. The column was washed thoroughly with DC buffer plus 50mM NaCl, then with two bed volumes of the same buffer containing 0.2mg/ml BSA for saturation of non-specific binding sites. 50-100μl of phosphocellulose column fraction made up to a final volume of 300μl with DC buffer containing 50mM NaCl, was loaded onto the column and the flow-through collected and re-loaded twice more. The column was then washed with 0.6ml DC buffer containing 50mM NaCl and subsequently with 0.3ml vols of DC buffer containing 0.1M, 0.2M, 0.3M, 0.6M and 2M NaCl. The eluate from each wash was collected separately. Proteins eluted were precipitated with 4 vols of acetone, lyophilised and resuspended in sample buffer for analysis by SDS-PAGE.

(b) Gel Retardation Assay for Binding to Single-Stranded DNA

The method used was essentially as described by Schneider *et al.* (1986).

A single stranded oligonucleotide (45mer, Section 2B.20) was 3'end labelled using terminal deoxynucleotide transferase in the presence of [α-^{32}P]dATP and purified on a Sephadex G-50 column. 0.1ng (5000 cpm) of labelled oligonucleotide was incubated with 2μg of sonicated calf thymus DNA and 10μl of enzyme fraction in a 20μl reaction mix containing buffer C plus 100mM NaCl. Incubation was at 25°C for 20 min then reactions were mixed with 5μl of GRA dyes (25% glycerol, 10mM DTT, 0.01% BPB in 5xTBE) and loaded onto a non-denaturing 5-8% polyacrylamide gel run in 1xTBE.

38. Immunofluorescent Staining of Proteins Expressed by *tsK* Recombinant Viruses

The method used for immunofluorescent staining of proteins followed that previously described by Randall and Dinwoodie (1986).

BHK cell monolayers in 2cm x 2cm tissue culture slide chamber wells were infected at a moi of 10 pfu/cell with *wt* HSV-1, *tsK* or *tsK* recombinant viruses at 38.5°C. 8-10hr post-infection, medium was removed from the wells and the cells washed with PBS. The cells were then fixed with 5% formaldehyde/2% sucrose in PBS for 10 min at room temperature, washed 3 times with PBS plus 1% NBCS and then permeabilised with 0.5% NP40/10% sucrose in PBS plus 1% NBCS for 5 min at room temperature. Following permeabilisation, the cells were washed as before and briefly air dried. 50-100µl of a 1:40 dilution of anti-UL5, anti-UL8, anti-UL9 or anti-UL52 antiserum (supplied by Dr M D Challberg) in PBS plus 1% NBCS was applied to the cells as required and incubated at room temperature for 1hr, shaking occasionally. The cells were then washed 3 times as before, briefly air dried and 50-100µl of a 1:30 dilution of swine anti-rabbit/FITC conjugated antiserum (Sigma Chemical Co.Ltd.) in PBS plus 1% NBCS applied for 1hr at room temperature. After a final 3 washes with PBS plus 1% NBCS and complete air drying, the chamber wells were removed from the slide and the cells mounted in 90% glycerol in PBS using a glass coverslip.

Slides were then viewed under the x25 and x40 objectives of a Leitz fluorescence microscope and photographed on Kodak TriX-pan 400 black and white 35mm film.

As described in the Introduction, in 1988 Challberg and his colleagues reported the identification of a set of seven HSV-1 open reading frames (ORFs) which are necessary and sufficient for origin-dependent HSV-1 DNA synthesis in tissue culture, i.e. ORFs UL5, UL8, UL9, UL29, UL30, UL42, and UL52 (Wu *et al.*, 1988; McGeoch *et al.*, 1988; See Introduction, Section 1C.2(a)).

The protein products of three of these genes had already been recognised in HSV-1 infected cells and been relatively well characterised. These were the single-stranded DNA binding protein, encoded by UL29, the viral DNA polymerase, encoded by UL30, and a polypeptide of apparent M_r 65 000 which binds non-specifically to double-stranded DNA, encoded by UL42.

At the outset of the work presented in this thesis, the protein products of the remaining four genes had not been identified, presumably due to their lower abundance in HSV-1 infected cells.

3A. EXPRESSION OF UL5, UL8, UL9 AND UL52 ORFs IN *tsK* RECOMBINANT VIRUSES

The first series of experiments aimed to identify the products encoded by replication genes UL5, UL8, UL9 and UL52 by over-expressing these ORFs in BHK cells using a herpes simplex virus vector.The virus expression system used was based on the HSV-1 temperature-sensitive mutant, *tsK*. This mutant has a lesion in immediate-early gene 3 which results in overproduction of immediate-early (IE) gene products and failure to produce other classes of proteins at the non-permissive temperature (NPT)(Preston 1979a). To generate recombinant viruses replication genes were initially cloned under the control of the IE3 promoter within the coding region of a plasmid-borne copy of the HSV-1 TK gene. The resulting plasmids were co-transfected into BHK cells with *tsK* DNA at the permissive temperature (PT) to allow insertion of the replication gene construct into the TK locus by homologous recombination as illustrated in **Figure 13.** TK deficient recombinant viruses were selected, purified and checked for the presence of the inserted copy of the replication gene. They were then examined to determine whether this additional gene copy was expressed at NPT.

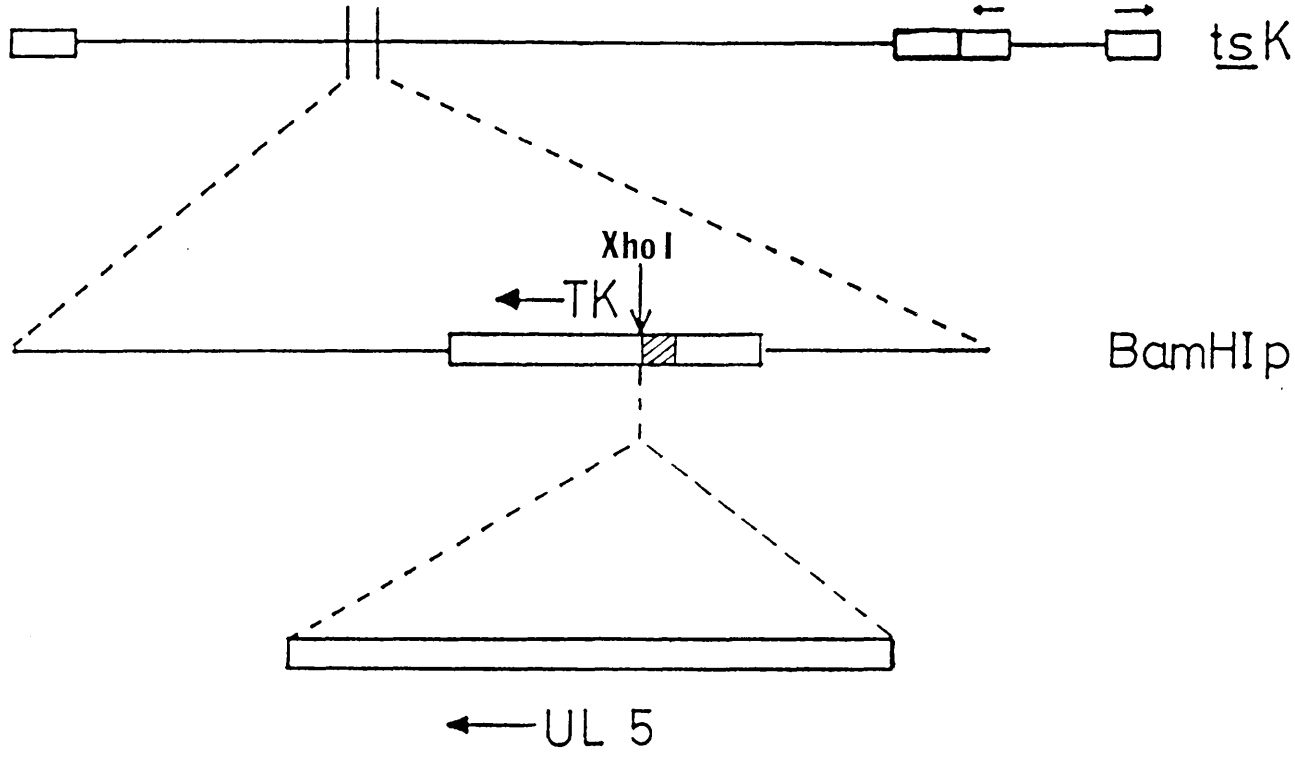

tsK
Xho I
TK
BamHI p
UL 5

Figure 13. Construction of *ts*K Recombinant Viruses

The figure schematically illustrates construction of *ts*K recombinant viruses individually expressing HSV-1 DNA replication genes. UL5, UL8, UL9 and UL52 ORFs were cloned downstream of the IE3 promoter within a cloned copy of the HSV-1 TK gene. The resulting plasmids and *ts*K viral DNA were recombined at the permissive temperature in co-transfected BHK cells, and TK^- recombinant viruses were isolated and screened for the presence of an inserted gene. The open box within the *Bam*HI *p* fragment represents the TK ORF and the hatched region, the IE3 gene promoter. Transcription of inserted replication genes from the IE3 promoter is in the same direction as TK (arrows).

1. Construction of Plasmids to Allow Insertion of UL5, UL8, UL9 and UL52 ORFs Into the TK Locus of *tsK*

Fragments of DNA containing the genes to be expressed were cloned in the correct orientation into the unique *Xho*I site of plasmid p23. The construction of this plasmid, which was provided by Dr C M Preston, is described in **Figure 14.**

Briefly, plasmid p23 consists of a cloned copy of the HSV-1 *Bam*HI *p* fragment into which has been inserted, at the SstI site within the TK coding sequence, a 357bp fragment containing the sequences corresponding to the 5' end, promoter and upstream regulatory sequences of gene IE3. The promoter and upstream regulatory sequences direct transcription in the same direction as the TK gene and the unique *Xho*I site lies immediately downstream of this fragment. The TK gene polyadenylation site is therefore available for use if cloned fragments lack their own polyadenylation signal.

The UL5, UL8, and UL9 ORFs had already been cloned by Dr N D Stow into the unique *Xho*I site of plasmid p23, yielding plasmids pUL59, pC6, and pIE9 respectively. These are described in **Table 2.**

The UL52 ORF was similarly inserted into p23 by utilizing plasmid pUL522 which contains the HSV-1 *Hpa*I *q* fragment blunt-end ligated into the filled-in *Sal*I site of the vector plasmid, pUC8 (**Figure 15**). Initial manipulations were aimed at obtaining the UL52 ORF as a *Bam*HI fragment and at shortening the 165bp untranslated region between the *Hpa*I site and the initiator codon for UL52.

*Hin*dIII linearised pUL522 was digested with exonuclease Bal31 for various times between 2 and 20 min at 31°C. The extent of deletion was monitored by re-digestion of these DNAs with *Eco*RI and agarose gel electrophoresis, as shown in **Figure 16.** *Eco*RI digestion of *Hin*dIII linearised pUL522 yields two large fragments of approximately the same size (*Eco*RI and *Hin*dIII-*Eco*RI fragments of 2450bp and 2600bp, respectively), and one smaller *Eco*RI-*Hin*dIII fragment of 1230bp. Nuclease Bal31 deletion from the *Hin*dIII site was monitored by the reduction in size of the small *Eco*RI-*Hin*dIII fragment which was estimated using *Hin*fI-cut pAT153 molecular weight marker fragments. Digestion for 14 and 20 minutes with Bal31 was estimated to shorten the small fragment by approximately 100bp. The ends of the molecules deleted by this amount were treated with calf-intestinal phosphatase and re-circularised by ligation in the presence of phosphorylated *Bam*HI 8bp linker oligonucleotides. The resulting molecules were used

Gene	ORF	Parent plasmid	Insert	Flanking sites	Recombinant plasmid
UL5	15133-12488	pUL51	*Mlu*I/*Mlu*I 15162-12127	*Bam*HI	pUL59
UL8	20478-18229	pUL82	*Mst*II/*Sst*I 20488-17850	*Bam*HI	pC6
UL9	23261-20709	p301	*Nar*I/*Sst*II 23539-20666	*Eco*RI	pIE9
UL52	109048-112221	pJC1	*Hpa*I/*Hpa*I 108967-112512	*Bam*HI	p206

Table 2. Plasmids Containing the UL5, UL8, UL9 and UL52 ORFs

Column 2 shows nucleotide co-ordinates of the UL5, UL8, UL9 and UL52 ORFs. The co-ordinates of gene-containing fragments sub-cloned into p23 are given in column 4 and the restriction sites flanking these subfragments in column 5. Recombinant plasmids derived from p23 which were used in co-transfections with *ts*K viral DNA are listed in column 6.

Figure 14. Cloning of ORFs Under IE Control

The figure illustrates (**a**) plasmid p23 and (**b**) the 357bp fragment containing the IE3 gene promoter. Restriction enzyme sites and nucleotide positions within the HSV-1 genome (McGeoch *et al.*, 1988b) are indicated.

Plasmid p23 comprises a copy of the HSV-1 *Bam*HI *p* fragment cloned into the *Bam*HI site of the vector pAT153. A 357bp fragment containing sequences corresponding to the 5' untranslated region, promoter and upstream regulatory region of the IE3 gene (striped region) has been inserted within the TK coding region (stippled region) such that a unique *Xho*I site is generated downstream of the promoter. In addition, a second *Hin*dIII site is present at the other end of the inserted 357bp fragment. ORFs to be expressed were cloned into the *Xho*I site in the appropriate orientation. Transcription from the IE3 promoter is in the same direction as TK (arrow). The TK polyadenylation site (closed circle) is available for use if cloned fragments lack their own polyadenylation signal. In (**b**), the directions of transcription originating from the IE3 and TK promoters are shown above and below the bar, respectively.


a
Hind III
Bam HI
(48634)
Sst I (Hind III)
(47358)
Xho I
pAT 153
p23
Sca I
Bam HI
(45055)
b
Sma I
131760
Bam HI
131399
cap site
131429
IE3 mRNA

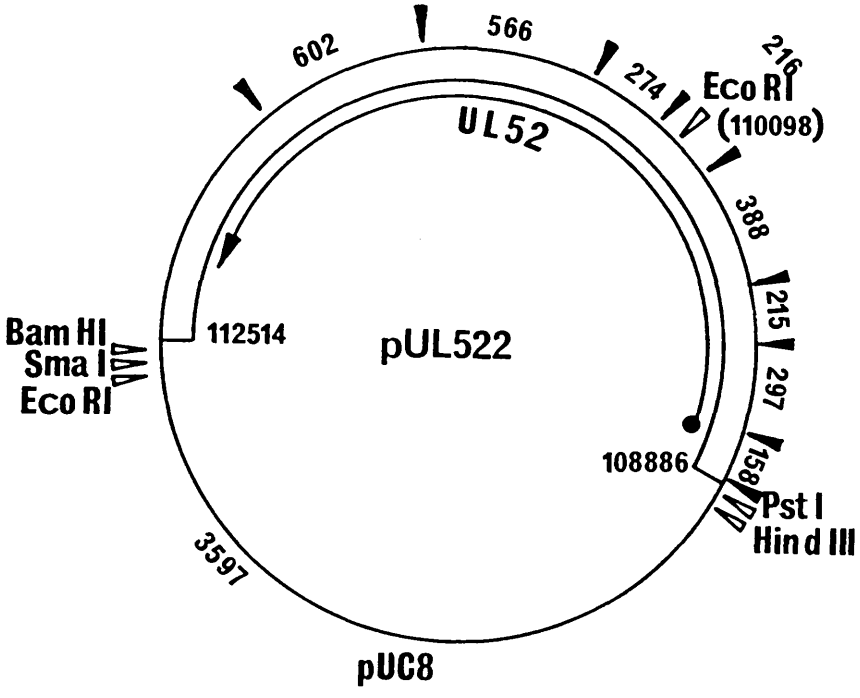


Figure 15. Plasmid pUL522

Plasmid pUL522 comprises the HSV-1 *Hpa*I *q* fragment cloned into the filled-in *Sal*I site of the vector pUC8.

Following linearization at the *Hin*dIII site, pUL522 was digested with exonuclease *Bal* 31 and progression of the deletion monitored by restriction enzyme analysis. Key restriction enzyme sites are labelled and indicated by the open arrows. *Sst*II sites (marked by filled arrows) used in restriction analyses are also shown and the sizes of *Sst*II fragments generated are indicated. Nucleotide positions within the HSV-1 genome for the ends of the *Hpa*I fragment and the *Eco*RI site are also indicated. The *Sst*II site closest to the *Hin*dIII site lies at nucleotide 108910 in the HSV-1 genome (24bp from the *Hpa*I site).

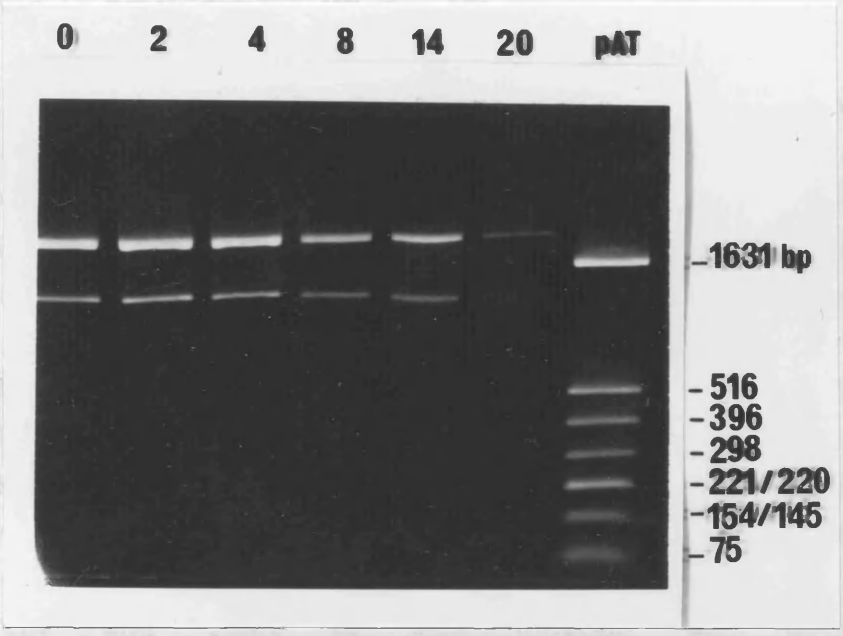


Figure 16. Exonuclease Bal 31 digestion of pUL522

*Hin*dIII linearized pUL522 DNA, digested for various times with exonuclease Bal 31, was phenol extracted then digested with *Eco*RI and analysed on a 1% agarose gel. Minutes of exonuclease digestion at 31°C are indicated above the lanes. The sizes of *Hin*fI digested pAT153 molecular weight marker fragments are indicated. Deletion was monitored by the reduction in size of the smallest pUL522 fragment.

to transform competent *E.coli* DH5 bacteria to ampicillin resistance. Plasmids from colonies were screened for the presence of two *Bam*HI sites and an appropriate degree of Bal31 deletion using small scale plasmid preparations. One such plasmid, pJC1, was analysed in further detail.

*Hin*dIII digested parent plasmid, pUL522, and *Bam*HI digested pJC1 were cut with *Eco*RI and the products analysed by agarose gel electrophoresis. This revealed that between 60bp and 100bp of the inserted fragment adjacent to the *Hin*dIII site of pUL522 had been deleted in plasmid pJC1 (**Figure 17**).

Further restriction analyses on a non-denaturing 5% polyacrylamide gel, shown in **Figure 18**, revealed that an *Sst*II site 138bp 5' to the initiation codon of the UL52 gene had been deleted in pJC1. Digestion with *Sst*II and *Bam*HI facilitated a more precise determination of the extent of Bal31 deletion. Comparison of lanes 2 and 4 in **Figure 18** reveals the loss of a 158bp fragment (present in pUL522) from pJC1. This indicates the deletion of the *Sst*II site closest to the *Hin*dIII site of pUL522 (**Figure 15**). The generation of a novel *Bam*HI-*Sst*II fragment in pJC1 (**Figure 18**, lane 3), estimated to be 105bp in length, demonstrates the removal of approximately 80bp from the end of the *Hpa*I fragment.

The end point of this deletion was determined, by dideoxynucleotide DNA sequence analysis. A 15mer oligonucleotide primer (nucleotides 109062-109047 of the HSV-1 genome) was used to determine the nucleotide sequence upstream of the initiator codon of gene UL52 in pUL522 and pJC1. As shown in **Figure 19**, the nucleotide sequences of pUL522 and pJC1 diverge at nucleotide 108967. A *Bam*HI site occurs at this point in pJC1, marking the limit of Bal31 digestion and the ligation of a *Bam*HI linker. This indicates that 84bp have been deleted from the *Hpa*I site leaving an untranslated leader sequence of 81bp preceding the initiation codon of the UL52 gene.

(a) Cloning of the UL52 ORF Into p23

5μg of DNA from a large scale plasmid preparation of pJC1 was digested with *Bam*HI. The *Bam*HI ends of the fragments were filled in and the fragment containing the UL52 gene was isolated and blunt-end ligated into the cut and filled in unique *Xho*I site of plasmid p23. DH5 bacteria were transformed with the ligation products and selected for

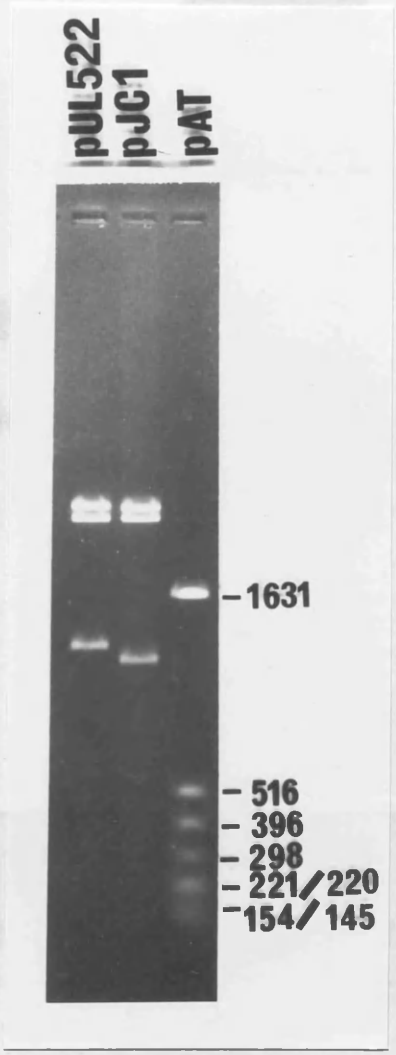


Figure 17. Mapping of *Bal* 31 deletion in pJC1 (I)

*Hin*dIII linearized pUL522 and *Bam*HI digested pJC1 were digested with *Eco*RI and the fragments separated on a 1% agarose gel. The sizes of *Hin*fI digested pAT153 molecular weight marker fragments are indicated. The reduced size of the smallest fragment in pJC1 indicates a deletion of 60-100bp of the inserted fragment from the *Hin*dIII site of pUL522.

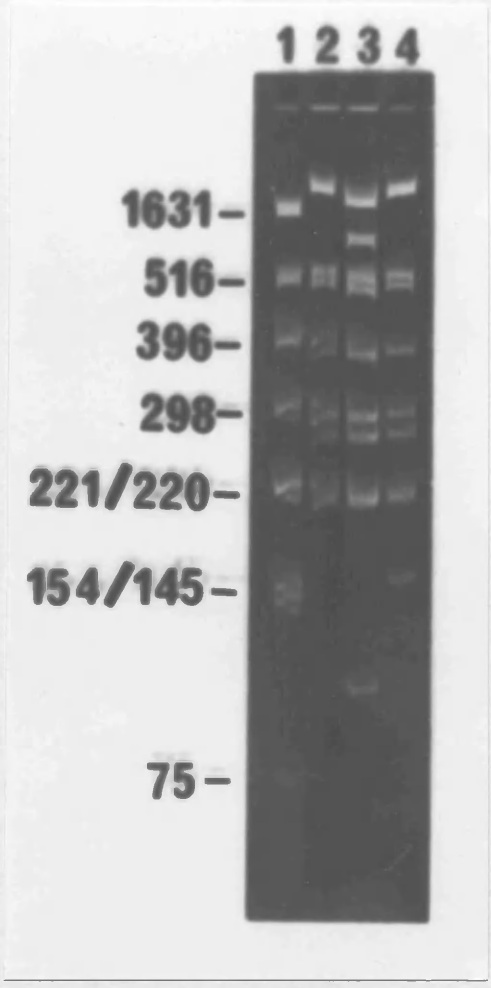
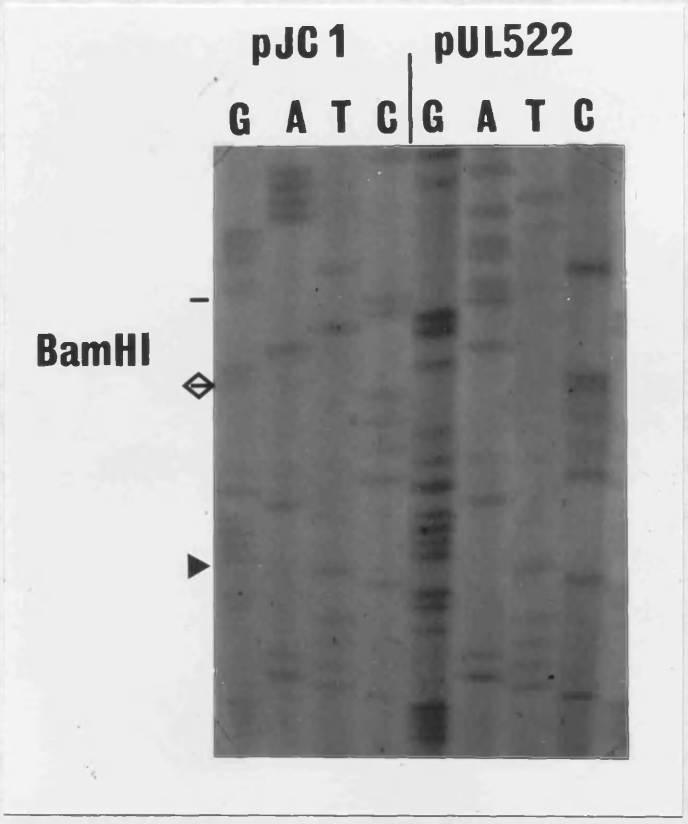


Figure 18. Mapping of *Bal* 31 deletion in pJC1 (II)

The figure shows restriction digests of pUL522 and pJC1 separated by PAGE. Lane 2) *Sst*II digest of pJC1, lane 3) *Sst*II/*Bam* HI digest of pJC1, lane 4) *Sst*II digest of pUL522. *Hinf*I digested pAT153 molecular weight marker fragments are indicated in lane 1. Sizes of *Sst*II fragments are shown in **Figure 15.**

Figure 19. Mapping of *Bal* 31 deletion in pJC1 (III)

The figure shows dideoxynucleotide sequencing performed on pUL522 and pJC1, as indicated. Reaction products were resolved on a 6% urea-denaturing polyacrylamide gel. The sequences read from the position of the arrow (nucleotide 108980) are shown below. The end-point of the deletion was determined as nucleotide 108967 (indicated by the open diamond). Beyond this nucleotide the sequences of pUL522 and pJC1 diverged. The introduction of a novel *Bam*HI site was apparent in pJC1 sequence.



Nucleotide Sequence from Position 108980

pUL522: G G G G A G C G C G C C C C G A G G A A C

◇

PJC1: G G G G A G C G C G C C C G G A T C C G G

ampicillin resistance. Colonies were picked and mini-plasmid DNA preparations tested for the presence and correct orientation of an insert by *Kpn*I and *Hin*dIII digestion (**Figure 20a**). The position of a unique *Kpn*I site within the UL52 ORF relative to the *Hin*dIII site immediately 5' of the IE3 promoter indicates the orientation of the insert (**Figure 20b**). Fragments of 6140bp, 3459bp and 1626bp are predicted which indicate the correct orientation for transcription of the UL52 ORF from the IE3 promoter. Fragments of 8715bp, 1626bp and 884bp indicate the reverse orientation. Plasmid p206, which contains the UL52 fragment in the appropriate orientation, was chosen for attempted recombination with *ts*K (**Figure 20**).

2. Selection and Isolation of *ts*K Recombinant Viruses

The thymidine analogue 5'bromo-deoxycytidine (BCdR) can be used to select for the growth of TK deficient HSV. The viral, but not the host TK, is capable of phosphorylating BCdR generating BrdCTP which inhibits viral replication. Because BCdR is not phosphorylated in cells infected with TK deficient HSV, virus replication is not inhibited. TK deficient viruses arising from homologous recombination between the p23-derived plasmids, which carry an interrupted TK gene, and the *Bam*HI *p* region of the *ts*K genome were therefore selected and enriched for by passaging in the presence of BCdR.

Duplicate sets of confluent monolayers of BHK cells in 50mm Petri dishes were co-transfected, using the calcium phosphate precipitation method (Corsalo and Pearson 1981), with *ts*K viral DNA and restriction enzyme cut plasmid DNA. Plasmids pUL59, pC6, and p206 were cut with *Bam*HI which cleaves at either end of the inserted *Bam*HI*p* fragment. Because the UL9 gene contains a *Bam*HI site, plasmid pIE9 was linearised within the vector sequences with *Sca*I. Following treatment of the transfected cells with 20% DMSO solution at 4hr post-transfection one set of monolayers was overlaid with 1xBHK medium supplemented with 5% newborn calf serum (set A), and the remaining set (set B) overlaid with the same medium plus 2% human serum.

After 3 days incubation at 31°C set B were stained with Giemsa and examined for the presence of plaques. Plaques were observed on the monolayers co-transfected with *ts*K viral DNA and each of the cut plasmid DNAs or transfected with *ts*K viral DNA alone. Therefore, *ts*K DNA is infectious and its ability to give rise to progeny is not

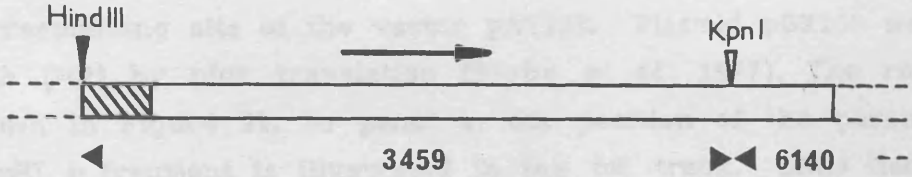
Figure 20. Cloning of the UL52 ORF into p23

*Kpn*I/*Hin*dIII digestion of mini-plasmid DNAs prepared from colonies obtained following transformation with the products of ligation between the insert-containing *Bam*HI fragment of pJC1 and plasmid p23 is shown in part **a**. Lanes 2-16 respectively contain mini-plasmid preps p201, 202, 204, 206, 207, 209, 211, 213, 214, 217, 218, 220, 221, 222 and 224. Lane 1 contains *Hin*dIII digested p23 which yields fragments of 5970bp and 1626bp (**Figure 14**). No *Kpn*I sites are present within p23. The position of a unique *Kpn*I site within the UL52 ORF relative to the *Hin*dIII site at the 5' end of the IE3 gene control region reveals the orientation of the insert, as indicated by the map in part **b**, in which the sizes (bp) of predicted fragments are shown. The IE3 promoter region is hatched and the direction of the UL52 ORF indicated. Plasmid p206, which exhibits the correct orientation (panel **i**), was used in subsequent co-transfection experiments.

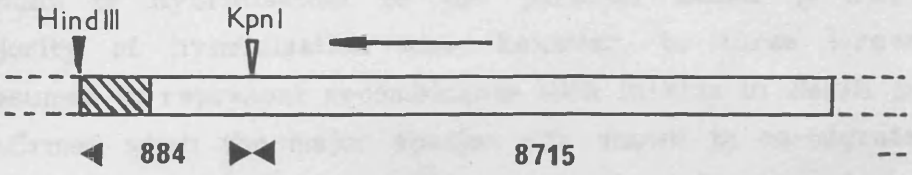
a


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

b i


HindIII
KpnI
3459
6140

ii


HindIII
KpnI
884
8715

inhibited by the cut plasmid DNAs. As expected, no plaques were observed on monolayers transfected only with cut plasmid DNA.

The cells and medium from the plates in set A were harvested and sonicated and the virus progeny titrated at 31°C, in duplicate, on BHK cell monolayers in 35mm Petri dishes. After virus absorption, one set of monolayers was overlaid with EC5Hu2 and the other set with the same medium additionally supplemented with 100μg/ml BCdR. This allowed determination of total virus progeny and TK deficient progeny respectively.

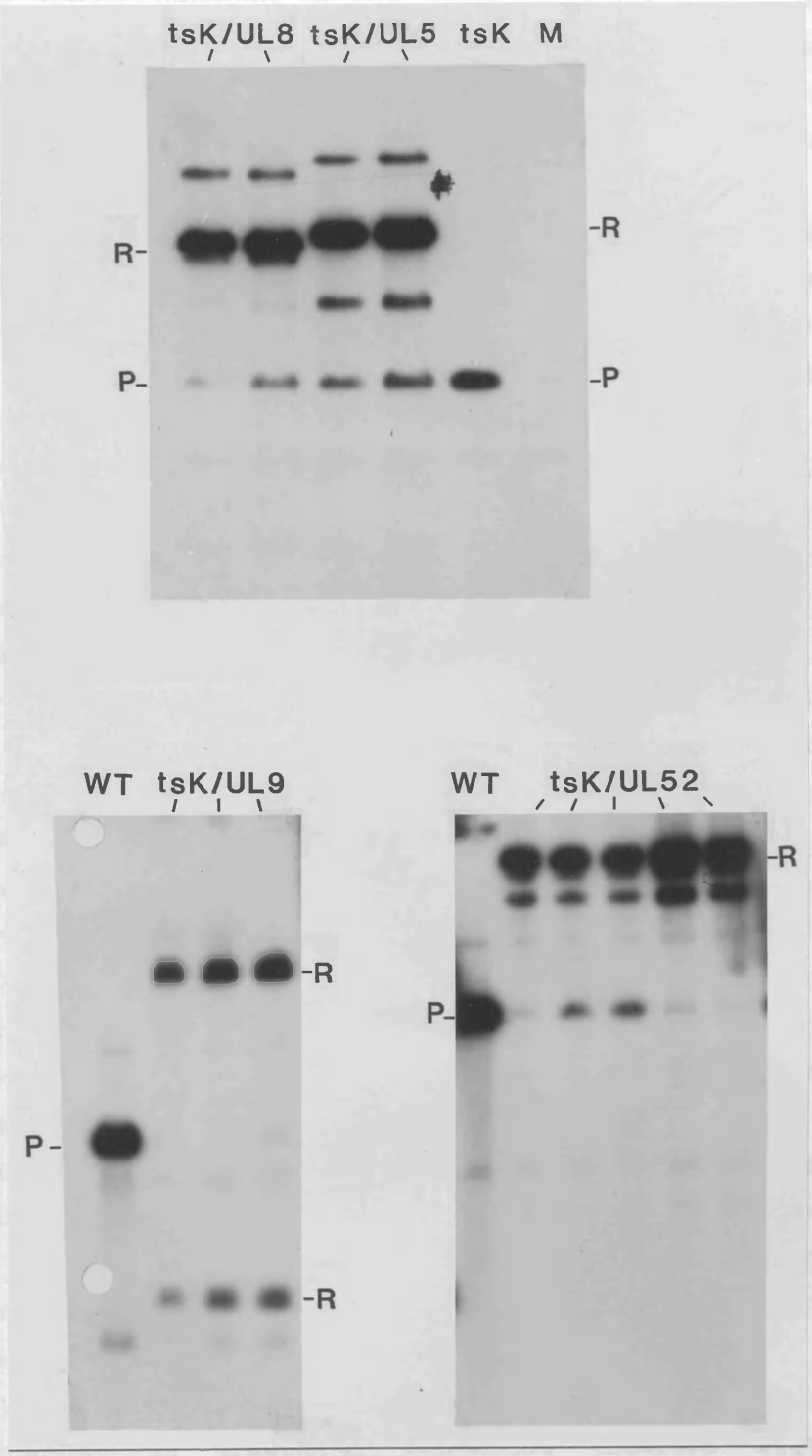
After 3 days the monolayers were stained and plaques counted. Approximately 0.5% of the virus progeny from cells co-transfected with *tsK* and the plasmid DNAs were TK deficient, although this may include TK deficient viruses other than the desired recombinants.

The TK deficient recombinant virus progeny from each co-transfection was enriched by a further two passages at approximately 0.1pfu/cell in the presence of BCdR. From the final passage, the medium of each plate was removed and retained as virus stock. DNA was prepared from the infected cell monolayer and the proportion of recombinant virus present estimated by restriction analyses. DNAs were cut with *Bam*HI or *Sst*II and electrophoresed through a 0.8% agarose gel in 1xLB, then transferred to a nitrocellulose membrane by the method of Southern, (1975) and probed with the plasmid pGX153 which contains the HSV-1 *Bam*HI *p* fragment inserted into the corresponding site of the vector pAT153. Plasmid pGX153 was labelled with [^{32}P] by nick translation (Rigby *et al.*, 1977). The results are shown in **Figure 21.** In panel **a**, the position of the parental HSV-1 *Bam*HI *p* fragment is illustrated in the *tsK* track. DNAs derived from co-transfections of *tsK* with pUL59, or with pC6, exhibited a weak amount of hybridisation to the parental *Bam*HI *p* fragment. The majority of hybridisation was, however, to three larger species, presumed to represent recombinants with inserts in *Bam*HI *p*. This was confirmed when the major species was shown to co-migrate with the *Bam*HI *p* derived fragment generated from the transfected plasmid (**Figure 22**). The migration difference between the major species was as expected from the different sizes of the inserted ORFs. The ratio of parental *Bam*HI *p* fragment to major insert-containing fragment suggested that in each case at least 80% of the progeny was recombinant virus containing an additional copy of either the UL5 or

Figure 21. Enrichment of *tsK* Recombinant Viruses

Southern blots hybridized to radio-labelled pGX153 are shown.

DNA was prepared from BHK cells mock-infected (M) or infected at the PT with wild-type HSV-1, *tsK* or enriched pools containing *tsK* recombinant viruses (*tsK*/UL5, *tsK*/UL8, *tsK*/UL9 or *tsK*/UL52) as indicated. Panels **a** and **c** show DNAs digested with *Bam*HI. Hybridization to to parental *Bam*HI *p* fragment (P) and the slower migrating recombinant fragment (R) is indicated. Panel **b** shows DNAs digested with *Sst*II and hybridization to parental (P) and recombinant (R) fragments. For each of the enriched pools hybridization to the fragment(s) corresponding to recombinant virus is substantially greater than to the parental virus fragment indicating that the enriched stocks contain at least 80% recombinant virus.


a
tsK/UL8
tsK/UL5
tsK
M
R-
-R
P-
-P
b
WT
tsK/UL9
-R
P-
-R
c
WT
tsK/UL52
-R
P-

UL8 ORF. The significance of the weaker hybridisation to the two other novel sized DNA fragments is not clear. It may possibly be due to inversions and/or deletions of the virus genome arising from the presence of second copies of the inserted viral genes (Pogue-Geile *et al.*, 1985). The very weak hybridisation to bands common to all tracks, including mock-infected,may be due to some homology between pGX153 and host DNA sequences.

Figure 21, panel **c** shows DNA from co-transfections of *ts*K with p206 hybridised to pGX153. Weak hybridisation to the parental *Bam*HI *p* fragment is apparent as well as hybridisation to two larger species which were presumed to represent an insert within *Bam*HI *p*. The largest species was subsequently shown to co-migrate with the *Bam*HI fragment generated from the transfected plasmid (**Figure 22**), thus confirming the presence of an inserted UL52 ORF. The ratio of parental *Bam*HI *p* to this insert-containing species suggested that over 80% of the progeny were recombinant viruses.

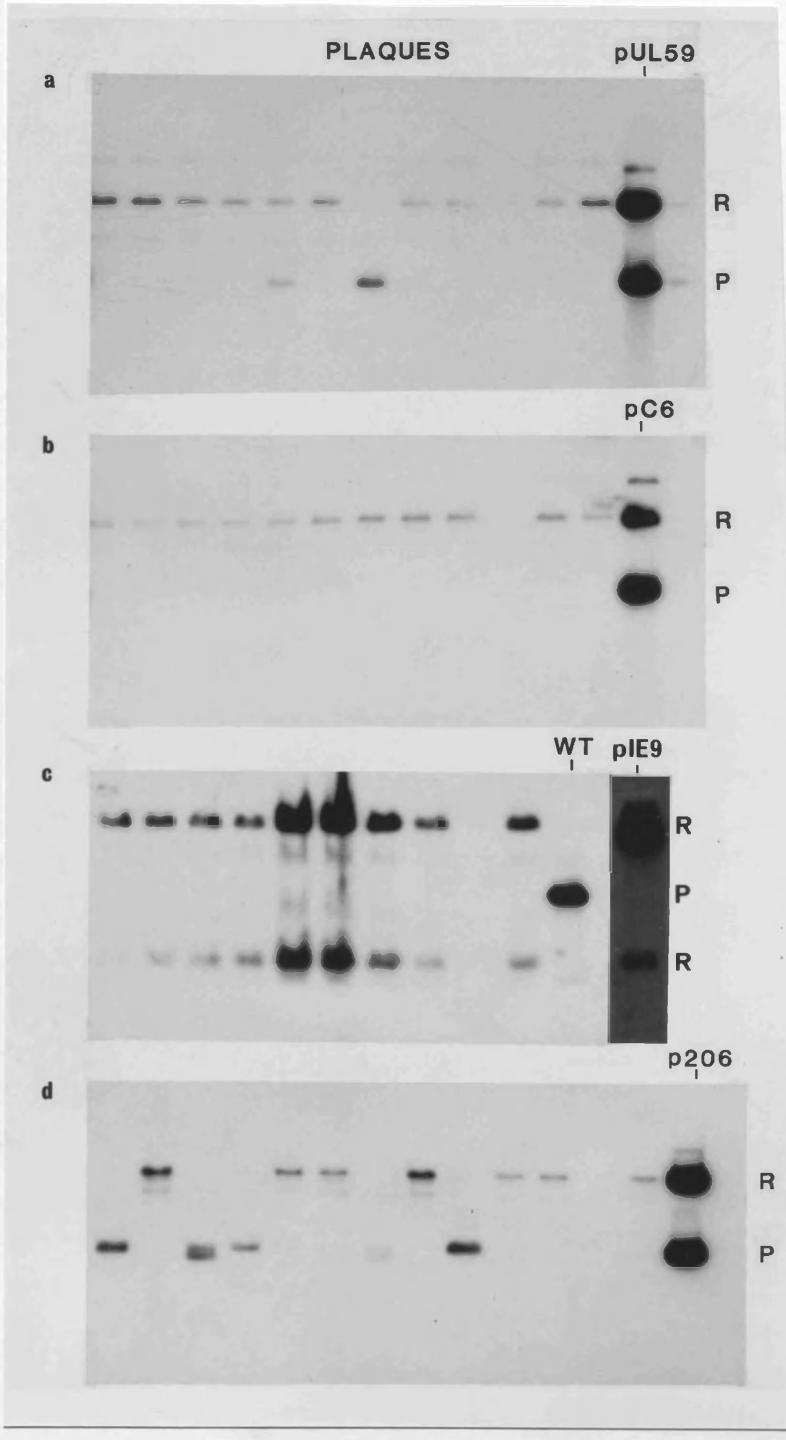
Figure 21, panel **b** shows analysis of DNA of the progeny derived from the co-transfection of *ts*K with pIE9. Since there is a *Bam*HI site within the UL9 gene coding region which would give rise to two predicted fragments fairly close in size to HSV-1 *Bam*HI *p* (3517bp and 3292bp) DNAs were cut with *Sst*II. *Sst*II HSV-1 fragments which hybridize to pGX153 are indicated in **Figure 23.** Digestion of plasmid pIE9 DNA gives rise to four major hybridising species (4513bp, 3987bp, 1073bp and 756bp; **Figure 22**). The 4513bp and 1073 bp fragments are generated from within the inserted HSV-1 sequences (**Figure 23c**). The large fragment migrates slightly slower than the 3987bp fragment which is detected because of hybridisation between pAT153 vector sequences in pIE9 and pGX153. *Sst*II digested *wt* HSV-1 DNA exhibits major hybridisation to two species (2259bp and 756bp; **Figure 23a**). The 2259bp fragment was regarded as diagnostic of the presence of parental *Bam*HI *p* sequences and the presence of 4513bp and 1073bp fragments as representing an insertion of the UL9 gene within *Bam*HI *p*. Hybridization to DNA derived from co-transfections was almost exclusively to fragments containing inserted sequences suggesting that the progeny consists of greater than 90% recombinant viruses (**Figure 21b**).

The above enriched progeny virus pools were plaque-purified by limiting dilution on microtitre-well BHK monolayers. The cells and medium of wells containing a single plaque were harvested and used

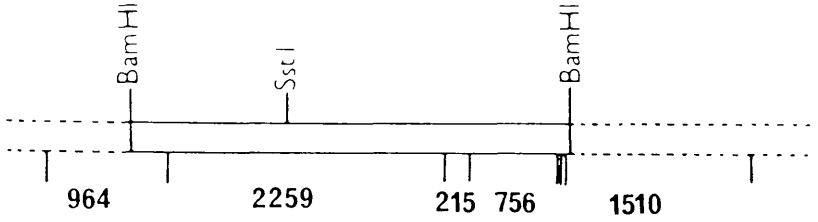
Figure 22. Purification of *ts*K Recombinant Viruses

Southern blots hybridized to radio-labelled pGX153 are shown.

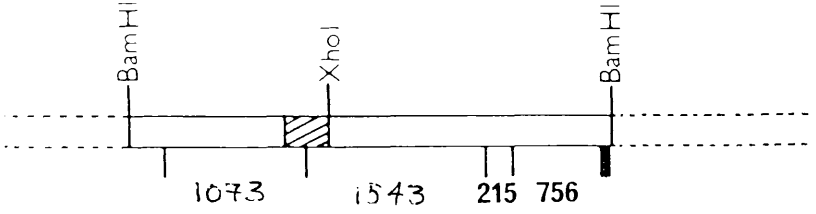
DNA was prepared from microtitre well monolayers of BHK cells infected at the PT with progeny of single plaques isolated from the enriched pools. In panels **a** (*ts*K/UL5 pool), **b** (*ts*K/UL8 pool) and **d** (*ts*K/UL52 pool) DNAs were digested with *Bam*HI. Marker plasmid DNAs, pUL59, pC6 and P206 respectively contain copies of the UL5, UL8 and UL52 genes inserted into the *Bam*HI *p* fragment. In panel **c** (*ts*K/UL9 pool), DNAs were digested with *Sst*II with wild-type HSV-1 and marker plasmid pIE9 DNAS serving as a control. Hybridization to parental (P) and recombinant (R) sequences is indicated. In the case of marker plasmids pUL59, pC6 and p206, the position of the pAT153 vector sequences, which co-migrate with the HSV-1 *Bam*HI *p* fragment, were taken as indicating the position of the parental virus fragment.


PLAQUES
pUL59
a
R
P
pC6
b
R
P
WT
pIE9
c
R
P
R
p206
d
R
P

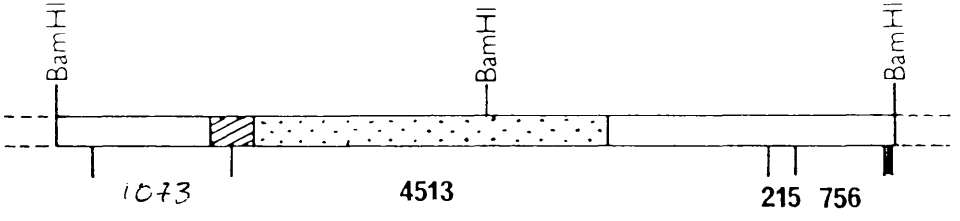
a. wt HSV-1



b. p23



c. pIE9



d. tsK / UL9

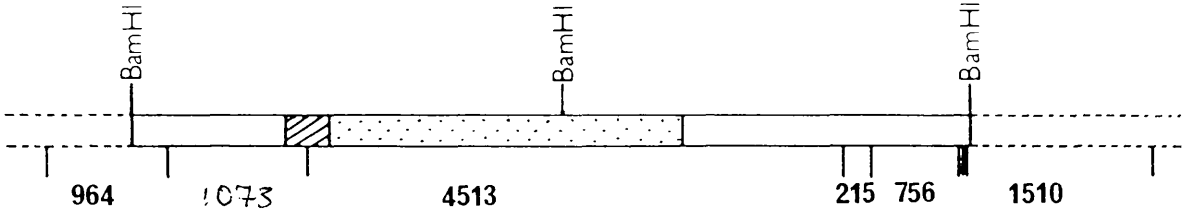


Figure 23. *Sst*II Restriction Fragments Hybridizing to pGX153

The sizes (bp) of major *Sst*II fragments of *wt* HSV-1 (**a**), p23 (**b**), pIE9 (**c**) and *ts*K/UL9 (**d**) DNA capable of hybridization to HSV-1 *Bam*HI *p* sequences within pGX153 are indicated. *Sst*II sites are marked below the solid horizontal lines. Striped and stippled regions respectively represent inserted fragments containing the IE3 promoter and the UL9 ORF. The restriction sites at which these fragments were inserted are also indicated.

to infect linbro well monolayers of BHK cells which were then incubated at 31°C for 3 days at which time an extensive CPE was observed. Virus released into the medium of the wells was stored at -70°C. From the infected cell monolayer total cellular DNA was prepared and analysed by Southern blotting as above. **Figure 22** illustrates the identification of purified recombinant viruses. Purified *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 viruses yielded *Bam*HI fragments which hybridised to a labelled pGX153 probe and co-migrated with the larger of the two fragments resulting from *Bam*HI digestion of plasmids pUL59, pC6 and p206 (respectively, panels **a**, **b** and **d**). Purified *ts*K/UL9 viruses (panel **c**) similarly yielded insert-containing fragments which co-migrated with the 4513bp and 812bp fragments of *Sst*II digested pIE9 DNA and lacked the corresponding *ts*K parental *Sst*II fragment. For progeny from *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 pools, virus was detected in 9 of 11, 11 of 11, 9 of 9 and 7 of 12 wells respectively, which exhibited the structure expected for recombinants containing the required inserted gene.

Elite stocks of the four recombinant viruses were grown from selected purified linbro well supernatants and stored at -70°C. From these elite stocks, working stocks of virus were propagated and titrated on monolayers of BHK cells at 31°C. The titres of the working stocks were generally between 10^8 and $5x10^8$ pfu/ml. Recombinant virus stocks used were named *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52.

To examine the genomic structures of the viruses virion DNA was prepared from each recombinant. Samples of each, as well as *ts*K and *wt* HSV-1 DNAs, were digested with *Bam*HI and run through 0.8% and 0.6% agarose gels, transferred to a nitrocellulose membrane and probed with nick-translated [^{32}P]-labelled pGX153 (**Figure 24**, panel **a**)) or *wt* HSV-1 DNA (**Figure 24**, panel **b**)) respectively. The results show that in the *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 genomes, the parental *Bam*HI *p* fragment (3579bp) is replaced by a larger fragment, and in the *ts*K/UL9 genome by two novel smaller *Bam*HI fragments (predicted sizes 3517 and 3292bp). The remaining *Bam*HI bands detected with the HSV-1 probe are essentially unchanged from the parental viruses showing that the recombinant viruses contain no major structural changes other than the insertion of the additional replication gene. Hybridization of the HSV-1 probe to the novel *Bam*HI fragments is most easily observed in *ts*K/UL9 in which the bands migrate close to the position of the parental *Bam*HI *p* fragment. For the other viruses the

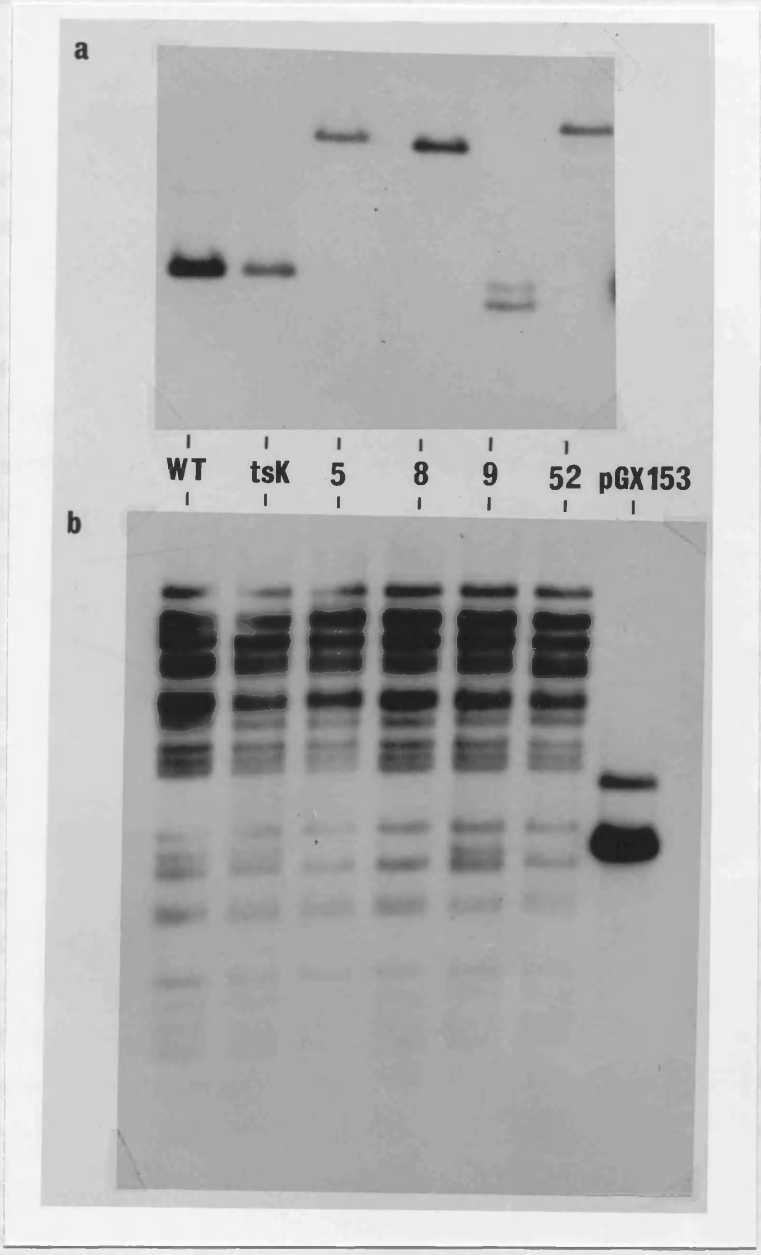


Figure 24. *Bam*HI Restriction Profile of *ts*K Recombinant Viruses

Southern blots hybridized to radio-labelled pGX153 (panel a) and *wt* HSV-1 DNA (panel b) are shown. DNA was prepared from BHK cells infected at the PT with *wt* HSV-1, *ts*K or individual *ts*K recombinant viruses, as indicated, and digested with *Bam*HI.

novel fragments would be expected to comigrate with with other HSV-1 *Bam*HI fragments.

3B. IDENTIFICATION OF THE POLYPEPTIDES ENCODED BY THE EXPRESSED ORFS

1. Over-expression of UL5, UL8, UL9 and UL52 Proteins at the NPT

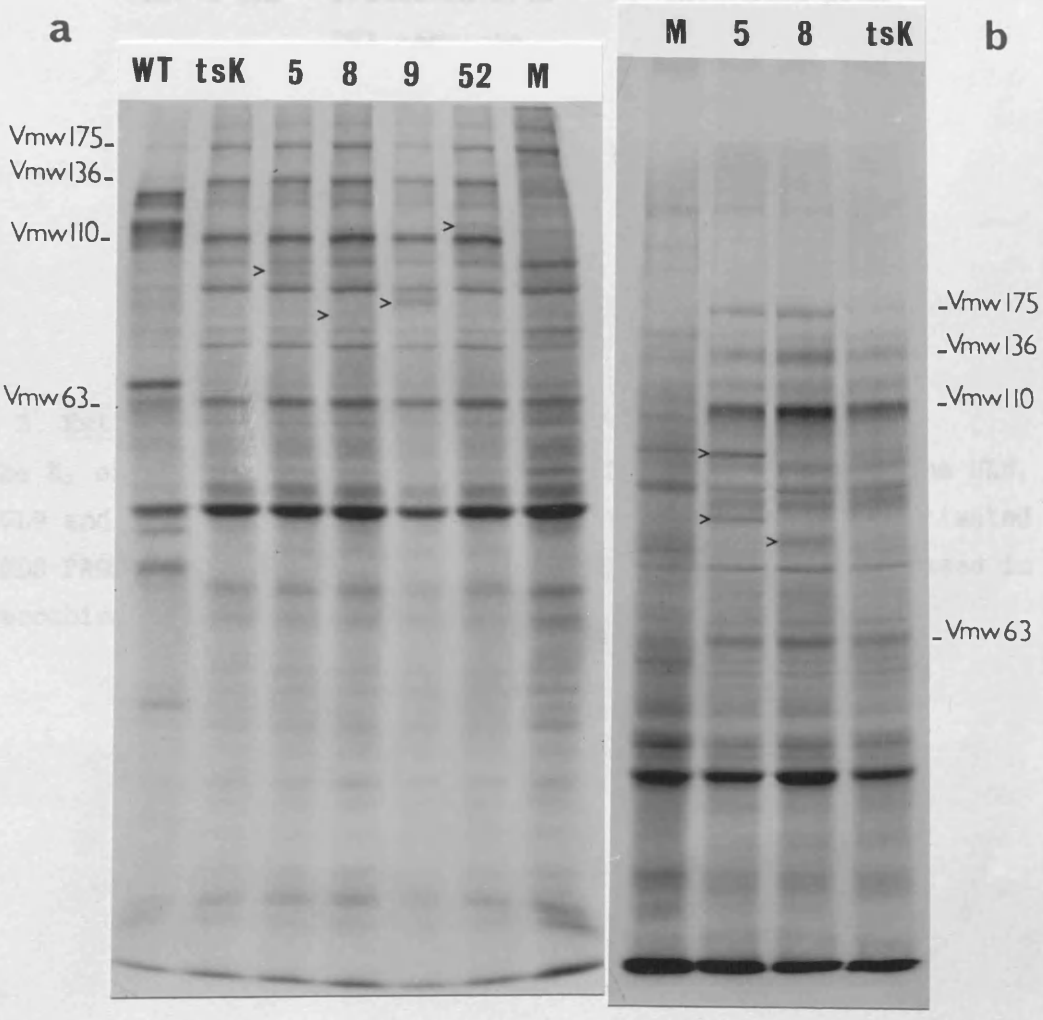
The HSV-1 mutant *ts*K over-produces immediate-early gene products at NPT (Preston,1979a). The products of the inserted ORFs expressed under the control of the IE3 gene promoter and upstream regulatory sequences were therefore examined.

Linbro well monolayers of BHK cells were either mock-infected or infected with *wt* HSV-1, *ts*K or one of the four recombinant viruses, *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 or *ts*K/UL52 at 10 pfu/cell and incubated at the NPT of 38.5°C. At 8hr post-infection the medium of each well was replaced with PBS containing 15μCi of [^{35}S]-L-methionine. At 10hr post-infection this labelling solution was removed and the cells harvested into 200μl of sample buffer. **Figure 25a** shows the analysis of the resulting mock and virus-infected cell extracts by SDS-PAGE. The *ts*K profile shows the major viral IE polypeptides induced by the parental virus ie. Vmw175, Vmw136, Vmw110 and Vmw63. The four recombinant viruses exhibited polypeptide profiles at the NPT indistinguishable from that of *ts*K, with the exception that each produced an additional single polypeptide corresponding to the product of the inserted gene. The UL8 product is more clearly seen in panel **b**. The positions of these unique polypeptide bands are indicated. Molecular weights were estimated for these proteins from a plot of SDS-PAGE mobility against $\log_{10}$ M_r for the major *ts*K induced polypeptides, Vmw175, Vmw136, Vmw110 and Vmw63, whose M_r had been previously independently estimated by other workers (Marsden *et al.*, 1976; Preston, 1979a;b). The values obtained correlate well with the M_r predicted for these proteins from DNA sequence analysis (**Table 3**; McGeoch *et al.* 1988a; b).

On some occasions a minor novel polypeptide band of lower molecular weight was also observed in cells infected at the NPT with the recombinant virus *ts*K/UL5. This may be a proteolytic degradation product of the overproduced protein (**Figure 25b**).

Figure 25. Identification of the UL5, UL8, UL9 and UL52 Polypeptides

Panel a shows ^{35}S-labelled polypeptides prepared from BHK cells mock-infected (M) or infected at the NPT with *wt* HSV-1, *ts*K or individual *ts*K recombinant viruses, *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52, as indicated, and analysed by SDS-PAGE. *ts*K recombinant viruses each exhibited a polypeptide profile indistinguishable from that of *ts*K with the exception of a single, novel band (indicated by the arrows). Panel b shows a similar experiment in which the UL8 protein was more clearly visible and a possible breakdown product of the L5 polypeptide was observed (intact UL8 and UL5 proteins, and the possible breakdown product are indicated). The estimated molecular weight of each novel polypeptide is shown in **Table 3**.


a
WT
tsK
5
8
9
52
M
Vmw175
Vmw136
Vmw110
Vmw63
b
M
5
8
tsK
Vmw175
Vmw136
Vmw110
Vmw63

HSV-1 ORF	Protein M_r	
	Predicted from DNA sequence	Estimated from SDS-PAGE
UL5	98,710	100,000
UL8	79,921	80,000
UL9	94,246	89,000
UL52	114,416	120,000

Table 3. Estimated Mr of Over-Expressed HSV-1 Proteins

The M_r of the polypeptides predicted to be encoded by the UL5, UL8, UL9 and UL52 genes are given in column 2. M_r values estimated from SDS-PAGE for these proteins when individually over-expressed in *ts*K recombinant viruses are given in column 3.

2. Immunoprecipitation of Expressed Polypeptides

The identities of the products of the expressed genes were confirmed by immunoprecipitation with cognate anti-sera provided by Dr M D Challberg. Extracts were prepared as described in Methods (Section 2B.30) from BHK cell monolayers infected with the four recombinant viruses, *ts*K or from mock infected cells. Each expressed protein was specifically precipitated by its cognate anti-serum from recombinant virus infected cells only (**Figure 26**). Possible proteolytic degradation products were also detected in some cases by this procedure (e.g. of UL9 and UL52 proteins). The presence of higher molecular weight proteins precipitated from the UL9 and UL52 extracts is difficult to explain. However, this may be due to their association either specifically or non-specifically with the over-expressed proteins. Unfortunately, pre-immune sera with which to perform control immunoprecipitations were not available.

3. Preliminary Characterization of the UL5, UL8, UL9 and UL52 Proteins

(a) Intracellular Localisation

Initial studies of the four over-expressed proteins investigated their intracellular localisation. BHK cell monolayers were infected at the NPT with each of the four recombinant viruses, labelled with [^{35}S]-L-methionine at 8hpi and harvested at 10hpi. The cells were fractionated into cytoplasm and nuclei as described in Methods (Section 37). As shown in **Figure 27a** the UL5 and UL52 gene products appeared to be present in both cytoplasm and nuclei whereas the UL9 gene product appeared to localise almost exclusively in the nuclei. The UL8 protein is not readily visible in this experiment. Panel **b** shows polypeptides extracted from BHK cells infected with *ts*K/UL8. Lane 1 contains total cell extract. Lanes 2 contains polypeptides present in cytoplasm and lanes 3 and 4 contain nuclear proteins. Salt-extractable nuclear polypeptides are shown in lane 3 and the remaining insoluble pellet in lane 4. The UL8 protein, indicated by the arrow, appears to be present in both cytoplasm and nuclei. Since nuclear polypeptides may leach from cell nuclei during fractionation or cytoplasmic proteins adhere to the surface of nuclei, studies of the intracellular localisation of these proteins also included the use of indirect immunofluorescent techniques. The results of these experiments are described in Section 3G.

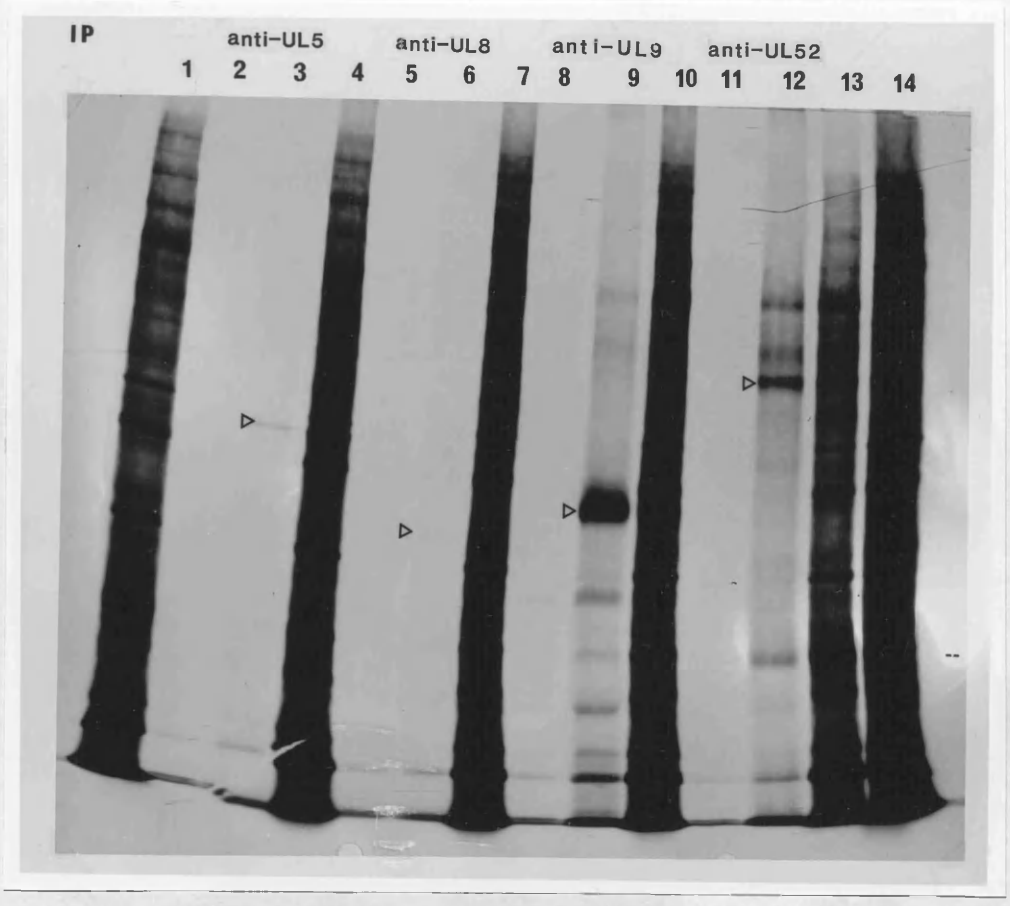


Figure 26. Immunoprecipitation of the UL5, UL8, UL9 and UL52 Proteins

Protein extracts were prepared from BHK cells either mock-infected or infected at the NPT with *ts*K or individual *ts*K recombinant viruses. Polypeptide profiles of each starting extract are shown; lane 1) *ts*K, lane 4) *ts*K/UL5, lane 7) *ts*K/UL8, lane 10) *ts*K/UL9, lane 13) *ts*K/UL52 and lane 14) mock-infection. Extracts were reacted with antisera and analysed in parallel. Lanes 2 and 3: *ts*K and *ts*K/UL5 extracts reacted with anti-UL5 antiserum, lanes 5 and 6: *ts*K and *ts*K/UL8 extracts reacted with anti-UL8 antiserum, lanes 8 and 9: *ts*K and *ts*/UL9 extracts reacted with anti-UL9 anti-serum and lanes 11 and 12: *ts*K and *ts*K/UL52 extracts reacted with anti-UL52 antiserum. The positions of the over-expressed UL5, UL8, UL9 and UL52 proteins precipitated by their cognate antisera are indicated by the arrows in lanes 3, 6, 9, and 12 respectively.

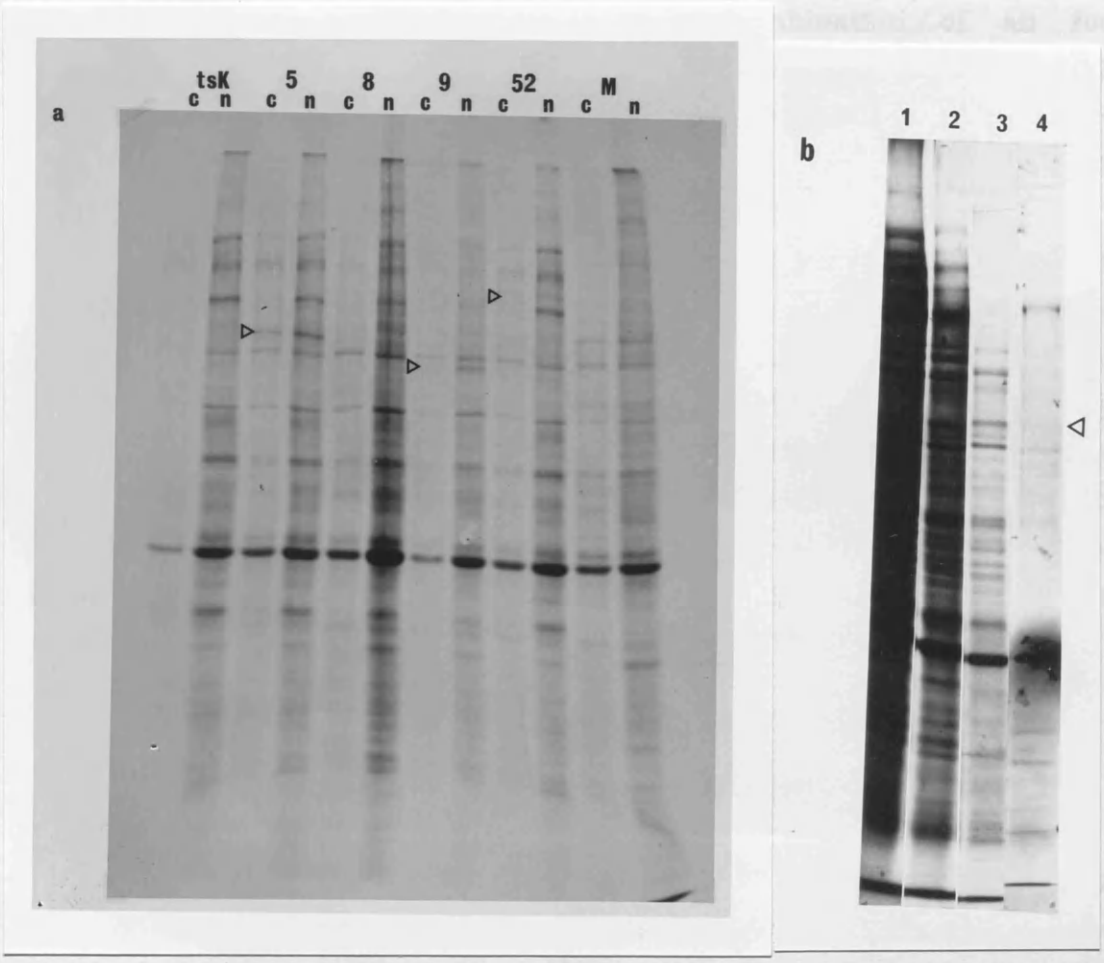


Figure 27. Fractionation of BHK cells Infected at the NPT

Panel a shows SDS-PAGE analysis of ^{35}S-labelled BHK cells mock-infected or infected at the NPT with *ts*K or individual *ts*K recombinant viruses, as indicated, and subsequently fractionated into cytoplasm (c) and nuclei (n). Panel b shows fractionation of ^{35}S-labelled BHK cells infected at the NPT with *ts*K/UL8. Lanes contain polypeptides present in 1) total cells, 2) cytoplasm, 3) nuclear extract and 4) nuclear pellet.

The over-expressed proteins are indicated by the arrows. UL5, UL8 and UL52 polypeptides are present in both cytoplasm and nuclei whereas the UL9 protein localizes largely within the nucleus.

(b) **Post-translational Modification by Phosphorylation**

To investigate whether the over-expressed UL5, UL8, UL9 and UL52 polypeptides were modified by phosphorylation a comparison of ^{35}S-methionine and ^{32}-orthophosphate labelling was performed. BHK cells in Linbro wells were mock-infected or infected with *wt* HSV-1, *ts*K, a single recombinant virus or a combination of all four recombinant viruses (*ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52). The Linbro well monolayers were labelled with ^{35}S-methionine or ^{32}P-orthophosphate at 8hpi and harvested at 10hpi. Extracts were prepared and analysed by SDS-PAGE (**Figure 28**).

Major labelled phosphoproteins in *ts*K infected cells included Vmw175, Vmw110, Vmw68 and Vmw63. In contrast, none of the over-expressed replication proteins was detected as a ^{32}P-labelled band indicating that the UL5, UL8, UL9 and UL52 proteins are not phosphorylated to a significant extent under these conditions. Additionally, these proteins were unlabelled in the multiple recombinant infection which suggests that they remain unphosphorylated when co-expressed and do not phosphorylate each other. It remains possible that during lytic infection under permissive conditions phosphorylation of these proteins may occur.

3C. FUNCTIONAL CHARACTERIZATION OF GENE PRODUCTS OVER-EXPRESSED BY *ts*K RECOMBINANT VIRUSES

At the time at which this work was being performed functions had been assigned to the products of some of the seven HSV-1 genes required for the replication of viral DNA. These included the viral DNA polymerase holoenzyme which contains subunits encoded by genes UL30 and UL42 (Powell and Purifoy 1977, Vaughan *et al.* 1985, Gallo *et al.* 1988, Crute and Lehman 1989), and a single-stranded DNA binding protein specified by the UL29 gene (Olivo *et al.* 1989 , Weller *et al.* 1983b, Quinn and McGeoch 1985). The UL9 gene product was subsequently characterized as an origin binding protein which binds to specific DNA sequences within the viral origins of DNA synthesis (Olivo *et al.* 1988, Weir *et al.* 1989). This activity was shown to be induced in cells infected at the NPT with the recombinant virus *ts*K/UL9 (Weir *et al.* 1989). In these experiments the four *ts*K recombinant viruses described above were assayed for origin-binding activity but only *ts*K/UL9 was capable of the induction.

Other possible functions for HSV-1 replication proteins included

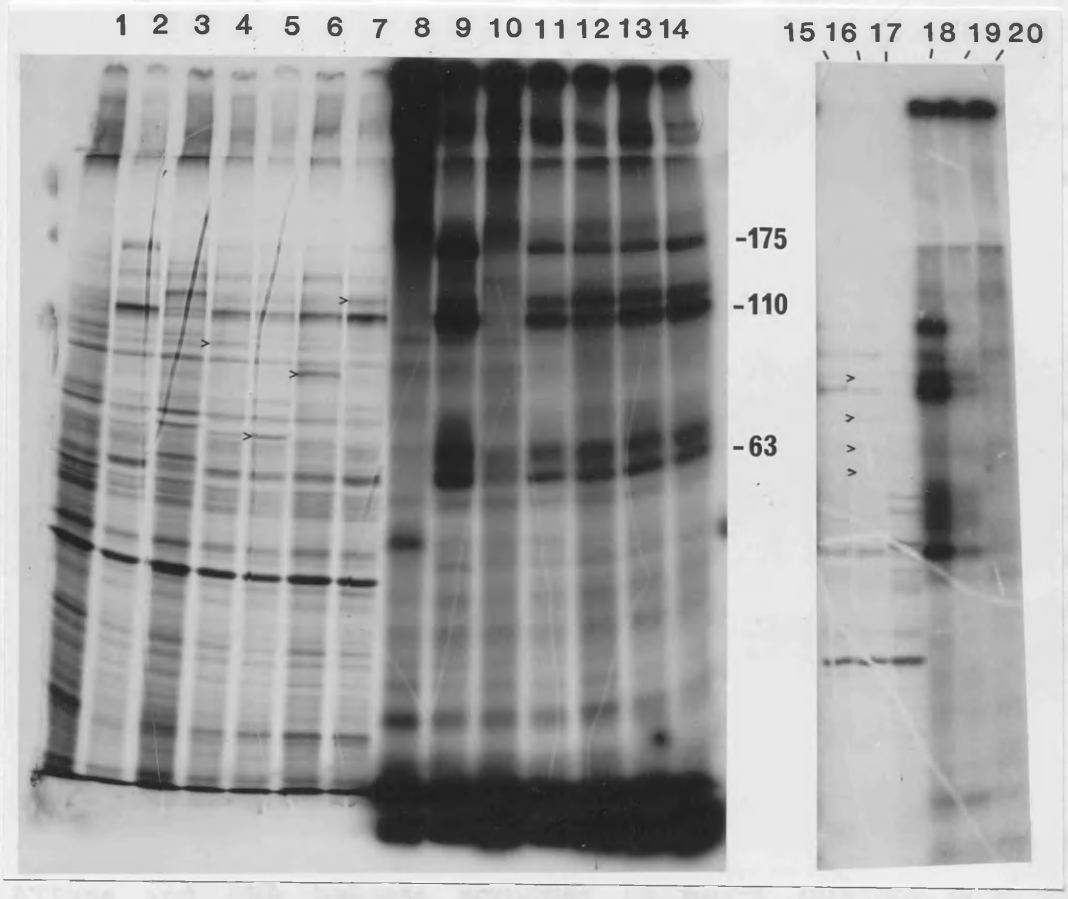


Figure 28. <u>^{32}P-Orthophosphate Labelling of Infected Cell Polypeptides at the NPT</u>

The figure shows analysis of BHK cells infected at the NPT and labelled with ^{35}S-L-methionine (lanes 1-7 and 15-17) or ^{32}P-orthophosphate (lanes 8-14 and 18-20). Infections were as follows: Mock-infection (1 and 8), *ts*K (2, 9, 15 and 18), *wt* HSV-1 (3, 10, 17 and 20), *ts*K/UL5 (4 and 11), *ts*K/UL8 (5 and 12), *ts*K/UL9 (6 and 13), *ts*K/UL52 (7 and 14) and all four *ts*K recombinants (16 and 19).

Comparison of respective ^{35}S- and ^{32}P-labelled lanes shows that none of the over-expressed proteins become strongly phosphorylated under these conditions.

DNA helicase, DNA primase, DNA ligase and topoisomerase activities. When the *ts*K recombinant viruses described above were isolated, these and origin-binding activity were considered likely candidate activities of the over-expressed replication proteins UL5, UL8, UL9 and UL52.

Assays for possible enzymatic activities involved in DNA replication were therefore developed and used in the analysis of extracts from cells infected either singly or multiply, with the recombinant viruses as a means to identifying the functions of the expressed proteins. These studies were initiated with an investigation of the enzymatic activities associated with DNA unwinding.

1. Unwinding of Duplex DNA

Unwinding of the DNA strands during replication is facilitated by DNA helicases (Geider and Hoffman-Berling 1981). This is accomplished in a reaction coupled to the hydrolysis of a nucleoside 5'triphosphate (NTP). Thus all helicases described to date are also DNA-dependent nucleoside 5'triphosphatases (NTPases). Presumably the energy released in the NTP hydrolysis reaction is utilised in the unwinding reaction although how the two reactions are coupled is not clear (Matson and Kaiser-Rogers 1990).

(a) DNA Helicase and DNA-Dependent ATPase Activities

It was considered possible that induction of DNA-dependent ATPase and DNA helicase activities in HSV-1 infected cells might require one or more of the UL5, UL8, UL9 and UL52 gene products. By infecting cells with recombinant viruses individually or in combination, it was hoped to assign these functions to a specific protein or combination of proteins.

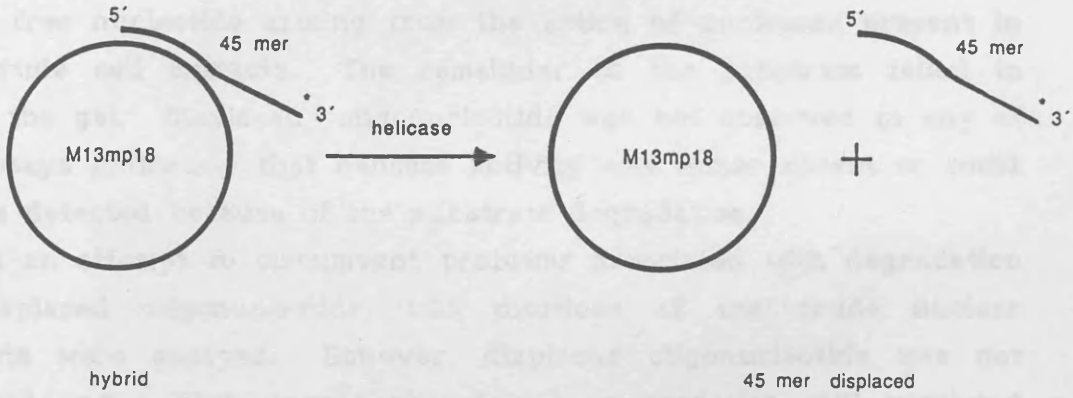
Induction of DNA helicase activity in cells infected with the recombinant viruses was assayed using a substrate consisting of a synthetic labelled oligonucleotide annealed to single-stranded M13mp18 DNA so as to leave an unannealed tail at its 3' end (**Figure 29a**). This structure of substrate had been shown to be required for the activity of a DNA helicase induced in cells infected with HSV-1 (Crute *et al.* 1988). When subjected to electrophoresis through a polyacrylamide gel this substrate barely enters the gel. However, the free labelled oligonucleotide, having been displaced from the single-stranded circle by heat denaturation or helicase activity, migrates rapidly through the gel (**Figure 29b**).

Figure 29. Partial Duplex Substrate for DNA Helicase Assay

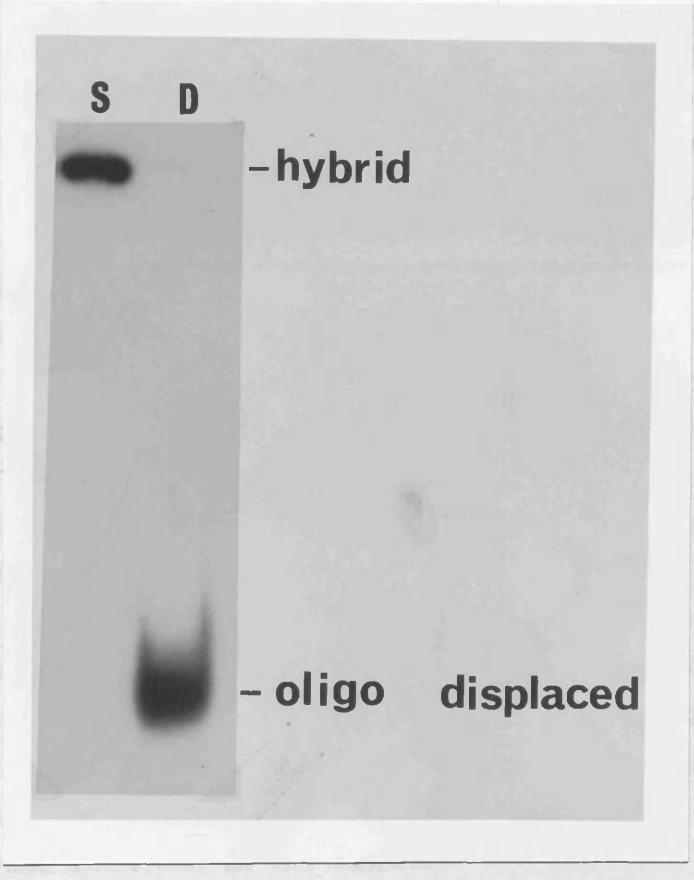
Part (a) of the figure represents the substrate used for assay of HSV-1 DNA helicase (Crute *et al.*, 1988). It comprises a 45 base radio-labelled single-stranded oligonucleotide which is complementary at its 5' end for 23 bases with a single-stranded M13mp18 DNA molecule, to which it is annealed. Unwinding of the duplex region by DNA helicase causes displacement of the oligonucleotide from the circular molecule allowing it to rapidly migrate through a polyacrylamide gel.

(b) The substrate was either unreacted (S) or denatured by heat (D) (90°C for 2 min) and then analysed on a non-denaturing polyacrylamide gel. The positions of the annealed hybrid molecule and the displaced oligonucleotide are indicated.

a


5′
45 mer
3′
M13mp18
helicase
M13mp18
5′
45 mer
3′
hybrid
45 mer displaced

b


S
D
-hybrid
- oligo displaced

Total cell extracts were prepared from BHK cells, either uninfected or infected with *wt* HSV-1, *ts*K, or one of the four recombinant viruses *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 or *ts*K/UL52 and assayed for DNA helicase activity using this substrate. The buffer used was helicase buffer 1, described in Materials and Methods (Section 2B.35). In **Figure 30** the positions of annealed substrate and free oligonucleotide (displaced by heat) are shown. In each sample a proportion of the substrate was degraded generating a fast migrating species, presumed to be free nucleotide arising from the action of nucleases present in the crude cell extracts. The remainder of the substrate failed to enter the gel. Displaced oligonucleotide was not observed in any of the assays indicating that helicase activity was either absent or could not be detected because of the substrate degradation.

In an attempt to circumvent problems associated with degradation of displaced oligonucleotide, 1:25 dilutions of the crude nuclear extracts were assayed. However, displaced oligonucleotide was not detected and a high degree of substrate degradation still persisted (data not shown).

As an alternative approach the induction of DNA-dependent ATPase activity, associated with DNA helicases was measured. An assay for the detection of DNA-dependent ATPase activity was developed based upon procedures used by Crute *et al.* 1988 and Clark *et al.* (1981). The amount of [gamma-^{32}P]ATP hydrolysed to [^{32}Pi] in a reaction containing infected cell extract and activated calf thymus DNA was estimated by removal of unhydrolysed [gamma-^{32}P]ATP from the reaction by adsorption to activated charcoal. Liberated [^{32}Pi] in the supernatant was quantified by liquid scintillation counting.

Total cell extracts were prepared from mock-infected BHK cells and from cells infected with *wt* HSV-1, *ts*K or *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52, either individually or in combination. Each crude extract exhibited DNA-dependent ATPase activity (**Table 4**). However, a significant increase in ATPase activity above levels detected in the control infections was not observed for any of the infections with recombinant viruses. This is probably due to the presence of many host ATPase activities in the extracts.

In an attempt to separate and identify specific enzymatic activities encoded by the expressed genes limited purification was performed on the infected cell extracts.

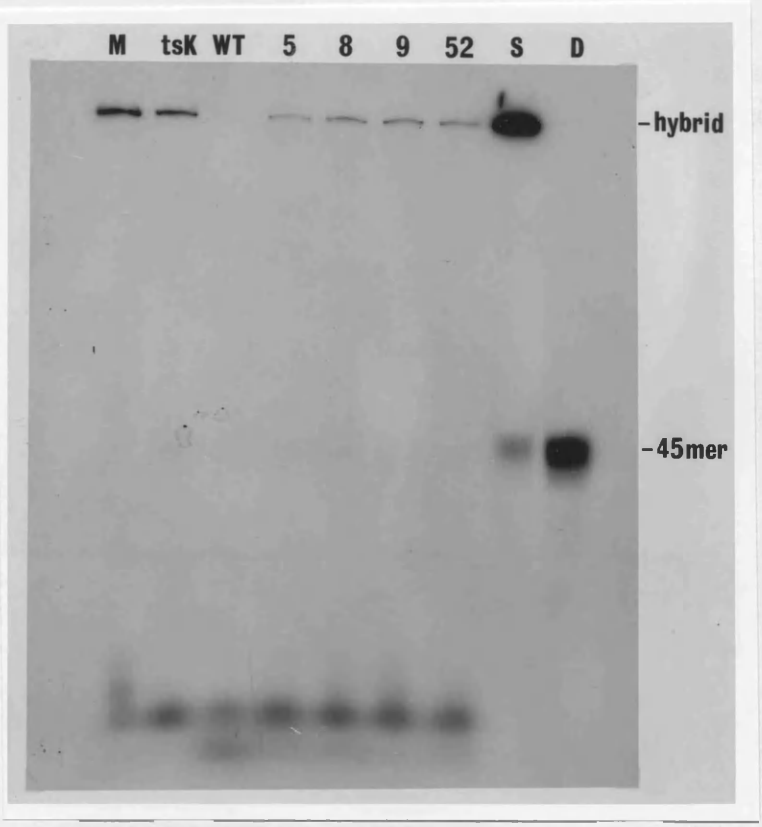


Figure 30. DNA Helicase Assay of Crude Infected-Cell Extracts

Total cell extracts were prepared from BHK cells mock-infected or infected at the NPT with *wt* HSV-1, *tsK* or individual *tsK* recombinant viruses (as indicated) and assayed for DNA helicase activity. Positions of hybrid substrate (S) and displaced oligonucleotide (D) are indicated. No displaced oligonucleotide was observed in any extract assayed. All reactions exhibited degradation of the substrate DNA.

Infection	nmoles ATP hydrolysed per 2.5μg protein
mock	1.53
tsK	1.73
wt HSV-1	1.82
tsK/UL5	1.40
tsK/UL8	1.76
tsK/UL9	1.11
tsK/UL52	1.80
tsK/UL5+*tsK*/UL8	0.85
tsK/UL5+*tsK*/UL9	2.10
tsK/UL5+*tsK*/UL52	2.26
tsK/UL8+*tsK*/UL9	2.50
tsK/UL8+*tsK*/UL52	0.50
tsK/UL9+*tsK*/UL52	0.95
tsK/UL5+*tsK*/UL8+*tsK*/UL9	1.34
tsK/UL5+*tsK*/UL8+*tsK*/UL52	1.52
tsK/UL5+*tsK*/UL9+*tsK*/UL52	2.04
tsK/UL8+*tsK*/UL9+*tsK*/UL52	1.27
tsK/UL5+*tsK*/UL8+*tsK*/UL9+*tsK*/UL52	1.67

Table 4. DNA-dependent ATPase Activity of Crude Cell Extracts

Total cell extracts were prepared from BHK cells infected as indicated in column 1 and assayed for DNA-dependent ATPase activity. Levels of ATP hydrolysis (nmoles) by each extract are indicated in column 2.

Total cell extracts from BHK cells infected either with *ts*K or mixedly with all four recombinants, *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 were , after dialysis, fractionated by column chromatography using the cation exchange medium, phosphocellulose, as described in Materials and Methods (Section 2.41a). Each eluted fraction of approximately 250μl was assayed for ATPase activity. In **Figure 31**, comparison of panels a) and b) reveals a diffuse peak of ATPase activity induced in cells infected with all four recombinants that was not observed in cells infected with *ts*K. This suggests that a specific DNA-dependent ATPase activity may be encoded by one or more of the HSV-1 genes UL5, UL8, UL9, and UL52. Cellular and/or other virally induced ATPases were also detected.

Fractions 7-18 were subsequently assayed for DNA helicase activity, using a substrate essentially as before but with the synthetic oligonucleotide labelled at the 3'end by terminal deoxynucleotide transferase. Comparison of panels **a** and **b** in **Figure 32** reveals that, much greater displacement of the labelled oligonucleotide was detected in fractions from cells mixedly infected with the four recombinants. This suggests the presence of a novel DNA helicase which may be encoded by one or more of the genes UL5, UL8, UL9 and UL52.

SDS-PAGE analysis of these fractions, shown in **Figure 33**, revealed that polypeptides present in fractions possessing ATPase activity included the over-produced UL5, UL8 and UL52 proteins. The UL9 protein was eluted to some extent around 200mM NaCl, perhaps co-inciding with the helicase activity in fractions 15-18, but mostly above 350mM. The above results suggested that the novel DNA-dependent ATPase and DNA helicase activities may be encoded by one or more of these three genes UL5, UL8 and UL52 and that UL9 may also have helicase activity.

3D. THE IDENTIFICATION OF AN HSV-1 ENCODED HELICASE-PRIMASE COMPLEX

In support of the above conclusion a complex possessing DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities was purified from HSV-1 infected cells and shown to comprise three polypeptides of M_r 120,000, 97,000 and 70,000 which were immunochemically identified as the products of the HSV-1 genes UL52, UL5 and UL8 respectively (Crute *et al.*, 1989). Functions of this

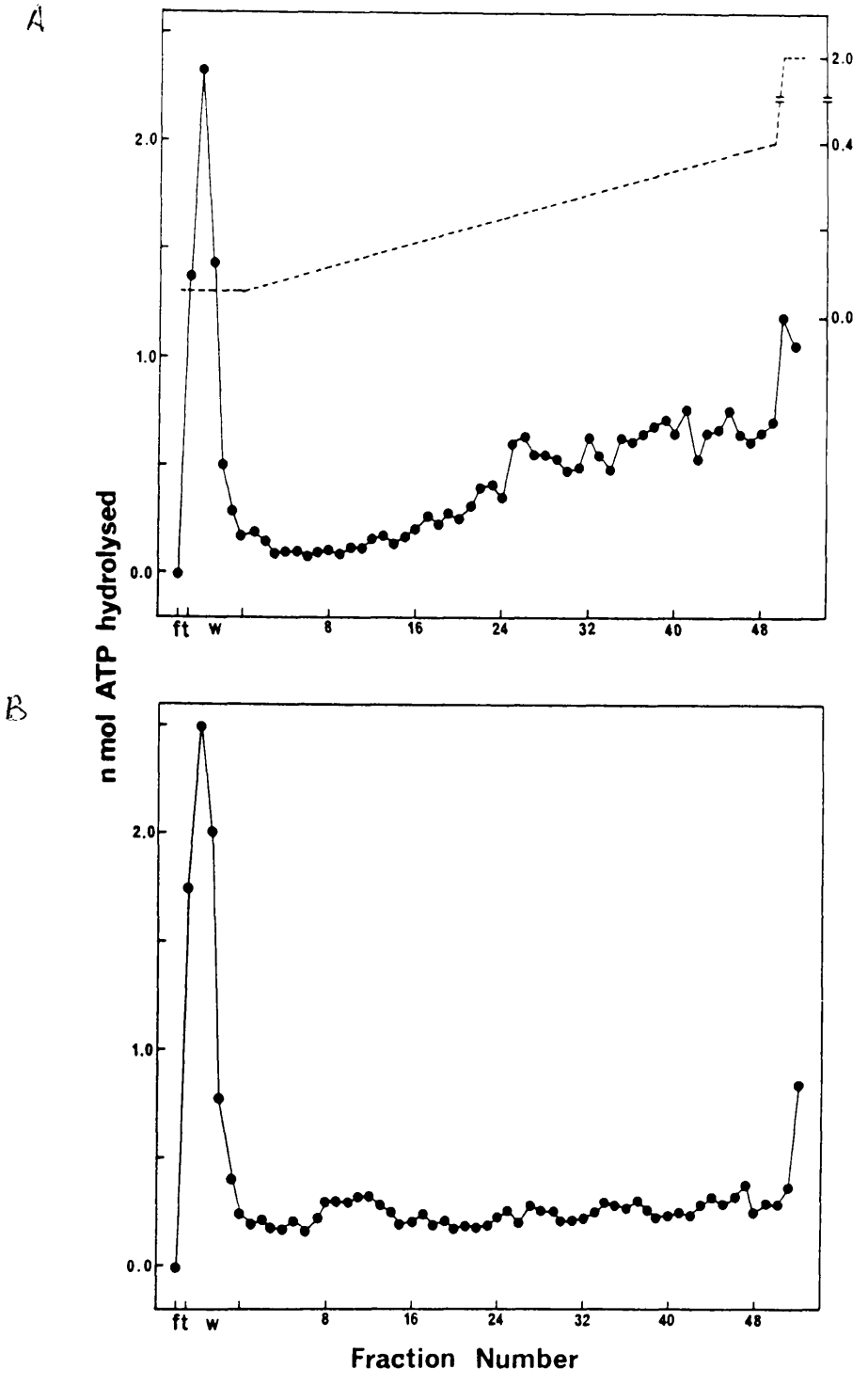


Figure 31. ATPase Activity Induced by All Four *ts*K Recombinant Viruses

Phosphocellulose column fractions of total cell extracts were prepared from BHK cells infected at the NPT, either with *ts*K (a) or mixedly with *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 (b) and assayed for DNA-dependent ATPase activity. The elution gradient applied to the column (M NaCl) is indicated by the broken line in panel a.

A diffuse peak of activity was induced in cells infected with all four *ts*K recombinant viruses (fractions 8-15; 150-180mM NaCl) which was not present in cells infected with *ts*K.

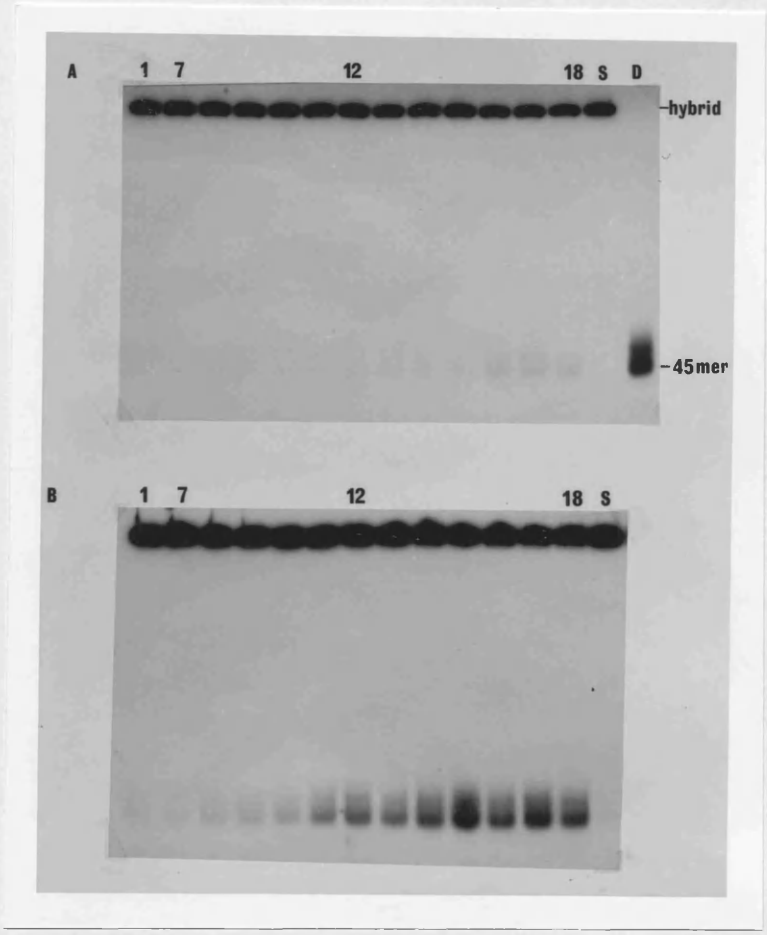


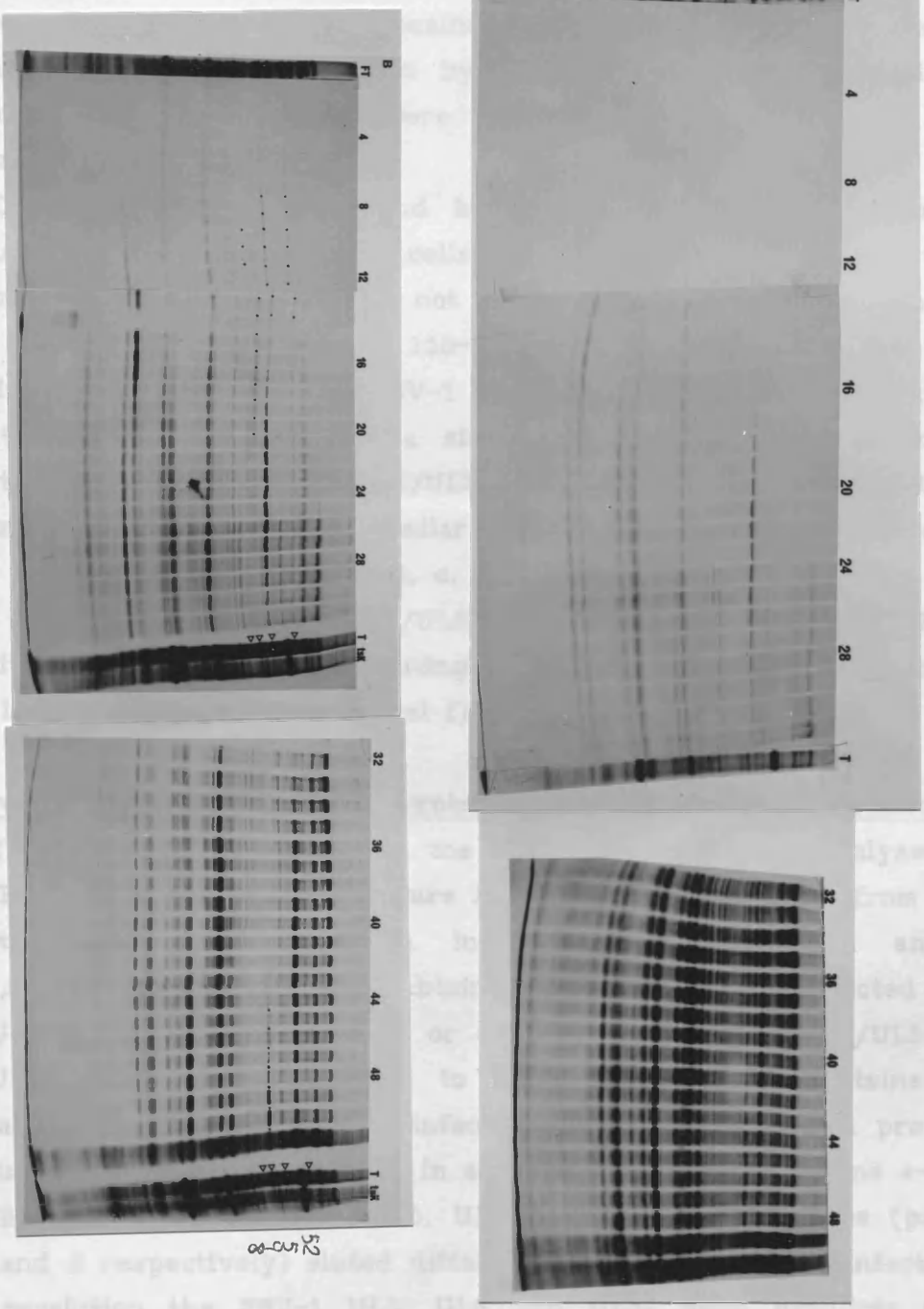
Figure 32. **DNA Helicase Activity Induced by All Four *ts*K Recombinant Viruses**

Selected phosphocellulose fractions previously assayed for ATPase activity (**Figure 31**) were subsequently assayed for DNA helicase activity. Low levels of displacement were apparent in fractions from cells infected with *ts*K (panel a). In panel b, fractions from cells mixedly infected with *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 exhibited greater amounts of oligonucleotide displacement. Displacement occurred chiefly in fractions 11-18, which overlapped the peak previously shown to contain ATPase activity (**Figure 31**).

Figure 33. Polypeptides Present in Phosphocellulose Fractions

The figure shows SDS-PAGE analysis of phosphocellulose fractions from BHK cells infected at the NPT with either *ts*K (panel **a**) or mixedly with *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 (panel **b**). Flow-through (FT) and eluted fractions (numbered) are indicated. Marker lanes containing total cell extract (T) was from cells infected with either *ts*K (panel **a**) or all four *ts*K recombinants (panel **b**). Positions of the over-expressed polypeptides are indicated by the arrows and dots.

The polypeptide profiles obtained reveal the UL5, UL8 and UL52 proteins co-elute in fractions 8-13 (150-180 mM NaCl). Most UL9 protein was eluted above 350mM NaCl although small amounts appeared to elute around 200mM NaCl.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complex and its subunits were therefore further investigated using the *ts*K recombinant viruses.

1. DNA-Dependent ATPase

Total cell extracts were prepared from BHK cells infected with either *ts*K or the three recombinants, *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52, either individually or in all possible combinations. Following dialysis extracts were partially purified by fractionation on phosphocellulose columns and 1ml fractions were collected and assayed for DNA-dependent ATPase activity.

Comparison of panels **a** and **h** in **Figure 34** reveals a peak of ATPase activity induced in cells triply infected with all three recombinant viruses which was not induced in cells infected with *ts*K. This peak of activity eluted at 150-200mM NaCl, a value similar to that previously reported for the HSV-1 helicase-primase complex (Crute et al. 1988). Cells infected with a single recombinant virus or doubly infected with *ts*K/UL5 plus *ts*K/UL8, or with *ts*K/UL8 plus *ts*K/UL52, all exhibited ATPase profiles similar to that observed in cells infected with *ts*K (respectively, panels **b**, **c**, **d**, **e** and **g**). However, where cells were doubly infected with *ts*K/UL5 plus *ts*K/UL52 a peak of ATPase activity was again observed eluting at the same salt concentration as that in the triple infection (panel **f**).

2. Presence of Over-Expressed Proteins in Phosphocellulose Fractions

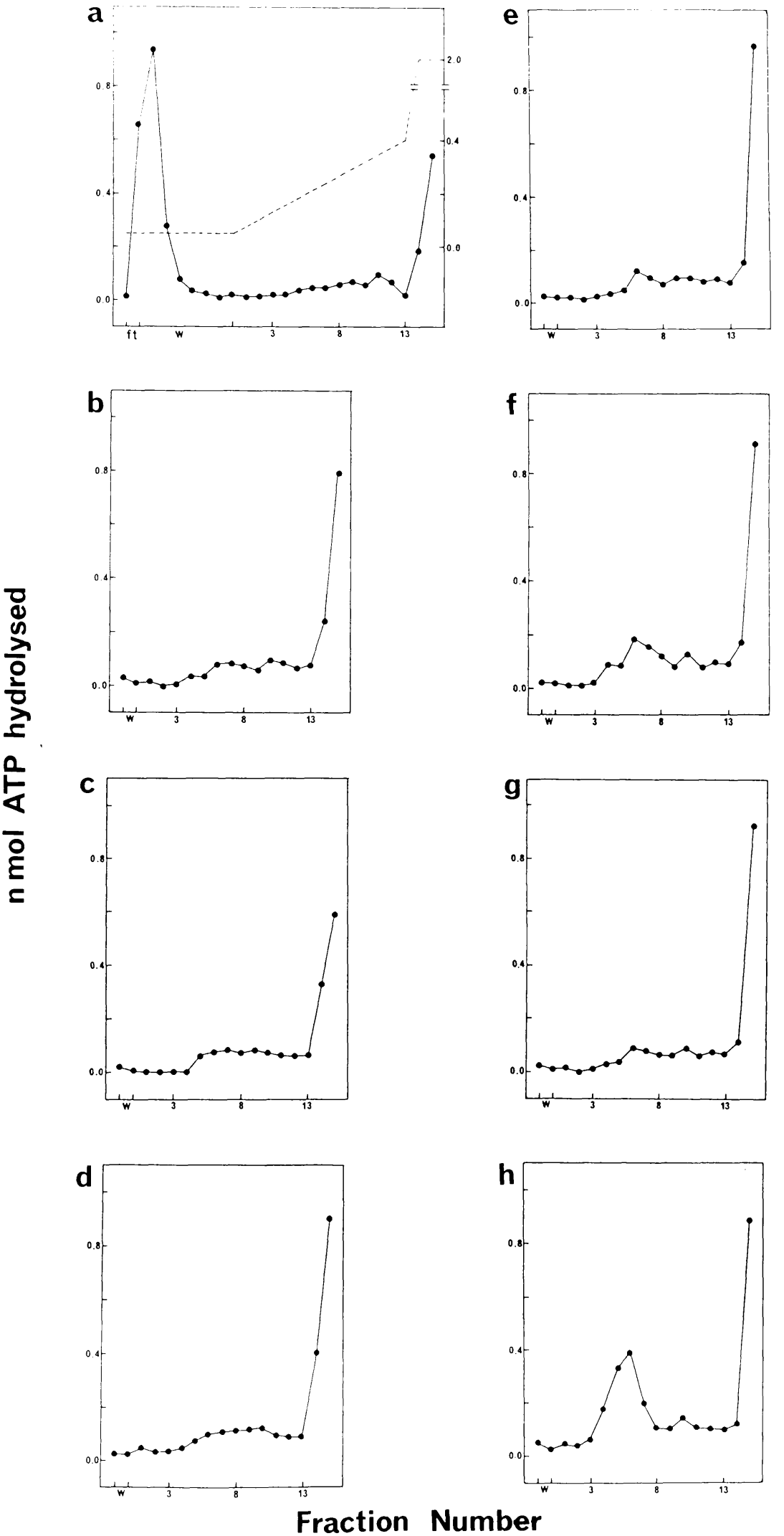
The polypeptides present in the above fractions were analysed by SDS-PAGE and are shown in **Figure 35**. The pattern obtained from cells infected with *ts*K is shown in panel **a**. Panels **h** and **f** show,respectively, the profiles obtained from cells triply infected with *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 or doubly infected with *ts*K/UL5 and *ts*K/UL52. Bands corresponding to the over-produced proteins are indicated. In both recombinant infections the over-expressed proteins co-eluted, coincident with peaks in enzymatic activity (fractions 4-6).

In single infections the UL5, UL8 and UL52 polypeptides (panels **b**, **c** and **d** respectively) eluted differently from these mixed infections. The co-elution the HSV-1 UL5, UL8 and UL52 gene products upon fractionation of triply infected cell extracts therefore suggests that they form a complex which is responsible for the induction of DNA-dependent ATPase activity. Moreover, the results indicate that the UL8 protein is not required for this activity. It is possible that the UL8 protein may enhance the ATPase activity although comparison of

Figure 34. A Novel ATPase Induced in BHK Cells Multiply Infected with *ts*K Recombinant Viruses

Phosphocellulose column fractions of total cell extracts prepared from BHK cells infected at the NPT with either *ts*K (**a**), *ts*K/UL5 (**b**), *ts*K/UL8 (**c**), *ts*K/UL52 (**d**), *ts*K/UL5 plus *ts*K/UL8 (**e**), *ts*K/UL5 plus *ts*K/UL52 (**f**), *ts*K/UL8 plus *ts*K/UL52 (**g**) or *ts*K/UL5 plus *ts*K/UL8 plus *ts*K/UL52 (**h**). The column elution gradient (M NaCl) is indicated as a broken line in panel **a**. Flow-through (ft), wash (w) and eluted fractions (numbered) are also indicated. ATPase activity present in the column flow-through fractions are shown as a peak in panel **a** but the corresponding peak has been omitted from panels **b-h**.

A novel peak of DNA-dependent ATPase was eluted between 150-200mM NaCl from cells infected with all three *ts*K recombinants. This activity was also detected but to a lesser degree, in cells doubly infected with *ts*K/UL5 plus *ts*K/UL52 but absent from all other infections.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ft
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n mol ATP hydrolysed
Fraction Number

Figure 35. Fractionation of Polypeptides From Infected BHK Cells: Elution of the HSV-1 Helicase-Primase Complex

The figure shows SDS-PAGE analysis of phosphocellulose fractions of total cell extracts prepared from BHK cells infected at the NPT and labelled with ^{35}S-L-methionine. Column flow-through (FT), eluted fractions (numbered) and appropriate total cell extract (T) are indicated. Bands corresponding to the over-expressed proteins are indicated by the arrows and dots.

Fractionated ^{35}S-labelled polypeptide profiles were obtained from cells infected with *ts*K (**a**), *ts*K/UL5 (**b**), *ts*K/UL8 (**c**), *ts*K/UL52 (**d**), *ts*K/UL5 plus *ts*K/UL8 (**e**), *ts*K/UL5 plus *ts*K/UL52 (**f**), *ts*K/UL8 plus *ts*K/UL52 (**g**) and *ts*K/UL5 plus *ts*K/UL8 plus *ts*K/UL52 (**h**).

In panel **h**, co-elution of the UL5, UL8 and UL52 proteins in fractions 4-7 (150-220mM NaCl) is apparent. Similarly, in panel **f**, the UL5 and UL52 proteins co-elute. In single infections over-produced proteins each tended to elute at a higher salt concentration than that at which they eluted when expressed together.

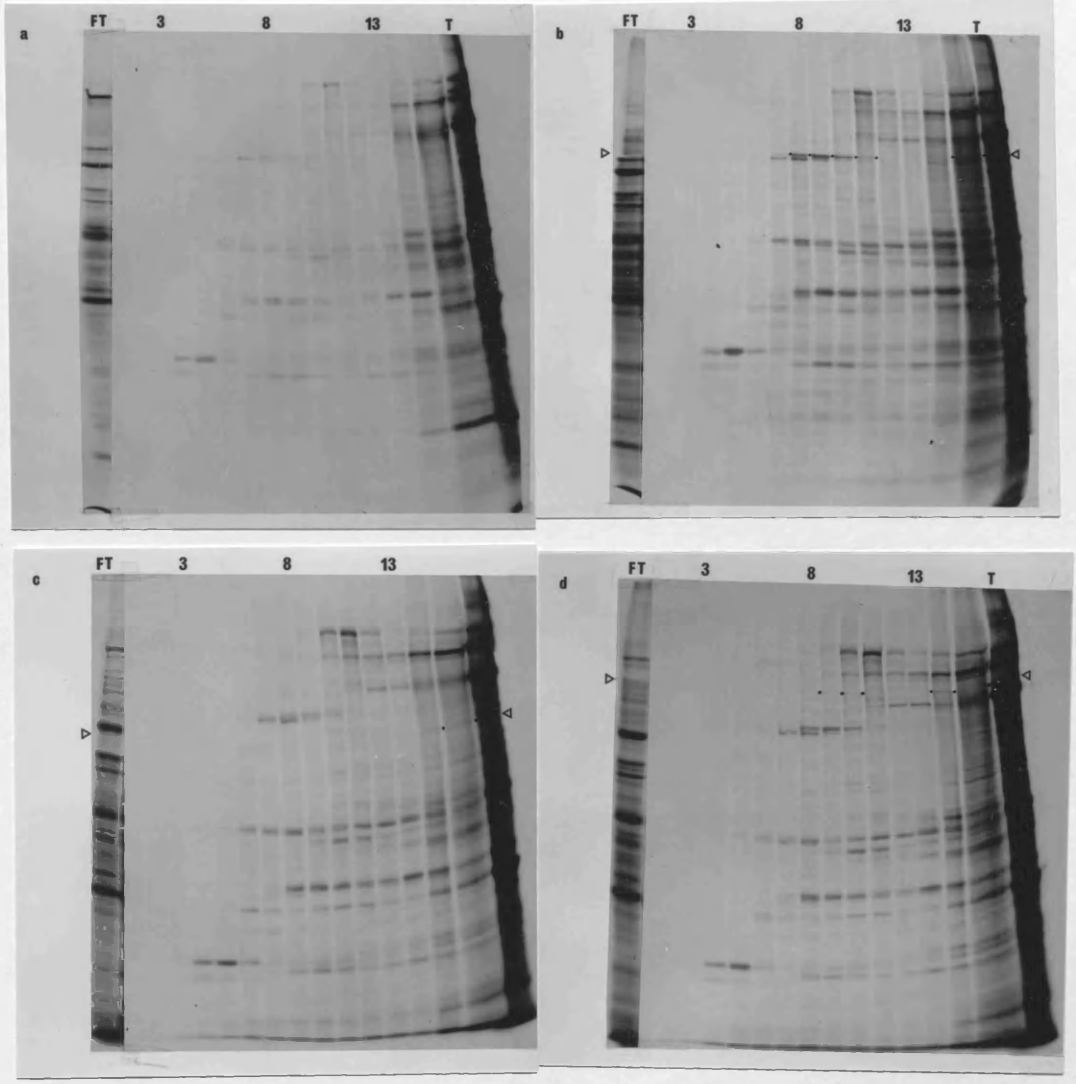
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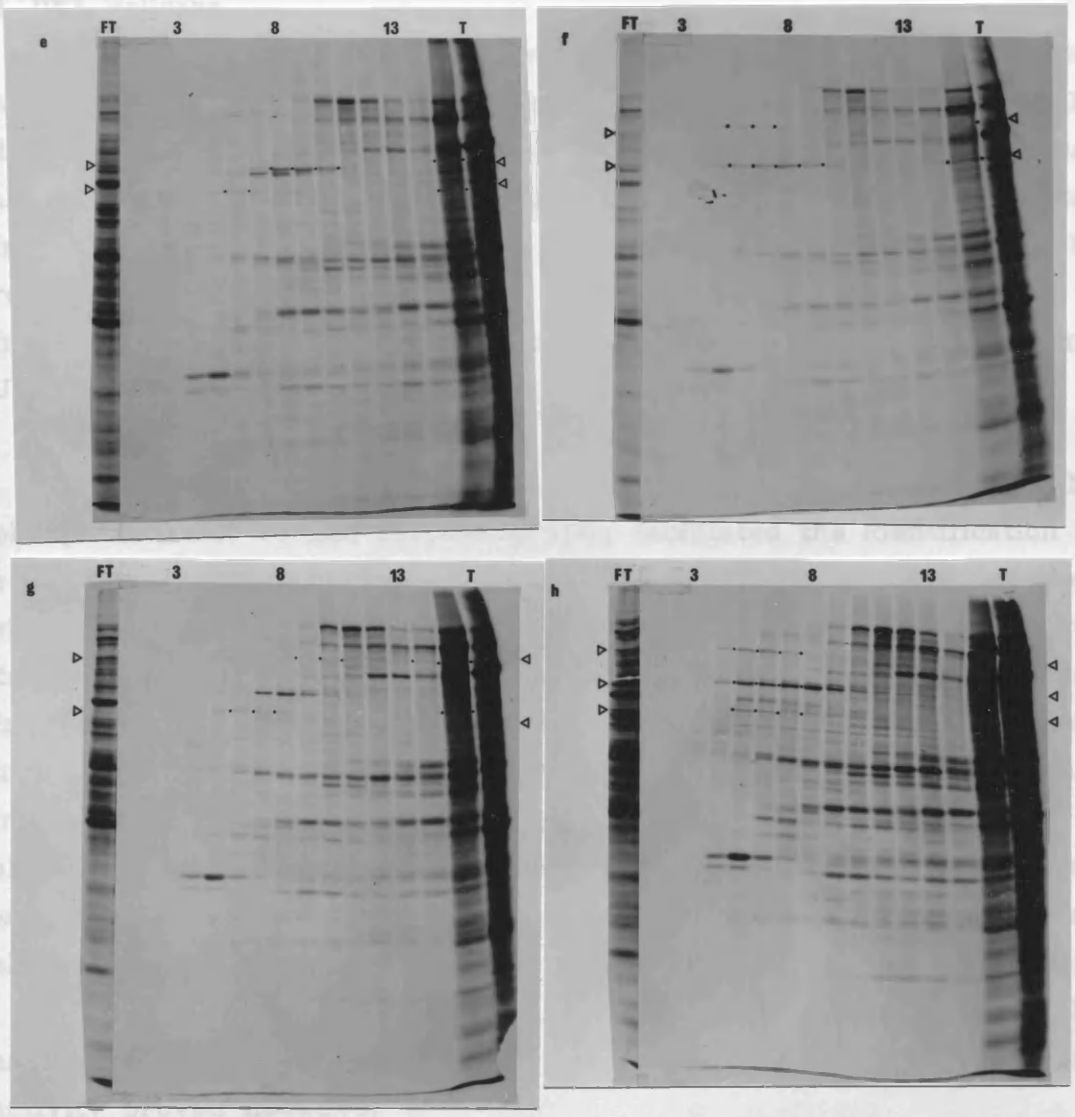
Figure 35. Panels **e - h**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panels f and h of **Figure 35** suggest that there may actually be slightly more of the UL5 and UL52 proteins present in the peak fractions from the triple infection than in the corresponding fractions from the double infection.

3. DNA Helicase

The above phosphocellulose fractions were also assayed for DNA helicase activity as described previously. No novel activity could be detected for any of the recombinant virus infections above that was above a background level of unwinding detected with *ts*K column fractions, similar to that observed in **Figure 32a** (data not shown). The reason for this is not clear since helicase activity was detectable in fractions from the quadruple infection which contained the UL5, UL8 and UL52 proteins (**Figure 32b**).

In summary, fractionation of infected cell extracts by phosphocellulose column chromatography facilitated the identification of a DNA-dependent ATPase induced in BHK cells which were infected either triply with *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 or doubly with *ts*K/UL5 and *ts*K/UL52. SDS-PAGE analysis revealed this activity to co-fractionate with the expressed UL5, UL8 and UL52 proteins in these infections. The formation of a complex is suggested by the fact that individually expressed UL5, UL8 and UL52 proteins each tended to elute at a higher salt concentration than that at which all three eluted when expressed together, or at which the UL5 and UL52 proteins eluted following a dual infection.

Although this DNA-dependent ATPase activity is likely to be associated with a DNA helicase activity, assay of fractions for helicase activity proved negative.

Further purification of infected cell extracts coupled with an increase in the sensitivity of the DNA-dependent ATPase and DNA helicase assays appeared necessary to allow further progress in the study of enzymatic activity using the *ts*K recombinant viruses. However, since baculovirus recombinants which synthesized greater amounts of the UL5, UL8 and UL52 proteins became available in the laboratory, these were also examined.

3E. EXPRESSION OF THE UL5, UL8, UL9 AND UL52 ORFS IN THE RECOMBINANT BACULOVIRUS SYSTEM

Concurrent with the work described in the preceding sections, Dr N D Stow constructed four recombinant baculoviruses each expressing one of the four proteins UL5, UL8, UL9 or UL52. Recombinant viruses were isolated and purified by limiting dilution from *Spodoptera frugiperda* insect cells which had been co-transfected with *wt* AcNPV DNA and a plasmid containing the appropriate gene inserted downstream of the strong, late promoter of the polyhedrin gene within the polyhedrin gene locus.

1. Identification of the UL5, UL8, UL9 and UL52 Gene Products Expressed by Recombinant Baculoviruses

Linbro well monolayers of *Spodoptera frugiperda* (*S.f.*) cells were infected at a multiplicity of 10 p.f.u./cell with either *wt* AcNPV or one of the four recombinant viruses AcUL5, AcUL8, AcUL9 or AcUL52. After 1hr at room temperature the infected cells were overlaid with TC100/5 and incubated at 28°C. At 24 hpi the infected cells were labelled in TC100 salts containing 30μCi/well [^{35}S]-L-methionine for 5-6hr at 28°C after which the cells were lysed in sample buffer. SDS-PAGE analysis of these extracts (**Figure 36**) shows that the products of the inserted genes were readily apparent. Comparison of the equivalent recombinant baculovirus and *ts*K infections in panels **a** and **b** respectively shows the electrophoretic mobility of the UL5, UL8, UL9 and UL52 proteins to be the same in both cell types. This suggests that these proteins do not undergo any detectable differential post-translational modifications when expressed in insect cells. The appearance of additional low molecular weight bands specific to infections with different recombinant baculoviruses may indicate a degree of proteolytic degradation of the expressed proteins.

Figure 37, panels **a** and **b**, show polypeptides from infections with the baculovirus and *ts*K recombinants stained by Coomassie Brilliant Blue. The products of the inserted genes were readily identified in recombinant baculovirus infections. In the *ts*K recombinant infections, only the UL9 gene product was visible. The results indicate that much greater levels of expression are provided by the baculovirus system, and further experiments were therefore performed on the HSV-1 enzymatic activities expressed in this system. It should also be noted that **Figures 36** and **37** show *S.f.* infected cells harvested at a time

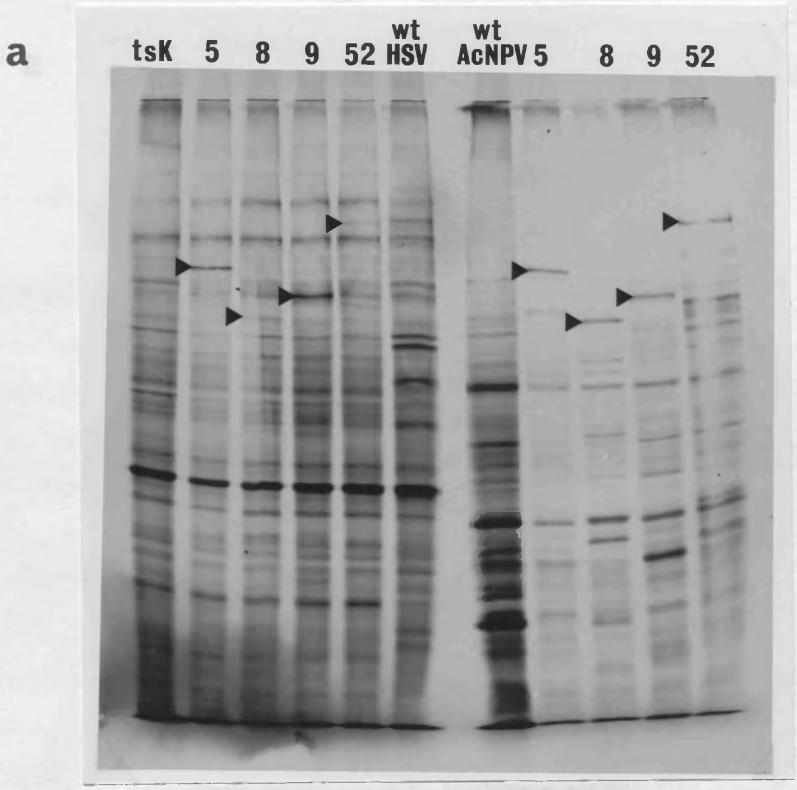


Figure 36. <u>**Over-Expression of HSV-1 Polypeptides in Recombinant *ts*K and Baculovirus Systems**</u>

The figure shows SDS-PAGE analysis of ^{35}S-labelled polypeptides prepared from a) BHK cells infected with either *wt* HSV-1, *ts*K or individual *ts*K recombinant viruses, *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/U152, as indicated, and b) *S.f.* cells infected with either *wt* AcNPV or individual recombinant baculoviruses, as indicated. The over-expressed HSV-1 polypeptides are indicated by the arrows.

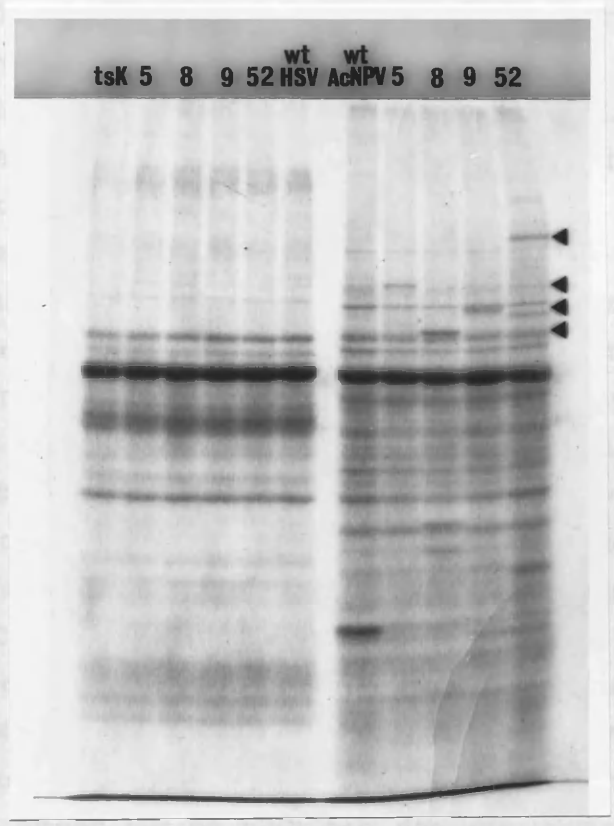


Figure 37. Coomassie-Blue Staining of Infected-Cell Polypeptides

The figure shows a coomassie-blue stained SDS-PAGE analysis of polypeptides prepared from a) BHK cells infected at the NPT with either *wt* HSV-1, *tsK* or individual *tsK* recombinant viruses, as indicated, and b) *S.f.* cells infected with either *wt* AcNPV or individual recombinant baculoviruses and lysed in sample buffer at 8 hpi (a) or 30 hpi (b). HSV-1 polypeptides expressed in the baculovirus system are indicated by the arrows. HSV-1 proteins over-expressed in the *tsK* system were barely visible indicating that a greater level of expression of the replication proteins is achieved in insect cells than in BHK cells.

considerably earlier than that at which maximum levels of foreign protein are expressed from the polyhedrin promoter (48-72 hpi). The reasons for harvesting at 30 hpi are outlined below.

(a) Insolubility of the UL52 Protein and the Preparation of Extracts from infected *S.f.* cells

Although yields of the HSV-1 proteins were much greater in extracts prepared at later times post-infection, for example 50hpi, fractionation experiments showed that the UL52 protein became insoluble and could only be isolated in a rapidly sedimenting form. When extracts were harvested at 30hr yields of the HSV-1 proteins were, as expected, reduced but the UL52 gene product was found predominantly in a soluble form in the cytoplasmic fraction. Co-expression of the UL5, UL8 and UL52 proteins in the same cell also tended to increase the proportion of UL52 protein which was soluble late in infection. This is probably because of the association of the three proteins in a complex (Crute *et al.* 1989). The proportion of UL52 protein recovered from the soluble fraction under these conditions, however, varied and on occasions a substantial amount remained insoluble. Experiments examining the induction of enzymatic activities by these recombinant viruses were therefore all performed on extracts prepared 30hpi. Efficient recovery of the over-expressed HSV-1 proteins was achieved by lysis of infected cells with NP40 in the presence of either low or high salt concentrations to give cytoplasmic or total cellular extracts, respectively.

3F. FUNCTIONAL CHARACTERISATION OF GENE PRODUCTS OVER-EXPRESSED BY RECOMBINANT BACULOVIRUSES

Experiments similar to those described above using the *ts*K recombinant viruses were carried out with the recombinant baculoviruses in an attempt to determine whether the insect cell-expressed proteins were functional and to confirm and extend the previously described findings.

Assembly of the HSV-1 Helicase-Primase Complex in Insect Cells

1. DNA-Dependent ATPase

S.f. cells were infected with AcNPV or the three recombinant viruses AcUL5, AcUL8 and AcUL52 either individually or in all possible combinations. Infections were labelled with [^{35}S]-L-methionine at 24hpi and the cells harvested at 30hpi. Over-expressed HSV-1 proteins were recovered in total cell extracts which were subsequently dialysed and fractionated on phosphocellulose columns. Collected fractions were assayed for ATPase activity in the presence of activated calf thymus DNA.

Comparison of **Figure 38**, panels **a** and **h**, reveals a distinct peak of ATPase activity, not present in cells infected with the vector, AcNPV, was induced in cells triply infected with AcUL5, AcUL8 and AcUL52. This peak of activity eluted at 150-200mM NaCl, a value similar to that observed for the ATPase activity from BHK cells triply infected with *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 (**Figure 34**), and also similar to that reported for the HSV-1 helicase-primase complex (Crute *et al.* 1988). The novel ATPase was shown to be stimulated by the presence of activated DNA in the reaction (**Figure 39**). A 10-fold increase in activity was observed in the presence of 20μg/ml activated calf thymus DNA compared with its absence.

ATPase profiles obtained from *S.f.* cells infected with either AcUL5, AcUL8 or AcUL52 alone or doubly infected with AcUL5 and AcUL8, or with AcUL8 and AcUL52, were each almost identical to that obtained from the control AcNPV extract (**Figure 38** panels **b**, **c**, **d**, **e** and **g** respectively).

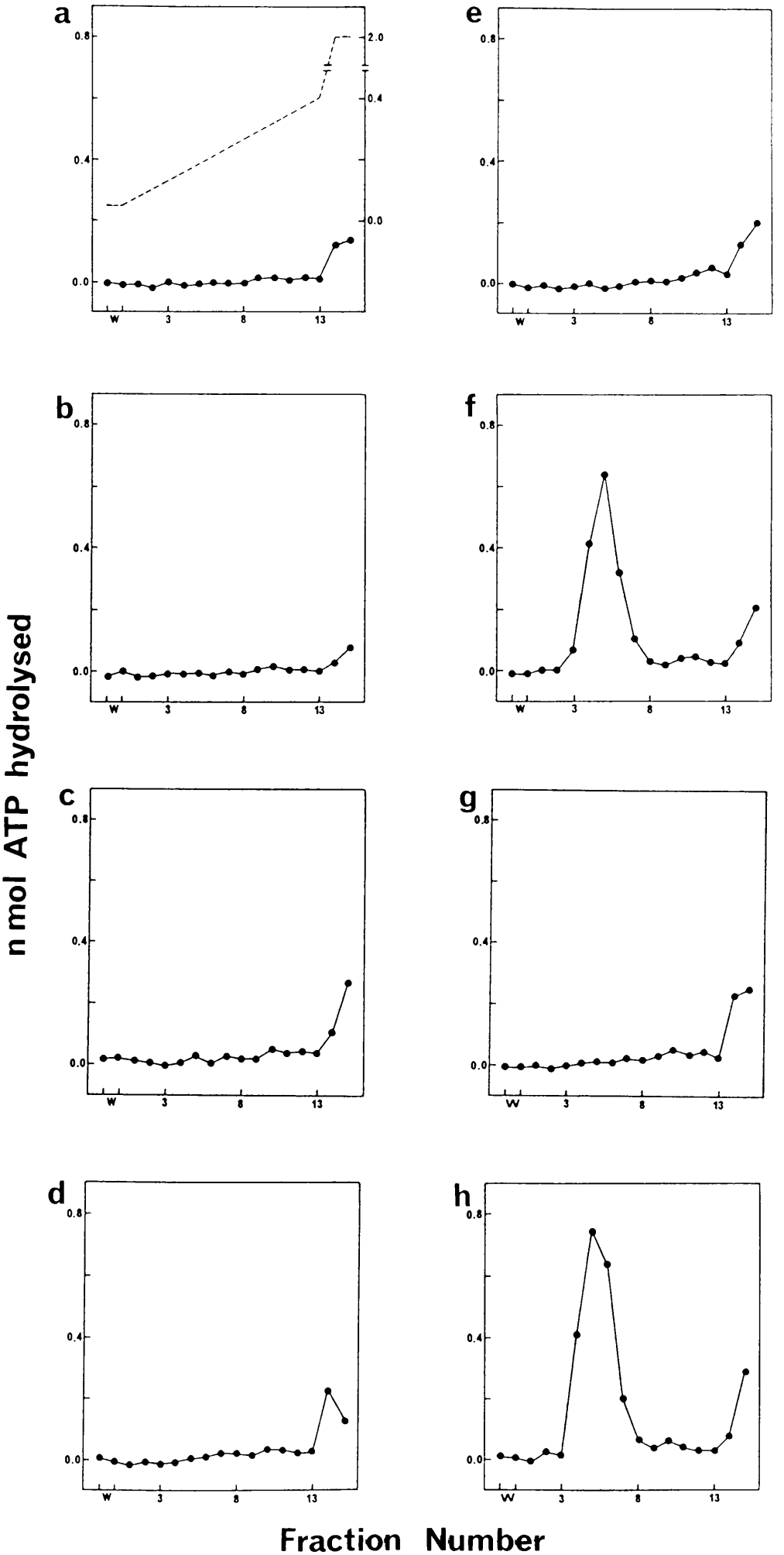
However, a distinct peak of ATPase activity was obtained from *S.f.* cells doubly infected with AcUL5 and AcUL52 which eluted at the same salt concentration as that from the triply infected cells (**Figure 38**, panel **c**).

These results (Calder and Stow, 1990) confirm and extend the work of Dodson *et al.*(1989), who demonstrated that the HSV-1 helicase-primase complex could assemble *in vivo* in insect cells mixedly infected with baculoviruses expressing the UL5, UL8 and UL52 gene products. They additionally show that the expression of the HSV-1 UL8 gene product is not required for the induction of the novel DNA-dependent ATPase activity associated with the complex. The absence of the UL8 protein resulted in only a small decrease in the level of ATPase detected.

Figure 38. **A Novel DNA-dependent ATPase Induced in *S.f.* Cells Multiply Infected with Recombinant Baculoviruses**

Total cell extracts were prepared from *S.f.* cells infected with either *wt* AcNPV (**a**), AcUL5 (**b**, AcUL8 (**c**), AcUL52 (**d**), AcUL5 plus AcUL8 (**e**), AcUL5 plus AcUL52 (**f**), AcUL8 plus AcUL52 (**g**) or AcUL5 plus AcUL8 plus AcUL52 (**h**), fractionated on phosphocellulose columns and assayed for DNA-dependent ATPase activity. ATPase activity in the column flow-through fractions (peak 0.8 nmol ATP hydrolysed)has been omitted from these graphs. Wash (w) and eluted (numbered) fractions are indicated. The column elution gradient (M NaCl) is indicated as the broken line in panel **a**.

Novel peaks of ATPase activity were eluted between fractions 4-7 (150-220mM NaCl) from cells triply infected with all three recombinant baculoviruses (**h**) and from cells doubly infected with AcUL5 plus AcUL52 (**f**). Induction of this activity was not observed in any other infection.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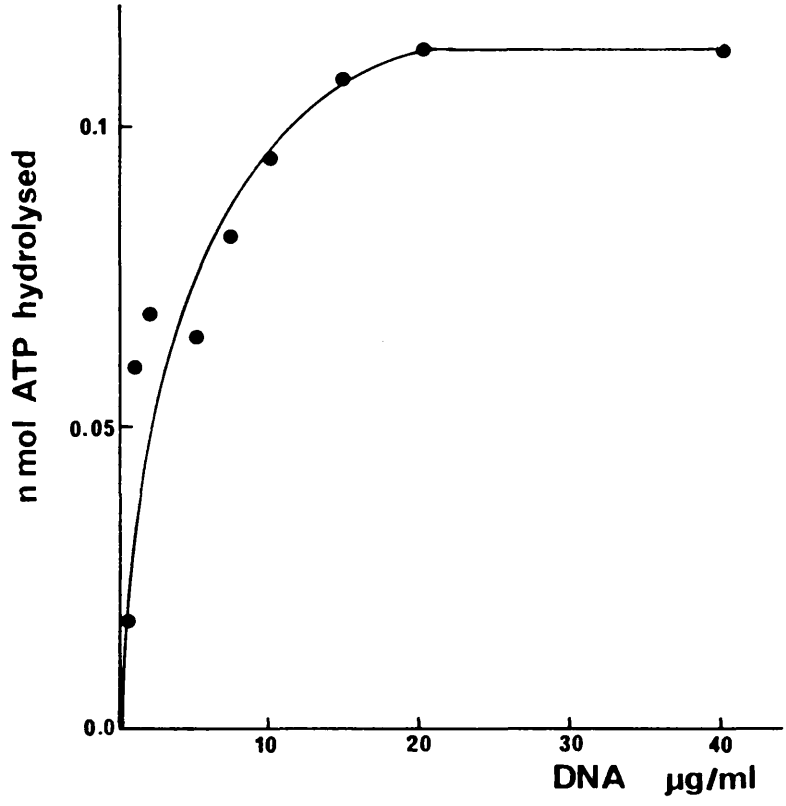

Figure 39. DNA Dependence of ATPase Induced in *S.f.* Cells Triply Infected with AcUL5, AcUL8 and AcUL52

The figure shows ATP hydrolysis exhibited by phosphocellulose column fraction 4 (see **Figure 38**) from cells triply infected with recombinant baculoviruses AcUL5, AcUL8 and AcUL52 in the presence of increasing amounts of activated calf thymus DNA.

2. DNA helicase

The above column fractions were also assayed for DNA helicase activity. An improvement to the protocol used with the *ts*K system was to implement the use of helicase buffer 2 (described in Materials and Methods, Section 2B.35). Conditions altered included a decrease in the concentration of Mg^{2+} ions, an increase in the concentration of ATP and DTT, and the addition of glycerol to a concentration of 10% in the reaction (Crute *et al.*, 1989). Fractions 6 - 8 from *S.f.* cells infected with *wt* AcNPV exhibited a very low level of displacement of labelled oligonucleotide, indicative of a low level of helicase activity (**Figure 40**, panel **a**). Small variations in the amount of hybrid substrate present at the top of the gel probably result from the action of nucleases. Very similar patterns were also obtained from cells singly infected with AcUL5, AcUL8 or AcUL52, or co-infected with AcUL5 plus AcUL8, or with AcUL8 plus AcUL52 (data not shown).

However, major novel peaks of helicase activity were obtained from cells infected either with all three recombinant viruses or with AcUL5 plus AcUL52 (**Figure 40**, panels **b** and **c**). In both cases maximum helicase activity was present in fractions 4 - 6, coincident with the peak of ATPase activity . Therefore, as with the ATPase activity, the novel DNA helicase does not require the presence of the UL8 protein.

The novel DNA helicase produced in both double and triple infections was dependent upon the presence of ATP and Mg^{2+} ions in the assay for activity (**Figure 41**).

3. Presence of HSV-1 Specified Proteins in Phosphocellulose Column Fractions

Figure 42 shows SDS-PAGE analysis of fractions from the phosphocellulose columns described above. The patterns obtained from *S.f.* cells infected with AcNPV, triply infected with AcUL5, AcUL8 and AcUL52, and doubly infected with AcUL5 and AcUL52 are represented in panels **a**, **h** and **f** respectively. Bands corresponding to the expressed HSV-1 proteins are indicated. In both recombinant virus infections the HSV-1 proteins co-eluted and were present in maximal amounts in fractions 4 - 6, coincident with peaks of ATPase and helicase activity.

In cells singly infected with either AcUL5, AcUL8 or AcUL52 the HSV-1 polypeptides each tended to elute at a higher salt concentration (200-300mM, and in the 2M wash) than in a mixed infection. A second

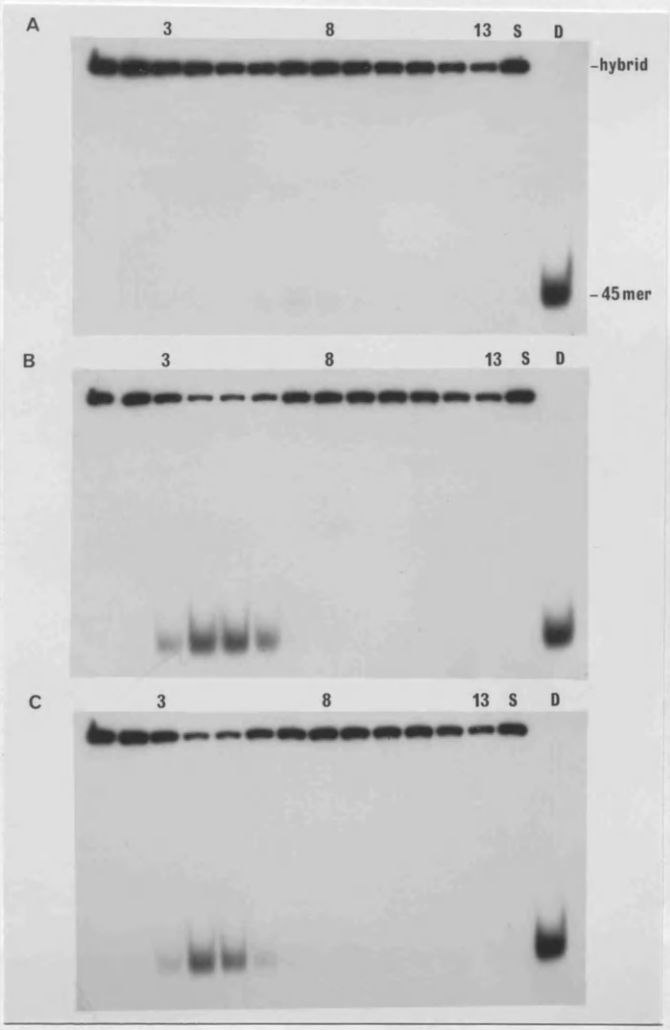

Figure 40. A Novel DNA Helicase Induced in *S.f.* Cells Multiply Infected With Recombinant Baculoviruses

Phosphocellulose column fractions of total cell extracts prepared form *S.f.* cells infected with either *wt* AcNPV (A), AcUL5 plus AcUL8 plus AcUL52 (B) or AcUL5 plus AcUL52 (C) were assayed for DNA helicase activity using the DNA substrate described in the text. The profiles of helicase activity in the phosphocellulose column fractions from the other extracts (AcUL5, AcUL8, AcUL52, AcUL5 plus AcUL8 and AcUL8 plus AcUL52) were essentially identical to panel A (data not shown).

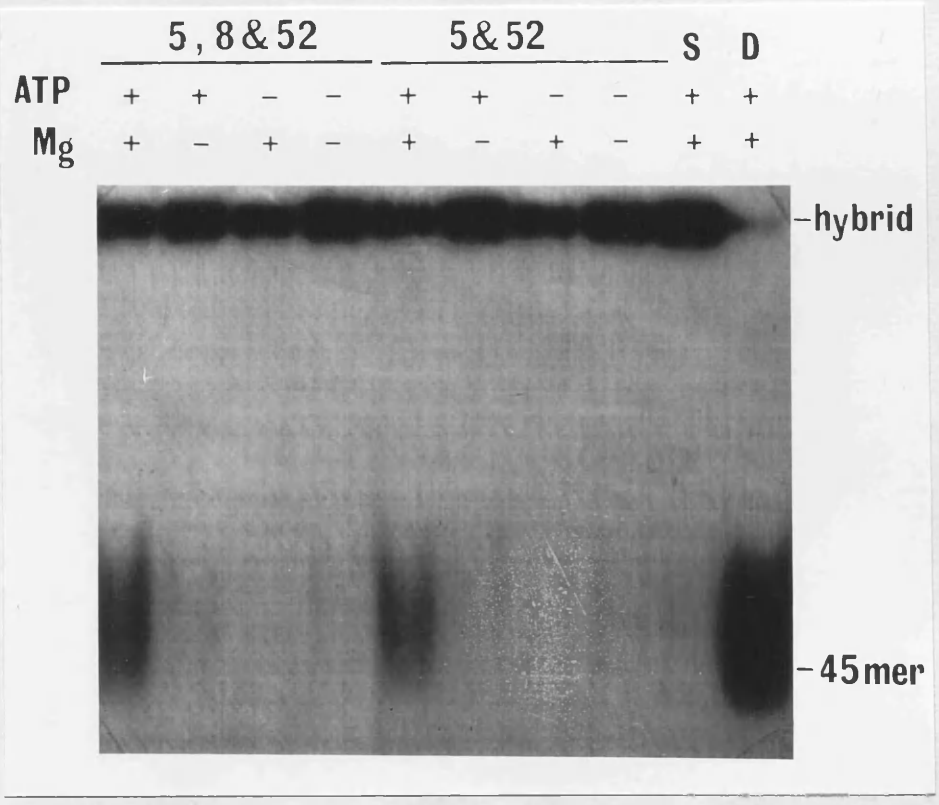

Figure 41. <u>**The Novel DNA Helicase is Dependent upon ATP and Mg^{2+}**</u>

DNA helicase activity of phosphocellulose column fractions containing the UL5 and UL52 proteins or the UL5, UL8 and UL52 proteins was assayed, using the tailed substrate described in the text, in the presence and absence of ATP (3mM) and Mg^{2+} (3.5mM) as indicated.

Figure 42. Fractionation of Polypeptides Present in *S.f.* Cells Infected with Recombinant Baculoviruses

The figure shows SDS-PAGE analysis of phosphocellulose column fractions of total cell extracts prepared from *S.f.* cells infected with either *wt*AcNPV (**a**), AcUL5 (**b**), AcUL8 (**c**), AcUL52 (**d**), AcUL5 plus AcUL8 (**e**), AcUL5 plus AcUL52 (**f**), AcUL8 plus AcUL52 (**g**) or AcUL5 plus AcUL8 plus AcUL52 (**h**). Column flow-through (FT), eluted fractions (numbered) and appropriate total cell extract (T) are indicated. Bands corresponding to over-expressed HSV-1 polypeptides are indicated by the arrows.

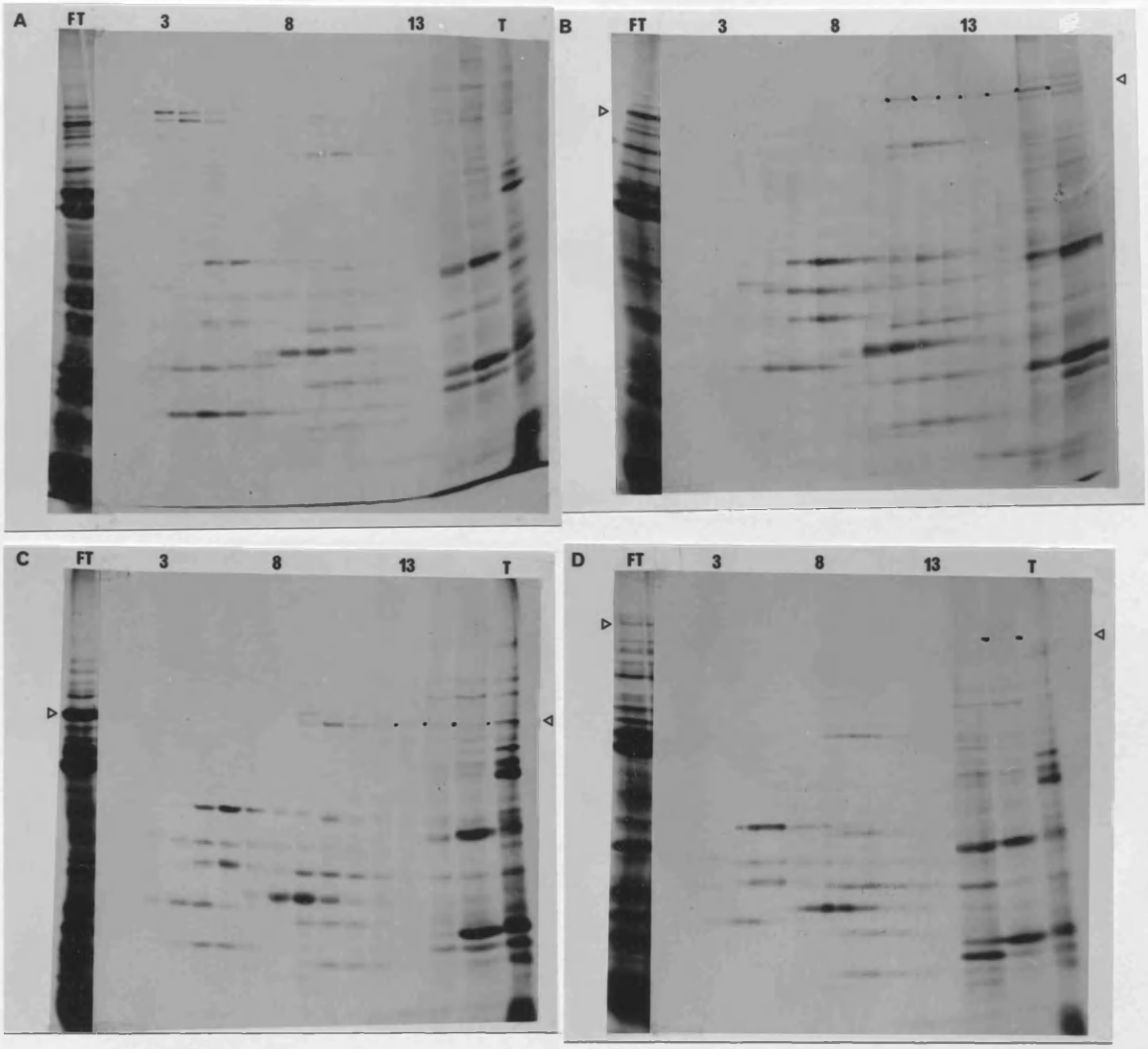
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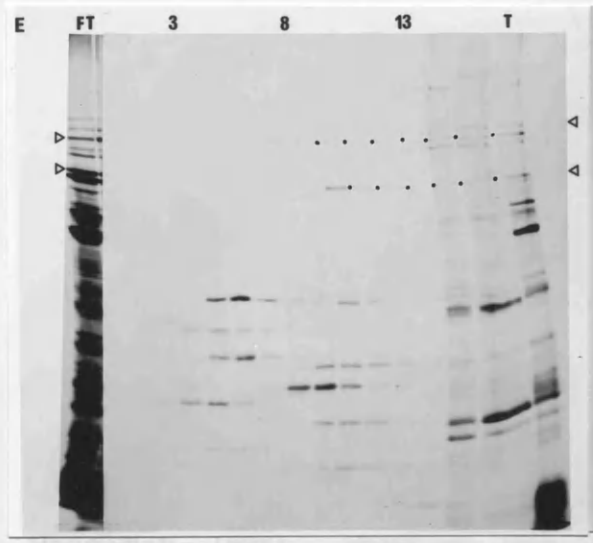
Figure 42. Panels **e - h**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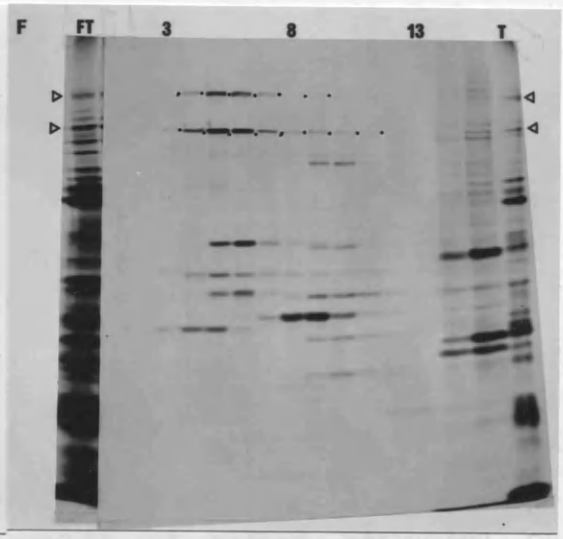
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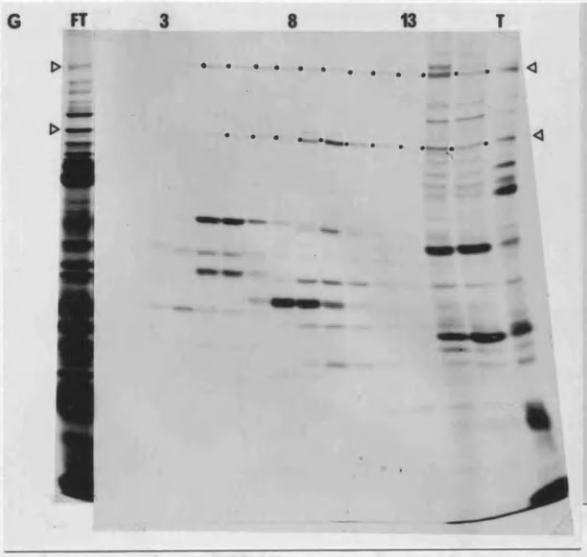
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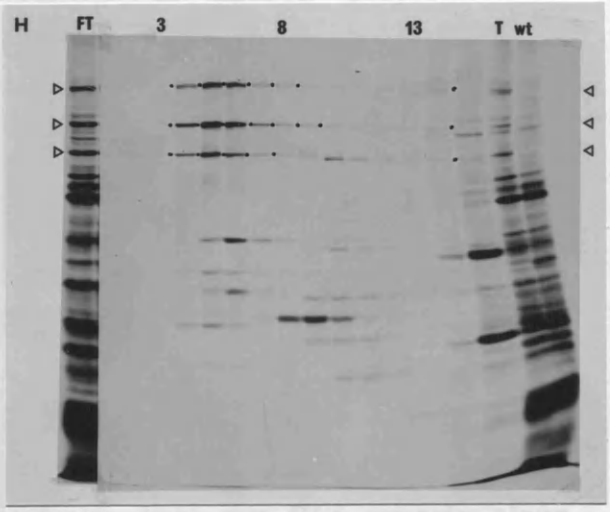
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feature of these proteins when expressed alone was that a larger proportion of protein failed to bind the column (**Figure 42**, panels **b**, **c** and **d** respectively).

These results confirm previous reports that the UL5, UL8 and UL52 proteins form a complex in infected cells (Crute et al. 1989, Dodson et al. 1989). Moreover, they strongly suggest that the UL5 and UL52 proteins are also able to form a complex in the absence of the UL8 protein. A recent report by Dodson and Lehman (1991) supports this conclusion.

4. Is the UL5 Protein the DNA Helicase?

Amino acid sequence analysis has revealed that the UL5 gene product possesses an NTP-binding site and shares sequence motifs with members of a family of proteins known to have DNA and RNA helicase activities (Gorbalenya and Koonin 1989, Gorbalenya et al. 1989, Hodgeman 1988). However, the results of assays performed on phosphocellulose column fractionated extracts from cells singly infected with either a baculovirus or *tsK* recombinant virus expressing the UL5 protein provided no evidence that the UL5 protein alone exhibited helicase activity. Extract prepared from *S.f.* cells infected with AcUL5 was alternatively fractionated by MONO-Q ion-exchange FPLC (Materials and Methods, Section 2B.33(b)). SDS-PAGE analysis of collected fractions (**Figures 43**) show that the UL5 protein eluted in a broad peak between fractions 16 and 28 (250-500mM NaCl). Fractions were assayed for DNA-dependent ATPase and DNA helicase activities as described previously for the phosphocellulose fractions (**Figures 44** and **45** respectively). The results indicate that the UL5 protein alone does not exhibit any detectable ATPase or helicase activity.

The significance of more slowly migrating bands in fractions 17 and 22 is unclear. It is possible that some protein in the fraction may bind displaced oligonucleotide and thereby retard its mobility in the gel. However, further experiments would be necessary to support this hypothesis.

5. The UL9 Protein is a DNA Helicase

Amino acid sequence analysis reveals that the UL9 gene product also contains an NTP-binding site and characteristic helicase motifs suggesting that the UL9 protein may have NTPase and DNA helicase activities. The characteristic motifs are present within the N-terminal

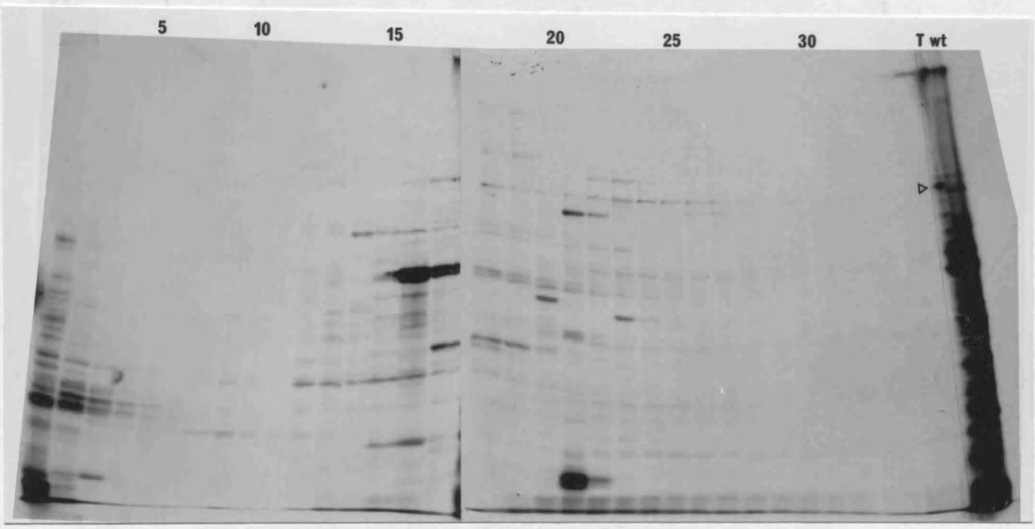


Figure 43. Presence of the UL5 Protein in FPLC Fractions: SDS-PAGE

The figure shows SDS-PAGE analysis of ^{35}S-labelled polypeptides present in FPLC fractionated total cell extract prepared from *S.f.* cells infected with AcUL5. Fractions (1-34), which include flow-through and wash, and marker lanes containing total cell extract prepared from *S.f.* cells infected with *wt*AcNPV (wt) or AcUL5 (T) are indicated. The position of the UL5 protein is indicated in the total cell extract by the arrow. This protein elutes from the 'Mono-Q' column in a broad peak (fractions 16-28; 250-500mM NaCl).

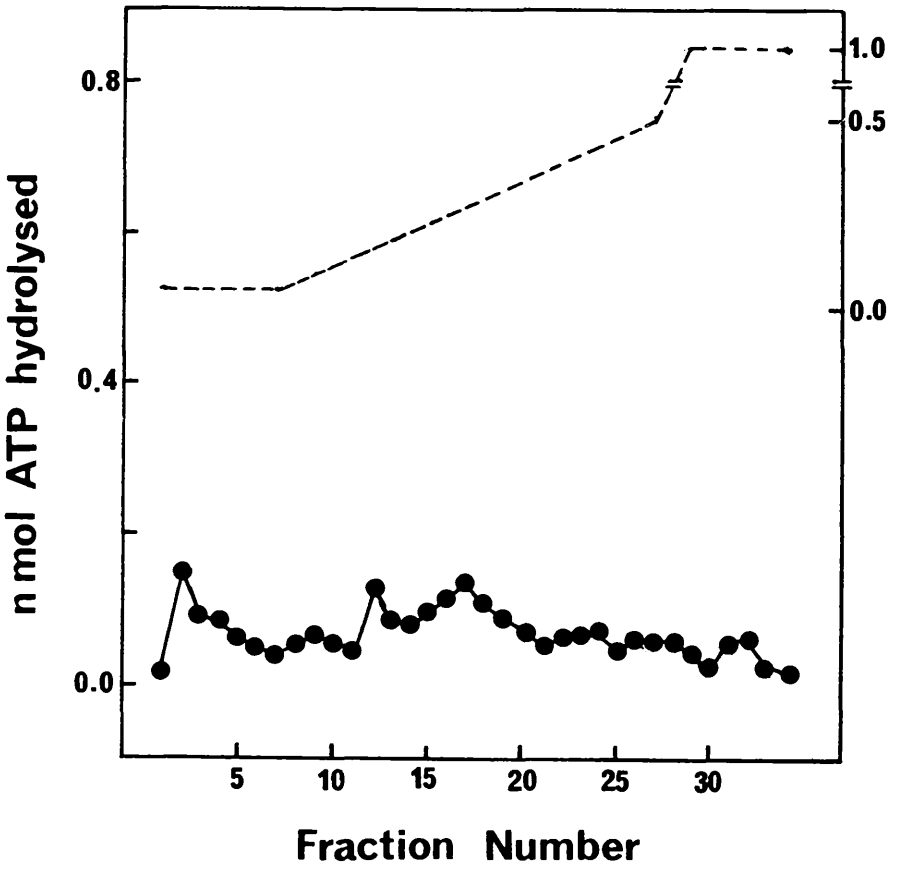


Figure 44. Purified UL5 Protein Is Not An ATPase

The FPLC fractions from AcUL5-infected *S.f.* cells (**Figure 43**) were assayed for DNA-dependent ATPase activity. The column elution gradient is indicated as the broken line. No significant peak of ATPase activity was detected in the fractions containing the UL5 protein (fractions 16-28).

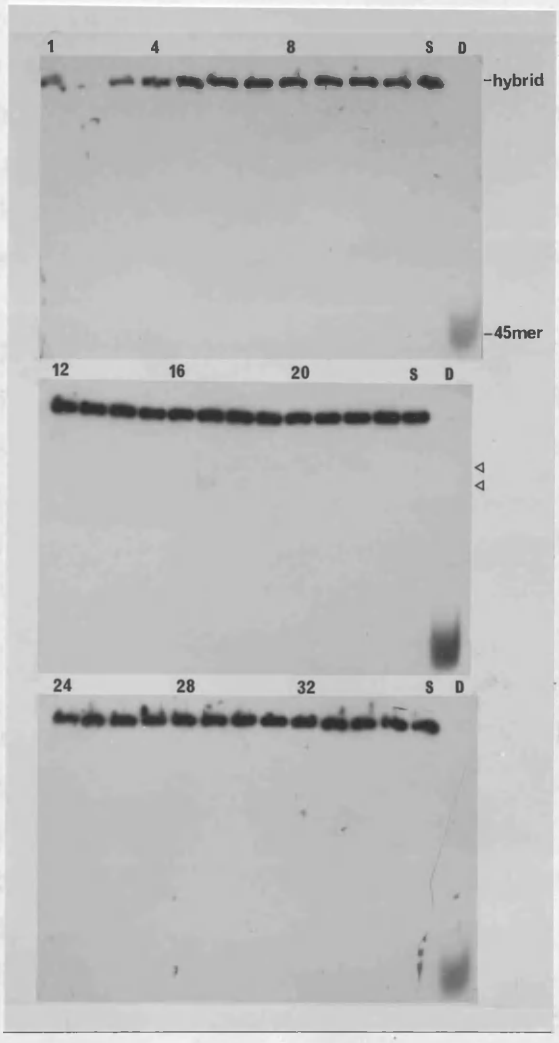


Figure 45. Purified UL5 Protein Is Not A DNA Helicase

The FPLC fractions (1-34, as indicated) of total cell extracts prepared from *S.f.* cells infected with AcUL5 (**Figure 43**) were also assayed for DNA helicase activity. Positions of hybrid substrate (S) and displaced oligonucleotide (D) are indicated. Displacement of single-stranded 45mer was not observed with any fraction. The significance of slower migrating bands present in fractions 17 and 22 (indicated by the arrows) is not clear.

400 amino acids of the 851 amino acid UL9 polypeptide. Gorbalenya and Koonin (1989) include the UL9 protein in superfamily 2 of known and putative DNA and RNA helicases. Intact UL9 protein expressed by the recombinant baculovirus AcUL9 has been purified by affinity chromatography on λ column containing a covalently linked double-stranded oligonucleotide corresponding to the UL9 recognition sequence (N D Stow, unpublished results). The C-terminal 317 amino acids (approximately one-third) of the UL9 protein (UL9CT), which possesses the sequence-specific DNA-binding domain but lacks an NTP-binding site (Weir *et al.*, 1989), was also expressed in a recombinant baculovirus and similarly purified (N D Stow, unpublished results).

The ability of the UL9 protein to unwind duplex DNA was investigated. Aliquots of affinity purified UL9 protein and UL9CT proteins, provided by Dr N D Stow, were assayed for DNA helicase as previously described for the phosphocellulose column fractions (Section 3F.2). **Figure 46**, panel **b** shows that the UL9 protein functions as a DNA helicase and displaces the labelled oligonucleotide. This activity was found to be dependent upon the presence of ATP and Mg^{2+} ions, as shown in **Figure 47**. DNA helicase activity was not detected when the helicase motifs were absent, as demonstrated by the inability of the UL9CT protein to displace the labelled oligonucleotide. Intact UL9 protein was also able to displace a labelled oligonucleotide from a helicase substrate lacking a 3' tail (**Figure 46a**). Neither helicase substrate contained the recognition sequence a DNA binding site for the UL9 protein. These results therefore show that the origin-binding protein exhibits a non-sequence specific DNA helicase activity, which is not, however, essential for its sequence-specific binding to the viral origins of replication.

6. DNA Primase

The UL5, UL8 and UL52 protein complex has also been reported to exhibit DNA primase activity (Crute et al. 1989, Dodson et al. 1989). Extracts were prepared from *S.f.* cells infected with *wt* AcNPV, with the three recombinants AcUL5, AcUL8 and AcUL52 or with AcUL5 plus AcUL52. Fractions from a phosphocellulose column were collected and assayed for ATPase and helicase activities as before, and additionally for DNA primase activity. DNA primase activity was assayed indirectly using the protocol previously employed by Crute *et al.* (1989) (Materials and Methods, Section 2B.36). This involves measuring incorporation of [^{32}P]dAMP into poly(dA) using a template of

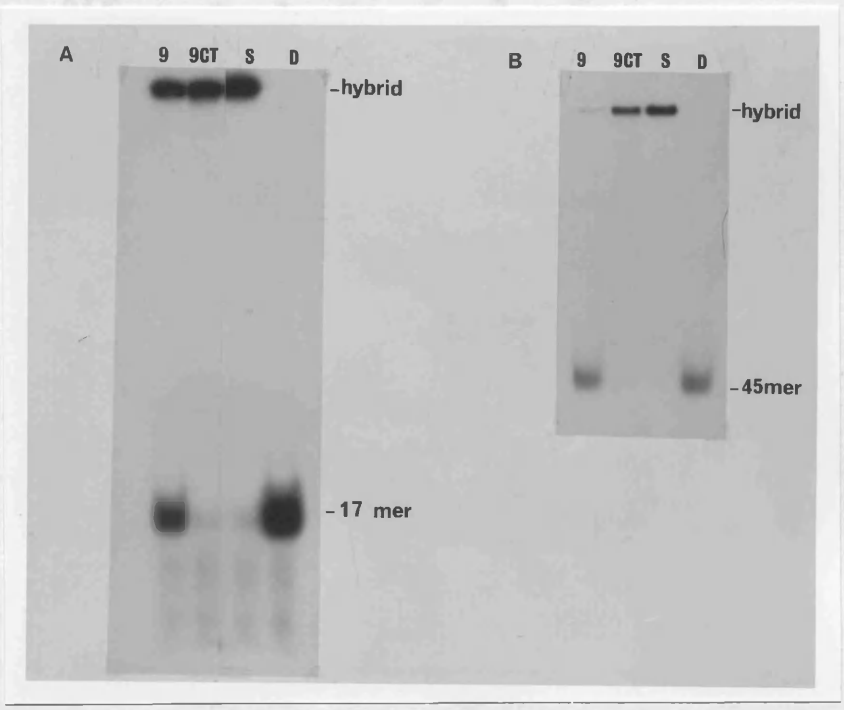


Figure 46. The UL9 Protein Is A DNA Helicase

Affinity purified intact UL9 protein and the C-terminal one-third fragment (UL9CT) were assayed for DNA helicase activity using two different partial duplex substrates. The positions of hybrid substrates (S) and displaced oligonucleotides (D) are indicated. Tracks marked (D) contain heat denatured substrate. In Panel a the annealed fragment (17 mer) was complementary throughout its length to the single-stranded M13 DNA. In panel b the annealed fragment (45 mer) had an unannealed 3'tail of 22 bases (Section 3C.1(a)).

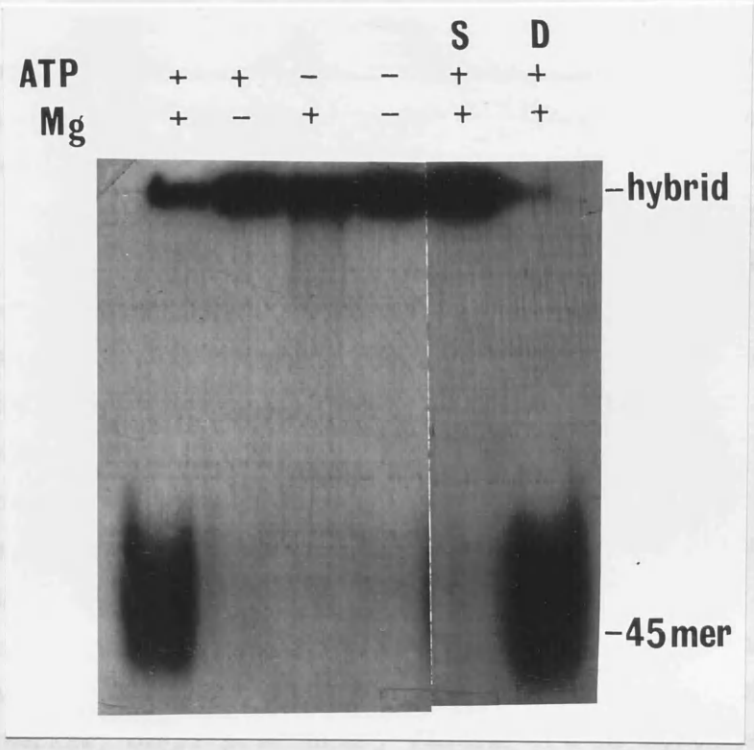


Figure 47. UL9 Protein Helicase Activity is Dependent upon ATP and Mg^{2+}

DNA helicase activity of affinity purified intact UL9 protein was assayed, using the tailed substrate described in the text, in the presence and absence of ATP (3mM) and Mg^{2+} (3.5mM) as indicated.

poly(dT) in the presence of the large fragment *E.coli* DNA polymerase I. This incorporation is dependent upon the synthesis of oligo (rA) primers by primase activity utilizing ATP in the reaction. Products of the primase assays were de-proteinised, phenol and chloroform extracted and ethanol precipitated prior to electrophoresis through a 0.35mm thick denaturing 6% acrylamide gel in the presence of urea.

Figure 48 shows the reaction products. All infections exhibited a major heavy smear of [^{32}P]-labelled poly(dA) in fractions 10 - 12. This is suggestive of a primase or RNA polymerase activity, presumably cell or AcNPV encoded and is in agreement with other reports (Dodson *et al.*, 1989; Dodson and Lehman, 1991).

A smear of [^{32}P]-labelled material was also observed in fractions 3-8 in cells either triply infected with all three recombinants or co-infected with AcUL5 and AcUL52 (panels **b** and **c** respectively) which was not present in the control *wt* AcNPV infection (panel **a**). This indicates the probable induction of a novel DNA primase activity specific to the over-expressed HSV-1 proteins.

Elution of these novel activities was coincident with peaks in ATPase and helicase activity and with the elution of the UL5, UL8 and UL52 proteins in the triple infection and the UL5 and UL52 proteins in the double infection, (similar to Figures 39, 40 and 42). The UL8 protein is clearly dispensable for this putative primase activity as well as ATPase and helicase activities.

Although the assay requires further refinement and additional experiments to exclude the possibility of the activity being due to an RNA polymerase, these preliminary results are in agreement with those of Dodson *et al.* (1989), who demonstrated exhibition of DNA primase activity by the UL5/UL8/UL52 protein complex assembled in insect cells using recombinant baculoviruses. Moreover, in support of the above conclusions a subassembly of UL5 and UL52 proteins purified from insect cells has been conclusively shown to possess DNA primase activity as well as DNA-dependent ATPase, DNA-dependent GTPase and DNA helicase activities (Dodson and Lehman, 1991). Thus the UL8 protein is dispensable for all the enzymatic activities characteristic of the HSV-1 helicase-primase complex.

7. DNA Binding Properties of the HSV-1 Helicase-Primase Complex

DNA helicases have been shown to bind non-sequence-specifically to single stranded DNA (reviewed by Matson and Kaiser-Rogers, 1990). DNA-binding properties of the HSV-1 helicase-primase complex were

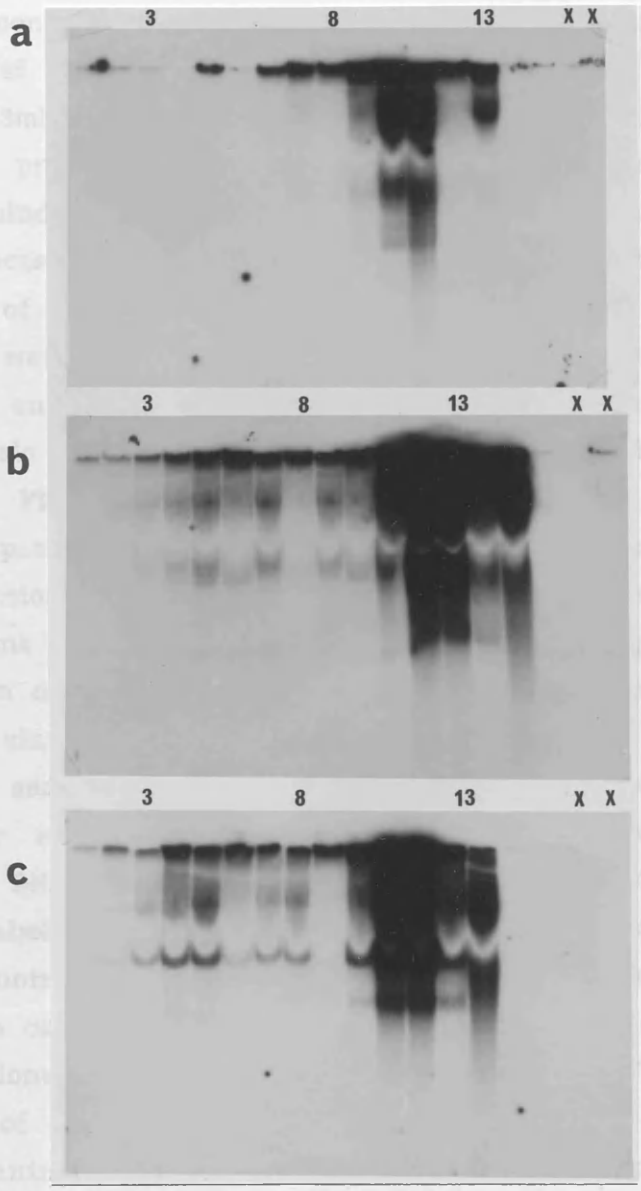


Figure 48. A Novel DNA Primase Induced in *S.f.* Cells Multiply Infected with Recombinant Baculoviruses

Phosphocellulose column fractions (numbered) of total cell extract prepared from *S.f.* cells infected with *wt* AcNPV (a), AcUL5 plus AcUL8 plus AcUL52 (b) or AcUL5 plus AcUL52 (c) were assayed for DNA primase activity as described in Materials and Methods. Control lanes (X) contain reactions lacking any phosphocellulose column fraction.

investigated by two approaches. In the first, single-stranded DNA cellulose columns were used. An aliquot of a phosphocellulose column fraction containing the UL5, UL8 and UL52 proteins from a triple infection of BHK cells with *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 was loaded onto a 0.3ml bed volume single-stranded DNA cellulose column which had been pre-run with a BSA solution in an attempt to reduce non-specific binding of proteins. After loading the sample, flow-through was collected, re-loaded twice and bound proteins eluted with a gradient of increasing NaCl concentration in DC buffer. The column fractions were run on SDS-PAGE, transferred to nitrocellulose and the UL5, UL8 and UL52 proteins detected by immunoblotting, as described in Materials and Methods, Sections 2B.25 and 2B.27. The results are shown in **Figure 49.** A large proportion of the UL5, UL8 and UL52 proteins passed directly through the column in the flow-through and wash fractions. Small amounts of the three proteins also appeared to be present in the highest salt fractions suggesting that a small proportion of the UL5, UL8 and UL52 protein complex may be able to bind the single-stranded DNA matrix.

In a second approach, a gel retardation assay (as described by Schneider *et al.*, 1986) was used in an attempt to detect single stranded DNA binding. A 45mer single stranded oligonucleotide was 3' end labelled with [^{32}P] using terminal transferase and incubated with aliquots of equivalent phosphocellulose column fractions (fraction 4 in each case, which contained the peak of helicase activity induced in infections with AcUL5 plus AcUL52 and AcUL5 plus AcUL8 plus AcUL52) of extracts from *S.f.* cells infected with AcNPV or the three recombinants AcUL5, AcUL8 and AcUL52 either alone or in all possible combinations. Reactions were analysed by electrophoresis through a native polyacrylamide gel to resolve any protein-DNA complexes. Formation of a retarded protein-DNA complex was detected on some occasions. However, the effect was not reproducible and the electrophoretic mobility of the complex did not vary between the double and triple recombinant virus infections (data not shown). These observations suggest that the formation of this protein-DNA complex was not due to specific binding of the UL5/UL52 or UL5/UL8/UL52 protein complexes.

Neither approach, therefore, provided clear evidence of the ability of the UL5, UL8 and UL52 proteins (individually or in combination) to bind to single-stranded DNA.

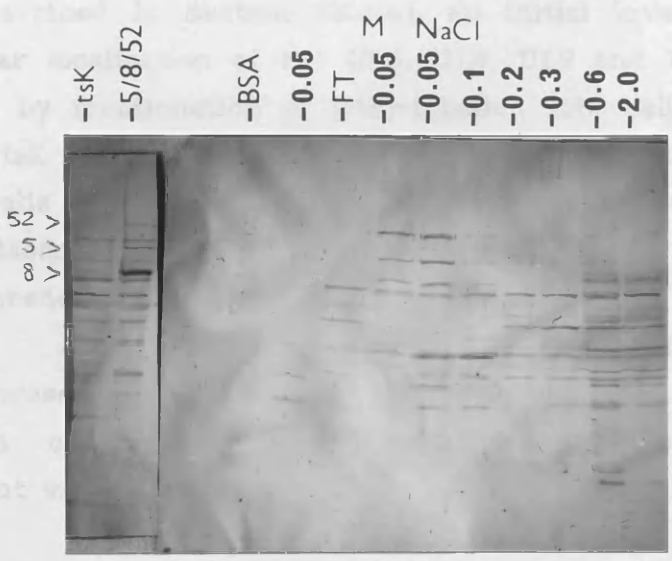


Figure 49. <u>DNA-binding Properties of the UL5, UL8 and UL52 Proteins Single-Stranded DNA Cellulose Columns</u>

Total cell extract prepared from BHK cells mixedly infected with *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 was fractionated on a single-stranded DNA cellulose column as described in Materials and Methods. Column eluates were run on a mini-SDS-polyacrylamide gel, electro-transferred to a nitrocellulose membrane and probed with a mixture of anti-UL5, anti-UL8 and anti-UL52 antisera. Polypeptides eluted in sample flow through (FT) and at increasing salt concentrations are indicated. Marker lanes to the left, contain total cell extract from BHK cells infected with *ts*K or the three *ts*K recombinants. Although other proteins were recognised, the UL5, UL8 and UL52 polypeptides are easily identifiable and are indicated.

3G. INTRACELLULAR LOCALISATION OF THE UL5, UL8, UL9 AND UL52 PROTEINS

1. Cell Fractionation Studies

As described in Section 3B.4(a), an initial investigation into the intracellular localisation of the UL5, UL8, UL9 and UL52 proteins was performed by fractionation of [^{35}S]-labelled BHK cells infected at the NPT with *ts*K recombinant viruses into cytoplasm and nuclei. In singly infected cells the UL5, UL8 and UL52 proteins were present in both the cytoplasmic and nuclear fractions whereas the UL9 protein was detected predominantly in the nuclei.

These studies were extended to include indirect immunofluorescence as a method of determining the intracellular localization of the replication proteins expressed by the *ts*K recombinant viruses.

2. Indirect Immunofluorescence

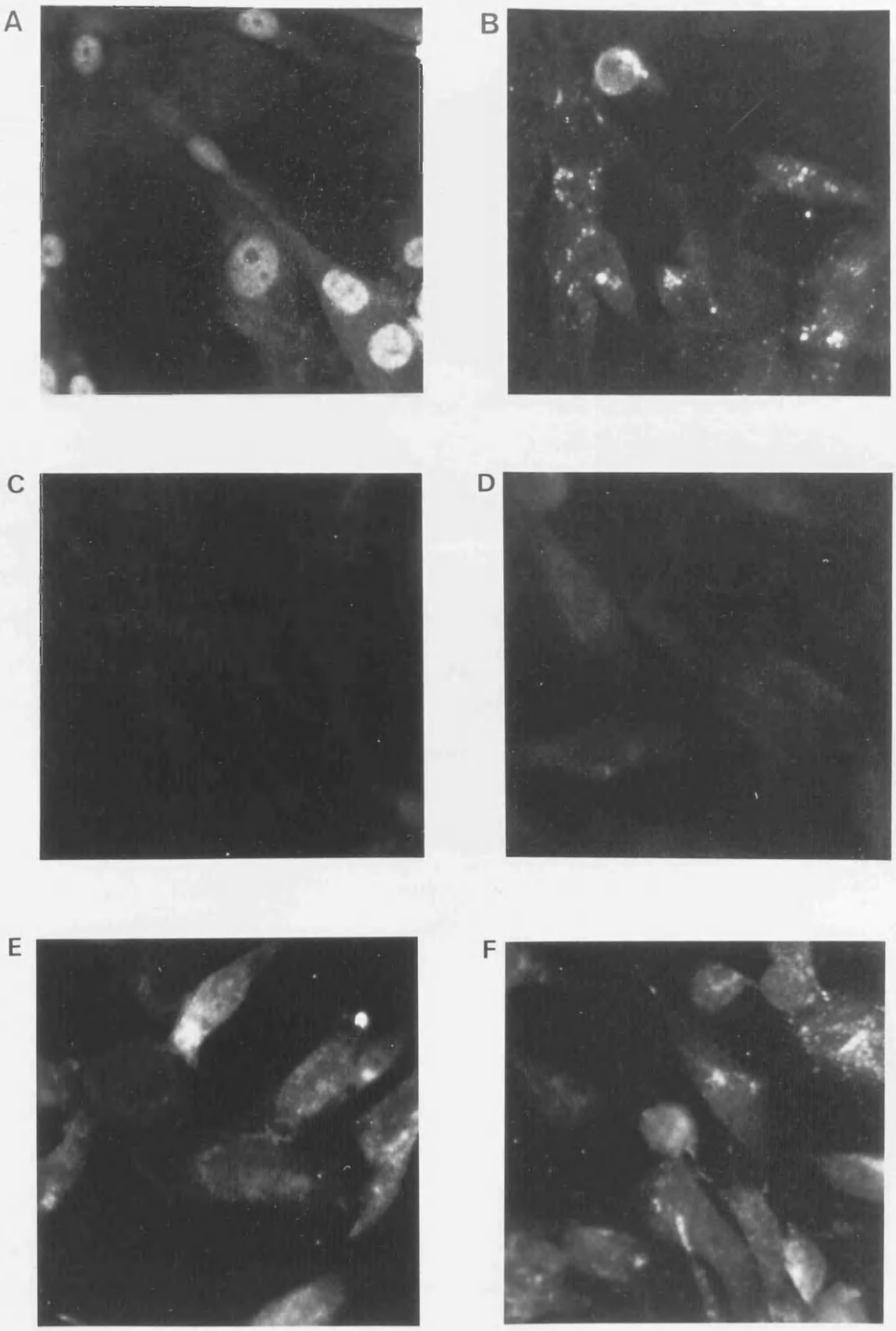
In initial experiments monolayers of BHK cells in slide chambers were infected at the NPT with 20 pfu/cell of either *wt* HSV-1, *ts*K, or one of the four recombinant viruses *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 or *ts*K/UL52. At 10 hpi the cells were fixed with formaldehyde and reacted with rabbit anti-peptide antisera (provided by Dr M D Challberg) to the UL5, UL8, UL9 and UL52 proteins. Primary antibodies were detected using FITC-conjugated swine anti-rabbit IgG. These experiments revealed that two of the anti-peptide antisera used, ie. anti-UL9 and anti-UL52, gave good fluorescent staining of the cognate proteins whereas anti-UL5 and anti-UL8 anti-sera did not. The UL9 protein expressed by *ts*K/UL9 localized in the nucleus of infected cells, exhibiting a diffuse pattern with some discrete foci (**Figure 50**, panel **a**). As shown in panel **b**, the UL52 protein expressed by *ts*K/UL52 exhibited a cytoplasmic perinuclear staining pattern. In comparison, cells infected with *ts*K exhibited only background fluorescence with either anti-UL9 or anti-UL52 antiserum (panels **c** and **d** respectively).

In *wt* HSV-1 infection neither the UL9 or UL52 protein appeared to be expressed in great enough quantities for specific fluorescence to be readily detected (panels **e** and **f**).

Figure 50. Intracellular Localization of the UL9 and UL52 Proteins

BHK cell monolayers infected at the NPT with *wt* HSV-1 (**e** and **f**), *ts*K (**c** and **d**), *ts*K/UL9 (**a**) or *ts*K/UL52 (**b**) were fixed and permeabilized as described in Materials and Methods and incubated with either anti-UL9 (**a**, **c** and **e**) or anti-UL52 (**b**, **d** and **f**) antiserum.

Cells were photographed under x40 magnification.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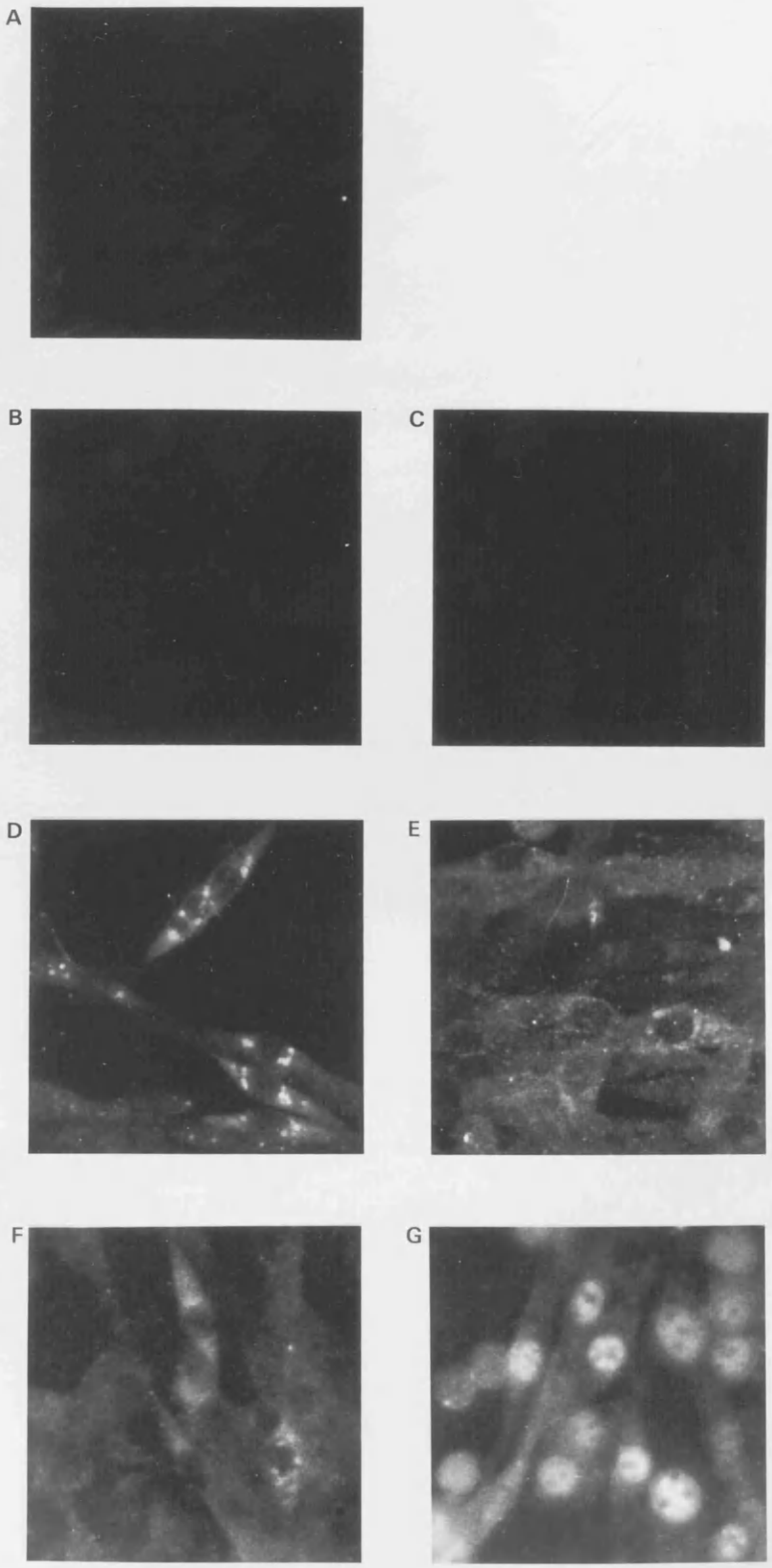
(a) Intracellular Localisation of the HSV-1 Helicase-Primase Complex

The intracellular localisation of the UL5, UL8 and UL52 proteins when expressed alone may differ from their localisation when co-expressed, when they interact to form the helicase-primase complex. This possibility is further suggested by the observation that the UL52 protein is predominantly cytoplasmic, whereas its expected site of activity is within the cell nucleus (**Figure 50**, panel **b**). Localisation of the UL52 protein was studied in single and mixed infections of BHK cells with the *ts*K recombinant viruses. Monolayers of BHK cells in slide chambers were infected at the NPT with *ts*K or the three recombinants *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52 either individually or in combination. At 10hpi infected cells were fixed and reacted with anti-UL52 antiserum. Staining patterns observed are shown in **Figure 51**. In control infections where the UL52 protein was not expressed, only background fluorescence was observed (panels **a**, **b** and **c**). Cells expressing the UL52 protein alone exhibited a cytoplasmic perinuclear staining pattern (panel **d**). However, in cells infected with all three recombinants, the UL52 protein became localised in the nucleus exhibiting strong diffuse nuclear staining with discrete, densely stained foci also present (nucleoli appeared unstained) (panel **g**). Cells which co-expressed the UL52 protein with either the UL5 protein or the UL8 protein, exhibited cytoplasmic perinuclear staining (panels **e** and **f**), similar to UL52 protein expressed alone. Previous experiments (Calder and Stow, 1990; Dodson and Lehman, 1991; This thesis) demonstrated that co-expressed UL5 and UL52 proteins form a complex, the immunofluorescence experiments, however, suggest that this complex may be unable to enter the cell nucleus. When the UL8 protein was also expressed with the UL5 and UL52 proteins, the localisation of the UL52 protein clearly became nuclear (panel **g**). A possible role for the UL8 protein in facilitating entry of the UL5 and UL52 subunits which specify helicase-primase activities into the cell nucleus may therefore be proposed. However, since UL52 protein co-expressed with UL8 protein also exhibited a cytoplasmic perinuclear staining pattern (panel **f**) it would seem that the UL8 protein is not able to facilitate transport of uncomplexed UL52 protein into the nucleus. DNA sequence analysis of the UL5, UL8 and UL52 ORFs has not revealed the presence of sequences obviously resembling those involved in the nuclear localisation of proteins (McGeoch *et al.*, 1988a).

Similar indirect immunofluorescence experiments were attempted

Figure 51. Intracellular Localization of the UL52 Protein in BHK cells Infected with *ts*K Recombinant Viruses (I)

BHK cell monolayers were infected at the NPT with *ts*K (**A**), *ts*K/UL5 (**B**), *ts*K/UL8 (**C**) and *ts*K/UL52 (**D**), *ts*K/UL5 plus *ts*K/UL52 (**E**), *ts*K/UL8 plus *ts*K/UL52 (**F**) or *ts*K/UL5 plus *ts*K/UL8 plus *ts*K/UL52 (**G**), fixed, permeabilized and incubated with anti-UL52 antiserum as described in Materials and Methods, Section 2B.38.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using anti-UL5 and anti-UL8 antisera. However, several attempts failed to yield convincing data on the intracellular localisation of the UL5 and UL8 protein subunits of the HSV-1 helicase-primase complex in single or mixed infections.

Although this work on the intracellular localisation of the HSV-1 helicase-primase complex and its subunits therefore remains incomplete, recent experiments (N D Stow and E C Stow, *per. comm.*) have confirmed and extended these findings. In particular they have shown that for each of the three constituent subunits efficient nuclear entry is only detected when co-expressed with the other two subunits. These results suggest that the UL8 protein may have a role in assisting transportation of the enzymatically active subunits of the complex (i.e. UL5 and UL52 proteins) from cytoplasm to nucleus but do not exclude the possibility that it may also be required for some other function such as stability, an additional, as yet unidentified enzymatic activity, or interaction with host components within the nucleus.

CHAPTER 4:DISCUSSION

1. Identification of the UL5, UL8, UL9 and UL52 polypeptides

At the outset of the work presented in this thesis the HSV-1 ORFs, UL5, UL8, UL9 and UL52, had been identified as encoding products essential for the replication of viral DNA. However, due to their low abundance in HSV-1 infected cells the identification of these polypeptides had not been achieved. By expressing the UL5, UL8, UL9 and UL52 ORFs individually using recombinant *ts*K viruses four unique, novel polypeptides were identified, each with an apparent molecular weight corresponding closely to that predicted. The identity of the UL5, UL8, UL9 and UL52 proteins was confirmed by immunoprecipitation and immunoblotting with antisera raised against peptides corresponding to their predicted C-terminal amino acid sequences. Phosphate labelling experiments showed that the UL5, UL8, UL9 and UL52 proteins, expressed under IE control at the NPT, were not significantly phosphorylated. The electrophoretic mobility of the UL5, UL8, UL9 and UL52 proteins in SDS-PAGE was constant whether expressed using *ts*K or baculovirus as a vector. This suggests that any other form of post-translational processing of the over-expressed proteins which may occur is similar in mammalian and insect cells.

The results of experiments described in this thesis show that three of the HSV-1 replication proteins (UL5, UL8 and UL52), when co-expressed, form a complex which exhibits two activities associated with DNA-strand unwinding and in addition possesses a DNA primase activity. Although the UL8 protein is not essential for these enzyme activities it appears to be required for efficient nuclear uptake of the enzymatically active subunits (UL5 and UL52).

2. Identification of the HSV-1 Helicase-Primase Complex

(a) DNA-Dependent ATPase

Multiple infection of BHK cells with the four recombinant viruses, *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52, at the non-permissive temperature allowed the identification of a novel viral-induced DNA-dependent ATPase activity in fractionated cell extracts. This activity was not induced in BHK cells infected with the parental virus, *ts*K. This result (Section 3C.1) suggested that the novel activity was encoded by one or more of the HSV-1 DNA replication genes UL5, UL8, UL9 and UL52. SDS-PAGE analysis of phosphocellulose column fractions

revealed that the UL5, UL8 and UL52 proteins co-eluted with the unique ATPase. One or more of these three polypeptides, therefore, appears likely to specify the induced activity.

Following the identification of this HSV-1 induced ATPase a series of single and mixed infections were performed to determine the viral protein(s) responsible. These experiments (Results, Sections 3D.1 and 3F.1) used infection of BHK cells with *ts*K recombinant viruses expressing the replication proteins, and infection of *S.f.* insect cells with recombinant baculoviruses.

Induction of a unique peak of ATPase activity was demonstrated in BHK cells triply infected at the NPT with *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52, and in *S.f.* cells triply infected with AcUL5, AcUL8 and AcUL52. This activity was not detected in BHK cells infected with *ts*K or in *S.f.* cells infected with wild-type AcNPV. Fractions which exhibited ATPase activity also contained the three over-expressed HSV-1 proteins. Because the UL5, UL8 and UL52 polypeptides did not elute at this salt concentration when expressed individually these observations suggest they form a protein complex.

The DNA-dependent ATPase activity was also induced in *S.f.* cells doubly infected with AcUL5 and AcUL52. The level of activity induced was similar to that observed in triply infected cells indicating that the UL8 protein is dispensable for this activity. In single infections with AcUL5 or AcUL52 no ATPase induction was observed suggesting that both proteins are required for activity.

Although a novel ATPase was similarly induced in BHK cells co-infected at NPT with *ts*K/UL5 and *ts*K/UL52 the level of activity was somewhat reduced from that observed in BHK cells infected with all three recombinant viruses. This suggests that although the viral ATPase induced in BHK cells does not require the presence of the UL8 protein, this polypeptide may play some role in ensuring maximum activity. One possibility is that it could be involved in stabilising the enzymatically active subunits at the higher temperature of 38.5°C.

(b) DNA Helicase

DNA helicases enzymatically unwind duplex DNA in a reaction coupled to the hydrolysis of ATP (or another NTP or dNTP). DNA helicases are thus also DNA-dependent ATPases. In Section 3F.2, the results of co-infection experiments with recombinant baculoviruses showed induction of a novel DNA helicase in insect cells either triply

infected with AcUL5, AcUL8 and AcUL52 or co-infected with AcUL5 and AcUL52. This activity was absent from infections with wild-type AcNPV, individual recombinants or double infections with AcUL5 and AcUL8 or AcUL8 and AcUL52. The novel DNA helicase eluted in column fractions which also exhibited DNA-dependent ATPase activity and contained the over-expressed HSV-1 proteins. The DNA helicase was of similar activity when isolated from cells co-expressing the UL5 and UL52 proteins or expressing all three helicase-primase subunits, indicating that the UL8 protein is also dispensable for helicase activity in this system.

(c) DNA Primase

Co-infection experiments with AcUL5, AcUL8 and AcUL52 additionally demonstrated the induction of a novel DNA primase activity in *S.f.* cells co-expressing either all three over-expressed HSV-1 proteins or just the UL5 and UL52 proteins (Section 3F.6). The elution profile of this activity corresponded to that of the previously identified DNA-dependent ATPase and DNA helicase activities and to that of the co-expressed UL5, UL8 and UL52 (or UL5 and UL52) polypeptides.

Crute *et al.* (1989) purified an induced helicase-primase activity from HSV-1 infected cells and showed that it comprises three polypeptide subunits encoded by the viral genes UL5, UL8 and UL52. The results presented in this Thesis are in agreement with this observation, and furthermore show that the functional complex can be assembled *in vivo* following triple infection of cells with either appropriate *ts*K or AcNPV recombinant viuses. Production of a functional complex therefore does not require any other HSV-1 protein. Dodson *et al.* (1989) have also recently reported assembly of a functional UL5, UL8 and UL52 protein complex in insect cells infected with recombinant baculoviruses. The results presented in this thesis show that a two subunit assembly, comprising the UL5 and UL52 polypeptides, also possesses the same enzymatic activities as the three subunit complex. The UL8 protein subunit is therefore not required for any enzymatic activity so far attributed to the HSV-1 helicase-primase complex (Calder and Stow, 1990; Dodson and Lehman, 1991).

Recent experiments with highly purified UL5/UL8/UL52 or UL5/UL52 protein complexes from insect cells detected no significant differences between their *in vitro* enzymatic activities (Dodson and

Lehman, 1991). It is possible, however, that *in vivo* the presence of the UL8 protein may be important for helicase and primase activities which act upon the HSV-1 genome rather than upon model substrates.

Polypeptide subunits expressed individually do not exhibit any enzymatic activity indicating that the minimum requirement for ATPase, helicase and primase activities is a complex of the UL5 and UL52 proteins (This thesis; Dodson and Lehman, 1991).

3. Counterparts of Helicase-Primase Subunits in Other Herpesviruses

Homologues of the UL5 and UL52 genes have been identified by sequencing studies in other herpesviruses belonging to the alpha-, beta- and gammaherpesviruses subfamilies (VZV, HCMV and EBV respectively). These genes therefore appear to be well conserved throughout the herpesvirus family. Although functional studies have not been reported for any of these herpesvirus homologues, the high degree of conservation is consistent with the encoded proteins having essential roles in viral DNA synthesis. It is interesting to note that although a VZV homologue of the UL8 protein has been identified, no proteins with recognisable amino acid sequence similarity to the UL8 protein are encoded by the genomes of HCMV and EBV. The UL8 gene may therefore be specific to the alphaherpesvirus lineage. This is clearly in marked contrast to the UL5 and UL52 protein subunits. A corresponding helicase-primase complex in EBV and HCMV may contain an equivalent of the UL8 protein which has diverged to such an extent as to be unrecognisable, or the essential function of the UL8 protein in the HSV-1 complex may be carried out by the UL5 and/or UL52 homologue, or by some other polypeptide. These possibilities await biochemical characterization of the HCMV and EBV enzymes.

4. The Role of The UL8 Protein

The UL8 gene is essential for the replication of HSV-1 DNA (Wu *et al.*, 1988; Carmichael and Weller, 1989) and, as the results in Sections 3D.3 and 3F.3 show, its polypeptide product is a subunit of the viral helicase-primase complex. Experimental evidence to date has indicated that this protein is not required for enzymatic activity of the complex. The role of the UL8 protein within the helicase-primase complex and in replication of viral DNA is, therefore, somewhat unclear. No outstanding features are apparent within the amino acid sequence predicted to be encoded by the UL8 gene and no obvious homologues have been identified in recent data base searches. Although

dispensable for the activities so far attributed to the complex, the UL8 subunit may confer an additional, as yet unidentified, enzyme function. A candidate activity which is likely to be required on the lagging strand is DNA ligase. Alternatively, this subunit may play an important role in the formation, translocation or stability of the complex. Intracellular localization studies, described in Section 3G.2(a), show that when the UL52 protein is expressed in BHK cells in the absence of other HSV-1 replication proteins it exhibits a cytoplasmic perinuclear staining pattern. Only when all three subunits of the helicase-primase complex are co-expressed does the UL52 protein become efficiently localized within the cell nuclei. These observations, together with the recent results of other localization experiments (N D Stow and E C Stow, *pers. comm.*), suggest that all three HSV-1 protein subunits must be present together for efficient localization of any one of them to the cell nucleus. An important function of the UL8 protein may therefore be to facilitate transport of the complex following its assembly in the cytoplasm, into the cell nucleus.

Many nuclear proteins have specific nuclear loacalization sequences (short stretches composed mainly of basic amino acids) (Silver, 1991). No such signal is evident in the polypeptides encoded by UL5, UL8 or UL52. It is possible that only a complex formed of all three subunits exists in a conformation such that a signal is presented which is recognized by the nuclear transport machinery of the cell.

Further studies are required to determine the factors responsible for localization of the HSV-1 helicase-primase complex within the cell nucleus during lytic infection. Of particular interest will be the intranuclear distribution of the complex in the presence of HSV-1 mDBP. This essential viral replication protein (encoded by gene UL29), in addition to binding single-stranded DNA, appears to play an important role in the organization of other viral and cellular replication proteins within the HSV-1 infected cell (Quinlan *et al.*, 1984; deBruyn Kops and Knipe, 1988; Wilcock and Lane, 1991). Under conditions which permit viral DNA synthesis, the HSV-1 DNA polymerase, UL42 protein and origin-binding protein each exhibit an intranuclear distribution similar to that of mDBP, being confined to the so-called replication compartments where viral DNA synthesis occurs (Olivo *et al.*, 1989). In contrast, in the absence of viral DNA synthesis these proteins are found at numerous small foci in the nucleus which are termed pre-replicative sites (Quinlan *et al.*, 1984; deBruyn Kops

and Knipe, 1988). Studies with HSV-1 mutants reveal that a functional mDBP is essential for the localization of the POL and UL42 proteins to these sites (Goodrich *et al.*, 1990; Bush *et al.*, 1991). By expressing the helicase-primase complex in the presence and absence of mDBP it may be possible to determine whether distribution of the complex is similarly affected by mDBP.

5. The UL5, UL8 and UL52 Proteins as Antiviral Targets

There are apparently no significant differences between the helicase-primase assembled in insect cells and that purified from HSV-1 infected cells (Crute and Lehman, 1991). The readily available recombinant enzyme may therefore be regarded as essentially identical to the helicase-primase complex which participates in the replication of the HSV-1 genome. Studies of the enzymatic mechanisms of this complex and of the structure and assembly of its protein subunits may aid in the development of specific antiviral agents. The helicase-primase complex is an attractive target because all three proteins are essential for lytic infection. In addition, because enzymatic activity appears to depend minimally upon a complex of the UL5 and UL52 subunits, it may be possible to inhibit viral replication by blocking assembly or causing dissociation of these subunits. The complex may also possibly be targeted through the use of substrate analogues, e.g. ribonucleotides, single-stranded or forked DNA substrates.

6. The UL5 Protein Amino Acid Sequence

By analysis of predicted polypeptide sequences Gorbalenya and Koonin (1989) have identified four superfamilies of cellular and viral proteins involved, or likely to be involved, in DNA or RNA helicase activity. The HSV-1 UL5 and UL9 proteins are members of superfamilies one and two respectively. Proteins in both of these families contain seven relatively well conserved motifs spanning the length of the polypeptide chain.

Of the seven conserved regions, motifs I and II respectively coincide with domains A and B of the well-known NTP-binding site present in many classes of proteins and described by Walker *et al.* (1982). The remaining motifs appear to be distinctive features of these known and putative helicases (Gorbalenya *et al.*, 1988; Gorbalenya and Koonin, 1989; Hodgman, 1988). Motif I exhibits the consensus sequence G/AXXGXGKS/T and is found almost exclusively in ATP- and GTP-binding proteins. X-ray crystallography studies suggest that this

domain forms a loop which binds a phosphate group (reviewed by Hodgman, 1988). Motif II contains a hydrophobic region preceding a conserved aspartate residue which is implicated in phosphate binding via a magnesium ion.

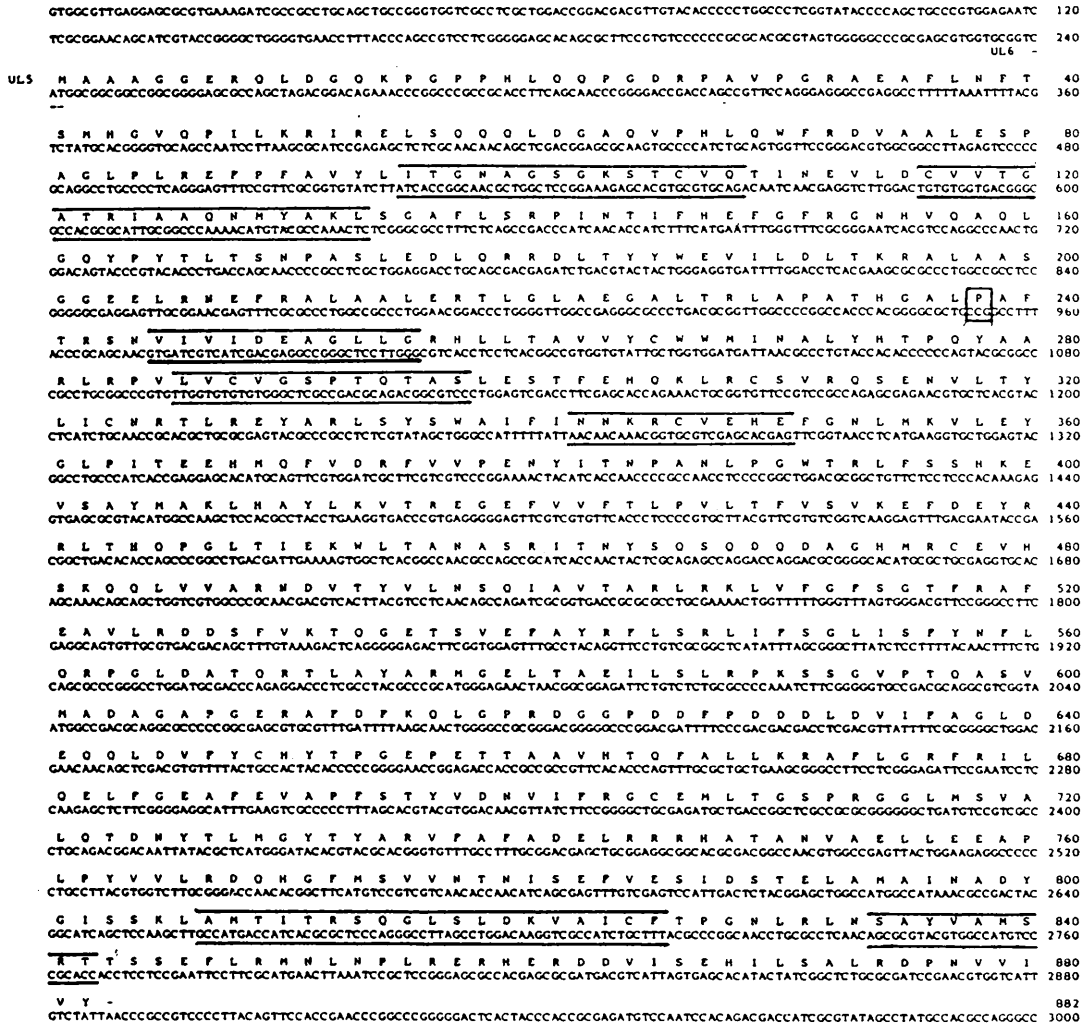
The positions of the seven conserved motifs in the UL5 sequence are shown in **Figure 52.** Motif I contains the sequence GNAGSGKS, which fits the consensus of a classical NTP-binding site. Also, within motif II, four hydrophobic residues precede conserved aspartate and glutamate residues. In addition, the UL5 sequence contains another motif, Ia, and a conserved tyrosine residue at the third position within motif VI, both of which are associated with DNA or polynucleotide binding (reviewed by Hodgman, 1988).

A mutant of HSV-1, *ts*K13, which is defective for DNA synthesis at the NPT, contains a single point mutation in the UL5 gene resulting in a change of the proline residue at position 238 to a leucine (**Figure 52**; Zhu and Weller, 1988). The temperature sensitive phenotype of this mutant is presumed to have arisen as a consequence of this amino acid substitution which lies close to motif II, the presumed NTP-binding site. Whether the *ts*K13 mutation affects binding or hydrolysis of ATP by the UL5 subunit awaits a functional comparison of wild-type and mutant polypeptides.

The presence of these motifs within the UL5 protein suggest that DNA helicase may be an intrinsic property of this subunit of the HSV-1 helicase-primase complex. However, the results shown in Sections 3D and 3F and reported by Dodson and Lehman (1991) indicate that neither the UL5 nor UL52 protein expressed individually is a DNA helicase or DNA primase. The highly conserved sequences present in the UL5 polypeptide are, nevertheless, likely to be important for helicase activity of the complex. The role of the UL52 protein in mediating this activity is unknown. Whether the NTP-binding site of the UL5 polypeptide is relevant to the primase activity of the complex is also unknown.

7. Coupling of Helicase and Primase Functions

The coupling of DNA helicase and DNA primase activities occurs frequently in prokaryotes. The complex comprising two HSV-1 polypeptides (UL5 and UL52) which exhibits helicase and primase activities may, for example, be analogous to the gene 41/61 primosome complex of bacteriophage T4 (Nossal and Alberts, 1983; Matson and



I: ITGNAGSGKSTCVQ

Ia: CVVTGATRIAAQNMYAKL

II: VIVIDEADGLLG

III: LVCVGSPTQTAS

IV: NNKRCVEHE

V: AMTITRSQGLSLDKVAICF

VI: SAYVAMSRT

Figure 52. Predicted Amino Acid Sequence Encoded by Gene UL5

The figure shows the DNA sequence of the UL5 gene (coding strand only)(McGeoch *et al.*, 1988a), with the predicted amino acid sequence encoded given in single-letter code above the DNA sequence. Also shown are 240 residues 5' and 114 residues 3' to the UL5 ORF. The translation initiation codons for adjacent genes UL4 and UL6 (CAT on UL5 coding strand) are marked.

Amino acid sequence motifs conserved in proteins belonging to the proposed helicase superfamily 1 are indicated by the solid, horizontal lines. These motifs (from the N-terminus I, Ia, II, III, IV, V and VI) are listed at the side (McGeoch *et al.*, 1988a; Gorbalenya and Koonin, 1989).

The proline residue at position 238, which is replaced by a leucine in the mutant *ts*K13 (Zhu and Weller, 1988), is boxed.

Kaiser-Rogers, 1990). In T4, DNA helicase activity is encoded by gene 41 and gene 61 protein is associated with DNA primase activity. When these proteins are dissociated, however, both activities are greatly reduced. By analogy, in HSV-1, helicase and primase activities may be performed by the products of genes UL5 and UL52 repectively but only under conditions where the two polypeptides associate to form a specific complex. If this is the case it may be possible to dissociate the two activities by mutagenesis.

In eukaryotes primase activity is frequently coupled with DNA polymerase in a tight complex. For example, mammalian DNA POLα, which comprises four polypeptide subunits possesses a catalytic domain for DNA synthesis, contained within the largest subunit, and a DNA primase activity supplied by the two smallest subunits (Fry and Loeb, 1986; Campbell, 1986). An example of coupling of DNA helicase and DNA primase functions may occur during replication of the SV40 genome. A specific interaction between mammalian DNA POLα and SV40 T antigen at the replication fork has been demonstrated (Smale and Tjian, 1986; Gannon and Lane, 1987) This may facilitate coupling of the DNA primase activity associated with POL α with the DNA helicase function of T antigen at the replication fork.

8. DNA Helicase Activity of the HSV-1 Origin-Binding Protein

The results described in Section 3F.5 show that the HSV-1 OBP, encoded by gene UL9, is also a DNA helicase. In addition the results show that the C-terminal one-third of the protein, which contains the origin-specific DNA-binding domain (Weir *et al.*, 1989) is incapable of DNA-strand unwinding. This indicates that sequences within the N-terminal two-thirds of the HSV-1 OBP are important for DNA helicase activity. The HSV-1 DNA-negative mutant, *ts*S, harbours a lesion within the N-terminal two-thirds of the UL9 protein and appears to express similar levels of origin-binding activity at the NPT to *wt* HSV-1 (Weir, 1990). This suggests that sequences in this part of the protein, which are distinct from those involved in origin-binding perform a function essential or important for replication of viral DNA. Whether the *ts*S mutation affects the DNA helicase activity of the UL9 protein awaits further study. In agreement with the properties of the C-terminal one-third fragment of the UL9 protein, the amino acid sequence predicted to be encoded by the UL9 gene contains, within the N-terminal 400 amino acids, seven motifs which have lead to its inclusion in superfamily two of proteins with helicase or putative helicase

activity (Gorbalenya and Koonin, 1989). The sequence APMGSGKT, which fits the consensus of an NTP-binding site, is present near the N-terminus of the UL9 protein within motif I.

In agreement with the results presented in this Thesis, Bruckner *et al.* (1991) have very recently reported that DNA-dependent ATPase and DNA helicase activities are exhibited by the HSV-1 UL9 protein in addition to its origin-binding properties. ATP hydrolysis by the OBP was reported to be strongly dependent upon the structure and sequence of the DNA present in the assay, with apparent preferences for single-stranded over double-stranded DNA and for poly(dT) over M13mp18 DNA sequences.

As shown in Section 3F.5, the UL9 DNA helicase is active on partial duplex substrates with or without a 3' single-stranded tail on the fragment to be displaced. This is also in agreement with the findings of Bruckner *et al* (1991). The UL9 protein, therefore, in addition to recognising and binding the HSV-1 origin, may locally unwind a short stretch of DNA at the origin in a manner similar to the separation of DNA strands at the SV40 origin by T antigen. However, there is no evidence to date for the unwinding of duplex ori_S or ori_L sequences by the UL9 protein.

In addition to its DNA unwinding properties at the SV40 origin of replication, T antigen is also capable of acting as a DNA helicase at the SV40 DNA replication fork (Stahl et al, 1986). Unwinding of duplex DNA at advancing HSV-1 replication forks could potentially be mediated by the OBP or the helicase-primase complex. Although helicase activities of the OBP and the helicase-primase complex have been demonstrated only on model substrates it seems likely that the helicase-primase complex plays a major role in the progression of the HSV-1 DNA replication fork whilst the helicase activity of OBP may be more important for initial opening of the ori_S and ori_L regions.

The DNA helicase activity of the HSV-1 helicase-primase complex is dependent upon the presence within the substrate of a 3' single-stranded tail on the fragment to be displaced (Crute *et al.*, 1988). This suggests that duplex DNA may only by unwound if a region where the DNA strands are already separated exists. Melting of a region of duplex DNA at the origin, perhaps facilitated by the DNA helicase action of the UL9 protein, may allow the relatively large helicase-primase complex access to the template DNA strands at the HSV-1 origin. The UL9 protein may additionally be involved in the assembly of a replicative complex at the HSV-1 origin. A general feature of OBP-

origin complexes may be to enable interaction of other replication proteins at this region.

Amplification of heterologous sequences by HSV-1 replication proteins has been reported in transformed cell lines and transfected cells in which functional SV40 T antigen and linked SV40 origin are present. In these instances all the HSV-1 replication proteins except the UL9 protein are required for amplification of heterologous DNA (Matz, 1989; Heilbron and zurHausen, 1989). The data suggest that a T antigen-SV40 origin complex may also exhibit some affinity for the remaining HSV-1 replication proteins resulting in their recruitment to the SV40 origin region and amplification of the atta ched sequences. It is not known whether this phenomenon is based primarily upon protein-protein interactions or whether the structure of locally unwound DNA may be important.

Following initial strand separation at the origin, the HSV-1 mDBP, which is essential for the replication of viral DNA, may contribute to maintaining the melted region as separate DNA strands in order to provide a loading site for the helicase-primase complex and establish a stable HSV-1 replication fork. In the presence of mDBP, the viral helicase-primase complex is capable of completely unwinding a nicked, double-stranded circular DNA template (Crute and Lehman, 1991). The DNA helicase component of the complex is proposed to bind the lagging strand template and to unwind the duplex whilst moving in a 5'-3' direction. The DNA primase activity, which synthesizes oligoribonucleotides 8-12 bases in length (Crute and Lehman, 1991), is available to prime discontinuous synthesis on the lagging strand whilst the displaced leading strand is available for continuous synthesis. Replication of both strands can therefore be co-ordinated by coupled activities of helicase and primase.

9. The HSV-1 DNA Replication Fork

The experimental evidence to date indicates that the only DNA polymerase required for the replication of HSV-1 DNA is the viral UL30 gene product. As described in the Introduction (Section 1D.3(a)) models for the concurrent synthesis of leading and lagging strands in *E. coli* and SV40 have been proposed which utilize one or two polymerases respectively.

In *E. coli*, Kornberg (1982; 1988) proposes that two DNA polymerase III molecules form an asymmetric dimeric complex by differential association of accessory proteins with one or other catalytic subunit

such that leading strand synthesis by one and lagging strand synthesis by the other is favoured. Looping of the lagging strand template around one polymerase molecule facilitates concurrent synthesis of both strands.

Similarly, in the replication of SV40 DNA, mammalian DNA polymerases α and δ, differentially complexed with specific accessory protein subunits, respectively synthesize lagging and leading strands. The lagging strand template has been proposed to be looped around POL α or alternatively maintained as an extended loop extended by single-stranded DNA-binding protein (RP-A/HSSB) (Stillman, 1989; Hurwitz *et al.*, 1990).

Events at the *E. coli* and SV40 replication forks are therefore co-ordinated by interactions between strand-specific DNA polymerase activities, accessory proteins, DNA helicase and DNA primase.

Fractionation studies of HSV-1 infected cells have shown that the catalytic subunit of the viral DNA polymerase (POL), encoded by gene UL30, co-purifies with the product of the HSV-1 gene UL42 (Gallo *et al.*, 1988; Parris *et al.*, 1988). In the absence of UL42 protein the catalytic subunit acts as a moderately processive DNA polymerase. In the presence of the UL42 protein, however, the enzyme is highly processive (Gallo *et al.*, 1989; Gottlieb *et al.*, 1990; Hernandez and Lehman, 1990), and comprises a heterodimer of the UL30 and UL42 gene products.

It is not known whether the replication of HSV-1 DNA involves dimerization of DNA polymerase catalytic subunits. However, by analogy with the models proposed for other systems, a complex of two polymerase molecules may facilitate concurrent synthesis of both strands of the viral DNA. A model can be proposed in which the leading strand is synthesized processively by the POL-UL42 heterodimer whilst primers synthesized on the lagging strand by the helicase-primase complex are elongated by the less processive POL alone. This polymerase molecule may therefore also interact directly with the helicase-primase complex. The main argument against this model is the 20-fold molar excess of UL42 protein over POL (Gottlieb *et al.*, 1990). However, different processed forms of UL42 protein are known to exist (Marsden *et al*, 1987) and perhaps these may regulate the activity of POL on the two strands.

In addition to its DNA polymerase function POL possesses intrinsic 3'-5' exonuclease proofreading and 5'-3' exonuclease/RNase H activities. Following synthesis of nascent fragments on the lagging strand

primers may be removed by the RNase H activity associated with POL and replaced by deoxyribonucleotides. HSV DNA synthesis is also likely to require additional functions, most importantly a DNA ligase to join nascent fragments, and topoisomerases (types I and/or II) for the release of torsional strain ahead of the advancing replication fork and perhaps also in the separation of daughter molecules. It is not clear whether the topoisomerase type I activity induced in HSV-1 infected cells is encoded by the virus or host cell genome (Biswal *et al.*, 1983; Muller *et al.*, 1985), whereas the induced topoisomerase type II activity is apparently specified by the host cell (Ebert *et al.*, 1990). The source of DNA ligase activity is also unclear. A specific strand nicking activity may also be important in switching from a mechanism involving bi-directional movement of replication forks to a rolling-circle mode in which only a single replication fork is active.

Other viral and cellular activities are also likely to be involved in regulating replication of the HSV-1 genome. These may include enzymes involved in nucleotide metabolism, protein kinases and phosphatases which might regulate the activity of the replicative enzymes and factors which control viral gene expression, particularly expression of the essential DNA replication proteins.

10. Further Work

The work presented in this Thesis has raised several interesting questions which it should now be possible approach experimentally. These include:

1. Attempting to separate the helicase and primase functions of the UL5/UL52 protein complex by site-directed mutagenesis, and hence possibly assign roles to the individual subunits.

2. Investigation of the requirements for interaction of the complex with single-stranded DNA.

3. Investigation of possible functions of the UL8 protein and in particular its role in facilitating nuclear entry.

4. Investigation of possible interactions of the helicase-primase complex with other components of the replicative machinery.

5. Investigation of whether the helicase activity of intact UL9 protein can unwind duplex DNA at the HSV-1 origin.

These studies should contribute to our understanding of the functions of the HSV-1 replication proteins and their mechanisms of action. Complete resolution of the factors involved in the replication of the

HSV-1 genome and their mechanisms of action, however, probably awaits the development of a cell-free system of HSV-1 origin-dependent DNA synthesis.

References

Abazua, P., Soeller, W. and Marians, K.J. (1984). Mutational analysis of primosome assembly sites I. Distinct classes of mutants in the pBR322 *Escherichia coli* factor Y DNA effector sequences. J. Biol. Chem. 259, 14286-14292.

Ace, C.I., Dalrymple, M.A., Ramsay, F., Preston, V.G. and Preston, C. M. (1988). Mutational analysis of the herpes simplex virus type 1 trans-inducing factor Vmw65. J. Gen. Virol. 69, 2595-2605.

Ace, C.I., McKee, T., Ryan, J.M., Cameron, J.M. and Preston, C.M. (1989). Construction and characterisation of a herpes simplex virus type 1 mutant unable to transinduce immediate early gene expression. J. Virol. 63, 2260-2269.

Ackermann, M., Braun, D.K., Pereira, L. and Roizman, B. (1984). Characterisation of the herpes simplex virus 1 alpha proteins 0, 4 and 27 with monoclonal antibodies. J.Virol. 52, 108-118.

Ackermann, M., Chou, J., Sarmiento, M., Lerner, R.A. and Roizman, B. (1986). Identification by antibody to a synthetic peptide of a protein specified by a diploid gene located in the terminal repeats of the L component of herpes simplex virus genome. J. Virol. 58, 843-850.

Addison, C. (1986). Characterisation of herpes simplex virus type 1 ts mutants which have structural defects. PhD Thesis, University of Glasgow.

Addison, C., Rixon, F.J., Palfreyman, J.W., O'Hara, M. and Preston, V.G. (1984). Characterization of a herpes simplex virus type 1 mutant which has a temperature-sensitive defect in penetration of cells and assembly of capsids. Virology 138, 249-259.

Al-Kobaisi, M. F. (1989). Identification and characterisation of herpes simplex virus genes required for encapsidation of DNA. Ph.D. Thesis. Glasgow University.

Alberts, B.M. (1984) The DNA Enzymology of protein replication machines. C.S.H. Symp. Quant. Biol. 49, 1-42.

Alberts, B.M., Barry, J., Bedinger, P., Formosa, T., Jongeneel, C.V., and Kreuzer, K.N. (1983). Studies on DNA replication in the T4 bacteriophage system. Cold Spring Harbour Symposia on Quantitative Biology. 47, 655-668.

Alberts, B. and Herrick, G. (1971) DNA cellulose chromatography. In: Methods in Enzymology XXI, (eds. Colowick, S.P. and Kaplan, N.O.) 198-217.

Alwine, J.C., Steinhart, W.L. and Hill, C.W. (1974). Transcription of the herpes simplex virus type 1 DNA in nuclei isolated from infected Hep-2 and KB cells. Virology 60, 302-307.

Aro, G.M., Purifoy, D.M. and Schaffer, P.A. (1975). DNA synthesis and DNA polymerase activity of herpes simplex virus type 1 temperature sensitive mutants. J. Virol. 16, 498-507.

Asher, Y., Heller, M. and Becker, Y. (1969). Incorporation of lipids into herpes simplex virus particles. J. Gen. Virol. 4, 65-76.

Bacchetti, S., Evelegh, M.J., Muirhead, B. and Spector, T., (1984). Immunological characterisation of herpes simplex virus type 1 and 2 polypeptides involved in viral ribonucleotide reductase activity. J. Virol. 49, 591-593.

Baer, R., Bankier, T., Biggin, M.D., Deininger, P.L., Farrell, P.J., Gibson, T.J., Hatfull, G., Hudson, G.S., Satchwell, S.C., Sequin, C., Tuffnell, P.S. and Barrell, B.G. (1984). DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature 310, 207-211.

Baker, T. and Kornberg, A. (1988). Transcriptional activation of initiation of replication from the *E.coli* chromosomal origin. An RNA-DNA hybrid near oriC. Cell 55, 113-123.

Baker, T.A., Funnel, B.F. and Kornberg, A. (1987). Helicase action of dnaB protein during replication from the *Escherichia coli* chromosome

origin *in vitro*. J. Biol. Chem. 262, 6877-6885.
Baker, T.A., Sekimizu, K., Funnell, B.E. and Kornberg, A. (1986). Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. Cell 45, 53-64.
Baringer, J.R. and Swoveland, P. (1973). Recovery of herpes simplex virus from human trigeminal ganglia. New Engl. J. Med. 288, 648-650.
Batterson, W. and Roizman, B. (1983). Characterization of the herpes simplex virion-associated factor responsible for the induction of alpha genes. J. Virol. 40, 371-377.
Bauer, G.A. and Burgers, H.M. (1988). DNA polymerase from *Saccharomyces cerevisiae*. 1. Purification and characterization. J. Biol. Chem. 263, 917-924.
Baur, C. and Knippers, R. (1988). Protein induced bending of the simian virus 40 origin of replication. J. Biol. Chem. 203, 1009-1019.
Bayliss, G.J., Marsden, H.S. and Hay, J. (1975). Herpes simplex virus proteins. DNA binding proteins in infected cells and in the virus structure. Virology 68, 124-134.
Beard, P., Faber, S., Wilcox, K.W. and Pizer, L.I. (1986). Herpes simplex virus immediate early infected cell polypeptide 4 binds to DNA and promotes transcription. Proc. Natl. Acad. Sci. USA 83, 4016-4020.
Becker, Y., Dym, H. and Sarov, I. (1968). Herpes simplex virus DNA. Virology 36, 184-192.
Ben-Porat, T. and Rixon, F.J. (1979). Replication of herpesvirus DNA. IV. Analysis of concatemers. Virology 94, 61-70.
Ben-Porat, T. and Tokazewski, S. (1977). Replication of herpesvirus DNA. II. Sedimentation characteristics of newly-synthesised DNA. Virology 79, 292-301.
Ben-Porat, T. and Veach, R.A. (1980). Origin of replication of the DNA of a herpesvirus (pseudorabies). Proc. Natl. Acad. Sci. USA 77, 172-175.
Bernstein, J.A. and Richardson, C.C. (1988a). A 7kDa region of the bacteriophage T7 gene 4 protein is required for primase but not helicase activity. Proc. Natl. Acad. Sci. USA 85, 396-400.
Bernstein, J.A. and Richardson, C.C. (1988b). Purification of the 56kDa component of the bacteriophage T7 primase/helicase and characterization of its nucleoside 5'-triphosphatase activity. J. Biol. Chem. 263, 14891-14899.
Bernstein, J.A. and Richardson, C.C. (1989). Characterization of the helicase and primase activities of the 63kDa component of the bacteriophage T7 gene 4 protein. J. Biol. Chem. 264, 13066-13073.
Biswal, N., Feldan, P. and Levy, C.C. (1983). A DNA topoisomerase activity copurifies with the DNA polymerase induced by herpes simplex virus. Biochim. Biophys. Acta 740, 379-389.
Blue, W.T. and Stobbs, D.G. (1981). Isolation of a protein kinase induced by herpes simplex virus type 1. J.Virol. 38, 383-388.
Bond, V.C., Person, S. and Warner, S.C. (1982). The isolation and characterization of mutants of herpes simplex virus type 1 that induces cell fusion. J. Gen. Virol. 61, 245-254.
Borowiec, J.A. and Hurwitz, J. (1988). Localized melting and structural changes in the SV40 origin of replication induced by T-antigen. EMBO J. 1, 3149-3158.
Bolet, A., Simon, M. Faye, G., Bauer, G.A. and Burgers, P.M.J. (1989). Structure and function of the *Saccharomyces cerevisiae* CDC2 gene encoding the large subunit of DNA polymerase III. EMBO J. 8, 1849-1854.
Bramhill, D. and Kornberg, A. (1988a). Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the E. coli chromosome. Cell 52, 743-755.
Bramhill, D. and Kornberg, A. (1988b). A model for initiation at origins

of DNA replication. Cell 54, 915-918.
Bravo, R. and Macdonald-Bravo, (1985). Changes in the nuclear distribution of cyclin (PCNA) but not its synthesis depend on DNA replication. EMBO J. 4, 655-661.
Briggs, M.R., Ikadonaga, J.T., Bell, S.P. and Tjian, R. (1986). Purification and biochemical characterization of the promoter-specific transcription factor Sp1. Science 234, 47-52.
Brown, M. and Faulkner, P. (1977). A plaque assay for nuclear polyhedrosis virus using a solid overlay. J. Gen. Virol. 36, 361-364.
Brown, S.M. and Harland, J. (1987). Three mutants of herpes simplex virus type 2: one lacking the genes US10, US11 and US12 and two in which R_S has been extended by 6kb to 0.91 map units until loss of U_S sequences between 0.94 and the U_S/TR_S junction. J.Gen.Virol. 68, 1-18.
Brown, S.M., Ritchie, D.A. and Subak-Sharpe, J.H. (1973). Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. J. Gen. Virol. 18, 329-346.
Bruckner, R.C., Crute, J.J., Dodson, M.S. and Lehman, I.R. (1991). The herpes simplex virus 1 origin binding protein: a DNA helicase. J. Biol. Chem. 266, 2669-2674.
Busby, D.W.G., House, W. and MacDonald, J.R. (1964). In: Virological Techniques. Churchill. London.
Bush, M., Yager, D.R., Gao, M., Weisshart, K., Marcy, A.I., Coen, D.M. and Knipe, D.M. (1991). Correct intranuclear localization of herpes simplex virus DNA polymerase requires the viral ICP8 DNA-binding protein. J. Virol. 65, 1082-1089.
Byrnes, J.J. (1985). Differential inhibitors of DNA polymerases alpha and delta. Biochem. Biophys. Res. Commun., 132, 628-634.
Byrnes, J.J., Downey, K.M., Black, V.L. and So, A.G. (1976). A new mammalian DNA polymerase with 3' to 5' exonuclease activity: DNA polymerase delta. Biochemistry. 15, 2817-2823.
Bzik, D.J. and Preston, C.M. (1986). Analysis of DNA sequences which regulate the transcription of herpes simplex virus immediate early gene 3: DNA sequences required for enhancer-like activity and response to transactivation by a virion polypeptide. Nucl. Acids Res. 14, 929-943.
Bzik, D.J., Fox, B.A., DeLuca, N.A. and Person, S. (1984). Nucleotide sequence specifying the glycoprotein gene, gB, of herpes simplex virus type 1. Virology 133, 301-314.
Cai, W., Gu, B. and Person, P. (1988). Role of glycoprotein B of herpes simplex virus type 1 in viral entry and cell fusion. J. Virol. 62, 2569-2604.
Calder, J.M. and Stow, N.D. (1990). Herpes simplex virus helicase-primase: the UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities. Nucl. Acids Res. 18, 3573-3578.
Cameron, I.R., Park, M., Dutia, B.M., Orr, A. and Macnab, J.C.M. (1985). Herpes simplex virus sequences involved in the initiation of oncogenic morphological transformation of rat cells are not required for maintenance of the transformed state. J. Gen. Virol. 66, 517-527.
Cameron, J.M., McDougall, I,. Marsden, H.S., Preston, V.G., Ryan, D.M. and Subak-Sharpe, J.H. (1988). Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target. J. Gen. Virol. 69, 2607-2612.
Campadelli-Fiume, G., Qi, S., Avitabile, E., Foa-Tomasi, L., Brandimarti, R and Roizman, B. (1990). Glycoprotein D of herpes simplex virus encodes a domain which precludes penetration of cells expressing the glycoprotein by superinfecting herpes simplex virus. J. Virol. 64, 6070-6079.

Campbell, J.L. (1986). Eukaryotic DNA replication. Annu. Rev. Biochem, 55, 733-771.

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

Capson, T.D., Benkovic, S.J. and Nossal, N.G. (1991). Protein-DNA cross-linking demonstrates stepwise ATP-dependent assembly of T4 DNA polymerase and its accessory proteins on the primer-template. Cell 65, 249-258.

Caradonna, S.J. and Cheng, Y.-C. (1981). Induction of uracil-DNA glycosylase and dUTP nucleotidohydrolase activity in herpes simplex virus-infected human cells. J. Biol. Chem. 256, 9834-9837.

Carmichael, E.P. and Weller, S.K. (1988). Herpes simplex virus type 1 DNA synthesis requires the product of the UL8 gene: isolation and characterization of an ICP6::lacZ insertion mutation. J. Virol. 63, 591-599.

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

Challberg, M.D. and Kelly, T.J. (1989). Animal virus DNA replication. Ann. Rev. Biochem. 58, 671-717.

Chartrand, P., Crumpacker, C.S., Schaffer, P.A. and Wilkie, N.M. (1980). Physical and genetic analysis of the herpes simplex virus DNA polymerase locus. Virology 103, 311-326.

Chartrand, P., Stow, N.D., Timbury, M.C. and Wilkie, N.M. (1979). Physical mapping of paa^r mutations of herpes simplex virus type 1 and type 2 by intertypic marker rescue. J. Virol. 31, 265-276.

Chee, M.S., Bankier, A.T., Beck, S., Bohni, R., Brown, C.M., Cerny, R., Horsnell, T., Hutchinson III, C.A., Kouzarides, T., Martignetti, J.A., Preddie, E., Satchwell, S.C., Tomlinson, P., Weston, K.M. and Barrell, B.G. (1990). Analysis of the protein coding content of the sequence of human cytomegalovirus strain AD169. In: Current Topics in Microbiology and Immunology. (ed, J. K. McDougall). 154, 129-173.

Chen, M.S. and Prusoff, W.H. (1978). Association of thymidylate kinase activity with pyrimidine deoxyribonucleoside kinase induced by herpes simplex virus. J. Biol. Chem. 253, 1325-1327.

Chiou, H.C., Weller, S.K. and Coen, D.H. (1985). Mutations in the herpes simplex virus major DNA-binding protein gene leading to altered sensitivity to DNA polymerase inhibitors. Virology 145, 213-226.

Chou, J. and Roizman, B. (1985). Isomerization of herpes simplex virus 1 genome: identification of the cis-acting and recombination sites within the domain of the a sequence. Cell 41, 803-811.

Chou, J. and Roizman, B. (1986). The terminal a sequence of the herpes simplex virus genome contains the promoter of a gene located in the repeat sequences of the L component. J. Virol 57, 629-637.

Chou, J. and Roizman, B. (1989). Characterization of DNA sequence-common and sequence-specific proteins binding to cis-acting sites for cleavage of the terminal a sequence of the herpes simplex virus 1 genome. J. Virol. 63, 1059-1068.

Chou, J. and Roizman, B. (1990). The herpes simplex 1 gene for ICP34.5, which maps in inverted repeats, is conserved in several limited-passage isolates but not in strain 17 syn+. J. Virol. 64, 1014-1020.

Clarke, P. (1991). Mutagenesis occurring following infection with herpes simplex virus and the contribution of virus ribonucleotide reductase. PhD Thesis, University of Glasgow.

Clark, R., Lane, D.P. and Tjian, R. (1981). Use of monoclonal antibodies as probes of simian virus 40 T antigen ATPase activity. J. Biol. Chem. 256, 11854-11858.

Clements, J.B., McLauchlan, J. and McGeoch, D.J. (1979). Orientation of

herpes simplex virus type 1 immediate-early mRNAs. Nucl. Acids Res. 7, 77-91.

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

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

Coen, D.M., Aschman, D.P., Gelep, P.T., Retondo, M.J., Weller, S.K. and Schaffer, P.A. (1984). Fine mapping and molecular cloning of mutations in the herpes simplex virus DNA polymerase locus. J. Virol 49, 236-247.

Coen, D.M., Furman, P.A., Gelep, P.T. and Schaffer, P.A. (1982). Mutations in the herpes simplex virus DNA polymerase gene can confer resistance to 9-beta-arabino-furanosyladenine. J. Virol. 41, 909-918.

Coen, D.M., Weinheimer, S.P. and McKnight, S.L. (1986). A genetic approach to promoter recognition during trans-induction of viral gene expression. Science 234, 53-59.

Cohen, G.H. (1972). Ribonucleotide reductase activity of synchronized KB cells infected with herpes simplex virus. J. Virol. 9, 408-418.

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

Cook, M.L., Bastone, V.B. and Stevens, J.G. (1974). Evidence that neurons harbour latent herpes simplex virus. Infection and Immunity 9, 946-951.

Cordingley, M.G., Campbell, M.E.M. and Preston, C.M. (1983). Functional analysis of a herpes simplex virus type 1 promoter: identification of far upstream regulatory sequences. Nucl. Acids Res. 11, 2347-2365.

Corsalo, C.M. and Pearson, M.L. (1981). Enhancing the efficiency of DNA-mediated gene transfer in mammalian cells. Somatic Cell Genetics 7, 603-616.

Costa, R.H., Draper, K.G., Kelly, T.J. and Wagner, E.K. (1985). An unusual spliced herpes simplex virus type 1 transcript with sequence homology to Epstein-Barr virus. J. Virol. 54, 317-328.

Costanzo, F., Campadelli-Fiume, G., Foa-tomasi, L. and Cassai, E. (1977). Evidence thatherpes simplex virus DNA is transcribed by cellular RNA polymerase II. J. Virol. 21, 996-1001.

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

Crumpacker, C.S., Chartrand, P., Subak-Sharpe, J.H. and Wilkie, N.M. (1980). Resistance of herpes simplex virus to acycloguanosine - genetic and physical analysis. Virology 105, 171-184.

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

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

Crute, J.J., Mocarski, E.S. and Lehman, I.R. (1988). A DNA helicase induced by herpes simplex virus type 1. Nucl. Acids Res. 16, 6585-6596.

Crute, J.J., Tsurumi, T., Zhu, L., Weller, S.K. Olivo, P.D., Challberg, M.D., Mocarski, E.S. and Lehman, I.R. (1989). Herpes simplex virus type 1 helicase-primase. A complex of three herpes-encoded gene products.

Proc. Natl. Acad. Sci. USA 86, 2186-2189.
Crute, J.J., Wahl, A.F. and Bambara, R.A. (1986). Purification and characterization of two new high molecualr weight forms of DNA polymerase. Biochemistry. 25, 26-36.
Dalrymple, M.A., McGeoch, D.J., Davison, A.J. and Preston, C.M. (1985). DNA sequence of the herpes simplex virus type 1 gene whose product is responsible for transcriptional activation of immediate-early promoters. Nucl. Acids Res. 13, 7865-7879.
Davison, A.J. (1984). Structure of the genome termini of varicella-zoster virus. J. Gen. Virol. 65, 1969-1977.
Davison, A.J. and Rixon, F.J. (1985). Cloning of the DNA of alphaherpesvirinae. In: Recombinant DNA Research and Virus. (Ed. Y. Becker). pp 103-124. Martinius Nijhoff Publishing, Boston.
Davison, A.J. and Scott, J.E. (1986). The complete DNA sequence of varicella-zoster virus. J. Gen. Virol. 67, 1759-1816.
Davison, A.J. and Taylor, P. (1987). Genetic relations between varicella-zoster virus and Epstein-Barr virus. J. Gen. Virol. 68, 1067-1079.
Davison, A.J. and Wilkie, N.M. (1981). Nucleotide sequences of the joint between the L and S segments of herpes simplex virus types 1 and 2. J. Gen. Virol. 55, 315-331.
Davison, A.J. and Wilkie, N.M. (1983). Location and orientation of homologous sequences in the genomes of five herpesviruses. J. Gen. Virol. 64, 1927-1942.
Dean, F.B., Borowiec, J.A. Ishimi, Y. Deb, S., Tegtmeyer, P. and Hurwitz, J. (1978a). Simian virus 40 large tumour antigen requires three core rplication origin domains for DNA unwinding and replication in vitro. Proc. Natl. Acad. Sci. USA 84, 8276-8281.
Dean, F.B., Dodson, M., Echols, H. and Hurwitz, J. (1987c). ATP-dependent formation of a specialized nucleoprotein structure by simian virus 40 (SV 40) large tumour antigen at the SV40 replication origin. Proc. Natl. Acad. Sci. USA 84, 8981-8985.
de Bruyn Kops, A. and Knipe, D.M. (1988). Formation of DNA replication structures in the herpes virus-induced cells requires a viral DNA binding protein. Cell 55, 857-868.
Decker, R.S., Yamaguchi, M. Possenti, R. Bradley, M.K. and DePamphilis, M.L. (1987). In vitro initiation of DNA replication in simian virus 40 chromosomes. J. Biol. Chem. 262, 10863-10872.
Deb, S. and Deb, S.P. (1989). Analysis of Ori-S sequence of HSV-1. Identification of one functional DNA binding domain. Nucl. Acids Res. 17, 2733-2754.
Deb, S., DeLucia, A., Baur, C., Koff, A. and Tegtmeyer, P. (1986a). Domain structure of the simian virus 40 core origin or replication. Mol. Cell. Biol. 6, 1663-1670.
Deb, S., DeLucia, A., Koff, A., Tsui, S. and Tegtmeyer, P. (1986b). The adenine-thymine domain of the simian virus 40 core origin directs DNA bending and coordinately regulates DNA replication. Mol. Cell. Biol. 6, 4578-4584.
Deb, S. and Doelberg, M. (1988). A 67 base pair segment from the Ori-S region of herpes simplex virus type 1 encodes origin function. J. Virol. 62, 2516-2519.
Deb, S. and Tegtmeyer, P. (1987). ATP enhances the binding of simian virus 40 large T antigen to the origin of replication. J. Virol. 61, 3649-3654.
Deiss, L.P., Chou, J. and Frenkel, N. (1986). Functional domains within the 'a' sequence involved in the cleavage-packaging of herpes simplex virus DNA. J. Virol. 59, 605-618.
Deiss, L.P. and Frenkel, N. (1986). Herpes simplex virus amplicon: cleavage of concatemeric DNA is linked to packaging and involves amplification of the terminally reiterated 'a' sequence. J. Virol. 57, 933-941.
Delius, H. and Clements, J.B. (1976). A partial denaturation map of

herpes simplex virus type 1 DNA. Evidence of inversions of the unique DNA region. J. Gen. Virol. 33, 125-133.
DeLuca, N.A., Bzik, D.J., Bond, V.C., Person, S. and Snipes, W. (1982). Nucleotide sequences of herpes simplex virus type 1 (HSV-1) affecting virus entry, cell fusion and production of glycoprotein gB (VP7). Virology 122, 411-423.
DeLuca, N.A. and Schaffer, P.A. (1985). Activation of immediate-early, early, and late promoters by temperature-sensitive and wild-type forms of herpes simplex virus type 1 protein ICP4. Mol. cell. Biol. 5, 1997-2008.
DeLuca, N.A. and Schaffer, P.A. (1988). Physical and functional domains of the herpes simplex virus transcriptional regulatory protein ICP4.J. Virol. 62, 732-743.
DeLucia, A.L., Lewton, B.A., Tjian, R. and Tegtmeyer, P. (1983). Topography of simian virus 40 A protein-DNA complexes: arrangement of pentanucleotide interaction sites at the origin of replication. J. Virol. 46, 143-150.
Denniston, K.J., Madden, M.J., Enquist, L.W. and Vande-Woude, G. (1981). Characterisation of coliphage lambda hybrids carrying DNA fragments from herpes simplex virus type 1 defective interfering particles. Gene 15, 365-378.
DePamphilis, M.L. (1988). Transcriptional elements as components of DNA replication. Cell 52,631-638.
Digard, P. and Coen, D.M. (1990). A novel functional domain of an α-like DNA polymerase. J. Biol. Chem. 265, 17393-17396.
Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983). Accurate transcription initiated by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucl. Acids Res. 5, 1475-1489.
Dixon, N. and Kornberg, A. (1983). Protein HU in the enzymatic replication of the chromosomal origin of Escherichia coli. Proc. Natl. Acad. Sci. USA 81, 424-428.
Dixon, R.A.F., Sabourin, D.J. and Schaffer, P.A. (1983). genetic analysis of temperature sensitive mutants which define the genes for the majorherpes simplex virus type 2 DNA binding protein and a new late function. J. Virol. 45, 343-353.
Dixon, R.A.F. and Schaffer, P.A. (1980). Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex type 1 immediate early protein VP175. J. Virol. 36, 189-203.
Dodson, M.S. and Lehman, I.R. (1991). Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus type 1 helicase-primase composed of the UL5 and UL52 gene products Proc. Natl. Acad. Sci. USA 88, 1105-1109.
Dodson, M.S., Crute, J.J., Bruckner, R.C. and Lehman, I.R. (1989). Overexpression and assembly of the herpes simplex virus type 1 helicase-primase in insect cells. J. Biol. Chem. 264, 20835-20838.
Dorsky, D.I. and Crumpacker, C.S. (1988). Expression of herpes simplex virus type 1 polymerase gene by in vitro translation and effects of gene deletions on activity. J. Virol. 62, 3224-3232.
Downey, K.M., Tan, T.-K., Andrews, D.M., Li, X. and So, A.G. (1988). Proposed roles for DNA polymerases α and delta at the replication fork. In: Cancer cells 6, Eukaryotic DNA replication (Eds. Kelly, T. and Stillman, B.) Cold Spring Harbor Labs, New York. pp403-410.
Draper, K.G., Devi-Rao, G., Costa, R.H., Blair, E.D., Thompson, R.L. and Wagner, E.K. (1986). Characterization of the genes encoding herpes simplex virus type 1 and type 2 alkaline exonucleases and overlapping proteins. J. Virol. 57, 1023-1036.
Dutia, B.M. (1983). Ribonucleotide reductase induced by herpes simplex virus has a virus specified constituent. J. Gen. Virol. 65, 513-521.
Ebert, S.N., Shtrom, S.S. and Muller, M.T. (1990). Topoisomerase II cleavage of herpes simplex virus type 1 DNA in vivo is replication

dependent. J. Virol. 64, 4059-4066.
Efstathiou, S., Kemp, S., Darby, G. and Minson, A.C. (1989). The role of herpes simplex virus type 1 thymidine kinase in pathogenesis. J. Gen. Virol. 70, 869-879.
Efstathiou, S., Minson, A.C., Field, H.J., Anderson, J.R. and Wildy, P. (1986). Detection of herpes simplex virus specific sequences in latently infected mice and humans. J. Virol. 57, 446-455.
Eisenberg, S.P., Coen, D.M. and McKnight, S.L. (1985). Promoter domains required for expression of plasmid-borne copies of the herpes simplex virus thymidine kinse gene in virus infected mouse fibroblasts and microinjected frog oocytes. Mol. Cell. Biol. 5, 1940-1947.
Elias, P., Gustafssom, C.M. and Hammarsten, O. (1990). The origin binding protein of herpes simplex virus 1 binds cooperatively to the viral origin of replication ori_S. J. Biol. Chem. 265, 17167-17173.
Elias, P. and Lehman, I.R. (1988). Interaction of origin binding protein with an origin of replication in herpes simplex virus 1. Proc. Natl. Acad. Sci. USA 85, 2959-63.
Elias, P., O'Donnel, M.E., Mocarski, E.S. and Lehman, I.R. (1986). A DNA binding protein specific for an origin of replication of HSV-1. Proc. Natl. Acad. Sci. USA 83, 6322-6326.
El Kareh, A., Murphy, A.J.M., Fichter, T., Efstratiadis, A. and Silverstein, S. (1985). "Transactivation" control signals in the promoter of the herpesvirus thymidine kinase gene. Proc. Natl. Acad. Sci. USA 82, 1002-1006.
Epstein, M.A. (1962). Observations on the fine structure of mature HSV and on the compostion of its nucleoid. J. Exp. Med. 115, 1-12.
Epstein, M.A. and Achong, B.G. (1986). The Epstein-Barr virus. - Recent advances. Heinemann.
Everett, R.D. (1984a). Trans-activation of transcription by herpes virus products; requirements for two HSV-1 immediate-early gene polypeptides for maximum activity. EMBO J. 3, 3135-3141.
Everett, R.D. (1984b). A detailed analysis of an HSV-1 early promoter: sequences involved in trans-activation by viral immediate-early gene products are not early-gene specific. Nucl. Acids Res. 12, 3037-3056.
Everett, R.D. (1985). Activation of cellular promoters during herpes virus infection of biochemically transformed cells. EMBO J. 4, 1973-1980.
Everett, R.D. (1986). The products of herpes simplex virus type 1 (HSV-1) immediate early genes 1, 2 and 3 can activate HSV-1 gene expression in trans. J. Gen. Virol. 67, 2507-2513.
Everett, R.D. (1989). Construction and characterisation of herpes simplex virus type 1 mutants with defined lesions in immediate-early gene 1. J. Gen. Virol. 70, 1185-1202.
Faber, S.W. and Wilcox, K.W. (1986). Association of herpes simplex virus regulatory protein ICP4 with specific nucleotide sequences in DNA. Nucl. Acids Res. 14, 6067-6083.
Faber, S.W. and Wilcox, K.W. (1988). Association of herpes simplex virus regulatory protein ICP4 with sequences spanning the ICP4 gene transcription initiation site. Nucl. Acids Res. 16, 555-570.
Fairman, M.P. and Stillman, B. (1988). Cellular factors required for multiple stages of SV40 DNA replication in vitro. EMBO J. 7, 1211-1218.
Fanning, E., Traut, W., Dornreiter, I., Dende, S., Alliger, P. and Posch, B. (1988). Sequence-specific binding of a cellular protein associated with DNA polymerase α to the SV40 core origin of DNA replication. In: Cancer cells 6, Eukaryotic DNA replication (Eds. Kelly, T. and Stillman, B.) Cold Spring Harbor Labs, New York. pp177-181.
Feinberg, A.P. and Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity.

Analytical Biochem. 132, 6-13.
Fenwick, M.L. (1984). The effects of herpes virus on cellular macromolecular synthesis. In: Comprehensive Virology 19, 359-390. Fraenkel-Conrat, H. and Wagner, R.R. (eds.) Plenum Press, N.Y.
Fenwick, M.L. and Everett, R.D. (1990). Transfer of UL41, the gene controlling virion-associated host cell shut off, between different strains of herpes simplex virus. J. Gen. Virol. 71, 411-418.
Fenwick, M.L. and Owen, S.A. (1988). On the control of immediate early (alpha) mRNA survival in cells infected with herpes simplex virus. J. Gen. Virol. 69, 2869-2877.
Fenwick, M.L. and Walker, M.J. (1979). Phosphorylation of ribosomal protein and of virus specific proteins in cells infected with herpes simplex virus. J. Gen. Virol. 45, 397-405.
Fenwick, M.L., Walker, M.J. and Petkevich, J.M. (1978). On the association of virus proteins with the nuclei of cell infected with herpes simplex virus. J. Gen. Virol. 39, 519-529.
Field, H.J. and Wildy, P. (1978). The pathogenicity of thymidine kinase deficient mutants of herpes simplex virus in mice. J Hyg. 81, 267-277.
Fisher, F.B. and Preston, V.G. (1986). Isolation and characterisation of herpes simplex type 1 mutants which fail to induce dUTPase activity. Virology 148, 190-197.
Fletcher, C., Heintz, N. and Roeder, R.G. (1987). Purification and characterization of OTF-1, a transcription factor regulating cell cycle expression of a human histone H2b gene. Cell 57,773-781.
Frame, M.C., Marsden, H.S. and Dutia, B.M. (1985). The ribonucleotide reductase induced by herpes simplex virus type 1 involves minimally a complex of two polypeptides (136K and 38K). J. Gen. Virol. 66, 1581-1587.
Frame, M.C., Purves, F.C., McGeoch, D.J., Marsden, H.S. and Leader, D.P. (1987). Identification of the herpes simplex virus protein kinase as a product of the viral gene US3. J. Gen. Virol. 68, 2699-2704.
Francke, B. and Garrett, B. (1982). The effect of a temperature-sensitive lesion in the alkaline DNase of herpes simplex virus type 2 on the synthesis of viral DNA. Virology 116, 116-127.
Fraser, N.W., Lawrence, W.C., Wroblewska, Z., Gilden, D.H. and Koprowski, H. (1981). Herpes simplex type 1 DNA in human brain tissue. Proc. Natl. Acad. Sci. 78, 6461-6465.
Freidmann, A., Shlomai, J. and Becker, Y. (1977). Electron microscopy of herpes simplex virus DNA molecules isolated from infected cells by centrifugation in CsCl density gradients. J. Gen. Virol. 34, 507-522.
Frenkel, N., Jacob, R.J., Honess, R.W., Hayward, G.S., Locker, H. and Roizman, B. (1975). Anatomy of herpes simplex virus DNA III. Characterization of defective DNA molecules and biological properties of virus populations containing them. J. Virol. 16, 153-167.
Frenkel, N., Locker, H., Batterson, W., Hayward, G.S. and Roizman, B. (1976). Anatomy of herpes simplex virus DNA VI. Defective DNA originates from the S component. J. Virol. 20, 527-531.
Frenkel, N., Locker, H. and Vlazny, D.A. (1980). Studies of defective herpes simplex viruses. Ann. New York Acad. Sci. 354, 347-370.
Frenkel, N., Locker, H. and Vlazny, D. (1981). Herpesvirus DNA: recent studies on the internal organization and replication of the viral genomes. (Ed, Y. Becker) Nijhoff, The Hague, The Netherlands.
Frenkel, N., Schirmer, E.C., Wyatt, L.S., Katsafanos, G., Roffman, E., Danovian, R.M. and June, C.H. (1990a). Isolation of a new herpes virus from human CD4+ T cells. Proc. Natl. Acad. Sci. USA 87, 748-752.
Frenkel, N., Schirmer, E.C., Katsafanos, G. and June, C.H. (1990b). T-cell activation is required for efficient replication of human herpesvirus 6. J. Virol. 64, 4598-4602.
Fuller, A.O. and Spear, P.G. (1985). Specificities of monoclonal and

polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by neutralizing antibodies. J. Virol. 55, 475-482.

Fuller, R.S., Funnell, B. E. and Kornberg, A. (1984). The dnaA protein complex with the *E. coli* chromosomal replication origin (oriC) and other DNA sites. Cell 38, 889-900.

Fuller, R.S. and Kornberg, A. (1983). Purified dnaA protein in initiation of replication at the *Eschericichia coli* chromosomal origin of replication. Proc. Natl. Acad. Sci. USA 80, 5817-5821.

Funnell, B.E., Baker, T.A. and Kornberg, A. (1986). Complete enzymatic replication of plasmids containing the origin of the *Escherichia coli* chromosome. J. Biol. Chem. 261, 5616-5624.

Furman, P.A., Coen, D.M., St.Clair, M.H. and Schaffer, P.A. (1981). Acyclovir-resistant mutants of herpes simplex virus type 1 express altered DNA polymerase or reduced acyclovir phosphorylating activities. J. Virol. 40, 936-941.

Gaffney, D.F., McLauchlan, J., Whitton, J.L. and Clements, J.B. (1985). A modular system for the assay of transcription regulatory signals the sequence TAATGARAT is required for herpes simplex virus immediate-early gene activation. Nucl. Acids Res. 13, 7847-7863.

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

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

Galloway, D.A., and McDougall, J.K. (1983). The oncogenic potential of herpes simplex viruses: evidence for a 'hit and run' mechanism. Nature 302, 21-24.

Gannon, J.V. and Lane, D.P. (1987). p53 And DNA polymerase α compete for binding to SV40 T antigen. Nature 329, 456-458.

Gao, M., Bouchey, J., Curtin, K. and Knipe, D.M. (1988). Genetic identification of a portion of the herpes simplex virus ICP8 protein required for DNA binding. Virology 163, 319-329.

Gao, M. and Knipe, D.M. (1989). Genetic evidence for multiple nuclear functions of the herpes simplex virus ICP8 DNA-binding protein. J. Virol. 63, 5258-5267.

Geider, K. and Hoffman-Berling, H. (1981). Proteins controlling the helical structural of DNA. Annu. Rev. Biochem. 50, 233-260.

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

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

Gelman, I. and Silverstein, S. (1987b). Dissection of immediate-early gene promoters from herpes simplex virus: sequences that respond to the viral transcriptional activators. J. Virol. 61, 3167-3172.

Gerster, T. and Roeder, R.G. (1988). A herpesvirus trans-activating protein interacts with transcription factor OTF1 and with other cellular proteins. Proc. Natl. Acad. Sci. USA 85, 6347-6351.

Giacherio, D. Haggar, L.P. (1979). A poly(dT) stimulated ATPase activity associated with simian virus 40 large T antigen. J. Biol. Chem. 254, 8113-8116.

Gibbs, J.S., Chiou, H.C., Bastow, K.F., Cheng, Y.-C. and Coen, D.M. (1988). Identification of amino acids in herpes simplex virus DNA polymerase involved in substrate and drug regulation. Proc. Natl.

Acad. Sci. USA 85, 6672-6676.
Gibbs, J.S., Chiou, H.C., Hall, J.D., Mount, D.W., Retondo, M.J., Weller, S.K. and Coen, D.M. (1985). Sequence and mapping analysis of the herpes simplex virus DNA polymerase gene predict a C-terminal substrate binding domain. Proc. Natl. Acad. Sci. USA 82, 7969-7963.
Gibson, M.G. and Spear, P.G. (1983). Insertion mutants of herpes simplex virus have a duplication of the glycoprotein D gene and express two different forms of glycoprotein D. J. Virol. 48, 396-404.
Godowski, P.J. and Knipe, D.M. (1983). Mutations in the major DNA-binding protein gene of herpes simplex virus type 1 result in increased levels of viral gene expression. J. Virol. 47, 478-486.
Godowski, P.J. and Knipe, D.M. (1985). Identification of a herpes simplex virus function that represses late gene expression from parental viral genomes. J. Virol. 55, 357-365.
Godowski, P.J. and Knipe, D.M. (1986). Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation. Proc. Natl. Acad. Sci. USA 83, 256-260.
Goldstein, D.J. and Weller, S.K. (1988a). Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. J. Virol. 62, 196-205.
Goldstein, D.J. and Weller, S.K. (1988b). Factor(s) present in herpes simplex virus type 1-infected cells can compensate for the loss of the large subunit of the viral ribonucleotide reductase: characterization of an ICP6 deletion mutant. Virology 166, 41-51.
Goldstein, D.J. and Weller, S.K. (1988c). An ICP6::lacZ insertional mutagen is used to demonstrate that the UL52 gene of herpes simplex virus type 1 is required for virus growth and DNA synthesis. J. Virol. 62, 2970-2977.
Gompels, U. and Minson, A.C. (1986). The properties and sequence of glycoprotein H of herpes simplex virus type 1. Virology 153, 230-247.
Goodrich, L.D., Schaffer, P.A., Dorsky, D.I., Crumpacker, C.S. and Parris, D.S. (1990). Localization of the herpes simplex virus type 1 65-kilodalton DNA-binding protein and DNA polymerase in the presence and absence of viral DNA synthesis. J. Virol. 64, 5738-5749.
Gorbalenya, A.E. and Koonin, E.V. (1989). Viral proteins containing the purine NTP-binding sequence pattern. Nucl. Acids Res. 17, 8413-8435.
Gorbalenya, A.E., Koonin, E.V., Donchenko, A.P. and Blinov, V.M. (1989). Two related superfamilies of putative helicases involved in replication, recombination, repair and expression of DNA and RNA genomes. Nucl. Acids Res. 17, 4713-4730.
Gottleib, J., Marcy, A.I., Coen, D.M. and Challberg, M.D. (1990). The herpes simplex virus type 1 UL42 gene product: a subunit of DNA polymerase that functions to increase processivity. J. Virol. 64, 5976-5987.
Graves, B.J., Johnson, P.F. and McKnight, S.L. (1986). Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. Cell 44, 565-576.
Gray, C.P. and Kaerner, H.C. (1984). Sequence of the putative origin of replication in the U_L region of herpes simplex virus type 1 ANG DNA. J. Gen. Virol. 65, 2109-2119.
Greaves, R. and O'Hare, P. (1989). Separation of the requirements for protein-DNA complex assembly from those for functional activity in the herpes simplex virusregulatory protein Vmw65. J. Virol. 63, 1641-1650.
Haffey, M.L., Stevens, J.T., Terry, B.J., Dorsky, D.I., Crumpacker, C.S. Wietstock, S.M., Ruyechan, W.T. and Field, A.K. (1988). Expression of herpes simplex virus type 1 DNA polymerase in *Saccharomyces cerevisiae* and detection of virus-specific enzyme activity in cell-free lysates. J. Virol. 62, 4493-4498.
Hall, J.D., Wang, Y., Pierpoint, J., Berlin, M.S., Rundlett, S.E. and

* Harrison P T, Thompson R and Davison A J, 1991. Evolution of herpesvirus thymidine kinase from cellular deoxycytidine kinase. J. Gen. Virol. 72, 2583-2586

Woodward, S. (1989). Aphidicolin resistance in herpes simplex virus type 1 reveals features of the DNA polymerase dNTP binding site. Nucl. Acids Res. 17, 9231-9244.

Hall, J.D. and Woodward, S. (1989). Aphidicolin resistance in herpes simplex virus type 1 appears to alter substrate specificity in the DNA polymerase. J. Virol. 63, 2874-2876.

Hamilton, J.D. (1982). Cytomegalovirus and Immunity. In: Monographs in Virology. (ed. Melnick). Vol 12.

Harris, R.A., Everett, R.D., Zhu, X., Silverstein, S. and Preston, C.M. (1989). Herpes simplex virus type 1 immediate-early protein Vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. J. Virol. 63, 3513-3515.

Hay, R.T. and DePamphilis, M.L. (1982). Initiation of SV40 DNA replication *in vivo*: location and structure of 5' ends of DNA synthesized in the ori region. Cell 28, 767-779.

Hay, R.T. and Hay, J. (1980). Properties of herpesvirus-induced "immediate-early" polypeptides. Virology 104, 230-234.

Hay, J., Moss, H. and Halliburton, I. W. (1971). Induction of deoxyribonucleic acid polymerase and deoxyribonuclease activities in cells infected with herpes simplex virus type II. J. Biochem. 124, 64-76.

Hay, J. and Subak-Sharpe, J.H. (1976). Mutants of herpes simplex virus types 1 and 2 that are resistant to phosphonoacetic acid induce altered DNA polymerase activities in infected cells. J. Gen. Virol. 31, 145-148.

Hayward, G.S., Jacob, R.J., Wadsworth, S.C. and Roizman, B. (1975). Anatomy of herpes simplex virus DNA. Evidence for four populations of molecules that differ in the relative orientation of their long and short components. Proc. Natl. Acad. Sci. USA 72, 4243-4247.

Heilbronn, R. and Zur Hausen, H. (1989). A subset of herpes simplex virus replication genes induces DNA amplification within the host cell genome. Virology 63, 3683-3692.

Heilbronn, R., Weller, S.K. and Zur Hausen, H. (1990). Herpes simplex virus type 1 mutants for the origin-binding protein induce DNA amplification in the absence of viral replication. Virology 179,478-481.

Herold, B.C., WuDunn, D., Soltys, N. and Spear, P.G. (1991). Glycoproteins C of herpes simplex virus type 1 plays a principal role in the adsorbtion of virus to cells and in infectivity. J. Virol. 65, 1090-1098.

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

Hill, J.M., Sedarati, F., Javier, R.T., Wagner, E.K. and Stevens J.G. (1990). Herpes simplex virus latent phase transcription facilitates in vivo reactvation. Virology 174, 117-125.

Hill, T.M., Sadler, J.R. and Betz, J.L. (1985). Virion component of herpes simplex virus type 1 KOS interferes with early shut off of host protein synthesis induced by herpes simplex virus type 2. J. Virol. 56, 312-316.

Hill, T.M., Sinden, R.R. and Sadler, J.R. (1983). Herpes simplex virus types 1 and 2 induce shut-of of host protein synthesis by different mechanisms in Friend leukaemia cells. J. Virol. 45, 241-250.

Hodgeman, T.C. (1988). A new superfamily of replicative proteins. Nature 333, 22-23.

Hoffman, P.J. (1981). Mechanism of degradation of duplex DNA by the DNase induced by herpes simplex virus. J. Virol. 38, 1005-1014.

Hoffman, P.J. and Cheng, Y.-C. (1979). DNase induced after infection of KB cells by herpes simplex virus type 1 or type 2. II. Characterization of an associated endonuclease activity. J. Virol. 32, 449-457.

Hohn, K.T. and Grosse, F. (1987). Processivity of the DNA polymerase α-

primase complex from calf thymus. Biochemistry 26, 2870-2880.
Holland, L.E., Anderson, K.P., Shipman,C. and Wagner, E.K. (1980). Viral DNA synthesis is required for the efficient expression of specific herpes simplex virus type 1 mRNA species. Virology 101, 10-24.
Holland, L.E., Sandri-Goldin, R.M., Goldin, A.L., Glorioso, J.C. and Levine, M. (1984). Transcriptional and genetic analyses of the herpes simplex virus type 1 genome: coordinates 0,29 to 0.45. J. Virol. 49, 947-959.
Holmes, A.M., Wietstock, S.M. and Ruyechan, W.T. (1988). Identification and characterization of a DNA primase activity present in herpes simplex virus type-1 infected HeLa cells. J. Virol. 62, 1038-1045.
Homa, F.L., Otal, T.M., Glorioso, J.C. and Levine, M. (1986). Transcriptional control signals of a herpes simplex virus type 1 late ($gamma_2$) gene lie within bases -34 to +124 relative to the 5' terminus of the RNA. Mol. Cell. Biol. 6, 3652-3666.
Honess, R.W., Buchan, A., Halliburton, I.W. and Watson, D.H. (1980). Recombination and linkage between structural and regulating genes of herpes simplex virus type 1: study of the functional organization of the genome. J. Virol. 34, 716-742.
Honess, R.W. and Roizman, B. (1973). Proteins specified by herpes simplex virus. XI. Identification and relative molar rates of synthesis of structural and non-structural herpes virus polypeptide in the infected cell. J. Virol. 12, 1347-1365.
Honess, R.W. and Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. J. Virol. 14, 8-19.
Honess, R.W. and Roizman, B. (1975). Regulation of herpesvirus macromolecular synthesis. Sequential transition of polypeptide synthesis requires functional viral polypeptides. Proc. Natl. Acad. Sci. USA 72, 1276-1280.
Honess, R.W. and Watson, D.H. (1977). Herpes simplex virus resistance and sensitivity to phosphonoacetic acid. J. Virol. 21, 584-600.
Huang, C.-C., Hearst, J.E. and Alberts, B.M. (1981). Two types of replication protein increase the rate at which T4 DNA polymerase traverses the helical regions in a single stranded DNA template. J. Biol. Chem. 256, 4087-4094.
Hubenthal-Voss, J. and Roizman, B. (1988). Properties of two 5'-coterminal RNAs transcribed part way and across the S component origin of DNA synthesis of the herpes simplex virus 1 genome. Proc. Natl. Acad. Sci. USA 85, 8454-8458.
Hubenthal-Voss, J., Starr, L. and Roizman, B. (1987). The herpes simplex virus origins of DNA synthesis in the S component are each contained in a transcribed open reading frame. J. Virol. 61, 3349-3358.
Huber, H.E., Tabor, S. and Richardson, C.C. (1987a). *Eschericia coli* thioredoxin confers processivity on the DNA polymerase activity of the gene 5 protein of bacteriophage T7. J. Biol. Chem. 262, 16212-16223.
Huber, H.E., Tabor, S. and Richardson, C.C. (1987b). *Eschericia coli* thioredoxin stabilizes complexes of bacteriophage T7 DNA polymerase and primed templates. J. Biol. Chem. 262, 16224-16232.
Huberman, J.A. (1987). Eukaryotic DNA replication: a complex picture partially clarified. Cell 48, 7-8.
Hurwitz, J. Dean, F.B., Kwong, A.D. and Lee, S.H. (1990). The *in vitro* replication of DNA containing the SV40 origin. J. Biol. Chem. 265, 18043-18046.
Jacob, R.J., Morse, L.S. and Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head to tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. J. Virol. 29, 448-457.
Jacob, R.J. and Roizman, B. (1977). Anatomy of herpes simplex virus

DNA. VIII. Properties of the replicating DNA. J. Virol. 23, 394-411.
Jamieson, A.T. and Subak-Sharpe, J.H. (1974). Bichemical sudies on the herpes simplex virus-specified deoxypyrimidine kinase activity. J. Gen. Virol. 24, 481-492.
Jamieson, A.T., Gentry, G.A. and Subak-Sharpe, J.H. (1974). Induction of both thymidine and deoxycytidine kinase activity by herpes viruses. J. Gen. Virol. 24, 465-480.
Jarvis, T.C., Paul, L.S. and von Hippel, P.H. (1989a). Structural and enzymatic studies of the T4 DNA replication system. I. Physical characterization of the polymerase accessory protein complex. J. Biol. Chem. 264, 12709-12716.
Jarvis, T.C., Paul, L.S., Hockensmith, J.W. and von Hippel, P.H. (1989b). Structural and enzymatic studies of the T4 DNA replication system. II. ATPase properties of the polymerase accessory protein complex. J. Biol. Chem. 264, 12717-12729.
Javier, R.T., Stevens, J.G., Disette, V.B. and Wagner, E.K. (1988). A herpes simplex virus transcript abundant in latently infected neurins is dispensable for establishment of the latent state. Virology 166, 254-257.
Jenkins, F.J., Casadaban, M.J. and Roizman, B. (1985). Application of the mini-Mu-phage for target-sequence-specific insertional mutagenesis of the herpes simplex virus genome. Proc. Natl. Acad. Sci. USA 82, 4773-4777.
Jenkins, F.J. and Roizman, B. (1986). Herpes simplex virus 1 recombinants with non-inverting genomes frozen in different isomeric arrangements are capable of independent replication. J. Virol. 59, 494-499.
Jofre, J.T., Schaffer, P.A. and Parris, D.S. (1977). Genetics of resistance to phosphonoacetic acid in strain KOS of herpes simplex virus type 1 J. Virol. 23, 833-836.
Johnson, D.C. and Ligas, M.W. (1988). Herpes simplex viruses lacking glycoprotein D are unable to inhibit virus penetration: quantitative evidence for virus-specific cell surface receptors. J. Virol. 62, 4605-4612.
Johnson, D.C., Wittels, M. and Spear, P. G. (1984). Binding to cells of virosomes containing herpes simplex virus type 1 glycoproteins and evidence for fusion. J. Virol. 52, 235-247.
Johnson, P.A. (1987). The control of herpes simplex virus late gene transcription. PhD Thesis, University of Glasgow.
Johnson, P.A., Best, M.G., Friedman, T. and Parris, D. (1991), Isolation of a herpes simplex virus type 1 mutant deleted for the essential UL42 gene and characterization of its null phenotype. J. Virol. 65, 700-710.
Johnson, P.A. and Everett, R.D. (1986a). DNA replication is required for abundant expression of a plasmid-borne late US11 gene of herpes simplex virus type 1. Nucl. Acids Res. 14, 3609-3625.
Johnson, P.A. and Everett, R.D. (1986b). The control of herpes simplex virus type-1 late gene transcription: a 'TATA-box'/cap site region is sufficient for fully efficient regulated activity. Nucl. Acids Res. 14, 8247-8264.
Johnson, P.A., MacLean, C.A., Marsden, H.S., Dalziel, R.G. and Everett, R.D. (1986). The product of the gene US11 of herpes simplex virus type 1 is expressed as a true late gene. J. Gen. Virol. 67, 871-883.
Jones, K.A. and Tjian, R. (1985). Sp1 binds to promoter sequences and activates herpes simplex virus immediate-early gene transcription in vitro. Nature 317, 179-182.
Jones, K.A., Yamamoto, K.R. and Tjian, R. (1985). Two distinct transcription factors bind to the HSV thymidine kinase promoter in vitro. Cell 42, 559-572.
Jones, P.C. and Roizman, B. (1979). Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of

three phases during which both the extent of transcription and accumulation of RNA in the cytoplasm are regulated. J. Virol. 31, 299-314.
Jongeneel, C.V. and Bachenheimer, S.L. (1981). Structure of replicating herpes virus DNA. J. Virol. 39, 656-660.
Kaerner, H.C., Maichle, I.B., Ott, A. and Schroder, C.H. (1979). Origin of two different classes of defective HSV-1 Angelotti DNA. Nucl. Acids Res. 6, 1467-1478.
Kaerner, H.C., Ott-Hartman, A., Schatten, R., Schroder, C.H. and Gray, C.P. (1981). Amplification of a short nucleotide sequence in the repeat unit of defective herpes simplex virus type 1 Angelotti DNA. J. Virol. 39, 75-81.
Kaguni, J.M. and Kornberg, A. (1984). Replication initiated at the origin (oriC) of the *E. coli* chromosome reconstituted with purified enzymes. Cell 38, 183-190.
Kaner, R.J. Baird, A., Marisukhani, A, Basilico, C., Summers, B.D., Florkiewicz, R.Z. and Haggar, D.P. (1990). Fibroblast growth factor receptor is a portal of cellular entry for herpes simplex virus type 1. Science 248, 1410-1413.
Kaplan, A.S. and Ben-Porat, T. (1963). The pattern of viral and cellular DNA synthesis in pseudorabies virus-infected cells in the logarithmic phase of growth. Virology 19, 205-214.
Kattar-Cooley, P. and Wilcox, K.W. (1989). Characterization of the DNA-binding properties of herpes simplex virus regulatory protein ICP4. J. Virol. 63, 696-704.
Keir, H.M. and Gold, E. (1963). Deoxyribonucleic acid nucleotidyl transferase and deoxyribonuclease from cultured cells infected with herpes simplex virus. Biochem. Biophys. Acta 72, 263-276.
Keir, H.M., Subak-Sharpe, J.H., Shedden, N.I.H., Watson, D.H. and Wildy, P. (1966). Immunological evidence for a specific DNA polymerase produced after infection by herpes simplex virus. Virology 30, 154-157.
Keller, S.W. (1975). Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. J. Immunol. 115, 1617-1624.
Kemble, G.W. and Mocarski, E.S. (1989). A host cell protein binds to a highly conserved sequence element (pac-2) within the cytomegalovirus 'a' sequence. J. Virol. 63, 4715-4728.
Kemp, L.M., Preston, C.M., Preston, V.G. and Latchman, D.S. (1986). Cellular gene induction during herpes simplex virus infection can occur without viral protein synthesis. Nucl. Acids Res. 14, 9261-9270.
Kennedy, P.G.E. (1987). Neurological complications of varicella-zoster virus. In: Infections of the nervous system. (Eds. P.G.E. Kennedy and R.T. Johnson). pp 177-208. Butterworth and Co.
Kieff, E.D., Bachenheimer, S.L. and Roizman, B. (1971). Size, composition and structure of the deoxyribonucleic acid of HSV subtypes 1 and 2. J. Virol. 8, 125-132.
Kit, S. and Dubbs, D. (1963). Acquisition of thymidine kinase activity by herpes simplex virus-infected mouse fibroblast cells. Biochem. Biophys. Res. Commun. 11, 55-66.
Knipe, D.M. (1989) The role of viral and cellular nuclear proteins in herpes simplex virus replication. Advances in Virus Research 37, 85-123.
Knipe, D.M., Batterson, W., Nosal, C., Roizman, B. and Buchan, A. (1981). Molecular genetics of herpes simplex virus. VI. Characterization of a temperature sensitive mutant defective in the expression of all early viral gene products. J. Virol. 38, 539-547.
Knipe, D.M., Ruyechan, W.T. and Roizman, B. (1979). Molecular genetics of herpes simplex virus. III. Fine mapping of a genetic locus determining resistance to phosphonoacetate by two methods of

marker transfer. J. Virol. 29, 698-704.
Knopf. C.W. (1986) Nucleotide sequence of the DNA polymerase gene of herpes simplex virus type 1 strain Angelotti. Nucl. Acids Res. 14, 8225-8226.
Knopf, K.-W. (1979). Properties of herpes simplex virus DNA polymerase and characterization of its associated exonuclease activity. Eur. J. Biochem. 98, 231-244.
Koff, A. and Tegtmeyer, P. (1988). Characterization of major recognition sequences for a herpes simplex virus type 1 origin-binding protein. J. Virol. 62, 4096-4103.
Kornberg, A. (1980, 1982). DNA replication. (With Supplement 1982). W.H.Freeman & Co., San Francisco.
Kornberg, A. (1988) DNA replication. J. Biol. Chem. 263, 1-4.
Kozak, M. and Roizman, B. (1974). Regulation of herpes virus macromolecular synthesis: nuclear retention of non-translated viral RNA sequences. Proc. Natl. Acad. Sci. USA 71, 4322-4326.
Kristie, T.M., LeBowitz, J.H. and Sharp, P.A. (1989). The octamer-binding proteins form multi-protein-DNA complexes with the HSV αTIF regulatory protein. EMBO J. 8, 4229-4238.
Kristie, T.M. and Roizman, B. (1984). Separation of sequences defining basal expression from those conferring alpha gene recognition within the regulatory domains of herpes simplex virus 1 alpha genes. Proc. Natl. Acad. Sci. USA 81, 4065-4069.
Kristie, T.M. and Roizman, B. (1986). DNA-binding site of major regulatory protein alpha 4 specifically associated with promoter-regulatory domains of alpha genes of herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA 83, 4700-4704.
Kwong, A.D. and Frenkel, N. (1987). Herpes simplex virus-infected cells contain a function(s) that destabilises both host and viral mRNA. Proc. Natl. Acad. Sci. USA 84, 1926-1930.
Kwong, A.D. and Frenkel, N. (1989). The herpes simplex virus virion host shutoff function. J. Virol. 63, 4834-4839.
Kwong, A.D., Kruper, J.A. and Frenkel, N. (1988). Herpes simplex virus host shutoff function. J. Virol. 62, 912-921.
Ladin, B.F., Blankenship, M.L. and Ben-Porat, T. (1980). Replication of herpesvirus DNA. V. Maturation of concatameric DNA of pseudorabies virus to genome length is related to capsid formation. J. Virol. 33, 1151-1164.
Langeland, N., Oyan, A.M., Marsden, H.S., Cross, A., Glorioso, J.C., Moore, L. and Haarr, L. (1990). Localization on the herpes simplex virus type 1 genome of a region encoding proteins involved adsorbtion to the cellular receptor. J. Virol. 64, 1271-1277.
Larder, B.A., Lisle, J.J. and Darby, G. (1986). Restoration of wild-type pathogenicity to an attenuated DNA polymerase mutant of herpes simplex virus type 1. J. Gen. Virol. 67, 2501-2506.
Larder, B.A., Kemp, S.D. and Darby, G. (1987). Related functional domains in virus DNA polymerases. EMBO J. 6, 169-175.
Lathangue, N.B., Shriver, K., Dawson, C. and Chan, W.L. (1984). Herpes simplex virus infection causes the accumulation of a heat shock protein. EMBO J. 3, 267-272.
Laux, G., Freese, U.K. and Bornkamm, G.W. (1985). Structure and evolution of two related transcription units of Epstein-Barr virus carrying small tandem repeats. J. Virol. 56, 987-995.
Lawrence, G.L., Chee, M., Craxton, M.A., Gompels, U.A., Honess, R.W. and Barrell, B.G. (1990). Human herpesvirus 6 is closely related to human cytomegalovirus. J. Virol. 64, 287-299.
Leary, K. and Francke, B. (1984). The interaction of a topoisomerase-like enzyme from herpes simplex virus type 1 infected cells with non-viral circular DNA. J. Gen. Virol. 65, 1341-1350.
Lebowitz, J.H. and McMacken, R. (1986). The *Escherichia coli* dnaB replication protein is a DNA helicase. J. Biol. Chem. 261, 4738-4748.

Lee, C.K. and Knipe, D.M. (1983). Thermolabile in vivo DNA-binding activity associated with a protein encoded by mutants of herpes simplex virus type 1. J. Virol 46, 909-919.

Lee, M.S. and Marians, K.J. (1989). The *Escherichia coli* primosomes can translocate actively in either direction along a DNA strand. J. Biol. Chem. 264, 14531-14542.

Lee, M.Y.W.T., Tan, C.-K., Downey, K.M. and So, A.G. (1984). Further studies on calf thymus DNA polymerase delta purified to homogeneity by a new procedure. Biochemistry 23, 1906-1913.

Lee, M.Y.W.T., Tan, C.-K., So, A.G. and Downey, K.M. (1980). Purification of deoxyribonucleic acid polymerase *δ* from calf thymus: partial characterization of phsical properties. Biochemistry 19, 2096-2101.

Lee, S.-H., Ishimi, Y., Kenny, M.K., Bullock, P., Dean, F.B. and Hurwitz, J. (1988). An inhibitor of the in vitro elongation reaction of simian virus 40 DNA replication is overcome by proliferating cell nuclear antigen. Proc. Natl. Acad. Sci. USA 85,9469-9473.

Leib, D.A., Coen, D.M., Bogard, C.L., Hicks, K.A., Yager, D.R., Knipe, D.M., Tyler, K.C. and Schaffer, P.A. (1989a). Immediate early regulatory gene mutants define different stages in the establishment and reactivation of herpes simplex virus latency. J. Virol. 63, 759-768.

Leib, D.A., Bogard, C.L., Kosz-Vnenchak, M., Hicks, K.A., Coen, D.M., Knipe, D.M. and Schaffer, P.A. (1989b). A deletion mutant of the latency associated transcript of herpes simplex virus type 1 reactivates from the latent state with reduced frequency. J. Virol. 63, 2893-2900.

Leinbach, S.S., Casto, J.F. and Pickett, T.K. (1984). Deoxyribonucleoprotein complexes and DNA synthesis of herpes simplex virus type 1. Virology 137, 287-296.

Leinbach, S.S. and Heath, L.S. (1988). A carboxy-terimnal peptide of the DNA binding protein ICP8 of herpes simplex virus contains a single-stranded DNA-binding site. Virology 166, 10-16.

Lemaster, S. and Roizman, B. (1980). Herpes simplex virus phosphoproteins. II. Characterisation of the virion protein kinase and of the polypeptides phosphorylated in the virion. J. Virol. 35, 798-811.

Li, J.J. and Kelly, T.J. (1984). Simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA 81, 6973-6977.

Li, J.J. and Kelly, T.J. (1985). Simian virus 40 DNA replication in vitro: Specificity of initiation and evidence for bidirectional replication. Mol. Cell. Biol. 5, 1238-1246.

Li, J.J., Peden, K.W.C., Dixon, R.A.F. and Kelly, T. (1986). Functional organization of the simian virus 40 origin of DNA replication. Mol. Cell. Biol. 6, 1117-1128.

Ligas, M.W. and Johnson, D.C. (1988). A herpes simplex virus mutant in which glycoprotein D sequences are replaced by β-galactosidase sequences binds to but is unable to penetrate cells. J. Virol. 62, 1486-1494.

Littler, E., Purifoy, D., Minson, A. and Powell, K.L. (1983). Herpes simplex virus non-structural proteins. III. Function of the major DNA-binding protein. J. Gen. Virol. 64, 983-995.

Liu, C.C. and Albert, B.M. (1981). Characterization of the DNA dependent GTPase activity of T4 gene 41 protein an essential component of the T4 bacteriophage DNA replication apparatus. J. Biol. Chem. 256, 2813-2820.

Locker, H., Frenkel, N. and Halliburton, I.W. (1982). Structure and expression of class II defective herpes simplex virus genomes encoding infected cell polypeptide number 8. J. Virol. 43, 574-593.

Lockshon, D. and Galloway, D. (1986). Cloning and characterization of ori_{L2}, a large palindromic DNA replication origin of HSV2. J. Virol. 58, 513-521.

Lockshon, D. and Galloway, D. (1988). Sequence and structural

requirements of a herpes simplex viral DNA replication origin. Mol. Cell. Biol. 8, 4018-4027.
Longnecker, R. and Roizman, B. (1986). Generation of an inverted HSV-1 mutant lacking L-S junction a sequences, an ori of DNA synthesis and several genes including those specifying glycoprotein E and the alpha 47 gene. J. Virol. 58, 583-591.
Longnecker, R. and Roizman, B. (1987). Clustering of genes dispensable for growth in culture in the S component of the HSV-1 genome. Science 236, 573-576.
Lopez, C., Pellet, P., Stewart, J., Goldsmith, C., Sanderlin, K., Black, J., Warfield, D. and Feorino, P. (1988). Characteristics of human herpesvirus 6. J. Infect. Dis. 157, 1271-1273.
MacPherson, I. and Stoker, M. (1962). Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence. Virology 16, 147-151.
McCarthy, A.M., McMahan, L. and Schaffer, P.A. (1989). Herpes simplex virus type 1 ICP27 deletion mutants exhibit altered patterns of transcription and are DNA deficient. J. Virol. 63, 18-27.
McGeoch, D.J. (1987). The genome of herpes simplex virus: structure, replication and evolution. J. Cell. Sci. Suppl. 7, 67-94.
McGeoch, D.J. (1989). The genome of the human herpesvirus: contents, relationships and evolution. Ann. Rev. Microbiol. 43, 235-265.
McGeoch, D.J., Dalrymple, M.A., Dolan, A., McNab, D., Perry, L.J., Taylor, P. and Challberg, M.D. (1988a). Structures of herpes simplex type 1 genes required for replication of virus DNA. J. Virol. 69, 444-453.
McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. and Taylor, P. (1988b). The complete DNA sequence of the long unique sequence of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69, 1531-1574.
McGeoch, D.J. and Davison, A.J. (1986). Alphaherpesviruses possess a gene homologous to the protein kinase gene family of eukaryotes and retroviruses. Nucl. Acids Res. 14, 1765-1777.
McGeoch, D.J., Dolan, A., Donald, S. and Rixon, F.J. (1985). Sequence determination and genetic content of the short unique region in the genome of HSV-1. J. Mol. Biol. 181, 1-13.
McGeoch, D.J., Dolan, A. and Frame, M.C. (1986). DNA sequence of the region in the genome of herpes simplex virus type 1 containing the exonuclease gene and neighbouring genes. Nucl. Acids Res. 14, 3435-3448.
McGeoch, D.J., Moss, H.W.M., McNab, D. and Frame, M.C. (1987). DNA sequence and genetic content of the *Hind*III L region in the short unique component of the herpes simplex virus type 2 genome: identification of the gene encoding glycoprotein G and evolutionary comparisons. J. Gen. Virol. 68, 19-38.
McKnight, J.L.C., Kristie, T.M. and Roizman, B. (1987). Binding of the virion protein mediating alpha gene induction in herpes simplex virus 1-infected cells to its *cis* site requires cellular proteins. Proc. Natl. Acad. Sci. USA 84, 7061-7065.
McKnight, S.L. and Kingsbury, R. (1982). Transcriptional control signals of a eukaryotic protein-coding gene. Science 217, 316-324.
McLauchlan, J. and Clements, J.B. (1983). Organisation of the herpes simplex virus type 1 transcription unit encoding two early proteins with molecular weights of 140,000 and 40,000. J. Gen. Virol. 64, 997-1006.
Mace, M. and Alberts, B.M. (1984). Characterization of the stimulatory effect of T4 gene 45 protein and the gene 44/62 protein complex on DNA synthesis by T4 DNA polymerase. J. Mol. Biol. 177, 313-327.
Mackem, S. and Roizman, B. (1980). Regulation of herpesvirus macromolecular synthesis. Transcription-initiation sites and domains

of alpha genes. Proc. Natl. Acad. Sci. USA 77, 7122-7126.
Mackem, S. and Roizman, B. (1981). Regulation of herpesvirus macromolecular synthesis. Temporal order of transcription of alpha genes is not dependent on the stringency of inhibition of protein synthesis. J. Virol. 40, 319-322.
Mackem, S. and Roizman, B. (1982a). Differentiation between alpha-promoter and regulatory regions of herpes simplex virus 1: the functional domains and sequence of a movable alpha-regulator. Proc. Natl. Acad. Sci. USA 79, 4917-4921.
Mackem, S. and Roizman, B. (1982b). Regulation of alpha genes of herpes simplex virus: the alpha-27 gene promoter-thymidine kinase chimera is positively regulated in converted L cells. J. Virol. 43, 1015-1023.
Mackem, S. and Roizman, B. (1982c). Structural features of the herpes simplex virus alpha gene 4, 0 and 27 promoter-regulatory sequences which confer alpha-regulation on chimeric thymidine kinase genes. J. Virol. 44, 939-949.
Macnab, J.C.M. (1987). Herpes simplex virus and human cytomegalovirus: their role in morphological transformation and genital cancers. J. Gen. Virol. 68, 2525-2550.
Madsen, P. and Celis, J.E. (1985). S-phase patterns of cyclin (PCNA) antigen staining resemble topographical patterns of DNA synthesis. FEBS Lett. 193, 5-11.
Manservigi, R., Spear, P.G. and Buchan, A. (1977). Cell fusion induced by herpes simplex virus is promoted and suppressed by different viral glycoproteins. Proc. Natl. Acad. Sci. USA 74, 3913-3917.
Marchetti, M.E., Smith, C.E. and Schaffer, P.A. (1988). A temperature-sensitive mutation in a herpes simplex virus type 1 gene required for viral DNA synthesis maps to coordinates 0.609 through 0.614 in U_L. J. Virol. 62, 715-721.
Marcy, A.I., Olivo, P.D., Challberg, M.D. and Coen, D.M. (1990). Enzymatic activities of overexpressed herpes simplex virusDNA polymerase purified from recombinant baculovirus infected insect cells. Nucl. Acids Res. 18, 1207-1215.
Marsden, H.S., Campbell, M.E.M., Haarr, L., Frame, M.C., Parris, D.S., Murphy, M., Hope, R.G., Muller, M.T. and Preston, C.M. (1987). The 65,000 M_r DNA binding and trans-inducing proteins of herpes simplex virus type 1. J. Virol. 61, 2428-2437.
Marsden, H.S., Crombie, I.K. and Subak-Sharpe, J.H. (1976). Control of protein synthesis in herpesvirus-infected cells: analysis of the polypeptides induced by wild type and sixteen temperature-sensitive mutants of HSV strain 17. J. Gen. Virol. 31, 347-372.
Marsden, H.S., Lang, J., Davison, A.J., Hope, R.G. and McDonald, D.M. (1982). Genomic location and lack of phosphorlyation of the HSV immediate-early polypeptide IE-12. J. Gen. Virol. 62, 17-27.
Marsden, H.S., Stow, N.D., Preston, V.G., Timbury, M.C. and Wilkie, N.M. (1978). Physical mapping of herpes simplex virus-induced polypeptides. J. Virol. 28, 624-642.
Martin, M.E.D., Thomson, B.J., Honess, R.W., Craxton, M.A., Gompels, U.A., Liu, M.-Y., Littler, E., Arrand, J.R., Teo, I. and Jones, M.D. (1991). The genome of human herpesvirus 6: maps of unit-length and concatemeric genomes for nine restriction endonucleases. J. Gen. Virol. 72, 157-168.
Mastrangelo, I.A., Hough, P.V.C., Wall, J.S., Dodson, M., Dean, F. and Hurwitz, J. (1989). ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. Nature 338, 658-662.
Mastrangelo, I.A., Hough, P.V.C., Welson, V., Wall, J., Hainfeld, J. and Tegtmeyer, P. (1985). Monomers through trimers of large tumour antigen bind in region I and monomers through tetramers bind in region II of simian virus 40 origin of replication as stable

structures in solution. Proc. Natl. Acad. Sci. USA 82, 3626-3630.
Matson, S.W. and Kaiser-Rogers, A. (1990). DNA Helicases. Ann. Rev. Biochem. 59, 289-329.
Matsumoto, K., Moriuchi, T., Koji, T. and Nakane, P.K. (1987). Molecular cloning of cDNA coding for rat proliferating cell nuclear antigen (PCNA)/cyclin. EMBO J. 6, 637-642.
Mathews, R.E.F. (1982). Classification and nomenclature of viruses. Intervirology 17, 1-200.
Matz, B. (1989). Herpes simplex virus causes amplification of recombinant plasmids containing simian virus 40 sequences. J. Gen. Virol. 70, 1347-1358.
Matz, B., Schlehofer, J.R., zur Hausen, H., Huber, B. and Fanning, E. (1985). HSV- and chemical carcinogen- induced amplification of SV40 DNA sequences in transformed cells is cell line dependent. Int. J. Cancer 35, 521-525.
Mavromara-Nazos, P., Silver, S., Hubenthal-Voss, J., McKnight, J.L.C. and Roizman, B. (1986). Regulation of herpes simplex virus 1 genes: alpha gene sequence requirements for transient induction of indicator genes regulated by beta or late ($gamma_2$) promoters. Virology 149, 152-164.
Mavromara-Nazos, P. and Roizman, B. (1987). Activation of herpes simplex virus 1 $gamma_2$ genes by viral DNA replication. Virology 161, 593-598.
Meignier, B., Longnecker, R., Mavromara-Nazos, P., Sears, A.E. and Roizman, B. (1988). Virulence of and establishment of latency by genetically engineered deletion mutants of herpes simplex virus type 1. Virology 162, 251-254.
Mellerick, D.M. and Fraser, N.W. (1987). Physical state of the latent herpes simplex virus genome in a mouse model system: evidence suggesting an episomal state. Virology 158, 265-275.
Metzler, D.W. and Wilcox, K.W. (1985). Isolation of herpes simplex virus regulatory protein ICP4 as a homodimeric complex. J. Virol. 55, 329-337.
Michael, N., Spector, D., Mavromara-Nazos, P., Kristie, T.M. and Roizman, B. (1988). The DNA-binding properties of the major regulatory protein alpha 4 of herpes simplex viruses. Science 239, 1531-1533.
Mocarski, E.S. and Roizman, B. (1981). Site-specific inversion sequence of the herpes simplex virus genome: domain and structural features. Proc. Natl. Acad. Sci. USA 78, 7047-7051.
Mocarski, E.S. and Roizman, B. (1982a). Herpesvirus-dependent amplification and inversion of cell-associated viral thymidine kinase gene flanked by viral a sequences and linked to an origin of viral DNA replication. Proc. Natl. Acad. Sci. USA 79, 5626-5630.
Mocarski, E.S. and Roizman, B. (1982b). Structure and role of the herpes simplex virus DNA temini in inversion, circularization and generation of virion DNA. Cell 31, 89-97.
Morgan, C., Rose, H.M., Holden, M. and Jones, E. P. (1959). Electron microscopic observations on the development of herpes simplex virus. J. Exp. Med. 110, 643-656.
Morgan, C., Rose, H.M. and Mednis, B. (1968). Electron microscopy of herpes simplex virus. I. Entry. J. Virol. 2, 507-516.
Morrison, J.M. and Keir, H.M. (1968). A new DNA-exonuclease in cells infected with herpes virus: partial purification and properties of the enzyme. J. Gen. Virol. 3, 337-347.
Moss, H. (1982). The herpes simplex virus specified deoxyribonuclease and DNA polymerase activities. PhD Thesis, University of Glasgow.
Moss, H. (1986). The herpes simplex virus type 2 alkaline DNase activity is essential for replication and growth. J. Gen. Virol. 67, 1173-1178.
Moss, H., Chartrand, P., Timbury, M.C. and Hay, J. (1979). Mutant of herpes simplex virus type 2 with temperature-sensitive lesions

affecting virion thermostability and DNase activity: identification of the lethal mutation and physical mapping of the nuc⁻ lesion. J. Virol. 32, 140-146.
Mosig, G. (1987). The essential role of recombination in phage T4 growth. Ann. Review Genetics, 21, 347-371.
Mullaney, J., Moss, H.W.McL. and McGeoch, D.J. (1989). Gene UL2 of herpes simplex virus type 1 encodes a uracil-DNA glycosylase. J. Gen. Virol. 70, 449-454.
Muller, M.T. (1987). Binding of the herpes simplex virus immediate-early gene product ICP4 to its own transcription start site. J. Virol. 61, 858-865.
Muller, M.T., Bolles, C.S. and Parris, D.S. (1985). Association of type 1 DNA topoisomerase with herpes simplex virus. J. Gen. Virol. 66, 1565-1574.
Murakami, Y. Wobbe, C.R., Weissbach, L. Dean, F.B. and Hurwitz, J. (1986). Role of DNA polymerase α and DNA primase in simian virus 40 DNA replication in vitro. Proc. Natl. Acad. Sci. USA 83, 2869-2873.
Murchie, M.-J. and McGeoch, D.J. (1982). DNA sequence analysis of an immediate-early gene region of the herpes simplex virus type 1 genome (map coordinates 0.950 -0.978). J. Gen. Virol. 62, 1-15.
Nagata, K., Guggenheimer, R.A. and Hurwitz, J. (1983a). Specific binding of a cellular DNA repair protein to the origin of replication of adenovirus DNA. Proc. Natl. Acad. Sci. USA 80, 6177-6181.
Nagata, K., Guggenheimer, R.A. and Hurwitz, J. (1983b). Adenovirus DNA replication in vitro: synthesis of full length DNA with purified proteins. Proc. Natl. Acad. Sci. USA 80, 4266-4270.
Nakai, H. and Richardson, C.C. (1988). Leading abd lagging strand synthesis at the replication fork of bacteriophage T7. J. Biol. Chem. 263, 9818-9830.
Nii, S., Morgan, C. and Rose, H.M. (1968). Electron microscopy of herpes simplex virus. J. Virol. 2, 517-536.
Nishioka, Y. and Silverstein, S. (1977). Degradation of cellular mRNA during infection by herpes simplex virus . Proc. Natl. Acad. Sci. USA 74, 2370-2374.
Nishioka, Y. and Silverstein, S. (1978). Requirement of protein synthesis for the degradation of host mRNA in Friend erythroleukemia cells infected with herpes simplex virus type 1. J. Virol. 27, 619-627.
Nossal, N.G. and Alberts, B.M. (1983). Mechanism of DNA replication catalyzed by purified T4 replication proteins. In: Bacteriophage T4. (Eds. Mathews, C.K., Kutter, E.M., Mosig, G. and Berget, P.B. American Society Microbiology. Washington, DC.) pp71-81.
O'Donnell, M.E., Elias, P. and Lehman, I.R. (1987a). Processive replication of single-stranded DNA templates by the herpes simplex virus induced DNA polymerase. J. Biol. Chem. 262, 4252-4259.
O'Donnell, M.E., Elias, P., Funnell, B.E. and Lehman, I.R. (1987b). Interaction between the DNA polymerase and single-stranded DNA binding protein (infected cell protein 8) of herpes simplex virus 1. J. Biol. Chem. 262, 4260-4266.
Ogawa, T. Baker,T.A., van der Ende, A. and Kornberg, A. (1985). Initiation of enzymatic replication at the origin of the *Escherichia coli* chromosome: contributions of RNA polymerase and primase. Proc. Natl. Acad. Sci. USA 82, 3562-3566.
O'Hare, P. and Goding, C.R. (1988). Herpes simplex virus regulatory elements and the immunoglobulin octamer domain bind a common factor and are both targets for virion transactivation. Cell 52, 435-445.
O'Hare, P. and Hayward, G.S. (1985a). Evidence for a direct role for both the 175,000- and 110,000- molecular weight immediate-early proteins of herpes simplex virus in the trans-activating of delayed early promoters. J. Virol. 53, 751-760.
O'Hare, P. and Hayward, G.S. (1985b). Three trans-activating regulatory

proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. J. Virol. 56, 723-733.
O'Hare, P. and Hayward, G.S. (1987). Comparison of upstream sequence requirements for positive and negative regulation of a herpes simplex virus immediate-early gene by three virus-encoded trans-acting factors. J. Virol. 61, 190-199.
Olivo, P.D., Nelson, N.J. and Challberg, M.D. (1988). Herpes simplex virus DNA replication: the UL9 gene encodes an origin-binding protein. Proc. Natl. Acad. Sci. USA 85, 5414-5418.
Olivo, P.D., Nelson, N.J. and Challberg, M.D. (1989). Herpes simplex virus type 1 gene products required for DNA replication: identification and overexpression. J. Virol. 63, 196-204.
Oka, A., Sasaki, H., Sugimoto, K. and Takanami, M. (1984). Sequence organisation of replication origin of the *Escherichia coli* K-12 chromosome. J. Mol. Biol. 176, 443-458.
Oka, A., Sugimoto, K., Takanami, H. and Hirota, Y. (1980). Replication origin of the *Escherichia coli* K-12 chromosome: the size and structure of the minimum DNA segment carrying the information for autonomous replication. Mol. Gen. Genet. 178, 9-20.
Oroskar, A.A. and Read, G.S. (1987). A mutant of herpes simplex virus type 1 exhibits increased stability of immediate early (alpha) mRNAs. J. Virol. 61, 604-606.
Para, M.F., Baucke, R.B. and Spear, P.G. (1982). Glycoprotein gE of herpes simplex virus type 1: effects of anti-gE on virion infectivity and on virus-induced Fc-binding receptors. J. Virol. 41, 129-136.
Parris, D.S., Cross, A., Haarr, L., Orr, A., Frame, M.C., Murphy, M., McGeoch, D.J. and Marsden, H.S. (1988). Identification of the gene encoding the 65-kilodalton DNA binding protein of herpes simplex virus type 1. J. Virol. 62, 818-825.
Patel, P., Chan, W.L., Kemp, L.M., Lathangue, W.B. and Latchamn, D.S. (1986). Isolation of cDNA clones derived from a cellular gene transcriptionally induced by HSV. Nucl. Acids Res. 14, 5629-5640.
Paterson, T. and Everett, R.D. (1988). The regions of the herpes simplex virus type 1 immediate early protein Vmw175 required for site-specific DNA-binding closely correspond to those involved in transcriptional regulation. Nucl. Acids Res. 16, 11005-11025.
Pederson, M., Tally-Brown, S. and Millet, R.L. (1981). Gene expression of herpes simplex virus III. Effect of arabinosyladenine on viral polypeptide synthesis. J. Virol. 38, 712-719.
Pereira, L., Wolf, M.H., Fenwick, M. and Roizman, B. (1977). Regulation of herpesvirus macromolecular synthesis. V. Properties of alpha polypeptides made in HSV-1 and HSV-2 infected cells. Virology 77, 733-749.
Perry, L.J. and McGeoch, D.J. (1988). The DNA sequences of the long repeat region and adjoining parts of the long unique region in the genome of herpes simplex virus type 1. J. Gen. Virol. 69, 2831-2846.
Perry, L.J., Rixon, F.J., Everett, R.D., Frame, M.C. and McGeoch, D.J. (1986). Characterization of the IE110 gene of herpes simplex virus type 1. J. Gen. Virol. 67, 2365-2380.
Poffenberger, K.L. and Roizman, B. (1985). A non-inverting genome of a viable herpes simplex virus 1: presence of head-to-tail linkages in packaged genomes and requirements for circularization after infection. J. Virol. 53, 587-595.
Poffenberger, K.L., Tabares, B. and Roizman, B. (1983). Characterization of a virable non-inverting herpes simplex virus 1 genome derived by insertion and deletion of sequences at the junction of components L and S. Proc. Natl. Acad. Sci. USA 80, 2690-2694.
Pogue-Geile, K.L., Lee, G.T.-Y. and Spear, P.G. (1985). Novel rearrangements of herpes simplex virus DNA sequences resulting from duplication of a sequence within the unique region of the L

component. J. Virol. 53, 456-461.
Polvino-Bodnar, M., Orberg, P.K. and Schaffer, P.A. (1987). Herpes simplex virus type 1 ori_L is not required for virus replication or for establishment and reactivation of latent infection in mice. J. Virol. 61, 3528-3535.
Post, L.E. and Roizman, B. (1981). A generalized technique for deletion of specific genes in large genomes: alpha gene 22 of herpes simplex virus 1 is not essential for growth. Cell 25, 227-232.
Powell, K.L. and Courtney, R.J. (1975). Polypeptides synthesized in herpes simplex virus type 2-infected HEp-2 cells. Virology 66, 217-228.
Powell, K.L., Littler, E. and Purifoy, D.J.M. (1981). Nonstructural proteins of HSV1. II. Major virus-specific DNA binding protein. J. Virol. 39, 894-902.
Powell, K.L. and Purifoy, D.J.M. (1976). DNA-binding proteins of cells infected by herpes simplex virus type 1 and type 2. Intervirology 7, 225-239.
Powell, K.L. and Purifoy, D.J.M. (1977). Non-structural proteins of herpes simplex virus. I. Purification of the induced DNA polymerase. J. Virol. 24, 618-626.
Powell, K.L., Purifoy, D.J.M. and Courtney, R.J. (1975). The synthesis of herpes simplex virus proteins in the absence of DNA synthesis. Biochem. Biophys. Res. Commun. 66, 262-271.
Prelich, G., Kostura, M., Marshak, D.R., Mathews, M.B. and Stillman, B. (1987a). The cell-cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication in vitro. Nature 326, 471-475.
Prelich, G., Tan, C.K., Kostura, M., Mathews, M.B., So, A.G., Downey, K. and Stillman, B. (1987b). Functional identity of proliferating cell nuclear antigen and a DNA polymerase delta auxiliary protein. Nature 326, 517-520.
Prelich, G. and Stillman, B. (1988). Coordinated leading and lagging straind synthesis during SV40 DNA replication in vitro requires PCNA. Cell 53, 117-126.
Preston, C.M. (1979a). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsk. J. Virol. 29, 275-284.
Preston, C.M. (1979b). Abnormal properties of an immediate early polypeptides in cells infected with the herpes simplex virus type 1 mutant tsK. J. Virol. 32, 357-369.
Preston, C.M., Cordingley, M.G. and Stow, N.D. (1984) Analysis of DNA sequences which regulate the transcription of a herpes simplex virus immediate early gene. J. Virol. 50, 708-716.
Preston, C.M., Frame, M.C. and Campbell, M.E.M. (1988). A complex formed between cell components and an HSV structural polypeptide binds to a viral immediate early gene regulatory DNA sequence. Cell 52, 425-434.
Preston, C.M. and Newton, A.A. (1976). The effects of herpes simplex virus type 1 on cellular DNA-dependent RNA polymerase activities. J. Gen. Virol. 33, 471-482.
Preston, V.G., Davison, A.J., Marsden H.S., Timbury, M.C., Subak-Sharpe, J.H., and Wilkie, N.M. (1978). Recombinants between HSV types 1 and 2: analysis of genome structures and expression of immediate-early polypeptides. J. Virol. 28, 499-517.
Preston, V.G. and Fisher, F.B. (1984). Identification of the herpes simplex virus type 1 gene encoding the dUTPase. Virology 138, 58-68.
Preston, V.G., Palfreyman, J.W. and Dutia, B.M. (1984). Identification of a herpes simplex virus type 1 polypeptide which is a component of the virus-induced ribonucleotide reductase. J. Gen. Virol. 65, 1457-1466.
Pruijn, G.J.M., Van Driel, W. and Van der Vliet (1986). Nuclear factor III, anovel sequence-specific DNA-binding protein from HeLa Cells

stimulating adenovirus DNA replication. Nature 322, 656-659.
Purifoy, D.J.M., Lewis, R.B. and Powell, K.L. (1977). Identification of the herpes simplex virus DNA polymerase gene. Nature 269, 621-623.
Purifoy, D.J.M. and Powell, K.L. (1976). DNA-binding proteins induced by herpes simplex virus type 2 in HEp-2 cells. J. Virol. 19, 717-731.
Purifoy, D.J.M. and Powell, K.L. (1981). Temperature-sensitive mutants in two distinct complementation groups of herpes simplex virus type 1 specify thermolabile DNA polymerase. J. Gen. Virol. 54, 219-222.
Purves, F.C., Longnecker, R.M., Leader, D.P. and Roizman, B. (1987). Herpes simplex virus 1 protein kinase is encoded by open reading frame US3 which is not essential for virus growth in cell culture. J. Virol. 61, 2896-2901.
Quinlan, M.P., Chen, L.B. and Knipe, D.M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. Cell 36, 857-868.
Quinlan, M.P. and Knipe, D.M. (1983). Nuclear localization of herpesvirus proteins: potential role for the cellular framework. Mol. Cell. Biol. 3, 315-324.
Quinlan, M.P. and Knipe, D.M. (1985). Stimulation of expression of a herpes simplex virus DNA-binding protein by two viral functions. Mol. Cell. Biol. 5, 957-963.
Quinn, J.P. and McGeoch, D.J. (1985). DNA sequence of the region in the genome of herpes simplex virus type 1 containing genes for DNA polymerase and the major DNA binding protein. Nucl. Acids Res. 13, 8143-8163.
Raab-Traub, N., Dambaugh, T. and Kieff, E. (1980). DNA of Epstein-Barr virus VIII. B95-8, the previous prototype is an unusual deletion derivative. Cell 22, 257-267.
Rabkin, S.D. and Hanlon, B. (1990). Herpes simplex virus DNA synthesis at a preformed replication fork in vitro. J. Virol. 64, 4957-4967.
Randall. R.E. and Dinwoodie, N. (1986). Intranuclear localization of herpes simplex virus immediate-early and delayed-early proteins: evidence that ICP4 is associated with progeny virus. J. Gen. Virol. 67, 2163-2177.
Rapp, F. and Wigdahl, B. (1983). Herpesvirus target considerations for the design of antiviral agents. In: Targets for the design of antiviral agents. (Eds. E. De Clerq and R.T. Walker) Plenum Press, NY. pp29-60.
Rawls, W.E. (1985). Herpes simplex virus. In: Virology. (ed) Fields, B.N. Raven Press, N.Y. pp527-561.
Read, G.S. and Frenkel, N. (1983). Herpes simplex virus mutants defective in the virion-associated shut-off of host polypeptide synthesis and exhibiting abnormal synthesis of alpha (immediate-early) viral polypeptides. J. Virol. 46, 498-512.
Reyes, G.R., LaFemina, R., Hayward, S.D. and Hayward, G.S. (1979) Morphological transformation by DNA fragments of human herpesviruses: evidence for two distinct transforming regions in HSV-1 and HSV-2 and a lack of correlation with biochemical transfer of the thymidine kinase gene. Cold Spring Harbor Symposia on Quantitative Biology 44, 629-641.
Rice, S.A. and Knipe, D.M. (1988). Gene-specific transactivation by herpes simplex virus type 1 alpha protein ICP27. J. Virol. 62, 3814-3823.
Rigby, P.W.J., Dickman, M., Rhodes, C. and Berg, P. (1977). Labelling deoxyribonucleic acid to high specific activity by nick translation with DNA polymerase. J. Mol. Biol. 113, 237-251.
Rixon, F.J., Campbell, M.E. and Clements, J.B. (1982). The immediate-early mRNA that encodes the regulatory polypeptide Vmw175 of herpes simplex virus type 1 is unspliced. EMBO J. 1, 1273-1277.
Rixon, F.J., Atkinson, M.A. and Hay, J. (1983). Intracellular distribution of herpes simplex virus type 2 DNA synthesis: examination by light

and electron microsopy. J. Gen. Virol. 64, 2087-2092.
Rixon, F.J. and McGeoch, D. J. (1985). Detailed analysis of the mRNAs mapping in the short unique region of the herpes simplex virus type 1. Nucl. Acids Res. 13, 953-973.
Roberts, M.S., Boundy, A., O'Hare, P., Pizzorno, M.C., Ciufo, D.M. and Hayward, G.S. (1988). Direct correlation between a negative autoregulatory response element at the cap site of the herpes simplex virus type 1 IE175 (alpha 4) promoter and a specific binding site for the IE175 (ICP4) protein. J. Virol. 62, 4307-4302.
Rock, D.L. and Fraser, N.W. (1983). Detection of HSV-1 genome in central nervous system of latently infected mice. Nature 302, 523-525.
Rock, D.L. and Fraser, N.W. (1985). Latent herpes simplex virus type 1 DNA contains 2 copies of the virion DNA joint region. J. Virol. 55, 849-852.
Rock, D.L., Nesburn, A.B., Ghiasi, H., Ong, J., Lewis, T.L., Lowensgard, J.R. and Weschler, S.L. (1987). Detection of latency-related transcripts in trigeminal ganglia of rabbits infected with herpes simplex virus type 1. J. Virol. 61, 3820-3826.
Roizman, B. (1979). The structure and isomerisation of herpes simplex virus genomes. Cell 16, 481-494.
Roizman, B. (1982). The family herpesviridae: general description, taxonomy and classification. In: The Herpesviruses, 1, pp1-23, (ed) Roizman, B. Plenum Press, N.Y.
Roizman, B. and Batterson, W. (1985). Herpesviruses and their replication. In: Virology. (ed. B. N. Fields), pp 497-526. Raven Press, N.Y.
Roizman, B. and Furlong, D. (1974). The replication of herpesviruses. In: Comprehensive Virology. (Eds, H. Fraenkel-Conrat and R.R. Wagner), vol 13 pp 229-403. Plenum Press, N.Y.
Roizman, B. and Roane, P.R. (1964). The multiplication of herpes simplex virus. II. The relation between protein synthesis and the duplication of viral DNA in infected HEp2 cells. Virology 22, 262-269
Roller, R.J., McCormick, A.L. and Roizman, B. (1989). Cellular proteins specifically bind single and double stranded DNA and RNA from the i nitiation site of a transcripti that crosses the origin of DNA replication of herpes simplex virus 1 Proc. Natl. Acad. Sci. USA 86, 6518-6522.
Russell, J. and Preston, C.M. (1986). An in vitro latency system for herpes simplex virus type 2. J. Gen. Virol. 67, 397-403.
Russell, J., Stow, N.D., Stow, E.C. and Preston, C.M. (1987) Herpes simplex virus genes involved in latency in vitro. J. Gen. Virol. 68, 3009-3018.
Ruyechan, W.T. (1983). The major herpes simplex virus DNA-binding protein holds single-stranded DNA in an extended figuration. J. Virol. 46, 661-666.
Ruyechan, W.T., Morse, L.S., Knipe, D.M. and Roizman, B. (1979). Molecular genetics of herpes simplex virus. II. Mapping of the major viral glycoproteins and of the genetic loci specifying the social behaviour of infected cells. J. Virol. 29, 677-697.
Ruyechan, W.T. and Weir, A.C. (1984). Interaction with nucleic acids and stimulation of the viral DNA polymerase by the herpes simplex virus type 1 major DNA-binding protein. J. Virol. 52, 727-733.
Sacks, W.R., Greene, C.C., Aschman, D.P. and Schaffer, P.A. (1985). Herpes simplex virus type 1 ICP27 is an essential regulatory protein. J. Virol. 55, 796-805.
Sacks, W.R. and Schaffer, P.A. (1987). Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein ICP0 exhibit impaired growth in cell culture. J. Virol. 61, 829-839.
Sadowski, I., Ma, J., Triezenberg, S. and Ptashne, M. (1988). GAL4-VP16 is an unusually potent transcriptional activator. Nature 335, 563-564.
Salahuddin, S.Z., Ablashi, D.V., Markham, P.D., Josephs, S.F.,

Sturzenegger, S., Kaplan, M., Kramarsky, B. and Gallo, R. (1986). Isolation of a new virus, HBLV, in patients with lymphoproliferative disorders. Science 234, 596-601.
Sanger, F., Nicklen, S. and Coulsen, A.R. (1977). DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463-5467.
Sarmiento, M., Haffen, M. and Spear, P.G. (1979). Membrane proteins specified by herpes simplex viruses. III. Role of glycoprotein VP7 (B2) in virion infectivity. J. Virol. 29, 1149-1158.
Schaffer, P.A., Carter, V.C. and Timbury, M.C. (1978). Collaborative complementation study of temperature-sensitive mutants of herpes simplex virus types 1 and 2. J. Virol. 27, 490-504.
Schaffer, P.A., Preston, V.G., Wagner, E.K. and Deri-Rao, G.B. (1987). Herpes simplex virus. In: Genet. Maps 4, 93-98. Cold Spring Harbor Laboratories.
Schaffer, P.A., Tevethia, M.J. and Benyesh-Melnick, M. (1974). Recombination between temperature-sensitive mutants of herpes simplex virus type 1. Virology 58, 219-228.
Scheck, N. and Backenheimer, S.L. (1985). Degradation of cellular mRNAs induced by a virion-associated factor during herpes simplex virus infection of Vero cells. J. Virol. 55, 601-610.
Schneider, R., Gander, T., Muller, U., Mertz and Winnacker, E.L. (1986). A sensitive and rapid gel retention assay for nuclear factor I and other DNA-binding proteins in crude nuclear extracts. Nucl. Acids Res. 14, 1303-1317.
Schrag, J.D., Ven Kataramprasad, B.V., Rixon, F.J. and Chiu, W. (1989). Three-dimensional structure of the HSV-1 nucleocapsid. Cell 56, 651-660.
Schroder, C.H., Stegmann, B., Lauppe, H.F. and Kaerner, H.C. (1975). An unusual defective genotype derived from herpes simplex virus strain ANG. Intervirol. 6, 270-284.
Schwartz, J. and Roizman, B. (1969). Similarities and differences in the development of laboratory strains and freshly isolated strains of herpes simplex viruses in HEp-2 cells: electron microscopy. J. Virol. 4, 879-889.
Sears, A. E., Halliburton, I.W., Meignier, B., Silver, S. and Roizman, B. (1985). Herpes simplex virus 1 mutant deleted in the alpha 22 gene: growth and gene expression in permissive and restrictive cells and establishment of latency in mice. J. Virol. 55, 338-346.
Sekulovich, R.E., Leary, K. and Sandri-Goldin, R.M. (1988). The herpes simplex virus type 1 alpha protein ICP27 can act as a trans-represor in combination with ICP4 and ICP0. J. Virol. 62, 4510-4522.
Shapira, M., Homa, F.L., Glorioso, J.C. and Levina, M. (1987). Regulation of the herpes simplex virus type 1 late (gamma) glycoprotein C gene: sequences beteween base pairs -34 to +39 control transient expression and responsiveness to transactivation by the products of the immediate early (alpha) 4 and 0 genes. Nucl. Acids Res. 15, 3097-3111.
Shiraki, K. and Rapp, F. (1986). Establishment of herpes simplex virus latency in vitro with cycloheximide. J. Gen. Virol. 67, 2497-2500.
Silver, P.A. (1991). How proteins enter the nucleus. Cell 64, 489-497.
Skinner, G.B.R. (1976). Transformation of primary rat embryo fibroblasts by type 2herpes simplex virus: evidence for a hit and run mechanism. British Journal of Experimental Pathology 57, 361-376.
Smale, S.T. and Tjian, R.T. (1986). T-antigen-DNA polymerase, a complex implicated in simian virus 40 DNA replication. Mol. Cell. Biol. 6, 4077-4087.
Smiley, J.R., Fong, B.S. and Leung, W.-C. (1981). Construction of a double-jointed herpes simplex viral DNA molecule: inverted repeats are required for segment inversion, and direct repeat promoter-deletions. Virology 113, 345-362.
Smiley, J.R., Wagner, M.J., Summers, W.P. and Summers, W.C. (1980).

Genetic and physical evidence for the polarity of transcription of the thymidine kinase gene of herpes simplex virus. Virology 102, 83-93.

Smith, C.A., Marchetti, M.E., Edmonson, P. and Schaffer, P.A. (1989). Herpes simplex virus type 2 mutants with deletions in the intergenic region between ICP4 and ICP22/47: identification of nonessential cis-acting elements in the context of the viral genome. J. Virol. 63, 2036-2047.

Smith, I.L. and Sandri-Goldin, R.M. (1988). Evidence that transcriptional control is the major mechanism of regulation for the glycoprotein D in herpes simplex virus type 1-infected cells. J. Virol. 62, 1474-1477.

Smith, R.F. and Smith, T.F. (1989). Identification of new protein kinase-related genes in three herpesviruses, herpes simplex virus, varicella-zoster virus and Epstein-Barr virus. J. Virol. 63, 450-455.

So, A.G. and Downey, K.M. (1988). Mammalian DNA polymerase α and δ: current status in DNA replication. Biochemistry 27, 4591-4595.

Soeller, W., Abarazua, P. and Marian, K.J. (1984). Mutational analysis of primosome assembly sites. II. Role of secondary structure in the formation of active sites. J. Biol. Chem. 259, 14293-14300.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Spaete, R.R. and Frenkel, N. (1982). The herpes simplex virus amplicon: a new eucaryotic defective-virus cloning-amplifying vector. Cell 30, 295-304.

Spaete, R.R. and Mocarski, E.S. (1985). The a sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes. J. Virol. 54, 817-824.

Spear, P.G. and Roizman, B. (1972). Proteins specified by herpes simplex virus. V. Purification and structural proteins of the herpesvirion. J. Virol. 9, 143-159.

Spivack, J.G. and Fraser,N.W. (1987). Detection of herpes simplex virus type 1 transcripts during latent infection of mice. J. Virol. 61, 3841-3847.

Stahl, H., Droge, P. and Knippers, R. (1986). DNA helicase activity o f SV40 large tumour antigen. EMBO J. 5, 1939-1944.

Staden, R. and McLachlan, A.D. (1982). Codon preferences and its use in identifying protein coding regions in long DNA sequences. Nucl. Acids Res. 10, 141-156.

Steiner, I, Spivack, J.G., Lirette, R.P., Brown, S.M., MacLean, A.R., Subak-Sharpe, J.H. and Fraser, N.W. (1989). Herpes simplex virus type 1 latency-associated transcripts are evidently not essential for latent infection. EMBO J. 8, 505-511.

Steiner, I, Spivack, J.G., O'Boyle, D.R., Lavi, C. and Fraser, N.W. (1988). Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. J. Virol. 62, 3493-3496.

Stevens, J.G. and Cook, M.L. (1971) Latent herpes simplex virus in spinal ganglia of mice. Science 173, 843-845.

Stevens, J.G., Wagner, E.K., Devi-Rao, G.B., Cook, M.L. and Feldman, L.T. (1987). RNA complementary to a herpesvirus alpha mRNA is prominent in latently infected neurons. Science 235, 1056-1059.

Stillman, B. (1989). Initiation of eukaryotic DNA replication in vitro. Annu. Rev. Cell Biol. 5, 197-245.

Stillman, B.W. and Gluzman, Y. (1985). Replication and supercoiling of simian virus 40 DNA in cell extracts from human cells. Mol. Cell. Biol. 5, 2051-2060.

Stow, N.D. (1982). Localization of an origin of DNA replication within the TR_S/IR_S repeated region of the HSV-1 genome. EMBO J. 1, 863-867.

Stow, N.D. and Davison, A.J. (1986). Identification of a varicella-zoster virus origin of DNA replication and its activation by HSV-1 gene products. J. Gen. Virol. 67, 1613-1623.

Thomas M S, Banks L M, Purifouy D J M and Powell K L, 1988. Production of antibodies of predetermined specificity against herpes simplex virus DNA polymerase and their use in characterizationof the enzyme. J. Virol. 62, 1550-1557.

Stow, N.D. and McMonagle, E.C. (1983). Characterization of the TR_S/IR_S origin of DNA replication of HSV-1. Virology 130, 427-438.

Stow, N.D., McMonagle, E.C. and Davison, A.J. (1983). Fragments from both termini of the herpes simplex virus type 1 genome contain signals required for the encapsidation of viral DNA. Nucl. Acids Res. 11, 8205-8220.

Stow, N.D., Murray, M.P. and Stow, E.C. (1986). Cis-acting singals involved in the replication and packaging of herpes simplex virus type 1 DNA. In: Cancer Cells, Vol. 4. DNA Tumour Viruses. (Eds, M. Botchan, T. Grodzicker and P.A. Sharp), pp 497-567. Cold Spring Laboratory, USA.

Stow, N.D. and Stow, E.C. (1986). Isolation and characterization of a HSV-1 mutant containing a deletion within the gene encoding the immediate early polypeptide Vmw110. J. Gen. Virol. 67, 2571-2585.

Stow, N.D., Weir, H.M. and Stow, E.C. (1990). Analysis of the binding site for the varicella-zoster virus gene 51 product within the viral origin of DNA replication. Virology 177, 570-577.

Stow, N.D. and Wilkie, N.M. (1976). An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. J. Gen. Virol. 33, 447-458.

Stow, N.D. and Wilkie, N.M. (1978). Physical mapping of temperature-sensitive mutations of herpes simplex virus type 1 by intertypic marker rescue. Virology 90, 1-11.

Strobel-Fidler, M. and Francke, B. (1980). Alkaline deoxyribonuclease induced by herpes simplex virus type 1: composition and properties of the purified enzyme. Virology 103, 493-501.

Sundin, O. and Varshavsky, A. (1980). Terminal stages of SV40 DNA replication procees via multiply intertwined catenated dimers. Cell 21, 103-114.

Swain, M.A. and Galloway, D.A. (1983). Nucleotide sequence of the herpes simplex virus type 2 thymidine kinase gene. J. Virol. 46, 1045-1050.

Swain, M.A., Peet, R.W. and Galloway, D.A. (1985). Characterization of the gene encoding herpes simplex virus type 2 glycoprotein C and comparison with the type 1 counterpart. J. Virol. 53, 561-569.

Swanstrom, R.L. and Wagner, E.K. (1974). Regulation of synthesis of herpes simplex virus type 1 virus mRNA during productive infection. Virology 60, 522-533.

Sydiskis, R.J. and Roizman, B. (1967). The dissaggregation of host polysomes in productive and abortive infection with herpes simplex virus. Virology 32, 678-686.

Sydiskis, R.J. and Roizman, B. (1968). The sedimention profiles of cytoplasmic polyribosome in mammalian cells productively and abortively infected with herpes simplex virus. Virology 34, 562-565.

Taha, M.Y., Brown, S.M., Clements, G.B. and Graham, D.I. (1990). The JH2604 deletion variant of herpes simplex virus type 2 (HG52) fails to produce necrotizing encephalitis following intracranial inoculation of mice. J. Gen. Virol. 71, 1597-1601.

Tan, C.-K., Castillo, C. So, A.G. and Downey, K.M. (1986). An auxiliary protein for DNA polymerase delta from foetal calf thymus. J. Biol. Chem. 261, 12310-12316.

Tegtmeyer, P. (1972). Simian virus 40 deoxyribonucleic acid synthesis: the viral replicon. J. Virol. 10, 591-598.

Tjian, R. (1978). The binding site on SV40 DNA for a T antigen-related protein. Cell 13, 165-179.

Tjian, R. and Robbins, A. (1979). Enzymatic activites associated with a purified simian virus 40 T antigen related protein. Proc. Natl. Acad. Sci. USA 72, 610-614.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76, 4350-4354.

Wang Y and Hall J D, 1990. Characterization of a major DNA-binding domain in the herpes simplex virus type 1 DNA-binding protein. J. Virol. 64, 2082-2089.

Traut, W. and Fanning, G. (1988). Sequence specific interactions between a cellular DNA binding protein and the simian virus 40 origin of DNA replication. Mol. Cell. Biol. 8, 903-911.

Triezenberg, S.J., LaMarco, K.L. and McKnight, S.L. (1988b). Evidence of DNA:protein interactions that mediate HSV-1 immediate early gene activation by VP16. Genes and Dev. 2, 730-742.

Triezenberg, S.J., Kingsbury, R.C. and McKnight, S.L. (1988a). Functional dissection of VP16, the trans-activator of herpes simplex virus immediate-early gene expression. Genes and Dev. 2, 718-722.

Tsurumi, T. and Lehman, I. R. (1990). Release of RNA polymerase from vero cell mitochondria after herpes simplex virus type 1 infection. J. Virol. 64, 450-452.

Tsurimi, T. Maeno, K. Nishiyamo, Y. (1987). A single base change within the DNA polymerase locus of herpes simplex virus type 2 can confer resistance to aphidicolin. J. Virol. 61, 388-394.

Tsurimoto, T., Melendy, T. and Stillman, B. (1990). Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin. Nature 346, 534-539.

Tsurimoto, T. and Stillman, B. (1989a). Purification of a cellular replication factor, RF-C, that is requires for coorfinated synthesis of leading and lagging strands during SV40 DNA replication in vitro Mol. Cell. Biol. 9, 609-619.

Tsurimoto, T. and Stillman, B. (1989b). Multiple replication factors augment DNA synthesis by the two eukaryotic DNA polymerases, alpha and delta. EMBO J. 8, 3883-3889.

Twigg, A.J. and Sherratt, D. (1980). Trans-complementable copy number mutants of plasmid ColE1. Nature 283, 216-218.

Umene, K. (1986). Conversion of a fraction of the unique sequence to part of the inverted repeats in the S component of the HSV type 1 genome. J. Gen. Virol. 67, 1035-1048.

Varmuza, S.L. and Smiley, J.R. (1985). Signals for site-specific cleavage of HSV DNA: maturation involves two separate cleavage events at sites distal to the recognition sequences. Cell 41, 793-802.

Vaughan, P.J., Purifoy, D.J.M. and Powell, K.L. (1985). DNA binding protein associated with herpes simplex virus DNA polymerase. J. Virol. 53, 501-508.

Venkatesan, M., Silver, L.L. and Nossal, N.C. (1982). Bacteriophage T4 gene 41 protein, required for the synthesis of RNA primers,is also a DNA helicase. J. Biol. Chem. 257, 12426-12434.

Viera, J. and Messing, J. (1982). The pUC plasmids, on M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-268.

Vlazny, D.A. and Frenkel, N. (1981). Replication of HSV DNA: localization of replication recognition signals within defective virus genomes. Proc. Natl. Acad. Sci. USA 78, 742-746.

Vlazny, D.A., Kwong, A. and Frenkel, N. (1982). Site-specific cleavage/packaging of HSV DNA and the selective maturation of nucleocapsids containing full-length viral DNA. Proc. Natl. Acad. Sci. USA 79, 1423-1427.

Wagner, E.K. (1985). Individual HSV transcripts. In: The Herpesviruses, Vol. III (Ed., B. Roizman), pp 45-104. Plenum Press.

Wagner, E.K., Devi-Rao, G.B., Feldman, L.T., Dobson, A.T., Zhang, Y.-F., Flanagan, W.M. and Stevens, J.G. (1988a). Physical characterization of the herpes simplex virus latency-associated transcript in neurons. J. Virol. 62, 1194-1202.

Wagner, E.K., Flanagan, W.M., Devi-Rao, G.B., Zhang, Y.-F., Hill, J.M., Anderson, K.P. and Stevens, J.G. (1988b). The herpes simplex virus latency-associated transcript is spliced during the latent phase of infection. J. Virol. 62, 4577-4585.

Watson, R.J. and Clements, J.B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA

synthesis. Nature 285, 329-330.
Weber, P.C., Challberg, M.D., Nelson, N.J., Levine, M. and Glorioso, J. C. (1988). Inversion events in the HSV-1 genome are directly mediated by the viral DNA replication machinery and lack sequence specificity. Cell 54, 369-381.
Weber, P.C., Levine, M. and Glorioso, J.C. (1987). Rapid identification of non-essential genes of herpes simplex virus type 1 by Tn5 mutagenesis. Science 236, 576-579.
Weinberg, D,H., Collins, K.L., Simancek, P., Russo, A. Wold, M.S., Virshup, D.M. and Kelly, T.J. (1990). Reconstitution of simianvirus 40 DNA replication with purified proteins. Proc. Natl. Acad. Sci. USA 87, 8692-8696.
Weinheimer, S.P. and McKnight, S.L. (1987). Transcription and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. J. Mol. Biol. 195,819-833.
Weir, H.M., Calder, J.M. and Stow, N.D. (1989). Binding of the herpes simplex virus type 1 UL9 gene product to an origin of viral DNA replication. Nucl. Acids Res. 17, 1409-1425.
Weir, H.M. and Stow, N.D. (1990). Two binding sites for the herpes simplex virus type 1 UL9 protein are required for efficient activity of the ori_S replication origin. J. Gen. Virol. 71, 1379-1385.
Weissbach, A., Hong, S.-C.L. and Aucker, J. (1973). Characterization of herpes simplex virus-induced deoxyribonucleic acid polymerase. J. Biol. Chem. 248, 6270-6277.
Weller, S.K., Aschman, D.P., Sacks, W.R., Coen, D.M. and Schaffer, P.A. (1983a). Genetic analysis of temperature-sensitive mutants of HSV-1: the combined use of complementation and physical mapping for cistron assignment. Virology 130, 290-305.
Weller, S.K., Lee, K.J., Sabourin, D.J. and Schaffer, P.A. (1983b). Genetic analysis of ts mutants which define the gene for the major HSV-1 DNA-binding protein. J. Virol. 45, 354-366.
Weller, S.K., Carmichael, E.P., Aschmand, P., Goldstein, D.J. and Schaffer, P.A. (1987). Genetic and phenotypic characterization of mutants in four essential genes that map to the left half of HSV-1 U_L DNA. Virology 161, 198-210.
Weller, S.K., Seghatoleslami, M.R., Shao, L., Rowse, D. and Carmichael, E.P. (1990). The herpes simplex virus type 1 alkaline nuclease is not essential for viral DNA synthesis: isolation and characterization of a lacZ insertion mutant. J. Gen. Virol. 71, 2941-2952.
Weller, S.K., Spadaro, A., Schaffer, J.E., Murray, A.W., Maxam, A.M. and Schaffer, P.A. (1985). Cloning, sequencing and functional analysis of ori_L, a herpes simplex virus type 1 origin of DNA synthesis. Mol. Cell. Biol. 5, 930-942.
Werstuck, G., and Capone, J.P. (1989a). Mutational analysis of the herpes simplex virus trans-inducing factor Vmw65. Gene 75, 213-224.
Werstuck, G., and Capone, J.P. (1989b). Identification of a domain of the herpes simplex virus trans-activator Vmw65 required for protein-DNA complex formation through the use of protein A fusion proteins. J. Virol. 63, 5509-5513.
Westrate, M.W., Geelen, J.L.M.C. and Van der Noordaa, J. (1980). Human cytomegalovirus DNA: physical maps for the restriction endonucleases BgII, HindIII, XbaI. J. Gen. Virol. 49, 1-21.
Whitley, R.J. (1985). Epidemiology of herpes simplex viruses. In: The Herpesviruses, Vol III. (Ed. B. Roizman), pp 1-44. Plenum Press.
Whitton, J.L. and Clements, J.B. (1984a). The junctions between the repetitive and the short unique sequences of the herpes simplex virus genome are determined by the polypeptide-coding regions of two spliced immediate-early mRNAs. J. Gen. Virol. 65, 451-460.
Whitton, J.L. and Clements, J.B. (1984b). Replication origins and a sequence involved in coordinate induction of the IE gene family are

conserved in an intergenic region of herpes simplex virus. Nucl. Acids Res. 12, 2061-2079.
Whitton, J.L., Rixon, F.J., Easton, A.E. and Clements, J.B. (1983). Immediate-early mRNA-2 of herpes simplex viruses types 1 and 2 is unspliced; conserved sequences around the 5' and 3' termini correspond to transcription regulatory signals. Nucl. Acids Res. 11, 6271-6287.
Wigdahl, B.L., Scheck,A.C., DeClerq, E., and Rapp, F. (1982). High efficiency latency and activation of herpes simplex virus in human cells. Science 217, 1145-1146.
Wilcock, D. and Lane, D.P. (1991). Localization of p53 retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. Nature 349, 429-431.
Wilcox, K.W., Kohn, A., Skylanskay, E. and Roizman, B. (1980). Herpes simplex virus phosphoproteins. I. Phosphate cycles on and off some viral polypeptides and can alter their affinity for DNA. J. Virol. 33, 167-182.
Wildy, P., Russell, W.C. and Horne, R.W. (1960). The morphology of herpes virus. Virology 12, 204-222.
Wildy, P. and Watson, D.H. (1962). Electron microscopic studies on the architecture of animal viruses. Cold Spring Harbor Symp. Quant. Biol. 27, 25-47.
Wilkie, N.M. (1973). The synthesis and substructure of herpesvirus DNA: the distribution of alkali-labile single strand interruptions in HSV-1 DNA. J. Gen. Virol. 453-467.
Wilkie, N.M. and Cortini, R. (1976). Sequence arrangement in herpes simplex virus type 1 DNA: identification of terminal fragments in restriction endonuclease digests and evidence for inversions in redundant and unique sequences. J. Virol. 20, 211-221.
Wobbe, C.R., Dean, F.B., Murakami, Y., Weissbach, L. and Hurwitz, J. (1985). In vitro replication of duplex circular DNA containing the SV40 DNA origin site. Proc. Natl. Acad. Sci. USA 83, 5710-5714.
Wohlrab, F. and Francke, B. (1980). Deoxyribopyrimidine triphosphatase activity specific for cells infected with herpes simplex virus type 1. Proc. Natl. Acad. Sci. USA 77, 1872-1876.
Wold, M.S. and Kelly, T. (1988). Purification and characterization of replication protein A, a cellular protein required for an in vitro replication of simian virus 40 DNA. Proc. Natl. Acad. Sci. USA 85, 2523-2527.
Wold, M.S., Li, J., Weinberg, D.H., Virshup, D.M., Sherley, J.L., Verheyen, E. and Kelly, T. (1988). Cellular proteins required for SV40 DNA replication in vitro. In: Cancer Cells 6: Eukaryotic DNA Replication. (Eds. T. Kelly and B. Stillman) Cold Spring Harbor Laboratory, N.Y. pp133-141.
Wong, S.M., Wahl, A.F., Yuan, P.-M., Arai, N., Pearson, B.E., Arai, K.-I., Korn, D., Hunkapiller, M.W. and Wang, T.S.F. (1988). Human DNA polymerase alpha gene expression is cell proliferation dependent and its primary structure is similar to both prokaryotic and eukaryotic replicative DNA polymerases. EMBO J. 7, 37-47.
Wong, S.W. and Schaffer, P.A. (1991). Elements in the transcriptional regulatory region flanking herpes simplex virus type 1 oriS stimulate origin function. J. Virol. 65, 2601-2611.
Worrad, D.M. and Caradonna, S. (1988). Identification of the herpes simplex virus uracil-DNA glycosylase. J. Virol. 62, 4774-4777.
Wu, C.A., Nelson, N.J., McGeoch, D.J. and Challberg, M. D. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. J. Virol. 62, 435-443.
WuDunn, D. and Spear, P.G. (1989). Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. J. Virol. 63, 52-58.
Wymer, J.P., Chung, T.D., Chang, Y.-N., Hayward, G.S. and Aurelian, L. (1989). Identification of immediate-early-type cis-response elements

in the promoter for the ribonucleotide reductase large subunit from herpes simplex virus type 2. J. Virol. 63, 2773-2784.
Yamanishi, K., Shiraki, K., Kondo, T., Okuno, T., Takahashi, M., Asano, Y. and Kurata, T. (1988). Identification of human herpes virus-6 as a causal agent for exanthem subitum. Lancet i, 1065-1067.
Yarranton, G.T. and Gelter, M.L. (1979). Enzyme-catalyzed DNA unwinding: studies on *Eschericia coli* rep protein. Proc. Natl. Acad. Sci. USA 76, 1658-1662.
Zhang, Y., Sirko, D.A. and McKnight, J.L.C. (1991). Role of herpes simplex virus type 1 UL46 and UL47 in αTIF-mediated transcriptional induction: characterisation of three viral deletion mutants. J. Virol. 65, 829-841.
Zhang, Y.-F. and Wagner, E.K. (1987). The kinetics of expression of individual herpes simplex virus type 1 transcripts. Virus Genes 1, 49-60.
Zhu, L. and Weller, S.K. (1988). UL5, a protein required for HSV DNA synthesis: genetic analysis, overexpression in *Escherichia coli*, and generation of polyclonal antibodies. Virology 166, 366-378.
Zhu, X., Chen, J. and Silverstein, S. (1991). Isolation and characterization of a functional cDNA encoding ICP0 from herpes simplex virus type 1. J. Virol. 65, 957-960.
Zyskind, J.W., Cleary, J.M., Brusilow, W.S.A., Harding, N.E. and Smith, D.W. (1983). Chromosomal replication origin from the marine bacterium *Vibrio harveyi* functions in *Escherichia coli*: oriC consensus sequence. Proc. Natl. Acad. Sci. USA 80, 1164-1168.

J. Gen. Virol. in press.

ON THE CELLULAR LOCALIZATION OF THE COMPONENTS OF THE HERPES SIMPLEX VIRUS TYPE 1 HELICASE-PRIMASE COMPLEX AND THE VIRAL ORIGIN-BINDING PROTEIN

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SUMMARY

We have constructed virus recombinants based upon the herpes simplex virus type 1 (HSV-1) mutant *ts*K which are individually able to express the products of four viral DNA replication genes (*UL5*, *UL8*, *UL9* and *UL52*) in the absence of any of the other proteins required for viral DNA synthesis. These viruses have been used in immunofluorescence experiments to investigate the cellular localization of the four expressed replication proteins. The results demonstrate that all three components of the viral helicase-primase complex (UL5, UL8 and UL52 proteins) must be co-expressed to allow their efficient localization to the nucleus. Since the UL5 and UL52 proteins together form a complex which is enzymatically indistinguishable from a complex formed from all three proteins, a possible role of the UL8 protein may be in facilitating nuclear uptake. The UL9 protein (origin-binding protein) efficiently entered the cell nucleus when expressed alone. Both UL9 protein and the tri-partite helicase-primase complex exhibited patterns of fluorescence which resembled the previously described "pre-replicative sites".

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) genes *UL5*, *UL8*, *UL9*, *UL29*, *UL30*, *UL42*, and *UL52* encode a set of seven proteins which have essential roles in viral DNA replication. Each of these genes identifies a complementation group whose members exhibit defects in viral DNA synthesis (reviewed by Challberg & Kelly, 1989; Weller, 1991). Moreover, in a transient assay in transfected tissue culture cells, the seven genes are both necessary and sufficient for viral origin-dependent DNA replication (Wu *et al.*, 1988; Heilbronn & zur Hausen, 1989). Genes *UL30* and *UL42* encode the catalytic and accessory subunits of the viral DNA polymerase (Gallo *et al.*, 1989; Hernandez & Lehman, 1990; Gottlieb *et al.*, 1990). A single-stranded DNA binding protein is specified by the *UL29* gene (Weller *et al.*, 1983; Quinn & McGeoch, 1985), and the product of the *UL9* gene binds to specific DNA sequences within the viral origins of replication (Olivo *et al.*, 1988; Weir *et al.*, 1989). The proteins encoded by genes *UL5*, *UL8*, and *UL52* form a complex in HSV-1 infected cells which exhibits DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities (Crute *et al.*, 1988; 1989).

The UL5, UL8, and UL52 proteins have been expressed by recombinant baculoviruses and shown to assemble into a functional complex in triply infected insect cells (Dodson *et al.*, 1989; Calder & Stow, 1990). Although no enzymatic activity has been attributed to any of the individual subunits of the helicase-primase complex, it has been shown that a sub-assembly of the UL5 and UL52 proteins formed in doubly infected insect cells exhibits each of the activities associated with the UL5/UL8/UL52

complex (Calder & Stow, 1990; Dodson & Lehman, 1991). Therefore, despite its well documented requirement for viral DNA synthesis (Wu *et al.*, 1988; Carmichael and Weller, 1989), no role can yet be assigned to the UL8 protein.

We previously constructed four recombinant viruses, *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 in which additional copies of the *UL5*, *UL8*, *UL9* or *UL52* gene had been inserted into the genome of the HSV-1 temperature-sensitive mutant *ts*K under the control of the immediate early (IE) gene 3 promoter (Weir *et al.*, 1989). At the non-permissive temperature *ts*K accumulates IE polypeptides but fails to induce the expression of early and late genes (Preston, 1979; Watson & Clements, 1980). Under these conditions the recombinant viruses over-express the product of the inserted gene but do not synthesize any of the other DNA replication proteins since these remain under their normal early control. In this paper we report the use of the recombinant viruses to investigate whether, in the absence of other DNA replication proteins, the UL5, UL8, UL9, and UL52 proteins localize to the nucleus of mammalian tissue culture cells. The results suggest that the UL8 protein may play a role in facilitating efficient nuclear entry of the helicase-primase complex.

METHODS

Cells and Viruses

Baby hamster kidney 21 clone 13 (BHK) cells (MacPherson & Stoker, 1962) were grown in Eagle's medium supplemented with 10% tryptose phosphate broth and 10% newborn calf serum. The viruses used were the HSV-1 temperature sensitive mutant *ts*K (Preston, 1979) and recombinant viruses *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 (Weir *et al.*, 1989). These viruses contain additional copies of the early genes *UL5*, *UL8*, *UL9* and *UL52* inserted under the control of the immediate early (IE) gene 3 promoter within the thymidine kinase gene of *ts*K, and were constructed using an approach similar to that described by Hummel *et al.* (1986). Briefly, plasmid p23, a gift of Dr C Preston, contains a 361 bp fragment (nucleotides 131759-131399; McGeoch *et al.*, 1988a) specifying the IE gene 3 promoter, 5' end and upstream regulatory sequences inserted at nucleotide 47358 within the TK gene coding region of the HSV-1 *Bam*HI p fragment. The inserted fragment is orientated so that transcription from the IE gene 3 promoter is in the same direction as the TK gene, and therefore the TK gene mRNA processing signals are potentially available for any transcript expressed from the IE3 promoter. Plasmid p23 contains a unique *Xho*I site immediately downstream of the IE3 promoter into which were inserted, in the correct orientation, DNA fragments containing the complete *UL5*, *UL8*, *UL9* and *UL52* ORFs. The fragments inserted correspond to nucleotides 15166-12128, 20492-17855, 23542-20670 and 108969-112514 respectively. The recombinant plasmids were co-transfected into BHK cells with intact *ts*K DNA and incubated at

the permissive temperature (31°C). TK^- virus progeny containing additional copies of the *UL5*, *UL8*, *UL9* and *UL52* genes inserted into the TK locus were enriched by growth in the presence of 100 μg/ml bromodeoxycytidine and purified. The structures of the recombinant virus genomes were confirmed by restriction endonuclease digestion and Southern blotting using appropriate probes. Stocks of the parental virus *ts*K and the resulting recombinant viruses *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 and *ts*K/UL52 were propagated in BHK cells at 31°C.

Preparation and Analysis of [^{35}S]methionine-labelled polypeptides

BHK cells in the 15 mm diameter wells of multi-well plates were mock-infected or infected with 10 p.f.u./cell of *ts*K or the recombinant viruses and incubated at 38.5°C for 6 h. The medium was then replaced with 200 μl PBS containing 15 μCi [^{35}S]methionine (Amersham; sp. act. >800 Ci/mmol). Incubation was continued for a further 2.5 h at 38.5°C. At the end of the labelling period the supernatant was removed and the cultures lysed and harvested as described by Marsden *et al.* (1978). Samples were analysed by electrophoresis through an SDS-polyacrylamide gel containing 9% acrylamide cross-linked with 1 part in 40 (w/w) N,N'-methylene bisacrylamide. Gel fixation, drying and autoradiography were as previously described (Marsden *et al.*, 1979).

Immunofluorescence

Sparse monolayers of BHK cells ($4x10^5$ cells) in 35 mm plastic petri dishes were infected with 10^7 p.f.u./dish of *ts*K or each recombinant virus as indicated and incubated at 38.5°C. (Because the monolayers were only approximately 25% confluent the actual

input multiplicity for each virus is likely to have been less than 25 p.f.u./cell). 9 h post-infection the cells were washed with PBS and fixed for 2 min with a 1:1 methanol/acetone mixture. The monolayers were further washed with PBS and allowed to become almost dry. A 50 μl drop of a 1 in 30 dilution (in PBS containing 1% foetal calf serum) of the primary antibody was applied to a marked region in the middle of the dish. The primary antisera were prepared in rabbits against synthetic decapeptides from the C-termini of the UL5, UL8, UL9 and UL52 proteins (Olivo *et al.*, 1989) and were the kind gift of Dr M D Challberg. After 1 h at room temperature, the monolayers were washed four times with PBS and similarly reacted with a 1 in 80 dilution of FITC-conjugated goat anti-rabbit IgG (Sigma). The cells were washed once with 10% sucrose, 0.5% NP40 in PBS and four times with PBS, and examined using a Leitz UV fluorescence microscope. Photographs were taken on Tri-X Pan 400 film with 25x or 40x objective lenses. Exposure times and printing conditions were kept constant for all the samples within any one experiment.

RESULTS

Polypeptide synthesis in cells infected with *ts*K recombinant viruses

Recombinant viruses in which additional copies of the HSV-1 replication genes *UL5*, *UL8*, *UL9* and *UL52* had been individually inserted under immediate-early control into the *ts*K genome were generated as described in Methods. At the NPT *ts*K over-expresses immediate-early (IE) genes, but, with the exception of Vmw136, the product of "psuedo-IE" gene *UL39* (Wymer *et al.*, 1989), does not synthesize any of the remaining early or late viral proteins (Preston, 1979). Since *UL5*, *UL8*, *UL9* and *UL52* are all early genes (Olivo *et al.*, 1989), the copies inserted under IE control, but not the normally regulated resident copies, should be expressed at NPT.

The polypeptides synthesized at 38.5°C in mock-infected BHK cells or cells infected with *ts*K or the four recombinant viruses *ts*K/UL5, *ts*K/UL8, *ts*K/UL9 or *ts*K/UL52 were labelled and analysed as described in Methods. Figure 1 demonstrates that in addition to the major viral polypeptides detected in *ts*K-infected cells (IE proteins Vmw175, Vmw110, Vmw68 and Vmw63 plus Vmw136), the recombinant viruses each produce a single novel species. Several observations confirm these novel proteins to be the products of the inserted *UL5*, *UL8*, *UL9* and *UL52* ORFs. First, their apparent molecular weights approximate to the values of 98710, 79921, 94246 and 114,416 predicted from DNA sequence data for the UL5, UL8, UL9 and UL52 proteins, respectively (McGeoch *et al.*, 1988b). Second, the proteins co-migrate on gels with the products expressed from the same ORFs in baculovirus vectors (unpublished results).

Third, they react with antisera raised against peptides predicted from the DNA sequence of the ORFs (immunofluorescence data presented in this paper, and unpublished results of immunoprecipitation experiments).

Immunofluorescence studies on the UL5, UL8 and UL52 proteins synthesized by recombinant viruses

The ability of the *ts*K recombinant viruses to synthesize the UL5, UL8, UL9 and UL52 proteins at NPT allowed examination of their localization in a cell line permissive for HSV-1 replication when expressed in isolation from the other DNA replication proteins. Because the UL5, UL8 and UL52 proteins exist as a complex in HSV-1 infected cells we also performed experiments expressing these proteins either individually or in all possible combinations and examined the cellular location of the expressed proteins by immunofluorescence.

The results for single infections are shown in Figure 2. Replicate sets of sparse monolayers of BHK cells were infected at NPT with *ts*K, *ts*K/UL5, *ts*K/UL8 or *ts*K/UL52 and subsequently processed as described in Methods. One set was reacted with anti-UL5 antibody (panels a-d), a second set with anti-UL8 antibody (panels e-h) and a third with anti-UL52 antibody (panels i-l). Following incubation with FITC-conjugated goat anti-rabbit IgG the cells were examined by UV fluorescence microscopy. The anti-UL5 antibody gave a weak background staining with the *ts*K, *ts*K/UL8 and *ts*K/UL52 infected cells (panels a, c and d) but a much brighter cytoplasmic fluorescence was apparent in the cells infected with recombinant *ts*K/UL5 (panel b). Similarly, the anti-UL52 antibody reacted specifically with cells

infected with *ts*K/UL52 revealing a bright cytoplasmic fluorescence (panel l). The pattern observed was more discrete than in panel b, and distinct, predominantly perinuclear, foci were apparent in the majority of cells. The result was less clear-cut with the anti-UL8 antibody but nevertheless a slightly increased intensity of fluorescence was apparent in the *ts*K/UL8 infection (panel g). The specificity of the reaction between the anti-UL8 antibody and *ts*K/UL8 infected cells is also demonstrated in Figure 3, panels f and g. These results therefore indicate that the anti-UL5, anti-UL8 and anti-UL52 antibodies are each capable of specifically detecting the corresponding gene product in cells infected with the *ts*K recombinant viruses. Furthermore, they demonstrate that the UL5, UL8 and UL52 proteins, when expressed individually in the absence of any other HSV-1 DNA replication protein, do not localize to the cell nucleus, the site of viral DNA synthesis (Rixon *et al.*, 1983) and the location of these proteins during the normal course of HSV-1 infection (Olivo *et al.*, 1989). In additional experiments, which serve as controls for the mixed infections described below, it was found that increasing the virus inoculum two- or three-fold did not significantly affect either the pattern or intensity of fluorescent staining (data not shown).

To investigate whether co-expression of the UL5, UL8 and UL52 proteins affected cellular localization, mixedly infected cells were similarly examined by immunofluorescence microscopy. The results are shown in Figure 3. UL5 protein was predominantly cytoplasmic when expressed alone or in combination with UL8 protein (panels b and c). In the presence of UL52 protein a slightly increased proportion appeared to enter the nucleus

(panel d), but only in the presence of both UL8 and UL52 proteins was efficient localization to the nucleus detected (panel e). Similarly, co-expression of all three proteins was necessary for efficient nuclear localization of the UL8 and UL52 proteins (panels j and o). The UL8 protein, however, appeared to exhibit a small increase in nuclear uptake in the presence of UL52 alone, and the UL52 protein exhibited a similar response in the presence of either UL5 or UL8 (panels i, m and n). These results indicate a mutual dependence amongst the UL5, UL8 and UL52 proteins for fully efficient nuclear localization, suggesting that they may be transported into the nucleus as a complex. In cells co-expressing UL52 and either UL5 or UL8 there appeared to be a small enhancement of nuclear uptake of both proteins, although this may reflect the presence of protein which is on the surface rather than inside the nucleus. The observation is nevertheless consistent with some form of interaction between these pairs of polypeptides in the doubly infected cells.

Cellular localization of the UL9 polypeptide

Recombinant virus *ts*K/UL9 was similarly used to determine the cellular localization of the UL9 polypeptide when expressed in the absence of other HSV-1 DNA replication proteins. Sparse monolayers of BHK cells were infected at NPT with *ts*K, *ts*K/UL9 or a combination of *ts*K/UL5, *ts*K/UL8 and *ts*K/UL52. Duplicate plates were stained with either anti-UL9 antibody or anti-UL52 antibody and examined by immunofluorescence. The results are shown in Figure 4. The anti-UL9 antibody reacted efficiently with only the *ts*K/UL9 infected cells (panel b), demonstrating the specificity of the antibody for the UL9 polypeptide, and revealed specific

localization of the UL9 protein within the nucleus. The cells exhibited a speckled staining pattern with distinct foci which was very similar to the pattern exhibited by UL52 protein in the triply infected cells (panel f). This result indicates that the UL9 protein, in contrast to the individually expressed UL5, UL8 and UL52 proteins, efficiently enters the nucleus in the absence of any other viral DNA replication protein.

DISCUSSION

We have utilised temperature-sensitive HSV-1 recombinant viruses to study the localization of four viral DNA replication proteins UL5, UL8, UL9 and UL52 in a cell type permissive for virus growth. The parental virus, *tsK* , is very tightly blocked at the IE stage of infection (Preston, 1979; Watson & Clements, 1980), and the recombinants differ from it at the non-permissive temperature (NPT) only by additionally expressing the products of the inserted genes. The observation that HSV-1 IE proteins are dispensable for viral DNA synthesis in transfected cells (Heilbronn & zur Hausen, 1989) further suggests that the proteins expressed by *tsK* at NPT are unlikely to have an important role in determining the localization of the viral DNA replication proteins. Our results show that of the UL5, UL8, UL9 and UL52 proteins only UL9, when individually expressed, is able to enter the cell nucleus. Nuclear uptake of the UL9 protein therefore appears to be independent of any of the other components of the viral DNA replication machinery, and it is quite likely that the UL9 polypeptide contains a specific nuclear localization sequence (reviewed by Silver, 1991).

In HSV-1 infected cells the UL5, UL8 and UL52 proteins interact to form the viral helicase-primase complex (Crute *et al.*, 1989), and our results demonstrate that all three proteins of the complex must be present to allow efficient nuclear uptake of any one. The most probable explanation is that the three proteins interact in the cytoplasm to form a complex which presents a signal for nuclear localization which is not recognizable on any of the individual subunits. This could potentially be present on

one of the subunits but masked, or in an inappropriate conformation until complex formation occurs. Alternatively, the recognized signal may be distributed over more than one subunit. The electrophoretic mobilities of the UL5, UL8 and UL52 proteins did not differ between singly and triply infected cells (data not shown) suggesting that post-translational polypeptide processing is unlikely to have a determining role in nuclear transport.

Recent experiments in which the UL5, UL8, and UL52 proteins were expressed in various combinations in insect cells using recombinant baculoviruses have demonstrated that the UL5 and UL52 proteins together can form a complex which exhibits all the enzymatic activities associated with the UL5/UL8/UL52 complex found in HSV-1 infected cells (Calder & Stow, 1990; Dodson & Lehman, 1991). Moreover, the UL5/UL8/UL52 and UL5/UL52 complexes purified from infected insect cells are virtually indistinguishable in their enzymatic properties (Dodson & Lehman, 1991). Experiments using the *ts*K recombinant viruses yielded results very similar to those reported previously for the recombinant baculoviruses (Calder & Stow, 1990) in so far as formation of a complex with an associated DNA dependent ATPase activity occurred in cells co-expressing either the UL5 and UL52 or the UL5, UL8 and UL52 proteins (J Calder & N Stow, unpublished observations). Since the UL8 protein is essential for viral DNA synthesis (Wu *et al.*, 1988; Carmichael & Weller, 1989) these data raise the question of its function. Our immunofluorescence results suggest that an important role of the UL8 protein may be to facilitate entry into the nucleus of the enzymatically active subunits (ie the UL5 and UL52 proteins). This, however, seems rather unlikely to be its sole function, since nuclear targetting

of proteins is readily achievable with signals consisting of short sequences relatively rich in basic amino acids (reviewed by Silver, 1991). The UL8 protein might also be involved in interacting with other components of the replicative machinery (eg the origin-binding protein or the viral DNA polymerase) or modulating the helicase and primase activities on their natural template, the viral genome. It is interesting to note that although Epstein-Barr virus and human cytomegalovirus (human herpesviruses belonging to the gamma- and beta-herpesvirus subfamilies, respectively) encode obvious homologues of the HSV-1 UL5 and UL52 proteins, they do not contain ORFs showing significant homology to UL8 (McGeoch *et al.*, 1988b; Chee *et al.*, 1990). Whether, in these viruses, the UL8 homologue has diverged to such an extent as to be unrecognizable, or whether its function is performed by another viral protein (including possibly the UL5 and/or UL52 homologue) or a host factor is not known.

When expressed using the *ts*K recombinant viruses the UL9 protein and the UL5/UL8/UL52 complex exhibited similar nuclear staining patterns in which protein was detected in quite numerous small foci (Figure 4). This pattern resembles the arrangement of of "pre-replicative sites" to which the UL29 product, the major DNA binding protein, localizes when viral DNA synthesis is blocked by the addition of phosphonoacetic acid (Quinlan *et al.*, 1984). When viral DNA synthesis is permitted UL29 protein migrates to the larger replication compartments where viral genome replication occurs (Rixon *et al.*, 1983; Quinlan *et al.*, 1984). Replicating host cell DNA and a number of host cell proteins have also been shown to relocate to the pre-replicative sites or replication compartments following infection with HSV-1 (de Bruyn Kops &

Knipe, 1988; Wilcock & Lane, 1991). Moreover, UL29 protein expressed from a transfected plasmid exhibits the "pre-replicative site" pattern (Quinlan & Knipe, 1985), suggesting that this protein may play an important role in organizing the viral replicative machinery and determining the sites at which viral genome replication will occur (reviewed by Knipe, 1989). Further experiments are necessary to determine whether the sites to which the UL9 protein and the helicase-primase complex localize are the same, and whether they are functionally equivalent to the pre-replicative sites defined by the presence of UL29 protein. Although recent studies suggest that the presence of functional UL29 protein is important for specific localization of the UL42 protein and viral DNA polymerase (Goodrich *et al*, 1990; Bush *et al*, 1991), it is possible that other HSV-1 replication proteins may have an intrinsic ability to locate to appropriate sites. It may also be possible to extend the approaches used in this paper in order to elucidate the minimum requirement of viral proteins for the intranuclear redistribution of replicating host cell DNA and host proteins described above.

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REFERENCES

BUSH, M., YAGER, D.R., GAO, M., WEISSHART, K., MARCY, A.I., COEN, D.M. & KNIPE, D.M. (1991). Correct intranuclear localization of herpes simplex virus DNA polymerase requires the viral ICP8 DNA-binding protein. *Journal of Virology* **65**, 1082-1089.

CALDER, J.M. & STOW, N.D. (1990). Herpes simplex virus helicase-primase: the UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities. *Nucleic Acids Research* **18**, 3573-3578.

CARMICHAEL, E.P. & WELLER, S.K. (1989). Herpes simplex virus type 1 DNA synthesis requires the product of the UL8 gene: isolation and characterization of an ICP6::*lacZ* insertion mutation. *Journal of Virology* **63**, 591-599.

CHALLBERG, M.D. & KELLY, T.J. (1989). Animal virus DNA replication. *Annual Review of Biochemistry* **58**, 671-717.

CHEE, M.S., BANKIER, A.T., BECK, S., BOHNI, R., BROWN, C.M., CERNY, R., HORSNELL, T., HUTCHISON, C.A. III, KOUZARIDES, T., MARTIGNETTI, J.A., PREDDIE, E., SATCHWELL, S.C., TOMLINSON, P., WESTON, K.M. & BARRELL, B.G. (1990). Analysis of the protein-coding content of the sequence of human cytomegalovirus strain AD169. *Current Topics in Microbiology and Immunology* **154**, 125-169.

CRUTE, J.J., MOCARSKI, E.S. & LEHMAN, I.R. (1988). A DNA helicase induced by herpes simplex virus type 1. *Nucleic Acids Research* **16**, 6585-6596.

CRUTE, J.J., TSURUMI, T., ZHU, L., WELLER, S.K., OLIVO, P.D., CHALLBERG, M.D., MOCARSKI, E.S. & LEHMAN, I.R. (1989). Herpes

simplex virus 1 helicase-primase: a complex of three herpes-encoded gene products. *Proceedings of the National Academy of Sciences, USA* **86**, 2186-2189.

DE BRUYN KOPS, A., & KNIPE, D.M. (1988). Formation of DNA replication structures in herpes virus-infected cells requires a viral DNA binding protein. *Cell* **55**, 857-868.

DODSON, M.S. & LEHMAN, I.R. (1991). Association of DNA helicase and primase activities with a subassembly of the herpes simplex virus 1 helicase-primase composed of the UL5 and UL52 gene products. *Proceedings of the National Academy of Sciences, USA* **88**, 1105-1109.

DODSON, M.S., CRUTE, J.J., BRUCKNER, R.C & LEHMAN, I.R. (1989). Overexpression and assembly of the herpes simplex virus type 1 helicase-primase in insect cells. *Journal of Biological Chemistry* **264**, 20835-20838.

GALLO, M.L., DORSKY, D.I., CRUMPACKER, C.S. & PARRIS, D.S. (1989). The essential 65-kilodalton DNA-binding protein of herpes simplex virus stimulates the virus-encoded DNA polymerase. *Journal of Virology* **63**, 5023-5029.

GOODRICH, L.D., SCHAFFER, P.A., DORSKY, D.I., CRUMPACKER, C.S. & PARRIS, D.S. (1990). Localization of the herpes simplex virus type 1 65-kilodalton DNA-binding protein and DNA polymerase in the presence and absence of viral DNA synthesis. *Journal of Virology* **64**, 5738-5749.

GOTTLIEB, J., MARCY, A.I., COEN, D.M. & CHALLBERG, M.D. (1990). The herpes simplex virus type 1 UL42 gene product: á subunit of DNA polymerase that functions to increase processivity. *Journal of Virology* **64**, 5976-5987.

HEILBRONN, R. & ZUR HAUSEN, H. (1989). A subset of herpes simplex virus replication genes induces DNA amplification within the host cell genome. *Journal of Virology* **63**, 3683-3692.

HERNANDEZ, T.R. & LEHMAN, I.R. (1990). Functional interaction between the herpes simplex-1 DNA polymerase and UL42 protein. *Journal of Biological Chemistry* **265**, 11227-11232.

HUMMEL, M., ARSENAKIS, M., MARCHINI, A., LEE, L., ROIZMAN, B. & KIEFF, E. (1986). Herpes simplex virus expressing Epstein-Barr virus nuclear antigen 1. *Virology* **148**, 337-348.

KNIPE, D.M. (1989). The role of viral and cellular nuclear proteins in herpes simplex virus replication. *Advances in Virus Research* **37**, 85-123.

McGEOCH, D.J., DALRYMPLE, M.A., DAVISON, A.J., DOLAN, A., FRAME, M.C., McNAB, D., PERRY, L.J., SCOTT, J.E. & TAYLOR, P. (1988a). The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1. *Journal of General Virology* **69**, 1531-1574.

McGEOCH, D.J., DALRYMPLE, M.A., DOLAN, A., McNAB, D., PERRY, L.J., TAYLOR, P. & CHALLBERG, M.D. (1988b). Structures of herpes simplex virus type 1 genes required for replication of virus DNA. *Journal of Virology* **62**, 444-453.

MacPHERSON, I. & STOKER, M. (1962). Polyoma transformation of hamster cell clones - an investigation of genetic factors affecting cell competence. *Virology* **16**, 147-151.

MARSDEN, H.S., STOW, N.D., PRESTON, V.G., TIMBURY, M.C. & WILKIE, N.M. (1978). Physical mapping of herpes simplex virus-induced polypeptides. *Journal of Virology* **28**, 624-642.

OLIVO, P.D., NELSON, N.J., & CHALLBERG, M.D. (1988). Herpes simplex virus DNA replication: the UL9 gene encodes an

origin-binding protein. *Proceedings of the National Academy of Sciences, USA* **85**, 5414-5418.

OLIVO, P.D., NELSON, N.J. & CHALLBERG, M.D. (1989). Herpes simplex virus type 1 gene products required for DNA replication: identification and overexpression. *Journal of Virology* **63**, 196-204.

PRESTON, C.M. (1979). Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant *ts*K. *Journal of Virology* **29**, 275-284.

QUINLAN, M.P., CHEN, L.B. & KNIPE, D.M. (1984). The intranuclear location of a herpes simplex virus DNA-binding protein is determined by the status of viral DNA replication. *Cell* **36**, 857-868.

QUINLAN, M.P. & KNIPE, D.M. (1985). A genetic test for expression of a functional herpes simplex virus DNA-binding protein from a transfected plasmid. *Journal of Virology* **54**, 619-622.

QUINN, J.P. & McGEOCH, D.J. (1985). DNA sequence of the region in the genome of herpes simplex virus type 1 containing the genes for DNA polymerase and the major DNA binding protein. *Nucleic Acids Research* **13**, 8143-8163.

RIXON, F.J., ATKINSON, M.A. & HAY, J. (1983). Intranuclear distribution of herpes simplex virus type 2 DNA synthesis: examination by light and electron microscopy. *Journal of General Virology* **64**, 2087-2092.

SILVER, P.A. (1991). How proteins enter the nucleus. *Cell* **64**, 489-497.

WATSON, R.J. & CLEMENTS, J.B. (1980). A herpes simplex virus type 1 function continuously required for early and late virus RNA synthesis. *Nature* **285**, 329-330.

WEIR, H.M., CALDER, J.M. & STOW, N.D. (1989). Binding of the herpes simplex virus type 1 UL9 gene product to an origin of viral DNA replication. *Nucleic Acids Research* **17**, 1409-1425.

WELLER, S.K. (1991). Genetic analysis of HSV genes required for genome replication. In *Herpesvirus transcription and its regulation*, pp. 105-135. Edited by E.K. Wagner. Boca Raton, CRC Press.

WELLER, S.K., LEE, K.J., SABOURIN, D.J. & SCHAFFER, P.A. (1983). Genetic analysis of temperature-sensitive mutants which define the gene for the major herpes simplex virus type 1 DNA-binding protein. *Journal of Virology* **45**, 354-366.

WILCOCK, D. & LANE, D.P. (1991). Localization of p53, retinoblastoma and host replication proteins at sites of viral replication in herpes-infected cells. *Nature* **349**, 429-431.

WU, C.A., NELSON, N.J., McGEOCH, D.J. & CHALLBERG, M.D. (1988). Identification of herpes simplex virus type 1 genes required for origin-dependent DNA synthesis. *Journal of Virology* **62**, 435-443.

WYMER, J.P., CHUNG, T.D., CHANG, Y-N., HAYWARD, G.S. & AURELIAN, L. (1989). Identification of immediate-early-type *cis*-response elements in the promoter of the ribonucleotide reductase large subunit from herpes simplex virus type 2. *Journal of Virology* **63**, 2773-2784.

FIGURE LEGENDS

Figure 1

Polypeptide synthesis in cells infected with the recombinant viruses. Polypeptides synthesized at NPT in mock-infected (MI) BHK cells or cells infected with *ts*K (K), *ts*K/UL5 (5), *ts*K/UL8 (8), *ts*K/UL9 (9), *ts*K/UL52 (52) were labelled and analysed as described in Methods. The *ts*K-induced polypeptides (Vmw175, Vmw136, Vmw110, Vmw68 and Vmw63) are indicated. Novel polypeptides synthesized by the recombinant viruses are marked with arrowheads.

Figure 2

Immunofluorescence microscopy of BHK cells infected with recombinant viruses expressing the UL5, UL8 or UL52 proteins. Cells were infected with *ts*K (panels a, e, i), *ts*K/UL5 (b, f, j), *ts*K/UL8 (c, g, k) or *ts*K/UL52 (d, h, l) and processed as described in Methods. The primary antibodies used were anti-UL5 (a-d), anti-UL8 (e-h) and anti-UL52 (i-l). The cells were photographed using a 25x objective; the edge of each square corresponds to 0.15 mm.

Figure 3

Immunofluorescence microscopy of cells expressing various combinations of the UL5, UL8 and UL52 proteins. Cells were infected with 10^7 p.f.u./dish of each of the following viruses and processed as described in Methods: *ts*K (panels a, f, k); *ts*K/UL5

(b); *ts*K/UL8 (g); *ts*K/UL52 (l); *ts*K/UL5 plus *ts*K/UL8 (c, h); *ts*K/UL5 plus *ts*K/UL52 (d, m); *ts*K/UL8 plus *ts*K/UL52 (i, n) or *ts*K/UL5 plus *ts*K/UL8 plus *ts*K/UL52 (e, j, o). The antibodies used were anti-UL5 (a-e), anti-UL8 (f-j) and anti UL52 (k-o). The cells were photographed using a 25x objective; the edge of each square corresponds to 0.15mm.

Figure 4

Immunofluorescence microscopy of BHK cells expressing the UL9 protein. Cells were infected as described in the legend to Figure 3 with *ts*K (a, d), *ts*K/UL9 (b, c) or *ts*K/UL5 plus *ts*K/UL8 plus *ts*K/UL52 (c,f). The antibodies used were anti-UL9 (a-c) or anti-UL52 (d-f). The cells were photographed using a 40x objective; the shorter edges of the rectangular panels correspond to 0.11 mm.

Fig 1

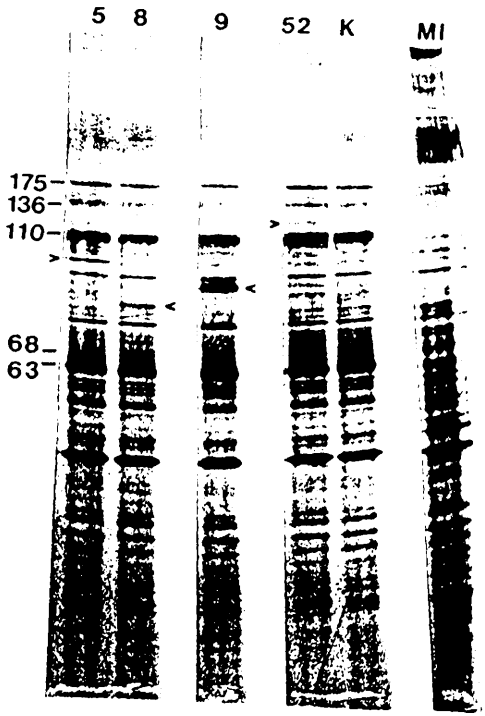


Fig 2 a, b c d
e f g h
i j k l

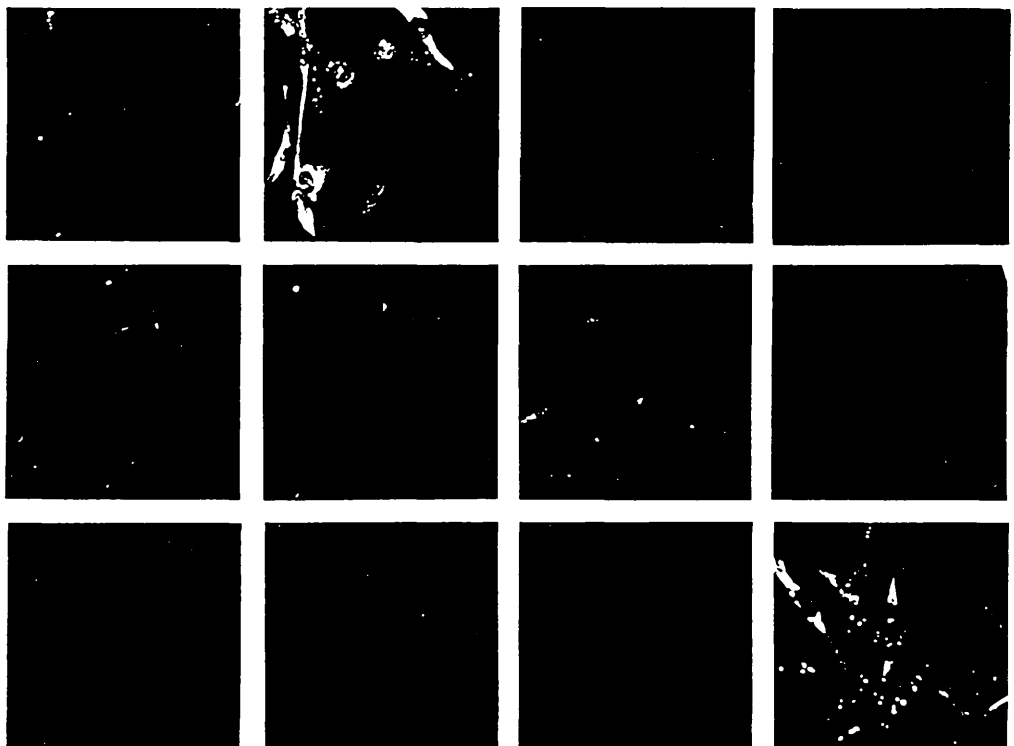
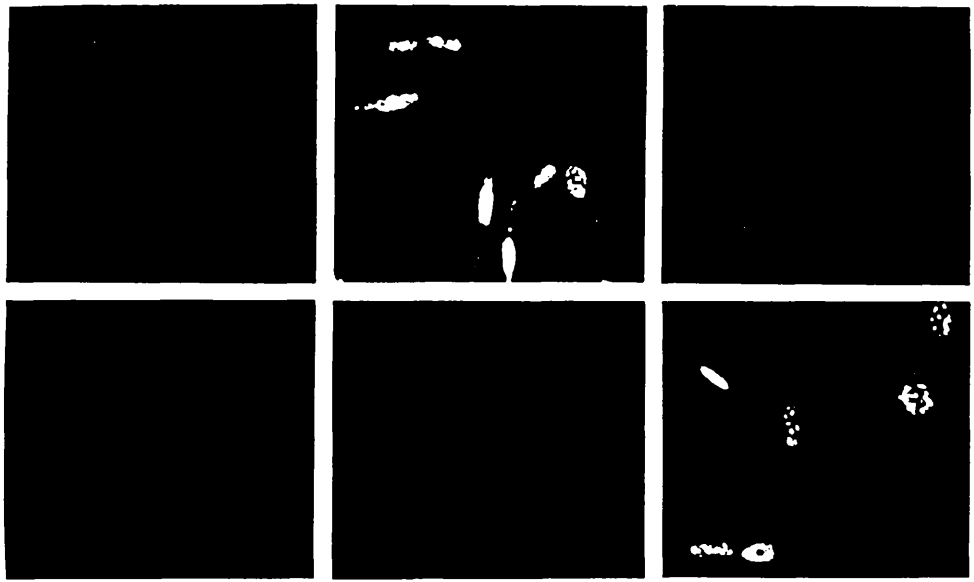


Fig 3

Fig 4 a b c
d e f



Binding of the herpes simplex virus type 1 UL9 gene product to an origin of viral DNA replication

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ABSTRACT

The binding of a herpes simplex virus type 1 (HSV-1) encoded polypeptide to a viral origin of DNA replication has been studied by using a gel retardation assay. Incubation of nuclear extract from HSV-1 infected cells with a labelled origin-containing fragment resulted in the formation of a specific retarded complex, the migration of which was further reduced in the presence of an antibody reactive with the UL9 gene product. Introduction of an additional copy of the UL9 gene, under the control of an immediate early (IE) promoter, conferred the ability to express origin binding activity at the non-permissive temperature upon an HSV-1 ts mutant blocked at the IE stage of infection. Endogenous or exogenous proteolytic activity revealed the presence of a relatively protease-resistant domain which retained sequence-specific DNA binding activity. The C-terminal 317 amino acids of the UL9 gene expressed as a fusion protein in Escherichia coli also bound to the origin. Our results demonstrate that the UL9 gene product binds to a viral origin and that sequence specific recognition and binding are specified by the C-terminal 37% of the polypeptide.

INTRODUCTION

The herpes simplex virus type 1 (HSV-1) genome is a linear double-stranded DNA of approximately 152 kb. Replication of this genome requires the presence of cis-acting elements which function as origins of DNA replication and of virus-encoded trans-acting proteins which perform essential roles in DNA synthesis.

The complete sequence of the HSV-1 genome has been determined and open reading frames capable of encoding 70 distinct polypeptides identified (1). Two approaches have indicated that the products of seven of these genes (UL5, UL8, UL9, UL29, UL30, UL42 and UL52) are involved in viral DNA

replication. Firstly, genetic analyses of HSV-1 mutants have shown that seven complementation groups containing mutants which exhibit defects in DNA synthesis map to these genes (2-10). Secondly, Challberg and his colleagues have demonstrated, using a plasmid amplification assay for HSV-1 origin-dependent DNA replication, that the products of these seven genes are both necessary and sufficient for DNA synthesis (11-13). Three of these seven DNA replication genes encode previously well-characterized proteins; the major single-strand-specific DNA binding protein is encoded by UL29 (3,14), the viral DNA polymerase by UL30 (14,15), and a 65,000 molecular weight protein which binds sequence-independently to double stranded DNA by UL42 (16,17). The products of the other four genes (UL5, UL8, UL9 and UL52) have not been so well characterized and are presumed to be present in much lower abundance in infected cells.

The viral origins of DNA replication are specified by two distinct but related sequences. One (ori_L) lies close to the centre of the long unique (U_L) region whilst the other (ori_S) is present within the inverted repeats, TR_S and IR_S (18-21 and Figure 1a). DNA fragments of 100 bp or less which specify a functional ori_S have been identified (22-24) and include a 45 bp near perfect palindrome with a central A+T-rich region. Although the sequence specifying ori_L contains a much longer (144 bp) palindrome there is high sequence similarity between the two origins covering the region of the ori_S palindrome and approximately 40 bp to one side of it.

Using a DNase I footprinting assay Elias and his colleagues (25,26) have demonstrated the presence of two specific binding sites for a virus-induced polypeptide within ori_S. Site I consists of an 18 bp region which overlaps the left end of the ori_S palindrome (Figure 1b). Lower affinity binding occurs to site II which is present in the opposite orientation to site I and is identical in 15 of the corresponding 18 positions. Two copies of a sequence which differs in only one position from that of site I are similarly located within ori_L and are also assumed to act as binding sites. Elias and Lehman (26) showed that the origin-binding

protein (OBP) had a molecular weight of 83,000, but the viral gene encoding it was not identified. It seemed very likely however that OBP would be the product of one of the four genes essential for HSV-1 DNA synthesis to which a function had not yet been ascribed. We therefore developed a gel retardation assay to facilitate the testing of the UL5, UL8, UL9 and UL52 gene products for origin-binding activity. Our results confirm and extend the very recent report by Olivo et al. (27) that the UL9 gene specifies OBP.

MATERIALS AND METHODS

Cells and viruses: Baby hamster kidney 21 clone 13 (BHK) cells (28) were grown in Eagle's medium supplemented with 10% tryptose phosphate broth and 10% foetal calf serum. Extracts were prepared from cell monolayers (10^7 cells/90 mm plastic Petri dish) infected with 5-10 p.f.u./cell and incubated for 8 h at 37°C or 38.5°C. The viruses used were wild type (wt) HSV-1 (Glasgow strain 17, syn^+), the HSV-1 temperature-sensitive mutant tsK (29) and recombinant viruses tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52. The recombinant viruses contain additional copies of the early genes UL5, UL8, UL9 and UL52 inserted under the control of the immediate early (IE) gene 3 promoter within the thymidine kinase gene of tsK. They were constructed essentially as described by Hummel et al. (30).

Preparation of nuclear extracts: In initial experiments nuclear extracts were prepared by the method of Piette et al. (31) from 3 infected cell monolayers. Proteins eluted from nuclei with buffer B containing 600 mM NaCl were precipitated with $(NH_4)_2SO_4$ and resuspended in 100 µl buffer B containing 100 mM NaCl. Subsequently it was noted that a procedure essentially as described by Dignam et al. (32) and modified by Preston et al. (33) gave greater reproducibility in terms of yield and activity. The nuclear pellet from 3 cell monolayers was eluted with 100 µl buffer C (20 mM Hepes pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT) containing 600 mM NaCl and after centrifugation the supernatant extract was flash frozen and stored at -70°C. Protein concentrations were determined by using a Bio-Rad Protein Assay kit.

Expression in Escherichia coli: Fusion proteins containing parts of the UL9 polypeptide were synthesised using the plasmid vector system pRIT2T (supplied by Pharmacia; ref. 34). Two additional plasmids were constructed such that the SmaI, BamHI, SalI and PstI cloning sites (Figure 6a) were positioned in the 2 other reading frames. EcoRI cleaved pRIT2T DNA was treated with mung bean nuclease to remove precisely the single-stranded overhangs and the plasmid recircularised either in the absence or presence of an 8 bp EcoRI linker (GGAATTCC). Resulting plasmids pRIT2T-4 and pRIT2T+4 respectively contain deletions and insertions of 4 bp and their identities were confirmed by DNA sequencing. DNA fragments were inserted into the appropriate plasmid as described in Figure 6 and the constructs used to transform E. coli strain K12ΔH1Δtrp (35) to ampicillin resistance. Bacteria containing the desired plasmids were isolated and propagated at 28°C. To induce synthesis of the fusion protein bacteria were grown to an A630 value of 0.4 in 50 ml L broth and rapidly shifted to 42°C for 75 min. The cells were harvested by centrifugation, washed twice with 10mM Tris-HCl pH 7.5, 1mM EDTA and resuspended in 0.5 ml buffer C containing 600 mM KCl. The suspension was extensively sonicated and centrifuged at 11,600 g for 10 min. The resulting supernatant was used as extract in the binding assays.

DNA fragments and oligonucleotides: The 100 bp BamHI/SalI ori_S-containing fragment from plasmid pS19 (22) was recloned between the corresponding sites of pTZ19U (36) and used as a source of the HSV-1 ori_S sequence (pS19 fragment). Plasmid pBY3 contains an unrelated but similarly sized HaeIII fragment of HSV-1 DNA originating from within BamHI y (37) and cloned into the SmaI site of pTZ19U. Oligonucleotides were synthesised using a model 8600 Biosearch DNA synthesiser and purified on a 15% acrylamide DNA sequencing gel. Duplex oligonucleotides were formed by mixing equivalent masses of the two complementary strands, heating to 95°C and allowing to cool slowly to room temperature. The duplex oligonucleotides were end-labelled and blunt-ended using the filling-in reactions of the Klenow fragment of DNA polymerase I (38). The following duplex oligonucleotides were used:

oligonucleotide I 5' GATCCGCGAAGCGTTCGCACTTCGTCCCA
GCGCTTCGCAAGCGTGAAGCAGGGTCTAG 5'

oligonucleotide II 5' GATCTGGGGCGAAGTGCGAGCACTTCGCG
ACCCCGCTTCACGCTCGTGAAGCGCCTAG 5'

oligonucleotide X 5' GATCGGATATGCTAATTAAATACAT
GCCTATACGATTAATTTATGTG 5'

Oligonucleotides I and II respectively contain the sequences of binding sites I and II within HSV-1 ori_S (see Figure 1 and ref. 26). Oligonucleotide X, containing an unrelated DNA sequence, was kindly provided by Dr A. Bailey.

Gel retardation analysis (39): Probe fragments, 3' end-labelled with [^{32}P]deoxyribonucleoside triphosphates, were eluted from 8% non-denaturing acrylamide gels, extracted sequentially with phenol and chloroform and precipitated with ethanol. Either 1 ng labelled oligonucleotide or 5 ng of labelled 100 bp ori_S fragment were incubated at 25°C with 2 µg sonicated calf thymus DNA and 5 µg extract in a 20 µl reaction mix containing 1 x buffer C plus 100 mM NaCl. After 20 min 5 µl loading buffer (25% glycerol, 0.05% bromophenol blue in 1 x TBE [90 mM Tris-borate, 2.5 mM EDTA]) were added and the samples loaded onto 5% polyacrylamide gels (55:1, acrylamide:N,N'-methylene bisacrylamide) containing 1 x TBE. Gels were run in 1 x TBE at 25 mA for 1.5-2 h, fixed in 10% acetic acid, dried and exposed for autoradiography.

Antisera: Rabbit antisera raised against decapeptides from the C-termini of the UL5, UL8, UL9 and UL52 gene products and reactive with the corresponding polypeptides (27) were kindly provided by Dr M.D. Challberg. 1 µl of antiserum was added to binding reactions.

RESULTS

Detection of ori_S binding activity in nuclear extracts of cells infected with wt HSV-1. The sequence of part of the 100 bp HSV-1 DNA insert of plasmid pS19, which specifies functional ori_S activity (22), is shown in Figure 1b together with the two binding sites for a viral polypeptide mapped by Elias and Lehman (26). Double-stranded oligonucleotides containing the sequences of binding sites I and II (oligonucleotide I and oligonucleotide

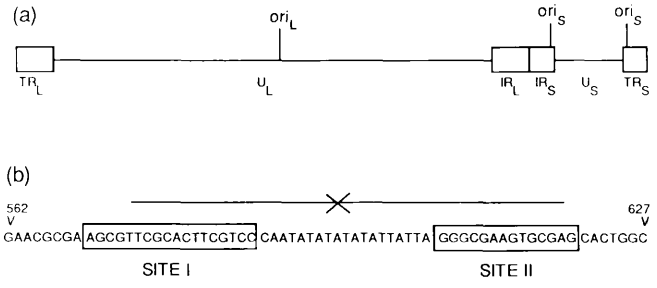

Figure 1. (a) Structure of the HSV-1 genome showing the locations of the origins of DNA replication. (b) DNA sequence from the ori$_S$ region (22). The 45 bp palindrome is indicated by arrows and the two binding sites identified by Elias & Lehman (26) are boxed.

II respectively) were synthesised and used with the 100 bp pS19 fragment in a gel retardation assay for the presence of proteins binding to these sites.

In initial experiments proteins eluted with 600 mM NaCl from nuclei of mock-infected and HSV-1 infected cells were precipitated with $(NH_4)_2SO_4$ and redissolved in buffer B plus 100 mM NaCl (31). These extracts were incubated with end-labelled pS19 fragments in the presence of sonicated calf thymus DNA and sequence specific competitor DNAs. The reaction products were analysed by electrophoresis through 5% non-denaturing polyacrylamide gels.

Figure 2 shows the results of a typical experiment. In the absence of added competitor DNA two retarded bands (A and B) representing protein-DNA complexes were detected (track 1). To verify the specificity of binding, 100-fold molar excesses of unlabelled ori$_S$ fragment (track 2), oligonucleotides I and II (tracks 3 and 4) and the 100 bp insert of plasmid pBY3 (containing unrelated HSV-1 DNA sequences [track 5]) were added to the assay. The ori$_S$ fragment and oligonucleotides I and II competed efficiently for binding resulting in a great decrease in the signal intensity of bands A and B. In contrast the pBY3 fragment did not compete. Complexes A and B therefore result from site specific binding to DNA sequences present within oligonucleotides I and II, and probably represent the same activity as previously detected by DNase footprinting (26). No

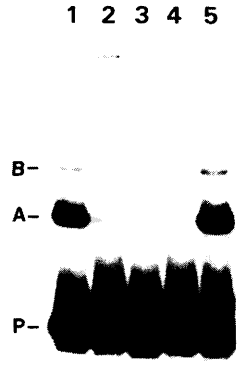

Figure 2. Gel retardation analysis of extracts of wt HSV-1 infected cells. Nuclear extract was incubated with ^{32}P end-labelled pS19 fragment either in the absence of competitor DNA (track 1) or in the presence of 100-fold molar excesses of unlabelled pS19 fragment (track 2), oligonucleotide I (track 3), oligonucleotide II (track 4) or pBY3 fragment (track 5). The products were separated by electrophoresis in a 5% non-denaturing polyacrylamide gel. A and B indicate specific retarded complexes and P the free probe.

similar activity was detected in extracts from mock-infected cells (e.g. Figure 3a, track 1).

The product of the UL9 gene interacts with binding site I. The recombinant viruses tsK/UL5, tsK/UL8, tsK/UL9 and tsK/UL52 contain additional copies of the early genes UL5, UL8, UL9 and UL52 inserted into the genome of the HSV-1 temperature-sensitive mutant tsK under the control of the immediate early (IE) gene 3 promoter. At the non-permissive temperature (38.5°C) tsK accumulates IE polypeptides but fails to induce the expression of early and late genes (29,40). The 4 recombinant viruses exhibit polypeptide profiles at 38.5°C indistinguishable from tsK with the exception that each induces a single additional polypeptide corresponding to the product of the inserted gene (J.M. Calder and N.D. Stow, manuscript in preparation).

Proteins were eluted with 600 mM NaCl from the nuclei of mock-infected cells and from cells infected at the non-permissive temperature with wt HSV-1, tsK or the four recombinant viruses. The extracts were tested in a gel

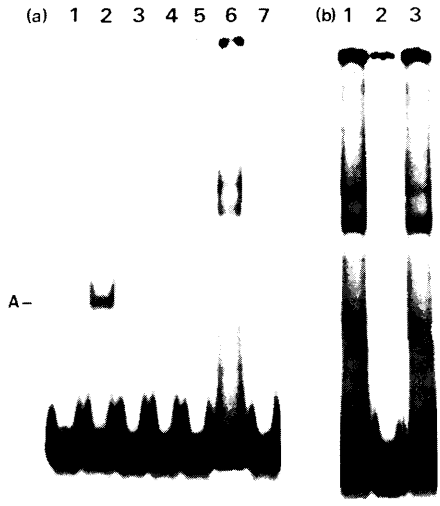

Figure 3. Gel retardation analysis of extracts from cells infected with recombinant viruses. Nuclear extract was prepared from cells incubated at 38.5°C and reacted with ^{32}P-labelled oligonucleotide I. (a) track 1, mock-infected cells; tracks 2-7, cells infected with wt HSV-1, tsK, tsK/UL5, tsK/UL8, tsK/UL9 or tsK/UL52 respectively. "A" indicates the major complex detected with wt HSV-1 extract. (b) Nuclear extract from cells infected with tsK/UL9 was incubated in the absence of competitor DNA (track 1) or in the presence of 100-fold molar excesses of oligonucleotide I (track 2) or oligonucleotide X (track 3).

retardation assay using ^{32}P-labelled oligonucleotide I as probe (Figure 3a). As expected, extract from cells infected with wt HSV-1 generated a major complex (A) and some minor bands (track 2). The only other extract which caused significant retardation of the probe fragment was from cells infected with recombinant virus tsK/UL9 (track 6). Although the pattern of retardation observed with extracts from cells infected with this virus varied from assay to assay the presence of material which failed to enter the gel and of a smear extending down most of the track were consistently observed. In addition, whereas somewhat diffuse bands migrating more slowly than complex A were usually seen, bands co-migrating with complex A were absent.

Figure 3b demonstrates that the pattern of retarded DNA probe represents sequence specific binding. Addition of a 100-fold excess of unlabelled oligonucleotide I but not

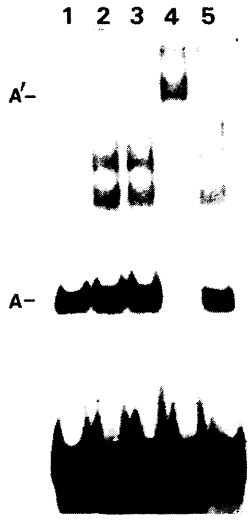

Figure 4. Effect of antisera on the wt HSV-1 complex. Nuclear extract from cells infected with wt HSV-1 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of added antiserum (track 1) or in the presence of antisera reactive with the UL5, UL8, UL9 or UL52 gene products (tracks 2, 3, 4 and 5 respectively). The products were analysed as described in Figure 2. A and A' indicate the major complexes formed in the absence of antiserum or in the presence of antiserum against the UL9 gene product respectively.

oligonucleotide X (which is a similar size but of unrelated DNA sequence) efficiently competed for binding.

Because the tsK/UL9 recombinant differs from tsK only in its ability to express the UL9 gene product at the non-permissive temperature the data indicate that this polypeptide participates in the formation of a complex with binding site I.

The UL9 gene product is present in complex A formed with extracts from cells infected with wt HSV-1. Nuclear extract from cells infected with wt HSV-1 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of added antibody or in the presence of antibodies reactive with the UL5, UL8, UL9 or UL52 polypeptides and the products analysed on a non-denaturing polyacrylamide gel. Figure 4 shows that the antibodies which react with UL5, UL8 or UL52 had little effect

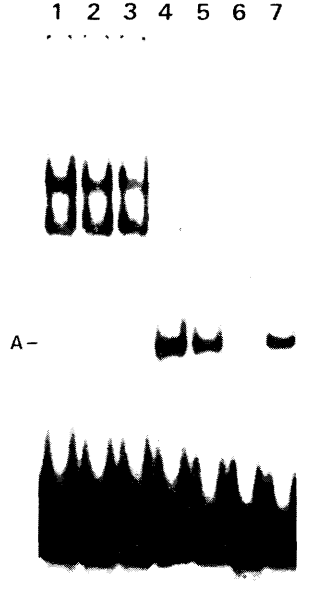

Figure 5. Effect of proteinase K on the tsK/UL9 complex. Nuclear extract from cells infected with recombinant tsK/UL9 was incubated with ^{32}P-labelled oligonucleotide I either in the absence of proteinase K (track 1) or in the presence of 1, 10, 100, 1000 or 2000 ng proteinase K (tracks 1-6 respectively). Wt HSV-1 extract was assayed in parallel in the absence of proteinase (track 7). The products were analysed as described in Figure 2. "A" indicates the major complex formed with wt extract.

on the pattern of retarded fragments. In contrast addition of antibody to the UL9 polypeptide resulted in the loss of the major retarded complex, A, and its replacement by a more slowly migrating form, A', indicative of the additional presence of antibody molecules in the complexes. Therefore at least a part of the UL9 polypeptide is contained within complex A, and since the antibody was raised against a C-terminal oligopeptide (27) this region of the protein must be present.

Effect of protease digestion on the binding activity from cells infected with the tsK/UL9 recombinant. Given that the major retarded complex from cells infected with wt HSV-1 reacted with an antibody against the UL9 polypeptide it was rather surprising that the pattern of retarded fragments observed using extract

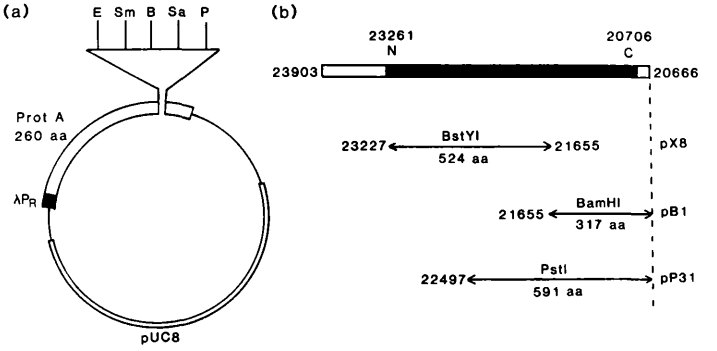

Figure 6. Expression of UL9 gene fragments in E. coli. (a) Structure of plasmid pRIT2T. pRIT2T-4 and pRIT2T+4 are essentially similar except that in pRIT2T-4 the EcoRI site is replaced by an ApaI site. The positions of the phage lambda promoter, P_R, and coding sequences for the N-terminal region of the Staphylococcus aureus A protein are indicated. (b) Fragments inserted into vectors. The upper line shows the SstI fragment containing the UL9 gene which was initially cloned into the EcoRI site of pUC8. The N- and C-terminal regions of the coding sequences (black bar) are indicated. Plasmids pX8, pB1 and pP31 contain the indicated fragments inserted in frame and in the appropriate orientation into pRIT2T-4, pRIT2T-4 and pRIT2T respectively. The C-terminal ends of the pB1 and pP31 inserts lie within the pUC8 polylinker. The nucleotide numbering is from McGeoch et al. (1).

from cells infected with the tsK/UL9 recombinant was very different. One possible explanation is that complex A contains only a fragment of the UL9 polypeptide.

Our attempts to size complex A using pore-gradient polyacrylamide gels (41) had suggested a molecular weight of approximately 45,000, clearly inconsistent with the binding of an intact UL9 polypeptide (molecular weight 94,000) to the labelled oligonucleotide (data not shown). We therefore examined the effect of proteinase K addition to binding reactions containing tsK/UL9 nuclear extract and labelled oligonucleotide I. The results (Figure 5) show that with increasing amount of added proteinase K there is a reduction in the amount of slowly migrating complexes and a concomitant appearance of a faster migrating species which exhibits a mobility very similar to that of complex A. No band of this mobility was detected when extracts from mock-infected cells or

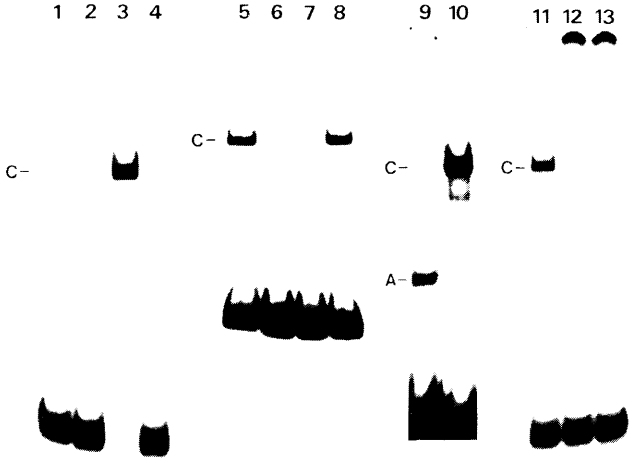

Figure 7. Gel retardation analysis of the pBl encoded fusion protein. Binding reactions were performed without extract (track 1) or with extracts from untransfomed bacteria (track 2), bacteria transformed with pRIT2T (track 4) or pBl (tracks 3, 5-8, 10-13), or from wt HSV-1 infected BHK cells (track 9). The probe fragments were ^{32}P-labelled oligonucleotide I (tracks 1-4, 9-13) and pS19 fragment (tracks 5-8). Unlabelled oligonucleotide I (track 6), oligonucleotide II (track 7) and oligonucleotide X (track 8) were added as competitors. Antisera reactive with UL9 and UL52 respectively were added to the reactions shown in tracks 12 and 13. C and A indicate the major complexes from bacteria containing pBl and from wt HSV-1 infected cells.

cells infected with tsK were similarly treated with proteinase K (data not shown).

The data thus suggest that the addition of proteinase K to extracts containing the UL9 polypeptide results in the cleavage of the polypeptide and the generation of a relatively protease-resistant domain which retains DNA binding activity.

Expression of the DNA binding domain of UL9 in Escherichia coli. Three DNA fragments representing approximately the N-terminal 2/3 and C-terminal 2/3 and 1/3 of the UL9 coding sequences were cloned in the correct reading frame into the vector pRIT2T or an appropriate frameshift derivative (see Figure 6 and Materials and Methods). The constructs were introduced into E. coli strain K12ΔH1Δtrp, extracts prepared from heat induced bacteria, and tested for binding activity using ^{32}P-labelled

oligonucleotide I. No binding activity was detected in extracts from the host bacteria alone or following the introduction of the vector pRIT2T (Figure 7, tracks 2 and 4). Of the three hybrid plasmids pB1 (track 3) and pP31 but not pX8 encoded binding activity. The activity encoded by pB1 also binds to the 100 bp ori_S-containing fragment and is efficiently competed by the addition of unlabelled oligonucleotides I and II but not oligonucleotide X (tracks 5-8). The C- terminal 317 amino acids of the U9 polypeptide therefore contain all the amino acid sequences required for sequence-specific binding to sites I and II within ori_S.

Figure 7 also shows that the fusion protein encoded by pB1 (predicted molecular weight 63,000) forms a complex which exhibits a considerably lower mobility than complex A formed using extract from cells infected with wt HSV-1 (tracks 9 and 10), in agreement with complex A containing only a proteolytic fragment of the UL9 polypeptide. The mobility of the pB1 complex was further reduced by the addition of antibodies reactive with the UL9 or UL52 polypeptides to the binding reaction (tracks 11-13). The ability of both these antibodies to bind to the DNA protein complex results from the presence of the protein A moiety in the pB1 fusion protein, and verifies that the hybrid protein is responsible for the observed binding.

DISCUSSION

The initial objective of the experiments presented in this paper was to identify the HSV-1 gene product which recognises the two specific binding sites within HSV-1 ori_S characterised by Elias and Lehman (26). The expression of binding activity in E. coli indicates that the only viral polypeptide essential for this activity is encoded by the UL9 gene and very strongly suggests that this gene product is directly responsible for sequence specific binding. Our identification of the UL9 polypeptide as an origin-binding protein is in complete agreement with the recent results of Olivo et al. (27) who expressed this viral protein in a baculovirus expression system and assayed for binding by an immunoprecipitation assay and DNase footprinting.

In addition to expressing binding activity in E. coli we also detected binding using nuclear extracts from cells infected with wt HSV-1 or the recombinant virus tsK/UL9. Competition experiments with synthetic oligonucleotides demonstrated that in each instance the binding target contained sequences homologous to binding sites I and II initially characterised by Elias and Lehman (26). The baculovirus-expressed UL9 polypeptide protected similar DNA sequences in DNase footprinting experiments (27).

By employing a gel retardation assay we were able to distinguish different sizes of complex formed by the addition of extracts from various sources. These results are interesting to compare with other recent reports which examined origin-binding activity by the same technique. The rapidly migrating complex A detected with extracts from cells infected with wt HSV-1 clearly contains only a portion of the UL9 gene product. This complex appears to be similar to the major complex described recently by Koff and Tegtmeyer (42). In contrast Elias and Lehman (26) purified origin binding activity from virus infected cells and observed a major (presumably intact) polypeptide of molecular weight 83,000 and two smaller components. This preparation gave a very different pattern, more closely resembling that obtained with tsk/UL9 extracts, in a gel retardation assay. This suggests that in extracts of cells infected with wt HSV-1 the UL9 polypeptide may be subjected to varying extents of proteolytic cleavage. We do not know whether the small forms observed by ourselves and by Koff and Tegtmeyer (42) are of any physiological significance or whether they merely reflect proteolytic activity occurring during the isolation procedure. The observation that extracts made in parallel from cells infected with the tsk/UL9 recombinant do not contain the cleaved form of the polypeptide responsible for the formation of complex A suggests that the proteolytic activity occurs as a consequence of infection proceeding from the IE to early and late stages, and could thus possibly be virus encoded.

Although interaction of intact UL9 polypeptide with labelled probe fragments produces a smeared pattern in a gel retardation assay this nevertheless represents sequence-specific

binding (Figure 3 and ref. 26). The smearing may occur because of aggregation and/or dissociation of complexes during incubation of the binding reactions and gel electrophoresis. This behaviour is not so noticeable in complexes containg only proteolytic fragments of UL9 or fragments synthesized in E. coli where much "tighter" bands are observed. The smearing may therefore be a consequence of interactions involving the N-terminal region of the UL9 polypeptide.

By expressing a fragment of the UL9 polypeptide in E. coli we have demonstrated that the C-terminal 317 amino acids specify all the structural information required for sequence-specific recognition and binding. A similar situation has previously been noted for another herpesvirus sequence-specific DNA binding protein, EBNA-1 of Epstein-Barr virus, the C-terminal domain of which is able to bind to its target recognition sequence (43). In addition, discrete domains of several other proteins are known to be responsible for sequence-specific binding to DNA and these are often relatively resistant to the action of added protease (41, 44-48).

It is likely that sequence-specific recognition of the HSV-1 origins of DNA replication is a very early event in initiation of HSV-1 DNA synthesis. Following binding of the UL9 polypeptide to the origins, regions of the protein not involved in direct interaction with DNA probably participate in other functions which may include interacting with other replication proteins, or unwinding or nicking DNA to facilitate the initiation of DNA synthesis. Expression systems for the UL9 polypeptide such as those described in this paper should prove useful in the study of these functions.

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REFERENCES

1. McGeoch, D.J., Dalrymple, M.A., Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. and Taylor, P. (1988) J. Gen. Virol. 69, 1531-1574.
2. Purifoy, D.J.M. and Powell, K.L. (1981) J. Gen. Virol. 54, 219-222.
3. Conley, A.J., Knipe, D.M., Jones, P.C. and Roizman, B. (1981) J. Virol. 37, 191-206.
4. Chartrand, P., Stow, N.D., Timbury, M.C. and Wilkie, N.M. (1979) J. Virol. 31, 265-276.
5. Matz, B., Subak-Sharpe, J.H. and Preston, V.G. (1983) J. Gen. Virol. 64, 2261-2270.
6. Weller, S.K., Lee, K.J., Sabourin, D.J. and Schaffer, P.A. (1983) J. Virol. 45, 354-366.
7. Weller, S.K., Carmichael, E.P., Aschman, D.P., Goldstein, D.J. and Schaffer, P.A. (1987) Virology 161, 198-210.
8. Carmichael, E.P., Kosovsky, M.J. and Weller, S.K. (1988) J. Virol. 62, 91-99.
9. Marchetti, M.E., Smith, C.A. and Schaffer, P.A. (1988) J. Virol. 62, 715-721.
10. Goldstein, D.J. and Weller, S.K. (1988) J. Virol. 62, 2970-2977.
11. Challberg, M.D. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 9094-9098.
12. Wu, C.A., Nelson, N.J., McGeoch, D.J. and Challberg, M.D. (1988) J. Virol. 62, 435-443.
13. McGeoch, D.J., Dalrymple, M.A., Dolan, A., McNab, D., Perry, L.J., Taylor, P. and Challberg, M.D. (1988) J. Virol. 62, 444-453.
14. Quinn, J.P. and McGeoch, D.J. (1985) Nucl. Acids Res. 13, 8143-8163.
15. Gibbs, J.S., Chiou, H.C., Hall, J.D., Mount, D.W., Retondo M.J., Weller, S.K. and Coen, D.M. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 7969-7973.
16. Marsden, H.S., Campbell, M.E.M., Haarr, L., Frame, M.C., Parris, D.S., Murphy, M., Hope, R.G., Muller, M.T. and Preston, C.M. (1987) J. Virol. 61, 2428-2437.
17. Parris, D.S., Cross, A., Haarr, L., Orr, A., Frame, M.C., Murphy, M., McGeoch, D.J. and Marsden, H.S. (1988) J. Virol. 62, 818-825.
18. Spaete, R.R. and Frenkel, N. (1982) Cell 30, 295-304.
19. Weller, S.K., Spadaro, A., Schaffer, J.E., Murray, A.W., Maxam, A.M. and Schaffer, P.A. (1985) Mol. Cell. Biol. 5, 930-942.
20. Vlazny, D.A. and Frenkel, N. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 742-746.
21. Stow, N.D. (1982) EMBO Journal 1, 863-867.
22. Stow, N.D. and McMonagle, E.C. (1983) Virology 130, 427-438.
23. Deb, S. and Doelberg, M. (1988) J. Virol. 62, 2516-2519.
24. Lockshon, D. and Galloway, D.A. (1988) Mol. Cell. Biol. 8, 4018-4027.
25. Elias, P., O'Donnell, M.E., Mocarski, E.S. and Lehman, I.R. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 6322-6326.
26. Elias, P. and Lehman, I.R. (1988) Proc. Natl. Acad. Sci.

U.S.A. 85, 2959-2963.
27. Olivo, P.D., Nelson, N.J. and Challberg, M.D. (1988) Proc. Natl. Acad. Sci. U.S.A. 85, 5414-5418.
28. MacPherson, I. and Stoker, M. (1962) Virology 16, 147-151.
29. Preston, C.M. (1979) J. Virol. 29, 275-284.
30. Hummel, M., Arsenakis, M., Marchini, A., Lee, L., Roizman, B. and Kieff, E. (1986) Virology 148, 337-348.
31. Piette, J., Kryszke, M.-H. and Yaniv, M. (1985) EMBO Journal 4, 2675-2685.
32. Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucl. Acids Res. 11, 1475-1489.
33. Preston, C.M., Frame, M.C. and Campbell, M.E.M. (1988) Cell 52, 425-434.
34. Nilsson, B., Abrahmsen, L. and Uhlen, M. (1985) EMBO Journal 4, 1075-1080.
35. Bernard, H.-U., Remaut, E., Hershfield, M.V., Das, H.K. and Helinski, D.R. (1979) Gene 5, 59-76.
36. Mead, D.A., Szczesna-Skorupa, E. and Kemper, B. (1986) Protein Engineering 1, 67-74.
37. Murchie, M.-J. and McGeoch, D.J. (1982) J. Gen. Virol. 62, 1-15.
38. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning (a laboratory manual), pp. 113-116. Cold Spring Harbor Laboratory, New York.
39. Fried, M. and Crothers, D.M. (1981) Nucl. Acids Res. 9, 6505-6525.
40. Watson, R.J. and Clements, J.B. (1980) Nature, London 285, 329-330.
41. van Huijsduijnen, R.A.M.H., Bollekens, J., Dorn, A., Benoist, C. and Mathis, D. (1987) Nucl. Acids Res. 15, 7265-7282.
42. Koff, A. and Tegtmeyer, P. (1988) J. Virol. 62, 4096-4103.
43. Rawlins, D.R., Milman, G., Hayward, S.D. and Hayward, G.S. (1985) Cell 42, 859-868.
44. Smith, D.R., Jackson, I.J. and Brown, D.D. (1984) Cell 37, 645-652.
45. McBride, A.A., Schlegel, R. and Howley, P.M. (1988) EMBO Journal 7, 533-539.
46. de Vargas, L.M., Pargellis, C.A., Hasan, N.M., Bushman, E.W. and Landy, A. (1988) Cell 54, 923-929.
47. Hall, M.N. and Johnson, A.D. (1987) Science 237, 1007-1012.
48. Tsai, S.Y., Carlstedt-Duke, J., Weigel, N.L., Dahlman, K., Gustafsson, J.-A., Tsai, M.-J. and O'Malley, B.W. (1988) Cell 55, 361-369.

Herpes simplex virus helicase-primase: the UL8 protein is not required for DNA-dependent ATPase and DNA helicase activities

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ABSTRACT

The herpes simplex virus type 1 helicase-primase complex consists of the products of the UL5, UL8 and UL52 genes. We have expressed these proteins in insect cells using baculovirus vectors and studied the requirements for enzymatic activities associated with the DNA unwinding function of the complex. In agreement with a recent report (Dodson, M.S., Crute, J.J., Bruckner, R.C. and Lehman, I.R. 1989, J. Biol. Chem. 264, 20835 – 20838) we find that DNA-dependent ATPase and DNA helicase activitites are assembled *in vivo* in insect cells triply infected with viruses expressing the UL5, UL8 and UL52 proteins. Moreover, these activities were also detected in cells in which only the UL5 and UL52 products were expressed indicating that the presence of the UL8 protein is essential for neither the ATPase nor helicase activity of the complex.

INTRODUCTION

The herpes simplex virus type 1 (HSV-1) genome is a double-stranded DNA of approximately 152 kbp (1). Recent studies have identified a set of seven HSV-1 gene products which, in transient replication assays in transfected tissue culture cells, are necessary and sufficient for origin dependent DNA replication (2–4). HSV-1 mutants with lesions in these genes all exhibit defects in DNA synthesis under non-permissive conditions indicating that the seven proteins play important roles in genome replication (5–12).

The seven replication proteins have now been identified either in HSV-1 infected cells or by making use of expression vectors (13–18), and roles in DNA synthesis have been assigned to them. The viral DNA polymerase holoenzyme contains subunits encoded by genes UL30 and UL42 (14,20–22). A single-stranded DNA binding protein is specified by the UL29 gene (16,23,24), and the product of the UL9 gene binds to specific DNA sequences within the viral origins of DNA synthesis (18,19). The remaining three proteins, encoded by genes UL5, UL8 and UL52, form a complex in HSV-1 infected cells which exhibits DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase activities (17,25,26). These enzymatic activities have not yet been assigned to individual subunits of the complex.

By analogy to other replication systems it would be expected that the DNA-dependent ATPase activity of the UL5/UL8/UL52 complex reflects the coupling of ATP hydrolysis to the strand unwinding activity of the helicase. In order to further investigate these activities we have utilised the baculovirus expression system (27-29), and have isolated recombinant viruses which individually overproduce the three HSV-1 proteins.

In agreement with the recent results of Dodson *et al.* (17) we demonstrate that the DNA-dependent ATPase and DNA helicase activities can be reconstituted by triple infection of *Spodoptera frugiperda* insect cells. Moreover co-expression of the UL5 and UL52 proteins is sufficient for production of these activities suggesting that the UL8 protein may play a separate role in the complex.

MATERIALS AND METHODS

Isolation of recombinant baculoviruses

Spodoptera frugiperda (Sf) cells were maintained at 28°C in TC100 medium (Gibco) supplemented with 5% foetal calf serum. The propagation of *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the preparation of viral DNA were as previously described (28,30). HSV-1 DNA fragments encoding the UL5, UL8 and UL52 genes were initially subcloned from appropriate plasmids containing larger inserts of wt HSV-1 (Glasgow strain 17) DNA, and subsequently inserted into the unique BamHI site of the baculovirus transfer vector pAcYM1 (28). Resulting plasmids containing HSV-1 inserts in the correct orientations were cotransfected with intact AcNPV DNA into Sf cells as previously described (28,30). Recombinant viruses were isolated and purified by performing serial dilutions essentially as described by Elliott and McGregor (31) except that double stranded DNA was prepared from infected cells and screened for the presence of recombinants by restriction enzyme digestion and Southern blot hybridisation to appropriate probes (32). The resulting recombinant viruses AcUL5, AcUL8 and AcUL52 contain and express the UL5, UL8 and UL52 genes respectively. The HSV-1 DNA sequences present in these viruses

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are nucleotides 12128–15166 (UL5), 17855–20492 (UL8) and 108961–112514 (UL52) (ref. 1), in each instance inserted via BamHI cohesive ends.

Preparation of cell extracts

Subconfluent Sf cell monolayers in 90 mm diam. plastic Petri dishes were infected with 10 p.f.u./cell of each of the recombinant viruses under test or with parental AcNPV and incubated at 28°C for 30 h. Cells from 3 monolayers were pooled for the preparation of each extract and all manipulations were performed at 4°C. Infected cells were harvested by scraping into the medium, pelleted and washed 3 times with tris saline solution. The final pellet of washed cells was resuspended in 0.75 ml buffer containing 0.5% Nonidet P40 (NP40) and incubated on ice. Two different buffers were employed: RSB (10 mM Tris-HCl pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM PMSF) for the preparation of cytosol, and buffer C (20 mM hepes pH 7.9, 25% glycerol, 600 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF) for the preparation of a fraction including salt-elutable nuclear proteins. Cells resuspended in RSB/NP40 were left on ice for 10 min and centrifuged at 11,000×g for 30 sec to pellet nuclei. Particulate material was removed by further centrifugation of the supernatant at 200,000×g for 30 min in a Beckman TLA 100.2 rotor. The final supernatant was applied directly to a phosphocellulose column. Cells resuspended in buffer C/NP40 were left on ice for 20–30 min then directly centrifuged at 200,000×g for 30 min. The resulting supernatant was dialysed against buffer B (20 mM hepes pH 7.6, 10% glycerol, 1 mM dithiothreitol, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF) containing 50 mM NaCl prior to column chromatography. In practice the two procedures gave almost indistinguishable results.

Phosphocellulose chromatography

Phosphocellulose chromatography was performed essentially as described (25). Extracts were applied to 2 ml bed volume columns of phosphocellulose (Whatman P11) equilibrated with buffer B containing 50 mM NaCl. The columns were washed with 10 ml of this buffer and proteins eluted with a linear gradient (15 ml) from 50 to 400 mM NaCl in buffer B. Finally 2 ml buffer B plus 2M NaCl was applied. 1 ml fractions were collected.

Assay for DNA-dependent ATPase activity

Reaction mixes (50 μl) contained 20 mM Tris-HCl pH 7.5, 10% glycerol, 3.5 mM $MgCl_2$, 100 μg/ml BSA, 5 mM dithiothreitol, 0.05 mM ATP containing 1.5 μCi [γ-^{32}P]ATP, 0.1 μg activated calf thymus DNA and 20 μl enzyme fraction. Incubation was for 40 min at 33°C. 150 μl activated charcoal in 50 mM HCl, 5 mM H_3PO_4 was added, the mixtures vortexed and left to stand for 5 min (33). The reactions were then centrifuged in a microfuge for 2 min and the free phosphate liberated by hydrolysis of ATP was determined by counting the radioactivity present in a 120 μl sample of the supernatant. The amount of ATP hydrolysed was calculated by comparison with the total radioactivity in an equivalent amount of the initial reaction mix.

Assay for DNA helicase activity

Helicase activity was assayed as previously described (25). The substrate consisted of a 45 base oligonucleotide with a 3′ tail annealed to M13mp18 single stranded DNA (25). The oligonucleotide was labelled using terminal transferase in the presence of [α-^{32}P]dATP (34), annealed to the M13mp18 DNA and the hybrid substrate purified by filtration through a Bio-gel A-1.5m column. Reaction mixes (40 μl) contained 20 μl enzyme fraction, 20 ng helicase substrate and 3 mM ATP in buffer A (25). Incubation was for 2 h at 37°C and the products were analysed by electrophoresis through a 10% acrylamide gel run in 89 mM Tris base, 89 mM boric acid, 2 mM EDTA. After electrophoresis gels were dried and exposed to autoradiographic film.

Assay for the UL5, UL8 and UL52 polypeptides

Two approaches were used to identify column fractions containing the UL5, UL8 or UL52 proteins. In some experiments one infected monolayer from a set of 3 was incubated from 24 to 30 h p.i. in 1.5 ml TC100 salt solution (43 mM KCl, 10 mM $CaCl_2$, 6 mM glucose, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, 4.6 mM $NaHCO_3$, 8 mM NaH_2PO_4 pH 6.2) containing 150 μCi ^{35}S-methionine. 40 μl samples of labelled column fractions were subsequently analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 9% gels (35). Gels were fixed, treated with EnHance (NEN), dried and exposed to autoradiographic film. Alternative immunological detection procedures were used in other experiments (not shown) with unlabelled extracts. Samples of column fractions were applied to nitrocellulose filters using a dot blot apparatus or electrophoretically transferred from an SDS polyacrylamide gel. Fractions containing the HSV-1 proteins were identified by using rabbit antipeptide antibodies directed against the UL5, UL8 or UL52 polypeptides (kindly provided by Dr M.D. Challberg; ref. 16) or an appropriate control serum, in conjunction with a detection kit (Promega) containing horseradish peroxidase conjugated second antibody.

RESULTS

Expression of UL5, UL8 and UL52 polypeptides by recombinant baculoviruses

Sf cells were infected with 10 p.f.u./cell wt AcNPV, AcUL5, AcUL8 or AcUL52 and incubated at 28°C for 50 h. The infected cells were then washed and samples containing total cellular proteins and the cytoplasmic and nuclear fractions analysed by SDS-PAGE. Fig. 1 shows the proteins visualised by Coomassie brilliant blue staining, each lane containing the material recovered from 1.5×10^5 cells. The products of the UL5, UL8 and UL52 genes are readily apparent in the lanes containing total cellular proteins indicating efficient expression by the recombinant viruses. Both the UL5 and UL8 proteins were present almost exclusively in the soluble cytoplasmic fraction, whereas the UL52 protein sedimented with the nuclei. Subsequent experiments have shown that this fractionation of the UL52 protein is a consequence of its insolubility rather than its translocation into the nuclei. Furthermore, when extracts were prepared at 24–30 h after infection, the yields of the HSV-1 proteins were, as expected, much lower but the UL52 gene product was found predominantly in the soluble cytoplasmic fraction (data not shown).

The observation of Crute *et al.* (26) that in HSV-1 infected cells the UL5, UL8 and UL52 polypeptides form a complex suggested that co-expression of these three proteins might increase the solubility of the UL52 product at late times after infection with AcUL52. This indeed occurred but the proportion of UL52 protein recovered in the soluble fraction was variable and on all occasions a significant amount remained insoluble. Experiments examining the induction of enzymatic activities by the

Fig. 1. Expression of HSV-1 UL5, UL8 and UL52 proteins by recombinant viruses. Sf cells were harvested 50 h after infection with AcNPV, AcUL5, AcUL8 or AcUL52 and washed in tris saline solution. Total cellular proteins (T) were prepared by direct lysis of a portion of the cells in sample buffer (35). The remainder of the cells were resuspended in RSB/NP40, incubated on ice for 10 min and centrifuged at 11,000×g for 10 min. The cytoplasmic supernatant (C) and nuclear pellet (N) were similarly prepared for SDS-PAGE. Each lane of a 9% polyacrylamide gel was loaded with material recovered from 1.5×10^5 cells. After electrophoresis the gel was stained with Coomassie brilliant blue, destained, dried and photographed. The positions of the UL5, UL8 and UL52 proteins are indicated.

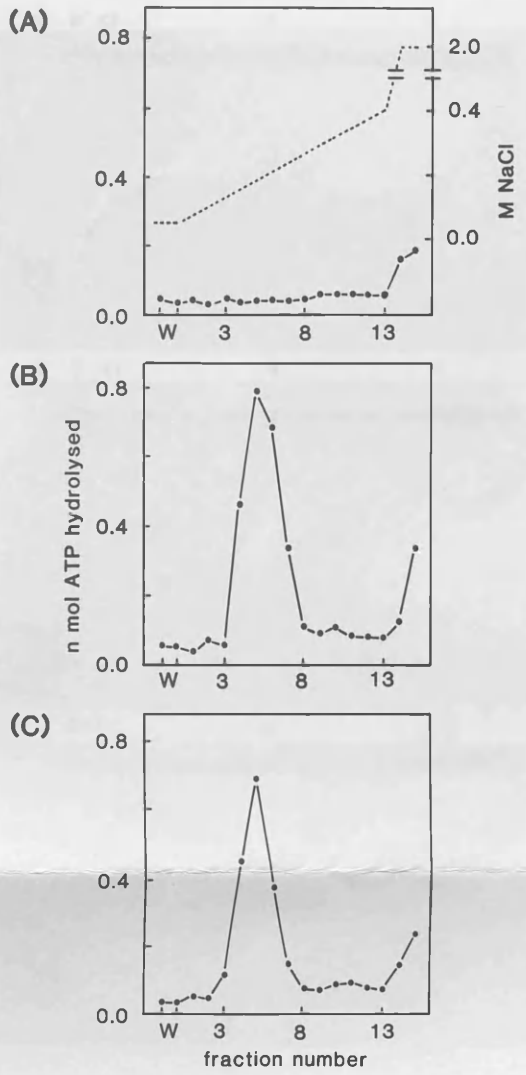

Fig. 2. Assay of phosphocellulose column fractions for ATPase activity. Extracts were prepared from cells infected with AcNPV (panel A), AcUL5 plus AcUL8 plus AcUL52 (panel B) or AcUL5 plus AcUL52 (panel C) and fractionated on phosphocellulose columns as described in the text. The ATPase activities of the final 2 fractions of the wash (W) and the gradient fractions (1−15) were determined. The profile of the NaCl gradient is shown in panel A. One plate of each set of 3 had been labelled with ^{35}S-methionine.

recombinant viruses were therefore performed on extracts prepared 30 h p.i. Efficient recovery of the over-expressed HSV-1 proteins was achieved by lysing infected cells with NP40 in the presence of either low or high salt concentrations. The resulting extracts (cytoplasmic or total cellular respectively) were centrifuged at high speed prior to column chromatography.

Fractionation of ATPase activities by phosphocellulose chromatography

Total cellular extracts were prepared from Sf cells infected with AcNPV or the three recombinant viruses AcUL5, AcUL8 and AcUL52 either alone or in all possible combinations. After dialysis the samples were fractionated on phosphocellulose columns as described in Materials and Methods. Fractions were collected and assayed for ATPase activity in the presence of activated calf thymus DNA.

Comparison of Fig. 2 panels A and B reveals that a distinct peak of ATPase activity, not present in cells infected with the vector, AcNPV, was induced in cells triply infected with recombinant viruses AcUL5, AcUL8 and AcUL52. This peak of activity eluted at 150−200 mM NaCl, a value similar to that previously reported for the HSV-1 helicase-primase complex (150 mM; ref. 25), and was dependent upon the presence of DNA in the assay.

The ATPase profiles obtained from cells infected with AcUL5, AcUL8 or AcUL52 alone or with AcUL5 plus AcUL8 or AcUL8 plus AcUL52 were each almost identical to that obtained with the control AcNPV extract (data not shown). In contrast, a distinct peak of ATPase activity was obtained from cells which received AcUL5 and AcUL52 in combination (panel C), and this activity eluted between the same salt concentrations as the activity from triply infected cells. The expression of the HSV-1 UL8 product is therefore not required for the induction of the novel DNA-dependent ATPase.

DNA helicase activity of phosphocellulose column fractions

Fractions from the phosphocellulose columns described above

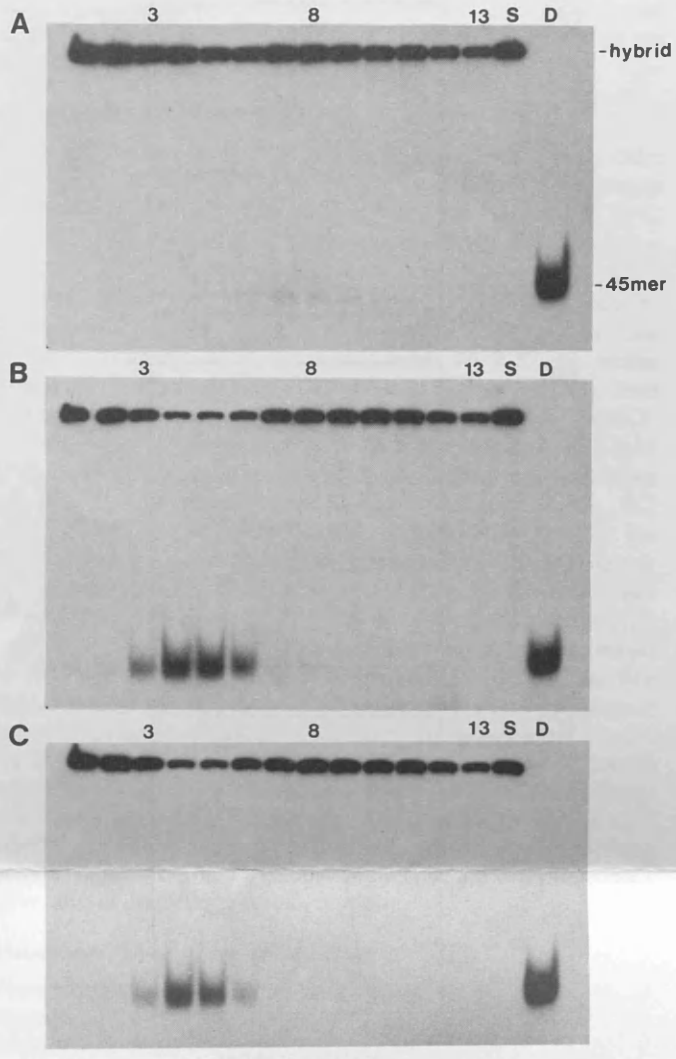

Fig. 3. Assay of phosphocellulose column fractions for DNA helicase activity. Helicase activity was determined for gradient fractions 1–13 of the 3 sets of extracts described in Fig. 2 (panel A, AcNPV; panel B, AcUL5 plus AcUL8 plus AcUL52; panel C, AcUL5 plus AcUL52). The reaction products were analysed on a 10% polyacrylamide gel. Marker lanes contain undenatured (S) or heat denatured (D) helicase substrate, and the positions of the undenatured hybrid substrate and displaced 45 base oligonucleotide are indicated.

were also assayed for DNA helicase activity using a substrate consisting of a synthetic oligonucleotide annealed to single-stranded M13mp18 DNA and containing a 3′ single-stranded tail.

Fig. 3, panel A shows that fractions 6–8 from cells infected with the vector AcNPV showed a low level of displacement of labelled oligonucleotide indicative of helicase activity. (Small variations in the amount of hybrid substrate present at the top of the gel probably result from the action of nucleases). Very similar patterns were also obtained with the extracts from cells singly infected with AcUL5, AcUL8 or AcUL52 or co-infected with AcUL5 plus AcUL8 or AcUL8 plus AcUL52 (data not shown). Major novel peaks of helicase activity were however obtained from cells infected with all three recombinant viruses or a combination of AcUL5 and AcUL52 (panels B and C). In both cases maximum helicase activity was present in fractions 4–6, coincident with the peak of ATPase activity (Fig. 2). Therefore, as with the ATPase activity, the novel DNA helicase activity does not require the UL8 protein.

Presence of HSV-1 specified proteins in phosphocellulose column fractions

The polypeptides present in phosphocellulose column fractions were analysed by SDS-PAGE. Fig. 4, panel A shows the pattern obtained with extracts from cells infected with AcNPV, and panels B and C the corresponding analysis of fractions from cells triply infected with AcUL5, AcUL8 and AcUL52 or doubly infected with AcUL5 and AcUL52. Bands corresponding to the expressed HSV-1 proteins are indicated. For both sets of recombinant virus infections the HSV-1 proteins co-eluted and were present in maximum amounts in fractions 4–6, coincident with the peaks of ATPase and helicase activity. In single infections the UL5, UL8 and UL52 polypeptides each eluted at a higher NaCl concentration (200–350 mM and in the 2 M wash) than the peaks of enzymatic activity described above (data not shown). The amounts of UL5 and UL52 protein recovered in the peak fractions from the double infection (panel C) appear to be slightly reduced compared with the triple infection (panel B) and this may possibly account for the slightly lower levels of ATPase and helicase activities observed in the doubly infected cells (Figs. 2 and 3).

DISCUSSION

The results presented in this paper show that HSV-1 polypeptides synthesised in insect cells by means of a baculovirus expression vector exhibit two activities associated with DNA strand-unwinding which are intrinsic to the viral helicase-primase complex. In agreement with the recent report of Dodson *et al.* (17) novel DNA-dependent ATPase and DNA helicase activities were induced in Sf cells triply infected with recombinant viruses expressing the UL5, UL8 and UL52 gene products, and these activities co-fractionated with the HSV-1 polypeptides. These results, and further purification and sizing of the enzyme by Dodson *et al.* (17) indicate that the UL5, UL8 and UL52 proteins synthesised in insect cells are able to assemble into a functional complex as occurs in cells infected with HSV-1 (26).

In testing the possible combinations of single and double infections with AcUL5, AcUL8 and AcUL52 we demonstrated that the novel ATPase and helicase activities were also detected in cells in which only the UL5 and UL52 proteins were expressed. The presence of the UL8 protein therefore appears to have no significant effect upon the expression of the HSV-1 ATPase and helicase activities. Two observations suggest that complex formation between the UL5 and UL52 proteins is important for the ATPase and helicase activities. Firstly, phosphocellulose column fractions from singly infected cells which were known to contain the UL5 or UL52 polypeptide exhibited neither enzymatic activity, and secondly when the two proteins were co-expressed they co-eluted from the column at a salt concentration lower than that at which either eluted when expressed alone. The UL5 and UL52 proteins present in fractions 8–13 (Fig. 4, panels B and C), which exhibited insignificant ATPase or helicase activity (Figs. 3 and 4), probably represent uncomplexed polypeptide molecules.

Examination of the amino acid sequence encoded by the UL5 gene led to the prediction that the protein might function as a helicase (36,37) and recent experiments confirm its presence in the viral helicase-primase complex (17,26 and this manuscript).

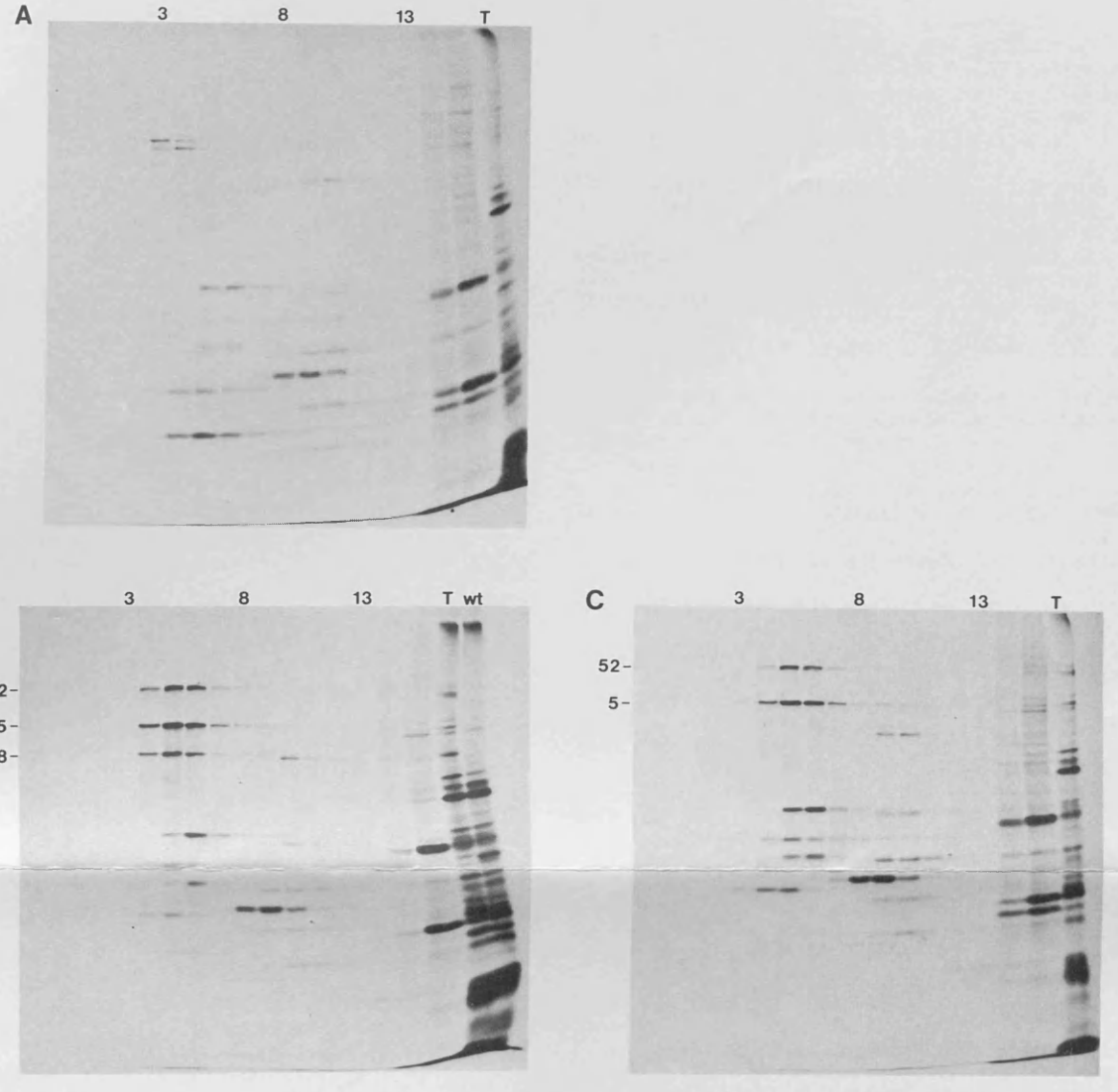

Fig. 4. SDS-PAGE of phosphocellulose column fractions. The final 2 fractions of the wash and gradient fractions 1–15, described in Fig. 2, were analysed by SDS-PAGE (panel A, AcNPV; panel B, AcUL5 plus AcUL8 plus AcUL52; panel C, AcUL5 plus AcUL52). Lanes labelled T contain total proteins present in the relevant extracts prior to phosphocellulose chromatography and wt (panel B) the corresponding extract from cells infected with AcNPV. The positions of the UL5, UL8 and UL52 proteins are indicated.

The reason why we did not detect helicase activity with UL5 protein in the absence of the UL52 product, or the role of the UL52 polypeptide in potentiating this activity are unknown.

An important question now concerns the role of the UL8 protein present alongside the UL5 and UL52 polypeptides in the helicase-primase complex of HSV-1 infected cells. The most attractive hypothesis is that its presence is necessary for the primase function of the complex (26). Alternatively, it may play an important role in formation, translocation or stability of the complex under normal conditions of infection (which are at a temperature 9°C higher than used for the growth of Sf cells in these studies). Finally, we can not rigorously exclude the unlikely possibility that an Sf cell or AcNPV protein can substitute for the UL8 protein within a functional complex.

Examination of Fig. 4, however, provides no indication that this may have occurred. Our current experiments aim to distinguish between these possibilities.

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REFERENCES

1. McGeoch, D.J., Dalrymple, M.A, Davison, A.J., Dolan, A., Frame, M.C., McNab, D., Perry, L.J., Scott, J.E. and Taylor, P. (1988) J. Gen. Virol. **69**, 1531–1574.
2. Wu, C.A., Nelson, N.J., McGeoch, D.J. and Challberg, M.D. (1988) J. Virol. **62**, 435–443.

3. McGeoch, D.J., Dalrymple, M.A., Dolan, A., McNab, D., Perry, L.J., Taylor, P. and Challberg, M.D. (1988) J. Virol. **62**, 444–453.
4. Challberg, M.D. and Kelly, T.J. (1989) Ann. Rev. Biochem. **58**, 671–717.
5. Chartrand, P., Stow, N.D., Timbury, M.C. and Wilkie, N.M. (1979). J. Virol. **31**, 265–276.
6. Conley, A.J., Knipe, D.M., Jones, P.C. and Roizman, B. (1981) J. Virol. **37**, 191–206.
7. Purifoy, D.J.M. and Powell, K.L. (1981) J. Gen. Virol. **54**, 219–222.
8. Matz, B., Subak-Sharpe, J.H. and Preston, V.G. (1983) J. Gen. Virol. **64**, 2261–2270.
9. Weller, S.K., Carmichael, E.P., Aschman, D.P., Goldstein, D.J. and Schaffer, P.A. (1987) Virology **161**, 198–210.
10. Carmichael, E.P., Kosovsky, M.J. and Weller, S.K. (1988) J. Virol. **62**, 91–99.
11. Marchetti, M.E., Smith, C.A. and Schaffer, P.A. (1988) J. Virol. **62**, 715–721.
12. Goldstein, D.J. and Weller, S.K. (1988) J. Virol. **62**, 2970–2977.
13. Powell, K.L., Littler, E. and Purifoy, D.J.M. (1981) J. Virol. **39**, 894–902.
14. Powell, K.L. and Purifoy, D.J.M. (1977) J. Virol. **24**, 618–626.
15. Parris, D.S., Cross, A., Haarr, L., Orr, A., Frame, M.C., Murphy, M., McGeoch, D.J. and Marsden, H.S. (1988) J. Virol. **62**, 818–825.
16. Olivo, P.D., Nelson, N.J. and Challberg, M.D. (1989) J. Virol. **63**, 196–204.
17. Dodson, M.S., Crute, J.J., Bruckner, R.C. and Lehman, I.R. (1989) J. Biol. Chem. **264**, 20835–20838.
18. Olivo, P.D., Nelson, N.J. and Challberg, M.D. (1988) Proc. Natl. Acad. Sci. USA **85**, 5414–5418.
19. Weir, H.M., Calder, J.M. and Stow, N.D. (1989) Nucl. Acids. Res. **17**, 1409–1425.
20. Vaughan, P.J., Purifoy, D.J.M. and Powell, K.L. (1985) J. Virol. **53**, 501–508.
21. Gallo, M.L., Jackwood, D.H., Murphy, M., Marsden, H.S. and Parris, D.S. (1988) J. Virol. **62**, 2874–2883.
22. Crute, J.J. and Lehman, I.R. (1989) J. Biol. Chem. **264**, 19266–19270.
23. Weller, S.K., Lee, K.J., Sabourin, D.J. and Schaffer, P.A. (1983) J. Virol. **45**, 354–366.
24. Quinn, J.P. and McGeoch, D.J. (1985) Nucl. Acids Res. **13**, 8143–8163.
25. Crute, J.J., Mocarski, E.S. and Lehman, I.R. (1988) Nucl. Acids Res. **16**, 6585–6596.
26. Crute, J.J., Tsurumi, T., Zhu, L., Weller, S.K., Olivo, P.D., Challberg, M.D., Mocarski, E.S. and Lehman, I.R. (1989) Proc. Natl. Acad. Sci. USA **86**, 2186–2189.
27. Smith, G.E., Summers, M.D. and Fraser, M.J. (1983) Mol. Cell Biol. **3**, 2156–2165.
28. Matsuura, Y., Possee, R.D., Overton, H. and Bishop, D.H.L. (1987) J. Gen. Virol. **68**, 1233–1250.
29. Miller, L.K. (1988) Ann. Rev. Microbiol. **42**, 177–199.
30. Overton, H.A., Ihara, T. and Bishop, D.H.L. (1987) Virology **157**, 338–350.
31. Elliott, R.M. and McGregor, A. (1989) Virology **171**, 516–524.
32. Stow, N.D., McMonagle, E.C. and Davison, A.J. (1983) Nucl. Acids Res. **11**, 8205–8220.
33. Clark, R., Lane, D.P. and Tjian, R. (1981) J. Biol. Chem. **256**, 11854–11858.
34. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Publications, Cold Spring Harbor.
35. Marsden, H.S., Stow, N.D., Preston, V.G., Timbury, M.C. and Wilkie, N.M. (1978) J. Virol. **28**, 624–642.
36. Gorbalenya, A.E. and Koonin, E.V. (1989) Nucl. Acids Res. **17**, 8413–8435.
37. Hodgman, T.C. (1988) Nature **333**, 22–23.