PURIFICATION AND CHARACTERISATION OF THE HERPES SIMPLEX VIRUS TYPE 1 DNA REPLICATION PROTEIN UL8

By

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SUMMARY

The genome of herpes simplex virus type 1 (HSV-1) contains seven genes whose products are directly involved in viral DNA replication. These are UL5, UL8, UL9, UL29, UL30, UL42 and UL52. I investigated the products of genes UL5, UL8, UL9, and UL52, as these were the least characterised. Peptides were synthesised with sequences corresponding to portions of the amino acid sequence of these proteins and the peptides were used to generate antisera in rabbits. The peptides produced sera reacting with two of the four proteins — UL8 and UL9. Sera were produced that reacted with both the N and C termini of UL8.

The project now focused on UL8 protein which was purified to homogeneity to allow its further characterisation. An existing recombinant baculovirus/*Spodoptera frugiperda* expression system was used as a source of the protein. The protein was extracted from the cells in a soluble form and was stable in the extraction buffer (neither denatured nor proteolysed) for at least 4 hours at room temperature. The protein was also stable during freezing and thawing, dilution, and incubation at 4 °C. A rapid and simple purification scheme was developed using DEAE sepharose and phenyl sepharose columns. Ten per cent glycerol was required in all buffers for successful chromatography. The purification produced UL8 more than 95% pure in only $3^1/_2$ hours beginning with the crude extract. The pure protein was intact and free from degradation products as determined by reaction with the N and C termini-specific sera.

Gel filtration chromatography established that the molecular mass of the purified protein was 75 +/-10 kD, indicating that it is a monomer in solution. (The protein's predicted molecular mass is 80 kD.)

The possible interaction of UL8 with nucleic acids was investigated using a gel band shift assay. Despite extensive investigation of different conditions this revealed no interaction of UL8 with fully or partially duplex DNA, single stranded DNA, or with a DNA/RNA hybrid.

An ELISA assay was established using purified UL8 as antigen. Optimum conditions were found for detecting antibodies to UL8 so that the assay could be used to screen hybridoma cell lines for UL8-specific antibody production. A cell fusion was performed using splenocytes from a mouse immunised with purified UL8 and this produced eighty one hybridoma lines of which 25 produced antibodies specific for UL8 in the ELISA assay. From these, nineteen ascites fluids were produced which contained antibodies specific for UL8 protein (again measured in ELISA assays). Six of these ascitic fluids reacted with UL8 in Western blotting experiments and seven immune precipitated UL8 from solution. Rabbit polyclonal sera specific for the protein were also produced.

Possible future work is discussed as well as the implications of these experiments for studies of HSV-1 DNA replication.

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Naturally the results presented in this thesis are the product of the author's own work, except where noted. The author was the recipient of a Medical Research Council award for training in research.

It was a slow day
And the sun was beating on the soldiers by the side of the road
There was a bright light
A shattering of shop windows
The bomb in the baby carriage was wired to the radio

These are the days of miracle and wonder
This is the long distance call
The way the camera follows us in slo-mo
The way we look to us all
The way we look to a distant constellation
That's dying in a corner of the sky
Medicine is magical and magical is art
The Boy in the Bubble
And the baby with the baboon heart

These are the days of miracle and wonder
And don't cry baby, don't cry
Don't cry

Paul Simon

CONTENTS

MATERIALS

METHODS

TABLES AND FIGURES

Introduction.

Results.

ABBREVIATIONS

aa	amino acid
AcNPV	*Autographa californica* nuclear polyhedrosis virus.
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
DTT	dithiothreitol
dUTP	deoxyuridine triphosphate
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunoabsorbant assay
HCMV	human cytomegalovirus
HHV	human herpesvirus
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
HSV	herpes simplex virus
IE	immediate early
IU	International units
M	molar
mM	millimolar
M_r	relative molar mass
ng	nanograms
nm	nanometres
NP40	non-ionic detergent P40
PAGE	polyacrylamide gel electrophoresis
pfu	plaque forming units
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecyl sulfate
Sf	*Spodoptera frugiperda*
TBS	tris buffered saline
TFA	trifluoroacetic acid
tris	tris(hydroxymethyl)aminomethane
TTBS	tris buffered saline-tween 20
VZV	varicella zoster virus

AMINO ACIDS

	Three letter code	One letter code
Alanine	Ala	A
Asparagine	Asn	N
Aspartate	Asp	D
Arginine	Arg	R
Cysteine	Cys	C
Glutamate	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

INTRODUCTION

The work presented in this thesis concerns DNA replication in herpes simplex virus type 1 (HSV-1). The introduction will describe relevant aspects of HSV-1 biology with emphasis on DNA replication. DNA replication in some other systems will also be discussed.

I) TAXONOMY OF THE HERPESVIRIDAE AND MEDICAL ASPECTS OF HSV-1 INFECTION.

i) The Herpesviridae.

Herpesviruses are distributed very widely in nature, their host range extending from humans to fish. It is possible that herpes-type viruses are present in all forms of eukaryotes. Herpesviruses are therefore of great antiquity and may have evolved their basic form long before the evolution of the hosts they currently parasitise.

Almost 100 viruses have been sufficiently well characterised to merit inclusion in the family Herpesviridae. Membership of the family is based on three types of characteristics: the composition and morphology of the virus particle; aspects of virus replication; and more general biological properties. An outline of the defining characteristics is found below (adapted from: International Committee on the Taxonomy of Viruses, 1981).

1) Properties of the virus particle:

Nucleic acid: a double stranded linear DNA molecule of relative molar mass (M_r) 80-150x10^6.

Proteins: more than 20 structural proteins are found in the virion.

Morphology: the virion is 120-200 nm in diameter and has three components: an icosahedral DNA-containing capsid 100-110 nm in diameter; a bilayered membrane containing surface projections; and an amorphous layer of variable thickness between these two structures called the tegument.

2)Virus replication:

The virus envelope binds to and fuses with the cell membrane, the capsid enters the cytoplasm and a nucleoprotein complex is transported to the nucleus. DNA replication is nuclear and infectious particles are produced by the budding of the capsid through a membrane, usually the nuclear membrane.

3) Biological properties:

These are very variable but the ability to remain latent in the host is ubiquitous.

The family Herpesviridae is currently divided into three subfamilies the Alpha-, Beta-, and Gammaherpesvirinae based on biological characteristics. However these divisions are rather subjective. A classification based on objective criteria (such as the conservation of genes or gene clusters) has not yet been achieved. The division between the Alpha- and Gammaherpesvirinae is primarily according to their host range *in vitro* and the characteristics of their latent infections. That is, alphaherpesviruses generally have a wide host range *in vitro* and their latent infections are established predominantly in sensory ganglia whereas the gammaherpesviruses have a narrow *in vitro* host range and latency is generally established in lymphoid tissue. The division between the betaherpesviruses and the other groups is based primarily on the length of the betaherpesviruses reproductive cycle and their slowness in producing cytopathology in cell cultures.

Seven human herpesviruses have been identified. These have been designated human herpesviruses 1-7. The first five are also called herpes simplex virus type 1, herpes simplex virus type 2, varicella-zoster virus, Epstein-Barr virus, and human cytomegalovirus respectively. The results of infection with these viruses range from a complete absence of morbidity to life threatening disease.

ii) Medical aspects of HSV-1 infections.

Herpes simplex virus type one is spread in the human population by intimate contact when the virus is juxtaposed to mucous membranes or abraded skin. The virus replicates at the site of infection and latency is established in dorsal root ganglia (sensory ganglia). Most infections are asymptomatic although severe symptoms may result if primary infection occurs during adolescence. The virus occasionally causes life threatening disease.

HSV-1 is found generally in oral sites, in which case latency is established in trigeminal ganglia. It may occur however in genital sites in which case latent infections are established in sacral ganglia. The average time of primary infection varies with socioeconomic status, but is generally during childhood (Whitley, 1990). Recurrent disease (following reactivation of virus from latent sites) occurs with varying frequency. Re-infection with the same or a different strain of virus may also occur. Herpes simplex virus type 2 (HSV-2) is transmitted sexually between genital sites.

The most common disease resulting from HSV-1 infection is herpes labialis a blistering of the lips around the infection site. The lesions are usually self limiting (that is

immune system limited) except in the immuno-compromised host in which case the disease may become disseminated widely around the body.

A more serious consequence of infection is herpes simplex keratoconjunctivitis following eye infection of which 300,000 cases are diagnosed each year in the United States (approximately 1 per 850 members of the population per year) a figure likely to be equalled or exceeded around the world. In this case recurrent disease may cause opacification of the cornea and, again in the United States, HSV keratoconjunctivitis is second only to accidental injury as a cause of corneal blindness (Whitley, 1990).

Infections of the central nervous system are the most serious outcome of HSV-1 infection. They are the most common cause of sporadic, fatal encephalitis in the US (Olson *et al*, 1967) with a rate of 1 per 200,000 people per year (Whitley *et al*, 1981). The exact symptoms reflect the areas of the brain involved but include a focal encephalitis usually localised to the temporal lobe, fever, altered consciousness, bizarre behaviour and disordered reasoning. The route by which the virus enters the brain is not known but the localised nature of the initial disease makes a blood borne infection unlikely. This being the case, direct neuronal transmission via trigeminal or olfactory tracts remains a possibility. Innoculation of the virus by the olfactory route in experimental animals has led to a fatal encephalitis similar to that seen in humans (Schlitt *et al*, 1986; 1988).

Antiviral therapy for HSV-1 infection.

Early attempts to develop effective antiviral therapy for HSV were spurred by an increasing incidence of genital HSV infections. The majority of effective drugs developed so far, and all those currently in clinical use, are nucleoside analogues.

Vidarabine, a purine nucleoside analogue, is used intravenously for neonatal herpes simplex infections and for HSV encephalitis, and topically for HSV keratitis. It is phosphorylated to its active form by cellular kinases and is therefore effective against thymidine kinase negative strains of HSV that are resistant to acyclovir (see below). The drug inhibits viral DNA polymerase at concentrations lower than those inhibiting cellular polymerases (Shipman *et al*, 1976) and its mechanism of action is through the viral polymerase (Coen *et al*, 1982). Due to the development of acyclovir the more toxic vidarabine is now less commonly used.

Acyclovir is used intravenously or orally for genital herpes, neonatal HSV, HSV encephalitis and HSV in the immuno-compromised patient. Acyclovir is a guanosine analogue and is very specific for HSV and VZV. The drug is activated specifically to the monophosphate by the viral thymidine kinase (TK) and is the first drug to require a viral enzyme for activation (Elion, 1982). In addition to this specificity it also has a higher affinity for the viral DNA polymerase than cellular DNA polymerases and inactivates the viral polymerase by trapping it in a complex with the terminated DNA chain (St Clair *et al*, 1980; Reardon and Spector, 1989). Resistance can develop *in vivo* by mutations in the

TK gene or the UL30 (*pol*) gene (Coen and Schaffer, 1980; Schnipper and Crumpacker, 1980).

Foscarnet (trisodium phosphonoformate), a pyrophosphate analogue also acting through the viral DNA polymerase, has been used to treat recurrent herpes labialis and genitalis. It is of particular interest clinically due to its activity against both HIV and herpesviruses and is in clinical trials for use with AIDS and AIDS-related complex patients (Hirsch and Kaplan, 1990).

A number of triterpenoid compounds have been shown to possess anti-viral activity *in vitro* including activity against herpesviruses (Dargan and Subak-Sharpe, 1985; Pompei *et al*, 1979). These include glycyrrhyzin, carbenoxolone sodium and cicloxolone sodium. Their mechanism of action involves binding to cell and viral membranes and they have been shown to reduce the yield of viral particles produced in cell culture systems and also to reduce the viability of these particles (Dargan and Subak-Sharpe, 1985). Trials have also shown that carbenoxolone sodium is beneficial for treatment of HSV infections in man (Poswillo and Roberts, 1981). Some of these compounds may eventually be used regularly for the treatment of HSV infections.

There is likely to be continued interest in the development of antivirals active against HSV infections especially due to the seriousness of such infections for immuno-compromised patients and the appearance of strains resistant to the drugs currently in use.

Immunisation against HSV.

Immunisation to prevent primary or recurrent HSV infection is an attractive prospect, but no vaccine is currently in use. Vaccination may be possible by several different methods: e.g. a subunit vaccine using purified viral glycoproteins; a killed whole virus vaccine or a live attenuated vaccine. As HSV recurs in the presence of high titres of antibody a vaccine must stimulate both humoral and cell mediated immunity. Considerable effort is being expended, in both academic and pharmaceutical laboratories, in the development of effective vaccines.

II) THE LIFE CYCLE OF HSV-1.

i) Virion structure.

The HSV-1 virion consists of four parts: the icosahedral capsid containing the viral genome; a surrounding layer called the tegument; and the envelope, an outer membrane studded with the viral glycoproteins. The full complement of virion proteins has not yet been established. Early studies suggested that there were approximately 33 virion proteins (Heine *et al*, 1974; Marsden *et al*, 1976). Of these, 7 have been shown to be capsid proteins, probably 10-15 are integral to or strongly associated with the envelope and five have been located in the tegument. The remainder are presumably components of the tegument or envelope. For a review of the structure of the virion see Dargan, 1986.

The viral genome.

The HSV-1 genome is a linear double stranded DNA molecule whose complete sequence has been determined for the Glasgow 17 *syn*$^+$ strain. The genome of this strain comprises 152,260 base pairs with a base composition of 68.3% G+C (McGeoch *et al*, 1988a; McGeoch *et al*, 1985; Davison and Wilkie, 1981; McGeoch *et al*, 1986; Perry and McGeoch, 1988). The molecule may be viewed as consisting of two covalently linked segments, the long (L) and short (S) regions, each of which comprises a unique sequence flanked by inverted repeats (Sheldrick and Berthelot, 1974). The long repeat (R_L) and the short repeat (R_S) are distinct. The genome also has a terminal redundancy of approximately 400 base pairs termed the *a* sequence which is present, in the opposite orientation, at the joint between the L and S components (Wagner and Summers, 1978). See figure 1. Preparations of HSV-1 DNA contain four sequence-orientation isomers generated by inversion of the unique segments relative to the repeats (Hayward *et al*, 1975; Delius and Clements, 1976; Wilkie, 1976). The significance of this genome organisation is unknown but may be a consequence of the mode of DNA replication. One genome isomer has been chosen arbitrarily as the prototype for purposes of representation.

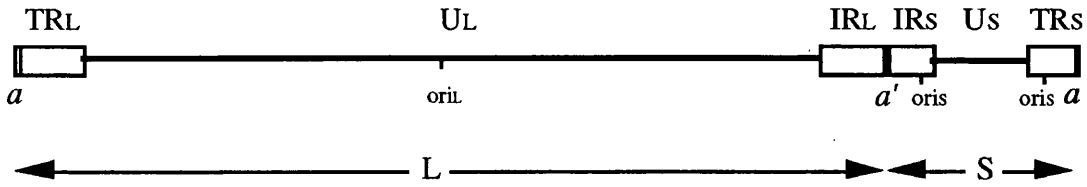

Figure 1. The genome of HSV-1 is shown in the prototype orientation. Unique sequences are represented by a single line and repeated sequences as boxes. The following features are labelled: L: long component; S: short component; *a* and *a'*: the *a* sequence in its two orientations; TRL and IRL: the terminal and internal long repeats respectively; TRs and IRs: the terminal and internal short repeats respectively; UL: the long unique region; Us: the short unique region; ori_L and ori_S: the origins of replication located within the long and short components respectively. After McGeoch *et al*, 1988a.

It was originally proposed (McGeoch *et al*, 1988a) that the genome contained 72 open reading frames encoding 70 distinct proteins. Only a proportion of these genes were predicted without any previous knowledge of coding potential. Many genes had previously been located approximately or precisely through the use of *ts* mutants, intertypic (HSV-1 x HSV-2) recombinants and mRNA mapping. However the availability of the complete genome sequence has greatly facilitated research on HSV-1 and related herpesviruses. Many of the predicted open reading frames are dispensable for virus growth in tissue culture although they are doubtless required for survival in the natural state. Four more distinct genes have since been proposed which encode: a putative latency associated transcript (LAT) product (2 copies, Stevens *et al*, 1987; Wagner *et al*, 1988a; Wagner *et al*, 1988b); UL26.5 (Liu and Roizman, 1991a); UL49A or UL49.5 (Barker and Roizman, 1992; Barnett *et al*, 1992); and ICP34.5 (2 copies, Ackermann *et al*, 1986b; Chou and Roizman, 1986).

The genome also contains three origins of DNA replication: ori_S located in the short repeat and ori_L located near the middle of UL. These were located by the following workers: (Stow, 1982; Mocarski and Roizman, 1982a; Stow and McMonagle, 1983; Spaete and Frenkel, 1982; Weller *et al*, 1985). See HSV DNA replication section.

The capsid.

The HSV-1 capsid appears in the electron microscope as an icosahedron with 150 hexavalent and 12 pentavalent capsomeres (Wildy *et al*, 1960). The capsid appears to have no internal structure (Gibson and Roizman, 1972; Schrag *et al*, 1989; Booy *et al*, 1991).

Mature capsids from purified virions comprise six proteins of which five were identified by Gibson and Roizman (1972) - VP5, VP19C, VP21*, VP23 and VP24, and a sixth by Heilman *et al* (1979) and Cohen *et al* (1980) - p12 or VP26. One form of immature capsid contains in addition a seventh protein VP22a (see later). Firm gene assignments have been made for all but one of the capsid proteins (see table 1).

Table 1. Capsid proteins.

Protein	**Molar mass (kD)**	**Gene**
VP5	155	UL19
VP19C	53	UL38
VP21	45	UL26?
VP22a	38	UL26.5
VP23	36	UL18
VP24	24	UL26
VP26	12	UL35

References for gene assignments are as follows: VP5: Costa *et al*, 1984; Davison and Scott, 1986b. VP19C and VP23: Rixon *et al*, 1990. VP21 (proposed as C-terminus of UL26) and VP24 (N-terminus of UL26): Davison *et al*, 1992. VP22a: Liu and Roizman, 1991b. VP26: McNabb & Courtney, 1992.

The functions of capsid proteins have not been defined with the exception of VP5 the major capsid protein, which is the major component of the capsid hexamers (Vernon *et al*, 1981). VP5, VP19C and VP23 have been shown to be located on the surface of the capsid while VP21, VP22a and VP24 are internal (Newcomb and Brown, 1989; 1991).

* It should be noted that VP21 is actually present only in immature capsids (Booy *et al.*, 1991).

① Sullivan & Smith, (1988)

② Roop *et al.*, (1993)

③ Glycoprotein M is also found in the virion: Baines & Roizman, (1993); MacLean *et al.*, (1993).

The virion envelope.

The envelope of the HSV-1 virion consists of a lipid membrane studded with viral proteins, mostly glycoproteins. Prominent spikes of at least three different types have been seen on the surface of virions (Wildy *et al*, 1960; Stannard *et al*, 1987). Each type of spike differed in size, shape and distribution in the virion membrane. Glycoproteins B, C and D were shown to be constituents of these spikes using monoclonal antibodies, each glycoprotein being present in only one type of spike (Stannard *et al*, 1987).

The number of envelope proteins is not finally established but there are at least 9 viral glycoproteins all of which are present in the virion envelope: gB, gC, and gD (Spear, 1976); gE (Baucke and Spear, 1979; Para *et al*, 1980); gG (Marsden *et al*, 1976; 1984; Roizman *et al*, 1984; Richman *et al*, 1986; Ackermann *et al*, 1986a; Frame *et al*, 1986a); gH (Buckmaster *et al*, 1984, Showalter *et al*, 1981); gI (Johnson and Feenstra, 1987; Longnecker *et al*, 1987; Johnson *et al*, 1988); gK (Hutchinson *et al*, 1992b; Ramaswarmy and Holland, 1992); and gL (Hutchinson *et al*, 1992a). Glycosylation of HSV-1 glycoproteins is predominantly N-linked (Spear, 1985; Pizer *et al*, 1980) although O-linked glycosylation does occur (Oloffson *et al*, 1981; Wenske and Courtney, 1983). In particular glycoprotein G of HSV-2 is heavily O-glycosylated (Serafini-Cessi *et al*, 1985). Cellular enzymes are thought to be responsible for these modifications. The role of carbohydrate moieties in glycoprotein function has not been defined. However, glycosylation plays some role in infectivity as virions produced in the presence of tunicamycin, which prevents N-linked glycosylation, are not infectious. Virions containing partially glycosylated glycoproteins are infectious (Kousoulas *et al*, 1983).

In addition to roles in attachment and penetration (see later) several of the viral glycoproteins exhibit other interesting properties. Glycoprotein C binds to the C3b fragment of the third component of complement (Friedman *et al*, 1984; Eisenberg *et al*, 1987; McNearney *et al*, 1987). Also gC can accelerate the decay of the C3 convertase of the alternative complement pathway (Fries *et al*, 1986). This activity could account for the protection against complement-mediated damage to virions and infected cells observed when gC is present but not when it is absent (Harris *et al*, 1990; McNearney *et al*, 1987). Although these results, obtained *in vitro*, suggest a role for gC in protecting virions and infected cells from the immune system, this has yet to be demonstrated *in vivo*.

An antibody binding activity is induced in cells by HSV-1 infection (Watkins, 1964). This IgG Fc binding activity was partially purified from infected cells and virions by Baucke and Spear (1979) and Para *et al* (1980) and shown to be a glycoprotein which was designated gE. The gene encoding gE was mapped to genome coordinates 0.924 - 0.951 (Para *et al*, 1982; Hope *et al*, 1982; Lee *et al*, 1982), which corresponds to gene US8 (McGeoch *et al*, 1985). Subsequently another glycoprotein was identified that,

* It has recently been reported that UL10 is present in virions as noted earlier: Baines & Roizman, 1993; MacLean *et al.*, 1993.

† plus five phosphoproteins

together with gE, bound IgG (Johnson and Feenstra, 1987; Johnson *et al*, 1988) and this was designated gI (Longnecker *et al*, 1987). Its gene was identified as US7.

No specific *in vivo* role for Fc binding has been demonstrated. In fact the Fc portion of antibody may not even be the relevant ligand, since homologous domains are highly conserved throughout the immunoglobulin superfamily. However, Adler *et al* (1978) found that pre-incubation of HSV-1 infected cells with non-immune IgG greatly reduced complement-mediated and cell-mediated lysis of the cells. This suggests a role for the Fc-binding complex in protecting virions and infected cells from immune attack.

With regard to non-glycoprotein envelope components, a number of genes were predicted by McGeoch *et al* (1988a) to encode membrane spanning proteins: UL3, UL10, UL20, UL34, UL43, and UL45. These are either singly or multiply hydrophobic in portions of their predicted amino acid sequence and may represent virion envelope constituents. UL3 and UL43 also have potential N-linked glycosylation sites. The UL45 gene product has been shown to be present in virions (McLean, 1990) but there have been no reports whether any of the other proteins are present.

The tegument.

The tegument is defined as the region between the capsid and the envelope and comprises approximately 65% of the virion volume (data from Schrag *et al*, 1989). It contains a number of proteins important to the virus life cycle. The total number of proteins present is unknown. Seen in the electron microscope the tegument has no apparent structure but its size is consistent between preparations and is independent of cell type leading to the conclusion that this feature is determined by the virus (McCombs *et al*, 1971). It is not known whether the tegument has any structure but the constituents may have some ability to self-associate. This idea is supported by analysis of the recently discovered L particles (Szilagyi and Cunningham, 1991) which consist of tegument and envelope. Disruption of the L particles with detergent leaves amorphous blobs of material thought to comprise tegument proteins alone (McLauchlan and Rixon, 1992).

To date five proteins have been identified in the tegument including the α-trans inducing factor (αTIF, also known as Vmw65 or VP16), the product of gene UL48 (Post *et al*, 1981; Cordingley *et al*, 1983; Batterson and Roizman, 1983; Campbell *et al*, 1984; Pellett *et al*, 1985; and Dalrymple *et al*, 1985); VHS the virion host shut off protein the product of gene UL41 (Sydiskis and Roizman, 1966; Fenwick and Walker, 1978; Kwong *et al*, 1988; McGeoch *et al*, 1988a); Vmw 81/82 the product of gene UL47 (McLean *et al*, 1990); and a 10kD phosphoprotein, the product of gene US9 (Frame *et al*, 1986b).

A number of investigators have reported protein kinase activity associated with the tegument or capsid of HSV-1 (Rubenstein *et al*, 1972; Stevely *et al*, 1985; Lemaster and Roizman, 1980) however the activities reported had very diverse properties and it was uncertain whether they were encoded by the virus. The first viral protein kinase to be

identified (the US3 gene product) appears to be distinct from the virion associated protein kinase (Frame *et al*, 1987; Purves *et al*, 1987). The UL13 gene product was predicted to be a serine and threonine specific protein kinase on the basis of its amino acid sequence (Smith and Smith, 1989). The gene has homologues in all three subgroups of the herpesviruses. It has since been shown that UL13 (a virion protein) is a protein kinase and that its major reaction is autophosphorylation (Cunningham *et al*, 1992; Overton *et al*, 1992).

ii) Entry into the cell.

Entry of HSV-1 into cells occurs by attachment of the virion to cell surface receptors followed by penetration via *p*H-independent fusion of the viral and cellular membranes (Morgan *et al*, 1968; Para *et al*, 1980; Fuller and Spear, 1987; Wittels and Spear, 1990). The nucleocapsid and the tegument components then enter the cell cytoplasm.

Initial attachment of the virus occurs through binding of viral glycoprotein gC to cell surface heparan sulfate proteoglycans. A number of lines of evidence support this conclusion:

1) Heparan sulfate is required for efficient entry of virus as cells enzymatically stripped of heparan sulfate or unable to produce heparan sulfate are very difficult to infect with HSV-1. Virions also bind to heparin (a compound structurally very similar to heparan sulfate) and heparin and heparin binding proteins also inhibit HSV-1 adsorption and plaque formation (WuDunn and Spear, 1989; Herold *et al*, 1991; Shieh *et al*, 1992).

2) Herpes simplex virus has a very broad host range *in vitro* suggesting that any receptor must be both ubiquitous and highly conserved, criteria satisfied by heparan sulfate. In addition no other receptor has been demonstrated.

3) Virions lacking gC bind more slowly to cells although they remain infectious (Langeland *et al*, 1990; Herold *et al*, 1991) and gC is the HSV-1 glycoprotein with the strongest heparin binding activity (Herold *et al*, 1991).

4) gC forms part of the longest spikes seen projecting from HSV-1 virions (Stannard *et al*, 1987).

The role of the C3b binding activity of gC in attachment is uncertain but the functions are likely to be unrelated. gC^- virus can still adsorb to cells via heparan sulfate. This and the fact that gB binds to heparin in affinity chromatography experiments suggests that this secondary binding is mediated by gB (Herold *et al*, 1991). Comparison of the properties of gC^- gB^+ and gC^- gB^- viruses should clarify this point. gD also appears to have some role in adsorption in addition to its established role in penetration (Fuller and Spear, 1985).

Earlier reports (Kaner *et al*, 1990; Baird *et al*, 1990) that the high affinity receptor for basic fibroblast growth factor could serve as a portal of entry for HSV-1 have not been confirmed (Shieh *et al*, 1992; Mirda *et al*, 1992; Muggeridge *et al*, 1992).

Penetration of the cell by fusion of the viral and cellular membranes follows adsorption and is probably mediated by at least 3 components of the virion gB, gD and gH, each of which is required for virus replication in tissue culture (Sarmiento *et al*, 1979; Cai *et al*, 1987; 1988; Ligas and Johnson, 1988; Desai *et al*, 1988). Evidence that gB, gD and gH are all required for penetration comes from studies of mutants producing virions

†As gH and gL form a functional unit and neither can be included in the virion alone, it may be said that both are required for penetration.
Gompels & Minson, (1989); Hutchinson *et al*, (1992a)

lacking one of these proteins. All three types of virions were able to adsorb to cells as strongly as wild-type virus but no plaques were produced (Cai *et al*, 1987; 1988; Desai *et al*, 1988; Fuller and Spear, 1987; Campadelli-Fiume *et al*, 1988; Fuller *et al*, 1989; Ligas and Johnson, 1988; Sarmiento *et al*, 1979). Addition of polyethylene glycol, a chemical fusogen, results in the production of a much greater number of plaques by these viruses. The precise roles of gB, gD and gH in fusion are not known. Fuller and Lee (1992) suggest that gD and gH are involved in the initiation of fusion.

iii) Events immediately after entry.

Upon fusion of the viral and cellular membranes the viral nucleocapsid and the tegument components enter the cell. The nucleocapsid is transported to the nucleus which the genome enters, and the virion host shut-off (VHS) function is activated.

Release of the tegument components of the virion into the host cell cytoplasm allows the virion host shut-off protein to begin functioning. A rapid inhibition of host protein synthesis (Fenwick and Walker, 1978; Nishioka and Silverstein, 1978; Smibert *et al*, 1992) is accompanied by degradation of host mRNA (Fenwick & McMenamin, 1984; Schek and Bachenheimer, 1985). The isolation of shut-off defective mutants showed that the *vhs* gene mapped to gene UL41 (Kwong *et al*, 1988). In addition to the expected effects, inactivation of the *vhs* gene was associated with increased stability of viral mRNAs (Kwong *et al*, 1988; Oroskar and Read, 1989). Degradation of mRNAs is non-specific, affecting host and viral species equally (Nishioka and Silverstein, 1978; Oroskar and Read, 1989) suggesting that VHS operates by a mechanism which involves stimulation of the cellular mRNA degradation machinery.

Nucleocapsid transport to the nucleus is mediated by microtubules (Kristensson *et al*, 1986) presumably using host transport mechanisms. Release of the viral DNA into the nucleus occurs by an unknown mechanism involving the product of gene UL36 (predicted molecular mass 336kD) a function identified using a temperature sensitive mutant (Batterson *et al*, 1983).

iv) Transcription in the infected cell.

Following infection of cells with HSV-1, transcription of cellular genes is greatly reduced whilst transcription of viral genes is strongly activated (Everett, 1987; Smibert and Smiley, 1990). The mechanism by which this process operates is complex and not fully understood. For example a cellular gene may behave differently to an identical gene and promoter combination placed in the viral genome (Smibert and Smiley, 1990). However, viral genes inserted in the cell genome are activated by infecting virus (Sandri-Goldin *et al*, 1983) and plasmid-borne cell or viral genes can be transactivated by viral products (Everett, 1984).

Viral genes are transcribed in the nucleus by cellular RNA polymerase II (Costanzo *et al*, 1977; Ben-Zeev and Becker, 1977). Various classifications of viral genes have been proposed based on their time of synthesis or presumed controls on their transcription (Jean *et al*, 1974; Honess and Roizman, 1974). However, only two classes of viral genes have been defined by experimental criteria: the immediate early (IE) or α genes and the true late genes. Transcription of IE genes is not inhibited by addition of protein synthesis inhibitors (Honess and Roizman, 1974). Transcription of true late genes

is greatly reduced by the inhibition of DNA replication, 95% reduction being suggested as a working definition (Johnson *et al*, 1986). These differences presumably reflect different controls on the transcription of these genes (see below). The remainder of HSV-1 genes have been called early, delayed early, leaky late, β, γ and a number of other names but they do not form a well defined class as each is expressed at different times after infection. For convenience they will be referred to here as early genes.

Transcription of IE genes.

The immediate early proteins and genes of HSV-1 are listed in table 2, with alternative nomenclatures. Transcription of IE genes does not require prior protein synthesis, but is stimulated by the virion tegument component Vmw65 (αTIF or VP16) (Post *et al*, 1981; Batterson and Roizman, 1983; Campbell *et al*, 1984; Pellett *et al*, 1985; Dalrymple *et al*, 1985). When released into the infected cell Vmw65 complexes the

Table 2: Nomenclature of immediate early (IE) proteins and genes.

Proteins	**Molar mass (kD)**	**Genes**
IE175 (Vmw175, ICP4)	175	IE175 (IE3, α4)
IE110 (Vmw110, ICP0)	110	IE110 (IE1, α0)
IE68 (Vmw68, ICP22)	68	US1 (IE4, α22)
IE63 (Vmw63, ICP27)	63	UL54 (IE2, α27)
IE12 (Vmw12, ICP47)	12	US12 (IE5, α47)

cellular transcription factor Oct 1 causing it to bind to the sequence TAATGARAT which is found in the regulatory regions of all IE genes (Mackem and Roizman, 1982; Kristie and Roizman, 1984; Preston, C.M. *et al*, 1984; Gaffney *et al*, 1985; O'Hare and Gooding, 1988; Preston, C.M. *et al*, 1988). Thus activation of IE transcription involves recognition of class-specific cis-acting regulatory signals that are distinct from basal promoter elements. In addition to the genes just mentioned, UL39 (encoding the large subunit of the HSV-1 ribonucleotide reductase) is regulated partly as an immediate early gene (Watson *et al*, 1979; Sze and Herman, 1992)

In addition to activation by Vmw65, IE genes are subject to autoregulation. IE175 down regulates its own promoter by binding across the cap site and may down regulate the promoters of other IE genes as well (O'Hare and Hayward, 1985a; Deluca and Schaffer, 1985; Everett, 1987).

Transcription of other genes; the role of the immediate early gene products.

HSV-1 IE gene products are involved in transactivation of early and true late promoters (see below) but the mechanisms of activation remain unclear as the promoters do not contain obviously conserved class-specific sequences analogous to the IE TAATGARAT (reviewed in Everett, 1987). Some early genes are partially dependent on DNA replication for their transcription, while true late genes are completely or virtually completely dependent on it. DNA replication induces a cis-acting modification of the template that allows true late genes to be transcribed (Johnson and Everett, 1986; Mavromara-Nazos and Roizman, 1987). Expression of true late genes appears also to be influenced by sequences downstream of the gene's TATA box (Mavromara-Nazos and Roizman, 1987; Kibler *et al*, 1991).

Transcription of both early and true late genes during infection is dependent on the continuous presence of functional IE175 the major HSV-1 transcriptional regulator (Preston, 1979; Dixon and Schaffer, 1980; Watson and Clements, 1980). IE175 has also been shown to transactivate the transcription of early and true late gene products in transfection assays (Gelman and Silverstein, 1986; O'Hare and Hayward, 1985a;1985b; Quinlan and Knipe, 1985). IE175 binds to DNA and a consensus sequence has been proposed (Faber and Wilcox, 1986) although the protein can bind to unrelated sites (Imbalazano *et al*, 1990; Tedder *et al*, 1989). The region of the protein involved in DNA binding has been located (DeLuca and Schaffer, 1988; Paterson and Everett, 1988; Wu and Wilcox, 1990). Transactivation by IE175 may be mediated by direct interaction of the protein with DNA or interaction through a cellular protein. IE175 may alternatively have multiple modes of action (Shepard and DeLuca, 1991a; 1991b; Imbalazano *et al*, 1990; Smiley *et al*, 1992).

IE110 is a powerful and wide ranging activator of transcription in transfection based assays (Everett, 1984; O'Hare and Hayward, 1985b; Quinlan and Knipe, 1985; Gelman and Silverstein 1985) and was therefore expected to play a crucial role in virus growth. However a virus with a deletion in the IE110 gene was viable, the only apparent phenotype being poor growth at low multiplicities of infection (Stow and Stow, 1986). It was later found that purified IE110 protein did not bind stably to DNA in solution although the sequence of the protein suggests the presence of a highly conserved zinc finger domain (Everett *et al*, 1991). Despite this IE110 is the major transactivator of gene UL39 coding for the large subunit of ribonucleotide reductase (Sze and Herman, 1992).

Temperature-sensitive mutants with lesions in the gene for IE63 protein were shown to exhibit altered levels of IE and true late transcripts (increased and decreased respectively) by Sacks *et al* (1985). However investigation of the effects of IE63 on viral transcription in transfection experiments showed a limited influence (Everett, 1986). That is, IE 63 was active only on some promoters and then only in the presence of IE175 and IE110. IE63 has recently been shown to act at least in part post transcriptionally,

affecting mRNA processing (Smith *et al*, 1992; McLauchlan *et al*, 1992). The other IE gene products (IE68 and IE12) have not been shown to have any effect on the transcription of viral genes.

v) Viral DNA replication.

Following transport of the nucleocapsid to the nucleus the viral DNA is replicated in the nucleus starting at approximately 3 hours after infection. As this happens cellular chromatin is displaced to the edge of the nucleus (Munk and Sauer, 1964; Rixon *et al*, 1983). Replication of viral DNA is described in more detail in section IV.

Multiple reduplication of the nuclear membrane is also seen (Nii *et al.*, 1968).

vi) Virion assembly.

HSV-1 virions are assembled by packaging the viral genome into preformed capsids in the nucleus of the infected cell. The nucleocapsids then bud through the nuclear membrane acquiring tegument and an envelope.

Encapsidation of the genome.

Three forms of capsids have been seen in HSV-1 infected cells. Large-cored capsids (sometimes referred to as type B) have a large electron-translucent core and do not contain DNA. Small-cored capsids (type C) have an electron-dense core made up of DNA. Empty capsids (type A) have no internal structure. Large-cored capsids are probably scaffold containing species as removal of the internal structure of large-cored capsids is required for the packaging of DNA (Ladin *et al*, 1980; Preston *et al*, 1983; Addison *et al*, 1984). After DNA packaging the complete capsid is then enveloped. Empty capsids may be capsids that have lost their scaffold but not yet acquired DNA or a dead-end species and not in the developmental pathway. The scaffold of large-cored capsids is believed to be composed of protein VP22a (Newcomb and Brown, 1989; 1991). The processing of VP22a by the UL26 protease is required for capsid maturation (Preston *et al*, 1983; Rixon *et al*, 1988; Liu and Roizman, 1991b; Preston *et al*, 1992).

Herpes simplex virus DNA replication produces high molecular mass concatenated genomes which must be processed into single genomes for packaging. The *a* sequence which is present at genome termini and at the joint between the L and S segments of the genome contains the cleavage and packaging signals. If extra copies of this sequence are inserted into the viral genome and cells are infected with the mutant the progeny virus includes novel forms resulting from cleavage and recombination at the new *a* sequence (Smiley *et al*, 1981; Mocarski and Roizman, 1982a; Varmuza and Smiley, 1985). Defective viral genomes also require the *a* sequence (along with an origin of

† Strain 17+ has 1.5 bp of DR1 at the S terminus and the remainder at the L terminus (Davison and Rixon, 1985).

replication) if they are to propagate in virus stocks (Stow *et al*, 1983; Deiss and Frenkel, 1986). The structure of the *a* sequence is shown in figure 2.

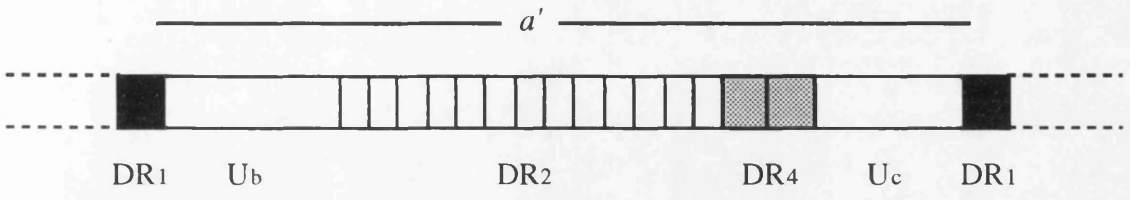

Figure 2. Structure of the *a* sequence.

The junction region of the viral genome is shown, the dotted lines representing IR_L and IR_S (left and right respectively). The following elements are present:

U_b unique sequence of 64 bp containing pac1 (see below).

U_c unique sequence of 58 bp containing pac2.

DR1 17-21 bp direct repeat shared between adjacent *a* sequences. Genome termini if ligated would share one DR1 element between them. Strain F has one 3' overhanging nucleotide at the S terminus and the remainder of the DR1 element at the L terminus. (Mocarski & Roizman, 1982b; Mocarski *et al.*, 1985).

DR2 directly repeated 12 bp element present in 1–22 copies.

DR4 repeat element present in present in 1–3 copies.

Data for this figure is taken from: Davison and Wilkie, 1981; Mocarski and Roizman, 1981.

Despite the highly variable nature of the *a* sequence between virus strains two highly conserved regions have been noted. These are called pac1 and pac2 and are required for packaging of HSV DNA (Deiss *et al*, 1986). Analysis of a complex formed at the *a* sequence suggested that three proteins were involved (Chou and Roizman, 1989). These were the viral alkaline exonuclease (Hay *et al*, 1971); ICP1, the product of gene UL36; and an unidentified 140 kD protein. While the exonuclease bound non-specifically to viral DNA, ICP1 and the 140 kD protein formed a complex that bound specifically to a combination of the pac2 and DR1 portions of the *a* sequence. This suggests that these proteins are involved in recognition and packaging of viral DNA. Additional evidence for the involvement of the alkaline nuclease in packaging comes from a null mutant. When grown on cells that did not express the nuclease this mutant gave very poor yields of virus despite producing large quantities of viral DNA and late proteins (Weller *et al*, 1990).

Envelopment and the release of mature virions.

Nucleocapsids associate with regions of the inner nuclear membrane modified by viral glycoproteins and then bud into the perinuclear space (Darlington and Moss, 1968; Morgan *et al*, 1959; Nii *et al*, 1968). Two routes have been proposed for the movement of virus to the cell surface from the perinuclear space (Morgan *et al*, 1959; Stackpole, 1969; Johnson and Spear, 1982; Spear, 1985). Enveloped HSV-1 particles may be carried to the endoplasmic reticulum and the Golgi apparatus and then to the plasma membrane in transport vesicles similar to those which carry membrane and secreted proteins. Prevention of budding from the Golgi apparatus with the ionophore monensin completely inhibited the production of extracellular virus, causing virions to accumulate in cytoplasmic vacuoles derived from the Golgi (Johnson and Spear, 1982). Alternatively, capsids may lose their envelope at the outer nuclear membrane and be re-enveloped into cytoplasmic vesicles before transport to the cell surface. Non-enveloped capsids seen in the cytoplasm and apparently budding into cytoplasmic vesicles may be produced by this route (Nii *et al*, 1968; Rixon *et al*, 1992).

vii) Latency.

Herpes simplex virus is able to persist in its host despite an active immune response due to its ability to become latent. Viral latency is defined as a state in which the genome of the infecting virus is present but infectious particles are not produced except during intermittent periods of reactivation (Ahmed and Stevens, 1990). HSV latency is established in the sensory ganglia which innervate the site of initial infection. After the virus replicates at the periphery the viral nucleocapsid is transported to the ganglion by retrograde axonal transport (Cook and Stevens, 1973; Para *et al*, 1980; Whitley, 1990; Lycke *et al*, 1984). There viral DNA is harboured in sensory neurons (Cook *et al*, 1974; Kennedy *et al*, 1983; McLennon and Darby, 1980).

Examination of HSV DNA in latently infected neurons showed that the ends of the genome were covalently joined both in a mouse model of the latent state and in humans (Rock and Fraser, 1983; 1985; Efstathiou *et al*, 1986). It was also shown that in the mouse, the DNA existed in an extrachromosomal form (probably a circle) and that it was associated with nucleosomes (Mellerick and Fraser, 1987; Deshmane and Fraser, 1989). Efstathiou *et al* (1986) estimated that latently infected human ganglia contained 0.01–0.1 HSV genomes per cell although, of course, not all cells are infected. By scoring neurons expressing viral latency associated transcripts (see below) to establish the proportion of neurons infected, quantitating viral DNA by slot blot, and assuming that 10% of ganglion cells are neurons Stevens (1989) estimated that approximately 20 genomes were present per latently infected neuron in mouse spinal ganglia.

Only a small part of the HSV genome is transcribed in the latent state. The transcribed region was first identified by Stevens *et al* (1987) in experimentally infected mice and its products were termed the latency associated transcripts or LATs. LATs were subsequently detected in human ganglia and in other experimental systems (Croen *et al*, 1987; Steiner *et al*, 1988; Wechsler *et al*, 1988; Rock *et al*, 1987; Wagner *et al*, 1988b). The transcription pattern of the LAT region is complex and not fully understood. 50–90% of LATs are an approximately 2 kb species. A minor 1.5 kb species and various other minor or unstable forms have also been detected (figure 3).

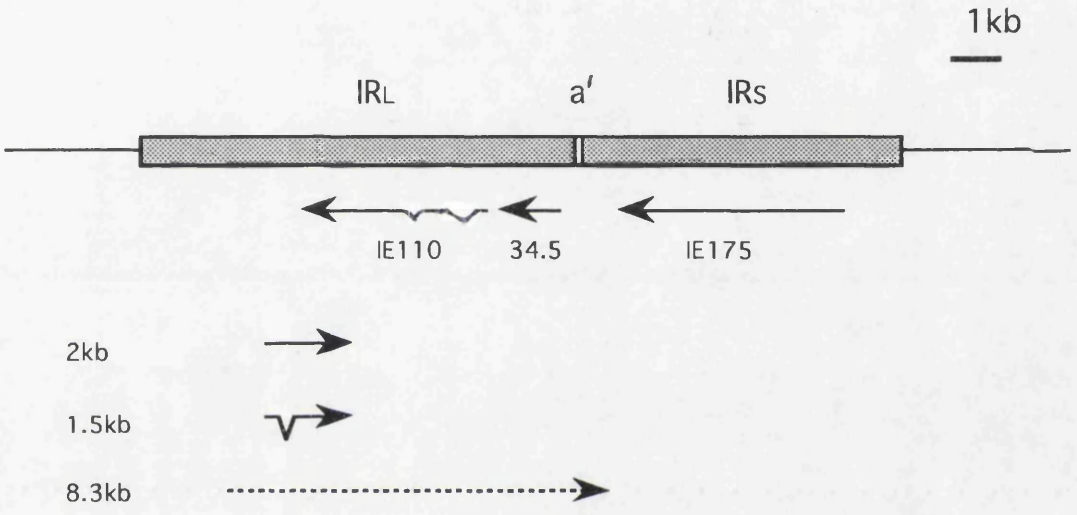

Figure 3. Transcripts from the LAT region of HSV-1. Transcripts found in latently infected cells are shown below a representation of the IR_L–U_L junction of HSV-1 DNA (unbroken line — major species; dashed line — minor species). The acute phase transcripts of genes IE110, ICP34.5, and IE175 are shown for comparison. A comparable arrangement exists in the other long repeat.

LATs are located predominantly in the nucleus and are not polyadenylated (Spivak and Fraser, 1987; Wagner *et al*, 1988b). It has been estimated that each latently infected murine neuron contains $2x10^4 - 5x10^4$ LATs in total (Wagner *et al*, 1988a). Promoter elements for RNA polymerase II were not apparent in the DNA sequence immediately upstream of the major LAT, the first LAT to be discovered. This led to a search for the promoter and a proposal that the major LAT was actually a stable intron spliced from the largest (c.8.3 kb) LAT, transcribed from a promoter 600–800 base pairs upstream of the 5' end of the major LAT (Dobson *et al*, 1989; Batchelor and O'Hare, 1990; Zwaagstra *et al*, 1989; Farrell *et al*, 1991). Two open reading frames have been postulated in the major LAT and a protein product of one of them was reported in neurons

latently infected *in vitro* (Wagner *et al*, 1988a; Doerig *et al*, 1991). The function of this putative gene product has yet to be identified.

LATs are antisense to the mRNA for the transactivating gene product IE110. Therefore a function for them might be to down regulate gene expression to induce a latent infection. However a number of experimenters have shown that LATs are not required for the establishment or maintenance of latency (Javier *et al*, 1988; Steiner *et al*, 1989). Experiments to determine if LATs are required for reactivation from latency have given conflicting results (Leib *et al*, 1989; Trousdale *et al*, 1991; Block *et al*, 1990; Ho and Mocarski, 1989). Trousdale *et al* used *in vivo* reactivation from rabbit eyes as an assay. They found that reactivation occurred in only 4% of eyes infected with LAT negative virus whereas reactivation occurred in 70% of eyes infected with wild type virus. They also found that significantly more wild type than mutant virus reactivated if explant co-cultivation was used as an assay. Experiments showing no difference between LAT^+ and LAT^- virus have used only explant co-cultivation as an assay. It is possible that due to the complex nature of the LAT region of the genome, the mutant viruses used in these studies behaved differently in latently infected neurons.

A previously unidentified gene RL1 (ICP34.5) has recently been demonstrated in the long repeats of the HSV-1 genome (Ackermann *et al*, 1986b; Chou and Roizman, 1986). This gene has a strong influence on the neurovirulence of HSV-1; viruses lacking both copies of the gene have their neurovirulence reduced by a factor of 10^5 despite showing unimpaired growth *in vitro* (Taha *et al*, 1989a; 1989b). The gene shows strong homology over part of its sequence to a gene, MyD116, associated with differentiation of myeloid cells (McGeoch and Barnett, 1991). Comparison of the growth of wild type and RL1 deficient virus in one neuroblastoma cell line suggests that RL1 may prevent the shutoff of protein synthesis that seems to be triggered in these cells in its absence (Chou and Roizman, 1992). These results suggest that ICP34.5 may be important during latency of HSV-1. However MacLean *et al* (1991) found that mutants of HSV-2 lacking RL1 behaved identically to wild type virus in establishing latency in mice and reactivating when infected ganglia were explanted. However, HSV-1 mutants in RL1 have impaired reactivation (Robertson *et al.*, 1992) and therefore it appears that HSV-1 and -2 behave differently in this respect.

III) DNA REPLICATION.

In the forty years since the double helical structure of DNA was proposed, considerable progress has been made in discovering how DNA is replicated. In almost all organisms the main features of the process are identical. Replication initiates at specific DNA sequences called origins and proceeds by the copying of the separated strands of the duplex. Nucleotides complementary to the existing sequence are added by a DNA polymerase, the new nucleotide chain being synthesised in the 5'–3' direction by the addition of nucleotide monomers. DNA polymerases can extend existing polynucleotide chains but not begin the synthesis of new ones and therefore a mechanism for priming polymerisation is required. Priming generally involves the synthesis, by a dedicated primase, of a short RNA chain which is then extended by the DNA polymerase. As replication proceeds away from an origin the duplex is opened ahead of the polymerase by a helicase enzyme. This results in the formation of a replication fork (see figure 4). Superhelical strain induced in the DNA by this process is relieved by a topoisomerase enzyme which nicks and rejoins one strand of the DNA ahead of the replication fork allowing untwisting to occur. To avoid lengthy exposure of DNA in a vulnerable single stranded form, both strands of the duplex must be copied in the same overall direction as the replication fork advances. As nucleotide polymerisation can proceed only in the 5'–3' direction, one strand is copied in short sections as it is exposed by the action of the helicase, while the other is copied continuously. Copying of the two strands is probably coordinated by a multiprotein complex called a replisome. This is proposed to contain the polymerases acting on the two strands, together with the helicase, the primase for repeated initiation on the discontinuously synthesised strand, and other accessory factors.

Table 3. The components of some well characterised replication systems.

Function	***E. coli***	**T7**	**T4**	**SV40**
Initiation	dnaA, RNA pol	T7 RNA pol	?	T antigen
Helicase	dnaB	gp4	gp41	T antigen
Primase	dnaG	gp4	gp61	pol α primase
Polymerase				
Core	pol III (α, ε, θ)	gp5	gp43	pol α, pol δ
Accessory subunits	β, γ complex, τ	thioredoxin	gp44, 62, 45	PCNA, RF-C
ssDNA-binding	SSB	gp2.5	gp32	RF-A
Swivel	gyrase		T4 topo	topo I or topo II
Decatenation	gyrase	?	?	topo II

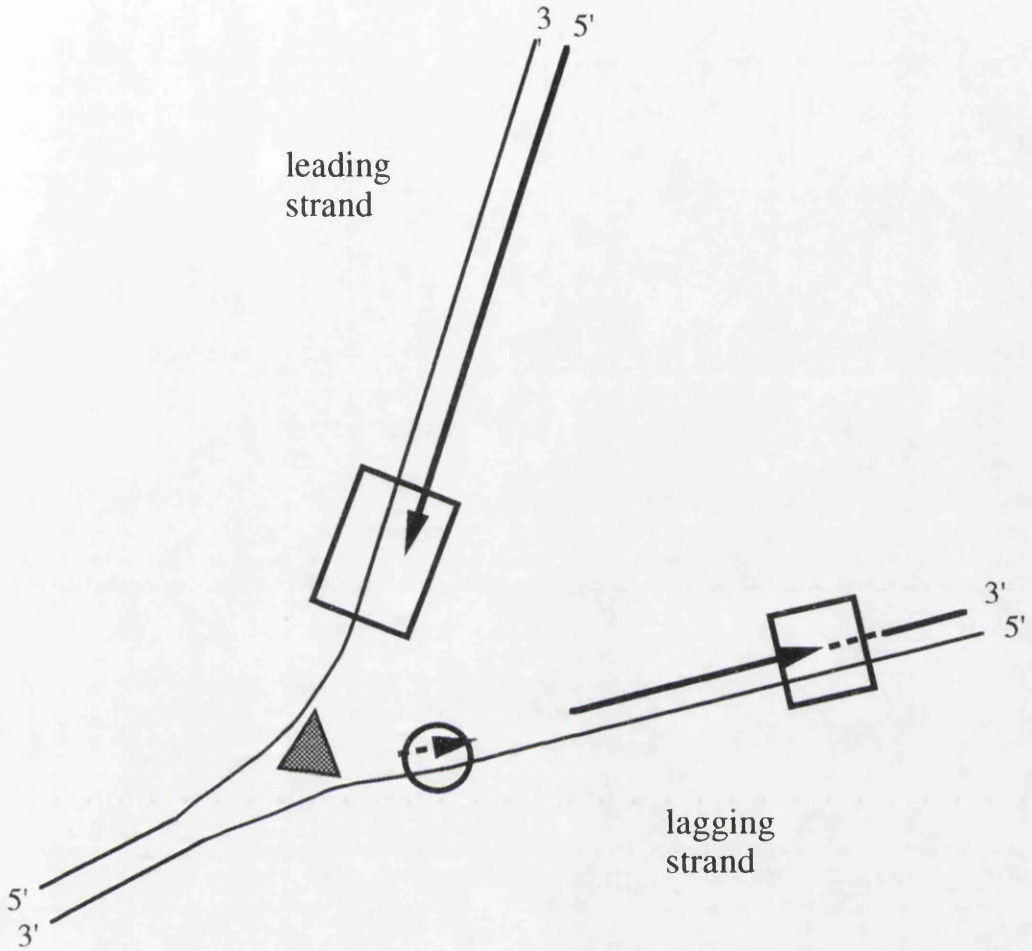

Figure 4. Cartoon of a replication fork. Parental DNA is represented by thin lines and newly synthesised DNA by thick lines. A polymerase with accessory factors (rectangle) synthesises the leading strand as the parental duplex is opened by the helicase (triangle). On the lagging strand, a polymerase (square) completes a fragment of newly synthesised DNA. A primase (circle) synthesises a primer (dotted line) for polymerase to extend as the next fragment. All regions of single stranded DNA would be covered by single stranded DNA-binding protein (not shown). In reality all the proteins may form a single complex, the DNA bending to allow this. Topoisomerase and ligase enzymes are not shown.

The whole process may be visualised as a machine drawing in duplex DNA and extruding two new duplexes at its far side. There is even some evidence that the replisome is attached to the nuclear matrix in some or all organisms and is therefore stationary relative to the cell as a whole while the DNA is drawn through the complex. Primers are removed in the course of chain elongation and the ends of the new chains are joined by the enzyme DNA ligase. Disentangling of the new duplexes by a topoisomerase completes replication. The details of the process, as determined in various organisms, will be described in more detail below. Table 3, listing the components of some well characterised replication systems, is provided for reference. A more complete description of DNA replication may be found in Kornberg and Baker, 1991.

i) Initiation.

Studies of prokaryotes, lower eukaryotes and eukaryotic viruses have revealed considerable similarity in the basic mechanisms of initiation. Three events must occur sequentially for initiation of DNA replication. These are:

i) binding of a protein to specific DNA sequences in the origin;
ii) local opening of the helix by this protein, a process that consumes energy;
iii) loading of a helicase to further open the DNA and allow priming and the assembly of the replication machinery.

Initiation in prokaryotes.

Initiation of DNA replication in *E. coli* is understood better than initiation in any other organism. *In vivo*, replication in *E. coli* begins only at oriC the unique chromosomal replication origin. Deletion analysis has defined the minimal size of OriC as 245 bp. The essential region contains four 9 bp boxes and three adjacent 13 bp boxes which are highly conserved. These boxes, where substitutions are not tolerated, are separated by portions of DNA of fixed length but random sequence (Asada *et al*, 1982; Oka *et al*, 1984). Initiation begins with the recognition of the 9 bp boxes by dnaA protein (Fuller *et al*, 1984). Twenty to forty monomers of dnaA bind cooperatively to the origin, protecting approximately 200 bp of DNA from nuclease attack. The helix adjacent to the dnaA boxes is then opened by dnaA protein (Bramhill and Kornberg, 1988). Opening of the helix allows loading of the dnaB helicase from a dnaB.dnaC.ATP complex, forming the pre-priming complex (Funnell *et al*, 1987). HU, a double stranded DNA-binding protein, is also required for the formation of the pre-priming complex, probably to stabilise a preferred bend in the DNA. The DNA is further opened by the helicase so that replication can commence (Baker *et al*, 1986). Initiation at other prokaryotic replication origins appears to follow the same three step sequence as in *E. coli*.

Initiation in eukaryotes.

Initiation in eukaryotic viruses is probably similar to initiation in *E. coli.* Certainly in SV40, the most studied, the same pattern occurs — binding of specific sequences by a protein resulting in helix melting at an adjacent site and the entry of other replication proteins. In these genomes the melted region is usually a palindrome or AT-rich region (Challberg and Kelly, 1989). The SV40 origin is contained within a 450 bp region, the minimal origin being 64 bp. The minimal origin contains three components: 1) four short repeats which bind T antigen; 2) seventeen AT base pairs which may participate in origin melting; and 3) a 15 bp palindrome which is the first region melted during initiation (Borowiec *et al,* 1990). T antigen opens the origin by its intrinsic helicase action. It is not known whether T antigen participates in loading other replication proteins into the opened origin but this is likely as a complex can be formed between T antigen and the host DNA polymerase α-primase (Smale and Tjian, 1986). Binding of transcription factors to sites in the origin region stimulates replication although the mechanism by which this occurs is uncertain (Guo *et al,* 1989).

In yeast, initiation begins at autonomous replication sequences (ARS) which confer autonomous replication on plasmids introduced into *Saccharomyces cerevisiae.* Autonomous replication sequences consist of two domains A and B both of which are essential. Domain A is an 11 bp sequence which is the binding site for a complex called the origin recognition complex (ORC), which is probably the initiator *in vivo* (Bell and Stillman, 1992; Diffley and Cocker, 1992). Domain B is a 50–100 bp AT-rich region adjacent to domain A, which binds an unidentified protein (Marahrens and Stillman, 1992; Kearsey, 1984; Umek and Kowalski, 1990). Some, but not all, autonomous replication sequences function as chromosomal origins *in vivo,* although the reasons for this are unknown (Linskens and Huberman, 1988).

Attempts to identify functional origins of replication in the genomes of higher eukaryotes have not been successful. The most promising system examined to date is Chinese hamster ovary (CHO) cells. CHO cells resistant to methotrexate show vast amplification of the dihydrofolate reductase gene region — as much as 200 kb is amplified up to 1000 fold. By identification of the restriction fragments which are replicated earliest in S phase, initiation has been localised to a 28 kb region (Leu and Hamlin, 1989; Anachkova and Hamlin, 1989). Initiation also appears to begin in the same region in non mutant cells (Burhans *et al,* 1990). However experiments attempting to localise an origin or origins of replication in this region have given conflicting results. It is possible that a large region, perhaps specified by packing rather than sequence, functions as a replication origin *in vivo* (Vaughn *et al,* 1990).

ii) Priming.

Priming is required for all DNA replication as no DNA polymerase can begin a DNA chain. Priming probably evolved to contribute to high fidelity DNA replication as the proof reading and error correcting mechanisms of DNA polymerases may not operate efficiently during chain initiation. Priming by short RNAs, synthesised for the purpose by a specific primase, is by far the most common method of priming. Beginning chains with RNA allows the beginning of the chain to be recognised as prone to errors, excised, and the gap filled by a high fidelity DNA polymerase. Only the extreme 3' ends of linear DNA cannot be copied by this mechanism. Prokaryotic priming is generally very different from that occurring in eukaryotes but has some aspects that are instructive.

Priming of the genomes of *E. coli* and its bacteriophages.

Priming of DNA replication in *E. coli* and many of its bacteriophages is carried out by the dnaG gene product, the *E. coli* primase (Kornberg and Baker, 1991). The dnaG gene product is a 60 kD protein which synthesises primers of approximately 11 nucleotides *in vivo*, recognising the sequence 3'-GTC (Rowen and Kornberg, 1978; Kitani *et al*, 1985; Hiasa *et al*, 1989a). When acting alone the primase appears to recognise a specific stem loop structure in some bacteriophage single stranded DNA, as well as the 3'-GTC sequence (Lambert *et al*, 1986; Hiasa *et al*, 1989b; Sims and Benz, 1980). However during replication of the *E. coli* chromosome dnaG primase probably acts as part of a mobile multiprotein complex called a primosome. Two types of primosome have been found, the dnaG-dnaB primosome and the ϕX174-type primosome. Other primosome components may induce a similar stem loop-type conformation in the chromosomal DNA.

DnaB-dnaG primosome.

On most single stranded substrates dnaG will synthesise primers only in the presence of *E. coli* dnaB protein (Arai and Kornberg, 1979). The dnaB protein is a helicase which has associated DNA-dependent ATPase activity, and almost certainly melts the DNA ahead of the advancing replication fork *in vivo* (Matson and Kaiser-Rogers, 1990; LeBowitz and McMacken, 1986). To be active as a helicase dnaB requires a pre-formed fork in its substrate DNA. The protein moves 5'–3' on the strand to which it is bound with a speed of 35–60 bp per second. SSB, the *E. coli* single stranded DNA binding protein, stimulates the helicase activity if added after helicase binds the DNA, probably by keeping the separated strands apart (LeBowitz and McMacken, 1986). DnaB and the primase associate with the template in a distributive manner, that is dissociation and reassociation occur at a high rate. When the reassociation of dnaB with the template is limiting the rate of priming can be increased by the addition of dnaC protein which increases the affinity of dnaB for DNA (Arai and Kornberg, 1981; Wahle *et al*, 1989). It

is not clear how dnaB stimulates priming but use of non-hydrolysable ATP analogues has shown that the helicase activity is not required. While the functional interaction of dnaB and primase is not in doubt, a physical association between the two proteins has not been demonstrated. It is possible that only a weak interaction exists or that the two proteins interact through a DNA structure. Priming by dnaG and dnaB is completely inhibited by previously added SSB (Arai and Kornberg, 1979). Despite this the dnaG-dnaB primase perhaps provides a model for priming of the leading strand at oriC because at oriC the melted DNA may be kept free of SSB by the action of dnaA protein.

The ϕX 174-type primosome.

During lagging strand synthesis primase must act on SSB-coated single stranded DNA. As the dnaB.dnaG complex is inactive on SSB-coated single stranded DNA, priming must occur by another mechanism under these conditions. This may involve the ϕX174-type primosome (Arai *et al*, 1981). The ϕX174-type primosome is a complex assembly of proteins which supports multiple priming events and is insensitive to inhibition by SSB. It primes conversion of the single stranded form of the ϕX174 genome to the double stranded form, acting at a specific site the primosome assembly site *pas*, a 70 nt stretch of DNA which may form a stem loop structure (Greenbaum and Marians, 1985). Sequences analogous to *pas* have been found in the *E. coli* chromosome but it is not known whether they function *in vivo*. The proteins required for primosome assembly, in addition to dnaB protein, primase, (and SSB bound to the template), are PriA, PriB, PriC, dnaC, and dnaT, all *E. coli* proteins. Although all of these proteins are required for the assembly of the primosome, it is not clear what part many of them play in assembly or which proteins become stably associated with the complex. The mobile primosome tracks along single stranded DNA synthesising multiple primers. Movement may be powered by either the PriA or dnaB helicase which translocate in opposite directions along a DNA strand (Lee and Marians, 1989). Primase is not required for either the integrity or the mobility of the complex. In fact the preprimosome may be the real processive entity with primase cycling on and off the complex to synthesise primers.

Several features of the ϕX174–type primosome make it an attractive model for priming of discontinuous strand synthesis *in vivo*. Firstly, the primosome is made up entirely of *E. coli* replication proteins, five of which (dnaB, dnaC, dnaT, PriA, and primase) have been implicated genetically in chromosomal replication, and three of which (dnaB, dnaC, and primase) are essential for oriC plasmid replication *in vitro*. Secondly, the complex generally moves 5'–3' on the DNA strand to which it is bound i.e. it is powered by dnaB protein. On replicating DNA this would place it on the discontinuously copied strand moving in the same direction as the advancing replication fork. Thirdly, the complex is processive, remaining stably associated with the template through multiple rounds of priming. The ability of the primosome to apparently move in both directions

along a DNA strand suggests that it in fact loops the DNA. This would permit it to act as the motive force for the two halves of the DNA pol III holoenzyme dimer thought to operate at the replication fork, allowing them to synthesise both strands in a coordinated manner (Lee and Marians, 1989; Lasken and Kornberg, 1988). The ϕX174-type primosome is involved in the replication of the discontinuous strand of the plasmid pBR322 (Minden and Marians, 1985), further strengthening the suggestion that it is the discontinuous strand primosome during replication of the *E. coli* chromosome.

Gene 41 and 61 proteins of phage T4.

The products of genes 41 (gp41) and 61 (gp61) of phage T4 synthesise pentaribonucleotides on single stranded DNA which can be extended by the phage DNA polymerase (Keppel *et al*, 1988). Gp41 functions as a 5'–3' helicase in isolation but both gene products are required for the synthesis of primers *in vivo*. Gp41 does not form a stable complex with single stranded DNA despite its helicase activity but is stabilised on the DNA by gp61 protein suggesting that the proteins associate. Optimum activity of the two proteins occurs at a ratio of five gp41 to one gp61 (Richardson and Nossal, 1989). The primer recognition sequence is 3'-TTG *in vivo* (Keppel *et al*, 1988).

Gene 4 protein of bacteriophage T7.

Gene 4 protein (gp4) of bacteriophage T7 is a helicase and a primase and is required for phage DNA replication (Keppel *et al*, 1988; Matson and Kaiser-Rogers, 1990). There are 56 kD and 63 kD forms of this protein, both of which have 5'–3' helicase activity and stimulate the T7 DNA polymerase. Only the larger of the two has primase activity. On reaching a specific recognition site gp4 catalyses the synthesis of tetra- and pentanucleotides. The consensus recognition site is 3'-CTG(G/T)(G/T). During DNA synthesis the helicase is highly processive while the primase shows no processivity indicating that more than one molecule of gene 4 protein is required at the replication fork (Nakai and Richardson, 1988). A heteromultimer is the most likely form with the primase continually associating and dissociating as primers are synthesised. The absence of a primase activity from the 56 kD form of the protein may allow it to translocate on the leading strand without stalling at primase recognition sites. Gene 4 protein stimulates DNA synthesis only by T7 DNA polymerase: the primers it synthesises are extended only by this enzyme. The polymerase also facilitates binding of gp4 to DNA. These results suggest a direct interaction between the proteins *in vivo*. Such an interaction has been detected *in vitro* (Nakai and Richardson, 1986a; 1986b). The polymerase-primase complex may stabilise primers on the template and speed primer recognition by the polymerase.

Comparison of prokaryote replicative helicases.

Opening of the replication fork by helicases is closely linked to priming. It is therefore appropriate to consider at this point prokaryotic helicases involved in replication. The *E. coli* dnaB protein, bacteriophage T7 gp4 protein, and bacteriophage T4 gp41 protein form a family of replicative helicases with similar properties in unwinding duplex DNA and in complex formation at the replication fork. All three proteins unwind DNA processively, moving with 5'–3' polarity. At a replication fork this would place them on the discontinuous strand moving in the same direction as the advancing fork. Melting of a duplex by these enzymes requires a pre-formed fork suggesting that the helicases bind both strands of DNA as they advance. These helicase proteins also stimulate primer synthesis. In contrast, the properties of PriA protein are quite distinct in terms of polarity of movement, processivity, and response to DNA polymerase, suggesting a different role for this protein in replication. The properties of the helicases are summarised below .

Table 4. Comparison of *E. coli* and phage replicative helicases (see Matson and Kaiser-Rogers, 1990).

Protein	**Mass (kD), form**	**Polarity**	**Rate (bp/s)**	**Processivity**	**Effect of primases**	**Effect of DNA polymerase**
dnaB	50 hexamer	5'–3'	35–60 750[a]	high[a] >50kb	stimulated by E. coli dnaG.	stimulates rolling-circle synthesis.
T7 gp4	63, 56 multimer	5'–3'	300[a]	high[a]	same protein.	specific for T7 pol.
T4 gp41	59 multimer	5'–3'	250[a]	high[a]	stimulated by T4 gp61.	accessory proteins stimulate.
PriA	76 monomer	3'–5'	50	<500bp	none	none

[a] When DNA polymerase is present.

Priming of DNA replication in eukaryotes.

Eukaryotic α DNA polymerases, which are involved in chromosomal replication, also have primase activity (Lehman and Kaguni, 1989). Pol α purified from numerous sources, including human cells and yeast, is composed of four subunits of 180, 70, 60, and 50 kD. Primase activity is detected in preparations of the 60 and 50 kD subunits (Kaguni *et al*, 1983). These proteins sediment as a heterodimer and are immunoprecipitated by an antibody reacting with the smaller protein. Neither protein is active alone and the active site of the enzyme appears to be made up of sequences from each subunit (Foiani *et al*, 1989). The genes encoding the two subunits are both essential in yeast (Lucchini *et al*, 1987; Lehman and Kaguni, 1989).

When DNA synthesis is proceeding, the *Drosophila* primase makes primers of 12–14 nt and the yeast and mouse enzymes primers of 8–12 nt (Conaway and Lehman, 1982; Tseng and Ahlem, 1983; Singh *et al*, 1986). Primers are initiated with a purine, however beyond this little if any sequence preference is apparent in the placing of primers. Thus eukaryotic primases appear to copy a certain length rather than reading a particular sequence as prokaryotic primases do. Deoxyribonucleotides rather than ribonucleotides can be incorporated in all but the first position of the primer and the exact composition of primers is influenced by the composition of the nucleotide triphosphate pools (Komberg and Baker, 1991). The fidelity of copying, at least for *Drosophila* and calf thymus primases, is low (Zhang and Grosse, 1990; Cotterill *et al*, 1987a).

If the primase subunits are assayed in the absence of polymerase, multiples of the characteristic primer length are produced (i.e. 24–mers and 36–mers) (Cotterill *et al*, 1987a; Brooks and Dumas, 1989). Also primer synthesis is blocked after an initial burst. No multiple length primers are produced in the presence of an active DNA polymerase subunit. These results suggest that the polymerase and primase activities of the complex are coordinated although it is not known how the primer terminus is moved from the primase to the polymerase active site.

The close coupling of helicase and primase activities in prokaryotic replication suggests that the same coupling might occur in eukaryotes. However very little is known about replicative helicases in eukaryotes and in these organisms primase is more usually coupled to DNA polymerase as described above. SV40 virus T antigen is a helicase and can associate directly with DNA polymerase α-primase but whether this interaction is required for priming of SV40 DNA replication is unknown (Smale and Tjian, 1986). Apart from the SV40 system, the HSV-1 UL5/UL8/UL52 assembly is the only known eukaryotic primase with an associated helicase activity.

iii) Elongation of DNA chains.

The elongation of DNA chains is carried out by template-directed DNA polymerases. A number of DNA polymerase have been found in all organisms examined and they all have the same basic properties. *E. coli* contains three DNA polymerases (I, II, and III) and other prokaryotes have analogous enzymes. Eukaryotes contain five DNA polymerases α, β, γ, δ and ε. *E. coli* DNA polymerase I is understood in more detail than the other polymerases and is the only DNA polymerase whose structure has been determined. Many of its properties are common to all DNA polymerases. *E. coli* pol II is primarily a repair enzyme, active in DNA damage-induced (SOS) repair (Campbell *et al*, 1972; Bonner *et al*, 1988). Description of its properties is limited therefore to table 5, which summarises the properties of the *E. coli* DNA polymerases.

***E. coli* DNA polymerase I**

E. coli DNA polymerase I is a 103 kD protein (928 amino acid residues) (Kornberg *et al*, 1956; Jovin *et al*, 1969; Joyce *et al*, 1982). It is a globular molecule with a diameter of 65 Å and can therefore contact approximately 2 turns (20 bp, c.70 Å) of helical DNA (Joyce and Steitz, 1987). Three activities are present in the intact protein: template-directed nucleotide polymerisation; proof-reading 3'–5' exonuclease; and 5'–3'

Table 5. The DNA polymerases of *E. coli*. Data from Kornberg and Baker, 1991.

	pol I	**pol II**	**pol III (core)**
Polymerisation: 5'–3'	+	+	+
Exonuclease: 3'–5'	+	+	+
Exonuclease: 5'–3'	+	–	–
Stimulation by β	–	+	+
Size (kD)	103	90	130, 27.5, 10
Molecules / cell	400	?	10–20
Turnover number[a]	600	30	9000
Structural genes	*polA*	*polB*	*dnaE* or *polC*[b]

[a] Number of nucleotides polymerised per minute per molecule at 37 °C.
[b] Gene for α subunit.

exonuclease. The protein is easily separated into two fragments by proteases, the small N terminal portion (35 kD) containing the 5'–3' exonuclease activity and the large C terminal

portion (68 kD) containing the polymerisation and 3'–5' exonuclease activities (Setlow *et al*, 1972). Inhibitors or point mutations can inactivate the 5'–3' exonuclease, the 3'–5' exonuclease, or the polymerase and leave the other activities intact, showing that each is in an independent domain of the protein (Derbyshire *et al*, 1988; Lehman and Uyemura, 1976).

The structure of the large fragment of DNA polymerase I has been determined by X-ray crystallography (Ollis *et al*, 1985; Freemont *et al*, 1988). The structure obtained at 2.75 Å resolution shows the fragment folded into two domains: a large domain (residues 521–928 of the intact protein) and a small domain (residues 324–517 of the intact protein). The large domain contains a cleft, 20–24 Å wide and 25–35 Å deep, with sides made up of many α helices and a base of β sheets. Eight base pairs of B form DNA can fit into the cleft and numerous studies have demonstrated that the cleft is the site of DNA binding (Freemont *et al*, 1988; Kornberg and Baker, 1991). Electrostatic field calculations for the large fragment also show that the greatest positive potential is located appropriately within the cleft (Warwicker *et al*, 1985). The α helices in the cleft may provide threads that direct one groove of the DNA along a spiral path so that the 3' terminus of the growing strand remains in the active site. A representation of the crystal structure of DNA polymerase I may be found in Joyce and Steitz (1987).

The polymerisation activity of DNA pol I operates on the 3'-OH of a primer DNA or RNA annealed antiparallel to a template strand. Pol I is tolerant of a number of template structures, accepting short or long 5' overhangs of the template strand, or duplex DNA with short single stranded gaps. Sedimentation and electron microscope studies with model templates have revealed the polymerase's preferences for binding (Englund *et al*, 1969; Griffith *et al*, 1971). The polymerase binds to the 3'-OH of single strand–double strand junctions and at the end of duplexes, but never in the centre of fully duplex molecules. Single stranded DNA is also bound as are nicks in double stranded DNA. DNA pol I has low processivity; only 20 nucleotides are added to the primer per binding event (Matson and Bambara, 1981). In addition to acting at conventional primer termini, DNA pol I is one of the few DNA polymerases able to initiate polymerisation at a nick in an otherwise fully duplex DNA unaided by any other factors (Lundquist and Olivera, 1982). This process involves helix melting and displacement of the 5' end of the chain. Coupled with the 5'–3' exonuclease activity, the polymerising activity results in nick translation and is exactly that required to remove primers and complete Okazaki fragments during DNA replication.

The proof-reading 3'–5' exonuclease activity of DNA pol I cleaves a mismatched terminus and is vital to the fidelity of the enzyme (Brutlag and Kornberg, 1972; Kunkel, 1988). The exonuclease works preferentially on a frayed end of double stranded DNA. The active site is contained in the small domain of the Klenow fragment of the polymerase as revealed by the presence of an inhibiting nucleotide monophosphate and

certain divalent metal ions, in the crystal structure. Mutation of the appropriate amino acid residues prevents metal binding and also destroys proof-reading exonuclease activity (Derbyshire *et al*, 1988; 1991). Mutations which destroy the proof-reading activity have a strong mutator phenotype.

The primary phenotypes of *E. coli* with mutations in *polA*, the DNA polymerase I gene, are delayed joining of nascent DNA fragments and an inability to repair damaged DNA. These defects are due to the impaired gap-filling and excision functions of the enzyme. Delayed joining of nascent DNA fragments during replication is due to the failure to remove RNA primers and strongly suggests that this process is performed *in vivo* by pol I (Kornberg and Baker, 1991).

E. coli DNA polymerase III holoenzyme.

E. coli DNA polymerase III holoenzyme is the principal replicative enzyme of *E. coli* (Gefter *et al*, 1971; Wickner *et al*, 1973). Each cell contains approximately 10 holoenzyme complexes. The holoenzyme contains 10 different subunits (α, ε, θ, τ, γ, δ, δ', χ, ψ, and β) and has a molar mass of >900 kD (Maki *et al*, 1988). It dissociates easily on extraction from cells into a number of sub-assemblies, the smallest of which is the core. Reconstitution of holoenzyme on SSB coated single stranded DNA leads to a rate of DNA synthesis of 700 nt per second at 37 °C (Mok and Marians, 1987). This is close to the rate of 1000 nt per second observed *in vivo*. The holoenzyme also has a processivity of greater than 10^5 (Mok and Marians, 1987).

The catalytic core of the holoenzyme, which cannot be dissociated without denaturing the constituent proteins, comprises the proteins α, ε, and θ (Maki and Kornberg, 1985; 1987). Subunit α contains the polymerase active site while the 3'–5' exonuclease is contained within the ε subunit, mutation of which leads to a 10^5-fold increase in cell mutations (Scheuermann and Echols, 1984; Ehrlich and Cox, 1980). The role of the θ subunit is unknown, as its absence does not alter the polymerase or exonuclease activities. A structural role may perhaps be inferred.

Accessory subunits of the holoenzyme.

The accessory subunits of the holoenzyme are τ, the γ complex (γ, δ, δ', χ, ψ), and β. The τ accessory factor plus the core forms the pol III' subassembly of the holoenzyme. Pol III' can be isolated in the form $core_2\tau_2$ (McHenry, 1982) and this dimer is also present in higher assemblies (Maki *et al*, 1988). τ is a single stranded DNA dependent ATPase and increases the processivity of the core about six-fold (Lee and Walker, 1987).

The γ complex was originally identified as a factor needed with the core and the β subunit to reconstitute the high processivity of the holoenzyme (McHenry and Kornberg, 1977). Judging from its molar mass the complex contains 2 copies each of proteins γ, δ,

δ', χ, and ψ. The complex is active in reconstituting high processivity at a level of ten core and β subunits to one γ complex, suggesting that the γ complex has a catalytic role (Maki and Kornberg, 1988a). The functions of the individual subunits are unknown.

The β component is the holoenzyme's most important processivity factor (Johanson *et al*, 1986; Lasken and Kornberg, 1987). It is a dimer of 40.6 kD subunits. β dissociates readily from the other nine proteins of the holoenzyme in the absence of ATP but is firmly bound in its presence. β is not demonstrably a DNA-binding protein but becomes attached to circular DNA duplexes in the presence of the γ complex and ATP. Scission of the DNA releases β (Stukenberg *et al*, 1991). These results have been explained now that the structure of β has been determined by X-ray diffraction (Kong *et al*, 1992). This has revealed that the protein dimer is a ring, with a hole in the centre large enough to accommodate a B form DNA helix suggesting that β closes around duplex DNA. The central hole is lined by alpha helices with their long axes perpendicular to the grooves of the B form helix which should allow β to slide easily on duplex DNA.

Holoenzyme dynamics.

DNA pol III holoenzyme can be reconstituted from its core, the β subunit, the γ complex and ATP. A pre-initiation complex is formed when the γ complex causes β to bind to the template-primer using ATP as an energy source (Burgers and Kornberg, 1982a; 1982b; O'Donnell, 1987; Maki and Kornberg, 1988b). Binding presumably involves opening the ring formed by β and closing it around the DNA. The reaction is slow, taking 1–2 minutes, and the action of the γ complex is catalytic. Within a few seconds the pre-initiation complex binds a core, with or without τ, forming an initiation complex in which the γ complex is present in sub-stoichiometric amounts. With τ, formation of the initiation complex is more rapid, the complex is more stable, and the processivity of the elongation complex is greater — similar to that of the holoenzyme. With τ present the enzyme does not pause at secondary structures in the template or at annealed strands, consistent with the helicase activity of τ. Elongation complexes isolated by incubating the reconstituted enzyme with only three dNTPs have a similar composition to initiation complexes. Dissociation of the holoenzyme from a completed duplex takes several minutes. However the enzyme is able to traverse a duplex region of 15 bp (in the direction of elongation) to reach the terminus of a new primer in less than a second (O'Donnell and Kornberg, 1985). The core can move from one pre-initiation complex to another. This movement is very rapid, taking about ten seconds, and may reflect recycling of the holoenzyme on the lagging strand *in vivo* (O'Donnell, 1987).

Higher order protein assemblies.

With a molar mass near 900 kD, pol III holoenzyme probably contains two β dimers and two of each of its other subunits (Maki *et al*, 1988; Johanson and McHenry, 1984). The holoenzyme is thought to be an asymmetric dimer of the form (core-β_2-τ_2)(core-β_2-γ complex) possibly joined through θ. A τ dimer on one half of the complex would increase processivity to allow copying of the leading strand of the template, while a γ complex attached to the other half would allow repeated recycling of the polymerase on the lagging strand. Both the structure and the dynamics of the holoenzyme *in vitro* support this conjecture (Maki *et al*, 1988; Johanson and McHenry, 1984).

Comparison with other systems suggests that the holoenzyme is likely to interact with other proteins at the replication fork although such interactions have not been detected. For example bacteriophage T7 DNA polymerase is coupled to the phage helicase and primase during DNA replication and mammalian polymerase α is closely linked to primase (see later). Polymerases from a number of organisms also interact with the organisms single stranded DNA-binding proteins — for example T4 bacteriophage (Huberman *et al*, 1971).

T4 DNA polymerase.

The proteins constituting T4 bacteriophage DNA polymerase are the products of genes 43, 44, 45, and 62 (Marians, 1992; Morris *et al*, 1975). The product of gene 43 (gp43) is the catalytic subunit and is homologous to the eukaryotic α DNA polymerase family. Operating alone gp43 can copy only primed single strands, not nicked duplexes, but its turnover rate of 400 nucleotides per second is similar to the rate of replication fork movement *in vivo* (Huang *et al*, 1981; Mace and Alberts, 1984). The three polymerase accessory proteins gp44, gp45, and gp62, complex with gp43 and stimulate it forty-fold when the T4 single stranded DNA binding protein is also present. This enzyme system is able to replicate through any secondary structure and has high processivity — more than 20,000 nucleotides are added per binding event. Formation of the processive complex is similar to the analogous process in *E. coli* in that hydrolysis of ATP to ADP is required. Once the complex is bound, a non-hydrolysable ATP analogue can support elongation. The presence of the T4 helicase stimulates the rate of movement of the 'holoenzyme' complex by a factor of three (Cha and Alberts, 1988).

The DNA polymerase has a very active 3'–5' exonuclease which gives the enzyme a high fidelity (Sinha, 1987; Bessman *et al*, 1974). There is no apparent 5'–3' exonuclease but sequences similar to those in the 5'–3' exonuclease domain of *E. coli* pol I are found in the N-terminus of the protein (Reha-Krantz, 1990).

T7 DNA polymerase.

T7 DNA polymerase is a 1:1 complex between the phage gene 5 protein (gp5) and the host thioredoxin (Tabor *et al*, 1987; Modrich and Richardson, 1975). In addition to its polymerising activity gp5 has a 3'-5' proof-reading exonuclease activity (Hori *et al*, 1979; Adler and Modrich, 1979). A 5'–3' exonuclease is encoded by phage gene 6 (Kerr and Sadowski, 1972a; 1972b). Gp5 alone has a maximum processivity of 50 nucleotides but this is increased 1000-fold by the thioredoxin (Tabor *et al*, 1987). Thioredoxin increases processivity by binding to gp5, stabilising the gp5-primer-template complex 20–80 fold (Huber *et al*, 1987). The action of the polymerase is coordinated to the phage helicase/primase by a direct interaction between the proteins. The polymerase also interacts with the gene 6 protein (Nakai and Richardson, 1986a; 1986b).

DNA polymerases of eukaryotes

Five DNA polymerases have been detected in mammalian cells of which four (α, β, δ, and ε) are nuclear and one (γ) is mitochondrial. α and δ are required for replication, β is involved in repair and the function of ε is unclear. γ is responsible for replicating the mitochondrial chromosome. Only α, δ and ε will be described here. Studies of the polymerases of the yeast *S. cerevisiae* have provided important insights into mammalian polymerases due to the availability of yeast mutants. The yeast polymerases I, II, and III are analogous to the mammalian polymerases α, ε, and δ respectively. The properties of eukaryotic polymerases are summarised in table 6 (overleaf).

Table 6. Eukaryotic DNA polymerases[a].

	α	δ	ε
Yeast designation	pol I	pol III	pol II
Yeast gene	POL1 (CDC17)	POL3 (CDC2)	POL2
Mass (kD)			
Native	>250	170	256
Catalytic core	165–180	125	215
Other subunits	70, 50, 60	48	55
Associated functions			
3'–5' exonuclease	yes[b]	yes	yes
Primase	yes	no	no
Properties			
Preferred template	gap	poly dA. oligo dT	poly dA. oligo dT
Processivity	low	high[c]	high
Fidelity	high	high	high
Replication	yes	yes	uncertain
Repair	no	uncertain	uncertain

[a] Data from Lehman and Kaguni, 1989; So and Downey, 1988; Burgers, 1989; Bambara and Jessee, 1991.

[b] Only detected with *Drosophila* polymerase α.

[c] In the presence of proliferating cell nuclear antigen (PCNA).

DNA Polymerase α.

This enzyme is a complex of four proteins: a polymerising subunit of 165–180 kD; subunits of 60 and 50 kD with primase activity; and a 70 kD subunit of unknown function (for a review see Lehman and Kaguni, 1989). Polymerase α is essential for chromosome replication as conditional lethal mutants of yeast and of mouse cells in culture are unable to replicate DNA at the non-permissive temperature (Murakami *et al*, 1985; Johnson *et al*, 1985). Also depletion of cell extracts with anti-pol α antibodies prevents replication (Murakami *et al*, 1986). In addition the level of pol α in proliferating cells changes according to the stage of the division cycle the cells are in (Bertazzoni *et al*, 1976; Craig *et al*, 1975). The relatively low processivity of pol α and its associated primase activity make it an attractive candidate for the replicator of the discontinuously synthesised strand *in vivo*.

The C-terminal portions of eukaryotic α polymerases contain six regions of highly homologous sequence (I–VI) whose order has also been conserved (Larder *et al*, 1987; Wong *et al*, 1988; Wang *et al*, 1989). These regions of homology are found in other DNA polymerases: yeast δ and ε polymerases; human polymerase δ; adenovirus, vaccinia virus, and herpesvirus polymerases; and the polymerases of bacteriophages T4, ϕ29 and PRD1. Analysis of drug resistant mutants of HSV-1 has predicted that regions I, II, and III of the polymerases are involved in substrate binding and nucleotide polymerisation (see HSV DNA replication section). The N-terminal portions of the α polymerases contain three regions with homology to the 3'–5' exonuclease domains of bacteriophage T4 and ϕ29 DNA polymerases (Spicer *et al*, 1988; Bernad *et al*, 1989). However exonuclease activity is detectable only in preparations of *Drosophila* DNA polymerase α and this only on removal of the 70 kD subunit (Cotterill *et al*, 1987b). An exonuclease has been detected complexed to human DNA polymerase α (Skarnes *et al*, 1986).

DNA polymerase α operates by an ordered mechanism binding sequentially to the single stranded template, the primer, and finally to the complementary dNTP (Wong *et al*, 1986). The activity of the enzyme is dependent on *p*H and Mg^{2+} concentration, *p*H 7.0 and 5mM Mg^{2+} being optimum. The processivity of the enzyme varies from 2 to 2000 nucleotides polymerised per association, rising with a decrease in temperature, *p*H, or Mg^{2+} concentration (Tan *et al*, 1987). Accessory factors may enhance the processivity *in vivo*. Fidelity is very high, at least for the *Drosophila* enzyme, with as few as one error per 10^6 nucleotides incorporated (Cotterill *et al*, 1987b).

A number of proteins that stimulate polymerase α have been identified. Protein A (also known as RF-A or RP-A) is a single stranded DNA binding protein that stimulates polymerase α during the replication of the SV40 chromosome *in vitro* (Ishimi *et al*, 1988). It has three subunits of 70, 32, and 13 kD and may assist the helicase activity of T antigen. Replication factor C (RF-C or RP-C) is a multimeric factor required for SV40 DNA replication. RF-C is a DNA-dependent ATPase and facilitates binding of polymerase α to the primer terminus (Tsurimoto and Stillman, 1989; 1990). Another distinct α-accessory factor (AAF), isolated from cultured mouse cells, contains proteins of 132 and 45 kD (Goulian *et al*, 1990; Goulian and Heard, 1990). It stimulates self-primed synthesis by polymerase α on single stranded DNA and increases the affinity of the enzyme for primers, raising processivity ten-fold so that the polymerase produces newly synthesised DNA the size of mammalian Okazaki fragments. Polymerase α has also been isolated as part of a large complex containing a diadenosine-tetraphosphate ($A(p_4)A$)-binding protein, a 3'–5' and 5'–3' exonuclease, and primer recognition proteins which stimulate the rate of primer usage (Vishwanatha *et al*, 1986; Skarnes *et al*, 1986).

DNA polymerase δ

DNA polymerase δ was discovered as a new type of DNA polymerase which at the time was unique among mammalian DNA polymerases in possessing an intrinsic 3'–5' exonuclease activity (Byrnes *et al*, 1976; for reviews see Burgers, 1989; Bambara and Jessee, 1991). The human enzyme consists of two proteins with molar masses of 125 and 48 kD, the larger being the catalytic subunit (Lee *et al*, 1991). The gene for human pol δ has been isolated and sequenced (Yang *et al*, 1992). Polymerase δ plays a critical role in DNA replication as shown by the following evidence: (1) Studies in permeabilised cell systems have implied that pol δ is involved in replication and repair (Hammond *et al*, 1987; Dresler and Kimbro, 1987). (2) A 36 kD protein identified as a processivity factor for pol δ was shown to be proliferating cell nuclear antigen (PCNA) or cyclin, a protein whose expression is strongly tied to the S phase of the cell cycle (Tan *et al*, 1986; Bravo *et al*, 1987; Prelich *et al*, 1987b). (3) Pol δ and PCNA are necessary for replication of the SV40 chromosome *in vitro* (Prelich *et al*, 1987a; Lee *et al*, 1989). (4) The gene for the yeast protein equivalent to pol δ (pol III) is CDC2, a critical cell division cycle gene (Boulet *et al*, 1989; Sitney *et al*, 1989).

PCNA is a key accessory factor for pol δ raising its processivity forty fold, thus enabling it to replicate a singly primed phage DNA circle (Tan *et al*, 1986; Bravo *et al*, 1987; Bauer and Burgers, 1988). It is a dimer of 37 kD subunits that has no enzymatic activity of its own and does not bind single or double stranded DNA. However mixing of PCNA with pol δ increases the affinity of the enzyme for primed DNA. Two other proteins are needed, along with PCNA, for pol δ action during replication of the SV40 chromosome. These are replication factor A (RF-A) (Kenny *et al*, 1989), and RF-C, which binds the primer terminus. Isolated pol δ is very active on poly dA.oligo dT but only weakly active on DNA of high sequence complexity. However reconstitution of a complex of pol δ with PCNA, RF-A, RF-C, and ATP does support replication of complex DNA at the physiological rate of greater than 100 nucleotides per second (Tsurimoto and Stillman, 1990; Tsurimoto *et al*, 1990; Lee and Hurwitz, 1990).

DNA polymerase ε

DNA polymerase ε was initially thought to be a form of pol δ due to their many shared properties. These are: an overt 3'–5' exonuclease activity; lack of primase activity; preference for poly dA.oligo dT as a template; and high processivity (Bambara and Jessee, 1991). The two enzymes also cross-react immunologically (Wong *et al*, 1989). The yeast gene for pol δ and that thought to encode pol ε are nevertheless distinct (Yang *et al*, 1992). Despite this, determination of the relationship between mammalian polymerases δ and ε must await the isolation of the mammalian pol ε gene. Human pol ε has a molar mass of 215 kD and the yeast equivalent a predicted molar mass of 256 kD (Syvaoja and Linn, 1989; Morrison *et al*, 1990). The clearest distinction between polymerases δ and ε

is the high processivity of ε in the absence of PCNA on the preferred poly dA.oligo dT template (Syvaoja and Linn, 1989; Syvaoja *et al*, 1990; Lee *et al*, 1991). A function for pol ε in replication or repair *in vivo* has not been established. Pol ε (but not pol α) is able to restore DNA repair synthesis to permeabilised UV-irradiated HeLa cells and the enzyme may therefore function as a repair enzyme *in vivo* (Nishida *et al*, 1988). However a role for pol ε in replication has not been ruled out.

iv) Completion of replication.

With the original DNA strands completely copied, the new DNA fragments must be joined and the daughter duplexes separated for replication to be complete. These two steps are carried out by DNA ligase and DNA topoisomerase respectively.

DNA ligases.

DNA ligases join the 3'-hydroxyl of a nucleotide chain to a 5'-nucleotide phosphate juxtaposed to it during all processes involving cleavage and joining of DNA i.e. replication, repair, and recombination (Lehman, 1974). *E. coli* and bacteriophage T4 each produce one ligase while two types (I and II) are found in eukaryotes of which the first is the replicative enzyme. Ligases catalyse the coupling of their cofactor (NAD^+ or ATP) to the 5'-phosphate of one nucleotide via a phosphate-phosphate bond. The cofactor is then displaced by the 3'-hydroxyl of the other nucleotide as it forms the phosphodiester bond. A DNA ligase is encoded by vaccinia virus and shows 30% amino acid homology to the *S. cerevisiae* and *S. pombe* ligases (Smith *et al*, 1989). No DNA ligase gene has been found in the genomes of the herpesviruses.

DNA topoisomerases.

DNA topoisomerases interconvert topological isomers of DNA by cleaving and rejoining the phosphodiester backbone of the molecule. The number of superhelical turns present in the DNA may be altered, linked rings may be decatenated, and knots may be removed. There are two types of topoisomerases: type I enzymes make single stranded breaks in DNA and do not require an energy source; type II enzymes make double stranded breaks using ATP. Both types are required during replication. Either can provide a swivel ahead of the advancing replication fork preventing overwinding of the DNA, and type II enzymes can decatenate daughter molecules. Topoisomerases are also involved in transcription and recombination (Wang, 1985).

In *E. coli*, gyrase (a type II enzyme also known as topoisomerase II) is the main replicative topoisomerase providing a swivel for the replication fork and decatenating duplexes. The type I enzymes topoisomerase I and III are also involved in replication (Wang, 1985).

Two topoisomerases (I and II) are found in yeast and these are type I and II enzymes respectively. Either can function as the swivel at the replication fork but only the type II enzyme can decatenate daughter molecules (Brill *et al*, 1987; Holm *et al*, 1985; Uemura and Yanagida, 1986). A similar result has been found in cell extracts replicating the SV40 chromosome *in vitro* (Yang *et al*, 1987). Vaccinia virus encodes a type I topoisomerase whose function is unknown (Shuman and Moss, 1987).

IV) HSV DNA REPLICATION.

Due to the complexity of the HSV-1 genome the overall mechanism of viral DNA replication has not been determined. The structure of replicating viral DNA has been analysed by velocity gradient sedimentation, electron microscopy, and by use of restriction enzymes.

Sedimentation studies and restriction enzyme analysis suggest that parental DNA loses its free ends very soon after infection, probably becoming circular (Davison and Wilkie, 1983; Poffenberger and Roizman, 1985; Jacob and Roizman, 1977; Jacob *et al*, 1979; Marks and Spector, 1988; Mocarski and Roizman, 1982b). This seems to occur by direct ligation of the ends. Fusion of the ends is rapid and does not require new protein synthesis which suggests that ligation can be carried by a cellular enzyme or a viral enzyme brought into the cell during infection (Poffenberger and Roizman, 1985).

Sedimentation of newly replicated viral DNA shows that it is larger than unit length and subsequently matures into virion DNA (Hirsch *et al*, 1976; 1977; Ben-Porat *et al*, 1976; Ben-Porat and Tokazewski, 1977). Electron microscopy revealed that replicating viral DNA is comprised of a variety of complex structures, many of which are greater than unit size (Jacob and Roizman, 1977; Ben-Porat and Tokazewski, 1977; Ben-Porat and Rixon, 1979). These include forked molecules, rings, linear molecules with terminal or internal loops and very large tangles. Restriction enzyme analysis demonstrated that in common with parental DNA, new viral genomes lack termini (Jacob *et al*, 1979; Jongeneel and Bachenheimer, 1981). Available data suggests that replication produces head to tail concatamers of genomes although how these are related to the circular parental molecules is not clear.

i) Initiation.

The origins of replication.

In common with other genomes, HSV-1 DNA is replicated from specific origins of replication. The position of origins of DNA replication in the HSV-1 genome was first suggested by the DNA sequences contained in defective interfering particles. These are found after repeated passage of undiluted virus stock and contain either sequences from the S component or sequences from the middle of U_L plus the L-S junction (Bronson *et al*, 1973; Schroder *et al*, 1975; Frenkel *et al*, 1976; Locker *et al*, 1982). The origin sequences (named ori_S and ori_L) were localised more precisely and cloned into plasmids which were found to replicate autonomously on transfection into cells infected with HSV-1 (Stow, 1982; Mocarski and Roizman, 1982a; Stow and McMonagle, 1983; Spaete and Frenkel, 1982). Ori_S is located in the R_S region of the genome and is therefore present in two

copies; ori_L is located near the centre of U_L, between genes U_L29 and U_L30. The locations of ori_S and ori_L are shown in figure 1. L-S junction sequences are invariably found in defective interfering particles because they contain the *a* sequence which is required for cleavage and packaging of viral DNA.

Table 7. The HSV-1 DNA replication proteins.

Gene	**Estimated M_r from SDS gels**	**Function**
UL5	99,000	helicase/primase
UL8	70,000	helicase/primase
UL9	85,000	origin binding
UL29 (ICP8)	130,000	ssDNA binding
UL30 (*pol*)	140,000	catalytic subunit of pol
UL42	65,000	pol processivity factor
UL52	114,000	helicase/primase

The ability of ori_S-containing plasmids to replicate transiently in cells infected with HSV-1 was used to develop an assay to uncover the viral genes required for origin-dependent DNA replication (Challberg, 1986; Wu *et al*, 1988; McGeoch *et al*, 1988b). Fragments of the viral genome produced by restriction enzyme digestion were transfected into cells along with the origin-containing plasmid and shown to support its replication. Subcloning of each fragment, together with DNA sequence analysis, identified 7 genes that were necessary and sufficient for the amplification of origin-containing plasmids. These included the genes for the viral DNA polymerase (UL30), the major DNA-binding protein ICP8 (UL29), and the 65 kD DNA-binding protein (UL42), all of which had been previously identified. The remaining 4 genes UL5, UL8, UL9, and UL52 had not been characterised previously. The positions of these genes in the viral genome correspond closely to the mapped mutations of DNA^- mutants falling into 7 complementation groups (Purifoy *et al*, 1977; Chartrand *et al*, 1980; Coen *et al*, 1984; Conley *et al*, 1981; Weller *et al*, 1987; Carmichael *et al*, 1988; Goldstein and Weller, 1988a; Marchetti *et al*, 1988; Carmichael and Weller, 1989; reviewed by Weller, 1990). This close concordance of results suggests that the products of the 7 genes identified are genuinely involved in DNA replication. The plasmid amplification assay has recently been shown to function in insect cells infected with baculoviruses overexpressing the 7 replication proteins (Stow, 1992). This system provides a convenient method by which mutated replication proteins can be screened for activity and produced in amounts sufficient for biochemical studies. It also

indicates that any host cell functions required for HSV-1 DNA replication are conserved between mammalian cells and insect cells.

The sequences of ori_S and ori_L contain similar regions. (The structure of ori_S is shown in figure 5.) In particular both origins contain an extensive palindrome of which the central 18 base pairs are solely AT residues (Murchie and McGeoch, 1982; Quinn and McGeoch, 1985; Knopf *et al.*, 1986). The ori_L palindrome is much larger than that in ori_S (144 and 46 residues respectively), and cloned ori_L is unstable in *E. coli* for this reason (Weller *et al.*, 1985). Due to this instability most analyses have been performed on ori_S. The minimum sequence required for ori_S function is similar to sequences contained within the ori_L palindrome suggesting that the two types of origin may be equivalent. It is not clear why HSV-1 possesses three origins of replication. Mutants viruses lacking ori_L or one copy of ori_S have been isolated and have no apparent growth defect either in cultured cells or in animal models (Longnecker and Roizman, 1986; Polvino-Bodnar *et al.*, 1987). A virus lacking ori_L and containing two partially defective copies of ori_S has been isolated and has a significant growth defect compared to an analogous virus containing a wild type ori_L (Challberg, 1991). These data strongly suggest that ori_S and ori_L function in the context of the viral genome.

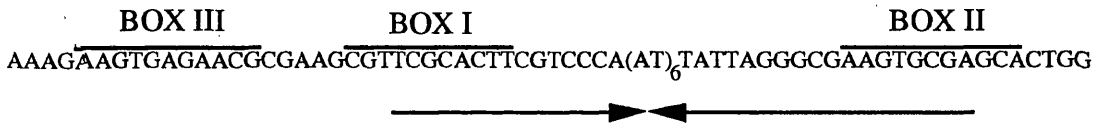

Figure 5. The structure of ori_S. Ori_S is shown as it would appear in the internal long repeat in the prototype orientation of the genome. Boxes I, II, and III are shown with the origin palindrome.

Analysis of ori_S suggests that it consists of two parts: a core origin, and flanking regions that enhance replication by methods not yet established (Lockshon and Galloway, 1988; Deb and Doelberg, 1988; Weir and Stow, 1990; Hernandez *et al.*, 1991; Wong and Schaffer, 1991). The core origin probably consists of 4 domains: three regions named box or site I, II and III, and the central AT-rich region (see figure 5). Boxes I and II, and the AT-rich region have been shown by genetic approaches to be essential for function *in vitro* (Lockshon and Galloway, 1988; Weir and Stow, 1990; Hernandez *et al.*, 1991). Boxes I and II (each 11 base pairs) bind the origin-binding protein (UL9 gene product) with high affinity. Box III is also a binding site but has much reduced affinity, despite differing from the box I sequence at only one position (Koff and Tegtmeyer, 1988; Olivo *et al*, 1988; Elias *et al*, 1990; Dabrowski and Schaffer, 1991). Mutations in boxes I or II that prevent UL9 binding also destroy or greatly inhibit origin function *in vitro*.

Mutations in box III reduce replication efficiency 5-fold. Inspection in the electron microscope of UL9 bound to DNA molecules containing the origin sequences suggests that UL9 covers approximately 110 nucleotides of the DNA (Rabkin and Hanlon, 1991), a region much larger than the 11 base pairs protected in nuclease footprinting assays. However the number of UL9 molecules bound to the DNA seen in the electron microscope could not be determined and it is possible that the central 11 base pairs of each binding site are complexed in a way that protects them from nuclease attack while a larger portion of the DNA is in less direct contact with the origin-binding protein. The origin-binding protein also appears to shorten the DNA by 20-30 nucleotides and introduce a bend into the molecule. This may reflect wrapping of the DNA around the protein.

The origin binding protein UL9.

The UL9 gene product was identified as the origin-binding protein by using immunoprecipitation and gel retardation assays (Olivo *et al*, 1988; Weir *et al*, 1989). Origin-containing DNA sequences bound to the origin-binding protein were specifically precipitated by antibodies against UL9 protein but not antibodies against other replication proteins. The number of molecules of UL9 that binds to each box in the origin has not been established. The observation that each box contains the inverted repeat GT(G or T)CG suggests the possibility that UL9 binds as a symmetrical dimer to each site although this is by no means certain (Koff and Tegtmeyer, 1988). It is known that UL9 is a dimer in solution with a dissociation constant of less than 10^{-10} M (Bruckner *et al*, 1991; Fierer and Challberg, 1992). The origin-binding function has been localised to the C-terminal third of UL9, amino acids 534-851 (Weir *et al*, 1989). The binding of the whole molecule to sites I and II is cooperative, cooperativity being ascribed to the N-terminal portion of the molecule (Elias *et al*, 1990; 1992). The cloned C-terminal third of UL9 did not show cooperative binding and was a monomer in solution. Footprinting analyses indicate that binding of UL9 induces a major change of conformation at the origin, the protein possibly forming a higher order structure by winding the DNA around itself (Elias *et al*, 1990; Koff *et al*, 1991; Fierer and Challberg, 1992). UL9 is a helicase displacing an oligonucleotide bound to M13 in the presence of ATP. Inclusion of a free 3' or 5' tail on the oligonucleotide facilitated the action of the helicase suggesting that it is displaced from one end rather than the middle, and indicating a 3'–5' polarity of helicase action (Bruckner *et al*, 1991; Fierer and Challberg, 1992). If the action of UL9 was analogous to that of SV40 large T antigen, UL9 would unwind the DNA around the origin after binding. However UL9 does not cause unwinding of a plasmid containing origin sequences, although a conformational change is induced. This change occurs in the absence of ATP (Fierer and Challberg, 1992). Thus UL9's helicase activity is independent of its origin-

binding activity. Much remains to be discovered about UL9 and its interaction with origin sequences.

Some of the sequences flanking the core origin of ori_S stimulate replication (Stow and McMonagle, 1983; Wong and Schaffer, 1991; Dabrowski and Schaffer, 1991). The sequences required were the promoter regulatory elements of the IE175 and IE68 / IE12 genes (which are divergently transcribed around ori_S) but not the transcript start sites themselves. Cotransfection of the flanking sequences on another plasmid titrated out the stimulating effect of flanking sequences in the origin-containing plasmid, suggesting removal of a diffusible factor mediating the stimulation. Replacement of the sequences with another promoter (HCMV immediate-early promoter) produced wild type levels of replication. These results are consistent with an unknown cellular protein (or proteins) binding to the required sequences thereby stimulating replication. The flanking regions do contain consensus binding sites for a number of transcriptional activator proteins such as Sp1 and NF1 but binding of these proteins has not been examined. Reconstruction of origin-dependent HSV-1 DNA replication *in vitro* should allow detailed examination of stimulation by the flanking regions.

ii) Priming.

During HSV DNA replication the parental DNA helix must be continually unwound and the lagging strand primed for the DNA polymerase. These activities can probably be assigned to the viral helicase-primase enzyme.

A virus specific helicase activity was partially purified from infected cells in 1988 (Crute *et al*, 1988). This enzyme has since been purified to homogeneity and been shown to have helicase and primase activity and to consist of the proteins UL5, UL8, and UL52, three of the viral gene products required for origin dependent DNA replication (Crute *et al*, 1989; Wu *et al*, 1988). The enzyme's catalytic properties have been thoroughly examined (Crute *et al*, 1989; Crute and Lehman, 1991; Sherman *et al*, 1992) and it has been overexpressed using the baculovirus expression system and purified from this source (Dodson *et al*, 1989). The purified enzyme has a molar mass of approximately 263,000 daltons and is a 1:1:1 heterotrimer of UL5, UL8, and UL52 (Crute and Lehman, 1991). The catalytic properties of the enzyme are listed below (table 8).

Table 8. Properties of the HSV-1 helicase-primase.

	Primase	**Helicase**
*p*H optimum:	8.5-9.0	active 5.5-9.0
Free Mg^{2+} requirement:	1.5 mM	1-10 mM
Template:	poly dT and M13 suitable	unpaired region required
Size of primers synthesised:	10-12 nt or 6 nt	
Rate and polarity[a] of helicase activity:		1 base pair per second, 5'–3'

[a] With respect to the strand to which it is bound.

The primase has very sharp *p*H and magnesium concentration optima, but no apparent template specificity being active on M13 or poly dT. Conflicting results have been published concerning the predominant size of the primers synthesised, 10–12 nucleotides and 6–8 nucleotides respectively being claimed (Crute and Lehman, 1991; Sherman *et al*, 1992). Thus the HSV-1 primase is similar to other eukaryotic primases.

The helicase activity of the helicase-primase complex is tolerant of wide *p*H and magnesium concentration changes. It displaces annealed oligonucleotides from M13 in an ATP-dependent manner, functioning better if displacing an oligonucleotide with a 3' unpaired tail than one with a 5' unpaired tail. The helicase activity is stimulated in the presence of the HSV-1 single stranded DNA-binding protein ICP8. Altogether, studies suggest that the helicase moves 5' to 3' along the strand to which it is bound. The measured rate of helicase action *in vitro* is much slower than the rate of movement off a pseudorabies virus replication fork which was measured at 50 nucleotides per second at 37 °C (Ben-Porat *et al*, 1977). (Pseudorabies virus is a member of the α herpesvirus family and shows many similarities to HSV.) However the rate of the HSV-1 helicase might be expected to increase substantially in the presence of other HSV-1 replication proteins. The helicase has two NTPase sites; site I hydrolyses GTP or ATP and site II ATP only. They have different turnover rates and K_m's and each is activated by a distinct DNA effector site which supports helicase action (Crute *et al*, 1991). The helicase may therefore bind both arms of a DNA fork.

A major step forward in localising the different activities of the complex to particular protein chains has been the observation that a stable subassembly (composed of proteins UL5 and UL52) has helicase and primase activities, and that these activities are indistinguishable from those seen with the full complex (Calder and Stow, 1990; Dodson and Lehman, 1991), with one exception to be discussed below. So far none of the

polypeptides composing the complex have been shown to be active alone as either helicase or primase. It has been observed that the amino acid sequence of the UL5 protein contains six regions that are conserved in all members of a superfamily of DNA and RNA helicases. One of these sequences is a consensus ATP-binding site (McGeoch *et al*, 1988b; Zhu and Weller, 1992). The six conserved regions, but not non-conserved regions, have been shown by directed mutation to be essential for the helicase function of the complex (Zhu and Weller, 1992). By exclusion it would appear that UL52 is primarily involved in primase activity but it is possible that UL5 and UL52 together constitute both the helicase and primase active sites. UL8 influences the cellular localisation of the UL5/UL8/UL52 complex (Calder *et al*, 1992). UL5 and UL52 form a complex in HSV infected cells in the absence of UL8 but this complex is not efficiently localised to the nucleus. Therefore one role of UL8 may be to facilitate nuclear transport.

As noted above, the UL5/UL8/UL52 complex and the UL5/UL52 subassembly have identical helicase and primase activities. Primase activity is measured in an indirect assay, in which a poly dT template primed by the enzyme under test, is the substrate for a DNA polymerase incorporating radiolabelled dNTPs. When the same assay was performed with M13 as template a much lower activity was observed for the UL5/UL52 subassembly than for the full complex (Sherman *et al*, 1992). This was puzzling as the size of the primers made by the two complexes on M13 was the same. Further experiments showed that DNA synthesis by HSV-1 polymerase (UL30/UL42 complex) on ICP8-coated, single stranded M13 could be primed by UL5/UL8/UL52 complex but not by the UL5/UL52 complex. The full reaction was referred to as lagging strand synthesis. Mixing of partially purified UL8 with UL5/UL52 restored most of the activity of the full complex in the lagging strand synthesis reaction. Effective priming by UL5/UL8/UL52 but not by UL5/UL52 was also seen when *E. coli* pol I was used instead of HSV-1 polymerase. Two explanations for this effect are possible: (i) a specific link between the primase and HSV-1 DNA polymerase mediated by UL8 and (ii) stabilisation of bound primers by the UL5/UL8/UL52 complex, again mediated by UL8. The results with the *E. coli* DNA polymerase suggest that the former is not the correct explanation unless considerable structural homology has been maintained in these distantly related polymerases. Primer stabilisation by UL8 seems the most likely explanation and would explain the template dependence of the UL8 effect. With a template of low complexity (such as poly dT) the short primers synthesised by the HSV-1 primase would reassociate quickly despite their high rate of dissociation at physiological temperatures. On a template of high sequence complexity such as M13 or natural HSV-1 DNA, reassociation at the specific site to which the primer was complementary would be so slow as to render priming ineffective. Therefore a mechanism for maintaining the integrity of primer template complexes is required. This function appears to be performed by UL8.

The T4 bacteriophage helicase/primase, comprising proteins 41 and 61, may have a similar system to HSV-1 for stabilising primers (Nossal and Hinton, 1987; Sherman *et al*, 1992). Together these proteins synthesise 5 nucleotide primers which are elongated by the viral polymerase. Gene 61 protein alone makes mostly 2 nucleotide primers, with traces of 5–45 nucleotide primers. Only the longer primers are elongated by the viral polymerase, presumably due to their greater stability. Nossal and Hinton propose that the gene 41 protein stabilises the 5 nucleotide primers synthesised by the full helicase/primase on the template. This seems analogous to the system proposed for HSV-1.

It has been proposed that a complex of two proteins C_1 and C_2 operates in eukaryotic cells as a primer recognition system (Pritchard and DePamphilis, 1983; Pritchard *et al*, 1983; Vishwanatha *et al*, 1992). The C_1C_2 complex increases the efficiency with which eukaryotic polymerase α-primase uses substrates with low primer to template ratios. The C_1C_2 complex binds to the polymerase α-primase so that a close association of the complex with primase is possible. However unlike UL8, the complex binds to single stranded (but not double stranded) DNA and exerts its effects even on substrates of low complexity. Therefore a different mechanism of action is probably involved.

iii) Elongation.

The viral DNA polymerase.

HSV-1 DNA is synthesised by the viral polymerase (Pol) the product of gene UL30 (*pol*). Drug resistant mutants with an altered DNA polymerase map to this gene strongly suggesting that its product is the catalytic protein (Purifoy *et al*, 1977; Chartrand *et al*, 1980; Coen *et al*, 1984). This suggestion was confirmed by the expression of the cloned UL30 gene which gave the HSV-1 polymerase activity (Dorsky *et al*, 1987; Marcy *et al*, 1990). There has been considerable interest in the UL30 gene product due to its potential as a drug target and the insight it gives into related eukaryotic DNA polymerases. The UL30 gene product possesses catalytic activity in the absence of other proteins but has low processivity on templates with significant regions of single stranded DNA (Gottlieb *et al*, 1990; Hernandez and Lehman 1990). It has a 3'-5' exonuclease (Knopf, 1979; O'Donnell *et al*, 1987) and also a 5'-3' exonuclease or RNaseH activity which may act to remove primers (Crute and Lehman, 1989). The regions of the protein chiefly responsible for the catalytic activities have been localised by studies of drug resistant mutants and by comparing the amino acid sequence of UL30 to homologous DNA polymerases of other organisms. As mentioned earlier comparison reveals six regions of homology (named I to VI, in order of decreasing similarity) which are conserved between UL30 and the polymerases of a number of eukaryotes and viruses

(Larder *et al*, 1987; Wong *et al*, 1988). UL30 has greatest similarity to human polymerase δ. UL30 mutants with altered sensitivity to nucleotide and pyrophosphate analogues and aphidicolin, have mutations predominantly in regions I, II, and III (residues 881-896, 694-736, and 805-845 respectively) (Tsurumi *et al*, 1987; Gibbs *et al*, 1985; 1988; Hall *et al*, 1989; Marcy *et al*, 1990). It is therefore likely that these regions are directly involved in substrate and drug binding and the polymerising activity of the enzyme. Limited homology to conserved regions of bacterial and bacteriophage DNA polymerases has led to the proposal that region IV (residues 432-479) is essential for 3'–5' exonuclease activity, and that sequences near the amino terminus of the protein (248-262) are involved in RNaseH activity (Bernad *et al*, 1989; Reha-Krantz, 1989;1990).

The product of gene UL42 was identified initially as a DNA-binding protein with a molar mass of 65,000 daltons (Bayliss *et al*, 1975; Vaughan *et al*, 1985; Marsden *et al*, 1987; Parris *et al*, 1988). The protein is required for viral growth and DNA synthesis (Marchetti *et al*, 1988; Johnson *et al*, 1991) and forms a heterodimer with the viral DNA polymerase (Gallo *et al*, 1988; Crute and Lehman, 1989; Gottlieb *et al*, 1990). The protein functions as an accessory factor of Pol, increasing the rate of incorporation of deoxynucleotide triphosphates into activated DNA (Gallo *et al*, 1989) and increasing the processivity of Pol on a defined template-primer (Gottlieb *et al*, 1990; Hernandez and Lehman, 1990). The physical association of Pol and UL42 parallels that observed previously between HSV-2 ICP34,35 and HSV-2 DNA polymerase (Vaughan *et al*, 1985). ICP34,35 has subsequently been shown to be the HSV-2 equivalent of UL42 protein (Gallo *et al*, 1989). The effect of UL42 on the rate of catalysis of the enzyme is dependent on the concentration of salt in the assay (Hart and Boehme, 1992). Footprinting of the full enzyme and its individual subunits on a defined template (a short double stranded DNA with a 5' single stranded overhang) showed that the isolated catalytic subunit binds to a region centred on the recessed 3' end, protecting 15 base pairs of double stranded DNA and 18 nucleotides of the unpaired region. Inclusion of UL42 protects an additional 5 base pairs of the double stranded region next to the UL30 binding site without affecting the unpaired region. UL42 alone bound non-specifically to the double stranded DNA. Filter binding and gel shift assays suggest that UL42 increases the affinity of UL30 for the model substrate by 5–10 fold (Challberg, 1991).

Physical interaction of UL30 and UL42 is required for DNA replication but not for basal polymerase activity (Digard *et al*, 1993; Stow, 1993). The final 227 residues of UL30 are sufficient for its interaction with UL42 (Digard and Coen, 1990) although attempts to map the region of interaction more precisely have given conflicting results (Tenney *et al*, 1993; Digard *et al*, 1993; Stow, 1993). Some portion of the last 60 amino acids of the polymerase is probably required. The portion of UL42 forming the interface with UL30 has not been determined (Owsianka *et al*, 1993).

The major DNA-binding protein ICP8.

The major HSV-1 DNA-binding protein ICP8 was identified as a 130 kD nuclear protein that bound tightly to single stranded DNA columns (Bayliss *et al*, 1975; Purifoy and Powell, 1976; Powell *et al*, 1981; Fenwick *et al*, 1978; Knipe and Spang 1982). It has many of the properties of helix destabilising proteins, binding more tightly to single than double stranded DNA and binding to single stranded DNA in a cooperative and sequence independent manner (Ruyechan, 1983; Ruyechan and Weir, 1984). Therefore ICP8 may be analogous to gene 32 protein of T4 bacteriophage and *E. coli* SSB protein. It is a matter of dispute whether processive replication of long single stranded DNA molecules by the UL30/UL42 complex requires ICP8 (Hernandez and Lehman, 1990; Gottlieb *et al*, 1990). *E. coli* single stranded DNA-binding protein SSB may be able to substitute for ICP8 in this reaction (O'Donnell *et al*, 1987). Interestingly, affinity chromatography experiments show that ICP8 interacts strongly with the viral alkaline nuclease and rather more weakly with UL42 (Vaughan *et al*, 1984). This result has been confirmed for alkaline nuclease by co-immunoprecipitation (Thomas *et al*, 1992). It is possible that ICP8 also interacts with other HSV-1 proteins.

ICP8 appears to play a role in organisation of viral DNA replication in the infected cell. Early electron microscopic autoradiography experiments (Rixon *et al*, 1983) showed that HSV DNA replication began at small, discrete sites that grew into large globular replication compartments that eventually filled the whole nucleus. It was later shown that ICP8 localised to probably identical early 'pre-replicative sites' and relocalised to 'replicative sites' as DNA replication progressed (Quinlan *et al*, 1984). Both types of site are strongly associated with the nuclear matrix. Other HSV-1 DNA replication proteins localised to replicative sites in a process that was dependent on functional ICP8 (De Bruyn Kops and Knipe, 1988; Bush *et al*, 1991; Calder *et al*, 1992). A number of cellular proteins with DNA- or cell proliferation-associated functions also co-localised with ICP8 at these sites. These included cellular single stranded DNA binding protein (RF-A); proliferating cell nuclear antigen (PCNA) the polymerase δ processivity factor; retinoblastoma protein; p53 'anti-oncogene' protein; DNA ligase I and cellular DNA polymerase α (Wilcock and Lane, 1991). These results are especially interesting given that it is not known whether any cellular proteins are involved in HSV-1 DNA replication, either during initiation or at the replication fork. HSV-1 does not appear to encode a ligase or topoisomerase, enzymes that are likely to be required for complete replication of the viral genome, and so these may be supplied by the cell.

iv) Enzymes of nucleotide metabolism.

In addition to proteins directly involved in DNA replication, HSV-1 encodes a number of enzymes that are involved in DNA and nucleotide metabolism. These are thymidine kinase (Kit and Dubbs, 1963); ribonucleotide reductase (Cohen, 1972); deoxyuridine triphosphatase (dUTPase, Wohlrab and Francke, 1980; Fisher and Preston, 1986); uracil-DNA glycosylase (Caradonna *et al*, 1987); and alkaline nuclease (Keir and Gold, 1963).

Thymidine kinase.

The HSV-encoded thymidine kinase (TK) phosphorylates thymidine (Kit and Dubbs, 1963), other deoxypyrimidine nucleosides, and some nucleoside analogs (Jamieson *et al*, 1974; Elion, 1982). Several anti-HSV drugs, for example acyclovir, are activated by TK, and mutations in the TK gene can lead to resistance to such drugs (Fyfe *et al*, 1978; Field *et al*, 1980; Coen and Schaffer, 1980; Schnipper and Crumpacker, 1980). The involvement of TK in viral pathogenesis is therefore of continuing interest.

TK is not required for virus growth in rapidly growing cultured cells, but TK minus mutants are impaired for growth in serum-starved cells (Field and Wildy, 1978; Jamieson *et al*, 1974). TK mutants grow inefficiently in nervous system tissues and have reduced pathogenicity when innoculated intracerebrally (Field and Darby, 1980). Available evidence suggests that TK minus mutants can establish latency but that they reactivate less frequently (Coen *et al*, 1989; Efstathiou *et al*, 1989).

Ribonucleotide reductase.

Ribonucleotide reductase (RR) plays a vital role in producing precursors for DNA synthesis by reducing ribonucleotides to deoxyribonucleotides. The HSV enzyme (Cohen, 1972) is composed of two subunits R1 (large) and R2 (small). These are homodimers of 140 kD and 38 kD proteins, the products of genes UL39 and UL40 (Huszar and Bacchetti, 1981; Anderson *et al*, 1981; Dutia, 1983; Preston, V.G. *et al*, 1984; Frame *et al*, 1985). Unlike cellular enzymes the HSV ribonucleotide reductase is not feedback inhibited by deoxynucleotide triphosphates (Huszar and Bacchetti, 1981).

Studies of temperature sensitive mutants and deletion mutants show that RR is not required in exponentially growing monkey cells at 37 °C, but is required in serum starved cells or those growing at 39.5 °C (Preston, V.G. *et al*, 1984; Goldstein and Weller, 1988b). The latter result appears to be due to the thermolability of some cellular or viral component that substitutes for RR during growth of mutants at 37 °C (Preston, V.G. *et al*, 1988; Goldstein and Weller, 1988c). This component may be the cellular ribonucleotide reductase.

Analysis of RR mutants in animal models (Cameron *et al*, 1988) indicates that the enzyme is required for virulence in mice and reactivation from latency (Jacobsen *et al*, 1989) but not for virulence in guinea pigs (Turk *et al*, 1989). RR mutants grow to 100-fold lower titre in primary human corneal fibroblasts suggesting that the enzyme is a valid antiviral target in humans (Brandt *et al*, 1991). There is considerable interest in inhibiting RR with targeted antiviral drugs, and more active compounds have been developed from a lead peptide which specifically inhibits the viral enzyme (Dutia *et al*, 1986; Cohen *et al*, 1986; reviewed in Marsden, 1992).

Deoxyuridine triphosphatase (dUTPase).

HSV-1 dUTPase catalyses the conversion of dUTP to dUMP and pyrophosphate (Wohlrab and Francke, 1980; Caradonna and Cheng, 1981). This reaction reduces the quantity of uridine incorporated into replicating DNA and also provides a source of dUMP for conversion to dTMP by thymidylate synthetase. The reaction is important for HSV-1 as the viral DNA polymerase is able to use dUTP as a substrate (Williams, 1984).

The viral dUTPase is not required for HSV-1 to grow in cultured cells even when the cells are restricted in their growth (Fisher and Preston, 1986). Pyles and colleagues (1992) investigated the growth of dUTPase deletion mutants in mice. The mutant viruses had reduced virulence and grew to reduced titres in the central nervous system despite growing to wild type levels in epithelial and peripheral nervous system tissue. Latent infections were established but reactivation was much less frequent.

Uracil DNA glycosylase.

This DNA repair enzyme removes uracil residues from DNA. Such residues can arise by misincorporation of dUTP during replication or via the deamination of cytosine. The HSV-1 uracil DNA glycosylase, first reported by Caradonna and Cheng (1981), is encoded by gene UL2 (Worrad and Caradonna, 1988; Mullaney *et al*, 1989). A mutant lacking the enzyme grows to the same titre as wild type virus in a single step growth experiment (Mullaney *et al*, 1989). Studies of mutant viruses in animal models have not been carried out.

Alkaline nuclease.

Infection of cells by HSV-1 or HSV-2 results in the induction of a novel enzyme activity, alkaline nuclease (Keir and Gold, 1963; Hoffmann and Cheng, 1978). Alkaline nuclease purified from HSV-infected cells exhibits exo- and endonuclease activities and differs from host cell nucleases in its biochemical properties, especially its high *p*H optimum (Morrison and Keir, 1968; Hoffman and Cheng, 1978; 1979; Francke *et al*, 1978; Streobel-Fidler and Francke, 1980). The isolation of a temperature-sensitive mutant showed that the enzyme was encoded by the virus (Francke *et al*, 1978). This finding was

confirmed by the induction of alkaline nuclease activity in *Xenopus* oocytes following injection of HSV DNA (Preston and Cordingley, 1982). The nuclease is not involved in viral DNA synthesis but probably plays a role in processing and packaging of viral DNA (Weller *et al*, 1990).

MATERIALS

All chemicals were Analar grade or equivalent and were supplied by BDH or Sigma unless noted.

Peptide synthesis chemicals.

Dimethyl formamide (DMF), piperidine, ether, trifluoroacetic acid (TFA) and acetonitrile were supplied by Rathburn Chemicals. Anisole and ethanedithiol (EDT) were from Aldrich. Derivatised amino acids were supplied by Pharmacia LKB or Novabiochem. Amino acid-linked resins were purchased from Peptide and Protein Research Consultants, Exeter, UK.

Production of antisera and monoclonal antibodies.

Rabbits and mice were supplied by Hylyne. Freund's adjuvant (complete and incomplete) was supplied by Sigma or Life Technologies. Pristane was supplied by Sigma.

ELISA assays.

Immulon 1 plates and strips were supplied by Dynatech. Conjugates for detection purposes were supplied by Sigma.

SDS polyacrylamide gels.

Acrylamide was supplied by Koch-Light Ltd or BDH and ammonium persulfate, tetramethylethylenediamine (TEMED) and Coomassie Brilliant Blue R250 were from Bio-Rad.

Tissue culture reagents.

Glasgow modified Eagles medium, Dulbecco's modified Eagles medium, TC100 medium, sodium bicarbonate solution, glutamine, penicillin, streptomycin, new born calf serum, and foetal calf serum for hybridoma cells were all supplied by Life Technologies. Foetal calf serum for *S. frugiperda* cells was supplied by Advanced Protein Products. Tissue culture flasks were from Falcon.

Western blotting.

Nitrocellulose for Western blotting was supplied by Schleicher and Schuell. Gelatin and colour development reagent 4-chloro-1-naphthol were from Bio-Rad. Horseradish peroxidase conjugates were obtained from Sigma.

Radiochemicals.

All radiochemicals were supplied by Amersham.

Chromatography of proteins.

All chromatography matrices and pre-packed columns were obtained from Pharmacia.

DNA band shift assays.

Oligonucleotides were synthesised on a Biosearch 8600 automated DNA synthesiser (Applied Biosystems) 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.

T4 polynucleotide kinase was obtained from New England Biolabs. T4 bacteriophage gene 32 protein and T7 bacteriophage RNA polymerase were obtained from Boehringer Mannheim and New England Biolabs respectively. HSV-1 UL42 protein was purified by the method of Gallo *et al* (1988).

BUFFER SOLUTIONS

T buffer:
10 mM triethanolamine *p*H7.5 (HCl)
10 mM KCl
1.5 mM $MgCl_2$
1 mM DTT
0.5 mM PMSF
10 μg/ml leupeptin
10 μg/ml pepstatin A

Reticulocyte standard buffer (RSB):

10 mM tris *p*H7.5 (HCl)
10 mM KCl
1.5 mM $MgCl_2$
1 mM DTT

Phosphate buffered saline (PBS):

136 mM NaCl
2.6 mM KCl
8 mM Na_2HPO_4
1.4 mM KH_2PO_4
0.7 mM $CaCl_2$
0.5 mM $MgCl_2$

Tris buffered saline (TBS):

20 mM tris *p*H7.5
0.5 M NaCl

Towbin blotting buffer:

25 mM tris
192 mM glycine
20% methanol (v/v) *p*H 8.3 (HCl)

METHODS

Synthesis of peptides.

Synthesis:

Peptides were synthesised using Fmoc solid phase chemistry (Atherton and Sheppard, 1989) and its associated standard procedures on a Biolynx automated peptide synthesiser (LKB Biochrom now Novabiochem) with software provided by the company. The resins used were Dimacryl KA resin with the carboxy terminal amino acid attached for single chain peptides and K_7A-Dimacryl KA resin for branched chain peptides.

Peptides were cleaved using 95% TFA in water plus the appropriate scavengers for the peptide's sequence. After cleavage peptides were washed by ether extraction, dissolved in water and lyophilised. The lyophilised peptides were stored at -20 °C.

Analysis:

Single chain peptides were analysed by Fast Atom Bombardment mass spectrometry (FAB-MS) by M-Scan Ltd, Ascot, Berkshire, UK.

Amino acid analysis of branched peptides was performed by Cambridge Research Biochemicals Ltd.

A Beckman System Gold instrument was used for high pressure liquid chromatography (HPLC) with a Dynamax reversed phase column (C_8, 4.1 ml bed volume; Anachem). A gradient from 0.1% TFA in water to 0.05% TFA, 95% acetonitrile in water, was applied over 10 ml at a flow rate of 0.5 ml/min. Peptides were detected by their absorbance at 205 nm and peaks were scanned (190-304 nm) to confirm that their absorbance profiles were characteristic of peptides.

Production of rabbit antipeptide and antiprotein sera.

Half sandy lop rabbits were pre-bled and their sera screened by Western blotting for reactivity against HSV-1-infected cell extracts and baculovirus-infected cell extracts. Rabbits whose sera showed no reactivity were used for immunisations. Peptide or protein solutions emulsified 1:1 with Freund's complete adjuvant (for the initial injection) or incomplete adjuvant (for subsequent injections) were injected intramuscularly into the hind limb. One hundred micrograms of peptide or 8–50 μg of protein were used per rabbit per injection. Test bleeds were screened for positive reaction on Western blot. Typically, animals were injected on days 0, 10, 30, and 40, test bled on day 39 and ex sanguinated under deep anaesthesia on day 50.

SDS polyacrylamide gels.

Sodium dodecyl sulfate (SDS) polyacrylamide gels for proteins used the buffer system of Laemli (1970). All resolving gels were 10% acrylamide with $^1/_{40}$th the quantity of N,N' methylenebisacrylamide unless otherwise stated. Resolving gels were made up to give a final buffer concentration of 375 mM tris, 3.5 mM SDS, *p*H 8.9 (HCl). Stacking gel with wells for samples was 5% acrylamide in 125 mM tris, 3.5 mM SDS, *p*H 6.7 (HCl). Gels were polymerised using a final concentration of 0.1% ammonium persulfate and 0.1% tetramethylethylenediamine (TEMED). Running buffer for the gels was 50 mM tris, 50 mM glycine and 3.5 mM SDS. Samples to be loaded onto gels were mixed 2:1 with boiling mix (0.15 M tris *p*H 6.7, 30% glycerol, 0.21 M SDS, 2.1 M β-mercaptoethanol) and heated for 5 minutes at 90 °C before loading.

Proteins were detected by staining with 0.2% Coomassie Brilliant Blue R250 in 47% methanol, 6% acetic acid in water and destained in water containing 5% methanol, 7% acetic acid. Scanning of stained SDS polyacrylamide gels was performed using a Hoefer Scientific Instruments (San Francisco, California, USA) GS 300 transmittance/reflectance scanning densitometer with a GS 360 data system from the same company run on an IBM personal computer.

Gels for figures were copied onto Kodak Electrophoresis duplicating paper before drying or dried and then photographed. Gels were dried for preservation onto Whatman filter paper using heat and vacuum.

Preparation of HSV-1 infected cell extracts for protein gels.

Baby hamster kidney (BHK) 21 clone 13 cells (Macpherson and Stoker, 1962) were grown in 850 cm^2 tissue culture roller bottles in Glasgow modified Eagle's medium supplemented with 0.25% $NaHCO_3$, 4 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, 10% tryptose phosphate broth, and 10% new-born calf serum. Medium was removed from 80% confluent monolayers and 10 plaque forming units (pfu) per cell of low passage working stock of HSV-1 17^+ (Brown *et al*, 1973) was added in identical medium except that it contained only 20% of the methionine and only 2% serum. Two hours post infection (2 hpi) the infecting stock was removed and 10 MBq of ^{35}S-methionine was added in the same medium. Sixteen hours later the cell monolayer was washed three times with PBS and the cells were removed by heating in 50 mM tris *p*H 6.7, 10% glycerol, 70 mM SDS, 0.7 M β-mercaptoethanol. These extracts were used at a concentration of 1.3 x 10^6 cell equivalents per cm width of gel.

*(1 hour at 37°C; all other Western blotting procedures were carried out at room temperature except where noted)

*4-chloro-1-naphthol and hydrogen peroxide were used at 0.05% and 0.015% respectively in TBS containing 16% methanol.

Western blotting.

Protein mixtures separated on SDS polyacrylamide gels were transferred to nitrocellulose membrane by electroblotting using a Bio-rad transblot tank run at 0.25 amps for three hours. The buffer system of Towbin *et al*, (1979) was used. Non-specific binding sites on membranes were blocked using 3% gelatin in TBS* before the addition of antisera diluted in TBS containing 0.05% Tween 20 and 1% gelatin. Diluted antisera were incubated on the membrane for 2 hours at 37 °C or overnight at room temperature. After extensive washing in TBS containing 0.05% Tween 20 (TTBS), HRP conjugated protein A (for rabbit antisera) or HRP conjugated goat anti-mouse IgG was added, diluted in 1% gelatin TTBS, and incubated for 1 hour. After further washing in TTBS followed by TBS, blots were developed using 4-chloro-1-naphthol and hydrogen peroxide*. Development was stopped by washing with deionised water and blots were dried and conserved in the dark.

Growth of *S. frugiperda* cells and *Autographa californica* nuclear polyhedrosis virus.

Spodoptera frugiperda Sf9 cells (strain IPLB-Sf-21, Summers and Smith, 1987) (abbreviation Sf) were grown at 28 °C in tissue culture dishes or flasks in TC100 medium supplemented with 5% foetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. Cells were routinely divided 1 to 9 every three days.

Wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV or baculovirus), and its recombinants, were grown on monolayers of Sf cells. Virus was inoculated onto approximately 75% confluent monolayers of cells and the medium harvested as virus stock three days later. Virus stock was stored at 4 °C or at -70 °C.

Precipitation of proteins with ammonium sulfate.

Finely ground solid ammonium sulfate was added slowly with agitation to protein solutions on ice. Solutions were then incubated for 10 minutes at room temperature or 4 °C with end over end mixing before being centrifuged for 10 minutes at room temperature in a benchtop microfuge.

Purification of UL8 protein.

Harvesting of infected cells and production of crude extract:

Sf cells (eight 175 cm^2 flasks) were infected with recombinant baculovirus AcUL8 (Calder and Stow, 1990) and harvested 60-70 hours later by shaking the cells into the medium and pelleting at 1860g. Medium was retained as virus stock. The cells were washed by resuspension in 10 ml of TBS per flask and repelleted. The cell pellet was resuspended in ice cold buffer G (see overleaf) without NP40, and then NP40 was added to give a final volume of 400 µl of buffer per flask. The cell suspension was incubated for 10 minutes on ice, centrifuged for 2 minutes at 10,000g and the supernatant removed and centrifuged at 175,000g for 30 minutes at 4 °C. The supernatant from this second centrifugation step (the crude extract) was frozen at -70 °C until required.

Fractionation of the crude extract:

Initial fractionation was performed using diethylaminoethyl (DEAE) sepharose. Thawed crude extract (4 ml) was centrifuged at 10,000g and loaded onto a 2 ml column equilibrated in buffer A. The column was washed with 10 ml of the same buffer and a 14.3 ml gradient of 100 mM to 350 mM NaCl in buffer B was run. UL8 protein eluted between 240 mM and 340 mM NaCl. A new column was packed for each purification. The UL8 containing fractions (seen as a distinct peak in the UV trace and previously identified by SDS polyacrylamide gel electrophoresis) were loaded onto a 5 ml phenyl sepharose column (Pharmacia, Low substitution Fast flow) equilibrated in buffer B containing 100 mM NaCl. The column was washed with 10 ml of this buffer before a 10 ml gradient to 100% buffer C was applied. UL8 protein eluted at the end of this gradient as a symmetrical peak in the 280 nm absorbance profile. Yield was approximately 1.5 mg of UL8 protein. Purity was estimated at 95% by scanning of a Coomassie blue stained SDS polyacrylamide gel.

Some early preparations of UL8 were passed through a superose 12 gel filtration column. However as this did not significantly increase purity, this step was not employed for subsequent preparations.

Buffers used in the purification:

Buffer G
20 mM triethanolamine *p*H7.5 (HCl)
10% glycerol
10 mM KCl
1.5 mM $MgCl_2$
1 mM dithiothreitol (DTT)
0.5 mM phenylmethylsulfonyl fluoride (PMSF)
0.5% NP40
10 µg/ml leupeptin
10 µg/ml pepstatin A

Buffer A
20 mM triethanolamine *p*H7.5 (HCl)
10% glycerol
0.1 M NaCl

Buffer B
20 mM tris *p*H8.0 (HCl)
10% glycerol

Buffer C
H_2O, 10% glycerol, 4 °C

Measurement of protein concentrations.

Protein concentrations were measured using a Pierce Bicinchoninic acid (BCA) protein assay kit with a solution of bovine serum albumin (BSA) also from Pierce as standard. Colour changes were detected at 562 nm using a Beckman DU-62 UV/Visible spectrophotometer.

Gel filtration chromatography.

Approximately 60 µg of purified UL8 protein in 150 µl of buffer was added to a 25 ml Pharmacia superose 12 gel filtration column equilibrated in 20 mM triethanolamine *p*H 7.5, 10% glycerol, 0.2 M NaCl. The column was run at 0.2 ml/minute in the same buffer. Molecular mass standards (Sigma) were dissolved at a concentration of 1 mg/ml in water and clarified by centrifugation before use.

DNA band shift assays.

Preparation of DNA:

The oligonucleotides used are represented below. When annealed they are identical to oligonucleotide I of Weir and Stow (1990), and the boxed portion represents box I of ori_S (a UL9 binding site) identified by these authors. However the DNA was used as a non-sequence-specific probe.

The oligonucleotides were supplied by Dr N. D. Stow in purified form.

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1          GATCCGCGA AGCGTTCGCACTTCGTC CCA
2              GCGCT TCGCAAGCGTGAAGCAG GGTCTAG
```

5' labelling:

Single stranded DNA (oligonucleotide 2; 1 μg), partially double stranded DNA (annealed oligonucleotides 1 and 2; 1 μg), and DNA/RNA hybrids (poly(rA).oligo$(dT)_{12-18}$, Pharmacia, 10 μg) were 5' labelled using T4 bacteriophage polynucleotide kinase and γ-^{32}P ATP (20 μCi per reaction) in kinase buffer: 50 mM tris HCl *p*H 7.6, 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM spermidine, 5 mM DTT for 30 minutes at 37 °C. Oligonucleotides were purified by phenol/chloroform extraction after labelling, ethanol precipitated, and resuspended in water for storage at -20 °C.

3' labelling:

Fully double stranded DNA was produced by 3' labelling annealed oligonucleotides 1 and 2 (200 ng) using Klenow fragment of *E. coli* polymerase and α-^{32}P GTP (20 μCi) the first complementary nucleotide, for 30 minutes at room temperature followed by a chase with excess of all four cold nucleotides for 15 minutes also at room temperature. The buffer used was: 50 mM tris HCl *p*H 7.6, 10 mM $MgCl_2$, 1 mM DTT, 50 μg/ml BSA (restriction enzyme grade). The 3' labelled oligonucleotide was purified and stored in the same manner as the 5' labelled oligonucleotides.

DNA-protein binding reactions:

DNA-protein binding reactions were carried out in GRA buffer: 50 mM HEPES *p*H 7.8 (NaOH), 10% glycerol, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT or the same buffer without sodium chloride or magnesium chloride (low salt GRA). All reactions also contained 100 μg/ml BSA and had a final volume of 20 μl. The reaction mixtures were incubated at 37 °C for 20 minutes, unless otherwise stated. Five microlitres of loading buffer (25% glycerol, 10 mM DTT, 0.01% bromophenol blue, all in TBE) was then added before electrophoresis.

* Wells were coated with 50 µl of protein solution and incubated overnight at 37°C (Where necessary UL8 was diluted in water containing 10% glycerol). The wells were washed five times with 'ELISA' PBS tween (EPT) and blocked with 100µl of 0.5% BSA in EPT for 1 hour at 37°C. The first antibody was added diluted in 0.5% BSA-EPT (50µl) and was left in the wells for 1 hour at 37°C. The wells were again washed five times with EPT. Bound mouse antibodies were detected with goat anti-mouse HRP conjugate (Sigma) diluted 1:1000 in 0.5% BSA-EPT (50 µl, 1 hour at 37°C); rabbit antibodies were detected similarly with protein A HRP conjugate. Wells were again washed five times with EPT before addition of the detection reagents dissolved in citrate buffer (100 µl): 0.1M Na_2HPO_4, 0.08M citric acid *p*H 4.0.

EPT is 0.15 M NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 0.05% Tween 20, stored as a 10X solution.

DNA gels:

DNA was electrophoresed through 5% polyacrylamide gels containing $^1/_{60}$th bis-acrylamide, made up in TBE: 90 mM tris, 89 mM boric acid, 1 mM EDTA. Gels were electrophoresed at 100-150 V using TBE as tank buffer, until the dye front was half to two thirds of the way to the bottom of the gel.

Mouse immunisation and monoclonal antibody production.

Female balb/c mice were immunised intraperitoneally with solutions of purified UL8 protein emulsified 1:1 with complete Freund's adjuvant (for the initial injection) or incomplete adjuvant (for subsequent injections). 5 or 10 μg of purified protein was used per injection. Orbital bleeds were screened for UL8 reactive antibodies by Western blotting. One positive mouse was selected, boosted with 35 μg of protein in 10% glycerol, killed three days later and its spleen removed.

Sple nocytes were fused at a ratio of approximately 10:1 with Sp2/0 Ag14 balb/c mouse myeloma cells using 33% polyethylene glycol, and plated out in 96 well plates in Dulbecco's modified Eagle's medium containing 5-20% foetal calf serum, 1-4% glutamine, 1% penicillin/streptomycin, 100 μM hypoxanthine, 0.42 μM aminopterin, 63 μM thymidine. Colonies were screened at the 24 well plate stage by ELISA. Lines with reactive culture supernatants were grown on in the same medium but without hypoxanthine, aminopterin, or thymidine and stored in liquid nitrogen.

Ascites fluid was produced by injecting each positive line into mice primed intraperitoneally with 0.5 ml of pristane (c.5x10^6 cells per mouse). When swelling became apparent mice were killed and their peritoneal cavities drained.

ELISA assays.

ELISA assays were performed in duplicate using Immulon 1 strips or plates.* Bound antibodies were detected using horseradish peroxidase conjugated to protein A or goat anti-mouse immunoglobulin. The chromogenic substrate ABTS and hydrogen peroxide were employed as detection reagents (used at final concentrations of 0.5 mg/ml and 0.015% respectively). These were mixed immediately before use. Optical density at 405 nm was read using a Titertek Multiskan Plus plate reader.

Immune precipitations.

The antigen for immune precipitations was a crude extract of Sf cells infected with AcUL8 (c.10 pfu per cell) and labelled with ^{35}S methionine from 25–32 hours post infection.

Cells were harvested at 32 hours post infection, lysed in RSB containing 0.5% NP40,

0.5 mM PMSF, and 1.0 mM DTT, and the extract clarified by centrifugation at 50,000 rpm. Approximately 1 x 10^5 cell equivalents of the labelled extract (20 µl) were mixed with 1 µl of serum or ascites fluid and incubated on ice for 1 hour. (1 µl of sheep anti-mouse antibody was added to tubes containing ascites fluid after 40 minutes.) Thirty microlitres of immunoprecipitin (suspended, freeze-dried *Staphlococcus aureus,* Bethesda Research Laboratories) was added to the mixture which was then incubated for a further 45 minutes. The pellet was centrifuged, separated from the supernatent and washed three times with the following buffer: 0.1 M Tris *p*H 8.0, 0.5 M LiCl, 1% β mercaptoethanol. The final pellet was dissolved in boiling mix (see 'SDS polyacrylamide gels') and run on a 10% SDS polyacrylamide gel.

RESULTS

The initial aim of this project was to identify the products of the HSV-1 genes UL5, UL8, UL9, and UL52 by using antisera raised against peptides with sequences corresponding to portions of the predicted amino acid sequence of these proteins.

I) SYNTHESIS AND ANALYSIS OF PEPTIDES.

Peptides were synthesised with sequences corresponding to the predicted amino acid sequence of parts of the proteins UL5, UL8, UL9, and UL52 (table 1). The regions chosen were terminal as termini are generally on the protein's surface. This was intended to ensure that in the full protein the epitope recognised by the sera would not be obscured by secondary structure. In addition, regions containing amino acid residues that would cause problems during peptide deprotection were avoided. For example, peptide 272 omits the N-terminal methionine of UL8 as the combination of methionine and tryptophan in a peptide can lead to modification of the methionine (Atherton and Sheppard, 1989). The peptides were synthesised as single chain peptides for analysis of molecular mass and as 'branched' peptides (figure 1, overleaf) to allow the generation of antisera without conjugation to carrier proteins.

Table 1. Sequence of peptides and their position in the corresponding protein.

Peptide	Sequence	Residues of protein	Number of residues in the protein
UL5 238	HILSALRDPNVVIVY	868–882	882
UL8 272	DTADIVWVEESVSAI	2–16	750
UL8 219	KFVYPFDDKMSFLFA	736–750	750
UL9 218	EAWPMMQGAVNFSTL	837–851	851
UL52 220	PCSPSVPCSTSQPSS	1044–1058	1058

Peptides were analysed by three methods. The molecular mass of single chain peptides was determined by Fast Atom Bombardment Mass Spectrometry (FAB-MS, by M Scan Ltd); the branched peptides were analysed for amino acid composition (by CRB Ltd); and both forms of peptide were analysed for purity by reversed phase HPLC.

The measured molecular masses of peptides UL5 238 and UL8 219 agreed exactly with the predicted values, (table 2). The value of 1677 rather than 1680 obtained for

peptide UL9 218 is not easily explained, the difference being too small to correspond to any of the species, such as a sodium ion, commonly found associated with peptides. The discrepancy may represent a counting error by M-Scan Ltd as the exact M_r of each peak in the spectrum was determined manually. The company reported that they were not able to maintain peptide UL8 272 in solution under the conditions required for FAB-MS and so no data was obtained for this peptide. The spectrum of peptide UL52 220 contained three

Figure 1. Structure of the branched peptides. See Tam (1988).

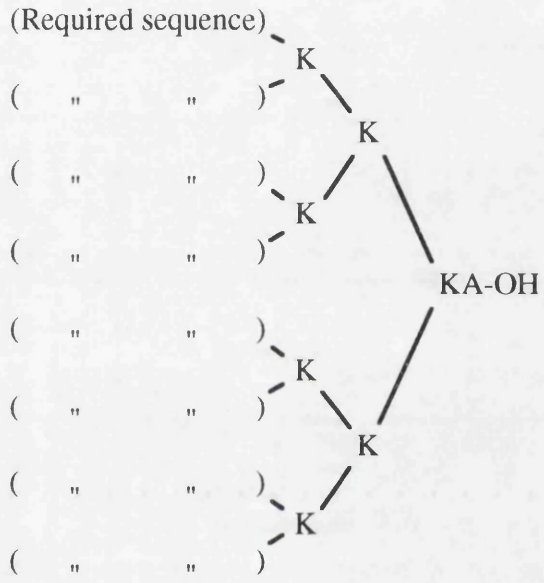


Table 2. Molecular mass and approximate purity of peptides. The measured relative molecular masses are listed, with the predicted values in parentheses. The measurement is accurate to +/- 1.

Peptide	**Purity by HPLC**		**M_r**
	Single chain	**Branched**	
UL5 238	N.D.[a]	N.D.	1707 (1707)
UL8 272	76%	97%	N.D.
UL8 219	60%	91%	1853 (1853)
UL9 218	42%	86%	1677 (1680)
UL52 220	42%	79%	1463[b] (1462)

[a] N.D. = not determined.

[b] Analysis showed three peaks corresponding to molecular masses of 1463, 1519, and 1635.

Table 3. Amino acid content of peptides. The quantity of each amino acid observed was compared to the expected quantity calculated from the number of residues in the sequence and the quantity of peptide hydrolysed. Thus, for example, peptide UL5 238 contained one arginine residue and 75% of the expected quantity of arginine was found in the analysis. The determination has an error of +/– 15%, and so values from 0.85–1.15 are regarded as equal to one. Major deviations from the expected values are discussed in the text.

	Peptides				
	UL5 238	**UL8 272**	**UL8 219**	**UL9 218**	**UL52 220**
Alanine[a]	1.22 (1.12)	2.73 (2.12)	1.41 (1.12)	2.22 (2.12)	- (0.12)
Asx[b]	2.17 (2)	2.03 (2)	2.33 (2)	0.96 (1)	-
Arginine	0.75 (1)	-	-	-	-
Cysteine	-	-	-	-	1.70 (2)
Glx[b]	-	2.33 (2)	-	2.04 (2)	1.22 (1)
Glycine	-	-	-	1.00 (1)	-
Histidine	0.94 (1)	-	-	-	-
Isoleucine	1.69 (2)	1.49 (2)	-	-	-
Leucine	2.11 (2)	-	1.02 (1)	1.32 (1)	-
Lysine[c]	1.00 (0.87)	0.89 (0.87)	2.71 (2.87)	0.84 (0.87)	0.91 (0.87)
Methionine	-	-	0.67 (1)	1.66 (2)	-
Phenylalanine	-	-	3.78 (4)	0.99 (1)	-
Proline	1.09 (1)	-	1.33 (1)	1.39 (1)	4.27 (4)
Serine	0.89 (1)	1.94 (2)	0.95 (1)	0.66 (1)	3.19 (6)
Threonine	-	1.02 (1)	-	0.80 (1)	0.88 (1)
Tryptophan[d]	-	- (1)	-	- (1)	-
Tyrosine	0.98 (1)	-	0.82 (1)	-	-
Valine	1.89 (3)	2.57 (3)	0.99 (1)	1.05 (1)	0.98 (1)

[a] The structure of the branched peptides causes 0.125 moles of alanine to be present per mole of the required sequence, in addition to any alanine in that sequence (see figure 1).
[b] The values found against Asx correspond to the amino acids aspartate and asparagine as asparagine is hydrolysed to aspartate during the analysis. Glx accounts similarly for glutamate and glutamine.
[c] The structure of the branched peptides causes 0.875 moles of lysine to be present per moles of the required sequence, in addition to any lysine in that sequence (see figure 1).
[d] Tryptophan is not detected as it is destroyed during hydrolysis.

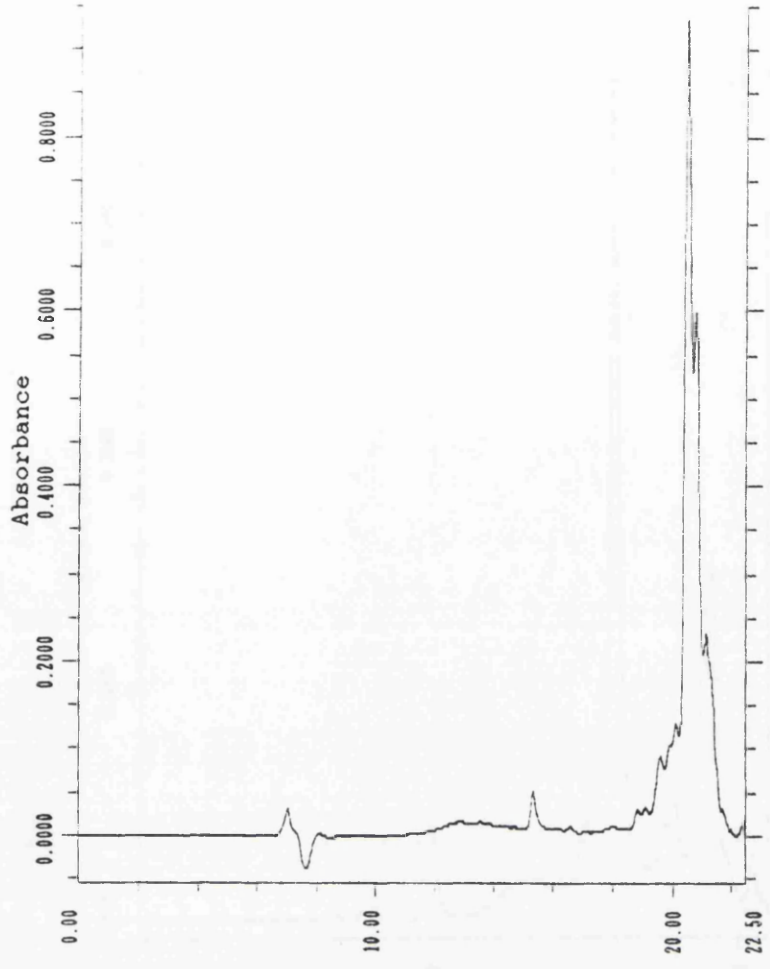
peaks corresponding to species with molecular masses of 1463, 1519, and 1635. The first of these is the molecular ion. The molecular masses of the other species suggest that they may be peptides with one or three t-butyl groups still attached. t-butyl groups were present as side-chain protectors during the synthesis of the peptide and presumably were not removed completely during cleavage. It is not possible to determine the extent of cleavage from the FAB-MS results.

Amino acid analysis of peptides UL9 218 and UL8 219 gave results in accordance with expectations as did analysis of peptides UL5 238, UL8 272, and UL52 220, with the following exceptions (see also table 3). The quantity of isoleucine and valine detected in peptides UL5 238 and UL8 272 was low due to incomplete hydrolysis of Ile-Val and Val-Val bonds. An anomalously large quantity of alanine seemed to be present in peptide UL8 272. Slightly greater than 3 molar equivalents of serine were detected in peptide UL52 220 rather than the 6 expected. The incomplete deprotection deduced from the molecular mass measurement may account for this as t-butyl groups are side-chain protectors for serine residues.

Peptides were analysed by reversed phase HPLC and the main peptide peak in the chromatogram (recorded at 225 nm) was assumed (on the basis of appropriate amino acid analysis and molecular mass data) to be the required sequence. The purity of peptides listed in table 2 is the area of the main peak as a percentage of the total area of peaks in the chromatogram (see figure 2). Peaks were also scanned over the wavelength range 190–340 nm to determine if their absorbance profiles were characteristic of peptides. In all cases the main peak had the characteristics of a peptide. That is, the absorbance peaked around 195 nm with a subsidiary peak at 280 nm if the peptide contained aromatic residues. The main impurities in the peptides run very close to the main peak and also have an absorbance profile characteristic of peptides. These probably represent species having one amino acid more or less than the required sequence. In branched peptides these alternative sequences will often form part of the same molecule and therefore will not be resolved by HPLC. Therefore purity is assessed better on single chain peptides. No data were obtained for peptide UL5 238 as it could not be dissolved in solvents suitable for chromatography. However amino acid analysis and M_r measurement suggest that the sample contains principally the correct sequence. Inclusion of peaks probably due to t-butyl containing species in the calculation of the purity of peptide UL52 220 raises the value obtained to 69%. Based on the three analyses, the purity of all peptides was judged suitable for the production of antisera.

Figure 2. Chromatography of peptides by reversed phase HPLC using a Dynamax C_8 column. Absorbance at 225 nm is plotted versus elution time in minutes. In each case the main peak is due to the required peptide. A) peptide UL9 218 single chain; B) peptide UL8 219 single chain; C) peptide UL52 220 single chain; D) peptide UL8 272 single chain.

A)


Absorbance
0.0000
0.2000
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0.6000
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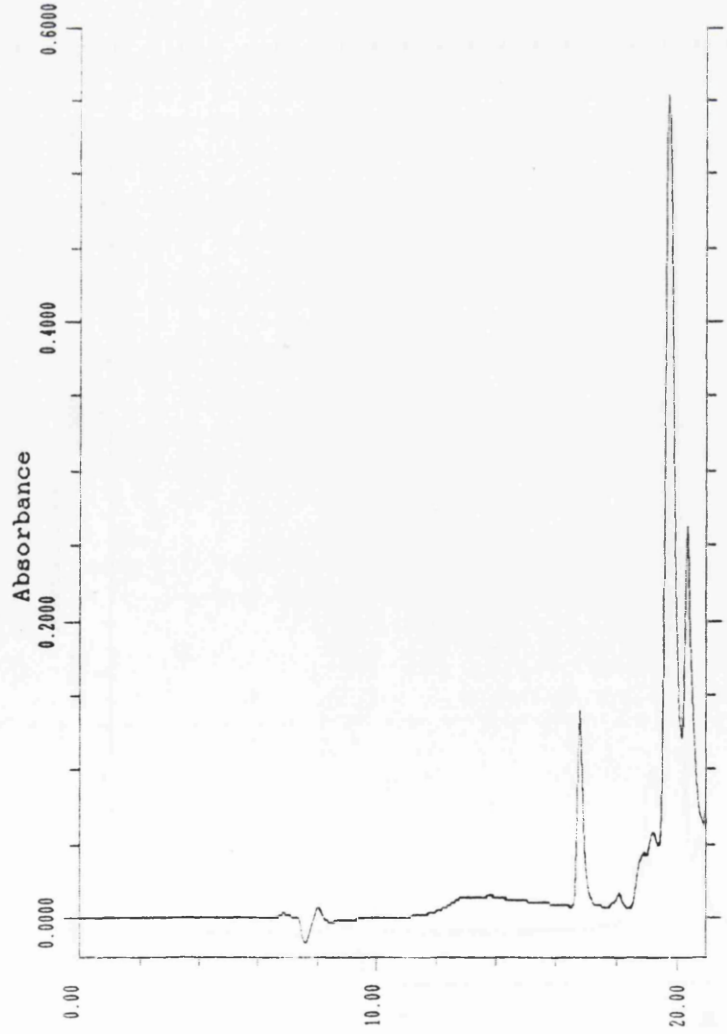
0.00
10.00
20.00
22.50

B)


Absorbance
0.0000
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10.00
20.00

II) PRODUCTION OF ANTIPEPTIDE ANTISERA.

Emulsified solutions of branched peptides were injected into rabbits and the resulting sera were tested on Western blots against extracts of cells infected with the recombinant baculoviruses AcUL5, AcUL8, AcUL9, and AcUL52 (Calder and Stow, 1990; Stow, 1992), which express the named protein. The results are shown in figure 3 and summarised in table 4.

Table 4. Titre of antipeptide sera on Western blots. The titre is defined as the greatest dilution of the serum that produced a visible band.

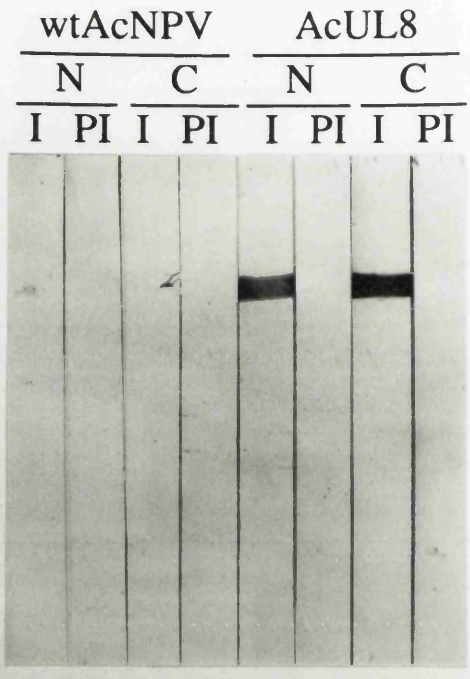
Peptide	Number of reactive sera[a]	Approximate Titre	Serum number
UL5 238	0 (2)	-	-
UL8 272	2 (2)	both 1:300	063, 064
UL8 219	2 (2)	1:64, 1:250	007, 008
UL9 218	1 (6)	1:64	021
UL52 220	0 (3)	-	-

[a] The values in parentheses are the number of rabbits used in attempts to produce positive sera.

Attempts to produce antipeptide sera reactive with proteins UL5 and UL52 were not successful. One serum reacting with protein UL9 was produced (serum 021; figure 3B). Pre-immune serum from rabbit 021 did not recognise proteins in cell extracts containing UL9, and the immune serum did not react with extracts of cells infected with wild type AcNPV. High titre sera recognising both the N- and C-termini of UL8 were produced. That they are UL8 specific is shown in figure 3A. The protein recognised by these sera corresponds to a protein seen in SDS polyacrylamide gels that is specific for AcUL8-infected cells.

Figure 3. Reactivity of antipeptide sera on Western blots. A) Extracts of cells infected with AcUL8 or wild type baculovirus (AcNPV) were electrophoresed on 10% SDS polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with antipeptide sera. The figure shows the reactivities of the pre-immune (PI) and immune (I) sera from rabbits 064 (N-terminus reactive) and 008 (C-terminus reactive), both used at a dilution of 1:200. Similar results were obtained with sera 007 and 063. B) Western blot using an extract of cells infected with AcUL9 as antigen. Sera used were: lane 1: positive control (anti-UL9 serum at 1:50 dilution, gift of M.D. Challberg, National Institutes of Health; USA); lane 2: buffer only; lanes 3–6: serum 021 at dilutions 1:4, 1:16, 1:64, and 1:256 respectively.

A)



B)

III) PURIFICATION OF UL8.

As UL5, UL9, and UL52 began to be examined by other workers but the function of UL8 remained unknown, this project focused more closely on purifying and characterising UL8. This choice was supported by the fact that the best sera produced were those reacting with UL8.

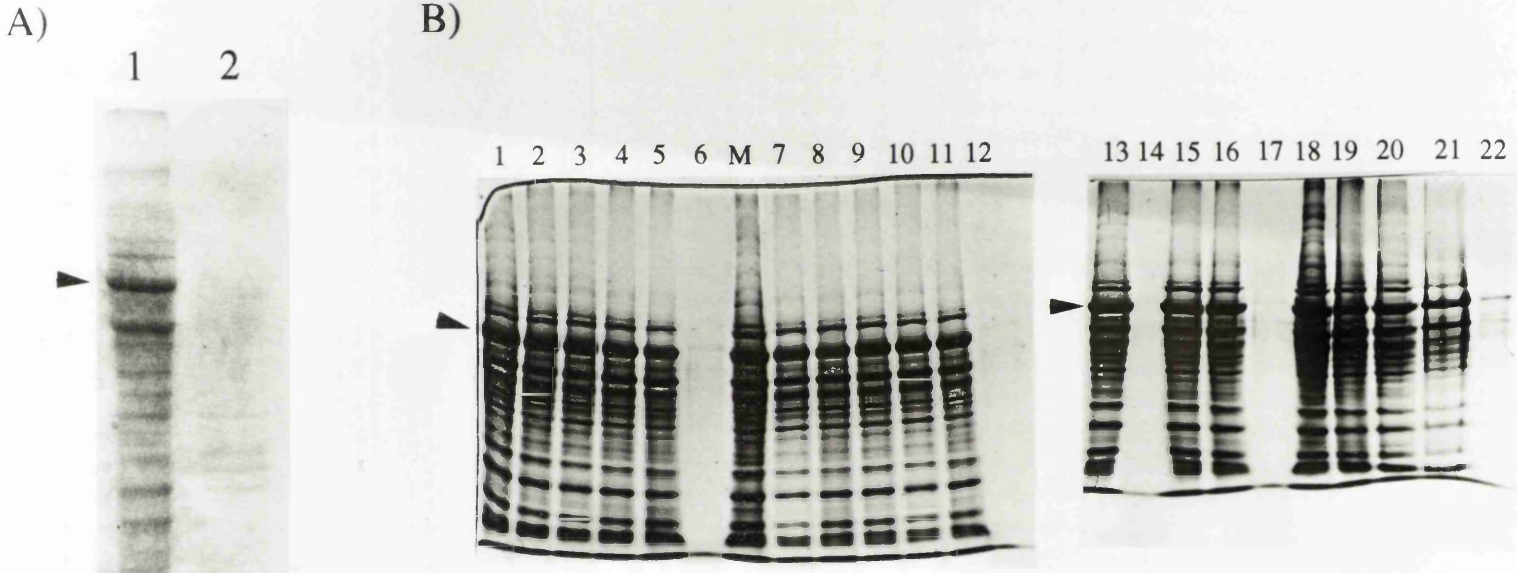
Production of crude cell extract

To overcome the difficulties caused by the tiny quantities of UL8 present in HSV-1 infected cells, UL8 protein was purified from *Spodoptera frugiperda* (Sf) cells infected with the recombinant baculovirus AcUL8 (Calder and Stow, 1990). UL8 expressed in Sf cells appears to be identical to that produced in HSV-1 infected cells as it is able to support the replication of a transfected plasmid containing an HSV-1 origin of replication (Stow, 1992).

Initial experiments focused on producing a crude extract containing UL8 in a stable, soluble form. UL8 expressed in Sf cells can be extracted in the soluble cytoplasmic fraction (Calder and Stow, 1990). In initial attempts to produce a crude extract Sf cells expressing UL8 were lysed in T buffer (10 mM triethanolamine *p*H 7.5 (HCl), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml leupeptin, 10 µg/ml pepstatin A) using a Dounce homogeniser. The extract was centrifuged at 10,000g for 30 seconds to pellet nuclei and the supernatant clarified by centrifugation at 175,000g for 30 minutes at 4 °C. It became apparent that due to the use of small sample volumes, losses in the homogeniser were large and so this was abandoned in favour of using the detergent NP40 at a concentration of 0.5% (v/v) to lyse the cells. This modification resulted in most UL8 being detected in the supernatant fraction after centrifugation (figure 4A), provided the cells were resuspended in the buffer before the detergent was added. If buffer containing detergent was added to the cell pellet the cells could not be resuspended. The presence of UL8 in the supernatant was confirmed by Western blotting.

The stability of UL8 in the crude extract was investigated. Portions of the crude extract were incubated at 25 °C or 4 °C, subjected to a number of cycles of freezing and thawing or diluted two, four, or eight-fold. Portions were centrifuged at 10,000g for 10 minutes at 4 °C at each stage to pellet denatured protein and part of the supernatant was electrophoresed on a protein gel (figure 4B). The results show that UL8 remained in the supernatant after all these treatments and therefore was probably not denatured. The stability experiments also show that no substantial proteolysis was occurring in three and a half hours at room temperature under these conditions. Initial attempts to identify suitable chromatography matrices for purification of the UL8 protein were thwarted by the observation that addition of the crude extract to any of the matrices investigated caused most

Figure 4. Initial experiments on the solubility and stability of the UL8 protein.
A) Soluble and insoluble material obtained after extraction of *S. frugiperda* cells with T buffer containing NP40. Portions of the supernatant (lane 1) and the pellet (lane 2) from low speed centrifugation were dissolved in gel sample buffer and run on a 10% SDS polyacryamide gel. Proteins were visualised with Coomassie Blue. UL8 is indicated by the arrow.
B) Stability of UL8 undergoing various treatments. The position of UL8 on both gels is indicated by the arrows. Incubation at 25 °C: lanes 1–5 show the crude extract at the start of the experiment and after 1, 2, 3, and $3^1/_2$ hours incubation respectively. Lane 6 shows the pelllet from the final sample. Similarly, lanes 7–12 show the results of incubation at 4 °C for these times. Lane M is a marker track (untreated material). Lanes 13-17 show the effects of freezing and thawing. Lane 13: starting material; lane 14: blank; lane 15: frozen and thawed twice; lane 16: frozen an d thawed three times; lane 17: pellet from the final sample. Lanes 18–20 show the effects of dilution with the initial extraction buffer (T buffer). Lane 18: starting material; lane 19: diluted two-fold; lane 20: diluted four-fold; lane 21: diluted eight-fold; lane 22: pellet.


A)
1
2
B)
1 2 3 4 5 6 M 7 8 9 10 11 12
13 14 15 16 17 18 19 20 21 22

of the UL8 protein to precipitate in the column. This problem was eventually solved by the inclusion of 10% glycerol in the harvesting buffer and all chromatography buffers.

Fractionation of the crude extract.

i) Precipitation with ammonium sulfate.

A crude extract of proteins in T buffer was precipitated with ammonium sulfate. Concentrations of 0, 25%, 40%, 60% and 80% saturating ammonium sulfate were initially tested. Portions of each supernatant and pellet were electrophoresed on SDS poly-acrylamide gels and Western blotted. The results of the experiment are shown in figure 5A. The experiment shows that UL8 precipitates slightly at 25% salt and totally at 40% or higher concentrations. The amount of salt required to precipitate UL8 was refined using 28%, 32%, 36%, and 40% saturating salt concentrations (fig 5B) until an optimal value of 36% was determined that gave maximum UL8 precipitation with minimum precipitation of other proteins. The purification achieved was excellent as UL8 precipitated at such a low salt concentration. Not all UL8 is precipitated by 28% or 32% saturating ammonium sulfate, but the protein is not found in the supernatant fraction of samples treated with these concentrations of salt. The reason for this is not clear, but may involve a salt activated protease.

Despite the substantial purification achieved with ammonium sulfate fractionation, this technique could not be used as UL8 protein appeared to be irreversibly denatured by it. While the precipitated protein could be resuspended in SDS containing buffer for gel electrophoresis, no non-denaturing buffer was able to resuspend it. Buffers used included RSB and RSB containing 0.05% NP40 or 10% glycerol.

ii) Anion exchange chromatography: MonoQ column.

Ion exchange chromatography was investigated as a purification method. To determine if a cation or an anion exchanger should be used, two attempts were made to determine the isoelectric point of UL8. A chromatofocusing column from Pharmacia was used, run from *p*H 7 to *p*H 4. The vast majority of the UL8 loaded was lost on the column and the small amount of UL8 that eluted formed a very broad peak eluting between *p*H 5.0 and 4.5. However as UL8 bound to the column its isoelectric point was clearly less than *p*H 7 and an anion exchanger was selected. (The calculated isoelectric point of UL8 is approximately 5.8.)

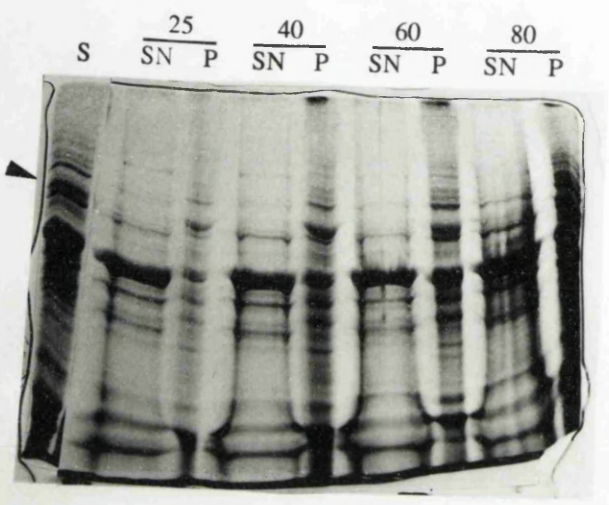
Initial experiments used a 1 ml MonoQ anion exchange column. The column was equilibrated in 20 mM triethanolamine buffer *p*H 7.5 and a crude cell extract was added. The column was washed with the same buffer, and bound proteins were eluted with a gradient from the starting buffer to 20 mM triethanolamine 1 M NaCl *p*H 7.5. UL8 eluted

Figure 5A. Ammonium sulfate precipitation of UL8 protein.

I) Crude cell extract containing UL8 was fractionated with 25%, 40%, 60%, or 80% saturating ammonium sulfate as indicated and portions of the supernatant (SN) and pellet (P) fractions were electrophoresed on SDS polyacrylamide gels. Proteins were detected by Coomassie blue staining. The lane marked S contains the starting material.

II) Crude cell extract containing UL8 was fractionated with 0, 25%, 40%, or 60% saturating ammonium sulfate as indicated and portions of the supernatant (SN) and pellet (P) fractions were electrophoresed on SDS polyacrylamide gels and immunoblotted with serum 008 at 1:200 dilution.

I)



II)

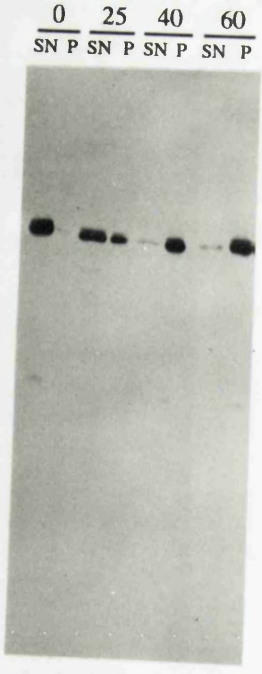
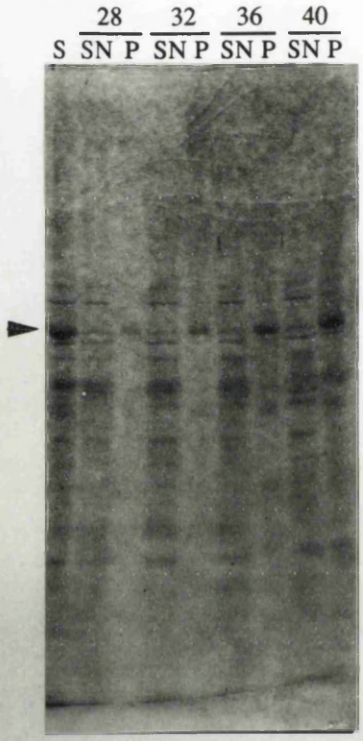
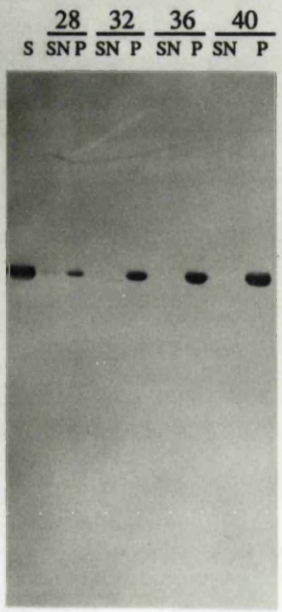


Figure 5B. Ammonium sulfate precipitation of UL8 protein – fine cuts.
I) UL8 was precipitated with 28%, 32%, 36%, or 40% saturating ammonium sulfate and the supernatant (SN) and pellet (P) from each fractionation were electrophoresed on 10% SDS polyacrylamide gels. The lane marked S contains the starting material. Proteins were detected by Coomassie blue staining.
II) Proteins were precipitated with 28%, 32%, 36%, or 40% saturating ammonium sulfate and electrophoresed on SDS gels. UL8 in supernatants (SN) and pellets (P) was detected by immunoblotting with serum 008 at 1:200 dilution. The lane marked S contains the starting material.

I)



II)



at c.0.3 M NaCl in 3 ml of buffer. Considerable purification was achieved, although recovery was low (c. 50 %). (Fig 6).

Increasing the concentration of triethanolamine to 40 mM to improve buffering and the inclusion of 10% glycerol or 0.5% NP40 in the starting and elution buffers did not improve the yield. The use of buffers at *p*H 6.5 or 6.1 to run the column also did not improve the yield and a lower *p*H could not be used to titrate the protein closer to neutrality as UL8 is not stable at such low *p*Hs. There was no reason to try a buffer of higher *p*H as this would only increase any negative charge on the protein and so the *p*H required to reduce the charge of the column substituents and thus their affinity for the protein would be unreasonably high.

One attempt was made to bind the UL8 protein to a MonoS cation exchange column in 50 mM HEPES buffer *p*H 7.5. However under these conditions most UL8 passed straight through the column without interacting with the matrix. As many other proteins also did not bind to the column, little purification was achieved and this approach was not pursued.

iii) Fractionation using a phenyl superose column.

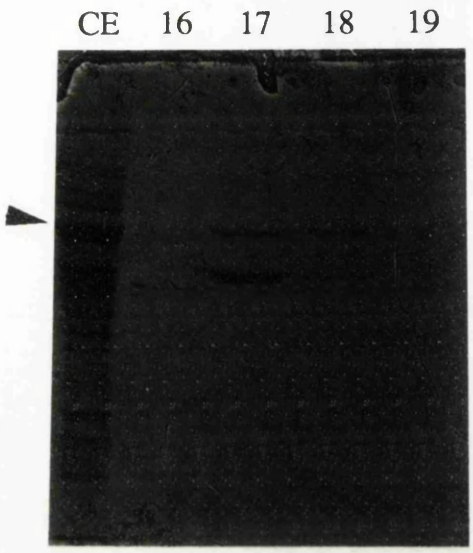
Hydrophobic interaction chromatography was investigated as a method of purification. Crude extracts of Sf cells expressing UL8 were added to a phenyl superose column equilibrated in 20 mM triethanolamine *p*H 7.5, 0.5 M NaCl and the column was developed with a gradient to 20 mM triethanolamine *p*H 7.5, 2% glycerol. In initial experiments the loading buffer contained 2 M NaCl but the use of this concentration of salt was found to be unnecessary. The majority of the proteins eluted in the flow through but UL8 eluted, considerably purified, at the end of the gradient (figure 7). Recovery was approximately 50% in this experiment but was unpredictable in later experiments due to variations between different crude extract preparations.

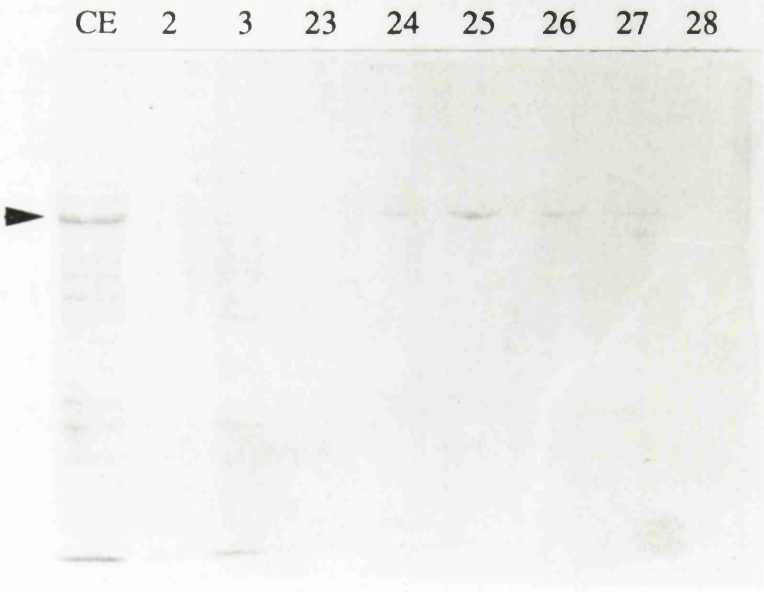
Several other chromatography matrices were tested for use in purifying UL8 but none proved suitable. UL8 either failed to bind to the matrix or could not be eluted without being denatured. The matrices tested were: heparin affigel; DE52 anion exchanger; cibacron blue 3GA-agarose; CTP agarose; blue dextran agarose; reactive red 120 agarose. Attempts to use hydroxy apatite were also unsuccessful. The matrix bound UL8 and the protein could be eluted to some extent, but the flow characteristics of the matrix were very poor.

Two purification steps had now been developed although each was of only moderate yield. The effectiveness of the steps varied between different preparations of crude extract, probably due to variations in total protein concentration, a factor which seemed to affect the stability of UL8 in the harvesting buffer (T buffer).

Figure 6. MonoQ fractionation of crude extract containing UL8. A Coomassie blue stained gel of the crude extract (CE) loaded onto the column and fractions 16–19. Fraction size was 1.5 ml. Western blotting showed that these fractions were the only ones in which UL8 was detected. See the text for experimental details.

Figure 7. A Coomassie blue stained gel of crude cell extract (CE) chromatographed on a phenyl superose column and of the fractions eluted from the column. Fractions 2 and 3 are the flow through and fractions 23 to 28 are the only ones containing UL8, again demonstrated by Western blotting. Fractions were 1 ml.


CE
16
17
18
19


CE
2
3
23
24
25
26
27
28

iv) Fractionation using diethylaminoethyl (DEAE) sepharose.

At this stage in the work the publication of a method for the purification of UL8 (Dodson and Lehman, 1991) suggested some changes to the purification scheme. The method of Dodson and Lehman used heparin agarose, DEAE sepharose, hydroxyapatite, and gel filtration columns. Ten per cent glycerol was used in all buffers except the harvesting buffer, presumably to increase the stability of the protein.

A DEAE sepharose column was packed and equilibrated in 20 mM triethanolamine *p*H 7.5, 10% glycerol, 0.1 M NaCl. Crude extract harvested in T buffer was loaded, the column washed with the start buffer, and the proteins eluted with a gradient from 0.1 M to 0.5 M NaCl in 20 mM tris *p*H 8.0, 10% glycerol. UL8 protein eluted around 0.3 M NaCl but yields were still low, comparable to those achieved with the MonoQ column. This was unexpected as the published method had achieved a high yield. The experiment was repeated using crude extract harvested in a buffer containing 10% glycerol, G buffer: 20 mM triethanolamine *p*H 7.5 (HCl), 10% glycerol, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5% NP40, 1 mM DTT, 0.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A. This modification gave an approximately quantitative yield (figures 8 & 9A).

v) Fractionation using phenyl sepharose.

Due to an observed progressive deterioration in the chromatographic properties of the phenyl superose column, hydrophobic interaction chromatography was continued with a phenyl sepharose matrix. The inclusion of 10% glycerol in the buffers for this matrix also improved the yield of UL8 obtained. Initial experiments with loose matrix in an eppendorf tube suggested that UL8 would bind and could be eluted from the matrix by buffers containing NP40. Use of a column however showed that water at 4 °C containing 10% glycerol was sufficient to elute the protein. Therefore the fractions from the DEAE sepharose column that contained UL8 were pooled and loaded without further treatment onto a phenyl sepharose column equilibrated in 20 mM tris *p*H 8.0 (HCl), 10% glycerol, 0.1 M NaCl. The column was washed with the starting buffer and a gradient to 10% glycerol in water at 4 °C was applied. UL8 eluted, virtually pure, at the end of the gradient (figures 8 & 9A). A nearly quantitative recovery of UL8 protein was achieved. Scanning of a Coomassie blue stained SDS polyacrylamide gel of the peak fractions showed that UL8 purified through DEAE sepharose and phenyl sepharose columns in the manner described produced protein that was 95% pure.

Western blotting of purified UL8 with N and C terminal antisera showed that the protein was intact and that no degradation products were present (fig 9B). The purified protein was probably not denatured as centrifugation at 10,000g did not precipitate it.

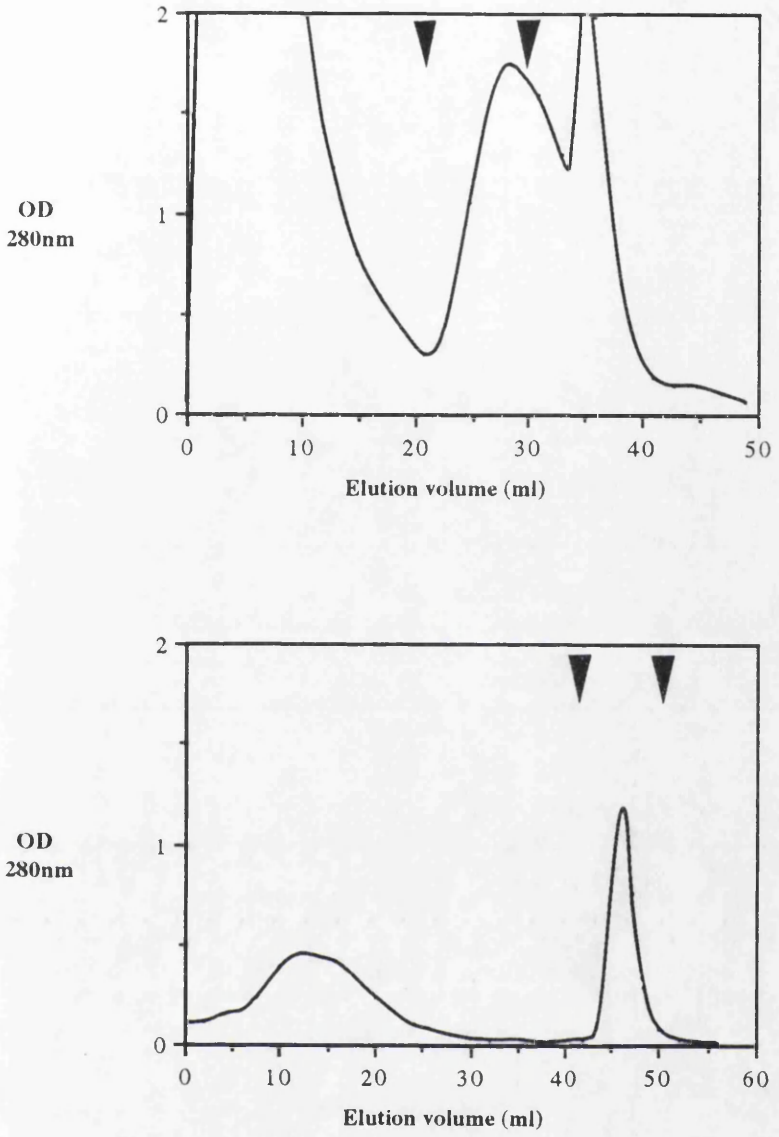
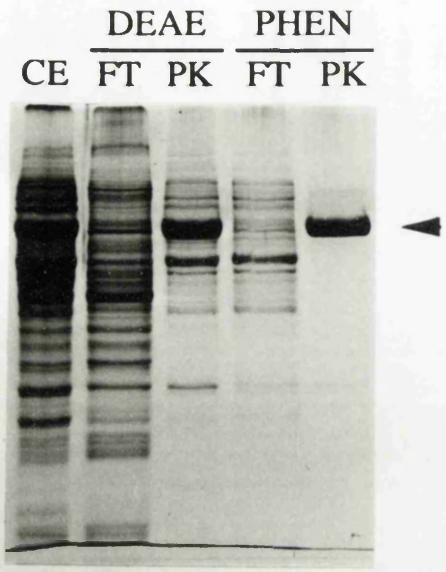


Figure 8. UV absorbance of material eluting from DEAE sepharose (upper panel) and phenyl sepharose columns (lower panel). Optical density at 280 nm is plotted versus elution volume in ml. Fractions containing UL8 are those between the arrows. The absorbances recorded are from an experiment using 4 ml of crude extract as starting material. Fractions from the DEAE column which contained UL8 were loaded onto the phenyl sepharose column.

Figure 9. A) Proteins present at different stages of the purification procedure, visualised by Coomassie staining of a 10% SDS polyacrylamide gel. The position of the UL8 protein is shown by the arrow. The five tracks show the proteins in the crude extract (CE) and the proteins that flowed through (FT) the DEAE sepharose (DEAE) or phenyl sepharose (PHENYL) columns or that eluted in the peak fractions (PK). The fractions from the DEAE column that contained UL8 were pooled and added to the phenyl sepharose column. B) Recognition of purified UL8 protein by antisera reacting with the N and C termini of the protein (sera 064 and 008). This panel shows a Western blot of proteins transferred from a 10% SDS gel.

A)



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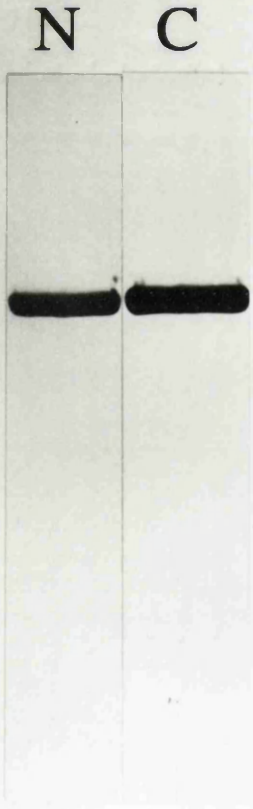


A summary of the full purification scheme, as used to produce the protein used for the rest of the experiments described in this thesis, is included in the methods section.

IV) PROPERTIES OF UL8.

i) Structure of UL8 protein in solution.

In HSV-1 infected cells UL8 protein exists as part of a 1:1:1 complex with the UL5 and UL52 proteins. The complex has a molecular mass of 263,000 daltons (Crute and Lehman, 1991). Whether purified UL8 was a monomer in solution or formed a complex was determined using gel filtration chromatography. UL8 eluted from a gel filtration column between 11.7 ml and 12.8 ml with a peak at 12.3 ml (see figure 10) giving an estimated molecular mass of 75,000 +/- 10,000 daltons. While this is an imprecise molecular mass determination the result shows unambiguously that purified UL8 exists as a monomer in solution.

ii) Investigations of possible interactions of UL8 protein with nucleic acids.

UL8 has an important role in nuclear localisation of the helicase primase complex of HSV-1 (Calder *et al*, 1992) but this is unlikely to be its only function. In searching for additional functions of UL8, its interaction with nucleic acids was investigated using a DNA band shift assay. As part of the viral helicase-primase complex UL8 has the opportunity to interact with a number of different nucleic acids: double stranded DNA; single stranded DNA; partially double stranded DNA; and DNA/RNA hybrids. Binding of UL8 to all these nucleic acids was examined.

Double stranded DNA.

Figure 11A shows the results of incubating UL8 with double stranded DNA. In contrast to UL42, up to 60 ng of UL8 in the reaction has no effect on the mobility of the probe after an incubation at 37 °C in low salt GRA buffer (50 mM HEPES *p*H 7.8 (NaOH), 10% glycerol, 0.1 mM EDTA, 0.5 mM DTT) (lanes 2-4). Experiments using up to 600 ng of UL8 also showed no effect of UL8 on probe mobility, nor did experiments carried out in GRA buffer (50 mM HEPES *p*H 7.8 (NaOH), 10% glycerol, 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT) or experiments carried out at room temperature or 4 °C. A number of different preparations of UL8 were used. Similar experiments performed with partially duplex DNA also failed to reveal any binding of UL8 (figure 11B).

Single stranded DNA.

Figure 12A shows the results of a band shift experiment using a single stranded DNA probe. As can be seen, addition of up to 600 ng of UL8 causes no shift in the position of the probe relative to reactions containing only water or buffer. Inclusion in the reaction mixture of gene 32 protein of T4 bacteriophage which binds cooperatively to single

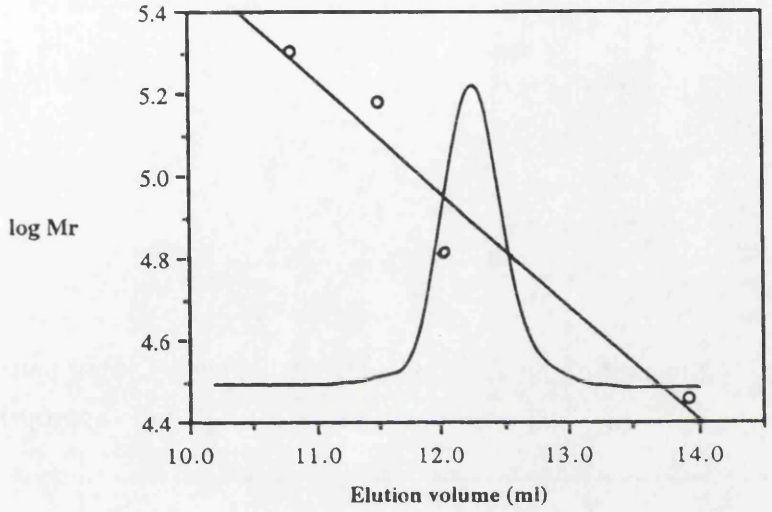


Figure 10. UL8 molecular mass determination. The elution of purified UL8 from a gel filtration column is shown (peak) together with the positions of elution of molecular weight standards (circles). Proteins used as molecular mass standards were: β-amylase (M_r 200,000); alcohol dehydrogenase (M_r 150,000); bovine serum albumin (M_r 66,000); and carbonic anhydrase (M_r 29,000). The straight line is a best fit line for the four points drawn by computer.

Figure 11. DNA mobility shift of double stranded DNA (A, lanes 1–6) or partially double stranded DNA (B, lanes 7–12). All reactions contained 1 ng of the appropriate nucleic acid.

A) Lane 1 shows the position of the complex (arrow) formed by duplex oligonucleotide and 170 ng of purified HSV-1 UL42 protein. The free probe is seen in lane 6 which had only water added to the binding reaction and in lane 5 which had only the buffer used to dilute UL8 protein added to the reaction mixture. 60, 6, or 2 ng of UL8 were added to the reactions shown in lanes 2–4.

B) Labels are as for panel A except for the quantities of UL8 added to the reaction. Also, only 0.25 ng of UL42 was used in the postive control reaction (lane 7).

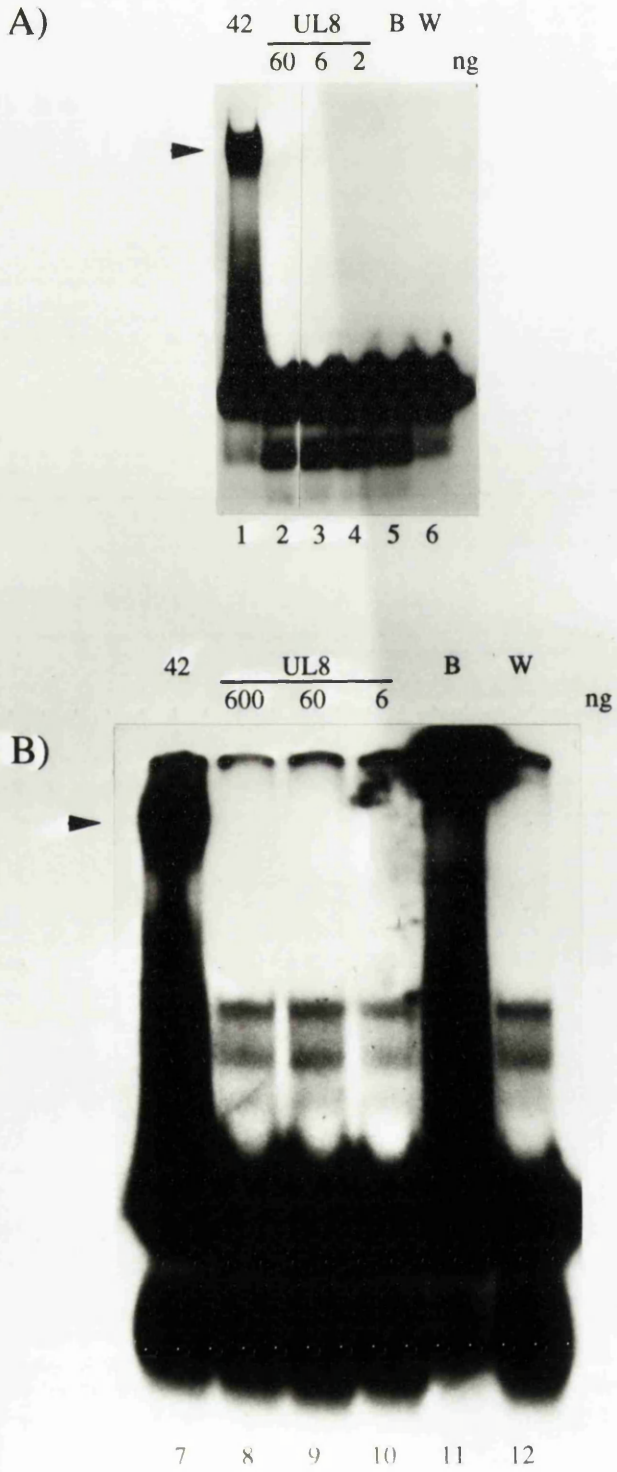
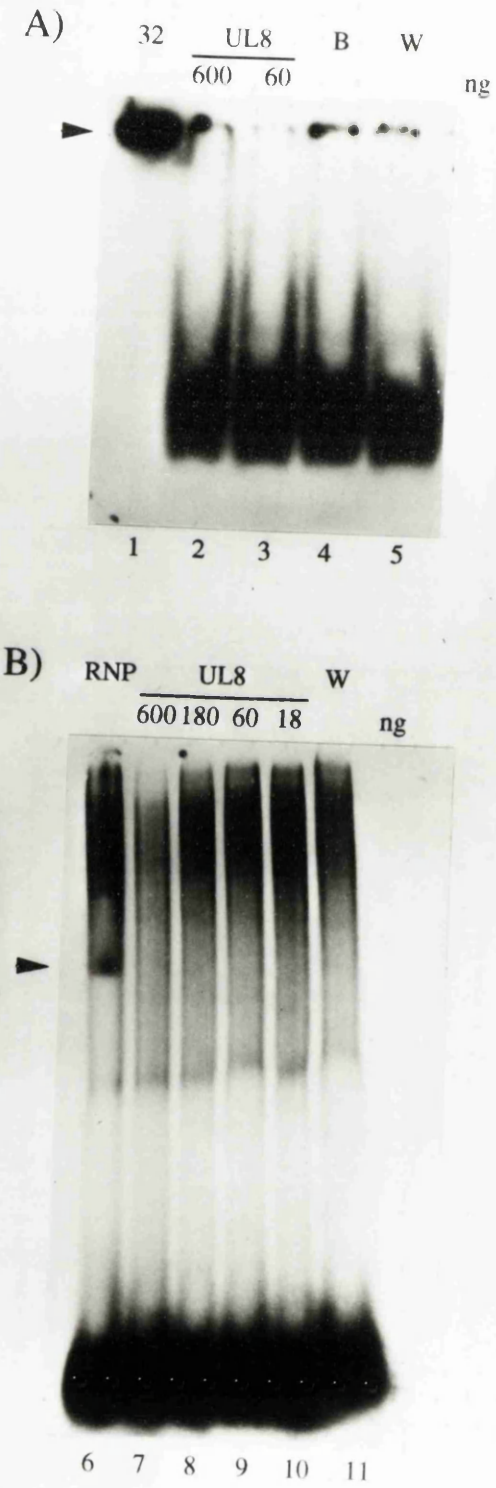

A)
42
UL8
B W
60 6 2
ng
1 2 3 4 5 6
42
UL8
B W
600 60 6
ng
B)
7 8 9 10 11 12

Figure 12. DNA mobility shift of single stranded DNA (A, lanes 1–5) or a DNA/RNA hybrid (B, lanes 6–11). All reactions contained 1 ng of the appropriate nucleic acid.

A) Reactions mixtures contained: 8500 ng of bacteriophage T4 gene 32 protein (lane 1); 600 or 60 ng of purified UL8 (lanes 2 and 3 respectively); UL8 dilution buffer (lane 4); or water (lane 5). The arrow indicates the position of the complex formed between gene 32 protein and the probe.
B) One unit of bacteriophage T7 RNA polymerase (lane 6); 600, 180, 60 or 18 ng of purified UL8 protein (lanes 7–10); or water (lane 11) was added to the basic reaction mixture. The complex formed by T7 RNA polymerase is indicated by the arrow.


A)
32
UL8
600 60
B
W
ng
1 2 3 4 5
B)
RNP
UL8
600 180 60 18
W
ng
6 7 8 9 10 11

stranded DNA causes the probe (arrow) to run just below the position of the gel loading slot. This experiment was performed in GRA buffer at 37 °C and similar experiments performed in low salt GRA buffer or at 25 °C or 4 °C produced identical results.

DNA/RNA hybrid.

Possible interaction of UL8 with a DNA/RNA hybrid [poly(rA).oligo(dT)$_{12-18}$] was investigated. The results are shown in figure 12B. No specific band due to UL8 can be seen although T7 RNA polymerase binds to the probe under the reaction conditions (arrow). The experiment shown in figure 12B was performed in low salt GRA buffer at 37 °C, however further experiments at different temperatures or in GRA buffer also failed to detect binding of UL8 to the probe.

V) PRODUCTION OF MONOCLONAL ANTIBODIES.

Purified UL8 protein was used to immunise mice to produce monoclonal antibodies. It was necessary to test conditions for screening hybridoma supernatants by ELISA against purified UL8 and to find a suitable positive control.

i) Development of ELISA screen for hybridoma supernatants.

Only antipeptide sera reactive to UL8 protein were available as a positive control in the initial stages of the development of an ELISA screen. ELISA assays were performed to test sera 008 and 064, using a crude extract of Sf cells as antigen. The results obtained with antiserum 008 are shown in figure 13. Optical density falls off as expected with increasing dilution of the antiserum. However, the optical density readings were inversely proportional to antigen concentration. Due to this unexpected result a greater range of antigen concentrations was investigated. (As virtually identical results were obtained with serum 064, experiments were continued with serum 008 alone.) In the second experiment (figure 14) the same range of antiserum dilutions were used but the quantity of antigen covered a greater range. It is clearly seen that there is a drop in absorbance with antiserum dilution and that the highest absorbance was seen with approximately 100 ng of UL8 per well. The drop in absorbance at higher antigen concentrations is presumably due to some interfering compound in the cell extract. The observed absorbance was sufficient for the ELISA screen and no further experiments were performed to determine the quantity of UL8 required for maximum absorbance. It should be noted that the quantity of UL8 used as antigen is an estimate. The estimate is based on the total protein concentration of the extract (measured using a commercial assay kit) and the proportion of the proteins thought to be UL8 from examination of Coomassie stained gels. For example, the total concentration of proteins in crude extracts was usually of the order of 4 mg/ml and UL8 comprised approximately 5% of the cell protein. The optical density of all these ELISA assays was measured after half an hour. A linear increase in optical density with time was observed in all cases.

Five mice were immunised with purified UL8 protein. Sera from these mice were tested on Western blots (figure 15). Serum from mouse 3 had the strongest and cleanest anti-UL8 reactivity, and so this mouse was chosen for use in the fusion. Serum from mouse 3 was tested for use as a positive control in ELISAs. Purified UL8 was used as antigen (figure 16). This shows that a strong reaction can be obtained even at a dilution of 102,400 with a UL8 concentration of 100 ng per well. In general, for use as a positive control, the serum was diluted 10,000 fold.

As a result of this experiment hybridoma supernatants were screened using 1 μg or 400 ng of purified UL8 per well. Optical

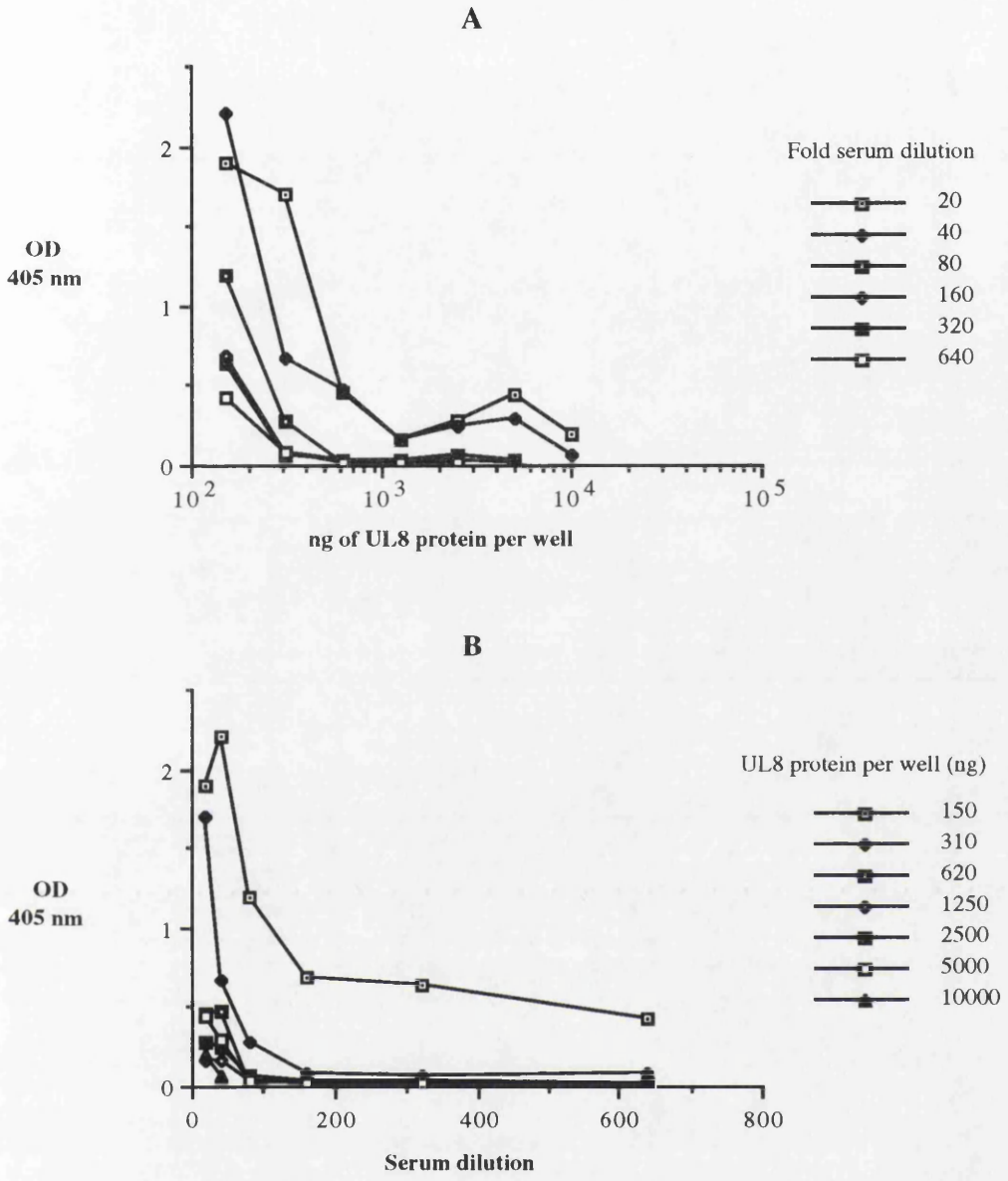


Figure 13. Results of ELISA assays of serum 008 tested against a crude extract of cells containing UL8. A) Optical density at 405 nm is plotted versus estimated quantity of UL8 protein per well for a number of serum dilutions, as given next to the figure. B) Optical density at 405 nm is plotted versus serum dilution for a number of quantities of antigen. These are given in the figure in ng per well.

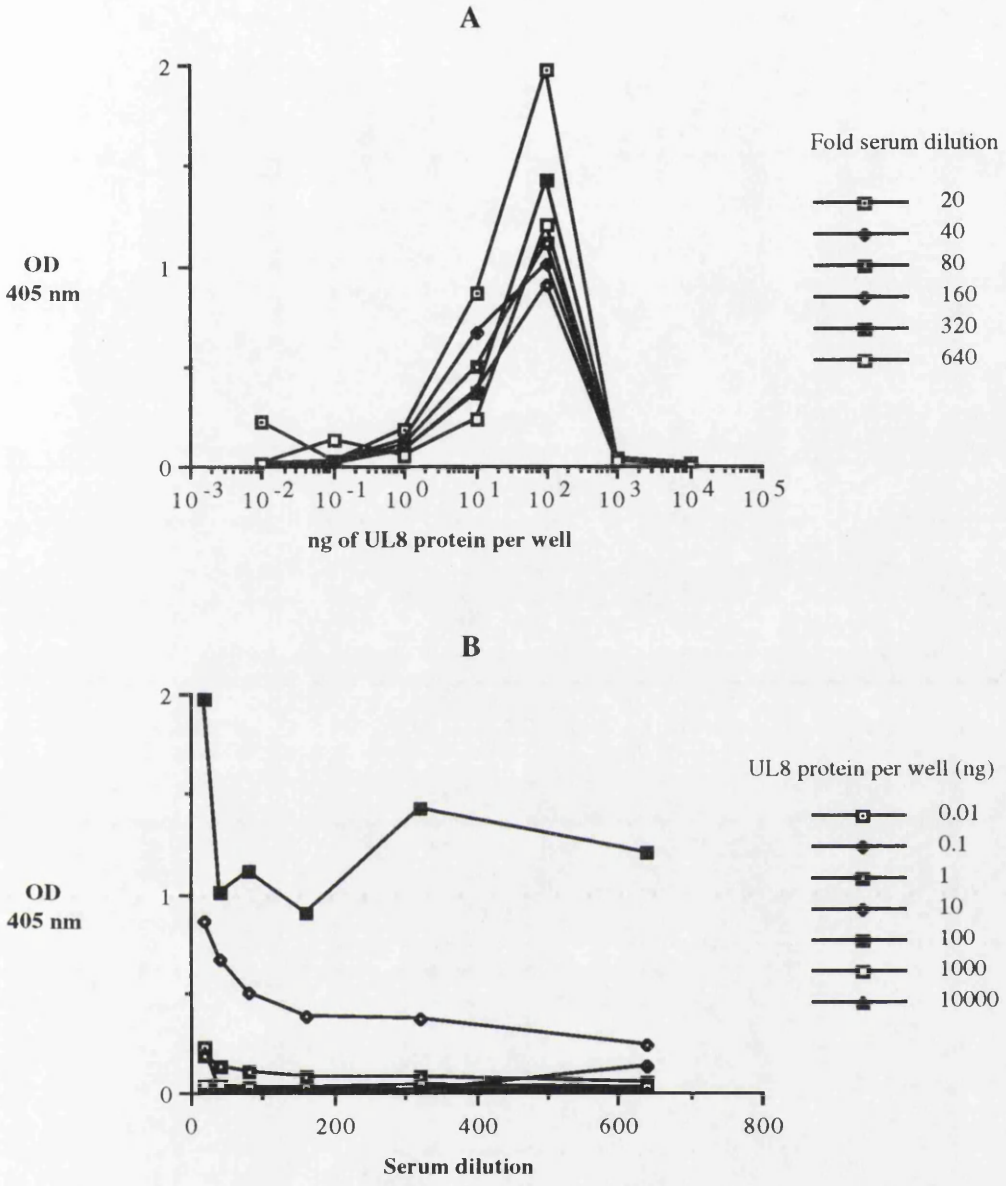
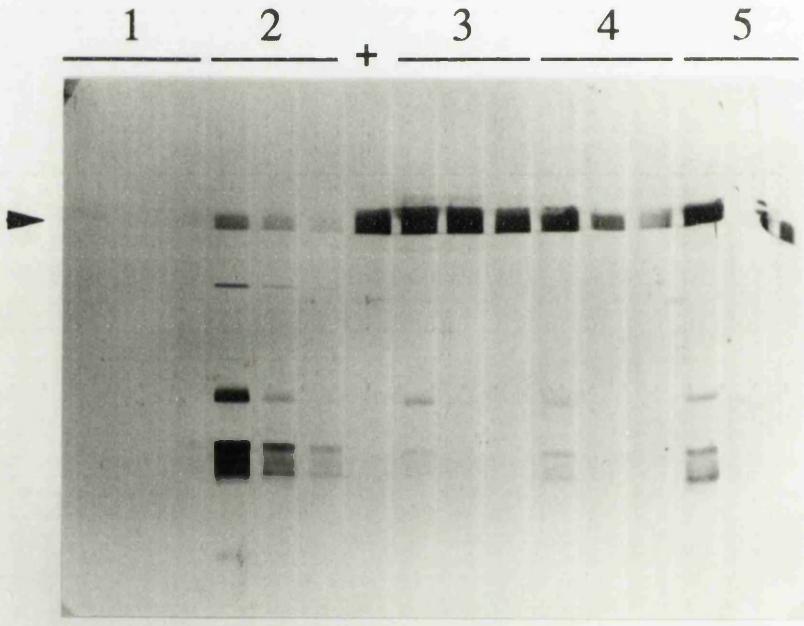


Figure 14. Results of ELISA assays of serum 008 tested against a crude extract of cells containing UL8. A) Optical density at 405 nm is plotted versus estimated quantity of UL8 protein per well for a number of serum dilutions, as given in the figure. B) Optical density at 405 nm is plotted against serum dilution for a number of quantities of antigen. These are given in the figure in ng per well.

Figure 15. Western blot of sera from mice immunised with purified UL8 protein. Each serum (1–5 as indicated) were screened at dilutions 1:100; 1:500; and 1:2000. These are shown successively from left to right for each serum. '+' indicates the positive control which was serum 008 used at a dilution of 1:200.


1
2
+
3
4
5

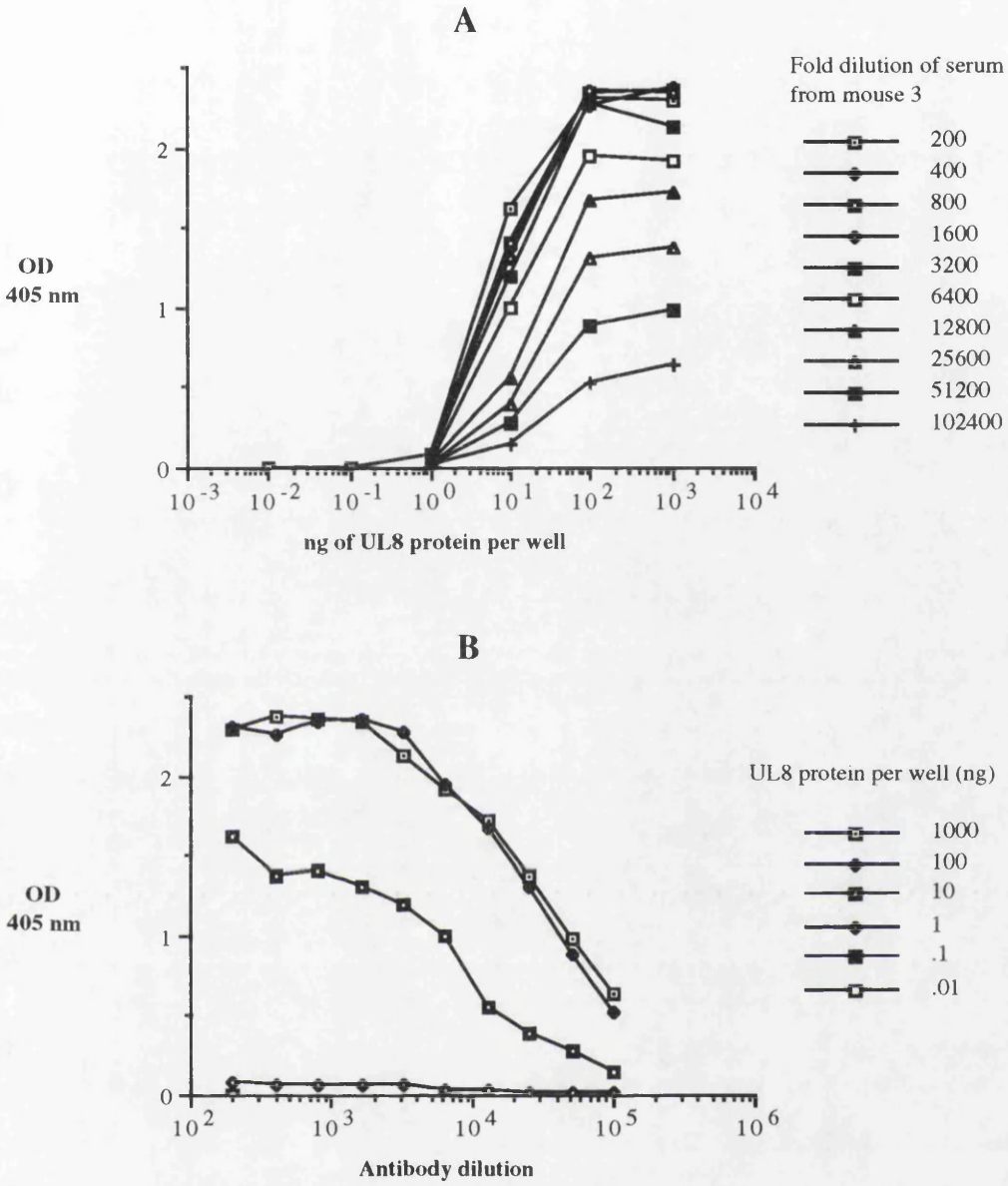


Figure 16. Results of ELISA assays of serum from mouse 3 tested against purified UL8. A) Optical density at 405 nm is plotted against quantity of UL8 protein per well for a number of serum dilutions, as given in the figure. B) Optical density at 405 nm is plotted against serum dilution for a number of quantities of antigen per well. These are given in the figure.

density readings of > 0.4 after 15 minutes were regarded as positive. All blanks were of negligible absorbance (< 0.05).

ii) Production of monoclonal antibodies and ascites fluid.

Sple nocytes from mouse 3 were fused to produce hybridoma lines. Eighty one hybridoma lines were produced of which 25 (31%) were positive when assayed by ELISA versus purified UL8 protein in the initial screen. Twenty of the positive lines continued to make UL8-specific antibody until they could be injected into mice to produce ascitic fluid. Nineteen of the resulting ascitic fluids were positive by ELISA against UL8.

iii) Characterisation of monoclonal antibodies.

The monoclonal antibodies produced were characterised by testing whether they reacted with UL8 protein in immune precipitations and Western blotting. Representative results are shown in figure 17 and summarised in table 5. Twelve of the ascitic fluids were tested in Western blots and immune precipitations; 7 of those tested were positive in Western blotting and 6 reacted with UL8 in immune precipitations. Western blot titres of the positive ascitic fluids varied from 10^3 to 10^5. As can be seen in figure 17 some UL8 is precipitated in the control lane. For this reason it is not possible to distinguish between antibodies reacting weakly in immune precipitations and those that do not react at all. Therefore only strongly reacting antibodies are regarded as positive.

iv) Rabbit anti-protein sera.

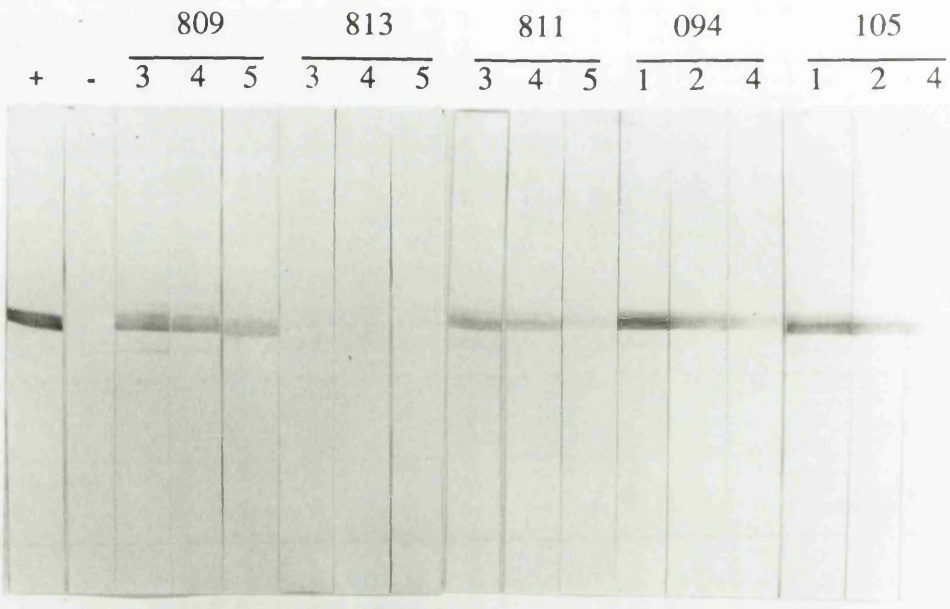
Two rabbits were injected with purified UL8. The resulting sera (094 and 105) reacted specifically with UL8 in Western blots at dilutions of 1:4000 and also precipitated UL8 from solution (figure 17).

Figure 17. A) Western blot of ascites fluids and rabbit sera. An extract of *Spodoptera* cells expressing UL8 was used as antigen. The positive control (+) is serum 008 at 1:200 dilution; the negative control (–) is buffer. The reactivity of ascites fluids 809, 813, and 811 at 10^3, 10^4, and 10^5-fold dilution is shown. Reactivity of rabbit sera 094 and 105 at 1000, 2000, and 4000-fold dilution is shown.

B) An autoradiograph showing immune precipitation of ^{35}S-labelled UL8 (arrow) by polyclonal and monoclonal antibodies specific for the protein. Lanes 1 and 2 show precipitation by sera from rabbits 094 and 105 respectively. Lanes 3-15 correspond to the following ascites fluids: 3: MP0809; 4: MP0810; 5: MP0811; 6: MP0812; 7: MP0813; 8: MP0814; 9: MP0815; 10: MP0816; 11: MP0817; 12: MP0818; 13: MP0819; 14: MP0820; 15: control ascites. The starting material is electrophoresed in the lane marked st.

A more accurate reproduction of the original autoradiograph may be found in the pocket at the back of the thesis.

A)


809
813
811
094
105
+ - 3 4 5 3 4 5 3 4 5 1 2 4 1 2 4

B)

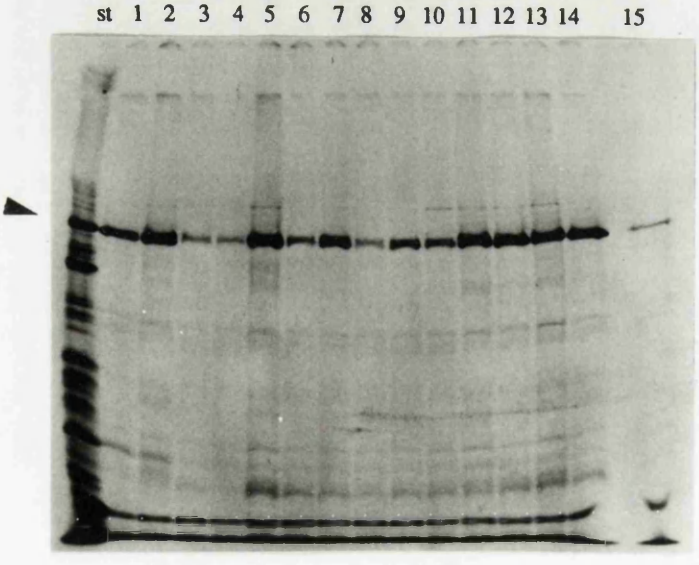

st 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Table 5. Anti-UL8 monoclonal antibodies. The reactivity of each ascites fluid in ELISA, Western blot and immune precipitation is shown (+/-) with approximate titres given in parentheses for those testing positive in Western blot. The titre is defined as the greatest dilution of the ascitic fluid that produced a visible band. N.D. indicates not determined.

Cell line	**Ascites positive by:**		
	ELISA	**Western blot**	**Immune precipitation**
MP0801	+	N.D.	N.D.
MP0802	+	N.D.	N.D.
MP0803	+	N.D.	N.D.
MP0804	+	N.D.	N.D.
MP0805	+	N.D.	N.D.
MP0806	+	N.D.	N.D.
MP0807	+	N.D.	N.D.
MP0808	–	N.D.	N.D.
MP0809	+	+ (10^5)	–
MP0810	+	–	–
MP0811	+	+ (10^3)	+
MP0812	+	+ (10^4)	–
MP0813	+	–	+
MP0814	+	+ (10^4)	–
MP0815	+	–	–
MP0816	+	–	–
MP0817	+	+ (10^4)	+
MP0818	+	+ (10^4)	+
MP0819	+	+ (10^4)	+
MP0820	+	–	+

DISCUSSION

The initial aim of this project was to identify the products of the HSV-1 open reading frames UL5, UL8, UL9, and UL52 which were known to be involved in viral DNA replication but were otherwise uncharacterised. Subsequently, as UL5, UL9, and UL52 were examined by other workers but the function of UL8 remained unknown, the project focused more closely on purifying and characterising UL8. Development of reagents to aid detailed analysis of UL8 was another aim. These aims were achieved.

Peptides and antipeptide sera.

Peptides have been synthesised with sequences corresponding to parts of the predicted sequences of proteins UL5, UL8, UL9, and UL52. Analysis of these peptides by HPLC, FAB-MS spectrometry, and amino acid analysis showed that they were sufficiently pure to use for generating antisera. Branched peptides were used to produce antisera as generally they are more antigenic than peptides conjugated to carrier proteins (McLean *et al*, 1991). Also they do not require conjugation to a carrier protein. Antisera raised against these peptides recognised two of the corresponding proteins, UL8 and UL9, at dilutions from 1:64 to 1:300. It is noteworthy that these sera confirm the accuracy of the HSV-1 predicted amino acid sequence in these regions (McGeoch *et al*, 1988a), including the minimum length of the coding region designated as gene UL8. The availability of sera recognising UL8 allowed the purification of that protein. Repeated attempts to raise sera recognising the proteins UL5 and UL52 in Western blots were unsuccessful. This is somewhat surprising as Olivo *et al* (1989) raised sera recognising proteins UL5 and UL52 on Western blots using peptides corresponding to the same part of the protein. For UL5 residues 874–882, and for UL52 residues 1051–1058 (compare with table 1 of the results). Thus these sequences do not appear to be inherently of low immunogenicity, nor are the epitopes of the proteins resistant to exposure even during denaturation. Olivo *et al* used peptides coupled to keyhole limpet hemocyanin rather than branched peptides. However, as noted above, branched peptides are generally more antigenic than peptides conjugated to carrier proteins (McLean *et al*, 1991). As the anti-peptide titres of the sera were not measured it is not known whether the peptide synthesis and purification, the immunisation or the screening of the sera was at fault.

Purification of UL8 protein.

A procedure for purifying the HSV-1 UL8 protein was developed. The purification is simple, requiring only two chromatography columns (DEAE and phenyl sepharose), and may be completed in $3^1/_2$ hours beginning with the crude extract. The purified protein is

greater than 95% pure and a yield of 1.5 mg of protein is obtained from 2.4 x 10^8 cells, equivalent to eight 175 cm^2 tissue culture flasks. The protein is not significantly proteolysed and is probably not denatured. The DEAE sepharose step is very similar to one step of a four column purification (heparin agarose, DEAE sepharose, hydroxyapatite, and gel filtration) described by Dodson and Lehman (1991). UL8 protein purified by Dodson and Lehman (1991) was described as greater than 95% pure although no data were presented. Those workers obtained approximately half the yield reported here. In addition my two step purification is likely to be simpler and faster. A partial purification producing UL8 30% pure, was reported by Sherman *et al* (1992). This purification was from cells expressing UL5, UL8, and UL52 so that the UL8 produced might contain other components of the helicase primase complex, an obvious disadvantage.

Initial experiments demonstrated that UL8 was stable in the harvesting buffer then being used (T buffer). The protein was stable while standing at 25 °C or 4 °C and was also stable to freezing and thawing and dilution. It was not possible to assay for activity but the protein was not denatured or significantly proteolysed during these treatments. Initial attempts to purify the protein showed that it was less resilient than it had seemed. Experiments using ammonium sulfate precipitation as a method of purification were unproductive as UL8 is denatured by this salt. In addition, interaction of the protein with all matrices tested resulted in large losses and the behaviour of the protein was unpredictable, varying from preparation to preparation. The stability of UL8 may be dependent on the total concentration of proteins in the preparation. The inclusion of glycerol at 10% by volume in the harvesting buffer and all chromatography buffers reduced losses of UL8 to undetectable levels. The value of glycerol in stabilising proteins in solution is well established (Scopes, 1982).

Conclusions may be drawn about the surface of UL8 from its chromatographic properties. The behaviour of UL8 in anion exchange chromatography suggests that it has a surface that is strongly negatively charged or with one region which is strongly negatively charged. UL8 bound very firmly to mono Q columns (substituted with quaternary ammonium groups) and to a diethyl amino ethyl-substituted matrix (DEAE sepharose). This interaction is presumably mediated by the side chains of aspartate or glutamate residues. Examination of the predicted sequence of the protein shows that acidic residues are not concentrated in any region of the protein (see overleaf). While the primary structure of the protein says little about the distribution of charges in the three dimensional structure, the distribution suggests that the binding is probably due to the overall negative charge of the surface. Elution of the protein from DEAE sepharose by a combination of increasing sodium chloride concentration and *p*H was probably due to disruption of salt bridges by the sodium chloride ions but also due to titration of column substituents closer to neutrality, as DEAE matrices do titrate in the region *p*H 7.5 – 8.0 (Scopes, 1982). As UL8 did not bind to the cation exchange column mono S, there is presumably no region of

The primary structure of UL8 protein of HSV-1 (750 amino acids). Overleaf is a plot of the physicochemical characteristics of the amino acids of UL8 against their position in the protein. This plot was compiled using the University of Wisconsin Genetics Computer Group software.

```
  1  MDTADIVWVE ESVSAITLYA VWLPPRAREY FHALVYFVCR NAAGEGRARF
 51  AEVSVTATEL RDFYGSADVS VQAVVAAARA ATTPAASPLE PLENPTLWRA
101  LYACVLAALE RQTGPVALFA PLRIGSDPRT GLVVKVERAS WGPPAAPRAA
151  LLVAEANIDI DPMALAARVA EHPDARLAWA RLAAIRDTPQ CASAASLTVN
201  ITTGTALFAR EYQTLAFPPI KKEGAFGDLV EVCEVGLRPR GHPQRVTARV
251  LLPRDYDYFV SAGEKFSAPA LVALFRQWHT TVHAAPGALA PVFAFLGPEF
301  EVRGGPVPYF AVLGFPGWPT FTVPATAESA RDLVRGAAAA YAALLGAWPA
351  VGARVVLPPR AWPGVASAAA GCLLPAVREA VARWHPATKI IQLLDPPAAV
401  GPVWTARFCF PGLRAQLLAA LADLGGSGLA DPHGRTGLAR LDALVVAAPS
451  EPWAGAVLER LVPDTCNACP ALRQLLGGVM AAVCLQIEET ASSVKFAVCG
501  GDGGAFWGVF NVDPQDADAA SGVIEDARRA IETAVGAVLR ANAVRLRHPL
551  CLALEGVYTH AVAWSQAGVW FWNSRDNTDH LGGFPLRGPA YTTAAGVVRD
601  TLRRVLGLTT ACVPEEDALT ARGLMEDACD RLILDAFNKR LDAEYWSVRV
651  SPFEASDPLP PTAFRGGALL DAEHYWRRVV RVCPGGGESV GVPVDLYPRP
701  LVLPPVDCAH HLREILREIE LVFTGVLAGV WGEGGKFVYP FDDKMSFLFA
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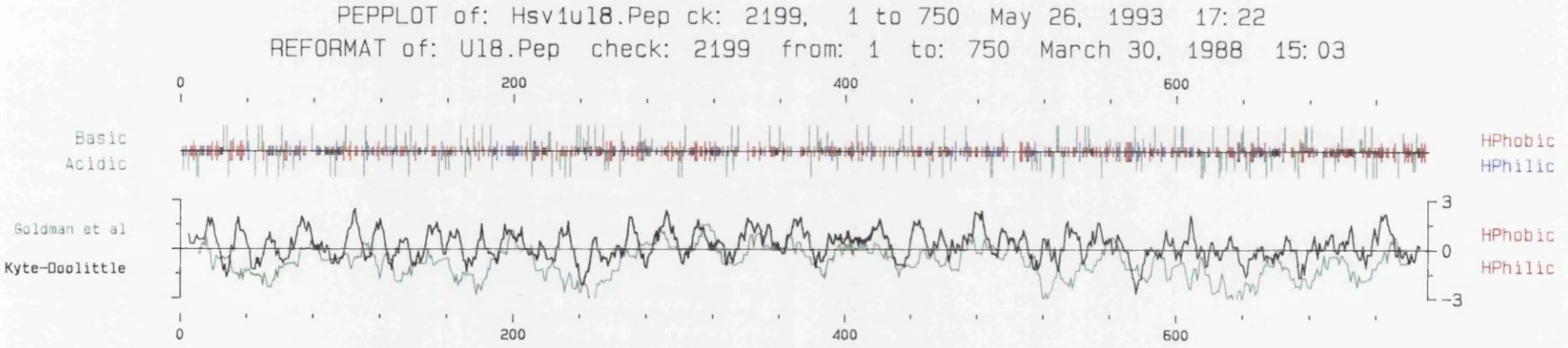

PEPPLOT of: Hsv1ul8.Pep ck: 2199, 1 to 750 May 26, 1993 17:22
REFORMAT of: U18.Pep check: 2199 from: 1 to: 750 March 30, 1988 15:03
0
200
400
600
Basic
Acidic
HPhobic
HPhilic
Goldman et al
Kyte-Doolittle
3
0
-3
HPhobic
HPhilic
0
200
400
600

concentrated positive charge on its surface. As UL8 bound to a phenyl sepharose column at only 0.1–0.3 M sodium chloride the surface of the protein must contain a high proportion of hydrophobic residues. It will be interesting to learn whether hydrophobic or negatively charged amino acids, or both, are important in the interaction of UL8 with UL5 and UL52.

Structure of UL8 in solution.

It has been demonstrated that purified UL8 is a monomer in solution. This result is perhaps not surprising as UL8 exists in infected cells as a 1:1:1 complex with the UL5 and UL52 proteins (Crute and Lehman, 1991). An approximate value for the protein's molecular mass (75,000 +/- 10,000 daltons) was obtained by gel filtration. (The error is based on a maximum error in the elution volume of +/- 0.1 ml.) Attempts to obtain a more accurate value for the molecular mass using glycerol gradient sedimentation were not successful. Purified UL8 was centrifuged through 10–30% glycerol gradients with standards run in parallel. This experiment was performed three times but in each case UL8 appeared in all fractions from approximately half way down the tube — corresponding to a molecular mass of 150 kDal — to the bottom of the tube. The standard proteins centrifuged as expected from their molecular masses. Thus it appears that UL8 is not stable in glycerol concentrations greater than about 20% by volume. It may be possible to determine the precise molecular mass of UL8 using sucrose gradient centrifugation.

Nucleic acid binding properties.

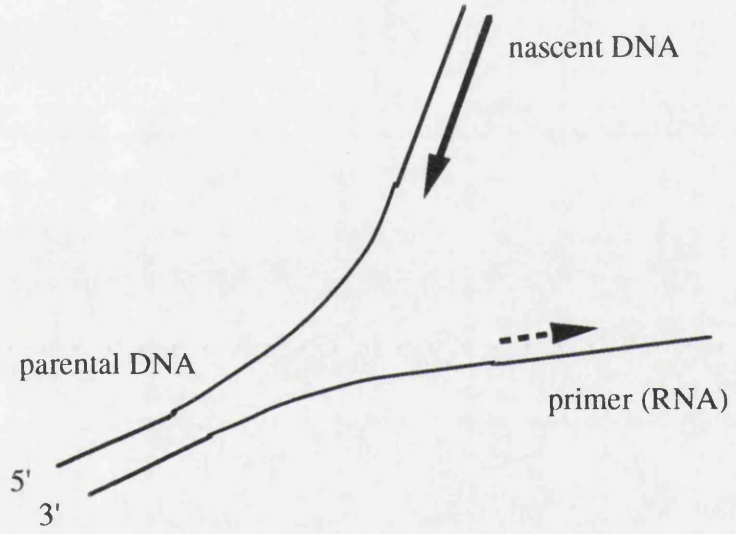
The possible interaction of UL8 with duplex and single stranded DNA and DNA/RNA hybrids was investigated using DNA band shift assays. No binding was detected under a number of conditions in which known nucleic acid binding proteins did bind. At first sight this is a surprising result as UL8 probably stabilises nascent primers on DNA *in vitro*, when contained in the viral helicase-primase complex (Sherman *et al*, 1992). One explanation is that UL8 binds nucleic acids only as part of the helicase-primase complex, or only in conjunction with other viral or cellular proteins. The possible additional cofactors are limited to ICP8 and either the UL30/UL42 complex or *E. coli* DNA polymerase I as these were the only other proteins present in the assay of Sherman *et al*. A specific interaction between UL8 and *E. coli* DNA polymerase I seems unlikely and so ICP8 is probably the only possible cofactor. If binding of the UL5/UL8/UL52 complex to DNA can be blocked by a UL8 specific monoclonal antibody, this would be strong evidence that UL8 does bind to DNA as part of the full complex.

Alternatively, UL8 may be similar to other proteins whose interactions with nucleic acids can only be detected under certain conditions. For example, binding of the human

DNA replication protein RF-C to DNA is revealed in a gel shift assay only after glutaraldehyde crosslinking and the binding is greatly enhanced by the presence of ATP (Tsurimoto and Stillman, 1991).

A third explanation of the failure of UL8 to bind nucleic acids is that it has properties analogous to proliferating cell nuclear antigen (PCNA) or the β subunit of *E. coli* DNA polymerase III holoenzyme. These are processivity factors for their cognate polymerases but do not bind DNA (see introduction). Thus UL8 may alter the DNA binding properties of UL5 or UL52 without contacting DNA itself.

One further structure might be investigated for interaction with UL8: a preformed single stranded/double stranded DNA fork which would correspond to the leading point of a replication fork (see diagram). Demonstrating conclusively whether purified UL8 binds nucleic acids will require further experiments designed to examine these possibilities.



DNA structures at a replication fork which UL8 might interact with.

Production of monoclonal antibodies and polyclonal anti-protein sera.

This thesis reports the first monoclonal antibodies specific for UL8. Nineteen cell lines producing monoclonal antibodies to UL8 have been developed. The variation in reactivity of the antibodies in the different assays (ELISA, Western blotting, and immune precipitations) suggests that a number of different epitopes are recognised (table 5). For example, reactivity in immune precipitation but not Western blotting (e.g. MP0813) suggests a conformational epitope, whereas the inverse reactivity (e.g. MP0809) suggests recognition of an internal sequential epitope. Antibodies reactive in Western blots and immune precipitations suggest an external sequential epitope (e.g. MP0811) while antibodies reactive only in ELISA suggests a fourth type of epitope. Preliminary localisation of epitopes using C terminal deletions of UL8 and Western blotting (being

carried out by E. Barnard, Institute of Virology) has produced results for two monoclonal antibodies. Antibody MP0817 fails to recognise a deleted protein lacking only the last 70 amino acids and antibody MP0811 recognises all deletions examined so far, even one which retains only the first 350 amino acids of the protein. Examination of further antibodies is planned. The epitopes recognised can be localised more precisely using synthetic peptides and N terminal deletions. Two UL8 specific polyclonal sera have been produced by injection of the purified protein into rabbits. These sera precipitate UL8 from solution and recognise the protein in Western blots at a dilution of 1:4000 (figure 16). The availability of these antibodies will greatly facilitate future studies of UL8.

Other possible future work.

Only four possible activities have been assigned to UL8: i) a role in viral DNA replication — ascribed through transient assays and viral mutants (Wu *et al*, 1988; Carmichael and Weller, 1989), ii) nuclear localisation (Calder *et al*, 1992), iii) primer utilisation (Sherman *et al*, 1992), iv) interaction with the UL5/UL52 complex. There is no evidence that UL8 affects the helicase activity of the UL5/UL8/UL52 complex. Future work should be directed with these facts in mind.

Assaying the ability of purified UL8 to participate in HSV-1 DNA replication.

Purified UL8 must be assayed to see if it can participate in viral DNA replication. There are four potential assays: the coupled primase/polymerase assay of Sherman *et al*, (1992); a variation of the plasmid amplification assay of either Wu *et al* (1988) or Stow (1992); and complementation of the UL8 negative mutant *hr*80 (Carmichael and Weller, 1989).

The coupled primase/polymerase assay (also called a lagging strand synthesis assay) consists of single stranded M13 DNA primed by the test enzyme. The activity of the enzyme is revealed by extending any primers with a polymerase which incorporates radiolabelled deoxynucleotide triphosphates. *E. coli* DNA polymerase I or HSV-1 UL30/UL42 complex may be used. In this assay UL5/UL8/UL52 complex is active while UL5/UL52 complex is not unless UL8 is added separately. Thus purified UL8 could be assayed. Use of the lagging strand synthesis assay would require the proteins mentioned above and ICP8 and only some of these proteins are available in purified form in this Institute. Although complex, this system represents an important step on the road to a full *in vitro* HSV-1 DNA replication system and should be pursued. The availability of pure UL8 contributes to this aim.

The two plasmid amplification assays are similar. The assay of Wu *et al* involves transfecting tissue culture cells with plasmids expressing the seven replication proteins and a plasmid containing an HSV-1 origin. The assay of Stow involves transfecting Sf cells

with a plasmid containing HSV-1 ori_S and superinfecting with seven recombinant baculoviruses each expressing one of the seven HSV-1 DNA replication proteins. In both assays the origin-containing plasmid is amplified and can be detected by its resistance to a methylation sensitive nuclease. Omitting the virus expressing UL8 might allow assay of purified UL8 by introducing the protein into the cells by electroporation, inclusion in the transfection mixture, liposome based transfer, or another method. The success of such an assay would depend on whether UL8 can be introduced into cells in a functional form, and whether exogenous protein can take the place of protein synthesised in the cell. With reference to the former, antibodies and other proteins can be introduced into cells by electroporation and retain function (Chen *et al*, 1993; Kashanchi *et al*, 1992).

Complementation of the UL8 negative mutant might be achieved by similar attempts to introduce UL8 into cells which are permissive for growth of wild type HSV-1 but not the mutant virus. Detection of viral DNA replication or a true-late viral protein would indicate that UL8 was complementing the mutation.

Crystallography.

Proteins homologous to UL8 are found in varicella zoster virus (McGeoch *et al*, 1988a; Davison and Scott, 1986a) and equine herpesvirus 1 (Telford *et al*, 1992), but not in human cytomegalovirus (Chee *et al*, 1990) or in Epstein-Barr virus (Baer *et al*, 1984), although positional homologues of the gene may exist in the last two organisms. Also, UL8 is the first primase subunit isolated from any organism that stabilises primers but apparently has no other role in the catalytic activity of the primase (Sherman *et al*, 1992). Therefore it would be very interesting to determining the three dimensional structure of the protein. This is practicable given the protein's size (80kDal). Crystallisation of a protein requires 10-100 mg of the protein as starting material (Wood, 1990), and at least the lower of these two figures can readily be achieved with the current purification. Growth of Sf cells in roller bottles or spinner cultures would make the 100 mg target realistic. The purification reported here yields UL8 with at least 95% purity, a suitable starting point for crystallography. The purified protein does not precipitate or become proteolysed for at least 24 hours at room temperature. In addition, a number of relevant properties of UL8 have been deduced from the development of the purification scheme: UL8 is stable at *p*H 6.1 or above (although the upper limit has not been determined); the protein is sensitive to ammonium sulfate, at least at 1 M and possibly below; up to 2 M sodium chloride does not adversely affect the protein; and 10% glycerol is required for stability but some concentration between 10% and 30% is too great. Given all these advantages, attempts should be made to crystallise UL8.

Interaction of UL8 with other proteins.

If the ability of UL8 to participate in DNA replication cannot be measured, it may be possible to establish that the purified protein is in native conformation by reconstituting the helicase/primase by mixing UL8 and the UL5/UL52 complex. It may be that the UL5/UL8/UL52 complex assembles with maximum efficiency only by simultaneous *de novo* synthesis of all three proteins but this possibility can now be tested. Precipitation of all three proteins by a monoclonal antibody to UL8 would show that the full complex had been reconstituted. Alternatively, chromatographing a mixture of UL8 and UL5/UL52 complex on a gel filtration column would show what proportion of UL8 had bound to the other proteins. In addition, mixing radiolabelled UL5/UL8/UL52 complex with excess unlabelled UL8 followed by immunoprecipitation or gel filtration would show whether UL8 dissociates from and reassociates with the other members of the complex at any appreciable rate. Naturally, if immune precipitation is employed the antibody used must not interfere with the interaction of the proteins. It has already been demonstrated that UL8 does not form a stable complex with either UL5 or UL52 alone (Dodson and Lehman, 1991).

If UL8 stabilises primers, the protein may interact with the viral polymerase as the primer terminus must be transferred to the polymerase, perhaps into a groove on its surface. The DNA duplex containing the nascent strand is contained in such a groove on the surface of *E. coli* DNA polymerase I (Freemont *et al*, 1988). Primer-template duplexes may interact similarly with the surface of UL8 allowing the primer's 3' terminus to be transferred into the polymerase active site. A physical association between UL8 and the polymerase during this process is possible rather than probable however as both *E. coli* DNA polymerase I and HSV-1 DNA polymerase can extend primers stabilised by UL8 (Sherman *et al*, 1992), and a site of interaction with UL8 is unlikely to have been conserved between the two polymerases. No interaction of UL8 and ICP8 has been demonstrated but such an interaction is also possible as ICP8 may coat all single stranded DNA at a replication fork.

The availability of monoclonal antibodies to UL8 will allow regions of the protein involved in particular functions to be identified. Interaction of UL8 with four groups of macromolecules may be examined: nucleic acids; UL5/UL52 complex; viral UL30/UL42 complex; and ICP8, the HSV-1 single stranded DNA binding protein. If UL8 binds primer-template complexes when part of the helicase-primase, this interaction might be blocked by appropriate monoclonal antibodies, identifying the region or regions involved. If UL8 binds strongly to UL5/UL52 then disrupting the preformed complex may not be possible. However, preventing the two portions of the complex associating using an antibody may still be possible. Some monoclonal antibodies bind only free UL8 and not UL8 contained in the UL5/UL8/UL52 complex, supporting this proposal (G. McLean,

Institute of Virology, personal communication).

It may be possible, with appropriate peptides, to disrupt protein-protein interactions identified using monoclonal antibodies. If the interaction is essential the disruption has the potential to prevent viral DNA replication. Disruption by peptides has been achieved for other protein-protein interactions (reviewed by Marsden, 1992) and such peptides are potential lead compounds for the development of antiviral drugs.

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