PEPTIDE INHIBITION OF HERPES SIMPLEX VIRUS TYPE 1 DNA POLYMERASE

BY

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Unless otherwise stated, all the results described represent my own work.

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SUMMARY

Seven proteins encoded by herpes simplex virus have been shown to be essential for the replication of virus DNA and for virus growth. This study is concerned with two of these proteins: Pol and UL42. Pol is the catalytic subunit of DNA polymerase and is encoded by gene *UL30*; UL42 is an accessory protein which serves to increase polymerase processivity and is encoded by gene *UL42*. Together these two proteins form the polymerase holoenzyme, and evidence suggests that the interaction between them is an essential one and hence a potential target for antiviral drugs.

To identify regions of the UL42 protein of herpes simplex virus type 1 which may affect viral DNA polymerase activity, a series of 96 overlapping pentadecapeptides spanning the entire 488 amino acid residues of the UL42 protein were synthesised and tested for their ability to inhibit polymerase activity on a primed single-stranded M13 DNA template. Two assays were used: (i) formation of full length double-stranded M13 molecules; (ii) rate of incorporation of deoxyribonucleoside triphosphates. Both of the assays were optimised to ensure that any inhibition by the peptides would be detected.

Peptides from five non-contiguous regions of the UL42 protein were found to inhibit polymerase activity in both the presence and absence of the UL42 protein. The most active peptides from each region correspond to amino acid residues 23 to38 (peptide 6), 64 to 78 (peptide 14), 89 to 102 (peptide 19), 229 to 243 (peptide 47), and 279 to 293 (peptide 57). By two different methods (DNA mobility shift and DNA precipitation), peptides 14, 19, 47 and 57 were found to bind DNA; they most probably inhibit enzyme activity by this mechanism. Peptide 6 did not bind DNA and must act by some mechanism other than competing for DNA.

The inhibitory peptides were also tested for activity against mammalian polymerase α and the Klenow fragment of *Escherichia coli* polymerase. Although some limited specificity was demonstrated (up to 10-fold for peptide 6), all the peptides showed significant activity against both polymerase α and *E. coli* polymerase.

UL42 peptides other than those which inhibited polymerase were also found to bind DNA. Twenty-six peptides were shown to have DNA-binding properties by gel mobility shift assay. Twenty-two of these were positively charged, suggesting that

non-specific electrostatic interactions were largely responsible for the observed binding.

There is evidence that the carboxy-terminus of Pol is required for the formation of a functional complex with UL42. Two peptides spanning the carboxy-terminal 42 amino acid residues of Pol were tested for their ability to inhibit the processivity of the Pol–UL42 holoenzyme. They had no detectable effect.

ABBREVIATIONS

AIDS	acquired immunodeficiency syndrome
ATP	adenosine 5'-triphosphate
Boc	butyloxycarbonyl
bp	base pairs
BSA	bovine serum albumin
C-	carboxy-
CCV	channel catfish virus
Ci	Curie(s)
cpm	scintillation counts per minute
CZE	capillary zone electrophoresis
dATP	2'-deoxyadenosine 5'-triphosphate
DCM	dichloromethane
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DHBT	3,4-dihydro-3-hydroxy-4-oxobenzotriazin-3-yl
DMF	dimethylformamide
dNTP	any deoxyribonucleoside triphosphate
dsDNA	double-stranded deoxyribonucleic acid
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
dUMP	2'-deoxyuridine 5'-monophosphate
dUTP	2'-deoxyuridine 5'-triphosphate
E	early
EBV	Epstein-Barr virus
EDT	ethanedithiol
EHV	equine herpes virus
ELISA	enzyme-linked imunosorbent assay
EMS	ethylmethylsulphide
FAB	fast atom bombardment
Fmoc	9-fluorenyl-methoxycarbonyl
gp X	gene X product
gX	glycoprotein X
HCMV	human cytomegalovirus
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid
HHV-6	human herpesvirus 6
HHV-7	human herpesvirus 7

HOBt	1-hydroxybenzotriazole
HPLC	high pressure liquid chromatography
HSV	herpes simplex virus
HSV-1	herpes simplex virus type 1
HSV-2	herpes simplex virus type 2
HVS	herpesvirus saimiri
IC_{50}	50% inhibitory concentration
ICP	infected cell polypeptide (HSV-1, HCMV)
ICSP	infected cell-specific polypeptide (HSV-2)
IE	immediate early
IgG	immunoglobulin G
IR	internal repeat
K_d	dissociation constant
kDa	kilodaltons
kDa	kilodaltons
K_i	inhibition constant
K_m	Michaelis-Menten constant
L	late
λ	bacteriophage λ
LAT	latency-associated transcript
m.o.i.	multiplicity of infection
M13	bacteriophage M13mp18 vector
MAb	monoclonal antibody
MCMV	murine cytomegalovirus
M_r	relative molecular mass
MS	mass spectrometry
Mtr	methoxytrimethylbenzene sulphonyl
N-	amino-
ori	origin of DNA replication
PAA	phosphonoacetic acid
PFP	pentafluorophenyl
Pmc	pentamethylchromane sulphonyl
Pol	catalytic subunit of HSV-1 polymerase (also abbreviation for polymerase, as in *E. coli* Pol I and III)
PRV	pseudorabies virus
PyBOP	benzotriazolyloxy-tris[pyrrolidino]-phosphonium-hexafluorophosphate
R1	large subunit of ribonucleotide reductase

R2	small subunit of ribonucleotide reductase
RP	reverse phase
RR	ribonucleotide reductase
ssDNA	single-stranded deoxyribonucleic acid
SV40	simian virus 40
T4	bacteriophage T4
T7	bacteriophage T7
TFA	trifluoroacetic acid
TK	thymidine kinase
T_m	midpoint melting temperature (of DNA)
TMSB	trimethylsilylbromide
TR	terminal repeat
Trt	trityl
ts	temperature sensitive
UL42	product of HSV-1 gene *UL42*
V_{max}	initial enzyme rate at saturating substrate concentration
VZV	varicella zoster-virus

ONE AND THREE LETTER ABBREVIATIONS FOR AMINO ACIDS

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
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

1. The Herpesviruses

1.1 Description and classification

Herpesviruses are found in a wide variety of host species, ranging from fish to humans (reviewed by Roizman, 1990). Membership of the family *Herpesviridae* is based on the structure of the virion. A herpes virion consists of a double stranded DNA genome contained in an icosahedral capsid 100-110nm in diameter; the capsid is surrounded by an amorphous proteinaceous layer of tegument which in turn is enveloped in a lipid membrane containing glycoprotein spikes.

Although they differ widely in their pathogenic potential, herpesviruses share several important biological properties. Their most notable feature is the ability to persist in a latent form in an infected individual; following an initial infection the viral DNA remains in a quiescent state in specific tissues of the host organism, from which it can be re-activated to produce a lytic infection. Most studies on latency have been carried out on herpes simplex virus type 1 (reviewed by Stevens, 1989; Ho, 1992). Other biological features common to all herpesviruses are as follows:

1) They encode many virus-specific enzymes, particularly those involved in nucleic acid metabolism and DNA replication.
2) Synthesis of viral DNA and assembly of capsids takes place in the nucleus.
3) Production of infectious virus always results in death of the infected cell.

Members of the family *Herpesviridae* are divided, mainly on the basis of their differing biological properties, into three sub-families:

Alphaherpesvirinae are neurotropic viruses which establish latent infections primarily in sensory ganglia. They are relatively fast-growing and have a variable host range in tissue culture and experimental animal systems. Members of this sub-family include herpes simplex virus type 1 and type 2 (HSV-1, HSV-2), varicella-

zoster virus (VZV), pseudorabies virus (PRV) and equine herpes virus type 1 (EHV-1).

Betaherpesvirinae are slow-growing viruses with a restricted experimental host range. Infected cells in culture may fuse to form giant cells or *cytomegalia.* They can establish latency in secretory glands, lymphoreticular cells, kidneys and other tissues. Members of this class include human cytomegalovirus (HCMV) and murine cytomegalovirus (MCMV).

Gammaherpesvirinae are lymphotropic viruses which infect and establish latency in T or B lymphocytes. They have a very restricted host range in experimental animals. Members of this sub-family include Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS).

The complete sequences have been published of EBV (Baer *et al,* 1984), VZV (Davison & Scott, 1986), HSV-1 (McGeoch *et al* 1985, 1986, 1988b; Perry & McGeoch 1988), HCMV (Chee *et al,* 1990), EHV-1(Telford *et al,* 1992), HVS (Albrecht *et al,* 1992), and channel catfish virus (Davison, 1992). Partial sequence data are available for many others. These data allow a re-evaluation, based on conservation of gene arrangement and coding content, of the original classification. On the whole, a high degree of genetic relatedness is found to be reflected in similar biological characteristics, confirming the original groupings, but there are exceptions. The sequence of Marek's disease virus places it in the alphaherpesvirinae, but its biological properties more closely resemble those of the gammaherpesvirinae (Buckmaster *et al,* 1988). Human herpesvirus 6 (HHV-6), being a lymphotropic virus, would also appear to belong in the gammaherpesvirinae, but in fact its genome structure and sequence is closely related to HCMV, the prototype betaherpesvirus. The sequencing of channel catfish virus (CCV), classified on the basis of morphology and biology as an alphaherpesvirus (Roizman, 1982), showed it to be completely unrelated to the herpesviruses of higher verterbrates (Davison, 1992). Thus, on the basis of the genetic data, CCV constitutes a new subfamily, or possibly a new virus family altogether.

1.2 Pathology and epidemiology of human herpesviruses

Seven herpesviruses which infect man have been identified to date: HSV-1, HSV-2, VZV, EBV, HCMV, HHV-6 and human herpesvirus 7 (HHV-7). They appear to be ubiquitous, and serological studies show that a large proportion of people throughout

the world have been exposed to and may be latently infected with one or more herpesviruses.

HSV-1 and HSV-2 (reviewed by Whitley, 1990), usually cause cold sores and genital lesions, respectively. Primary infection can be asymptomatic or result in virus replicating at the site of infection to form a localised lesion. Virions are transported via the axons to the sensory ganglia that innervate the site of infection. The virus thereafter remains in a latent state in the ganglia, and can reactivate spontaneously to produce recurrent infection. The frequency of reactivation is increased by physical or emotional stress, fever, exposure to bright sunlight or UV light, tissue damage or immune suppression. The virus can occasionally cause encephalitis or disseminated disease in newborn infants. HSV-2 has long been suspected of an association with cervical carcinoma but, despite numerous studies, its role as a causative agent of transformation is not clear.

VZV infection (reviewed by Gelb, 1990) has two distinct clinical manifestations: chickenpox (varicella) and shingles (zoster). Varicella represents the primary infection, usually in childhood; zoster is caused by reactivation later in life of virus from sensory ganglia, and is limited to the skin innervated by a single sensory ganglion. The most common complication of herpes zoster is postherpetic neuralgia (pain lasting over a month).

EBV (reviewed by Miller, 1990) infects B lymphocytes in the peripheral blood and lymphoid organs, causing infectious mononucleosis or 'glandular fever'. EBV is also associated with two specific cancers: Burkitt's lymphoma, a tumour affecting children in East Africa, and nasopharyngeal carcinoma, which has a high incidence in southern China.

HCMV infections (reviewed by Alford & Britt, 1990) are most often asymptomatic, and latency is established in the absence of any associated disease. Virulence is seen only in the absence of a competent immune system. The virus is a threat *in utero*, since it has the ability to cross the placenta and cause congenital infection which can lead to birth defects. It is frequently reactivated after organ transplants. It is one of the more common opportunistic infections associated with AIDS, and acts as a co-factor in AIDS progression.

HHV-6 (reviewed by Lopez & Honess, 1990) is a lymphotropic virus acquired by most individuals during their first two years of life. It can cause exanthem subitum, a mild fever and rash, but in most cases seroconversion is asymptomatic.

HHV-7 was recently isolated from the T lymphocytes of a healthy individual (Frenkel *et al*, 1990). The virus has not yet been associated with a known disease.

The virulence of all these viruses is generally controlled by an intact immune system, but they are able to cause serious disease in immunocompromised individuals. Therefore, with the use of immunosuppressive drugs for the control of malignant disease, the increase in organ transplants, and the advent of AIDS, they pose a growing problem.

2. Herpes Simplex Virus Type 1

2.1 The structure and content of the HSV-1 genome

The HSV-1 genome is a linear DNA molecule about 152kbp in length and comprises a long (L) and a short (S) region, which are covalently linked. The long region consists of a unique sequence (U_L) flanked by a repeat element (R_L); the terminal (TR_L) and internal (IR_L) copies of the repeat are oriented in oppos ite directions (Sheldrick & Berthelot, 1974). The short region similarly consists of IR_S, U_S and TR_S (Figure 1a). The sequences of R_L and R_S are distinct, and contain short tandem reiterations which vary in number (Davison & Wilkie, 1981; Rixon *et al*, 1984; McGeoch *et al*, 1988b; Perry & McGeoch, 1988). At the very ends of the genome there is a 400bp direct repeat called the *a* sequence, of which one or more copies are also found in an inverted orientation at the junction between L and S (Roizman, 1979; Davison & Wilkie 1981). The genome terminates at each end with an unpaired base which has a free 3'-hydroxyl group (Mocarski & Roizman, 1982). During DNA replication the L and S segments can adopt different relative orientations, so that HSV DNA isolated from virions is composed of equimolar amounts of four isomers (Figure 1a) (Hayward *et al*, 1975; Wilkie, 1976; Delius & Clements, 1976; Roizman, 1979). The average base composition of HSV-1 DNA is 68.3% G+C (McGeoch *et al*, 1988b).

The complete sequence of the HSV-1 genome has been determined (McGeoch *et al* 1985, 1986, 1988b; Perry & McGeoch 1988). The original analysis (McGeoch *et al*, 1988b) identified a total of 72 genes encoding 70 distinct proteins (Figure 1b). Since then, three additional ORFs have been identified and designated according to their position: UL26.5 (Liu & Roizman, 1991a, b), UL49.5 or 49A (Barker & Roizman, 1992; Barnett *et al*, 1992), and $\gamma_1$34.5 or RL1 (Chou & Roizman, 1990; McGeoch *et al*, 1991; Dolan *et al*, 1992). In addition, a region of R_L encodes the latency associated transcripts (LATs), which are the only viral transcripts detectable in latently infected cells (Stevens *et al*, 1987; Wagner *et al*, 1988; reviewed by Fraser *et al*, 1992). The products of over 50 genes have been identified and

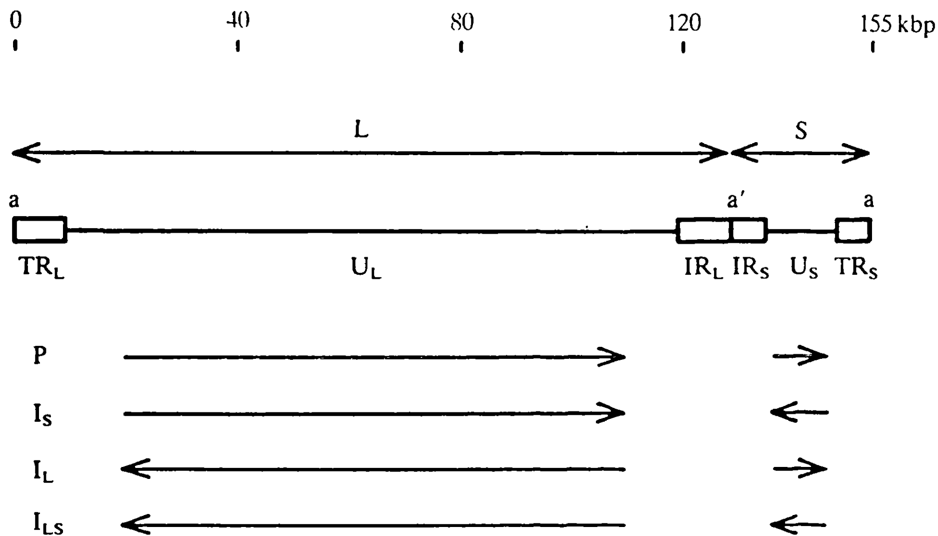
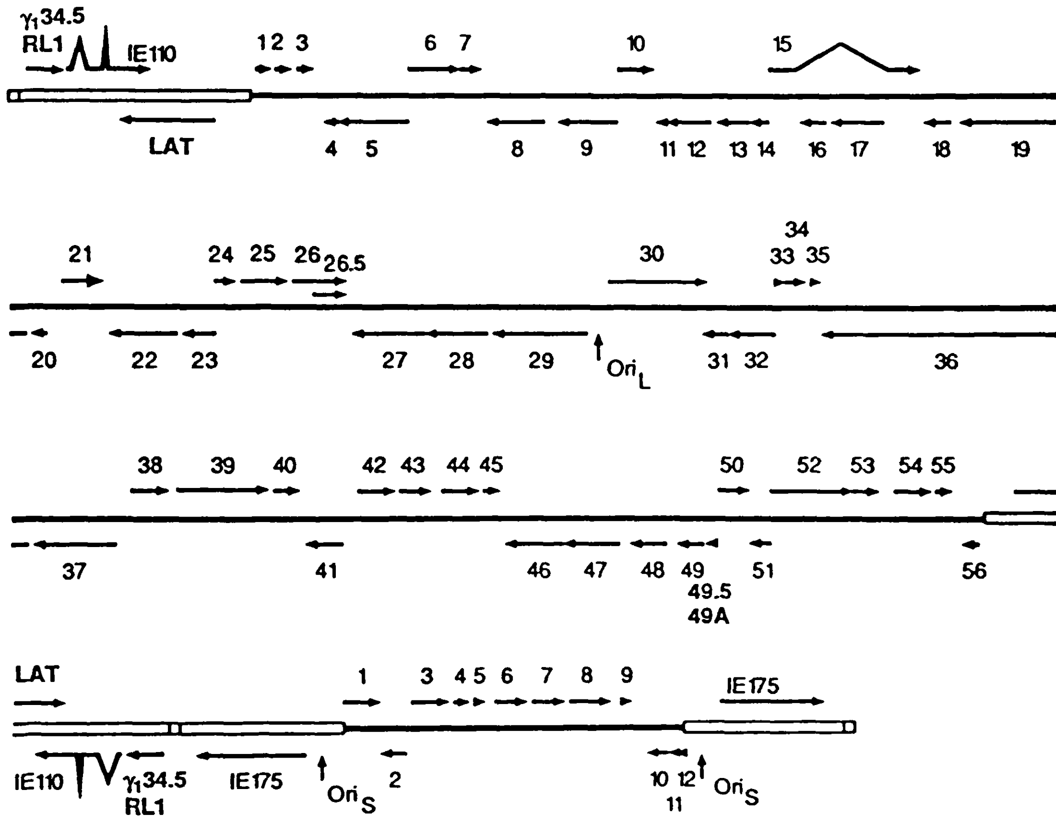


Figure 1. Organization of the HSV-1 genome.

a. A representation of HSV-1 DNA as two covalently joined segments, L and S. Unique sequences (U_L and U_S) are shown as solid lines, and major repeat elements (TR_L and IR_L, IR_S and TR_S) as open boxes. Terminal *a* sequences and the internal, opposite orientation *a'* sequence are indicated. The relative orientations of the L and S segments in the four isomers are indicated by the four lines of arrows. These are designated P (an arbitrary prototype), I_S (inverted S), I_L (inverted L) and I_{LS} (both inverted). Reproduced from McGeoch, 1987.



b. The genome in the P orientation, represented on four lines. The locations of open reading frames are shown by arrows, with splicing within coding regions indicated. In the top four lines, genes UL1–UL56 are shown as 1–56, and, in the bottom line, genes US1–US12 as 1–12. Locations of origins of DNA replication are indicated. Reproduced from McGeoch, 1989, with the addition of UL26.5 (Liu & Roizman, 1991a, b), UL49.5/49A (Barker & Roizman, 1992; Barnett et al, 1992), γ1 34.5/RL1 (Chou & Roizman 1990; McGeoch et al, 1991; Dolan et al, 1992), and LATs (Stevens et al, 1987; Wagner et al, 1988).

characterised, and their functions in the viral life cycle determined to varying extents, as described in the following sections.

2.2 The Lytic Cycle

2.2.1 Attachment and penetration

HSV-1 enters cells by a pH-independent fusion of the virion envelope with the cell plasma membrane (Morgan *et al*, 1968; Para *et al*, 1980; Wittels & Spear, 1991). This is a complex, multi-step process mediated by specific interactions between components of the virion envelope and the cell surface (reviewed by Marsden, 1987; Spear, 1992).

HSV-1 specifies the synthesis of ten glycoproteins (UL1-gL, UL22-gH, UL27-gB, UL44-gC, UL53-gK, US4-gG, US5-gJ, US6-gD, US7-gI and US8-gE), which are probably all found in the virion envelope. The architecture of the envelope is not well understood, but high resolution electron microscopy of virions shows at least two kinds of projections: rod-like spikes about 14nm long, and short "fuzzy" material (Stannard *et al*, 1987). Use of gold-labelled monoclonal antibodies (MAbs) showed that the long spikes are composed of gB, and at least some of the shorter protrusions are gD, while gC extends even further than gB from the surface of the envelope (Stannard *et al*, 1987). There is evidence for specific associations between pairs of glycoproteins, which probably reflect a functional interaction: gI and gE can be coprecipitated from extracts of infected cells with antibodies specific for either, or with normal IgG (Johnson & Feenstra, 1987; Johnson *et al*, 1988). The formation of a stable complex between gH and gL is required for their correct processing and cell surface expression (Hutchinson *et al*, 1992). A number of other HSV-1 gene products (UL10, UL20, UL34, UL43, UL45 and UL49A) are predicted from their characteristic hydropathy profiles to be membrane proteins, but they are still poorly characterised.

The initial step in attachment is an interaction with the heparan sulphate which is covalently linked to cell surface proteoglycans (WuDunn & Spear, 1989; Shieh *et al*, 1992). [Heparan sulphate is one of the most common sulphated glycosaminoglycans found on the cell surface (Kjellén & Lindahl, 1991).] Cells that fail to produce heparan sulphate, or that have been stripped of it, are highly resistant to HSV infection (WuDunn & Spear, 1989; Shieh *et al*, 1992). Both heparin (Nahmias &

Kibrick, 1964) and heparin-binding proteins (WuDunn & Spear, 1989; Kaner *et al*, 1990) can block the binding of virus to the cell surface.

There is evidence that gC, which has an affinity for heparin (WuDunn & Spear, 1989), plays a role in the initial attachment, since virions lacking gC adsorb more slowly to cells than wt virions (Langeland *et al*, 1990; Herold *et al*, 1991). Although it facilitates attachment, gC is not essential for this process, and gC$^-$ mutants can still infect cells (Heine *et al*, 1974; Langeland *et al*, 1990; Herold *et al*, 1991). Neomycin and polylysine inhibit infectivity, at least in part by blocking binding of the virus to a cellular receptor (Langeland *et al*, 1987, 1988). These compounds block the attachment of both wt and gC$^-$ virus, and therefore affect a receptor–ligand interaction which is independent of gC (Campadelli-Fiume, 1990; Langeland *et al*, 1990).

Glycoproteins B, D and H are essential for virus growth in cell culture. [The recent finding that gL is essential for correct expression of gH (Hutchinson *et al*, 1992) would imply that it too is an essential glycoprotein.] Virus mutants with lesions in the genes for gB (Sarmiento *et al*, 1979; Little *et al*, 1981; Cai *et al*, 1988), gD (Ligas & Johnson, 1988), or gH (Desai *et al*, 1988; Forrester *et al*, 1992), have severely reduced infectivity, but can still attach to cells, indicating that these glycoproteins are required for some other step. There is evidence that gD may stabilise attachment by interacting with a specific cell surface receptor, since gD$^-$ virions are unable to block superinfection by wt virus (Johnson & Ligas, 1988), and soluble gD blocks infection (Johnson DC *et al*, 1990). Arrest of virus penetration by gH-specific MAbs suggests that gH initiates membrane fusion (Fuller & Lee, 1992). Numerous experiments with *syn* (syncytial) mutants indicate that gB, gD, gH, gL, UL20, UL24 and possibly gK are involved in membrane fusion (reviewed by Spear, 1992). The cell surface molecules with which they interact remain to be identified.

It was recently reported that the basic fibroblast growth factor (bFGF) receptor was a specific portal of entry for HSV (Kaner *et al*, 1990; Baird *et al*, 1990), but this has now been disproved (Shieh & Spear, 1991; Mirda *et al*, 1992; Muggeridge *et al*, 1992). The observed effects were probably due to the interaction of bFGF with cell-surface heparan sulphate (Burgess & Maciag, 1989; Shieh & Spear, 1991; Muggeridge *et al*, 1992).

Having entered the cell, the capsid is transported, possibly via the microtubules (Kristensson *et al*, 1986), to the nuclear pores, and the viral DNA is released into the nucleus. A function essential for this process has been mapped to a region within UL36, which encodes the tegument protein VP1 (Batterson *et al*, 1983).

2.2.2 Effects on host cell macromolecular synthesis

Several components of the virion, apart from the viral genome, influence the course of infection. One effect of infection is the inhibition of cellular protein, RNA and DNA synthesis, which can be seen within an hour of infection. As infection proceeds, the synthesis of host cell macromolecules steadily declines and the synthesis of virus-specific material increases (reviewed by Fenwick, 1984). The first phase of the "host shutoff" is mediated by a component of the virion, and does not require viral gene expression (Fenwick & Walker, 1978). A later phase of host shutoff, which reduces the remaining levels of host protein synthesis, is dependent on the expression of viral genes (Fenwick & Clark, 1982).

The immediate effect of virion host shutoff is the disaggregation of cellular polyribosomes (Sydiskis & Roizman, 1966, 1967; Nishioka & Silverstein, 1978) and the degradation of cellular mRNA (Fenwick & Walker, 1978; Schek and Bachenheimer, 1985). Several *vhs* mutants have been isolated, which fail to induce early shutoff, but are not defective in the secondary shutoff function (Fenwick & Clark, 1982; Read & Frenkel, 1983). In cells infected with one such mutant, *vhs1*, the half-lives of all mRNA species examined — both viral and cellular — are longer than in cells infected with wt virus (Oroskar & Read, 1989). This indicates that the vhs function reduces the stability of all mRNAs, without discriminating between cellular and viral messages, and may thus contribute to the downregulation of early viral gene expression as infection proceeds (Oroskar & Read, 1989).

The vhs function is mediated by the product of gene UL41 (Kwong *et al*, 1988; Fenwick & Everett, 1990; Smibert & Smiley, 1990), a 58kDa protein, found in virions (Smibert *et al*, 1992), and more precisely in the tegument (McLauchlan *et al*, 1992a). It is not, however, an essential structural component (Fenwick & Everett, 1990).

The vhs function of most HSV-2 strains is more potent than that of HSV-1 (Schek and Bachenheimer, 1985), but for strain 17^{+} of HSV-1 and strain HG52 of HSV-2, the converse is true. The lack of shutoff by HG52 is due to a frameshift mutation within the UL41 coding sequence, which results in a truncated protein (Everett & Fenwick, 1990).

2.2.3 Regulation of gene expression

HSV-1 genes are expressed in a temporally-controlled way, and have been designated α, β and γ (Honess & Roizman, 1974), or immediate early (IE), early (E) and late (L) (Clements *et al*, 1977). Experiments using metabolic inhibitors showed that IE genes are transcribed in the absence of viral protein synthesis, whereas transcription of early genes requires *de novo* protein synthesis, and that of late genes requires viral DNA synthesis (Honess & Roizman, 1974, 1975; Clements *et al*, 1977; reviewed by Wagner, 1985, 1991). This is, however, a generalisation, and not all genes fall neatly into these groupings. The late genes are subdivided into two classes: leaky-late (βγ, γ_1) and true-late (γ_2). Both require DNA replication for maximal expression, but leaky-late genes are expressed at detectable levels before the onset of DNA replication, whereas true-late genes are not (Wagner, 1985). In general, IE genes encode transcriptional regulators, E genes encode proteins involved in DNA replication — all the replication proteins and nucleotide metabolism enzymes fall into this category, while L gene products are virion structural and assembly proteins. There are, however, exceptions to this generalisation. For example, US11 is expressed as a true-late gene (Johnson *et al*, 1986), but the product is not found in virions (Marsden *et al*, 1976); R1, the large subunit of ribonucleotide reductase (Preston VG *et al*, 1984) is regulated as an IE protein (Clements *et al*, 1980; Wymer *et al*, 1989).

2.2.3.1 α-TIF

The first effector in the cascade of transcriptional activation is a virion protein (Batterson & Roizman, 1983). This *trans*-inducing factor is encoded by gene *UL48* and was identified as Vmw65 (α-TIF, VP16) (Campbell *et al*, 1984; Dalrymple *et al*, 1985; Pellet *et al*, 1985), a 65kDa phosphoprotein which is a major component of the tegument (Marsden *et al*, 1978; Roizman & Furlong, 1974; Szilágyi & Cunningham, 1991; McLauchlan & Rixon, 1992). The effect of α-TIF is to induce transcription of the IE genes by cellular RNA polymerase II (Post *et al*, 1981; Batterson & Roizman, 1983; Campbell *et al*, 1984).

All the IE gene promoters contain one or more copies of a far-upstream element with the sequence TAATGARAT (R=purine) which is necessary for α-TIF transactivation (Mackem & Roizman, 1982; Murchie & McGeoch, 1982; Cordingley *et al*, 1983; Campbell *et al*, 1984). The α-TIF, which in isolation does not bind to DNA

(Marsden *et al*, 1987), associates with cellular factors to form a complex that binds specifically to the TAATGARAT motif (Preston CM *et al*, 1988; O'Hare & Goding, 1988). One of the factors in this complex is the cellular protein OTF-1 (OCT-1, NFIII, TRF), which normally binds to an octamer motif similar to TAATGARAT (O'Hare *et al*, 1988; Gerster & Roeder, 1988). It appears that α-TIF alters the specificity of OTF-1, so that it binds to the viral *cis*-acting element (O'Hare *et al*, 1988; Gerster & Roeder, 1988; Aphrys *et al*, 1989). Mutational studies have shown that the acidic C-terminal domain of α-TIF is responsible for the transactivation (Sadowski *et al*, 1988; Triezenberg *et al*, 1988; Greaves & O'Hare, 1989), while N-terminal residues are required for complexing with cellular proteins (Ace *et al*, 1988; Triezenberg *et al*, 1988; Greaves & O'Hare, 1990).

The IE genes contain other upstream *cis*-acting elements, apart from the TAATGARAT motif, which enhance their transcription even in the absence of α-TIF (Lang *et al*, 1984; Preston CM *et al*, 1984; Kristie & Roizman, 1984). Experiments with UL48 mutants confirm that α-TIF is not absolutely required for the initiation of infection, although it greatly increases its efficiency, especially at low m.o.i. (Ace *et al*, 1989).

2.2.3.2 The IE genes and their products

Several nomenclatures exist for the IE genes and their products. The most commonly used are listed in Table 1.

Table 1. The IE genes of HSV-1 and their products

Glasgow nomenclature			Chicago nomenclature	
Gene*	Gene†	Product	Gene	Product
IE-1	IE110§	Vmw110	α0	ICP0
IE-2	UL54	Vmw63	α27	ICP27
IE-3	IE175§	Vmw175	α4	ICP4
IE-4	US1	Vmw68	α22	ICP22
IE-5	US12	Vmw12	α47	ICP47
	UL39	R1		ICP6

* Original designation (Clements *et al*, 1979).

† Later designation in complete HSV-1 sequence (McGeoch *et al*, 1988b).

§ IE110 and IE175 are located in the terminal and internal repeats (Figure 1b).

All the IE polypeptides except Vmw12 are phosphorylated proteins which are found in the nucleus (Pereira *et al*, 1977; Hay & Hay, 1980; Marsden *et al*, 1982). The functions of Vmw12 and Vmw68 are poorly understood; Vmw110, Vmw175 and Vmw63 control the expression of E and L genes (reviewed by Everett, 1987; Wagner, 1991).

Vmw175 is considered to be the major regulatory protein of HSV-1. It is required for the activation of E and L promoters (Preston, 1979; Everett, 1984a, 1984b; DeLuca, 1985; Gelman & Silverstein, 1985; O'Hare & Hayward, 1985a; DeLuca & Schaffer, 1985; Quinlan & Knipe, 1985) and is essential for viral growth (Preston, 1979). It also represses its own synthesis and that of other IE gene products (O'Hare & Hayward, 1985b; DeLuca & Schaffer, 1985).

The accumulated evidence from many studies indicates that Vmw175 acts by binding to DNA at specific sites and interacting with cellular transcription factors. It binds to the sequence ATCGTC, but also to other sequences, and a degenerate 13-base consensus has been proposed to more accurately describe the recognition site, of which there are over 500 copies throughout the HSV-1 genome (DiDonato *et al*, 1991). Binding of Vmw175 at its own transcriptional start site is the likely mechanism of self-repression (Roberts *et al*, 1988; DeLuca & Schaffer, 1988), whereas binding within a promoter region leads to transcriptional activation (Tedder *et al*, 1989).

The DNA-binding domain is located within a highly conserved region, encompassing about 230 amino acid residues, near the N-terminus of the protein. Mutations within this domain drastically affect transactivation, repression and DNA binding (Paterson & Everett, 1988a, 1988b; Shepard *et al*, 1989; Paterson *et al*, 1990). A polypeptide corresponding to this region retains the sequence-specific DNA-binding properties of the whole protein (Wu & Wilcox, 1991; Everett *et al*, 1990, 1991a). The regions responsible for interactions with other proteins have not been clearly defined.

Vmw110 is a potent and promiscuous transactivator of gene expression (reviewed by Everett *et al*, 1991b). In transfection assays it activates transcription from IE, E and L promoters of HSV-1 (O'Hare & Hayward, 1985a, 1985b; Gelman & Silverstein, 1985; Quinlan & Knipe, 1985; Mavromara-Nazos, 1986; Everett, 1986), and from a variety of heterologous promoters, including the SV40 early promoter (O'Hare *et al*, 1986; Everett, 1988a) and the HIV LTR (Mosca *et al*, 1987).

In some transfection systems, joint activation by Vmw110 and Vmw175 is up to 20-fold greater than by either protein alone (Everett, 1984b), but the magnitude of this synergistic effect depends very much on the experimental conditions (Everett, 1988b). Another indication that the two proteins may interact is the observation that *ts* mutations in Vmw175 can affect the transport of Vmw110 into the nucleus (Knipe & Smith, 1986).

Vmw110 is not essential in tissue culture, but it confers a growth advantage on the virus (Stow & Stow, 1986; Sacks & Schaffer, 1987). An outstanding property of Vmw110 is its ability to reactivate latent virus. In an *in vitro* latency system, Vmw110 alone can replace whole virus as the reactivating agent (Harris *et al*, 1989). However, its role in reactivation is not so clearly demonstrable *in vivo*, since virus lacking Vmw110 is able to spontaneously reactivate from latently infected mouse ganglia, albeit more slowly than wt virus (Clements & Stow, 1989).

Vmw63 is essential for virus growth; lack of it results in overexpression of IE genes and very reduced expression of L genes (Sacks *et al*, 1985; McCarthy *et al*, 1989; reviewed by Sandri-Goldin, 1991). In transfection experiments, Vmw63 has little effect on its own, but it can repress the transactivation of IE and E genes by Vmw110 and Vmw175, and augment their transactivation of L genes (Everett, 1986; Sekulovich *et al*, 1988; Su & Knipe, 1989). It appears to be involved in the switch from early to late gene expression during HSV-1 infection. There is growing evidence that it acts post-transcriptionally (Smith *et al*, 1992; Sandri-Goldin & Mendoza, 1992), probably by selectively stimulating mRNA 3' processing of late gene polyA sites (McLauchlan *et al*, 1992b).

It is interesting that, unlike the IE genes, the E and L genes do not contain any virus-specific promoter sequences. The sequences required for transactivation by IE proteins are the same as those required for *cis* activation in the absence of viral products, and these sequences correspond to the binding sites of cellular transcription factors (Everett, 1984a, b; Eisenberg *et al*, 1985; McKnight & Tijan, 1986; Johnson & Everett, 1986; Homa *et al*, 1988). Moreover, cellular genes, *eg* transfected copies of a rabbit β-globin gene,can be efficiently transactivated by HSV-1 IE proteins (Everett, 1984a, b, 1985). The mechanisms which determine activation of viral genes and concomitant repression of cellular genes during infection are still conjectural.

2.2.4 Synthesis of viral DNA

2.2.4.1 Sites of DNA synthesis

Viral DNA synthesis starts three to five hours after infection and increases to a peak between nine and eleven hours after infection (Rixon *et al*, 1983). Initially, synthesis is localised to discrete sites within the nucleus, but these grow and coalesce as infection proceeds, until the entire nucleus of the infected cell is filled with replicating viral DNA (Rixon *et al*, 1983). The inital sites of viral DNA replication appear to be virus-specific structures induced by infection, and have been called "replication compartments" (Quinlan *et al*, 1984; reviewed by Knipe, 1989). They contain viral replication proteins (Quinlan *et al*, 1984; de Bruyn Kops, 1988; Goodrich *et al*, 1990; Bush *et al*, 1991), and also various host cell replication proteins, which relocate from sites of cellular DNA synthesis (Wilcock & Lane, 1991). Cellular replication protein A (RPA), proliferating cell nuclear antigen (PCNA), polymerase α, DNA ligase I, p53 and retinoblastoma protein are found in the viral replication compartments (Wilcock & Lane, 1991), but which, if any, of these are required to complete the HSV replication apparatus remains to be determined.

2.2.4.2 Replicative intermediates

Within 30 minutes of infection, the linear DNA genome loses its free ends, most probably by circularising (Jean & Ben-Porat, 1976; Jacob *et al*, 1979; Poffenberger & Roizman, 1985). With the onset of DNA synthesis, molecules of more than unit length appear (Jacob & Roizman, 1977; Hirsch *et al*, 1977). At later stages of infection, large, tangled masses of viral DNA can be seen (Jacob & Roizman, 1977; Ben-Porat & Rixon, 1979). Analysis by restriction enzyme digestion shows that this DNA consists of multiple head-to-tail concatemers (Jacob *et al*, 1979; Jongeneel & Bachenheimer, 1981). It is thought that the concatemeric DNA is generated by a rolling-circle mechanism of replication, like that of bacteriophage λ (reviewed by Kornberg & Baker, 1992). Alternatively, it could arise by homologous recombination, which may be linked to replication, as in bacteriophage T4 (Formosa & Alberts, 1986; Mosig, 1987).

2.2.4.3 Nucleotide metabolism enzymes

HSV-1 encodes many of the proteins required to replicate its DNA genome. These include proteins which are directly involved in DNA synthesis, and enzymes involved in nucleotide metabolism. The seven viral proteins which act directly at the replication fork are essential for the viral lytic cycle. (These, and the *cis*-acting origins of replication, are described in Section 4.) In contrast, the enzymes involved in nucleotide metabolism are largely dispensable for viral replication in exponentially growing cultured cells, but may be essential for growth in animals and particularly in non-dividing cells such as neurones. This makes them potential targets for antiviral therapy (reviewed by Weller, 1991).

Thymidine kinase (TK) was one of the first virus-induced enzymes to be identified (Kit & Dubbs, 1963) and has been intensively studied since then. The HSV TK, unlike its cellular counterpart, phosphorylates not only thymidine (dT), but also deoxyuridine (dU), deoxycytidine (dC), thymidilate (dTMP), and a variety of nucleoside analogues, including acyclovir (Jamieson *et al*, 1974; Fyfe *et al*, 1978; Field *et al*, 1980). The specific antiviral action of acyclovir is due largely to its selective phosphorylation by the viral TK (Section 5.1).

Functional TK is not required for virus growth in dividing cells, but is necessary for growth in serum-starved cells (Jamieson *et al*, 1974). A requirement for the enzyme *in vivo* has not been so clearly demonstrated. Several studies have shown that tk^- virus has a reduced ability to establish latent infections in mice (Field & Wildy, 1978; reviewed by Price, 1985), whereas, in the guinea pig or rabbit, tk^- virus can establish reactivatable latent infections (Stanberry *et al*, 1985; Meignier *et al*, 1988). There is other evidence that TK may have a role in reactivation, but not in the establishment of latency (Coen *et al*, 1989a, 1989b; Efstathiou *et al*, 1989).

Ribonucleotide reductase (RR) maintains a pool of DNA precursors by the conversion of ribonucleotides to the corresponding deoxyribonucleotides (reviewed by Thelander & Reichard, 1979). The viral enzyme (Cohen, 1972) is composed of two non-identical subunits of 136kDa and 38kDa (Dutia, 1983; Bacchetti *et al*, 1984; Frame *et al*, 1985), encoded by genes *UL39* and *UL40*, respectively (Preston VG *et al*, 1984; McLauchlan & Clements, 1983). Mutants with lesions in *UL39* or *UL40* are able to grow in cells under normal culture conditions, but not in resting cells, nor in cells at 39.5°C (Goldstein & Weller, 1988a, c; Preston VG *et al*, 1988). Studies in mice show that RR-deficient viruses are much less pathogenic than wt virus, and are unable to establish latent infections (Cameron *et al*, 1988; Jacobson *et*

al, 1989; Brandt *et al*, 1991). Although care must be taken in extrapolating from the mouse model to human infections, since there is evidence that the growth of RR-deficient virus is more severely impaired in mouse than in human cells (Jacobson *et al*, 1989), recent experiments have demonstrated a requirement for the enzyme in primary human corneal fibroblast cultures (Brandt *et al* 1991). These observations have stimulated considerable interest in the potential of herpesvirus RR as an antiviral target, particularly since the viral enzyme can be specifically inhibited by subunit disruption (Section 6.2).

Deoxyuridine triphosphatase (dUTPase) catalyses the hydrolysis of dUTP to dUMP and pyrophosphate (Wohlrab & Francke, 1980). It reduces the intracellular concentration of dUTP and thus prevents the incorporation of uridine into DNA. The dUMP it generates can be converted to dTMP by thymidilate synthase. Mutants in *UL50*, the gene encoding dUTPase (Preston & Fisher, 1984), grow normally in exponentially growing and in serum-starved cells (Fisher & Preston, 1986). In a mouse *in vivo* model, dUTPase-deficient mutants are moderately attenuated for neurovirulence, neuroinvasiveness and reactivation from latency (Pyles *et al*, 1992), indicating a role for dUTPase in pathogenesis.

Uracil-DNA glycosylase is a DNA repair enzyme that removes uracil residues from DNA. These arise by the misincorporation of dUTP during replication, or by the deamination of cytosine, and lead to GC to AT transition mutations. The virus-specific enzyme, first reported by Caradonna & Cheng (1981), is encoded by gene *UL2* (Caradonna *et al*, 1987; Mullaney *et al*, 1989). Disruption of the *UL2* gene does not affect viral growth in cultured cells (Mullaney *et al*, 1989). Its requirement *in vivo* has not been tested, but the high degree of conservation between the homologous genes in VZV and EBV may indicate a role for uracil-DNA glycosylase in pathogenesis.

Alkaline nuclease, encoded by gene *UL12* (Francke & Garrett, 1982; Moss, 1986), is a virus-specific deoxyribonuclease with an unusually high pH optimum (Morrison & Keir, 1968; Hoffman & Cheng, 1978; Francke *et al*, 1978). The precise role of this enzyme in the viral life cycle is unknown. Although initial studies suggested it might be required for DNA synthesis (Moss *et al*, 1979; Francke & Garrett, 1982; Moss, 1986), subsequent experiments showed that it was not, since a *UL12* deletion mutant was able to synthesise wt levels of DNA and late viral proteins (Weller *et al*, 1990). This mutant produced very low numbers of infectious virions, indicating that the alkaline nuclease may be required for processing or packaging of virion DNA (Weller *et al*, 1990). It has been shown to form a specific complex with

the major DNA binding protein, ICP8 (Vaughan *et al*, 1984; Thomas *et al*, 1988, 1992) (Section 4.5), but the significance of this interaction is not known.

2.2.5 Virion structure and assembly

The three structural components of the virion — capsid, tegument and envelope — are each composed of numerous proteins. More than half of the 73 genes of HSV-1 code for proteins which are known or suspected to be present in the virion, or to participate in its assembly. (For reviews see Dargan, 1986; Roizman & Sears, 1990).

2.2.5.1 Capsid and core

The capsid is an icosahedral shell, composed of 162 capsomeres, (Wildy *et al*, 1960; Schrag *et al*, 1989), each of which are hexamers or pentamers of the major capsid protein, VP5 (Steven *et al*, 1986). Three different types of capsids, distinguishable by their appearance in electron micrographs and by their protein composition, are found in the nuclei of virus-infected cells. Type A (empty) capsids lack any internal structure; they are composed of five polypeptides: VP5 (UL19, 155kDa), VP19c (UL38, 53kDa), VP23 (UL18, 36kDa), VP24 (UL26, 24kDa) and VP26 (UL35, 12kDa) (Marsden *et al*, 1978; Morse *et al*, 1978; Rixon *et al*, 1990; Davison *et al*, 1992; McNabb & Courtney, 1992). Type B (intermediate) capsids contain an internal scaffold of two additional polypeptides, VP21 (UL26, 45kDa) and VP22a (UL26.5, 38kDa) (Preston *et al*, 1983; Braun *et al*, 1984; Liu & Roizman, 1991b). Type C (full) capsids contain a core of genomic DNA, and their protein composition is essentially like that of A capsids.

It was thought for many years that the DNA in the core was spooled around a protein plug, since in many electron micrographs it appeared to be a toroid (Furlong *et al*, 1972). However, Booy *et al* (1991) have shown, using cryoelectron microscopy and image reconstruction, that the DNA forms a uniformly dense ball. It appears to be in a liquid-crystalline state, like the encapsidated DNA of the bacteriophages T4 and λ (Lepault *et al*, 1987). The condensed, toroidal shapes seen previously may have resulted from the fixation procedures used for preparing specimens (Puvion-Dutilleul *et al*, 1987).

2.2.5.2 Capsid morphogenesis

The currently favoured model is that the capsid shell assembles with the scaffolding protein and protease, in a process of co-condensation, to form a B capsid. The scaffolding protein is then degraded by proteolysis and is absent from the mature capsid. Analogous mechanisms for head assembly are used by the large DNA phages (Wood & King, 1980). The relationship between the scaffolding protein and the protease is an interesting one, and parallels are found in other herpesviruses (Welch *et al*, 1991). *UL26* and *UL26.5*, which encode the protease and VP22a, respectively, are nested 3' co-terminal genes which utilise the same reading frame (Figure 1b). Thus the VP22a protein represents the C-terminal portion of the protease. VP22a is cleaved by the protease at a specific site near the C-terminus (Liu & Roizman, 1991b, 1992; Preston *et al*, 1992). The protease cleaves itself at the same position, since it shares the same sequence (Liu & Roizman, 1991b), and also at another site in the unique N-terminal portion, generating two fragments: the C-terminal fragment is VP21, and the N-terminal fragment is another capsid protein, VP24. The *ts*1201 mutant, which has a lesion in the N-terminal part of *UL26* affecting the proteolytic activity (Preston *et al*, 1983, 1992), has been particularly useful for elucidating the pathway of capsid morphogenesis. At the non-permissive temperature, capsids of the *ts*1201 virus are arrested at the stage of large-cored B capsids. Following downshift to the permissive temperature, proteolytic processing of VP22a and protease occurs, and this is accompanied by the appearance of small-cored B capsids, C and A capsids (Rixon *et al*, 1988).

It has been suggested that the outer shell might condense around a pre-formed scaffold, on the evidence that VP22a purified from virions can self-assemble into 60nm-diameter structures (Newcomb & Brown, 1991). However, no such structures have been detected in infected cells, and therefore large cored B capsids appear to be the earliest identifiable stage in the pathway of capsid assembly.

2.2.5.3 DNA packaging

Mature C capsids are formed by the removal of VP22a and VP21 from B capsids and the packaging of DNA. These two processes appear to be linked, as shown by experiments with the *ts*1201 mutant, described above, and similar observations with other *ts* mutants deficient in DNA packaging (Sherman & Bachenheimer, 1988). Empty A capsids arise from failed attempts to package DNA. The newly replicated,

concatemeric DNA is cleaved into unit-length genomes as part of the packaging process (Ladin *et al*, 1980; Deiss & Frenkel, 1986). The signals required for cleavage and encapsidation are located in particular regions of the *a* sequence (Stow *et al*, 1983; Varmuza & Smiley, 1985; Deiss *et al*, 1986; Deiss & Frenkel, 1986) (Section 2.1).

Two viral proteins of >250kDa and 140kDa have been identified which bind specifically to sites within the *a* sequence, and which might play a role in the encapsidation process (Chou & Roizman, 1989). A number of gene products may be involved in the packaging process, since *ts* mutations in genes *UL6, UL25, UL28, UL32* and *UL33* affect the formation of full capsids (Addison *et al*, 1984; Sherman & Bachenheimer, 1987; Addison *et al*, 1990; Al-Kobaisi *et al*, 1991). The transcriptional activator, α-TIF (UL48) may also have a function in encapsidation, since a *UL48* deletion mutant is defective in this function (Weinheimer *et al*, 1992).

2.2.5.4 Tegument and envelope

The tegument is the most poorly characterised part of the virion. It is usually described as an "amorphous" layer between the capsid and the envelope. Its complete protein composition has not yet been defined; virion proteins which are not components of the capsid or envelope are assigned to the tegument. The discovery of light (L) particles, which consist of tegument and envelope without a core (Szilágyi & Cunningham, 1991), has facilitated the identification and study of tegument proteins (McLauchlan *et al*, 1992a; McLauchlan & Rixon, 1992). The protein composition of the tegument of L particles and virions is very similar, but not identical (Szilágyi & Cunningham, 1991); in particular, Vmw175 is present in L particles but not in virions (McLauchlan & Rixon, 1992). None of the tegument proteins are absolutely required for infectivity since transfection of cells with viral DNA results in productive infection. However, vhs (UL41) and α-TIF (UL48), play important roles during the early stages of infection (Sections 2.2.2, 2.2.3). A major tegument protein of unknown function is Vmw82/81 (UL47) (McLean *et al*, 1990). The structure of the tegument component of L particles is largely unaffected by the removal of the envelope, which shows that it possesses a degree of inherent structure.(McLauchlan & Rixon, 1992).

The envelope is a cell-derived membrane, modified by the presence of numerous viral proteins and glycoproteins, described in Section 2.2.1. None of the membrane

constituents appears to be essential for the process of virion assembly or envelopment, but some may be essential for viral egress (Section 2.2.5.5).

2.2.5.5 Tegumentation, envelopment and egress

Capsids acquire a tegument and envelope *en route* from the nucleus to the outside of the cell, but there is still controversy over the details of these events. It is clear that capsids leave the nucleus by budding through the inner nuclear membrane into the space between the inner and outer nuclear membranes (Darlington & Moss, 1968; Nii *et al*, 1968). The site of tegumentation of HSV-1 capsids is not known. In HHV-6 it appears to take place within an invagination of the cytoplasm into the nucleus, termed a tegusome, where capsids can be seen surrounded by varying amounts of tegument material (Roffman *et al*, 1990).

There is good evidence that the Golgi complex and Golgi-derived vesicles are involved in virion maturation and transport to the outside of the cell. Disruption of Golgi function by monensin inhibits the processing of envelope glycoproteins, their transport to the cell surface, and the egress of virions (Johnson & Spear, 1982). The virions, which accumulate in the cytoplasm, contain immature forms of the envelope glycoproteins (Johnson & Spear, 1982). Maturation of glycoproteins and egress of virus is similarly hindered in cells with defects in the Golgi glycosyltransferases (Campadelli-Fiume, 1982; Serafini-Cessi, 1983). The UL20 membrane protein may be involved in the egress of virus. In some cell types, a virus deleted in this gene produces mature infectious virions but fails to transport them to the cell surface (Baines *et al*, 1991).

In fully permissive cells, mature virions are released from 18–20 hours after the initial infection.

3. DNA replication: mechanisms and enzymology

The faithful duplication of genetic material is achieved by the co-ordinated action of a large number of enzymes and proteins. Studies of *E. coli*, coliphages, eukaryotic viruses and mammalian cells show that the general mechanism of DNA replication appears to be shared across the whole spectrum of living organisms from bacteria to mammals. It can be broken down into a number of distinct steps (Table 2), although *in vivo* these steps are closely linked and the proteins which mediate them act in concert.

Table 2. The roles of enzymes and proteins at the replication fork

Process	Protein/enzyme involved
Initiation at origin	Origin-binding protein
Strand separation	DNA helicase
Release of topological stress	Topoisomerase
Helix destabilisation	Single-stranded DNA binding protein
RNA priming	Primase
Template-guided addition of nucleotides	Polymerase and processivity factors
Proof-reading	3'–5' exonuclease
Removal of primers	5'–3' exonuclease, RNase H
Joining of Okazaki fragments	DNA ligase

3.1 Initiation of replication

The characteristics of an origin of replication are (i) a recognition site for an origin-binding protein and (ii) a region of AT-rich DNA which is prone to melting (Kornberg & Baker, 1992). The *E. coli* origin, *ori C*, consists of a minimal sequence of 245 bp containing four copies of a 9bp recognition sequence and three

AT-rich stretches of 13 nucleotides (Hirota *et al*, 1981). ATP-activated binding of the dna A protein to *ori C* promotes melting of the adjacent AT-rich region and allows the helicase to enter (Fuller *et al*, 1984; Sekimuzu *et al*, 1987; Bramhill & Kornberg, 1988).

The minimal essential sequence of the SV40 origin is only 64bp and consists of (i) four repeats of a 5bp sequence to which the large T-antigen binds (Jones & Tijan, 1984), (ii) a 15bp palindrome and (iii) 17 AT base pairs (Dean *et al*, 1987). ATP is required for the duplex to melt (Borowiec & Hurwitz, 1988).

3.2 Strand separation

Helicases use the energy of NTP hydrolysis to break the bonds between the strands of duplex DNA and thus open up the replication fork. A helicase is therefore a DNA-dependent ATPase.

Of the ten helicases isolated from *E. coli*, three play a role in replication: dnaB, PriA and Rep (reviewed by Matson & Kaiser-Rogers, 1990). The dnaB protein, a hexamer of 50 kDa subunits, interacts with single-stranded DNA as a dnaB-dnaC complex, and is activated by the ATP-dependent release of dnaC from the complex (Wahle *et al*, 1989). It is responsible for fork movement, and translocates processively 5'–3' along the DNA, tethered to the template of the lagging strand (LeBowitz & McMacken, 1986). PriA (protein n', replication factor Y) translocates 3'–5' and probably threads the lagging strand "backwards" through the replisome. (Figure 4) (Lee & Marians, 1987; Kornberg & Baker, 1992). The gene 4 protein (gp4) of phage T7 and the gene 41 protein (gp41) of phage T4 are helicases with similar properties to dnaB; all three interact with a primase, and activate primer synthesis (Liu & Alberts,1981; Venkatesan *et al*, 1982; Matson *et al*, 1983; LeBowitz & McMacken, 1986).

The multifunctional SV40 large T-antigen is both a helicase and an origin-binding protein (reviewed by Fanning & Knippers, 1992). It is active as two hexamers of the 92kDa protein (Mastrangelo *et al*, 1989) and melts DNA bi-directionally from the origin, translocating with 3'–5' polarity along the leading strand template (Goetz *et al*, 1988; Wiekowski *et al*, 1988). T-antigen associates directly with the catalytic subunit of pol α/primase (Dornreiter *et al*, 1990). Since T-antigen is the only viral protein required for replication, the establishment of an *in vitro* SV40 replication system (Li & Kelly, 1984) has allowed host proteins essential for viral DNA replication to be identified (reviewed by Challberg & Kelly, 1989).

3.3 Release of topological stress

Extensive helicase action requires a topoisomerase to relieve the torsional stress introduced by unwinding: for every 10.5 bases melted by a helicase, the two strands must be untwisted by one turn, otherwise the DNA in front of the replication fork becomes supercoiled, and the helicase stalls (Kornberg & Baker 1992). A topoisomerase introduces a transient break in the phosphodiester backbone through the formation of a covalent protein-DNA intermediate, and allows the DNA strands to pass through each other.

There are two types of topoisomerase found in both prokaryotic and eukaryotic cells: type I enzymes introduce a break in one strand of the duplex in the absence of ATP, while type II enzymes generate a break in both strands and require the energy of ATP hydrolysis (reviewed by Wang, 1985). Either type of enzyme can relax supercoiled DNA, but only topoisomerase II can unlink catenated DNA molecules.

E. coli DNA gyrase, a type II topoisomerase, is required at several stages of replication:

i) To maintain a negatively supercoiled template for assembly of the initiation complex and opening of the duplex at the origin (Funnell *et al*, 1987).
ii) To provide the swivel for the progress of the replication fork (Baker *et al*, 1986).
iii) To decatenate and supercoil the daughter molecules during the terminal stages of genomic replication (Marians, 1987).

Mammalian topoisomerase II is both functionally and structurally related to *E. coli* gyrase, whereas eukaryotic and bacterial type I topoisomerases are unrelated. In the SV40 *in vitro* replication system, either type of topoisomerase can provide the swivel for fork movement, but topoisomerase II is essential for decatenation of daughter molecules at the end of replication (Yang *et al*, 1987). Experiments in yeast suggest the same division of labour (DiNardo *et al*, 1984; Brill *et al*, 1987).

3.4. Helix destabilisation

The movement of a replication fork requires the concerted action of a helicase, a topoisomerase and a single-stranded DNA binding protein (SSB). SSBs bind tightly and co-operatively to single-stranded DNA, with no sequence specificity, coating an extensive region of the DNA. They convert duplex DNA to single strands at a temperature far below the T_m for the DNA, without requiring the energy of ATP

hydrolysis. They also facilitate the converse process of helix renaturation, by melting out regions of secondary structure (Kornberg & Baker, 1992).

E. coli SSB is an extremely stable tetramer of 18.9kDa subunits which increases the fidelity and processivity of *E. coli* pol II and pol III holoenzymes (reviewed by Chase & Williams, 1986; Meyer & Laine, 1990). The rate of replication of phage T4 depends on the amount of available gp 32, the phage-encoded SSB (Chase & Williams 1986).

Replication protein A (RPA) (formerly called RF-A) is the human SSB, made up of three tightly associated polypeptides of 70kDa, 32–34kDa and 11kDa (Fairman & Stillman, 1988). The 70kDa subunit binds to single-stranded DNA (Kenny *et al*, 1990). The 34kDa subunit is phosphorylated in a cell cycle-regulated manner, but its specific function is unknown (Din *et al*, 1990). RPA is required for the initiation and elongation stages of SV40 replication *in vitro* (Wold *et al*, 1989; Fairman & Stillman, 1988).

3.5 Priming

No DNA polymerase is able to start a DNA chain, and therefore a priming mechanism is necessary (Kornberg & Baker, 1992). RNA priming is the predominant mechanism, but alternatives exist in some organisms.

The *E. coli* primase (dnaG product) associates with the dnaB helicase and at least five other proteins to form a 'primosome' that translocates along the DNA, fuelled by ATP hydrolysis (Weiner *et al*, 1976; Arai & Kornberg, 1981). The activity of primase alone is distributive, and is therefore stimulated by association with the pre-primosome, which is processive (Lee & Marians, 1989).

Phage T7 helicase-primase is made up of two distinct polypeptides which are both encoded by gene 4 (Dunn & Studier, 1983). The larger form of the gene 4 protein has primase activity and acts distributively, while the smaller form is a highly processive helicase (Nakai & Richardson, 1988). The phage T4 primosome is composed of two proteins, a DNA helicase (gp 41) and an RNA primase (gp 61) (Nossal, 1980; Liu & Alberts, 1981). They are both fully active only when complexed (Cha & Alberts, 1989; Richardson & Nossal, 1989).

Eukaryotic primase activity is tightly associated with pol α. The primase activity resides in the two smallest subunits (48- and 58kDa) of the heterotetrameric enzyme

(Plevani *et al,* 1985) (Section 3.6). The characteristic length of eukaryotic primers is 8–12 nucleotides (Singh *et al,* 1986; Campbell, 1986). The fidelity of primer synthesis by pol α/primase is low, with an error rate of 1/300 to 1/500 (Zhang & Grosse, 1990).

The exclusive 5'–3' polarity of synthesis by DNA polymerases means that the leading (continuous) strand needs to be primed once only, at the origin, whereas the lagging (discontinuous) strand must be primed repeatedly. Extension by DNA polymerase of primers on the lagging strand yields Okazaki fragments (Okazaki *et al,* 1968; Sugino *et al,* 1972), which are 100-200 bases in eukaryotes and 1000-2000 bases in prokaryotes (Kornberg & Baker,1992).

Alternative strategies to RNA priming exist in certain viruses: in phage φX174 a 3'-OH terminus is generated by endonucleolytic cleavage of the genome (Ikeda *et al,* 1976); adenoviruses and certain *B. subtilis* phages employ a terminal protein in which a 3'-OH group of a Ser, Thr or Arg provides the primer terminus (Arnberg & Arwert, 1976; Rekosh *et al,* 1977; Challberg *et al,* 1980).

3.6 Template-dependent polymerisation of DNA

Polymerisation of DNA has 2 basic features:

i) Each deoxynucleotide added is selected by base-pair matching to the template strand.

ii) A phosphodiester bond is formed between the 5'-phosphate group of the deoxynucleotide and the 3'-hydroxyl group of the primer terminus. The reaction consists of a nucleophilic attack by the 3'-OHgroupof the primer terminus on the α-phosphate of the dNTP, resulting in linkage of the nucleotide to the primer by a 3'–5'-phosphodiester bond and the concomitant release of inorganic pyrophosphate. Chain growth is exclusively 5'–3', and opposite in polarity to the template. Polymerase activity invariably requires a divalent metal ion, preferably Mg^{2+} (Kornberg & Baker, 1992).

DNA polymerase activity may be assayed on various DNA templates: (i) "activated" dsDNA from a source such as calf thymus or salmon sperm (dsDNA is made into an active template by treatment with endo- and exonucleases to generate short single-stranded regions and 3'-OH termini); (ii) a synthetic homopolymer such as poly(dA), multiply primed with oligo(dT); (iii) a defined genomic sequence such as single-stranded circular phage DNA, singly primed with a synthetic oligonucleotide.

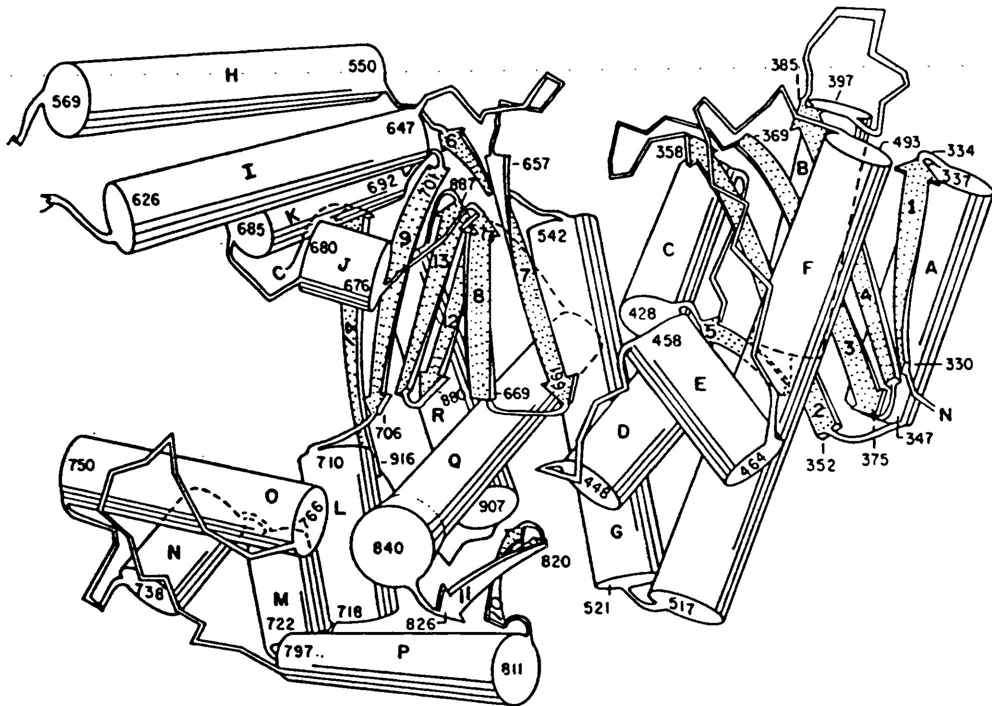


Figure 2. The tertiary structure of the Klenow fragment of *E. coli* Pol I. α-helices are represented by tubes (lettered) and β-sheet by arrows (numbered). The break between helices H and I shows the position of an approximately 50-residue disordered region. The division between the large and small domains is the loop between helices F and G. Reproduced from Ollis *et al*, 1985.

The activity of a polymerase on these different types of template provides a useful characterisation.

DNA Pol I of *E. coli* was the first polymerase to be recognised (Kornberg *et al,* 1956; Lehman *et al,* 1958). It contains three enzyme activities in one polypeptide chain: the polymerase, a 3'–5' exonuclease and a 5'–3' exonuclease (reviewed by Joyce *et al,* 1986). The enzyme is readily cleaved by proteases into a small N-terminal domain containing the 5'–3' exonuclease activity, and a large C-terminal fragment — the Klenow fragment — which contains the polymerase and 3'–5' exonuclease activities (Klenow & Henningsen, 1970; Setlow *et al,* 1972). The crystal structure of the Klenow fragment (Ollis *et al,* 1985) shows two domains, corresponding to the two enzyme activities (Figure 2):
i) A large domain folded into a deep cleft, which is the binding site for the template-primer (reviewed by Steitz & Joyce, 1987), and a proximal dNTP binding site (Polesky *et al,* 1990).
ii) A small domain which contains the 3'–5' exonuclease activity, the binding site for NMP (Ollis *et al,* 1985), and two binding sites for divalent metal cations (Derbyshire *et al,* 1988).

The *in vivo* function of Pol I is probably repair rather than replication (Steitz & Joyce, 1987), since it has low processivity and contains 5'–3' exonuclease activity which excises base-paired nucleotides ahead of the growing chain (Klett *et al,* 1968).

The replicative polymerase of *E. coli* is DNA Pol III (Wickner *et al,* 1973). The holoenzyme is a large complex (>1MDa) of at least ten subunits (α, ε, θ, τ, γ, δ, δ', χ, ψ and β) (reviewed by McHenry, 1988). The smallest active subassembly is a heterotrimer of α, ε and θ (Kornberg & Gefter, 1971). It possesses the polymerase and proofreading activities of the holoenzyme, which reside in the α and ε subunits respectively, but lacks the processivity of the holoenzyme. Processivity is conferred by the remaining subunits (Section 3.7), which interact with the core to form an asymmetric hetero-oligomer (Section 3.8).

There are five known eukaryotic polymerases,α, β, γ, δ and ε, which have been isolated from a variety of sources; the genes which encode them have been identified in budding yeast and in mammalian cells (reviewed by Wang, 1991).

Pol α, the first eukaryotic polymerase to be identified, is a heterotetramer composed of a 165kDa DNA polymerase subunit, two subunits of 58kDa and 48kDa which make up the primase, and a 70kDa subunit of unknown function (reviewed by Lehman & Kaguni, 1989; Wang, 1991). It is the only eukaryotic polymerase with a

tightly associated primase activity and should therefore be designated pol α/primase. It is essential for replication and for many years was considered to be the only replicative polymerase. Unlike the other replicative polymerases, it appears to have no proofreading activity (Section 3.9). The moderate processivity of pol α (Wang, 1991) is modulated by several accessory proteins (Section 3.7).

Pol β, a protein of 39kDa, is the smallest of the polymerases. It has a high affinity for nicked duplex DNA and is considered to be a repair enzyme, but may also have a role in filling in the gaps between Okazaki fragments after primer excision (Section 3.10) (reviewed by Wang, 1991; Kornberg & Baker, 1992).

The catalytic subunit of pol γ is a protein of 140 kDa and is found only in mitochondria, although it is encoded by a nuclear gene (reviewed by Wang, 1991; Kornberg & Baker, 1992).

Pol δ is composed of a 125kDa catalytic subunit and an associated 48kDa subunit of unknown function (Lee *et al*, 1984; Boulet *et al*, 1989; Yang *et al*, 1992). It has an intrinsic 3'–5' exonuclease activity (Bauer *et al*, 1988), and is highly processive in conjunction with proliferating cell nuclear antigen (PCNA) (Prelich *et al*, 1987b). It is one of the principal replicative polymerases, originally identified as an essential factor for the complete replication *in vitro* of SV40 DNA (Prelich *et al*, 1987a, b; Weinberg & Kelly, 1989).

The catalytic subunit of pol ε is a protein of 255kDa and is the largest of the polymerases. Like pol δ, it has a tighly linked 3'–5' exonuclease activity (Syvaoja & Linn, 1989), and the two enzymes have some immunological and structural similarities (Wong *et al*, 1989), but are encoded by distinct genes (Morrison *et al*, 1990). Pol ε was initially characterised as a PCNA-independent processive polymerase, and thus distinguished from pol δ (Nishida *et al*, 1988; So & Downey, 1988). More recently, it has been recognised that PCNA has a stimulatory effect on pol ε (Yoder & Burgers, 1991; Lee *et al*, 1991). In budding yeast, pol ε has been identified as a third essential replicative polymerase (Morrison *et al*, 1990).

3.7 Processivity

A non-processive or *distributive* polymerase dissociates from the DNA template-primer after the addition of one nucleotide, while a *processive* enzyme remains associated with the template and translocates along it for repeated additions. The processivity of a polymerase is defined as the number of nucleotides incorporated

into the template-primer before polymerase and template-primer dissociate. It can be determined on a defined template such as oligo(dT)/poly(dA), or singly-primed, phage ssDNA. All the replicative polymerases have a degree of intrinsic processivity, which is greatly increased by accessory factors (Kornberg & Baker, 1992).

The processivity of *E. coli* Pol III increases with the complexity of the subassembly (Table 3; from McHenry, 1991; Kornberg & Baker, 1992).

Table 3. The processivity of subassemblies of E.coli Polymerase III

Designation	Subunit composition	Processivity	Stimulatory factors
Pol III core	α, ε, θ	10	None
Pol III'	α, ε, θ, τ	60	Spermidine
Pol III*	α, ε, θ, τ, γ, δ, δ', χ, ψ	200	SSB
Pol III holoenzyme	α, ε, θ, τ, γ, δ, δ', χ, ψ, β	$>10^5$	SSB

In the presence of the τ subunit the Pol III core forms a dimeric complex designated Pol III', which is several times more processive than the core (Fay *et al*, 1982). Pol III*, which lacks only the β subunit of the holoenzyme, is still more processive, and its activity can be further stimulated by SSB (Fay *et al*, 1982; Meyer & Laine, 1990). The addition of the β subunit to Pol III* restores completely Pol III holoenzyme activity and increases processivity by over 1000-fold (Mok & Marians, 1987).

The 40.6kDa β subunit exists as a dimer in solution (Johanson & McHenry, 1980) and is not demonstrably a DNA-binding protein (Stukenberg *et al*, 1991). The highly processive holoenzyme can be reconstituted in two distinct stages (O'Donnell, 1987; Maki & Kornberg, 1988). In the first stage, the γ complex (γδδ'χψ) hydrolyzes ATP and transfers the β subunit onto the primed template, forming a preinitiation complex. In the second stage, the core polymerase interacts with the β subunit to form the processive polymerase. The γ complex is needed only in catalytic amounts and can be subsequently removed, leaving β tightly bound to the DNA (Stukenberg *et al*, 1991). The β subunit slides freely along duplex DNA and binds directly to the α subunit of the core, tethering it to the template (Stukenberg *et al*, 1991).

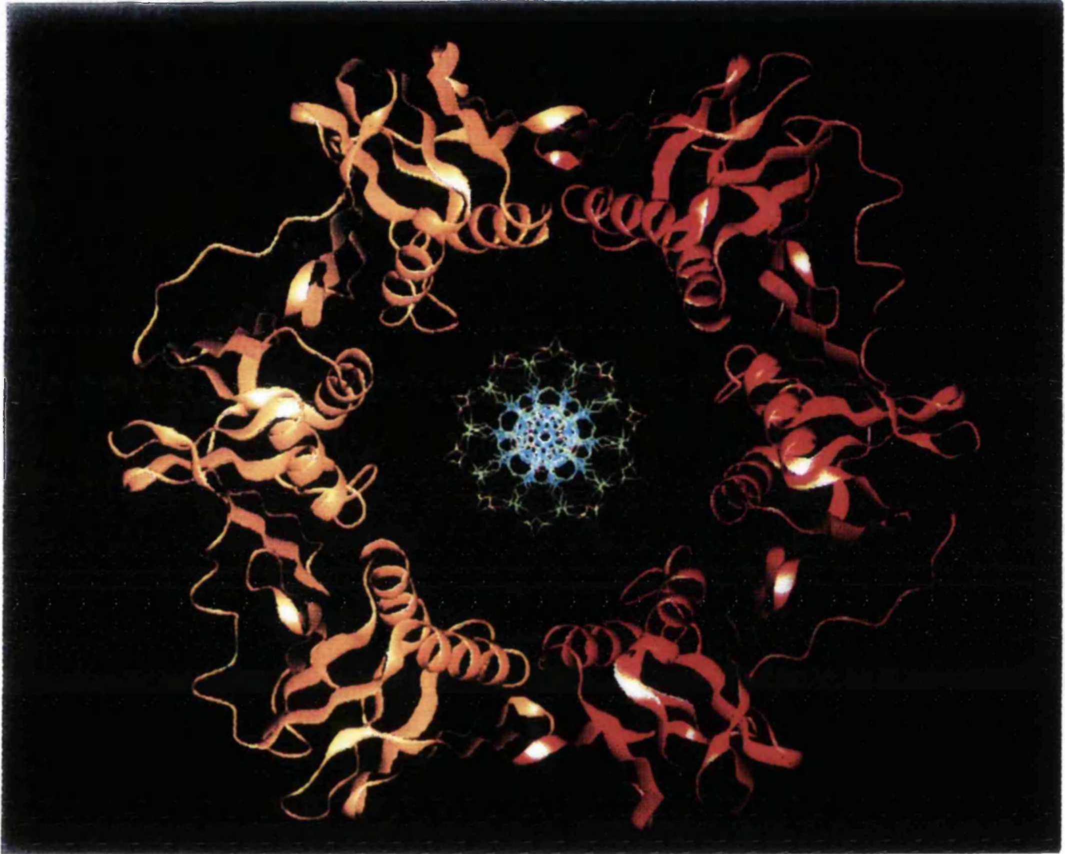


Figure 3. The tertiary structure of the β-clamp of *E. coli* DNA polymerase III holoenzyme. A ribbon representation of the polypeptide chain of a β subunit dimer is shown, looking down the 2-fold axis of the ring. The α-helices are shown as spirals and the β-sheets as flat ribbons. The two monomers are coloured yellow and red. A standard model of B-form DNA is shown in the geometric centre of the ring. Reproduced from Kong, 1992.

The structure of the β dimer has recently been determined by X-ray crystallography to be a closed ring that can encircle duplex DNA (Kong *et al*, 1992). Each monomer consists of three structural domains of identical topology and very similar three-dimensional structure. The head-to-tail dimerisation of two monomers results in a highly symmetrical, rigid toroid with 12 α helices lining its inner surface and six β sheets forming a star-shaped outer scaffold (Figure 3). At the dimer interface, the hydrogen bonded β sheet continues across the molecular boundary. The interface is further stabilized by the formation of a small hydrophobic core and six ion pairs in which all the positively charged residues are contributed by one monomer and all the negatively charged residues by the other. ATP hydrolysis by the γ complex may provide the energy to break these interactions in order to open the ring and close it again around the DNA (Kong *et al*, 1992).

Several features indicate that the protein is designed to wrap around duplex DNA without sequence-specific interactions. The hole in the middle of the ring (diameter ~35Å), is large enough to accommodate A or B form DNA (diameter ~25Å). The α helices lining the ring are perpendicular to the major and minor grooves of the DNA helix. The electrostatic charge of the protein is distributed in such a way that the surface of the hole has strongly positive electrostatic potential, which would stabilize it around the DNA, while the outer edge and faces of the ring are negatively charged (Kong *et al*, 1992).

While the crystal structure reveals much about the interaction of β with DNA, it reveals little about its interactions with the α subunit and the other components of the Pol III* subassembly. One face of the ring is less strongly charged than the other and bears six prominent loops, which may reflect asymmetric protein-protein interactions (Kong *et al*, 1992).

There is evidence that cycling of the holoenzyme from one template to another is achieved by the dissociation of Pol III* from the β clamp, followed by reassociation with a fresh β at the new primer terminus — a process which is not yet understood but which probably involves interactions between the auxiliary factors. The association between Pol III core and β is stabilized by the presence of ATP (Lasken & Kornberg, 1987) and by the τ subunit (Maki & Kornberg, 1988). The rate of holoenzyme cycling to a new template depends on the concentration of the β-DNA preinitiation complex (O'Donnell, 1987). At low concentrations of β, primer utilization by polymerase on the lagging strand is less efficient than at higher concentrations (Zechner *et al*, 1992). It is estimated that β exists in a 10 to 20-fold

excess over Pol III* in the cell (Kornberg & Baker, 1992), which would support this model.

It is intriguing that there is very little (<16%) sequence identity between the domains of the β subunit (Ohmori *et al*, 1984; Kong *et al*, 1992). One consequence of this sequence plasticity may be a lack of significant amino acid homology between β and other processivity factors, even if they were of similar structure. An alignment of the sequences of the three domains of β shows that the hydrophobic core of each domain forms a weakly conserved region, in as much as only uncharged residues are found at buried positions. A plausible alignment between β and PCNA and phage T4 gp 45 can be made by matching the buried residues in β with uncharged residues in the other sequences (Kong *et al*, 1992).

The phage T4 holoenzyme consists of a simpler assembly of proteins which have analogous functions to those of the host Pol III. The catalytic gp 43 core polymerase associates with three accessory factors: the products of genes 44, 45 and 62 (reviewed by Cha & Alberts, 1988; Young *et al*, 1992). The gp 44/62 complex is a DNA-dependent ATPase and gp 45 is a processivity factor which stimulates it (Piperno & Alberts, 1978; Mace & Alberts, 1984a). These accessory proteins assemble with the polymerase on a primer-template junction (Munn & Alberts, 1991). ATP hydrolysis is required for the accessory proteins to form a "sliding clamp" that tethers the polymerase to the template (Piperno & Alberts, 1978; Mace & Alberts, 1984b). The gene 32 protein stabilizes the binding of the accessory proteins to a hairpin template DNA, both by smoothing out secondary structures in the single stranded portion of the template and by direct interactions with the gp 44/62 complex (Mace & Alberts, 1984a) and with polymerase (Huberman *et al*, 1971; Formosa *et al*, 1983; Burke *et al*, 1980). T4 is a particularly attractive model system, since the polymerase catalytic subunit has significant sequence similarity to human pol α and to HSV-1 Pol (Section 4.5).

The processivity of phage T7 polymerase (gp 5) is achieved simply by association with the a host protein,thioredoxin, in a 1:1 complex (Huber *et al*, 1987). Formation of the complex requires neither ATP nor DNA template-primer (reviewed by Marians, 1992). Thioredoxin stabilises the binding of gp 5 to a primer-template by 20- to 80-fold and increases its processivity by 1000-fold (Tabor *et al*, 1987; Huber *et al*, 1987).

Processive synthesis by the eukaryotic pol δ requires the cooperative action of proliferating cell nuclear antigen (PCNA/cyclin), replication factor C (RFC) and

replication protein A (RPA), and hydrolysis of ATP (reviewed by Wang, 1991; Kornberg & Baker, 1992).

PCNA is the functional equivalent of the *E. coli* Pol III β subunit and of the phage T4 gp 45 (Tan *et al*, 1986; Tsurimoto & Stillman, 1990). It is a cell cycle-regulated protein of 36kDa (Mathews *et al*, 1984), which was found to be essential for SV40 replication *in vitro*, and this led to the initial recognition of pol δ as a replicative polymerase (Prelich *et al*, 1987a&b). PCNA is a highly conserved protein (Matsumoto *et al*, 1987; Bauer & Burgers, 1990), and has some homology with T4 gp 45 (Tsurimoto & Stillman, 1990), but none with *E. coli* β, although a tentative alignment of non-polar residues based on the structure of β has been made (Kong *et al*, 1992) (Section 3.6).

RPA is the human SSB (Section 3.4). In addition to its role in unwinding DNA, RPA can stimulate the activity of polymerases α and δ (Kenny *et al*, 1989, 1990). RPA alone can increase the processivity of pol α (Tsurimoto & Stillman, 1989b; 1991b), but it has no effect on pol δ in the absence of PCNA and RFC (Tsurimoto & Stillman, 1989b).

RFC is a complex of three polypeptides of 140kDa, 41 kDa and 37kDa, and is essential for leading strand synthesis during SV40 replication *in vitro* (Tsurimoto & Stillman, 1989a). It is a DNA-dependent ATPase and binds to DNA at a primer-template junction (Yoder & Burgers, 1991; Tsurimoto & Stillman, 1991a). The 41kDa subunit binds ATP, while the 140kDa subunit binds primer-template DNA (Tsurimoto & Stillman, 1991a). The ATPase activity is stimulated by PCNA and by RPA (Tsurimoto & Stillman, 1990). RFC and PCNA form an ATP-dependent primer recognition complex at the primer-template junction in the presence of RPA (Tsurimoto & Stillman, 1991a&b). Interaction of pol δ with the complex initiates processive DNA synthesis. This replicative system has strong functional similarities to the T4 holoenzyme, in which the gp 44/62 complex fulfils a role analogous to that of RFC (Tsurimoto & Stillman, 1990).

A heterodimeric factor which specifically stimulates pol α/primase on single-stranded DNA templates but has no effect on any other polymerases has been reported (Goulian *et al*, 1990). Alpha accessory factor (AAF) increases the affinity of pol α/primase for the DNA template, increasing its processivity from ~15 to ~115 nucleotides, and allowing it to traverse double-stranded DNA regions (Goulian & Heard, 1990).

3.8 Coordination of leading and lagging strand replication

The replicative polymerases of *E. coli*, phage T4 and eukaryotic cells all share a number of structural and funtional features that allow them to fulfil the asymmetric requirements at a replication fork, where the lagging strand polymerase needs to dissociate from the template on completion of each Okazaki fragment and reassociate with the next primer, while the leading strand polymerase needs to be highly processive. The idea that coordinated replication of the leading and lagging strands is achieved by the physical association of the leading and lagging strand polymerases — the asymmetric dimer hypothesis (Sinha *et al*, 1980) — has won widespread acceptance and is supported by studies of the subassemblies of *E. coli* Pol III, T4 polymerase, SV40 replication *in vitro* and other systems (reviewed by Thömmes & Hübscher, 1990; Kornberg & Baker, 1992; Marians, 1992).

The core of *E. coli* Pol III can be isolated as a dimer (McHenry, 1982), which suggests that the functional holoenzyme is also dimeric (Johanson & McHenry,1984; Maki *et al*, 1988). The τ and γ subunits are similar but distinct, and may be responsible for structural and functional asymmetry in a dimeric holoenzyme (Maki *et al*, 1988; O'Donnell & Studwell, 1990; Wu *et al*, 1992). It has been demonstrated using holoenzyme reconstituted from individually purified subunits that the lagging strand polymerase remains associated with the replication fork, presumably via protein-protein interactions with the leading strand assembly (Wu *et al*, 1992). Figure 4 shows the dimeric polymerase and other replication proteins associated in a "replisome" at the replication fork, with the lagging strand DNA being translocated through the replisome in the opposite direction to the movement of the replication fork. The replisome should be compared to a sewing machine through which the DNA is threaded, rather than a train travelling along the template (Kornberg & Baker, 1992).

Phage T4 holoenzyme is effective in the synthesis of both strands. The holoenzyme, made up of gp 43 (pol) and gp 44/62 and 45 (accessory proteins), requires only the gp 41 (DNA helicase) for leading strand synthesis. Addition of gp 61 (primase) allows lagging strand synthesis, and the two assemblies become coupled in the presence of gp 32 (SSB) (Cha & Alberts, 1988, 1989).

A dimeric, asymmetric replisome could be achieved in eukaryotic cells by various combinations of multi-subunit replicative polymerases. The simplest model, supported by studies of SV40 replication *in vitro*, is that the highly processive pol δ synthesises the leading strand, and the less processive, primase-containing pol α

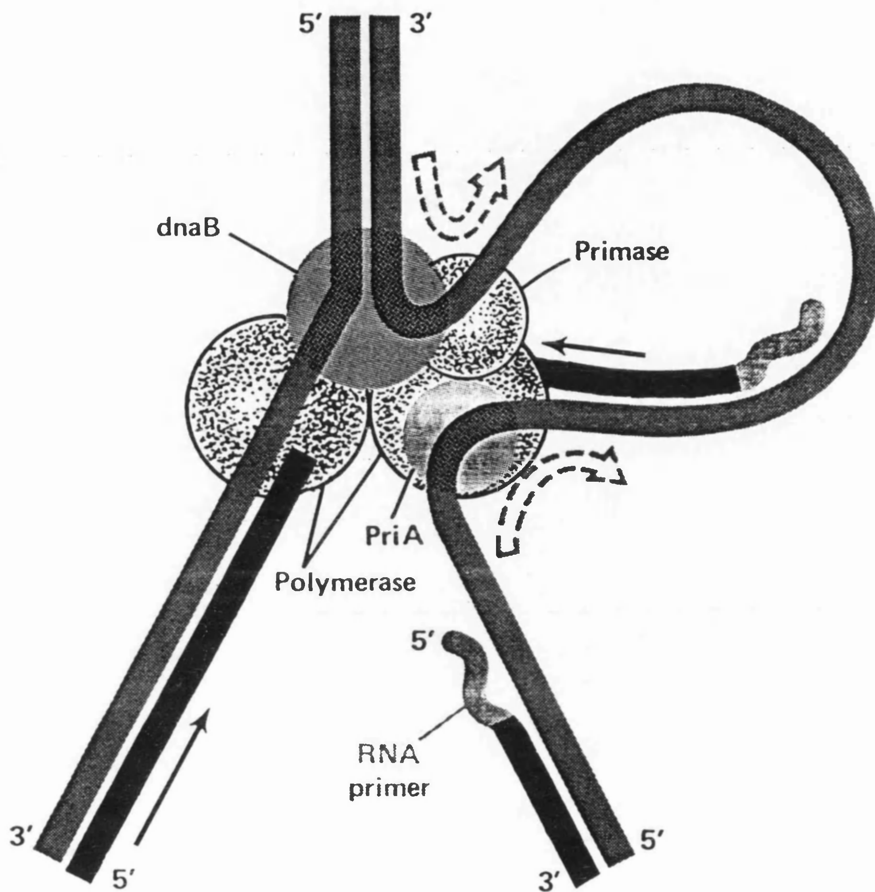


Figure 4. A hypothetical model of the *E. coli* replication fork, showing the association of leading and lagging strand polymerases. Primase is associated with the lagging strand polymerase. The dnaB and PriA helicases are translocated in opposite directions along the DNA template, as indicated by the dashed open arrows. The SSB associated with the single stranded DNA is not shown. Reproduced from Kornberg & Baker, 1992.

synthesises the lagging strand (Lee *et al*, 1989; Weinberg & Kelly, 1989). A more complex model proposes that pol ε is the leading strand polymerase, while polymerases α and δ are responsible for lagging strand replication; each Okazaki fragment is initiated by pol α and elongated by δ (Morrison *et al*, 1990). The roles of δ and ε in this tri-polymerase system may be reversed (Burgers, 1991). The latter model, in which pol ε completes Okazaki fragment synthesis, is supported by the finding that calf pol ε functionally interacts with a 5'–3' exonuclease (Siegal *et al*, 1992) which may be involved in the removal of primers.

3.9 Proof-reading

The ability to proof-read and excise mismatched nucleotides at the primer terminus is an essential function of the replication machinery, and is performed by a 3'–5' exonuclease (reviewed by Kunkel, 1988; Kornberg & Baker, 1992). The 3'–5' exonuclease of *E. coli* Pol I resides in a distinct domain of the catalytic subunit (Ollis *et al*, 1985), while the 3'–5' exonuclease activity of *E. coli* Pol III is in the ε subunit (Scheuermann & Echols, 1984), which associates with the α catalytic subunit to form the core polymerase. Phage T4 gp43 has both polymerase and 3'–5' exonuclease activities (Bessman *et al*, 1974).

The eukaryotic polymerases γ, δ and ε each have an associated 3'–5' exonuclease activity (reviewed by Wang, 1991). The pol δ exonuclease resides in the catalytic subunit (Simon *et al*, 1991; Boulet *et al*, 1989; Yang *et al*, 1992), and sequence comparison suggests that the same is true for pol ε (Morrison *et al*, 1990). It has not been clearly resolved whether or not pol α possesses a proofreading 3'–5' exonuclease activity. A proofreading activity was uncovered in the catalytic subunit of *D. melanogaster* pol α by separating it from the 70kDa subunit (Cotterill *et al*, 1987). The catalytic subunit of yeast pol α was shown to have exonuclease activity in one study (Brooke *et al*, 1991) but not in another (Kunkel *et al*, 1989).

The distance between the polymerase and exonuclease sites of *E. coli* Pol I (25–30Å) is such that the primer terminus must shuttle between the two active sites to allow excision of a mispaired base (Ollis *et al*, 1985; Joyce, 1989). It may be that this relocation is accomplished by the dissociation and reassociation of polymerase and template-primer (Joyce, 1989). Experiments with the catalytic subunit of T4 polymerase, however, show that proofreading is intrinsically processive, *ie* the enzyme can excise one or two mismatched bases and then continue synthesis on the same template, without dissociation (Reddy *et al*, 1992).

3.10 Removal of primers, ligation of Okazaki fragments

The final steps of DNA replication are the removal of RNA primers, filling of the gaps, and ligation of the Okazaki fragments. The first of these is mediated by ribonuclease H and 5'–3' exonuclease, the second by DNA polymerase and the third by DNA ligase. RNase H is a ubiquitous enzyme which can specifically hydrolyze RNA from RNA/DNA hybrids (reviewed by Crouch & Dirksen, 1982). *E. coli* RNase H cannot remove the ribonucleotide esterified to DNA, and so this requires an additional 5'–3' exonuclease, such as *E. coli* Pol I (Westergaard *et al,* 1973). Similarly, in the *in vitro* SV40 replication system, covalently closed circular DNA can only be synthesised if an RNase H, a double strand-specific 5'–3' exonuclease and a DNA ligase are present (Ishimi *et al,* 1988). Unlike a 3'–5' exonuclease, which acts specifically on unpaired bases, a 5'–3' exonuclease excises base-paired nucleotides, and can excise short oligonucleotides of up to 10 bases (Crouch & Dirksen, 1982).

Pol α alone is sufficient to fill the gaps left after primer excision in the SV40 *in vitro* system (Ishimi *et al,* 1988). This does not however exclude a role for polymerases β, δ or ε *in vivo* (Kunkel, 1992).

In the last formal step of DNA replication, a DNA ligase mediates the formation of a phosphodiester bond between the 3'-hydroxyl and 5'-phosphate termini of adjacent base-paired nucleotides (Kornberg & Baker, 1992).

4. HSV-1 DNA replication

A fairly recent milestone in this field was the identification of the complete set of viral genes that are required for HSV-1 DNA replication. Mark Challberg showed that restriction fragments of HSV-1 DNA cloned into plasmid vectors can support the replication of HSV-1 origin-containing plasmids when co-transfected into cells (Challberg, 1986). This led to the identification of seven genes (*UL5, UL8, UL9, UL29, UL30, UL42* and *UL52*) which were necessary and sufficient for origin-dependent DNA replication (Wu *et al*, 1988; McGeoch *et al*, 1988a). Analyses of various mutant viruses had shown that all lesions which rendered the virus unable to synthesise DNA mapped to one of these seven genes (Purifoy & Powell, 1981; Chartrand *et al*, 1980; Conley *et al*, 1981; Weller *et al*, 1983; Littler *et al*, 1983; Weller *et al*, 1987; Carmichael *et al*, 1988; Goldstein & Weller, 1988b; Zhu & Weller, 1988; Marchetti *et al*, 1988), corroborating the results of the transient replication assay.

Genes *UL30* and *UL29* respectively encode the viral polymerase and the major DNA-binding protein, which had been previously identified by biochemical and genetic analyses (Sections 4.4 and 4.5). The functions of the remaining gene products have since been identified as a helicase-primase (UL5, UL8, UL52), a polymerase accessory protein (UL42), and an origin-binding protein (UL9) (reviewed by Challberg & Kelly, 1989; Challberg, 1991). The seven replication proteins are described in detail below, with particular attention given to the polymerase and UL42 proteins.

Two functions essential for replication which appear not to be encoded by HSV-1 are a topoisomerase (Section 3.3) and a DNA ligase (Section 3.10). These may be supplied by the host cell in both the mammalian (Challberg, 1986) and insect (Stow, 1992) *in vivo* replication systems. The topoisomerase activity induced upon infection of cells by HSV-1 (Leary & Francke, 1984) or HSV-2 (Bapat *et al*, 1987) is indistinguishable from cellular topoisomerases (Bapat *et al*, 1987). Inhibitors of topoisomerase block both HSV-1 replication and reactivation from latency (Spivac *et al*, 1987). Several host cell replication proteins, including DNA ligase I, are seen to

re-locate to sites of viral DNA synthesis in infected cells (Wilcock & Lane, 1991; Section 2.2.4.1). However, an *in vitro* system that supports origin-dependent HSV-1 DNA replication has not yet been established, and cellular factors essential for viral DNA replication remain to be determined.

4.1 Origins of replication

The HSV-1 genome contains three *cis*-acting replication origins: ori_L, located near the centre of U_L, and two copies of ori_S, one in IR_S and one in TR_S. These origin sequences were originally identified by their presence as tandem repeats in defective HSV DNA species, which arise when virus is repeatedly passaged at high multiplicity (Frenkel *et al*, 1975; Kaerner *et al*, 1979; Vlazny & Frenkel, 1981; Spaete & Frenkel, 1982). Plasmids containing the origin sequences were amplified in cells infected with HSV (Stow, 1982), allowing the precise localisation of ori_S (Stow & McMonagle, 1983). A similar analysis of ori_L was hampered by its inherent instability in bacterial plasmid vectors, and was achieved by the sequencing of non-cloned viral DNA fragments (Weller *et al*, 1985; Quinn & McGeoch, 1985).

The sequences of ori_S and ori_L are closely related. Both contain an extensive palindrome, the centre of which consists exclusively of AT base pairs. The ori_S palindrome has 21 residues in each arm and is imperfect; the ori_L sequence is a perfect inverted repeat of 72 residues. The biological significance of multiple origins is unclear. Mutant viruses lacking ori_L or one copy of ori_S have been isolated and have no obvious growth defect either in cultured cells or in animals (Longnecker & Roizman, 1986; Polvino-Bodnar *et al*, 1987). Both origins are located between divergently transcribed genes: ori_S between *IE-3* and *IE-4* (IR_S) or *IE-5* (TR_S), and ori_L between *UL30* and *UL29* (Figure 1b). The significance, if any, of their proximity to genes encoding an important transcriptional activator and the major replication proteins is not understood.

Due to the instability of ori_L, most analyses of origin function have been done on ori_S. Mutational analyses have shown that both the central AT-rich sequence (Stow, 1985) and the palindromic arms (Lockshon & Galloway, 1988; Weir & Stow, 1990) are essential for function, while flanking transcriptional regulatory elements enhance the efficiency of replication (Wong & Schaffer, 1991). The palindromic arms of both origins contain binding sites for the origin-binding protein (Elias *et al*, 1986; Elias & Lehman, 1988), product of the *UL9* gene (Olivo *et al*, 1988; Weir *et al*, 1989) (Section 4.2).

4.2 UL9 (Origin-binding protein)

Prior to the identification of *UL9* as one of the seven genes required for HSV-1 origin-dependent replication (Wu *et al*, 1988; McGeoch *et al*, 1988a), a protein of apparent M_r 83 000, which bound to the viral origin of replication, had been isolated from HSV-1-infected cells (Elias *et al*, 1986; Elias & Lehman, 1988). This origin-binding protein was then shown to be the product of the *UL9* gene (Olivo *et al*, 1988; Weir *et al*, 1989).

The UL9 protein binds to two high affinity sites, designated site I and site II, which are present in opposite orientations on either side of the central AT domain of the origin sequence (Elias & Lehman, 1988; Olivo *et al*, 1988; Weir *et al*, 1989). The protein specifically recognises an 11bp sequence, CGTTCGCACT (Elias *et al*, 1990; Koff & Tegtmeyer, 1988). In *ori*$_S$ (but not in *ori*$_L$) the sequence of site II differs from that of site I in two positions, which results in a 10-fold lower binding affinity for UL9 (Elias & Lehman, 1988; Elias *et al*, 1990). There is also a third site of much lower affinity, which differs from site I by a single base pair. All three UL9 binding sites are necessary for optimum DNA replication in transient assays (Lockshon & Galloway, 1988; Hernandez *et al*, 1991; Weir & Stow, 1990). The binding of UL9 to sites I and II is cooperative (Elias *et al*, 1990, 1992), and results in looping and distortion of the DNA (Koff *et al*, 1991; Fierer & Challberg, 1992). Purified UL9 exists predominantly as a dimer in solution (Fierer & Challberg, 1992), which may reflect a capacity to form a higher-order nucleoprotein complex when bound to sites I and II at the origin. The N-terminal region of the protein is required for dimerisation (Elias *et al*, 1992).

Like other origin-binding proteins, UL9 also has helicase and DNA-dependent ATPase activities (Bruckner *et al*, 1991; Fierer & Challberg, 1992). Assays using short oligonucleotides annealed to single-stranded DNA show that the helicase translocates 3'–5' along the DNA, and that unwinding is dependent upon hydrolysis of ATP, dATP, CTP or dCTP (Fierer & Challberg, 1992).

Sequence comparisons and site-directed mutagenesis show that the helicase activity resides in the N-terminal two-thirds of the protein. The N-terminal 400 amino acid residues of UL9 contain six conserved motifs which characterise a superfamily (SF2) of helicases (Gorbalenya *et al*, 1989). Two of the motifs define a nucleotide binding domain (Walker *et al*, 1982), while the functions of the other regions are unknown. The nucleotide binding domain and at least three of the other regions are necessary for UL9 function, as tested by the ability of mutant UL9 protein expressed from a transfected plasmid to complement DNA replication by a UL9-deficient virus

(Martinez *et al*, 1992). The functions of the UL9 protein have also been studied in a replication assay in which insect cells are transfected with an HSV-1 origin-containing plasmid and superinfected with recombinant baculoviruses expressing the 7 DNA replication proteins (Stow, 1992). The nucleotide binding site (and by implication helicase activity) is important for origin-dependent DNA replication (Stow *et al*, 1993). However, there is no direct evidence that binding of UL9 to the origin brings about local strand separation (Fierer & Challberg, 1992).

The DNA-binding domain of UL9 has been localised to the C-terminal one-third of the protein (Weir *et al*, 1989; Deb & Deb, 1991). Analysis of UL9 proteins altered in this region shows that DNA-binding is essential for replication (Arbuckle & Stow, 1993; Stow *et al*, 1993). The DNA-binding domain alone has a dominant inhibitory effect on replication, showing that DNA binding alone is insufficient for UL9 function and that the helicase activity is also required (Stow, 1992; Stow *et al*, 1993).
No direct protein-protein interactions between UL9 and any of the other replication proteins have yet been described.

4.3 UL5, UL8, UL52 (Helicase-primase)

A novel DNA helicase, initially identified as a DNA-dependent ATPase activity, was found to be induced in HSV-1 infected cells (Crute *et al*, 1988). A complex of three polypeptides of 120kDa, 97kDa and 70kDa was found to be responsible for this activity (Crute *et al*, 1989). Rabbit antisera were used to identify the components of the complex as the products of the UL52, UL5 and UL8 genes, respectively (Crute *et al*, 1989). The M_r of the complex was determined to be 263 000, indicating that it is a heterotrimer of the UL5, UL8 and UL52 proteins (Crute & Lehman, 1991).

The helicase can utilise ATP or GTP as a cofactor. When assayed on a model substrate, it preferentially unwinds DNA duplexes that have a 3'-OH single-stranded tail, suggesting that it translocates in a 5'–3' direction, *ie* bound to the lagging strand template at the replication fork (Crute *et al*, 1989). In the presence of ICP8 (Section 4.5) and ATP, it can fully unwind a nicked 2.3kbp circular plasmid DNA, at a rate of 2 nucleotides/s (Crute & Lehman, 1991); in the absence of ICP8, only limited unwinding occurs. The complex contains two separate nucleoside triphosphatase sites; one catalyses the hydrolysis of both ATP and GTP, the other hydrolyses only ATP. These two sites are activated by independent DNA effector sites that support the helicase activity of the enzyme (Crute *et al*, 1991).

In addition to helicase activity, the enzyme was found to have a tightly associated DNA primase, which synthesises oligoribonucleotide primers 10–12 nucleotides in length on an M13 template, or 6–8 nucleotides in length on a poly(dT) template (Crute & Lehman, 1991). A similar coupling of helicase and primase activities is found in bacteriophages T4 and T7 (Section 3.5).

A complex with identical physical and enzymatic properties assembles in insect cells triply-infected with baculoviruses expressing the *UL5, UL8* and *UL52* genes (Dodson *et al*, 1989). The contribution of the individual subunits to the activity of the complex is only partially understood. Co-expression of the UL5 and UL52 proteins results in a stable sub-assembly with all the enzymatic activities — DNA-dependent ATPase, DNA-dependent GTPase, DNA helicase and DNA primase — of the trimeric complex (Calder & Stow, 1990; Dodson & Lehman, 1991). The *UL5* gene is highly conserved among the herpesviruses, and contains a consensus ATP-binding site (McGeoch *et al* 1988a). It also contains six sequence motifs found in all the members of the SF1 superfamily of DNA and RNA helicases (Hodgman, 1988; Gorbalenya *et al,* 1988a, 1988b). [SF1 and SF2 are distinct but distantly related superfamilies of helicases (Gorbalenya *et al,* 1989)]. It has been demonstrated by mutational analysis that all six conserved motifs are essential for helicase function, but not for binding of UL5 to the other subunits (Zhu & Weller, 1992). Therefore, helicase activity can be assigned with some confidence to the UL5 subunit, and, by default, primase activity to the UL52 subunit. The isolated UL5 protein is inactive, a behaviour which resembles that of the phage T4 gene 41 helicase, which is fully active only when complexed with the gene 61 primase (Section 3.5). The difficulty of obtaining the isolated UL52 protein in a soluble form has hampered its study.

Only the intact trimeric complex is transported into the nucleus of an infected cell. In the absence of any one of the subunits, the other two remain in the cytoplasm (Calder *et al,* 1992). Therefore, a function of UL8 may be to facilitate entry of the enzymatically active UL5–UL52 complex into the nucleus, but this is unlikely to be its only role. Another possible role for the UL8 subunit has been demonstrated in a "lagging strand synthesis assay", in which DNA polymerase elongates primers synthesised by the helicase-primase complex on an M13 ssDNA template (Sherman *et al,* 1992). In the absence of UL8, the efficiency of primer utilisation by either HSV or *E. coli* polymerase was greatly decreased. On this basis, it was proposed that UL8 stabilises the association of nascent primers with the DNA template (Sherman *et al,* 1992).

4.4 UL30 and UL42 (DNA polymerase)

The HSV-1 DNA polymerase is currently considered to be a heterodimer of the UL30 and UL42 polypeptides. The part played by the UL42 protein in the holoenzyme complex is still not fully understood (Section 4.4.3).

4.4.1 UL30 (DNA polymerase catalytic subunit)

The induction of a DNA polymerase by HSV was first recognised by Keir and Gold (1963). The viral enzyme could be distinguished from its cellular counterpart immunologically (Bolden *et al*, 1975; Keir *et al*, 1966a), by its greater sensitivity to phosphonoacetic acid (PAA) (Mao *et al*, 1975; Hay & Subak-Sharpe, 1976) and by a higher optimum salt concentration (Weissbach *et al*, 1973; Keir *et al*, 1966b).

Purification of the virus-induced activity showed the catalytic subunit of the polymerase (Pol) to be a polypeptide of ~140kDa (Powell & Purifoy, 1977). The associated 3'–5' exonuclease activity (Knopf, 1979; O'Donnell *et al*, 1987) has been shown to reside in the same polypeptide (Marcy *et al*, 1990). The genetic locus of Pol was identified by mapping *ts* and PAA-resistant mutants (Honess & Watson, 1977; Chartrand *et al*, 1979, 1980). Sequencing of the *pol* gene — later designated *UL30* (McGeoch *et al*, 1988b) — showed Pol to be a protein of 1235 amino acid residues, with a predicted M_r of 136 272 (Quinn & McGeoch, 1985).

The HSV-1 Pol belongs to a conserved family of replicative polymerases (reviewed by Wang *et al*, 1989; Wang, 1991). A central region of about 400 amino acid residues contains six motifs conserved between the herpesviruses, vaccinia virus, budding yeast polymerases α, δ and ε, and human polymerases α and δ (reviewed by Wang, 1991). Five of these motifs are also conserved in adenovirus and phage T4 polymerases, and three are found in the polymerases of *B. subtilis* phage φ29 and *E.coli* phage PRD1.

The conserved regions are designated I to VI, in order of decreasing similarity (Wong *et al*, 1988). Their relative position in the polypeptide is unaltered throughout the family of α-like polymerases (Figure 5). An additional short but highly conserved motif between regions I and V has been designated region VII (Hwang *et al*, 1992). The catalytic subunits of yeast polymerases δ and ε (Boulet *et al*, 1989; Morrison *et al*, 1990), human pol δ (Yang *et al*, 1992) and the herpesvirus polymerases (Baer *et al*, 1984; Quinn & McGeoch, 1985; Davison & Scott, 1986; Kouzarides *et al*, 1987; Telford *et al*, 1992) form a subfamily with more extensive homology. This is reflected in their greater enzymatic similarity: they all have an

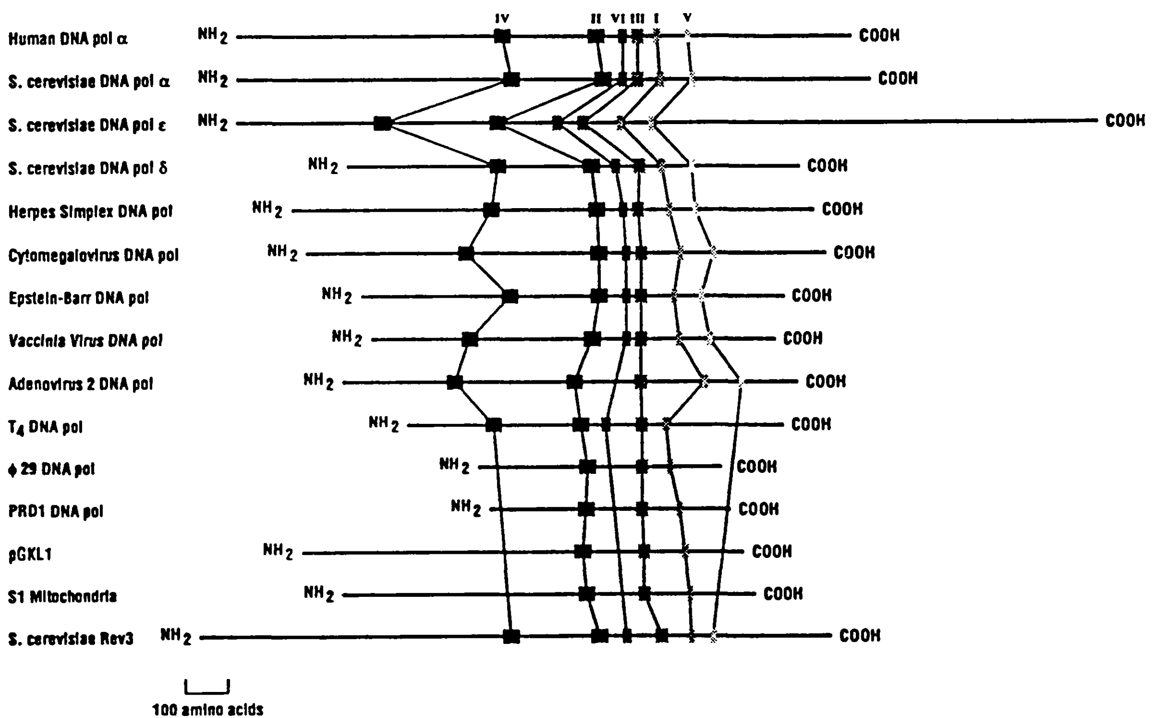


Figure 5. The conserved regions of α-like DNA polymerases, designated I–VI in order of decreasing homology. The boxes represent the consensus sequences of each region. Similar regions of each DNA polymerase polypeptide are joined by vertical lines, showing the conservation of their linear spatial arrangement. Reproduced from Wang, 1991.

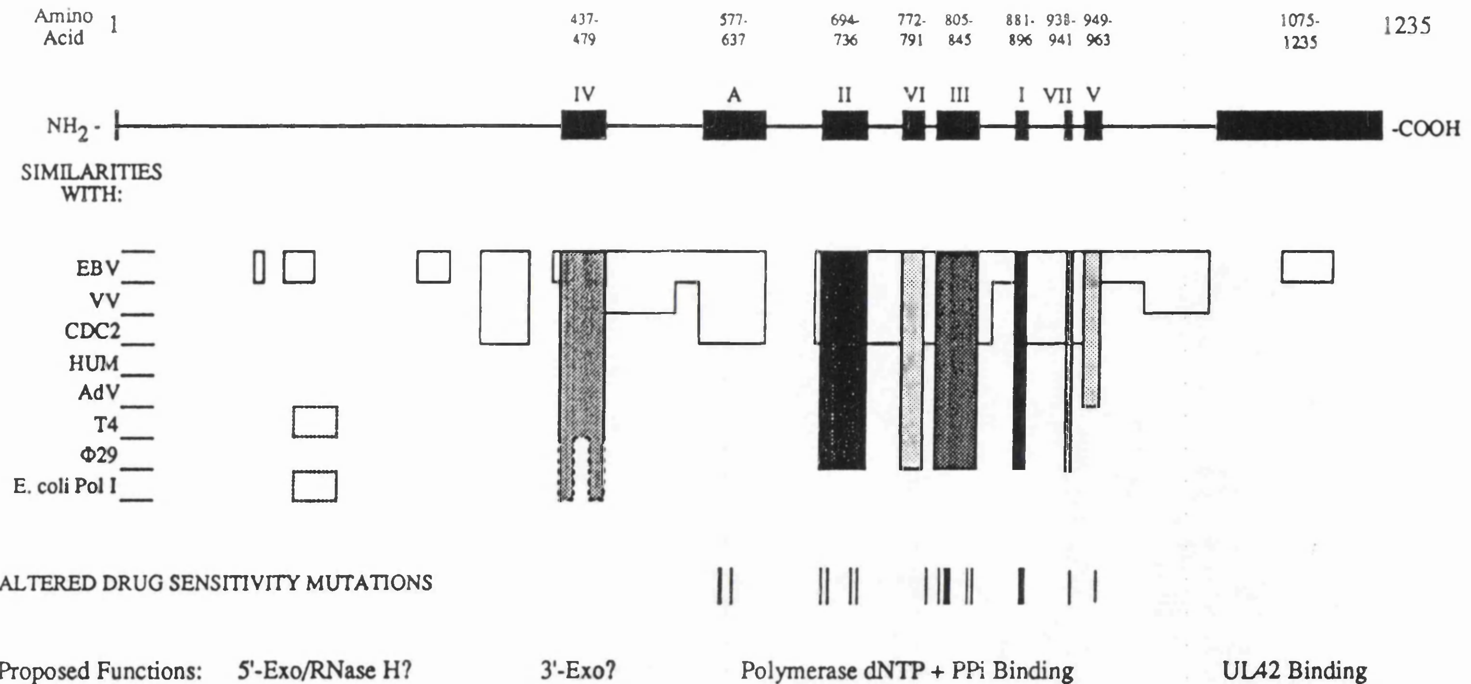


Figure 6. HSV Pol: location of functional sites and regions of sequence similarity. The top line is a schematic of the Pol polypeptide showing, as filled boxes, the conserved regions I–VII, region A, and the UL42 binding domain. The numbers above the line refer to amino acid residues, while the vertical lines below show the positions of mutations that confer resistance or hypersensitivity to polymerase substrate analogues. The degree of shading below boxes I–VII roughly indicates the degree of sequence similarity found with the polymerases listed on the left: Epstein Barr virus; VV, vaccinia virus; CDC2, yeast DNA polymerase δ; HUM, human DNA polymerase α; AdV, adenovirus; T4; ϕ29; *E. coli* Pol I. Reproduced from Coen, 1992.

associated 3'–5' exonuclease activity, proposed, on the basis of sequence comparisons with T4 polymerase and *E.coli* Pol I, to reside within region IV and adjoining sequences (Reha-Krantz, 1988; Bernad *et al*, 1989).

HSV-1 Pol is a major target of anti-viral compounds (Section 5). Many drug-resistant or hypersensitive mutants of HSV-1 have been isolated that contain mutations in the *pol* gene. Sequence analyses show that most of these contain point mutations within regions I, II, III, V and VII, and a region designated A, which is shared only among herpesvirus polymerases and polymerases δ and ε (Gibbs *et al*, 1988; Hwang *et al*, 1992; reviewed by Coen, 1992). It is therefore reasonable to suppose that these highly conserved regions are directly involved in substrate and drug interactions.

Unlike the host polymerases δ and ε, HSV Pol has an associated 5'–3' exonuclease with ribonuclease H activity (Crute & Lehman, 1989; Hernandez & Lehman, 1990; Marcy *et al*, 1990), proposed to reside in the N-terminal region of the polypeptide (Bernad *et al*, 1989; Haffey *et al*, 1990). The HSV-1 Pol therefore has the same three enzyme activities as *E.coli* Pol I. It has been suggested that HSV-1 Pol may have a three-domain structure resembling that of *E.coli* Pol I, although the sequences show no gross homology (Haffey *et al*, 1990). Opposed to this model is the finding that point mutations in the proposed 3'–5' exonuclease site severely impair polymerase activity (Gibbs *et al*, 1991).

Deletion studies have shown that the extreme C- and N-termini of the Pol polypeptide are not required for catalytic activity (Haffey *et al*, 1990), but that C-terminal sequences are involved in the interaction of Pol with its accessory protein, the *UL42* gene product (Section 4.4.3). The conserved regions and functional domains of HSV-1 Pol are shown in Figure 6.

4.4.2 UL42 (DNA polymerase accessory protein)

HSV-1 infection of cells induces the synthesis of a number of proteins that bind to DNA, as demonstrated by their ability to bind to dsDNA-cellulose. One of the major protein species thus identified is a 65kDa protein (BP4, Bayliss *et al*, 1975), which was designated $65K_{DBP}$ (Marsden *et al*, 1987). It is heavily phosphorylated (Marsden *et al*, 1987), and displays strong, non-cooperative and sequence-independent binding to DNA in the absence of any other protein (Gallo *et al*, 1988; Gottlieb *et al*, 1990).

UL42 was identified as the gene encoding $65K_{DBP}$ by using a $65K_{DBP}$-specific MAb to immunoprecipitate the products of *in vitro* translation from mRNA selected with oligonucleotide sequences from three candidate ORFs (Marsden *et al*, 1987; Parris *et al*, 1988). The assignment was confirmed by showing that antisera produced against two peptides from the C-terminal region of UL42 reacted with $65K_{DBP}$ (Parris *et al*, 1988). This map location corresponds to that of the HSV-2 DNA-binding protein ICSP34,35 (Powell & Courtney, 1975; Vaughan *et al*, 1985). Analysis of a series of HSV-1/HSV-2 intertypic recombinants, using type-specific MAbs reactive with $65K_{DBP}$ or ICSP34,35, showed that the two proteins were equivalent (Gallo *et al*, 1988).

The first suggestion that this DNA-binding protein might form part of a functional complex with polymerase came from the observation that ICSP34,35 consistently co-purified with DNA polymerase from HSV-2 infected cells (Vaughan *et al*, 1985). An interaction between HSV-1 DNA Pol and $65K_{DBP}$ was subsequently recognised (Gallo *et al*, 1988). The two proteins copurified through successive chromatography steps and were separated only by glycerol gradient centrifugation. Other DNA-binding proteins, including the viral ICP8 and alkaline nuclease, and cellular topoisomerase, also co-eluted with the polymerase from DEAE-Sephacel and Blue Sepharose, but were separable by chromatography on Mono-Q. The most tenacious and specific association was between Pol and $65K_{DBP}$, demonstrated by the retention of Pol on an immunoaffinity column containing MAb 6898, which is specific for $65K_{DBP}$ (Gallo *et al*, 1988).

The evidence that UL42 is essential for viral replication comes not only from the results of transient replication assays (Wu *et al*, 1988; Stow, 1992) but also from the finding that lesions in the *UL42* gene result in viruses that are defective for DNA replication (Marchetti *et al*, 1988; Johnson *et al*, 1991).

4.4.3 The functional interaction between Pol and UL42

The study of the Pol–UL42 interaction has been stimulated by the idea that it may prove to be a novel target for anti-viral drugs. The extreme difficulty of obtaining a pure preparation of Pol, uncontaminated with UL42, was overcome by cloning the *UL30* and *UL42* genes and and obtaining small amounts of protein by *in vitro* transcription and translation (Digard & Coen, 1990), or overproducing the proteins in the heterologous baculovirus expression system (Crute & Lehman, 1989; Marcy *et al*, 1990; Gottlieb *et al*, 1990; Stow, 1992) This has allowed the study of their interaction.

A comparison of the hydrodynamic properties of isolated Pol and UL42, with those of the Pol–UL42 complex purified from infected cells, indicated that the complex is most probably a heterodimer, of M_r 181 000–190 000 (Crute & Lehman, 1989; Gottlieb *et al*, 1990). A complex with identical physical and enzymatic properties is assembled in insect cells co-infected with baculoviruses expressing the two proteins, showing that the formation of a functional complex does not require any other viral proteins (Gottlieb *et al*, 1990).

Several groups have studied the role of the UL42 subunit in the heterodimer. Gallo *et al* (1989) purified polymerase from HSV-1-infected cells and separated the two subunits by centrifugation through a glycerol gradient, a step which resulted in an apparent loss of much of the polymerase activity. When the UL42 protein was added back to the Pol, polymerase activity (assayed on an activated DNA template in high salt conditions) was stimulated 4–10-fold. A similar stimulation was seen on mixing Pol and UL42 that had been produced by *in vitro* transcription and translation of cloned *UL30* and *UL42* (Gallo *et al*,1989).

Gottlieb *et al* (1990) did not find that UL42 stimulated Pol activity on an activated DNA template, but demonstrated instead that it increased the processivity of Pol on a defined template. When assayed on singly-primed M13 ssDNA, the Pol–UL42 complex utilised fewer primers and synthesised much longer products than did Pol alone (Gottlieb *et al*, 1990). Hernandez & Lehman (1990) reported that the UL42 protein increased both the processivity and the activity of Pol, but only if the ssDNA template was coated with ICP8 (Section 4.5). A stimulation by *E. coli* SSB of Pol-UL42 activity and processivity on ϕX ssDNA has also been reported (O'Donnell *et al*, 1987).

In a template challenge experiment, the presence of UL42 reduced the rate of dissociation of the polymerase from the DNA (Gottlieb *et al*, 1990). Gel shift and filter binding assays showed that the affinity of Pol for a synthetic hairpin oligonucleotide increased five to ten-fold in the presence of UL42 (Gottlieb & Challberg, unpublished). These data indicate that the role of UL42 in the holoenzyme may be to "clamp" the polymerase to the DNA template, in a manner analogous to other processivity factors, such as the *E.coli* β-clamp, or PCNA (Section 3.7).

Some of the discrepancies described above arise from the use of different assay conditions in different laboratories. The effect of UL42 on Pol is critically dependent on the nature and concentration of the salt present in the assay: The apparent K_m for Pol–UL42 for the DNA template remains unchanged over a range of salt

concentrations, whereas the K_m for Pol alone increases with salt concentration (Hart & Boehme, 1992). Concomitantly, at low salt concentrations, the V_{max} for Pol alone is greater than that for Pol–UL42. This complex kinetic relationship is manifest as an *inhibition* by UL42 of polymerase activity in low salt conditions, an *activation* in high salt (as used by Gallo *et al,* 1989), and *little effect* at intermediate concentrations of salt (as used by Gottlieb *et al,* 1990 and Marcy *et al,* 1990) (Hart & Boehme, 1992). The discrepancies in the observed effects of ICP8 on processivity remain to be resolved.

There is as yet no published quantitation of the processivity of HSV-1 Pol (in terms of the number of nucleotides incorporated per binding event) in the presence and absence of UL42. The HSV-1 polymerase is usually described as being exceptionally slow (0.25 nucleotides/s), but highly processive, on the basis of experiments carried out with Pol–UL42 before the functional contribution of UL42 to the holoenzyme was recognised (O'Donnell *et al,* 1987). [The addition of *E. coli* SSB increased the rate 20-fold (O'Donnell *et al,* 1987), which is still an order of magnitude below the observed *in vivo* rate of PRV replication fork movement of 50 nucleotides/s (Ben-Porat *et al,* 1977).] The predominant products synthesised by Pol on an M13 ssDNA template in the presence of UL42 are about 10 times longer than those synthesised in its absence (Gottlieb *et al,* 1990).

4.4.4 Regions involved in the Pol–UL42 interaction

The UL42-binding domain of Pol has been investigated by testing the ability of mutated Pol proteins to interact with UL42. Co-immunoprecipitation of the proteins translated *in vitro* in rabbit reticulocyte lysates showed that the C-terminal 227 amino acid residues of Pol are necessary and sufficient for specific interaction with UL42 (Digard & Coen, 1990). Three subsequent studies have extended this finding to show that amino acid residues close to or at the the very C-terminus of this region are essential for the interaction, but different conclusions were reached regarding exactly which residues are important (Diggard *et al,* 1993a; Tenney *et al,* 1993; Stow, 1993).

A detailed analysis of deletion and insertion mutants showed that only ~35 amino acid residues at the extreme C-terminus of Pol (residues 1201–1235) are necessary for interaction with UL42 as detected by co-immunoprecipitation (Digard *et al,* 1993a). Moreover, the processivity of a Pol frameshift mutant in which the C-terminal 27 residues were replaced with an unrelated sequence could not be

stimulated by UL42, and mutants lacking the C-terminal 19 or 27 residues were unable to complement a *pol* null mutant in a transfection assay (Digard *et al,* 1993a). However, the correlation is not absolute: a mutant with a four-amino acid in-frame insertion at residue 1216 was unaffected in its ability to complement a *pol* null mutant, but was severely impaired in its ability to interact with UL42 as detected by co-immunoprecipitation (Digard *et al,* 1993a).

An analysis of several truncated Pol proteins expressed in yeast cells yielded substantially different results, indicating that the region essential for interaction with UL42 is not at the extreme C-terminus of Pol, but between residues 1176 and 1195 (Tenney *et al,* 1993). C-terminal truncations of 19 and of 40 residues did not affect the ability of Pol to be stimulated by UL42, but removal of 59 residues abolished UL42 stimulation. The Pol lacking 19 residues interacted with UL42 in a co-immunoprecipitation assay, but the mutants lacking 40 and 59 residues were not co-immunoprecipitated with UL42 (Tenney *et al,* 1993).

A third study (Stow, 1993) showed that Pol lacking the extreme C-terminal 27 amino acid residues was unable to support origin-dependent DNA replication in insect cells in the presence of the other six replication proteins. Full-length but not truncated Pol could be co-immunoprecipitated with UL42 from extracts of co-infected cells. The baseline catalytic activity of the truncated Pol was unimpaired, but, unlike the full-length control, it could not be stimulated by UL42 (Stow, 1993).

Both Digard *et al* and Tenney *et al* observed a lack of binding to UL42 of one or two Pol mutants which exhibited a functional interaction. This apparent contradiction may be due to such mutants forming complexes of reduced affinity, which are sufficient for a functional interaction, but do not survive the relatively high stringency of the co-immunoprecipitation assay. The major contradiction in these three studies, however, is that Digard *et al* and Stow find that the presence of the extreme C-terminus of Pol is essential for function, whereas Tenney *et al* find that it is not. This controversy will need to be resolved.

An important implication of the finding that a functional interaction between Pol and UL42 is essential for viral origin-dependent DNA replication and for virus growth (Stow, 1993; Digard *et al,* 1993) is that the Pol–UL42 interface is indeed a potential target for antiviral drugs.

It is interesting that the site of interaction of polymerase δ with PCNA is proposed to be located in the C-terminus of the protein (Yang *et al,* 1992), in a region where the

sequence of polymerase δ diverges completely from that of its herpesvirus counterparts.

The region of the UL42 protein involved in the interaction has not been identified, and is the subject of intense investigation by several groups. Digard *et al* have analysed an extensive set of mutant UL42 proteins (obtained by *in vitro* transcription and translation of cloned *UL42*), testing (i) their ability to associate with Pol, (ii) to increase Pol processivity and (iii) to bind to DNA. They found that the N-terminal 340 amino acid residues of UL42 were sufficient for all three activities, whereas deletions of more than 35 residues anywhere within this region abolished all three activities (Digard *et al*, 1993b). In contrast, single in-frame insertions of 4–5 residues at any position had no effect, with the exception of an insertion at residue 160, which destroyed Pol-binding and the capacity to increase processivity, but did not affect DNA-binding. This mutant was unable to complement a *UL42* null virus *in vivo* (Digard *et al*, 1993b). Thus the main conclusions of this analysis are (i) that the DNA-binding and Pol-binding properties of UL42 are separable; (ii) that stimulation of processivity requires a direct interaction with Pol; (iii) that this interaction is essential *in vivo*. However, a more precise delineation of the functional domains of UL42 remains elusive. It seems that the N-terminal two-thirds of the protein constitute a single domain which cannot be subdivided without loss of structure and concomitant loss of both Pol- and DNA-binding properties.

The amino acid sequence of UL42 (McGeoch *et al*, 1988a) contains no recognised functional motifs and few interpretable features. In contrast to Pol, which is highly conserved among the herpesviruses, UL42 is the least conserved of the seven essential replication proteins. It has significant homology with its counterparts in PRV (29%) (Alberto Epstein, personal communication), in EHV-1 (27.8%; gene 18) (Telford *et al*, 1992), and in VZV (23.5%; gene 16) (Davison & Scott, 1986). The processivity factors of HCMV (ICP36) and HHV-6 (p41), share 41% sequence identity with each other, but have no significant sequence homology with UL42 (Chee *et al*, 1990; Chang & Balachandran, 1991a, 1991b; Ertl & Powell, 1992). There is a positional homologue in EBV (BMRF1), with barely significant homology (21%) (Baer *et al*, 1984).

An alignment of the PRV, EHV-1 and VZV homologues with UL42 shows that, in the N-terminal two-thirds of the protein, there are several regions where conservative substitutions predominate (Figure 7). At the C-terminus there is no homology, and the proteins differ both in length and in sequence. This division of UL42 into a relatively conserved N-terminal domain and a unique C-terminal domain is reflected

```
1                                                                                                        100
HSV   .......... .MTDSPGGVA PASPVEDASD ASLGQPEEGA PCQVVLQGAE LNGILQAFAP LRTSLLDSLL VMGDRGILIH NTIFGEQVFL PLEHSQFSRY
VZV   .......... MDLRSRTDDA LDMELHAGFD APE....... IARAVLTEKT LTGLISSISP LVNRLRDSIL IFSDEGLIIH CSLETEQLYI PIPANMFDQY
EHV   .......MAL PRAMRPGGSH PNNFLFNTLP VIDNPVERQR .AMAVFERES LRDAFEMLTP IAPSLKNAFL IFNEDGLLIH TSVGGEQVYI PIQTNNMESY
PRV   MSLFDDGLED LDRHPTHAHH PAQVIHDGPF VLEDGEPLQR TGMLVLSDEH LEHARAAIAP LAAHLAHAFL VFSEAGLLVH ASVRGEQVYV TLAPDQFSTF

101                                                                                                      200
HSV   RW RGPTAAF LSLVDQKRSL LSVFR.ANQY PDLRRVELAI TGQAPFRTLV QRIW...... .....TTTSD GEAVELASET LMKRELTSFV VLVPQGTPDV
VZV   NW.TGPRMVV LAATEGRSSL IDAFRHTKDP STPTRLYFKF TGQPPERSII QTMVWQR... .....PGDCG PDDQVQCYKQ VVKRELACYT MMFPNLTPDI
EHV   SWKKAPPAVF LANVDGRRGL LDAFK.AKTQ TNVSKVVFEI ENYSPSRILT QTVFSARDQT EEDTEMGSDA EGATQTVSSR LVKHEFNNYA LMLPTRQPDV
PRV   VW.SGPQAVF LGNVDGSGGV LDALK.VDRR RTVFNVTFEV YGAFPARLLT RRAYFA.... ....DAGVLA TGPGSPSVAC VYKHEFNDYC IMLPSRAPDV

201                                                                                                      300
HSV   QLRLTRPQLT KVLNATGADS ATPTTFELGV NGKFSVFTTS TCVTFAAR.E EGVSSSTSTQ VQILSNALTK AGQAAANAKT VYGENTHRTF SVVVD.DCSM
VZV   SICLKRDQFT RLQRLL.KTF GFTTCFILTA TDMYIQTAGG GFISFNVSLD INGSKPTPYN ...LIRSITN SKRILNNV.. VYGSGSMREF GVLLETHSGF
EHV   SMSLSKAQLN KIL.GVCKQA GDPITFQCLF DDTLQVRSGD RQVVFSVDYQ HADNCGVESS SSLLEKMPMK TKKSAPEP.. IRGISGRRLF TLVLDEDTNF
PRV   SLTLSRPQVA KLA.AVAKGA AAGTTFALAR GLDFSVSSSA GVVTFPARDR DG........ TAVLERASQR .....RQG.. VDAVGATEPF AMTLETAHGL

301                                                                                                      400
HSV   RAVLRRLQV. .GGGT.LKFF LTTPVPSLCV TATGPNAVSA VFLLKPQKIC LDWLGHSQGS PSAGSSASRA SGSEPTDSQD SASDAVSHGD PEDLDGAARA
VZV   RSAVQNLKLT RDETCYINFY LALTNSPMVG LYIQRSAPVH SFFYATFLSP KDLKEKLTSM QLFANMESVK DEPPLKKRRN LLTKRNEKNT GNKMGGKLPE
EHV   KQLIQKLKLK NAGAV.LNFF LDPDSIPMIG LSTKQPFSVM MFFMCSYPTQ PCQVGFSP.. .AAFSSTPMG AGVKRRASEE EESDQPPK.. ..........
PRV   LTLLQRLRAG NAELT.FNFF TTPRQAPLFS VTTCGPVRAT TFFFCA.PAD PATVPAAPEG AAATVAAACG AGASAAPAAG DKRPAAPR.. ..........

401                                                                                                      500
HSV   GEAGALHACP MPSSTTRVTP TTKRGRSGGE DARADTALKK PKTGSPTAPP PADPVPLDTE DDSDAADGTA ARPAAPDARS GSRYACYFRD LPTGEASPGA
VZV   TTWQEGIGIR EYCVAPPVDP AGTLDYSELS RESDVICTVK .......... .......... .......... .......... .......... ..........
EHV   .......... ......KLFP DGKLFKSNFV LLMDKTGAKI PCPEQPMHF. .......... .......... .......... .......... ..........
PRV   .......... ......MYTP IAKRPRAASG EGGHAYGDLF .......... .......... .......... .......... .......... ..........

501              517
HSV   FSAFRGGPQT PYGFGFP
VZV   .......... .......
EHV   .......... .......
PRV   .......... .......
```

Figure 7. An alignment of the HSV-1 UL42 protein sequence with the homologous proteins of VZV, EHV and PRV. Asterisks indicate identical residues and dots indicate conservative substitutions. The positions of the inhibitory peptides are indicated above the sequence. The vertical arrow marks the approximate boundary, at residue 320 of UL42, between the relatively conserved N-terminal domain and the unique C-terminal domain. The alignment was made using the GCG PileUp programme. The numbering is of the consensus sequence and not of HSV-1 UL42. Courtesy of Dan Tenney (personal communication).

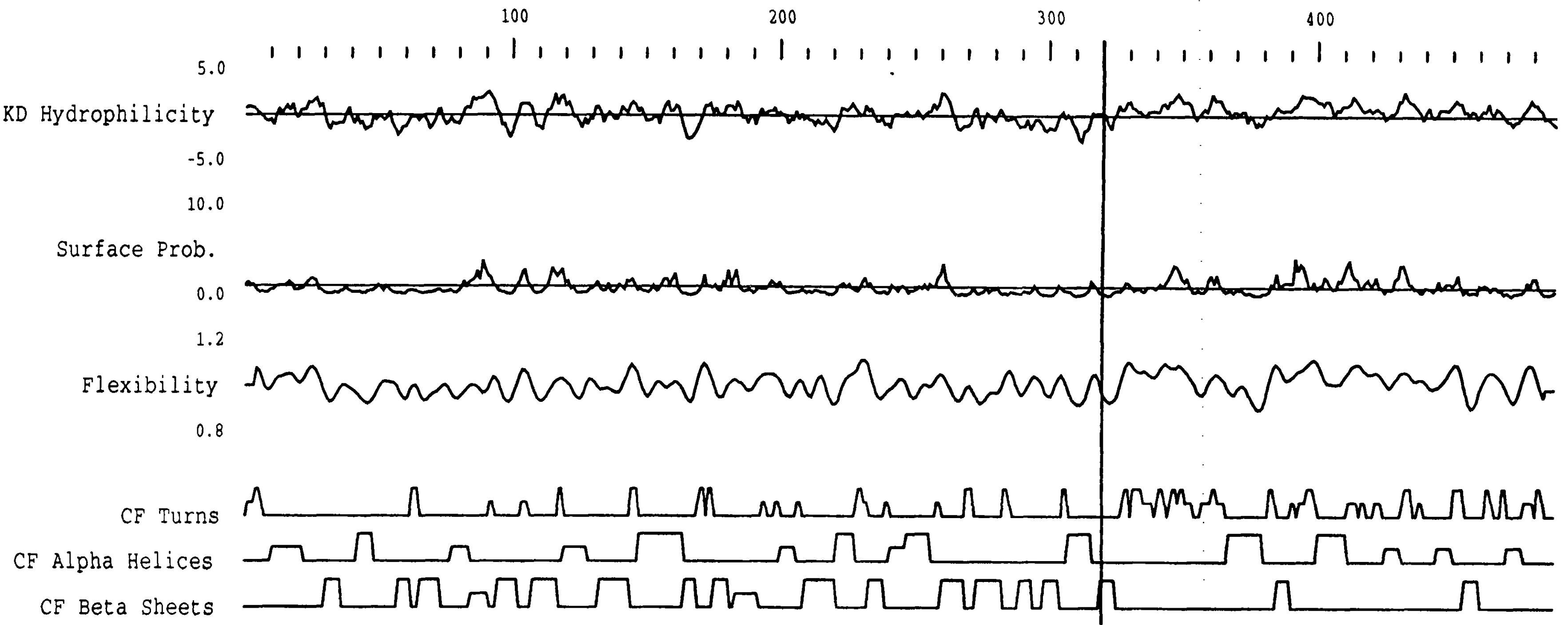


Figure 8. Predicted features of the UL42 amino acid sequence. The amino acid residues are numbered in the top line. The quantitated attributes of hydrophilicity, surface probability, flexibility, and secondary structure (helices, β-sheets and turns) are plotted in separate panels. The vertical line at residue 320 marks the approximate boundary between the conserved N-terminal and unique C-terminal domains.

The plots were generated using the GCG PeptideStructure and PlotStructure programmes. The secondary structure prediction is according to Chou & Fasman, 1978; Nishikawa, 1983; the hydropathicity is calculated according to Kyte & Doolittle, 1982; surface probability according to Emini, 1985; flexibility according to Karplus-Schulz, 1985.

in the predicted structural features: the N-terminal region of ~325 amino acid residues is more hydrophobic, less flexible, has a higher probability of β-sheet structure and a lower surface probability — all of which would indicate a more compact structure — than the C-terminal region (Figure 8). The region of UL42 found to be necessary for function (Digard *et al*, personal communication) coincides precisely with the conserved N-terminal domain.

4.4.5 A comparison of UL42 with other processivity factors

At the current level of knowledge, there appear to be interesting similarities but also important differences between the UL42 protein and other processivity factors, such as PCNA, the *E. coli* β-clamp, T4 gp 45 and thioredoxin.

There is a molar excess (at least 20-fold) of UL42 relative to Pol in infected cells (Gottlieb *et al*, 1990), which has led to suggestions that the free UL42 may have an additional role in infection. However, the β subunit of *E. coli* Pol III is also found in a 10–20-fold excess relative to the catalytic core (Kornberg & Baker, 1992). Studies of *E. coli* Pol III indicate that the mechanism by which polymerase cycles from one replication fork to another requires a relative abundance of the processivity factor (Section 3.7).

One feature shared by UL42 and all the above-mentioned processivity factors is a direct interaction with the catalytic subunit of the respective polymerase (Section 3.7). Another property that they may have in common is a plasticity of sequence. There is virtually no sequence homology between the three domains of the β-clamp, despite their strikingly similar three-dimensional architecture (Section 3.7). The high divergence between the herpesvirus UL42 homologues may indicate a similarly low restriction on amino acid substitutions. Not surprisingly, there is no detectable amino acid homology between UL42 and PCNA or the prokaryotic processivity factors. It is conceivable, however, that UL42 should be compared to the small subunit of polymerase δ, rather than to PCNA (Gottlieb *et al*, 1990). The sequence and function of the 48kDa subunit are as yet unknown, but it is seen to co-purify with the catalytic subunit through numerous chromatographic steps (Tan *et al*, 1986), in a manner reminiscent of UL42.

The DNA-binding property of the UL42 protein sets it apart from the other processivity factors. The *E. coli* β-clamp (Stukenberg *et al*, 1991), the T4 gp 45 (Mace & Alberts, 1984a), PCNA (Tan *et al*, 1986; Prelich *et al*, 1987a), and thioredoxin (Huber *et al*, 1986), do not bind to DNA in the absence of other factors.

The binding of PCNA, of the β-clamp, and of gp 45 to DNA is mediated by RFC, by the γ-complex, and by the gp 44/62 protein complex, respectively, in an ATP-dependent process (Section 3.7). In contrast, the formation of a processive, template-bound Pol–UL42 complex does not require ATP hydrolysis (O'Donnell *et al*, 1987), and resembles the T7 gp 5–thioredoxin complex in this respect.

The β-clamp is a dimer, both in solution and bound to DNA (Section 3.7), but there is no evidence for dimerisation of UL42 (Gottlieb *et al*, 1990).

4.5 UL29 (Major DNA-binding protein; ICP8)

The *UL29* gene product, commonly designated infected-cell polypeptide 8 (ICP8) (Honess & Roizman, 1973), is the major viral DNA-binding protein (Bayliss *et al*, 1975; Purifoy & Powell, 1976). It is an abundant protein of about 130kDa which binds more tightly to single-stranded than to double-stranded DNA (Ruyechan & Weir, 1984). Its binding is cooperative and sequence-independent (Ruyechan, 1983; Ruyechan & Weir, 1984). These properties are characteristic of the prokaryotic helix-destabilising proteins like the *E. coli* SSB and T4 gp 32 (Section 3.4), and suggest that ICP8 plays an analogous role in replication. Conditional lethal mutants mapping in the gene for ICP8 (Conley *et al*, 1981), or its HSV-2 equivalent, ICSP11,12 (Littler *et al*, 1983), were found to display a DNA-negative phenotype, confirming that the protein is essential for DNA replication.

There is clearly a functional interaction between ICP8 and the UL5/UL8/UL52 helicase-primase (Section 4.3). There are also several lines of evidence that ICP8 interacts with DNA polymerase. It stimulates polymerase activity on activated DNA (Ruyechan & Weir, 1984; Gottlieb *et al*, 1990), and on singly-primed M13 ssDNA (Hernandez & Lehman, 1990). Hernandez and Lehman (1990) found that ICP8 potentiates the enhancement by UL42 of Pol processivity, but Gottlieb *et al* (1990) did not see this effect (Section 4.4). The stimulation of HSV-1 polymerase by *E.coli* SSB (O'Donnell *et al*, 1987) may reflect the functional similarity of *E. coli* SSB and ICP8. A direct interaction between the major DNA binding protein and polymerase is indicated by the specific retention of the latter on an immunoaffinity column containing a MAb specific for HSV-2 ICSP 11,12 (Vaughan *et al*, 1984). Several *ts* mutants with defects in ICP8 have an altered sensitivity to the DNA polymerase inhibitors PAA and aphidicolin (Chiou *et al*, 1985). Immunofluorescent studies have shown that, at early times (2–3h) after infection, ICP8 localises to the cell nucleus where it assumes a punctate distribution at "pre-replicative sites" (Quinlan *et al*,

1984). As viral DNA replication proceeds, these develop into large globular "replication compartments" where viral DNA replication occurs (Rixon *et al*, 1983; de Bruyn Kops & Knipe, 1988) (Section 2.2.4.1). ICP8 shows identical localisation in the absence of any other viral proteins (Quinlan *et al*, 1984), but the localisation of Pol and of cellular replication proteins to the pre-replicative sites requires a functional ICP8 (Bush *et al*,1991). This indicates that it may play a key role in organising cellular and viral components into structures related to HSV replication (de Bruyn Kops & Knipe, 1988; Wilcock & Lane, 1991).

Studies of the major DNA-binding protein of both HSV serotypes have shown that it also interacts with the viral alkaline nuclease (Section 2.2.4.3). The two proteins interact specifically on immunoaffinity columns (Vaughan *et al*, 1984) and are co-immunoprecipitated by MAbs specific for either protein (Thomas *et al*, 1988, 1992). The significance of this interaction is not known.

Some functional domains of ICP8 have been identified. The C-terminus contains a nuclear localisation signal (Gao & Knipe, 1989). A "zinc finger" motif is found in the central region of the sequence, and recently it was shown that zinc is tightly associated with the protein (Gupte *et al*, 1991). A 300bp region proximal to the zinc finger is absolutely required for DNA binding (Leinbach & Heath, 1988; Gao & Knipe, 1989; Wang & Hall, 1990). There is a weak but detectable homology between the N-terminal regions of ICP8 and PCNA (Matsumoto *et al*, 1987). Mutants deleted in this region fail to replicate DNA, showing that it has an essential function (Gao & Knipe, 1989). Whether this region is involved in interactions with Pol or with cellular proteins remains to be seen.

5. Anti-herpetic drugs

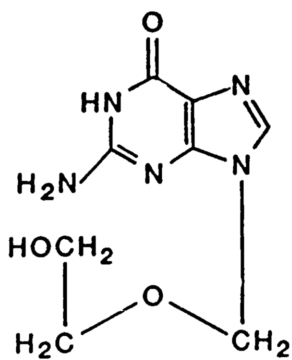
There are a number of compounds which are used to control herpesvirus infections. The most successful antiviral drugs to date are nucleoside analogues, which specifically block viral DNA replication. The structures of several anti-herpetic drugs are shown in Figure 9a. The ultimate target of all these drugs is the viral DNA polymerase. The ability of nucleoside analogues to selectively inhibit viral polymerases is apparently due to the inherently lower precision of base selection by the viral enzymes compared to their cellular counterparts (Hall *et al*, 1985). The selectivity of a drug determines its therapeutic index, *ie* the effective antiviral dose relative to the dose which has an adverse effect on the host. The molecular basis of anti-herpetic drug action has been succinctly reviewed by Coen (1992).

5.1 Acyclovir

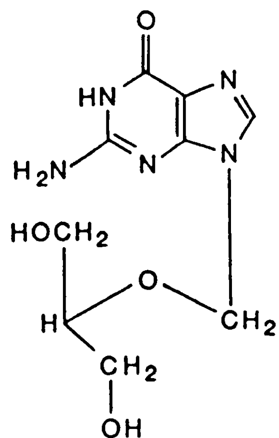
ACV; acycloguanosine; 9-[(2-hydroxy-ethoxy)methyl]guanine

ACV has the highest therapeutic index (300–3000) of any anti-herpetic drug (Crumpacker *et al*, 1979; Dorsky & Crumpacker, 1987), and the molecular basis of its action is well understood. ACV consists of a guanine base linked to a molecule representing an incomplete sugar ring (Figure 9a). It is a potent inhibitor of HSV, VZV and EBV DNA synthesis (Elion *et al*, 1977; Schaeffer *et al*, 1978; Colby *et al*, 1980). The selectivity of this drug is determined by its interactions with two viral enzymes, thymidine kinase (TK) and DNA polymerase.

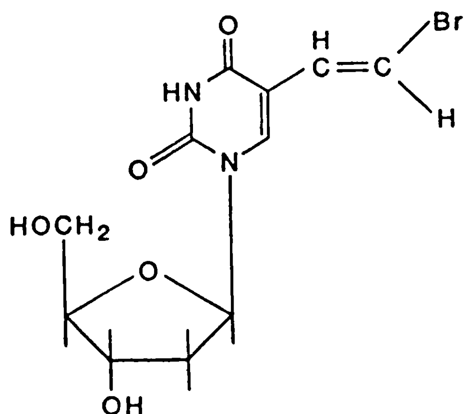
As described in Section 2.2.4.3, the viral TK, being less specific than its cellular counterpart, is able to phosphorylate a range of nucleosides and nucleoside analogues, including ACV (Elion *et al*, 1977; Fyfe *et al*, 1978). The ACV 5'-monophosphate is then converted to the di- and triphosphate forms by cellular kinases. ACV triphosphate (ACVTP) selectively inhibits HSV polymerase (Furman *et al*, 1979; Derse *et al*, 1981) in a three-step process: (i) ACVTP competes with dGTP for binding to the polmerase; (ii) it is incorporated into the DNA chain; (iii)



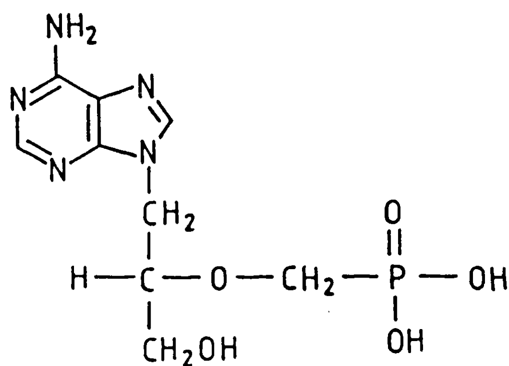
Acyclovir



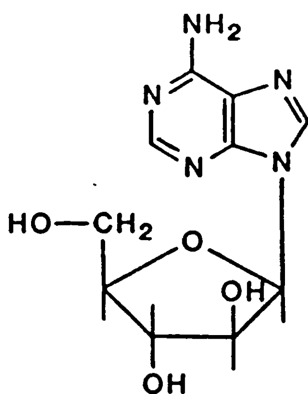
Ganciclovir



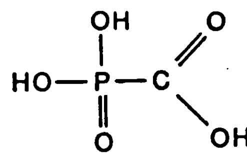
BVdU



(S)-HPMPA



Vidarabine



Foscarnet

Figure 9a. Structures of some anti-herpetic drugs

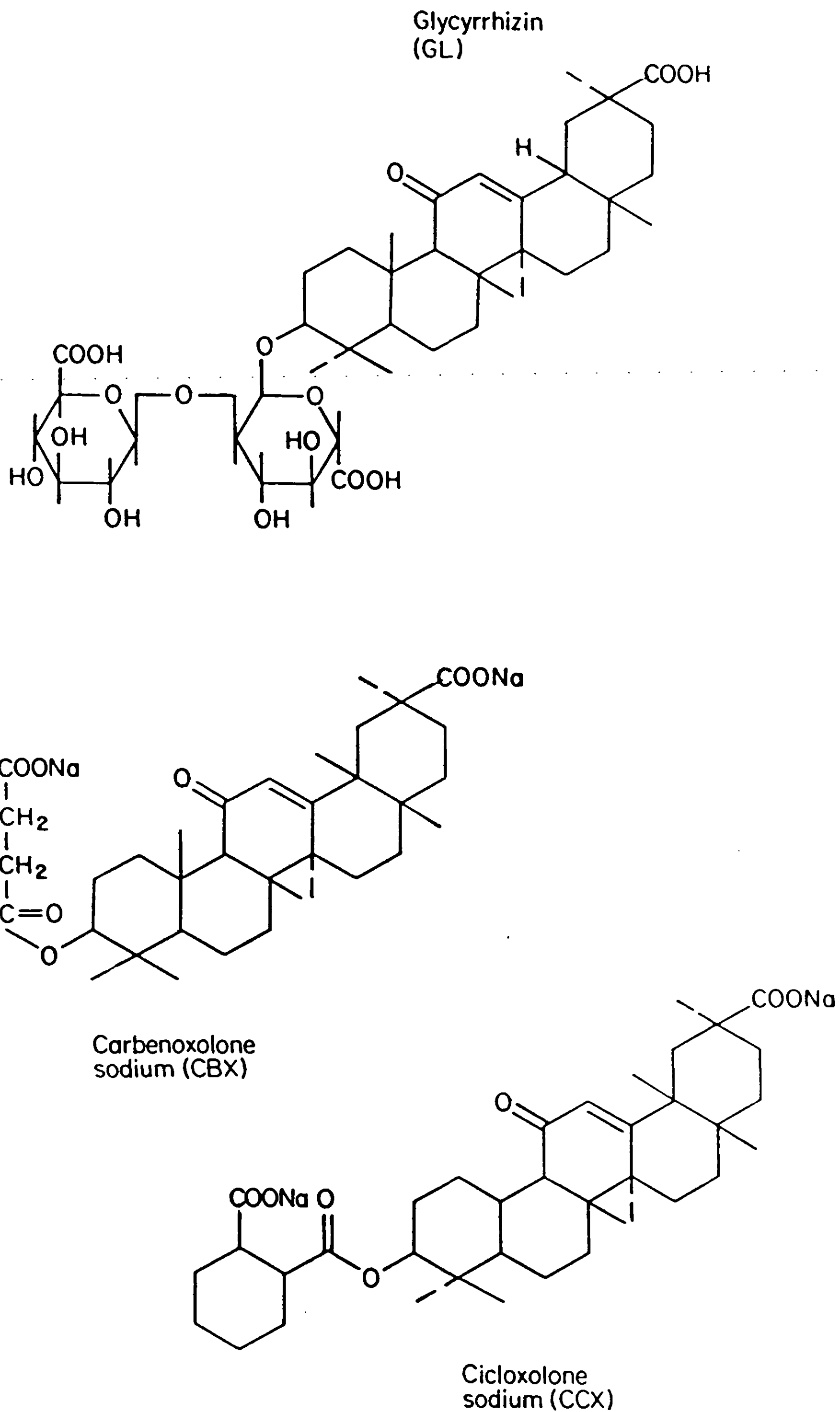


Figure 9b. Structures of some anti-herpetic drugs.

Reproduced with modification from Dargan & Subak-Sharpe, 1992

the polymerase translocates to the next position and binds the next required nucleotide, but cannot catalyse the formation of a phosphodiester bond in the absence of a 3'-OH on the ACVTP, which results in the formation of a tight dead-end complex and apparent inactivation of the enzyme (Reardon & Spector, 1989 and references therein).

That the viral TK and Pol are the molecular targets with which ACV selectively interacts is confirmed by the analysis of mutant viruses resistant to the drug — which arise in patients treated with ACV and in laboratory culture. The mutations conferring ACV resistance map exclusively to the *pol* and/or *tk* genes (Coen & Schaffer, 1980; Schnipper & Crumpacker, 1980; Field *et al*, 1980; Furman *et al*, 1981). Many mutants are TK^-, but others encode TK and polymerase enzymes which have altered substrate specificities (Furman *et al*, 1981; Larder *et al*, 1983; Coen *et al*, 1984; Darby *et al*, 1984).

5.2 Ganciclovir

DHPG; 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine

Ganciclovir is a nucleoside analogue similar in structure to acyclovir (Figure 9a). It is one of the few drugs effective against HCMV, and is also active against HSV, VZV and EBV (Cheng *et al*, 1983). HCMV does not encode a TK (Chee *et al*, 1990), and is therefore relatively refractory to the action of ACV. It does, however, possess another pyrimidine kinase activity capable of phosphorylating ganciclovir (Biron *et al*, 1985). This enzyme has been identified, by the study of ganciclovir-resistant HCMV isolates, as the product of gene UL97 (Littler *et al*, 1992; Sullivan *et al*, 1992).

Cellular enzymes convert the ganciclovir 5'-monophosphate to the triphosphate, which selectively inhibits the viral polymerase (Mar *et al*, 1985). Incorporation of ganciclovir into DNA causes the polymerase to pause or to dissociate from the template, but does not inactivate it in the manner of acyclovir (Frank *et al*, 1984; Reid *et al*, 1988).

5.3 Other nucleoside analogues

5.3.1 Vidarabine

araA, 9-(β-D-arabinofuranosyl) adenine

This is an analogue of adenosine, in which an adenine base is attached to the sugar arabinose (Figure 9a). It is 5'-phosphorylated to an active triphosphate only by cellular enzymes, and is therefore far less selective than ACV. It does, however, have sufficiently selective activity against HSV, VZV, CMV and EBV to be used as a drug. Its antiviral activity is probaly entirely due to its interaction with viral polymerases and incorporation into DNA at a lower concentration than that which affects cellular replication (reviewed by North & Cohen, 1984). The isolation of araA-resistant mutants of HSV with lesions in the *pol* gene confirms that the inhibition of the viral polymerase is selective (Coen *et al*, 1982, 1985).

5.3.2 Halogenated pyrimidine nucleoside analogues

Numerous halogenated analogues of pyrimidine nucleosides have been evaluated for their antiviral effects (reviewed in De Clercq, 1985), and some of the earliest antiviral drugs, such as idoxuridine and trifluridine, are in this category. These compounds are phosphorylated by both cellular and viral TKs, and their major selective target is the viral polymerase (reviewed by Prusoff *et al*, 1984; De Clercq, 1985).

An extensively studied compound of this type is (E)-5-(2-bromovinyldeoxyuridine) (BVdU) (Figure 9a), which is active against HSV-1 and VZV, but not so active against HSV-2. BVdU is phosphorylated to the 5'-monophosphate by the viral TK, and then to the diphosphate by the thymidilate kinase activity of HSV-1 and VZV (but not HSV-2) TK (Fyfe *et al*, 1982). The 5'-triphosphates of BVdU and other similar compounds are competitive inhibitors of thymidine triphosphate utilisation by viral DNA polymerases (Allaudeen *et al*, 1981; Ruth & Cheng, 1981). They are used as alternative substrates and incorporated quite efficiently into viral DNA (Allaudeen *et al*, 1982; Kowalzick *et al*, 1982). This results in deleterious effects such as DNA fragmentation (Chen *et al*, 1976; Mancini *et al*, 1983) and transcriptional defects (Ruth & Cheng, 1982; Sági *et al*, 1982).

5.3.3 Phosphonomethylalkyl derivatives

These phosphorylated nucleoside analogues are a newer class of antiviral drug. The prototype compound, (S)-HPMPA (Figure 9a), is active against a broad spectrum of DNA viruses (De Clercq *et al*, 1986). The clinical importance of these compounds lies in the fact that, being already phosphorylated, they are effective against TK$^-$ mutant viruses which are resistant to other drugs (De Clercq *et al*, 1986). These compounds are further phosphorylated by cellular kinases and almost certainly act by selectively inhibiting viral DNA polymerase, as indicated by the isolation of a *pol* mutant of HSV-1 resistant to a phosphonmethylalkyl compound (Foster *et al*, 1991).

5.4 Foscarnet
Phosphonoformic acid, PFA

Like phosphonoacetic acid, foscarnet is an analogue of pyrophosphate (Figure 9a), which is a product of the polymerisation of DNA. It is a potent inhibitor of herpesvirus replication (Helgstrand *et al*, 1978). It acts by non-competitive inhibition, interacting directly with Pol at the pyrophosphate binding site (Erikkson *et al*, 1980; Ostrander & Cheng, 1980). The existence of foscarnet-resistant *pol* mutants shows that it acts selectively on the viral enzyme (Erikkson & Oberg, 1979).

5.5 Triterpenoid compounds

These compounds exhibit broad-range antiviral activity, both in tissue culture and in man (reviewed by Dargan & Subak-Sharpe, 1992). The synthetic triterpenoids carbenoxolone (CBX) and cicloxolone (CCX) are based on the structure of glycyrrhizin (GL), which is the active constituent of licorice (Revers, 1946) (Figure 9b). GL is routinely used in Japan for the treatment of viral hepatitis. Clinical trials have shown that topical treatment with CCX or CBX accelerates the healing of HSV lesions (Poswillo & Roberts, 1981; Csonka & Tyrrell, 1984).

The antiviral activity of GL, CBX and CCX is largely a result of their disruption of host cell membrane functions, particularly the postranslational modification and transport of glycoproteins by the Golgi, which in turn affects the replication and assembly of a wide range of viruses — *in vitro* studies have shown that viruses from at least 12 different virus families are sensitive to one or more of these compounds.

A major potential advantage of the triterpenoid compounds is that — since they do not selectively target a particular virus protein but act by altering the cellular environment — the likelihood of drug-resistant mutants arising is minimal.

6. Disruption of protein interactions

Any biological process which relies on the specific interaction between two macromolecules could, in principle, be inhibited by interfering with that interaction. Recognition sites on proteins are exquisitely specific, and there are various examples of peptides, derived from the recognition site on one of the ligands, which retain sufficient structure to compete with the whole protein for a binding site. This may indicate that a particular sequence is directly involved in an interaction; it also identifies a compound which is capable of specifically disrupting that interaction.

Essential interactions between viral protein subunits are potential targets for antiviral agents. Described below are some examples of interactions involving virus proteins that can be specifically inhibited. This topic has been more comprehensively reviewed by Marsden (1992). The challenging task of developing therapeutically useful drugs from lead peptides is briefly considered.

6.1 F1 protein of Paramyxoviridae

Membrane fusion by *Paramyxoviridae* is an example of a virus-host cell interaction that can be disrupted by a peptide fragment of the virus component. *Paramyxoviridae* — the family to which measles and mumps viruses belong — have two surface glycoproteins: an attachment protein and a fusion (F) protein. The F protein is made fusogenic by proteolytic cleavage of an inactive precursor (F_0) to give two disulphide-linked polypeptides, F_1 and F_2. The newly-generated N-terminus of F_1 is very hydrophobic and can insert into the cell membrane (reviewed by Kingsbury, 1990a). Short peptides with amino acid sequences similar to the N-terminal region of F_1 specifically inhibit virus-induced cell fusion and virus infection (Nicolaides *et al*, 1968; Miller *et al*, 1968; Richardson *et al*, 1980; Richardson & Choppin, 1983). The cell membrane components of this interaction are as yet undetermined.

6.1a The α-TIF–Oct-1 transcription complex

The HSV-1 virion protein α-TIF (Vmw65, VP16) plays a central role in the assembly of a virus-specific transcription complex responsible for the expression of IE genes (Section 2.2.3.1). The complex is composed of α-TIF and at least two cellular proteins: the cellular transcription factor Oct-1(OTF-1, NFIII, TRF), and the complex forming factor CCF (HCF, VCAF-1, C1) (Gerster & Roeder, 1988; O'Hare & Goding, 1988; Preston *et al*, 1988; Kristie *et al*, 1989; Katan *et al*, 1990; Xiao & Capone, 1990).

Formation of the complex can be inhibited by peptides corresponding to a region of α-TIF involved in the interaction (Haigh *et al*, 1990; Hayes & O'Hare, 1993). The region of α-TIF encompassing amino acid residues 360–388 was found, by mutational analysis, to be critical for complex assembly (Greaves & O'Hare, 1990). The susceptibility of this region of α-TIF to proteolysis, and its selective protection from proteolysis upon complex assembly, indicated that it was (i) exposed on the surface of the protein, and (ii) likely to be involved in protein–protein interactions within the complex (Hayes & O'Hare, 1993). A peptide corresponding to amino acids 360–373 was found to inhibit complex assembly without affecting the DNA-binding property of Oct-1 alone (Haigh *et al*, 1990). The peptide, proposed to act by competing with α-TIF for binding to the cellular components of the complex, inhibited complex formation when added to a nuclear extract prior to the addition of α-TIF, but was not able to disrupt a pre-formed complex (Haigh *et al*, 1990). Single amino acid substitutions within this region which reduced the complex-forming ability of the whole protein (Greaves & O'Hare, 1990) correspondingly reduced the inhibitory properties of the peptide (Hayes & O'Hare, 1993). By using overlapping peptides, the inhibitory sequence has been narrowed down to eight amino acids, REHAYSRA, corresponding to residues 360–367 (Hayes & O'Hare, 1993). Interestingly, this sequence does not encompass all the residues shown to be essential for complex formation (Greaves & O'Hare, 1990). It remains to be tested whether the presence of this peptide in a cell prevents the expression of IE genes upon HSV-1 infection.

6.2 Herpesvirus ribonucleotide reductase (RR)

HSV RR provides the best-studied example of an interaction between two viral protein subunits that can be dissociated by a peptide. It is likely that the enzyme plays an important role in pathogenesis, and is therefore a valid antiviral target (Section 2.2.4.3).

RR consists of two dissociable, homodimeric proteins: a large subunit (R1) and a small subunit (R2) (Thelander & Reichard, 1979; Averett *et al*, 1984; Ingemarsson & Lankinen, 1987). A nonapeptide (YAGAVVNDL), corresponding to the very C-terminus of the small subunit, specifically inhibits the viral enzyme and does not affect its cellular counterpart (Dutia *et al*, 1986; Cohen *et al*, 1986). The peptide acts by competing for the R2 binding site on the R1 protein, and thus inhibits the normal association of the two subunits (Figure 10) (Dutia *et al*, 1986; Cohen *et al*, 1986; McClements *et al*, 1988; Paradis *et al*, 1988; Darling *et al*, 1990).

The mammalian and *E. coli* enzymes can be similarly inhibited by the corresponding C-terminal peptides(Yang *et al*, 1990; Cosentino *et al*, 1991). Moreover, peptides from the HSV, mammalian and *E. coli* C-terminal R2 sequences — which are totally diverse — can each inhibit the homologous, but not the heterologous enzymes (Cosentino *et al*, 1991).

Although effective at inhibiting HSV RR enzyme activity *in vitro*, the YAGAVVNDL peptide does not inhibit virus growth in tissue culture, probably because it is too large to cross the cell membrane (Dutia *et al*, 1986). Nevertheless, it provides a starting point for the development of therapeutically useful compounds. The most active derivatives yet reported have 500-fold greater potency (IC_{50} for inhibition of RR activity = 100nM) and are significantly smaller (M_r = 682) than the lead peptide (Biomega Inc, 1990). Their antiviral activity, if any, has not been reported.

The likely spectrum of activity of such compounds has been investigated. The RRs induced by PRV and EHV-1 are inhibited by the YAGAVVNDL peptide (Cohen *et al*, 1987; Telford *et al*, 1990), indicating that a drug based on the peptide would also be active against these alphaherpesviruses. It is doubtful whether the more distantly related HCMV encodes a RR. Several workers have been unable to detect an HCMV-induced RR activity (Lankinen, Darling, personal communication). The predicted protein sequence of HCMV gene *UL45* shares significant homology with the C-terminal one-third of other herpesvirus R1 subunits (Chee *et al*, 1990). However, it lacks all of the motifs — including a C-terminal pair of redox shuttle cysteines, essential for enzyme activity — which are conserved in the R1 subunits of

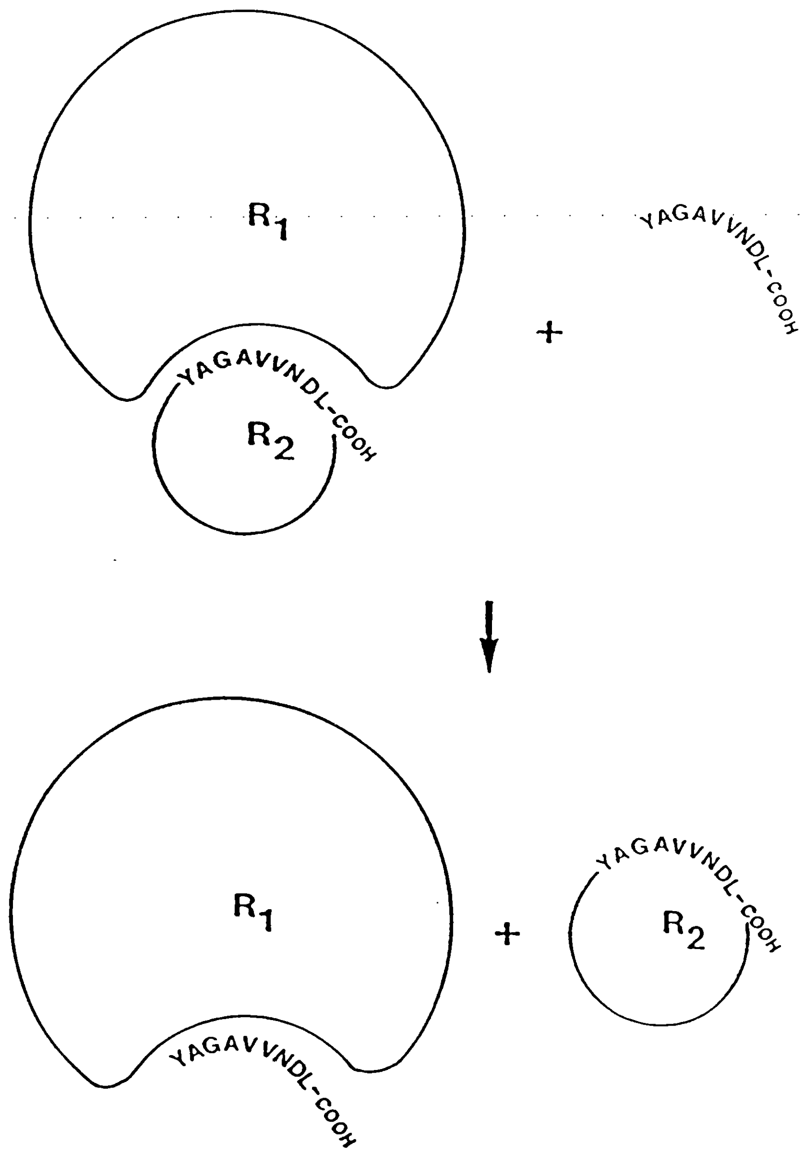


Figure 10. A schematic representation of the mechanism by which HSV ribonucleotide reductase is inhibited by the peptide YAGAVVNDL. The figure is a simplification of the enzyme in which both the large and small subunits are homodimers. Reproduced with modifications from Dutia *et al*, 1986.

mouse, *E. coli*, T4, Vaccinia, VZV, HSV and EBV (Eriksson & Sjörberg, 1989). The R2 subunits also contain highly conserved residues, which are postulated to make up the iron centre of the enzyme (Stubbe, 1989), but no HCMV gene encodes these motifs. These (unpublished) data indicate that HCMV does not encode a functional RR, and therefore virus growth would not be susceptible to inhibition by the blocking of this enzyme.

6.3 Semliki forest virus (SFV) spike glycoprotein

SFV is one of the two well-studied members of the alphavirus genus. Virions of these small, enveloped RNA viruses consist of a nucleocapsid surrounded by a host-cell derived envelope, which contains spikes of the viral glycoproteins E1 and E2. The virion is assembled at the inner surface of the plasma membrane, by a process in which the nucleocapsid protein associates specifically with the cytoplasmic domain of the E2 glycoprotein. Mature virions then bud from the plasma membrane (reviewed by Schlesinger & Schlesinger, 1986, 1990).

By using a network of anti-E2 anti-idiotype and anti-anti-idiotype antibodies, the region which interacts with the nucleocapsid was localised to the very C-terminus of the glycoprotein (Vaux *et al*, 1988; Kail *et al*, 1991). An octapeptide corresponding to E2 C-terminal sequence (CCAPRAHA) interacted specifically with nucleocapsids, and blocked binding of a MAb specific for the nucleocapsid recognition site (Kail *et al*, 1991). The effect of the octapeptide on virus growth was tested by co-microinjecting cells with peptide and infectious SFV RNA. In the presence of the peptide, budding and spread of virus was inhibited and virus yield was reduced by at least an order of magnitude (Kail *et al*, 1991). It remains to be seen whether this is a route to developing an antiviral therapy.

6.4 Influenza virus haemagglutinin

Inhibition of influenza virus, apparently similar to the inhibition of SFV described above, has also been reported (Collier *et al*, 1991). Influenza A, an orthomyxovirus, contains two membrane glycoproteins, haemagglutinin (HA) and neuraminidase (N). HA consists of two disulphide-linked chains, HA_1 and HA_2. The protein is anchored in the cell membrane close to the C-terminus of HA_2, with a short (11 amino acid residue) region on the internal side of the membrane. Virion assembly is poorly characterised, but appears to involve interactions between viral matrix

proteins, the nucleocapsid, and the cytoplasmic domains of the viral glycoproteins at the plasma membrane (reviewed by Kingsbury, 1990b).

A 10 amino acid peptide (NGSLQCRICI), corresponding to the cytoplasmic domain of HA_2, was shown to inhibit the release of infectious virions when added to the culture medium during infection (Collier *et al*, 1991). The peptide acted specifically on influenza virus and had no effect on two other enveloped RNA viruses, Sindbis and vesicular stomatitis virus. It was postulated to cross the plasma membrane and act by competing with the hemagglutinin for sites recognised by nucleocapsids or matrix proteins.

6.5 Development of useful drugs from lead peptides

Inhibitory peptides are not therapeutically useful because they are rapidly metabolised and are generally too large to cross cell membranes. This means that any lead peptide requires extensive modification through a process of molecular mimicry whereby overall size is reduced, labile and flexible peptide bonds are replaced by bonds which are more resistant to degradation and have a more restricted conformation, and side-chains are substituted by more potent groups. The success rate is low, the time scale is long, and an element of luck is involved (reviewed by Johnson AL *et al*, 1990).

One reason for persevering with the development of compounds which act by subunit dissociation is that, potentially, the incidence of drug-resistant mutations could be orders of magnitude lower than that seen with drugs such as nucleoside analogues. Drug resistance would require simultaneous, complementary mutations in both of the protein subunits such that a functional complex could be formed in the presence of the drug. It will be very interesting to see if this proves to be true in practice.

MATERIALS

1. Chemicals and reagents

Unless specifically stated below, all chemicals and reagents were obtained from BDH Chemicals Ltd, Poole, Dorset, or from Sigma (London) Ltd and were of analytical grade or better.

Ammonium persulphate, N,N,N',N-tetramethylethylenediamine (TEMED)	Bio-Rad Laboratories, Richmond, California, USA.
Bio-Rad protein assay reagents and standards	Bio-Rad Laboratories, Richmond, California, USA.
Ecoscint A	National Diagnostics, Manville, New Jersey, USA.
Acetic acid, acetonitrile (HPLC-grade S), *t*-amyl alcohol, diethyl ether, dimethylformamide, formic acid, piperidine, trifluoroacetic acid	Rathburn Chemicals Ltd., Walkerburn, Scotland, UK.
Ethanedithiol, ethylmethylsulphide, *m*-cresol, tetrabutylammonium fluoride, thioanisole, trimethylsilylbromide	Aldrich Chemical Co Ltd, Gillingham, Dorset, UK.
Fmoc amino acid active esters, 1-hydroxybenzotriazole (HOBt), Fmoc-Ultrosyn C resin	Novabiochem (UK) Ltd, Nottingham, UK.
Ultrosyn A resin with Fmoc Pro attached	Peptide & Protein Research, Exeter, Devon, UK.

^{32}P-labelled nucleotide	Activity	Concentration	Code
[$\alpha^{32}P$]dATP	~3000Ci/mmol	10mCi/ml	PB 10204
[$\alpha^{32}P$]dCTP	~3000Ci/mmol	10mCi/ml	PB 10205
[$\gamma^{32}P$]ATP	~5000Ci/mmol	10mCi/ml	PB 10218

Materials

λ DNA	BRL, Paisley, Scotland,UK.
M13 mp18 ssDNA	1) New England Biolabs, Bishops Stortford, UK. 2) Cambridge Bioscience, Cambridge, UK.
M13 sequencing primer no. 1211: 5'-GTAAAACGACGGCCAGT	New England Biolabs, Bishops Stortford, UK.
Duplex oligonucleotide: 5'-GATCCGCGAAGCGTTCGCACTTCGTCCCA GCGCTTCGCAAGCGTGAAGCAGGGTCTAG-5'	Synthesised and purified in house by Nigel Stow.
Radiochemicals	Amersham International PLC, Bucks, UK.
Deoxynucleoside triphosphates, ATP (100mM solutions, pH 7.5)	Pharmacia LKB, Uppsala, Sweden

2. Miscellaneous materials

Whatman DE81 ion exchange paper	Whatman International Ltd, Maidstone, UK.
Kodak X-Omat XS1 film	Kodak Ltd, UK.
Dynamax HPLC columns	Anachem, Luton, Bedfordshire, UK.

3. Enzymes

Restriction enzymes	Boehringer Mannheim, Lewes, E. Sussex, UK.
T4 polynucleotide kinase	New England Biolabs, Bishops Stortford, UK.
Klenow polymerase	Purified in house by Moira Watson from *E. coli* cells expressing a plasmid construct (Joyce & Grindley, 1983).

HSV-1 Pol	Gift from J Gottlieb and M Challberg, purified from insect cells infected with recombinant baculovirus (Gottlieb *et al*, 1990).
HSV-1 UL42 protein	1) Gift from J Gottlieb and M Challberg, purified from insect cells infected with recombinant baculovirus (Gottlieb *et al*, 1990).
	2) Purified in house by Mary Murphy from insect cells infected with recombinant baculovirus (Stow, 1992).

4. Buffers and solutions

Annealing buffer	20mM Tris-HCl pH7.5, 25mM NaCl
Alkaline electrophoresis buffer	30mM NaOH, 2mM EDTA
Alkaline loading buffer	100mM NaOH, 1mM EDTA, 2.5% Ficoll, bromophenol blue
Binding buffer	20mM HEPES pH7.6, 0.5mM DTT, 0.5mM EDTA, 10% glycerol, 50µg/ml BSA
Buffer B	10mM Tris-HCl pH 8, 5mM $MgCl_2$, 100 mM NaCl, 1mM 2-mercaptoethanol
Klenow buffer	50mM Tris-HCl pH7.5, 10mM $MgCl_2$, 1mM DTT
Loading buffer	25% glycerol, 10mM DTT, 0.01% bromophenol blue in TBE
Pol reaction buffer	20mM HEPES pH7.6, 1mM $MgCl_2$, 4% glycerol, 0.1mM EDTA, 1mM DTT, 75mM NaCl

Materials

Pol storage buffer	20mM HEPES pH7.6, 0.5mM DTT, 0.5mM EDTA, 10% glycerol, 50μg/ml BSA
PK (T4 polynucleotide kinase) buffer	40mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 5mM DTT
Stop solution	20μg/ml of sonicated calf thymus DNA, 0.67% SDS, 300mM NaCl
TBE	90mM Tris, 89mM boric acid, 1mM EDTA
TE	10mM Tris-HCl pH 7.4, 1mM EDTA

METHODS

1. Oligopeptides

1.1 Synthesis of overlapping peptides

Ninety six pentadecapeptides spanning the entire 488 amino acids of the UL42 protein were synthesised by continuous-flow Fmoc chemistry (reviewed by Atherton & Sheppard, 1989) using a Novabiochem peptide synthesiser. DHBT amino acid esters were used for coupling Arg, Thr and Ser; PFP esters in conjuction with HOBt were used for coupling all the other amino acids. The progress of the synthesis was monitored by measuring the UV absorption of the fluorenylmethyl chromophore during each cycle of acylation and deprotection, and remedial action taken whenever necessary.

Each peptide was 15 amino acids long and overlapped the adjacent one by 10 amino acids, with some exceptions, discussed in the results. The three in-series columns of the synthesiser were used for simultaneous coupling of each five-amino-acid epitope to three consecutive peptides. With the exception of the carboxy-terminal peptide, they were synthesised on Ultrosyn C Kieselguhr polyamide resin, to which the first residue is attached on the machine under standard conditions for peptide bond formation. Ultrosyn C is specifically designed to make peptide carboxy-amides. The scale of synthesis was 0.12mmol, *ie* 0.04mmol of functionalised resin (400mg) was used in each column. Pre-packed vials containing 0.5mmol of Fmoc amino acid esters were used, giving a 4x excess of amino acid during coupling reactions.

Synthesis of the C-terminal peptide

The C-terminal peptide was synthesised as a peptide acid on Ultrosyn A resin to which a proline had been coupled via an acid-labile linkage. Synthesis of a peptide with a C-terminal proline posed a potential problem, since C-terminal Pro dipeptide esters tend to undergo spontaneous cleavage from the resin support due to base-catalysed intramolecular aminolysis during deprotection of the second residue with 20% piperidine in dimethylformamide (DMF). An alternative deprotection reagent,

0.02M tetrabutyl ammonium fluoride in DMF, was therefore used for the second deprotection (Ueki & Amemiya, 1987).

Extra peptides

Other peptides, as described in the text, were also synthesised. Unless stated otherwise, all were purified to greater than 95% homogeneity and had an M_r (determined by FAB MS analysis — 1.3, below) in agreement with that expected.

1.2 Cleavage and deprotection

The peptide resin was transferred from the column into a sintered glass funnel and washed with the following solvents: DMF, *t*-amyl alcohol, acetic acid, *t*-amyl alcohol and diethyl ether. It was then transferred into a 100ml round-bottomed flask and dried in an evacuated dessicator. As a precaution against an unsuccessful cleavage or any mishap, in most cases only half of the peptide resin was cleaved initially while the rest was stored at –20°C. 95% trifluoroacetic acid(TFA) (1ml/10mg resin) was used to cleave the peptide from the resin and remove the sidechain protecting groups.

For peptides which did not contain Arg(Mtr), Cys(Trt), Met or Trp, a 95% aqueous solution of TFA was used, while for peptides containing any combination of the above amino acids, appropriate scavengers were added (Table 4). The cleavage reaction was carried out in a stoppered flask at room temperature for the time shown in Table 4. The resin was removed by filtration under reduced pressure through a sintered glass funnel and washed with 5ml of TFA. The peptide solution was reduced on a rotary evaporator to a volume of 1–2ml (except in the case of peptides containing Cys-Trt, which, to avoid reattachment of the Trt group, were only reduced to 8–10ml). The peptide was then precipitated out of solution by the addition of 20ml of diethyl ether. The precipitate was centrifuged, the ether removed and the precipitate thoroughly washed twice with ether. The peptide was then dissolved in 25ml of Milli-Q water and, after removal of any residual ether by rotary evaporation, it was shell-frozen and lyophilised.

Cleavage of peptides containing Arg and Trp

Peptides containing Arg(Mtr) and an unprotected Trp residue can be cleaved successfully in 95% TFA/ 5% phenol/ 1% EDT, but occasionally problems occur due to the irreversible modification of Trp residues, either by dithioketal formation or by transfer of the Mtr group from arginine to the indole ring of tryptophan (Atherton & Sheppard, 1989). An alternative two-step method recommended by Peter White of Novabiochem was therefore used for all peptides containing both Arg and Trp.

Table 4. CONDITIONS FOR CLEAVAGE OF PEPTIDES

Arg	Cys	Met	Trp	TFA (%)	Scavengers (%)	Time (hrs)
–	–	–	–	95	Water (5)	1–1.5
+	–	–	–	95	Phenol (5)	>6
–	+	–	–	95	EDT (5)	1–1.5
–	–	+	–	95	EMS (5)	1–1.5
–	–	–	+			1–1.5
–	+	–	+	94	Phenol (5) EDT (1)	1–1.5
+	+	–	–			>6
+	+	+	–	93	Phenol (3)	>6
+	–	+	–		EDT (1) EMS (3)	
+	–	+	+		TMSB (12.5)	
+	+	–	+	71	EDT (4.5)	0.5
+	+	+	+		*m*-cresol (1)	
					Thioanisole (11)	

Scavenger abreviations

EDT	ethanedithiol
EMS	ethylmethylsulphide
TMSB	trimethylsilylbromide

The peptide was first cleaved from the resin by allowing 5% TFA in dichloromethane (DCM) to drip through the resin in a sintered glass funnel for an hour. The TFA and DCM were removed on a rotary evaporator. The cleavage mixture of TMSB, EDT, *m*-cresol and thioanisole in TFA (Table 4) was made up under under nitrogen, cooled to 0°C and added to the protected peptide. This was allowed to stand at 0°C under a blanket of nitrogen for 20–30 minutes. The mixture was then evaporated using a stream of nitrogen and the peptide precipitated in ether as above.

1.3 Analysis of peptides

Mass spectrometry

The molecular weight of each peptide was determined by mass spectrometry (MS). Several were analysed by M-Scan Ltd, Ascot, England, using the Fast Atom Bombardment (FAB) ionisation technique (Barber *et al*, 1981). The majority were analysed by plasma desorption ionisation on a time-of-flight mass spectrometer by John Kitchin at Glaxo Group Research. These MS techniques of peptide analysis have been reviewed by Biemann (1992).

Reverse phase high pressure liquid chromatography (HPLC)

The purity of each peptide was determined by reverse phase HPLC monitored at 225nm, on a Beckman System Gold HPLC using a Dynamax 300Å C8 analytical column (4.6mm internal diameter x 250mm length, catalogue number 83-303-C) and a gradient of 0–95% acetonitrile (plus 0.05%TFA) in water (plus 0.1%TFA) run over 20 minutes at a flow rate of 0.5ml/min.

Capillary zone electrophoresis (CZE)

Where indicated, purified peptides were analysed by CZE by Barry Coomber at Glaxo Group Research. The physical basis of separation in CZE is different from that in reverse phase HPLC, and is therefore an appropriate way of checking their purity.

1.4 Purification of Peptides

Where indicated, peptides were purified by reverse phase chromatography on a Beckman System Gold HPLC using a Dynamax 300Å C8 preparative column (21.4mm internal diameter x 250mm length, catalogue number 83-323-C) and a gradient of 0-95% acetonitrile (plus 0.05%TFA) in water (plus 0.1%TFA) run over

20 minutes at a flow rate of 10ml/min. Fractions were analysed by reverse phase HPLC as above, the peak fractions pooled, lyophilised and reanalysed by reverse phase HPLC.

1.5 Storage of peptides

Lyophilised peptides were stored at –20°C in dry universal bottles. On removal from storage, they were warmed to room temperature before opening.

1.6 Dissolution of peptides

Peptides were dissolved in Milli-Q water for analysis, purification and addition to assays. Those which were not easily soluble in water were treated as follows: Suspensions of acidic peptides in water were bubbled with ammonia vapour (typically 1–2 volumes of vapour/volume of water) in order to raise the pH of the solution. Basic peptides were dissolved in a small amount of 100% formic acid and then water was added to the required volume.

2. DNA polymerase assays

2.1 HSV-1 DNA polymerase

HSV-1 DNA polymerase activity was assayed using a singly primed, single stranded circular M13 (7.25kb) DNA template under conditions described previously (Gottlieb *et al*, 1990) with minor modifications. The template was prepared by hybridising 1pmol of synthetic oligonucleotide primer to 400fmol of M13 mp18 ssDNA in 20µl of annealing buffer. The mixture was incubated at 65°C for 2 minutes and allowed to cool slowly to room temperature for 2 hours.

The assay was performed at 37°C in a 50µl volume of pol reaction buffer containing 0.5mM ATP, 60µM of each of three unlabelled deoxyribonucleoside triphosphates and 2µM of the fourth [$\alpha^{32}P$]-labelled nucleotide (either dATP or dCTP at 9×10^4 cpm/pmol), 2.5µg BSA (protease free) and Pol andUL42 proteins as described in the Results. M13 DNA template-primer was used at 10µg/ml (4nM) for measuring the activity of Pol alone, and at 1µg/ml for all other assays, unless specified otherwise. Peptides were included as indicated and the mixture pre-incubated at 37°C for 10 min

(3 min in assays of full length M13 DNA synthesis). Reactions were initiated by the addition of [α^{32}P] dNTP.

2.1.1 Measurement of processive activity

To measure the synthesis of full-length M13 DNA the reactions were terminated after 30 minutes by adding 150µl of stop solution. The reaction products were extracted with an equal volume of phenol (pre-equilibrated with TE buffer) followed by chloroform/isoamyl alcohol (24:1), precipitated in ethanol and re-dissolved in 40µl of alkaline loading buffer, then analysed by electrophoresis in alkaline 1% agarose gels. The DNA was denatured to single strands under these conditions (Sambrook *et al*, 1989). Gels were run overnight at 40V in alkaline electrophoresis buffer, dried and exposed to X-Omat XS1 film at –70°C for autoradiography.

Densitometry

A Hoeffer GS-300 Scanning Densitometer and GS-360 data analysis system was used to quantitate the density of bands on the autoradiographs, by scanning through the centre of each band and integrating the absorbance.

Radiolabelled marker DNA

Full length linearised M13 DNA was radiolabelled and used as a size marker on all gels. Double stranded M13mp8 DNA, linearised with *Sma* I and treated with calf intestinal phosphatase, was obtained from Aidan Dolan. 100ng of DNA were incubated with 0.5 unit T4 polynucleotide kinase for 20 min at 37°C in a 5µl volume of PK buffer containing 5µCi [γ^{32}P] ATP (6.6×10^6 cpm/pmol). M13mp8 DNA is 30 base-pairs shorter than mp18, but this difference is undetectable at the top of a 1% agarose gel.

Radiolabelled fragments obtained by *Hin*d III digestion of λ DNA were used as molecular size markers. 4µg of λ DNA were incubated for 90 min at 37°C with 10 units of *Hin*d III in a 20µl volume of buffer B. Then 0.5µg of the digested DNA was incubated for 15 min at 30°C in a 7.5µl volume of Klenow buffer containing 5mM dGTP, 5mM dCTP, 5mM dTTP, 10µCi [α^{32}P] dATP (6.6×10^6 cpm/pmol) and 2 units of Klenow polymerase (methods generally according to Sambrook *et al*, 1989).

2.1.2 Measurement of total activity

To measure the rate of DNA synthesis, samples of 10μl were removed at intervals and spotted onto Whatman DE81 ion-exchange filters, which had been previously soaked in 0.1M EDTA and air-dried. (This pre-treatment of the filters had the effect of stopping immediately the polymerase reaction and prevented high blank counts.) The discs were washed for 3 x 10 min with 5% (w/v) Na_2HPO_4, then for 2 x 5 min with water and finally for 2 x 30 sec with industrial methylated spirits. They were dried in a current of warm air and transferred to scintillation vials. Ecoscint was added (4ml/vial) and the radioactivity counted in a Beckman LS5000CE scintillation counter.

2.2 Polymerase α

Mammalian DNA polymerase α activity was assayed at 37°C in 200μl of 75mM Tris-HCl buffer pH 7.5, containing 6.5mM $MgCl_2$, 83μM dATP, 83μM dCTP, 83μM dGTP, 1.67mM 2-mercaptoethanol, 11μg/ml activated calf thymus DNA, 0.42mg/ml BSA, 3.4μM [^{3}H] dTTP (10.2 Ci/mmol), and 30mM NaCl carried over into the assay from the enzyme solution. Peptides were included as appropriate and assay mixtures were pre-incubated at 37°C for 10 min in the absence of [^{3}H] dTTP prior to initiation of reaction with the labelled nucleotide. Samples of 20μl were removed at intervals over the first 16 min of reaction and spotted onto a DEAE-filter mat, previously soaked in 0.1M EDTA and air-dried. The filter mat was washed as described in 2.1.2 above and counted in an LKB beta-plate scintillation counter.

2.3 Klenow polymerase

The activity of the Klenow fragment of *E. coli* Pol I was assayed at 37°C in a 50μl volume of Klenow buffer containing 60μM dATP, 60μM dGTP, 60μM dTTP, 20μM [α^{32}P] dCTP (9×10^3 cpm/pmol), 20μg BSA, 4nM primed M13 DNA and 0.1 unit of enzyme. Peptides were included as indicated and the mixture pre-incubated at 37°C for 10 min. Reactions were initiated by the addition of [α^{32}P] dCTP. Samples were removed at intervals onto Whatman DE81 filters as described above.

3. Assays for DNA binding

3.1 DNA mobility shift assay

A duplex oligonucleotide was radiolabelled by incubating 200ng of oligonucleotide in a 50µl volume of Klenow buffer containing 20µCi [α^{32}P]-dGTP (6.6×10^6 cpm/pmol) with 2 units of Klenow polymerase at room temperature for 30 min. The single stranded ends were then filled by adding 100µM dATP, dCTP, dGTP and dTTP to the reaction mix and incubating for a further 15 min at room temperature. The DNA was extracted with 1:1 phenol/chloroform followed by chloroform, precipitated in ethanol, lyophilised, dissolved in water and stored at –20°C.

The assay was performed at 37°C in a 20µl volume of binding buffer containing 0.2ng of radiolabelled DNA. Peptides were added as described in the text. After 20 min incubation, 5µl of loading buffer were added and the samples analysed by electrophoresis in 8% polyacrylamide gels (40:1 acrylamide:N,N'-methylenebisacrylamide) containing TBE. Gels were run in TBE at 120V for 3 hrs, dried and exposed to X-Omat XS1 film at –70°C for autoradiography.

3.2 DNA precipitation assay

Primed M13 ssDNA and activated salmon sperm DNA were labelled with ^{32}P by incubating 2µg of DNA in a volume of 180µl for 30 min with Klenow polymerase under the conditions described in Section 2.3 above. The assay was performed in a volume of 50µl containing 50ng of radiolabelled DNA. Peptides were added as described and the mixture incubated for 20 min at 37°C. The tubes were spun for 10 min at 13000*g* in a bench centrifuge, a 10µl sample of the supernatant was removed onto Whatman DE81 filters as described in Section 2.1.2 above and the radioactivity measured by scintillation counting.

4. Measurement of protein concentration

The Bio-Rad Protein Assay (Micro-assay) was carried out according to the manufacturer's instructions, using bovine γ-globulin as a standard.

RESULTS

1. Oligopeptides

The peptides were numbered 1–96: the *n*th peptide contained amino acids $5n-6$ to $5n+8$ in the published amino acid sequence of UL42 (McGeoch *et al*, 1988a). There were a few exceptions: peptide 1 contained residues 1–13; peptides 6, 22, 38 and 67 were extended by one residue at the N-terminus to 16 amino acids, in order to avoid peptides with an N-terminal glutamine which could cyclize spontaneously in aqueous solution to form pyroglutamic acid (Grant, 1992); peptides 3, 19, 35 and 64 were correspondingly shortened to 14 amino acids (Table 5).

Analysis by mass spectrometry (MS) showed that all 96 peptides were of the correct molecular weight (Table 5). Most were analysed by time-of-flight-MS which is not always accurate to within one mass unit. This accounts for the differences of 2 or 3 mass units between the calculated and the measured M_r values (Table 5). Several peptides were reanalysed by FAB-MS, which is an inherently more accurate technique, and the predicted and measured M_r values corresponded to within one mass unit (peptides 18, 27, 53, 54, 94, 95, 96).

The yields were mostly within 50% of the maximum expected yield (Table 5). The yields of peptides containing both Arg and Trp, which were cleaved by the TMSB method, were exceptionally low: only 10% of the expected amount. The first step of this method used 5% TFA in DCM to cleave the peptide from the resin, which was in fact too low a concentration of acid for complete cleavage. Thus a large proportion of the peptide remained attached to the resin and was lost.

Of the 96 peptides, 93 were soluble in water, although at least 19 required the solution to be acidified or made alkaline, depending on their charge. Peptides 43, 54 and 61, were very poorly soluble. To check that this was a sequence-dependent characteristic and not caused by impurities or a poor synthesis, these three peptides were resynthesised individually on single columns. The resynthesised peptides were as insoluble as the original ones.

Table 5. UL42 PEPTIDES

Peptide no	Sequence	Residues in UL42	Calculated M_r	Measured M_r	Yield (mg)	Purity (%)
1	MTDSPGGVAPASP	1–13	1185	1186	19	96.4
2	SPGGVAPASPVEDAS	4–18	1339	1340	22*	66.2
3	APASPVEDASDASL	9–22	1328	1329	18*	75.2
4	VEDASDASLGQPEEG	14–28	1502	1503	19*	91.1
5	DASLGQPEEGAPCQV	19–33	1499	1500	24	94.6
6	GQPEEGAPCQVVLQGA	23–38	1581	1581	32	62.2
7	APCQVVLQGAELNGI	29–43	1510	1511	20	73.2
8	VLQGAELNGILQAFA	34–48	1542	1544	25*	53.6
9	ELNGILQAFAPLRTS	39–53	1628	1629	30*	86.9
10	LQAFAPLRTSLLDSL	44–58	1643	1645	60*	93.9
11	PLRTSLLDSLLVMGD	49–63	1629	1629	23*	51.0
12	LLDSLLVMGDRGILI	54–68	1627	1628	25*	75.2
13	LVMGDRGILIHNTIF	59–73	1698	1699	67*	87.6
14	RGILIHNTIFGEQVF	64–78	1743	1746	59	64.8
15	HNTIFGEQVFLPLEH	69–83	1780	1780	19*	50.7
16	GEQVFLPLEHSQFSR	74–88	1773	1773	36*	87.8
17	LPLEHSQFSRYRWRG	79–93	1931	1931	57	50.0
18	SQFSRYRWRGPTAAF	84–98	1828	1827	6	74.5
19	YRWRGPTAAFLSLV	89–102	1636	1636	5	81.6
20	PTAAFLSLVDQKRSL	94–108	1644	1645	36*	70.7
21	LSLVDQKRSLLSVFR	99–113	1759	1761	37*	53.6
22	DQKRSLLSVFRANQYP	103–118	1920	1922	43*	39.7
23	LSVFRANQYPDLRRV	109–123	1832	1834	39*	74.2
24	ANQYPDLRRVELAIT	114–128	1757	1759	34*	79.9
25	DLRRVELAITGQAPF	119–133	1684	1683	33*	79.4
26	ELAITGQAPFRTLVQ	124–138	1643	1643	33*	85.3
27	GQAPFRTLVQRIWTT	129–143	1772	1771	22	72.2
28	RTLVQRIWTTTSDGE	134–148	1761	1762	37	78.5
29	RIWTTTSDGEAVELA	139–153	1647	1648	30	76.8
30	TSDGEAVELASETLM	144–158	1551	1553	35	38.8
31	AVELASETLMKRELT	149–163	1689	1691	39	47.8

contd...

Peptide no	Sequence	Residues in UL42	Calculated M_r	Measured M_r	Yield (mg)	Purity (%)
32	SETLMKRELTSFVVL	154–168	1752	1753	32*	26.6
33	KRELTSFVVLVPQGT	159–173	1672	1672	33*	33.1
34	SFVVLVPQGTPDVQL	164–178	1597	1597	32*	84.0
35	VPQGTPDVQLRLTR	169–182	1579	1579	31*	83.1
36	PDVQLRLTRPQLTKV	174–188	1762	1763	37*	76.9
37	RLTRPQLTKVLNATG	179–193	1666	1666	38*	46.8
38	PQLTKVLNATGADSAT	183–198	1585	1585	31*	58.9
39	LNATGADSATPTTFE	189–203	1494	1494	19*	97.9
40	ADSATPTTFELGVNG	194–208	1479	1479	29*	87.3
41	PTTFELGVNGKFSVF	199–213	1642	1641	31*	68.9
42	LGVNGKFSVFTTSTC	204–218	1559	1558	30*	34.8
43	KFSVFTTSTCVTFAA	209–223	1608	1610	30*	NS†
44	TTSTCVTFAAREEGV	214–228	1571	1570	31*	45.0
45	VTFAAREEGVSSSTS	219–233	1526	1528	23*	44.7
46	REEGVSSSTSTQVQI	224–238	1606	1608	28*	54.0
47	SSSTSTQVQILSNAL	229–243	1534	1537	40	58.4
48	TQVQILSNALTKAGQ	234–248	1570	1572	40	64.2
49	LSNALTKAGQAAANA	239–253	1399	1400	49	80.0
50	TKAGQAAANAKTVYG	244–258	1449	1450	51	83.1
51	AAANAKTVYGENTHR	249–263	1601	1601	30*	86.7
52	KTVYGENTHRTFSVV	254–268	1736	1739	43*	72.0
53	ENTHRTFSVVVDDCS	259–273	1707	1707	70	62.0
54	TFSVVVDDCSMRAVL	264–278	1640	1640	65	NS†
55	VDDCSMRAVLRRLQV	269–283	1759	1762	60	74.0
56	MRAVLRRLQVGGGTL	274–288	1625	1626	68	76.3
57	RRLQVGGGTLKFFLT	279–293	1691	1691	37*	63.5
58	GGGTLKFFLTTPVPS	284–298	1520	1520	29*	88.5
59	KFFLTTPVPSLCVTA	288–303	1622	1622	37*	61.5
60	TPVPSLCVTATGPNA	294–308	1426	1426	27*	57.8
61	LCVTATGPNAVSAVF	299–313	1448	1448	26*	NS†
62	TGPNAVSAVFLLKPQ	304–318	1540	1540	25*	89.1
63	VSAVFLLKPQKICLD	309–323	1672	1672	35*	40.2
64	LLKPQKICLDWLGH	314–327	1622	1622	12	69.0
65	KICLDWLGHSQGSPS	319–333	1626	1626	28	67.1
66	WLGHSQGSPSAGSSA	324–338	1427	1426	44	87.2

contd...

Peptide no	Sequence	Residues in UL42	Calculated M_r	Measured M_r	Yield (mg)	Purity (%)
67	SQGSPSAGSSASRASG	328–343	1392	1392	55	69.0
68	AGSSASRASGSEPTD	334–348	1378	1378	55	84.3
69	SRASGSEPTDSQDSA	339–353	1493	1493	46	79.8
70	SEPTDSQDSASDAVS	344–358	1494	1494	43	69.4
71	SQDSASDAVSHGDPE	349–363	1500	1500	39	90.6
72	SDAVSHGDPEDLDGA	354–368	1483	1483	48	84.4
73	HGDPEDLDGAARAGE	359–373	1508	1508	58	72.2
74	DLDGAARAGEAGALH	364–378	1422	1422	54	90.2
75	ARAGEAGALHACPMP	369–383	1450	1450	51	83.2
76	AGALHACPMPSSTTR	374–388	1498	1498	57	74.1
77	ACPMPSSTTRVTPTT	379–393	1548	1548	56	80.8
78	SSTTRVTPTTKRGRS	384–398	1633	1633	67	61.6
79	VTPTTKRGRSGGEDA	389–403	1530	1530	52	72.1
80	KRGRSGGEDARADTA	394–408	1545	1546	51	86.2
81	GGEDARADTALKKPK	399–413	1555	1555	66	81.9
82	RADTALKKPKTGSPT	404–418	1569	1569	70	89.4
83	LKKPKTGSPTAPPPA	409–423	1488	1489	59	95.8
84	TGSPTAPPPADPVPL	414–428	1415	1415	51	83.6
85	APPPADPVPLDTEDD	419–433	1547	1547	62	74.1
86	DPVPLDTEDDSDAAD	424–438	1573	1573	58	64.0
87	DTEDDSDAADGTAAR	429–443	1508	1508	54	74.2
88	SDAADGTAARPAAPD	434–448	1384	1384	52	76.0
89	GTAARPAAPDARSGS	439–453	1383	1383	43	88.8
90	PAAPDARSGSRYACY	444–458	1584	1584	59	68.7
91	ARSGSRYACYFRDLP	449–463	1760	1760	70	55.3
92	RYACYFRDLPTGEAS	454–468	1747	1747	59	83.9
93	FRDLPTGEASPGAFS	459–473	1550	1550	56	96.3
94	TGEASPGAFSAFRGG	464–478	1410	1410	51	56.5
95	PGAFSAFRGGPQTPY	469–483	1551	1551	52	89.1
96	AFRGGPQTPYGFGFP	474–488	1598	1598	35	82.8

* Only half (200mg) of the resin was cleaved.

† These peptides were not soluble enough to be analysed.

The soluble peptides were analysed by reverse phase HPLC monitored at 225nm (Table 5). By this method, their average purity was 72%.

2. Optimisation of assay conditions

The effect of the UL42 protein on Pol is detectable as (i) an increase in the rate of incorporation of radiolabelled nucleotides into a template DNA — measured in an "activity" assay — and (ii) an increase in the proportion of full-length DNA synthesised on a singly-primed, single-stranded circular M13 DNA template — measured in a "processivity" assay.

Both of these assays were used to investigate the inhibitory potential of the UL42 peptides. Initially, only the processivity assay was used; in later experiments, the activity assay was introduced. Here, the optimisation of the two assays is combined because taken together the experiments illustrate more fully the effect of UL42 on Pol. An undefined template, such as activated calf thymus DNA is standardly used in polymerase activity assays. However, in the experiments described here, a defined template was used in both types of assay, to allow a direct comparison of the data. The assay conditions used were closely based on those described by Gottlieb *et al* (1990). The following experiments were carried out to ensure that any inhibition would be reflected by a corresponding reduction in the amount of product.

2.1 Titration of UL42

Figure 11 (parts a and b) shows the effect of adding increasing amounts of UL42 to a fixed amount (140fmol) of Pol. As observed previously (Gottlieb *et al,* 1990), the amount of full-length M13 DNA product increases with UL42 concentration up to an amount above which there is no more stimulation, and there is a corresponding decrease in the amount of shorter products. [In this experiment the amount of full-length product apparently falls below the maximum at the highest concentrations of UL42, but this drop was not consistently observed in other experiments (Figure 14).]

Figure 12 (parts a and b) shows the effect of a parallel titration in which the rate of incorporation of [$\alpha^{32}P$] dCTP into M13 DNA was measured. The rate of incorporation (measured from 0–5 min) increases with UL42 concentration up to a

a

UL42(fmol)

0 .08 0.2 0.7 2.1 6.4 19 58 173 520

b

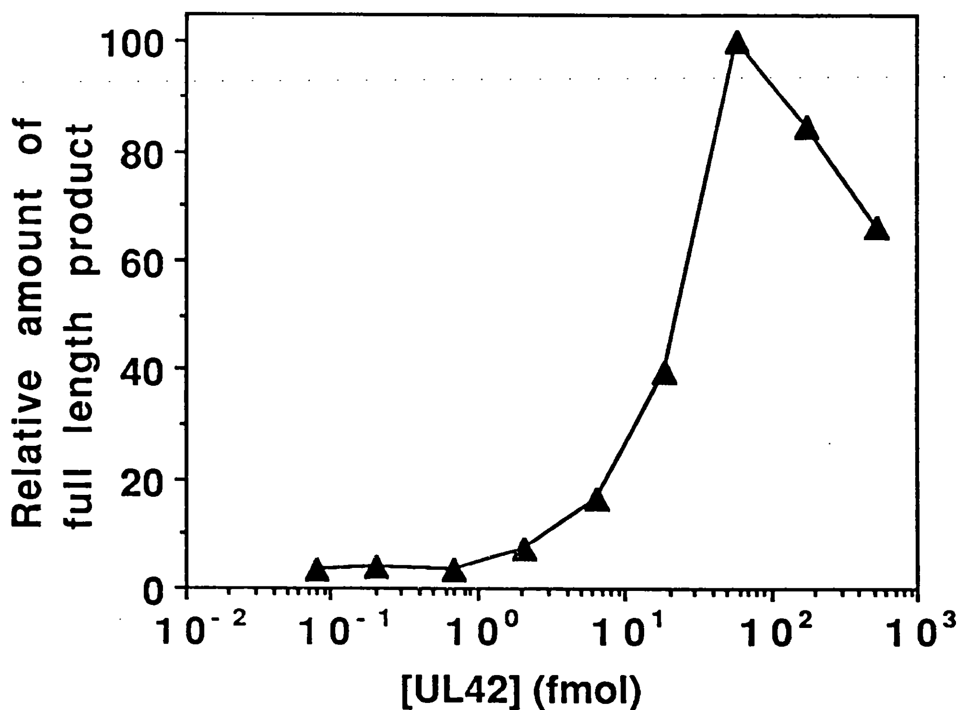


Figure 11. The effect on the processivity of HSV-1 polymerase of adding increasing amounts of UL42 protein to a fixed amount (140fmol) of Pol. The concentration of M13 template-primer DNA was 2μg/ml.

a. An autoradiograph showing the products of reaction analysed by electrophoresis in an alkaline 1% agarose gel. The top band in each track corresponds to full length M13 (not shown). The numbers above each track indicate the amount of UL42 protein (fmol) added to the reaction.

b. A plot of the relative amounts of full length product, obtained by densitometry of the bands in the above autoradiograph, against UL42 concentration.

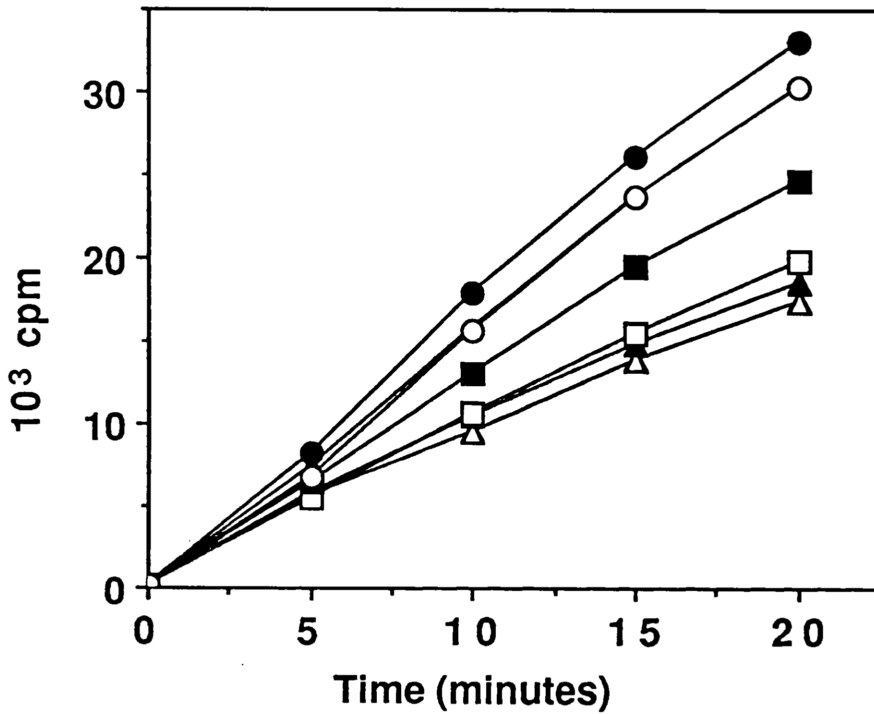
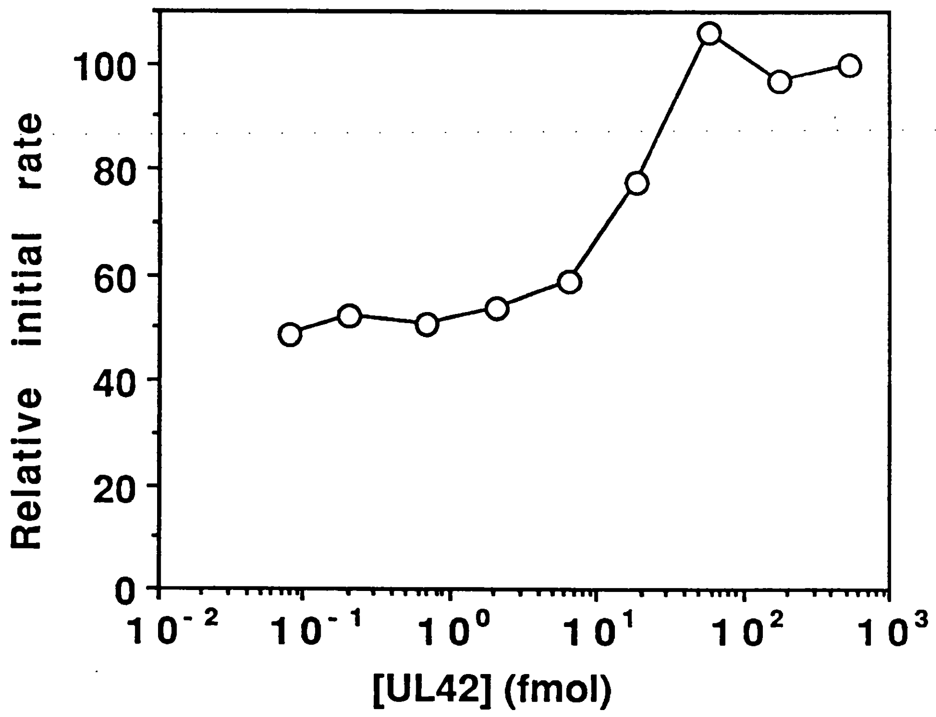


Figure 12. The effect on the activity of HSV-1 polymerase of adding increasing amounts of UL42 protein to a fixed amount of Pol.

a. The rate of incorporation of [α^{32}P] dCTP into DNA by 140fmol of Pol in the absence (△) and in the presence of 2.1fmol (▲), 6.4fmol (□), 19fmol (■), 58fmol (●), 173fmol (○) and 520fmol (masked behind 173fmol) of UL42 protein. The concentration of M13 template-primer DNA was 2μg/ml.



b. A plot of the rates shown in **a** against UL42 concentration.

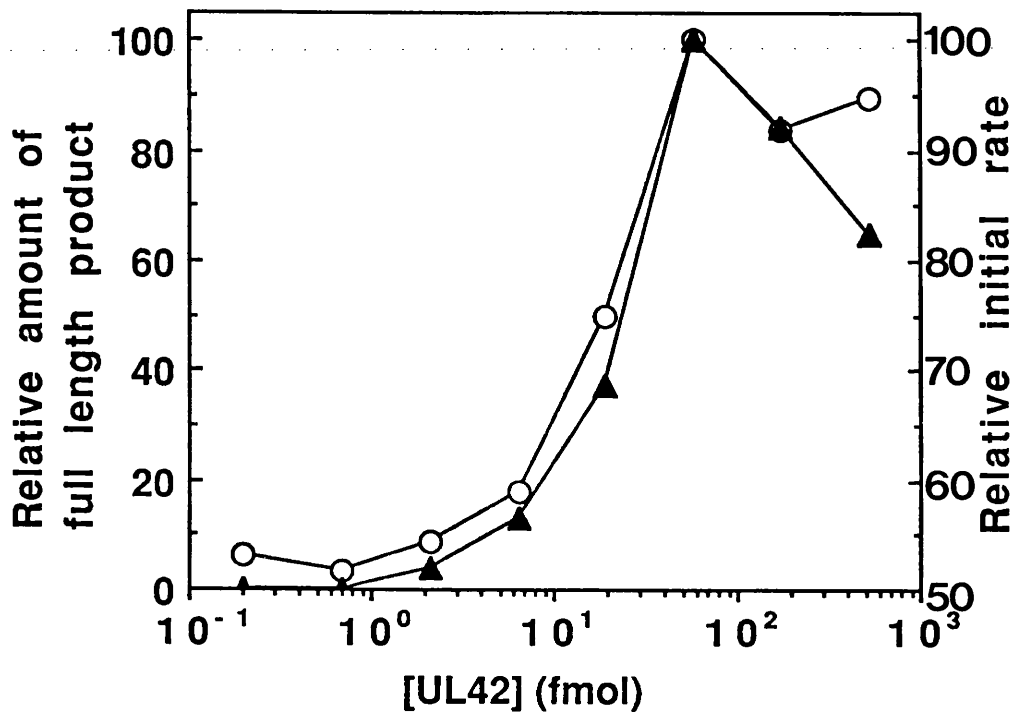


Figure 13. The correlation between the increase in full length product (▲) and the increase in enzyme rate (○) with UL42 concentration. The values for Pol alone have been subtracted and the plots normalised to the maximum at 58fmol UL42.

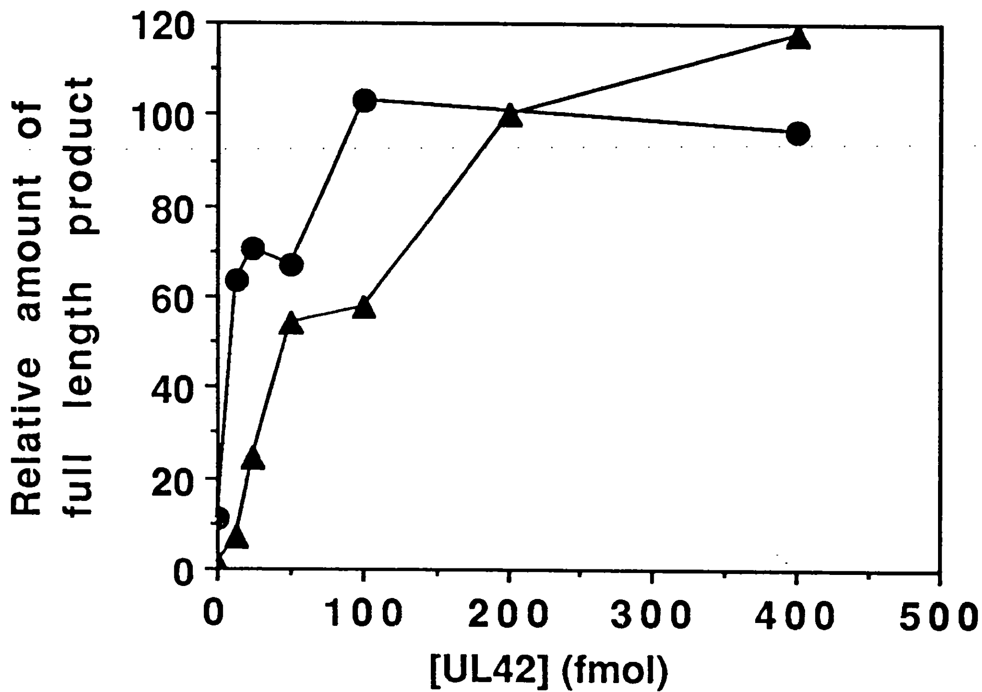


Figure 14. The effect on the processivity of HSV-1 polymerase of adding increasing amounts of UL42 protein (batch 1) to a fixed amount (70fmol) of Pol. The results of two independent experiments are shown, in each of which the amount of product represents the average densitometric measurement of the full length M13 bands in two duplicate samples. The concentration of M13 template-primer DNA was 1μg/ml.

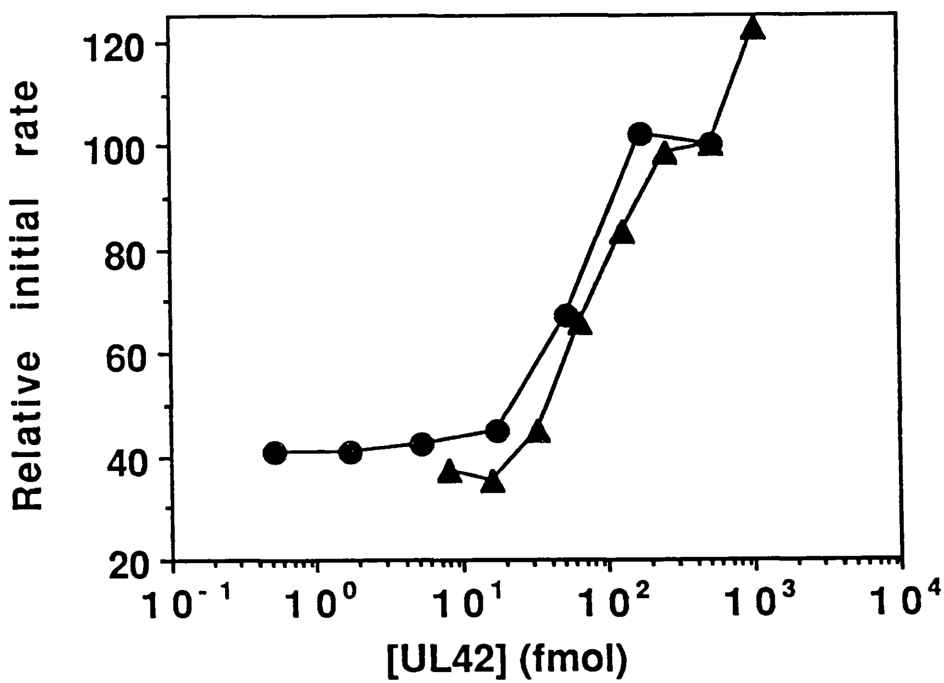


Figure 15. The effect on the activity of HSV-1 polymerase of adding increasing amounts of UL42 protein (batch 2) to a fixed amount of Pol. The rate of incorporation of [α^{32}P] dCTP into singly-primed M13 DNA (2μg/ml) by 140fmol (▲) and 350fmol (●) Pol, in the presence of the indicated concentrations of UL42 protein, was measured in two independent experiments.

a
M13 0 5 10 20 25 30 35 40 M13

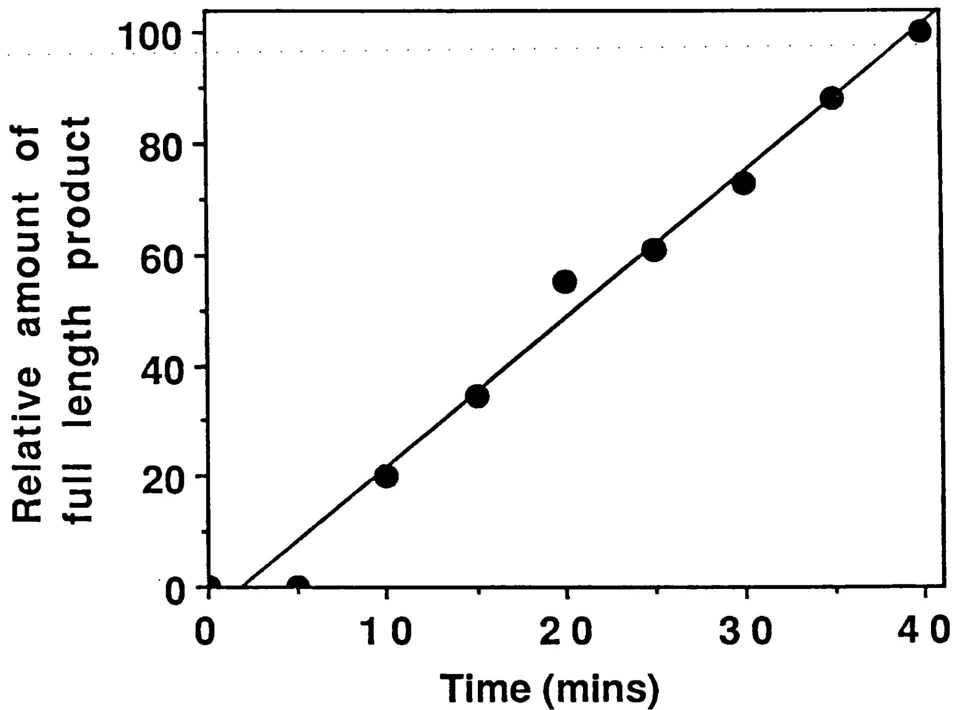


Figure 16. Time course of accumulation of full length product.

a. An autoradiograph showing the products of reaction at 5 min intervals from 0 to 40 min, analysed by electrophoresis in an alkaline 1% agarose gel. The numbers above each track show the time of reaction in minutes. The outermost tracks contain full length, linearised M13 DNA end-labelled with ^{32}P.

b. A plot of the relative increase in the amount of full length product with time. The plotted values represent the average densitometric readings of duplicate samples.

level above which there is no further stimulation. A superimposition of the plots of full-length product and initial rate against UL42 concentration (Figure 13) shows the correlation between the increase in the rate of incorporation of $\alpha^{32}P$ dCTP and the increase in the amount of full-length product, indicating that the two assays detect a stimulation of Pol at similar concentrations of UL42.

The two batches of UL42 used in these experiments were titrated several times using the activity and processivity assays (Figures 14 and 15), and an amount which gave near maximal stimulation was used for further experiments: 70fmol of batch 1 (from John Gottlieb) and 130fmol of batch 2 (from Mary Murphy).

2.2 Time course of accumulation of full-length product

The rate of accumulation of full-length product was monitored. It was found to be linear up to 40 min, after an initial lag of several minutes, as shown in Figure 16 (parts a and b). The initial lag during which there was no appearance of full-length product is due to the time taken by the Pol–UL42 complex to traverse the template. An incubation time of 30 min was chosen for further experiments.

2.3 Synthesis of full-length product by Pol alone

In the absence of UL42 protein, HSV Pol is processive, although, to produce the same amount of full-length, product 5- to 10-fold greater amounts of Pol alone than of Pol–UL42 are required (Gottlieb *et al*, 1990). The rate of accumulation of full-length product was again linear up to 35 minutes after an initial lag, and increased with Pol concentration (data not shown). An incubation time of 30 mins, and 350fmol of Pol (5 times the amount used with UL42) were used for further experiments.

2.4 Substrate concentration and measurement of K_m

To ensure that the DNA substrate was in excess, the rates of accumulation of full-length product in the presence of different concentrations of primed M13 template DNA were measured. A concentration of 0.2µg/ml DNA was found to be rate-limiting, while a concentration of 1µg/ml allowed the maximum rate of

accumulation of full-length product (data not shown). On this basis a concentration of 1μg/ml was used in processivity assays.

The effect of template-primer concentration on enzyme activity was studied in more detail by using the activity assay to measure the K_m for Pol and that for Pol–UL42 for primed M13 DNA. Figure 17a shows progress plots of the rate of incorporation of [$\alpha^{32}P$] dCTP into primed M13 DNA by Pol (350fmol) at four different DNA concentrations. A Lineweaver-Burk double-reciprocal plot of the rate against the substrate concentration is shown in Figure 17b. The K_m derived from the plot is 1nM (2.5μg/ml). The data from a similar experiment to determine the K_m for Pol–UL42 are shown in Figure 18 (parts a and b). The K_m derived from the Lineweaver-Burk plot is 0.14nM (0.35μg/ml).

These results show that the K_m for Pol for the template-primer is about 7-fold lower in the presence of UL42 than in its absence. This is consistent with the observed 5- to 10-fold increase in the affinity of Pol for a synthetic "hairpin" template in the presence of UL42 (Gottlieb and Challberg, unpublished data). Accordingly, M13 template-primer was used at 1μg/ml to assay Pol–UL42 activity and at 10μg/ml to assay Pol alone, which resulted in approximately equal rates of incorporation by Pol–UL42 and Pol (Figure 24).

It is also evident from these data that the concentration of the DNA substrate is one factor which determines the observed magnitude of stimulation of Pol by UL42.

2.5 Salt concentration

All the above experiments were done in the presence of 75mM NaCl. During the course of these experiments it became clear that the ability of UL42 to stimulate Pol was critically dependent on salt concentration (Hart & Boehme, 1992; see Introduction, 4.4.3). The effect of salt concentration on the relative amounts of full-length M13 synthesised by Pol and Pol–UL42 was therefore examined. The results (Figure 19a and b) show that at NaCl concentrations of 76mM and 120mM there is a large difference in the amount of full-length product synthesised in the presence and absence of UL42. In contrast, at lower salt concentrations of 1mM and 41mM NaCl, there is very little difference in the amount of full-length product: Pol alone is almost as processive as Pol–UL42. This is consistent with the finding that the K_m for Pol for the DNA template increases sharply with salt concentration, while that for Pol–UL42 remains relatively unaffected (Hart & Boehme, 1992).

a

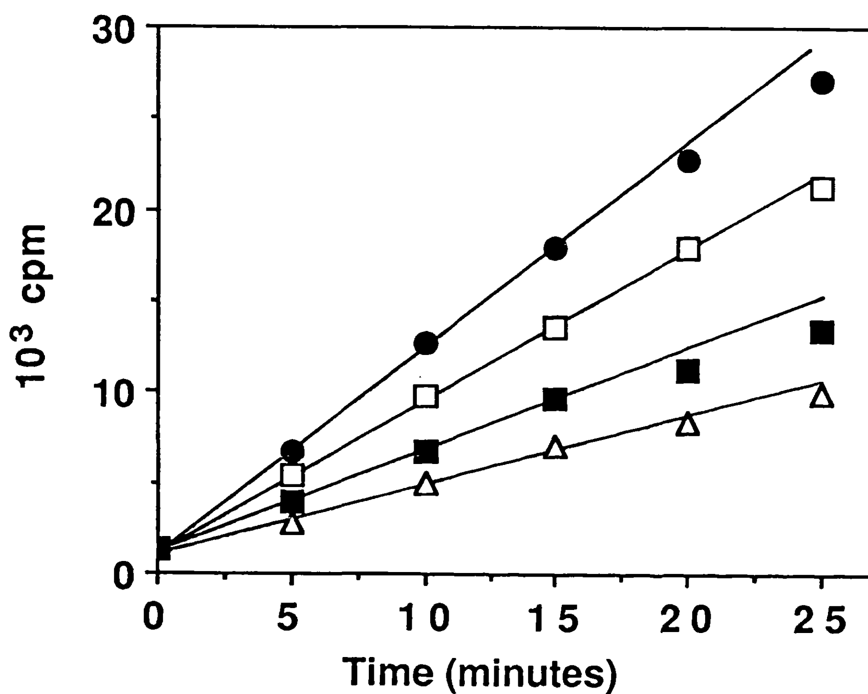
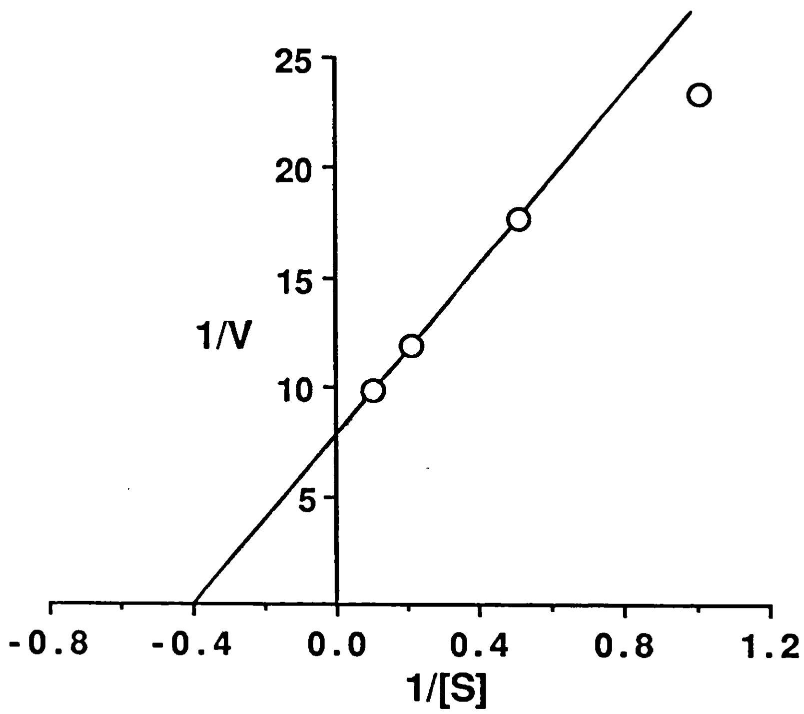


Figure 17. Measurement of K_m for HSV-1 Pol.

a. Progress plots of the rate of incorporation of [$\alpha^{32}P$] dCTP by Pol into singly-primed M13 DNA at 1μg/ml (△), 2μg/ml (■), 5μg/ml (□) and 10μg/ml (●).

b



b. A Lineweaver-Burk double-reciprocal plot of the rate *vs* the substrate concentration. More weight is given to the data at higher substrate concentrations. The negative intercept on the abcissa gives the reciprocal of the K_m.

a

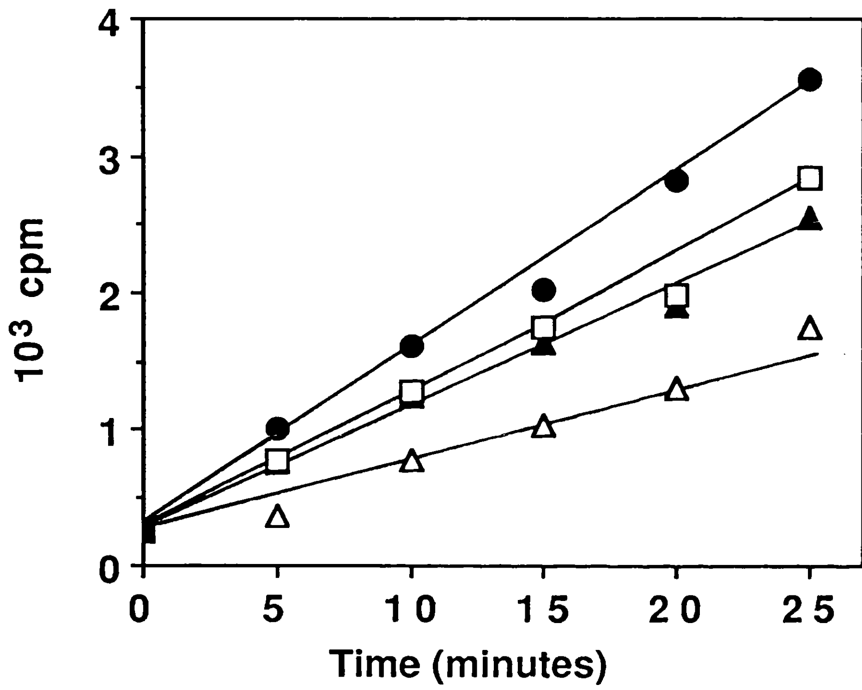
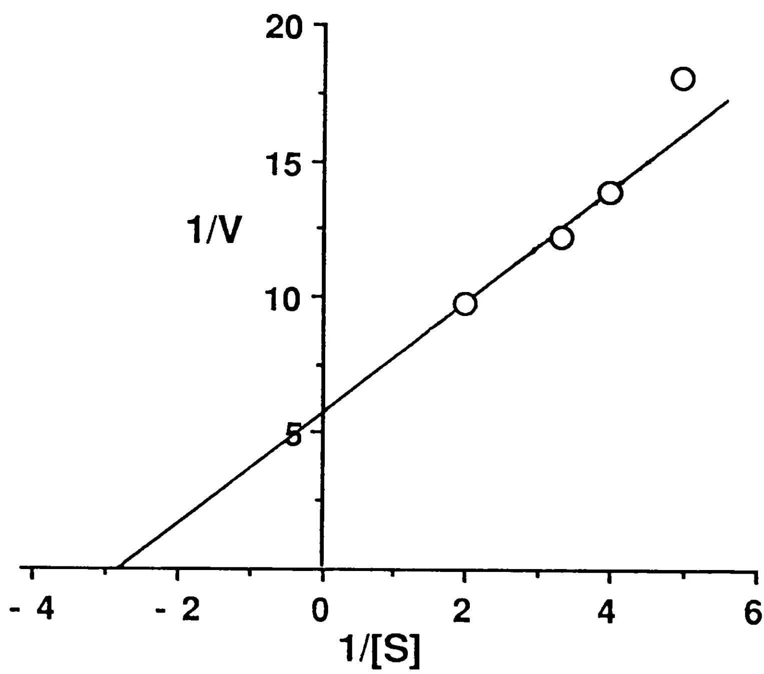


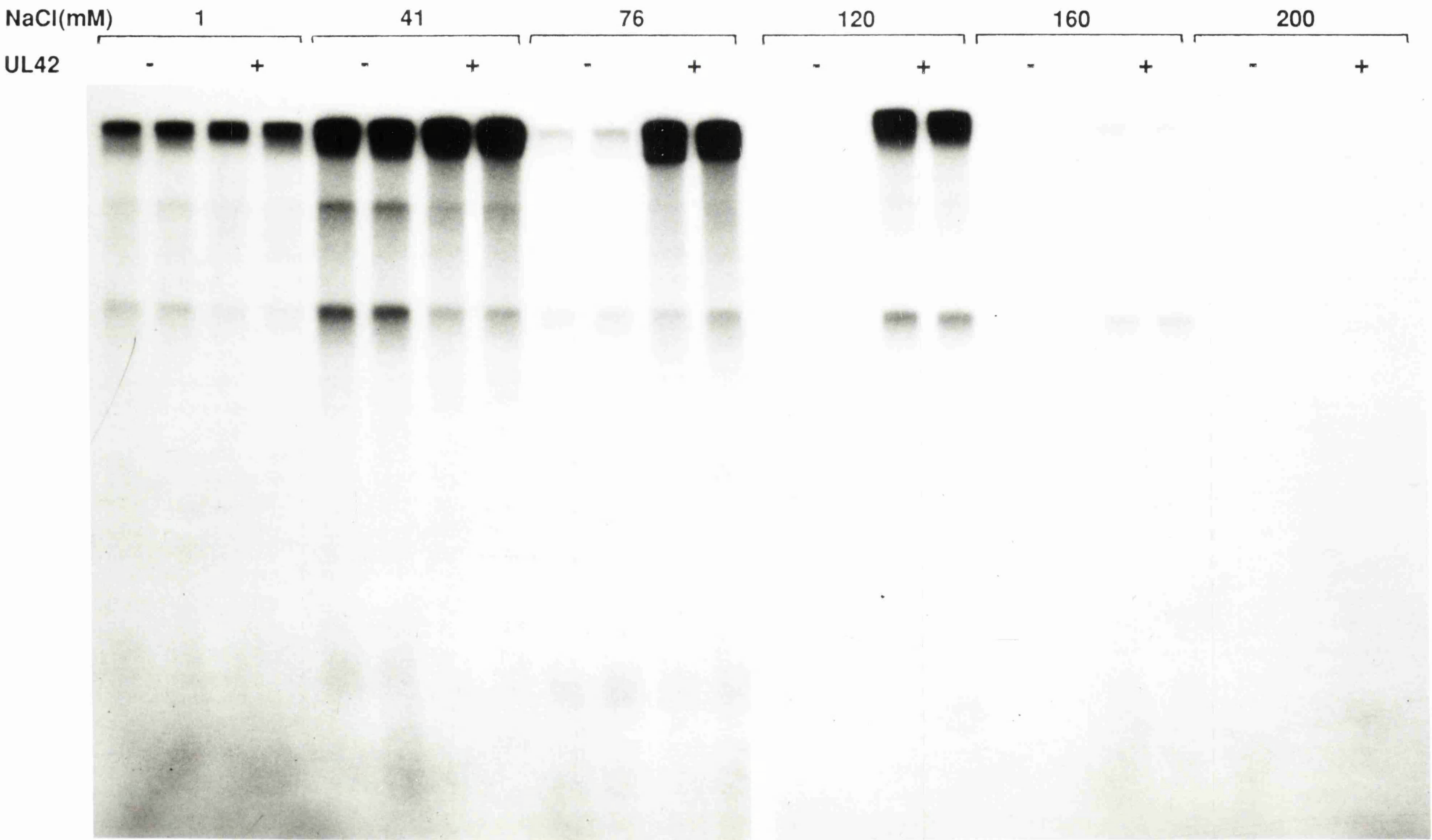
Figure 18. Measurement of K_m for Pol/UL42.

a. Progress plots of the rate of incorporation of [α^{32}P] dCTP by Pol/UL42 into singly-primed M13 DNA at 0.2µg/ml (△), 0.25µg/ml (▲), 0.3µg/ml (□) and 0.5µg/ml (●).

b



b. A Lineweaver-Burk double-reciprocal plot of the rate *vs* the substrate concentration. More weight is given to the data at higher substrate concentrations. The negative intercept on the abcissa gives the reciprocal of the K_m.


a
NaCl(mM)
1
41
76
120
160
200
UL42
- + - + - + - + - + - +

b

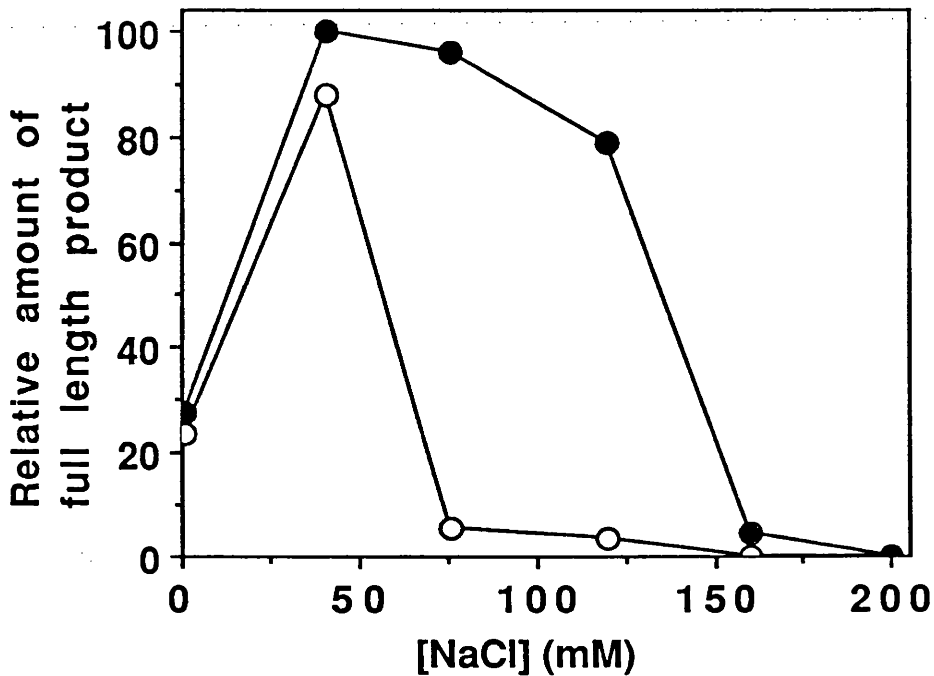


Figure 19. The effect of NaCl concentration on the processivity of Pol in the presence and absence of UL42 protein.

a. An autoradiograph showing the amount of full length product synthesised by Pol in the presence (+) and absence (–) of UL42 at six different concentrations of NaCl. The numbers above the tracks indicate the mM concentration of NaCl in the reactions. The products of the reaction were analysed by electrophoresis in alkaline 1% agarose gels.

b. A plot of the relative amounts of full length product in the presence (●) and absence (○) of UL42, obtained by densitometry of the bands in the autoradiograph shown in **a**, against NaCl concentration.

These data show that 75mM is a suitable concentration of NaCl to test for inhibition of UL42-mediated Pol processivity. It should be noted, in addition, that the K_m values determined in 2.4 above are valid only in the presence of 75mM NaCl.

3. Peptide inhibition of the synthesis of full-length M13 DNA by the Pol–UL42 complex

3.1 Screening of peptides

Peptides were initially screened at a concentration of 50µM, without purification, for their effect on the synthesis of full-length M13 DNA. Each peptide was tested on at least two occasions, first as a single sample and then in duplicate. If the result was unclear, the test was repeated until a reliable result was obtained. Reactions with Pol–UL42 and Pol were always included as controls. Figure 20 shows the results of two such experiments (parts a and b) which identify peptides 47, 48, 57, 58 and possibly 46 as inhibitory. Using this assay, peptides 6, 7, 8, 14, 15, 18, 19, 34, 41, 42, 46, 47, 48, 57 and 58 were found to be inhibitory.

Graham Hart, at Glaxo Group Research, carried out an independent screen of the peptides, testing them at a concentration of 100µM for inhibition of Pol–UL42 activity on an activated DNA template. Only peptides 8, 14, 41, 47 and 48 inhibited Pol–UL42 by more than 50%; a number of others, including peptides 6, 7, 15, 19, 42, 46, and 57 showed some degree of inhibition. The two screens therefore gave essentially similar results.

3.2 Purification of peptides

Two or more peptides from each overlapping set were purified by reverse phase (RP) HPLC for further study (Table 6). Peptides 34, 41 and 42 were found to be non-inhibitory upon purification, so are not included in the table and were not investigated further. Peptide 95 was purified as a non-inhibitory control (purity = >99%). Figure 21 shows an analysis of peptide 15 before and after purification, as an example of the degree of purification typically obtainable by preparative RP-HPLC. The purity of all peptides was determined by analytical RP-HPLC. The purity of peptides 47, 57 and 95 was further checked by capillary zone

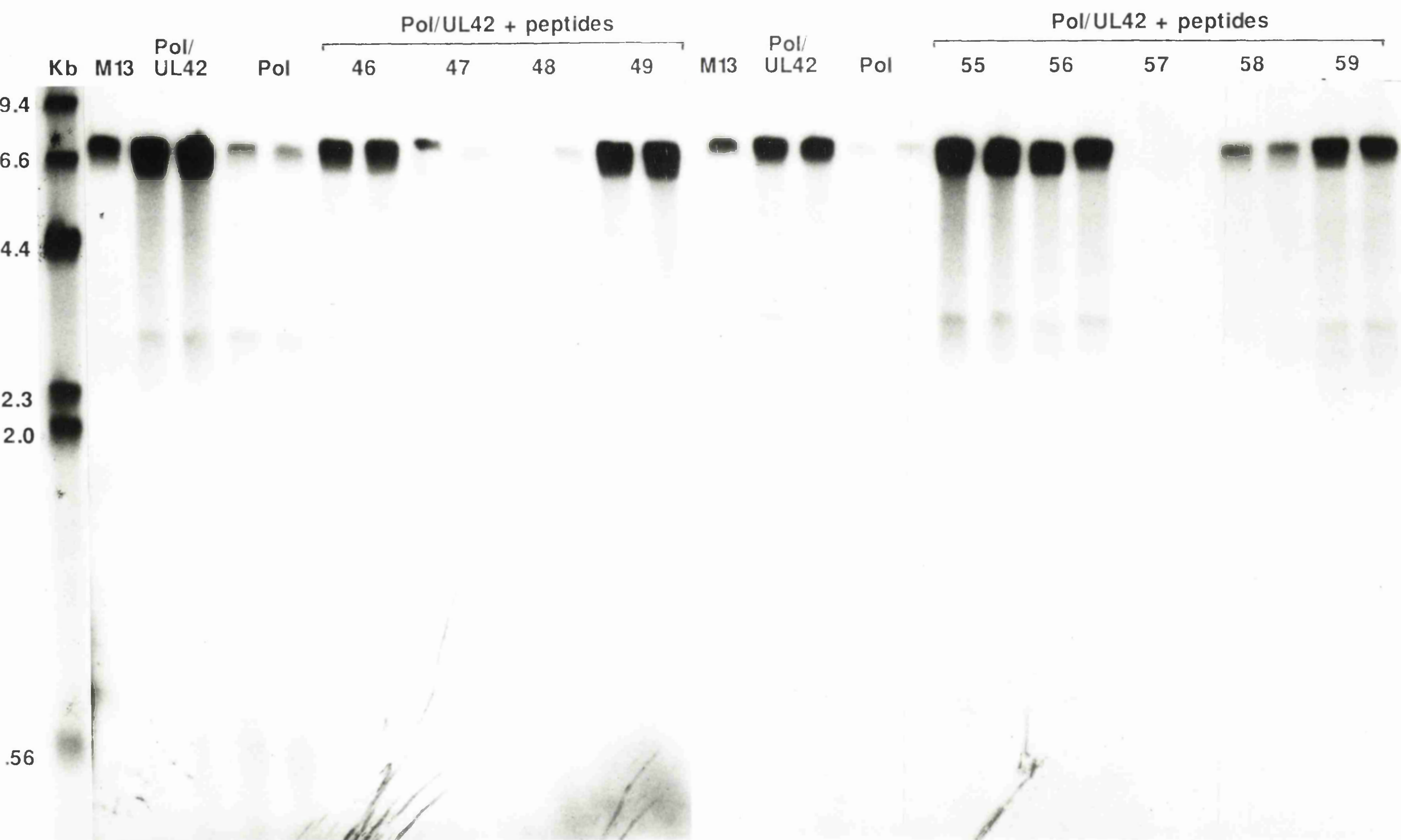
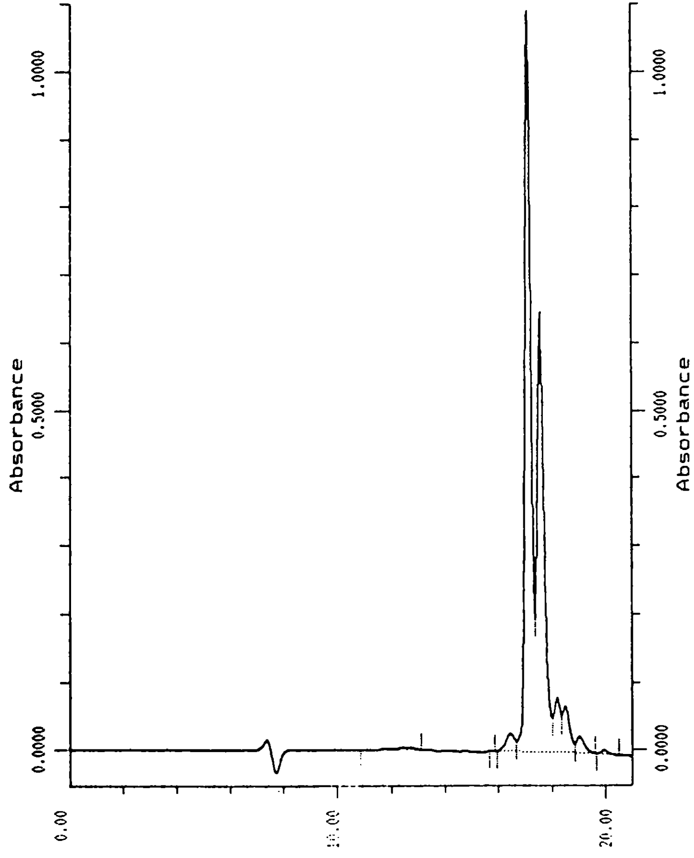

a
b
Kb
M13
Pol/
UL42
Pol
Pol/UL42 + peptides
46
47
48
49
M13
Pol/
UL42
Pol
Pol/UL42 + peptides
55
56
57
58
59
9.4
6.6
4.4
2.3
2.0
.56

Figure 20. The effect of peptides (50μM) on the formation of full length M13 DNA by HSV-1 Pol/UL42. Two separate experiments (parts **a** and **b**) are shown in both of which the formation of full length M13 DNA in the presence of UL42 protein (Pol/UL42) and absence of UL42 protein (Pol) is demonstrated. The effect of peptides 46, 47, 48 and 49 on the formation of full length M13 DNA by 70fmol of Pol/UL42 (Pol/UL42 + peptides) is shown in part **a**, while the effect of peptides 55, 56, 57, 58 and 59 is shown in part **b**. All reactions were performed in duplicate. Also shown are molecular weight markers: λ DNA digested with *Hin*d III (Kb, part **a**) and full length, linearised M13 DNA (M13, parts **a** and **b**) both end-labelled with ^{32}P. The numbers on the left of the figure give the sizes of the products in kilobases. The products of the reaction and marker DNAs were analysed by electrophoresis in alkaline 1% agarose gels.

a



b

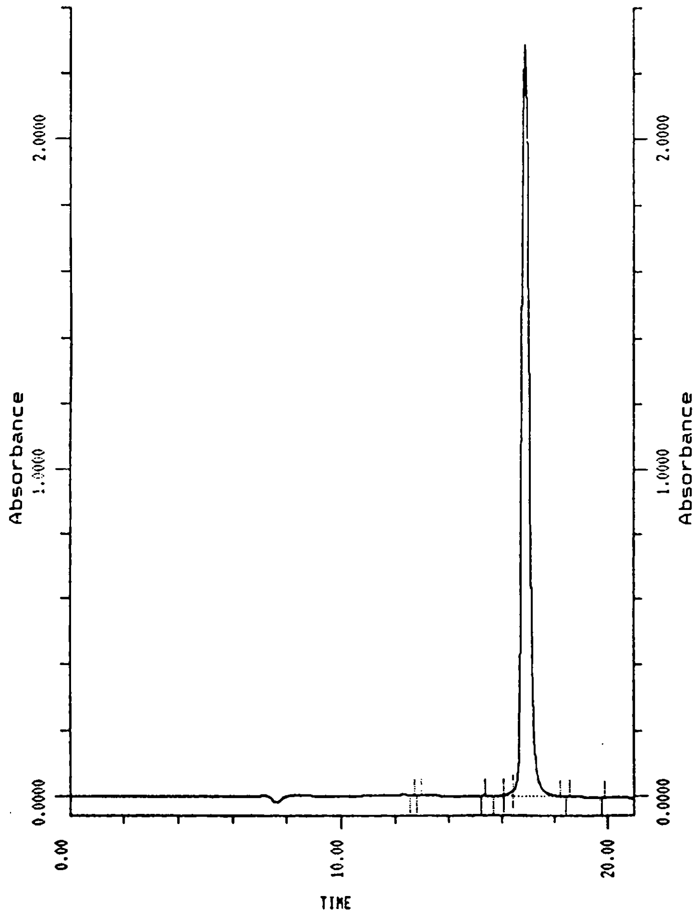


Figure 21. An example of the purification of a peptide by RP-HPLC.

a. A chromatogram of peptide 15 before purification, analysed by RP-HPLC monitored at 225nm. The main peptide peak represents 52.1% of the total material.

b. A similar analysis of the same peptide after purification. The peptide peak represents 99.6% of the total material.

Table 6. INHIBITORY PEPTIDES PURIFIED BY REVERSE PHASE HPLC

Peptide	Sequence	Residues in UL42	Purity (%)
5	DASLGQPEEGAPCQV	19–32	>99
6	GQPEEGAPCQVVLQGA	23–38	96
7	APCQVVLQGAELNGI	29–43	91
14	RGILIHNTIFGEQVF	64–78	97
15	HNTIFGEQVFLPLEH	69–83	>99
18	SQFSRYRWRGPTAAF	84–98	97
19	YRWRGPTAAFLSLV	89–102	>99
46	REEGVSSSTSTQVQI	224–238	97
47	SSSTSTQVQILSNAL	229–243	95–99
48	TQVQILSNALTKAGQ	234–248	>99
57	RRLQVGGGTLKFFLT	279–293	>99
58	GGGTLKFFLTTPVPS	284–298	95

electrophoresis, by Barry Coomber at Glaxo Group Research Ltd, which showed them to be homogeneous.

Purified preparations of peptides 6 and 7 contained two peaks of material when re-analysed after standing in solution for a few hours. The relative proportions of the two peaks varied and could be influenced by time or pH: the later eluting peak increased with time, and also when the pH of the solution was raised by bubbling with ammonia vapour. The nature of the two species was not established but they possibly represent different states of oxidation of the cysteine residue. Independently synthesised batches of peptide 6 displayed identical behaviour.

Peptide 47 and related sequences (truncated peptides and peptides containing one or two amino acid substitutions) showed very unusual properties when subjected to reverse phase HPLC: only a fraction of the peptide (15-20%) was eluted from the column using the standard elution conditions (a 20 min gradient of 0-95% acetonitrile in water). The remainder of the peptide could be eluted using repeated gradients. About 10 consecutive gradients were required to elute all of the peptide from either the analytical or the preparative columns. This behaviour remained essentially unaltered in various chromatographic conditions as follows:

1) doubling the TFA concentration in the solvents;
2) replacing TFA with 30mM ammonium formate;
3) replacing acetonitrile with methanol;
4) replacing acetonitrile with isopropanol;
5) changing from a C8 to a C3 column;
6) eluting at elevated temperatures: 50˚C, 60˚C.

Consequently, purification was achieved by using the standard conditions and eluting the peptide with three consecutive gradients, then washing the column with a sufficient number of gradients to remove all of the residual peptide. Similar behaviour has apparently been observed with strongly α-helical peptides (Peter White, personal communication).

3.3 Determination of IC_{50}

IC_{50} values for the inhibition of full-length M13 DNA synthesis by purifired peptides were measured. Figure 22 shows titrations of peptides 6, 14, 47 and 48 from which IC_{50} values of 3.5μM, 7.5μM, 0.8μM and 9μM, respectively, were determined. The values for peptides 6, 14, 19, 47, 57 and 95 are shown in Table 7.

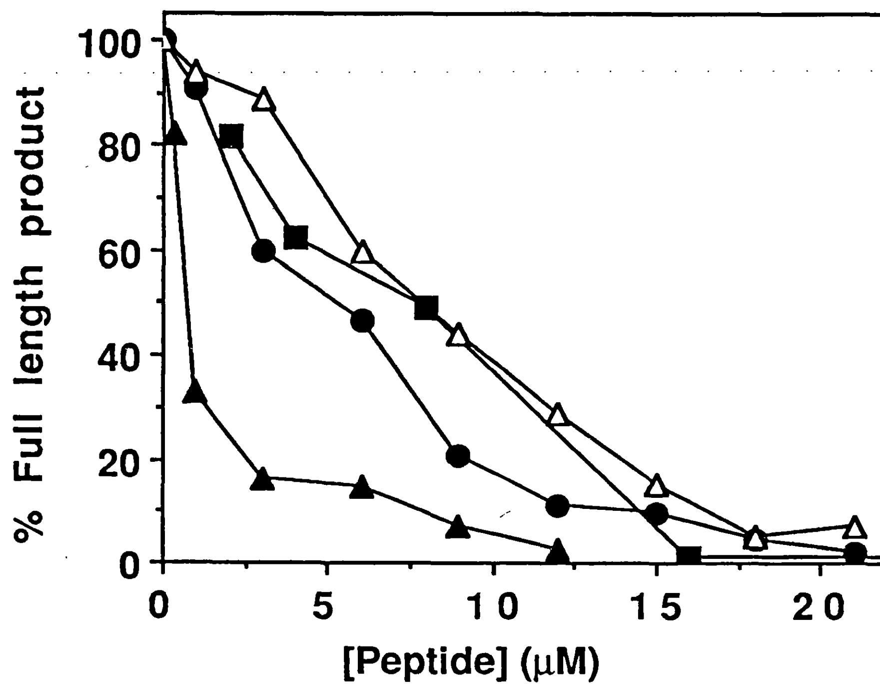


Figure 22. Inhibition of full length product by peptides 6 (●), 14 (■), 47 (▲) and 48 (△). The data were obtained by densitometry of autoradiographs of duplicate titrations, and the amount of full length M13 DNA produced in the presence of peptide was expressed as a percentage of that produced in the uninhibited reaction.

4. Peptide inhibition of the synthesis of full-length M13 DNA by Pol

Peptides were tested for inhibition of full-length M13 DNA synthesis by Pol alone. The results with purified peptides 5, 47, 56, 57 and 95 at a concentration of 50µM are shown in Figure 23 (part a) and show peptides 47 and 57 to be completely inhibitory at this concentration. An IC_{50} of 1.5µM for inhibition by peptide 47 was obtained by densitometric analysis of the data shown in Figure 23b. This value and those for other peptides are listed in Table 7. Two shorter M13 DNA products (5 Kb and 3Kb) accumulate to a greater extent in the absence of UL42 protein (compare Figures 19 and 23) and probably arise from the polymerase 'pausing' at specific sites on the template (compare Figures 20 and 23). Densitometric analysis showed these species to be inhibited to the same extent as the full-length product (data not shown). Synthesis of these shorter molecules by Pol–UL42 was also inhibited by the peptides (Figure 20) as was the accumulation of DNA products of sizes 0.5Kb to 0.7Kb.

5. Peptide inhibition of the rate of DNA synthesis by Pol and Pol–UL42

The action of these peptides was further investigated by determining their effect on the rate of incorporation of [$\alpha^{32}P$] dCTP into DNA. To achieve linear incorporation peptides were pre-incubated for 10 minutes at 37°C with the assay mixture lacking dCTP. The inhibition by peptides 6, 14 and 47 of polymerase activity in the presence and absence of UL42 is shown in Figure 24. The progress plots at various concentrations of all three peptides show virtually linear kinetics over the first 10 minutes of reaction. These rates were plotted against peptide concentration (Figure 27) to determine IC_{50} values (Table 7). The IC_{50} values for peptides 19 and 57 were similarly determined (Figure 27 and Table 7).

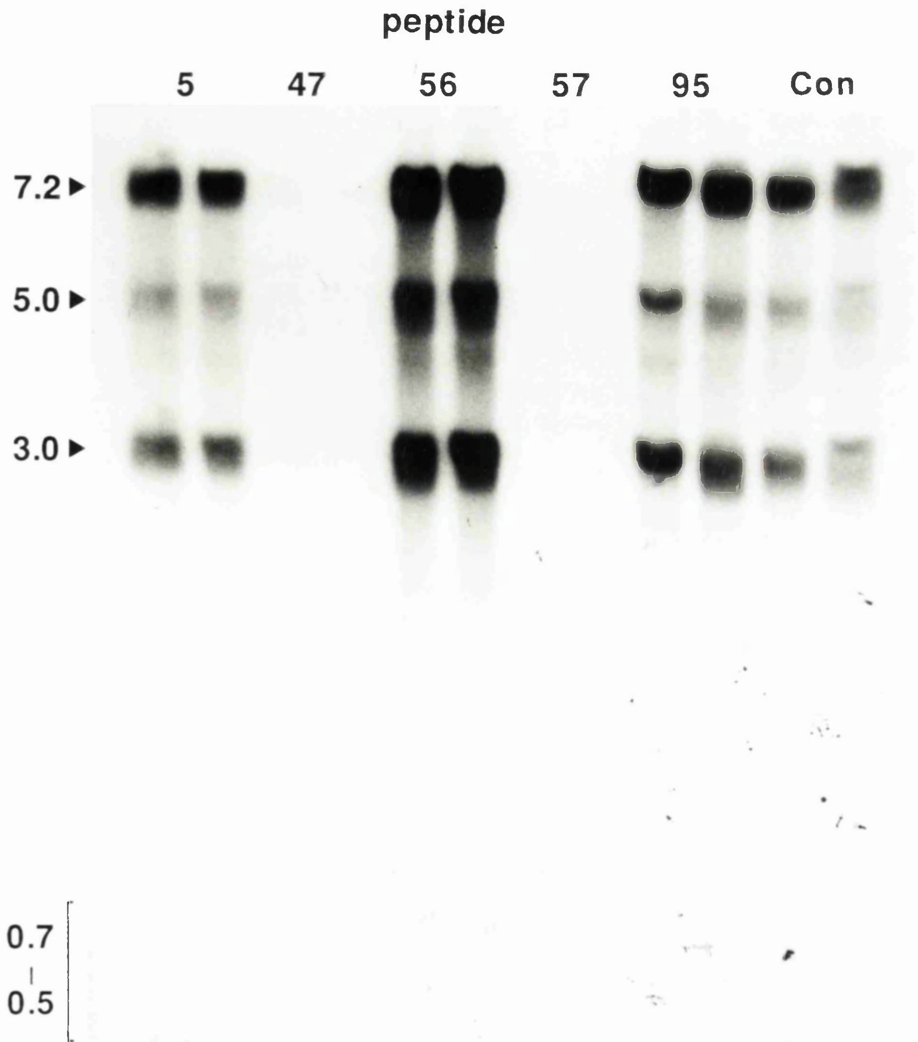

a
peptide
5
47
56
57
95
Con
7.2
5.0
3.0
0.7
0.5
b
peptide 47 concentration (uM)
0
0.3
1
3
6
9
12
M13

Figure 23. The effect of peptides on the synthesis of full length M13 DNA by 350fmol of Pol alone. Part **a** shows the effect of peptides 5, 47, 56, 57 and 95, all at a concentration of 50μM, as well as a control which lacks peptide (Con). Part **b** shows the products formed in the presence of various concentrations of peptide 47. All reactions were performed in duplicate. The single track on the right shows full length, linearised M13 DNA end-labelled with ^{32}P. The DNAs were analysed by electrophoresis in alkaline 1% agarose gels. The numbers on the left of the figure give the sizes of the products in kilobases.

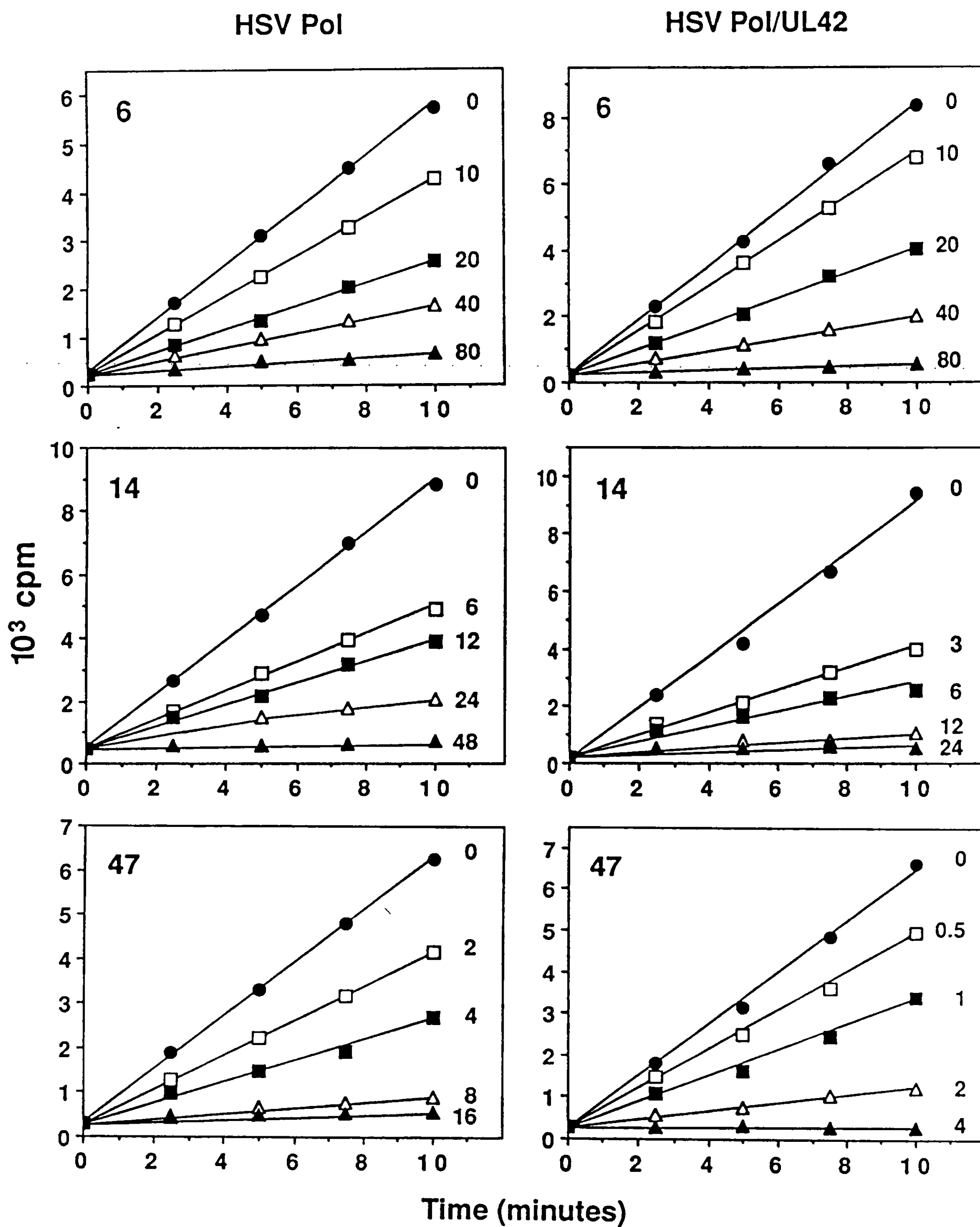


Figure 24. Progress plots of [$\alpha^{32}P$] dCTP incorporation into M13 DNA by Pol (left panels) and Pol/UL42 (right panels) in the presence of peptides 6, 14 and 47 at the indicated concentrations (μM).

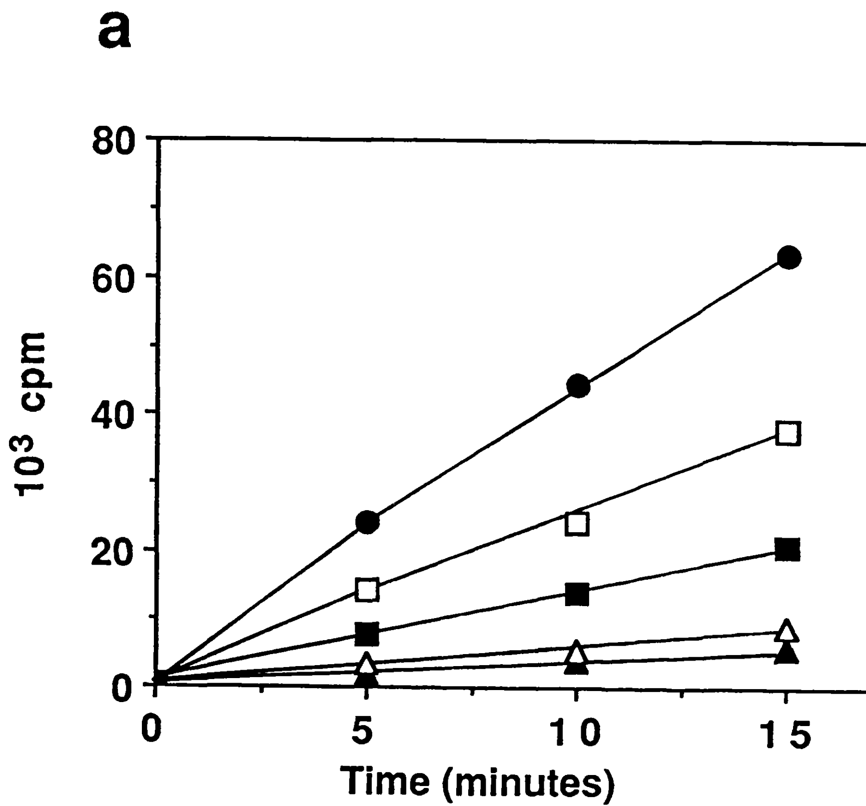
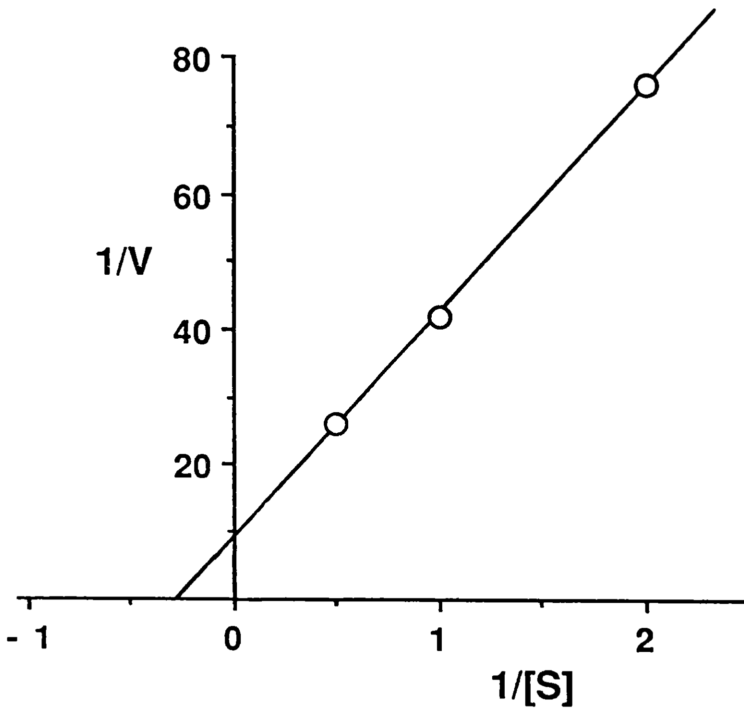


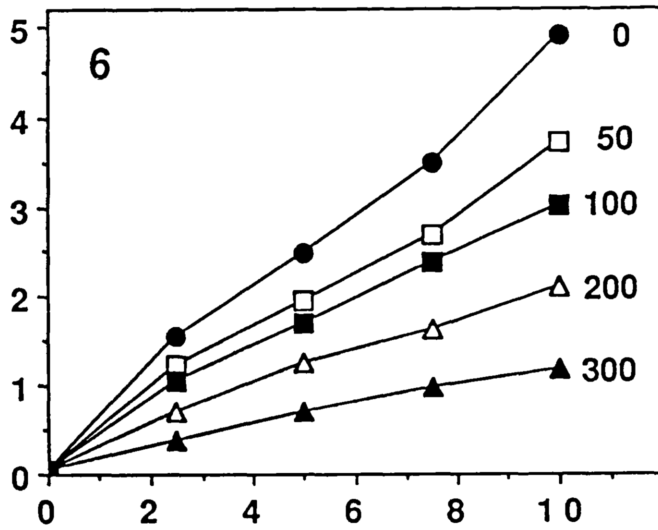
Figure 25. K_m for *E. coli* Klenow polymerase.

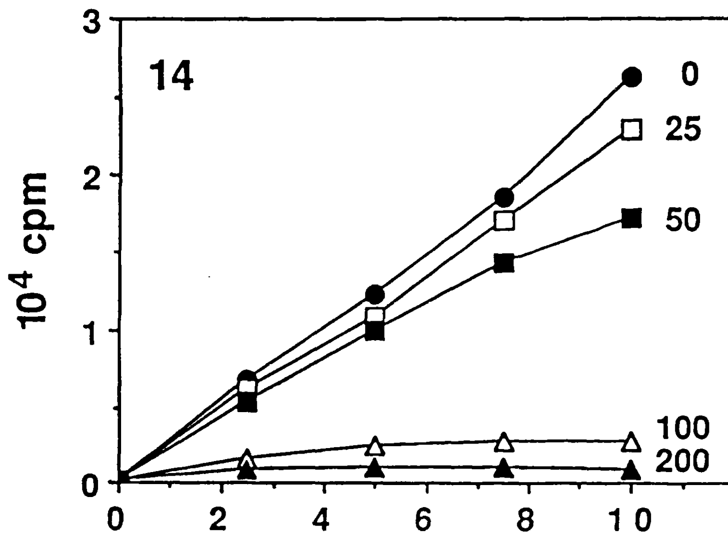
a. Progress plots of the rate of incorporation of [$\alpha^{32}P$] dCTP by Klenow polymerase into primed M13 DNA at 0.08μg/ml (▲), 0.17μg/ml (△), 0.5μg/ml (■), 1μg/ml (□) and 2μg/ml (●).



b. A double-reciprocal plot of the rate *vs* the substrate concentration. (The rates for the two lowest substrate concentrations have not been included.)

Klenow





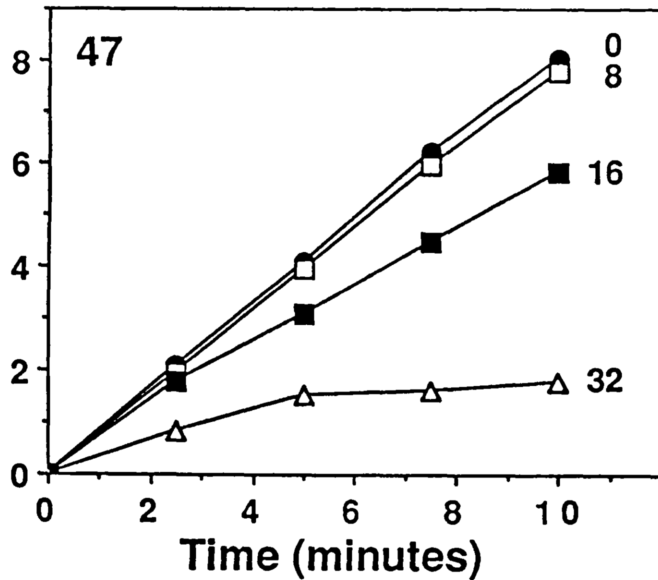


Figure 26. Progress plots of [$\alpha^{32}P$] dCTP incorporation into M13 DNA by Klenow polymerase in the presence of peptides 6, 14 and 47 at the indicated concentrations (μM).

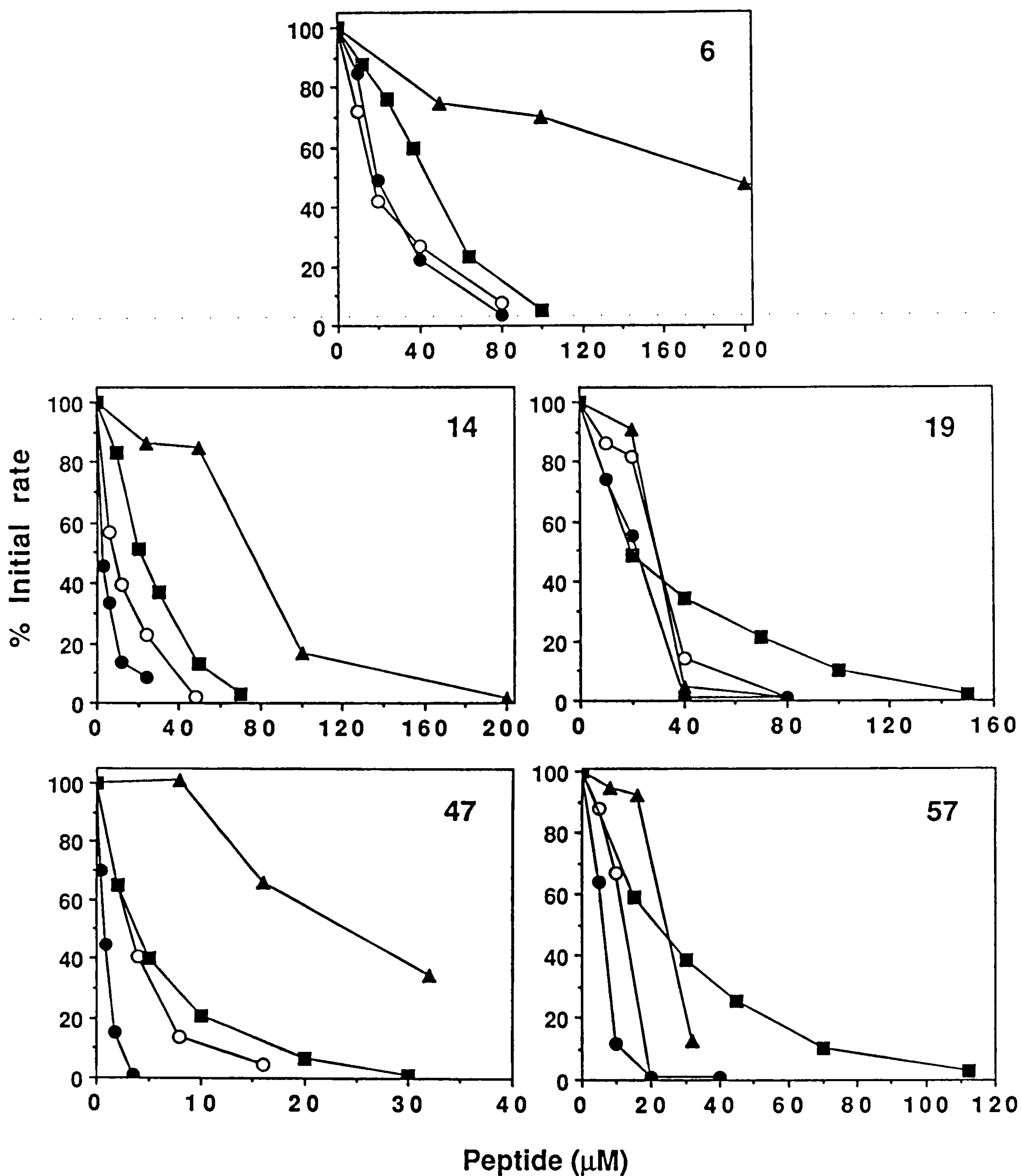


Figure 27. A comparison of the inhibition of HSV-1 Pol (○), Pol/UL42 (●), polymerase α (■) and Klenow polymerase (▲) by peptides 6, 14, 19, 47 and 57. The rate of incorporation of [$\alpha^{32}P$] dCTP into M13 DNA in the presence of peptide is expressed as a percentage of the uninhibited rate.

Table 7. **IC_{50} VALUES (μM) FOR INHIBITION OF POLYMERASE ACTIVITIES**

Peptide	HSV Pol: synthesis of full length M13 DNA		HSV Pol: activity on M13 template		*E. coli* Klenow pol activity on M13 template	Mammalian pol α activity
	–UL42	+UL42	–UL42	+UL42		
6	3.5	3.5	20	18	190	43
14	7.5	7.5	13	5	75	21
19	20	9	30	22	32	19
47	1.5	0.8	3	0.8	21	3.6
57	9	12	12.5	5.5	29	70
95	NI(50)*	NI(50)	NI(100)	NI (100)	NI (300)	NI (200)

* No inhibition was observed at the concentration indicated in the brackets

6. Peptide inhibition of *E. coli* Pol I and mammalian polymerase α

The specificity of these peptides for HSV-1 polymerase was assessed by testing their effect on mammalian polymerase α, and also on the Klenow fragment of *E. coli* Pol I, the catalytic domain of which bears no sequence homology to HSV-1 Pol (Quinn & McGeoch, 1985).

Incorporation of [α^{32}P] dCTP into DNA by the *E. coli* enzyme was linear under the conditions described. The K_m for the Klenow polymerase for the M13 template was found to be 1.6nM (4μg/ml) (Figure 25 a and b), and a concentration of 10μg/ml was used for inhibition experiments.

Progress plots for the Klenow polymerase in the presence of peptides 6, 14 and 47 are shown in Figure 26. Inhibition by peptides 14 and 47 increases with time, particularly at the higher peptide concentrations. To determine IC_{50} values for these peptides, the rates of incorporation between 2.5 and 5 min were used, and the derived IC_{50} values are given in Table 7.

Incorporation of [^{3}H] dTTP into activated calf thymus DNA by pol α in the absence of peptide and in the presence of various concentrations of peptides 6, 14, 19, 47 and 57 was linear with respect to time. The rates of incorporation are plotted in Figure 27 and the derived IC_{50} values are shown in Table 7. The data for pol α inhibition were generated by Graham Hart at Glaxo Group Research.

Figure 27 shows a comparison of the inhibition of HSV-1 Pol, Pol–UL42, pol α and Klenow polymerase by peptides 6, 14, 19, 47 and 57. Inhibition is largely non-specific, although peptides 6, 14, 47 and 57 display some limited specificity. Peptide 6 shows the greatest specificity for the HSV polymerase, being 10-fold less active against Klenow polymerase and 2-fold less active against polymerase α (Table 7).

7. Interaction of peptides with DNA

7.1 Binding of inhibitory peptides to DNA

One mechanism by which the inhibitory peptides could interfere with polymerase would be by blocking its binding to DNA. A gel mobility shift assay was used to investigate their DNA-binding properties. Peptides were tested at a concentration of 50μM, in similar buffer conditions to those used for the polymerase assays, for their ability to complex with a synthetic duplex oligonucleotide. Peptides 14, 18, 19, 47, 48 and 57 formed complexes which had various mobilities, while peptides 6, 7, 15 and 95 did not bind (Figure 28). Identical binding was seen in the presence of 76mM NaCl and in the absence of salt (data not shown). Further binding experiments were done in the absence of salt. Peptide 6, at concentrations of up to 250μM, showed no evidence of binding to DNA (data not shown).

7.2 Screening of the UL42 peptides for DNA-binding

The remaining UL42 peptides were tested for their ability to bind to DNA in the gel mobility shift assay. Peptides were screened in duplicate at a concentration of 50μM, without purification. Twenty peptides, in addition to those already identified above, were found to form complexes with the DNA. All the DNA-binding peptides are listed in Table 8.

The peptides shown in Table 8 (with the exception of peptides 17, 33, 37, 55 and 90) were purified by RP-HPLC to >90% homogeneity and re-tested for DNA binding. Only peptide 67 was found not to bind in a purified form. All further experiments were done with purified peptides.

7.3 Measurement of K_d

The dissociation constant, K_d, for a DNA-binding protein can be estimated from the concentration of protein required to bind half of the DNA in a gel mobility shift assay (provided that the molar DNA concentration is at least 100-fold lower than the protein concentration required to bind half of the DNA) (Carey, 1991). This method was used to obtain an approximate K_d value for the DNA-binding peptides. One such experiment is shown in Figure 29. Part **a** shows an autoradiograph of four-fold dilutions of peptides 46, 47 and 48 tested in the gel mobility shift assay;

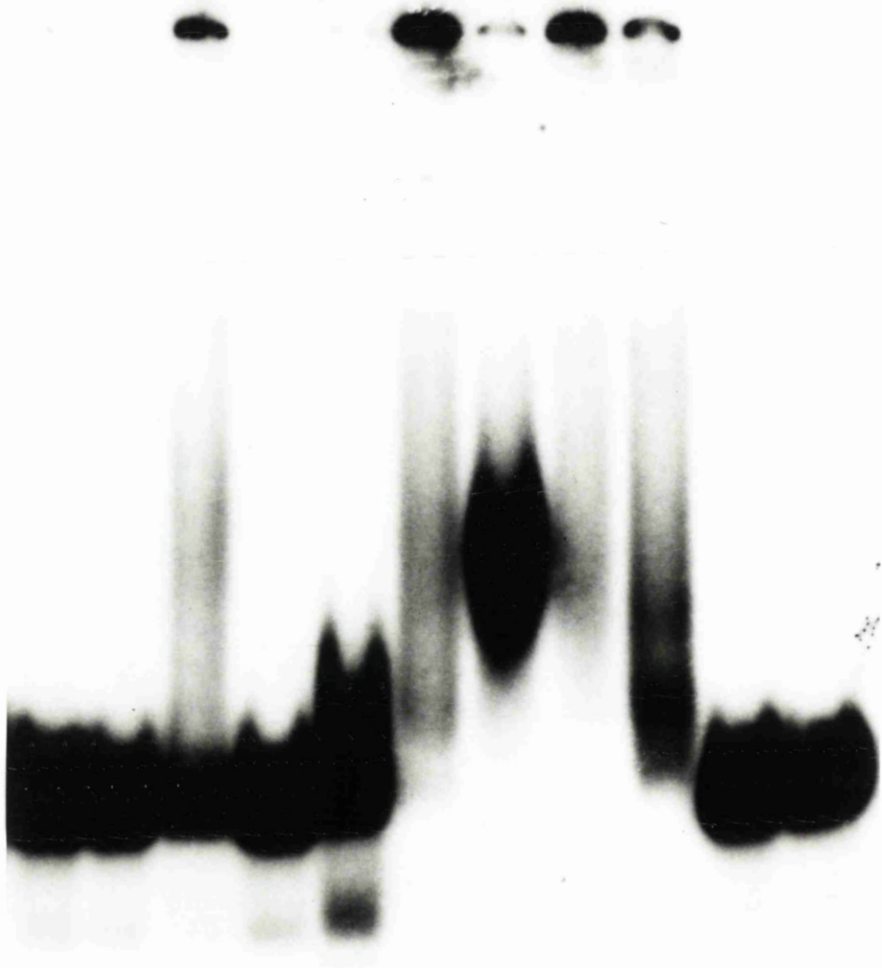

6
7
14
15
18
19
47
48
57
95
Con

Figure 28. The mobility of a duplex oligonucleotide in the absence of peptide (Con) and in the presence of peptides 6, 7, 14, 15, 18, 19, 47, 48, 57 and 95, all at a concentration of 50μM.

a

46 (μM)

64 16 4 1 .25 .06 .016 0

47 (μM)

64 16 4 1 .25 .06 .016 0

48 (μM)

64 16 4 1 .25 .06 .016 0

b

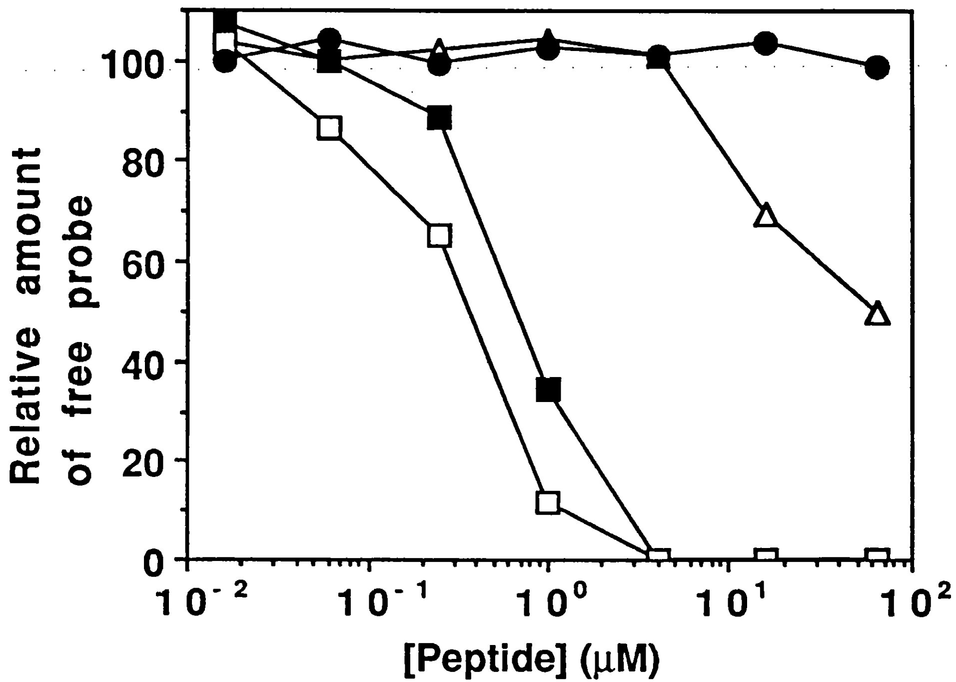


Figure 29. The effect of peptides on the mobility of a duplex oligonucleotide.

a. Autoradiograph showing the complexes formed with four-fold dilutions of peptides 46, 47 and 48. The numbers above the tracks indicate the μM concentration of peptide.

b. Plots of the fraction of free DNA *vs* log of peptide concentration for peptides 46 (△), 47 (■), 48 (□) and 49 (●). The data were obtained by densitometry of the bands in the autoradiograph shown in a, and a similar titration of peptide 49.

Table 8. DNA-BINDING PEPTIDES

Peptide	Sequence	Charge	K_d (μM)
13	LVMGDRGILIHNTIF	+1	8.5
14	RGILIHNTIFGEQVF	+1	15
17	LPLEHSQFSRYRWRG	+3	ND*
18	SQFSRYRWRGPTAAF	+3	>50
19	YRWRGPTAAFLSLV	+2	1.2
27	GQAPFRTLVQRIWTT	+2	5
33	KRELTSFVVLVPQGT	+1	ND
35	VPQGTPDVQLRLTR	+1	12
36	PDVQLRLTRPQLTKV	+2	5
37	RLTRPQLTKVLNATG	+3	ND
41	PTTFELGVNGKFSVF	0	0.9
42	LGVNGKFSVFTTSTC	+1	0.5
46	REEGVSSSTSTQVQI	–1	64
47	SSSTSTQVQILSNAL	0	0.9
48	TQVQILSNALTKAGQ	+1	0.4
55	VDDCSMRAVLRRLQV	+1	ND
56	MRAVLRRLQVGGGTL	+3	15
57	RRLQVGGGTLKFFLT	+3	0.2
58	GGGTLKFFLTTPVPS	+1	0.4
59	KFFLTTPVPSLCVTA	+1	1
62	TGPNAVSAVFLLKPQ	+1	4.5
63	VSAVFLLKPQKICLD	+1	0.8
67	SQGSPSAGSSASRASG	+1	ND
90	PAAPDARSGSRYACY	+1	ND
91	ARSGSRYACYFRDLP	+2	11
92	RYACYFRDLPTGEAS	0	ND

* K_d not determined

part **b** displays the densitometric data from the same autoradiograph as a Bjerrum plot of the fraction of free DNA *vs* log of peptide concentration.

Peptides 47 and 48 form different types of peptide-DNA complexes (Figure 29a). Peptide 48 precipitates most of the DNA into a complex that does not enter the gel. A similar phenomenon has been observed with very basic proteins (Carey, 1991). Several of the basic UL42 peptides precipitated the DNA in this way. Peptide 47 forms a soluble complex which migrates as a discrete band at the higher peptide concentrations, but which increases in mobility and becomes less defined at lower peptide concentrations (see also Figures 30 and 31). Other peptides formed complexes which smeared throughout the length of the gel.

The K_d values obtained by this method (Table 8) are probably not very accurate, but they give some indication of the relative binding strengths. Only one peptide (47) was titrated more than once: the values from three independent titrations were 0.4μM, 0.7μM and 1.7μM.

7.4 Peptide 47

The following experiments were designed to test the possibility that peptide 47 might represent a major DNA-binding region of UL42, and were carried out with purified peptides.

7.4.1 Competition of peptide 47 with UL42 protein

Titration of peptide 47 in the presence of UL42 protein showed that, at a 70-fold molar excess, the peptide prevented the protein from binding to DNA, but at an excess of 23-fold or lower it did not compete (Figure 30).

7.4.2 Mapping of residues important for binding

To identify the residues important for binding to DNA, truncated peptides were synthesised. However, none of the peptides truncated by more than two amino acids was soluble and therefore could not be adequately tested. The shortest soluble peptides were:

301: STSTQVQILSNAL

302: SSSTSTQVQILSN

K_d values were 2μM for peptide 301 and 9μM for peptide 302, compared with 0.9 μM for peptide 47 itself. The relative binding affinities of peptides 46, 47, 48 and

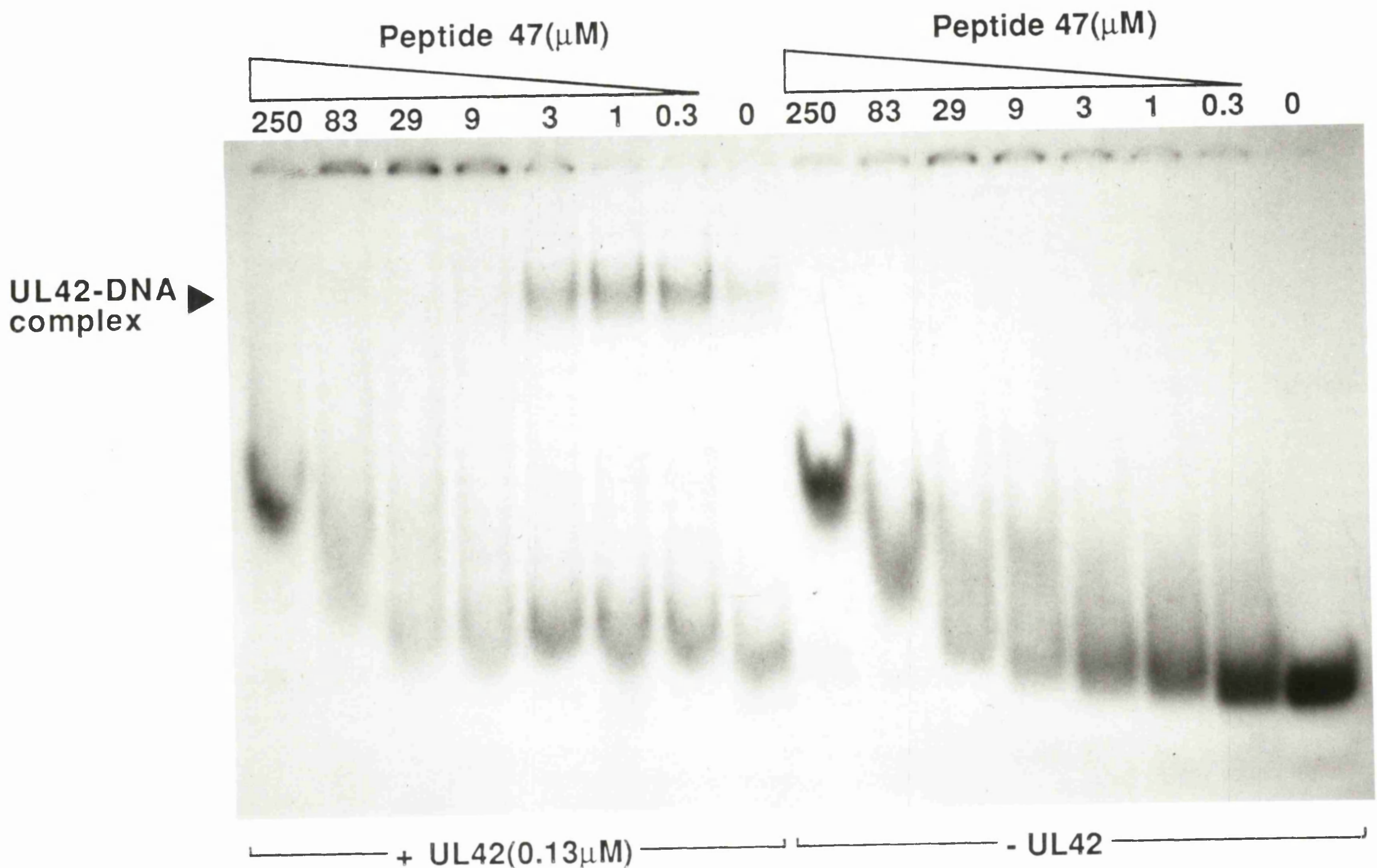
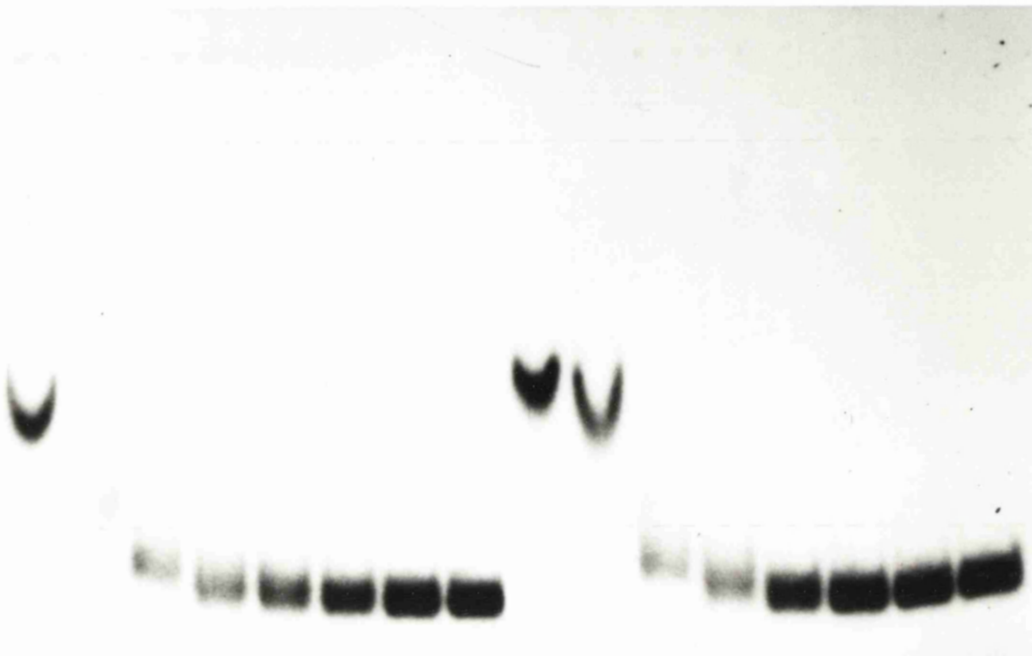
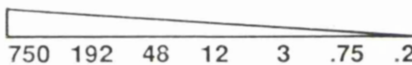

Peptide 47(μM)
250 83 29 9 3 1 0.3 0
Peptide 47(μM)
250 83 29 9 3 1 0.3 0
UL42-DNA complex
+ UL42(0.13μM)
- UL42

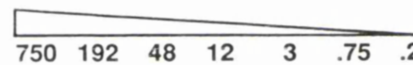
Figure 30. The binding of three-fold dilutions of peptide 47 to a duplex oligonucleotide in the presence and absence of UL42 protein (0.13μM), as indicated below the tracks. The numbers above each track indicate the μM concentration of peptide present in the reaction. The position of the UL42–DNA complex is indicated.

301(μM): STSTQVQILSNAL
750 192 48 12 3 .75 .2 0

372(μM): LQASNVSTLQITS
750 192 48 12 3 .75 .2 0




371A(μM): STSTEVEILSNAL
750 192 48 12 3 .75 .2 0


371B(μM): STSTEVEILSDAL
750 192 48 12 3 .75 .2 0

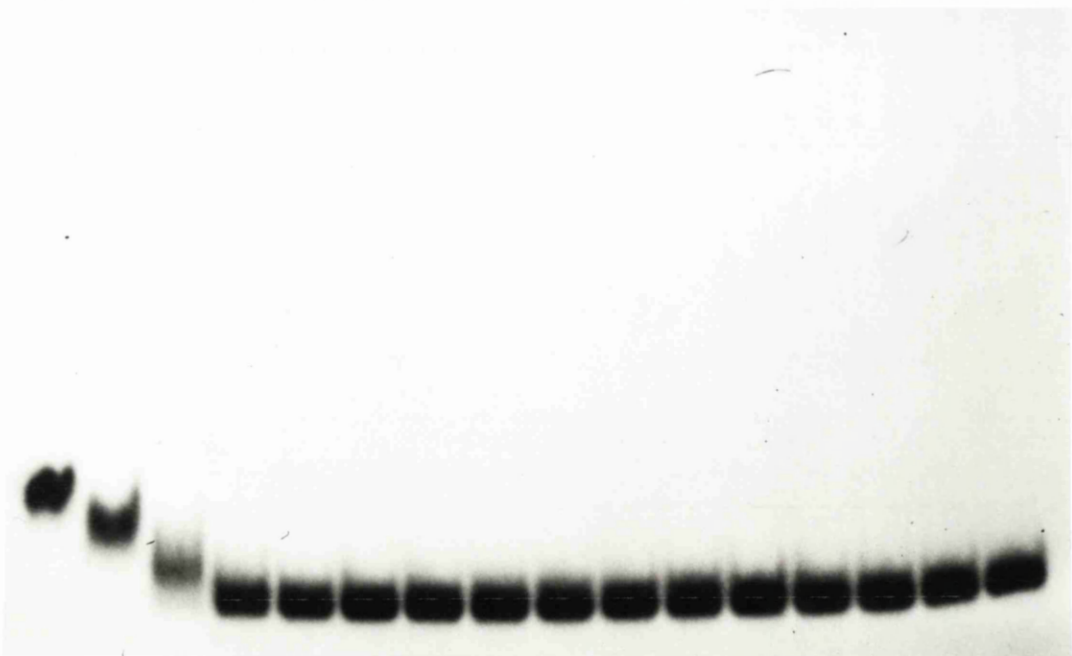


Figure 31. The binding of four-fold dilutions of peptide 301 and peptides with variant sequences to a duplex oligonucleotide. The sequence and μM concentration of peptide present in each reaction is indicated above the tracks.

Table 9. DNA BINDING OF SUBSTITUTED AND SCRAMBLED PEPTIDES

Peptide	Sequence	K_d (μM)
47	SSSTSTQVQILSNAL	0.9
301	STSTQVQILSNAL	2
363A	STSTQVEILSNAL	75
363B	STSTEVQILSNAL	17
371A	STSTEVEILSNAL	62
371B	STSTEVEILSDAL	>750
372	LQASNVSTLQITS	6

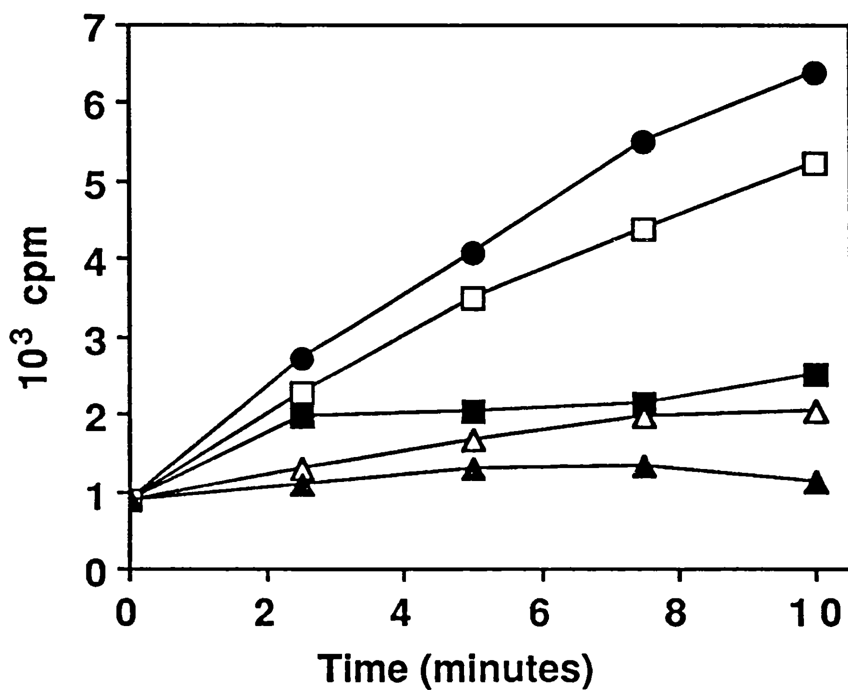


Figure 32. Progress plots of [α^{32}P] dCTP incorporation into M13 DNA by HSV-1 Pol in the absence of peptide (●) and in the presence of peptides 301 (■), 371A (△), 371B (□) and 372 (▲), all at a concentration of 100μM.

49 (Figure 29 and Table 8) suggest that the whole of the sequence common to peptides 47 and 48 (TQVQILSNAL) is necessary for strong DNA binding, while neither half of it, present in peptides 46 and 49, is sufficient.

Acidic residues were substituted for the glutamine and asparagine residues in the sequence of peptide 301, generating the series of peptides shown in Table 9. The DNA binding of all of the substituted peptides was lower than that of the original sequence. DNA binding was reduced more by substituting the central glutamine, Q_7, (peptides 363A and 371A) than by substituting Q_5 (peptide 363B) with a negatively charged glutamic acid (Table 9). Binding was only abbrogated completely by the introduction of three negatively charged residues, in peptide 371B (Figure 31 and Table 9).

The relative importance of amino acid sequence and composition for binding was investigated by synthesising a scrambled sequence with the same amino acid composition as 301. The scrambled peptide (372) bound to DNA almost as efficiently as the native sequence (Figure 31 and Table 9). The measured K_d was 3-fold higher, but this is within the margin of error of this technique.

An experiment was carried out to investigate whether the DNA-binding properties of the substituted and scrambled peptides correlated with their capacity to inhibit HSV-1 Pol. Peptides 301, 371A, 371B and 372 were tested at 100μM for their effect on Pol activity. The results (Figure 32) showed that the peptides which bound to DNA to a measurable extent, *ie* 301, 371A and 372 (Table 9), substantially inhibited Pol activity, whereas peptide 371B, which did not bind to DNA, had only a slight, and probably insignificant, effect on Pol.

7.5 DNA precipitation

All the DNA-binding experiments had been carried out using a short synthetic duplex oligonucleotide unrelated in sequence to the M13 or activated DNA templates used in the polymerase assays. To demonstrate unequivocally that the DNA-binding peptides were capable of precipitating template DNA, peptides were incubated with radio-labelled M13 and activated salmon sperm DNAs. The radioactivity remaining in solution was measured after centrifugation. Incubation with a 50μM concentration of peptides 14, 19, 47 and 57 removed over 95% of the DNA from solution (Table 10). Peptide 6, in contrast, appeared to increase the

Table 10. PRECIPITATION OF DNA BY PEPTIDES

Peptide	% DNA remaining in solution	
	M13	Activated SS
None	100	100
6	109.7	132.7
14	0.2	1.0
19	0.02	0.6
47	2.5	2.7
57	0.1	0.1
95	53.5	30.9

number of counts in the sample, probably by preventing binding of the DNA to the walls of the plastic Eppendorf tube.

The finding that peptide 95 also precipitated about half of the DNA was unexpected, since it did not appear to bind to DNA in the gel retardation assay, nor did it inhibit in the polymerase assay, in fact it was used throughout as a non-inhibitory control. Peptide 95 has a charge of +1 and could therefore be expected to interact with DNA, as observed with many of the other positively-charged peptides.

8. The effect on Pol–UL42 of peptides from the C-terminus of Pol

Peptides corresponding to the C-terminus of Pol were tested in the processivity assay for their ability to disrupt the Pol–UL42 interaction. Peptide 384 encompassed residues 1209–1235, and peptide 386, residues 1194–1223 (Figure 33a).

These peptides were first tested, at 10μM–100μM, for their ability to precipitate DNA. Peptide 384 (charge = +1) was found to precipitate about 50% of the DNA, and peptide 386 (charge = –1) apparently increased the amount of DNA in solution by 30% (data not shown). The extent of DNA precipitation by peptide 384 is similar to that of peptide 95 (Table 10), and would not therefore be expected to interfere with the polymerase assay.

The peptides were then tested for their effect on the synthesis of full-length M13 DNA by Pol–UL42. No inhibition was observed at concentrations of up to 50μM under the standard assay conditions (data not shown). To increase the sensitivity of the assay, the experimental conditions were adjusted from the standard protocol as follows: i) the amount of UL42 in the reaction was reduced to 33fmol — which results in less than maximal stimulation of processivity; ii) the peptides were incubated for 3 min at 37°C with UL42 in the assay mixture lacking both Pol and dCTP, then Pol was added and the reaction initiated by the addition of [α^{32}P] dCTP. Again, there was no detectable difference in the processivity of Pol–UL42 in the presence of peptides 384, 386, and 95, at concentrations of up to 200μM (Figure 33b).

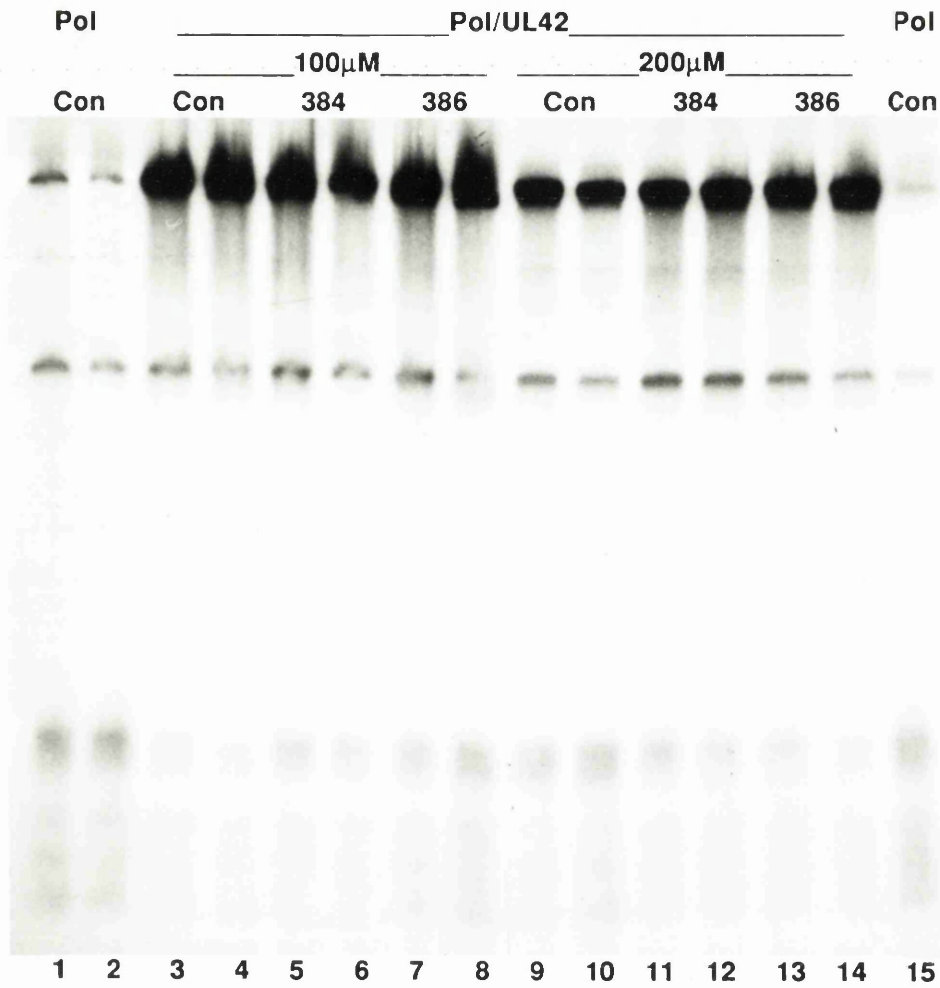
a

Figure 33.

a. The C-terminal 50 amino acid residues of Pol. The horizontal lines show the parts of the sequence synthesised as Pol peptides 384 (residues 1209–1235) and 386 (residues 1194–1223).

b. The effect of Pol peptides 384 and 386 on the synthesis of full length M13 DNA by Pol/UL42. Tracks 1, 2 and 15 show the products of polymerisation by Pol alone, in the presence of 200μM peptide 95 (which does not affect polymerase activity). Tracks 3–14 show the products of polymerisation by Pol/UL42 in the presence of 100μM and 200μM peptides 384, 386, and 95 (Con), each tested in duplicate, as indicated above the tracks.

b



DISCUSSION

The aim of this project was to identify the region(s) of the UL42 protein involved in interacting with Pol, the catalytic subunit of HSV-1 polymerase. At the time when the project was started, there was no information about the regions of UL42 likely to be involved in the interaction. The use of overlapping peptides to address such a question was a novel approach, and one which had the potential advantage that peptides identified at an early stage of the investigation might serve as leads for anti-viral drug development. The application of this approach was stimulated by the observation that the HSV ribonucleotide reductase is specifically inhibited by the nonapeptide YAGAVVNDL, which corresponds to the C-terminus of the small subunit of the enzyme. The peptide competes with the small subunit for a binding site on the large subunit and thus disrupts their interaction (Introduction 6.2). The aim of this project was also, therefore, to answer the more general question of whether this phenomenon is peculiar to ribonucleotide reductase, or whether a similiar disruption of other interacting subunits can be effected by peptides from the interface.

1. Oligopeptides

The only problems encountered during the synthesis, cleavage and analysis of the 96 UL42 peptides (Table 5) were (i) a low yield of peptides containing arginine plus tryptophan — this was due to using the TMSB cleavage method under suboptimal conditions (Results 1); (ii) the heterogeneity of peptides 22, 30, 31, 32, 33, 37, 42, 44 and 63, of which less than 50% represented the desired product (Table 5); (iii) the poor solubility of peptides 8, 13, 20, 34, 41, 42, 43, 45, 47, 52, 54 and 61.

The first two of these problems have since been alleviated by the development of new reagents for solid-phase peptide synthesis. The successful synthesis of peptides containing arginine plus tryptophan has been aided by the availability of Trp(Boc) — the Boc side-chain protecting group prevents modification of the indole ring of tryptophan during cleavage —, and by the introduction of Arg(Pmc), which is

deprotected far more rapidly than Arg(Mtr). Peptides containing arginine plus tryptophan, synthesised with these reagents, can be cleaved using a standard cocktail of TFA and chemical scavengers. The replacement of PFP and DHBT amino acid esters by the novel, more reactive PyBOP® chemistry (developed by Novabiochem) yields peptides of higher purity.

Only peptides 43, 54 and 61 were insoluble (except in concentrated acid). Several others, *eg* peptides 13, 34, 41, 42 and 47 were incompletely soluble in water in their crude state, but completely soluble after purification, which suggests that impurities accounted for the insoluble material in the crude preparation.

In principle, the purification and analysis of peptides should be carried out by two diverse chromatographic techniques, eg reverse phase HPLC and capillary zone electrophoresis (CZE), since not all peptides with closely related sequences are resolved by RP HPLC (Grant, 1992). We did not have regular access to CZE, however, and therefore used reverse phase chromatography for both purification and analysis. That this can achieve adequate results in most cases was shown by a CZE analysis of several of the purified peptides, which confirmed their purity (Results 3.2).

The decision to screen the unpurified peptides was made on the assumption that impure peptides are more likely to yield false positive than false negative results in an enzyme inhibition assay. One advantage of purifying all of the peptides before screening would have been the apparent improvement in solubility. Recent improvements in the capacity of reverse phase columns would make it possible to purify all peptides before screening, and this would be the approach of choice.

2. Optimisation of assay conditions

The experiments described in Section 2 of the Results were carried out to ensure that the peptides were tested under conditions that would allow the detection and measurement of their inhibitory properties. Suitable concentrations of UL42, of salt and of DNA template-primer were established as described, and the rate of accumulation of product was observed to be linear for the duration of the assay. The experiments described in Section 2 touch on some interesting questions regarding the HSV polymerase — such as the rate of polymerisation, or the relative affinity for DNA of Pol and Pol–UL42 — but do not provide enough data to make a real contribution towards answering them. For instance, the measurements of K_m for Pol and for Pol–UL42 are probably not very accurate, since they were based on a

minimum amount of data. They were adequate as a basis for establishing the concentrations of DNA template that would be in an approximately four-fold excess over K_m, but they should not be taken as an accurate measure of the relative affinities of Pol and of Pol–UL42 for a DNA template-primer. [It should be pointed out that the measured K_m values are *apparent* K_ms, composed of the intrinsic affinity of the protein(s) for DNA, and the relative probabilities of their dissociating from the DNA template or translocating along it for successive rounds of polymerisation.]

3. Identification of inhibitory peptides

The 96 UL42 peptides were screened for their ability to inhibit the synthesis of full-length M13 DNA by Pol–UL42 (Results 3.1). None of the peptides was seen to reduce the processivity of Pol–UL42 to that of Pol, *ie* to cause a reduction in the amount of full-length M13 product and a corresponding increase in the amount of shorter products. The inhibitory peptides which were identified reduced the amount of full-length product without causing an evident increase in the amount of shorter products, as seen in Figure 20. Nevertheless, the observed inhibition was worthy of further investigation.

Purified peptides 6, 7, 14, 15, 18, 19, 47, 48, 57, and 58 — representing five discrete regions of the UL42 protein — were consistently inhibitory, whereas peptides 34, 41 and 42 were no longer inhibitory upon purification, and thus proved to be "false positives". The inhibition by these crude peptides may have been associated with their poor solubility. The very insoluble peptides 43, 54 and 61 also caused an apparent inhibition when added to the assay as an undissolved precipitate, but this cannot be interpreted as an adequate test of their inhibitory potential. It is possible, but unlikely, that these peptides contain inhibitory sequences, since all the sequences present in the insoluble peptides were tested in the adjacent soluble ones.

The presence of a number of peptides, for example peptides 55 and 56 (Figure 20), appeared to increase the amount of full-length product. This effect was seen with peptides 4, 10, 55, 56, 77, 78, 79 and 91, but was not investigated further.

4. Investigation of polymerase inhibition by UL42 peptides

The potency of the peptides was assessed by determining IC_{50} values for their inhibition of Pol–UL42 and of Pol, obtained by measuring (i) the amount of full-

length product and (ii) the rate of incorporation of triphosphates into a primed M13 DNA template in the presence of a range of peptide concentrations (Results 3.3, 4, 5) (Table 7). The most potent was peptide 47, and the least potent was peptide 19. More reliance should be attached to the values from the incorporation experiments for the following reasons. First, the values for the formation of full-length product are based on a single datum point (the average of two samples, taken at 30 min). Second, it is not known whether the rate of formation of product in the presence of peptide is linear. Third, the method used for densitometry was less accurate than is radioactivity counting in a scintillation counter. In general, the extent of inhibition by the peptides of formation of full length product or incorporation of triphosphates was similar (within a factor of 2.4). However, peptide 6 was five- to six-fold more inhibitory of the formation of full length product.

The data obtained with both assays indicated that the peptides inhibited both Pol and Pol–UL42, and therefore provided no evidence that any of the peptides acted by disruption of the Pol–UL42 interaction. A peptide which acted in this manner might have been expected to reduce the level of Pol–UL42 activity to that of Pol alone and to have no effect on Pol itself.

The specificity of the peptides was assessed by comparing their inhibition of polymerase α and of Klenow polymerase with their inhibition of HSV-1 polymerase (Results 6) (Table 7 and Figure 27). Inhibition was found to be largely non-specific, although peptides 6, 14, 47 and 57 displayed some limited specificity. Peptide 6 showed the greatest specificity for the HSV polymerase, being 10-fold less active against Klenow polymerase and 2-fold less active against polymerase α. Whether this reflects a specificity for α-like polymerases remains to be tested.

5. Interaction of UL42 peptides with DNA

The investigation of DNA-binding as a mechanism of non-specific polymerase inhibition by the peptides provided clear evidence that peptides 14, 19, 47 and 57 interfered with polymerase activity by interacting with the DNA template (Results 7). By incubation of template DNA (1μg/ml) with a 50μM concentration of peptides 14, 19, 47 and 57, over 95% of the DNA was removed from solution (Table 10). The K_d values for oligonucleotide-binding (Table 8) for peptides 14 and 47 were close to their IC_{50}s for inhibition of HSV polymerase; the K_ds for peptides 19 and 57 were substantially lower than their IC_{50}s for enzyme inhibition. These data indicate that

the DNA-binding activity of peptides 14, 19, 47 and 57 probably accounts for the observed polymerase inhibition.

The finding that the rate of DNA synthesis by Pol–UL42 was more inhibited by peptides 14, 19, 47 and 57 than was the rate of synthesis by Pol alone (Figure 27) was probably a reflection of the lower concentration of DNA used in the Pol–UL42 assay (1μg/ml) than that used in the Pol assay (10μg/ml). In contrast, peptide 6 — which does not bind to DNA — showed the same activity against Pol–UL42 and Pol alone. The limited specificity of peptides 14, 47 and 57 for the different polymerases may reflect the different affinities of the enzymes for DNA. The specificity of peptide 6 must be due to another mechanism.

The detection of the DNA-binding properties of these peptides prompted further investigations to address the following questions: Did these peptides represent the only DNA-binding sequences among the 96 UL42 peptides? Did they represent DNA-binding regions of the protein?

Screening of all the UL42 peptides for DNA-binding in a gel mobility shift assay (Results 7.2) showed that 26 out of the 96 peptides exhibited a detectable level of binding to a radiolabelled oligonucleotide (Table 8). It is evident that the majority of peptides that bind to DNA are positively-charged: out of the 26 DNA-binding peptides, 22 are positively charged. The segregation of the 96 UL42 pepides into basic, acidic and uncharged, and the number in each category that bind to DNA is as follows:

		Total	DNA-binding
Basic	:	45	22
Acidic	:	34	1
Uncharged	:	17	3

Almost 50% of all the basic peptides, less than 20% of the uncharged peptides and less than 3% of the acidic peptides have DNA-binding properties detectable by this method. Therefore the peptides almost certainly bind to DNA predominantly by means of electrostatic interactions between basic amino acid side-chains and the acidic phosphate groups of the sugar-phosphate backbone of the DNA. The only DNA-binding peptides containing no basic groups were peptides 46 and 47.

It is evident that there is a rather poor correlation between the relative DNA-binding strengths and the relative potency of polymerase inhibition by the peptides. Several possibilities might account for the discrepancy. First, this method of quantitating DNA-binding may be inappropriate for peptides. Second, the relative strengths of

interaction of peptides with M13 DNA and with the test oligonucleotide may differ. Third, polymerase inhibition might involve a mechanism in addition to binding of the peptides to DNA. However, the single, double and triple substitution of acidic residues for polar residues in the sequence of peptide 47 (Results 7.4.2) showed that the presence of three acidic residues abbrogated both DNA-binding and polymerase inhibition. This adds weight to the argument that, in the case of peptide 47, the observed polymerase inhibition can be accounted for solely by the DNA-binding capacity of the peptide.

Some of the DNA-binding peptides — 55, 56 and 91 — were seen to enhance, rather than inhibit, polymerase activity in the original screen. Subtle differences in the way that peptides interact with DNA may determine whether they enhance, inhibit, or have little effect on polymerase activity. An enhancement of protein–DNA interactions by basic peptides has been observed: Bannister & Kouzarides (1992) report that the sequence-specific DNA-binding of a transcription factor in a gel mobility shift assay was enhanced by the addition of poly L-Lys or another basic peptide, but not by the addition of a neutral or acidic peptide. However, in contrast to the results presented here, they found that the two basic peptides did not themselves bind to DNA.

The limited data obtained by DNA precipitation (Results 7.5) suggest that this may be a better method than DNA mobility shift for assaying the DNA-binding properties of peptides. The precipitation assay may be more sensitive than the gel retardation assay, since the interaction of peptide 95 (charge =+1) with DNA is detectable by precipitation but not by gel retardation (Results 7.5). The prediction that an interaction of all basic peptides with DNA would be detectable by this method was not tested. The precipitation assay is also quicker to carry out and could be expected to give more accurate quantitation than the gel mobility shift assay. It would provide a simple way of screening peptides for DNA-binding before testing them for some other activity in a DNA-containing enzyme assay.

It is difficult to comment on the extent to which the DNA-binding peptides might represent DNA-binding regions of the UL42 protein. Considering the high proportion of DNA-binding peptides, and their distribution throughout the length of the protein, it seems improbable that they all represent portions of the UL42–DNA interface, although the tight but non-specific DNA-binding of UL42 would predict an extensive surface of interaction. The binding of overlapping peptides which differed in charge was seen to be determined by their charge, and by implication to be largely independent of sequence or other features. This is exemplified by peptides 33–36, the charge and binding properties of which are shown overleaf:

Peptide	Charge	DNA-binding
33	+1	yes
34	–1	no
35	+1	yes
36	+1	yes

However, it is also likely that many of the positively charged residues, particularly arginines, are to be found on the surface of the protein (Chothia, 1976) and may therefore contribute to its DNA-binding by electrostatic interactions.

6. Peptide 47

Throughout this study, peptide 47 generated a lot of interest: it was the most potent of the inhibitory peptides; peptides 47, 48, 49 and 50 represented a region of UL42 (residues 239–256) predicted to be a strongly amphipathic helix which might be involved in a coiled-coil hydrophobic interaction with the C-terminus of Pol (Digard *et al*, 1993a); its anomalous behaviour in reverse phase chromatography suggested unusual structural or binding properties; it stood out among the DNA-binding peptides because it formed a more discrete complex than any of the others, and it was the only peptide containing no charged residues which bound to DNA. These considerations suggested that it might represent a major DNA-binding region of UL42. The experiments described in Results 7.4 were carried out to test this possibility.

Attempts to identify the minimum binding region were frustrated by the insolubility of peptides truncated by more than two residues at either the N- or the C-terminus. To test the idea that the polar residues in the sequence TQVQILSNAL (residues 234–243, represented in both peptides 47 and 48) might be be involved in interactions with DNA, the glutamine and asparagine residues were substituted singly and in combination. Substitution by alanine residues resulted in insoluble peptides which could not be tested. Substitution of glutamine by glutamic acid and of asparagine by aspartic acid resulted in a progressive loss of DNA-binding (Table 9). Peptide 371A (STSTEVEILSNAL), containing two acidic residues, still bound detectably to DNA, whereas peptide 371B (STSTEVEILSDAL), containing three acidic residues, showed no evidence of binding (Figure 31 and Table 9). However, since the loss of binding is almost certainly caused by a general electrostatic repulsion between the peptide and the oligonucleotide, it is highly probable that substituting any other

residues in the peptide sequence by acidic residues would have produced similar effects.

In some cases, the DNA-binding properties of a peptide may be determined solely by its amino acid composition. A comparison of the binding characteristics of a 44-residue peptide corresponding to an RNA-binding domain of the yeast poly(A) binding protein, and of peptides with randomised sequences but the same amino acid composition, showed that the randomised peptides retained the binding properties of the peptide with the natural sequence (Nadler *et al* 1992a, 1992b). These observations prompted an experiment to investigate the relative importance of amino acid sequence and composition in determining the DNA-binding properties of peptide 47/301. The scrambled peptide (372) bound to DNA almost as efficiently as the native sequence (Figure 31 and Table 9). Unfortunately, the HPLC-purified preparation of peptide 372 was found by FAB-MS analysis to contain several species of different M_rs. These are almost certainly deletion peptides, resulting from the incomplete coupling of several residues during the synthesis of this peptide. A firm conclusion cannot therefore be drawn from this experiment, but it gives a preliminary indication that the sequence of peptide 301/47 is immaterial and that the observed DNA binding is entirely attributable to its amino acid composition. Such a conclusion would not rule out the possibility of residues 234–243 of UL42 playing a role in the DNA-binding of the protein. A similar scrambled sequence containing the 15 amino acid residues of peptide 47 was synthesised, but was found to be very poorly soluble, and so the experiment to confirm this conclusion was not repeated.

An investigation of the DNA-binding properties of a UL42 mutant protein with residues 234–243 deleted or altered should determine whether this epitope constitutes a major DNA-binding determinant. This experiment has not been done, but sequences immediately adjacent to this epitope have been mutated: neither the deletion of residues 242–250, nor the replacement of A_{242} by a 5-amino acid insertion, had any effect on the DNA- or Pol-binding properties of UL42 (Digard *et al*, 1993b).

7. Pol C-terminal peptides

As the experiments described in this thesis were nearing completion, evidence became available to us that the extreme C-terminus of Pol is crucial for the interaction with UL42 (Digard *et al*, 1993a; Stow, 1993; see Introduction, 4.4.4). The prediction that peptides from this region of Pol could inhibit the interaction had been

tested in a preliminary experiment, which showed that a peptide corresponding to residues 1194–1223 of Pol inhibited processive synthesis by Pol–UL42 (Don Coen, personal communication). To further investigate this finding, two peptides were synthesised and tested (Figure 33a and b). Peptide 384 (residues 1209–1235) corresponded to the residues deleted by Nigel Stow, and peptide 386 (residues 1194–1223) to the peptide found by Don Coen to be inhibitory. Tested at a concentration of 200μM, the peptides had no detectable effect on Pol–UL42 processivity (Figure 33b). This result is at variance with that communicated by Coen.

On the evidence of the available data, there are several alternative possible interpretations of the lack of inhibition by the Pol C-terminal peptides:

(i) If the extreme C-terminal ~30 residues of Pol are an essential component of the Pol–UL42 interface (Digard *et al*, 1993a; Stow, 1993), then the lack of inhibition by the Pol peptides indicates that the nature of the Pol–UL42 interaction is such that it cannot be disrupted by a peptide corresponding to the region of interaction on one of the proteins.

(ii) The lack of inhibition by the Pol peptides is consistent with the finding of Tenney *et al* (1993) — published after the completion of the experiments described here — that the C-terminal 40 residues of Pol may not be essential for the interaction of Pol and UL42 (Introduction 4.4.4).

(iii) Some of the data from the mutagenesis studies suggest that the physical interaction of Pol and UL42 may not be essential for their functional interaction. A mutant Pol with a four-amino-acid insertion at residue 1216 is not co-immunoprecipitated with UL42, but it is fully functional in vivo, as tested by its ability to complement a *pol* null mutant (Digard *et al*, 1993a). Similarly, deletion of the C-terminal 40 residues of Pol results in a mutant protein which cannot be co-immunoprecipitated with UL42, but which can be stimulated by UL42 to the same extent as the full-length protein, as tested by its activity on an activated DNA template (Tenney *et al*, 1993). The lack of inhibition by the Pol peptides is consistent with this idea.

Whilst inhibition of UL42-stimulated processivity would have provided further evidence that the extreme C-terminus of Pol interacts with UL42, the observed lack of inhibition by the Pol peptides does not contribute to resolving the controversy over the Pol–UL42 interface.

8. Strength of complex formation

A clearer understanding of whether it is possible to disrupt the Pol–UL42 complex requires not only the identification of the regions of the proteins involved in the interaction, but also the determination of the strength of that interaction. When equimolar amounts of purified Pol and UL42 proteins were co-incubated for 1 hour at 37°C and then subjected to gel filtration chromatography, all of the protein was eluted in the form of the heterodimeric complex, which is indicative of a strong interaction (John Gottlieb, personal communication).

E. coli ribonucleotide reductase (RR), like the HSV RR, is disrupted by peptides corresponding to the C-terminal 19 or more amino acid residues of the small subunit (R2) (Climent *et al*, 1991). The intrinsic dissociation constant, K_d, for a single molecule of R2 binding to R1 is 13µM , and the inhibition constant, K_i, for a 30-residue peptide is 15–18µM, suggesting that no other parts of R2 contribute to its binding to R1 (Climent *et al*, 1992). Moreover, it is believed that the C-terminus of R2 is a rather flexible region, since it could not be resolved by X-ray crystallography (Nordlund *et al*, 1990), and this suggests that it could be effectively mimicked by an inherently flexible peptide. A K_d of 13µM does not indicate a very strong association between the subunits — very strong complexes are considered to have K_d values below 10^{-7}M (Suelter, 1985). The overall binding affinity of R1 for R2 is increased to 0.18µM (Climent *et al*, 1992) by the dimeric nature of the subunits. It may be, therefore, that the characteristics of the RR subunit interface render it particularly susceptible to this type of inhibition by subunit dissociation.

It is interesting in this regard that the Pol–UL42 interface is also proposed to consist of the C-terminus of one of the proteins, since protein termini are often more flexible than internal sequences (Karplus-Schulz, 1985). However, one would predict that if the affinity of Pol and UL42 were orders of magnitude greater than that of R1 and R2, then the Pol–UL42 complex would not be susceptible to a similar dissociation.

9. Future work

Consideration of some of the questions outlined above, and of the difficulties of unambiguously interpreting results obtained using co-immunoprecipitation, identifies the need for a more quantitative method of detecting the physical association between Pol and UL42. Relatively large amounts of both Pol and UL42 proteins are now available, prepared by Mary Murphy, from insect cells infected with recombinant baculovirus vectors (Stow, 1992). This has allowed the development of an enzyme-

linked immunosorbent assay (ELISA): Pol protein is bound to the plastic surface of a microtitre well; after removal of the unbound protein and blocking of unoccupied sites on the plastic, UL42 is added; this is followed by a UL42-specific MAb, Z1F11, which in turn is bound by protein A coupled to horseradish peroxidase (HRP); finally, the addition of a colorimetric substrate allows the quantitative detection of the bound HRP. The MAb Z1F11 (Schenk *et al*, 1988) reacts with an epitope of UL42 (residues 363–369) which is not required for its interaction with Pol (Murphy *et al*, 1989; Digard *et al*, 1993b; Introduction 4.4.4), and therefore would not interfere with Pol–UL42 complex formation.

Such an assay can be used to answer the following types of questions, which would advance the understanding of the Pol–UL42 interaction and of its potential as an antiviral target:

How is the Pol–UL42 interaction affected by the presence of DNA?

Is the affinity of Pol and UL42 increased by the formation of a ternary complex with the DNA template–primer?

Can any of the UL42 peptides disrupt the physical association of Pol and UL42?

Which C-terminal sequences of Pol are involved in the interaction with UL42?

This last question can be addressed by testing in the ELISA

(i) whether C-terminally truncated Pol proteins can form a complex with UL42;

(ii) whether peptides covering a more extensive region of the Pol C-terminus are able to disrupt the Pol–UL42 interaction;

(iii) whether antisera raised against Pol C-terminal sequences are able to disrupt the Pol–UL42 interaction.

What are the relative strengths of interaction with UL42 of full-length and of C-terminally truncated Pol?

Given the existing evidence for the involvement of the C-terminus of Pol in the interaction with UL42, another line of investigation would be to chemically cross-link the Pol C-terminal peptides to the UL42 protein and thus attempt to identify the regions of UL42 which contribute to the interface.

Many of the interactions that are likely to exist between the replication proteins of HSV-1 have yet to be identified and characterised. The interaction between Pol and UL42 has been studied more extensively than any other interactions between the seven essential replication proteins. The cloning and heterologous expression of UL5, UL8, UL9, UL29 and UL52 (Stow, 1992), allows their purification in relatively large amounts, and the production of reagents — MAbs and polyclonal antibodies of high specificity — essential for investigating their interactions.

Discussion

A library of random peptide sequences, generated using the fusion phage system (Parmley & Smith, 1988), could be screened for sequences which disrupt protein interactions. The R1–R2 interaction would provide a suitable model system for subsequent investigation of interactions between replication proteins. Such an approach, however, may identify inhibitory sequences unrelated to the sequence of the ligand under investigation (Scott & Smith, 1990; Christian *et al*, 1992).

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